Reversal of New-Onset Diabetes through Modulating Inflammation and Stimulating β-Cell Replication in Nonobese Diabetic Mice by a Dipeptidyl Peptidase IV Inhibitor

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Inhibition of dipeptidyl peptidase IV (DPP-IV) activity by NVP-DPP728, a DPP-IV inhibitor, improves the therapeutic efficacy of glucagon-like peptide-1 (GLP-1). CD26 is a membrane-associated glycoprotein with DPP-IV activity and is expressed on lymphocytes. We investigated the effect of NVP-DPP728 on reversing new-onset diabetes in nonobese diabetic (NOD) mice and modulating the inflammatory response and stimulating β-cell regeneration. New-onset diabetic NOD mice were treated with NVP-DPP728 for 2, 4, and 6 wk. Blood glucose level was monitored. Regulatory T cells in thymus and secondary lymph nodes, TGF-β1 and GLP-1 in plasma, and the insulin content in the pancreas were measured. Immunostaining for insulin and bromodeoxyuridine (BrdU) were performed. The correlation of β-cell replication with inflammation was determined. In NVP-DPP728-treated NOD mice, diabetes could be reversed in 57, 74, and 73% of mice after 2, 4, and 6 wk treatment, respectively. Insulitis was reduced and the percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells was increased in treated NOD mice with remission. Plasma TGF-β1 and GLP-1, the insulin content, and both $insulin^+$ and $BrdU^+$ β-cells in pancreas were also significantly increased. No significant correlations were found between numbers of both $insulin^+$ and $BrdU^+$ β-cells in islets and β-cell area or islets with different insulitis score in NOD mice with remission of diabetes. In conclusion, NVP-DPP728 treatment can reverse new-onset diabetes in NOD mice by reducing insulitis, increasing $CD4^+CD25^+FoxP3^+$ regulatory T cells, and stimulating β-cell replication. β-Cell replication is not associated with the degree of inflammation in NVP-DPP728-treated NOD mice. **(*Endocrinology* 151: 3049–3060, 2010)**

Development of type 1 diabetes is due to an imbalance between β-cell regeneration and β-cell destruction. Therefore, preventing β-cell destruction and promoting β-cell regeneration would have a significant therapeutic impact. At the clinical onset of type 1 diabetes, human patients still have a certain percentage of their β-cell mass remaining. If autoimmunity against β cells were controlled in these patients, diabetes could be reversed by increasing the β-cell mass sufficiently to restore normoglycemia.

Glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP) are two potent glucose-dependent insulinotropic peptide hormones that stimulate insulin secretion, promote β-cell regeneration, and prevent β-cell apoptosis (1). Our earlier study showed that exendin-4, a GLP-1 analog, can enhance the reversal of diabetes

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Abbreviations: BrdU, Bromodeoxyuridine; DPP-IV, inhibition of dipeptidyl peptidase IV; GIP, glucose-dependent insulinotropic polypeptide; GLP, glucagon-like peptide; NOD, nonobese diabetic.

through stimulating β-cell replication in nonobese diabetic (NOD) mice after restoring self-tolerance (2). However, GLP-1 and GIP have extremely short half-lives after secretion because of rapid degradation by dipeptidyl peptidase IV (DPP-IV). DPP-IV is a serine-type protease that cleaves N-terminal dipeptides from polypeptides containing proline or alanine as the penultimate amino acid. Inhibition of DPP-IV activity could potentially improve the therapeutic effectiveness of GLP-1 and GIP (3).

CD26 is a membrane-associated peptidase with DPP-IV activity in its extracellular domain and is expressed in lymphocytes (4, 5). It contributes to the regulation of development, maturation, and migration of T cells, cytokine production, and T cell-dependent antibody production (6). Inhibition of surface DPP-IV not only suppresses T cell proliferation and Th1 cytokine productions but also stimulates TGF-β1 secretion (7, 8). DPP-IV inhibitor treatment prevents and ameliorates experimental allergic encephalomyelitis in mice through up-regulating TGF-β1 (9).

Using CD26-deficient mice, a recent study showed that TGF-β1-mediated control of inflammation and autoimmunity through CD26/DPP-IV (10). TGF-β1 plays a critical role in ameliorating autoimmune diabetes (11–13). It protects against diabetes via regulation of expansion of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells (14). TGF-β signaling is also required for the function of regulatory T cells (12, 15). Regulatory T cells are indispensable for maintaining self-tolerance and preventing autoimmune diseases (16). Thus, targeting CD26/DPP-IV may have beneficial effects in human patients with type 1 diabetes by stimulating β-cell replication through potentiation of the incretin response and ameliorating the autoimmune response.

NVP-DPP728 is an orally active and selective DPP-IV inhibitor that inhibits degradation of GLP-1 (17, 18) and improves glucose tolerance and preserves islet function (19). Its effect is directly due to the inhibition of plasma DPP-IV activity (20–22). In this study, we investigated the effects of NVP-DPP728 in NOD mice on reversing diabetes, modulating inflammation, and stimulating β-cell regeneration. Although it has been shown that DPP-IV-inhibitor treatment stimulates TGF-β1 secretion, whether DPP-IV inhibitors up-regulate $CD4^+CD25^+FoxP3^+$ regulatory T cells has not been previously addressed. We therefore also investigated the effects of NVP-DPP728 treatment on increasing $CD4^+CD25^+FoxP3^+$ regulatory T cells in diabetic NOD mice.

Materials and Methods

Mice

Female NOD/Lt mice ($H\text{-}2^{g7}$) and male NOD.scid mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were housed in pathogen-free animal facilities at the University of Minnesota. The animal use protocol was approved by the Institutional Animal Care and Use Committee at our university. Spontaneous diabetes in NOD mice was monitored after the age of 12 wk, using a Glucometer Elite blood glucose monitor (Bayer, Tarrytown, NY). New-onset diabetes was diagnosed when the nonfasting blood glucose level was greater than 250 mg/dl on at least two consecutive measurements. Remission was defined as the blood glucose levels consistently remaining at less than 200 mg/dl.

Treatment

NVP-DPP728 was obtained from IQsynthesis (St. Louis, MO). New-onset diabetic NOD mice, which had the nonfasting blood glucose level greater than 250 mg/dl on at least two consecutive measurements, were given 30 mg/kg NVP-DPP728 orally, twice daily, for 2, 4, or 6 wk. All diabetic mice also received insulin at 1 U/d. Insulin treatment was stopped if the blood glucose level was less than 200 mg/kg.

Flow cytometric analysis

NOD mice, with or without NVP-DPP728 treatment, were randomly selected to detect $CD4^+CD25^+FoxP3^+$ regulatory T cells. Single-cell suspensions were prepared and incubated with 1 μg anti-CD4, anti-CD25, and anti-FoxP3 conjugated with fluorescein isothiocyanate, phycoerythrin, or allophycocyanin (BD PharMingen, San Diego, CA) and then washed in PBS-2% fetal calf serum. Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

In vivo adoptive transfer

Spleens were harvested from NVP-DPP728-treated diabetic NOD mice with remission. Lymphocytes were isolated from the spleen and were resuspended in PBS at 1×10^7/ml. A total of 0.5 ml of lymphocytes was injected in the tail vein of each NOD.scid mouse. Lymphocytes from the spleen of diabetic NOD mouse also were obtained and were injected into each NOD.scid mouse as a control group. The onset of diabetes was monitored by measuring the blood glucose.

Insulin content

Pancreases were collected and minced in acidified ethanol (75% ethanol, 0.1 N HCl) until tissues became homogenates. Samples were incubated for 24 h at 4 C to extract insulin from the tissue. The extracts were centrifuged and the supernatant was resuspended in 1 ml H_2O and serially diluted in buffer of 10 mM Tris (pH 7.4) and 1 mM EDTA. Insulin was measured using insulin ELISA kits for mouse insulin (Mercodia, Uppsala, Sweden).

