On the Pathogenicity of Autoantigen-Specific T Cell Receptors

Amanda R. Burton1, Erica Vincent1, Greig P. Lennon1, Matthew Smeltzer2, Chin-Shang Li3, Kathryn Haskins4, John Hutton5, Roland M. Tisch6, Eli E. Sercarz7, Pere Santamaria8, Creg J. Workman1,* and Dario A.A. Vignali1,*

1Department of Immunology, St. Jude Children’s Research Hospital, Memphis, TN 38105.
2Department of Biostatistics, St. Jude Children’s Research Hospital, Memphis, TN 38105
3Division of Biostatistics, MS 1C, Room 145, Department of Public Health Sciences, University of California, Davis, CA 95616.
4Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80206.
5Barbara Davis Center for Childhood Diabetes, University of Colorado, Aurora, CO 80045.
6Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599.
7Division of Immune Regulation, Torrey Pines Institute for Molecular Studies, San Diego, CA 92121.
8Julia McFarlane Diabetes Research Centre (JMDRC) and Department of Microbiology and Infectious Diseases, Institute of Infection, Immunity and Inflammation, Faculty of Medicine, The University of Calgary, Calgary, Alberta T2N 4N1, Canada.
* Shared senior authorship.

Running Title: Autoreactivity vs pathogenicity in TCR Rg mice

Corresponding Author:
Dr. Dario Vignali
Department of Immunology, St. Jude Children’s Research Hospital, 332 N. Lauderdale, Memphis, TN 38105-2794, USA.
dario.vignali@stjude.org

Received for publication 12 August 2007 and accepted in revised form 20 February 2008.
ABSTRACT

Objective: Type 1 diabetes is mediated by T cell entry into pancreatic islets and destruction of insulin-producing β cells. The relative contribution of T cells specific for different autoantigens is largely unknown as relatively few have been assessed in vivo.

Research Design and Methods: We generated mice possessing a monoclonal population of T cells expressing one of 17 T cell receptors (TCR) specific for either known autoantigens (GAD65, IA2, IA2β/phogrin and insulin), unknown islet antigens or control antigens on a NOD.scid background using retroviral-mediated stem cell gene transfer and 2A-linked multicistronic retroviral vectors [referred to herein as retrogenic mice (Rg)]. The TCR Rg approach provides a mechanism by which T cells with broad phenotypic differences can be directly compared.

Results: Neither GAD- nor IA2-specific TCRs mediated T cell islet infiltration or diabetes even though T cells developed in these Rg mice and responded to their cognate epitope. IA2β/phogrin and insulin-specific Rg T cells produced variable levels of insulitis, with one TCR producing delayed diabetes. Three TCRs specific for unknown islet antigens produced a hierarchy of insulitogenic and diabetogenic potential (BDC-2.5>NY4.1>BDC-6.9), while a fourth (BDC-10.1) mediated dramatically accelerated disease with all mice diabetic by day 33, well before full T cell reconstitution (days 42-56). Remarkably as few as 1000 BDC-10.1 Rg T cells caused rapid diabetes following adoptive transfer into NOD.scid mice.

Conclusions: Our data show that relatively few autoantigen-specific TCR can mediate islet infiltration and β cell destruction on their own, and that autoreactivity does not necessarily imply pathogenicity.

ABBREVIATIONS. GAD, glutamic acid decarboxylase; Tg, transgenic; Rg, retrogenic.
Autoimmune Type 1 diabetes is a chronic, complex autoimmune disease that is mediated by the infiltration of autoreactive lymphocytes into the pancreas resulting in the destruction of the insulin-producing β cells in the islets of Langerhans (1). The non-obese diabetic (NOD) mouse is a spontaneous murine model of type I diabetes and involves many of the same autoantigens targeted by human T cells (1-5). CD4⁺ T cells appear to be essential in both the early and late stages of the disease as evidenced by the ability of multiple CD4⁺ T cell clones to transfer disease (6-8). Additionally, anti-CD4 therapy can prevent the onset of disease in NOD mice (9).

Over two dozen islet specific autoantigens have been implicated in the initiation and/or pathogenesis of diabetes (6). While T cell responses to many of these antigens have been studied [e.g. glutamic acid decarboxylase (GAD) (10-13) insulin (14;15) the tyrosine phosphatase-like protein IA2 (islet cell antigen 512) (16-18), IA2β (phogrin) (19;20)], their relative contribution to disease initiation or pathogenesis remains unclear. Furthermore, the specificities of the majority of diabetogenic T cell clones isolated from NOD mice are still unknown (8). A key question that remains unresolved is the extent to which autoantigenicity and pathogenicity are linked.

