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Alpha-GalCer Administration after Allogeneic Bone Marrow Transplantation Improves Immune Reconstitution in Mice[△]

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Key words: immune reconstitution; α -galactosylceramide; bone marrow transplantation

Objective To explore the effect of α -galactosylceramide (α -GalCer) on immune reconstitution under acute graft-versus-host disease (aGVHD).

Methods BALB/c mice were transplanted with allogeneic C57BL/6 bone marrow cells and splenocytes (both 1×10^7) after receiving lethal total-body irradiation. α -GalCer (100 μ g/kg) or vehicle (dimethylsulfoxide) was administered intraperitoneally immediately after transplantation. The effects of α -GalCer on immune reconstitution, proliferation of T cells and B cells, hematopoiesis, and thymic microenvironment were assessed.

Results The α -GalCer group exhibited higher percentages of CD3⁺, CD4⁺, CD8⁺, B220⁺, CD40⁺, and CD86⁺ cells compared with the vehicle group. The number of colony forming unit per 1000 CD34⁺ cells in the α -GalCer group was higher than in the vehicle group ($P=0.0012$). *In vitro* proliferation assays showed that the α -GalCer group had higher percentages of CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells compared with the vehicle group. As for the results of *in vivo* proliferation assays, the numbers of CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells were higher in the α -GalCer group than in the normal group, especially the number of B220⁺ cells ($P=0.007$). Significant difference was not found in thymocyte count between the α -GalCer group and the vehicle group, nor in the percentages of CD3⁺, CD4⁺, and CD8⁺ cells.

Conclusion Administration of α -GalCer after allogeneic bone marrow transplantation may promote immune reconstitution in the presence of aGVHD.

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A LLOGENEIC hematopoietic stem cell transplantation (HSCT) is a therapy for some hematologic disorders. However, a series of severe complications of HSCT, such as infection, graft rejection, disease recurrence, and graft-versus-host disease (GVHD), are major life-threatening risks for patients after transplantation. These complications of HSCT have been found closely correlated with immune reconstitution.¹ Several approaches have been reported as capable to improve immune reconstitution by promoting mature T cell expansion or lymphopoiesis, such as subcutaneous injection of interleukin (IL)-7, IL-2, keratinocyte growth factor (KGF), and growth hormone (GH), and donor lymphocyte infusion.²⁻⁶ However, these approaches may worsen acute GVHD (aGVHD) while enhancing immune reconstitution. It is therefore necessary to discover novel strategies that can specifically promote immune reconstitution without aggravating aGVHD.

α -galactosylceramide (α -GalCer), an activator of natural killer T (NKT) cells, has been found to stimulate host NKT cells and ameliorate aGVHD.⁷⁻⁹ In addition, NKT cells activation by α -GalCer promotes the trans-activation of bystander cells, including T cells, B cells, dendritic cells, and natural killer cells,¹⁰⁻¹³ as well as regulates hematopoiesis.¹⁴ Therefore, we investigated whether α -GalCer can enhance immune reconstitution with a mouse model of HSCT.

MATERIALS AND METHODS

Mice

Male C57BL/6 (H-2K^b) mice and female BALB/c (H-2K^d) mice aged 8 to 10 weeks, weighing 18-22 g, were purchased from the Institute of Experimental Animals (Chinese Academy of Military Medical Sciences) and maintained in a specific pathogen-free environment. Sterilized food and hyperchlorinated drinking water were provided. The maintenance and treatment of the mice were in full compliance with the regulations for the protection of animal rights. The study protocol was approved by institutional Animal Use and Care Committee.

Bone marrow transplantation

BALB/c mice were conditioned with lethal total-body irradiation of 7.5 Gy ⁶⁰Co, and inoculated intravenously within 4-6 hours with 1×10^7 bone marrow cells plus 1×10^7 splenocytes derived from C57BL/6 mice. The recipient mice ($n=50$) were injected intraperitoneally with α -GalCer (100 μ g/kg) immediately after HSCT, whereas mice in the vehicle group ($n=50$) received the diluent [dimethylsulfoxide

(DMSO)] only.¹³ To assess whether α -GalCer promoted the differentiation of hematopoietic stem cells (HSCs), we performed white blood cell (WBC) counts with a hematology analyzer (Sysmex XT-2000iv, Sysmex Corporation, Kobe, Japan) on day 3, 8, and 15 after transplantation.

