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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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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

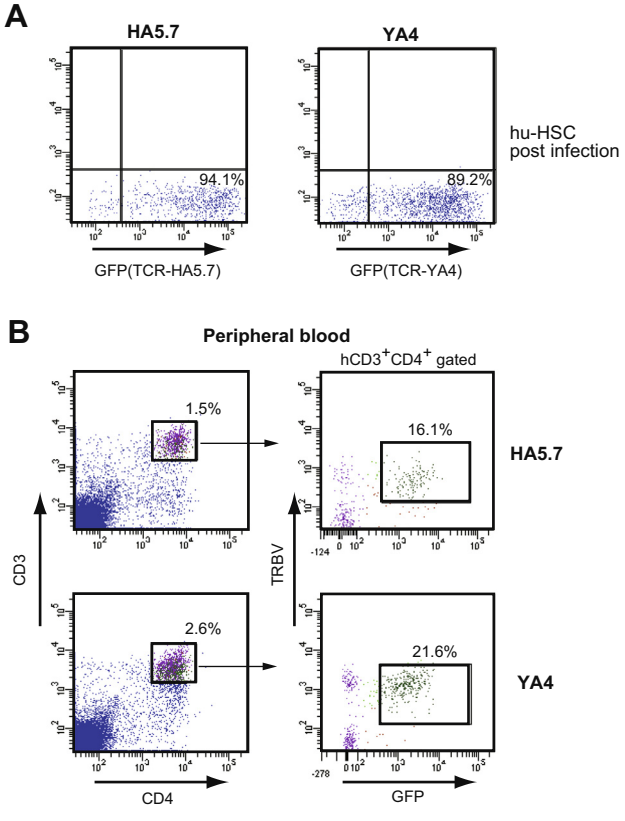

Fig. 1. (A) Introduction of the BLG-specific TCR gene into human HSCs (hu-HSC). *In vitro* activated human HSCs (3×10^5) were spin-infected four times with retroviral vectors encoding BLG-specific TCR (HA5.7 or YA4). GFP expression in HSCs was detected 12 h after the last infection using a flow cytometer. (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.

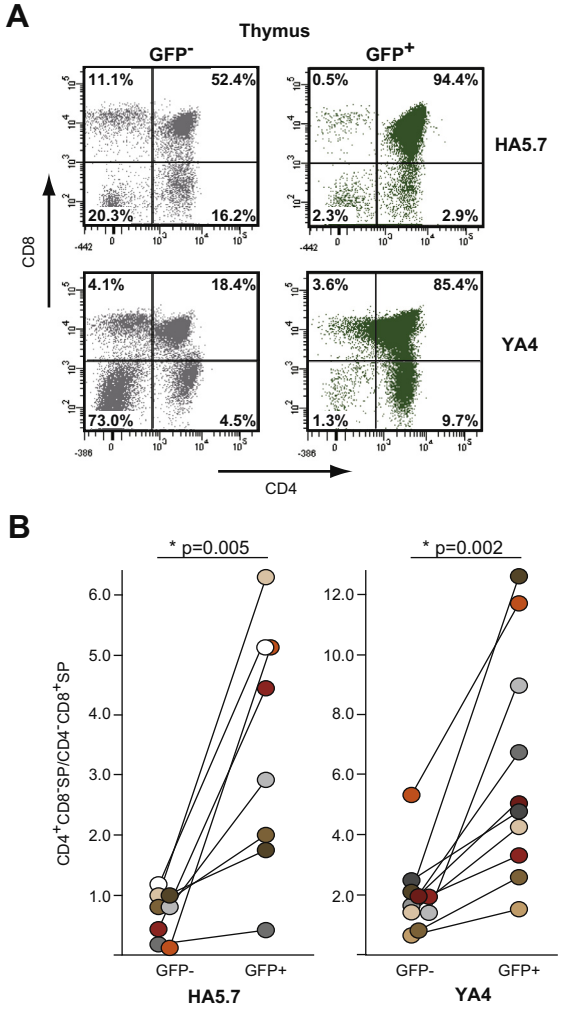

Fig. 2. (A) Analysis of thymocytes in NOG-DR4/I-A^o mice. Thymocytes from hu-HSC (TCR) NOG-DR4/I-A^o mice were stained with anti-CD4 and -CD8 antibodies at 20–24 weeks post-transplantation. Differentiation in GFP$^-$ and GFP$^+$ subpopulations was shown. (B) The ratios of CD4$^+$CD8$^-$ SP to CD4$^-$CD8$^+$ 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 SI and SII). 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

