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Detection and Characterization of T Cells Specific for BDC2.5 T Cell-Stimulating Peptides[1]

Sylvaine You,* Cyndi Chen,* Wen-Hui Lee,* Chun-Hua Wu,* Valeria Judkowski,[†] Clemencia Pinilla,[†] Darcy B. Wilson,[†] and Chih-Pin Liu[2]*

Nonobese diabetic (NOD) mice expressing the BDC2.5 TCR transgene are useful for studying type 1 diabetes. Several peptides have been identified that are highly active in stimulating BDC2.5 T cells. Herein, we describe the use of I-Ag7 tetramers containing two such peptides, p79 and p17, to detect and characterize peptide-specific T cells. The tetramers could stain CD4+ T cells in the islets and spleens of BDC2.5 transgenic mice. The percentage of CD4+, tetramer+ T cells increased in older mice, and it was generally higher in the islets than in the spleens. Our results also showed that tetAg7/p79 could stain a small population of CD4+ T cells in both islets and spleens of NOD mice. The percentage of CD4+, tetramer+ T cells increased in cells that underwent further cell division after being activated by peptides. The avidity of TCRs on purified tetAg7/p79+ T cells for tetAg7/p79 was slightly lower than that of BDC2.5 T cells. Although tetAg7/p79+ T cells, like BDC2.5 T cells, secreted a large quantity of IFN-γ, they were biased toward being IL-10-producing cells. Additionally, <3% of these cells expressed TCR Vβ4. In vivo adoptive transfer experiments showed that NOD/_scid_ recipient mice cotransferred with tetAg7/p79+ T cells and NOD spleen cells, like mice transferred with NOD spleen cells only, developed diabetes. Therefore, we have generated Ag-specific tetramers that could detect a heterogeneous population of T cells, and a very small number of NOD mouse T cells may represent BDC2.5-like cells. *The Journal of Immunology,* 2003, 170: 4011–4020.

T ype 1 diabetes, insulin-dependent diabetes mellitus (IDDM),[3] is an autoimmune disease. Previous studies using the nonobese diabetic (NOD) mouse, an experimental model for human IDDM, have clearly demonstrated that both CD4+ and CD8+ T cells play a central role in the pathogenesis of the disease (1–4). These T cells are probably activated by self-Ags present in the islets of the pancreas. Autoreactive T cells that participate in IDDM may recognize one of several candidate autoantigens that include proteins such as insulin and glutamic acid decarboxylase 65 (GAD) (5–7).

The cells of the diabetogenic CD4+ T cell clone, BDC2.5, can rapidly induce insulitis and hyperglycemia in NOD or NOD/_scid_ mice within 2 wk after birth. However, they cannot transfer the disease to NOD mice older than 3 wk of age or to adult NOD/_scid_ mice (8–10). NOD mice expressing the BDC2.5 TCR transgene have been a very useful model for studying the role of these cells in the pathogenesis of IDDM, while T cells derived from BDC2.5 mice may behave differently from the original T cell clone (11–13). Although BDC2.5 TCR transgenic NOD mice (BDC2.5 mice) can develop an aggressive form of IDDM, only ~10–20% of BDC2.5 mice develop the disease (12). This may be due to the fact that regulatory T cells, such as those that express the surface

marker DX5, are present in these animals, and they can prevent β islet cells from being destroyed (14). However, activated T cells from these animals can transfer disease to NOD/_scid_ recipients. Additionally, all NOD/scid mice that express the BDC2.5 TCR transgene are diabetic by 4 wk of age (15).

Previous studies have shown that the BDC2.5 T cells are specific for a β islet granule membrane Ag presented by the NOD class II MHC I-Ag7 (16). However, the identity of this Ag remains unknown. While searching for the Ag recognized by BDC2.5 T cells using a positional scanning combinatorial peptide library, a series of peptide analogs were identified that stimulate T cells from BDC2.5 mice (17). Some of these peptides include sequences similar to the GAD$_{528-539}$ peptide or the longer GAD$_{526-541}$ peptide, suggesting that the GAD peptide is a candidate autoantigen for BDC2.5 T cells. After being activated by these synthetic peptide analogs, BDC2.5 transgenic T cells can adoptively transfer the disease to adult NOD/_scid_ recipients. In addition to T cells from transgenic mice, T cells derived from NOD mice also respond spontaneously to these peptides in culture.

To better understand the development of BDC2.5 T cells in the animals and their role in the pathogenesis leading to IDDM, we have generated Ag-specific I-Ag7 tetramers to detect, isolate, and characterize BDC2.5 T cells or T cells from NOD mice that were stimulated by the peptides. In a previous report we described the generation of an I-Ag7 tetramer specific for the GAD$_{524-543}$ peptide (which includes both the 528–539 and 526–541 epitopes that can weakly stimulate BDC2.5 T cells) (18). However, the tetramer stained GAD$_{524-543}$ peptide-specific hybridoma cells weakly and failed to detect a significant number of T cells in NOD spleens and islets (18). This may be due to the fact that the autoreactive T cells specific for GAD epitopes are present at a low frequency in NOD mice and that their TCRs have low affinities for their ligands. Therefore, the binding affinity of the tetAg7/GAD$_{524-543}$ tetramer for its cognate TCRs could be too low for the T cells to be detected. In this study we have generated new I-Ag7 tetramers

specific for the previously identified peptide analogs that could stimulate BDC2.5 T cells (17). Among these peptides, the 1040-79 peptide (p79), one of the most active peptides identified in the previous report, stimulates BDC2.5 T cells with an EC_{50} of 0.5–0.7 nM. Because the p79 peptide can stimulate BDC2.5 T cells at a very low concentration, we hypothesize that the tetramer specific for the p79 peptide, tetAg7/p79, is a better reagent to detect T cells that share the same Ag specificity as that of BDC2.5 T cells in NOD mice. We also generated an additional tetramer specific for another peptide, 1136-17 (p17), which, like p79, stimulates BDC2.5 T cells at low concentrations. We report here the use of these tetramers to stain and characterize T cells from BDC2.5 and NOD mice.

Materials and Methods

Mice

NOD and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Biozzi/ABH (ABH) mice, which also express the class II MHC I-Ag7, were purchased from Harlan Co. (Indianapolis, IN). BDC2.5 transgenic mice were a gift from Drs. D. Mathis and C. Benoit (11). Less than 10% of mice in the NOD background and 100% of mice in the NOD/*scid* background in our colony develop diabetes by 6 mo and 4 wk of age, respectively. All the animals used were housed in a specific pathogen-free environment in the animal facility at Beckman Research Institute, City of Hope.

Peptides and production of class II MHC tetramers

The p79 and p17 peptides were synthesized at Beckman Research Institute, City of Hope, and purified to at least 90% using reverse phase HPLC. The p79 peptide has been described as 1040-79 (17). A detailed description of the p17 peptide will be reported by Judkowski et al. (unpublished observations). The method of class II MHC tetramer production has been described previously (18–20).

Isolation of pancreatic islet-infiltrating cells

Pancreases from BDC2.5 or NOD mice were perfused with collagenase P (Roche, Indianapolis, IN), removed from the animal, and incubated for 16–18 min in a water bath at 37°C. Pancreatic tissues were washed several times with HBSS plus 10% FCS, then filtered through a strainer, and islets were isolated after Histopaque density-gradient purification (Sigma-Aldrich, St. Louis, MO). Islets were dissociated into single cells by trypsin/EDTA treatment. The islet cell suspension was then either directly stained with tetramers and Abs or cultured with peptides and irradiated APC for 7 days before staining.

Flow cytometry and confocal microscopy

Staining of cells using I-Ag7 tetramers has been described previously (18). Briefly, islet or spleen cells were stained with PE-labeled tetramers plus unconjugated H57 Ab at 37°C for 3 h. Anti-CD4 Abs (BD PharMingen, San Diego, CA) were added during the last 30 min of incubation. Cells were washed and either analyzed by FACS using FACSCalibur (BD Biosciences, San Jose, CA), or fixed with 4% paraformaldehyde overnight for confocal microscopic analysis. All other Abs and annexin V-FITC were purchased from BD PharMingen, except for F4/80 Ab which was a gift from Dr. S. Kovats (Duarte, CA).

