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Presence of Diabetes-Inhibiting, Glutamic Acid Decarboxylase-Specific, IL-10-Dependent, Regulatory T Cells in Naive Nonobese Diabetic Mice

Sylvaine You,* Cyndi Chen,* Wen-Hui Lee,* Todd Brusko,† Mark Atkinson,† and Chih-Pin Liu2*

Immunization of NOD mice with autoantigens such as glutamic acid decarboxylase (GAD) 221–235 peptide (p221) can induce Ag-specific CD4+ T regulatory (Tr) cells. However, it is unclear whether these Tr cells acquire their regulatory capacity due to immunization or whether they are constitutively harbored in unimmunized naive mice. To address this question, we used an I-Ag7 tetramer to isolate p221-specific T cells from naive NOD mice (N221+ cells) after peptide-specific in vitro expansion. The N221+ T cells produced IFN-γ and IL-10, but very little IL-4, in response to p221 stimulation. These T cells could function as regulatory cells and inhibit in vitro proliferation of diabeticogenic BDC2.5 cells. This suppressive activity was cell contact-independent and was abrogated by Abs to IL-10 or IL-10R. Interestingly, IL-2 produced by other T cells present in the cell culture induced unactivated N221+ T cells to exhibit regulatory activities involving production of IL-10. In vivo, N221+ cells inhibited diabetes development when cotransferred with NOD splenocytes into NOD/Scid recipients. Together, these results demonstrate that p221-specific IL-10-dependent Tr cells, including Tr type 1 cells, are present in naive NOD mice. The use of spontaneously arising populations of GAD peptide-specific Tr cells may represent a promising immunotherapeutic approach for preventing type 1 diabetes. The Journal of Immunology, 2004, 173: 6777–6785.

Taken collectively, these findings suggest that the modulation of GAD autoimmunity can influence the development of type 1 diabetes in NOD mice.

Indeed, treatment of NOD mice with β cell autoantigens can prevent the progression toward overt disease. Immunization of prediabetic NOD mice via intrathymic, oral, or nasal administration of GAD protein or peptides can protect them from developing type 1 diabetes (10, 16–20). Treatment with recombinant vaccinia virus expressing GAD can also prevent type 1 diabetes in NOD mice (21). The disease protection observed in these studies is associated with the induction of Ag-specific T regulatory (Tr) cells, which may elicit immune tolerance by suppressing the diabeticogenic activity of T cells and other β cell-specific autoimmune responses (16–21).

Despite these studies, the role of GAD in the actual pathogenesis of type 1 diabetes in NOD mice remains controversial. Only one diabeticogenic, GAD-specific clone has been identified to date, and diabetes develops normally in GAD65 knockout NOD mice (22, 23). Additionally, a recent report indicated that transgenic NOD mice tolerant to GAD were not protected from type 1 diabetes and exhibited normal incidence and kinetics of insulin-dependent diabetes mellitus onset (24). Finally, CD4+ T cells specific for different epitopes of GAD65 are not pathogenic, but, rather, can inhibit diabetes development (18–20, 25). Therefore, the roles of GAD and its peptides in the development of T cell-mediated β cell autoimmunity remain to be determined.

Previous studies have identified several I-Ag7-binding, GAD-immunodominant epitopes (26). To date, we have focused our studies on the GAD221–235 peptide (p221), one of the most immunogenic GAD determinants. Our previous studies have shown that immunization of NOD mice with p221 led to the generation of p221-specific Tr cells that could prevent diabetes development (20). However, those studies left unclear whether p221-specific Tr cells arise after immunization with p221 or whether such cells
develop spontaneously in NOD mice. Attempts to address this question have been hampered by the fact that autoantigen-specific T cells are present at a very low frequency in unimmunized naive NOD mice (20). To avoid this problem, we used a novel approach involving class II MHC I-Ag7 tetramers specific for p221 to identify and isolate a large number of p221-specific T cells from unimmunized naive NOD mice. These cells, like T cells isolated from p221-immunized NOD mice, exhibit regulatory activities and can inhibit diabetes development.

Materials and Methods

Mice

NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BDC2.5 transgenic mice were a gift from Drs. D. Mathis and C. Benoist (Harvard University, Boston, MA). In our colony, >75% of the female NOD mice develop type 1 diabetes by 6 mo. All animals used were 7–8 wk old and were housed in a specific pathogen-free environment in the animal facility at Beckman Research Institute, City of Hope (Duarte, CA). The naive mice refer to young prediabetic NOD mice that have not been manipulated or treated with i.p., i.v., i.m., s.c., oral, or nasal administration of peptides or adjuvants.

Peptides and class II MHC tetramers

The GAD221–235 (p221), GAD206–220 (p206), and p79 (one of the most active peptide analogs that can stimulate BDC2.5 cells; previously described as 1040–79 (27)) peptides were synthesized at Beckman Research Institute, City of Hope, and brought to >90% purity using reverse phase HPLC. Production of class II MHC tetramer has been previously described (28).

