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Early Autoimmune Destruction of Islet Grafts Is Associated with a Restricted Repertoire of IGRP-Specific CD8+ T Cells in Diabetic Nonobese Diabetic Mice

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β cell replacement via islet or pancreas transplantation is currently the only approach to cure type 1 diabetic patients. Recurrent β cell autoimmunity is a critical factor contributing to graft rejection along with alloreactivity. However, the specificity and dynamics of recurrent β cell autoimmunity remain largely undefined. Accordingly, we compared the repertoire of CD8+ T cells infiltrating grafted and endogenous islets in diabetic nonobese diabetic mice. In endogenous islets, CD8+ T cells specific for an islet-specific glucose-6-phosphatase catalytic subunit-related protein derived peptide (IGRP 206–214) were the most prevalent T cells. Similar CD8+ T cells dominated the early graft infiltrate but were expanded 6-fold relative to endogenous islets. Single-cell analysis of the TCR α and β chains showed restricted variable gene usage by IGRP 206–214-specific CD8+ T cells that was shared between the graft and endogenous islets of individual mice. However, as islet graft infiltration progressed, the number of IGRP 206–214-specific CD8+ T cells decreased despite stable numbers of CD8+ T cells. These results demonstrate that recurrent β cell autoimmunity is characterized by recruitment to the grafts and expansion of already prevalent autoimmune T cell clonotypes residing in the endogenous islets. Furthermore, depletion of IGRP 206–214-specific CD8+ T cells by peptide administration delayed islet graft survival, suggesting IGRP 206–214-specific CD8+ T cells play a role early in islet graft rejection but are displaced with time by other specificities, perhaps by epitope spread. The Journal of Immunology, 2006, 176: 1637–1644.
was initiated to gain insight into the nature of β cell-specific CD8+ clonotypes in autoimmune- mediated islet graft rejection.

Materials and Methods

Mice

NOD/LtJ, NOD.Cg-Tg(TcarTcrB/NY8.3)Pesa (8.3 TCR transgenic), and NOD.CB17-Pkdcl–/– (J/NOD, scid) mice were bred and housed under specific pathogen-free conditions. Diabetes was monitored weekly by measuring urine glucose levels with Diamix (Bayer). Mice were diagnosed as diabetic when the level of urine glucose exceeds 0.25% for two successive measurements according to manufacturer’s guidelines. A urine glucose level of 0.25% is equivalent to a blood glucose value of ≥250 mg/dl as determined by an Autokit Glucose CII assay (WAKO) (data not shown).

BALB/c and FVB/J mice were bred and housed in filter-covered isolator cages. Animals were maintained at an American Association of Laboratory Animal Care-accredited animal facility. All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Peptides

MHC class I peptides NRP-V7 (KYKNKAVFL), IGRP206–214 (VYLLKT NVFL), GAD546–554 (SYQPLGDKV), InsB15–23 (LYLVCGERG), and nucleoprotein (NP147–155, TYQRTRALV) were synthesized (Avidity). Tetramers, Abs, and flow cytometry reagents were obtained from BD Biosciences (BD Pharmingen), and Use Committee.

Tetramers, Abs, and flow cytometry

H2Kb tetramers were prepared as described (28). Briefly, peptide/MHC monomers were purified by HPLC and biotinylated using biotin-protein ligase (Avidity). Tetramers were assembled by conjugating MHC monomers with streptavidin-PE (Molecular Probes). Fluorescent-conjugated anti-mouse Abs used for cell surface staining include anti-CD4 (CF-595, FITC), and nuclear stain (CF-147–155, YOYRTLAL) were synthesized at the University of North Carolina A1a RiboNucleic Core Facility. The InsB-G9V peptide was modified from its native sequence to increase MHC class I stability (27).

Single-cell suspensions from spleens, lymph nodes, islets, and islet grafts were prepared in PBS. Peripheral blood was collected via the tail vein and RBC lysed where appropriate. T cells were costained with tetramers and Abs in PBS containing 3% FBS, 10 mM HEPEs, and 1 mM EDTA for 1 h on ice. Flow cytometry data were acquired on FACS Calibur (BD Biosciences) and analyzed using Summit software (DakoCytomation).

