

HLA-A2–Matched Peripheral Blood Mononuclear Cells From Type 1 Diabetic Patients, but Not Nondiabetic Donors, Transfer Insulinitis to NOD-*scid*/ γ ^{null}/HLA-A2 Transgenic Mice Concurrent With the Expansion of Islet-Specific CD8⁺ T cells

Fatima Whitfield-Larry,¹ Ellen F. Young,¹ Garrick Talmage,¹ Elizabeth Fudge,² Anita Azam,² Shipra Patel,² Joseph Largay,² Warren Byrd,² John Buse,² Ali S. Calikoglu,² Leonard D. Shultz,³ and Jeffrey A. Frelinger¹

OBJECTIVE—Type 1 diabetes is an autoimmune disease characterized by the destruction of insulin-producing β -cells. NOD mice provide a useful tool for understanding disease pathogenesis and progression. Although much has been learned from studies with NOD mice, increased understanding of human type 1 diabetes can be gained by evaluating the pathogenic potential of human diabetogenic effector cells in vivo. Therefore, our objective in this study was to develop a small-animal model using human effector cells to study type 1 diabetes.

RESEARCH DESIGN AND METHODS—We adoptively transferred HLA-A2–matched peripheral blood mononuclear cells (PBMCs) from type 1 diabetic patients and nondiabetic control subjects into transgenic NOD-*scid*/ γ ^{null}/HLA-A*0201 (NOD-*scid*/ γ ^{null}/A2) mice. At various times after adoptive transfer, we determined the ability of these mice to support the survival and proliferation of the human lymphoid cells. Human lymphocytes were isolated and assessed from the blood, spleen, pancreatic lymph node and islets of NOD-*scid*/ γ ^{null}/A2 mice after transfer.

RESULTS—Human T and B cells proliferate and survive for at least 6 weeks and were recovered from the blood, spleen, draining pancreatic lymph node, and most importantly, islets of NOD-*scid*/ γ ^{null}/A2 mice. Lymphocytes from type 1 diabetic patients preferentially infiltrate the islets of NOD-*scid*/ γ ^{null}/A2 mice. In contrast, PBMCs from nondiabetic HLA-A2–matched donors showed significantly less islet infiltration. Moreover, in mice that received PBMCs from type 1 diabetic patients, we identified epitope-specific CD8⁺ T cells among the islet infiltrates.

CONCLUSIONS—We show that insulinitis is transferred to NOD-*scid*/ γ ^{null}/A2 mice that received HLA-A2–matched PBMCs from type 1 diabetic patients. In addition, many of the infiltrating CD8⁺ T cells are epitope-specific and produce interferon- γ after in vitro

peptide stimulation. This indicates that NOD-*scid*/ γ ^{null}/A2 mice transferred with HLA-A2–matched PBMCs from type 1 diabetic patients may serve as a useful tool for studying epitope-specific T-cell-mediated responses in patients with type 1 diabetes. *Diabetes* 60:1726–1733, 2011

NOD mice develop spontaneous diabetes due to autoimmune destruction of pancreatic β -cells. These mice have served as a useful tool for understanding many aspects of type 1 diabetes (1,2). For example, the identification of certain pathogenic epitopes were originally found in NOD mice and subsequently observed in the blood of type 1 diabetic patients (3). The major shortcoming of these studies is their inability to evaluate the human autoreactive effector cells directly. Researchers have identified a number of pathogenic autoreactive epitopes of CD4⁺ and CD8⁺ T cells that recognize and result in β -cell killing. Epitopes for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), insulin, and preproinsulin have been identified in the islets of NOD mice (4). T cells specific for epitopes from these proteins and other islet proteins, including islet amyloid polypeptide (IAPP), have been found in the blood of type 1 diabetic patients (5). At the same time, however, many of the findings in NOD mice have not directly translated to human type 1 diabetes. Importantly, a large number of therapies appear to “cure” diabetes in NOD mice, but these therapies have not readily translated to humans. Anti-CD3 antibody therapy, which is extremely effective in NOD mice (6,7), is less effective in patients (8,9). These immunomodulatory therapies still leave concerns about their effects, as discussed by Santamaria (10). Other immune therapies aimed at targeting B cells, such as anti-CD20 monoclonal antibody treatment, also offer short-term CD19⁺ B-cell depletion and partially preserve β -cell function (11).

Development of humanized mice in which HLA-matched peripheral blood mononuclear cells (PBMCs) from type 1 diabetic patients are adoptively transferred into immune-deficient mice would provide a means of studying human immune cells without the restrictions inherent to human studies (12). Specifically, it would be possible to identify human autoreactive epitope-specific T cells that infiltrate the islets directly ex vivo. A small-animal model that recapitulates the clinical manifestations of type 1 diabetes would also assist in identifying novel therapeutic targets

From the ¹Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; the ²Division of Endocrinology, Departments of Medicine and Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and ³The Jackson Laboratory, Bar Harbor, Maine.

Corresponding author: Fatima Whitfield-Larry, fwhit@email.unc.edu.

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F.W.-L. is currently affiliated with the Division of Endocrinology, Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

J.A.F. is currently affiliated with the Department of Immunobiology, University of Arizona, Tucson, Arizona.

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and in developing and testing novel immunotherapeutic agents. Furthermore, we would be able to investigate the mechanisms involved in disease pathogenesis of many other autoimmune diseases, especially those with disease-associated epitopes, shared between humans and mice (13,14).

During the past several years, many investigators have used human hematopoietic stem cells (HSCs) for engraftment into immunodeficient mice (15–17). These studies have attempted to develop a complete human immune system in a murine host. In many cases, successful engraftment and cell differentiation was observed. Most recently, investigators showed that functional human CD4⁺ and CD8⁺ T cells developed after being transferred into immune-deficient HLA transgenic mice and that these T cells demonstrated HLA-restricted responses (18). In contrast, our goal was not to recapitulate the entire autoimmune process; rather, we sought to develop a humanized mouse model that would be useful for identifying pre-existing autoreactive diabetogenic circulating T cells from type 1 diabetic patients that are important in the direct pathogenesis of type 1 diabetes.

