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Ex Vivo Analysis of Thymic CD4 T Cells in Nonobese Diabetic Mice with Tetramers Generated from I-A<sup>g7</sup>/Class II-Associated Invariant Chain Peptide Precursors<sup>1</sup>

Mei-Huei Jang<sup>2</sup>, Nilufer P. Seth<sup>2</sup> and Kai W. Wucherpfennig<sup>3</sup><sup>++</sup>

The MHC determines susceptibility and resistance to type 1 diabetes in humans and nonobese diabetic (NOD) mice. To investigate how a disease-associated MHC molecule shapes the T cell repertoire in NOD mice, we generated a series of tetramers from I-A<sup>g7</sup>/class II-associated invariant chain peptide precursors by peptide exchange. No CD4 T cell populations could be identified for two glutamic acid decarboxylase 65 peptides, but tetramers with a peptide mimetic recognized by the BDC-2.5 and other islet-specific T cell clones labeled a distinct population in the thymus of young NOD mice. Tetramer-positive cells were identified in the immature CD4<sup>+</sup>CD<sup>8</sup><sup>low</sup> population that arises during positive selection, and in larger numbers in the more mature CD4<sup>+</sup>CD<sup>8</sup><sup>+</sup> population. These results demonstrate that a T cell population can expand in the thymus of NOD mice to levels that are at least two to three orders of magnitude higher than estimated for a given specificity in the naive T cell pool. Based on these data, we propose a model in which I-A<sup>g7</sup> confers susceptibility to type 1 diabetes by biasing positive selection in the thymus and later presenting peptides from islet autoantigens to such T cells in the periphery. The Journal of Immunology, 2003, 171: 4175–4186.

The MHC represents a principal susceptibility locus for many autoimmune diseases, but the mechanisms by which particular MHC alleles increase the risk for a given autoimmune disease are not fully understood. The disease-associated polymorphisms map to the peptide binding site and crystallographic studies have shown that they determine the shape and charge of key pockets which accommodate peptide side chains (1). Many alleles confer susceptibility differ from non-associated alleles at only one or a few positions in the peptide binding site, implying a high degree of specificity. Peptide binding experiments have demonstrated that disease-associated MHC molecules bind peptides from candidate autoantigens, but other peptides from the same autoantigens can be bound by MHC molecules that do not confer susceptibility to the disease (2–4). The high degree of specificity implied by the genetic data could, however, be explained by a two-stage model in which the disease-associated MHC polymorphisms determine the outcome of two critical Ag presentation events: presentation of peptides in the thymus that promote positive selection of potentially pathogenic T cell populations, followed later by presentation of peptides from autoantigens to the same T cells in the target organ. This hypothesis has been difficult to test because conventional assays of T cell function are not suitable for analysis of the thymic T cell repertoire.

Type 1 diabetes is particularly relevant for addressing this general question because a wealth of information is available on MHC genetics for the human disease as well as the nonobese diabetic (NOD)<sup>4</sup> mouse model. The MHC is the most important susceptibility locus for human type 1 diabetes and the disease-associated human HLA-DQ<sub>8</sub> and DQ<sub>2</sub> molecules share significant structural similarities with the I-A<sup>g7</sup> molecule expressed in NOD mice (5–10). A key polymorphic residue (β<sup>57</sup>) lacks a negative charge in both DQ<sub>8</sub> and I-A<sup>g7</sup> and therefore does not form a salt bridge with an arginine from the α-chain (α<sup>76</sup>) (5, 6). The positively charged arginine is therefore exposed in the P9 pocket of DQ<sub>8</sub> and I-A<sup>g7</sup>, resulting in a pocket with a preference for acidic peptide side chains. The structural similarities between DQ<sub>8</sub> and I-A<sup>g7</sup> suggest that similar Ag presentation events are relevant in the human disease and the NOD mouse model (8–10). Other MHC class II alleles provide dominant protection from type 1 diabetes in humans and NOD mice. In humans, the DR2/DQ6 haplotype as well as several other MHC class II haplotypes significantly reduce the risk for type 1 diabetes (5, 11). In NOD mice, I-E molecules are not expressed due to a deletion in the E<sub>α</sub> promoter and transgenic expression of the E<sub>α</sub> gene results in protection from the disease (12–15). Protective MHC class II molecules could affect thymic selection of relevant T cell populations and/or modulate the immune response in the periphery.

Investigation of the mechanisms by which MHC molecules confer susceptibility or protection from autoimmunity thus requires analysis of CD4 T cell populations in the thymus and the target organ. Tetrameric forms of MHC class II/peptide complexes may

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Abbreviations used in this paper: NOD, nonobese diabetic; CLIP, class II-associated invariant chain peptide; MOG, myelin oligodendrocyte glycoprotein; GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; PLP, proteolipid protein.

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be useful for this purpose because labeling does not depend on a particular T cell effector function. Tetramers of murine MHC class II/peptide complexes have been generated by covalently attaching the peptide of interest to the N terminus of the MHC class II β-chain, but this approach requires the generation of a new transfectant/recombinant virus for every peptide of interest (16–19).

Investigation of T cell populations in a spontaneous model of autoimmune disease requires analysis of a panel of candidate peptides and peptide conjugates. We initially developed this technique with human MHC class II molecules and were able to visualize virus-specific CD4 T cells in peripheral blood without prior in vitro culture (20). We have now examined whether this approach is applicable to the generation of tetrameric forms of murine MHC class II/peptide complexes for ex vivo characterization of CD4 T cells in a murine model of autoimmunity.