Plasma TGF-β1 assay

Blood samples were obtained by cardiac puncture. Plasma samples were stored at −20 C until analyzed. Plasma TGF-β1 were measured using a mouse TGF-β1 ELISA kit (R&D Systems Inc., Minneapolis, MN).

Plasma active (7-36 amide) GLP-1 assay

Diabetic NOD mice were treated with 30 mg/kg NVP-DPP728. Blood samples were collected after half-hour treatment, and after 12–16 h treatment. Plasma active GLP-1 was measured by using active GLP-1 ELISA kit (Millipore, Billerica,

MA) that is specifically for the GLP-1(7-36) amide form, the majority of circulating biologically active GLP-1.

Pathology

Pancreases of NOD mice, with or without NVP-DPP728 treatment, were harvested and fixed with 4% paraformaldehyde and embedded in paraffin. The pancreas sections were stained with hematoxylin and eosin for histological scoring of insulitis. Insulitis scoring was done by the pathologist (T.D.O.) who was blinded as to treatment group. The level of leukocyte infiltration in the islets was scored as follows: 1) well-preserved and completely intact islet without leukocyte infiltrate, 2) well-preserved islet with periislet infiltrate or few leukocytes intraislet, 3) islet partially disrupted by leukocyte infiltrate, and 4) islet extensively disrupted and destroyed with only single endocrine cells discernible in the infiltrate.

Immunofluorescence

To label replicated β-cells, we injected bromodeoxyuridine (BrdU; 100 mg/kg · d, Sigma-Aldrich, St. Louis, MO) into NOD mice for 4 wk, starting from the day of NVP-DPP728 treatment. Double-immunofluorescence staining for insulin and BrdU was done on formalin-fixed and paraffin-embedded pancreatic sections. For insulin labeling, sections were first deparaffinized, rehydrated, and incubated with guinea pig antiswine insulin (Dako A/S, Glostrup, Denmark). Next, sections were incubated with fluorescein isothiocyanate-conjugated, goat anti-guinea pig Ig. For BrdU labeling, sections were incubated with biotinylated anti-BrdU (BrdU kit; BD Biosciences, San Jose, CA). Sections were then incubated with streptavidin-AlexaFluor-594. Finally, nuclear staining was done using TOPRO-3 (Molecular Probes, Eugene, OR). All $BrdU^+$ and $BrdU^-$ nuclei in $insulin^+$ β cells were counted. The percentage of replicated β-cells was calculated as the mean ± SD of $insulin^+$ and $BrdU^+$ β-cells over the total of $insulin^+$ β-cells in islets.

Statistics

The significance of differences between groups was determined by the Kaplan-Meier analysis, one-way ANOVA, and a Student's t test. Data were shown as mean ± SD. A value of $P < 0.05$ was considered statistically significant.

Results

NVP-DPP728 treatment reversed new-onset diabetes in NOD mice

Remission of diabetes was not achieved in the insulin therapy-only group. All NOD mice in the control group

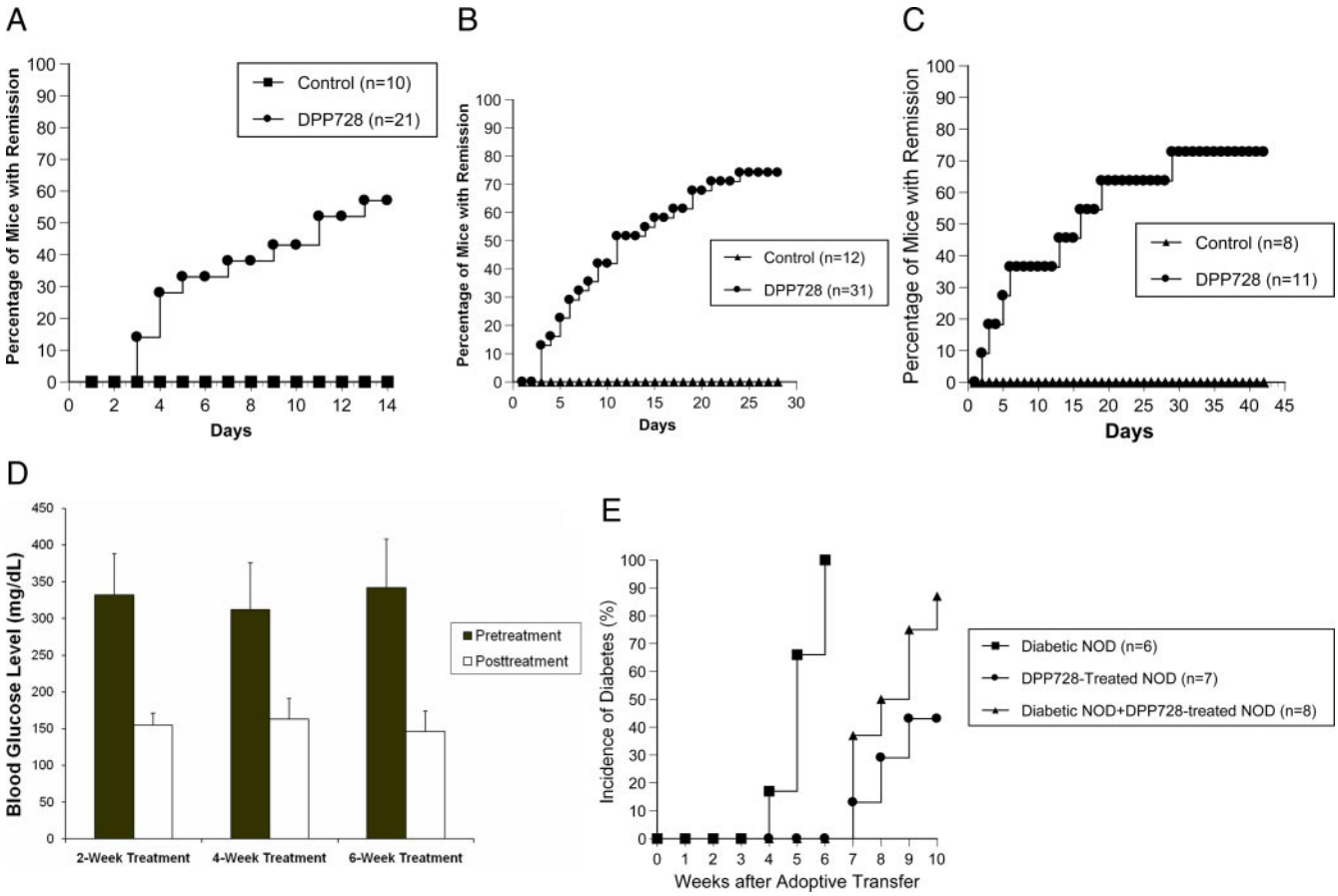

FIG. 1. NVP-DPP728 treatment reversed new-onset diabetes in NOD mice. A, Incidence of remission in 2-wk-treated NOD mice. B, Incidence of remission in 4-wk-treated NOD mice. C, Incidence of remission in 6-wk-treated NOD mice. D, Blood glucose levels in treated diabetic NOD mice with remission before and after 2, 4, and 6 wk treatment. E, Incidence of diabetes in NOD.scid mice after adoptive transfer of lymphocytes from control diabetic NOD mice, from NVP-DPP728-treated NOD mice with remission, and after coadoptive transfer of lymphocytes from control diabetic NOD mice with lymphocytes from NVP-DPP728-treated NOD mice with remission.

