Human Natural Regulatory T Cell Development, Suppressive Function, and Postthymic Maturation in a Humanized Mouse Model

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CD4+ regulatory T cells (Tregs) control adaptive immune responses and promote self-tolerance. Various humanized mouse models have been developed in efforts to reproduce and study a human immune system. However, in models that require T cell differentiation in the recipient murine thymus, only low numbers of T cells populate the peripheral immune systems. T cells are positively selected by mouse MHC and therefore do not function well in an HLA-restricted manner. In contrast, cotransplantation of human fetal thymus/liver and i.v. injection of CD34+ cells from the same donor achieves multilineage human lymphohematopoietic reconstitution, including dendritic cells and formation of secondary lymphoid organs, in NOD/SCID mice. Strong Ag-specific immune responses and homeostatic expansion of human T cells that are dependent on peripheral human APCs occur. We now demonstrate that FOXP3+ “natural” Tregs develop normally in human fetal thymic grafts and are present in peripheral blood, spleen, and lymph nodes of these humanized mice. Humanized mice exhibit normal reversal of CD45 isoform expression in association with thymic egress, postthymic “naive” to “activated” phenotypic conversion, and suppressive function. These studies demonstrate the utility of this humanized mouse model for the study of human Treg ontogeny, immunobiology and therapy. The Journal of Immunology, 2011, 187: 3895–3903.

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CD4+ regulatory T cells (Tregs) expressing the IL-2Rα-chain (CD25) and the master regulator FOXP3 transcription factor play a pivotal role in controlling adaptive immune responses and maintaining self-tolerance (1). Many studies have demonstrated the ability of Tregs to suppress autoimmunity (2–5), transplant rejection (6), tumor rejection (7, 8), responses to infections (9–11), and graft-versus-host (GVH) disease (12). Tregs are now being used in early clinical trials. However, Tregs cannot be readily studied in vivo in humans and there are significant differences between mouse and human Treg immunobiology. The availability of an in vivo animal model in which to study human Tregs could greatly advance our understanding and allow evaluations of the impact of immunotherapies on this important cell population.

Various humanized (HU) mouse models have been developed in efforts to reproduce a human immune system and allow its study. These have involved reconstitution of immunodeficient mice (SCID or RAG-deficient) and their derivatives, including NOD/SCID mice and NOD/SCID/IL-2Rγnull mice, with human hematopoietic cells (13, 14), with lymphocytes (15, 16), or with fetal thymus and liver grafts under the kidney capsule (17). Immunodeficient mice receiving human hematopoietic cells partially support the maturation of human lymphocytes, as evidenced by the development of Ig-producing human B cells and CD4+ and CD8+ T cells in secondary lymphoid organs (14, 18, 19). Virus-specific T cell responses have also been reported when human hematopoietic cells are given to newborn immunodeficient mice (20). However, only low numbers of T cells populate the peripheral immune systems of these mice. These T cells are positively selected by mouse MHC and therefore do not function well in an HLA-restricted manner (21).

We have recently shown that cotransplantation of human fetal thymus/liver and i.v. injection of CD34+ cells from the same donor achieves long-term repopulation with multilineage human lymphohematopoietic cells, including dendritic cells and formation of secondary lymphoid organs, in NOD/SCID mice (22, 23). In this model, human fetal thymic grafts and hematopoietic stem cells originate from the same human fetus. Therefore, HLA-restricted responses between T cells and human APCs in the periphery occur. Indeed, these HU mice demonstrate homeostatic expansion of human T cells in vivo that is dependent on the presence of human APCs in the periphery (24). The immune systems of these HU mice show T and B cell responses to HIV infection (25), strong Ag-specific immune responses with class-switched Ig responses (26), and they spontaneously reject porcine skin (22) and islet xenografts (27). However, the development and function of Tregs in this HU mouse model has not been investigated.

In this study, we show that FOXP3+ “natural” Tregs (nTregs) develop normally in human fetal thymic grafts and are present in...
peripheral blood, spleen, and lymph nodes (LN)s in HU mice. Similar to normal humans, a shift in CD45R isoform expression between thymic and peripheral “naive” Tregs occurs in HU mice. Human Tregs in HU mice show phenotypic conversion in the periphery that suggests they have been activated and exhibit similar suppressive function to Tregs from healthy adult human peripheral blood.

