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CNS Destruction Mediated by GAD-Specific CD4+ T Cells[1]

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Abstract

High titers of autoantibodies against glutamic acid decarboxylase 65 (GAD65) are commonly observed in patients suffering from type 1 diabetes (T1D) as well as Stiff Person syndrome (SPS), a disorder that affects the central nervous system, and a variant of SPS, progressive encephalomyelitis with rigidity and myoclonus (PERM). While there is a considerable amount of data focusing on the role of GAD65-specific $CD4^+$ T cells in T1D, little is known about their role in SPS. Here we show that mice possessing a monoclonal GAD65-specific $CD4^+$ T cell population (4B5, PA19.9G11 or PA17.9G7) develop a lethal encephalomyelitis-like disease in the absence of any other T cells or B cells. GAD65-reactive $CD4^+$ T cells were found throughout the CNS in direct concordance with GAD65 expression and activated microglia: proximal to the circumventricular organs at the interface between the brain parenchyma and the blood brain barrier. In the presence of B cells, high titer anti-GAD65 autoantibodies were generated but these had no effect on the incidence or severity of disease. In addition, GAD65-specific $CD4^+$ T cells isolated from the brain were activated and produced IFN-γ. These findings suggest that GAD65-reactive $CD4^+$ T cells alone mediate a lethal encephalomyelitis-like disease that may serve as a useful model to study GAD65-mediated diseases of the CNS.

INTRODUCTION

Glutamic acid decarboxylase (GAD) catalyzes the conversion of glutamic acid to γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS). There are two different isoforms of GAD, GAD65 and GAD67 that are generated from two different genes. GAD is expressed in the pancreas and central nervous system and has been implicated as a target antigen in Type 1 Diabetes (T1D) (1,2), Stiff- Person Syndrome (SPS), a rare autoimmune disorder thought to occur because of an impairment of GABA production,

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and a variant of SPS, progressive encephalomyelitis with rigidity and myoclonus (PERM) (3,4).

Autoantibodies against GAD are a hallmark of T1D, with 80% of new-onset T1D patients showing detectable levels of anti-GAD antibodies prior to clinical onset of disease (5). High titers of anti-GAD antibodies are also detected in SPS patients suggesting a prominent role of the GAD antigen in this disease. In fact, there is some suggestion that the anti-GAD antibodies may inhibit the function of GABA, leading to the neurological symptoms observed in these patients (6,7). While there is considerable evidence for the presence of anti-GAD antibodies in SPS and PERM, far less is known about the role and relative importance of GAD reactive T cells in these diseases.

We had previously generated a number of GAD65-reactive $CD4^+$ T cell hybridomas or clones from NOD mice either immunized with a series of peptides comprising the major immunogenic GAD65 epitopes (8,9) or left untreated (10). The TCRs were cloned and expressed using a retroviral-mediated stem cell gene transfer system (referred to herein as retrogenic [Rg] mice) in which sublethally irradiated NOD.*scid* mice were reconstituted with NOD.*scid* bone marrow transduced with retrovirus containing a self-cleaving 2A-peptide linked TCR and a green fluorescent protein (GFP) in the same vector (11–14). Our previous studies have shown that T cells expressing GAD65-specific TCRs did not mediate diabetes nor cause insulitis (8,9), consistent with other studies (15). In the present study, we describe the surprising finding that three of these GAD65-reactive clonotypes (4B5, PA19.9G11 and PA17.9G7) induced a lethal encephalomyelitis-like disease and ataxia in Rg mice. In addition to detailing these observations, we also addressed the following questions: (1) Do the GAD65 reactive T cells infiltrate the CNS and cause inflammation? (2) In the presence of B cells, are GAD65 antibodies generated and do these affect the disease phenotype? (3) Is there a link between T cell pathogenesis, GAD65 reactivity and cytokine secretion in the inflammation observed in the CNS?

MATERIALS AND METHODS

Mice

NOD.*scid*, NOD.*Tcra7*$^{-/-}$ and *Rag1*$^{-/-}$ mice were obtained from The Jackson Laboratory and bred in-house. B6^{g7} mice were a gift from C. Benoist and D. Mathis (Harvard Medical School, Boston, MA). All mice were bred and housed at the St. Jude Animal Resources Center (Memphis, TN) in a Helicobacter-free SPF facility following state, national and institutional mandates. The St. Jude Animal Resources Center is accredited by the American Association for the Accreditation of Laboratory Animal Care. All animal experiments followed animal protocols approved by the St. Jude Institutional Animal Care and Use Committee.

