Dipeptidyl Peptidase IV Inhibition With MK0431 Improves Islet Graft Survival in Diabetic NOD Mice Partially via T-Cell Modulation

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OBJECTIVE—The endopeptidase dipeptidyl peptidase-IV (DPP-IV) has been shown to NH_2-terminally truncate incretin hormones, glucose-dependent insulinotropic polypeptide, and glucagon-like peptide-1, thus ablating their ability to potentiate glucose-stimulated insulin secretion. Increasing the circulating levels of incretins through administration of DPP-IV inhibitors has therefore been introduced as a therapeutic approach for the treatment of type 2 diabetes. DPP-IV inhibitor treatment has also been shown to preserve islet mass in rodent models of type 1 diabetes. The current study was initiated to define the effects of the DPP-IV inhibitor sitagliptin (MK0431) on transplanted islet survival in nonobese diabetic (NOD) mice, an autoimmune type 1 diabetes model.

RESEARCH DESIGN AND METHODS—Effects of MK0431 on islet graft survival in diabetic NOD mice were determined with metabolic studies and micropositron emission tomography imaging, and its underlying molecular mechanisms were assessed.

RESULTS—Treatment of NOD mice with MK0431 before and after islet transplantation resulted in prolongation of islet graft survival, whereas treatment after transplantation alone resulted in small beneficial effects compared with nontreated controls. Subsequent studies demonstrated that MK0431 pretreatment resulted in decreased insulitis in diabetic NOD mice and reduced in vitro migration of isolated splenic $CD4^+$ T-cells. Furthermore, in vitro treatment of splenic $CD4^+$ T-cells with DPP-IV resulted in increased migration and activation of protein kinase A (PKA) and Rac1.

CONCLUSIONS—Treatment with MK0431 therefore reduced the effect of autoimmunity on graft survival partially by decreasing the homing of $CD4^+$ T-cells into pancreatic β-cells through a pathway involving cAMP/PKA/Rac1 activation. ***Diabetes*** **58: 641–651, 2009**

The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), exert a number of actions that improve glucose homeostasis, including potentiation of glucose-stimulated insulin secretion (GSIS), promotion of β-cell proliferation and survival, and inhibition of glucagon secretion (1–7). Because GIP and GLP-1 are rapidly degraded by the endopeptidase dipeptidyl peptidase IV (DPP-IV; CD26), it has not been possible to directly take advantage of their beneficial actions for the treatment of type 2 diabetes (8,9). Therefore, a number of strategies have been explored to circumvent this problem, including the development of small molecule (10,11) and DPP-IV–resistant peptide (12,13) incretin receptor agonists, and DPP-IV inhibitors (14,15). Members of two classes of compounds have recently been approved by the Food and Drug Administration as type 2 diabetes therapeutics: the DPP-IV–resistant GLP-1 receptor agonist (incretin mimetic) exenatide (Byetta) and the DPP-IV inhibitor sitagliptin (Januvia).

Although DPP-IV inhibitors have been extensively studied in treatment of type 2 diabetes, little is known about their potential for treatment of type 1 diabetes. Infusion of GLP-1 was shown to reduce glycemic excursions in type 1 diabetic patients, and this result was attributed to reduced glucagon levels and delayed gastric emptying (16,17). In preclinical studies, the DPP-IV inhibitor isoleucine thiazolidide was shown to improve glucose tolerance in both streptozotocin (STZ)-induced (18,19) and BioBreeding (BB) (19) diabetic rats, associated with increased β-cell survival and possibly islet neogenesis (18). Additionally, we recently showed that the DPP-IV inhibitor MK0431 prolonged islet graft survival in STZ-induced diabetic mice (20). In the current study, we show that MK0431 pretreatment resulted in the prolongation of islet graft survival in an autoimmune type 1 diabetes model, the nonobese diabetic (NOD) mouse, through a mechanism that includes modulation of $CD4^+$ T-cell migration.

RESEARCH DESIGN AND METHODS

NOD/LtJ mice (NOD, $H2^{g7}$) were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice (8–10 weeks old) were placed on either a normal chow diet (Purina Rodent Chow 5015) or diet containing Sitagliptin (MK0431) (21) (Purina Rodent Chow 5015 plus 4 g MK0431/kg; Research Diets, New Brunswick, NJ) ad libitum. From the mice that had been fed a normal chow diet, two groups of diabetic mice were studied. Immediately after the islet transplantation, group 1 mice received the normal chow diet for the remainder of the study (NCD Tx), whereas group 2 mice were fed the MK0431-containing diet (Post MK0431 Tx). Group 3 mice were animals that had received the MK0431 diet for ~1 month before surgery and for the remainder of the study (Pre MK0431 Tx). In a second study, mice were fed either normal chow diet or MK0431 for a period of 4 weeks. All animal experiments were conducted in accordance with the guidelines put forth by the University of British Columbia Committee on Animal Care and the Canadian Council on Animal Care.

DPP-IV activity assays. To measure the plasma DPP-IV activity, a fluorometric assay was used as previously described (20).

Islet isolation and islet gene transfer. Islets were isolated from nondiabetic male NOD/LtJ mice (8–12 weeks old) by collagenase digestion (22). Recombinant adenovirus-expressing HSV1-Sr39TK was produced and expanded by infection of human embryonic kidney-293 cells (23). Islets were exposed to rAD-TK as previously described (20,24,25).

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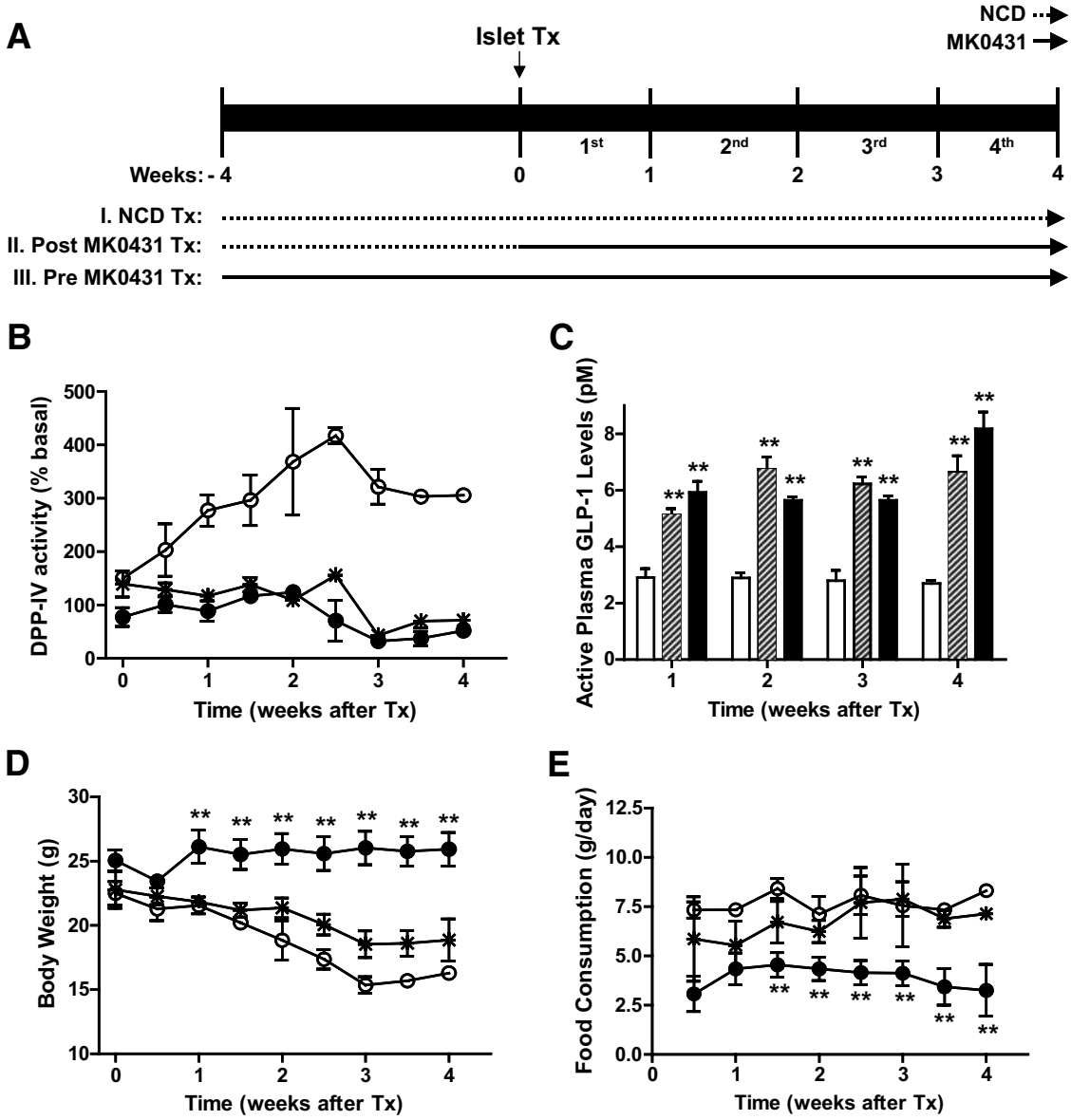

