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Proinsulin Expression Shapes the TCR Repertoire but Fails to Control the Development of Low-Avidity Insulin-Reactive CD8$^+$ T Cells

Diabetes 2016;65:1679–1689 | DOI: 10.2337/db15-1498

NOD mice, a model strain for human type 1 diabetes, express proinsulin (PI) in the thymus. However, insulin-reactive T cells escape negative selection, and subsequent activation of the CD8$^+$ T-cell clonotype G9C8, which recognizes insulin B15-23 via an αβ T-cell receptor (TCR) incorporating *TRAV8-1/TRAJ9* and *TRBV19/TRBJ2-3* gene rearrangements, contributes to the development of diabetes. In this study, we used fixed TRAV8-1/TRAJ9 TCRα-chain transgenic mice to assess the impact of PI isoform expression on the insulin-reactive CD8$^+$ T-cell repertoire. The key findings were: *1*) PI2 deficiency increases the frequency of insulin B15-23–reactive TRBV19$^+$CD8$^+$ T cells and causes diabetes; *2*) insulin B15-23–reactive TRBV19$^+$CD8$^+$ T cells are more abundant in the pancreatic lymph nodes of mice lacking PI1 and/or PI2; *3*) overexpression of PI2 decreases TRBV19 usage in the global CD8$^+$ T-cell compartment; *4*) a biased repertoire of insulin-reactive CD8$^+$ T cells emerges in the periphery regardless of antigen exposure; and *5*) low-avidity insulin-reactive CD8$^+$ T cells are less affected by antigen exposure in the thymus than in the periphery. These findings inform our understanding of the diabetogenic process and reveal new avenues for therapeutic exploitation in type 1 diabetes.

Autoreactive T cells are key players in the process of immune-mediated β-cell destruction, which culminates in the development of type 1 diabetes. Immature CD4$^+$CD8$^+$ thymocytes that recognize self-derived peptide-loaded major histocompatibility complex (MHC) molecules via high-affinity interactions with a clonotypically expressed T-cell receptor (TCR) are typically removed by negative selection to prevent the egress of such autoreactive T cells. Similar deletional events may also occur in the periphery (1). However, these tolerogenic mechanisms are not infallible, and self-derived antigen-specific T-cell escape likely underpins a number of autoimmune diseases.

Proinsulin (PI) is a major diabetogenic autoantigen in humans (2–5) and mice (6–10). PI is generated from a larger preprohormone by cleavage of the signal peptide and then further processed in the pancreatic β-cells to insulin, which is the metabolically active hormone. Self-derived antigens, including PI, are expressed in the thymus (11,12) under the control of the autoimmune regulator (AIRE) protein, a transcription factor found in medullary thymic epithelial cells (13). Central exposure to these antigens limits the development of autoreactive T cells. Although the MHC locus, encompassing both class I and II genes, is the most important genetic susceptibility factor for type 1 diabetes (14), the insulin 5′-variable number tandem repeat region also plays a significant role by regulating PI expression in the thymus and pancreas (15). There are two types of PI in mice, known as PI1 and PI2, which are separately encoded in the genome. PI2 is expressed in the thymus and pancreas, whereas PI1 is thought to occur primarily in the pancreas (11,16), with only low-level expression in the thymus (17). For this reason, it has been suggested that PI2 may be more pivotal in the development of T-cell tolerance. Investigation of individual PI1 knockout ($PI1^{-/-}$) mice backcrossed to

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Received 30 October 2015 and accepted 29 February 2016.
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db15-1498/-/DC1.

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the NOD background has shown that loss of PI1 reduces the incidence of diabetes (18). In contrast, loss of PI2 in the corresponding *PI2*$^{-/-}$ model accelerates the development of diabetes in 100% of mice (19). Conversely, thymic overexpression of PI2 on the MHC class II promoter (NOD*PI2*^{tg}) leads to a decreased incidence of diabetes (20,21). These findings suggest that PI2 is important for both central and peripheral tolerance.

Insulin-reactive CD4$^+$ and CD8$^+$ T cells have been shown to be important for the development of diabetes in humans and mice (6–8,10,22). The dominant CD8$^+$ T-cell epitope in NOD mice, insulin B15-23 (8,22), overlaps with a longer peptide recognized by diabetogenic CD4$^+$ T cells (23). In a study where both PI1 and PI2 were eliminated and an altered insulin expressing alanine at position B16 instead of tyrosine was substituted for native insulin, the resulting *PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice were protected from diabetes due to removal of the cognate CD4$^+$ and CD8$^+$ autoreactive T-cell epitopes (9). However, if both PI1 and PI2 are deleted without insulin substitution, mice die rapidly from metabolic problems related to insulin deficiency. It is also notable that insulin autoimmunity is required for the development of islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) reactivity (24,25). Modifying the development of insulin autoimmunity may therefore prove to be a key therapeutic intervention.

We previously generated a highly diabetogenic murine CD8$^+$ T-cell clone (G9C8) that expresses an αβ TCR encoded by *TRAV8-1/TRAJ9* and *TRBV19/TRBJ2-3* gene rearrangements (22). In vitro, this clone displayed potent cytotoxic and proliferative activity in response to islet cells. In vivo, G9C8 caused diabetes within 5–10 days in young prediabetic NOD and NOD.*scid* mice (22). Moreover, T cells that recognize the H-2K^d–restricted insulin B15-23 epitope targeted by G9C8 infiltrate the islets of NOD mice at 4 weeks of age, a time when very few T cells with other specificities are present (8,26). These observations further suggest that CD8$^+$ T cells are important in the pathogenesis of autoimmune diabetes in the NOD mouse model. The insulin B15-23 peptide (LYLVCGERG) binds poorly to H2-K^d (27,28). Consequently, relatively high peptide concentrations are required for exogenous recognition of this antigenic complex, which typically elicits low-avidity T cells. Of note, the native B15-23 sequence is conserved in humans and common to murine PI1 and PI2.

It is established that CD8$^+$ T cells in humans can recognize antigenic peptides derived from preproinsulin and destroy β-cells (4,5). However, the underlying mechanisms that allow the development and expansion of such disease-relevant CD8$^+$ T-cell populations remain obscure. In this study, we generated fixed TRAV8-1/TRAJ9 TCRα-chain NOD mice (designated A22 for simplicity to reflect usage of mouse line 22) (29) with either normal PI1 and PI2 levels (*A22Cα*$^{-/-}$ mice), PI2 overexpression (*A22Cα*$^{-/-}$*PI2*^{tg} mice), PI2 deficiency (*A22Cα*$^{-/-}$*PI2*$^{-/-}$ mice), PI1 deficiency (*A22Cα*$^{-/-}$*PI1*$^{-/-}$ mice), or both PI1 and PI2 deficiency with a mutant transgene preventing recognition of the insulin B15-23 peptide (*A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice) (Table 1). These unique models were then used to investigate the role of PI expression on the insulin B15-23–reactive CD8$^+$ T-cell repertoire.

