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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

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\( Ptpn22 \) 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 (T\(_{\text{regs}}\)). 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-\( \gamma \)-producing T cells, and an increased ratio of CD4\(^+\)Foxp3\(^+\) T\(_{\text{regs}}\) to CD4\(^+\)IFN-\( \gamma \) or to CD8\(^+\)IFN-\( \gamma \) 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 T\(_{\text{regs}}\) or their suppressive activity. Adoptive transfer experiments showed that transgenic splenocytes exhibited attenuated diabetogenic 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 diabetogenic T cells but not in T\(_{\text{regs}}\). The Journal of Immunology, 2013, 191: 594–607.

**Type 1 diabetes (T1D)** is a T cell–mediated autoimmune disease characterized by selective destruction of insulin-producing \( \beta \) cells in the pancreas. A widely used animal model, the NOD mouse, spontaneously develops autoimmune diabetes resembling human T1D (1, 2). In these mice, autoreactive T cells are activated and differentiate into effector cells upon TCR engagement with peptides derived from \( \beta \) cell proteins and captured by APCs in pancreatic lymph nodes (PLNs) during developmental remodeling of the pancreas, eventually causing the destruction of insulin-producing \( \beta \) cells (3–5). Regulatory T cells (T\(_{\text{regs}}\)) play a pivotal role in maintaining peripheral tolerance and inhibiting the development of diabetes, and deficiency of this regulatory subset significantly exacerbates the disease process (6–9). Several strategies to manipulate T\(_{\text{regs}}\) can delay or cure autoimmune diabetes (10, 11), supporting the concept that the diabetogenic process is based on the counterbalancing net effect between effector T cell–triggered autoimmunity and T\(_{\text{reg}}\)-mediated tolerance.

A single nucleotide polymorphism (SNP) in \( PTPN22 \) (C1858T) leads to an amino acid (R620W) substitution in humans and was identified as a critical risk factor for many autoimmune diseases such as T1D (12), rheumatoid arthritis (13), and systemic lupus erythematosus (14). \( PTPN22 \) encodes lymphoid tyrosine phosphatase in humans, the ortholog of mouse PEST domain–enriched tyrosine phosphatase (Pep), and is expressed exclusively in hematopoietic cells (15). Pep contains a N-terminal catalytic domain and C-terminal PEST domain, comprises proline-, glutamic acid-, serine-, and threonine-rich motifs, and can interact with the Src homology 3 domain of C-terminal Src kinase (16). The Pep–C-terminal Src kinase complex cooperatively inhibits TCR signaling by dephosphorylating Src family kinases FynT (Y417), Lck (Y394), and Zap70 (Y319) (17, 18). The negative role of Pep in TCR-mediated signaling and responses was illustrated in vivo in a study of \( Ptpn22^{-/-} \) mice that exhibited increased effector/memory T cell proliferation and cytokine production upon TCR stimulation. Although \( Ptpn22^{-/-} \) mice showed an increase in the number of germinal centers and effector/memory T cells, and concentrations of serum IgG1, IgG2a, and IgE, they did not develop autoimmune disease (19), suggesting that more complicated regulatory mechanisms are involved in Pep-mediated immune tolerance.
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 autoimmune encephalomyelitis (21). The authors suggested that the increased CD25 expression and TCR-induced calcium flux observed in Ptpn22−/− thymic Tregs 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 infected 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/Syjuw mice (K4, D0, L1, and L-AI) 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 pBlueScript II vector. The pBlueScript II–Ptpn22 was digested with EcoRI and subcloned into the 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)F1 females. dLck–Ptpn22 transgenic mice were typed by PCR using P1 (5′–ATCAGGATGG-TTCGCTCCA-3′) and P2 (5′–CAAAGGGCATCTGTTGACAGAT-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/proteinase 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 insulinis 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 insulinis was scored blindly on sections stained with H&E, as described previously (23).

Purification of T cells

Splenocytes from female or male NOD mice were treated with Tris-buffered ammonium chloride to eliminate the erythrocytes, washed, and resuspended in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, and 10 mM HEPES (number No. 41966-029; Life Technologies, Grand Island, NY). Mouse T lymphocytes were isolated from splenocytes by positive selection with anti-CD90.2 or anti-CD4 (L3T4) magnetic beads using the MACS magnet system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity for isolated cells was >90%.

