β Cell-Specific CD4+ T Cell Clonotypes in Peripheral Blood and the Pancreatic Islets Are Distinct

Li Li, Qiuming He, Alaina Garland, Zuoan Yi, Lydia T. Aybar, Thomas B. Kepler, Jeffrey A. Frelinger, Bo Wang and Roland Tisch

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Type 1 diabetes (T1D) is marked by the breakdown of self-tolerance to β cells within the T cell compartment (1–3). Defects in thymic selection coupled with aberrant peripheral immunoregulation are believed to contribute to the development and expansion of pathogenic, β cell-specific CD4^+ and CD8^+ T cells (4–7). Studies conducted using the NOD mouse, a spontaneous model of T1D, indicate that early stages of the diabetic response involve effector T cells targeting only a limited number of β cell autoantigens including insulin B chain and glutamic acid decarboxylase 65 (GAD65) (1, 8–10). Islet inflammation or insulitis is first detected in NOD mice 3–4 wk of age. Intramolecular and intermolecular epitope spread occurs as insulitis continues to recruit additional β cell-specific T cell clones that amplify the autoimmune response (11–13). Overt diabetes is diagnosed typically in NOD mice older than 12 wk of age when ~90% of the β cell mass has been destroyed. In prediabetic subjects, insulitis may proceed for a number of years before clinical diabetes is diagnosed. The relative role of different β cell-specific clonotypes in the disease process, and the molecular basis underlying recognition of β cell autoantigens and subsequent expansion of pathogenic T effectors is poorly understood.

The progression of β cell autoimmunity in prediabetic (e.g., at risk) individuals or diabetic patients undergoing immunotherapy can be monitored by assessing β cell-specific T cell reactivity (14, 15). The source of these T cells is limited to PBL because the islets are generally inaccessible. Accordingly, an important question is whether clones of β cell-specific T cells detected in PBL accurately reflect those T cells infiltrating the islets, and in turn provide a reliable readout to evaluate disease progression. Studies have demonstrated that clones of β cell-specific CD8^+ T cells found in the islets also reside in PBL of NOD mice (16, 17). For instance, analyses of CDR of TCR expressed by CD8^+ T cells specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) demonstrated that identical clones reside in both PBL and the islets at differing frequencies (16). Furthermore, increased IGRP-specific CD8^+ T cells found in PBL of prediabetic NOD mice have been shown to correspond with the progression to overt diabetes (18). In contrast, whether clones of islet-infiltrating CD4^+ T cells are also found in PBL has yet to be determined. In celiac disease, for example, gliadin-specific CD4^+ T cells are also found in PBL but the islets are generally inaccessible. Accordingly, an important question is whether clones of β cell-specific T cells detected in PBL accurately reflect those T cells infiltrating the islets, and in turn provide a reliable readout to evaluate disease progression. Studies have demonstrated that clones of β cell-specific CD8^+ T cells found in the islets also reside in PBL of NOD mice (16, 17). For instance, analyses of CDR of TCR expressed by CD8^+ T cells specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) demonstrated that identical clones reside in both PBL and the islets at differing frequencies (16). Furthermore, increased IGRP-specific CD8^+ T cells found in PBL of prediabetic NOD mice have been shown to correspond with the progression to overt diabetes (18). In contrast, whether clones of islet-infiltrating CD4^+ T cells are also found in PBL has yet to be determined. In celiac disease, for example, gliadin-specific CD4^+ T cells are also found in PBL but the islets are generally inaccessible. Accordingly, an important question is whether clones of β cell-specific T cells detected in PBL accurately reflect those T cells infiltrating the islets, and in turn provide a reliable readout to evaluate disease progression. Studies have demonstrated that clones of β cell-specific CD8^+ T cells found in the islets also reside in PBL of NOD mice (16, 17). For instance, analyses of CDR of TCR expressed by CD8^+ T cells specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) demonstrated that identical clones reside in both PBL and the islets at differing frequencies (16). Furthermore, increased IGRP-specific CD8^+ T cells found in PBL of prediabetic NOD mice have been shown to correspond with the progression to overt diabetes (18).
Haskins and colleagues (21, 22). The BDC2.5 T cell clone is diabetogenic upon adoptive transfer into young NOD or NOD.scid recipients (23, 24). In addition, NOD.scid mice transgenic for the BDC2.5 TCR α-chain (Vα1) and β-chain (Vβ4) rapidly develop diabetes, further demonstrating disease relevance of this T cell cloneotype (24–26). The β cell autoantigen recognized by BDC2.5 CD4+ T cells is unknown; however, a panel of stimulatory mitogenic peptides has been established (27). In this study, soluble MHC class II (Iaαβ)-Ig multimers (sIAγ7-Ig) covalently linked to a mimetic BDC2.5 epitope (mBDC) were used to isolate and characterize the properties of BDC2.5 clonotypic CD4+ T cells from NOD mice. Our results demonstrate that sIAγ7-mBDC multimer binding CD4+ T cell clones (g7-mBDC+ T cells) from PBL and islets differ in phenotype, diabetogenic capacity, and TCR repertoire.