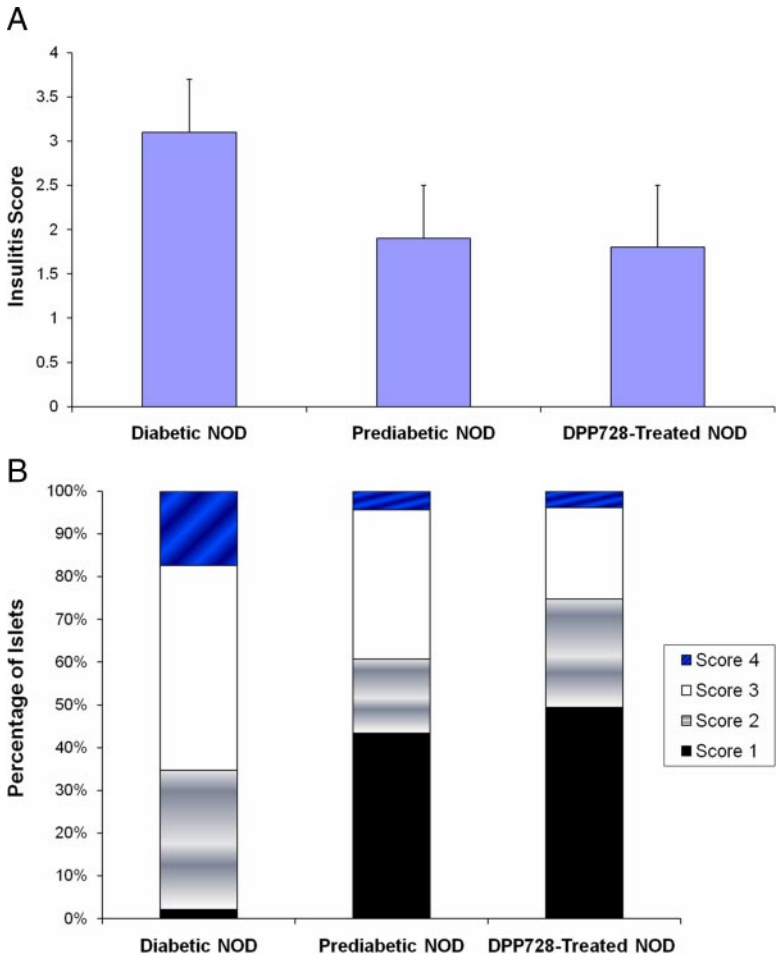

FIG. 2. A, Insulitis score in control diabetic NOD mice, prediabetic NOD mice, and 4-wk NVP-DPP728-treated NOD mice. B, Percentage of islets in the pancreas of diabetic, control NOD mice, prediabetic NOD mice, and 4-wk NVP-DPP728-treated NOD with different insulitis scores.

became progressively more hyperglycemic. However, in the group treated with NVP-DPP728 for 2 wk, 11 of 20 (57%) diabetic NOD mice became normoglycemic at 2 wk (Fig. 1A; $P < 0.01$). The mean blood glucose level of these 11 diabetic NOD mice at the time of initiation of treatment was 332 ± 56 mg/dl but decreased to 155 ± 16 mg/dl at 2 wk ($P < 0.01$). To determine whether short-term NVP-DPP728 treatment could induce long-term remission, we continued to monitor blood glucose in these 11 mice with remission after discontinuing NVP-DPP728 treatment. The mean blood glucose level among these mice increased to 341 ± 211 mg/dl, and only two of 11 mice remained normoglycemia at 6 wk after treatment. Thus, our data showed that NVP-DPP728 treatment reversed new-onset diabetes in NOD mice. However, long-term remission could not be induced in most NOD mice by 2 wk treatment.

To determine whether the rate of remission could be further increased, we extended our treatment to 4 and 6 wk. Diabetic NOD mice in the control groups became progressively more hyperglycemic. They either died spontaneously or were euthanized due to significant debilitation. In contrast, 23 of 31 diabetic NOD mice treated with NVP-DPP728 for 4 wk (74%) became normoglycemic at 4 wk (Fig. 1B; $P < 0.01$); and eight of 11 (73%) mice treated with NVP-DPP728 for 6 wk became normoglycemic at 6 wk after initiation of treatment (Fig. 1C; $P < 0.01$). The blood glucose levels of 23 NOD mice with remission after 4 wk treatment and eight NOD mice with remission after 6 wk treatment were significantly decreased, compared with the levels at the time of initiation of treatment (Fig. 1D).

To determine whether long-term remission was induced after extended 6 wk treatment, we randomly monitored four mice after stopping treatment. Hyperglycemia returned between 7 and 42 d (28 ± 15 d) after treatment. Thus, extended NVP-DPP728 treatment could increase the rate of reversal of new-onset diabetes in NOD mice. However, diabetes gradually returned after stopping treatment.

Significant decrease of body weight was not found in eight mice with remission after 6 wk treatment. The mean body weight was 23.8 ± 1.6 g before treatment and 22.3 ± 2.0 g after treatment.

Immunoregulation mediated reversing new-onset diabetes in NVP-DPP728-treated NOD mice

To determine whether immunoregulation plays a role in NVP-DPP728-treated NOD mice with remission, we adoptively transferred lymphocytes from NVP-DPP728-treated NOD mice with remission. Lymphocytes from NVP-DPP728-treated NOD mice could transfer diabetes to some NOD.scid mice. All six NOD.scid mice that received lymphocytes from diabetic NOD mice developed diabetes at 6 wk after adoptive transfer (Fig. 1E). However, the development of diabetes was significantly delayed compared with induction of diabetes by the transfer of lymphocytes from control diabetic NOD mice. Diabetes did not develop at 6 wk ($n = 7$; $P < 0.05$), and in only three of seven NOD.scid mice at 10 wk, after receiving the same amount of lymphocytes from NVP-DPP728-treated NOD mice. The onset of diabetes in NOD.scid mice was also significantly delayed after cotransfer of lymphocytes from diabetic NOD mice with lymphocytes from NVP-DPP728-treated NOD mice. Diabetes did not develop in

