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Original Article

Postnatal donor lymphocytes enhance prenatally-created chimerism at the risk of graft-*versus*-host disease

Jeng-Chang Chen[1], Liang-Shiou Ou[2], Hsiu-Yueh Yu[3], Ming-Ling Kuo[4], Pei-Yeh Chang[1], Hsueh-Ling Chang[3]

[1]Department of Surgery, Chang Gung Children's Hospital, College of Medicine, Chang Gung University, Taoyuan, Taiwan; [2]Department of Allergy, Asthma and Rheumatology, Chang Gung Children's Hospital, College of Medicine, Chang Gung University, Taoyuan, Taiwan; [3]Pediatric Research Center, Chang Gung Children's Hospital, Taoyuan, Taiwan; [4]Department of Microbiology and Immunology, Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan

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Abstract: The major barrier to clinical application of *in utero* hematopoietic stem cell transplantation is insufficient chimerism for phenotypic correction of target diseases or induction of graft tolerance. Postnatal donor lymphocyte infusion (DLI) may enhance donor cell levels so as to further facilitate tolerance induction. We created murine mixed chimeras *in utero*. Chimeras with <10% donor cells were subjected to postnatal DLI to evaluate the effects of DLI on chimerism augmentation and skin tolerance induction. Within one day after DLI, recipients experienced a transient peaking of donor chimerism, which could be as high as 20~40%. However, the transient chimerism peaking didn't benefit donor skin survivals despite immediate skin placement after DLI. In case of fruitful DLI, chimerism augmentation was usually observed after a latent period of 2~4 weeks. Otherwise, chimerism would return to around pre-DLI levels by days 7~14. Peripheral chimerism of >3% could be consistently boosted up to >10%, whereas chimerism of <0.2% hardly showed any significant enhancement. As for chimerism levels of 0.2~3%, chimerism augmentation up to >10% succeeded in 3(15%) of 20 recipients. Notably, chimerism augmentation by postnatal DLI was often associated with unexpected death or graft-*versus*-host disease (GVHD). In conclusion, transient chimerism augmentation by DLI played no role in facilitating graft tolerance. Substantial augmentation by DLI demanded a threshold chimerism level and posed a serious risk of GVHD to the recipients. It raised the concern about using postnatal DLI to broaden therapeutic horizons of *in utero* hematopoietic stem cell transplantation.

Keywords: Chimerism augmentation, donor lymphocyte infusion, graft-*versus*-host disease, *in utero* transplantation, tolerance induction

Introduction

Hematopoietic chimerism is well-known for its immunological relevance to donor-specific tolerance [1, 2]. Although the establishment of donor cell chimerism as a major step towards allograft tolerance in organ transplantation is at the frontier of clinical application [3], there is no shortage of conflicting experiments or clinical observations pertaining to chimerism and tolerance [4-6]. This debate might be related to the observation that the relevance of hematopoietic chimerism to donor-specific graft tolerance lies in the induction rather than the maintenance phase [6, 7]. In our previous studies, peripheral chimerism of >3% at skin graft placement consistently conferred lasting skin tolerance [6], suggesting that induction of graft tolerance did not always necessitate donor-dominant cellular chimerism. It has been long known that *in utero* marrow transplantation mostly yielded limited or even no chimerism [8, 9], far below the threshold level of 3%. This also reflected limited attainability for induction of graft tolerance following *in utero* marrow transplantation [4, 6, 10].

Donor lymphocyte infusion (DLI) is a kind of adoptive immunotherapy in which lymphocytes from the original stem cell donor are infused after allogeneic bone marrow transplantation for hematopoietic malignancies [11]. This thera-

