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CXCL10 Impairs β Cell Function and Viability in Diabetes through TLR4 Signaling

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SUMMARY

In type 1 and type 2 diabetes (T1/T2DM), β cell destruction by apoptosis results in decreased β cell mass and progression of the disease. In this study, we found that the interferon γ-inducible protein 10 plays an important role in triggering β cell destruction. Islets isolated from patients with T2DM secreted CXCL10 and contained 33.5-fold more CXCL10 mRNA than islets from control patients. Pancreatic sections from obese nondiabetic individuals and patients with T2DM and T1DM expressed CXCL10 in β cells.

Treatment of human islets with CXCL10 decreased β cell viability, impaired insulin secretion, and decreased insulin mRNA. CXCL10 induced sustained activation of Akt, JNK, and cleavage of p21-activated protein kinase 2 (PAK-2), switching Akt signals from proliferation to apoptosis. These effects were not mediated by the commonly known CXCL10 receptor CXCR3 but through TLR4. Our data suggest CXCL10 as a binding partner for TLR4 and as a signal toward β cell failure in diabetes.

INTRODUCTION

In both type 1 and type 2 diabetes (T1/T2DM), the major mechanism leading to the decreased β cell mass is increased β cell apoptosis (Butler et al., 2003; Kurrer et al., 1997). A lack of proliferation may be a reason for insufficient compensation for a higher insulin demand in T2DM (Meier et al., 2008), e.g., under the situation of obesity, pregnancy, or cortisol excess.

In T1DM, β cell destruction is mediated through the autoimmune system and the resulting high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species (ROS), and other inflammatory products (Eizirik and Mandrup-Poulsen, 2001). Several mechanisms have been postulated to contribute to increased β cell apoptosis in T2DM. These include endoplasmic reticulum (ER) stress, hyperglycemia, hyperlipidemia, oxidative stress, islet amyloid polypeptide (IAPP) oligomers, and activation of proinflammatory cytokines (Donath et al., 2005).

The role of inflammation in the pathogenesis of T2DM is supported by several recent prospective studies. Inflammatory markers are found in obesity, insulin resistance, and diabetes and link the pathology of the metabolic diseases (Wellen and Hotamisligil, 2005). Low-grade inflammation and activation of the innate immune system can lead to β cell failure in T2DM (Pickup, 2004). Although the markers of immune activation are below the levels of acute inflammation, there are increases in acute phase proteins such as CRP and factors associated with endothelial activation, including ICAM; VCAM and t-PA; cytokines, e.g., IL-1β, IL-6, and TNFα; and chemokines, e.g., CCL5 (former RANTES), MCP-1, IL-8, and CXCL10 (Kolb and Mandrup-Poulsen, 2005), which could directly lead to impaired β cell survival and function, shown for cytokines in numerous publications in vitro (Rabinovitch and Suarez-Pinzon, 1998). The role of chemokines remains to be elucidated.

Interferon γ-inducible protein (IP-10), also known as chemokine (C-X-C motif) ligand 10 (CXCL10), is a 10 kDa secreted protein produced in a variety of cells, including endothelial cells, monocytes, fibroblasts, and keratinocytes (Luster, 1998). Isolated human islets produce CXCL10 in response to IFN-γ (Cardozo et al., 2003). Increased levels of CXCL10 have been detected in serum of patients with newly diagnosed as well as longstanding T1DM (Nicoletti et al., 2002; Shimada et al., 2001), T2DM (Xu et al., 2005), and in patients with high risk for developing T1DM (Nicoletti et al., 2002) and T2DM (Giulietti et al., 2006). In the nonobese diabetic (NOD) mouse model, CXCL10 is produced in pancreatic islets even before detectable insulitis (Cardozo et al., 2003; Li et al., 2005). In a model of LCMV-induced diabetes, CXCL10 has been shown to be among the first chemoattractant factors expressed in the pancreas (Christen and Von Herrath, 2004). These data suggest that upregulated CXCL10 synthesis takes place either prior to or during an early phase of the disease. Inhibition of CXCL10 delayed immune-mediated diabetes in the NOD mouse (Morimoto et al., 2004), and transgenic mice expressing CXCL10 in β cells show spontaneous infiltration of lymphocytes as well as impairment of β cell function (Rhode et al., 2005). In mice deficient for the CXCL10 receptor CXCR3, T cell-mediated diabetes is substantially delayed (Frigerio et al., 2002). Collectively, these studies imply

that CXCL10 is important in mediation of β cell apoptosis in T1DM and a potential candidate in T2DM. In order to address this possibility, we first posed the question of whether CXCL10 expression is increased in the islet in T1DM, in T2DM, or in obesity. Having affirmed that postulate, we investigated whether increased CXCL10 expression in the islet in T2DM is secondary to most common metabolic milieus in T2DM, specifically increased glucose or fatty acid concentrations. Because the answer is negative, we further postulated that the expression of CXCL10 may be a primary event in the evolution of β cell dysfunction in T2DM. Finally, we examined the effects of CXCL10 on β cell function and viability in human islets and the underlying mechanisms.

RESULTS

CXCL10 Is Expressed and Secreted in Isolated Pancreatic Islets from Organ Donors with T2DM

Healthy human islets neither secreted nor expressed CXCL10 (Figures 1A and 1C, panel 1). In contrast, human isolated islets from organ donors with T2DM secreted 65.62 ± 14.6 pg/ml/20 islets within 24 hr, which were accumulated after 96 hr (196 ± 58 pg/ml/20 islets; $p < 0.001$ versus control islets) (Figure 1A). As a positive control for CXCL10 secretion, we incubated healthy islets in the presence of the cytokine IFN-γ (1000 U/ml). These islets secreted 999.1 ± 127.2 pg/ml CXCL10/20 islets into the culture medium during the 24 hr culture period.

The expression of CXCL10 mRNA by T2DM islets was confirmed by quantitative RT-PCR. We found a 33.5-fold increase of CXCL10 expression in the T2DM group ($p < 0.01$) (Figure 1B). When we compared CXCL10 expression from islets from lean and obese nondiabetic individuals, we found a small but significant 3.3-fold increase in the obese islets ($p < 0.05$) (Figure 1B). Islets were isolated from three organ donors with diabetes (average HbA1c was 8.1, glucose was between 132 and 224 mg/dl, age was 60.7, and BMI was 27.2; cause of death was subarachnoidal hemorrhage and stroke), from four obese organ donors (average age was 48.5, and BMI was 34.3), and from four lean control organ donors (average age was 54.3, and BMI was 21.6).

Marselli et al. have compared several genes for their changes during isolation by comparison of islets from laser capture dissection and conventional islet isolation, and they found that CXCL10 expression was unchanged by islet isolation (Marselli et al., 2008). Therefore, it is unlikely that CXCL10 expression was induced by the islet isolation.

We also investigated the islet cellular localization of CXCL10 in pancreatic sections obtained at autopsy from seven poorly controlled type 2 diabetic patients, all with documented fasting plasma glucose > 145 mg/dl (see Table S1 available online for age, gender, BMI, and glucose levels), as well as from one pancreas biopsy obtained from a patient newly diagnosed with T1DM (Meier et al., 2005). We have also included five autopsy sections from obese and lean nondiabetic individuals. Double-immunostaining for CXCL10 in red (Figure 1C, panels 1, 3, 5, and 7) and insulin in green (Figure 1C, panels 2, 4, 6, and 8) revealed the presence of CXCL10 localized in the β cells in human pancreatic sections from patients with T2DM (Figure 1C, panels 5 and 6) and T1DM (Figure 1C, panels 7 and 8), but not in lean nondiabetic controls (Figure 1C, panels 1 and 2). In contrast, in the nondiabetic obese controls, CXCL10-positive cells were observed (Figure 1C, panels 3 and 4). The duration of T2DM in the different patients ranged from 1 to 26 years, and we did not detect differences in CXCL10 expression among them. CXCL10 was absent in α cells, δ cells, and exocrine cells. Also, isolated human islets exposed to IFN-γ stained positive for CXCL10 (data not shown).

To investigate the processes that mediate CXCL10 expression in vitro, human islets from nondiabetic organ donors were exposed for 48 hr to a diabetic milieu (0.5 mM palmitate, 0.5 mM palmitate plus 0.5 mM oleate, 2 ng/ml IL-1β, 1000 U/ml IFN-γ at 5.5 or 33.3 mM glucose) (Figure 1D, left panel), the combination of the three cytokines IL-1β plus IFN-γ plus TNF-α (Figure 1D, middle panel), or for 24 hr to 20 or 40 μM h-IAPP or 100 U/ml TNF-α alone (Figure 1D, right panel). Western blot analysis revealed that CXCL10 expression was independent of glucolipotoxicity or h-IAPP. CXCL10 expression was induced by TNF-α and by IFN-γ, confirming our RT-PCR and immunostaining results (data not shown), and by the cytokine mixture IFN-γ plus IL-1β (Figure 1D, middle panel). Addition of TNF-α potentiated CXCL10 expression induced by IFN-γ plus IL-1β. In contrast, elevated glucose levels did not further induce IFN-γ-induced CXCL10 expression (Figure 1D, lower panel).