Tetramer binding kinetics

The association and dissociation kinetics measured using the tetramers were determined using a previously described method (21), with minor modifications. For analysis of tetramer staining at equilibrium, BDC2.5 spleen cells were stained for 1 h at room temperature with increasing concentrations of tetAg7/p79 (0–35 nM) and with anti-CD4 Ab (BD PharMingen) in RPMI medium. Apparent K_d values were derived from the negative reciprocal of the slope of the regression line fit to Scatchard plots of bound tetramer/free tetramer (fluorescence units per nanomolar concentration of tetramer) vs bound tetramer (fluorescence units). The median tetramer staining intensity was used as the measurement of bound tetramer.

To measure the half-life of tetramer binding to cells, cells were stained for 1 h at room temperature with a suboptimal concentration of the tetAg7/p79 tetramer plus anti-CD4 Ab in RPMI medium. The blocking anti-I-A^{g7} Ab was used at 30 µg/ml (American Type Culture Collection, Manassas, VA). Aliquots were taken at appropriate time points and analyzed by flow cytometry. The binding half-life ($t_{1/2}$) is equal to ln (2) divided by the slope value of the natural logarithm (ln) of the normalized fluorescence plotted vs time. The normalized fluorescence was determined by calculating the percentage of total fluorescence (sum of fluorescence intensity of tetramer$^+$ CD4$^+$ T cells normalized per CD4$^+$ cell at each data point) with respect to the initial time point.

Cytokine assay

The IL-2 production bioassay has been previously described (18). For ELISA, cell culture supernatant was harvested after incubating cells with peptides or tetAg7/p79 and irradiated APC for 24 h. Mouse IFN-γ, IL-4, and IL-10 OptEIA ELISA assay kit sets (BD PharMingen) were used to measure the amount of cytokines according to the manufacturer's instruction.

CFSE labeling and in vitro T cell stimulation

CD4$^+$ T cells from NOD mouse spleens were purified using magnetic beads (Miltenyi Biotec, Auburn, CA) and labeled with CFSE as previously described (22, 23). Briefly, CD4$^+$ T cells were resuspended at a concentration of 10×10^6 cells/ml in serum-free HBSS or PBS and incubated with CFSE (0.8 µM, final concentration) for 10 min a 37°C. CFSE labeling was stopped by adding an equal volume of heat-inactivated FCS. After washes, the cells were cultured with peptides (50 µg/ml) and irradiated NOD mouse CD4$^+$ T cell-depleted APC in RPMI medium containing 10% FCS for 3 days. Live cells were further incubated and maintained in medium supplemented with IL-2. After 10–13 days in culture, cells were stained with tetAg7/p79 and anti-CD4 Ab. Following activation with peptides and expansion in vitro, CD4$^+$, tetramer$^+$ T cells were isolated using FACS and magnetic beads (Miltenyi Biotec).

Intracellular cytokine staining

Intracellular cytokine staining was performed according to a previously described method (24). Briefly, T cells were incubated with PMA plus ionomycin and monensin, stained with surface Abs, fixed with paraformaldehyde, and resuspended in 0.1% saponin buffer (w/v; Sigma-Aldrich, St. Louis, MO). The cells were then intracellularly stained using Abs against cytokines or negative isotype control Abs.

Adoptive transfer of T cell into NOD/scid mice

Four- to 5-wk-old NOD/*scid* mice received a single i.v. injection of tetramer$^+$ T cells, tetramer$^+$ T cells plus NOD splenocytes, or NOD splenocytes alone. Recipient mice were monitored up to 26 wk of age and were considered diabetic after 2 consecutive wk of glycosurea >2% and blood glucose level >250 mg/dl.

Results

We have generated a new class II MHC tetramer, tetAg7/p79, in which the I-Ag7 β-chain is covalently linked to the previously identified p79 peptide (17). This tetramer was first used to stain T cells from islets and spleens of BDC2.5 mice. Initial studies showed that this tetramer could detect a majority of CD4$^+$ T cells in spleens (82.5%) and pancreatic islets (90.5%) of the BDC2.5 mice (Fig. 1A). The tetramer was specific for BDC2.5 T cells because it did not detect a population of T cells in the spleens of control ABH mice (Fig. 1A). ABH mice also express the same class II MHC I-Ag7 as that expressed in NOD mice, but they do not develop diabetes (25). The BDC2.5 TCR bears a Vβ4 fragment in its β-chain (26). The percentage of CD4$^+$, tetramer$^+$ T cells correlates with that of CD4$^+$ T cells bearing TCR Vβ4, although some Vβ4$^+$ T cells (~15%) were not stained by the tetramer. This is consistent with previous fndings that some Vβ4$^+$ T cells bear a different TCR α-chain (11). The tetramer could also detect essentially all CD4$^+$ T cells from NOD/scid mice expressing the BDC2.5 TCR transgene (data not shown). Additionally, the tetramer did not stain NOD mouse T cells specifically for the pGAD$_{206}$ (p206) peptide isolated using the tetAg7/p206 tetramer (data not shown) (18). In comparison, the tetAg7/p206 tetramer did not stain T cells derived from the BDC2.5 TCR transgenic mice (Fig. 1B). TetAg7/p206 is considered as a good control tetramer because it contains an irrelevant GAD peptide, p206, whose amino acid sequence is not related to that of p79. The advantages
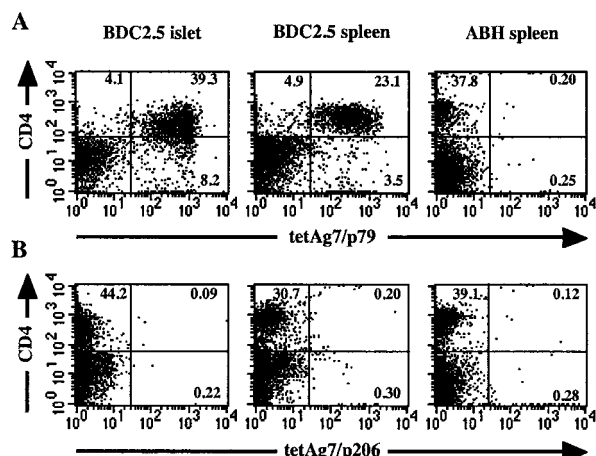
FIGURE 1. Detection of BDC2.5 T cells by tetAg7/p79 tetramer. *A*, FACS analyses of cells detected by the tetramer. Spleen- and islet-infiltrating cells from BDC2.5 transgenic mice were stained with tetAg7/p79 tetramer and anti-CD4 Ab, as described in *Materials and Methods*, and analyzed by flow cytometry. ABH spleen cells were used as the negative control. The cells shown represent lymphocyte population after they were electronically gated based on their forward and side scatter profiles. The number in each quadrant represents the percentage of cells. *B*, FACS analyses of cells from BDC2.5 mice and from ABH mice that were stained with a control tetAg7/p206 tetramer.

FIGURE 2. Confocal analyses of cells detected by the tetAg7/p79 and tetAg7/p206 tetramer. *A* and *B*, Purified CD4$^+$ T cells from BDC2.5 mouse islets and spleens were stained with tetAg7/p79 (*A*), and cells from BDC2.5 mouse spleens were stained with tetAg7/p206 (*B*). The yellow cells represent cells that were costained by the FITC-conjugated anti-CD4 Ab and the PE-labeled tetAg7/p79 tetramer. *C* and *D*, Purified CD4$^+$ T cells from NOD mouse islets and spleens were stained with tetAg7/p79 (*C*), and cells from NOD mouse spleens were stained with tetAg7/p206 (*D*). The single yellow cell surrounded by green cells (the CD4$^+$ T cells) represents cells that were costained by FITC-anti-CD4 Ab and the PE-tetramer. The results are representative of at least three experiments.

of having p206 as an irrelevant peptide in the tetramer are that it is a mouse self-peptide, but its amino acid sequences are different from those of p79. Furthermore, p206 does not stimulate BDC2.5 T cells, nor does it stimulate p79-specific NOD mouse T cells (data not shown, also refer to Fig. 9). Besides the GAD p206 peptide, we have also performed additional control experiments using another tetramer, tetAg7/pcOVA, to stain T cells from BDC2.5 TCR transgenic mice. This control tetAg7/pcOVA tetramer contained a non-self peptide, cOVA$_{323–339}$, as an irrelevant peptide and it also did not stain BDC2.5 T cells (see Fig. 5B). Therefore, it appears that tetAg7/p79 tetramer is a good staining reagent to detect essentially all BDC2.5 T cells from transgenic mice. In addition to using FACS to analyze T cells stained with the tetramer, we also used a confocal microscope to observe CD4$^+$, tetramer$^+$ T cells from spleens and islets (Fig. 2, *A* and *B*). Similar to the FACS analyses, the majority of T cells in the spleens and islets of BDC2.5 mice were positively stained by both FITC-conjugated anti-CD4 Ab and PE-labeled tetAg7/p79 tetramer and appeared as yellow colored cells (Fig. 2A). In comparison, the green CD4$^+$, BDC2.5 T cells were not stained by the tetAg7/p206 tetramer (Fig. 2B).

