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Induction of Autoantigen-Specific Th2 and Tr1 Regulatory T Cells and Modulation of Autoimmune Diabetes

Cyndi Chen,* Wen-Hui Lee,* Pen Yun,* Peter Snow,† and Chih-Pin Liu2*

Autoantigen-based immunotherapy can modulate autoimmune diabetes, perhaps due to the activation of Ag-specific regulatory T cells. Studies of these regulatory T cells should help us understand their roles in diabetes and aid in designing a more effective immunotherapy. We have used class II MHC tetramers to isolate Ag-specific T cells from nonobese diabetic (NOD) mice and BALB/c mice treated with glutamic acid decarboxylase 65 peptides (p206 and p221). Based on their cytokine secretion profiles, immunization of NOD mice with the same peptide induced different T cell subsets than in BALB/c mice. Treatment of NOD mice induced not only Th2 cells but also IFN-γ/IL-10-secreting T regulatory type 1 (Tr1) cells. Adoptive transfer experiments showed that isolated tetramer+ T cells specific for p206 or p221 could inhibit diabetes development. These cells were able to suppress the in vitro proliferation of other NOD mouse T cells without cell-cell contact. They performed their regulatory functions probably by secreting cytokines, and Abs against these cytokines could block their suppressive effect. Interestingly, the presence of both anti-IL-10 and anti-IFN-γ could enhance the target cell proliferation, suggesting that Tr1 cells play an important role. Further in vivo experiments showed that the tetramer+ T cells could block diabeticogenic T cell migration into lymph nodes. Therefore, treatment of NOD mice with autoantigen could induce Th2 and Tr1 regulatory cells that can suppress the function and/or block the migration of other T cells, including diabeticogenic T cells, and inhibit diabetes development. The Journal of Immunology, 2003, 171: 733–744.

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2 Address correspondence and reprint requests to Dr. Chih-Pin Liu, Division of Immunology, Beckman Research Institute, City of Hope, 1450 East Duarte Road, Duarte, CA 91010-3000. E-mail address: cliu@coh.org
3 Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; GAD, glutamic acid decarboxylase; Tr1, T regulatory type 1; ICS, intracellular cytokine staining; UW, upper well; LW, lower well; L, ligand.

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other T cells in vitro. They could also block the migration of diabetogenic T cells in vivo.

Materials and Methods

Mice

The NOD and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The BDC2.5-transgenic mice were a gift from Drs. D. Mathis and C. Benoît (26). All animals were housed in a specific pathogen-free environment in the animal facility at the Beckman Research Institute, City of Hope.

Peptides and immunization

GAD65 p206 (TYEIAPVFVLLEYVT), the previously published BDC2.5 T cell-stimulating peptide p79 (1040–79) (27) were synthesized at the Beckman Research Institute, City of Hope, and purified using reversed-phase HPLC to a purity of >98%. Animals were injected i.p. with 100 μg of a peptide emulsified in an equal volume of IFA (Sigma-Aldrich, St. Louis, MO) on days 0 and 7. Splenocytes were removed from animals on day 14 for further analyses.

Production of class II MHC tetramers

This method has been described previously (20–22). Production and initial use of the tetAg7/p206 and tetAg7/p79 has been previously described (22, 23). The C-terminal end of the α- and β-chain of I-Ad/p206 heterodimer contained a leucine zipper for heterodimer pairing (28).

Isolation and staining of tetramer+ T cells and cytokine assays

Splenocytes were pooled from at least four immunized animals and cultured in Click’s medium (Life Technologies, Grand Island, NY) plus peptide for 3 days. Live cells were further incubated in IL-2/RPMI 1640 medium before being separated into tetramer+ and tetramer− cells using FACS and magnetic beads (Miltenyi Biotec, Auburn CA). Both tetramer+ and tetramer− cells were maintained in the IL-2 medium and, for longer cultures, the tetramer− cells were stimulated with the Ag in the IL-2 medium 2–3 wk after the last Ag stimulation in vitro. The tetramer+ cells were maintained in the same culture condition without the GAD peptides.

Staining of cells using tetramers has been described previously (22). Briefly, T cells were stained with PE-labeled tetramers plus the anti-TCR Cβ Ab, H57, at 37°C for 2 h and analyzed by FACS using FACSCalibur (BD Biosciences, San Jose, CA) (29). All of the Abs used for FACS and for in vitro inhibition assays were purchased from BD PharMingen (San Diego, CA).

The IL-2 bioassay has been described previously (22). Capture ELISA (assay kit from BD PharMingen or from Promega, Madison, WI) was used to measure the amount of cytokines according to the manufacturer’s instruction using cell culture supernatant harvested after incubating cells with Ags for 24 h. The GAD65 protein was purchased from Diamyd Diagnostic (Stockholm, Sweden).

Intracellular cytokine staining

Intracellular cytokine staining (ICS) was performed according to a previously described method (30). Briefly, T cells were incubated with PMA plus ionomycin and brefeldin A, stained with surface Abs, fixed with paraformaldehyde, and resuspended in 0.1% saponin buffer (w/v) (Sigma-Aldrich). The cells were then intracellularly stained using Abs against cytokines or negative isotype control Abs (BD PharMingen).

Adoptive transfer of T cells into NOD/scid mice and migration inhibition assays

Four- to 7-wk-old NOD/scid mice received a single i.v. injection of tetramer+ cells, tetramer− cells plus NOD splenocytes, or NOD splenocytes alone. Recipient mice were considered diabetic when mice were glycocotic for 2 consecutive weeks.

