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Adipose tissue derived-factors impaired pancreatic β -cell function in diabetes

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ABSTRACT

Inflammatory factors produced and secreted by adipose tissue, in particular peri-pancreatic adipose tissue (P-WAT), may influence pancreatic β -cell dysfunction. Using the ZDF Rat model of diabetes, we show the presence of infiltrating macrophage (ED1 staining) on pancreatic tissue and P-WAT in the pre-diabetes stage of the disease. Then, when the T2D is installed, infiltrating cells decreased. Meanwhile, the P-WAT conditioned-medium composition, in terms of inflammatory factors, varies during the onset of the T2D. Using chemiarray technology, we observed an over expression of CXCL-1, -2, -3, CCL-3/MIP-1 α and CXCL-5/LIX and TIMP-1 in the 9 weeks old obese ZDF pre-diabetic rat model. Surprisingly, the expression profile of these factors decreased when animals become diabetic (12 weeks obese ZDF rats). The expression of these inflammatory proteins is highly associated with inflammatory infiltrate. P-WAT conditioned-medium from pre-diabetes rats stimulates insulin secretion, cellular proliferation and apoptosis of INS-1 cells. However, inhibition of conditioned-medium chemokines acting via CXCR2 receptor do not change cellular proliferation apoptosis and insulin secretion of INS-1 cells induced by P-WAT conditioned-medium. Taken together, these results show that among the secreted chemokines, increased expression of CXCL-1, -2, -3 and CXCL-5/LIX in P-WAT conditioned-medium is concomitant with the onset of the T2D but do not exerted a direct effect on pancreatic β -cell dysfunction.

1. Introduction

Pancreatic β -cells play a central role in the development of type 2 diabetes (T2D). In patients with metabolic syndrome or during the silent phase of T2D pathogenesis, pancreatic β -cell is able to compensate for insulin resistance by increasing β -cell mass and function in order to maintain normoglycaemia. However, during the transition to T2D, β -cell is exposed to metabolic and inflammatory stresses, which gradually lead to secretory dysfunction and a loss in β -cell mass over time. The mechanisms involved in the process of compensation and decompensation are poorly understood and still debated [1].

These mechanisms could be influenced by a dialog between endocrine pancreas and peripheral tissues, especially the adipose tissue. This latter tissue displays a wide range of physiological functions: regulation of fat mass and nutrient homeostasis, blood pressure control, bone

mass, and thyroid/reproductive function [2]. All these processes are coordinated by adipose tissue mainly through the synthesis and release of adipokines, such as inflammatory cytokines, chemokines, growth factors and others proteins. For these reasons, adipose tissue is now considered as an endocrine organ and part of an innate immune system [3].

In this respect, there is growing evidence that obesity is associated to a chronic systemic low grade state of inflammation [4]. In obesity, the increased visceral adiposity modifies adipocyte function and secretory profile [5]. Adipokines with pro-inflammatory properties are overproduced, while others with anti-inflammatory or insulin-sensitizing properties, like adiponectin, are decreased. Inflammation biomarkers, such as Tumor Necrosis Factor α (TNF α), Interleukin-6 (IL-6) and C-reactive protein (CRP), are increased in obesity and predict the development of type 2 diabetes [6]. These adipose tissue-derived

Abbreviations: T2D, type 2 diabetes; P-WAT, peri-pancreatic adipose tissue; CM, conditioned-medium; INS-1, insulin secreting β -cell line originated from a rat insulinoma

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factors are secreted by the different cells of the adipose tissue. Macrophages, which infiltrate obese adipose tissue, are necessary and sufficient to perpetuate the obese state and insulin resistance [7]. Although the involvement of pro-inflammatory cytokines in pancreatic β -cell dysfunction and T2D pathogenesis is emerging, it remains unclear how fat-derived factors are able to early destabilize and counteract β -cell compensation and finally lead to its dysfunction.

Obesity is associated to the development of an ectopic adipose tissue which penetrates the pancreas and establishes direct contacts with islets of Langerhans, called peri-pancreatic adipose tissue (P-WAT). Recently, Rebuffat SA et al. [8,9] showed that factors secreted by this P-WAT modulate proliferation of β -cells in an animal model of obesity and insulin resistance. These arguments suggest a paracrine communication between P-WAT and β -cell, partially responsible for adaptation of β -cells.

Our hypothesis is that β -cell plasticity and function could be influenced by the dialog between β -cell and the adipose tissue surrounding the pancreas. Therefore, to address changes in β -cell mass and function during the onset of T2D, we focused on fat-derived factors proceeding from the P-WAT in an animal model of T2D, the obese ZDF rat. In this study, we showed that macrophages infiltration and adipocytes size of P-WAT were linked to changes in the profile of secreted factors. In addition, we demonstrated that conditioned-medium of P-WAT influence β -cell mass and function, leading to the development of T2D.

2. Materials and methods

2.1. Ethical approval

Animals were housed according to institutional guideless for animal use and care. The animals have been handled in the laboratory animal house and used in accordance with the "Principles of Laboratory Animal Care" (NIH Publication no. 85-23, revised 1985) and according to national law. Our laboratory was habilitated to perform experiments on vertebrate animals (approval D34-172-25 from the French Agriculture Ministry) and our study was approved by the ethics committee of our institution.

2.2. Zucker Diabetic Fatty (ZDF) rats

Male, leptin receptor-deficient ZDF rats (ZDF/Gmi, fa/fa) and homozygous lean ZDF rats (ZDF/Gmi, +/+) were purchased from Charles River Laboratories (L'Arbresle, France). Animals were housed on a 12–12 h light–dark cycle at room temperature (21–22 °C) with free access to food (Purina 5008; Purina Mills, St. Louis, MO, USA) and water. Eight obese ZDF rats from each groups of age were sacrificed; 6 weeks-old (insulin-resistant rats), 9 weeks old (pre-diabetic rats) and 12 weeks old (T2D rats). Male lean ZDF rats were used as control littermate. For biochemical measurement, blood was collected from the tail vein, centrifuged and plasma aliquots sampled and frozen. Rats from each group were anesthetized and killed by decapitation. For morphological studies, pancreases and adipose tissue were removed, fixed in 10% buffered formalin, dehydrated, embedded into paraffin and cut into 5 μ m sections with a Leica RM 2145 (Leica Instruments, Nussloch, Germany). White adipose tissue surrounding the pancreas, P-WAT, was also excised, weighed, cut and used for the preparation of conditioned-medium.

