Type 1 diabetes of both humans and NOD mice is characterized by selective destruction of β-cells within pancreatic islets (1–4). For the NOD mouse, multiple studies demonstrate that insulin is a primary autoantigen for triggering anti-islet autoimmunity (5,6). To date, with the exception of preproinsulin (7), deletion of several characterized islet target antigens does not alter progression to diabetes in the NOD mouse (8–10). Even the prominent CD8 targeting of the molecule islet glucose-6-phosphatase catalytic subunit-related protein indicate that the insulin B chain peptide, amino acids 9–23 (insulin B9–23) containing a native tyrosine at position 16, is essential for development of diabetes (13). NOD mice lacking native insulin but producing an insulin with a mutation of the B9–23 sequence (B16A) do not develop diabetes (7), and elimination of insulin-reactive T cells results in the dramatic prevention of diabetes (14,15).

Most T-cell receptor (TCR) interactions with peptide-major histocompatibility complex (MHC) complexes occur through binding of six complementarity determining regions (CDR; three each for α- and β-chains). The CDR3 region is most often crucial for antigen recognition (16). In this region, which includes the N region for α-chains and nDN region for β-chains, highly variable amino acid sequences are generated from gene rearrangements of V and J segments (plus D segment for β-chains) (16). The CDR1 and CDR2 regions are germline-encoded by the V segments and, for many TCRs, predominantly interact with the α helices of the MHC molecule (17,18). In NOD mice, TCRs targeting the insulin B9–23 peptide presented by the I-A^d MHC class II molecule frequently use the Vα gene segment TRAV5D-4^04 (formerly termed Vα 1383) rearranged to the Ja gene segments TRAJ53 and 42 (19,20). Among these TCRs, the N region sequences of the α-chains were highly variable, and no consistent TCR β-chain usage was apparent. Two anti-insulin B9–23 TCR α-chains (derived from T-cell clones 12–4.1 and 12–4.4) using the same Vα (TRAV5D-4^04) and Ja (TRAJ53) gene segments, but having unique N region sequences, were capable of inducing insulin autoimmunity in Ca knockout NOD mice (21). In this article, we show that the sequences underlying such induction of insulin autoimmunity are relatively simple. Namely, the germline-encoded sequences of Vα TRAV5D-4 CDR1 and CDR2 combined with many CDR3 sequences and diverse Ja elements are sufficient to induce anti-insulin autoimmunity.

RESEARCH DESIGN AND METHODS

Mice. NOD.scid mice (NOD.CB17-Prkdc^csd/J, 001303) and Ca knockout NOD mice (NOD.129P2(C)-Tcra^tm1Mjo/Doj, 004444) were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.A double insulin-knockout NOD.scid mice were generated in the Eisenbarth laboratory (13). All three strains, NOD.scid, and NOD.scid mice, and retrogenic mice were maintained in a pathogen-free animal colony at the Barbara Davis Center satellite animal facility and the Center for Comparative Medicine. All animal experiments were approved by the Animal Care and Use Committee of the University of Colorado Denver.

