

# T-Cell Promiscuity in Autoimmune Diabetes

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**OBJECTIVE**—It is well established that the primary mediators of  $\beta$ -cell destruction in type 1 diabetes are T-cells. Nevertheless, the molecular basis for recognition of  $\beta$ -cell-specific epitopes by pathogenic T-cells remains ill defined; we seek to further explore this issue.

**RESEARCH DESIGN AND METHODS**—To determine the properties of  $\beta$ -cell-specific T-cell receptors (TCRs), we characterized the fine specificity, functional and relative binding avidity/affinity, and diabetogenicity of a panel of GAD65-specific CD4<sup>+</sup> T-cell clones established from unimmunized 4- and 14-week-old NOD female mice.

**RESULTS**—The majority of GAD65-specific CD4<sup>+</sup> T-cells isolated from 4- and 14-week-old NOD female mice were specific for peptides spanning amino acids 217–236 (p217) and 290–309 (p290). Surprisingly, 31% of the T-cell clones prepared from 14-week-old but not younger NOD mice were stimulated with both p217 and p290. These promiscuous T-cell clones recognized the two epitopes when naturally processed and presented, and this dual specificity was mediated by a single TCR. Furthermore, promiscuous T-cell clones demonstrated increased functional avidity and relative TCR binding affinity, which correlated with enhanced islet infiltration on adoptive transfer compared with that of monospecific T-cell clones.

**CONCLUSIONS**—These results indicate that promiscuous recognition contributes to the development of GAD65-specific CD4<sup>+</sup> T-cell clones in NOD mice. Furthermore, these findings suggest that T-cell promiscuity reflects a novel form of T-cell avidity maturation. *Diabetes* 57:2099–2106, 2008

**T**ype 1 diabetes is characterized by the autoimmune-mediated destruction of the insulin-producing  $\beta$ -cells of the islets of Langerhans (1–3). Based on studies in the nonobese diabetic (NOD) mouse, a spontaneous model of type 1 diabetes, the primary effectors of  $\beta$ -cell destruction are CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (1,4–6). Early during  $\beta$ -cell autoimmunity, a select panel of autoantigens, including proinsulin, insulin, GAD65, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and dystrophin myotonia kinase, are targeted by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in NOD mice (7–12). As the diabetogenic response proceeds,  $\beta$ -cell-specific T-cell reactivity “spreads” in a relatively defined pattern (13,14). Additional autoantigenic determinants are sequentially recognized within a single protein

(intramolecularly) and among different antigens (intermolecularly) to effectively amplify the diabetogenic response.

The key events involved in the breakdown of self-tolerance within the T-cell compartment and which shape the T-cell receptor (TCR) repertoire of  $\beta$ -cell-specific T-cells remain ill defined. Studies have suggested that defective thymic negative selection contributes to increased production of  $\beta$ -cell-specific T precursors (15–17). Furthermore, the peptide-binding properties of major histocompatibility complex (MHC) class II and class I molecules that are associated with type 1 diabetes susceptibility are thought to shape the TCR repertoire of diabetogenic T-cell effectors (18–22). Properties intrinsic to  $\beta$ -cell-specific TCRs may also contribute to the pathogenicity of T-cell effectors. For instance, avidity maturation promotes the expansion of IGRP-specific CD8<sup>+</sup> T-cells that in turn display increased TCR avidity/affinity and enhanced pathogenicity (23). One intriguing possibility is that the pathogenicity of an autoreactive T-cell is influenced by the degree of TCR cross-reactivity. In this model, a T-cell expressing a TCR that cross-reacts with multiple  $\beta$ -cell-derived epitopes would be selectively expanded and exhibit increased pathogenicity.

Antigen recognition by TCRs is inherently degenerate (24–26). Furthermore, a number of studies have reported cross-reactive T-cell responses between synthetic peptides that exhibit little if any sequence homology with the natural ligand (27–30). Allogeneic recognition by T-cells provides a biologically relevant example of the flexibility associated with TCR recognition (31,32). Gorman and colleagues (33) demonstrated the presence of CD4<sup>+</sup> T-cells recognizing two nonoverlapping epitopes of myelin basic protein (MBP) in a murine model of experimental autoimmune encephalomyelitis (EAE). This finding suggests that cross-reactive or “promiscuous” T-cell clonotypes may promote tissue-specific autoimmunity. Other studies have reported promiscuous recognition of nonoverlapping epitopes within the same viral or foreign antigen by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (34–36). The molecular basis and functional impact of T-cell promiscuity remains largely undefined.

In an effort to determine the properties of  $\beta$ -cell-specific TCRs, we prepared a large panel of GAD65-specific CD4<sup>+</sup> T-cell clones isolated from unimmunized 4- and 14-week-old NOD female mice. We demonstrate that a significant frequency of these T-cell clones recognize nonoverlapping peptides derived from GAD65, and that this promiscuity correlates with increased pathogenicity.

## RESEARCH DESIGN AND METHODS

NOD/LtJ and NOD.CB17.Prkdc<sup>scid</sup>/J (NOD.scid) mice were maintained and bred under specific-pathogen-free conditions, in a facility accredited by the American Association of Laboratory Animal Care. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

**Establishment of GAD65-specific CD4<sup>+</sup> T-cell clones.** GAD65-specific T-cell clones were established from unimmunized 4- and 14-week-old NOD female mice with full-length murine GAD65 and cloned by limiting dilution as

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described previously (37). Subclones were expanded and monoclonality was confirmed via staining with available anti-variable (V) $\alpha$  and -V $\beta$ -specific antibodies (Abs) and/or inverse RT-PCR (see below). T-cell clones were maintained on a 21-day growth cycle in which  $2 \times 10^6$  T-cells were stimulated with  $2 \times 10^7$  irradiated (3,000 rad) splenocytes and 10  $\mu$ g/ml GAD65. On day 3, base medium containing 10% FBS and 20 units/ml interleukin (IL)-2 was added, and cultures were expanded accordingly up to 21 days.