2.3. Pancreas and peripancreatic adipose tissue morphological studies

Pancreas and P-WAT sections were stained with hematoxyline-eosine, following standard protocols, for light microscopy. The size of adipocytes were estimated with the Leica DRM software (Leica Microsystems GmbH, Wetzlar, Germany), based on $\times 200$ magnification of histological sections. For insulin staining, pancreatic tissue sections (5 μ m) were fixed with 3% paraformaldehyde for 30 min,

permeabilized 5 min in 0.1% Triton X-100 and quenched with 50 mM NH_4Cl for 10 min. After two washings with PBS, the slides were saturated with 2% BSA-gelatine solution and then incubated with an anti-insulin antibody diluted at 1/200 overnight at 4 °C. After three washings, sections were incubated for 1 h with Fluorescein isothiocyanate (FITC) - conjugated antibodies. After three additional washings, the slides were mounted in citifluor (Citifluor, U.K.) and observed with the BioRad MRC 1024 confocal microscope using the facilities of RIO imagery platform (Montpellier, France). The negative control was performed using only the conjugated antibody by incubating the cells with the secondary antibody alone.

2.4. Immunohistochemistry of infiltrated macrophages in tissues

Pancreas and P-WAT sections were deparaffinized, blocked for endogenous peroxidases and incubated with the anti-CD68 (ED1) antibody (diluted 1:100; Dako, Trappes, France) at 4 °C overnight. Antibody distribution was visualized by a streptavidin–biotin complex assay and a Diaminobenzidine (DAB) substrate kit (Dako), and analyzed with Leica DRM software (Leica Microsystems GmbH), based on $\times 100$ magnification of histological sections. Sections incubated without primary antibody were used as negative control.

2.5. Conditioned-medium preparation

The conditioned-medium of adipose tissue was prepared as previously described [8,10], with some modifications. P-WAT was finely minced in PBS. Under sterile conditions, the tissue aliquots were filtered through nylon gauze (70 μ m mesh, Cell Strainer; BD Biosciences, Bedford, MA), washed in PBS, and cultured for 24 h in RPMI 1640 (Biosera, Ringmer, UK) containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mmol/l L-glutamine, 11.1 mmol/l D-glucose. The tissue to medium ratio was set at 1 g of tissue to 5 ml of medium. The conditioned-medium was subsequently collected, carefully avoiding the lipid floating on the top, and then filtered with a 0.22 μ m sterile filter (Millipore, Bedford, MA) and kept frozen at -80 °C until use.

2.6. Rat cytokine array

This array, Cat # ARY008 (R&D System, Minneapolis, MN) is able to analyze 29 different cytokines (CXCL-1/CINC-1, CXCL-2/CINC-3, CXCL-3/CINC-2, ICAM-1, GM-CSF, $\text{IFN}\gamma$, IL-1 α , IL-1 β , IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IL-17, CXCL-10/IP-10, CXCL-5/LIX, L-selectin, CXCL-9/MIG, CCL3/MIP-1 α , CCL5/RANTES, CXCL-7/Thymus chemokine-1, TIMP-1, TNF- α , VEGF). Briefly, conditioned-medium from P-WAT of obese and lean ZDF rats (6, 9 and 12 week old animals) were mixed with the detection antibody cocktail at room temperature for 60 min, added to saturated array membranes and incubated overnight at 4 °C. The membranes were washed and then, incubated with the streptavidin-horseradish peroxidase (HRP) solution for 30 min at room temperature. After washings, the antibodies were detected by chemiluminescence using the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA). The intensities of signals were quantified directly with a chemiluminescence imager (Vilber-Lourmat, Marne la Vallée, France).

2.7. CXCL-1 assay

C-X-C motif chemokine ligand-1 (CXCL-1) concentration was determined in P-WAT conditioned-medium using Quantitative Rat CXCL-1 immunoassay (R&D System), following the manufacturer's protocol.

2.8. INS-1 cells culture

INS-1 rat insulinoma β -cells used in the study have been obtained by CB Wollheim [11] and used between the 10th and 16th cell culture

passage. Cells were maintained in RPMI 1640 supplemented with 10% FBS (vol./vol.), 1 mmol/l sodium pyruvate, 50 μ mol/l 2-mercaptoethanol, 2 mmol/l glutamine, 10 mmol/l HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Sigma-Aldrich).

2.9. Proliferation assay

INS-1 cells growth was assessed using the Calbiochem® BrdU Cell proliferation Assay according to the manufacturer's instructions. Cells were cultured in triplicate in 96-well culture microplates at 1×10^4 cells per well in 200 μ l of culture medium during 24 h. After serum deprivation, the cells were incubated in the presence of the P-WAT conditioned-medium diluted at 1:3 in INS-1 cell medium for 24 h. To assess the effects of exogenous CXCL-1 on cellular proliferation, recombinant rat chemokine (R&D System) was added to the culture medium at the desired concentration. For blockade experiments, CXCR2 antagonist (SB225002, Sigma) was added at the desired concentration to P-WAT conditioned-medium. Microplates were then incubated for an additional 24 h at 37 °C in a wet atmosphere containing 5% CO₂. Bromodeoxyuridine (BrdU, dilution 1:100) was then added, and microplates incubated for an additional 24 h. BrdU incorporation was measured using a HRP-conjugated anti-BrdU antibody and *o*-phenylenediamine as substrate. The colorimetric reaction was stopped by addition of 4 N sulphuric acid per well and absorbance measured at 490 nm.

2.10. Apoptosis assay

INS-1 cells apoptosis was assessed using the homogeneous caspases assay, fluorimetric (Roche) according to the manufacturer's instructions. Cells were cultured in duplicate in 96-well culture microplates at 1×10^4 cells per well. They were incubated in the presence of the P-WAT conditioned-medium (1:3 in the culture medium) for 24 h. Pre-diluted apoptosis inducing agents were then added, and microplates incubated for an additional 24 h. Caspase substrate pre-diluted in incubation buffer is added and incubated for 2 h at 37 °C. The incubation buffer is lysing the cells during this incubation. Free R110 is determined fluorimetrically at 521 nm; the developed fluorochrome is proportional to the concentration of activated caspases.

2.11. Insulin secretion

INS-1 cells were cultured for 24 h in the absence or in the presence of (1) different P-WAT conditioned-medium (1:3 in the culture medium), (2) recombinant chemokine CXCL-1 (R&D system), (3) CXCR2 inhibitor (SB225002, Sigma) alone or with P-WAT conditioned-medium. After 24 h, cells were then washed and deprived in glucose during 1 h and incubated in the presence of 2.8 and 8.3 mM glucose. Cell supernatants were then collected and insulin content was extracted with acid/alcohol mixture (1.5%/75%). Quantification of insulin was performed using HTRF (Homogeneous Time Resolved Fluorescence) assay kit (Cisbio, Marcoule, France).