Generation of α-chain retrogenic mice. Retrogenic mice were generated using the modified version of the protocol described previously (22,23). TCR α-chain constructs were either generated by PCR using cDNA from original T-cell clones (12–4.4, 12–4.1, BDC-6.9, BDC-10.1, BDC-2.5, 14H4, 5F2, and 6C5) or were assembled based on sequences. For the NY4.1 α-chain, a sequence published in the National Center for Biotechnology Information was used (accession number U80816). The 2H6 sequence was kindly provided from Dr. Li Wen (Yale University, New Haven, CT). TCR α-chain constructs encoding all α-chains detected by the 454 high-throughput sequencing and chimeric human Vα TRAV3–1 α-chains were also assembled by PCR with overlapping primers. TCR α-chain constructs were cloned into marine stem cell virus (MSCV)-based retroviral vectors carrying green fluorescent protein (GFP) (pMiGID) (22). Phoenix cells were cotransfected with the pMiGID plasmids and the pCL-Eco packaging vector using Lipofectamine 2000 (Life Technologies/Invitrogen) to produce replication-incompetent retrovirus encoding TCR α-chain sequences. Bone marrow cells were prepared from Ca knockout NOD.
mice treated with 5-fluorouracil (Sigma-Aldrich) and spin-injected with the retroviral supernatant daily for 4 consecutive days. The bone marrow cells were cultured in complete DMEM containing 20% heat-inactivated fetal bovine serum, 20 ng/mL IL-3, 50 ng/mL IL-6, and 50 ng/mL stem cell factor (Life Technologies/Invitrogen). NOD.scid mice received 210 rad of radiation from the IBL 437C10 irradi (Ciba International) or 225 rad from the RS2000 X-ray irradiator (Radiation Source Technologies, Suwanee, GA) and were injected with the bone marrow cells (2 × 10^6 cells) intravenously. For all experiments, cultured bone marrow cells were assessed for GFP expression by flow cytometry prior to the injection. 2 × 10^6 cells were positive for GFP. Peripheral blood mononuclear cells (PBMC) from all recipient mice were assessed for T-cell generation 5 weeks post-bone marrow transplantation; generally, 5%–20% of PBMC expressed GFP and CD4. PBMC were stained with anti-CD4, anti-CD8, and anti-TCRαβ antibodies (eBioscience) and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA).

**Measurement of micro-insulin autoantibody assay.** All retrogenic mice were bled at 8, 12, 16, and 20 weeks after bone marrow transfer. Female NOD mice were bled at 8, 12, 10, 12, and 16 weeks of age for comparison. Islet autoantibody (IAA) levels were measured with the 96-well filtration plate micro-IAA assay previously described and expressed as an index (24). A value ≥0.01 is defined as positive.

**Assessment of diabetes incidence.** The blood glucose levels were measured weekly. Retrogenic mice considered diabetic after two consecutive blood glucose values ≥250 mg/dL.

**Assessment of insulin of antigens.** The pancreata were obtained when killed (8–20 weeks after bone marrow transplant) and were fixed in 10% formalin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin, and sections from islet grafts were also stained with polyclonal guinea pig anti-insulin antibodies (Linco Research Immunology, St. Charles, MO), followed by incubation with a peroxidase-labeled antiguinea pig IgG antibody (Kirkegaard and Perry Laboratories). More than 10 pancreatic islets from an individual mouse were randomly selected and evaluated for lymphocytic infiltration (no insulin, peri-inslet infiltrates, intraislet infiltrates) by the same reader (M.N.) blinded to the group of mice.

**IFN-γ enzyme-linked immunospot assay.** IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed according to the manufacturer's instructions (BD Biosciences). Retrogenic mice (100 pancreatic islets/well), harvested from NOD.scid mice along with spleen cells from young NOD mice (1 × 10^6 cells/well) overnight. IL-2 secretion in supernatants was measured with multiple CDR3-β hybridomas that were selected by ELISPOT assay. The pancreata were obtained when killed (8–20 weeks after bone marrow transplant) and were fixed in 10% formalin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin, and sections from islet grafts were also stained with polyclonal guinea pig anti-insulin antibodies (Linco Research Immunology, St. Charles, MO), followed by incubation with a peroxidase-labeled antiguinea pig IgG antibody (Kirkegaard and Perry Laboratories). More than 10 pancreatic islets from an individual mouse were randomly selected and evaluated for lymphocytic infiltration (no insulin, peri-inslet infiltrates, intraislet infiltrates) by the same reader (M.N.) blinded to the group of mice.