**Materials and Methods**

**Expression and purification of IA^γ/CLIP complexes**

To express soluble I-A^γ, the transmembrane and cytoplasmic segments of the I-Aγ and I-Aβ chains were replaced with leucine zipper dimerization domains from the transcription factors Fos and Jun, respectively, as previously described (21). For the purpose of the present work, several changes were made to the original constructs. To permit biotinylation of I-A^γ^β^β^ molecules, a recognition site for the Escherichia coli BirA enzyme (GLNDIFEQKIEWHE, single amino acid code; Ref. 22) was inserted at the C terminus of the I-Aα-Fos chain through a linker with a thrombin cleavage site permitting replacement of the CLIP peptide following purification of the protein. The protein sequence encoded by the 5' coding segment of the construct was: MALQIPSLLLSAAVVVLMVLSSPGTEG (signal peptide)-GDSGT (signal peptide cleavage site and Kpnl restriction site)-PVSQMRMAT PLLMRP (CLIP peptide)-GGGGSLVPRGSGSGS (linker with thrombin cleavage site and BamHI restriction site)-GDSER (N terminus of mature I-Aβ chain). These I-Aα and I-Aβ constructs were cloned into the BglII-EcoRI and BamHI sites of the pC dendritic cells (23) or PerCP-labeled anti-CD3 (for cells isolated from lymph nodes and spleen) or PerCP-labeled anti-CD8 (for cells isolated from thymus; all Abs were obtained from BD Pharmingen). Apoptotic cells were excluded using Alexa 488-labeled annexin V.

**Labeling of T cells with MHC class II tetramers and enrichment of tetramer-positive cells with anti-PE microbeads**

For tetramer formation, biotinylated I-A^γ^β^β^/peptide complexes were incubated with R-PE-labeled streptavidin (Molecular Probes, Eugene, OR) for at least 1 h on ice at a 4:1 molar ratio of I-A^γ/β^β/ to streptavidin and a final I-A^γ^β^β^ concentration of 0.1 mg/ml unless described otherwise. Four-color FACs analysis was performed with the PE-labeled tetramer and allophycocyanin-labeled anti-CD4, as well as PerCP-labeled anti-CD3 (for cells isolated from lymph nodes and spleen) or PerCP-labeled anti-CD8 (for cells isolated from thymus; all Abs were obtained from BD Pharmingen). Magnetic enrichment of tetramer-labeled cells was used to confirm the presence of cell populations present at low frequencies. Cells were stained with tetramers (typically at 10 μg/ml) in RPMI 1640, 10% FCS for 30 min at room temperature with gentle rocking; labeled Abs were then added and the reaction was kept on ice for another 20 min. Cells were then washed twice with PBS/1% FCS, cell pellets were resuspended in 80–100 μl of PBS/1% FCS and 15 μl of the anti-PE microbeads (Miltenyi Biotec, Auburn, CA) were added to each sample. Following incubation for 20 min in the refrigerator, unbound beads were washed away with PBS/1% FCS and cells were resuspended in 1 ml of PBS/1% FCS. At this point, each sample was split and 900 μl were used for magnetic enrichment while 100 μl were kept as a nonenriched, but otherwise identically treated, sample. For enrichment, 900 μl of the cell suspension were loaded onto the MS column (Miltenyi Biotec), the column was washed three times and cells were eluted in 2 ml of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 (pH 7.4). Annexin V Alexa 488 conjugate (2 μl; Molecular Probes) was added and cells were incubated for 15 min at room temperature and then placed on ice until FACs analysis. Cells that were not magnetically enriched were resuspended in 200 μl of the diluted annexin V solution (1/1000) and treated like the magnetically enriched cells.

**FACS analysis** was performed using a FACSCalibur (BD Biosciences, San Diego, CA) and the CellQuest software (BD Biosciences). Lymphocytes were gated based on forward and side scatter and CD3/CD4, CD4/CD8, or CD4 expression as indicated in the figure legends.

**Enrichment of CD4 single-positive thymocytes**

CD4 single-positive thymocytes were enriched from single cell suspensions by negative selection with a mixture of mAbs to CD8, CD11b (Mac-1), CD45R (B220), an erythroid marker (TER119) and a myeloid differentiation Ag (Gr-1) (SpinPe se murine CD4 T cell enrichment kit; StemCell Technologies, Vancouver, British Columbia, Canada). The cell suspension was incubated with the Ab mixture for 20 min in the refrigerator (4–10°C). Free Abs were washed away with PBS/2% FCS, and SpinSep Dense Particles (StemCell Technologies) which bind to Ab-labeled cells were then added. Following incubation for 20 min on ice, cells bound to the particles were removed by centrifugation through the SpinSep Density Medium and nonlabeled cells were recovered from the interface, washed, and resuspended in RPMI 1640, 10% FCS for tetramer staining.
Tetramer labeling of T cells from pancreatic lymph nodes and spleen

Pancreatic lymph nodes were dissected and single cell suspensions were prepared. Cells were washed twice with PBS and then resuspended in RPMI 1640, 10% FCS for staining. For splenocytes, the single cell suspension was first washed twice with PBS and then resuspended in red cell lysis buffer (Sigma-Aldrich). Following an incubation for 10–15 min at room temperature, cells were washed twice with PBS, and resuspended in RPMI 1640, 10% FCS for staining. Magnetic enrichment of tetramer-labeled cells was performed as described above for thymocytes.

