

Co-Transplantation of Fetal Bone Tissue Facilitates the Development and Reconstitution in Human B Cells in Humanized NOD/SCID/IL-2R γ^{null} (NSG) Mice

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Abstract

Background In terms of the function and reconstitution efficacy of human immune cells, co-transplantation of human fetal tissues, such as thymus and liver, with CD34⁺ hematopoietic stem cells (HSCs) has potential advantages in the generation of humanized mice.

Objective and Methods To examine the effects of bone tissues in the reconstitution of human immune cells, particularly in B cells, we generated a new humanized

mice co-transplanted with human fetal thymus (hFT)/fetal bone (hFB) tissues and human fetal liver-derived CD34⁺ cells.

Results Humanized mice exhibited effective reconstitution of human immune cells earlier compared to control humanized mice. In terms of quantity, the number of immune cells, such as human T, B, and monocyte/macrophages was significantly increased. Furthermore, significant increase of B cell progenitors and immature/naïve B cells could be detected in the bone marrow and spleen of humanized mice.

Conclusion Our results demonstrate that co-transplantation of hFB tissue may facilitate the reconstitution of human B and T cells, and therefore the humanized model may be used to develop therapeutic human antibodies for clinical use.

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Introduction

Humanized mice are considered a valuable tool for developing new therapeutics and for studying human immune responses to infections and the development of human immune cells [1–11]. More than 20 years after the first successful engraftment of human leukocytes and hematopoietic organs in mice [12, 13], many different humanized mice have been reported and are being used for a variety of purposes [6–18]. Among them, recent reports have shown that transplantation of fetal human thymus and liver tissues, along with intravenous administration of CD34⁺ cells, into combined immunodeficiency

(NOD/SCID) mice leads to the development of multiple lineages of human lymphohematopoietic cells and formation of secondary lymphoid organs with normal architecture [6, 19, 20]. Furthermore, humanized mice exhibited effective antigen-specific human T cell responses and T cell-dependent production of human antibodies against the T-dependent antigen, 2,4-dinitrophenyl hapten-keyhole limpet hemocyanin (DNP23-KLH) [20]. Although the cellular and molecular mechanisms by which the T cell-dependent antibody response is facilitated in the humanized mice generated with fetal thymus/liver/CD34⁺ cells remain to be addressed, human tissue transplantation might be helpful in the development of immunocompetent human immune cells and thereby improve humanized mice for the development of therapeutics for clinical use.

In this study, we developed humanized mice transplanted with human fetal thymus (hFT)/fetal bone (hFB)/fetal liver (hFL)-derived CD34⁺ cells. Considering the essential roles of bone tissues, which provide niche environments to regulate B cell development, and the thymus in determining the major histocompatibility complex (MHC) restrictions of human T cells, we initially expected that co-transplantation of hFT and hFB tissues with hematopoietic stem cells (HSCs) may exert synergistic effects on the reconstitution of HSC-derived human immune cells, particularly T and B cells. We found that humanized mice transplanted with hFT/hFB tissues and hFL-derived hCD34⁺ cells exhibited effective reconstitution of human immune cells compared to other humanized mice transplanted with hFL-derived hCD34⁺ cells alone. Moreover, hFT/hBT-engrafted humanized mice exhibited effective reconstitution of human B cells, with associated increase of pro-B, pre-B, immature B, and naïve B cells in their bones, strongly indicating an important role for fetal bone tissue in facilitating human B cell development in mice. Taken together, the data demonstrate that hFT/hBT/hCD34⁺ cells-transplanted humanized mice might be useful for studying the entire process of human B-lymphocyte development and the production of specific human antibodies.

Methods

Mice

NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NOD-scid IL-2R γ ^{null}, NSG) mice were obtained from the Jackson laboratory (Bar Harbor, ME, USA). All mice were bred as a homozygous line and maintained under specific-pathogen-free (SPF) conditions in accordance with the ethical guidelines for the care of mice at the Laboratory Animal Research Center (LARC) at the Samsung Biomedical Research Institute in Seoul, the Republic of Korea.

Preparation of Human Fetal Thymus/Bone (Thy/Bon) and Isolation of Fetal Liver (FL)-Derived CD34⁺ Cells

Human fetal liver (FL), thymus, and bones at gestational age of 20 weeks were obtained from the department of obstetrics and gynecology, Samsung Medical Center (Seoul, Korea), along with informed parental consent, according to the guidelines of the institutional review board (IRB) established by the Samsung Medical Center. For the isolation of FL-derived CD34⁺ cells, pieces of fetal liver from the identical donor of fetal thymus and bone were collected in RPMI-1640 medium and ground using a 70- μ m nylon cell strainer (BD Falcon, Bedford, MA, USA). The cells were transferred to several new 50 ml tubes, suspended in RPMI-1640 medium (Hyclone, South Logan, UT, USA), and then centrifuged at 400 \times g for 10 min at room temperature. After washing, the cells were resuspended in RPMI-1640 medium. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation, and then hCD34⁺ cells were further purified by using CD34 MicroBead Kit (Miltenyi Biotec, GlodBach, Germany) according to the manufacturer's instructions. Purified hCD34⁺ cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human (h) lineage cocktail 1 (Becton Dickinson, Franklin Lakes, NJ, USA), phycoerythrin (PE)-conjugated anti-hCD34 (BD PharmingenTM, San Jose, CA, USA), and PE-Cyanine 7 (Cy7)-conjugated anti-hCD38 (eBioscience, San Diego, CA, USA), and then the purity was measured by flow cytometry. hLin⁺hCD34⁺hCD38⁻ cells (>95%) were used in this study.

Generation of Humanized NSG Mice

Busulfex (busulfan, Ben Benue Laboratories, Inc., Bedford, OH, USA) was dissolved in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO, USA) and diluted with 0.9% saline. The liquid busulfan solution was injected into NSG mice at a dose of 15 mg/kg body weight in a total volume of 50 μ l. Twenty-four hours after busulfex injection, FL-derived CD34⁺ cells (2×10^5) isolated from the same donor of fetal thymus and bone were intrahepatically injected into conditioned neonatal NSG mice. For the generation of humanized mice transplanted with human fetal thymus/bone (Thy/Bon), FL-derived CD34⁺ cells (2×10^5) were intrahepatically injected into conditioned neonatal NSG mice with simultaneous subcutaneous implantation of 1 mm³ pieces of human fetal thymus and bone (Thy/Bon).