**RESULTS**

Retronogic NOD mice expressing Vα TRAV5D-4 α-chains with multiple CDR3α sequences develop anti-insulin autoimmunity. To investigate which α-chain TCR sequences are sufficient to induce insulin autoantibodies, we first created a series of retrogenic mice expressing TCR α-chain sequences derived from NOD CD4 T-cell clones into TCR α-chain knockout NOD mice (Supplementary Table 1). In these mice, individual T cells express the introduced TCR receptor α-chain that associates with endogenously selected β-chain sequences produced in vivo. The α-chain TCR sequences used are derived from clones that react to the insulin B9–23 peptide, unknown islet antigens, or completely unrelated antigens. Despite different N regions and Jα sequences, six out of seven α-chain retrogenic strains expressing the TRAV5D-4 sequence developed high levels of insulin autoantibodies (Fig. 1A–G and Supplementary Table 1). Of note, α-chains originally derived from diabetogenic T-cell clones that react with unknown islet antigens (i.e., NY4.1 described by Santamaria et al. (26) and BDC-6.9 established by Haskins et al. (27)), or even a non-diabetogenic clone reactive with an HEL peptide [14H4 established by Vignali and colleagues (28)], could induce insulin autoantibodies. Three strains expressing α-chains originally reacting with the insulin B9–23 peptide developed relatively the higher level of insulin autoantibodies compared with female wild-type NOD mice (Fig. 1A–C and N; P = 0.03 [12–4.4], 0.04 [12–4.1], and <0.01 [8–1.1] versus wild-type NOD). In contrast, only one of the retrogenic strains expressing TCR α-chains other than Vα TRAV5D-4 developed insulin autoantibodies (Fig. 1H–M; P < 0.02 versus strains that express α-chains with TRAV5D-4). Retrogenic mice expressing TCR α-chains derived from clones known to be highly diabetogenic, but that do not use Vα TRAV5D-4 [BDC-2.5 and BDC-10.1 established by Haskins et al. (27)], and those expressing TCR α-chains from clones that respond to the insulin B9–23 peptide [2H6 established by Wen and colleagues (29) and 12–2.35 established by
by Daniel and Wegmann (30) did not lead to the production of insulin autoantibodies. The only non–TRAV5D-4 α-chain that induced insulin autoantibodies is derived from a diabetogenic insulin B:9–23-reactive T-cell clone [12–1.19 established by Daniel and Wegmann (30)]. It is notable that none of the three additional retrogenic mouse strains expressing α-chains containing this Vα segment (TRAV13–1) used by this 12–1.19 α-chain developed insulin autoantibodies (see below; Fig. 4D). Although TRAV5D-4 sequences induced insulin autoantibodies, only a subset of the Vα TRAV5D-4 retrogenic mice developed overt diabetes (Supplementary Fig. 1), and the severity of lymphocytic infiltration in the islets was consistent with the diabetes incidence (Fig. 2). Of note, although α-chain retrogenic mice are lymphopenic compared with wild-type mice in general, and peripheral CD4 and CD8 T-cell populations have broad variations even in the same strain (Supplementary Fig. 2), the number of T cells in the periphery did not correlate with the development of insulin autoantibodies, insulinitis, and diabetes, and the percentages of CD4 and CD8 T cells do not statistically differ by strains by one-way ANOVA. Expression of the insulin B:9–23 sequence is required for the development of anti-insulin autoimmunity. We next asked whether development of insulin autoimmunity in TRAV5D-4 α-chain retrogenic mice is associated with the recognition of the insulin B:9–23 peptide. Spleen cells from strains expressing TRAV5D-4 α-chains that produced high levels of insulin autoantibodies (from clones 12–4.1, 12–4.4, 8–1.1, and BDC-6.9) responded to insulin B:9–23, but did not respond to either the insulin B:9–23 peptide with B16:A mutation or a control tetanus toxin 830–843 peptide (Fig. 3A). Responses to the insulin B:9–23 peptide by spleen cells from 14H4, 5F2, and NY4.1 α-chain retrogenic mice were undetectable. The levels of insulin autoantibodies in these strains are not as high as those of other TRAV5D-4 α-chain strains showing reactivity to the insulin
B:9–23 peptide, which may suggest the low probability of B:9–23-reactive T cells in the strains without the response to B:9–23. In the retrogenic mice that express an α-chain other than TRAV5D-4, spleen cells from only the TRAV5D-4–23 peptide. As shown in Fig. 3C, 17 out of 30 5KC CD4+ T-cell lines responded to islets isolated from NOD.scid mice, and 14 of the 17 islet-responding lines (82%) were insulin B:9–23-reactive. Thus, the majority of TCRs of CD4 T cells infiltrating and responding to pancreatic islets of TRAV5D-4 α-chain retrogenic mice reacted with the insulin B:9–23 peptide.