CFSE labeling and isolation of p221-specific T cells

NOD mouse splenic CD4+ T cells were purified using magnetic beads (Miltenyi Biotec, Auburn, CA) and labeled with CFSE. After washing, the cells were cultured with p221 (50 μg/ml) or a control p206 peptide (50 μg/ml) plus irradiated NOD mouse CD4+ T cell-depleted APCs for 3 days in RPMI 1640 medium plus 10% FCS (HyClone, Logan, UT). Live cells were also incubated in the medium supplemented with IL-2 for 2 days. Then the cells were stained with tetAg7/p221 and anti-CD4 Ab to evaluate the presence of p221-specific T cells in the culture. The CD4+ tetAg7/p221+ T cells were isolated using FACS and magnetic beads. The cell yield of N221+ T cells after sorting was ~0.5–1% of the CD4+ T cells initially used. Isolated p221-specific T cells were maintained in IL-2-supplemented medium and are named N221+ T cells in this study. Resting N221+ T cells were used for in vivo and in vitro experiments. For immediate ex vivo staining experiments, there were ~1×106 CD4+ T cell events gated and analyzed using FACS. Cells were stained with tetrablotors (20 μg/ml) for 3 h at 37°C in RPMI 1640 medium, and anti-CD4 Ab was added during the last 30 min of incubation before the cells were analyzed by FACS. Dead cells were carefully excluded by containing with 7-aunaomycinomycin D, and the analysis was performed on gated live CD4+ T cells. In these assays, the tetAg7/p221 did not detect a significant population of these cells compared with cells obtained from ABH mice and BDC2.5 mice, which were used as negative controls. Similar results were obtained using T cells recovered from 6-, 8-, and 12-wk-old or diabetic NOD mice and from islet-infiltrating lymphocytes.

Flow cytometry

T cells were stained with PE-labeled tetrablotors (tetAg7/p221, tetAg7/p206, or tetAg7/p79) plus the unconjugated anti-TCR CB3 H57 Ab at 37°C for 3 h. Anti-CD4, anti-CD5, anti-CD25, anti-CD40, anti-CD44, anti-CD152, anti-CD69, or anti-isotype controls (BD Pharmingen, San Diego, CA) were added during the last 30 min of incubation. Cells were washed and analyzed by FACS using FACSCalibur (BD Biosciences, San Jose, CA).

Cytokine assays

The IL-2 production bioassay has been previously described (28). For ELISA, cell culture supernatant was harvested after incubating N221+ T cells with peptides and irradiated APCs for 24 h. Mouse IFN-γ, IL-4, and IL-10 OPELIA ELISA kits (BD Pharmingen) were used to measure the quantity of secreted cytokines according to the manufacturer’s instructions.

For intracellular cytokine staining, N221+ T cells were incubated with PMA, ionomycin, and monensin; stained with Abs for cell surface staining; 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 isotype controls.

Tetramer binding kinetics

The association and dissociation kinetics measured with the tetrablotors were determined using a previously described method (29), with minor modifications. For analysis of tetrablotor staining at equilibrium, N221 T cells were incubated for 3 h at room temperature with increasing concentrations of tetAg7/p221 (from 0–560 nM) and with anti-CD4 Ab (BD Pharmingen) in RPMI 1640 medium. Apparent Kd values were derived from the negative reciprocal of the slope of the regression line fit to Scatchard plots of bound tetrablotor/free tetrablotor (fluorescence units per nanomolar concentration of tetrablotor) vs bound tetrablotor (fluorescence units). The median tetrablotor staining intensity was used as the measurement for the bound tetrablotor. Because the tetrablotor was in vast excess even at the lowest concentration, the concentration of free tetrablotor simply reflects the staining concentration.

For the analysis of tetrablotor staining decay, the t1/2 of tetrablotor binding to cells was measured. N221+ T cells were stained for 1 h at room temperature with a suboptimal concentration of tetAg7/p221 plus anti-CD4 Ab. Cells were then washed and incubated with saturating amounts of blocking anti-I-Ag7 Ab used at 30 μg/ml (American Type Culture Collection, Manassas, VA). Aliquots were taken at appropriate time points and analyzed using FACS. The binding t1/2 is equal to ln2 divided by the slope of the natural logarithm (ln) of normalized fluorescence plotted vs time. The normalized fluorescence was determined by calculating the percentage of the total fluorescence (sum of the fluorescence intensity of tetrablotor CD4+ T cells normalized per CD4+ cell at each data point) with respect to the initial time point. In other words, the slope is equivalent to ln(Fa/Fb)/t, where Fa is the normalized fluorescence at the start point of the interval, Fb is the normalized fluorescence at the end point of the interval, and t is the length of the interval (minutes).

In vitro inhibition assays

CD4+ T cells obtained from BDC2.5 transgenic NOD mice (target cells) were labeled with CFSE and incubated with irradiated APCs and p79 (1 μg/ml), one of the most active peptide analog that can stimulate BDC2.5 cells (27). Increasing numbers of N221+ T cells were added in the presence or the absence of p221 (10 μg/ml). After 4 days at 37°C, cells were stained with anti-CD4 and class II MHC I-Ag7 tetrablotor specific for p79 (tetAg7/p79). BDC2.5 cell proliferation was monitored by analyzing CFSE intensity within the CD4+ tetAg7/p79+ T cell population.

