Supporting Information

Pang et al. 10.1073/pnas.1322009111

SI Materials and Methods

ELISAs. These assays were performed as previously described (1). ELISA plates (MaxiSorp Nunc; Thermo Fisher Scientific) were coated with 5 mg/mL candidate dipeptidyl peptidase 4 (DPP4) peptides in carbonate buffer overnight at 4 °C. The peptides were conjugated to the carrier protein BSA (Peptide Institute). After blocking with PBS containing 3% skim milk, the sera were diluted from 100- to 325,000-fold in blocking buffer. After incubation (overnight at 4 °C) and subsequent washing, the plates were incubated with horseradish peroxidase (HRP)-conjugated antibodies specific for mouse IgG (GE Healthcare) for 3 h at room temperature. For the IgG subclass determination assay, anti-mouse IgG subclass-specific HRP-conjugated antibodies (IgG1, IgG2b, and IgG2c) were used. After washing with PBS, color was developed with the peroxidase chromogenic substrate 3,3′-5,5′-tetramethyl benzidine (Sigma-Aldrich), and the reaction was halted using 0.5 N sulfuric acid. Absorbance was detected using a microplate reader (Bio-Rad) at 450 nm. The half-maximal antibody titer was determined according to the highest value in the dilution range of each sample.

Western Blot Analysis. Recombinant DPP4 (R&D Systems) and the BSA–DPP4 conjugate were separated electrophoretically by SDS/PAGE and blotted onto poly(vinylidene difluoride) membranes (Millipore). The blots were incubated with sera from mice immunized with the candidate DPP4 peptides or keyhole limpet hemocyanin (KLH) or with commercially available anti-DPP4 antibody [CD26 (T-19); sc-7044; Santa Cruz Biotechnology]. For evaluation of DPP4 and positive-control CD20 expression, mouse splenocyte lysate was collected in RIPA lysis buffer (Millipore) and then subjected to SDS/PAGE. After electrophoresis, the membrane was incubated with anti-mouse DPP4 antibody (R&D Systems) or anti-mouse CD20 antibody (eBioscience), respectively. After incubation with HRP-conjugated antibodies specific for mouse IgG (GE Healthcare), chemiluminescence signal was detected with a FujiFilm LAS 1000 camera and analyzed with MultiGauge version 3.2 software.

Pancreatic Insulin Content and Histological Analysis. The whole pancreas, free of fat and other nonpancreatic tissue, was rapidly isolated as described previously (2). For the insulin content assay, the isolated pancreas was frozen in liquid nitrogen and stored at −80 °C until use. The pancreas was thawed and homogenized with an acid/ethanol solution (0.18 M HCl, 70% ethanol). The homogenates were stored overnight at 4 °C and then centrifuged (10,000 × g, 20 min, 4 °C), and the supernatant was prepared for the insulin assay (Morinaga). The concentration of total protein was measured using a protein assay kit from Bio-Rad. Pancreatic insulin content was normalized to total protein content in each sample. For the immunohistochemistry (IHC) analysis, the isolated pancreas was fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sliced in 4-μm sections. The sections were reacted with a primary antibody [guinea pig anti-insulin antibody (1:200 dilution), Dako; rabbit anti-Ki76 antibody (1:500 dilution), Thermo Fisher Scientific] and secondary antibody (biotinylated anti-guinea pig IgG or biotinylated anti-rabbit IgG; Vector Laboratories). For insulin immunostaining, sections were counterstained with hematoxylin and mounted for microscopic observation (FSX100; Olympus). For the histological examination, the jejunum, liver, and kidney were dissected, fixed in 4% paraformaldehyde overnight, embedded in paraffin, sliced in 4-μm sections, and stained with hematoxylin and eosin. To measure the β-cell proliferation ratio in each mouse ~65 islets were counted, and the β-cell proliferation per islet was calculated by dividing the Ki67-positive β-cell number by the total islet number.

T-Cell Proliferation Assay. T-cell proliferation was evaluated as described previously (3). Immunized mice were euthanized at the end of the experiment, and splenocytes (10^6 cells per well) were stimulated with RPMI medium 1640 (Life Technologies) containing DPP4 peptide, KLH, and phytohemagglutinin (PHA) (Wako) at a concentration of 10 μg/mL. After incubation for 48 h at 37 °C, 1 μCi [^{3}H]thymidine (PerkinElmer) was added to each well and incubated for 8 h. [^{3}H]Thymidine uptake was determined using a MicroBeta 1450 TriLux scintillation counter (Wallac).

