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Sensitive *in vivo* Assay System for Human Stem Cells Using NOD/SCID/γ_c^{null} mice

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Recent advances in stem cell research present us promising possibilities for regenerative medicine. However, investigation on human stem cells is behind from those of murine mostly due to the lack of appropriate analyzing systems. Our NOD/SCID/γ_c^{null} (NOG) mice model realized complete reconstitution of lympho-hematopoietic system as well as liver regeneration by human hematopoietic stem cell transplantation. This model will provide a versatile tool to investigate not only human hematopoietic system but also other human stem cell systems along with stem cell plasticity.

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Introduction

Stem cells are characterized by their capacity for self-renewal and multi-lineage differentiation. Although various in vitro assays have been exploited, experimental transplantation is the most reliable method for the analysis of stem cell activity.[1] With regard to human stem cells, immuno-compromised mice, such as nude mice or severe combined immuno-deficient (SCID) mice have been used as surrogate *in vivo* assay with limited successes. Recently, non-obese diabetes/severe combined immuno-deficient (NOD/SCID) mice are widely used as recipients of human hematopoietic stem cells because they can sustain human hematopoietic cell with relatively high chimerism.[2] However, the murine environment is not adequate enough for human stem cell activities and engraftability of human cells in these mice is usually not satisfactory. To develop more sensitive mice model, we introduced common gamma chain null (γ_c^{null}) phenotype into NOD/SCID mice (NOD/SCID/γ_c^{null}) and evaluate their efficacy as recipient of human cells. We observed excellent engraftability of human hematopoietic stem cell in our model, which prompted us to explore the possibility whether this model can support other human stem cell system.

Materials and Methods

Mice

Mice used in this study were NOD/Shi-*scid* (NOD/SCID) mice and newly developed NOD/SCID/γ_c^{null} (NOG) mice, both of which were developed at the Central Institute for Experimental Animals.[3,4] All mice were maintained in vinyl isolators under specific pathogen free conditions. All materials including bedding, food and tap water were autoclaved at 128 ºC for 30 min in a laminar firm-capped container and moved to the vinyl isolators in a sterile manner.

Transplantation

Human cord blood (CB) was collected during normal full-term deliveries after obtaining informed consent. Mononuclear cells (MNCs) were separated by Ficoll-Hypaque® density gradient centrifugation after depletion of phagocytes with Silica® (Immuno Biological Laboratories, Fujioka, Japan). $CD34^+$ cell fraction was isolated using AutoMACS® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of selected cells was typically about 98%. Xeno-transplantation of purified CB $CD34^+$ cells was done using a modification of a previously reported method.[5,6] Briefly, 8- to 12- week-old mice were irradiated with 240 cGy. The indicated dose of CB $CD34^+$ cells was injected through

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the tail vein. NOD/SCID mice were treated with 400 µL of PBS containing 20 µL of anti-asialo GM1 antiserum (Wako, Osaka, Japan) shortly before cell transplantation and every 11th day thereafter. NOG mice were given no treatment. Mice were prophylactically provided sterile acidified water with neomycin sulfate (Gibco BRL, Grand Island, NY) and kept under specific pathogen free conditions.

Liver injury

Some mice were subjected to liver damage. For the induction of acute liver damage, mice were given sublethal 0.4 mg/kg of anti-Fas antibody (BD, Franklin Lakes, NJ) intraperitoneally. For chronic liver damage, they were treated with 2 mL/kg of carbon tetrachloride (CCl_4) intraperitoneally 4 times a week.

Flow cytometric analysis of mice with human cell transplants

Peripheral blood was collected through heparinized calibrated pipettes and transferred to EDTA-2Na containing tube. At indicated time, the mice were killed by cervical dislocation. Bone marrow, spleen, and thymus were taken for analysis. Lineage analysis of human hematopoietic cells in various tissues was carried out using FACS Calibur® (BD) according to the manufacturer's protocol. Antibodies used for flow cytometric analysis were anti-human CD45-FITC, CD3-FITC, CD34-PE, CD10-PE, CD3-PE, TCR $\gamma\delta$-PE, anti-mouse CD45-allo-phycocyanin (APC) (all from BD) and anti-human CD19-PC5, TCR $\alpha\beta$-PC5 (Beckman Coulter, Fullerton, CA)

Histological examination

Mice were killed by cervical dislocation. Livers from mice were taken out, fixed in 10% formalin and embedded in paraffin blocks. After removal of paraffin, slides were rehydrated, washed and stained with primary antibodies diluted with 1% bovine serum albumin/PBS (-). After overnight staining of primary antibodies, anti-mouse or anti-rabbit antibodies were used as secondary antibodies. Hoechst 33324 (Molecular Probes, Eugene, OR) was used for nuclear staining.