RESEARCH DESIGN AND METHODS

Mice

Insulin B15-23–reactive TRAV8-1/TRAJ9 TCRα-chain transgenic mice were generated as described previously (29). Line 22 was selected for further characterization, hence the designation A22. TCRα-chain transgenic mice were crossed with NOD*Cα*$^{-/-}$ mice to generate *A22Cα*$^{-/-}$ mice exclusively expressing the *TRAV8-1/TRAJ9* transgene. These *A22Cα*$^{-/-}$ mice were then crossed with NOD mice overexpressing PI2 under the MHC class II promoter (NOD*PI2*^{tg}) to generate *A22Cα*$^{-/-}$*PI2*^{tg} mice. In addition, *A22Cα*$^{-/-}$ mice were crossed with NOD mice lacking PI2, NOD mice lacking PI1, and NOD mice lacking PI1 and PI2 but reconstituted with a mutant transgene encoding a tyrosine-to-alanine mutation at position 16 of the insulin B chain (9) to generate *A22Cα*$^{-/-}$*PI2*$^{-/-}$ mice, *A22Cα*$^{-/-}$*PI1*$^{-/-}$ mice, and *A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice, respectively (Table 1). Mice were housed in microisolators or scantainers in the specific pathogen–free facility at Cardiff University. All procedures were performed in accordance with protocols approved by the U.K. Home Office.

Flow Cytometric Analysis of TCRVβ Expression

Thymus, spleen, pancreatic lymph nodes (PLNs), and mesenteric lymph nodes (MLNs) were harvested from mice aged 4–7, 8–10, and 12–16 weeks. Single-cell suspensions were generated and stained with monoclonal antibodies (mAbs) specific for CD4, CD8α, CD19, and 14 distinct TCRVβ chains. Live single CD8$^+$ T cells were then gated to visualize TCRVβ expression patterns. Data were analyzed with FlowJo software version 7.6.5 (Tree Star Inc.).

Tetramer Analysis

Thymus, spleen, PLNs, and MLNs were harvested from TCRα transgenic mice and polyclonal NOD mice at 4–8 weeks of age. Single-cell suspensions were generated and stained serially with pretitrated concentrations of the H-2K^d-LYLVCGERG tetramer (National Institutes of Health Tetramer Core Facility), a viability dye, and the following mAbs: αCD4-PECy7, αCD8α-FITC, αCD11b-APC, and αCD19-PerCPCy5.5. Data were analyzed with FlowJo software version 7.6.5. G9C8 transgenic T cells (29) were used as a positive control. The minimal H-2K^d-AYAAAAAAV tetramer was used to determine nonspecific background.

TRBV19$^+$CD8$^+$ T-Cell Isolation and Expansion

Single-cell PLN suspensions from 6-week-old *A22Cα*$^{-/-}$, *A22Cα*$^{-/-}$*PI2*$^{-/-}$, and *A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice were sorted by flow cytometry for live CD4$^-$ CD8$^+$CD11b$^-$CD19$^-$TCRVβ6$^+$ events. Cells were initially

Table 1—Transgenic and knockout mice used in this study

Genotype	TCRα-chain	TCRβ chain	PI1 expression	PI2 expression	Diabetes
A22Cα$^{-/-}$	G9 TCRα transgene (insulin B15-23–specific)	Endogenous TCRβ chains	Normal	Normal	No diabetes
A22Cα$^{-/-}$*PI2^{tg}*	G9 TCRα transgene (insulin B15-23–specific)	Endogenous TCRβ chains	Normal	Overexpression (under MHC class II promoter)	No diabetes
A22Cα$^{-/-}$*PI1*$^{-/-}$	G9 TCRα transgene (insulin B15-23–specific)	Endogenous TCRβ chains	Deficient	Normal	No diabetes
A22Cα$^{-/-}$*PI2*$^{-/-}$	G9 TCRα transgene (insulin B15-23–specific)	Endogenous TCRβ chains	Normal	Deficient	Accelerated diabetes in males
A22Cα$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A^{tg}*	G9 TCRα transgene (insulin B15-23–specific)	Endogenous TCRβ chains	Lack both native PI genes but express a mutated insulin transgene		No diabetes

cultured in bulk with the addition of interleukin (IL)-2 (20 units/mL), IL-7 (2 ng/mL), and insulin B15-23 peptide (1 μg/mL) in RPMI complete medium (2 mmol/L L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 5% FBS, and 0.05 mmol/L 2-mercaptoethanol in RPMI medium). Expanding cells were replated after limiting dilution and grown to sufficient numbers for functional analysis.

Cytotoxicity Assays

Expanded TRBV19$^+$CD8$^+$ T cells were washed twice in RPMI complete medium to remove cytokines from the culture. P815 cells (targets) were labeled with PKH-26 (Sigma) and incubated with TRBV19$^+$CD8$^+$ T cells (effectors) in the presence of insulin B15-23 peptide at an effector-to-target ratio of 10:1 for 16 h at 37°C. TO-PRO-3 iodide was added immediately before flow cytometric analysis. Single PKH-26$^+$TO-PRO-3$^+$ P815 cells were gated, and cytotoxicity was expressed as the percentage of dead cells/total targets (30). Specific target cell killing was corrected for spontaneous background death by subtracting the percentage of dead cells in the control sample comprising targets with T cells in the absence of peptide.

ELISAs

TRBV19$^+$CD8$^+$ T cells were cocultured with irradiated bone marrow–derived dendritic cells in the presence of insulin B15-23 peptide at an effector-to-target ratio of 10:1 for 48 h at 37°C. Supernatants were analyzed for macrophage inflammatory protein-1β (MIP1β) and interferon-γ (IFNγ) levels according to the manufacturer's instructions (R&D Systems and eBioscience, respectively).

