A Humanized Mouse Model Generated Using Surplus Neonatal Tissue

Matthew E. Brown,1,2 Ying Zhou,1 Brian E. McIntosh,3 Ian G. Norman,1 Hannah E. Lou,1 Mitch Biermann,4 Jeremy A. Sullivan,1 Timothy J. Kamp,4,5 James A. Thomson,2,5,6 Petros V. Anagnostopoulos,7
and William J. Burlingham1,*

1Division of Transplantation/Department of Surgery, University of Wisconsin, Madison, WI 53792, USA
2Regenerative Biology, Morgridge Institute for Research, Madison, WI 53715, USA
3Covance Laboratories Inc., Madison, WI 53704, USA
4Department of Medicine, University of Wisconsin, Madison, WI 53792, USA
5Department of Cell & Regenerative Biology, University of Wisconsin, Madison, WI 53792, USA
6Department of Molecular, Cellular, & Developmental Biology, University of California, Santa Barbara, CA 93106, USA
7Division of Cardiothoracic Surgery/Department of Surgery, University of Wisconsin, Madison, WI 53792, USA
*Correspondence: burlingham@surgery.wisc.edu
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SUMMARY

Here, we describe the NeoThy humanized mouse model created using non-fetal human tissue sources, cryopreserved neonatal thymus and umbilical cord blood hematopoietic stem cells (HSCs). Conventional humanized mouse models are made by engrafting human fetal thymus and HSCs into immunocompromised mice. These mice harbor functional human T cells that have matured in the presence of human self-peptides and human leukocyte antigen molecules. Neonatal thymus tissue is more abundant and developmentally mature and allows for creation of up to ~50-fold more mice per donor compared with fetal tissue models. The NeoThy has equivalent frequencies of engrafted human immune cells compared with fetal tissue humanized mice and exhibits T cell function in assays of ex vivo cell proliferation, interferon γ secretion, and in vivo graft infiltration. The NeoThy model may provide significant advantages for induced pluripotent stem cell immunogenicity studies, while bypassing the requirement for fetal tissue.

INTRODUCTION

Recent years have seen great progress in deriving immuno-deficient mouse strains capable of being engrafted with human cells and tissues to create humanized mouse models (Lan et al., 2006; Lavender et al., 2013). These include mice modified to produce human cytokines (Rongvaux et al., 2014; Saito et al., 2016), that express specific human major histocompatibility complex (MHC) types (Shultz et al., 2010) and that do not require irradiation for xenoengraftment (McIntosh et al., 2015). While the non-T cell immune compartments within the animals are biologically relevant, humanizing these host strains via surgical implantation of human thymus tissue is required for creation of de novo T cells that can recognize a full complement of human MHC molecules presenting antigens in vivo (Shultz et al., 2012; Theocharides et al., 2016; Zhao et al., 2015). Humanized mice such as the bone marrow, liver, thymus (BLT) mouse, generated by co-transplantation of hematopoietic stem cells (HSCs) along with human fetal thymus tissue, offer a powerful translational system to study human immune responses (Hu and Yang, 2012; Kalscheuer et al., 2012; Lan et al., 2006). They are particularly useful for virology research, induced pluripotent stem cell (iPSC) immunogenicity studies, and other research requiring functional T cells selected on human self-antigen complexes (Lavender et al., 2013; Rong et al., 2014; Yu et al., 2007; Zhao et al., 2015). Humanized models incorporating human thymus fragment implantation are uniquely suited for investigating questions relating to patient-specific immune responses to iPSC cell therapies, as self-tolerance is largely dictated by thymus-dependent mechanisms (Griesemer et al., 2010; Zhao et al., 2015).

There are multiple barriers preventing more-widespread use of the above-mentioned humanized mouse models. For example, limited fetal specimen size necessitates multiple tissue samples from divergent genetic backgrounds over an experimental course and each specimen typically yields only 15–20 humanized mice (Hassini et al., 2014). This results in significant experimental variability and discourages robust characterization of sparse and ephemeral tissue supplies. In addition, fetal tissue’s immature developmental status may influence gene expression patterns, phenotype, and function of fetal tissue-derived immune cells; BLT models may not reliably represent clinical patient immune responses (Beaudin et al., 2016; Lee et al., 2011; McGovern et al., 2017; Mold and McCune, 2012; Mold et al., 2010; Notta et al., 2016).

We developed the NeoThy humanized mouse model, which utilizes abundant non-fetal human thymus tissue from neonatal cardiac surgery patients, paired with umbilical cord blood HSCs from autologous or unrelated donors. We evaluated human immune cell engraftment kinetics and their phenotype and function.
RESULTS AND DISCUSSION

Human thymus tissue was obtained from neonatal cardiac surgeries after receiving informed consent. Neonatal thymus samples provided more tissue (mean 9.3 ± 2.9 g, n = 7 samples, 7-day-old median age patients) compared with fetal sources (mean 0.58 g at 20 weeks gestation) (Hasini et al., 2014). This enabled cryopreservation and banking of hundreds of thymus fragments from each donor to generate humanized mice (Figure 1A). NeoThy mice were made from multiple neonatal thymus and cord blood samples and compared with fetal tissue control animals. Humanization with a 1 × 1 mm neonatal thymus fragment and intravenous (i.v.) injection of 0.5 × 10^5–1.5 × 10^5 cord blood hCD34+ HSCs resulted in thymic organoid formation across all four donors tested. These first-generation animals are distinguished from second-generation animals that received zhCD2 antibody (see below). The resulting thymic organoids were significantly smaller than those arising from fetal tissue (Figure 1B), yet, like fetal controls, they maintained thymic anatomy, including Hassall’s corpuscles, indicating an active role in human thymopoiesis. We hypothesize that size differences between fetal and neonatal organoids may be due to variations in thymic epithelial cell progenitors within the two tissue types, rather than being the result of differential thymopoiesis efficiencies (Bleul et al., 2006).

To explore organoid function in more detail, we investigated whether the smaller organoid size affected engraftment of human immune cells in NeoThy mice. We were not able to isolate >4,000 cells from harvested NeoThy organoid explants, preventing flow cytometric analysis of the developing thymocytes. However, no significant differences in peripheral hCD45+, hCD19+, and hCD3+ immune cell frequency were observed between NeoThy mice and animals reconstituted with fetal thymus and cord blood HSCs (Figures 1C and 1D). Further, organoid size differences did not impact absolute numbers of CD3+ T cells (Figures S1A and S2B). HSC-only controls had significantly more hCD19+ B cells and little to no hCD3+ T cells at 15–18 weeks post-humanization, compared with both fetal and NeoThy mice, indicating that both models require a thymic organoid for robust T cell reconstitution. These results argue against the dysplastic murine thymus of the host animal playing a role in thymopoiesis (McDermott et al., 2010). However, we cannot conclusively rule out murine thymic tissue contributing to human T cell repopulation and function in our model.

Two strains of mice, NSG receiving 250 RAD sublethal irradiation, and NSG-W not requiring irradiation, were successfully humanized with neonatal tissues (Figure S2A), suggesting that the humanization capacity of neonatal tissues may translate well to other immunocompromised strains. The phenotype of the engrafted human immune systems may differ depending on available cytokines, hematopoietic niches, irradiation requirements, and other specific host attributes (Brehm et al., 2010).

We used the NSG-W immunocompromised mouse for NeoThy creation because of the convenience of not having to irradiate animals. That also eliminated toxicity associated with irradiation, e.g., death and graft versus host disease (GVHD) (McIntosh and Brown, 2015). The second-generation NeoThy mice described in Figure 2 received i.v. injection of 100 µg zhCD2 antibody on days 0 and 7 post-surgery. This method was described previously by Kalscheuer et al. (2012) for removing GVHD-associated passenger thymocytes from fetal thymic fragments. zhCD2 antibody injections delayed T cell development by approximately 4 weeks compared with first-generation NeoThy mice (Figure S3A). This delay in T cell emergence indicates that early arising T cells in BLT and other first-generation humanized mice may be partially derived from passenger thymocytes rather than de novo from injected HSCs educated on thymic fragment epithelial cells. As passenger thymocytes within the fragment may have already reached their final stages of development without encountering mouse xenoantigens, first-generation mice may be more prone to GVHD than second-generation mice in which de novo human T cells mature in the presence of mouse xenoantigens (Kalscheuer et al., 2012; Lockridge et al., 2013).