Human serum albumin assay

Peripheral blood was collected and subjected to ELISA assay for determining human albumin levels using Human ELISA Quantification Kit (BETHYL laboratories, Montgomery, TX) according to the manufacturer's protocol.

Results

NOG mice are able to support multi-lineage differentiation including T cells with very high chimerism

After transplantation of 1×10^5 CB $CD34^+$ cells into NOD/SCID and NOG mice, the engraftment of human hematopoietic cells was monitored by analyzing human $CD45^+$ cells in peripheral blood (PB) of each mouse. At any time point, PB of NOG mice had far more human cells than that of NOD/SCID mice (Figure 1 A, B). At 5 months after transplantation, as much as 80% cells in bone marrow

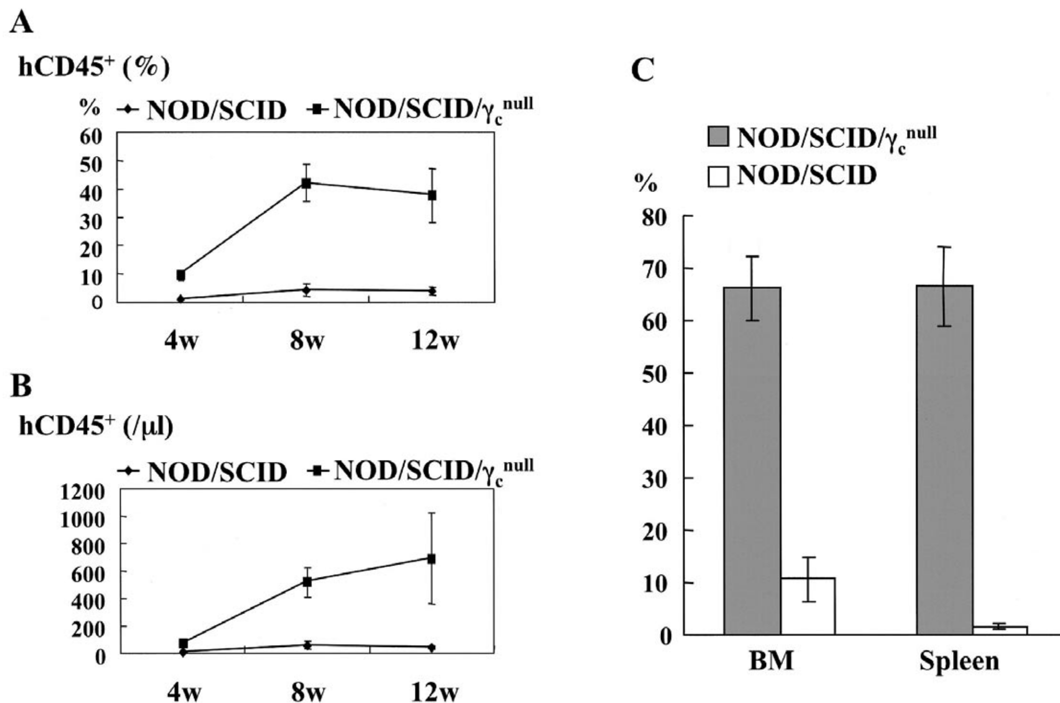

Figure 1. Human $CD45^+$ cells in NOD/SCID and NOG mice. Percentage and absolute number of human $CD45^+$ cells in PB of mice were monitored every 4 week after transplantation (A, B). Five months after transplantation, mice were sacrificed and human $CD45^+$ cells in BM and spleen were evaluated (C).

(BM) and spleen were of human origin (Figure 1 C). The lineage analysis of human hematopoietic cells engrafted in NOG mice showed that BM was populated with granulocytes, B cells, megakaryocytes, erythroblast (Figure 2) while spleen was mainly populated with mature B and T cells (Figure 3). The development of T cells was exclusively seen in NOG mice. These T cells had similar surface markers seen in human PB, that is, CD4 or CD8 single positive T cells and CD45 RA$^+$ naïve or CD45RO$^+$ memory T cells (Figure 4). Of interest, there were considerable amount of CD4 and CD8 double positive T cells in murine thymus and CD45RA$^+$ naïve T cells were mostly found in thymus. Moreover, human T cells appeared in thymus earlier than any other lymphoid organs (data not shown).

