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Age-Dependent Loss of Tolerance to an Immunodominant Epitope of Glutamic Acid Decarboxylase in Diabetic prone RIP-B7/DR4 Mice

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Abstract

We have identified for the first time an age-dependent spontaneous loss of tolerance to two self-antigenic epitopes derived from putative diabetes associated antigens glutamic acid decarboxylase (GAD65) and glial fibrillary acidic protein (GFAP) in RIP-B7/DRB1*0404 HLA transgenic mice. Diabetic and older non-diabetic mice exhibited a proliferative response to an immunodominant epitope from GAD65 (555-567) and also from GFAP (240-252) but not from an immunogenic epitope from diabetes associated islet-specific glucose-6-phosphatase catalytic subunit-related protein. The response to both of these self-antigens is not observed in young mice but is observed in older non-diabetic mice, and is accompanied by histological evidence of insulinitis in the absence of overt diabetes. Islet infiltrates in older non-diabetic mice and diabetic mice contain CD4⁺/FoxP3⁺ cells and suggest the presence of a regulatory mechanism prior and during diabetic disease. Diabetes penetrance in RIP-B7/DR0404 mice is 23% with a mean onset age of 40 weeks and is similar to that reported for RIP-B7/DR0401 mice. A gender preference is observed in that 38% of female mice become diabetic compared to 8% of male mice.

Keywords

Diabetes; Tolerance; MHC; T cells; Transgenic

INTRODUCTION

Type I diabetes (T1D) is an autoimmune disease characterized by pancreatic islet tissue destruction mediated by autoreactivity within both the cellular and humoral arms of the immune system. The disease has a strong association in both humans and the primary mouse model of

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diabetes the non-obese diabetic mouse (NOD) with specific polymorphic antigen-presenting MHC class II molecules suggesting a direct involvement of CD4⁺ T cells and their stimulating self-antigens in disease progression. Extensive MHC peptide-binding studies indicate that MHC molecules, polymorphic in their peptide binding regions, will have unique peptide binding profiles. Based upon these observations, proposed hypotheses by which these diabetes-associated peptide-binding MHC molecules predispose towards diabetes includes: 1.) aberrant thymic selection (age-dependent) resulting in ineffective negative selection of autoreactive T cells or a skewed selection of regulatory T cell elements (not mutually exclusive) and 2.) environmentally-driven temporal changes in the presentation of MHC-dependent pancreatic specific tissue antigens resulting in T cell activation. Further support for the importance of specific human MHC class II molecules in the pathogenesis of diabetes comes from transgenic mice carrying human susceptible and non-susceptible MHC class II genes [1]. These class II deficient (I-A β ^{0/0}) mice expressing the B7-1 costimulatory molecule (CD80) under the rat-insulin-promotor (RIP-B7), develop diabetes if they also express the human-diabetes associated MHC genes DQA1*0301/B1*0302 (DQ8) while those carrying the non-diabetes associated MHC genes DQA1*0103/B1*0601 or the mouse endogenous class II genes are diabetes-free [2]. RIP-B7 mice transgenically expressing the diabetes associated DRA1*0101/B1*0401 or DRA1*0101/B1*0301 genes are also diabetic prone [3,4]. These humanized mouse models of diabetes have provided unique reagents in which to identify human class II restricted antigens within putative autoantigens under immunization conditions that may be pertinent in human diabetes and subsequently be targets for testing immune interventions aimed at ameliorating or preventing diabetes [5-8].

