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A model for personalized in vivo analysis of human immune responsiveness

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Abstract

Studies of human immune diseases are generally limited to the analysis of peripheral blood lymphocytes of heterogenous patient populations. Improved models are needed to allow analysis of fundamental immunologic abnormalities predisposing to disease and in which to assess immunotherapies. Immunodeficient mice receiving human fetal thymus grafts and fetal CD34+ cells i.v. produce robust human immune systems, allowing analysis of human T cell development and function. However, to use humanized mice to study human immune-mediated disorders, immune systems must be generated from adult hematopoietic cells. Here, we demonstrated robust immune reconstitution in mice with hematopoietic stem cells (HSCs) aspirated from bone marrow

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of adults with Type 1 diabetes (T1D) and healthy control volunteers. In these humanized mice, cryopreservation of HLA allele-matched fetal thymic tissue prevented allogeneic adult HSC rejection. Newly generated T cells, which included regulatory T cells, were functional, self-tolerant, and had a diverse repertoire. The immune recognition of these mice mimicked that of the adult CD34⁺ cell donor, but the T cell phenotypes were more predominantly “naïve” than those of the adult donors. HSCs from T1D and control donors generated similar numbers of natural Tregs intrathymically; however, peripheral T cells from T1D subjects showed increased proportions of activated or memory cells compared to controls, suggesting possible HSC-intrinsic differences in T cell homeostasis that might underly immune pathology in T1D. This “Personalized Immune” (PI) mouse provides a new model for individualized analysis of human immune responses that may provide new insights into not only T1D, but other forms of immune function and dysfunction as well.

Introduction

While large-scale studies of human populations have provided important clues to the genetic basis of immune diseases and responses, little is known about the mechanisms by which these genes exert their effects. The ability to dissect these mechanisms in patient populations is currently limited largely to the analysis of peripheral blood lymphocytes from individuals with diverse disease characteristics, duration, treatments and environments, and in whom immunological causes and effects of inflammatory cascades cannot be readily distinguished. Thus, there is a need for models that eliminate all of these inter-individual variables while allowing analysis of individuals with demonstrated disease. While human peripheral blood mononuclear cells (PBMC) can populate immunodeficient mice (1), the function of T cells is limited in this setting and complicated by xenogeneic graft-vs-host reactivity (2). Human T cells develop in human fetal thymus (THY) grafts implanted with fetal liver under the kidney capsule (3). The combination of intravenous human hematopoietic stem cell (HSC) infusion with human fetal thymus and liver (THY/LIV) grafts under the kidney capsule allows human immune reconstitution with high levels of peripheral human T cells, B cells, and both myeloid and plasmacytoid dendritic cells (4), with antigen-specific immune responses *in vivo* (4-6). Normal thymic development of regulatory T cells (Tregs) with suppressive function (7) and homeostatic peripheral expansion of human T cells occurs (8). However, this model is limited to the analysis of fetal HSC-derived immune systems.

Humanized mice provide the opportunity to analyze the effects of autoimmunity-associated genetic polymorphisms on immune regulation. Recently-defined non-HLA-linked genes collectively confer substantial autoimmune disease risk (9-12). In humans with autoimmune diseases, however, underlying immunoregulatory defects arising from non-HLA-associated genes are largely undefined. Given that many of these loci contain immunoregulatory genes, such as cytokines, costimulatory and inhibitory molecules (9-12), intrinsic abnormalities in the cells of the immune system, which originate from hematopoietic stem cells (HSCs), likely contribute to the development of autoimmunity. Consistently, diabetes disease susceptibility is transferred via hematopoietic cells in NOD mice (13) and possibly in humans (14). However, studies of patients with disease cannot distinguish underlying causes from effects of disease evolution, disease treatment or precipitating environmental factors.

Fulfillment of the above basic research potentialities of humanized mice would require achievement of human immune reconstitution and function with adult HSCs obtained from patients. However, these cells are not available in large quantities from study volunteers, and adult HSCs engraft less efficiently than fetal CD34⁺ cells in immunodeficient mice (15). Furthermore, even if obtained in large quantities for therapeutic applications, adult HSCs may be rejected by allogeneic thymocytes pre-existing in fetal thymus grafts. Here, we report the development of a new humanized mouse model that supports robust peripheral

reconstitution of T cells and APCs from small numbers of adult, allogeneic bone marrow CD34+ cells. This “Personalized Immune” (PI) mouse model could potentially be used to identify HSC-intrinsic immune abnormalities predisposing to autoimmunity as well as model individual human immune responses in both health and disease.

