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Analysis of T cell receptor beta chains that combine with dominant conserved TRAV5D-4*04 anti-insulin B:9-23 alpha chains

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Abstract

Objective—The objective of this study was to define the spectrum of TCR beta chains permissive for T cells with alpha chains containing the conserved TRAV5D-4*04 sequence to target the insulin B:9-23 peptide, a major epitope for initiation of diabetes in the NOD mouse.

Materials and Methods—We produced T cell hybridomas from mice with single T cell receptors (BDC 12-4.1 TCR $\alpha^+ \beta^+$ double transgenic mice and BDC12-4.4 TCR $\alpha^+ \beta^+$ double retrogenic mice) or from mice with only the corresponding alpha chains transgene or retrogene and multiple endogenous TCR beta chains.

Results—Hybridomas with the complete BDC12-4.1 and BDC12-4.4 T cell receptors, despite having markedly different TCR beta chains, responded to similar B:9-23 peptides. Approximately 1% of the hybridomas from mice with the fixed TRAV5D-4*04 alpha chains and multiple endogenous beta chains responded to B:9-23 peptides while the majority of hybridomas with different beta chains did not respond. There was no apparent conservation of TCR beta chain sequences in the responding hybridomas.

Conclusions—Approximately 1 percent of hybridomas utilizing different TCR β chains paired with the conserved TRAV5D-4*04 containing alpha chains respond to insulin peptide B:9-23. Therefore, TCR beta chain sequences make an important contribution to insulin B: 9-23 peptide recognition but multiple beta chain sequences are permissive for recognition.

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Keywords

Insulin; T cell Receptor; Type 1 Diabetes

INTRODUCTION

The major genetic determinant of type 1 diabetes of man and the NOD mouse are HLA class II alleles and autoimmunity directed at insulin is hypothesized to be a key determinant [1]. In animal models it is possible to mutate the insulin B:9-23 sequence and prevent diabetes. The insulin B:9-23 peptide can be used to both induce [2, 3] and prevent [4, 5] autoimmune diabetes in mice [6–9]. This peptide is presented by antigen-presenting cells from both NOD mice (I-A^{g7}) [10] and BALB/c (I-A^d) mice [11, 12]. Despite its potential immunologic importance the B:9-23 peptide binds with low affinity to the class II allele I-A^{g7} of the NOD mouse [13], [14]. Studies of the original Wegmann anti-B:9-23 clones has documented predominantly production of Th1 cytokines with 5/6 IFN- γ but not IL-4 and the sixth clone producing both IFN- γ and IL-4 [15]. All of these clones accelerated diabetes in young NOD mice and the T cell receptor BDC 12-4.1 as a transgenic produces diabetes [16].

There are multiple known and uncharacterized islet autoantigens recognized by CD4 and CD8 T cells [16–21]. Nevertheless there is considerable evidence in the NOD mouse that proinsulin/insulin is a crucial target of autoimmunity leading to islet beta cell destruction [6, 22–27] and patients with type 1 diabetes also have T cells that react with proinsulin/insulin [27–33]. In contrast to other currently known islet autoantigens, only elimination of the response to insulin, by insulin gene deletion or altering specific insulin sequences, leads to dramatic prevention of diabetes in the NOD mouse model [25]. Krishnamurthy and coworkers [34] have recently reported that the prominent CD8 T cell immune response to the molecule, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), is “downstream” of the immune response to insulin and that abrogating immune responses to insulin prevents diabetes, while abrogating immune responses to IGRP does not influence progression to diabetes in NOD mice. Nakayama and coworkers [25, 35] have replaced both the insulin 1 and insulin 2 genes in NOD mice with a transgene expressing a mutated sequence of proinsulin 2 (alanine at B chain position 16 rather than tyrosine). Substitution of alanine for tyrosine was chosen because the anti-B:9-23 CD4 T cell clones of Wegmann and coworkers (e.g. BDC12-4.1, BDC12-4.4) did not proliferate when incubated with the B16 alanine mutated B:9-23 insulin peptide [24]. The transgenic proinsulin B16 alanine mouse lacking both native proinsulin genes does not develop diabetes and the majority of mice have neither insulinitis nor insulin autoantibodies. If these insulin 1 and insulin 2 double knockout transgenic mice receive islets expressing the native insulin B16 tyrosine sequence, they rapidly develop insulin autoantibodies and insulinitis [6, 25].