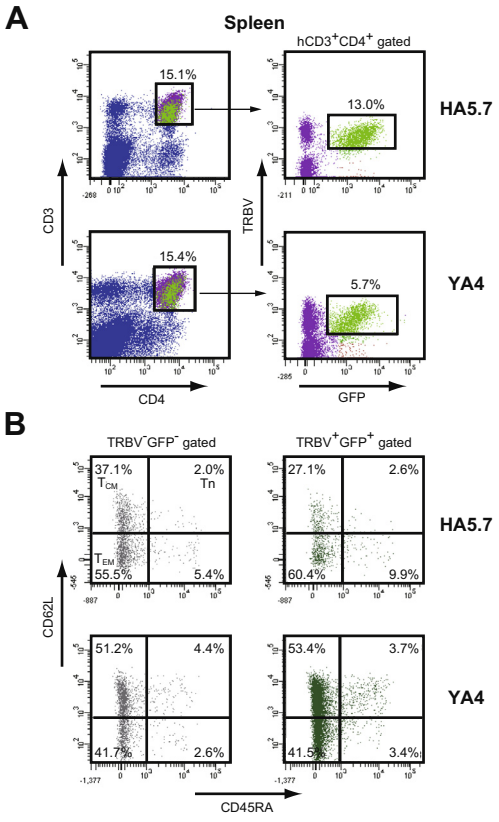

Fig. 3. (A) Accumulation of BLG-specific TCR-expressing human CD4$^+$ T cells in the spleen. Spleen cells from hu-HSC (TCR) NOG-DR4/I-A^o mice were prepared at 20–24 weeks post-transplantation and stained with anti-human CD45, CD3, CD4 and TRBV antibodies for flow cytometric analysis. (B) Staining with 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.)

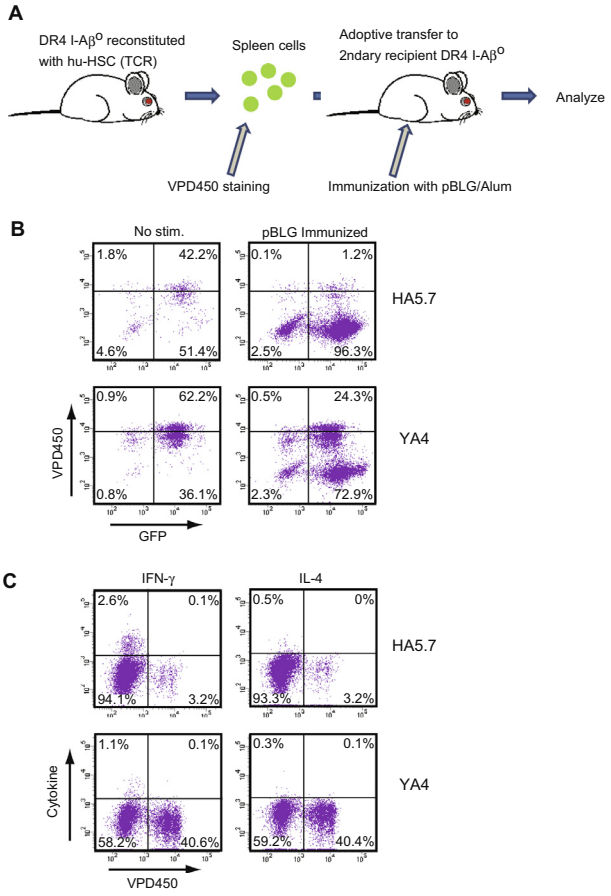

Fig. 4. (A) Schema of adoptive transfer. Spleen cells were prepared from hu-HSC (TCR) NOG-DR4/I-A^o mice at 20–24 weeks post-transplantation and were labeled with VPD450 dye. The cells were transplanted into new NOG-DR4/I-A^o mice, which were subsequently immunized with the specific BLG peptide. The 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.

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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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