Results

Overcoming the immune barrier imposed by mature T cells in fetal thymus grafts

To assess human immune reconstitution from adult HSCs in immunodeficient mice, CD34+ cells were isolated from discarded human bone marrow infusion filters and given i.v. to sublethally irradiated nonobese diabetic-severe combined immunodeficient (NOD/SCID) mice receiving fetal THY transplantation. Recipients of untreated fetal human THY grafts showed low peripheral T cell reconstitution during the first weeks after transplantation, which declined markedly over time, suggesting that these cells emigrated from the graft (average CD3+ cell reconstitution $7.0\% \pm 8.22\%$ of PBMC at 6 weeks, $n=5$ vs $2.34\% \pm 2.26\%$ at 16 weeks post-transplantation). Non-T cells did not reconstitute from injected allogeneic CD34+ cells, suggesting that these were rejected. Moreover, some animals reconstituted with fetal human THY grafts and CD34+ cells have developed a late-onset graft-versus-host disease (GVHD)-like wasting syndrome that resulted in mortality (Fig. S1). We hypothesized that thymocytes pre-existing in the THY grafts might reject allogeneic CD34+ cells and expand to attack recipient tissues, preventing immune reconstitution and causing xenogeneic GVHD, respectively. We therefore tested methods for depleting graft thymocytes in an effort to prevent these phenomena.

Fetal thymus organ culture (FTOC) with 2'-deoxyguanosine (dGuo) depletes thymocytes while preserving stromal elements (16) that can support thymopoiesis (17). NOD/SCID mice received allogeneic adult CD34+ cells plus fetal THY tissue cultured for 7 or 21 days in the presence of dGuo. Control animals received fetal liver CD34+ cells from the thymic tissue donor. Mice that received 5×10^5 adult CD34+ cells without a THY graft reconstituted an average of 20% human PBMCs by Week 10 (Figure 1A), with robust B cell reconstitution (Figure 1B), but CD3+ cells were undetectable (Figure 1C). In mice that received 7 day dGuo-cultured THY tissue plus allogeneic CD34+ cells, CD3+ levels averaging ~7% of PBMC were detectable by 6 weeks and subsequently declined (Figure 1C), but CD19+ cells did not appear (Figure 1B). These data suggest that mature T cells escaping dGuo depletion may have rejected the infused allogeneic CD34+ cells. Cells within the THY grafts did not achieve human non-T cell reconstitution or a high level of T cell reconstitution (Figure 1C).

In contrast, successful thymic engraftment with human thymopoiesis as well as peripheral CD19+ cell reconstitution occurred after intravenous infusion of 5×10^5 allogeneic adult CD34+ cells with a 21-day dGuo-cultured THY graft, (average ~25% human CD3+ cells among PBMC at 20 weeks, Figure 1B,C). Since recipients of CD34+ cells without a THY graft did not reconstitute T cells and THY grafts treated with dGuo for even 7 days did not contain enough progenitors to permit long-term (>14 weeks) T cell reconstitution (Figure 1C), we conclude that progenitors derived from peripherally-infused allogeneic adult CD34+ cells populated 21-day dGuo-treated thymi, underwent thymopoiesis, and emigrated to the periphery. Control recipients of dGuo-treated fetal thymus tissue with 4×10^5 autologous fetal liver CD34+ cells instead of allogeneic adult marrow CD34+ cells showed more rapid T cell reconstitution (Figure 1C) than was achieved with allogeneic adult CD34+ cells.

While the above recipients of 21-day FTOC grafts plus 5×10^6 adult CD34+ cells exhibited high levels of long-term (>20 weeks) human chimerism (Figure 1), a lower dose of adult CD34+ cells did not achieve robust immune reconstitution (average $3.89\% \pm 9.89\%$ human

cells in PBMC at 20 weeks in recipients of 2×10^5 CD34+ cells, $n=9$). Only limited HSCs are available through volunteer bone marrow aspiration. Therefore, we used NOD/SCID/IL2 receptor γ chain^{null} (NSG) mice, which lack NK cells and are more permissive for engraftment of human HSCs (18), for the ensuing experiments.

We evaluated irradiation of THY grafts to deplete pre-existing thymocytes. NSG mice receiving 7 Gy irradiated THY plus 3×10^5 adult CD34+ cells showed excellent B cell and monocyte reconstitution, but low numbers of peripheral T cells by 20 weeks (Fig. S2). THY grafts were so small as to be barely visible under the kidney capsule upon laparotomy (Fig. S3A). T cells eventually reconstituted the periphery by 34 weeks post implantation (Fig. S2).

