Diabetologia (2013) 56:1098–1107
DOI 10.1007/s00125-013-2862-8

ARTICLE

Role of endogenous IL-6 in the neonatal expansion and functionality of Wistar rat pancreatic alpha cells

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Received: 29 October 2012 / Accepted: 28 January 2013 / Published online: 23 February 2013
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Abstract
Aims/hypothesis Plasma glucagon concentrations rise sharply during the early postnatal period. This condition is associated with increased alpha cell mass. However, the trophic factors that regulate alpha cell turnover during the perinatal period have not been studied. Macrophage infiltrations are present in the neonatal pancreas, and this cell type releases cytokines such as IL-6. Alpha cells have been identified as a primary target of IL-6 actions. We therefore investigated the physiological relevance of IL-6 to neonatal pancreatic alpha cell maturation.
Methods Histochemical analyses were performed to quantify alpha cell mass, replication and apoptosis. Pancreatic *Il6* expression was determined by quantitative RT-PCR. The biological effect of IL-6 was tested in two in vivo rat models of IL-6 blockade and chronic undernutrition.
Results Alpha cell mass increased sharply shortly after birth but decreased significantly after weaning. Pancreatic alpha cell proliferation was as high as 2.5% at the beginning of suckling but diminished with time to 1.2% in adulthood. Similarly, alpha cell neoformation was remarkably high on postnatal day (PN) 4, whereas alpha cell apoptosis was low throughout the neonatal period. Moreover, *Il6* mRNA exhibited developmental upregulation in the pancreas of suckling rats, with the highest expression on PN2. Neutralisation of IL-6 reduced alpha cell mass expansion and glucagon production. IL-6 staining was detected within the islets, mainly in the alpha cells. Finally, undernourished neonates showed altered alpha cell number and function and delayed appearance of IL-6 in the pancreas.
Conclusions/interpretation These data point to a potential role for local IL-6 in the regulation of alpha cell growth and function during suckling.

Keywords Alpha cell mass · Glucagon · Interleukin-6 · Maternal food restriction · Pancreas development · Suckling

Abbreviations

CD68	Cluster of differentiation 68
IL-6Ab	IL-6-neutralising antibody
IL-6R	IL-6 receptor
IUGR	Intrauterine growth retardation
PN	Postnatal day
STAT3	Signal transducer and activator of transcription 3
UN	Undernourished

Introduction

The control of glucose homeostasis by the islets of Langerhans depends mainly on the coordinated secretion of glucagon and insulin by alpha and beta cells, respectively. Consequently, the circulating levels of these hormones are determined by the number and function of these two endocrine cell types. There is a large body of evidence indicating that beta cell mass shows considerable plasticity, increasing or decreasing in response to nutrients, hormones and growth factors [1–3]. By contrast, there is scant information

Electronic supplementary material The online version of this article (doi:10.1007/s00125-013-2862-8) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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regarding the identity of the signals regulating alpha cell number. Studies have reported that plasma glucagon concentrations rise sharply in humans [4] and rats [5] during the early postnatal period. This condition is associated with an increase in alpha cell mass [6]; however, the trophic factors that regulate alpha cell function and turnover during the perinatal period have not been studied. This time is considered a critical window for endocrine pancreas remodelling [7, 8], and macrophage infiltrations have been observed as a normal feature of fetal and neonatal human [9, 10] and murine pancreas [11]. The functional significance of this macrophage accumulation remains to be elucidated, but it has been suggested that macrophages may participate in postnatal pancreas development because they are able to secrete numerous substances, such as cytokines and growth factors, which are known to be implicated in pancreas regeneration/organogenesis [12].

Cytokines, especially IL-6, merit special attention. In addition to its immunomodulatory effects, several studies have shown that IL-6 has a significant impact on non-immune events, such as glucose homeostasis and metabolism [13]. Indeed, systemically elevated IL-6 levels in obesity are considered a predictive factor for the development of type 2 diabetes [14]. Local islet IL-6 levels are also elevated under conditions of metabolic stress in models of obesity and type 2 diabetes [15], supporting a potential paracrine/autocrine role for IL-6 in the pancreatic islet under these conditions. However, the specific role of IL-6 in the pancreatic islet is still unclear because a potential role for IL-6 in beta cell survival has also been reported [16]. Recently, pancreatic alpha cells have been identified as a primary target of IL-6 actions [17], regulating alpha cell mass expansion and glucagon production and release. However, knowledge concerning the islet cellular origin of this cytokine during physiology is sparse. In investigating this premise in the present study, we found transitory islet-derived IL-6 in the neonatal rat pancreatic alpha cell.

Increasing evidence points to the fact that cytokine production in general, and levels of IL-6 in particular, respond to dietary regulation. In this context, intrauterine growth retardation (IUGR) has been linked to the development of type 2 diabetes in adulthood. The mechanisms underlying this phenomenon are not fully described, but pancreatic beta cell dysfunction and beta cell mass reduction are common findings of studies conducted with nutrition-restricted animal models [18, 19]. However, despite the importance of alpha cells and glucagon secretion in the regulation of glycaemia and nutrient homeostasis, little is known about the contribution of this endocrine cell type to the relationship between poor fetal and early postnatal nutrition and the susceptibility to diabetes later in life. In the present study, using a rat model of maternal undernutrition, which shows altered alpha cell growth and function, we assessed the physiological relevance of IL-6 to normal pancreatic alpha cell development and its contribution to islet adaptation to nutrient insult.