**Expression and purification of soluble IA<sup>sE7</sup>-Ig fusion proteins.** Soluble (s) IA<sup>sE7</sup>-Ig dimers were engineered as previously described (38). Briefly, IA<sup>sE7</sup>  $\alpha$ - and  $\beta$ -chain extracellular domains were attached to fos and jun leucine zippers, respectively. Peptide epitopes were covalently linked to the NH<sub>2</sub> terminus of the IA<sup>sE7</sup> $\beta$ -chain by a flexible thrombin-GGGGS linker. cDNAs encoding the respective sIA<sup>sE7</sup>-Ig chains were subcloned into the pMT-Bip vector (Invitrogen, Carlsbad, CA) and transgene expression driven by a metallothionein-inducible promoter. Expression vectors were cotransfected via calcium phosphate into *Drosophila* S2 cells with pHygro, and stable transfectants were selected in hygromycin-containing Schneider's medium. sIA<sup>sE7</sup>-Ig dimer protein expression was induced by 500  $\mu$ mol/l CuSO<sub>4</sub> for 7–10 days and purified by affinity chromatography on a Protein A column (GEBioscience, Pittsburgh, PA).

**Flow cytometry.** sIA<sup>sE7</sup>-Ig dimers were multimerized using biotin- or Alexa 647-coupled Protein A (Molecular Probes, Invitrogen, Eugene, OR) for flow cytometric analyses (38). Cells were incubated with sIA<sup>sE7</sup>-Ig multimers at 37°C for 1 h, followed by streptavidin-phycoerythrin, anti-CD3 (fluorescein isothiocyanate), and CD4 (peridinin-chlorophyll-protein complex) antibodies staining on ice for 30 min (eBioscience, San Diego, CA). Data were acquired on a Cyan flow cytometer (DakoCytometry, Carpinteria, CA) and analyzed using Summit software (DakoCytometry).

**T-cell proliferation and cytokine assays.** Proliferation assays were performed as previously described (37). Briefly,  $2 \times 10^4$  T-cells were cultured in triplicate with  $2 \times 10^5$  irradiated (3,000 rad) splenocytes per well (0.2 ml) plus antigen in a 96-well round-bottom microtiter plate for 72 h. Proliferation was measured by uptake of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) during the last 16 h of culture using a Trilux 1450 Microbeta Wallac Harvester (Wallac, Turku, Finland).

A capture enzyme-linked immunosorbent assay (ELISA) using paired antibodies purchased from BD Pharmingen was used to measure cytokine secretion from supernatants harvested after 48 h of culture. The concentration of interferon- $\gamma$  (IFN- $\gamma$ ), IL-2, and IL-4 was determined in triplicate in 0.1 ml culture supernatant by comparing with a standard curve of the respective cytokines. The lower limits of detection for IFN- $\gamma$ , IL-2, and IL-4 were 50, 25, and 30 pg/ml, respectively.

**Antigens.** Full-length murine GAD65 containing a histidine tag on the COOH terminus was expressed via *Baculovirus* and purified as previously described (37). Histidine-tagged fragments encoding murine GAD65 spanning amino acids 112–282 (f217), 240–439 (f290), and 160–364 (f217+f290) or chloroform acetyltransferase (fCAT) were expressed in *Escherichia coli* using the TrcHis expression system (Invitrogen) and purified under denaturing conditions via Ni<sup>2+</sup> affinity chromatography and preparative SDS-PAGE. Peptides were synthesized using standard F-moc chemistry on a Rainin Symphony (Rainin Instruments) at the peptide synthesis facility of University of North Carolina. The purity of the peptides was verified by reverse-phase high-performance liquid chromatography and mass spectroscopy.

**Inverse PCR.** Total RNA was extracted from  $1 \times 10^6$  T-cells via Trizol (Invitrogen) and reverse transcribed using oligo-dT primers. Second-strand DNA was synthesized with 1 unit RNase H, 5 units *E. coli* ligase, and 25 units *E. coli* DNA polymerase I for 16 h at 14°C. Double-stranded DNA (dsDNA) was treated with T4 DNA polymerase to blunt both 5' and 3' ends and facilitate intramolecular ligation by T4 DNA ligase. Primers encoding the constant region of the TCR  $\alpha$ - and  $\beta$ -chains were used to amplify circularized TCR dsDNA. PCR amplicons were cloned into pCR2.1 Topo vector (Invitrogen) and sequenced by the University of North Carolina Genomics Core Facility.

**Construction, expression, and purification of single-chain TCR.** Single-chain TCR (scTCR) was constructed as previously described (39). Briefly, cDNA encoding the 11H11 clonotypic TCR was amplified by RT-PCR. The TCR V $\alpha$ - and V $\beta$ -chain gene segments were engineered with a 4 $\times$ GGGGS flexible linker via overlapping PCR and a human Ig constant  $\kappa$  domain and 12 $\times$ -histidine tag added to the COOH terminus. The scTCR was subcloned into the expression vector pAK400, and the plasmid was transformed into BL21 *E. coli* (Stratagene, La Jolla, CA). Expression of scTCR was induced with 1 mmol/l IPTG for 16 h, after which the periplasm was extracted via osmotic shock. scTCR was then purified under native conditions by Co<sup>2+</sup> affinity chromatography.

**Surface plasmon resonance.** A Biacore 2000 instrument (Biacore, Piscataway, NJ) was used to measure binding interactions between purified sIA<sup>sE7</sup>-Ig dimers and scTCRs. sIA<sup>sE7</sup>-Ig dimer was immobilized by amine-coupling chemistry on a CM5 research-grade sensor chip. Surface densities for individual experiments are indicated in Fig. 4. scTCRs were prepared in filtered and

degassed PBS and injected at a flow rate of 20  $\mu$ l/min. In all experiments, one blank channel was used as a negative control.  $K_D$  values as well as on and off rates were obtained by nonlinear curve fitting of subtracted curves using the Langmuir 1:1 binding model with the BIAevaluation program (version 3.0.2; Biacore) and the global-fitting software Clamp (version 3.3). The equilibrium  $K_D$ , under steady-state conditions, was also determined using the BIA evaluation program.

**Measurement of relative TCR binding affinity.** The association kinetics measured using the multimers were determined using a previously described method (40). Briefly, T-cell clones were stained with anti-TCR $\alpha\beta$  antibody and increasing concentrations of sIA<sup>sE7</sup>-Ig multimers for 1 h at room temperature. Data were normalized to the level of TCR expression. The apparent  $K_D$  values were derived from the negative reciprocal of the slope of the regression line fit to Scatchard plots of bound multimer/free multimer (normalized fluorescence units per nanomolar concentration of multimer) versus bound multimer (normalized fluorescence units).