TCR retroviral constructs

All TCRs were generated as 2A-linked single ORFs using recombinant PCR and cloned into an MSCV-based retroviral vector with a green fluorescent protein (GFP) marker as previously described (11,12,14). Details of cloning strategies and primer sequences are available upon request (vignali.lab@stjude.org). The PA21.14H4, PA19.9G11 and PA17.9G7 TCR were cloned from hybridomas generated by immunizing NOD mice with HEL 11–25 (PA21.14H4), GAD 221–237 (PA19.9G11), and GAD 284–300 (PA17.9G7), respectively (8). The 4B5 TCR was cloned from a GAD65-specific $CD4^+$ T cell clone established from unimmunized 4 wk-old NOD female mice using recombinant GAD65 protein for *in vitro* expansion (10). The PA21.14H4 and 4B5 TCRs have a glycine only linker between the Cα and TaV.2A (PA21.14H4) or PTV1.2A (4B5) sequence. The PA19.9G11 and PA17.9G7 TCRs have a glycine-serine-glycine linker between the Cα and PTV1.2A sequences.

Retroviral-mediated stem cell gene transfer

Retroviral-mediated stem cell gene transfer was performed as previously described (11–14). Briefly, bone marrow was harvested from the humerus, pelvis, tibia and femur of 5FU treated mice. The bone marrow was incubated with mIL-3, hIL-6 and mSCF for 48 h and then cultured on plates seeded with irradiated TCR retroviral producer cells. After 48 h, the transduced bone marrow was harvested from the plates and 4×10^6 bone marrow cells were injected into the tail veins of NOD.*scid* mice that had been irradiated with 300 Rads.

For experiments requiring both T cells and B cells, NOD.*scid* bone marrow was transduced with TCR^+GFP^+ virus and either NOD.*Tcra*$^{-/-}$ (B cells) or NOD.*scid* (control) bone marrow transduced with vector containing YFP^+ only virus. Mice received 2×10^6 NOD.*scid* TCR transduced bone marrow and either 2×10^6 NOD.*Tcra*$^{-/-}$. YFP^+ transduced bone marrow or 2×10^6 NOD.*scid* YFP^+ transduced bone marrow.

Flow cytometric analysis and cell sorting

For flow cytometric analysis, cells were stained with CD4-APC Cy7 or CD4-PE and TCRβ-APC or TCRβ–PE (BD Biosciences and eBioscience, San Diego, CA). Live, GFP^+ cells were gated for analysis unless otherwise indicated. For analysis of activation markers, cells were stained with CD69-PerCPCy5.5 (BD Biosciences San Diego, CA). For intracellular cytokine staining, the spleen or pituitary were teased into a single cell suspension. All cells were activated with anti-CD3 and anti-CD28 coated beads overnight. Following activation, Golgi plug (BD Biosciences, San Diego, CA) was added and incubated with the cells for 5.5 h. Cells were stained with CD4-Cychrome (BD Biosciences, San Diego, CA), fixed and permeablized with a cytofix/cytoperm solution kit (BD Biosciences, San Diego, CA) and stained with IFN-γ-APC (BD Biosciences, San Diego, CA). For purification of cells by FACS, splenocytes were stained with CD4-APC and TCRβ-PE (BD Biosciences and eBioscience, San Diego, CA) and gated on $GFP^+CD4^+TCR^+$ cells.

Isolation of lymphocytes from brain

Brains were extracted and homogenized through a 70 μm filter, rinsed with PBS, re-filtered and centrifuged at 1200 rpm for 5 min. Cells were then resuspended in 70% Percoll (GE Healthcare) and placed in a 40%–70% Percoll gradient and centrifuged at 2400 rpm for 30 min with no brake. The interface was collected and washed twice with PBS. The cells were then stained as outlined above.

Functional assays and cytokine analysis

Functional assays were performed by culturing 2.5×10^4 $GFP^+CD4^+TCR^+$ cells purified by FACS with 5×10^5 irradiated NOD splenocytes and a titration of GAD65 whole protein or peptides (Diamyd, Pittsburg, PA) in 10% FBS-supplemented Eagle's minimum essential medium for 48 h. Cells were pulsed with 1μCi of [^{3}H]-thymidine for 24 h and harvested. All values were corrected for background. Prior to pulsing, 50 μl of supernatant was removed and analyzed for IL-2, , IL-17 and IFN-γ using a Milliplex kit (Millipore, Billerica, MA) and Bioplex machine (BioRad, Philadelphia, PA).