Further analyses of T cells in BDC2.5 mice showed that the tetramer could detect ~72 and ~68% of the CD4$^+$ T cells from the islets and spleens, respectively, of 4-wk-old BDC2.5 mice (Table I). The percentage of CD4$^+$, tetAg7/p79$^+$ T cells increases with age and can reach an average of ~93% in the islets and ~82% in the spleen of 9-wk-old mice. These results suggest that while other T cells are present in islets and spleens of BDC2.5 mice, BDC2.5 T cells eventually accumulate or expand in both organs as the mice age.

Previous studies have suggested that autoreactive T cells bear TCRs with relatively lower avidity for their ligands (27, 28). It has been possible to use MHC tetramers to measure the avidity between TCRs and their ligands (20, 21). It would be interesting to determine the avidity of the BDC2.5 TCR for its ligands, but its natural antigenic peptide is not known. As an alternative, we determined the avidity of the BDC2.5 TCR for the I-Ag7/p79 complex using the tetramer with a previously described method (21).
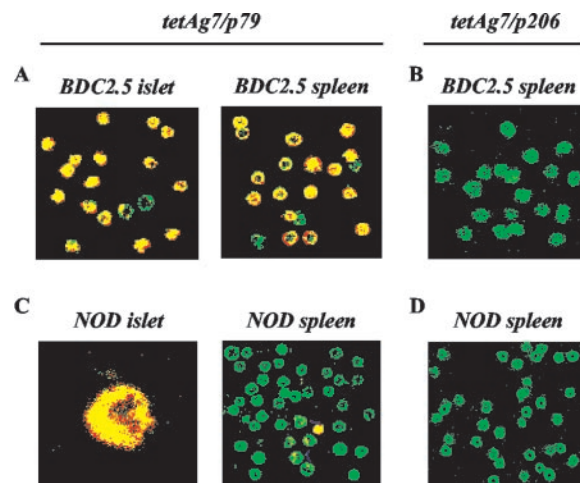
We have analyzed T cells from spleens of 4- and 9-wk-old animals. Our results showed that the apparent K_d values of BDC2.5 T cells from 4- and 9-wk-old animals for the I-Ag7/p79 ligand were 22.5 and 16.4 nM, respectively (Fig. 3A). The $t_{1/2}$ values were 32.4 and 48.4 min for 4- and 9-wk-old animals, respectively (Fig. 3B). Compared with other CD4$^+$ T cells, the BDC2.5 TCR appears to have a high binding affinity for the I-Ag7/p79 ligand (20, 29–31). This may be because the p79 peptide was not the natural peptide ligand, but a superagonist for BDC2.5 T cells, in contrast to the previously described peptides that were agonist or partial agonist for their cognate TCRs. To determine whether the increased avidity of BDC2.5 TCRs on T cells in older mice was due to increased TCR expression level, we stained T cells from 4- and 9-wk-old mice using an anti-TCR Vβ Ab, H57 (32). The results showed that there was no significant difference in the TCR expression level found on T cells from 4- or 9-wk-old mice (data not shown).

We next studied whether the tetAg7/p79 tetramer could also stimulate BDC2.5$^+$ T cells. The results demonstrated that, like the p79 peptide, the tetramer showed a dose-dependent stimulation of T cells from BDC2.5 mice, but not from control ABH mice, in IL-2 production assays (Fig. 4). The tetramer also stimulated splenic CD4$^+$ T cells from BDC2.5 mice to produce a large quantity of IFN-γ and, to a lesser extent, IL-4, and IL-10. However, it seems that the tetramer stimulated the cells to produce lower amounts of the cytokines, especially IL-4 and IL-10, than in cells stimulated with the p79 peptide. The difference may be due to the presence of a lower concentration of the antigenic peptide in the recombinant protein compared with that of the synthetic peptide used in the assays. To determine whether this is the case, we compared the IL-2 production response to equal molarities of the synthetic peptide and the recombinant peptide present in the tetramer used in the assays. The results shown in the bar chart in Fig. 4 demonstrated that after being adjusted to the concentration of the peptide rather than the amount of the protein used in the assays, the

Table I. *Percentage of CD4$^+$ T cells in BDC2.5 mice detected by the tetAg7/p79 tetramer^a*

Mouse Age (wk)	% of Tetramer$^+$ Cells in Islets	% of Tetramer$^+$ Cells in Spleens
4	72.3 ± 1.5	68.1 ± 2.8
6	83.1 ± 2.7	77.1 ± 0.8
9	93.3 ± 2.0	81.6 ± 2.3

^{a} The cells were isolated from the spleen or pancreatic islets of 4-, 6-, and 9-wk-old BDC2.5 transgenic mice. The numbers are the average of at least three experiments ± SEM.

tetramer could actually induce a stronger ($\sim$2- to 5-fold) IL-2 production response than did synthetic peptide (Fig. 4). Therefore, it seems that the tetramer is a more active reagent than the synthetic peptide in stimulating BDC2.5 T cells.

To determine whether the tetAg7/p79 tetramer could detect T cells not only from the BDC mice but also from NOD mice, we stained cells from islets and spleens of NOD mice. Interestingly, the tetramer could detect a small population of noncultured CD4$^+$ T cells from NOD mouse islets (0.75%) and spleens (0.46%) compared with cells from control BALB/c (0.27%; Fig. 5A, *upper panel*) or ABH animals ($\leq$0.2%; data not shown; also refer to Figs. 1A and 5B). Although 0.27% of BALB/c mouse cells were stained by tetAg7/p79, they did not appear as a distinct population compared with that in NOD spleen cells. Additionally, $\leq$0.2% of ABH mouse CD4$^+$ cells were stained by tetAg7/p79. Therefore, compared with control cells, $\sim$0.2–0.3 and $\sim$0.4–0.5% of NOD mouse spleen and islet CD4$^+$ T cells, respectively, were stained by tetAg7/p79. In addition to control cells, we performed experiments using control tetramers containing irrelevant peptides. As a control tetramer, consistent with our previous findings (18), the tetAg7/p206 tetramer did not detect a significant population of NOD mouse T cells in islets and spleens (Fig. 5A). The reason for this is not clear, but it could be that the TCR affinity of p206-specific T cells for their ligands or the frequency of such cells in NOD mice was too low to be detected by tetAg7/p206. We have performed additional control experiments using the tetAg7/pcOVA tetramer to stain NOD mouse T cells to further confirm that the tetAg7/p79$^+$ cell population detected in NOD mice was a small, but real, population. The results shown in Fig. 5B demonstrated that the percentage of NOD mouse CD4$^+$ T cells stained by the tetAg7/pcOVA control tetramer (0.21%) was at least 2-fold less than the percentage of T cells stained by the tetAg7/p79 tetramer (0.52%).

It is conceivable that autoreactive T cells could have been activated previously by self-Ags and became larger blast cells, which could be distinguished from other naive T cells. To determine whether this is true for T cells detected by the tetramer, we reanalyzed the results by electronically gating on the cells of a larger size according to their forward and side scatter profiles. Interestingly, the percentage of CD4$^+$ T cells positively stained by the tetramer increased 2- to 3 fold to $\sim$1.9 and $\sim$1.8% in islets and spleens, respectively (Fig. 5A, *lower panel*). Furthermore, the frequency or number of tetramer$^+$ cells did not increase in older animals or diabetic animals (data not shown). Additionally, we have performed further control experiments to determine whether the CD4$^+$ T cells detected by tetAg7/p79 were not T cells but were macrophages, dendritic cells, or apoptotic cells that may be nonspecifically stained by the tetramer. The results showed that tetAg7/p79 did not detect a population of CD4$^+$ T cells that were positively stained by annexin V, which stains apoptotic cells; by anti-CD11C Ab, which stains dendritic cells; or by F4/80, an Ab which stains macrophages (Fig. 5C) (33). Together, these results suggest that tetAg7/p79 can detect a small population of T cells in
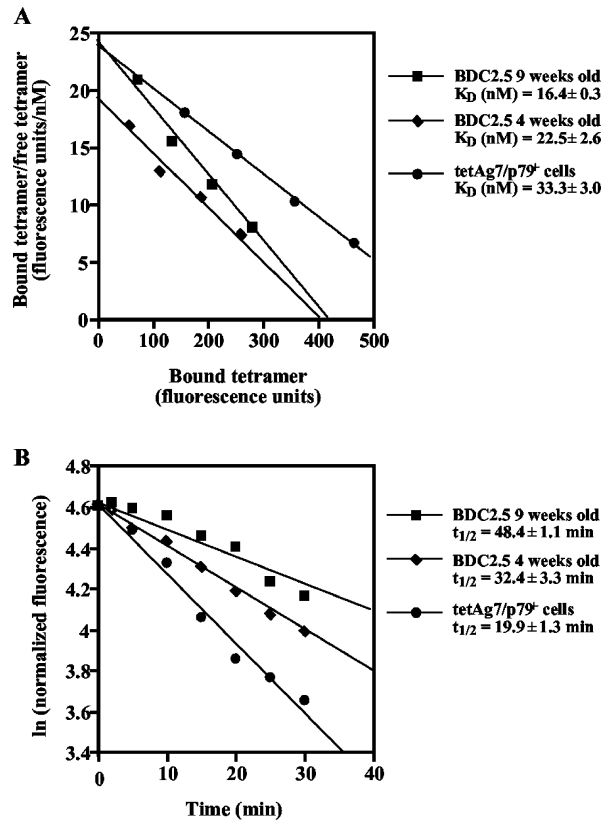