Antibody-Dependent Cell-Mediated Cytotoxicity Assay. Antibody-dependent cell-mediated cytotoxicity (ADCC) was evaluated using a CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega). Peripheral blood mononuclear cells (PBMCs) were isolated from C57BL/6J mice using Ficoll-Paque Plus (GE Healthcare) as effector cells. The target cells (splenocytes from C57BL/6J mice) in 96-well plates at 10^4 cells per well were treated with a positive-control antibody (anti-mouse CD20 functional grade-purified; eBioscience) or purified IgG antibody from E3-immunized or KLH control mice at various concentrations from 0.01 to 10 μg/mL at 37 °C for 30 min. After incubation, PBMCs were added to the individual wells at effector-to-target cell ratios of 10:1 and 1:1. The plates were then incubated for 4 h at 37 °C, and the release of lactate dehydrogenase in the supernatants was analyzed using a Spectra Fluor at 490 nm (SH-9000; Corona Electric). Cell death was evaluated as the percentage of total cell lysis.

1. Gardiner SM, et al. (2000) Active immunization with angiotensin I peptide analogue vaccines selectively reduces the pressor effects of exogenous angiotensin I in conscious rats. *Br J Pharmacol* 129(6):1178–1182.
2. Furuta Y, et al. (2010) Chronic administration of DSP-7238, a novel, potent, specific and substrate-selective DPP IV inhibitor, improves glycaemic control and beta-cell damage in diabetic mice. *Diabetes Obes Metab* 12(5):421–430.
3. Yip HC, et al. (1999) Adjuvant-guided type-1 and type-2 immunity: Infectious/noninfectious dichotomy defines the class of response. *J Immunol* 162(7):3942–3949.
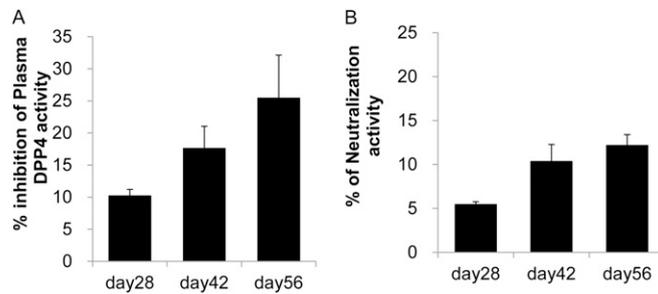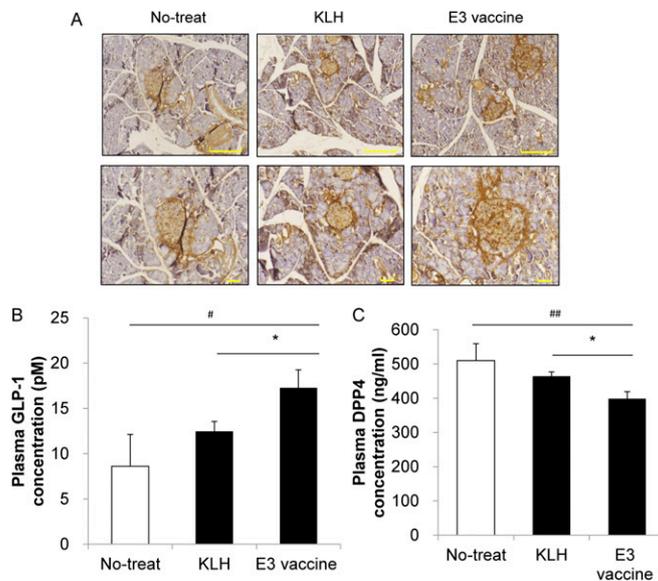
Fig. S1. Evaluation of DPP4 inhibition activity and neutralization activity in a time-dependent manner in normal diet-fed mice. Percent inhibition of plasma DPP4 activity (*A*) and neutralization activity (*B*) was measured on days 28, 42, and 56 after E3 vaccine (20 μg per mouse) immunization in normal diet-fed mice. All data are expressed as the mean ± SEM.
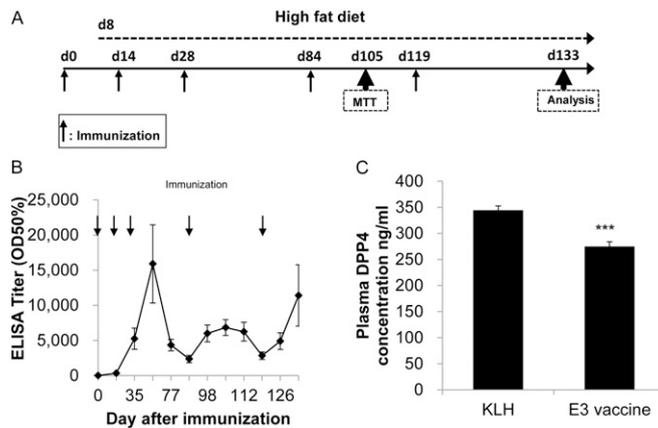
Fig. S2. Evaluation of titer and DPP4 concentration in high-fat diet (HFD) mice. (*A*) The time course of the experiment is shown for the HFD-induced insulin resistance mouse model. Male C57BL/6J mice were immunized with the E3 vaccine on days 0, 14, 28, 84, and 119. The HFD was administered from day 8, and the meal tolerance test (MTT) was performed on day 105. (*B*) In HFD mice immunized with the E3 vaccine (20 μg per mouse) on days 0, 14, 28, 84, and 119 (arrows), the titer of anti-DPP4 antibody was determined at the indicated times. The titer is expressed as the serum dilution that exhibited half-maximal binding (optical density: OD50%). (*C*) Plasma DPP4 concentration was measured on day 133 after E3 (20 μg per mouse) or KLH-only immunization. The data are expressed as the mean ± SEM. ***$P < 0.001$ versus the KLH control group.