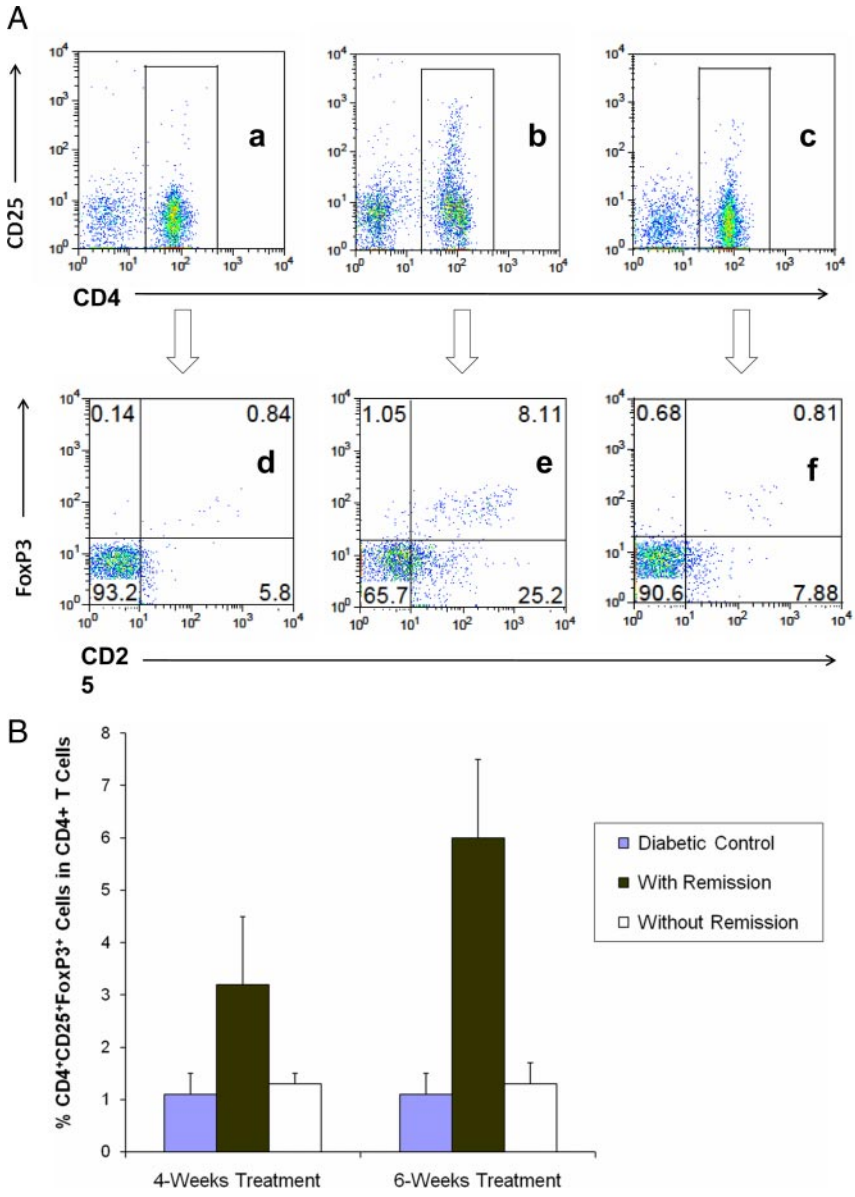

FIG. 3. NVP-DPP728 treatment increased $CD4^+CD25^+FoxP3^+$ regulatory T cells in thymus of diabetic NOD mice with remission. A, $CD4^+CD25^+$ T cells in thymus of control diabetic NOD mouse (a), 6-wk-treated NOD mouse with remission (b), and treated NOD mouse with relapse after stopping treatment (c); $CD25^+FoxP3^+$ cells in the total $CD4^+$ T cells in the thymus of control diabetic NOD mouse (d), 6-wk-treated NOD mouse with remission (e), and treated NOD mouse with relapse after stopping treatment (f). B, Percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in the total $CD4^+$ T cells in the thymus of control diabetic NOD mice and 4- and 6-wk-treated NOD mice, with and without remission.

NOD.scid mice at 6 wk (n = 8; $P < 0.05$), after cotransfer of 1×10^7 lymphocytes from diabetic NOD mice with 5×10^6 lymphocytes from NVP-DPP728-treated NOD mice. However, diabetes did develop in seven of eight NOD.scid mice at 10 wk.

NVP-DPP728 treatment reduced insulitis in diabetic NOD mice

To determine whether insulitis was reduced by NVP-DPP728 treatment in these mice with remission, we evaluated the degree of insulitis in the pancreas of 12 NVP-DPP728-treated NOD mice. The mean insulitis score was significantly lower in prediabetic NOD mice and diabetic NOD mice treated with NVP-DPP728 for 4 wk than in diabetic NOD mice (Fig. 2A). The mean insulitis score was 1.9 ± 0.6 in prediabetic NOD mice, 3.1 ± 0.6 in diabetic NOD mice, and 1.8 ± 0.7 in diabetic NOD mice treated with NVP-DPP728 for 4 wk (n = 12 *vs.* diabetic NOD mice, $P < 0.01$). Mice treated with NVP-DPP728 had the highest percentage of intact islet, with a score of 1 (Fig. 2B).

NVP-DPP728 treatment increased $CD4^+CD25^+FoxP3^+$ regulatory T cells in diabetic NOD mice with remission

Because naturally occurring $CD4^+CD25^+FoxP3^+$ T cells develop in the thymus as a normal process of T cell ontogenesis, we measured the percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the thymus of diabetic NOD mice treated with NVP-DPP728 for 4 and 6 wk. The percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the thymus of treated NOD mice with remission significantly increased after 4 and 6 wk treatment, compared with untreated control NOD mice and treated NOD mice without remission (Fig. 3, A and B). After 4 wk of treatment, the mean percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the thymus was 1.1 ± 0.4% in control diabetic NOD mice without treatment (n = 9), 1.3 ± 0.2% in diabetic NOD mice without remission (n = 4), and 3.2 ± 1.3% in diabetic NOD mice with remission (n = 12; $P < 0.01$).

Extending treatment for 6 wk significantly increased $CD4^+CD25^+FoxP3^+$ regulatory T cells in the thymus of diabetic NOD mice with remission (Fig. 3B, $P < 0.01$). After 6 wk of treatment, the mean percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the thymus 6.0 ± 1.5% in diabetic NOD mice with remission (n = 4 *vs.* 4 wk treatment; $P < 0.01$), and 1.3 ± 0.4% in diabetic NOD mice without remission (n = 3; $P > 0.05$). However, a significant decrease was observed in

NOD mice with a relapse after stopping treatment (*vs.* 6 wk treatment, $P < 0.01$). There was no significant change of $CD4^+$ T cells in the thymus (data not shown). Thus, NVP-DPP728 treatment could increase the induction of naturally occurring regulatory T cells in the thymus, and continuing treatment is required for maintaining the increase. Relapse in mice with remission was associated with the decrease of naturally occurring regulatory T cells in the thymus.

The percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the pancreatic lymph nodes also was significantly increased after 4 wk treatment (Fig. 4A). The mean percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the pancreatic lymph nodes was 6.1 ± 1.6% in control diabetic NOD mice without treatment (n = 9) and 10.2 ± 2.5% in diabetic NOD mice with remission after 4 wk treatment (n = 12; $P < 0.05$). However, no significant increase of these cells was found in neck lymph nodes, spleen, and peripheral blood after 4 wk treatment ($P > 0.05$).

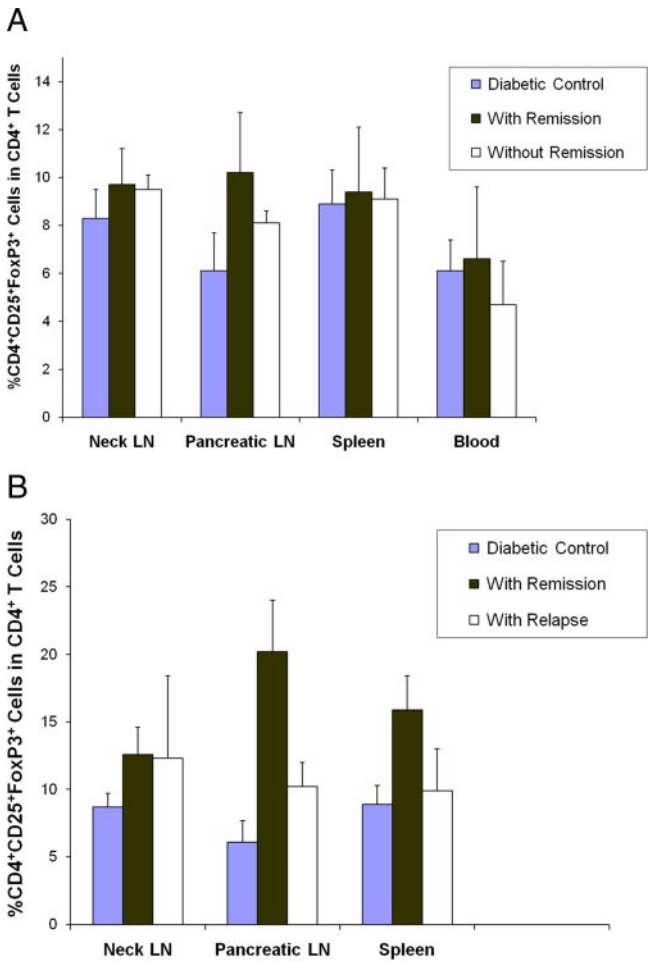

FIG. 4. NVP-DPP728 treatment increased $CD4^+CD25^+FoxP3^+$ regulatory T cells in secondary lymph organs of diabetic NOD mice with remission. A, Percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells from the neck lymph nodes, pancreatic lymph nodes, spleen, and peripheral blood in control diabetic NOD mice and treated diabetic NOD mice with and without remission, after 4 wk of treatment. B, Percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells from the neck lymph nodes, pancreatic lymph nodes, and spleen of control diabetic NOD mice, 6-wk-treated NOD mice with remission, and 6-wk-treated NOD mice with relapse after stopping treatment.

