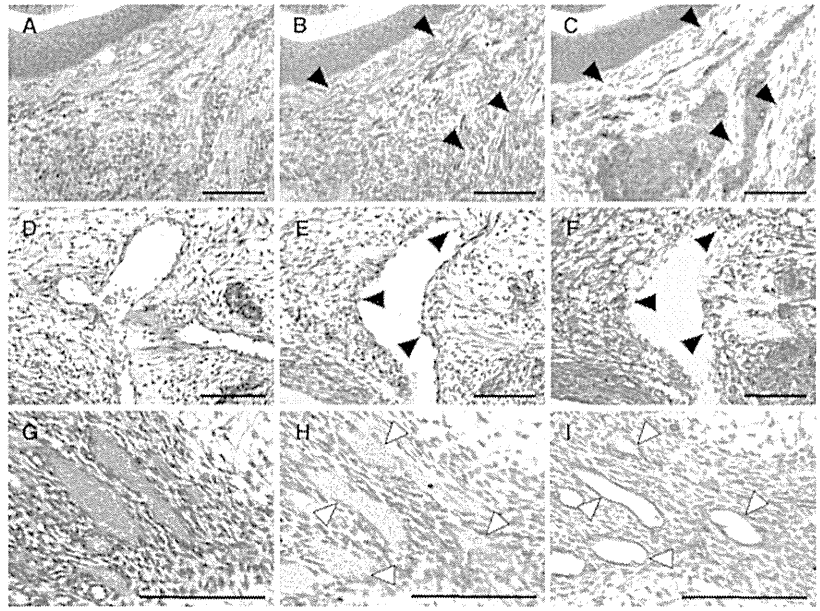

Fig. 3. The microenvironment in human iPS cell-derived teratomas. The human iPS cells were subcutaneously transplanted into PgkEGFP-NOG mice. Formed teratomas were recovered 8 weeks after transplantation. We stained the paraffin sections of the teratomas with HE stain (A, D, and G), and serial sections were counterstained with GFP (B, E, H), HLA (C), or human vimentin (F and I). The microenvironment is composed of EGFP-expressing fibroblasts (B and C; black arrowheads) or vascular cells (E and F; black arrowheads), and iPS cell derivatives, which were positively stained using human-specific markers. Vimentin-stained human vessels were not stained with EGFP (H and I; white arrowheads). *Bar=200 μm.

tively for HLA and vimentin (Figs. 3 C and F). On the other hand, vimentin-positive human vascular cells were not EGFP-positive (Figs. 3 H and I). Collectively, these results indicated that human iPS cell-derived teratomas contained components of host stromal tissues that composed the complicated microenvironment.

Discussion

In the present study, we established a PgkEGFP-NOG mouse that displayed systemic EGFP expression and equivalent immunodeficiency to the NOG mouse. Previously, we reported another EGFP-expressing NOG mouse line, which was established by the congenic method using a marker-assisted selection protocol (NOG-EGFP mouse) [18]. We summarized the systemic EGFP expression patterns of PgkEGFP-NOG and NOG-EGFP mice in Figs. S1, S2, and S3. Quantification of EGFP fluorescence revealed that the NOG-EGFP mouse line showed enhanced EGFP expression, especially in the heart, skeletal muscle, and pancreas. On the other hand, EGFP expression in the liver and ductal epithelium of PgkEGFP-NOG mice was stronger than that of NOG-EGFP mouse. One of the differences between PgkEGFP-NOG and NOG-EGFP mice was the transgene driver. In the NOG-EGFP mice, EGFP is expressed under the control of the cytomegalovirus early enhancer element and chicken beta-actin (CAG) promoter [12]. Moreover, transgene expression might be affected by the genomic environment. These factors reflect the differences in transgene expression between PgkEGFP-NOG and NOG-EGFP mice.

The use of PgkEGFP-NOG mice makes it easy to investigate formation of the microenvironment in the recipient liver. Transplantation of human cancer cells into the liver of PgkEGFP-NOG mice demonstrated that the tumor microenvironments in the xenograft tissues were composed of host fibroblasts and vessels (Fig. 2). In addition, the tumor microenvironment was not observed in the necrotic areas of the xenograft colonies. A previous study demonstrated that angiogenesis is induced from transplanted tumor cells, which is essential for

tumor growth [21]. Our results indicate that formation of a suitable microenvironment is essential for xenotransplanted tumor cell growth and maintenance.

Teratoma formation is regarded as a landmark of the pluripotency of human iPS cells. Since the teratoma contains three germ layer derivatives, it is difficult to distinguish between host tissues and iPS cell derivatives by morphological characterization. However, the origin of iPS cell derivatives can be easily identified in PgkEGFP-NOG mice (Fig. 3). Moreover, teratoma formation in the PgkEGFP-NOG mouse demonstrated that EGFP-expressing stromal tissue components were the primary components of the microenvironment in the teratomas (Fig. 3). Our results indicated that formation of a suitable microenvironment is induced simultaneously with teratoma formation.

Taken together, our established PgkEGFP-NOG mouse demonstrated severe immunodeficiency and systemic EGFP expression. Transplanted cell derivatives in the PgkEGFP-NOG mice were readily identified by the absence of EGFP expression, suggesting that the PgkEGFP-NOG mouse presents a useful tool to analyze the interactions between xenotransplanted cells and host stromal tissues.

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—Original—

NOD-*Rag2*^{null} *IL-2Rγ*^{null} Mice: An Alternative to NOG Mice for Generation of Humanized Mice

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Abstract: We have developed NOD-*Rag2*^{null} *IL-2Rγ*^{null} (NR2G) mice similar to NOD-*scid IL-2Rγ*^{null} (NOG) mice that are known as an excellent host to generate humanized mice. To evaluate the usefulness of NR2G mice as a host for humanized mice, the engraftment rates and differentiation of human cells after human hematopoietic stem cell (HSC) transplantation were compared among NR2G, NOG, and NOD-*scid* mice. For this purpose, the appropriate irradiation doses to expand the niche for human stem cells in the bone marrow were first determined. As a result, 8 and 2.5 Gy in adult, and 4 and 1 Gy in newborn NR2G and NOG mice, respectively, were found to be appropriate. Next, 5 × 10^4 human umbilical cord blood CD34$^+$ cells were intravenously inoculated into irradiated adult or newborn of the immunodeficient mice. These HSC transplantation experiments demonstrated that both NR2G and NOG mice showed high engraftment rates compared with NOD-*scid* mice, although NOG mice showed a slightly higher engraftment rate than that for NR2G mice. However, no difference was found in the human cell populations differentiated from HSCs between NR2G and NOG mice. The HSC transplantation experiments to adults and newborns of two immunodeficient mice also revealed that the HSC transplantation into newborn mice resulted in higher engraftment rate than those into adults. These results showed that NR2G mice could be used as an alternative host to NOG mice to generate humanized mice.

Keywords: humanized mice, immunodeficient mice, NOD-*Rag2*^{null} *IL-2Rγ*^{null} mice, NOG mice

Introduction

Immunodeficient mice harboring human cells or tissues are considered useful for analyzing human biology *in vivo* without the ethical constraints associated with using humans themselves. These mouse models, termed humanized mice, would significantly advance our understanding of various human diseases and would facilitate development of new drugs against human diseases [18].

Recent advances in the development of newly immunodeficient mice such as NOD-*scid IL-2Rγ*^{null} (NOG/NSG) and BALB/c-*Rag2*^{null} *IL-2Rγ*^{null} (BR2G) mice have enabled the generation of humanized mice in which various human immune cells are successfully developed, and have promoted research in human biology and dis-

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Abbreviations: RAG2/1: recombination activating gene 2/1, HSC: hematopoietic stem cell, RBC: red blood cell, WBC: white blood cell, SPF: specific pathogen free, PB: peripheral blood, CB: umbilical cord blood, BM: bone marrow, SPL: spleen

eases [6, 14, 16]. Indeed, those studies not only showed an extremely high engraftment efficacy but also the development of multi-lineage human cells, including various subsets of T cells after transplantation of human umbilical cord blood (CB)-derived $CD34^+$ stem cells (HSCs) [4].

To generate appropriate humanized mice, the choice of immunodeficient mouse strains, human stem cell source, inoculation route, and mouse age used for cell transplantation are considered to be important. For a stem cell source, Lepus *et al.* [8] recently compared the engraftment and differentiation of human cells from various sources using three types of immunodeficient mice—NSG, BR2G, and C.B-17-*scid/bg* mice—and concluded that the use of $CD34^+$ stem cells from fetal liver and CB is suitable for studying human hematopoietic cell lineage development and function in humanized mice. However, the use of cells from aborted fetuses is not always feasible because of ethical issues in some countries. In this sense, the use of stem cells from CB may be more convenient for collection to circumvent ethical issues. For an inoculation route, intravenous inoculation has been used commonly for adult mice; by contrast, intrahepatic or intravenous inoculation has been used for newborn mice [4, 15]. However, it remains unresolved which route generates humanized mice more efficiently because of the different conditions, such as cell sources and mouse strains, used by researchers.

Regarding mouse strains, NOG/NSG and BRG mice are presently generally used for the generation of humanized mice. Pearson *et al.* [12] recently reported radioresistant NOD-*Rag1*^{null} *IL-2Rγ*^{null} (NR1G) mice, as well as NSG mice, showing high engraftment of human cells. Brehm *et al.* [2] compared the engraftment of human cells among NSG, NR1G, and BALB/c-*Rag1*^{null} *IL-2Rγ*^{null} (BR1G) mice generated based on *Rag1*^{null} mice and concluded that NSG and NR1G mice showed more efficient engraftment than BR1G mice. Both *Prkdc* and *Rag1/2* genes are responsible to compose T and B cell receptor genes resulting in T and B cell deficiency in mice mutated with both genes. SCID mice mutated with *Prkdc* gene but not with RAG1/2 genes have disadvantages in which irradiation sensitivity and T/B cell leakiness occurred [1, 3]. Therefore, Introduction of RAG1/2 mutation for *Prkdc*^{scid} mutation into mice may provide more stable immunodeficient strain of mice for xenotransplantation. We also independently developed NOD-*Rag2*^{null} *IL-2Rγ*^{null} (NR2G) mice based on *Rag2*^{null} mice. In the present study, we evaluated the usefulness of NR2G mice as humanized mice by comparing human cell engraftment and differentiation among NOG, NR2G, and NOD-*scid* mice after HSC transplantation.

