Systemic Human T Cell Developmental Processes in Humanized Mice Cotransplanted With Human Fetal Thymus/Liver Tissue and Hematopoietic Stem Cells

Sung-Yeon Joo,[1,2] Yun Shin Chung,[1] Bongkum Choi,[1,2] Miyoung Kim,[1] Jong-Hwa Kim,[5] Tae-Gook Jun,[6] Jun Chang,[7] Jonathan Sprent,[8] Charles D. Surh,[9] Jae-won Joh,[4] and Sung Joo Kim[1,2,4,10]

Background. In many humanized mouse models, there are few T cells in the engrafted human cell, whereas the number of B cells is high. We attempted to overcome this limitation and investigate whether the entire process of human T cell development arose similarly to the process in humans, as previously reported.
Methods. To produce an advanced humanized mice model, we transplanted human fetal liver/thymus tissue subrenally and injected human $CD34^+$ stem cells intravenously into NOD/SCID/IL2Rgamma null (NSG) mice.
Results. Humanized mice transplanted with fetal thymus/liver tissues and fetal liver-derived $CD34^+$ stem cells (FLT+FLCD34) showed higher levels of human cells and T cells than mice transplanted with fetal liver-derived $CD34^+$ stem cells only (FLCD34). In the transplanted thymus tissue of FLT+FLCD34 mice, thymus seeding progenitors (TSPs), early thymic progenitors (ETPs), pre-T cells, and all the other human T cell populations were identified. In the periphery, FLT+FLCD34 mice have high levels of $CD45RA^+$ T cells; conversely, FLCD34 mice have higher levels of $CD45RO^+$ T cells. The $CD45RO^+$ T cells of FLCD34 mice proliferated rapidly after stimulation and exhibited innate T cells properties, expressing PLZF (promyelocytic leukemia zinc finger protein).
Conclusion. Human T cells educated by mouse MHC II in mice without a human thymus differ from normal human T cells. On the basis of these findings, numerous T cell-tropic human diseases could be explored in our humanized mice and molecular aspects of human T cell development could be also studied extensively.

Keywords: Human T cell development, Fetal thymus/liver, NOD/SCID/IL2Rgamma null mice, Innate T cell.

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Recently, a number of groups have attempted to develop animal models that possess a human immune system by various immunodeficient mice. When Mamoru Ito established a highly efficient immunodeficient mouse model, NOD/SCID/$\gamma_c^{-/-}$, studies using humanized mouse models were accelerated in many fields (*1–3*). Many researchers have generated humanized mice models by transplanting human hematopoietic stem cells from various sources, such as umbilical cord blood, human bone marrow, and fetal liver (*2, 4*). The levels of human cell engraftment in the periphery of those humanized mice are high compared with those of NOD/SCID mice.

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[1] Transplantation Research Center, Samsung Biomedical Research Institute, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.
[2] Department of Molecular Medicine, Sungkyunkwan University School of Medicine, Seoul, Korea.
[3] Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Seoul, Korea.
[4] Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.
[5] Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.
[6] Department of Thoracic and Cardiovascular Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.
[7] Division of Life and Pharmaceutical Sciences and the Center for Cell Signaling and Drug Discovery Research, Ewha Womans University, Seoul, Korea.
[8] Department of Immunology, The Garvan Institute of Medical Research, Sydney, Australia.
[9] Department of Immunology and Microbial Science, The Scripps Research Institute, San Diego, CA.
[10] Address correspondence to: Sung Joo Kim M.D., Ph.D., Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong, Gangnam-gu, Seoul, 135-710, Korea.
E-mail: kmhyj111@hotmail.com

S.Y.J. designed the study, performed the experiments and analyses, and wrote the manuscript; Y.S.C. discussed the results; B.C. and M.K. assisted in the transplantations and experiment; J.H.K. provided the human fetal tissues at Samsung Medical Center; T.G.J. provided the juvenile human thymus; J.C. performed the analysis and discussed the results; J.S. discussed the results; C.D.S. discussed the results; J.J. discussed the results; and S.J.K. designed and supervised the study.

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However, in many previously published humanized models using hematopoietic stem cells, most of the initially engrafted human cells were B cells until 16 weeks after transplantation. Human T cells appeared at low levels at 12∼16 weeks after transplantation, and the level of engrafted T cells was low compared with the levels of B cells at the initial stage of transplantation (*1–3*). Moreover, Watanabe et al. (*5*) reported the insufficient function of positively selected thymic T cells in NOD/shi-scid/γ_c^{null} (NOG) mice reconstituted with $CD34^+$ hematopoietic stem cells. Based on this and other reports, many researchers have suggested that these abnormalities occur because the thymic selection of human T cells by thymic epithelial cells requires human molecules that are absent in the mouse thymus (*6, 7*). To enhance human T cell development in humanized mice, several groups have used a mouse model designed by McCune et al. (*8*). Trials in these mice show improved levels of T cell development in NOD/SCID mice that received grafts of human fetal thymus and liver tissues (*9, 10*).