Materials and Methods

Animals and human tissues and cells

NOD/SCID mice were housed in a specific pathogen-free microisolator environment and used at 6–10 wk old. Discarded human fetal thymus and liver tissues of gestational age of 17–20 wk were obtained from Advanced Biosciences Resources (Alameda, CA). Protocols involving the use of human tissues and animals were approved by the Massachusetts General Hospital and Columbia University Medical Center Human Research Committee and Subcommittees on Research Animal Care, respectively, and all of the experiments were performed in accordance with the protocols. Human peripheral blood was obtained under an Institutional Review Board-approved protocol from healthy adult donors aged 29–40 y, and PBMCs were isolated by Ficoll separation.

Generation of HU mice

HU NOD/SCID mice were created as previously described (23, 24, 26). Briefly, female NOD/SCID mice (6–10 wk old) were conditioned with 2.5 Gy total body irradiation. Human fetal thymus and liver fragments measuring ∼1 mm^3 were implanted together under the recipient kidney capsule. CD34^+ fetal liver cells (FLCs) from the same human fetal liver were isolated by the MACS separation system using anti-human CD34 microbeads (Miltenyi Biotec, Auburn, CA). Within 24 h surgery, 1–5 × 10^5 CD34^+ FLCs were injected i.v. In a slight modification of the protocol, animals used in studies of Helios expression received cryopreserved/thawed human fetal thymus and i.v. injection of anti-CD2 mAb at the time of transplantation, a method that we have shown allows robust human thymopoiesis/T cell reconstitution and eliminates preexisting mature T cells from the thymic graft (H. Kalscheuer, N. Danzl, T. Onoe, T. Faust, R. Winchester, T.R. Spitzer, H. Tahara, Y.G. Yang, and M. Sykes, submitted for publication).

Flow cytometry

Human hematopoietic cell repopulation in the HU mice and the profiles of T cells were assessed via flow cytometry (FCM) analysis. Human T cells were bled to measure the profile of Tregs in PBMCs 16–18 wk posttransplantation and sacrificed to harvest organs 20–21 wk posttransplantation. Mononuclear cells were isolated from blood or single-cell suspensions of lymphoid tissues by Ficoll separation. The following mAbs, purchased from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA), were used in different combinations: anti-mouse CD34 (30-F11), anti-mouse Ter-119 (Ter-119), anti-human CD4 (RPA-T4), anti-human CD8 (RPA-T8), anti-human CD14 (M5E2), anti-human CD19 (HIB19), anti-human CD45 (H130), anti-human CD3 (SK7), anti-human CD45RA (HI100), anti-human CD45RO (UCHL1), anti-human CD25 (M-A251), anti-human CD127 (hiL-7R-M21), anti-human FOXP3 (236A/E7), anti-HLA-DR (L234), allophycocyanin-streptavidin, PE-Cy5-streptavidin, PE-Cy5-7-streptavidin, and isotype control mAbs. All samples were acquired using a FACS caliber or LSRII BD Biosciences, Mountain View, CA, and analyses were performed with FlowJo software (Tree Star, San Carlos, CA). Tregs were assayed in the thymic grafts, spleens, and LN s with the Abs above and anti-mouse/human Helios (22F6; BioLegend, San Diego, CA) and acquired on a FACS Canto. Dead cells were excluded from the analysis by gating out cells with low forward scatter and high propidium iodide-retaining cells or with low forward and side scatter cells in analyses of intracellular staining.

For intracellular staining, cells were washed twice with wash buffer and surface Ags were stained with Abs. The cells were then fixed and permeabilized with a FOXP3 staining buffer set (eBioscience, San Diego, CA), followed by incubation with anti-human FOXP3 (256A/E7; eBioscience) alone or with anti-mouse/human Helios (22F6; BioLegend) for 30 min at 4°C according to the manufacturers’ instructions. Cells were washed twice in permeabilization buffer (eBioscience) and resuspended in PBS before analysis.

In vitro suppression assay

To quantify suppression capacity of CD4^+CD25^+CD127^+ human T cells of HU mice, we performed suppression assays in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 10% AB human serum (Sigma-Aldrich, St. Louis, MO), 1% HEPEs buffer, and 1 × 10^−5 M 2-ME (Sigma-Aldrich). Splenocytes and LN cells were harvested from each HU mouse, and live mononuclear cell suspensions were isolated by Ficoll separation. Human cells were enriched by depletion of mouse-derived cells using anti-mouse CD45 and Ter-119 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. To purify CD4^+CD25^+ (Tregs) and CD4^+CD25^−CD127^− (suppressor) T cells, enriched human cells were stained with anti-human CD4 (RPA-T4), CD8 (RPA-T8), CD25 (M-A251), and CD127 (hiL-7R-M21) mAbs and sorted on a FACS Aria (BD Biosciences). Healthy human control CD4^+CD25^+ and CD4^+CD25^−CD127^− T cells were purified from PBMCs by the same method. The purities of both subsets were >97%.

