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## Human and rodent pancreatic $\beta$ -cells express IL-4 receptors and IL-4 protects against $\beta$ -cell apoptosis by activation of the PI-3-Kinase and Jak/STAT pathways

**Short title:** IL-4 receptors and IL-4 cytoprotection in pancreatic islet cells

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## Synopsis

Secretion of pro-inflammatory cytokines is associated with loss of pancreatic  $\beta$ -cell viability and cell death. Interleukin-4 (IL-4) has been reported to mediate a protective effect against the loss of pancreatic  $\beta$ -cells and IL-4 receptors have been found in rat pancreatic  $\beta$ -cells at both the RNA and protein level. The aim of this study was to investigate IL-4 receptor expression on human islet cells and to examine the signalling pathways by which IL-4 exerts its effects using the rat  $\beta$ -cell lines, BRIN-BD11 and INS-1E. By means of immunohistochemistry, it was demonstrated that IL-4 receptors are present on human islet cells. Using a flow cytometric method for evaluating cell death, it was confirmed that incubating  $\beta$ -cells with IL-4 attenuated cell death induced by interleukin-1 $\beta$  and interferon- $\gamma$  by approximately 65%. This effect was abrogated by the presence of the PI-3-kinase inhibitor, wortmannin, suggesting that activation of the PI-3-kinase pathway is involved. In support of this, Western blotting revealed that incubation of cells with IL-4 resulted in increased phosphorylation of Akt, a downstream target of PI-3-kinase. Increased tyrosine phosphorylation of STAT6 also occurred in response to IL-4 and a selective Jak3 inhibitor reduced the cytoprotective response. Both effects were prevented by over-expression of the tyrosine phosphatase, PTP-BL. We conclude that IL-4 receptors are functionally competent in pancreatic  $\beta$ -cells and that they signal via PI-3-kinase and Jak/STAT pathways. These findings may have implications for future therapeutic strategies for the management of diabetes.

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## INTRODUCTION

Autoimmune destruction of pancreatic beta-cells in Type I diabetes (T1D) is thought to occur as a result of the disruption of normal pancreatic immunoregulation which may, in part, reflect a functional polarisation of the T helper (Th) subset of CD4<sup>+</sup> lymphocytes [1]. Secretion of pro-inflammatory cytokines, such as interleukin (IL)-1 $\beta$  IL-6, interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$ , by Th1 lymphocytes and macrophages in the pancreatic environment are associated with increasing loss of insulin producing  $\beta$ -cells in the pancreatic islets [2,3,4]. This is exacerbated by a concomitant reduction of secretion of anti-inflammatory cytokines, such as IL-4, by Th2 lymphocytes [5,6,7]. In animal models, IL-4 has been proposed to play a protective role in the development of diabetes [8,9], while patients with elevated levels of IL-4 may be resistant to the development of diabetes [10].

IL-4 is a multifunctional cytokine that plays a critical role in the regulation of immune responses. Its effects depend upon binding to and signalling through a receptor complex consisting of the IL-4 receptor alpha (IL-4R $\alpha$ ) chain and either the cytokine receptor common gamma chain ( $\gamma$ c) (in type I IL-4 receptors) or the  $\alpha$ 1-chain of IL-13 receptors (in type II IL-4 receptors), resulting in a series of phosphorylation events mediated by receptor-associated kinases [11]. In turn, these signal to promote cell growth, resistance to apoptosis, and/or gene activation and differentiation. In other cell types, heterodimerisation of the IL-4R $\alpha$  chain with the  $\gamma$ c (common- $\gamma$ ) chain results in tyrosine phosphorylation of the Jak family of protein kinases in response to IL-4 [11,12]. The activation of Jak kinases culminates in the phosphorylation of the transcription factor STAT6, to propagate the signal arising from binding of IL-4. In addition, IRS-1 and IRS-2 can also become phosphorylated as a result of interaction of IL-4 with the IL-4R $\alpha$  [13,14]. This response promotes their high affinity association with the p85 regulatory subunit of PI-3-kinase leading, in turn, to activation of PI-3-kinase and downstream kinases such as Akt [15].

In a previous study we confirmed, at both the RNA and protein level, the presence of each component of the Type I and Type II IL-4 receptors in rat pancreatic  $\beta$ -cells [16]. In addition we showed that IL-4 had the capacity to attenuate the cytotoxic effects of IL-1 $\beta$ /IFN $\gamma$  on pancreatic  $\beta$ -cells [16]. However it was not established which signaling pathway is responsible for the anti-apoptotic effects of IL-4 in  $\beta$ -cells. Therefore, the aim of the present study was to confirm that IL-4R are found on human pancreatic  $\beta$ -cells and to investigate the signaling pathways by which IL-4 exerts its effects in  $\beta$ -cells.