For all tetramer analyses, CD8+ T cells were gated based on forward and side scatter and CD3 and CD8 expression.

For single-cell analyses, Kα-NRPV7 tetramer-binding CD8+ T cells were sorted by a MoFlo high-speed sortor (DakoCytomation) into 25 µl of RVerse plus Ab (Cell Analysis) (final concentration of 10 µg/ml) and 25 µl of 1× RVerse plus cytokine mAb (anti-IFN-γ, anti-IL-4, or anti-IL-10) (BD Pharmingen). Plates were seeded with islet-infiltrating lymphocytes at 1 × 10^6 cells per well in HL-1 medium (BioWhittaker), and 5 × 10^5 irradiated splenocytes were added. Peptides were added at a final concentration of 10 µg/ml. Cultures were incubated for 24 h at 37°C. Cells were removed by washing, and the plates were incubated with the appropriate biotinylated anti-mouse cytokine Abs overnight at 4°C. Plates were then washed, incubated with streptavidin-HRP (BD Pharmingen) for 2 h at room temperature, and developed using a 100-mM sodium acetate buffer containing 0.3 mg/ml 3-amin-9-ethylcarbazole (Sigma-Aldrich) and 0.015% hydrogen peroxide. An ImmunoSpot plate reader (Cellular Technology) was used to count the spot-forming cells (SFC) per well.

Peptide immunization

Diabetic NOD mice were immunized i.v. with 200 µg of IGRP or HA peptide in PBS. A total of five immunizations were given at 2, 4, and 6 days before islet implantations, and at 5 and 12 days postimplantation. Levels of IGRP206–214-specific CD8+ T cells in peripheral blood were determined by flow cytometry before the first peptide immunization and after the third injection before islet transplantation using Kβ-NRPV7 tetramer. Alternatively, peptide-treated diabetic NOD mice received islet grafts, and islet infiltrates were analyzed 7 days postimplantation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Values of p were calculated using Student’s t test. Survival curves were compared using Kaplan Meier log-rank test.

Results

IGRP206–214-specific CD8+ T cells predominate the early infiltrates of syngeneic islet grafts

To gain insight into the mechanism of recurrent β cell autoimmunity, the specificity and frequency of CD8+ T cells that infiltrate grafted vs endogenous islets were measured. Initially, the predominate CD8+ clonotype(s) residing in the endogenous pancreas was assessed in nondiabetic 20-wk-old NOD female mice, which represent a late preclinical stage of T1D. ELISPOT was used to measure the relative frequency of IFN-γ, IL-4, and IL-10-secreting CD8+ T cells specific for a panel of known β cell autoantigenic epitopes. This included IGRP206–214 and the corresponding NRPV7 mimotope, in addition to InsB15–23, and DMK138–146-H2Kb-restricted peptides derived from ProInsB25–C34 (37) and GAD65 (GAD65246–254) (38) were also tested. Pooled pancreatic islets from groups of four 20-wk-old NOD female mice were cultured for 3 days in IL-2-containing medium. Lymphocyte infiltrates were harvested and stimulated in vitro with the panel of RBC, removing debris using a 70-µm nylon filter, and resuspending in FACSC buffer for flow cytometric analysis. For a negative control, a similar sized tissue sample was dissected from the nontransplanted kidney and processed accordingly.

Single-cell RT-PCR and TCR repertoire analyses

TCR usage was analyzed by a single-cell PCR protocol previously described (29) with the following modifications. Single-cell RT-PCR was performed using a Qiagen OneStep RT-PCR kit (Qiagen) according to the manufacturer’s protocol. A panel of primers specific for all known TCR α- or β-chain variable regions and respective constant regions were used for reverse transcription and first-round PCR amplification. RT-PCR amplimers (2.5 µl) were used as templates for second-round PCR amplification using a panel of nested TCR α- or β-chain-specific primers. All oligonucleotides were synthesized at the Nucleic Acids Core Facility at the University of North Carolina. PCR products were treated with Exonuclease I (NEB Biolabs) and shrimp alkaline phosphatase (Roche), and sequenced at the University of North Carolina Genome Analysis Facility. TCR sequence alignments were performed using Sequencher software (Gene Codes). TCR α- and β-chain (TRA and TRβ, respectively) gene family usage was identified and assigned using the international ImMunoGeneTics (IMGT) information system (http://imgt.cines.fr/); Refs. 30–35 and former nomenclature based on Arden et al. (36).

