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A Peptide of Glutamic Acid Decarboxylase 65 Can Recruit and Expand a Diabetogenic T Cell Clone, BDC2.5, in the Pancreas

Yang D. Dai,* Kent P. Jensen,† Agnes Lehuen,§ Emma L. Masteller,¶ Jeffrey A. Bluestone,‖ Darcy B. Wilson,‡ and Eli E. Sercarz*‡

Self peptide-MHC ligands create and maintain the mature T cell repertoire by positive selection in the thymus and by homeostatic proliferation in the periphery. A low affinity/avidity interaction among T cells, self peptides, and MHC molecules has been suggested for these events, but it remains unknown whether or how this self-interaction is involved in tolerance and/or autoimmunity. Several lines of evidence implicate the glutamic acid decarboxylase 65 (GAD-65) peptide, p524–543, as a specific, possibly low affinity, stimulus for the spontaneously arising, diabetogenic T cell clone BDC2.5. Interestingly, BDC2.5 T cells, which normally are unresponsive to p524–543 stimulation, react to the peptide when provided with splenic APC obtained from mice immunized with the same peptide, p524–543, but not, for example, with hen egg white lysozyme. Immunization with p524–543 increases the susceptibility of the NOD mice to type 1 diabetes induced by the adoptive transfer of BDC2.5 T cells. In addition, very few CFSE-labeled BDC2.5 T cells divide in the recipient’s pancreas after transfer into a transgenic mouse that overexpresses GAD-65 in B cells, whereas they divide vigorously in the pancreas of normal NOD recipients. A special relationship between the BDC2.5 clone and the GAD-65 molecule is further demonstrated by generation of a double-transgenic mouse line carrying both the BDC2.5 TCR and GAD-65 transgenes, in which a significant reduction of BDC2.5 cells in the pancreas has been observed, presumably due to tolerance induction. These data suggest that unique and/or altered processing of self Ags may play an essential role in the development and expansion of autoreactive T cells.


Division of Immune Regulation, and †Division of Experimental Medicine, Torrey Pines Institute for Molecular Studies, San Diego, CA 92121; ‡Autoimmunity and Transplantation Division, The Walter and Eliza Hall Institute, Royal Melbourne Hospital, Parkville, Australia; §Institut National de la Santé et de la Recherche Médicale, Unité 561, Hopital Cochin-Saint Vincent de Paul, Paris, France; and ‖Diabetes Center, University of California, San Francisco, CA 94143

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2 Address correspondence and reprint requests to Dr. Eli Sercarz, Division of Immune Regulation, Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121. E-mail address: esercarz@tpims.org

3 Abbreviations used in this paper: GAD, glutamic acid decarboxylase; HEL, hen egg white lysozyme.

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proliferation assay in vitro (our observation). However, Judkowski et al. (22) showed that a smaller GAD-65 peptide, p526–541, could activate BDC2.5 T cells at high concentrations. By scanning combinatorial peptide libraries, this group has identified several strong agonistic peptides that are highly homologous to a region within the GAD-65 p524–543 peptide and can activate BDC2.5 T cells at nanomole concentrations. Subsequently, Yoshida et al. (23) identified a panel of mimotopes that are quite similar to those eluted by Judkowski et al. (22) and also strongly stimulate BDC2.5 T cells as well as several other diabetes-related clones. This scenario fits with our finding that peptide p524–543 contains two registers at least, each of which stimulates a distinct T cell population. The N-terminal register, p524–538, is dominant and stimulates for regulatory T cells, whereas the C-terminal determinant, p530–543, is cryptic, and cells endogenously stimulated by this determinant appear early in the NOD mouse (1, 17). It is possible that the BDC2.5 T cell recognizes a previously undescribed cryptic determinant using residues located within p524–543 and created after anomalous, unconventional Ag processing.