FIG. 1. The effects of MK0431 in diabetic NOD mice after islet transplantation. *A*: Schematic diagram of group design. Islets isolated from nondiabetic NOD mice were infected with 250 MOI of rAD-TK, 200 of which were transplanted under the right kidney capsule of diabetic female NOD mice. Islet-transplanted diabetic NOD mice were fed as follows: group 1, normal chow diet throughout (NCD Tx); group 2, MK0431 diet after transplant (Post MK0431 Tx); group 3, MK0431 diet ~1 month before and after transplant (Pre MK0431 Tx). *B* and *C*: The effects of MK0431 on plasma DPP-IV activity (*B*) and plasma active GLP-1 levels (*C*) in diabetic NOD mice after islet transplantation. *D–I*: The effects of MK0431 on food consumption (*D*), body weight (*E*), water intake (*F*), nonfasting (*G*) and fasting (*H*) glycemic control, and survival rates (*I*) in diabetic NOD mice after islet transplantation. Number of animals: $n = 5\sim7$ per group: NCD Tx group (○), Post MK0431 Tx group (*), and Pre MK0431 Tx group (●). All data represent means ± SE, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where $^{**}P < 0.05$ vs. NCD Tx. □ and ○: NCD T. ▨ and *: Post MK0431 Tx. ■ and ●: Pre MK0431 Tx.

Islet transplantation. A cohort of 70 female NOD/LtJ mice was divided into two randomly selected groups of 35, and from the age of 8–10 weeks, one group of mice received a normal chow diet while the second received a diet containing MK0431 (4 g/kg). When mice developed diabetes, defined as a blood glucose level of ≥15 mmol/l for 3 consecutive days, they received an islet transplant. Islets (200 obtained from nondiabetic male NOD/LtJ mice and infected with 250 multiplicities of infection [MOI] of rAD-TK) were transplanted under the kidney capsule as described previously (20,24,25).

Plasma glucose determinations, intraperitoneal glucose tolerance tests, and plasma hormone measurements. Nonfasting blood glucose levels were measured in mouse-tail blood using a SureStep Glucose analyzer (LifeScan) at the time points indicated in Figs. 1 and 2. For the intraperitoneal glucose tolerance tests (IPGTTs), mice were fasted for 4 h, and blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min after the glucose challenge (2 g/kg). Blood samples with glucose levels ≥27.8 mmol/l were diluted with blood from nondiabetic mice and levels calculated. Plasma insulin, glucagon, and active GLP-1 levels were determined using a Multiplex assay kit (Linco Research).

Synthesis of 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine. 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine ([^{18}F]FHBG) was synthesized by a modification of the method of Ponde et al. (26) as previously described (24).

Micropositron emission tomography. A PET reporter gene (PRG)/PET reporter probe (PRP) system was used, as previously described (20,24,25). *HSV1-sr39tk* was expressed in islets as described above. The probe, [^{18}F]F-HBG, was systemically administered and its retention in islets, after phosphorylation by rAD-TK, was quantified by micropositron emission tomography (microPET) imaging. Transplanted mice were scanned using a Focus120 (CTI Concorde) system that yielded 63 slices, 1.2 mm apart with an in-plane resolution of 2 mm full-width at half-maximum (27). Image reconstruction and data analysis were performed as previously described (20,24,25).

Insulitis scoring and measurement of relative β-cell area. Paraffin-embedded pancreatic sections were stained with hematoxylin-eosin (H-E), and islets were examined in a blinded manner under the microscope to evaluate the degree of mononuclear cell infiltration. The degree of insulitis was scored in the following five categories: 0, intact islet; <25% of the islet infiltrated; 25–50% of the islet infiltrated; 50–75% of the islet infiltrated; and

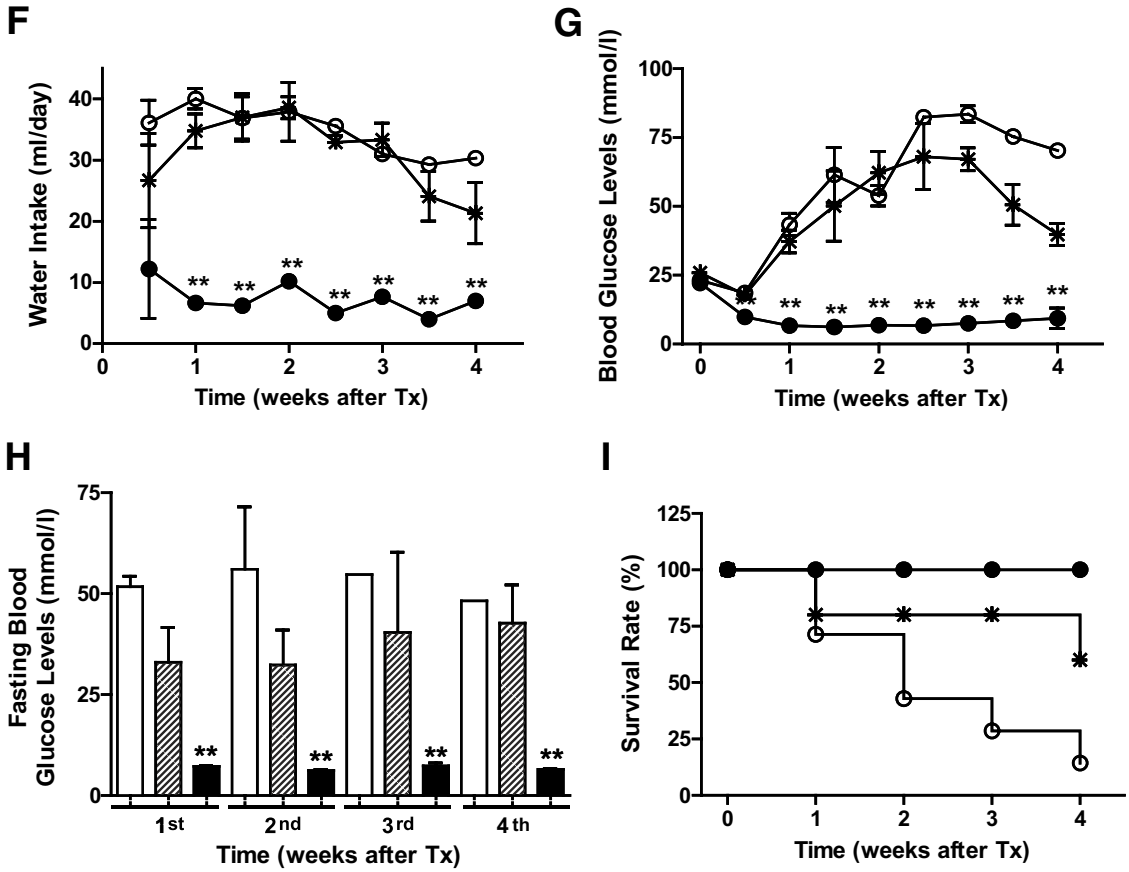

FIG. 1. Continued.