2.12. Antibody arrays

Panorama antibody microarray containing 224 different antibodies spotted in duplicate on nitrocellulose-coated slides was purchased from Sigma-Aldrich. Protein extracts (1 mg/mL) from pancreatic islets of obese ZDF rats ($n = 2$ per group) were labeled with Cy3 and Cy5 (Amersham Biosciences, Buckingham, UK) as described by the manufacturer (Sigma-Aldrich, Steinheim, Germany). Samples labeled with a dye/protein molar ratio 2 were applied to the antibody microarray in Array Incubation Buffer (Sigma) and incubated for 45 min protected from light with gentle shaking. The array was then washed three times with 5 ml of Washing Buffer (Sigma) and air-dried. Cy3 and Cy5 signals were read on the Gene Pix Pro 4.0 (MDS Analytical Technologies). Each

experiment was carried out twice and analyzed for both Cy3 and Cy5 signals. Proteins whose expression was found down or upregulated by 2 fold or more between 9 versus 6 weeks and 12 versus 9 weeks old obese ZDF rats were considered as significant.

2.13. Statistical analysis

All data are expressed mean \pm SEM, unless otherwise stated. Statistical analyses were performed using GraphPad Prism v5.0 (San Diego, CA, USA). Differences between groups were analyzed by paired or unpaired Student's *t*-tests. A two- or three-way analysis of variance (ANOVA) was carried out for the factors strain (lean ZDF and obese ZDF) and cohort (6, 9 or 12 weeks old) using the general linear models procedure. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characteristics of the study model

The obese ZDF rats develop obesity and insulin resistance in the young age and then, with age, they progressively develop hyperglycemia associated with impaired pancreatic β -cell function and loss of β -cell mass [12]. As expected, body weight was increased with age and significantly greater at the age of 9 and 12 weeks for obese ZDF ($P < 0.05$) (Table 1). Blood glucose was significantly elevated in 12-week-old obese ZDF rats as compared with 6-week-old obese ZDF (2.78 g/L \pm 1.9 vs. 1.06 g/L \pm 0.03, $*P < 0.05$). Insulinemia was significantly decreased in 12-week-old obese ZDF rats (2.20 ng/mL \pm 0.78) compared to 6- and 9- week-old obese ZDF (8.11 ng/mL \pm 2.51 and 6.93 ng/mL \pm 3.57, respectively) attesting of an impaired insulin secretion.

In order to determine whether the passage from pre-diabetes to diabetes in this model is associated with morphological alteration of pancreatic islets and P-WAT, we performed histological studies. In obese ZDF animals, an increased islet size was observed during the development of the disease. Islets from 6-week-old obese ZDF rats had a rounded morphology, as observed for all lean ZDF rat islets (Fig. 1A). At 9 weeks, when obese ZDF rats become pre-diabetic, islets were enlarged with a more shapeless appearance (Fig. 1A). Islet hypertrophy correlated with an increase in the total insulin islet content as observed by a dense staining for insulin. Then at the diabetic stage, at 12 weeks, islet morphology is highly disorganized with extensions into the surrounding exocrine tissue (Fig. 1A), and a decrease in insulin content was evidenced by a weak and diffuse staining (Fig. 1B). In parallel with evolution of diabetes, adipocytes progressively infiltrated the pancreas (Fig. 1C). The size of adipocytes was increasing at 12 weeks of age. As the number of adipocytes did not vary from 6 weeks to 12 weeks, we can conclude that adipose tissue growth is due to an enlargement of

Table 1
Body weight and metabolism-related biochemical parameters in Obese Zucker Diabetic Fatty (ZDF) rats ($n = 5-15$ per group).

	Obese ZDF, 6 weeks	Obese ZDF, 9 weeks	Obese ZDF, 12 weeks
Body weight (g)	198.00 \pm 30.10	334.00 \pm 25.00	381.70 \pm 31.30
Blood glucose (g/L)	1.06 \pm 0.03	1.28 \pm 0.26*	2.78 \pm 1.9*
Insulin (g/L)	8.11 \pm 2.51	6.93 \pm 3.57	2.20 \pm 0.78**
Leptin (ng/mL)	36.12 \pm 4.01	41.99 \pm 4.60	33.76 \pm 4.96
HbA _{1c} (%)	ND	3.72 \pm 0.08	6.58 \pm 0.92

Values are expressed as mean \pm sem.

ND, not determined.

* $P < 0.05$.

** $P < 0.01$.

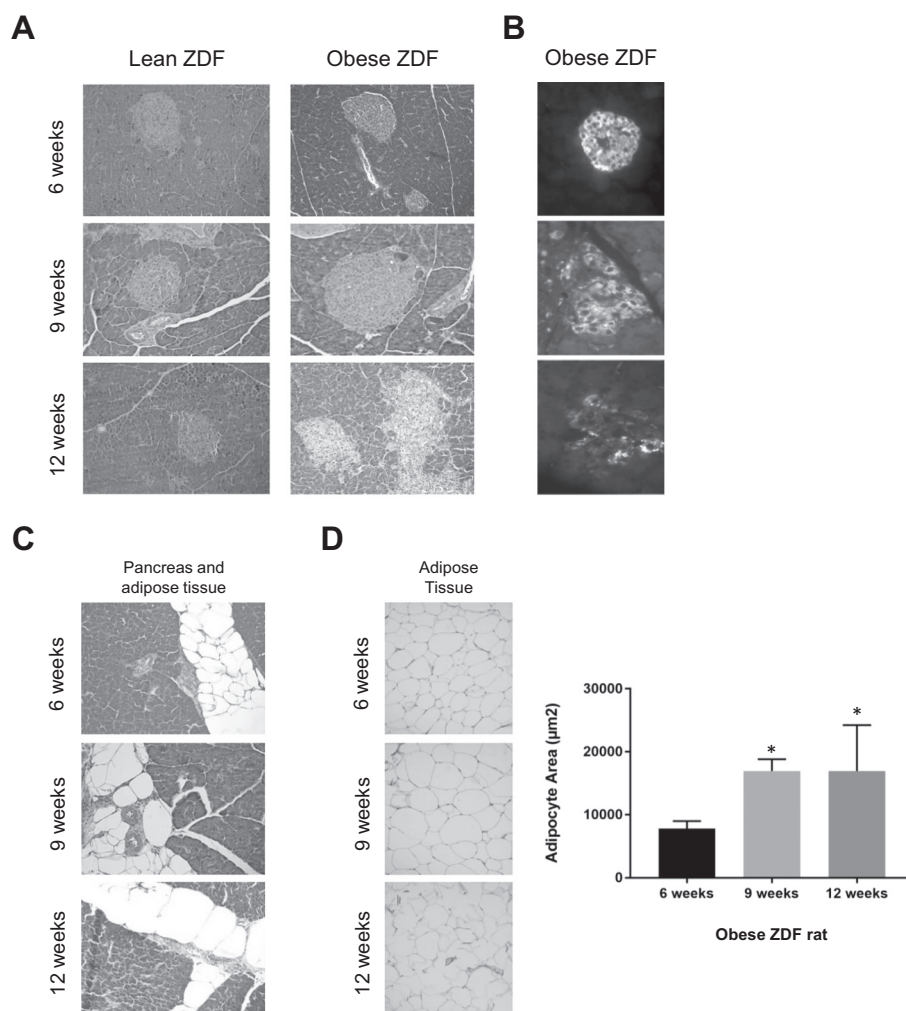


Fig. 1. Morphological alteration of pancreatic islets and P-WAT. Hematoxylin and eosin staining of pancreas from Lean and obese ZDF rats (A). Immunofluorescence of pancreatic islet from obese ZDF rat aged of 6, 9 and 12 weeks (B). Adipose tissue infiltration in pancreas of obese ZDF rats (C). Hematoxylin and eosin staining of adipose tissue from obese ZDF rats, aged of 6, 9 and 12 weeks (D). In each group, adipocytes area was measured and the results are the mean \pm S.E.M. * $P < 0.05$ compared to 6 weeks animals.

existing fat cells (Fig. 1D). For comparison, P-WAT adipocytes from lean ZDF animals kept identical cell-size from 6 to 12 weeks of age (data not shown).