CXCL10 Induces β Cell Death and Proliferation and Impaired β Cell Function

To investigate the consequence of CXCL10 expression in human type 2 diabetic islets, we added human recombinant CXCL10 to cultured islets from healthy organ donors. We used 0.1 ng/ml CXCL10, a concentration that we measured from islets from donors with T2DM and that has been found in the serum from patients with diabetes, as well as 50 ng/ml, the ED_{50} for the ability of CXCL10 to chemoattract T lymphocytes cultured in the presence of IL-2 (Loetscher et al., 1996).

Both 0.1 and 50 ng/ml CXCL10 increased β cell apoptosis by 1.6-fold ± 0.3-fold (n.s.) and 2.0-fold ± 0.4-fold ($p < 0.01$) after 1 day (data not shown) and 2.7-fold ± 0.5-fold and 2.9-fold ± 0.6-fold ($p < 0.001$) after 4 days of culture compared to untreated control islets at 5.5 mM glucose (Figures 2A and 2C). After 1 day of culture, CXCL10 showed a tendency to induce β cell proliferation (data not shown), which was significant after 4 days of treatment (1.5-fold with 0.1 ng/ml and 2.7-fold with 50 ng/ml, $p < 0.05$) compared to controls (Figures 2B and 2D). Basal apoptosis and proliferation rates measured during the culture period in control conditions did not change. To verify a specific CXCL10-mediated effect and to exclude an endotoxin contamination of the recombinant protein, we preincubated human recombinant CXCL10 for 30 min with a neutralizing CXCL10 antibody and then exposed it to the islets. Treatment with anti-CXCL10 Ab fully inhibited CXCL10-induced β cell apoptosis after 4 days ($p < 0.001$) compared to CXCL10-treated cells alone (Figure 2C). Potential LPS contamination of the recombinant CXCL10 was further investigated by preincubation of CXCL10 for 30 min at 37°C in 5% CO_2 with 2 μg/ml of polymyxin B, which inhibits LPS (Giamarellos-Bourboulis et al., 2009). This did not change CXCL10-mediated β cell apoptosis, confirming a CXCL10-specific effect (β cell apoptosis was 2.5-fold induced by CXCL10 pretreated with polymyxin, $p < 0.0001$).

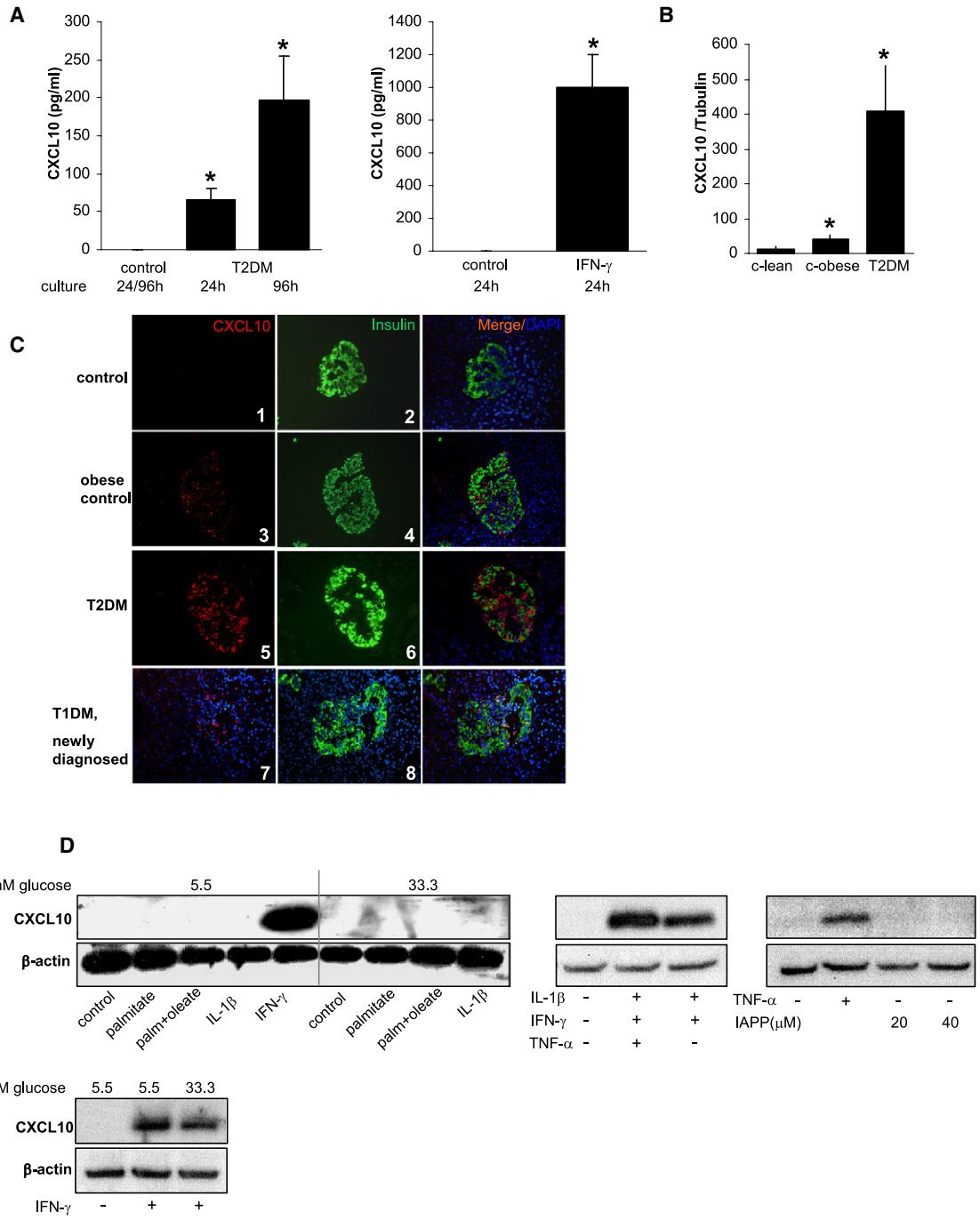

Figure 1. CXCL10 Is Increased in Islets from Patients with T2DM

(A) CXCL10 secretion from isolated human pancreatic islets from five healthy controls and three donors with T2DM.

(B) RT-PCR quantification of CXCL10 mRNA expression in human islets. Results represent means ± SE from four lean nondiabetic, four obese nondiabetic, and three organ donors with T2DM.

(C) Double/triple-immunostaining in pancreatic tissue sections from a representative lean nondiabetic control (1 and 2), obese control (3 and 4), and a patient with T2DM (5 and 6) or T1DM (7 and 8) (magnification, ×200).

(D) Human islets were cultured at 5.5 or 33.3 mM glucose for 48 hr (six experiments from six healthy organ donors). *$p < 0.05$ versus healthy untreated control islets.

We also show that, after 1 day culture in suspension dishes, apoptosis is induced by CXCL10 using western blot analysis of cleaved caspase 3 (Figure 3G). To confirm that CXCL10 actions were mediated by apoptosis and to investigate a matrix independent effect, we quantified cleavage of caspase 3. Human islets were cultured in suspension for 48 hr, Bouin's