**T-cell adoptive transfers and histopathology.** "Resting" T-cell clones ( $10 \times 10^6$ ) stimulated with peptide 21 days prior were injected intraperitoneally into 5- to 8-week-old NOD.scid male mice. Recipient mice were monitored for diabetes incidence. Pancreases were harvested and fixed with 10% formalin. Serial cross-sections (5  $\mu$ m) were cut and stained with hematoxylin-eosin (H-E).

**Islet isolation.** Pancreases were perfused with 2 mg/ml collagenase P and incubated for 30 min at 37°C, and the homogenate was applied to a Ficoll gradient. Islets were washed, handpicked, and then dissociated by treatment with 0.5 mg/ml collagenase D for 30 min at 37°C.

**Statistical methods.** Student's *t* test and  $\chi^2$  analyses were carried out to determine statistical significance.

## RESULTS

**GAD65-specific CD4<sup>+</sup> T-cells recognize two nonoverlapping epitopes.** To investigate the properties intrinsic to  $\beta$ -cell-specific TCRs and autoantigen recognition, we used GAD65-specific CD4<sup>+</sup> T-cell clones established from the spleens of unimmunized 4- or 14-week-old NOD female mice.  $\beta$ -Cell autoimmunity and GAD65-specific CD4<sup>+</sup> T-cell reactivity are initially detected in NOD female mice at 3–4 weeks of age. In contrast,  $\beta$ -cell autoimmunity is well established in 14-week-old NOD female mice. Eighty-nine CD4<sup>+</sup> T-cell clones were established from 4-week-old NOD female mice using intact murine GAD65 for T-cell stimulation. Using a panel of overlapping GAD65-derived 20-mer peptides, 88 clones were specific for a peptide spanning amino acid residues 217–236 (p217) (Table 1; Fig. 1A). These clones exhibited Th0-like (IL-2<sup>+</sup>) or Th1-like (IL-2<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>) phenotypes based on ELISA (for cytokine secretion by a subset of these clones, see Supplementary Table 1, which is detailed in the online appendix [available at <http://www.dx.doi.org/10.2337/db08-0383>]). A minimum of 25 distinct clonotypes was identified based on flow cytometric analysis with available TCR V $\alpha$ - and V $\beta$ -specific antibodies and oligonucleotide sequencing of RT-PCR amplified V $\alpha$  and V $\beta$  gene segments. A single clone (6H1) specific for GAD65 290–309 (p290) (Table 1; Fig. 1A) was identified, which exhibited a Th2-like phenotype (IL-2<sup>+</sup>, IL-4<sup>+</sup>) (see Supplementary Table 1) (37).

A total of 150 GAD65-specific CD4<sup>+</sup> T-cell clones were established from 14-week-old NOD female mice. Several different GAD65-derived peptides, including p217 and p290, were recognized by these CD4<sup>+</sup> T-cell clones (Table 1). The majority (>90%) of these T-cell clones exhibited a Th1-like phenotype (IL-2<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>), whereas the remainder of clones were Th0-like (IL-2<sup>+</sup>) (for cytokine secretion by a subset of these clones, see Supplementary Table 1). Surprisingly, 50 of the T-cell clones (33%) were stimulated by two nonoverlapping GAD65-specific peptides (Table 1). Most of these promiscuous T-cell clones responded to p217 and p290 (47 of 150); 3 T-cell clones recognized both peptides p290 and 400–420 (p400) (Table 1). Furthermore,

TABLE 1  
GAD65 peptide specificity of CD4<sup>+</sup> T-cell clones established from unimmunized NOD female mice

GAD65 peptide	Amino acid sequences	Clones
4-week-old NOD female mice*		
p217-236	EYVTLKKMREIIGWPGGSGD	88 (98.9)
p290-309	AALGIGTDSVILIKCDERGK	1 (1.1)
Total		89
14-week-old NOD female mice*		
p16-36	GSGDSENPGTARAWCQVAQKF	5 (3.3)
p202-221	TNMFYIEIAPVFLLEYVTL	13 (8.7)
p217-236	EYVTLKKMREIIGWPGGSGD	36 (24)
p232-251	GGSGDGIFSPGGAISNMYAM	14 (9.3)
p290-309	AALGIGTDSVILIKCDERGK	22 (14.7)
p400-420	VPLQCSALLVREEGLMQNCNQ	6 (4.0)
p561-575	ISNPAATHQDIDFLI	4 (2.7)
p217-236 + p290-309		47 (31.3)
p290-309 + p400-420		3 (2.0)
Total		150

Data are *n* (%) of T-cell clones specific for single or dual GAD65-derived peptides. \*GAD65-specific T-cell clones were established from two independent cloning experiments in which spleens from three to five donor NOD female mice either 4 or 14 weeks of age were pooled in a single experiment.

all of the promiscuous T-cell clones displayed a Th1-like phenotype (IL-2<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>) (for cytokine secretion by a subset of these clones, see Supplementary Table 1).

To rule out the possibility that T-cell promiscuity was an in vitro artifact of cloning, multimerized sIA<sup>G7</sup>-Ig dimers were used to detect promiscuous CD4<sup>+</sup> T-cells in the

spleen, pancreatic lymph nodes (PLNs), mesenteric lymph nodes (MLNs), and islets of nondiabetic 12-week-old NOD female mice. The sIA<sup>G7</sup>-Ig dimers contain covalently linked p217, p290, or a hen egg lysozyme-derived peptide (pHEL) as a control. As shown in Fig. 1B and C, CD3<sup>+</sup>CD4<sup>+</sup> T-cells binding both sIA<sup>G7</sup>-p217 and sIA<sup>G7</sup>-p290 multimers were