Materials and Methods

Mice

NOD mice were purchased from The Jackson Laboratory and bred for two generations at the Torrey Pines Institute for Molecular Studies. BDC2.5 TCR transgenic mice on a NOD background (BDC2/NOD) were provided by Dr. A. Lehuen (Institut National de la Sante et de la Recherche Medicale, Paris, France). Briefly, mouse GAD-65 cDNA was cloned from a c57BL/6 brain library (Stratagene) using a PCR-generated probe. The cDNA was inserted into the pC4DHG8 plasmid (a gift from K. Karjalainen, Basel, Switzerland). The plasmid contains a mouse Igκ promoter and genomic human FcγR sequence (Fc). GAD-65 cDNA was inserted into the plasmid between the promoter and Fc sequences. The original stop codon of the GAD-65 cDNA was replaced with a splice donor site by PCR. For generation of GAD-65 transgenic mice, purified GAD-65-Fc plasmids were digested by restriction enzyme to obtain GAD-65-Fc fragments, which were purified by agarose gel electrophoresis for microinjection. Fertilized NOD oocytes injected with GAD-65-Fc fragments were reimplanted into F(nNOD × C57BL/6) foster mothers. The first offspring were screened on the BDC2.5-TgAg transgene by testing the urine glucose level using Chemstrip uG (Roche). Mice with a glucose level of 96-Well, flat-bottom plates together with various concentrations of Ag in 10% FCS complete RPMI 1640 medium, containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 10 mM HEPES buffer, and 0.1 mM MEM nonessential amino acids (Invitrogen Life Technologies). After 3 days of culture, [3H]thymidine (International Chemical and Nuclear) was added for another 18–24 h of culture. Cells were harvested with a Micro Cell Harvester (Skatron Instruments), and incorporation of [3H]thymidine was measured on a Wallac MicroBeta Trilux counter (PerkinElmer).

Production of Ag-specific APC and generation of T cell lines

To prepare Ag-primed APC, GAD-65 peptides or HEL were emulsified in CFA or IFA and injected i.p. After 2 wk, the mice were boosted with the same peptide or Ag emulsified in IFA i.p., and spleens were collected 4 wk after the first immunization. After lysing RBC with RBC-lysing buffer (Sigma-Aldrich), splenic APC cells were either purified using MACS beads coated with anti-B220 Ab (Milenyi Biotec) or irradiated at 3000 rad by a Gammacell 40 irradiator (Atomic Energy). To generate T cell lines, 6- to 8-wk-old female NOD mice were immunized with a GAD-65 peptide emulsified in CFA i.p. After 2 wk, spleens were collected, and single-cell suspensions were prepared. Stimulation and expansion of peptide-specific T cells were conducted in vitro by adding 10 μg/ml peptide(s) in culture for 5 days, followed by a period in culture without Ag but with 25 U/ml IL-2 (PeproTech). After three cycles of stimulation and rest, >90% of cells were CD4 positive. The specificity of the line was tested by proliferation assay with peptides or control Ags in the presence of irradiated NOD spleenocytes. A BDC2.5 T cell line was generated by stimulating naive BDC2.5 splenocytes collected from BDC2.5 TCR transgenic mice with 0.2 μg/ml of the mimotope, m1040-31, for 5 days, followed by culture in complete RPMI 1640 containing 25 U/ml IL-2 for 1 wk.

FACS

Staining of lymphocytes for surface markers was performed using a 1/100 dilution of respective Abs. Abs specific for mouse CD4, TCRβ4/8, CD44, CD69, and IgG2b were purchased from BD Pharmingen. To detect binding of specific MHC-Ag complexes by BDC2.5 T cells, an I-Aβ'-Ig-dimer, covalently linked to a BDC2.5 mimotope, m1040-31, was generated by Dr. J. Bluestone and his colleagues (San Francisco, CA) (26). The dimer was visualized by secondary protein G-coupled FITC fluorescence (Sigma-Aldrich). A BDC2.5-specific clonotypic mAb was provided by Dr. O. Kanagawa (Washington University, St. Louis, MO) to stain the BDC2.5 T cells, followed by a secondary anti-IgG2b-FITC Ab.

