Increased serum CXCL1 and CXCL5 are linked to obesity, hyperglycemia, and impaired islet function

Craig S Nunemaker1, H Grace Chung1,2, Gretchen M Verrilli1,3, Kathryn L Corbin1, Aditi Upadhye1 and Poonam R Sharma1

1Division of Endocrinology and Metabolism, Department of Medicine; 2Department of Biomedical Engineering and 3Department of Biology, University of Virginia, PO Box 801413, Charlottesville, Virginia 22908, USA

Abstract

Proinflammatory cytokines are thought to play a significant role in the pathogenesis of type 2 diabetes (T2D) and are elevated in the circulation even before the onset of the disease. However, the full complement of cytokines involved in the development of T2D is not known. In this study, 32 serum cytokines were measured from diabetes-prone BKS.Cg-m+/-Leprdb/db (db/db) mice and heterozygous age-matched control mice at 5 weeks (non-diabetic/non-obese), 6–7 weeks (transitional-to-diabetes), or 11 weeks (hyperglycemic/obese) and then correlated with body weight, blood glucose, and fat content. Among these 32 cytokines, C-X-C motif ligand 1 (CXCL1) showed the greatest increase (78%) in serum levels between db/db mice that were hyperglycemic (blood glucose: 519 ± 23 mg/dl, n=6) and those that were non-hyperglycemic (193 ± 13 mg/dl, n=8). Similarly, increased CXCL1 (+68%) and CXCL5 (+40%) were associated with increased obesity in db/db mice; note that these effects could not be entirely separated from age. We then examined whether islets could be a source of these chemokines. Exposure to cytokines mimicking low-grade systemic inflammation (10 pg/ml IL1β+20 pg/ml IL6) for 48 h upregulated islet CXCL1 expression by 53 ± 3-fold and CXCL5 expression by 83 ± 10-fold (n=4, P<0.001). Finally, overnight treatment with the combination of CXCL1 and CXCL5 at serum levels was sufficient to produce a significant decrease in the peak calcium response to glucose stimulation, suggesting reduced islet function. Our findings demonstrated that CXCL1 and CXCL5 i) are increased in the circulation with the onset of T2D, ii) are produced by islets under stress, and iii) synergistically affect islet function, suggesting that these chemokines participate in the pathogenesis of T2D.

Introduction

Chronic low-grade inflammation is increasingly considered as a contributing factor to many metabolic diseases including type 2 diabetes (T2D). Obesity leads to excess fatty acids and lipids in the body, which have toxic and damaging effects on the body’s metabolism (Paolisso et al. 1995, Poitout & Robertson 2002, Robertson et al. 2004) and also cause low-grade systemic inflammation (Wellen & Hotamisligil 2003). This inflammation, particularly of fat tissue, increases the circulating levels of proinflammatory cytokines in the blood. These cytokines
Contribute to the two defining characteristics of T2D: i) insulin resistance and ii) defective insulin secretion, which lead to hyperglycemia (Greenberg & McDaniel 2002). Our hypothesis is that increased levels of circulating cytokines caused by obesity can have potentially damaging effects in distal tissues, such as the pancreatic islets of Langerhans. We have recently demonstrated that exposure to circulating levels of certain cytokines (interleukin 1β (IL1B) and IL6 in combination) is sufficient to trigger dysfunction and apoptosis in islets from diabetes-prone mice without significantly disrupting islet function in healthy mice (O’Neill et al. 2013).

Our objective in this study was to identify other circulating cytokines and chemokines associated with T2D that could also contribute to changes in normal islet function. We examined 32 different cytokines/chemokines in serum from leptin-receptor-deficient db/db mice during the development of diabetes in order to identify additional cytokines that could directly contribute to or protect against islet decline in T2D. We identified two chemokines of interest that increased in serum with the development of obesity and hyperglycemia: C-X-C motif ligand 1 (CXCL1) and CXCL5. Both CXCL1 and CXCL5 bind with the CXCR2 receptor and are associated with processes as diverse as angiogenesis, wound healing, and tumorigenesis. CXCL1 is expressed in macrophages, neutrophils, and epithelial cells, and it plays a key role in inflammation as a chemoattractant for neutrophils. CXCL5 is expressed in eosinophils as well as epithelial cells and has a well-established role in stimulating the chemotaxis of angiogenic neutrophils, particularly in response to inflammation.