Regarding mouse age, when HSCs are inoculated, adult or newborn mice have been used. Particularly, HSC inoculation into newborn mice is considered to be efficient because Traggiai *et al.* [16] reported humanized mice by HSC inoculation into newborn BRG mice. However, it is unclear what differences exist in the engraftment and differentiation of human cells from HSCs upon inoculation into newborn and adult mice. To address this issue, we compared the engraftment and differentiation of human cells in the mice when the same lot of CB $CD34^+$ cells was transplanted into newborn and adult NOG, NR2G, and NOD-*scid* mice.

Materials and Methods

Mice

NOD.CB17-*Prkdc*^{scid}/ShiJic (NOD-*scid*), NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/Jic (NOD-*scid IL-2Rγ*^{null}: NOG), NOD.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug}/ShiJic (NOD-*Rag2*^{null} *IL-2Rγ*^{null}: NR2G, here termed to distinguish from NR1G of NOD-*Rag1*^{null} *IL-2Rγ*^{null} mice), and BALB/c-*Rag2*^{null} *IL-2Rγ*^{null} (BR2G) were used in the present study. NOD-*scid* mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). NOG and NR2G mice were maintained at the Central Institute for Experimental Animals (CIEA). NR2G mice, first described in the present study, were generated as follows. NOD.Cg-*Rag2*^{tm1Fwa}/Jic mice were generated by the six-generation backcross-mating of B6.129S1-*Rag2*^{tm1Fwa}/JJic mice [13], which were originally a gift from Dr. Alt F. of Columbia University, into NOD mice using a speed congenic technique combining a marker-assisted selection protocol and *in vitro* fertilization [15]. NR2G mice were obtained by intercross mating among the offspring (NOD-*Rag2*$^{+/-}$ *IL-2Rγ*$^{+/-}$) between NOD-*Rag2*^{null} and NOD-*IL-2Rγ*^{null} mice.

For HSC transplantation experiments, adult mice were obtained by natural mating, and newborn mice were obtained by Cesarean section from recipient IQI females transplanted with NR2G embryos into the oviduct after *in vitro* fertilization. The newborn mice were nursed by IQI foster mothers until weaning after irradiation and cell transplantation.

The present study was performed in accordance with institutional guidelines and was approved by the Animal Experimentation Committee of CIEA.

Irradiation

Adults and newborns of NOG, NR2G, and BR2G mice were irradiated with 0–12 Gy using an X-ray irradiation device (MBR-1505R; Hitachi Medical Co., Tokyo, Japan) to determine an appropriate dose for the respective mice. The body weight of NOG, NR2G, and BR2G mice was measured each week for 4 or 8 weeks after irradiation using a scale (Pocket Scale 80; BOMSO, Tokyo, Japan).

Transplantation of human HSCs

The same lot of commercially available human CB derived $CD34^+$ cells (Lonza, Basel, Switzerland) was used in the current study. The frozen cells were incubated for a few minutes in a 37°C water bath, and then were moved quickly into phosphate-buffered saline (PBS) containing 2% fetal bovine serum. After washing with PBS, the viability of $CD34^+$ cells was examined by dye exclusion using 2.5% Trypan blue solution, and the cells with more than 80% viability were used for transplantation into the mice. For HSC transplantation into adult mice at 8 to 9 weeks of age, 5×10^4 of $CD34^+$ cells were inoculated intravenously via the tail vein at 24 h after irradiation. Newborn mice were irradiated at a day after birth, and 5×10^4 $CD34^+$ cells were inoculated intravenously via the facial vein at 24 h after irradiation.

Flow cytometry

To identify human cells in mouse peripheral blood (PB), the spleen, and the bone marrow (BM), multicolor flow cytometric analysis was performed using a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with FACS Diva software (ver. 5.0.2; Becton Dickinson).

PB was collected periodically from the retro-orbital venous plexus using a capillary pipette (Drummond Scientific, Broomall, PA, USA) coated with heparin (Novo-Heparin 5000 units for Injection; Mochida Pharmaceutical Co., Tokyo, Japan) under anesthesia with isoflurane between 8 and 20 weeks after transplantation.

Mice were sacrificed by exsanguinating under anesthesia between 22 and 23 weeks after HSC transplantation. PB was collected from an abdominal vein, and the femurs, spleens and thymuses were also removed. After preparation of single cell suspensions and following treatment with red blood cell (RBC) lysis solution (0.154 M NH_4Cl, 13.770 mM $NaHCO_3$, 0.102 mM EDTA-2Na) to eliminate RBCs, white blood cells (WBCs) were suspended in PBS containing 2% fetal bovine serum. The cells were incubated with human-specific antibodies for 30 min at 4°C under protection from light.

The anti-human antibodies used for staining were fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 (clone HI30; Becton Dickinson), anti-human CD33 (clone HIM3-4; BD Pharmingen, Franklin Lakes, NJ, USA), and anti-human CD4 (clone RPA-T4; eBioscience, Inc., San Diego, CA, USA); phycoerythrin (PE)-conjugated anti-human CD3 (clone UCHT1 555333; BD Pharmingen), PE-Cy7-conjugated anti-human CD3 (clone UCHT1; Beckman Coulter, Brea, CA, USA), and anti-human CD19 (clone J4.119; Beckman Coulter); allophycocyanin (APC)-conjugated anti-human CD8a antibody (clone OKT8; eBioscience, Inc.), anti-mouse CD45 antibody (clone 30-F11; BD Pharmingen), and APC-Cy7-conjugated anti-human CD45 (clone 2D1; BD Pharmingen). The engraftment rate of human cells was expressed as a percentage of human $CD45^+$ cells in the total human $CD45^+$ and mouse $CD45^+$ cells. The ratio of human immune cells was expressed as a percentage of human $CD3^+$, $CD19^+$, $CD4^+$, and $CD8^+$ cells in human $CD45^+$ cells.

Immunochemistry

To identify human cells in the spleen of transplanted mice, the spleens were removed from the mice after blood removal, and tissues were fixed with 10 nM formalin (10 nM Mildform; Wako, Tokyo, Japan), embedded in paraffin, and stained with hematoxylin and eosin (HE), mouse anti-human CD45 antigen monoclonal antibody (clone 2B11+PD7/26; Dako Cytomation, Glostrup, Denmark), rabbit anti-human CD3 monoclonal antibody (clone SP7; Nichirei, Tokyo, Japan), and mouse anti-human CD79a monoclonal antibody (clone JCB117; Nichirei). Briefly, 5-μm-thick sections of femurs and spleens on amino-silane coated glass slides (Matsunami glass, Osaka, Japan) were immunostained by the universal immuno-enzyme polymer method (Nichirei). Each of the anti-human antibodies was incubated overnight at 4°C. Sections were serially incubated with peroxidase-labeled polymer-conjugated goat anti-mouse antibody (Histofine Simplestain Max-PO; Nichirei) for 30 min at room temperature. Immunoreaction products were visualized by incubation with 0.02% 3, 3'-diaminobenzidine (DBA; Dojindo, Kumamoto, Japan) containing 0.006% H_2O_2. Immunostained sections were counterstained with hematoxylin for visualization of nuclei.

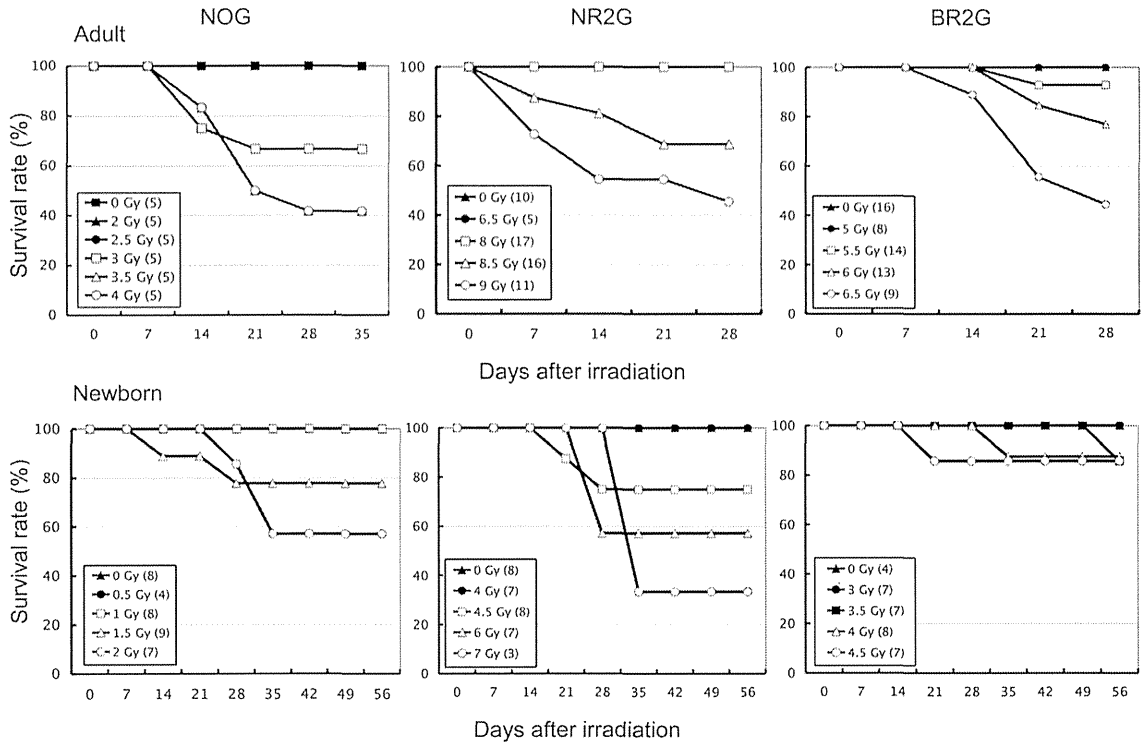

Fig. 1. Sensitivity of immunodeficient mice to total-body irradiation. Adult and newborn NOG, NR2G, and BR2G mice were irradiated with 0–12 Gy using an X-ray irradiation device to determine the appropriate dose. The body weight of NOG, NR2G, and BR2G mice was measured every week beginning 4 or 8 weeks after irradiation using a scale.

