SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 6 (STAT6) AS A REGULATOR OF PANCREATIC BETA CELL HEALTH

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Declaration

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Abstract

The incidence of type 1 diabetes (T1DM) is increasing annually and the disease pathophysiology remains to be completely understood. The current understanding suggests a complex interplay of genetic factors, environmental factors and the immune system that ultimately converge to cause selective destruction of the pancreatic beta cells. As part of this process immune cells (e.g. cytotoxic T-cells), infiltrate the islets and release pro-inflammatory cytokines (e.g. IL-1β, TNF-α and IFN-γ) which cause beta cell dysfunction and cell death. Increasing evidence suggests that there is an imbalance between the levels of pro-inflammatory and anti-inflammatory cytokines at the islet site in T1DM and this may contribute to beta cell death. Anti-inflammatory cytokines (e.g. IL-4, IL-13 and IL-10) are reported to be protective in certain animal models of the disease, but their roles in pancreatic beta cells have been less extensively studied.

The work in this thesis primarily examines the pathway stimulated by the anti-inflammatory cytokines IL-13 and IL-4 and its role in mediating the cytoprotectivity in beta cells. Particularly, we examine whether STAT6 is central to these protective effects.

It was confirmed that STAT6 plays a crucial role in IL-4 and IL-13 mediated protection of beta cells against a range of cytotoxic stimuli (pro-inflammatory cytokines, palmitate and serum withdrawal). Additionally, It was shown that IL-13 and IL-4 induce the upregulation of anti-apoptotic genes such as *MCL1, BCLXL* and *SOCS1,* in a STAT6 regulated manner in beta cells. Surprisingly, we observed the STAT6-dependent upregulation of a gene not previously

associated with this pathway, *SIRPα.* This was further investigated as a regulator of beta cell viability and the results revealed that knockdown of *SIRPα* resulted in beta cell apoptosis, implicating this protein as a novel regulator of beta cell viability.

Taken together, this work shows that loss of STAT6 renders beta cells vulnerable to cell death and suggest that therapeutic targeting of this pathway may offer a potential treatment for T1DM.

Table of content

Lists of Figures

List of Tables

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Abbreviations

ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCL2L1	B-cell lymphoma 2 like 1
BSA	Bovine serum albumin
CD	Cluster of differentiation
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
FFPE	Formalin-fixed paraffin embedded
GWAS	Genome-wide association study
HRP	Horseradish peroxidase
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL	Interleukin
JAK	Janus Kinase
LB	Luria Broth
MCL-1	Myeloid leukaemia cell differentiation protein
MHC	Major histocompatibility complex
MODY	Mature onset diabetes of the young
mRNA	Messenger RNA

NO	Nitric oxide
NOD	Non-obese diabetic
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium iodide
PI-3K	Phosphoinositide-3 Kinase
PVDF	Polyvinylidene difluoride
RISC	RNAi inducing silencing complex
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering RNA
SIRPα	Signal regulatory protein alpha
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TBST	Tris-buffered saline +tween
TNF	Tumour necrosis factor
TRADD	TNF receptor-associated death domains

Publications from this thesis

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Full papers

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Chapter 1.0: General Introduction

1.0 Introduction

1.1 Diabetes Mellitus

The word “diabetes” was first used in 250 BC by a Greek physician, Aretaeus. Diabetes is a Greek word, which means 'syphon' because the disease usually causes fluid to drain from patients. In this context, Aretaeus noticed that the disease caused patients to pass excessive amounts of urine but It was not until 1674 that Thomas Willis, personal physician to King Charles I, completed the term 'Diabetes Mellitus'; imbued with the Latin word “mellitus” meaning honey (Dean and McEntyre, 2004).

Diabetes mellitus describes an assemblage of metabolic disorders arising from defects in the control of insulin secretion or in responses to the hormone causing hyperglycaemia (American Diabetes Association, 2010). Two distinct forms of the disease have been resolved and were initially accredited to a French scientist, Lancereaux, although this distinction was earlier recognised by a British scientist, called Harley (1866). In one of the subtypes, it was observed that patients were obese and in the other, patients were lean (Lancereaux, 1880). These have since been named type 1 (occurring in lean persons) and type 2 (typically in obese persons) diabetes mellitus. We know now that type 1 diabetes mellitus (T1DM) arises from a significant loss of insulin secretion requiring administration of exogenous insulin, whereas type 2 diabetes mellitus (T2DM) is more prevalent and, arises from a complex combination of insufficient insulin secretion by beta cells and peripheral insulin resistance. T2DM in some individuals can be reversed by lifestyle changes such as exercise and reducing intake of glucose-rich meals, without a requirement for exogenous insulin. Recent findings suggest that the

classification of diabetes needs to be more complex although the vast majority of the pathology falls in these two broad groups (American Diabetes Association, 2010). Other forms of diabetes have been established and will be discussed later in the chapter.

According to the World Health Organisation (WHO, 2016 global report on diabetes), an estimated 422 million adults were living with diabetes in 2014 and the number is estimated to rise to 642 million by 2040. Diabetes UK estimates that approximately 4.7 million people live with diabetes in the UK (Diabetes, 2019) (https://www.diabetes.org.uk/about_us/news/new-stats-people-living-with-diabetes). It is suggested that the increase in the global diabetes burden will mostly affect countries experiencing the economic transition from low to middle-income levels and that the prevalence is underreported in these settings (Yach et al., 2004). T2DM makes up 90% of all diabetes and type 1 diabetes mellitus with other forms of diabetes make up the remaining 10% (Zheng et al., 2017).

1.1.1 Type 2 Diabetes Mellitus (T2DM)

T2DM (formally referred to as non-insulin dependent diabetes mellitus) is the most common form of diabetes, and is usually associated with hyperglycaemia due to insulin resistance and may require insulin therapy as a last resort. Insulin resistance is defined as the inability of target tissues such as liver and muscle to respond appropriately to insulin stimulation (Freeman and Pennings, 2018). The rise in the number of people with T2DM is largely attributed to the increase in the numbers of obese people worldwide including children and is suggested to be the main cause that is driving the increased incidence of T2DM. The disease is diagnosed using serological techniques in which either

random plasma glucose, fasting blood glucose or oral glucose tolerance tests are measured from blood samples. In these tests a random blood sample, a fasting blood sample (12h fasting) or blood sample after a 2h glucose challenge administered orally or intravenously, is used to measure blood glucose levels. Alternatively, glycated haemoglobin measurements can be used to indicate the average blood glucose over the previous three months. Glycated or glycosylated haemoglobin is formed by a non-enzymatic reaction between glucose and haemoglobin at the N-terminal valine of either the beta chain or alpha chain of the haemoglobin molecule (Makris and Spanou, 2011). A random blood glucose test which detects ≥7mmol/L (normal values 5.6-6.9mmol/L), a fasting blood glucose test of ≥7mmol/L or a 2h glucose tolerance test (following either oral or intravenous administration of 75g glucose) reporting values of ≥11.1mmol/L supports the diagnosis of diabetes (Kahn et al., 2014). Additionally, a glycated haemoglobin concentration ≥48mmol/mol (or ≥6.5%) may also indicate that an individual is diabetic, however this is not a strict rule since certain individuals from South East Asian or African background differ greatly in their values of glycated haemoglobin due to genetic factors enriched in these populations (Canadian Diabetes Association, 2013).

The combined effects of insulin resistance in target tissues and beta cell dysfunction result in beta cells being unable to match the metabolic demand of the body for appropriate levels of insulin in response to rising blood glucose (Kahn et al., 2014). Beta cell dysfunction in T2DM follows from a plethora of factors, which include obesity, an individual's genetic susceptibility and epigenetics, environmental factors and alterations within the immune system.

1.1.1.1 Lifestyle and obesity

Obesity is said to be the most blamed factor responsible for insulin resistance but a combination of genetic, and environmental factors also play a role (Eguchi and Manabe, 2013). Obesity is a disease that can be defined as the excess accumulation of fat or adipose tissue leading to an increased in body weight (≥ 20% of the ideal weight) (Hruby and Hu, 2015). The body mass index (BMI) is commonly used to determine obesity in an individual; it is a ratio of the weight in kilograms and the square of the height in metres. An individual is classed obese if they have a BMI of ≥30kg/m^2 (Ofei, 2005). Lifestyle factors that have been implicated in the development of obesity or overweight include excessive consumption of alcohol, physical inactivity, and high calorific intake. Nutritional factors or dietary risk factors for T2DM include processed foods, potatoes, trans-fatty acids, irregular eating habits, consumption of white rice, beverages with high sugar content and unsaturated fats (Ayala et al., 2008, McNaughton et al., 2008). On the contrary, brown rice, zinc, vitamin D, dietary fibre, dairy produce, vegetables, peanut butter and healthy food habits appear to reduce the risk of T2DM (Steyn et al., 2004). Being obese contributes about 55% to the disease aetiology, although a proportion of lean people develop diabetes and many obese people do not. Currently, about 80-95% of white Europeans with type 2 diabetes are obese and clinical trial interventions to reduce obesity through weight loss or dieting and exercise can reverse diabetes (Franks, 2012, Lean et al., 2018, Steven et al., 2016). Obesity induces a chronic low-grade inflammation in tissues, which can lead to the increased release of adipokines and pro-inflammatory cytokines such as TNF-α and IL-1β. These cytokines affect lipid metabolism detrimentally leading to

an increase in circulating free fatty acids and a decrease in overall insulin sensitivity (Boden, 2008).

Aside from these, an age-associated decrease in beta cell response, insulin sensitivity, and glucose intolerance have also been established (Karakelides et al., 2010). This age-association might be attributed to sarcopenia since older people tend to be less active (Kirkman et al., 2012). Additionally, there is decreased islet proliferation and increased sensitivity of beta cells to apoptosis with age due to an increase in the expression of the death receptor Fas (Maedler et al., 2006). All of the above probably contribute to beta cell dysfunction and a reduction in beta cell mass.

The emphasis on lifestyle cannot be overstated (Kolb and Martin, 2017), however, there are also genetic and environmental components that cumulate in the dysfunctional beta cells. For example, beta cell dysfunction has been suggested to be caused by glucolipotoxicity and amyloid deposition in the islet (Porte and Diabetes, 2001).

1.1.1.2 Genetic and epigenetic factors associated with T2DM

More than 175 genetic loci have been associated with T2DM most of which are highly linked to beta cell function (Grarup et al., 2014). It is estimated that an individual's risk of developing diabetes (if one parent is diabetic) is 40% and this risk increases to 70% if both parents have T2DM (Ali, 2013). In support of this, twin studies have shown a concordance rate of 70% in monozygotic twins and 20-30% in dizygotic twins (Kaprio et al., 1992). Single-nucleotide polymorphisms (SNPs) in genes controlling insulin synthesis and secretion (such as transcription factor 7-like 2 (*TCF7L2*) which was discovered by linkage studies and GWAS) have been reported to increase the susceptibility

of an individual to T2DM. *TCF7L2* is a transcription factor that influences Wnt signalling which is known to modulate proinsulin synthesis and glucose stimulated insulin secretion in beta cells (Zhou et al., 2014). Other genes associated with T2DM include peroxisome proliferator-activated receptor (*PPARG*), insulin receptor substrate 1 and 2 (*IRS1* and *IRS2*), inwardly-rectifying potassium voltage-gated channel subfamily J 11 (*KCNJ11*), hepatic nuclear factor 1 alpha (*HNF1A)*,hepatocyte nuclear factor 1-beta (*HNF1B)* and hepatocyte nuclear factor 4 alpha (*HNF4A)* and Wolfram syndrome 1 (WFS1), fat-mass and obesity-associated gene (*FTO)* (Ali, 2013). Although these genes have been identified, not everyone with the susceptibility alleles develops diabetes and only about 20% of diabetes is linked directly to heritability (Prasad and Groop, 2015).

T2DM risk can also be conveyed by epigenetic changes caused predominantly by environmental factors such as intrauterine malnutrition or intrauterine hyperglycaemia (Kwak and Park, 2016). Epigenetic changes refer to changes in gene function (upregulation or silencing of a gene) without nucleotide alterations usually due to methylation of specific bases, and histone modifications (Halban et al., 2014, Kwak and Park, 2016).

Evidence of epigenetic changes have been found in T2DM including in genes influencing beta cell function and survival such as peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1),* insulin (*INS),* pancreatic and duodenal homebox 1 (*PDX1),* suppressor of cytokine signalling 2 *(SOCS2),* aristaless related homebox1 *(ARX),* and histone deacetylase 7 *(HDAC7)* (Davegårdh et al., 2018). In a study using islets from individuals with T2DM, hyper DNA methylation of the insulin promoter negatively regulated the

INS gene expression and glucose stimulated insulin secretion compared to control donors (Yang et al., 2011). Similarly, islets from individuals with T2DM have been reported (Yang et al., 2012) to have methylation at CpG sites of the *PDX1* promoter and enhancer regions which correlated with *PDX1* expression. *Pdx1* is a key transcription factor that is essential for beta cell development, function and survival. Loss of this transcription factor has been shown in *pdx1* null mice to cause pancreatic agenesis (Fujimoto and Polonsky, 2009).

In some studies, researchers found differences in DNA methylation levels in the islet beta cells when compared to peripheral blood cells. Most of these studies could not be replicated *in vitro* by stimulating beta cells with high glucose (Yang et al., 2011, Yang et al., 2012), raising the question as to whether DNA methylation is a cause or consequence of hyperglycaemia.

1.1.1.3 Environmental factors in T2DM

It is now established that certain environmental factors can induce epigenetic changes linked to diseases, implying that the environmental effects interact to regulate cellular responses. Exposure to land and air pollutants such as exhaust fumes and herbicides have been associated with up to a 20% increased risk of developing T2DM (Dendup et al., 2018). Many pollutants are lipophilic and thus obese individuals tend to store more toxic pollutants in their adipose tissue (Zeliger, 2013). An example of one such pollutant is atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) a herbicide, which has been shown to promote mitochondrial dysfunction, insulin resistance and obesity (Murea et al., 2012). Atrazine was shown to change mitochondria structure and function by decreasing the oxygen consumption rate through the inhibition of a key mitochondria complex I & II enzymes (Lim et al., 2009). In

this same study, mice exposed to atrazine significantly gained visceral fat irrespective of whether or not they were on a high fat diet. Insulin resistance was induced in these mice through inhibition of the insulin-Akt signalling pathway (Lim et al., 2009). In this context, a recent systematic review associated higher levels of walkability (a measure of how friendly an area is to walk) and green space with a lower risk of developing T2DM. This same review showed that increased levels of noise pollution and air pollution were associated with a greater risk of developing T2DM (Dendup et al., 2018).

1.1.1.4 Immune system

The involvement of the immune system in T2DM almost always comes as a complication arising from hyperglycaemia or obesity (Spranger et al., 2003). A number of studies have shown an increase in the levels of circulating TNF-α in the blood of persons with T2DM compared to controls (Spranger et al., 2003, Chen et al., 2013). It has also been shown that increased glucose and TNF-α have an additive effect by amplifying NF-kB activation and hence exacerbating inflammation through the generation of reactive oxygen species (Iwasaki et al., 2007). Other cytokines that are dysregulated in T2DM include IL-1β, and IL-6 (Spranger et al., 2003). The increase in cytokine release seems to be a consequence of oxidative stress resulting from hyperglycaemia and the release of obesity-induced adipokines. This however, is more likely to be a consequence for the complications in the disease rather than being the main cause of the disease (Graves and Kayal, 2008).

1.1.2 Other forms of Diabetes

1.1.2.1 Gestational diabetes

Glucose intolerance can arise during pregnancy, in which case it is termed gestational diabetes and occurs in 5% of women during the second or third trimester of pregnancy. Although it resolves immediately after delivery in most cases, the development of gestational diabetes increases the risk of developing T2DM later in life, especially in obese women (Gilmartin et al., 2008). Up to 40% of women who were diagnosed with gestational diabetes develop T2DM after 10years of follow-up (Kaaja and Rönnemaa, 2008). The disease may stem from a variety of factors such as increases in placental lactogen secretion, insulin secretion, and growth hormone and insulin resistance (Kampmann, 2015, Kaaja and Rönnemaa, 2008). Together these re-orient maternal metabolism towards a lipid dependent metabolism in order to favour glucose reaching the foetus. Consequently, the profile of maternal inflammatory cytokines (TNF-α, IL-1β, and IL-6) increases and together with increased blood glucose can cause further deterioration of insulin sensitivity (Baz et al., 2016). Gestational diabetes is tested by performing an oral glucose tolerance test at the beginning of the third trimester (24-28 weeks) at least twice and is positive if the results are ≥7.2mmol/L in both tests (Kampmann, 2015, Rani, 2016). Gestational diabetes can be managed by pre-conception counselling, exercise and diet (reduction in daily caloric intake) during pregnancy, as well as the use of oral hypoglycaemics such as metformin, and Glibenclamide (Anwar et al., 2018).

1.1.2.2 Maturity Onset Diabetes of the Young (MODY)

Another form of diabetes that is non-insulin dependent but differs from T2DM, is Maturity Onset Diabetes of the Young (MODY) which is reported to make up approximately 1.2-3% of diabetes diagnosed in children (Hattersley and Patel, 2017). However, MODY is often misdiagnosed as T1DM thus the prevalence may be under represented (Ledermann, 1995). MODY is a group of heterogeneous monogenic inheritable diabetes that result from mutations in transcription factors and enzymes that modulate beta cell maturation and function (Anık et al., 2015). Most patients present with autosomal dominant inheritance by the age of 25. The clinical features of these patients are variable depending on the mutation causing the disease. At least 13 gene mutations have been associated with the disease (Chambers et al., 2016). The most common mutations described lie in the coding regions of genes encoding hepatocyte nuclear factor 1α (*HNF1*α), hepatocyte nuclear factor 4α (HNF4α) and glucokinase (GCK), and these account for 52%, 10% and 32% of MODY cases in the UK respectively (Thanabalasingham and Owen, 2011). Other genes include *HNF1B, INS, PDX1,* paired box 4 (*PAX4),* neuronal differentiation 1 *(NEUROD1), KCNJ11* and ATP binding cassette subfamily C member 8 (*ABCC8),* but their incidence is less common (Thanabalasingham and Owen, 2011, Gardner and Tai, 2012). The misdiagnosis of MODY as T1DM has a massive impact on treatment. Given the correct diagnosis, MODY patients can often be effectively treated with anti-hyperglycaemic drugs which are preferable to exogenous insulin administration (Boyd and Byrne, 2018).

1.1.2.3 Latent Autoimmune Diabetes of Adulthood (LADA)

Latent autoimmune diabetes of adulthood (LADA) was first reported in 1986 in a group of individuals previously diagnosed with T2DM. These persons

present with more typical features of T1DM such as the production of islet autoantibodies but they also had preserved beta cell function (Groop et al., 1986). Due to the challenges of classifying LADA, there is controversy over its existence: the only difference between LADA and T1DM is the dependence on insulin in T1DM individuals (Laugesen et al., 2015). Since in the past T1DM was mostly diagnosed in the young, and now at any age, it is argued that LADA is simply T1DM of the old (Thomas et al., 2018). LADA makes up approximately 10% of type 2 diabetes individuals between the ages of 35-75 years. Individuals diagnosed with LADA and with autoantibodies experience beta cell failure within 5 years and those without autoantibodies after around 12 years. LADA patients have dietary treatment similar to those of T1DM individuals as well as Metformin. The use of exogenous insulin typically follows beta cell dysfunction (Stenström et al., 2005).

1.1.2.4 Neonatal Diabetes Mellitus (NDM)

Neonatal diabetes mellitus (NDM) is a rare genetic form of diabetes that occurs in children below the age of 6 months and with incidence rates of 1 in 100 000 (Hattersley and Patel, 2017). There exist two clinical forms of NDM, permanent NDM (PNDM) which requires lifelong exogenous administration of insulin, and transient NDM (TNDM) which is sporadic sometimes recurring in an individual's twenties or thirties (Marin et al., 2016, Aguilar-Bryan and Bryan, 2008). These two forms can only be distinguished by genetic analysis. The genetic causes of more than 90% of TNDM are now known and over 68% of these cases are due to abnormalities in the imprinted region of chromosome 6q24. The rest are due to mutations in the *KCNJ11* and *ABCC8* genes (Sood et al., 2017, Marin et al., 2016). PNDM is mostly due to mutations in the *KCNJ11*, *ABCC8* and the *INS* genes (Marin et al., 2016), with KCNJ11 and

ACC8 accounting for 50% of cases (Hattersley and Patel, 2017). NDMs are generally managed by the use of exogenous insulin and sulfonylurea in order to improve neurodevelopment (Marin et al., 2016).

1.1.3 Type 1 Diabetes Mellitus (T1DM)

The main focus of this thesis relates to type 1 diabetes mellitus (T1DM) rather than the other types described above. T1DM is an autoimmune disease in which there is selective destruction of the pancreatic beta cells of the islets of Langerhans. This leads to lifelong exogenous insulin dependence to control blood glucose (Insel et al., 2015). Beta cell destruction usually occurs during a subclinical period in which insulin-containing beta cells are selectively destroyed by the immune system in persons that are genetically susceptible. In the past, T1DM was mainly diagnosed in children hence it acquired the name Juvenile-onset diabetes although it can be diagnosed at any age (Atkinson et al., 2013, Thomas et al., 2018).

It is estimated that approximately 1,106,500 children under the age of 20 live with type 1 diabetes worldwide (Snouffer, 2018). More than 30 000 children in the UK live with the disorder and trends suggest that the disease incidence is steadily increasing in many European countries and the world at large (Patterson et al., 2009, Patterson et al., 2018). However, the reason for this increase in T1DM incidence is not clear.

In a similar manner to T2DM, the disease is diagnosed serologically by measuring fasting blood glucose concentration (≥7.0mmol/L positive), random blood glucose (≥11.1mmol/L means diabetic) or oral glucose tolerance tests. Glycated haemoglobin levels above 48mmol/mol can be used but this test is usually less sensitive since dysglycemia is rapid in T1DM (DiMeglio et al.,

2018). A combination of the serological tests above, and the clinical symptoms are most commonly used for diagnosis, hence 50% of adults with new-onset T1DM tend to be misdiagnosed as T2DM (DiMeglio et al., 2018). This has important implications since the treatments used for these two diseases are different. During insulin biosynthesis insulin is first synthesized as preproinsulin. A signal peptide is cleaved in the ER to give proinsulin. Proinsulin is then cleaved to yield active insulin in vesicles from where it is released with the other cleaved component, c-peptide in equimolar quantities. C-peptide levels can be useful for disease diagnosis; since very low concentrations indicate severe beta cell assault. C-peptide concentrations (normal range: 0.26-1.03nmol/L) can be a sensitive measure of beta cell function but should be used for definitive diagnosis in combination with other tests (Yi et al., 2018). The gold standard for distinguishing type 1 diabetes from the others, is a serological measurement of two or more auto-antibodies (which recognise beta cell specific proteins). These are normally present in more than 90% of patients at diagnosis and indicate ongoing disease pathophysiology.

There are five main islet autoantibodies which have been identified to date, these are Islet cell autoantibodies (ICA), Insulin autoantibodies (IAA), antibodies raised against glutamic acid decarboxylase 65 (GAD65), protein tyrosine phosphatase related antigen 2 molecule (IA-2A) and zinc transporter 8 autoantibody (ZnT8) (Törn et al., 2008, Lampasona et al., 2011, Schlosser et al., 2011). Recently, a 38kDa glycoprotein autoantigen was identified as tetraspanin-7 was also shown to be a target of islet autoantibodies in T1DM (McLaughlin et al., 2016).The functions of these antigens are on Table 1.

Table 1: Functions of autoantigens in T1DM

Antigen	Function	Reference
GAD65	An enzyme essential in gamma-aminobutyric acid (GABA) biosynthesis which is an inhibitor of neurotransmission in the central nervous system with paracrine functions. Its function in the islet is not known but local paracrine effects on glucagon secretion are suggested	(Lampasona and Liberati, 2016, Wendt et al., 2004)
IA-2A	A protein tyrosine phosphatase-like protein promotes mobilisation of secretory granules to the plasma membrane	(Mziaut et al., 2008)
ZnT8	Regulates Zinc content in beta cells. Stabilizes insulin crystals in secretory vesicles	(Lemaire et al., 2012)
IAA	Insulin is triggers glycogenesis hence responsible for glucose homoeostasis	(Lampasona and Liberati, 2016)
ICA	Non-specific antigens on the islets	(Lampasona and Liberati, 2016)
Tetraspanin 7	Function as a molecular scaffold and facilitate signalling on receptors such as EGFR	(Termini and Gillette, 2017)

A number of factors have been identified that may promote the development of T1DM, although the explanation as to how they lead to the disease remains a conundrum. Both genetic and environmental factors have been implicated and possibly combine to provoke the immune system into attacking pancreatic beta cells in a selective manner (Roep and Tree, 2014, Russell and Morgan, 2014). In the subsequent sections, various factors associated with the development of T1DM will be examined.

1.1.3.1 Genetic factors

The evidence that genetic factors play a role in T1DM arises from the observation that a significant number of individuals with the disorder have

close relatives with T1DM. There is a 15 fold higher risk of developing T1DM among siblings of a person with T1DM compared to the general population. Additionally, the risk is 3% if the mother has T1DM individual and 5% if the father has T1DM (Pociot and Lernmark, 2016). There is also a greater than 50% chance of concordant development of T1DM in monozygotic twins (Steck and Rewers, 2011). Together, these data reveal a significant genetic component to the disease.

The human leukocyte antigen- DQ (HLA-DQ) locus accounts for about a half of the most important susceptibility genes in T1DM, however, an additional 50 non-HLA genes also contribute to the risk of T1DM (Knip and Simell, 2012). The HLA class II region on chromosome 6p21 (precisely loci HLA-DRB1 and HLA-DQB1) is known to exert the highest T1DM risk, with the alleles DR3-DQA1*0501-DQB1*0201 (DR3) and DR4-DQA1*0301-DQB1*0302 (DR4) accounting for 30-50% of the genetic T1DM risk (Steck and Rewers, 2011). At least one of these alleles is present in 95% of all T1DM patients in the US (Aly et al., 2006). Although the HLA-DR/DQ locus confers a high risk of developing T1DM, some haplotypes of these have been reported to be protective such as HLA-DQA1*0102, DQB1*0602 (Steck and Rewers, 2011). Other HLAs associated with T1DM include alleles A24, A30 and HLA I. HLA-B18, B39, B44, C3, C8, and C16. There also exist non-HLA gene markers and currently, more than 50 such markers have been confirmed with the most significant being in *INS* (11q15), interferon induced with helicase C domain 1 (*IFIH1*) (2q24), cytotoxic T-lymphocytes associated protein 4 (*CTLA*-4), protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) and IL2RA (Eizirik et al., 2012).

To understand the genetics of T1DM, two main approaches have been employed. In the first, children born of a first-degree family history of T1DM have been monitored for development the disease. And in the other, new-borns are screened to identify children at increased genetic risk. They are then followed up to detect autoantibodies known to be associated with increased risk for T1DM (Størling and Pociot, 2017, Pociot and Lernmark, 2016). These approaches have helped to record the natural history of T1DM and to categorise the alleles with various risk levels.

There is only a 33% chance of developing the disease in individuals with the highest genetic susceptibility and in conjunction with evidence from pair-wise twin studies, it is acceptable to argue that genetic susceptibility alone is not sufficient to develop T1DM and additional environmental factors are required to trigger the disease (Barnett et al., 1981).

1.1.3.2 Environmental factors as triggers of T1D

A growing number of potential environmental factors have been reported which may act as triggers for T1DM (or at least influence this process). These include demographic, viral infection, early introduction of cow's milk, vitamin D, and the intestinal microbiome. Evidence for each of these factors has been generated to support their involvement (Knip and Simell, 2012). A study by Barnett et al. (1981) and another by Kaprio et al. (1992) suggested that only about 13%-33% of paired monozygotic twins concordantly develop T1DM indicating that other factors influencing the disease exist.

The Geography of T1DM is also an indicator of a possible environmental factor with the highest number of cases recorded in northern Europe. Also, the rate of increase in T1DM over 15 years cannot be explained by genetic factors

alone and points to contributions from the environmental changes (Patterson et al., 2009). In the subsequent sections, the various environmental factors associated with the development of T1DM will be examined.

Viruses

The involvement of viruses in the pathogenesis of T1DM was first suggested over a century ago by Harris (1899). Recently the evidence has become more compelling, with certain enterovirus serotypes receiving the most attention (Morgan and Richardson, 2014, Harris, 1899, Richardson and Morgan, 2018). A number of viruses have been implicated in T1DM including, mumps, cytomegalovirus (CMV), rotavirus, enteroviruses particularly coxsackie virus B (CVB), herpesvirus and rubella virus (Filippi and von Herrath, 2008, Richardson and Horwitz, 2014).

The link between enteroviruses and T1DM have been established by meta-analysis suggesting a clinically significant association (Yeung et al., 2011). Enteroviruses are a group of non-enveloped RNA viruses from the picornavirus family which induce mainly mild and asymptomatic infections in infants and newborns (Knip and Simell, 2012). There are over 100 different serotypes of enteroviruses but the most commonly implicated in T1DM is Coxsackie virus B4 (although B1 to B6 have also been implicated) (Filippi and von Herrath, 2008). Coxsackie B3 has been shown to infect and accelerate insulitis in NOD mice at 8 weeks (Drescher et al., 2004). In the Finnish diabetes protective study, investigators showed that enteroviral infection occurred in susceptible children prior to the appearance of islet auto-antibodies (Lönnrot et al., 2000). This appearance has been deemed seasonal with the highest infections occurring between autumn and winter (Kimpimäki et al., 2001). Evidence of enteroviral infection in the pancreas has been obtained by staining

tissue sections for the viral capsid protein VP1 in children and young adults soon after the diagnosis of T1DM (Richardson et al., 2013, Richardson et al., 2009). It is proposed that the virus gets to the pancreas through the blood (or via the pancreatic duct), and that beta cells are targeted by these viruses because they express an isoform of the Coxsackie-adenovirus receptor (CAR) (Ifie et al., 2018), which enteroviruses can use to gain entry into the cells. The virus might induce beta cell death by cytolysis after replication, although very little evidence of large scale islet cell lysis has been reported in T1DM. Rather, It has been suggested that a persistent form of infection might develop and affect the immune system by upregulation of MHC I or through molecular mimicry (Knip and Simell, 2012, Richardson and Horwitz, 2014).

Other viruses have been implicated in the pathoaetiology of T1DM with the first report dating back to 1899; a case of T1DM after mumps infection (Harris, 1899). An Australian study showed an association of rotavirus infection and the beta cell autoimmunity in T1DM susceptible children (Honeyman et al., 2000), but another Finnish study did not support the same conclusion (Blomqvist et al., 2002). A strong correlation has also been reported between CMV and islet cell autoantibodies in individuals with T1DM implicating persistent CMV infections in T1DM (Pak et al., 1988).

Bacteria

Emerging evidence suggests that the microbiome homeostasis may play a crucial role in the development of metabolic disorders such as type 2 diabetes, dyslipidaemia, non-alcoholic fatty liver disease (Fabbiano et al., 2017), and T1DM. In a report on NOD mice that lacked the Myeloid differentiation primary response 88 (MYD88), (a toll-like receptor adaptor protein) developed diabetes according to the microbial constituents of the intestine (Wen et al., 2008). A

comparative study on the oral microbiota and faecal microbiota of T1DM persons versus controls showed a significant difference in certain species (e.g. *Chirstensenella*) associated with T1DM (de Groot et al., 2017). However, these changes in microbiota seem to be largely due to a change in metabolism and not the cause of the disease. In one report, to illustrate this bariatric surgery to induce weight loss was performed and the gut microbiota of the individuals studied. The results revealed rapid changes in the gut microbiota as an adaptation to a starvation-like situation (Furet et al., 2010).

Dietary factors

A variation in exposure to complex diets at different stages in life such as the early introduction of cow milk, vitamin D, cereals (gluten) and short-term breastfeeding have been implicated in T1DM (Antvorskov et al., 2016). In a breastfeeding study performed involving T1DM susceptible infants, it was observed that infants breastfed for a minimum of 4 months had a lower risk of developing IA-2A autoantibodies when compared to infants breastfed for ≤2 months (Kimpimäki et al., 2001). In another study by the same group, early introduction of cow's milk to T1DM susceptible children was associated with increased risk of seroconversion (Kimpimäki et al., 2001) although others did not find any association in children under the age of 5 (Wadsworth et al., 1997).

Two recently published case reports of T1DM susceptible children have reported remission from T1DM with the use of high doses of vitamin D (1000IU/day) and omega 3 fatty acids (50-60mg/kg/day) (Cadario et al., 2018). Additionally, taking high concentrations of vitamin D in the last trimester of pregnancy was associated with a decreased risk of developing T1DM in susceptible offspring (Virtanen, 2016).

Studies using wheat based feed and gluten free diet performed with NOD mice have suggested a role for gluten in the development of diabetes. NOD mice on a gluten free diet had a reduced incidence of diabetes (15%) when compared to those fed a gluten containing diet (64%) (Funda et al., 1999). In humans, first-degree relatives of T1DM individuals put on a gluten free diet for 6 months followed by a gluten containing diet for a further 6 months had a significantly better insulin response to glucose during the gluten free period compared to when fed the gluten containing diet. This decreased with a return to a gluten containing diet (Pastore et al., 2003). Furthermore, the case study of a 6 year old boy diagnosed with T1DM who was then placed on a gluten free diet revealed better fasting blood glucose levels and no change in GAD65 autoantibody titres providing anecdotal evidence that such a diet might preserve beta cells (Sildorf et al., 2012).

1.1.3.3 The immune system involvement in T1DM

It is established that the immune system plays a key role in T1DM pathogenesis since autoantibodies against beta cell proteins can be readily detected in the blood of patients, and thus are generally used as biomarkers of the disease (Yi et al., 2018). However, studying the pancreas provides much more direct evidence for the role of the immune system in the disease. Unfortunately, studies on the human pancreas are limited by the lack of access to the pancreas due to its retroperitoneal location thus making it hard to study the disease pathogenesis completely (Morgan, 2017).

The NOD mouse model has been used to gain an improved understanding of the disease pathogenesis in T1DM. Pancreatic sections reveal the infiltration of various immune cell subsets (DC, macrophages, natural killer cells, $CD4^+$

and $CD8^+$ T-cells) with similar phenotypes to those found in humans (Pearson et al., 2016). Examination of the human pancreas from patients with T1DM reveals the presence of various immune cells associated with and infiltrating into the islets of Langerhans, termed 'insulitis'. Recently a consensus view was generated on the definition of insulitis which states that three or more islets contain greater than or equal to fifteen CD45+ cells/islet (Vecchio et al., 2018, Leete et al., 2016, Willcox et al., 2009b, Campbell-Thompson et al., 2013). A more detailed assessment of the immune cell subtypes present in insulitic pancreatic islets revealed that CD8+ cytotoxic T-cells were predominant, with lower numbers of CD4+ and $CD20^+$ B-cells. Other studies also noted the occasional presence of macrophages, natural killer cells, dendritic cells and FoxP3 positive cells at the islet site (Willcox et al., 2009b, Rodriguez-Calvo et al., 2014). The infiltrating immune cells are likely mediators of beta cell death in T1DM primarily mediated through the secretion of protein factors from the influent T-cells (cytokines, perforins, granzymes) (Pirot et al., 2008).

1.2 The Pancreas; its Architecture and function

The pancreas is a hammer-shaped organ that lies beneath the stomach, measuring between 14-20cm in length in adult humans and with an average weight of 100g. It develops from the foregut endoderm and appears by the end of the seventh week of gestation. Functionally, the pancreas is responsible for the maintenance of glucose homeostasis and the secretion of pancreatic enzymes (e.g. amylase) for digestion. The pancreas connects to the duodenum via the Ampulla of Vater, which is a duct formed by the union of the bile duct and the pancreatic duct. The pancreas is anatomically divided into the head, which links to the digestive system, the body and the tail which are closest to the spleen (Fig. 1.1). The bulk of the pancreas (85%) is made up of acinar cells responsible for the exocrine functions of the organ with only approximately 2% responsible for the endocrine function (Campbell and Verbeke, 2013, Longnecker, 2014). Blood flows through the portal system first from the endocrine pancreas then into the exocrine capillaries with a high concentration of islet hormones which may exert trophic effects since pancreatic exocrine cells have receptors for hormones such as insulin (Pandol, 2010).

