Weak proinsulin peptide-MHC complexes are targeted in autoimmune diabetes in mice

Matteo G. Levisetti, MD¹,², Danna M. Lewis, BS¹, Anish Suri, PhD² and Emil R. Unanue, MD².

Departments of Medicine¹, Pathology and Immunology².
Washington University School of Medicine
Saint Louis, MO 63110

Running title: C-peptide reactive T cells

Corresponding Author:
Matteo G. Levisetti
Campus Box 8127
660 S. Euclid Av.
Saint Louis, MO 63110
Phone: 314-362-1265
Fax: 314-362-7641
mleviset@im.wustl.edu

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Objective. Weak MHC binding of self peptides has been proposed as a mechanism that may contribute to autoimmunity by allowing for escape of autoreactive T cells from the thymus. We examined the relationship between the MHC binding characteristics of a beta cell antigen epitope and T cell autoreactivity in a model of autoimmune diabetes.

Research Design and Method. The binding of a proinsulin epitope, PI-1(47-64), to the MHC class II molecules I-A\textsuperscript{g7} and I-A\textsuperscript{k} was measured using purified class II molecules. T cell reactivity to the proinsulin epitope was examined in I-A\textsuperscript{g7} and I-A\textsuperscript{k} positive mice.

Results. C-peptide epitopes bound very weakly to I-A\textsuperscript{g7} molecules. However, C-peptide reactive T cells were induced following immunization in I-A\textsuperscript{g7} bearing mice (NOD and B6.g7) but not in I-A\textsuperscript{k} bearing mice (B10.BR and NOD.h4). T cells reactive with the proinsulin-1(47-64) peptide were found spontaneously in the peri-pancreatic lymph nodes of prediabetic NOD mice. These T cells were activated by freshly isolated beta cells in the presence of antigen presenting cells (APC) and caused diabetes when transferred into NOD.scid mice.

Conclusions. These data demonstrate an inverse relationship between self peptide-MHC binding and T cell autoreactivity for the proinsulin-1(47-64) epitope in autoimmune diabetes.
Defective negative selection caused by weak interactions between self peptides and MHC molecules has been proposed as a mechanism that may contribute to autoimmunity. We have been interested in examining the relationship between peptide-MHC interactions and T cell autoreactivity for disease relevant antigens in autoimmune diabetes. In the case of the nonobese diabetic (NOD) mouse, the class II MHC molecule, I-A\(^{g7}\), has been shown to be a weak peptide binder (1). While examining the CD4 T cell response to peptides derived from the insulin beta chain in the NOD mouse, we were struck by its low binding affinity to the I-A\(^{g7}\) class II MHC molecule (2). Many of the spontaneous T cells that were identified reacted to a segment previously identified as a focus for spontaneous T cell reactivity (3-6). Such a peptide apparently bound in two registers to I-A\(^{g7}\), but binding was in the low micromolar range and showed a fast dissociation rate. Many of the amino acids in the peptide affected both binding and T cell recognition, implying a very loose peptide-MHC complex (2).

The relationship of low affinity of a peptide to an MHC molecule and autoimmune reactivity has been noted also in the encephalitogenic peptide from myelin basic protein (7; 8). This has led to the speculation that low affinity peptides may not be conducive to the normal mechanisms in the thymus that control autoreactivity. A study by McNeil and Evavold noted that peptides with fast dissociation rates and poor binding to MHC molecules were ineffective at deleting thymocytes even though the same ligands were capable of eliciting proliferation of mature T cells in the periphery (9). Similarly in a tumor model system, low-affinity epitopes were shown to activate tumor-specific CTLs and could confer anti-tumor immunity upon immunization (10). Lastly, a study by Roep and colleagues that measured in vitro binding interactions of proinsulin epitopes to various HLA-DR molecules noted that DR alleles associated with protection from diabetes bound various proinsulin epitopes with a higher affinity when compared to DR molecules that predispose to T1DM (11).

