Cutting Edge: CD4 T Cells Reactive to an Islet Amyloid Polypeptide Peptide Accumulate in the Pancreas and Contribute to Disease Pathogenesis in Nonobese Diabetic Mice

Rocky L. Baker, Thomas Delong, Gene Barbour, Brenda Bradley, Maki Nakayama and Kathryn Haskins

*J Immunol* published online 16 September 2013
http://www.jimmunol.org/content/early/2013/09/14/jimmunol.1301480

---

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/09/16/jimmunol.1301480.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: CD4 T Cells Reactive to an Islet Amyloid Polypeptide Peptide Accumulate in the Pancreas and Contribute to Disease Pathogenesis in Nonobese Diabetic Mice

Rocky L. Baker, Thomas Delong, Gene Barbour, Brenda Bradley, Maki Nakayama, and Kathryn Haskins

We previously reported a peptide KS20 from islet amyloid polypeptide (IAPP) to be the target Ag for a highly diabetogenic CD4 T cell clone BDC-5.2.9. To track IAPP-reactive T cells in NOD mice and determine how they contribute to the pathogenesis of type 1 diabetes, we designed a new I-Ag7 tetramer with high affinity for BDC-5.2.9 that contains the peptide KS20. We found that significant numbers of KS20 tetramer+ CD4 T cells can be detected in the pancreas of prediabetic and diabetic NOD mice. To verify pathogenicity of IAPP-reactive cells, we sorted KS20 tetramer+ cells and cloned them from uncloned T cell lines isolated from spleen and lymph nodes of diabetic mice. We isolated a new KS20-reactive Th1 CD4 T cell clone that rapidly transfers diabetes. Our results suggest that IAPP triggers a broad autoimmune response by CD4 T cells in NOD mice. The Journal of Immunology, 2013, 191: 000–000.

Type 1 diabetes (T1D) is an autoimmune disease in which CD4 T cells play a central role in the destruction of insulin-producing β cells. The NOD mouse is a widely used animal model for T1D and has been of great value for our understanding of the genetic basis, immune pathogenesis, and regulation of disease. Our BDC panel of autoreactive CD4 T cell clones was generated from spleen and lymph node cells of diabetic NOD mice, and has provided a useful set of tools for rapid transfer of disease and for investigating effector function of Th1 T cells in the pancreas (1, 2). We have also exploited the BDC panel of T cell clones for identifying new autoantigens in T1D. A peptide from chromogranin A (ChgA) was identified as an antigenic epitope for the T cell clone BDC-2.5 (3), and KS20, a 20-aa peptide from islet amyloid polypeptide (IAPP), was found to stimulate the diabetogenic T cell clone BDC-5.2.9 (4).

MHC tetramers have been used to track how T cells respond to particular Ags in a variety of health-related areas such as infectious disease, tumor immunology, and vaccines (5). In T1D, where disease is driven, in part, by CD4 T cells, the development of MHC class II reagents to characterize autoreactive CD4 T cell responses is of great interest. Although >30 islet autoantigens have been identified in the NOD mouse (many of which are detected in human T1D patients as well) (6), there has been only limited success producing I-Ag7 tetramers. One exception is the I-Ag7 tetramer that detects BDC-2.5–like T cells in NOD mice, a reagent that was produced with a peptide mimotope highly stimulatory for BDC-2.5 (7). In this study, our goal was to determine whether pathogenic CD4 T cells with reactivity to IAPP could be identified in NOD mice by means of a new I-Ag7 tetramer reagent incorporating KS20, a natural peptide sequence. We report in this article on the binding specificity of the I-Ag7/KS20 tetramer and demonstrate that IAPP-reactive T cells accumulate in the pancreas of diabetic mice and contribute to disease development. IAPP is a previously uncharacterized autoantigen for CD4 T cells in T1D, and our studies suggest that IAPP-reactive T cells are important players in the pathogenesis of T1D.