In suppression assays, responders (2 × 10^5 cells/well) were cultured in U-bottom 96-well plates with suppressors at indicated ratios in the presence of plate-bound anti-CD3 mAb (UCHT1, 1 μg/ml) and soluble anti-CD28 mAb (CD28.2, 2.5 μg/ml) for 4 or 5 d in the presence of irradiated human allogeneic PBMCs (3000 rad, 4 × 10^5 cells/well) for 5 d at 37°C in 5% CO_2. Cells were harvested after 16 h incubation with 1 μCi[^3]H]thymidine.[^3]H]Thymidine incorporation was measured by beta-counter. Data are expressed as actual cpm or stimulation index (cpm of allostimulated culture/cpm of autostimulated culture). In autostimulated control cultures, responder cells were incubated with autologous PBMCs. All data are shown as mean of[^3]H]thymidine incorporation or stimulation index in triplicate cultures.

Statistical analysis

Statistical analysis and comparisons were performed with Prism software version 4.0 (GraphPad Software, San Diego, CA). Data in bar graphs are expressed as means ± SEM. A Mann–Whitney U test, Kruskal–Wallis test, and two-way ANOVA were used to compare groups. A p value < 0.05 was considered statistically significant.

Results

Human nTregs develop in thymus grafts of HU mice and have a similar phenotype as Tregs in control fetal human thymus

We analyzed human thymocytes in thymic grafts of HU mice 20 wk posttransplantation. Macroscopically, the size of thymic grafts increased from 1 mm^3 to ~1 cm in length and contained ~100–300 × 10^6 thymocytes (Fig. 1A). FCM analysis revealed a similar phenotypic distribution in the percentage of double-negative, double-positive, and CD4 and CD8 single-positive (SP) human thymocytes in all mice to those in human fetal thymus (Fig. 1B). Among both CD4 and CD8 SP thymocytes, most were CD45RO^+CD45RA^- (Fig. 1C), consistent with results reported for normal human thymus (28). Additionally, ~60 and 80%, respectively, of CD4 and CD8 SP thymocytes wereCCR7^+ (Fig. 1C).

Expression of α-chains of IL-2 and IL-7 receptors (CD25 and CD127, respectively) discriminates between human Tregs and activated T cells (29), and CD127 expression inversely correlates with FOXP3 expression and suppressive function of human Tregs (30). Therefore, CD25^+CD127^− CD4^+CD8^- thymocytes were analyzed as putative Tregs. The proportions of CD25^+CD127^- cells among CD4 SP thymocytes in normal human fetal thymus and in HU mice were equivalent (Fig. 1D, Supplemental Fig. 1). High frequencies of FOXP3^+ nTregs were detected among CD25^+CD127^- CD4^+CD8^- thymocytes in both human fetal thymus and thymus grafts of HU mice (Fig. 1D, Supplemental Fig. 1). Similar to the general SP thymocyte population (Fig. 1C), most thymic Tregs in both the unmanipulated fetal thymus and the 20-wk grafts were CD45RO^-CD45RA^- (Fig. 1D, Supplemental Fig. 1). Moreover, the proportions of Tregs expressing HLA-DR, which identify the Tregs with maximum suppressive ability (31), were also similar between human fetal thymus and thymic grafts in HU mice (Fig. 1D, Supplemental Fig. 1).

Recent studies have demonstrated that expression of the Helios transcription factor distinguishes nTregs from those generated in the periphery from conventional T cells (induced Tregs) in humans and mice (32–35). To directly address the interpretation that...
thymic and peripheral Tregs in HU mice were largely nTregs, we assessed Helios expression. As shown in Fig. 1E, most thymic graft CD4+CD8−FoxP3+ cells also expressed Helios, consistent with the interpretation that these are nTregs.

Memory conversion of peripheral CD4 and CD8 T cells in HU mice

Peripheral reconstitution with human T cells in adult NOD/SCID mice requires implantation of a human fetal thymus graft (24), and human thymopoiesis does not occur to a significant extent in recipient mouse thymi of these animals (data not shown). The expression of CD45 isoforms on peripheral CD4 and CD8 T cells that developed in and emigrated from the human fetal thymus graft showed a markedly different pattern from SP thymocytes of the same animals, which were largely CD45RA+CD45RO− (Fig. 1C). Similar to adult control human PBMCs, both CD4+ and CD8+ T cells in PBMCs of HU mice included naive-type (CD45RA+CD45RO−) and “memory” or previously activated cells (CD45RA−CD45RO+) (36) (Fig. 1C, Supplemental Fig. 2). Thus, despite the relative youth of the immune systems in the HU mice
compared with the adult human PBMC donors, a high proportion of naive human T cells originating in the human thymus grafts converted to the memory phenotype in the periphery of HU mice.