CXCL1 and CXCL5 are also associated with diabetes. Increased serum levels of CXCL1 have been reported in patients with type 1 diabetes (Takahashi et al. 2011, Hakimizadeh et al. 2013) and more recently in T2D (Sajadi et al. 2013). Increased serum CXCL5 has also been observed in obesity and insulin resistance in mice and humans (Chavey et al. 2009), and it is associated with complications of diabetes including nephropathy (Higurashi et al. 2009) and atherosclerosis (Chen et al. 2011). CXCL1 has also been reported to be a CD40-induced chemokine in isolated human pancreatic islets (Klein et al. 2008), which is also expressed in response to amyloid in T2D (Westwell-Roper et al. 2011). CXCL1 expression is associated with increased rates of islet transplant failure (Citro et al. 2012, Cowley et al. 2012), and blocking the CXCL1 receptor CXCR2 improves transplant outcomes (Citro et al. 2012). In this study, we demonstrated that both CXCL1 and CXCL5 increase in serum as diabetes-prone db/db mice become obese and hyperglycemic. Further, we demonstrated for the first time that exposure to CXCL1 and CXCL5 at circulating levels can directly affect islet function and that islet cell stress can induce the expression of CXCL1 and CXCL5. Our findings together suggest a role for these chemokines in T2D both systemically and within the pancreatic islet.

**Materials and methods**

**Mice**

Studies of serum cytokine levels were conducted using male BKS.Cg-Dock7m+/-/Leprdb/db (db/db) mice as a model of T2D, with age-matched heterozygous (het) mice as controls (Jackson Laboratories, Bar Harbor, ME, USA). Mice were used at ages of 5, 6–7, and 11 weeks. Follow-up studies of cytokine effects on normal pancreatic islets were conducted using outbred CD1 mice at ages of 8–12 weeks (Charles River Laboratories, Wilmington, MA, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia (UVA).

**Dual-energy X-ray absorptiometry analysis**

DEXA scans were performed using a Lunar PixiMUS densitometer (GE Medical Systems, Waukesha, WI, USA) to determine fat and bone mineral content (BMC). Mice were anesthetized by i.p. injection of 20 mg/ml xylazine and 100 mg/ml ketamine in 0.9% sterile saline at a dose of 5 μl/g body weight and then placed on the Lunar PIXImus tray for scanning, as described previously (Roland et al. 2010). After running a quality control test, the animal was scanned to measure area, bone mineral density (BMD), BMC, lean body mass, fat body mass, total body mass, and percent body fat. Following the scan, mice were injected subcutaneously with 1 ml of 0.9% sterile saline for hydration and then placed on a heating pad and monitored until recovery from anesthesia.

**Cytokine panel**

For this study, 34 db/db and 34 het mice varying in age from 5, 6–7, and 11 weeks old were killed by CO₂. Blood was immediately collected by heart puncture, allowed to clot at room temperature, and then centrifuged to collect serum. Samples were frozen at −20°C for long-term storage. Serum was thawed briefly to extract 60 μl and shipped to Millipore, Inc. (St Charles, MO, USA) on dry ice for use in a 32-plex mouse cytokine assay. Samples were
diluted 1:2 by Millipore and were run in singlet following the bead-based assay protocol.

**Islet isolation and treatment**

Pancreatic islets were isolated by collagenase-P digestion (Roche Diagnostics) followed by centrifugation with Histopaque 1100 (Sigma–Aldrich) as described previously (Carter et al. 2009). Islets were incubated overnight in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin to allow recovery from collagenase digestion before further treatment. Various islet stressors were prepared as follows: rotenone and thapsigargin were purchased from Sigma–Aldrich and prepared in stocks of DMSO to final concentrations <0.1%. Glucose-free RPMI medium (10% FBS and 1% penicillin/streptomycin) was supplemented with 1 M glucose stock to produce the 28G condition. Stocks of the murine forms of IL1B, IL6, CXCL1, and CXCL5 (purchased from R&D Systems, Inc., Minneapolis, MN, USA) were prepared in PBS with 0.1% BSA. Oleate, linoleate, and palmitate were purchased from Sigma–Aldrich and were prepared in 100 mM stock concentrations in methanol and stored at −80 °C. To treat the islets, required amount of each fatty acid was taken in a glass tube and dried under a stream of nitrogen to remove methanol. The dried mixture of fatty acids thus obtained was resuspended in the RPMI medium with 0.1% BSA followed by vortexing and sonication. All experiments were completed within 48–72 h after isolation.