Antibodies and flow cytometry analysis

In murine HSCT models, the numbers of CD3⁺, CD4⁺, CD8⁺, B220⁺, CD40⁺, CD86⁺, CD11c⁺, and CD80⁺ cells of splenocytes were compared between the α -GalCer group and the vehicle group on day 2, 7, 14, 28, and 70 after HSCT. The number of thymocytes was also compared between the α -GalCer group and the vehicle group on day 14, 28, and 70 post HSCT, and the numbers of CD3⁺, CD4⁺, and CD8⁺ of thymocytes were compared between age-matched normal group and the α -GalCer group on day 70 post HSCT. Antibodies used were PE-conjugated anti-mouse H-2K^b, CD40 and CD86, FITC-conjugated anti-mouse B220, CD4 and CD80, PE-CY5-conjugated anti-mouse CD3 and CD8, isotype control and CD16/32 (BD Pharmingen, San Diego, CA, USA), and PE-CY5-conjugated anti-mouse CD11c (eBioscience, San Diego, CA, USA) antibodies. Splenocytes were preincubated with unlabeled CD16/32 antibodies before incubation with the relevant monoclonal antibodies. Finally, the cells were analyzed using a Beckman Coulter Epics XL flow cytometer (Brea, CA, USA) and EXPO32ADC software (version 12, Beckman Coulter).

Human G-CSF mobilized peripheral blood stem cell colony forming unit (CFU) assays

Since T cell and B cell progenitors originate from HSCs, we investigated whether the accelerated immune reconstitution of T cells and B cells correlated with the effects of α -GalCer on HSCs. For cytokine-replete CFU assays, G-CSF mobilized peripheral blood mononucleocytes separated by the COBE Spectra Apheresis System (CaridianBCT Corporation, Lakewood, CO, USA) were plated onto Human GM-Colony Methylcellulose medium (Beijing Anapure Bioscientific, Beijing, China), supplemented with 100 ng/mL α -GalCer or vehicle (DMSO). All the cultures were performed in duplicates, and the numbers of CFU were scored after 10 days.

Proliferation assays

In the early phase following transplantation, expansion of mature donor T cells is a primary source of immune reconstitution. *In vitro* proliferation assays used normal C57BL/6 splenocytes (1×10^6) cultured in complete RPMI1640 medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), supplemented with

100 ng/mL α -GalCer or vehicle (DMSO). After 72 hours, the cells were collected and counted using a hemocytometer (Sysmex XT-2000iv, Sysmex Corporation) and the percentages of CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells were detected using the Beckman Coulter flow cytometer.

In vivo proliferation assays were performed with C57BL/6 mice ($n=5$) administered intraperitoneally with 100 μ g/kg α -GalCer. The mice that did not receive α -GalCer administration served as the normal group ($n=5$). The mice were sacrificed 72 hours later and their spleens harvested. Using fluorescent flow cytometry, the percentages of CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells, and the mean fluorescence intensity of CD86 and CD40 on B220⁺ cells were analyzed and compared between α -GalCer group and normal group.

Statistical analysis

SPSS version 13.0 was used for statistical processing. The data were expressed as means $\pm$ SD, and the standard two-tailed, unpaired Student's *t*-test was performed to compare donor chimerism, CFU number, WBC counts, T cell number and B cell number in *in vivo* proliferation assays, and thymocyte counts between two groups. $P<0.05$

was defined as statistically significant.

RESULTS

α -GalCer accelerated immune reconstitution of T cells and B cells.

The α -GalCer group exhibited higher percentages of CD3⁺, CD4⁺, CD8⁺, B220⁺, CD40⁺, and CD86⁺ cells, but not CD11c⁺ and CD80⁺ cells, than those in the vehicle group at same time point. The difference was most significant on day 70. The mice from the α -GalCer group exhibited expansion of donor B220⁺ cells (29.89%), CD3⁺ cells (61.46%), CD4⁺ cells (50.43%), and CD8⁺ cells (19.32%) on day 70 after HSCT, while mice from the vehicle group had a lower engraftment of donor B220⁺ cells (5.37%), CD3⁺ cells (25.98%), CD4⁺ cells (13%), and CD8⁺ cells (5.67%) (Fig. 1A). The expression of costimulatory molecule CD86 in the α -GalCer group and the vehicle group was displayed on 30.67% and 9.36% cells, respectively; while CD40 expression was detected on 30.02% and 1.15% cells, respectively (Fig. 1B). These data indicated that α -GalCer accelerated immune reconstitution of T cells and B cells after transplantation.