Recognition of the insulin B:9-23 peptide is predominately determined by a conserved Valpha sequence [36, 37]. The anti-B:9-23 T cells cloned by Wegmann and coworkers were isolated directly from islets of prediabetic NOD mice [22, 23] and were unusual in that they had marked conservation of their V α (TRAV5D-4) and J α (TRAJ53) gene segments, but no conservation of the TCR alpha chain N-region and no apparent conservation of their TCR

beta chain repertoire [10, 38]. We have produced a T cell receptor transgenic mouse expressing one of these anti-B:9-23 T cell receptors, BDC 12-4.1 [16], and retrogenic mice expressing another TCR (BDC12-4.4), with both receptors having TRAV5D-4*04 and TRAJ53 but with different N-region sequences. The complete transgenic 12-4.1 T cell receptor can induce spontaneous diabetes on a NOD RAG^{-/-} background as can the retrogenic on a SCID background [16]. The BDC12-4.1 TCR $\alpha^+ \text{Ca}^{-/-}$ transgenic, which utilizes the fixed alpha chain and endogenous beta chains, develops insulin autoantibodies but not diabetes [39]. We hypothesized that multiple TCR beta chains are able to complement the conserved alpha chain and play an important role in TCR recognition of the B:9-23 insulin peptide. To directly test the contribution of multiple beta chains given a fixed alpha chain, in this manuscript we describe the creation of a series of T cell hybridomas from mice expressing the TRAV5D-4*04 containing TCR α chains either in combination with their specific beta chains (from BDC 12-4.1 TCR $\alpha^+ \beta^+$ RAG^{-/-} mice and BDC12-4.4 TCR $\alpha^+ \beta^+$ NOD/SCID retrogenic mice) or with endogenous beta chains (from BDC 12-4.1 TCR $\alpha^+ \text{Ca}^{-/-}$ RAG^{+/-} mice and BDC12-4.4 TCR $\alpha^+ \text{Ca}^{-/-}$ NOD/SCID retrogenic mice). We utilized a hybridoma fusion partner that expresses LacZ upon T cell receptor stimulation to facilitate screening of a large number of hybridomas [40] and analyzed the TCR endogenous beta chain repertoire of the hybridomas and their response to multiple insulin peptides.

Basically in the current study we have produced T cell receptor hybridomas recognizing insulin peptide B:9-23 from two different alpha chain fixed transgenic mice: BDC 12-4.1 TCR alpha chain single transgenic mice and BDC 12-4.4 TCR alpha chain retrogenic mice. These mice with a single alpha chain utilized endogenous beta chains to produce T cell receptors that recognized insulin peptide B:9-23. We first studied the BDC 12-4.1 hybridomas and then the BDC 12-4.4 hybridomas. The hybridomas were analyzed for their response to a series of insulin B:9-23 peptides and the beta chain T cell receptors of the positive hybridomas were sequenced.

Research Design and Methods

Generation of insulin B:9-23 peptide specific T-cell hybridomas

Hybridomas were generated utilizing spleen cells or pancreatic lymph node cells from transgenic mice with either the BDC12-4.1 or BDC12-4.4 TCR alpha and beta chain double transgenes or only the alpha chain transgene (summarized in table 1). The alpha chains of BDC12-4.1 and 12-4.4 are different only in their N region (GAN versus A) while they have markedly different beta chains (Table 2). For the alpha chain only transgenic and retrogenic mice, a mutation of the C α constant region ($\text{Ca}^{-/-}$) prevented utilization of other alpha chains while allowing use of endogenous TCR β chains. For BDC12-4.1 TCR $\alpha^+ \beta^+$ RAG^{-/-} mice, the RAG knockout prevented utilization of any other T cell receptor chains. BDC12-4.4 TCR $\alpha^+ \beta^+$ NOD/SCID retrogenic mice expressed only the introduced TCR due to the SCID mutation. The splenocytes from BDC12-4.1 TCR $\alpha^+ \beta^+$ RAG^{-/-} and BDC12-4.4 TCR $\alpha^+ \beta^+$ NOD/SCID mice were fused with BWZ.36 cells [40] to produce TCR expressing hybridomas. Splenocytes from four different BDC12-4.1 TCR $\alpha^+ \text{Ca}^{-/-}$ mice (2 mice with B:9-23 peptide immunization and 2 mice without immunization), were also utilized to generate hybridomas. BDC12-4.1 TCR $\alpha^+ \text{Ca}^{-/-}$ mice were immunized twice (one week apart) with