In NOD mice, β -cell-specific CD8⁺ T-cell clones are found in the peripheral blood and pancreatic islets (19). In patients with type 1 diabetes, epitope-specific T cells display T-lymphocyte cytotoxic activity toward human β -cells (20). Humans and mice also share many of the protein sequences of identified epitopes (21). Therefore, because it is likely that lymphocytes that have been exposed to islet antigens circulate within the blood of patients with type 1 diabetes, we adoptively transferred peripheral blood mononuclear cells (PBMCs) from HLA-A2-matched type 1 diabetic patients into transgenic NOD-*scid*/ γ c^{null}/HLA-A*0201 (NOD-*scid*/ γ c^{null}/A2) mice (Table 1). NOD-*scid*/ γ c^{null} mice provide a lymphopenic environment that supports the survival of human cells. We

used mice expressing a full-length human *HLA* gene to allow engagement of the human CD8 expressed on the transferred cells with exogenous HLA on the mouse cells. Engagement of CD8 enhances both recognition and survival of CD8 T cells (22).

After transfer of human PBMCs, we were able to show engraftment and islet cell infiltration of the PBMCs from the type 1 diabetic donors. Further, the islet-infiltrating cells were enriched in T cells that recognize diabetogenic epitopes and underwent additional expansion after transfer to a secondary recipient.

RESEARCH DESIGN AND METHODS

Animals. Mice were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care at the University of North Carolina (UNC) Animal Facility and handled according to the UNC Office of Animal Care and Use. All experimentation was approved by the UNC Institutional Animal Care and Use Committee. The original breeding pairs of NOD.Cg-*Prkdc*^{scid} *Il2* γ ^{m1W^{fl}} Tg(HLA-A2.1)Enge/Sz and NOD.Cg-*Prkdc*^{scid} *Emv30P*. Tg(HLA-A/H2D/B2M)1Dvs/DvsJ mice were previously described (23,24). Animals were housed in autoclaved microisolator cages and fed autoclaved food and chlorinated water. Mice were used at 8–14 weeks old and defined as diabetic if two consecutive weekly blood glucose measurements were ≥ 250 mg/dL.

PBMCs. All protocols involving the collection and use of PBMCs were approved by the UNC Office of Human Research Ethics and conducted according to Institutional Review Board Approval of Human Subjects Research.

Peripheral blood from patients diagnosed with type 1 diabetes or nondiabetic donors (NDD) was collected under sterile conditions in heparinized tubes. PBMCs were isolated on Ficoll-Hypaque. After isolation and purification, cells were tested for expression of HLA-A2 by flow cytometry using fluorescein isothiocyanate-labeled BB7.2 monoclonal antibody (Abcam, Cambridge, MA). HLA-A2⁺ PBMCs from type 1 diabetic patients or NDDs were adoptively transferred into mice.

Isolation of human lymphocytes from mice. NOD-*scid*/ γ c^{null}/A2 mice were intraperitoneally injected with $17\text{--}52 \times 10^6$ HLA-A2⁺ PBMCs in 400 μ L of PBS from type 1 diabetic patients or NDD. Spleen, PLN, blood, and islets were removed from recipient mice 2 to 6 weeks after transfer. Islets were isolated as previously described (25). Briefly, pancreata were perfused with a 2 mg/mL solution of collagenase P (Sigma-Aldrich, St. Louis, MO), dissected, and incubated at 37°C for 20 min. Islets were purified using a Ficoll PM 400 (Sigma-Aldrich) gradient, handpicked, counted, and then cultured overnight in RPMI-1640 containing 10% FBS and 4 ng/mL recombinant human interleukin (IL)-2 (Peprotech, Rocky Hill, NJ). Infiltrating lymphocytes were collected and filtered through a 40- μ m nylon filter. Each sample constituted all of the islet cells isolated from an individual mouse. Human cell populations were obtained by gating on human CD45⁺/mouse CD45⁻. Cell suspensions were stained using fluorescently-labeled mouse antibodies to human surface markers: CD45 (PerCp), CD19 (PeCy7), CD8 (PB), CD3 (antigen-presenting cell [APC]), and CD4 (APC-Cy7) (ebioscience, San Diego, CA), or CD3 (PO), CD3 (PTR), and CD4 (PO) (Invitrogen, Carlsbad, CA). Samples were analyzed on a Beckman-Coulter (Dako, Glostrup, Denmark) CyAn ADP using Summit V4.3.01 software. Mice that had $>2 \times 10^6$ human CD45⁺ cells in their spleen were included in our analysis.

Peptides and tetramers. Peptides IGRP_{265–273} (VLFGLGFAD), IA-2_{797–805} (MVWESGCTV), IAPP_{5–13} (KLQVFLIVL), and insulin_{B10–18} (HLVEALYLV) were purchased from GenScript (Piscataway, NJ) at $>90\%$ purity, dissolved in DMSO, and stored at -20°C . A2/IGRP, A2/IA-2, A2/IAPP, and A2/insulin tetramers were assembled with these peptides as described (26). Briefly, HLA-A2 and human β_2 -microglobulin were produced in *Escherichia coli* and refolded with peptide in vitro. Refolded peptide major histocompatibility complex (MHC) monomer was purified by high-performance liquid chromatography, biotinylated using Biotin Protein Ligase, and assembled into tetramers by conjugation with UltraAvidin-PE (Leinco, St. Louis, MO).