Flow Cytometric Analysis

Peripheral blood mononuclear cells (PBMCs) were collected from tail veins 8, 12, and 16 weeks after transplantation. Red blood cells (RBCs) were removed with 1 \times lysis buffer (eBioscience) according to the manufacturer's instructions,

and cells were stained with antibodies mentioned below. Flow cytometric analysis was then performed. Briefly, 2 ml of pre-heated $1\times$ RBC lysis buffer was added to ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO, USA) coated tube containing 200 μ l of hu-NSG mice whole blood and then inverted to mix. After incubating for 10 min, samples were centrifuged at $400\times g$ for 5 min. After washing, cell pellets were resuspended in $1\times$ PBS containing 0.2% bovine serum albumin (BSA, Invitrogen, CA, USA), 0.05% sodium azide and analyzed by FACS Aria (BD Biosciences, CA, USA). Twenty weeks after transplantation, the hu-NSG mice were sacrificed. Their bone marrow (BM), spleen, and liver were subsequently isolated. The BM and spleen cells were treated with $1\times$ RBC lysis buffer (eBioscience) to remove the RBCs. Pieces of liver derived from each humanized mouse were pressed through a 70- μ m nylon cell strainer (BD Falcon), and then cells were collected by centrifugation at $400\times g$ for 5 min. The cells were washed several times with RPMI-1640 medium, and then mononuclear cells (MNCs) were isolated by Ficoll-Hypaque density gradient centrifugation. The isolated cells were suspended in $1\times$ PBS containing 0.2% BSA and 0.05% sodium azide and subjected to flow cytometry. For flow cytometry analysis, cells were stained with the following antibodies: FITC-conjugated anti-human (h) CD45, -hCD34, and -hIgD; PE-conjugated anti-hIgM and anti-hCD20; peridinin chlorophyll protein (PerCP)-Cyanine (Cy) 5.5-conjugate anti-hCD19; PE-Cy7-conjugated anti-hCD3 and -hCD19; allophycocyanin (APC)-conjugated hCD19. These antibodies were purchased from eBioscience. Samples were incubated with an appropriate volume of the indicated antibodies for 30 min on ice. Stained cells were acquired on FACS Aria (BD Biosciences, San Jose, CA, USA) and analyzed with FACS Diva software (BD Biosciences).

Statistical Analysis

Data are expressed as mean \pm SD. The Student's *t*-test was used to calculate statistical differences, and statistical significance was defined by $p < 0.05$. All statistical analyses were conducted using GraphPad Prism v5.01 software (GraphPad Software Inc., CA, USA).

Results

Generation of humanized NOD/SCID/IL-2R γ^{null} (NSG) mice co-transplanted with human fetal thymus (hFT)/human fetal bone (hFB), and human fetal liver (hFL)-derived CD34 $^{+}$ cells.

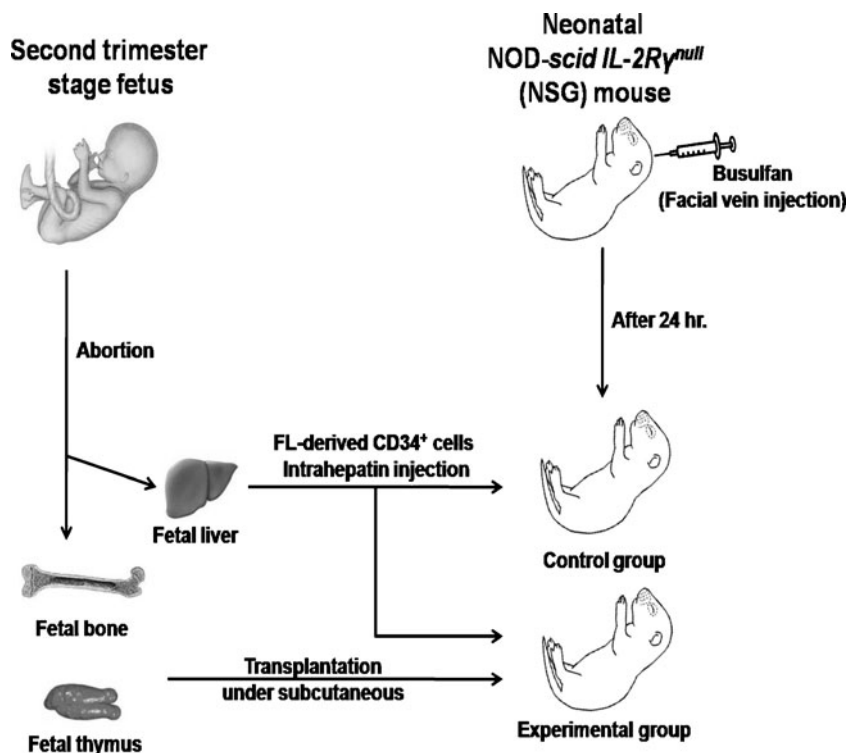
Previous studies have shown that grafting hFT/ hFL tissues into NOD/SCID mice can lead to successful reconstitution of human immune cells *in vivo* [6, 19, 20].

