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Different Modulation of Ptpn22 in Effector and Regulatory T Cells Leads to Attenuation of Autoimmune Diabetes in Transgenic Nonobese Diabetic Mice

Li-Tzu Yeh,* Shi-Chuen Miaw,† Ming-Hong Lin,‡ Feng-Cheng Chou,§ Shing-Jia Shieh,§ Yi-Ping Chuang,‡ Shih-Hua Lin,§ Deh-Ming Chang,§ and Huey-Kang Sytwu*‡

Ptpn22 encodes PEST domain–enriched tyrosine phosphatase (Pep), which negatively regulates TCR proximal signaling and is strongly associated with a variety of autoimmune diseases in humans. The net effect of Pep on the balance of immunity and tolerance is uncertain because of the simultaneous inhibition of TCR-mediated signaling of effector and regulatory T cells (Tregs).

In this study, we generated transgenic NOD mice that overexpressed Pep in T cells. The transgenic mice had a significantly lower incidence of spontaneous autoimmune diabetes, which was accompanied by fewer IFN-γ–producing T cells, and an increased ratio of CD4+Foxp3+ Tregs to CD4+IFN-γ+ or to CD8+IFN-γ+ T cells, respectively, in pancreatic islets. Transgenic T cells showed markedly decreased TCR-mediated effector cell responses such as proliferation and Th1 differentiation. By contrast, the inhibitory effect of transgenic Pep on TCR signaling did not affect the differentiation of Tregs or their suppressive activity. Adoptive transfer experiments showed that transgenic splenocytes exhibited attenuated diabeticogenic ability. To examine further the pathogenic features of transgenic T cells, we generated Ptpn22/BDC2.5 doubly transgenic mice and found reduced proliferation and Th1 differentiation in CD4+ T lymphocytes with additional Pep in pancreatic lymph nodes but not in inguinal lymph nodes of NOD/SCID recipients. This finding indicates that transgenic Pep attenuates T cell functions in an islet Ag–driven manner. Taken together, our results demonstrate that Pep overexpression in T cells attenuates autoimmune diabetes in NOD mice by preferentially modulating TCR signaling–mediated functions in diabeticogenic T cells but not in Treg.

The online version of this article contains supplemental material.

Abbreviations used in this article: dLPC, dLPE, dLck; Ptpn22; ILN, inguinal lymph node; PTPN22; PTPN22, encoding lymphoid tyrosine phosphatase in humans, the ortholog of mouse PEST domain–enriched tyrosine phosphatase (Pep), and is expressed exclusively in hematopoietic cells; Pep, PEST domain–enriched tyrosine phosphatase; PLN, pancreatic lymph node; SNP, single nucleotide polymorphism; Treg, inducible regulatory T cell; TID, type 1 diabetes; Tconv, conventional T cell; T1D, type 1 diabetes; T1D, type 1 diabetes; T1D, type 1 diabetes.
The contribution of Ptpn22 deficiency to the development of autoimmune diseases was first shown in a study of Ptpn22+/−/CD45 E613R mice, which exhibit a lupus-like disease on a non-autoimmune background (20). In these double-mutant mice, loss of Pep expression affected T cells intrinsically and provoked mutant B cells to produce autoantibodies in a cell-extrinsic fashion, suggesting that Ptpn22 deficiency can cooperate with CD45 E613R in the context of susceptible microenvironment to break down immune tolerance. However, a recent study of Ptpn22+/−/ mice showed that Pep deficiency positively mediated Treg development and further reduced the severity of experimental autoimmunoneuromyelitis (21). The authors suggested that the increased CD25 expression and TCR-induced calcium flux observed in Ptpn22+/−/ thymic Treg may promote Foxp3 expression and Treg development. These paradoxical results suggest that Pep regulates effector T cells and Tregs simultaneously but causes different effects on the maintenance of immune homeostasis and/or induction of autoimmunity.

A recent report (22) indicated that NOD mice exhibit lower Ptpn22 transcription in PLNs compared with nondiabetic NOD. B10 mice in the early stage of autoimmune diabetes, implying an association between a lower Pep expression level and the diabetic process in NOD mice. Several in vitro studies have demonstrated that Ptpn22 overexpression attenuates TCR-mediated signaling and IL-2 production (17, 18); however, its role in autoimmunity is uncertain because of the simultaneous attenuation of Pep in effector T cells and Tregs (20, 21). In this study, we generated a transgenic NOD mouse model to investigate whether overexpression of Pep in T cells protects mice from the development of autoimmune diabetes and to dissect further the different effects of transgenic Pep on effector T cells and Tregs. We found that transgenic Pep significantly protected NOD mice from diabetes by preferentially attenuating the activation of effector T cells upon encountering islet Ags in PLNs. Interestingly, although additional Pep expression attenuated TCR signaling of Tregs their development, differentiation, and suppressive activity were not changed. Our study demonstrates the protective effect of increased expression level of Pep in autoimmune diabetes and identifies different regulation of Pep-mediated TCR signaling in effector T cells and Tregs in NOD mice.

Materials and Methods

Mice

NOD/Syju mice (K4, D10, L1, and I-Aβ) and NOD.BDC2.5 TCR transgenic mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME), NOD/SCID mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and subsequently bred at the Animal Center of the National Defense Medical Center (Taipei, Taiwan) under specific pathogen-free conditions. Experiments were conducted in accordance with institutional guidelines and were approved by the National Defense Medical Center Institutional Animal Care and Use Committee.

Generation of Ptpn22-transgenic mice and genotyping

Ptpn22 was cloned from cDNA of NOD mouse spleenocytes and inserted into pbBlueScript II vector. The pbBlueScript II–Ptpn22 was digested with EcoRI and subcloned into the pcDNA3.1(B)–HA plasmid to generate pcDNA3.1(B)–HA–Ptpn22. The pcDNA3.1(B)–HA–Ptpn22 was digested with BamHI and subcloned into the distal Lck promoter expression vector to generate the dLck–HA–Ptpn22 construct (13). The linearized DNA fragment with dLck–Ptpn22 was purified and microinjected into the pronuclei of one-cell NOD embryos. These injected embryos were then implanted into pseudopregnant (BALB/c × FVB/Bj) females. dLck–Ptpn22 transgenic mice were typed by PCR using P1 (5′-ATCAGGATGG- TCGACCTCCA-3′) and P2 (5′-CAAGGGCCCAGAGTCAGAAGAT- 3′) primers. The existence and copy numbers of transgenic Ptpn22 were evaluated by Southern blot analysis. In brief, the genomic DNA was isolated from Ptpn22 transgenic founders by SDS/protease K digestion and phenol/chloroform extraction and digested with NotI. The digested DNA was electrophoresed, transferred to nitrocellulose, and probed with 32P-labeled probe DNA. All transgenic mice used in our study were hemizygous for the Ptpn22 transgene.

Detection of transgenic Ptpn22 expression

Total RNA was prepared from different organs of NOD female mice using the TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s protocols. Five micrograms of total RNA was reverse transcribed with oligo-dT by SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was used as template to perform PCR using P1 and P2 primers to detect the transcription of transgenic Ptpn22. Total cell lysates isolated from thymocytes or purified T cells were analyzed by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with indicated Abs to detect the translation of transgenic and total Ptpn22.

Assessment of insulitis and diabetes

Urine glucose concentration was measured weekly or every second day using the Chemstrip (Boehringer Mannheim, Indianapolis, IN). NOD female mice with glycosuria > 500 mg/dl on two consecutive tests were classified as diabetic. For histological analysis, pancreases from female mice at the age of 13–14 wk were fixed in 10% buffered formalin. The severity of insulitis was scored blindly on sections stained with H&E, as described previously (23).