Interferon- γ production assay. Cells ($2\text{--}3 \times 10^6$) were incubated overnight in complete RPMI-1640 containing 10% FBS and 4 ng/mL recombinant human IL-2 (Peprotech). After 24 h, the cells were incubated with IGRP, IA-2, IAPP, and insulin peptide pool or individually at 40 μ g/mL of each peptide. A2/IGRP, A2/IA-2, A2/IAPP, and A2/insulin PE-conjugated tetramers (2.5 μ L each) along with anti-CD28 (1 μ g/mL) was added. Cells were incubated at 37°C, 5% CO₂ for 2 h. Brefeldin A was added to inhibit protein secretion, followed by an additional 4-h incubation, placed on ice, stained with fluorescently labeled surface antibodies, fixed, permeabilized, and stained with human anti-interferon (IFN)- γ monoclonal antibody (ebioscience).

TABLE 1
Type 1 diabetic and nondiabetic haplotype-matched PBMC donors used in transfer experiments*

Patient ID	Age	Sex	Diabetes duration (years)
Type 1 diabetes (<i>n</i> = 10)			
A1	Adult	F	48
A19	Adult	F	41
A21	Adult	M	22
A25	Adult	M	12
A27	Adult	M	12
A31	Adult	M	25
A33	Adult	M	12
A37	Adult	M	22
H82	Child	F	5
H96	Child	F	13
NDD (<i>n</i> = 9)			
NDD1	Adult	M	NA
NDD2	Adult	M	NA
NDD3	Adult	F	NA
NDD4	Adult	F	NA
NDD5	Adult	M	NA
NDD6	Adult	F	NA
NDD7	Adult	M	NA
NDD8	Adult	F	NA
NDD9	Adult	M	NA

ID, identification; NA, not applicable. *All PBMC donors were HLA-A2 haplotype.

RESULTS

PBMCs from NDD and type 1 diabetic patients engraft in NOD-*scid*/ γ ^{null}/A2 mice. Previous studies using human HSCs have shown NOD-*scid*/ γ ^{null} mice support growth, survival, and differentiation of CD34⁺ HSC (17). HLA-A2*0201 is the most commonly expressed HLA class I allele in Europeans (45%) and is permissive for the development of type 1 diabetes in humans and transgenic mice. Therefore, we evaluated the ability of HLA-A2-matched PBMCs from NDD and type 1 diabetic patients to successfully engraft in NOD-*scid*/ γ ^{null}/A2 mice. Flow cytometry analyses confirmed the presence of human CD45⁺ cells in the spleen of mice receiving PBMCs from NDD or type 1 diabetic patients (Fig. 1A–C). Human CD45⁺ cells were also found in the blood and PLN of NOD-*scid*/ γ ^{null}/A2 mice (Fig. 1D and F), albeit at lower levels. The number of human CD45⁺ cells in both groups of mice that received NDD or type 1 diabetic PBMCs appear to be similar in the spleen ($P = 0.206$, Fig. 1G) and PLN ($P = 0.3177$, Fig. 1H). Therefore, we determined that both NDD and type 1 diabetic patient PBMCs survive and home to the spleen (primary site of engraftment) and PLN of NOD-*scid*/ γ ^{null}/A2 mice after transfer. Our data

are consistent with previously reported findings that the primary site of engraftment by human cells in NOD-*scid*/ γ ^{null} mice is the spleen (27). Interestingly, the level of engraftment appears to increase markedly in mice receiving $>20 \times 10^6$ PBMCs from patients with type 1 diabetes. Successful engraftment was defined as recovering $\geq 2 \times 10^6$ human CD45⁺ cells from the spleen of mice that received PBMCs. In our studies, transfer of $\sim 30 \times 10^6$ PBMCs allows for successful engraftment. This demonstrates the ability of NOD-*scid*/A2/ γ ^{null} mice to support survival of PBMCs from patients with type 1 diabetes after transfer.

PBMCs from type 1 diabetic donors proliferate in NOD-*scid*/ γ ^{null}/A2 mice. To evaluate proliferation potential of type 1 diabetic PBMCs in NOD-*scid*/ γ ^{null}/A2 mice, we labeled PBMCs with carboxyfluorescein succinimidyl ester (CFSE) and confirmed the presence of human CD45⁺ cells in the peripheral blood, spleen, PLN, and islets of mice after transfer. Human CD45⁺ cells reconstituted in all compartments tested. Most of the human CD45⁺ cells in the peripheral blood, spleen, PLN, and islets of NOD-*scid*/ γ ^{null}/A2 mice were CFSE low, implying that many of cells underwent at least five rounds of cell division (Fig. 2A–L).