100 µg insulin2 B:9-23 peptide in 100 µl IFA (Incomplete Freund's Adjuvant). Five days after the second immunization, the mice were euthanized and a single cell suspension of spleen or pancreatic lymph node cells were cultured and activated with plate-bound anti-CD3 and anti-CD28 monoclonal antibodies (10 µg/ml each) in round 96-well plates for 48~72 hours in DMEM media with 10% FBS (DMEM-10), with 5% CO₂ at 37°C. Cells from BDC12-4.4 TCR α^+ C $\alpha^{-/-}$ NOD/SCID retrogenic mice were activated in vitro similarly without immunization in donor animals. Activated T cells were harvested from the fluid phase by pipetting, and T cells were separated with a one-step gradient method with Ficoll-Hypaque™ Plus (GE Healthcare). The cells were mixed with an equal number of log phase BWZ.36 cells (BWZ.36 cells are transfected with a LacZ construct driven by a nuclear factor of activated T-cells (NFAT) promoter [40, 41]). The mixtures of cells were twice centrifuged and supernant were removed from the cell pellet. The BWZ.36 cells have no functional TCR (α or β chain) [42]. An out-of-frame TRBV12 sequence was present in the parental fusion partner BWZ.36 and in all the hybridomas [43].

One milliliter of PEG1450 (0.5 g/ml. Sigma, P7181) was added to the cell pellet, drop by drop, over 45 seconds. Fetal Bovine Serum-free DMEM media was added to the cells slowly to a final volume of 50 ml. Cells were mixed by inverting the tube several times and then incubated at 37°C for 10 minutes to complete fusion. After washing with Hanks Balanced Salt Solution (HBSS), cells were diluted to different densities (3×10^5 /ml, 3×10^4 /ml and 3×10^3 /ml) with DMEM-10. Cells (100µl) were plated and cultured in round bottom 96-well-plates followed with supplementation of 100 µl 2x HAT (Hypoxanthine H, 2×10^{-4} M; Aminopterin A, 0.8×10^{-6} M; Thymidine T, 3.6×10^{-5} M) and 2x hygromycin B (2mg/ml, Sigma Cat: H0654) selection media on the next day. Seven to ten days after fusion, cell colonies were visible. Response to a variety of native and altered insulin B:9-23 peptides were tested with a LacZ activity assay. Cells were cultured in 1× HA media (Hypoxanthine H, 10^{-4} M; Aminopterin A, 0.4×10^{-6} M); and hygromycin B for at least 10 days and then were transferred to regular DMEM -10 media for maintenance. Colonies positive by the LacZ activity assay were subcloned.

BDC12-4.4 retrogenic mice were produced as previously described following published techniques [39, 44]. Mice were bred and housed under specific pathogen-free conditions at the University of Colorado Denver Center for Comparative Medicine (CCM) with an approved protocol from the University of Colorado Denver Animal Care and Use Committee.

T cell Receptor stimulation LacZ assay

Antigen specific hybridomas stimulation was estimated by measuring LacZ activity using chlorophenol red- β -D-galactopyranoside (CPRG) as substrate [40]. NOD spleen cells (1×10^5 /100 µl /well) were used as antigen presenting cells. Insulin2 B:9-23 peptide (SHLVEALYLVCGERG) as well as a mutated peptide insulin2 B:19A (SHLVEALYLVAGERG, 100 µg/ml final concentration) were used as standard stimulating peptides. Fluid phase anti-CD3 antibodies or anti-TCR beta chain antibodies (1µg/ml) were used as positive controls. Tetanus Toxin (TT, QYIKANSKFIGITEL) peptide, a BDC2.5 mimotope HRPI-RM peptide (EKAHRPIWARMDAKK) [45], and B:9-23 peptide with

amino acid 16 mutated to alanine (B:16A) were used as negative controls. NOD splenocytes were cultured separately in media with negative control peptides, B:9-23 peptides, or anti-CD3 antibodies in flat bottom 96-well plates for at least 1 hour in DMEM-10 at 37°C to allow binding of peptide to antigen presenting cells. Twenty thousand T cell hybridomas were then added to each well with the peptide loaded splenocytes. After overnight incubation at 37°C, cells were washed with PBS to remove the media and 100 µl of CPRG reagents (91mg/L CPRG, 0.125% NP-40, 1mM MgCl₂ in 10mM phosphate buffer) were added to each well. This caused cell lysis and intracellular LacZ was released into the supernatant. Cleavage of CPRG by LacZ resulted in production of chlorophenol red. After 4 hours at 37 °C the absorbance was read at 575 nm and 655 nm (reference wavelength). Antigen-specific hybridoma responses were increased several fold over the background level. If the response was weak, the plate was left to develop overnight at room temperature. The OD value was reread the next day. The stimulation index (SI) was calculated as LacZ activity compared to background without stimulation. The TT peptide never induced a stimulation index greater than 2, and the positive hybridomas responded to peptide with SI greater than or equal to 3. All positive hybridomas were confirmed responsive to B:9-23 in a repeat experiment.