Materials and Methods

Mice

NOD and NOD.scid breeding mice were acquired from The Jackson Laboratory, and were bred and housed in specific pathogen-free conditions at National Jewish Health. NOD.IAPP+ mice were bred in our colony by backcrossing C57BL6.IAPP+ mice (8) onto the NOD background. Breeding mice and experimental animals were monitored for development of disease by urine glucose (Diastix; Bayer), and hyperglycemia was confirmed by OneTouch Ultra glucometer (LifeScan). Prediabetic mice used in this study were 10–28 wk of age and had normal blood glucose readings (4–9 mmol/l) at the time of analysis. Mice were considered diabetic when blood glucose levels were >15 mmol/l (270 mg/dl) for two consecutive readings. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.
**Peptides**

HRPI-RM is a BDC-2.5 mimotope (9), full sequence: EKAHRPIWARMDAK, and was obtained from Kurabo Industries (Osaka, Japan). All other synthetic peptides described in Table I and Fig. 3C were obtained from CHI Scientific at a purity >98%.

**Culture and isolation of T cell clones**

T cell clones (BDC-2.5, BDC-10.1, and BDC-5.2.9) were restimulated in complete medium, which is supplemented DMEM, as described previously (10). We isolated the Vβ10 BDC-5/S3.4 and the Vβ12 BDC-9/S3.5 CD4 T cell clones by FACS sorting using the Synergy cell sorter (Cyto, IL; three consecutive single-cell sorts were performed to purify the Vβ10 or Vβ12 populations) followed by cloning by limiting dilution. These T cell lines were stimulated biweekly with Ag/MHC as described earlier.

**Competitive peptide binding assay**

Soluble I-Ag7 with covalently attached Class II–associated invariant chain peptide (CLIP) was obtained from the National Institutes of Health tetramer core, and competitive peptide binding assays were performed as previously described by Stadinski and colleagues (3). To quantify binding to I-Ag7, we compared inhibition curves for each peptide with the curve obtained with the HEL (hen egg lysozyme) peptide (GGGMKRHGLDNYRGYSL) using the MKASSAY software (J. Kappler, National Jewish Health, Denver, CO). Results are presented as the binding capacity of each peptide relative to that of HEL, which was set at 100% for each concentration tested. I-Ag7 binding ability of each peptide relative to HEL is expressed as a percent. Data are from two independent experiments, and individual binding results varied <30% from the averaged value.

**Flow cytometry**

Surface and intracellular cytokine staining were performed as previously described (11) with combinations of the indicated reagents and were then analyzed on a Cyan flow cytometer (DakoCytomation). Data were analyzed using FlowJo software (TreeStar). For tetramer staining, cells were resuspended in complete medium with NaN3 and Fc block and containing 20 μg/ml tetramer. Cells were then cultured at 37°C and resuspended every 30 min. After 2 h, surface staining with Abs was performed at room temperature for 20 min.

**Adoptive transfer**

For T cell clones, cells from expansion subcultures were washed three times in HBSS, and 1 × 10⁶ cells were injected i.p. into young (<14-d-old) NOD or NOD.scid pups.

**Ex vivo analysis of pancreas**

Pancreata were harvested and tissue was digested to obtain a single-cell suspension using collagenase (Sigma) or liberase (Roche, Germany) according to the manufacturer’s protocol. Single-cell suspensions were washed and stained with an appropriate master mix of Abs and tetramers before flow cytometry analysis.

**Ag assay**

T cell clones (2 × 10⁶) were cultured with peritoneal exudate cells (2.5 × 10⁵) and Ag, as described previously (4), to test responses to Ag. Ags used were islet cells (1 × 10⁶) from either NOD or NOD.IAPP−/− mice, a granule-enriched membrane fraction obtained from β cells, referred to as β-membrane (12), or synthetic peptides (HRPI-RM), B9-23 (Insulin B chain 9-23; SHLVEALYLVCGERG), or as described in Table I. After 24 h, IFN-γ concentrations were determined in culture supernatants of duplicate wells by ELISA.

**Statistics**

Statistical significance was determined by a two-tailed Student test. The p values ≤0.05 were considered significant.