Flow cytometric analysis

Lymphocytes from spleens and PLNs were stained with fluorochrome-conjugated Abs specific for murine CD4 (RM4-5), CD25L (MEL-14), CD44 (IM7), Foxp3 (FJK-16a), CD25 (PC61.5), IFN-γ (XMG1.2), and IL-4 (11B11), which were purchased from eBioscience (San Diego, CA). Fluorochrome-conjugated Abs to murine CD3 (17A2), CD8a (53-6.7), TCRβ (H57-597), CD19 (1D3), ERK1/2 (pT202/pY204), and TCRVB4 (KT4) were purchased from BD Biosciences (San Jose, CA). Fluorochrome-conjugated Abs to murine CD5 (53-7.3) and CXCXR3 (CXCXR3-173) were purchased from BioLegend (San Diego, CA). Cells were analyzed using a FACS caliber flow cytometer and CellQuest software (BD Pharmingen, San Jose, CA).

Cell stimulation and Western blot analysis

CD4+ T cells were stimulated at 37°C by coating with anti-CD3ε (1 μg/ml; 145-2C11; DB Pharmingen) and anti-CD28 (1 μg/ml; 37.51; BioLegend) and then cross-linking with mouse anti-hamster IgG mixture (25 μg/ml; G94-56 and G70-204; DB Pharmingen) during the indicated periods of time. After stimulation, cells were immediately resuspended in lysis buffer (50 mM Tris [pH 7.4], 10% glycerol, and 150 mM NaCl) containing protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor mixture (Roche, Mannheim, Germany). Cell lysates were analyzed by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with Abs to hemagglutinin tag (3F10; Roche); Zap70 (2705), Zap70 phosphorylated at Tyr195 (2701), Lck (2752), Lck phosphorylated at Tyr394 (2101), AKT, AKT phosphorylated at S473 (4068), Foxo1 (9462), Foxo1 phosphorylated at S256 (9461), S6 (2217), S6 phosphorylated at S235/S236 (4858) (Cell Signaling Technology, Beverly, MA); ERK (3739), ERK phosphorylated at Tyr204 (2219), JNK2 (2037-1), JNK2/1/2/3 phosphorylated at Tyr223 and Tyr235 (2155-1), p38 (1544-1), p38 phosphorylated at Thr180 and Tyr182 (9222), and Threonine (Thr) and Tyrosine (Tyr) (1229-1) (Epitomics, Burlingame, CA); and β-actin (AC-5; Sigma-Aldrich). Abs to Pep were polyclonal Abs provided by Dr. S.-C. Miu (Graduate Institute of Immunology, National Taiwan University College of Medicine, Taipei, Taiwan).

Intracellular staining for phospho-ERK

Splenocytes or lymph node cells were harvested into serum-free RPMI 1640 medium at 37°C for at least 1 h before stimulation (as described above) for 2 min. After stimulation, the cells were fixed and permeabilized using
Cytotox/Cytopenr kit (eBioscience) and stained with surface markers and anti–phospho-ERK for 1 h. Data were collected on a FACS Calibur flow cytometry.

Calcium flux
Single-cell suspensions of lymphocytes were loaded with 4 μg Flu-4 AM per 10^7 cells (number 41966-029; Life Technologies). After 30 min at 37°C, cells were kept at room temperature for 30 min to allow cleavage of the AM esters and then washed twice with complete RPMI 1640 medium. Flu-4 AM–labeled T cells were stained with 10 μg/ml CD3ε–biotin Ab on ice for 30 min. Cells were warmed to 37°C, the sample was analyzed for 30 s to establish a baseline. 2 μg/ml streptavidin–PerCP Cy5.5 (eBioscience) was added at 30 s to cross-link CD3, and the reaction proceeded for 30 min on a FACS Calibur flow cytometer. Intracellular calcium concentration was analyzed using the FlowJo kinetic function.

T cell proliferation assay
For proliferation assays, splenocytes or purified CD90.2^+ T cells were cultured in triplicate wells of 96-well flat-bottom plates (2–5 × 10^3 cells/200 μl/well) with indicated amounts of soluble anti-CD3ε, soluble anti-CD3ε, and anti-CD28 or Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen). After 24 h, the cultured cells were pulsed with 1 μCi [3H] thymidine–biotin (PerlinElmer, Shleton, CT) per well and harvested after 16–18 h. The plates were harvested onto UniFilter-96, GFC microplate (PerkinElmer), and the incorporated [3H] thymidine was detected with a Packard TopCount Microplate Scintillation Counter. For cell division, splenocytes from 8- to 10-week-old female NOD mice were isolated and labeled with CFSE by incubating 10^7 cells in 1 ml PBS with 2.5 mM CFSE for 10 min at 37°C. The CFSE reaction was quenched by adding 10 μl cold complete RPMI 1640 medium, and the cells were washed once. The CFSE-labeled splenocytes were cultured in 96-well flat-bottom plates (5 × 10^3 cells/200 μl/well) and stimulated with soluble anti-CD3ε (0.25 μg/ml) and anti-CD28 (0.125 μg/ml) for 3 d. Data were collected on a FACS Calibur flow cytometry.

T cell differentiation
CD4^+CD2L2^+CD25^− naive T cells were isolated using a CD4^+CD2L2^+T Cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer’s instructions. Purity for isolated cells was >95%. The naive CD4^+ T cells were stimulated with indicated amount of plate-coated anti-CD3ε and anti-CD28 (1 μg/ml) Abs under Th1 (10 ng/ml IL-12, 5 ng/ml IL-2, and 10 μg/ml anti–IL-4) or induced Tregs (Th3) (10 ng/ml IL-2, 5 ng/ml IL-10) for 3 d. For intracellular cytokine staining, the T cells were stimulated with PMA (Sigma, St Louis, MO, USA) and ionomycin (Sigma, St Louis, MO, USA) in the presence of monensin (Sigma, St Louis, MO, USA) for 4–5 h. The chemical inhibitors SB203580 and SP600125 (Calbiochem, San Diego, CA, USA) were added as indicated concentration. The percentage of IFN-γ-producing T cells was analyzed by gating on CD4^+ T cells. Data were collected on a FACS Calibur flow cytometry.

CD4^+CD25^+ Treg suppression assays
CD4^+CD25^+ Treg cells were isolated using a CD4^+CD25^+ Regulatory T Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer’s instructions. Purity for isolated cells was >90%. To measure the suppressive activity, CD4^+CD25^+ effector T cells (5 × 10^5/well) were cultured in round-bottom 96-well plates in the presence of Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen) and the indicated ratio of CD4^+CD25^+ Treg cells. After 72 h, the cultured cells were pulsed with 1 μCi [3H] thymidine/well and analyzed 16–18 h after.

Isolation of pancreas-infiltrating cells
Pancreases were isolated from female NOD mice by collagenase XI (Sigma-Aldrich) digestion and Histopaque 1077 (Sigma-Aldrich) gradient purification. Marginal cells were collected and incubated with Cell Dissociation Buffer (number 41966-029; Life Technologies). The solution was passed through a cell strainer, and suspended single cells were counted and stimulated (as described above) for intracellular cytokine staining. The percentage of IFN-γ–producing T cells was analyzed by gating on CD4^+ and CD8^+ T cells. The percentage of CD4^+Foxp3^+ T cells was analyzed by gating on CD4^+ T cells.

