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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+Helios+ “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.

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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

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Abbreviations used in this article: FCM, flow cytometry; FLC, fetal liver cell; GVH, graft-versus-host; HU, humanized; LN, lymph node; nTreg, natural regulatory T cell; SP, single-positive; Treg, regulatory T cell.

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peripheral blood, spleen, and lymph nodes (LN) 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 Bioscience 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 Committees 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. 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 mm3 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 × 107 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 tissue and i.v. injection of anti-CD2 mAb at the time of transplantation, a method that we have shown allows robust human thymopoesis/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 using flow cytometry (FCM) analysis. HU mice 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 CD45 (30-F11), anti-CD4 (RPA-T4), anti-human CD4 (RPA-T4), anti-human CD8 (RPA-T8), anti-human CD14 (M5E2), anti-human CD19 (HB19), anti-human CD45 (HI30), anti-human CD3 (SK7), anti-human CD45RA (HI100), anti-human CD45RO (UCHL1), anti-human CD25 (M-A251), anti-human CD25 (HI100), anti-human CD45RO (UCHL1), anti-human CD45RA−(HI100), anti-human FOXP3 (236A/E7), anti-HLA-DR (L234), allopurinol and streptavidin, PE-Cy5-streptavidin, and isotype control mAbs. All samples were acquired using a FACS caliber or LSR II (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 LNs with the Abs above and anti-mouse/human HELIOS (22P6; 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 Abs 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 (236A/E7; eBioscience) alone or with anti-mouse/human HELIOS (22P6; BioLegend) for 30 min at 4°C according to the manufacturer’s 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 × 10−3 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+ (responder) 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 (HI-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 × 106 cells/well) were cultured in 12-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 d or in the presence of irradiated human allogeneic PBMCs (3000 rad, 4 × 105 cells/well) for 5 d at 37°C in 5% CO2. Cells were harvested after 16 h incubation with 1 μCi [3H]thymidine. [3H]Thymidine incorporation was measured by beta-counter. Data are expressed as actual cpm or stimulation index (cpm of all stimulated culture/cpm of autostimulated culture). In autostimulated control cultures, responder cells were incubated with autologous PBMCs. All data are shown as mean of [3H]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 mm3 to ~1 cm in length and contained ~100–300 × 106 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 were CCRIλ+ (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 CD45+CD4+CD8+CD25+CD127low thymocytes were held to contain 210 × 10^6 thymocytes. A, Representative macroscopic appearance of thymic implant in HU mouse. NOD/SCID mice that received human fetal thymus/liver and i.v. injection of CD34+ FLCs were sacrificed to evaluate the development of human thymocytes in human thymic grafts 18 wk after implantation. The HU mouse graft was harvested and stained with CD4 and CD8 T cells. B, Phenotype of thymocytes in thymic grafts of HU mouse (left) and human fetal thymus (right) are shown. C, Comparison of phenotypic distribution of CD4 or CD8 SP T cells of thymus graft and PBMCs of the same animals. Humanized mice (n = 4) were sacrificed 21 wk posttransplantation. Mononuclear cells of thymic graft and blood were isolated from each individual mouse by Ficoll separation. Single-cell suspensions were stained for markers of T cells (CD4 and CD8) and phenotypic markers of T cells (CD45RA, CD45RO, and CCR7) and analyzed by FCM. Open bars and filled bars represent distribution of indicated phenotypic subpopulations (CD45RO-RA+, CD45RO-RA-, CD45RO-RA+, and CCR7+ cells) among CD4 and CD8 SP T cells in thymus grafts and PBMCs, respectively. All data are expressed as means ± SEM. Experiments were repeated twice with similar results. ***p < 0.001, comparing thymus grafts and PBMCs (two-way ANOVA, Bonferroni test). D, Phenotypes and Tregs of CD4 SP thymocytes in thymic grafts of HU mouse (lower panels) and human fetal thymus (upper panels). The thymic graft specimen shown in A and human fetal donor thymus were analyzed. The bold line represents staining of the indicated gated subpopulations with fluorochrome-labeled mAb for the indicated molecule. The filled area represents staining with isotype control mAb. Representative profiles are shown. E, Helios expression in human CD45+CD4+CD8+CD25+CD127low thymocytes was assessed by FCM in the thymic graft of HU mice 18 wk posttransplant. Representative FCM profile is shown (left) and total FOXP3^+Helios^+ and FOXP3^+Helios^- cells were calculated from n = 2 (right).

**FIGURE 1.** Normal human thymocyte populations and Treg phenotype of CD25^+CD127^-CD4^-CD8^- thymocytes in thymic grafts of HU mouse. A, Representative macroscopic appearance of thymic implant in HU mouse. NOD/SCID mice that received human fetal thymus/liver and i.v. injection of CD34+ FLCs were sacrificed to evaluate the development of human thymocytes in human thymic grafts 18 wk after implantation. The HU mouse graft shown in the photomicrograph contained 210 × 10^6 thymocytes. B, Phenotype of thymocytes in thymic grafts of HU mouse (right) and human fetal thymus (left) are shown. C, Comparison of phenotypic distribution of CD4 or CD8 SP T cells of thymus graft and PBMCs of the same animals. Humanized mice (n = 4) were sacrificed 21 wk posttransplantation. Mononuclear cells of thymic graft and blood were isolated from each individual mouse by Ficoll separation. Single-cell suspensions were stained for markers of T cells (CD4 and CD8) and phenotypic markers of T cells (CD45RA, CD45RO, and CCR7) and analyzed by FCM. Open bars and filled bars represent distribution of indicated phenotypic subpopulations (CD45RO^-RA+, CD45RO^-RA-, CD45RO^-RA+, and CCR7^+ cells) among CD4 and CD8 SP T cells in thymus grafts and PBMCs, respectively. All data are expressed as means ± SEM. Experiments were repeated twice with similar results. ***p < 0.001, comparing thymus grafts and PBMCs (two-way ANOVA, Bonferroni test). D, Phenotypes and Tregs of CD4 SP thymocytes in thymic grafts of HU mouse (lower panels) and human fetal thymus (upper panels). The thymic graft specimen shown in A and human fetal donor thymus were analyzed. The bold line represents staining of the indicated gated subpopulations with fluorochrome-labeled mAb for the indicated molecule. The filled area represents staining with isotype control mAb. Representative profiles are shown. E, Helios expression in human CD45+CD4+CD8+CD25+CD127^- thymocytes was assessed by FCM in the thymic graft of HU mice 18 wk posttransplant. Representative FCM profile is shown (left) and total FOXP3^+Helios^+ and FOXP3^+Helios^- cells were calculated from n = 2 (right).
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+FOXP3- 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+FOXP3+ 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 PBMCs expressed HLA-DR by their CellSearch (n = 6).
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 ± 5.77% 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

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**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. 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⁺, 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 peripheral reconstitution (42, 43). Infusion of human hematopoietic 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 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 hematopoietic stem cells, but phenotypic subsets and peripheral Tregs were not described (46).

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
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 alloreactive 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+FOXP3low cells are activated Tregs that are derived from CD45RA-CD45RO-CD25+CD4+FOXP3high 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, grafted, 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+ in 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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