T cells to a dominant epitope of GAD65 express a public CDR3 motif

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Abstract

Non-obese diabetic (NOD) mice spontaneously develop autoimmune diabetes, and serve as a model for type 1 diabetes (T1D) and natural autoimmunity. T cell responses to the pancreatic islet antigen glutamic acid decarboxylase 65 (GAD65) can be detected in the spleens of young prediabetic NOD mice, which display a unique MHC class II molecule. Here, we report that a distinct TcR β chain and CDR3 motif are utilized by all NOD mice in response to a dominant determinant on GAD65, establishing a public repertoire in the spontaneous autoimmunity to an important islet cell antigen. GAD65 530–543 (p530)-reactive T cells preferentially utilize the Vβ4, Dβ2.1 and Jβ2.7 gene segments, with a CDR3 that is characterized by a triad of amino acids, DWG, preceded by a polar residue. In addition, we used CDR3 length spectratyping, CDR3-specific reverse transcriptase–PCR and direct TcR sequencing to show that the TcR β chain structural patterns associated with p530-specific T cells consistently appeared in the islets of young NOD mice with insulitis, but not in the inflamed islets of streptozotocin-treated C57BL/6 mice, or in inflamed NOD salivary glands. To our knowledge, this is the first report to demonstrate that a public T cell repertoire is used in spontaneous autoimmunity to a dominant self-determinant. These findings suggest that defined clonotypes and repertoires may be preferentially selected in haplotypes predisposed to spontaneous autoimmunity.

Introduction

Type 1 diabetes (T1D) is an inflammatory autoimmune disease characterized by cellular infiltration into the pancreatic islets (insulitis) and the destruction of insulin-producing beta cells (1). The ability to maintain glucose homeostasis is lost when a sufficient number of beta cells have been eliminated, followed by numerous secondary complications that are related to chronic hyperglycemia (1). Animal models such as the non-obese diabetic (NOD) mouse have provided the basis for our understanding of the histological progression from insulitis to overt diabetes, including the phenotypic nature of leukocytes that comprise the inflammatory lesions in the pancreatic islets (2, 3). However, while an influx of T cells seems to be critical to both the inflammatory and destructive phases of insulitis, the antigenic specificity of such cells is still being defined.
to be determined if the early infiltrating autoreactive T cells in the inflamed lesion in spontaneous autoimmune disease are composed of lower affinity clones that survived thymic education, or if particular clonotypes and repertoires are preferentially selected in those individuals (haplotypes) predisposed to autoimmune disease. Given the natural progression to both autoimmunity and autoimmune disease in the NOD mouse, it is well suited for addressing such issues.

To gain insight into the specificity of islet-invasive T cells in NOD mice, previous studies analyzed TcR Vβ expression in the islets of young mice to determine if there is a restricted TcR structural pattern in the inflamed organ (13–16). A narrow TcR Vβ gene usage would be suggestive of a limited determinant specificity and a focused immune response. This type of analysis has provided interesting, but mixed results, with even the most extensive studies falling short of identifying the antigenic specificity of invasive T cells. The challenge has been the recovery of antigen-specific T cells from the earliest stages of inflammation in the islet. The discovery of the antigenic specificity of early arriving T cells could be extremely informative; however, the early stage is also the time point when the number of infiltrating T cells would be most limited, making the harvest of T cells from the inflammatory lesion impractical. Here, we report that a distinct T cell public repertoire is used in the spontaneous autoimmunity to an important beta cell antigen, and that the repertoire is present in the islets of prediabetic NOD mice.

Methods

Mice

Female NOD.Mrk.Tac mice were purchased from Taconic Farms (Germantown, NJ, USA) and bred at the University of Toledo or the La Jolla Institute for Allergy and Immunology. NOD/M\textsuperscript{a} (17) are maintained in a breeding colony at the University of Toledo. C57BL/6 and NOR/Lt mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were age matched and sex matched in all experiments. NOD.BDC2.5 TcR Tg mice (18) were kindly provided by Nora Sarvetnick (The Scripps Research Institute, La Jolla, CA, USA).

Peptides and antigens

GAD65 peptides were synthesized at the UCLA Peptide Synthesis Laboratory on an Advanced Chemtech 395 synthesizer, using F-moc chemistry, and purified using HPLC. Peptide purity was determined by capillary electrophoresis and the composition was verified by mass spectrometry.

Recombinant mGAD65 was produced from BL21(DE3)-pLysS Escherichia coli containing the bacterial expression vector pET3a810-mGAD65. The bacteria and vector were kindly provided by Drs. Agnes Lehuen and Nicolas Glachenhaus. The vector contains the full-length mGAD65 cDNA preceded by an N-terminal haemagglutinin and a 6X-histidine tag. The recombinant protein was purified on Ni-NTA beads as previously described (19).

T cell proliferation assay

Spontaneous and induced proliferative responses were determined as previously described (4, 19). For spontaneous responses, spleen cells (SPCs) were plated at 8 × 10\textsuperscript{3} nucleated cells per well in 96-well flat-bottom plates, using HL-1 serum-free medium (Bio-Whittaker, Walkersville, MD, USA). Peptides were added to a final concentration of 10–40 μg ml\textsuperscript{-1} for 5 days at 37°C in 7% CO\textsubscript{2}, and [\textsuperscript{3}H]-thymidine ([\textsuperscript{3}H]-TdR) (International Chemical and Nuclear, Irvine, CA, USA), 1 μCi per well, was added for the last 16 h. The cells were harvested from microtiter plates using a Micro Cell Harvester (Skatron Instruments, Sterling, VA, USA) and incorporation of label was measured by liquid scintillation counting in an LKB 1205 Betaplate counter. The results were expressed as mean counts per minute of triplicate wells; the standard deviation was <15% in all experiments.

