Original article

MOLECULAR METABOLISM

Fractalkine (CX3CL1), a new factor protecting β-cells against TNFα

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ABSTRACT

Objective: We have previously shown the existence of a muscle—pancreas intercommunication axis in which CX3CL1 (fractalkine), a CX3C chemokine produced by skeletal muscle cells, could be implicated. It has recently been shown that the fractalkine system modulates murine β-cell function. However, the impact of CX3CL1 on human islet cells especially regarding a protective role against cytokine-induced apoptosis remains to be investigated.

Methods: Gene expression was determined using RNA sequencing in human islets, sorted β- and non-β-cells. Glucose-stimulated insulin secretion (GSIS) and glucagon secretion from human islets was measured following 24 h exposure to 1—50 ng/ml CX3CL1. GSIS and specific protein phosphorylation were measured in rat sorted β-cells exposed to CX3CL1 for 48 h alone or in the presence of TNFα (20 ng/ml). Rat and human β-cell apoptosis (TUNEL) and rat β-cell proliferation (BrdU incorporation) were assessed after 24 h treatment with increasing concentrations of CX3CL1.

Results: Both CX3CL1 and its receptor CX3CR1 are expressed in human islets. However, CX3CL1 is more expressed in non-β-cells than in β-cells while its receptor is more expressed in β-cells. CX3CL1 decreased human (but not rat) β-cell apoptosis. CX3CL1 inhibited human islet glucagon secretion stimulated by low glucose but did not impact human islet and rat sorted β-cell GSIS. However, CX3CL1 completely prevented the adverse effect of TNFα on GSIS and on molecular mechanisms involved in insulin granule trafficking by restoring the phosphorylation (Akt, AS160, paxillin) and expression (IRS2, ICAM-1, Sorcin, PCSK1) of key proteins involved in these processes.

Conclusions: We demonstrate for the first time that human islets express and secrete CX3CL1 and CX3CL1 impacts them by decreasing glucagon secretion without affecting insulin secretion. Moreover, CX3CL1 decreases basal apoptosis of human β-cells. We further demonstrate that CX3CL1 protects β-cells from the adverse effects of TNFα on their function by restoring the expression and phosphorylation of key proteins of the insulin secretion pathway.

Keywords Islets; Insulin secretion; Myokines; Survival; Insulin signaling pathway; Inflammation

1. INTRODUCTION

Type 2 diabetes (T2D) pathophysiology is a complex process combining 2 major mechanisms: insulin resistance and β-cell failure. During the prediabetic period, characterized by peripheral insulin resistance, euglycemia is maintained by a compensatory increase of insulin secretion. However, the addition to this prediabetic state of β-cell failure, affecting both cell function and survival, leads to hyperglycemia and clinically manifest T2D [1]. This is exacerbated by abnormal α-cell function resulting in pathologically elevated levels of glucagon [2]. Several studies provide evidence that T2D is associated with elevated levels of the proinflammatory cytokine tumor necrosis factor-α (TNFα) in adipose tissue [3], skeletal muscle [4], and plasma [5—7]. In β-cells, TNFα on its own induces insulin resistance mediated by nitric oxide [8], a reduction of glucose-stimulated Ca^{2+} influx [9] and a decrease of glucose-stimulated insulin secretion (GSIS) [10].

CX3CL1, also named Fractalkine, is the only member of the CX3C chemokine family. It is first synthesized in a plasma membrane bound form and a soluble form is shed through enzymatic cleavage by Adam 10 and Adam 17 [11,12]. CX3CL1 is the specific ligand for a G protein coupled receptor (GPCR) named CX3CR1. Upon binding to its receptor, CX3CL1 is implicated in chemotaxis, cellular adhesion and in increased cell survival during hemostasis and inflammatory episodes [13]. Reporter gene studies have shown that CX3CL1 is principally expressed in neurons, lung epithelial cells, kidney and in the intestine [13—15], whereas CX3CR1 is mainly expressed in monocytes, natural killer cells and T cells [15,16]. Intriguingly, CX3CL1 has recently been described as a novel adipokine positively regulated by obesity and diabetes [17].

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Several studies strongly suggest that CX3CL1 could be implicated in the low-grade inflammation status of adipose tissue associated with obesity and T2D development. Indeed, CX3CL1 is involved in monocyte adhesion to adipocytes in vitro and its expression in adipocytes is stimulated by inflammatory cytokines [17]. We have observed that CX3CL1 expression is dramatically increased in primary human skeletal muscle cells treated with TNF-α [18]. The CX3CL1/CX3CR1 system could also be implicated in some diabetes-associated pathologies such as painful neuropathy or extracellular matrix accumulation in diabetic nephropathy [19].

Interestingly, it has recently been shown that CX3CL1 could have beneficial metabolic effects [20]. It was thus observed that in CX3CR1 KO mice, GSIS was decreased. CX3CL1 itself increased insulin secretion during glucose tolerance tests in mice and in GSIS from ex vivo isolated islets. These acute actions of CX3CL1, mediated by an MEK dependent pathway, were associated with chronic effects maintaining the expression of genes necessary to preserve fully functional β-cells. This study also showed that T2D risk factors such as aging and obesity are associated with a decrease in CX3CL1 expression within islets [20].

In the present study, we demonstrate for the first time that human islets secrete CX3CL1 and that CX3CL1 protects β-cells from the adverse effects of TNFα on their function by restoring phosphorylation of key proteins of the insulin secretion pathway. We further demonstrate that CX3CL1 impacts human islets by decreasing glucagon secretion without affecting that of insulin. Moreover, CX3CL1 decreases basal apoptosis of human β-cells.

2. MATERIAL AND METHODS

2.1. Antibodies and reagents

Recombinant TNFα, CX3CL1 and anti-CX3CL1 were obtained from R&D Systems Europe Ltd (Abingdon, United Kingdom). Anti-phospho-paxillin (Y118) was obtained from Invitrogen (Carlsbad, CA); Anti-paxillin from Becton Dickinson (San Jose, CA); Anti-phospho-Akt (Ser473) and anti-phospho-ERK-1/2 (Thr202 and Tyr204) were purchased from New England Biolabs (Beverly, MA). Anti-ERK1/2, anti-Akt, anti-AS160 and anti-phospho-(Ser/Thr)-AS160 were obtained from Cell Signaling Technology (Beverly, MA). Anti-p65 subunit of NF-κB (C-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and monoclonal anti-actin from Sigma. Anti-IRS-1 and IRS-2 were a gift from Dr. M.F. White (Children's Hospital, Harvard University, Boston, MA). Anti-insulin and anti-glucagon were from Dako (Glostrup, Denmark).

2.2. Human islets, sorted-cells and non-β-cells

Human islets were kindly provided by the Cell Isolation and Transplant Centre of the University of Geneva, through JDRF award 31-2008-413 (ECIT Islet for Basic Research Program). Human β-cells were sorted by FACS after labeling with Newport Green using a FACS Vantage (Becton Dickinson, USA) as described before in Ref. [21]. The purity of the β-cells and non-β-cells fractions were assessed for each preparation using immunofluorescence staining for insulin and glucagon.

2.3. RNA extraction and real-time PCR

Total RNA was extracted from 100 islets equivalents plated on extracellular matrix-coated culture dishes with a total RNA extraction kit (ReliaPrep RNA Cell Miniprep System, Promega, USA) and reverse transcribed with random hexamers. Commercially available human primers to 18s rRNA and CX3CL1 were purchased and assayed according to the manufacturer's protocol using the ABI 7000 system (Applied Biosystems, Foster City, CA). Changes in mRNA expression were calculated using the difference of cycle threshold values.