To determine the in vivo inhibitory effect of tetramer+ T cells on the migration of adoptively transferred BDC2.5 T cells, the BDC2.5 mouse splenocytes (1 × 10⁶/mouse) were mixed with equal numbers of the tetramer+ T cells and then co-transferred into NOD/scid mice. In addition, the tetramer+ T cells were preactivated with PMA/ionomycin (50 and 2500 ng/ml) for 16 h, washed, and cultured with the BDC2.5 splenocytes for 2 h before being co-transferred into NOD/scid mice. Cells from lymph nodes (axillary, brachial, inguinal, lumbar, and caudal nodes) were isolated and pooled together from adoptively transferred mice at 4 and 24 h after the transfer and stained with anti-CD4 Ab and tetAg7/p79 (23). Pancreatic lymph nodes were also removed, but the total number of cells was too low to show reliably the presence of BDC2.5 cells.

CFSE labeling

CD4+ T cells from BDC2.5 TCR-transgenic mouse spleens were purified using magnetic beads (Miltenyi Biotec) and labeled with CFSE. Briefly, CD4+ T cells in serum-free PBS were incubated with CFSE (0.8 μM) for 10 min at 37°C. After washing, the labeled CD4+ BDC2.5 T cells were used for further analyses.

In vitro inhibition assays using cocultures

CFSE-labeled CD4+ T cells from BDC2.5 mice (the target cells) were cultured in the absence of tetramer+ cells and 1) without peptides or 2) with p79 alone or together with GAD peptides. The CFSE-labeled cells were also cultured with either N221+ or N206+ cells plus p79 and GAD peptides. Irradiated (3000 rad) NOD mouse CD4+ cells were used as APCs. After being cultured for 4 days, cells were stained with the tetAg7/p79 tetramer (23). The effect of tetramer+ cells on BDC2.5 T cell proliferation was analyzed using FACS to determine whether the CFSE intensity was altered as compared with that of control cells.

Transwell assays

NOD splenocytes were cultured outside of Transwells (Coming, Corning, NY) in the lower well using a 24-well plate. Tetramer+ cells were cultured in the Transwell (the upper well) in the presence of irradiated APCs. All of the cells were activated with 100 ng/ml MTA and 5000 ng/ml ionomycin in the culture. After 72 h, [3H]thymidine was added to the lower well and the cells were harvested 24 h later. Thymidine incorporation was determined using a Wallac cell harvester (Wallac, Gaithersburg, MD).

To determine the cytokine effect on target cell proliferation, anti-cytokine Abs were also added to the Transwell assays. The procedures were the same as described above, except that a saturating amount of Abs (20 μg/ml) was added to the culture.

Results

Isolation of CD4+ tetramer+ T cells

To isolate GAD peptide-specific CD4+ T cells derived from NOD mice and from BALB/c mice, we have produced I-Ag7 (tetAg7/p206 or tetAg7/p221) and I-Ad (tetAd/p206 or tetAd/p221) tetramers bound to GAD p206 or p221. We chose these two GAD peptides because they are major T cell epitopes and may play important roles in IDDM (12–14, 31, 32). Treatment of NOD mice with GAD protein or peptides reduced the incidence of diabetes (12–14, 33). To understand the function of regulatory T cells induced in Ag-treated animals and their roles in regulating IDDM development, we isolated CD4+ tetramer+ T cells from p206- or p221-immunized NOD mice (N206+ or N221+ cells). In addition to NOD mice, we also immunized BALB/c mice, whose cells express class II MHC I-Ad molecules. Both I-Ad and I-Ag7 use the same α-chain and their β-chains differ from each other only in the β1 domain. Furthermore, immunization of NOD and BALB/c mice could induce Ag-specific T cell responses. Therefore, despite their different genetic background, immunization of BALB/c mice with the same GAD peptides allows us to characterize peptide-specific T cells (B206+ or B221+ cells) selected by I-Ad. We have repeated the experiments of mouse immunization and T cell isolation and have isolated two to three tetramer+ T cell lines from peptide-treated mice for further analyses. Consistent with previous studies, NOD mice immunized with IFA alone could still develop diabetes (12, 14, 15). However, diabetes development in NOD mice could be inhibited by immunization with IFA plus the GAD peptides. Therefore, treatment of NOD mice with the GAD peptides and not the IFA may induce regulatory T cells that can inhibit diabetes development.

The I-Ag7 and I-Ad tetramers were Ag specific, as they did not detect a significant number of tetramer+ T cells specific for the other peptide (Fig. 1A). The isolated tetramer+ T cells were also Ag specific because only tetramer+ but not tetramer− T cells (N206−, N221−, B206−, and B221− cells) derived from NOD or BALB/c mice responded to both synthetic (Fig. 1, B and D) and recombinant (Fig. 1, C and E) peptides and secreted IL-2. These
We then determined the cytokine secretion profiles of Ag-stimulated tetramer$^+$ T cells. The cells were cultured in medium supplemented with IL-2 without selectively promoting either Th1 or Th2 cell differentiation. ELISA analyses showed that tetramer$^+$ but not tetramer$^-$ T cells secreted IFN-γ and IL-4 in response to the Ags. N206$^+$ cells secreted 2- to 3-fold less IFN-γ but 2-fold more IL-4 than did B206$^+$ cells (Fig. 2, A and B). In contrast, N221$^+$ cells secreted ~4-fold more IFN-γ and 1.5-fold more IL-4 than did B221$^+$ cells (Fig. 2, C and D). Compared with N206$^+$ cells, N221$^+$ cells secreted between 8- and 10-fold more IFN-γ and comparable amounts of IL-4. The IFN-γ:IL-4 ratio (at 25 μg/ml of the peptides used for stimulation) for N206$^+$ cells was 4-fold less than that for B206$^+$ cells (0.38 vs 1.6) and 8-fold less for N221$^+$ cells (0.38 vs 3.1; Fig. 2E). The ratio for B206$^+$ cells was similar to that for B221$^+$ cells (1.6 vs 1.3). Further ICS of tetramer$^+$ cells showed that although not all cells produced the cytokines, the results essentially correlate with those of ELISA studies except for B206$^+$ cells producing IL-4 (Fig. 3). The explanation for this observation is not clear. Taken together, the results showed that N206$^+$ cells were biased toward Th2-like cells compared with B206$^+$ cells. In contrast, N221$^+$ cells were more like Th1 cells compared with B221$^+$ cells. On the other hand, the cells from BALB/c mice are essentially nonpolarized. Additionally, a small population of these tetramer$^+$ cells (0.3–0.5%) might coproduce IL-4/IFN-γ, suggesting that they are Th0 cells (data not shown).