Adaptive transfer
Splenocytes (2 × 10^7 cells) isolated from normoglycemia female NOD donor mice at 12–14 wk of age were injected into female NOD/SCID recipients via the retro-orbital plexus. Diabetes was identified in NOD/SCID recipients as described previously (24). CFSE-labeled splenocytes or naïve CD4^+ T cells from transgenic Ptpn22/BDC2.5 or BDC2.5 normoglycemia female NOD mice at 12–16 wk of age were adoptively transferred into female NOD/SCID recipients via the retro-orbital plexus. Different lymphoid organs from the recipient mice were harvested 3 or 5 d following adoptive transfer, and the percentage of cell dividing and IFN-γ+ of transferred CD4^+ T cells were analyzed on a FACS Calibur flow cytometer. For analysis of the ability of CD4^+CD25^+ Treg cells to suppress the development of diabetes, 5 × 10^3 splenocytes from normoglycemia female BDC2.5 mice were cotransferred with 5 × 10^4 CD4^+CD25^+ Treg cells from transgenic or nontransgenic female NOD mice into NOD/SCID recipients via the retro-orbital plexus and monitored daily the diabetic development by measuring urine glucose concentration in these recipients. The recipient mice with glycosuria > 500 mg/dl on two consecutive tests were classified as diabetic.

Statistical analysis
The log-rank (Mantel–Cox) test was used for comparison of survival curves. Student unpaired t test was applied for statistical analysis of other experiments in this study.

Results
Generation of Ptpn22-transgenic NOD mice
Ptpn22 encodes Pep, which negatively regulates TCR proximal signaling and is strongly associated with a variety of autoimmune diseases in humans. The studies of Ptpn22−/− mice reveal that the role of Pep in autoimmunity is complex because of the simultaneous inhibition of TCR-mediated signaling in effector and Tregs.

To investigate the potential effect of Pep on the regulation of autoimmune diabetes, we established a T cell–specific Ptpn22–transgenic mouse model by injecting the Lck distal promoter–Ptpn22 construct into fertilized NOD eggs. The construct contains the mouse distal Lck promoter, partial first noncoding exon of Lck gene, hemagglutinin-tagged NOD mouse Ptpn22 cDNA, human growth hormone (hGH) gene with a poly(A) signal, and two chicken 5’-terminus β-globin hypersensitivity site 4 insulator elements (Fig. 1A). Two transgenic founders were obtained and denoted as dLk−Ptpn22 C (dLPC) and dLk−Ptpn22 E (dLPE). Southern blot analysis of tail genomic DNA indicated that dLPC and dLPE founder mice carried 2–5 and 10–20 copies of the chimeric transgene, respectively (Fig. 1B).

To identify the existence and transcription of transgenic Ptpn22, we performed PCR analysis using a pair of forward and reverse primers located in the coding region of dLk−Ptpn22 (P1) and the second exon of hGH (P2), as indicated in Fig. 1A. The transgenic mice were easily distinguished from nontransgenic littermates by PCR genotyping with P1 and P2 primers (Fig. 1C). The transcription signal of transgenic Ptpn22 appeared mainly in lymphoid organs such as the thymus, spleen, and PLNs (Fig. 1D). The transgenic signals shown in the intestine and brain may have been caused by some residual lymphocytes because of incomplete perfusion. Western blot analysis revealed that transgenic Pep was expressed in both thymocytes and splenic T cells of dLPC and dLPE mice. Moreover, the total amount of Pep protein in these cells increased in a stepwise fashion from nontransgenic littermates to dLPC mice to dLPE mice (Fig. 1E).

Reduction in the incidence of spontaneous diabetes and severity of insulitis in transgenic mice
To investigate the potential effect of transgenic Pep on the development of autoimmune diabetes, we first compared the diabetic kinetics and incidence between transgenic mice and their nontransgenic littermates. The dLPC mice became diabetic after 17 wk of age, and the nontransgenic littermates started to develop diabetes.
at 13 wk of age, indicating a transgenic Pep-mediated delay in disease onset. At ∼25 wk of age, the incidence of diabetes in nontransgenic littermates increased to 50%, but the incidence was 25% in dLPC mice. After 30 wk of age, ∼70% of nontransgenic mice had become diabetic, but only 35% of dLPC mice had developed diabetes (Fig. 2A), indicating a significant protection induced by transgenic Pep in T cells.

Although the initial incidence and kinetics of diabetes were similar in dLPC mice and dLPE mice before 30 wk of age, the overall diabetic rate at 40 wk was significantly lower in dLPE mice than in dLPC mice (40 versus 60%), suggesting that the Pep-mediated protection is transgene dosage dependent (Fig. 2A). We used histological analysis to evaluate the severity of insulitis in the transgenic mice and nontransgenic littermates at 13–14 wk of age. More intact islets (grade 0) and less leukocyte infiltration were observed in both dLPC and dLPE transgenic mice compared with their nontransgenic littermates (Fig. 2B), suggesting that additional expression of Pep in T cells attenuated the diabetogenic property of lymphocytes. These results indicate that transgenic Pep significantly protected NOD mice from diabetes by decreasing the infiltration of leukocytes into islets in a transgene dosage-dependent manner. Because the protective phenotype in dLPC mice is sufficient to discriminate the modulatory effects caused by different levels of Pep, we therefore used dLPC mice as one representative Ptpn22-transgenic line in all subsequent experiments.

Lymphocyte development in transgenic mice

A previous study (19) reported that Pep-mediated TCR signaling regulates positive selection of thymocytes and homeostasis of effector/memory T cells in the periphery. To investigate whether transgenic Pep attenuates diabetes by modulating the development or homeostasis of lymphocytes, we first counted cell numbers in the thymus, spleen, and PLNs and compared these between transgenic and nontransgenic mice at 12–14 wk of age. Cell numbers in these lymphoid organs did not differ between dLPC mice and nontransgenic littermates (Fig. 3A). We then analyzed the different populations of thymocytes in these transgenic mice. The percentages of different thymocyte subsets (CD4+CD8−, CD4+CD8+, and CD4−CD8+) were similar in dLPC mice and nontransgenic littermates (Fig. 3B). The expression of CD5 and TCRβ in the CD4+CD8+ double positive thymocyte stage was almost identical in the dLPC mice and their nontransgenic littermates (Fig. 3C). Collectively, our results indicated that transgenic Pep did not affect the development of thymocytes in dLPC mice.
To investigate whether transgenic Pep modulates the development of peripheral lymphocytes, we measured the percentages of different lymphocyte populations (CD4+, CD8+, and CD19+) in the spleens and PLNs of these transgenic mice. The percentage of each lymphocyte population in the spleens (Fig. 3D) and in PLNs (Fig. 3E) was similar in the dLPC mice and their nontransgenic littermates. Moreover, the percentages of CD44hiCD62LloCD4+ and CD44 hiCD62L hi-loCD8+ T cells in the spleen (Fig. 3F) and in PLNs (Fig. 3G) were indistinguishable between the transgenic and control mice, indicating that homeostasis of effector/memory T cells in peripheral lymphoid organs were not affected by transgenic Pep. Previous studies reported that Ptpn22 deficiency in NOD mice is associated with an increase in the percentage of these cells in the pancreas (19). To investigate whether the homeostasis those cells is also affected in aged dLPC mice, we measured the percentages of CD44 hiCD62L loCD4+ and CD44 hiCD62L hi-loCD8+ T cells in the spleen (Fig. 3F) and in PLNs of dLPC and nontransgenic mice at ages more than 6 mo. Our results reveal that the percentages of those cells were similar in transgenic mice and control littermates (Supplemental Fig. 1), indicating that the homeostasis of effector/memory T cells in peripheral lymphoid organs was not affected by transgenic Pep.