FIGURE 3. The association and dissociation kinetics of tetAg7/p79 for BDC2.5 T cells from 4- or 9-wk old BDC2.5 transgenic mice and for isolated tetAg7/p79$^+$ T cells. *A*, Scatchard analysis of binding of the tetAg7/p79 tetramer to BDC2.5 T cells and tetAg7/p79$^+$ T cells. Cells were stained with increasing concentrations of the tetramer. The apparent K_D values were determined by plotting the median staining intensity of bound tetramer/free tetramer vs bound tetramer. *B*, Analyses of decay kinetics of the tetramer bound to BDC2.5 T cells and to tetAg7/p79$^+$ T cells. The natural logarithm of the normalized fluorescence is plotted vs time (0–30 min) after addition of the anti-I-Ag7 Ab that competitively binds to the same BDC2.5 T cells. See *Materials and Methods* for details. The results are representative of at least three experiments.

NOD mice, and that the tetramer$^+$ T cells are activated in vivo, perhaps by endogenous peptides homologous to p79.

To further confirm the results obtained from FACS analyses, we used a confocal microscope to determine whether T cells from NOD mice were stained by the tetAg7/p79 tetramer. The results obtained from confocal microscope analyses were consistent with those of FACS analyses (Fig. 2, *C* and *D*). The estimated percentage of tetramer$^+$ T cells stained by tetAg7/p79 was comparable to that obtained using FACS, as shown in Fig. 5.

We then wanted to know whether the p79 peptide was able to stimulate and expand splenic T cells from NOD mice, and whether the stimulated and expanded T cell population could be detected more easily using the tetramer. First, we labeled purified splenic CD4$^+$ T cells with CFSE and then incubated the cells with the p79 peptide plus irradiated CD4-depleted APC. Ten days later, we stained the cells with anti-CD4 Ab plus the tetramer and analyzed the cells using FACS. Interestingly, some of the cells underwent at least six rounds of cell division after the first 10-day culture period (Fig. 6, *A* and *B*). The results show that the percentage of T cells detected by the tetramer increased in cells that went through more rounds of cell division (Fig. 6, *C* and *D*). The percentage of tetramer$^+$ T cells increased almost 20-fold, from 0.19% in cells that did not undergo division to 3.32% in cells that divided more than
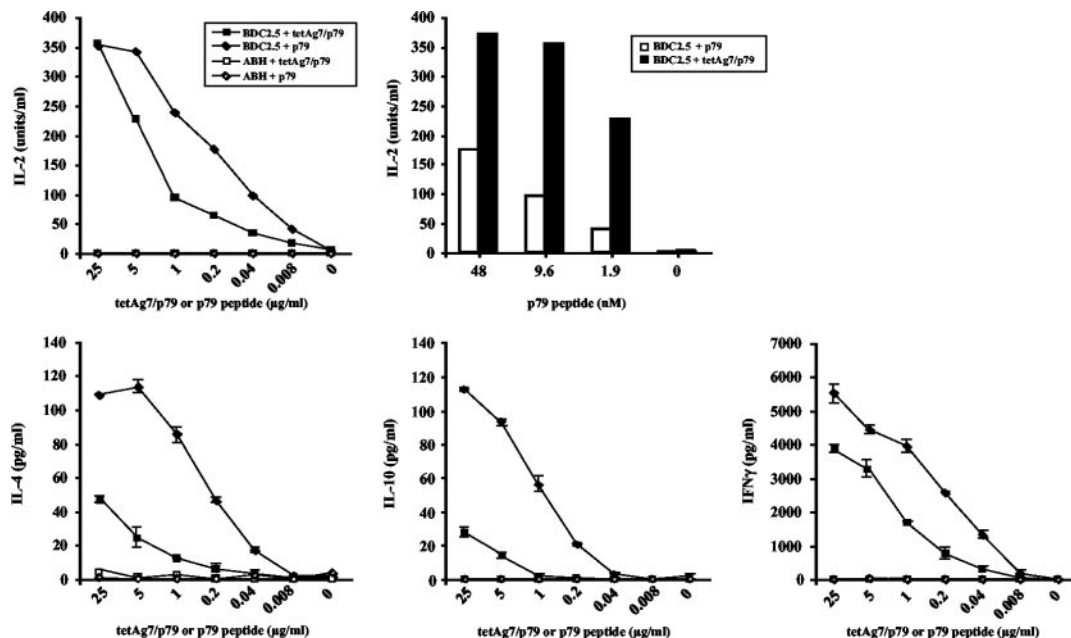
FIGURE 4. Analyses of cytokine production by CD4$^+$ splenic T cells from BDC2.5 mice stimulated by the p79 peptide or the tetAg7/p79 tetramer. The peptide and the tetramer were diluted at a 5-fold serial dilution starting from 25 μg/ml plus irradiated CD4$^+$ T cell-depleted BDC mouse spleen cells as the APC. After being cultured for 24 h, supernatants were collected for ELISA and IL-2 bioassay. CD4$^+$ T cells from ABH mice were used as the negative control, with irradiated CD4$^+$ T cell-depleted ABH mouse spleen cells as the APC. The bar chart shows a comparison of the effects of the synthetic peptide and the recombinant peptide present in the tetramer at equal molarities in stimulating BDC2.5 T cells. The results of the IL-2 secretion response to the same molar concentration of the synthetic peptide and the recombinant peptide show that the tetramer was actually more active than the synthetic peptide (~5-fold at 1.9 nM and ~2-fold at 48 nM) in stimulating the cells. The calculated total M_r of the peptides (~5 kDa) in the tetramer is ~1/110th that of the tetramer. The results represent the average of at least three experiments.

six times (Fig. 6D). As a control we also stained p79-stimulated cells with tetAg7/p206 tetramer, and the results showed that even cells that underwent more rounds of cell division were not stained by this tetramer (Fig. 6, C and D). It is not clear why tetAg7/p79 did not stain all dividing cells. It could be that other T cells proliferated in response to non-p79 peptides because the purity of the p79 peptide we used in the studies was ~90%, and that the culture medium contained FBS. Some T cells may respond to the 10% non-p79 peptides or FBS-derived peptides and further divide in cell culture containing IL-2. Autoreactive T cells specific for other autoantigens may also proliferate in the presence of APC and IL-2. Additionally, it is likely that many p79-stimulated T cells may bear low affinity TCRs and are not stained by tetAg7/p79. However, following initial stimulation, further culture of the cells with additional antigenic stimulation could result in more significant expansion of tetramer$^+$ T cells and increase the total percentage of the cells to at least 20% (data not shown). In our previous report we showed that Ag-specific NOD mouse T cells express higher levels of CD4 after they are activated by their Ags (18). Consistent with those studies, the activated tetAg7/p79 tetramer$^+$ T cells also expressed higher levels of CD4 on their surface. Therefore, the tetAg7/p79 tetramer$^+$ T cells responded to the p79 peptide and were enriched in the more divided cell population.