Flow cytometric analysis

Lymphocytes from spleens and PLNs were stained with fluorochrome-conjugated Abs specific for murine CD4 (RM4-5), CD45L, CD44 (IM7), Foxp3 (FJK-16s), CD25 (PC6.5), IFN-γ (XMG1.2), and IL-4 (11B11), which were purchased from eBioscience (San Diego, CA). Fluorochrome-conjugated Abs to mouse CD3 (17A2), CD8α (53-6.7), TCRβ (H57-597), CD19 (1D1), ERK1/2 (pT202/pY204), and TCRβV4 (KT4) were purchased from BD Biosciences (San Jose, CA). Fluorochrome-conjugated Abs to mouse CD5 (53-7.3) and CXCR3 (CXCR3-173) were purchased from BioLegend (San Diego, CA). Cells were analyzed using a FACSCalibur 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-CD3e (1 μg/ml; 145-2C11; BD 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; BD 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 Tyr 370 (2701), Lck (2752), Lck phosphorylated at Tyr 394 (2701), AKT (3734), AKT phosphorylated at S473 (3845), JNK2 (2037-1), JNK1/2/3 phosphorylated at Tyr 185 and Tyr 223 (2155-1), ERK (3739), ERK phosphorylated at Tyr204 (2219-1), S6 (2217), AKT phosphorylated at S473 (3845), JNK1 (2037), JNK phosphorylated at Tyr 183 and Tyr 185 (2235-1), p38 (1544-1), p38 phosphorylated at Thr 180 and Tyr 182 (1229-1) (Epitomics, Burlingame, CA); and β-actin (AC-15; Sigma-Aldrich). Abs to Pep were provided by Dr. S.-C. Miaw (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

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Cytotox/Cytoperm kit (eBioscience) and stained with surface markers and anti–phospho-ERK for 1 h. Data were collected on a FACSCalibur flow cytometer.

Calcium flux

Single-cell suspensions of lymphocytes were loaded with 4 μg Flu-o-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-o-4 AM–labeled T cells were stained with 10 μM 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 (eBioSciences) was added at 30 s to cross-link CD3, and the reaction proceeded for 10 min on a FACS Calibur flow cytometer. Intracellular calcium concentration was analyzed using the Fluo-4 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 × 10^5 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 [methyl-3H]thymidine (PerkinElmer, Shelton, CT) per well and harvested after 16–18 h. The plates were harvested onto UniFilter-96, GF/C microplate (PerkinElmer), and the incorporated [methyl-3H]thymidine was detected with a Packard TopCount Microplate Scintillation Counter. For cell division, splenocytes from 8- to 10-wk-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 ml cold complete RPMI 1640 medium, and the cells were washed once. The CFSE-labeled splenocytes were cultured in 96-well flat-bottom plates (5 × 10^5 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 cytometer.

T cell differentiation

CD4⁺CD26⁻CD25⁻ naive T cells were isolated using a CD4⁺CD26⁺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 2 μg/ml anti-IL-4) or induced Treg (Threg) conditions (TGF-β, 5 ng/ml; IL-2, 5 ng/ml) 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 cytometer.

CD4⁺CD25⁺ ICAM suppression assays

CD4⁺CD25⁺ Treg, 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. After 72 h, the cultured cells were pulsed with 1 μCi [methyl-3H] thymidine/well and analyzed after 16–18 h.

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.

Adoptive 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 naive 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⁺TCRF+ T cells were analyzed on a FACS Calibur flow cytometer. For analysis of the ability of CD4⁺CD25⁺ Treg, to suppress the development of diabetes, 5 × 10^5 splenocytes from normoglycemia female BDC2.5 mice were co transferred with 5 × 10^4 CD4⁺CD25⁺ Treg 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 autoimmune immunity is complex because of the simultaneous inhibition of TCR-mediated signaling in effector and Treg cells. 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 S'-terminus β-globin hypersensitivity site 4 insulator elements (Fig. 1A). Two transgenic founders were obtained and denoted as dLck–Ptpn22 C (dLPC) and dLck–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 dLck–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 incidence and severity 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 (CD4CD8, 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. 3A). Collectively, our results indicated that transgenic Pep did not affect the development of thymocytes in dLPC mice.
FIGURE 2. Reduction in the incidence of spontaneous diabetes and severity of insulitis in Ptpn22 transgenic NOD mice. (A) Reduction in the incidence of spontaneous diabetes in Ptpn22-transgenic NOD mice. Urine glucose concentrations of female dLPC or dLPE mice or control littermates were monitored weekly for spontaneous diabetes incidence. Diabetes was defined as glycosuria > 500 mg/dl at two consecutive tests. Significance was evaluated by log-rank test. (B) Reduction in the severity of insulitis in Ptpn22-transgenic NOD mice. The severity of insulitis was scored for the degree of mononuclear cell infiltration. Cumulative results in each group were determined from six normoglycemia female mice at 13–14 wk of age and at least 100 islets in total.