Materials and Methods

Mice

NOD/Jax and NOD.CB17-Prkdcscid/J (NOD.scid) mice were maintained and bred under specific pathogen-free conditions in an American Association for Laboratory Animal Care-accredited animal facility. All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Islet isolation

Pancreas specimens were perfused with 0.2 mg/ml Liberase (Roche) and digested for 30 min at 37°C. Islets were purified via Ficoll gradient, handpicked, and counted. For flow cytometry analysis, freshly isolated islets were dissociated into a single-cell suspension using enzyme-free cell dissociation solution (Sigma-Aldrich) before staining. Lymphocytes infiltrating the islets were collected, and cellular debris removed by 70-μm nylon filters.

sIAγ7-Ig dimer/multimer preparation

sIAγ7-Ig dimers were generated as described (13, 28). Briefly, sIAγ7-Ig were tethered via a thrombin GGGGS linker to the mBDC epitope (sIAγ7-mBDC) (RTRPLWVRME; see Ref. 27) or a hen egg lysozyme peptide (sIAγ7-HEL) spanning residues 12–26 as (MKRHRGLDNYRGYS). The sIAγ7-Ig dimers were expressed by Drosophila S2 cells, and purified from culture supernatant by affinity chromatography and HPLC as described (13). For staining purposes, Iaαβ-Ig multimers were prepared by incubating dimers with protein A conjugated with the Alexa Fluor 647 fluorochrome.

FACS analysis

Single-cell suspensions were prepared from islets isolated as described. Peripheral blood was collected via the tail vein and RBC lysed. T cells were stained with sIAγ7-Ig multimers in PBS containing 3% FBS, 10 mM HEPES, and 1 mM EDTA for 1 h at room temperature, and then incubated with Abs specific for CD3 (FITC) and CD4 (PE) (eBioscience) on ice for 30 min; PerCp-conjugated Abs specific for CD19, CD11c, and F4/80 (eBioscience) were used to gate out B cells, dendritic cells, and macrophages, respectively. In some experiments, g7-mBDC+ T cells were examined for intracellular expression of IFN-γ and TNF-α. In this analysis, single-cell suspensions were first stimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml) for 5 h at 37°C. Cells were washed and stained as described with sIAγ7-Ig multimers and the panel of Abs with the exception that anti-CD3 (Pe-Cy7) and anti-CD4 (PacBlue) Ab conjugates were used. Cells were then fixed, permeabilized, and stained with anti-IFN-γ (FITC) and anti-TNF-α (PE) Abs (eBioscience). Data were acquired on a Cyan flow cytometer (DakoCytomation), and analyzed using Summit software (DakoCytomation).

For single-cell analyses, g7-mBDC+ T cells were sorted by a MoFlo high-speed sorter (DakoCytomation) into 4 μl of RT-PCR buffer at 1 cell/well of a 96-well PCR plate (USA Scientific), and RT-PCR was performed immediately. All FACS analyses and single-cell sorting were performed at the University of North Carolina Flow Cytometry facility.