Cryopreservation and thawing of fetal THY grafts to allow peripheral reconstitution of T cells and multiple hematopoietic lineages from allogeneic, adult human hematopoietic stem cells

Transplantation of cryopreserved and thawed mouse thymus tissue can restore immune function (19;20). Since the studies above suggested that human fetal THY tissue contains viable, alloreactive and xenoreactive thymocytes, we evaluated the ability of cryopreservation of intact fetal thymic tissue fragments to deplete these thymocytes. As shown in Supplemental Figure 4, cryopreservation of thymic tissue indeed led to marked depletion (1567 fold decrease in total cellularity) of all thymocyte subsets from fetal thymic tissue.

To test the utility of cryopreserved fetal thymic tissue, sublethally irradiated NSG mice received cryopreserved and thawed fetal human THY grafts plus $3-5 \times 10^5$ allogeneic, adult CD34+ cells i.v. To further assure depletion of T cells derived from pre-existing graft thymocytes, the mice received a depleting anti-human CD2 mAb. As shown in Figure 2, all mice achieved human B cell and monocyte chimerism by 6 weeks. Unlike recipients of CD34+ cells alone, which showed minimal T cell reconstitution, recipients of cryopreserved and thawed THY grafts generated peripheral T cells, which appeared by 6 weeks and peaked at ~10% and ~30% of PBMC at 16 weeks following infusion with 3×10^5 or 5×10^5 CD34+ cells, respectively (Figure 2A, lower right panel). While it took 20 weeks to achieve T cell reconstitution with 21-day dGuo-treated grafts, similar T cell levels were reconstituted by 10 weeks with cryopreserved and thawed THY grafts. At the time of animal sacrifice, these cryopreserved grafts were markedly enlarged, showing evidence of robust human thymopoiesis with predominant CD4/CD8 double positive thymocytes (Figure 2B, C).

Control animals receiving cryopreserved THY grafts without i.v. CD34+ cells (with [$n=5$] or without [$n=4$] anti-CD2 mAb) did not repopulate significant human T cells or non-T cells in the periphery ($0.46\% \pm 0.34\%$ human cell reconstitution 20 weeks post-transplantation). Thus, the majority of pre-existing graft thymocytes were depleted by cryopreservation and administration of CD34+ cells was necessary for human T cell and non-T cell reconstitution. Additional control NSG animals receiving fresh thymic tissue with allogeneic CD34+ cells showed human T cells (mean 2.4% of PBMC) but no human chimerism in any other lineage by 5 weeks ($<0.006\%$), whereas recipients of autologous CD34+ cells showed significant human T cell (mean 3.4%), B cell (mean 2.1%) and monocyte (mean 0.2%) reconstitution by this time (Fig. S5). These data are consistent with the interpretation that T cells from fresh thymic tissue implanted into NSG mice rejected allogeneic CD34+ cells.

None of the long-term animals (17 of 17 followed for 20 weeks) that received cryopreserved and thawed THY plus anti-CD2 mAb developed wasting syndrome or other evidence of GVHD. Mouse class II+ cells were present in the long-term human THY grafts (Fig S6). Anti-CD2 mAb was not required to prevent rejection of allogeneic donor stem cells, as no

difference was seen in the level of human reconstitution by 15 weeks when groups of mice receiving cryopreserved thymic grafts and allogeneic CD34+ cells with or without anti-CD2 mAb treatment were compared (Fig. S7).

Human immune reconstitution from a bedside bone marrow aspirate from control and T1D volunteers

We next evaluated reconstitution capabilities of adult CD34+ cells isolated from bedside bone marrow aspiration. An aspiration of 15ml bone marrow yielded 3.6×10^5 and 2.7×10^6 CD34+ cells from an initial healthy control and T1D volunteer subject, respectively. Sublethally irradiated NSG mice received 1.8×10^5 adult CD34+ cells each plus a cryopreserved and thawed human fetal THY graft and anti-human CD2 mAb. Control irradiated mice received CD34+ cells without THY tissue. Human chimerism was detectable by Week 6 and peaked at ~25%-80%. Recipients of THY grafts plus i.v. CD34+ cells from the control and T1D volunteers developed substantial CD3+ cell levels by 8 weeks, while control mice (no THY graft) had markedly delayed T cell reconstitution (Figure 3). CD19+ cells and CD14+ cells also developed from the HSCs of the T1D and control volunteers. Similar results were obtained in 3 additional experiments, in each of which 4-14 NSG mice were each reconstituted with $2-3 \times 10^5$ CD34+ cells from a single volunteer aspirate. Composite data from these experiments are presented in Figure 3A. Splenic T cell reconstitution was also rapid and robust, with a mean of 2.47×10^6 (SEM 0.6×10^6) human CD3 cells per spleen in a group of 3 T1D cell-reconstituted mice and 10^6 CD3 cells in a healthy control-reconstituted mouse spleen that were sacrificed at 9 weeks. Figure 3B shows the robust thymopoiesis from adult CD34+ cells, including normal proportions of CD4/CD8 double positive and single positive cells and similar proportions of CD45RO+ and CD45RA+ cells among single positive thymocytes as was seen with fetal thymus and fetal CD34+ cells (7). Although thymocyte numbers tended to be lower in T1D compared to healthy control CD34 cell-reconstituted animals, no statistically significant differences were seen between the two groups.