Adoptive transfer experiments and histology

Seven- to 8-wk-old female NOD/scid mice received a single i.v. injection of 1×105 cells of N221+ T cells, N221+ T cells plus NOD splenocytes, or NOD splenocytes alone. Recipient mice were considered diabetic after 2 consecutive wk of glycosuria >2% and blood glucose levels >250 mg/dl. After diabetes onset or after 39 wk of age, mice were killed, and their pancreases were removed for histology. Pancreatic tissues were fixed in 10% formalin/PBS for 24 h, followed by 70–100% ethanol and xylene, then paraffin-embedded and sectioned. Sections were stained with H&E to detect islet-infiltrating lymphocytes.

Statistical analysis

The development of diabetes was plotted using the Kaplan-Meier method showing a nonparametric cumulative survival plot. Statistical comparisons between curves was performed using the log-rank (Mandel-Cox) test. Student’s t test was also used for group comparisons.
Results

Purification and characterization of p221-specific T cells from naive NOD mice

We generated a p221-specific class II MHC tetramer (tetAg7/p221) to isolate p221-specific CD4+ T cells from unimmunized naive NOD mice. Our initial experiments demonstrated that tetAg7/p221 did not detect a significant population of p221-specific CD4+ cells in NOD mouse spleen compared with that in control ABH mice and NOD mice expressing the BDC2.5 TCR transgene (data not shown), confirming data presented in our previous report (28). Furthermore, there was no significant population of p221-specific T cells detected in islet-infiltrating lymphocytes or in spleen cells isolated from 6-, 8-, or 12-wk-old or diabetic NOD mice (data not shown).

Therefore, we performed additional experiments to determine whether p221-specific T cells were present in unimmunized naive NOD mice. We first labeled purified naive NOD mouse splenic CD4+ T cells with CFSE, then stimulated them with p221 plus irradiated CD4+ T cell-depleted APCs. It was expected that if p221-specific CD4+ T cells were indeed present in naive NOD mice, these cells would respond to p221 stimulation and proliferate faster than the other non-p221-specific T cells. Ten days after stimulation of the cells with p221, we stained the cells with tetAg7/p221 plus an anti-CD4 Ab and analyzed the cell proliferation by FACS based on the CFSE intensity of labeled cells. The results showed that ~14% of CD4+ T cells that underwent more than five rounds of cell division in response to p221 stimulation were stained positively by tetAg7/p221 (Fig. 1A, R2). In comparison, the tetramer failed to detect a significant population of p221-specific T cells in the nondivided cells compared with that of control cells incubated with a control p206 peptide (Fig. 1A, R1). Additionally, the percentage of tetAg7/p221+ T cells increased in additionally divided cells compared with that of less divided cells in the presence of p221 (data not shown). In our control experiments we incubated CFSE-labeled cells with p206 and stained the cells with tetAg7/p221. The results showed that tetAg7/p221 did not detect a significant population of cells (<0.1%) that had divided more than five times. Another control experiments showed that no significant population of p221-stimulated T cells was stained with tetAg7/p206 (<1.0%). Detection of T cells specific for p221 peptide after p221-specific in vitro stimulation demonstrated that these cells were present in unimmunized naive NOD mice, although their frequency was too low to be detected using tetramer without additional in vitro stimulation.

To obtain sufficient number of p221-specific T cells for further in vitro and in vivo experiments, we performed experiments to expand p221-specific T cells in vitro and isolated these cells using tetAg7/p206 (the N221+ cells). The purity of isolated N221+ cells was ~99% (Fig. 1B). The tetAg7/p221 tetramer did not stain CD4+ T cells specific for an irrelevant peptide, p79, isolated using tetAg7/p79 (tetAg7/p79+ cells; Fig. 1B) (30). Additionally, these N221+ cells were not stained positively by another control tetramer, tetAg7/p206, specific for a control p206 peptide. Additional studies showed that N221+ cells secreted IL-2 in response to p221, but not in response to the control p206 peptide, indicating that these cells were specific for p221, but not for other irrelevant peptides (Fig. 2A). N221+ cells also proliferated in response to p221, but not to control insulin B9-23 peptide (data not shown).

Phenotypic studies showed that >90% of N221+ T cells expressed high levels of CD5 (~99%), CD25 (~92%), and CD44 (~100%), indicating the activation status of these cells (Fig. 1C). A significant population of these cells also expressed higher levels of CD69 (33%). We also determined whether these cells express cell surface markers implicated in pathogenic or regulatory processes. Previous studies have reported that CD40 may be expressed on diabetogenic T cells, and that CD62L may be expressed on regulatory T cells (31, 32). Our results showed that N221+ cells did not express CD40, CD40L, or CD62L, but they expressed a low level of CD45RB. In addition to these phenotypic studies, we determined TCR Vβ-chain usage, and the results showed that ~95% of N221+ T cells bore the Vβ chain (data not shown).