**Results and Discussion**

**An I-Ag7/KS20 tetramer binds the IAPP-reactive CD4 T cell clone BDC-5.2.9 with high specificity**

With the exception of GAD65 (13) and insulin (14), few I-Ag7 MHC class II tetramers have been described for investigating the Ag-specific CD4 immune response in T1D. We previously reported that KS20, a 20-mer peptide sequence from IAPP, is the peptide ligand for the diabetogenic CD4 T cell clone BDC-5.2.9 (4). To design an MHC class II tetramer capable of detecting IAPP-reactive CD4 T cells, we identified the optimal amino acid sequence from KS20 for activation of BDC-5.2.9. Peptide-mapping studies were performed in which we investigated truncated versions of pro-IAPP, or the KS20 sequence within, for ability to stimulate the T cell clone and to bind to I-Ag7 (Table I). For T cell activation, we measured IFN-γ responses to Ag/MHC as a readout, and EC₅₀ values were determined to compare the activity of the different peptides with KS20 (EC₅₀ = 0.2 nM). In Table I, the relative binding affinity of the different peptides is compared with an HEL peptide, previously reported to bind strongly to I-Ag7 (15). Our data established that the 20-aa IAPP peptide, KS20, is the optimal sequence for stimulation of the T cell clone BDC-5.2.9, and this peptide was therefore used for the production of the I-Ag7/KS20 tetramer with the KS20 sequence covalently attached to the β-chain of I-Ag7.

To determine whether the I-Ag7/KS20 tetramer was specific for IAPP-reactive T cells, we stained the diabetogenic CD4 T cell clone BDC-5.2.9, specific for KS20, with the tetramer and used as controls two non-IAPP–reactive T cell clones from the BDC panel, BDC-2.5 and BDC-10.1. Control tetramers were the I-Ag7/2.5mi tetramer specific for BDC-2.5 (7) and I-Ag7/CLIP. Fig. 1 shows that the I-Ag7/KS20 tetramer stains only the BDC-5.2.9 T cell clone, and the I-Ag7/2.5mi tetramer stains only BDC-2.5; none of the clones tested stained with the I-Ag7/CLIP tetramer. These data indicate that the I-Ag7/KS20 tetramer is specific for the IAPP-reactive T cell clone BDC-5.2.9, and are consistent with the ability of KS20 to activate BDC-5.2.9. However, because the I-Ag7/KS20 tetramer is loaded with a natural sequence from IAPP, we hypothesized that this tetramer might also be able to detect KS20-reactive CD4 T cells expressing a variety of TCRs.

**Table I. EC₅₀ and I-Ag7 binding of peptides derived from IAPP**

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide Sequence</th>
<th>EC₅₀ (nM)</th>
<th>I-Ag7 Binding (Relative to HEL %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG38</td>
<td>KCNTATCATQRLANFLVRSS</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>DS23</td>
<td>DKRCNTATCATQRLANFLVRSS</td>
<td>55</td>
<td>93.5</td>
</tr>
<tr>
<td>KS20</td>
<td>KCNTATCATQRLANFLVRSS</td>
<td>0.2</td>
<td>61.9</td>
</tr>
<tr>
<td>KV17</td>
<td>KCNTATCATQRLANFLVRSS</td>
<td>10</td>
<td>10.8</td>
</tr>
<tr>
<td>KN14</td>
<td>KCNTATCATQRLANFLVRSS</td>
<td>&gt;10⁵</td>
<td>44.1</td>
</tr>
<tr>
<td>KQ10</td>
<td>KCNTATCATQ</td>
<td>&gt;10⁵</td>
<td>12.3</td>
</tr>
<tr>
<td>CS19</td>
<td>CNTATCATQRLANFLVRSS</td>
<td>5</td>
<td>40.7</td>
</tr>
<tr>
<td>NS18</td>
<td>NTATCATQRLANFLVRSS</td>
<td>&gt;10⁵</td>
<td>30.9</td>
</tr>
<tr>
<td>TS17</td>
<td>TATCATQRLANFLVRSS</td>
<td>&gt;10⁵</td>
<td>36.8</td>
</tr>
</tbody>
</table>
IAPP-reactive T cells accumulate in the pancreases of NOD mice.