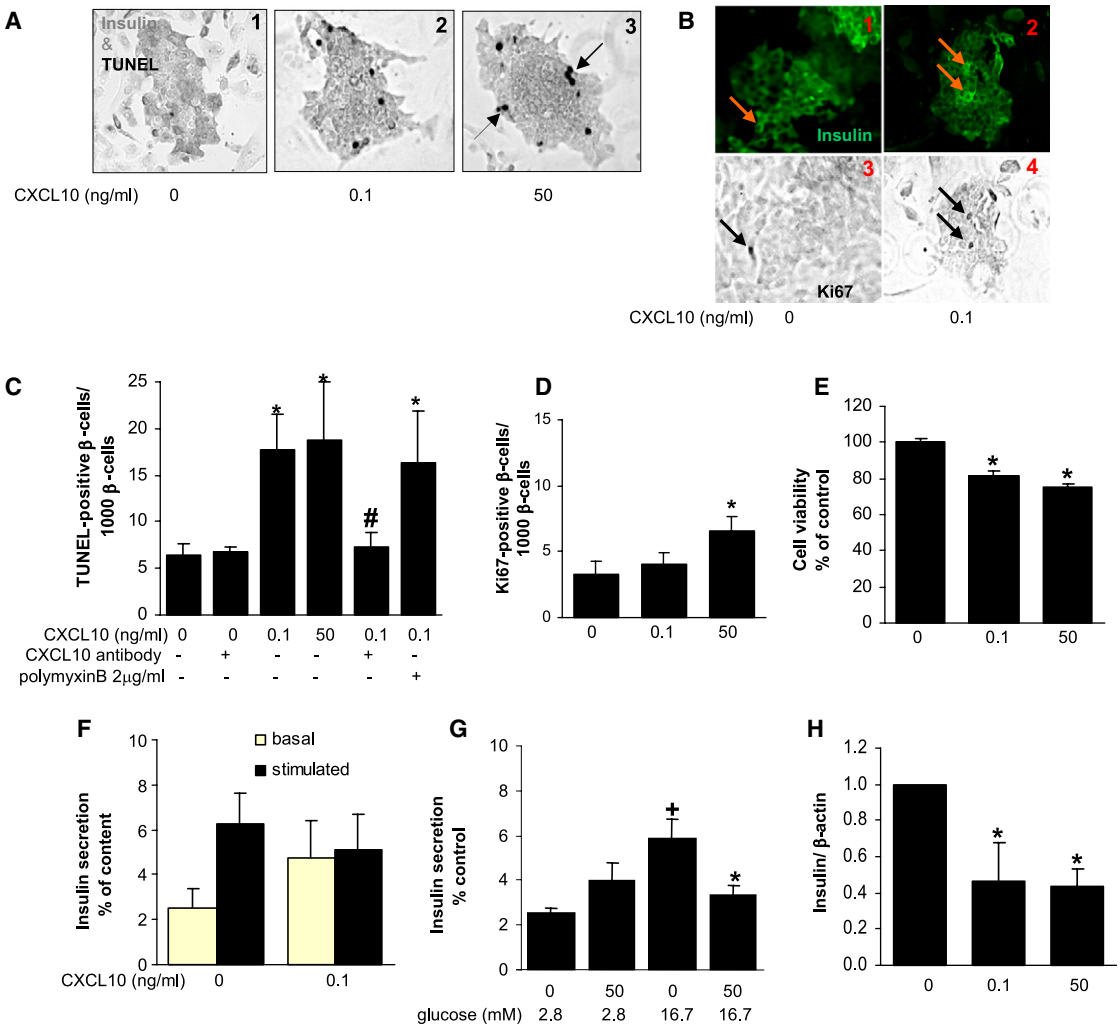

Figure 2. CXCL10 Induces β Cell Death and Impaired β Cell Function

(A–D) Human pancreatic islets cultured with increasing CXCL10 concentrations dishes for four days. Apoptosis was measured by the TUNEL assay (A and C) and proliferation by the Ki-67 antibody (B and D). Black arrows indicate doublets of apoptotic β cells indicative of postmitotic apoptosis (A3), and the red and black arrows mark nuclei stained positive for Ki-67 (B).

(C and D) Human recombinant CXCL10 was preincubated for 30 min with an antagonistic CXCL10 antibody or polymyxinB and then incubated with the islets. Results are means ± SE of TUNEL-positive (C) or Ki-67-positive (D) β cells per 1000 counted β cells (the average number of cells counted was 4400 for TUNEL and 6358 for Ki-67 for each treatment group in each experiment) from four different experiments in triplicate from three to seven different organ donors.

(E) MTT was performed on RINm5F cells exposed for 4 days to CXCL10.

(F) GSIS from islets exposed to CXCL10. Basal and stimulated insulin secretion indicate the amount secreted during 1 hr incubations at 2.8 (basal) and 16.7 mM (stimulated) glucose following the 6 day culture period and normalized to insulin content.

(G) Acute basal and stimulated insulin secretion for 1 hr, normalized to insulin content.

(H) Quantitative RT-PCR analysis of insulin expression. Total mRNA was isolated from human islets from three different organ donors and cultured for 1 day in 5.5 mM glucose with or without CXCL10.

Results are means ± SE of untreated controls at 5.5 mM glucose. $^{*}p < 0.05$ to untreated control; $^{+}p < 0.05$ to 2.8 mM glucose; $^{\#}p < 0.05$ to 0.1 ng/ml CXCL10.

fixed, and paraffin embedded, and islet sections were double-stained for cleaved caspase 3 and insulin. CXCL10 induced a 2.6-fold ($p < 0.05$) and 3-fold ($p < 0.001$) increase in caspase 3-positive β cells at 0.1 and 50 ng/ml CXCL10, respectively (data not shown). In order to quantify β cell number after CXCL10 exposure, we measured β cell viability in the β cell line RINm5F and detected an 18% and 25% decrease in viability by 0.1 and 50 ng/ml CXCL10, respectively ($p < 0.01$) (Figure 2E).

Chronic preincubation of CXCL10 for 6 days induced a 48% decrease in insulin stimulatory index (stimulated over basal insulin secretion), compared to control ($p < 0.05$) (Figure 2F). To determine the acute effect of CXCL10 on insulin secretion, we cultured the islets in the presence of CXCL10 at low and high glucose for 1 hr. At low glucose, CXCL10 had no significant effect; however, at high glucose, GSIS was 45% decreased by CXCL10 compared to control ($p < 0.05$), suggesting that the combination of high glucose and CXCL10, even in the short term, inhibits β cell function (Figure 2G).

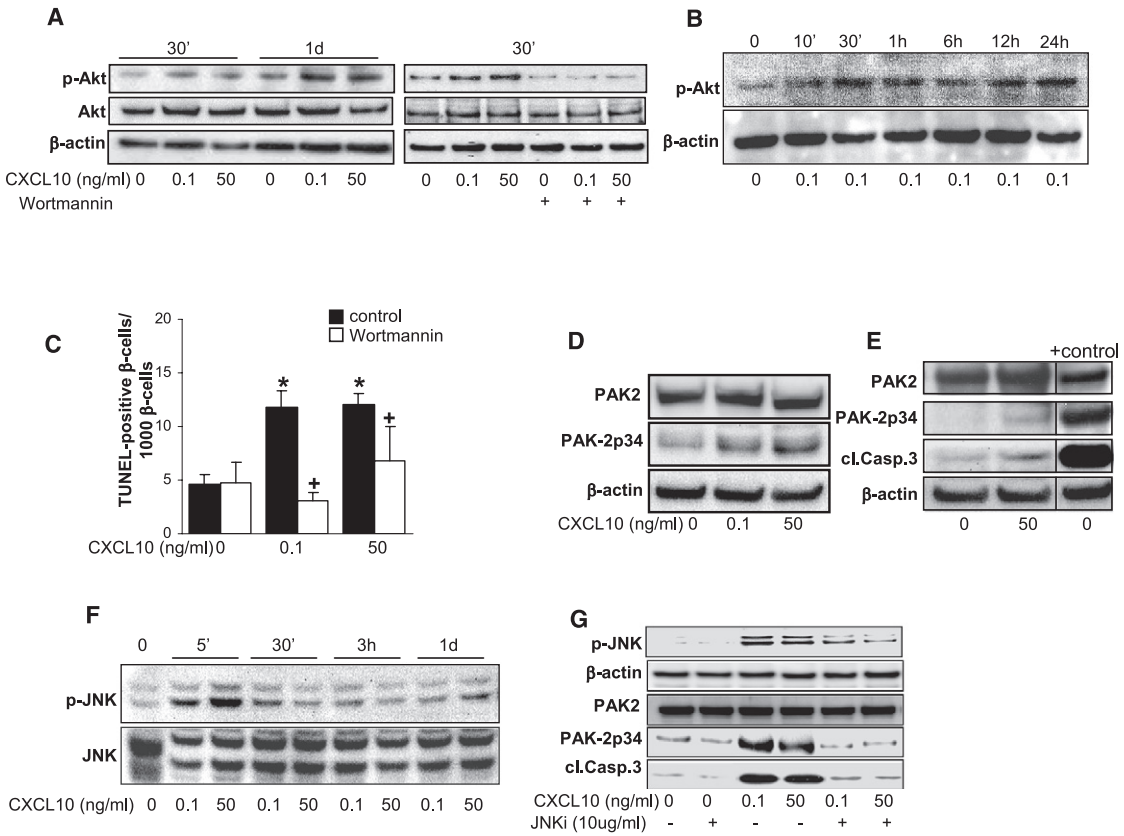

Figure 3. Activation of the PI3K/Akt Pathway by CXCL10 Subsequently Leads to PAK-2 Cleavage and Apoptosis in β Cells

(A and B) Western blot analysis of p-Akt in human islets.