CFSE-labeled adoptive transfer experiments

For CFSE labeling, 107 cells/ml suspended in RPMI 1640 medium were incubated at 37°C with 5 μM CFSE (Sigma-Aldrich). The labeling reaction was stopped after 10 min by addition of 5% FCS/RPMI 1640 medium, and residual CFSE was removed by washing with RPMI 1640 medium. Transfer experiments were performed by injecting 107 CFSE-labeled cells into the tail vein of irradiated (900 rad) or normal young NOD and GAD-Fc/-NOD mice. One week after the transfer injections, the spleen, pancreatic lymph nodes, inguinal lymph nodes, and pancreas were collected to study the distribution and proliferation of the CFSE-labeled cells. Pancreas-infiltrating cells were collected by Ficoll separation after collagenase digestion (0.46 mg/ml) for 15 min at 37°C as previously described (19).

Diabetes

Diabetes development was monitored once a week starting at 12 wk of age by testing the urine glucose level using Chemstrip uG (Roche). Mice with two consecutive measurements >250 mg glucose/dl were considered diabetic.

Results

Antigenic specificity of the BDC2.5 T cell clone: cross-reactivity to GAD-65 peptide p524–543

The quest to find the native Ag of the well-known diabetogenic T cell clone BDC2.5 (27) remains unsolved, although several strongly agonistic peptides (mimotopes) homologous to a region of the mouse GAD-65 molecule have been recently identified through combinatorial peptide library techniques (22). What is interesting to us is that the mimotopes cover a limited region within a dominant GAD-65 determinant (p524–543), which had been identified a decade ago as one of the earliest diabetes-related T cell determinants in the NOD mouse (1). To test whether p524–543-reactive
T cells are cross-reactive to BDC2.5 mimotopes, we generated T cells lines specific for peptide p524–543. Several of the mimotopes have shown activity in stimulating a T cell line specific for p524–543 (Fig. 1A), but no activity for a control, HEL-specific T cell line (data not shown), indicating a possible relationship between the ligands recognized by the p524–543-specific T cells and the BDC2.5 T cells. To directly measure the binding affinity of the mimotopes to p524–543-specific T cells, we used an I-A\(^\beta\) Ig dimer covalently linked with one of the mimotopes stimulating BDC2.5 T cells. The I-A\(^\beta\) Ig dimer binds strongly to BDC2.5 T cells, as expected, and also binds to the p543 line cells, but with a lower affinity than to the BDC2.5 T cells.

FIGURE 1. GAD–65 p524–543-specific T cells cross-react to a BDC2.5 mimotope(s). A p524–543 (p543) line was produced by stimulation of p524–543-primed LNC with p524–543 in vitro, followed by resting in the presence of IL-2; the stimulation and resting cycles were repeated twice. A, The p543 line cells are reactive to a BDC2.5 mimotope, 1040–31 (m31), in the presence of irradiated syngeneic APC. B, An I-A\(^\beta\)-mimotope Ig dimer clearly binds to the p543 line cells, but with a lower affinity than to the BDC2.5 T cells. C, BDC2.5 T cells failed to respond to short peptides within the p524–543 region, but respond well to the mimotope, m1040–31.

Previously, we and others (28–30) have shown that addition or deletion of flanking residues surrounding a core determinant peptide could influence the affinity between a peptide and its specific TCR. To test whether there was a high affinity ligand of BDC2.5 T cells within p524–543, 17 short peptides (12–18 mer) were synthesized covering different sequences of p524–543 (Fig. 2). By proliferation assays using native splenocytes from BDC2.5 transgenic mice as responders, we found that none of the 17 short peptides nor p524–543 itself could stimulate BDC2.5 cells, whereas BDC2.5 T cells responded well to the mimotope, m1040-31 (Fig. 1C). The question of a possible nested cryptic, high affinity determinant of BDC2.5 T cells within p524–543 remains open. If there had been such a high affinity ligand within p524–543, the flanking residues around it must have contributed a strong inhibitory role toward BDC2.5 recognition.

The p524–543-primed APC are crucial in activation of BDC2.5 T cells by the peptide

Instead of continuing to search for a possible high affinity ligand of BDC2.5 T cells within p524–543 using synthetic peptides, we took a different approach to study the processing and presentation of a possibly cryptic, high affinity ligand of BDC2.5 T cells within peptide p524–543. Because APC diversity and activity have been shown to be crucial in processing and presentation of cryptic T cell determinants (31), we decided to treat splenic APC with various inflammatory stimuli such as IFN-\(\gamma\), anti-CD40, and LPS, asking whether these activated APC could stimulate BDC2.5 T cells in the culture. However, none of these activated APC populations could stimulate BDC2.5 T cells after processing of p524–543 (data not shown).