Among an increasing number of candidate autoantigens, the pancreatic antigens insulin, glutamic acid decarboxylase 65 (predominantly GAD67 in the mouse islet), and islet antigen 2 (IA2) have been studied extensively, while other potential self-antigens such as islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and glial fibrillary acidic protein (GFAP) have either been studied less well or are relatively newcomers as potential self-antigens involved in diabetes. CD4⁺ GAD65 reactive T cells have been identified and correlated with type I diabetes in humans and GAD reactive CD4⁺ T cells and specific epitopes have also been identified and implicated in diabetes in the NOD mouse [9-12]. GAD65 reactivity has also been identified in diabetic RIP-B7/DRB1*0401 mice, however specific epitopes to which they react to are unknown. GFAP is an islet non-specific self-antigen with expression in the Schwann cells surrounding the islets whose CD4⁺ T cell reactivity to appears to precede lymphocyte infiltration into the NOD islet [13]. IGRP is a major self-protein target driving CD8 T cell expansion in the NOD islets and as a target in TcR transgenic settings is capable of inducing diabetes [14,15]. In this study we: 1.) show that RIP-B7 mice transgenic for the DR4 subtype DRA1*0101/B1*0404, reported in some human studies to be a diabetes protective allele, [16] are also spontaneously diabetic with kinetics and penetrance of disease similar to RIP-B7/DRB1*0401 mice and 2.) for the first time identify an age-dependent spontaneous loss of cellular tolerance in RIP-B7/DRB1*0404 mice to an immunodominant epitope in GAD65 555-567 (identical in sequence to mouse GAD65 & GAD67) and a DR4-binding epitope within GFAP (240-252) which precedes diabetes. Immune reactivity to a DR4-binding self-antigen epitope within the putative diabetes associated molecule IGRP was not observed. In addition we also demonstrate that while there is a cellular reactivity to GAD65 with age in these mice, a humoral response to GAD65 was undetectable at all ages tested using a standardized radio-labeled GAD65 assay. Cellular reactivity to these two self-antigens was observed in older but not younger non-diabetic mice and correlates with a CD4⁺ lymphocytic islet infiltrate containing both FoxP3⁻ and FoxP3⁺ cells. The finding of epitope-specific cellular reactivities in these diabetes-prone humanized mice that are mimics of the human disease emphasizes the utility of these mice in understanding and testing MHC-dependent antigen-specific interventions aimed at preventing human diabetes.

MATERIALS AND METHODS

Mice

Class II deficient C57Bl/6 mice (I-Ab^{0/0}) transgenic for the DRA1*0101 and DRB1*0401 genes (DR0401-IE mice) were obtained from Taconic (Germantown, NY) and kept in a specific pathogen free facility. DR0404-IE mice (containing the DRB1*0404 gene instead of DRB1*0401 on the I-Ab^{0/0} C57Bl/6 background) were made in the same manner as previously reported for DR0401-IE mice [17]. These C57Bl/6 I-Ab^{0/0} mice express a human-mouse chimeric class II molecule in which the TCR interacting and peptide binding domains of mouse I-E (domains α 1 and β 1, exon 2 in both genes) have been replaced with the α 1 and β 1 domains from DRA1*0101 and DRB1*0401 (or DRB1*0404) respectively. Retention of the murine α 2 and β 2 domains allows for the cognate murine CD4-murine MHC interaction. C57Bl/6 mice transgenic for the costimulatory molecule B7-1 driven by the rat-insulin-promoter (RIP-B7 mice) were obtained from Li Wen. RIP-B7 mice were crossed with DR0401-IE and also DR0404-IE mice to generate RIP-B7/DR0401-IE (B7/DR0401) and RIP-B7/DR0404-IE (B7/DR0404) mice. Mice were monitored weekly for blood glucose via saphenous veins bleeds using a One-Touch FastTake glucometer (LifeScan, Milpitas, CA). Mice with blood glucose readings above 250 mg/dl were rechecked 24 hours later and considered diabetic if both readings were above 250 mg/dl. At this point mice were sacrificed for experiments. All animal work was approved by the Benaroya Research Institute (BRI) Animal Care and Use Committee (ACUC) and animals were housed in the BRI AAALAC-accredited animal facility.

Histology

Fresh tissues were fixed in phosphate-buffered formalin for paraffin-embedding and hematoxylin and eosin staining (H & E) or frozen in Tissue-Tek OTC compound (Sakura Finetek, Torrance, CA 9050) for H&E staining and immunofluorescence on frozen sections. Frozen sections (5 μ m) were fixed in (-4°C) acetone for 10 minutes, dried and biotin blocked (Kit E-21390 Molecular Probes, Eugene OR, USA) followed by blocking with PBS-blocking buffer (PBS containing 0.1% NaN₃, 1% FBS and 2% Horse serum) for 30'. The following primary Ab were used at 1:100 dilution: CD4-Alexa-Fluor 488 (MCD0420), CD8-Alexa-Fluor 488 (MCD0820), control Rat IgG Alexa-Fluor 488 (R2a20, Caltag, Burlingame, CA, USA), and biotin-labeled CD45R (RA3-6B2), biotin-labeled CD4 (GK1.5), and Alexa-Fluor 488 FoxP3 (FJK-16s, eBioscience, San Diego, CA, USA). Alexa-Fluor 568-streptavidin (Molecule probes, Eugene, OR, USA) was used a secondary reagent at 1:100 for detecting biotinylated primary antibodies. For FoxP3 staining, tissues were pre-treated for 30 minutes in FoxP3 stain buffer (PBS, 1% goat serum, 1% BSA, 0.1% tween-20) containing 1 mg/ml RNase and 170 U/ml DNase. All subsequent staining on sections for FoxP3 were done with FoxP3 stain buffer containing 5% FBS. All immunofluorescence staining was done at room temperature. H&E and fluorescence images were taken on a Leica DM IRB microscope.