(C) Human islets were cultured for 4 days in 5.5 mM glucose. Results are mean ± SE of TUNEL-positive per 1000 β cells. *$p < 0.05$ relative to islets cultured at 5.5 mM glucose alone; $^{+}p < 0.05$ relative to islets in the absence of Wortmannin at the same CXCL10 concentration.

(D–G) Western blot analysis of human islets cultured for 1 day or from 5 min to 1 day (F) or from purified rat β cells (E). Heat shock-treated CHO cells were used as a positive control for activated PAK-2p34 (E).

Representative Western blots from four independent experiments from four different organ donors (A, B, D, and F), two different donors (G), or three different β cell isolations (E).

Insulin content decreased by 46% in the CXCL10-treated cells (0.01 ng/ml, $p < 0.05$) (data not shown). Insulin mRNA was decreased by 52% and 56% after 1 day of culture by 0.1 and 50 ng/ml CXCL10, respectively ($p < 0.05$) (Figure 2H). We then examined the effects of two other ligands of CXCR3—IFN-γ-inducible chemokines CXCL11 (former I-TAC) and CXCL9 (former Mig)—on β cell turnover in human isolated islets. In contrast to CXCL10, neither CXCL11 nor CXCL9 at comparable concentrations, based on their activity, affected β cell apoptosis and β cell proliferation (Figure S1A) after 4 days of culture. To investigate a functional response induced by CXCL11 and CXCL9, the mobilization of intracellular calcium was assessed by Fluo-3 in INS-1 cells. Both CXCL11 and CXCL9 induced the mobilization of $[Ca^{2+}]i$ in the cells, which confirms their activity (Figure S1B). As positive control for $[Ca^{2+}]i$ influx in the β cells, we used 20 mM glucose, which also resulted in Fluo-3 uptake (data not shown).

CXCL10-Mediated PAK-2 Cleavage Switches Akt Signaling into Apoptosis

Next, we examined the molecular mechanism of the deleterious effects of CXCL10 on cultured human islets. We detected increased Akt phosphorylation within 30 min of culture with CXCL10, which was sustained through the entire 24 hr culture period (Figures 3A and 3B). The PI3K inhibitor Wortmannin (100 nM) blocked this Akt phosphorylation in the presence of CXCL10, confirming that PI3K signaling was involved (Figure 3A). Furthermore, PI3K inhibition blocked CXCL10-induced β cell death (Figure 3C). Here, we show that sustained Akt activation can trigger apoptosis in the β cell. One of the direct downstream effectors of Akt is the p21-activated kinase 2 (PAK-2). Akt-mediated phosphorylation of PAK-2 mediates cell survival; however, if PAK-2 is proteolytically cleaved, apoptosis is induced (Jakobi et al., 2003). We found full-length PAK-2 constitutively expressed in human islets and rat β cells. However, upon CXCL10-induced caspase 3 cleavage (Figure 3G for human islets and 3E for rat β cells), we detected increased levels of the cleaved C-terminal fragment of PAK-2 (PAK-2p34) in human islets and in purified rat β cells (Figures 3D and 3E). Heat-shocked hamster ovary cells (CHO) were used as positive control for PAK-2 cleavage (Chan et al., 1998). Besides caspase 3 and PAK-2 activation, CXCL10 also triggered JNK activation, a pathway that is activated by inflammatory cytokines and free fatty acids in the β cell, leading to β cell apoptosis (Hotamisligil, 2005).

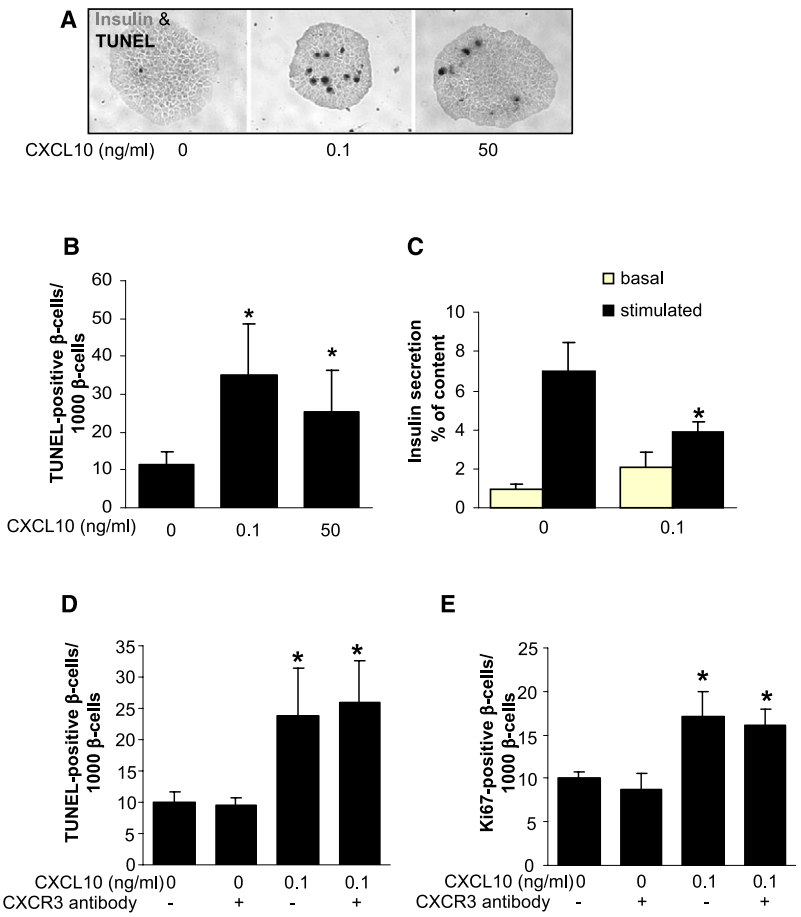

Figure 4. The Effects of CXCL10 Are CXCR3 Independent

(A–D) Analysis of β cell apoptosis and function in *CXCR3*$^{-/-}$ mice. Mouse islets were cultured on extracellular matrix-coated dishes for 4 days.

(A and B) TUNEL assay.

(C) Basal and stimulated insulin secretion during 1 hr incubation at 2.8 (basal) and 16.7 mM (stimulated) glucose following a 4 day culture period.

(D and E) Isolated islets from C57BL/6J mice were precultured for 1 hr with goat serum (control) or with goat anti-mouse CXCR3 neutralizing antibody prior to addition of 0.1 ng/ml recombinant mouse CXCL10.

Results are means ± SE of the percentage of TUNEL-positive (B and D) and Ki-67-positive (E) β cells per 1000 β-cells. Results are means ± SE of two independent experiments in quadruplicates from six mice, respectively. *$p < 0.05$ to untreated control islets.

(F) RT-PCR for CXCR3 was performed from purified human β cells and human islets cultured for 3 days.

(G) Immunohistochemical analysis for CXCR3 on sections from human isolated islets. Magnification, ×200.

to the pattern of Akt activation. Using the small molecule inhibitor of JNK (JNKi), we show that JNK inhibition protected from CXCL10 induced β cell apoptosis and PAK-2 cleavage (Figure 3G). This was confirmed by the costaining of human islets with insulin and the TUNEL assay, in which coculture of JNKi 30 min before exposure of the human islets with CXCL10 significantly abolished β cell apoptosis (data not shown).

Crosstalk between PI3K/Akt and JNK has been shown before (Aikin et al., 2000), and proteolytic activation of PAK-2 correlated with activation of JNK in previous reports (Rudel et al., 1998). Therefore, we aimed to investigate the link of the CXCL10-activated pathways through JNK.

After 5 min of exposure, CXCL10 induced JNK activation that still persisted after 1 day of culture (Figure 3F), which was similar

The Effect of CXCL10 on β Cells Is Independent of the CXCR3 Receptor

CXCL10 is the ligand for the receptor CXCR3. To further study the mechanisms of CXCL10-induced β cell destruction, we investigated the role of its receptor, CXCR3. We exposed islets isolated from CXCR3 knockout mice to CXCL10. Similar to our observations in human islets, CXCL10 induced β cell apoptosis in islets from wild-type C57Bl/6J mice (Figure 4A). Furthermore, we did not detect alterations of CXCL10-induced increase in β cell death (Figures 4A and 4B) and insulin secretion (Figure 4C) in islets isolated from CXCR3 knockout mice (0.1 and 50 ng/ ml CXCL10 increased β cell apoptosis 2.7-fold and 2.0-fold, respectively; $p < 0.05$). By preincubation with 0.1 ng/ml CXCL10 for 6 days, glucose-stimulated insulin secretion was decreased by 41% and stimulatory index by 77% compared to untreated control ($p < 0.05$).