In this study, we first demonstrated that the entire process of human T cell development occurs through education by human MHC molecules in our humanized mice co-transplanted with human fetal thymus/liver tissues and fetal liver-derived $CD34^+$ hematopoietic stem cells. Additionally, we showed that the development of these engrafted human T cells involved events similar to those occurring in the human thymus. Additionally, we revealed that the innate properties of human T cells grafted in mice transplanted only with hematopoietic stem cells significantly differ from those of T cells grafted in mice cotransplanted with fetal tissue and hematopoietic stem cells.

RESULTS

Increase of Human T Cells in Human Fetal Thymus/Liver Tissues and Fetal Liver-Derived $CD34^+$ Hematopoietic Stem Cells Co-Transplanted NSG Mice

Human fetal thymus/liver tissues were implanted in the kidney capsules of NSG mice and fetal liver-derived $CD34^+$ hematopoietic stem cells were cotransplanted intravenously. Figure 1A shows the percentage increases of human $CD45^+$ cells in the peripheral blood of FLCD34 mice and FLT+FLCD34 mice. Up to 24 weeks after transplantation, the average human cell engraftment for FLT+FLCD34 mice was 1.6–7.25-fold higher than FLCD34 mice at every time point.

Figure 1(B and C) shows the differences between FLT+FLCD34 and FLCD34 with respect to the ratios of human T cells and B cells among the total reconstituted human $CD45^+$ cells in FLT+FLCD34 and FLCD34 mice. Substantial amounts of human T cells were detected in the periphery of FLT+FLCD34 mice from 4 weeks after transplantations, and these levels increased up to 24 weeks after transplantation. In contrast, in FLCD34 mice, small percentages of human T cells were first detected in the periphery at 8 weeks after transplantation and increased very slowly. Additionally, instead of T cells, relatively high percentages of B cells were present in FLCD34 mice. The higher percentages of T cells in FLT+FLCD34 mice were also observed in the bone marrow, spleen, liver, and lymph nodes (see **Figure S1**, **SDC**, http://links.lww.com/TP/A720). During the entire period of the experiments, no mice showed symptoms of graft-versus-host disease (GvHD), such as rash, diarrhea, or a hunched back.

Enlargement of Implanted Human Fetal Thymus

Human fetal thymic tissues implanted in the kidney capsule of NSG mice were enlarged at 36 weeks after transplantation (Fig. 2Aa, Ab). At 12 weeks after transplantation, the cortical and medullary regions could be visualized, and the number of lobules was increased (Fig. 2Bb). In addition, they were more distinguishable after an additional 10 weeks (Fig. 2Bc). This enlargement of implanted human fetal thymus tissue and the appearance of a thymic structure reflect the vigorous selections and maturation of T cells. In Figure 2C, we confirmed the existence of human medullary thymic epithelial cells (mTECs). As shown in Figure 2C, cytokeratin 8 (CK8) was expressed in human mTECs, Hassall's corpuscles (a) and the subcapsular region (b) of juvenile human thymus tissue. Likewise, CK8 was expressed in mTECs, Hassall's

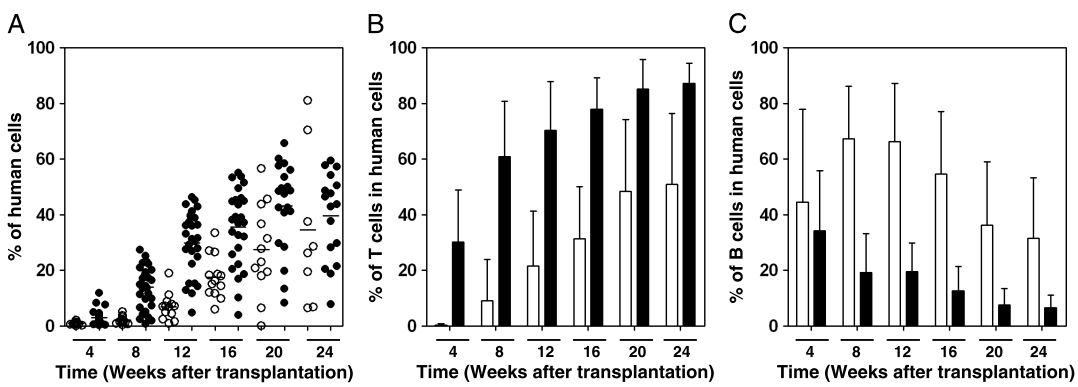

FIGURE 1. Comparison of human cell engraftment levels and the proportion of T cells and B cells in FLCD34 (n=10) and FLT+FLCD34 mice (n=25). (A) Time course of the reconstitution of human $CD45^+$ cells in the peripheral blood (○: FLCD34, •: FLT+FLCD34). (B) Time course of changes in human $CD3^+$ T cells in the peripheral blood. (C) Time course of changes in human $CD19^+$ B cells in the periphery (□: FLCD34, ■: FLT+FLCD34).