Single-cell RT-PCR and TCR repertoire analyses

TCR usage was analyzed by a single-cell PCR protocol previously described (16, 29, 30). Briefly, single-cell RT-PCR was performed using a OneStep RT-PCR kit (Qiagen) according to the manufacturer’s protocol. A panel of primers specified for all known TCR β-chain variable (TRBV) regions, and respective constant regions were used for reverse transcription and first-round PCR amplification. RT-PCR amplions (2 μl) were used as templates for second-round PCR amplification using nested TCR β-chain-specific primers. For single-cell TCR Vα chain analyses, islet-derived g7-mBDC+ T cells were sorted from 16-wk-old NOD female mice, and RT-PCR performed using primers specific for all known TCR α-chain variable regions and Co region, and TRBV-15-specific primers. The efficiency of RT-PCR amplification was ≥75% for a given experiment. All oligonucleotides were synthesized at the Nucleic Acids Core Facility at the University of North Carolina. PCR products were treated with Exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (Roche), and sequenced at the University of North Carolina Genome Analysis Facility. TCR sequence alignments were performed using Sequencer software (Gene Codes). TRBV-D-J gene family usage was identified and assigned using the SoDA software (Texas). TCR clonotype analyses takes into account unseen species to address incomplete sampling.

Isolation and adoptive transfer of g7-mBDC+ T cells

The g7-mBDC+ T cells were purified from PBL and the islets via MACS as described with some modifications (33). Whole blood was collected from a total of 50 NOD female mice 16 wk of age via cardiac puncture and whole body perfusion with PBS and 2 mM EDTA; greater than 2.0 ml of blood was typically obtained from a single mouse. PBL were separated using Vacutainer CPT tubes (BD Biosciences) and the manufacturer’s directions and resuspended in cold MACS buffer (PBS plus 0.5% FBS and 2 mM EDTA). CD4+ T cells were isolated by negative selection with a mixture of Abs specific for CD8, CD11b, CD45R, TER119, and Gr-1 (StemCell Technologies). Purified CD4+ T cells were preincubated with 0.5 mg/ml protein A with MACS buffer for 30 min at 4°C. Cells were then incubated at room temperature for 1 h with 10 μM sIAγ7-Ig multimers prepared with a protein A-biotin conjugate. The suspension was washed twice, and incubated with anti-biotin magnetic microbeads (Miltenyi Biotech) on ice for 15 min. The g7-mBDC+ T cells were washed twice, and purified by magnetic isolation twice through LS columns (Miltenyi Biotech). CFSE-labeled g7-mBDC+ T cells stimulated in vitro with mBDC pulsed DC proliferated analogously to BDC2.5 CD4+ T cells isolated from nonobese diabetic (NOD) BDC2.5 mice lacking the TCR α gene (data not shown).

Islet-infiltrating T cells were released from hand-picked islets from the same 50 NOD female mice used for PBL isolation, with enzyme-free cell dissociation solution (Sigma-Aldrich). CD4+ T cells were prepared via negative selection, and sIAγ7-mBDC multimer binding T cells purified as described for PBL-derived g7-mBDC+ T cells. Normally, 175 islets are obtained from each nonobese diabetic mouse. PBL (6 × 106) or islet-derived (4 × 106) g7-mBDC+ T cells resuspended in PBS were injected i.v. into 5- to 8-wk-old NOD.scid male mice, and diabetes monitored by measuring blood glucose levels. Recipients with >250 mg/dl blood glucose were diagnosed as diabetic. Pancreas specimens were harvested and fixed with 10% formalin. Serial cross-sections (5 μm) were cut and stained with H&E.

Statistical methods

Student’s t test and χ2 analyses were conducted to determine statistical significance.