2.4. RNA sequencing dataset

Median gene expression levels (RPKM) of CX3CL1 and CX3CR1 in human islets vs. sorted β-cells or non-β-cells were derived from the RNA sequencing data obtained from Nica et al. [22].

2.5. CX3CL1 measurement in islet supernatant

Human islets were cultured 24 h in the presence or not of 20 ng/ml TNFα. To determine human CX3CL1 concentrations, islet supernatants were assayed using a multi-well sandwich immunoassay kit from Meso Scale Diagnostics (Gaithersburg, MD).

2.6. Rat sorted β-cells

Islets of Langerhans were isolated by collagenase digestion of pancreas from adult male Wistar rats followed by Ficoll (Histopaque-1077, Sigma–Aldrich, St. Louis, MO) purification [23]. Rat β-cells were sorted as previously described in Ref. [10].

2.7. Immunofluorescence

CX3CL1, insulin, glucagon, phospho-paxillin and paxillin were detected with specific antibodies according to standard procedures for fixation and confocal fluorescence microscopic observation [24]. Cell basal membranes were observed by confocal microscopy using a Zeiss LSM 510 or LSM 700 Meta microscope with a 63× oil immersion lens, and the images were acquired using LSM 510 or LSM 700 software (Carl Zeiss, Iena, Germany) and processed using Image J software (NIH).

2.8. Immunochemistry

Human islets treated or not with 20 ng/ml TNFα for 24 h were washed in PBS and fixed in 2% paraformaldehyde and sectioned at 5 μm. Before immunofluorescence, sections were rehydrated, incubated at room temperature with the necessary antibodies and observed by confocal microscopy.

2.9. Proteomic analysis

Six-plex TMT labeling was performed for protein quantification, according to manufacturer's instruction (Thermo Scientific). Briefly, 40 μg of each of the 6 samples were digested with trypsin, labeled, and pooled together, according to standard procedures [25]. Peptides were separated by off-gel electrophoresis, desalted and solubilized in an appropriate amount of 5% ACN/0.1% formic acid for mass spectrometry analysis.

Nano LC-MS/MS analyses were performed on a nanoAcquity system (Waters) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Raw data were converted, and peak lists were submitted to Easyprot for identification, and Isobar for quantification [26].

2.10. Western blotting

Phosphorylation and expression of various proteins were determined by Western blot analysis. Rat β-cell protein lysate was separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with the indicated primary antibody as previously described in Ref. [10]

2.11. Insulin secretion

Acute insulin release was measured as previously described in Ref. [10]. In brief, islets or cells were preincubated 2 h in Krebs–Ringer bicarbonate Hepes buffer with 0.5% BSA (KRB) and 2.8 mM glucose followed by 1 h at 2.8 mM glucose (basal secretion) and a further 1 h at 16.7 mM glucose (stimulated secretion) in a sequential manner.

Cellular insulin was extracted with 0.18 M HCl in 70% ethanol (acid ethanol). Insulin was measured by radioimmunoassay in supernatants and cell extracts using rat or human insulin standards as appropriate.

2.12. Glucagon secretion

Human islets were washed and preincubated for 2 h in KRB, 2.8 mM glucose, and then incubated containing 16.7 mM glucose (basal secretion) for 1 h followed by 1 h incubation in KRB containing 2.8 mM glucose. Islet glucagon was extracted in acid ethanol for determination of glucagon content. Secreted glucagon and glucagon content were measured by radioimmunoassay (Glucagon RIA kit, Millipore, USA).

2.13. Detection of apoptosis and proliferation

Cell death was measured by TUNEL assay and proliferation was assessed by BrdU incorporation, as previously described in Ref. [10].

2.14. Statistical analyses

Data are expressed as means ± SEM, with the number of individual experiments presented in the figure legends. All data were tested for normality and analyzed with PRISM (GraphPad, San Diego, CA). Differences were evaluated using Student's *t* test and ANOVA with Bonferroni post hoc test for multiple comparison analysis. Significance was set as $p < 0.05$.

3. RESULTS

3.1. CX3CL1 is expressed and secreted in human islet cells and regulated in response to TNFα

Using RNA sequencing (Figure 1A and B, data derived from Ref. [22]) and immuno-staining (Figure 1C), we report for the first time that CX3CL1 and its receptor CX3CR1 are differentially expressed in human islet cells. CX3CL1 is more expressed in the non-β-cell population (composed of approximately 60% α-cells with <5% β-cells) than in the β-cell population (approximately 90% β-cells). Immunofluorescence staining of dispersed human islet cells, confirmed that CX3CL1 is present in both β-cells and α-cells. Surprisingly, CX3CL1 co-localizes with glucagon but not insulin granules (Figure 1C). The receptor CX3CR1 is expressed in human β-cells and in non-β-cells, though at a lower absolute level compared with the ligand (Figure 1B). However, mRNA levels may not be faithfully reflected by those of the corresponding protein.

Interestingly, CX3CL1 is up-regulated in human islets treated with TNFα (20 ng/ml) for 24 h as shown here by immunofluorescence (Figure 1D) and mRNA expression (Figure 1E). Moreover, CX3CL1 released in the medium is also increase by TNFα (Figure 1F), indicating that TNFα regulates islet cell CX3CL1 expression and cleavage, leading us to explore the impact of CX3CL1 on pancreatic islet cells in the absence or in the presence of this cytotoxic cytokine.

3.2. CX3CL1 decreases human α-cell glucagon secretion and β-cell apoptosis

We analyzed the impact of increasing concentrations of CX3CL1 on human islet insulin and glucagon secretion and on sorted rat β-cell insulin secretion. The concentrations were chosen in order to cope with the amount released after TNFα stimulation. Neither insulin secretion (Figure 2A and E) nor cellular insulin content (Figure 2B and F) was significantly influenced by any concentration of CX3CL1 in either cell preparation. By contrast, low glucose stimulation of glucagon secretion was abolished by CX3CL1 across the entire concentration range studied without any effect on basal secretion at high glucose (Figure 2C) and without affecting total glucagon content (Figure 2D). We have previously demonstrated in different studies that focal adhesions (FA) are important molecular assemblies involved in insulin granule trafficking and secretion [27]. Indeed, glucose stimulation