py exerts so-called graft-*versus*-tumor effects to eradicate any residual disease after a transplant, prevent disease relapse, and even treat tumor recurrence [12]. Conventional allogeneic bone marrow transplantation demands preparative myeloablative conditioning to maximize tumor cytoreduction [13] and facilitate donor stem cell engraftment [14] at the expense of transplant-related toxicities and graft-*versus*-host disease (GVHD) [15, 16]. Less toxic non-myeloablative conditioning potentially minimizes fatal adverse effects [17], but usually confers a state of mixed chimerism [18]. Under this circumstance, DLI can be employed for converting mixed to full chimerism [12, 19, 20]. However, the prerequisite donor cell levels for significant chimerism augmentation by DLI remains unknown. Besides, it's feasible to have a small-scale enhancement of donor cell chimerism up to more than 3% by DLI so that micro- or low-level mixed chimeras may be endowed with a graft tolerance-inducible state. We then prompted this study to evaluate the effects of postnatal DLI on the facilitation of hematopoietic chimerism and graft tolerance in prenatally-created micro- or low-level chimeras. It was found that transient chimerism augmentation had no benefit for graft tolerance. Substantial augmentation demanded a threshold chimerism level before DLI and often ensued at the cost of GVHD.

Materials and methods

Mouse husbandry

Inbred FVB/N (H-2^q) and C57BL/6 (H-2^b) mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) at the age of 6-8 weeks, and housed in the Animal Care Facility at Chang Gung Memorial Hospital (CGMH) under the standard guidelines from "Guide for the Care and Use of Laboratory Animals" and with the approval of the CGMH Committee on Animal Research. *In utero* transplantation was performed in a C57BL/6-into-FVB/N strain combination. 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 splenic lymphocytes and bone marrow cells (BMCs)

Under sterile conditions, adult C57BL/6 mice were sacrificed. BMCs were flushed out from the tibias and femurs with phosphate buffer saline (PBS) using a 26-gauge needle, and splenocytes were obtained by dissociating spleens in 70 μm cell strainers (BD Biosciences). Light-density BMCs and splenic lymphocytes were then enriched with NycoPrep 1.077A density gradient (Nycomed, Pharma AS, Oslo, Norway). Marrow T-cells were depleted using anti-CD3 FITC (BioLegend, San Diego, CA) and anti-FITC microbeads (Miltenyi Biotec, Auburn, CA) following the manufacture's instructions. Splenic lymphocytes and T-cell-depleted BMCs contained 20~30% and <0.5% of $CD3^+$ cells respectively by staining their aliquots with anti-CD3 FITC (BioLegend).

In utero transplantation

T-cell-depleted BMCs of $5\sim10\times10^6$ in 5~10 μl PBS were intraperitoneally injected into gestational day 14 fetuses by transuterine approach [21]. Following the transplantation, pregnant mice were intraperitoneally given 1 ml warm saline and had their abdomens closed by 5-0 vicryl sutures. Then, they were housed in an undisturbed room without bedding changes until the pups were 1 week old. Pups were weaned at 3 weeks of age.

Postnatal DLI

Within one day after the determination of peripheral chimerism at the age of 1 month, recipients under restraint were given a dose of $2.5\sim5\times10^7$ donor lymphocytes in 0.1 ml PBS via tail veins using a 3/10 cc insulin syringe, ULTRA-FINR II 30 gauge 5/16" short needle (Becton Dickinson). Recipients with any signs of GVHD after lymphocyte infusion were enrolled for GVHD assessment.

Analyses of chimerism and lineages

Blood was sampled via the tail tip after restraint and depleted of red cells using ACK buffer, pH 7.2-7.4, consisting of 0.15 M NH_4CL, 1.0 mM $KHCO_3$ and 0.1 mM Na_2EDTA (Sigma Chemical Co., St. Louis, MO). Cells were first incubated with anti-mouse CD16/32 antibody (Clone 93, BioLegend) to lessen nonspecific Fc-mediated binding of monoclonal antibodies, and then stained with anti-H-$2K^q$ FITC (Biolegend) and anti-H-$2K^b$ PE. A negative control consisted of anti-H-$2K^q$ FITC and mouse IgG2a PE (Biolegend) to define background staining. Leukocyte lineages were analyzed using anti-H-$2K^b$ PE and

Table 1. Outcome of postnatal DLI

Group	Undetectable	0.01~0.19%	0.20~3%	>3%
Case number	15	28	20	4
Death within 1 month after DLI	0	2†	3†	0
Chimerism at 2 months old				
Undetectable	15	16	2	-
0.01-0.19%	-	5	1	-
0.20-3%	-	3	5+3†+2*	-
3.01-10%	-	1*	1*	-
>10%	-	1*	1†+2*	1+3*

†: Unexpected death without post-mortem autopsy, *: Histologically-proved GVHD.

either anti-CD3, CD4, CD8, CD45R, CD11b and Gr1 FITC (BioLegend). Cell events (50,000~100,000) were acquired for analysis after gating out dead cells using propidium iodide.