In addition to using the p79 peptide, which was identified as a synthetic peptide analog, we studied whether NOD mice contained T cells that can be stimulated by the p17 peptide. The p17 peptide, like p79, can stimulate BDC2.5 T cells at low concentrations. We generated tetAg7/p17 tetramer and found out that, like tetAg7/p79, it could detect a large population of CD4$^+$ T cells from BDC2.5 mice (Fig. 7A). However, the background of cells stained with tetAg7/p17 was higher than that of tetAg7/p79, and the percentage of brightly stained T cells (67.7%) was lower than that stained by tetAg7/p79 (82.5%; Figs. 1A and 7A). In addition, the percentage

of NOD mouse splenic CD4$^+$ T cells (2.25%) stained by tetAg7/p17 was even lower than that of control ABH mouse splenic CD4$^+$ T cells (5.18%; Fig. 7A). These results suggest that the tetramer failed to detect a significant population of splenic T cells from NOD mice. It has been shown that cross-reactivity is an essential feature of TCRs (34); therefore, tetAg7/p79 may detect not only BDC2.5 cells, but also other T cells specific for various BDC2.5 T cell-stimulating peptides. For further studies, we used tetAg7/p79 to determine whether it could also detect NOD mouse T cells that were stimulated by the p17 peptide. We first labeled purified CD4$^+$ NOD mouse splenic T cells with CFSE and stimulated the cells with the p17 peptide. The cultured cells were then analyzed by staining with the tetAg7/p79 tetramer and anti-CD4 Ab. Interestingly, the cells proliferated in response to p17 more slowly than in response to p79, because fewer p17-stimulated T cells (9.5%) than p79-stimulated T cells (20.5%) underwent more than six rounds of cell division (Fig. 6, A and B, and Fig. 7, B and C). These results suggest that the two peptides may stimulate different T cell populations in NOD mice or that p17 is less active in stimulating the same T cells (Figs. 6 and 7). However, after being stimulated and cultured for 13 days, the percentage of tetramer$^+$ T cells increased in cells that had undergone the most rounds of cell division. The tetAg7/p79 tetramer could stain a small population of cells (0.9%) that underwent at least six rounds of cell division (Fig. 7, D and E). In comparison, the tetAg7/p79 tetramer detected only 0.07 and 0.6% of cells that did not divide or underwent two divisions, respectively. As a control, we also used tetAg7/p206 to stain p17-stimulated cells, and the results showed that the tetramer did not stain a significant population of the cells (Fig. 7, D and E). Therefore, NOD mouse splenic T cells could respond to p17, and tetAg7/p79 could detect T cells that responded to this peptide. To determine whether p17-specific T cells were present in NOD mouse islets, islet-infiltrating cells were cultured with p17 peptide
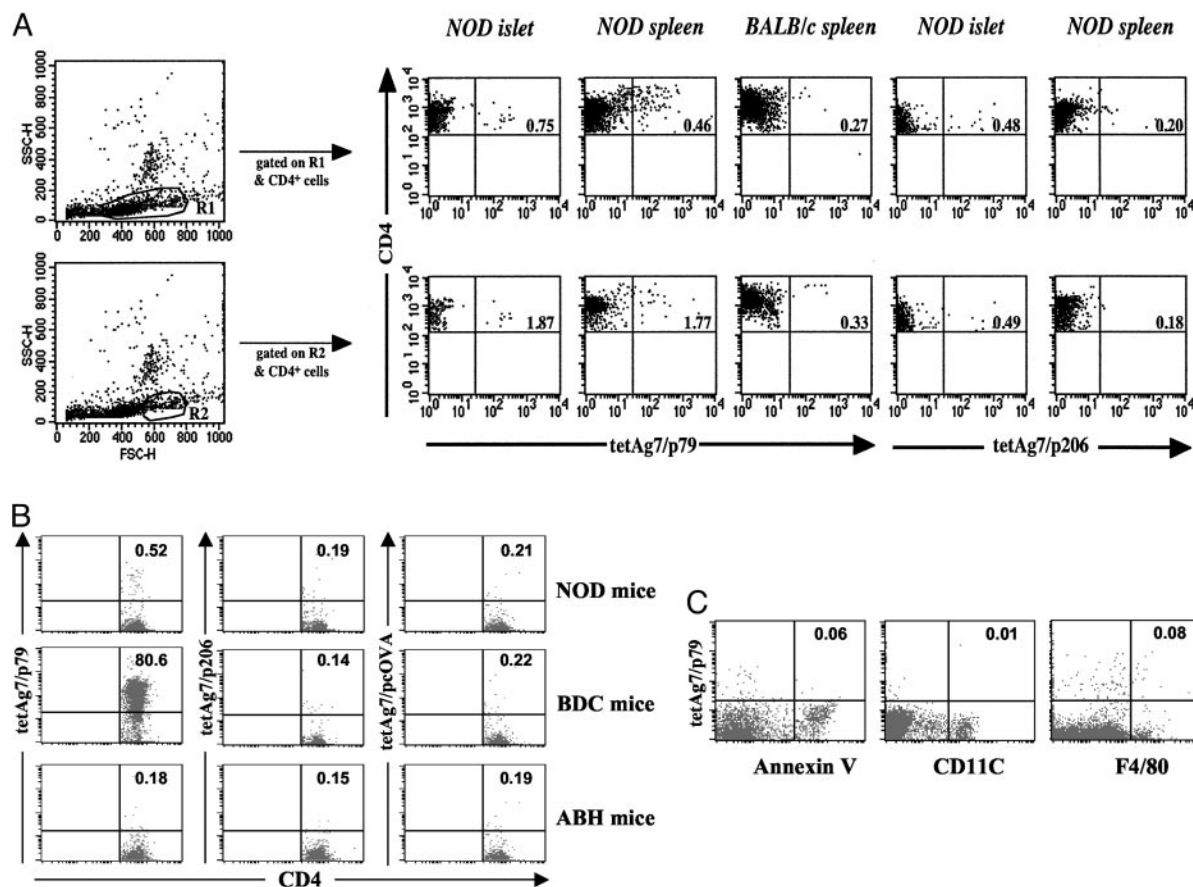
FIGURE 5. Detection of CD4$^+$, tetAg7/p79$^+$ cells in NOD spleens and pancreatic islets. *A*, Noncultured cells from spleens and islets were stained with tetAg7/p79 or the control tetAg7/p206 tetramers and anti-CD4 Ab as described in *Materials and Methods*. Only CD4$^+$ T cells are shown. The percentage of CD4$^+$ T cells detected with the tetAg7/p79 tetramer increased in a population of larger cells that were electronically gated based on their forward and side scatter profiles. The large cells are usually considered as being previously activated, perhaps by self-Ags. The R1 and R2 gates were set to exclude as much as possible the very large cells, such as macrophage or apoptotic cells in the analyses. *Upper panel*, Cells were first electronically gated in the R1 gate (total lymphocyte population) and then gated on CD4$^+$ T cells and analyzed for CD4 and tetramer staining. *Lower panel*, Analyses of cells that were electronically gated in the R2 gate (cells of larger size). *B*, Staining of noncultured spleen cells from NOD mice, BDC mice, and ABH mice with anti-CD4 Ab (*x*-axis) and tetramers (*y*-axis), including tetAg7/p79, tetAg7/p206, and tetAg7/pcOVA. The number in the quadrant represents the percentage of cells stained by both anti-CD4 and the tetramer. *C*, Staining of NOD mouse spleen T cells with tetAg7/p79 plus annexin V, anti-CD11C Ab, or F4/80 Ab. The numbers represent the percentage of cells stained by both the tetramer and the other reagents. The number in each quadrant represents the percentage of cells. The results shown are representative of at least three experiments.

and APC for 7 days. Interestingly, we could detect a distinct CD4$^+$ T cell population stained with tetAg7/p79 (0.49%; Fig. 8*A*). The percentage of tetramer$^+$ cells increased to 1.32% if the cells were electronically gated only on CD4$^+$ T cells (Fig. 8*B*). These results show that p17 peptide-specific T cells were present not only in spleens but also in islets of NOD mice.