## EXPERIMENTAL

### Materials

RPMI 1640, trypsin/EDTA, phosphate-buffered saline (PBS), penicillin and streptomycin were purchased from Invitrogen, UK. Anti-human IL-4 receptor antibody was purchased from R&D Systems, UK. DNase free ribonuclease was from Roche, Lewes, UK. Rabbit anti-mouse HRP, DakoREAL Envision/HRP rabbit anti-mouse antibody, REAL DAB chromogen and DAKO

REAL substrate buffer were from DAKO, Ely, UK. DPX Mountant was purchased from Fluka, UK. IL-4, IL-1 $\beta$ , IFN $\gamma$ , goat anti-rabbit Alkaline Phosphatase IgG antibody, protease inhibitors, CDB-Star Chemiluminescent Substrate and Wortmannin were from Sigma, Poole, UK. CellTiter 96 AQueous One Solution Cell Proliferation (MTS) Assay was from Promega, Southampton, UK. Rabbit anti-rat pAkt antibody was from Santa Cruz, UK. Jak3 inhibitor II (WHI-P154) was from Calbiochem, Nottingham, UK.

### **Growth and incubation of rodent clonal $\beta$ -cells**

BRIN-BD11 and INS-1 cells were used in the studies. The cells were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 400 U/ml penicillin and 200  $\mu$ g/ml streptomycin. INS-1E Flp-In-TREx cells were established and grown as described by Welters et al [17]. Cells inducibly expressing the tyrosine phosphatase PTP-BL were generated using Flp recombinase [17, 18] and were grown in the presence of hygromycin to maintain selection pressure. When required, over-expression of PTP-BL was achieved by culture in the presence of tetracycline for 24h.

### **Immunocytochemical staining for IL-4 receptor expression**

Paraffin embedded human pancreatic sections obtained at autopsy from non-diabetic individuals. They were kindly provided by Dr AK Foulis (Glasgow) and were used with full ethical permission (North Glasgow NHS Trust). The sections were de-waxed in xylene (2 x 4 minutes). Slides were then rehydrated through 100% ethanol for 1 min, 90% ethanol for 1 min, 70% ethanol for 1 min, 100% methanol for 1 min and H<sub>2</sub>O for 5 min. Slides were blocked with 0.5ml normal goat serum for 5 minutes and primary mouse anti-human IL-4 receptor antibody, isotype-specific control IgG or anti-insulin serum applied and incubated overnight at 4C°. After washing in Tris-buffered saline pH 7.4, secondary antibody conjugated to horseradish peroxidase was applied and the slides were incubated for further 1hr. After washing in Tris-buffered saline DAB substrate was applied for 10 minutes. After further washing, slides were stained with haematoxylin for 5 minutes. Dehydration of the slides was performed by immersion in 50% ethanol (5 min) 70% ethanol (5 min) 90% ethanol (5 min) and finally 100% ethanol (2 x 5 min). Slides were cleared in xylene (2 x 5 min) and mounted in DPX medium for examination.

### **Flow cytometric analysis of cell death**

$\beta$ -cell death was analysed using flow cytometry. Following incubation with test reagents, the culture medium containing detached cells was harvested. Adherent cells were trypsinised and both the detached and adherent cells were collected by centrifugation (300g; 5min). They were resuspended in 200  $\mu$ l of phosphate-buffered saline (PBS) and fixed with ice-cold ethanol: PBS (70 : 30 v:v) for 30 min on ice. After fixation the cells were centrifuged for 5 min at 300g and resuspended in 820  $\mu$ l of PBS. 80 $\mu$ l of propidium iodide (0.5 mg/ml) and 5 $\mu$ l of DNase free ribonuclease were added and incubation continued for 30 min at 37°C. Cellular DNA was analysed using a flow cytometer and the extent of DNA fragmentation determined as an index of loss of viability. Experiments were performed in triplicate and were repeated on at least three occasions.

## Western blotting analysis

$\beta$ -cells were incubated with appropriate reagents and homogenised in lysis buffer (20mM Tris, 150mM NaCl, 1 mM EDTA, 1% Triton X and protease inhibitors; pH 7.4) for protein extraction. The homogenate was then centrifuged at 2000g for 10 min at 4°C and the supernatant was collected, aliquoted and stored at -80°C until use. The protein concentration was determined using bovine serum albumin (BSA) as standard. Immunoblotting was carried out using 70 $\mu$ g of protein loaded into BioRad Criterion™ Precast 12% gels. Separated proteins were transferred into PVDF membranes. Membranes were then blocked in Tris buffered saline 0.1% Tween-20 (TBS-T, pH 7.5) containing 2% BSA and 5% non-fat dried milk for 1h at room temperature before adding primary antibody. After overnight incubation at 4°C, the membranes were washed three times in TBS-T and secondary goat anti-rabbit IgG HRP conjugated antibody added for 1 h. The membranes were washed 3 times for 5 min in TBS-T. Protein bands were detected using CDP Star chemiluminescent Substrate.