>75% of the islet infiltrated. The degree of insulitis in 20–25 islets per mouse was evaluated. β-Cell area was measured on the sections of each pancreas stained for insulin. Morphometric evaluation of the total β-cell area was performed by computer-assisted image analysis (Northern Eclipse ver. 6) using light and immunofluorescence microscopy. The area of insulin-positive cells and the total area of the tissue section were evaluated for each section, and the percentage β-cell area was calculated by the ratio of the area occupied by insulin-positive cells to the total pancreatic area.

$CD4^+$ T-cells isolation. For isolation of T-cells, spleen cell suspensions were prepared from female NOD mice. $CD4^+$ T-cells were enriched using the SpinSep Mouse $CD4^+$ T Cell separation kit (Stem Cell Technologies), and the purity of $CD4^+$ T-cells was confirmed by flow cytometry.

Migration assay. $CD4^+$ T-cells were plated on membrane inserts (8-μm pore size) in serum-free RPMI 1640, and cell migration was assayed using Transwell chambers (Corning), in the presence or absence of purified porcine kidney DPP-IV (32.1 units/mg; 100 mU/ml final concentration; provided by Dr. Hans-Ulrich Demuth, Probiodrug, Halle, Germany) (28) ± DPP-IV inhibitor (100 μmol/l). After 1 h, cells on the upper surface were removed mechanically, and cells that had migrated into the lower compartment were counted. The extent of migration was then expressed relative to the control sample.

Rac1 guanosine triphosphate (GTP) binding assay. Total cellular extracts were isolated from $CD4^+$ T-cells and Rac1 GTP binding assays performed using the fluorophore-based RhoGEF exchange assay kit (Cytoskeleton, Denver, CO), and data were normalized to protein concentration.

cAMP measurements. $CD4^+$ T-cells were incubated for 30 min with GIP, 100 nmol/l GLP-1 or 100 mU/ml purified porcine DPP-IV in the presence of 0.5 mmol/l IBMX. cAMP concentration in the cell extracts was determined using the Parameter cyclic AMP assay kit (R&D Systems).

Protein kinase A activity assay. Activity was measured using a protein kinase A (PKA) kinase activity assay kit (Stressgen, Mississauga, ON) according to the manufacturer's protocol. The enzyme activity was normalized to protein concentration, and data are shown as the relative activity to control.

Western blot analysis. Total cellular extracts were separated on a 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad). Probing of the membranes was performed with antibodies to phospho-p38 mitogen-activated protein kinase (MAPK) (threonine-180/tyrosine-182), p38 MAPK, phospho-p42/44 MAPK (threonine-202/tyrosine-204), p42/44 MAPK, phospho–stress-activated protein kinase/Jun NH_2-terminal kinase (SAPK/JNK) (threonine-183/tyrosine-185), phospho–protein kinase B (PKB) (serine-473), phospho-PKB (threonine-308), PKB, and β-actin (Cell Signaling Technology, Beverly, MA). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) using horseradish peroxidase–conjugated IgG secondary antibodies.

Statistical analysis. Data are expressed as means ± SE. Data were analyzed using the linear regression analysis program PRISM (GraphPad, San Diego, CA), and area under the curves were calculated using the algorithm provided in the Prism software package. Significance was tested using ANOVA with Newman-Keuls hoc test ($P < 0.05$) as indicated in figure legends.

RESULTS

MK0431 treatment improves islet graft survival in diabetic NOD mice. Islet-transplanted, diabetic NOD mice were studied to determine the effect of MK0431 treatment on graft survival. Three groups of mice were studied, as described in RESEARCH DESIGN AND METHODS: group 1 received normal chow diet throughout the study period (NCD Tx), group 2 received MK0431 only after transplant (Post MK0431 Tx), and group 3 received MK0431 before and after transplant (Fig. 1*A*). Importantly, of mice that were treated with MK0431 before receiving the transplant, only 22.8% (8 of 35) became diabetic, compared with 40% (14 of 35) of the mice receiving normal chow diet.

For the transplants, islets were infected with a rAD-TK, recombinant adenovirus expressing *HSV1-sr39tk*, the gene for a mutant form of herpes simplex virus 1 thymidine kinase, to enable PET imaging (29,30), and inserted under the kidney capsule of diabetic female NOD mice. The effect of recombinant adenoviral infection on NOD islet function was addressed with in vitro studies. Insulin secretory capacity was preserved in 250 MOI rAD-TK–treated islets, compared with untreated or control rAD-β-