Analysis of CD4 T cells from mice immunized with peptides

NOD mice or B10.H-2<sup>ez</sup> mice (both from Taconic Farms, Germantown, NY) were immunized s.c. with peptide emulsified in CFA (1:1 emulsion of CFA and peptide at 4 mg/ml). Popliteal lymph nodes were isolated 10 days following immunization and single cell suspensions were prepared. Cells were washed twice with PBS, resuspended in RPMI 1640 with 10% FCS, and subjected to tetramer staining. The remaining cells were cultured for 3–4 days at a density of 5 × 10<sup>6</sup>/ml in the presence of the respective peptide (100 nM) in AIM-V medium (Invitrogen) supplemented with 2 mM l-glutamine, 55 μM 2-ME. Viable cells were purified by Ficoll density gradient centrifugation prior to tetramer labeling.

Peptide binding assays

Unlabeled peptides were used as competitors for binding of a biotinylated reference peptide to thrombin-cleaved I-A<sup>ez</sup>/CLIP (21). Thrombin-cleaved I-A<sup>ez</sup> molecules (200 nM) were incubated with biotinylated mouse transferrin peptide (1 μM) in the presence of nonbiotinylated competitor peptides (40 nM to 30 μM) overnight at 37°C in 50 mM sodium phosphate (pH 6.0), 100 mM sodium chloride, 1 mM EDTA, 100 μg/ml BSA, and 1× protease inhibitor mixture (Sigma-Aldrich). I-A<sup>ez</sup> bound biotinylated peptide was then quantitated with europium-labeled streptavidin and then washed four times with 50 mM Tris, 150 mM sodium chloride, 20 mM EDTA, 0.05% Tween 20 to remove unbound Ab. Nonspecific binding sites were then blocked at room temperature for 2 h using 150 μl/well assay buffer (Wallac). Plates were washed twice and the reaction samples (diluted 1/1 in assay buffer) were added to the plates (100 μl/well). Following an incubation for 2 h at room temperature, plates were washed four times and 100 μl of europium-labeled streptavidin (Wallac) was added to each well (1/2000 in 50 mM Tris, 150 mM sodium chloride, 20 μM EDTA) for detection of the I-A<sup>ez</sup> bound biotinylated peptide. The plates were incubated for another hour and washed six times. One-hundred microliters of enhancement solution (Wallac) was then added to each well and the signal was quantitated in a fluorescence plate reader (Delphi Fluorometer; Wallac) following 30 min of incubation at room temperature.

Results

Generation of tetramers from I-A<sup>ez</sup>/CLIP precursors

We previously demonstrated that soluble I-A<sup>ez</sup> heterodimers could be expressed in insect cells using DNA constructs in which the transmembrane and cytoplasmic domains were replaced with leucine zipper dimerization domains from the transcription factors Fos and Jun. These soluble I-A<sup>ez</sup> molecules bound peptides known to represent I-A<sup>ez</sup> restricted T cell epitopes, and several I-A<sup>ez</sup>/peptide complexes displayed a long half-life in the absence of detergent, suggesting that tetramers generated by peptide loading would be stable. The invariant chain-derived CLIP peptide bound to I-A<sup>ez</sup> at a neutral pH but rapidly dissociated (t<sub>1/2</sub> of <5 min) at pH 5.2 (21). Because “empty” MHC class II molecules have a tendency to aggregate we reasoned that the CLIP peptide could protect the hydrophobic binding site at a neutral pH during biosynthesis and be effectively exchanged with any peptide of interest at the acidic pH characteristic for the endosomal peptide-loading compartment. We expressed I-A<sup>ez)/CLIP complexes as inactive precursors by attaching CLIP to the N terminus of the I-Aβ chain through a linker with a thrombin cleavage site, and converted this precursor to the peptide-receptive form by linker cleavage. In APCs, the MHC class II/CLIP complex represents the natural substrate for a DM-accelerated peptide exchange reaction and is generated by proteolytic cleavage of the MHC class II bound invariant chain in the endosomal/lysosomal compartment (23–26).

I-A<sup>ez</sup>/CLIP complexes were affinity purified from the supernatant of insect cells infected with a recombinant baculovirus, with a typical yield of 1.8–2.5 mg/L of culture. Cleavage of the linker with thrombin was visualized by SDS-PAGE (Fig. 1) and peptide.
binding experiments confirmed that the CLIP peptide could be released (Fig. 2). New I-A\(^{\beta7}\)/peptide complexes were generated by exchange of CLIP with peptides synthesized with an N-terminal DNP affinity tag. Two purification steps were used to isolate defined I-A\(^{\beta7}\)/peptide complexes: I-A\(^{\beta7}\) molecules were first separated from free peptide by HPLC gel filtration chromatography (Fig. 1B), followed by affinity purification of the complex using an anti-DNP HPLC column (Fig. 1C).

We used this peptide loading procedure to generate tetramers with peptides known to represent CD4 T cell epitopes in NOD mice. The glutamic acid decarboxylase (GAD) (206–220) peptide was identified as a major epitope for a large panel of murine T cell hybridomas that were generated from NOD mice immunized with recombinant GAD65, and we previously showed that the GAD hybridomas that were generated from NOD mice immunized with the myelin basic protein (MBP) (85–99) peptide served as a negative control. Thrombin-cleaved I-A\(^{\beta7}\) molecules (200 nM) were used to isolate de-

ation steps at 4°C, but was not stable; generation of this tetramer by peptide loading would therefore require modification of peptide anchor residue(s) that increase the affinity of binding to I-A\(^{\beta7}\). For all other peptides, efficient peptide loading was observed because >50% of I-A\(^{\beta7}\) molecules were occupied with DNP-labeled peptides, based on the surface area of the peaks in the DNP affinity purification step representing bound and unbound protein, respectively (Fig. 1C, Table I). The final protein yields were excellent considering that two purification steps were performed following the peptide loading reaction (Table I, Fig. 1).