We have now explored the possibility that engraftment of human fetal bone (hFB) tissue facilitates the reconstitution human B cells in humanized mice. We therefore designed a humanized mouse model in which hFT/hFB tissues and hFL-derived CD34 $^{+}$ cells were engrafted. As shown in Fig. 1, human fetal liver, bone, and thymus tissues were obtained from the second trimester stage fetus, and human CD34 $^{+}$ cells were purified from fetal liver tissues as described in “Methods”. Neonatal NSG mice were pre-conditioned with 15 mg/kg busulfan, and then intrahepatically injected with hFL-derived CD34 $^{+}$ cells [18]. For the transplantation of fetal tissues, hFT and hFB tissues were freshly sectioned to a size of 1 mm 3 within 24 h after abortion, and simultaneously transplanted into neonatal NSG mice after the injection of hFL-derived CD34 $^{+}$ cells as described in “Methods”. To determine an effective reconstitution time for human immune cells, peripheral blood was sampled from humanized mice engrafted with hFL-derived CD34 $^{+}$ cells alone (named by “Cells”) or hFT/hFB tissues and hFL-derived CD34 $^{+}$ cells (named by “Tissues+Cells”) at different times as indicated Fig. 2. Reconstitutions of hCD45 $^{+}$, hCD45 $^{+}$ hCD3 $^{+}$ T, and hCD45 $^{+}$ hCD19 $^{+}$ B cells were then analyzed by flow cytometry. The hCD45 $^{+}$ cells appeared in significant amount in both humanized mice lines in a time-dependent manner (Fig. 2a, b). Reconstitution was highly effective and occurred at an earlier time (from 8 weeks after transplantation) in fetal tissue-engrafted mice compared to humanized mice generated by hFL-derived CD34 $^{+}$ cells (Fig. 2c: closed bars, Tissues+Cells; open bars, Cells alone). Moreover, when we measured the proportions of human T (represented by hCD45 $^{+}$ hCD3 $^{+}$) and B (represented by hCD45 $^{+}$ hCD19 $^{+}$) cells in the hCD45 $^{+}$ population, the balanced proportion of human T and B cells in tissue-engrafted mice was proficiently established 8 weeks after transplantation and significantly maintained until 16 weeks (Fig. 2b, d). These results indicate that engraftment of hFT and hFB tissues induced effective reconstitution of human T and B cells.

Human Fetal Thymus and Bone Tissues Enhance Differentiation of Human T and B Cells in Humanized Mice

Since the reconstitution of human T cells in peripheral blood was more efficient in hFT and hFB tissues-transplanted mice, we examined whether the effect was associated with transplanted hFT. Based on the kinetic analysis shown in Fig. 2, humanized mice were sacrificed 20 weeks after transplantation, and then thymocytes were isolated and stained with different antibodies to evaluate T cell differentiation. The total numbers of thymocytes were significantly higher in tissue-engrafted humanized mice

Fig. 1 Schemes for the generation of humanized-NSG (hu-NSG) mice. Neonatal NSG mice were injected with 15 mg/kg busulfan into the facial vein. hCD34⁺ cells were purified from fetal liver (FL) of second trimester stage fetus, as described in “Methods”. As a control group ($n=5$), 2×10^5 FL-derived hCD34⁺ cells were injected intrahepatically into neonatal NSG mice conditioned with busulfan. As an experimental group ($n=5$), 2×10^5 FL-derived CD34⁺ cells were injected intrahepatically into neonatal NSG mice conditioned with busulfan and fetal tissues (fetal bones and thymuses) were simultaneously implanted subcutaneously in the conditioned neonatal NSG mice, as described in “Methods”



than in humanized mice generated by hFL-derived CD34⁺ cells alone (Fig. 3b, bar graph). The proportion of hCD45⁺hCD3⁺hCD8⁺hCD4⁻ single-positive (CD8 SP), and hCD45⁺hCD3⁺hCD8⁻hCD4⁺ single-positive (CD4 SP) cells were significantly increased in tissue-engrafted humanized mice (Fig. 3a, b; $33 \pm 3\%$ vs. $23 \pm 2\%$ in hCD8 SP and $22 \pm 2\%$ vs. $10 \pm 1\%$ in hCD4 SP). The absolute quantity of hCD4⁺ SP and hCD8⁺ SP cells was markedly higher in tissue-engrafted humanized mice than in humanized mice generated by hFL-derived CD34⁺ cells alone (Fig. 3c, bar graphs of hCD4⁺ SP and hCD8⁺ SP, respectively). Although the proportion of hCD45⁺hCD3⁺hCD8⁺hCD4⁺ (DP) was reduced in tissue-engrafted humanized mice (Fig. 3a, b), the absolute number of cells was significantly higher than humanized mice generated by hFL-derived CD34⁺ cells alone (hCD4⁺hCD8⁺ DP in Fig. 3c). It might have been due to increase in total thymocytes, as shown in Fig. 3b. The results suggest that, although engrafted fetal tissues may impact development of immature T cells originated from the tissues, engraftment of fetal thymus tissue induces human T cell differentiation in the thymus of humanized mice.

We next assessed the impact of engrafted hFB tissue on B cell differentiation. Mononuclear cells from bone marrow were isolated and stained with antibodies specific for hCD45, hCD3, hCD19, hCD11b, and hCD14. The proportion of hCD45⁺ cells was markedly higher in tissue-engrafted humanized mice than in humanized mice generated by hFL-derived CD34⁺ cells alone (Fig. 3d, e; $84 \pm 6\%$ vs. $58 \pm 5\%$). Moreover,

hCD45⁺hCD19⁺ B cells, hCD45⁺hCD3⁺ T cells, and hCD45⁺hCD11b⁺hCD14⁺ macrophages were significantly increased (Fig. 3d, e; $56 \pm 4\%$ vs. $47 \pm 3\%$ in B cells; $10 \pm 2\%$ vs. $2 \pm 0.3\%$ in T cells; $4 \pm 2\%$ vs. $1 \pm 0.2\%$ in macrophages). In addition, the absolute numbers of cells were also increased in tissue-engrafted humanized mice (Fig. 3f). The results indicate that engrafted bone tissue enhances human B cell development and facilitates reconstitution of human immune cells in the bone marrow.