We then asked whether the expression of native insulin B:9–23 sequence is required for TRAV5D-4 α-chain retrogenic mice to develop anti-insulin autoimmunity in vivo. To test this, we used B16:A double insulin-knockout NOD.scid mice (both native Ins1 and Ins2 genes knocked out) as bone marrow recipients (13). With the knockout of native insulin genes (Ins1 and Ins2) and a preproinsulin transgene producing insulin in islets with alanine rather than tyrosine at position B16, retrogenic mice do not express native insulin in their islets and lymphoid epithelial cells. The lack of native insulin B:9–23 expression in the recipient mice abrogated development of insulin autoantibodies (Fig. 3E; \( P < 0.01 \)), diabetes, and lymphocyte reactivity to the insulin B:9–23 (Fig. 3D; \( P < 0.02 \)). Thus, development of anti-insulin autoimmunity of TRAV5D-4 α-chain retrogenic mice is dependent upon the expression of the native insulin B:9–23 sequence.

**TRAV5D-4 α-chains with variable CDR3 sequences induce anti-insulin autoimmunity.** To characterize the utilization of the \( V_{\alpha} \) TRAV5D-4 in NOD pancreatic islets, we PCR-amplified TCRs from cDNA of 20-week-old NOD islets and sequenced these amplicons using high-throughput sequencing. Within ~100 islets extracted from two mice, we found >1,000 unique in-frame TRAV5D-4 sequences with relatively few expanded identical CDR3 sequences. Individual mice had notably different TRAV5D-4 repertoires and TCR variant frequencies in islets (Fig. 4A); for example, we detected approximately 1,400 counts of the same TRAV5D-4 sequence in one mouse (x-axis), whereas that sequence was not detected at all in the second mouse (y-axis). To assess whether multiple TRAV5D-4 α-chain sequences observed in these islets would induce insulin autoantibodies in retrogenic mice, we produced five TRAV5D-4 α-chain retrogenic strains using sequences obtained from the 454 sequencing (Supplementary Table 3), and four out of five strains developed insulin autoantibodies (Fig. 4B). Of the sequences chosen, two were observed at high frequency and three were singletons (observed once) in the
high-throughput sequencing experiments. Thus, multiple intra-islet α-chain-containing VαTRAV5D-4 with different CDR3 sequences are capable of inducing anti-insulin autoimmunity. As controls, we created retrogenics with TRAV6 or TRAV13–1a-chain sequences that were also observed at high frequency in islets based on 454 sequencing (Supplementary Table 3). None of the strains with these control Vα segments developed insulin autoantibodies (Fig. 4C and D; P, 0.01 versus strains that express α-chains with TRAV5D-4).