The functionality of tetramers generated by this peptide exchange procedure was assessed by immunization of NOD mice with the GAD (206–220) or BDC-11 peptide in CFA and labeling of short-term T cell lines generated from draining lymph nodes with the respective tetramers (the peptides used for immunization did not carry the DNP group). The tetramer with the GAD (206–220) peptide labeled only CD4 T cells from mice immunized with the GAD peptide, but not from BDC-11 immunized mice (Fig. 3). Conversely, the tetramer with the BDC-11 peptide labeled CD4 T cells from mice immunized with the BDC-11 peptide, but not from GAD (206–220) immunized mice. In addition, cells from neither T cell line were labeled by the CLIP control tetramer. These results demonstrated that the peptide exchange procedure was suitable for the generation of tetramers of I-A\(^{\beta7}\)/peptide complexes.

FIGURE 2. Peptide binding to I-A\(^{\beta7}\)/CLIP precursors. A competition assay was used to compare the binding of GAD (206–220) and BDC-11 peptides to I-A\(^{\beta7}\)/CLIP, with the myelin basic protein (MBP) (85–99) peptide serving as a negative control. Thrombin-cleaved I-A\(^{\beta7}\) molecules (200 nM) were incubated with biotinylated mouse transferrin peptide (1 \(\mu M\)) in the presence of nonbiotinylated competitor peptides (40 nM to 30 \(\mu M\)) overnight at 37°C. I-A\(^{\beta7}\)-bound biotinylated peptide was then detected with europium-labeled streptavidin following capture of I-A\(^{\beta7}\) with mAb 10-2.16. Even though the affinity of the BDC-11 peptide for I-A\(^{\beta7}\) was lower than for GAD (206–220), tetramers could be generated with both peptides based on the exchange procedure.
Ex vivo analysis of T cell populations in NOD mice by magnetic enrichment of tetramer-labeled cells

We next determined whether such tetramers were suitable for ex vivo analysis of murine CD4 T cell populations. To examine these cell populations independent of the autoimmune process in NOD mice, we first immunized B10.H-2^fl^ mice with the GAD (206–220) or BDC-11 peptides and labeled cells from draining lymph nodes 10 days following immunization, without prior in vitro culture (Fig. 4). The GAD (206–220) tetramer labeled a substantial population of CD4 T cells (0.65%) from lymph nodes of GAD (206–220) immunized mice, while background labeling with the CLIP tetramer was low (0.01%). The tetramer-positive population was highly enriched by use of anti-PE microbeads prior to FACS analysis (18.89% of CD4 T cells). A large population was present in draining lymph nodes of mice immunized with the BDC-11 peptide (1.78% tetramer-positive cells), and a striking enrichment to 77.65% of CD4 T cells was observed following isolation with anti-PE microbeads. The BDC-13 tetramer was used because it typically provided brighter staining than the BDC-11 tetramer; increasing peptide length to a 15 mer (BDC-15) did not result in a further increase in the intensity of staining (data not shown). Magnetic enrichment thus resulted in a clear delineation of tetramer-positive and -negative populations and enhanced detection of brightly labeled cells that may express TCRs with relatively high avidities for the respective I-A^fl^/peptide complex.

Analysis of pancreatic lymph nodes from young, nonimmunized NOD mice demonstrated that the enrichment procedure was also suitable for the detection of naturally expanded T cell populations (Fig. 5). T cells that bound the BDC-13 tetramer were detected at a frequency of 0.07%, which was substantially higher than for the control tetramers. Magnetic enrichment of cells labeled with the BDC-13 tetramer yielded a distinct, brightly labeled population (5.1% of CD4 T cells), while no discrete population was identified with the three control tetramers.

Detection of T cells labeled with the BDC tetramer in the thymus of young NOD mice

In newborn mice, very few T cells can be recovered from peripheral lymphoid structures. Only 0.14 × 10^6 CD4 T cells were isolated from the spleen of 2-day-old mice, compared to 16.75 × 10^6 from 6- to 8-wk-old C57BL/6 mice (37). Peripheral T cell numbers thus increase >100-fold during the first weeks of life and the initial development of insulitis at 3–4 wk of age in NOD mice may

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**Table I. I-A^fl^ Tetramers used for analysis of CD4 T cells in NOD mice**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Loading Efficiency (%)</th>
<th>Final Yield (µg)</th>
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</thead>
<tbody>
<tr>
<td>CLIP</td>
<td>PVSQMRMATPLNLMP</td>
<td>NA</td>
<td>490</td>
</tr>
<tr>
<td>OVA</td>
<td>ISQAVIDVAHAEINEAGR</td>
<td>58</td>
<td>230</td>
</tr>
<tr>
<td>HIV p24</td>
<td>SPEVIMFSEALSEG</td>
<td>63</td>
<td>240</td>
</tr>
<tr>
<td>MOG (1–20)</td>
<td>GGFRVIGPRHIPHALGDEV</td>
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<td>180</td>
</tr>
<tr>
<td>BDC-11</td>
<td>AVRPFLVREMA</td>
<td>78</td>
<td>230</td>
</tr>
<tr>
<td>BDC-11 W→R</td>
<td>AVRPFLVREMA</td>
<td>58</td>
<td>210</td>
</tr>
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<td>AAAVPPLVREMA</td>
<td>66</td>
<td>280</td>
</tr>
<tr>
<td>BDC-Osa</td>
<td>AAHHHPIMARSDA</td>
<td>81</td>
<td>290</td>
</tr>
<tr>
<td>GAD (206–220)</td>
<td>TEYIAVPVLLEYVT</td>
<td>ND</td>
<td>460</td>
</tr>
<tr>
<td>GAD (471–490)</td>
<td>VKCLELAELYLYNIIKREG</td>
<td>ND</td>
<td>180</td>
</tr>
</tbody>
</table>