Flow cytometry assay

Hybridomas were incubated with monoclonal antibodies (APC conjugated anti-mouse CD3e (145-2C11); PE anti-mouse TCRβ chain (H57-597); FITC conjugated anti-mouse TCRVβ2 chain (B20.6) or isotype antibodies in flow cytometry staining buffer (2% FBS, 1mM EDTA, 0.1% Na Azide in Hank's buffered salt solution) at 4°C for 30 minutes. After washing, cells were analyzed with a FACSCalibur flow cytometer. Hybridomas from 12-4.1 TCRα⁺β⁺Ca^{-/-} mice and 12-4.4 TCRα⁺Ca^{-/-} retrogenic mice, cells were analyzed by flow cytometry with monoclonal Abs specific for the different Vβ family chains (Mouse Vβ TCR Screening Panel, CAT: BD 557004) identifying their specific beta chains.

Gene Analysis and Sequencing of TCRs

Total RNA was isolated from 1–5 × 10⁶ T cell hybridomas using the SuperTCR Express Mouse T cell Receptor Vβ Repertoire Clone Screening Assay Kit (BioMed Immunotech), and cDNA was prepared by reverse transcription and PCR. Multiple beta chains were amplified following the protocol supplied with the Vβ Repertoire Clone Screening Assay Kit. The amplified products were visualized on 2% agarose gels and extracted with a QIAquick Gel Extraction kit. DNA was inserted into the TA cloning vector PCR2.1 (Invitrogen) followed by transformation of One Shot E. coli cells and plating on LB plus ampicillin with X-gal. White colonies were selected to expand at 37°C in LB plus ampicillin overnight. The plasmid was extracted from 1.5ml of cultures. Two to four DNA clones from each T cell hybridoma were sequenced in both directions using M13 Reverse and M13 Forward primers (Forward: 5'-GTA AAA CGA CGG CCA G-3', Reverse: 5'-CGA GAA ACA GCT ATG AC-3') on the AB1373A DNA sequencer (Applied Biosystems). Random hexamer primers (Pharmacia) were used to reverse transcribe hybridomas. The α chain genes were amplified by PCR using specific primers (BDC12-4.1 alpha chain: Forward: 5'-CAATGAAAACATATGCTCCTAC-3', Reverse: 5'-TGTCCTGAGACCGAGGAT-3';

BDC12-4.4 alpha chain: Forward: 5'-GGCGAGCAGGTGAGACAG-3', Reverse: 5'-CAGGCAGAGGGTGCTGTCCTG-3').

Results

BDC12-4.1 and BDC12-4.4 complete TCR hybridomas respond to similar multiple insulin peptides

In order to generate TCR hybridomas, splenocytes and lymph node cells were fused with the BWZ.36 cell line. BWZ.36 lacks functional TCR genes. We initially created T cell hybridomas from BDC12-4.1 TCR $\alpha^+\beta^+$ RAG $^{-/-}$ mice and BDC 12-4.4 TCR $\alpha^+\beta^+$ SCID retrogenic mice [44]. To confirm TCR expression, hybridomas were stained with monoclonal antibodies specific to their relevant V β chains. The TCR beta chains from both hybridomas were sequenced as described above. As expected, only TRBV1 was used by BDC12-4.1 hybridomas and TRBV5 by BDC12-4.4 hybridomas. The sequences of beta chains were confirmed (Table 1) [16] and the alpha chain was the expected TRAV5D-4*04 (GeneBank Access numbers. [U80816](#), [DQ172905](#).) for both BDC12-4.1 TCR hybridomas [16] and BDC12-4.4 TCR [10].

Both BDC12-4.1 and BDC12-4.4 T cell clones were initially derived from non-diabetic NOD mice and were discovered subsequently to respond to insulin B:9-23 peptides. To test the responsiveness of the BDC12-4.1 and BDC12-4.4 T cell hybridomas produced in the current study, we tested a series of B:9-23 peptides including altered and truncated versions. The BDC12-4.1 and BDC12-4.4 hybridomas responded similarly to the altered and truncated insulin B:9-23 peptides (Figure 1). N-terminal truncated peptides B:10–23, B:11–23 and B:12–23 but not B:13–23 stimulated both BDC12-4.1 TCR hybridomas and BDC12-4.4 hybridomas which indicated that their minimal epitope contains the 12th amino acid valine at its N-terminus. At the C-terminus, both hybridomas responded to the truncated peptide B:9–22 but the BDC12-4.1 hybridomas responded to B:9-21 (SI =4.0 $\pm$ 0.38, Figure 1C) while the BDC12-4.4 hybridomas did not. The insulin B:12–22 peptide stimulated both BDC12-4.1 and BDC12-4.4 hybridomas. None of the truncated peptides lacking both the 12th amino acid residue (valine) and 22nd residue (arginine) stimulated the hybridomas. The BDC12-4.1 and BDC12-4.4 hybridomas failed to respond to the B16A mutated (alanine replacing tyrosine at position B16) B:9-23 peptide. Figure 1C summarizes the response of the two hybridomas to multiple peptides. The similar peptide recognition by both BDC12-4.1 TCR $\alpha^+\beta^+$ and BDC12-4.4 TCR $\alpha^+\beta^+$ hybridomas is likely determined by the conserved Valpha and Jalpha sequences, despite markedly different V β sequences.