Statistical analysis

Mean values and standard deviations were calculated using the Excel software (Microsoft, Redmond, WA, USA). Significant differences were identified using Student's *t*-test, and a *P*-value less than 0.05 was deemed to indicate statistical significance.

Results

Radiation sensitivity of immunodeficient mice

Prior to transplantation experiments of human HSCs, the radiation sensitivity of adult and newborn mice of NOG, NR2G, and BR2G mice was investigated to determine an appropriate irradiation dose for HSC transplantation in each mouse strain. After irradiation into the mice with a dose ranging from 0.5 to 12 Gy, the body weight was periodically measured, and general observation was also performed for 26 days in adults and 56 days in newborns after irradiation, respectively. Non-irradiated mice in each strain were used as controls. As shown in Fig. 1, the minimum irradiation doses for the survival of all mice of each strain were 2.5 Gy, 8 Gy, and 5.5 Gy in adults, and 1 Gy, 4 Gy, and 3.5 Gy in newborns of NOG, NR2G, and BR2G mice, respectively. Thus extremely high resistance to irradiation was observed in NR2G mice.

Engraftment of human cells after HSC transplantation into adults and newborns of immunodeficient mice

The engraftment and differentiation of human cells were investigated when HSCs were transplanted into adult or newborn NOG, NR2G, and NOD-*scid* mice using the experimental protocol shown in Table 1. The 2.5-Gy dose of irradiation for adult NOD-*scid* mice used here was described previously [17], and this dose was equivalent with that of adult NOG mice. Therefore, the 1-Gy dose for newborn NOG mice was used for newborn NOD-*scid* mice.

Table 2 summarizes the engraftment rates of human cells into the adults and newborns of three strains of mice at 20 weeks after HSC transplantation. Twenty weeks after cell transplantation into NOG mice, 3 (30%) of 10 adult mice died, but none of the six newborn mice died during the same period. By contrast, all of the trans-

Table 1. Experimental conditions for human cell engraftment in immunodeficient mice

Mouse strain	Age for injection	No. of mice	Dose of irradiation (Gy)	Route of injection
NOG	Adult	10	2.5	Tail vein
	Newborn	6	1	Facial vein
NR2G	Adult	10	8	Tail vein
	Newborn	6	4	Facial vein
NOD-*scid*	Adult	9	2.5	Tail vein
	Newborn	5	1	Facial vein

Table 2. Human cell engraftment in adults or newborns of three strains of immunodeficient mice at 20 weeks after $CD34^+$ cell transfer

Age for injection	Mouse strain	No. of mice	Survival rate (%)	Frequency of engrafted mice (%)	Human $CD45^+$ cells (%)*
Adult	NOG	10	70 (7/10)	100 (7/7)	50.8 ± 17.2
	NR2G	10	100 (10/10)	100 (10/10)	40.0 ± 12.4
	NOD-*scid*	9	55.6 (5/9)	100 (5/5)	43.4 ± 14.6
Newborn	NOG	6	100 (6/6)	100 (6/6)	69.9 ± 4.5
	NR2G	6	83.3 (5/6)	100 (5/5)	43.1 ± 9.9
	NOD-*scid*	5	40 (2/5)	100 (2/2)	12.3 ± 2.1

*Percentages of human $CD45^+$ cells in mononuclaer cells of peripheral blood.

planted adult NR2G mice survived, but 1 (17%) of 6 newborn mice died. Human cells could be engrafted successfully in all surviving mice. When the engraftment rate of human cells was compared among the three strains of the mice, no significant difference was observed among adult mice of the three mouse strains. However, a significant difference was noted among the mouse strains when human cells were transplanted into newborn mice—namely, engraftment was higher in NOG, NR2G, and NOD-*scid* mice, in that order.

To examine the time course of human cell engraftment and differentiation from HSCs, the peripheral blood of the transplanted mice were collected at 8, 12, 16, and 20 weeks after HSC transplantation into adults and newborns mice, and then were analyzed by flow cytometry (Figs. 2 and 3).

In NOG and NR2G mice, only $CD19^+$ B cells were detected at 8 weeks after HSC transplantation. $CD3^+$ T cells were detected at 12 weeks after transplantation and reached 20% at 16 weeks in the NOG and NR2G mice. By contrast, only $CD19^+$ B cells, not $CD3^+$ T cells, developed during all periods tested in NOD-*scid* mice. The same results were obtained in transplanted adult and newborn mice, suggesting no significant difference in the differentiation of human cells. Conversely, engraftment of human cells was higher in transplanted NOG mice than in transplanted NR2G mice for both newborns and adults.

Human cells in the BM, the spleen, and PB of HSC transplanted mice

The human cell engraftment rates in the BM, the spleen, and PB at 22–23 weeks after cell transplantation into adult and newborn mice are shown in Supplemental Data Fig. 1. Extremely high engraftment rates were observed in both NOG and NR2G mice regardless of the transplanted age of the mice. Human cell engraftment rates were higher in the spleen, the BM, and PB, in that order, and this tendency was identified regardless of the transplanted age of the mice. The transplanted newborn mice showed higher engraftment rates than transplanted adult mice. However, no difference in the engraftment rate was observed between NOG and NR2G mice, except in PB from the newborn transplanted mice. The percentage of T, B cells and other lineage cells in human $CD45^+$ cells of the BM, the spleen, and PB are shown in Fig. 4. $CD3^+$ T cells were dominant in PB, but $CD19^+$ B cells were dominant in the BM and spleen of both NOG and NR2G mice. Particularly, a few $CD3^+$ cells were detected in the BM. No difference in the T- and B-cell subsets was noted in transplanted adult and newborn mice. For other lineage cells including NK cells and

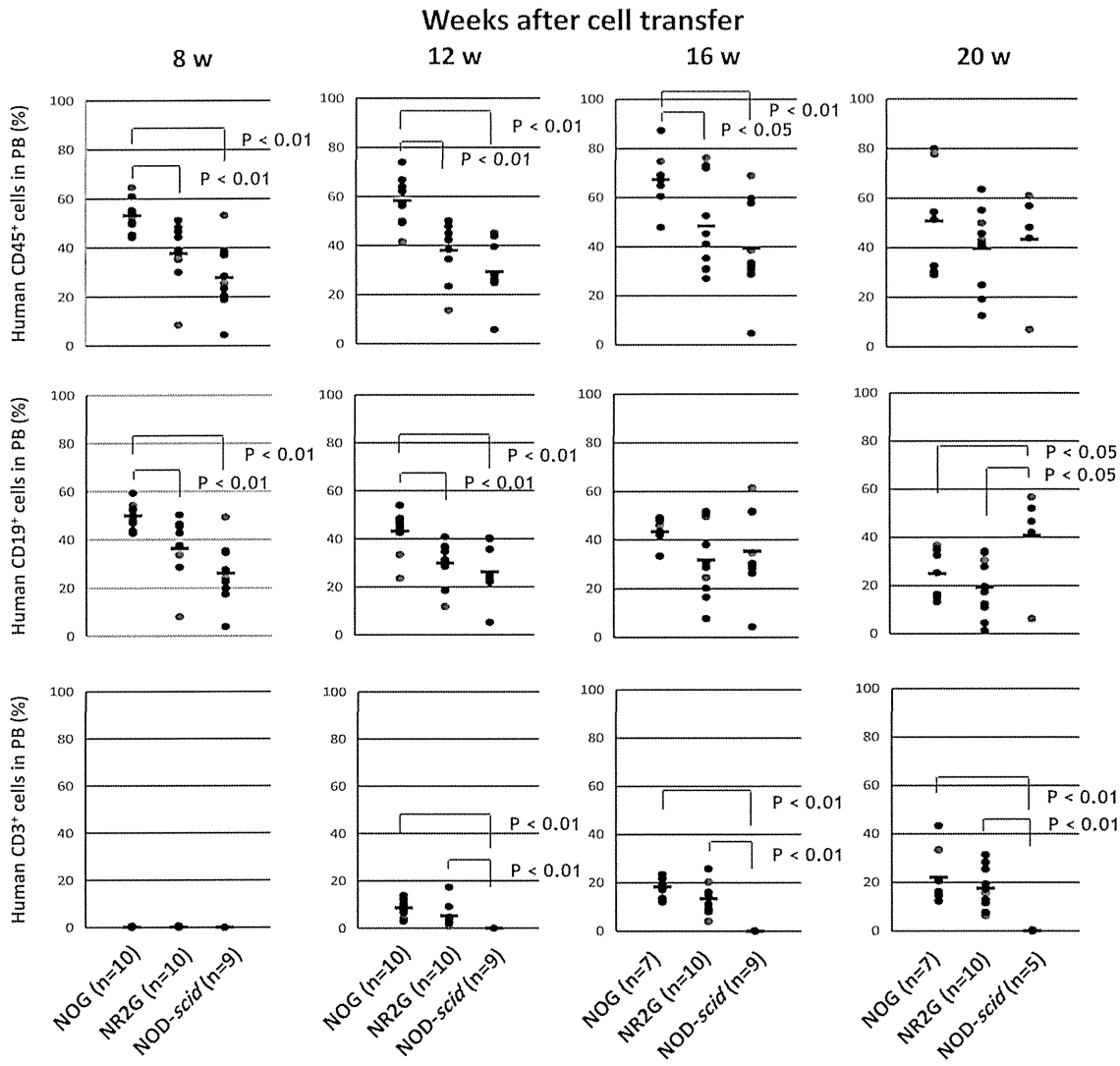

Fig. 2. Human cells in mouse peripheral blood after HSC transplantation into adult immunodeficient mice. A total of 5×10^4 commercially available human CB $CD34^+$ cells was intravenously inoculated via the tail vein in adult mice at 24 h after irradiation. After HSC transplantation, PB was collected periodically from the retro-orbital venous plexus using a capillary pipette coated with heparin under anesthesia with isoflurane at 8–20 weeks after transplantation. Human cells in mouse PB were analyzed by flow cytometry.

myeloid cells, only a small numbers of them were observed in NOG and NR2G mice, and there was also no difference among them (Data not shown).