3.2. Macrophages infiltration into P-WAT was correlated to changes in secreted factors

To determine whether the level of inflammation in P-WAT and pancreatic islets varies during the development of the disease, the presence of macrophages was investigated using ED1 staining. In obese ZDF (Fig. 2A, B) compared to lean ZDF animals from the same age. In these tissues, macrophage infiltration was more pronounced in 9-week-old and decreased in 12-week-old obese ZDF rats when they became diabetic. In 9-week-old obese ZDF rat pancreas, macrophages were mainly localized at the periphery of the islets. However, some infiltrated macrophages were also observed in the exocrine pancreatic tissue.

Then, we examined P-WAT conditioned-medium expression level of 29 cytokines and chemokines in 6-, 9- and 12-week-old obese ZDF rats and age-matched lean ZDF rats as controls, in order to obtain a cytokine profile in obesity and T2D. The profile of P-WAT secreted factors was determined by cytokine antibody array (Fig. 3A). Interleukine-1 α (IL-1 α), Tissue inhibitor of metalloproteinase-1 (TIMP-1), L-selectin and soluble Intercellular adhesion molecule-1 (sICAM-1) were detected in

all lean ZDF rat conditioned-medium (Fig. 3C) and in 6- and 12-week-old obese ZDF rats (Fig. 3B). However, in 9-week-old obese ZDF rats, the cytokine profile varies. CXCL-1, CXCL-2, CXCL-3, CXCL-5/LIX and MIP-1 α /CCL3 appeared greatly expressed; IL1- β to a lesser extent and VEGF were increased in P-WAT conditioned-medium (Fig. 3B). On the contrary, in epididymal (E)-WAT the same cytokines, plus IL-1ra, IL-6, IP10, RANTES and MIG were observed mainly at 6 weeks and disappeared at 9 weeks in CM from obese ZDF rats (data not shown). In all cases, CNTF, Fractalkine, GM-CSF, IL-2, IL-3, IL-4, IL-10, IL-13, IL-17 and MIP-3 were not expressed.

During the development of T2D, P-WAT and pancreatic islets exhibit increased state of inflammation, associated with macrophages infiltration and modifications in expression and level of secreted cytokines from P-WAT.

3.3. The conditioned-medium of P-WAT modify beta-cells mass and function

In order to investigate the paracrine effect of secreted factors from P-WAT on pancreatic β -cell, INS-1 cells were incubated with P-WAT conditioned-medium from 6, 9 or 12 week old obese ZDF or lean ZDF rats (Fig. 4). Proliferation of INS-1-cells was greater in the presence of conditioned-medium from obese ZDF compared to that of lean ZDF, and this, for all stages of the disease (pre-diabetes and T2D). In addition, the

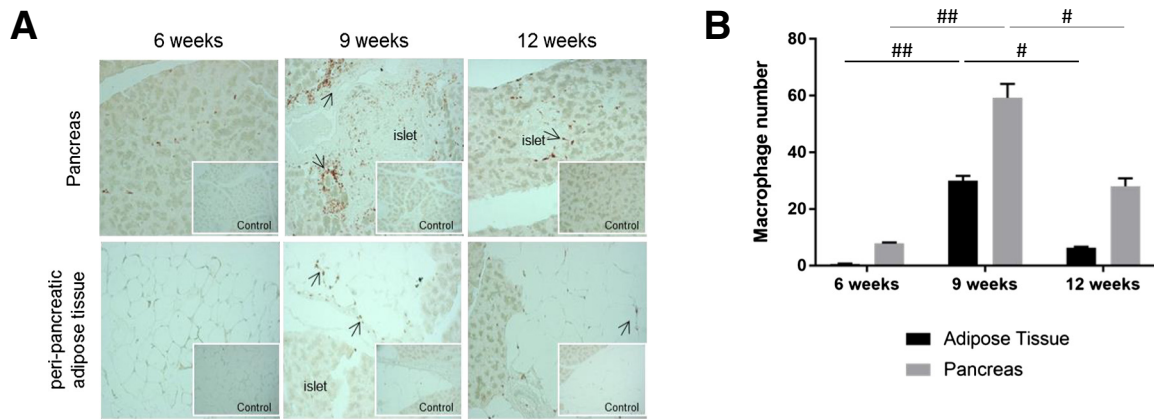


Fig. 2. Macrophages infiltration into P-WAT was correlated to changes in secreted factors. Representative immunostaining of macrophages infiltrating the P-WAT from obese ZDF rats, aged of 6, 9 and 12 weeks. Macrophages infiltration (A). Quantification of macrophages number in P-WAT and Pancreas (B). # $P < 0.05$, ## $P < 0.01$.

proliferation rate of INS-1 was significantly increased by conditioned-medium from 6-week-old obese ZDF (2.95 ± 0.13 versus 2.36 ± 0.15 at 6-week-old, * $P < 0.05$), but not by the one from 9-week-old obese ZDF (NS versus 9-week-old). This result indicated lower effect on β -cell proliferation of P-WAT conditioned-medium from diabetic in comparison to pre-diabetic rats (Fig. 4A). In contrast, proliferation was significantly enhanced by P-WAT conditioned-medium from lean ZDF rats between 9 and 12 weeks old. Overall, proliferation rate was 1.4 fold more important in obese ZDF than in lean ZDF rats at 6 weeks and decreased to 0.8 at 12 weeks.

The effect of P-WAT conditioned-medium from lean or obese ZDF animals of the same age, on apoptosis, is comparable, except at the age of 6 weeks (Fig. 4B). An enhanced apoptosis could be observed with the conditioned-medium from pre-diabetic and diabetic obese ZDF animals: a 2 fold increase with conditioned-medium from 9-week-old rats and a 1.4 fold increase with the conditioned-medium from 12-week-old rats as compared to 6-week-old rats. Nevertheless, the same variation was noted with age-matched controls lean ZDF animals, demonstrating that P-WAT conditioned-medium apoptosis effect is age dependent with a maximum effect on apoptosis at the age of 9-weeks, but independent on the metabolic status.