Assessment for GVHD

Clinical signs of GVHD included weight loss, hair loss or ragged fur, hunched appearance, diarrhea, or noticeably decreased activity. Recipients with any signs of GVHD were sacrificed to obtain the liver and lung for further histopathological examinations. The organs were fixed in 4% formaldehyde/PBS and then embedded in paraffin. Tissue sections in 10 μm thickness were mounted on glass slides, stained with hematoxylin & eosin, and examined under an Olympus light microscope. Images were captured by a Nikon D90 digital camera.

Skin transplantation

Skin transplantation was performed within one day after the examination of chimerism, or one or 14 days after DLI. Briefly, tail skins from C57BL/6 donors were transplanted on the backs of FVB/N recipients, and monitored as described before [22]. Engrafted skin was defined by good hair growth. Rejection was defined as when ≤20% of the original graft remained. A tolerant state was defined by skin engraftment for at least 120 days.

Statistical analyses

In survival analyses of skin transplants, the survival time was defined by estimating the length of time from the date of skin transplantation to the date of graft rejection or the date of last follow-up. Otherwise, the graft was considered censored at last follow-up. When a mouse died before skin rejection was observed, it was counted as a withdrawal and treated the same way as cases lost to follow-up. Plots of survival time were constructed by Kaplan-Meier method. The log rank test was employed to compare survival curves. Differences were regarded as significant in case of $P<0.05$.

Results

Mixed chimeras created by in utero marrow transplantation

Following *in utero* transplantation of allogeneic marrow, 123 recipients with first month peripheral chimerism of <10% were collected. The immune response of mixed chimeras to the donor can be classified as "reliable tolerance", "possible tolerance", and "hyporesponsiveness" based upon peripheral chimerism of >3%, 0.2~3%, and <0.2% at the time of graft placement [6]. As a result, these recipients were grouped by their first month chimerism levels into the categories of ">3%", "0.20~3%" and "<0.2%". There were 4, 38, and 81 cases for each group. The group "<0.2%" was further categorized into "0.01-0.19%" (n=52) and "undetectable" (n=29).

Postnatal donor lymphocyte infusion in mixed chimeras

In order to evaluate whether postnatal DLI could augment chimerism and/or facilitate tolerization in recipients that were categorized as "possible tolerance" and "hyporesponsiveness", we subjected 20, 28 and 15 recipients arbitrarily in groups "0.20~3%", "0.01~0.19%" and "undetectable" to DLI within one day after the determination of first month chimerism (**Table 1**). DLI was also given in all the 4 recipients of group ">3%". Following DLI, 2 group "0.20~3%" and 3 group "0.01~0.19%" recipients died unexpectedly within 1 month before their post-DLI peripheral chimerism could be examined for the first time.

Monthly follow-up of peripheral chimerism after postnatal DLI

All recipients with postnatal DLI were examined for peripheral chimerism monthly from 2