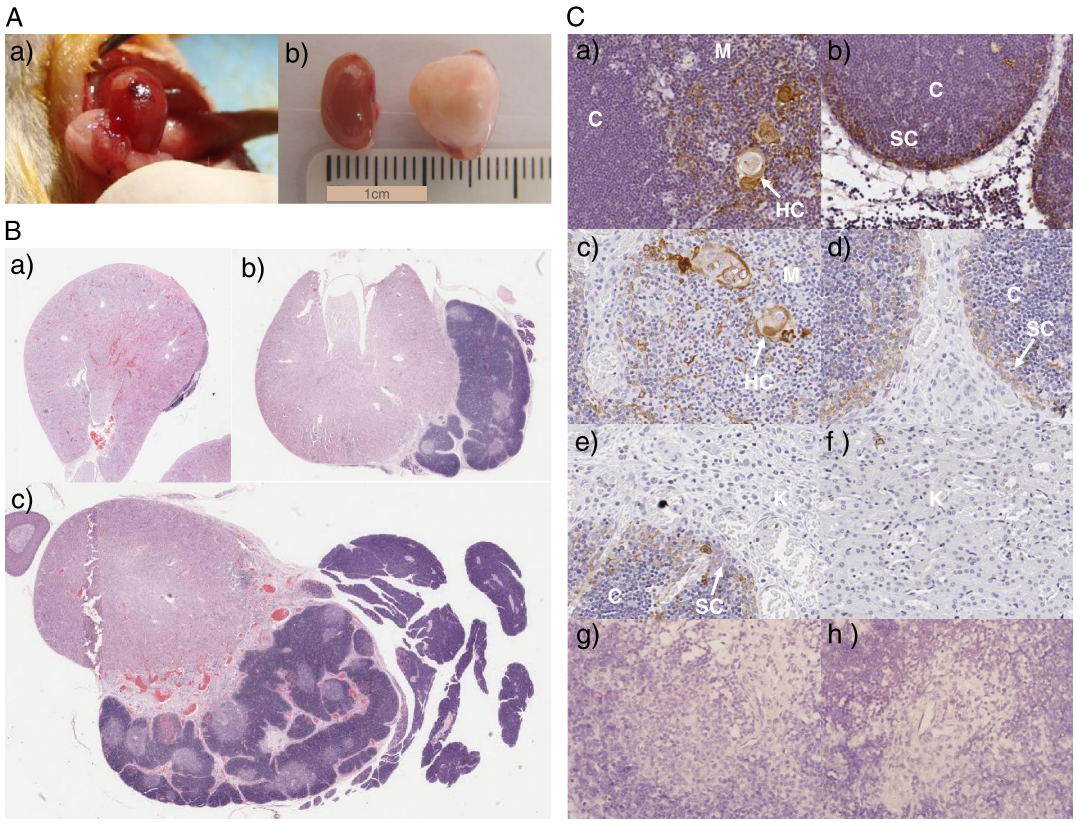

FIGURE 2. Histologic analysis of fetal tissues implanted in FLT+FLCD34 mice and the thymic tissues of FLT+FLCD34 and FLCD34 mice. A, Macroscopic images of human fetal tissue implants in the kidneys of FLT+FLCD34 mice at day 0 (a) and 36 weeks (b) after transplantation. B, Volumetric growth of implanted fetal thymic tissues at 1 week (a), 12 weeks (b), and 22 weeks after transplantation (c) (hematoxylin-eosin; magnification ×10). C, Immunohistochemistry of human cytokeratin 8 in human thymic epithelial cells (magnification ×200); CK8 expression in the medulla (a) and subcapsular region (b) in juvenile human thymus as a positive control. CK8 expression in the medulla (c), subcapsular region in implanted fetal thymic tissue under the kidney capsule of NSG mice (d), junctional between the implanted fetal thymic tissue(lower left side) and kidney (upper right side), (e) and an NSG kidney as a negative control (f). CK8 expression in the thymus of FLT+FLCD34 (g) and FLCD34 (h) mice. C, cortex; M, medulla; HC, Hassall's corpuscle; SC, subcapsular region; K, kidney.

corpuscles (c) and the subcapsular region (d and e) of the implanted human fetal thymus. CK8 was not expressed in the native thymi of FLT+FLCD34 (g) and FLCD34 (h) mice, as expected. Therefore, we concluded that the implanted human fetal thymus became enlarged because of the proliferation of human thymic epithelial cells (*11*).

Human T Cell Developmental Process in FLT+FLCD34 Mice

We first examined common lymphoid progenitors (CLPs) in bone marrows obtained from the tibias and femurs of hind legs. As shown in Figure 3A, among the Lin^- population from total bone marrow cells, $CD34^+CD38^+$ cells were gated as primitive cells. $CD127^+CD10^+$ populations were distinctly identified as approximately 1.9–3.7% of the total bone marrow population. As shown in Figure 3 (B and C), the FLT+FLCD34 mice had more CLPs in the bone marrow than did FLCD34 mice. These data suggest that previously injected human fetal liver-derived $CD34^+$ hematopoietic stem cells homes to the bone marrow and can differentiate into various lineages.