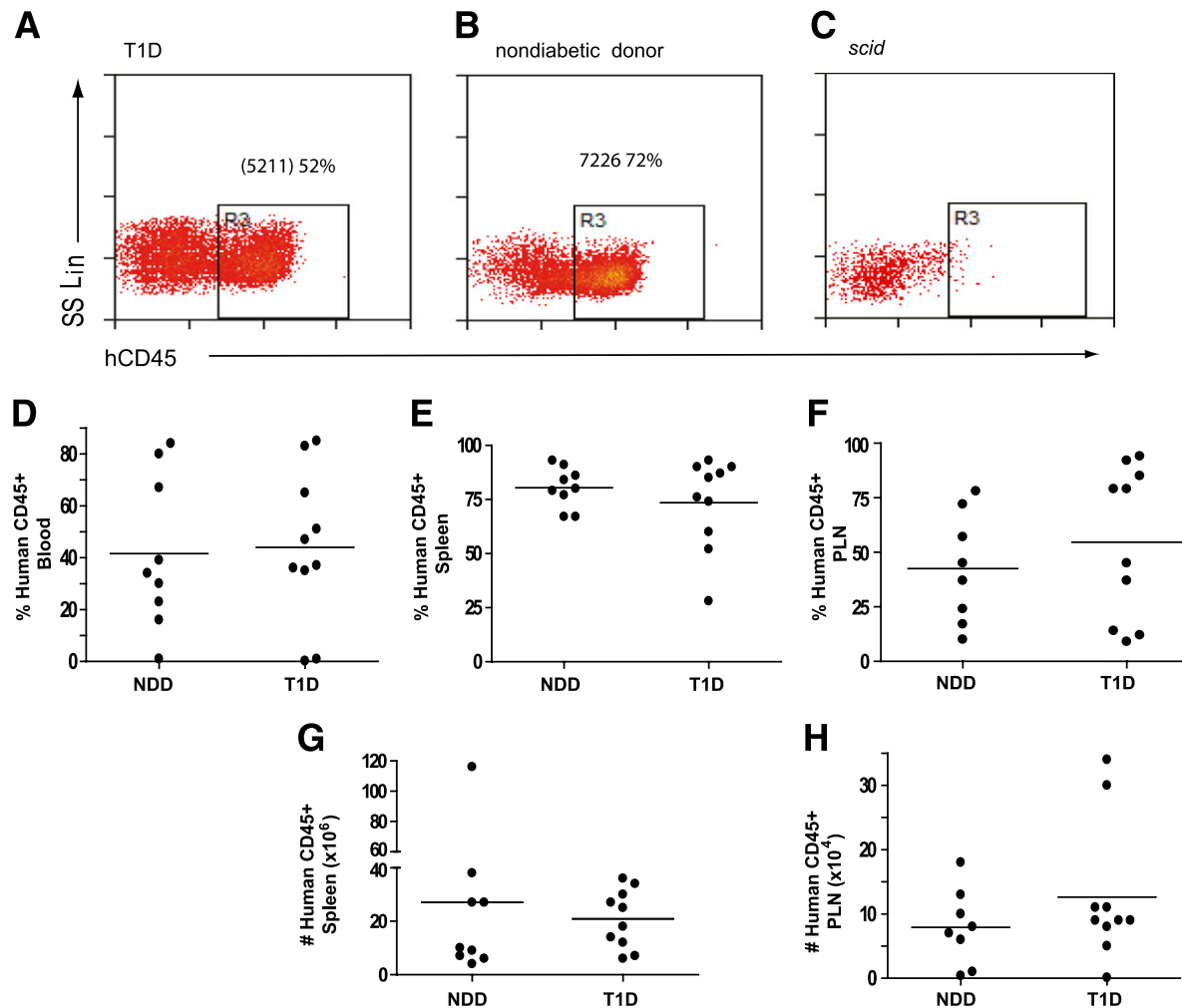


FIG. 1. Human cells from type 1 diabetic (T1D) patients and NDDs engraft in NOD-*scid*/ γ ^{null}/A2 mice. Representative histograms of NOD-*scid*/ γ ^{null}/A2 splenocytes after intraperitoneal injection of PBMCs from type 1 diabetic patients (A), NDDs (B), or scid (C). Cells were gated on human CD45⁺. At 2 to 6 weeks after transfer, summary of the percentages of human CD45⁺ cells in the blood (NDDs, $n = 9$; type 1 diabetes, $n = 10$) (D), spleen (NDD, $n = 9$; type 1 diabetes, $n = 10$) (E), and draining PLN (NDD, $n = 8$; type 1 diabetes, $n = 10$) (F) were determined by flow cytometry. The number of human CD45⁺ cells in the spleen (NDD, $n = 9$; type 1 diabetes, $n = 10$) (G) and draining PLN (NDD, $n = 8$; type 1 diabetes, $n = 10$) (H) was also evaluated. (A high-quality color representation of this figure is available in the online issue.)