Additional cytokine secretion assays showed that both N206$^+$ and N221$^+$ cells also secreted a large amount of IL-10 (Fig. 4, A and B). In contrast to the IL-2 results, N206$^+$ and N221$^+$ cells secreted ~3- to 4-fold more IL-10 than did B206$^+$ and B221$^+$ cells (Fig. 4, A and B). All tetramer$^+$ T cells secreted very little IL-10. The calculated IL-4:IL-10 ratio of the amount of cytokines that were secreted at the Ag concentration of 25 μg/ml is shown.

**FIGURE 1.** Staining and IL-2 secretion of purified tetramer$^+$ T cells derived from NOD or BALB/c mice. A, Staining of tetramer$^+$ T cells by 1-Ag7 or 1-Ad tetramers plus an anti-CD4 Ab. Tetramer$^+$ cells isolated from cultured splenocytes of 7- to 8-wk-old female NOD mice (left) or BALB/c mice (right) that had been immunized with the indicated peptides were stained with the corresponding tetramer as described in Materials and Methods. The percentage of N206$^+$ and N221$^+$ T cells was ~1–3% in the spleen cells of immunized mice as compared with <1% in nonimmunized mice before in vitro culture. After being cultured for 3–4 wk, the cells were purified using tetramers. Purified cells were stained with anti-CD4 and tetramers as indicated and were analyzed by FACS. The numbers shown in each quadrant represent the percentage of CD4$^+$ T cells stained by the tetramer. The results were typical of at least four different experiments. B–E, Analyses of IL-2 production by tetramer$^+$ and tetramer$^-$ T cells stimulated by Ags. Tetramer$^+$ or tetramer$^-$ cells (1 x 10⁵ cells/well) were incubated with APCs (3 x 10⁶ cells/well) from NOD mouse spleen plus either soluble synthetic peptides (B and D) or plate-bound recombinant peptides covalently linked to I-Ag7 or I-Ad heterodimers (C and E). Ags were 5-fold serially diluted from 25 μg/ml (synthetic peptides) or 25 μg/ml (recombinant peptides) to 25 pg/ml (recombinant peptides). Cell culture supernatant was harvested after 24 h and HT2 cells were used as the indicator in a MTT assay. The results were an average of at least four independent experiments (except for B206$^+$ cells which were from two experiments).

**FIGURE 2.** ELISA of tetramer$^+$ T cells. A–D, ELISA of IFN-γ and IL-4 production by Ag-stimulated tetramer$^+$ and tetramer$^-$ T cells derived from NOD or BALB/c mice. Cells were stimulated with various concentrations of peptides as described in Fig. 1. Cell culture supernatant was harvested after 24 h for ELISA. The results shown are an average of at least four independent experiments except for B206$^+$ cells. On the other hand, the cells from BALB/c mice are essentially nonpolarized. Additionally, a small population of these tetramer$^+$ cells (0.3–0.5%) might coproduce IL-4/IFN-γ, suggesting that they are Th0 cells (data not shown).
TGF-β (Fig. 4, C and D). Additional ICS studies showed that essentially all IL-4 producing N206+ or N221+ cells coproduced IL-10, indicating that they represent Th2 cells (Fig. 4E). Interestingly, IFN-γ-producing N206+ cells also produced IL-10, suggesting that they probably are not the classical Th1 cells but more likely to be the Tr1 cells as described before (35). Additionally, the majority of IFN-γ-producing N221+ cells coproduced IL-10 while a small portion of the cells did not, indicating that the former cells were like Tr1 cells and the later ones like Th1 cells. Therefore, the IFN-γ-secreting N221+ cells were mostly Tr1 cells rather than Th1 cells. The results also showed that N206+ cells contained nearly 2-fold more Th2 cells than did N221+ cells, whereas N221+ cells contained 40-fold more Tr1 cells and 10-fold more Th1 cells than did N206+ cells. Therefore, GAD p206 and p221 could stimulate T cells that differ from each other in that they contained various Th and Tr cell populations. N206+ cells were relatively more biased toward Th2 cells and N221+ cells toward Tr1 cells. These results also suggest that the indication of a Th1, Th2, or Tr1 cell response can be better defined with experiments, including both ICS and ELISA.

Because the tetramer+ T cell lines were derived from T cells of immunized mice and were restimulated in vitro with the peptides, we also studied the cytokine secretion profile of T cells derived from GAD protein-immunized NOD and BALB/c mice before further in vitro Ag stimulation. Our results demonstrated that T cells isolated from GAD-immunized NOD mice secreted a significant amount of IFN-γ, IL-4, and IL-10 after they were restimulated in vitro by GAD and p206 (Fig. 5, A–C). When the T cells were restimulated with p221, they also secreted a large amount of IFN-γ and IL-10 but secreted a small amount of IL-4. Although the total amount of cytokines secreted by these mixed populations of T cells (usually containing <3% tetramer+ cells) was smaller than that of purified tetramer+ cells shown above in Figs. 2 and 4, the amount of cytokines secreted at a per cell base is comparable to that of tetramer+ cells. Furthermore, the IFN-γ:IL-4 ratio of T cells was 3.3, 0.4, and 4.1 from GAD-immunized mice in response to GAD (50 μg/ml), p206 and p221 (40 μg/ml), respectively, similar to that of tetramer+ cells in response to the peptides (25 μg/ml) shown above (Fig. 2E). These results suggest that the cytokine secretion profile of NOD mouse T cells specific for p206 or p221 remain the same before and after they were restimulated in vitro with the peptides. Additionally, both N206+ and N221+ T cells could respond to the GAD protein and secrete IL-2, suggesting that these cells were similar to those T cells that develop spontaneously in NOD mice (Fig. 5D). On the other hand, the T cells from GAD protein-immunized BALB/c mice did not show a significant cytokine secretion response to GAD, p206, and p221 (data not shown). The reason for this weak response is not clear. It could be that the frequency of T cells specific for these peptides in GAD-immunized BALB/c mice was too low to show a significant response if the