Compared to human islets, the deleterious effect of chronic CXCL10 pretreatment on GSIS was greater in mouse islets. The stimulatory index under control conditions was higher in mouse than in human islets (2.6 versus 6.9). This could be due to different islet isolation procedures. Mouse islets were isolated

in our lab and put in culture immediately after isolation, whereas human islets were shipped to our lab and had to be precultured after shipment.

Then, we precultured isolated pancreatic islets from C57Bl/6J mice with goat anti-mouse CXCR3, an antagonistic antibody to CXCR3 that has been shown previously to block CXCL10-mediated effects (Molesworth-Kenyon et al., 2005). After 1 hr preculture, CXCL10 was added to the islets for the 3 day culture period. CXCL10 increased apoptosis 2.4-fold without and 2.6-fold with CXCR3 neutralization compared to serum-treated control ($p < 0.05$) (Figure 4D). In parallel, proliferation was 1.7-fold increased without and 1.6-fold increased with CXCR3 neutralization compared to serum treated control ($p < 0.05$) (Figure 4E). There were no differences between the treatment groups with or without the neutralizing antibody.

Because CXCL10 exhibits similar effects in the presence and absence of CXCR3, we assume that its effect is CXCR3 independent in pancreatic β cells. Subsequently, we analyzed CXCR3 gene expression in human islets. CXCR3 mRNA was detected in islets as well as in purified β cells (Figure 4F). Sequence analysis of the PCR products confirmed CXCR3 expression according to published sequences. By fluorescent immunocytochemistry, we found CXCR3 expression in human islets (1.08 ± 0.16 positive cells/islet). We observed CXCR3-positive β cells (Figure 4G, panels 1 and 2), but most CXCR3-positive cells were negative for insulin. No CXCR3 staining was observed in α and δ cells, as assessed by double-immunostaining with anti-glucagon or anti-somatostatin antibodies, respectively (Figure 4G, panels 3–6). Synaptophysin and chromogranin A (alternative markers for endocrine cells) only partially colocalized with CXCR3 (data not shown). Because CXCR3 is expressed in activated T cells, we performed triple staining for insulin (Figure 4G, panel 7), CXCR3 (Figure 4G, panel 8), and a T cell marker CD3 (Figure 4G, panel 9) and found CXCR3-positive T cells in human islets. However, we detected a large variation in the number of these CXCR3-positive β and T cells in the six different human islet isolations studied. The expression levels of CXCR3 in the islets were similar in untreated cells and cells treated with CXCL10 or IL-1β and IFN-γ (data not shown).

CXCL10 Binds to TLR4 in Pancreatic β Cells and Mediates β Cell Apoptosis

In the search for the specific receptor for the CXCL10-mediated effects in β cells, we analyzed gene expression of a number of receptors in islets by PCR. Initially, we looked for GPR1 (a functional CXCL10 receptor detected in epithelial and endothelial cells [Soejima and Rollins, 2001]), CCR3 (CXCR3 ligands were found to bind to CCR3 in T cells [Booth et al., 2002; Loetscher et al., 2001] but exhibit antagonistic effects), CCR5 (which is highly upregulated by TNF-α and IFN-γ, together with upregulation of CXCL10 [Croitoru-Lamoury et al., 2003]), and TLR4 (upon its activation, CXCL10 production is induced). Untreated human islets expressed all of the four receptors (Figure 5A). To identify the binding capacity of CXCL10 to these receptors, we used the Dynabead technology (Invitrogen). Beads were coated with CXCL10 prior to incubation with human islet lysates. While CCR5 and GPR1 failed to bind CXCL10 and CCR3 had a limited capacity to bind CXCL10, we identified TLR4 as the receptor for CXCL10 (Figure 5B).

To confirm the hypothesis that CXCL10 is a ligand for TLR4, a classical radioactive receptor-binding assay has been performed (Bernhagen et al., 2007). As cell models for the binding assay, the HEK293 and the macrophage cell lines RAW 264.7 have been chosen. Whereas HEK293 cells completely lack TLR4 (Erridge et al., 2007) as well as CXCR3 (Lu et al., 1999) expression, TLR4 is constitutively expressed in RAW 264.7 cells (Fang et al., 2004). The 293 cells were cotransfected with a combination of expression constructs TLR4/MD2/CD14 in order to reconstitute the complete receptor apparatus. For the assay, we used a constant 1 nM concentration of ^{125}I-labeled CXCL10. The concentration of unlabeled competitor lipopolysaccharide (LPS), a well-known specific ligand for TLR4 (Palsson-McDermott and O'Neill, 2004), was between 0.1 ng/ml and 100 μg/ml, and the concentration of IFN-γ as negative control was between 10 and 1000 U/ml. CXCL10 was able to bind the TLR4 receptor in the presence of MD2 and CD14 (Figure 5C) and in the presence of MD2 alone (similar results; data not shown), whereas CXCL10 binding did not occur in the presence or absence of unlabeled LPS in either untransfected (Figure 5E) or transfected 293 cells with only the vector expressing TLR4 (data not shown). Unlabeled LPS competed for the binding site and was able to displace radiolabeled CXCL10 at concentrations higher than 50 μg/ml in both cell lines 293 (Figure 5C) and RAW264.7 (Figure 5D). IFN-γ as cold competitor was used under the same experimental conditions and was unable to displace hot CXCL10 from the binding site, confirming the specific interaction between CXCL10 and TLR4 (Figures 5C and 5D, insert). The efficiency of transfection has been determined using a GFP reporter plasmid, and the expression of the TLR4, MD2, and CD14 has been confirmed by western blot analysis (data not shown). We next examined the ability of CXCL10 to regulate TLR4 receptor expression. An amount of 0.1 ng/ml CXCL10 caused a 2-fold induction of TLR4 mRNA (Figure 5F). Since TLR4 signaling results in upregulation and secretion of CXCL10 (Giarratana et al., 2004; Hoshino et al., 2002; Selvarajoo, 2006), we examined CXCL10 production downstream of CXCL10 signaling and measured a 4.5-fold and 33.3-fold induction of CXCL10 mRNA in human islets after treatment with 0.1 and 50 ng/ml CXCL10, respectively (Figure 5G). To investigate whether CXCL10 signaling is TLR4 dependent, we exposed human islets to a TLR4-specific siRNA (siTLR4) or scrambled control siRNA (siScr). For evaluating transfection efficiency, islets were treated with FITC-labeled nontargeted siRNA and green fluorescence monitored over the 4 day culture period. Transfection efficiency was 5% after 24 hr and 75% after 48 hr, which was maintained during the 4 day experiment (data not shown).

We achieved an average downregulation of 48% TLR4 protein expression by the siRNA, shown by western blot analysis (Figure 5H, insert). After a 4 day culture period with 0.1 ng/ml CXCL10, we analyzed β cell apoptosis. CXCL10 treatment induced a 4-fold induction of β cell apoptosis in human islets pretreated with scramble control siRNA (siSCR). Pretreatment with siTLR4 induced a 39.6% reduction of β cell apoptosis ($p < 0.05$) (Figure 5H), supporting a specific involvement of TLR4 in CXCL10 signaling.

TLR4, CD14, and MD2 are expressed in β cells (Vives-Pi et al., 2003). This was confirmed in our study in isolated human islets