Finally, we isolated from nonimmunized NOD mice the T cells that are specific for the p79 peptide using the tetAg7/p79 tetramer. Although the p79 peptide could stimulate T cells from NOD mice, it may stimulate polyclonal T cell subsets that share the Ag specificity of BDC2.5 T cells. Therefore, it is likely that the tetAg7/p79 tetramer detects not only BDC2.5 T cells in NOD mice, but also other cells in the animals, which may include T cells responding to p79 or p17 peptide or to other BDC2.5 T cell-stimulating peptides. To determine whether this is the case, we stimulated T cells from NOD mouse spleens with p79, isolated CD4$^+$ T cells using the tetAg7/p79 tetramer, and compared the isolated T cells with the BDC2.5 T cells. The isolated p79-specific CD4$^+$, tetramer$^+$ T cells were maintained in IL-2 medium, and the culture condition was not biased toward the differentiation of either Th1 or Th2 cells. The purity of the cells was >99%, and they were not stained

by the tetAg7/p206 tetramer (see below in Fig. 10*A*). We determined the avidity of the isolated tetramer$^+$ T cells using the tetAg7/p79 tetramer (Fig. 3). The K_d and $t_{1/2}$ of tetramer$^+$ T cells were about one-half those of T cells from 9-wk-old BDC2.5 mice. The results showed that the avidity of tetramer$^+$ T cells was lower than that of BDC2.5 T cells for the same tetAg7/p79 ligand.

We then determined their cytokine secretion profile in response to the p79 peptide. The IL-2 secretion assay results showed that the isolated tetramer$^+$ T cells were indeed specific for the p79 peptide, and surprisingly, they did not respond to the other peptides used, including p17, pGAD$_{206}$, and pGAD$_{526}$ peptide (Fig. 9). Therefore, although these peptides can stimulate BDC2.5 T cells, they stimulate different population of T cells from NOD mice. In addition, we determined the cytokine secretion pattern of the isolated p79-specific CD4$^+$, tetramer$^+$ T cells. These cells, like BDC2.5 T cells, secreted a large quantity of IFN-γ in response to the p79 peptide (Fig. 9). However, unlike BDC2.5 T cells, tetramer$^+$ T cells also secreted a large amount of IL-10 and, to a lesser extent, IL-4 (Fig. 9). To study in more detail the cells secreting IL-4, IL-10, and IFN-γ, we performed intracellular cytokine staining using Abs against IL-4, IL-10, or IFN-γ (Fig. 10). The results
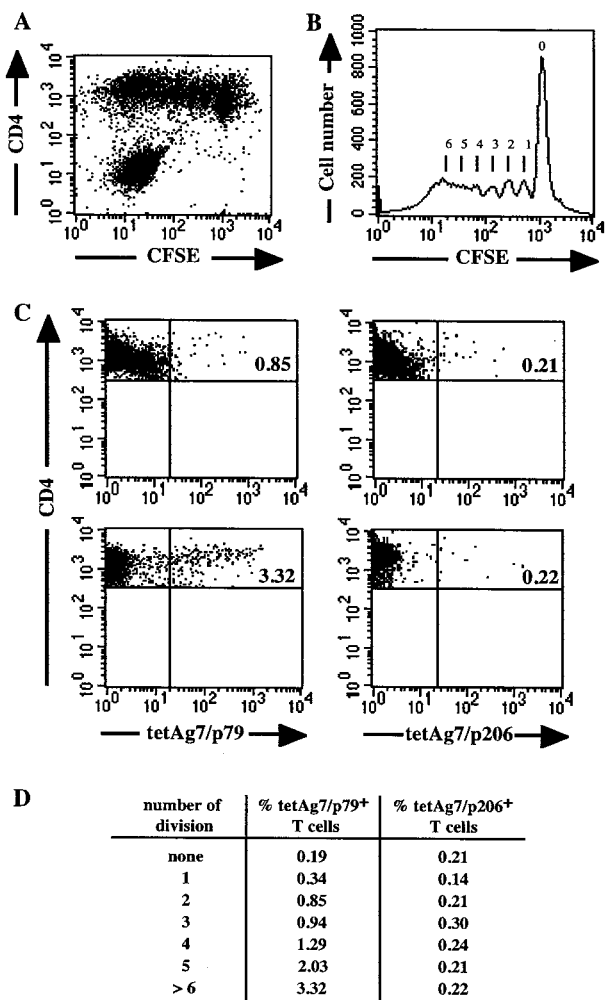
FIGURE 6. In vitro stimulation and expansion of T cells stimulated by the p79 peptide from NOD mouse spleens. CD4+ T cells from naive NOD mice were labeled with CFSE and cultured with the p79 peptide and irradiated APC from NOD mice. After incubation for 10 days, cells were stained with tetAg7/p79 or a control tetAg7/p206 tetramer and anti-CD4 Ab. *A* and *B*, Proliferation of CD4+ T cells in response to p79 peptide stimulation. As shown by CFSE staining, some CD4+ T cells were able to divide at least six times following peptide stimulation. *A*, Dot plot of CFSE-labeled cells stained with anti-CD4 Ab. *B*, Histogram of cells labeled with CFSE; the number above each peak indicates the number of cell division after stimulation. *C*, Staining of CD4+ cells that have had two (*upper panel*) and more than six (*lower panel*) rounds of cell division with tetAg7/p79 (*left column*) or with tetAg7/p206 (*right column*). *D*, Percentage of tetAg7/p79+ CD4+ T cells in populations that underwent different numbers of cell division. The results show that the percentage of tetramer+ T cells increases when cells undergo further rounds of cell division. The cells were also stained with a control tetAg7/p206 tetramer. The results showed that the percentage of cells stained by tetAg7/p206 did not increase in the population of cells that underwent more rounds of cell division. The results shown are representative of at least two experiments.

FIGURE 7. In vitro stimulation and expansion of T cells stimulated by the p17 peptide from NOD mouse spleens. *A*, BDC2.5 T cells from islets and spleens were stained with tetAg7/p17 tetramer. Spleen cells from NOD and ABH mouse were used as controls. Also shown is the BDC2.5 spleen cells stained with tetAg7/p206. *B–E*, CD4+ T cells from naive NOD mice were labeled with CFSE and cultured with the p17 peptide and irradiated NOD mouse APC. After incubation for 13 days, cells were stained with tetAg7/p79 or tetAg7/p206 and anti-CD4 Ab. *B* and *C*, Proliferation of CD4+ T cells in response to p17 peptide stimulation. As shown by CFSE staining, some CD4+ T cells were able to divide at least six times following peptide stimulation. *B*, Dot plot analysis of CFSE-labeled cells stained with anti-CD4 Ab. *C*, Histogram analysis of cells labeled with CFSE. The number above each peak indicates the number of cell divisions after stimulation with p17. *D*, Staining of CD4+ cells that have had two (*upper panel*) and more than six (*lower panel*) rounds of cell division with tetAg7/p79 (*left column*) or tetAg7/p206 (*right column*). *E*, Percentage of CD4+, tetAg7/p79+ T cells in populations that have undergone different numbers of cell division. The tetramer could detect a small population of T cells in cells that underwent at least six rounds of cell division. The cells were also stained with a control tetAg7/p206 tetramer. The results showed that the percentage of cells stained by tetAg7/p206 did not increase in the population of cells that underwent more rounds of cell division. The results shown were representative of at least two experiments.

showed that 17–18% of the tetramer+ cells produced IL-10, 4–5% of the cells produced IFN-γ, and ~1% of the cells produced both cytokines (Fig. 10*B*). In addition, although the cells secreted some IL-4, essentially no cell that produced IL-4 was detected using this method compared with the isotype control staining. Therefore, the tetramer+ cells seem to be biased toward being IL-10-producing cells, and a smaller population produced IFN-γ, while very few, if any, cells produced IL-4. Furthermore, we also analyzed the TCR Vβ usage of tetramer+ T cells, and the results showed that tet-
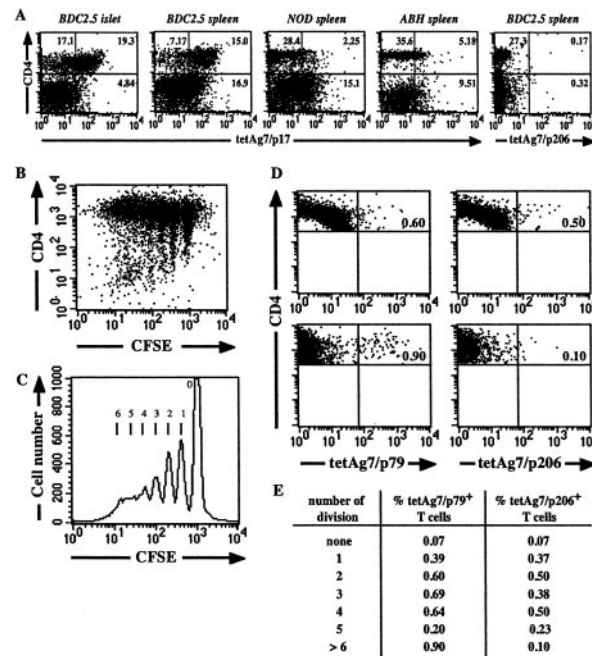
ramer+ T cells contained a heterogeneous population of T cells that express different TCRs. There were <3% of the cells that bore TCR Vβ4, whereas ~30% of the cells expressed TCR Vβ7 (data not shown). Therefore, the population of splenic T cells that responded to the p79 peptide in vitro is not biased toward the BDC2.5-like, TCR Vβ4+ T cells that secreted a large amount of IFN-γ and much less IL-4 and IL-10. The results suggest that although the tetAg7/p79 tetramer can detect BDC2.5 T cells, only a small portion of the p79 peptide-stimulated T cells from NOD mice represent BDC2.5 T cells.