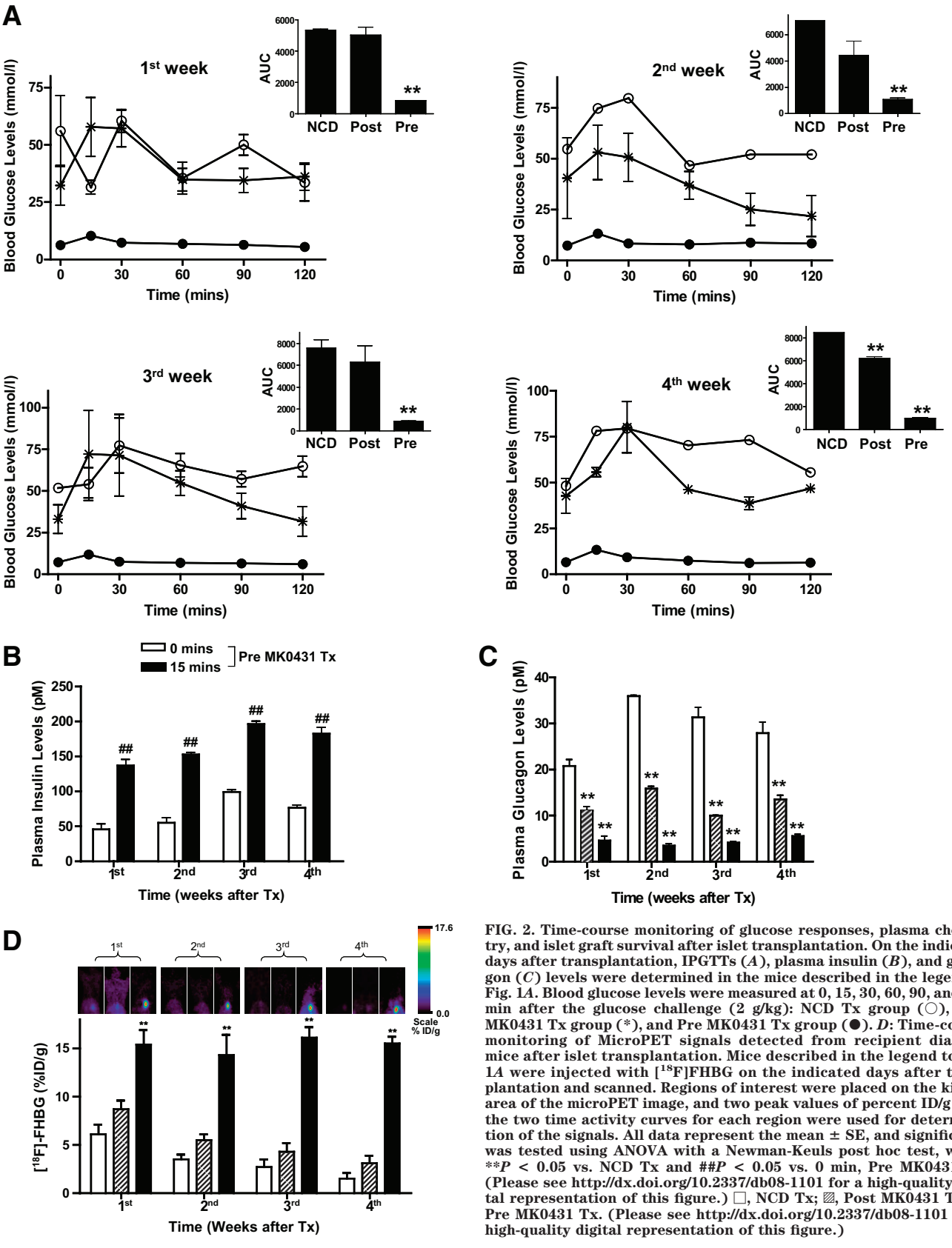

FIG. 2. Time-course monitoring of glucose responses, plasma chemistry, and islet graft survival after islet transplantation. On the indicated days after transplantation, IPGTTs (*A*), plasma insulin (*B*), and glucagon (*C*) levels were determined in the mice described in the legend to Fig. 1*A*. Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min after the glucose challenge (2 g/kg): NCD Tx group (○), Post MK0431 Tx group (*), and Pre MK0431 Tx group (●). *D*: Time-course monitoring of MicroPET signals detected from recipient diabetic mice after islet transplantation. Mice described in the legend to Fig. 1*A* were injected with [^{18}F]FHBG on the indicated days after transplantation and scanned. Regions of interest were placed on the kidney area of the microPET image, and two peak values of percent ID/g from the two time activity curves for each region were used for determination of the signals. All data represent the mean ± SE, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ***P* < 0.05 vs. NCD Tx and ##*P* < 0.05 vs. 0 min, Pre MK0431 Tx. (Please see http://dx.doi.org/10.2337/db08-1101 for a high-quality digital representation of this figure.) □, NCD Tx; ▨, Post MK0431 Tx; ■, Pre MK0431 Tx. (Please see http://dx.doi.org/10.2337/db08-1101 for a high-quality digital representation of this figure.)

gal virus–treated islets (supplementary Fig. 1*A*, available in an online appendix at http://dx.doi.org/10.2337/db08-1101). Furthermore, although small decreases in mean cell viability were observed with both rAD-TK– and rAD-β-gal—treated islets compared with untreated islets, these reductions did not reach statistical significance (supplementary Fig. 1*B*), indicating that 250 MOI was an appropriate dose for further studies. The DPP-IV activity in plasma from nondiabetic NOD mice was 67.2 ± 4.3 mU/ml, whereas levels measured at the time of islet transplanta-

tion were 100.7 ± 7.1 mU/ml in the NCD diabetic group, 93.8 ± 16.5 mU/ml in the post-MK0431 Tx group, and 52.1 ± 11.8 mU/ml in the pre-MK0431 Tx group (n = 5∼7 per group). Plasma DPP-IV activity in the NCD Tx group progressively increased to levels that were ∼300–400% greater than at the initiation of the study. Both pre- and post-MK0431 Tx groups showed significantly reduced DPP-IV activity compared with the NCD group (Fig. 1*B*). Plasma levels of active (NH_2-terminally intact) GLP-1 were significantly increased in both pre- and post-MK0431 Tx groups, demonstrating protection of circulating incretin from DPP-IV–mediated degradation (Fig. 1*C*). For the first 0.5 week after transplantation, the pre-MK0431 Tx group tended to be slightly heavier (Fig. 1*D*), eat less food (Fig. 1*E*), and drink less water (Fig. 1*F*) than the post-MK0431 Tx group; however, this difference was not statistically significant. The NCD and post-MK0431 Tx groups then lost body weight (Fig. 1*D*) despite their greater food and water intake (Fig. 1*E* and *F*), whereas the pre-MK0431 Tx group maintained body weight and food and water intake remained stable (Fig. 1*D–F*). By 0.5 weeks after transplantation, nonfasting blood glucose levels were normalized in the pre-MK0431 Tx group, whereas levels in NCD and post-MK0431 Tx groups progressively increased from 1 week on (Fig. 1*G*). A similar trend was evident for fasting blood glucose (Fig. 1*H*). During the course of the study, ∼80% of the NCD Tx group and 40% of the post-MK0431 Tx group died, probably as a result of the severe hyperglycemia, whereas all of the pre-MK0431 Tx group survived (Fig. 1*I*).

IPGTTs were performed after transplantation (n = 5∼7 animals/group). As expected, with the suboptimal transplanted islet mass, IPGTTs showed a rapid deterioration of glucose handling in NCD and post-MK0431 Tx groups. By contrast, the pre-MK0431 Tx group preserved the capacity to regulate blood glucose levels normally until the end of the study (Fig. 2*A*). Both basal and glucose-stimulated plasma insulin levels in the NCD and post-MK0431 Tx groups were below 6.2 pmol/l (the detection limit of the assay), whereas the pre-MK0431 Tx group showed stable insulin secretory responses to glucose stimulation (Fig. 2*B*). Although plasma glucagon levels increased substantially in the NCD Tx group over the 3 weeks after transplantation, glucagon levels in the post-MK0431 Tx group remained low and were further decreased in the pre-MK0431 Tx group (Fig. 2*C*). The effect of MK0431 on islet graft survival was further assessed by microPET imaging. The PET signal from transplanted islets in the NCD and post-MK0431 Tx groups decreased dramatically within 1 week, whereas mice in the pre-MK0431 Tx group showed sustained PET signals for up to 4 weeks after transplantation (Fig. 2*D*). These results indicated that MK0431 pretreatment resulted in prolongation of islet graft survival.

Modulation of the immune system in MK0431-treated mice. In view of the reduced incidence of diabetes in the pre-MK0431 Tx group and the prolonged islet graft survival, we considered it likely that the action of MK0431 in NOD mice involved modulation of the immune system during the 1-month pretreatment period. To determine whether MK0431 administration could impact immune cell infiltration of transplanted islets, the development of insulitis was examined in female NOD mice receiving normal chow diet or MK0431 diets for 1 month starting at 8–10 weeks of age (Fig. 3*A*). By 12–14 weeks of age, the incidence of diabetes was substantially decreased in the MK0431 group compared with the NCD group; 17.6% (3 of 17) of the mice developed diabetes in the MK0431 group and 35% (6 of 17) in the NCD group. Animals with glucose <15 mmol/l were grouped as "normal" and those with glucose ≥15 mmol/l as "diabetic." There were no significant differences in blood glucose levels between the diabetic mice that had been fed normal chow diet 21.1 ± 2.5 (n = 6) and those receiving MK0431 diet 20.7 ± 2.8 (n = 3) (Fig. 3*B*). However, histochemical analysis revealed that islets in sections from the diabetic MK0431 group exhibited a more intact structure, whereas the majority of islets in the diabetic NCD group showed severe insulitis: ∼90 and 70% of the islets in NCD diabetic and MK0431 diabetic mice, respectively, were infiltrated by >25% (Fig. 3*C*). Treatment with MK0431 resulted in significantly increased islet β-cell area in both the mice with normal plasma glucose and the diabetic mice: normal mice β-cell area, NCD: 0.37 ± 0.03%; MK0432: 0.61 ± 0.05%; diabetic mice β-cell area, 0.01 ± 0.003%; and MK0431: 0.12 ± 0.02% (Fig. 3*D*).