Results

Islet- and PBL-derived mBDC-specific CD4+ T cells increase with age in NOD female mice but differ in phenotype

In an initial effort to determine whether BDC2.5 clonotypic CD4+ T cells differ between PBL and islets as β cell autoimmunity progresses, the frequency and phenotype of g7-mBDC+ T cells was examined in the respective tissues of NOD female and male mice of varying ages. In our colony, 75% and 20% of female and male NOD mice, respectively, develop overt diabetes by the age of 30 wk. Temporal analysis of purified islets in NOD female mice demonstrated that at 4 wk of age, the time at which insulitis is initially detected histologically, ~1.7% of CD4+ T cells bound sIAγ7-mBDC (Fig. 1A). By 6 wk of age, a 2-fold decrease in the
frequency of g7-mBDC⁺ T cells was detected, which in turn was maintained up to 24 wk of age (Fig. 1A). However, the absolute number of g7-mBDC⁺ T cells per islet increased with age (Fig. 1B). In contrast, the frequency and number of g7-mBDC⁺ T cells infiltrating the islets of NOD male mice were reduced compared with age-matched females (Figs. 1, A and B).

In the PBL of 4-wk-old NOD female mice, ~0.4% of CD4⁺ T cells bound sIA⁻²-mBDC (Fig. 1C). This frequency of g7-mBDC⁺ T cells increased by 8 wk of age and was maintained up to 24 wk of age in NOD female mice. In contrast, the frequency of g7-mBDC⁺ T cells in the PBL of age-matched NOD male mice was reduced, especially in older animals (Fig. 1C). The frequency of g7-mBDC⁺ T cells detected in NOD males was similar to that in PBL of age-matched NOD female mice, which decreased with age (Fig. 1D). This frequency of g7-mBDC⁺ T cells was maintained up to 24 wk of age (Fig. 1D). However, the absolute number of g7-mBDC⁺ T cells expressed FoxP3, with a 10-fold increase in the frequency of g7-mBDC⁺ T cells expressing IFN-γ (57%) and TNF-α (43%) was detected in the islets (Fig. 1D). In both PBL and islets, 3–5% of g7-mBDC⁺ T cells expressed FoxP3 consistent with an earlier report for NOD.BDC2.5 mice (34). Taken together, these findings demonstrate that g7-mBDC⁺ T cells are detected in PBL and the islets early in the disease process, and that the frequency or number of sIA⁻²-mBDC binding CD4⁺ T cells is increased in age-matched female compared with male NOD mice. Furthermore, the frequency of g7-mBDC⁺ T cells exhibiting a typical type 1 effector phenotype is significantly increased in the islets compared with PBL at a late preclinical stage of TID.

Islet- but not PBL-derived g7-mBDC⁺ T cells are diabetogenic
Adoptive transfer experiments were conducted to determine whether differences in pathogenicity existed between islet- and
PBL-derived g7-mBDC⁺ T cells. Specifically, g7-mBDC⁺ T cells isolated from the islets or PBL of 16-wk-old NOD female mice were injected into NOD.scid recipients, and the development of diabetes monitored. Greater than 90% of the purified PBL-derived CD4⁺ T cells isolated from PBL or islets of 16-wk-old prediabetic NOD female donors, and monitored for diabetes. *, p = 0.04, for islet g7-mBDC⁺ CD4⁺ T cells alone vs PBL g7-mBDC⁺ CD4⁺ T cells alone by Kaplan-Meier Log Rank test. B. Pancreas specimens from diabetes-free NOD.scid recipients of PBL or islet-derived g7-mBDC⁺ CD4⁺ T cells were examined for insulitis as determined by H&E staining. †, p = 0.0003; ‡, p = 0.0005 by χ², C. Splenocytes from n = 3 individual NOD.scid mice receiving purified PBL- or islet-derived g7-mBDC⁺ CD4⁺ T cells were cultured with 20 μg/ml mBDC or hen egg lysozyme peptide (pHEL) or medium alone. The frequency of IFN-γ-secreting T cells was determined via ELISPOT. §, p = 0.017, for cultures stimulated with mimetic BDC vs hen egg lysozyme peptide or medium only by Student’s t test. Data are representative of two experiments.