To assess whether second-generation NeoThy mice were susceptible to GVHD, we followed a cohort of five NeoThy mice for 32 weeks post-humanization (Figure 2A). At 32 weeks, five of five mice survived while maintaining human immune cell reconstitution of hCD45+ cells. Three of five animals showed no symptoms of GVHD, two of five showed minor GVHD scores (Lockridge et al., 2013). Conversely, first-generation NeoThy mice that did not receive zhCD2 antibody died at multiple time points, beginning at 15 weeks post-humanization. As immuno-compromised mice are prone to infections and other causes of death besides GVHD, large longitudinal cohorts of mice necropsied at time of death would be required to determine general susceptibility of the NeoThy to GVHD. As T cells begin to emerge at ~10–12 weeks post-humanization (Figure 2B), the experimental window for transplantation studies in the NeoThy may allow monitoring of transplanted grafts >20 weeks in vivo.

NeoThy mice created in NSG-W hosts using autologous thymus and HSCs, or with thymus and allogeneic HSCs, and in multiple donors with varying degrees of human leukocyte antigen (HLA) matching (Figure S2B) all robustly engrafted with human CD45+, CD19+, and CD3+ cells (Figure 2B). Both first- and second-generation protocols for NeoThy mice produce a typical component of hCD19+
B cells and hCD3+ T cells, including hCD4+ and hCD8+ subsets (Figure 2C, additional data not shown). Additional human immune cell types relevant to transplant tolerance and rejection were consistently detected. Human CD4+CD127loCD25+FOXP3+ regulatory T cells were observed in blood, spleen, and lung (Figure 2D), along with CD11b+, CD11c+, and CD14+ myeloid cells relevant for in vivo antigen presentation (Figure 2E). These data

Figure 1. Engraftment of Human Thymus Tissue and Immune Cells
(A) Human neonatal thymus is abundant (e.g., 14.75 g, shown). Membrane, adipose, and blood vessels were removed and tissue processed into large (I), then medium (II), then 1 x 1 mm fragments (III) for cryopreservation. More than 1,000 fragments suitable for transplantation can be obtained from a single thymus.
(B) Implanted thymus fragments develop into organoids under the kidney capsule when co-transplanted i.v. with hCD34+ cells, +/- ahCD2 antibody depletion (second- and first-generation mice, respectively). Histological analysis of first-generation fetal humanized mouse (NSG) (left) and second-generation neonatal (NSG-W) (right) thymic organoids, including Hassall’s corpuscles, are shown (4X scale bar, 500 μm; inset is 10X scale bar, 100 μm).
(C) Humanized mice were generated from various human tissue samples in irradiated NSG mice w/o ahCD2 antibody depletion (first generation) and compared for human immune cell engraftment (hCD45+), including B cells (hCD45+hCD19+) and T cells (hCD45+hCD3+) at early (6–7 weeks post-surgery) and late (15–18 weeks) time points. In four independent experiments, n = 12 animals received fetal thymus and allogeneic cord blood CD34+ cells (Fet Thymus + Allo Cord), n = 9 animals received neonatal thymus and allogeneic cord (Neo Thymus + Allo Cord), n = 3 allogeneic cord only (Allo Cord Only) and n = 12 neonatal thymus alone from 3 donors (Thymus Only). The Thymus Only condition did not receive hCD34+ cells. The Allo Cord Only condition received hCD34+ cells only.
(D) Splenocytes from fetal (n = 6) and neonatal tissue-derived (n = 13) first- and second-generation mice, using both NSG and NSG-W strains (seven independent experiments), were compared for human engraftment markers as in (C). Statistics were conducted using ANOVA, analyzed with GraphPad Prism 7.00. The Thymus Only control was included for comparison and was not analyzed for significance. ****p < 0.0001, ***p = 0.0001, **p < 0.01, *p < 0.05; ns, not significant; no bar, not analyzed. See also Figures S1, S2, and S3.
Figure 2. Long-Term Survival and Engraftment of Human Immune Cells in NeoThy Mice

(A) Five second-generation and four first-generation (+/− CD2 antibody, respectively) NeoThy (NSG-W) mice from the same experiment were humanized with neonatal thymus and allogeneic cord blood from one donor and followed for 32 weeks post-humanization, with no further experimental manipulations. Second-generation mice human immune cell reconstitution of hCD45⁺ cells was a mean of 61.6% ± 17.0%, hCD45⁺hCD19⁺ cells, mean of 39.4% ± 11.6%, and hCD45⁺hCD3⁺ cells, mean of 49.1% ± 15.6% at experiment endpoint. First-generation mice reconstitution at 14 weeks (latest measurement prior to first animal death) for hCD45⁺ cells was a mean of 46.7% ± 34.2%, hCD45⁺hCD19⁺ cells, mean of 24.2% ± 21.2%, and hCD45⁺hCD3⁺ cells, mean of 41.1% ± 26.8%. Statistics were conducted using Kaplan-Meier estimator method, compared with a log rank test, analyzed by GraphPad Prism 7.00 (p = 0.0221).

(B) Second-generation NeoThy (NSG-W) mice were humanized via implantation of neonatal thymus fragment and co-injection with either autologous (PED05, see Table S1) hCD34⁺ cells (n = 10 mice to start, 1 mouse removed at 17 weeks + 4 mice removed at 20 weeks → n = 5 at endpoint, 2 pooled experiments) (left) or allogeneic hCD34⁺ cells matched at 1 allele each for HLA-A, -B, and -DR (n = 12 mice to start, 1 mouse removed at 15 weeks + 6 mice removed at 20 weeks/ n = 5 at endpoint, 1 experiment) (right) (PED05 + donor Allo Cord 212, see Table S1). Total human immune cell engraftment is shown over time. Error bars were determined using SD.

(C) Representative distributions of B and T cells are observed in blood of mice from (B) 18 weeks post-surgery.

(D) Human CD4⁺ hCD127⁻hFOXP3⁺ surface and intracellular flow cytometry staining for regulatory T cells are shown compiled from one representative experiment of 13 second-generation NeoThy (NSG-W) mice engrafted with autologous PED05 tissues at 15 weeks and in representative second-generation NeoThy (NSG-W, allogeneic tissues) blood, spleen, and lungs at 17–29 weeks post-humanization.

(E) Peripheral blood from 1 experiment using 10 second-generation NeoThy (NSG-W, autologous PED05 tissue) mice at 15 weeks were compiled to show reproducibility within experiments (top panel) and representative staining from second-generation NeoThy (NSG-W, allogeneic tissues) is shown for blood, spleen, and lung cells stained for the myeloid cell markers hCD11b, hCD14, and hCD11c gated on viable single hCD45⁺ cells at 17–29 weeks (bottom panel). See also Figures S1, S2, and S3.
indicate that the NeoThy model harbors relevant cell populations for T cell responses; a primary mediator of transplant tolerance and allorejection.

Having shown robust engraftment of human T cells and other immune cells, we examined T cell function in the NeoThy model. Ex vivo engagement of T cell receptors with zCD3, zCD28, and rHIL2 stimulated T cell proliferation, indicated by blast-like clustering, dilution of CSFE dye in both CD4+ and CD8+ T cell populations, and production of Th1 cytokine interferon γ (Figures 3A and S2C). NeoThy mice reconstituted with human B and T cells also produced immunoglobulin G (IgG) and IgM antibodies (Figure 3B), indicating a functional T helper role of CD4+ T cells as well as functional B cells. Models of human T cell-mediated tolerance and rejection must demonstrate antigen-specific T cell function. Using the trans vivo delayed-type hypersensitivity assay, we show that NeoThy T cells mount a collagen V-specific effector response to co-injected antigen in the absence of anti-transforming growth factor β neutralizing antibody. This demonstrates the utility of the NeoThy for study of innate-like Th17 cell function in the context of iPSC immunogenicity (Sullivan et al., 2017). Last, NeoThy mice challenged with a B6 skin graft mounted rejection responses characterized by the two metrics of (1) hCD3+ T cell infiltration and (2) visible graft loss over time, similar to fetal tissue controls (Figures 3D and 3E). These data demonstrate the robust functional attributes of the T cell repertoire and the suitability of the NeoThy for pre-clinical investigations of iPSC immunogenicity.