Mechanisms underlying the DPP-IV–mediated reduction in lymphocytic infiltration. Potential mechanisms by which DPP-IV inhibitor treatment protected islets from severe lymphocytic infiltration were next assessed. $CD4^+$ T-cells have been considered to be essential for the development of diabetes through recognition of β-cell antigens in the context of the class II major histocompatibility complex (MHC) $I\text{-}A^{g7.}$ The identification of an islet-specific $CD4^+$ T-cell clone for the acceleration of diabetes in young NOD mice (31) led us to consider the potential involvement of DPP-IV–mediated $CD4^+$ T-cell migration. The in vitro migration of splenic $CD4^+$ T-cells isolated from the diabetic NCD group was significantly increased when compared with the normal NCD group, and MK0431 treatment partially restored the levels toward normal (Fig. 3*E*). The extent of $CD4^+$ T-cell migration correlated well with plasma DPP-IV activity and blood glucose levels (r^2 = 0.85, $P < 0.0001$ for plasma DPP-IV activity and $r^2 = 0.79$, $P < 0.0001$ for fasting blood glucose levels; Fig. 3*F* and *G*). These results suggested that the prolonged islet graft survival observed in the pre-MK0431 Tx group was at least partially due to immunomodulation.

The effect of MK0431 treatment on signaling modules potentially involved in the regulation of $CD4^+$ T-cell migration was then studied. Rac1 is a member of the ρ-GTPase family and has been shown to play an important role in cytoskeletal reorganization, membrane trafficking, cell growth, and development (32,33). In view of this role, we examined the Rac1 GTP binding activity in splenic T-cells from normal chow diet and MK0431-treated NOD mice. As shown in Fig. 4*A*, Rac1 GTP binding activity in $CD4^+$ T-cells of the diabetic NCD group was substantially reduced in the diabetic MK0431-treated group. On the other hand, there were no significant changes in the level of phosphorylation of a number of other signaling molecules, including p38 MAPK (Thr180/Tyr182), SAPK/JNK (Thr183/Tyr185), p42/44 MAPK (Thr202/Tyr204), PKB (Ser473), or PKB (Thr308) (Fig. 4*B–F*). Further in vitro studies were then performed to determine whether elevated circulating incretins or DPP-IV itself were likely responsible for the increase in T-cell migration and Rac1 activity. $CD4^+$ T-cells were isolated from the spleen of nondiabetic female NOD mice and treated with DPP-IV, GIP, or GLP-1 in the presence or absence of DPP-IV inhibitor. As shown in Fig. 5*A*, treatment of $CD4^+$ T-cells with DPP-IV resulted in ∼1.6-fold increase in T-cell migration that was abolished by DPP-IV inhibitor treatment.

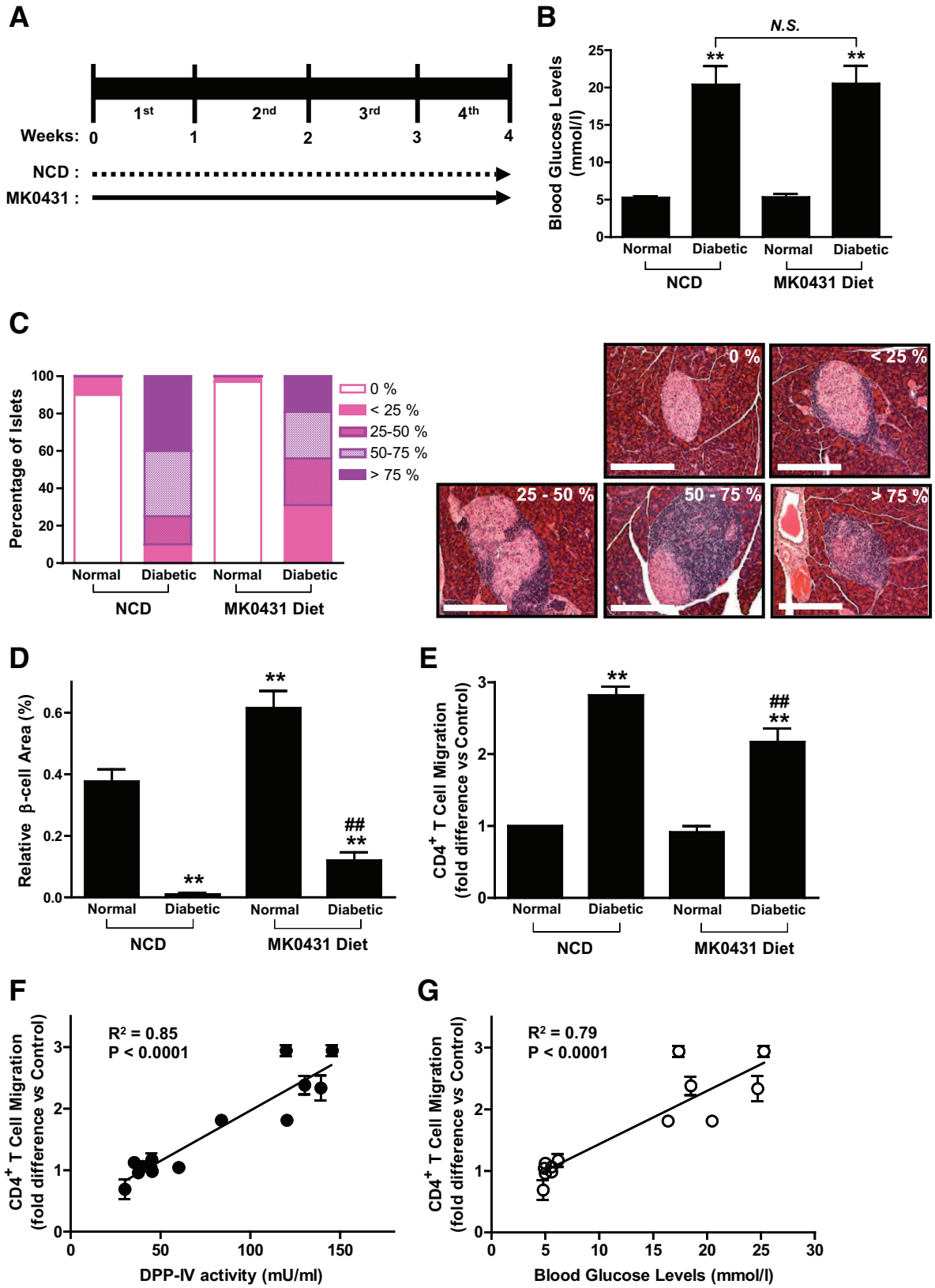

FIG. 3. MK0431 regulates the migration of CD4$^+$ T-cells. *A*: Schematic diagram of group design. Female NOD mice (8–10 weeks old) were placed on normal chow diet (NCD group) or MK0431 diet (MK0431 group) for 1 month. *B*: Nonfasting blood glucose levels. *C*: Representative pancreatic sections from 12- to 14-week-old littermate female NOD mice stained with H-E were shown. The extent of insulitis was assessed as described in RESEARCH DESIGN AND METHODS. *D*: Relative β-cell area. β-Cell area was expressed as the percentage of sectional pancreas area as described in RESEARCH DESIGN AND METHODS. *E*: CD4$^+$ T-cells were isolated from the spleen of NCD and MK0431 group. The migration of CD4$^+$ T-cells was determined using Transwell chamber as described in RESEARCH DESIGN AND METHODS. Correlation between migration of splenic CD4$^+$ T-cells and plasma DPP-IV activity (*F*) and blood glucose levels (*G*). Data were analyzed using the linear regression analysis program PRISM. All data represent mean ± SE, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where **$P < 0.05$ vs. normal NCD group and ##$P < 0.05$ vs. diabetic NCD group. (Please see http://dx.doi.org/10.2337/db08-1101 for a high-quality digital representation of this figure.)