The hallmark of preclinical type I diabetes is the infiltration of CD4⁺ T cells into the pancreatic islets. While many cell types participate in the disease process, CD4⁺ T cells command a central role in the initiation, regulation and progression of the disease. Clearly autoantigen specificity plays a critical role in governing CD4⁺ T cell entry into islets. However, the ability to study these processes using unmanipulated T cell populations that have a broad range of insulititic and diabetogenic potential has been greatly limited. T cell receptor (TCR) transgenic mice (Tg) are powerful tools for the analysis of autoimmune diseases such as type I diabetes. However, only five MHC class II-restricted, autoantigen specific Tg NOD lines have been described (BDC-2.5, BDC-6.9, NY4.1,12-4.1 and a GAD286-300 Tg), providing a limited phenotypic range (21-25). We developed a new approach for the rapid generation of TCR Tg mice, referred to as retrogenic (Rg) mice, using retroviral-mediated stem cell gene transfer and novel ‘self-cleaving’ 2A peptide-linked multicistronic retroviral vectors that express both TCR chains, plus a fluorescent protein marker, in a single vector (26-30). This approach allows us to generate and analyze multiple TCR Rg mice on a variety of defined genetic backgrounds in less than two months. This is particularly important in studying diabetes for which background genes have a significant effect on disease outcome (31). Furthermore, the phenotype of Tg mice can be affected by additional variables such as the level and timing of TCR expression and the possibility of insertional mutagenesis by the integrated transgene. These issues are eliminated in Rg mice as the same expression system is used for all TCRs, multiple, independently generated mice are analyzed, and retroviral integration is widespread. This facilitates the direct comparison between multiple TCRs.

The goal of this study was to assess the link between autoreactivity versus pathogenicity of multiple autoantigen-specific TCRs using the Rg system. We chose to clone and generate a panel of Rg mice expressing twelve different TCRs specific for four known autoantigens (various epitopes of GAD65, IA2, phogrin and insulin) and four different TCRs specific for unknown islet antigens. All four known autoantigens have been implicated in the disease onset, but their relative contribution is still controversial. With this panel of Rg mice we addressed two important questions. First, are all autoreactive TCRs pathogenic and capable of initiating insulitis and diabetes in the absence of other T cells and B cells? Second, is there a defined relationship between the extent of insulitis, the
time of diabetes onset and the incidence of diabetes?

**RESEARCH DESIGN AND METHODS**

**Mice.** NOD.scid mice were obtained from The Jackson Laboratory and bred in-house. B6.H2\(^{b7}\) mice were provided by Christophe Benoist and Diane Mathis (Joslin Diabetes Center, Boston, MA)(31) and crossed onto a RAG-1\(^{-/-}\) background. Diabetes incidence was monitored on a bi-weekly, weekly, or more frequent basis by testing for the presence of glucose in the urine by Clinistix (Bayer, Elkhart, IN). Mice testing positive by Clinistix were then tested with a One Touch Ultra glucometer (Lifescan, Milpitas, CA) for blood glucose levels and were considered diabetic if their blood glucose was greater than 200mg/dL for retroviral-mediated stem cell gene transfers or 400mg/dL for adoptive transfers. 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 ORF inserts using recombinant PCR and cloned into an MSCV-based retroviral vector with a green fluorescent protein (GFP) marker as previously described (26;28-30). Details of cloning strategies and primer sequences are available upon request (amanda.burton@stjude.org). TCRs were cloned by PCR using plasmid or cDNA obtained from: Phogrin13 and Phogrin18 T cell clones, and 10.23 hybridoma from John Hutton (Barbara Davis Center for Diabetes, Aurora, Colorado); BDC-6.9 and BDC-10.1 T cell clones from Kathryn Haskins (University of Colorado Health Sciences Center, Denver, Colorado); BDC-2.5 TCR plasmids from Luc Teytou (The Scripps Research Institute, La Jolla, CA); NY4.1 TCR plasmids from Pere Santamaria (University of Calgary, Alberta, Canada); 1A4 TCR plasmids from Roland Tisch (University of North Carolina, Chapel Hill, NC); S30.45.19 hybrid from Eli Sercarz (Torrey Pines Institute for Molecular Studies, San Diego, CA); and PA15.14B12, PA19.5E11, PA18.10F10, PA17.9G7, PA18.9H7 and PA21.14H4 hybridomas generated in our lab by Paula Arnold. The 12-4.4\(^{v1}\) and 12-4.1 TCR constructs were generated de novo from the published sequence data (12-4.1 accession numbers: DQ172905 for \(\alpha\) chain and DQ180320 for \(\beta\) chain) and information generously provided by George Eisenbarth (Barbara Davis Center for Childhood Diabetes, Aurora, CO). For TCR with unknown \(V_{\alpha}\) and \(V_{\beta}\) usage, \(V_{\beta}\) was determined by flow cytometry using \(V_{\beta}\)-biotinylated antibodies and streptavidin-PE. \(V_{\alpha}\) usage was determined by RT-PCR with \(V_{\alpha}\) primers, cloning into the PCR -Blunt II-TOPO vector (Invitrogen, Carlsbad, CA) and sequencing as previously described (32). All TCR 2A-linked vectors utilize the PTV1.2A sequence except for PA21.14H4, NY4.1, and PA18.10F10 which utilize the TaV.2A sequence (27;28). The BDC-10.1 and BDC-6.9 vectors contain a gly-ser-gly linker between the \(C_{\alpha}\) region and the PTV1.2A sequences. The BDC-2.5 vector lacks this linker, while the remaining vectors contain a gly between the \(C_{\alpha}\) region and the 2A sequences.