For induced responses, 6- to 10-week old mice were immunized subcutaneously in a hind footpad with 20 μg of peptide emulsified in CFA (DIFCO, Detroit, MI, USA). Popliteal and inguinal lymph nodes and spleens were removed 9–11 days later to prepare single-cell suspensions. Lymph node cells (LNCs) and SPCs were plated in 96-well microtiter plates at 5 × 10\textsuperscript{4} or and 8 × 10\textsuperscript{5} nucleated cells per well, respectively, in X-VIVO-10 medium (Bio-Whittaker) supplemented with 2 × 10\textsuperscript{-5} M 2-mercaptoethanol. The 10 μg ml\textsuperscript{-1} level of peptide was usually optimal for inducing proliferation, which was determined by the incorporation of [\textsuperscript{3}H]-TdR during the last 16 h of a 4-day culture. Tuberculin-purified protein derivative (1:40) was used as a positive control for proliferation.

To measure the proliferative response of T cell lines or clones, 2 × 10\textsuperscript{3} T cells were plated with 2 × 10\textsuperscript{5} irradiated syngeneic SPCs, with or without antigen, in 96-well plates. The plates were pulsed with [\textsuperscript{3}H]-TdR during the last 16 h of a 3-day culture, and then treated in the same manner as the proliferation assays described above. To confirm GAD65 reactivity, T cells were challenged with irradiated SPCs, plus recombinant mGAD65, or with M12C3 cells transfected with the I-A\textsuperscript{β7} beta chain and mouse GAD65, M12C3.g7.GAD65.

T cell lines, clones and hybridomas

T cell lines and clones were produced from the SPCs of untreated NOD mice (spontaneous) or LNCs from peptide-immunized mice (induced). In vitro T cell stimulation was performed using irradiated syngeneic SPCs as antigen-presenting cells (APCs), plus the peptide (10 μg ml\textsuperscript{-1}) of interest. Three to five days later, the cells were expanded in rIL-2-containing medium (10 U ml\textsuperscript{-1}), and subsequently cloned by limiting dilution in 96-well plates containing 2 × 10\textsuperscript{3} peptide-pulsed, irradiated, syngeneic SPCs in IL-2-containing medium.

Antigen-specific T cell hybridomas were created by fusing lymphocytes from peptide-immunized mice with the BWS147 αβ TcR (−/−) cell line (American Type Tissue Collection), as previously described (20). Antigen-responsive hybridomas were identified using the HT-2 bioassay (20) and were cloned by limiting dilution prior to further analysis and TcR gene sequencing. For analysis of BDC2.5 T cells, SPCs from NOD.BDC2.5 TcR Tg mice were stimulated in vitro with 2.5 μg ml\textsuperscript{-1} of Con A for 48 h. mRNA was then purified from the responding blasts.

Cytokine ELISA

Supernatants collected from T cell lines and clones, 24–72 h after antigenic stimulation, were tested for the presence of
IFN-γ, IL-4, IL-5 and IL-10 by ELISA. IL-2 was measured using the HT-2 bioassay (20). Recombinant murine cytokines were used as standards.

**Flow cytometry analysis**

For analysis of TcR Vβ expression, T cell lines, clones and hybridomas were stained with FITC or PE-conjugated antibodies (PharMingen, San Diego, CA, USA) specific for CD3, CD4, αβ TcR or particular TcR Vβ chains, including Vβ2, 3, 4, 5.1, 5.2, 6, 7, 8, 1.1, 8.2, 8.3, 9, 10, 12, 13, 14 and 17a. Stained cells were analyzed on a FACSscan flow cytometer (Becton Dickinson, Mansfield, MA, USA) using CellQuest software (Becton Dickinson).

**Islet extraction and reverse transcription–PCR**

Pancreatic islets were collected and pooled from groups of four to six naive NOD.Mrk.Tac or NOD.M12 mice of various ages (1, 2, 3, 4, 6, 8, 12 and 20 weeks). As a control, pancreatic islets were collected from naive NOD or streptozotocin (STZ)-treated C57BL/6 mice. Briefly, 6- to 10-week old female C57BL/6 mice were treated with STZ (40 mg kg⁻¹, Sigma Chemical, St Louis, MO, USA), as prepared immediately prior to intra-peritoneal injections, for five consecutive days, and were monitored biweekly for glucosuria. STZ-treated mice were sacrificed 3 weeks after the first dose of STZ or after the onset of T1D. Pancreatic tissue from NOD, NOR or STZ-treated C57BL/6 mice was digested with collagenase, as described previously (21), followed by handpicking of the islets under a dissecting microscope. Total RNA was isolated from purified islets using Rneasy or Trizol (GIBCO/BRL, Grand Island, NY, USA), as per the manufacturer’s instructions, and first-strand cDNA synthesis was performed using an oligo-dT primer (dT)15. cDNA fragments were then amplified using Vβ primers (23) and a primer specific for the residues found in the CDR3 of the TcR β chain of GAD530–543-reactive T cells (5'-GTACGTTCAGTGCCCCC-CCAGTC-3') or a primer specific for the residues found in the CDR3 of the TcR β chain of the BDC2.5 T cell clone (5'-GTAGTCGGAGTTGTTGTTGTC-3'). To enhance the amplification of specific PCR products/template touchdown PCRs were performed using Amplitaq Gold (Applied Biosystems, Foster, CA, USA) and annealment temperatures close to that of the melting temperature (Tm) for the oligo with the lowest Tm. During the touchdown PCR, eight cycles were performed as follows: 94°C for 5 min, 94°C for 45 s, 66°C for 45 s (0.5°C decrease per cycle) and 72°C for 45 s. The PCR conditions were then adjusted to twenty-five cycles of 94°C for 45 s, 61°C for 45 s and 72°C for 45 s.