Proliferation assays

Single cell suspensions of lymph node cells (LNC) from inguinal, mesenteric and brachial lymph nodes and spleen cells were prepared by gently pressing through 0.40 μ m nylon cell strainers (BD-Falcon REF 352340, Bedford MD) in Hanks buffer (Gibco, Rockville MD) and spun down (1000 rpm, 200g). Splenic RBC were lysed using ACK lysis buffer [18] for 5' at 37°C at which time ~25 ml of media was added and cells spun down (200g). Splenocytes were resuspended in DMEM-10 (DMEM cat 11965-092 (Gibco, Rockville MD.) supplemented with 10% FBS (Hyclone, Logan Utah), 100 μ g/ml Penicillin, 100U/ml Streptomycin, 50 μ M β me, 2mM glutamine and 1mM sodium pyruvate (Gibco, Rockville MD)). Lymph node response assays were carried out in 96 round-bottom plate wells with 2x10⁵ LNC and cultured with 2x10⁵ splenocytes that had been irradiated with 3000 Rads from a Cesium- γ source, in a volume of 150 μ l. Supernatants for cytokine analysis were taken (50 μ l) at 72 hrs. and 1 μ Ci/well

of ^3H -thymidine (Perkin/Elmer Life Sciences, Boston MA) was added at 72 hours. Thymidine incorporation was assayed at 96 hours using a liquid scintillation counter analyzed on a scintillation counter (Wallac-Perkin/Elmer Life Sciences, Boston MD.) at 96 hours. Splenocyte responses were measured in the same manner using 5×10^5 splenocytes per well. Stimulation indices were calculated as the quotient of specific antigen response at 100 $\mu\text{g/ml}$ divided by control peptide (HA 307-319) response at 100 $\mu\text{g/ml}$. Cytokines in supernatants from soluble CD3 (clone 145-2C11, 2.0 $\mu\text{g/ml}$)/CD28 (clone 37.51, 0.2 $\mu\text{g/ml}$) stimulated splenocytes at 72 hours were measured using Mouse Th1/Th2 Cytokine Kit (BD Bioscience Pharmingen, San Diego, CA)

Peptides

The following peptides were synthesized on an Applied Biosystems 432A peptide synthesizer (Foster City, CA): mouse GAD65 (555-567) NFFRMVISNPAAT, mouse GFAP (240-252) TQYEAVATSNMQE, and mouse IGRP (247-258) DWIHIDSTPFAG. Peptides were resuspended in DMSO at 50mg/ml and diluted in DMEM-10 prior to use in assays.

Detection of mouse GAD65 antibodies

The mouse GAD65 (mGAD65) binding capacity of mouse sera was determined as previously described [19]. The protocol was modified by using protein-G in place of protein-A for the immunoprecipitation of mouse antibodies. Briefly, in vitro translated ^{35}S -labeled mGAD65 was immunoprecipitated, in triplicate, overnight at 4°C with different dilutions of sera from B7/DR0404 mice or sera from GAD65 immunized DR0401-IE/DR0404-IE mice. After 18hr the antibody-mGAD65 complex was separated from unbound antigen by 7.5% protein G sepharose (Zymed, San Francisco, CA) using a multiwell-adapted procedure (Millipore, Bedford, MA). Plates were washed 8 times with washing buffer containing 0.15% Tween 20 and 0.1% bovine serum albumin, dried, and counted using a Wallac 1450 Micro Beta Liquid Scintillation Counter. Concentrations of mGAD65 antibody were expressed as an antibody index calculated from the following formula: mGAD65Ab index = (cpm of unknown samples - average cpm of negative standards)/(cpm of positive control sample - average of negative standards). The positive control standard used was sera from the GAD65 immunized DR0404 mouse diluted at 1:25 to give an Ab index of 1.0. An antibody positive serum against mGAD65 was obtained from DR0401-IE/DR0404-IE mice that were immunized with 50 μg of recombinant *E. Coli* generated human GAD65 (courtesy of Zymogenetics, Seattle, WA) in CFA and boosted on days 14 and 28 in IFA. Serum was taken on day 38.