TCR Clonotyping

Clonotypic analysis of H-2K^d-LYLVCGERG tetramer-positive CD8$^+$ T cells and oligoclonal lines was performed as described previously (31). Briefly, viable tetramer-positive CD8$^+$ T cells (n = 43–2,353) from individual mice or TRBV19$^+$CD8$^+$ T cells (n = 5,000) from individual oligoclonal lines were sorted directly into 1.5-mL microtubes (Sarstedt) containing 100 μL RNAlater (Applied Biosystems) using a custom-modified FACSAria II flow cytometer (BD Biosciences). Unbiased amplification of all expressed *TRBV* gene products was conducted using a template-switch anchored RT-PCR with a 3′ constant region primer (5′-TGGCTCAAACAAGGAGACCT-3′). Amplicons were subcloned, sampled, sequenced, and analyzed as described previously (32). Concatenated data are shown for each genetic strain. The IMGT nomenclature is used in this report (33).

Histology

Immunohistochemistry was performed on fixed pancreata as described previously (29). Sections were scored for insulitis after staining with hematoxylin-eosin (Vector Laboratories).

Statistics

Statistical analyses were conducted using ANOVA or a Student t test with R software. Bonferroni correction was used for multiple comparisons such that $P < 0.05$ is

equivalent to $P < 0.0000022907$ and $P < 0.01$ is equivalent to $P < 0.0000004581$.

RESULTS

CD4$^+$ and CD8$^+$ T-Cell Numbers Are Not Affected by PI Expression

In preliminary experiments, we used flow cytometry to assess the impact of PI expression on T-cell lineage development in our mouse models (*A22Cα*$^{-/-}$, *A22Cα*$^{-/-}$*PI2*^{tg}, *A22Cα*$^{-/-}$*PI2*$^{-/-}$, *A22Cα*$^{-/-}$*PI1*$^{-/-}$, and *A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg}). Groups of mice were screened at 4–7 weeks and 12–16 weeks. No significant differences in CD4$^+$ or CD8$^+$ T-cell numbers were apparent among strains, regardless of age, either in the thymus (Fig. 1) or in the periphery (Supplementary Fig. 1). However, a substantial reduction in the total number of CD4$^+$ T cells was observed for all TCRα-chain transgenic strains relative to polyclonal NOD mice (Fig. 1 and Supplementary Fig. 1).

PI Expression Alters the Proportion of TRBV19$^+$CD8$^+$ T Cells

To evaluate the global influence of PI expression on the CD8$^+$ T-cell repertoire, 14 TCRVβ-specific mAbs were used to assess TRBV usage by CD8$^+$ T cells isolated from the thymus, spleen, and lymph nodes of the various *A22Cα*$^{-/-}$ mice aged either 4–7 weeks or 12–16 weeks. A strong PI-mediated effect was observed on the expression of TRBV19 (Vβ6), which encodes the TCRVβ chain used by the G9C8 clone (Fig. 2 and Supplementary Fig. 2). Specifically, the proportion of TRBV19$^+$CD8$^+$ T cells, both in the thymus and in the periphery, decreased with age in *A22Cα*$^{-/-}$ mice (Fig. 2). This effect was more pronounced in mice with PI2 overexpression (*A22Cα*$^{-/-}$*PI2*^{tg}), which also displayed lower proportions of TRBV19$^+$CD8$^+$ T cells compared with *A22Cα*$^{-/-}$ mice, notably approximating those observed in polyclonal NOD mice. However, mice lacking PI1 (*A22Cα*$^{-/-}$*PI1*$^{-/-}$), PI2 (*A22Cα*$^{-/-}$*PI2*$^{-/-}$), or both PI genes (*A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg}) showed no significant changes in the proportion of TRBV19$^+$CD8$^+$ T cells with age. Similar trends were observed with respect to the absolute number of TRBV19$^+$CD8$^+$ T cells, which declined with age but differed among strains only in PLNs at 12–16 weeks, where a substantial reduction was observed in *A22Cα*$^{-/-}$*PI2*^{tg} mice (Supplementary Fig. 2). Less marked effects were noted with other TRBV segments. In particular, the proportion of CD8$^+$ T cells expressing TRBV13 (Vβ8), the most commonly used gene segment in NOD mice, was unaffected by PI expression (data not shown).

Insulin B15-23–Reactive CD8$^+$ T Cells Are Increased in Mice Lacking PI1 or PI2

As the TRAV8-1/TRAJ9 chain present in these mice specifically recognizes the insulin B15-23 epitope, we used H-2K^d-LYLVCGERG tetramers to quantify the effect of PI expression on the development of insulin B15-23–reactive CD8$^+$ T cells (Supplementary Fig. 3*A*). Increased numbers and percentages of tetramer-positive CD8$^+$ T cells were detected in the thymus, spleen, and PLNs of PI2-deficient (*A22Cα*$^{-/-}$*PI2*$^{-/-}$) mice compared with wild-type *A22Cα*$^{-/-}$ mice (Fig. 3 and Supplementary Fig. 3). Of note, *A22Cα*$^{-/-}$*PI1*$^{-/-}$ mice also showed an increased proportion of insulin B15-23–reactive CD8$^+$ T cells in PLNs compared with *A22Cα*$^{-/-}$ mice, whereas a smaller increase was observed in PLNs from *A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice despite greater enhancements in the thymus and spleen. However, only the *A22Cα*$^{-/-}$*PI2*$^{-/-}$ and

