Original Article

The Effects of DPP-IV Inhibition in NOD Mice with Overt Diabetes

(type 1 diabetes / sitagliptin / NOD mice / regulatory T cells / DPP-IV)

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**Abstract. Sitagliptin is a dipeptidyl peptidase IV (DPP-IV) inhibitor that exerts an anti-hyperglycaemic effect by preventing degradation of glucagon-like peptide 1 with subsequent β-cell stimulation and potential regeneration. We tested whether sitagliptin therapy in symptomatic non-obese diabetic (NOD) mice would lead to changes in the immune cell profile, improve β-cell survival and induce diabetes remission. Flow cytometry analysis of immune cells in the spleen and peripheral lymph nodes, immunohistology of the pancreas and DPP-IV activity were investigated in diabetic NOD mice, either treated or non-treated with sitagliptin, at 0, 7, 14 and 28 days after hyperglycaemia onset, and in non-diabetic NOD controls. While compared to diabetic controls sitagliptin prevented increase of the CD8$^+$/CD4$^+$ ratio in pancreatic nodes after four weeks (0.443 ± 0.067 vs. 0.544 ± 0.131; P < 0.05), the population of Tregs in lymph nodes increased from day 0 to 28 in both treated and non-treated diabetic groups (8 ± 1.76 vs. 13.45 ± 5.07 % and 8 ± 1.76 vs. 13.19 ± 5.58 %, respectively). The severity of islet infiltration was similar in both diabetic groups and decreased in parallel with β-cell loss. Surprisingly, sitagliptin blocked the DPP-IV activity only temporarily (on day 7, 277.68 ± 89.2 vs. 547.40 ± 94.04 ng/ml in the diabetic control group) with no apparent effect later on. In conclu-
sion, sitagliptin administered after the onset of overt hyperglycaemia in NOD mice had only a marginal immunological effect and did not lead to diabetes remission. Failure to block DPP-IV over time represents an important finding that requires further explanation.**

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Abbreviations: DPP-IV – dipeptidyl peptidase IV, GIP – glucose-dependent insulinotropic peptide, GLP-1 – glucagon-like peptide 1, NOD – non-obese diabetic, Tregs – CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells among CD4$^+$ lymphocytes.

Introduction

Type 1 diabetes mellitus is a chronic disease caused by autoimmune destruction of pancreatic β cells. T-cell mediated destruction of β cells results in insulin deficiency and hyperglycaemia. It is becoming increasingly clear that β-cell-specific CD8$^+$ cells play a pivotal role in the destruction process and constitute a significant portion of insulitis (Roep and Peakman, 2012). To abrogate the autoimmune destructive process, many immunological approaches to restore immune tolerance were performed. However, it has been demonstrated that the mere suppression of autoimmunity is not sufficient to reverse the manifested diabetes as most of the insulin-producing cells have already been destroyed at this stage (Ablamunits et al., 2007). Several immunointerventional studies showed that proliferation and/or regeneration of β cells were necessary to restore normoglycaemia in animal models (Ogawa et al., 2004; Shoda, 2005). In humans at least some of the β cells were detected as long as 50 years after the type 1 diabetes onset (Keenan et al., 2010). This finding suggests that sustained β-cell regeneration is possible in principle.

Recently, so-called incretin-based therapies started a new way of treatment for patients with type 2 diabetes. The incretin effect is mediated mainly by two gut hormones, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1). Both hormones enhance insulin secretion from pancreatic β cells in a glucose-dependent way. In addition, GLP-1 is capable to suppress α-cell glucagon secretion, enhances β-cell proliferation and inhibits β-cell apoptosis (Suen and Burn, 2012). Above that, the therapies targeted to stimulate insulin secretion by incretins also exhibit considerable immunological effects. Regulation of lymphocyte

proliferation and maintenance of peripheral regulatory T cells seems to be mediated through GLP-1 receptor, as its mRNA was found in T cells in the spleen, thymus and lymph nodes of NOD mice (Hadjiyanni et al., 2008). Therapy of NOD mice with GLP-1 analogue exendin 4 led to up-regulation of regulatory T cells and induction of IL-10 production (Xue et al., 2008). Incretin-mediated down-regulation of autoimmune response was also shown after syngeneic islet transplantation. This was evident from the change of prevailing pro-inflammatory (interferon- γ) to suppressive (TGF-β 1) cytokine expression (Suarez-Pinzon et al., 2008). The use of GLP-1 analogues or inhibitors of their degradation seemed to be attractive for human use in type 1 diabetes of recent onset. Although several authors demonstrated a positive effect (Ellis et al., 2011; Kielgast et al., 2011; Kutoh, 2011), the underlying mechanism remained unclear. No methods to prove possible β-cell regeneration and changes of immune profiles were used.