A second pathway for inducing visibility of previously cryptic determinants that we have proposed previously (32) requires Ag processing in Ag-specific B cells. To examine the role of Ag-primed B cells in activation of BDC2.5 T cells, we immunized and boosted NOD mice 2 wk later with the p524–543 peptide, and these peptide-primed splenocytes were then used as APC for activation of BDC2.5 T cells in the presence of p524–543 in culture. Surprisingly, after earlier in vivo priming with p524–543, the NOD splenocytes became efficient in activating the BDC2.5 T cells (Fig. 3A), whereas control splenocytes challenged with a second GAD–65 peptide, p246–66 (p246), or HEL protein were inefficient. A similar result was observed in four separate experiments. These data suggest that p524–543 priming generates an activity within a population of APC, perhaps through special

FIGURE 2. BDC2.5 T cells failed to respond to short peptides within p524–543. Seventeen short peptides covering the p524–543 region of GAD65 were synthesized and purified. A proliferation assay of BDC2.5 transgenic mice with 1–100 \(\mu\)g/ml of the peptides shown. Proliferation was monitored by incorporation of \(^{3}\)Hthymidine. None of the 17 peptides, except the mimotope, could stimulate BDC2.5 cells to proliferate (stimulation index, <3; data not shown).
processing, which favors the proliferation of BDC2.5 T cells, although BDC2.5 does not respond to splenic dendritic cells or peritoneal macrophages directly pulsed with p524–543 (data not shown). CFA is not required for generation of this unique APC activity, because splenocytes challenged with p524–543 peptide emulsified in IFA were also sufficient in activating BDC2.5 T cells in the presence of p524–543 peptide (Fig. 3A). Therefore, non-specific activation of APC, such as macrophages or dendritic cells, may not be responsible; alternatively, we propose that Ag-specific B cells generated by p524–543 priming are the agents for BDC2.5 activation by p524–543. This hypothesis is supported by the observation that magnetically sorted B cells, via the B220 marker, from p524–543-primed, but not p246–66-primed, splenocytes are as good as primed splenocytes in activation of BDC2.5 cells by p524–543 (Fig. 3B). The activation cannot be attributed to an in vitro activation by some contaminated T cells, generated by p524–543 immunization, because 1) in the absence of BDC2.5 T cells, purified B220+ cells or irradiated Ag-primed splenocytes were unresponsive to the p524–543 peptide; and 2) similarly primed B cells specific for a second Ag, e.g., GAD65 p246–266 or HEL, could not present the same priming Ag and p524–543 peptide to activate BDC2.5 T cells.

The data indicate that an unusual APC activity may be required for recruiting BDC2.5 T cells into islets to induce diabetes. It is also supported by three independent in vivo experiments in attempting the adoptive transfer of diabetes with BDC2.5 cells, in which challenge with p524–543 significantly enhanced the susceptibility of prediabetic NOD recipients to diabetes shortly after adoptive transfer of naive BDC2.5 T cells (Table I). In two experiments, all four NOD mice, including two male mice that were immunized with p524–543, developed diabetes within 2 wk after receiving naive BDC2.5 cells, whereas mice from the same litter, but not preimmunized with the peptide, were resistant to BDC2.5-induced diabetes. The data indicate that priming NOD mice with p524–543 accelerates the disease process initiated by transferring BDC2.5 T cells.