ELISPOT

ELISPOT plates (Millipore) were coated overnight at 4°C with purified rat anti-mouse cytokine Abs in PBS (anti-IFN-γ, anti-IL-4, or anti-IL-10) (BD Pharmingen). Plates were seeded with islet-infiltrating lymphocytes at 1 × 10^6 cells per well in HL-1 medium (BioWhittaker), and 5 × 10^5 irradiated splenocytes were added. Peptides were added at a final concentration of 10 µg/ml. Cultures were incubated for 24 h at 37°C. Cells were removed by washing, and the plates were incubated with the appropriate biotinylated anti-mouse cytokine Abs overnight at 4°C. Plates were then washed, incubated with streptavidin-HRP (BD Pharmingen) for 2 h at room temperature, and developed using a 100-mM sodium acetate buffer containing 0.3 mg/ml 3-amin-9-ethylcarbazole (Sigma-Aldrich) and 0.015% hydrogen peroxide. An ImmunoSpot plate reader (Cellular Technology) was used to count the spot-forming cells (SFC) per well.
peptides. IFN-γ-secreting CD8⁺ T cells were detected in response to IGRP₂₀₆₋₂₁₄ and NRP-V7, but not InsB₁₅₋₂₃, DMK₁₃₈₋₁₄₆, ProInsB₂₅-C₃₄, GAD₆₅₅₋₆₅₄, or the control influenza NP peptide (Fig. 1A). No IL-4 or IL-10-secreting T cells were detected above background in response to any of the peptides tested. Similar results were obtained when lymphocyte infiltrates isolated from islets of individual 20 wk-old NOD female mice were examined (data not shown). Consistent with the ELISpot data, H₂Kd tetramers complexed with NRP-V7 (Kd-NRPV7) bound CD8⁺ T cells from islets prepared from four individual non-diabetic 20-wk-old NOD female mice (Fig. 1B). K₄-NRPV7 bound 7.9 ± 2.8% of islet-infiltrating CD8⁺ T cells, whereas only minimal binding was observed with K₄-InsB₁₅₋₂₃ (0.7 ± 0.3%) or K₄-NP (0.4 ± 0.1%) (Fig. 1B). K₄-NRPV7⁺ CD8⁺ T cells were also detected in the pancreatic lymph nodes (PLN) (0.4 ± 0.1%) and spleen (0.5 ± 0.2%), albeit at lower frequencies than that seen in the islets (Fig. 1B). Because increased binding to CD8⁺ T cells prepared from 8.3 TCR NOD transgenic mice was detected for K₄-NRPV7 compared with H₂Kd tetramer complexed with IGRP₂₀₆₋₂₁₄ (Kd-IGRP) (data not shown), NRP-V7 tetramers were used in subsequent experiments to detect IGRP₂₀₆₋₂₁₄-specific clonotypes ex vivo.

The aforementioned results indicated that IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells were the most prevalent of the known MHC class I-restricted β cell-specificities in the islets; therefore, efforts initially focused on K₄-NRPV7 binding in syngeneic islet grafts. Recent onset diabetic NOD female mice were transplanted with islets prepared from NOD.scid donor mice. Recurrent diabetes was typically detected ~2 wk postimplantation. The infiltrates from grafted and endogenous islets were compared 7 days postimplanta-
tion within individual recipients. Strikingly, a marked increase in the frequency of K₄-NRPV7⁺ CD8⁺ T cells was detected in islet grafts (42.1%) (Fig. 2A) compared with the endogenous islets (8.9%) (Fig. 2B). Few K₄-NRPV7⁺ CD8⁺ T cells were detected in the draining renal lymph node (0.7%), PLN (0.9%), or spleen (1.4%) of islet graft recipients (Fig. 2, C–E). In 10 recipients analyzed, a >6-fold increase in the frequency of K₄-NRPV7⁺ CD8⁺ T cells was detected in grafted vs endogenous islets (p = 0.003) (Fig. 2F). Minimal staining (<0.6%) was observed using the control K₄-NP tetramer in all samples analyzed. Furthermore, no significant staining above background was detected with K₄-InsB (0.8 ± 0.3%) or K₄-ProIns (0.6 ± 0.2%) tetramers. Consistent with a role as effector cells, 98% of K₄-NRPV7⁺ CD8⁺ T cells infiltrating the islet graft were CD62L⁻lowCD44high (data not shown).