Next, human CD4 and CD8 DN thymocytes from the enlarged human fetal thymus shown in Figure 3D were sorted, and their surface phenotypes were analyzed. As summarized in Table 1 based on Blom B and Spits H's review (*12*), and Terstappen LW's paper (*13*), $CD34^+$ DN thymocytes included three major populations: $CD1a^-CD38^{low}$, $CD1a^-CD38^+$, and $CD1a^+CD38^+$ (Fig. 3D). These three populations can be referred to as TSPs, ETPs, and pre-T cells according to Table 1. In addition, when the level of CD34 expression in these three, each populations was evaluated, the intensity of CD34 expression was decreased from TSPs to pre-T cells according to developmental stage (Fig. 3E).

$CD3^-CD4^+$ ISP (immature single positive), $CD3^-CD4^+CD8^+$ EDP (early double positive), $CD3^+CD4^+CD8^+$ DP (double positive), $CD3^+CD4^+$ SP (single positive), and $CD3^+CD8^+$ SP populations were founded in the sorted positive fraction (Fig. 3F). Furthermore, the analysis of additional surface markers revealed sequential changes in CD7, CD5, CD10, CD45RA, and CD127 on the surface of human thymocytes developed in the implanted human fetal thymus, similar to the pattern observed in thymocytes in a

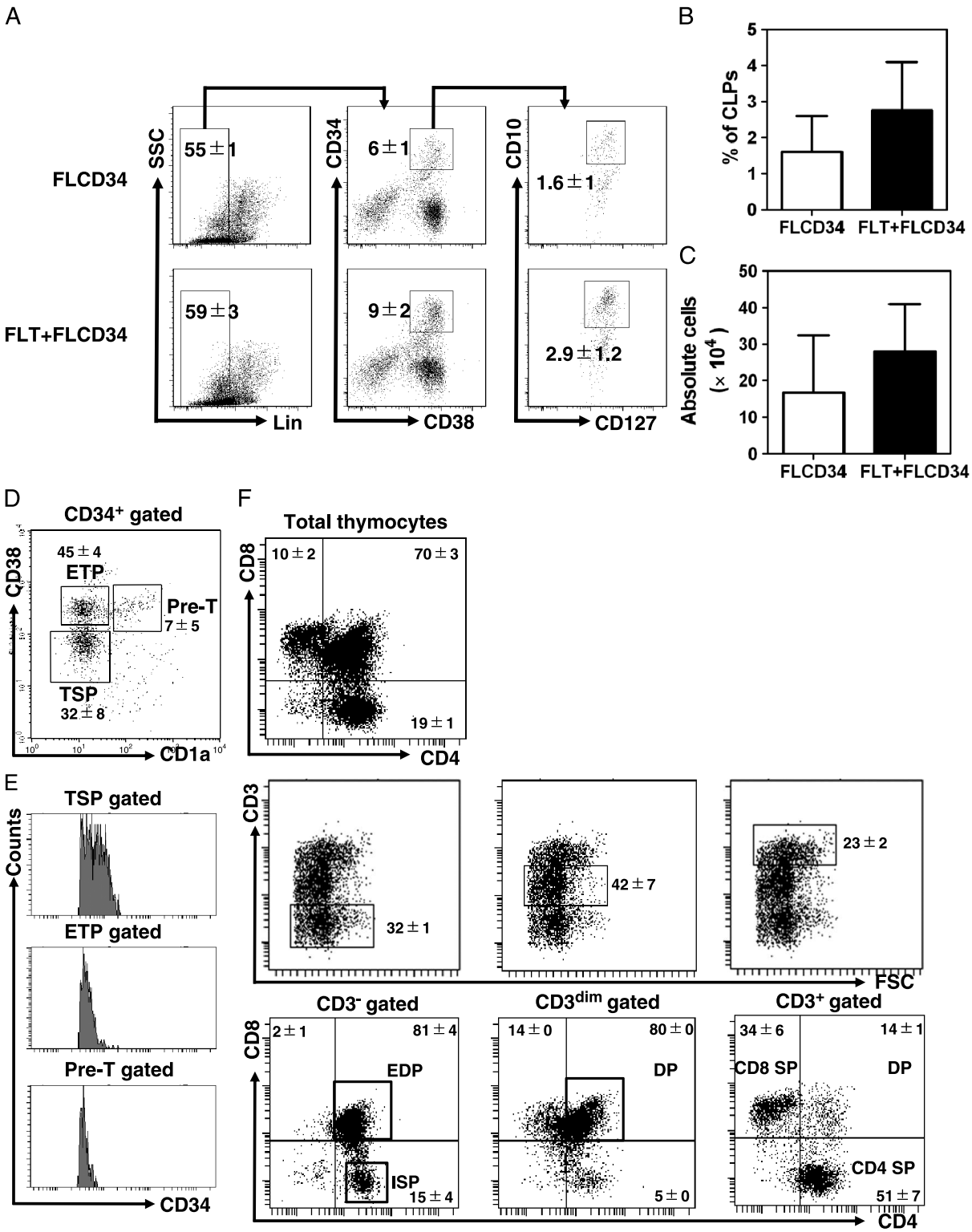

FIGURE 3. Representation of the entire human T cell development processes in bone marrow and implanted human fetal thymus tissue (n=2). A, Human CLPs in the bone marrow of FLCD34 (n=3) and FLT+FLCD34 mice (n=3) at 18 weeks after transplantation. Lineage-negative (Lin^-) bone marrow cells in FLT+FLCD34 mice (middle rows) include more $CD34^+CD38^+CD127^+CD10^+$ CLPs than does the Lin^- population in FLCD34 (top rows). B, The percentage of CLPs in the total live bone marrow cell population. C, The absolute number of CLPs in bone marrow (□: FLCD34, ■: FLT+FLCD34). (D) Human primitive thymocyte populations in the sorted $CD4^-CD8^-$ DN population from enlarged fetal thymocytes. (E) Serial expression levels of the human CD34 marker in each primitive population. (F) Maturing T cells in implanted human fetal thymus tissue.