Histology

Spleens were harvested and mice perfused with 2.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS pH 7.4. The brain, pituitary and spinal cord were removed from each mouse and placed in fixative overnight at 4°C. All tissues were cryoprotected in 30% sucrose in PBS for 3 days, embedded in freezing medium (Triangle Biomedical Sciences, Durham, NC), flash frozen on dry ice and stored at −80°C for later use. 20μm thick sections of brain and spinal cord and 10μm sections of pituitary were cut on a HM 560 cryostat (Microm,

Walldorf, Germany) then thaw-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were air-dried overnight then stored at −20°C. The following primary antibodies were used: rat anti-CD4 (1:50; eBiosciences, San Diego, CA), rabbit anti-Iba-1 (1:500; Wako Chemicals, Japan) and rabbit anti-GAD65 (1:500; Chemicon, Temecula, CA). To demonstrate the presence of $CD4^+$ cells in the brains of mice possessing GAD65-reactive T cells, sagittal sections were treated with anti-CD4 primary then biotinylated goat anti-rat secondary (1:500; BD Biosciences). Visualization was by diaminobenzidine (DAB) reaction using Vectastain ABC kit as per manufacturer directions. For double labeling of GAD65 with CD4, goat anti-rat Alexa Fluor 546 and goat anti-rabbit Alexa Fluor 647 (Invitrogen, Carlsbad, CA) was used on all tissues. Slides were coverslipped with Prolong Gold Antifade Reagent with DAPI (Invitrogen) to counterstain nuclei.

GAD65-specific autoantibody detection

GAD65 autoantibodies were measured using a radiobinding assay as previously described (16,17). Briefly, sera from the TCR retrogenic mice detailed were incubated with [^{3}H]-thymidine-labeled GAD65 and precipitated with protein A Sepharose (Amersham). The assay was performed on a 96-well filtration plate (Fisher) and radioactivity was counted on a Topcount 96-well plate beta counter (PerkinElmer). Antibody levels were expressed as an index. The inter-assay coefficient of variation is 10% (n=50) for GAD65 autoantibodies. The upper limits of normal controls (0.032 for GAD65 autoantibodies) were established as the 99th percentile of 198 healthy controls. In the most recent DASP (Diabetes Autoantibody Standardization Program) workshop, the sensitivity and specificity were 76% and 99%, respectively, for GAD65 autoantibodies.

RESULTS

GAD65-specific TCRs cause an encephalomyelitis-like disease

In order to investigate the role of GAD65-specific $CD4^+$ T cells in T1D, we had previously established Rg mice expressing ten different GAD65-specific TCRs (8, 9 and data not shown)). While none of the GAD65-specific TCRs mediated insulitis or T1D, surprisingly, NOD.*scid* Rg mice expressing three of these GAD65-specific monoclonal TCRs, 4B5, PA19.9G11 and PA17.9G7, developed a lethal encephalomyelitis-like disease (Table I, Fig. 1, *A* and *B*). As a control, Rg mice expressing an H-2A^{g7}-restricted TCR specific for hen egg lysozyme (HEL) (PA21.14H4) were used. It is important to note that because retroviral-transduced bone marrow from NOD.*scid* mice was used, no additional T or B cells were present in the recipient animals. Beginning on day 28 post-bone marrow transplant, the mice expressing the GAD65-specific TCRs, 4B5, PA17.9G7 and PA19.9G11 began to lose weight. Strikingly, weight loss was followed by symptoms that resemble experimental autoimmune encephalomyelitis (EAE) including a flaccid tail, abnormal righting reflex, impaired balance, involuntary movements, paralysis and eventual death (Fig. 1 *A*). Rg mice expressing the three GAD65-reactive TCRs exhibited a similar severity and time of onset of disease (Fig. 1, *A* and *B*). In contrast, NOD.*scid* Rg mice expressing the HEL-specific TCR PA21.14H4 remained asymptomatic. It is important to note we have never observed diabetes in Rg mice expressing any GAD-specific TCR in this or previous studies (9, 18 and data not shown). In addition, Rg mice expressing 4B5, PA17.9G7 and PA19.9G11 TCRs did not develop any insulitis nor peri-insulitis.

We also tested the ability of 4B5 to cause disease in a non-autoimmune prone background using *Rag1*$^{-/-}$.B6^{g7} bone marrow and sub-lethally irradiated *Rag1*$^{-/-}$.B6^{g7} mice as recipients. This resulted in a disease incidence of 60% with all mice that displayed symptoms progressing to Grade 5 (Fig. S1). However, this incidence was less than that seen on a NOD.*scid* background.