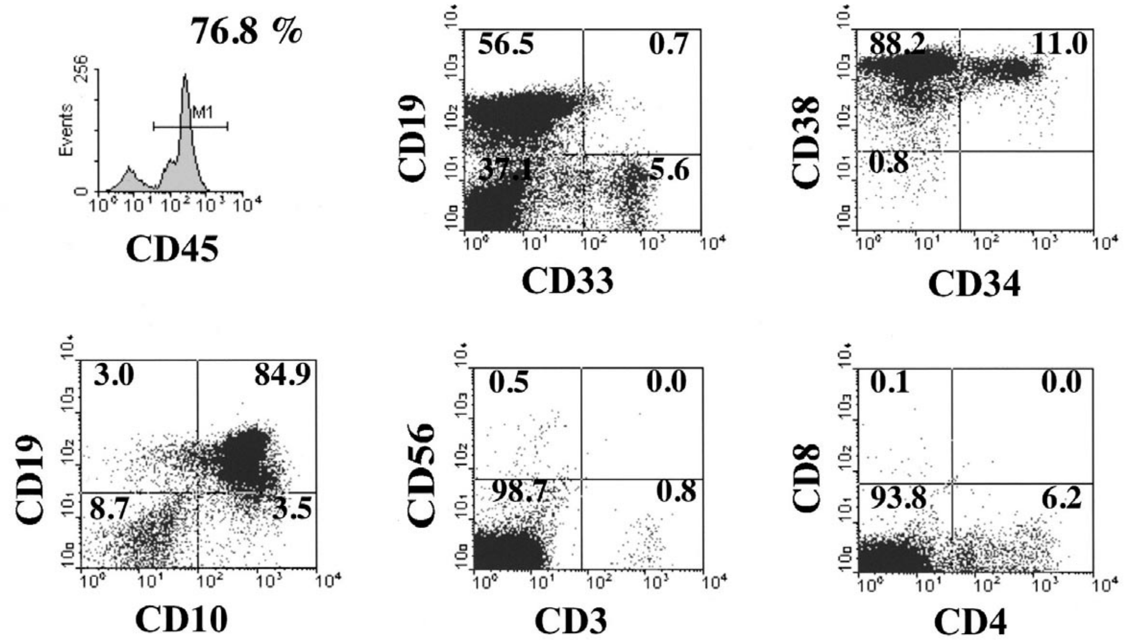

Figure 2. Flowcytometric analysis of BM of NOG mice transplanted with CD34$^+$ CB cells. Representative FACS analysis of BM of NOG mice with transplants is shown.