To determine the role of isolated tetAg7/p79+ T cells in diabetes, we adoptively transferred the cells into NOD/*scid* mice with or without NOD spleen cells (Fig. 11). The results showed that mice receiving only tetramer+ T cells did not develop diabetes. However, all mice that received both tetramer+ T cells and NOD spleen cells (cotransfer), like mice receiving only NOD spleen cells (single transfer), developed diabetes by the age of 25 wk. Although cotransferred mice showed slightly delayed (by 2 wk) onset and
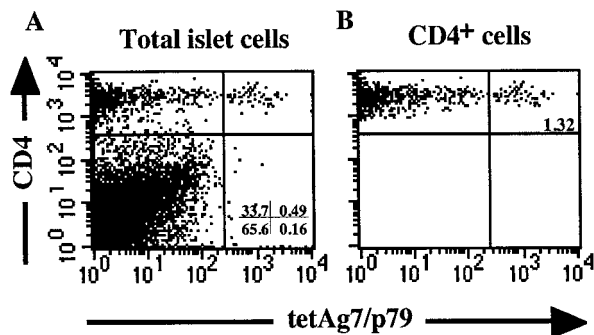
FIGURE 8. In vitro stimulation and expansion of T cells stimulated by the p17 peptide from 9-wk-old NOD mouse islets. Islet-infiltrating cells from naive NOD mice were cultured with the p17 peptide and irradiated NOD mouse APC. *A*, After incubation for 7 days, cells were stained with tetAg7/p79 tetramer and anti-CD4 Ab. The tetAg7/p79 tetramer can detect ~0.5% of islet cells. *B*, The percentage of cells detected by the tetramer increased to 1.32% when the cells were electronically gated only on CD4+ T cells. The results shown are representative of at least two experiments.

FIGURE 10. Intracellular cytokine staining of tetAg7/p79+ T cells. *A*, Isolated tetramer-positive cells were stained with both anti-CD4 Ab and the tetAg7/p79 or the tetAg7/p206 tetramer. *B*, The stimulated tetramer-positive cells were also stained intracellularly with isotype control Abs, anti-IL-10 plus anti-IFN-γ Abs, and anti-IL-4 plus anti-IFN-γ Abs. The number in each quadrant represents the percentage of cells stained with the Abs.

relatively synchronized development of diabetes compared with single-transferred mice, the difference in disease development between single-transferred and cotransferred mice was not statistically significant. Therefore, although the isolated tetAg7/p79+ T cells secreted some IL-10, they did not significantly inhibit diabetes development in recipient mice.

Discussion

We have shown in this report that the tetAg7/p79 tetramer can detect CD4+ T cells from islets and spleens of BDC2.5 mice. Furthermore, we have demonstrated that in addition to FACS, confocal microscopy can be used to detect a similar frequency of these cells with the tetramer. Our results show that a large percentage of CD4+, tetAg7/p79+ T cells are present in pancreatic islets and spleens of BDC2.5 mice. More than 70% of the CD4+ T cells infiltrating the islets were stained positively for the tetramer by the age of 4 wk, and ~68% of CD4+ splenic T cells were detected by the tetramer. In addition, the percentage of CD4+, tetAg7/p79+ T

cells increases with the age of the mice, suggesting that these cells gradually expand and accumulate in both spleens and islets. However, diabetes occurs spontaneously in <10% of the BDC2.5 mice in our colony, as reported by others (12). The BDC2.5 mouse-derived T cells were functional, because after stimulation with the p79 peptide or the tetAg7/p79 tetramer, the BDC2.5 T cells produced cytokines such as IL-2 and IFN-γ (Fig. 4). Therefore, the low incidence of diabetes shown in previous reports could not be due to the fact that BDC2.5 T cells were anergic to stimulation, because they respond to the p79 peptide and the tetAg7/p79 tetramer. This could be due to a recently identified CD4+, DX5+ T cell that can regulate the development of diabetes in BDC2.5 mice by protecting islet cells from damage (14).

Our results also have shown that the avidity of BDC2.5 TCR for the tetramer slightly increases with age. The reason for the increase is not clear. Because the BDC2.5 TCR is expressed as a rearranged TCR transgene in these mice, its binding affinity for its ligand should not change with age. Therefore, the increase in the avidity of BDC2.5 TCRs for their ligands may not be due to the avidity maturation of autoreactive T cells proposed previously for studies of CD8+ T cells in NOD mice (35). An explanation for the increased avidity is that it is due to the increased BDC2.5 TCR levels in T cells in older animals. Because the avidity of TCR represents
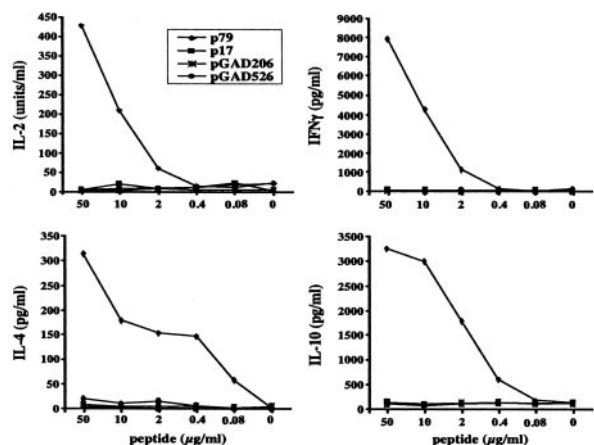

FIGURE 9. Analyses of cytokine production by tetAg7/p79+ T cells. NOD mouse splenic T cells were isolated (>99% purity) with the tetramer after they were stimulated in vitro with the p79 peptide. Cytokine production by these T cells was determined after they were stimulated with the p79, p17, GAD206, or GAD526 peptides. The peptides were diluted at a 5-fold serial dilution starting from 50 μg/ml plus irradiated NOD mouse APC. After being cultured for 24 h, supernatants were collected for ELISA and IL-2 bioassay. The results are representative of at least three experiments.
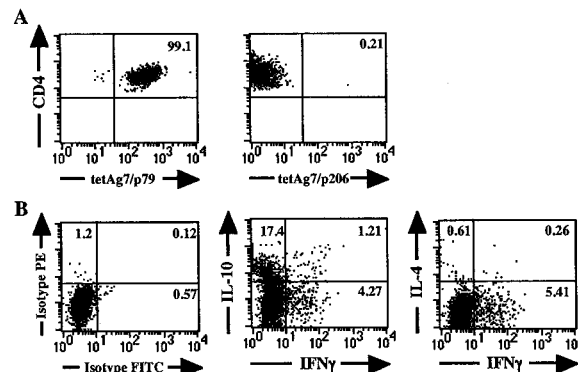

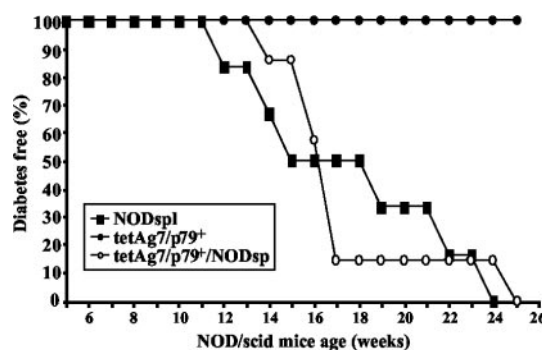**FIGURE 11.** Adoptive transfer of tetAg7/p79+ T cells into NOD/*scid* mice. The tetramer+ T cells (1 × 10⁷/mouse) were i.v. injected into young NOD/*scid* mice (4–5 wk old) with or without NOD spleen cells (1 × 10⁷/mouse). NOD mouse spleen cells without tetramer+ T cells were also adoptively transferred into NOD/*scid* mice as controls. There were six or seven mice used per group in these experiments.