**Retroviral-mediated stem cell gene transfer.** Retroviral mediated stem cell gene transfer was performed as previously described (26-30).

**Flow cytometric analysis and cell sorting.** At 35, 70, and 140 days post-transplant, spleens, inguinal lymph nodes (ILN) and pancreatic lymph nodes (PLN) were harvested, processed, counted and stained with TCR\(\beta\)(H57)-PE/ and CD4-APC (BD Biosciences Pharmingen, San Diego, CA). For functional assays, splenocytes were stained with CD4-PE (BD Biosciences Pharmingen, San Diego, CA) and FACS sorted for GFP\(^+/\)CD4\(^+\) on a MoFlow (Dako, Fort Collins, CO).
Adoptive transfers. At 28 days post-transplant, GFP^+CD4^+TCR^+ splenocytes from NOD.scid TCR Rg mice were purified by FACS and injected i.v. into NOD.scid recipient mice.

Islet isolation. Pancreata were perfused by injecting 3 ml collagenase P (1.5 µg/ml in HBSS) (Roche, Indianapolis, IN) harvested and placed in 3-5 ml collagenase P on ice. The pancreata were then incubated at 37°C for 16-20 min, after which 7 ml of HBSS with 5% FBS was added and the tissues pelleted at 1000 rpm for 3 min. The pellet was washed twice with 7 ml of 5% FBS/HBSS and resuspended in 7 ml of 5% FBS/HBSS. Islets were handpicked and dissociated with 1 ml cell dissociation buffer (Invitrogen, Carlsbad, CA) and incubated at 37°C for 5 min. After vortexing, the dissociation process was repeated two times. Cells were placed in 10 ml 5% FBS/HBSS, centrifuged for 10 min at 1300 rpm, resuspended in 5% FBS/HBSS and irradiated at 3000 rads.

Functional assays. To determine the activation index of TCRs with known specificities, 5 x 10^5 splenocytes of mice 70 or 140 days post-transplant were cultured with or without peptide in either supplemented Eagle's minimum essential medium (SMEM) or RPMI with 10% FBS for 48 hours in 96-well flat bottom plates. In some instances, splenocytes were FACS sorted as described above, and 2.5 x 10^4 purified T cells were cultured with irradiated (3000 rads) NOD/LtJ splenocytes with or without peptide for 48 hours. The wells were then pulsed with 1µCi/well of [3H]-thymidine for the final 8 or 24 hours and then harvested. The peptides used were: Ia2 678-688, GAD 217-236, GAD 284-300, GAD 206-220, GAD 510-524, GAD 524-538, GAD 530-543, HEL 11-25, Insulin B 9-23, IA2β 640-659, and IA2β 755-777. For TCRs of unknown specificity, 2.5 x 10^4 purified GFP+/CD4+ splenic T cells were cultured with 5.0 x 10^4 irradiated purified islets from 11-19 week old NOD/LtJ mice in SMEM with 10%FBS in 96 well round bottom plates for 72 hours, pulsed with 1µCi/well of [3H]-thymidine for 18 hours and then harvested.

Insulitis scores. Pancreata of NOD.scid TCR retrogenic mice were harvested at 28, 70, and 140 days post-transplant, placed into 10% buffered formalin, embedded in paraffin, 4 µm-thick sections cut at 150 µm step-sections and stained with hematoxylin and eosin (H&E) at the St. Jude Histology Core Facility. An average of 90-100 islets per mouse were scored in a blinded manner using the following metric: no insulitis (normal islet; no infiltration), peri-insulitis (infiltration on edges of islet or 0-20 % of islet infiltrated) or insulitis (infiltration of 30-100% of islet).