Efficient Reconstitution of Human Immune Cells Occurred in Liver and Spleen of Tissue-Engrafted Humanized Mice

hFT and hFB tissue engraftments induced T and B cell differentiation in the thymus and bone marrow, respectively, of humanized mice (Fig. 3). We subsequently assessed whether reconstitution of human immune cells appeared in the liver and spleen of the tissue-engrafted humanized mice. The liver-resident mononuclear cells were isolated and stained with antibodies specific for hCD45, hCD3, hCD19, hCD20, hCD11b, and hCD14. As shown in Fig. 4, reconstitution of hCD45⁺ cells in the liver was significantly higher in tissue-engrafted humanized mice than in humanized mice generated by hFL-derived CD34⁺ cells. The proportion and absolute number of hCD45⁺hCD3⁺ T and hCD45⁺hCD19⁺ B cells were markedly higher in tissue-engrafted humanized mice than in humanized mice generated by hFL-derived CD34⁺ cells alone (Fig. 4a, b, second dot plots; $27 \pm 2\%$ vs. $18 \pm 3\%$ in hCD45⁺hCD3⁺ T cells and $27 \pm 3\%$ vs. $10 \pm 2\%$ in

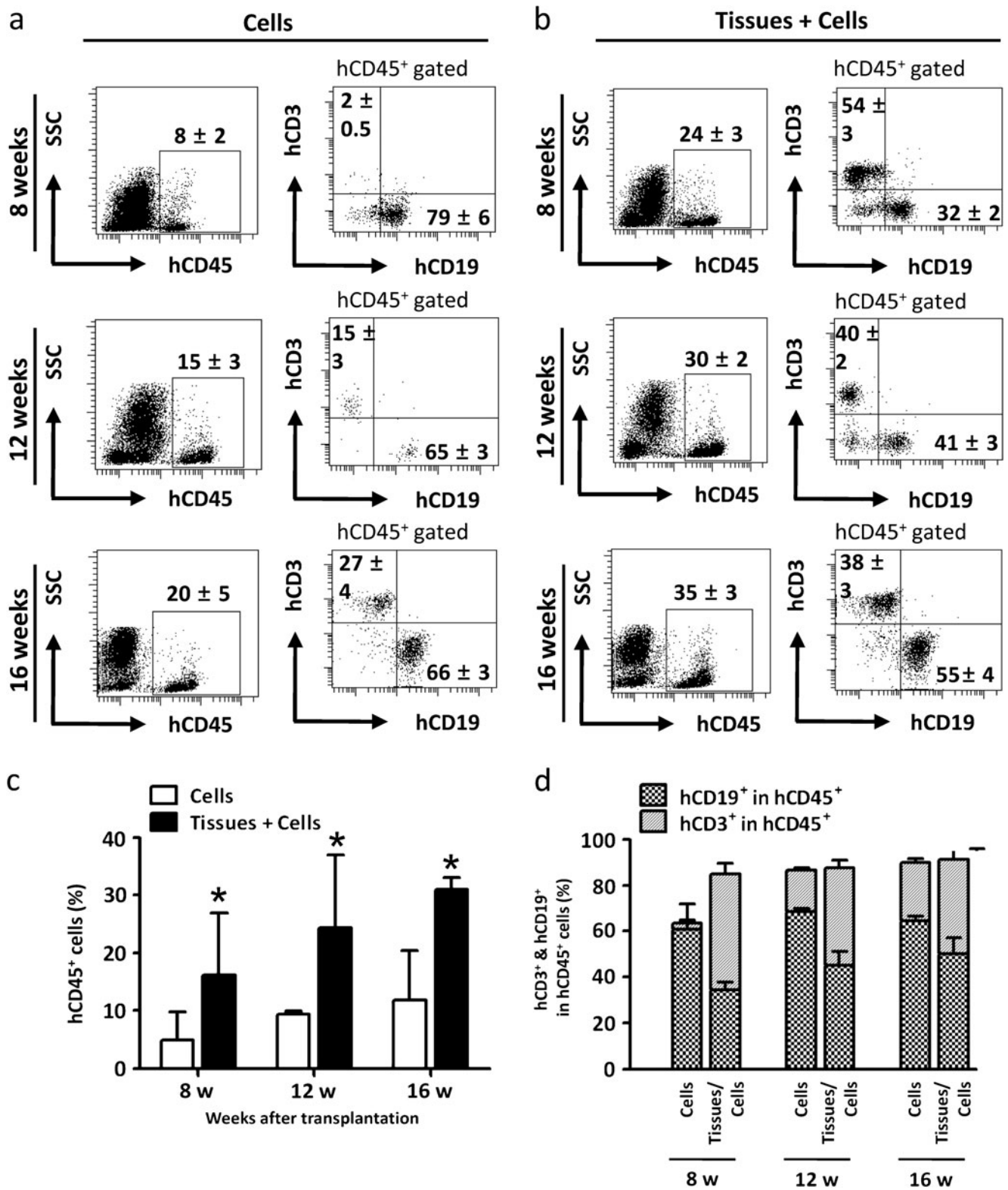
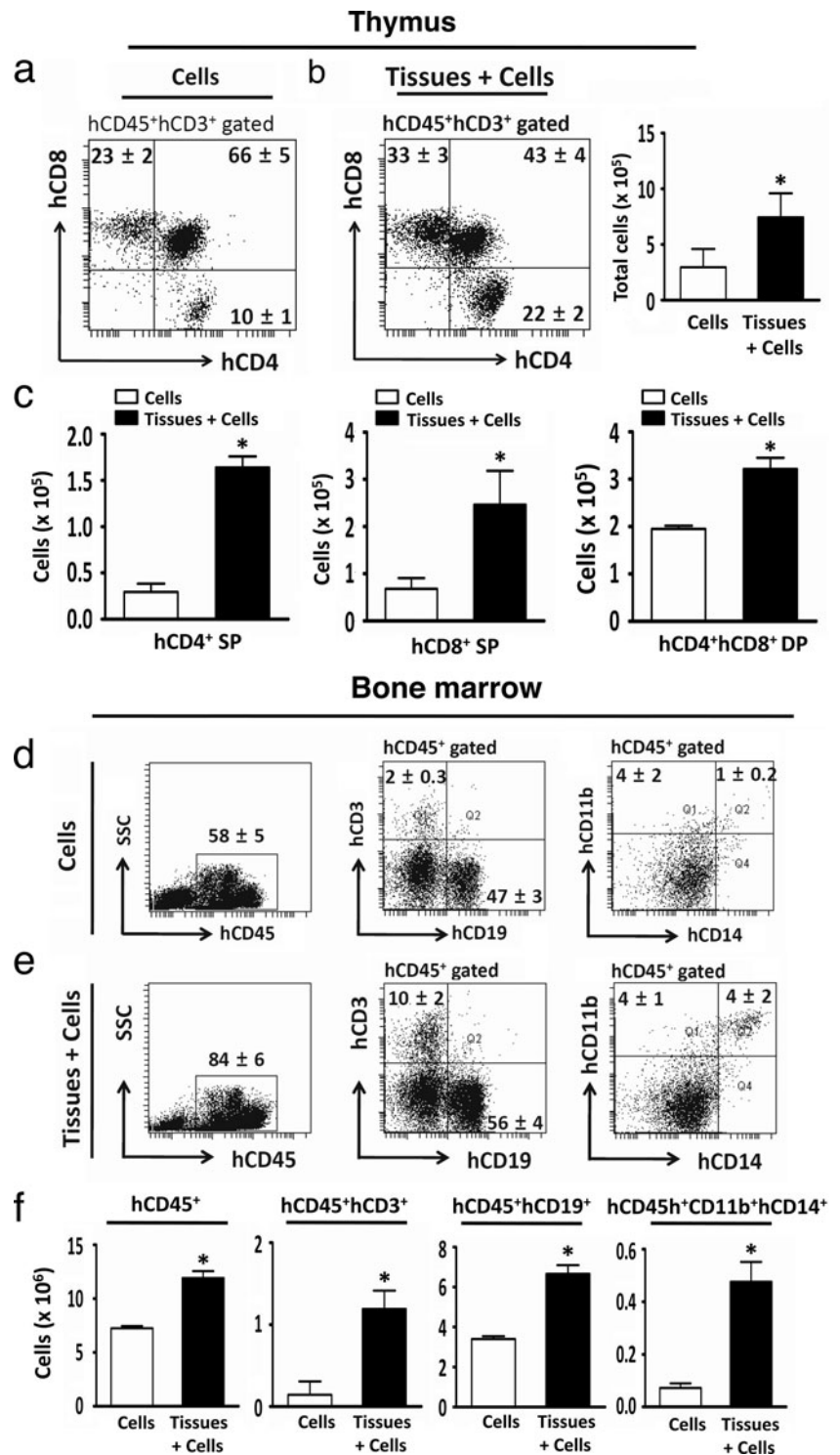