In BDC12-4.1 $\alpha^+\beta^+$ hybridomas, native B:9-23 peptide sequences from both insulin1 and insulin 2 (proline versus serine at position B9) induced strong stimulation (average SI 18.8 $\pm$ 1.1 and 15.38 $\pm$ 0.8, Figure 1C). Figure 2A illustrates the dose response of BDC12-4.1 hybridomas to the peptide with or without B:19 cysteine replaced by alanine. The dose response curve of the B:19A peptide was shifted to lower concentrations compared to the response to the native insulin2 B:9-23 peptide. Three μ g/ml of B:19A stimulated while nearly 50 μ g/ml of native B:9-23 was required for a significant stimulation. In addition, the 100 μ g/ml response to the B:19A peptide was greater (SI: 20.8 $\pm$ 1.5 vs. 7.5 $\pm$ 0.8). In contrast,

the BDC12-4.4 hybridomas responded better to native insulin_{2B:9-23} than to the B19A peptide (Figure 2B).

The major aim of the current study was to determine if other TCR beta chains function as well as the specific BDC12-4.1 or BDC12-4.4 beta chains with their conserved alpha chains in recognizing the insulin B:9-23 peptide. We generated hybridomas from BDC12-4.1 TCR α^+ C $\alpha^{-/-}$ mice. In these mice, all T cells have the transgenic alpha chain, but utilize multiple endogenous beta chains. We fused splenocytes or pancreatic lymph node cells from two BDC12-4.1 TCR α^+ C $\alpha^{-/-}$ mice with BWZ.36 cells in independent fusions. All hybridomas were evaluated for response to a control tetanus toxin (TT) peptide, insulin B:9-23, insulin B:19A peptide, and anti-CD3 antibodies. None of the 161 CD3 antibody-responsive hybridomas from non-immunized mice responded to insulin B:9-23 peptides. In order to generate insulin B:9-23 peptide responsive hybridomas, we immunized BDC12-4.1 TCR α^+ C $\alpha^{-/-}$ mice with insulin B:9-23 in IFA. From two independent fusions, 14 out of 746 CD3 antibody-responsive hybridomas responded to the insulin B:19A peptide. In total, 14 hybridomas out of 907 (1.54%) anti-CD3 responsive hybridomas from BDC12-4.1 TCR α^+ C $\alpha^{-/-}$ mice responded to insulin peptides (Figure 3A). Table 3 summarizes the numbers of hybridomas studied for responses to insulin peptides.

To analyze the frequencies of endogenous TCR beta chains that can pair with a second conserved anti-B:9-23 TCR alpha chain, we created hybridomas by fusing splenocytes from BDC 12-4.4 TCR α^+ C $\alpha^{-/-}$ NOD/SCID retrogenic mice with BWZ.36 cells. Bone marrow cells from donor BDC12-4.4 TCR α^+ C $\alpha^{-/-}$ NOD mice were used to create retrogenic mice. After screening with the LacZ activity assay stimulated with insulin B:9-23 peptide, four out of 496 anti-CD3 responsive hybridomas (0.8%) responded to insulin B:9-23 peptides. Figure 3B illustrates the response of positive hybridomas in the LacZ activity assay.

In summary, approximately one percent of anti-CD3 responsive hybridomas from TRAV5D-4 alpha chain fixed transgenic mice (BDC12-4.1 or 12-4.4) respond to the insulin B:9-23 peptides.

Multiple beta chains pair with TRAV5D-4 conserved alpha chain in insulin peptide-responsive hybridomas

To define the beta chain repertoires of the TRAV5D-4 alpha chain T cell hybridomas with endogenous beta chains, we analyzed insulin B:9-23 responsive hybridomas and non-responsive hybridomas with antibodies specific for different beta chains by flow cytometry. Responsive hybridomas used multiple different beta chains in both BDC12-4.1 and BDC12-4.4 alpha chain fixed mice. Table 4 summarizes the beta chain sequences of the responsive hybridomas that were single clones. The sequences of cell lines which had more than one T cell receptor are not included in the table. In individual BDC12-4.1 TCR α^+ C $\alpha^{-/-}$ hybridomas, TRBV3, TRBV13-3, TRBV15, TRBV16, and TRBV19 were utilized. In addition, N region and beta chain usage varied. In BDC12-4.4 TCR α^+ C $\alpha^{-/-}$ NOD hybridomas, TRBV1 and TRBV5 were used to recognize the insulin B:9-23 peptide, again with different N and J regions (Table 4). No conserved sequence was found among the beta chains paired with the conserved TRAV5D-4 alpha chain and recognized the insulin B:9-23

peptide. In conclusion, multiple beta chains pair with the conserved TRAV5D-4 alpha chain and permit TCR recognition of insulin B:9-23 peptides.