Methods

Animals and diets Pregnant Wistar rats were used throughout the study. At birth, litters were uniformed to eight pups per nursing dam to minimise the effects of litter size on postnatal growth. The experiments were carried out in at-term fetuses, in neonates on postnatal days (PN) 4, 14 and 23, and in 3-month-old adult rats. All animals were weighed before being killed by decapitation, blood samples were collected and serum was separated and stored frozen at −20°C. Pancreases were removed, weighed and stored at −70°C. One series of animals was submitted to food restriction. Pregnant rats received 35% of the standard chow ingested by a control pregnant rat during the third week of gestation and the suckling period. Four to six pups from at least three separate litters were studied at each time point and condition.

All studies were conducted according to the principles and procedures outlined in the National Institute of Health Guidelines for Care and Use of Experimental Animals and approved by the Animal Ethics Committee of the Complutense University of Madrid.

Immunohistochemistry and morphometry Immunohistochemistry was performed as previously described [20]. Sections at fixed intervals were incubated with rabbit anti-glucagon antibody (Millipore, Temecula, CA, USA) using the indirect peroxidase method. Alpha cell relative volume was determined by calculating the ratio between the area occupied by glucagon immunoreactive cells and that occupied by total pancreatic cells. Total alpha cell mass per pancreas was derived by multiplying the total pancreatic weight by the alpha cell relative volume. Alpha cell neogenesis was quantified as the number of single alpha cells and alpha cell clusters (two to four) incorporated into the ductal epithelium per μm^2 of the pancreatic area. Results represent the average of five to six sections per animal from four to six animals from each experimental group and age.

Alpha cell apoptosis was estimated by TUNEL assay (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Millipore) coupled to glucagon staining. For alpha cell replication, animals were injected with BrdU 50 mg/kg body weight i.p. (Sigma-Aldrich, St Louis, MO, USA) 1 h before killing. BrdU-positive cells were detected with a cell proliferation kit (GE Healthcare, Amersham, UK). Alpha cell apoptosis or replication rate was expressed as the percentage of apoptotic or BrdU-positive alpha cells, respectively.

For cluster of differentiation 68-positive (CD68+) cell staining, mouse anti-rat CD68 antibody was used (Abcam,

Cambridge, UK). The number of CD68+ cells was counted and expressed per mm^2 of total pancreatic surface area. For pancreatic IL-6/IL-6 receptor (IL-6R) localisation, correlative sections were incubated with the primary antibodies goat anti-mouse IL-6 or rabbit anti-mouse IL-6R (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-human glucagon, and visualisation was carried out using appropriate secondary antibodies coupled to peroxidase or alkaline phosphatase. Specificity of the antibodies against IL-6 and IL-6R was confirmed by isotype controls (goat IgG I-5000 and rabbit IgG I-1000, both from Vector Laboratories, Burlingame, CA, USA).

RNA extraction and quantitative RT-PCR Total RNA was extracted from frozen pancreas or isolated islets using TRIzol Reagent (Invitrogen, Life Technologies, Madrid, Spain) and reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies). Real-time quantitative PCR analyses were performed using TaqMan probes (Applied Biosystems) to determine the relative abundance of *Il6* (Rn01410330_m1), *Il6r* (Rn00566707_m1) and *Gcg* (Rn00562293_m1). The comparative threshold cycle method was used to calculate the relative expression. The target gene values were normalised to the expression of the endogenous reference (18S; Rn01428913_gH).

IL-6 neutralisation Each rat was injected i.p. with 1, 5, 10 or 15 μg neutralising goat anti-rat IL-6 antibody (IL-6Ab; Abcam) or PBS on alternate days immediately after birth until the day they were killed. Serum was collected every other day and IL-6 concentration was determined by ELISA (R&D Systems Europe, Abingdon, UK).

Islet isolation and culture, and measurement of glucagon secretion and content Islets were isolated as previously described [19, 21] (electronic supplementary material [ESM] Methods). Glucagon secretion was measured by RIA in isolated islets after exposure to different glucose concentrations (ESM Methods).

Western blot Protein samples from islets were prepared as previously described [22], separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) Immobilon membranes and incubated overnight with the corresponding primary antibodies (phosphorylated signal transducer and activator of transcription 3 [STAT3] [Tyr 705]/STAT3 from Cell Signaling Technology, Barcelona, Spain; and IL-6R from Santa Cruz Biotechnology). SaOS-2 and HeLa cell extracts, known to express *Il6r*, were used as positive controls. Immunoreactive bands were visualised using the enhanced chemiluminescence (ECL) western blot protocol (GE Healthcare). Normalisation of western blot was ensured by β-actin (Sigma-Aldrich), and the intensity of bands was quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

Analytical determinations For details, see ESM Methods.

Statistics Data are expressed as means±SEM. Significance was tested using Student's *t* test (two-tailed). For multiple comparisons, one-way ANOVA, followed by Tukey's or Tamhane's test, was performed. The level of significance was $p<0.05$. Statistical analysis was performed with SPSS software, version 19.0 (www.spss.com).