Fig. 2 Kinetic analysis of reconstituted human T and B cells in the peripheral blood of humanized NSG mice. Peripheral blood was sampled from the tails of each group ($n=5$) of humanized NSG mice at indicated time points. PBMCs were stained with antibodies specific for hCD45, hCD3, and hCD19. After hCD45⁺ cells were gated in a dot plot of SSC vs. hCD45, the gated cells were further analyzed by

the expression of hCD3 vs. hCD19 (**a**, humanized mice generated with FL-derived hCD34⁺ cells alone; **b**, humanized mice generated with fetal Thy/Bon tissues and FL-derived hCD34⁺ cells). The proportion of hCD45⁺ cells and the absolute number of hCD45⁺hCD3⁺ and hCD45⁺hCD19⁺ are presented in **c** and **d**, respectively. Data are mean ±SD in humanized mice ($n=5$, each group); * $p<0.05$)

Fig. 3 Analysis of human T and B cell development in the thymuses and bone marrow of humanized NSG mice. At 20 weeks after transplantation, thymuses were isolated from humanized mice generated with FL-derived hCD34⁺ cells alone (a) and humanized mice generated with fetal Thy/Bon tissues and FL-derived hCD34⁺ cells (b). Total thymocytes were counted and stained with antibodies specific for hCD45, hCD3, hCD4, and hCD8. After hCD45⁺hCD3⁺ cells were gated in a dot plot of hCD45 vs. hCD3, the gated cells were further analyzed by the expression of hCD4 vs. hCD8. c The absolute numbers of hCD45⁺hCD3⁺hCD4⁺hCD8⁻ (CD4 SP), hCD45⁺hCD3⁺hCD4⁻hCD8⁺ (CD8 SP), and hCD45⁺hCD3⁺hCD4⁺hCD8⁺ (DP) cells are presented. Data are mean±SD in humanized mice (*n*=5, each group; **p*<0.05). Twenty weeks after transplantation, bone marrow (d, e, and f) was isolated, mononuclear cells were purified as described in “Methods”. The cells were counted, stained with antibodies specific for hCD45, hCD3, hCD14, hCD19, and hCD11b, and analyzed by flow cytometry. After hCD45⁺ cells were gated in a dot plot of SSC vs. hCD45, the gated cells were further analyzed by the expression of hCD3 vs. hCD19 and hCD14 vs. hCD11b. The absolute numbers of hCD45⁺, hCD45⁺hCD3⁺, hCD45⁺hCD19⁺, and hCD45⁺hCD11b⁺ cells are presented. Data are mean±SD in humanized mice (*n*=5, each group; **p*<0.05)



hCD45⁺hCD19⁺ B cells; Fig. 4c, second and third bar result, respectively). Consistent and significant increases of hCD45⁺hCD19⁺hCD20⁻ (4±1% vs. 2±1%) and hCD45⁺hCD19⁺hCD20⁺ B cells (24±2% vs. 9±1%) could be detected in the liver of tissue-engrafted humanized mice

(Fig. 4a, b). The absolute number of cells were also increased (Fig. 4c). In the spleen of humanized mice, the reconstitution efficacy of hCD45⁺ cells was significantly higher in tissue-engrafted humanized mice than in humanized mice generated with hFL-derived CD34⁺ cells alone