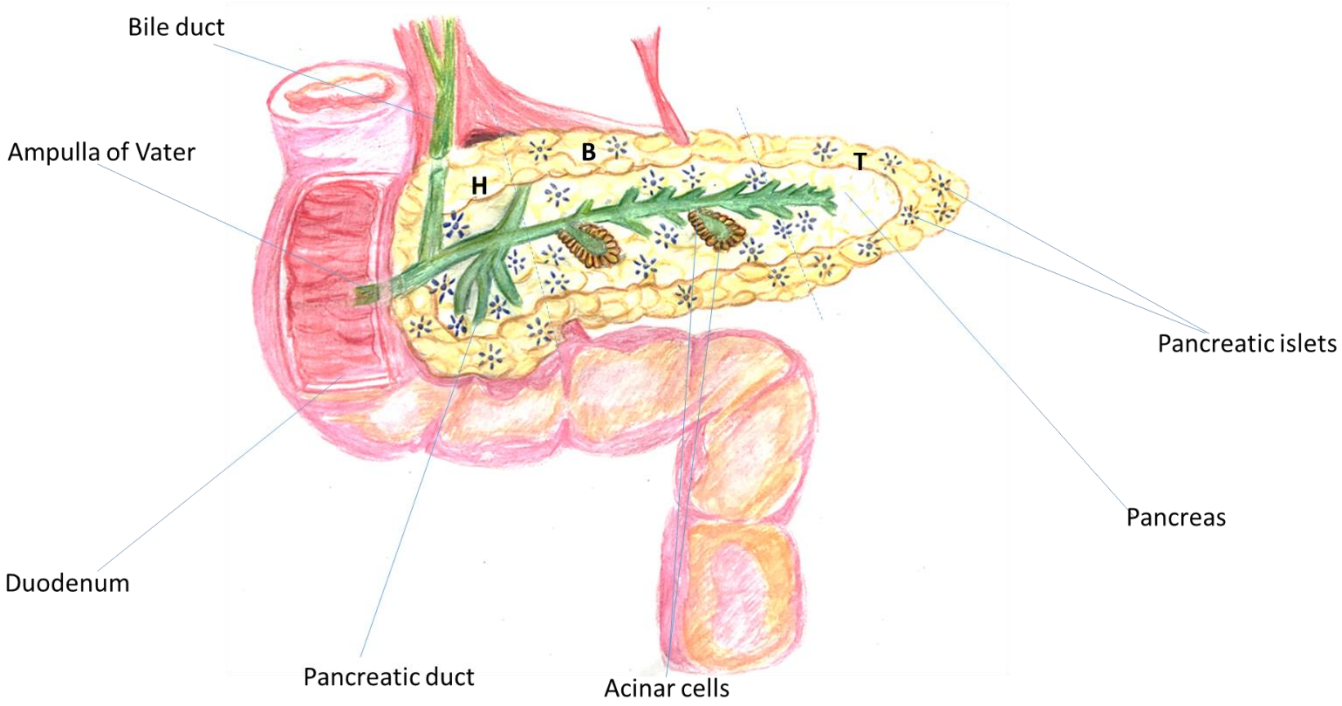


Figure 1. 1: The pancreas and surrounding organs

The pancreas is divided into the pancreatic head (H), body (B) and the tail (T)

Courtesy of *Mrs Nikki Archer*

1.2.1 The Exocrine Pancreas

The exocrine pancreas makes up about 85% of the whole pancreas and is responsible for secretion of digestive enzymes through its ductal system into the duodenum. These enzymes flow with water and ions also secreted by the exocrine cells. The exocrine pancreas is composed mainly of the ductal system, and the lumen which extends from the duodenum to the acinus made up of acinar cells (exocrine cells) which are in berry-shaped clusters (Pandol, 2010). Acinar cells through this ductal system secrete over 20 different enzymes involved in chemical digestion. These include proteases, amylases, hydrolases, lipases, and ribonucleases and each is normally stored in zymogen granules in their proenzymes forms. The enzymes are activated once in the duodenum by an enterokinase, which cleaves a peptide from the N-terminal of the proenzyme. Activated trypsin is a primary target, which then activates other pro-enzymes (α-Amylase, procarboxypeptidase, proelastase, phospholipase, mesotrypsin).

The existence of the exocrine and endocrine pancreas in one organ usually means that inflammatory damage to the exocrine compartment could result in collateral damage to the endocrine compartment and vice versa. In this context, 30% of people with acute pancreatitis display glycosuria and 50% of these have elevated blood glucose although their symptoms resolve upon resolution of pancreatitis. Chronic pancreatitis, however, depending on the pathology can lead to overt diabetes (Gorelick, 1993). Endocrine malfunction can also lead to a pancreatic exocrine insufficiency in more than 50% of T1DM cases (Campbell-Thompson et al., 2015).

1.2.2 The Endocrine pancreas

The endocrine pancreas is made up of the pancreatic islets of Langerhans; these structures are disseminated throughout the pancreas and makeup about 2% of the pancreas volume (10^5-10^6 islets) in a human pancreas. Each pancreatic islet consists of various cell types (endocrine, endothelial, nerve, fibroblast) and is usually about 40-107μm in diameter (Seino and Bell, 2008, Komatsu et al., 2017). Pancreatic islets contain five different endocrine cell types: insulin-secreting beta cells, glucagon-producing alpha cells, pancreatic polypeptide-secreting PP cells, somatostatin secreting delta cells and ghrelin-secreting epsilon cells (Figure 1.2, PP and ε cells have not been stained).

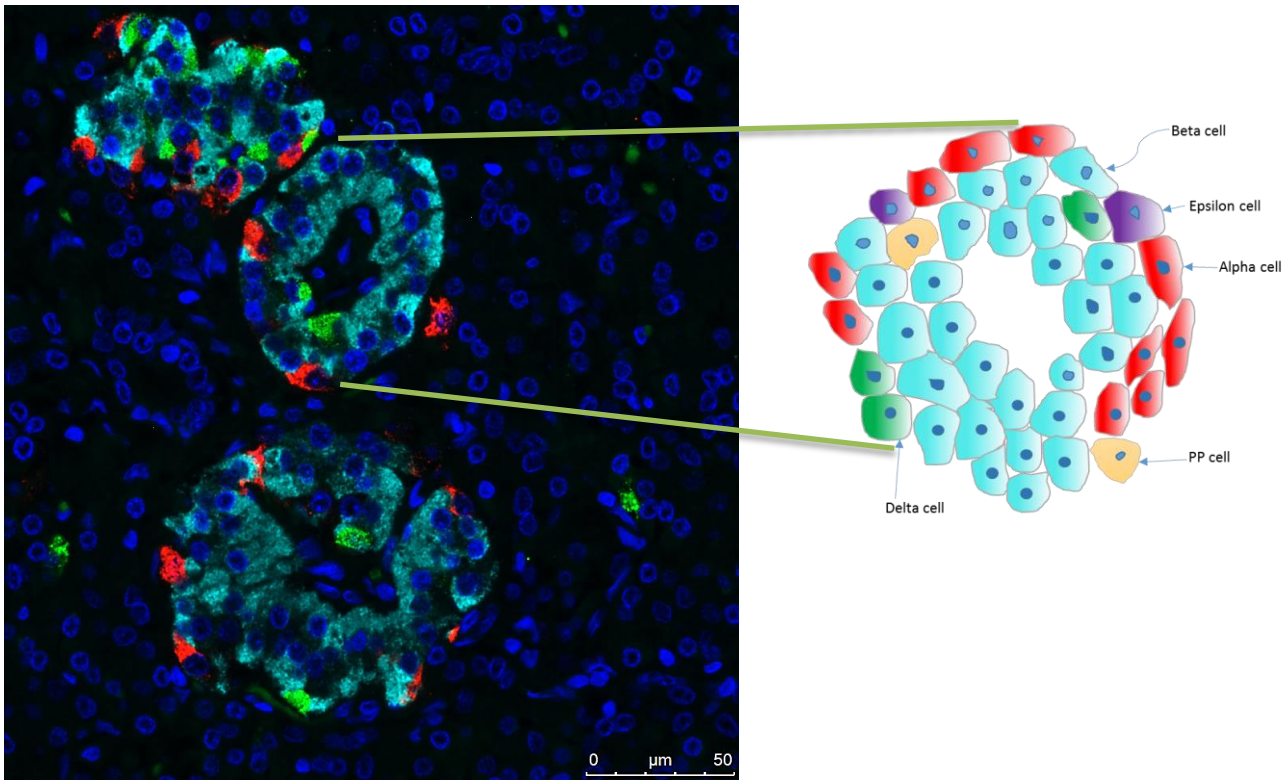


Figure 1.2: Representative image of human pancreas showing immunofluorescent stained alpha (red), beta (cyan) and delta cells (green).

Human FFPE section was cut, placed on a slide, cleared, rehydrated and probed with antisera against insulin (cyan), glucagon (red) and somatostatin (green). Islets were observed under the fluorescent microscope at 20X magnification. Scale bar 550μm.

Courtesy: *Dr Sarah Richardson*

Alpha cells (α cells)

The alpha cells makeup about 30% of the islet cell mass in humans and are responsible for the secretion of glucagon, a hormone which promotes the release of glucose into the blood. Glucagon protects the body against hypoglycaemia by stimulating hepatocytes to produce glucose by initiating glycogenolysis and gluconeogenesis. Glucagon is synthesized from a large precursor proglucagon, which is 178 amino acids in length and is cleaved by prohormone convertase 2 to yield active glucagon (29 amino acids) in alpha cells. The control of glucagon secretion is thought to be both intrinsic (by glucose) and paracrine (somatostatin, insulin and neuronal) although the exact mechanisms are still under debate.

It has been shown that glucagon secretion is predominantly modulated by the prevailing glucose concentration, but it can also be affected by somatostatin and insulin. Briefly, at low glucose concentrations (hypoglycaemia, glucose ≤3.3mmol/L), alpha cell plasma membrane depolarisation as T-type channels open, leading to the activation of voltage-gated Na+ and calcium channels. The Ca^{2+} channels open leading to an increase in intracellular Ca^{2+}. The Increase in intracellular Ca^{2+} causes the migration of secretory granules and their fusion to the cell membrane, and thus the exocytosis of glucagon (Briant et al., 2016, Quesada et al., 2008). It is also suggested that incretins, such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) can suppress glucagon secretion (Xavier, 2018). Furthermore, under certain circumstances, alpha cells may secrete GLP-1, an alternative cleavage product of proglucagon. Under such conditions, this may lead to autocrine effects on glucagon release (Marchetti et al., 2012).

Delta cells (δ cells)

Somatostatin is secreted by delta cells and these cells make up roughly 11% of the human islet (Seino and Bell, 2008). Delta cells are not only present in the pancreatic islet, but in the gastrointestinal tract and, the central nervous system. Somatostatin has a broad inhibitory effect on the secretion of many hormones, including glucagon, and insulin. Somatostatin is synthesized as preprosomatostatin (116 amino acids) which is cleaved into prosomatostatin (92 amino acids) by proconvertase 1. Prosomatostatin is then enzymatically cleaved by proconvertase 2 to yield somatostatin-14(14 amino acids) and somatostatin-28 (28 amino acids) both of which are biologically active. Somatostatin receptors are present on both beta cells and alpha cells hence its actions directly affect these two cell types. Furthermore, somatostatin has been suggested to inhibit PP secretion in addition to its own secretion (Brereton et al., 2015).

Pancreatic Polypeptide-producing (PP) cells

Pancreatic polypeptide-producing cells (PP) also called F-cells were thought to be the smallest population of cells in the human islets (<2%) prior to the discovery of epsilon cells. More than 90% of PP cells are located at the head of the pancreas (Rahier et al., 1983). These cells produce a 36-amino acid peptide pancreatic polypeptide, which is released postprandially upon stimulation by the vagus nerve. Alternatively, secretion can be stimulated by other factors such as arginine. Pancreatic polypeptide is known to act directly in the gut where it reduces gastric secretion and the motor activities of the intestines. Surprisingly, our current understanding suggests that this hormone has a limited impact on other islet cells. That said, It has been shown to inhibit glucagon secretion at low glucose concentrations but no effects on insulin

release have been reported (Xavier, 2018). These cells have received limited attention compared to pancreatic beta cells.

Epsilon cells (ε cells)

Epsilon cells make up the smallest population of islet cells (about 1% in adult human islet) and secrete the hormone ghrelin. Ghrelin is known to inhibit insulin secretion from rat, mouse and human beta cells and hence it acts as a paracrine inhibitor of insulin in the islet, similar to somatostatin. The major sources of ghrelin are the pancreas and stomach (Wierup et al., 2014). Ghrelin has been identified in the hypothalamus precisely in the arcuate nucleus where it is suggested to regulate appetite leading to increases in food intake, a decrease in energy use, all this cumulating to body weight gain (Sato et al., 2012).

In diabetes research, the central focus has been on the beta cell due to the obvious dysregulation in insulin function and action, however, other islet cells have been implicated in the disease pathogenesis. For example, it has been reported that alpha cells increase in mass in T1DM and hyper secretion of glucagon (hyperglucagonemia) is a common feature of T1DM. Similar to alpha cells, the number of delta and PP cells have also been shown to increase in both T1DM and T2DM (Brereton et al., 2015). The potential sources of these endocrine cell types in diabetes are neogenesis of progenitor cells, self-replication to replenish the pancreatic islet volume, and transdifferentiation from other endocrine cells or exocrine acinar cells (Corritore et al., 2016).

Beta cells (β cell)

The beta cells are the most abundant and the most studied of all the islet cells and makeup about 60% of the islet population in human. By three weeks of

gestation, the first endocrine cells to appear are the beta cells, these are then followed by the alpha cells (at 8weeks), then the delta cells and then the epsilon cells (at week 9). PP cells begin to appear at about week 17 (Jeon et al., 2009). The primary function of beta cells is the synthesis and secretion of insulin (Xavier, 2018, Seino and Bell, 2008). Many metabolites (amino acids, free fatty acids, and ketones), neurotransmitters (acetylcholine and norepinephrine) and hormones (GLP-1, GIP, Glucagon, Gastrin, Secretin, CCK and PACAP) induce insulin secretion but the most important is glucose. Glucose induces and amplifies insulin secretion.

The secreted form of insulin has 51aa, although insulin is initially translated as a 110aa protein known as preproinsulin. Preproinsulin has a hydrophobic N-terminal 24aa residue signal peptide which interacts with a signal recognition particle (ribonucleoprotein particle) in the cytosol. The nascent preproinsulin is translocated into the lumen of the rough endoplasmic reticulum (RER). Signal peptidases on the inner surface of the RER then cleave the signal sequence to form proinsulin. Following this event, the signal sequence is degraded rapidly. Under the catalysis of enzymes and associated chaperone proteins such as protein-thiol reductase, proinsulin is folded and three disulphide bonds are formed within the molecule. In a rate-limiting step, proinsulin is transported to the Golgi apparatus where it is packaged into immature secretory vesicles. In these vesicles, proinsulin is cleaved by trypsin-like enzymes PC1/3 and PC2 into mature insulin and a 31aa residue connecting peptide (C-peptide) (Seino and Bell, 2008, Fu et al., 2013).

Glucose enters the beta cells through a facilitated form of transport via the glucose transporters, GLUT1 and GLUT2. Once glucose enters it becomes

phosphorylated by glucokinase to form glucose-6-phosphate (G6P) to initiate glycolysis. Metabolism via glycolysis yields pyruvate which is converted to acetyl-CoA catalysed by pyruvate dehydrogenase. Acetyl-CoA then enters the tricarboxylic acid cycle in which ATP is produced and transported to the cytoplasm. An increase in cytoplasmic ATP causes the closure of ATP sensitive potassium channels, which prevents K+ efflux from the cell and induces depolarisation of the plasma membrane. This event promotes the opening of the L-type voltage-dependent Ca^{2+} channels leading to an influx of Ca^{2+} into the cytosol. Ca^{2+} influx causes insulin secretory granules to move towards the plasma membrane where they dock and fuse for exocytosis of insulin to occur (Marchetti et al., 2017b).

Since the thesis focuses on the role of STAT6 in beta cells, I will expand more on beta cell models and some advantages and disadvantages.

Beta cell study models

The study of beta cells has been limited by the accessibility and availability of pancreatic tissue (Morgan, 2017). Primary beta cells obtained from individuals are rare to find and usually undergo various treatments to isolate beta cells, which changes their functionality and potentially skew experiments. Additionally, maintaining these primary cells out of their *in vivo* habitat can be laborious and will require the technical expertise, hence research on beta cells is mostly dependent on cell lines and animal models (Skelin et al., 2010).

The use of cell lines is advantageous in that experiments performed with these are generally reproducible over a limited number of passages. Cell lines can also be transfected and manipulated to investigate gene expression or the cytotoxicity of different agents (Ulrich et al., 2002). Many different rodent cell

lines derived from virus or radiation induced insulinomas. The rat insulinoma cell line (RIN) and inulinoma cell line (INS-1E) were both derived by x-ray irradiation of rat insulinomas (Asfari et al., 1992, Skelin et al., 2010). RIN cells differ in their glucose response (insulin decreases with passages) and transport compared to the native beta cells and hence not appropriate for glucose stimulated insulin secretion assays (Halban et al., 1983). The INS-1E cells respond to glucose stimulated insulin secretion within physiological range but differ in total insulin content (about 20% compared to 60% in the native beta cell of which 10% can be released) and require the use of toxic beta mercaptoethanol to culture (Asfari et al., 1992). The mouse insulinoma (MIN6) cell line are derived from neoplastic islet cells transduced with SV40 under the insulin promoter. These cell response to glucose stimulated insulin secretion within physiological ranges in the presence of nicotinamide and sometimes this response is lost between passages (Miyazaki et al., 1990). Other cell lines have been reported but disadvantaged by their relatively low response to glucose stimulated insulin secretion or no insulin content (Skelin et al., 2010). Some human derived beta cell lines have been developed, for example, the Flatt lab through electrofusion of isolated human pancreatic beta cells and immortalised human ductal PanC-1 epithelial cell line developed the 1.1B4, 1.4E7, and 1.1E7 (McCluskey et al., 2011). Although these cells have been reported to respond to glucose stimulation within physiological ranges, in our hands these cells have produced inconsistent results. Another human cell line developed is the EndoC βH1 which is herein detailed in materials and methods (section 2.2.1.2).

The rationale for the use of animal models in research lies in the similarities between the pathologies in humans, dogs, cats, monkey, pigs, mice and rats

(O'Kell et al., 2017). Animal models provide information on the various pathways and their possible role in T1DM. For instance, poor antigen presenting cell maturation reported in both NOD mice and humans was a head start to beta cell auto-antibodies (Serreze and Chen, 2005). Several animal models for T1DM have been reported and are generated by either chemically inducing insulin deficiency (Streptozotocin-induced), genetic induction (AKITA mice), virally induced (using coxsackie B, encephalomyocarditis, Kilham rat or LCMV virus) and spontaneous autoimmune (NOD mice and BB rats) (King, 2012). The difficulty with the use of animal models in T1DM research comes from the heterogeneity of the disease and the fact that the pathogenesis of the human T1DM is not completely understood. T1DM heterogeneity arises from ages of onset, differences in the genetics and differences between individuals (von Herrath and Nepom, 2009). A few translating successes and failures have been reported in animal models. For example, the use of anti-CD20 in NOD mice has now been reported in humans to have a considerable success in subjects with high C-peptide (Herold et al., 2011). The Finish trial in which oral insulin was administered to children was first established in NOD mice before translating to human failed to have an effect in children as recorded in mice (https://clinicaltrials.gov/ct2/show/NCT00336674) (Funda et al., 2014). The differences in T1DM between mice, rats and humans possibly account for most of the failed translations. The heterogeneous nature of T1DM in humans with varying in age of onset and different genetics (Leete et al., 2016) and hence the choice of the animal model should be representative of the type of T1DM one seeks to understand (von Herrath and Nepom, 2009). The pathophysiology in mice and rat models differs from humans in terms of the autoantibodies detected, mice and rats mostly have the ICA autoantibody (with

the exception of NOD mice which can also have IAA and GAD) while humans have 7 types of antibodies (CHATZIGEORGIOU et al., 2009). Additionally, the pancreatic architecture and insulin levels differ between humans and animals. The rat and mouse islets are reported to have alpha cells on the outside surrounding a core of beta cells (homotypic contact) while human beta cells and alpha cell intermingle in no particular pattern (heterotypic contact) (Abdulreda et al., 2013). The human islet has more alpha cells (35%) when compared to the rodent models (18%) (Seino and Bell, 2008). Physiological differences have been reported such as the effect of ATP on human islets, which leads to insulin release, but in rodents have had conflicting reports (Abdulreda et al., 2013). Additionally, mice have a higher fasting blood glucose compared to humans, while rats show a closer figure to humans. Rats have a fasting blood glucose of about 5.65 mmol/L (Wang et al., 2010), mice 6.1 mmol/L (Sun et al., 2016) and humans 5.6 mmol/L (Kahn et al., 2014).

Although differences exist between animal models, these models have contributed extensively to the understanding of beta cell pathophysiology.

1.3 Mechanisms of beta cell death in Type 1 diabetes

Gaining a full understanding of the mechanisms of beta cell death in T1DM has been complicated by many factors including the limited access to the whole pancreas from human donors (and thus an over-reliance on rodent data). When human samples are available there is variability between donors, fixation methods and autolysis occurring during the time of death that can obscure the disease pathology (Morgan et al., 2014). Therefore, the precise mechanisms involved in beta cell death remain uncertain but are probably varied and depend on a myriad of factors including both the underlying cause of diabetes, genetic factors and the extent of immune system activation. In the two main forms of diabetes (T1DM and T2DM), there is beta cell death, with the key difference being that there is an autoimmune attack of beta cells in type I which is not seen in T2DM (Cnop et al., 2005). For the purpose of this thesis, I will expand more on the mechanisms of beta cell death in T1DM.

1.3.1 Genetic susceptibility

HLA

As already described, polymorphisms within the HLA class ll gene family have the strongest association with T1DM, and these genes are found in the human leukocyte antigen region on chromosome 6p21.3. The HLA region codes for homologous cell-surface proteins that are known to be the most polymorphic in the human genome. Their polymorphic nature gives the immune system an advantage against a wide range of microorganisms, allowing them to present a large diversity of peptides to T-cells (Janeway et al., 2001). HLA II is expressed on specialised antigen presenting cells and is made up of α and β chains, which together are used to present antigens to CD4+ T-cells (Nerup et al., 1974). Alleles that confer genetic risk of T1DM are structurally different at

the molecular level. A well-known example is the replacement of aspartic acid residue at position 57 of the beta chain of the DQ molecule by either valine, alanine or serine in people with T1DM. This modification causes a change in electric charge within the peptide-binding groove of HLA-DQ8 and changes its ability to bind insulin epitopes (Todd et al., 1987). It is suggested that the weak binding of some auto-epitopes by diabetogenic class II alleles fails to generate tolerance at the thymus or peripherally leading to the perpetuation of self-recognising immune cells (Wong and Li, 2003).

HLA class I, on the other hand, is expressed by all nucleated cells and presents intracellular antigens to CD8+ T-cells. HLA class I has been associated with T1DM independent of the HLA class II DR/DQ allele after adjusting analysis for linkage equilibrium (Noble et al., 2002).

NON HLA loci

In humans, the expression of insulin is modulated by the transcription of a highly polymorphic insulin gene variable number tandem repeat associated with a proinsulin promoter. One such polymorphism is in the VNTR I which causes a peripheral increase in *INS* mRNA but a much lower expression in the thymus (Durinovic-Belló et al., 2010). Low expression of *INS* mRNA in the thymus affects the negative selection process in which less bound T-cells are not destroyed creating insulin autoreactive T-cells. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is another T1DM candidate gene which encodes for the T-cell receptor which is expressed on T-cells 48h after their activation. Although the exact mechanism by which polymorphisms in this gene contribute to T1DM is unclear, it is suggested that mechanism might involve alternative spliced variants of the gene. Targeted disruption of PTPN22

another candidate gene has been shown to lead to an increase in the number of memory T-cells although the mechanism remains elusive (Kim and Polychronakos, 2005). It is reported that the SNP R620W in PTPN22 causes a decrease in the B and T cell receptors, hence preventing the removal of autoreactive cells in the thymus (Sharp et al., 2015). At least 8 IL-4R SNPs have been reported and together with IL-4 and IL-13 haplotypes have been suggested to be involved in persistent islet autoimmunity and T1DM (Mirel et al., 2002, Erlich et al., 2009) although the precise mechanism is not completely understood (Steck et al., 2005). Others have not found an association between these SNPs and T1DM (Maier et al., 2003).

Conversely, other SNPs in the IL-4R, IL-4 and IL-13 lead to protection from T1DM (Bugawan et al., 2003).

1.3.2 Apoptosis

Apoptosis is a form of programmed cell death in which somatic cells die in a genetically controlled fashion without damaging neighbouring cells. In this form of cell death, the cell shrinks, undergoes pyknosis, the nuclear envelope collapses and DNA fragmentation occurs (Prause et al., 2016). The cell membrane integrity is altered to display properties that allow them to be phagocytosed easily. Apoptosis differs from necrosis, a process in which cells are lysed in an unregulated manner causing collateral damage to neighbouring cells (Alberts et al., 2002b). Apoptosis serves as a homeostatic process to maintain cell numbers and it is also used by the immune system to eliminate infected or damaged cells. T1DM is a chronic slowly-progressing autoimmune destruction of beta cells, which presents clinically after about 70% ablation of the beta cells. It has been suggested that most probable cause of beta cell

death is due to apoptosis although evidence for this is limited in tissue (Pirot et al., 2008, Cnop et al., 2005).

Apoptosis is the most studied form of beta cell death and can be 'extrinsic' (via death receptors) or 'intrinsic' (BCL-2- mediated or mitochondrial). There is an additional pathway that is T-cell mediated through perforins and granzyme-dependent killing of the cell. This perforin/granzyme pathway induces apoptosis through granzyme A or granzyme B (Thomas et al., 2009b).

The extrinsic pathway of apoptosis

The extrinsic pathway is regulated by the activation of TNF receptor superfamily members including FasL/FasR, and TNF-α/TNFR1 that are transmembrane receptors whose extracellular domains are rich in cysteine residues. The intracellular domain is made up of about 80amino acids and termed the death domain. Activation of FasR by the binding of its ligand (FasL) leads to the formation of a trimer which induces the formation of a Fas-associated death domain (FADD) complex. This event leads to the recruitment of procaspase-8 to FADD to form the death-inducing complex (DISC). Procaspase-8 undergoes autocatalysis to form active caspase 8 and this activated form induces the cleavage of procaspase 3 to active caspase 3. Alternatively, caspase 8 can activate mitochondrial death signalling through the cleavage of the BH3-interacting domain death agonist (BID) to a truncated bid (tBID)(Kawasaki et al., 2004). This process can be inhibited by a protein called c-FLIP that binds to FADD and caspase 8 making them ineffective (Elmore, 2007). The truncated tBID then activates BCL2 antagonist/killer 1 (Bak) and BCL2 associated X, apoptosis regulator (Bax) activation. Activated Bax and Bak form pores within the mitochondria membrane leading to

cytochrome c release. Cytochrome c reacts with Apaf1 forming an apoptosome, leading to the activation of caspase 9 which in turn activates the execution caspases 3,6 and 7 induction of apoptosis (Dewson and Kluck, 2009). Execution caspases translocate to the nucleus and trigger proteolysis and fragmentation of DNA through the activation of caspase activated DNase (CAD) (Liu et al., 1998b) (Fig 1.3).

The intrinsic pathway of apoptosis

The intrinsic pathway, on the contrary, is activated by withdrawal of growth factors, hormones and cytokines that suppress death signals. Importantly, it is non-receptor mediated but can also be activated by viral infections, radiation, toxins, and free radicals (Elmore, 2007). The intrinsic pathway relies on the delicate balance between the pro-apoptotic BCL-2 members Bax, Bak, (BCL2 interacting mediator of cell death (Bim), BCL2 associated agonist of cell death (Bad), BID, and Egl-1 and anti-apoptotic BCL-2 members; Bcl-XL, Mcl-1, and Bfl-1 of mitochondrial proteins (Thomas et al., 2009a). BCL-2 proteins reside in the outer mitochondrial membrane and regulate mitochondrial membrane permeability. A stimulus that promotes intrinsic apoptosis will initiate the loss of mitochondrial transmembrane potential, which is irreversible, and thus lead to programmed cell death. Transcriptional upregulation of pro-apoptotic BCL-2 family members leads to the activation of Bax/Bak that diminishes the mitochondrial transmembrane potential leading to the release of cytochrome c and Smac (also known as Diablo) from the mitochondria into the cytosol. Cytochrome c interacts with the adapter protein Apaf1 and together form the apoptosome, this once formed recruits and activates procaspase-9. Under normal conditions, prosurvival BCL-2 members prevent Bax/Bak activity by binding to them (Prause et al., 2016) (Fig 1.3).

The perforin/granzyme apoptosis

The killing of beta cells by CD8+ cytotoxic T-cells is suggested to be mediated via perforins and granzymes or through the secretion of pro-inflammatory cytokines. Perforins are cytolytic proteins expressed by CD8+ T-cells and are localised in granules together with granzymes which are released by exocytosis in response to stimulation of T-cells following antigen recognition of HLA I molecules. Released perforins form transmembrane pores in a Ca^{2+} dependent manner, this enables the entry of granzymes (serine proteases) which activate apoptotic caspases (Thomas et al., 2009b). Interestingly, a report on perforin-deficient NOD mice revealed that these mice were delayed from developing diabetes, however, they displayed similar levels of insulitis compare to wild type mice. These data suggested the killing of beta cells mediated by T-cells does not require perforins but might be propagated by other mechanisms such as FAS ligand binding and cytokine secretion (Kägi et al., 1997).

Granzyme A, activates DNA degradation through a DNAse NM23-H1 which is a tumour suppressor gene. The gene is responsible for immune surveillance of tumours wherein they induce apoptosis in tumour cells. A nucleosome assembly known as SET normally inhibits this gene. Granzyme A acts by cleaving the SET complex releasing NM23-H1 which leads to DNA damage and inhibition of repairs (Martinvalet et al., 2005).

The granzyme B pathway activates apoptosis in a caspase-dependent manner via a single-stranded DNA damage. Granzyme B like caspases cleaves proteins at aspartate residues leading to the activation of procaspase-10 or cleavage of inhibitor of caspase-activated DNAse (CAD). It has also been shown that granzyme B can cleave BID leading to the subsequent release of

cytochrome c (act of intrinsic pathway) or can directly cleave caspase-3 bypassing the upstream signalling pathway (Martinvalet et al., 2005). The protection of beta cells from granzyme induced cell death by IL-13 remains to be investigated but it is reported that loss in STAT6 leads to increase in *NM23-H1* mRNA (Li et al., 2012).

Both extrinsic and intrinsic apoptosis have a common endpoint, the execution phase. It involves the execution caspases (caspase 3, 6 and 7). Caspase-3 is pivotal to execution as it cleaves endonuclease CAD from its inhibitor. CAD degrades chromosomal DNA and causes chromatin condensation (Elmore, 2007) Fig 1.3. Caspase 3 has been reported to be the main executioner caspase, firstly because it activates other executioner caspases. Secondly, the final stages of apoptosis such as nuclear condensation, chromatin margination and DNA fragmentation are only performed by caspase 3. Caspase 6 and 7 seem to have more specialised roles in apoptosis upstream of the final stages which are, proteolysis of Lamin A (a supporting scaffolding protein of the nuclear envelop) by caspase 6 and proteolysis of poly-ADP ribose polymerase (PARP) by caspase 7 (Slee et al., 2001). (Fig 1.3).

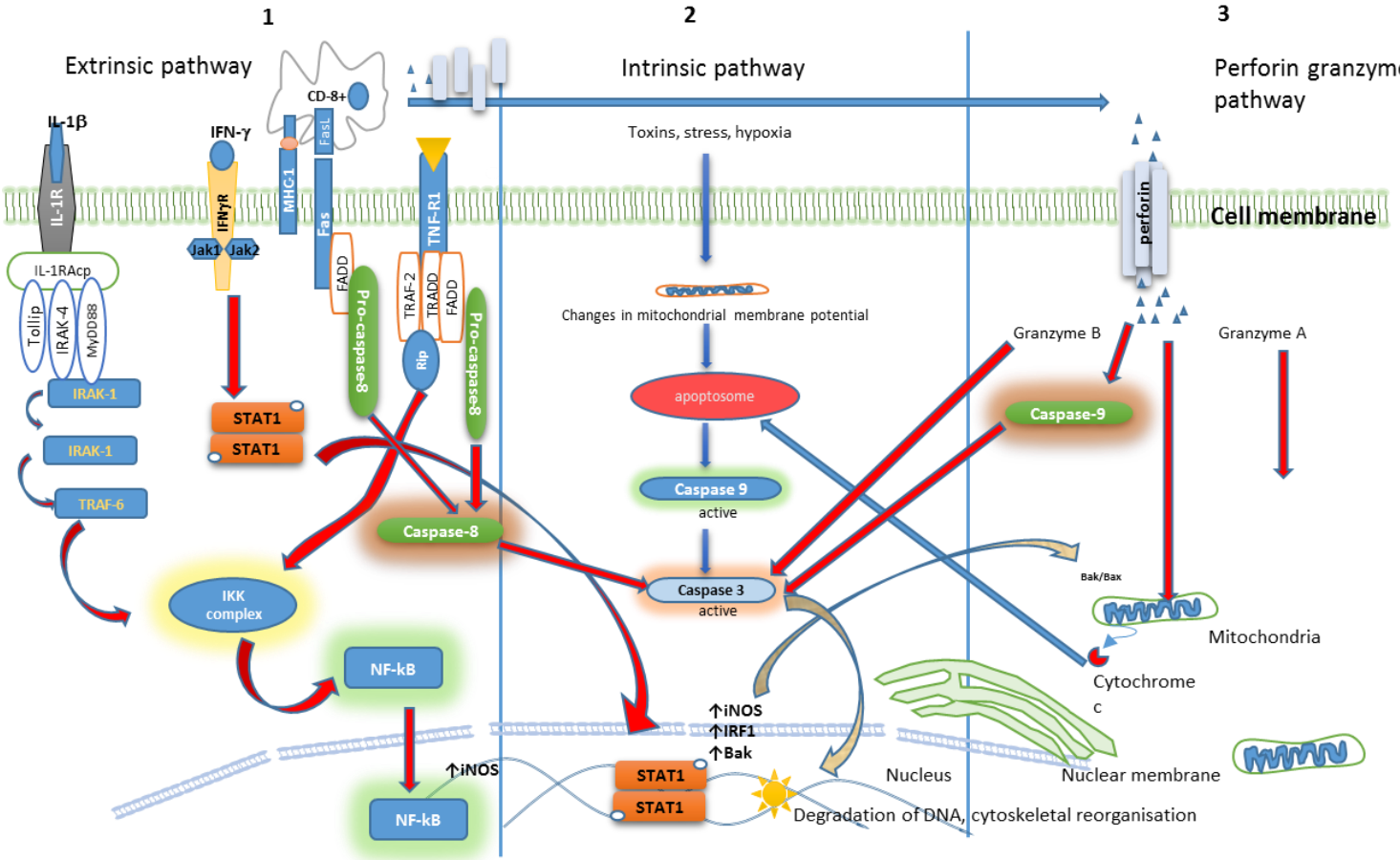


Figure 1. 3: Intrinsic, Extrinsic and perforin granzyme pathways for apoptosis

Apoptosis can occur by either by **1)** extrinsic mechanisms where cytokines and CD8+ T-cells induce death through cell surface receptors, **2)** intrinsic mechanisms, which is induced by toxins or stress in a mitochondria driven fashion or by **3)** perforin/granzyme mechanisms usually secreted by CD8+ cells. All of these pathways ultimately converge in some way at the level of the mitochondria. Modified from Pirot et al., (2008)

1.3.3 Pro-inflammatory cytokines and beta cells

Although the mechanism of beta cell death in T1DM is still not completely clear, the mechanisms implicated include a) expression of the Fas ligand or death receptors on beta cells. b) CD8+ T-cell release of granzymes and perforins. c) immune cell secretion of pro-inflammatory cytokines such as IL-1β, TNF-α, IFN-γ by CD4+, CD8+ T-cells (Prause et al., 2016). d) auto-antibody mediated cellular cytotoxicity (Wong and Wen, 2005).

Persons with T1DM have a significantly higher proportion of circulating pro-inflammatory cytokines (IL1β, IL-6, and TNF-α) than matched healthy controls (Chatzigeorgiou et al., 2010, Pham et al., 2011, Thomas et al., 2013, Gouda et al., 2018). In *in vitro* studies, the cytotoxic effects of these cytokines on beta cells involves the production of nitric oxide, reactive oxygen species and activation of apoptotic pathways (Thomas et al., 2013).

It is well established that beta cell death can occur due to chronic exposure to pro-inflammatory cytokines. Human pancreatic beta cells express the IL-1β receptor and are reported to secrete IL-1β under high glucose (33.3mM) (Maedler et al., 2002). Activated macrophages have also been suggested to secrete IL-1β in the pancreas of T1DM individuals (Eizirik et al., 2009). IL-1β has been shown to induce human and rodent beta cell dysfunction (by inhibiting insulin biosynthesis) and death in *in vitro* studies (Russell et al., 2013, Palmer et al., 1989, Spinas et al., 1987, Cetkovic-Cvrlje and Eizirik, 1994). Specifically, IL-1β activates a cascade of signalling processes when it binds to its receptor, leading to the formation of multi-protein complex cytoplasmic domain made up of MyD88, Tollip, IL-1R, IL-1 receptor associated kinase (IRAK)-1 and IRAK-4. IRAK-4 activates IRAK-1, which interact with TNF receptor associated factor (TRAF)-6

that in turn activates nuclear factor kappa B subunit 1 (NF-κB) or mitogen-activated protein kinase 1 (MAPK) and inhibitor of nuclear factor kappa B kinase subunit beta (IKK). IKK then activates inhibitors of NF-κB leading to translocation of NF-κB to the nucleus. Once in the nucleus, it modulates the expression of inducible Nitric Oxide (iNOS) which leads to beta cell death through oxidative stress and mitochondrial damage (Collier et al., 2011) (Fig 1.3).

TNF-α on its own has a little effect on beta cell cytotoxicity but in combination with IL-1β can induce beta cell death and inhibit insulin biosynthesis (Cetkovic-Cvrlje and Eizirik, 1994). Specifically, TNF-α binds to its receptor (TNF-R1) and this leads to the formation of trimer with the TNF receptor-associated death domain protein (TRADD). TRADD recruits TRAF-2 and then together with TRAF-6, this protein complex activates NF-κB leading to P38 and JNK activation (Elmore, 2007). Rodent beta cells express low levels of TNF-R1 but not TNF-R2 and have been shown to respond to TNF-α stimulation accordingly inducing NF-κB (Stephens et al., 1999, Kwon et al., 1999). TNF-α has been reported to be secreted by human islets (Hanley et al., 2006) and also by NKT cells, CD4+ T-cells and some neurons (Eizirik et al., 2009).

