



# Type 1 diabetes induction in humanized mice

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**There is an urgent and unmet need for humanized *in vivo* models of type 1 diabetes to study immunopathogenesis and immunotherapy, and in particular antigen-specific therapy. Transfer of patient blood lymphocytes to immunodeficient mice is associated with xenogeneic graft-versus-host reactivity that complicates assessment of autoimmunity. Improved models could identify which human T cells initiate and participate in beta-cell destruction and help define critical target islet autoantigens. We used humanized mice (hu-mice) containing robust human immune repertoires lacking xenogeneic graft-versus-host reactivity to address this question. Hu-mice constructed by transplantation of HLA-DQ8<sup>+</sup> human fetal thymus and CD34<sup>+</sup> cells into HLA-DQ8-transgenic immunodeficient mice developed hyperglycemia and diabetes after transfer of autologous HLA-DQ8/insulin-B:9–23 (InsB:9–23)-specific T-cell receptor (TCR)-expressing human CD4<sup>+</sup> T cells and immunization with InsB:9–23. Survival of the infused human T cells depended on the preexisting autologous human immune system, and pancreatic infiltration by human CD3<sup>+</sup> T cells and insulinitis were observed in the diabetic hu-mice, provided their islets were stressed by streptozotocin. This study fits Koch's postulate for pathogenicity, demonstrating a pathogenic role of islet autoreactive CD4<sup>+</sup> T-cell responses in type 1 diabetes induction in humans, underscores the role of the target beta-cells in their immunological fate, and demonstrates the capacity to initiate disease with T cells, recognizing the InsB:9–23 epitope in the presence of islet inflammation. This preclinical model has the potential to be used in studies of the pathogenesis of type 1 diabetes and for testing of clinically relevant therapeutic interventions.**

type 1 diabetes | insulin | humanized mice

**T**ype 1 diabetes mellitus (T1D) is an autoimmune disease resulting from immune destruction of insulin-producing  $\beta$  cells in the pancreatic islets. Autoreactive T cells recognizing  $\beta$ -cell antigens (e.g., insulin) are believed to play a critical role in disease onset and progression, but direct evidence for this is still lacking (1). The loss of  $\beta$  cells and the subsequent reduction or lack of insulin result in chronic elevation of blood glucose levels, leading to severe complications such as heart disease, stroke, and kidney failure. Patients with T1D rely on exogenous insulin to control the disease (2), but insulin therapy does not eliminate the risk for T1D complications and may be associated with life-threatening hypoglycemia (3). Thus, new therapies are urgently needed for T1D treatment. Recent insight into the human disease lesion in the pancreas indicated profound differences in immunopathology between patients with T1D and the nonobese diabetic mouse model that is regarded as the nearest preclinical model of autoimmune diabetes (4). These observations, combined with a range of other differences in the immune systems of humans versus mice, prompted us to design preclinical models in which the immune system is humanized to study both disease mechanisms and immunotherapeutic strategies (5).

In this study, we sought to overcome this issue, using humanized mice (hu-mice), by infusing autologous hu-mouse-derived human

T cells engineered with a TCR that recognizes the HLA-DQ8-restricted insulin B chain peptide consisting of amino acids 9–23 (InsB:9–23). InsB:9–23 is a dominant MHC class II-restricted antigen recognized by islet-infiltrating insulin-specific T cells and serves as an essential target of the immune destruction of pancreatic  $\beta$  cells in nonobese diabetic (NOD) mice (6, 7). Previous observations suggested this epitope may also serve as a key autoantigenic target in humans, as it does in mice. HLA class II-restricted T-cell response to InsB:9–23 peptide is highly associated with T1D in humans (8). A recent study using HLA-DQ8/B:11–23<sup>R22E</sup> tetramers further confirmed the presence of CD4 T cells recognizing the HLA-DQ8-restricted B:11–23 peptide in patients with T1D (9). More recently, 2.35% of CD4 T-cell clones isolated from inflamed islets of patients with T1D were found to recognize InsB:9–23 (10). However, there has been no direct evidence for human InsB:9–23-reactive T-cell-mediated *in vivo* destruction of pancreatic  $\beta$  cells in humans. Our data showed that adoptive transfer of HLA-DQ8-restricted InsB:9–23-specific human CD4 T cells is capable of inducing diabetes in HLA-DQ8-Tg hu-mice, consistent with the potential of T-cell responses to the InsB:9–23 epitope to initiate T1D in humans.