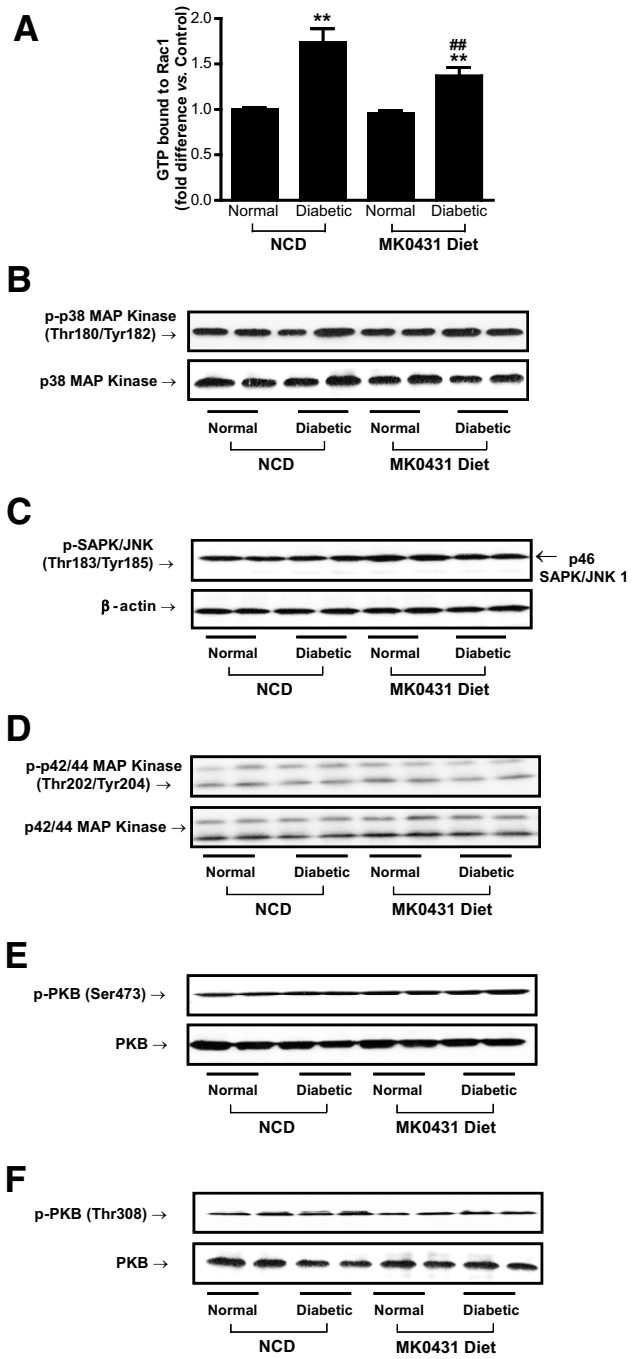

FIG. 4. Signaling modules potentially involved in the effect of MK0431 on splenic CD4$^+$ T-cells. *A*: Rac1 GTP binding activity. Total cellular extracts were isolated from CD4$^+$ T-cells described in Fig. 3 legend; Rac1 GTP binding activity was determined as described in RESEARCH DESIGN AND METHODS; and the data are expressed as fold difference vs. normal NCD group. Western blot analyses were performed with antibodies against phosphorylated p38MAPK (Thr180/Tyr182) (*B*), SAPK/JNK (Thr183/Tyr185) (*C*), p42/44 MAPK (Thr202/Tyr204) (*D*), PKB (Ser473) (*E*), PKB (Thr308) (*F*), and actin. Western blots are representative of $n = 3$, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where $^{**}P < 0.05$ vs. normal NCD group and $^{\#\#}P < 0.05$ vs. diabetic NCD group.

Neither GIP nor GLP-1 exhibited significant effects on T-cell migration. In preliminary studies, similar effects of DPP-IV on migration of human T-cell lymphoma Jurkat cells were observed. Treatment resulted in ~3.8-fold increases in migration that were abolished by DPP-IV inhibitor treatment (supplementary Fig. 2, available in the online appendix).

Treatment with DPP-IV also increased Rac1 GTP binding activity, whereas both GIP and GLP-1 had no effect (Fig. 5*B*). There were also no significant effects of DPP-IV, GIP, or GLP-1, in the presence or absence of DPP-IV inhibitor, on phosphorylation of other signaling proteins examined (Fig. 5*C*–G), strongly suggesting that DPP-IV regulates the migration of CD4$^+$ T-cells directly via a pathway involving Rac1.

Both PKA activation of Rac1 (34) and Rac1 activation of PKA (35) have been described in different cell types. Involvement of the cAMP/PKA pathway in DPP-IV action on CD4$^+$ T-cells was therefore studied. Treatment of splenic CD4$^+$ T-cells with 100 mU/ml DPP-IV for 30 min resulted in ~2.7-fold increase in cAMP concentration compared with control, and DPP-IV inhibitor abolished the effect (Fig. 6*A*). By contrast, treatment with GIP or GLP-1 (100 nmol/l) had no significant effects on cAMP levels (Fig. 6*A*). MK0431 decreased DPP-IV–mediated PKA activation in a concentration-dependent manner (Fig. 6*B*). The low concentrations that resulted in inhibition support a specific action of MK0431 on DPP-IV. Additionally, MK0431 did not inhibit the PKA stimulatory effect of 6-Bnz-cAMP, a PKA-selective, PDE-resistant cAMP analog (Fig. 6*C*). This suggests that the activation is upstream of cAMP production, probably at an extracellular level. As expected, when PKA was inhibited by either H-89 or Rp-cAMPs, DPP-IV was no longer capable of stimulating PKA, and DPP-IV inhibition exhibited no further effect (data not shown). Together, these results strongly support a mechanism for DPP-IV–mediated PKA activation proximal to, or at the level of, adenylate cyclase. Forskolin, a direct activator of adenylyl cyclase, was found to mimic the effect of DPP-IV on CD4$^+$ T-cell migration, and responses to both DPP-IV and forskolin were greatly reduced by administration of the selective PKA inhibitor H-89 (Fig. 6*D*). Similarly, DPP-IV and forskolin both increased Rac1 GTP binding, whereas H-89 abolished their effects (Fig. 6*E*). Together, these results strongly suggest that DPP-IV activates a pathway involving the production of cAMP and activation of PKA and Rac1, resulting in increased CD4$^+$ T-cell migration.

DISCUSSION

Two major developments have been responsible for dramatically improving the long-term success of islet transplantation and allowing islet-alone grafts: the development of automated methods for human islet isolation (36) and improved immunosuppression (37), culminating in what is known as the "Edmonton Protocol" (37,38). However, despite major successes, with current procedures, at least two donor pancreata are generally needed to achieve insulin independence because of the loss of islet viability both during isolation and after transplantation. Additionally, only 67% (39) and 10% (40) of transplant recipients have been shown to be insulin independent at the end of 1 and 5 years, respectively. The causes of loss after transplant are multiple, and apoptosis is thought to play a critical role. Because the incretin hormones GIP and GLP-1 have been reported to stimulate β-cell proliferation and survival (1–3,5–7), increasing the concentrations of bioactive incretin hormones through DPP-IV inhibitor treatment could offer therapeutic advantages in type 1 diabetic patients receiving islet transplants. In the present study, MK0431 pretreatment was shown to exert beneficial effects on glycemia in mice receiving islet transplants, as