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 CD44^hiCD62L^hiCD4+ and CD44^loCD62L^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 mice revealed an accumulation of effector/memory T cells in peripheral lymphoid organs at ages > 6 mo (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^loCD62L^hi-^loCD8+ T cells in the spleen (Fig. 3F) and in PLNs of dLPC 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 pancreas 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+ Tregs are crucial to controlling pancreatic autoimmunity in situ and to maintaining immune tolerance in NOD mice. We next counted the number of CD4+Foxp3+ cells in pancreas infiltrates in these transgenic mice. Although there were fewer infiltrating CD4+Foxp3+ cells (Fig. 4E), the ratio of CD4+Foxp3+ Tregs 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 Tregs 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,
18). Although the role of Pep in vivo was illustrated in the study of Ptpn22 transgenic NOD 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+IFN-γ+ and CD8+IFN-γ+ T cells of normoglycemia female dLPC mice or control littersmates at 12–14 wk of age were analyzed by flow cytometry.

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 Tregs, have not been elucidated. To address this issue, we first analyzed the phosphorylation status of ERK in CD4+Foxp3+ 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 their 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 their nontransgenic littersmates, 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 further investigate 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 × 10^4 BDC2.5 splenocytes and 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.
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 394, respectively. The phosphorylation status of both Zap70 Y319 and Lck Y394 sites 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.

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 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 attenuated in transgenic lymphocytes (Fig. 7B). To investigate whether the decrease in MAPK activation is directly involved in decrease in IFN-γ-producing T cell differentiation, we purified naive CD4+ T cells (CD4+CD62L+CD25−) from dLPC mice or their nontransgenic littermates and cultured those cells in a Th1-differentiated condition in the presence or absence of inhibitors of p38 (SB203580) and JNK (SB600125) as indicated concentration. Our data reveal that the percentage of IFN-γ-producing T cells of nontransgenic mice was significantly decreased in the presence of p38 or JNK inhibitors (Supplemental Fig. 3), indicating that a decrease in MAPK activation negatively regulates Th1 cell differentiation, thus abrogating the difference in percentage of IFN-γ-producing cells between the dLPC and nontransgenic cells. However, no significant difference was observed in dLPC T cells treated with these two inhibitors (Supplemental Fig. 3, right panel), suggesting that transgenic Pep and MAPK inhibitors do not cause an additional inhibitory effect on Th1 cell differentiation at these indicated concentrations.

We next evaluated the TCR-induced calcium mobilization of T cells from dLPC mice. Our data reveal that the TCR-induced calcium flux was decreased in both transgenic CD4+CD25+ (Fig. 7C, upper panel) and CD4+CD25− (Fig. 7C, lower panel) T cells compared with their nontransgenic T cells, respectively. These results indicate that transgenic Pep downregulated TCR proximal molecules, the MAPK pathway, and calcium flux, which subsequently contributed to the attenuation of proliferation and differentiation of Th1 cells in NOD mice.