IFN-γ has been reported to inhibit insulin release and to induce beta cell death (Cetkovic-Cvrlje and Eizirik, 1994, Laffranchi and Spinas, 1997). IFN-γ binding to its cognate receptor (IFNGR1) leads to the autophosphorylation of a Janus Kinase and recruitment and activation of STAT-1 which homodimerizes, migrates to the nucleus and modulates the expression of genes such as Myc proto-oncogene protein (c-*Myc)*, BCL2 and IRF-1, Bak expression which leads to mitochondrial permeability and release of cytochrome c eventually leading to cell apoptosis (Zhou et al., 2008). Knockdown of STAT-1 and interferon regulatory

factor (IRF)-1 in IFN-γ stimulated INS-1E beta cells protected them from apoptosis. Knockdown of STAT1 also downregulated the production of NO. Exposure of rodent beta cells to IFN-γ leads to the downregulation of beta cell functional genes glucokinase, Pdx1 and Nkx2.2 that seem to be IRF-1 modulated (Moore et al., 2011). It is suggested that dendritic cells and CD4+ and CD8+ T-cells secrete IFN-gamma (Ablamunits et al., 1998, Eizirik et al., 2009).

Fas seem to be absent from the normal human islet according to immunohistochemistry on pancreatic sections but is upregulated in IL-1β treated islets (Loweth et al., 2000). Fas expression has been induced in rodent beta cells using IL-1β, TNF-α and IFN-γ. Transgenic NOD mice with the dominant negative form of Fas were protected from developing diabetes and from cytokine induced Fas upregulation (Allison et al., 2005).

Whilst the roles of IL-1β, IFN-γ and TNF-α are well established in inducing beta cell death, the role of IL-6 remains somewhat controversial. IL-6 in combination with IL-1β has been shown to potentiate beta cell death in response to a variety of cytotoxic stimuli (Prause et al., 2016, Russell et al., 2013, Ellingsgaard et al., 2008), whilst studies by Choi et al. (2004) have shown the reverse suggesting that IL-6 reduces the incidence of diabetes in NOD mice (Kristiansen and Mandrup-Poulsen, 2005). The source of IL-6 at the islet site is also a matter of debate. Many investigators assume that IL-6 will primarily be released by influent immune cells, however, it has also been shown that IL-6 is expressed by islet endocrine cells and that its release can be stimulated under certain conditions (Campbell et al., 1989).

These are not the only cytokines suggested to induce beta cell death. IL-17A has been shown to increase the expression of IL-1β, IFN-γ, and TNF-α mRNA and

proteins in human pancreatic islets, potentially exacerbating beta cell apoptosis (Grieco et al., 2014, Arif et al., 2011). Moreover, IL-12, IL-23, IL-24 and IL-33 have been studied in pancreatic beta cells and have each shown the capacity to induce oxidative and endoplasmic reticulum(ER) stress which eventually leads to beta cell dysfunction and death (Taylor-Fishwick et al., 2013, Weaver et al., 2015, Prause et al., 2016).

1.3.4 Anti-inflammatory cytokines and beta cells

Whilst the actions of pro-inflammatory cytokines are extensively studied, the actions of anti-inflammatory cytokines (IL-2, IL-4, IL-10, IL-13 and IL-22) on beta cells have received much less attention. Anti-inflammatory cytokines can be secreted by a variety of immune cells such as CD4+ T-cells, M2 macrophages and regulatory B cells (Russell and Morgan, 2014). IL-10 and IL-22 have been shown to protect beta cells from apoptosis, to suppress ER stress and to promote glucose-stimulated insulin secretion (Xu et al., 2010, Hasnain et al., 2014).

The actions of IL-4 and IL-13 have been reported *in vitro* to protect human and rodent beta cells from cytotoxicity induced by pro-inflammatory cytokines and *in vivo* to protect NOD mice from developing diabetes (Russell et al., 2013, Manna and Aggarwal, 1998, Rutti et al., 2016, Zaccone et al., 1999, Cameron et al., 1997). Systematically, It is suggested that IL-4 might act by increasing the regulatory T-cell population and that IL-13 might act by suppressing NF-κB activity, while also upregulating beta cell survival genes (Cameron et al., 1997, Manna and Aggarwal, 1998, Rütti et al., 2016).

This thesis is focused on studying the mechanism by which IL-13 (and the related cytokine IL-4) protects beta cells from cytotoxicity. Treatment of cells with these cytokines is reported to stimulate Jak/STAT signalling pathway.

1.4 The Jak/STAT signalling Pathway

Cells receive external signals from different proteins such as growth factors, cytokines and hormones through a myriad of cell surface receptors, which connect to different intracellular pathways in order to communicate information to the nucleus. The Jak/STAT signalling pathway is one such signalling pathway, and it is responsible for signalling in response to different soluble factors such as erythropoietin, IL-13 and growth hormone (Sehgal et al., 2003). The canonical pathway is constituted of two key families of proteins; these are the Janus kinase (Jak) proteins and the Signal Transducer and Activators of Transcription (STAT) protein families. The pathway is negatively regulated by endogenous inhibitors including suppressors of cytokine signalling (SOCS), SH2 containing protein tyrosine phosphatase (SHP) 1 and 2 proteins and protein inhibitors of activated STATs (PIAS) (Liongue and Ward, 2013).

Janus Kinases

There exist four members of the mammalian Jak family: Jak1, Jak2, Jak3 and Tyrosine Kinase 2 (Tyk2). Jaks are large non-receptor tyrosine kinases ranging in size from 120-140kDa (Zouein et al., 2011). Structurally, Jaks are divided into seven Jak homology domains (JH1-7) (Fig 1.4). JH1 is the tyrosine kinase domain and is responsible for the kinase function of the protein. The JH2 is a pseudokinase domain, which has no kinase activity and is reported to play a role in the functional regulation of the kinase domain. This structural architecture of the kinase domains gave the Jaks their name “Janus” derived from the two-faced Roman god of beginnings, endings and duality (Yamaoka et al., 2004). The protein also contains an SH2 like domain (between JH3-JH4) whose function remains unknown and a FERM domain (JH4 to JH7). The FERM domain mediates the binding of Jaks to various cytokine receptors and together with the

pseudokinase domains can modulate the activity of the kinase domain (Sehgal et al., 2003, Stark and Darnell, 2012, Yamaoka et al., 2004). Jaks are activated when associated with various receptors of the cytokine superfamily, or other receptors such as certain tyrosine kinases, or G-protein coupled receptors (Table 2). Canonically, Jaks are mostly associated with receptors, but non-receptor associated Jaks exist and have been reported to have other functions such as gene regulation (Zouein et al., 2011, Noon-Song et al., 2011).

Table 2 : Jaks their associated receptors and growth factors

Receptors	Cytokines and/or growth factors	Associated Jaks	Ref
Type 1 cytokine receptors			
γC receptors	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21	Jak1, Jak3	1
gp130	IL-6, IL-11, IL-12, IL-23	Jak1, Jak2	1
EPOR	EPO	Jak2	2
PrlR	Prolactin	Jak2	3
OSMR	OSM	Jak2	4
LIFR	LIF	Jak1, Jak2	1
GHR	GH	Jak2	5
Type II cytokine receptors			
IFNGRA	IFN-γ	Jak1, Jak2, Tyk2	1
IFNAR1&2	IFN-α	Jak1, Tyk2	1
IL10RA	IL-10	Jak1, Tyk2	6
IL20RA	IL-20	Jak1, Jak2	7
IL22RA1	IL-22	Jak1, Tyk2	8
Chemokine receptors			
CXCR4	SDF-1α	Jak2, Jak3	9
Seven membrane Receptors			
CCK2R	Gastrin	Jak2	10
Angiotensin II Receptor	Angiotensin II	Jak2, Tyk2	1
Receptor Tyrosine Kinases			
VEGF R1, R2, R3	VEGF	Jak2	1
EGFR	EGF	Jak1, Jak2	11
PDGFR	PDGF	Jak2	12
FGFR	FGF	Jak2	13
IGF-1R	IGF	Jak1, Jak2	14
TrkBR	TrkB	Jak2	15

1(Sehgal et al., 2003) 2. (Choi et al., 2010) 3. (Fujinaka et al., 2007) 4. (Dharminder Chauhan et al., 1995) 5. (Waters and Brooks, 2015) 6. (Williams et al., 2004) 7. (Lee et al., 2013) 8. (Lejeune et al., 2002) 9. (Vila-Coro et al., 1999) 10. (Ferrand et al., 2005) 11. (Andl et al., 2004) 12. (Masamune et al., 2005) 13. (Dudka et al., 2010) 14. (Gual et al., 1998) 15. (Kim et al., 2015)

Every member of the Jak family has been implicated in T1DM and since the thesis is centred on the Jak/STAT pathway, the various Jaks have been illustrated on table 3 below and their link to T1DM described.

Table 3: Jaks and beta cell health

Jak	Role in beta cells or T1DM	Reference
Jak1	• Blockade of Jak1 protected NOD mice from rapidly developing T1DM	(Trivedi et al., 2017)
Jak2	• Blockade of Jak2 inhibits IFN-γ signalling hence protects NOD mice from diabetes	(Trivedi et al., 2017)
	• Phosphorylation induced by EPO and lactogens protects beta cells from cytotoxicity	(Fujinaka et al., 2007)
	• Inhibition reversed IL-13 protection of INS-1E during serum withdrawal	(Russell et al., 2013)
Jak3	• Inhibition of Jak3 reverses IL-4 protection of INS-1E from cytokine-induced death	(Kaminski et al., 2010)
	• Inhibition of Jak3 protected islets from pro-inflammatory cytokine mediated cell death	(Lv et al., 2009)
Tyk2	• SNP mutations in Tyk2 are associated with T1DM	(Marroqui et al., 2015)
	• Tyk2 knockdown reduces MHCI expression and cell death in human islets induced by poly-IC	(Marroqui et al., 2017)
	• Tyk2 transduces IFN-α signals	

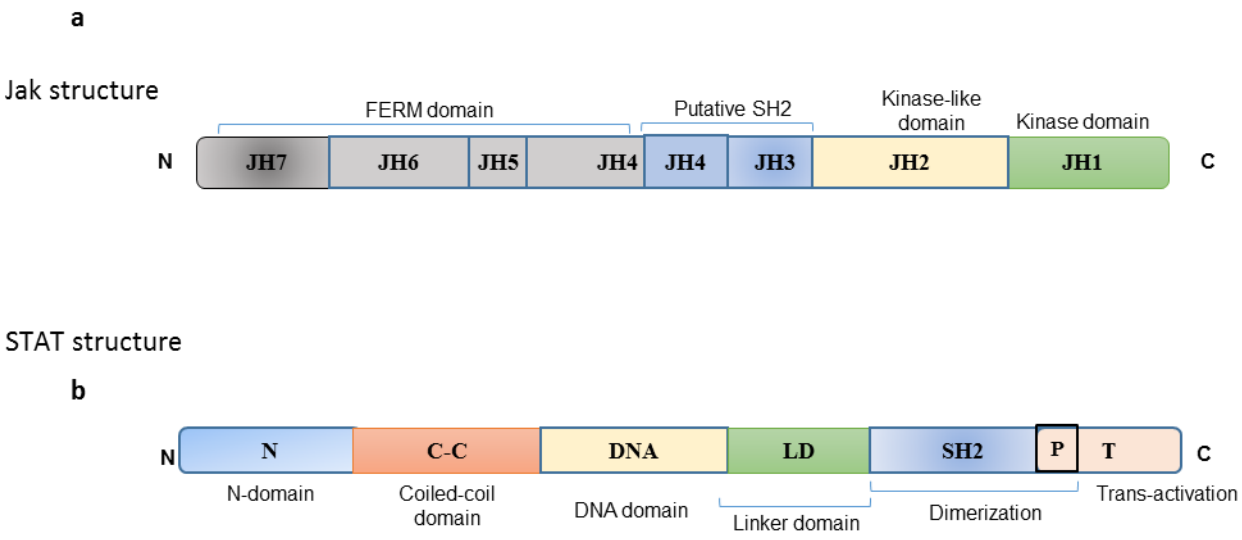


Figure 1. 4: Janus kinase and STAT domain structures

a. Domain structure of Janus Kinases **b.** Domain structure of STAT, C= C-terminal, N= N-terminal domain, P= phosphorylated tail, T=transactivation domain

Signal Transducers and Activators of Transcription (STAT)

The STAT proteins are a family of cytoplasmic transcription factors that are activated in response to various latent cytokines and growth factors. There are seven identified members of the STAT family in humans; these are STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STATs are between 750-800 amino acids in length (shorter isoforms have been reported that lack a functional transcriptional activation domain, although they can bind and occupy certain binding sites) (Stark and Darnell, 2012). Each of the STAT proteins has a similar structure, containing an N-terminal domain, a coiled-coil domain, a DNA binding domain, a Linker domain, an SH2 domain, and a trans activation domain (Fig 1.4b).

The N-terminal domains of the different STAT family proteins vary subtly in length (124-145 amino acids). Although most STATs use the SH2 domain for dimer formation, STAT4 has been suggested to use the N-terminal domain instead (Sehgal et al., 2003, Hossain et al., 2013, Vinkemeier et al., 1998). The N-terminal domain is also important for post-translational modification of STATs (such as FoxP3-STAT3) (Hossain et al., 2013), and may negatively regulate the phosphorylation status of STAT1 at Y701 (Shuai and Liu, 2003).

The coiled-coil domain has been reported to have a series of functions. For example, it is the binding site for other proteins including transcription factors (e.g. IRF9, or other STATs such as STAT3) (Vogt et al., 2011). Furthermore, the coiled-coil domain has been reported to be essential for nuclear translocation of STAT proteins (Ma et al., 2003). The C-C domain of STAT3 has been reported to be important for its SH2 domain binding to gp130 receptor upon activation by EGF or IL-6 (Zhang et al., 2000). The DNA binding domain has immunoglobulin variable fold structures, and it is through these regions that STAT dimers bind to

DNA at palindromic response elements or GAS motifs TTCN3/4GAA (Heim, 2003).

The linker domain (LD) is a ridged spacer which connects the DNA binding domain and the SH2 domain (Liongue and Ward, 2013). The SH2 domain is critical in mediating the binding of STATs to receptors and mediates the homo and hetero dimerization of STATs. Specifically, the SH2 domain of an activated STAT protein facilitates its binding to other phosphorylated STAT monomers (Heim, 2003).

The transcriptional activation domain (or trans-activation) can contain conserved tyrosine, serine or threonine residues that are phosphorylated at the receptor complex depending on the STAT molecule. In STAT6, the tyrosine at position 641 is essential for transcriptional activity, while Threonine 645 is a negative regulator of DNA binding and transcription. The role of serine 756 is not completely understood but is suggested to interact with other molecules such as PP2A (Wang et al., 2004). The trans-activation domain is the least conserved domain and has been reported to interact with many proteins such as BRCA1 however; its biological significance is not completely understood (Sehgal et al., 2003, Lim and Cao, 2006).

The 7 members of the STAT family have in one way or other been implicated in T1DM but for the purpose of this thesis, STAT6 will be the main focus. Table 4 illustrates the 7 STAT members and the literature reporting their potential role in type 1 diabetes.

Table 4 : Signal Transducers and Activators of Transcription family and beta cell function

STAT	Role in T1D	References
STAT1	• Knockout of STAT1 protects NOD mice from diabetes • Upregulated in the insulin containing islets of patients with T1DM • Drives apoptosis in rat beta cells stimulated with pro-inflammatory cytokines	(Kim et al., 2007) (Richardson et al., 2016) (Moore et al., 2011)
STAT2	• Propagates IFN-α signalling in T1D • Induces inflammation and ER stress in human beta cells	(Santin et al., 2012) (Marroqui et al., 2017)
STAT3	• Activating mutations in STAT3 induce an autoimmune form of neonatal diabetes • Activated in T-cells of T1DM individuals • Important for pancreatic development and insulin secretion	(Flanagan et al., 2014) (Hundhausen et al., 2016) (Kostromina et al., 2010)
STAT4	• Enhances IL-12 mediated beta cell death • SNPs in STAT4 have been suggested to be involved in early development of T1DM	(Weaver et al., 2015) (Lee et al., 2008)
STAT5(A &B)	• Important for beta cell insulin secretion • Lactogens protect rodent and human cells via STAT5 from STZ induced cytotoxicity • Important for beta cell growth	(Jackerott et al., 2006, Lee et al., 2007)
STAT6	• IL-4 and IL-13 induce STAT6 phosphorylation and protect rodent beta cells but the mechanism is unclear • Whole body knockout decreases insulin action and IL-4 administration improves insulin action and prevented autoimmunity	(Russell et al., 2013, Kaminski et al., 2007, Kaminski et al., 2010) (Ricardo-Gonzalez et al., 2010, Lau et al., 2012)

1.4.1 Central dogma of IL-13 and IL-4 signalling

Like many cytokines, the actions of IL-13 are dependent on the type of cell being stimulated, for example, in B cells it induces the synthesis of IgE and in monocytes, it inhibits pro-inflammatory cytokine synthesis (Punnonen et al., 1998). IL-13 shares similar functions to IL4, although it has only approximately 25% amino acid sequence homology. Immune cells such as invariant natural killer T-cells (iNKT) (Usero et al., 2016) and innate lymphoid type 2 (Hams and Fallon, 2012) cells have been shown to secrete IL-4 and IL-13. Although most of the secretion of IL-4 and IL-13 is thought to be by infiltrating immune cells, the human pancreatic islet has also been shown to express IL-13 mRNA (Olsson et al., 2005).

IL-4 signals by binding either to type I or type II cell surface receptors. Type I receptors are comprised of an IL-4Rα subunit and the common gamma chain (γC). However, IL-4Rα dimerises with IL-13Rα1 to generate a type II receptor. IL-13 signals by binding to the type II receptor (made up of IL-13Rα1 and IL-4Rα). Alternatively, IL-13 can also bind to another receptor IL-13Rα2 the role of which has not been completely elucidated. However, IL-13Rα2 has a higher affinity for IL-13 than the type II receptor; as such, it acts as a negative regulator of the canonical IL-13 signalling.

Unstimulated receptors exist as monomers with their associated Jaks. Binding of cytokines leads to receptor dimerization (IL-4Rα with γC or IL-13Rα), bringing the associated receptor Jaks in close proximity leading to trans-phosphorylation of each other. Canonically, It is suggested that IL-4 activates Jak1/Jak3 whereas IL-13 activates Jak1, Jak2 or Tyk2 (Jiang et al., 2000). However, the exact complement of Jaks activated in response to these cytokines depends on the cell

type. Once activated the Jaks phosphorylate the key tyrosine residues on the cytoplasmic tail of the cytokine receptor. It is suggested that signalling of both IL-13 and IL-4 occurs on the IL-4Rα which contains five tyrosine residues, Y497 for the recruitment of the IRS2, Y575, Y603, and Y631 for STAT6 docking and Y713 as a negative regulator of signalling (Hershey, 2003). It is also known that STAT3 is activated in response to the stimulation of these cytokines by docking on the cytoplasmic tail of IL-13Rα1 (Y402) (Umeshita-Suyama et al., 2000). STAT6 binding to the receptor leading to its phosphorylation by Jak proteins, which induces the dissociation of STAT6 from the receptor and promotes the dimerization of phospho-STAT6 proteins and sometimes heterodimerisation with STAT3 (Delgoffe and Vignali, 2013). Alternatively, the activation of IRS-2 leads to the activation of the PI3-K/AKT pathway (Russell et al., 2013). Homodimers of STAT6 are then translocated to the nucleus where gene transcription occurs (McCormick and Heller, 2015) Fig. 1.5.

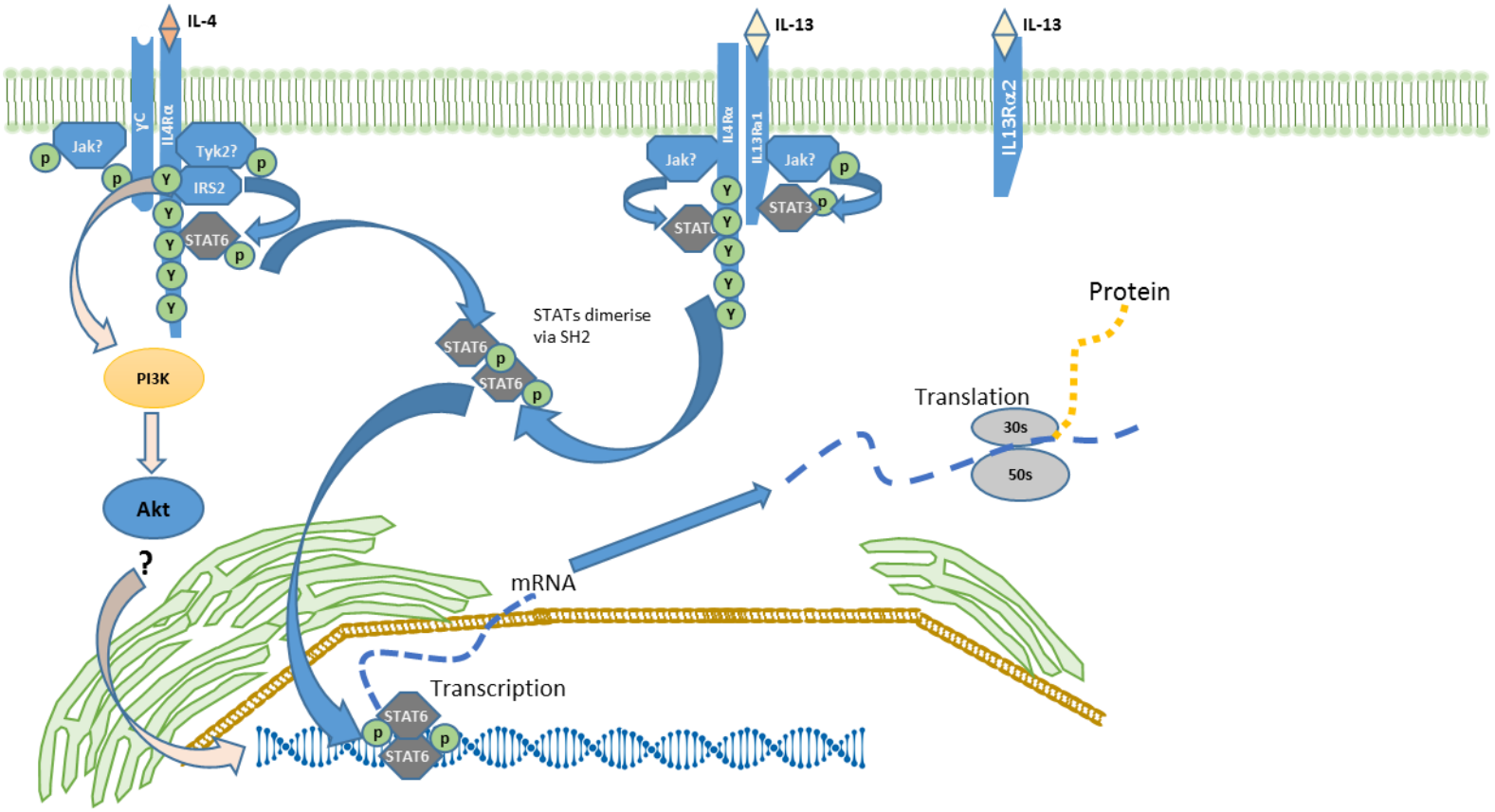


Figure 1. 5: IL-13 and IL-4 Signalling pathway

Schematic representation of Jak/STAT signalling. Cytokine binding leads to receptor dimerization, the activation of receptor-associated Jaks, followed by phosphorylation of tyrosine residues on the receptor tail to which STAT6 docks. Following docking STAT6 is phosphorylated, detaches from the receptor and dimerizes in the cytosol into the nucleus where it transcribes target genes.

Negative regulation of Jak/STAT signalling pathway

The Jak/STAT pathway can be negatively regulated at two major levels; either at the level of the Jaks or at the level of STATs.

Negative regulation of Jaks

The most extensively researched negative regulator of Jaks is the suppressor of cytokine signalling (SOCS). There are at least 8 members of this family SOCS1-7 and cytokine-inducible SH2 containing protein (CISH) (Shuai and Liu, 2003). The precise mechanism by which SOCS proteins inhibit Jak kinase activity remains to be completely understood, however, a number of possibilities have been suggested. For example, it is reported that a conserved sequence of SOCS called the extended SH2 domain with high affinity for phosphopeptides might be the way the inhibitory mechanism works (Babon et al., 2006). Specifically, SOCS1 and SOCS3 have an extended SH2 domain which binds directly to the activation loop of Jak2 (Y1007 and Y1008) preventing the phosphorylation of tyrosine residues on the cytoplasmic tail of the receptor (Yoshimura and Yasukawa, 2012). Additionally, others have suggested that the SH2 domain of SOCS3 and CISH can bind directly to tyrosine residues on the cytoplasmic tail of the receptor inhibiting the binding of STATs (Nicholson et al., 2000). Alternatively, SOCS proteins contain another conserved region of 40 amino acids at C-terminus called the SOCS box, this region interacts with molecules that recruit ubiquitin transferase and this can lead to the degradation of the SOCS-Jak complex (Shuai and Liu, 2003).

Additionally, Jaks are regulated by protein tyrosine phosphatases such as SHP1 and SHP2. These proteins dephosphorylate Jak2 and Jak1 at their activation loops (Alicea-Velázquez et al., 2013, You et al., 1999). SHP1 regulates EPO signalling by dephosphorylating any Jak2 which is associated with the EPO

receptor (Jiao et al., 1996). SHP1 has also been reported to dephosphorylate Jak1 in IFN-α signalling in macrophages since SHP1 deficient macrophages show an increased Jak1 and STAT1 phosphorylation (David et al., 1995). Additional phosphatases have also been shown to interact with Jaks, for example, CD45 negatively regulates EPO and IFN signalling by dephosphorylating Jak1 and Jak3 (Irie-Sasaki et al., 2001).

Regulation of STATs

STAT activity can be regulated by post-translational modifications (e.g. phosphorylation, methylation and, ubiquitylation) or through their interaction with certain proteins, such as protein inhibitors of activated STATS (PIAS) and protein tyrosine phosphatases (PTP) (Shuai and Liu, 2003).

As already described, the stimulation of cells by certain cytokines induces the tyrosine phosphorylation of STAT proteins at the transactivation domain, leading to dimerization and subsequent translocation to the nucleus. However, there exist threonine and, serine phosphorylation sites in some STATs proteins, which can also modify protein activity. For example, STAT1 full transcriptional activity and function after IFN activation requires serine phosphorylation (Sadzak et al., 2008). However, in the context of STAT6, serine phosphorylation at the transactivation domain has been suggested to negatively regulate DNA binding (Maiti et al., 2005). Methylation also has a role in controlling STAT activity, for example, methylation of the Arg31 residue of STAT1 by arginine methyl-transferase 1 increases DNA binding activity of STAT1 and is suggested to inhibit the interaction of PIAS1 with the protein (Shuai and Liu, 2003). This effect was independent of tyrosine and serine phosphorylation. Methylation of a similar residue (Arg27) in STAT6 has also been reported to enhance STAT activity with

increased tyrosine phosphorylation, nuclear translocation and DNA binding all observed (Chen et al., 2004).

There are four members of the protein inhibitors of activated STATs (PIAS) family PIAS1, PIAS3, PIASX (PIAS2) and PIASY (PIAS4) and these are constitutively expressed within the beta cells (Segerstolpe et al., 2016). PIAS proteins can regulate STAT activity by; 1) Blocking DNA binding of STAT molecules, for example, STAT1 and STAT3 binding are blocked by PIAS1 and PIAS3 respectively (Chung et al., 1997, Liu et al., 1998a). 2) PIAS2 and PIAS4 enhance the activity of HDACs to inhibit transcription of STAT2 and STAT4 respectively (Arora et al., 2003, Liu et al., 2001) 3) STAT activity can be modulated by sumoylation of the STAT proteins, but this binding can either enhance or inhibit the activity of STAT molecules (Shuai, 2006). PIAS proteins contain a serine/threonine rich domain suggested to be responsible for targeted binding, they include a Zn-binding RING-finger like domain for SUMO transfer, a scaffold attachment factor A/B for binding to scaffold and a matrix attachment regions in the nuclear matrix. PIAS proteins operate by binding to their STAT dimers thereby preventing STATs from interacting with the DNA (Seif et al., 2017).

The Jak/STAT signalling pathway can also be regulated by cross talk between the STATs. For instance, pre-treatment of cells with one cytokine can lead to upregulation of genes that negatively regulate pathways induced by a different cytokine. For example, pre-treatment with IFN-γ inhibits IL-4 induced STAT6 activation through the inhibition of transcription of IL-4R mRNA (So et al., 2000).

Viruses have also been shown to target STATs for degradation; this is mediated through the polyubiquitin-proteasome pathway and provides a method of evading the immune system. Specifically, simian virus 5 has been reported to target

STAT1, parainfluenza virus targets STAT2 and mumps virus targets STAT1 and STAT3 through the assembly of STAT-specific ubiquitin ligase complexes (Ulane et al., 2005). These are then degraded by the proteasomal pathway. Additionally, enteroviruses have been reported to inhibit the translocation of IFN activated STAT1/2 heteromers to the nucleus by downregulating the karyopherin-α1, a p-STAT1 nuclear localisation receptor (Wang et al., 2017).

In addition to the above mentioned regulations, STAT molecules can be regulated at the level of alternative splicing. For instance, alternative splicing of the STAT3 gene generates two isoforms, STAT3α and STAT3β through a conserved acceptor site on exon 23, which leads to the addition of seven amino acids and a termination codon (Aigner et al., 2018). These isoforms compared to the wild-type STAT3 have an altered C-terminal transactivation domain and the serine 727 phosphorylation site. STAT3β was until recently suggested to be a dominant-negative regulator of STAT3 but now known to have different transcription and regulatory activities (Caldenhoven et al., 1996). STAT3α and STAT3β are not the only isoforms, two other STAT3 isoforms exist STAT3γ and STAT3δ formed by proteolytic processing (Nakajima et al., 2003). Proteolytic processing is a post translation modification of proteins by protease cleavage of a single of multiple peptides leading to the activation or inhibition of the target protein (Neurath, 1989). In STATs, proteolytic processing usually generates a C-terminally truncated STAT protein commonly referred to as STATγ (Hendry and John, 2004). Proteolytic processing of STAT6 has been described in mice to occur between amino acid 685-686 (Aspartate and methionine). The forced expression of this mutant forms have been reported to prolong the accumulation of STAT6

in the nucleus and induce apoptosis and cell growth suggesting a dominant negative effect (Hendry and John, 2004).

1.4.2 The role of IL-13 and Jak/STAT6 in pancreatic beta cell health

In T1DM IL-13 levels are reduced along with other anti-inflammatory cytokines (IL-4, IL-10) (Berman et al., 1996, Rapoport et al., 1998), however pro-inflammatory cytokines secreted from infiltrating CD4+ and CD8+ lymphocytes or macrophages are increased during insulitis (Mandrup-Poulsen, 1996, Eizirik and Mandrup-Poulsen, 2001). One possibility is that the reduction in IL-13 secretion might be due to a dysfunction in the invariant NKT-cells (Usero et al., 2016). In NOD mice models the cytokine is reported to reduce the incidence of diabetes (Zaccone et al., 1999).

In vitro studies have shown the expression of IL-13Rα1 and IL-4Rα in both human and rodent islets (Russell et al., 2013). The precise Jaks associated with the IL-4Rα or IL-13Rα1 in beta cells are not clearly defined but the involvement of Jaks in T1DM has been proposed (see table 2). IL-13 has been shown to activate STAT6, STAT3 and IRS2 and to protect rodent and human islets from the cytotoxic effects of pro-inflammatory cytokines (Russell et al., 2013, Rütti et al., 2016). STAT6 is more highly expressed in beta cells in the islets when compared to other islet cells and the exocrine pancreas and its expression diminishes in T1DM persons as seen on stained tissue sections (Leslie et al, 2018). Although IL-13 protects against cytotoxicity the mechanism by which this is accomplished is not completely understood. Additionally, the role of STAT6 in this pathway has not been reported in beta cells.

1.4.3 Therapeutic inhibition of Jak/STAT pathway

The role of the Jak/STAT pathway in cell communication, growth and survival is established, as well as its role in autoimmune diseases and malignancy reported (Liu et al., 2015b). Mutations that lead to inactivation of Jak1 and Jak2 are lethal in mice due to neurological and erythropoiesis dysfunction respectively the reason why these forms of mutations have not been seen in humans (Sehgal et al., 2003). Jak3 and Tyk2 inactivating mutations cause severe immunodeficiency disease and viral infections respectively, linking both of these to the immune system (Aittomäki and Pesu, 2014). Activating mutations of these pathways on the other hand have been reported in malignancies or proliferative disease. For instance the change in the amino acid valine to phenylalanine at position 617 in Jak2 (pseudokinase domain) leads to a constitutively active Jak2/STAT5 signalling and heighten haematopoiesis and reported in patients with polycythaemia vera (Pesu et al., 2005, James et al., 2005). These gains in function indicate that inhibitory therapeutic intervention could play an important role in addressing these. Ruxolitinib is a potent Jak1 and Jak2 inhibitor FDA approved for the treatment of rheumatoid arthritis and polycythaemia vera irrespective of Jak2V617F mutations (Clark et al., 2014). Oclacitinib a pan-Jak inhibitor has also been approved for use in the treatment of atopic dermatitis in dogs (Cosgrove et al., 2013). Tofacitinib originally designed to specifically inhibit Jak3 has been reported to inhibit Jak1, Jak2 and to a lesser extend Tyk2 have been approved for the use in the treatment of severe rheumatoid arthritis. It is also suggested to be used for immunosuppression in autoimmune diseases and transplantations (Changelian et al., 2003).

STATs are known to also have a gain or loss in function with mutations. For example, inactivating mutations in STAT5B A630P have been reported to cause

growth retardation (Kofoed et al., 2003). STAT3 inhibitory mutations have been reported to be important for immune response against bacterial (staphylococcus, pneumococcus) and fungal infections (candiditis) and an autosomal dominant hyper IgE syndrome (Job's disease) due to a loss in IL-17 production (Milner et al., 2008). Activating mutations in various STATs have been reported in multiple leukaemias and lymphomas. For instance it has been shown that STAT6 D419G mutation enhances the expression of CD23 in some non-Hodgkin lymphomas (Bösl et al., 2015). STAT molecules are non-enzymatic proteins and hence therapeutic targeting is challenging, however, theoretically they can be targeted by preventing their phosphorylation, disrupting their SH2 domain hence preventing dimerization and receptor association and inhibiting DNA binding (Aittomäki and Pesu, 2014, O'Shea et al., 2015). One major challenge of specifically targeting STAT molecules lies in the similarities in their sequence homology. For examples, targeting of STAT1 is likely to interfere with STAT3 activity which could be severe (O'Shea et al., 2015). A few molecules have been developed to target STATs although none has yet been approved for use in humans (Furqan et al., 2013).

1.5 Aims

IL-4 and IL-13 have been shown to protect NOD mice from developing T1DM and protect human islets and rodent beta cells from cytotoxic stimuli (Zaccone et al., 1999, Cameron et al., 1997, Russell et al., 2013). However, the mechanism by which this protection is mediated remains undefined and the genes altered following stimulation of these cells are unknown. Furthermore, no study has clearly defined the complement of Jaks activated by IL-13 and IL-4 in beta cells and it is difficult to infer this information from other cell models since this differs between cell types. These cytokines signal by activating the transcription factor STAT6 and so the goal of this thesis was to investigate to the role of STAT6 in beta cell cytoprotection. This has been divided into the following aims;

1. Assess the protective effect of IL-4 and IL-13 in rodent and human-derived beta cell lines (Chapter 3)
2. Determine the complement of Jaks expressed in beta cells and reveal which are activated by IL-13 and IL-4 treatment (Chapter 4)
3. Investigate the functional consequences of altered STAT6 expression and activity in beta cells (Chapter 5)
4. Study the genes regulated by IL-13 stimulation of rodent and human beta cells and to assess whether these changes are dependent on STAT6 activity (Chapter 4 and 5)
5. Study the impact of novel genes induced by IL-13 and IL-4 stimulation on beta cells viability (Chapter 6).

1.6 Study hypothesis

1. IL-13 protects beta cells from serum withdrawal, palmitic acid and pro-inflammatory cytokines

2. Loss of STAT6 attenuates the protection of beta cells by IL-13 from cytotoxic stimuli

3. IL-13 and IL-4 protect beta cells from cytotoxicity by upregulating anti-apoptotic genes in a STAT6 dependent manner

Chapter 2.0: Materials and Methods

2.0 Materials and Methods

This chapter explains in detail the different methods and materials used in the various studies reported in this thesis. There are, however, chapters with specific methods that are detailed therein.