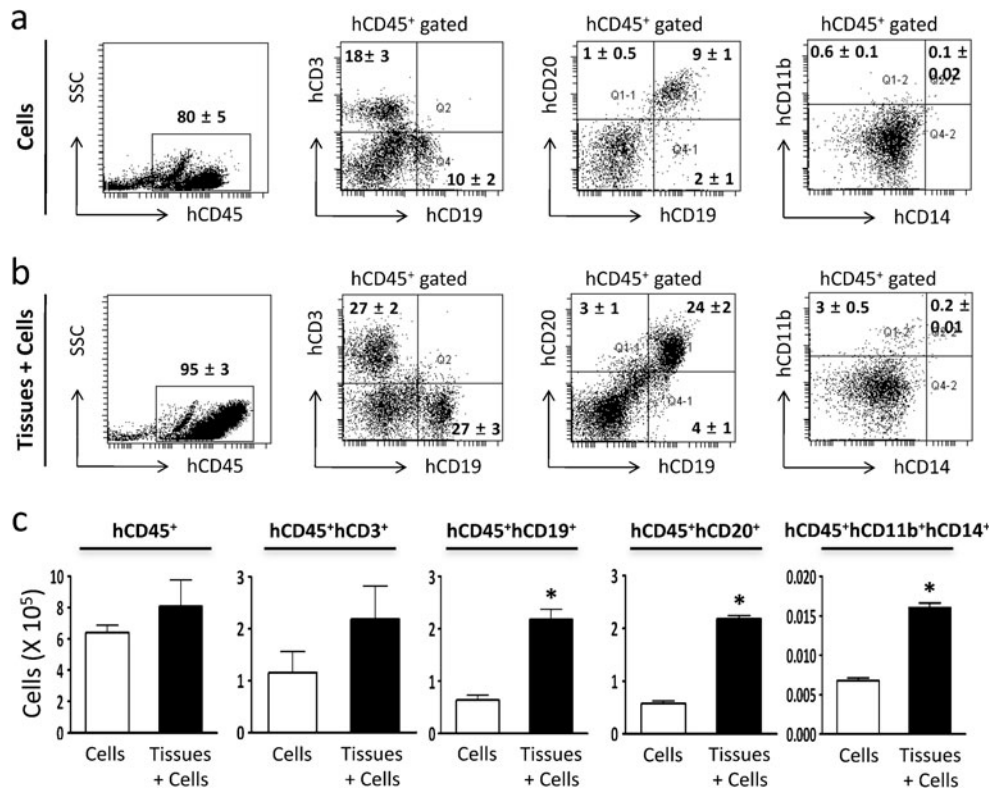


Fig. 4 Reconstitution of human cells in the liver of humanized NSG mice. Twenty weeks after transplantation, liver tissues were isolated. Mononuclear cells were purified as described in “Methods”. Cells were counted, stained with antibodies specific for hCD45, hCD3, hCD14, hCD19, hCD20, and hCD11b, and analyzed by flow cytometry. After hCD45⁺ cells were gated in a dot plot of SSC vs. hCD45, the gated cells were further analyzed by the expression of

hCD3 vs. hCD19 and hCD14 vs. hCD11b (a, humanized mice generated with FL-derived hCD34⁺ cells alone; b, humanized mice generated with fetal Thy/Bon tissues and FL-derived hCD34⁺ cells). The absolute numbers of hCD45⁺, hCD45⁺hCD3⁺, hCD45⁺hCD19⁺, and hCD45⁺hCD11b⁺ cells are presented (c). Data are mean±SD in humanized mice (n=5, each group; *p<0.05)

(Fig. 5a,b, first dot plots; 79±3% vs. 55±4%; Fig. 5c, first bar result). hCD45⁺hCD3⁺ T cells were also dramatically higher in tissue-engrafted humanized mice than in humanized mice generated by hFL-derived CD34⁺ cells alone (Fig. 5a, b, second dot plots; 20±2% vs. 9±2%; Fig. 5c, second bar result). Although little difference in the proportion of cells could be detected, the absolute number of hCD45⁺hCD19⁺ B cells, hCD45⁺hCD20⁺ B cells, and hCD45⁺hCD11b⁺ hCD14⁺ macrophages was markedly higher in tissue-engrafted humanized mice than in humanized mice generated with hFL-derived CD34⁺ cells alone (Fig. 5a–c). These results demonstrate that co-transplantation of human fetal thymus and fetal bone tissues might effectively induce reconstitution of human immune cells in tissue and organs.

Analysis of Human B Cell Maturation and Development in the Bone Marrow and Spleen of Tissue-Engrafted Humanized Mice

As shown in Figs. 4 and 5, reconstitution of human B cells occurred efficiently in tissue-engrafted humanized mice.

Based on the results, we further examined whether fetal bone tissue affected the developmental and differentiation stages of B cells. Mononuclear cells derived from bone marrow and spleen were isolated as described in “Methods” and then stained with antibodies specific for hCD19, hCD10, hCD34, hIgM, hCD27, and hIgD to identify pre-B cells (represented by hCD19⁺hCD10⁺hCD34[−]hIgM[−]), pro-B cells (represented by hCD19⁺hCD10⁺hCD34⁺hIgM[−]), memory B cells (represented by hCD19⁺hIgM⁺hCD27⁺hIgD[−]), activated B cells (represented by hCD19⁺hIgM⁺hCD27[−]hIgD⁺), immature B cells (represented by hCD19⁺hIgM⁺hCD27[−]hIgD[−]), and naïve B cells (represented by hCD19⁺hIgM[−]hCD27[−]hIgD[−]) [10]. There were no significant changes in the proportion of their subset cells in the bone marrow and spleen (dot plots: bone marrow, Fig. 6a, b; spleen, Fig. 6c, d). However, the absolute number of pro-B, pre-B, immature B, and naïve B cells was significantly increased in the bone marrow of hFB tissue-transplanted humanized mice compared to that in humanized mice generated with hFL-derived CD34⁺ cells (Fig. 6a, b, bar graph). Similarly, in the spleen, the absolute number of pre-B, memory B, activated B, and naïve B cells were significantly higher in hFB tissue-transplanted human-