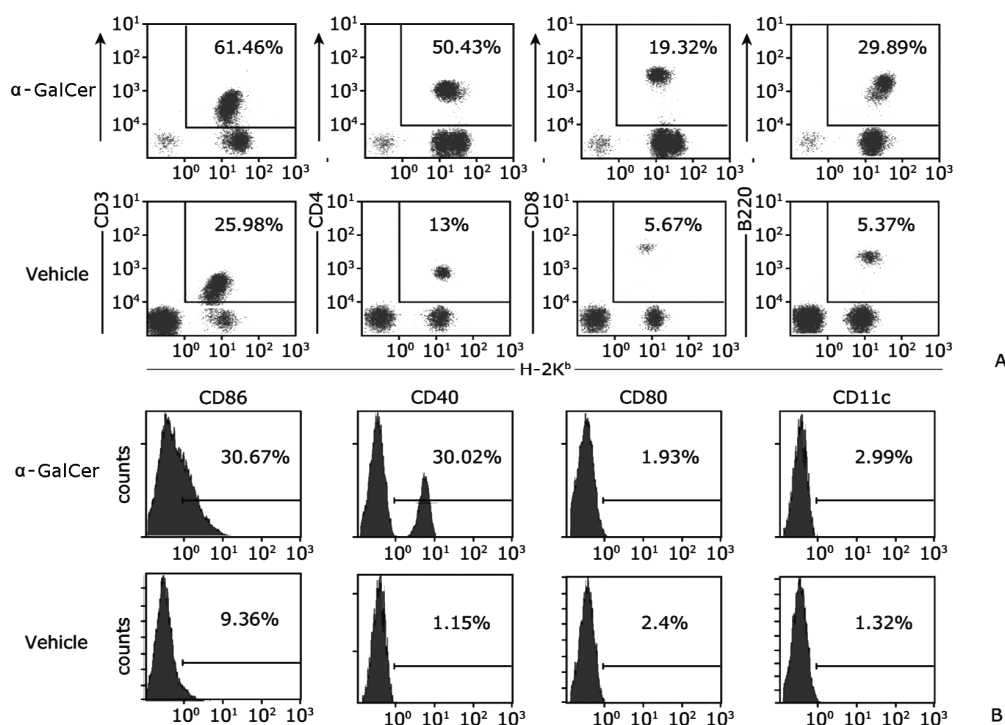


Figure 1. α -GalCer accelerated immune reconstitution of T cells and B cells.

- Splenocytes of BALB/c mice in the α -GalCer group and the vehicle (dimethylsulfoxide) group were stained with anti-H-2K^b and anti-CD3, anti-CD4, anti-CD8, anti-B220 monoclonal antibodies (mAbs) on day 70 after allogeneic hematopoietic stem cell transplantation (HSCT). The α -GalCer group exhibited higher percentages of CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells compared with the vehicle group.
- Splenocytes of mice in the α -GalCer group and the vehicle group were stained with mAbs of CD86, CD40, CD80, and CD11c on day 70 after HSCT. The α -GalCer group had higher percentages of CD40⁺ and CD86⁺ cells compared with the vehicle group.

α -GalCer promoted engraftment, proliferation, and differentiation of HSCs

After HSCT, splenocytes were stained with anti-H-2K^b monoclonal antibody to determine donor chimerism. On day 7, donor chimerism of the α -GalCer group was higher than that of the vehicle group ($43.3\% \pm 6.5\%$ vs. $17.6\% \pm 1.4\%$, $P < 0.001$). The two groups showed equal donor chimerism on day 14 ($P > 0.05$). Both groups had almost complete donor chimerism ($>95\%$) on day 28, yet on day 70, the donor chimerism of the vehicle group decreased to approximately 55%, whereas maintained at $>95\%$ in the α -GalCer group ($P < 0.001$) (Fig. 2A). These findings suggested that α -GalCer might not only promote short-term engraftment but also maintain long-term engraftment.