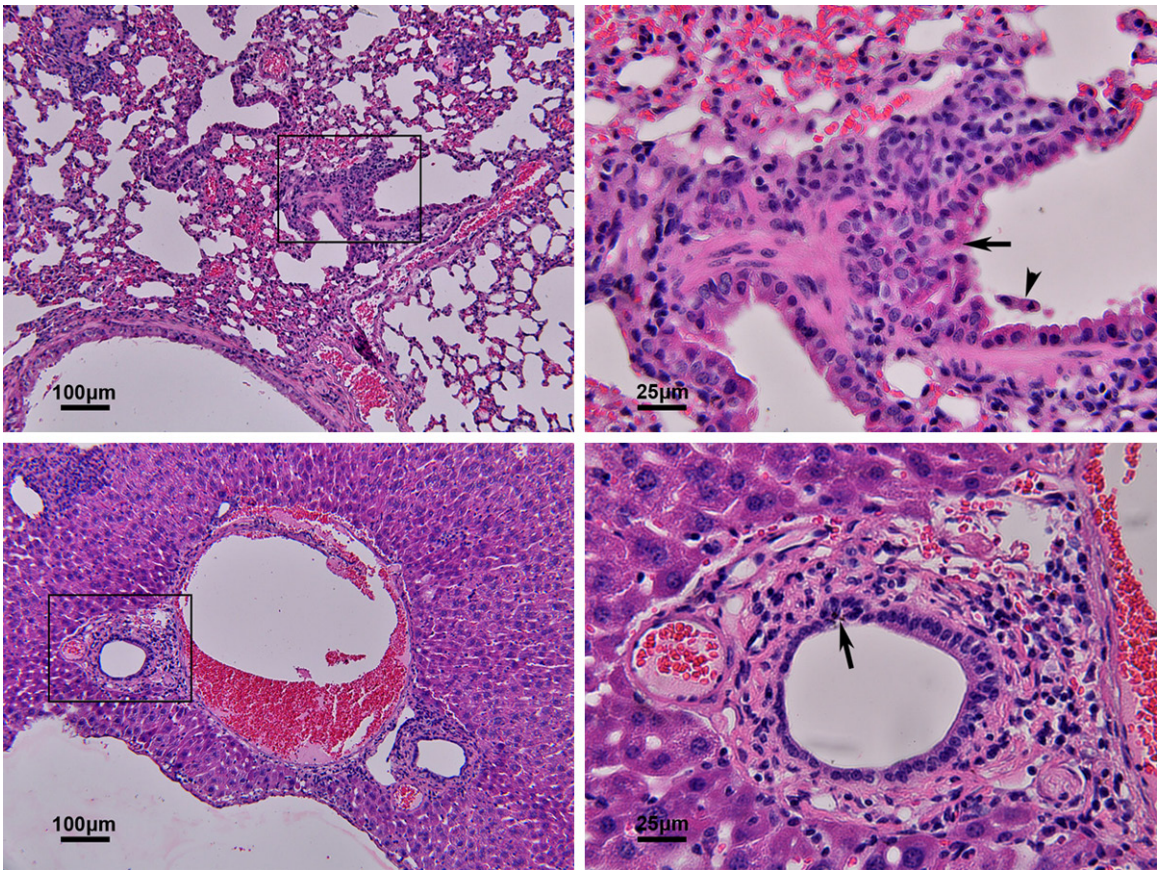

Figure 1. Lung and liver involvement of GVHD after postnatal DLI. A representative recipient with low-level chimerism of 0.10% was subjected to postnatal DLI at 1 month old and had chimerism augmentation up to 12.94% at 2 months old. However, the mouse showed signs of GVHD. Histological examinations by hematoxylin-eosin staining showed mononuclear cell infiltration in the peribronchial area of the lung (upper left panel) and the portal area of the liver (lower left panel). The lung involvement of GVHD was destructive to airway epithelia (arrow, upper right panel) with epithelial shedding (arrowhead). The liver involvement of GVHD also damaged endothelia of bile ducts (arrow, lower right panel).

months old (one month after DLI) onwards (**Table 1**). None of 15 group "undetectable" cases exhibited any detectable donor cells in the circulation at 2 months old. Among 28 recipients of group "0.01~0.19%", sixteen still lost circulating donor cells within one month after DLI. Chimerism was kept within 0.01~0.19% in 5 mice and 0.2-3% in 3 mice by the age of 2 months, but finally turned out to be undetectable by 3~5 months of age. Two with the first month chimerism of 0.18% and 0.10% were found to have remarkable augmentation of chimerism up to 3.96% and 12.94% at 2 months old, which rapidly dropped to around 1% at 3 months old. Notably, both presented with signs of GVHD at the age of 3~4 months. Histopathological examinations revealed peribronchial mononuclear cell infiltration with airway epithelial destruction in the lung, and mononuclear cells infiltration around the portal area with bile duct destruction in the liver (**Figure 1**), compatible with the occurrence of GVHD.