T cell function and self-tolerance in mice reconstituted with volunteer donor bone marrow CD34+ cells

We assessed T cell function by transplanting allogeneic human and xenogeneic (pig) skin to THY-grafted mice that received adult CD34+ cells. These mice rapidly rejected allogeneic human and xenogeneic pig skin grafts (Figure 4A), while naive, untreated NSG mice accepted allogeneic human and xenogeneic skin grafts for the duration of follow-up (106 and 50 days, respectively) (Figure 4A) with no infiltrates or evidence for rejection on histology (Fig S8).

To assess self-tolerance of T cells generated from adult CD34+ cells of T1D and healthy volunteers, mixed lymphocyte reactions (MLR) were performed using purified T cells isolated from the spleens and lymph nodes. T cells from mice reconstituted from T1D and control subjects showed self-tolerance along with strong responses to allogeneic human stimulators in MLR (Figure 4B, Table S1). Notably, fresh adult donor T cells and T cells from a mouse reconstituted from the same healthy control bone marrow donor showed similar, robust responses to the allogeneic stimulator and similar self-tolerance. Thus, immune responsiveness and self-tolerance to the adult volunteer were recapitulated in these mice. Because they generate immune function from an individual adult bone marrow donor, we refer to these animals henceforth as “Personalized Immune” (PI) mice.

Similar Treg development from T1D and control bone marrow CD34+ cells

We assessed the presence of Tregs in thymus grafts and the periphery of reconstituted mice. As shown in Figure 5A, CD25^{high}FoxP3+ Tregs were present among CD4+CD8-

thymocytes of PI mice. Analysis of CD4+CD8-CD25+CD127^{lo} thymocytes demonstrated that the majority of FoxP3+ cells were also Helios+, indicative of thymically-derived “natural” Tregs. Similar numbers and proportions of Tregs were detected in THY grafts reconstituted from control and T1D volunteers. Furthermore, while some studies have suggested that Treg numbers are reduced in the blood of T1D subjects compared to healthy controls (21), similar proportions of Tregs were detected in the peripheral blood of both groups of reconstituted mice (Figure 5B).

Diverse TCR repertoire in single positive (SP) thymocytes derived from adult donor CD34+ cells

At 20 weeks post-transplantation, we performed spectratyping analysis on CD4 and CD8 SP thymocytes of mice reconstituted from T1D CD34+ cells and a normal volunteer (Figure 6). These human T cells showed a diverse repertoire, with similar utilization of the BV families and a polyclonal CDR3 length distribution for each BV. The reconstituted repertoires resembled those of the average CD4 T cell repertoires of 12 healthy adults, with average Hamming distances for all analyzed BV families in each sample ranging from 14.2 to 26.2, mean 20.6 (Table S2). This is indicative of typical T cell polyclonality as seen in healthy control peripheral blood lymphocytes (PBL).

Naïve vs memory T cell phenotype in PI mice

The T cells populating the peripheral tissues of mice reconstituted from CD34+ bone marrow cells of healthy control and T1D volunteers included both “naïve”-type CD45RA+CD45RO- and “memory”-type CD45RA-CD45RO+ cells (Figure 7A). Comparison of T cells in the blood of a control CD34+ cell donor revealed a marked increase in the proportion of naïve-type CD45RA+CD45RO- CD4, CD8 and Treg subsets in the PI mouse reconstituted from the same donor (Figure 7A,B). Thus, a rejuvenated version of the adult donor’s immune system is generated in PI mice. As shown in Figure 7C, the human thymus was necessary for this rejuvenation, as the proportion of naïve-type T cells in PBMC of recipients of CD34+ cells alone was markedly lower than that in mice that also received thymus grafts.

When we compared the proportions of naïve-type and memory-type CD4 and CD8 T cells in the blood of PI mice generated simultaneously from T1D or healthy control donors, the T cells derived from T1D CD34+ cells showed significantly reduced proportions of naïve-type cells compared to those derived from healthy controls (Figure 7A). Tregs derived from CD34+ cells of T1D donors tended more toward the “memory” phenotype than those from healthy controls, but this trend did not achieve statistical significance (Figure 7B; $p=0.07$).