It was apparent from the symptoms that there was a high probability of encephalopathy in the experimental animals, therefore we examined brain sections from experimental and control NOD.*scid* Rg mice to determine if the GAD65-reactive T cells were infiltrating the brain. Interestingly, analysis of mouse brain sagittal sections revealed that there were $CD4^+$ T cells infiltrates throughout the brain of experimental but not control animals (Fig. S2). The location of the cellular infiltrates in the brain was consistent with the position of the circumventricular organs, the structures lining the cavity of the third and fourth ventricles where the blood brain barrier is incomplete. These regions include the pituitary gland, the vascular organ of the lamina terminalis, the subfornical organ, the pineal gland and the area postrema (19,20). Brains from control NOD.*scid* Rg mice expressing the HEL-specific TCR PA21.14H4 were devoid of $CD4^+$ cells (Fig. S2).

Next, we determined if $CD4^+$ T cell infiltration correlated with areas of GAD65 expression in the brain. T cell infiltration was localized to the regions within the brain that have been shown to express GAD65, including the pituitary gland, thalamus (Fig. 1*C*), olfactory bulb (Fig. S2) and spinal cord (Fig. S3) (21). $CD4^+$ T cell infiltration induced localized inflammation as determined by expression of Iba-1, a marker for microglial and macrophage activation predominantly in the intermediate and posterior lobes of the pituitary gland (Fig. 1*C*) (22). Taken together these data suggest that some GAD65-reactive T cells infiltrate the brain in the absence of B cells and cause localized inflammation concordant with GAD65 expression that results in a lethal encephalomyelitis-like disease.

GAD-reactive T cell development appears normal

Given the striking phenotype of the GAD65-specific TCR Rg mice, we first verified that 4B5, PA17.9G7 and PA19.9G11 T cell reconstitution was equivalent by GFP and TCR expression to the PA21.14H4 HEL-specific control (Fig. 2, *A* and *B*). While the percentage and number of T cells in the spleens of 4B5 and the control PA21.14H4 TCR NOD.*scid* Rg mice were comparable, significant reductions were seen in PA17.9G7 and PA19.9G11 Rg mice (Fig. 2, *A* and *C*). While these differences may underlie the basis for the reduced incidence of disease in PA17.9G7 and PA19.9G11 compared with 4B5 Rg mice (Fig. 1*A*), the rate of disease onset and severity was nevertheless comparable in all three GAD-specific TCR Rg mice (Fig. 1, *A* and *B*). The basis for the reduced splenic cellularity is unknown but may be due to inefficient thymic selection due to the availability of selecting peptides, differential homeostatic expansion and/or antigen-driven stimulation in the periphery, or partial exertion of central or peripheral tolerance. The level of TCR expression on the T cells was also slightly lower in PA17.9G7 and PA19.9G11 compared to 4B5 and the PA21.14H4 control Rg mice (Fig. 2*B*). It is important to point out that while there was some variability in the levels and expression of 4B5, PA17.9G7 and PA19.9G11, all three were broadly equivalent to the other GAD65-reactive TCRs expressed that did not cause the lethal encephalomyelitis-like disease ((8, 9) and data not shown)).

GAD-reactive B cells do not alter the disease incidence or severity

In addition to anti-GAD antibodies being a hallmark of T1D, they are also prevalent at high levels in SPS and are thought to be instrumental in mediating the disease. Thus we determined if the incidence and/or severity of the encephalomyelitic-like disease observed in 4B5, PA17.9G7 and PA19.9G11 Rg mice was enhanced by the presence of B cells and potentially anti-GAD65 antibodies. Accordingly, chimeric Rg mice were generated by transferring a 1:1 ratio of NOD.*scid* bone marrow transduced with retrovirus containing TCR.GFP and either NOD.*Tcrα* $^{-/-}$ bone marrow transduced with YFP only or NOD.*scid* bone marrow transduced with YFP only into NOD.*scid* mice. The NOD.*Tcrα* $^{-/-}$ mice, which lack expression of the TCRα chain, fail to develop T cells but not B cells. The chimeric Rg mice expressing 4B5, PA17.9G7, PA19.9G11 and the control PA21.14H4 TCR that received the YFP transduced

NOD.*Tcrα* $^{-/-}$ bone marrow exhibited equivalent numbers of T cells and B cells in the spleen (Fig. 3*A*). All of the GAD65-specific TCR chimeric Rg mice which had B cells developed encephalitic disease that was comparable to B cell-deficient mice. Notably, there were no differences in the incidence, rate or severity of disease (Fig. 3*B*).