**Sequencing Vβ genes**

The Vβ4-Cβ expansions discovered by immunoscope were re-amplified with a Vβ4-Jβ2.7 or Vβ4-Jβ1.2 set of primers and cloned into the pCR2.1 cloning vector from the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Individual colonies were grown up in 5 ml Luria–Bertani medium and plasmid DNA was isolated using a Qiagen miniprep kit. Sequencing reactions were carried out directly on the plasmid DNA using an M13 primer and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit following the manufacturer’s instructions (Applied Biosystems). Reaction products were then loaded on a DNA sequencer (ABI PRISM 310; Applied Biosystems), and CDR3 region sequences were analyzed using standard software (Applied Biosystems).

**Adoptive transfer**

The NOD-derived T cell clone NOD.530.1 was re-stimulated with peptide-pulsed (p530) irradiated syngeneic SPCs. Seventy-two hours later, the cells were washed twice with serum-free RPMI-1640 and then adoptively transferred into the peritoneal cavities of three to six NOD.scid mice. Thereafter, the mice were tested twice a week for urine glucose, while blood glucose levels were tested biweekly by glucometer (Roche Diagnostics, Mannheim, Germany). As a control, SPCs from newly diabetic NOD mice were transferred into NOD.scid mice. Mice were considered diabetic when blood readings resulting products were then denatured in formamide and analyzed on an Applied Biosystems 310 Prism sequencer (Applied Biosystems, Foster, CA, USA) using GeneScan 2.0 software. The relative index of stimulation (RIS) value was calculated as the area under the experimental peak divided by the area under the control peak found within a Gaussian distribution. Peaks were normalized prior to division. Control peaks were obtained from CFA-immunized animals, naive animals or unaffected lymph nodes, which all typically gave equivalent RIS values.

**CDR3-specific RT-PCR**

For CDR3-specific analysis, a nested PCR was performed using Vβ4 and Jβ2.7 primers for GAD530–543-reactive T cells or Vβ4 and Jβ1.2 primers for BDC2.5-like T cells, as described for the RT-PCR protocol above. One microliter of product from this first-round reaction was used as a template in a second round of amplifications using Vβ4 and a primer specific for the conserved residues in the CDR3 of the TcR β chain of GAD530–543-reactive T cells (5'-GTACGTTCAGTGCCCCC-CCAGTC-3') or a primer specific for the residues found in the CDR3 of the TcR β chain of the BDC2.5 T cell clone (5'-GTAGTCGGAGTTGTTGTTGTC-3'). To enhance the amplification of specific PCR products/template touchdown PCRs were performed using Amplitaq Gold (Applied Biosystems, Foster, CA, USA) and annealment temperatures close to that of the melting temperature (Tm) for the oligo with the lowest Tm. During the touchdown PCR, eight cycles were performed as follows: 94°C for 5 min, 94°C for 45 s, 66°C for 45 s (0.5°C decrease per cycle) and 72°C for 45 s. The PCR conditions were then adjusted to twenty-five cycles of 94°C for 45 s, 61°C for 45 s and 72°C for 45 s.

**TCR repertoire analysis (CDR3 length spectratyping)**

Repertoire analyses were performed using a modified protocol described by Pannetier et al. (22). Total RNA was isolated from antigen-induced blasting LNC suspensions of individual mice (RNasey, Qiagen) and cDNA syntheses were then performed using an oligo-dT primer (dT)15 (GIBCO BRL, Carlsbad, CA, USA). From each cDNA, RT–PCRs were then performed using Vβ primers (23) and a common Cβ primer (5'-CAGTC-3'), using Vβ genes and then 12 cycles of amplification (94°C for 45 s, 60°C for 45 s and 72°C for 45 s) followed by a 1-min extension at 72°C. The resulting products were then denatured in formamide and analyzed on an Applied Biosystems 310 Prism sequencer (Applied Biosystems, Foster, CA, USA) using GeneScan 2.0 software. The relative index of stimulation (RIS) value was calculated as the area under the experimental peak divided by the area under the control peak found within a Gaussian distribution. Peaks were normalized prior to division. Control peaks were obtained from CFA-immunized animals, naive animals or unaffected lymph nodes, which all typically gave equivalent RIS values.
were >250 mg dl⁻¹. Prior to sacrifice, the blood glucose level on all recipients was tested. The pancreases from all recipients were harvested, fixed in buffered formalin, and then paraffin embedded. Hematoxylin- and eosin-stained sections from each tissue block were examined for insulinitis by blinded observers.

Results

A TcR β chain structural motif is associated with the response to p530 in NOD mice