Among 20 recipients subjected to postnatal DLI in group "0.20~3%", 3 had a decline of peripheral chimerism below 0.2% by the age of 2 months. Peripheral chimerism fluctuated within 0.20~3% for 5 months after DLI in 10, of which 4 died unexpectedly and one showed signs of GVHD with subsequent histological confirmation. Four with the first chimerism of 1.54%, 2.40%, 1.22% and 1.87% showed fruitful chimerism augmentation, which reached their highest levels of 10.05%, 17.09%, 49.14% and 58.99% at the age of 2-3 months before

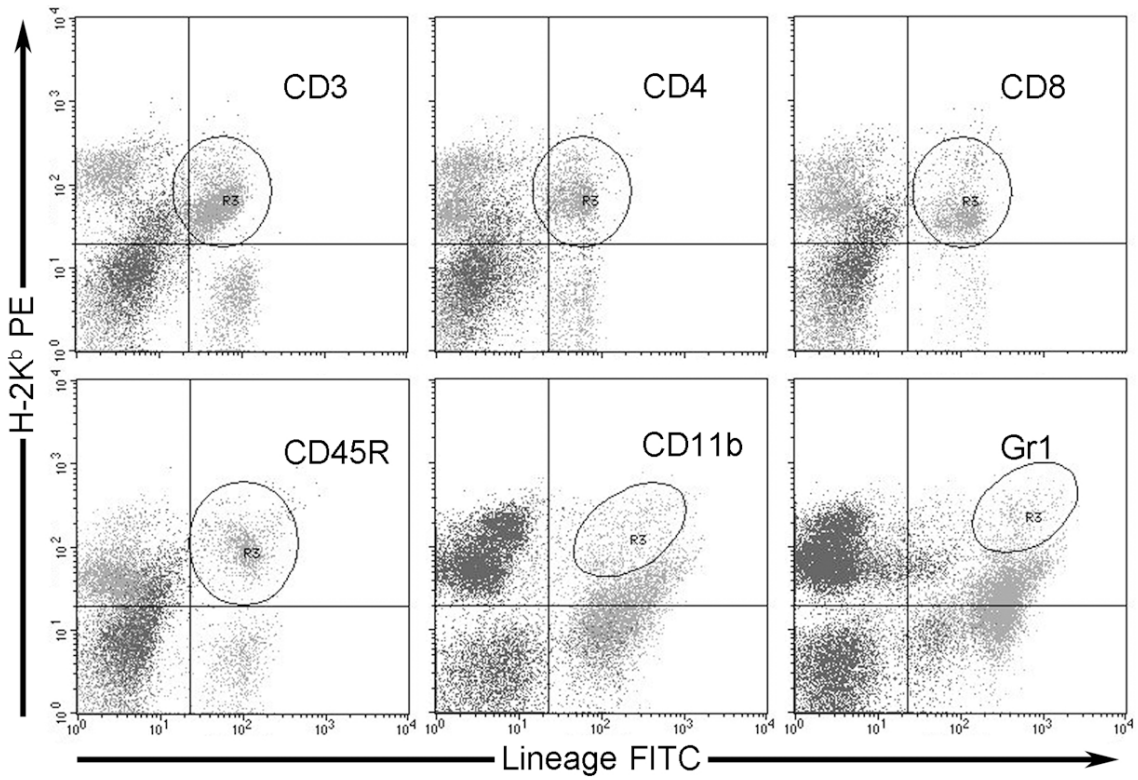

Figure 2. Multilineage expression of donor leukocytes following chimerism augmentation by postnatal DLI. A mixed chimera with peripheral chimerism of 4.01% at 1 month old had chimerism augmentation by postnatal DLI up to 26.2% at 4 months old. This augmentation displayed multilineage expression of donor H-2^b leukocytes.

they died unexpectedly (3 cases) or of histologically-proven GVHD following GVHD presentation (1 case). Among 7 recipients that died unexpectedly, 3 could be subjected to histological examinations. All showed mononuclear cell infiltration either in the liver or lung, supporting the diagnosis of GVHD.