Discussion

We previously reported that an alpha chain only (BDC12-4.1 TCR $\alpha^+Ca^{+/-}$) transgenic NOD mouse develops insulin autoantibodies but not diabetes [39]. In contrast, insulin autoantibodies are suppressed in mice with a transgene encoding the BDC12-4.1 beta chain [39]. In this study, we have produced T cell receptor hybridomas from mice expressing the alpha and beta chains of insulin B:9-23 responsive hybridomas. The results indicate that multiple different TCR beta chains are able to pair with the conserved TRAV5D-4 alpha chains to create T cell receptors that recognize B:9-23 peptides. Most endogenous beta chains do not permit T cells to recognize the insulin B:9-23 peptide, even though the TCRs are expressed on the cell surface and the hybridomas can be stimulated by anti-TCR beta chain and anti-CD3 antibodies.

Having responsive BDC12-4.1 and BDC12-4.4 hybridomas driving LacZ expression allowed us to characterize the response to multiple mutated and truncated insulin B:9-23 peptides in detail. The BDC12-4.1 hybridomas and BDC12-4.4 hybridomas share the same TRAV5D-4 and J alpha gene segment while their beta chain sequences are very different. In this study, both BDC12-4.1 hybridomas and BDC12-4.4 hybridomas respond similarly (but not identically) to B:9-23 peptides. Both cells respond to insulin1 and insulin2 B: 9-23 peptides. The BDC12-4.1 T cell hybridoma responds better to a mutated B:9-23 peptide with the native cysteine at position 19 replaced with an alanine. In contrast, BDC12-4.4 hybridomas responded less well to B:19A peptide. The BDC 12-4.1 hybridomas were stimulated by a B:9-21 peptide while BDC12-4.4 hybridomas were not and both hybridomas responded to insulin B:12-22. The similar peptide recognition profiles of both BDC12-4.1 TCR $\alpha^+\beta^+$ and BDC12-4.4 TCR $\alpha^+\beta^+$ hybridomas is likely determined by their conserved Valpha and Jalpha sequences. Similar to the original BDC12-4.1 TCR, all 14 B:9-23 peptide responsive hybridomas with the BDC12-4.1 TCR alpha chain (despite different beta chains) respond to the B:19A peptide better than native B:9-23. In contrast, the insulin peptide responsive BDC12-4.4 hybridomas respond better to the native B:9-23 peptide than to the mutated B:19A peptide.

Having hybridomas with TCRs stimulating LacZ expression facilitated screening a large number of T cell hybridomas from alpha chain single transgenic mice. Multiple different TCR beta chains with different Vbeta, N regions, D, and J regions were able to combine with the conserved anti-B:9-23 Valpha chains and respond to insulin peptide B:9-23. Most hybridomas generated from alpha chain only transgenic mice responded to anti-TCR antibodies but not insulin peptides. Our study indicates that both TCR alpha and beta sequences make important contributions to the responsiveness of T cells with the conserved anti-B:9-23 alpha chain. In our previous report [39], mice carrying both BDC 12-4.1 TCR α and β chain ($Ca^{+/-}$) develop insulinitis, whereas mice with the BDC 12-4.1 TCR α chain only developed peri-islet insulinitis. The lack of insulinitis in BDC12-4.1 alpha chain mice may be related to a small number of T cells with “permissive” beta chains in these mice.

We hypothesize that the conserved alpha chains with multiple different permissive beta chains targeting B:9-23 presented by I-Ag7, are a necessary component for the development of spontaneous anti-insulin autoimmunity of the NOD mouse, given the NOD strain's inability to maintain tolerance. The great diversity of beta chains able to complement TRAV5D-4 containing conserved alpha chains for recognition of the B:9-23 peptide suggest that the TCR topography of binding to I-Ag7-peptide is likely to be unusual as has been reported for a few other “autoimmune” T cell receptors[46].

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Abbreviations

RAG	recombinase activating gene
LacZ	beta-D-galactosidase
SI	stimulation index
TT	Tetanus Toxin
IFA	incomplete Freund's adjuvant

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Figure 1.