## MTS assay

$\beta$ -cells were seeded in 96 well plates at 10<sup>4</sup> cells/well and, after 24 h incubation, IL-4 added with or without relevant inhibitors (wortmannin (500nM), Jak3 (78nM)). After a further 24 h incubation, MTS assay was performed according to manufacturer's instructions. Briefly, 20ul of MTS reagent was added to each well and the mixture incubated for 1h at 37°C. Absorbance was read at 490nm.

## Statistical analysis

Statistical analysis was performed using the Student's t-test or by analysis of variance, and results were considered significant when P<0.05

# RESULTS

## Expression of IL-4 receptors in human pancreas

In a previous study we demonstrated that rat pancreatic  $\beta$ -cell lines express receptors for IL-4 at both the RNA and protein level [16]. However, it has not been established whether primary  $\beta$ -cells also express the receptor and, therefore, immunohistochemical analysis was undertaken in sections of normal adult human pancreas to verify this (Fig.1 – upper panel). Intensely positive staining with anti-IL-4R $\alpha$  antibodies was seen in the majority of islet endocrine cells, consistent with the presence of IL-4 receptors on human  $\beta$ -cells. Similar data were obtained in pancreas samples from 4 separate individuals and, in each case, the majority of islets were stained with anti-IL4 receptor antibody. Immunostaining of the adjacent section for insulin (Fig.1; middle panel) suggested that fewer cells stained positively for the hormone than for IL-4 receptor implying that the receptors may be present on both  $\beta$ -cells and on other islet endocrine cell types.



In addition to the labelling of islet endocrine cells, immunopositivity for IL-4 receptor was also detected in the surrounding exocrine tissue, although this was less intense than on islet cells. The staining intensity was lost when the primary antibody was omitted (not shown) or replaced with isotype control mouse IgG (Fig.1- lower panel).

#### **Activation of the PI-3-kinase pathway in beta-cells treated with IL-4**

Previous studies by our own and other groups have established that the cytotoxic actions of pro-inflammatory cytokines in  $\beta$ -cells are attenuated by pre-incubation with IL-4 [16, 22]. This was confirmed in the present work (Fig.2) where the extent of cell loss caused by incubation of  $\beta$ -cells with IL-1 $\beta$  & IFN $\gamma$  was attenuated by approximately 65% in the presence of 20ng/ml IL-4. This effect was prevented by the presence of the PI-3-kinase inhibitor wortmannin (Fig.2) suggesting that activation of the PI-3-kinase pathway is involved. In support of this, Western blotting revealed that incubation of cells with IL-4 resulted in increased phosphorylation of Akt, a downstream target of PI-3-kinase (Fig.3). The extent of this increase was less marked than that induced in parallel studies with 10nM IGF-1 (Fig.3) but, nevertheless, the response to IL-4 resulted in a clear increase in Akt phosphorylation.

#### **Activation of Jak/STAT signalling in beta-cells treated with IL-4**

In immune cells, the activation of IL-4 receptors leads to alterations in gene transcription as well as to increases in PI-3-kinase activity and it is thought that these effects may reflect the activation of different signalling pathways by IL-4 receptors [11]. In particular, IL-4R activation is frequently associated with changes in the phosphorylation status of STAT proteins, especially STAT6. Therefore we next examined whether the Jak-STAT pathway may also play a role in mediating the response to IL-4 in  $\beta$ -cells. Initially, the effects of an inhibitor of Jak-3 kinase (WHI-P154) were investigated and it was observed that this significantly reduced the cytoprotective response to IL-4 in beta-cells exposed to pro-inflammatory cytokines (Fig. 4).

A second index of improved viability was also measured in these experiments, MTS reduction, which provides information on the functional status of mitochondrial dehydrogenases. It was observed that exposure of beta-cells to IL-4 resulted in a marked increase in MTS reduction, consistent with an enhanced metabolic propensity of the cells in the presence of the anti-inflammatory cytokine (Fig.5). Importantly, this response was progressively reduced in cells over-expressing the tyrosine phosphatase, PTP-BL, which is active against phospho-STAT proteins (Fig.5). In order to confirm this more directly, Western blotting of cell extracts was undertaken using an antiserum raised against tyrosine phosphorylated STAT6. The results revealed a marked increase in phospho-STAT6 formation in  $\beta$ -cells exposed to IL-4 (Fig.6). Strikingly, as in the case of IL-4-induced MTS reduction (Fig.5), this response was entirely lost in cells over-expressing the tyrosine-specific phosphatase, PTP-BL (Fig.6).