Essential amino acid sequences in the TRAV5D-4 CDR1 and CDR2 for the recognition of insulin B:9–23. Evidence that TRAV5D-4 α-chains with diverse CDR3 sequences are capable of inducing anti-insulin autoimmunity (in response to the insulin B:9–23 peptide) led to the hypothesis that the TRAV5D-4 CDR1 and/or CDR2 sequences might be crucial to the recognition of the insulin B:9–23/I-Ag7 complex. To assess which specific amino acids in CDR1 and CDR2 are important for response to the insulin B:9–23, we performed an in vitro alanine scan of the α-chain CDR1 and CDR2 regions in the 8–1.1 TRAV5D-4 a-chain retrogenic mouse a-chain containing VαTRAV5D-4 with different TCR. For this experiment, we used 5KC CD4+ T cells (31) as recipients of TCR reconstitution, and the complete set of alanine mutations at each position of CDR1 and CDR2 were tested for response to insulin B:9–23 peptides in 5KC T-cell lines. As shown in Fig. 5A, mutations of asparagine in CDR1 (position 5) and arginine in CDR2 (position 2) abrogated the response to the insulin B:9–23 peptide. Notably, reactivity of other insulin B:9–23-reactive TCRs (12–4.1 and 12–4.4) also depended on these two amino acid sequences at positions CDR1-5 and CDR2-2 (Fig. 5B). In contrast, the two anti-HEL:11–25-reactive TCRs that also contain VαTRAV5D-4 (14H4 and 5F2) responded to their peptide target presented by I-Ag7, despite replacing either CDR1-5 or CDR2-2 with alanine (Fig. 5C). Importantly, retrogenic mice with these TRAV5D-4 chains containing alanine mutations at CDR1-5 and CDR2-2 positions did not develop anti-insulin autoimmunity in response to insulin B:9–23 peptides. Spleen cells from α-chain retrogenic mice (A, B, and D mice expressing an α-chain with TRAV5D-4, B:9–23) were tested for response to insulin B:9–23 peptide (closed bar) and B:9–23 negative (double insulin knockout) mice expressing an α-chain with 8–1.1 TRAV5D-4 (open bar). Data are mean ± SEM and cumulative from two independent experiments. E: The peak value of insulin autoantibodies of 8–1.1 TRAV5D-4 α-chain retrogenic mice with/without native insulin B:9–23 expression. Insulin autoantibodies were measured every 4 weeks after bone marrow transplantation. Symbols represent individual mice (wild-type retrogenic recipient mice, n = 9; insulin B:9–23-negative (double insulin-knockout) retrogenic recipient mice, n = 3). IAA index ≥ 0.01 is defined as positive.
develop any anti-insulin antibodies (Fig. 5D; for each position, \(P < 0.01\) versus no mutated TCR), and T-cell responsiveness to insulin B:9–23 in these mice, as measured by IFN-\(\gamma\) ELISPOT, was completely abrogated (Fig. 5E; for each position, \(P < 0.02\) versus no mutated TCR). This lack of anti-insulin autoimmunity was not due to a lack of T cells in the periphery as both CD4 and CD8 T cells were present at levels comparable to the nonmutated 8–1.1 \(\alpha\)-chain retrogenic mice. Chimeric \(\alpha\)-chains with the human ortholog of murine TRAV5D-4 are capable of inducing anti-insulin autoimmunity.

To explore the potential relevance of our findings to human type 1 diabetes, we produced retrogenic NOD mice with a chimeric human V\(\alpha\) TRAV13–1 \(\alpha\)-chain. TRAV13–1 is the human ortholog of murine TRAV5D-4 (70% amino acid identity) with identical amino acids at the key positions of CDR1\(\alpha\)-5 and CDR2\(\alpha\)-2 (asparagine and arginine) as the murine TRAV5D-4 (Supplementary Fig. 3). In these retrogenic experiments, we engineered mice to express chimeric \(\alpha\)-chains composed of the V\(\alpha\) sequences from human TRAV13–1, but with N, J\(\alpha\), and C\(\alpha\) regions from mice, specifically 8–1.1 or 12–4.1 \(\alpha\)-chain CDR3 sequence. These chimeric \(\alpha\)-chain retrogenic mice developed insulin autoantibodies (Fig. 6A) and diabetes (Fig. 6B).

**DISCUSSION**

It is generally assumed that for typical immune responses, CDR3 sequences of both \(\alpha\)- and \(\beta\)-chains dominate the specificity to peptide recognition. In our experiments, different levels of insulin autoantibodies were induced in retrogenic mice by TRAV5D-4 \(\alpha\)-chains with diverse CDR3 sequences (Fig. 1). The quantitative differences between retrogenic strains expressing TRAV5D-4 \(\alpha\)-chains are likely determined by influence of varying CDR3 and J\(\alpha\) sequences contributing to recognition of the insulin B:9–23-I-A\(^{\beta7}\) complex. Our study provides evidence that amino acids asparagine and arginine in CDR1 and CDR2 (respectively) in the TRAV5D-4 gene segment are essential for anti-insulin reactivity. Mutating a single amino acid of CDR1 and CDR2 in vitro and in vivo abrogated targeting of insulin B:9–23. This suggests that the CDR1 and CDR2 regions of the specific germline-encoded V\(\alpha\) sequence predispose TCRs to target the insulin B:9–23 peptide presented by I-A\(^{\beta7}\) and are usually sufficient for the induction of anti-insulin autoimmunity in NOD mice. Such a simple TCR motif may relate to the ease and frequency of activating islet autoimmunity when other diabetes-promoting genetic and/or environmental factors exist.