It has been shown that IFN-γ–producing CD4+ T (Th1) cells play a positive role in the pathogenesis of autoimmune diabetes and that IL-4–producing CD4+ T (Th2) cells are critical to suppressing the development of diabetes (25). To investigate whether the attenuation of autoimmune diabetes occurs through modulation of the development or balance of Th1 and Th2 cells by additional Pep, we measured the percentages of CD4+ IFN-γ+ and CD4+IL-4+ T cells in peripheral lymphoid organs of transgenic mice. The percentages of these cells in the spleens (Fig. 3H) and PLNs (Fig. 3I) did not differ between dLPC mice and their nontransgenic littermates. Collectively, our results demonstrate that additional expression of transgenic Pep in T cells did not interfere with the development of lymphocytes in thymus, spleen, and PLNs or homeostasis of effector/memory T cells or with the Th1/2 cell balance in transgenic NOD mice.

Decrease in number and pathogenic features of pancreas-infiltrating T cells in transgenic mice

Our data indicated that the severity of insulitis was decreased significantly in dLPC mice (Fig. 2B). We counted the cells and different lymphocyte populations infiltrating into the pancreases of transgenic mice at 12–14 wk of age. The numbers of total cells (Fig. 4A) and CD4+ and CD8+ T cells (Fig. 4B) were lower in dLPC mice, and the reduction in these cell numbers correlated with alleviation of insulitis in transgenic NOD mice. It has been well reported that pathogenic T cells inside the pancreas of the NOD mouse are mainly IFN-γ–producing cells. We therefore counted the IFN-γ–producing CD4+ and CD8+ T cells in pancreas infiltrates. Our data reveal that there was no significant difference in the percentage of these cells between dLPC and nontransgenic mice (Fig. 4D). However, the number of these pathogenic T cells was significantly lower in dLPC mice compared with their nontransgenic littermates (Fig. 4C).

A recent study (9) demonstrated that CD4+Foxp3+ Treg cells are crucial to controlling pancreatic autoimmunity in situ and to maintaining immune tolerance in NOD mice. We next counted the number of CD4+Foxp3+ T cells in pancreas infiltrates in these transgenic mice. Although there were fewer infiltrating CD4+Foxp3+ cells (Fig. 4E), the ratio of CD4+Foxp3 Treg to CD4+IFN-γ+ (Fig. 4F, left panel) or to CD8+IFN-γ+ cells (Fig. 4F, right panel) was significantly higher in transgenic mice compared with their nontransgenic littermates, suggesting a better balance between regulatory and pathogenic lymphocytes in the pancreas of dLPC mice. Our results indicated that the numbers of T cells and IFN-γ–producing CD4+ or CD8+ T cells were lower, and the ratio of Treg to CD4+IFN-γ+ or to CD8+IFN-γ+ cells was higher in pancreas infiltrates of dLPC mice. Collectively, these changes may have contributed to the Pep-mediated protection in NOD mice.

Attenuation of TCR-mediated cell proliferation and Th1 differentiation in transgenic T lymphocytes

Previous studies have reported that Pep overexpression attenuates TCR signaling transduction and IL-2 production in T cell lines (17,
Although the role of Pep in vivo was illustrated in the study of Ptpn22−/− mice, the biological function and physiological effects of Pep overexpression in TCR-mediated responses of primary T cells are still unclear. We first investigated whether transgenic expression of Pep in T lymphocytes attenuates T cell proliferation in NOD mice. Splenocytes were isolated from dLPC mice at 8–10 wk of age, and TCR-mediated cell proliferation was evaluated. Proliferation was significantly lower in splenocytes of dLPC mice upon anti-CD3ε or anti-CD3ε and anti-CD28 stimulation compared with their control littermates (Fig. 5A), demonstrating transgenic Pep-mediated attenuation of T cell proliferation. The proliferation of purified transgenic T cells stimulated with anti-CD3ε and anti-CD28 was markedly lower in T cells from dLPC compared with control mice (Fig. 5B), indicating an inhibition of T cell–intrinsic proliferation in dLPC mice. To determine whether the decrease in proliferation in transgenic T cells results from less division of CD4 or CD8 populations, we examined cell division by measuring the dilution of the cytosolic dye CFSE upon TCR stimulation. Both CD4+ and CD8+ T cells of splenocytes from dLPC mice divided less upon stimulation compared with cells from their nontransgenic littermates (Fig. 5C). We also found that the decrease in proliferation in Ptpn22-transgenic T cells was not caused by a loss of the TCR or CD3 molecule expression (data not shown).

CD4+IFN-γ+ T cells play a pivotal role in the pathogenic development of T1D. Previous studies have demonstrated that TCR signal transduction initiates the differentiation of naive CD4+ T cells (26) and that the signaling strength regulates the development of distant Th cell phenotypes (27). To investigate whether additional expression of transgenic Pep attenuates the differentiation of naive CD4+ T cells into IFN-γ–producing Th1 cells, we cultured purified naive CD4+ T cells (CD4+CD62L+CD25−) from dLPC mice with anti-CD3/CD28 Dynabeads or different concentrations of plate-bound anti-CD3ε and fixed concentration of plate-bound anti-CD28 in the presence of IL-12, IL-2, and anti–IL-4 for 3 d. The percentage of CD4+IFN-γ+ cells was significantly lower in T cells from dLPC mice than in their nontransgenic littermates (Fig. 5D). However, the degree of attenuation of Th1 differentiation in T cells from dLPC mice was reduced at higher concentrations of anti-CD3ε Ab (Fig. 5E), suggesting that higher TCR signaling strength can override the transgenic Pep-mediated attenuation of Th1 differentiation in NOD mice. A previous study (28) reported that the expression of CXCR3 is critical to the trafficking of Th1 cells to the site of inflammation. We therefore investigated whether additional expression of Pep affects the expression of CXCR3 in differentiated Th1 cells. Our results revealed a decreased expression of CXCR3 on transgenic CD4+ IFN-γ+ T cells compared with nontansgenic CD4+IFN-γ+ T cells after 3-d culture in Th1-differentiated condition (Supplemental Fig. 2), suggesting that an additional expression of transgenic Pep interferes with the migration of converted Th1 cells to pancreas by attenuating the induction of CXCR3 during Th1 cell

FIGURE 3. Lymphocyte development in Ptpn22 transgenic NOD mice. (A) The absolute cell numbers of thymus, spleen, and PLNs of normoglycemia female dLPC mice or control littermates at 12–14 wk of age were counted. (B) The percentages of lymphocyte populations in the thymus of normoglycemia female dLPC mice or control littermates at 12–14 wk of age were analyzed by flow cytometry. (C) Histogram of CD5 and TCRβ expression among CD4+CD8+ thymocytes from normoglycemia female dLPC mice or control littermates at 12–14 wk of age were analyzed by flow cytometry. Data are representative of three independent experiments. (D–G) The percentages of different lymphocyte populations in spleens (D, F) and PLNs (E, G) of normoglycemia female dLPC mice or control littermates at 12–14 wk of age were analyzed by flow cytometry. (H and I) The cells from spleens (H) or PLNs (I) of normoglycemia female dLPC mice or control littermates at 12–14 wk of age were stimulated with PMA and ionomycin in the presence of monensin for 4–5 h. The percentages of CD4+IFN-γ+ and CD4+IL-4+ T cells were analyzed by flow cytometry. Data are presented as the mean ± SEM.
differentiation. Collectively, our data demonstrate that transgenic Pep attenuated TCR-mediated responses in terms of proliferation of CD4+ and CD8+ T cells, differentiation of naive CD4+ T cells into Th1 cells, and expression of CXCR3 on Th1 cells, contributing to the protection against autoimmune diabetes in NOD mice.