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**FIGURE 3.** Functionality of tetramers generated by peptide exchange. NOD mice were immunized s.c. with either GAD (206–220) or BCD-11 peptides in CFA. Cells from popliteal lymph nodes were cultured for 3 (BDC-11) or 4 days (GAD 206–220) in serum-free medium with the immunizing peptide at 100 nM (peptides used for immunization and culture were not DNP-labeled). Following isolation of viable cells by Ficoll density gradient centrifugation, cells were labeled with tetramers (20 µg/ml) for 2 h at 37°C, and PerCP-labeled CD3 and allophycocyanin-labeled CD4 Abs were added during the last 30 min of incubation. Cells were then stained with Alexa 488 annexin V to permit exclusion of apoptotic cells and gated based on forward light and side light scatter, annexin V, and anti-CD3 labeling. Histograms show staining with PE-labeled tetramers vs allophycocyanin-labeled anti-CD4.
thus coincide with seeding of the periphery by thymic emigrants. Therefore, we examined whether the initial expansion of T cells labeled with the BDC-13 tetramer had occurred in the thymus (Fig. 6). Because the majority of thymocytes represent CD4−CD8+ T cells, we enriched CD4+CD8− cells by negative selection using a mixture of Abs to CD8 (depletion of CD8 single-positive and double-positive cells), B220, CD11b, and other markers. This enrichment procedure reduced background staining and also permitted analysis of larger numbers of CD4+CD8− cells. A distinct population of tetramer-positive CD4+CD8− T cells was only identified with the BDC-13 tetramer, but none of the control tetramers. Following enrichment, a brightly labeled discrete cell population was observed for the BDC-13 tetramer (0.74% of CD4 T cells), but none of the control tetramers (0.01 or less of CD4 T cells). Therefore, the enrichment procedure provided a clear distinction between the BDC-13 tetramer and the control tetramer, and also permitted clear separation of tetramer-positive and -negative cells. No T cell populations were detected in the thymus of NOD mice with the GAD (471–490) tetramer or the MOG (1–20) control tetramer (data not shown).

FIGURE 4. Enrichment of T cells labeled ex vivo with tetramers. B10.H-2kg congenic mice were immunized with the GAD (206–220) or BDC-11 peptides in CFA and cell suspensions from popliteal lymph nodes were labeled with tetramers without prior in vitro culture. To minimize tetramer internalization, tetramer labeling (20 μg/ml for A; 10 μg/ml for B) was performed at room temperature for 30 min, followed by incubation on ice for 20 min after addition of PerCP-labeled CD3 and allophycocyanin-labeled CD4 Abs. Cells were then washed and labeled with anti-PE microbeads. A total of 5 × 10⁶ cells were used per staining reaction and 90% of these cells were used for magnetic enrichment while the remaining 10% of cells were not enriched following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD3−CD4− population.

FIGURE 5. Ex vivo analysis of T cells in pancreatic lymph nodes. Cells from pancreatic lymph nodes of 4-wk-old NOD mice (30 days old) were stained with PE-labeled CLIP, OVA (323–339), GAD (206–220), and BDC-13 tetramers. Cells were first stained with tetramers (10 μg/ml) for 30 min at room temperature, followed by incubation on ice for 20 min after addition of an allophycocyanin-labeled CD4 Ab. A total of 3 × 10⁶ cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD4-positive population.
Because the molecular identity of the islet Ag recognized by BDC T cells is not known, a critical question relates to the specificity of tetramer labeling. We have used two approaches to address this question (Fig. 7). The first is based on the fact that a number of different peptide mimetics have been identified that activate T cells specific for this islet Ag (28, 31). For two of these peptides, only four critical residues in the nine amino acid core are identical but both peptides nevertheless stimulate BDC-2.5 T cells. Tetramers with both mimic peptides (BDC-13 and BDC-Osa) labeled a discrete population of CD4 T cells in the thymus of young NOD mice, showing that the TCR specificity of these cells corresponded to that reported for BDC-2.5 and other T cell clones. As a second approach, we generated a tetramer with a single amino acid analog peptide in which a critical TCR contact residue was substituted. Analysis of T cell clones and hybridomas demonstrated that the tryptophan present at position 5 (relative to the P1 anchor residue) was critical and that a number of substitutions, including a change to arginine, abrogated T cell activation (28, 31). Therefore, we generated a tetramer with this single amino acid substitution (BDC-11 W→R) and found that this substitution greatly reduced the frequency of T cells that were detected compared to the BDC-11 tetramer (from 0.56 to 0.04% in samples enriched with anti-PE beads). Among this set of tetramers, the BDC-13 tetramer gave the brightest staining and labeled the largest cell population, while the staining intensity of the BDC-Osa tetramer was similar to BDC-11. These experiments thus demonstrate a high degree of specificity of tetramer labeling.