## Results

The TCR  $\alpha$  ( $V_{\alpha 21}$ ) and  $\beta$  ( $V_{\beta 11}$ ) chain cDNA was extracted from an InsB:9–23-specific human T-cell line (clone #5), which was established from blood from an 18-y-old Caucosoid HLA-DQ8 homozygous man diagnosed with T1D at the age of 8 y (11, 12), and linked by a P2A self-cleaving peptide gene. The TCR $\alpha$ -P2A- $\beta$  gene fragment was then linked to a F2A-AcGFP gene fragment, and then cloned into a lentiviral vector (LV-insTCR; Fig. S1). To assess the

## Significance

**Type 1 diabetes (T1D) is known to be caused by immune destruction of insulin-producing  $\beta$  cells, but the disease pathogenesis remains poorly understood largely because of limitations in animal models to study the immunopathology. Here we established a humanized mouse T1D model, in which diabetes is driven by human T cells recognizing the HLA-DQ8-restricted insulin B chain peptide consisting of amino acids 9–23 (InsB:9–23). This study not only demonstrates the capacity of InsB:9–23-specific human CD4 T cells to initiate diabetes but also provides a preclinical humanized mouse model that has the potential to be used in studies of the immunopathogenesis and immunotherapy of T1D.**

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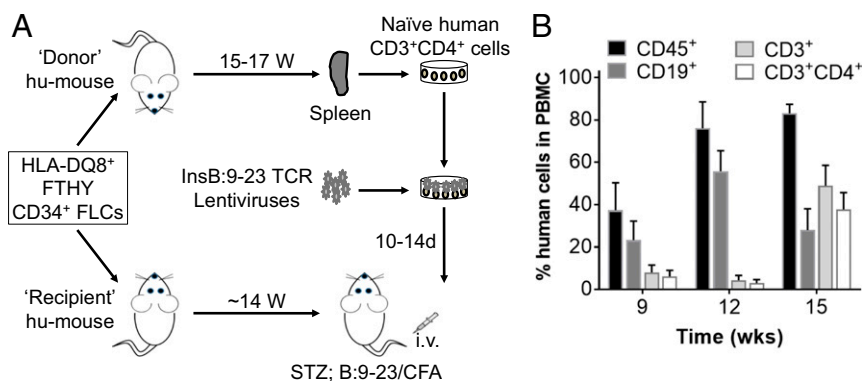
The authors declare no conflict of interest.

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**Fig. 1.** Preparation of hu-mice for generating InsB:9-23-specific T cells and for induction of diabetes. (A) Schematic showing preparation of both donor hu-mice with HLA-DQ8<sup>+</sup> human immune reconstitution, from which splenic human CD4<sup>+</sup>-naive T cells are prepared, transduced with LV-insTCR, expanded, and injected into recipient hu-mice for diabetes induction, and recipient HLA-DQ8-Tg hu-mice reconstituted with human immune cells autologous to the donor hu-mice, which are used as the recipients of LV-insTCR-transduced CD4<sup>+</sup> T cells. (B) Levels (mean ± SEM) of human hematologic and immune cell chimerism in PBMCs of hu-mice from a representative experiment.

efficacy of cell surface TCR expression of the engineered human T cells, we measured TCR expression on the TCR-negative human cell line J.RT3-T3.5 cells after transduction with LV-insTCR. Flow cytometric (FCM) analysis revealed that all transduced, that is, GFP<sup>+</sup>, J.RT3-T3.5, cells gained cell surface expression of human CD3 (Fig. S24) and αβTCR (Fig. S2B). All GFP<sup>+</sup> cells were also stained positively for human TCR<sub>vβ11</sub>, further confirming the expression of the engineered B:9-23-specific TCR on the transduced cells (Fig. S2C). Together, these results confirmed that efficient cell surface expression of the diabetogenic TCR in human T cells can be achieved via lentiviral transduction.