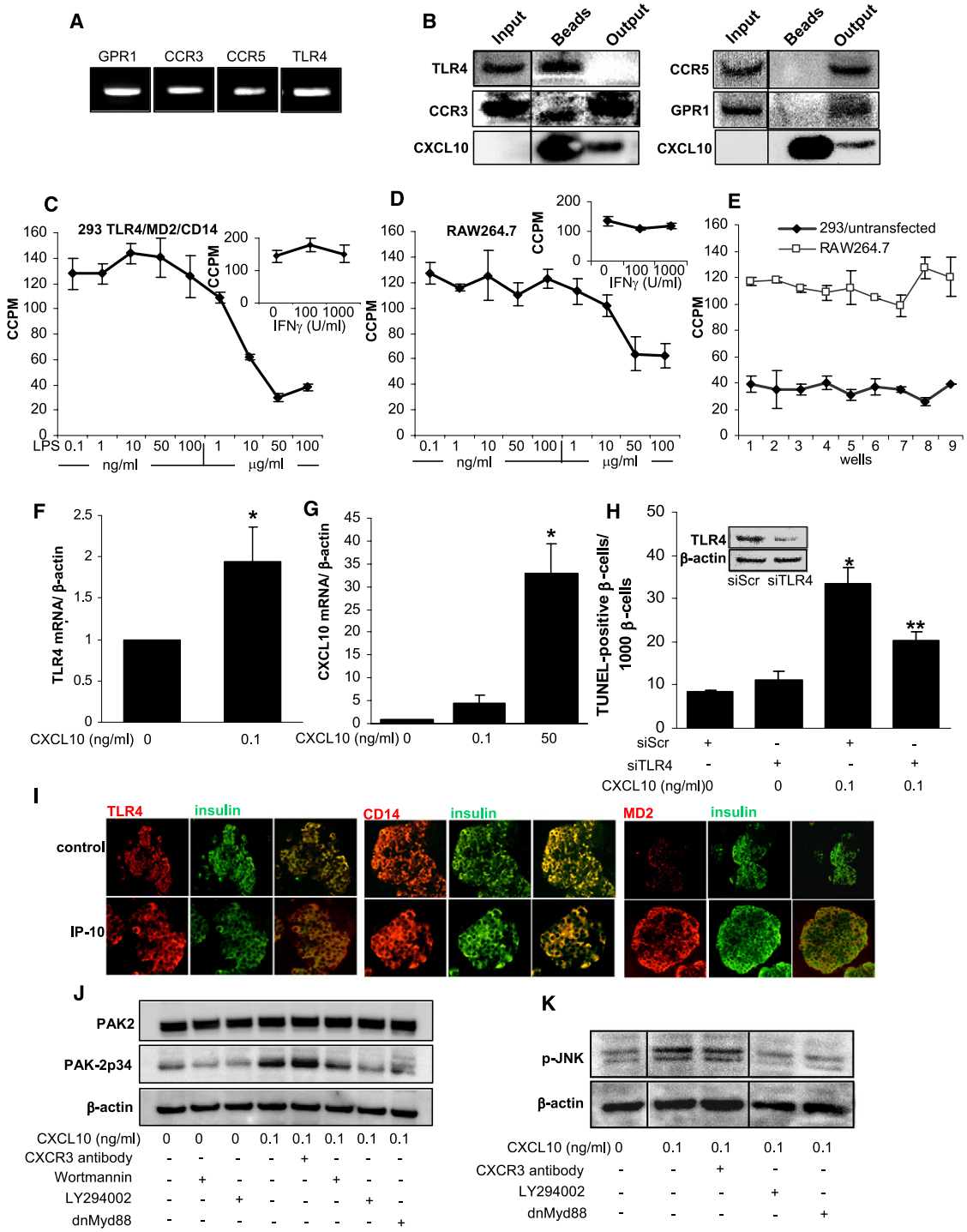

Figure 5. CXCL10 Binds to TLR4 in Pancreatic β Cells and Mediates β Cell Apoptosis

(A) PCR analysis of GPR1, CCR3, CCR5, and TLR4 in isolated human islets.

(B) Paramagnetic pull-down assay in human isolated islets. CXCL10 was bound to magnetic beads and incubated with human islet lysates. After magnetic separation, the bead fraction, supernatants (output) together with the original islet fraction before the pull-down (input), was transferred to lysate buffers, and western blot analysis was performed (three experiments from three different islet donors).

(C–E) Radioactive receptor-binding assay was performed in 293 and RAW264.7 cell lines with 1 nM radioactive-labeled CXCL10 and increasing concentrations of nonlabeled LPS.

(C) The 293 cells were cotransfected with different plasmids expressing TLR4, MD2, and CD14.

(D) Results of the RAW264.7 cells are shown. In both cases, the specificity of the binding is confirmed by the ability of increasing concentrations of cold-LPS to displace CXCL10 from the binding site. Control for the specific interaction of CXCL10 and TLR4 has been confirmed using increasing concentrations of

by double-staining for insulin and TLR4, CD14, and MD2 (Figure 5I). We treated the islets with CXCL10 and detected a slightly enhanced expression of TLR4 and an increased MD2 expression in the β cells. In contrast, CD14 was unchanged by CXCL10.

We next investigated whether CXCL10-mediated TLR4 signaling is linked to the activation of the Akt/PAK-2/JNK pathway by western blot analysis. Addition of a neutralizing antibody against CXCR3 changed neither CXCL10-induced PAK-2 cleavage nor activation of JNK. In contrast, inhibition of TLR4 signaling by addition of a dominant-negative virus for myeloid differentiation primary response protein 88 (Myd88), a key adaptor molecule in TLR4 signaling, prevented both CXCL10-induced PAK-2 cleavage and activation of JNK (Figures 5J and 5K). Also, blocking Akt activation by two different PI3K inhibitors (Wortmannin and LY294002) blocked PAK-2 cleavage as well as JNK activation, which suggests that Akt is upstream of PAK and JNK and confirms their interaction.

TLR4$^{-/-}$ Mice Are Protected from the Diabetogenic Effect of CXCL10

To further confirm the involvement of TLR4 in CXCL10-mediated β cell destruction, we analyzed β cell apoptosis from islets isolated from C57Bl/6 and *TLR4*$^{-/-}$ mice (8 to 12 weeks old). We used LPS as positive control to induce TLR4-dependent apoptosis, which induces β cell damage in vitro (Arnush et al., 1998) and in vivo (Saitoh et al., 2004). In our study, 20 μg/ml LPS induced a 2.9-fold induction of β cell apoptosis, which was prevented in isolated islets from TLR4 knockout mice (data not shown). CXCL10 exposure of the islets resulted in a 2.2-fold increase in β cell apoptosis in the wild-type, but not in the *TLR4*$^{-/-}$ mice (Figure 6A). Since we have used C57Bl/10ScCr mice, which were reported to have a mutation in the IL-12Rbeta2 chain in addition to TLR4 deletion (Poltorak et al., 2001), the TLR4-specific effect was confirmed by overexpressing TLR4 in the *TLR4*$^{-/-}$ islets. Treatment of the *TLR4*$^{-/-}$ with CXCL10 did not result in caspase 3 activation. In contrast, when *TLR4*$^{-/-}$ islets were transfected with construct for mouse TLR4, CXCL10 induced caspase 3 activation. Western blot analysis showed the absence of TLR4 in the *TLR4*$^{-/-}$ mice and confirmed TLR4 plasmid overexpression (Figure 6B). To answer the question of whether the CXCL10 effects on β cells in vitro would also result in impaired glucose homeostasis in vivo, we performed glucose tolerance tests in 10-week-old WT and *TLR4*$^{-/-}$ mice injected with 100 ng CXCL10 over 8 days. CXCL10 resulted in impaired fasting glucose and elevated glucose levels 15, 30, and 60 min after glucose injection (Figure 6C). In parallel, glucose-stimulated insulin secretion was reduced by 39% ($p < 0.05$) (Figure 6D) in the CXCL10-injected WT mice. Such effects were not observed in the *TLR4*$^{-/-}$ mice (Figures 6C and 6D). We posed the question of whether islet survival was affected in vivo by CXCL10 injections. In line with our in vitro data, isolated WT mouse islets injected with CXCL10 showed cleaved caspase 3 expression, whereas it was neither detected in solvent-treated isolated islets from WT mice nor in both solvent and CXCL10-treated *TLR4*$^{-/-}$ mice (Figure 6E).

DISCUSSION

Cytokines and chemokines produced and secreted by activated macrophages, adipocytes, and also by pancreatic β cells have been suggested to initiate β cell apoptosis in T1DM and T2DM (Donath et al., 2003). Understanding the mechanisms of secretion and function of these proinflammatory factors is important for the treatment of diabetes.

In the present study, we report that the chemokine CXCL10 is produced and secreted by cultured human islets of patients with T2DM. Immunostaining shows CXCL10 in pancreatic sections from patients with T1 and T2DM predominantly localized in β cells. Interestingly, we also could find some CXCL10 staining in the islet sections from obese individuals. This is in line with findings from a recent study that shows that CXCL10 is associated with both BMI and waist circumference (Herder et al., 2007) and may provide a link from obesity to diabetes through CXCL10 expression.

Elevated serum CXCL10 levels have been observed in T2DM and are between 184 and 248 pg/ml (Herder et al., 2006; Xu et al., 2005) and between 200 and 600 pg/ml in recent onset type 1 diabetic patients (Nicoletti et al., 2002; Shimada et al., 2001).

We found a similar CXCL10 concentration of ~100 pg/ml secreted by isolated islets from donors with T2DM. An amount of 100 pg/ml CXCL10 induced an ~3-fold induction of β apoptosis in human and mouse islet isolations, which correlates with the induction of apoptosis observed in vivo in pancreatic sections from autopsy from patients with T2DM (Butler et al., 2003) as well as with T1DM (Meier et al., 2005). We frequently observed doublets of apoptotic β cells, indicative of postmitotic apoptosis, supporting the concept that replicating β cells are more vulnerable to apoptotic stimuli (Donath et al., 1999; Ritzel and Butler, 2003). A critical regulator of β cell proliferation and survival is Akt (also known as protein kinase B [PKB]), which is activated

nonlabeled IFN-γ under the same experimental conditions, which did not compete for CXCL10 binding (inserts in [C and D]). As control, radioactivity was measured in cells treated with CXCL10 alone in RAW267.4 cells and in nontransfected 293 cells, where only background radioactivity was measured. The binding activity has been calculated as centicounts per minute (CCPM).