FIGURE 2. Islet- but not PBL-derived g7-mBDC⁺ T cells from 16-wk-old NOD female mice exhibit a diabetogenic capacity. A, Groups of n = 5 NOD.scid mice were i.v. injected with g7-mBDC⁺ CD4⁺ T cells isolated from PBL or islets of 16-wk-old prediabetic NOD female donors, and monitored for diabetes. *, p = 0.04, for islet g7-mBDC⁺ CD4⁺ T cells alone vs PBL g7-mBDC⁺ CD4⁺ T cells alone by Kaplan-Meier Log Rank test. B, Pancreas specimens from diabetes-free NOD.scid recipients (see supplemental Fig. 1A). NOD.scid recipients injected with PBL-derived g7-mBDC⁺ T cells remained diabetes-free 25 wk posttransfer (Fig. 2A), and histological analysis of the islets demonstrated only a low frequency of peri-insulitis (Fig. 2B). CD4⁺ T cells binding sIA⁺/mBDC multimers, however, were readily detected in the blood and spleen, whereas a limited number of islet-infiltrating g7-mBDC⁺ T cells were found in the PLN and islets of these NOD.scid recipients (see supplemental Fig. 1B). Despite injection of 10-fold fewer cells, 3 of 5 NOD.scid mice receiving islet-infiltrating g7-mBDC⁺ T cells developed diabetes (Fig. 2A), and the majority of islets (~70%) in the diabetes-free recipients were heavily infiltrated (Fig. 2B). The sIA⁺/mBDC multimer staining demonstrated that >95% of the islet-infiltrating CD4⁺ T cells were g7-mBDC⁺ (see supplemental Fig. 1C). ELISPOT showed that a marked increase in the frequency of IFN-γ-secreting T cells in response to mBDC peptide was detected in the spleen of NOD.scid recipients of islet- but not PBL-derived g7-mBDC⁺ T cells (Fig. 2C). These results demonstrate that islet- but not PBL-derived g7-mBDC⁺ T cells are diabetogenic.

Islet- and PBL-derived mBDC-specific CD4⁺ T cells express distinct TCR Vβ repertoires

Differences in pathogenicity of islet- and PBL-derived g7-mBDC⁺ T cells may reflect distinct TCR repertoires. To test this hypothesis, TCR use by mBDC-specific CD4⁺ T cells was assessed at the single cell level. The sIA⁺/mBDC binding CD3⁺CD4⁺ T cells were sorted from PBL and the islets of 16-wk-old NOD female mice, and TCR Vβ gene usage was detected in the spleen of these NOD.scid recipients (see supplemental Fig. 1A). Despite injection of 10-fold fewer cells, 3 of 5 NOD.scid mice receiving islet-infiltrating g7-mBDC⁺ T cells developed diabetes (Fig. 2A), and the majority of islets (~70%) in the diabetes-free recipients were heavily infiltrated (Fig. 2B). The sIA⁺/mBDC multimer staining demonstrated that >95% of the islet-infiltrating CD4⁺ T cells were g7-mBDC⁺ (see supplemental Fig. 1C). ELISPOT showed that a marked increase in the frequency of IFN-γ-secreting T cells in response to mBDC peptide was detected in the spleen of NOD.scid recipients of islet- but not PBL-derived g7-mBDC⁺ T cells (Fig. 2C). These results demonstrate that islet- but not PBL-derived g7-mBDC⁺ T cells are diabetogenic.

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The online version of this article contains supplemental material.
Table I. Diversity among g7-mBDC<sup>+</sup> T cells in CDR3β sequences<sup>a</sup>

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>TRBV</th>
<th>CDR3</th>
<th>TRBJ</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>CASS</td>
<td>KDSSYEQ</td>
<td>YFG</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>CASS</td>
<td>LGWPGAEQ</td>
<td>FFG</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>CASS</td>
<td>PGGRDYAEQ</td>
<td>FFG</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>CASS</td>
<td>PGGRDYAEQ</td>
<td>FFG</td>
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<td>15</td>
<td>CASS</td>
<td>LQGQDTQ</td>
<td>FFG</td>
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<tr>
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<td>CASS</td>
<td>LADSYEQ</td>
<td>YFG</td>
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<td>15</td>
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<td>YFG</td>
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<td>CASS</td>
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<td>CASS</td>
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<tr>
<td>10</td>
<td>15</td>
<td>CASS</td>
<td>PDRGQDTQ</td>
<td>YFG</td>
</tr>
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</table>

<sup>a</sup> The diversity among TRBV-15-expressing (TRBV) and TCR β-chain J variable (TRBJ)-expressing g7-mBDC<sup>+</sup> T cells is assessed by comparison of the CDR3β sequences of individual T cells.