MHC tetramers are capable of detecting Ag-specific T cells and are thus useful for exploring the immune response toward a particular Ag. Using the I-Ag7/KS20 tetramer, we investigated whether KS20-reactive CD4 T cells could be detected in the spleen, pancreatic lymph nodes, and pancreas of prediabetic NOD female mice. Compared with staining with the negative control (I-Ag7/CLIP), we observed a significantly higher percentage of I-Ag7/KS20 (p < 0.01) and I-Ag7/2.5mi tetramer+ cells (p = 0.01) in the pancreas of prediabetic NOD mice (Fig. 2A). Few to no tetramer+ cells were observed in the spleen or pancreatic lymph nodes (Supplemental Fig. 1).

Because I-Ag7/KS20+ cells were observed primarily in the pancreas, we compared the cellular infiltrate in prediabetic female NOD mice, diabetic NOD mice, and NOD. IAPP−/− mice as a negative control (there are no IAPP-reactive T cells in NOD.IAPP−/− mice, but incidence of diabetes is the same as in IAPP+/+ mice). As shown in Fig. 2A and 2B, none of the mice showed infiltration of I-Ag7/CLIP tetramer+ cells in the pancreas. In the pancreases of NOD. IAPP−/− mice, we observed a significantly higher percentage of I-Ag7/2.5mi tetramer+ cells compared with the CLIP control (p < 0.001), but no I-Ag7/KS20+ T cells (p = 0.08). Multiple Ags may contribute to disease pathogenesis and T cells with other Ag specificities (e.g., BDC-2.5−“like” T cells) in the pancreatic infiltrates of IAPP−/− mice is likely the reason why the absence of one Ag does not alter spontaneous disease progression. Prediabetic and diabetic NOD mice showed an increase of both I-Ag7/KS20+ and I-Ag7/2.5mi+ cells over I-Ag7/CLIP control (p < 0.02), and compared with prediabetic mice, there were higher percentages of I-Ag7/KS20+ cells in diabetic mice (p < 0.01).

Our results indicate that KS20-reactive T cells accumulate in the pancreas of prediabetic NOD mice and even more so in diabetic mice. Because tetramers detect only T cells with high affinity for peptide–MHC complexes (16), the percentage of tetramer+ cells reported in our study may underestimate the actual percentage of these cells. Overall, the data indicate that IAPP is an important autoantigen in NOD mice: significant numbers of IAPP-reactive T cells are found in the pancreas of ∼40% of prediabetic mice and >80% of diabetic NOD mice.

KS20-reactive T cells are diverse in TCR usage.

Our data indicate that KS20-reactive T cells in the pancreas can be detected using the I-Ag7/KS20 tetramer, but because of the relatively low numbers of cells and their poor viability in culture after ex vivo isolation, determination of TCR usage, functional phenotype, and diabetogenic potential of KS20-reactive T cells derived from the pancreas is difficult. Therefore, to address this question, two of the original uncloned BDC T cell lines (BDC-5 and BDC-9) were thawed, analyzed for antigenic reactivity, and tetramer screening was carried out to determine whether I-Ag7/KS20+ T cells were present. We found that a Vβ10 population in the BDC-5 line and a Vβ12 population in the BDC-9 line stained with the I-Ag7/KS20 tetramer and responded to KS20 by secreting high amounts of IFN-γ (data not shown). As shown in Fig. 3A and 3B, we isolated and cloned two additional I-Ag7/KS20 tetramer+ cells.
T cell clones bearing different TCR Vβ regions than BDC-5.2.9. Intracellular cytokine staining indicated that the new CD4+ T cell clones BDC-5/S3.4 and BDC-9/S3.5 had a Th1 phenotype similar to BDC-5.2.9 (Supplemental Fig. 2). The graph in Fig. 3C shows that although BDC-5/S3.4 and BDC-9/S3.5 reacted to NOD islets, these clones did not react to islets lacking IAPP. As a control, we used the BDC-2.5 T cell clone, which reacted with islets from both mouse strains. BDC-5/S3.4 and BDC-9/S3.5 were also strongly activated in response to KS20, confirming that IAPP/KS20 was the Ag for these T cell clones. To investigate pathogenicity, we adoptively transferred the new KS20-reactive T cell clone BDC-5/S3.4 into either young NOD or NOD.scid mice, and as illustrated in Fig. 3D, this clone is highly diabetogenic. Our results demonstrate that the I-Ag7/KS20 tetramer can detect KS20-reactive diabetogenic CD4 T cells isolated from diabetic NOD mice that vary in TCR Vβ usage. Sequencing analysis of the new T cell lines indicates that TCR Vα and β junction regions are also variable among IAPP-reactive CD4 T cells (Table II). Because each line (BDC-5 and BDC-9) was generated from different diabetic mice, our data also suggest that IAPP leads to a broad autoimmune response in NOD mice. Like BDC-5.2.9, both BDC-5/S3.4 and BDC-9/S3.5 respond to KS20 at nanomolar concentrations (data not shown), indicating that this peptide strongly stimulates these T cells. Others have found that the strength of signals induced by TCR ligands might influence the fate of Th cells (as reviewed in Ref. 17). For example, Gottschalk et al. (18) have found that a low dose of a strongly agonistic peptide could induce persistent Foxp3 induction, whereas, in contrast, it has been reported that high doses of strongly agonistic ligands favor Th1 differentiation (19). Because of the high abundance of IAPP in the secretory granules (20) and the strong stimulatory activity of KS20, we hypothesize that this islet protein is likely to promote a pathogenic Th1 phenotype in vivo.