2.1 Reagents

Table 5: Chemicals and reagents

Reagent	Company	Catalogue number
2-Mercaptoethanol	Fisher(UK)	BP176-100
Attractene	Qiagen(USA)	301005
Bovine Serum Albumin (BSA)	Roche (Switzerland)	10775835001
CDP-Star	Sigma(USA)	C0712
Dimethyl sulfoxide	Sigma(USA)	PH1309
DMEM	Fisher(UK)	12491015
Extracellular matrix	Sigma(USA)	E1270
Fibronectin	Sigma(USA)	F1141
Foetal Bovine Serum (FBS)	Fisher(UK)	10270
Glycine	Sigma(USA)	G8898
LDS	Invitrogen(USA)	NP0007
L-Glutamine 200mM	Life Tech (USA)	21051040
Lipofectamine Ltx	Fisher(UK)	100014470
Lipofectamine RNAi Max	Fisher(UK)	13778030
MOPS SDS running buffer	Life Tech (USA)	B0001
Nicotinamide	Sigma(USA)	N5535
Optimem	Fisher(UK)	31985070
Palmitic acid	Sigma(USA)	P0500
Penicillin/Streptomycin	Life Tech(USA)	15140122
Phosphatase inhibitor cocktail 2	Sigma(Israel)	P2850
Phosphatase inhibitor cocktail 3	Sigma(Israel)	P0044
Phosphate buffered saline	Lonza(Switzerland)	10010023
Propidium iodide	Sigma (USA)	P4170
Protease inhibitor cocktail	Sigma(Israel)	P8340
Re-blot strong plus	Millipore (USA)	2504
RNeasy mini kit	Qiagen(USA)	74104
RPMI 1640	Lonza (Belgium)	21875034
RT2 First Strand	Qiagen(USA)	330401
Skim milk powder	Sigma(USA)	70166
Sodium Selenite	Sigma (USA)	S5261
SYBRGreen Master Mix	Qiagen (USA)	330500
Tris-Acetate gels 12%	Invitrogen (USA)	EA03552BOX
Trizma base	Sigma(USA)	T1503
Trypan blue	Sigma(USA)	T6146
Trypsin EDTA	Fisher(UK)	SM-2003

2.2 Cell cultures

2.2.1 Cell culture conditions

2.2.1.1 Rat insulinoma β-cell line (INS-1E)

INS-1E cells are a rat cell line derived by X-ray irradiation of rat insulinoma cells and respond to glucose stimulation by increased insulin secretion within the functional ranges (3-11mM glucose) (Špaček et al., 2008). INS-1E cells possess a high insulin content although, they require the use of β-mercaptoethanol for their proliferation (Skelin et al., 2010, Asfari et al., 1992). Unlike many beta cells, INS-1E cells remain close to the parental cell phenotype over at least 116 passages where they still respond with a glucose-stimulated insulin response similar to that of the parent cell (Asfari et al., 1992). The cells were cultured in RPMI-1640 medium containing 11mM glucose and supplemented with 10% FBS, 100U/ml penicillin, 2mM L-glutamine, 100U/ml Streptomycin and 50μM β-mercaptoethanol. The cells were plated in 75cm^2 flasks, 25cm^2 flask, 6well plates or 12well plates depending on the experiment and incubated in 5% CO_2 in a humid incubator at 37^{0}C.

2.2.1.2 Human EndoC βH1 beta cell line

EndoC βH1 cells are a human beta cell line generated by transducing foetal pancreatic buds with a lentiviral vector expressing SV40LT (simian vacuolating virus 40 T antigen) under an insulin promoter (Ravassard et al., 2011). Lentiviral vector transduction of cells enables long-term expression of a target gene, herein expressing SV40LT under the insulin promoter. SV40LT is an oncoprotein, which induces malignancy in cells by binding to tumour suppressor proteins such as P53 and P105-Rb causing cells to replicate (promotes cells to leave the G1 phase to the S-phase) (Ali and DeCaprio, 2001). Hence, the transducing of foetal

pancreas with a lentivirus expressing SV40LT enabled the selective formation of insulin positive cells. SCID mice were then grafted with these buds to obtain mature pancreatic tissue that differentiated into beta cells expressing SV40LT and formed insulinomas. The beta cells were re-grafted into other SCID mice after transducing them with a lentiviral vector expressing the human telomerase reverse transcriptase (hTERT). The human telomerase reverse transcriptase together with the telomerase RNA component form the telomerase complex which lengthens telomeres in DNA immortalizing cells by permitting them to exceed their dividing thresholds (Hayflick limit). Hence, the lentiviral vector will permit long-term expression of the hTERT hence preventing these cells from apoptosis (Weinrich et al., 1997). The resulting beta cells were then expanded *in vitro* to generate the cell line (Ravassard et al., 2011).

The use of primary beta cells in research is challenging due to the availability of these cells and the labour involved in isolating beta cells from pancreata. Also, rodent beta cells experiments do not always translate to humans in several ways. For example, humans only have a single gene that encodes for insulin while rodents have two. In addition, sensitivity to treatments such as palmitate between human beta cells and rodent beta cells is different. EndoC βH1 cells are not killed by palmitate treatment while rodent beta cells are. These differences and many more suggest that we need to use human beta cell lines to fill in the gaps in knowledge. EndoC βH1 cells have the advantage of expressing insulin and responding to glucose stimulated secretion in a dose dependent manner. EndoC βH1 cells express beta cell specific genes such as MAFA, NKX6-1, PAX6, PDX1, NEUROD1, GCK, SLC2A2, KCNJ11, GAD2 and PTPRN. Although EndoC βH1 expressed the right markers for human beta cells, they have a very slow growth rate (144h to divide) (Tsonkova et al., 2017), express relatively low insulin per

cell compared to humans (0.46 pg in EndoC βH1 vs 20pg in humans) (Marchetti et al., 2017a). Lastly, EndoC βH1 are a recommended cell line but still fall short by the fact that, they are a mono-layer cell culture and do not exactly replicate the in vivo environment in which beta cells co-exist with other endocrine cell types. Furthermore, genetic manipulation of cells by the addition of various plasmids alter some functions although only the IAPP protein has been reported in EndoC βH1 cells at the moment (Skelin et al., 2010, Ravassard et al., 2011).

EndoC βH1 cells were cultured in plates pre-coated with ECM/fibronectin, 100U/mL Penicillin, 100U/ML streptomycin and 25mmol/L glucose within Dulbecco's Modified Eagle's Medium (DMEM) for at least 1hour. The coating medium was then replaced with culture medium which consisted of DMEM containing 5.6mmol/L glucose and supplemented with 2mM L-glutamine, 100U/mL Penicillin, 100U/mL streptomycin, 50μM β-2-Mercaptoethanol, 10mM Nicotinamide, 5.5μg/mL transferrin, 2% fatty acid free albumin power (bovine serum albumin) and 6.6ng/mL sodium selenite. The cells were mainly cultured in a 25cm^2 flask or in 6, 12 or 24 well plates depending on the experiment and incubated at 5% CO2 at 37^{0}C in a humidified incubator (Ravassard et al., 2011).

2.2.1.3 HEK293 cells

HEK293 cells are a human embryonic kidney cell line developed by exposing embryonic kidney cells to an adenovirus type 5 DNA (Graham et al., 1977). These cells were cultured as previously described (Graham et al., 1977) with few modifications. They were cultured in 11mM glucose RPMI-1640 medium supplemented with 10% FBS, 100U/ml penicillin, 2mM L-glutamine, and 100U/ml Streptomycin.

2.2.2 Passaging cells

INS-1E and HEK293 cell lines were grown to about 80% confluency before passaging. The medium was aspirated and the cells washed with phosphate buffered saline (PBS), then incubated in 0.05% trypsin-0.53mM EDTA (Fisher, UK) for 5min at 37^{o}C to detach the cells from the plates. The trypsin was neutralised with culture medium and then spun (200g, 5min) to pellet the cells. Pelleted cells were resuspended in 10mL of fresh medium, counted with a haemocytometer and seeded as required per experiment.

EndoC βH1 cells were seeded at about 60-70% confluency and cultured to a confluency of over 90% before passaging them in a similar manner to the INS-1 with a slight modification of using PBS containing 10% FBS as the neutralising medium after trypsinisation.

2.3 Silencing gene expression with small interference RNA

Small interference RNAs (siRNA) are small (20-30 nucleotides) noncoding double-stranded RNAs that can regulate gene expression by binding to the complementary messenger RNAs of the gene of interest. Specifically, the newly formed double-stranded RNA is processed into siRNA by RNAse II-like enzymes (Dicer). The siRNA then binds to an RNA inducing complex (RISC) where the more stable 5' strand is incorporated into the RISC and separated from the other. The single-stranded siRNA then guides the RISC complex to align and bind to its target mRNA. A combination of catalytic RISC protein and Argonuate 2 cleaves the mRNA (Hassan D. et al., 2017). Knockdown experiments exploited the use of small interference RNA (siSTAT6, & siSIRPα all from Thermofisher, USA) while scrambled siRNA sequences were employed for control treatments (Eurofins, Germany). The siRNA transfection was performed with lipofectamine

RNAi max (a liposome delivery system) transfection reagent (Invitrogen, USA), Optimem (Lifetechnologies, USA) according to the manufacturer's instructions with only slight modifications. siRNA (10nM/well) was diluted in Optimem medium (60μL/mL culture medium) for 10min followed by the addition of lipofectamine (3.4μl/mL of culture medium). The solution was then mixed and applied cells in a dropwise fashion and then mixed gently by swirling the plate.

Table 6: siRNA mix for transfection

Reagent/ml	Control	Treatment
Optimem	60μl	60μl
siRNA	10nM*	10nM
Lipofectamine	3.5μl	3.5μl

*the scrambled siRNA is used

2.4 Assessment of cell viability

2.4.1 Using trypan blue as a vital dye

Following treatment, medium containing detached cells was collected from culture plates into 10mL tubes and this was later supplemented with medium containing any remaining adherent cells after these were harvested by trypsinisation. The cell suspension was then centrifuged at 188g for 5min and the supernatant aspirated. The cell pellet was reconstituted with an equal volume of trypan blue (0.4% in PBS) in culture medium. Trypan blue stains dead cells (but not live cells) because live cells actively pump it out whereas it is retained in non-viable cells (Tran et al., 2011).

2.4.2 Propidium Iodide staining

Propidium iodide (PI) is a fluorescent DNA intercalating agent which binds to short non-specific sequences dsDNA (4-5 DNA base pairs). PI has an excitation

wavelength between 491-495nm and a maximum emission wavelength of 636nm. Unlike trypan blue, PI cannot penetrate an intact plasma membrane, and thus can only get into the cell when the plasma membrane has become permeable. Once bound to DNA its fluorescence increases by about 30 fold with a shift in the excitation maximum and emission maximum in both the red and blue respectively. This enables its use for rapid detection of dead cells whose DNA is accessible due to a permeable plasma membrane. PI staining was performed as previously described (Crowley et al., 2016). Both attached and detached cells were harvested as described above (section 2.4.1), centrifuged and re-suspended in FACS buffer (Table 11). Cells were stained with a solution of 20μg/mL of propidium iodide diluted in FACS buffer and then incubated for 10min at 4^0C (on ice). Cells were then analysed using a BD Accuri™ C6 flow cytometer in which single cells flow through a nozzle and pass a laser beam to detect forward and side scatter (FSC and SSC). The extent of forward scatter relates to the cell size while the side scatter relates to the granularity of the cell. The instrument was calibrated to separate cells according to size and granularity which allowed gating to detect both live and dead cells (P1 see fig. 2.1) while excluding cellular debris. Cells that were permeable to PI were detected by measuring the light emitted at 575nm. By the use of appropriate gating, the proportions of live and dead cells were estimated under each incubation condition (Fig. 2.1).

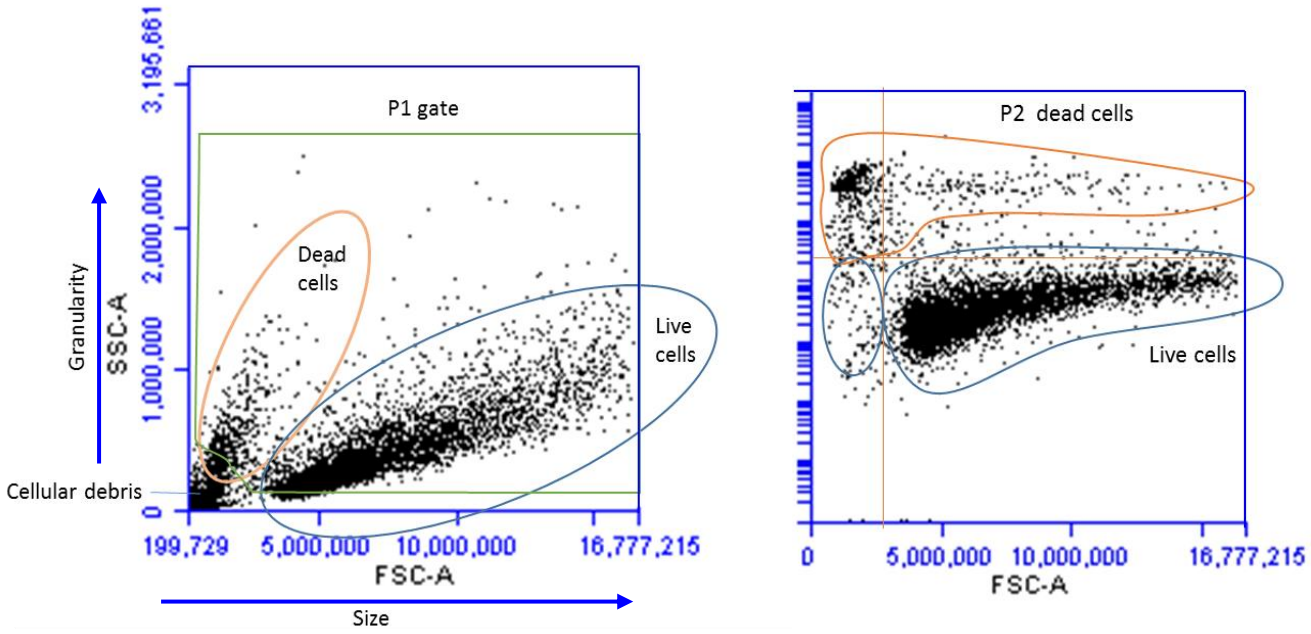


Figure 2. 1: Flow cytometer gating showing the populations P1 (both live and dead cells) and P2 (dead cells).

INS-1E or EndoCβHC1 cells were treated with 20μg/mL of PI /Facs buffer for 10min on Ice and analysed on the flow cytometer. Ungated cells (P1 left panel) comprise of two populations, which are resolved as viable, or non-viable cells respectively (right panel).

2.5 Cell cycle analysis

Cell cycle analysis at a single time point was performed using a flow cytometer as described by Pozarowski & Darzynkiewicz (2014). After treatment, cells were trypsinised and pelleted by spinning at 300g for 5min. The pellets were re-suspended in 250μl cold saline GM (Table 11) and then fixed with 750μL 95% cold ethanol for at least 30min at 4^0C. The fixative was removed and replaced with staining solution (50μg/ml of PI and 100μg/ml of RNase A in PBS) for at least 1h at room temperature before 25000 events were counted using the Accuri C6™ flow cytometer. The data were analysed graphically (Fig. 2.2) to reveal the proportions of apoptotic cells, those in the G0/G1 phase of the cell cycle, the S phase and the G2/M phase.

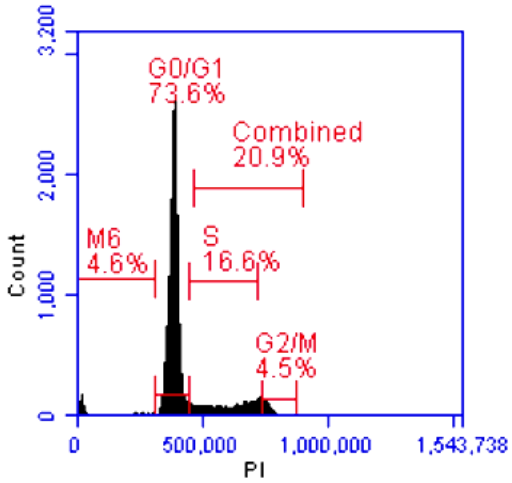


Figure 2. 2: Cell cycle analysis showing the apoptotic cells as M6, synthesis (S), G1/G0 and G2/M phases.

Untreated cells were harvested, fixed and stained to show the apoptotic cells (M6, 4.6%), Synthesis (S, 16.6%), G0/G1 (73.6%), Gap2 and mitosis phase (M)(G2/M, 4.5%).

2.6 Gene Expression (RT-PCR) studies

2.6.1 RNA extraction and estimation from cultured cells

RNA extraction was performed using the RNeasy™ Mini kit by Qiagen. Cells were washed with PBS. Then, adherent cells were disrupted using RLT buffer (350μL for 6well plates). With the help of a scraper, unattached cells were disrupted and transferred to a shedding column (QIA Shredder™) and centrifuged for a 2min at 13000g. An equivalent amount of ethanol (70%) (350μl) was added to the spin-through and the eluate transferred to an RNeasy Spin column. Ethanol precipitates the RNA enabling it to bind to the RNeasy membrane. The samples were briefly centrifuged at 8000g, before washing with RPE and RW1 buffers. The RNA was eluted from the spin column using 50μl DNase/RNase free water and stored at -20^{0}C until needed for cDNA synthesis.

RNA quality and quantity were measured using a NanoDrop™ 8000 Spectrophotometer (Thermo Scientific, UK). The 260/280nm ratio is used to assess the quality of the sample and a ratio of greater than or equal to 2.0 was regarded as appropriate. Alternatively, the Agilent bioanalyser was used to confirm the quality of the RNA by electrophoretically measuring the RNA integrity (RIN) of samples. The RNA integrity is a measure of the ratio of 28S to 18S ribosomal subunits which indicates the level of RNA degradation in each sample. It also can detect the presence of genomic DNA in the samples and compute a RIN number to grade the quality of the RNA (from the best quality of 10 to the poorest quality 1).

2.6.2 cDNA synthesis

cDNA synthesis was performed using the RT^2 First Strand kit (Qiagen, USA), according to the manufacturer's instructions. Using 100ng of total RNA, genomic

DNA was first eliminated with a DNA elimination buffer. Ten microliters of reverse transcription mix (Table 8: Reverse-transcription mix) was added and synthesis performed at 42^{0}C for 15mins followed by a stop step of 95^{0}C for 5mins:

Table 7: Genomic DNA elimination mix

Component	Amount/reaction
RNA	100ng
Buffer GE	2µL
RNase-free water	variable
Total volume	10µL

5mins at 42^{o}C then placed on ice for 1min

Table 8: Reverse-transcription mix

Component	Volume/reaction
5x Buffer BC3	4µL
Control P2	1µL
RE3 Reverse Transcriptase	2µL
RNase-free Water	3µL
Total volume	10µL

2.6.3 Quantitative RT-PCR.

Relative gene expression was measured by quantitative real-time PCR using RT^2 SYBRGreen master mix (Qiagen, USA) following the manufacturer's protocol. Amplicons were read on the Applied Biosystem^{TM} Quant Studio 12K flex. HPRT1 and YY1 were used as housekeeping genes for all PCR reactions and were used to normalise the data during analysis. Analysis of data was performed using the change in cycle threshold (Ct) formula to obtain the fold change in mRNA level.

$$Fold\ change = 2^{-(\Delta\Delta Ct)}$$

$$\Delta\Delta Ct = \Delta Cttreatment - \Delta Ctcontrol$$

$$\Delta Ctsample = Ct(GOItreatment) - Ct(HKGtreatment)$$

$$\Delta Ctcontrol = Ct(GOI\ control) - Ct(HKG\ control)$$

GOI: gene of interest, HKG: Housekeeping gene.

Table 9: PCR mix for one reaction and cycling conditions

Component	Volume
RT2 SYBR Green Mastermix	12.5 µL
cDNA synthesis reaction	1 µL
RT2 qPCR Primer Assay (10µMStock)	1 µL
RNase-free water	10.5µL
Total volume	25µL

Cycling conditions

Cycles	Duration	Temperature
1	10min	95^0C
40	15 s	95^0C
	1min	60^0C

2.7 Protein detection by western blotting

2.7.1 Protein extraction

In order to study the expression and phosphorylation of proteins, western blotting was performed as described by Russell et al. (2013) with some modifications. Briefly, after treatment each flash of cells was washed with cold PBS. This was removed and replaced with lysis buffer (Table 11) containing Protease and Phosphatase inhibitors for 10min on ice. The contents of each flask were scraped and transferred into a 1.5mL tube on ice, mixed and then centrifuged for 10min at 2500g at 4^0C. The supernatant was transferred into a new 1.5mL tube and stored at -20^0C until used.

2.7.2 Protein estimation

The concentration of protein within samples was estimated using bicinchoninic acid (BCA) (ThermoFisher, UK). This test exploits the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium resulting in a change in colour from blue to purple. The test is made up of a two-step reaction; the first involves chelating of

Cu^{2+} by various amino acid residues (Cysteine or cystine, tyrosine, and tryptophan) to form a blue complex in an alkaline environment with sodium potassium tartrate. The next step is the colour formation step in which bicinchoninic acid (BCA) reacts with Cu^{1+} to form a BCA/copper complex soluble in water with a linear absorbance as protein concentration increases at 562nm (Huang et al., 2010). In order to perform the test, 10μL of each sample (1:5 and 1:10 dilutions in lysis buffer) and BSA standards (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2mg/mL) were pipetted into a 96well plate. This was followed by 200μL BCA reagent. The BCA reagent was prepared by diluting Reagent A (sodium tartrate) with Reagent B (copper (II) sulphate pentahydrate) at 1:50 ratio. The plate was then incubated for 15min on a mixer before reading at 562nm on a PheriSTAR™ instrument. The protein concentration of samples was calculated using from a standard curve.

Preparation of protein samples and running of the gel

Equal amounts of protein (10-50μg/well) were loaded into each well of polyacrylamide gel after dilution with lysis buffer. Each sample contained 65% protein extract, 25%LDS loading buffer (4 x) and 10% β-mercaptoethanol. Before loading the mixture, the samples were heated at 70^0C for 10min. 20μL of each sample was loaded into a pre-cast 12% poly-acrylamide Bis-Tris gel (Invitrogen). The gel was run at 120V in 1X MOPS (Life Tech, USA) running buffer alongside a rainbow molecular marker for 2h.

Transfer of proteins and blotting.

PVDF membrane and blotting paper (Millipore, UK) were cut to approximately the size of the gel. The PVDF membrane was pre-soaked in methanol; rinsed 3x with dH_2O, and then placed on the gel. The gel and membrane were sandwiched

between pre-soaked (in transfer buffer) blotting paper and two pre-soaked blotting pads. The anode core was placed on top and then locked into place. The module was filled with transfer buffer (Table 11: Buffers and their Contents1) to cover the membrane. The outer chamber was then filled with water to help cool the membrane during the transfer process and the module run at 30V, 170mA for 2h.

After electrophoresis, the membrane was blocked with 5% milk or BSA diluted in TBST for 1h at room temperature, followed by addition of primary antibody in 5% milk diluted in TBST at 4^0C. After incubation, three 15min washes with TBST were performed, the membrane was then probed with alkaline phosphatase conjugated secondary antibody diluted 1:25000 in TBST containing 1%milk for 1hour at room temperature. The secondary antibody depended on the species in which the primary antibody was raised. Another 3X TBST wash was performed followed by incubation of the membrane in chemiluminescent reagent alkaline phosphatase substrate (Sigma Aldrich, UK) for 5min at room temperature and then detection using the Licor C-Digit blot scanner. In cases where the secondary antibody was conjugated to a fluorophore, no detection fluid was used and the blots were scanned directly on the Licor clx Odyssey^{TM} instrument.

Re-probing membranes

Western blotting membranes were reprobed when two or more proteins of interest were to be detected on a single blot. To reprobe, the previously bound primary antibody was removed using a stripping buffer (reblot strong plus, Merck Millipore, UK) (diluted using dH_2O (Promega, UK)). The membrane was incubated in reblot strong plus buffer for 13min followed by two 5min washes in

TBST. The membrane was then blocked with 5% milk TBST before adding the next primary antibody.

2.8 Protein detection by Immunohistochemistry and Immunofluorescence

2.8.1 Immunohistochemistry

Immunostaining was performed on human formalin-fixed paraffin embedded pancreas samples from the Exeter Archival Diabetes Biobank (http://foulis.vub.ac.be/). Serial sections were cut (4μm) and mounted on to glass slides. Immunohistochemistry was performed as previously described (Arif et al., 2014, Willcox et al., 2009a). Samples were dewaxed using two changes of histoclear (D-limonene) (National Diagnostic, Nottingham UK), then rehydrated in decreasing ethanol concentration (from 100% to 50%) before putting the slides into ddH_20 in 50mL Coplin jars.

Fixing of tissues with formalin can cause epitope masking hence reducing the immunoreactivity of antigens of interest with antibodies. This occurs because formalin forms cross-links between proteins and nucleic acids and the formation of these bonds lead to steric hindrance of antibody binding sites (Werner et al., 2000). Heat Induced Epitope retrieval (HIER) was performed to re-expose blocked epitopes in a process suggested to break the cross-linking bonds formed by formalin fixation. Sections of pancreas on glass slides were placed in a pressure cooker containing citrate buffer (pH6, see Table 11: Buffers and their Contents) in a microwave (800W) then heated for 20min before cooling for a further 20min. Samples were blocked with 5% (v/v) normal goat serum (NGS) and then probed with primary antibody diluted in Dako antibody diluent and left overnight at 4^0C.

Following primary antibody incubations, slides were given 3 washes in TBS before blocking with 300μL of Dako Real Peroxidase™ for 5min in order to arrest any endogenous tissue peroxidase activity within the sample. The blocking reagent was washed once in TBS, then a relevant secondary antibody added. The Dako REAL EnVision™ HRP conjugated secondary antibody was added for 1h and unbound antibody removed by three 5min washing in TBS. After washing, sections were incubated in 300μL of Dako Real EnVision™ high-sensitivity diaminobenzidine (DAB+) chromogenic substrate for 10min followed by a 5min wash in dd$H_2$0 to stop the reaction. DAB is catabolised by horseradish peroxidase (HP) to produce a brown-coloured product at places where secondary antibodies are bound to the tissue. The tissue sections were then stained with haematoxylin and eosin for 1min and washed with running tap water for 5min. Each tissue section was briefly dipped in STWS (40mM $NaHCO_3$, 170mM $MgSO_4$) to "blue" the tissue. The sections were then washed in in ddH_2O followed by incubation in $CuSO_4$ solution (20mM $CuSO_4$/150mM NaCl) for 5min to intensify the chromogenic stain (DAB). Sections were given a final wash in ddH_2O followed by dehydration by dipping slides in increasing concentration of ethanol 50%, 70%, 90% and twice in 100% (5min each). The sections were then cleared of any remaining alcohol and water by 2x5min incubation in histoclear and mounted under a coverslip with use of distyrene/xylene (DPX) (Dako, UK) before leaving to dry at RT.

2.8.2 Immunofluorescent staining

In order to study multiple antigens on a tissue section, slides were sequentially probed with the different primary antibodies and fluorescent-tagged secondary antibodies. To do this, slides were dewaxed, rehydrated, and blocked as described in section 2.8.1. The first antiserum (diluted in antibody diluent) was

put on sections for 1h at room temperature followed by three 5min PBS washes. Secondary fluorescent conjugated antibody was applied to the sections in opaque staining trays at room temperature for 1h. Secondary antibody was then washed 3X with PBS in opaque Coplin jars and the next sequence of stain performed. On the last sequence, the secondary antibody was added together with a nuclear stain 4′,6-diamidino-2-phenylindole (DAPI). Following the last stain, slides were washed and mounted using Dako fluorescent mounting media and then left to dry overnight in a dark cupboard. The slides were visualised on a fluorescent microscope (Leica Microsystems, Milton Keynes, UK).

2.9 DNA cloning

2.9.1 Agar plates, and Broth

Luria Broth (LB) was used for *E.coli* amplification and was prepared by weighing 20g of broth, which was then dissolved in 1L of ddH_20; this was autoclaved at 121^{0}C for 15min and left to cool to a temperature lower than 50^{0}C before adding an antibiotic (100μg/mL ampicillin, 50μg/mL kanamycin(Sigma, UK)). LB with agar was used for inoculating competent *E.coli.* It was prepared by weighing 35g in 1L of ddH_20 then autoclaved. Following autoclaving, the agar was left to cool before adding 100μg/mL of ampicillin or 50μg/mL kanamycin depending on the plasmid, mixed and then poured under the biosafety cabinets into 20mL Petri dishes.

Competent E.coli

Bacteria can obtain genetic information by three mechanisms, conjugation from other bacteria, transduction by the help of a bacteriophage and by transformation which is uptake of DNA through the cell wall. Bacterial uptake of plasmid or DNA (transformation) is possible if the recipient bacteria is made “competent” through

heat shock or electroporation both of which precede $CaCl_2$ treatment (Rahimzadeh et al., 2016, Bergmans et al., 1981). The mechanism by which $CaCl_2$ helps in DNA uptake is still unclear but it is thought to bring the DNA close to the bacterial cell wall (Rahimzadeh et al., 2016).

2.9.2 Bacterial Transformation, amplification and plasmid extraction

Transformation and expansion

Following resuspension of each plasmid, it was transformed into competent *E.coli* (New England Biolabs, UK) using a heat shock method as described with some modifications by Froger and Hall (2007). Into a 1.5mL Eppendorf 100μL of competent *E.coli* was transferred and mixed with 100ng of plasmid by flicking. The mixture was then left on ice for 10min prior to being heat shocked at 42^0C for 50s in a water bath, and then incubated on ice for 2min. The transformed bacteria were transferred into a universal tube containing 900μL LB without ampicillin. The LB broth with transformed bacteria was cultured at 37^0C in a shaker for 1h at 150rpm. Fifty microliters of solution were then spread on an LB agar plate with ampicillin or kanamycin (in a biosafety cabinet). The plates were grown overnight in a non-CO_2 incubator at 37^0C to allow bacterial colonies to form.

Fifty millilitres of LB+ Ampicillin (or Kanamycin) was placed into a universal tube, then colonies were picked from the plate and inoculated into the broth and incubate for 8h in at 37^0C shaking incubator.

Plasmid extraction

After expansion by overnight liquid culture, the culture was transferred into 50mL tubes for extraction. The tubes are spun down at 4000rpm for 15min and the supernatant decanted. Extraction was performed using a Midiprep as required by

the manufacturer (Qiagen, USA). Briefly, cells were resuspended in 4mL of buffer (P1), and then lysed with 4mL of lysis buffer (P2) and mixed by inverting the capped tube 4-6times. The mix was then neutralised with 4mL prechilled buffer (P3). The solution was transferred to a filter (QIAfilter) and balanced with QBT buffer before filtering with the aid of a plunger into a column (Qiagen-Tip). The column was washed twice before eluting the plasmid with 5mL of buffer QF. The DNA was then precipitated with 3.5mL Isopropanol and spun at 4000rpm for 45min. The supernatant was discarded before DNA was precipitated with 70% ethanol then spun for 4000rpm for 10min. The supernatant was discarded and the tubes air dried before dissolving the DNA in 100μL DNase free water. DNA was then quantified using a Nanodrop instrument (Thermofisher, USA).

2.9.3 Transfection of INS-1E and HEK293 cells

Plasmids were then transfected into cells using Attractene (Qiagen, USA) or Lipofectamine LTX (Fisher, UK) and Optimem (Fisher, UK) as transfection reagents according to table 10:

Table 10: Plasmid transfection in INS-1E or HEK293 Cells

Reagent	Control	Treatment
Optimem	60μl/mL	60μl/mL
DNA (STAT6, CA, DN)	0ng/mL	400ng/mL
DNA (Reporter firefly luciferase)for Dual Luciferase experiments	200ng/mL	200ng/mL
DNA (*Renilla* control) for Dual luciferase experiments	200ng/mL	200ng/mL
eGFP (for viability experiments)	500ng/mL	500ng/mL
Attractene or	2μl/mL	2μl/mL
Lipofectamine	3.5μl/mL	3.5μl/mL

2.9.4 Dual-Luciferase Reporter assay

The Dual-Luciferase Reporter Assay (DLR) is an assay system that measures the activity of a given promoter for a transcription factor, gene or intracellular signal (McNabb et al., 2005). The term 'Dual' simple refers to the assay's capacity to measure the expression of two luciferase enzymes at the same time. The assay exploits the differences in the biochemistry of two enzymes, a luciferase (61kDa) from the firefly (*Photinus pyralis*) and a (36kDa) luciferase from the sea pansy (*Renilla reniformis*). The enzymes are different in structure and substrate requirements permitting measurement of two protein products in the same reaction. These two enzymes do not require any post-translational modification for their functioning and so act efficiently as reporters. The photon emission reaction of the firefly luciferase occurs by the oxidation of beetle luciferin in the presence of ATP, Mg^{2+} and a co-enzyme to increase the speed of reaction. The luminescence reaction for the *Renilla* occurs by the oxidation of coelenterazine by *Renilla* luciferase to coelenteramide and CO_2 (Fig. 2.3.). In the DLR, the activity of the firefly enzyme is directly proportional to that of the promoter (experimental reporter) whilst the *Renilla* acts as the control and allows the number of cells transfected to be controlled for. These activities were measured simultaneously from the same lysate, such that as one reaction was quenched (Firefly), the other was activated *Renilla.* The experimental activity (firefly luciferase) was then normalised against the control (*Renilla)* (Sherf et al., 1996).

DLR was performed following the manufacturer's instructions. Briefly, 24h after STAT6 mutant plasmid transfection alongside the luciferase reporter and *Renilla* plasmids, cells were given two PBS washes before lysing with 100μL of 1x

passive lysis buffer at room temperature for 15min with gentle shaking. Lysates were transferred into 1.5mL Eppendorf tubes and placed on ice. The luciferase assay reagent II (LAR II) and the Stop and GloR substrate were reconstituted as required and used to prime the plate reader. 20μl of each sample was transferred into a 96-well white plate and the plate placed into the PHERstar luminometer. The instrument was set to inject the LARII reagent at 0s then read for 12s before the injection of the STOP and GloR with another 12s there after making a total of 24s recording. The readings with the Firefly enzyme were normalised to the *Renilla* data and expressed relative to the relevant controls.

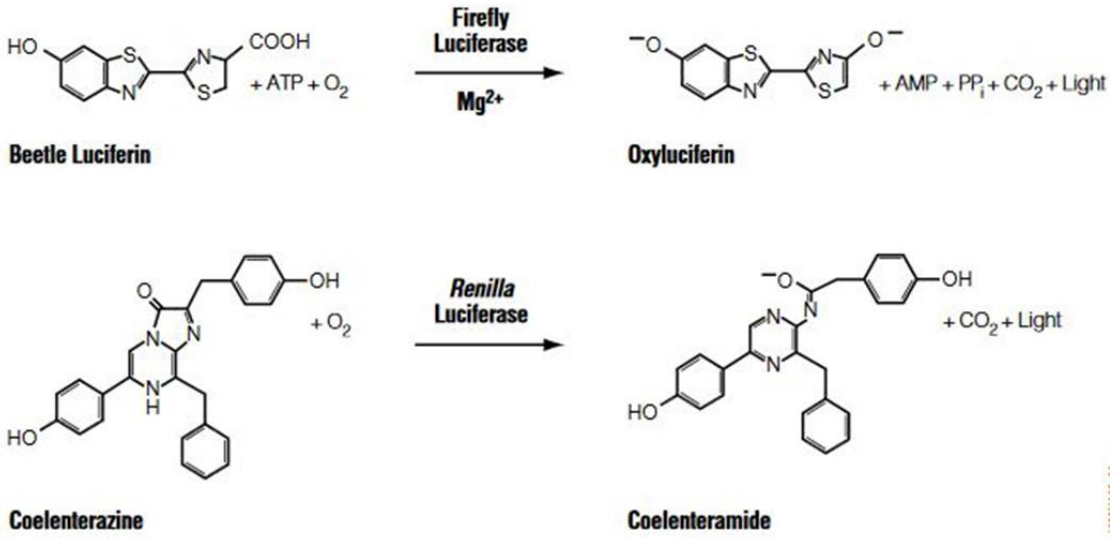


Figure 2. 3: Reactions of Luciferin and coelenterazine catalysed by firefly and *Renilla* luciferase enzymes

Source: www.promega.com

2.10 Statistical Analysis

All statistical analyses were performed on GraphPad Prism 7.03 for Windows software. To compare two means, a Student's t-test was performed and when more than two means were compared, an ANOVA was used with a Tukey post-test. ANOVA stands for the analysis of variance and is usually used to compare if the means of two or more unrelated data sets are different. It differs from the t-test in that it examines the variability of between the groups and within the groups. Even though the ANOVA test can tell that there is a difference in the means of the data set, it is limited by the fact that it cannot tell which group is different or how big the difference (Ennos, 2007). To identify which group is different and how big the difference is a post-hoc test is performed. There are several post hoc test but for the purpose of this thesis the Tukey post-test was used. The Tukey test is used to determine if there is a significant difference between experimental group on a studentized range distribution. It compares the mean of each group to the mean of every other group within a given data set (Ennos, 2007). Data in this thesis is presented as mean values ± standard error of the mean (SEM).

Table 11: Buffers and their Contents

Unless or otherwise stated in text or on table 5, the chemicals on this table were acquired from Sigma (USA).

Buffer	Contents
BCA Protein assay	Reagent A: Sodium carbonate, sodium bicarbonate, bicinchoninic acid & sodium tartrate in 0.1N sodium hydroxide. Reagent B: 4% cupric sulphate pentahydrate
FACS Buffer	PBS, 2% FBS
LDS sample buffer (4X)	4M glycerol, 0.56M Tris Base, 0.42M Tris HCL, 0.3M Lithium dodecyl sulphate, 2mM EDTA, 0.075% serva blue G250, 0.025% Phenol red
Lysis Buffer	20mM Tris, 150mM NaCl, 1mM EDTA, 1% Triton-X
MOPS running buffer (20X)	50 mM MOPS (3-(N-morpholino) propanesulfonic acid), 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7
Saline GM	- D-Glucose (Sigma) 6.1mM - KCl (Sigma) 5.4mM - Na_2HPO_4 (Sigma) 2.75mM - KH_2PO_4 (Sigma) 1.1mM - NaCl (Sigma) 6.9mM - 50μL 0.5M EDTA (Sigma) All into 50mL tube and top up to the mark with ddH_2O water
TBS	137mM NaCl, 2.6mM KCl, 49.5mM Tris (pH 7.6)
TBST	TBS+0.05% Tween (pH 7.6)
Transfer Buffer	25mM Tris, 0.19M Glycine, 20% Methanol (pH 8.3)
Whole cell lysis buffer	20mM Tris, 0.15M NaCl, 1mM EDTA and 1% Triton-X 100 (pH 7.4)
Citrate buffer(10mM)	Citric acid 1.92g, 1L ddH_20 and add 2N NaOH to pH6

Chapter 3.0: IL-13 and IL-4 protect pancreatic beta cells from cytotoxicity

3.0 IL-13 and IL-4 protect pancreatic beta cells from cytotoxicity

3.1 Introduction

T1DM is a pancreatic islet inflammatory disease in which the insulin-producing beta cells of the pancreas are specifically destroyed by autoreactive T-cells. Developing therapies to resolve the disease pathology has been unsuccessful probably because the pathogenesis of the disease itself remains to be fully understood (Grunnet and Mandrup-Poulsen, 2011).