Fig. 2A). Despite a reduced frequency, TRBV-15 also predominated among the Vβ genes in mouse number four (7/20), mouse number five (5/21), mouse number six (12/21), and mouse number nine (6/20) (see supplemental Fig. 2A). Overall, an ~6-fold increase in the frequency of TRBV-15 usage was detected in islet vs PBL g7-mBDC<sup>+</sup> T cells (Fig. 3). To further assess the diversity among TRBV-15-expressing g7-mBDC<sup>+</sup> T cells, CDR3β sequences were compared in the individual T cells. In 7 of 10 NOD female mice, one to two clones predominated within the pool of TRBV-15-expressing g7-mBDC<sup>+</sup> T cells infiltrating the islets (mouse number one, number two, number three, number six, number seven, eight, number ten) (see supplemental Table I). Notably, in NOD male mice in which TRBV-15 clones were detected in both the islets and PBL (mouse number one, three, four, five, six, seven, eight, and ten), the respective TRBV-15-expressing clones were distinct between the two sets of g7-mBDC<sup>+</sup> T cells (see supplemental Table I). These results demonstrate that a limited number of mBDC-specific clones expressing TRBV-15 reside in the islets of NOD female mice, whereas the repertoire of g7-mBDC<sup>+</sup> T cells in PBL exhibits no marked clonotypic skewing and no overlap in TCR-β expression.

To determine whether preferential expansion of TRBV-15-expressing g7-mBDC<sup>+</sup> T cells in the islets of NOD female mice correlated with the general development of "pathogenic" β cell autoimmunity, TCR usage of g7-mBDC<sup>+</sup> T cells in a group of nine individual NOD male mice 16 wk of age was also investigated. In one of these NOD male mice (mouse number four), no g7-mBDC<sup>+</sup> T cells were detected in either the islets or PBL. Sequencing results demonstrated that in the islets of 8 NOD male mice, only 19.6% of 175 islet g7-mBDC<sup>+</sup> T cells used TRBV-15 (Fig. 3) which in turn was ~3-fold less than that detected in islet infiltrating g7-mBDC<sup>+</sup> T cells of NOD female mice (Fig. 3). Furthermore, with the exception of mouse number three in which 86.5% of g7-mBDC<sup>+</sup> T cells expressed TRBV-15, the frequency of TRBV-15-expressing g7-mBDC<sup>+</sup> T cells in the islets was typically <20% among the individual animals (see supplemental Fig. 3A). Vβ gene usage by g7-mBDC<sup>+</sup> T cells residing in PBL was also diverse (Fig. 3) (and see supplemental Fig. 3B). Interestingly, 20.5% and 9.9% of g7-mBDC<sup>+</sup> T cells expressed TRBV-13.2 and TRBV-15, respectively (Fig. 3), similar to that detected in PBL of NOD female mice (Fig. 3). These findings indicate that g7-mBDC<sup>+</sup> T cell clonotypes are diverse in both PBL and the islets of the majority of NOD male mice.

The Shannon entropy was calculated to quantitate the level of CDR3β gene diversity among single islet and PBL g7-mBDC<sup>+</sup> T cells examined in 16-wk-old NOD female (see supplemental Table I) and male (see supplemental Table II) mice. The posterior distribution of the Shannon entropy in a given population represents the entropy of diversity for a given CDR3β sequence. As demonstrated in Fig. 4, a significant decrease in diversity was detected in NOD female mice for g7-mBDC<sup>+</sup> T cells infiltrating the islets (2.84 ± 0.41) vs PBL (5.59 ± 0.16) (p = 0.0014). Furthermore, this reduced diversity in islet g7-mBDC<sup>+</sup> T cells in NOD female mice was significantly lower compared with g7-mBDC<sup>+</sup> T cells infiltrating the islets of NOD male mice (4.48 ± 0.57; p = 0.045) (Fig. 4). Moreover, a similar degree of diversity was detected in NOD male mice for g7-mBDC<sup>+</sup> T cells isolated from either islets (4.48 ± 0.57) or PBL (5.48 ± 0.14) (Fig. 4). These results indicate TCR-β gene usage by g7-mBDC<sup>+</sup> T cells in PBL of both male and female NOD mice is diverse. However, in the islets of NOD female mice, the TCR-β repertoire of g7-mBDC<sup>+</sup> T cells is much less diverse and largely consists of a limited number of TRBV-15-expressing clones.