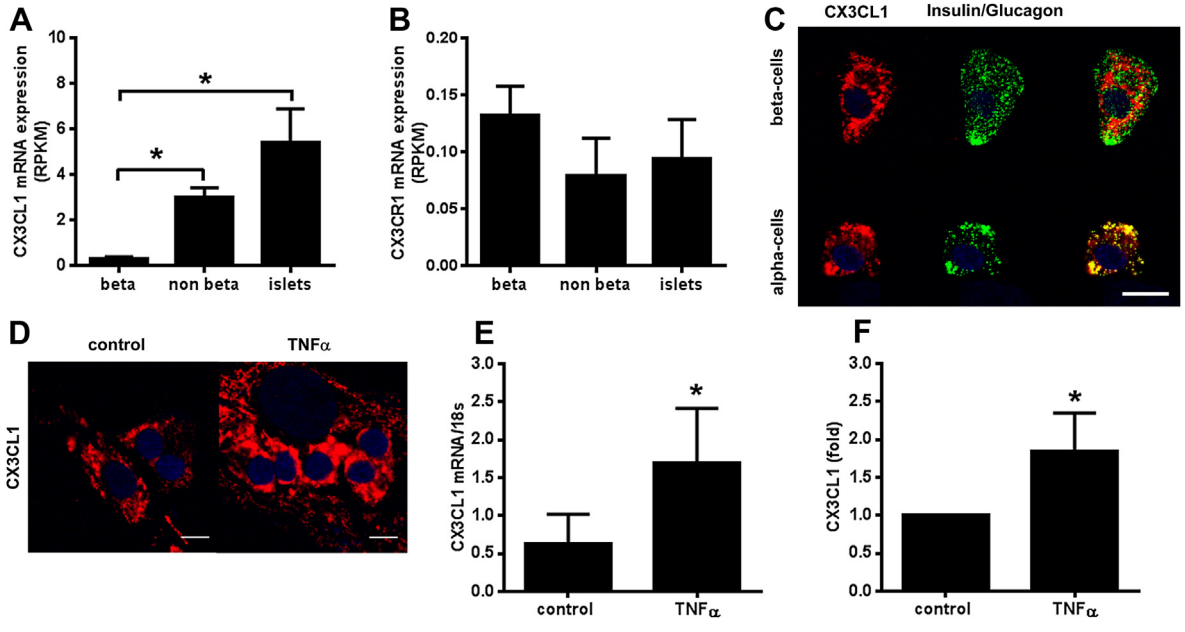

Figure 1: CX3CL1 is expressed in human islet cells and regulated in response to TNFα. A: CX3CL1 mRNA expression and B: CX3CR1 mRNA expression in human islets ($n = 11$), sorted β-cells ($n = 6$) and non-β-cells ($n = 6$) presented as reads per kilobase per million mapped reads (RPKM). Data derived from Ref. [22]. C: Representative images of human non-sorted islet cells co-stained for CX3CL1 (red), insulin (green) or glucagon (green) and DAPI (blue). D: Representative images of human non-sorted islet cells cultured 24 h in the presence or not of 20 ng/ml TNFα co-stained for CX3CL1 (red) and DAPI (blue). E: CX3CL1 mRNA expression in human islets after 24 h culture in the presence of 20 ng/ml TNFα ($n = 5$). F: CX3CL1 released in the media from human islets after 24 h culture in the presence of 20 ng/ml TNFα ($n = 5$). *$p < 0.05$ as indicated, tested by ANOVA followed by Bonferroni post hoc test or student's *t* test.

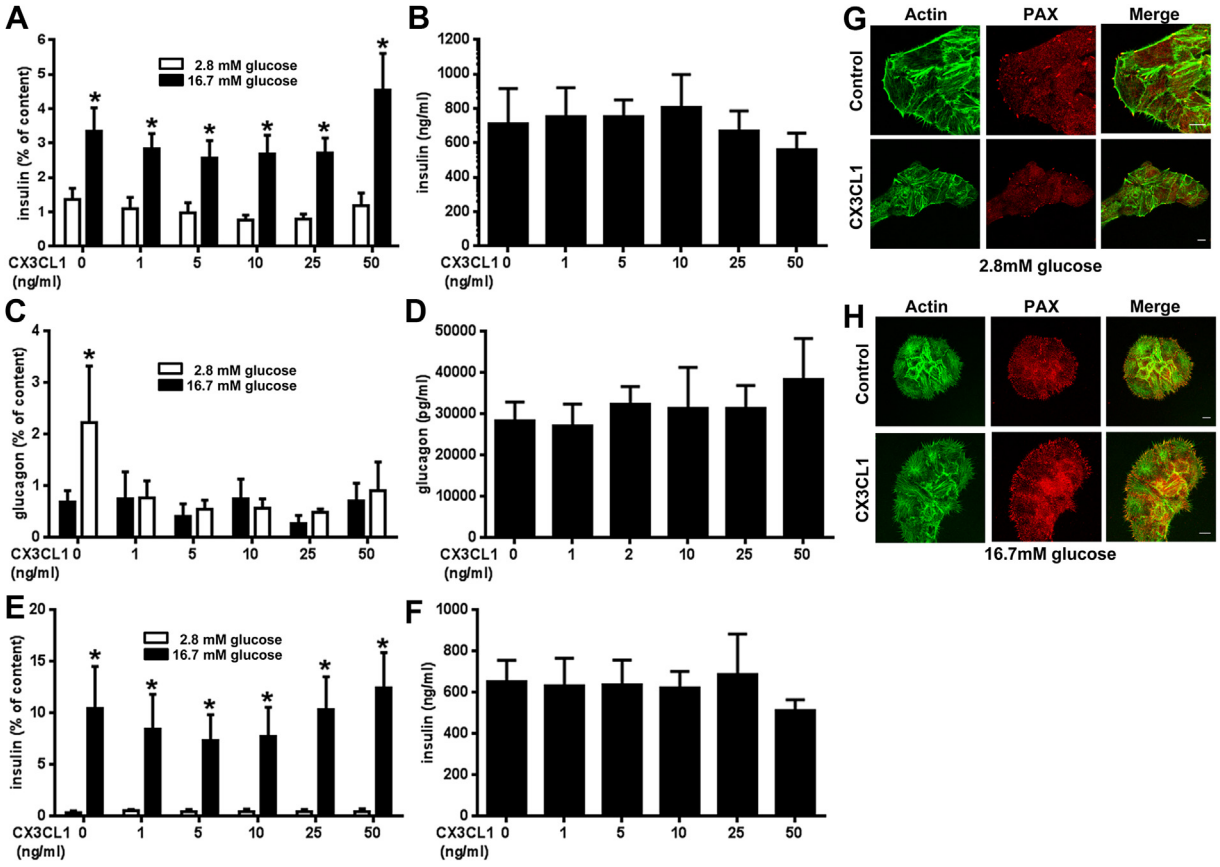

Figure 2: CX3CL1 decreases human islet glucagon secretion without affecting insulin secretion. Human islets (A–B) and sorted rat primary β-cells (E and F) were cultured for 24 h on 804G matrix-coated dishes in the presence of increasing concentrations of CX3CL1. A: Insulin secretion: human islets were incubated for 60 min at 2.8 mM glucose (open bars) followed by 60 min at 16.7 mM glucose (closed bars) ($n = 8$). B: Total insulin content of human islets ($n = 8$). C: Glucagon secretion: human islets were incubated for 60 min at 16.7 mM glucose (closed bars) followed by 60 min at 2.8 mM glucose (open bars) ($n = 5$). D: Total glucagon content of human islets ($n = 5$). E: Insulin secretion: rat sorted β-cells were incubated for 60 min at 2.8 mM glucose (open bars) followed by 60 min at 16.7 mM glucose (closed bars) ($n = 5$). F: Total insulin content of rat sorted β-cells ($n = 5$). G and H: Representative images of rat primary β-cells cultured for 24 h with or without 25 ng/ml CX3CL1. Prior to fixation, cells were incubated for 3 h at 2.8 mM and then incubated in either 2.8 mM or 16.7 mM glucose for 1 h. Cells were subsequently fixed and stained for actin (with phalloidin, green), anti-paxillin (red) and DAPI (blue). Scale bar: 10 μm. *$p < 0.05$ as tested by ANOVA followed by Bonferroni post hoc test.

induces FA remodeling by the formation of small protrusions at basal membranes containing paxillin which are necessary for insulin secretion [28] (Figure 2H). In agreement with our results obtained on insulin secretion (Figure 2A and E), CX3CL1 treatment had no effect on focal adhesion morphology (Figure 2G–H).