The aim of our study was to prove whether administration of dipeptidyl peptidase IV (DPP-IV) inhibitor sitagliptin leads to relevant changes in the immune system in a rodent model of autoimmune type 1 diabetes and to morphological changes in the endocrine pancreas. In NOD mice, similar studies have already been performed, but the therapy was started well before hyperglycaemia onset and diabetes manifestation (Hadjiyanni, 2008). In contrast, in our study the intervention started in the stage of fully developed diabetes. This situation is closer to the clinical situation, where most of the β cells are already destroyed or impaired.

Material and Methods

Mice and monitoring for diabetes

Female mice of NOD/LtJ (H-2^{g7}) purchased from Jackson Laboratory (Bar Harbor, ME) were utilized in the study. Mice were housed in specialized ventilated breeding boxes equipped with air filtration, and provided with autoclaved water and food ad libitum. Diabetes was diagnosed when the non-fasting blood glucose level was > 13 mmol/l on two consecutive days. The blood glucose level was measured from the tip of the tail and was monitored twice a week throughout the whole study period using a Performa Nano (Roche, Basel, Switzerland) glucometer. The animals were terminated under general anaesthesia. This study was approved by the local Committee for the Protection of Animals against Cruelty in IKEM.

Study groups

Three groups of NOD mice were included in the study. In a sitagliptin group (N = 28) diabetic NOD mice were treated with 100 mg of sitagliptin (Januvia, MSD, Hoddesdon, UK) dissolved in 200 ml of drinking water. Mice were kept with free access to drinking water. In a diabetic control group (N = 28) diabetic NOD mice

were kept without treatment. As a non-diabetic control group, non-diabetic NOD mice with sustained normoglycaemia (non-fasting blood glucose up to 9 mmol/l) in 26th week of age were used (N = 5). As soon as sustained diabetes was diagnosed, the animals were included either into the sitagliptin or diabetic control group within three days (day 0). The animals were terminated on days 0, 7, 14 and 28. No exogenous insulin was administered.

Flow cytometry

After termination, the spleen and pancreatic lymph nodes were harvested. Splenocytes isolated by mild dissociation in Iscove's modified Dulbeco's medium were separated by gradient centrifugation in Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Lymph node cells were isolated by dissociation of nodes through 60-μm mesh. Isolated cells were labelled for flow cytometric analysis. We used fluorescent antibodies against surface antigens CD3, CD4, CD8, CD25 (A488 anti-CD3, eBioscience, San Diego, CA; Qdot605 anti-CD4, Invitrogen, Eugene, OR; PerCP-Cy5.5 anti-CD8, eBioscience; APC anti-CD25, produced in the Institute of Microbiology ASCR, Prague, Czech Republic). Subsequently, the fixed cells were permeabilized (FoxP3 Staining Buffer Set, eBioscience) and the intracellular antigen FoxP3 was labelled using a PE-conjugated antibody (eBioscience). Appropriate PE-conjugated isotype control (eBioscience) was applied in control samples. The labelled cells were analysed using the flow cytometer LSR II with FACSDiva software (BD, San Jose, CA).

T-lymphocyte subpopulations and CD8$^+$ and CD4$^+$ T-lymphocyte ratios were determined as well as the population of CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells among CD4$^+$ lymphocytes (Tregs).

Immunofluorescence labelling of islet cells

Approximately half of each pancreas was fixed in 4% formaldehyde and embedded in paraffin. The immunofluorescence staining was performed in 5-μm sections. Prior to antigen detection, tissue sections were incubated in Target Retrieval Solution, Citrate pH 6, (Dako, Glostrup, Denmark) according to the manufacturer's instructions, briefly incubated in the solution for 30 min at 97 °C in a water bath and allowed to cool for 15 min at room temperature. Subsequently, the non-specific binding sites were blocked by 5% donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA). Specific antibodies against C-peptide, glucagon and Ki-67 were applied overnight at 4 °C (rabbit anti-C-peptide and rabbit anti-glucagon antibodies; Cell Signaling Technology, Danvers, MA, rat anti-mouse Ki 67 antibody; Dako). Anti-rat secondary antibodies conjugated with AlexaFluor 555 and anti-rabbit antibodies conjugated with AlexaFluor 488 (both Invitrogen) were used. Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO). The sections were mounted in the solution of

1,4-diazabicyclo[2.2.2]octane and poly-vinyl alcohol (Sigma-Aldrich) in glycerol and examined under an Olympus BX41 fluorescence microscope equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan).