**Discussion**

Characterizing β cell-specific T cell reactivity in individuals at risk or diabetic patients undergoing immunotherapy can provide important insight into the nature and progression of the diabetogenic response (14, 15). Because PBL are typically used as a source of T cells, a key issue is whether peripheral β cell-specific T cell clones reflect those found infiltrating the islets. The current study demonstrates that despite recognizing the same peptide, PBL-derived g7-mBDC<sup>+</sup> T cells represent a distinct pool of clones relative to mBDC-specific CD4<sup>+</sup> T cells infiltrating the islets.

Marked differences in the phenotype and pathogenicity of PBL- and islet-derived g7-mBDC<sup>+</sup> T cells were detected. Only a low
frequency of PBL-derived g7-mBDC+ T cells expressed IFN-γ and TNF-α (Fig. 1C), with ~55% of T cells having a typical naive phenotype (e.g., CD62Lhigh, CD44low; data not shown). Furthermore, PBL-derived g7-mBDC+ T cells failed to efficiently traffic to the islets and mediate diabetes upon adoptive transfer into NOD.scid recipients (Fig. 2 and supplemental Fig. 1). This finding is consistent with work by Liu and colleagues (35) who demonstrated that transfer of NOD splenic CD4+ T cells that bind sAγ7 tetramers complexed to a different mimetic BDC peptide also failed to mediate diabetes in NOD.scid recipients. Islet-infiltrating g7-mBDC+ T cells conversely exhibited a predominant type 1 effector phenotype (Fig. 1C), and induced insulin and diabetes despite injection of 10-fold less T cells compared with PBL-derived g7-mBDC+ T cells (Fig. 2). Direct evidence that clones of PBL- vs islet-derived mBDC-specific CD4+ T cells differed was provided by TCR analysis. The TCR repertoire of PBL-derived g7-mBDC+ T cells was diverse (Fig. 4), consisting of multiple clonotypes based on TRBV and CDR3γ gene usage (Fig. 3 and supplemental Fig. 2 and supplemental Table I). In contrast, only limited diversity was detected in the TCR repertoire of islet-infiltrating g7-mBDC+ T cells (Fig. 4), and somewhat surprisingly no overlap of CDR3γ gene usage was seen with PBL-derived mBDC-specific CD4+ T cells (see supplemental Table I).4 The latter observation is in contrast to studies demonstrating that dominant clonotypes of β cell-specific CD8+ T cells found infiltrating the islets were also detected in the PBL of wild-type or transgenic NOD mice expressing HLA-A2 (16, 17). Trafficking properties intrinsic to tissue-specific CD4+ and CD8+ T cells may in part explain the distribution of the respective T cells. Whether the differences detected between PBL- and islet-derived g7-mBDC+ T cell clones are common among other β cell-specific CD4+ T cells needs to be ascertained. In this regard it is noteworthy that analyses of patients with other autoimmune diseases such as celiac disease have also demonstrated clonotypic differences between CD4+ T cells found in PBL and the target tissue (19, 20).

Expansion of g7-mBDC+ T cells in the islets corresponds with the preferential development of destructive insulitis and overt diabetes in female NOD mice. An elevated number of g7-mBDC+ T cells progressively increased with age as seen in the islets of female but not age-matched NOD mice (Fig. 1B). These results are consistent with the diabetogenicity seen for the in vitro established BDC2.5 T cell clone, and primary T cells expressing the corresponding BDC2.5 clonotypic TCR as a transgene (22–24). Islet-infiltrating g7-mBDC+ T cells may represent an important pool of effectors driving insulitis and eventual β cell destruction. Interestingly, despite the lack of pathogenic effector function, the frequency of g7-mBDC+ T cells found in PBL also increased with age in female but not male NOD mice (Fig. 1C). This age-dependent increase of PBL-derived g7-mBDC+ T cells may reflect a general increase of thymic output or accumulation of β cell-specific T precursors in female vs male NOD mice. Alternatively, ongoing β cell destruction in NOD female mice may result in the systemic release of native autoantigen resulting in limited expansion of a diverse pool of nonpathogenic g7-mBDC+ T cells.