Naive CD4+CD25+FOXP3+ Tregs are enriched in the CD4 cell population in the periphery of HU mice compared with adult control human PBMCs. The proportions of CD25+CD127− cells (8.12 ± 1.52% versus 3.49 ± 0.60%) and of CD25+FOXP3+ cells (5.96 ± 1.40% versus 2.77 ± 0.80%) among CD4+ T cells in PBMCs of HU mice were significantly greater than those in adult human PBMCs (Fig. 2A, 2B, top left and bottom left, respectively). Equivalent expression of FOXP3 was detected among CD4+CD25+CD127− T cells in PBMCs of both HU mice and adult humans (86.61 ± 4.00% versus 86.68 ± 2.58%; Fig. 2B, left middle). Because Tregs (and T cells and other lineages) did not require the fetal liver fragment for their development (Supplemental Figs. 3, 4) and the thymus graft and CD34 cell injection are both required for optimal T cell reconstitution (23, 24), these Tregs apparently originated from thymocytes developing de novo in the graft from progeny of injected CD34 cells.

Most CD4+CD25+FOXP3+ Tregs in adult human PBMCs showed the previously activated CD45RO+CD25+ phenotype (Fig. 2A, 2B, middle right), and Tregs in HU mouse PBMCs also included both naive-type and memory-type cells. However, a significantly greater proportion of Tregs in PBMCs of HU mice had the naive CD45RO−CD25+ phenotype compared with Tregs in adult human PBMCs (Fig. 2A, 2B, top right). In HU mouse PBMCs, 39.94% (±12.38) of Tregs were CD45RA+, compared with only 13.86% (±3.90) of Tregs in adult human PBMCs (Fig. 2B, top right). Conversely, only 38.90% (±9.79) of Tregs in HU

**FIGURE 2.** Human Tregs circulating in the periphery of HU mice have increased proportions of resting cells compared with adult humans. Humanized mice (● in B and C; n = 26) or healthy human volunteers (○ in B and C; n = 6) were bled to measure distribution of Tregs and their phenotype in PBMCs. Blood samples of HU mice were obtained 16–18 wk posttransplantation and isolated PBMCs were stained for CD4, CD8, CD25, CD127, CD45RA, CD45RO, and HLA-DR. Samples were fixed and further stained intracellularly for FOXP3. A, Phenotypic characteristics of Tregs in PBMCs from representative healthy adult human (upper row) and HU mouse (lower row), respectively. CD4+CD25+FOXP3+ human T cell populations were gated and their surface expression of CD45RA, CD45RO, and HLA-DR is shown in the histograms. The bold line represents staining with fluorochrome-labeled mAb for the indicated molecule, and the filled area represents staining with isotype control mAb. B and C, Comparison of proportion of CD25+CD127− cells among total CD4+ T cells, FOXP3+ cells among CD4+CD25+CD127− T cells, and of CD25+FOXP3+ Tregs among total CD4+ T cells (B) and proportion of CD45RO−CD25+FOXP3+ activated Tregs among total CD4+ T cells (C) in PBMCs of HU mice (●) or healthy adult human volunteers (○). Each circle represents results from an individual animal or donor and each line represents mean value. Results are shown from the combination of three similar experiments. **p < 0.001, comparing HU mice and human volunteers (Mann-Whitney U test).
mouse PBMCs were CD45RO+, compared with 79.58% (±6.84) of Tregs in adult human PBMCs (Fig. 2B, middle right). Additionally, a significantly lower proportion of Tregs in HU mouse PBMCs expressed HLA-DR than was observed in adult human PBMCs (20.90 ± 6.84% and 38.95 ± 4.56%, respectively; Fig. 2B, bottom right). Overall, these results suggest that Treg activation and/or homeostatic expansion had occurred in HU mice, but to a lesser extent than in healthy adult PBMCs. However, when taking into account the increased proportion of Tregs in PBMCs of HU mice compared with adult human donors, the percentage of CD45RO+CD25+FOXP3+ memory Tregs among total CD4+ T cells was similar between the groups (Fig. 2C).