Islet morphology

All detected islets on randomly selected pancreatic sections were examined in each animal. Separately, distribution of β and α cells was scored as follows: 0) no hormone-positive cell detected at the site of a former islet, 1) from one positive cell to 10 % of positive area in the residue of the islet, 2) 10–50 % of positive area in the islet, 3) 50–100 % of positive area in the islet. The grade of immune cell infiltration inside and around the islets was scored on sections counterstained with DAPI according to Zhang et al. (2007): 0) no apparent immune cell infiltration, 1) 1–10 % of the area occupied by infiltration, 2) 10–25 % of the area, 3) 25–50 % of the area and 4) more than 50 % of the islet residue area occupied with immune cell infiltration. The Ki-67-positive cells occurring in the sections were not quantified because co-localization of the C-peptide or glucagon with the Ki-67 marker was very rare.

Plasma DPP-IV activity

Blood samples were obtained by cardiac puncture on the day of study termination. Fifty µl of blood was mixed with natrium citrate (Sigma-Aldrich) and centrifuged to obtain the plasma. To measure the DPP-IV activity in the plasma sample, the DPP-IV-Glo™ Protease Assay (Madison, WI) was used. The activity was determined in relation to the activity of recombinant DPP-IV enzyme (R&D Systems, Minneapolis, MN).

Statistical analysis

Data were analysed using the GraphPad InStad 3 (San Diego, CA). Student's *t*-test and analysis of variance (ANOVA) were used to assess the differences between data groups. All data are presented as mean values ± standard deviation. P values < 0.05 were considered to be significant.

Results

Sitagliptin had no effect on diabetes remission rate

Fully developed diabetes with sustained hyperglycaemia was diagnosed in all mice at the time of inclusion into the sitagliptin or the diabetic control groups. The glycaemia control was not improved in the sitagliptin group compared to the diabetic control group. Treatment with sitagliptin did not increase the number of remissions (sustained glycaemia at levels lower than 13 mmol/l) as it is seen in the curves of glycaemia (Fig. 1). In both diabetic groups, one case of remission was observed out of 28 mice.

Pancreatic lymph node Tregs were up-regulated after diabetes manifestation

To investigate the effects of sitagliptin on regulatory immune cells, we analysed the population of Tregs in splenocytes and pancreatic lymph nodes. The changes in the Treg population in the diabetic mice groups were compared with those in non-diabetic NOD mice.

The course of Treg population development in the pancreatic lymph nodes is shown in Fig. 2A. The number of Tregs gradually increased in both diabetic groups from day 0 to day 28 with no difference between them. The average Treg rate in both groups tended to be higher than in the non-diabetic control group; however, the difference was not statistically significant.

We did not observe any significant changes in the populations of Tregs isolated from splenocytes of diabetic, sitagliptin-treated and non-diabetic NOD mice during the entire study period (Fig. 2B).

Sitagliptin prevented increase of CD8+/CD4+ ratio in pancreatic lymph nodes

As CD8+ cytotoxic T lymphocytes represent the main cellular elements in autoimmune islet destruction, we evaluated changes in their population in relation to the CD4+ helper T-lymphocyte population. We expressed
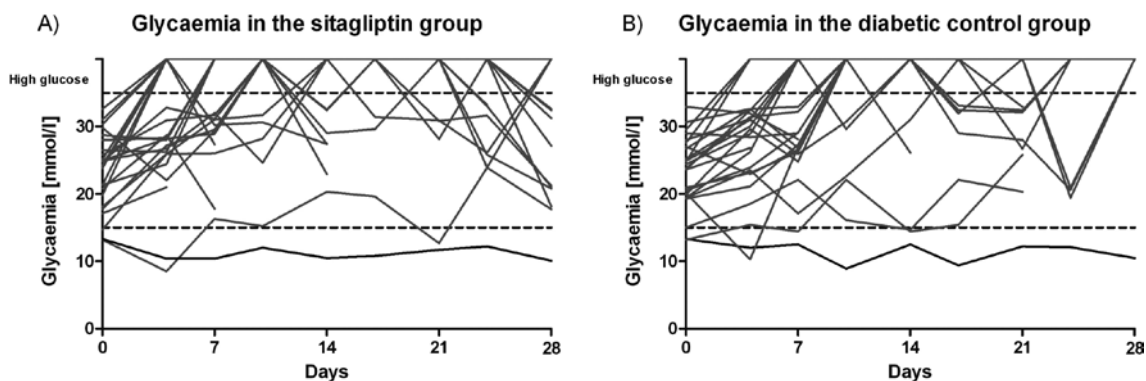