TABLE 1. The phenotypic changes in developing human T cells (*15, 20*)

	CLP	TSP	ETP	Pre-T	Small ISP	Large ISP	EDP	DP	DP	SP
CD34	+++	++	+	+	+/−	−	−	−	−	−
CD1a	−	−	−	+		+	+	+	+	+/−
CD38	+	+++	+++	+++	+++	+++	+++	+++	+	+/−
CD45RA	+++	+	+	−	−	−	−	−	−	+
CD3	−	−	−	−	−	−	−	+++	+++	+++
CD4	−	−	−	−	+	+++	+++	+++	+++	+++
CD8	−	−	−	−	−	−	+++	+++	+++	+++
CD8α	−	−	−	−	−	−	+	+	+	+
CD8β	−	−	−	−	−	−	−	+	+	+
CD2	−	+	+/−	+	+	+	+	+	+	+
CD5	−	+	+	+	+	+	+	++	++	+++
CD7	+	+	+	+	+	+	+	+	+	+
CD10	+	−		+/−		+	−	−	−	−
CD127	+		+	+	+	+	+	+	+	+

CLP, common lymphoid progenitor; TSP, thymus seeding progenitor; ETP, early thymic progenitor; Pre-T, pre-T cell; ISP, immature single positive; EDP, early double positive; DP, double positive; SP, single positive.

human infant, as shown in Table 1 (see **Figure S2, SDC**, http://links.lww.com/TP/A720).

Humanized Mice Without Fetal Tissues Have More Memory-Type T Cells in the Periphery

We found that the $CD3^+$ T cells in the periphery of these two groups have distinctly different phenotypes with respect to CD45RA and CD45RO (Fig. 4A). In FLT+FLCD34 mice, approximately one third of $CD3^+$ T cells showed $CD45RA^+$ naive phenotypes. This result is in sharp contrast to the T cell phenotype of FLCD34 mice, which had a relatively high percentage of $CD45RO^+$ T cells. Specifically, almost half of the $CD3^+$ T cells in FLCD34 mice exhibited the $CD45RO^+$ memory T cells phenotype at 24 weeks after transplantation (Fig. 4B).

At 29 weeks after transplantation, CFSE (carboxyfluorescein diacetate, succinimidyl ester)-labeled splenocytes were stimulated with anti-CD3 and anti-CD28 antibodies for 5 days (Fig. 4C). Beginning on day 3, some proliferated cells were observed in both groups. On day 4, the T cells of FLCD34 mice showed a more abundant proliferating fraction than that of FLT+FLCD34 mice. On day 5, most of the T cells from the FLCD34 group had shifted to the CFSE-diluted fraction after stimulation with anti-CD3 and anti-CD28 antibodies. This result reflects the tendency of $CD45RO^+$ memory T cells to proliferate rapidly in response to stimulatory conditions (*14*).

Upon incubation with the strong stimulators anti-CD3 and anti-CD28 antibodies, the proliferating cells entered apoptosis (Fig. 4D). FLCD34 splenocytes showed a sharp decrease in cell viability after day 1. With respect to the well-known properties of memory T cells, this result suggests that the splenic T cells of FLCD34 mice are more susceptible to activation-induced cell death. These results indicate that the T cells of FLCD34 mice developed within mouse thymic environments had features of memory-type T cells.

Human T Cells of Humanized Mice Without Human Fetal Tissues Transplantation Exhibited Properties of Innate T Cells

PLZF is an important transcriptional factor that is expressed in invariant NKT cells and $CD4^+$ T cells after an MHC class II-dependent thymocyte-thymocyte (T-T) interaction (T-T $CD4^+$) (*15*). Additionally, it was recently reported that the presence of $PLZF^+CD4^+$ T cells regulates the development of innate $CD8^+$ T cells (*16*).

Therefore, FLCD34 T cells with memory-type cell characteristics were further investigated for the expression of PLZF, which is a property of innate T cells. More than 30% of the CD4 SP thymocytes from the native thymi of FLCD34 mice were clearly expressed intracellular PLZF (Fig. 4E). The $PLZF^+$ fraction decreased after release into the periphery, and 10% of $CD4^+PLZF^+$ splenocytes in FLCD34 mice were PLZF positive; however, this rate is still higher than that observed in FLT+FLCD34 mice.