Previously, we demonstrated that the earliest arising GAD65-specific T cells in NOD mice recognized the determinant 530–543 (p530) and preferentially utilized the Vβ4 TcR gene (19). In this report, the genes encoding the TcR β chain of p530-specific T cells were sequenced to further characterize the structure of the TcR used in this response. A panel of distinct T cell hybridomas was produced from the SPCs of three separate p530-immunized female NOD mice. In addition, the TcR genes of two spontaneously arising p530-reactive T cell lines were also sequenced—the T cell line NOD-spont-10 and the T cell clone NOD-spont-6 were developed from the spleens of untreated, prediabetic, female NOD mice. Prior to mRNA extraction and cDNA preparation, the T cell populations were analyzed by FACS to confirm αβ TcR expression (data not shown). As expected, each of the p530-reactive T cells utilized the Vβ4 TcR gene segment (Table 1). This was not due to a general preference of Vβ4+ T cells in the NOD repertoire, as flow cytometry revealed that Vβ8+ T cells were the most prevalent family in the spleen and lymph nodes of naive mice (data not shown). When we examined the expression pattern of the other TcR chain-encoding elements, we found that the DJβ2.1 gene was also exclusively utilized by p530-reactive T cells (Table 2). Furthermore, there was a distinct preferential usage of the Jβ2.7 gene (Table 2); although the gene segments Jβ2.3 and Jβ2.1 were also utilized, it was far less frequently than Jβ2.7 (Table 1). These results reveal a TcR gene utilization pattern in which there is restriction in two of the three TcR gene segments that encode the CDR3 of the β chain, and a strong preference in the third. Additionally, an analysis of the CDR3, and the VD and DJ junctions, revealed an amino acid motif, comprised of an aspartic acid, tryptophan and glycine triad (DWG), which was preceded by a polar residue (glutamine or arginine) in all of the Jβ2.7-expressing T cells that were sequenced (Table 2), regardless of the length of the CDR3 region, or whether the T cells were from p530-immunized mice or naive NOD mice (Table 2). The glutamine residues were encoded by two nucleotides from the Vβ4 gene, ca, while the third nucleotide, a or g, was encoded by a non-template mechanism (N addition) (Table 2). In contrast, the arginine was encoded by a single nucleotide from the Vβ4 gene, c, and two non-template-encoded nucleotides, gg (Table 2). An exception to this CDR3 motif was observed in two p530-reactive T cells (530.38.3 and 530.6.2), which utilized a leucine residue in the position immediately preceding the DWG motif (Table 2). Similar to the previous T cells, the leucine in the CDR3 was encoded by Vβ4 and non-template elements (Table 2). The CDR3 lengths in the β chain of the p530-reactive T cells varied between 8 and 10 residues (Table 2), as did the nucleotide composition of the CDR3s, confirming that these were distinct T clones; however, CDR3 lengths of eight residues were most prevalent among the T cells. Interestingly, the hybridoma 530.97.3 and two transcripts from the spont-10 line expressed identical Vβ TcR chains (Table 2), suggesting that immunization with p530 did not alter the repertoire but led to the expansion of the same T cell population that was spontaneously primed in vivo. The DWG motif was also seen in the few clones that did not express Jβ2.7 (data not shown).

Table 1. p530-reactive T cells demonstrate a limited TcR structural pattern

<table>
<thead>
<tr>
<th>Clone</th>
<th>T cella</th>
<th>Sourceb</th>
<th>Strainc/colony</th>
<th>Responsive to GAD65d</th>
<th>TcR</th>
<th>Vβ</th>
<th>Jβ</th>
</tr>
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<tr>
<td>530.38.3</td>
<td>hyb</td>
<td>Imm</td>
<td>Tac/LIAI</td>
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<td>4</td>
<td>2.7</td>
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<td>530.6.2</td>
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<td>Imm</td>
<td>Tac/LIAI</td>
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<td>2.7</td>
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<tr>
<td>530.97.3</td>
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<td>Imm</td>
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<td>2.7</td>
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<td>4a</td>
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<td>530.170.6</td>
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<td>530.136.2</td>
<td>hyb</td>
<td>Imm</td>
<td>Tac/LIAI</td>
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<td>4a</td>
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n.d. = not determined. ahyb = T cell hybridomas, clone = T cell clone or line = T cell line. bT cells were produced from the spleens of naive or p530–543-immunized NOD female mice. cT cells were produced from NOD Mrk.Tac (Tac) or NOD.M² (M²) mice, housed at the La Jolla Institute for Allergy and Immunology (LIAI) or the University of Toledo (UT). dT cells were tested for reactivity with recombinant mGAD65 protein by proliferation and/or cytokine ELISA. eFACS analysis revealed that >50% of CD4+ T cells expressed Vβ4 after an initial in vitro stimulation with p530–543, and >70% after a second stimulation.
Table 2. TcR transcripts similar to p530-reactive T cells are found in the islets of young NOD mice

<table>
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<tr>
<th>Clone</th>
<th>Vβ4 FWb</th>
<th>CDR3a</th>
<th>Jβ</th>
<th>CDR3 length</th>
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<td>8</td>
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<td>YFG 2.7</td>
<td>8</td>
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aSequences found in the CDR3, according to Arden et al. (70). bSequences from the framework region of the TcR. cNucleotides encoded by DJβ2.1 are bolded. dhyb, sequence of T cell hybridomas isolated from p530-immunized mice. eSequence of T cell line produced from spleens of naive NOD female mice. fTcR transcripts harvested from the islets of 4-week-old NOD mice.

GAD65-like TcR sequences in pancreases

To determine if the TcR sequences that display motifs associated with p530-reactive T cells were present in the islets of young NOD mice, cDNA preps from the islets of 4- or 5-week-old female NOD mice were PCR amplified using Vβ4 and Jβ2.3, Jβ2.7 or a Cβ-specific primer and were then directly sequenced. Two TcR sequences were detected that displayed the prototypical DWG motif found in the CDR3 of p530-specific T cells (Table 2). An islet-derived clone, islet-3067.A, differed from two p530-reactive T cells recovered from NOD mice, 530.97.3 and spont-10 (Table 2) by a single amino acid substitution (arginine to glutamine) at the second position of the CDR3. Like the p530-reactive T cell hybridomas, the glutamine residue in the islet-derived TcR β chain is encoded by non-template bases (Table 2). A second islet-derived sequence, 3067.JB1.2-8, differed from p530-reactive T cells by the insertion of a non-template-encoded histidine, between the second and third residues of the CDR3, resulting in a slightly longer CDR3 sequence (Table 2). Thus, both islet-derived sequences displayed p530-like features in their TcR β chain, including the polar amino acid that precedes the DWG. It is worth noting again that the TcR clone, spont-10, was obtained from untreated NOD mice.