**Glucose-stimulated calcium measurements**

Intracellular calcium ([Ca^{2+}]_i) was measured using the ratiometric [Ca^{2+}]_i indicator fura-2 AM as described previously (Crim et al. 2010, Corbin et al. 2011). Briefly, Cell Tracker Red CMTPX (CTR, Invitrogen), a membrane-penetrating fluorescent probe, was used in order to distinguish cytokine-treated islets from control islets by selectively labeling only one of the two groups, thus enabling simultaneous comparison of the two treatment groups (see Corbin et al. (2011)) for additional details). All islets were loaded with 1 μM fura-2 AM for 30 min in the presence or absence of 200 nM CTR, washed, and then recorded using a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan) attached to an Olympus BX51WI fluorescence microscope (Olympus, Tokyo, Japan) using 340 and 380 nm excitation light and 510 nm emission as described previously (Jahanshahi et al. 2009). Data were recorded and analyzed using IP Lab software Version 4.0 (Scanalytics, Rockville, MD, USA).

**Glucose-stimulated insulin secretion**

After overnight incubation, islets were tested for insulin secretion as described previously (Crim et al. 2010). Briefly, islets were preincubated at 37 °C in an atmosphere of 5% CO_2 for 1 h in a standard KRB solution, then washed, and incubated in KRB supplemented with 3 mM glucose for 1 h followed by treatment with KRB containing 11 mM glucose for 1 h. The supernatant was collected after each treatment and insulin concentration in the supernatant was measured by ELISA (Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions.

**Real-time PCR**

RNA was collected from islets treated with cytokines associated with low-grade inflammation (IL6+IL1β). Untreated islets served as a control. RNA and cDNA preparation and real-time PCRs (RT-PCRs) were performed as described previously (O’Neill et al. 2013). RNA purity and concentration were determined using a Nanodrop spectrophotometer.

**Statistical analysis**

An unpaired two-tailed t-test was used to compare het and db/db groups, with a P value of P<0.05 used as an indication of statistical significance. All comparisons of serum cytokine levels assumed a minimum of four detectable samples. Cytokines with less than four detectable samples were considered undetectable for statistical evaluation.

**Results**

**Observed differences in weight, blood glucose, and body composition in db/db mice**

We first measured body weight and blood glucose for db/db mice and heterozygous controls at each of the key developmental stages of T2D (5, 6–7, and 11 weeks). As shown in Fig. 1, db/db mice showed both increased body weight (Fig. 1A) and blood glucose (Fig. 1B) as early as 6–7 weeks of age. By 11 weeks, there was a more substantial difference in weight (P<0.001) and blood glucose (P<0.001) when compared with het mice. There were no significant differences in either parameter between het and db/db mice at 5 weeks.

To further examine whether there could be more nuanced differences between strains in the early stages
of disease progression, we examined body composition by DEXA for the 5-week-old mice only. As can be observed from Table 1, substantial differences in fat pads, BMC, and percent body fat were found between het and db/db mice ($P<0.001$). There were also variations in BMD ($P<0.01$) and, to a lesser extent, lean body mass and body fat ($P<0.05$). These differences in lean vs fat mass may account for the similarity in total body weight at this age.

**Serum cytokine differences between strains at each age**

Cytokine milieu differences between strains at various ages were very few. Among 32 cytokines tested, 12 were not detectable ($n<4$ samples/strain) across all age groups. The remaining cytokines that could be evaluated showed no differences between db/db and het at either 6–7 or 11 weeks. The only statistical difference between strains was observed for serum IL1A from the 5-week-old mice. IL1A was $322\pm67$ ng/ml in db/db mice ($n=15$) vs $574\pm86$ ng/ml in het controls ($n=14$, $P=0.03$).