To further characterize the relationship between peptide-MHC binding strength and immune reactivity we have examined here other regions of the proinsulin molecule targeted by CD4 T cells. The insulin molecule is derived from the processing of the prohormone, proinsulin, and is secreted from the pancreatic beta cell with equimolar amounts of the connecting peptide, C-peptide (Table I) (12). We examined the PI-1(47-64) (SPGDLQTLALEVARQKR) segment since reactivity to it had been found in NOD mice (5; 13). These data extend our understanding of the role of proinsulin as an autoantigen in type 1 diabetes and demonstrate an inverse relationship between peptide-MHC affinity and T cell autoreactivity against a defined beta cell antigen epitope.

**RESEARCH DESIGN AND METHODS**

**Mice.** NOD, NOD.scid, NOD.h4, B6.g7 and B10.BR mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). All mice were housed and cared for in accordance with the guidelines of the Washington University Committee for the Humane Care of Laboratory Animals and with National Institutes of Health guidelines on laboratory animal welfare.

**Peptides.** Synthetic peptides were purchased from Biosynthesis, Inc.
(Lewisville, Texas). All peptides were >95% pure, confirmed by HPLC and MS analysis.

**PPLN assays.** Spleens, axillary and inguinal lymph nodes, and peripancreatic lymph nodes were isolated from 10-16 week old male and female NOD mice and dispersed into single-cell suspensions by passage through cell strainers. The splenocytes or lymph node cells (3-5x10^4/well) were subsequently cultured with irradiated NOD splenocytes (5x10^5/well), and 1-5 μM peptide for 7 days in round bottom 96-well plates in a final volume of 200 μl. On day 7 the cultures were re-stimulated with fresh irradiated NOD splenocytes (5x10^5/w), IL-2 (25 units/ml), and 1-5 μM peptide. T cell positive wells were tested days 14-17 with APCs and peptide (10 μM). Only data from cells with greater than 200 CPM were considered, the stimulation index (SI) was calculated by dividing values for APCs+peptide/APCs alone. The data presented in figure 2A was pooled from seven independent experiments. All tissue culture was done in DMEM supplemented with 10% fetal calf serum (DMEM 10% FCS).

**Primary T cell lines.** The anti-C-peptide primary T cell lines were generated by immunization of 8-10 week old NOD mice with 10 nmoles of PI-1(47-64) in complete Freund’s adjuvant (CFA). Immunized lymph nodes were harvested at day 7 and cultured at 5 x 10^5 cells/well in DMEM with 10% FCS and 1 μM peptide. On day seven, and at 7-10 day intervals thereafter, the T cells (1-2 x 10^4/w) were restimulated with fresh irradiated NOD splenocytes (5 x 10^5 cells/well), IL-2 (25 U/ml) and peptide (1μM). Hybridomas were generated from these primary lines by fusion to the BW5147 α-β- thymoma partner cell line following standard techniques (14).

**T cell assays.** Primary T cell proliferation assays were performed by incubation of T cells (2x10^4/well) with irradiated NOD splenocytes (5x10^5/well) in the presence of peptide or dispersed mouse beta cells. Primary T cell proliferation assays were pulsed with thymidine (1 μCi/well) on day 2 and harvested day 3. T cell hybridoma assays were performed with NOD splenocytes or the I-A^87 expressing cell line, C3G7 (2.5x10^4/well) as APCs and supernatants were collected at 24 hours for IL-2 quantitation by CTLL assay. Fixation of the C3G7 cell line was performed by incubation in 1% paraformaldehyde for 15 minutes followed by incubation in 0.2M DL-lysine for 10 minutes and then washing 3 times in DMEM 10% FCS.

**Islet isolation.** Islets of Langerhans were isolated from mice of various ages and dispersed using standard techniques (15). The digestion of the pancreatic tissue was performed with Type XI collagenase (Sigma, St. Louis, MO) without DNase.