We determined the cytokine secretion profile of N221+ T cells using ELISA after peptide-specific activation (Fig. 2A). Our results demonstrated that they produced large amounts of IL-10 and IFN-γ, very little IL-4, and little IL-10 and IFN-γ, very little IL-4, and little IL-12 and IL-10, both p221-specific T cell lines secreted MIP-1α and GM-CSF (>10,000 pg/ml) and a lower level of RANTES (~500–1,000 pg/ml), TNF-α (~1,500–2,500 pg/ml), and IL-6 (~400 pg/ml; data not shown). N221+ T cells from naive NOD mice also secreted small quantities of IL-5, IL-12, and IL-13 (~150 pg/ml). These results demonstrate that p221-specific T cells isolated from either naive or p221-immunized NOD mice had similar patterns of cytokine secretion. Additional intracellular cytokine staining analyses showed that ~30% of the cells produced...
respectively (Fig. 3). These results suggest that N221/H11001 and N221/H9253 T cells bear CD4+ BDC2.5 T cells were stimulated with p79 and incubated with increasing amounts of N221+ T cells in the presence or the absence of p221. In the absence of N221+ cells, BDC2.5 cells proliferated well in response to p79, but not to p221 (Fig. 4A). Only ~8% of them underwent fewer than two cell divisions after p79 stimulation compared with ~93% of the cells that did not divide in response to p221 stimulation (Fig. 4A). Interestingly, coculture of BDC2.5 cells with N221+ T cells significantly increased the percentage of nondividing or less frequently dividing BDC2.5 T cells in response to p79 (Fig. 4B and Table I). This inhibitory effect was proportional to increasing numbers of N221+ cells in the cell culture. The percentage of nondividing CD4+ BDC2.5 T cells increased from 3.7% in the absence of N221+ cells to ~43% in the presence of an equal number of p221-activated N221+ cells (1:1 ratio; Table I). Similarly, the frequency of BDC2.5 cells that underwent fewer than two cell divisions increased to ~76% in the presence of an equal number of p221-activated N221+ cells. Interestingly, without p221 stimulation, N221+ cells also inhibited BDC2.5 cell proliferation, although the inhibitory effect was significantly enhanced in the presence of p221 (Fig. 4B and Table I). Together, these results demonstrated that N221+ T cells could function as Tr cells in vitro by inhibiting the proliferation of other T cells.

To further investigate the regulatory function of these N221+ cells, we performed Transwell assays. In these assays we measured the proliferation of BDC2.5 cells using two different methods, labeling the cells with CFSE (Fig. 5A) or incubating the cells with [3H]thymidine for the last 16–18 h during a 4-day assay (Fig. 5B). The study of CFSE-labeled BDC2.5 cells showed that although N221+ T cells were physically separated from the target BDC2.5 cells, they were still able to efficiently inhibit BDC2.5 cell proliferation (Fig. 5A). The percentage of BDC2.5 cells that underwent fewer than two rounds of cell division increased from 8% in the absence of N221+ cells to 33% in the presence of an equal number of N221+ cells in the Transwell. The inhibitory effect was comparable to that observed without using a Transwell-based assay. Additional results obtained from studying [3H]thymidine-labeled BDC2.5 cells demonstrated that the presence of an equal number of N221+ T cells in Transwells could result in a 60% inhibition of BDC2.5 cell proliferation in response to p79 (Fig. 5B). Additional similar experiments using Transwells showed that N221+ cells could inhibit the proliferation of target NOD mouse splenic T cells stimulated by PMA/ionomycin (data not shown). These studies suggest that the regulatory function of N221+ T cells is not cell-specific.

In vitro regulatory function of N221+ T cells

We then performed in vitro studies to determine whether these N221+ T cells can indeed function as Tr cells. CFSE-labeled BDC2.5 T cells were stimulated with p79 and incubated with increasing amounts of N221+ T cells from naive NOD mice could produce IFN-γ, the majority of them produced only IL-10. Based on their cytokine secretion profiles, it appears that these N221+ T cells were neither Th1 nor Th2 cells. They may have contained two populations of Tr cells. Some of them were more likely to be the Tr1 cells producing a large amount of IL-10 and a smaller amount of other effector cytokines, such as IFN-γ, whereas the other N221+ Tr cells produced only IL-10 and no IFN-γ (33, 34). In addition to secreting cytokines, N221+ T cells proliferated to p221 in a dose-dependent manner in the presence of irradiated APCs (Fig. 2C). In contrast, N221+ T cells did not respond to the control insulin B9-23 peptide. We also used tetAg7/p221 to determine the TCR avidity of N221+ cell for its ligand. We measured the apparent Kd and the t1/2 for the binding of tetramer to N221+ cells. These studies found that the Kd and the t1/2 values were 333.3 nM and 30.1 min, respectively (Fig. 3). These results suggest that N221+ cells bear TCRs that have relatively low avidity for their ligand compared with T cells selected in nondiabetic animals. This observation is consistent with previous studies suggesting that TCRs of autoreactive T cells have low avidity for their MHC/peptide ligands (35, 36).