The marked increase in K₄-NRPV7⁺ CD8⁺ T cells infiltrating the transplant was dependent on H₂Kd expression by the islet graft. In diabetic NOD (H₂DbKd) recipients of BALB/c (H₂DdKd) islets, a 5-fold increase of K₄-NRPV7⁺ CD8⁺ T cells was detected in grafted (13.9 ± 0.9%) vs endogenous (2.8 ± 1.0%) islets (p = 0.003) (Fig. 3). In contrast, in NOD recipients of FVB (H₂KbKb) islets, K₄-NRPV7⁺ CD8⁺ T cells were detected in the graft, but the frequency of tetramer binding CD8⁺ T cells was equivalent to that detected in the endogenous islets (2.7 ± 0.6% vs 3.3 ± 1.9%, respectively) (Fig. 3). These results demonstrate that IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells dominate the early infiltrate of syngeneic islet grafts, and that the frequency of this set of clonotypes is significantly expanded in grafts compared with the endogenous islets.

The TCR repertoire of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells in grafted and endogenous islet infiltrates is restricted and shared To determine the diversity of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells residing in grafted vs endogenous islets, the TCR repertoire of K₄-NRPV7⁺ CD8⁺ T cells was examined in four individual recipients 7 days postimplantation via single-cell sorting and RT-PCR. A total of 53 Vβ TCR sequences were analyzed from K₄-NRPV7⁺ CD8⁺ T cells isolated from grafted and endogenous islets, all of which used the Vα17-Jα42 segment (IMGT nomenclature, TRAV16-TRAJ42) characteristic of IGRP₂₀₆₋₂₁₄-specific clonotypes with a conserved N junction. Analysis of the TCR β-chain revealed preferential usage of Vß8.1 (TRBV13–3), and Jß2.4 (TRBJ2–4) and Jß2.7 (TRBJ2–7) (Fig. 4, A and B). Alignment of the CDR3β segments indicated a restricted number of T cell clones in each recipient, with one or two dominant clonotypes comprising up to 87% of K₄-NRPV7⁺ CD8⁺ T cells analyzed (Fig. 4C). Notably, these clonotypes were found to be dominant in both grafted and endogenous islets of individual recipients (Fig. 4C). However, when the TCR repertoires of K₄-NRPV7⁺ CD8⁺ T cells were compared among the recipients, different sets of clones were detected in each recipient (Fig. 4C). The identity of the dominant clones also differed among the four recipient mice analyzed. Indeed, only two clonotypes with the respective CDR3β usage of SDSAQQNTL and SDCGTYEQ were repeatedly observed (Fig. 4C).

T cells analyzed (Fig. 4C). Taken together, these results indicate that in diabetic NOD mice, the TCR repertoire of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells infiltrating grafted and endogenous islets is shared and limited to a few dominant clonotypes. Furthermore, clonotypic variation exists within IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells among individual recipient mice.

**FIGURE 1.** IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells are the prevalent β cell-specific clonotypes in the islets of 20-wk-old NOD female mice. A. Pooled islet T cell infiltrates from four 20-wk-old NOD female mice were expanded in IL-2-containing medium. ELISpot was used to measure the frequency of IFN-γ-secreting T cells upon restimulation with a panel of MHC class I-restricted peptides (NP, NRP-V7, IGRP₂₀₆₋₂₁₄, InsB₁₅₋₂₃, GAD₆₅₅₋₆₅₄, ProInsB₂₅-C₃₄, and DMK₁₃₈₋₁₄₆). IFN-γ-specific SFC per 10,000 islet-infiltrating lymphocytes is shown after subtraction of background (approximately six SFC) in medium-only wells. Data are representative of four separate experiments. B. The average percentage ±SEM of K₄-NRPV7⁺ (■) and K₄-InsB₁₅₋₂₃⁺ (□) CD8⁺ T cells isolated from islet infiltrates, PLN, and spleens of four individual 20-wk-old NOD female mice was determined. K₄-NP served as a negative control (□).
The specificity of CD8+ T cells infiltrating an islet graft varies in a temporal manner