Homeostasis, TCR-mediated ERK phosphorylation, suppressive function, and differentiation of Ptpn22-transgenic CD4+Foxp3+ T cells

Studies using different mouse models have demonstrated that the integrity of TCR signaling is crucial for the production, homeostasis, and function of Tregs (29). A recent study (21) indicated that the numbers of Tregs in the thymus and periphery were increased in Ptpn22−/− mice, suggesting that Pep negatively regulates the development of Tregs. To investigate whether transgenic Pep affects the development and homeostasis of Tregs, we measured the percentages of CD4+Foxp3+ cells in the thymus, spleen, PLN, and pancreas infiltrates of transgenic NOD mice at 12–14 wk of age. The percentages of pancreas-infiltrating CD4+Foxp3+ and CD8+Foxp3+ T cells of normoglycemia female dLPC mice or control littermates at 12–14 wk of age were analyzed by flow cytometry. (F) Increase in ratio of pancreas-infiltrating Tregs to pathogenic T cells in Ptpn22-transgenic mice. The ratio of pancreas-infiltrating CD4+Foxp3+ to CD4+Foxp3− cells and CD8+Foxp3+ to CD8+Foxp3− cells of normoglycemia female dLPC mice or control littermates at 12–14 wk of age were analyzed according to the cell numbers of each population. Significance was evaluated by two-tailed Student unpaired t test. *p<0.05, **p<0.01.

Previous studies have demonstrated that Tregs are activated through TCR stimulation to execute suppressive functions (30–32); however, the regulatory role of Pep in TCR signaling and its subsequent function in Treg, have not been elucidated. To address this issue, we first analyzed the phosphorylation status of ERK in CD4+Foxp3+ (Fig. 6B, right upper panel) and CD4+Foxp3− T cells from dLPC mice. ERK phosphorylation in both CD4+Foxp3+ (Fig. 6B, right upper panel) and CD4+Foxp3− T cells (Fig. 6B, right lower panel) from dLPC mice was significantly inhibited compared with that from nontransgenic littermates, indicating that additional expression of transgenic Pep effectively blocked the ERK phosphorylation in both CD4+Foxp3+ Tregs and CD4+Foxp3− T cells upon TCR stimulation.

To investigate further whether transgenic Pep-mediated attenuation of TCR signaling modulates the suppressive function of Tregs, we isolated CD4+CD25+ Tregs from dLPC mice and measured their ability to suppress TCR-mediated proliferation of nontransgenic CD4+CD25− conventional T cells (Tconv). Interestingly, although the TCR-mediated ERK phosphorylation decreased markedly in transgenic Tregs (Fig. 6B, right upper panel), their suppressive effect on Tconv proliferation was similar to that of nontransgenic Tregs (Fig. 6C). However, consistent with the reduction in ERK phosphorylation (Fig. 6B, right lower panel), the transgenic Tconv significantly decreased TCR-mediated proliferation compared with nontransgenic Tconv (Fig. 6D). Furthermore,
we investigate whether transgenic Pep modulates the ability of Tregs to suppress the development of diabetes. We adoptively transferred splenocytes from 10- to 12-wk-old female BDC2.5 TCR transgenic mice with CD4^+CD25^+ Tregs from female dLPC or nontransgenic mice in a ratio of 10 to 1 (5 × 10^3 Tregs) into NOD/SCID mice and monitored daily the diabetic development in these recipients. Our data reveal that 100% of the mice received BDC2.5 splenocytes alone developed diabetes before 30 d after transfer. Notably, a delay onset of diabetes and significant lower incidence was observed in recipients received BDC2.5 splenocytes combined with nontransgenic or dLPC Tregs, (Fig. 6E), indicating that the suppressive function of dLPC Tregs to the development of diabetes is comparable with that of nontransgenic Tregs in vivo. These results suggest that transgenic Pep-mediated inability of ERK phosphorylation in Tregs was not sufficient to interfere with their immunosuppressive function. Our results also suggest that transgenic Pep simultaneously attenuated TCR-mediated ERK phosphorylation in both Tregs and Tconv but modulated their subsequent responses differently.

Recent studies (33, 34) have demonstrated that the strength of TCR signaling inversely regulates the differentiation of iTregs. The ratio of CD4^+Foxp3^+ Tregs to CD4^+IFN-γ^- or to CD8^+IFN-γ^- T cells increased in pancreas infiltrates of dLPC mice (Fig. 4F); we therefore investigated whether transgenic Pep promotes the differentiation of iTregs under different TCR signaling strengths. We cultured purified naive CD4^+ T cells (CD4^+CD62L^+CD25^- Foxp3^-) from dLPC mice with different concentrations of plate-bound anti-CD3ε and 1 μg/ml plate-bound anti-CD28 (E) in the presence of 10 ng/ml IL-12, 5 ng/ml IL-2, and 10 μg/ml anti-IL-4 for 3 d. The percentages of CD4^+IFN-γ^- T cells were measured by flow cytometry. Data are representative of two independent experiments. Significance was evaluated by two-tailed Student unpaired t test. *p < 0.05.

**FIGURE 5.** Attenuation of TCR-mediated cell proliferation and Th1 differentiation in Ptpn22-transgenic T lymphocytes. (A and B) Attenuation of TCR-mediated cell proliferation in transgenic T cells. Splenocytes (A) or purified T cells (B) isolated from female dLPC mice or control littermates at 8–10 wk of age were stimulated with the indicated amounts of soluble anti-CD3ε, soluble anti-CD3ε, and anti-CD28 or anti-CD3/CD28 Dynabeads in a ratio of 1:2 (beads to cells). The cell proliferation was measured by the incorporation of [methyl-^3H]thymidine. Data are representative of three independent experiments, presented as the mean ± SEM. Significance was evaluated by two-tailed Student unpaired t test. **p < 0.005. (C) Attenuation of TCR-mediated cell division in transgenic T cells. CFSE-labeled splenocytes from female dLPC mice or control littermates at 8–10 wk of age were stimulated with soluble 0.25 μg/ml anti-CD3ε and 0.125 μg/ml anti-CD28. The cell division was measured by flow cytometry by gating on CD4^+ or CD8^+ T cells. Data are representative of three independent experiments. The percentages of dividing cells are presented as the mean ± SEM. Significance was evaluated by two-tailed Student unpaired t test. (nontransgenic CD4^+ T cells versus dLPC CD4^+ T cells [p < 0.05]; nontransgenic CD8^+ T cells versus dLPC CD8^+ T cells [p < 0.05]). (D and E) Attenuation of Th1 differentiation in transgenic T cells. Naive CD4^+ T cells from female dLPC mice or control littermates were stimulated with anti-CD3/CD28 Dynabeads (D) or indicated concentrations of plate-bound anti-CD3ε and 1 μg/ml plate-bound anti-CD28 (E) in the presence of 10 ng/ml IL-12, 5 ng/ml IL-2, and 10 μg/ml anti-IL-4 for 3 d. The percentages of CD4^+IFN-γ^- T cells were measured by flow cytometry. Data are representative of two independent experiments. Significance was evaluated by two-tailed Student unpaired t test. *p < 0.05.
found that transgenic Pep attenuated TCR-mediated ERK phosphorylation (Fig. 6B), subsequent cell proliferation (Fig. 5A–C), and naive T cell differentiation into Th1 cells (Fig. 5D). To elucidate the regulatory effects of transgenic Pep on TCR signaling pathways in NOD mice, we first examined the TCR-mediated phosphorylation of Zap70 and Lck at tyrosines (Y) 319 and Y394, respectively. The phosphorylation status of both Zap70 Y319 and Lck Y394 sites was significantly reduced in transgenic Pep expressing NOD T cells compared with nontransgenic CD4 + T cells (Fig. 7A), indicating a decrease in MAPK activation negatively regulates Th1 cell differentiation, thus abrogating the difference in percentage of IFN-γ-producing T cells in NOD mice.