To determine whether the NeoThy model could be used for in vivo investigation of allogeneic human iPSC immunogenicity, we reprogrammed NeoThy tissue donor cells into iPSCs. We successfully reprogrammed donor umbilical cord blood CD34-depleted cells (Figures S4A–S4F), and thymic stromal cells (data not shown) into iPSCs via a non-integrating episomal vector method (Yu et al., 2011). These iPSCs displayed conventional markers of pluripotency and were karyotypically normal. The cells were directly differentiated into cardiac troponin T-positive (cTNT) cardiomyocytes (CMs) (mean 75.5% cTNT+, ten differentiations of two donor lines) (Figure S4E). The iPSC-CMs spontaneously contracted in both 2D and 3D suspension cultures (Movies S1 and S2). iPSC-CMs from donor PED04 (Table S1) were transplanted under the kidney capsule of three second-generation NeoThy mice made with PED05 tissues and sacrificed 26 days later (Figure 4A).

NeoThy mice engrafted with allogeneic iPSC-CMs showed macroscopic and histological retention of implanted cells (3/3) at the terminal time point and were infiltrated by human CD4+ and CD8+ T cells (Figure 4B). The lower degree of CD8+ infiltration observed could reflect the higher ratio of peripheral blood CD4+ to CD8+ T cells seen in NeoThy (Figure 2C) and BLT (Rajesh et al., 2010) mice, and/or it could be due to the HLA-A2 match (associated with CD8+ T cell recognition) and HLA-DR mismatch (associated with CD4+ T cell recognition) between graft and recipient in the experiments.

Metrics for assessing rejection/tolerance of PSC therapies, in humanized mice and other models, vary among research groups and cell types (de Almeida et al., 2014; Kooreman et al., 2017; Sugita et al., 2016; Zhao et al., 2011). Progression of the field will require cell-type-specific criteria, informed by clinical solid organ transplantation guidelines (Haas, 2016; Solez et al., 1993). Our metric of graft infiltration can indicate a tolerance (de Almeida et al., 2014; Ling et al., 2015) or rejection response (Zhao et al., 2011). We cannot make a definitive conclusion about iPSC-CM transplant rejection, as this will require future studies at multiple time points corroborated with in vitro assays, such as the mixed lymphocyte reaction (Bach and Voynow, 1966) and other observations, such as graft destruction or disruption of function. Assessing mechanisms of graft infiltration in the NeoThy will be a key component of PSC immunogenicity studies going forward.

One previous report described humanizing animals with neonatal thymus, albeit without HSC co-injection (Barry et al., 1991). Our control data from Figure 1C (thymus-only) confirm their observations that thymic fragments are retained but without appreciable circulating human CD45+ cells. We did not observe human immune cells in our controls at early time points (6–7 weeks), and only 2 of 12 animals showed very low peripheral blood engraftment by 15–18 weeks (time point not measured by Barry et al., 1991). These later-emerging cells were overwhelmingly T cells, suggesting the emigration and homeostatic proliferation of passenger thymocytes from the thymic graft. In contrast, the robust circulating immune cells in the NeoThy are likely due to HSC co-injection coupled with human thymic education in vivo, as described below. The NSG-W host strain’s more thorough immuno-depletion and/or use of quickly processed neonatal patient thymic explants may also have influenced our results.

A recently published report illustrated deficiencies in the T cell function of fetal BLT mice resulting in impaired alloimmune rejection of endothelial cells (Kooreman et al., 2017). This contrasts with our ex vivo assay of T cell proliferation following TCR stimulation (Figures 3A and S2C). We also tested the NeoThy versus fetal tissue control animals for markers associated with naive versus memory T cells (Figure S1C). We observed statistically significant differences in levels of CD45RA+ T cells among CD4+, but not CD8+, cells. In addition, there were smaller but statistically significant differences in the CD45RO+ T cells among...
CD8+, but not CD4+, cells. Future studies will explore whether these differences are biologically significant, e.g., if they impact the alloimmune response to various iPSC-derived cell types, and whether there are meaningful differences in lymphopenia-induced proliferation between the NeoThy and fetal models.

Interestingly, HLA-B58 disparity tracking experiments show that passenger thymocytes from thymus fragments

Figure 3. Human Immune Cell Function in the NeoThy
(A) Ex vivo splenic T cells from a representative second-generation NeoThy (NSG-W, allogeneic tissues) mouse were labeled with CFSE dye and stimulated with 5 µg/mL αhCD3 (OKT3) antibody + αhCD28 + 300 IU/mL hIL2 and compared with unstimulated control with hIL2 alone. Flow cytometric analysis of CFSE dye dilution, gated on CD3+ cells, is shown: black line, unstimulated control; red line, stimulated condition, both at day 4 ex vivo. ELISA for human interferon γ (IFN-γ) was performed on day 4 cell culture supernatants. Error bars were determined using SD. ND, indicates no detection.

(B) Serum samples from 4 pooled independent experiments of second-generation NeoThy (NSG-W) (n = 9) and fetal thymus mice (n = 7) (both with allogeneic tissues) with verified B and T cell engraftment, 10–23 weeks post-humanization, were analyzed by ELISA for the concentration of human immunoglobulin G (IgG) and IgM antibodies. Healthy adult human serum (Hu Control) was used as a positive control. Statistics were conducted using ANOVA and no significant differences were found between fetal and NeoThy models for IgG or IgM.

(C) Antigen-specific T cell functionality in second-generation NeoThy (NSG-W) mice is measured by tvDTH assay via uncovering of a collagen V (Col V)-specific response after α transforming growth factor β (αTGF-β) Ab blocking.

(D and E) In one experiment, B6 mouse skin was grafted onto 12–14 weeks first-generation fetal (n = 3 mice) and NeoThy mice (n = 6, NSG, both allogeneic tissues), and non-humanized NSG controls (n = 3). Visible graft destruction (top right panel) and histological infiltration of hCD3+ T cells into rejecting B6 skin grafts (bottom right panel) is shown. Scale bars, 100 µm (20×). Fetal tissue engrafted mice had a mean human immune cell reconstitution of 17.9% ± 3.0% hCD45+ and 46.2% ± 15.3% hCD45+hCD3+ and NeoThy mice had a mean of 68.6% ± 27.3% hCD45+ and 49.2% ± 27.6% hCD45+hCD3+ just prior to skin transplant surgery. Graft rejection was classified as presence of macroscopic lesions and hair loss for >5 days, coupled with hCD3+ infiltration. Statistics were conducted using Kaplan-Meier estimator method, compared with a log rank test, analyzed by GraphPad Prism 7.00. Differences were not significant. See also Figures S1 and S2.
repopulate first-generation mice (not given zhCD2 antibody), in an early wave of hCD3⁺ T cell emergence (Figure S3). These mice have a mix of B58-positive and -negative T cells (Figure S3B, left plot), indicating both de novo T cell development from HSCs as well as passenger thymocyte emigration and proliferation. In contrast, second-generation mice (with zhCD2 antibody) (Figure S3B, right plot) exhibited a delay in T cell emergence and T cells uniformly expressed the HLA type of HSC source. This observation indicates the antibody-depleted passenger thymocytes from the thymic graft and these mice were reconstituted primarily with de novo T cells, as reported previously (Kalscheuer et al., 2012).

Using the clinically relevant metrics of immune cell frequency, and ex vivo and in vivo T cell function, the NeoThy humanized mouse model is a viable alternative to fetal tissue humanized mice. In addition to using the NeoThy for investigation of the immunogenicity of iPSC-derived cell therapies, the shear abundance of neonatal thymic tissue allows for other intriguing applications. Recent breakthroughs in developing PSC-derived HSCs could be validated in the NeoThy (Sugimura et al., 2017). By investigating the T lymphopoietic potential of iPSC-derived HSCs educated by iPSC donors’ thymic fragments, comparing them with autologous primary cord blood HSC controls (Wang et al., 1997).

The NeoThy model can play an integral role, in conjunction with in vitro assays of immunogenicity, in future studies investigating patient immune responses to self and allogeneic iPSC therapies and will also be of great value for hematopoiesis, transplant immunology, and virology research.

**EXPERIMENTAL PROCEDURES**

This work was approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health and the Health Sciences Institutional Review Board, and complied with federal and state law. Humanized mice were generated similarly to previously published reports (Kalscheuer et al., 2012; Lan et al., 2006). In brief, 6- to 10-week-old immune-compromised 250 RAD irradiated NOD.PrikdcscidIl2rg⁻/⁻ (NSG) and non-irradiated NOD.B6.Scid Il2rg⁻/⁻ KIh41/W41 (NBSGW, described in text as NSG-W) mice were used as host animals. Cyropreserved CD34-enriched HSCs were plated in SFEM medium (STEMCELL Technologies) plus 100 ng/mL rhStem Cell Factor (Miltenyi Biotech), and incubated at 37°C in 5% CO₂ overnight. The next day, 0.5–1.5 × 10⁵ HSCs were injected (i.v.) into anesthetized mice, coinciding with cryopreserved thymus fragment implantation surgery into the kidney capsule. A subset of mice also received i.v. zhCD2 antibody (100 μg) at days 0 and 7 post-surgery. Detailed methodology is available in the online Supplemental Experimental Procedures.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and two movies and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.02.011.