**ELISPOT.** Mice were immunized with 10 nmoles PI-1(47-64) peptide in CFA and lymph nodes were examined on day 7 by ELISPOT analysis for IL-2 positive cells. Lymph nodes cells (1x10^6/well) were plated in triplicate on anti-IL-2 capture antibody coated filter plates in the presence of 10 μM antigen and developed 24 hours later with anti-IL detection antibodies (BD Biosciences, San Jose, CA). Plates were read by C.T.L. Cellular Technology Ltd. and analyzed with ImmunoSpot 3.2 software.

**Peptide-binding assays.** Soluble I-A^87 or I-A^k was produced using the recombinant baculovirus system as previously described (16). Peptide binding assays were done under acidic (pH 5.5) conditions. Briefly, 0.5-1 ug of I-A^87 or I-A^k/class II-associated invariant chain peptide was treated with 0.1 U of
thrombin to cleave both the zipper tails and peptide linker (Novagen, Madison, WI) and simultaneously incubated with 0.125 pmol of I\(^{125}\)-radiolabeled mimotope reference peptide (GKKVATTVHAGYG) and increasing doses of unlabeled peptides in 200 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 20 mM MES, and 150 mM sodium chloride. Binding reactions were incubated overnight at 25°C in 30 \(\mu\)l volumes. Complexes were purified from free peptide by gel filtration Bio-spin columns (Bio-Rad). The percentage of bound peptide was evaluated by gamma counting. Usually about 25-35% of input peptide was bound, while less than 0.5% of peptides nonspecifically passed through the Bio-spin columns. The IC\(_{50}\) value is very close to the binding equilibrium constant. The IC\(_{50}\) values shown in Table II are averages of 3-5 independent binding experiments.

**Biologic K\(_{off}\) rates.** The C3G7 APC line was incubated with peptide (10 \(\mu\)M) for 2 hours and washed. Anti-C-peptide T cells (2x10\(^4\)/well) were added to the APC containing wells (2.5x10\(^4\)/well) cultures at time zero, and at 4 and 8 hours after washing. The supernatants were collected at 24 hours and IL-2 was measured by CTLL assay.

**Adoptive T cell transfer.** Two of the three primary ACP T cell lines were expanded in vitro and transferred into 8-12 week old NOD.scid recipients by tail vein injection. Diabetes was defined by the measurement of blood sugars >250 mg/dl on two separate occasions. All recipients were followed for 30 weeks or up to disease onset.

**RESULTS**

*The PI-1(47-64) peptide bound weakly to I-A\(^{\beta7}\).* Binding assays were done with soluble I-A\(^{\beta7}\) and I-A\(^k\) and synthetic peptides. The IC\(_{50}\) value indicates the concentration of unlabeled peptide required to affect binding of a reference peptide to the class II molecules, the higher the value, the weaker the binding of the peptide to the MHC. The PI-1(47-64) and PI-2(49-66) peptides bound poorly to I-A\(^{\beta7}\), with IC\(_{50}\) values of 98.9 \(\mu\)M and 135.9 \(\mu\)M, respectively (Table II). In contrast, these peptides bound to I-A\(^k\), with IC\(_{50}\) values of 4.8 \(\mu\)M and 2.1 \(\mu\)M, respectively. The PI-1(49-61) peptide bound slightly better to I-A\(^{\beta7}\) with an IC\(_{50}\) of 20 \(\mu\)M, perhaps due to the loss of the positively charged P62Lys, which is likely disfavored at the carboxy-end of the peptide binding groove of I-A\(^{\beta7}\). As a point of reference, the mouse insulin-2 beta-chain (9-23) peptide bound to I-A\(^{\beta7}\) with an IC\(_{50}\) of 3 \(\mu\)M.