CDR3 length spectratyping (immunoscope) reveals a public repertoire in the NOD GAD65 p530 T cell response

Next, CDR3 spectratyping was performed on p530-responding blasts in individual NOD mice to determine the prevalence of the structural pattern associated with the clones in Tables 1 and 2. RNA was extracted from a p530-specific T cell line that displayed the most common CDR3 length, NOD-spont-10 (Tables 1 and 2), to establish the CDR3 spectratypic signature pattern for the β chain used by our p530-T cell clones, which was then compared with the LNCs of four p530-immunized NOD mice. The T cells and LNCs were re-stimulated in vitro with p530 prior to mRNA isolation. cDNA transcribed from the mRNA was subjected to CDR3 length spectratyping using primers specific for the Vβ4 TcR gene segment, in combination with primers specific for each of the 12 Jβ segments (Fig. 1a). When mRNA from the NOD-spont-10 T cell line was analyzed, expansions were only detected when a combination of Vβ4 and Jβ2.7 specific primers was used, and it produced the prototypical eight amino acid CDR3 seen in the immunized mice (Fig. 1b). Interestingly, reproducible expansions were observed in the LNCs of all p530-immunized mice when the combination of Vβ4 with Jβ2.7 specific primers were used (Fig. 1a); which amplify the predominant TcR gene segments selected by our panel of T cells (Table 2). On the other hand, three of four animals showed expansions when Vβ4 and Jβ2.3 primers were used in the analysis (Fig. 1a), a combination used by two of the T cells in our panel (Table 1). In multiple experiments, these Vβ4-Jβ2.3- and Vβ4-Jβ2.7-containing expansions were identified by the characteristic 9- to 8-amino acid long CDR3’s, respectively (data not shown). The specific expansion of Vβ4/Jβ2.7-expressing p530-reactive T cells in all NOD mice tested indicates that it represents a public repertoire, and suggests that the TcR β chain motif encodes a selective advantage for these particular T cell clones. A mouse TcR public repertoire is defined as a subset of antigen-specific T cells, with a defined TcR structure, which are present in all members of a given mouse strain. In addition to the focused public repertoire (Vβ4/Jβ2.7), numerous private repertoires (Vβ × Jβ combinations) were also present in the mice, some of which were only displayed in a single animal (Fig. 1a).

To investigate if p530-reactive T cells might contribute to the early stages of inflammation in the pancreatic islets, pooled purified pancreatic islets from six NOD mice was used as a source of mRNA templates for cDNA synthesis. A comparison
of the spectratype from the cDNA of islets from 5-week-old naive NOD mice demonstrated that T cells expressing TcR β chains with the p530-responsive profile were present in prediabetic mice and in the early stages of islet inflammation (Fig. 1b).

To examine the temporal expression of these TcR transcripts in the islets, mRNA was extracted from the pooled islets of female NOD mice, 2–20 weeks of age. Initially, the islet cDNA was examined for the expression of CD3e, Vb4 and β-actin by RT-PCR (Fig. 2) to serve as an indicator of T cell infiltration, or as an internal template control. It should be noted that we chose to limit the sensitivity of our PCRs (cycles) to decrease the likelihood of generating false positives. We were not able to consistently detect CD3e-specific amplicons in the islets of mice <3 weeks of age (Fig. 2) and only those samples that were positive for CD3e were selected for further examination.

Using CDR3 spectratyping, we observed significant p530-like expansions in the islets of 4-week-old NOD mice (Fig. 3; relative stimulation index = 5.0) when the primers were specific for Vb4 and Jb2.7. These responses became Gaussian or normalized as the mice aged and the CDR3 expression pattern became more heterogeneous, such that by 8 weeks of age, no selective expansion of p530-like amplicons (RIS < 3) could be detected (Fig. 3) when compared with the controls (quiescent SPCs or Con A blasts, data not shown). This TcR β chain expression pattern was found in both NOD.Mrk.Tac and NOD.M2 mice, and the experiments were performed at least twice for each age group. Similar patterns of responses were found in male NOD.Mrk.Tac mice (data not shown). These results clearly indicate that T cells with TcR structural features identical to those of the public p530-specific T cell clones could be found in the islets of very young NOD mice.

**BDC2.5-like clones are detected in the islets by spectratypic analysis**

To determine if the influx of p530-reactive T cells into the pancreas was part of a catastrophic breakdown in self-tolerance in the islets, we characterized the temporal appearance of another islet-specific T cell in the pancreas. The BDC2.5 T cell clone was originally recovered from the peripheral lymphoid tissue of a diabetic NOD mouse and was subsequently shown to be diabetogenic in adoptive transfer experiments (24). To establish the CDR3 spectratypic pattern of the BDC2.5 clone, we performed analyses using RNA recovered from the peripheral lymphoid tissue of a diabetic NOD mouse and was subsequently shown to be diabetogenic in adoptive transfer experiments (24). To establish the CDR3 spectratypic pattern of the BDC2.5 clone, we performed analyses using RNA recovered from the BDC2.5 T cell clone or Con A blasts produced from the splenocytes of BDC2.5 TcR transgenic mice. Spectratypic analysis revealed a single peak for BDC2.5...
when primers for Vβ4 and Jβ1.2 were used (Fig. 4a). No amplicons were observed when using the BDC2.5 cDNA in conjunction with any other combination of Vβ4 and Jβ1.2 primers (data not shown), which is in accordance with the published TcR sequence of the BDC2.5 clone showing that it utilizes the Vβ4 and Jβ1.2 gene segments (25). When we analyzed the panel of cDNAs from the NOD islets, we were unable to detect significant BDC2.5-like expansions in islets of young mice 4–5 weeks of age (RIS < 3) (Fig. 4a), suggesting that the emergence of BDC2.5 clonotype was not coincident with the p530-like T cells. To confirm this point, CDR3-specific primers were used to study the temporal expression of the BDC2.5 TcR idiotypic expansions in islets of young mice 4–5 weeks of age (Fig. 4b). In contrast to the p530 motif, BDC2.5-like amplification products were only detected consistently in mice that were more than 10 weeks of age, thus supporting the results from spectratyping analysis. Together, these findings suggest that during the early stages of islet inflammation in our young NOD mice, T cells with a limited number of antigenic specificities can be detected.