To investigate the potential impact of CX3CL1 on human β-cell survival, cell death was quantified using TUNEL assay on dispersed human islet cells. After 24 h treatment, CX3CL1 decreased human β-cell apoptosis. This decrease in apoptosis was concentration dependent and could only be observed with 5 and 10 nM CX3CL1 (Figure 3D). When cell death of FACS-purified rat β-cells (Figure 3B) or of β-cells of dispersed rat islets (Figure 3C) was evaluated, no significant effect of CX3CL1 could be observed at any concentration studied. These results could suggest that the positive impact of CX3CL1 on human β-cells is species specific. However, there are other potentially confounding factors. Specifically, basal apoptosis (in the absence of CX3CL1) was 11.6 ± 4.5% for human β-cells but only 0.7 ± 0.3% and 0.05 ± 0.02%, respectively, for sorted rat β-cells or β-cells within an unsorted rat islet cell population.

We investigated also the impact of CX3CL1 on rat β-cell proliferation (note that this was not possible using adult human β-cells since they do not replicate to any meaningful extent in vitro [29]). Replication was assessed by BrdU incorporation over 24 h. As for survival, CX3CL1 did not affect rat β-cell proliferation (Figure 3A).

3.3. CX3CL1 treatment prevents the impact of TNFα on glucose-stimulated insulin secretion and on the insulin signaling pathway in rat primary β-cells

The impact of CX3CL1 on insulin secretion was further investigated in primary rat sorted β-cells following 24 h of treatment with TNFα to induce insulin resistance and decrease GSIS [8]. Neither CX3CL1 (48 h pre-treatment) nor TNFα (24 h treatment) had an impact on subsequent short-term (1 h) basal (2.8 mM glucose) insulin secretion, whereas TNFα treatment dramatically reduced GSIS (1 h, 16.7 mM glucose) and this was prevented by CX3CL1 (Figure 4A). As observed previously for TNFα [10], the total insulin content was not influenced by the different conditions (data not shown). We have previously shown that several proteins from the canonical insulin signaling pathway are involved in GSIS in primary β-cells [30]. Therefore, we next explored the impact of CX3CL1 and/or TNFα on Akt, AS160, and IRS-2 protein expression in the basal condition (2.8 mM glucose) or after glucose stimulation (16.7 mM glucose). In rat primary sorted β-cells treated with TNFα for 24 h, glucose action on Akt Ser473 and AS160 phosphorylation was completely prevented (Figure 4B and C). By contrast,

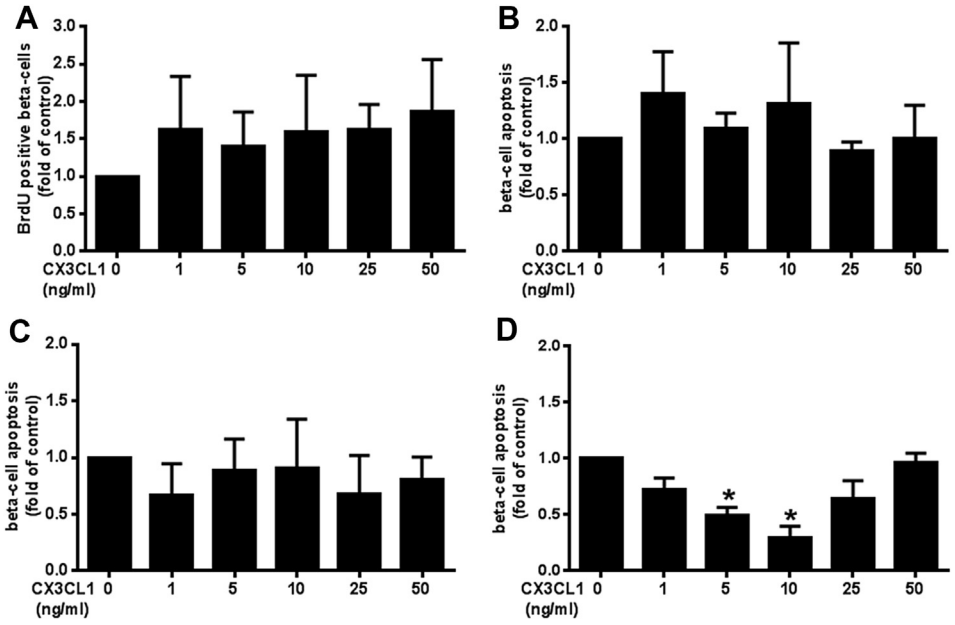

Figure 3: CX3CL1 decreases human β-cell apoptosis. A: BrdU-positive rat β-cells. Sorted rat primary β-cells were cultured on 804G matrix-coated dishes for 24 h in the presence of increasing concentrations of CX3CL1. Proliferation was measured by BrdU incorporation over 24 h ($n = 4$). B: Rat β-cell death. Sorted rat primary β-cells were cultured on 804G matrix-coated dishes for 24 h in the presence of increasing concentrations of CX3CL1. Cell death is expressed as TUNEL-positive β-cells normalized to Control (absolute value 0.7 ± 0.3%); $n = 4$. C: Rat β-cell death. Rat islets were dispersed into single cells and cultured (not sorted) on 804G matrix-coated dishes for 24 h in the presence of increasing concentrations of CX3CL1. Cell death is expressed as TUNEL-positive β-cells normalized to Control (absolute value 0.05 ± 0.02%); $n = 3$. D: Human β-cell death. Human islets were dispersed into single cells and cultured (not sorted) on 804G matrix-coated dishes for 24 h in the presence of increasing concentrations of CX3CL1. Cell death is expressed as TUNEL-positive β-cells normalized to Control (absolute value 11.6 ± 4.5%); $n = 3$. *$p < 0.05$ vs. 0 ng/ml CX3CL1 as tested by ANOVA followed by Bonferroni post hoc test.