The spleen and LNs of HU mice were analyzed for the presence and phenotype of Tregs. Analysis of CD25+FOXP3+ cells among CD4+ T cells revealed a significantly increased frequency of Tregs in LNs of HU mice (4.72 ± 0.63%) compared with spleen and PBMCs of the same animals (2.49 ± 0.36% and 3.43 ± 0.64%, p < 0.001 and p < 0.05, respectively; Fig. 3A). Strikingly, most CD4+CD25+FOXP3+ Tregs in LNs showed a CD45RO+CD45RA− memory phenotype (Fig. 3B, 3C, top and middle), and the proportion of such cells among Tregs was significantly greater (and the proportion of CD45RO−RA+ Tregs significantly lower) than that in PBMCs of the same mice (62.35 ± 3.73% versus 20.78 ± 6.18% and 36.23 ± 3.96% versus 70.25 ± 3.96%, respectively; Fig. 3C). Furthermore, a significantly greater proportion of Tregs in LNs and spleen expressed HLA-DR than in PBMCs (64.23 ± 5.43% and 46.33 ± 1.10% versus 9.66 ± 1.58%, respectively; Fig. 3C). Thus, an increased proportion of Tregs in secondary lymphoid organs, especially LNs, of HU mice had the activated/memory phenotype compared with those in PBMCs.

To determine whether peripheral Tregs in spleens and LNs of HU mice were nTregs and/or induced Tregs, we assessed Helios expression. As shown in Fig. 3D, the vast majority of splenic and peripheral LN Tregs were Helios+, suggesting that they were indeed nTregs.

Human CD4+CD25+CD127+ T cells from HU mice are functional Tregs

To determine the suppressive capacity of Tregs from HU mice, we performed in vitro suppression assays. We isolated Tregs and effector T cells from pooled spleen and LNs of HU mice 20 wk posttransplant by FACS. CD4+CD25+CD127− Tregs and CD4+CD25− effector T cells were purified (Fig. 4A). As shown in Fig. 4A, these purified CD4+CD25+CD127− T cells expressed high levels of FOXP3 whereas purified CD4+CD25− T cells did not. Responder cells were mixed with Tregs in the indicated ratios and cultured in the presence or absence of irradiated allogeneic

**FIGURE 3.** Distribution and phenotypes of Tregs in secondary lymphoid organs of HU mice. Humanized mice were sacrificed 21 wk posttransplantation and mononuclear cells of spleen, LNs, thymic grafts, and blood isolated from each individual mouse were analyzed by FCM. Mononuclear cells from each organ were isolated by Ficoll separation and analyzed by FCM as in Fig. 3A. A, Comparison of proportion of CD25+FOXP3+ Tregs among CD4+ T cells in indicated organs. Each circle represents cells from an individual animal or donor. B, Phenotypic characteristics of CD4+CD25+FOXP3+ Tregs in indicated organs from representative HU mouse. Histograms show expression of each indicated molecule. The bold line represents staining with fluorochrome-labeled mAb for the indicated molecule, and the filled area represents staining with isotype control mAb. C, Comparison of phenotypic distribution of Tregs among CD4+CD25+FOXP3+ cells. Each circle represents a result from an individual animal. D, nTregs were assessed in splenic (SPL) and peripheral LN (PLN) populations by FCM, gating on human CD45+CD4+CD8−CD25−CD127+ cells, and analyzing FOXP3+ cells for expression of Helios as shown (right panels). Total cells are calculated in FOXP3+Helios+ and FOXP3+Helios− cells from n = 4 HU mice. *p < 0.05, **p < 0.01, ***p < 0.001, comparing indicated combination (Kruskal–Wallis test, Dunn’s multiple comparison test).
PBMCs (Fig. 4C) or plate-bound anti-CD3 mAb and soluble anti-CD28 mAb (Fig. 4B), Tregs isolated from HU mice suppressed the proliferation of responder T cells to both allogeneic and non-specific stimulation, and their suppressive ability was comparable to that of Tregs isolated from adult human PBMCs. These results indicated that functional Tregs are present in peripheral lymphoid organs of HU mice.

Discussion

In this study, we report that functional nTregs develop in large numbers in a HU mouse model. Studies of human lymphocyte development and function are limited largely to PBMCs, as ethical considerations limit the use of invasive procedures and interventions that confer risk without potential benefit to the study volunteer. Thus, the development of HU mice, or mice with human immune systems, has been pursued by many groups as an approach to performing such studies, but each system has shown limitations. For example, administration of mature human lymphocytes has been useful for studies of infection and anti-tumor immunity but does not allow lymphocyte development to be analyzed. Additionally, the utility of this model may be limited by xenogeneic GVH responses, which skew T cell reactivity (16) and can cause GVH disease (37–39), and by development of EBV-related lymphomas (40, 41). Fetal human thymus and liver grafts have been used to achieve normal thymopoiesis but extremely limited peripheral reconstitution (42, 43). Infusion of human hematopoietic stem cells has allowed B cell reconstitution and low-level T cell development in the mouse thymus of neonatal immunodeficient mice (13, 14, 20). Whereas human thymopoiesis in the mouse thymus can be somewhat enhanced by promoting signaling through the IL-7 pathway (44), postthymic T cell function is likely to be limited by the species incompatibility between the thymus and the human APC populations that populate the periphery. Virus-specific T cell responses have been reported when such cells are given to newborn immunodeficient mice (14, 20). However, only low numbers of T cells repopulate the peripheral immune systems of these mice. The development of functional Tregs has been described in one of these models (45), but the T cells developed in the native thymus of RAG2-γc knockout mice, and only very small numbers were detectable in the periphery. In another model, intrathymic Tregs were demonstrated in the human thymus grafts of NOD/SCID mice receiving human fetal thymus grafts and i.v. human hemopoietic stem cells, but phenotypic subsets and peripheral Tregs were not described (46).