**Reactivity to PI-1(47-64) is I-A\(^{\beta7}\) dependant.** The role played by the MHC class II molecule, I-A\(^{\beta7}\), and background genes, in autoreactivity to C-peptide was examined by characterizing the T cell responses in NOD, NOD.h4, B6.g7, and B10.BR mice. Mice were immunized with the PI-1(47-64) peptide and lymph nodes were examined on day 7 by ELISPOT analysis for IL-2 positive cells. Both I-A\(^{\beta7}\) containing strains, NOD and B6.g7, had peptide reactive T cells (Figure 1A,C) compared to the I-A\(^k\) positive strains that did not (Figure 1B,D). The frequency of PI-1(47-64) reactive cells detected under these conditions was low: approximately 1/30,000 in the NOD mice and 1/50,000 in the B6.g7 mice. However, PI-1(47-64) specific T cells were not detected in the NOD.h4 and B10.BR mice. The T cell reactivity against PI-1(47-64) present in the NOD and B6.g7 mice was clearly I-A\(^{\beta7}\) restricted as evidenced by inhibition with MHC class II blocking antibody, AG2.42.7 (17). These data demonstrate
that I-A\(^{87}\) alone is necessary and sufficient for the generation of PI-1(47-64) T cells independent of other background genes.

**Peri-pancreatic lymph nodes harbor C-peptide reactive T cells.** Given the central importance of the PPLNs in the priming of diabetogenic T cells, we began by examining the PPLNs of prediabetic mice for the presence of T cells specific for the PI-1(47-64) peptide. Like others (5; 18), we did not detect reactivity to proinsulin peptides in primary proliferation assays with PPLNs or spleens from 10-16 week-old NOD mice (data not shown). Given this difficulty in detecting primary T cells responses, we expanded PPLN T cells, in addition to T cells from the spleen and peripheral lymph nodes, for two weeks in vitro with the PI-1(47-64) peptide, and then tested all T cells in the expanded cultures for peptide reactivity. T cells specific for the PI-1(47-64) peptide were identified in the PPLNs from 10-16 week old mice, but were not found in the spleen or axillary and inguinal lymph nodes (Figure 2A, B). 5 out of 246 expanded T cell lines from the PPLNs reacted with the PI-1(47-64) peptide (stimulation index >5). In contrast, no PI-1(47-64) reactive T cells were detected from the 454 T cell lines expanded from the spleen or axillary and inguinal lymph nodes. The presence of C-peptide reactive T cells in the PPLNs was in agreement with previous data that indicated these nodes as the site for priming of diabetogenic T cells (19-21). Moreover, this finding also suggested a possible role for these T cells in the early pathogenesis of the disease. To further characterize the biology of C-peptide reactive T cells we first generated several CD4+ T cell lines were generated, including the anti-C-peptide (ACP) #2 line (Figure 3A). All lines demonstrated similar reactivity in vitro, maximally stimulated by the PI(47-64) peptide from proinsulin-1 and also reactive with the PI(49-66) peptide from proinsulin-2. The T cell lines also recognized the PI-1(49-61) and the PI-2(51-63) segments, which differ only by one residue (Arg for PI-1 at position 60 and Gln for PI-2 at position 62). The T cell lines were stimulated by the full length C-peptides from proinsulin 1 and 2, although less than by the PI-1(47-64) peptide.

In order to determine if the ACP T cell lines were reactive with the naturally processed and presented forms of the proinsulin epitope, T cells were tested for reactivity with freshly isolated mouse beta cells. All three primary ACP lines, including ACP #2, reacted to dispersed mouse beta cells from young NOD mice (4-6 weeks of age), as well as, beta cells from other strains such as 129/Svj (Figure 3B). Furthermore, the ACP T cells reacted to dispersed islets isolated from 5 month old NOD mice demonstrating that antigen presenting cells from the islet infiltrate present the C-peptide epitope (Figure 3C).