RESULTS

RIP-B7/DR0401 and RIP-B7/DR0404 mice spontaneously become diabetic

C57Bl/6 class II deficient (I-Ab^{0/0}) mice transgenic for DRA1*0101/B1*0401 (DR0401-IE mice) and DR0404-IE mice were bred with I-Ab^{0/0}/RIP-B7 (on C57Bl/6 background) mice to generate RIP-B7/DR0401-IE (B7/DR0401) and RIP-B7/DR0404-IE (B7/DR0404) mice. Beginning at eight weeks of age, 22 B7/DR0401 mice (10 ♀, and 12 ♂) and 26 B7/DR0404 (13 ♀, 13 ♂) mice were monitored weekly for blood glucose (non-fasting) via saphenous vein bleeds. Mice in which blood glucose was greater than 250 mg/dl were assayed again 24 hours later and considered diabetic if the second blood glucose reading was also greater than 250 mg/dl. Normal blood glucose in non-diabetic mice was 115 ± 15 mg/dl (n=10, ages 6-40 weeks of age, averaged over 5 weeks). B7/DR0401 and B7/DR0404 mice become diabetic starting at about 20 weeks of age (Figure 1) with 2nd day blood glucose readings in diabetic mice averaging 468 ± 55 mg/dl and 380 ± 76 in B7/DR0401 and B7/DR0404 mice respectively. Diabetes penetrance over the 54-week study was 27% with a mean diabetic onset age of 37 ± 9 weeks in B7/DR0401 mice and 23% with a mean diabetic onset age of 40 ± 9 weeks in B7/DR0404 mice. Gender skewing towards diabetes was observed in B7/DR0401 mice where

50% of female mice (5 of 10) and only 8% (1 of 12) male mice became diabetic (Table 1). The same trend was also observed in B7/DR0404 mice where 38% of female mice (5 of 13) and only 8% (1 of 13) male mice became diabetic. Diabetic mice also exhibited an extensive cellular infiltrate into the islets of the pancreas that correlated with diabetes (Figure 2A, 2B) that was not observed in 8 week old female non-diabetic mice (Figure 2C, 2D). Characterization of the infiltrate using immunofluorescence on frozen diabetic pancreatic tissue indicated an infiltrate consisting of CD4, CD8, and CD45R (B220) positives cells (Figures 2F, 2G, and 2H) and is consistent with what has been observed in RIP-B7/DQ8 and RIP-B7/DR0401 mice [2,3]

Diabetic mice respond to GAD65 (555-567) and GFAP (240-252)

Diabetic mice were assayed for LN responses to three DR4-binding pancreatic self-antigen epitopes GAD65 (555-567), GFAP (240-252), and IGRP(247-258). A mouse-human sequence comparison of these three peptides is shown in table II. GAD65 (555-567) is identical in sequence in mouse and human and was chosen as it was the only GAD65 epitope identified from MHC class II eluted peptides from a GAD65 transfected DR4 cell line [20]. Mouse GFAP (240-252) and IGRP(247-258) were identified as putative DR0404-binding epitopes through the use of the MHC peptide-binding predictive algorithmic program Tepitope [21]. Mouse GFAP (240-252) and IGRP (247-258) differ from their human sequences by two residues that reside in the Tepitope-predicted P4 and P6 (GFAP) and P6 (IGRP) MHC-binding pockets respectively and suggest that the predicted TcR contact residues in both of these epitopes are identical in human and mouse sequences. However, an alternative binding profile for mouse IGRP(247-258) is also predicted that would change the P5 TcR contact residue between the mouse and human epitopes.

As shown in Figure 3A, diabetic B7/DR0404 mice exhibit a spontaneous response to GAD65 (555-567) and GFAP (240-252) but not to IGRP (247-258). Human GAD65 (555-567) is also identical in sequence to mouse GAD65 and mouse GAD67. The epitope-specific response to GAD65 found here extends previous findings in B7/DR0401 mice where a weak response to whole GAD65 was reported but specific epitopes were not identified [3]. A summary of the response in all diabetic mice tested to GAD65 (555-567) and GFAP (240-252) is shown in figure 3B and is compared to the response in 8-week-old non-diabetic B7/DR0404 mice. Diabetic B7/DR0401 mice also exhibited a spontaneous response to GAD65 (555-567) (data not shown). Both DR0401-IE and DR0404-IE mice are capable of responding to IGRP (247-258) peptides under immunizing conditions (Figure 3C) suggesting that the spontaneous response to GAD65 (555-567) and GFAP (240-252) is antigen-specific, and is not due to a loss of global tolerance to these pancreatic proteins.