Histological analysis of sagittal sections of the brain further demonstrated no significant difference in pathology between Rg mice that receive only T cells versus T plus B cells (Figs. S2 and S4). While there was no difference in the disease phenotype, B cells infiltrating the brain and the pituitary gland were clearly detected in the chimeric Rg mice (Fig. 3*C* and data not shown). In the pituitary gland, the majority of the cells were found in the intermediate and posterior lobes. Although the localization of cells was similar, there was a difference in the number of T cells present in the brain of 4B5 Rg mice deficient of or replete with B cells (Figs. 1*C* and 3*C*). Interestingly, the location of B cell infiltrates correlated directly with T cell localization and GAD65 expression (Fig. 3*C*). Similar to T cell-only Rg mice, infiltration resulted in localized inflammation as determined by expression of Iba-1 by activated microglia and/or macrophages. Finally, we assessed if anti-GAD65 antibodies were generated in the presence of B cells and GAD65-reactive T cells in the chimeric Rg mice. High titers of anti-GAD specific antibodies were generated in all three groups compared to the PA21.14H4 + B cell only control (Fig. 3*D*). These results suggest that GAD65-reactive T cells alone mediate an EAE-like disease, and that the presence of B cells and anti-GAD65 antibodies have no marked effect on the phenotype and severity of the disease.

GAD65-reactive CD4$^+$ T cells in Rg mice exhibit a pro-inflammatory phenotype

Although the GAD-specific 4B5, PA17.9G7 and PA19.9G11 TCR Rg mice described here develop a lethal encephalomyelitis-like disease, this was not observed with other GAD-specific TCR Rg mice analyzed previously (8,9). The PA17.9G7 and PA19.9G11 TCRs were cloned from T cell hybridomas that were generated by immunizing NOD mice with the GAD65 peptides 284–300 and 221–237, respectively (Table I). PA19.9G11 recognizes GAD221–237, whereas PA17.9G7 exhibits dual specificity for GAD221–237 and GAD284–300. On the other hand, the GAD65-specific 4B5 CD4$^+$ T cell clone was established from unimmunized NOD mice using recombinant GAD65 *in vitro*, and is specific for both the GAD217–236 and GAD290–309 peptides (10,23). While CD4$^+$ T cells from 4B5, PA17.9G7 and PA19.9G11 Rg mice proliferated strongly to their respective peptides, only 4B5 and PA17.9G7 Rg T cells proliferated in response to GAD65 protein (Fig. 4*A* and unpublished data). Interestingly, whereas the three CD4$^+$ T cell populations produced comparable amounts of IL-2, 4B5 and PA19.9G11 produced significant amounts of IFN-γ upon stimulation with GAD65 protein *in vitro*, and 4B5 and PA17.9G7 produced IL-17 (Fig. 4*B*).

We next determined if the GAD65-specific T cells were activated and secreting IFN-γ in the brain and pituitary of TCR Rg mice. T cells in the brain and pituitary expressed elevated levels of CD69, indicating an activated phenotype (Fig. S5). Furthermore, an increased frequency of GAD65-specific CD4$^+$ T cells expressing intracellular IFN-γ was detected in the brain and pituitary of 4B5 and PA19.9G11 TCR Rg mice (Fig. 4, *C* and *D*). These results suggest that CD4$^+$ T cells expressing the 4B5, PA17.9G7 and PA19.9G11 clonotypic TCRs recognize GAD65 in the brain and pituitary, and exhibit a pro-inflammatory phenotype.

DISCUSSION

GAD is a critical enzyme involved in the production of GABA, the main inhibitory neurotransmitter in the CNS, and is a primary target for autoantibodies in patients suffering from SPS and PERM. These patients present with symptoms ranging from muscular rigidity and superimposed spasms, stiff limbs and progressive encephalomyelitis with rigidity (24). However, with the exception of a few publications (25–28), little information is available on

the extent of T cell involvement in SPS and PERM despite reports of lymphocyte infiltration into the CNS of these patients (29,30).