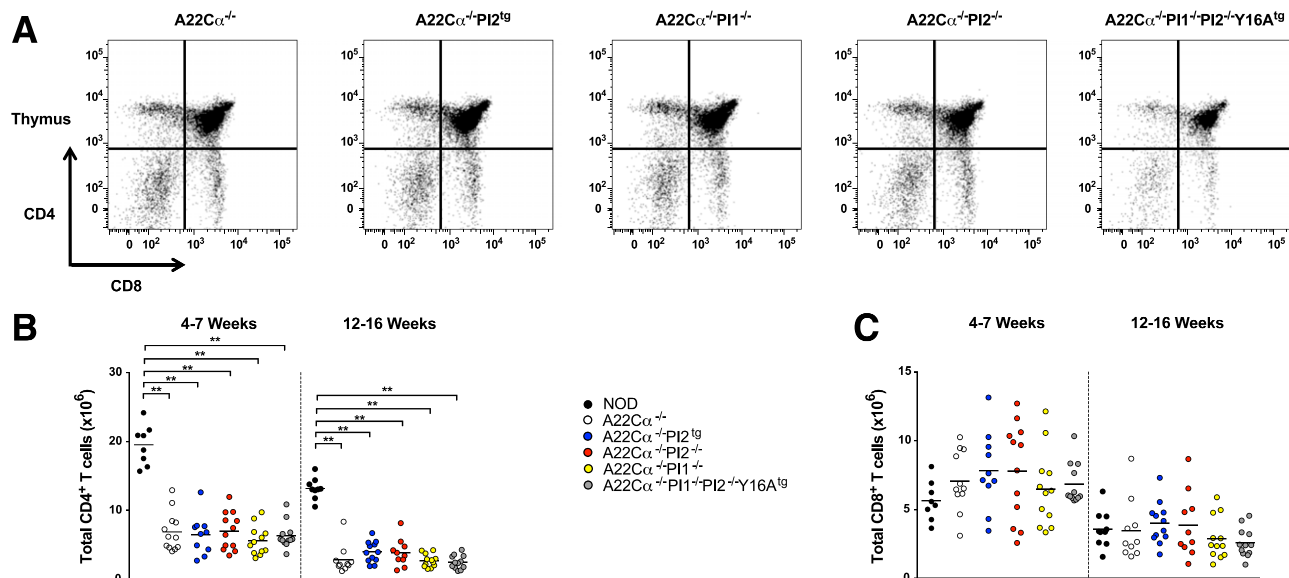

Figure 1—CD4$^+$ and CD8$^+$ T-cell numbers in the thymus are unaffected by PI expression. Live single-positive CD4$^+$ and CD8$^+$ T cells were quantified in single-cell suspensions of thymus from mice aged 4–7 or 12–16 weeks. Representative flow cytometry plots (*A*), total CD4$^+$ T-cell numbers (*B*), and total CD8$^+$ T-cell numbers (*C*) are displayed for all strains. Data are shown for 9–12 mice per group (encompassing three to four experiments). Males and females were included because no differences were detected when analyzed separately. $^{**}P < 0.01$.

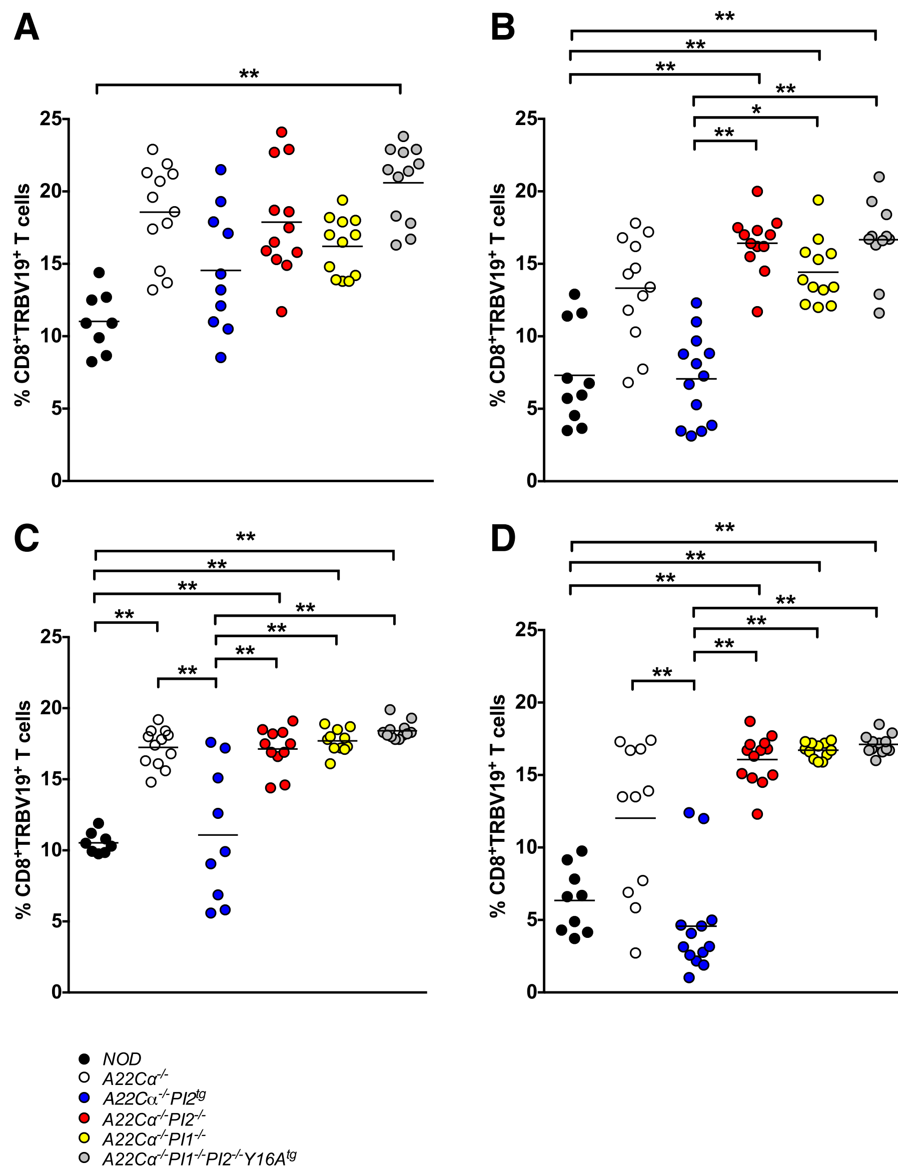

Figure 2—PI-specific and age-related effects on TRBV19 expression. CD8$^+$ T cells expressing TCRVβ6 (TRBV19) were quantified in single-cell suspensions of thymus (*A* and *B*) and PLNs (*C* and *D*) from mice aged 4–7 (*A* and *C*) or 12–16 (*B* and *D*) weeks. Data are shown for 9–12 mice per group (encompassing three to four experiments). Males and females were included because no differences were detected when analyzed separately. *$P < 0.05$, **$P < 0.01$.

A22Cα$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} strains exhibited an increase in the number of insulin B15-23–reactive CD8$^+$ T cells in both the thymus and the periphery (Supplementary Fig. 3*B*). No differences in tetramer staining intensity were detected among strains, indicating equivalent levels of cognate TCR expression (Supplementary Fig. 3*C*).

Insulin B15-23–Reactive CD8$^+$ T Cells Cause Spontaneous Diabetes in PI2-Deficient Mice

To assess the biological relevance of these proportional and numerical differences in insulin B15-23–reactive CD8$^+$ T cells, we examined the incidence of spontaneous diabetes across strains. Strikingly, diabetes occurred spontaneously only in male PI2-deficient (*A22Cα*$^{-/-}$*PI2*$^{-/-}$) mice (Fig. 4). Moreover, disease onset was considerably accelerated in this group compared with male wild-type NOD mice, which develop diabetes after 20 weeks of age in our colony, with a final incidence of 20% by 35 weeks (data not shown). Mild insulitis was detected in male *A22Cα*$^{-/-}$ and nondiabetic *A22Cα*$^{-/-}$*PI2*$^{-/-}$ mice, whereas normal pancreatic histology was observed in male *A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice (Supplementary Table 1). These findings suggest that PI2 deficiency permits the development of diabetogenic insulin B15-23–reactive CD8$^+$ T cells.