There is an important difference in the education of developing human T cells between the FLCD34 and FLT+FLCD34 mice. FLT+FLCD34 human T cells developed in the implanted human fetal thymus and the mouse's native thymus, whereas FLCD34 T cells developed only in the mouse's native thymus. It has been reported that T cells that develop in the murine thymus of humanized mice have an innate T cell phenotype. Rapid secretion of cytokines, such as IFN-γ, is one of the traits of innate T cells (*17*). Thus, we compared the secretion of IFN-γ between FLCD34 and FLT+FLCD34 T cells, and as expected, FLCD34 splenocytes expressed intracellular IFN-γ in response to stimulation with PMA/ionomycin at a more than twofold higher rate than did FLT+FLCD34 splenocytes (Fig. 4F).

DISCUSSION

The use of humanized mice has become an established technique that is now applied to the study of various human diseases, such as cancer and viral infection. However,

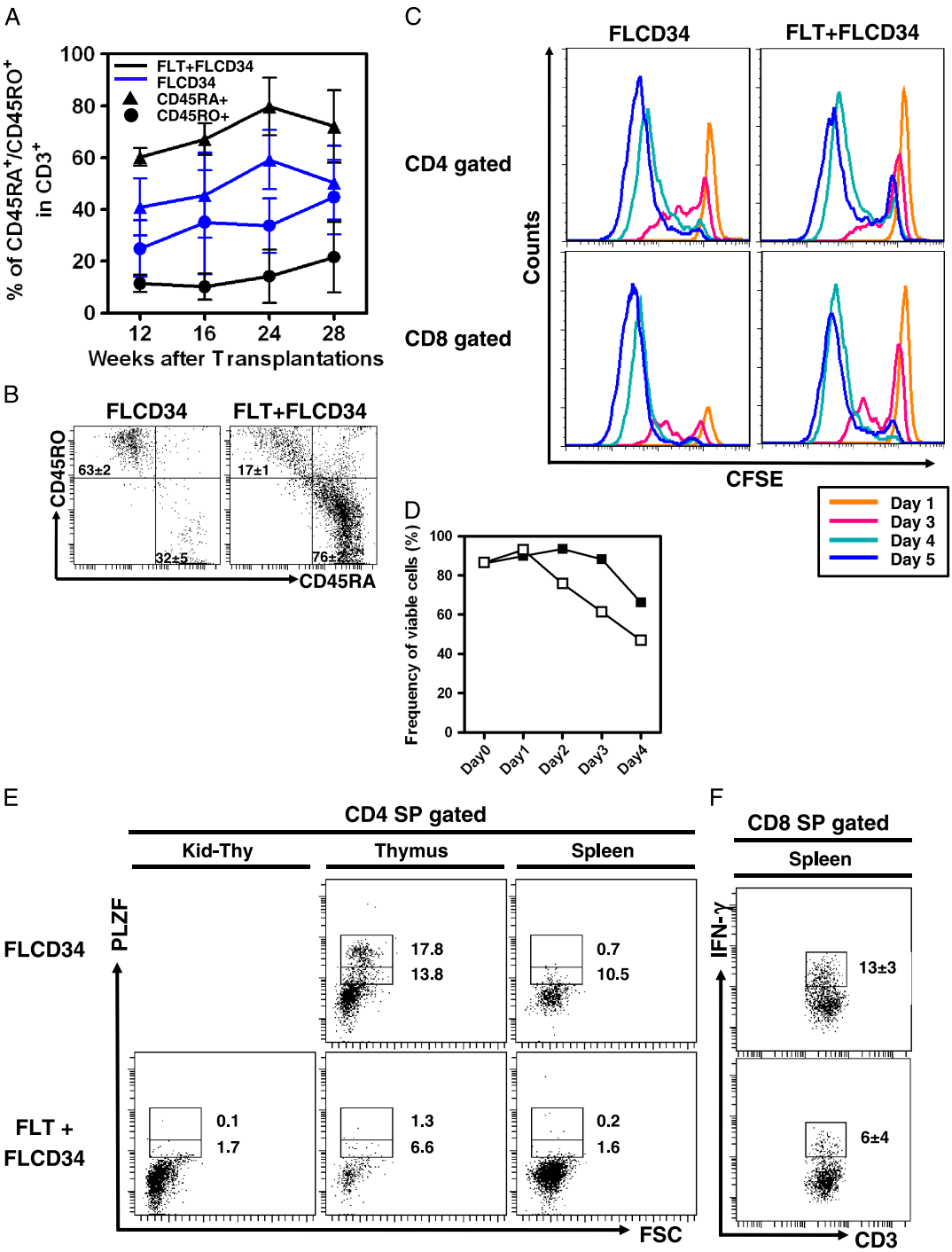

FIGURE 4. Representative expression levels of CD45RA$^+$ naïve T cells and CD45RO$^+$ memory T cells and the characteristics of innate T cell in FLCD34 (n=10) and FLT+FLCD34 mice (n=12). (A) Differences in the percentage of CD45RA$^+$ and CD45RO$^+$ cells in the peripheral blood plotted against time after transplantations. (B) Representative percentages of CD45RA$^+$ and CD45RO$^+$ cells in the splenocytes of FLCD34 (n=3) and FLT+FLCD34 mice (n=3) at 24 weeks after transplantation. (C) Comparison of CFSE-labeled splenocyte proliferation levels after stimulation with anti-CD3 and anti-CD28 antibodies in vitro 24 weeks after transplantation in FLCD34 (n=3) and FLT+FLCD34 mice (n=3). (D) In vitro measurements of cell viability after stimulation with anti-CD3 and anti-CD28 antibodies 24 weeks after transplantation (□: FLCD34, ■: FLT+FLCD34). (E) Intracellular expression of PLZF in the thymocytes of implanted human fetal thymus and the endogenous mouse own thymus and spleen at 24 weeks after transplantation. (F) Comparison of the percentage of intracellular IFN-γ in FLCD34 (n=3) and FLT+FLCD34 mice (n=3) after 6 hours of stimulation with PMA/ionomycin at 29 weeks after transplantation.