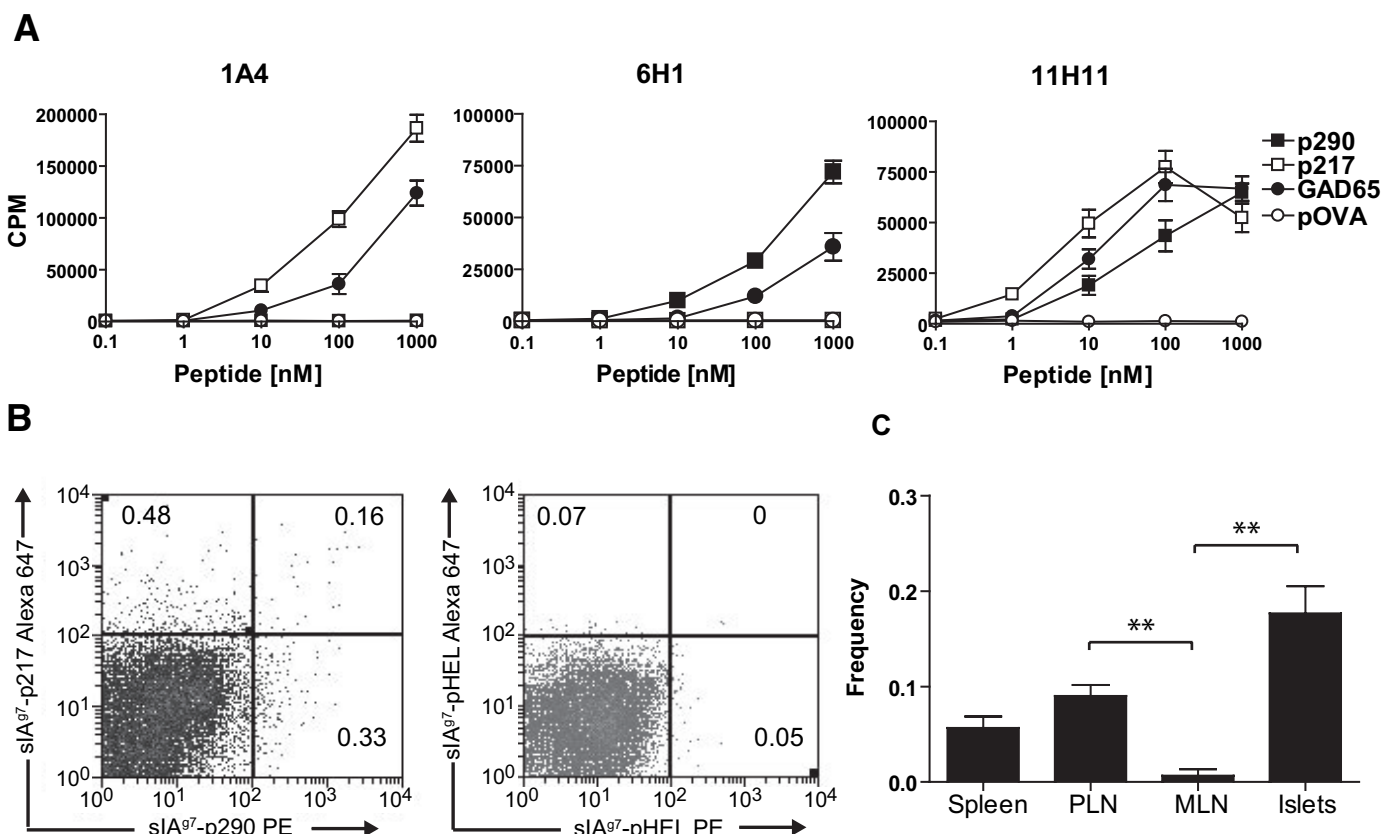


FIG. 1. GAD65-specific CD4<sup>+</sup> T-cells exhibit promiscuous recognition of p217 and p290. **A**: Clones representative of p217 (1A4) and p290 (6H1) monospecific or promiscuous (11H11) T-cells were stimulated with p217, p290, GAD65 protein, or OVA peptide (321-339) as negative control, and proliferation was measured via [<sup>3</sup>H]thymidine uptake. **B**: Representative flow cytometric data for CD4<sup>+</sup> T-cells isolated from the islets of a 12-week-old NOD female mouse and co-stained with sIA<sup>G7</sup>-p217 and sIA<sup>G7</sup>-p290 multimers or sIA<sup>G7</sup>-pHEL multimers as a control. **C**: The frequency of promiscuous CD4<sup>+</sup> T-cells detected ex vivo in the spleen, PLNs, MLNs, and islets of 12-week-old NOD female mice. The average percentages of promiscuous CD4<sup>+</sup> T-cells  $\pm$  SD calculated from five independent experiments are presented. \**P* < 0.004, PLNs vs. MLNs; \*\**P* < 0.003, islets vs. MLNs; Student's *t* test.

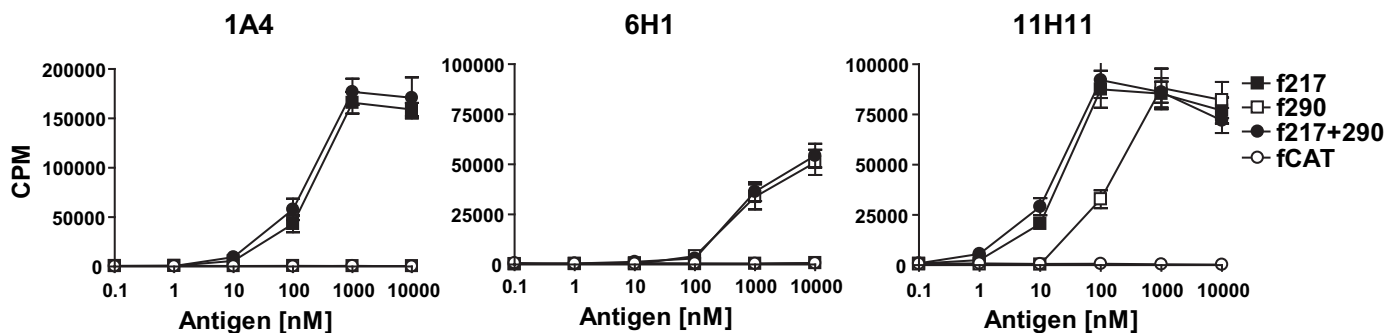


FIG. 2. Promiscuous CD4<sup>+</sup> T-cell clones recognize naturally processed and presented p217 and p290 epitopes. Clones representative of p217 (1A4) and p290 (6H1) monospecific or promiscuous (11H11) T-cells were stimulated with GAD65 fragments containing p217 (f217), p290 (f290), or both p217 and p290 (f217 + 290) or an irrelevant protein (fCAT), and proliferation was measured via [<sup>3</sup>H]thymidine uptake. Data are representative of a minimum of six independent experiments.

detected in the islets and to a lesser extent the PLNs and spleen, but not the MLNs. Together these findings indicate that GAD65-specific CD4<sup>+</sup> T-cells recognizing nonoverlapping epitopes are present in NOD female mice at a late preclinical stage of type 1 diabetes.