Response of B7/DR4 mice to GAD65 (555-567) and GFAP (240-252) precedes diabetes

Because insulinitis precedes overt diabetes in the NOD mouse [22] and most likely also in humans, suggestive of a self-antigen driven ongoing immune response prior to overt diabetes, we investigated whether non-diabetic mice would also respond to GAD65 (555-567), GFAP (240-252), and IGRP (247-258). While young non-immunized non-diabetic B7/DR0404 mice do not exhibit a detectable response to these epitopes at 8-12 weeks of age (Fig 3B and 4), older non-diabetic B7/DR0404 mice (54, 36, and 23 week old mice) were responsive to hGAD65 (555-567) and GFAP (240-252) but not IGRP (247-258). Similar results were obtained from 46 and 17 week old B7/DR0401 mice (data not shown). All of these mice were non-diabetic with blood glucose measurements between 100-130 mg/dl. The cellular reactivity to GAD65 (555-567) and GFAP (240-252) was not the result of a loss of global tolerance as IGRP (247-258) (which is capable of mounting a response in DR0404 mice under immunizing conditions, figure 3C) did not show any stimulatory capacity in these non-diabetic mice (Figure. 4C.). A summary of the age-dependent response to GAD65 (555-567) and GFAP (240-252) in B7/DR0404 mice is shown in figure 4. Interestingly, the magnitude of the response

to GAD65 (555-567) in non-diabetic mice is greatest in 36 week-old mice and is less in 54 week-old non-diabetic mice and also in the diabetic mice. A similar type of response to GAD65 (and GAD67) is also observed in NOD mice where the response to GAD65 is greatest in 8 week-old non-diabetic mice [9]. Cytokine responses from direct ex-vivo self-antigen stimulated B7/DR0404 splenocytes were at the detection limits of the assay (data not shown) precluding a determination of Th1/Th2 skewing to these self-antigens. However, a Th1-type of response was observed in B7/DR0404 mice (Figure 5) upon CD3/CD28 stimulation and is contrary to a report of a Th2 response to CD3 only stimulation observed in B7/DR0401 mice [3]. While the majority of mice did not become diabetic over the 54-week study, several of the older non-diabetic mice (3 of 6 and 1 of 3 for 54 and 46-week old mice respectively) did have insulinitis (in less than 10% of their islets, Figure 6), which was not observed in any islets in 17-week old mice. The CD4⁺ infiltrate in these older non-diabetic mouse islets contained CD4⁺/FoxP3⁻ and the T-cell regulatory type CD4⁺/FoxP3⁺ cells (Figure 6).

Anti-mGAD65 antibodies are not detected in non-diabetic nor diabetic B7/DR0404 mice

As GAD65 antibodies are detected in the prediabetic stage in both rodents and humans [9,23, 24] we assayed serum samples from young non-diabetic, older non-diabetic, and diabetic B7/DR0404 mice for antibodies against mouse GAD65 (mGAD65). GAD65 antibody-positive control serum was obtained from DR0401-IE/DR0404-IE mice that had been immunized and boosted twice with *E. Coli* generated recombinant GAD65 protein. As shown in figure 7, recombinant GAD65 immunized mice, but not non-immunized DR0404-IE mice exhibit an antibody response to radio-labeled in vitro translated mGAD65 at serum dilutions as low as 1:6400. Anti-mouse GAD65 antibodies were not detected in 1:11 diluted serum of young nor older non-diabetic B7/DR0404 mice nor from serum taken from diabetic B7/DR0404 mice (Figure 7). Undetectable anti-mGAD65Ab were also obtained 1:25 and 1:100 diluted serum from B7/DR0404 mice, the latter tested to eliminate the possibility of a prozone effect.

DISCUSSION

The combination of class II deficient (I-A^{0/0}) non-autoimmune prone C57Bl/6 mice and transgenic expression of autoimmune-correlated human MHC genes creates a host environment in which the animals are prone to spontaneous diabetes when the co-stimulatory molecule CD80 (B7-1) is driven by the rat-insulin-promotor (RIP). Forced expression of the costimulatory molecule B7-1 bypasses early indirect events, most likely due to environmental insults which are believed to initiate diabetes in humans [25,26], such that RIP-B7/HLA mice display a CD4 and CD8 T cell spontaneous infiltrate into the pancreas correlating with diabetes [2]. The strength of this model is also underscored in that these same mice transgenic for non-diabetes associated HLA genes are not spontaneously diabetic. We have extended studies on these HLA transgenic diabetic prone mice by: 1.) showing that mice transgenic for the DR4 subtype DRA1*0101/B1*0404 genes are also diabetes prone and 2.) identified an age-dependent spontaneous loss of tolerance to two islet antigens GAD65 (555-567) and GFAP (240-252) during the pre-diabetic and diabetic phases.