Discussion

In this study, we sought to develop a means of generating functional immune systems from HSCs of adult humans in immunodeficient mice in order to overcome the limitations of current models for the analysis of human immunoregulation in health and disease. We demonstrate here that adult, bone marrow-derived CD34+ cells can reconstitute NSG mice grafted with cryopreserved and thawed allogeneic thymus tissue, generating multiple hematopoietic lineages, including T cells, B cells and myeloid cells. Cryopreserving and thawing the fetal thymus plus administering anti-CD2 mAb depletes mature T cells from the graft, preventing rejection of allogeneic CD34+ cells and GVHD, while preserving thymic function. Thymopoiesis, growth of the thymus graft and reconstitution of a functional, diverse and rejuvenated immune system is achieved. Self-tolerance of the adult donors is recapitulated. While fetal liver fragments were included in the humanized mouse model upon which our studies are based (4;6), these fragments are not required, as thymocyte

progenitors from infused CD34+ cells populated the human thymic grafts in the current study.

While *in vivo* thymopoiesis and peripheral reconstitution were also achieved from dGuo-treated human thymi, T cell reconstitution from infused adult CD34+ cells was slow when thymi were dGuo-treated sufficiently long (21 days) to prevent rejection of allogeneic CD34+ cells. Slow T cell recovery has also been observed in patients with complete DiGeorge syndrome receiving thymic tissue cultured for several weeks in dGuo (22). Our results suggest that cryopreservation of thymic tissue might support more rapid T cell recovery while preventing GVHD.

Cryopreservation of fetal thymus tissue permits HLA typing of tissue for use with adult CD34+ cells sharing HLA alleles, which is important for optimal immune function. The use of NSG mice allows the engraftment of relatively small numbers of allogeneic adult HSC, allowing reconstitution of multiple mice from a bedside bone marrow aspirate.

The specific tolerance to CD34+ cell donor “self” antigens and the absence of GVHD in our studies most likely reflects intrathymic deletion due to the presence of APCs from the human HSC donor and the murine recipient, respectively, in the human thymus graft, as previously suggested in another thymic xenograft model (23). Although not tested directly, we hypothesize that the inclusion of anti-CD2 mAb was important for the prevention of a wasting syndrome induced by residual xenogeneic GVH-reactive mature T cells emigrating from fetal human thymus grafts. This possibility was suggested by the development of a late-onset (at 22 weeks) GVHD-like syndrome (severe alopecia, skin inflammation, hunched posture and weight loss) in the only mouse that did not receive anti-CD2 mAb within a group of NSG mice receiving cryopreserved THY grafts (plus allogeneic CD34+ cells i.v.).

Immune reconstitution from adult bone marrow CD34+ cells of patients in NSG mice provides an immune system unaltered by disease, allowing comparison of individuals in a controlled and prospective manner. Human immune analyses are typically limited to peripheral blood samples, and underlying immune dysregulation cannot be distinguished from the ensuing cascade of inflammatory events that culminate in disease. Defects in Treg numbers and function have been reported for T1D (21;24-26), systemic lupus erythematosus (27) and rheumatoid arthritis (28), but this has been controversial in T1D (29-31). We observed no gross abnormalities in the T cell populations generated from T1D subjects' CD34+ cells, which generated Tregs intrathymically in similar proportions as healthy control CD34+ cells. However, we observed significantly reduced proportions of naïve-type T cells in the blood of PI mice generated from T1D compared to healthy control donors, suggesting that abnormalities of T cell homeostasis, as described in NOD mice (32), might be a feature of T1D-derived HSCs. Our model will allow assessment of genetically-programmed, HSC-intrinsic immunoregulatory abnormalities in T1D in relation to predisposing gene alleles.

HLA-transgenic immunocompetent mice have provided insight into the pathogenesis of autoimmune diseases such as rheumatoid arthritis (33), multiple sclerosis (34), celiac disease (35) and T1D (36-39). However, none of these models permit analyses of human HSC-intrinsic, genetically determined immune abnormalities that may contribute to autoimmune pathogenesis. In contrast, the combined administration of i.v. CD34+ cells and fetal THY tissue in immunodeficient mice generated functional human T cells, T-B interactions, class-switched antibody responses, with secondary lymphoid organs containing both plasmacytoid and myeloid dendritic cells (4-6). Since Tregs develop normally (7) and T cell homeostasis can be studied in this model (8), it will allow assessment of HSC-intrinsic immunoregulatory abnormalities associated with autoimmune diseases in HSC donors. The

ability to HLA type the thymus before transplantation allows selection for thymi with disease-associated HLA alleles.

Our model has not yet been developed to allow the study of diabetes pathogenesis, as animals reconstituted with HSCs from T1D patients did not develop evidence of insulinitis (Figure S9). While we would not expect transplantation of T1D HSCs to cause autoimmune disease in unmodified NSG mice, further development of the model using HLA transgenic NSG mice might permit studies of autoimmune disease pathogenesis.