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** ICS of tetramer+ T cells. The cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 6 h at 37°C. Cells were then stained for cell surface Ag (CD4), fixed, permeabilized, and then intracellularly stained with anti-IFN-γ or anti-IL-4 Abs. The Ab isotype controls are also shown below the cytokine Ab staining. The results are representative of at least four independent experiments.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Analyses of cytokine production by tetramer+ cells. Analyses of IL-10 (A and B), TGF-β cytokine production by tetramer+ and tetramer– cells using ELISA (C and D). The lower detection limit for IL-10 and TGF-β was 30 and 15 pg/ml, respectively. The results shown are the average of at least four independent experiments. E, N206+ and N221+ cells were costained intracellularly with anti-IL-10 plus anti-IL-4 or anti-IFN-γ Abs. The numbers shown represent the percentage of cells in each quadrant.
cells were not restimulated with the peptides and further expanded in vitro. Additionally, as we have shown in this report, the number of Ag-specific T cells from GAD peptide-immunized BALB/c mice could be significantly increased and isolated using the tetramers after they were further cultured in vitro with the peptides.

Adoptive transfer of N206\textsuperscript{+} and N221\textsuperscript{+} T cells reduced diabetes incidence

To determine the role of N206\textsuperscript{+} and N221\textsuperscript{+} T cells during diabetes development, we transferred these cells into NOD/scid mice. Because NOD/scid mice do not have T and B cells and do not develop diabetes, the initial studies will show whether the transferred tetramer\textsuperscript{+} T cells alone can induce diabetes. Nondiabetic NOD mouse splenocytes were used as the control. The results showed that although all of the mice that received splenocytes developed diabetes, none of those receiving tetramer\textsuperscript{+} T cells alone became diabetic (Fig. 6A). Therefore, N206\textsuperscript{+} and N221\textsuperscript{+} T cells alone could not induce diabetes in recipient mice. One possibility may be that other types of cells such as CD8\textsuperscript{+} T cells or B cells are required for tetramer\textsuperscript{+} cells to expand and function in these animals. Therefore, we cotransferred (double transfer) either N206\textsuperscript{+} or N221\textsuperscript{+} cells along with NOD splenocytes into NOD/scid mice. Interestingly, both cells inhibited the development of diabetes (Fig. 6B). Mice that received N206\textsuperscript{+} cells showed a slightly delayed...
The disease progressed between mice double transferred with N206\(^+\)/H11001 and N221\(^+\)/H11001 cells, as determined by using the Wilcoxon test and the chi\(^2\) test. These differences are probably not due to the number of tetramer\(^+\) T cells present in recipient animals as a comparable percentage of N206\(^+\) or N221\(^+\) cells was detected in the recipient animals (2–3% of total splenocytes) 20–30 wk after the cell transfer. Cotransfer (double transfer) of N206\(^+\) cells and N221\(^+\) cells along with NOD mouse splenocytes did not show a synergistic effect on diabetes development (Fig. 6B). The results were essentially identical to those of the double-transferred mice receiving N206\(^+\) cells in combination with NOD splenocytes. In addition, nondiabetic animals that received T cells did not develop detectable insulitis (data not shown), suggesting that the tetramer\(^+\) T cells may prevent cell infiltration into islets.

We have performed additional experiments to determine whether the disease suppression was specifically induced by N206\(^+\) and N221\(^+\) cells but not by other activated NOD mouse T cells. First, we studied whether Con A-stimulated NOD splenocytes, in addition to T cells activated by the GAD peptides, were able to suppress diabetes development in adoptive transfer experiments. The results showed that, unlike the tetramer\(^+\) T cells, Con A-activated cells did not inhibit diabetes development and all of the mice receiving these cells developed diabetes by the age of 26 wk (Fig. 6C). Second, in a separate study, we have generated a new I-A\(\gamma\) tetramer (tetAg7/p79) that could detect essentially all of the BDC2.5 T cells from BDC2.5 TCR-transgenic NOD mice (23). BDC2.5 T cells were diabetogenic CD4\(^+\) T cells and BDC2.5 TCR-transgenic NOD mice could develop an aggressive form of diabetes (26, 36). The tetAg7/p79 tetramer contains the p79 peptide that is highly active in stimulating BDC2.5 T cells and its sequence is unrelated to the GAD p206 and p221 peptides (27). Therefore, p79 can serve as a good control peptide and tetAg7/p79 as a good control tetramer containing an irrelevant peptide. We have isolated BDC2.5 T cells from BDC2.5 TCR-transgenic mice with this tetramer, although not all CD4\(^+\) T cells in these animals bear the BDC2.5 TCR. The BDC2.5 T cells were stimulated with p79 in vitro and transferred into NOD/\(\text{scid}\) mice. The results showed that recipient mice that received p79-activated but not nonactivated BDC2.5 T cells developed diabetes within 10 days after adoptive transfer of the cells (Fig. 6D). Third, we have used tetAg7/p79 to isolate and characterize NOD mouse CD4\(^+\) T cells specific for p79 (23). In those studies we found that tetAg7/p79\(^+\) T cells did not inhibit diabetes development after being cotransferred with NOD splenocytes into NOD/\(\text{scid}\) mice. Altogether, these in vivo adoptive transfer experiments showed that N206\(^+\) and N221\(^+\) T cells but not Con A-activated T cells or T cells specific for an unrelated Ag could suppress diabetes development.