InsB:9-23 is a dominant epitope recognized by autoreactive T cells in both patients with T1D and NOD diabetic mice (8, 13). Because the TCR used in this study recognizes an HLA-DQ8-restricted InsB:9-23 peptide, we used HLA-DQ8<sup>+</sup> human fetal thymus (FTHY) and CD34<sup>+</sup> cells isolated from fetal liver cells (FLCs) to construct hu-mice and used HLA-DQ8-Tg hu-mice as the recipients of the engineered human T cells (Fig. 1A). Adoptive transfer of peripheral blood mononuclear cells (PBMCs) from patients with T1D (14, 15) or human T cells that were virally transduced to express β-cell-specific TCRs (16) into immunodeficient mice has been explored previously to characterize the β-cell-specific autoimmune responses of human T cells, and the results demonstrated the potential of these models to facilitate understanding of the immunopathogenesis of human T1D. However, although survival and islet infiltration of the transferred human T cells were clearly detected in the recipient mice carrying the diabetogenic HLA transgene matched to the diabetic patient donors, none of the recipient mice developed diabetes. The presence of human T cells reactive to xenogeneic mouse antigens and varying degrees of graft-versus-host disease (GVHD) were confounding factors in the evaluation of antigen-specific immune responses in these studies (14-16). Indeed, previous studies have shown that xeno-GVH responses can result in global T-cell anergy of transfused human T cells even when they do not cause overt GVHD (17). To avoid both anti-human allogenic and anti-recipient mouse xenogeneic immune responses, self- and mouse-tolerant human T cells (18) from autologous hu-mice were used to generate InsB:9-23-specific T cells, which were then adoptively transferred into HLA-DQ8-Tg hu-mice with an autologous immune system (i.e., hu-mice used as the recipients and those used as the source of InsB:9-23-specific T cells were made by FTHY and CD34<sup>+</sup> FLCs from the same fetus; Fig. 1A). Human lymphohematopoietic cell reconstitution in hu-mice was monitored by FCM analysis of peripheral blood human cells. Fig. 1B shows the kinetics of human CD45<sup>+</sup>, CD19<sup>+</sup>, CD3<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>

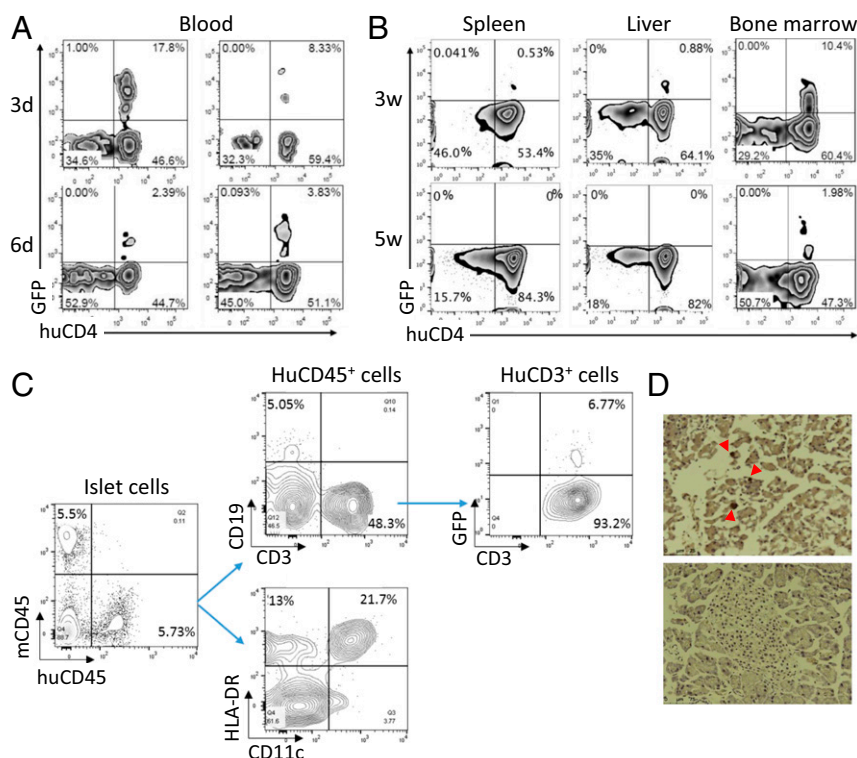
T cells in PBMCs from a representative experiment, in which the level of human CD3<sup>+</sup> T cells in all hu-mice examined was greater than 30% in PBMCs by 15 wk after humanization.