**CDR3-specific RT-PCR**

The clear TcR Vβ, Dβ, and Jβ restriction patterns observed in the p530-specific response of NOD mice provide an opportunity to use CDR3-specific primers to determine the age at
which the prototypic p530 TcR motif first appears in the islets of NOD mice. cDNA prepared from the pooled islets of 2-, 3-, 4-, 5-, 8-, 12- or 20-week-old NOD mice were first amplified by PCR using a Vβ4 primer and a Jβ2.7 primer (Fig. 5). The amplicons were then re-amplified using the Vβ4 primer and a primer that contained nucleotide sequences complementary to the bases that encode the CDR3 of the spont-10 T cell line—DWG amino acid triad in the Dβ2.1 gene and a portion of the Jβ2.7 gene. These primers were shown to specifically amplify p530-like TcR transcripts (Fig. 5). The resulting amplicons would only come from mRNA templates that were encoded by Vβ4, Dβ2.1 and Jβ2.7 gene segments. Amplicons were detected in islets as early as 4 weeks of age, and persisted until at least 9 weeks of age (Fig. 5). In some experiments, amplicons were detected in mice as old as 28 weeks of age (Fig. 6a); however, the responses consistently peaked in 4- to 8-week old mice. The amplicons were detectable in both the NOD.Mrk.Tac and NOD.M1T colonies. As a control, mRNA collected from the salivary glands of 10-week-old NOD mice were analyzed to determine if p530-induced expansions could be detected in other inflamed tissues.

Despite the fact that salivary glands in NOD mice become inflamed (26), and here, showed signs of infiltration (positive responses in CDS3-specific RT–PCR analysis, data not shown), no amplicons were seen when p530-CDR3-specific primers were used to analyze the salivary tissues (Fig. 6a). Spectratypic analysis of salivary gland-derived cDNA with primers specific for Vβ4 and Jβ2.7 also failed to detect p530-like expansions (Fig. 6b). These findings demonstrated that the p530-like expansions seen in the pancreas were not the result of random accumulations of T cells into inflamed sites, but show that they were target organ specific.

NOD mice are unique in their spontaneous development of insulitis and T1D; however, diabetes can be induced in otherwise normal C57BL/6 mice following multiple injections of selected doses of STZ. To determine if p530 responses were associated with islet inflammation in other mouse strains or haplotypes, C57BL/6 mice were treated with STZ (40 mg kg$^{-1}$) on five consecutive days. Three weeks after the first treatment, the point at which insulitis appeared histologically in C57BL/6 mice, mRNA was extracted from islet cells and examined by RT–PCR. In addition, islet mRNA was collected from 4- to 8-week old NOD female mice and 4-week-old NOR mice. The islets of NOR/Lt mice are a good control for non-specific amplifications since these mice share almost 90% of their genes with NOD mice, yet they remain free of insulinis and diabetes. While we could find expression of 3C3 (Fig. 6c) in the C57BL/6 sample, no Vβ4 TCR expression was detectable (Fig. 6c), even upon re-amplification (data not shown). This indicates that preferential expression of the p530 CDR3 motif is not associated with the early stages of pancreatic insulitis in C57BL/6 mice. Additionally, we find that C57BL/6 mice are unable to mount proliferative responses to the GAD p530 peptide, even in response to active immunization with the peptide in CFA (Fig. 6d). Analysis of NOR islets showed that CD3e, Vβ4 (Fig. 6c) or CDR3 specific amplicons (data not shown) were not detected in the islets of young NOR mice, while CD3e and Vβ4 amplicons were easily found in NOR SPCs (data not shown). These results show that the Vβ4 amplicons detected in NOD mice are specific for inflamed islets and a characteristic of the NOD strain.

**Fig. 5.** Expression of CDR3-specific TcR transcripts in the islets. The islet-specific cDNAs from Fig. 2 were amplified in RT–PCRs to examine the expression pattern of TcR transcripts that contained the CDR3 elements (Vβ4, Dβ2.1 and Jβ2.7), which are displayed in the p530-specific response. PCRs were first performed with a combination of primers specific for Vβ4 and Jβ2.7. Products from those reactions were then re-amplified using a primer specific for complementary for the sequences found in the CDR3 of p530-specific T cells (5’-GTACTGTTCAGTGCCCCCCCAGTC-3’, residues in Dβ2.1 and Jβ2.7) in conjunction with the Vβ4-specific primer. T cell blasts from NOD.BDC2.5 TcR Tg mice, p530-immunized NOD mice and the p530-reactive T cell line ‘Spont-10’ served as controls.

**p530-specific T cell clone transfers insulin into NOD.scid mice**

We have isolated a p530-reactive T cell clone that expresses the canonical TcR Vβ4 chain structure. The NOD-derived Tn clone NOD.530.1 proliferates and produces IFN-γ in the presence of GAD65-expressing APCs (M12C3.g7.GAD65) (Fig. 7a) or when cultured with GAD65 protein and syngeneic APC (data not shown). In three separate experiments, this clone induced periinsulitis when adoptively transferred into NOD.scid mice (Fig. 7b). However, in spite of the pronounced infiltration, the NOD.scid recipients did not progress to T1D. In parallel experiments, we saw no insulitis in NOD.scid mice that received the Th cell clone 268.11 (Fig. 7b), which produces IFN-γ in the presence of GAD65 peptide 268–288 (Fig. 7a).