In models involving development of human T cells in a mouse thymus, T cells are positively selected by mouse MHC and may therefore not function optimally with human APCs in the periphery. Positive selection of Tregs is mediated by thymic epithelial cells (47–50). Encounters with selecting self-peptide/MHC Ags in the periphery support their expansion (51), and postthymic encounters with MHC (52) and with cognate Ag (53) allow Tregs to acquire normal regulation of host-reactive T cells. This defect was partly reversible by the addition of host-type thymic epithelial cells to the graft (54). Thus, species incompatibility between the thymus and the human APCs with which human T cells can interact is likely to limit the function of any human Tregs that develop in the host mouse thymus in HU mouse models.

In this study, we describe the development, peripheral phenotypic conversion, accumulation, and function of human natural Tregs that are produced from i.v. injection of human CD34+ fetal liver cells into NOD/SCID mice cotransplanted with human fetal
thymus. In this model, hemopoietic stem cell-derived T cells develop in an autologous human thymus. The human thymus grafts in these mice grow markedly and their peripheral hematolymphoid tissues populate with multilineage human lymphohematopoietic cells, including both myeloid and plasmacytoid dendritic cells, and secondary lymphoid organs develop (22, 23). Because the human thymic grafts are autologous to the human hematopoietic cells in the periphery, HLA-restricted responses between T cells and human APCs in the periphery are associated with strong Ag-specific immune responses, including class-switched Ig responses (26) and anti-infectious responses (25). Previous studies have shown that human fetal thymic tissue alone is insufficient to achieve long-term thymopoiesis in immunodeficient mice (55), and i.v. injection of CD34+ cells is needed to achieve high levels of long-term T cell reconstitution in HU mice receiving fetal thymus grafts (23). Moreover, the human thymus graft is essential for the achievement of peripheral human T cell reconstitution in our model (24), and the fetal liver graft is not required for long-term T cell reconstitution, including Tregs (Supplemental Figs. 3, 4). Therefore, our data strongly suggest that population of the human thymus with progenitors from the injected CD34 cells is the major source of long-term T cell reconstitution, including nTregs. Consistent with this interpretation, most of these Tregs expressed the Helios transcription factor, which has been shown to be present in nTregs of mice and humans, but not in induced Tregs that are generated from conventional T cells extrathymically (32). In the experiment in which Helios expression was analyzed, the thymus graft was first cryopreserved/thawed and the mice were treated with depleting anti-CD2 mAb immediately after transplant. These two procedures have been shown to eliminate pre-existing aloreactive thymocytes without compromising thymic growth and function (H. Kalscheuer, N. Danzl, T. Onoe, T. Faust, R. Winchester, T.R. Spitzer, H. Tahara, Y.G. Yang, and M. Sykes, submitted for publication). Because no fetal liver fragment was implanted in these mice and a hemopoietic stem cell source is required for long-term thymopoiesis in human thymic grafts (55), the robust Helios+ Treg repopulation detected in long-term grafts and in the periphery demonstrates that the Tregs were derived de novo in the grafts from progeny of the CD34 cells injected i.v.

This model has allowed adoptive transfer studies to be performed that assessed the fate of naive human T cells in a lymphopenic environment, demonstrating that such cells expand, convert to the effector/memory phenotype, and develop effector functions. These activities were dependent on and proportional in magnitude to the number of autologous human APCs in the periphery of the adoptive recipients (24), supporting the notion that, as in rodents (56–67), lymphopenia-driven expansion of human T cells requires interaction with a peptide/MHC complex in the periphery that was also present on the positively selecting thymic epithelium. In addition to demonstrating high levels of Treg reconstitution in the periphery in this HU mouse model, we demonstrate in this article that human thymus-derived natural Tregs undergo phenotypic changes in the periphery that likely reflect interactions with autologous human APCs. Consistent with the role reported in rodents for interactions with peripheral (thymic) self-peptide/MHC complexes to confer function on Tregs (52, 53), the Tregs in peripheral tissues of our HU mice demonstrated function that was, on a per-cell basis, similar to that of Tregs obtained from healthy adult human PBMCs.