FIGURE 6. Adoptive transfer of tetramer\(^+\) T cells into NOD/\(\text{scid}\) mice. A, Tetramer\(^+\) T cells (5 \(\times\) 10\(^6\) cells/mouse) were transferred alone (single transfer). None of the recipient animals developed diabetes, except for the positive control mice that received NOD mouse splenocytes (Nspl; 10 \(\times\) 10\(^6\) cells/mouse). B, NOD/\(\text{scid}\) mice received NOD mouse splenocytes (10 \(\times\) 10\(^6\) cells/mouse) cotransferred with either N206\(^+\) or N221\(^+\) T cells (double transfer; 5 \(\times\) 10\(^6\) cells/mouse), or with both N206\(^+\) (5 \(\times\) 10\(^6\) cells/mouse) and N221\(^+\) T cells (5 \(\times\) 10\(^6\) cells/mouse; triple transfer). The difference in the disease progress of double-transferred mice receiving NOD spleen cells plus N206\(^+\) cells or N221\(^+\) cells was statistically significant, as determined by using the Wilcoxon test and the \(\chi^2\) test (\(p < 0.05\)). The disease progress between mice double transferred with N206\(^+\) cells and NOD splenocytes and triple-transferred mice was not significantly different from each other. C, NOD/\(\text{scid}\) mice received NOD mouse splenocytes (10 \(\times\) 10\(^6\) cells/mouse) that were preactivated with Con A for 24 h. Those without Con A activation were used as the controls. D, NOD/\(\text{scid}\) mice received BDC2.5 T cells (10 \(\times\) 10\(^6\) cells/mouse) with or without p79 peptide activation. The BDC2.5 cells were isolated from BDC2.5 TCR-transgenic mice. We have determined the phenotype of the CD4\(^+\) T cells of Con A-stimulated splenocytes and BDC2.5 cells stimulated with peptide using Abs against several different surface Ags. Briefly, \(>92\%\) of the Con A-stimulated CD4\(^+\) cells expressed CD5, CD25, CD44, CD45RB, CD62 ligand (L), and CD69, and some of these cells also expressed CD40 (\(\sim 37\%\)) and CD40L (\(\sim 67\%\)). Similarly, \(>92\%\) of the peptide-stimulated BDC2.5 T cells expressed CD5, CD25, CD44, and CD69, and a majority of cells also expressed CD40 (\(\sim 77\%\)), CD45RB (\(\sim 83\%\)), and CD62L (\(\sim 67\%\)). But only a small percentage of cells expressed CD40L. At least 10 animals were used in each transfer experiment.
In vivo regulatory function of N206+ and N221+ cells

To determine the potential in vivo mechanisms of how the tetramer+ T cells may inhibit diabetes development in adoptive transfer experiments, we determined whether the tetramer+ T cells could block the migration of the diabetogenic BDC2.5 T cells into lymph nodes after they were cotransferred into recipient animals. We did the adoptive transfer experiments in two different ways. First, we mixed and cotransferred the BDC2.5-transgenic mouse spleen cells with equal numbers of tetramer+ T cells into NOD/scid mice. The presence of BDC2.5 T cells in the lymph nodes of recipient mice was monitored using the tetAg7/p79 tetramer. Our results demonstrated that, compared with that of mice receiving just the BDC2.5 splenocytes, the number of BDC2.5 cells (CD4+ tetAg7/p79+ T cells) present in the lymph nodes of recipient mice was significantly reduced at 4 and 24 h after the transfer (Fig. 7A). The number of BDC2.5 T cells in the lymph nodes was reduced 3-fold at both 4 and 24 h when they were cotransferred with the N206+ cells, and was reduced 8- and 4-fold at 4 and 24 h, respectively, when they were cotransferred with N221+ cells. Second, the tetramer+ T cells were preactivated by PMA/ionomycin and then incubated with BDC2.5 cells for 2 h at 37°C to find out whether incubation of BDC cells with activated tetramer+ cells may have a better inhibitory effect than nonactivated cells. The results showed that, like the nonactivated cells, activated tetramer+ cells also blocked the migration of BDC2.5 T cells into lymph nodes, except that the activated cells may result in a better effect at significantly reducing the number of BDC2.5 cells in lymph nodes at 24 h (10- and 12-fold for N206+ cells and N221+ cells, respectively; Fig. 7B). Therefore, these results show that the tetramer+ T cells could block the migration of the diabetogenic BDC2.5 cells into lymph nodes. The results are also consistent with our data that there was essentially no insulitis detected in the protected mice.

Furthermore, by tracking the localization of the transferred tetramer+ cells, we found that the tetramer+ T cells could migrate into the lymph nodes and be detected at 4 and 24 h after the transfer (Fig. 7C). The results showed that a significant number of both N206+ and N221+ cells were present in the lymph nodes at 4 h and that the number of tetramer+ cells increased at 24 h. Additionally, more N206+ cells than N221+ cells were present in the lymph nodes at 4 h, whereas more N221+ cells than N206+ cells were present in the nodes at 24 h. It is not known why these cells showed a different migration rate into lymph nodes and whether such a difference in migration and/or accumulation in lymph nodes may contribute to the difference in their effect on diabetes development. Altogether, these in vivo studies demonstrated that, in addition to suppressing the proliferation of other T cells as shown in the in vitro assays (see Figs. 8 and 9), inhibition of diabetogenic T cell migration could also be a mechanism of these tetramer+ regulatory T cells to inhibit diabetes development in recipient animals.