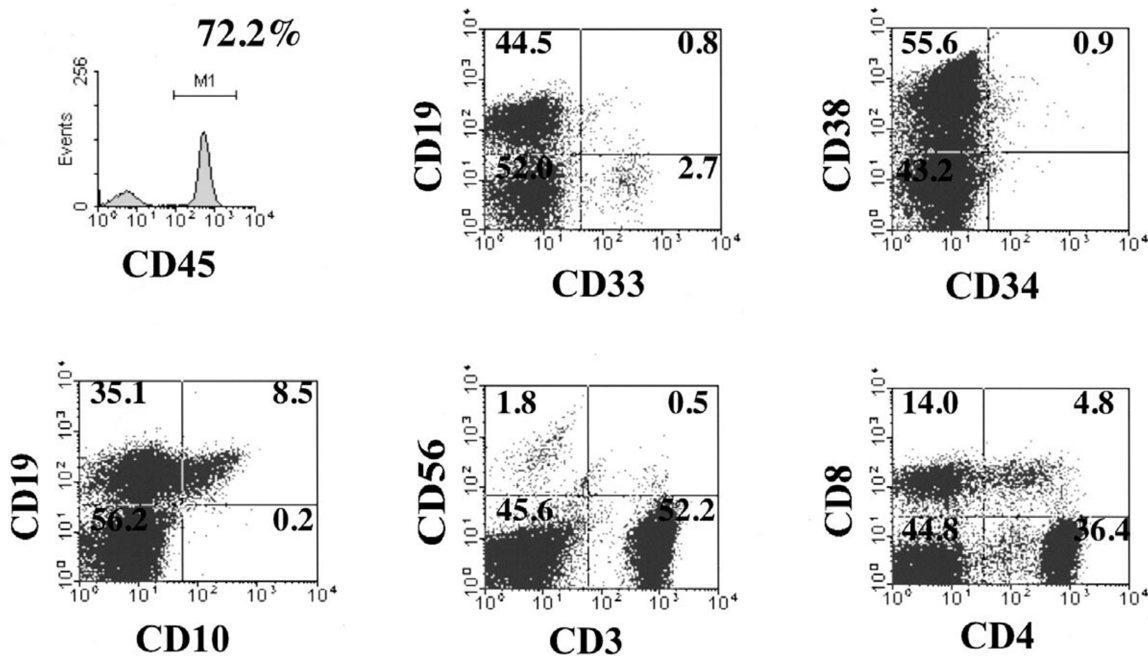

Figure 3. Flowcytometric analysis of spleen of NOG mice transplanted with CD34$^+$ CB cells. Representative FACS analysis of spleen of NOG mice with transplants is shown. Note the abundant engraftment of human T cells.

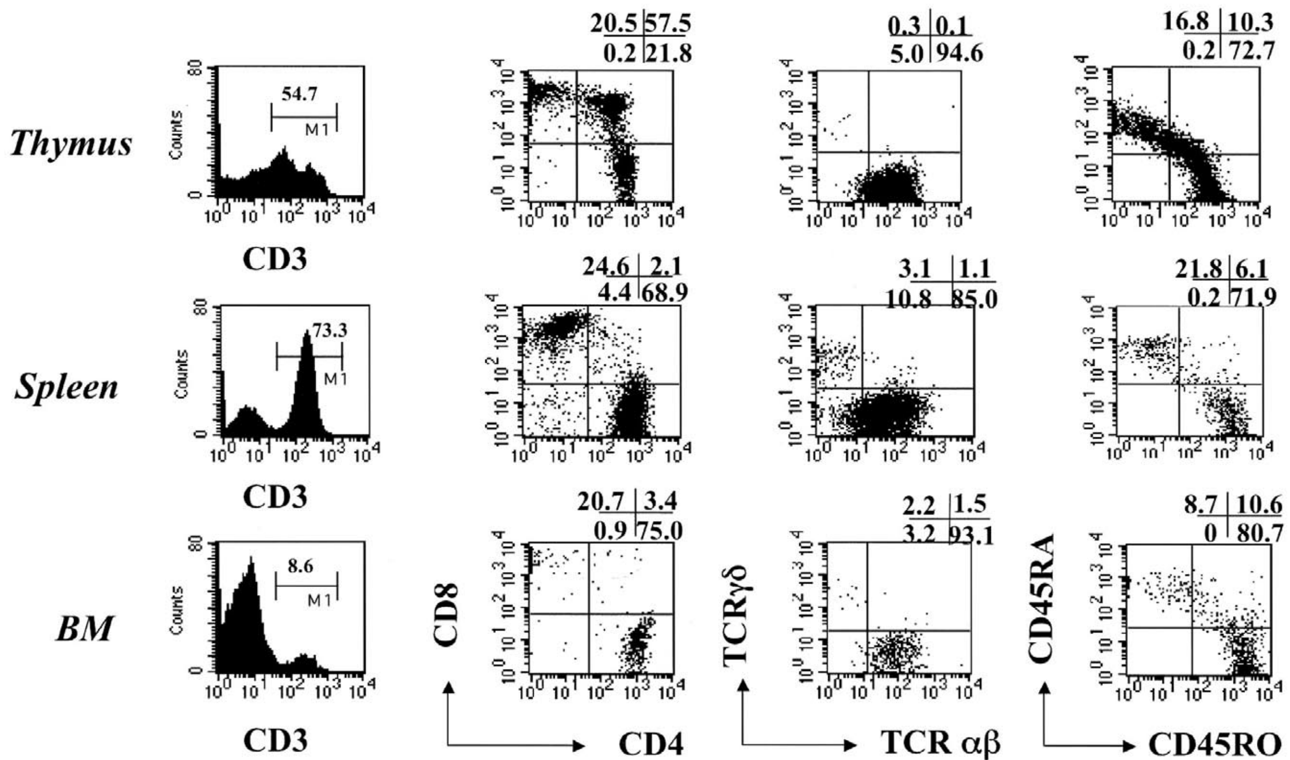

Figure 4. Human T cell analysis of various lymphoid organs in NOG mice. Representative FACS analysis of T cell specific markers in thymus, spleen and BM of NOG mice is shown.