When treatment was extended to 6 wk, we detected a significant increase in $CD4^+CD25^+FoxP3^+$ regulatory T cells, not only in the pancreatic lymph nodes but also in the neck lymph nodes and the spleen in NOD mice with remission (Fig. 4B). Although a significant increase was found in both pancreatic and neck lymph nodes, the level was significantly higher in the pancreatic lymph nodes than the neck lymph nodes ($P < 0.01$). The percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells was 20.2 ± 3.8% in the pancreatic lymph nodes (n = 4 *vs.* diabetes control; $P < 0.01$), 12.6 ± 2.0% in the neck lymph nodes (n = 4 *vs.* diabetes control; $P < 0.05$), and 15.9 ± 2.5% in the spleen (n = 4 *vs.* diabetes control; $P < 0.01$). Our data indicate that more regulatory T cells in the draining lymph nodes of pancreas. However, the level of these cells significantly declined in pancreatic lymph nodes and spleen in NOD mice with normoglycemia but relapsed after stopping treatment, compared with mice with remission after 6 wk treatment. After stopping treatment, the level was 10.2 ± 1.8% in pancreatic lymph nodes ($P < 0.01$) and 9.9 ± 3.1% in the spleen of NOD mice with normoglycemia but returned to hyperglycemia after stopping treatment (n = 4 *vs.* with remission after 6 wk treatment; $P < 0.01$).

Because regulatory T cells migrating to the inflammatory site is required for optimal control of inflammation (23), we also measured the $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in the pancreas of treated NOD mice. Although the percentage of $CD4^+$ T cells in total pancreas of 6 wk NVP-DPP728-treated NOD mice with remission was significantly lower than in control diabetic NOD mice (Fig. 5A, $P < 0.05$), significant increases of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells were found in the pancreas of NVP-DPP728-treated NOD mice with remission, compared with the control diabetic NOD mice (Fig. 5B). However, percentage of these cells significantly declined in pancreases of NOD mice with normoglycemia but relapsed after stopping treatment, compared with the mice with remission after 6 wk treatment. The percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells in pancreases was 20.9 ± 4.7% in NVP-DPP728-treated NOD mice with remission (n = 4), 7.5 ± 1.4% in control diabetic NOD mice (n = 6; $P < 0.01$), and 5.9 ± 1.9% in treated NOD mice with a relapse after stopping treatment (n = 4; $P < 0.01$).

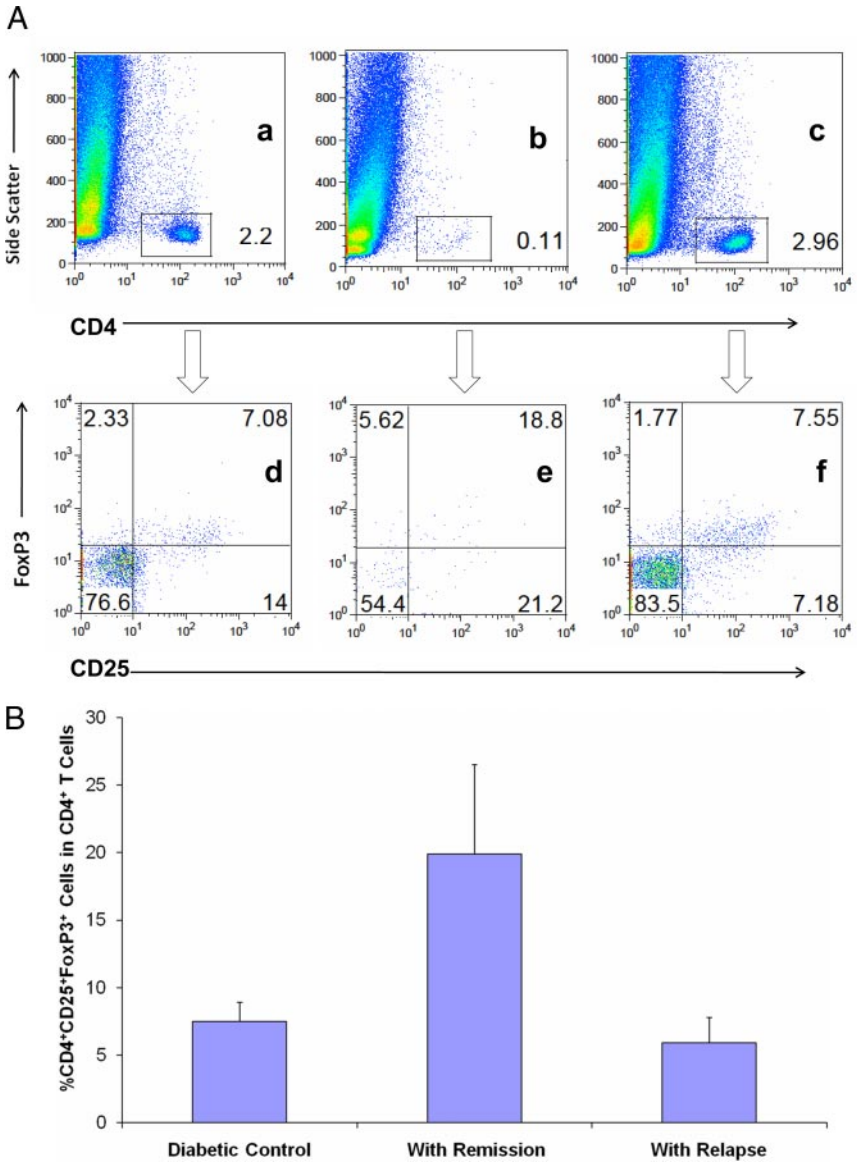

FIG. 5. NVP-DPP728 treatment increased $CD4^+CD25^+FoxP3^+$ regulatory T cells in the pancreas of diabetic NOD mice with remission. A, $CD4^+$ T cells in the pancreas of control diabetic NOD mouse (a), 6-wk-treated NOD mouse with remission (b), and treated NOD mouse with relapse after stopping treatment (c); $CD25^+FoxP3^+$ cells in the total $CD4^+$ T cells in the pancreas of control diabetic NOD mouse (d), 6-wk-treated NOD mouse with remission (e), and treated NOD mouse with relapse after stopping treatment (f). B, Percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells from the pancreas of control diabetic NOD mice, 6-wk-treated NOD mice with remission, and treated NOD mice with relapse after stopping treatment.

NVP-DPP728 treatment increased TGF-β1 production in the plasma of NOD mice with remission

Because inhibition of surface DPP-IV stimulates TGF-β secretion (7, 8), we determined plasma TGF-β1 levels in NVP-DPP728-treated NOD mice. After 4 wk treatment, the plasma TGF-β1 levels in NVP-DPP728-treated NOD mice with remission were significantly increased compared with control mice (Fig. 6A). However, a significant increase was not found in NVP-DPP728-treated NOD mice without remission. TGF-β1 plasma level was 8.1 ± 1.2 ng/ml in untreated diabetic NOD mice (n = 6), 21.0 ± 10.6 ng/ml in NVP-DPP728-treated NOD mice with remission (n = 8 *vs.* control diabetic mice; $P < 0.05$), and 10.2 ± 2.3 ng/ml in NVP-DPP728-treated NOD mice without remission (n = 5, $P > 0.05$).