**CXCL1 and CXCL5 associated with obesity and hyperglycemia**

We hypothesized that serum levels of cytokines might also vary within the diabetes-prone strain as the disease progresses to obesity and hyperglycemia. We thus made comparisons of serum cytokine levels between the most vs least obese db/db mice or between the most vs least hyperglycemic db/db mice. As there were no cases of hyperglycemia or obesity among the youngest mice, transition to diabetes in the 6–7- and 11-week-old groups was focused for analysis. As shown in Fig. 2A, serum levels of CXCL1 and CXCL5 were both higher in obese mice (body weight $>40$ g, $n=6$) compared with non-obese mice (body weight $<35$ g, $n=10$, $P<0.001$). Notably, as serum levels of different cytokines can differ by orders of magnitude, data are presented for cytokine levels in obese mice as a percent of the cytokine levels in non-obese controls. Scatter plots for CXCL1 and CXCL5 vs body weight are shown in Fig. 2B and C respectively. As shown in Fig. 2D, serum levels of CXCL1 were similarly higher among hyperglycemic mice (blood glucose $>400$ mg/dl, $n=5$) compared with non-hyperglycemic mice (blood glucose $<250$ mg/dl, $n=8$, $P<0.001$). CXCL5 was increased as well, but not significantly ($P=0.098$). Scatter plots for each chemokine vs blood glucose are shown in Fig. 2E and F. See also Supplementary Figure 1, see section on supplementary data given at the end of this article, for serum cytokine levels reported in pg/ml.

**Table 1** Key body composition and dual-energy X-ray absorptiometry (DEXA) results for db/db (pre-diabetic) and het mice at 5 weeks of age

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Heterozygous</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>$20.7\pm0.9$</td>
<td>$21.2\pm1.0$</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>$154\pm3$</td>
<td>$182\pm28$</td>
</tr>
<tr>
<td>Fasting BG (mg/dl)</td>
<td>$146\pm5$</td>
<td>$128\pm22$</td>
</tr>
<tr>
<td>Fat pads (mg)‡</td>
<td>$189\pm26$</td>
<td>$620\pm57$</td>
</tr>
<tr>
<td>BMD (mg/cm²)†</td>
<td>$35\pm1$</td>
<td>$30\pm1$</td>
</tr>
<tr>
<td>BMC (mg)‡</td>
<td>$237\pm15$</td>
<td>$136\pm6$</td>
</tr>
<tr>
<td>Lean body mass (g)‡</td>
<td>$15.8\pm0.7$</td>
<td>$13.9\pm0.5$</td>
</tr>
<tr>
<td>Body fat (g)‡</td>
<td>$3.8\pm0.3$</td>
<td>$6.6\pm0.7$</td>
</tr>
<tr>
<td>% Body fat†</td>
<td>$19\pm1$</td>
<td>$32\pm1$</td>
</tr>
</tbody>
</table>

$^*P<0.05$, $^\dagger P<0.01$, $^\ddagger P<0.001$. 

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**Figure 1** Compared with het mice, db/db mice have progressively higher (A) body weight and (B) blood glucose with increasing age. **$P<0.01$, ***$P<0.001$. 

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Increased gene expression of Cxcl1 and Cxcl5 in pancreatic islets in response to cell stress

We then examined various stressors hypothesized to trigger β-cell failure in T2D (Montane et al. 2014) for their effects on Cxcl1 and Cxcl5 gene expression. Islets from CD1 mice were exposed for 48 h to one of the following stressors: 20 nM rotenone as a model of oxidative stress (Hoehn et al. 2009), 100 nM thapsigargin as a model of ER stress (Eizirik et al. 2008), 10 pg/ml IL1β + 20 pg/ml IL6 as a model of low-grade inflammation (cytokines; Spranger et al. (2003) and O’Neill et al. (2013)), 28 mM glucose as a model of glucotoxicity (28G, Tang et al. (2012)), or free fatty acids (FFAs: 50 μM palmitate + 100 μM oleate + 50 μM linoleate) as a model of lipotoxicity (Watt et al. 2012). Islets incubated in standard RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin were used as controls. As shown in Fig. 3, we found that cytokine treatment substantially stimulated Cxcl1 (~50-fold increase, P < 0.001) and Cxcl5 expression (~80-fold, P < 0.001). Thapsigargin also appeared to consistently stimulate Cxcl1 expression (Fig. 3A), but the degree of stimulation was highly variable across trials and did not reach significance (3.7-, 7.8-, 16.5-, and 55.5-fold increase; P = 0.14). As shown in Fig. 3B, thapsigargin-induced Cxcl5 expression showed a similar variability (2.3-, 11.8-, 23.6-, and 44.0-fold increase; P = 0.07). FFA treatment also mildly, but significantly, upregulated Cxcl5 expression by approximately eightfold (Fig. 3B, P < 0.05). Overall, exposure to low-dose cytokines had the most robust effect on Cxcl1 and Cxcl5 expression, suggesting that the downstream effects of low-grade inflammation may be mediated, at least in part, by these chemokines.