(F and G) Quantitative RT-PCR was performed in human islets exposed to CXCL10 for 3 days. The increases in TLR4 (F) and CXCL10 (G) mRNA expression are expressed as relative change from the untreated control (four experiments from four islet donors).

(H) Isolated human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to 50 nM scrambled control siRNA (siScr) or siRNA to TLR4 (siTLR4). At 1 day after transfection, CXCL10 was added, and apoptosis was measured by double-staining for TUNEL and insulin after the 4 day culture period. Results are means ± SE of TUNEL-positive β cells per 1000 β cells. Western blot analysis of TLR4 shows 48% downregulation in the siTLR4-treated human islets (insert).

(I) Islet sections were double-stained for insulin and TLR4 (left panel), CD14 (middle panel), and MD2 (right panel).

(J and K) Western blot analysis from human isolated islets after 24 hr exposure to 0.1 ng/ml CXCL10 with or without preincubation with an antagonistic CXCR3 antibody, 100 nM Wortmannin, 50 μM LY294002, or a dominant-negative virus for Myd88 (three independent experiments from three different organ donors).

*$p < 0.05$ to untreated control; **$p < 0.05$ to CXCL10 treated control islets.

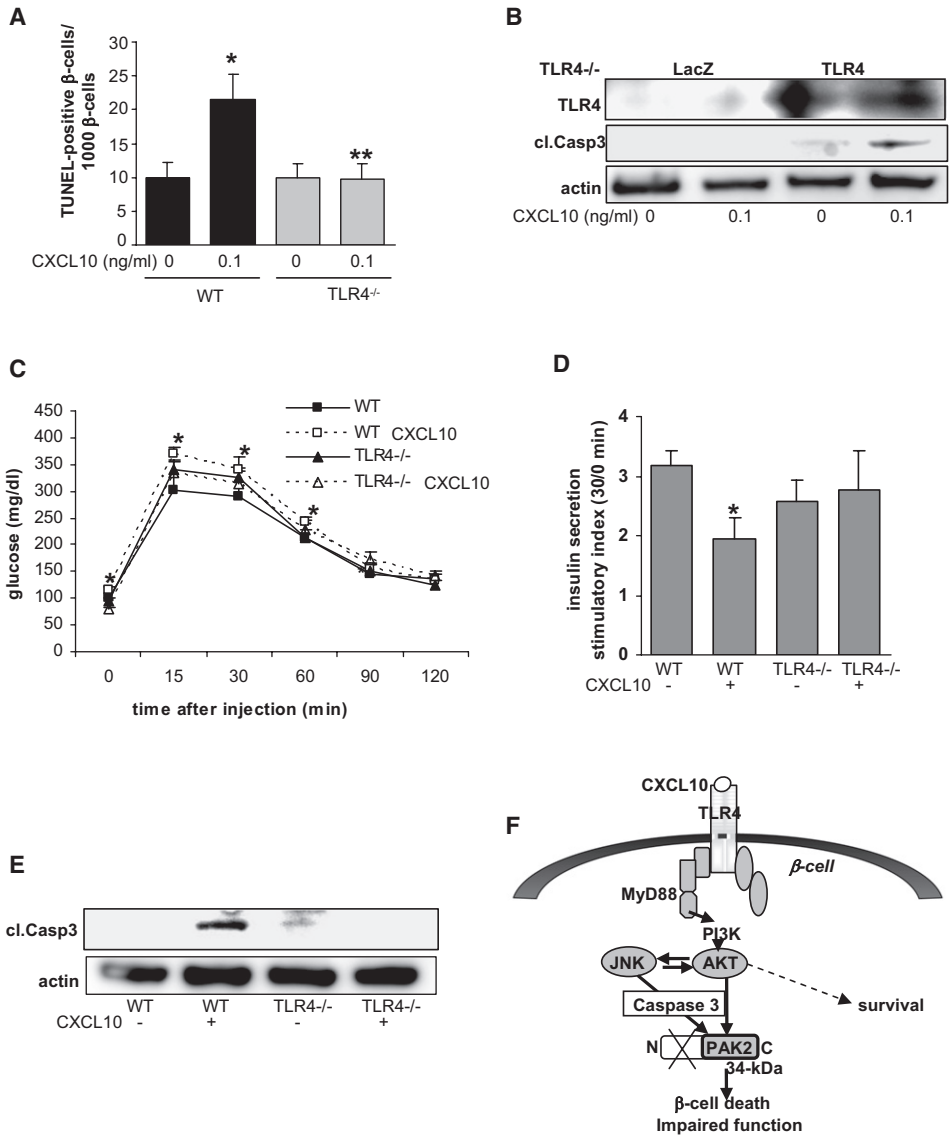

Figure 6. *TLR4*$^{-/-}$ Mice Are Protected from the Diabetogenic Effect of CXCL10

(A) Analysis of β cell apoptosis in wild-type and *TLR4*$^{-/-}$ mice. Mouse islets were cultured for 4 days.

(B) Western Blot analysis of isolated islets from *TLR4*$^{-/-}$ mice transfected with an expression vector for LacZ (negative control) or mouse TLR4 and exposed to 0.1 ng/ml CXCL10 for 3 days.

(C–E) WT and *TLR4*$^{-/-}$ mice (10 weeks old) were injected with 100 ng CXCL10 or solvent over 8 days.

(C and D) IPGTTs were performed at the end of the experiment, and (**C**) glucose levels were measured during 120 min. (D) Plasma insulin levels were measured from the same mice during the IPGTT 30 min before and after glucose injection, expressed as stimulated (30 min) over basal (0 min) insulin secretion. Results shown are the means ± SE (3 independent experiments with a total of 12 mice in each group). *$p < 0.05$ CXCL10 injected WT mice to untreated WT mice.

(E) Western blot analysis from isolated islets from the same mice.

(F) Our view on the pathways of CXCL10 in the β cell. Binding of CXCL10 to TLR-4 leads to MyD88-mediated long-term activation of Akt. Akt crosstalks with JNK, and its activation leads to cleavage of caspase 3. The signal for proliferation is converted to apoptosis initiated by the cleavage of PAK2.

downstream of PI3K (Aikin et al., 2000). In previous reports, blocking Akt phosphorylation renders islets more susceptible to β cell apoptosis (Dickson and Rhodes, 2004). Akt undoubtedly plays an important role in regulating β cell growth and survival (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001). It is a convergent point in cell signaling, i.e., induced by insulin and growth factors, and is involved in the regulation of cell cycle by altering its components, e.g., it increases protein stability of cyclin D and p21 (Fatrai et al., 2006). In contrast, CXCL10 induced apoptosis despite upregulated p21 levels (Zhang et al., 2005).

We have observed a sustained Akt activation by CXCL10. A similar role for Akt in promoting rather than preventing apoptosis has been shown before (Castino et al., 2008; Lu et al., 2006). Administration of the PI3K inhibitor Wortmannin inhibited Akt

phosphorylation as well as CXCL10-induced β cell death, suggesting that CXCL10 mediated its effects through sustained Akt activation. The role of Akt in the maintenance of β cell mass and survival has been debated recently. Mice carrying an 80% depletion in Akt do not display loss of islet mass, in contrast to the phenotype of IRS-2-deficient mice, which display a severe β cell loss (Bernal-Mizrachi et al., 2004). Therefore, downstream effectors of this signaling pathway may play a more critical role than Akt activation itself. CXCL10-induced Akt activation may first lead to proliferation and survival, but, at an event, when β cells are highly vulnerable to apoptosis, it is finally switched toward β cell death by other downstream signals. Among such downstream targets of Akt are p21-activated kinases (PAK) (Tang et al., 2000). PAKs are activated by a variety of GTPase-dependent and independent mechanisms (Bokoch, 2003). Among them, PAK-1 and PAK-2 regulate cell survival and apoptosis. PAK-1 protects from apoptosis (Faure et al., 1997), whereas PAK-2 has a unique dual function: activation of full-length PAK-2 stimulates cell survival (Jakobi et al., 2001), whereas, upon cleavage into a 34 kDa activated form (PAK-2p34), it directs apoptosis (Rudel and Bokoch, 1997). Here, we show the presence of the cleaved fragment PAK-2p34 upon caspase 3 activation in β cells. Such proteolytic activation of PAK-2 correlated with activation of the c-Jun amino terminal kinase (JNK) in our study as well as in previous reports (Rudel et al., 1998). Similar to the pattern of Akt activation, we observed that stimulation of human islets with CXCL10 elicits a prolonged activation of JNK, and blocking JNK could inhibit cleavage of caspase 3 and PAK-2 (a suggested mechanism of the Akt-JNK-PAK2 pathway is summarized as a cartoon in Figure 6F).