While the fortuitous discovery that certain GAD65-reactive $CD4^+$ T cell populations can cause a lethal encephalomyelitis-like disease in mice was important, the phenotype of the disease versus the clinical features of PERM/SPS is different. However this may be the consequence of anti-GAD TCR clonality in the Rg system and/or the severity and rapid, lethality of the disease, which may supersede the typical clinical features associated with PERM/SPS. Consequently, the GAD65-reactive $CD4^+$ T cell-induced encephalomyelitis-like disease may provide some insight into the encephalomyelitis observed in PERM. The three GAD65-specific T cell clonotypes, 4B5, PA17.9G7 and PA19.9G11, infiltrated the brain, secreted IFN-γ and mediated inflammation as determined by microglial activation resulting in a lethal encephalomyelitis-like disease. Although seen throughout the CNS, the primary sites of T cell infiltration were localized in and around the GAD positive GABAergic cells located proximal to the circumventricular organs, where there is an interface between the brain parenchyma and the blood (19,20). Additionally, throughout the experiments it appeared that the number of $CD4^+$ T cells as well as the depth of their penetration into the parenchyma increased with disease progression. However further analysis is required to validate these observations. We also found that the encephalomyelitic disease was mediated in the absence of B cells and importantly GAD65-specific autoantibodies. Indeed, despite high titers of anti-GAD65 autoantibodies in B cell-replete chimeric Rg mice, no significant differences were detected in disease frequency or severity relative to B cell-deficient Rg mice. Taken together these findings show that GAD65-specific autoantibodies are neither required nor participate in mediating the lethal encephalomyelitis-like disease observed in GAD65-specific Rg mice. It is tempting to speculate, that the high titer GAD65-specific autoantibodies seen in PERM patients may not be the only contributors to disease onset or progression and that perhaps GAD65-specific T cells may also play a role in mediating pathology at sites were GABAergic cells are located.

It is currently unclear why T cells expressing these three particular GAD65-specific TCRs mediate the lethal encephalomyelitic disease observed whereas other GAD65-specific TCR Rg mice that we have previously analyzed fail to develop this disease ((8,9) and data not shown). There are four possibilities. First, it is interesting to note that with the exception of one TCR, PA19.9G11, PA17.9G7 and 4B5 were the only TCRs from a panel of 10 GAD65-specific TCR that recognize the 217–236 epitope. The other TCR, 1A4, not studied in detail here as it is expressed poorly, caused an encephalomyelitic disease albeit at a reduced incidence compared to PA19.9G11, PA17.9G7 and 4B5 (data not shown). This suggests that GAD65 217–237 may represent a key immunogenic epitope in the disease. Second, 4B5 and PA17.9G7 TCRs, which were particularly pathogenic, share the unusual feature of exhibiting dual specificity (GAD 217–236/GAD 290–309 and GAD 221–237/GAD 284–300 respectively), which might contribute to their escape from tolerance and their ultimate pathogenicity. Third, relatively few of the GAD65-specific Rg T cell populations we have tested respond strongly to the whole GAD65 protein. However, it seems less likely that this alone is a deciding factor as 4B5, PA17.9G7 and PA19.9G11 Rg T cells exhibit quite different reactivities to GAD65 protein despite mediating comparable levels of disease incidence and severity (Figs. 1 and 4*A*). Lastly, it is important to note that the avidity/affinity of the respective TCRs could be a key determinant in regulating T cell pathogenicity. For example, GAD65-specific T cell clones exhibiting poly-specificity appear to express TCR with increased avidity/affinity relative to mono-specific TCRs (23). It is clear that more studies will be required to establish a link between epitope specificity, TCR avidity/affinity and/or GAD protein reactivity, and disease pathogenicity. It is noteworthy that the versatility of the TCR Rg system provides an ideally flexible and powerful platform to test these issues in the future (9,11,12).

In summary, we show for the first time that GAD65-specific $CD4^+$ T cells directly mediate pathology in the CNS leading to a lethal encephalomyelitis-like disease. This system may represent a useful model for studying GAD65-related CNS diseases. Furthermore, given that GAD65-specific T cells have been observed in T1D patients, our observations question whether these might be implicated in some of the neurological complications that are occasionally observed in T1D patients. In addition, there has recently been increased interest in the potential use of antigen-specific therapies in T1D. Even though a recent trial involving GAD immunization did not report any SPS-like observations (31), our data suggest that caution should be exercised in the use of any therapy involving GAD or GAD-specific T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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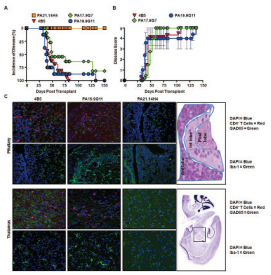

Figure 1.