**Discussion**

We have identified a public T cell repertoire in the cellular immune response to a dominant determinant of GAD65 in NOD mice. Earlier studies showed that T cells of specificities
directed against the p524-543 fragment of GAD65 were among the first to spontaneously appear in the NOD spleen (4, 27). Previously, we mapped the fine specificity of the endogenous p524-543 response to the p530–543 moiety of the longer peptide, and discovered that the responding T cells utilized the Vβ4 TcR gene almost exclusively (19). Here, we demonstrate that the p530-specific T cells in the NOD mouse are not only restricted in Vβ gene usage but also there is an apparent selection pressure for clones of limited diversity in DJβ gene and Jβ gene usage. A major finding of this report is that a distinct TcR β chain and CDR3 motif are used by all NOD mice in response to p530–543, thus establishing a public repertoire in response to an important beta cell antigen. To our knowledge this is the first report to demonstrate that a public T cell repertoire is used in spontaneous autoimmunity to a dominant self-determinant.

A critical aspect of autoimmunity is the selection and expansion of autoreactive T cells. There is an established association between the expression of unique MHC alleles and susceptibility to particular autoimmune diseases (28–31); yet the precise role of the susceptible alleles is still unclear. Of significance is whether susceptibility to autoimmune disease relates to the activation and expansion of autoreactive lymphocytes in general, or do specific T cell repertoires become engaged in individuals that express the relevant MHC molecules. If the spontaneous T cell response to a self-epitope is dominated by a public repertoire, this would indicate that the responding clones are not comprised of a random array of clones of lesser affinity that happen to have escaped thymic selection, but instead suggests that the clones have been selected on specific MHC molecules, and preserved in certain genetic backgrounds. The immune response to GAD65 is used as an indicator of disease progression in T1D (32–34), and it continues to be a source of interest for predicting susceptibility in humans (35, 36). Interestingly, HLA-DQ8, a human class II MHC molecule associated with increased risk of T1D, shares structural features with the NOD I-Ag7 molecule (37, 38). Remarkably, these similarities may extend to functional relevance, as the DQ8 molecule can substitute for I-Ag7 in the selection of islet-reactive T cells (39, 40), including those with specificity for GAD65 (39, 41). Our findings provide an incentive to investigate the structure of TcRs in DQ8-restricted responses to islet antigens such as GAD65 to determine if public repertoires contribute to beta cell autoimmunity in human T1D as well.

Limitations in TcR diversity to self-antigens is not a novel observation—cellular immune responses in some inducible autoimmune diseases are dominated by Vβ8+ T cells, including experimental autoimmune encephalomyelitis (42, 43) and collagen induced arthritis (44–46). However, our report is unique in that each of the three gene elements that encode

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**Fig. 6.** Expression of p530-like TcR transcripts is organ- and strain specific. p530-like TcR transcripts are not found in the salivary glands of NOD mice. (a) mRNA extracted from purified islets of NOD mice at various ages or from inflamed salivary glands of 10- to 11-week old NOD mice were used to synthesize cDNA. CDR3-specific RT–PCRs were performed as in Fig. 5. (b) Salivary gland cDNA was examined by CDR3 length spectratyping, using primers specific for the Vβ4 and and Jβ2.7 gene segments, as described in Figs 1 and 3. The arrow points to the peak that would be expected with p530-specific T cells. (c) Vβ4 TcRs are not associated with insulitis in C57BL/6 mice. mRNA extracted from islets of three naive female NOD (4–8 weeks old), NOR (4 weeks old) or STZ-treated female C57BL/6 mice (6 weeks old) was used to synthesize cDNA and perform RT–PCR with a combination of primers specific for CD3ε or Vβ4/Jβ. (d) The p530 determinant is not immunogenic in C57BL/6 mice. LNCs from groups of three female, p530-immunized, NOD or C57BL/6 mice were tested for proliferative recall response to p530 in a thymidine incorporation assay. The results are expressed as stimulation indices in response to in vitro challenge with p530 peptide or anti-CD3.
the V region of the TcR β chain was restricted in the p530 autoimmune response. Furthermore, this idioptic TcR structure was observed in p530-reactive T cells isolated from both naive and p530-immunized NOD mice. T cells recognizing an epitope on the insulin beta chain reportedly show a constrained TcR α chain pattern in the NOD mouse (47, 48); however, it remains to be determined if it represents a public repertoire. Based on sequence data from five p530-specific T cell clones, we found that at least three different Va gene (Va2, 9 and 13) and three different Ja genes (Ja20, 44 and 47) can contribute to the CDR3α in the 530–543 response in NOD mice (data not shown). The heterogeneity of the TcR α chain suggests that the majority of the specificity for 530-543–I-A^7 complex is encoded in the TcR β chain. This structural phenotype contrast with that of insulin β9-23-specific T cells, where a number of different TcR Vβ chains are paired with TcR Va13, suggesting that the TcR α chain encodes majority of the specificity in the anti-insulin response in NOD (47). Therefore, restricted TcR Vβ usage is not a general mechanism used by autoreactive T cells in NOD mice, but may be unique to certain specificities. The preference for the Vβ4 chain in p530 reactivity is not seen in NOD-derived T cells specific for hen egg lysozyme or other self-antigen such as myelin oligodendrocyte glycoprotein (S. Mayo and A. Quinn, unpublished results).