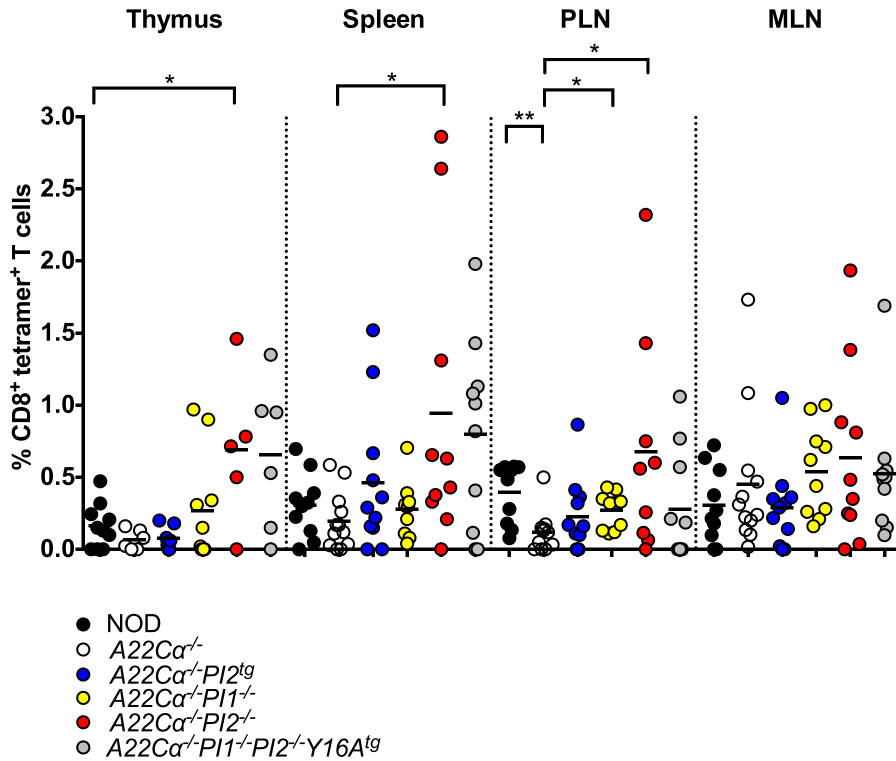

Figure 3—PI2 deficiency increases the proportion of insulin B15-23–reactive CD8+ T cells. H-2K^d-LYLVCGERG tetramer-positive CD8+ T cells were enumerated in single-cell suspensions of thymus, spleen, PLNs, and MLNs from mice aged 4–8 weeks. Nonspecific staining was quantified in parallel experiments with the minimal H-2K^d-AYAAAAAAV tetramer. Data are shown after background subtraction for 5–10 mice per group. Males and females were included because no differences were detected when analyzed separately. *P < 0.05, **P < 0.01.

Insulin B15-23–Reactive CD8$^+$ T Cells Express Multiple TRBV Chains and Exhibit PI-Specific Changes in TRBV19 Usage

To further our understanding of the diabetogenic process, we evaluated the TCR repertoire specifically within the insulin B15-23–reactive CD8$^+$ T-cell population. Data are shown for wild-type mice (*A22Cα*$^{-/-}$), PI2 overexpressing mice (*A22Cα*$^{-/-}$*PI2*^{tg}), PI2-deficient mice (*A22Cα*$^{-/-}$*PI2*$^{-/-}$), and mice lacking both PI1 and PI2 (*A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg}). By sequencing expressed *TRB* gene transcripts in H-2K^d-LYLVCGERG tetramer-positive CD8$^+$ T cells from PLNs, we identified a restricted TCR repertoire in all four groups of mice (Fig. 5). Alterations in PI expression clearly influenced *TRBV* gene selection in insulin B15-23–reactive CD8$^+$ T cells. Most notably, the proportion of TRBV19$^+$CD8$^+$ T cells increased when PI2 or both PI1 and PI2 were absent. Moreover, no TRBV19 sequences were detected when PI2 was overexpressed. These observations suggest that PI expression critically affects the selection of insulin B15-23–reactive TCRs, even in the context of a highly restricted clonotypic repertoire.

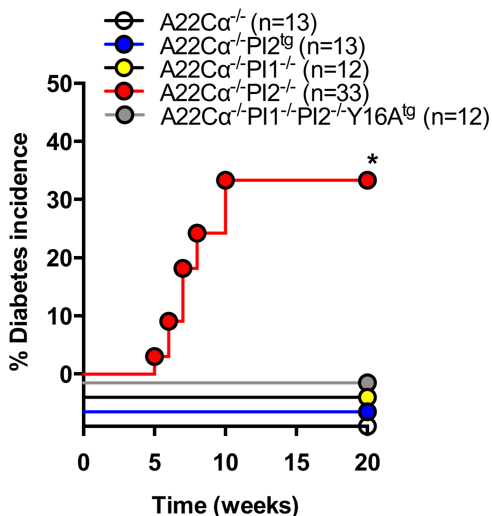

Figure 4—Mice deficient in PI2 develop spontaneous diabetes. Mice were housed together from weaning and tested weekly for glycosuria using Diastix. Positive results were validated 24 h later, and diabetes was confirmed by a blood glucose level >13.9 mmol/L. Only males were included because no females developed diabetes. *P < 0.05.