Concluding remarks

Important questions remain to be answered regarding the role of IAPP in autoimmune diabetes. After insulin and ChgA, IAPP is the third β-cell secretory granule protein found to induce activation of pathogenic CD4+ T cells in NOD mice. One question is how does a strong peptide ligand such as the KS20 peptide from IAPP influence the immunological fate of IAPP-reactive cells, and is this a late disease stage Ag? Because disease is diagnosed in humans only at a late stage, it could be that Ag-specific tolerance induction strategies would be more effective targeting late-arising Ags than Ags found early in disease. Second, is IAPP processed differently or modified in the autoimmune environment? Like insulin and ChgA, IAPP has a propensity to aggregate, and particularly in the pancreas, it is likely to promote a pathogenic Th1 phenotype in vivo.

Table II. Comparison of TCR sequences of three IAPP-reactive T cell clones

<table>
<thead>
<tr>
<th>T Cell Clone</th>
<th>Junction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ</td>
<td></td>
</tr>
<tr>
<td>Vβ6</td>
<td>BDC-5.2.9</td>
</tr>
<tr>
<td>Vβ10</td>
<td>BDC-5/S3.4</td>
</tr>
<tr>
<td>Vβ12</td>
<td>BDC-9/S3.5</td>
</tr>
<tr>
<td>Vα</td>
<td></td>
</tr>
<tr>
<td>Vα12</td>
<td>BDC-5.2.9</td>
</tr>
<tr>
<td>Vα8</td>
<td>BDC-5/S3.4</td>
</tr>
<tr>
<td>Vα4</td>
<td>BDC-9/S3.5</td>
</tr>
</tbody>
</table>
creatic inflammatory environment, this property could contribute to these proteins being a good source of potentially autoantigenic peptides. Our results with a peptide from ChgA suggest that the diabetogenic ligand from this protein is posttranslationally modified (21). Finally, there is evidence to indicate that ChgA and IAPP are also important Ags in human patients. We have found a significant difference in CD4 T cell responses to ChgA between T1D patients and control subjects (Gottlieb et al. submitted for publication), and IAPP has been shown to be an autoantigen, both as a target of autoantibodies (22) and of CD8 T cells (23). Therefore, another question is whether the human analog of KS20 elicits T cell responses from T1D patients, and could these responses provide a new biomarker for human disease?

Acknowledgments
I-Ag7 tetramers and monomers used in this study were provided by the National Institutes of Health tetramer core (Atlanta, GA). We thank John Kappler and Fran Crawford for helpful discussion and assistance with the peptide binding assay.

Disclosures
The authors have no financial conflicts of interest.

References
The authors have no financial conflicts of interest.