In vitro regulatory function of N206+ and N221+ cells

In addition to the in vivo adoptive transfer experiments, we further studied the in vitro regulatory function of N206+ and N221+ cells and determined whether they could suppress the proliferation of other T cells. First, N206+ or N221+ cells were cocultured with CFSE-labeled BDC2.5 T cells with or without p79, a peptide that is highly active in stimulating BDC2.5 cells (27). After stimulated by p79 for 4 days, BDC2.5 cells could proliferate ≥3 rounds of cell division as shown by the decrease of CFSE intensity (Fig. 8). However, BDC2.5 cell proliferation was suppressed in the presence of N206+ or N221+ cells and, therefore, more BDC2.5 cells still expressed higher intensity of CFSE and divided <3 rounds. Coculture of BDC2.5 cells with equal numbers of N206+ (Fig. 8A) or N221+ (Fig. 8B) cells activated with their antigenic peptides increased the percentage of cells that underwent <3 rounds of cell division from ~14 to 23% in the absence of tetramer+ cells to ~80 or ~70% in the presence of N206+ or N221+ cells, respectively. It is worth noting that, although most of the BDC2.5 cells have not divided when they were cocultured with tetramer+ cells, the total number of BDC2.5 cells are much lower than the number of cells present in the other cell cultures analyzed (Fig. 8). Therefore, the results suggest that the N206+ and N221+ cells may have in some way decreased the total number of BDC2.5 cells in the culture, perhaps by cytotoxicity due to the release of cytokines.

We then wanted to know whether cell-cell contact between tetramer+ cells and target cells was necessary for their regulatory functions. We addressed this question using Transwell assays that tetramer+ T cells were cultured in a Transwell (upper well or UW) and separated from the target NOD splenocyte cultured outside of the Transwell in the lower well (LW). Both tetramer+ cells and NOD splenocytes were stimulated with PMA/ionomycin. The results showed that the proliferation of NOD splenocytes (5 × 105...
cells/well) in the lower wells could be suppressed by as little as 2% of activated tetramer<sup>+</sup> T cells (1 × 10<sup>4</sup> cells/well) present in the Transwell (Fig. 9, A and B). The suppression effect was proportional to the number of activated tetramer<sup>+</sup> cells present in the Transwell because splenocyte proliferation was further inhibited in the presence of more activated tetramer<sup>+</sup> T cells. Therefore, the percent inhibition of splenocyte proliferation increased from an average of 22.5% in the presence of 2% N206<sup>+</sup> cells to 74.5% in the presence of 50% N206<sup>+</sup> cells (Table I). Additionally, the percent inhibition of NOD mouse T cell proliferation increased from an average of 22.5% in the presence of 2% N221<sup>+</sup> cells to 70% in the presence of 50% N221<sup>+</sup> cells (Table I). In summary, both N206<sup>+</sup> and N221<sup>+</sup> cells were able to suppress the proliferation of other NOD T cells, including diabetogenic T cells. Furthermore, they could perform their regulatory function without the need to be in contact with the target cells.

**Blocking the regulatory function of N206<sup>+</sup> and N221<sup>+</sup> cells**

It has been shown that IL-4 and IL-10 can regulate autoimmune response (11, 37–40). Because both N206<sup>+</sup> and N221<sup>+</sup> T cells produced IL-4, IL-10, and IFN-γ, it is likely that these cells performed their regulatory function by secreting these cytokines. To find out whether this is true, we repeated the Transwell assays with the addition of saturating amounts of Abs against IL-4, IL-10, and IFN-γ in the cell culture. We included anti-IFN-γ Ab in these assays because the tetramer<sup>+</sup> T cells, especially the N221<sup>+</sup> cell, contained Tr1 cells that could produce a large amount of both IL-10 and IFN-γ. Additionally, it has not been shown before that autoantigen-specific Tr1 cells may play a role in diabetes development. Studies on N206<sup>+</sup> cells showed that anti-IL-10, and, to a lesser extent, anti-IFN-γ, but not anti-IL-4 Abs, could partially restore the proliferation of the NOD spleen target cells (Fig. 9C). Compared with that of cells cultured with N206<sup>+</sup> cells in the absence of the Abs, the proliferation of target cells increased 1.7- and 1.3-fold in the presence of anti-IL-10 and anti-IFN-γ Abs, respectively (Fig. 9C and Table II). The use of a combination of two Abs against different cytokines did not result in a synergistic effect that further increased the target cell proliferation. Therefore, N206<sup>+</sup> cells may perform their regulatory function at least partly by secreting IL-10 and perhaps also IFN-γ. It is possible that another unknown factor(s) also plays a role for N206<sup>+</sup> cell to perform their regulatory functions.

On the other hand, all three Abs could partially increase the target cell proliferation suppressed by N221<sup>+</sup> cells (Fig. 9D and Table II). Interestingly, anti-IFN-γ Ab had the best effect and resulted in a 1.7-fold increase of target cell proliferation compared with that of cells cultured with N221<sup>+</sup> cells without the Ab. The presence of Abs against IL-4 and IL-10 had the same effect as that with just the anti-IL-10 Ab (Table II). However, surprisingly, the presence of both anti-IL-10 and anti-IFN-γ Abs resulted in a 2.6-fold increase of the target cell proliferation. Therefore, the presence of these two Abs not only fully restored but also enhanced the target cell proliferation by ~30% compared with that of cells cultured in the absence of N221<sup>+</sup> cells and Abs (Fig. 9D). In summary, it appears that a majority of N221<sup>+</sup> cells and some N206<sup>+</sup> cells could function not only as Th2 cells but also as Tr1 regulatory T cells by secreting both IL-10 and IFN-γ.