Although the proportion of Tregs expressing the activated/memory (CD45RO+HLA-DR+) phenotype was lower in the PBMCs of HU mice than of the (much older) adult control human volunteers, this was compensated by a greater proportion of Tregs overall in the T cells of HU mice compared with adult PBMCs, such that the overall percentages of these activated/memory Tregs was similar in both groups. The increased proportion of naive nTregs in PBMCs of HU mice compared with adult human donors may reflect the greater tendency of fetal compared with adult hemopoietic stem cells to generate Tregs (46). The percentage of memory-type non-Treg CD4+ and CD8+ T cells in PBMCs was similar in PBMCs of 20 wk HU mice to that in adult volunteers. Taken together, these studies suggest that conversion of recent thymic emigrants to the memory phenotype may be accelerated in the periphery of HU mice compared with that of healthy adult humans. This may reflect the absolute lymphopenia in these mice when the first T cells populate the periphery and a possible failure to completely fill up the peripheral lymphoid compartment, in which case “lymphopenia-driven” memory conversion, which we have demonstrated previously via adoptive transfer (24), may occur continually in HU mice. Conversion of CD45RA+ resting Tregs to the CD45RO+ activated phenotype in association with expansion has previously been demonstrated by adoptive transfer of the resting subset into immunodeficient mice (36). Although we cannot rule out the possibility that some of the conversion to the activated phenotype was in response to xenogeneic GVH reactivity, the mice in the present studies appeared healthy and were sacrificed well before any clinical evidence of wasting disease, which only appears after ~30 wk in association with loss of thymic cellularity in our model. The presence of significant xenogeneic GVH reactivity has been associated with global “anergy” (16), which is inconsistent with the robust proliferative and class-switched IgG responses to protein Ags observed in our model (23, 25, 26).

The percentage of activated/memory-type Tregs in the spleen and LNs of HU mice was considerably higher than that in the PBMCs of the same mice. Recent studies have shown that human CD45RA−CD45RO−CD25−CD4+FOXP3high cells are activated Tregs that are derived from CD45RA−CD45RO−CD25−CD4+FOXP3low resting Tregs (36). Although both subsets were found to have suppressive activity in vitro, the activated subset also expresses HLA-DR (36), which has previously been reported to identify Tregs with increased suppressive activity compared with the HLA-DR−negative subset (31). A study comparing the proportions of Tregs in PBMCs versus other secondary lymphoid tissues in humans did not analyze differences between the tissues in detail, but the data shown are consistent with the pattern we observed in HU mice, with proportions of CD45RO−CD45RA−CD25− cells in LNs > spleen > PBMCs (68). Because HLA-DR identifies Tregs with the greatest functional suppressive activity (31), it would be predicted that the proportion of Tregs expressing these markers would correlate with the level of suppressive activity. Consistently, on a per-cell basis, Tregs from these lymphoid tissues of HU mice and from PBMCs of the healthy volunteers showed remarkably similar suppressive activity. Thus, our studies document the full thymic and postthymic maturation of functional human Tregs. The postthymic conversion to the activated phenotype presumably reflects tonic or Ag-specific interactions with self human APCs in the periphery and/or may reflect homeostatic expansion of early thymic emigrants. Although further studies are needed to distinguish among these possibilities, the ability to perform manipulations such as APC depletions, immunizations, graf- tectomy, and adoptive transfer studies to HU mice with autologous APCs but lacking T cells, as we have previously described (24), will allow direct assessment of the factors that drive human Treg homeostasis, phenotypic conversion, expansion, survival and functional maturation, and evaluation of Treg-based therapies.

Our studies provide a direct window onto human natural Treg development in the thymus and their fate in the periphery. The midgestational fetal thymus tissue used in this study contained
a significant fraction of Tregs, which mostly expressed a high level of CD45RO, without CD45RA, and this phenotype was recapitulated in 20 wk thymus grafts in our HU mice. This phenotype is consistent with that previously described for CD4+CD25+ cells in human infant thymus (68). Because cord blood CD4+CD25+ cells express mainly the CD45RO CD45RA phenotype, the data presented in this study, combined with human infant thymus studies, suggest that human thymic CD3+CD4+CD8+ CD25+CD127+ FOXP3+CD45RO+CD45RA- cells are immature nTregs and that, as seen for all SP CD4 and CD8 T cells (Fig. 1C and Ref. 28), loss of CD45RO and gain of CD45RA are terminal maturation events that either precede or follow emigration from the thymus to the periphery. Overall, our studies document that normal thymic Treg development occurs in human thymus grafts in our HU mouse model, permitting new insights into the development and function of natural human Tregs.