To collect human CD4<sup>+</sup> T cells for genetic engineering, spleens were harvested from hu-mice between 15 and 17 wk after humanization. After confirming high levels of human T-cell reconstitution by FCM analysis of PBMCs and spleen cells (Fig. 2A), human CD4<sup>+</sup>-naive T cells were enriched by depletion of mouse cells and human CD14<sup>+</sup>, CD19<sup>+</sup>, CD8<sup>+</sup>, CD45RO<sup>+</sup>, and CD25<sup>+</sup> cells. The purified cell population consisted of ≈85% human CD3<sup>+</sup>CD4<sup>+</sup> T cells, with the majority of the cells expressing a CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD25<sup>-</sup> naive phenotype (Fig. 2B). The human naive CD4<sup>+</sup> T-cell-enriched cells were preactivated by anti-human CD3/CD28 Dynabeads, followed by incubation with LV-insTCR. The transduced human T cells were then expanded for 10-14 d with irradiated feeder cells plus human cytokines (IL-2, IL-7, and IL-15) and PHA (phytohemagglutinin) or OKT3 (ortho Kung T-cell 3). Using this protocol, we were able to achieve ≈130-200-fold expansion in numbers (Fig. S3). At the end of culture, the majority of the expanded cells were CD3<sup>+</sup>CD4<sup>+</sup> T cells with more than 80% of the cells expressing the engineered TCR, as identified by coexpression of GFP and TCR<sub>vβ11</sub> (Fig. 2C). The expanded human CD4<sup>+</sup> T cells were used immediately in an effort to induce diabetes in HLA-DQ8-Tg hu-mice.

We initially injected the InsB:9-23-TCR-engineered human CD4<sup>+</sup> T cells into HLA-DQ8-Tg NSG mice or hu-mice that were grafted 14 wk earlier with autologous human CD34<sup>+</sup> FLCs (without FTHY; these hu-mice contained human antigen-presenting cells, but not T cells). FCM analysis revealed that the infused human GFP<sup>+</sup>CD4<sup>+</sup> T cells were detectable for at least 17 d in the spleen and 42 d in bone marrow from hu-mice grafted with human CD34<sup>+</sup> FLCs, but not in these tissues from control NSG mice (Fig. S4A). The lack of detectable GFP<sup>+</sup>CD4<sup>+</sup> T cells in NSG mice is consistent with our previous studies showing that the engrafted human CD34<sup>+</sup> FLC-derived cells, likely antigen-presenting cells, play an important role in the survival and/or expansion of infused human T cells when there is no xeno-GVH reactivity (19). However, neither HLA-DQ8-Tg hu-mice nor NSG mice developed diabetes or hyperglycemia after infusion of InsB:9-23-TCR-engineered human CD4<sup>+</sup> T cells (Fig. S4B). Injection of InsB:9-23-TCR-engineered human CD4<sup>+</sup> T cells also failed to induce diabetes or hyperglycemia in low-dose streptozotocin-treated HLA-DQ8-Tg hu-mice that were grafted 14 wk earlier with autologous human CD34<sup>+</sup> FLCs and FTHY (Fig. S4C).

Considering the fact that the TCR used in this study was isolated from a blood-derived T-cell clone that may not respond





**Fig. 3.** Survival of the infused LV-insTCR-transduced human CD4<sup>+</sup> T cells in hu-mice. (A) Survival of GFP<sup>+</sup>CD4<sup>+</sup> T cells in peripheral blood from hu-mice at 3 and 6 d after cell transfer. Data from two hu-mice at each point are shown. (B) Presence of GFP<sup>+</sup>CD4<sup>+</sup> human T cells in spleen, liver, and bone marrow cells of two representative hu-mice analyzed at 3 and 5 wk, respectively, after cell transfer. (C) Pancreatic islets were prepared from hu-mice 11 d after infusion of LV-insTCR-transduced human CD4<sup>+</sup> T cells, digested with trypsin, and stained with the indicated antibodies to detect human cell infiltration by flow cytometry. (D) Pancreatic tissue sections were prepared from hu-mice between 3 and 4 wk after injection of LV-insTCR-transduced GFP<sup>+</sup> human T cells ( $n = 3$ ), and stained with anti-GFP antibodies. Shown are representative immunohistochemistry images of pancreas sections from hu-mice receiving LV-insTCR-transduced GFP<sup>+</sup> (Top) or control (Bottom) human T cells.