Combined effect of CXCL1 and CXCL5 on islet function

We have previously shown that circulating levels of proinflammatory cytokines could directly affect islet function (O’Neill et al. 2013). We thus examined whether CXCL1 and CXCL5 could have a direct impact on pancreatic islet function at concentrations consistent with serum levels. Pancreatic islets were treated overnight with individual dosages of 100 pg/ml CXCL1, 10 ng/ml CXCL5, both, or neither of them; these doses approximate the serum levels measured in the 32-plex cytokine panel. Islet function was then assessed by glucose-stimulated insulin secretion. As shown in Fig. 4A, we did not observe any significant differences in insulin release during incubation in low (3 mM) glucose among treatment groups, although there was a slight tendency for more insulin release among chemokine-treated islets compared with untreated controls (not significant, P > 0.25). Chemokines also had no effect on insulin secretion in stimulatory glucose (11 mM) conditions (Fig. 4B). This lack of effect on insulin release is not necessarily
surprising. We have previously shown that other proinflammatory cytokines in combination at low levels do not significantly affect insulin release in normal healthy islets, but they do disrupt calcium handling (Dula et al. 2010, O’Neill et al. 2013).

We then examined islet calcium responses to glucose stimulation following overnight exposure to 100 pg/ml CXCL1, 10 ng/ml CXCL5, both chemokines, or neither of them. As shown in Fig. 5A, overnight exposure to CXCL1 had no effect on basal calcium (3 mM glucose), the phase 1 response to glucose stimulation (also called the peak response) or the phase 2 response (also called the plateau). As shown in Fig. 5B, overnight exposure to CXCL5 similarly had no effect on intracellular calcium. As both CXCL1 and CXCL5 bind to the same receptor, CXCR2, we hypothesized that these two chemokines could have a synergistic effect. As shown in Fig. 5C, combined treatment of 100 pg/ml CXCL1 and 10 ng/ml CXCL5 resulted in a significant decrease in the peak calcium response to glucose stimulation. The peak calcium response to glucose stimulation was reduced by ~40% (Fig. 5D, P<0.01) and the sustained plateau response by ~15% among six trials (Fig. 4D, P<0.05). This supports our hypothesis, suggesting that the presence of both serum CXCL1 and CXCL5 is necessary to affect islet function.

**Discussion**

In this study, we provide evidence that CXCL1 and CXCL5 are central to low-grade systemic inflammation associated with islet decline in T2D. We showed that i) only CXCL1 and CXCL5 were significantly associated with the onset of obesity and hyperglycemia in serum panel measurements of 32 cytokines/chemokines, ii) cytokine-induced stress in turn induced substantial
upregulation of Cxcl1 and Cxcl5 expression in islets, and iii) overnight exposure to CXCL1 and CXCL5 together could disrupt normal islet function. Collectively, these data suggest that these chemokines play a role both in systemic inflammation as evidenced by the increased levels in serum and in the inflammatory response within the islet itself.

The primary source of proinflammatory cytokines in obesity is adipose tissue. Both resident immune cells in adipose tissue and adipocytes themselves can contribute to the increase in circulating cytokines. Among 32 cytokines/chemokines tested in mouse serum, only CXCL1 and CXCL5 were positively correlated with the development of obesity and hyperglycemia. Our data are consistent with other studies showing that high-fat diet can increase CXCL1 and T2D (Chavey et al. 2013), for which age was not a factor.