Population studies addressing the contribution of the DR4 subtype DRA1*0101/B1*0404 (DR0404) in human diabetes susceptibility is divided as protective, neutral, and susceptible conclusions have been drawn for this particular DR4 subtype MHC gene [16,27-29]. Lack of consensus on the contribution of DR0404 in human diabetes can be partly explained by linkage disequilibrium, which makes it difficult to study a single MHC gene and correlating it to diabetes. We find that RIP-B7 mice transgenic for the HLA DR0404 gene complex like B7/DR0401 mice are diabetes prone with similar kinetics (average diabetes onset age of ~40weeks) and penetrance of disease. Diabetes in these mice was gender-dependent with 50% and 38% of females becoming diabetic in B7/DR0401 and B7/DR0404 mice respectively and only 8% of males becoming diabetic. This gender preference for susceptibility to diabetes in

B7/DR4 mice is similar to that observed in the NOD mouse but is in contrast to a previous report on diabetes in B7/DR0401 mice [3].

Using three DR4-binding self-antigen epitopes derived from sequences within the diabetes-associated autoantigens glutamic acid decarboxylase 65 (GAD65), Glial fibrillary acidic protein (GFAP), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) we find that at the time of diabetes B7/DR0404 mice exhibit a spontaneous loss of T cell tolerance to GAD65 (555-567) and GFAP (240-252) but not IGRP (247-258). While all three of these epitopes are capable of generating T cell immune responses in immunized DR0401 and DR0404 animals, these spontaneous responses to GAD65 (555-567) and GFAP (240-252) are absent in non-diabetic unimmunized 8-12 week old B7/DR4 mice. However, non-diabetic mice ages 23-54 weeks of age show evidence of a specific loss of tolerance to GAD65 (555-567) and GFAP (240-252) but not to IGRP (247-258) in the absence of overt diabetes. The natural processed DR4 epitopes within GFAP and IGRP are not yet known, however, the loss of tolerance to GFAP (240-252) in older B7/DR0404 mice is suggestive that this epitope is within a naturally processed GFAP epitope. Human GAD65 (hGAD65) (555-567) is a self-antigen DR4-binding peptide within a naturally processed epitope of GAD65 capable of eliciting T cell responses in both HLA-DR4 humans and mice [5,7,20,30]. The response to these two self-antigens precedes the average diabetes onset-age by at least 16 weeks. We have also observed that many mice beyond the average disease-onset age have normal blood glucose but have insulinitis in some of their islets, an observation reported in non-diabetic NOD mice as well [22]. In contrast to human diabetics and the NOD mouse, none of the B7/DR0404 mice examined in our study exhibited a detectable spontaneous humoral response to GAD65 as assayed by antibody detection to recombinant radio-labeled mouse GAD65.

DR0401 has been reported to have a protective effect for diabetes in B7/DQ8/DR0401 mice when compared to diabetic-prone B7/DQ8 mice [3]. The reduced incidence of diabetes in B7/DQ8/DR0401 mice was correlated with a shift in CD3-mediated cytokine response (IFN- γ :IL-4 ratio) from a Th1 in B7/DQ8 mice to a Th2 in B7/DQ8/DR0401 mice. We observe that B7/DR0404 mice (and also B7/DR0401 data not shown) respond in a Th1 manner to CD3/CD28 stimulation. The discrepancy in measured cytokine response may be due to a greater stimulation in our assay due to the addition of CD28 co-stimulation. Cytokine responses to self-antigen (GAD65 555-567 in our assay and whole GAD65 in the previous report) was near detection limits in both our experiments and those report by Wen[3].

The role of GAD in the pathogenesis in diabetes is ambiguous. Immune responses to GAD65 are correlated with insulinitis and diabetes [9], transfer of T cells specific to GAD65 can induce diabetes [11], intrathymic injection of GAD peptide can delay diabetes [31], and transgenically-driven antisense GAD can prevent diabetes [32]. On the other hand, I-Ag7 restricted GAD65 specific TcR transgenic mice on a NOD background do not get diabetes [33] and mice tolerized to GAD65 are not protected from diabetes [34]. These inconsistencies in the role that immunity to GAD plays in the pathogenesis to diabetes can be partly explained by selection/generation of regulatory T cell elements under specific conditions. The islet-antigen specific BDC2.5 CD4⁺ T cell clone is diabetes inducing in transfer experiments [35], and as a TcR transgene is diabetic in NOD.Scid but not NOD [36]. This conundrum for the BDC2.5 TcR mouse has recently been explain by demonstrating that effector and regulatory cells of the same clonotypic TcR do exist together [37]. The occurrence of this has also been shown in other pseudo-self antigen TcR transgenic models [38,39]. The reactivity to GAD65 (555-567) observed in nearly all non-diabetic B7/DR0404 mice (ages 23-54 weeks) along with insulinitis in the older mice (ages 46-54 weeks) suggest that a regulatory mechanism may be preventing overt diabetes in the majority of these mice as only a subset would be expected to become diabetic. Our finding of CD4⁺/FoxP3⁺ cells in infiltrated islets from non-diabetic mice supports this idea. The ability