We found that transgenic expression of Pep in T cells attenuated TCR-mediated ERK phosphorylation in both effector T cells and Tregs (Fig. 6B); however, the downregulation of subsequent cell responses was observed only in effector T cells and not in Tregs in Ptpn22-transgenic NOD mice. We therefore investigated the mechanism underlying the different modulation of transgenic Pep.
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, 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-Ag7 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+Vβ4+ 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+Vβ4+ cells when encountering antigenic peptides in the context of the
NOD I-A<sup>β<sup>7</sup></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 double-transgenic mice into NOD/SCID recipients, and measured the proliferation index of CD4<sup>+</sup> V<sub>b</sub>4+ T cells in PLNs. Significance was evaluated by two-tailed Student unpaired t test. *p < 0.05. (E) Attenuation of Ptpn22-transgenic T cell differentiation in an islet Ag–driven manner in vivo. Naïve CD4<sup>+</sup> T cells of dLPC/BDC2.5 or BDC2.5 mice were transferred into NOD/SCID recipients. Five days later, PLN cells were isolated and stimulated with PMA and ionomycin in the presence of monensin for 4–5 h. The IFN-γ-secreting CD4<sup>+</sup>V<sub>b</sub>4+ T cells were analyzed by flow cytometry. The graph (right panel) is a summary of three different experiments, showing the percentage of CD4<sup>+</sup>V<sub>b</sub>4+IFN-γ<sup>+</sup> T cells in PLNs. Significance was evaluated by two-tailed Student unpaired t test. *p < 0.05.

Notably, the division of dLPC/BDC2.5 double-transgenic CD4<sup>+</sup> Vβ<sup>4+</sup> T cells was significantly inhibited compared with that of BDC2.5 single-transgenic T cells in PLNs of recipients (Fig. 8E, right lower panel), suggesting that transgenic Pep attenuated the islet Ag–driven proliferation of autoreactive T cells in situ and prevented the diabetogenic process in NOD mice.

We also adoptively transferred naïve CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup>) from dLPC/BDC2.5 double-transgenic NOD mice into NOD/SCID recipients and measured the percentage of CD4<sup>+</sup>Vβ<sup>4+</sup>IFN-γ<sup>-</sup> T cells in PLNs. The percentage of IFN-γ-producing CD4<sup>+</sup>Vβ<sup>4+</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 diabetogenic 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 \textit{Ptpn22} \textsuperscript{−/−} \textit{CD45E613R} mice indicates that a \textit{Ptpn22} deficiency in cooperation with the \textit{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 \textit{Ptpn22} \textsuperscript{−/−} mice exhibited increased numbers of T\textsubscript{reg}, 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 \textit{Ptpn22}-transgenic lines as dLPC and dLPE. Although we used dLPC mice as one representative \textit{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 cell 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\textsuperscript{+} and CD8\textsuperscript{+}IFN-\gamma) T cells in pancreas infiltrates were significantly lower in both dLPC and dLPE female mice compared with their nontransgenic littermates 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 littermates to dLPC mice to dLPE mice, indicating that the inhibition of pathogenic T cells is Pep dosage dependent. Interestingly, the percentages of CD4\textsuperscript{+}Foxp3\textsuperscript{+} T\textsubscript{reg}, in thymus, spleen, PLN, and pancreas infiltrates did not differ significantly between dLPC mice and their nontransgenic littermates (Supplemental Fig. 4E). Moreover, the immunosuppressive activity of T\textsubscript{reg}, from dLPC (Fig. 6C) or dLPE mice (Supplemental Fig. 4F) was comparable with that from their nontransgenic littermates, indicating that the immunosuppressive function of T\textsubscript{reg}, 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 \textit{Ptpn22} \textsuperscript{−/−} mice revealed an accumulation of effector/memory T cells in peripheral lymphoid organs and increased number of T\textsubscript{reg}, 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 T\textsubscript{reg}, (Fig. 6A) were similar in the thymus and different lymphoid organs in 12- to 14-wk-old transgenic NOD mice and their control littermates. The percentages of those cells were also similar in transgenic mice and control littermates at ages > 6 mo (Supplemental Fig. 1), in contrast to age-matched \textit{Ptpn22} \textsuperscript{−/−} 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 T\textsubscript{reg}, 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-\gamma–producing T cells, and CD4\textsuperscript{+}Foxp3\textsuperscript{+} 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. 5D), 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\textsuperscript{+}IFN-\gamma T cells compared with nontransgenic CD4\textsuperscript{+}IFN-\gamma 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 (45) has reported that CXCR3 signaling induces Lck-dependent phosphorylation of Zap70 on Tyr\textsuperscript{519} and treatment of Syk/Zap70 inhibitor picetannol 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-\gamma–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 Tregs.

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 Pep22 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 attenuates the effector function of CD4+CD25− Tconv (Fig. 6D) 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

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