and are capable of mounting *in vivo* antigen-specific immune responses after antigen immunization or transplantation (29–31). The human immune cells from recipient hu-mice might also be involved in the immune destruction of pancreatic  $\beta$  cells. They were clearly essential for the prolonged survival of infused autoreactive T cells in our study (Figs. S44 and S5), consistent with the role that human antigen-presenting cells were shown to play in facilitating the survival, expansion, and phenotypic conversion of human T cells in hu-mice when xeno-GVH reactivity is absent (19). In addition, the presence of both GFP<sup>+</sup> and GFP<sup>-</sup> human CD3<sup>+</sup> T cells in the pancreatic islets from hu-mice receiving LV-insTCR-transduced (i.e., GFP<sup>+</sup>) human CD4 T cells (Fig. 3) suggests a possible contribution of recipient endogenous human T cells to the disease development. T cells recognizing numerous antigenic epitopes, including others of insulin, glutamate decarboxylase, islet specific glucose 6 phosphatase catalytic subunit related protein, and the islet tyrosine phosphatase IA-2, are associated with T1D in humans and NOD mice (32). Although T cells specific for InsB:9–23 may be required for ignition of T1D, the development and progression of the disease might also involve functional epitope spreading (7, 33). Further studies are needed to precisely understand the role of the recipient human immune cells in the development of diabetes in hu-mice infused with human diabetogenic T cells.

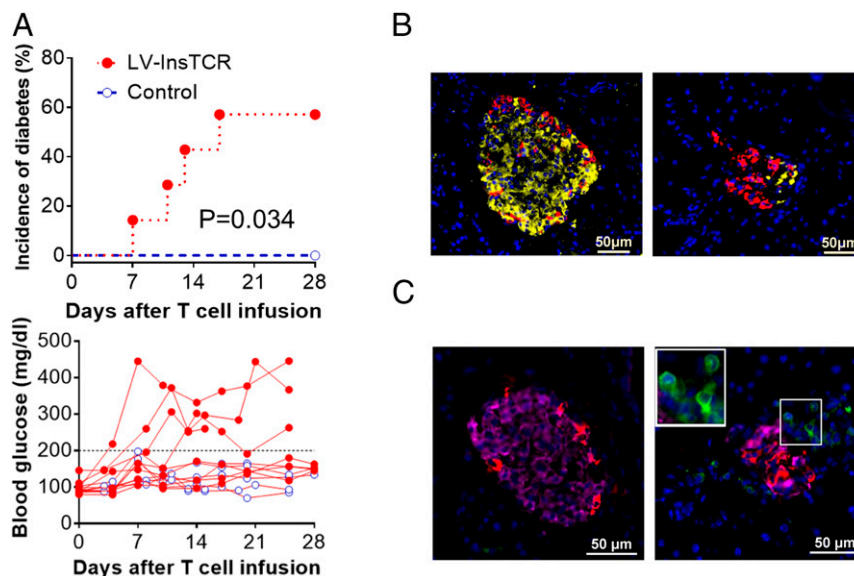
Our hu-mice model will allow investigation of the pathogenicity and recruitment of human islet autoreactive T-cells, as well as identify their potentially initiating or pathogenic target beta-cell autoantigens, rendering this model uniquely suited to investigate antigen-specific immunotherapy in T1D in preclinical models *in vivo* that hitherto was impossible with any other animal model.

## Materials and Methods

**Animals and Human Tissues and Cells.** The NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice and NOD.Cg-Prkdc<sup>scid</sup> H2-Ab1<sup>tm1Doi</sup> Tg(HLA-DQA1,HLA-DQB1) 1Dv/SzJ (HLA-DQ8-transgenic NOD/SCID) mice were purchased from the Jackson Laboratory. HLA-DQ8-transgenic NSG mice were generated by crossing HLA-DQ8-Tg NOD/SCID mice with NSG mice. All mice were housed in a specific pathogen-free microisolator environment and used between 6 and 12 wk of age. Human FTHY and liver tissues of gestational age of 17–21 wk were obtained from Advanced Bioscience Resource. J.RT3-T3.5 cell line, a TCR $\beta$  chain-deficient human T-cell line derived from the E6-1 clone of Jurkat cells that does not express CD3 or TCR $\alpha\beta$  heterodimers on the surface (34), was purchased from American Type Culture Collection (ATCC TIB-153). Protocols involving the use of discarded human tissues and animals were approved by the Columbia University Medical Center Institutional Review Board and the Institutional Animal Care and Use Committee, respectively, and all experiments were performed in accordance with these protocols.