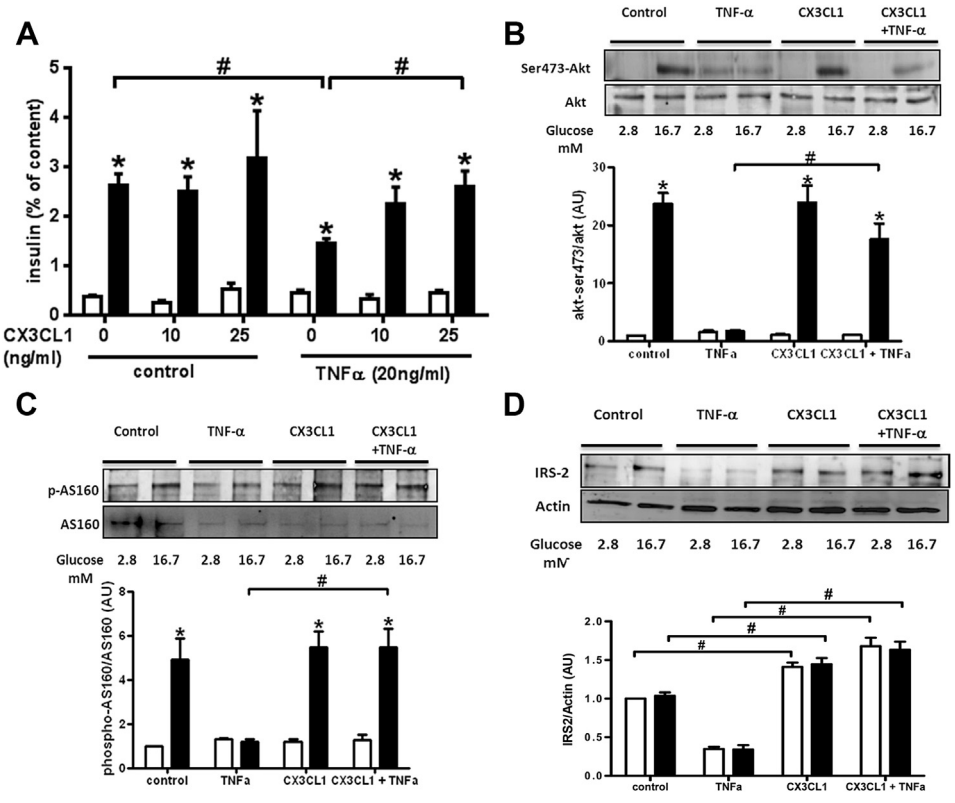

Figure 4: CX3CL1 treatment prevents the impact of TNFα on glucose-stimulated insulin secretion and on the insulin signaling pathway in rat primary β-cells. Rat primary β-cells were cultured in the presence of CX3CL1 (48 h, 25 ng/ml) and/or TNFα (20 ng/ml for the last 24 h) and then incubated for 60 min at 2.8 mM glucose (open bars) followed by 60 min at 16.7 mM glucose (closed bars) ($n = 5$). A: Insulin secretion (expressed as percentage of cell content/h). B and C: Representative western blots and quantification showing Akt Ser-473 (B) or AS160 (C) phosphorylation. D: Representative western blots and quantification showing IRS-2 protein expression. *$p < 0.05$ vs. 2.8 mM glucose; #$p < 0.05$ for indicated comparison as tested by ANOVA followed by Bonferroni post hoc test.

in cells pretreated with CX3CL1, glucose-induced Akt and AS160 phosphorylation was unaltered by TNFα exposure (Figure 4B and C). IRS-2 protein expression was increased by 48 h CX3CL1 treatment. In that condition IRS-2 protein degradation induced by TNFα was prevented (Figure 4D).

3.4. CX3CL1 treatment prevents TNFα action on mTOR and NFκB but has no effect on ERK-1/2 in primary sorted rat β-cells

Several pathways have been implicated in the negative impact of TNFα on insulin signaling in β-cells, adipose tissue and skeletal muscle [31–33]. Therefore we next explored the impact of CX3CL1 in TNFα induced mTOR, NFκB and ERK-1/2 signaling. Glucose stimulation increased mTOR, NFκB and ERK-1/2 phosphorylation in the control condition (Figure 5A–C) while TNFα increased basal phosphorylation of these same 3 proteins, and prevented further phosphorylation of mTOR and NFκB in the presence of high glucose (Figure 5A and B). In cells treated with CX3CL1, mTOR expression was reduced, glucose-induced mTOR and NFκB phosphorylation was prevented, whereas ERK-1/2 phosphorylation was increased (Figure 5). There was no change in total NFκB or ERK-1/2 (Figure 5B and C). Primary rat β-cells pre-treated with CX3CL1 were resistant to TNFα action on mTOR and NFκB phosphorylation (Figure 5). Total mTOR protein expression was reduced in cells pre-treated with CX3CL1 and exposed to TNFα (Figure 5A). Interestingly, CX3CL1 pre-treatment had no impact on TNFα induced (basal) ERK-1/2 phosphorylation (Figure 5C).

3.5. CX3CL1 treatment prevents TNFα effects on FA and paxillin phosphorylation in β-cells

As mentioned above, we have shown that FA remodeling, which involves focal adhesion kinase and paxillin phosphorylation and paxillin recruitment to nascent protrusions at the basal membrane, is crucial for glucose-stimulated insulin secretion in rat primary β-cells. While CX3CL1 had no impact on FA morphology on its own (Figure 2), we next investigated the impact of TNFα on these events and any possible protective role of CX3CL1. Here we show for the first time that glucose induces paxillin phosphorylation either in human dispersed islets (Figure 6A and B) or in human whole islets (Figure 6C) to the same extent as observed previously in rat primary β-cells [28]. TNFα treatment prevents the glucose impact on FA remodeling and paxillin phosphorylation (Figure 6A–D). We next investigated if CX3CL1 treatment could impact on FA remodeling when primary rat β-cells were challenged with TNFα. Under the basal condition (2.8 mM glucose) we observed

