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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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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 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 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 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 × 105 CD34+ FLCs were injected i.v. In a slight modification of the protocol, animals used in studies of Helios expression received cryopreserved/thawed fetal thymic tissue and i.v. injection of anti-CD2 mAb 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 (HIL-7R-M21) mAbs and sorted on a FACSARia (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%.


Flow cytometry

Human hematopoietic cell repopulation in the HU mice and the profiles of T cells were assessed by 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 CD34 (30-F11), anti-mouse Ter-119 (Ter-119), anti-human CD4 (RPA-T4), anti-human CD8 (RPA-T8), anti-human CD14 (M52E2), anti-human CD19 (HIB19), anti-human CD45 (HI30), anti-human CD3 (SK7), anti-human CD45RA (H100), anti-human CD45RO (UCHL1), anti-human CD25 (M-A251), anti-human CD127 (HIL-7R-M21), anti-human FOXP3 (236A/E7), anti-IL-2 receptor (IL-2R), anti-CD28 (CD28.2), and soluble anti-CD28 mAb (CD28.2, 2.5 µg/ml) and soluble anti-CD25 mAb (CD28.2, 2.5 µg/ml) for 4 or 5 d 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 allstimulated 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. Macrosopically, 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 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).

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 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 CD45RO+CD45RA- (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 (CD45RO+CD45RA-) (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 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+CD45RA- 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+CD45RA+ 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 ± 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

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−, 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).
FIGURE 4. Suppressive activity of Tregs developing in HU mice is comparable to that of normal human donors. A, Representative phenotypic profile and purity of CD4+CD25+ (responders, left) and CD4+CD25+CD127+ (suppressors, middle) T cells of a HU mouse after FACS sorting. The frequency of CD25+CD127+ cells among gated CD4 SP T cells in each cell fraction is shown. The right panel shows intracellular FOXP3 expression of the indicated fraction. The shaded line represents the staining with isotype control mAb, and the thick line and dashed line represent the test staining of suppressor and responder cells, respectively. B and C, In vitro suppression of proliferation of CD4+CD25− T cells (responders) by FACS-sorted CD4+CD25+CD127+ T cells (suppressors) isolated from normal human PBMCs or pooled spleen and LNs of HU mice 20 wk posttransplantation. CD4+CD25+ T cells (2 × 10^5) isolated from human PBMCs or pooled spleen and LN cells of 3 HU mice were incubated with autologous CD4+CD25+CD127+ T cells in indicated ratios in the presence of plate-bound anti-CD3 mAb (1 μg/ml) and soluble anti-CD28 mAb (2.5 μg/ml) for 4 d (B) or in the presence of 30 Gy-irradiated allogeneic PBMCs as stimulators for 5 d (C). Cultures were pulsed with [3H]thymidine at day 3 (B) or 4 (C) and harvested 16 h later. Data are expressed as mean of triplicates.
thymus. In this model, hematopoietic 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 plasmacytid 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 human thymic 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 extrathympically (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 allogeneic thymocytes without compromising thymic growth and function (H. Kalscheuer, N. Danzl, T. Ono, 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 hematopoietic 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 hematopoietic 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, 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...
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...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+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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