As for group ">3%", four cases with the first month peripheral chimerism of 4.01%, 4.22%, 4.81% and 5.67% were enrolled for DLI. All had significant increment of peripheral chimerism >10% at 2 months old. However, two died unexpectedly at the age of 2~4 months with postmortem microscopic findings of mononuclear cell infiltration in hepatic portal area, suggestive of GVHD. One with weight loss and hunched posture proved to have GVHD by histological examinations. The fourth recipient had progressive increment of chimerism up to around 40% at 6 months old, and then fluctuated within 40~70% by the age of 1 year. This chimerism augmentation exhibited multilineage expression of leukocytes in the circulation (**Figure 2**).

Kinetics of peripheral chimerism after postnatal DLI

For dynamics studies of circulating donor cells, 15 recipients were additionally examined for peripheral chimerism at earlier time points within 30 days after DLI (**Figure 3**). Two group "0.2~3%" and 1 group "0.01~0.19%" recipients died unexpectedly within 3 days after DLI. Donor cell chimerism could be rapidly boosted up within 2 hours after DLI, and might increase to as high as 20~40% within one day. For recipients with first month chimerism of 0~0.19%, chimerism mostly returned to pre-booster levels by days 7~14 and universally became undetectable by post-DLI day 30. For recipients with chimerism of 0.2~3%, 3 had a decline of chimerism to around the pre-booster levels from day 7 onwards. In the two others, chimerism showed a slight decline within days 2~5 after the initial uprising, and then progressively increased to around 20% and 60% respectively on day 30 (2 months old). However, both died by 3 months old.

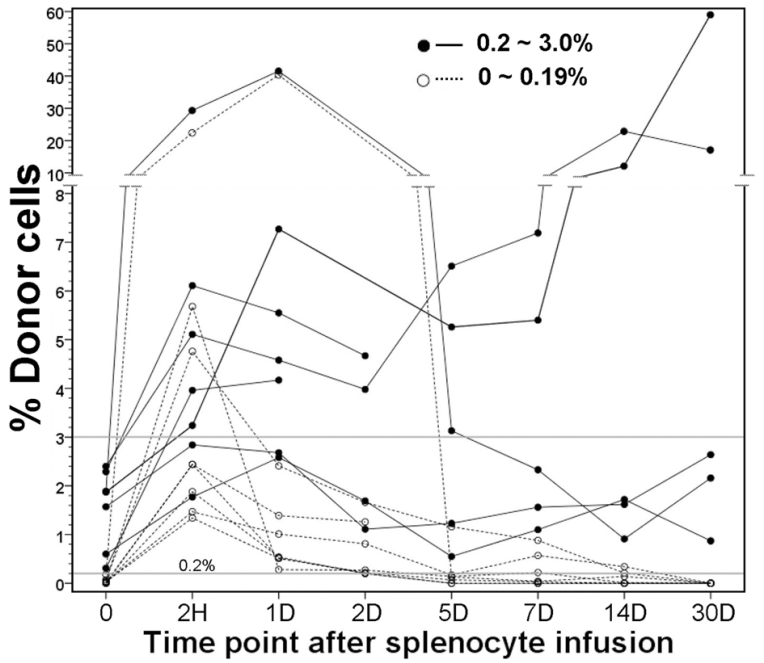

Figure 3. Dynamics of donor cell levels within 1 month after postnatal DLI. 15 recipients (7, 6 and 2 respectively from groups "0.2~3%", "0.01~0.19%" and "undetectable") were examined for circulating donor cells levels within 30 days after postnatal DLI at the time points of 2 hours (2 H), 1, 2, 5, 7, 14 and 30 day (1~30 D).