Results

Alpha cell mass increases sharply during suckling Relative alpha cell mass exhibited a threefold increase from the fetal period to PN4 and then declined progressively with age (Fig. 1a). These structural changes in alpha cells were reflected in circulating glucagon levels (Table 1). The increase in alpha cell mass during suckling was due to alpha cell hyperplasia, not only because islet density was enhanced (Fig. 1b) but also because the alpha cell:beta cell ratio per islet was higher at this time (Fig. 1c). We also observed a significant number of isolated clusters of alpha cells with no contact with beta cells during the neonatal period. These clusters may be a sign of active alpha cell turnover. Therefore, to understand the mechanisms that determine the changes in alpha cell mass that occur with age, we first conducted an estimate of alpha cell neoformation. We observed that this process notably increased on PN4 and then progressively decreased until reaching undetectable values in adulthood (Fig. 1d). Moreover, the percentage of self-replicating alpha cells diminished with age, being as high as 3.5% at the end of gestation, 2.5% at the start of suckling but only 1.2% in adulthood (Fig. 1e). Finally, the alpha cell apoptosis rate was low throughout the neonatal period but doubled in adulthood (Fig. 1f).

Neonatal pancreas shows a transitory enhancement of Il6 mRNA The above results clearly indicate that the physiological expansion of alpha cells during development follows a different schedule from the one described for beta cells and that other trophic factors are probably involved [7]. Following this premise and because alpha cells have been recently described as primary targets of IL-6 [17], we explored whether the structural changes in alpha cells observed with age were correlated with variations in serum IL-6 levels. However, circulating IL-6 did not show any regulation with

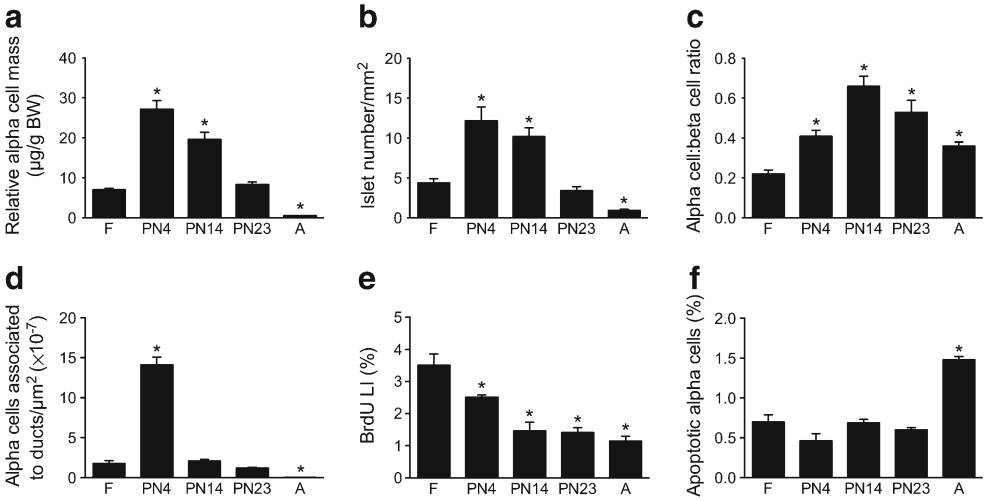

Fig. 1 Pancreatic alpha cell ontogeny. (**a**) Relative alpha cell mass under physiological conditions from the fetal period (F) to adulthood (A) expressed as alpha cell mass in μg/g body weight (BW). (**b**) Islet density. (**c**) Changes in islet conformation during the first 3 months of age. (**d**) Alpha cell neoformation from ducts was evaluated through quantification of single alpha cells and alpha cell clusters (two to four) incorporated into the ductal epithelium per unit of total tissue area. (**e**) BrdU labelling index (LI) of alpha cells as a representation of alpha cell replication rate. (**f**) Alpha cell apoptosis rate. Apoptotic alpha cells were quantified on sections double-immunostained for apoptosis and glucagon. Data are means±SEM (n=4–6 at each age). $^{*}p<0.05$ relative to F

age under normal physiological conditions (Table 1). By contrast, local pancreatic expression of *Il6* experienced a twofold increase on PN2 compared with fetal values, followed by a subsequent decrease (Fig. 2a). We found a simultaneous and notable accumulation of CD68+ cells (a macrophage transmembrane protein) [23] in perivascular areas, in interlobular connective tissue and in the vicinity of islets throughout the suckling period (Fig. 2b, c). Immunohistochemical analysis revealed no association between pancreatic IL-6 and CD68+ cells. Interestingly, IL-6 staining was detected in the periphery of islets coinciding with glucagon-positive labelling and being highly evident until PN4 but barely detectable afterwards (Fig. 2c). Finally, IL-6R immunoreactivity was observed within islets throughout the suckling period, although staining was weak in the centre of the islet compared with the periphery, suggesting higher levels of IL-6R in alpha cells (Fig. 2d).

Alpha cell mass expansion was partly reduced after IL-6 neutralisation To confirm the role of IL-6 during neonatal alpha cell mass expansion, the biological activity of this cytokine was neutralised. Because only neonates that were administered 15 μg IL-6Ab displayed serum IL-6 concentrations below detectable levels throughout the experiment (Fig. 3a), we used this dose for the following experiments.