**Minimal epitope mapping and antigen processing requirements for C-peptide reactive T cells.** Analysis of ACP T cell hybridomas showed heterogeneity in their reactivity to truncated peptides (Figure 4A and Table III). The likely minimal T cell epitope spanned the PI-1(51-61) sequence (LQTLALEVARQ), since loss of the P51Leu resulted in a noticeable decrease in T cell activation for all hybridomas examined (Figure 4A and Table III). Some T cells responded well to the PI-1(49-60) peptide lacking the P61Gln, such as ACP2.1, where as other did not, see...
ACP1.28, indicating variable requirements for carboxy terminal residues. Identification of the nonamer canonical core within the PI-1(51-61) segment could not be determined by T cell reactivity, and all T cells tested (0/8) with the three possible nonamers spanning the PI-1(51-61) peptide were negative; (51-59:LQTLALEVA, 52-60:QTLALEVAR, 53-61:TLALEVARQ). These results may indicate a need for amino or carboxy flanking residues that serve as TCR contacts, as was seen with the beta-chain (9-23) epitope (2), or possibly loss of MHC binding affinity with the truncated peptides, or both.

In order to assess the requirements for antigen processing of the C-peptide T cell epitopes, T cells were tested for reactivity with synthetic peptides presented by fixed APCs. We found that ACP T cell hybridomas were stimulated by fixed APCs presenting the PI-1(47-64), PI-1(49-61), and full length C-peptide (Figure 4B), indicating that all three peptides, even the full length C-peptide-1 29-mer, could form recognizable complexes without the need for further processing.

We next examined the stability of the peptide-MHC complexes formed by PI-1(47-64) and I-A\(^{g7}\) by functional experiments in which T cells were applied to antigen pulsed and washed APCs over an 8 hour time course (Figure 4C). The antigen pulsed APCs quickly lost their ability to stimulate C-peptide reactive T cells. The T cell response, for both PI-1(47-64) and C-peptide-1 pulsed APCs, was reduced to 50% of its maximal value in 3 hours, indicating that the C-peptide/MHC complexes disassociated rapidly.

**C-peptide reactive T cells induce diabetes.** The pathogenicity of the ACP primary T cell lines was assessed by adoptive transfer into NOD.scid recipient mice. Three separate transfer experiments were performed: group 1 (n=8) received 9.4x10\(^6\) ACP#1 T cells; group 2 (n=4) received 17x10\(^6\) ACP#2 T cells; group 3 (n=4) received 3 infusions of ACP#1 and #2 T cells. The incidence of diabetes was 50% for all groups; group 1 (4/8) by 20 weeks post transfer, group 2 (2/4) at 10 and 20 weeks post transfer, and group 3 (2/4) at 20 and 22 weeks post transfer (Figure 5A). The induction of diabetes in 8/16 NOD.scid mice, which never develop diabetes, indicated that the ACP T cells were pathogenic in the adoptive transfer model. Although the kinetics of disease induction in these experiments is less than some observed with activated T cells from TCR transgenic mice, BDC2.5 for example, the incidence is similar to the spontaneous incidence seen in wild-type NOD mice. Furthermore, all recipient mice, including those that were not overtly hyperglycemic at 30 weeks, had severe destructive insulitis and loss of beta cell mass (Figure 5B).

**DISCUSSION**

The main findings from these studies are 1) binding of the PI-1(47-64) and PI-1(49-61) peptides to I-A\(^{g7}\) is incredibly poor, and the peptide-MHCs are short-lived; 2) I-A\(^{g7}\) plays a central role in the generation of the anti-C-peptide T cell repertoire in that autoreactivity was found to strains bearing I-A\(^{g7}\) but not I-A\(^{k}\) molecules; 3) C-peptide reactive T cells are found in the peri-pancreatic lymph nodes of prediabetic NOD mice; 4) anti-C-peptide T cells are reactive with mouse beta cells and respond to full length C-peptide presented by live, and fixed APCs; 5) despite this weak peptide-MHC interaction, C-peptide reactive T cells are
capable of causing diabetes in an adoptive transfer model.