Insulin B15-23–Reactive TRBV19$^+$CD8$^+$ T Cells Exhibit Low Functional Sensitivity Irrespective of PI Expression During Development

As the selection of insulin B15-23–reactive TRBV19$^+$CD8$^+$ T cells was influenced by PI expression, we next investigated the role of antigen exposure as a determinant of cellular function. TRBV19$^+$CD8$^+$ T cells from wild-type (*A22Cα*$^{-/-}$), PI2-deficient (*A22Cα*$^{-/-}$*PI2*$^{-/-}$), or PI1 and PI2–deficient (*A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg}) mice were isolated and cultured briefly with the cognate peptide in

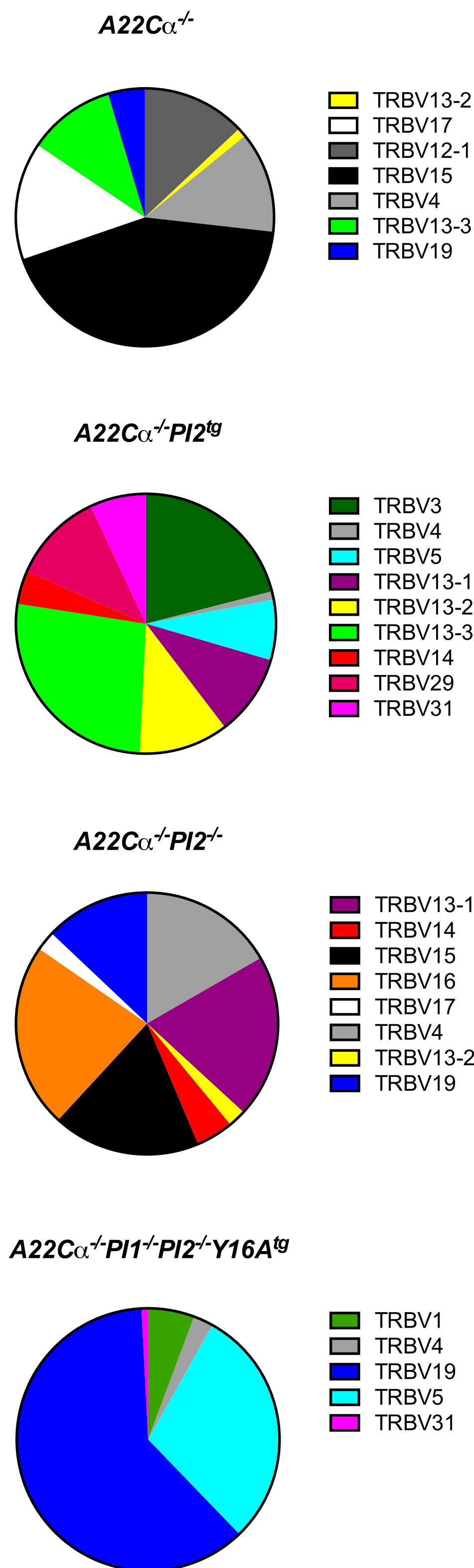

Figure 5—The TCRVβ repertoire of insulin B15-23–reactive CD8$^+$ T cells. H-2K^d-LYLVCGERG tetramer-positive CD8$^+$ T cells were sorted by flow cytometry from single-cell suspensions of PLNs from mice aged 4–8 weeks and analyzed for *TRB* gene expression. Data are shown for five to seven mice per group, comprising 6–20 distinct clonotypes.

the presence of IL-2 and IL-7 to generate a panel of insulin B15-23–reactive oligoclonal T-cell lines. The functional profile of these lines was assayed by measuring cytotoxicity and proinflammatory cytokine (MIP1β and IFNγ) production in response to the insulin B15-23 peptide. Similar cytotoxic responses were observed for all strains regardless of PI expression, and target cell lysis required high doses of exogenous peptide (Fig. 6*A*). Lines generated from *A22Cα*$^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice displayed enhanced MIP1β responses (Fig. 6*B*) but produced less IFNγ in parallel comparisons with wild-type (Fig. 6*C*). Overall, these results suggest that insulin B15-23–reactive TRBV19$^+$CD8$^+$ T cells exhibit intrinsically low levels of functional sensitivity and that altered reactivity to cognate antigen does not underlie the development of spontaneous diabetes in PI2-deficient (*A22Cα*$^{-/-}$*PI2*$^{-/-}$) mice.

Insulin B15-23–Reactive TRBV19$^+$CD8$^+$ T Cells Use TRBJ2-3 and Exhibit a Common TCR CDR3β Motif

The highly diabetogenic G9C8 clone expresses a *TRBV19/TRBJ2-3* gene-encoded TCRβ chain (22). Although no obvious differences in functional sensitivity were observed between insulin B15-23–reactive CD8$^+$ T-cell clonotypes selected in mice with differing levels of PI expression, we nonetheless conducted a molecular analysis of *TRB* gene rearrangements in our oligoclonal antigen-specific lines to assess the impact of PI expression on TCR selection. All lines were dominated by TRBV19$^+$ TCRs. We obtained 11 unique TRBV19-associated CDR3β sequences, five of which were private and six of which were shared (Table 2). Ten of these distinct CDR3β sequences incorporated TRBJ2-3 with a fixed loop length of 13 amino acids generating a motif (CASS-XXXX-GAETLY) in common with the original G9C8 clone (CASS-IRDR-GAETLY). Moreover, arginine was universally conserved at position 6 in these CDR3β sequences, likely providing an important contact residue for recognition of the insulin B15-23 peptide. Amino acids with nonpolar side chains were preferred at position 5 (with the exception of arginine), whereas amino acids with acidic side chains (aspartic acid or glutamic acid) or uncharged polar side chains (glutamine or threonine) were preferred at position 7. Little preference was observed for any particular residue at position 8. In contrast, we found very limited sequence similarity across the CDR3β loop within the tetramer-positive CD8$^+$ T-cell population as a whole (Fig. 3). One common CDR3β sequence (CASSLGGYEQY) was found in *A22Cα*$^{-/-}$ and *A22Cα*$^{-/-}$*PI2*$^{-/-}$ mice, and another (CASSRVPGEQY) was found in more than one *A22Cα*$^{-/-}$ mouse (Supplementary Table 2). It is also notable that the CASS-XXXX-GAETLY motif was not detected in a parallel analysis of noninsulin B15-23–reactive TRBV19$^+$CD8$^+$ T cells (data not shown). Collectively, these findings demonstrate that insulin B15-23 elicits a highly biased TRBV19$^+$ repertoire consistent with a strict docking mode for TCR recognition dictated by structural constraints.