In 4-wk-old mice, T cells labeled with the BDC-13 tetramer could already be detected in both thymus and spleen. To define the site of initial expansion, we compared thymic and splenic cell populations in 15-day-old NOD mice (Fig. 8). T cells labeled by the BDC-13 tetramer could only be detected in the thymus but not in

FIGURE 6. Ex vivo detection of T cells in the thymus of 4-wk-old NOD mice. CD4+/CD8− thymocytes from 4-wk-old (28 days old) NOD mice were enriched by negative selection with a mixture of mAbs to CD8, CD11b, CD45R, an erythroid marker (TER119), and a myeloid differentiation Ag (Gr-1). Cells were first stained with tetramers (10 μg/ml) for 30 min at room temperature, followed by incubation on ice for 20 min after addition of PerCP-labeled CD8 and allophycocyanin-labeled CD4 Abs. A total of 4 × 10^6 cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD4+/CD8− population.

FIGURE 7. Specificity of tetramer labeling of thymocytes. CD4+/CD8− thymocytes were enriched from the thymus of 4.5-wk-old NOD mice (32 days old) as described in Fig. 6 and labeled with six different tetramers: two negative control tetramers (CLIP and HIV p24), two tetramers with mimic peptides that both represent agonists for BDC-2.5 T cells (BDC-13 and BDC-Osa), and two tetramers with 11-mer peptides (BDC-11 and BDC-11 W→R) in which one of the peptides carried a substitution (W→R) at a critical TCR contact residue. A total of 4 × 10^6 cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD4+/CD8− population.
the spleen at this age. In the thymus, the following numbers of tetramer$^{+}$/CD4$^+$ cells were detected following enrichment: CLIP and HIV p24 control tetramers, 0 cells; BDC-13 tetramer, 133 cells. Following enrichment, the following numbers of tetramer$^{+}$/CD4$^+$ cells were detected among splenocytes: CLIP and HIV p24 control tetramers, 0 cells; BDC-13 tetramer, 1 cell (data not shown). This result demonstrated that the T cell population detected with the BDC tetramers originated in the thymus and had not recirculated from secondary lymphoid organs.

Positive selection occurs during the transition from CD4$^+$CD8$^-$ double-positive to single-positive thymocytes (38) and we therefore examined at which point T cells could be detected with the BDC-13 tetramer. Suspensions of nonfractionated thymocytes were labeled with CLIP or BDC-13 tetramers and four populations were defined based on CD4 and CD8 expression, with P1 corresponding to CD4$^+$CD8$^-$ and P2 to CD4$^+$CD8$^{low}$ cells (Fig. 9). T cells labeled with the BDC-13 tetramer could not be identified in the double-positive population (P3) or CD8$^+$CD4$^-$ single-positive cells (P4). However, BDC-13 tetramer-positive cells were present in the intermediate CD4$^+$CD8$^{low}$ population (P2) and a significant enrichment was observed with anti-PE microbeads (from 0.01 to 0.25%; 25-fold enrichment). Substantially larger numbers were present in the more mature CD4$^+$CD8$^-$ population (0.08%, enriched to 9.47%; >100-fold enrichment).

We also examined tetramer labeling for thymocytes from B10 control mice (H-2b haplotype) and B10 mice congenic for the MHC locus of NOD mice (B10.H-2$^{es}$) (Fig. 10). The BDC-13 tetramer did not identify a cell population in the thymus of B10 mice, but labeled a distinct population in the thymus of B10.H-2$^{es}$ mice. These results demonstrate that the presence of the MHC locus of NOD mice on a different genetic background is sufficient for positive selection of this T cell population in the thymus.

Discussion
These results demonstrate that positive selection in the thymus of NOD mice creates substantial numbers of CD4$^+$CD8$^-$ T cells that bind I-A$^e$/BDC tetramers. The frequency of these cells is at least two to three orders of magnitude higher than the average precursor frequency estimated for T cells with a given MHC/peptide specificity in the naive T cell pool (1:10$^5$ to 1:10$^6$ or lower). An expanded population of these T cells was also observed in the thymus of B10 mice congenic for H-2$^{es}$, indicating that the NOD MHC genes were sufficient on a different genetic background. Tetramer labeling was specific, based on a number of criteria: 1) discrete cell populations were not detected in the thymus of NOD mice with a panel of control tetramers; 2) the tetramer-labeled cell population could be significantly enriched with anti-PE microbeads, while no enrichment of cells labeled with control tetramers was observed; 3) the cell population was present in the thymus of NOD and B10.H-2$^{es}$, but not B10 control mice; 4) staining was greatly reduced by a single amino acid substitution in the peptide known to affect activation of T cell clones/hybridomas reactive with the islet autoantigen; 5) two mimic peptides known to stimulate the same islet-specific T cell clones labeled this thymic T cell population, even though these peptides only shared sequence identity at four positions within the nine amino acid core. Even though activated T cells can recirculate into the thymus (39, 40), T cells detected with the I-A$^e$/BDC tetramers had expanded in the thymus due to efficient positive selection and did not represent a recirculating population because they could be detected in the thymus, but not the spleen, of 2-wk-old NOD mice (Fig. 8). You et al. (17) previously reported detection of a CD4 T cell population labeled with an I-A$^e$/BDC tetramer in pancreatic lymph nodes and the spleen of NOD mice. They demonstrated that these cells proliferated in...
vitro and produced IFN-γ and other cytokines, indicating that the cells were functional (17).