Fig. 1. Glycaemia: A) after sitagliptin administration, B) in the diabetic control group. Data represent blood glucose values from individual animals. The bottom line represents our threshold of initial glucose level crucial for remission. The upper line represents glucose levels up to 35 mmol/l. No animals received any exogenous insulin.
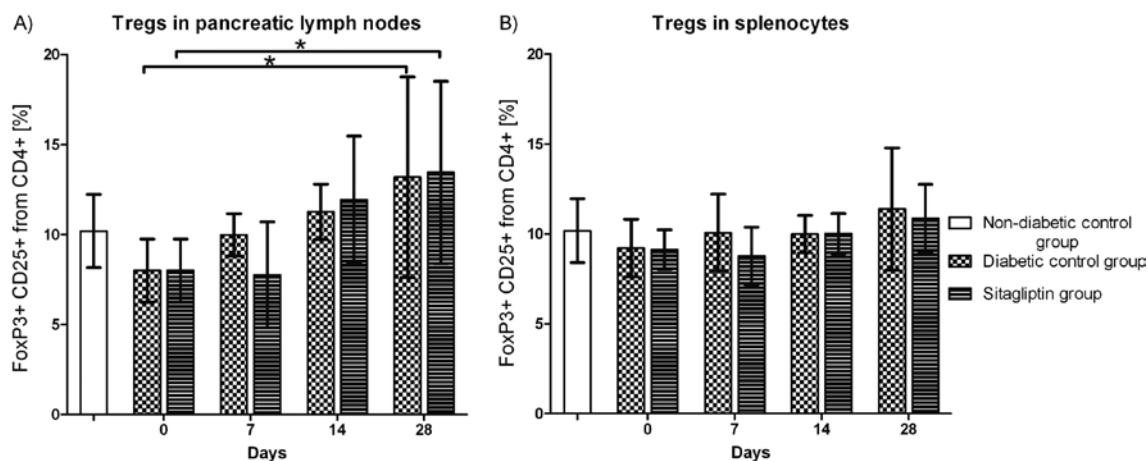
Fig. 2. Population of Tregs in pancreatic lymph nodes (A) and in splenocytes (B). A) In pancreatic lymph nodes the population of Tregs increased in the diabetic (13.19 ± 5.58 vs. 8 ± 1.76 %) and the sitagliptin (13.45 ± 5.07 vs. 8 ± 1.76 %) group on day 28 in comparison to day 0, $P < 0.05$. B) In the spleen we did not observe any difference between the diabetic (9.22 ± 1.9 %), the sitagliptin (9.13 ± 1.1 %) and the non-diabetic (10.18 ± 1.78 %) group on day 0 and neither on day 28 (11.39 ± 3.39 %; 10.86 ± 1.9 %; 10.18 ± 1.78 %).
The data are presented as means $\pm$ SD, * represents $P < 0.05$.

the relation of these populations in the form of $CD8^+/CD4^+$ T-lymphocyte ratio. The $CD8^+/CD4^+$ T-lymphocyte ratio decreased in splenocytes in the diabetic control group within two weeks after the onset of diabetes (Fig. 3A). Sitagliptin therapy had no effect on the $CD8^+/CD4^+$ T-lymphocyte ratio in splenocytes as compared with the diabetic as well as non-diabetic control groups.

In pancreatic lymph nodes, we observed pronounced changes in the $CD8^+/CD4^+$ T-lymphocyte ratio in the diabetic control group. The ratio increased significantly at four weeks. In contrast to the situation in diabetic control mice, the $CD8^+/CD4^+$ T-lymphocyte ratio in the sitagliptin group was not changed during the study period and was significantly lower at the end of the study in comparison with the diabetic control group. Moreover,

the ratio in the sitagliptin group did not differ from that in the non-diabetic control group (Fig. 3B).