NVP-DPP728 treatment increased insulin content in diabetic NOD mice with remission

We measured the insulin content in whole pancreases of 4 wk NVP-DPP728-treated diabetic NOD mice with remission, in prediabetic NOD mice, diabetic NOD mice, and NOD.scid mice. The insulin content in NOD mice treated with NVP-DPP728 was significantly increased, as compared with the control diabetic NOD mice (Fig. 6B). Insulin content was significantly higher in NVP-DPP728-treated NOD mice and prediabetic NOD mice than diabetic NOD mice ($P < 0.01$). However, the insulin content of control NOD.scid mice was significantly higher than in the prediabetic NOD mice and NVP-DPP728-treated NOD mice with normoglycemia ($P < 0.01$).

NVP-DPP728 treatment increased plasma active GLP-1 levels in diabetic NOD mice

To determine whether NVP-DPP728 treatment increases the plasma active GLP-1 levels, we measured the plasma active GLP-1 concentrations in NOD mice, at 0.5 and 12–16 h after treatment. The plasma active GLP-1 levels were significantly increased at 0.5 h but not 12–16 h after treatment, compared with the control diabetic NOD mice (Fig. 6C). The plasma GLP-1 was 3.4 ± 0.5 pM in control diabetic NOD mice (n = 6), 17.3 ± 9.3 pM in NVP-DPP728-treated mice at 0.5 h after treatment (n = 6 *vs.* control; $P < 0.01$), and 4.9 ± 1.9 pM in NVP-DPP728-treated mice at 12–16 h after treatment (n = 16 *vs.* control; $P > 0.05$).

NVP-DPP728-stimulated β-cell replication in NOD mice

Because there are few β-cells remaining in diabetic NOD mice after 4 wk, we used prediabetic NOD mice as

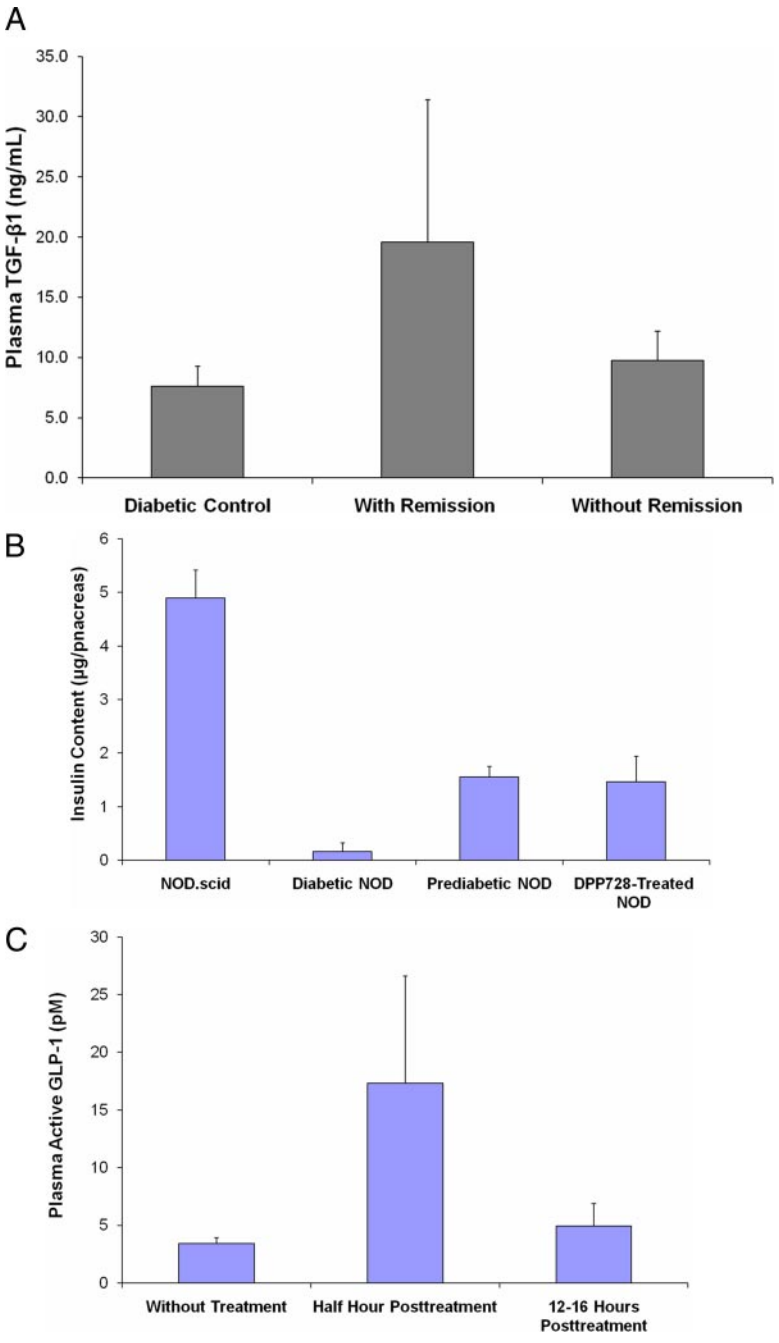

FIG. 6. A, TGF-β1 plasma level in NVP-DPP728-treated NOD mice after 4 wk treatment. B, The insulin content of whole pancreas in control NOD.scid mice, diabetic NOD mice, prediabetic NOD mice, and NVP-DPP728-treated diabetic NOD mice with remission. C, Active GLP-1 in plasma of diabetic NOD mice and of NVP-DPP728-treated diabetic NOD mice.

controls and also treated them BrdU daily. Those mice with normoglycemia were killed 4 wk later, and their pancreases were processed for double-immunofluorescent staining for insulin and BrdU. Insulin$^+$ and BrdU$^+$ β-cells could be detected in islets of NOD mice (Fig. 7A). Diabetic mice with remission after treated with NVP-DPP728 for 4 wk had significantly more insulin$^+$ and BrdU$^+$ β-cells than control NOD mice (Fig. 7B). The mean percentage of insulin$^+$ and BrdU$^+$ β-cells was 2.0 $\pm$ 2.3% in prediabetic NOD mice (n = 6). In contrast, diabetic mice treated with NVP-DPP728 for 4 wk had significantly more replicated β-cells, with a mean percentage of 24.2 $\pm$ 7.8% insulin$^+$ and BrdU$^+$ β-cells in these pancreases (n = 10; $P < 0.01$).

β-Cell replication did not correlate with inflammation in NVP-DPP728-treated NOD mice

To determine whether β-cell replication correlates with inflammation, we analyzed insulitis and insulin$^+$ and BrdU$^+$ β-cells in the pancreases of NVP-DPP728-treated mice with remission (n = 10). No significant difference was found between insulitis score and the percentage of insulin$^+$ and BrdU$^+$ β-cells (Fig. 8, A and B). The mean percentage of insulin$^+$ and BrdU$^+$ β-cells was 22.1 $\pm$ 9.1% in islets with insulitis score 1; 24.8 $\pm$ 6.5% in islets with insulitis score 2; 26.7 $\pm$ 5.5% in islets with insulitis score 3; and 21.6 $\pm$ 9.5% in insulitis score 4 ($P > 0.05$). We also assessed for correlations between the percentage of insulin$^+$ and BrdU$^+$ β-cells with an estimate of remaining β-cell area. We divided islets of 10 mice into three different groups: group 1 was less than one third of islet area with insulin$^+$ β-cells; group 2 was greater than one third but less than two thirds islet area with insulin$^+$ β-cells; and group 3 was greater than two thirds of islet area with insulin$^+$ β-cells. No significant correlations were found between the percentage of insulin$^+$ and BrdU$^+$ β-cells in islets and remnant β-cell area estimate in NOD mice with normoglycemia (Fig. 8C). The mean percentage of insulin$^+$ and BrdU$^+$ β-cells is 23.5 $\pm$ 9.0% in islets with less than one third insulin$^+$ β-cell area; 23.6 $\pm$ 6.3% in islets with greater than one third and less than two thirds insulin$^+$ β-cell area; and 24.9 $\pm$ 7.8% insulin$^+$ β-cell area ($P > 0.05$).