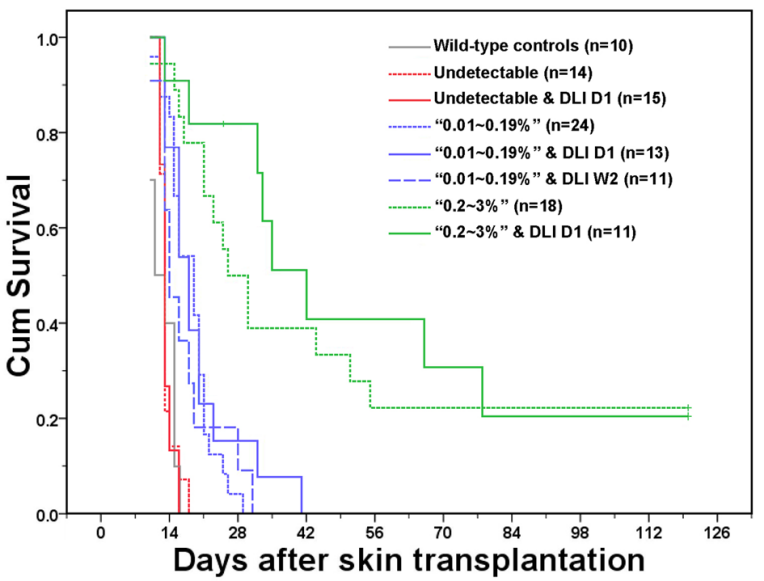

Figure 4. Survival curves of donor skin grafts. H-2^{q} murine recipients in groups "0.2~3%", "0.01~0.19%" and "undetectable" were subjected to H-2^{b} donor skin transplantation within one day (DLI D1) or 2 weeks (DLI W2) after postnatal DLI. Their skin graft survivals were compared with those from wild-type controls and their counterparts without DLI. DLI either on day 1 (DLI D1) or week 2 (DLI W2) did not show beneficial effect on donor skin survivals. Regardless of DLI, skin graft survivals were better in group "0.2~3%" than in groups "0.01~0.19%" (P<0.001~P=0.004), "undetectable" (P<0.001 for all) and wild-type controls (P<0.001 for both). Also, groups "0.01~0.19%" had better skin survivals than group "undetectable" (P<0.001~P=0.023). Skin survivals did not differed between group "undetectable" and wild-type controls.

Effects of postnatal DLI on donor skin survivals

We demonstrated in previous studies that chimerism levels at the time of skin transplant related to skin survivals with >3% for consistent skin tolerance of >120 days, 0.2~3% for a 34% chance of skin tolerance, and <0.2% for prolonged skin survivals only within 60 days [6]. In order to evaluate whether postnatal DLI could improve donor skin survivals, we subjected the recipients that had received postnatal DLI to donor skin transplant. Recipients of group "0.01~0.19%" were randomized to undergo skin transplant either within one day or after two weeks following DLI. The counterpart controls were recipients from the same group, subjected to skin transplant directly without DLI. All recipients receiving donor skin transplant either within one day or 2 weeks after DLI rejected donor skins within 6 weeks without significant difference from their counterpart controls (**Figure 4**).

As for groups "0.2~3%" and "undetectable", recipients were subjected to donor skin transplant within one day after DLI. Both showed comparable survival experience of donor skins to their counterparts without DLI. Regardless of DLI, group "0.2~3%" compared favorably in donor skin survivals with group "0.01~0.19%", which in turn was superior to group "undetectable" and wild-type controls without *in utero* marrow transplantation (**Figure 4**).

In group ">3%", the only one long-term survivor following DLI was rendered tolerant of

donor-specific skin for more than 120 days without the emergence of any clinical GVHD signs.

Discussion

Despite the claimed privilege and advantage of fetal environment for stem cell engraftment, cures for target diseases amenable to hematopoietic stem cell transplantation remains barely achievable *in utero* [9]. Therefore, prenatal cellular therapy has not yet become fully implemented reality and routine so far. The major barrier is micro- to low-level or even absent donor cell engraftment in most animal models and clinical trials [9, 21, 22]. It's reported that phenotypic correction for murine beta-thalassemia by cellular therapy necessitates a chimerism level of at least 10% [23]. This therapeutic threshold could be reached only in about 5% of murine recipients that received *in utero* transplantation of allogeneic T-cell-depleted marrow [22]. Encouragingly, postnatal DLI was reported to effectively enhance donor cell levels of low-level mixed chimeras following *in utero* marrow transplantation, wherein DLI of 3×10^7 lymphocytes caused complete chimerism [24]. However, the prerequisite donor cell levels for chimerism augmentation by DLI remained obscure. With similar DLI doses in this study, chimerism could be enhanced up to >10% in all the cases of group ">3%" with the first month chimerism of 4~6% as well as in some recipients of group "0.20~3%" with the first month chimerism of 1~3%, but augmentation was hardly observed in recipients of groups "0.01~0.19%" and "undetectable". Thus, donor cell levels required for chimerism augmentation by postnatal DLI essentially paralleled those required for induction of graft tolerance [6]. It suggests that chimerism augmentation by DLI demanded a tolerance-inducible chimeric state.