## DISCUSSION

The present results demonstrate the presence of IL-4 receptors on human adult pancreatic islet cells by immunohistochemistry (Fig 1). Since the majority of islet cells were stained by the anti-IL-4 receptor antibodies and, in serial sections, by anti-insulin serum (Fig.1 middle panel), this suggests that human  $\beta$ -cells express the receptor. As such, these results provide support to our earlier conclusion that IL-4 receptors are expressed on clonal pancreatic  $\beta$ -cells derived from rat [16] and they show that expression of these molecules is not restricted to rodent islet cells. However, the pattern of immunohistochemical staining implies that, at least in man, other islet endocrine cells may also express IL-4 receptors since positive immunostaining for these molecules did not appear to be restricted only to  $\beta$ -cells. Nevertheless, the presence of IL-4 receptors on normal human pancreatic  $\beta$ -cells is significant since it implies that, as in rat [16] these receptors may play a protective role against cell damage mediated by pro-inflammatory cytokines. As such, it is clear that the balance between the influence of pro- and anti-inflammatory cytokines (including IL-4) on  $\beta$ -cells might be an important determinant of their ultimate fate during the progression towards type 1 diabetes. In this context, it is interesting to note that a relative lack of IL-4 production has been reported in patients with type 1 diabetes in a number of studies. For example, peripheral blood mononuclear cells (PBMC) from human diabetic patients were shown to produce a decreased level of IL-4 when challenged with polyclonal activators such as anti-CD3 antibody or phytohemagglutinin [5]. Moreover, others have reported that elevated IFN $\gamma$ /IL-4 ratios correlate with the presence of destructive insulinitis and impaired Th2 cell cytokine production from cells that infiltrate the pancreas of diabetic patients [19,20,21].

In view of these considerations, it is important to determine the intracellular signalling pathways by which IL-4 may mediate its effects in  $\beta$ -cells. Accordingly, we have employed clonal rat  $\beta$ -cell lines for functional analysis in the present work since we showed previously that recombinant IL-4 significantly reduced apoptotic  $\beta$ -cell death induced by pro-inflammatory cytokines in these cells [16]. In addition, another group has recently demonstrated a protective effect of IL-4, IL-10 and IL-13 in blocking pro-inflammatory cytokine-induced activation of caspase-3 [22]. Previous work in other cells, has established that different signalling pathways can be activated upon addition of IL-4 [13,14] and, in particular, it has been proposed that IL-4 mediated heterodimerisation of the IL-4R $\alpha$  chain with the  $\gamma$ c (common- $\gamma$ ) chain results in tyrosine phosphorylation of the Jak family of protein kinases, particularly Jak1 and Jak3. This appears to reflect the binding of Jak1 to the IL-4R $\alpha$  chain, while Jak3 associates with the  $\gamma$ c chain [11,12]. The activation of Jak kinases culminates in the phosphorylation of the transcription factor STAT6, to propagate the signal arising from binding of IL-4. However, in addition to the Jak kinases, a second group of molecules that often become phosphorylated as a result of interaction of IL-4 with the IL-4R $\alpha$  chain are the insulin receptor substrates, particularly IRS-1 and IRS-2 [13,14]. This response leads to the tyrosine phosphorylation of sites located within both IRS1 and IRS2, thereby promoting their high affinity association with the p85 regulatory subunit of PI-3-kinase. This in turn leads to activation of PI-3-kinase and thence to activation of downstream kinases such as Akt that are important in mediating cytoprotection and cell proliferation [15].



In the present work we report that the ability of IL-4 to attenuate  $\beta$ -cell death induced by pro-inflammatory cytokines can be ameliorated by the addition of the PI-3-kinase inhibitor, wortmannin (Fig.2). This suggests strongly that the PI-3-kinase pathway is activated in rat  $\beta$ -cells exposed to IL-4 and that it can contribute to cytoprotection. In support of this, Western blotting analysis revealed that incubation of  $\beta$ -cells with IL-4 results in increased phosphorylation of Akt (Fig.3). These results imply that functional activation of IL-4R $\alpha$  complexes occurs during exposure of  $\beta$ -cells to IL-4 and that subsequent activation of the PI-3-kinase pathway plays a role in mediating the effects of the cytokine. However, it is clear that activation of a second signalling pathway involving Jak kinases is also associated with IL-4R activation. Thus, flow cytometry was used to measure the percentage of cells undergoing apoptosis after exposure to pro-inflammatory cytokines and it was observed that a selective inhibitor of Jak3 significantly reduced the cytoprotective response to IL-4 (Fig.4). Evidence for the involvement of Jak3/STAT6 was reinforced by the observation that IL-4 directly promotes STAT6 phosphorylation in  $\beta$ -cells, as measured by Western blotting (Fig.6). Thus, we conclude that both PI-3-kinase and Jak3/STAT6 are activated by IL-4 in  $\beta$ -cells and that both signalling pathways may contribute to cytoprotection. Interestingly, this conclusion differs from data obtained with mouse T-cells where these pathways are also activated but appear to be dispensable for cytoprotection [23]. Thus, the primary signalling mechanism(s) mediating the protective effects of IL-4 may differ between pancreatic  $\beta$ -cells and cells of the immune system.