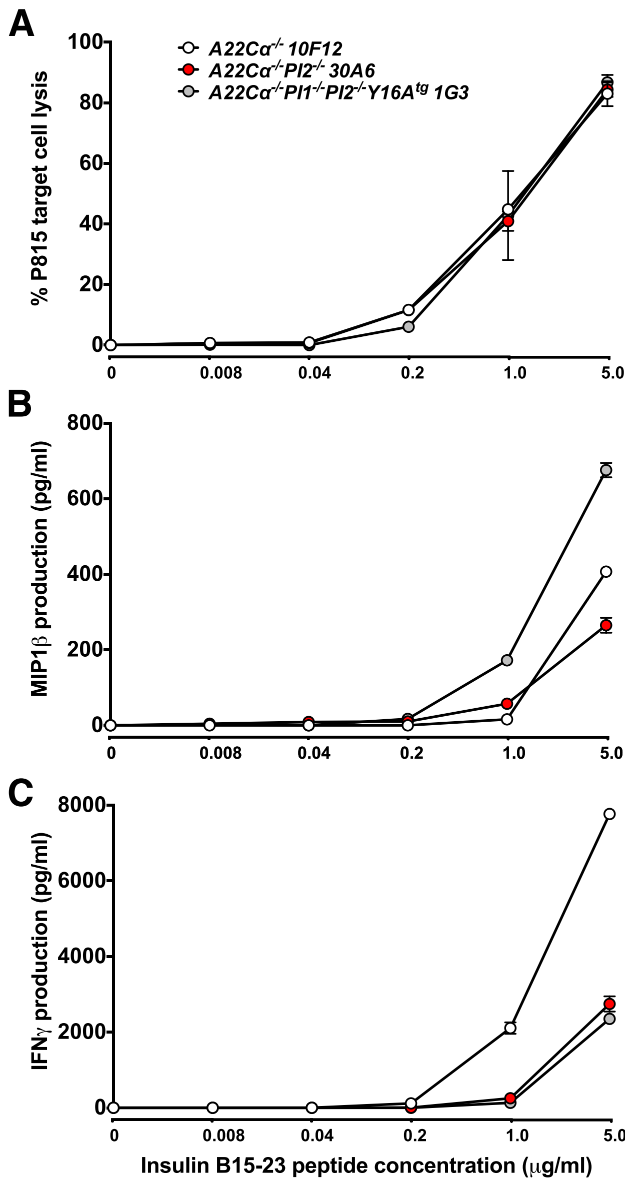

Figure 6—TRBV19^{+}CD8^{+} T cells exhibit low levels of functional sensitivity to insulin B15-23. Oligoclonal TRBV19^{+}CD8^{+} T-cell lines expanded in the presence of insulin B15-23 were analyzed for cytotoxic activity (*A*), MIP1β (*B*), and IFNγ (*C*) production. Data are shown for representative clones (*A22C*$\alpha^{-/-}$: 10F12; *A22C*$\alpha^{-/-}$*PI2*$^{-/-}$: 30A6; *A22C*$\alpha^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg}: 1G3).

DISCUSSION

In this study, we report five principal findings based on an in-depth analysis of fixed TCRα-chain NOD mice. First, PI1 or PI2 deficiency is associated with significant expansions of insulin B15-23–reactive CD8^{+} T cells in the draining lymph node for the pancreas, the target organ in type 1 diabetes. Second, the proportion of insulin B15-23–reactive CD8^{+} T cells expressing TRBV19 increases in the absence of PI. This repertoire shift potentially contributes to the development of autoimmune diabetes. Third, variations in PI expression alter global *TRBV* gene usage in the CD8^{+} T-cell compartment. In particular, TRBV19^{+}CD8^{+} T-cell numbers were reduced significantly upon increased exposure to PI2 through transgenic overexpression, although deletion of either the PI1 or PI2 gene in isolation did not change the frequency of this population. Fourth, insulin B15-23–reactive TRBV19^{+}CD8^{+} T cells display low levels of functional sensitivity for the cognate peptide irrespective of PI expression, consistent with a lack of epitope-regulated negative selection in the thymus. Fifth, antigen recognition by insulin B15-23–reactive TRBV19^{+}CD8^{+} T cells is driven by a highly biased set of TCRs characterized by *TRBJ2-3* gene usage and the presence of a conserved non–germline-encoded arginine residue at position 6 in the CDR3β loop. Collectively, these data show for the first time that PI expression can directly affect the development of autoreactive TCRs.

PI2 is expressed in the thymus and has been shown to promote tolerance (17,20,21,34). As a consequence, NOD mice with PI2 deficiency develop accelerated diabetes (19). However, NOD mice lacking PI1 do not develop autoimmune diabetes, presumably because PI2 tolerizes potentially autoreactive T cells (18). Although PI1 is also expressed in the thymus, PI2 is the predominant isoform; equivalent levels of PI1 and PI2 are found in the periphery (17,35,36). It is notable in this regard that PI deficiency enhanced the development of TRBV19^{+}CD8^{+} T cells in the thymus and minimized corresponding age-related declines in the periphery. These data suggest that both PI1 and PI2 have tolerogenic properties, with the weak effects on disease likely related to the low frequency of insulin B15-23–reactive cells in the overall TRBV19^{+}CD8^{+} T-cell population.

Antigen encounter in the thymus preferentially deletes high-avidity T cells (37,38). In accordance with this process, the current data show that increased numbers of insulin B15-23–reactive CD8^{+} T cells are present in mice lacking either PI2 alone or both PI1 and PI2. Moreover, only PI2-deficient mice developed diabetes in this study. It is remarkable that male mice were exclusively affected in this regard, despite equivalent increases in insulin-reactive CD8^{+} T cells in female mice lacking PI2. Recent data suggest that nonimmune factors, including interactions between gut microbiota and androgens (39,40), contribute to such sexual dimorphism in the development of diabetes. Irrespective of the underlying mechanism, however, these findings suggest that insulin B15-23–reactive CD8^{+} T cells are important determinants of disease because *A22C*$\alpha^{-/-}$*PI1*$^{-/-}$*PI2*$^{-/-}$*Y16A*^{tg} mice express a mutant transgene that prevents recognition of the cognate peptide.