**Promiscuous GAD65-specific CD4<sup>+</sup> T-cell clones recognize naturally processed and presented p217 and p290 epitopes with increased avidity.** Various studies have shown that T-cells may respond to a synthetic peptide but not to the corresponding naturally processed epitope, thereby bringing into question the physiological relevance of the former. To ensure that the promiscuous CD4<sup>+</sup> T-cell clones recognized naturally processed and presented p217 and p290 epitopes, the stimulatory capacity of recombinant fragments of GAD65 containing the p217 (f217) or p290 (f290) epitopes only or both p217 and p290 epitopes (f217 + 290) was tested. As expected, monospecific p217 (1A4) and p290 (6H1) T-cell clones responded to the corresponding GAD65-specific recombinant proteins, but a fCAT recombinant used as a negative control did not (Fig. 2). Significant proliferation of the promiscuous 11H11 clone was induced by both f217 and

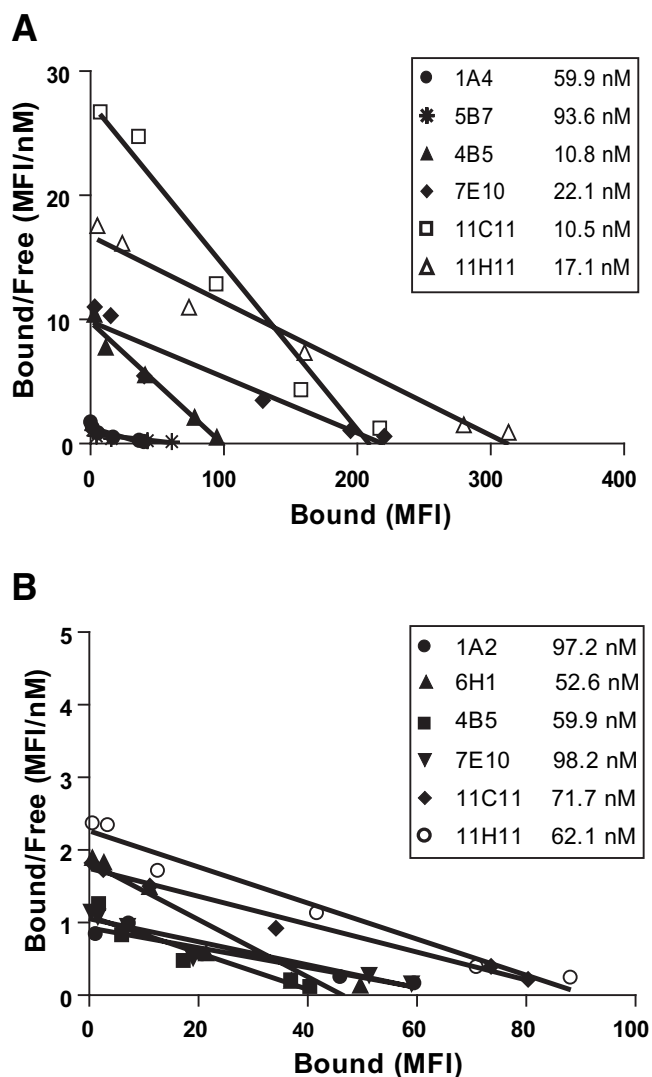
f290 but not fCAT (Fig. 2). These results demonstrate that promiscuous T-cell clones are readily stimulated by protein containing the p217 and p290 epitopes.

Interestingly, the promiscuous 11H11 clone exhibited a more robust proliferative response to f217 and f290 relative to the monospecific 1A4 and 6H1 clones (Fig. 2). This finding suggested that promiscuous TCR recognition enhanced T-cell responsiveness to antigen. To further investigate this possibility, proliferation of several monospecific and promiscuous T-cell clones in response to the panel of GAD65-specific recombinant proteins was measured in greater detail. Specifically, the concentration of antigen that elicited 50% of the maximum proliferative response (e.g., [EC<sub>50</sub>]) was determined. The average [EC<sub>50</sub>] measured in response to f217 and f290 for the monospecific p217 and p290 T-cell clones was 452 ± 173 and 668 ± 163 nmol/l, respectively (Table 2). Strikingly, the average [EC<sub>50</sub>] of the panel of promiscuous T-cell clones was increased 4.9- and ~2-fold for f217 (93 ± 47 nmol/l) and f290 (367 ± 71 nmol/l), respectively, relative to the corresponding panel of monospecific T-cell clones (Table 2).

TABLE 2  
[EC<sub>50</sub>] determination for GAD65-specific T-cell clones

Specificity	Clone*	f217 (nmol/l)	f290 (nmol/l)	f217 + 290 (nmol/l)
p217-236	1A4	240 ± 36	NR	200 ± 21
	2C11	564 ± 76	NR	530 ± 90
	5B7	385 ± 42	NR	310 ± 38
	1C9	620 ± 99	NR	604 ± 85
	Average	452 ± 173	NR	411 ± 188
p290-309	6H1	NR	460 ± 63	424 ± 55
	1A2	NR	854 ± 105	807 ± 122
	5A7	NR	710 ± 86	765 ± 93
	6C10	NR	650 ± 79	680 ± 102
Average	NR	668 ± 163	669 ± 171	
p217-236 + p290-309	11H11	40 ± 19	250 ± 38	35 ± 14
	7E10	54 ± 23	390 ± 57	33 ± 10
	4D10	125 ± 41	440 ± 63	62 ± 25
	10C1	58 ± 15	357 ± 50	40 ± 20
	4B5	94 ± 36	330 ± 48	60 ± 28
	11C11	59 ± 20	320 ± 76	32 ± 8
	8A9	161 ± 34	369 ± 85	144 ± 37
	11A10	149 ± 30	480 ± 106	83 ± 45
	Average	93 ± 47†	367 ± 71†	61 ± 38

Data are means ± SE. The concentration of f217, f290, and f217 + 290 inducing [EC<sub>50</sub>] measured by [<sup>3</sup>H]thymidine uptake is provided for the respective T-cell clones. \*Clones 1A4 and 6H1 were established from 4-week-old NOD female mice; all other clones were established from 14-week-old NOD female mice. †*P* < 10<sup>-3</sup>, the average [EC<sub>50</sub>] of the group of promiscuous vs. p217-236- or p290-309-specific T-cell clones (Student's *t* test). NR, no response.