humanized mice must be assessed for similarity with human traits to ensure that these mice are appropriately used for various disease models and treatment developments. Watanabe et al. (*5*) conducted a detailed comparison of immune cell functions between hu-HSC NOG mice and humans. In response to stimulation, humanized models established with only transplanted $CD34^+$ hematopoietic stem cells showed partially blocked B cell development and reduced T cell function in the periphery. Therefore, this study suggested that further improvements in the function of the immune system of humanized mice were necessary.

Our study shows that humanized mice reconstituted via the cotransplantation of human fetal thymus/liver tissues and fetal liver–derived $CD34^+$ hematopoietic stem cells have adequate numbers of human T cells, similar to that of humans and unlike humanized mice models reconstituted with hematopoietic stem cells only. The mouse immune system differs from the human immune system with respect to several T cell developmental stages. Before the identification of $CD4^+CD8^+$ DP cells, immature T cells in mice are classified as $CD4^-CD8^-$DN1 to DN4 using the surface markers CD44 and CD25. However, in humans, some studies have subdivided immature thymocytes using CD1a, CD34, and CD38 (*18*). Blom and Spits (*12*) reviewed the development of human lymphoid cells and reported more surface markers that can be used to subdivide immature T cells into TSPs, ETPs, and pre-T cells. We summarized the detailed phenotypes of phenotypes of developing human T cells in Table.1. Subsequently, we analyzed all of the populations involved in human T cell development in the enlarged human fetal thymus in the kidney capsule of FLT+FLCD34 mice. The development of human T cells in the implanted human fetal thymus mimicked the reported steps for human T cell development. All of the T cell populations, from DN to SP, found in the implanted fetal thymus were compared with those in a human fetal thymus and in juvenile human thymi from children of various ages (see **Figure S3**, **SDC**, http://links.lww.com/TP/A720). The implanted human fetal thymus showed populations of TSPs, ETPs, ISPs, and SPs that were much better separated than those in the human fetal and juvenile human thymi. Therefore, our FLT+FLCD34 mouse model could be effectively used for research on immature T cell populations.

Although the description of murine T cell development is well established, the information available about human T cell development is not as comprehensive (*12, 18, 19*). Rothenberg et al. (*20*) reviewed the wide array of results regarding the developmental program of the T cell lineage and related genes. However, most of these results were from the mouse immune system. Because the regulation of specific genes is easier to investigate in mice than in humans, large differences in the amount of research in humans and mice might be inevitable. Our humanized mouse model that was cotransplanted with both human thymus/liver and hematopoietic stem cells may allow more extensive research on human T cells in vivo.

The human T cells in our humanized mouse model using fetal tissues (FLT+FLCD34) proceed through the entire developmental process and are educated by human thymic epithelial cells, producing a T cell pool similar to that in humans. The T cell population in the periphery contained more naive T cells than memory T cells, similar to the normal humans periphery. In addition, these T cells were more viable in response to stimulating conditions. $CD45RO^+$ memory T cells, which are dominant in $CD34^+$ hematopoietic stem cell-transplanted humanized mice (FLCD34), proliferated rapidly in response to stimulation, but showed a striking loss of viability and several innate T cell properties. Therefore, T cells educated by mouse MHC II in mice lacking a human fetal thymus differ from that of normal human T cells in their characteristics and functions. These differences could limit the applicability of these models to studying human diseases.

Using the model in our study, numerous T cell–tropic human diseases could be explored, and the molecular mechanisms underlying human T cell development could be studied extensively.

MATERIALS AND METHODS

Animals, Human Fetal Tissues, and Human Children's Thymus

Approximately 6- to 8-week-old NSG mice (Jackson Laboratory) were maintained in a specific pathogen-free facility at the Laboratory of Animal Research Center in Samsung Medical Center. The Laboratory of Animal Research Center is approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Human fetal thymus and liver tissues of gestational age 17 to 24 weeks were obtained from the Advanced Bioscience Resource Institute (Alameda, CA) and Samsung Medical Center (Seoul, Korea). To prevent the attack of residual mature T cells from the 2 mm^3 piece of fetal tissue after transplantation, we used frozen and thawed human fetal thymic tissues (*21*). The number of mature T cells in frozen fetal thymic tissue was lower compared with fresh fetal thymic tissue (see **Figure S4**, **SDC**, http://links.lww.com/TP/A720). Juvenile human thymus glands were obtained from patients who underwent surgery for congenital heart disease at Samsung Medical Center. All of the samples from Samsung Medical Center were obtained with written informed consent in accordance with the guidelines set forth by the institutional review board at Samsung Medical Center. Donated fetal tissues were freshly isolated within 24 hours of abortion and frozen until use.