Our data confirms, and extends the observations of other groups that have identified the C-peptide region of proinsulin as a CD4+ T cell target in the NOD mouse (5; 13; 22; 23). The recovery of C-peptide reactive T cells from the PPLNs, and not from other locations, suggests that these T cells may encounter their antigen in this critically important site of diabetogenic T cell priming (19-21; 24). Intra-islet APCs, most likely dendritic cells, must encounter large amounts of C-peptide in the islets and may traffic to the PPLNs where they could prime and activate naïve C-peptide reactive T cells. Alternatively, lymphatic drainage of the pancreas might deliver high concentrations of C-peptide to the PPLNs, allowing uptake and presentation by nodal dwelling APCs. Indeed, the islets readily provided antigen to APCs-figure 3B, and APCs recovered from insulitic islets stimulated the T cells-figure 3C. In either case it is not difficult to imagine a scenario in which PPLNs are exposed to large amounts of C-peptide, which would be required for activation of the T cells by these very weak MHC-binding peptides. Although C-peptide circulates, its concentration in blood, 2-5 nM, would not be sufficient for either activation or regulation in APCs at peripheral sites.

Why aren’t C-peptide reactive T cells deleted in the thymus? Weak MHC-binding peptides may well be the best at fostering autoimmunity in great part by avoiding negative selection in the thymus, or by failure of regulatory mechanisms, although other factors are likely to play a role. The PI-1(47-64) epitope represents a striking example of a very poor binding MHC ligand, IC50 values ~100 μM (1), that gives rise to T cells capable of causing diabetes in adoptive transfer experiments. The relationship between poor peptide-MHC binding and the fostering of an autoimmune, pathogenic T cell repertoire has been suggested in studies on experimental allergic encephalomyelitis (7; 8) and in our recent work on the insulin beta-chain (9-23) epitope (2) and in other model systems (9-11).

The obligatory role for the diabetogenic I-A<sup>g7</sup> molecule in eliciting C-peptide-reactive CD4 T cells remains a key issue. Our results with the four mouse strains: NOD (I-A<sup>g7</sup>+), NOD.h4 (I-A<sup>k</sup>+), B6.g7 (I-A<sup>g7</sup>+ and B10.BR (I-A<sup>k</sup>+), demonstrated that expression of I-A<sup>g7</sup>, independent of the genetic background, was sufficient to generate C-peptide reactive CD4 T cells (Figure 1). This is in agreement with previous data from Kanagawa et al. (25) and Ridgway et al. (26), indicating that the expression of I-A<sup>g7</sup> in different strains of mice allowed for escape of autoreactive T cells. A more recent study from Stratmann et al. (27) used MHC tetramers to trace islet-reactive CD4+ T cells in I-A<sup>g7</sup>-expressing mice of different genetic backgrounds. Here again, the expression of I-A<sup>g7</sup> in different strains selected for tetramer-reactive CD4+ T cells. The NOD strain has also been shown to be susceptible to other autoimmune diseases (28-31). Taken together, these data point to a central role for I-A<sup>g7</sup> in selection (or escape) of autoreactive T cells. We believe that the explanation lies in the structural features of I-A<sup>g7</sup>, characterized by an overall low peptide-binding affinity and instability (32), in addition to other susceptibility genes. Indeed these findings do not lessen the importance of other factors in NOD mice that play a role in the loss of self tolerance (33-35). The fact that B6.g7 mice do not develop diabetes highlights
the need of both MHC and non-MHC encoded susceptibility genes.

A second important factor in the lack of T cell tolerance may be specific to the insulin molecule. It may be that the peptide-MHC complexes formed in the thymus, do not recapitulate the complexes that appear in the periphery. The deleting complexes are presumably formed by the traditional processing and presentation of proinsulin peptides in thymic medullary epithelial cells. Here, peptides are selected by the biological and physical forces that govern peptide presentation by I-A\textsuperscript{g7} molecules in thymic cells, and are not the same as events that might occur in the periphery. Although proinsulin is clearly expressed in the thymus (36; 37) perhaps largely under the control of the AIRE transcription factor (38), there is no evidence for the expression of the prohormone processing machinery, such as the prohormone convertases 1 and 2, which would be required to generate the actual C-peptide fragment that is secreted by the beta-cell. The absence of tolerance to C-peptide, as it is presented in the pancreas, may be explained, in a sense, by the lack of a secretory apparatus in the thymus.