Our “Personalized Immune” mouse model will also allow the analysis of individual responsiveness of an adult marrow donor to immunotherapeutic agents. In addition, the reconstitution of multiple mice with naïve T cells with a diverse repertoire derived from adult HSCs could potentially provide patients with thymic insufficiency due to immunosuppressants, chemotherapy, irradiation or HIV, with functional, self-tolerant T cells for adoptive transfer. Mice receiving human fetal THY and CD34+ cell grafts generate anti-HIV and other antigen-specific immune responses (5; 40), suggesting the immunotherapeutic potential of this approach. However, robust methods of overcoming the infectious risks of murine pathogens, including endogenous retroviruses (41), would be needed before this approach could be considered.

In summary, we have established a model that permits the development of multilineage peripheral human hematopoietic cells from adult HSCs. The “Personalized Immune” mouse provides an immune system unaltered by disease or its treatment that should allow the analysis of intrinsic defects in immunoregulation associated with autoimmune disorders and of genetically-controlled responses to immunotherapies.

Materials and Methods

Animals and human tissues and cells

Nonobese diabetic-severe combined immunodeficient (NOD/SCID) and NOD/SCID/IL2 receptor γ chain^{null} (NSG) mice were obtained from Jackson Laboratory, and housed in a specific pathogen-free microisolator environment. Human fetal thymus and liver tissues (gestational age 17-20 weeks) were obtained from Advanced Biosciences Resource. Fetal thymus fragments were cryopreserved in 10% DMSO and 90% human AB serum (Atlanta Biologicals), irradiated or cultured, depending on the experimental design. CD34+ cells were isolated from a 15ml bone marrow aspirate, or from discarded human bone marrow filters obtained from the Massachusetts General Hospital (MGH) Bone Marrow Processing Laboratory, or from fetal human liver tissue using a magnetic-activated cell sorter (MACS) separation system with anti-human CD34+ microbeads (Miltenyi Biotec). Human skin was obtained from the National Disease Research Interchange (NDRI) and pig skin kindly provided by Dr. David H. Sachs (MGH). The use of human tissues/cells and animals were approved by the MGH and Columbia University Medical Center (CUMC) Human and Animal research review committees, respectively, and the experiments were performed in accordance with the approved protocols.

Blood samples and bone marrow aspirates from T1D and control volunteers were recruited through the Human Studies Core of the Harvard JDRF Autoimmunity Center from the Joslin Diabetes Institute or from the Naomi Berrie Diabetes Center at Columbia University Medical Center and were collected with written informed consent under protocols approved by the IRBs of the Massachusetts General Hospital and the Columbia University Medical Center, respectively.

Fetal thymus organ culture

Human fetal thymus culture was performed as previously published (16). Briefly, thymus fragments were placed on 0.8µm isopore membrane filters (Millipore) on 1cm² Gelfoam sponges (Pharmacia & Upjohn Co). To eliminate endogenous thymocytes, organ cultures were grown in the presence of 1.35 mM 2'-deoxyguanosine (Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) at 37°C for 7 or 21 days.

Human tissue transplantation

Mice were conditioned with sublethal (2.5 Gy) total-body irradiation. Human fetal thymus fragments measuring about 1mm³ were implanted underneath the recipient kidney capsule. Within 24 hours, 1-5×10⁵ human CD34+ cells were injected intravenously. Some recipients were treated intravenously with anti-human CD2 mAb (BTI322 (42); 100µg) on Days 0 and 7.

Skin grafting

Split thickness (2.3mm) skin samples from a MHC miniature pig and an allogeneic human donor were grafted on the lateral thoracic wall 39 weeks after human tissue transplantation. Skin grafts were evaluated daily from day 7 onward to 4 weeks and then at least one inspection every third day thereafter. Grafts were defined as rejected when less than 10% of the graft remained viable.

Flow Cytometry (FCM)

Levels of human hematopoietic cells in transplanted mice were assessed by multicolor flow cytometry. Mice were tail bled at regular intervals after transplantation to obtain peripheral blood mononuclear cells (PBMC), which were prepared with Histopaque-1077 (Sigma-Aldrich). Fluorochrome-labelled mAbs, purchased from BD Pharmingen, were used in different combinations: anti-mouse CD45, anti-mouse Ter119, anti-human CD4, anti-human CD8, anti-human CD14, anti-human CD19, anti-human CD45, anti-human CD3, anti-human CD45RA, anti-human CD45RO, anti-human CD127, anti-human FoxP3, anti-human CD25 and isotype control mAbs. FCM analysis was performed using a FACSCalibur, FACSCanto or LSR II (BD), and analysis was carried out by FlowJo software (TreeStar). Dead cells were excluded from the analysis by gating out low forward scatter and high propidium iodide (PI) – retaining cells. Murine erythroid cells were excluded by gating out mouse Ter119+ cells.