**Expression of CTLA-4 by a subpopulation of tetramer<sup>+</sup> T cells**

It has been shown that T cells expressing increased levels of CTLA-4 could regulate the function of other T cells (41–43). Expression of higher levels of CTLA-4 has also been implicated as a potential mechanism that GAD peptide-specific T cells could perform their regulatory functions (44). Therefore, although N206<sup>+</sup> and N221<sup>+</sup> cells could exert their regulatory functions through cytokine-mediated suppression rather than cell-cell contact, we also determined whether a subpopulation of the tetramer<sup>+</sup> regulatory T cells expressed CTLA-4. We stained the N206<sup>+</sup> and N221<sup>+</sup> T cells with an anti-CTLA-4 Ab. The results showed that ~18.2% of N206<sup>+</sup> cells and ~17.1% N221<sup>+</sup> cells expressed CTLA-4 on the cell surface (Fig. 10). Therefore, a subpopulation of these tetramer<sup>+</sup> T cells did express CTLA-4.

**Discussion**

These studies show that treatment of NOD mice with autoantigenic peptides can induce Ag-specific CD4<sup>+</sup> regulatory T cells with cytokine secretion profiles that varied depending on the Ag specificity. Both N206<sup>+</sup> and N221<sup>+</sup> T cells were able to inhibit IDDM, which could be because these cells contained IL-4/IL-10 coproducing Th2 cells and IFN-γ/IL-10 coproducing Tr1 cells (15, 16,
However, N221 cells contained 10-fold more Th1 cells, 40-fold more Tr1 cells, and nearly 2-fold less single IL-10-producing cells than did N206 cells. It has been shown in previous studies that Tr1 cells could inhibit not only Th1 but also Th2 cell responses (45). Therefore, IFN-γ/IL-10 coproducing N221 cells may inhibit both pathogenic Th1 cells and other T cells such as Th2 cells during IDDM development. The presence of Tr1 cells could also be one of the reasons why blocking both IFN-γ and anti-IL-10 Abs was statistically significant with p < 0.01 as determined by Student’s t test. The results were obtained from at least three different experiments. Nspl, NOD mouse splenocytes.

Our results also showed that the cytokine secretion profile of the T cells from similarly treated BALB/c mice were different from that of NOD mouse tetramer + T cells. Further studies to determine the cytokine secretion profiles of T cells before being restimulated and expanded in vitro showed that both p206-specific and p221-specific T cells isolated from GAD-immunized NOD mice, like the N206 + and N221 + cells, were biased toward IL-4-producing and IFN-γ-producing cells, respectively. On the other hand, we could not detect a significant cytokine secretion response by T cells derived from GAD-immunized BALB/c mice, suggesting that the frequency of such T cells was too low to be detected without further restimulation with the peptides in vitro. These studies suggest that the function of the tetramer + T cells from NOD mice was...
probably polarized in vivo rather than in vitro during cell culture because the cells were cultured under the same conditions. Furthermore, N206+ and N221+ cells can also respond to the GAD protein presented by irradiated NOD splenic APCs, suggesting that these T cells are similar to those that develop spontaneously in NOD mice. Therefore, it seems that the properties of the tetramer+ Tr cells in vivo are similar to those observed in the in vitro analyses and that the atypical cytokine secretion profile of autoreactive T cells such as N221+ and N206+ cells is likely intrinsic to NOD mice. The reason for the differences seen in these animals is not clear but it may be due to the difference in I-Ad vs I-Ag7 molecules that could result in the selection of T cells with distinct functions. One might have predicted that BALB/c mouse T cells were more likely to become Th2 cells due to their genetic backgrounds (46, 47). However, in addition to the difference in producing IFN-γ/IL-4, the B206+/B221+ cells secreted more IL-2 but less IL-4/IL-10 than did N206+/N221+ cells. It is likely that these differences are due to the unique features of I-Ag7 structure and its property in binding and presenting peptides or due to the nature of the autoantigen-MHC complex (48–50). Although the weaker IL-2 response may be interpreted as an indication that N206+/N221+ cells bore TCRs of lower affinity than B206+/B221+ cells for their ligands, it is not clear whether the same rationale can be applied to explain the differences in IL-4/IL-10 production. In addition, the decreased IL-2 secretion response of N206+ and N221+ cells is consistent with the notion that they contain Tr1 cells. Alternatively, the difference could be due to altered APCs in NOD mice (51, 52).

An interesting finding in this report is that treatment of NOD mice using the GAD peptides would induce not only Th2 but also Tr1 regulatory T cells. The tetramer+ regulatory T cells not only could inhibit diabetes but could also suppress the proliferation of other T cells in vitro by secreting different cytokines, and their cell-cell contact with target cells was not required. Whereas one may expect that anti-IL-4 and anti-IL-10 Abs can inhibit the regulatory function of the tetramer+ cells, it is not clear why anti-IFN-γ can also do so. Additionally, it is not clear why blocking both IFN-γ and IL-10 not only restore but even enhance the NOD spleen cell proliferation suppressed by N221+ cells. Previous studies on NOD mice deficient in IFN-γ, IL-10, or IL-4 expression showed that elimination of any one of these cytokines did not significantly alter the rate of IDDM development (53, 54). However, it is not known whether NOD mice deficient in, e.g., both IFN-γ and IL-10 expression, will have altered IDDM development. Interestingly, inhibition of IDDM development in NOD mice by the nonspecific immunostimulatory agents CFA or bacillus Calmette-Guérin vaccine treatment was dependent on the presence of IFN-γ (54). Therefore, it is likely that IFN-γ can be involved in the regulation of IDDM in NOD mice. Our studies to determine the potential in vivo mechanisms underlying the inhibition of diabetes development by the tetramer+ T cells showed that these regulatory T cells could block the migration of the diabetogenic BDC2.5 T cells into lymph nodes of recipient mice. This blocking effect may be reminiscent of the findings described in a recent report that low-dose IFN-γ exerts anti-inflammatory properties, such as suppression of T cell trafficking (55). Therefore, incubation of T cells in vitro with a low concentration of IFN-γ blocked the migration of treated T cells into lymph nodes after they were adoptively transferred into recipient mice. In our studies, we have shown that both N206+ and N221+ regulatory T cells secreted IFN-γ. However, considering the amount of IFN-γ produced by these tetramer+ Tr cells, it seems that this level of IFN-γ is relatively low when compared with the higher concentration of IFN-γ (~100 ng/ml) normally secreted by Th1 cells. Therefore, the tetramer+ regulatory T cells may block the migration of BDC2.5 T cells because they secrete low concentrations of IFN-γ. Further studies should help determine whether these and other mechanisms are responsible for the blockade of BDC cell migration by the tetramer+ cells. In summary, these results suggest that, in addition to suppressing T cell proliferation as shown in the in vitro assays, inhibition of diabetogenic T cell migration into lymph nodes may be one of the in vivo regulatory mechanisms that the tetramer+ cells could inhibit diabetes development in recipient mice. These results are also consistent with our observations that essentially no insulin was detected in the protected mice.