These facts are highly suggestive of human T lymphopoiesis taking place in thymi of NOG mice.

NOG mice are very sensitive recipient model for human hematopoietic stem cell

To confirm the efficiency of human HSCs engraftment in NOG mice, limiting dose of CD34$^+$ cells were transplanted (Table 1). Three months after transplantation, human CD45$^+$ cells in BM was analyzed and mice with more than 1% of CD45$^+$ cells were defined as successful engraftment. Surprisingly, only 1000 CD34$^+$ cells resulted in successful engraftment in 5 of 5 NOG mice. Even as few as 100 CD34$^+$ cells could be engrafted in 2 of 6 mice. This fact illustrates that NOG mice is extremely sensitive for human hematopoietic activity.

Table 1. Efficient engraftment of human HSCs in NOG mice

CD34$^+$ cell dose	Ratio of successful engraftment
1000	5/5
200	2/3
100	2/6

Limiting doses of CB CD34$^+$ cells were transplanted into NOG mice. Three months after transplantation, BM was analyzed for engraftment. Identification of more than 1% of human CD45$^+$ cells was defined as successful engraftment.

NOG mice transplanted with CD34$^+$ cells develop human type hepatocytes

To explore the possibility to utilize NOG mice for the analysis of other stem cell system, we turned our attention to liver regeneration. According to the previous reports, we gave hepatic injury to NOG mice with human CD34$^+$ cell transplant and looked for human hepatocytes in the liver. Human hepatocyte specific antibody (HepPar1) positive cells were rich especially around portal veins (data not shown). Although human CD45$^+$ cells were also rich in NOG mice, their distribution in liver was totally different from those of HepPar1$^+$. Staining of anti human albumin showed basically the same pattern, that is, mostly rich around portal veins. Double staining of HepPar1 antibody and anti-human albumin revealed that considerable amount of the cells in the liver was double positive, suggesting HepPar1$^+$ cells' functional maturation. Furthermore, ELISA revealed that PB of the most of the NOG mice with CB CD34$^+$ cells was positive for human albumin (data not shown), suggesting that these human hepatocytes were fully mature.

Discussion

Compared to the tremendous progress in understanding murine stem cell systems, human stem cell research has been lagging mainly due to the lack of appropriate assay system or ethical issues. Introduction

of immunodeficient mice such as NOD/SCID mice in particular, has enabled us to study human hematopoietic system *in vivo*. However, the engraftment level achieved in these models is generally low and not all hematopoietic cell lineages are observed. Therefore more sensitive model has been sought to study human hematopoiesis in detail. We have already reported that anti-asialo GM1 antibody treatment enhanced human hematopoietic stem cell engraftment in conventional NOD/SCID mice model.[4] Consequently, we assumed that complete eradication of murine NK cells would lead to further improvement and decided to introduce γ_c^{null} phenotype into NOD/SCID mice. Indeed, NOD/SCID/γ_c^{null} (NOG) mice had not only all immune dysfunction seen in conventional NOD/SCID mice but also lacked NK cell activity completely. Transplantation of CB CD34$^+$ cells resulted in far better engraftment in NOG mice model. Moreover, T lymphopoiesis was also observed, which is another very unique character of this model. With these advantages, we examined the possibility of this novel model as the recipient for other human stem cell system. We first began with hepatic stem cell system because there had already been reports on liver regeneration by hematopoietic stem cells using murine transplantation model. Currently, the mechanism by which hematopoietic cells repair damaged hepatocytes is a hot topic of controversy which is intensively investigated.[7-13] It was a great surprise that NOG mice can support plenty of human hepatocyte development by CB CD34$^+$ cell transplantation only with simple liver injury. The functional maturation of these hepatocytes were demonstrated by human albumin production histologically as well as serum human albumin detection by ELISA. We are now investigating the mechanism by which these cells developed. Our NOG mice model will provide a unique *in vivo* assay system for the investigation of a variety of human stem cell systems, which will be a versatile tool for the preclinical evaluation of the forthcoming human cell therapy.

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