**Humanized Mouse Preparation.** Hu-mice were constructed as described in our previous studies (29, 35). Briefly, NSG and HLA-DQ8-Tg NOD/SCID or NSG mice were conditioned with sublethal (1.5 Gy) total body irradiation and received human CD34<sup>+</sup> FLCs ( $1 \times 10^5$ , *i.v.*) and a FTHY tissue fragment ( $\approx 1$  mm<sup>3</sup>; under the kidney capsule) from the same fetus.

**HLA-DQ8/InsB:9–23-Specific TCR Isolation and Lentiviral Vector Construction.** After informed consent, peripheral blood was drawn from an 18-y-old Caucasoid HLA-DQ8/8 homozygous man diagnosed with T1D at the age of 8 y, and a T-cell line (clone #5) was generated by stimulation with InsB6-22 (the epitope being identical in B:9–23, and the T cells cross-react with B:9–23) (11, 12). The TCR  $\alpha$  and  $\beta$  chain cDNA were extracted and linked by a P2A self-cleaving peptide gene (36). The TCR  $\alpha$ -P2A- $\beta$  gene fragment was then linked with a F2A-AcGFP (*aequorea coerulea* green fluorescent protein) gene fragment and then cloned into a lentiviral vector (LV-insTCR; Fig. S1). Pseudotyped lentiviruses were produced by transfection, using Lipofectamine



**Fig. 4.** Induction of diabetes in HLA-DQ8-Tg hu-mice by adoptive transfer of autologous diabetogenic human CD4<sup>+</sup> T cells. HLA-DQ8-Tg hu-mice were treated with low-dose streptozotocin and injected 1–2 d later with  $5 \times 10^6$  expanded LV-insTCR-transduced or control (i.e., the same hu-mouse-derived human CD4<sup>+</sup> T cells that were similarly expanded in vitro as the LV-insTCR-transduced CD4<sup>+</sup> T cells) human CD4<sup>+</sup> T cells, followed 1 d later by immunization with InsB:9–23 peptides. (A) Cumulative incidence of diabetes (Top) and levels of blood glucose (Bottom) in hu-mice receiving LV-insTCR-transduced (solid symbol;  $n = 7$ ) or control (opened symbol;  $n = 6$ ) human T cells. Mice were defined as hyperglycemia if two consecutive blood glucose measurements  $>200$  mg/dL (B and C) Immunofluorescent staining of pancreas samples prepared between 3 and 4 wk after injection of CD4<sup>+</sup> T cells from hu-mice receiving control (Left) or LV-InsTCR-transduced (Right) human T cells ( $n = 3$  per group). (B) Staining of mouse insulin (yellow) and glucagon (red). (C) Staining of human CD3<sup>+</sup> cells (green), mouse insulin (pink) and glucagon (red). Nuclear is stained blue by DAPI.

2000 (Invitrogen) of 293T cells with a four-plasmid system consisting of the transfer vector (LV-insTCR) and three packaging plasmids (pMD2.G, pMDLg/pRRE, and pRSV-Rev). The supernatant with viral particles was collected 48 and 72 h posttransfection and concentrated by ultracentrifugation at  $50,000 \times g$  for 2 h. Lentiviruses were stored at  $-80^\circ\text{C}$  until use.

**Flow Cytometric Analysis.** Levels of human hematopoietic cells in humanized mice were determined by FCM analysis of PBMCs and splenic cells, using various combinations of the following monoclonal antibodies: anti-human CD45, CD19, CD3, CD4, CD8, CD45RA, CD45RO; anti-mouse CD45 and Ter119; and corresponding isotype controls (all purchased from Biolegend). Human T cells transduced with B:9–23-specific TCR (using  $V_{\alpha 21}$  and  $V_{\beta 11}$ ) were identified by staining with anti-human TCR $V_{\beta 11}$ -FITC (mouse IgG2a; Beckman Coulter) followed by anti-mouse IgG2a-PE secondary mAb (Biolegend). The secondary anti-mouse IgG2a-PE was required to distinguish between GFP-positive cells with and without TCR $V_{\beta 11}$  expression, as all virally transduced cells are expected to express AcGFP (Fig. S1). To detect human immune cell infiltration in pancreatic islets, islets were prepared and dissociated with trypsin and stained with anti-human CD45, CD3, CD19, CD11c, HLA-DR, and anti-mouse CD45. Samples were collected on a FACS LSR II (Becton Dickinson) and analyzed with FlowJo software (TreeStar). Dead cells were excluded from the analysis by gating out DAPI-positive cells.