AUTHOR CONTRIBUTIONS

M.E.B. designed/ performed experiments, analyzed data, and wrote the manuscript. Y.Z. designed/ performed experiments and analyzed data. B.E.M. designed/ performed experiments. I.G.N., H.E.L., M.B., and J.A.S. performed the experiments. P.V.A. performed cardiac surgery. T.J.K. and J.A.T directed research, and W.J.B. directed research and edited the manuscript.

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REFERENCES

Bach, F.H., and Voynow, N.K. (1966). One-way stimulation in mixed leukocyte cultures. Science 153, 545–547.

Barry, T.S., Jones, D.M., Richter, C.B., and Haynes, B.F. (1991). Successful engraftment of human postnatal thymus in severe combined immune deficient (SCID) mice: differential engraftment of thymic components with irradiation versus anti-asialo GM-1 immunosuppressive regimens. J. Exp. Med. 173, 167–181.

Beaudin, A.E., Boyer, S.W., Perez-Cunningham, J., Hernandez, G.E., Derderian, S.C., Jujuvarapu, C., Aaserude, E., MacKenzie, T., and Forsberg, E.C. (2016). A transient developmental hematopoietic stem cell gives rise to innate-like B and T cells. Cell Stem Cell 19, 768–783.

Bleul, C.C., Corbeaux, T., Reuter, A., Fisch, P., Monting, J.S., and Boehm, T. (2006). Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature 441, 98–99.

Brehm, M.A., Cuthbert, A., Yang, C., Miller, D.M., Dilorio, P., Landgren, J., Burzenski, L., Gott, B., Foreman, O., Kavirayani, A., et al. (2010). Parameters for establishing humanized mouse models to study human immunity: analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2r(null) mutation. Clin. Immunol. 135, 84–98.

de Almeida, P.E., Meyer, E.H., Kooreman, N.G., Diecke, S., Dey, D., Sanchez-Freire, V., Hu, S., Ebert, A., Odegaard, J., Mordwinkin, N.M., et al. (2014). Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance. Nat. Commun. 5, 3903.

Griesemer, A.D., Sorenson, E.C., and Hardy, M.A. (2010). The role of the thymus in tolerance. Transplantation 90, 465–474.

Haas, M. (2016). The revised (2013) Banff classification for antibody-mediated rejection of renal allografts: update, difficulties, and future considerations. Am. J. Transplant. 16, 1352–1357.

Hasini, H.S., Velichety, S.D., Thyagaraju, K., Jyothirmayi, K., and Jaipal Ch. (2014). Morphological features and morphometric parameters of human fetal thymus glands. Int. J. Anat. Res. 2, 202–207.

Hu, Z., and Yang, Y.G. (2012). Full reconstitution of human platelets in humanized mice after macrophage depletion. Blood 120, 1713–1716.

Kalscheuer, H., Danzl, N., Onoe, T., Faust, T., Winchester, R., Golland, R., Greenberg, E., Spitzer, T.R., Savage, D.G., Tahara, H., et al. (2012). A model for personalized in vivo analysis of human immune responsiveness. Sci. Transl. Med. 4, 125ra130.

Kooreman, N.G., de Almeida, P.E., Stuck, J.P., Nolakanti, R., Diecke, S., Shao, N.-Y., Swijnenburg, R.-J., Sanchez-Freire, V., Matsa, E., Liu, C., et al. (2017). Alloimmune responses of humanized mice to human pluripotent stem cell therapeutics. Cell Rep. 20, 1978–1990.

Lan, P., Tonomura, N., Shimizu, A., Wang, S., and Yang, Y.G. (2006). Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. Blood 108, 487–492.

Lavender, K.J., Pang, W.W., Messer, R.J., Duley, A.K., Race, B., Phillips, K., Scott, D., Peterson, K.E., Chan, C.K., Dittmer, U., et al. (2013). BLT-humanized C57BL/6 Rag2-/-gammac-/-CD47-/- mice are resistant to GVHD and develop B- and T-cell immunity to HIV infection. Blood 122, 4013–4020.

Lee, H.M., Bautista, J.L., and Hsieh, C.S. (2011). Thymic and peripheral differentiation of regulatory T cells. Adv. Immunol. 112, 25–71.

Ling, C., Li, Q., Brown, M.E., Kishimoto, Y., Toya, Y., Devine, E.E., Choi, K.O., Nishimoto, K., Norman, I.G., Tsegyal, T., et al. (2015). Bioengineered vocal fold mucosa for voice restoration. Sci. Transl. Med. 7, 314ra187.

Lockridge, J.L., Zhou, Y., Becker, Y.A., Ma, S., Kenney, S.C., Hematti, P., Captini, C.M., Burlingham, W.J., Gendron-Fitzpatrick, A., and Gumperz, J.E. (2013). Morphological features and morphometric parameters of human fetal thymus glands. Int. J. Anat. Res. 2, 202–207.

McDermott, S.P., Eppert, K., Lechman, E.R., Doedens, M., and Dick, J.E. (2010). Comparison of human cord blood engraftment between immunocompromised mouse strains. Blood 116, 193–200.

McGovern, N., Shin, A., Low, G., Low, D., Duan, K., Yao, L.J., Msallam, R., Low, I., Shadan, N.B., Sumatoh, H.R., et al. (2017). Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. Nature 546, 662–666.
McIntosh, B.E., and Brown, M.E. (2015). No irradiation required: the future of humanized immune system modeling in murine hosts. Chimerism 6, 40–45.

McIntosh, B.E., Brown, M.E., Duffan, B.M., Maufort, J.P., Vereide, D.T., Slukvin, I.I., and Thomson, J.A. (2015). Nonirradiated NOD, B6.SCID II2gamma-/- Kit(W41/W41) (NBSGW) mice support multilineage engraftment of human hematopoietic cells. Stem Cell Reports 4, 171–180.

Mold, J.E., and McCune, J.M. (2012). Immunological tolerance during fetal development: from mouse to man. Adv. Immunol. 115, 73–111.

Mold, J.E., Venkatasubrahmanyam, S., Burt, T.D., Michaelsson, J., Rivera, J.M., Galkina, S.A., Weinberg, K., Stodart, C.A., and McCune, J.M. (2010). Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. Science 330, 1695–1699.

Notta, F., Zandi, S., Takayama, N., Dobson, S., Gan, O.I., Wilson, G., Kaufmann, K.B., McLeod, J., Laurenti, E., Dunant, C.F., et al. (2016). Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science 351, aab2116.

Rajesh, D., Zhou, Y., Jankowska-Gan, E., Roenneburg, D.A., Dart, M.L., Torrealba, J., and Burlingham, W.J. (2010). Th1 and Th17 immunocompetence in humanized NOD/SCID/IL2rgammnull mice. Hum. Immunol. 71, 551–559.

Rong, Z., Wang, M., Hu, Z., Stradner, M., Zhu, S., Kong, H., Yi, H., Goldrath, A., Yang, Y.G., Xu, Y., et al. (2014). An effective approach to prevent immune rejection of human ESC-derived allografts. Cell Stem Cell 14, 121–130.

Rongvaux, A., Willinger, T., Martinek, J., Strowig, T., Gearty, S.V., Teichmann, L.L., Saito, Y., Marches, E., Halene, S., Palucha, A.K., et al. (2014). Development and function of human innate immune cells in a humanized mouse model. Nat. Biotechnol. 32, 364–372.

Saito, Y., Ellegast, J.M., Rafiei, A., Song, Y., Kull, D., Heikenwalder, M., Rongvaux, A., Halene, S., Flavell, R.A., and Manz, M.G. (2016). Peripheral blood CD34 cells efficiently engraft human cytokine knock-in mice. Blood 128, 1829–1833.

Shultz, L.D., Brehm, M.A., Garcia-Martinez, J.V., and Greiner, D.L. (2012). Humanized mice for immune system investigation: progress, promise and challenges. Nat. Rev. Immunol. 12, 786–798.