Cytokines are small protein molecules involved in intercellular communication and implicated in almost every biological process in the body. They can be secreted by most types of cells (almost every nucleated cell) although immune cells are an important source. The actions of cytokines can be anti-inflammatory or pro-inflammatory (Charles, 2007).

A long list of cytokines have been implicated in the pathogenesis of T1DM and in many cases, their effects on beta cells have been extensively studied. For instance, IL-1β has been shown to inhibit insulin biosynthesis and induce beta cell death by apoptosis through NO production. Cytokines such TNF-α and IFN-γ on their own induce very little beta cell death but in combination with IL-1β exacerbate beta cell death. IFN-γ inhibits insulin secretion and induces beta cell dysfunction independent of NO production. (Eizirik and Mandrup-Poulsen, 2001, Rabinovitch and Suarez-Pinzon, 2003, Thomas et al., 2013, Laffranchi and Spinas, 1997). Other pro-inflammatory cytokines have been shown to inhibit insulin secretion by induction of beta cell ER stress through increased production of reactive oxygen species and nitrites include IL-23, IL-24 and IL-33 (Hasnain et al., 2014). Circulating levels of these cytokines have been monitored in T1DM and their changes studied extensively (He et al., 2014, Alnek et al., 2015).

Increasing evidence suggests that in T1DM there is a paradigm shift in the Th1/Th2 CD4+ T-cells cytokine secretion to a Th1 secretion model (Walker and von Herrath, 2016, He et al., 2014). Other reports show a decrease in circulating Th1 cytokines; in one such report, they studied mRNA levels of these cytokines which might be different from protein expression levels (Vaseghi and Jadali, 2016). In another report, wherein T1DM patients were grouped into under 15 years and over 15 years of age, an initial increase in Th1 cytokines (individuals under 15 years) was followed by a decrease (over 15 years individuals) suggesting that duration of T1DM might have an influence on serum cytokines levels (Fatima et al., 2016). Serum cytokine levels (IL-2, IL-5, IL-10, IL-6, IL-1β, IL-8, IL-10, IL-17, IL-23, and GM-CSF) of patients compared to their matched controls seem to be elevated irrespective of whether Th1 or Th2 derived (Alnek et al., 2015). While many studies have been published on the role of pro-inflammatory cytokines in beta cell biology (Choi et al., 2010, Collier et al., 2011, Stanley et al., 2017, Souza et al., 2008), few have looked at their "anti-inflammatory" counterparts. The levels of these anti-inflammatory cytokines (IL-4 and IL-13) have only been studied at a serological level which gives us a picture of the alterations in circulating cytokines but does not provide evidence of events in the islet environment which could be different (Russell and Morgan, 2014).

The protective role of IL-13 and related IL-4 have been previously studied in NOD mice by treating them with human recombinant IL-13 three times weekly which led to a delay in their development of type 1 diabetes. Use of anti-CD3 monoclonal antibody to induce the release of cytokines (IFN-γ and TNF-α) by T-cells in these mice was also diminished by IL-13 administration when compared to their controls. IL-4 treatment of NOD mice was suggested to protect them from diabetes by diminishing insulitis and promoting a Th2 response while decreasing

the number of T-cells infiltrating the pancreatic islets (Zaccone et al., 1999, Cameron et al., 1997). Similarly, α–galactosylceramide (α-GalCer) treated NOD mice were protected from developing T1DM mainly by modulating IL-4 secretion by invariant natural killer cells (NKT). NOD mice treated with α-GalCer and anti-IL-4 mAB or NOD IL-4 -/- mice treated with α-GalCer still developed T1DM. In these experiments, α-GalCer was used to activate invariant NKT-cells (shown to be deficient in T1DM) which then produce IL-4 and IL-10. The use of NODIL-4 -/- showed that the protection offered by α-GalCer was mainly due to IL-4 (Mi et al., 2004).

We and others, have shown that IL-13 and related IL-4 protect beta cells from inflammatory cytokines (IL-1β, TNF-α, IFN-γ and IL-6), palmitic acid and serum deprivation induced death (Kaminski et al., 2010, Kaminski et al., 2007, Russell et al., 2013, Rutti et al., 2016). Human islets stimulated with pro-inflammatory cytokines had diminished insulin secretion while their co-incubation with Th2 (IL-4 and IL-10) and Th3 (TGF-1β) cytokines reversed this inhibition. In these experiments, cell death was prevented by the addition of Th2, but not Th3 cytokines (Marselli L and M, 2001).

The objectives of this chapter were as follows;

- To investigate the protection of INS-1E beta cells by IL-13 (and IL-4), from serum withdrawal, pro-inflammatory cytokines, and palmitic acid induce cytotoxicity
- To investigate the effect of IL-6 with and without pro-inflammatory cytokines on EndoC βH1 beta cells

- To investigate the protection of EndoC βH1 cells by IL-13 from pro-inflammatory cytokine induced cytotoxicity

3.2 Materials and Methods

Rat INS-1E and human EndoC βHC1 beta cell lines were used in these experiments. Culture of these cells has been described in section 2.2.2.1 and 2.2.2.2. All cytokines (IL-1β, IL-6, TNF-α, IL-4, IL-13, and IFN-γ) were purchased from R&D systems (Abingdon, Oxford, UK). Palmitic acid and Bovine serum albumin were purchased from Sigma (Dorset, UK). The concentrations used in these experiments were previously optimised in our group (Russell et al., 2013, Kaminski et al., 2007).

3.2.1 Viability assessment

Viability of cells was assessed with either trypan blue or propidium iodide as described in section 2.4. Cell viability analysis was performed to assess the response to various cytotoxic stimuli in this chapter.

3.2.3 Serum withdrawal assay

Serum withdrawal experiments were performed according to (Russell et al., 2013). Briefly, INS-1E cells were seeded at $2.0x10^5$ cells/mL in a 12-well plate and complete medium was replaced after 4h with serum free medium in the presence or absence of IL-13 or IL-4. Cells were incubated for 96h, with a medium change performed after 48h. Cells were then harvested and viability measured as described in section 2.4.2.

3.2.2 Cytokine induced cytotoxicity

INS-1E or EndoC βH1 cells were pre-incubated with 20ng/mL of IL-13, for 48hours. The medium was then changed and replaced with medium containing 20ng/mL of each of the following cytokines IL-6, IL-1β, TNFα and IFNγ with and without IL-13 for 48hours (Russell et al., 2013). Both floating and attached cells were then harvested and cell viability measured.

3.2.3 Palmitic acid induced cytotoxicity

Palmitic acid induced cytotoxicity assays were performed as described by (Russell et al., 2013). Palmitic acid was prepared in 50% ethanol and dissolved by heating for 10min at 70^0C. The palmitic acid was introduced to INS-1E cells by using fatty acid free BSA as a carrier, to which palmitic acid was bound upon incubation at 37^0C for 1h. Prior to palmitic acid treatment of cells, INS-1E were stimulated with and without 20ng/mL of IL-13 for 48hours, followed by a further 48hours of palmitic acid treatment. Cells were then harvested and viability measured.

3.3 Results

In order to demonstrate that IL-13 protects beta cells from cytotoxicity, cultured beta cells were incubated under cytotoxic conditions comprising of: serum withdrawal, palmitic acid or pro-inflammatory cytokines. The cytoprotective potential of IL-13 was investigated in rodent INS-1E and human EndoC βH1 cells.

3.3.1 IL-13 partially protects INS-1E beta cells from serum withdrawal induced cytotoxicity

Serum withdrawal (sometimes called serum starvation or serum deprivation or serum free conditoins) involves the use of cell culture medium without FBS and has been used to induce cell death in various cell types including beta cells (Higuchi et al., 2006, Charles et al., 2005, Russell et al., 2013). FBS contains growth factors and lack of these leads to the activation of intrinsic apoptotic pathways, culminating in cell death (Charles et al., 2005).

Initially, the level of beta cell death induced by serum withdrawal in INS-1E cultures was assessed over a 96h to determine a suitable time point at which to conduct subsequent experiments. Serum withdrawal for up to 48h did not induce significant cell death when compared to serum containing medium (24h Con 4.1±0.6% cell death, SW 7.7±1.26% p=0.88; 48h Con, 13.3±3.4% SW 21.6±7.8% p=0.99). However, there was a significant rise in cell death at 72h and 96h in serum free conditions when compared to cells incubated in serum containing medium (72h Con 10.16±2.2%, SW 31.8±4.1% cell death p<0.001; 96h Con 21.1±2.8%, SW 44.5±5.3% cell death). With reference to the previously established time point, 96h was chosen for all other serum withdrawal experiments (Fig. 3.1) (Russell et al., 2013).

IL-13 protected INS-1E cells from serum withdrawal induced cell death at 96h (SW, 47.9±1.8%; SW+IL-13, 36.4±2.4% cell death, $p<0.001$) (Fig. 3.2). IL-4 also significantly improved beta cell viability after removal of serum from the culture medium (SW, 52.8±1.6%; SW+IL-4, 46.7±1.3% cell death, $p<0.01$) (Fig.3.3). Earlier reports had also shown that IL-13 and related IL-4 can protect pancreatic beta cells from cytotoxic effects of serum withdrawal (Kaminski et al., 2007, Russell et al., 2013, Sternesjo and Sandler, 1997).

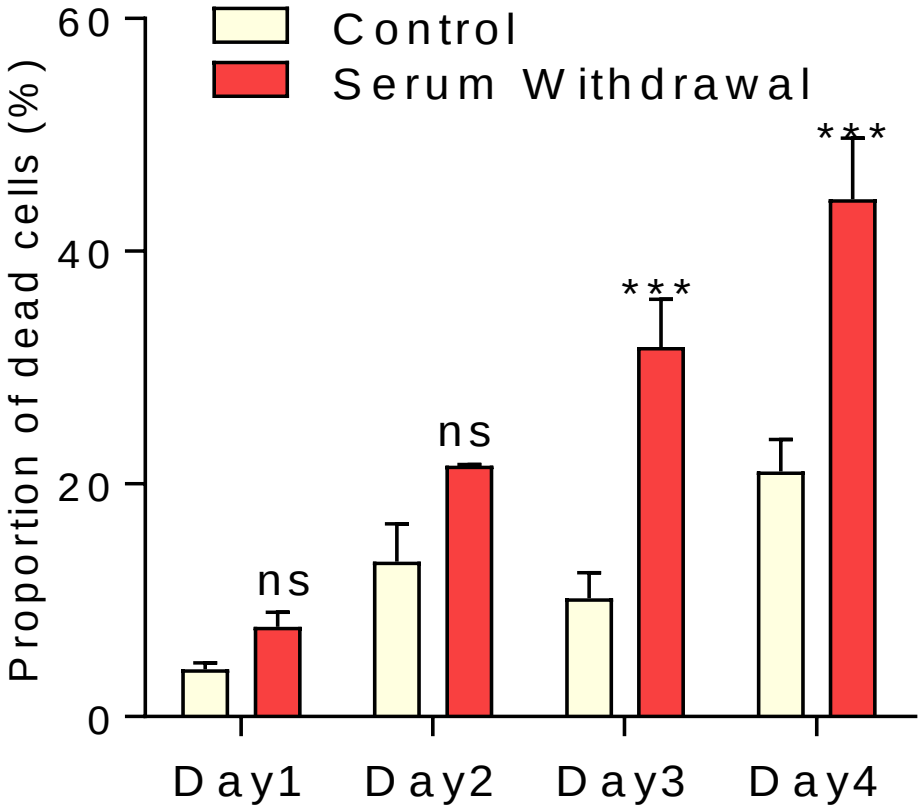


Figure 3. 1 Serum withdrawal induces cell death in INS-1E beta cells

INS-1E cells were incubated in serum withdrawn conditions for 96h and cell viability assessed using trypan blue. Data represents mean values ±SEM of three replicates in two experiments. ***p<0.001, ns: not significantly different from serum containing culture medium determined by student *t*-test.

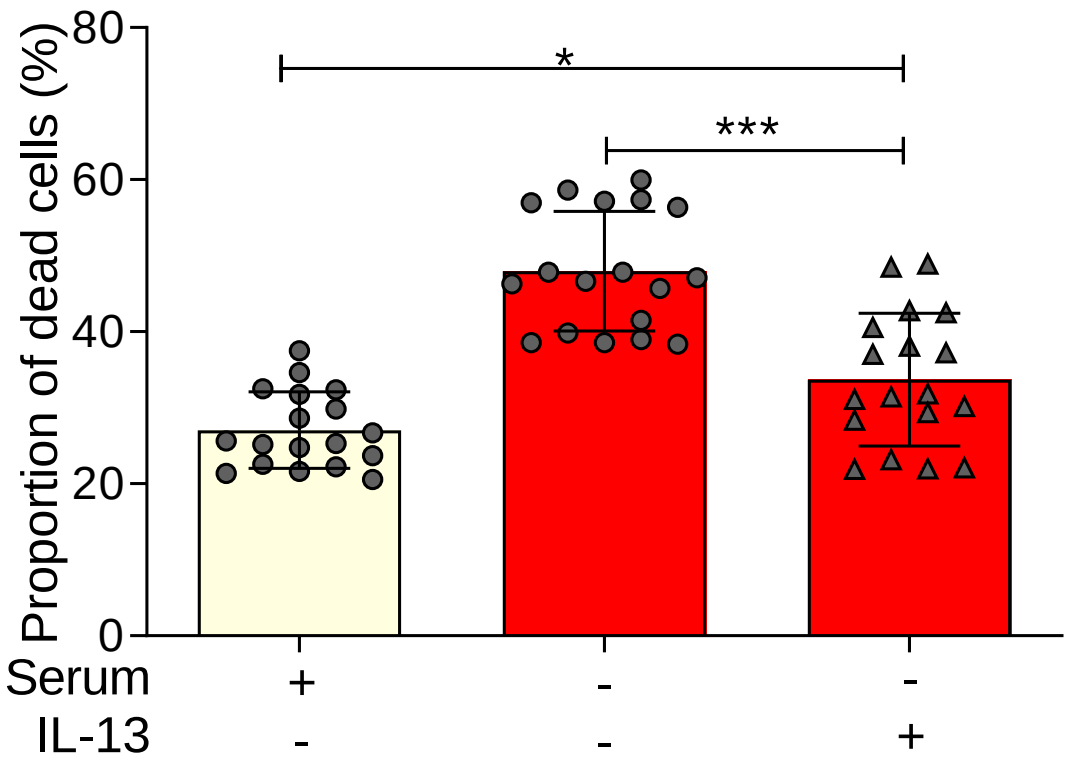


Figure 3. 2 IL-13 treatment partially protects INS-1E beta cells from serum withdrawal induced cell death

Cell death was induced by incubation of INS-1E beta cells in serum withdrawn media for 96h. These cells were untreated or treated with 20ng/mL of IL-13 and cell viability assessed by trypan blue staining.

Data represent mean values ±SEM of 6 replicates of three different experiments, ***p<0.001 determined by student *t*-test.

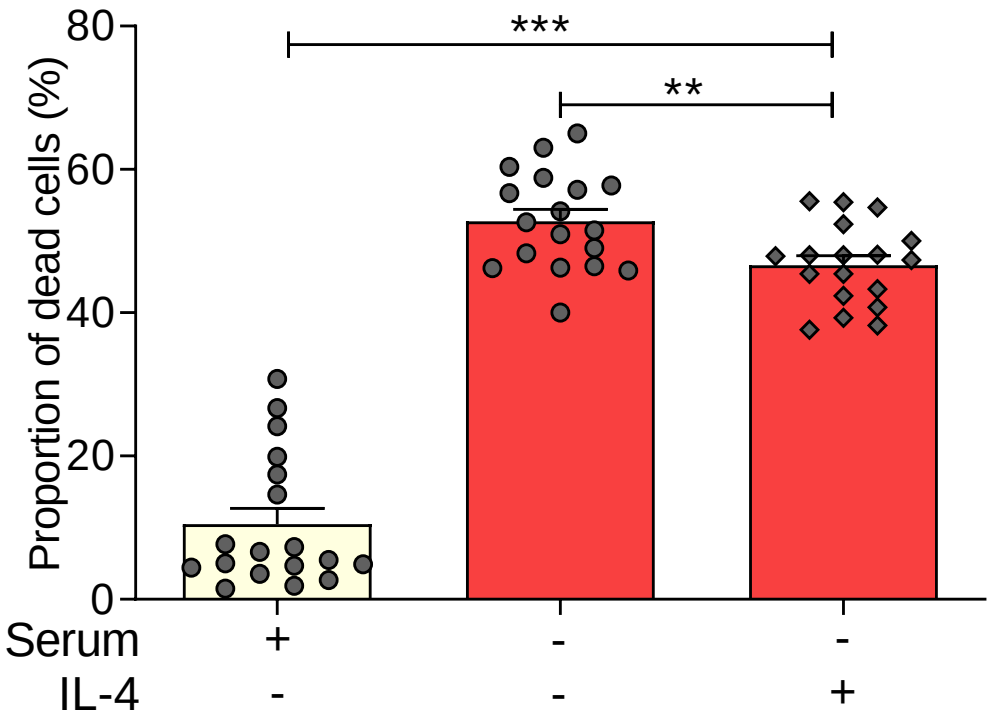


Figure 3. 3 IL-4 treatment partially protects INS-1E beta cells from serum withdrawal induced cell death

Cell death was induced by incubation of INS-1E beta cells in serum withdrawn media for 96h. The cells were untreated or treated with 20ng/mL of IL-4 and cell viability assessed by trypan blue staining.

Data represent mean values ±SEM of 6 replicates of three different experiments, **p<0.01 determined by student *t*-test.

3.3.2 IL-13 partially protects INS-1E beta from pro-inflammatory cytokines induce cell death

Emerging evidence suggests that an imbalance between pro-inflammatory and anti-inflammatory cytokines occurs in T1DM and that this may play an important role in modulating the viability of pancreatic beta cells in the disease (Fatima et al., 2016, Fitas et al., 2018). Pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ are widely reported to induce beta cell death and dysfunction and these effects have been extensively studied in pancreatic islets and beta cells (Rutti et al., 2016, Collier et al., 2011, Cetkovic-Cvrlje and Eizirik, 1994, Marselli L and M, 2001). IL-1β alone is reported to induce cell death, however, this depends on the concentration used and the length of exposure (Spinas et al., 1986, Palmer et al., 1989). Conversely, TNF-α, IFN-γ and IL-6 do not induce any potent cytotoxic effects on their own, but when applied in combination with IL1-β, these cytokines are cytotoxic (Cetkovic-Cvrlje and Eizirik, 1994).

Previous experiments by Russell et al. (2013) revealed that pre-incubation of INS-1E with IL-13 was crucial to its mediated protection from pro-inflammatory cytokines induced cell death. This revelation was assessed here and confirmed by treatment of INS-1E beta cells with pro-inflammatory cytokines (IL-1β, TNF-α IFN-γ and IL-6) for 48h simultaneously with IL-13 and following pre-treatments of IL-13. INS-1E beta cell treatment with pro-inflammatory cytokines induced significant cell death (pro-cytokines (IL-1β, TNF-α IFN-γ and IL-6), 64.6±4.3%) which was reduced by 48h pre-incubation with IL-13 (pro-cytokines+pre-IL-13: 47.4±2.2% $p<0.001$). However, under conditions where IL-13 was added concurrently with pro-inflammatory cytokines, there was a less significant reduction in cell death (pro-cytokines+pre-IL-13: 47.4±2.2%, pro-cytokines+ IL-

13: 60.4±3.5%, cell death $p<0.05$) Fig. 3.4. On the basis of these results, all subsequent experiments were performed with a 48h pre-incubation period with IL-13. INS-1E cells were then treated with four pro-cytokines (IL-1β, TNF-α, IL-6 and IFN-γ) with and without IL-13 for 48h. As expected in experiments with a in other experiments, IL-13 significantly reduced cell death induced by these cytokines (pro-cytokines: 78.7±2.8%, pro-cytokines+IL-13: 58.1±4.0%, cell death $p<0.001$) Fig 3.5. These results are in agreement with those of Kaminski et al., (2007), Russell et al., (2013) who showed that pre-incubation for 48h with IL-4 or IL-13 significantly reduced cell death induced by pro-inflammatory cytokines.

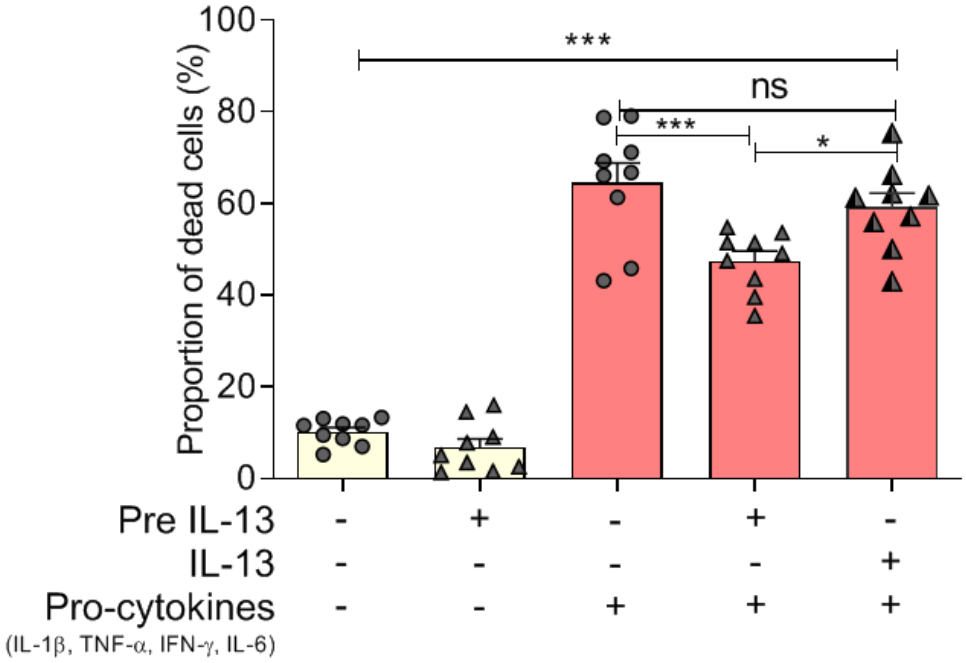


Figure 3. 4 IL-13 pre-incubation partially protected INS-1E cells from pro-inflammatory cytokine induced cell death.

INS-1E beta cell death was induced with pro-inflammatory cytokines, IL-1β, IFN-γ, TNF-α and IL-6 for 48h with and without pre-treatment with IL-13. Cell death was assessed by trypan blue. Data represent mean values of ±SEM of three technical replicates of experiments repeated thrice.

*p<0.05, ***p<0.001 determined by student *t*-test.

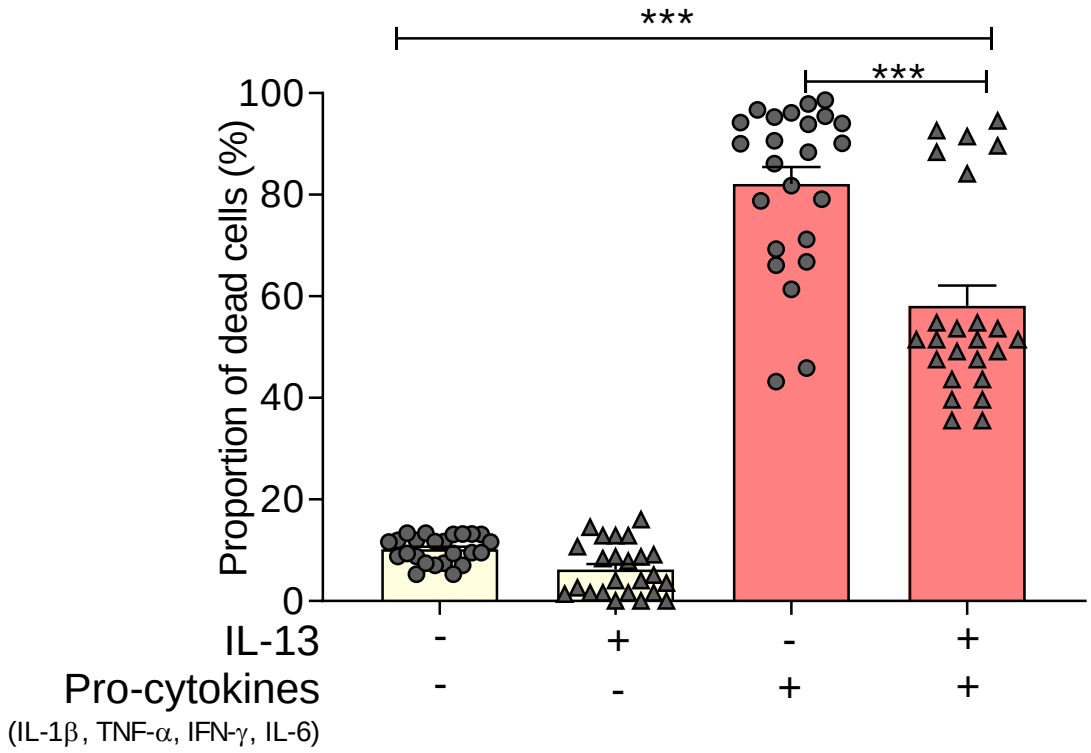


Figure 3. 5 IL-13 partially protects INS-1E beta cells from pro-inflammatory cytokine induced cell death

INS-1E cells were cultured in the presence of 20ng/mL of IL-1β, IFN-γ, TNF-α, & IL-6 with and without IL-13. Cell death was assessed by trypan blue. Data represent mean values ±SEM of 8 technical replicates of experiments performed three times. ***p<0.001 determined by student *t*-test**.**

3.3.3 IL-13 partially protects beta cells from palmitic acid induced cell death

Palmitic acid has been shown to induce cell death in various beta cell lines (Morgan et al., 2008, Diakogiannaki et al., 2007, Welters et al., 2004, Lenzen, 2008). The exact way by which this fatty acid induces cell death remains elusive but it is an extremely effective cytotoxin. Experiments with palmitic acid were performed in serum free medium since FBS contains various fatty acids (Gregory et al., 2011) including palmitic acid, that might alter the overall concentration of the fatty acid. Initially, two concentrations of the fatty acid were chosen based on literature (0.25mM, and 0.5mM) (Maedler et al., 2003, Diakogiannaki et al., 2007) to treat INS-1E for 24h and 48h to assess the cell death profile. Incubation of cells in 0.5mM palmitic acid for 24h induced significantly more cell death when compared to the 0.25mM (control:15.8±1.0%, 0.25mM 24h:24.7±3.2%, 0.5mM 24h: 64.4±4.0%, cell death $p<0.001$). Forty-eight hours incubation with palmitic acid led to a significant increase in cell death in the 0.25mM treatment when compared to 24h (0.25mM 24h: 24.7±3.2%, 0.25mM 48h:79.5±1.6%, cell death $p<0.001$). There was a significant increase in cell death in the 0.5mM 48h treatment (0.5mM 24h: 64.4±4.0%, 0.5mM 48h: 83.4±2.7% cell death $p<0.01$) but this was not significantly different from the 0.25mM 48h treatment (79.5±1.6%), as previously noted (Diakogiannaki et al., 2007, Russell et al., 2013), Fig. 3.6. Experiments with the chosen concentration (0.25mM palmitic acid) were then performed after pre-incubation with IL-13 for 48h. As expected palmitic acid treatment of INS-1E cells for 48h led to significant cell death (Control: 9.4±1.0, PA: 86.18±2.0% cell death, $p<0.001$) that was significantly reduced by 48h pre-incubation with IL-13 (PA: 86.2±2.0%, PA+IL-13: 72.9±2.6%, $p<0.01$) Fig 3.7.

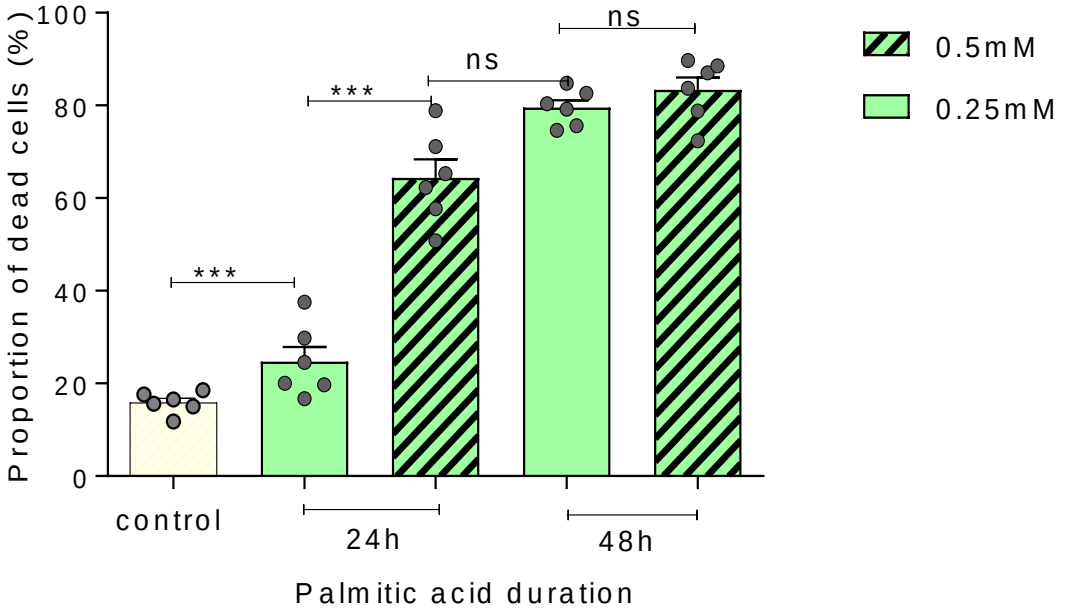


Figure 3. 6 Palmitate induces INS-1 cell death at different concentrations and times

INS-1E beta cells were treated with 0.25mM and 0.5mM palmitic acid for 24h and 48h and cell viability assessed with trypan blue. Data represent mean values ±SEM of triplicates of experiments performed twice. ***p<0.001 determined by student *t*-test.

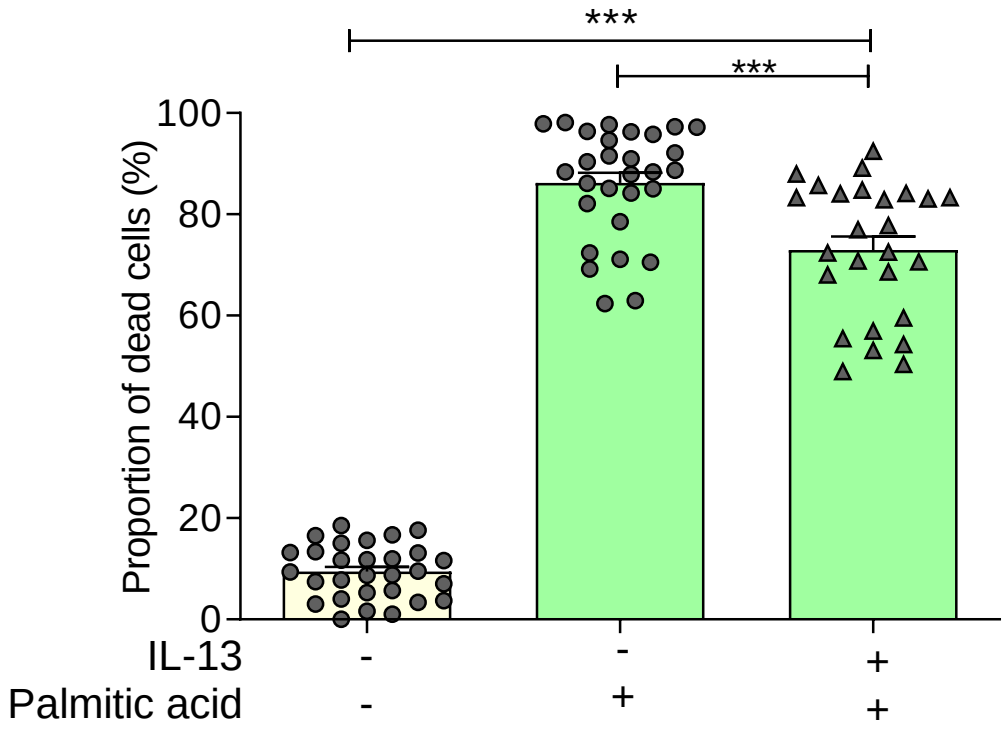


Figure 3. 7 IL-13 partially protects INS-1E cells from palmitic acid induced cytotoxicity

Cell death was induced by treating INS-1E beta cells with 0.25mM of palmitic acid for 48h with and without IL-13 and cell death assessed by trypan blue.

Data represent mean values of ±SEM of 6 technical replicates of three experimental repeats. **p<0.01, ***p<0.001 determined by student *t*-test.

3.3.4 IL-13 partially protects EndoC βH1 from pro-inflammatory cytokines

Then EndoC βH1 cell line has been shown to respond glucose with a rise in insulin secretion and respiration in a similar manner to human beta cells (Krizhanovskii et al., 2017). It has been shown by others (Cunha et al., 2017, Lenzen, 2008) that pro-inflammatory cytokines induce cell death in EndoC βH1 beta cells.

It is well established that IL-1β, IFN-γ and TNF-α are detrimental to beta cell survival but the role of IL-6 has been controversial (Eizirik et al., 2009, Prause et al., 2016). Whilst some reports show a protective response during inflammation (Choi et al., 2004, Kristiansen and Mandrup-Poulsen, 2005), our group and others have revealed that it potentiates beta cell death (DiCosmo et al., 1994, Russell et al., 2013). Experiments using EndoC βH1 cells were performed to understand the role of IL-6 in inducing cell death. EndoC βH1 cells were cultured in the presence of cytokines (IL-1β, IFN-γ and TNF-α) with and without IL-6 and with and without IL-13. Based on the results from experiments using INS-1E cells, pro-inflammatory cytokine stimulation of EndoC βH1 to show IL-13 protection were performed following IL-13 pre-treatment Fig 3.4. As previously shown in rodent beta cells by Russell et al. (2013), IL-6 significantly increased cell death by pro-inflammatory cytokines (pro-cytokines-IL6; 66.1±1.6%, pro-cytokines+IL6; 74.3±1.0%, cell death $p<0.01$). Interestingly, IL-13 protected EndoC βH1 cells from cell death induced by the pro-inflammatory cytokine treatment without IL-6 (pro-cytokines-IL6; 66.1±1.6%, pro-cytokines-IL6+IL13; 52.9±0.7%, cell death $p<0.01$). Additionally, IL-13 significantly protected Endo βH1 cells from pro-inflammatory cytokine treatment with IL-6 (pro-cytokines+IL6; 74.3±1.0%, pro-cytokines+IL6+IL-13; 60.3±2.0%, cell death $p<0.01$) Fig 3.8.

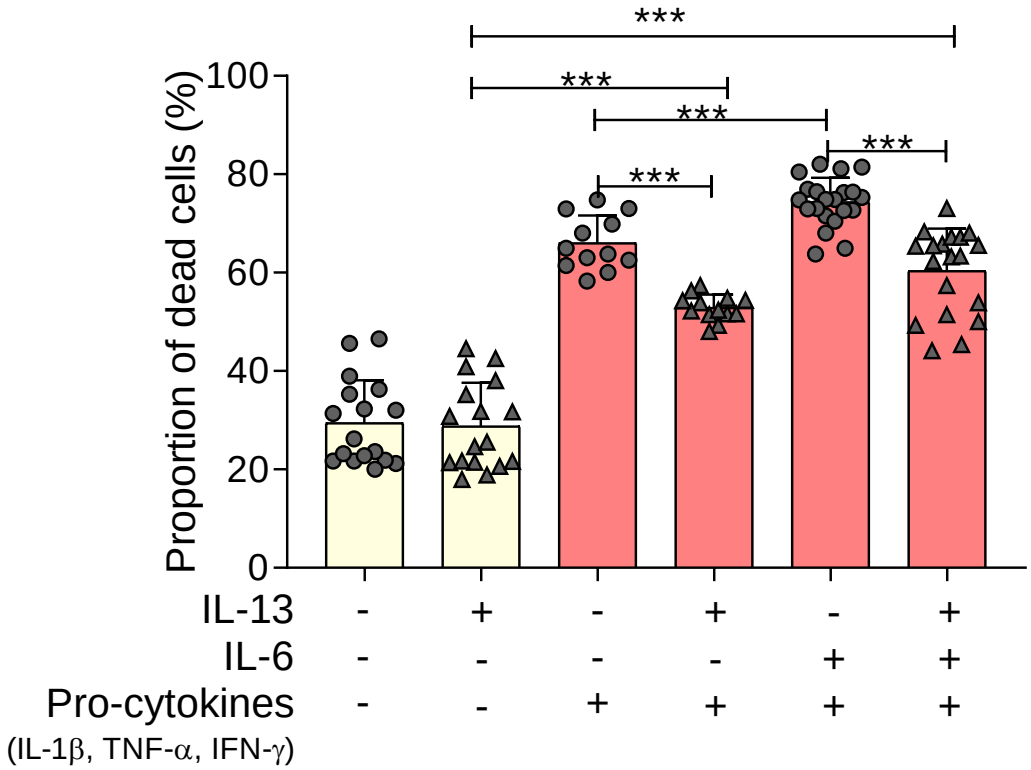


Figure 3. 8 IL-13 partially protects EndoC βH1 from pro-inflammatory cytokines and pro-inflammatory cytokines with IL-6

EndoC βH1 cells were cultured in the presence of 20ng/mL of IL-1β, IFN-γ, TNF-α, ± IL-6, in the presence and absence of IL-13 and cell viability assessed by flow cytometry after propidium iodide staining. Data represent mean values ±SEM of 6 replicates of experiments performed three times. ***p<0.001 determined by student *t*-test.