Self tolerance in BDC2.5 and GAD-65 double-transgenic mice
To examine whether GAD-65 Ag plays a role in the development and activation of BDC2.5 T cells during the course of diabetes in NOD mice, we generated a double-transgenic mouse line, BDC2.5/GAD-65, by breeding BDC2.5 TCR transgenic mice with a GAD-65 transgenic mouse line in which the GAD-65 transgene was fused to a human IgG-Fc region and driven by an Igκ promoter; therefore, transgene overexpression is restricted to B cells. Interestingly, the overexpression of GAD-65 does not seem to affect the development and maturation of BDC2.5 T cells, because the double-transgenic mice exhibit normal development of thymus and spleen, with >80% CD4+ T cells carrying the BDC2.5 TCRBV transgene (TCRBV4), similar to those observed in the BDC2.5 TCR single-transgenic mouse, although we do not know whether the percentage of T cells with a different endogenous TCR α-chain in the double-transgenic mouse is different from that in the BDC2.5 mouse. There are also no significant differences in total cell number or CD4/CD8 ratios in the spleen. In addition, splenocytes from double-transgenic mice show comparable percentages of T cell subpopulations, such as CD4+CD25+, CD4+CD69+, CD4+CD62L-, and CD4+CD44+, with those in BDC2.5 single-transgenic mice (data not shown). However, when we compared cells isolated from pancreatic lymph nodes or the pancreas from the double-transgenic mice, we found that BDC2.5/GAD-65 mice consistently show a significant decrease in total lymphocyte number in the pancreatic lymph nodes and pancreas at the age of 12–16 wk. Fig. 4A shows one typical example of more than five similar observations. The total cell number in two pancreatic lymph nodes in a 12- to 16-wk-old BDC2.5/GAD-65 mouse is ~106, but in a BDC2.5 mouse of the same age, this value can be 3 times higher. In addition, the percentage of BDC2.5 T cells exhibiting the clonotypic phenotype (stained positively by a clonotypic Ab) also decreased significantly in the pancreases of BDC2.5/GAD-65 mice from 19.6% in the BDC2.5 mouse to 11.6% in the BDC2.5/GAD-65 mouse (Fig. 4B). These data suggest that peripheral overexpression of GAD-65 in B cells can inhibit the infiltration and activation of BDC2.5 T cells during the course of diabetes in GAD-65 double-transgenic mice.

Table I. Priming NOD recipients with p524-543 (p543) peptide increases susceptibility to the transfer of diabetes by BDC2.5

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Recipients</th>
<th>Priming (Age at Injections in Weeks)</th>
<th>Age at Transfer (Weeks)</th>
<th>Diabetes in Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 Male</td>
<td>p543 + IFA (12 and 15)</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2 Male</td>
<td>p543 + IFA (12 and 15)</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3 Male</td>
<td>PBS + IFA (12 and 15)</td>
<td>16</td>
<td>No (18)</td>
</tr>
<tr>
<td></td>
<td>4 Male</td>
<td>PBS + IFA (12 and 15)</td>
<td>16</td>
<td>No (18)</td>
</tr>
<tr>
<td>2</td>
<td>1 Female</td>
<td>p543 + IFA (9 and 10)</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2 Female</td>
<td>p543 + IFA (9 and 10)</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>3 Female</td>
<td>PBS + IFA (9 and 10)</td>
<td>14</td>
<td>No (16)</td>
</tr>
<tr>
<td></td>
<td>4 Female</td>
<td>PBS + IFA (9 and 10)</td>
<td>14</td>
<td>No (16)</td>
</tr>
<tr>
<td>3</td>
<td>7 Females</td>
<td>p543 + IFA (9)</td>
<td>10</td>
<td>2/7 (15)</td>
</tr>
<tr>
<td></td>
<td>6 Females</td>
<td>HEL + IFA (9)</td>
<td>10</td>
<td>0/6 (15)</td>
</tr>
</tbody>
</table>

*Adoptive transfer of diabetes in NOD mice by BDC2.5 cells. Peptide p524-543(p543) (50 μg per mouse) and HEL (500 μg per mouse) emulsified in IFA were injected i.p. into normal NOD recipients 1 or 2 wk before the transfer of 106 lymphocytes isolated from BDC2.5 transgenic mice. Disease development was monitored by urine-glucose level.