Our data, whether derived from fat tissue, islets, immune cells, or other sources, suggest that CXCL1 and CXCL5 can directly affect islet function. Although neither CXCL1 nor CXCL5 alone appeared to affect islets, overnight exposure to both CXCL1 and CXCL5 at concentrations detected in serum significantly reduced the calcium response to glucose stimulation, a key component of the stimulus-secretion pathway for insulin (Straub & Sharp 2002, Ramadan et al. 2011). As CXCL1 and CXCL5 both bind to the chemokine receptor CXCR2, it is possible that both ligands are required to activate CXCR2 to produce downstream effectors that act on islet cells. Ours is the first evidence that these chemokines can directly affect islet function.

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Whether the primary purpose of CXCL1 and CXCL5 in islets is to initiate an immune response or to modify islet activity remains an open question. Many endocrine tissues, including the endocrine cells within pancreatic islets, have been shown to express the CXCR2 receptor (Tecimer et al. 2000), leaving open the possibility that the effects of these chemokines are mediated in an autocrine or paracrine manner entirely within the islet. Blockade of the CXCR1/2 pathway improved islet engraftment in mouse and human transplant recipients, and blockade also reduced recruitment of leukocytes and NKT cells to the site of the islet graft in mice (Citro et al. 2012). These findings suggest that blocking CXCR2 directly reduces inflammatory responses, but the improved islet engraftment could also be the due effects of blockade within the islet improving islet function. In a cyclophosphamide-induced transgenic mouse model of type 1 diabetes, marked upregulation of both CXCL1 and CXCL5 was thought to derive from inside the inflammatory infiltrate, not the islet cells themselves, suggesting primarily an immune-mediated response (Matos et al. 2004). Our findings suggest that CXCL1 and CXCL5 can synergistically inhibit the function of islets in vitro, where the islets are isolated from immune input. However, various resident immune cells, particularly macrophages, have been observed in isolated islets (Bonishi-Schnetzler et al. 2008), hence we cannot rule out these possible effects.

Regarding the regulation of CXCL1 and CXCL5 within islets, we examined a number of stressors that are hypothesized to trigger islet decline and failure in T2D (Donath et al. 2005). The combination of IL1β and IL6 at the low levels associated with low-grade inflammation had by far and away the strongest effect on Cxcl1 and Cxcl5 gene expression. This is because IL1β has been previously shown to upregulate Cxcl1 and this upregulation is dependent upon activation of NFκB (a target of IL1β signaling) and STAT signaling (associated with IL6) (Burke et al. 2014). Increased circulating levels of IL1B and IL6 are associated with an increased risk of T2D (Spranger et al. 2003), and we have shown previously that circulating levels of these cytokines can increase islet dysfunction and apoptosis in islets from diabetes-prone mice (O’Neill et al. 2013). We also observed in this study that exposure to FFAs upregulated Cxcl5 (and Cxcl1 to a minor extent), which is consistent with palmitate-induced CXCL1 expression observed in human islets (Igoillo-Esteve et al. 2010). Finally, thapsigargin-induced ER stress also appeared to upregulate Cxcl1 and Cxcl5, though the degree of upregulation was highly variable from trial to trial. This is intriguing as silencing of the transcription factor C/EBPdelta has been linked to increased Cxcl1 expression and increased Ddit3 expression (also known as CHOP, a key proapoptotic factor in ER stress) in rat islets and β-cell lines (Moore et al. 2012).

CXCL1 and CXCL5 are thus highly sensitive to low levels of inflammation in islets, and these same chemokines appear to be capable of synergistically affecting islet function. Our data suggest that CXCL1 and CXCL5 are markers of islet dysfunction, and they may be key instigators of islet failure in T2D. More work is required to determine why inflammation causes such strong upregulation of CXCL1 and CXCL5 in islets and to determine whether CXCL1 and CXCL5 act on endocrine cells, immune cells, and/or other cell types within the islet to inhibit islet function.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0126.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
C S N wrote the manuscript, designed experiments, acquired and analyzed data, edited the manuscript, and provided funding. C S N is a guarantor of this article and, as such, has full access to all the data in the study and takes responsibility for the integrity and accuracy of the data. H G V acquired and analyzed data. K L C acquired data. A U acquired data and edited the manuscript. P R S acquired and analyzed data, and developed methods.

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