Although DLI marks significant advances in the field of marrow transplantation, it also leads to a high rate of GVHD [25]. It was reported that delayed DLI, 5 weeks after bone marrow transplantation, might effectively augment chimerism in the absence of adverse GVHD in murine mixed chimeras [26]. In a C57BL/6-into-Balb/c murine model of *in utero* marrow transplantation on day 14 or 15 of gestation, postnatal DLI at 4~8 weeks old (about 5~9 weeks after transplantation) indeed enhanced chimerism with minimal GVHD [24]. Additionally, the reduced GVHD was attributed to the lack of irradiation with accompanying proinflammatory cytokine milieu in the fetal recipients. In this prenatal model, we experienced high incidence of GVHD or unexpected death following postnatal DLI especially when chimerism augmentation could be achieved. In contrast, recipients without any chimerism augmentation were totally absent from GVHD or even unexpected death. The high correlation between GVHD and chimerism augmentation suggested that the graft-versus-host effects from DLI played an important role in chimerism augmentation. Thus, GVHD remains a matter of great concern following postnatal DLI in conditioning-free models of prenatal marrow transplantation despite minimal GVHD claimed in previous studies. The difference in strain combinations might have an influence on the incidence of post-DLI GVHD.

Dynamics studies of circulating donor cell levels following DLI revealed that DLI might create a transient upsurge of donor cells within one day. It suggested an additive effect of donor lymphocytes on chimerism levels. As for futile DLI, donor chimerism rapidly returned to around pre-DLI levels by days 7~14, whereas in case of fruitful DLI chimerism augmentation of >10% did not ensue until 2~4 weeks after DLI. This made it clear that substantial chimerism augmentation necessitated a latent period of 2~4 weeks after DLI. As we know, tolerance induction is another appealing result for prenatal cellular immunotherapy. In the study of Hayashi et al. [24], the conversion to full chimerism by DLI occurred in mixed chimeras with donor cell levels of <10%. However, individual's donor cells levels were not specified in their cases. Thus, it remained unknown whether postnatal DLI could facilitate induction of graft tolerance in mixed chimeras with chimerism levels lower than the threshold of 3%. In this regard, such recipients were collected, subjected to postnatal DLI, and evaluated for induction of skin tolerance in our studies. Following DLI, chimerism might peak at over 3% within one day, but often dropped to around pre-DLI levels by days 7~14. Skin transplantation was undergone during the period of chimerism peaking in order to meet the most appropriate timing of higher chimerism levels for graft tolerance induction. However, the transient chimerism peaking was ineffective to promote donor skin survivals, let alone 2 weeks away from the peaking. As a

result, postnatal DLI provided no substantial benefit for donor skin survivals regardless of the timing of skin placement in mixed chimeras without sufficient donor cell levels for induction of graft tolerance.

Although *in utero* marrow transplantation generally created micro- to low-level chimerism, postnatal DLI might elicit a transient chimerism peaking regardless of the final DLI outcome. At skin transplantation, this additive and transient increment of donor cells played no role in promoting skin tolerance. Hematopoietic chimerism could be consistently enhanced up to therapeutically significant levels of >10% when its levels could cross the threshold of 3% at the time of DLI. This threshold level was similar to that required for consistent induction of skin tolerance [6]. It's worthwhile to note that chimerism augmentation by postnatal DLI often ensued at the expense of GVHD. This raises qualms about using postnatal DLI as a strategy for chimerism augmentation in low-level mixed chimeras generated by *in utero* marrow transplantation.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jeng-Chang Chen, Department of Surgery, Chang Gung Children's Hospital, College of Medicine, Chang Gung University, 5, Fu-Shin Street, Kweishan, 333, Taoyuan, Taiwan. Tel: +886-3-3281200 Ext. 8227; Fax: +886-3-3287261; E-mail: bx9619@cgmh.org.tw; jch.chen@msa.hinet.net

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