Shultz, L.D., Saito, Y., Najima, Y., Tanaka, S., Ochi, T., Tomizawa, M., Doi, T., Sone, A., Suzuki, N., Fujiwara, H., et al. (2010). Generation of functional human T-cell subsets with HLA-restricted immune responses in HLA class I expressing NOD/SCID/IL2rgamma(null) humanized mice. Proc. Natl. Acad. Sci. USA 107, 13022–13027.

Solez, K., Axelsson, R.A., Benediktsson, H., Burdick, J.E., Cohen, A.H., Colvin, R.B., Croker, B.P., Droz, D., Dunnill, M.S., Halloran, P.F., et al. (1993). International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. Kidney Int. 44, 411–422.

Sugimura, R., Jha, D.K., Han, A., Soria-Valles, C., da Rocha, E.L., Lu, Y.F., Goettel, J.A., Serao, E., Rowe, R.G., Malleshaiah, M., et al. (2017). Haematopoietic stem and progenitor cells from human pluripotent stem cells. Nature 545, 432–438.

Sugita, S., Iwasaki, Y., Makabe, K., Kimura, T., Futagami, T., Suegami, S., and Takahashi, M. (2016). Lack of T cell response to iPSC-derived retinal pigment epithelial cells from HLA homozygous donors. Stem Cell Reports 7, 619–634.

Sullivan, J.A., Jankowska-Gan, E., Hegde, S., Pestrak, M.A., Agashe, V.V., Park, A.C., Brown, M.E., Kernien, J.F., Wilkes, D.S., Kaufman, D.B., et al. (2017). Th17 responses to collagen type V, kalphi-tubulin, and vimentin are present early in human development and persist throughout life. Am. J. Transplant. 17, 944–956.

Theocharides, A.P.A., Rongvaux, A., Fritsch, K., Flavell, R.A., and Manz, M.G. (2016). Humanized hematolymphoid system mice. Haematologica 101, 5–19.

Wang, J.C.Y., Doedens, M., and Dick, J.E. (1997). Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 89, 3919–3924.

Yu, J., Chau, K.F., Vodyanik, M.A., Jiang, J., and Jiang, Y. (2011). Efficient feeder-free episomal reprogramming with small molecules. PLoS One 6, e17557.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frané, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1892–1897.

Zhao, T., Zhang, Z.N., Rong, Z., and Xu, Y. (2011). Immunogenicity of induced pluripotent stem cells. Nature 474, 212–215.

Zhao, T., Zhang, Z.N., Westenskow, P.D., Todorova, D., Hu, Z., Lin, T., Rong, Z., Kim, J., He, J., Wang, M., et al. (2015). Humanized mice reveal differential immunogenicity of cells derived from autologous induced pluripotent stem cells. Cell Stem Cell 17, 353–359.
Supplemental Information

A Humanized Mouse Model Generated Using Surplus Neonatal Tissue

Matthew E. Brown, Ying Zhou, Brian E. McIntosh, Ian G. Norman, Hannah E. Lou, Mitch Biermann, Jeremy A. Sullivan, Timothy J. Kamp, James A. Thomson, Petros V. Anagnostopoulos, and William J. Burlingham
Supplemental Data
Figure S1: Immune Cell Reconstitution and Characterization in Peripheral Blood, Spleen, Lymph Node, Lung and Bone Marrow, Related to Figures 1, 2 and 3. (A) To investigate absolute cell number in humanized mice, second generation NeoThy (NSG-W) mice with autologous immune systems (N=12 mice, 15 weeks post-humanization surgery) were compared to BLT fetal tissue mice (first generation, NSG) with autologous immune systems (N=9, 12 weeks post-humanization surgery) in two separate experiments. Each group of mice = one donor tissue set. Groups selected based on similar mean percent CD3+(all individuals in comparison >1% hCD3+ cells), to compare effect of fetal vs neonatal thymic organoid on T cell frequency. Absolute number comparisons used equal blood volumes, with consistent blood processing and flow cytometry analysis. The y-axis is the typical volume in a mouse blood draw. Experiments were done by the same person, within 4 weeks of each other, to minimize variability. (B) Percentage positive and absolute numbers were compared, using the counts in A, for hCD45 and hCD3 (of hCD45+). (C) NeoThy and Fetal BLT mice, as in A, were analyzed for hCD45RA and hCD45RO by flow cytometry. Events gated on viable single hCD4+ (left panel) or hCD8+ cells. (D) Splenocytes (N=10 mice), (E) Lymph Nodes (N=4), (F) Lung (N=4) and (G) Bone Marrow (N=3) were isolated from first and second generation NeoThy (NSG-W) mice from five neonatal thymus donors at time points 17-45 weeks post-humanization ([D] used data from 4 independent experiments, other tissues were one experiment). Statistics conducted using student’s t-tests, analyzed by GraphPad Prism 7.00. Differences that were analyzed were noted for significance (**** = p<0.0001, * = p<0.05, ns = not significant, no bar = not analyzed).
Figure S2: Humanized Mice Host Strains, NeoThy Donor Reproducibility and T Cell Proliferation. Related to Figures 1, 2 and 3. (A) NSG (+250 RAD X-ray irradiation, N=10, three independent experiments) or NSG-W (non-irradiated, N=8, two independent experiments) mice were reconstituted with neonatal human thymus tissue and allogeneic cord blood CD34+ cells (neither condition was given anti-CD2 antibody i.e. both were first generation). Mice were from single donors. Reconstituted mice were compared for human immune cell engraftment (hCD45+, including B cells (hCD19+, of hCD45+) and T cells (hCD3+, of hCD45+) at an early (6-7 weeks post-humanization surgery) and late (15-18 weeks) time point. Statistics were conducted using ANOVA/student’s t-tests, analyzed by GraphPad Prism 7.00. Differences were not significant unless otherwise denoted (**** = p<0.0001, *** = p<0.001, ** = p<0.01, * = p<0.05). (B) Peripheral blood from second generation NeoThy (NSG-W) mice made from three thymus donors and three cord blood CD34+ cell donors was analyzed by flow cytometry for hCD45, hCD19 and hCD3 at 14-17 weeks post-humanization surgery. Mouse replicate numbers (N) indicated on graph. The PED05 - Autologous Cord CD34+ HSC condition was pooled from three experiments, other conditions were single experiments. Error bars were determined using S.D. (C) Ex vivo splenic T cells from a representative second generation NeoThy (NSG-W) mouse (left panel) were labeled with CFSE proliferation dye and stimulated with αhCD3 + αhCD28 antibodies + 300IU/ml hIL2 and compared to adult human PBMCs (right panel). 4x, scale bar=500um. Flow cytometric analysis of CFSE dye dilution, gated on viable singlet CD4+ vs CD8+ cells, is shown at day 4. Cells were gated by comparison to unstimulated CFSE labeled PBMCs.
Figure S3: Elimination of Passenger Thymocytes via Anti-hCD2 Antibody. Related to Figure 1 and 2. To determine whether reconstituted T cells developed de novo from injected CD34+ cells (and thus are likely to be tolerant of the animal host) or resulted from passenger T cell emigrating from the thymic fragment (predeveloped cells, potentially mediating GVHD), first and second generation NeoThy (NSG-W) mice were created using an HLA disparity MA2.1 antibody tracking method. Host mice were implanted with HLA-B58 negative neonatal thymus (donor “PED01”, see Table S1) plus HLA-B58 positive allogeneic cord blood CD34+ cells (donor “Allo Cord 522”, see Table S1), with or without αhCD2 antibody to deplete thymic passenger T cells. (A) Peripheral blood from the mice was analyzed at the indicated early and late time points. NeoThy mice with (second generation) or without (first generation) αhCD2 antibody treatment were compared for human immune cell engraftment (hCD45+), including B cells (hCD19+, of hCD45+) and T cells (hCD3+, of hCD45+) at early (8w, N=6 for without αCD2, N=11 with αCD2) and late (15w, N=3 for without αCD2, N=11 with αCD2) time points (Data pooled from three independent experiments). Statistics were conducted using student’s t-tests, analyzed by GraphPad Prism 7.00 (*** = p<0.001, ** = p<0.01). Differences were not significant unless otherwise denoted. Error bars were determined using S.D. (B) At week 15 post-surgery B58 negative (thymus donor HLA) hCD3’hCD4+ T cells and B58 positive (HSC donor HLA) T cells are shown in first generation mice that did not receive hCD2 depleting antibody (left) compared to second generation mice that received hCD2 depleting antibody (100ug) at day 0 and day 7 post-surgery (right).
Figure S4: Neonatal Tissue Donor iPSC Characterization and Differentiation Potential. Related to Figure 4.