Concerning the effect of P-WAT conditioned-medium on insulin secretion (Fig. 4C), we observed an increased insulin release in response to 8.3 mM glucose with conditioned-medium from 9-week-old obese ZDF rats but a decreased one with conditioned-medium from 12-week old obese ZDF rats compared to 6-week-old obese ZDF animals. P-WAT conditioned-medium from obese ZDF rats (6, 9 and 12 weeks) had no effect on INS-1 cells in the presence of basal glucose. Control medium alone have no effect on proliferation, apoptosis nor insulin secretion in INS-1 cells.

All functional effects of the conditioned-medium on INS1 cells are in line with the antibody array data obtained from obese ZDF rat pancreatic islets (Table. 2). Between 6 and 9 weeks, we observed an up-regulation of proteins involved in survival (Cdk6, Cyclin D3, MAPK, PKB/Akt) and apoptosis (CHOP-10, i-NOS, NF κ B), whereas the down-regulation of the same proteins occurred between 9 and 12 weeks. However, JNK and p38 MAPK, both stress-activated kinases, are expressed at each age, and more activated in diabetic rats.

3.4. Beta cells mass and function are not improved by CXCL-1 or others cytokines through CXCR2 receptor

As detected among the secreted factors, CXCL-1 was shown to increase in P-WAT conditioned-medium from pre-diabetic animals. This cytokine was closely associated with increased obesity [13]. We hypothesized that CXCL-1 could be responsible, at least in part, for the

conditioned-medium effect on β -cell mass and function. First, in order to determine the concentration of cytokine to be used in our tests and to get closer to the patho-physiological context, we quantified CXCL-1 present in each P-WAT conditioned-medium of obese ZDF rats by ELISA. The cytokine concentration was $21.1 \text{ pg/mL} \pm 29.5$ in conditioned-medium of 6-week-old, $628.4 \text{ pg/mL} \pm 102.3$ in conditioned-medium of 9-week-old obese ZDF animals and was not detectable in conditioned-medium of 12-week-old obese ZDF rats. Thus, CXCL-1 concentration present in P-WAT conditioned-medium varies with the disease status, with a peak at 9 weeks at the pre-diabetic stage.

Next, we determine whether this cytokine plays a role in β -cell dysfunction during the development of diabetes. INS-1 cells were incubated with increased concentration of CXCL-1, and then proliferation (Fig. 5A), apoptosis (Fig. 5B) and insulin secretion (Fig. 5C) were analyzed. Regardless of the concentration used, CXCL-1 has no effect on INS-1 cells proliferation, apoptosis, and function.

Others cytokines such as CXCL-2, CXCL-3, and CXCL-5/LIX are present (or released) concomitantly with CXCL-1 in P-WAT conditioned-medium from pre-diabetic animals (Fig. 3A, B). All these cytokines could act synergistically on the β -cells mass and function via the CXCR2 receptor. After checked the expression of CXCR2 on INS1 cells (data not shown), we investigated whether the effects of the conditioned-medium on β -cell could be inhibited by the use of a selective CXCR2 antagonist, the SB225002. This antagonist was devoid of any effects when used alone (Fig. 6A, C, E). We observed the same profile of proliferation (Fig. 6B), apoptosis (Fig. 6D) and insulin secretion (Fig. 6F) in presence or absence of CXCR2 antagonist. P-WAT conditioned-medium effects on β -cell mass and function did not act through the receptor CXCR2.

4. Discussion

Recently, using a model for human obesity and metabolic syndrome [14], we showed that factors produced by adipose tissue surrounding the pancreas (P-WAT) have been involved in β -cell mass adaptation [8]. As T2D pathogenesis clearly involves bio-communication between metabolic tissues, we have hypothesized that both β -cell compensation in insulin resistance and its progressive dysfunction in T2D are governed by signals emanating from P-WAT. We used as a model, the obese ZDF rat, which progressively develops diabetes between the ages of 9–10 weeks.

In the obese ZDF rat, we observed that adipose tissue penetrates the pancreas and enters in close contact with islets during the development of the disease. In addition, morphological alteration of islets and P-WAT was associated with macrophage infiltration and changes in P-WAT secreted factors. Indeed, the P-WAT expansion is characterized by a