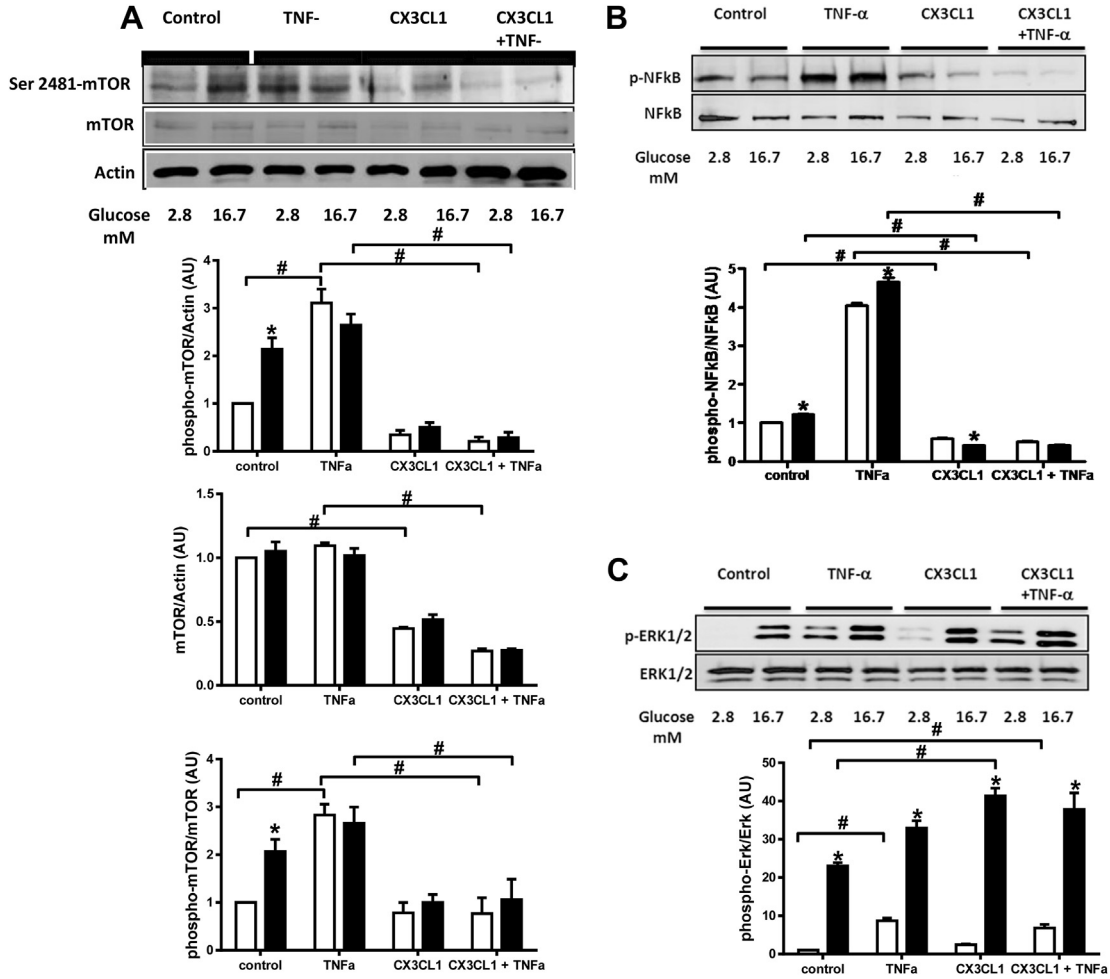

Figure 5: CX3CL1 treatment prevents TNFα action on mTOR and NFκB but not ERK-1/2 in primary sorted rat β-cells. Rat primary β-cells were cultured in the presence of CX3CL1 (48 h, 25 ng/ml) and/or TNFα (20 ng/ml for the last 24 h) and then incubated for 60 min at 2.8 mM glucose (open bars) followed by 60 min at 16.7 mM glucose (closed bars) ($n = 5$). Representative Western blots and quantification showing mTOR Ser-2481 (A), NFκB p65 (B) or ERK1/2 (C) phosphorylation. *$p < 0.05$ vs. 2.8 mM glucose; #$p < 0.05$ for indicated comparison as tested by ANOVA followed by Bonferroni post hoc test.

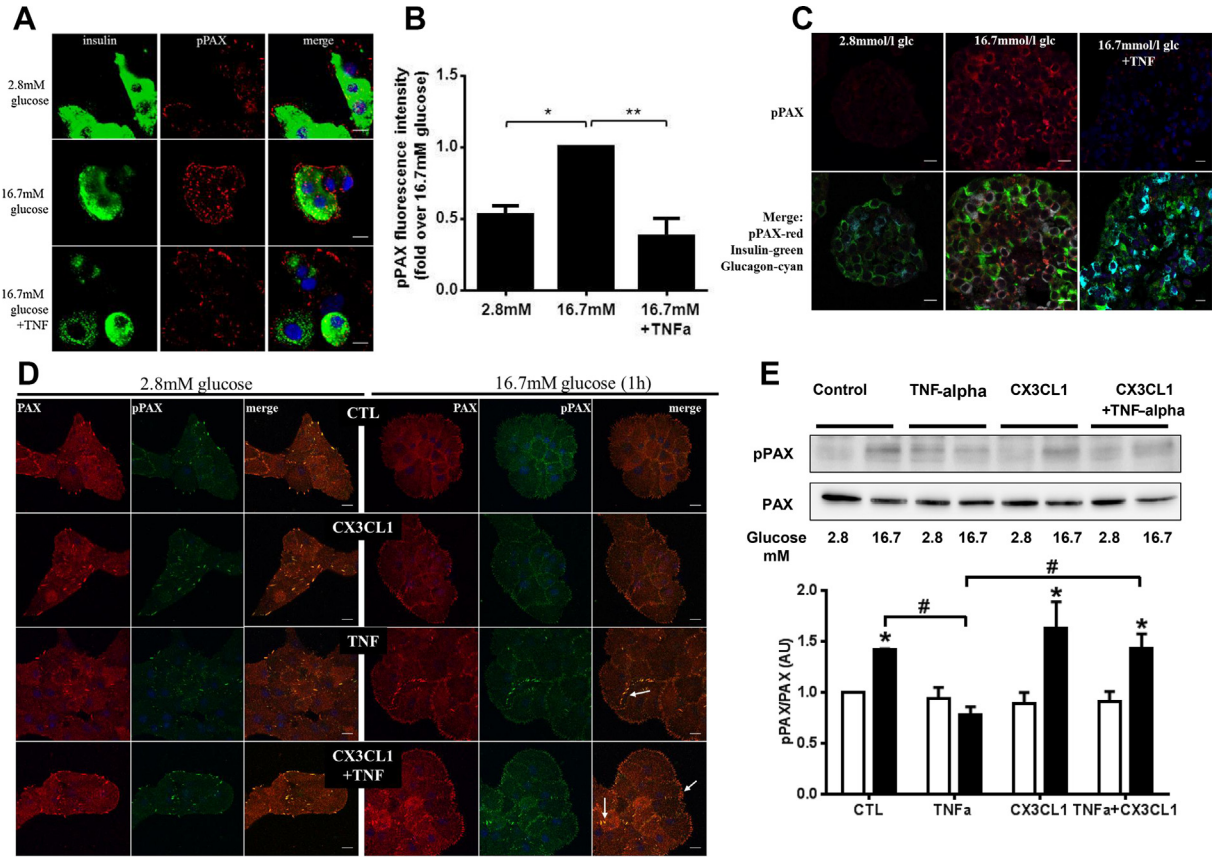

Figure 6: Impaired glucose-induced FA remodeling by TNFα is prevented by CX3CL1. A: Human dissociated pancreatic cells were cultured on 804G matrix-coated dishes in the presence or not of TNFα for 24 h followed by 1 h incubation at 2.8 mM glucose and stimulated by 16.7 mM glucose for 1 h. Cells were subsequently fixed and stained for insulin (green), phospho-paxillin (red) and DAPI (blue). B: Fluorescence intensity corresponding to phospho-paxillin in insulin positive cells was quantified using Image J and expressed as intensity per total cell surface. C: Human islets maintained in suspension were treated with TNFα for 24 h and then incubated in 2.8 mM glucose solution followed by 1 h in 16.7 mM glucose and then embedded in paraffin. Islet sections (5 μm) were then fixed and stained for insulin (green), glucagon (cyan), phospho-paxillin (red) and DAPI (blue). D and E: Rat primary β-cells were cultured in the presence of CX3CL1 (48 h, 25 ng/ml) and/or TNFα (20 ng/ml for the last 24 h) and then incubated for 1 h at 2.8 mM glucose or 16.7 mM glucose as indicated on the figure. Representative images (D) of rat β-cells stained for paxillin (with phalloidin, red), anti-phospho-paxillin (green) and DAPI (blue), scale bar: 10 μm. Representative western blots and quantification (E) showing paxillin phosphorylation. *$p < 0.05$ vs. 2.8 mM glucose; #$p < 0.05$ for indicated comparison as tested by ANOVA followed by Bonferroni post hoc test.

large FAs containing phospho-paxillin at the periphery and in the center of the cell (Figure 6D). The number and morphology of these FAs were unchanged at 2.8 mM glucose regardless of TNFα and/or CX3CL1 treatment. When primary rat β-cells were stimulated with 16.7 mM glucose, FA remodeling was altered by TNFα and the number of large FAs was maintained at levels normally observed at 2.8 mM glucose. However, when cells were previously treated with CX3CL1, TNFα was no longer able to impact FA morphology (Figure 6D). In line with these results and our previous work on FAs and paxillin, we further observed that TNFα prevented glucose induced paxillin phosphorylation, while CX3CL1 protected against this effect of TNFα (Figure 6E).