As shown in human G-CSF mobilized peripheral blood stem cell CFU assay, the number of CFU per 1000 CD34⁺ cells in the α -GalCer group exceeded that in the vehicle group (57.5 ± 9.8 vs. 19 ± 9.0 , $P = 0.0012$, Fig. 2B). In addition, the volume of CFU in the α -GalCer group was larger (Fig. 2C), which indicated that CFU of the α -GalCer group consisted of more cells than that of the vehicle group. Based on those observations, it could be inferred that α -GalCer may promote HSC proliferation.

The WBC count in the α -GalCer group was significantly higher than that in the vehicle group on day 3

($P = 0.002$, Fig. 2D). The WBC counts of both groups were similar on day 8 ($P = 0.6$), and rose to the normal level on day 15. The results suggested that α -GalCer promoted rapid HSC differentiation.

α -GalCer stimulated proliferation of T cells and B cells

The effect of α -GalCer on the proliferation of T cells and B cells was assessed with *in vitro* proliferation assays. There was no significant difference in the number of cells between the two groups. However, compared with the vehicle group, the α -GalCer group exhibited higher percentages of CD3⁺ (25.5% vs. 20.2%), CD4⁺ (13.0% vs. 5.8%), CD8⁺ (9.8% vs. 4.2%), and B220⁺ cells (47.1% vs. 32.2%) (Fig. 3A).

In vivo proliferation assays showed that the spleens of the α -GalCer mice appeared bigger and plumper compared with those of the normal mice. The numbers of splenocytes, CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells were higher in the α -GalCer group than in normal mice, especially the amount of splenocytes ($P = 0.003$) and B220⁺ cells ($P = 0.007$) (Fig. 3B). The expression of CD86 and CD40 on B220⁺ cells was also higher in the α -GalCer group, especially that of CD86 ($P < 0.0001$, Fig. 3B). These data indicated that α -GalCer promoted proliferation of T cells and B cells both *in vitro* and *in vivo*.

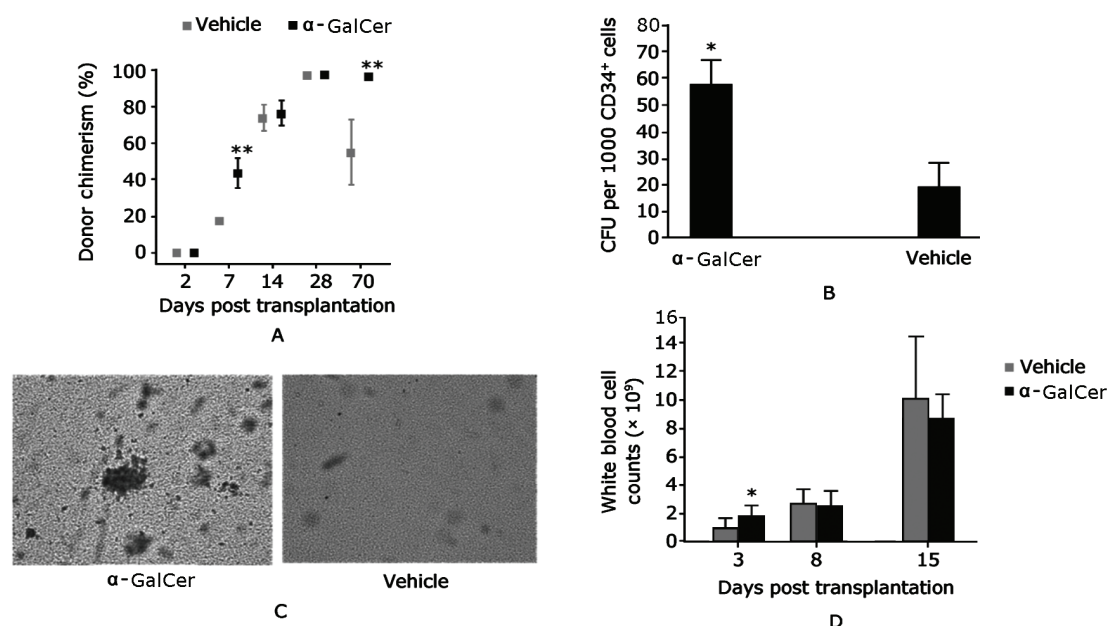


Figure 2. α -GalCer promoted hematopoietic stem cell engraftment, proliferation, and differentiation.