Mixed lymphocyte reactions

Splenocytes and lymph nodes were harvested from humanized mice and mononuclear cell suspensions were isolated by Ficoll separation. Human T cells were enriched by depletion of mouse cells using anti-mouse CD45 and anti-Ter-119 microbeads (Miltenyi Biotec) followed by T cell purification using the Pan T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. Purity was >90%. Responder T cells (10⁵ per well) were cultured with irradiated human allogeneic PBMCs (3000rad, 10⁵ cells per well) as stimulators for 5 days and proliferation was measured via [³H] thymidine incorporation as we have described (43). In self-stimulated control cultures, responder cells were incubated with autologous PBMCs from the same humanized mouse, depleted of mouse CD45+ and Ter119+ cells. Data are shown as mean [³H] thymidine incorporation in triplicate cultures.

Spectratyping

Total RNA was extracted directly from 1 to 2×10⁴ CD4 or CD8 single positive thymocytes (purity >80%), reverse transcribed and single-strand complementary cDNA synthesis was performed as described (44). Amplification reactions were performed using a TCR β-chain

constant region primer and individual variable region primers as described (44). Products were then used in run-off reactions with a C β -specific FAM-labeled primer (Integrated DNA Technologies) as described (44). The labelled products were then used to determine the length distribution of the TCR β -chain length. The size and area of the peaks corresponding to the DNA products were determined using an ABI 3100 Genetic Analyzer (Applied Biosystems) and analyzed using Applied Biosystems Genotyper 3.7 NT. Hamming distances to assess the quantitative difference between the experimental and reference β -chain length distributions of peripheral blood CD4 T cells in normal humans were calculated as described (44).

Statistical analysis

Statistical analysis and comparisons were performed with GraphPad Prism version 4.0 (GraphPad Software). Data in bar graphs are expressed as mean $\pm$ SEM. Student's t-test for parametric data sets, or Mann-Whitney test for nonparametric data sets were used to compare groups. ANOVA was used to resolve overall effects between transplant groups over time, and Mann-Whitney test used for individual time point comparisons. A p value less than 0.05 was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Peripheral human cell reconstitution in NOD/SCID mice following transplantation of dGuo-treated thymic tissue

Fetal thymic tissue was treated with dGuo for 7 (black diamonds, n=4) or 21 days (black triangles, n=5) before transplantation into sublethally irradiated NOD/SCID that received 5×10^5 allogeneic, adult CD34+ cells or 4×10^5 autologous fetal liver CD34+ cells (open circles, n=5) intravenously. Age-matched control animals received 5×10^5 adult CD34+ cells alone (open squares, n=5). The mice were bled to measure human (hu) cell reconstitution in (total mouse plus human) peripheral blood mononuclear cells (PBMCs) at the indicated time points. A: total human chimerism; B: percentage of human B cells; C: percentage of human T cells among PBMCs at indicated time points.

Figure 2. Multilineage human cell reconstitution in NSG mice receiving cryopreserved and thawed thymic grafts and allogeneic, adult CD34+ cells

A) Sublethally irradiated NSG mice that received a cryopreserved and thawed fetal thymus graft in combination with 3×10^5 (black squares, $n=6$) or 5×10^5 (black triangles, $n=6$) adult CD34+ cells and two doses of anti-CD2 mAb i.v. were bled to measure human cell reconstitution in (total mouse plus human) PBMCs at the indicated time points. Age-matched control animals received 3×10^5 adult HSCs alone (white squares, $n=6$). Single cell suspensions of PBMCs were stained for markers of human hematopoietic cells (CD45), T cells (CD3), B cells (CD19) and monocytes (CD14). Dead cells and mouse red blood cells were excluded from the analysis. B) Representative THY graft appearance 20 weeks post-transplantation. NSG mice that were transplanted with cryopreserved and thawed fetal thymus tissue had abundant, viable thymic tissue underneath the kidney capsule. C) FCM analysis of thymocytes (representative of 12 grafts in a single experiment).