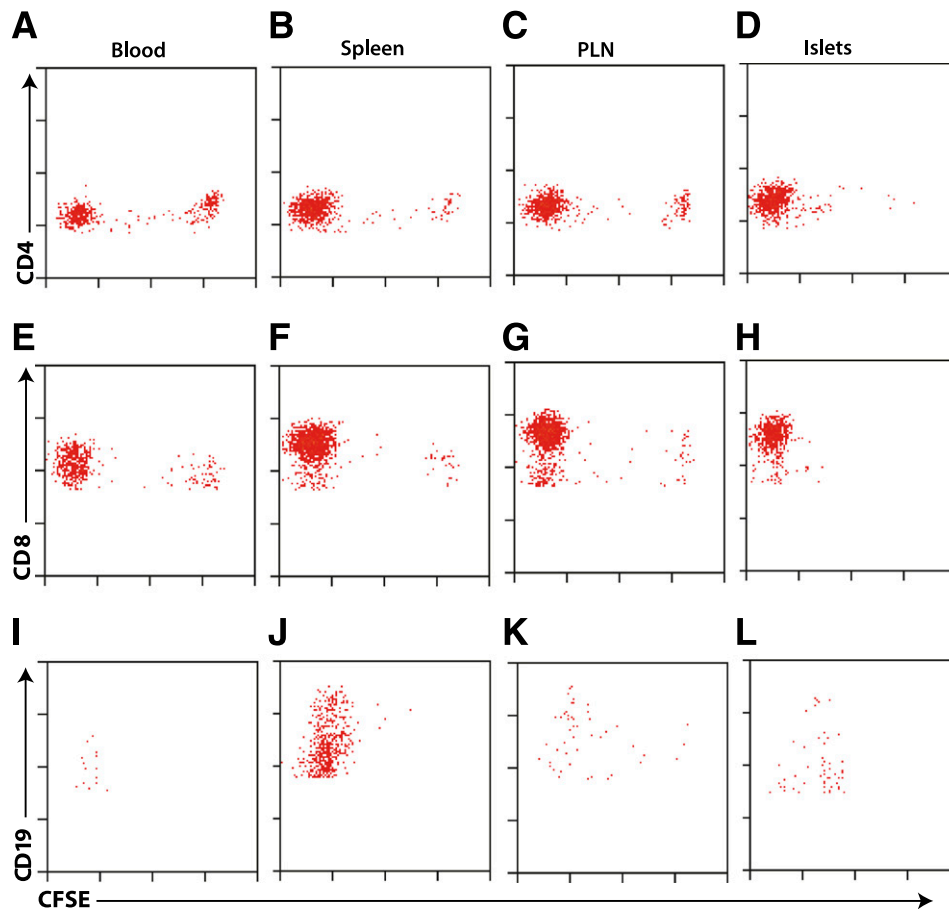


FIG. 2. Proliferation of human lymphocytes from type 1 diabetic patients in NOD-*scid*/ γ ^{null}/A2 mice is shown 2 weeks after transfer. PBMCs from patients with type 1 diabetes were labeled with CFSE and transferred intraperitoneally into 8- to 14-week-old NOD-*scid*/ γ ^{null}/A2 mice. At 2, 3, and 6 weeks after transfer of PBMCs, the proliferation of CD4⁺ (top row), CD8⁺ (middle row), and CD19⁺ (bottom row) cells was determined by CFSE dye dilution in cells from the blood (A, E, and I), spleen (B, F, and J), draining PLN (C, G, and K), and islets (D, H, and L) using flow cytometry. (A high-quality color representation of this figure is available in the online issue.)

Human T and B cells are present in the spleen and PLN of NOD-*scid*/ γ ^{null}/A2 mice after transfer. Human cells were observed in the spleen and PLN after adoptive transfer in NOD-*scid*/ γ ^{null}/A2 mice. CD3⁺ T cells and CD19⁺ B cells were observed in the spleen and PLN (Fig. 3). In fact, the predominant human lymphocyte subset in the spleen and PLN of mice was CD3⁺ T cells. CD3⁺ T cells in the spleen of mice receiving NDD or type 1 diabetic PBMCs were $97 \pm 1.8\%$ and $82 \pm 17.4\%$, respectively, of the human CD45⁺ cell population (Supplementary Fig. 1A). The level of CD3⁺ T cells in the spleen and PLN was similar in mice receiving cells from NDDs or type 1 diabetic patients (Fig. 3A and B). The number of CD19⁺ B cells in the spleen and PLN was also similar in both groups of mice.

We compared the level of CD4⁺ and CD8⁺ T cells in the spleen and PLN after transfer. Cells were gated from hCD45⁺/CD3⁺ populations. The number (Fig. 3C and D) and percentage (Supplementary Fig. 1C and D) of CD4⁺ and CD8⁺ T cells in the spleen and PLN was similar between both groups. In addition, both groups of mice had similar CD4/CD8 ratios in the spleen (Supplementary Fig. 1E) and PLN (Supplementary Fig. 1F) after transfer.

PBMCs from type 1 diabetic donors infiltrate the islets of NOD-*scid*/ γ ^{null}/A2 mice at a higher frequency than PBMCs from NDD. We determined the level of islet infiltration in NOD-*scid*/ γ ^{null}/A2 mice receiving PBMCs

from NDD or type 1 diabetic patients. We isolated islets from individual mice after engraftment and determined the presence of cellular infiltrates. The total number of human CD45⁺ cells was significantly higher in mice that received type 1 diabetic PBMCs ($P = 0.0006$, Fig. 4A). Among these infiltrates, although not widely appreciated, B cells are found in the islets of both diabetic mice (25) and humans (28). The level of CD3⁺ T cells was significantly higher in mice that received type 1 diabetic PBMCs ($P = 0.0238$, Fig. 4B), whereas the level of CD19⁺ B cells was similar ($P = 0.1310$, Fig. 4B). The percentage of CD3⁺ T cells and CD19⁺ B cells was similar between the two groups (Supplementary Fig. 1G).

On a per-islet basis, the number of human CD45⁺ cells ($P = 0.0006$, Fig. 4C) and CD3⁺ T cells ($P = 0.0175$, Fig. 4D) was also increased. The level of CD19⁺ B cells ($P = 0.0256$, Fig. 4E) was also significantly increased. This suggests that although the percentages of infiltrating lymphocytes are similar, the actual numbers of T and B cells are increased in mice that received PBMCs from type 1 diabetic patients. **Human CD4 and CD8 T cells are present in the islets of NOD-*scid*/ γ ^{null}/A2 mice after transfer.** We determined whether CD4⁺ and CD8⁺ T cells were present in the islets of mice after transfer because both are required for the development of type 1 diabetes in mice and the issue cannot be tested in humans. The number of CD4⁺

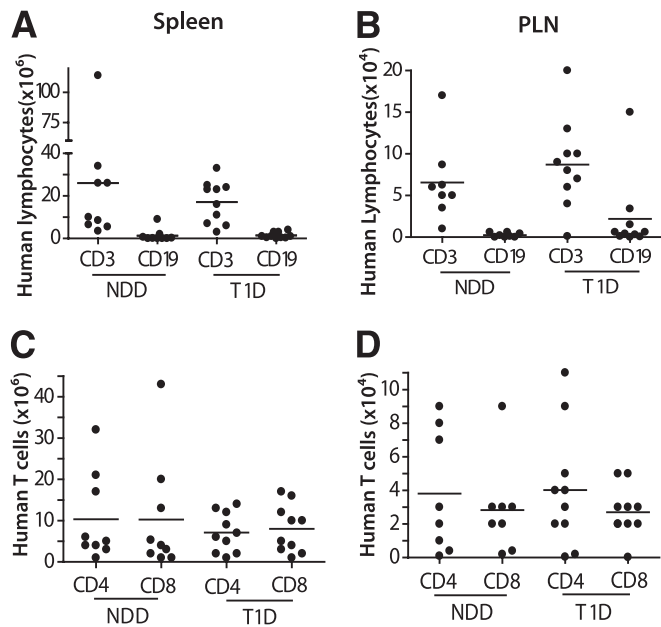


FIG. 3. Human T and B cells were recovered from the spleen and draining PLN of NOD-*scid*/ γ c^{null}/A2 mice after transfer. Beginning at 2 weeks after transfer, flow cytometry was used to determine the number of human CD3⁺ T cells and CD19⁺ B cells in the spleen (A) and PLN (B) of mice that received PBMCs from type 1 diabetic (T1D) patients or NDDs. Cells were gated on human CD45⁺ lymphocytes. The number of CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells were calculated in the spleen (C) and draining PLN (D).