The spontaneous development of autoimmune disease in the NOD mouse not only provides us with a remarkable model of T1D diabetes but also provides a framework for understanding T cell-mediated autoimmune diseases in general. One of the essential questions concerning inflammatory autoimmune disease is the nature of the initial infiltrating cells. Discovering whether spontaneous insulitis, which precedes T1D in mice and rats, begins by a random accumulation of lymphocytes into the pancreatic islets or if it is initiated by organ-specific lymphocytes is fundamental to our understanding of susceptibility and progression of the disease. In addressing this issue, it is reasonable to select a limited number of antigenic specificities for analysis. Although several islet cell antigens are likely involved in the development of overt diabetes, because of its spontaneous and early appearance (4, 27) we have utilized the response to the 530–543 determinant on GAD65 as a model to investigate the initiation beta cell-specific autoimmunity. A chief obstacle to providing answers regarding the initial inflammatory lesion rests with the difficulty of isolating antigen-specific T cells at time points when there are few T cells invading the islets—a systemic total of 100 islet-specific T cells may be sufficient to instigate inflammatory disease (49). We face a conundrum wherein

Fig. 7. The p530-reactive T cell clone NOD.530.1 induces insulitis. (a) The p530-reactive T cell clone NOD.530.1 and the p268-reactive T cell clone 268.11 were co-cultured with M12C3.g7.GAD65 cells (I-A^7 and GAD65 expressing), M12C3.g7 cells (I-A^7 expressing) or M12C3.g7 cells pulsed with GAD65 peptides 530–543 or 268–288 (10 μg ml^-1). Forty-eight hours later, the culture supernatants were assayed for IFN-γ by ELISA. (b) A total of 1.0 × 10^6 NOD.530.1 or NOD268.11 T cells were injected intra-peritoneally into 8- to 10-week old NOD.scid mice. Ten days later, the pancreas was collected, formalin-fixed and sectioned. Five micrometer sections were stained with hematoxylin and eosin and examined at 10× and 40×. The arrow points to an infiltration of T cells and perinsulitis. The experiments were repeated three times in groups of three to six mice each.
more cells are available at later time points, for example, 12 weeks of age, although, the invading T cells become more heterogeneous as the lesion progresses, failing to provide information about the earliest arriving T cells. To overcome this obstacle and to satisfy the need for both specificity and sensitivity, we utilized RT-PCR and immune spectratyping to help understand beta cell autoimmunity. RT-PCR has the advantage of being extremely sensitive while maintaining specificity. RT-PCR analysis has been successful in studying gene expression using as few as 10 cells (50, 51), while spectratyping is designed to measure major expansions (RT-PCR amplicons) whether they are limited to very few V genes or to a broader assortment. We examined islets from NOD mice at 4, 8 or 12 weeks of age and established that the TcR structural features associated with the anti-GAD65 response are present in the islets of very young mice, but not in the inflamed salivary glands. The pattern of early appearance and subsequent resolution has been observed with T cells of other specificities (52), both in the spleen and in the target organ. While we were able to isolate TcR transcripts from the islets that expressed all the canonical structural features of the TcRs of p530-reactive T cells (Vβ- and DJβ-1-encoded regions, the DWG motif in CDR3 and a Q or R residue preceding the DWG residues) as well as the predominant DJβ7-encoded elements and similar sized CDR3 lengths, we cannot definitively state that these Vβ chains are from p530-reactive T cells. However, the data are highly suggestive and consistent with such a conclusion. There are at least 24 Vβ, 12 Jβ and 2 DJβ murine TcR genes. We have shown that 1 out of the 588 possible combinations of Vβ, DJβ and Jβ genes is used in the public repertoire of p530-reactive T cells, and that this combination is naturally and significantly expanded in the islets of young NOD. Moreover, since the typical CDR3 of a murine TcR β chain is 6- to 13-amino acids long, there are 2304 possible combination of Vβ, Jβ and CDR3 lengths discernable by CDR3 spectratyping. We find that the predominant pattern of Vβ, Jβ and CDR3 size used by p530-specific T cells (1/2304) is significantly expanded in the islets of young NOD mice, but not in other inflamed tissues of these animals, or in the islets of C57BL/6 mice with insulitis.

The contribution of GAD65 to T1D in NOD mice remains controversial (53-56), despite the fact that GAD65-reactive T cells have been shown to transfer T1D to NOD.scid mice (57), and other reports showing an association with GAD65 and beta cell damage (39, 57-60) (Yang Dai and E. Sercarz, unpublished results). Furthermore, treatments that tolerize, deviate or alter the anti-GAD65 response in young mice seem to provide some measure of protection from diabetes (4, 19, 27). We have isolated a p530-reactive T cell clone that transfers insulitis into NOD.scid mice, but does not incite T1D. In parallel control experiments, we did not see islet infiltration in recipients that received T cells specific for another GAD65 determinant. We know that T1D does not occur in the absence of insulitis, yet insulitis does not always progress to T1D (61, 62). Therefore, it is possible that the mechanisms and clones that govern early peripheral insulitis are distinct from those that dominate invasive malignant insulitis and cause overt beta cell damage (63, 64). Collectively, previous findings suggest that immune responses to GAD65 make a contribution to the development of T1D, although other antigens are also diabetogenic, and may be able to prompt disease in the absence of GAD65. Similar to the NOD mouse, humoral and cellular responses to GAD65 have also been detected in the peripheral blood of recent-onset T1D patients (65). It is clear that GAD65 is one of a few beta cell antigens for which antibodies, Tn, and CTLs have been found in prediabetic NOD (4, 27, 66), recent onset T1D in humans (67) and in first-degree at-risk relatives of patients with T1D (68, 69). Thus, despite the controversy regarding its role in T1D in NOD mice, GAD65 remains an important auto-antigen in human T1D, particularly as a model for spontaneous beta cell autoimmunity to a protein that remains fairly sequestered. The prevention of diabetes necessitates definition of the events prior to the establishment and expansion of diabetogenic T cells. An understanding of the progression and consequences of these earliest events in islet inflammation is necessary to reveal the mechanisms that lead to the propagation of T1D.

Acknowledgements

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>GAD65</td>
<td>glutamic acid decarboxylase 65</td>
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<tr>
<td>[3H]TdR</td>
<td>[3H]-tritiated thymidine</td>
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<td>LNC</td>
<td>lymph node cell</td>
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<td>NOD</td>
<td>Non-obese diabetic</td>
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<td>relative index of stimulation</td>
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<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
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