Two groups have isolated a series of T cell clones reactive with an islet secretory granule Ag, with the T cells originating from islets of prediabetic NOD mice or spleen/lymph nodes of diabetic NOD mice (28, 30). These clones cause diabetes following transfer to NOD scid/scid mice, and the BDC-2.5 TCR has been used to generate TCR transgenic mice that develop spontaneous diabetes (32–34). The native autoantigen is not known, but analysis of combinatorial peptide libraries has provided a series of peptide mimetics that stimulate these T cell clones/hybridomas at low peptide concentrations (28, 31). Surprisingly, six of seven independent clones/hybridomas were stimulated by the same peptide mimetics, indicating that the majority of these clones have the same Ag specificity. This analysis also delineated key structural features of the peptides required for T cell activation. Within the nine amino acid core segment of these peptide mimetics, four positions were particularly important (P3, P5, P7, P8) while a number of substitutions were tolerated at the other five positions. The BDC-2.5 T cell clone was stimulated by two peptides in which only these four positions were conserved (P-W-R-M at P3, P5, P7, and P8) while the other positions were distinct (VR-L-V-E and HI-I-A-D at P1, P2, P4, P6 and P9). The substitutions at three of these five positions were relatively conservative (leucine/isoleucine at P4, valine/alanine at

**FIGURE 9.** Analysis of T cell subpopulations in the thymus of NOD mice. Total thymocyte cell suspensions from 40-day-old NOD mice were labeled with CLIP or BDC-13 tetrarsers as well as PerCP-labeled CD8 and allophycocyanin-labeled CD4 Abs. Because a large fraction of thymocytes were CD4$^+$CD8$^-$, a total of $5 \times 10^7$ cells were used per staining reaction to permit adequate analysis of the other subpopulations. Ninety percent of the cells were used for magnetic enrichment (B) while the remaining 10% were not enriched (A) following labeling with anti-PE microbeads. For the nonenriched population, only $1 \times 10^6$ labeled cells were analyzed. Based on CD4 and CD8 labeling, four populations (P1–P4) were defined and tetramer-PE vs CD4-allophycocyanin staining plotted for annexin V-negative cells.
P6, glutamic acid/aspartic acid at P9). The divergent positions are largely buried in the I-A\(^{d}\) binding groove (P1, P4, P6, and P9 pockets), and previous studies on other peptide mimetics have demonstrated that the MHC binding surface can be diverse (41). I-A\(^{d}\) tetramers with both mimetics labeled the CD4 T cell population in the thymus of NOD mice, indicating that the structural requirements for TCR recognition are similar for the thymic T cell population studied here and the T cell clones isolated by other research groups. Molecular characterization of the native target Ag will be required for tetramer-based analysis of this T cell population with the native peptide.

The identification of autoreactive CD4 T cells with tetramers of MHC class II/peptide complexes has represented a significant challenge, due to technical difficulties in the expression of MHC class II/peptide complexes and the relatively low frequency of autoreactive CD4 T cells. In addition, the avidity for self-peptide/MHC complexes may in general be lower than those for microbial peptide/MHC complexes due to thymic and peripheral tolerance mechanisms (42). Peptide mimetics may be useful for the detection of autoreactive CD4 T cells because peptides with the highest biological activity can be chosen from a large number of variants. This concept was elegantly illustrated by the use of the NRP-V7 peptide mimic recognized by an islet-specific CD8 T cell population. A tetramer with this peptide could be used to predict the development of type 1 diabetes in NOD mice based on labeling of CD8 T cells from peripheral blood (43). T cells labeled with this tetramer were also detected in islets, starting at 7 wk of age and peaking at 11–14 wk, at which time the frequency of these cells ranged from 1.61 to 36.8% of CD8 T cells in individual mice. The brightness of tetramer labeling and consequently the size of the detected cell population had been optimized based on single amino acid substitutions at position 7 of the peptide (W, A, or V) and the population labeled with the NRP-V7 tetramer was 4- to 5-fold larger than the one stained with the NRP-A7 tetramer. Recent work has demonstrated that the self-peptide recognized by these CD8 T cells is derived from the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and that tetramers loaded with the IGRP peptide label a CD8 T cell population in islets and blood of NOD mice that is similar in size to the population identified with the tetramer loaded with the NRP-V7 mimic peptide (44). Similarly, the BDC-13 tetramer used here yielded brighter staining and labeled a larger population of cells than the BDC-11 tetramer with the shorter peptide as well as the BDC-Osa tetramer. Therefore, it is possible that CD4 T cell populations could also be detected in the thymus for some of the other peptides investigated here, provided that the peptide sequences are optimized in such a fashion. A potential limitation of the peptide mimic approach is that not all T cells labeled with such a tetramer may recognize the native peptide, and this issue can in part be addressed by use of two or more mimetics that stimulate the same T cell population.