**FIGURE 2.** Analyses of cytokine production by N221+ T cells. A, N221+ cells were stimulated by increasing amounts of p221 or an irrelevant control peptide, p206, plus irradiated APCs for 24 h. Supernatants were collected for ELISA and IL-2 bioassay. B, After stimulation with PMA/ionomycin, N221+ T cells were fixed and stained intracellularly with isotype control Abs, anti-IL-10, anti-IFN-γ, or anti-IL-4 Abs. The numbers in each quadrant represent the percentage of cells. C, N221+ cells were stimulated by increasing amounts of p221 or an irrelevant control insulin B9-23 peptide plus irradiated APCs for 4 days. [3H]Thymidine was added to cell culture during the last 18–20 h.

IL-10, ~2% produced IFN-γ, and ~1% coproduced both cytokines (Fig. 2B). Although a small number of N221+ T cells from naïve NOD mice could produce IFN-γ, the majority of them produced only IL-10. Based on their cytokine secretion profiles, it appears that these N221+ T cells were neither Th1 nor Th2 cells. They may have contained two populations of Tr cells. Some of them were more likely to be the Tr1 cells producing a large amount of IL-10 and a smaller amount of other effector cytokines, such as IFN-γ, whereas the other N221+ Tr cells produced only IL-10 and no IFN-γ (33, 34). In addition to secreting cytokines, N221+ T cells proliferated to p221 in a dose-dependent manner in the presence of irradiated APCs (Fig. 2C). In contrast, N221+ T cells did not respond to the control insulin B9-23 peptide.

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To further investigate the regulatory function of these N221+ cells, we performed Transwell assays. In these assays we measured the proliferation of BDC2.5 cells using two different methods, labeling the cells with CFSE (Fig. 5A) or incubating the cells with [3H]thymidine for the last 16–18 h during a 4-day assay (Fig. 5B). The study of CFSE-labeled BDC2.5 cells showed that although N221+ T cells were physically separated from the target BDC2.5 cells, they were still able to efficiently inhibit BDC2.5 cell proliferation (Fig. 5A). The percentage of BDC2.5 cells that underwent fewer than two rounds of cell division increased from 8% in the absence of N221+ cells to 33% in the presence of an equal number of N221+ cells in the Transwell. The inhibitory effect was comparable to that observed without using a Transwell-based assay. Additional results obtained from studying [3H]thymidine-labeled BDC2.5 cells demonstrated that the presence of an equal number of N221+ T cells in Transwells could result in a 60% inhibition of BDC2.5 cell proliferation in response to p79 (Fig. 5B). Additional similar experiments using Transwells showed that N221+ cells could inhibit the proliferation of target NOD mouse splenic T cells stimulated by PMA/ionomycin (data not shown). These studies suggest that the regulatory function of N221+ T cells is not cell-specific.
Contact dependent and that these cells may exert their regulatory function by secreting cytokines.

**IL-10-dependent regulatory function of N221+ cells**

We then investigated whether N221+ cells may alter the cytokine secretion profile of target T cells. We analyzed the production of IL-4, IL-10, and IFN-γ by BDC2.5 cells cocultured, or not, with N221+ cells in vitro. As expected, when cultured alone, BDC2.5 cells secreted large amounts of IFN-γ and little IL-10 in response to p79 (Fig. 6A). Interestingly, when BDC2.5 cells were cocultured with unactivated N221+ cells, the amount of IFN-γ present in the cell culture was reduced ~3-fold (from 3600 to 1100 pg/ml) in the presence of p79, with a concomitant ~4-fold increase in IL-10 (from 400 to 1700 pg/ml; Fig. 6A). IL-4 production was not detected under any of these conditions (data not shown). Therefore, N221+ cells could inhibit IFN-γ production by BDC2.5 cells, perhaps due to the production of IL-10 by N221+ cells.

Table I. **Inhibitory effect of N221+ cells on BDC2.5 T cell proliferation**

<table>
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<tr>
<th>N221/BDC CD4+ Ratio</th>
<th>GAD221 Peptide</th>
<th>% BDC2.5 CFSE+CD4+ Cells</th>
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<td></td>
<td>+</td>
<td>43.1</td>
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* BDC2.5 CFSE+CD4+ T cells have been stimulated with p79 and APC and cultured with N221+ cells, with or without GADp221, for 4 days. The data represent the percentage of BDC2.5 CD4+ T cells that did not divide or underwent one round of cell division.

We then addressed the issue of how N221+ cells could produce IL-10 without being activated by p221. Because BDC2.5 cells produced IL-2 in response to p79 stimulation, we hypothesized that N221+ cells could produce IL-10 in the presence of IL-2 secreted by activated BDC2.5 T cells. To test this hypothesis, we determined whether N221+ cells could produce IL-10 when incubated with increasing amounts of IL-2. The results demonstrated that N221+ cells could produce IL-10 in a dose-dependent fashion, but they did not produce significant amounts of IFN-γ even at the higher concentration of IL-2 (Fig. 6B). Addition of p221 to the coculture could further increase the production of IL-10 (3-fold increase) by N221+ cells compared with that when cells were cultured in medium containing IL-2 alone (data not shown). These results suggest that IL-2 can induce unactivated N221+ T cells to exhibit regulatory activities by producing IL-10 and can inhibit the proliferation and cytokine production of target cells.