TCR-mediated activation of ERK, JNK, and p38 MAPK, three important arms of the MAPK family, plays a critical role in Th1 cell differentiation (35–37). We also found a decrease in Th1 cell differentiation in transgenic T cells in vitro (Fig. 5D, 5E). We next measured the phosphorylation of ERK, JNK, and p38 in transgenic CD4 + T cells upon TCR stimulation. TCR-induced phosphorylation of ERK, JNK, and p38 was significantly reduced in transgenic CD4 + T cells compared with nontransgenic CD4 + T cells (Fig. 7A), indicating that transgenic Pep attenuated TCR signaling by downregulating the activation of proximal signaling molecules.

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in effector T cells and Tregs. Several recent studies have demonstrated that TCR-mediated Akt activation results in the phosphorylation of S6 ribosomal protein (S6) and transcription factor Foxo1, which interferes with Foxp3 expression and suppressive function of Tregs, whereas the interruption of TCR-mediated activation of Akt, downstream S6, or Foxo1 favors the induction of Foxp3 (38-41). To investigate whether transgenic Pep-mediated attenuation of TCR signaling affects the activation of Akt, S6, or Foxo1, we examined the phosphorylation status of these molecules upon TCR stimulation. Phosphorylation of AKT serine (S) 473, S6 S235/236, and Foxo1 S256 sites decreased markedly in transgenic T cells compared with that of nontransgenic T cells (Fig. 7D). These results indicate that additional expression of transgenic Pep attenuated TCR-mediated activation of Zap70 and calcium flux, which interferes with Foxp3 expression, whereas transgenic Pep simultaneously downregulated TCR-induced activation of Akt, downstream S6, or Foxo1, which may all promote the induction of Foxp3. Collectively, the net effect of transgenic Pep in TCR signaling pathway may cause an “unchanged” function in the Tregs.

**Amelioration of diabetogenic properties in transgenic lymphocytes**

Our data demonstrated that transgenic Pep attenuates TCR-mediated cell proliferation and Th1 cell differentiation in vitro. To investigate further whether transgenic Pep modulates the diabetogenic properties of lymphocytes in vivo, we adoptively transferred splenocytes from 12- to 14-wk-old dLPC mice into NOD/SCID mice and monitored the development of autoimmune diabetes in these recipients. Approximately 80% of the mice that received nontransgenic splenocytes developed diabetes 50 d after transfer (Fig. 8A). Notably, <30% of the recipients transferred with transgenic splenocytes developed diabetes at the same time (p < 0.01; Fig. 8A). The incidence of diabetes was significantly lower in mice that received transgenic splenocytes 90 d after transfer compared with mice transferred with control splenocytes (Fig. 8A), indicating that transgenic Pep downregulated the diabetogenic properties of lymphocytes in NOD mice.

Because the development of T1D is initiated by autoreactive T cells through the early recognition of islet Ags, we investigated whether transgenic Pep could attenuate autoimmune diabetes by downregulating their responses to pancreatic Ags presented in the context of the NOD I-A^7 molecule. We crossed dLPC mice and BDC2.5 TCR transgenic mice, an accepted model to track the fate and/or responses of islet-reactive CD4^+ T cells, to generate dLPC/BDC2.5 double-transgenic mice. Flow cytometric analysis revealed that the percentages of CD4^+ Vß4^+ T cells in inguinal lymph nodes (ILNs) and PLNs of dLPC/BDC2.5 double-transgenic mice were similar to those in BDC2.5 mice (Fig. 8B), indicating that transgenic Pep did not interfere with the development of clonotype-positive cells in BDC2.5 NOD mice. Although the islet Ag recognized by the BDC2.5 TCR was identified as chromogranin A (42), several agonist mimotope peptides are still used to evaluate the TCR-mediated responses of clonotype-positive T cells (43). To investigate whether transgenic Pep downregulates the proliferative responses of CD4^+ Vß4^+ cells when encountering antigenic peptides in the context of the
NOD I-A<sup>β</sup><sup>1</sup> molecule, we stimulated splenocytes isolated from dLPC/BDC2.5 mice with an agonistic BDCmi peptide recognized by the BDC2.5 TCR. Cell proliferation was significantly lower in the splenocytes from dLPC/BDC2.5 NOD mice compared with those from their BDC2.5 single-transgenic littermates (Fig. 8C), indicating that transgenic Pep attenuated the proliferation of BDC2.5 T cells in an islet Ag–driven manner.

To investigate further whether transgenic Pep attenuates autoreactive T cell responses upon encountering natural islet Ags during the development of autoimmune diabetes, we adoptively transferred CFSE-labeled splenocytes from dLPC/BDC2.5 mice to NOD/SCID recipients and measured the proliferative status of CD4<sup>+</sup>V<sup>b</sup> T cells in adjacent PLNs but not in ILNs (3), the division of single-transgenic CD4<sup>+</sup>V<sup>b</sup> T cells was vigorous in PLNs compared with ILNs (Fig. 8D, left lower panel), indicating islet Ag-driven T cell proliferation. Notably, the division of dLPC/BDC2.5 double-transgenic CD4<sup>+</sup>V<sup>b</sup> T cells was significantly inhibited compared with that of BDC2.5 single-transgenic T cells in PLNs of recipients (Fig. 8D, right lower panel), suggesting that transgenic Pep attenuated the islet Ag–driven proliferation of autoreactive T cells in situ and prevented the diabetic process in NOD mice.

We also adoptively transferred naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>C<sup>CD62L<sup>−/−</sup></sup>C<sup>CD25<sup>−</sup></sup>) from dLPC/BDC2.5 double-transgenic NOD mice into NOD/SCID recipients and measured the percentage of CD4<sup>+</sup>V<sup>b</sup>IFN-γ<sup>+</sup> T cells in PLNs. The percentage of IFN-γ-producing CD4<sup>+</sup>V<sup>b</sup> T cells was lower in PLNs of recipients transferred with T cells from dLPC/BDC2.5 double-transgenic mice compared with recipients transferred with T cells from BDC2.5 single-transgenic littermates (Fig. 8E). Collectively, our results demonstrate that transgenic Pep attenuated the proliferation and Th1 differentiation of diabeticogenic T cells in PLNs, which contributed to the protection against autoimmune diabetes in Ptpn22-transgenic NOD mice.

**Discussion**

Although the PTPN22 (1858T) variant is strongly associated with a variety of autoimmune diseases in humans, the biological
function and pathophysiology of Pep in autoimmunity is controversial. Previous study of Ptpn22ΔCD45E613R mice indicates that a Ptpn22 deficiency in cooperation with the CD45 E613R-mutant allele induces autoimmune disease (20). These results support the idea that Pep negatively regulates the development of autoimmune diseases. However, a recent study demonstrated that Ptpn22Δ−/− mice exhibited increased numbers of Tregs in the thymus and spleen and that these contributed to the reduced severity of experimental autoimmune encephalomyelitis (21), implying a different role of Pep in autoimmune pathogenesis. In this study, we first generated transgenic NOD mice overexpressing Pep in T cells under the control of the mouse distal Lck promoter and investigated the potential role of Pep in a “gain-of-function” manner. Our data demonstrate that transgenic expression of Pep in T cells attenuated the incidence of spontaneous autoimmune diabetes and severity of insulitis in a transgene dosage–dependent manner by downregulating T cell proliferation and Th1 differentiation in an islet Ag–driven manner.