**FIG. 3.** Promiscuous CD4<sup>+</sup> T-cell clones exhibit an increased relative TCR avidity/affinity. sIA<sup>g7</sup>-p217 (A) and sIA<sup>g7</sup>-p290 (B) multimer binding by monospecific p217 (1A4 and 5B7) and p290 (6H1 and 1A2) and promiscuous (4B5, 7E10, 11C11, and 11H11) T-cell clones was measured by Scatchard plot analysis. The binding data were fitted to a standard Scatchard plot, and  $K_D$  was calculated by the slope of the line. Data are the average of two independent experiments.

**TABLE 3**  
TCR repertoire of the monospecific and promiscuous CD4<sup>+</sup> T-cell clones

Specificity	Clone	V $\alpha$ - J $\alpha$		CDR $\alpha$ 3		V $\beta$ -D $\beta$ -J $\beta$		CDR $\beta$ 3		
p290	6H1	V2-J44	YFCAA	SNTGANTGKGL	TFG	V14-D1-J2.5	YLCAW	SQLGANQDTQ	YFG	
	1A2	V4-J39	YFCAL	VDYANKM	IFG	V11-D1-J1.3	YLCAS	SRTGSSYEQ	YFG	
	5A7	V4-J39	YFCAL	VDYANKM	IFG	V8.2-D2-J2.5	YFCAS	GDAGGAQDTQ	YFG	
	6C10	V3-J26	YFCAG	GSNYQL	IWG	V8.2-D1-J2.6	YFCAS	GDRLGVYEQ	YFG	
p217	2C11	V17-J26	YFCAM	REDSNYQL	IWG	V1-D1-J1.5	YFCAS	SQEARNNQAP	LFG	
	1A4	V4-J20	YYCAL	GEGYAQG	TFG	V4-D1-J2.6	YFCAS	SHRDNYEQ	YFG	
	5B7	V1-J8	YLCAT	RNMGYK	TFG	V6-D1-J1.6	FLCAS	SRDRGGQ	YFG	
	1C9	V4-J20	YYCAL	RGSNAKL	TFG	V1-D1-J1.1	YFCAS	SQRGANTEV	FFG	
	7E10	V1-J17	YLCAA	PTSSGSWQL	IFG	V6-D1-J1.1	FLCAS	NIRQQGNTTEV	FFG	
	4D10	V4-J18	YYCAL	GEDYNGGKL	IFG	V1-D1-J2.3	YFCAS	SRDRGQGAETL	YFG	
	10C1	V13-J8	YFCAA	SEDMGYKL	TFG	V1-D1-J2.3	YFCAS	SPRDSAETL	YFG	
	p217 and p290	4B5	V2-J7	YFCAA	SALHYSNNRL	TLG	V6-D2-J2.3	FLCAS	TRGTGRAETL	YFG
		11C11	V13-J37	YFCAA	SEAGGADRL	TFG	V8.3-D2-J2.3	YFCAS	TNWGGGAEQ	FFG
		11H11	V2-J18	YFCAA	SGYNQGKL	IFG	V16-D1-J1.2	TFCAS	SPRQTNSDY	TFG
11A10		V13-J37	YFCAA	SEAGGADRL	TFG	V1-D2-J2.3	YFCAS	SPRDSAETL	YFG	

V $\alpha$ -J $\alpha$  and V $\beta$ -D $\beta$ -J $\beta$  gene segments and amino acid sequences of CDR3 $\alpha$ / $\beta$  are provided for the monospecific and promiscuous T-cell clones.

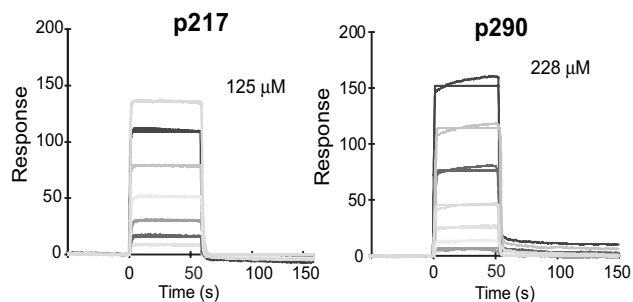
These findings indicate that promiscuous versus mono-specific T-cell clones have an increased [EC<sub>50</sub>].

To confirm the above observation, sIA<sup>g7</sup>-p217 and sIA<sup>g7</sup>-p290 binding by the respective T-cell clones was measured and analyzed via Scatchard plot. Normalizing for the level of TCR expression, the average relative TCR affinity of the promiscuous T-cell clones was increased fivefold for sIA<sup>g7</sup>-p217 ( $K_D = 15.1 \pm 2.8$  nmol/l) compared with p217-specific T-cell clones ( $K_D = 76.7 \pm 16.9$  nmol/l;  $P = 0.005$ ; Student's  $t$  test) (Fig. 3). No significant difference in relative TCR affinity for p290 was detected between the promiscuous ( $K_D = 72.9 \pm 17.6$  nmol/l) and p290-only-specific ( $K_D = 75.2 \pm 22.6$  nmol/l) T-cell clones (Fig. 3). Together, these results demonstrate that promiscuous TCR recognition correlates with an increased [EC<sub>50</sub>] and enhanced relative TCR affinity specific for p217.