T-cell subsets in PB, the spleen, and the thymus from HSC transplanted mice

To investigate the differentiation potential of T cell subsets, human $CD3^+$, $CD4^+$, and $CD8^+$ cells were examined in the BM, spleen, and thymus of both strains of mice. The ratios of $CD4^+$ cells were twofold those of $CD8^+$ cells in all organs tested. In the thymus, $CD4^+CD8^+$ T cells were dominant, as in humans. Here, no difference in the ratios of these T-cell subsets was observed in all organs of both mouse strains (Table 3). In addition, no difference in T-cell subset differentiation was observed between the adult and newborn transplanted mice. These results indicate that T-cell subsets can develop from HSCs in both strains, regardless of the transplanted age.

Distribution of human cells in the spleen

To investigate the distribution of human cells in the spleens of NOG and NR2G mice, we performed im-

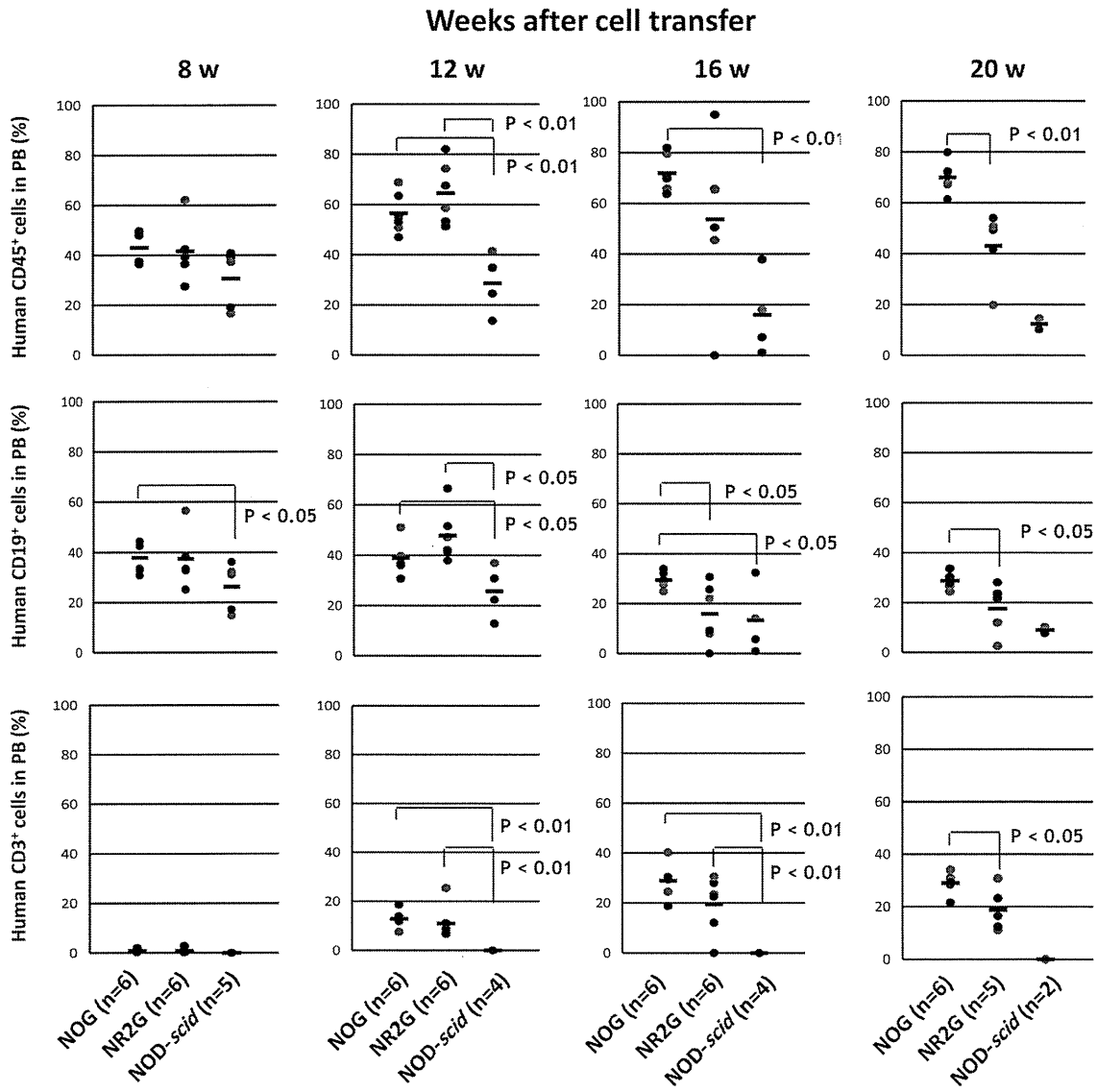

Fig. 3. Human cells in mouse peripheral blood after HSC transplantation into newborn immunodeficient mice. A total of 5×10^4 commercially available human CB $CD34^+$ cells was intravenously inoculated via the facial vein in newborn mice at 24 h after irradiation. After HSC transplantation, the analysis was done in the same manner as described in Figure 2. The number of NOD-*scid* mice (n=2) at 20 weeks after HSC transplantation was so small for statistical analysis that the mice were excluded in statistical analysis.

munohistological staining of the spleen using anti-human CD45, CD79a, and CD3 antibodies. As shown in Supplemental Data Fig. 2, human $CD45^+$ cells were broadly distributed in the spleen and formed strongly concentrated round-shaped areas. In these areas, $CD79a^+$ B cells were located in the periphery, whereas $CD3^+$ T cells were found in the central regions. Such structures were also observed in NOG and NR2G mice regardless of their transplanted age.

Discussion

In the present study, we established NR2G mice by introducing the *Rag2*^{null} gene into NOD-*IL-2Rγ*^{null} mice and investigated the capacity of the NR2G mice as recipients for human cell development after HSC transplantation in comparison to NOG mice, which are currently used extensively for generating humanized mice. In addition, we investigated the differences in human cell development and differentiation when HSCs are

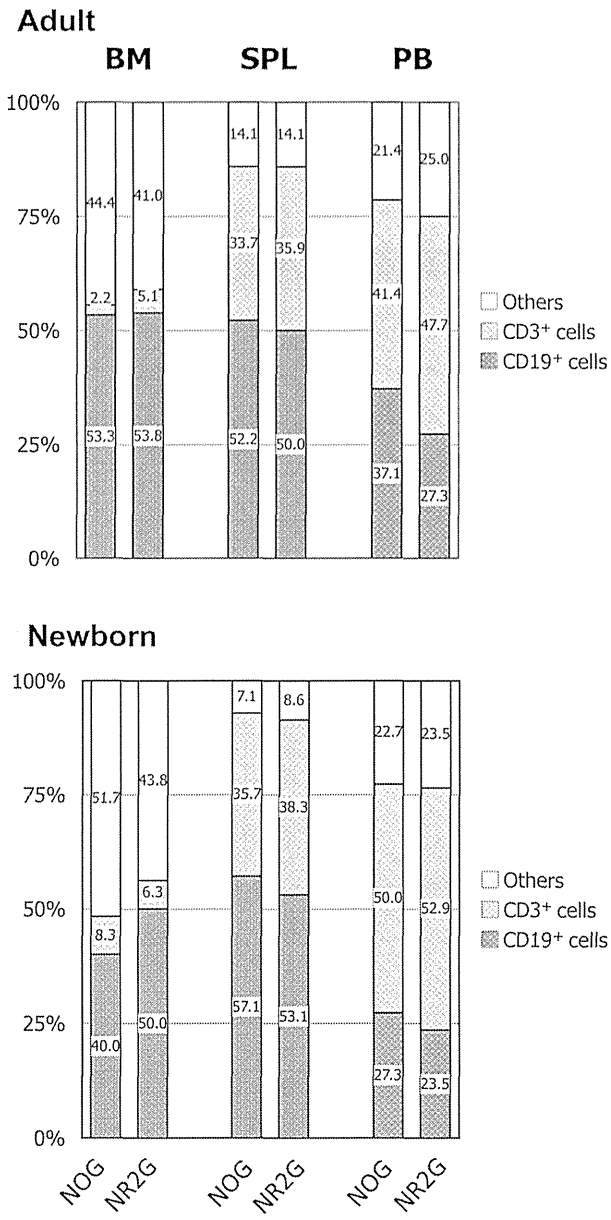

Fig. 4. Human T and B cells in the BM, spleen and PB of HSC-transplanted mice at 20 weeks after HSC transplantation. Figures in the column represent the percentage of each cell type among human CD45$^+$ cells. The number of NOG and NR2G mice used in this figure was 7 and 10 in adult, and 6 and 5 in newborn, respectively.

transplanted into newborns and adults of these immunodeficient mice.