In this study, we obtained two Ptpn22-transgenic lines as dLPC and dLPE. Although we used dLPC mice as one representative Ptpn22-transgenic line in our experiments, we observed a transgene dosage–dependent effect in the inhibition TCR-mediated cell responses and protection of diabetes. Our data reveal that the overall diabetic rate at 40 wk was significantly lower in dLPE mice than in dLPC mice (40 versus 60%; Fig. 2A). We observed a significant decrease in the number of spleens from dLPC mice compared with that from dLPC or nontransgenic mice (Supplemental Fig. 4A), indicating that additional expression of transgenic Pep significantly affects homeostasis of splenocytes in high (dLPE) expression. The numbers of pathogenic (CD4+/CD8− or CD8+IFN-γ+) T cells in pancreas infiltrates were significantly lower in both dLPC and dLPE female mice compared with their nontransgenic litters at 12–14 wk of age, respectively (Supplemental Fig. 4B). However, there was no significant difference of these cells in pancreas infiltrates between dLPC and dLPE mice, correlating with similar diabetic kinetics in these mice before 30 wk of age (Fig. 2A). Although there was no significant difference in the numbers of pancreas-infiltrating pathogenic T cells between dLPC and dLPE mice, the TCR-mediated cell proliferation (Supplemental Fig. 4C) and Th1 cell differentiation (Supplemental Fig. 4D) were decreased in a stepwise fashion from nontransgenic litters to dLPC mice to dLPE mice, indicating that the inhibition of pathogenic T cells is Pep dosage dependent. Interestingly, the percentages of CD4+Foxp3+ Tregs in thymus, spleen, PLN, and pancreas infiltrates did not differ significantly between dLPC mice and their nontransgenic litters (Supplemental Fig. 4E). Moreover, the immunosuppressive activity of Tregs from dLPC (Fig. 6C) or dLPC mice (Supplemental Fig. 4F) was comparable with that from their nontransgenic litters, indicating that the immunosuppressive function of Tregs is not affected by additional expression of transgenic Pep, no matter in low (dLPC) or high (dLPE) expression. In adoptive transfer of splenic cells in SCID, a significant decrease in diabetic incidence was observed in mice that received dLPC or dLPE splenocytes, compared with mice that received nontransgenic or dLPC splenocytes, suggesting that higher transgenic Pep expression mediates a better protection of diabetes in dLPC mice. Collectively, our data reveal that an additional expression of transgenic Pep attenuates pathogenic T cells in a Pep dosage–dependent manner further contributes to the differential protection of diabetes in dLPC and dLPE mice.

Previous studies showed that Ptpn22Δ−/− mice revealed an accumulation of effector/memory T cells in peripheral lymphoid organs and increased number of Tregs, from thymus to periphery, indicating that Pep deficiency affected the homeostasis and development of these cells (19, 21). In contrast to these reports, our data reveal that although an additional Pep was detected in transgenic thymocytes and peripheral T cells (Fig. 1E), the numbers and percentages of effector/memory T cells (Fig. 3F, 3G) or Tregs (Fig. 6A) were similar in the thymus and different lymphoid organs in 12- to 14-wk-old transgenic NOD mice and their control litters. The percentages of those cells were also similar in transgenic mice and control litters at ages > 6 mo (Supplemental Fig. 1), in contrast to age-matched Ptpn22Δ−/− mice, which show an obvious accumulation of effector/memory T cells in peripheral lymphoid organs (19). Collectively, our results demonstrate that transgenic expression of additional Pep in a T cell–specific manner does not interfere with the systemic development of effector/memory T cells or Tregs in NOD mice. Although transgenic Pep did not interfere with the systemic development of T cells in NOD mice, we observed a significant decrease in the numbers of CD4+/CD8+ T cells, IFN-γ–producing T cells, and CD4+/Foxp3+ T cells in pancreas infiltrates of dLPC mice at 12- to 14-wk-old (Fig. 4B, 4C, 4E). Our data reveal that transgenic Pep attenuated T cell proliferation in vitro (Fig. 5A–C) and in vivo (Fig. 6D), suggesting that Pep overexpression–mediated reduction of T cell proliferation contributes to the decreased infiltration of T cells in pancreas (Fig. 4B). In addition to TCR-mediated cell proliferation, we observed a decreased expression of CXCR3, which is critical to the trafficking of Th1 cells to the site of inflammation (28), on transgenic CD4+IFN-γ+ T cells compared with nontransgenic CD4+IFN-γ+ T cells after 3 d culture in Th1-differentiated condition (Supplemental Fig. 2). These results suggest that an additional expression of transgenic Pep attenuates the induction of CXCR3 during Th1 cell differentiation, which may further interfere with the migration of converted Th1 cells to pancreas. A previous study (44) has reported that CXCR3 signaling induces Lck-dependent phosphorylation of Zap70 on Tyr319 and treatment of Syk/Zap70 inhibitor piceatannol inhibits CXCR3-mediated T cell chemotaxis. Moreover, a recent study (45) demonstrated that Pep negatively regulates the migration of T cells by dephosphorylating G protein–coupled receptor kinase 2 that critical to the degradation or recycling of chemokine receptors. We therefore propose that an additional expression of transgenic Pep affects the signaling transduction and/or stability of chemokine receptors, further interfering with the chemotaxis of T cells. In the future, more studies are needed to investigate these hypotheses.

In our study, the activation status of TCR proximal molecules, Zap70 and Lck, and calcium flux was decreased in transgenic lymphocytes (Fig. 7A, 7C), a finding that is consistent with those of previous reports that Pep is a negative regulator of T cell activation (17, 18, 20). We first demonstrated that additional expression of transgenic Pep attenuated TCR-mediated activation of JNK and p38 (Fig. 7B). Consistent with several reports that the MAPK pathway positively regulates Th1 cell differentiation (34–36), our data suggest that Pep overexpression–mediated downregulation of MAPK pathway (Fig. 7B) contributes to the decrease in the percentage of IFN-γ–producing cells in differentiated T cells (Fig. 5D, 5E), subsequently inhibiting the diabetogenic process in transgenic NOD mice. In adoptive transfer experiments, we demonstrated further that transgenic Pep attenuated T cell proliferation and Th1 cell differentiation in an islet Ag–specific manner (Fig. 8D, 8E). Collectively, our results suggest that modulation of the TCR threshold by Pep overexpression preferentially affects
T cell proliferation and differentiation but is unlikely to interfere with the development and homeostasis of effector/memory or Treg.