T cells per islet was significantly higher in mice that received type 1 diabetic PBMCs compared with NDD PBMCs ($P = 0.0175$, Fig. 4F). Although the level was not statistically significant, CD8⁺ T cells were also increased in mice that received type 1 diabetic PBMCs ($P = 0.1820$, Fig. 4G). This shows that a higher number of CD4⁺ and CD8⁺ T cells from the PBMCs of type 1 diabetic patients infiltrate the islets of NOD-*scid*/ γ c^{null}/A2 mice compared with NDDs. Interestingly, the CD4/CD8 ratio was higher in mice that received PBMCs from type 1 diabetic patients (Supplementary Fig. 1I). **IGRP, IAPP, insulin, and IA-2-specific CD8⁺ T cells can be found in NOD-*scid*/ γ c^{null}/A2 mice transferred with PBMCs from type 1 diabetic patients.** The significant advantage of this transfer model is the potential to identify antigen-specific CD8⁺ T cells, which are important for the development of diabetes in patients with type 1 diabetes. To understand the specificity of T cells in the islets of NOD-*scid*/ γ c^{null}/A2 mice transferred with PBMCs from type 1 diabetic patients, we used IGRP, IAPP, insulin, and IA-2/A2 tetramers to identify epitope-specific T cells. Lymphocytes from the spleen and islets of NOD-*scid*/ γ c^{null}/A2 mice were incubated with pooled HLA-A2 tetramers individually loaded with IGRP, IAPP, insulin, and IA-2 peptides after transfer to maximize our sensitivity of detection. Figure 5A shows the number of tetramer⁺ CD8⁺ T cells in the islets and spleen. In fact, the level of CD8⁺/tetramer⁺ T cells was significantly higher in the islets (17–80%) compared with the spleen (5–24%) of mice engrafted with type 1 diabetic PBMCs. These results demonstrate that A2-epitope-specific CD8⁺ T cells primarily infiltrate the islets of mice that received type 1 diabetic PBMCs. Unfortunately, the sample size was not sufficient to examine the cells recovered from the islets for individual A2-tetramer specificity.

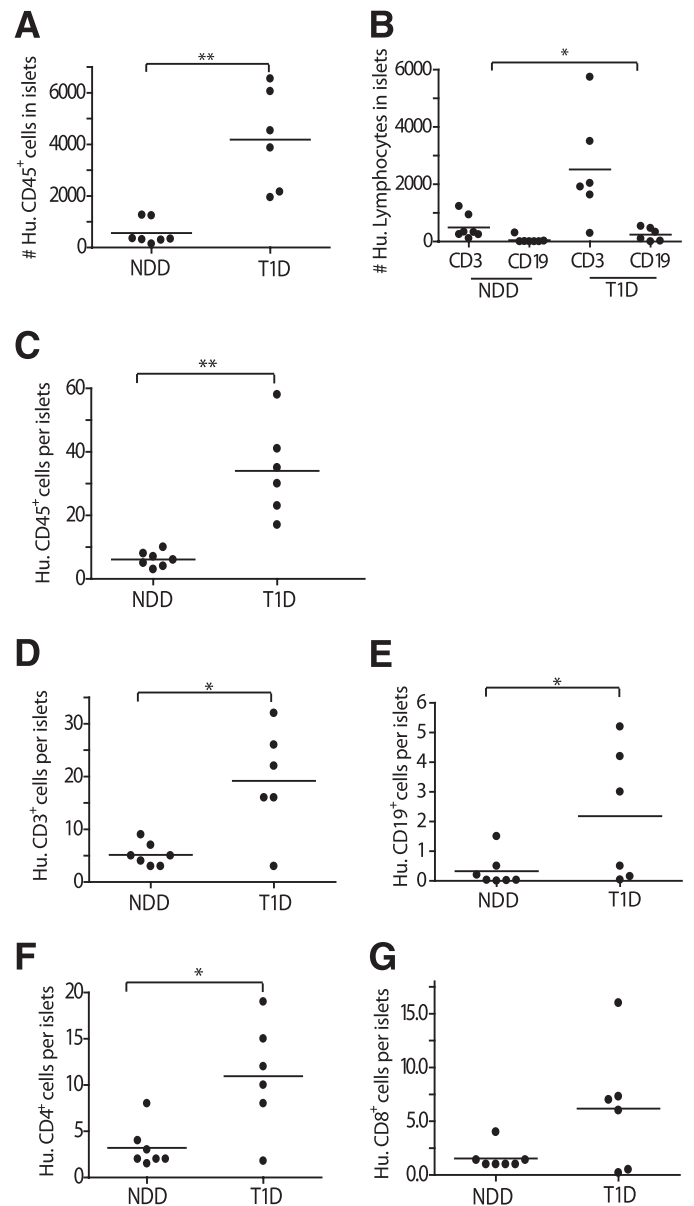


FIG. 4. Human T and B cells from patients with type 1 diabetes (T1D) infiltrate the islets of NOD-*scid*/ γ c^{null}/A2 mice after transfer. The total number (top) of CD45⁺ (A) and CD3⁺ (B) T and CD19⁺ B cells as well as the number of CD45⁺ (C), CD3⁺ (D), CD19⁺ (E), CD4⁺ (F), and CD8⁺ (G) cells per islet (bottom) was determined from the islets of individual mice at 2 to 6 weeks after transfer. Islets from individual mice were isolated as described in the RESEARCH DESIGN AND METHODS (NDDs, $n = 7$; type 1 diabetes, $n = 6$). * $P < 0.05$ and ** $P < 0.001$, one-tailed non-parametric Mann-Whitney test.