Next, the frequency of Kd-NRPV7+ CD8+ T cells was examined shortly before graft failure. The percentage of Kd-NRPV7+ CD8+ T cells present in the grafted islets was significantly reduced by day 13 postimplantation (Fig. 5A). An average of 4.7 ± 1.1% of CD8+ T cells bound Kd-NRPV7 tetramers compared with 24.1 ± 4.3% in infiltrates of day 7 grafted islets (p = 0.001). The former was not significantly expanded compared with that detected in the endogenous islets (2.9 ± 1.6%).

To determine whether this reduction was attributed to an influx of non-Kd-NRPV7+ CD8+ T cells, the number of CD4+, CD8+, and Kd-NRPV7+ CD8+ T cells present within the grafted and endogenous islets was analyzed. A 7-fold increase in CD4+ T cells was observed in the islet graft infiltrates between days 7 and 13 (p = 0.006) (Table I). In comparison, the number of CD8+ T cells increased only slightly (1.5-fold) during this period. Strikingly, there was a 3-fold decrease in the number of Kd-NRPV7+ CD8+ T cells detected between days 7 and 13 in the grafted islets (p = 0.02) despite a relatively constant number of CD8+ T cells in the islet graft. Furthermore, the number of Kd-NRPV7+ CD8+ T cells found in grafted and endogenous islets at 13 days postimplantation was equivalent (Table I). In contrast, at day 7 postimplantation, the number of Kd-NRPV7+ CD8+ T cells was increased >5-fold compared with the endogenous islets (Table I). No significant change in T cell numbers was observed in the endogenous islet infiltrates of the recipient mice between the two time points (Table I).

The reduction of Kd-NRPV7+ CD8+ T cells in grafted islets could not be attributed to the influx of InsB-specific or ProIns-specific CD8+ T cells, as staining with Kd-InsB (0.7 ± 0.1%) and Kd-ProIns (0.3 ± 0.3%) tetramers, respectively, was not significantly above that detected with Kd-NP tetramers (0.4 ± 0.04%).

Similar to results observed at 7 days postimplantation, there was a preferential usage of Vβ8.1 (TRBV13–13) with Jβ2.4 (TRBJ2–4) or Jβ2.7 (TRBJ2–7) among Kd-NRPV7+ CD8+ T cells detected in the grafted and endogenous islets (Fig. 5A). The TCR clonotypes of Kd-NRPV7+ CD8+ T cells detected in the grafted and endogenous islets were represented at similar frequencies (Fig. 5D), and the identity of the dominant clonotype(s) varied among the recipient mice. Collectively, these results demonstrate that the TCR repertoire of IGRP206–214-specific CD8+ T cells remains constant as islet graft destruction progresses, but that the number of these CD8+ T cells declines.

**Depletion of IGRP206–214-specific CD8+ T cells delays islet graft rejection**

Because IGRP206–214-specific CD8+ T cells dominated the early pool of graft-infiltrating CD8+ T cells, whether survival of the
were injected i.v. three times with soluble IGRP206–214 in PBS on days 2, 4, and 6 before islet implantation. Two more peptide immunizations were given at 5 and 12 days postislet implantation to ensure continued depletion. Circulating levels of Kd-NRPV7 T cells were detected in peripheral blood before islet transplantation were equally effective in near complete depletion of Kd-NRPV7 CD8+ T cells in peripheral blood and prevented infiltration of IGRP206–214-specific CD8+ T cells into the islet grafts.