To explore the importance of the Jak/STAT pathway further,  $\beta$ -cells over-expressing one specific member of the class of protein tyrosine phosphatases (PTP) PTP-BL [18] were employed. PTP-BL has recently been demonstrated to antagonise the phosphorylation of STAT6 mediated by IL-4 [24] and, consistent with this, the increase in tyrosine phosphorylation of STAT6 mediated by IL-4 was completely prevented in  $\beta$ -cells over-expressing PTP-BL (Fig. 6). Thus, as suggested for immune cells [24] these data imply that PTP-BL could be involved in the regulation of IL-4 signalling in  $\beta$ -cells. Moreover, the results demonstrate that the Jak3/STAT6 pathway can be functionally manipulated in  $\beta$ -cells and they suggest that it is involved in the regulation of  $\beta$ -cell survival in response to IL-4. Further support for this proposal derives from our observation that the increase in  $\beta$ -cell MTS reduction induced by IL-4 (an index of improved metabolic activity) was significantly attenuated by up-regulation of PTP-BL (Fig.5).

Therefore, we have provided evidence for the presence of IL-4 receptors on the surface of human pancreatic  $\beta$ -cells. In addition, we reveal that the functional activity of IL-4 involves activation of both PI-3-kinase and Jak3/STAT6 pathways and that these can be opposed by increased expression of the tyrosine phosphatase, PTP-BL.

Taken together, the data imply that targeting of IL-4 receptors may offer a means to attenuate the loss of pancreatic  $\beta$ -cells occurring during autoimmune diabetes. Furthermore, they may have similar implications for therapeutic intervention in type 2 diabetes since recent evidence has also implicated modest islet inflammation [25,26] and increased IL-1 $\beta$  production in mediating  $\beta$ -cell demise [27].

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## Figure legends

### Figure 1.

**Expression of IL-4 receptors (IL-4R) on human islet cells.** Sections of adult human pancreas were stained with anti-IL-4R $\alpha$  serum to detect expression of the receptor. (Upper panel) A typical islet is shown in which the majority of endocrine cells were stained (brown) consistent with expression of IL-4R $\alpha$ . (Middle panel) The adjacent section was stained for insulin (brown) to reveal the presence of  $\beta$ -cells. (Lower panel) Sections of pancreas were also exposed to control IgG (isotype specific IgG for the antiserum to IL-4 receptor) and were unstained.

### Figure 2.

**Involvement of the PI-3-kinase pathway in mediating the protective effects of IL-4 in  $\beta$ -cells.** Cells were incubated under control conditions (white bars) or with IL-4 (20ng/ml; grey bars) for 48h. After this time, IL-1 $\beta$  (2ng/ml) plus IFN $\gamma$  (2ng/ml) was added in the absence or presence of wortmannin (500nM) as shown. Cells were incubated for further 24h then harvested and analysed by flow cytometry. Each point is the mean ( $\pm$  S.E.M.) from at least four separate experiments. \*\* $p < 0.01$  relative to IL-1 $\beta$ /IFN $\gamma$  in the absence of IL-4.

### Figure 3.

**Phosphorylation of Akt in pancreatic  $\beta$ -cells exposed to IL-4.** Cells were incubated for 10 min under control conditions or in the presence of IGF-1 (10nM) or IL-4 (20ng/ml) as shown. Proteins were then extracted and fractionated by SDS-PAGE followed by immunoblotting with either anti-phospho-Akt antibody (p-Akt; upper panel) or total Akt antibody (lower panel). Data are from a representative experiment which was repeated twice with similar results.

### Figure 4.

**Effects of an inhibitor of Jak3 on the cytoprotective actions of IL4 in  $\beta$ -cells.** Cells were incubated under control conditions (white bar) or in the presence of pro-inflammatory cytokines (Cyto -IL-1 $\beta$  (2ng/ml) plus IFN $\gamma$  (2ng/ml)) IL-1 $\beta$  & IFN $\gamma$  plus IL-4 (20ng/ml); IL-1 $\beta$  & IFN $\gamma$  plus the Jak-3 inhibitor WHI-P154 (Jak; 1 $\mu$ M) or IL-1 $\beta$  & IFN $\gamma$ /IL-4 plus WHI-P154, as shown for 24h. After this time the cells were harvested and their viability determined by flow cytometry.

\* $p < 0.001$  relative to cells incubated under control conditions; \*\*  $p < 0.01$  relative to cells incubated in the presence of IL-1 $\beta$  & IFN $\gamma$  alone; \*\*\* $p < 0.001$  relative to cells incubated with IL-1 $\beta$ /IFN $\gamma$  and IL-4 in the absence of Jak-3 inhibitor.