A: Stimulation of BDC12-4.1 TCR α + β + hybridomas by truncated and mutated insulin B: 9-23 peptides. BDC12-4.1 TCR α + β + hybridomas respond to multiple insulin B:9-23 peptides. From left to right is from the 9th amino acid residue to the 23rd amino acid of the B:9-23 peptide (insulin 1 P at B9; insulin 2 S at B9). An empty box indicates that the amino acid is absent in the truncated peptide. The mutated amino acid residue is shown using standard single letter code. Peptides which stimulate the hybridomas are shown with green box; peptides which do not stimulate are shown with yellow box. B: Stimulation of BDC12-4.4 TCR α + β + hybridomas by multiple peptides as in panel A. C: The response of multiple insulin peptides in BDC12-4.1 complete TCR hybridomas or BDC12-4.4 complete TCR hybridomas. Data are the mean $\pm$ SEM of triplicate wells. A tetanus toxin peptide was used as negative control and anti-CD3 antibodies in fluid phase were used as positive control. Stimulation index is calculated relative to the PBS response.

Figure 2.

2A illustrates the OD values of the dose response of BDC12-4.1 TCR α + β + hybridomas to insulin2 B:9-23 and B:19A peptide. Triangle: insulin2 B:19A peptide; square: insulin2 B:9-23 peptide. Data shown are average values of triplicate wells. Figure 2B illustrates the OD values of the dose response of BDC12-4.4 TCR α + β + hybridomas to insulin2 B:9-23 and B:19A peptide. Triangle: insulin2 B:19A peptide; square: insulin2 B:9-23 peptide.

Figure 3.

The response to insulin peptide in hybridomas generated from insulin B:9-23 peptide immunized 12-4.1 TCR $\alpha^+ \text{Ca}^{-/-}$ mice (Figure 3A) and BDC12-4.4 TCR $\alpha^+ \text{Ca}^{-/-}$ retrogenic mice spleen cells or pancreatic lymph node cells (Figure 3B). Blank bar: stimulation index of tetanus toxin peptide. Black bar: stimulation index of insulin B19A or B:9-23 peptide. Names of hybridoma reflect source of cells (L: cells are from pancreatic lymph node lymphocytes. SP: cells are from splenocytes.)

Table 1

Summary of T cell receptors of mice used to generate hybridomas.

Source Mice of Hybridomas	TCR α Chain (TRAV, N, TRAJ)	TCR β Chain (TRBV, nDn, TRBJ)	Donor mice for retrogenic mice	Recipient mice as retrogenic
BDC12-4.1 TCR $\alpha^+\beta^+$ RAG $^{-/-}$	TRAV5D-4*04, GAN, TRAJ53	TRBV1, PGLGN, TRBJ2-7	NA *	NA
BDC12-4.4 TCR $\alpha^+\beta^+$ retrogenic NOD/SCID	TRAV5D-4*04, A, TRAJ53	TRBV5, DT, TRBJ2-2	SCID	SCID
BDC12-4.1 TCR $\alpha^+\text{Ca}^{-/-}$ mice	TRAV5D-4*04, GAN, TRAJ53	Endogenous beta chains	NA	NA
BDC12-4.4 TCR $\alpha^+\text{Ca}^{-/-}$ retrogenic NOD/SCID mice	TRAV5D-4*04, A, TRAJ53	Endogenous beta chains	C $\alpha^{-/-}$	SCID

* Not Applicable.

Table 2

Amino acid sequences of BDC12-4.1 and 12-4.4 T cell receptors.

Alpha Chains		CDR1	CDR2
4.1 α:	GEQVEQLPSILRVQEGSSASINCSYE	DSASNY	FPWYKQEPGENPKLIID IRSNME
4.4 α:	GEQVEQLPSILRVQEGSSASINCSYE	DSASNY	FPWYKQEPGENPKLIID IRSNME
		CDR3	N region J region
4.1 α:	RKQTQGLIVLLDKKAKRFLHITDTQPGDSAMYFC	AAS	GAN SGGSNYKLTFG
4.4 α:	RKQTQGLIVLLDKKAKRFLHITDTQPGDSAMYFC	AAS	A SGGSNYKLTFG
		C region	
4.1 α:	KGTLTIVTP	NIQNPEPAVYQLKDPRSQDSTLCCLFTDFDSQINVPKTMESGTFITDKTV	
4.4 α:	KGTLTIVTP	NIQNPEPAVYQLKDPRSQDSTLCCLFTDFDSQINVPKTMESGTFITDKTV	
4.1 α:	LDMKAMDSKSNGAIAWSNQTSTFTCDIFKETNATYPSSDVPCDATLTEKSFETDMNL		
4.4 α:	LDMKAMDSKSNGAIAWSNQTSTFTCDIFKETNATYPSSDVPCDATLTEKSFETDMNL		
4.1 α:	NFQNLVSMGLRILLKLVAGFNLLMTLRLWSS		
4.4 α:	NFQNLVSMGLRILLKLVAGFNLLMTLRLWSS		