3.4 Discussion

The data arising from the experiments in this chapter show that IL-13 protects INS-1E and EndoC βH1 cells against serum withdrawal (IL-4 protects against), palmitic acid and pro-inflammatory cytokine induced cell death. The protection of beta cells by IL-13 from serum withdrawal is in agreement with previous data (Russell et al., 2013, Rütti et al., 2016). The results in this chapter show for the first time, that IL-13 can also protect human-derived EndoC βH1 cells from pro-inflammatory cytokines induced cell death. Also, the use of palmitic acid to induce beta cell death was reduced by IL-13 as previously reported (Russell et al., 2013).

In the pancreas, anti-inflammatory cytokines such as IL-4 and IL-13 are likely to be secreted by infiltrating immune cells such as invariant natural killer T-cells (Usero et al., 2016, Mi et al., 2004) or the islet ductal cells (Prokopchuk et al., 2005) or pancreatic stellate cells (Xue et al., 2015), although the precise source is not entirely clear. Some of these sources have been reported to be dysregulated in T1DM leading to decreased availability of these cytokines (Usero et al., 2016).

In this chapter, serum withdrawal was exploited to induce cell death in INS-1E cells. Serum withdrawal ultimately induces cell death by apoptosis through the activation of caspase 3 and caspase 9 (Higuchi et al., 2006, Goyeneche et al., 2006). Serum withdrawal conditions have also been shown to induce death through the depletion of the pro-survival factor Bcl-2, which in turn caused the release of Bak/Bax that change the mitochondrial outer membrane potential by forming pores, followed by the discharge of cytochrome C leading to apoptosis in RINm5F cell (a rat insulinoma cell line)(Tejedo et al., 2001). We report in our experiment, the significant reduction of beta cell death by IL-4 and IL-13

stimulation due to serum withdrawal. This protection could be explained by results from other cell types wherein, IL-13 is reported to increase the expression of anti-apoptotic Bcl-2 protein while diminishing the expression of Bax, hence it is likely that IL-13 counteracts serum withdrawal depletion of Bcl-2 and increment of Bax to sustain the beta cells (Yang et al., 2015, Chand et al., 2018). It has also been suggested that serum withdrawal increase superoxide and nitrogen oxide production in INS-1 cells (Maestre et al., 2003), although there is controversy about the involvement of cytochrome C. Serum withdrawal has been shown to induce cell death in other cell types through the activation of P53. P53 is a tumour suppressor molecule that is activated during cell stress and trans activates Apaf-1 which forms a complex with pro-caspase 9 and cytochrome C leading to cleavage of caspase 9. Caspase 9 then cleaves caspase 3 that effects DNA degradation (Fridman and Lowe, 2003). IL-13 has been show to suppress P53 in T-cells, therefore, the protection of INS-1E from serum withdrawal could have been through the repression of p53 leading to the inhibition of Apaf-1 and hence inhibition of the pro-caspase 9/Apaf complex (Yang et al., 2015).

The accumulation of lipids in beta cells and other cells can lead to their dysfunction and death. This process is known as lipotoxicity. This is primarily associated with type 2 diabetes and can consequently lead to reduced insulin secretion, and pancreatic beta-cell failure (Kusminski et al., 2009, Robertson et al., 2004, DeFronzo, 2010). Palmitate is the most abundant circulating saturated fatty acid found in the body and can be acquired through diet or synthesized directly when needed (Carta et al., 2017). Palmitate may potentiate beta cell death by a variety of mechanisms, such as increased chemokine production (CXCL1, CCL2), ER stress leading to unfolded protein response (UPR) but also by enhancing the secretion and toxicity of IL-1β and other cytokines such as TNF-

α, IL-6 and IL-8 . (Sharma and Alonso, 2014, Russell et al., 2013, Igoillo-Esteve et al., 2010, Song et al., 2014). Palmitate also induces caspase 3 activation and other ER stress markers in rodent beta cells (Song et al., 2014). Other mechanisms reported include the upregulation of Programme Cell Death 2 Like (Pdcd2l), mitochondrial dysfunction, Bcl-2 mRNA downregulation, Caspase-2 increase in the cytosol, increase superoxide and NO production, increase in Bax and the arrest of G_0 phase of the cell cycle (Maestre et al., 2003). Taken together, it is clear that the precise mode of killing of beta cells by palmitate remains elusive but it might be a combination of these mechanisms. From the various reports cited above, it is suggestive that palmitate initiates the production of cytokines, which together with lipotoxicity lead to beta cell death. IL-13 therefore might be counteracting the actions of the pro-inflammatory cytokines either by upregulating pro-survival factors such as Bcl-2 or downregulating Bax (Yang et al., 2015). Fatty acids like pro-inflammatory cytokines both induce nitrite production which has been previous shown to be reduced by IL-4 or IL-13 co-stimulation in INS-1E cells (Russell et al., 2013) and suggest that IL-13 might protect against fatty acids in a like manner.

The present results reveal that IL-13 and IL-4 can protect both rat INS-1E cells and human-derived EndoC βH1 cells from pro-inflammatory cytokine induced beta cell death. These results are in agreement with the findings by us and others in *in vitro* experiments which show that IL-13 and IL-4 can act directly on the beta cells and protect human islets, rat and human-derived beta cells lines from cell death induced by pro-inflammatory cytokines (Rutti et al., 2016, Russell et al., 2013). The death of beta cells mediated by pro-inflammatory cytokines is one of the most researched in the context of type 1 diabetes pathogenesis (Eizirik et al., 2009, DiCosmo et al., 1994, Choi et al., 2004, Pirot et al., 2008, Souza et al.,

2008) and low chronic inflammation by cytokines in type 2 diabetes (Eguchi and Manabe, 2013).

TNF-α induces beta cell death through the activation of Caspase 8 and IKK complex which then leads to the activation of caspase 3 and NF-κB leading to apoptosis (Tomita, 2017). In immune cells IL-13 has been reported to reduce or limit caspase 3 cleavage although the precise mechanism is not known (Yang et al., 2015). IL-13 has been shown to inhibit TNF-α activities in other cell types by inhibiting nuclear translocation of NF-κB through decreasing cytoplasmic expression of its nuclear translocation protein p65 (Manna and Aggarwal, 1998). The reduction in NF-kB activity by IL-13 has been illustrated using clonal beta cell lines by Rutti and colleagues who suggest that is the most probably way IL-13 prevents the action of IL-1β (Rutti et al., 2016). IL-1β increases nitric oxide production by beta cells which enhances beta cell death. It has been shown that the addition of IL-13 or IL-4 decreases significantly the production of nitric oxide in beta cells (Russell et al., 2013). IFN-γ induces beta cell death through the activation of STAT1 that transcribes Interferon regulatory factor 1 (IRF-1) and iNOS which together alter the mitochondrial outer membrane permeabilization (MOMP) through upregulation of Bak which subsequently leads to caspase activation and cell death by apoptosis (Cnop et al., 2005). IRF-1 can also induce apoptosis in a p53 dependent manner (Tanaka et al., 1994). IL-13 has been shown to regulate BCLXL pro-survival molecule and likely helps maintain the balance of these molecules enabling a stable MOMP hence preventing cytochrome C release and apoptosis (Wurster et al., 2002). Also, IL-13 can probably counteract the action of IRF-1 should the alternative cell death pathway be activated by suppression of p53 (Yang et al., 2015).

In the present chapter, it was shown that IL-6 enhances the effects of pro-inflammatory cytokines on EndoC βH1 cells. These results are completely in accord with previous reports, which were generated using various rodent and human beta cell lines (Russell et al., 2013, Wadt et al., 1998, Southern et al., 1990). However, despite this, others have shown that IL-6 rescues beta cells from pro-inflammatory cytokines (Kristiansen and Mandrup-Poulsen, 2005, Choi et al., 2004). IL-6 signals via Jak/STAT3 and also through the MAPK/mTOR pathway and can be secreted by islet beta cells in vivo (Pilström et al., 1995).

In our experiments, 20ng/mL of IL-6 was used together with pro-inflammatory cytokines and this resulted in increased INS-1E and EndoC βH1 cell death. Similarly, use of 20ng/mL IL-6 on INS-832/13 in the presence of TNF-α and IL-1β, increase beta cell death and decreased glucose stimulated insulin secretion (Oh et al., 2011). In a recent report the use of 10ng/mL IL-6 to stimulate INS-1 832/3 cells for 1h did not affect glucose stimulated insulin secretion. Additionally, IL-6 did not rescue INS-1 832/3 dysregulation of glucose stimulated insulin secretion induced by IL-1β with and without conditioned medium (containing a cocktail of myokines, secretions from muscle cells) (Barlow et al., 2018). Contrary to these findings In experiments wherein IL-6 is proposed to improve beta cell function 1ng/mL of IL-6 was used to show improvement of glucose stimulated insulin secretion in MIN6 cells and had to be pre-incubated for 24h to observe this effect (Suzuki et al., 2011). Additionally, 24h Pre-incubation with 80μg/mL of IL-6 or serum from exercised mice (claimed to be rich in IL-6) protected INS-1E from cytokine induced cell death (Paula et al., 2015). In all these, IL-6 is suggested to protect at concentrations lower than 10ng/mL or greater than 200ng/mL, also depending on the source of the IL-6, and if pre-incubated 24h

before inflammatory stimulations. Pre-incubation of IL-6 in our experiments did not result to any improvement in beta cell viability (data not shown). The serum used by researchers from exercised mice arguably contains more than just IL-6 and hence might not be the main protective compound. Taken together, our data revealed that, IL-6 enhances beta cell death in pro-inflammatory cytokines, conceivably by a STAT3 dependent mechanism that leads to the cleavage of caspase 3 as reported by Oh et al. (2011).

A role for IL-4 and IL-13 in protecting NOD mice (a model for type 1 diabetes), from developing autoimmune diabetes has also been reported (Zaccone et al., 1999, Cameron et al., 1997) with the suggestion that the protection might be due to the skewing of CD4+ T-cells response towards a Th2 phenotype (Wong, 2011). Contrary to the protection offered by administration of IL-13 or IL-4 NOD mice deficient in the IL-4Rα/IL-13Rα1 hetero receptor showed resistance to developing type 1 diabetes, due to increase T-regs and decreased Th17 cells. However, the ablation of the hetero receptor does not take into account the circulating levels of the IL-13 neither was a comparison performed for IL-13 treated NOD mice (Ukah et al., 2017). Also, the role of IL-13 in glucose homeostasis has been illustrated using C57BL/6 and BALB/C mice in which IL-13 was ablated and glucose tolerance test performed with marked increases witnessed in IL-13-/- compared to controls. There was an upregulation in gluconeogenesis genes, with suggestions that this is possibly through the transcription factor STAT3 (Stanya et al., 2013) although a recent study by Rutti et al. (2016) suggests that IL-13 does not rescue IL-1β induced loss in glucose-stimulated insulin response in human islets and rodent cells.

The potentiating effect of IL-6 was illustrated using human EndoC βH1 cell lines, but other works seem to show the opposite effect (Choi et al., 2004). Although, in the experiments of Choi and colleagues, rat beta cells were pre-incubated with IL-6 for 24h prior to toxicity. Interestingly, the actions of IL-6 seem to depend on whether it was pre-cultured with the beta cells, and on the concentration used to stimulate the beta cells. We show in our present experiments that IL-13 still protects beta cells from pro-inflammatory cytokines with and without IL-6.

In conclusion, the actions of IL-13 seem to offer a 'versatile protection' against a variety of cytotoxic stimuli (pro-inflammation, palmitate, and serum withdrawal), which initiate beta cell death using different mechanisms but converge in ER stress or mitochondrial dysfunction. The underlying mechanism by which IL-13 is protecting beta cells still not completely understood. We have shown in our previous work that this is potentially through the Jak/STAT6 signalling (Russell et al., 2013), but others have suggested that it is potentially though, the PI3Kinase signalling pathway (Rutti et al., 2016). In the subsequent chapters, experiments seek to explore which signal transduction pathways are involved in the cytoprotective actions of IL-13.

Chapter 4.0: Characterisation of the IL-13 Jak/STAT6 Signalling Pathway in beta cells

4.0 Characterisation of the IL-13 Jak/STAT6 Signalling Pathway in beta cells

4.1 Introduction

In the previous chapter, it was shown that both IL-13 and IL-4 protect beta cells from various cytotoxic stimuli (cytokines, serum withdrawal and palmitic acid). In other published work it has been suggested that this protection is likely to be mediated by the Jak/STAT signalling pathway (Russell et al., 2013). The role of Jak/STAT has been studied in the context of various important aspects of pancreatic beta cell biology, for example with reference to the actions of insulin, growth factors, erythropoietin, cytokines, prolactin and other hormones (Choi et al., 2010, Fujinaka et al., 2007, Shen et al., 2010).

Upon cytokine binding to a cognate receptor, a conformational change occurs allowing the receptor to dimerize and bringing Jak proteins into close proximity with each other leading to their trans phosphorylation. Jaks phosphorylate specific tyrosine residues within the cytoplasmic tail of the receptor creating docking sites for the STAT proteins. Importantly, Jaks also activate bound STAT molecules (by phosphorylation) that then dissociate from the receptor and homo- or heterodimerize and migrate to the nucleus to induce the transcription of target genes (Dodington et al., 2018, Shuai and Liu, 2003).

4.1.1 Jak1

The roles for Jak1 and Jak2 in T1DM have been investigated recently by Trivedi and colleagues in experiments wherein they used a Jak1/Jak2 inhibitor (AZD1480) in NOD mice and on isolated human islets. The results showed a reduction in various hallmarks of T1DM in the mice, including IFN-γ signalling, MHC class I expression, and islet T-cell accumulation. Altogether the data

indicated a reduction in autoimmunity in NOD mice through a reduction in phospho-STAT1 (Trivedi et al., 2017). Another drug, Exenatide (Ex-4), a potent glucagon-like peptide 1 receptor (GLP-1) agonist, also significantly reduced Jak1 expression in INS-1E cells and this was associated with a decrease STAT1 (Couto et al., 2007). Data from these experiments were obtained by real-time PCR, but the conclusions support those of Trivedi et al (2017). These results implicate Jak1 as a regulator of beta cell viability.

4.1.2 Jak2

Atkinson and colleagues, who treated female NOD mice with a potent Jak2 inhibitor, AG490 (also known to inhibit Jak3), and found a reduced incidence of diabetes compared to vehicle treated controls. However, although pancreatic leukocyte infiltration was not diminished and euglycemia could only be achieved in mice whose initial blood glucose levels were below 17.5mM (Davoodi-Semiromi et al., 2012). In a separate study, it was found that treatment of C57BL/6 mice with recombinant Human (rHu) EPO for one week caused activation of Jak2 and STAT5 and that this was associated with a reduction in the incidence of diabetes in db/db mice exposed to streptozotocin (STZ). The PI3K/Akt pathways was also activated under these conditions and, in order to attempt to verify the involvement of Jak2, a knockout approach was taken in which RIPcre^{+}Jak2 $^{fl/fl}$ mice were treated with STZ and rHuEPO. There was no protection of rHuEPO from STZ induce diabetes in the mutant animals indicating that Jak is essential for the ability of rHuEPO to promote beta cell viability (Choi et al., 2010). We have shown that Jak2 is activated by IL-13 and have suggested that Jak2 could be involved in mediating the protective actions of IL-13 under condtions of serum withdrawal-induced cytotoxicity on INS-1E cells. A Jak2

inhibitor (hexabromocyclohexane) antagonised this protective response (Russell et al., 2013).

4.1.3 Jak3

Jak3's role in T1DM was illustrated by study of the actions of Janex-1, a Jak3 selective inhibitor, which was administered to NOD mice for 25weeks and the extend of insulitis assessed. Janex-1 treatment lowered the proportion of NOD mice developing T1DM without affecting their B cell and T-cell splenic populations (Cetkovic-Cvrlje et al., 2003). Janex-1 acts by suppressing NF-κB and upregulating SOCS1 and SOCS3 (Lv et al., 2009) which may account for its protective effects. Another Jak3 inhibitor WHI-P154, reversed the cytoprotection of IL-4 from pro-inflammatory cytokines in INS-1E (Kaminski et al., 2010). Similarly, the islets of C57BL/6J mice in which Jak3 had been knocked out were more resistant to cytokine toxicity compared to controls. Thus, Jak3 appears to play an important role in mediating beta cell cytotoxicity under certain conditions.

4.1.4 Tyk2

The fourth member of the Jak family is Tyk2, a kinase which is activated by IFN-α. In human EndoC βH1 cells this leads to upregulation of MHC class I molecules in a STAT1 and STAT2 dependent manner. Knockdown of Tyk2 reversed the IFN-α induced inflammatory response and also attenuated the induction of ER stress markers such as CHOP, CXCL10, ATF3, BIP and XBP1s (Marroqui et al., 2017). Given that loss of Tyk2 seems to reduce ER stress in the beta cells in the presence of IFN-α, it can be inferred that beta cells might also have an impaired antiviral response observed in the absence of Tyk2 (Størling and Pociot, 2017).

4.1.5 IL-4 and IL-13 regulated genes

The regulation of genes by IL -4 and IL-13 (mediated via Jak/STAT signalling) has been extensively studied in other cell types (Ritz et al., 2008, Jiang et al., 2000, Fichtner-Feigl et al., 2005) but gene regulation in beta cells has received limited attention. IL-13 has been shown to upregulate four beta cell genes *CISH, CD83, Galnt14* and *St6galnac3* using rat beta cells (Rütti et al., 2016). *CISH* is a negative regulator of the Jak/STAT signal transduction regulatory pathway belonging to SOCS family of proteins and binds phosphorylated tyrosine residues. *CD83* is an antigen presentation protein, while *Galnt14 and St6galnac3* encode for N-acetylgalactosaminyltransferase and sialyltransferases respectively (Rütti et al., 2016, Trengove and Ward, 2013). IL-4 and IL-13 are known to upregulate anti-apoptotic genes such as *BCLXL, MCL1* and *SOCS1* in other cell types (Ritz et al., 2008, Dunkle et al., 2011).

Our group has shown that IL-13 activates Jak2, STAT3, STAT6, and the PI3-AKT pathways in beta cells but have argued that the inhibition of the PI3-AKT pathway does not alter IL-13-induced cytoprotection in INS-1E cells (Russell et al., 2013). By contrast a study by Rutti and colleagues suggested that the PI3-AKT is the main pathway by which IL-13 protects beta cells (Rutti et al., 2016).

The main objectives of this chapter were as follows;

- To investigate and identify the Jaks that are activated by IL-4 and IL-13 stimulation and determine how important they are to IL-13 mediated protection of beta cells
- To investigate and identify the genes regulated by IL-13 and IL-4 using a Jak/STAT qPCR array system.

4.2 Materials and Methods

4.2.1 Cytokine Stimulation

20ng/ml of IL-4 and IL-13 were used to stimulate INS-1E cells for the desired time according to each experiment.

4.2.2 Gene expression studies using RT2 Profiler PCR array

Gene expression studies were performed as described in section 2.6. At the conclusion of the incubation periods, cells were lysed and RNA extracted according to the manufacturer's protocol, before being reverse transcribed to form cDNA. SYBRGreen was used in the qPCR protocol designed to amplify the cDNA using commercially synthesised primers targeted to the genes of interest.

4.2.3 Western blotting

Western blotting and small interference RNA studies were performed as previously described in section 2.7 and section 2.3 respectively. INS-1E and EndoC βHC1 were cultured as described in section 2.2.

Table 12: List of antibodies and dilutions

ANTIBODY	COMPANY	CATALOGUE NUMBER	DILUTION
STAT6	Cell signalling	5397	1:1000
Phospho STAT6	Santa Cruz	Sc-11762	1:200
JAK1	St John's Labs	STJ113441	1:500
JAK2	Santa Cruz	Sc-294	1:500
JAK3	Santa Cruz	Sc-6932	1:1000
TYK2	Fisher	720124	1:1000
PHOSPHO JAK1	St John's labs	STJ90314	1:500
PHOSPHO JAK2	Abcam	Ab219728	1:1000
PHOSPHO JAK3	St John's labs	STJ98188	1:500
PHOSPHO TYK2	Cell signalling	68790s	1:1000
SIRPA	Cell signalling	13379s	1:1000
GAPDH	Proteintech	60004-1	1:10000
MCL-1	Cell signalling	94296s	1:1000
BCL_{XL}	Biolegend	633902	1:1000
BETA ACTIN	Sigma	A5316	1:25000

4.3 Results

4.3.1 INS-1E express all four Janus kinases

In order to reveal which Jak proteins are involved in IL-13 signalling in INS-1E cells, it was important to study the complement of Jaks expressed in these cells. INS-1E cells were cultured, lysed and protein extracted as in section 2.7. A series of western blot analyses were then performed, probing with antisera directed against the different Jaks (see Table 12: List of antibodies and dilutions).

Western blotting analysis of INS-1E cells showed the presence of Jak1 with a molecular weight of about 130kDa (Fig. 4.1) and are in agreement with previous data obtained by qPCR in INS-1E and human islet beta cells (Couto et al., 2007). Immunohistochemical staining of INS-1E cells has also revealed the presence of Jak1 (Stout et al., 1997).

INS-1E cells also expressed two Jak2 bands at an approximate molecular weight of 125kDa and 110kDa respectively (Fig4.1). This confirms earlier data showing the expression of Jak2 in INS-1E cells by western blotting (Fujinaka et al., 2007, Russell et al., 2013). I also found that Jak3 is expressed in INS-1E beta cells and that the antigen appears as three bands with molecular weights of 53kDa, 70kDa and 115kDa (Fig. 4.1). Jak3 has previously been observed in INS-1E cells using immunohistochemistry by Stout et al., (1997).

The presence of Tyk2 was observed by western blotting with the band migrating at a molecular weight equivalent to about 134kDa (Fig 4.1).Tyk2 has previously been observed in INS-1E cells by immunostaining (Stout et al., 1997) and in EndoC βHC1 beta cells by western blotting (Marroqui et al., 2015).

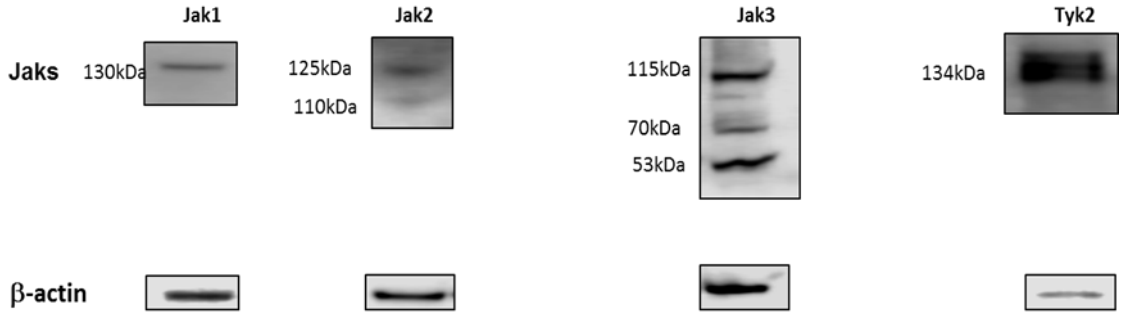


Figure 4. 1: All four Jaks are present in INS-1E beta cells.

INS-1E cells were lysed and supernatant collected. Western blotting was performed and membranes probed for anti-sera for Jak1, Jak2, Jak3 and Tyk2. The data represents three independent experiments.

4.3.2 Jak1, 2, 3 and Tyk2 are activated by IL-13 and IL-4 with a differing time course

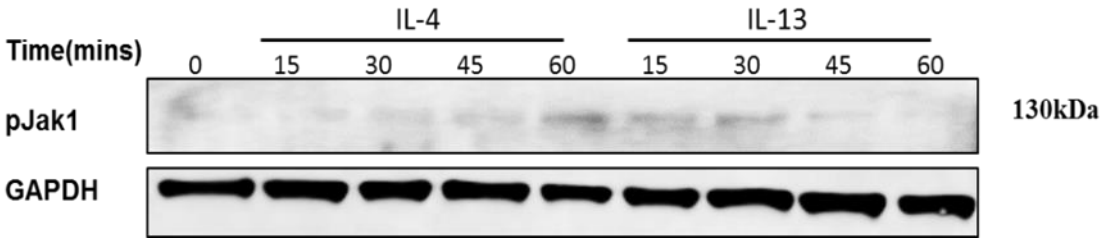
To determine whether Jak1 is activated by IL-13 and IL-4, INS-1E cells were stimulated with either IL-4 or IL-13 for periods of 15, 30, 45 and 60min prior to cell lysis. Following western blotting, membranes were probed with an anti-serum against the activated form of Jak1 (Y1022/1023). The results reveal that IL-4 induced the phosphorylation of Jak1 but that this was only seen at later points during the incubation period (45min and 60min). IL-13, on the other hand, stimulated Jak1 phosphorylation at much earlier time (15min), and phosphorylation then declined by at 45min and had completely disappeared by 60min (Fig 4.2). These data suggest a difference in the way these two cytokines induce Jak1 signalling.

Membranes were also probed for phospho-Jak2 (Y1007/1008). Initial results with IL-13 treatment showed a somewhat unexpected pattern in that basal levels of phospho-Jak2 were high even in the absence of stimulation. Addition of IL-13 induced an initial decline in phospho-Jak2 before the phosphorylation increased once more (Fig. 4.3a). In a repeated set of experiments, the stimulation time was expanded, and the data showed a similar initial pattern of Jak2 phosphorylation, with an initial decline followed by a later increase (75-120min after initial stimulation) exceeding the initial basal levels (Fig. 4.3b). IL-4 treatment induced a similar pattern of Jak2 phosphorylation, although there were some subtle differences in the kinetics of phosphorylation. In experiments with IL-4, Jak2 phosphorylation was initially depleted within 15min but then elevated between 30-75min before declining again (Fig. 4.3c).

In similar experiments, INS-1E cells were stimulated with IL-13 and IL-4 for 0-60min, samples were lysed and a Western blot performed probing for phospho-Jak3 (Y785). IL-13 induced the phosphorylation of Jak3 within 15min and this remained elevated over the rest of the 60min time-course. However, IL-4 stimulated Jak3 phosphorylation only after 45min treatment (Fig 4.4).

Probing of Western blot membranes from similar experiments to those described above with anti-phospho-Tyk2 (1054/1055), revealed that, IL-4 induced the phosphorylation of Tyk2 at 45min and 60min while IL-13 treatment induced the phosphorylation of Tyk2 strongly but transiently at 15min (Fig 4.5). IFN-γ treatment of INS-1E for 30min also induced phosphorylation of Tyk2. The phosphorylation patterns of the different Jaks in response to IL-4 and IL-13 stimulation have been represented in Fig 4.6.

a.



b

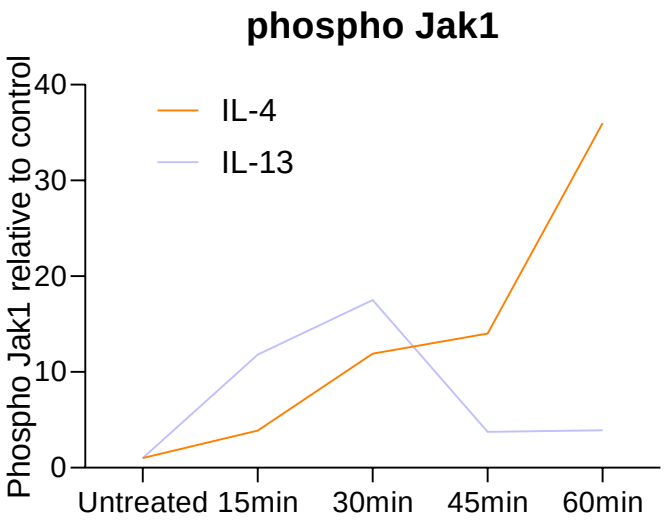


Figure 4. 2: Jak1 is activated by both IL-4 and IL-13.

INS-1E cells were treated with 20ng/mL of either IL-4 or IL-13 for 15-60mins, followed by a PBS wash. Cells were lysed and the supernatant collected. **a**. Western blotting was performed and membranes probed with anti-sera for phospho-Jak1. GAPDH was used as loading control. **b**. Densitometry analysis shows the pattern of phosphorylation

The data represents two independent experiments.

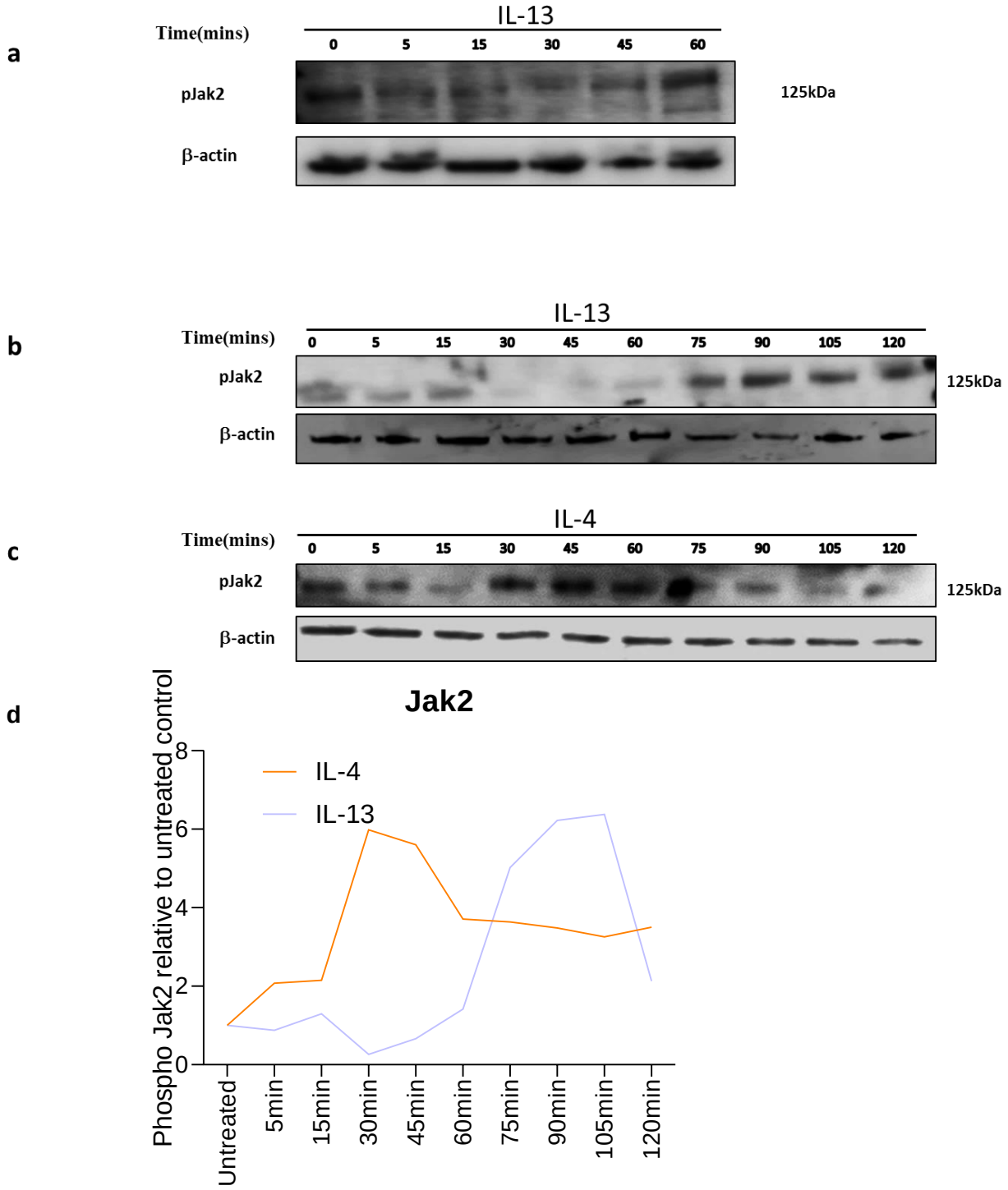


Figure 4. 3: Jak2 is activated by both IL-4 and IL-13 after initial depletion of phosphor-Jak2.

INS-1E cells treated with 20ng/mL of either (a-b) IL-13 or (c) IL-4 for 120min, washed with PBS. Cells were lysed and the supernatant collected. Western blotting was performed with 50μg of protein and membranes probed with anti-sera for phosphor-Jak2. Beta-actin was used as loading control. d. shows densitometric representation of Jak2 phosphorylation. The data represents three independent experiments.

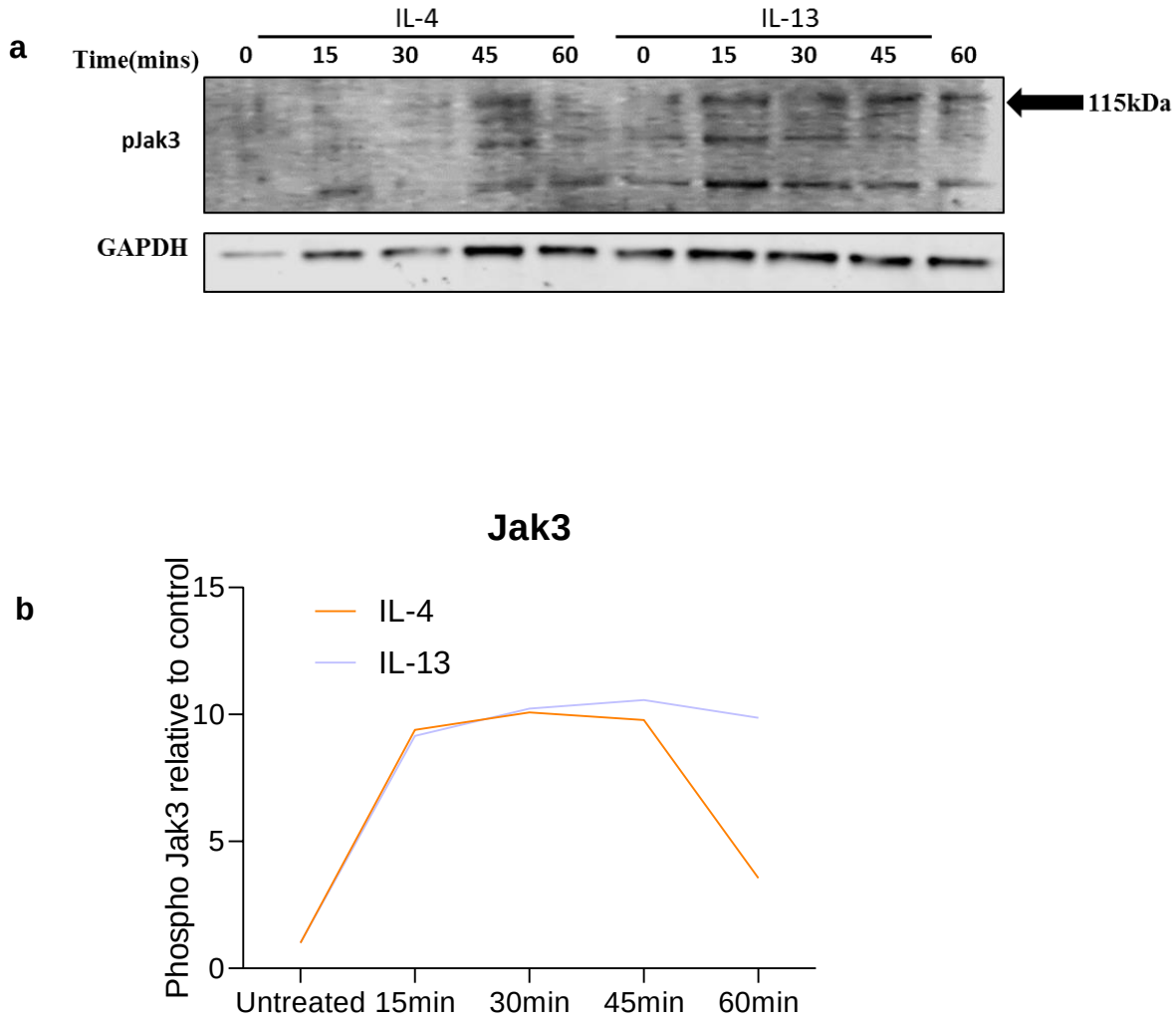


Figure 4. 4: IL-4 and IL-13 both activate Jak3 at different time points

a. INS-1E cells were treated with 20ng/mL of either IL-4 or IL-13 for 15-60mins, followed by a PBS wash. Cells were lysed and the supernatant collected. Western blotting was performed with 50μg of protein and membranes probed with anti-sera for phospho-Jak3. GAPDH was used as loading control. **b**. Densitometry analysis reveal Jak3 phosphorylation pattern upon IL-4 and IL-13 treatments. The data represents two independent experiments.