**Promiscuous T-cell clones recognize dual epitopes using a single TCR.** Next, TCRs expressed by the clones were analyzed to determine whether promiscuous peptide recognition 1) was attributed to expression of a single TCR versus dual/multiple TCRs and 2) correlated with selective TCR V $\alpha$  and/or V $\beta$  gene usage. An inverse PCR strategy was used to amplify full-length V gene regions from cDNA prepared from each T-cell clone. In this way, if a given T-cell clone expressed multiple  $\alpha$ - and/or  $\beta$ -TCR chains, functional (and aberrant) mRNA transcripts would be readily cloned independent of the V gene family. All monospecific and promiscuous T-cell clones expressed a single TCR (Table 3). Furthermore, there was no significant sharing of V $\alpha$  or V $\beta$  gene usage or V $\alpha$  and V $\beta$  complementary determining region (CDR3) motifs among the promiscuous (or monospecific) T-cell clones (Table 3).

To unambiguously demonstrate that a single TCR bound both p217 and p290, a soluble scTCR from the promiscuous 11H11 clone was engineered, and binding kinetics to sIA<sup>g7</sup>-p217 and sIA<sup>g7</sup>-p290 were measured via surface plasmon resonance. Both sIA<sup>g7</sup>-p217 and sIA<sup>g7</sup>-p290 bound to 11H11 scTCR with  $K_D$  values of  $38.8 \pm 3.9$  and  $117.6 \pm 15.2$   $\mu$ mol/l, respectively (Fig. 4). These results demonstrate that promiscuous T-cell clones express a single TCR and that TCR gene usage is heterogeneous.

**Promiscuous T-cell clones exhibit an enhanced capacity to mediate insulinitis.** Because the promiscuous T-cell clones demonstrated enhanced [EC<sub>50</sub>] and sIA<sup>g7</sup>-Ig binding avidity, we hypothesized that promiscuous versus



scTCR/pMHC IA <sup>g7</sup>	$k_{on}$ (mol <sup>-1</sup> s <sup>-1</sup> )	$k_{off}$ (s <sup>-1</sup> )	$t_{1/2}$ (s)	$K_D$ (μM)
11H11/p217	$1.57 \times 10^4$	0.61	1.14	$38.8 \pm 3.9$
11H11/p290	$4.59 \times 10^3$	0.54	1.28	$117.6 \pm 15.2$

**FIG. 4.** The promiscuous 11H11 clonotypic TCR binds both sIA<sup>g7</sup>-p217 and sIA<sup>g7</sup>-p290. BIAcore sensorgrams of 11H11 scTCR binding to sIA<sup>g7</sup>-p217 or sIA<sup>g7</sup>-p290 captured by surface-immobilized anti-mouse IgG<sub>2a</sub>-specific antibody at a flow rate of 20 μl/min. The corresponding background responses were subtracted. The latter were obtained by injecting the analyte over the same anti-mouse IgG<sub>2a</sub>-specific antibody surface in the absence of prior sIA<sup>g7</sup>-Ig capture. The highest concentrations of each scTCR are indicated, and the other lines are from twofold dilutions of the highest concentration.  $K_D$  values are the average of two independent experiments.

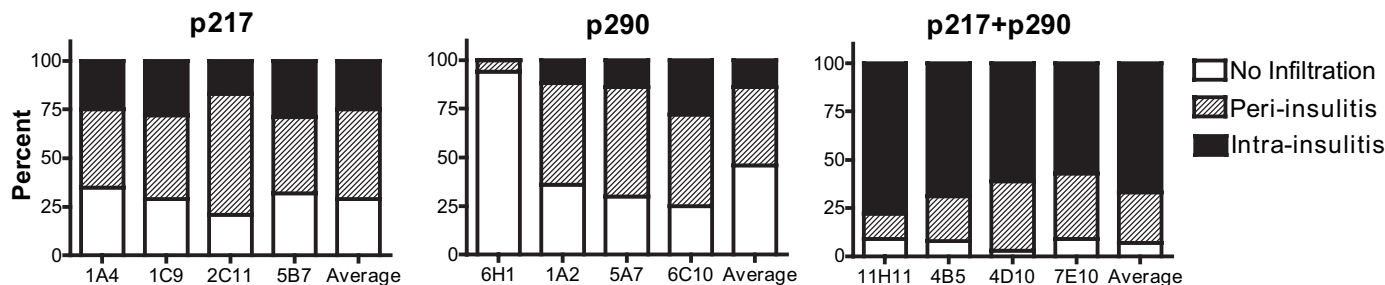
monospecific T-cell clones would also display increased pathogenicity. Accordingly, adoptive transfer experiments were carried out in which  $10 \times 10^6$  cells of the individual T-cell clones were injected into NOD.scid recipients, and insulinitis and diabetes were examined. Neither monospecific nor promiscuous T-cell clones induced diabetes 4 weeks after transfer. However, the frequency of intra-insulinitis was significantly increased in recipients of promiscuous versus p217- and p290-specific T-cell clones (Fig. 5). For example, on average, intra-insulinitis was detected in 67% (186 of 279) of the islets of mice receiving the promiscuous T-cell clones (Fig. 5). On the other hand, intra-insulinitis, on average, was detected in only 25% (63 of 252) and 14% (30 of 215) of NOD.scid mice receiving p217- and p290-specific T-cell clones, respectively (Fig. 5). These observations demonstrate that a more aggressive type of insulinitis is mediated by the promiscuous versus monospecific T-cell clones.

## DISCUSSION

Cross-reactivity is an intrinsic property of TCRs that plays a critical role in T-cell development and homeostasis (24–26). T-cell cross-reactivity was originally attributed to

molecular mimicry, in which two antigenic peptides share structural and chemical properties. Molecular mimicry has been linked to cross-reactive T-cell responses between self and microbial antigens in a variety of autoimmune diseases, including type 1 diabetes (41–44). However, TCRs have also been shown to recognize multiple epitopes that have little or no structural similarities (27–30,45). Furthermore, reports have demonstrated that TCRs specific for viral or other foreign antigens recognize nonoverlapping epitopes from the same antigen (34–36). These promiscuous T-cell clones may represent a subset of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells with enhanced effector functions (34–36). Together, these findings indicate that a high degree of inherent plasticity is associated with TCR epitope recognition that in turn may influence clonotypic selection/expansion in the periphery. The current study demonstrates that a relatively high frequency of GAD65-specific CD4<sup>+</sup> T-cell clones recognizes two nonoverlapping GAD65 peptides and that this promiscuity correlates with increased avidity/affinity and an enhanced capacity to mediate insulinitis. Detection of CD4<sup>+</sup> T-cells binding both sIA<sup>g7</sup>-p217 and sIA<sup>g7</sup>-p290 predominately in the islets of 12-week-old NOD female mice is noteworthy and is an argument against the possibility that promiscuous T-cell recognition is an artifact of cloning and that clones with this specificity are in fact positively selected in the thymus.