of these cells to keep autoimmunity in check, as has been shown in several models of autoimmunity (see Nishioka and references therein) [40], will depend on not only on their mere presence as shown here but their number and functionality. Recent reports have demonstrated that the suppressive activity of CD4⁺/CD25⁺ phenotypic regulatory cells can change their in vivo suppressive ability over time and also by their surroundings [41,42].

T cell responses to whole-protein GFAP have been observed in lymph nodes from NOD mice prior to overt diabetes and also in peripheral blood from human diabetics and ICA positive non-diabetic first degree relatives (FDR) but not from ICA negative FDR [13]. T cell reactivity to GFAP (240-252) was observed in older B7/DR0404 mice even though only a fraction (~25%) would have been expected to develop diabetes. While the role that reactivity to GFAP plays in diabetes pathogenesis is unknown, transfer of a GFAP T cell line into NOD/Scid mice has been shown to lead to peri-insulitis but not diabetes [13]. CD8 T cell reactivity to an IGRP epitope is highly pathogenic in NOD mice [14], however, T cell responses to IGRP have not been identified in human diabetics. In our studies we did not observe any spontaneous reactivity to an IGRP DR0404-binding epitope in B7DR0404 humanized mice.

While the decrease in T cell reactivity to GAD65 in diabetic mice could possibly be explained by a loss of stimulating antigen due to destruction of the pancreatic islets, harder to explain is the cellular responses to GAD65 seen in non-diabetic older mice when only a subset would be expected to progress on to diabetes. One possible explanation is that the cellular regulatory elements that maintain healthy mice from an autoimmune state (Treg) are slow to respond (or are delayed) to an expanding autoreactive state. Such is the case in the EAE model of multiple sclerosis where it has been shown that an increase in CD4⁺/CD25⁺ Treg cells into the CNS correlates with remission of the disease [43]. In the case of a foreign antigen where T cells (thymically selected on self-antigens and potentially autoreactive) are expanding to clear the pathogen a delayed regulatory mechanism is desired. However, once the pathogen is cleared a regulatory mechanism is needed to control the potentially autoreactive expanded T cells. It is possible that the cellular immunity to self antigens seen in older mice in the absence of diabetes is being held in check by regulatory mechanisms driven by the increase in self-antigens due to tissue destruction. Our data showing the presence of CD4⁺FoxP3⁺ (Treg) cells in non-diabetic infiltrated islets supports this notion. It has been demonstrated that transgenic TcR CD4⁺CD25⁺ Treg T cells are capable of expansion upon immunization with the TcR restricted antigen [44]. Such a mechanism may also explain the presence of insulitis in the absence of overt diabetes observed in non-diabetic older NOD mice [22] and the non-diabetic older B7/DR4 mice here. Clinical diabetes at this point may then result from an inadequate or ineffective regulatory response as has been suggested in some recent autoimmune patient studies [45, 46].

B7/HLA transgenic mice have been useful in demonstrating the importance of the human MHC in disease susceptibility and also in identifying antigenic epitopes within self-antigens. The finding of an age-dependent spontaneous loss in self-tolerance in these humanized mice prior to diabetes to putative human diabetes autoantigens lends more support to these models in studying the relevant human disease.

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Figure 1.

RIP-B7/DR4 mice are spontaneously diabetic. Incidence of diabetes in B7/DR0401 (solid thin line) and B7/DR0404 (solid heavy line) mice was determined by blood glucose monitoring weekly via saphenous vein bleeds over a 52-week period. Mice with blood glucose in excess of 250 mg/dl were measured again 24 hours later and considered diabetic if the second reading also exceeded 250 mg/ml.

Diabetic B7/DR0404

Non-diabetic B7/DR0404 (8 wk)

Figure 2.

Islets in diabetic mice exhibit a cellular infiltrate. Hematoxylin and Eosin staining of pancreatic islets from a 40 week-old diabetic B7/DR0404 mouse (A & B) and a non-diabetic 8-week old mouse (C & D). At least 10 islets were viewed for each mouse pancreas and representative pictures are shown. H&E (E) and immunofluorescence staining of an islet for CD4 (F), CD8 (G), and CD45R (H) on frozen section from a diabetic B7/DR0404 mouse.