**Figure 5.**

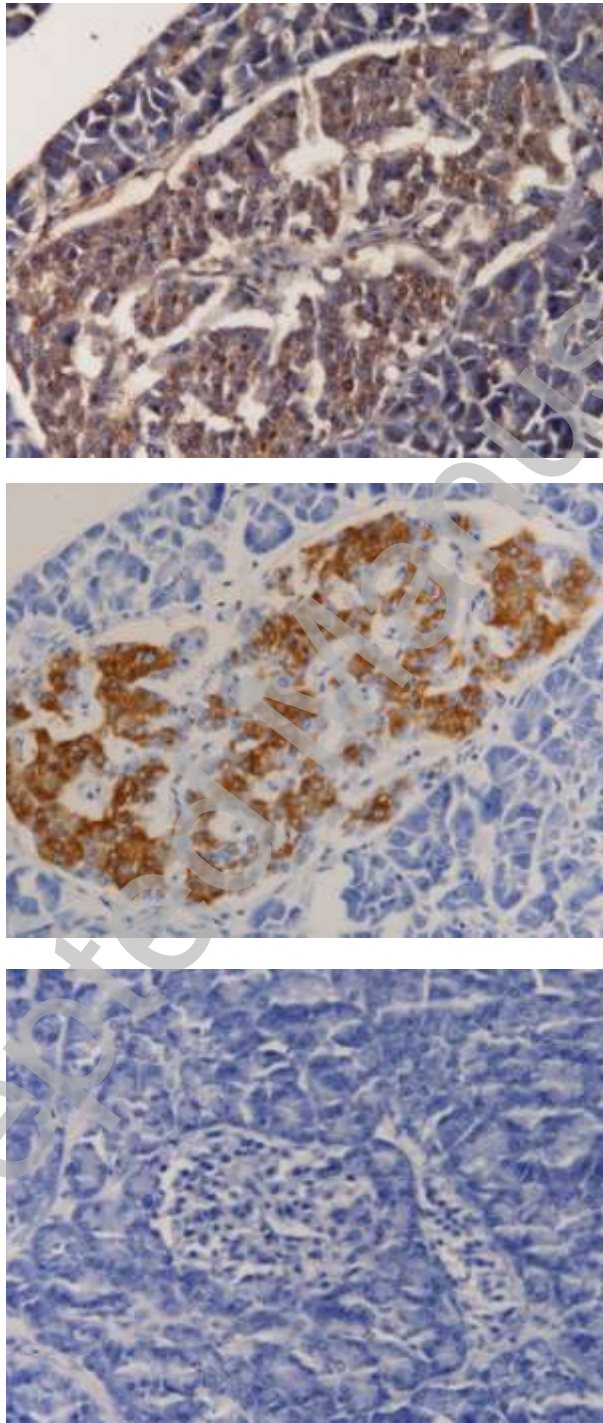
**MTS reduction in  $\beta$ -cells treated with IL-4.** Cells were treated with increasing concentrations of tetracycline (tet; 20ng/ml or 1000ng/ml) for 24h to induce the expression of PTP-BL. IL-4 (20ng/ml) was included during this incubation period, as shown. MTS reagent was then added and the absorbance measured 1h later. Results are expressed relative to control (100%) and are shown as mean values  $\pm$  SEM from replicates of 8. The experiment was repeated twice with similar results. \* $p$ <0.001 relative to the absence of IL-4; \*\* $p$ <0.05 relative to IL-4 alone; \*\*\* $p$ <0.001 relative to IL-4 alone.

**Figure 6.**

**Phosphorylation of STAT6 following incubation with IL-4.** INS-1 cells inducibly expressing the tyrosine phosphatase PTP-BL were generated using Flp recombinase. When required, over expression of PTP-BL was achieved by culture in the presence of 1 $\mu$ g/ml tetracycline for 24h. Un-induced cells received vehicle. IL-4 (20ng/ml) was included for the final 30 min of culture, as shown. Proteins were extracted, subjected to SDS-PAGE electrophoresis and immunostained with anti-pSTAT6 antibody.  $\beta$ -actin was used as a loading control (lower panel). Data are from a representative experiment which was repeated twice with similar results.