It is notable that we did not detect any significant functional differences in insulin B15-23–reactive TRBV19^{+}CD8^{+} T cells across strains of mice with altered levels of PI expression. This counterintuitive finding may reflect in vitro activation and expansion prior to assay, which could mask intrinsic differences in the ex vivo setting, or a convergence of functional properties due to the expression of PI in peripheral lymphoid tissue (13,41). Regardless of origin, these autoreactive cells expressed similar

Table 2—CDR3β sequences of insulin B15-23–reactive oligoclonal lines

Donor strain	ID	TRBV	CDR3β sequence	TRBJ	Frequency (%)
$A22C\alpha^{-/-}$	10F11	19	CASS**IRTG**GAETLY	2-3	100
	10F12	19	CASS**MRQG**GAETLY	2-3	100
	30B6	19	CASS**IRQG**GAETLY	2-3	94.74
		19	CASS**IRQG**DAETLY	2-3	5.26
	30C4	19	CASS**MRQG**GAETLY	2-3	88.89
		19	CASS**RRDR**GAETLY	2-3	11.11
	30D7	19	CASSSGLEQY	2-7	93.75
		19	CASS**FREE**GAETLY	2-3	6.25
	30F7	19	CASS**IRTG**GAETLY	2-3	94.12
		24	CASSRDSDTEVF	1-1	5.88
$A22C\alpha^{-/-}PI2^{-/-}$	1F9	19	CASS**IRQG**GAETLY	2-3	96
		19	CASS**FREE**GAETLY	2-3	4
	30A6	19	CASS**FREE**GAETLY	2-3	100
	30B9	19	CASS**MRQG**GAETLY	2-3	69.23
		19	CASS**FREE**GAETLY	2-3	23.08
		19	CASS**IREG**GAETLY	2-3	7.69
	30D4	19	CASS**IRQG**GAETLY	2-3	94.12
		19	CASS**VRQG**GAETLY	2-3	5.88
	30D8	19	CASS**FREE**GAETLY	2-3	50
		19	CASS**IRQG**GAETLY	2-3	25
		19	CASS**FRQE**GAETLY	2-3	16.67
		19	CASS**MRQR**GAETLY	2-3	8.33
	30H3	19	CASS**IRQG**GAETLY	2-3	100
$A22C\alpha^{-/-}PI1^{-/-}PI2^{-/-}Y16A^{tg}$	1G3	19	CASS**MRQG**GAETLY	2-3	100
	3F11	19	CASS**MRQG**GAETLY	2-3	63.64
		19	CASS**RRDR**GAETLY	2-3	31.82
		4	CASSQDGQDTQY	2-5	4.55
	3G5	19	CASS**IRQG**GAETLY	2-3	100
	10G2	19	CASS**MRQG**GAETLY	2-3	58.33
		19	CASS**FREE**GAETLY	2-3	33.33
		19	CASS**FRQE**GAETLY	2-3	8.33
	30A8	19	CASS**MRQG**GAETLY	2-3	100
	30B8	19	CASS**MRQG**GAETLY	2-3	100
	30E2	19	CASS**IRTG**GAETLY	2-3	61.9
		19	CASS**MRQG**GAETLY	2-3	33.33
		19	CASS**FREE**GAETLY	2-3	4.76
	30H1	19	CASS**IRQG**GAETLY	2-3	100

Colored sequences represent those found in more than one mouse. Recurrent motifs incorporating a non-germline–encoded arginine residue are underlined in bold.

TRBV19/TRBJ2-3 transcripts incorporating a CDR3β motif based around a central arginine residue. Arginine is degenerately encoded at the nucleotide level and readily incorporated on a probabilistic basis during the somatic recombination process (42). It can also play a key role in TCR recognition (43). The current data therefore suggest a highly conserved mode of antigen engagement focused on the G9C8 blueprint.

Several studies have previously identified a tolerogenic role for PI2 (17,19–21). In contrast, the role of PI1 has not been fully established. The only reported investigation in NOD mice found that PI1 deficiency protected against the development of diabetes (18). On a nonautoimmune background (129/SV), mice lacking PI2 showed increased PI1 gene transcripts as well as enhanced β-cell mass to compensate for lower insulin production, indicating the importance of PI1 in metabolism (44). Moreover, the PI2 B9-23 peptide can induce proliferation of PI1-reactive NOD T cells, and the PI1 B9-23 and C49-66 peptides can induce proliferation of PI2-reactive NOD T cells (21). Such cross-reactivity suggests that both isoforms can facilitate the expansion of PI-specific populations in the periphery. Further studies are therefore required to assess the relative contributions of PI1 and PI2 as determinants of tolerance and diabetogenicity.

Although the current model system addresses a single specificity, the epitope nonetheless derives from insulin, which is known to be an important early antigenic target in type 1 diabetes. Previous work by other investigators has focused on IGRP-specific NY8.3 $CD8^+$ T cells (45–47).

However, fundamental differences exist between these two diabetogenic epitopes. Most notably, IGRP is not expressed in the thymus (1). In contrast, studies of PI facilitate an understanding of repertoire development in the presence of an autoantigen that is naturally expressed both in the thymus and in the periphery. Moreover, IGRP reactivity depends on an autoimmune response to insulin (24,25). In humans, the key genetic susceptibility region IDDM2 relates to the level of PI expression. The current study is therefore informative because it describes the impact of variable PI expression on clonotype selection in response to a defined and biologically relevant autoimmune epitope in type 1 diabetes.

Two important conclusions emerge from the present data. First, PI expression shapes the insulin-reactive $CD8^+$ T-cell repertoire in a mouse model of type 1 diabetes. Second, an avidity threshold exists below which insulin-reactive $CD8^+$ T cells are less affected by antigen exposure in the thymus compared with the periphery. These findings have potential implications for the induction of antigen-specific tolerance as a therapeutic strategy against autoimmune diabetes.

Acknowledgments. The authors thank the National Institutes of Health Tetramer Core Facility for provision of the H-2K^d-LYLVCGERG tetramer and various control tetramers. L.C. Harrison and A. Lew (Walter and Eliza Hall Research Institute, Melbourne, VIC, Australia) provided NOD mice overexpressing PI2 (NOD*PI2^{tg}*) under the MHC class II promoter before they were available commercially.

Funding. This work was supported by the Medical Research Council (grant G0901155) and the Wellcome Trust (grant 100326Z/12/Z). J.A.P. was the recipient of a Diabetes UK Studentship (08/3767). D.A.P. is a Wellcome Trust Senior Investigator.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. J.A.P. contributed to the experiments, data analysis, and writing and editing of the manuscript. T.C.T. and K.L. contributed to the experiments and editing of the manuscript. J.E.M. contributed to the experiments, data analysis, and editing of the manuscript. E.D.L., A.P., J.D., D.K., and K.M. contributed to the experiments. P.M. contributed to the data analysis. L.W. contributed to the data analysis and editing of the manuscript. D.A.P. contributed to the data analysis and writing and editing of the manuscript. F.S.W. contributed to the study concept, data analysis, and writing and editing of the manuscript. F.S.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 13th International Congress of the Immunology of Diabetes Society, Lorne, VIC, Australia, 7–11 December 2013; the Diabetes UK Annual Professional Conference, Manchester, U.K., 13–15 March 2013, and Liverpool, U.K., 5–7 March 2014; and the British Society for Immunology Annual Congress, Brighton, U.K., 1–4 December 2014.

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