Analysis of the $CD8^+$ and $CD4^+$ populations showed that the changes in $CD8^+/CD4^+$ T-lymphocyte ratios were caused solely by changes in the population of $CD8^+$ T lymphocytes. The population of $CD4^+$ T lymphocytes did not change during the study period (data not shown).

Severity of immune cell infiltration in islets was not influenced by sitagliptin

Stimulation of β-cell regeneration and suppression of inflammation in pancreatic islets following sitagliptin treatment have been reported (Kim et al., 2010). There-
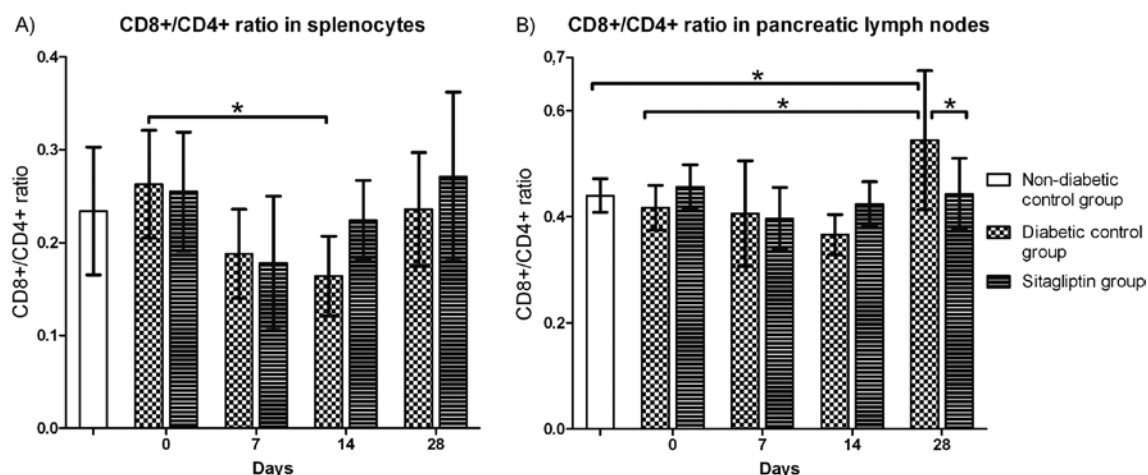

Fig. 3. $CD8^+/CD4^+$ T-lymphocyte ratio in splenocytes (A) and pancreatic lymph nodes (B). A) $CD8^+/CD4^+$ T-lymphocyte ratio decreased significantly in the diabetic control group on day 14 (0.164 ± 0.043) in comparison to day 0 (0.263 ± 0.058), $P < 0.05$. B) $CD8^+/CD4^+$ T-lymphocyte ratio increased significantly in the diabetic control group on day 28 (0.544 ± 0.131) in comparison to the sitagliptin (0.443 ± 0.067) and the non-diabetic group (0.44 ± 0.03), $P < 0.05$. In the diabetic control group the ratio increased from day 0 (0.417 ± 0.042) to day 28 (0.544 ± 0.131) $P < 0.05$.
The data are presented as means $\pm$ SD, * represents $P < 0.05$.
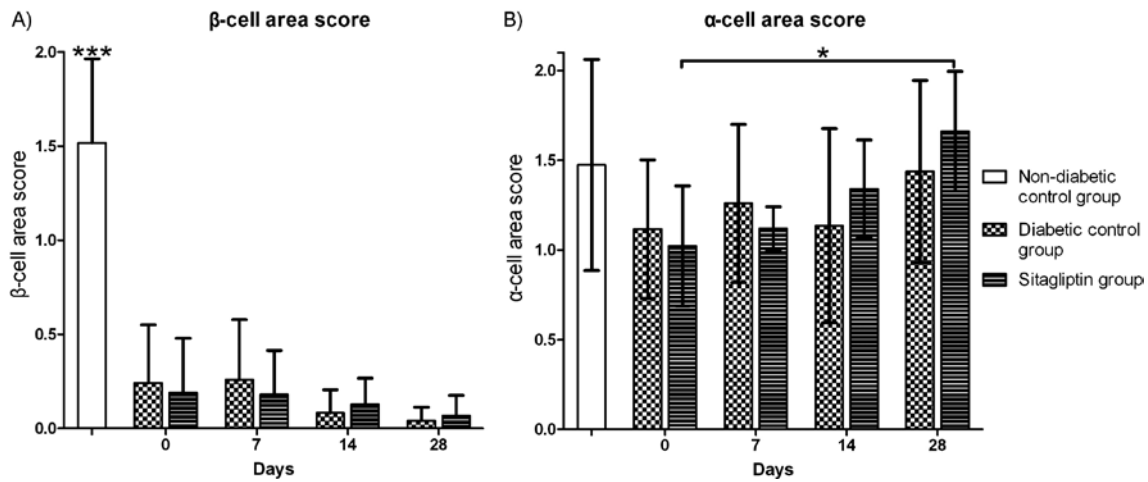
Fig. 4. Endocrine cell area scores: A) The area of C-peptide-positive cells in islets was apparently lower in both diabetic groups than in the non-diabetic control group on day 0 (0.19 ± 0.29 vs. 1.52 ± 0.45 in the sitagliptin group; 0.24 ± 0.31 vs. 1.52 ± 0.45 in the diabetic control group), P < 0.001. B) The area of glucagon-positive cells increased only in the sitagliptin group from day 0 to day 28 (1.02 ± 0.33 vs, 1.66 ± 0.33), P < 0.05, but it was not different from the diabetic and non-diabetic control groups.