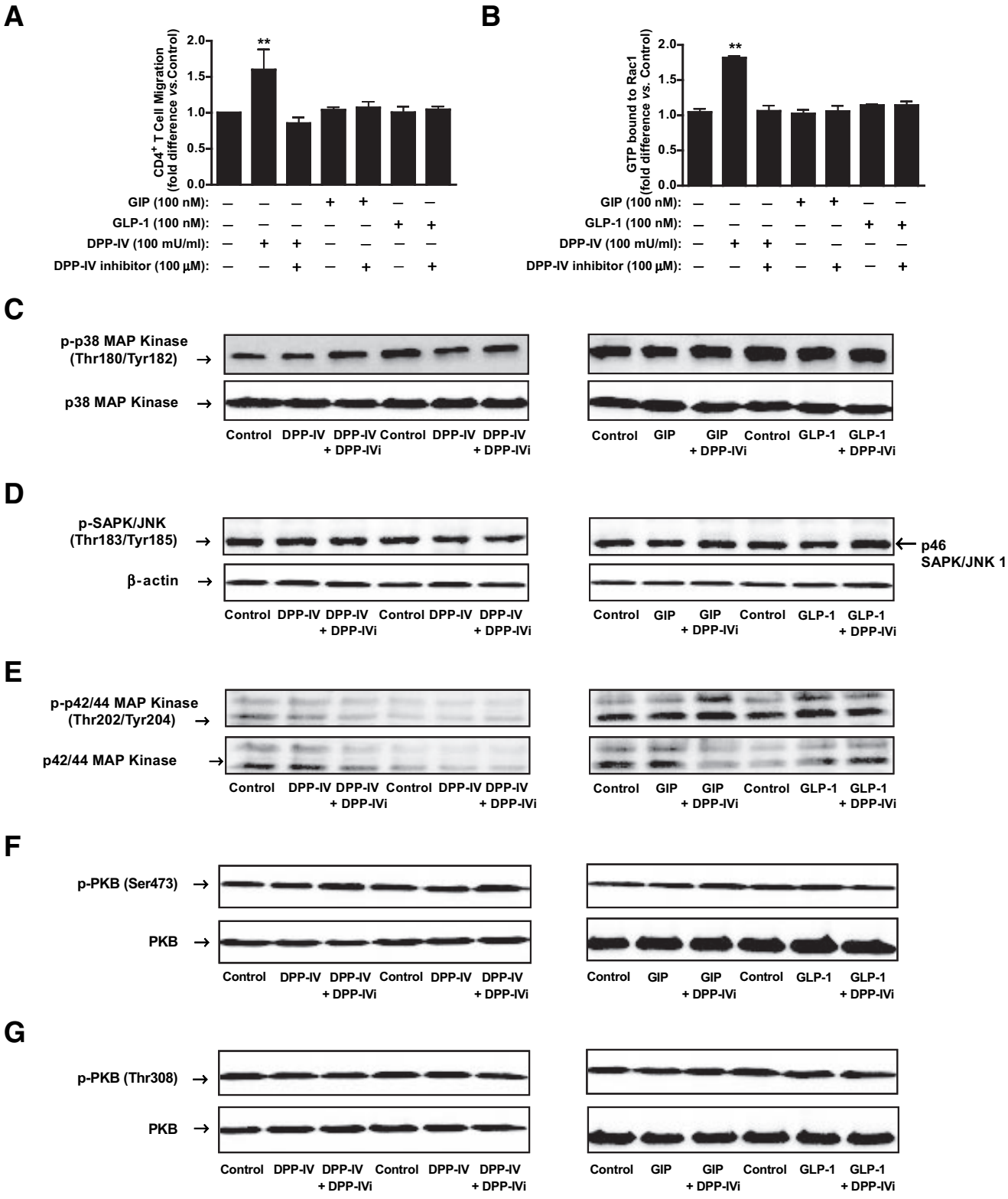

FIG. 5. The effect of sDPP-IV and incretins on splenic CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleen of nondiabetic female NOD mice placed on normal chow diet. T-cells were stimulated with purified porcine DPP-IV (100 mU/ml), GIP or GLP-1 (100 nmol/l) in the presence or absence of 100 μmol/l DPP-IV inhibitor for 1 h. *A*: Splenic CD4$^+$ T-cell migration. The migration of splenic CD4$^+$ T-cells were determined using Transwell chamber (Corning) as described in RESEARCH DESIGN AND METHODS. *B*: Rac1 GTP binding activity. Total cellular extracts were isolated, and Rac1 GTP binding activity was determined. *C–G*: Total cellular extracts were isolated, and Western blot analyses were performed with antibodies against phosphorylated p38MAPK (Thr180/Tyr182) (*C*), SAPK/JNK (Thr183/Tyr185) (*D*), p42/44 MAP Kinase (Thr202/Tyr204) (*E*), PKB (Ser473) (*F*), PKB (Thr308) (*G*), and actin. Western blots are representative of $n = 3$, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where $^{**}P < 0.05$ vs. control.

indicated by metabolic studies and microPET imaging (Figs. 1 and 2). In contrast to the preserved GSIS of the pre- MK0431 Tx group (Fig. 2*A*), glucose tolerance rapidly deteriorated in both the NCD and post-MK0431 Tx groups, and these two groups showed greatly attenuated PET signals (Fig. 2*D*). In a subsequent study, it was shown that