Prior to HSC transplantation, irradiation sensitivities were determined in NOG, NR2G, and BR2G mice. NR2G mice showed high resistance against irradiation, similar to that in NR1G mice reported by Pearson *et al.* [12], compared with NOG and NOD-*scid* mice with the *Prkdc*^{scid} gene in which DNA double-strand break repair is impaired [3]. However, the irradiation resistance of NR2G mice was higher than that of NR1G mice. Namely, all NR2G mice survived for at least 26 days after 8-Gy irradiation to adult mice and at least 56 days after 4-Gy irradiation to newborn mice. By contrast, 30% of NR1G adult mice died within 28 days after 7-Gy irradiation. The reason for this difference in irradiation resistance between the NR2G and NR1G mice remains unclear. The difference between the targeted *Rag2* and *Rag1* genes in both mouse types is unlikely to affect irradiation resistance because both genes are closely located on chromosome 2 [10], and the targeting strategy and resulting phenotypes are quite similar between them [9, 13]. One explanation may be the difference in the irradiation source, namely X-ray irradiation for NR2G mice and ^{137}Cs irradiation for NR1G mice. Another explanation may be the difference in environmental factors, including the intestinal flora. Ivanov *et al.* [7] reported that the intestinal flora influenced the appearance of T helper cells in the intestinal lymphoid apparatus and demonstrated that the responses differed among mice with different flora from different breeders. The difference in the irradiation resistance between the mouse strains may be due to differences in the intestinal flora because damage to the intestine—in which cell turnover is rapid—causes early death after irradiation. Differences in irradiation sensitivity among mouse strains have been reported [11]; however, these differences are not likely to exist between NOD/LtSz and NOD/ShiJic mice because they are closely related in genetics as substrains.

Table 3. Percentages of T-cell subsets in the spleen, thymus, and peripheral blood of HSC-transplanted mice

Age in injection	Mouse strain	No. of mice	Spleen			Peripheral blood			Thymus			
			hCD3	hCD4	hCD8	hCD3	hCD4	hCD8	hCD3	hCD4/CD8	hCD4	hCD8
Adult	NOG	7	28.9 ± 2.9	64.1 ± 6.1	30.0 ± 4.4	14.1 ± 4.9	70.9 ± 4.2	26.5 ± 3.6	90.2 ± 12.0	62.6 ± 26.4	22.0 ± 14.9	13.1 ± 10.3
	NR2G	10	27.7 ± 13.9	69.0 ± 8.5	28.6 ± 8.8	8.2 ± 5.8	76.1 ± 8.8	22.0 ± 9.0	89.3 ± 6.2	46.9 ± 29.0	29.9 ± 17.5	15.5 ± 9.8
Newborn	NOG	6	29.5 ± 6.0	56.8 ± 8.0	35.5 ± 7.5	31.4 ± 16.0	69.5 ± 3.6	28.5 ± 3.0	88.4 ± 3.7	69.9 ± 13.2	13.9 ± 6.5	12.4 ± 5.2
	NR2G	5	24.1 ± 7.1	63.9 ± 12.9	30.5 ± 10.0	17.4 ± 5.8	70.8 ± 7.4	27.8 ± 6.7	78.8 ± 15.0	83.1 ± 6.0	10.8 ± 3.5	4.4 ± 1.7

Human cells in spleen, thymus and peripheral blood from mice at 20 weeks after HSC transplnatation were analyzed by flow cytometry.

Pearson *et al.* [12] also reported that NR1G mice showed high human cell engraftment and differentiation after HSC transplantation, similar to that in NSG mice. In the current study, we also compared the degree of human cell engraftment and differentiation among NR2G, NOG, and NOD-*scid* mice. To eliminate the influence of different sources of $CD34^+$ cells and different inoculation periods on the results of this comparison, we used the same lot of commercially available $CD34^+$ cells and inoculated them into the mice on the same day. Particularly, for the comparison between adults and newborns at the transplanted age, we obtained the newborns by Cesarean section after transplantation of their embryos from *in vitro* insemination. High engraftment of human cells from HSCs was observed in both NOG and NR2G mice regardless of inoculation age, although the HSC population in the former was slightly higher than that in the latter during the test periods. No fundamental difference in the differentiation of human cells was observed between the two mouse strains. B cells developed earlier than T cells. Namely, B cells were dominant 8 to 12 weeks after cell transplantation, but T cells became dominant from 16 weeks after cell transplantation. By contrast, no T cells developed in NOD-*scid* mice during the test period. Immunohistochemical staining of human cells in the spleen revealed that differentiated human cells were located in the same area in both NR2G and NOG mice. These results indicate that NR2G mice can be used as an alternative to NOG mice for generating humanized mice.

Regarding the age of HSC transplantation, Traggiai *et al.* [16] reported the successful generation of humanized mice by intrahepatic inoculation of HSCs into newborn BR2G mice. Ishikawa *et al.* [5] also reported the high efficiency of human cell development by intravenous HSC inoculation into newborn NSG mice. The present study also revealed that the transplantation of HSCs into newborn mice resulted in a higher engraftment rate than transplantation into adult mice. However, the difference in the engraftment rates between the mouse strains was not marked, and the differentiation of human cells was fundamentally similar, suggesting that researchers can select the age for HSC inoculation into humanized mice according to the purpose of their research.

In conclusion, newly established NR2G mice can be used as an alternative host to NOG mice for generation of humanized adult and newborn mice by inoculation of HSCs.

Acknowledgments

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Visualization of the human CD4$^+$ T-cell response in humanized HLA-DR4-expressing NOD/Shi-*scid*/γc^{null} (NOG) mice by retrogenic expression of the human TCR gene

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ABSTRACT

The development of severe immunodeficient mouse strains containing various human genes, including cytokines or HLA, has enabled the reconstitution of functional human immune systems after transplantation of human hematopoietic stem cells (HSC). Accumulating evidence has suggested that HLA-restricted antigen-specific human T-cell responses can be generated in these humanized mice. To directly monitor immune responses of human CD4$^+$ T cells, we introduced β-lactoglobulin (BLG)-specific T cell receptor (TCR) genes derived from CD4$^+$ T-cell clones of cow-milk allergy patients into HSCs, and subsequently transplanted them into NOG-HLA-DR4 transgenic/I-Aβ deficient mice (NOG-DR4/I-A^o). In the thymus, thymocytes with BLG-specific TCR preferentially differentiated into CD4$^+$CD8$^-$ single-positive cells. Adoptive transfer of mature CD4$^+$ T cells expressing the TCR into recipient NOG-DR4/I-A^o mice demonstrated that human CD4$^+$ T cells proliferated in response to antigenic stimulation and produced IFN-γ *in vivo*, suggesting that functional T-cell reactions (especially Th1-skewed responses) were induced in humanized mice.

1. Introduction

An increasing number of studies have demonstrated that severe immunodeficient mice, including NOD/Shi-*scid* IL2rγ^{null} (NOG) [1], NOD/LtSz-*scid* IL2rγ^{null} (NSG) [2], and BALB/c Rag2^{null}IL2rγ^{null} (BRG) [3], can be used for long-term engraftment of human hematopoietic stem cells (HSC) and differentiation of various human hematopoietic lineages. Genetic modifications of these original mouse strains by introducing human cytokine- or HLA genes have improved both hematopoiesis and human immune systems [4]. For example, we established IL-3/GM-CSF transgenic (tg) NOG mice, which show enhanced human myelopoiesis including dendritic cells (DC), granulocytes (basophils and eosinophils), and mast cells [5]. Multiple replacements of M-CSF [6], IL-3, GM-CSF [7], and thrombopoietin (TPO) [8] genes with the corresponding human ortholog genes (MITRG mouse) have been used for human myeloid cell development [9]. In addition, transgenic strains containing HLA-A2 [10,11] or HLA-DR genes [12,13] showed that HLA-restricted immune reactions could be induced. Thus, these improved hu-HSC-engrafted mice (hu-HSC mice) can be used to study human hematopoiesis and immune reactions, which is important for developing novel clinical strategies.

Previously, we and others reported that the expression of HLA-DR4 [13] or -DR1 [12] in NOG or NSG, respectively, induced antigen-specific IgG responses only when the mice were transplanted with HSC from HLA-haplotype matched donors. These results suggest that human CD4$^+$ T cells in hu-HSC mice can recognize antigen peptides loaded on HLA-DR molecules in antigen-presenting cells (APC), and that the activated CD4$^+$ T cells can subsequently induce class switch recombination in the Ig locus in antigen-specific B cells. However, how the *in vivo* immune reactions of CD4$^+$ T cells proceed in hu-HSC mice remains unclear, due in part to the low availability of antigen-specific HLA-II tetramers. In this study, we utilized TCR genes from beta-lactoglobulin (BLG) specific human CD4$^+$ T cell clones restricted by HLA-DR4 [14] and examined whether the introduction of BLG-specific TCR genes into HSCs by retroviral vector led to differentiation of BLG-specific human CD4$^+$ T cells in HLA-DR4 transgenic mice. We also investigated the functions of these T cells using *in vitro* and *in vivo* assays.

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0006-291X/

2. Materials and methods

2.1. Mice

NOG-HLA-DR4-Tg I-A$^{-/-}$ mice (NOG-DR4/I-A^{o}) were described previously [13]. Mice were maintained in the Central Institute for Experimental Animals (CIEA) under specific-pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of CIEA (certification number, 11004A) and were performed according to CIEA guidelines.

2.2. CD34^{+} hematopoietic stem cells

Umbilical CD34^{+} cells were obtained from AllCells (Alameda, CA).

2.3. DNA constructs

BLG-specific CD4^{+} T-cell clones (HA5.7 and YA4) were kindly provided by Dr. Kondo [14]. The TCRα and β genes were isolated and the sequences of the CDR3 region were determined. A lentiviral vector containing the TCR1.9 gene (pCCL.PPT.hPGK.1.9.IRES.EGFP) was kindly provided by Dr. Scott G. Kitchen [15]. The VJ and VDJ region of TCRα and β, respectively, in the vector were replaced with those of TCR-HA5.7 or -YA4. The TCR was further sub-cloned into the pDΔNsam-IRES-EGFP [16].