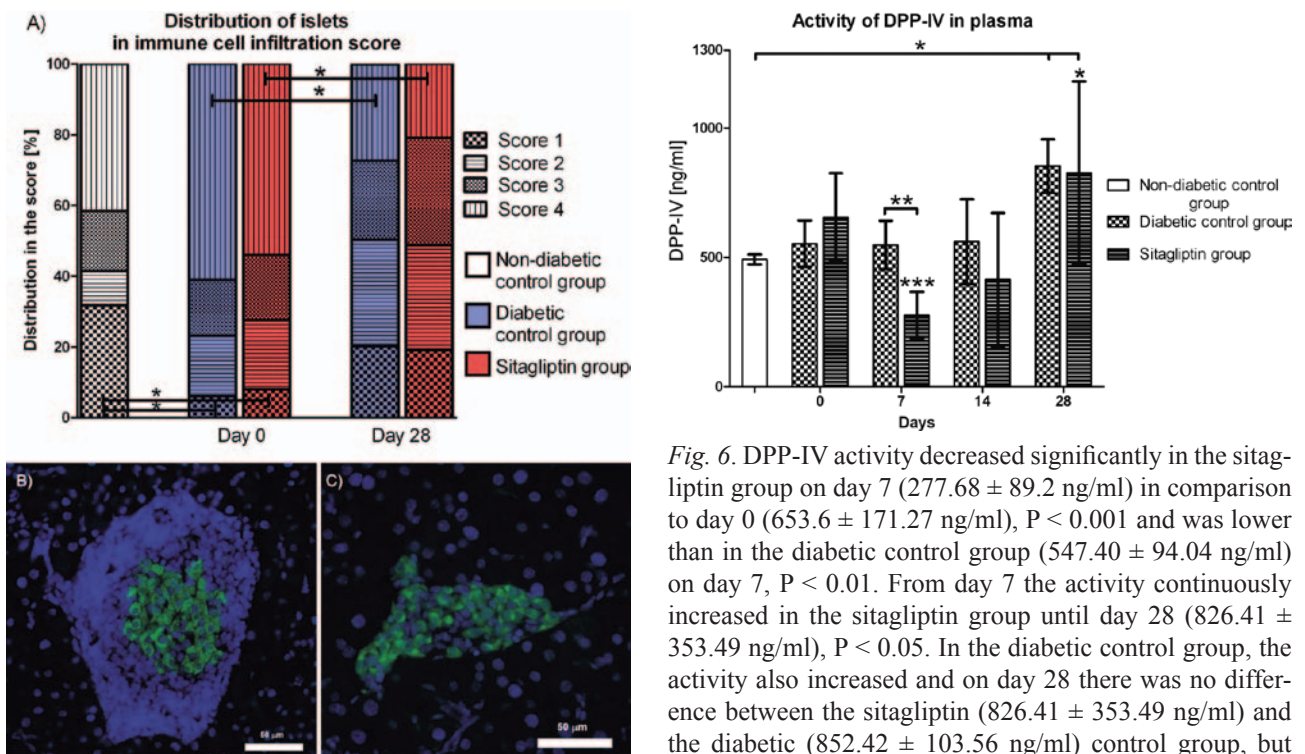The data are presented as means ± SD, * represents P < 0.05, *** represents P < 0.001.