Human Fetal Liver–Derived $CD34^+$ Hematopoietic Stem Cell Purification

Human fetal liver cells were fractionated on a Ficoll-Hypaque gradient (1.077 g/m^3). Isolated mononuclear cells were washed in phosphate-buffered saline several times, and $CD34^+$ cells were sorted from mononuclear cells by using AUTOMACS (Miltenyi Biotec, Germany). The purity of the sorted $CD34^+$ hematopoietic stem cells was 90% or greater.

Transplantation of Human Fetal Thymus/Liver Tissues and Fetal Liver–Derived $CD34^+$ Cells Into NSG Mice

Animals were irradiated with 2-Gys from a Cobalt source. At 24 hours after irradiation, 2 mm^3 human fetal liver and thymic tissues were implanted in the kidney capsule. After surgery, 2×10^5 fetal liver–derived $CD34^+$ hematopoietic stem cells were injected intravenously. Mice received 100 mg/L ciprofloxacin (CJ Pharma, Korea) in their drinking water for 4 weeks after transplantation. Ciprofloxacin was kindly donated by CJ Pharma. The data represent pooled observations from three separate experiments. A total of 25 FLT+FLCD34 and 10 FLCD34 mice were used.

Flow Cytometry Analysis of Human Lymphocytes Generated in NSG Mice

To confirm the engraftment of human cells, multicolor cytometric analyses were performed using FACS Vantage (BD Biosciences, San Jose, CA), FACS Calibur (Becton Dickinson), and FACS Aria (Becton Dickinson) instruments. Antihuman CD45-fluorescein isothiocyanate (CD45-FITC), CD3-FITC, CD1a-FITC, CD45RA-FITC, CD34-phycoerithrin (CD34-PE),

CD4-PE, CD11b-PE, CD19-PE, CD33-PE, CD45RO-PE, CD56-phycoerithrin 5-succinimidylester (CD56-PE-Cy5),CD3-PE-Cy5, CD8-PE-Cy5, CD38-PE-Cy5, CD3- peridinin-chlorophyll-Cy5.5 (CD3-PerCP-Cy5.5), CD-38-PerCP-Cy5.5, CD4-phycoerithrin 7-succinimidylester (CD4-PE-Cy7), CD127-PE-Cy7, CD8a-allophycocyanin (CD8a-APC), and CD10-APC were purchased from eBioscience (San Diego, CA). Antihuman Lin-FITC was purchased from BD Bioscience (San Jose, CA).

Isolation of a Human DN ($CD4^-CD8^-$) Population From Implanted Human Fetal Thymus

Thymocytes obtained from the fetal thyme implanted in FLT+FLCD34 mice were stained with anti-CD4 and anti-CD8 magnetic microbeads (Miltenyi Biotec, Germany) according to the manufacturer's instruction. Using AUTOMACS, double-negative fractions were sorted from stained thymocytes. Other positive fractions, excluding the double-negative fraction, were also obtained for analysis of the T cell developmental process.

Histology and Immunohistochemistry

Implanted human fetal thymic tissues were obtained and fixed with 10% formalin and processed for hematoxylin-eosin staining. After treatment with heated citrate buffer for antigen retrieval, formalin-fixed paraffin-embedded sections were immunostained with mouse anti-human cytokeratin 8 monoclonal antibody (4.1.18; Chemicon International, Germany) to detection mTECs, Hassall's corpuscles and the subcapsular region of the thymus (*11*).

CFSE Proliferation Assay and Apoptosis Measurements

Splenocytes were labeled with CFSE using the CellTrace CFSE cell proliferation kit (Molecular Probes, Eugene, Oregon) according to the manufacturer's instruction. CFSE-labeled splenocytes were incubated with anti-CD3 (10 μg/mL) and anti-CD28 (1 μg/mL) antibodies and analyzed using a FACS Calibur instrument.

The apoptosis rates of the CFSE-labeled cells during the incubation period were measured using a PE Annexin V Apoptosis Detection Kit I until day 4 after stimulation by anti-CD3 (10 μg/mL) and anti-CD28 (1 μg/mL) antibodies.

Intracellular Staining of PLZF and IFN-γ

To examine intracellular PLZF, thymocytes and splenocytes were stained as described previously (*17*). To stain for IFN-γ, splenocytes from each group of humanized mice were obtained and stimulated with PMA (50 ng/mL) and ionomycin (1 ug/mL) for 6 hours; brefeldin A (10 μg/mL) was added during the last 4 hours of stimulation. Stimulated splenocytes were harvested and surface stained with anti-CD3-PerCp-Cy5.5, anti-CD56-FITC, and anti-CD8α-APC. Splenocytes were then fixed and intracellularly stained with anti-IFN-γ-PE as described for PLZF and analyzed using a FACS Calibur instrument.

Statistical Analysis

The results are represented as individual data points or as the mean with SD. Statistical comparisons were performed using unpaired *t* tests.

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