2.4. Preparation of lentivirus

The 293T cells in complete DMEM were transfected with three plasmids; namely, pCAG-HIVgp, pCMV-VSV-G-RSV-rev, which were from RIKEN Bioresource center (Tsukuba, Japan), and pCCL.PPT.hPGK.TCR.IRES.EGFP. After 48 h, the supernatants were recovered and concentrated by ultracentrifugation (25,000 rpm, 2 h), subsequently stored as aliquots at −80 °C in a deep freezer.

2.5. Infection of human T cells by lentivirus

Human peripheral blood mononuclear cells (PBMC) were stimulated with immobilized anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies (Abs) for 24 h in complete RPMI medium. Concentrated lentivirus was added at a multiplicity of infection (MOI) of 20.

2.6. Dendritic cell (DC) preparation

Human CD14^{+} monocytes in PBMCs from a donor with HLA-DRB1*0405 were purified by MACS using anti-CD14 Microbeads and an LS column (Miltenyi Biotech). The cells were stimulated with recombinant human GM-CSF and IL-4 (50 ng/ml) (Miltenyi Biotech) for 7 days. The medium was replaced every 3 day and harvested at day 7. Maturation was induced with recombinant human TNFα (50 ng/ml) (Miltenyi Biotech).

2.7. ELISPOT

IFN-γ production by human CD4^{+} T cells was detected using an ELISA kit (BD Biosciences).

2.8. Preparation of retrovirus

A packaging cell line, PLAT-F [17], was cultured as previously described [33]. They were transfected using the retroviral constructs. The medium was replaced with fresh medium 12 h post transfection. The supernatants were recovered and used to infect HSCs at 48 h.

2.9. Infection of HSC using viral vectors

CD34^{+} cells were cultured in X-VIVO 15 (Lonza, Walkersville, MD) with 1% human serum albumin (HSA) (Kaketsuken, Kumamoto, Japan) and stimulated with cytokines (100 ng/ml stem cell factor (SCF), 100 ng/ml Flt-3 ligand (Flt-3L), 25 ng/ml thrombopoietin (TPO), and 100 ng/ml IL-6 (from Miltenyi Biotech, Gradbach, Germany)) in a 24-well plate (3×10^5/well) for 48 h. The stimulated CD34^{+} cells were transferred to six-well CH-296 (Retronectin, Takara, Otsu, Japan)-coated plates (3×10^5/well). After the addition of an equivalent volume of virus supernatants, the cells were spun at 2400 rpm for 120 min at 32 °C. The infection was repeated every 12 h, and after 48 h of culture, approximately 5×10^4 to 1×10^5 infected cells were injected intravenously into each mouse 24 h after X-irradiation (2.5 Gy) (MBR-1505R, Hitachi Medical Corp., Tokyo, Japan).

2.10. Flow cytometry

Thymus, spleen, and peripheral blood (PB) were obtained from the reconstituted mice 16–24 weeks after HSC transplantation. Thymus and spleen were minced with slide glasses and the debris was removed using nylon mesh. Red blood cells were eliminated using BD Pharm Lyse (BD Biosciences, San José, CA). The mononuclear cells (MNC) were suspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline (PBS) containing 1% FCS with 0.1% NaN_3). MNCs were stained with appropriate Abs for 15–20 min at 4 °C. After washing and resuspending in FACS buffer with propidium iodide (PI) solution, the cells were subjected to flow cytometric analysis using a FACSCanto multi-color flow cytometer (BD Biosciences). Data were analyzed using the FACSDiva software (BD Biosciences). The following Abs were used: anti-TCR Vβ5.1 (TRBV5-1)-phycoerythrin (PE) and anti-TCR Vβ12 (TRBV10-3)-PE, purchased from Beckman Coulter (Brea, CA). The following antibodies were purchased from Biolegend (San Diego, CA): anti-CD62L-PE-Cy7, anti-CD3-allophycocyanin (APC), anti-CD4-APC, anti-CD8a-APC, anti-CD3-APC-Cy7, anti-CD4-APC-Cy7, anti-CD45RA-APC-Cy7, and anti-human CD45-APC-Cy7. For intracellular cytokine staining, T cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 h in the presence of BrefeldinA (Biolegend). CytoFix™ and Cytofix/CytoPerm™ (BD Biosciences) were used for staining with anti-IFN-γ-PE-Cy7 and anti-IL-4 antibodies (Biolegend).

2.11. In vitro T-cell culture

Splenocytes were stained with VPD450 dye (BD Biosciences) according to the manufacturer's instructions and were cultured in complete RPMI containing recombinant human IL-2 (10 U/ml, Peprotech, Rocky Hill, NJ) in the presence or absence of the BLG_{97-117} peptide (Sigma–Aldrich Japan, Tokyo, Japan). After 1 week, cultured cells were recovered and T-cell proliferation was investigated using a FACS Aria II with a violet laser (BD Biosciences).

2.12. Adoptive transfer

VPD450-labeled splenocytes were intravenously injected into two or three new NOG-DR4/I-A^{o} mice as secondary recipients. On the same day, the recipient mice were immunized with 100 μg of BLG_{97-117} peptide with Alum (2 mg/mouse). The T-cell responses were analyzed at day 8.

2.13. Statistical analysis

Welch's *t*-test was used to identify significant differences after the *F*-test of equality of variances.

3. Results

To visualize immune responses of human CD4$^+$ T cells in NOG mice reconstituted with human hematopoietic cells (hu-HSC NOG), we used TCR genes from two independent BLG-specific CD4$^+$ T-cell clones (HA5.7 and YA4). These CD4$^+$ T clones were derived from cow-milk allergy patients and respond to BLG_{97-117} in a HLA-DRB1*0405 restricted manner [14]. We isolated transcripts for TCRα and TCRβ genes [14] (Fig. S1A) and constructed lentiviral vectors encoding BLG-specific TCRs. We next confirmed the expression of TCRs and GFP in J.RT3-T3.5, which is a derivative cell line from Jurkat T cells lacking endogenous TCRβ, using nucleofection (Fig. S1B). Regarding the TCR of HA5.7, we used TRAV8-3 because of the strong expression compared to TRAV16 (Fig. S1B). Human CD4$^+$ T cells were infected by the lentivirus and subsequently stimulated with specific BLG_{97-117} peptide with dendritic cells from an HLA-DR4$^+$ donor. We confirmed IFN-γ production in infected CD4$^+$ T cells, which indicated that exogenous TCR is functional (Fig. S1C).

To introduce BLG-specific TCR genes into human HSCs, we used a retroviral vector, pDΔNsam-IRES-EGFP [16,18]. After infection by retrovirus in *in vitro* cultures, the frequency of GFP$^+$ cells typically reached more than 80% (Fig. 1A). These HSCs were transferred into irradiated NOG-DR4/I-A^o mice (hu-HSC (TCR) NOG-DR4/I-A^o). Hematopoiesis from HSCs was confirmed in the peripheral blood (PB) around 6 weeks after HSC transplantation. T-cell development from transferred HSCs was detected in the PB around 3–4 months, as described previously, and T cells expressing BLG-specific TCR were detected as TRBV$^+$GFP$^+$CD3$^+$CD4$^+$ T cells (Fig. 1B, TRBV5-1 and TRBV10-3 for HA5.7 and YA4, respectively).

In the thymus from hu-HSC (TCR) NOG-DR4/I-A^o mice at 20 weeks post-HSC transplantation, a significant fraction of the human thymocytes were GFP$^+$ cells (data not shown). Differentiation into CD4$^+$CD8$^+$ double-positive (DP), CD4$^+$CD8$^-$, and CD4$^-$CD8$^+$ single-positive (SP) thymocytes was detected in both GFP$^-$ and GFP$^+$ groups (Fig. 2A). The ratio of CD4$^+$CD8$^-$ to CD4$^-$CD8$^+$ SP cells in GFP$^+$ cells was significantly higher than that in GFP$^-$ cells (Fig. 2B), which suggested that the exogenously introduced BLG-specific TCR preferentially induced the positive selection of thymocytes into CD4$^+$ T cells rather than CD8$^+$ T cells.

In the spleen, the frequency of GFP$^+$ cells in total human CD45$^+$ cells varied significantly among individual mice, despite the use of

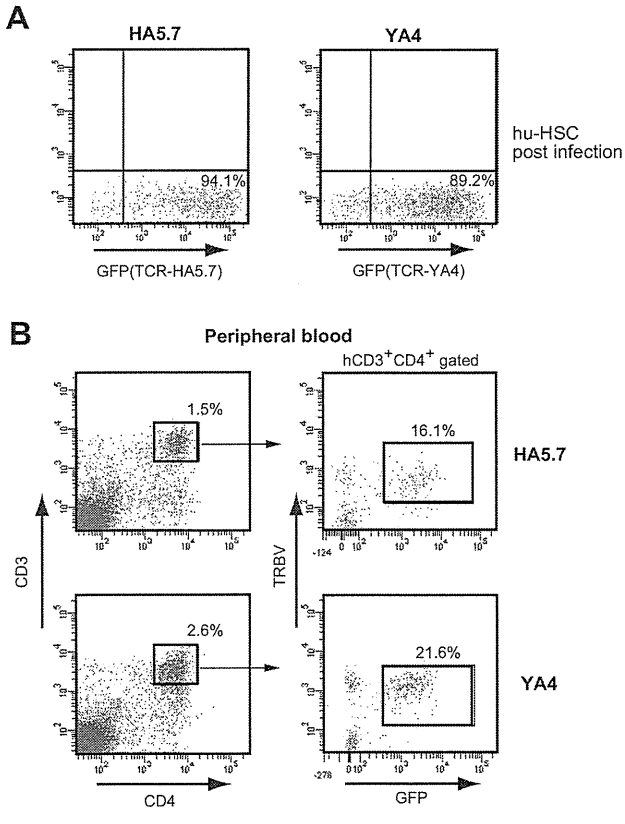

Fig. 1. (A) Introduction of the BLG-specific TCR gene into human HSCs (hu-HSC). *In vitro* activated human HSCs (3×10^5) were spin-infected four times with retroviral vectors encoding BLG-specific TCR (HA5.7 or YA4). GFP expression in HSCs was detected 12 h after the last infection using a flow cytometer. (B) Development of BLG-specific human CD4$^+$ T cells in hu-HSC (TCR) NOG-DR4/I-A^o mice. Peripheral blood retro-orbitally collected from hu-HSC NOG-DR4/I-A^o mice was analyzed using a flow cytometer at 20 weeks post-transplantation. Expression of GFP and TRBV from the retroviral vectors was analyzed after gating of human CD45$^+$CD3^{+-} CD4$^+$ T cells. Representative data from at least 10 individual mice are shown.