- Donor chimerism on day 2, 7, 14, 28, and 70 after transplantation. The donor chimerism of α -GalCer group was higher than that of vehicle group on day 7 and day 70 after HSCT.
- Human G-CSF mobilized peripheral blood stem cell colony forming unit (CFU) assays. The number of CFU per 1000 CD34⁺ cells in the α -GalCer group was higher than that in the vehicle group.
- CFU morphology. $\times 200$
- White blood cell (WBC) counts on day 3, 8, and 15 after transplantation. The WBC count in the α -GalCer group was significantly higher than that in the vehicle group on day 3.

* $P < 0.005$, ** $P < 0.001$ compared with the vehicle group.

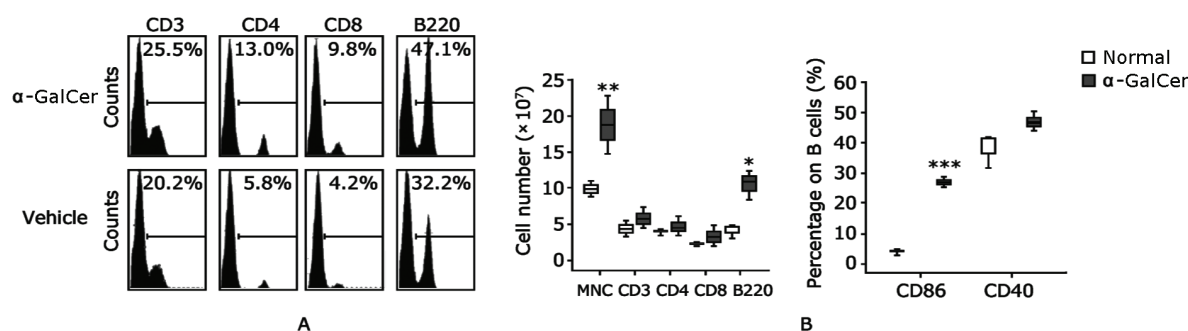


Figure 3. α-GalCer promoted proliferation of T cells and B cells.

- A. *In vitro* proliferation assay results. Splenocytes of the α-GalCer group and the vehicle group were stained with anti-CD3, anti-CD4, anti-CD8, and anti-B220 mAbs. The α-GalCer group had higher percentages of CD3⁺, CD4⁺, CD8⁺, and B220⁺ cells compared to the vehicle group.
- B. According to the result of *in vivo* proliferation assay. The numbers of splenocytes, CD3⁺, CD4⁺, CD8⁺, B220⁺, CD86⁺ and CD40⁺ cells were higher in the α-GalCer group than in normal mice. MNC stands for monocytes in spleen.

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$ compared with the normal group.

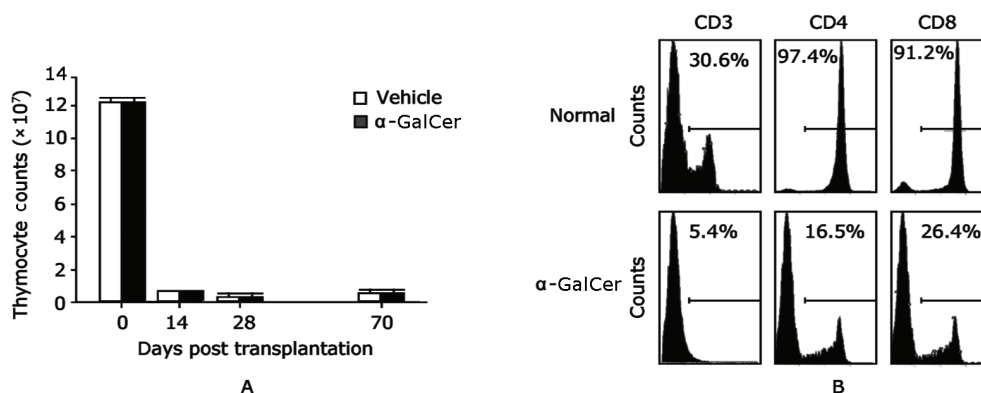


Figure 4. α-GalCer may not improve thymic microenvironment milieu.