In vitro stimulation with A2-tetramer and peptide-induced IFN- γ production by antigen-specific human CD8⁺ T cells from NOD-*scid*/ γ c^{null}/A2 mice after transfer. To determine if epitope-specific human CD8⁺ T cells from the spleen and islets of mice after transfer are functionally active, cells were incubated with pooled A2 tetramers and their respective peptides and assessed for IFN- γ production after short-term stimulation. Figure 5A shows that CD8⁺/tetramer⁺ T cells are present in the spleen (12%) and islets (15%). In addition, these epitope-specific cells from the spleen and islets produce IFN- γ after peptide stimulation (Fig. 5B). These results demonstrate that the islets of NOD-*scid*/ γ c^{null}/A2 mice contained

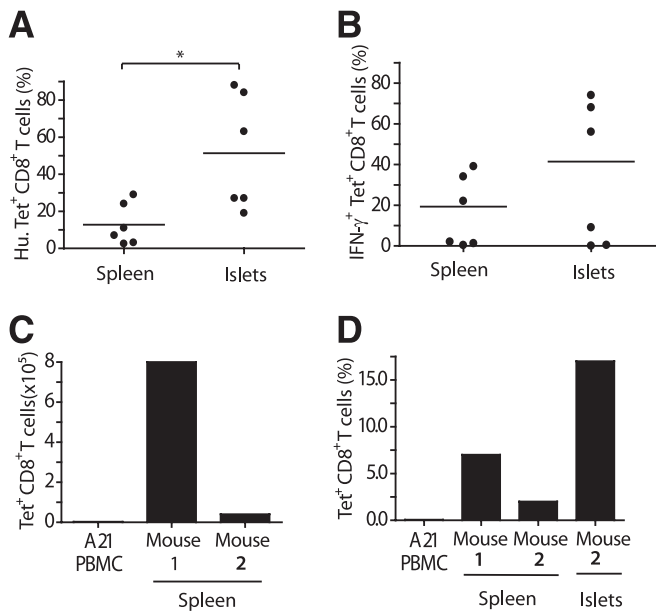


FIG. 5. A2-epitope-specific CD8⁺ T cells from type 1 diabetic patients in NOD-*scid*/ γ ^{null}/A2 mice. **A:** Percentages of CD8⁺/tetramer⁺ T cells in the spleen and islets of NOD-*scid*/ γ ^{null}/A2 mice engrafted with PBMCs from type 1 diabetic patients ($n = 6$). **B:** In vitro stimulation with A2 tetramers and peptides induced the production of IFN- γ from epitope-specific CD8⁺ T cells from the spleens and islets of NOD-*scid*/ γ ^{null}/A2 mice that received type 1 diabetic patient PBMCs ($n = 5$). Percentages (**C**) and numbers (**D**) of CD8⁺/tetramer⁺ T cells in the spleen of mouse 1 and mouse 2 compared with the amount in the peripheral blood of a type 1 diabetic patient. **D:** The percentages are shown of CD8⁺/tetramer⁺ T cells in the islets of mouse 2.

functional, human, epitope-specific CD8⁺ T cells that are capable of producing IFN- γ after restimulation. Although overt diabetes did not develop in any the mice transferred with PBMCs from patients with type 1 diabetes, we did observe by histology islet infiltration (Supplementary Fig. 2A) and elevated blood glucose levels up to 235 mg/dL (Supplementary Fig. 2D). Mice that received NDD or type 1 diabetic PBMCs displayed no clinical signs of graft-versus-host disease, as determined by histologic analysis of the liver and kidney (data not shown).

IGRP, IAPP, insulin, and IA-2-specific CD8 T cells survive after second passage into NOD-*scid*/ γ ^{null}/A2 mice. To determine whether epitope-specific T cells can continue to expand from one mouse to the next, we isolated splenocytes from a NOD-*scid*/ γ ^{null}/A2 mouse that received 20×10^6 type 1 diabetic PBMCs (mouse 1) and transferred 18×10^6 splenocytes intraperitoneally into a second NOD-*scid*/ γ ^{null}/A2 mouse (mouse 2). We compared the level of CD8⁺/tetramer⁺ T cells isolated from a patient with type 1 diabetes (A21) to 1) observe the level of epitope-specific CD8⁺ T cells after each transfer and 2) determine if they expanded. The number and percentage of CD8⁺/tetramer⁺ T cells increased from 3×10^3 to 7×10^5 cells and in percentage from 0.06 to 6.85% (Fig. 5C and D), in the spleen of the mouse transferred with type 1 diabetic PBMCs. In fact, these antigen-specific CD8⁺ T cells were not only in the spleen but also in the islets of mouse 2 (Fig. 5D), demonstrating the selective expansion of epitope-specific cells.

DISCUSSION

NOD-*scid*/ γ ^{null}/A2 mice have been widely used to study human immunology because these mice serve as a host for

human immune cells (13). In addition, immune-deficient mice are being used to study various human diseases, including graft-versus-host disease after the injection of MHC-mismatched human PBMCs (29) and also islet transplantation rejection by mismatching MHC class I molecules of donor PBMCs to recipient islet MHC class I molecules (12,30).

Our goal is to study human autoimmunity, specifically type 1 diabetes, using a humanized mouse model by transferring HLA-A2 matched PBMCs from type 1 diabetic patients and NDDs into NOD-*scid*/ γ ^{null}/A2 mice. We observed proliferation and survival of T cells (CD3⁺, CD4⁺, and CD8⁺) and B cells for at least 6 weeks in these mice. Similar levels of T cells in the spleen and PLN were found in mice engrafted with PBMCs from type 1 diabetic patients and NDDs; however, more B cells were found in the spleen and PLN in mice that received PBMCs from type 1 diabetic patients. This demonstrates the ability of human immune cell subsets to engraft secondary lymphoid organs after transfer. Moreover, the higher levels of B cells in the PLN and spleen may reflect what occurs in the PLN and spleen of type 1 diabetic patients. NOD-*scid* mice that express a chimeric *A2/K^b* gene and were wild-type at the IL2r- γ chain locus were poorly engrafted (data not shown).