Quantitative RT-PCR showed a 50% reduction in pancreatic *Il6* mRNA levels in PN2 neonates injected with IL-6Ab when compared with those administered PBS (Fig. 3b), together with a significant decrease (33%) in *Gcg* expression (Fig. 3c). In agreement, IL-6 blockade partially reduced (sham 55.6±1.6 μg/g; IL-6Ab 37.2±0.5 μg/g body weight) the relative alpha cell mass increase (Fig. 3d) as well as circulating glucagon levels (sham 397.0±23.2 ng/l; IL-6Ab 110.2±17.9 ng/l) (Fig. 3e) observed in non-treated suckling rats of the same age. These events triggered mild hypoglycaemia in PN2 neonates injected with IL-6Ab (Fig. 3f).

Table 1 Biological variables in Wistar rats on the last day of gestation (F), during postnatal life and in adulthood (A)

Variable	F	PN4	PN14	PN23	A
Body weight (g)	5.0±0.05	11.8±0.05*	31.2±0.30*,†	65.4±1.12*,†,‡	266.5±2.50*,†,‡,§
Pancreas weight (mg)	19.6±0.40	25.8±0.30*	73.0±4.51*,†	232.0±6.00*,†,‡	880.4±17.0*,†,‡,§
Glycaemia (mmol/l)	2.9±0.23	6.7±0.14*	7.7±0.29*	7.3±0.36*	4.5±0.14*,†,‡,§
Insulinaemia (pmol/l)	1,170.3±51.6	68.8±5.16*	103.3±10.3*	68.8±17.2*	533.5±17.2*,†‡,§
Glucagonaemia (ng/l)	98.7±3.70	458.0±5.50*	533.3±21.0*	114.9±44.0†,‡	89.3±3.70†,‡
Serum IL-6 (pg/ml)	155.2±45.6	207.1±60.7	163.1±51.3	266.7±85.2	167.6±49.8

Data are means±SEM (n=3 litters, 24 animals)

$p<0.05$: *relative to F, † relative to PN4, ‡ relative to PN14, § relative to PN23

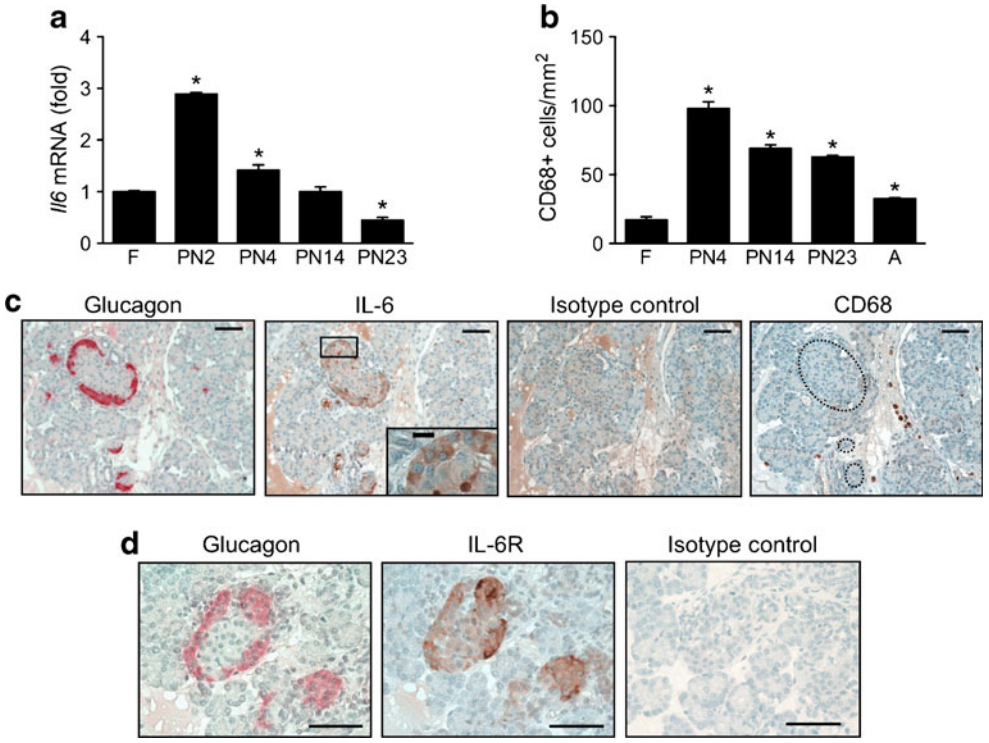

Fig. 2 *Il6* expression profile during pancreas development. (**a**) Quantitative RT-PCR analysis of *Il6* expression on rat pancreases of different ages normalised for 18S (n=4–6 pancreases at each age). Data are means±SEM. $^{*}p<0.05$ relative to at-term fetuses (F). (**b**) Number of CD68+ cells in pancreases from rats of different ages (A, 3-month-old adult rats). Cells were counted and expressed per mm^2 of total pancreatic surface area at each age. Data are means±SEM (one section per rat and age was analysed; n=4 rats at each age). $^{*}p<0.05$ relative to F. (**c**) Representative images of immunohistochemical localisation of glucagon (red), IL-6 (brown) and CD68 (brown) in serial sections of rat pancreases on PN4. Isotype control staining of IL-6 is indicated. (**d**) Representative images of immunohistochemical localisation of glucagon (red) and IL-6R (brown) in serial sections of rat pancreases on PN4. Isotype control staining of IL-6R is indicated. Scale bars on large images, 50 μm; on inset, 10 μm

IUGR disturbs alpha cell growth and function Maternal food restriction jeopardises the development of the endocrine pancreas, and circulating glucagon has been found to be decreased in undernourished (UN) neonates [22]. We therefore questioned whether UN animals showed altered alpha cell expansion capacity. The assessment of pancreatic alpha cell mass indicated a dramatic reduction in this variable in UN neonates on PN4. However, it was normalised on PN14 and experienced a significant increase at the end of suckling when compared with controls (Fig. 4a). These results could not be explained by changes in the rate of alpha cell replication because quantification of this variable showed defective alpha cell proliferation capacity in UN rats on PN4 and PN14 (Fig. 4b). By contrast, the number of alpha cells associated to ducts was markedly higher on PN14 and PN23 in UN vs controls (Fig. 4c).