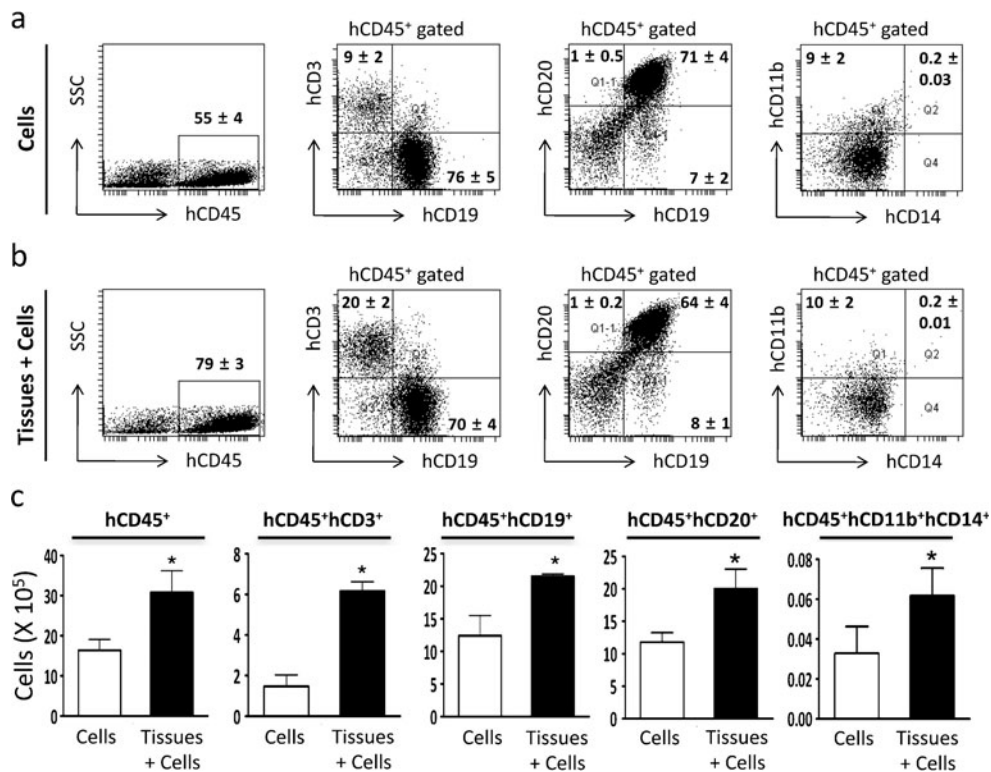


Fig. 5 Reconstitution of human cells in the spleen of humanized NSG mice. Twenty weeks after transplantation, spleens were isolated, and then splenocytes were purified as described in “Methods”. The cells were counted, stained with antibodies specific for hCD45, hCD3, hCD14, hCD19, hCD20, and hCD11b, and analyzed by flow cytometry. After hCD45⁺ cells were gated in a dot plot of SSC vs. hCD45, the gated cells were further analyzed by the expression of

hCD3 vs. hCD19 and hCD14 vs. hCD11b (**a**, humanized mice generated with FL-derived hCD34⁺ cells alone; **b**, humanized mice generated with fetal Thy/Bon tissues and FL-derived hCD34⁺ cells). The absolute numbers of hCD45⁺, hCD45⁺hCD3⁺, hCD45⁺hCD19⁺, and hCD45⁺hCD11b⁺ cells are presented (**c**). Data are mean±SD in humanized mice ($n=5$, each group; $*p<0.05$)

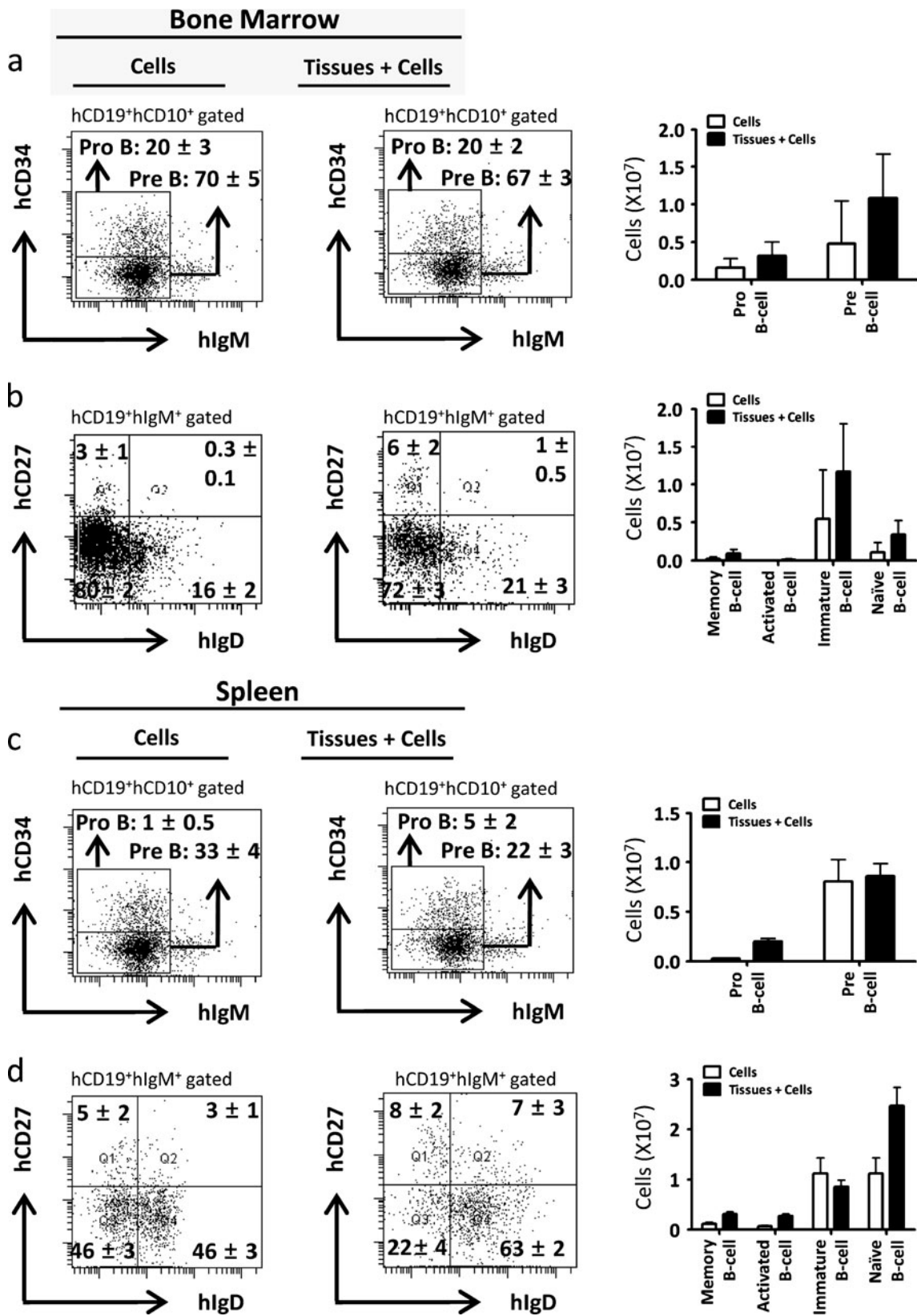
ized mice than in humanized mice generated with FL-derived CD34⁺ cells (Fig. 6c, d, bar graph). These results strongly indicate that transplantation of human fetal bone tissue facilitate B cell development and differentiation at a quantitative level, thereby enhance the effective reconstitution in periphery, tissues, and organs of humanized mice.