GAD65-reactive CD4$^+$ T cells cause a lethal encephalomyelitis-like disease. *A*, Incidence of disease in NOD.*scid* mice transplanted with bone marrow transduced with retrovirus encoding the TCR listed. *B*, Disease severity as determined by disease score. An EAE-based scoring system was used to measure disease severity: 0 - normal; 1 – lethargic, ruffled fur, weight loss of at least 10%, flaccid tail; 2 – 1 plus abnormal righting reflex; 3 – impaired balance and/or ataxia, involuntary movements, weakness or stiffness of limbs, usually asymmetric; 4 – paralysis and/or persistent tremors, possible incontinence; 5 – moribund or death (32). The number of mice followed for incidence is as follows: PA21.14H4 (9), 4B5 (17), PA19.9G11 (8), and PA17.9G7 (11) while 5 mice from each group were monitored for disease score as outlined in the materials and methods section. *C*, Pituitary (upper panel) and thalamus (lower panel) sections of mice with a Grade 3 score (Grade 0 for PA21.14H4) were stained with either anti-mouse CD4 (red), anti-GAD65 (green) and DAPI (blue), or anti-Iba-1 (green) and DAPI (blue). The diagrams on the right side depict the anterior (Ant), intermedia (Int) and posterior (Post) regions of the pituitary gland (upper panel) and a sagittal section of a brain and the location of the thalamus (lower panel). Data is representative of 6 experiments with 8–17 mice per TCR group.

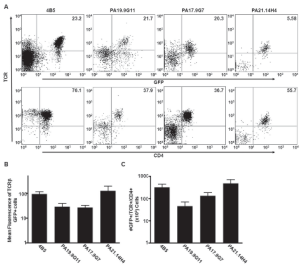

Figure 2.

T cell reconstitution is equivalent between Rg mice expressing GAD65-specific and control TCRs. *A*, Representative flow cytometric plot illustrating GFP^+TCR^+ cells (top panel) and $CD4^+TCR^+$ cells (bottom panel) in the spleens of representative TCR NOD.*scid* Rg mice. The GFP^+TCR^+ plots were gated on live cells and the $CD4^+TCR^+$ plots were gated on live, GFP^+ cells. *B*, Mean fluorescence of TCR expression of GFP^+ cells in spleens of Rg mice represented in *A*. *C*, Number of $GFP^+TCR^+CD4^+$ cells in the spleens of TCR Rg mice represented in *A*. Data is representative of 6 experiments with 4–11 mice per TCR group.

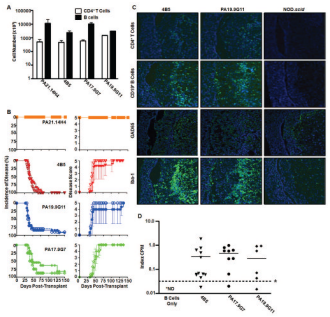

Figure 3.

The presence of B cells and GAD65 autoantibodies in Rg mice has no effect on the disease mediated by GAD65-specific CD4$^+$ T cells. *A*, Number of CD4$^+$ T cells and CD19$^+$ B cells in spleens of chimeric Rg mice receiving NOD.*scid* bone marrow transduced with retrovirus encoding the TCR and either NOD.*Tcra*$^{-/-}$ or NOD.*scid* bone marrow transduced with retrovirus encoding YFP only. Numbers were determined by gating on live, GFP$^+$ (T cells) or YFP$^+$ (B cells) cells. *B*, Left panel depicts incidence of disease and right panel indicates disease severity of chimeric Rg mice receiving 2 × 10^6 TCR retroviral-transduced bone marrow cells from NOD.*scid* and vector transduced bone marrow from either NOD.*scid* (T-cells alone) or NOD.*Tcra*$^{-/-}$ (T cells + B cells) mice. The T cells alone group is depicted as closed symbols and the T cells plus B cells group is depicted as open symbols with a dashed line. The number of mice followed for incidence is as follows: PA21.14H4 (4), PA21.14H4 + B cells (7), 4B5 (7), 4B5 + B cells (15), PA19.9G11 (4), PA19.9G11 + B cells (10), PA17.9G7 (5) and PA17.9G7 + B cells (8) while 5 mice from each group were monitored for disease score as outlined. *C*, Pituitary gland sections from NOD.*scid* mice receiving 4B5- or PA19.9G11-transduced NOD.*scid* bone marrow plus NOD.*Tcra*$^{-/-}$ YFP only transduced bone marrow, and an unmanipulated NOD.*scid* mouse as control. Sections were stained with anti-mouse CD4 (T cells), anti-mouse CD19 (B cells), anti-mouse GAD65 (GAD65) or anti-mouse Iba-1 (activated macrophages/microglial) (green) and counterstained with DAPI (blue). Mice in the 4B5 + B cells and PA19.9G11 + B cells groups displayed clinical signs of disease at the time of euthanasia. *D*, GAD65-specific autoantibody titres in serum from the chimeric Rg mice at various states of disease are depicted. Data is representative of 4 experiments with 4–15 mice per TCR group.