To determine whether IL-10 produced by N221+ cells could indeed contribute to their regulatory functions, we investigated the effects of anti-IL-10 and anti-IL-10R Abs on the function of these cells. We repeated the Transwell assay using NOD mouse splenocytes as the target cells present in the lower well plus Abs against IL-10 or IL-10R in the culture. The presence of both anti-IL-10 and anti-IL-10R Abs significantly restored the proliferation of NOD mouse splenocytes inhibited by N221+ cells (Fig. 7A). Inhibition of NOD splenocyte proliferation by N221+ cells decreased from ~45% in the absence of the Abs to ~10% and ~20% in the presence of anti-IL-10R and anti-IL-10 Ab, respectively. The stronger effect of anti-IL-10R Ab than anti-IL-10 Ab on restoring target cell proliferation could be due to a stronger neutralizing effect on IL-10. Similar results were obtained using BDC2.5 T cells as the target cells, demonstrating that N221+ cell-mediated suppression of the Ag-specific response could be abrogated in the presence of an anti-IL-10R Ab (Fig. 7B). Together, these data provide evidence that IL-10 is a key factor contributing to the immunoregulatory function of N221+ cells, suggesting that these cells are IL-10-dependent Tr cells.
mice (Fig. 8A). In comparison, all the NOD/scid mice receiving NOD mouse splenocytes alone (isolated from 10-wk-old NOD mice) became diabetic within 12 wk after the cell transfer. Interestingly, purified N221⁺ T cells afforded significant protection from disease transfer; ~50% of the recipients receiving both N221⁺ cells and NOD splenocytes remained free of diabetes for >20 wk after cell transfer (Fig. 8A). Histological analysis of pancreatic sections revealed the presence of extensive insulitis and massive islet destruction of mice receiving NOD splenocytes (Fig. 8B). In contrast, no lymphocyte infiltration was observed in animals receiving N221⁺ cells alone. In contrast to diabetic mice, protected animals cotransferred with NOD splenocytes and N221⁺ cells showed only mild nonaggressive peri-insulitis, leaving most islets free of inflammation (Fig. 8B). Consistent with our previous studies using T cells isolated from p221-immunized NOD mice, these results suggest that the migration of diabetogenic T cells into pancreatic islets was blocked by N221⁺ cells (20). Therefore, these N221⁺ T cells exhibit regulatory functions in vivo and efficiently prevent both insulitis and diabetes development.

Discussion

It has been shown that both humoral and cellular immune responses arise spontaneously against GAD in humans and NOD mice; therefore, the GAD protein is believed to be an important autoantigen in type 1 diabetes (11–14). However, the exact roles of T cells specific for various GAD peptides during the pathogenesis of diabetes development in NOD mice remain to be determined in more detail. To address this question, we have used the tetAg7/p221 tetramer to isolate from unimmunized naive NOD mice the T cells specific for p221. The p221 peptide has been identified as one of the most dominant epitopes derived from the GAD protein and may play an important role in diabetes development (19, 26). We have previously shown that immunization of NOD mice with p221 could activate p221-specific regulatory T cells, leading to the suppression and delay of diabetes development (20). In this report we provide evidences demonstrating that T cells specific for p221 are present in unimmunized naive NOD mice. These cells exhibit IL-10-dependent regulatory capacities, and immunization of NOD mice using p221 is not necessary for inducing their regulatory activities.

The number of p221-specific T cells present in the spleens of naive NOD mice was too low to be detected using the tetramer. However, a population of proliferating CD4⁺ T cells specific for p221 rapidly appeared within 10 days after peptide-specific in vitro stimulation. In fact, a small, but significant, population (~1%) of p221-specific CD4⁺ T cells could be detected within 5 days after p221 stimulation. This procedure allowed us to expand and isolate

**FIGURE 5.** Direct cell-cell contact is not required for N221⁺ cells to inhibit CD4⁺ BDC2.5 T cell proliferation. A, Increasing amounts of N221⁺ T cells (from 1 × 10⁵ to 5 × 10⁶ cells) were cultured in a Transwell, separating them from BDC2.5 cells (5 × 10⁵ cells), in the presence of APCs, p79, and p221 (lower panel). As a control, N221⁺ cells were also cocultured with BDC2.5 cells (middle panel). After being cultured for 4 days at 37°C, cells were stained with tetAg7/p79 and anti-CD4 Ab and analyzed for the CFSE intensity. B, [³H]Thymidine incorporation of BDC2.5 cells. These cells (5 × 10⁵ cells) were cultured for 4 days with various numbers of N221⁺ T cells in Transwell assays. [³H]Thymidine (1 μCi/well) was added to the cells during the last 24 h of culture. p < 0.008 for 2.5 × 10⁵ and 5 × 10⁶ N221⁺ cells.