Our results show that both N221+ and N206+ cells contain Tr1 cells and can secrete not only IFN-γ but also IL-10. Considering the suppressive effect of IL-10 (11), it is likely that secretion of both IL-10 and IFN-γ may be required for the proper regulatory function of Tr1 cells, and blocking of both cytokines could abolish the regulatory function of these cells. It has not been shown before that autoantigen-specific Tr1 regulatory cells can inhibit autoimmune diabetes development. Our results demonstrate that the GAD peptide-specific Tr1 cells can be induced in NOD mice and they may also play a role in regulating the development of diabetes. Because Tr1 cells could inhibit not only Th1 but also Th2 cell responses, further studies are necessary to determine the relative roles of IL-4/IL-10-coproducing Th2 cells vs IFN-γ/IL-10-coproducing Tr1 cells in regulating diabetes development in NOD mice (11, 14, 54, 56).

Table II. Effect of anti-cytokine Abs on the proliferation of NOD spleen cells suppressed by N206+ or N221+ cells in Transwell assays

<table>
<thead>
<tr>
<th>Abs Present in Cell Culture</th>
<th>Fold Increaseα</th>
<th>No Ab</th>
<th>Anti-IL-4</th>
<th>Anti-IL-10</th>
<th>Anti-IFN-γ</th>
<th>Anti-IL-4/Anti-IL-10</th>
<th>Anti-IL-10/Anti-IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>N206+ cells</td>
<td>1</td>
<td>0.9</td>
<td>1.7</td>
<td>1.3</td>
<td>1.5</td>
<td>13.5</td>
<td>13.5</td>
</tr>
<tr>
<td>N221+ cells</td>
<td>1</td>
<td>1.3</td>
<td>1.5</td>
<td>1.7</td>
<td>1.5</td>
<td>2.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

α Fold-Increase = (% from cell culture with Ab treatment)/(% from cell culture without Ab treatment). % = (cpm of NOD cells in LW with tetramer+ cells)/(cpm of NOD cells in LW without of tetramer+ cells) × 100%.

FIGURE 10. Expression of CTLA-4 on tetramer+ T cells. The N206+ and N221+ cells were stained with Abs against CTLA-4 and CD4. The numbers shown in each quadrant represent the percentage of cells stained positively by the anti-CTLA-4 Ab.
Several different populations of CD4+ T cells have been shown to regulate autoimmune diseases. These cells may be identified by their surface phenotype, e.g., CD25+, DX5+, or CD45RBlow cells, or by unique cytokine profiles, e.g., Th2, Th3, and Tr1 cells (35, 37–40, 57). Although the Ag specificity of these cells is largely uncharacterized, peripheral tissue-specific autotransplants could be responsible for activating these cells for their function in vivo (58). The tetramer regulatory T cells identified in the current studies were specific for different GAD peptides and expressed CD25 and low levels of CD45RB (data not shown). Interestingly, a subpopulation of the N206+ and N221+ T cells also expressed CTLA-4 on the cell surface, similar to the activated T cells specific for GAD p286 peptide in transgenic mice (44). It has been shown that increased expression of CTLA-4 by T cells, such as the CD4+CD25+ Tr cells, may regulate the function of other pathogenic T cells through TGF-β production (41, 42). However, the role of CTLA-4 expression and TGF-β production by CD4+CD25+ Tr cells in regulating the functions of other T cells has been controversial (43, 59). It is likely that the CTLA-4+ tetramer cells may exert regulatory functions through CTLA-4. However, our results showed that the tetramer T cells barely produced TGF-β. Therefore, it seems that, if CTLA-4 plays a role, secretion of TGF-β is not a major mechanism by which the tetramer T cells exert their regulatory functions. Further studies to isolate and characterize the CTLA-4 tetramer T cells should allow us to determine their role in diabetes development. In addition, the N206+ and N221+ cells seem to be different from the CD4+, CD25+, or CD45RBlow T cells. Although the tetramer cells expressed CD25, it may simply reflect their activation status. Furthermore, cell-cell contact with target cells was necessary for the regulatory function of CD4+CD25+ cells but not for the tetramer cell. Based on the cytokine secretion profiles, it is likely that N206+ and N221+ T cells are different from the other autotransplant-specific T cells described previously (14, 15, 44). In those studies, the autoreactive T cells may be Th2 cells or they may secrete a distinct profile of cytokines. Based on the current studies and those of others, it appears that T cells specific for different GAD peptides can differ in their capacity in the induction of varied populations of T cells with different effects on diabetes development. These studies also demonstrate the potential feasibility of inducing Ag-specific regulatory T cells with a diabetes-related autotransplant to prevent autoimmune diabetes.

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