(A) Umbilical Cord Blood CD34 negative fraction hematopoietic cells were preferentially expanded via StemSpan CD34 Expansion Supplement cytokine cocktail and reprogrammed with episomal factors. Resultant iPSCs display typical pluripotent stem cell morphology and stained positive for Tra-1-60 (Alexafluor 488) surface marker. (B) Neonatal donor iPSC lines were positive for Alkaline Phosphatase (10x, scale bar=100um), and (C) pluripotency markers: SSEA-4 surface marker expression and OCT4 pluripotency associated transcription factor intracellular expression. (D) iPSC lines made from multiple NeoThy tissue donors were co-injected with matrigel subcutaneously into the hind limb of non-humanized NSG-W mice and harvested 6-10 weeks later. Representative images show tissues derived from all three germ layers present in the teratomas: I, Gastrointestinal/Respiratory (Endoderm), II, Retinal Pigment Epithelium (Ectoderm), III, Cartilage (Mesoderm). 20x, scale bar=100um. (E) NeoThy tissue donor iPSC lines can be directly differentiated in vitro into contracting cardiac troponin T (cTNT) positive cardiomyocytes. Cardiomyocytes show typical morphology (left panel, 10x, scale bar=100um) and over 80% cTNT+ purity by intracellular flow cytometry. (F) Reprogrammed iPSC lines display a normal g-banding karyotype. 20 total cells were counted, 8 were analyzed, 4 karyogrammed. Band resolution was 450-475. No clonal abnormalities were detected at the stated band level of resolution.
| Donor / Tissue   | Sex | A   | B   | Bw | DRB |
|-----------------|-----|-----|-----|----|-----|
| PED01 Thymus    | M   | 26  | 28  | 35 | 38  |
| PED02 Thymus    | M   | 2   | 2   | 27 | 44  |
| PED04 Thymus    | M   | 2   | 11  | 27 | 62  |
| PED05 Thymus/Cord | M | 1  | 2   | 8  | 51  |
| Allo Cord 212   | M   | 2   | 3   | 7  | 8   |
| Allo Cord 522   | F   | 24  | 66  | 7  | 58  |
| Allo Cord 929   | N/A | 11  | 69  | 35 | 62  |

Table S1: Neonatal Tissue Donor HLA Typing. Related to Figures 1-4, S1-4. HLA Class I and Class II typing is shown for various neonatal thymus and umbilical cord donors used for the experiments in the manuscript. HLA typing was done via sequence-specific oligonucleotide method and reported as serologic equivalents. The HLA/Molecular Diagnostics Laboratory in the UW-Madison Department of Pathology and Laboratory Medicine performed the analysis.

Supplemental Movie File S1: Contracting iPSC-Cardiomyocytes. Representative video of contracting cardiomyocytes in 2D monolayer prior to aggregation and transplantation.

File: IPSC-CMs PED04iPSCB 2D 10x

Supplemental Movie File S2: Contracting iPSC-Cardiomyocytes. Representative video of contracting cardiomyocytes in 3D aggregate cell cultures prior to transplantation.

File: IPSC-CMs PED05iPSCAalt 3D Aggregates 4x
Tissue Processing for Humanization Experiments

All human tissue research was conducted with informed consent and the approval of University of Wisconsin-Madison Health Sciences Institutional Review Board and was performed in accordance with federal and state law. Human tissue for mouse humanization experiments were obtained and processed as follows. Fetal thymus and liver tissue for control experiments was obtained from Albert Einstein College of Medicine at Yeshiva University, Advanced Bioscience Resources (Alameda, CA) and the University of Washington. Tissue was shipped overnight on ice in antimycotic/antibiotic solution and processed the following day. Liver was processed by macerating the tissue over a 100µm cell strainer with the blunt end of a syringe (both BD Biosciences, San Jose, CA) in sterile X-Vivo 10 media (Lonza, Basel, Switzerland). Individualized cells were collected and purified by density centrifugation with Lymphocyte Separation Medium (Corning, Manassas, VA), and RBCs lysed with ACK buffer (Thermo Fisher Scientific, Waltham, MA). HSCs were enriched using hCD34+ MACS beads (Miltenyi, Bergisch Gladbach, Germany) and cryopreserved in CryoStor CS10 freezing medium (Stem Cell Technologies, Vancouver, BC Canada). Fetal thymus was processed similarly to neonatal thymus, described as follows. Neonatal thymus tissue was obtained from newborn cardiac surgery patients (median age of 7 days old) during their care at the University of Wisconsin Hospital and Clinics American Family Children’s Hospital. Immediately following surgical excision and receipt of tissue, thymus tissue was placed in cold, sterile X-Vivo 10 media and adipose and other extraneous tissue were removed working in a biological safety cabinet. Tissue was dissected into 1mm x 1mm fragments with a scalpel and forceps. Fragments were placed in CryoStor CS10 freezing medium, frozen within 4 hours of surgical excision using a controlled rate freezing box to -80°C, then placed in a liquid nitrogen freezer for long-term storage. Five to 20 thymus fragments were frozen per 1ml of CS10 media.

Cord Blood Processing

Autologous and allogeneic human umbilical cord blood was collected in Citrate Phosphate Dextrose (CPD) solution bag collection units (Pall Corporation, Port Washington NY), purified by density centrifugation with Lymphocyte Separation Medium (Corning, Manassas, VA), and RBCs lysed with ACK buffer (Thermo Fisher Scientific, Waltham, MA). HSCs were enriched using hCD34+ MACS beads (Miltenyi, Bergisch Gladbach, Germany) and cryopreserved in CS10 medium, as above.

Mouse Humanization

The Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health approved all experiments. Humanized mice were generated similarly to previous published reports.(Kalscheuer et al., 2012; Lan et al., 2006) Briefly, 6-10 week old male NOD.PrkdcscidIL2rg−/− (NSG) and NOD.B6.Scid IL2rg−/− KitwW41/W41 (NSG-W) mice were used as immune-compromised host animals. NSG mice were irradiated with 250 RAD via an X-RAD 320ix irradiator (Precision X-Ray, North Branford, CT). One day prior to humanization surgery, hCD34-enriched HSCs (see Cord Blood Processing, above) were thawed and plated in SFEM medium (Stem Cell Technologies, Vancouver, BC Canada) plus 100ng/ml recombinant human Stem Cell Factor (Miltenyi, Bergisch Gladbach, Germany), and incubated at 37°C in 5% CO2 overnight. Live cell numbers were determined via hemocytometer and trypan blue method. On the day of surgery, cells were collected, washed and resuspended in 10 mM HEPES-buffered Hank’s balanced salt solution (Thermo Fisher Scientific, Waltham, MA) for retro-orbital injection. 0.5e5-1.5e5 HSCs in a 100µl volume were injected intravenously into isoflurane anesthetized mice, coinciding with surgery to implant cryopreserved thymus fragments under the left mouse kidney capsule. “Second generation” mice also received i.v. injection of αCD2 antibody (100µg) at days 0 and 7 post-surgery to deplete passenger T cells emigrating from the thymus fragment. The αCD2 antibody-producing hybridoma was a generous gift of Dr. Pierre Gianello, and purified antibody (endotoxin level <2EU/mg) was custom manufactured by Bio X Cell (West Lebanon, NH). All mice were treated with Buprenorphine for post-operative pain management and their drinking water was supplemented with Baytril antibiotic for 10 days post-surgery.

Human Cell Engraftment Assessment
Peripheral mouse blood was sampled via retro-orbital eye bleeds into heparin-coated capillary tubes (Thermo Fisher Scientific, Waltham, MA). Blood samples were collected into Eppendorf tubes containing 150µl of 2% dextran solution (Sigma Aldrich, Saint Louis, MO) in Dulbecco’s phosphate buffered saline without calcium or magnesium (dPBS/-) (Corning, Manassas, VA) and 150µl of 0.5%-Heparin solution (Sigma Aldrich). Blood was settled for 20 minutes, then the translucent upper layer (containing leukocytes) was spun down at 400g x 5 minutes and resuspended in ACK red blood cell lysis buffer (Thermo Fisher Scientific, Waltham, MA) for 10 minutes. Cells were washed twice in cold FACS Buffer (10 mM HEPES-buffered Hank’s balanced salt solution [Thermo Fisher Scientific, Waltham, MA], 2% fetal bovine serum [Hyclone, Pittsburgh, PA], 0.1% Sodium Azide [Sigma Aldrich]) and spun down at 400g x 5 minutes.