The data presented here extends our understanding of the role of proinsulin as an autoantigen and again seems to illustrate an inverse relationship between weak peptide-MHC binding and T cell autoimmune pathogenicity. The degree to which C-peptide reactive T cells contribute to disease pathogenesis in the NOD mouse cannot be determined with certainty. The T cells were pathogenic in circumstances of adoptive transfer and they represent one set of the many proinsulin reactive T cells that seem to play a key role in the development of autoimmune diabetes. The identification of T cell reactivity against C-peptide epitopes in Type 1 patients suggests that C-peptide might also be an important autoantigen in the human disease (39-42).

ACKNOWLEDGEMENTS
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REFERENCES
17. Suri A, Vidavsky I, van der Drift K, Kanagawa O, Gross ML, Unanue ER: In APCs, the autologous peptides selected by the diabetogenic I-Ag7 molecule are unique and determined by the amino acid changes in the P9 pocket. *J Immunol* 168:1235-1243, 2002
**Table I.**  
HUMAN AND MURINE PROINSULIN SEQUENCES

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The sequences of the human, mouse-1 and mouse-2 proinsulin molecules are shown. The amino acid residues in red are cleaved off during the physiologic processing of the prohormone and C-peptide is secreted in equimolar amount with the mature insulin heterodimer. The amino acid residue numbering refers to the mouse proinsulin-1 sequence and the PI-1(47-64) segment is underlined.
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This panel shows the binding analysis of synthetic peptides to soluble I-A^g7 and I-A^k. Binding is expressed by IC_{50} values, μM. The mime peptide is a synthetic reference peptide that has been used in I-A^g7 binding assays (16). The binding of mouse insulin-2 B:(9-23), 3 μM, peptide is presented as a point of reference. Binding studies were performed at pH 5.5 as described in the Materials and Methods.
Table III.  ACP T cell SC$_{50}$ values.

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<td>—</td>
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<td>—</td>
<td>—</td>
<td>&gt;30</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GDLQTLAELVAQQ</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.6</td>
<td>0.5</td>
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Values shown are concentrations of peptide required to half maximally stimulate a given T cell (μM, -: none detected). Values are averages of 2-5 independent experiments.
FIGURE LEGENDS

Figure 1. The anti-C-peptide T cell repertoire in NOD mice is I-A<sup>g7</sup> dependant. Both I-A<sup>g7</sup>-containing strains, NOD and B6.g7, had peptide reactive T cells (A,C) compared to the I-A<sup>k</sup> positive strains, NOD.H4 and B10.BR that did not (B, D). The frequency of PI-1(47-64) reactive cells detected under these conditions was approximately 1/30,000 in the NOD mice and 1/50,000 in the B6.g7 mice. However, PI-1(47-64) specific T cells were not detected in the NOD.H4 and B10.BR mice. The T cell response to PI-1(47-64) in NOD and B6.g7 mice was MHC class II restricted as evidenced by inhibition with the MHC class II blocking antibody, AG.2.42.7. Data presented is representative of three independent experiments.

Figure 2. Peri-pancreatic lymph nodes contain T cells reactive with PI-1(47-64). (A) T cells from the PPLNs of NOD mice from 10-16 weeks of age contain PI-1(47-64) reactive T cells. 5/246 expanded T cell lines from the PPLNs reacted with the SPGDLQTLALEVARQKRG peptide with a stimulation index (SI) greater than 5. In contrast, 0/454 T cell lines expanded from the spleen, or axillary and inguinal lymph nodes were stimulated by the PI-1(47-64) peptide. (B) Data from one representative experiment in which 41 T cell lines from the PPLNs were tested for reactivity with the PI-1(47-64) peptide.