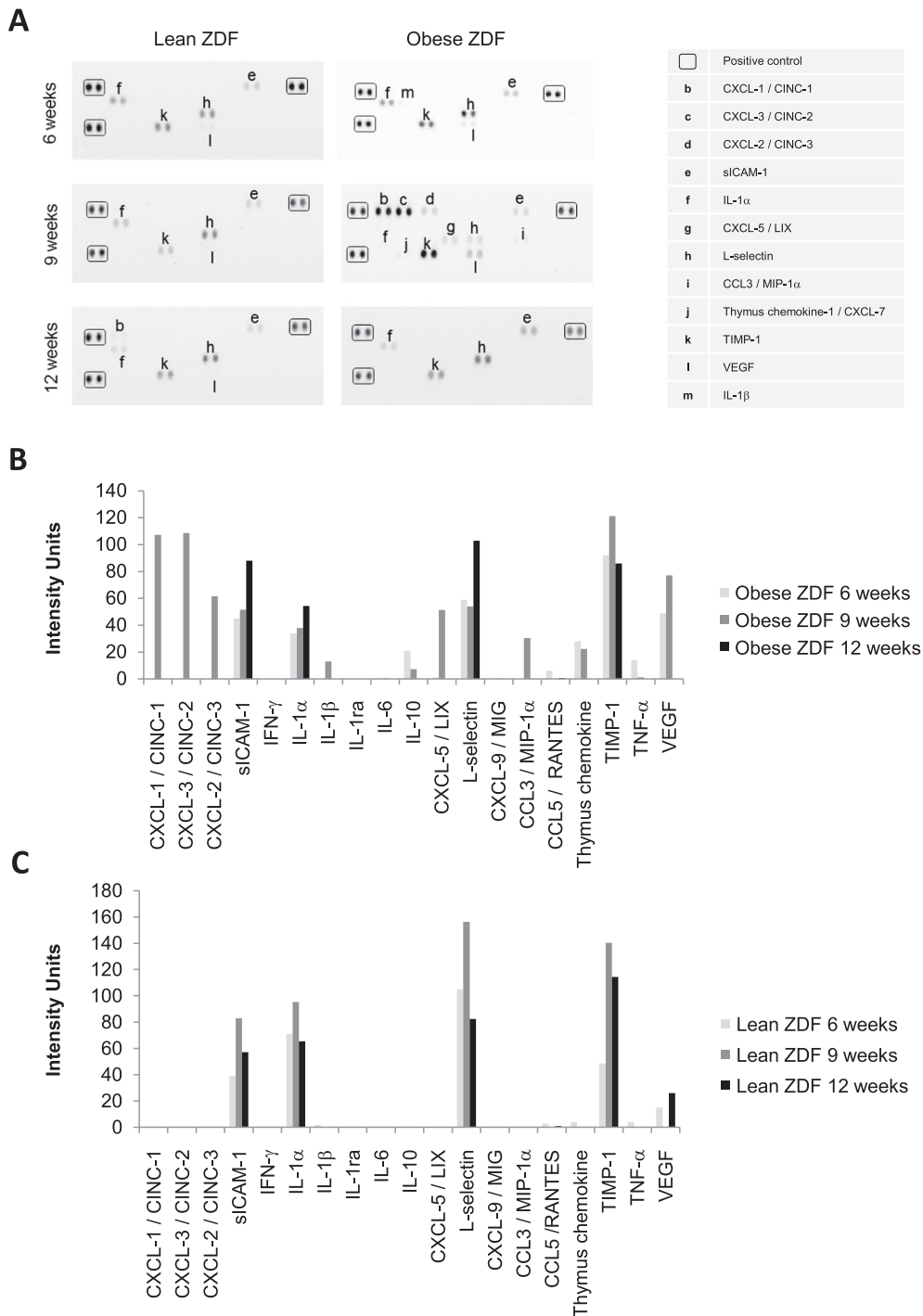


Fig. 3. Characterization of P-WAT conditioned-medium (CM). Presence of different cytokines and growth factors in CM from lean and obese ZDF rats was measured by cytokine array. Panel (A) illustrates representative dot blot/results from lean and obese ZDF rats, aged of 6, 9 and 12 weeks. Mean pixel density are represented in panels (B,C).

massive hypertrophy of adipocytes, a high level of macrophage infiltration with increased adipokines secretion such as MIP-1 α /CCL3, demonstrating that P-WAT undergoes pathological remodeling during the T2D. TIMP-1, secreted by P-WAT and shown to promote adipose tissue expansion, participates to this active remodeling [15]. In 9-week-old obese ZDF rats (pre-diabetic stage), islets become enlarged to compensate for insulin resistance and to maintain normoglycemia, as previously described in Zucker *fa/fa* rats (Jetton, et al. 2005). As previously shown in 12-week-old obese ZDF rats, when the disease is installed, islets appeared highly disorganized and are no more able to

secrete enough insulin to restore glucose homeostasis [16]. Furthermore the increased numbers of islet-associated macrophages in 9-week-old obese ZDF rats could be related to the presence of dying cells and could also lead to islet destruction by secreting pro-inflammatory cytokines in close proximity to islet cells.

There are investigated whether and how adipose tissue derived factors could influence β -cell plasticity and function throughout the natural history of T2D. It is now accepted that intra-organ communication plays an important role in systemic metabolic regulation and particularly adipose tissue has a central role in regulation of other

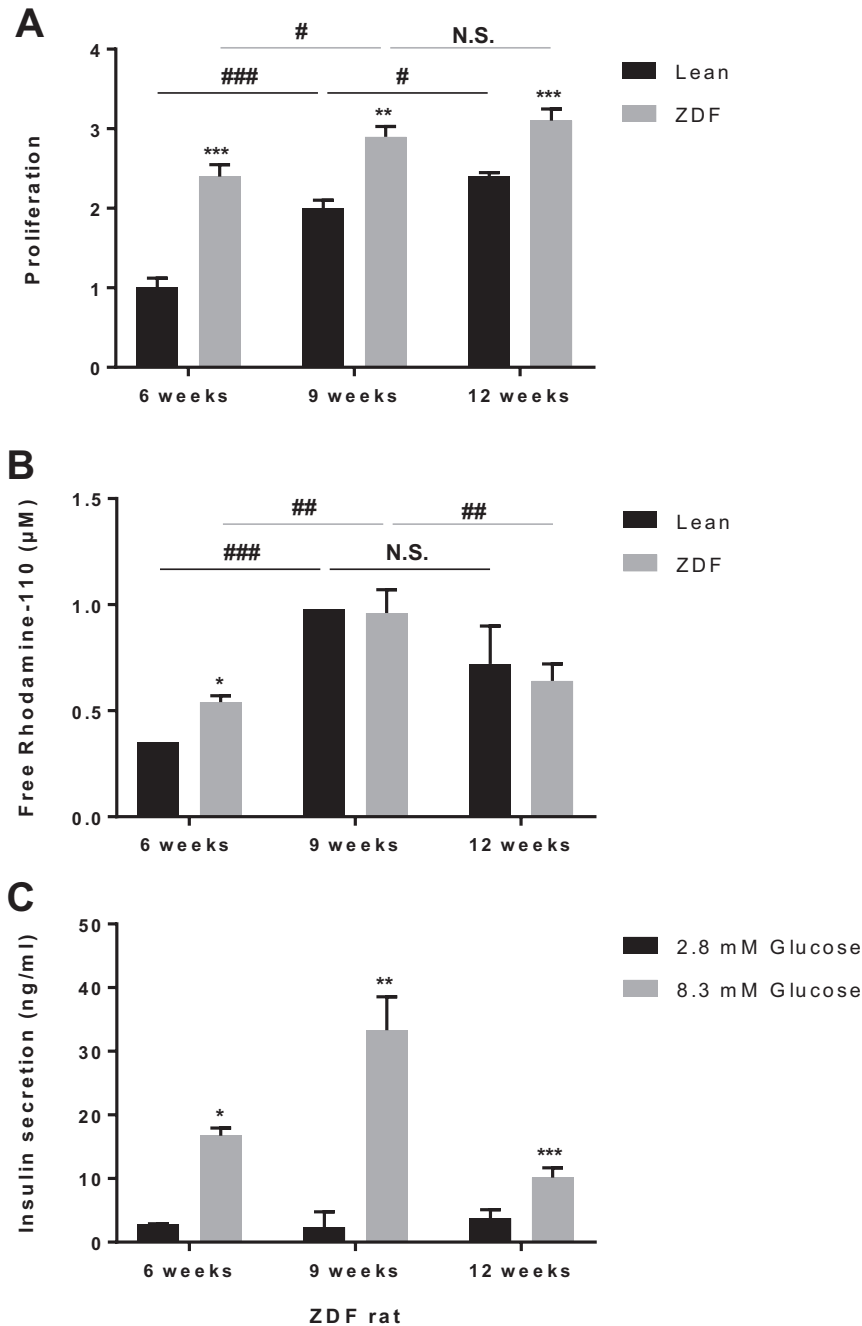


Fig. 4. Effects of P-WAT conditioned-medium on pancreatic β -cells. INS1 cells were incubated for 24 h with P-WAT conditioned-medium; then (A) proliferation, (B) apoptosis and (C) insulin secretion were measured. Values are means \pm S.D. for three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared obese ZDF rats vs. lean ZDF animals or compared 8.3 mM vs. 2.8 mM Glucose. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$. NS, not significant.