The duration of graft survival in untreated and HA peptide-treated transplant recipients was not significantly different, with median graft survival of 15 and 12 days, respectively (Fig. 6). In contrast, islet graft survival in IGRP206–214-treated mice was delayed with a median of 31 days (five mice per treatment group, \( p = 0.05 \), IGRP206–214 vs untreated; \( p = 0.03 \), IGRP206–214 vs HA; log-rank test) (Fig. 6). One IGRP206–214 treated-mouse remained euglycemic at 67 days postimplantation when the experiment was terminated. Recurrent diabetes in the remaining four IGRP206–214-treated mice was not due to reappearance of Kd-NRPV7 CD8+ T cells. For example, a reduced number of Kd-NRPV7 CD8+ T cells was detected in islets implanted in IGRP206–214 vs HA-treated recipient mice (\( p = 0.04 \)) at the time of onset of recurrent diabetes (Table II). No significant binding with Kd-InsB15–23 (0.5 ± 0.3%) or Kd-ProInsB (0.2 ± 0.1%) tetramers was detected in the graft infiltrates of IGRP206–214-treated recipients. These findings demonstrate that depletion of IGRP206–214-specific CD8+ T cells delays islet graft rejection.

**Discussion**

Established autoimmunity in diabetic islet (or pancreas) transplant recipients is an important factor contributing to the failure of subsequent β cell engraftment (23–26). CD4+ and CD8+ T cells have significantly reduced after IGRP206–214 (\( p = 0.002 \)) but not HA peptide immunization (Table II). The frequency of Kd-NRPV7+ CD8+ T cells was also markedly reduced (<0.3%) in graft infiltrates of IGRP206–214-treated recipient mice examined 7 days postislet implantation. This indicates that IGRP206–214 treatment effectively depleted Kd-NRPV7 CD8+ T cells in peripheral blood and prevented infiltration of IGRP206–214-specific CD8+ T cells into the islet grafts.

Transplanted islets could be enhanced by depleting these T cells was investigated. For this purpose, high doses of soluble peptide were administered. Injections of soluble IGRP206–214 or NRP-V7 peptides were equally effective in near complete depletion of Kd-NRPV7 CD8+ T cells (data not shown). Diabetic NOD mice were injected i.v. three times with soluble IGRP206–214 in PBS on 2, 4, and 6 days before islet implantation. Two more peptide immunizations were given at 5 and 12 days postislet implantation to ensure continued depletion. Circulating levels of Kd-NRPV7 CD8+ T cells in peripheral blood before islet transplantation were significantly reduced after IGRP206–214 (\( p = 0.002 \)) but not HA peptide immunization (Table II). The frequency of Kd-NRPV7+ CD8+ T cells was also markedly reduced (<0.3%) in graft infiltrates of IGRP206–214-treated recipient mice examined 7 days postislet implantation. This indicates that IGRP206–214 treatment effectively depleted Kd-NRPV7 CD8+ T cells in peripheral blood and prevented infiltration of IGRP206–214-specific CD8+ T cells into the islet grafts.

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The frequency of K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells decreases as islet graft destruction progresses but the TCR repertoire remains constant. The frequency of K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells in endogenous (□) and grafted (●) islets in diabetic islet recipients was analyzed 13 days postimplantation (n = 8). A, The percentage of staining using control K<sup>a</sup>-NP was <0.5%. The TCR β-chain repertoire of K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells present in grafted and endogenous islets from individual transplant recipients was determined using single-cell RT-PCR at 13 days postimplantation. Averaged frequencies of V<sup>β</sup> (B), J<sup>β</sup> (C), and CDR3<sup>β</sup> (D) gene usage of sorted K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells from four recipients are shown. V<sup>β</sup>8.1, V<sup>β</sup>8.3, V<sup>β</sup>10, and V<sup>β</sup>16 correspond to IMGT nomenclature of TRBV13–3, TRBV13–1, TRBV4, and TRBV3, respectively. A total of 11, 14, and 13 CDR3<sup>β</sup> sequences were analyzed from the endogenous islets of recipients 1, 3, and 4, respectively. TCR sequences were compared with a total of 28, 51, 36, and 20 CDR3<sup>β</sup> sequences derived from the grafted islets of the recipients 1–4, respectively. ND, Not done, islets were not recovered from recipient two for analysis.