Statistics. The time to diabetes (survivor function) for each group of mice was estimated using the Kaplan-Meier log-rank test both overall and pair-wise within each experiment. The overall Type I error rate for each experiment was controlled at the 0.05 level using the Bonferroni adjustment for multiple comparisons.

RESULTS

Cloning and expression of β cell-specific TCRs. GAD65 is one of the principal autoantigens in human TID and was one of the first autoantigens implicated in diabetes in NOD mice (10;11). Nevertheless, its role as a target autoantigen remains controversial (24;26;33;34). We cloned and expressed seven different TCRs specific for the major immunogenic GAD epitopes and others that have been proposed to modulate disease progression (Table 1) (35;36). All of the GAD specific TCRs, with the exception of 1A4, were derived by immunizing NOD mice with peptide. 1A4 was isolated from the islets of a non-immunized, non-diabetic NOD mouse. Four different TCRs based on relevant epitope immunogenicity were chosen: PA15.14B12 and PA19.5E11 (specific for GAD206-220, the primary immunogenic epitope of GAD65) and 1A4 and PA17.9G7 (which covered the second and third most immunogenic epitopes GAD 221-235 and GAD284-300, respectively) (35;36). We also cloned and expressed three different TCRs specific for the C terminus portion of GAD65 thought to be important in the initiation of islet
infiltration and β cell destruction, two of which, PA18.9H7 and 530.45.19, cover the GAD_{524-538} and GAD_{530-543} epitope, respectively, and PA18.10F10, specific for GAD_{510-524}, just upstream of epitope GAD_{524-543} (Table 1). 530.45.19 and PA18.9H7 are particularly intriguing as it has been reported that spontaneously arising, GAD-reactive T cells in NOD mice use \( \text{V}\beta^{4+} \) (530.45.19) and are specific for the 530-543 epitope while TCRs that recognize GAD_{524-543} are primarily generated by immunization, use \( \text{V}\beta^{12+} \) (PA18.9H7), and may have regulatory activity (10;13;34).

Insulin has also been implicated in the diabetogenic response. A considerable number of T cells infiltrating the islets react to insulin with more than 90% recognizing the insulinB_{9-23} epitope (15;37). Recently it has been shown that insulin I/II null NOD mice expressing an insulin transgene with a mutation in the critical insulin B_{9-23} epitope did not develop disease, suggesting that it is a primary autoantigenic epitope (38). T cell clones generated against insulinB_{9-23} both accelerate diabetes onset in NOD mice and confer disease following adoptive transfer into NOD.scid mice (15). The 12-4.1 and 12-4.4^{vl} TCRs were originally isolated from the islets of pre-diabetic NOD mice and were reconstructed by PCR from published sequences (see Materials and Methods and Table I for details) and expressed in NOD.scid mice.

Insulinoma-associated tyrosine phosphatase-like protein (IA2) or islet cell antigen 512 is found in the secretory granules of β cells (17;39) and is a potential target of CD4^{+} T cells (16;18;40). We cloned the TCR 10.23, which is specific for IA2, and was derived through immunizations with whole protein. We later identified the epitope as IA2_{676-688} (data not shown). In addition, given the implication of IA2β (phogrin)-reactive CD4^{+} T cells in disease pathogenesis (19;20;41), two phogrin specific TCRs, Phogrin13 and Phogrin18 were cloned from T cell lines established by immunization with whole protein. (41).

Included in this study were clonotypic BDC-2.5, BDC-6.9, BDC-10.1 and NY4.1 TCRs specific for undefined β cell epitopes. BDC-2.5, BDC-6.9 and BDC-10.1 were the first diabetogenic CD4^{+} T cell clones described and were originally isolated from the spleen and lymph nodes of diabetic NOD mice by Haskins and colleagues (8;42;43), while NY4.1 was one of six CD4^{+} T cells clones that were isolated from the islets of acutely diabetic NOD mice and were reactive to islet cells (44). These clones are highly diabetogenic upon transfer into NOD.scid mice or when expressed as a transgene in NOD mice (BDC-2.5, BDC-6.9 and NY4.1) (21-23;45;46). We have previously generated BDC-2.5 and NY4.1 Rg mice (26) which were included in this study as positive controls for the other TCR Rg mice. Likewise, Rg mice expressing PA21.14H4, an H-2A^{g7}-restricted, hen egg lysozyme (HEL_{11-25})-specific TCR, were included as negative controls.