Spleens were harvested from sacrificed humanized mice and dissociated through a 100µm cell strainer with the blunt end of a syringe (both BD Biosciences, San Jose, CA) into a petri dish containing sterile X-Vivo 10 media (Lonza, Basel, Switzerland). Dissociated cells were collected and purified by density centrifugation with Lymphocyte Separation Medium (Corning, Manassas, VA), and RBCs lysed with ACK buffer (Thermo Fisher Scientific, Waltham, MA). Live cell numbers were determined via hemocytometer counting after trypan blue staining. Cells were resuspended in cold FACS buffer and filtered through cell strainers additional times as needed to remove any residual cell clumps. Bone marrow was harvested from mouse femurs, flushed with sterile Dulbecco’s phosphate buffered saline without calcium or magnesium (dPBS/-) (Corning, Manassas, VA) and RBCs lysed similar to spleens above. Mesenteric lymph nodes were harvested as with spleens above, with no RBC lysis.

Lung was perfused with sterile dPBS/- to remove non-tissue resident blood cells, digested with 2mg/ml Collagenase D and 40U/ml DNase I (Roche Diagnostics, Indianapolis, IN) at 37°C for 60 minutes while being automatically rotated. Digested cell suspension was passed through a 100µm cell strainer (BD Biosciences, San Jose, CA) into a petri dish containing sterile X-Vivo 10 media (Lonza, Basel, Switzerland). Dissociated cells were collected and purified by density centrifugation with Lymphocyte Separation Medium (Corning, Manassas, VA), and red blood cells lysed with ACK buffer (Thermo Fisher Scientific, Waltham, MA). Live cell numbers were counted as above. Cells were resuspended in cold sterile FACS buffer and filtered through cell strainers additional times as needed to remove any residual cell clumps.

Combinations of the following antibodies were used in flow cytometry to detect specific human markers: anti-mouse CD45 (clone 30-F11, 553080); anti-human (αh) CD45 (clone HI30, 555485); αhCD3 (clone HIT3a, 555340); αhCD4 (clone RPA-T4, 561004, 560648); αhCD8α (clone RPA-T8, 561947); αhCD11b (clone ICRF44, 555388); αhCD11c (clone B-ly6, 560895); αhCD14 (clone HCD14, 325068); αhCD19 (clone HIB19, 561741, 555414, 560728); αhFOXP3 (clone 250D/C7, 560045, 560082) and αhHLA-B58 (clone MA2.1). Negative gating control (isotype) antibodies were clone MOPC-21 for IgG1 (565572, 554680, 557224, 565573, 554684), clone R35-95 for IgG2a (554688), and clone A95.1 for rat IgG2b (553988). Antibodies were purchased from BD Biosciences (San Jose, CA) directly conjugated with fluorochromes, except for αhCD14 (BioLegend). The αhHLA-B58 antibody was purified from a hybridoma line (MA2.1, ATCC, Manassas, VA) and conjugated to various fluorochromes using a Molecular Probes labeling kit (Thermo Fisher Scientific, Waltham, MA), per the manufacturer’s instructions. For flow cytometric staining, tissues were collected as indicated above from mice at various time points after implantation of human cells and tissue. Samples were stained in 100 µl FACS buffer and dead cells were excluded from the analysis. Flow cytometry was performed on BD Aria III and BD Accuri flow cytometers (BD Biosciences, San Jose, CA) and analyzed using BD Accuri C6 software and FlowJo Version 10 software (FlowJo, LLC, Ashland, OR). Positive human immune cell engraftment was determined by taking the percentage of αhCD45 within the total CD45+ (αhCD45 plus anti-mouse CD45+ fractions) to normalize against varying amounts of contaminating red blood cells not removed by ACK buffer, as previously described. (Saito et al., 2016)

CFSE Proliferation Assay

Ex vivo splenocytes were obtained from three antigen-matched NeoThy humanized mice sacrificed at 22 weeks of age after the “second-generation” procedure (see Figure 1C) and dissociated through a 100µm cell strainer using the blunt end of a syringe (both BD Biosciences, San Jose, CA) into a petri dish containing sterile X-Vivo 10 media (Lonza, Basel, Switzerland). Dissociated cells were collected and purified by density centrifugation with Lymphocyte Separation Medium (Corning, Manassas, VA), and red blood cells lysed with ACK buffer (Thermo Fisher Scientific, Waltham, MA). Live cell numbers were determined via hemocytometer counting after trypan blue.
staining. Cells were resuspended in cold FACS buffer and filtered through cell strainers additional times as needed to remove any residual cell clumps. One day prior to assay, 48 well plates (Corning, Manassas, VA) were coated with 5µg/ml αhCD3 antibody (clone OKT3, eBioscience/Thermo Fisher Scientific, Waltham, MA) in 250µl Dulbecco’s phosphate buffered saline without calcium or magnesium (dPBS-/-) (Corning, Manassas, VA) and incubated overnight at 4°C. Splenocytes were stained in RPMI 1640 basal medium (Gibco/Thermo Fisher Scientific, Waltham, MA) with 2 µl of a 1:20 stock dilution of Carboxyfluorescein succinimidyl ester (CSFE) dye (eBioscience/Thermo Fisher Scientific, Waltham, MA) for 10 minutes at 37°C. Cells were washed with RPMI 1640 + 20% Fetal Bovine Serum (FBS) (Hyclone, Pittsburgh, PA), followed by 2x with dPBS-/. Cells were resuspended at 2e6/ml in RPMI 1640 + 5% FBS + 1X Penicillin-Streptomycin + 1X Glutamax (both Thermo Fisher Scientific, Waltham, MA) and 500µl was plated onto αhCD3-coated and uncoated negative control wells. Cultures were supplemented with 2µg/ml αhCD28 antibody (clone 37.51, BD Biosciences, San Jose, CA) and both stimulated and unstimulated cultures received 300 IU/ml hiIL2 (PeproTech, Rocky Hill, NJ). Medium was not changed; 60µl samples were removed at day 4 and frozen at -80°C for future use (see ELISA for Human Interferon Gamma Production, below). Cultures were analyzed on a Nikon Eclipse Ti-S inverted microscope system with 4X/0.10, 10X/0.30 and 20X/0.50 objectives. Images acquired using a Nikon DS Qi2 camera and NIS Elements D4.30.02 64bit software.

**ELISA for Human Interferon Gamma Production and Human IgG/IgM**

Enzyme-linked immunosorbent assay (ELISA) for human interferon gamma production was performed on frozen cell culture supernatants from CFSE Proliferation Assay (above) using the Human IFN gamma ELISA Ready-SET-Go! Kit (eBioscience/Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s instructions. ELISA for human immunoglobulin IgG and IgM quantification was performed using human IgG and IgM ELISA kits (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s instructions. Plates were read on a ELx800 plate reader (BioTek Instruments, Winooski, VT) and results analyzed using Gen5 software (BioTek Instruments) and Excel software (Microsoft, Redmond, WA).

**Trans vivo Delayed-Type Hypersensitivity Assay (tvDTH)**

The tvDTH assay provides an experimental measurement of human donor-specific effector T cell responses and regulatory T cell responses in a convenient mouse model. Assay was performed as previously described.(Burlingham et al., 2007; Jankowska-Gan et al., 2013) Briefly, *ex vivo* splenocytes (processed as described above) from humanized mice were co-injected with antigen described by Sullivan et al to elicit an effect T cell response,27 with or without blocking antibody, into the footpad of NOD/SCID mouse (The Jackson Laboratories, Bar Harbor, ME). Swelling response was measured by calipers 18-24 hours later. Human Collagen Type V (5 µg) was a gift from Dr. David Brand (University of Tennessee, Memphis), and was prepared as described elsewhere.(Yoshida et al., 2006) Human Collagen type I was purchased from BD Biosciences (San Jose, CA). Neutralizing αTGFβ antibody (25µg) was purchased from R&D Systems (Minneapolis, MN).