A key observation made in this study is that autoimmune destruction of islet grafts is mediated by a restricted repertoire of CD8<sup>+</sup> T cells in the islet grafts. For example, a significant increase in K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells binding K<sup>a</sup>-NRPV7 tetramer (Fig. 2). Attempts to assess graft infiltrates at earlier posttransplantation times were unsuccessful due to insufficient T cell numbers. Detection of IGRP<sub>206–214</sub>-specific CD8<sup>+</sup> T cells in the islet grafts is consistent with reports demonstrating the importance of this set of clonotypes in mediating the progression of β cell destruction in endogenous islets (19, 20). The frequency of K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells at 7 days postimplantation represented an ~6-fold increase in grafted vs endogenous islets (Fig. 2). Expansion of IGRP<sub>206–214</sub>-specific CD8<sup>+</sup> T cells was dependent on H2K<sup>d</sup> expression by the transplanted islets. For example, a significant increase in K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells compared with endogenous islets was detected in BALB/c (H2K<sup>b</sup>) but not FVB (H2K<sup>b</sup>) islets (Fig. 3). This increase in K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells is likely due to direct and indirect

been reported to mediate autoimmune destruction of both allogeneic and syngeneic islet grafts (25, 26, 39–41). To develop effective strategies to induce and monitor islet transplantation tolerance in the clinic, knowledge of the β cell epitopes targeted by T cells and the dynamics of autoimmune-mediated destruction of an islet graft is needed. In the current study, these issues were examined by comparing the repertoire of β cell-specific CD8<sup>+</sup> T cells found infiltrating grafted and endogenous islets in diabetic NOD recipient mice.

A key observation made in this study is that autoimmune destruction of islet grafts is mediated by a restricted repertoire of β cell-specific CD8<sup>+</sup> T cells, which in turn evolves in a time-dependent manner. IGRP<sub>206–214</sub>-specific CD8<sup>+</sup> T cells predominated in graft infiltrates 7 days postimplantation with up to 42% of infiltrating CD8<sup>+</sup> T cells binding K<sup>a</sup>-NRPV7 tetramer (Fig. 2). Attempts to assess graft infiltrates at earlier posttransplantation times were unsuccessful due to insufficient T cell numbers. Detection of IGRP<sub>206–214</sub>-specific CD8<sup>+</sup> T cells in the islet grafts is consistent with reports demonstrating the importance of this set of clonotypes in mediating the progression of β cell destruction in endogenous islets (19, 20). The frequency of K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells at 7 days postimplantation represented an ~6-fold increase in grafted vs endogenous islets (Fig. 2). Expansion of IGRP<sub>206–214</sub>-specific CD8<sup>+</sup> T cells was dependent on H2K<sup>d</sup> expression by the transplanted islets. For example, a significant increase in K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells compared with endogenous islets was detected in BALB/c (H2K<sup>b</sup>) but not FVB (H2K<sup>b</sup>) islets (Fig. 3). This increase in K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells is likely due to direct and indirect

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Table I. CD4<sup>+</sup>, CD8<sup>+</sup>, and K<sup>a</sup>-NRPV7<sup>+</sup> CD8<sup>+</sup> T cells present in islet grafts and endogenous islets at 7 and 13 days posttransplantation