Fig. S3. Evaluation of the DPP4 vaccine in HFD mice. (*A* and *B*) In high-fat diet mice immunized with the E3 vaccine or KLH, body weight (*A*) and food consumption (*B*) were measured weekly. (*C*) A time-course experiment is shown for HFD-induced diabetic model mice. Male C57BL/6J mice were fed an HFD for 5 wk before the first E3 vaccination (20 μg per mouse). The mice were immunized with the E3 vaccine or KLH only on days 0, 14, 28, and 63. (*D*) In therapeutic model mice, DPP4 activity was evaluated on day 56 in mice immunized with the E3 vaccine or KLH by ELISA. The data are expressed as the mean ± SEM. *$P <$ 0.05 versus the KLH control group.

Fig. S4. Evaluation of E3 vaccine effectiveness on the onset of diabetes in db/db mice. (*A*) In overnight-fasted young db/db mice (6 wk of age; *n* = 5), the MTT [2 g carbohydrate (CHO)/kg] was performed on day 28 after E3 vaccine (20 μg per mouse) or KLH-only preimmunization. The plasma glucose level was measured at 0, 30, 60, 90, and 120 min after oral food administration. (*B*) The area under the curve (AUC) (time 0–120 min) was assessed during the MTT. (*C* and *D*) At 15 min after the meal challenge (2 g CHO/kg), plasma insulin level and pancreatic insulin content were determined by ELISA on days 28 and 35, respectively. All data are expressed as the mean ± SEM. *$P <$ 0.05 versus the KLH control group; #$P <$ 0.05 and ##$P <$ 0.01 versus the nontreated group.