To analyse the secretory capacity of alpha cells, isolated islets from PN4 rats were tested in a static assay at different glucose concentrations (Fig. 4d). At a low glucose concentration (0.5 mmol/l), islet alpha cells from control animals showed a characteristic pattern of glucagon secretion that was effectively inhibited by high glucose levels (3 and 21 mmol/l). Nevertheless, the release of glucagon in islets from UN neonates was only 35% of the value observed at 0.5 mmol/l glucose in control islets. Furthermore, alpha cells from UN islets did not respond to the increase in glucose concentration. These alterations seem to be related in part to changes in *Gcg* expression (Fig. 4e) and are reflected by marked hypoglucagonaemia at this age (Fig. 4f).

Islets from UN neonates show defective IL-6R signalling pathway To further assess the role of IL-6 on alpha cell expansion, we conducted quantitative RT-PCR analysis of *Il6* in the pancreas of UN rats. Compared with control animals, UN rats showed lower *Il6* mRNA expression at the end of gestation and on PN4 and PN14. However, pancreatic *Il6* expression significantly increased in UN rats at the end of suckling (Fig. 5a). Furthermore, immunostaining of IL-6 also showed delayed detection of this cytokine within islets from UN rats until PN23 (Fig. 5b).

Next, we performed in vitro studies to test the IL-6R signalling pathway in UN rats. When IL-6 binds to its receptor, signals are transduced via activation of STAT3 and extracellular signal-regulated kinase signalling, regulating several

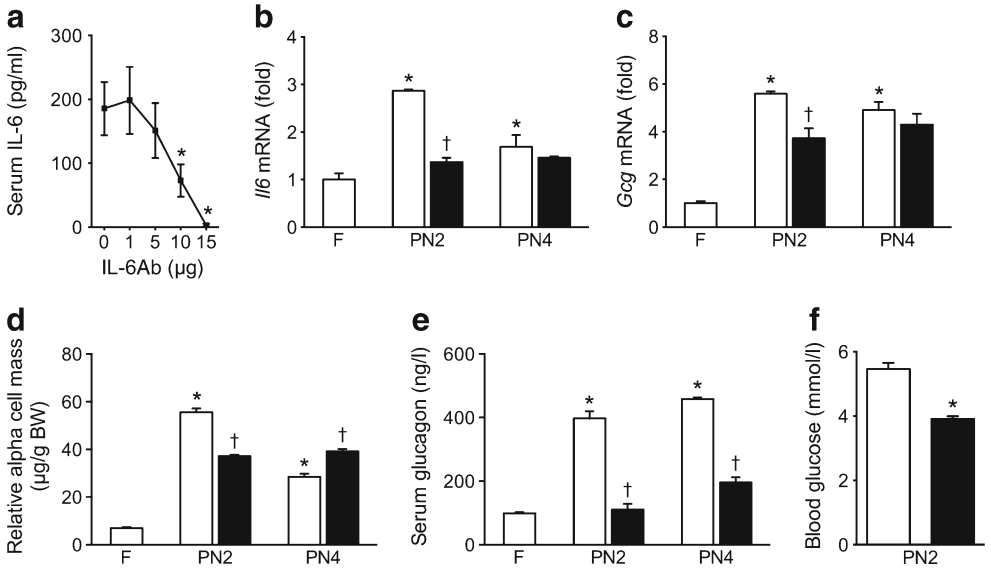

Fig. 3 Alpha cell mass and serum glucagon levels are reduced after IL-6 neutralisation. (**a**) Serum IL-6 levels after neutralisation. Different doses (1, 5, 10 or 15 μg per rat every 48 h) of IL-6Ab were administered i.p. immediately after birth (n=4–5). Data are means±SEM. $^{*}p<0.05$ relative to sham-injected neonates. (**b**) Quantitative RT-PCR analysis of *Il6* expression normalised for 18S on rat pancreases of different ages injected with IL-6Ab or sham-injected (n=5–6 for each condition); F, at-term fetuses. (**c**) Quantitative RT-PCR analysis of *Gcg* expression normalised for 18S on rat pancreases of different ages injected with IL-6Ab or sham-injected (n=5–6 for each condition). (**d**) Alpha cell mass and (**e**) serum glucagon levels from neonates injected with IL-6Ab or sham-injected (n=4–5). (**f**) Serum glucose levels from PN2 neonates injected on PN1 with IL-6Ab or sham-injected (n=4–5). Data are means±SEM. (**b**–**e**) $^{*,\dagger}p<0.05$, determined by ANOVA, where the asterisk compares different ages with the same treatment and the cross symbol compares sham neonates with neonates injected with IL-6Ab at the same age. White bars, neonates injected with sterile PBS (sham); black bars, neonates injected with 15 μg IL-6Ab

downstream targets. First, we detected higher levels of *Il6r* mRNA in islets from UN neonates compared with controls (Fig. 5c), whereas no differences at the protein level were observed between groups (Fig. 5d). However, time-dependent phosphorylation of STAT3 was observed after IL-6 treatment of cultured islets. Maximum phosphorylation of