**FIGURE 6.** Reduced IFN-γ production by BDC2.5 T cells in the presence of N221⁺ T cells. A, Analyses of IL-10 and IFN-γ production by BDC2.5 cells (5 × 10⁵ cells) cultured with or without p79 (1 μg/ml) plus irradiated APCs in the presence or the absence of N221⁺ cells (5 × 10⁶ cells). B, The effect of increasing amounts of IL-2 on the production of IL-10 and IFN-γ by N221⁺ cells. The supernatants were harvested after a 48-h coculture of the cells, and the amounts of cytokines produced were measured using ELISA.

**N221⁺ cells protect from adoptive transfer of diabetes**

We then determined whether N221⁺ T cells could exhibit regulatory capacities in vivo by regulating diabetes development in a series of adoptive transfer studies. The results showed that N221⁺ cells did not induce diabetes when transferred alone into NOD/scid mice (Fig. 8A). In comparison, all the NOD/scid mice receiving NOD mouse splenocytes alone (isolated from 10-wk-old NOD mice) became diabetic within 12 wk after the cell transfer. Interestingly, purified N221⁺ T cells afforded significant protection from disease transfer; ~50% of the recipients receiving both N221⁺ cells and NOD splenocytes remained free of diabetes for >20 wk after cell transfer (Fig. 8A). Histological analysis of pancreatic sections revealed the presence of extensive insulitis and massive islet destruction of mice receiving NOD splenocytes (Fig. 8B). In contrast, no lymphocyte infiltration was observed in animals receiving N221⁺ cells alone. In contrast to diabetic mice, protected animals cotransferred with NOD splenocytes and N221⁺ cells showed only mild nonaggressive peri-insulitis, leaving most islets free of inflammation (Fig. 8B). Consistent with our previous studies using T cells isolated from p221-immunized NOD mice, these results suggest that the migration of diabetogenic T cells into pancreatic islets was blocked by N221⁺ cells (20). Therefore, these N221⁺ T cells exhibit regulatory functions in vivo and efficiently prevent both insulitis and diabetes development.

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The number of p221-specific T cells present in the spleens of naive NOD mice was too low to be detected using the tetramer. However, a population of proliferating CD4⁺ T cells specific for p221 rapidly appeared within 10 days after peptide-specific in vitro stimulation. In fact, a small, but significant, population (~1%) of p221-specific CD4⁺ T cells could be detected within 5 days after p221 stimulation. This procedure allowed us to expand and isolate
a sufficient number of p221-specific T cells using tetAg7/p221. The isolated cells were specific for p221, but not for other peptides, and they bore TCRs with a relatively lower avidity for the I-Ag7/p221 complex, consistent with previous studies suggesting that autoreactive T cells express low avidity TCRs (35, 36). Future experiments will determine whether N221 cells exhibit regulatory function involving IL-10 production. A, NOD mouse splenocytes (in the lower well (L.W.)) were cultured with N221 T cells (in the Transwell (U.W.)) and stimulated with PMA (100 ng/ml) and ionomycin (5000 ng/ml). Anti-IL-10 or anti-IL-10R Abs were added to the culture (20 μg/ml), and NOD splenocyte proliferation was measured after 4 days. These Abs were able to significantly (p < 0.01) restore NOD splenocyte proliferation. The results represent the mean ± SEM of four different experiments. B, CFSE-labeled CD4+ BDC2.5 T cells (in L.W.) were cultured with N221 T cells (in U.W.) and were stimulated with p79 (1 μg/ml). Anti-IL-10R Ab was added to the culture, and the proliferation of BDC2.5 T cells was determined after 4 days in culture.