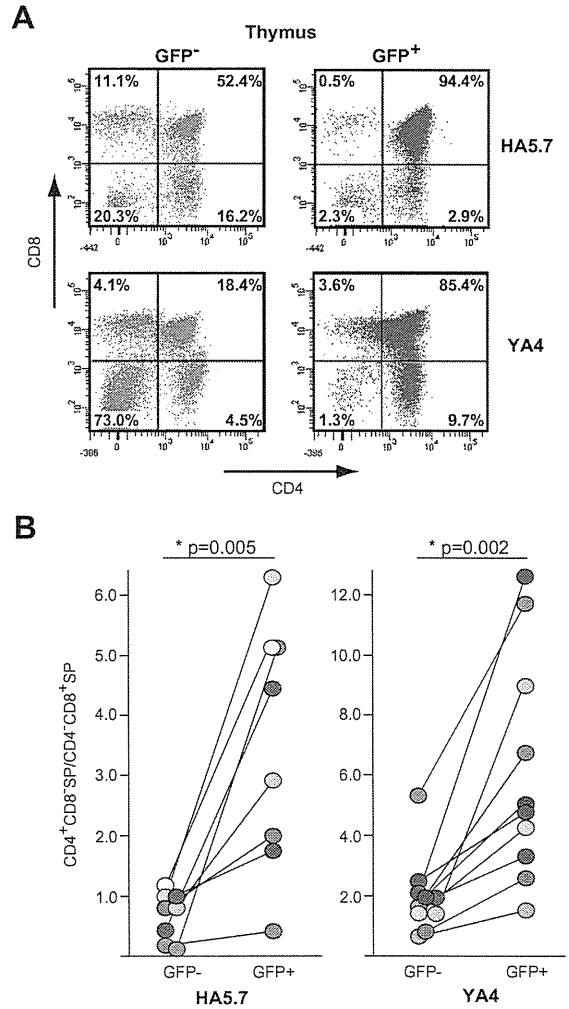

Fig. 2. (A) Analysis of thymocytes in NOG-DR4/I-A^o mice. Thymocytes from hu-HSC (TCR) NOG-DR4/I-A^o mice were stained with anti-CD4 and -CD8 antibodies at 20–24 weeks post-transplantation. Differentiation in GFP$^-$ and GFP$^+$ subpopulations was shown. (B) The ratios of CD4$^+$CD8$^-$ SP to CD4$^-$CD8$^+$ SP thymocytes were calculated based on flow cytometric analysis, and were subsequently plotted. Each circle represents one recipient NOG-DR4/I-A^o mouse. The *p*-value was obtained using Welch's *t*-test after the *F*-test of equality of variances.

mice from the same littermates and the same HSCs (Tables S1 and S2). A significant number of TCR-HA5.7$^+$ or TCR-YA4$^+$ CD4$^+$ T cells accumulated in the spleen (Fig. 3A). The majority of CD4$^+$ T cells in the spleen of hu-HSC (TCR) NOG-DR4/I-A^o showed the phenotype of CD45RA$^-$CD62L$^+$ central memory (T_{CM}) or CD45RA$^-$CD62L$^-$ effector memory T cells (T_{EM}), irrespective of the expression of BLG-specific TCR (Fig. 3B), which was consistent with previous reports.

To examine the functions of CD4$^+$ T cells with TCR-HA5.7 or -YA4, we stimulated whole splenocytes from hu-HSC (TCR) NOG-DR4/I-A^o with the specific BLG_{97-117} peptide *in vitro* after staining with VPD450 dye, which is an indicator of cell proliferation. In the presence of the BLG_{97-117} peptide, CD4$^+$ T cells with BLG-specific TCR showed robust proliferation, while culture without peptide induced only modest proliferation (Fig. S2). The frequency of TRBV$^+$GFP$^+$ populations in CD3$^+$CD4$^+$ T cells increased approximately three- to four-fold by peptide stimulation.

We next investigated whether CD4$^+$ T cells with BLG-specific TCR could mediate immune responses *in vivo*. We adoptively transferred VPD450-stained human cells prepared from hu-HSC (TCR) NOG-DR4/I-A^o into NOG-DR4/I-A^o mice secondary recipients (Fig. 4A). These recipient mice were immunized with Alum/BLG_{97-117} peptide, after which spleen cells were analyzed at day 8. The proliferation of TRBV$^+$GFP$^+$CD3$^+$CD4$^+$ T cells was detected in the mouse group with immunization (Fig. 4B), while a small

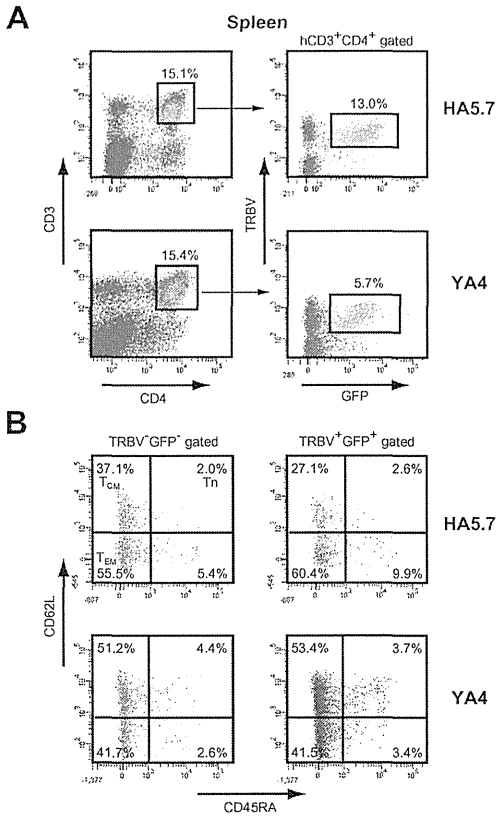

Fig. 3. (A) Accumulation of BLG-specific TCR-expressing human CD4$^+$ T cells in the spleen. Spleen cells from hu-HSC (TCR) NOG-DR4/I-A^o mice were prepared at 20–24 weeks post-transplantation and stained with anti-human CD45, CD3, CD4 and TRBV antibodies for flow cytometric analysis. (B) Staining with anti-TRBV, CD4, CD62L, and CD45RA. FACS plots showed differentiation of T-cell subsets including CD62L$^+$CD45RA$^+$ T-naive (T_n), CD62L$^+$CD45RA$^-$ central memory T(T_{CM}), CD62L$^-$CD45RA$^-$ effector memory T (T_{EM}) in BLG-specific TCR expressing CD4$^+$ T cells (TRBV$^+$GFP$^+$, green), and not-transduced CD4$^+$ T cells (TRBV$^-$GFP$^-$, gray). Representative data from at least 10 mice are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

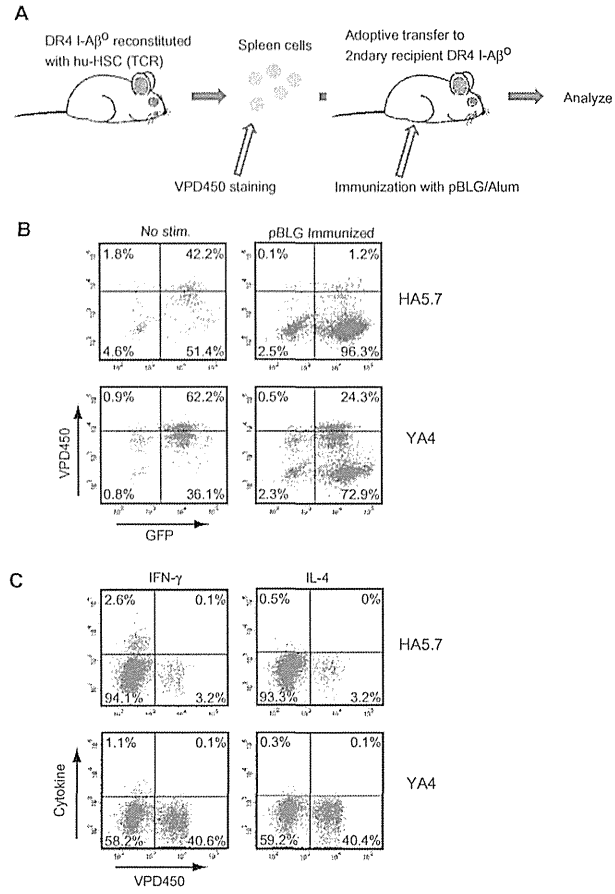

Fig. 4. (A) Schema of adoptive transfer. Spleen cells were prepared from hu-HSC (TCR) NOG-DR4/I-A^o mice at 20–24 weeks post-transplantation and were labeled with VPD450 dye. The cells were transplanted into new NOG-DR4/I-A^o mice, which were subsequently immunized with the specific BLG peptide. The spleen cells were recovered at day 8 and stained with anti-TRBV, -CD3, -CD4, and -CD45 antibodies. Dilution of VPD450 dye indicated proliferation of CD4$^+$ T cells. (B) Cytokine production by proliferating CD4$^+$ T cells. Spleen cells mentioned above were re-stimulated with PMA/ionomycin for 4 h *in vitro* in the presence of Brefeldin A. After fixation, intracellular IFN-γ and IL-4 was detected using flow cytometric analysis. FACS plots show representative data from four independent experiments.

number of GFP$^+$ cells were detected in the group without antigen challenge. PMA/ionomycin treatment induced production of a significant amount of IFN-γ, but only modest IL-4 production in TRBV$^+$GFP$^+$CD3$^+$CD4$^+$ T cells (Fig. 4C), suggesting that Th1 response was strongly induced in *in vivo* environments.