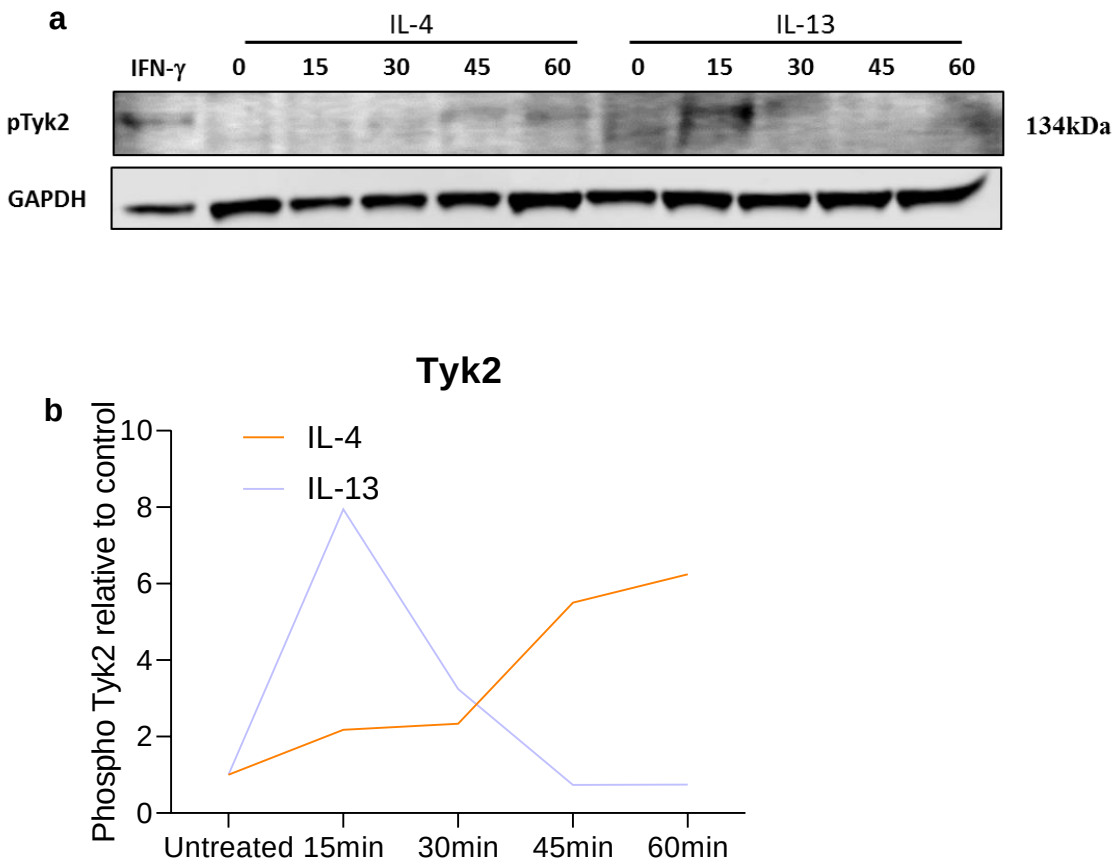


Figure 4. 5: IL-4 and IL-13 both activate Tyk2 at different time points

a. INS-1E cells were treated with 20ng/mL of either IL-4 or IL-13 for 15-60mins, followed by a PBS wash. Cells were lysed and the supernatant collected. Western blotting was performed on 50μg of protein and membranes probed with anti-sera for phospho-Tyk2. GAPDH was used as loading control. **b**. Densitometry analysis reveal Tyk2 phosphorylation pattern upon IL-4 and IL-13 treatments. The data represents two independent experiments

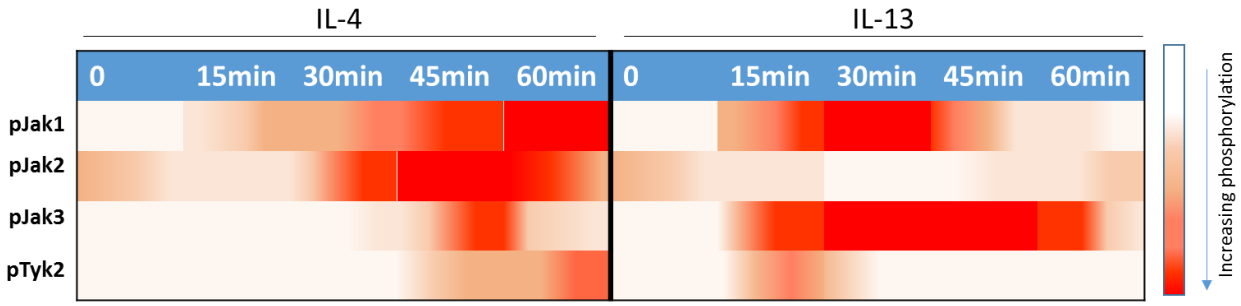


Figure 4. 6: Heat map highlighting phosphorylation pattern of Jaks upon IL-4 and IL-13 stimulation

Western blots probed for the various phospho Jaks and based on the densitometry analysis, the intensity of the band with respect to time graded accordingly from light pink to intense red using Microsoft Office PowerPoint.

4.3.3 IL-13 and IL-4 both activate STAT6

It has been shown previously that both IL-13 and IL-4 activate STAT6 in clonal pancreatic beta cell lines, human islets (Kaminski et al., 2007) and in monocytes (Roy et al., 2002). It was, therefore, important to confirm these data in the current study. Experiments were performed by stimulating INS-1E cells with IL-4 and IL-13 for 0-60min. Western blots were performed with cell lysates and probed with anti-phospho-STAT6 (Y641). IL-13 and IL-4 induced the phosphorylation of STAT6 at 15min to 60min. Western blotting analysis revealed the appearance of three bands, the lowest and expected band at 105kDa, the middle at 150kDa and the highest at 200kDa as previously observed in our group (Russell et al., 2013) (Fig. 4.7).

4.3.4 Jak2 knockdown reduced STAT6 phosphorylation

Since Jak2 has been previously implicated in IL-13 signalling (Russell et al., 2013), and we report changes in Jak2 phosphorylation in the current study (Fig. 4.3), we set out to examine whether Jak2 acts upstream of STAT6 in INS-1E cells. In order to achieve this goal, we depleted Jak2 expression levels using a small interference RNA system (siRNA). It was initially important to optimise the use of the siRNA molecules by targeting an abundantly expressed gene. GAPDH was chosen to test this. Knockdown was performed as described in section 2.3, initially using two transfection reagents, attractene and Lipofectamine, to determine which was better for siRNA transfections. A modestly successful knockdown of GAPDH was obtained using both attractene and Lipofectamine at 24h (Fig. 4.8a). An improved knockdown was obtained at 48h using Lipofectamine, with GAPDH expression depleted by approximately 75% (Fig 4.8b) compared to control. Cells transfected using attractene did not show a clear

change in GAPDH expression. This optimised protocol was used for experiments using STAT6 siRNA described later in chapter 5.

Following the optimisation of the siRNA protocol, silencing of Jak2 expression was attempted using the same approach. Transfection of INS-1E cells with Jak2 targeting siRNA for 48h successfully depleted Jak2 expression (Fig. 4.9). As previously reported a 30min IL-13 treatment induced a robust increased in phospho-STAT6 (Y641) expression under control conditions, however, this response was partially blocked by Jak2 knockdown (Fig. 4.9).

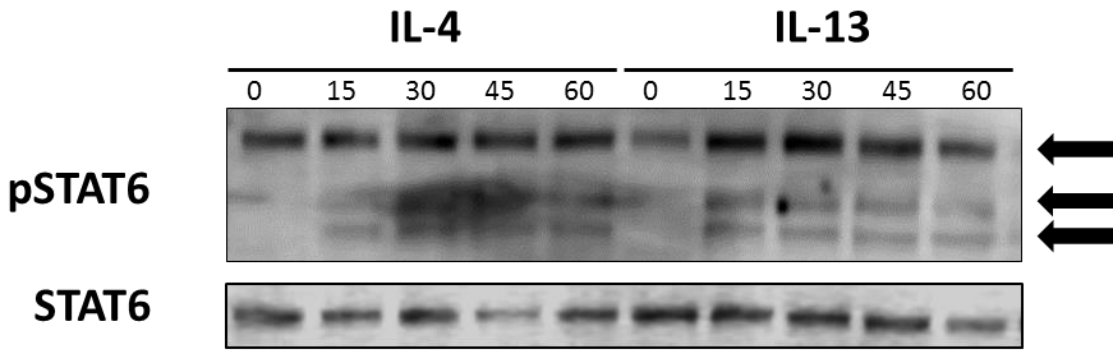


Figure 4. 7: IL-4 and IL-13 induce STAT6 phosphorylation in a similar manner.

INS-1E cells were treated with 20ng/mL of either IL-4 or IL-13 for 15-60mins, followed by a PBS wash. Cells were lysed and the supernatant collected. Western blotting was performed using 50μg of proteins and membranes probed with anti-sera for phospho-STAT6. Total STAT6 was used as loading control. The data represents three independent experiments.

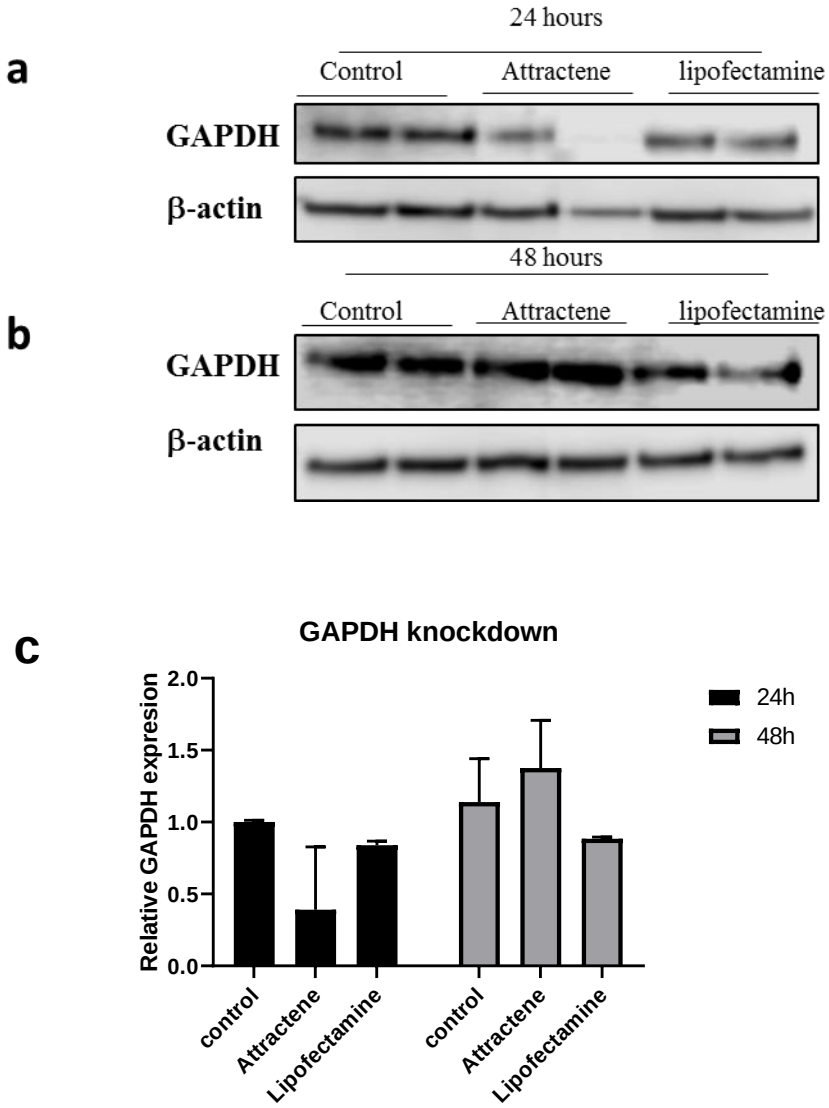


Figure 4. 8: Lipofectamine performed better for long duration transfections. INS-1E cells were treated with 10nM small interference RNA targeting (a-b) GAPDH using attractene and lipofectamine transfection reagents for 24h and 48h. Cells were then washed with PBS. Cells were lysed and the supernatant collected. Western blots were performed and membranes probed for (a-b) GAPDH. Beta-actin was used as the loading control. (c) Densitometry analysis reveal the extend of knockdown although no significant difference was observed. Data represents two independent experiments.

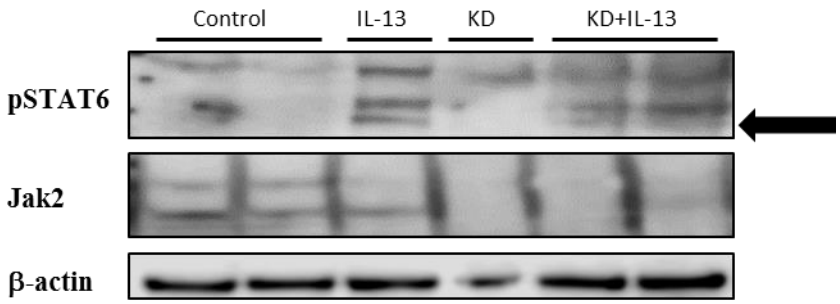


Figure 4. 9: Jak2 knockdown reduced STAT6 induced phosphorylation by IL-13.

INS-1E cells were transfected with 10nM of siJak2 for 48h followed by IL-13 stimulation for 30min. Cells were then harvested, lysed, centrifuged and the supernatant collected. Western blotting was performed and membranes probed for Jak2, phospho-STAT6 and beta actin as loading control.

Data represents results of experiments performed at least twice.

4.3.5 IL-13 stimulation leads to the upregulation of anti-apoptotic genes

In order to discover genes regulated by IL-13, a rat Jak/STAT targeted PCR array was used (RT2 Profiler PCR array from Qiagen). RT2 Profiler PCR arrays are pathway focused arrays with a panel of 84 genes, 5 housekeeping genes, 3 reverse transcription controls, 1 genomic DNA control and 3 positive controls all on a 96well plate. In these experiments, INS-1E cells were stimulated with IL-13 for 48h, RNA extracted, estimated and cDNA synthesized as described in section 2.6. cDNA was amplified on the Quantstudio 12K flex and analysed using the Qiagen online analysis tool which calculates fold change of each gene relative to the untreated control.

The most highly upregulated gene was *SIRPα,* a gene whose protein product is known to bind the surface marker CD47 leading to inhibition of phagocytosis in macrophages (Willingham et al., 2012). This gene has been studied only rarely in beta cells and has not previously been implicated in the response to IL-13 in these cells. The anti-apoptotic genes *MCL1* and *BCL2L1* were also upregulated, along with *EPOR* (erythropoietin receptor)*, IFNGR1* (interferon gamma receptor)*, SMAD1* (Mothers against decapentaplegic homolog 1), *CDKN1a* (Cyclin-dependent kinase inhibitor 1, known for inhibiting apoptosis), *SH2B1 (*SH2-domain adapter 1), *EGFR* (Epidermal growth factor receptor), and *SOCS1* a negative regulator of the Jak/STAT signalling pathway (amongst others represented in Fig. 4.10 & table 12). The RT2 Profiler arrays were used to identify the most upregulated genes and the results were then confirmed using specific SYBRGreen PCR primer assays in separate experiments. The upregulation of most of the genes seen by the array method was confirmed using samples obtained after 48h treatment of INS-1E cells followed by qPCR: *SIRPα* (Control: 1.0, IL-4: 3.0±0.4, IL-13:2.7±0.15, *p<0.001*)*, MCL1 (*Control: 1.0, IL-4: 1.5±0.12,

IL-13:1.46±0.07, *p<0.01*), and *SOCS1* (Control: 1.0, IL-4: 1.5±0.1, 1.7±0.2, *p<0.001*). *BCL2L1* was significantly upregulated by IL-4 and not by IL-13 (Control: 1.0, IL-4: 1.5±0.2 *p<0.05*, IL-13: 1.15±0.1) as was *EPOR* (control: 1.0, IL-4: 2.3±0.6 *p<0.05,* IL-13: 1.13±0.1). Although strongly upregulated in the PCR array, *SMAD1* and *IFNGR1* were not changed by IL-13 or IL-4 in the specific qPCR primer experiments (Fig. 4.11). Protein expression of SIRPα, MCL1, and BCLXL was determined by western blotting and as expected these proteins were upregulated following 48h exposure to IL-4 and IL-13 in INS-1E cells (Fig. 4.12). Densitometric analysis of the western blots revealed a significant upregulation of SIRPα in response to both IL-4 and IL-13 relative to the untreated control (Fig. 4.13). Densitometric analysis also revealed a significant upregulation of BCLXL by IL-4 but not by IL-13 while similar analysis of MCL1 expression showed no significant difference between treatments and controls (Fig. 4.13).

EndoC βH1 cells that were stimulated with IL-13 for 48h were harvested lysed and probed with anti-sera against SIRPα, MCL1 and BCLXL. SIRPα, and MCL1 showed an upregulation after IL-13 treatment while BCLXL was less affected (Fig. 4.12c).

Although qPCR experiments for total STAT6 were not performed, western blot experiments revealed that both IL-13 and IL-4 significantly increased STAT6 levels in INS-1E beta cells after 48h but STAT3, was not altered (Fig 4.14).

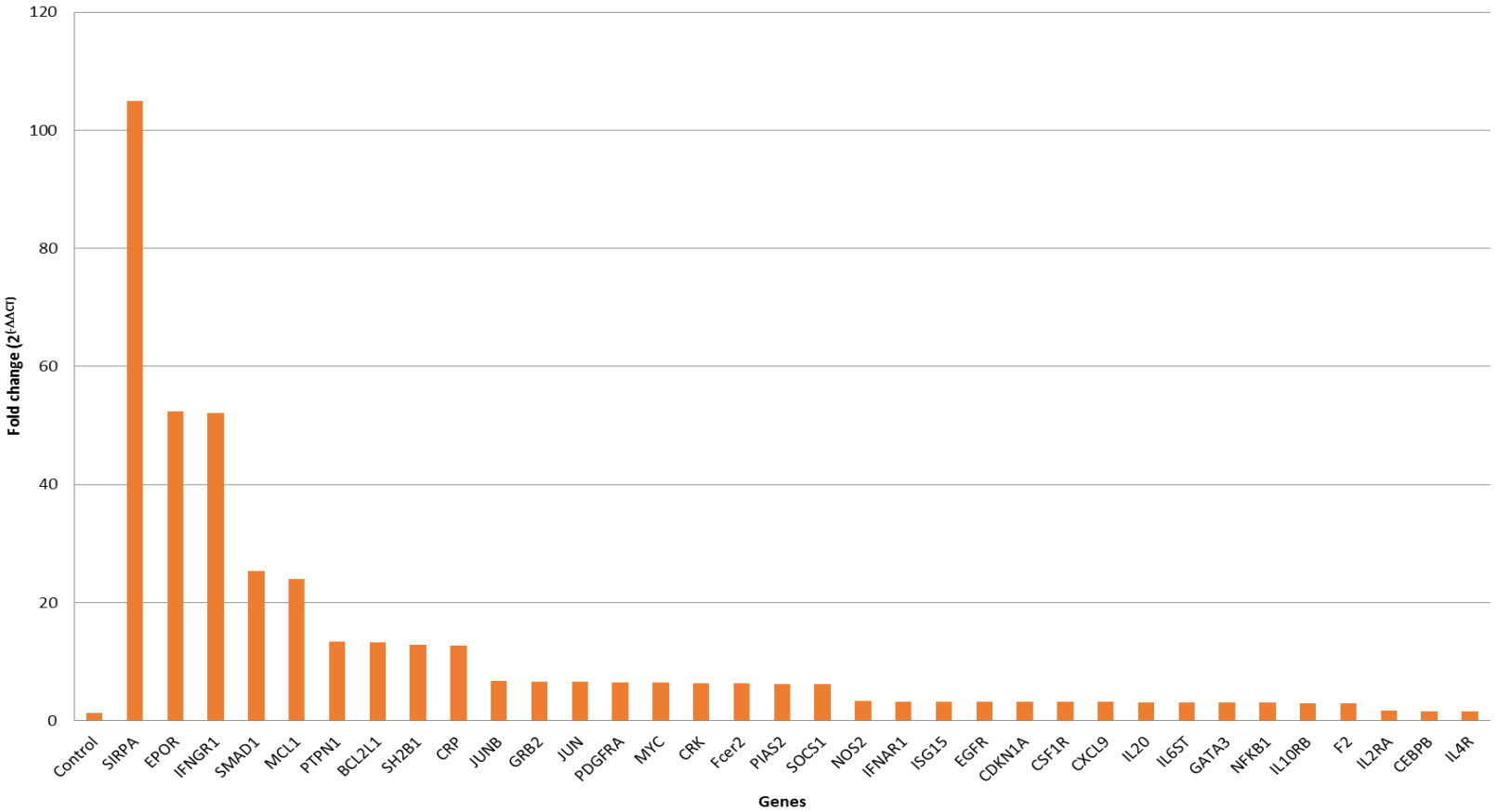


Figure 4. 10: IL-13 stimulation of INS-1E cells upregulates an array of genes.

INS-1E cells were stimulated with IL-13 for 48h, harvested and RNA collected using an RNeasy kit. RT-PCR was performed using RT2-First Strand from Qiagen. cDNA was then used to perform a qPCR on an RT2 Profiler PCR array. Data represents expression relative to unstimulated cells.

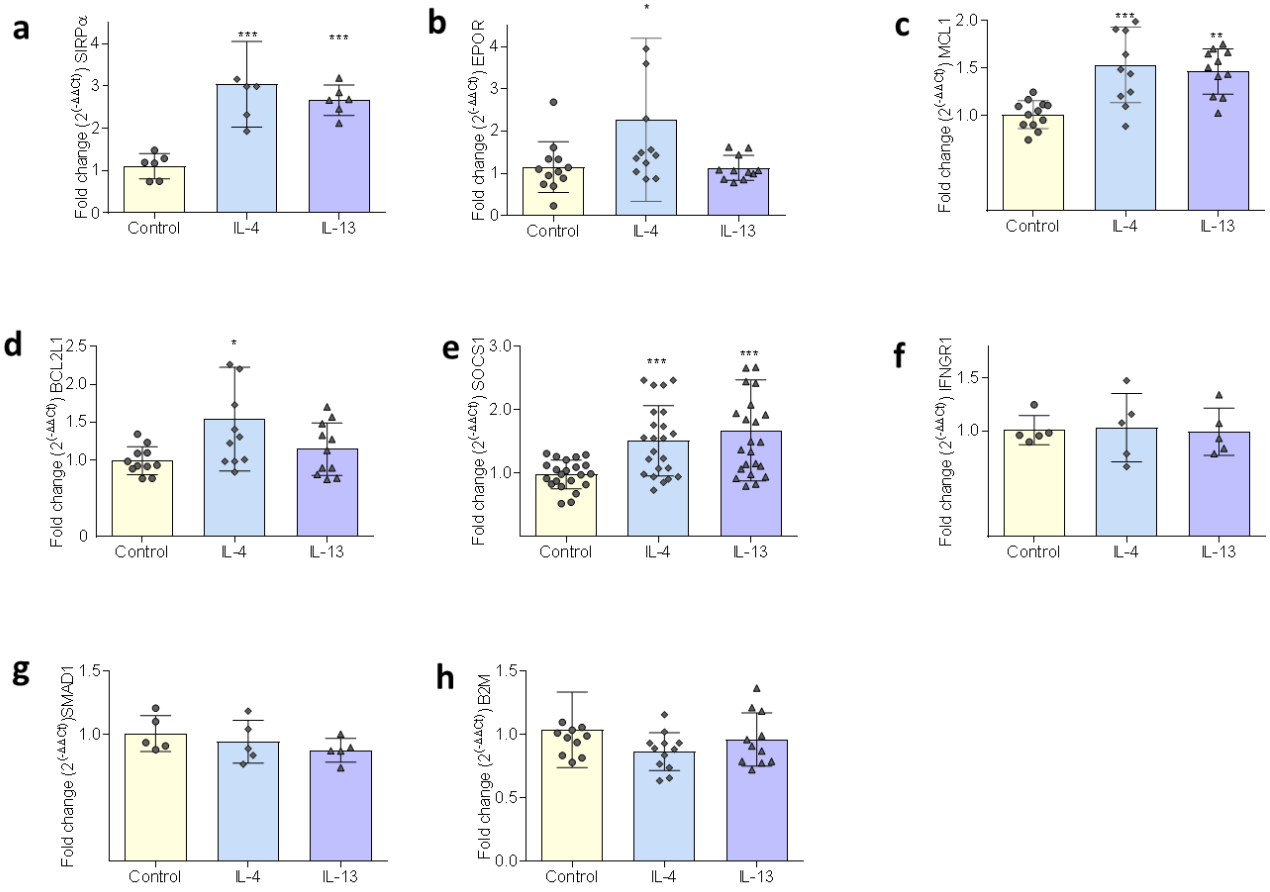


Figure 4. 11: Specific gene primer qPCR confirmed the upregulation of genes from the array.

INS-1E cells were stimulated with 20ng/mL of IL-4(♦) or IL-13(▲) for 48h, harvested and RNA extracted with RNeasy. cDNA was synthesized using the RT2 First Strand kit and amplicons amplified by SYBRgreen qPCR. a. *SIRP*α, b. *EPOR*, c. *MCL*-1 d. *BCL2L1* e. *SOCS*-1, f. *IFNGR1*, g. *SMAD1* and h. *B2M*. Data represents mean of ±SEM of three independent experiments each with three replicates *p<0.05, **p<0.01 and ***p<0.001 relative to control determined by student *t*-test.

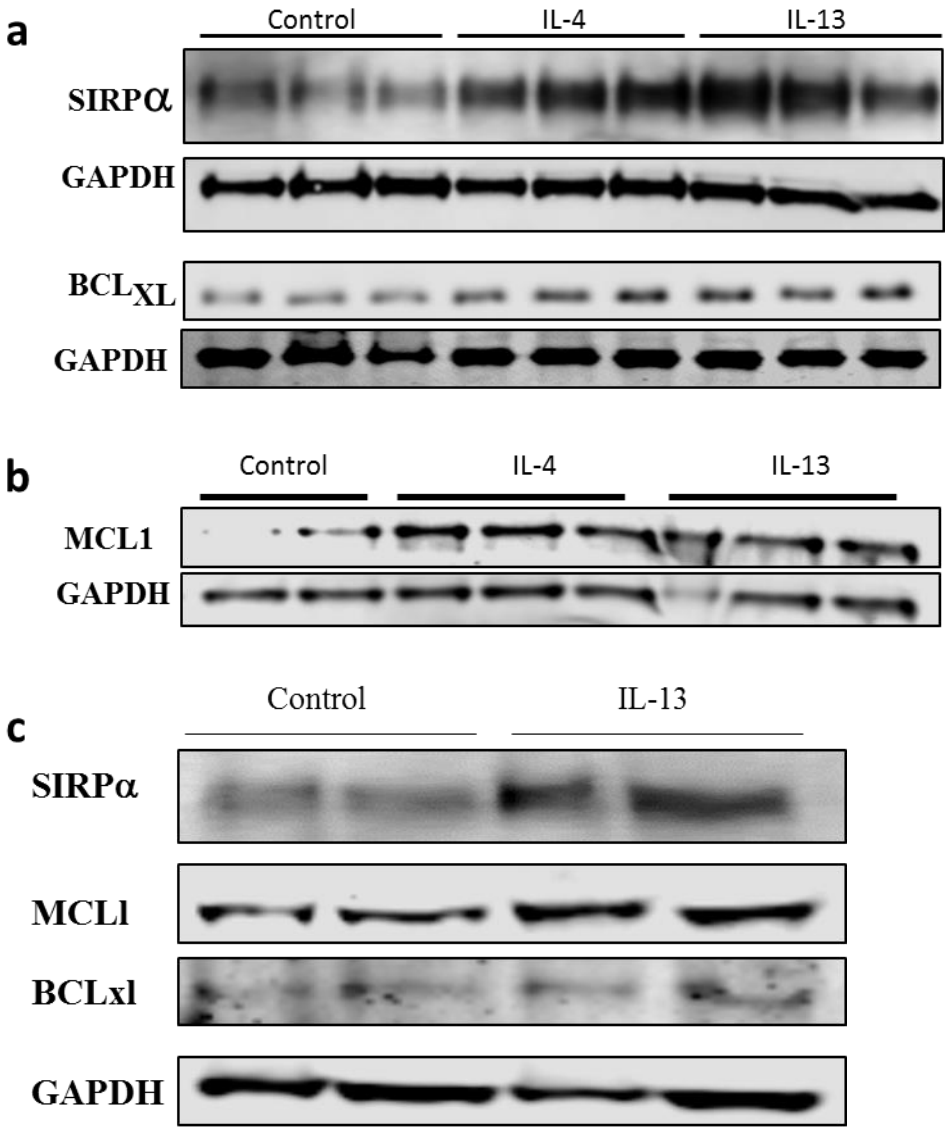
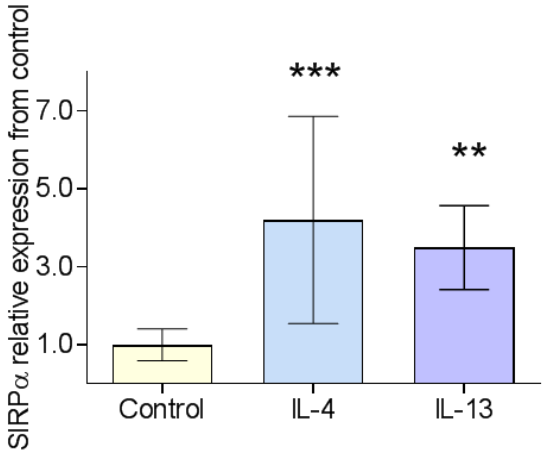


Figure 4. 12: IL-4 and IL-13 upregulate SIRPα, MCL1 in both INS-1 and EndoC βH1 cells and BCLXL in INS-1E.

(**a** & **b**) INS-1E and (**c**) EndoC βH1 cells were treated with 20ng/mL for 48h, lysed and the supernatant collected. Western blotting was performed with 50μg of protein and membranes probed with anti-sera against SIRPα, MCL-1, BCLXL, with GAPDH as loading control. Data represents three independent experiments.

a



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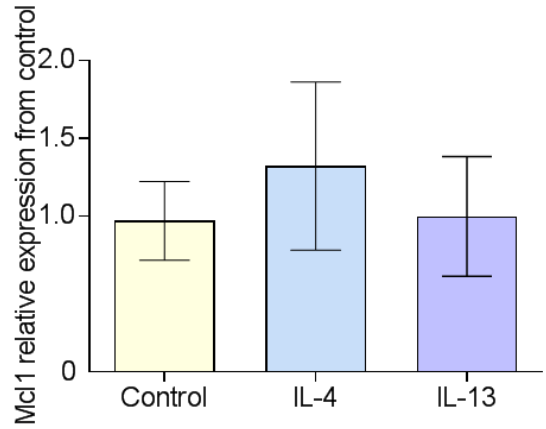

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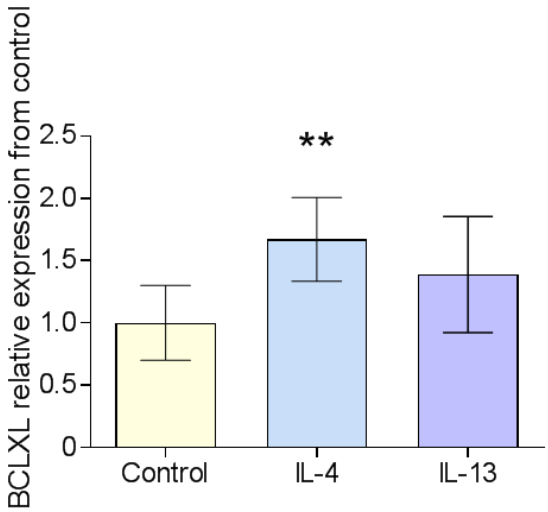


Figure 4. 13: Densitometric analysis on INS-1E Western blots revealed persistent SIRPα upregulation.

Western blot densitometry analysis were performed with the Licor software normalised with loading control and expressed relative to the control. Data represent means ±SEM of three independent experiments determined by student *t*-test.

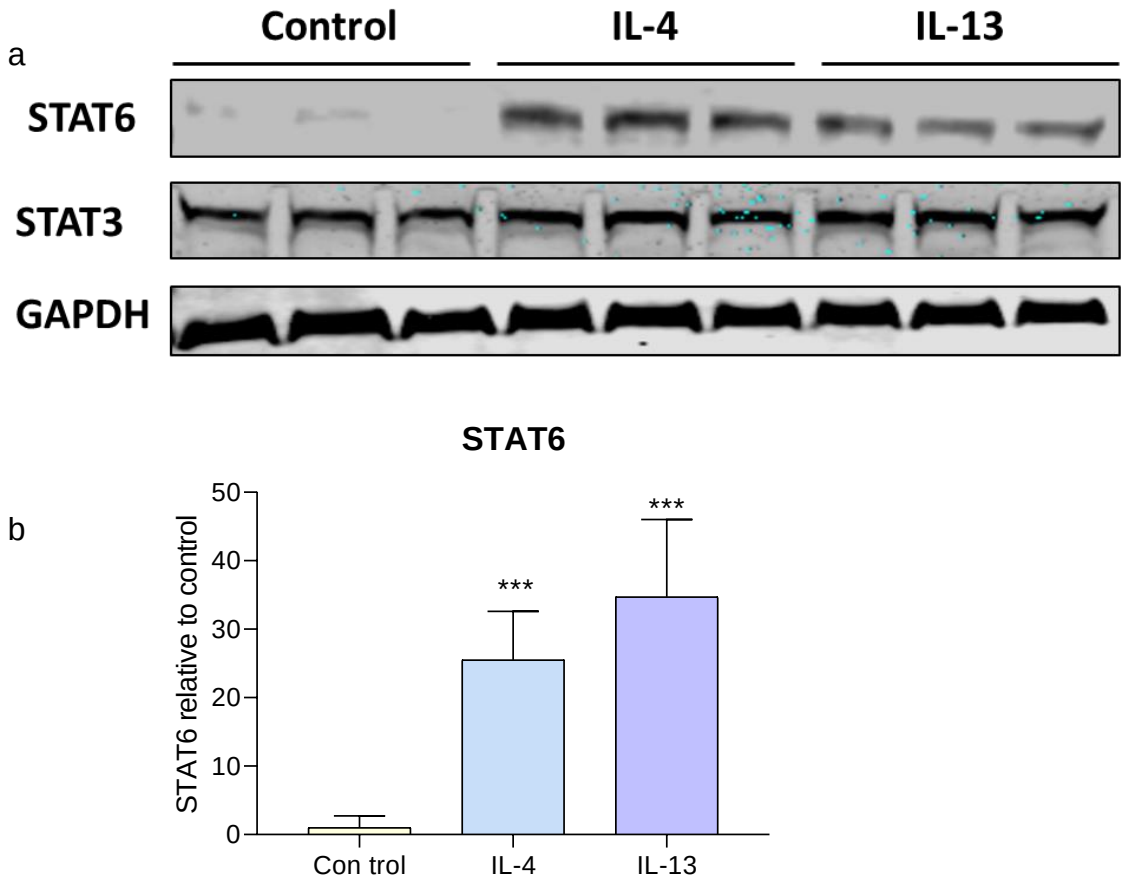


Figure 4. 14: IL-4 and IL-13 both upregulate STAT6 expression but not STAT3.

a. INS-1E cells were stimulated with 20ng/mL of IL-4 or IL-13 for 48h, cells harvested, lysed and the supernatant collected. Western blotting was performed with 50μg of proteins and membranes probed with anti-sera against STAT3, STAT6 and loading control GAPDH. b. Densitometry analysis confirm a significant increase in STAT6 upon IL-4 and IL-13 stimulations. Data represents three independent experiments

4.4 Discussion

4.4.1 The role of the Jaks

In the previous chapter, it was revealed that IL-13 and IL-4 protect pancreatic beta cells from a range of cytotoxic stimuli, but the protective mechanisms remain to be completely understood. IL-4 and IL-13 signal by binding to their cognate receptors leading to receptor dimerization and Jak trans-phosphorylation. IL-4 binds to IL-4Rα and forms a heterodimer with the common gamma chain (γC) receptor, while IL-13 binds to IL-13Rα1 and forms a heterodimer with IL-4Rα (Russell and Morgan, 2014). It has previously been shown that the IL-13 and IL-4 receptor components are present on both rat clonal beta cell lines and on human islet tissue sections (Kaminski et al., 2010). The present chapter sought to characterize the key components of the Jak/STAT6 pathway that might be involved in the protection of beta cells from cytotoxicity. The impact of IL-13 signalling on the PI3K/At pathway was not studied here since our previous work suggests that, although, this pathway is activated in response to IL-13, it is unlikely to be involved in the cytoprotective effects of the cytokine in beta cells (Russell et al., 2013).

In Jak/STAT signalling, the receptor associated Jaks are phosphorylated after receptor dimer formation and prior to the recruitment of STATs. However, it is unclear which Jak proteins act upstream of STAT6 in response to IL-13 treatment. It was shown by western blotting analysis that rat INS-1E beta cells express all four known Janus kinases (Jak1, Jak2, Jak3 and Tyk2). These data support initial findings by Stout et al. (1997) who also showed by immunohistochemistry that INS-1E expressed these proteins. These data are also in accord with other studies that show indirectly that Jak1 and Jak2 are expressed in INS-1E and NOD

mice using Jak1 and Jak2 inhibitors to influence downstream effects (Trivedi et al., 2017, Couto et al., 2007). Jak1 has been implicated in IFN-γ signalling in both beta cells and other cell types. However, a role for Jak1 in IL-4 and IL-13 mediated Jak/STAT signalling has not been previously reported in beta cells. It was shown here that both IL-4 and IL-13 activate Jak1 (Fig. 4.2) but with differing kinetics. Research on cytokines that use the common gamma chain, illustrates that these cytokines require Jak1 for signal transduction (Haan et al., 2011). IL-4 has previously been shown to activate Jak1 in a haematopoietic cell line (Friedrich et al., 1999) and other cell types through the IL-4R (Chen et al., 1997). IL-13 has also been suggested to activate Jak1 in other cell types (Keegan et al., 1995). Jak1 is known to also be activated by type 1 interferons leading to STAT1/STAT2 activation in other cell types (Ahmed et al., 2013). In future experiments, it will be important to determine the role of Jak1 is to IL-13 signalling by performing knockdown experiments with Jak1. Jak2 knockdown was performed instead of Jak1 based on previous experiments by our group demonstrating that Jak2 knockdown reversed IL-13 protection (Russell et al., 2013). It is important to note that the activation of Jak1 can also be induced by type 1 interferons (Ahmed et al., 2013). The association of Jaks to receptors is not specific as one type of Jak can be found on different receptors. The association of Jaks to the receptor is thought to occur after *de novo* synthesis of receptors (Usacheva et al., 2002) and according to the RT2 profiler array, the IL-4Rα was upregulated by IL-13 stimulation. It is tempting to speculate that IL-13 might protect from pro-inflammatory IFN-γ cytokine by competition for cytoplasmic Jak1, although research on receptor association dynamics is still needed to confirm this. Alternatively, signalling through Jak1 could lead to the upregulation of negative regulators of other signalling pathways such as SOCS1

and PIAS1 (Liu et al., 2016, Sun et al., 2013). This suggests that Jak1 might play a role in the IL-13 mediated protection of INS-1E from inflammatory cytokines in multiple ways.