organs' metabolism via cytokines and NEFA secretion [17,18]. For example, P-WAT secreted factors have been shown to increase β -cell proliferation in a rat model of diet-induced obesity [8,9]. Interestingly, we demonstrate here that P-WAT influences, not only β -cell proliferation but also insulin secretion. An increase in β -cell insulin secretion is observed (taking into account the age effect). The effect of P-WAT conditioned-medium of pre-diabetic animals on the increased islet insulin secretion allows compensating the relative decrease in proliferation rate of β -cells. However it is important to notify that compensation mechanisms involved in the two models of rats, the rat model of diet-induced obesity [8], and the obese ZDF rat, are different since the first increases its rate of cell proliferation and the second its capacity of β -cells to secrete insulin. Thus, β -cells gradually deplete, which explains

in part why the obese ZDF rat becomes diabetic whereas the rat model of diet-induced obesity never develops diabetes. Surprisingly, we found that P-WAT from lean ZDF rats lead to both proliferation and apoptosis of β -cells, suggesting that P-WAT secreted factors could induce a β -cell remodeling in maximum at 9 weeks of age [19]. The obese ZDF rat conditioned-medium, despite increasing β -cell proliferation, does not allow such remodeling in the young age [16].

Obesity is known to be associated to a general low-grade chronic inflammation characterized by macrophage activation and inflammatory cytokine production [20,21]. The primary source of pro-inflammatory cytokines in obesity is adipose tissue where macrophages and adipocytes themselves are able to secrete cytokines in response to various stress [22]. Thus, ectopic adipose tissue such as P-WAT that is

Table 2

Changes in the expression pattern of proteins involved in signal transduction, apoptosis, and cycle cell were examined by Antibody Array in pancreatic islets from obese ZDF rats.

	Obese ZDF rats, 9 weeks vs. 6 weeks	Obese ZDF rats, 12 weeks vs. 9 weeks
CHOP-10 (GADD 153)	1.67	<i>0.32</i>
i-NOS	2.21	<i>0.27</i>
n-NOS	1.59	<i>0.37</i>
Cdk6	3.65	<i>0.49</i>
Cyclin D3	1.59	<i>0.46</i>
NFκB	4.00	<i>0.52</i>
JNK activated	1.67	2.65
P38 MAPK	1.26	1.89
P38 MAPK activated	1.39	1.83
MAPK (Erk1 + Erk2)	3.68	1.40
MAPK activated	2.33	1.51
PKB/Akt	3.50	<i>0.03</i>
PKC γ	2.10	<i>0.33</i>

The level of protein expression was quantified and the most representative changes are summarized on this table. Up or down regulated pancreatic islets proteins from obese ZDF rats between 6 and 9 weeks of age and 9–12 weeks of age are presented in relative values. A 1.5-fold increase (**bold**) or decrease (*italic*) taken as cut-off value (positive or negative) is considered as significant.

in direct contact with the pancreas may influence the β-cells function by its secreted factors. We evidenced that P-WAT secretion of cytokine varies during the different stages of the disease, from pre-diabetic stage (6 weeks) to T2D (12 weeks) with an increased secretion when obese ZDF rats became obese (9 weeks). Among the cytokines/chemokines tested in P-WAT conditioned-medium from ZDF rats, only few have been secreted by P-WAT from 6-week-old obese ZDF rats (siCAM-1, IL-1α, L-selectin, and TIMP-1). However, at the age of 9 weeks, a major change in cytokine production by the adipose tissue has been observed: CXCL-1, -2, -3, CXCL-5/LIX, MIP-1α/CCL3 appeared in the conditioned-medium of adipose tissue and TIMP-1 level was found increased by 4 fold. Macrophage inflammatory protein (MIP)-1α/CCL3, a chemokine which is almost entirely derived from macrophages rather than adipocytes, correlate with the presence of macrophages in P-WAT from 9-week-old obese ZDF rats. In addition, secretion of the chemokines CXCL-1, -2, -3, and CXCL-5/LIX concomitantly with infiltration of macrophages into the P-WAT, suggest that macrophages participate to the secretion of CXCL-1 and CXCL-2. Conversely, CXCL-1, -2, -3 and CXCL-5/LIX are able to promote macrophage and neutrophil mobilization [23], and perpetuate and inflammation through attraction on new immune cells in the tissue.

The presence of CXCL-1,-2,-3 and CXCL-5/LIX is increased in P-WAT conditioned-medium and is positively correlated with obesity and the development of T2D. These data are consistent with other studies showing an increase of CXCL-1 levels in serum of T2D [24], or in adipose tissue [25] as well as an increased serum CXCL-5/LIX level in obesity and insulin resistance in diabetic mice and humans [26]. In vitro treatment with the combination of CXCL-1 and CXCL-5/LIX was sufficient to produce a decrease in calcium response to glucose stimulation suggesting reduced islet function [13]. These changes due to both depletion of ER calcium and increased calcium influx in basal glucose did not result in any significant effect on insulin release in low or high glucose [27]. One explanation could be that the cytokine effect depends on the concentration, time of exposure, and combination of several cytokines or factors. And even if we try to reproduce as closely as possible the conditions present in vivo, the experimental conditions, and interaction between different cytokines/factors will never be identical to that exists in the context of T2D. In addition, depending on time of contact (acute vs chronic) and concentration, cytokines could have protective or deleterious effects on the β-cell. This is well known for IL-1β which can induce a bimodal effect on insulin secretion: a stimulating and a suppressive effect depending on IL-1β concentration,