Beta Chains		CDR1	CDR2
4.1 β:	VTLLEQNPRWRLVPRGOAVNLRCLK	NSQYPWM	SWYQQDLQKQLQWLFT LRSPGD
4.4 β:	NTKITQSPRYLILGRANKSLECEQH	LGHNAM	YWYKQSAEKPPPELMFL YNLKQL
		CDR3	N region J region
4.1 β:	KEVKSLPGADYLATRVTDTELRLQVANMSQGRITLYC	TCS	PGLGN EQYFGPGTR
4.4 β:	IRNETVPSRFIECPDSSKLLHISAVDPEDSAVYFC	ASSQ	DT NTGQLYFGEYSK
		C region	
4.1 β:	LTVL EDLRNVTTPKVSLEFESKAEIANKQKATLVCLARGFFPDHVELSWVWNGKEVHSG		
4.4 β:	LTVL EDLRNVTTPKVSLEFESKAEIANKQKATLVCLARGFFPDHVELSWVWNGKEVHSG		
4.1 β:	VSTDPAQAYKESNYSYCLSSRLRVSAFWHNPNNHFRQVQFHGLSEEDKWPEGSPKPV		
4.4 β:	VSTDPAQAYKESNYSYCLSSRLRVSAFWHNPNNHFRQVQFHGLSEEDKWPEGSPKPV		
4.1 β:	QNISAEAWGRADCGITSASYHQGVLSATILYEILLGKATLYAVLVSGVLVLMAMVKKKNS		
4.4 β:	QNISAEAWGRADCGITSASYHQGVLSATILYEILLGKATLYAVLVSGVLVLMAMVKKKNS		

Table 3

Frequencies of B:9-23 and B:19A peptide-responsive hybridomas derived from mice with the transgenic TCR alpha chain paired with endogenous TCR beta chains.

Insulin peptide response*	12-4.1 TCR $\alpha^+ \text{Ca}^{-/-}$ Transgenic mice	12-4.4 TCR $\alpha^+ \text{Ca}^{-/-}$ Retrogenic mice
positive	14/907 (1.54%)	4/496 (0.8%)
negative	893/907 (98.46%)	491/496 (99.2%)

* Anti-insulin B:9-23 or B:19A peptide stimulation was evaluated with LacZ activity assay for all hybridomas. Both B:9-23 peptide responsive positive and non-responsive negative hybridomas responded to stimulation by anti-CD3 antibodies.

Table 4

Sequences of endogenous TCR beta chains of insulin 2 B:9-23 peptide-responsive hybridomas generated from BDC 12-4.1 TCR $\alpha^+\beta^-C\alpha^{-/-}$ transgenic mice or BDC12-4.4 TCR $\alpha^+C\alpha^{-/-}$ NOD/SCID retrogenic mice.

Source mice	V beta (CDR3)	n region	J beta
BDC12-4.1 alpha chain transgenic mice	TRBV3: VERPDGSYFTLKIQPTALED SAVYFCASSL	DGGVC	TRBJ2-3*01: AETLYFGSGTRLTVL
	TRBV13-3: KASRPSQENFSLILELASLSQTAVYFCASSD	APF	TRBJ2-7*01: YEQYFGPGTRLTVL
	TRBV13-3: KASRPSQENFSLILELASLSQTAVYFCASS	WDWGGVE	TRBJ2-5*01: DTQYFGPGTRLLVL
	TRBV15: AEMLNSSFSTLKIQPTPKDSAVYLCASSL	RP	TRBJ1-1*01: NTEVFFGKGTRLTVV
	TRBV16: AQMPNQSHSTLKIQTQPQDSAVYLCASSL	DRGQA	TRBJ1-1*01: NTEVFFGKGTRLTVV
	TRBV19: ASREKKSSFSLTVTSAQKNEMAVFLCASSI	RG	TRBJ1-1*01: TEVFFGKGTRLTW
BDC12-4.4 alpha chain retrogenic mice	TRBV19: ASREKKSSFSLTVTSAQKNEMAVFLCASSI	WDWG	TRBJ2-4*01: NTLYFGAGTRLSVL
	TRBV1: ATRVTDTELRLQVANMSQGRTLYCTCSA	VGC	TRBJ1-5*02: QPAPLFGEGTRLSVL
	TRBV5: PECPDSSKLLHISAVDPEDSAVYFCASSQ	ET	TRBJ2-2*01: NTGQLYFGEGSKLTVL
	TRBV5: PECPDSSKLLHISAVDPEDSAVYFCASSQ	ETGGGG	TRBJ2-5*01: DTQYFGPGTRLLV