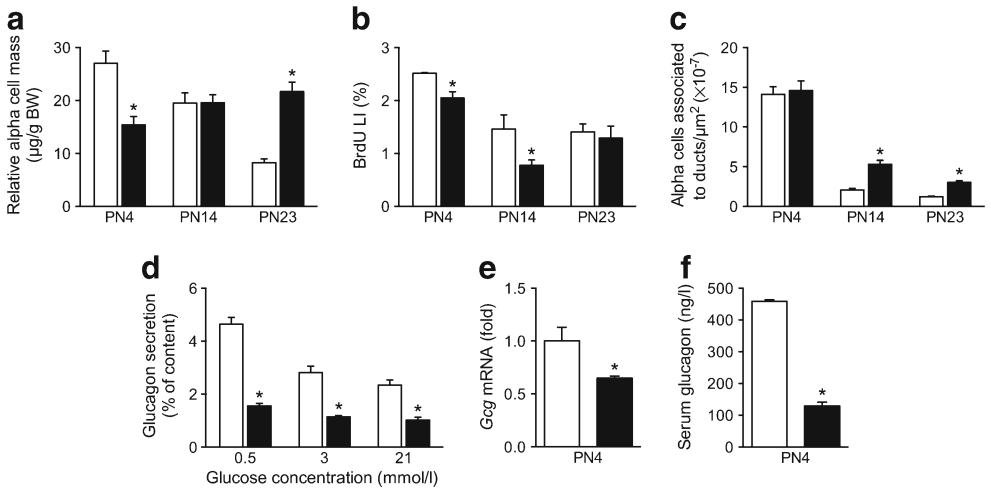

Fig. 4 Effect of maternal food restriction on offspring pancreatic alpha cell development. (**a**) Relative alpha cell mass expressed as alpha cell mass in μg/g body weight (BW) in neonatal rats fed ad libitum or UN (n=4–6 in each experimental group and age). (**b**) BrdU labelling index (LI) of alpha cells as a representation of alpha cell replication rate in control and UN neonates (n=4–6 in each experimental group and age). (**c**) Alpha cell neogenesis quantified as single alpha cells and alpha cell clusters (two to four) incorporated into the ductal epithelium per unit of total tissue area (n=4–6 in each experimental group and age). (**d**) Glucagon secretion in response to glucose from isolated islets of control and UN neonates on PN4 (n=8–10 in each group). (**e**) Quantitative RT-PCR analysis of *Gcg* expression on isolated islets of control and UN neonates on PN4 normalised for 18S (n=5 in each group). (**f**) Serum glucagon levels measured by RIA in control and UN rats on PN4 under 16 h fasting conditions (n=8 in each group). Data are means±SEM. $^{*}p<0.05$, determined by Student's t test comparing control rats with UN rats at each age. White bars, control neonates; black bars, UN neonates