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Disclosures
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Supplemental Data

Supplementary Figure 1. Similar phenotypes of Tregs in thymic grafts of humanized mice and human fetal thymus.

The phenotypes of the indicated gated subpopulation of CD4 single positive thymocytes in thymic grafts of humanized mice (closed circles, n = 4) and human fetal thymus (open circle, n = 1) are shown. Humanized mice were sacrificed 21 weeks post-transplantation. Mononuclear cells of thymic graft and human fetal thymus were isolated by ficoll separation. Isolated MNCs were stained for CD4, CD8, CD25, CD127, CD45RA, CD45RO and HLA-DR. Samples were fixed and further stained intracellulary for FoxP3. The proportion of CD25+CD127- cells among CD4+ single positive T cells and FoxP3+ cells among CD25+CD127-CD4+ single positive T cells were analyzed by FCM (left panels). CD25+FoxP3+CD4+ single positive T cell populations were gated and their surface expression of CD45RA, CD45RO and HLA-DR were analyzed (right panels). Each circle represents gated percentages from an individual animal or donor, and the line represents mean.

Supplementary Figure 2. Peripheral blood T cells in humanized mice show similar phenotypes to those in normal humans.

Humanized mice (closed bar, n = 26) and healthy human volunteers (open bars; n = 6) were bled to measure the phenotypes of CD4 (upper panel) and CD8 (lower panel) T cells, respectively. Blood samples of humanized mice were obtained 16-18wk post-transplantation and isolated PBMC were stained and analyzed by FCM. Open bars and closed bars represent distribution of indicated phenotypic subpopulation (CD45RA+RO-, CD45RA-RO+ and CD45RA+RO+ cells).
among CD4 and CD8 T cells in normal human and humanized mice, respectively. All data are expressed as mean ± SEM. Pooled results of two similar experiments are presented. There is no statistically significant difference between normal humans and humanized mice (two-way ANOVA).

**Supplementary Figure 3. Fetal liver fragment is not required for human T, B or monocyte reconstitution in NOD-scid mice receiving human fetal thymus graft and fetal liver-derived CD34+ cells i.v.**

Mean percentages of various human cell populations in PBMC at indicated time points are shown for mice that received human fetal thymus grafts and i.v. fetal liver CD34+ cells, with or without a fetal liver (Liv) fragment adjacent to the thymus (Thy) graft under the kidney capsule. N= 3-5 per group. There was no significant difference in human cell populations between the groups receiving or not receiving a fetal liver fragment.

**Supplementary Figure 4: Fetal liver fragment is not required for Treg reconstitution in NOD-scid mice receiving human fetal thymus graft and fetal liver-derived CD34+ cells i.v.**

Phenotype of human T cells in PBMC 18 weeks post-transplantation for mice that received human fetal thymus grafts and i.v. fetal liver CD34+ cells, with or without a fetal liver (Liv) fragment adjacent to the thymus (Thy) graft under the kidney capsule. N= 3-5 per group. A. Top panels: representative gated human CD4+CD3+ PBMCs, showing that about 10% of cells have the CD25+CD127- phenotype. Bottom panels: similar, high FoxP3 expression in gated CD25+CD127- CD4+CD3+ human T cells in PBMC of both groups. B. Summary data showing similar percentages of CD4+ cells among PBMCs and of CD25+CD127- cells among CD4+ T
cells in mice receiving human fetal Thy tissue and i.v. CD34+ cells, with or without fetal liver fragments. No significant differences were detected between the two groups.
Supplementary Figure 1
Supplementary Figure 2

CD4 T cells

- % among human CD4+ T cells
- Humanized mouse: black bars
- Human: white bars

CD8 T cells

- % among human CD8+ T cells
- Humanized mouse: black bars
- Human: white bars
Supplementary Figure 3

Human chimerism

- % hCD45* cells among PBMCs
- Weeks after Tx: 8, 12, 16, 20

Human T cell chimerism

- % hCD3* cells among PBMCs
- Thy/Liv plus CD34+ FLCs
- Thy plus CD34+ FLCs
- Weeks after Tx: 8, 12, 16, 20

Human B cell chimerism

- % hCD19* cells among PBMCs
- Weeks after Tx: 8, 12, 16, 20

Human monocytes chimerism

- % hCD14* cells among PBMCs
- Weeks after Tx: 8, 12, 16, 20