The pathogenicity of islet-infiltrating g7-mBDC+ T cells was characterized by a skewed TCR repertoire (Fig. 3 and Table I and supplemental Table I). Greater than 60% of islet infiltrating g7-mBDC+ T cells in 6 of 10 NOD female mice (mouse number one, number two, number three, number six, number seven, and number ten) expressed TRBV-15 (supplemental Fig. 2). Notably, the majority of islet-infiltrating g7-mBDC+ T cells that were transferred into NOD.scid mice and trafficked to the islets also preferentially expressed TRBV-15 (supplemental Fig. 4). TRBV-15-expressing g7-mBDC+ T cells residing in the islets typically consisted of one to two dominant clones based on CDR3β sequences (supplemental Table I). Interestingly, among the TRBV-15-expressing clones, two CDR3β motifs were shared between individual NOD female mice; namely the LAQQGQGYEQ (mouse number six, number seven, number eight, and number ten) and PGGRDIAEQ (mouse number three, number four, and number five) motifs (Table I). These findings are similar to earlier results reported by our group and others demonstrating that islet infiltrating IGRP-specific CD8+ T cells preferentially express a particular Vα gene motif, and consist of one to three dominant clones based on CDR3β usage (16, 29, 36, 37). These results also argue that the pathogenicity of g7-mBDC+ T cells is characterized by selective TRBV-15 gene usage. Indeed, nonpathogenic g7-mBDC+ T cells derived from the PBL of NOD female mice expressed a relatively diverse TCR repertoire characterized by a modest increase in TRBV13.2 usage (Fig. 3). In addition, the “nondestructive” insulitis typical of NOD male mice consisted of g7-mBDC+ T cells with a reduced frequency of TRBV-15, and a more diverse TCR repertoire relative to NOD female mice (Fig. 3). Interestingly, in male NOD mouse number three 89% of g7-mBDC+ T cells infiltrating the islets expressed TRBV-15 (see supplemental Table II), possibly reflecting “destructive” insulitis leading to overt diabetes that develops relatively infrequently in NOD male mice. Although diabetogenicity correlates with preferential TRBV-15-expression, other Vβ genes are also expressed by pathogenic g7-mBDC+ T cells. For instance, the diabetogenic BDC2.5 T cell clone expresses TRBV-2 (Vβ4) (25). Our results support a model in which TRBV-15-expressing islet-infiltrating g7-mBDC+ T cells are preferentially expanded due to avidity/affinity maturation. Increased TCR avidity would be expected to provide a selective advantage in expansion for clones compared with those expressing TCR with relatively weak avidity (38). Similar avidity/affinity maturation has been reported for IGRP-specific CD8+ T cells (37). Although the level of sAγ7-BDC multimer binding was not significantly different between PBL- and islet-derived CD4+ T cells (L. Li and R. Tisch, unpublished data), a concerted effort assessing the avidity/affinity of the respective clonotypes is warranted. Interestingly, more than 50% of TRBV-15-expressing g7-mBDC+ T cells sorted from the islets of NOD female mice were found to express TRAV-13 (supplemental Fig. 5), indicating that the TCR repertoire of pathogenic clones is further restricted by preferential Vα gene usage. Together these results suggest that insulitis leading to efficient β cell destruction is driven by the expansion of a limited number clones specific for a given autoantigen.

In conclusion, this study provides the first evidence that PBL-derived CD4+ T cells specific for a given β cell autoantigen may not be representative of corresponding pathogenic CD4+ effectors residing in the islets. Additional studies are needed to determine whether the distinction between PBL- and islet-derived g7-mBDC+ T cells in pathogenicity and TCR repertoire diversity are indeed common properties of other β cell-specific CD4+ T cells.

Disclosures
The authors have no financial conflict of interest.

References