Discussion

The outcomes of this study demonstrate that humanized mice generated by co-transplantation of human fetal thymus/bone tissues and fetal liver-derived CD34⁺ cells can effectively induce reconstitution and differentiation of human T and B cells. The major advantage of human tissue-engrafted mice is that human immune cells develop proficiently in an autologous human environment. Recent studies have shown that engraftment of autologous human fetal thymic tissue could critically contribute to the generation of a diverse V β -T cell antigen receptor (TCR) T cell repertoire by inducing de novo thymopoiesis and overall T cell homeostasis [5]. Moreover, human T cells that

developed in human thymic-engrafted mice generate human major histocompatibility complex class I- and class II-restricted adaptive immune responses to Epstein-Barr Virus (EBV) infection, suggestive that the engrafted fetal thymic

Fig. 6 Analysis of human B cell progenitors and mature B cells in the bone marrow and spleens of humanized NSG mice. Twenty weeks after transplantation, bone marrow (**a** and **b**) and spleens (**c** and **d**) were isolated from humanized mice generated with FL-derived hCD34⁺ cells alone and humanized mice generated with fetal Thy/Bon tissues and FL-derived hCD34⁺ cells, and then mononuclear cells were purified as described in “Methods”. The cells were counted, stained with antibodies specific for hCD19, hCD10, hCD34, hCD27, hIgM, and hIgD, and analyzed by flow cytometry. After hCD19⁺hCD10⁺ cells were gated in a dot plot of hCD19 vs. hCD10, the gated cells were further analyzed by the expression of hCD34 vs. hIgM (**a** and **c**, dot plots). The absolute numbers of hCD19⁺hCD10⁺hCD34⁺hIgM⁻ (Pro-B cells) and hCD19⁺hCD10⁺hCD34⁻hIgM⁻ (pre-B cells) are presented (**a** and **c**, bar graphs). After hCD19⁺hIgM⁺ cells were gated in a dot plot of hCD19 vs. hIgM, the gated cells were further analyzed by the expression of hCD27 vs. hIgD (**b** and **d**, dot plots). The absolute numbers of hCD19⁺hIgM⁺hCD27⁺hIgD⁻ (memory B), hCD19⁺hIgM⁺hCD27⁺hIgD⁺ (activated B), hCD19⁺hIgM⁺hCD27⁻hIgD⁻ (immature B) and hCD19⁺hIgM⁺hCD27⁻hIgD⁺ (naïve B) cells are presented (**b** and **d**, bar graphs). Data are mean±SD in humanized mice ($n=5$, each group)



tissue plays essential roles in the generation and maintenance of human T cells [5]. Although humanized mouse models are expected to be a valuable tool for studying the

human immune system and development of therapeutics targeted to human diseases, a critical hurdle for the functional reconstitution of human immune cells has been

reported [10, 21–24]. To induce successful immune reaction against exogenous antigens in humanized mice, immunological cooperation between humoral and cellular response is absolutely required. However, current humanized murine models present a challenge in humoral responses mediated by human B cells [21–24]. It appears to be related to the skewing of human B cells towards B-1 cell lineages, the lack of interaction between B and T cells due to mismatch of MHC molecules, and functional abnormalities [23]. To improve the current humanized model, we tried to make a new humanized murine model transplanted with fetal bone tissues to facilitate human B cells, along with fetal thymus tissues to support human T cells. The transplantation of autologous human fetal liver-derived CD34⁺ cells into NSG mice previously implanted with human fetal thymus and bone tissues results in systemic repopulation with human immune cells, including T and B cells. Interestingly, in contrast to the humanized mice generated by human fetal liver-derived CD34⁺ cells, humanized mice engrafted with human fetal thymus and bone tissues demonstrated enhancement of human B cell maturation, differentiation, and reconstitution in both mouse tissues and lymphoid organs. However, it is not completely ruled out that progenitor or lymphoid cells contained in human fetal thymus and bone tissues may be contributed to reconstitute human immune cells, such as human T and B cells, in the humanized mice. In regard to the issue, previous reports have shown that transplantation of NOD/SCID mice with human CD34⁺ cells results in much higher levels of systemic repopulation with human cells [25–27]. Despite the higher degree of human reconstitution, however, T cells generally fail to develop in this system [9, 28]. In contrast to the lack of human T cells in SCID or NOD/SCID mice transplanted with purified CD34⁺ cells, there is an abundance of human T cells in SCID mice previously implanted with human fetal thymic and liver tissues (SCID-hu thy-liv mice) [13, 29]. Although practical experiments that have been performed are absolutely required to rule out the contributory role of progenitor or lymphoid cells contained in the engrafted human tissue [30–32], these results suggest that the human fetal thymic tissue implantation might support or facilitate functional and efficient reconstitution of human T cells in the humanized mice. Along with these previous observations, we propose at least two hypotheses on how the engrafted human fetal thymus and bone tissue facilitate the reconstitution and development of human B and T cells in humanized mice: one is that niche environments to be provided by human fetal tissues that facilitate the development provided by the tissues might be involved in the effective reconstitution of T and B cells, similar to the functional role of engrafted human thymus in the development of human T cells [5, 33–35]. Another is that

progenitor, lymphoid, or HSCs present in human fetal bone and thymus tissues may also contribute to the reconstitution of human immune cells in humanized mice. Therefore, further studies that focus on these issues mentioned above must be undertaken. To apply for biomedical and scientific research fields as a humanized animal model, moreover, functional studies related to the production of successful IgG against exogenous antigens must be tested in the humanized mice. Taken together, we anticipate that humanized mice engrafted fetal bone tissues could be developed into a good model to investigate the mechanisms involved in the proper differentiation of human B cells and the study of the human immune system requiring B cell function.

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