FIGURE 3. GAD-65 p524–543-primed APC can activate BDC2.5 T cells. Splenocytes were collected from NOD mice after immunization with p524–543 or control Ags, emulsified in CFA or IFA. For activation of BDC2.5 cells in vitro, primed APC cells, either irradiated splenocytes (A) or B220+ B cells (B; sorted by MACS; 3 × 106), were mixed with 107 B2C.5 T cells that had been prestimulated with mimotope m1040-31 and incubated with rIL-2 in the presence of the p524–543 peptide or control Ags in culture for 72 h, followed by pulse with 0.5 μCi of [3H]thymidine for 24 h.
expansion of BDC2.5 T cells in the pancreas, possibly through a dose-dependent enhancement of self tolerance.

Inhibition of expansion of BDC2.5 T cells in the pancreas of GAD-65-Fc transgenic mice

The reduced recruitment of BDC2.5 T cells into the pancreas in the BDC2.5/GAD-65 double-transgenic mouse indicates that tolerance might have been induced in BDC2.5 T cells. We were unable to distinguish the BDC2.5 T cells isolated from the double-transgenic or single-transgenic mice by surface markers such as CTLA-4, CD25, CD44, or CD62L. However, it is possible that in the double-transgenic mouse, the T cells could be functionally altered during maturation despite undetectable phenotypic changes. We performed adoptive transfer experiments by injecting CFSE-labeled BDC2.5 T cells separately into either NOD mice or GAD-65 transgenic mice. In this way, we could address the question of whether peripheral tolerance after overexpression of GAD-65 contributes to the reduced recruitment and/or expansion of BDC2.5 in the pancreas of double-transgenic mice. Nonirradiated NOD or GAD-65 transgenic mice (12–16 wk old) were used as recipients in the transfer experiments, so that a competitive normal T cell repertoire was maintained in the recipients. Distribution and expansion of CFSE-labeled BDC2.5 donor cells in the recipient mice were monitored on days 2, 4, and 7 after injection. BDC2.5 cells cannot be activated in the periphery and remain undivided in the spleen and inguinal lymph nodes isolated from both NOD and GAD-65-Fc recipients at the time points that we examined, and there were no significant differences with respect to the percentage and number of donor BDC2.5 cells between the NOD and GAD-65 transgene recipients (data not shown). Interestingly, in the pancreatic lymph nodes, a vigorous division of CFSE-labeled BDC2.5 donor cells occurred; however, only in the NOD recipients (some labeled BDC2.5 cells performed over five divisions on day 7 after transfer), but not in the GAD-65-Fc recipients (Fig. 5). From days 2–7, a steady increase in the percentage of CFSE-positive BDC2.5 cells, including CFSElow and CFSEhigh in the pancreatic lymph nodes was observed in NOD recipients, whereas this CFSE-positive percentage remained barely changed in the GAD-65-Fc recipients (Fig. 5). This is an important finding that emphasizes a clear relationship between BDC2.5 cells and the GAD65 molecule. The data confirm that the pancreas provides the primary stimulatory signal for recruiting and activating BDC2.5 T cells and indicate that enhanced peripheral tolerance of BDC2.5 T cells by GAD-65 down-regulates this priming process of BDC2.5 T cells in the pancreas.

GAD-65 promotes lymphopenia-induced proliferation of BDC2.5 T cells

To further explore the tolerance mechanism of BDC2.5 T cells by GAD-65, we examined the proliferative response of naive T cells in lymphopenic recipients. It has been suggested that lymphopenia-induced, homeostatic proliferation might result from a low affinity interaction of T cells with compatible ligands (33). We have demonstrated that BDC2.5, a well-known autoreactive T cell clone, interacts with GAD-65, possibly with a low affinity. How would GAD65 affect BDC2.5 tolerance within a lymphopenic environment? To accomplish this, CFSE-labeled naive BDC2.5 T cells, isolated from BDC2.5 transgenic mice, were transferred into sublethally irradiated (900 rad) NOD or GAD-65-Fc transgenic mice. Three or 5 days later, spleens from recipients were collected to monitor the lymphopenia-induced homeostatic proliferation of the CFSE-labeled cells. We repeatedly observed that the total cell number collected from a GAD-65-Fc recipient was significantly higher than that from a NOD recipient, although an equal number of donor cells was transferred at the onset. More importantly, the percentage of CD4+ CFSE+ donor cells in GAD-65-Fc recipients was about twice the number in NOD recipients on days 3 and 5 after transfer (Fig. 6a), whereas the total number of CD4+ CFSE+ cells, which were most likely the residual recipient’s cells surviving from the irradiation, was about equal in the GAD-65-Fc and NOD recipients, suggesting a possibly enhanced homeostatic proliferation of BDC2.5 cells in GAD-65-Fc transgenic mice. In addition, we observed that BDC2.5-like precursor cells increased significantly in GAD-65-Fc transgenic mice, as detected using a BDC2.5-specific clonotypic Ab (data not shown). This increased frequency of a BDC2.5-like population in the GAD-65 transgenic mouse was also