- A. Thymocyte counts on day 14, 28, and 70 after transplantation show no significant inter-group difference.
- B. Thymocytes of age-matched normal group and the α-GalCer group were stained with anti-CD3, anti-CD4, and anti-CD8 mAbs on day 70 after transplantation. The results demonstrate significantly lower percentages of CD3⁺, CD4⁺, and CD8⁺ cells in the α-GalCer group compared with the normal group ($P < 0.0001$).

Thymus-independency of enhancement of immune reconstitution by α-GalCer

We investigated whether the accelerated immune reconstitution by α-GalCer correlated with an improved thymic microenvironment. As shown in Figure 4A, the thymus of mice from the α-GalCer group or the vehicle group declined from day 14 to 28, and had not fully recovered by day 70 after HSCT. There were no significant inter-group differences in the thymocyte count on day 14, 28, and 70 ($P > 0.5$). Compared with age-matched normal mice, mice treated with α-GalCer had significantly lower numbers of CD3⁺, CD4⁺, and CD8⁺ cells on day 70 after HSCT ($P < 0.0001$, Fig. 4B). It might indicate that enhancement of immune reconstitution by α-GalCer could be thymus-independent.

DISCUSSION

To date, α-GalCer has been used mainly for reducing aGVHD in transplantation. There is no systematic investigation yet on the effects of α-GalCer on immune reconstitution. Morecki et al⁷ have previously reported that α-GalCer promoted engraftment in a murine haploidentical myeloablative HSCT model, whereas Kuwatani et al¹⁵ reported that α-GalCer delayed engraftment in an allogeneic non-myeloablative HSCT model. We demonstrated in the present study that in an allogeneic myeloablative HSCT mouse model, α-GalCer accelerates immune reconstitution of CD3⁺, CD4⁺, CD8⁺ T cells and B220⁺ B cells by increasing their quantity and the expression levels of CD86 and CD40.

Immune reconstitution following transplantation in-

volves the expansion of both T-cell and B-cell compartments. The early phase of T cell reconstitution entails the expansion of mature donor T cells in response to cognate antigen and the "homeostatic" peripheral expansion induced by stimulation of low-affinity self-antigens. The second phase of T cell reconstitution is the maturation of lymphoid progenitors *via* T cell lymphopoiesis in a thymic-dependent and thymic-independent manner. On the other hand, B cell immune reconstitution includes the maturation of lymphoid progenitors *via* B cell lymphopoiesis into mature B cells in a T-cell-dependent and T-cell-independent manner in the bone marrow.¹⁶

Considering the course of immune reconstitution, we first investigated whether α -GalCer could accelerate immune reconstitution by regulating HSCs. It is well known that short-term engraftment is initiated by differentiating progenitors, while durable, long-term, multilineage engraftment relies on stem cells' homing to their specialized niches.¹⁷ We observed that the donor engraftment rate in early (day 7) and later (day 70) stages of transplantation was higher and hematopoietic reconstitution was faster in the α -GalCer group than in the vehicle group, and thus hypothesized that α -GalCer may promote (i) HSCs engraftment, proliferation, and differentiation; (ii) proliferation of mature T cells and B cells. It has been reported that NKT cells exert positive effects on proliferation and differentiation of HSCs by secreting cytokines granulocyte-macrophage colony-stimulating factor and IL-3.¹⁴ Thus we performed G-CSF mobilized peripheral blood stem cell CFU assays. As expected, α -GalCer promoted proliferation and differentiation of HSCs. α -GalCer also enhanced the proliferation of T cells and B cells, and the expression of CD86 and CD40 on B cells as well, which is in agreement with earlier reports that NKT cell activation trans-activates T cells and B cells.^{8,9} IL-7 and IL-2 display similar promoting effects on immune reconstitution as α -GalCer does.^{2,3}

Thymus is an important site for T cells expansion, selection, and maturation. Some substances that are used to enhance immune reconstitution, such as IL-7, KGF, and GH, have been reported to increase proliferation of early thymic progenitors or improve the thymic microenvironment milieu.²⁻⁵ We investigated whether the acceleration of immune reconstitution by α -GalCer correlated with improvement of thymus. The results suggested thymus-independency, but further studies are necessary to provide more solid evidence for this assumption.