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Figure 1



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Figure 2

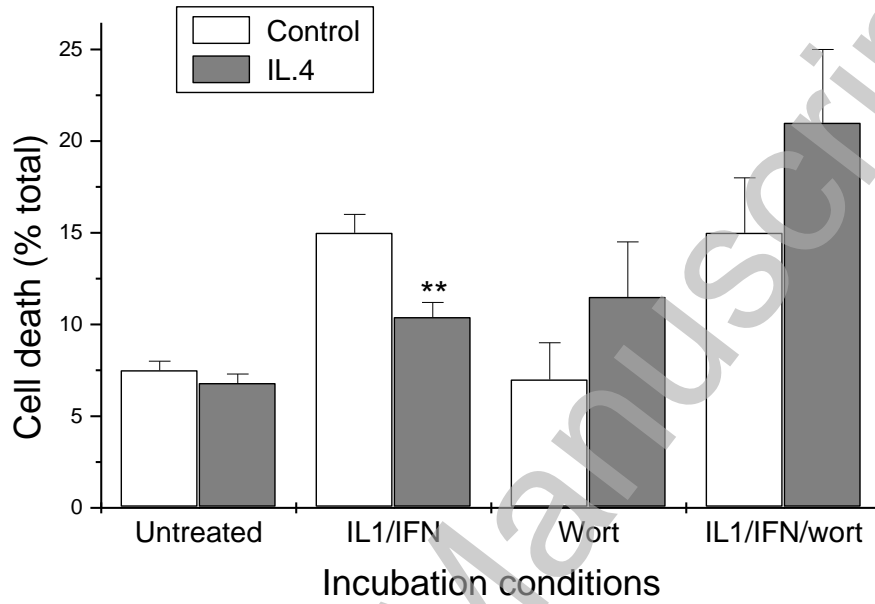
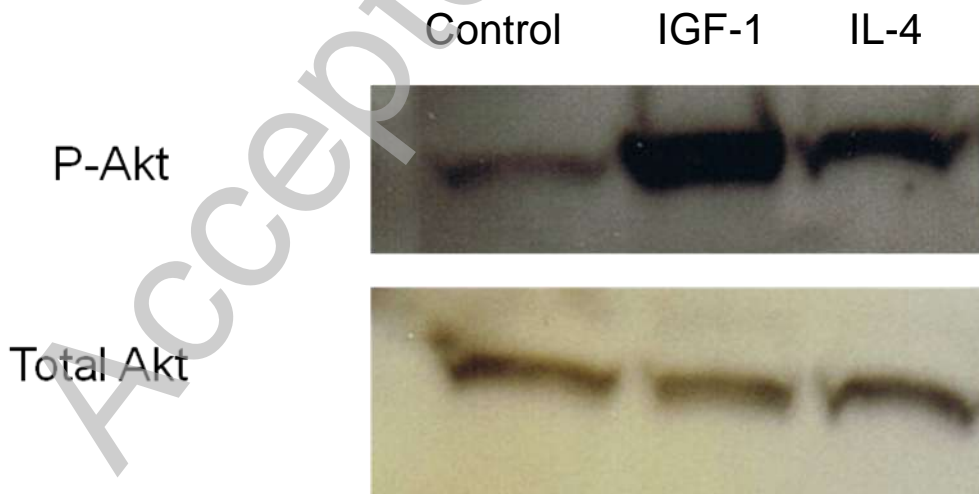
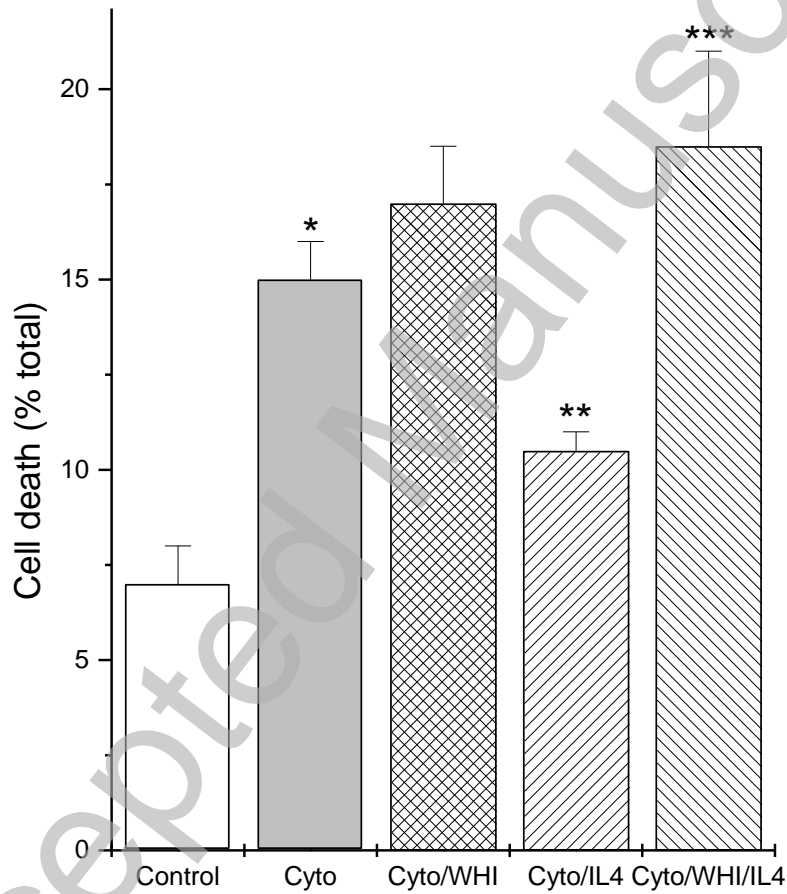