TCR Rg mice were established using NOD.scid mice as both bone marrow donors and recipients. This enabled us to examine the ability of Rg T cells to infiltrate the islets and mediate diabetes in the absence of other T cells or B cells. For each TCR, we examined expression and reconstitution levels, T cell proliferative capacity and the incidence of insulitis and diabetes in the Rg mice.

**Expression and function of β cell-specific TCRs**

All of the TCRs were expressed in mice and had comparable levels of GFP (Figure 1 and data not shown). However, there was variability in TCR expression levels compared to the NOD control, and in the number of GFP^{+}/TCR^{+}/CD4^{+} Rg T cells in the spleen, ILN and PLN (Figures 1 and 2). This is likely due to the influence of thymic selection and the availability of selecting ligands, as well as homeostatic and antigen driven proliferation in the periphery. Importantly, TCR expression level and T cell numbers were consistent within each group suggesting that this variability was due to intrinsic characteristics of each TCR rather then
experimental variance. Also, there was a correlation between the number of T cells in the spleen compared with the ILN and PLN within each TCR Rg group (Figure 2).

The GAD65-specific TCRs were reactive to and specific for their respective peptides, albeit with differing efficiencies (Table I and Figure 3A and B). While 12-4.1 and 12-4.4v1 recognize the same epitope, InsulinB9-23, the significant difference in stimulation index observed is likely due to differences in cognate ligand affinity caused by differences in CDR/Vβ usage (Table I). Furthermore, differences in IA2- and phogrin-specific TCR expression in 10.23, Phogrin13 and Phogrin18 Rg mice may explain the differing reactivity observed (Table I and Figure 1 and 3A). However, Rg T cells were antigen specific and T cells isolated from the spleen and PLN reacted equivalently, as exemplified with Phogrin18 T cells (Figure 3C and data not shown). Finally, all of the unknown islet antigen-specific TCRs, BDC-2.5, BDC-6.9, BDC-10.1, and NY4.1, were expressed at detectable levels (Figure 1). The level of TCR expression was more variable amongst some of the Rg mice. This was particularly evident with BDC-2.5 T cell and may, in part, be due to the relatively low level of TCR expression seen in these mice compared with other Rg T cells (Figures 1 and 2). The functionality of these Rg T cells was determined by measuring proliferation in response to purified, irradiated islets from 11-19 week old NOD mice. BDC-10.1 Rg T cells were strongly reactive to irradiated islets, while BDC-2.5 T cells responded weakly compared to the control HEL specific PA21.14H4 Rg T cells, Rg T cells alone and islets alone (Figure 3D and data not shown). In contrast, minimal reactivity was seen with BDC-6.9 and NY4.1 Rg T cells suggesting that either these islets express insufficient levels of the antigenic epitope to elicit proliferation in vitro or more likely that T cells derived from these Rg mice are less sensitive than the parent T cell clones.

**Insulitis and incidence of diabetes of Rg mice.** Next we evaluated the extent of insulitis 28, 70 and/or 140 days post-bone marrow transplant, and the incidence of diabetes for 140 days in the 17 TCR Rg mouse lines. Essentially no peri-insulitis/insulitis was observed in any of the GAD-specific Rg mice (Table I and Figure 4). Likewise, minimal infiltration of IA2β676-688-specific 10.23 T cells or IA2β/phogrin640-659-specific Phogrin13 T cells was seen in the respective Rg mice. This was surprising since the phogrin specific T cell clones, Phogrin15 and Phogrin12, infiltrate rat islets transplanted under the kidney capsule and destroy β cells (41). However, demonstrable peri-insulitis (~11%) and insulitis (~6%) was observed in IA2β/phogrin755-777-specific Phogrin18 Rg mice, although this was not manifested until 140 days post-transfer (Table I and Figure 4). The differential ability of these phogrin-specific T cells to infiltrate islets may be due to differences in epitope recognition or TCR affinity/avidity (Table I). Not surprisingly, none of these Rg mice developed diabetes (Table I).

Analysis of the two insulinB9,23 Rg mice was particularly intriguing. While significant peri-insulitis and insulitis were observed, insulitis was greater in the 12-4.1 versus the 12-4.4v1 Rg mice (Table I). Furthermore, diabetes developed in 33% of the 12-4.1 Rg mice (ID50 – 60 days) while none of the 12-4.4v1 Rg mice became diabetic. The difference in diabetes incidence may be due to differences in the relative avidity of the corresponding T cells to antigen (Table I). The peri-insulitis/insulitis score appeared to decline by 140 days post-transfer. Given that a proportion of the 12-4.1 Rg mice become diabetic, it is possible that this reduced insulitis score is due to the reduced disease incidence in the remaining mice. However, this cannot account for the decline in the 12-4.4v1 Rg mice and suggests that the insulitis resolves with time in these mice.