Discussion

Our results showed that NVP-DPP728 treatment could reverse new-onset diabetes in NOD mice by reducing insulitis, increasing CD4$^+$CD25$^+$FoxP3$^+$ regulatory T

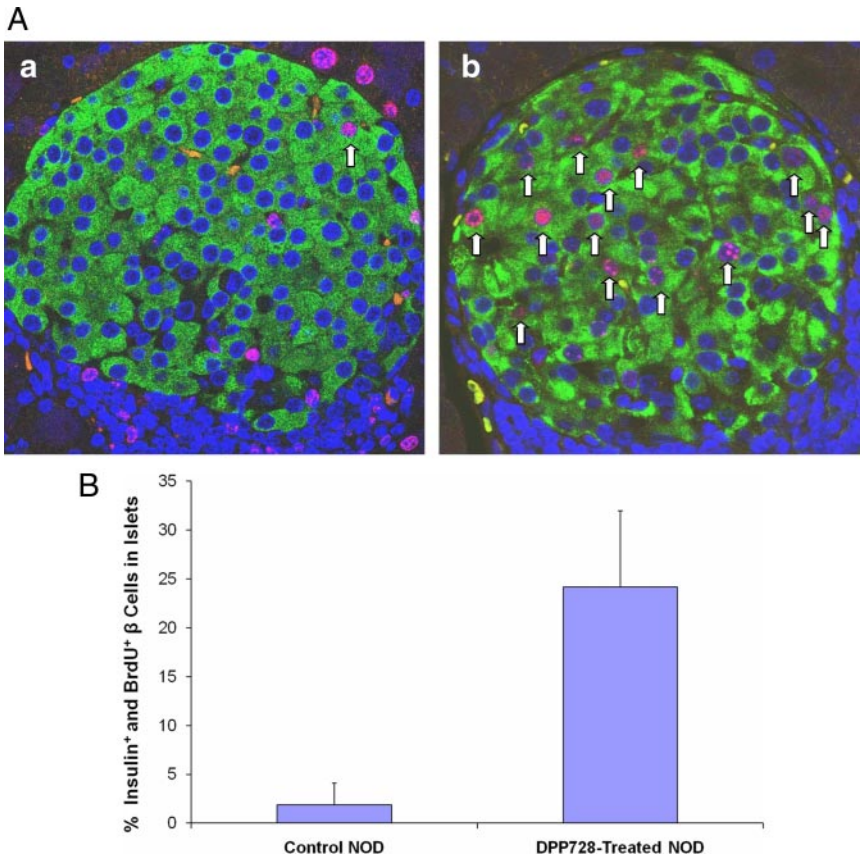

FIG. 7. NVP-DPP728 stimulated β-cell replication in NOD mice. A, Double immunofluorescent staining for insulin (*green color*) and BrdU (*red color*) in islets of prediabetic NOD mouse given only BrdU daily for 4 wk (a) and in the islet from NOD mice with remission after 4 wk treatment (b). Insulin$^+$ and BrdU$^+$ β cells in the islet (*arrowheads*) of control, prediabetic NOD mouse, and the islet from 4-wk-treated NOD mice can be seen. B, Percentage of insulin$^+$ and BrdU$^+$ β-cells in the islets of prediabetic NOD mouse given only BrdU daily for 4 wk and in the islet from NOD mice with remission after 4 wk treatment.

cells, and stimulating β-cell replication. Although an earlier study reported that treating new-onset diabetic NOD mice with a different DPP-IV inhibitor at 10 mg/kg · d restored normoglycemia in 38% of mice (24), the mechanisms were not addressed. The reason for the lower success rate in their study may be due to the low dose of the DPP-IV inhibitor they used and the treatment regimen. Because of the short half-life of NVP-DPP728 (20), we treated diabetic NOD mice twice daily. It is possible that some of variation in efficacy in our studies is related to the differences in actual drug consumption and the short half-life of NVP-DPP728. Sitagliptin, a Food and Drug Administration-approved DPP-IV inhibitor, could prolong syngeneic islet graft survival in diabetic NOD mice. Sitagliptin pretreatment decreased insulitis and suppressed the recurrent autoimmunity partially through decreasing the homing of CD4$^+$ T cells into islets (25). It will be interesting to directly compare the effect of NVP-DPP728 on the immune system with sitagliptin and other DPP-IV inhibitors with a long half-life.

The imbalance between effector T cells and regulatory T cells is a key factor that leads to the onset of diabetes in NOD mice. Our data show that immune regulation plays a critical role in NVP-DPP728-treated NOD mice with normoglycemia. Regulatory T cells can reverse new-onset diabetes in NOD mice (26). In our early study, we found that up-regulating CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells in the pancreatic lymph nodes was responsible for ameliorating autoimmunity in NOD mice (2). One source of regulatory T cells is naturally occurring CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells that developed in the thymus. We found that the frequency of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells was significantly increased in the thymus of diabetic NOD mice, with remission at 4 and 6 wk after NVP-DPP728 treatment. Whether these regulatory T cells are polyclonal or islet autoantigen specific is unknown.

A significant increase of these cells also was found in the pancreatic lymph nodes but not in neck lymph nodes, spleen, and peripheral blood after 4 wk treatment. In NVP-DPP728-treated NOD mice without remission, a significant increase of regulatory T cells was not detected. After 6 wk treatment, the frequency of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells was significantly increased in not only the thymus but also the neck lymph nodes, pancreatic lymph nodes, and spleen. The increase of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells in the thymus indicates that naturally occurring regulatory T cells were up-regulated by NVP-DPP728 treatment and suggests that these cells migrated to the secondary lymphoid organs. However, it seems more CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells migrated to the draining lymph nodes of the inflammatory site. In these 6-wk-treated mice, the frequency of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells in pancreatic lymph nodes was significantly higher than in the neck lymph nodes. Although the frequency of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells was increased in the thymus and secondary lymph organs after 6 wk treatment, a significant decrease of these cells was found in mice that had relapsed after stopping treatment. Our data demonstrate that up-regulation of regulatory T cells is associated with remission. Although NVP-DPP728 treatment could reverse new-onset diabetes in NOD mice, long-term remission was not induced in most NOD mice by short-term