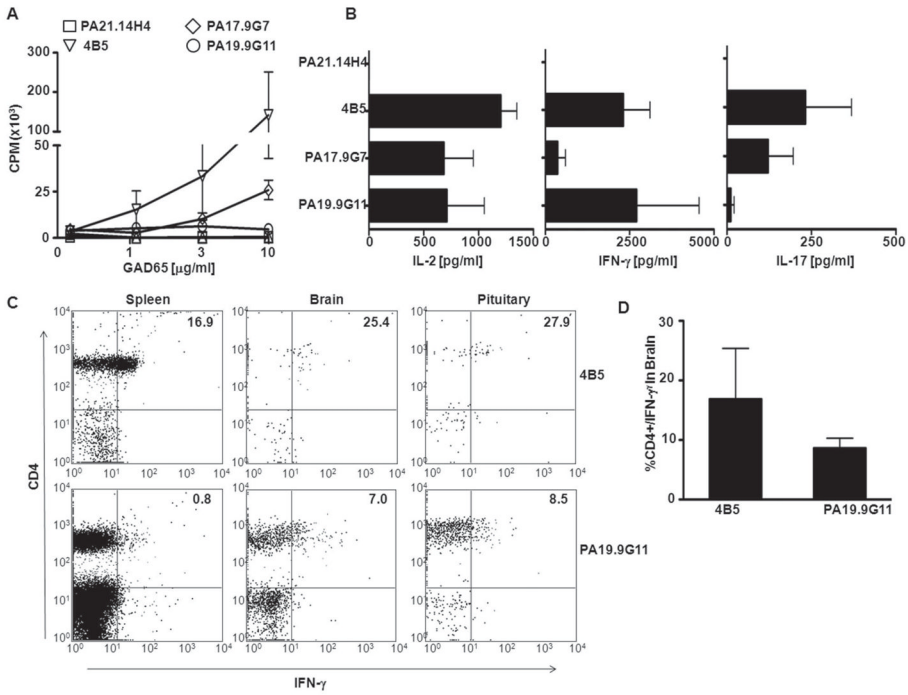

Figure 4.

GAD65-specific $CD4^+$ T cells isolated from the brain are activated and secreting IFN-γ. *A*, Reactivity of GFP^+CD4^+ splenic T cells (2.5×10^4) purified by FACS to GAD65 whole protein was determined in the presence of 5×10^5 irradiated APCs for 48 hr, pulsed with [^{3}H]-thymidine and harvested 24 hrs later. Background counts were subtracted from the data depicted. *B*, Concentration of IL-2, IFN-γ and IL-17 in supernatants of cells depicted in *A* following incubation with 10μg/ml GAD65. *C*, Representative flow cytometric plots of $CD4^+$ IFN-γ^+ cells in the spleen, brain and pituitary of 4B5 and PA19.9G11 retrogenic mice displaying a disease score of 3 which was six weeks post transplant. Plots were gated on GFP^+ cells. *D*, Percentage of GFP^+CD4^+IFN-γ^+ cells in brains of 4B5 and PA19.9G11 Rg mice six weeks post transplant, displaying a disease score of 3. For *A* and *B* data is representative of 2 experiments with spleens and lymph nodes of 3–4 mice pooled for each TCR. For *C* and *D* data is representative of 4 experiments with 4 mice for each TCR.

Table I

GAD specific T cell receptors

		TCR Usage		
TCR Name	**Antigen Specificity**	**Vα**	**Vβ**	**TCR generated from NOD mice by:**
PA17.9G7	GAD221–237 & GAD284–300	4.5	10	Immunization-GAD284–300
PA19.9G11	GAD221–237	17.1	5.2	Immunization-GAD221–237
4B5	GAD217–236 & GAD290–309	2.4	6	Unimmunized Mice
PA21.14H4	HEL11–25	13.1	6	Immunization with HEL 11–25