CXCL10 exerts its biological activity through the G protein-coupled receptor CXCR3. Also, CXCR3-independent effects of CXCL10 have been described (Cole et al., 2001; Luster et al., 1995; Soejima and Rollins, 2001). Our data support such CXCR3-independent effects in pancreatic islets. First, CXCL10 exhibited similar effects on the β cells in the presence and absence/neutralization of CXCR3, and, second, two other known CXCR3-binding chemokines, CXCL11 and CXCL9, did not affect β cell turnover. The possible mechanism of a CXCR3-independent CXCL10 signaling would, therefore, be the presence of another CXCL10 binding receptor. A novel functional receptor with unknown identity that binds CXCL10 has been identified recently, with abundant levels in epithelial and endothelial cells (Soejima and Rollins, 2001). Unlike CXCR3, this receptor does not bind CXCL9 or CXCL11, which would support our findings that CXCL9 and CXCL11 have no effect on the β cells in contrast to CXCL10. Possible candidates were a variety of seven-transmembrane-spanning G protein-coupled receptors with sequence motifs shared by chemokine receptors. Of these orphan receptors, we have tested GPR1, which was highly expressed in islets but failed to bind CXCL10. Another candidate, APJ (Marchese et al., 1994), was only marginally present in human islets (data not shown). CXCR3 ligands have been shown before to bind to CCR3, the receptor for eotaxin and several CC chemokines, but lack agonistic effects (Booth et al., 2002; Loetscher et al., 2001). CCR5 is highly upregulated by TNF-α and IFN-γ, together with upregulation of CXCL10 (Croitoru-Lamoury et al., 2003). Both CCR3 and CCR5 were expressed in islets. Though CCR3 bound to CXCL10 to a limited extent, CCR5 did not.

Interestingly, we observed activation of CXCL10 mRNA upon CXCL10 exposure, and such CXCL10 upregulation is an important downstream event of Toll-like receptor 4 (TLR4) activation (Giarratana et al., 2004; Hoshino et al., 2002; Selvarajoo, 2006). We confirmed TLR4 expression (Giarratana et al., 2004; Vives-Pi et al., 2003) in human islets, tested its binding capacity to CXCL10, and found CXCL10 as a ligand for TLR4. We have excluded a possible contamination of the recombinant protein with microbial products by preincubation of CXCL10 with polymyxin B, which blocks LPS activity. This had no effect on the CXCL10 actions. In contrast, preincubation of CXCL10 with a neutralizing CXCL10 antibody prevented its effects.

Signal transduction downstream of TLR4 is another mechanism that induces Akt activation. Other ligands of TLR4; LPS (Li et al., 2003) and lauric acid (Lee et al., 2003) showed this, resulting in NFκB activation and inflammation. TLR4 depletion prolongs islet graft survival of pancreatic islets after transplantation (Goldberg et al., 2007). Importantly, nonendotoxine ligands of TLR4 have been identified; Shi et al. show that free fatty acids induce TLR4 upregulation and activation in adipocytes and macrophages and induce inflammation and trigger insulin resistance (Shi et al., 2006). A possible explanation for the increase in CXCL10 in obese individuals could be that elevated circulating levels of free fatty acids (Wilding, 2007), which bind to TLR4, would consequently result in increased production of CXCL10. A loss-of-function mutation in TLR4 prevents diet-induced obesity and insulin resistance (Tsukumo et al., 2007). Furthermore, a TLR4 gene polymorphism in humans is associated with reduced CRP levels and a decreased risk of coronary heart disease and diabetes (Kolek et al., 2004). These earlier studies and our new observations suggest that TLR4 signaling plays an important role in diabetes and insulin resistance.

The present study provides further evidence that chemokines, in addition to other proinflammatory factors, directly trigger β cell destruction and contribute to the pathophysiology of T2DM. CXCL10, which is expressed relatively early in diabetes development as seen in the NOD mouse and in pancreata from obese nondiabetic individuals, may serve as a marker for β cell destruction, but more studies from different cohorts are required to confirm this hypothesis. Our data suggest a potential mechanism for the switch from proliferation into apoptosis in β cells. Using anti-inflammatory targets of the TLR4-signaling pathway will be of high importance to rescue the β cell from inflammation-induced self-destruction and to preserve β cell function and mass.

EXPERIMENTAL PROCEDURES

Details on procedures, animals, antibodies, and primers used are given in the Supplemental Data.

Animals

C57BL/6J and TLR4 knockout mice (C57BL/10ScCr) were obtained from Jackson Laboratory (Bar Harbor, ME). Homozygous CXCR3 on a C57BL/6J background were kindly provided by Dr. Craig Gerard (Perlmutter Laboratory, Children's Hospital and Harvard Medical School, Boston, MA). Animal experiments were conducted according to the protocols approved by the University of California Los Angeles Chancellor's Animal Research Committee and by the Bremen Senate in agreement with the National Institutes of Health animal care guidelines and §8 of the German animal protection law.

Islet Isolation and Culture

Human islets were isolated from pancreata of 11 healthy organ donors and 3 with T2DM as described previously (Oberholzer et al., 2000). Human islets from three isolations were purified into single β cells and cultured as described (Meyer et al., 1998). Mouse islets were isolated by bile duct perfusion and collagenase digestion as described (Maedler et al., 2001).

Measurement of CXCL10 Release

CXCL10 secretion into culture media of isolated islets from healthy and organ donors with T2DM was assessed by a Human Cytokine/Chemokine LINCO*plex* Kit (Linco Research, Inc., St. Charles, MO) according to the manufacturer's instructions.

Measurement of Changes in Intracellular Ca^{2+} Concentration

INS-1 cells were seeded on Fluoro dishes (WPI, Sarasota, FL) and cultured overnight in RPMI 1640 medium containing 3 mM glucose without FCS. Cells were loaded with 2.5 μM Fluo-3 AM by incubation for 30 min in KRB buffer.

Immunohistochemistry

Human islet sections were prepared as described before (Maedler et al., 2002b). Human pancreatic tissue was obtained at autopsy from nondiabetic subjects and from patients with T2DM (all with documented fasting plasma glucose > 145 mg/dl; see Table S1). A pancreas biopsy was obtained from an 89-year-old newly T1DM-diagnosed patient (with random blood glucose of 349 mg/dl; details described recently [Meier et al., 2006]).

Western Blot Analysis

Islet lysates were prepared as described previously (Maedler et al., 2006).

Protein Pull-Down Assay

Dynabeads M-270 Epoxy (Invitrogen) were coated with recombinant human CXCL10 (R&D Systems Inc., Minneapolis, MN). After washing, human islet cell lysates were added to the beads and incubated for 1 hr. Bound proteins were eluted in Laemmli buffer for SDS-PAGE. Whole-cell lysates from cultured islets, eluted samples, and flowthrough were electrically transferred to PVDF membranes.

Chemokine-Binding Assay

Binding assays were performed using RAW 264.7 cells (constitutively expressing TLR4 [Fang et al., 2004]) or HEK293 cells (not expressing TLR4 [Visintin et al., 2001]) transfected with or without the combination of pFLAG-TLR4, pFLAG-CD14 (kindly provided by Dr. Kirschning, TU Munich [Dziarski et al., 2001]), and pDUO-hMD2/TLR4A (kindly provided by Dr. Clett Erridge, Institute of Biomedical and Life Sciences, University of Glasgow [Erridge et al., 2008]) receptor expression plasmids using a standard policationic lipid reagent protocol.

RNA Extraction and Quantitative Reverse Transcription-PCR

Total RNA was isolated from the cultured islets and analyzed as described previously (Maedler et al., 2006). Primer sequences are published in the Supplemental Data.

Plasmid Overexpression and Small Interfering RNA

siRNA-Lipofectamine2000 complexes were prepared as described before (Shu et al., 2008) (Liptofectamine2000; invitrogen) using 50 nM siRNA to TLR4 (Stealth Select RNAi, invitrogen) and scramble siRNA (Ambion, Austin, TE) and added to the islet culture. Plasmid overexpression of TLR4 (kindly provided by Dr. Carsten Kirschning) was performed as described before (Maedler et al., 2002a).

Glucose-Stimulated Insulin Secretion, Intraperitoneal Glucose, and Insulin Tolerance Tests

Tests were performed as described previously in detail (Sauter et al., 2008).

Statistical Analysis

Samples were evaluated in a randomized manner by a single investigator (F.T.S. blinded to the treatment conditions). Data were analyzed by paired, Student's t test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, one table, and one figure and can be found with this article online at http://www.cell.com/cell-metabolism/S1550-4131(09)00004-7.

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