As previously reported, BDC-2.5 and NY4.1 Rg mice developed significant insulitis ~28 days post-transfer, which is remarkable given that it
takes ~50 days for complete bone marrow reconstitution and maximal T cell population of the periphery. There was also substantial insulitis in the BDC-10.1 Rg mice (87% for BDC-10.1 versus 93% for BDC-2.5 at 28 days post-transfer - Table I and Figure 4). Indeed, there was a correlation between the percentage of infiltrating CD4⁺ Rg T cells, and the severity of insulitis and incidence of diabetes (BDC-10.1 - 5.9% CD4⁺ T cells in the islets; BDC-2.5 - 5.1%; 12.4-1 - 3.6%; Phogrin18 - 0.6%; PA21.14H4 - 0.2%).

Diabetes incidence in the BDC-2.5 Rg mice was 100% with an ID₅₀ of 52 days. Curiously, the rate of diabetes development in the NY4.1 Rg mice was comparable (ID₅₀ – 46 days) but the incidence was only 71% (Table I and Figure 5A). Insulitis development in the BDC-6.9 Rg mice was delayed with no insulitis at day 28 but substantial insulitis at days 70 and 140 post-transfer. Consistent with this, diabetes onset in the BDC-6.9 Rg mice was delayed (ID₅₀ – 81 days; incidence – 56%), in accordance with observations made with their Tg counterparts (22). Diabetes onset in the BDC-10.1 Rg mice was rapid and highly penetrant with all the mice hyperglycemic by day 33 (ID₅₀ – 27 days) (Figure 5A). Given the diabetogenic potential of the BDC-10.1 TCR and the flexibility of the Rg system, BDC-10.1 Rg T cells were generated on a C57BL/6 background using the B6g7.RAG-1–/– mice. Interestingly, BDC-10.1 Rg T cells on a non-autoimmune background mediated diabetes, albeit at a slower rate (ID₅₀ - 76 days) and reduced frequency (80%) (Figure 5A).

To further evaluate the diabetogenic potency of the BDC-10.1 Rg T cells, GFP⁺ CD4⁺ T cells were sorted from BDC-10.1, NY4.1 and the control TCR PA21.14H4 Rg mice, and adoptively transferred into NOD.scid mice (Figure 5A). As expected, the HEL-specific T cell recipients failed to develop diabetes, while mice receiving NY4.1 Rg T cells initially developed diabetes by day 55 post-transfer and by day 138 all recipients were diabetic. Strikingly, all of the BDC-10.1 T cell recipients developed diabetes by day 13 post-transfer. Finally, we adoptively transferred titrated numbers of purified BDC-10.1 Rg T cells into NOD.scid mice to define the minimal number required to mediate disease (Figure 5C). Remarkably, only 50,000 BDC-10.1 Rg T cells were required to cause rapid diabetes onset in 100% of recipients by day 13, while as few as 1000 BDC-10.1 Rg T cells caused diabetes in 80% of recipients. Taken together, these data highlight the increased diabetogenic potential of BDC-10.1 T cells.

**DISCUSSION**

Type I diabetes is a complex disease mediated by T cells of multiple specificities, many of which have yet to be determined. Important questions remain concerning what governs T cell islet entry, what makes a T cell diabetogenic and what the relationship is between autoreactivity and pathogenicity. While the generation of TCR Tg mice has provided important insight into the disease process, these studies represent a limited repertoire of potential autoantigen epitope-specific TCRs that might mediate type I diabetes. Our study demonstrates the utility of the Rg approach for the generation and analysis of mice expressing a large panel of TCRs with varied specificity. These data show that for the panel of TCRs studied, relatively few promote islet entry and β cell destruction in the absence of other T cells and B cells. Indeed, it is noteworthy that four of the five TCRs that promoted diabetes when expressed in Rg mice recognize unknown antigens.