Figure 3. Primary anti-PI-1(47-64) T cell lines are reactive with beta cells. (A) Three primary anti-PI-1(47-64) T cell lines were generated by immunization of young NOD mice. One such line, ACP #2, recognized the PI-1(47-64) peptide, in addition to the PI-1(49-61) segment and full length C-peptide-1. The ACP#2 T cell line recognized peptides from the PI-2 sequence, although not as well as PI-1. (B) The ACP#2 T cell line responded to freshly isolated beta cells from 4-6 week old NOD mice. In both peptide and beta cell assays, irradiated NOD splenocytes were used as APCs and cultures were pulsed with labeled thymidine on day 2 and harvested on day 3. (C) ACP#1 reacted to dispersed islets from 5 month old NOD mice in the absence of exogenous APCs.

Figure 4. Anti-C-peptide T cells respond to PI-1(49-61) and full length C-peptide presented by fixed APCs and the pMHC complexes dissociate rapidly. (A) The ACP1.28 T cell responds well to PI-1(49-61):GDLQTLALEVARQ, however, reactivity was abolished with the loss of the P51Leu or the P61Gln. The ACP2.1 T cell, in contrast, appeared to tolerate truncations of the flanks and recognized PI-1(49-60): GDLQTALAEVAR rather well and responded weakly to both PI-1(52-61):QTLAELVARQ and PI-1(49-59):GDLQTALAEVA. (B) Both ACP1.28 and ACP2.1 responded strongly to fixed APCs pulsed with peptide, indicating that further processing of the peptide was not required. This was true for the PI-1(47-64) and PI-1(49-61) epitopes, in addition to the full length C-peptide-1 molecule. (peptide concentration: 30 μM, experiments performed in triplicate) (C) The stability of the pMHC complexes formed from PI-1(47-64) or C-peptide-1 was examined by adding T cells to antigen-pulsed and washed APCs over an 8 hour time course. The % maximal response was determined by dividing the T cell response at T<sub>4</sub> or T<sub>8</sub>, in CPM, by the value achieved when the T cells were added at T<sub>0</sub>. APCs pulsed with either PI-1(47-64) or C-peptide-1, lost 50% of their ability to maximally stimulate T cells in 3 hours, indicating that the pMHC complexes dissociated rapidly on the surface of the APCs.
Figure 5. Anti-C-peptide T cells transferred disease into recipient NOD.scid mice and cause severe insulitis. (A) Adoptive transfer of ACP T cells into NOD.scid recipient mice induced diabetes in 50% (8/16) by 22 weeks post transfer. See Materials and Methods for details. (B) Histology of islets from all recipient mice, diabetic and normoglycemic, showed severe, destructive insulitis. The islet histology shown in this figure was taken from a non-diabetic recipient mouse at 30 weeks post transfer.
Figure 2

A

<table>
<thead>
<tr>
<th>T cells analyzed</th>
<th>PI-1(47–64) reactive T cells (SI &gt; 5)</th>
<th>%</th>
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<tbody>
<tr>
<td>Ax/Ing LN</td>
<td>302</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>152</td>
<td>0</td>
</tr>
<tr>
<td>PPLN</td>
<td>246</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 3

A

ACP #2

- P1-1(47-64)SPGDLQTLALEVARQKR
- P1-2(49-66)GAGDLQTLALEVAQQKR
- P1-1(49-61)GDLQTLALEVARQ
- P1-2(51-63)GDLQTLALEVAQQ
- C-peptide-1 (1-29)
- C-peptide-2 (1-31)

B

ACP #2

- BETA CELLS

C

ACP #1

- II alone
- II + T
- II + T + anti-I-Aβ7
Figure 5

A

\[ \text{Weeks post transfer} \]

\[ \% \text{ Disease Free} \]

B

[Image of histological section]