4. Discussion

In this study, we introduced BLG-specific TCR genes derived from milk-allergy patients into human HSCs using a retrovirus and visualized immune responses of the BLG-specific human CD4$^+$ T cells in the HSC-transferred humanized mice.

Previously, we and others reported that antigen-specific IgG responses could be generated when HLA-DR transgenic mice were reconstituted with HSCs from HLA-haplotype matched donors [12,13]. However, no information on how the T-cell responses function *in vivo* is available. Our approach demonstrated the vigorous proliferation of CD4$^+$ T cells in response to antigen stimulation and production of IFN-γ in humanized mice *in vivo*. The simultaneous detection of CD4$^+$ T-cell proliferation and cytokine production demonstrated that CD4$^+$ T cells acquired effector functions to produce IFN-γ after multiple proliferations. On the other hand, the production of IL-4 was modest despite the use of Alum as a

strong Th2-inducing adjuvant. It has been shown that T cells in hu-HSC mice spontaneously differentiate into Th1 cells because of exposure to an extremely lymphopenic environment caused by the *scid* mutation and γc deficiency [19]. Another explanation for Th1-skewing is that the combination of TCR-HA5.7 or -YA4 and their specific peptide may provide excessive TCR signals, which may preferentially induce Th1 responses [20–22].

Weak IL-4 production by antigen-specific $CD4^+$ T cells may result in the inefficient IgG responses in hu-HSC mice. Indeed, our previous results demonstrated that all the mice immunized with exogenous antigens did not always show IgG production [13]. Based on these results, we need to explore the requirements for generating a microenvironment to promote Th2 responses in hu-HSC humanized mice. Multiple cytokines and cells are involved in inducing Th2 responses *in vivo*. For example, IL-4 derived from basophils, together with the antigen-presenting capacity of the population, is thought to be important for Th2 responses [23,24], but this remains controversial in humans [25,26]. Group 2 innate lymphoid cells (ILC2), including nuocytes [27] or natural helper cells (NH) [28], are considered a source of IL-13 [29]. In addition, TSLP, IL-25, and IL-33 from epithelial cells are believed to be important factors regulating Th2 differentiation [30]. Expression of these human cytokine genes using conventional transgenes or replacement of the mouse genes with human counterparts by homologous recombination would generate a suitable cytokine milieu for Th2 differentiation [31].

Our results suggested that human T cells with exogenous TCR were subjected to normal differentiation processes in the thymus. Indeed, TCR-HA5.7 and -YA4 induced skewed differentiation toward $CD4^+CD8^-$ SP cells, reflecting the original cellular sources. In previous reports, an exogenous TCR gene (TCR1.9) specific for HIV-Gag_{77-85}, which was derived from an HLA-A*0201 restricted T-cell clone, was introduced into human HSCs using lentiviral vectors [15]. When fetal-liver derived HSCs with the TCR1.9 were transferred into NOD/scid [15] or NSG mice [32], which were also implanted with the liver and thymus tissues from the same fetus (BLT-mouse), TCR1.9-expressing thymocytes differentiated into $CD4^-CD8^+$ SP cells in the implanted human thymus. These results suggest that exogenous TCR mediates the positive selection of thymocytes. Considering that our previous report demonstrated that mouse thymic epithelial cells (TEC) are critical for the positive selection of human thymocytes in hu-HSC NOG mice [33], which differ from BLT-mice, it remains unclear why mouse antigen-derived peptides and HLA-DR4 complexes in the mouse TEC induce human thymocyte differentiation into $CD4^+CD8^-$ SP cells. There may be overlap between mouse and human peptides in the amino acid sequences or configurations.

Successful expression of BLG-specific TCR in hu-HSC NOG-DR4/I-A^o mice suggests that pathogenic TCR can be introduced using similar methods. An association of HLA II with autoimmune diseases has been suggested in previous reports, and $CD4^+$ T cells are thought to play an important role in mediating diseases. For example, HLA-DR*0405 is known to be associated with type I diabetes [34], Vogt–Koyanagi–Harada (VKH) disease [35], or rheumatoid arthritis [36]. Isolation of pathogenic TCR restricted by HLA-DR4 from these patients followed by their introduction into HSCs would facilitate development of pathogenic T clones in NOG-DR4/I-A^o mice *in vivo*. When novel transgenic strains expressing target antigens are established in the background of NOG-DR4/I-A^o, adoptive transfer of T cells with pathogenic TCR into these mice may induce autoimmunity. These models can be used to develop novel strategies for treating autoimmune diseases.

Humanized mouse models are commonly used as important research tools for bridging 'bench' and 'bed' sides. The combination of patient-derived materials or genetic information with the generation of novel mice may lead to the establishment of useful models for human diseases and facilitate the development of novel therapeutic strategies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.062.

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Predominant Development of Mature and Functional Human NK Cells in a Novel Human IL-2–Producing Transgenic NOG Mouse

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We generated a severe immunodeficient NOD/Shi-*scid*-IL-2Rγ^{null} (NOG) mouse substrain expressing the transgenic human IL-2 gene (NOG–IL-2 Tg). Upon transfer of human cord blood–derived hematopoietic stem cells (hu-HSCs), CD3$^-$CD56^{high}CD16$^{+/-}$ cells developed unexpectedly, predominantly in the NOG–IL-2 Tg (hu-HSC NOG–IL-2 Tg). These cells expressed various NK receptors, including NKp30, NKp44, NKp46, NKG2D, and CD94, as well as a diverse set of killer cell Ig-like receptor molecules at levels comparable to normal human NK cells from the peripheral blood, which is evidence of their maturity. They produced levels of granzyme A as high as in human peripheral blood–derived NK cells, and a considerable amount of perforin protein was detected in the plasma. Human NK cells in hu-HSC NOG–IL-2 Tg produced IFN-γ upon stimulation, and IL-2, IL-15, or IL-12 treatment augmented the in vitro cytotoxicity. Inoculation of K562 leukemia cells into hu-HSC NOG–IL-2 Tg caused complete rejection of the tumor cells, whereas inoculation into hu-HSC NOG fully reconstituted with human B, T, and some NK cells did not. Moreover, when a CCR4$^+$ Hodgkin's lymphoma cell line was inoculated s.c. into hu-HSC NOG–IL-2 Tg, the tumor growth was significantly suppressed by treatment with a therapeutic humanized anti-CCR4 Ab (mogamulizumab), suggesting that the human NK cells in the mice exerted active Ab-dependent cellular cytotoxicity in vivo. Taken together, these data suggest that the new NOG–IL-2 Tg strain is a unique model that can be used to investigate the biological and pathological functions of human NK cells in vivo. *The Journal of Immunology*, **2015, 194: 000–000.**

Natural killer cells are members of the innate lymphoid cell family (1). These cells make up 5–20% of lymphocytes in normal human peripheral blood (PB) and are distributed in various organs, including the liver, lung, spleen, and lymph nodes. They are large granular lymphocytes and store perforin, granzymes, or IFN-γ in their intracellular granules, which they release in response to various immunological stimuli. These cells engage in the clearance of viral pathogens or intracellular microbes as the first line of defense. Because of their potent tumor-killing activity, NK cells are considered critical in tumor immunosurveillance. Indeed, the induction of Ab-dependent cell cytotoxicity (ADCC) (2) in NK cells by therapeutic mAbs has become a mainstream form of cancer treatment (e.g., rituximab [anti-CD20 mAb], trastuzumab [anti-Her2 mAb], and mogamulizumab [anti-CCR4 mAb]).

Although applications of NK cells have increased in line with the development of new mAbs, studies of human NK cells remain largely dependent on in vitro systems. Usually, human NK cells are isolated from healthy donors or are induced from CD34$^+$ hematopoietic stem cells (HSCs) in the presence of cytokines. These cells are subsequently used to assay cytotoxicity or cytokine-producing ability in vitro. Despite their ease of use, in vitro systems do not always provide sufficient information about the in vivo behavior of human NK cells, which is essential to understand the interactions between human NK cells and their target cells, such as tumor cells. Therefore, it is necessary to develop novel in vivo experimental systems to study human NK cells. Such systems must fulfill several requirements: long-term survival of a significant number of human NK cells, maintenance of their function, and the systems must be autonomous (i.e., not require exogenous manipulation or supplementation).

Recent progress in the development of extremely severe immunodeficient mouse strains has markedly improved engraftment of xenotransplants. This humanized mouse technology has been used to transfer human NK cells into immunodeficient mice to examine the cells' in vivo cytotoxicity against tumors (3). The in vivo development of human NK cells from human HSCs also was reported (4–6). However, the former approach was limited to

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I.K. performed all of the animal experiments and analyzed the data. I.K., T.T., R.I., and M.I. designed the study. Y. Kawakami advised on immunology. T.K. performed embryo manipulation. T.M., Y. Ka, and T.O. bred NOG–IL-2 Tg mice. H.S. supervised the genotyping of the Tg mice. M.I. organized the project. I.K., T.T., and M.I. wrote the manuscript.

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Abbreviations used in this article: ADCC, Ab-dependent cell cytotoxicity; BM, bone marrow; BRG, BALB/C-RAG2$^{-/-}$-IL-2Rγc$^{-/-}$; CB, cord blood; hPBMC, human PBMC; HSC, hematopoietic stem cell; hu-HSC, human cord-blood–derived hematopoietic stem cell; iNK, immature NK; KIR, killer cell Ig-like receptor; MNC, mononuclear cell; NCR, natural cytotoxicity receptor; NKG2C, NK group 2 membrane C; NOG, NOD/Shi-*scid*-IL-2Rγ^{null}; NOG–IL-2 Tg, NOG mouse substrain expressing transgenic human –IL-2; non-Tg, nontransgenic; PB, peripheral blood; PB-NK, PB-derived NK; qPCR, quantitative PCR.

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