the sum of affinities of individual TCR for their ligands, an increased TCR expression level on cells would therefore increase both the number of binding sites and their avidity for the ligands. An increased tetramer staining intensity on T cells correlates with an increase in the total affinity or avidity of TCR for their ligands (20). However, staining of T cells with the anti-TCR Cβ Ab, H57, or the tetramer showed that total TCR or the BDC2.5 TCR expression level on T cells did not change significantly when mice became older (data not shown). Another possible explanation may be that the BDC2.5 T cells in transgenic mice may bear different levels of a second TCR. It has been shown that >25% of CD4$^+$ T cells in BDC2.5 mice may express an endogenous TCR α-chain (11). Consistent with the previous studies, the tetAg7/p79 tetramer staining results showed that not all CD4$^+$ T cells bearing the TCR Vβ4 gene fragment were detected by the tetramer. Therefore, it is likely that more T cells of younger mice than older mice bear a second TCR other than the BDC2.5 TCR by expressing different TCR α-chains. In older animals, T cells bearing more or only the BDC2.5 TCR may expand faster and eventually predominate in the periphery, especially in the islets. This may be due to encounter of BDC2.5 T cells with self-Ags in the older animals. Alternatively, rearrangement and assembly of a second TCR α-chain gene may gradually shut down in older BDC2.5 animals.

It has been suggested that autoreactive T cells bear TCRs with low avidity for their ligands (27, 28). It is interesting that the avidity of BDC TCR for the tetAg7/p79 ligand was relatively higher than that of some of the CD4$^+$ T cell TCRs studied previously (20, 29–31). This may be because p79 is not the natural agonist peptide, but a superagonist peptide for BDC TCR, which results in a stronger binding affinity for the TCR. It is likely that the avidity of BDC TCR for its unknown natural ligand would be lower than its avidity for tetAg7/p79. It could also be that we used a tetramer to determine the avidity of TCR, whereas the previous studies used MHC/peptide monomers. The tetrameric ligand would have a much stronger binding affinity for the TCR than did the monomeric ligand. In addition, our avidity measurement studies showed that the avidity of TCR expressed on the isolated tetAg7/p79$^+$ T cells was about one-half that of BDC2.5 TCR. The reason for this is currently unknown. One possibility could be that the superagonist p79 peptide induced apoptosis of T cells that bear TCRs with higher avidity for I-Ag7/p79 during in vitro expansion. This would result in the expansion of T cells bearing TCR of lower affinity.

NOD splenic T cells responded spontaneously to the p79 peptide; however, the response was not very strong. This suggests that the frequency of splenic T cells specific for the peptide was not high, as shown in the FACS and confocal microscopy studies. These studies show that, compared with the controls, the tetAg7/p79 tetramer stained a small, but real, population of CD4$^+$ T cells present in both spleens (0.2–0.3%) and pancreatic islets (0.4–0.5%) of NOD mice. The percentage of tetramer$^+$ T cells did not increase in older or diabetic NOD mice. Although we were very careful in handling and staining cells, the cells that were positively stained by the tetramer may still include a small number of macrophages, dendritic cells, and apoptotic cells that may be nonspecifically stained by the tetramer. If that is the case, then the percentage of tetramer$^+$ cells may be even lower than those shown in Fig. 5. In addition, as shown in Fig. 5C, tetAg7/p79 did not stain annexin V$^+$ cells, CD11C$^+$ cells, and F4/80$^+$ cells. These results indicate that the cells detected by tetAg7/p79 were not apoptotic cells, dendritic cells, or macrophages. In addition, the very small percentage of cells stained by the tetramer probably represents a real population of live cells, because we could further expand and isolate such cells in vitro. It is conceivable that the tetAg7/p79

tetramer may detect more than one T cell population in NOD mice that include BDC2.5 T cells and other T cells that could also respond to p79

Indeed, our studies on characterizing the p79 peptide-stimulated T cells that were isolated with the tetAg7/p79 tetramer are consistent with this view. Studies of the isolated tetramer$^+$ T cells showed that although the T cells secreted a large quantity of IFN-γ in response to the p79 peptide, they also secreted a larger amount of IL-10 and, to a lesser extent, IL-4 than did BDC2.5 T cells. Further intracellular cytokine staining studies showed that these cells were biased toward being IL-10-producing cells rather than IFN-γ- or IL-4-producing cells. Additionally, <3% of the cells expressed TCR Vβ4. Therefore, considering the small percentage of CD4$^+$ T cells detected by the tetAg7/p79 tetramer, it seems that BDC2.5 T cells are present at very low numbers in NOD mice. The BDC2.5 T cells are probably present in NOD mice at a frequency even lower than 0.5% of the total CD4$^+$ T cells detected by the tetAg7/p79 tetramer in spleens or islets, as shown in Fig. 5. Alternatively, because the cells were cultured in vitro before analyses, it is likely that BDC2.5 T cells proliferate more slowly than other cells (such as those that bear TCR Vβ7) and are eventually present as a minor population in tetramer$^+$ T cells. Interestingly, the isolated p79-responsive T cells did not respond to other BDC2.5 T cell-stimulating peptides, such as p17 and pGAD$_{526}$ peptides, although tetAg7/p79 tetramer could detect T cells stimulated by the p17 peptide. This suggests that the tetramer may be able to detect a more heterogeneous T cell population than T cells stimulated in vitro by the peptide linked to the tetramer. Therefore, the p17 and other peptides, such as the p79 peptide, may also stimulate a unique population of T cells derived from NOD mice. Based on these results, it seems that T cells specific for more than one self-Ag may play critical roles in the pathogenesis of autoimmune diabetes. It is likely that these diabetogenic T cells, like BDC2.5 T cells, may also be present in NOD mice at a very low frequency. Alternatively, it is likely that T cells specific for different self-antigenic peptides may infiltrate the islets in NOD mice that are at different stages of the disease.

Although the isolated tetAg7/p79$^+$ T cells secreted a large quantity of IL-10, the tetramer$^+$ T cells did not inhibit the development of diabetes when they were cotransferred with NOD mouse spleen cells into NOD/scid mice. The in vivo adoptive transfer experiments showed that both cotransferred (receiving both tetramer$^+$ cells and NOD splenocytes) and single-transferred (receiving NOD splenocytes alone) NOD/scid mice developed diabetes by the age of 25 wk. The difference of diabetes development between cotransferred animals and single-transferred animals was not statistically significant. It is not clear why the tetAg7/p79$^+$ T cells did not enhance diabetes development in recipient animals. Several possibilities may explain these results. One possibility is that the number of BDC-like T cells present in the tetAg7/p79$^+$ population was too small to induce a faster development of diabetes. These BDC-like cells may also expand in vivo at a rate slower than the other cotransferred T cells, or they may undergo apoptosis and eventually constitute an even smaller portion of the cells in the recipient mice. In addition, the slightly delayed diabetes onset in cotransferred animals suggests that T cells specific for other self-Ags may play a role during early diabetes development. It is also possible that IL-10 produced by some tetAg7/p79$^+$ cells resulted in the delay.

Both p79 and p17 peptides could stimulate BDC2.5 T cells, but p79 was identified as a peptide analog using a positional scanning combinatorial peptide library. Interestingly, p17 could stimulate a smaller population of NOD splenic T cells than that stimulated by p79. Additionally, p17-stimulated T cells proliferated more slowly

than those cells that responded to p79. However, T cells from NOD mice can be stimulated spontaneously by the p17 peptide in vitro, suggesting that a peptide(s) with a sequence similar to or identical with that of the p17 epitope is present in these mice. Therefore, the p17 peptide or its homologues in NOD mice may be involved in diabetes. Future studies on T cells specific for p17 and other peptides, such as the GAD peptides, that can stimulate BDC2.5 T cells should provide more helpful information on the role of these cells in the pathogenesis of diabetes and show whether they are related to BDC2.5 T cells.

In summary, although it has been relatively easy to detect different populations of Ag-specific CD8$^+$ T cells using class I MHC tetramers, it has been difficult to detect and isolate autoreactive CD4$^+$ T cells. A major contribution of these studies is that we were able to generate class II MHC tetramers to detect and isolate a heterogeneous population of NOD mouse T cells specific for peptides that stimulate diabetogenic BDC2.5 T cells. The knowledge obtained from these studies and the use of similar reagents and approaches should facilitate further studies to determine the roles of T cells and different autoantigens in the development of autoimmune diabetes.

Acknowledgments

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