It has recently been suggested that GAD-reactive T cells may not be as critical to the initiation and progression of type I diabetes as originally thought (10;11;33). Our data support this view. Despite the high immunogenicity of GAD epitopes (206-220, 221-235 and 284-300) and the large number of Rg T cells in the spleens and lymph nodes, no β cell destruction was seen in the 7 GAD-reactive TCR Rg mouse lines analyzed. While some peri-insulitis was observed in some mice, particularly those
expressing TCR specific for distal GAD epitopes (eg. 530.45.19 specific for GAD\(_{530-543}\)), this was not significantly greater compared to HEL-specific Rg mice (PA21.14H4). While we cannot rule out a role for GAD-reactive T cells in the later stages of pathogenesis or in concert with other T cells (34), our data suggest that T cells expressing the current panel of clonotypic TCRs specific for GAD are not capable of initiating insulitis and diabetes on their own. Furthermore, several studies suggest that certain GAD-reactive TCRs are expressed by regulatory T cells, an issue that was not addressed in this study. While the IA2-specific Rg T cells did not infiltrate the islets, suggesting that IA2 may not be an important autoantigenic epitope in the early stages of type I diabetes, both phogrin-specific T cell populations infiltrated islets to varying degrees. However, although Phogrin18 mediated significant peri-insulitis/insulitis, likely due to increased TCR expression and T cell number, this required 140 days to develop.

Taken together these data suggest that some of the most commonly studied type I diabetes autoantigens, GAD, IA2 and IA2\(\beta\)/phogrin, may have a reduced role in initiating type I diabetes.

On the other hand, the importance of insulin as an autoantigen (38;47) was reaffirmed in this study. Both insulin-specific TCRs conferred significant insulitis, while only 12-4.1 caused diabetes. Given that these TCRs recognize the same epitope, the phenotypic distinctions observed are likely due to differences in TCR V\(\beta\) (V\(\beta\)2 versus V\(\beta\)12) and CDR usage, and/or affinity/avidity as the 12-4.1 Rg T cells have a considerably higher stimulation index against the insulinB\(_{9-23}\) epitope. This suggests that while autoantigen and epitope availability are important factors in mediating the disease process, TCR affinity is likely to be an important factor.

Differences in the diabetogenicity of TCRs from BDC-2.5, BDC-6.9 and BDC-10.1 in Rg mice would not have been predicted from their pathogenicity as T cell clones, which is very similar in adoptive transfers into young NOD or NOD.scid recipients (8;43). The hierarchy of insulitic and diabetogenic potential exhibited by the three TCRs that had previously been expressed as transgenes was largely as expected (BDC-2.5 > NY4.1 > BDC-6.9)(21-23). The most surprising observation was the diabetogenic potential of Rg T cells expressing the BDC-10.1 TCR. These data suggest that this TCR may recognize a critical epitope in disease etiology and/or may have an ‘optimal’ affinity/avidity for maximal islet infiltration and \(\beta\) cell destruction. It is important to note that despite the autoreactivity of the BDC-2.5, BDC-10.1 and NY4.1, all three TCRs were expressed and Rg T cells escaped thymic selection (albeit to a lesser extent in the spleen compared to other Rg T cells). This suggests that diabetogenicity was attributed to the specificity of the TCR and not to differences in the levels of TCR expression or the number of Rg T cell present in mice.

Taken together, these data suggest that autoantigen-specific TCRs can be segregated into three phenotypic groups: (1) TCRs that fail to mediate T cell islet entry but may play a role in the later stages of the disease (eg. TCRs specific for GAD and IA2); (2) TCRs that mediate islet infiltration but not \(\beta\) cell destruction and thus may only contribute significantly to type I diabetes if potentiated by diabetogenic T cells (eg. certain phogrin- and insulin-specific TCRs); and (3) TCRs that mediate (to varying degrees) insulitis and \(\beta\) cell destruction and thus may be key initiators/propagators of type I diabetes (eg. certain TCRs specific for insulin and unknown islet antigens). Understanding the molecular distinctions between these three populations will be critically important. In addition, gaining insight into the levels and timing of autoantigen expression/availability as well as the location/phenotype of the APCs presenting autoantigens will be important and may explain the differences we observed between the autoreactive Rg T cells. In conclusion, our data suggest that autoreactivity does not imply
pathogenicity as most autoreactive TCRs, on their own, would appear to be non-pathogenic.