<table>
<thead>
<tr>
<th>Days Posttransplantation</th>
<th>Islet Graft (absolute number per 10,000 gated events)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Endogenous Islets (absolute number per 10,000 gated events)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 7</td>
<td>655 (±241)</td>
<td>826 (±207)</td>
</tr>
<tr>
<td>Day 13</td>
<td>4731 (±707)</td>
<td>1275 (±130)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data represents averaged events (±SEM) from three recipient mice at each time point. A total of 10,000 events were analyzed within the lymphocyte gate based on forward and side scatter, and subsequently were gated on CD4<sup>+</sup>, CD8<sup>+</sup> and CD8<sup>+</sup> K<sup>a</sup>-NRPV7<sup>+</sup> T cells.
presentation of the IGRP206–214 epitope by H2Kd expressing donor β cells and APC residing in the graft, respectively. Albeit reduced relative to NOD and BALB/c islets, a significant frequency of Kd-NRPV7+ CD8+ T cells was also detected in infiltrates of MHC mismatched FVB islets (Fig. 3). This result suggests that, in fully MHC mismatched islet grafts, autoimmune-mediated destruction occurs via cross-presentation and -priming by recipient APC. Notably, the frequency and number of Kd-NRPV7+ CD8+ T cells varied in a temporal manner despite a relatively constant number of CD8+ T cells during infiltration and destruction of syngeneic islet grafts. For instance, a >3-fold reduction in the number of Kd-NRPV7+ CD8+ T cells was detected in NOD islet grafts 13 vs 7 days postimplantation (Table I). The progressive loss of Kd-NRPV7+ CD8+ T cells suggests that IGRP206–214-specific CD8+ T cells are recruited into the islet graft from a finite pool, and undergo expansion and subsequent contraction. A similar profile of expansion and contraction was detected in islet grafts after adoptive transfer of CD8+ T cells isolated from 8.3 TCR NOD transgenic mice (C. P. Wong and R. Tisch, unpublished results). The above findings also suggest that inter- (and intra-) molecular epitope spread occurs in an ordered progression during islet graft destruction. By 13 days postimplantation, IGRP206–214-specific CD8+ T cells are displaced as a major set of clonotypes in the islet graft by other CD8+ T cells that, however, do not include either InsB15–23- and ProInsB25-C34-specific CD8+ T cells. The specificity and diversity of these additional clonotypes are of obvious interest, and need to be defined. These results suggest a scenario in which IGRP206–214-specific CD8+ T cells promote early autoimmune destruction of islet grafts and subsequent epitope spread. Indeed, a delay (albeit short-lived) was detected in the onset of recurrent diabetes in islet graft recipient mice treated with high doses of soluble peptide (Fig. 6) and depleted of IGRP206–214-specific CD8+ T cells (Table II). This delay in islet graft rejection may reflect the recruitment and/or differentiation of sufficient numbers of other pathogenic effectors. These results also indicate that islet graft rejection can be mediated in the absence of IGRP206–214-specific CD8+ T cells.

Single-cell analysis of TCR Vα and Vβ gene usage by Kd-NRPV7+ CD8+ T cells demonstrated that the immunodominant clonotypes mediating β cell destruction in the endogenous islets were also recruited to the islet grafts. All of the sorted Kd-NRPV7+ CD8+ T cells expressed the canonical Vα17-Jα42 element characteristic of IGRP206–214-specific clonotypes (17, 36). However, as determined by CDR3β sequences, up to two dominant clonotypes were detected in the endogenous islets that, in turn, were also found to dominate the islet graft of an individual recipient (Figs. 4 and 5). The diversity of these immunodominant clonotypes may in fact be greater based on recent findings by Santamaria and colleagues (42) showing that three different Vα17 elements are used by IGRP206–214-specific clonotypes. Due to the positioning of primers used in our study, the sequence spanning CDR1α that contains the respective substitutions in the Vα17 elements could not be determined. These findings indicate that the IGRP206–214-specific CD8+ T cells driving early islet graft infiltration are recruited from an already established pool of effector and/or memory T cells as opposed to naive precursors. Immunodominance within the islet graft is likely to be established by clonotypes found at a relatively high frequency and/or exhibiting increased avidity/affinity. Indeed, progression toward overt diabetes in NOD mice corresponds with the expansion of IGRP206–214-specific CD8+ T cells having increased avidity/affinity (20). However, whether recruitment of other β cell-specific clonotypes to the islet graft follow the same “rules” as IGRP206–214-specific CD8+ T cells remains to be determined.

In summary, autoimmune destruction of islet grafts is characterized by a restricted repertoire of β cell-specific CD8+ T cells, and an apparent ordered progression of epitopes that are targeted. Early infiltrates are dominated by established effector and/or memory IGRP206–214-specific CD8+ T cells that are needed for efficient islet graft rejection. Finally, tolerogenic strategies targeting graft-infiltrating β cell-specific CD8+ T cells may prove to be of significant clinical value in preventing recurrent autoimmunity in islet transplantation.

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Disclosures

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References