Studies, including those with NOD mice, have previously reported that promiscuous T-cell recognition can be due to expression of dual TCRs (46,47). We show here that recognition of both p217 and p290 is mediated by a single TCR based on TCR α- and β-chain gene cloning (Table 3) and functional analysis of the promiscuous 11H11 scTCR (Fig. 4). Despite promiscuous epitope recognition, these promiscuous TCRs nevertheless display a high degree of specificity. For example, other β-cell derived autoantigens, such as insulin, GAD67, heat shock protein 60, carboxypeptidase H, and peripherin, or a large panel of previously identified IA<sup>g7</sup>-restricted peptides from foreign antigens fail to stimulate the promiscuous T-cell clones (R.T., unpublished results). The molecular basis for this promiscuity is unclear. No preferential usage of Vα or Vβ gene segments or CDR3α/β motifs was detected among the panel of promiscuous T-cell clones (Table 3). This finding is analogous to other reports demonstrating a high degree of heterogeneity among TCRs that cross-react with the same epitopes (48). The two properties shared by the promiscuous T-cell clones, however, were an increased [EC<sub>50</sub>] (Table 2) and relative TCR affinity (Fig. 3) compared with the monospecific T-cell clones. Importantly, increased TCR affinity has been correlated with enhanced



**FIG. 5.** Promiscuous T-cell clones exhibit enhanced pathogenicity compared with monospecific T-cell clones. The frequency of insulinitis as determined by H-E staining was assessed in groups of five NOD.scid mice receiving monospecific p217 and p290 and promiscuous T-cell clones 4 weeks after transfer. A minimum of 50 islets was counted for each mouse.  $P < 10^{-3}$ , number of islets containing intra-insulinitis detected in promiscuous vs. p217- or p290-monospecific T-cell clones;  $\chi^2$ . Data are representative of two independent experiments.

cross-reactivity due to the flexibility of the CDR loops that interact with the peptide-MHC complex (28,32,49).

Properties intrinsic to the respective peptides may also contribute to promiscuous TCR recognition. For instance, analysis of the core peptides that induce maximum T-cell proliferation demonstrated that whereas monospecific and promiscuous T-cell clones recognized the same p217 core peptides, this was not the case for p290 (Supplementary Table 2). All promiscuous T-cell clones recognized an "extended" p290 core peptide with additional amino acid residues on the NH<sub>2</sub> terminus compared with the minimal epitopes recognized by monospecific T-cell clones (Supplementary Table 2). This suggests that the flanking residues may alter the conformation of bound p290 and/or the IA<sup>g7</sup> molecule to promote recognition by a promiscuous TCR. In view of the strong association between MHC and type 1 diabetes susceptibility, it is tempting to speculate that properties intrinsic to IA<sup>g7</sup> preferentially promote the development and/or expansion of promiscuous  $\beta$ -cell-specific T-cells in NOD mice. In support of this hypothesis, an increased frequency of T-cell clones recognizing non-overlapping ovalbumin peptides is detected in NOD versus a NOD strain congenic for IA<sup>d</sup> (NOD.GD) immunized with ovalbumin (R.T., unpublished data).

Strikingly, promiscuous GAD65-specific T-cell clones were established from only 14- and not 4-week-old NOD female mice (Table 1). This observation suggests that increased T-cell promiscuity may in fact reflect a novel type of TCR avidity maturation. The Santamaria group (23) has shown that increased TCR avidity/affinity correlates with enhanced pathogenicity of IGRP-specific CD8<sup>+</sup> T-cells. Similarly, we have demonstrated that GAD65-specific promiscuous T-cell clones exhibited an increased [EC<sub>50</sub>] (Table 2) and relative TCR affinity (Fig. 3), which also correlated with an increased capacity to mediate insulinitis on adoptive transfer into NOD.scid recipients (Fig. 5). The four promiscuous T-cell clones individually mediated significantly more intra-insulinitis relative to the monospecific T-cell clones (Fig. 5). Presumably, promiscuous peptide recognition coupled with increased TCR avidity/affinity enhanced the capacity of the promiscuous T-cell clones to expand in vivo and efficiently penetrate the islets. The significant increase in the frequency of CD4<sup>+</sup> T-cells that bound both sIA<sup>g7</sup>-p217 and -p290 multimers in the islets and PLNs compared with the MLNs of 12-week-old NOD female mice is noteworthy (Fig. 1C), further suggesting antigen-driven expansion of promiscuous T-cells. A similar tissue distribution was detected for p217 and p290 monospecific CD4<sup>+</sup> T-cells (Supplementary Fig. 1). The lack of induction of diabetes by the T-cell clones tested is consistent with the notion that p217- and p290-specific CD4<sup>+</sup> T-cells play a role in supporting the diabetogenic response that does not involve, however, mediating direct  $\beta$ -cell destruction. One scenario is that these clones provide help for  $\beta$ -cell-specific CD8<sup>+</sup> (and CD4<sup>+</sup>) T-cells by conditioning the extracellular milieu or "licensing" resident antigen presenting cells in the islets.

Currently, it is unclear whether CD4<sup>+</sup> and CD8<sup>+</sup> T-cells specific for  $\beta$ -cell autoantigens other than GAD65 also exhibit promiscuous epitope recognition and if so, to what extent. Interestingly, the Goverman group showed in murine EAE that dual-specific T-cells recognizing nonoverlapping MBP-specific peptides could also be detected (33). Based on this and our own findings, T-cell promiscuity may in fact be a common feature of autoreactive T-cells. Further study is required to determine the relative contri-

bution of T-cell promiscuity in type 1 diabetes and other T-cell-mediated autoimmune diseases, in addition to defining the molecular basis for dual (multiple) peptide recognition by TCR.

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