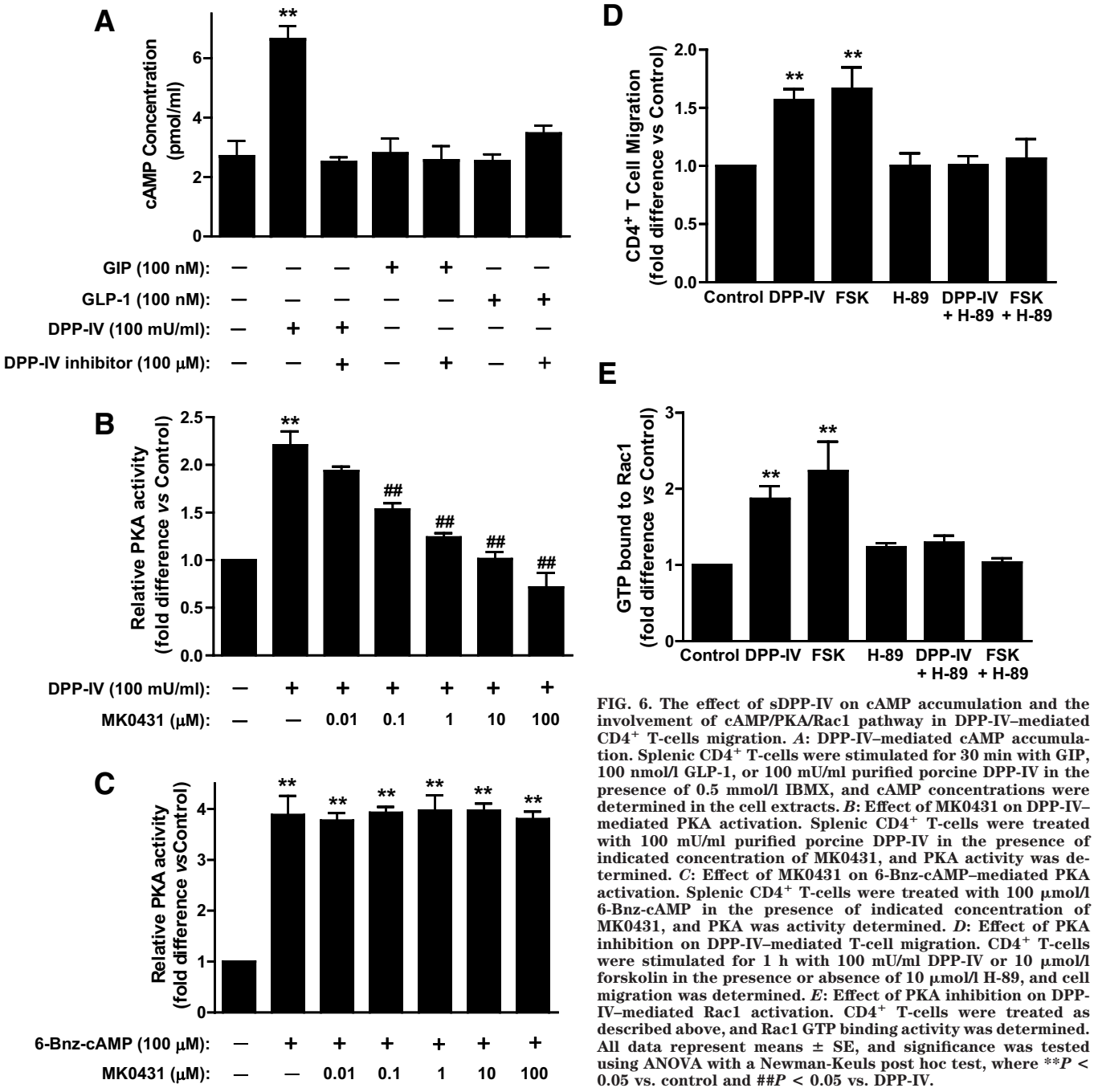

FIG. 6. The effect of sDPP-IV on cAMP accumulation and the involvement of cAMP/PKA/Rac1 pathway in DPP-IV–mediated $CD4^+$ T-cells migration. *A*: DPP-IV–mediated cAMP accumulation. Splenic $CD4^+$ T-cells were stimulated for 30 min with GIP, 100 nmol/l GLP-1, or 100 mU/ml purified porcine DPP-IV in the presence of 0.5 mmol/l IBMX, and cAMP concentrations were determined in the cell extracts. *B*: Effect of MK0431 on DPP-IV–mediated PKA activation. Splenic $CD4^+$ T-cells were treated with 100 mU/ml purified porcine DPP-IV in the presence of indicated concentration of MK0431, and PKA activity was determined. *C*: Effect of MK0431 on 6-Bnz-cAMP–mediated PKA activation. Splenic $CD4^+$ T-cells were treated with 100 μmol/l 6-Bnz-cAMP in the presence of indicated concentration of MK0431, and PKA was activity determined. *D*: Effect of PKA inhibition on DPP-IV–mediated T-cell migration. $CD4^+$ T-cells were stimulated for 1 h with 100 mU/ml DPP-IV or 10 μmol/l forskolin in the presence or absence of 10 μmol/l H-89, and cell migration was determined. *E*: Effect of PKA inhibition on DPP-IV–mediated Rac1 activation. $CD4^+$ T-cells were treated as described above, and Rac1 GTP binding activity was determined. All data represent means ± SE, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where $^{**}P < 0.05$ vs. control and $^{\#\#}P < 0.05$ vs. DPP-IV.

despite improved islet structural integrity in the diabetic MK0431 group compared with that in the diabetic NCD group (Fig. 3*C*), there were no significant differences in nonfasting blood glucose levels between the groups (Fig. 3*B*). Additionally, no significant differences in nonfasting blood glucose levels were observed even in 2-month MK0431-pretreated mice (data not shown). However, because MK0431 treatment resulted in the preservation of pancreatic β-cells in both mice with normal glucose tolerance and the diabetic group (Fig. 3*D*), we cannot exclude a significant contribution from the residual islets to the overall beneficial effects of MK0431 pretreatment on glycemia in the mice receiving islet transplants.

There is considerable evidence supporting a critical role for DPP-IV in immune regulation, including delivery of a costimulatory signal for T-cell activation (41). Acute rejection in experimental models of cardiac allograft transplantation was shown to be associated with increased serum DPP-IV activity, and inhibition resulted in abrogated acute and accelerated rejection (42,43). Additionally, inhibition of intragraft DPP-IV significantly reduced ischemia/reperfusion-associated pulmonary injury, allowing for successful lung transplantation (44). In the present study, islet graft survival was only prolonged significantly in the pre-MK0431 group. Despite similar active GLP-1 levels in the pre- and post-MK0431 Tx groups (Fig. 1*C*), the post-MK0431 Tx group showed only minor improvements in glucose homeostasis, with mean fasting glucose levels slightly lower than the NCD Tx group over the first 2 weeks (Fig. 1*H*) and a small enhancement in glucose tolerance at 4th week (Fig. 2*A*). Second, NCD and post-MK0431 Tx groups showed significantly attenuated PET

signals from the 1st week compared with those of the pre-MK0431 Tx group (Fig. 2*D*). These results strongly suggest that MK0431 modulated the immune function of DPP-IV during the 1-month pretreatment period, thus contributing to islet graft survival in the NOD mice. The pathogenesis of type 1 diabetes involves activation of autoimmune T-cells followed by their homing in on the pancreatic islets, resulting in the destruction of β-cells. It was previously reported that soluble DPP-IV (sCD26) had an enhancing effect on transendothelial T-cell migration mediated through its intrinsic DPP-IV enzyme activity (45). In the present study, migration of splenic $CD4^+$ T-cells prepared from the diabetic NCD group was significantly increased compared with that of the nondiabetic NCD group, and MK0431 treatment partially restored the levels toward normal (Fig. 3*E*).

Active, GTP-bound Rac1 plays an important role in the control of cell migration by regulating actin-rich lamellipodial protrusions that are critical for the generation of driving force of cell movement (46). Recently, it was shown that basal activation of Rac1 via MHC class II molecule stimulation is essential for $CD4^+$ T-cell motility, and self-ligand deprivation is associated with reduced levels of active Rac1 (47). In the present study, enhanced Rac1 GTP binding activity in $CD4^+$ T-cells of the diabetic NCD group was found to be decreased in the diabetic MK0431-treated group (Fig. 4*A*). Furthermore, DPP-IV directly influenced the migration of $CD4^+$ T-cells and Rac1 GTP binding activity (Fig. 5). It is therefore conceivable that increased plasma DPP-IV activity in diabetic NCD mice led to increased $CD4^+$ T-cell migration by regulating Rac1 GTP binding activity, whereas MK0431 attenuated autoimmune diabetes partially through decreasing $CD4^+$ T-cell migration. Strong evidence for the involvement of increased cAMP production and PKA activation in DPP-IV–mediated $CD4^+$ T-cell migration was obtained (Fig. 6*A–D*), although it is unclear as to the protein activation sequence. In the current studies, DPP-IV and forskolin activation of Rac1 (Fig. 6*E*) were all ablated by treatment with the PKA inhibitor H89, suggesting that Rac1 is downstream of PKA. Although the effect of cAMP/PKA on cell migration has been reported to be positive or negative depending on the cell type, the spatial-temporal distribution and activation of cAMP/PKA during the regulation of cell migration are likely to be critical components of its action (48).

Although the results reported in the present study suggest that direct inhibitor effects on DPP-IV, and not increased levels of active GIP and GLP-1, are mainly responsible for the improvements in graft retention, we have not completely ruled out a contribution from the incretins or other factors. Additionally, the reduced incidence of diabetes in the MK0431-treated group may well involve the incretins. The long-acting GLP-1 receptor agonist exendin-4, has been shown to synergize with anti-CD3 monoclonal antibody treatment in reversing diabetes in NOD mice by enhancing the recovery of β-cells (49). Furthermore, continuous administration of GLP-1 to prediabetic NOD mice reduced diabetic incidence by regulating β-cell proliferation and apoptosis (50). More recently, the GLP-1 receptor was shown to be expressed in lymphoid tissue, and exendin-4 treatment increased the number of $CD4^+$ and $CD8^+$ T-cells in the lymph nodes and reduced the number of $CD4^+CD25^+Foxp3^+$ regulatory T-cells in the thymus, suggesting direct effects of GLP-1 on the immune system (51). However, exendin-4 treatment was not associated with significant changes in the number of $CD4^+$ and $CD8^+$ T-cells or B-cells in the spleen (51). In the present study, neither GIP nor GLP-1 treatment produced significant effects on splenic $CD4^+$ T-cell migration in vitro (Fig. 5), whereas DPP-IV was stimulatory. The reason for the diverse effects of GLP-1/exendin-4 on the different subset of T-cells in vivo is not understood, but the potential for DPP-IV to modify additional subsets of lymphocytes, including those in lymph nodes and the thymus, requires further investigation. The beneficial actions of DPP-IV inhibitors and DPP-IV–resistant GLP-1 receptor agonists in type 2 diabetes have mainly been attributed to increased incretin receptor–mediated responses. Although it has been recognized that DPP-IV also plays a significant role in modulating immune function, no beneficial sequelae of inhibiting such actions in diabetes have been described. The ability of the DPP-IV inhibitor MK0431 to prolong islet graft survival by reducing immunocyte migration and islet infiltration suggests that the underlying cAMP/PKA/Rac1 could be an additional drug target.

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