Figure 3. Multilineage human cell reconstitution in NSG mice receiving cryopreserved/thawed thymic grafts and allogeneic, adult CD34+ cells isolated from bedside bone marrow aspirates

A) Sublethally irradiated NSG mice received cryopreserved/thawed fetal thymus tissue in combination with $1.8-3.0 \times 10^5$ adult CD34+ cells isolated from bone marrow aspirates from healthy volunteers (black squares, $n=3$ donors, 6 recipients) and T1D subjects (black triangles, $n=4$ donors, 29 recipients). Mean levels of human cell reconstitution in (total mouse plus human) PBMCs are shown over time. Control animals received adult HSCs alone from the T1D subjects (open circles, $n=2$ donors, 7 recipients). Thymus grafts and bone marrow donors were HLA-typed for T1D-associated DRB and DQB alleles and HLA A*201 using SNP genotyping assays. The thymic tissue and bone marrow donors shared at least HLA*A201 and DRB*0302 and/or DQB*0301. ANOVA revealed an effect of THY transplant on CD3 reconstitution comparing T1D CD34+ cells alone with T1D CD34+ plus THY transplantation at early (6-14 weeks) timepoints. Individual timepoint comparisons

with Mann-Whitney U test revealed significant differences at 8-14 weeks (* $p < 0.05$). B) Graft thymocytes were analyzed 22-25 weeks post-transplantation in T1D (n=5) and control (n=3) HSC-reconstituted animals. Mean + SEM are shown. No significant differences between T1D and control animals were noted.

Figure 4. Functional and self-tolerant immune systems in NSG mice receiving fetal thymus graft and adult CD34⁺ cells

A) NSG mice (n=3) that received a 7 Gy irradiated thymic graft plus 3×10^5 adult CD34⁺ cells reconstituted peripheral T cells >30 weeks after transplantation. Thirty-nine weeks after transplantation, they were grafted with allogeneic human and xenogeneic pig skin. Survival of pig and human skin grafts (n=3 and 4, respectively) on untreated control NSG mice “naïve NSG” are also shown. B) Human T cells (>90% pure) were enriched from the spleen and peripheral lymph nodes of NSG mice 20 weeks following transplantation of cryopreserved and thawed THY grafts and allogeneic bone marrow CD34⁺ cells from a healthy control (black bar) or T1D subject (dotted bars). Control T cells were isolated from

PBMC of the same healthy control volunteer (open bar). Supplementary Table 1 shows the naïve/memory cell distribution of CD4 cells in the 3 mice reconstituted from T1D CD34+ cells.

Figure 5. Tregs in thymus grafts and periphery of PI mice

A) 20-22 weeks after transplantation, single cell suspensions were prepared from thymus grafts of NSG mice that received a cryopreserved and thawed THY graft and allogeneic CD34⁺ cells from one of two healthy volunteers (circles) or one T1D subject (squares) and analysed for CD4⁺CD8⁻CD25⁺FoxP3⁺ cells by FCM (top row). As a marker for natural Tregs, Helios expression in CD4⁺CD8⁻CD25⁺CD127^{lo}FoxP3⁺ thymocytes is shown in NSG mice derived from a second human donor pair in the bottom row.

B) Similar proportions of Tregs in PBMCs 20 weeks after transplantation of cryopreserved/thawed fetal thymus grafts with CD34⁺ cells from one of two healthy controls (black squares) or one T1D subject (black triangles) compared to two healthy humans (black circles). Left plots show CD25 and FoxP3 staining on CD4⁺ T cells from PI mice generated from control and T1D donors.

Figure 6. Diverse repertoire of T cells in PI mice

Spectratyping (β -chain CDR3 length distribution) of human CD4 and CD8 SP T cells in THY grafts reconstituted with CD34⁺ cells from one T1D donor or one of two healthy controls 20 weeks after transplantation. Spectratype from one representative animal (#5700) of six (mice) is shown. The vertical axis is relative fluorescence units (full scale=6,000 units). The horizontal axis is nucleotide size. Reference size markers are low fainter peaks. Representative BV is shown from a total of 12 analyzed per sample. The Hamming distances of all 6 samples, a measure of the relative distances of the observed TCR β -chain length distribution from a reference distribution of healthy adult CD4 T cells, are shown in Supplemental Table 1, and each indicates the reconstitution of a polyclonal repertoire.

Figure 7. Naïve vs memory phenotype of T cells in PI mice

A) and B) Proportions of CD45RA⁺ CD4 and CD8 T cells (B) and Tregs (C) in PBMCs of healthy volunteers and of PI mice 20 weeks after THY implantation plus i.v. infusion of CD34⁺ cells from one T1D subject (black squares) or one of two healthy controls (black triangles), including (open circle) the donor of CD34⁺ cells for the control mouse indicated with an open triangle (*p<0.05, excluding the outlier in the CD8 population of controls from statistical analysis). C) T cell populations were assayed by FCM in NSG mice injected with CD34⁺ fetal liver cells with or without allogeneic thymus at 7 weeks post-transplant. Mean ± SEM are shown, n=4 for each group. *p<0.05, **p<0.005.