Fig. 5. Immune cell infiltration in islets: A) Percentage of islets in the pancreas of the non-diabetic, diabetic and sitagliptin control groups. In the non-diabetic control group the percentage of islets in score 1 was significantly higher than in both diabetic groups on day 0. At the end of the study the severity of immune cell infiltration decreased in the diabetic groups. The data are presented as means ± SD, * represents P < 0.05. B) Severe immune cell infiltration inside and around an islet with persistent β cells in a non-diabetic mouse (green – C-peptide; blue – DAPI staining of nuclei). C) An islet with expanded α-cell area and very low immune cell infiltration in a diabetic mouse (green – glucagon, blue – DAPI staining of nuclei).
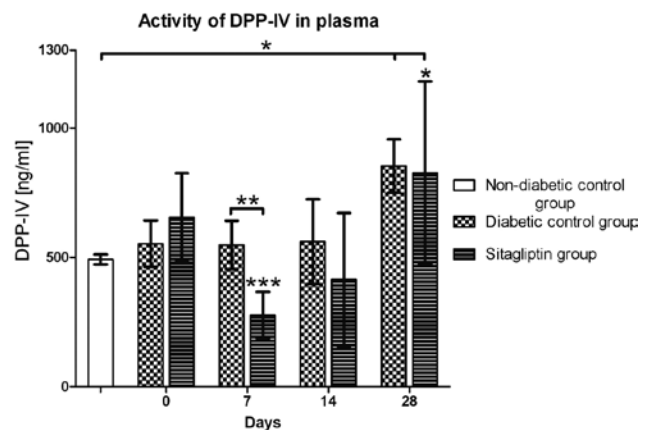

Fig. 6. DPP-IV activity decreased significantly in the sitagliptin group on day 7 (277.68 ± 89.2 ng/ml) in comparison to day 0 (653.6 ± 171.27 ng/ml), P < 0.001 and was lower than in the diabetic control group (547.40 ± 94.04 ng/ml) on day 7, P < 0.01. From day 7 the activity continuously increased in the sitagliptin group until day 28 (826.41 ± 353.49 ng/ml), P < 0.05. In the diabetic control group, the activity also increased and on day 28 there was no difference between the sitagliptin (826.41 ± 353.49 ng/ml) and the diabetic (852.42 ± 103.56 ng/ml) control group, but there was significant difference between these two groups and the non-diabetic group (491.93 ± 19.05 ng/ml).

The data are presented as means ± SD, * represents P < 0.05, ** represents P < 0.01, *** represents P < 0.001.

fore, our study was focused on changes of endocrine cell distribution and immune cell infiltration.

In both groups of diabetic NOD mice, the β-cell score was significantly lower on day 0 as compared to the non-diabetic control group. At the time of diabetes manifestation, most of the β cells had already been destroyed (Fig. 4A).

The α-cell score increased significantly from day 0 to day 28 only in the sitagliptin group. Despite this expansion, the score did not differ between all groups (Fig. 4B).

The severity of immune cell infiltration was apparently higher in diabetic animals than in the non-diabetic control group. The difference consisted mainly in higher frequency of islets with advanced infiltration (Fig. 5). In diabetic animals the severity of infiltration decreased over time, resulting in similar scores on day 28 to the non-diabetic controls.

Sitagliptin had only a transient effect on DPP-IV activity

To confirm the inhibitory effect of sitagliptin during the study, we measured the DPP-IV activity in the plasma. The expected drop in DPP-IV activity in the sitagliptin group was observed on day 7. Surprisingly, this decrease was not permanent and from day 14 the activity gradually increased. After two weeks, the difference between the sitagliptin and the diabetic control group was no more evident (Fig. 6). On day 28 the DPP-IV activity was significantly higher in both diabetic groups than in the non-diabetic group.

Discussion

In our study, we focused on the time-dependent effects of DPP-IV inhibitor sitagliptin in an animal model of type 1 diabetes. According to the results of previous studies, we expected improved glucose profiles, protection of pancreatic β cells and stimulation of regulatory T cells that might suppress the autoimmune β-cell destruction. However, in our setting in NOD mice, sitagliptin did not increase the rate of diabetes remission. In fact, the diabetes reversal rate was very low in both diabetic groups in the study. Similarly unsuccessful were also several studies using GLP-1 analogue exendin 4 which was started in the same stage of diabetes development as in our protocol (Xue et al., 2008; Tian et al., 2009). In successful studies, the DPP-IV inhibitor was used in NOD mice that were either still in the pre-diabetic stage, or the inhibitor was used together with exogenous insulin (Kim et al., 2010; Tian et al., 2010). Insulin therapy itself may contribute to lower activation of the autoimmune reaction by putting the persisting β cells into rest (Skowera et al., 2008). Other successful studies took advantage of combination therapy with a DPP-IV inhibitor or a GLP-1 analogue and another immunomodulatory agent (Ogawa et a., 2004; Tian et al., 2009) or with a proton pump inhibitor (Suarez-Pinzon et al., 2009).