α -GalCer therapy has already been used in clinical trials for the treatment of cancer patients and was well tolerated.¹⁸ According to the findings of this study,

α -GalCer also offers the possibility of a novel strategy for specifically enhancing immune reconstitution in HSCT under the condition of aGVHD. It may be beneficial to use α -GalCer therapeutically to separate graft-versus-leukemia and aGVHD.

REFERENCES

1. Cavazzana-Calvo M, André-Schmutz I, Dal Cortivo L, et al. Immune reconstitution after haematopoietic stem cell transplantation: obstacles and anticipated progress. *Curr Opin Immunol* 2009; 21:544-8.
2. Sportès C, Hakim FT, Memon SA, et al. Administration of rhIL-7 in humans increases *in vivo* TCR repertoire diversity by preferential expansion of naive T cell subsets. *J Exp Med* 2008; 205:1701-14.
3. Mackall CL. Enhancing immune reconstitution after stem cell transplants with cytokines. *Cytotherapy* 2002; 4: 427-8.
4. Seggewiss R, Loré K, Guenaga FJ, et al. Keratinocyte growth factor augments immune reconstitution after autologous hematopoietic progenitor cell transplantation in rhesus macaques. *Blood* 2007; 110:441-9.
5. Chen BJ, Cui X, Sempowski GD, et al. Growth hormone accelerates immune recovery following allogeneic T-cell-depleted bone marrow transplantation in mice. *Exp Hematol* 2003; 31:953-8.
6. Amrolia PJ, Mucioli-Casadei G, Huls H, et al. Add-back of allodepleted donor T cells to improve immune reconstitution after haplo-identical stem cell transplantation. *Cytotherapy* 2005; 7:116-25.
7. Morecki S, Paniqrahi S, Pizov G, et al. Effect of KRN7000 on induced graft-vs-host disease. *Exp Hematol* 2004; 32: 630-7.
8. Hashimoto D, Asakura S, Miyake S, et al. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. *J Immunol* 2005; 174:551-6.
9. Harauchi K, Takahashi T, Matsumoto A, et al. Host-residual invariant NKT cells attenuate graft-versus-host immunity. *J Immunol* 2005; 175:1320-8.
10. Hong C, Lee H, Park YK, et al. Regulation of secondary antigen-specific CD8(+) T-cell responses by natural killer T cells. *Cancer Res* 2009; 69:4301-8.
11. Tonti E, Galli G, Malzone C, et al. NKT-cell help to B lymphocytes can occur independently of cognate interaction. *Blood* 2009; 113:370-6.
12. Kuns RD, Morris ES, Macdonald KP, et al. Invariant natural killer T cell-natural killer cell interactions dictate transplantation outcome after alpha-galactosylceramide ad-

- ministration. *Blood* 2009; 113:5999-6010.
13. Kojo S, Seino K, Harada M, et al. Induction of regulatory properties in dendritic cells by Valpha14 NKT cells. *J Immunol* 2005; 175:3648-55.
 14. Kotsianidis I, Silk JD, Spanoudakis E, et al. Regulation of hematopoiesis *in vitro* and *in vivo* by invariant NKT cells. *Blood* 2006; 107:3138-44.
 15. Kuwatani M, Ikarashi Y, Iizuka A, et al. Modulation of acute graft-versus-host disease and chimerism after adoptive transfer of *in vitro*-expanded invariant Valpha14 natural killer T cells. *Immunol Lett* 2006; 106:82-90.
 16. Lacerda JF. Immunologic reconstitution after allogeneic stem cell transplant. *Acta Med Port* 2004; 17:471-80.
 17. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? *Blood* 2005; 106:1901-10.
 18. Kunii N, Horiguchi S, Motohashi S, et al. Combination therapy of *in vitro*-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* 2009; 100:1092-8.