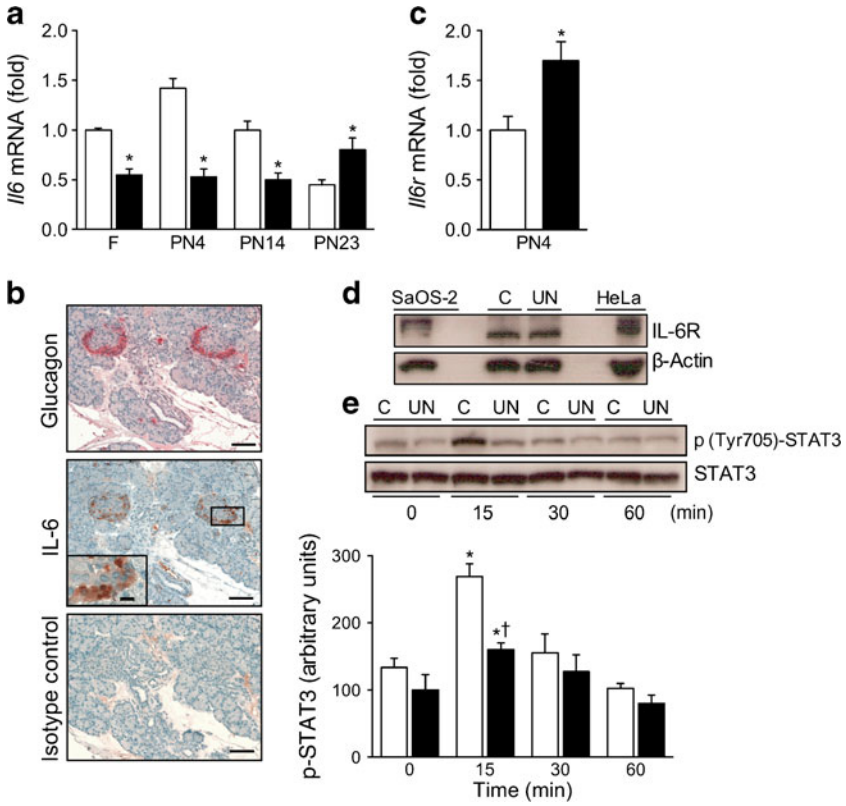

Fig. 5 Pancreatic islets from UN neonates show a defective IL-6R signalling pathway. (**a**) Quantitative RT-PCR analysis of *Il6* expression on rat pancreases of different ages normalised for 18S (n=4 in each group). Data are means±SEM. $^{*}p<0.05$, determined by Student's t test comparing control rats with UN rats at each age. (**b**) Representative images of immunohistochemical localisation of glucagon (red), IL-6 (brown) and isotype control of IL-6 in serial sections of UN rat pancreases on PN23. Scale bars on large images, 50 μm; on insets, 10 μm. (**c**) Quantitative RT-PCR analysis of *Il6r* expression on isolated islets of control and UN neonates on PN4 normalised for 18S (n=5–6 in each group). (**d**) Western blot of IL-6R in cultured islets from control (C) and UN rats on PN4. SaOS-2 and HeLa cell extracts were used as positive controls. (**e**) Time course of STAT3 phosphorylation (P; middle) in the presence of 100 ng/ml IL-6 with its graphical representation (n=4–6). Data are means±SEM. $^{*,\dagger}p<0.05$, determined by ANOVA, where the asterisk compares different times of stimulation within the same population and the cross symbol compares control values with values obtained in UN islets at the same time. White bars, control neonates; black bars, UN neonates

STAT3 protein was observed at 15 min of IL-6 treatment in both animal groups, although islets from UN neonates showed a very low response (Fig. 5e). No significant change in total STAT3 level was observed due to nutritional manipulation or IL-6 treatment (Fig. 5e). These data suggest that alpha cell/islet IL-6R might be dysfunctional following undernourishment. However, we cannot rule out that IL-6R-induced STAT3-independent signalling pathways could be mediating the transcriptional effects of IL-6 in UN islets.

Discussion

Our study had four main findings. First, *Il6* mRNA and protein production exhibited developmental regulation in rat pancreas. Second, pancreatic IL-6 localised within the islets, mainly in the alpha cells during the suckling period. Third, short-term IL-6 neutralisation after birth was associated with decreased alpha cell mass and circulating glucagon levels. Fourth, IUGR disturbs alpha cell growth and function and alters the ontogenetic profile expression of pancreatic *Il6*. These data point to the potential role of local IL-6 for regulation of alpha cell maturation during suckling.

During the perinatal period, important modifications in several physiological functions and, in particular, dramatic changes in nutrition occur. This condition is associated with a remarkable increase in alpha cell mass and hyperglucagonaemia. It has been suggested that the neonatal increase in plasma glucagon may be related to the stress of birth through activation of the sympathetic nervous system that results in the release of catecholamines [24], which are potent stimuli of glucagon release [25]. In addition, catecholamines elevate circulating IL-6 [26, 27], and IL-6 has been proposed as a factor in the increase in alpha cell mass in diabetes [17]. However, our study did not detect any increase in blood IL-6 levels with birth, and to our knowledge it has not been previously reported. By contrast, IL-6 levels exhibited developmental upregulation in the pancreas of newborn rats. Several studies have uncovered IL-6 as a

cytokine that regulates the growth and maturation of developing organs, such as the brain in rats [28] or sheep [29]. Accordingly, the suckling period can be considered a critical window for endocrine pancreas remodelling in rodents [8, 9]. Therefore, our results suggest that there could be an ontogenetic regulatory role for IL-6 in normal neonatal pancreas maturation. Interestingly, our study demonstrates that islet cells represent one of the major sources of IL-6 in the early perinatal period. A predilection of IL-6-positive cells in the periphery of the islet implied a predominant IL-6 production by alpha cells, which was confirmed by staining correlative sections for glucagon. Previous studies have also described pancreatic islet cell production of IL-6 in humans under physiological conditions [30] or in rodent models of obesity and type 2 diabetes [31, 32].

It seems that IL-6 does not exhibit toxicity to alpha cells, because the presence of IL-6R in alpha cells detected by us and others [17] suggests that IL-6 may be involved in the regulation of alpha cell fate during the neonatal period through an autocrine loop. Thus, in vitro IL-6 treatment induces alpha cell proliferation [17], and there is some evidence of increased islet cell neogenesis in transgenic mice overexpressing *Il6* in beta cells [33]. In support of this, our study shows an active alpha cell self-replication rate and a remarkable increase in the number of glucagon-positive cells budding from pancreatic ducts soon after birth, coinciding with a high expression of *Il6* in the pancreas.

We cannot ascertain from our temporal measurements whether the changes in *Il6* expression regulate neonatal pancreas development and, thus, subsequent alpha cell maturation. Therefore, to confirm the physiological role of IL-6 during suckling, IL-6Ab was administered to the neonates. IL-6 neutralisation partially reduced alpha cell mass expansion as well as *Gcg* expression. Consequently, suckling rats displayed decreased circulating glucagon levels and hypoglycaemia. This suggests that pancreatic IL-6 is a regulator of neonatal alpha cell growth and function.