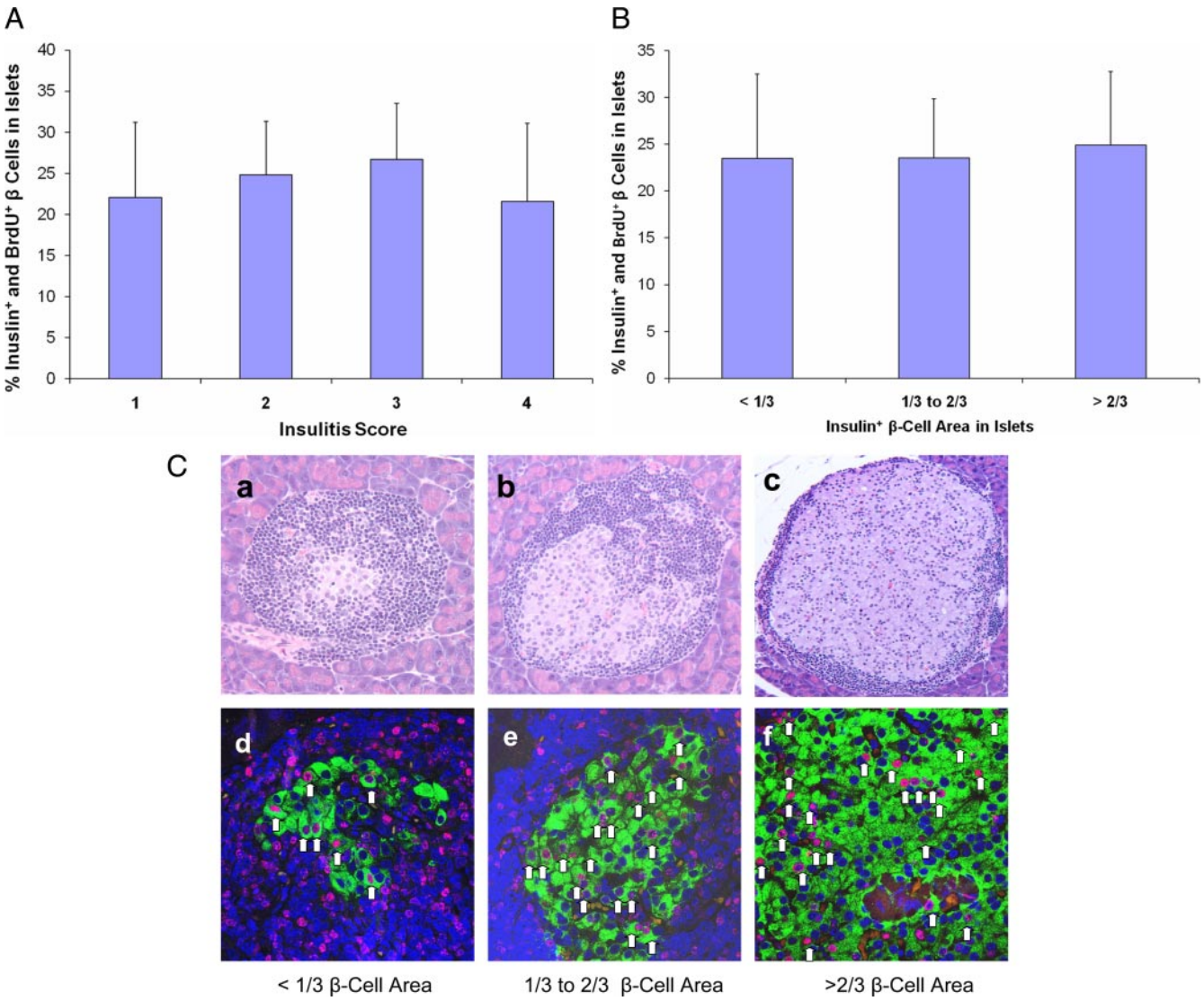

FIG. 8. β-Cell replication does not correlate with inflammation in NVP-DPP-728-treated NOD mice. A, Percentage of insulin$^+$ and BrdU$^+$ β cells in the islets of different insulitis score in the pancreas of NOD mice with remission after 4 wk treatment. B, Hematoxylin-and-eosin-stained islets with less than one third β-cell area (a), one third to two thirds β-cell area (b), and greater than two thirds β-cell area (c); and double-immunofluorescent insulin- (*green color*) and BrdU (*red color*)-stained islets with less than one third β-cell area (d), one third to two thirds β-cell area (e), and greater than two thirds β-cell area (f) in the pancreas from NOD mice with remission after 4 wk treatment. Insulin$^+$ and BrdU$^+$ β-cells in the islet (*arrowheads*) with different β-cell area from 4-wk-treated NOD mice can be seen. C, Percentage of insulin$^+$ and BrdU$^+$ β-cells in the islets with less than one third β-cell area, one third to two thirds β-cell area, and greater than two thirds β-cell area from 4-wk-treated NOD mice with remission.

treatment. Relapse after stopping treatment is linked to reducing regulatory T cells.

Acceleration of diabetes in NOD mice by cyclophosphamide is associated with reducing CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells and can be prevented by transfer of CD4$^+$CD25$^+$ T cells (27). The protection directly correlated with the increase of T regulatory proportions in the pancreas. We found that more CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells and less inflammatory cells in the pancreas of NOD mice with remission suggested that these cells suppress the accumulation and function of effector T cells in the pancreatic environment. T regulatory cells suppress effector T cell migration, accumulation, and proliferation in draining lymph nodes and inflamed tissues (23). Thus, our findings suggest that up-regulation of naturally occurring CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells in not only the thymus and the pancreatic lymph nodes but also the pancreas is a critical factor in the amelioration of autoimmunity. Because the balance between infiltrated effector T cells and regulatory T cells determines the ultimate outcome of immune responses to islet antigens in the pancreas, further studies are needed to determine how NVP-DPP728 treatment increases naturally occurring CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells and enhances the migration of these regulatory T cells.

T cell progenitors that are generated from hematopoietic stem cells, multiple progenitors, or common lymphoid

progenitors migrate from bone marrow to the thymus to generate mature T cells. This process is regulated by chemokines and its receptors. CD26/DPP-IV cleaves chemokines (5). Inhibition of CD26 promotes hematopoietic stem cell homing and engraftment (28–30). Multiple cell migration-related abnormalities occur in the thymus of NOD mice, and $CD4^+$ $FoxP3^+$ regulatory T cells were significantly reduced (31). CD26 deficiency results in altered thymic emigration patterns in CD26-deficient rats. An increase in regulatory T cells and a reduced memory T cells pool also were observed (32). CD26 is an endogenous inhibitor of T-cell motility, and chemokines stimulate cell polarity and migration through abrogation of the CD26-dependent inhibition (33). It suggests that T-cell motility is regulated by a CD26-controlled cell surface cascade. Chemokines and their receptors regulate the migration of regulatory T cells (23, 34–38). Thus, it is possible that NVP-DPP728 treatment modulates chemokines and their receptors in diabetic NOD mice and promotes T-cell progenitors and/or regulatory T cells to migrate to the thymus and then the secondary lymph organs as well as the pancreas. Further studies are needed to investigate the effect of NVP-DPP782 treatment on chemokines and their receptors in NOD mice.

Consistent with the earlier study that showed that DPP-IV inhibitor treatment increased TGF-β1 secretion *in vivo* (9), we found that NVP-DPP827 treatment significantly increased the plasma TGF-β1 levels in diabetic NOD mice with remission. The correlation between $CD4^+CD25^+Foxp3^+$ regulatory T cells and TGF-β1 production is not clear. Although the increase of TGF-β1 production is probably due to the up-regulated regulatory T cells, because $CD4^+CD25^+$ regulatory T cells secrete TGF-β1 (12, 15), we could not rule out that TGF-β1, induced by other types of immune cells, promotes expansion of the $CD4^+CD25^+Foxp3^+$ regulatory T cells and converts peripheral $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells (14).

In these 4-wk-treated NOD mice with remission, we found that $insulin^+$ and $BrdU^+$ β cells in islets significantly increased. When BrdU was given 24 h before harvesting pancreases, increased replication of β-cells was not detected in NOD mice treated with anti-CD3 monoclonal antibody (39). A significant increase of replicating β-cells also was not found in NOD mice treated with anti-CD3 monoclonal antibodies and exendin-4, when Ki67 was used as a marker (40). Because the replication rate of β-cells in adults is very slow (41), short-term BrdU labeling or Ki67 staining is not sufficient to label β-cells, and daily BrdU labeling is necessary for successfully labeling replicating β-cells. We found that DPP-728 treatment increased the plasma GLP-1 level in NOD mice. Therefore, stimulating β-cell replication in NOD mice by DPP-728 treatment probably is due to increasing plasma GLP-1 level.

Using adoptive transfer, a study reported that increased β-cell replication is associated with inflammation to islets in the pancreas (42). We analyzed the correlation of β-cell replication with inflammation in NVP-DPP728-treated mice with remission. Neither significant differences between insulitis and the percentage of replicated β-cells nor significant differences between the islets with different β-cell area and the percentage of replicated β-cells were found. Our data demonstrate that inflammation is not associated with β-cell replication in NVP-DPP728-treated NOD mice.

In conclusion, our study demonstrates that NVP-DPP728 treatment could reverse new-onset diabetes by increasing naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T cells and stimulating β-cell replication. Our studies also indicate that targeting CD26/DPP-IV has great clinical potential for reversal of the new-onset of human type 1 diabetes.

Acknowledgments

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