FIGURE 4. Self tolerance induced by GAD-65 reduces the recruitment of BDC2.5 cells to the pancreas. Total cell number (A) and BDC2.5-positive T cells (B) are significantly reduced in the pancreatic lymph nodes and the pancreas in BDC2.5/GAD-65-Fc double-transgenic mice. Two pancreatic lymph nodes were collected from each mouse (14–16 wk old), and islet-infiltrating lymphocytes (IIL) were isolated by Ficoll gradient separation from the pancreas. FACS analysis was performed using a clonotypic mAb (provided by Dr. O. Kanagawa, Washington University, St. Louis, MO) specific for BDC2.5 T cells. Similar results were observed in five female BDC2.5/GAD-65-Fc double-transgenic mice.

FIGURE 5. Peripheral tolerance induced in GAD-65 transgenic mice prevents the expansion of BDC2.5 T cells in the pancreas. CFSE-labeled BDC2.5 T cells were injected i.v. into young, nonirradiated NOD or GAD-65-Fc/NOD transgenic mice (107 cells/mouse). After 2, 4, and 7 days, the pancreatic and inguinal lymph nodes and spleens were harvested for FACS analysis. Fifty thousand cells were collected for each sample, except for the day 7 sample from the GAD-65-Fc recipient for which only 12,000 events were collected due to the limited cell number isolated. The CFSE division profile of the injected BDC2.5 cells in the pancreatic lymph nodes is shown, and the numbers indicate the percentage of CFSE-positive cells of the total CD4+ population.
confirmed by a proliferation assay in which splenocytes from GAD-65-Fc transgenic NOD mice responded more vigorously to a BDC2.5 mimotope, m1040-31, than cells from NOD mice (Fig. 6). The data support the idea that overexpression of self Ag in the periphery enhances homeostatic proliferation of autoreactive T cells and reinforce the accumulated evidence presented in this paper, that BDC2.5 T cells can recognize a GAD-65 epitope.

Discussion
This study was initiated after the observations that peptides agonistic for the BDC2.5 T cell clone mimic a region within a dominant GAD-65 region, p524–543 (1, 22). T cell lines specific for p524–543 cross-react weakly with the agonistic mimotopes of BDC2.5 cells. We hypothesized that a high affinity, possibly cryptic (9), determinant within the p524–543 region could be recognized by BDC2.5 T cells. Seventeen different short peptides, therefore, were synthesized, covering different sequences within the p524–543 region. Unfortunately, none of these short peptides, including peptide p526–541, which was shown previously (22) to be stimulatory to BDC2.5 cells, could activate BDC2.5 T cells in a proliferation assay using freshly isolated splenocytes or lymph node cells from BDC2.5 transgenic mice.