MHC class II tetramers have been generated by covalent attachment of the peptide of interest to the N terminus of the \(\beta\)-chain, but this approach requires the generation of a new recombinant virus/transfectant for every peptide (19). Generation of tetramers from MHC class II/CLIP precursors offers several significant advantages: 1) many different tetramers can be generated from the same MHC class II/CLIP precursor, permitting investigation of a number of different candidate peptides. This aspect is critical because the key T cell epitopes are not known with certainty in any autoimmune disease, except for animal models where disease is induced by deliberate immunization with a single Ag or peptide; 2) peptide variants that increase the affinity to the MHC molecule or the TCR can be investigated. The length of the peptide can also be optimized, an aspect that may be significant for peptides that have the potential to bind to the MHC class II molecule in more than one register; 3) binding occurs at the acidic pH of the endosomal compartment, rather than the neutral pH of the endoplasmic reticulum. This aspect may be relevant in select cases where different binding registers of the same peptides are possible and where the protonation state of the peptide or the MHC molecule may determine the preferred binding mode. Affinity purification of the final complex may not always be necessary, due to the high peptide loading efficiencies observed here. Nevertheless, this step can be...
used to confirm appropriate loading and to exclude the possibility that staining is due to a contaminating peptide. The peptide exchange procedure may also permit tetramers of DQ8/peptide complexes to be generated for the analysis of islet-specific T cells in the human disease. Empty DQ8 molecules have a strong tendency to aggregate (our unpublished data), indicating that protection of the peptide binding site by an exchangeable, low affinity peptide will be required for the generation of DQ8 tetramers by peptide loading.

These findings have important implications for thymic T cell repertoire development, in particular in terms of MHC-linked susceptibility to autoimmunity. The surprisingly high frequency of CD4 T cells identified with I-A<sup>E7</sup>/BDC tetramers demonstrates that the T cell repertoire in NOD mice can be highly biased, apparently because positive selection of this population is efficient while negative selection is either inefficient or largely absent. An important role of thymic repertoire selection in susceptibility to autoimmunity could explain the exquisite allele specificity observed for disease-associated vs nonassociated MHC class II alleles. A key aspect of MHC-associated susceptibility to type 1 diabetes is the presence of a nonaspartic acid residue at position 57 of both DQ and I-A β chains (5, 6). Based on these data, we propose that MHC class II molecules which confer susceptibility to type 1 diabetes act at two distinct sites: initially in the thymus by promoting efficient positive selection of potentially pathogenic T cell populations and later in pancreatic lymph nodes and islets by presenting islet-derived peptides that induce differentiation of these T cells into effector cells that initiate and propagate the inflammatory process. The stringent structural requirements for peptide presentation implied by the genetic data could thus be explained by the requirement for presentation of different peptides in the thymus and the periphery to the same T cell population. This two-stage model of MHC-linked susceptibility could thus explain the observation that particular structural properties of I-A<sup>E7</sup> and DQ8 are tied to disease susceptibility. In most other DQ and I-A molecules, the aspartic acid residue present at B57 forms a salt bridge with arginine α76, but this salt-bridge is not formed in DQ8 and I-A<sup>E7</sup>. Arginine α76 is instead available to form a salt bridge with acidic peptide side chains bound in the P9 pocket (3, 8–10). The B57 polymorphism may thus permit presentation of positively selecting peptides (with an acidic residue at P9) and simultaneously prevent binding of peptides that could induce negative selection of relevant T cell populations (peptides with side chains that cannot be accommodated in the P9 pocket). Experiments in transgenic NOD mice support this hypothesis because mice that coexpressed a mutant I-A<sup>E7</sup> β-chain with substitutions of residues β56 and 57 of the P9 pocket or other I-A molecules were protected from the disease (14, 45, 47).

Several other lines of evidence indicate that thymic repertoire selection is critical in the development of type 1 diabetes. In humans, susceptibility to the disease is influenced by the promoter region of the insulin gene (IDDM2 locus) and protective alleles are associated with higher levels of insulin mRNA in the thymus (48, 49). In NOD mice, a defect in thymic negative selection has been reported. Kishimoto and Sprent (50) demonstrated that negative selection in NOD mice was impaired for a population of semimature thymocytes in the medulla with a CD4<sup>+</sup>CD8<sup>+</sup>HLA<sup>high</sup>-chain phenotype (50). Reduced levels of apoptosis were observed for this cell population in vitro following stimulation with anti-CD3 or anti-CD28 plus anti-CD28 or in vivo following injection of the superantigen staphylococcus enterotoxin B. This defect in apoptosis was not observed in NOR, B6.H-<sup>2γ7</sup> or (B6.H-<sup>2γ7</sup> × NOD)<sub>F1</sub> mice. Lesage et al. (51) demonstrated a T cell intrinsic defect in thymic-negative selection in NOD mice based on a transgenic model in which a membrane-bound form of hen egg lysozyme (HEL) was expressed in islets, along with a HEL-specific TCR. Negative selection of HEL-specific T cells was defective on the NOD but not the B10 background, and experiments in bone marrow chimera demonstrated that the defect was T cell intrinsic. A failure of negative selection has also been implicated for the immunodominant T cell epitope of myelin proteolipid protein (PLP, residues 139–151) in SJL mice. Immunization with this peptide induces a severe, chronic form of experimental autoimmune encephalomyelitis. Only an alternatively spliced form that did not include the exon encoding the PLP (139–151) epitope was detected in the thymus, while both splicing variants were expressed in the target organ (52, 53). This failure of negative selection is evidenced by the fact that PLP (139–151)-specific T cells can be readily detected in nonimmunized mice in a T cell proliferation assay (52). It is possible that the same mechanism is responsible for the observation that T cells recognized by I-A<sup>E7</sup>/BDC tetramers are not deleted in the thymus. MHC class II molecules that confer susceptibility to an autoimmune disease may thus set the stage for disease development by permitting the emergence of potentially pathogenic T cell populations from the thymus.

References