ACKNOWLEDGMENTS

We are very grateful to Paula Arnold for her contributions during the early stages of this project, Kate Vignali, Yao Wang and Smaroula Dilioglou for technical assistance, the Vignali lab for assistance with harvesting bone marrow, Richard Cross, Jennifer Rogers and Yuxia He for flow cytometry analysis, St Jude Hartwell Center staff for oligo synthesis and DNA sequencing, staff of the St Jude ARC Histology Laboratory and Animal Husbandry Unit and Flow Cytometry and Cell Sorting Shared Resource facility staff for AutoMACS. We would like to thank Luc Teyton and Christophe Benoist for the BDC-2.5 TCR plasmids and Christophe Benoist and Diane Mathis for the B6.H2^{g7} mice. J.H was supported by NIH R01 DK052068 and NIH P30 DK57516. P.S. was supported by the Canadian Institutes of Health Research and is a Scientist of the Alberta Heritage Foundation for Medical Research. D.A.A.V. was supported by funds from the Juvenile Diabetes Research Foundation International (1-2004-141 – [The Robert and Janice Compton Research Grant, In Honor of Elizabeth S. Compton] and 1-2006-847), a pilot project from the Cooperative Study Group for Autoimmune Disease Prevention (U19 AI050864-05 - George Eisenbarth, PI), the St Jude Cancer Center Support CORE grant (CA-21765) and the American Lebanese Syrian Associated Charities (ALSAC).
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Expression of Rg T cells in NOD.scid mice. Flow cytometric analysis of splenocytes from female NOD.scid TCR retrogenic mice 32-39 days post-transplant was performed. Splenocytes were gated on live (based on forward/side scatter) GFP+ cells and dot plots of CD4 versus TCR (anti-TCRβ - H57) expression depicted. The numbers in the top right quadrant refer to the percentage of TCR+CD4+ T cells. Note that the level of TCR expression and the number of T cells was more variable amongst BDC-2.5 Rg mice, with the example depicted representative of those on the low end (See Figure 2 for a mean of all mice examined).

**Figure 2.** Reconstitution levels of TCR retrogenic mice. The spleens, ILN and PLN from the NOD.scid TCR retrogenic mice were removed, single cells suspensions prepared, counted and stained for TCR and CD4 expression. The number of Rg T cells was determined from on the percentage of GFP+/TCR+/CD4+ cells and the total number of cells in the spleen and lymph nodes. The data represents the mean ± SE of 3-6 mice per group. * Not Determined

**Figure 3.** T cells from TCR retrogenic NOD.scid mice are functional and specific for their cognate antigen. (A) 5 x 10^5 splenocytes from TCR NOD.scid retrogenic mice were incubated with varying concentrations of peptide for 48 h, pulsed with 1 µCi and harvested 24 hours later. Background counts from T cells alone were subtracted at each antigen concentration. Data are representative of 2 experiments. (B) GFP+/CD4+ T cells from PA17.9G7 and PA21.14H4 retrogenic mice were purified by FACS and incubated with or without 5µM GAD284-300 or HEL11-25 peptide presented by irradiated NOD splenocytes for 48 h, pulsed with 1 µCi and harvested 18 h later. Data represent mean ± SE of 3 independent experiments. (C) Phogrin18 Rg T cells were purified by FACS based on GFP and CD4 expression and incubated with a titration of various peptides presented by irradiated NOD splenocytes for 48 h, pulsed with 1 µCi and harvested 18 h later. Data represent mean ± SE of 3 independent experiments. (D) BDC2.5, BDC10.1 and PA21.14H4 Rg T cells were sorted and incubated with or without irradiated 11-19 week old NOD/LtJ islets for 72 h, pulsed with 1µCi and harvested 18 h later. The data represent the mean ± SE of 2-4 independent experiments.

**Figure 4.** Insulitis incidence in TCR Rg NOD.scid mice. H&E stained pancreata of NOD.scid retrogenic mice were scored for lymphocytic infiltration of the islets at 28, 70 and 140 days post-transplant. Insulitis was scored (~100 islets per mouse) using the following metric: no insulitis (normal islet; no infiltration), peri-insulitis (infiltration on edges of islet or 0-20 % of islet infiltrated) or insulitis (infiltration of 30-100% of islet). The average score of 4-17 mice (Table I) is presented with standard error (~800 slides in total). An alternative scoring system was also used for comparison, which gave comparable results [BDC10.1 – 0.85±0.04; BDC2.5 – 0.89±0.02; NY4.1 – 0.59±0.08; BDC6.9 and 14H4 – 0](48).

**Figure 5.** Diabetes incidence in TCR Rg NOD.scid mice. (A) NOD.scid and B6.g7.RAG-1−/− TCR retrogenic mice were monitored for diabetes onset for 140 days. (B) GFP+CD4+TCRβ+ cells were FACS purified from BDC-10.1, NY4.1 and PA21.14H4 NOD.scid Rg mice 28 days post-transplant, adoptively transferred into NOD.scid recipient mice and monitored for diabetes. (C) A titration of purified BDC-10.1 NOD.scid Rg T cells were transferred into NOD.scid mice and monitored for diabetes. The tables of p values on the right were generated using the log-rank test both overall and
pair-wise within each experiment. The p value was based at the 0.05 significance level using the Bonferroni adjustment for multiple comparisons.

**NOTE:** The figures for this article can be found using the link entitled “Figures”. (Available at http://dxdoi.org/10.2337/db07-1129.)