Figure 3



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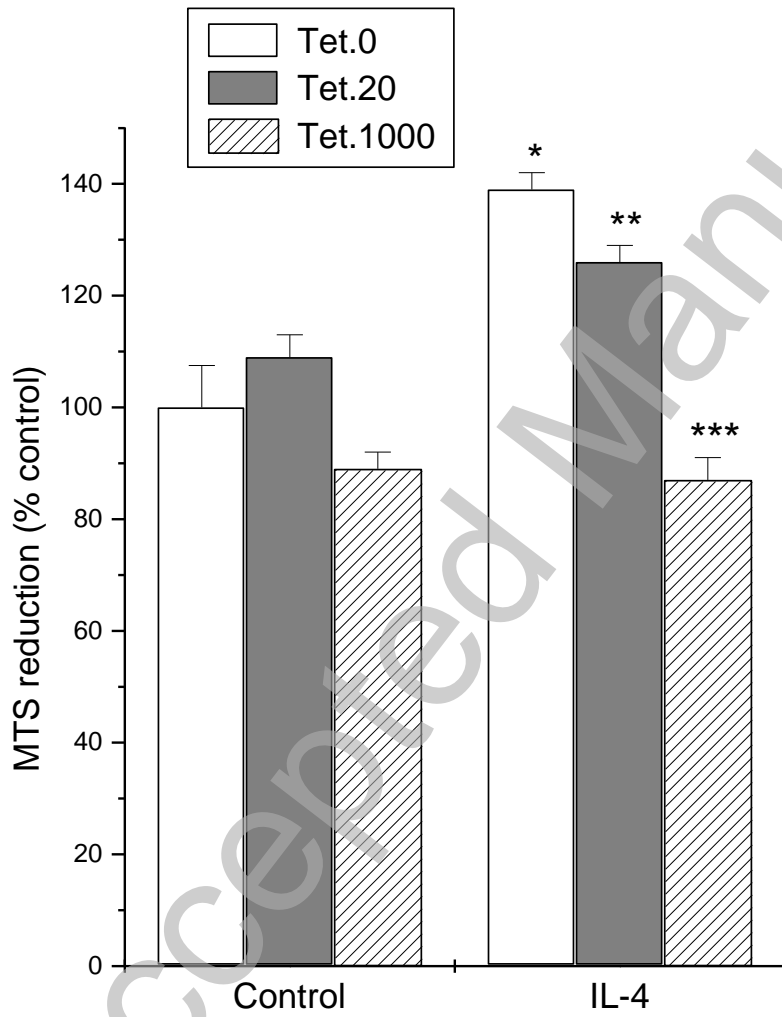
Figure 4



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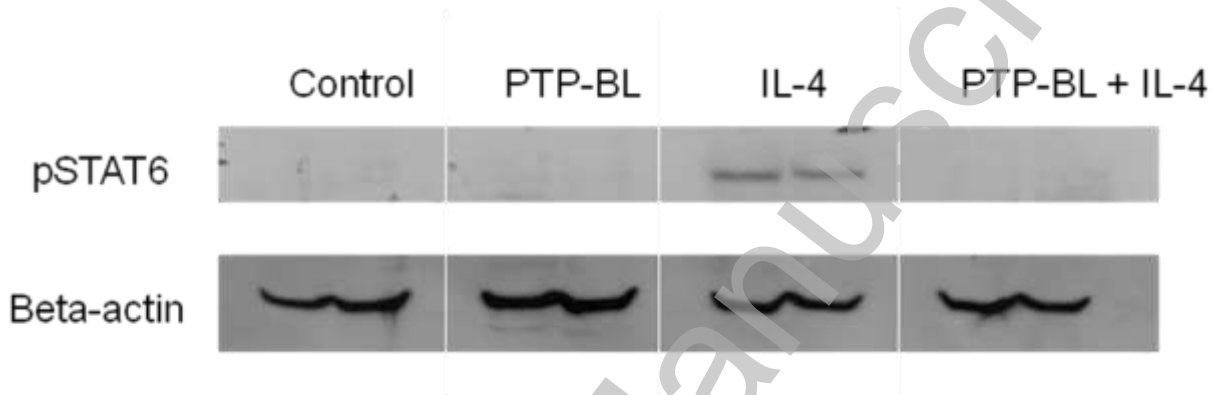
Figure 5



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Figure 6



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