3.6. Impact of TNFα on human islet protein expression

In order to understand in finer molecular detail the impact of TNFα on pancreatic cells, we have explored modification of protein expression of human islets treated with TNFα for 24 h. Protein expression was monitored using TMT approach. We were able to identify 1298 different proteins in human islets, among which 31 were significantly up or down regulated after TNFα treatment. Here we focused our attention on proteins for which the ratio TNFα-treated/untreated was <0.9 or >1.2 (Table 1). We were particularly interested in proteins where regulation induced by TNFα is known to be NFκB dependent, or in proteins known to mediate the effects of TNFα on β-cells or in proteins involved in β-cell physiology. The prohormone convertase PCSK1, previously known as PC1/3, is essential for proinsulin conversion. Moreover, in islets this protein is more highly expressed in β-than non-β cells (Table 2). Sorcin which belongs to the penta-EF-hand family, has been shown to be involved in GSIS [34]. ICAM-1 is expressed on many cell types including endothelium, hepatocytes, and leukocytes and was shown previously to be up-regulated in islets exposed to TNFα [35]. PCSK1 and sorcin had their protein expression reduced by TNFα exposure in human islet and sorted primary β-cells (Table 1, Figure 7A–D). Interestingly, this effect was reversed when primary rat β-cells were pre-treated with CX3CL1 (Figure 7B–D). Conversely, Intercellular adhesion molecule 1 (ICAM1), a glycoprotein know to interact with integrins, was increased after TNFα treatment, while CX3CL1 exposure did not prevent its up-regulation (Figure 7A).

4. DISCUSSION

Recently, we have shown that human skeletal muscle cells with different insulin sensitivity secrete different myokines that have a

Table 1 — Proteomic analysis of human islets treated with TNFα.

Description	Swissprot AC	Gene	Ratio TNF/control	*p* value ratio	*p* value sample
Glutamate decarboxylase 2	Q05329	GAD2	0.8	0.01834	0.03359
Spectrin beta chain, non-erythrocytic 2	O15020	SPTBN2	0.8	0.03830	0.02569
Neuroendocrine convertase 1	P29120	PCSK1	0.8	0.00076	0.03475
Sorcin	P30626	SRI	0.9	0.04345	0.01012
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O94760	DDAH1	0.9	0.01900	0.00141
Protein Hook homolog 1	Q9UJC3	HOOK1	0.9	0.01216	0.04452
Catalase	P04040	CAT	0.9	0.04172	0.01311
Sec1 family domain-containing protein 1	Q8WVM8	SCFD1	0.9	0.02080	0.00114
Endothelin-converting enzyme 1	P42892	ECE1	1.2	0.04623	0.00127
Proteasome activator complex subunit 1	Q06323	PSME1	1.2	0.02628	0.00568
Sterol O-acyltransferase 1	P35610	SOAT1	1.2	0.00001	0.00000
Putative RNA-binding protein Luc7-like 2	Q9Y383	LUC7L2	1.2	0.04923	0.00228
E3 ubiquitin-protein ligase RNF213	Q63HN8	RNF213	1.3	0.00206	0.01677
Solute carrier family 12 member 7	Q9Y666	SLC12A7	1.3	0.03840	0.01046
Tryptophan—tRNA ligase, cytoplasmic	P23381	WARS	1.4	0.00103	0.00001
Signal transducer and activator of transcription 1-alpha/beta	P42224	STAT1	1.5	0.04124	0.00996
Superoxide dismutase [Mn], mitochondrial	P04179	SOD2	2.3	0.02664	0.02274
Intercellular adhesion molecule 1	P05362	ICAM1	3.3	0.00003	0.00002

Proteins down- or up-regulated regulated in human islets by >10% after 24 h TNFα treatment compared with control. Data obtained by proteomic analysis of cultured human islets ($n = 3$).

Table 2 — PCSK1, Sorcin and ICAM1 mRNA expression.

	Islets	β-cells	Non-β-cells
ICAM1	27.53 ± 7.9	11.11 ± 2.5*	16.66 ± 3.9
SORCIN	12.5 ± 0.8	10.93 ± 0.7	9.2 ± 0.5
PCSK1	112.1 ± 24.7	232.9 ± 26*	56.9 ± 10.1#

ICAM1, Sorcin and PCSK1 mRNA expression in human islets ($n = 11$), sorted β-cells ($n = 6$) and non-β-cells ($n = 6$) presented as reads per kilobase per million mapped reads (RPKM).
*$p < 0.05$ β-cells vs. islets; #$p < 0.05$ non-β-cells vs. islets as tested by ANOVA followed by Bonferroni post hoc test.
Data derived from Ref. [22].

bimodal impact on β-cell insulin secretion, proliferation and survival [18]. Thereby, we identified CX3CL1 (fractalkine) as one of the major cytokines regulated by TNFα in human skeletal muscle cells. In the present study, we have investigated the effects of this novel myokine on islet cells and shown that CX3CL1 can protect β-cells from the negative impact of TNFα while also improving human β-cell survival in culture and blocking the stimulation of glucagon secretion by low glucose from human islets. Moreover, we demonstrate that human islets are able to produce and secrete CX3CL1.

CX3CL1 is a unique member of the CX3C subclass of the chemokine superfamily and signals only via one receptor, which is a G protein-coupled receptor named CX3CR1 [36]. CX3CL1 is synthesized in a membrane-bound form and is processed by Adam 10 and Adam 17 at its exposed N-terminus to generate the soluble form [15,37]. CX3CL1 interacts with its receptor either bound to the membrane to facilitate cell—cell interaction and communication or in its cleaved soluble form to exert paracrine and endocrine effects on different tissues [11,12,15—17]. Therefore, we tested the impact of CX3CL1 on islet cells under control conditions or after TNFα treatment, shown previously to decrease GSIS and to induce insulin resistance [10]. We have observed, using RNA sequencing in human islet cells, sorted β and non-β-cells [22], that CX3CL1 is expressed in islet cells. This expression is higher in non-β-cells and CX3CL1 co-localizes with glucagon but not insulin-containing secretory vesicles, suggesting a possible paracrine or autocrine role in islets further to secretion from α-cells. CX3CR1 receptor mRNA is expressed at comparable though extremely low levels in all islet cells. Our data extend previous findings by others showing by immunofluorescence that CX3CL1 and its receptor are expressed in human β-cells [20]. Low levels of expression in α-cells might have prevented the detection of the receptor protein by immunofluorescence in the earlier study [20]. Nevertheless, using lower concentrations (1—50 ng/ml vs. 100 ng/ml), different incubation time (48 h vs. 1 h), and different experimental setting (sequential vs. differential insulin secretion test) than the one used by Lee et al. [20], CX3CL1 did not potentiate GSIS in human islets or rat sorted β-cells. In agreement with their findings, we do, however, conclude that CX3CL1 does not affect basal insulin secretion. Our data are supported by the absence of FA remodeling induced by CX3CL1, which is normally necessary for insulin secretion [27].

Interestingly, CX3CL1 treatment blocks low glucose stimulation of glucagon secretion. This effect can be seen as a feedback loop regulation of glucagon secretion during and after exercise as CX3CL1 has been identified as a myokine with increased circulating levels after exercise [38]. Indeed, glucagon levels increase during exercise in order to stimulate hepatic glucose production and decrease rapidly after the training period [39,40] similarly to what we observed with CX3CL1 treatment. Moreover, as glucagon is known to impact on liver to lower lipid content, a decrease of glucagon secretion after CX3CL1 stimulation might increase lipid plasma level. High serum lipid levels have been shown to be strongly related to the development of atherosclerosis. Therefore, the impact of CX3CL1 on glucagon secretion might in part explain the pro-artherogenic effect of CX3CL1 [41].