It was notable that retrogenic strains expressing TRAV5D-4 \(\alpha\)-chains developed peri-islet insulitis or even intraislet insulitis, which indicates that even T cells expressing islet-unrelated TRAV5D-4 \(\alpha\)-chains are capable of recognizing islet antigens. TRAV5D-4 \(\alpha\)-chain retrogenic mice had greater lymphocytic infiltration than non-TRAV5D-4 \(\alpha\)-chain strains. TRAV5D-4 \(\alpha\)-chains, even ones derived from islet-unrelated antigens, paired with appropriate \(\beta\)-chains may have a higher chance to recognize islet antigens.
The evidence that TRAV5D-4 α-chains induce insulin autoantibodies may suggest that such TCRs recognize the insulin B:9–23 peptide and initiate islet infiltration. Indeed, NOD mice transgenic or retrogenic for the 12–4.1 and 12–4.4 TRAV5D-4 α-chains have the greater number of T cells reacting with the insulin B:9–23 peptide (32).

There are other examples of dominant V gene segment usage in autoimmune diseases. Tisch and colleagues (33) have found a preponderance of a specific TCR Vβ usage among T cells responding to the anti-islet BDC-2.5 mimotope. In the BB rat model, a specific Vβ sequence (Tcrb-VI3) that is polymorphic among rat strains contributes to disease...
The identical insulin B9–23 peptide sequence is present in human and mouse (Ins2), and an ortholog of mouse TRAV5D-4 (human TRAV13–1) is able to substitute for TRAV5D-4 in α-chain retrogenic mice. Of note, the structure of I-A^8 is similar to that of the diabetes-susceptible DQ8 MHC class II molecule. Both molecules lack an aspartic acid residue at position 57 of the β-chains (42) and select common peptides that are naturally processed (43). We propose the concept that an accident of nature with the insulin B9–23 peptide bound by the susceptible MHC class II and recognition of such a peptide–MHC complex by relatively large number of TCRs containing germline-encoded TRAV5D-4 (compared with TCRs with specific CDR3 sequences) may underlie the heightened risk of autoimmune diabetes for a species.

The current study indicates that despite dramatic diversity in N region and Jα sequences, producing retrogenic mice having a common Vα sequence, TRAV5D-4, results in insulin autoimmunity. In the presence of the appropriate class II MHC alleles, such a Vα may set the stage for generation of insulin-specific autoimmunity. Even the related human sequence (TRAV13–1) as a chimeric retrogenic with murine CDR3 and Cα region induces insulin autoantibodies. In conclusion, by use of an α-chain retrogenic mouse model, we identified a pathogenic α-chain sequence that elicits insulin autoimmunity via the recognition of insulin B9–23 peptides. It is notable that the Vα TRAV5D-4 sequence that is encoded in the germline with fixed CDR1 and CDR2, but a diversity of CDR3 sequences, recognizes one essential peptide to provoke autoimmunity. Targeting of the interaction of pivotal germline Vα or Vβ sequences with cognate peptide–MHC complexes may provide a disease-specific immunotherapeutic strategy for prevention of autoimmunity.

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M.N. is on a University provisional patent for treating islet autoimmunity targeting MHC–peptide complex with small molecules. G.S.E. is on two University provisional patents for treating islet autoimmunity targeting MHC–peptide complex with small molecules or antibodies. G.S.E. has a research grant from Novartis in the same area. No other potential conflicts of interest relevant to this article were reported.

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