**Skin Transplantation**

Donor skin was prepared from sacrificed C57BL/6J (The Jackson Labs, Bar Harbor, ME). Ear or belly dermis skin was prepared in oval sections, approximately 1-2cm x 1-2cm in size, and kept in cold sterile Dulbecco’s phosphate buffered saline without calcium or magnesium (dPBS-/-) (Corning, Manassas, VA) solution until the time of transplantation. Under anesthesia, a segment of same size, full thickness skin on the back of recipient humanized or non-humanized control mice was removed surgically and the wound quickly covered with the donor skin. Oasis TA5 tissue adhesive (Med-Vet International, Mettawa, IL) was applied around the edges of the skin graft, which was then covered with petroleum jelly-coated gauze and secured with a bandage. Bandages were removed on day 7 post-operation for graft evaluation. Drinking water was supplemented with Baytril antibiotic for a total of 10 days post-surgery. Mice were sacrificed at various timepoints, and tissues collected in 10% buffered formalin for histological sectioning. Images were acquired on an iPhone 5s camera.

**Reprogramming Somatic Cells into iPSC Lines**

Hematopoietic progenitor cells were expanded from the CD34-depleted fraction of donor cord blood using StemSpan CD34+ Expansion Supplement in SFEM (Stem Cell Technologies, Vancouver, CA). After 3-6 days of culture, cells were reprogrammed as previously described.(Yu et al., 2011; Yu et al., 2009) Briefly, the cells were
electroporated using an Amaxa Nucleofector II with the pEP4EO2SEN2K, pEP4EO2SET2K, and pCEP4-M2L plasmids. Cells were derived on both Vitronectin XF (Stem Cell Technologies) and growth factor reduced Matrigel (Corning, Corning, NY), using E8 medium (Stem Cell Technologies) culturing and Versene (ThermoFisher Scientific, Waltham, MA) passaging method.

**Flow Cytometric Characterization of Pluripotency**

iPSCs maintained on Matrigel were harvested and stained for the presence of the SSEA-4 (clone MC813-70, 560126) pluripotency marker. Intracellular OCT4 (clone 40/Oct3, 560186) staining was performed on cells fixed and permeabilized with BD Cytofix/Cytoperm (all BD Biosciences, San Jose, CA). Cells were stained 2 hours for intracellular markers and 30 minutes at 4 degrees Celsius for surface markers and analyzed on an Accuri flow cytometer.

**Live Cell Tra-1-60 Immunocytochemistry Staining for Pluripotency**

Colonies of iPSCs were stained for Tra-1-60 (Stemgent, Cambridge, MA) by diluting primary antibody to a concentration of 2.5ug/ml in fresh E8 culture medium (Stem Cell Technologies, Vancouver, CA) and then sterile filtering the solution. Cell culture medium was aspirated and staining medium was added and incubated for 30 minutes at 37 degrees Celsius. Staining medium was aspirated and cells washed two times with culture medium. Cells were then visualized on a fluorescent microscope for Tra-1-60 expression. Alternatively, cells were stain using a mouse anti-Tra-1-60 IgM primary antibody (R & D Systems, Minneapolis, MN) and then stained with an anti-mouse IgM secondary antibody conjugated to AlexaFluor488 (Invitrogen, Carlsbad, CA).

**Alkaline Phosphatase Assay**

Near-confluent iPSCs stained with Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, SK-5300, Burlingame, CA) according to the manufacturer’s instructions.

**Teratoma Formation**

Two confluent wells of a 6 well plate were co-injected with 100ul growth factor reduced Matrigel (Corning, Corning, NY) subcutaneously into female NSG-W mice. Teratomas were allowed to form and animals were sacrificed 6-10 weeks after initial cell injection. Teratomas were processed for histological sectioning and H+E staining.

**Cardiomyocyte Differentiation Protocol**

Cardiomyocytes (CMs) were differentiated from iPSCs using small molecule temporal modulation of the Wnt pathway (aka GiWi) protocol and stained for cTNT, as previously described. (Lian et al., 2012) Prior to transplantation, CMs were thawed into EB20 medium, containing DMEM/F12 basal medium, 20% FBS, 1% non-essential amino acids, and 0.007ul/ml of beta-mercaptoethanol (all sterile filtered) and placed in Ultra Low Attachment 6 well plates (Corning, Corning, NY) on a rotating platform for 24 hours. CM medium was changed to EB2 (same formulation as EB20 but with 2% FBS instead of 20%), feeding the cells every 2-3 days thereafter. After beating was visualized, CM aggregates were transplanted under the mouse kidney capsule, similar to humanization surgery described above.

**Histology and Immunohistochemistry**

Tissues were collected, fixed in 10% neutral buffered formalin and submitted for histological analysis by the University of Wisconsin Department of Surgery Histology CORE Service. Briefly, after 24 hours fixation, the samples were processed into paraffin blocks using a Leica ASP300S tissue processor (Leica Biosystems, Buffalo Grove, IL). Paraffin-embedded specimens were cut into 5µm sections and deparafinized in xylene. Next, specimens were stained with routine Hematoxylin & Eosin (H&E). Serial sections were subjected to immunohistochemical analysis using 1:500 polyclonal rabbit αhCD3 (Abcam, Cambridge, MA) primary antibody. Tissue sections
underwent antigen retrieval using a citrate buffer at pH 6.0 in an 80°C water bath for 2 hours. The sections were then blocked with 10% BSA and primary incubation for 1 hour at room temperature. Endogenous peroxidase activity was quenched and antibody detection was done with ImmPRESS anti-rabbit Ig HRP (Vector Laboratories, Burlingame, CA). Lastly, the signal was visualized with DAB and sections counterstained with Hematoxylin for contrast. Slides were analyzed on a Nikon Eclipse Ti-S inverted microscope system with 4X/0.10, 10X/0.30 and 20X/0.50 objectives. Images acquired using a Lumenera Infinity 1 camera and NIS Elements D4.30.02 64bit software.

**HLA Typing**

HLA Typing was performed by the HLA/Molecular Diagnostics Laboratory in the UW-Madison Department of Pathology and Laboratory Medicine using One Lambda (Canoga Park, CA) reagents. Approximately 1-5x10⁶ hCD34-negative cord blood cells were used for testing via sequence-specific oligonucleotide method, reported as serologic equivalents.

**Statistical Analysis**

Analysis of variance was used for comparing groups. Two tailed student’s t-tests (α=0.05 significance level) were used for comparisons of two samples with an assumption of equal variance. Where assumption of unequal variance was violated, we used a t-test assuming unequal variance. Kaplan-Meier method was used to estimate skin graft rejection rates, which were compared using a log-rank test. P values less than 0.01 were considered significant. Analysis was performed with Prism 7.00 (GraphPad Prism software, La Jolla, CA).

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Supplemental References

Burlingham, W.J., Love, R.B., Jankowska-Gan, E., Haynes, L.D., Xu, Q., Bobadilla, J.L., Meyer, K.C., Hayney, M.S., Braun, R.K., Greenspan, D.S., et al. (2007). IL-17–dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants. The Journal of Clinical Investigation 117, 3498-3506.

Jankowska-Gan, E., Hegde, S., and Burlingham, W.J. (2013). Trans-vivo Delayed Type Hypersensitivity Assay for Antigen Specific Regulation. e4454.

Kalscheuer, H., Danzl, N., Onoe, T., Faust, T., Winchester, R., Goland, R., Greenberg, E., Spitzer, T.R., Savage, D.G., Tahara, H., et al. (2012). A model for personalized in vivo analysis of human immune responsiveness. Sci Transl Med 4, 125ra130.

Lan, P., Tonomura, N., Shimizu, A., Wang, S., and Yang, Y.G. (2006). Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. Blood 108, 487-492.

Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L.B., Azarin, S.M., Raval, K.K., Zhang, J., Kamp, T.J., and Palecek, S.P. (2012). Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci U S A 109, E1848-1857.

Saito, Y., Ellegast, J.M., Rafiei, A., Song, Y., Kull, D., Heikenwalder, M., Rongvaux, A., Halene, S., Flavell, R.A., and Manz, M.G. (2016). Peripheral blood CD34 cells efficiently engraft human cytokine knock-in mice. Blood.

Yoshida, S., Haque, A., Mizobuchi, T., Iwata, T., Chiyo, M., Webb, T.J., Baldridge, L.A., Heidler, K.M., Cummings, O.W., Fujisawa, T., et al. (2006). Anti-Type V Collagen Lymphocytes that Express IL-17 and IL-23 Induce Rejection Pathology in Fresh and Well-Healed Lung Transplants. American Journal of Transplantation 6, 724-735.

Yu, J., Chau, K.F., Vodyanik, M.A., Jiang, J., and Jiang, Y. (2011). Efficient feeder-free episomal reprogramming with small molecules. PLoS One 6, e17557.

Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, II, and Thomson, J.A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. Science 324, 797-801.