Fig. S5. Glucagon-like peptide 1 (GLP-1) and DPP4 concentration in plasma in db/db mice. (*A*) Pancreatic sections from untreated and KLH- and E3-immunized mice were obtained on day 35 for insulin IHC. [Scale bars, 200 μm (*Upper*) and 50 μm (*Lower*).] (*B*) At 10 min after meal challenge (2 g CHO/kg), the plasma intact GLP-1 concentration was determined on day 42 in db/db mice immunized with the E3 vaccine or KLH. (*C*) The plasma DPP4 concentration was examined on day 42 by ELISA. The data are expressed as the mean ± SEM. *$P < 0.05$ versus the KLH control group; #$P < 0.05$ and ##$P < 0.01$ versus the nontreated group.
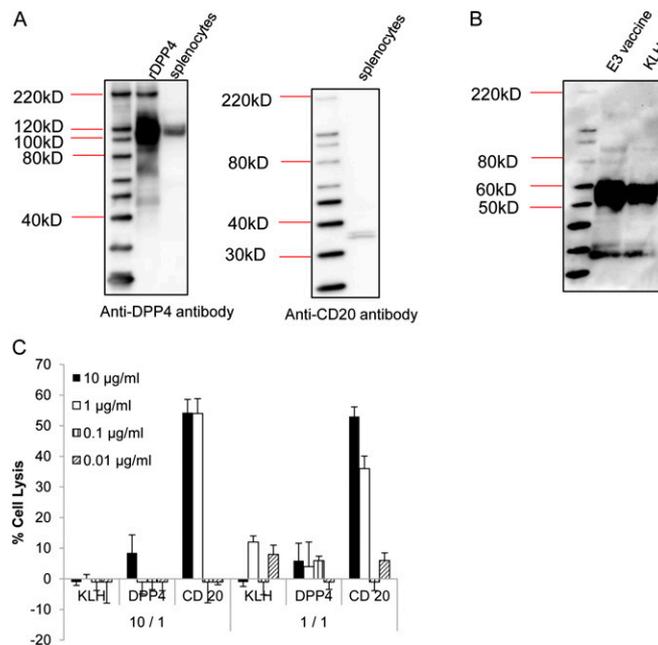
Fig. S6. Evaluation of cell damage using the ADCC assay. (*A*) Evaluation of DPP4 and CD20 expression in splenocytes was confirmed by Western blot using anti-DPP4 antibody or anti-CD20 antibody. "Splenocytes" indicates cell lysis from splenocytes; "rDPP4" indicates recombinant mouse DPP4 as a positive control. (*B*) The purification of IgG from mice treated with E3 peptide or KLH only was confirmed by Western blot with HRP-conjugated anti-mouse IgG. (*C*) The ADCC activity of anti-DPP4 antibody was assessed using PBMCs as an effector cell, and the ratios of effector cells and target cells were 10:1 and 1:1. The target cells (splenocytes; $n = 3$) were treated with anti-CD20 antibody (positive-control antibody: CD20) or purified IgG antibody from mice immunized with E3 peptide (DPP4) or KLH only at various concentrations from 0.01 to 10 μg/mL. Cell death was evaluated as the percentage of cell lysis (total cell death). The data are expressed as the mean ± SEM.
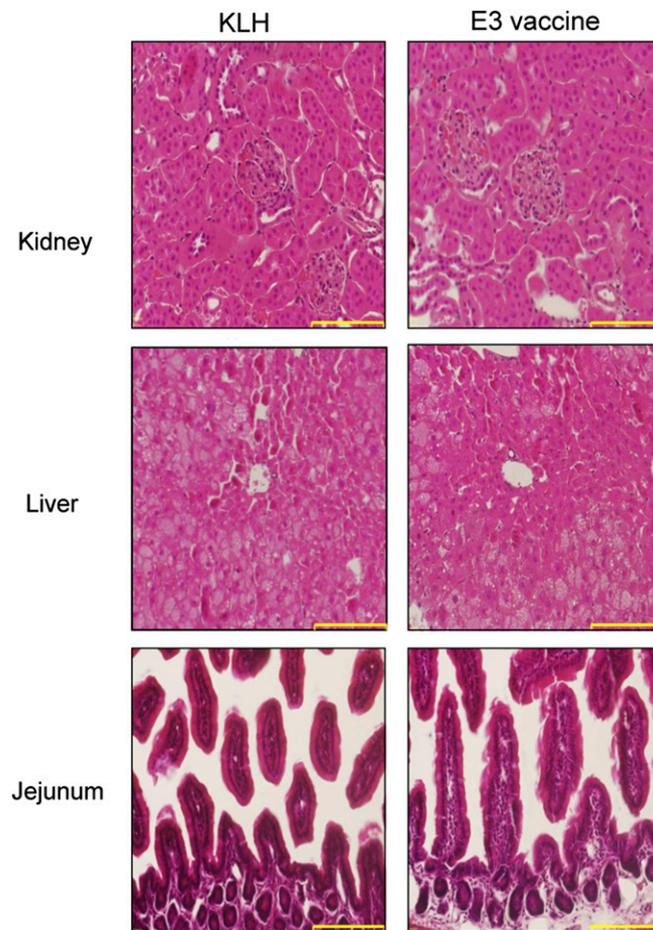
Fig. S7. Evaluation of tissue damage after E3 immunization. The kidney (*Top*), liver (*Middle*), and jejunum (*Bottom*) from mice immunized with E3 vaccine (20 μg per mouse) or KLH only were stained with H&E on day 70. (Scale bars, 50 μm.)