Studies of their cytokine secretion profiles demonstrated that N221 cells barely secreted some IL-4 and secreted no TGF-β. However, these cells produced a larger quantity of IFN-γ and predominantly IL-10 in response to p221 stimulation. Interestingly, unlike the p221-specific T cells isolated from immunized NOD mice, which contained a large percentage of IL-10/IFN-γ-coproducing cells, a significant population of N221 T cells from naive NOD mice produced only IL-10 and no IFN-γ. These results suggest that immunization of NOD mice using p221 only activates and expands a spontaneously arisen population of IL-10-producing T cells, but also induces a population of IL-10/IFN-γ-coproducing cells (20). Interestingly, both our in vivo and in vitro studies have demonstrated that these N221 cells, like those derived from p221-immunized NOD mice, are endowed with IL-10-dependent regulatory properties. Adoptive transfer experiments have shown that N221 T cells could prevent diabetes development. No invasive infiltration or destructive insulitis was detected in the pancreatic islets of the recipient mice, suggesting that the migration of diabetogenic T cells to the pancreas was blocked either directly or indirectly by a mechanism dependent on the presence of these N221 cells. Additional experiments with these N221 cells also showed that they could inhibit the proliferation of other NOD mouse T cells, including diabetogenic BDC2.5 T cells. This inhibitory function of N221 cells depends not on direct cell-cell interaction between N221 cells and target T cells, but on soluble factors secreted by these N221 cells. Blocking of IL-10 using both anti-IL-10 and anti-IL-10R Abs could significantly restore the proliferation of target T cells suppressed by N221 cells. Our studies also showed that N221 cells were able to inhibit the production of IFN-γ by BDC2.5 T cells, probably by secreting IL-10. Previous studies have demonstrated that Tr1 cells are defined by their ability to suppress Th1 or Th2 responses mainly via the production of a high level of IL-10 along with small amounts of other effector cytokines, such as IFN-γ (33, 34). Based on our results shown in Fig. 2B, it appears that N221 cells contained some IL-10/IFN-γ-coproducing Tr1 cells (1.2%), a larger population of Tr cells producing IL-10 alone (~30%), and a small population of IFN-γ-producing cells (2%). Because the N221 cells used in these studies are polyclonal, it remains to be determined which specific population(s) contributes to the observed IL-10-dependent regulatory function. However, because the N221 cells demonstrated IL-10-dependent regulatory function, these cells are named IL-10-dependent Tr cells. It is currently unclear why these cells are...
polarized to produce IL-10. Previous studies have provided evidence that CD4+ T cells specific for different epitopes of GAD65 are not pathogenic, but, rather, can inhibit diabetes development (18–20, 25). Our results are consistent with these previous findings. Moreover, our studies suggest that NOD mouse CD4+ T cells specific for the GAD p221 peptide may develop spontaneously as polarized IL-10-producing Tr cells that can protect animals from developing type 1 diabetes.

It has been previously reported that NOD mouse-derived, peptide-specific T cells, induced by administration of soluble β cell autoantigens such as GAD, inhibit, rather than accelerate, diabetes development, possibly mediated by Th2 cells (16–20, 39). To our knowledge, this is the first report describing the identification and characterization of autoantigen-specific Tr1 cells and Tr cells producing IL-10 alone present in prediabetic naive NOD mice. Because in vitro cell culture is necessary to detect and isolate a large number of these p221-specific T cells, the possibility exists that in vitro stimulation and expansion might have modified the phenotype and functional properties of the naturally arising p221-specific T cells. In autoimmune diabetes, the frequency of autoantigen-specific CD4+ T cells is so low that these autoreactive T cells from freshly isolated spleen cells or islets are not or are hardly detectable by FACS even with the use of tetramers. The best and perhaps the only way to visualize such cells and to obtain a sufficient number of these cells for additional experiments is to expand them in vitro in a peptide-specific manner. It is expected that if the cells specific for the peptide of interest are present in the mice, they will respond to peptide stimulation and proliferate at a higher rate than other non-Ag-specific T cells. That is what we have observed with T cells specific for p221, demonstrating that p221-specific T cells are present in naive NOD mice. In addition, although we are able to detect the presence of p221-specific T cells in naive NOD mice, it is essential to obtain sufficient numbers of T cells to perform further in vitro and in vivo experiments. To achieve these goals, in vitro stimulation and expansion are necessary for us to isolate a large number of p221-specific T cells and to characterize the function of these cells. It is possible that the culture conditions used to expand these T cells could have provided signals for the acquisition of regulatory functions. However, as described in our previous report, the same experimental conditions were used to isolate from naive NOD mice the CD4+ T cells specific for a B2DC2.5 T cell-stimulating peptide, p79 (30). Using the tetAg7/p79 tetramer, we could detect p79-specific T cells in unimmunized naive NOD mice, and then could use this tetramer to isolate p79-specific T cells. In these previous experiments, we have used the same cell culture protocol that we have used to expand and isolate N221+ cells described in this report. Additional functional studies showed that, unlike N221+ cells, these p79-specific T cells did not inhibit diabetes development (30). In addition, in another report we demonstrated that T cells cultured under the same culture conditions displayed the same cytokine secretion profiles as freshly isolated T cells before culture in vitro (20). These results suggest that the culture conditions used in these studies did not alter the phenotype or functionality of the isolated Ag-specific T cells. Together, these findings argue against the possibility that N221+ cells acquired regulatory properties during the in vitro culture process.

Our results have demonstrated that N221+ T cells are neither Th1 nor Th2 cells, but are IL-10-dependent Tr cells. These N221+ Tr cells are most likely different from previously identified regulatory cells defined by their surface expression of markers such as CD25 (40, 41). Although N221+ cells express high levels of CD25, it may simply reflect their activation status after the in vitro stimulation and expansion. In addition to expressing CD25, N221+ cells expressed a low level of CD45RB. It has been previously shown that CD45RBlow T cells could prevent colitis development (42). Interestingly, the presence of IL-2 produced by other activated T cells was able to induce IL-10 production by these N221+ Tr cells. This novel finding suggests that some GAD peptides can elicit spontaneous regulatory, rather than aggressive autoimmune, responses that inhibit diabetes progression. Activation and expansion of spontaneously arising autoantigen-specific Tr cells, such as N221+ cells, may represent a promising approach for providing durable protection against type 1 diabetes.

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References