Tian et al. (2010) demonstrated an association of diabetes remission with up-regulation of regulatory T cells. In our study, we did not find any difference between the Treg population in the sitagliptin and the diabetic control groups. This may explain the equal remission rate between these groups. Similar results were observed by Tian et al. (2009), who treated diabetic NOD mice with exendin 4. One week after the treatment, the population of Tregs was not significantly different between control diabetic and exendin 4-treated NOD mice. Comparing diabetic and non-diabetic control groups we found less Tregs in pancreatic lymph nodes in the non-diabetic control group. Also Mellanby et al. (2007) found that the proportion of CD4⁺CD25⁺ cells in pancreatic lymph node cells of diabetic mice was higher than that in non-diabetic NOD mice. In contrast to our data, Xue et al. (2008) demonstrated that therapy with exendin 4 led to an increase in the number of Tregs in the spleen.

The effector CD8⁺ cells represent the crucial cellular component in the autoimmune destruction of β cells. The cytotoxic autoimmune process is driven especially by autoantigens originating from the β cells. Under hyperglycaemia, the remaining β cells in the pancreatic islets are stimulated to higher synthesis of preproinsulin, which is one of the main diabetogeneic autoantigens. In consequence, the remaining β cells in hyperglycaemic conditions are subjected to a greater killing efficiency of cytotoxic T lymphocytes (Skowera et al., 2008). Therefore, the changes in the frequency of CD8⁺ cells could reflect the intensity of the autoimmune response. In our study we observed a significant increase in CD8⁺ T lymphocytes in pancreatic lymph nodes of diabetic NOD mice at the end of the study. Of note, in the sitagliptin group the frequency of CD8⁺ T lymphocytes was identical with that in the non-diabetic group and significantly lower than in the diabetic control group. This suggests a protective effect of sitagliptin treatment against expansion of cytotoxic T lymphocytes in the local lymph nodes. Contrary to that, the significant decrease of CD8⁺ T lymphocytes in splenocytes of the diabetic control NOD mice found in our study may be caused by their migration into the pancreatic islets during the first weeks after the diabetes onset.

In addition to the changes in CD8⁺ population, we evaluated the immune cell infiltration in the pancreatic islets. The time-dependent decrease of immune cell infiltration corresponded to the very low number of β cells remaining in the islets. From our results obtained at different time points we can conclude that the decrease of immune cell infiltration is probably caused by lower release of β-cell antigens rather than by treatment with sitagliptin, as it was assumed by Tian et al. (2010).

The gradual increase of the α-cell score observed in our study is in accordance with the replacement of destructed β cells by an increased number of α cells that was described by Takeda et al. (2011). Contrary to this study group we did not observe any effect of sitagliptin treatment on the decrease of the α-cell proliferation. Nevertheless, rarely detected expression of Ki-67 antigen did not prove changes in α-cell proliferation. In β cells, we did not detect the Ki-67 antigen at all. Similarly, data of Tian et al. (2009) indicated that therapy with exendin 4 alone did not stimulate replication of residual β cells. The ideal window for using incretin-based agents is likely to be before or immediately after diagnosis, when a significant β-cell mass is still viable (Bosi, 2010).

In our study, the most interesting effect of sitagliptin treatment was observed in DPP-IV activity. To our knowledge, the DPP-IV activity was not monitored at different time points of the intervention in any other study. To our surprise, we demonstrated that the inhibitory effect of sitagliptin was only temporary within the first seven days, although the drug was being administered throughout the study. Why DPP-IV inhibition does not work later on needs further investigation. As the loss of the inhibitory effect was uniformly found in all sitagliptin-treated animals, we consider this finding as important and worth testing in human pharmacology. An increase of DPP-IV activity after diabetes onset (also present in our study) has already been reported in humans (Mannucci et al., 2005), but a failure of sitagliptin or another DPP-IV inhibitor to block this activity over time has not been tested yet.

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