Figure 3.

Lymph node cells from diabetic B7/DR0404 mice exhibit a spontaneous response to GAD65 (555-567) and GFAP (240-252). Lymph node cells from diabetic mice were harvested and assayed for proliferative responses to class II restricted epitopes from GAD65, IGRP, and GFAP; black bars (100 µg/ml), dark gray bars (10 µg/ml), light gray bars (1.0 µg/ml), and white bars (no antigen) (A). ^3H -thymidine was added to cultures at 72 hours before scintillation counting of thymidine incorporation at 96 hour. Mean response to GAD65 (555-567) and GFAP (240-252) in diabetic B7/DR0404 mice compared to 8-12 week old non-diabetic mice (B). DR0404 mice can mount a response to IGRP (247-258) post immunization (C). DR0404

mice were immunized with 100 μ g of IGRP (247-258) peptide in CFA and after 10 days lymph nodes were assayed for recall response to the immunizing antigen.

Figure 4.

Statistical significance of lymph node spontaneous response to diabetes associated self-antigens in diabetic and older non-diabetic mice B7/DR0404 mice compared to 8-13 week old non-diabetic B7/DR0404 mice. Stimulation indexes were calculated from proliferations at 100 μ g/ml. Asterisks denote statistical difference compared to the 8-13 week old group of mice. T-test were calculated and all asterisks denote p values < 0.05 relative to stimulation index from 8 or 8-13 week old mice. Other p values are between datasets are shown on the graph.

Figure 5.

B7/DR0404 mice respond with a Th1 bias to CD3/CD28 stimulation. Splenocytes from B7/DR0404 mice were stimulated in vitro with soluble CD3/CD28 (2.0/0.2 ug/ml) for 72 hour at which time supernatant were assayed for IFN- γ and IL-4.

Figure 6.

Islets in non-diabetic older B7/DR0404 mice exhibit insulinitis with the presence of $CD4^{+}/FoxP3^{+}$ cells. Hematoxylin and Eosin staining of formalin-fixed paraffin-embedded pancreatic islets from 3-54-week old, 3-46 week old, and 2-17 week old B7/DR0404 non-diabetic mice (A.). $CD4^{+}/FoxP3^{+}$ cells are present in infiltrated islets of 44 week old diabetic B7/DR0404 (B) and 52 week old non-diabetic (C) mice. Immunofluorescence was done on frozen pancreatic sections. The presence of $CD4^{+}/FoxP3^{+}$ staining in infiltrated islets was observed in at least 3 non-diabetic older mice. FoxP3⁺ staining is shown in blue and $CD4^{+}$ in green.

Figure 7.

Antibodies to mouse GAD65 are detected in GAD65 immunized mice but not in diabetic B7/DR0404 mice. Anti-mouse GAD65 antibodies were assayed from serum samples from GAD65 immunized DR0404 mice (open circles, serum dilution shown on top x-axis), from young non-diabetic (black bar), older non-diabetic (white bar) and also from diabetic (gray bar) mice at 1:11 dilution.

Table 1

Diabetes in B7/DR0401 and B7/DR0404 mice

Mice	Mice in study		Total	%	Diabetes Avg. age (wk)	B. Glucose (mg/dl)	Diabetes by gender		%
	N	0-+					0-+	0->	
B7/DR0401	22	10	6 of 22	27%	37 +/- 9	468 +/- 55	of 10	1 of 12	8%
B7/DR0404	26	13	6 of 26	23%	+/- 9	380 +/- 76	5 of 13	1 of 13	8%

Table II

Name	aa	Species	Sequence			
Glutamic acid decarboxylase 65 (GAD65) 555-567		Human GAD65	P1 NFERMVISNPAAT	P4	P6	P9
		Mouse GAD65			same	
		Human GAD67			same	
		Mouse GAD67			same	
Glial fibrillary acidic protein (GFAP) 240-252		Human	P1 TQYEAMASSNMHE	P4	P6	P9
		Mouse		*	*	*
IGRP	247-258	Human	P1 TQYEAVAISNMQE	P4	P6	P9
			DW/HIDT/TFAG			
		Mouse	DW/HIDSTPEAG		*	

Asterisks - residue differences between mouse and human.

Underlined - amino acid residues predicted to bind in the MHC class II P1,P4,P6, and P9 peptide-binding pockets.

Italic - alternative P1,P4,P6, and P9 binding pockets