Interestingly, CX3CL1 treatment protects sorted rat β-cells from the effect of TNFα on GSIS, insulin sensitivity and FA remodeling. Our data are in agreement with the improvement of glucose tolerance in mice injected with CX3CL1 and the results obtained in mice lacking CX3CR1 [18]. Furthermore, we observed that the protective effect of CX3CL1 was not limited to TNFα. Indeed, CX3CL1 treatment protects β-cells from apoptosis induced through exposure to IL-1 β, interferon-gamma and TNFα together (data not shown). Moreover, CX3CL1 prevented the activation of mTOR and NFκB, two known TNFα targets implicated in the negative regulation of the insulin signaling cascade and glucose uptake [31,33]. mTOR protein expression is decreased by CX3CL1 treatment which explain in part the decrease of its phosphorylation

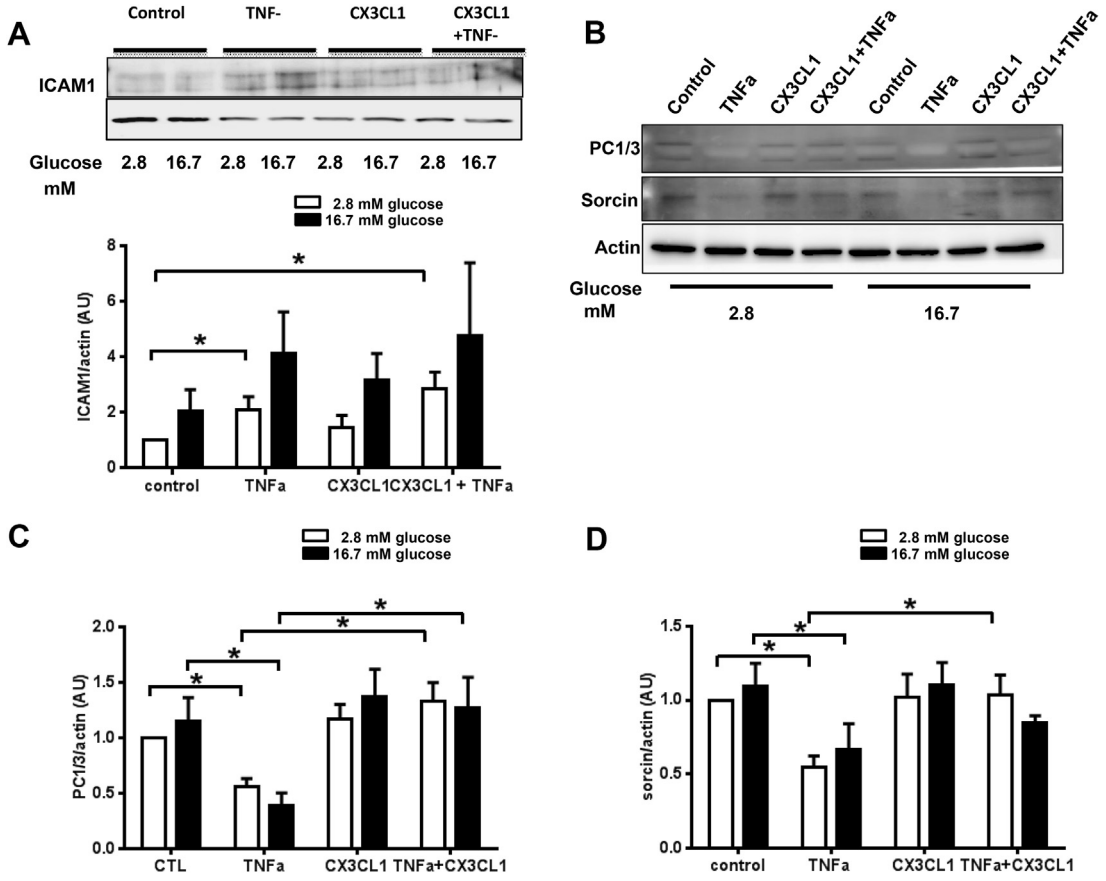

Figure 7: CX3CL1 prevents TNFα impact on sorcin, PCSK1 and ICAM-1 protein expression. A: Representative western blot and quantification of ICAM1 in rat primary β-cells cultured in the presence of CX3CL1 (48 h, 25 ng/ml) and/or TNFα (20 ng/ml for the last 24 h) and then incubated for 60 min at 2.8 mM glucose (open bars) followed by 60 min at 16.7 mM glucose (closed bars) ($n = 5$). B: Representative western blot of sorcin and PC1/3. C–D: Quantification showing PSC1/3 (C) and Sorcin (D) protein expression. *$p < 0.05$ for indicated comparison as tested by ANOVA followed by Bonferroni post hoc test.

when cells were treated with TNFα. Nevertheless, the action and impact of mTOR phosphorylation in primary islets is not clear [42]. Some reports show that inhibition of mTOR phosphorylation by rapamycin treatment has no impact on GSIS or apoptosis in human and/or porcine islets while a study performed in a cell line describes a decrease of GSIS after long treatment with rapamycin [43]. Our data suggest that a partial decrease of mTOR protein expression induced by CX3CL1 has no negative impact on β-cell function and survival. Interestingly, CX3CL1 increases ERK1/2 phosphorylation after glucose stimulation, in agreement with a previous study [20], whereas the impact of TNFα was unchanged. Our data indicate that the negative impact of TNFα on β-cells is not mediated by ERK1/2 as postulated in adipose tissue and skeletal muscle [31,44].

In order to gain insight into the protective impact of CX3CL1 on TNFα treated β-cells we analyzed all the proteins in human islets regulated by TNFα. Using a proteomic approach we have identified 31 proteins significantly regulated by TNFα among 1298 detected in human islets. In the present study, we decided to focus on proteins regulated by TNFα and known to be involved in β-cell function and survival. ICAM-1 was up-regulated in human islets and sorted primary rat β-cells after TNFα exposure. We have shown in the past that the beneficial impact on primary β-cell secretion and spreading induced by the 804G extracellular matrix used here as the culture substratum for islet cells, is mediated by interaction between laminin and β-1 integrins [45]. ICAM protein levels were increased 3.3-fold by TNFα, in keeping with our previous findings [35] but this increase was not prevented by CX3CL1. This data confirm our previous observation showing that ICAM-1 was not responsible for the effect of TNFα on β-cell [35]. Sorcin, which belongs to the penta-EF-hand family, has been shown to be involved in GSIS [34]. In our study, we show for the first time that sorcin is down regulated by TNFα in human islets and rat primary β-cells. Sorcin has been shown to interact with the ryanodine receptor (RyR) to inhibit RyR activity and therefore stopping the Ca^{2+} induced Ca^{2+} release process in the heart [46]. Interestingly, CX3CL1 treatment prevents sorcin degradation by TNFα, which could explain the rescue of GSIS, a calcium dependent process. Our hypothesis is supported by a recent study where sorcin silencing induced a decrease of GSIS to the same extent as what we observe in TNFα treated β-cells [46]. Moreover, CX3CL1 treatment prevents PCSK1 down regulation in human islets and primary sorted β-cells induced by TNFα. Since decreased levels of this prohormone convertase impact negatively on conversion of proinsulin to insulin [47], we speculate that conversion may be perturbed by TNFα and that CX3CL1 would protect against this.

5. CONCLUSIONS

Taken together, our data demonstrate that the recently discovered myokine CX3CL1 (fractalkine) is also produced in human islets and that such production is increased by TNF-α, perhaps as part of a local, intra-islet protective loop. Indeed, our data further indicate that CX3CL1 is a protective factor against the adverse effects of TNFα on β-cells, with specific interaction between the TNFα and CX3CL1

signaling pathways. CX3CL1-based therapy could therefore be a potential approach to prevent islet cell dysfunction in T2D.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest to disclose in relation to this work.

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