However, we were able to activate BDC2.5 T cells using APC cells isolated from mice immunized with p524–543, presumably because p524–543-specific APC might be efficient in processing the p524–543 peptide into a ligand suitable for BDC2.5 recognition. One possible mechanism that might mediate the generation of such a ligand by p524–543-primed APC cells would involve differential protein splicing (34, 35); p524–543 peptides could be spliced and religated by special protein ligases, omitting a few amino acids flanking the spliced small fragments. Interestingly, the best agonist mimotopes of BDC2.5 T cells seem to result from omission of a few amino acids within the p524–543 peptide (Fig. 2). Artificially spliced peptides can lead to new, dominant determinants (36), and it is probable that spliced determinants are often responsible for reactivity in autoimmune and tumor systems (37). A second mechanism could be due to differential Ag processing and presentation after receptor-mediated Ag internalization (38, 39); for example, the receptor could be surface Ig specific for the p524–543 peptide. Not only is enhanced Ag uptake relevant, but also a different protease activity in the specific B cells may play a crucial role in the presentation of a unique self Ag(s) and activation of autoreactive T cells (40, 41). The requirement for additional processing of the 20-mer peptide p524–543 for generation of a suitable ligand for BDC2.5 CD4+ T cells indicates a third mechanism that MHC class I may use through a possible TAP-independent cross-presentation in those Ag-primed APC (42). Finally, an additional mechanism may involve the sharing of the MHC groove by short peptides (43) derived from this region, and rendered highly immunogenic by an altered peptide ligand-type mechanism.

We have clearly shown that GAD-65 does play a role in tolerizing BDC2.5 T cells and preventing their infiltration into pancreatic islets in NOD mice by observing a reduced recruitment and expansion of BDC2.5 T cells in the pancreas of GAD-65 transgenic mice. We could not completely exclude that this reduced infiltration and expansion of BDC2.5 in the pancreas were due to bystander suppression through the induction of regulatory cells after overexpression of GAD-65 in the periphery instead of a direct interaction of GAD-65 with BDC2.5 cells. However, the GAD-65 transgenic mice that we used in this study have a similar susceptibility to diabetes as wild-type NOD mice (Dr. A. Lehuen, unpublished observation), suggesting that overexpression of GAD-65 does not tolerate all diabetogenic T cells. Therefore, the tolerance of BDC2.5 cells observed in this study most likely resulted from a specific interaction of GAD-65 in the periphery. Presumably, in the normal NOD mouse, GAD65 is expressed in thymus and peripheral tissue (44), and BDC2.5 T cells have experienced central and/or peripheral tolerance by interaction with GAD65, whereas overexpression of GAD65 did not change the phenotype of BDC2.5 cells and certainly could not deplete BDC2.5-like cells. In contrast, we observed an increased frequency and activity of BDC2.5-like cells in GAD65 transgenic cells, although their pathogenic activity seems partially damaged. Does enhanced homeostatic proliferation heighten or depress autoimmunity (45)?

One possible explanation of our observations could be that the requirements for stimulating homeostatic expansion are different from those for generating pathogenic effectors. Altered Ag processing of GAD65, as proposed in this study in Ag-primed APC, may create a different, high affinity ligand for activation of BDC2.5 cells in the pancreatic lymph nodes and the islets.

Finally, it is not known whether the BDC2.5 clone belongs to the earliest islet-infiltrating T cell family that can initiate a long process of diabetes progression. The BDC2.5 T cell clone can induce severe diabetes in young NOD recipients (46), whereas BDC2.5 TCR transgenic NOD mice are not very susceptible to type I diabetes (47). Using the I-A3β tetramer loaded with a mimotope of the BDC2.5 clone, several studies have shown that BDC2.5-like cells could be found early in the thymus, the periphery, and the pancreas of NOD mice (48–50). However, we could not identify a significant clonal expansion of BDC2.5 T cells in NOD islets until a late prediabetic stage of islet infiltration (9–10 wk), as detected by CDR3-length spectroscopy.4 BDC2.5 cells may be recruited to the inflammatory sites after activation of early, high affinity, disease-driving T cells. It needs to be considered that the late, possibly low affinity, interaction exemplified by BDC2.5 T cells with the GAD-65 peptide p524–543 or some other Ags expressed in the islets, attributable to a degeneracy of TCR specificity, may be sufficient to recruit or activate a second or third wave of islet-infiltrating T cells. Whether and how these late infiltrators cause pathology in the pancreas remain unresolved. We have suggested that their delayed reactivity may be related to the difficulty inherent in creating a suitable ligand from a self Ag such as GAD-65.

4 A. Quinn, M. McInerney, D. Huffman, B. McInerney, K. Haskins, and E. Sercarz. T cells to a dominant epitope of GAD65 express a public CDR3 motif. Submitted for publication.
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Disclosures

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References