Through this model we have revealed an increased level of islet infiltration in mice that received type 1 diabetic PBMCs compared with NDD PBMCs. Previous studies using NOD-*scid* mice and HLA-A1⁺ PBMCs from type 1 diabetic patients have demonstrated the presence of islet-reactive T cells in pancreatic tissue (31), but these T-cell clones were not capable of intraislet infiltration. We have shown here that T and B cells preferentially infiltrate the islets of NOD-*scid*/ γ ^{null}/A2 mice that received type 1 diabetic PBMCs and that a significant number of CD8⁺ T cells from the islets and spleen are specific for IGRP, IA-2, IAP, and insulin (known diabetogenic epitopes). These cells were found at a higher frequency in the islets compared with the spleen, indicating the islets are a preferred site of autoreactive T-cell expansion and accumulation. Therefore, the extravasation and accumulation of human autoreactive islet-specific T cells from circulation into the islets suggest that PBMCs from type 1 diabetic patients are capable of infiltrating the target organs necessary for the induction of diabetes.

Circulating cytotoxic T lymphocytes isolated from HLA-A2⁺ type 1 diabetic patients have been shown to kill β -cells through recognition of a glucose-regulated preproinsulin epitope (18). In our model, higher frequencies of diabetogenic epitope-specific CD8⁺ T cells from type 1 diabetic patients infiltrate the islets of NOD-*scid*/ γ ^{null}/A2 mice. Furthermore, these antigen specific cells are capable of producing IFN- γ after peptide stimulation.

Until now, no reported studies have demonstrated islet infiltration of NOD-*scid*/ γ ^{null}/A2 mice by antigen-specific T cells using HLA-A2-matched PBMCs from type 1 diabetic patients. This may be the result of poor engraftment in previous models that is often associated with natural killer cell activity or the lack of human costimulatory molecules such as human MHC molecules (32,33). We identified an engraftment threshold and determined that $20\text{--}30 \times 10^6$ PBMCs is ideal for maximum engraftment in NOD-*scid*/ γ ^{null}/A2 mice. This signifies a size limitation for transferring human cells into mice, which is similar to previous findings using NOD-*scid*/ γ ^{null} mice (34).

Although many genetic loci contribute to the development of diabetes, initial genome scans attribute particular

MHC class I and II alleles as major contributors to the development of autoreactive T-cell responses in both humans and NOD mice (35,36). The process of developing diabetes is accelerated in NOD HLA-A2.1 MHC class I transgenic mice and is mediated by pathogenic A2-restricted T-cell responses (37). In humans, two islet-associated epitopes, IA-2 and GAD65, have been identified as being recognized by HLA-A2-restricted CD8⁺ T cells, thus indicating that CD8⁺ T cells that are HLA-A2 restricted may contribute to β -cell death. Although it is not clear whether CD8⁺ T cells are involved during the early phases of type 1 diabetes, we do know that they are involved in the development of diabetes because diabetes does not develop in NOD mice lacking MHC class I. The importance of IGRP, IAPP, IA-2, and insulin-specific CD8⁺ T cells is still unknown in humans. However, GAD65 and IA-2 are major islet antigens targeted in human type 1 diabetes. The presence of an enriched population of epitope-specific CD8⁺ T cells in the islets after transfer may indicate the expansion of epitope-specific immunodominant T cells from patients with type 1 diabetes in these humanized mice.

Overt diabetes has not yet developed in any of the NOD-*scid*/ γ ^{null}/A2 mice that received PBMCs from HLA-A2⁺ type 1 diabetic patients, although one of the nine recipients of type 1 diabetes HLA-A2⁺ PBMCs did show elevated blood glucose levels up to 235 mg/dL. It is likely that in addition to the HLA-A2 molecule, this mouse model may be improved by the addition of human MHC class II molecules or other costimulatory molecules that would allow interaction of the donor cells with mouse cells in the spleen and lymph node and perhaps provide a better environment for engraftment in these tissues. In the current model, CD4⁺ T cells can interact with engrafted donor APCs via human class II only, whereas the CD8⁺ T cells can receive stimulation from both donor and recipient (NOD-*scid*/ γ ^{null}/A2 mouse) cells.

It is difficult to isolate large numbers of diabetogenic CD8⁺ T cells directly from the peripheral blood of patients with type 1 diabetes. Therefore, one of the advantages of this model is the 1,000-fold increase in epitope-specific CD8⁺ T cells observed in mice engrafted with type 1 diabetic PBMCs. Presumably, this will allow more studies of human diabetogenic T cells; for example, we can test the cross-reactivity of diabetogenic T cells to common pathogens. This model also allows us to identify, directly from type 1 diabetic patients, islet epitope-specific CD8⁺ T cells. The four epitopes tested here comprised 63–88% of the islet-infiltrating CD8⁺ T cells in three of six mice that received type 1 diabetic PBMCs. In the islets of the remaining mice engrafted with type 1 diabetic PBMCs, these epitopes were recognized by 19–27% of CD8⁺ T cell infiltrates. In the future, we plan to use larger pools of peptides derived from islet antigens, and this may lead to the identification of other islet-infiltrating T cells.

This model of autoimmunity may also be useful in predicting islet cell transplantation by analyzing the level of pre-existing diabetogenic T cells in candidate patients with type 1 diabetes. Therefore, with future developments of this model, researchers may investigate more directly the mechanisms underlying T cell responses. We can gain valuable insight into the pathogenesis of various autoimmune diseases and predict immunotherapeutic responses.

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