**Lentiviral Transduction of Human CD4<sup>+</sup> T Cells from Humanized Mice.** Hu-mouse spleen cells enriched for human CD4<sup>+</sup>-naive T cells were prepared by negative separation using magnetic-activated cell sorting (MACS; Miltenyi Biotec). Briefly, spleen cells were stained with PE-conjugated mAbs against mouse Ter119, mouse CD45, huCD14, huCD19, huCD8, huCD45RO, and huCD25, followed by incubation with anti-PE magnetic beads (Miltenyi) and MACS column purification. The purified human CD4<sup>+</sup>-naive T cells were stimulated for 3.5 d in medium containing human T-Activator CD3/CD28 Dynabeads (Gibco Life Technologies), followed by incubation with lentiviruses in 96-well plates pre-coated with retronectin (Takara Bio Inc) for 24 h at a multiplicity of infection of 40. Cells were washed twice and expanded for 10–14 d in vitro in T-cell expansion medium (RPMI 1640 supplemented with 10% human AB serum), irradiated feeder cells, recombinant human cytokines (20 U/mL IL-2, 10 ng/mL IL-7, and 10 ng/mL IL-15; R&D), plus PHA (1.5  $\mu\text{g}/\text{mL}$ , Cat# L1668-5 mg; Sigma) or anti-CD3 mAb (OKT3; 30 ng/mL, Cat# NC9195482; Thermo Fisher). Feeder cells were 35-Gy-irradiated pooled allogeneic PBMCs ( $3 \times 10^6/\text{mL}$ ) and 60-Gy-irradiated Epstein-Barr virus-transformed lymphoblastoid cell lines

( $1.5 \times 10^5/\text{mL}$ ). The expanded cells were injected into hu-mice immediately in efforts to induce diabetes.

**Induction of Diabetes in HLA-DQ8-Tg Hu-Mice.** The HLA-DQ8-Tg hu-mice on the NOD/SCID or NSG background were conditioned with two successive low doses of streptozotocin (50 mg/kg/d  $\times$  2 d, i.p.; Sigma) starting 1 or 2 d before adoptive transfer of lentivirally transduced human CD4<sup>+</sup> T cells derived from preestablished hu-mice with an autologous (same fetal tissue donor) immune system (i.e., hu-mice made with the same fetal tissues as the adoptive recipient hu-mice). In some experiments, the HLA-DQ8-Tg hu-mice were also immunized with 100  $\mu\text{L}$  (100  $\mu\text{g}$ ) InsB:9–23 peptide (SHLVEA-LYLVCGERG; AnaSpec, Inc.) in 100  $\mu\text{L}$  Freud's Complete Adjuvant (InvivoGen) by s.c. injection 1 d after human CD4<sup>+</sup> T-cell transfer. Blood glucose levels were monitored twice a week, using FreeStyle blood glucose test strips and a blood glucose meter (Abbott Diabetes Care Inc.). Mice were considered diabetic after two consecutive blood glucose measurements  $>200$  mg/dL (37).

**Immunofluorescent Staining of Pancreatic Tissues.** Pancreata were harvested from the hu-mouse recipients between 3 and 4 wk after injection of CD4<sup>+</sup> T cells, and fixed in 4% paraformaldehyde for histological analysis. Briefly, frozen sections were prepared and stained with anti-insulin (guinea pig IgG; Dako), anti-glucagon (rabbit IgG; Sigma), anti-human CD3-Alexa Fluor 488 (Biolegend), followed by staining with donkey anti-guinea pig IgG-Cy5 (H+L; Jackson ImmunoResearch) and donkey anti-rabbit IgG-Dylight (H+L; Jackson ImmunoResearch). All sections were mounted in Vectorshield with DAPI (Vector Laboratories). Images were obtained using Leica DMI 6000B wide-field microscope.

**Statistical Analyses.** The log-rank (Mantel-Cox) test was used to analyze the difference in the incidence of diabetes between groups. A  $P$  value of  $\leq 0.05$  was considered significant.

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