It should be noted that lactation is a specific metabolic condition characterised by a milk-based diet. The contribution of milk glucose to the total glucose requirements of the newborn is no more than 10–40% [24]. This implies that new gluconeogenic substrates must be supplied to maintain neonatal glucose homeostasis. Non-esterified fatty acids derived from the high-fat content of milk are excellent oxidative substrates for the liver. Glucagon is the dominant hormone involved in the maturation and regulation of liver gluconeogenesis and fatty acid oxidation [24]. Thus, we suggest that alpha cell mass expansion during suckling is the consequence of a physiological state of increased glucagon demand to ensure the metabolic adaptation of newborns to extrauterine life, and local IL-6 seems to play a positive role in this adaptation. Nevertheless, we cannot rule out that several other factors may also play a role during neonatal alpha cell expansion, since insulin and IGF-1 receptors are also expressed in islet alpha cells [34, 35].

The injection of a neutralising antibody during the early days of postnatal life represents a limitation in our study in that it restricts us to establishing a cause–effect relationship between the lack of IL-6 and the subsequent reduction in alpha cell mass and function, but it does not let us study the longer-lasting consequences on adult alpha cells. However, both alpha and beta cells from at-term fetuses can be considered as immature in their sensitivity to glucose compared with adult islets [36] and must undergo additional development during the postnatal period. Therefore, all dietary and metabolic events that occur during the perinatal period and the suckling–weaning transition may induce a change in the alpha cells from fetal to adult phenotype with altered glucose thresholds and the ability to release glucagon rapidly. Our data show a continuum in the maturation of glucagon secretion from birth to adulthood in response to glucose (ESM Fig. 1). Hence, any deregulation of these processes due to the abnormal availability of growth factors, such as IL-6, could be of great importance for the correct adult pancreatic physiology and might compromise the alpha cell capacity for adaptation to any later nutritional insult. Likewise, Ellingsgaard et al [17] described normal islet architecture and glucose homeostasis in young adult *Il6* knockout mice under standard fed conditions, suggesting that IL-6 may not be essential for normal alpha cell development. However, after an 18-week high-fat diet, *Il6* knockout animals exhibited glucose intolerance despite decreased fasting glucagon levels and no expansion of alpha cells.

The extensive changes experienced by islet cells throughout neonatal life may render them highly sensitive to any nutritional insult at this time. We and others have previously reported in rodent models that maternal food restriction significantly affects beta cell mass in the offspring neonates [22, 37]. Decreased beta cell mass correlates with hypoinsulinaemia and hypoglycaemia due to increased insulin sensitivity, as we previously demonstrated in our model [38, 39]. In addition, we have observed reduced circulating glucagon levels in suckling pups [22], suggesting that islet adaptations to chronic malnutrition also involve changes in alpha cells. These data prompted us to consider our rat model suitable for further study of the role of IL-6 in pancreatic alpha cell maturation and its contribution to nutritional islet adaptation. In the present work, we describe impaired alpha cell functionality at the level of both glucagon production and secretion in UN neonates. This feature, together with liver glucagon resistance previously reported in our model [38], is associated with marked compensatory alpha cell hyperplasia at the end of suckling. Similarly, alpha cell hyperplasia was observed in animal models in which glucagon signalling had been inhibited either by reduction of glucagon receptor expression [40] or by disruption of proglucagon processing [41, 42]. In both cases, the increase in

alpha cell mass was mainly due to alpha cell replication [41, 43]. By contrast, in the present study, UN neonates showed impaired alpha cell proliferation but enhanced neogenesis.

Interestingly, we also observed a differential expression pattern of pancreatic *Il6* throughout the neonatal period in food-restricted pups compared with controls. Very few and contradictory data exist in the literature regarding the regulation of IL-6 in IUGR subjects. Both reduced [44] and increased [45] fetal IL-6 levels have been documented in IUGR. In our hands, dietary regulation of IL-6 occurred both at the mRNA and protein level, with defective production of the cytokine in the early perinatal period and increased levels close to weaning. These changes in local IL-6 paralleled those observed in relative alpha cell mass, strengthening the argument that IL-6 contributes to alpha cell maturation. However, it would be reasonable to suggest that this differential regulation of IL-6 may influence the risk of developing chronic diseases later in life. Studies are underway to evaluate this possibility and to quantify accurately the dynamic changes in alpha cell population in our IUGR rats fed a high-fat diet.

In summary, using two in vivo rat models of IL-6 blockade and IUGR, we clearly demonstrated in the present study the physiological relevance of local IL-6 for normal neonatal alpha cell mass expansion and functionality. Our results illustrate for the first time that IL-6 exhibits developmental regulation in the rat pancreas. In particular, pancreatic *Il6* expression was enhanced acutely at the beginning of the suckling period and was localised within the islets, mainly in the alpha cells.

Finally, since therapies involving the attenuation of glucagon secretion and action are currently being developed for the treatment of type 2 diabetes, it will be important to determine the nature of the mechanisms and factors involved in alpha cell maturation and function. Accordingly, the suckling period in rodents represents an interesting state of hyperglucagonaemia and alpha cell hyperplasia to explore new targets for therapeutic purposes and to understand how perinatal nutrition determines the development of glucose intolerance in adulthood.

Funding This work was supported by Ministerio de Economía y Competitividad (BFU2011/25420), Comunidad de Madrid (S2010/BMD-2423) and CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM, ISCIII, Spain). EFM receives support from CIBERDEM.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement All authors contributed to the conception, design, analysis or interpretation of data, and drafting or critical revision of the article for important intellectual content. All authors have given their final approval of the version to be published.

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