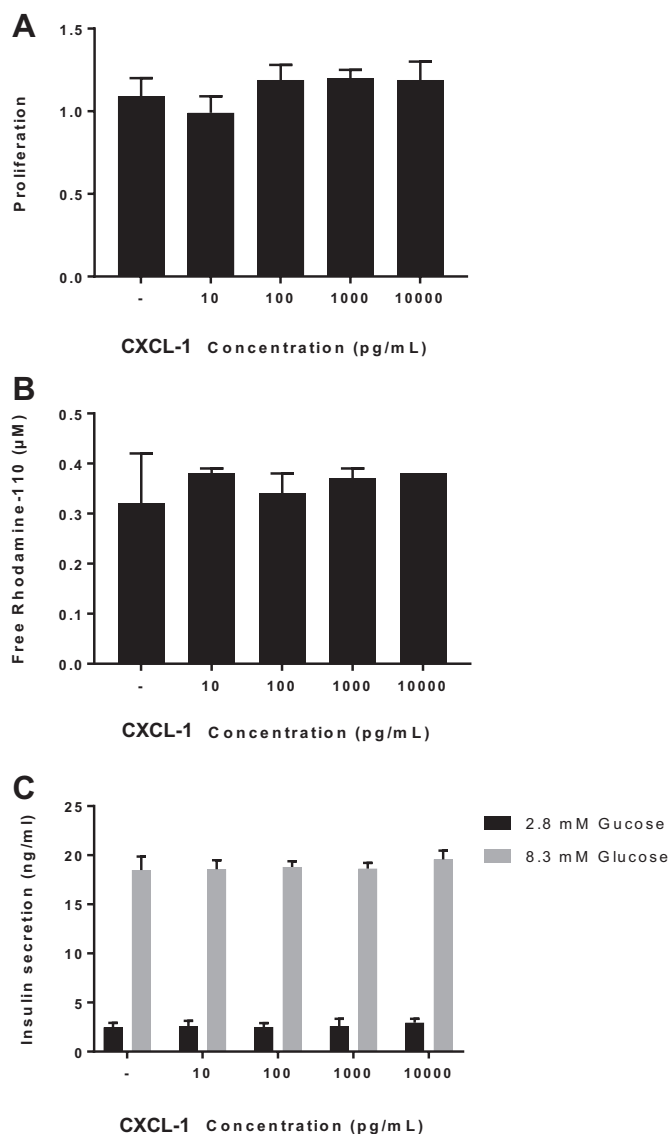


Fig. 5. Effect of CXCL-1 stimulation on pancreatic β-cells. INS1 cells were incubated with increasing CXCL-1 concentration for 24 h; then (A) proliferation, (B) apoptosis and (C) insulin secretion were measured. The results are the mean ± S.D. for three independent experiments.

duration of exposure and glucose concentration [28,29].

Furthermore, the use of CXCR2 antagonist does not restore the negative effect of 9-week-old rat conditioned-medium on β-cells function. This suggests that CXCL-1, -2, -3 and CXCL-5/LIX do not have a direct effect on β-cells. This hypothesis is supported in part by Chavey et al., study which demonstrates that CXCL-5/LIX mediates insulin resistance [26], through activation of the JAK/STAT/SOCS signaling pathway via the CXCR2 receptor in mice muscle.

In conclusion, we demonstrated in this study that secreted factors from P-WAT contribute to progressive β-cell dysfunction occurring in T2D. This dysfunction could be related to the indirect action of chemokines through CXCR2 receptor expressed on adjacent immune cells or endocrine cells which would secrete in turn factors acting directly on β-cells.

Authors' contributions

SAR and SR designed and performed the experiments, analyzed and interpreted the data and wrote the manuscript. ES and CG performed

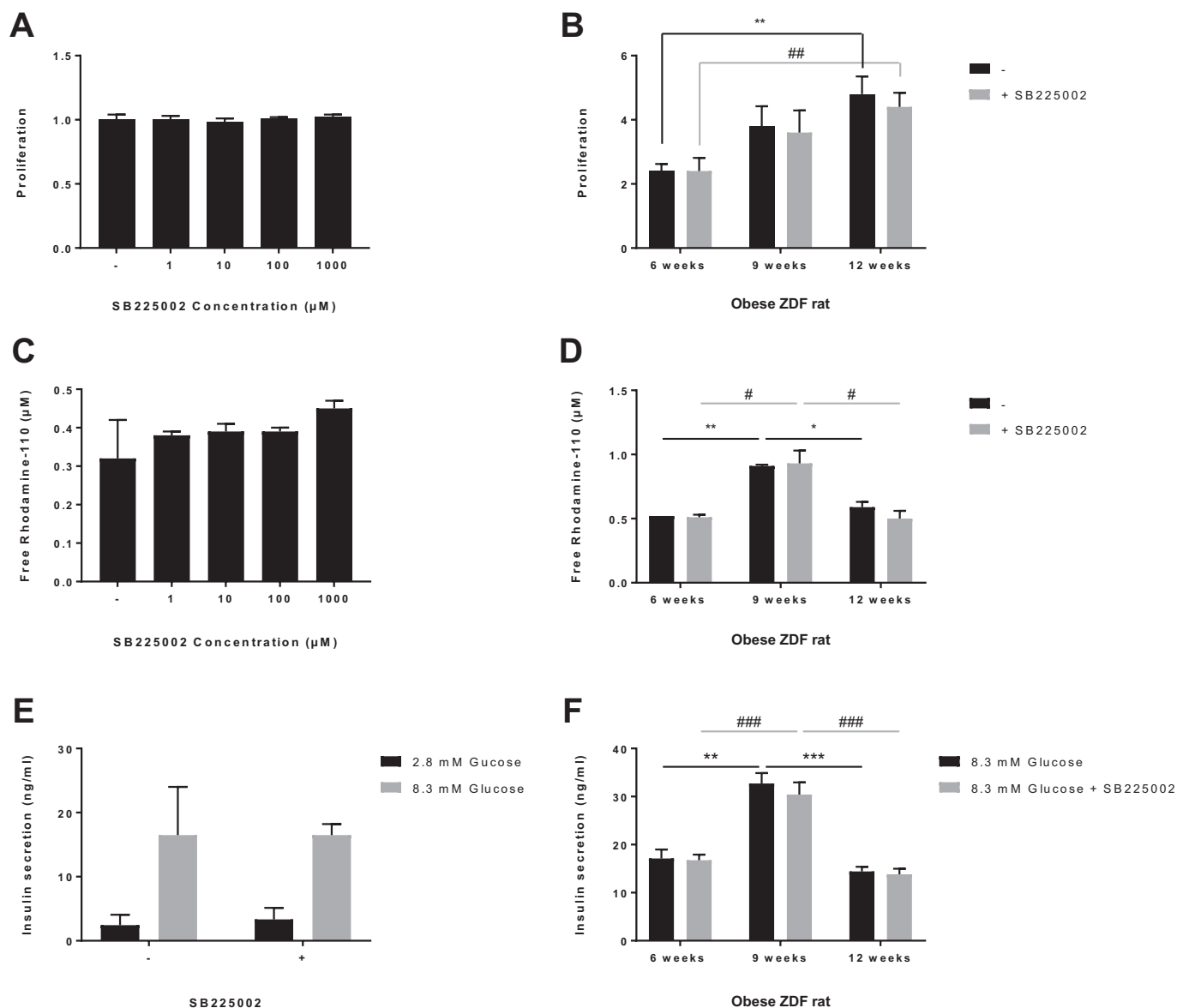


Fig. 6. Effect of CXCR2 inhibitor in presence or not of P-WAT conditioned-medium on pancreatic β -cells. INS1 cells were incubated for 24 h with CXCR2 inhibitor (SB225002) in presence or not of P-WAT conditioned-medium from lean and obese ZDF rats; then (A, D) proliferation, (B, E) apoptosis and (C, F) insulin secretion were measured. The results are the mean \pm S.D. for three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the experiments. ADL revised the manuscript. All authors approved the final version of the manuscript. SR is responsible for the integrity of the work as a whole.

Duality of interest

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

Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

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