Several studies have demonstrated that the perturbation of TCR signaling leads to a variety of defects in development, migration, or suppressive function of Tregs (32, 46, 47). We found that transgenic expression of Pep attenuated TCR-signaling–induced ERK phosphorylation in Tregs (Fig. 6B, upper panel); however, the transgene did not interfere with the development, differentiation, and suppressive activity of these cells (Fig. 6A, 6C, 6E, 6F). A very recent study (48) of mutant CD3ζ-chain knockin mice indicated that attenuation of TCR proximal signaling disproportionately impairs downstream signaling responses and subsequently favors Foxp3 expression and Treg lineage commitment. Consistent with this result, in our study, additional expression of transgenic Pep attenuated TCR proximal signaling and downstream Akt and Foxo1 activation in T cells (Fig. 7D). However, in contrast to their finding that the development and suppressive function of Tregs were significantly increased in mutant CD3ζ-chain knockin mice, the percentage, differentiation, and immunosuppression of Tregs were not affected in Ptpn22-transgenic mice (Fig. 6A, 6C, 6E, 6F). One possible explanation for this discrepancy is a reduction in TCR-mediated calcium flux in Ptpn22-transgenic Tregs (Fig. 7C, lower panel). Calcium signaling is crucial for the development and function of Tregs (49). Studies of gene-mutated mice indicate that mutations of TCR signaling molecules such as Zap70 and Lat downregulate calcium flux and subsequently lead to the impairment of development and/or suppressive function of Tregs (47, 50).

We propose that additional expression of transgenic Pep attenuates TCR-mediated Akt, S6, and Foxo1 activation, which may favor the induction of Foxp3 expression. However, transgenic Pep simultaneously downregulates calcium flux upon TCR stimulation, which subsequently impairs the expression of Foxp3. Therefore, the potential induction of Foxp3 by attenuated Akt, downstream S6, and Foxo1 in Ptpn22-transgenic T cells may be compensated by decrease in calcium flux, the net effect of which is “unchanged” development and function of Tregs in transgenic NOD mice.

The phenomenon observed in Ptpn22-transgenic NOD mice raises the question why TCR-mediated calcium flux is attenuated in T cells of Ptpn22-transgenic mice but not in mutant CD3ζ-chain knockin mice. Consistent with previous reports that Pep overexpression attenuated TCR-mediated calcium flux in Jurkat cell lines (20) and Ptpn22−/− lymphocytes showed increased calcium flux (19, 21), our results suggest that Pep-mediated signaling, different from CD3ζ-chain, selectively attenuates TCR-mediated calcium flux in T cells. Collectively, our data demonstrate that transgenic expression of additional Pep in T cells attenuates TCR signaling in both effector T cells and Tregs. However, the different effects of TCR-mediated Akt activation on effector T cells and Tregs cause differences in the functions of these two cells. We propose that Ptpn22 is a “reduction-of-function” allele (29) because the phosphorylation of ERK is reduced equally in Ptpn22-transgenic effector T cells and Tregs (Fig. 6B), and additional expression of transgenic Pep attenuated the effector function of CD4+CD25+ T cells (Fig. 6C) to a greater extent than interfering with the function of Tregs (Fig. 6C). This selective effect of transgenic Pep on effector T cells and Tregs should have led to a better counterbalance between these two cells and thereby contributed to the protection from autoimmune diabetes in Ptpn22-transgenic NOD mice.

Our study expands further the current knowledge of increased Pep levels in TCR-mediated signaling pathway, development, homeostasis, and functions of counterregulatory effector T cells and Tregs in autoimmune diabetes that have not been explored previously. In the future, more studies will examine the effect of increased Pep expression level in different subsets of T cells and autoimmune diseases.

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Disclosures
The authors have no financial conflicts of interest.

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**Supplementary Figure 1.** Lymphocyte development in 36-wk-old Ptpn22 transgenic NOD mice. The percentages of effector/memory CD4+(CD44hiCD62Llo) and CD8+(CD8αCD44hiCD62Lhilo) T cells in spleens and in PLNs of normoglycemia female dLPC mice and control littermates at 36 wk of age were analyzed by flow cytometry. Data are representative of two independent experiments. The percentages of effector/memory T cells of two independent experiments are presented as the mean±SEM.
Supplementary Figure 2. Decrease in CXCR3 expression on in vitro differentiated Th1 cells with Ptpn22 transgene. Naive CD4+ T cells from normoglycemia female dLPC mice or control littermates were stimulated with plate-bound 5 μg/ml anti-CD3ε and 1 μg/ml anti-CD28 in the presence of 10 ng/ml IL-12, 5 ng/ml IL-2 and 10 μg/ml anti-IL-4. The percentage of CXCR3 in CD4+IFN-γ+ T cells was measured by flow cytometry. Data show here are two independent experiments.
Supplementary Figure 3. Attenuation of TCR-mediated Th1 differentiation in the presence of MAPK inhibitors. Naive CD4+ T cells from normoglycemia female dLPC mice or control littermates were stimulated with plate-bound 5 μg/ml anti-CD3ε and 1 μg/ml anti-CD28, 10 ng/ml IL-12, 5 ng/ml IL-2 and 10 μg/ml anti-IL-4 in the presence of DMSO or 10 μM SB203580 or 5 μM SP600125 for 3 days. The percentages of CD4+IFN-γ+ T cells were measured by flow cytometry. Data are representative of three independent experiments.
Supplementary Figure 4. Attenuation of TCR-mediated cell proliferation and Th1 differentiation is Pep dosage-dependent. (A) The absolute cell numbers of thymus, spleen and PLNs of normoglycemia female dLPC, dLPE mice and control littersmates at 12–14 wk of age were counted. *p<0.05, **p<0.01 (B) Decrease in number of pancreas-infiltrating pathogenic cells in Pten22 transgenic mice. The absolute cell number of pancreas-infiltrating CD4+IFN-γ+, CD8+IFN-γ+ T cells of normoglycemia nontransgenic or dLPC or dLPE female mice at 12–14 wk of age was counted based on flow cytometric analysis. *p<0.05, **p<0.01 (C) Attenuation of TCR-mediated cell proliferation in transgenic T cells. Splenocytes isolated from nontransgenic or dLPC or dLPE female mice at 8–10 wk of age were stimulated with the indicated amounts of soluble anti-CD3ε, soluble anti-CD28 and anti-CD28. The cell proliferation was measured by the incorporation of [methyl-3H]thymidine. Data are representative of three independent experiments, presented as the mean±SEM. **p<0.01, ***p<0.001 (D) Attenuation of Th1 differentiation in transgenic T cells. Naive CD4+ T cells from normoglycemia nontransgenic or dLPC or dLPE female mice were stimulated with plate-bound 5 μg/ml anti-CD3ε and 1 μg/ml anti-CD28 in the presence of 10 ng/ml IL-12, 5 ng/ml IL-2 and 10 μg/ml anti-IL-4. The percentages of CD4+IFN-γ+ T cells were measured by flow cytometry. Data are representative of three independent experiments. (E) Foxp3+ expression among gating CD4+ T cells (%) in thymus (n=3), spleens (n=5), PLNs (n=9) and pancreas infiltrates (n=8) of normoglycemia female dLPE mice and control littersmates at 12–14 wk of age was measured by flow cytometry. (F) Suppressive activity of dLPE transgenic Treg in vitro. Purified CD4+CD25+ Treg from female dLPE mice or control littersmates were cultured with CD4+CD25+ Tcon, from female control mice at indicated ratio in the presence of anti-CD3/CD28 Dynabeads in a ratio of 1:2 (beads to Tcon). The cell proliferation was measured by the incorporation of [methyl-3H]thymidine. Data are representative of two independent experiments, presented as the mean±SEM. (G) Diabetes incidence in NOD/SCID recipients transferred with splenocytes. Adoptive transfer of splenocytes from normoglycemia female nontransgenic or dLPC or dLPE mice at 12–14 wk of age to NOD/SCID female recipients by i.v. injection. Glycosuria of recipients was measured every second day. Diabetes was defined as glycosuria >500 mg/ml at two consecutive tests. Significance was evaluated by log-rank test. *p<0.05