Jak2 is known to be activated by IL-4 and IL-13 in vascular endothelial cells (Palmer-Crocker et al., 1996). The results in this chapter shows the appearance of two Jak2 bands suggested to be isoforms and have been reported in other cell types (Feener et al., 2004, Robertson et al., 2009) although this has not been reported in beta cells. In INS-1E beta cells, it has been shown that prolactin activates Jak2 in a biphasic manner, with an increase in phosphorylation at 15mins, then a nadir at 30mins (Brelje et al., 2002). Jak2 has been implicated in beta cell proliferation, survival, and protection against beta cell cytotoxicity, and can be phosphorylated in response to EPO, Lactogens and prolactin (Choi et al., 2010, Fujinaka et al., 2007, Yao et al., 2015). Furthermore, Jak2 inhibitors have been used to reverse diabetes in NOD mice (Trivedi et al., 2017), where systemic delivery was used. Importantly, we have shown that Jak2 is activated upon IL-13 stimulation and the use of a Jak2 specific inhibitor revealed its role in mediating the protective effects of IL-13 (Russell et al., 2013). However, when the time-course of Jak2 activation was explored in the present work, these data revealed a nadir in Jak2 phosphorylation shortly after IL-13 (or IL-4) treatment, but this evolved into an elevated level of phosphorylation after 75min of stimulation. It is unclear if this initial change is due to enzymatic dephosphorylation of Jak2, as this process is known to be performed by a number of protein phosphatases including; protein tyrosine phosphatase 1B (PTPN1), Src homology region 2 domain containing phosphatase (SHP)-1 and SHP2 which can all cause a rapid dephosphorylation of the tyrosine 1008 and 1007 on Jak2 (Li et al., 2015a). Furthermore, all of these phosphatases have been reported to be present in beta

cells (Zhang et al., 2009, Fernandez-Ruiz et al., 2014). The dephosphorylation event almost coincides with the activation of STAT6 at 5min and a reported increase in the activity of PTPN1 (Li et al., 2015a), which might further implicate phosphatases. Building on previous results which had implicated Jak2 in IL-13 protection of beta cells (Russell et al., 2013), we decided to study Jak2 further. Jak2 knockdown reduced the activation of STAT6 by IL-13 (Fig. 4.8) providing very strong support for a possible role of this kinase in IL-13 mediated protection of beta cells from cytotoxicity.

The presence of Jak3 in INS-1E beta cells and its activation by both IL-4 and IL-13 has also been revealed in this chapter. Western blotting showed the presence of three bands which represents 3 spliced variants of Jak3 described in other cells (Lai et al., 1995). Jak3 phosphorylation is induced by IL-4 and IL-13 via the common gamma chain present in their receptors in other cell types (Malar et al., 1996). Jak3 inhibition using a highly specific inhibitor, WHI-P154 reversed the protection induced by IL-4 in rat beta cells cultured in the presence of pro-inflammatory cytokines (Kaminski et al., 2010). Inhibition of Jak3 using JANEX-1 in islets and NOD mice reduced NF-κB activation by blocking IL-1β and IFN-γ signalling and also reduced iNOS expression by a similar mechanism (Lv et al., 2009). One possibility is that, IL-4 and IL-13 activation of Jak3 leads to a signalling cascade that generates negative feedback molecules (such as SOCS1) which then counteract the signalling of pro-inflammatory cytokines such as IL-1β and IFN-γ.

The data presented here and that of others (Marroqui et al., 2015, Stout et al., 1997) suggest that INS-1E cells express Tyk2. Tyk2 can be activated by both IL-4 and IL-13 in other cell types (Millward-Sadler et al., 2006), but is also known to

be activated by type 1 interferons (Ahmed et al., 2013). More studies are needed to confirm which receptor Tyk2 associates with preferentially and whether or not it plays a role in IL-13 mediated protection. Tyk2 gene mutations are implicated in T1DM and in virus-induced diabetes (Nagafuchi et al., 2015) and knockdown of this gene leads to a decrease in MHC I expression and cell death in poly-IC stimulated human beta cells (Marroqui et al., 2015).

Until recently, the role of Jak proteins was understood to be restricted to the Jak/STAT signal transduction pathway. However, in the last few years, each of the Jak proteins has been shown, unexpectedly, to also translocate to the nucleus, where they bind and phosphorylate tyrosine residues on histones leading to altered promoter activity. This, in turn, leads to changes in the expression of various genes which could potentially be involved in viability, survival and other important cell processes. (Zhu et al., 2017, Ahmed et al., 2013, Dawson et al., 2009, Landires et al., 2013). This novel insight has however only been studied in very few cell types but not in pancreatic beta cells, and may illustrate a different but crucial role of the Janus kinases. It is possible that the activation of these kinases by different cytokines will lead to differential localisation at key nuclear binding sites eventually leading to diverse activities.

A question that remains unanswered is which receptor (IL-4Rα, γC, IL-13Rα1) mediates the activation of each specific isoform of Jak in beta cells? The present understanding indicates a large variation in the cytokine receptor sequence that interacts with Jaks (Ferrao and Lupardus, 2017). This suggests that different Jaks bind to specific receptors with variable affinities. Immuno-precipitation of the receptor components might be one way to define Jak associations. Answering this question will give a clearer picture as to which of the Jaks is involved in IL-

4/IL-13 signalling. Jak proteins use the FERM domain to bind to specific receptor components called box1 and box2 (Ferrao and Lupardus, 2017). The IL-4Rα and IL-13Rα1 have been reported to each have both box1 and box2 and can accommodate different Jaks (Usacheva et al., 2002, Ferrao and Lupardus, 2017), but whether these are activated simultaneously or in sequence (and which is most important) remains to be answered.

4.4.2 The genes regulated by IL-4 and IL-13 stimulation

Following the activation of Jaks, the appropriate STAT is recruited to the receptor cytoplasmic tail where it becomes activated. In the case of IL-4 and IL-13 signalling, canonically STAT6 is recruited and phosphorylated, and this model was confirmed here using INS-1E cells (and was previously reported in human islets (Russell et al., 2013)). However, it is also important to concede that additional pathways may be activated independent of STAT6. For example, it was previously shown that STAT3 is also phosphorylated in response to IL-13 treatment of INS-1E (Russell et al., 2013). In addition, activation of PI3K also occurs upon IL-4 and IL-13 stimulation (Rutti et al., 2016, Russell et al., 2013). It is established that activated STAT6 dimerises with other activated STAT6 monomers and migrates to the nucleus where it binds to the palindromic sequence TTC$(N)_{3\ or4}$ GAA to regulate gene expression (Goenka and Kaplan, 2011). In trying to understand the genes upregulated by activated by IL-13, a qPCR array kit was exploited.

Anti-apoptotic molecules such as BCLXL and MCL-1 were upregulated in response to IL-4 and IL-13 treatment at both gene and protein levels. These results concur with findings in other cell types (Lømo et al., 1997, Wurster et al., 2002). The importance of both BCLXL and MCL-1 as key anti-apoptotic proteins

is well established in beta cell survival, and thus it is likely that the upregulation of these proteins may contribute to the protective effects of IL-13 (Carrington et al., 2009, Miani et al., 2013). In keeping with this, activated STAT6 dimers have been shown to bind to the BCLXL gene promoter region to upregulate BCLXL expression (Wurster et al., 2002, Natoli et al., 2013). Data from our group has also shown a loss in MCL-1 in certain islets of people with T1DM when compared to non-diabetes controls (Richardson et al., 2013). This could imply that decreased anti-apoptotic signalling contributes to the death of beta cells in T1DM (Usero et al., 2016).

The results from the gene-specific qPCR assays revealed the dramatic upregulation of *SIRP*α (Signal regulatory protein alpha, also known as Src Homology domain-containing protein tyrosine phosphatase substrate 1 SHPS-1) in response to IL-13 and IL-4. These data were confirmed by qPCR and western blotting in EndoC βH1 and INS-1E beta cells. SIRPα is a transmembrane glycoprotein of the Immunoglobulin superfamily involved in cell-cell communication through binding to its ligand CD47 (Oshima et al., 2002). CD47 is a marker of self and is known to inhibit phagocytosis in macrophages through its interaction with SIRPα (Bian et al., 2016). However, this binding event sends a bidirectional signal and as such can have effects on both cells (Oshima et al., 2002). Results here suggest that CD47 is present in beta cells under control conditions and agrees with the results of Kobayashi and colleagues who showed the expression of both SIRPα and CD47 in the islets of C57BL mice. In that report, islet SIRPα knockout mice had a reduced ability to secrete insulin (Kobayashi et al., 2008). The role of SIRPα in beta cell viability remains to be

addressed and this is considered in chapter 6 of this thesis. The induction of SIRPα by IL-13 response has not been documented previously in beta cells.

Suppressor of cytokine signalling 1 (SOCS1) was also significantly upregulated by both IL-4 and IL-13, and a similar effect has been reported in other cell types (Hebenstreit et al., 2003). SOCS proteins are the most studied negative regulators of the Jak/STAT signalling pathway, wherein they are reported to target signalling proteins such as the Jaks for degradation through the ubiquitin pathway (Shuai and Liu, 2003). The role of SOCS1 in beta cell health has been extensively studied (Martinez-Nunez et al., 2011, Sun et al., 2013, Trengove and Ward, 2013, Li et al., 2015b) and may be protective to these cells under certain circumstances. Overexpression of SOCS1 protects NOD mice (a T1DM mouse model) from insulitis by preventing both MHCI expression and FASL expression and decreasing T-cell proliferation (Chong et al., 2004). Others have shown that SOCS1 protected NOD mice from diabetes by blocking IFN-γ signalling and preventing T-cells from attacking the islets (Flodstrom-Tullberg et al., 2003). In this context, SOCS1, and other members of the SOCS family (2&3) have been shown to be increased at the mRNA level in human islets from people with T1DM, possibly as a protective mechanism against inflammatory cytokines (Santangelo et al., 2005). Taken together, these data imply that beta cells might be protected from cytotoxicity, in part through the upregulation of SOCS1 which can negatively regulate pro-inflammatory cytokines signalling.

The gene-specific qPCR showed an increased expression of the erythropoietin receptor (EPOR) by IL-4, but not by IL-13. Little is known about the relationship between EPOR and IL-4, except for one report that showing that EPOR signalling protects against white adipose tissue inflammation and insulin resistance in a

STAT6 dependent on manner (Alnaeeli et al., 2014). Erythropoietin has been reported to protect db/db mice from streptozotocin induced diabetes in a beta cell specific manner through the upregulation of BCLXL (Choi et al., 2010).

The RT2 Profiler array also revealed the upregulation other genes such as *GATA3, PIAS2, SH2B1, PTPN1, GRB2, JUN, PDGFRA*, *CRK, CRP* and *MYC* however, these require further validation with specific primers. Some of these genes are known to be STAT6 regulated, such as *GATA3*, and *PIAS2*, (Shuai and Liu, 2003) and are implicated in diabetes and or IL-13/IL-4 signalling see.

Table 13: Other IL-13 regulated genes and their beta cell functions

Gene	Function	Reference
GATA3	• Reduced GATA3 upregulation in response to type 2 cytokines in cord blood T lymphocytes is a genetic risk for T1DM	(Luopajärvi et al., 2007)
PIAS2	• Upregulated in PBMCs of non-diabetic rats stimulated with serum from diabetic rats • Negative regulator of STAT2	(Kaldunski et al., 2010) (Shuai and Liu, 2003)
GRB2	• An adaptor protein essential for IGF PI3K signalling	(Hügl et al., 1998)
PTPN1	• Negative regulator of the STATs • Its deletion protects mice from T1DM	(Lu et al., 2008) (Herren et al., 2015)
cJUN	• Helps the beta cell recover from iNOS toxicity	(Scarim et al., 2003)
PDGFRA	• Essential for PDGF signalling important for age dependent proliferation of beta cells	(Chen et al., 2011)
CRK	• An adaptor protein Involved in glucose signalling	(Lee et al., 2004)
CRP	• An acute phase protein secreted in response to inflammation and helps in phagocytosis. Hence its levels are upregulated in T1DM	(Chase et al., 2004)
MYC	• Overexpression of the c-*MYC* in streptozotocin induced diabetes mice reversed the alter gene expression of liver metabolism genes	(Riu et al., 2002)

Collectively, this chapter set out to study the signalling pathway stimulated by IL-13 and IL-4 and in beta cells. Whilst a conclusion on which particular Jaks are involved in the signalling cannot be drawn pending receptor component experiments and viability experiments, the data suggest that all Jaks could potentially be involved. The activation of Jak proteins leads to STAT6 recruitment and phosphorylation, this in turn increased the expression of various genes, some

of which are well known, such as *MCL-1* and *BCL2L1* and another, not formally associated with IL-13, called *SIRP*α. And these might be contributing to the cytoprotection observed following IL-13 treatment.

I have confirmed in this chapter, the presence of all four Jaks in INS-1E beta cells and illustrated the different activation patterns of these kinases upon IL-4 and IL-13 stimulation. The dynamics of Jaks and receptors association is still not known and might be key to understanding the role of Jaks in beta cells. Importantly, the genes regulated by IL-13 were studied in beta cells and revealed the upregulation of anti-apoptotic MCL-1 and BCLXL. Additionally, the dramatic upregulation of SIRPα was observed whose role in beta cells has not been completely defined.

In the next chapter, the impact of manipulating STAT6 expression by either overexpression or knockdown on IL-13 protection in beta cells will be investigated.

Chapter 5.0: The Impact of STAT6 on beta cells

5.0 The Impact of STAT6 on beta cells

5.1 Introduction

Evidence in the previous chapter demonstrated that the ability of IL-13 to protect beta cells involves the upregulation of a variety of gene products. Leading on from this, the results described in this chapter establish whether these genes are regulated in a STAT6 dependent manner and evaluate the role played by STAT6 in the cytoprotection afforded by IL-13. Downstream targets of IL-4 and IL-13 have been extensively studied in immune cells and in other cell types, but relatively little is known about their roles in beta cells (Elo et al., 2010, Czimmerer et al., 2018, Chen et al., 2003, Szanto et al., 2010).

IL-13 signals by binding to its cognate receptor leading to receptor dimerization, Jak phosphorylation and the recruitment of STAT6. STAT6 is then phosphorylated, homo-dimerises and migrates to the nucleus (Jiang et al., 2000). According to a GWAS study conducted using human T helper (Th) cells, 80% of IL-4 activated genes (453 genes were studied) are regulated by STAT6 over a period of 48h. These included genes such as *GATA3, GIMAP4, IL-24, LTB, SPINT2* and *SOCS1* (Elo et al., 2010) but not those found in the present work in beta cells. In beta cells, some of the genes regulated by IL-13 have been identified by Rütti et al. (2016). In their experiments, INS-1E cells were stimulated for 48h and the regulation of 4 genes noted which include *CISH*, *CD83*, *Galnt14* and *St6galnac3* (a pseudogene). Although *CD83* has been linked to T1DM, Rutti and colleagues did not go on to confirm whether these genes are directly controlled by STAT6 since they concluded that the protection afforded by IL-13 was PI3 kinase-mediated (Rutti et al., 2016).

Whole body knockout of *STAT6* in Balb/CJ or C57BL/6j mice led to increases in serum cholesterol, triglycerides, and insulin resistance due to an increase in PPARα which caused the activation of fatty acid break down pathways. Hence, these mice had lean bodies but high serum fat levels. This suggested that STAT6 may regulate PPARα expression, hence controlling lipogenesis (Ricardo-Gonzalez et al., 2010). In another report where Balb/c Lyn knockout mice and Lyn knockout STAT6 knockout were used, the absence of STAT6 exacerbated autoimmunity by increasing serum auto-antibodies, increasing T-cell activation (increase CD69, CD44 and decrease CD62L), and increasing serum cytokines such as IFN-γ, suggesting that polarisation to a more Th1 response occurs in the absence of STAT6 to encourage autoimmunity (Lau et al., 2012).

The aims of this chapter were:

- To assess the impact of STAT6 on beta cell viability by suppressing its expression using small interference RNA. Additionally, to study if loss of STAT6 had any influence on IL-13 afforded protection of beta cells from cytotoxicity
- Alternatively, the impact of STAT6 was studied by enhancing its expression by transfecting a STAT6 plasmid vector into beta cells and performing cytoprotection experiments
- Gene and protein expression studies were performed after STAT6 knockdown to determine whether the genes upregulated in chapter three are controlled by STAT6.

5.2 Materials and methods

5.2.1 Real-time RT-PCR

After incubation of cells with relevant test reagents, RNA was extracted as described in section 2.6.1, and 500ng used for cDNA synthesis as described in section 2.6.2. qPCR was performed accordingly to the methods given in section 2.6.3.

5.2.2 Knockdown of STAT6

Knockdown of STAT6 was performed as described in section 2.3. Briefly, 10nmol /mL of STAT6 siRNA (5' CGAGAUCUUGCUCAAUUAATT, 3' UUAAUUGAGCAAGAUCUGGA from Life technologies, USA) was mixed with Optimem, and the transfection reagent Lipofectamine RNAi Max (Thermofisher, UK). The mixture was used to transfect plated cells in a dropwise manner, plates swirled and incubated at 37^0C.

5.2.3 Plasmids

All plasmids arrived on blotting paper or were lyophilised and were reconstituted following the manufacturers' protocols. Briefly, tubes containing the plasmids were centrifuged at 5000g for 5min and DNA suspended in RNAse/DNAse free water. The tubes were then left at room temperature for 10min and centrifuged at 5000g for one minute before storage at -20^0C or used to transform competent *Escherichia coli (E.coli)*.

STAT6 plasmid

The rat STAT6 gene ORF cDNA clone expression plasmid was obtained from a commercial source (Stratech, UK), (RefSeq NM_001044250.1). The STAT6 insert was positioned within the multiple cloning site of a pCMV3 vector conferring ampicillin resistance. The insert was flanked by various restrictions and two

recognised by; *KpnI* and *XbaI,* were employed to isolate and sub clone the insert. The structure of the STAT6 expression plasmid is shown in figure 5.1 below.

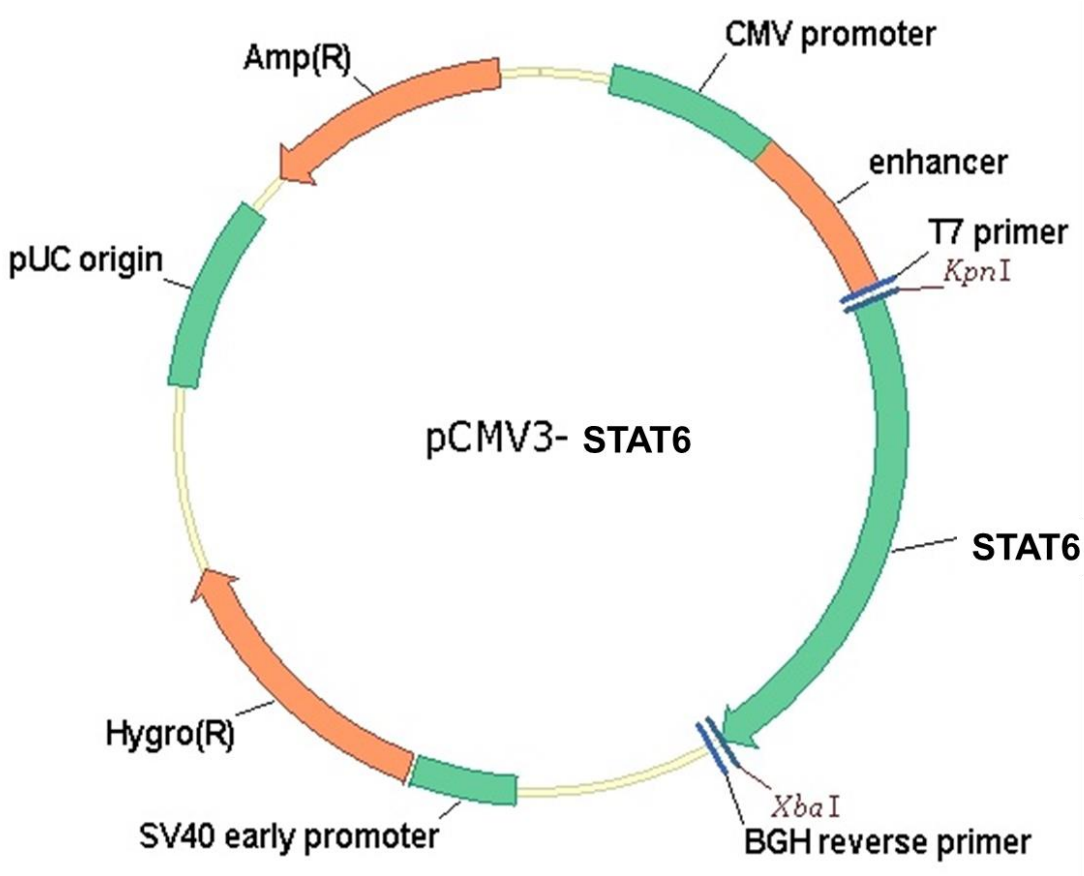


Figure 5. 1: STAT6 cDNA expression plasmid

(Courtesy of Sinobiological: http://www.sinobiological.com/Rat-STAT6-Gene-cDNA-Clone-full-length-ORF-Clone-expression-ready-untagged-p53097.html#img-1)

STAT6 reporters constructs

The STAT6 reporters plasmids were generously gifted by Mrs Laura Pelletier and Prof Karen Leroy of the French National Institute of Health and Medical Research (Paris, France). The STAT6 reporters (N3 or N4) were constructed to report the binding activity of STAT6 to either an N3 or an N4 GAS domains. The STAT6 reporters (each containing either an N4 or an N3) are positioned upstream of the luciferase gene (Fig. 5.2) thereby allowing their use to monitor promoter activity.

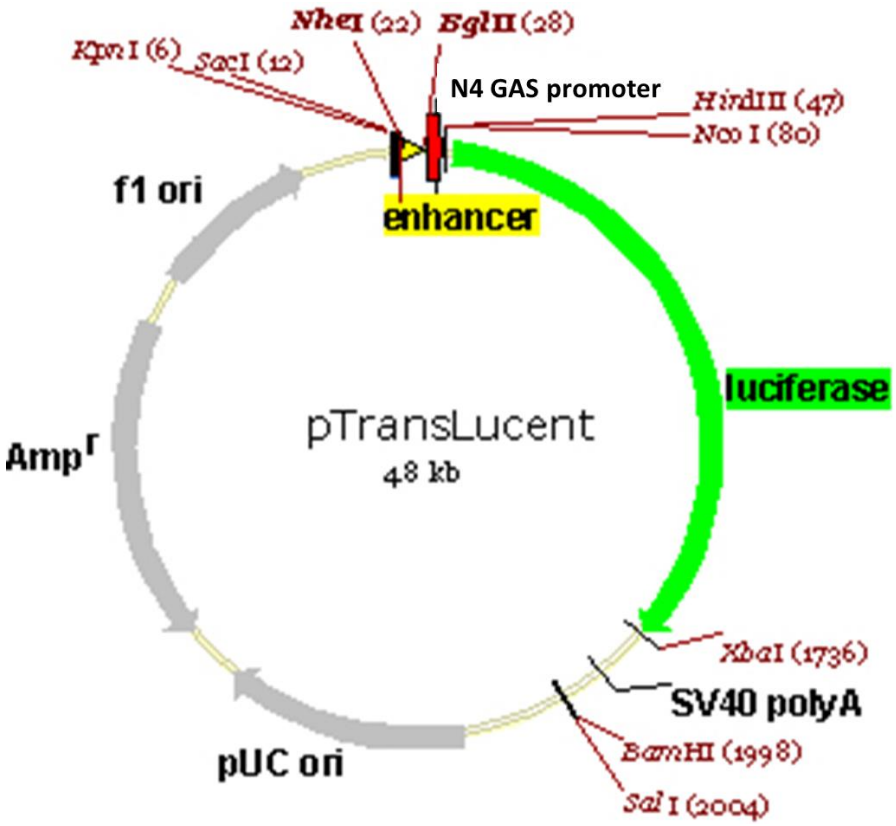


Figure 5. 2: STAT6 reporter construct

(Courtesy of panomics: www.panomics.com

***Renilla* plasmid**

The renilla plasmid was obtained commercially from Promega, UK. The vector encodes a luciferase reporter gene hluc (from *Renilla reniformis)* with an HSV-TK promoter which allows constitutive expression in mammalian cells Fig 5.3.

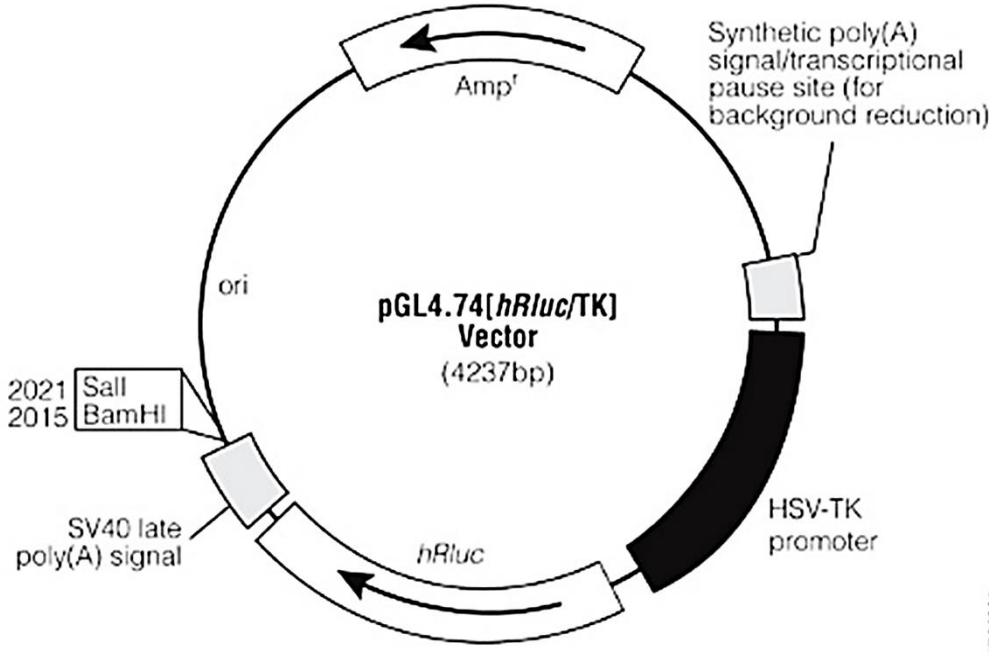


Figure 5. 3: Renilla reporter construct

Source https://www.promega.co.uk/resources/protocols/product-information-sheets/a/pgl474-vector-protocol/

GFP plasmid

In order to monitor the transfection efficiency of our cells, maxGFP was employed. This exploits a variant of green fluorescent protein (GFP) from the copepod *Pontellina plumata* (Shagin et al., 2004). The plasmid was commercially obtained from Lonza, UK and encodes a kanamycin resistance gene for use in selection (Fig.5.4).

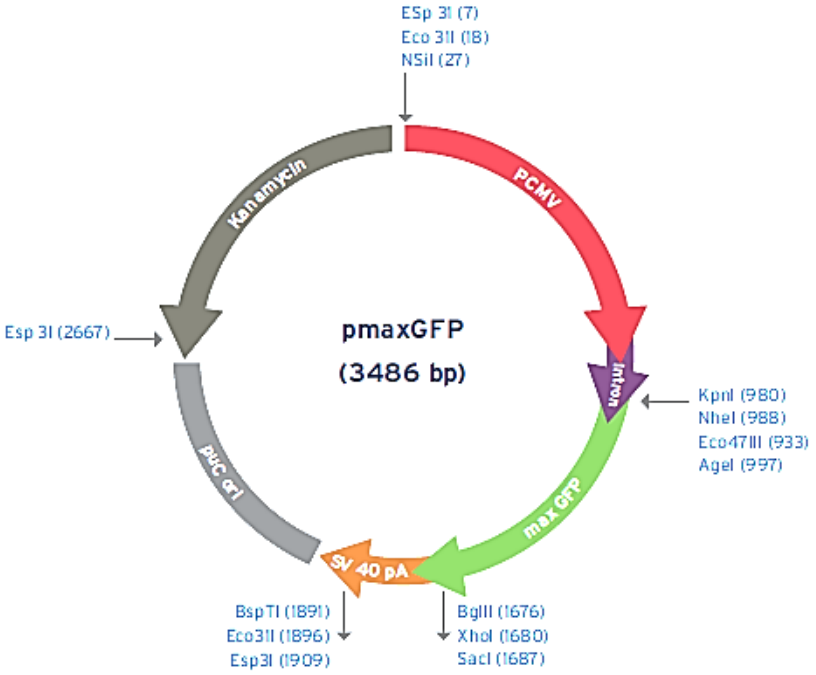


Figure 5. 4 pmaxGFP plasmid map

Source: https://bioscience.lonza.com/lonza_bs/US/en/Transfection/

5.2.6 Site-directed Mutagenesis

Site-directed mutagenesis (SDM) is a protocol performed to create targeted changes in DNA by either insertion, deletion or substitution. Such changes in the DNA sequence may lead to gain or loss of function in a target protein if designed appropriately. SDM was performed following a protocol previously described (Bachman, 2013) although pioneered originally by Kunkel (Kunkel, 1985). The most important step in carrying out SDM is the design of specific primers that will create the change required in the target sequence. The NEBasechanger website (www.nebasechanger.neb.com) was used to simulate substitutions in rat STAT6 cDNA in order to create constitutively active and dominant negative forms. The following primers were used to modify the STAT6 sequence; constitutively active STAT6 (STAT6CA): forward primer 5' AAGCAATATGCCGCTAGCCTTCTC3' reverse primer 3'ACTGATAAAGCCGATGATC5'; dominant negative STAT6 (STAT6DN): primer 5'GGGAGGGGTTCTGTCTCAACTAC3', reverse 3' GTCCTTTCCCATCTGTTC 5'. At protein level, the constitutively active STAT6 primer induced a change in the amino acids valine and threonine at position both to alanines at position 547 and 548. While the dominant negative primers induced a change of the tyrosine 641 to a phenylalanine (Daniel et al., 2000).

Following the acquisition of the primers, the SDM PCR was performed using a Q5® SDM kit (New England Biolabs (NEB), UK). An exponential amplification of the plasmid was done using a combination of Q5 master mix, the appropriate primers and the template DNA. Denaturation was performed for 30s at 98^0C followed by 25cycles of 98^0C 10s, 60^0C 30s, 72^0C 30s to anneal and a final extension step of 72^0C for 2min.

After the SDM, the PCR products were circularised by ligases, and template DNA removed by a Dpn1 enzyme (NEB, UK).

Following this step the plasmids products were sent off for sequencing to confirm the successful substitution of the nucleotides.

5.2.4 Confirming successful insertion or deletion of base pairs

Five microliters of each sample from the SDM was sent off to Source BioScience™ for sequencing using the appropriate primers depending on the vector in which the DNA was inserted. Sequencing results were analysed using an online sequence alignment tool (www.expasy.org) in which both the reference sequence and mutated sequence are aligned to observe the SDM.

5.2.5 Bacteria transformation and plasmid purification

Bacterial transformation to amplify plasmid DNA was performed as detailed in section 2.9.2.

5.2.6 Transfection of cells with plasmid DNA

Transfection of INS-1E cells or HEK293 cells was performed as detailed in section 2.9.3.

5.2.7 Viability assays

Cell viability assays were performed as described in section 2.4, using propidium iodide staining with some additional modifications. In these experiments, cells were transfected with STAT6 constructs prior to the viability assay. Green Fluorescent Protein (GFP) construct was co-transfected with STAT6 cDNA in order to select only the transfected cells, and thus only the GFP positive cells were used for viability counts. After harvesting the cultured cells and staining with

PI, the flow cytometer was gated to sort GFP positive cells. From the GFP positive gate, PI positive cells were quantified (Fig 5.6).

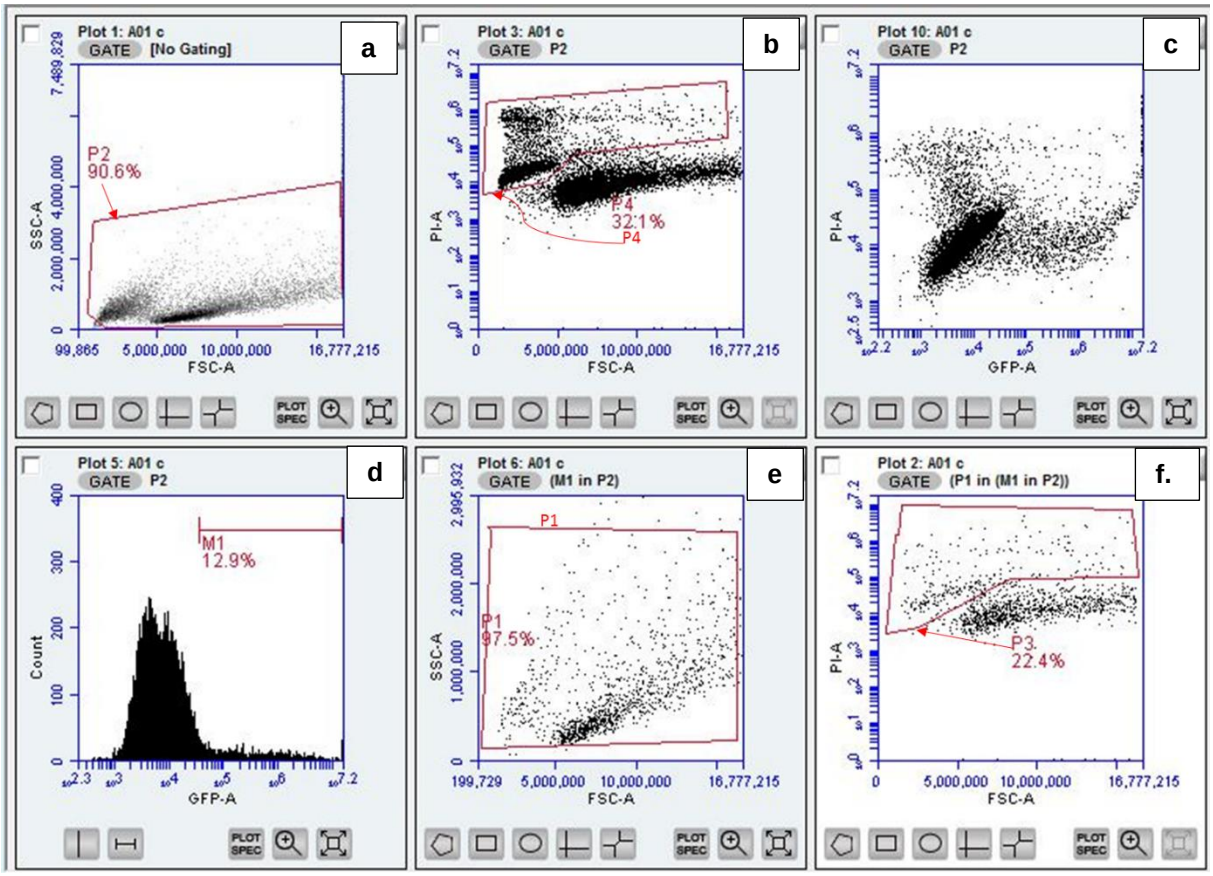


Figure 5. 5: Gating for cell viability read out on the flow cytometer.

After harvesting cells transfected and treated with various reagents using trypsin, cells were stained with 20μg/mL PI/Facs buffer and analysed on the flow cytometer. Panel **a**. shows the total population of cells by size (FSC) and granularity (SSC) gated P2. Panel **b.** shows the percentage of the total population P2 that is non-viable labeled P4. Panel **c**. shows the compensation plot of GFP and PI performed according manufacturer's recommendations. Panel **d**. shows a histogram GFP positive cells (M1). GFP positive cells were then plotted by size and granularity (P1), panel **e**. and on panel **f,** P1 cells were plotted for PI positive cells P3, which represents the percentage non-viable cells positive for GFP. "A" after each acronym on the plots stands for Area

5.2.8 Western blotting

Western blotting was performed to assess protein expression according to the protocol in section 2.7.

5.3 Results

5.3.1 STAT6 knockdown abrogated IL-13 protection

In the previous chapter, a knockdown protocol was established and exploited to deplete STAT6 expression in INS-1E cells. A comparison of the different transfection reagents revealed that Lipofectamine was more effective than attractene (Fig 5.6a). STAT6 levels were significantly reduced (compared to cells exposed to scrambled siRNA) in rodent beta cells by approximately 75% within 48h of introduction of STAT6 siRNA according to densitometric analysis (Fig. 5.6b). Using the Lipofectamine transfection reagent to monitor the duration of knockdown, it was confirmed that STAT6 expression was reduced for at least four days (Fig. 5.6c).

It was important to understand whether STAT6 depletion affected beta cell viability in the absence of a cytotoxic stimuli. To asses this, STAT6 knockdown was performed in INS-1E cells and cell viability compared with those exposed to a scrambled control for a period of 7days. Knockdown of STAT6 did not impact the viability of cells for at least six days (Fig.5.7). Observation of the transfected cells under the light microscope suggested that, STAT6 knockdown reduced the rate of cell growth compared to the scrambled control. Both scramble and knockdown treatments witnessed an increase in cell death by day 6 and 7 due to increase cell density relative to growth factors in the medium. These lead to cell starvation and induction of intrinsic apoptotic pathway and hence increase cell death (Alberts et al., 2002b, Alberts et al., 2002a).

To determine whether the protection offered by IL-13 was mediated via a STAT6-dependent pathway, IL-13 mediated protection was studied after STAT6 knockdown. Serum withdrawal was initially exploited to induce beta cell death

and was performed as described in section 3.2.3. As already established in chapter 3.0 and in other studies (Russell et al., 2013, Charles et al., 2005), serum withdrawal from INS-1E cells led to a significant increase in cell death, and this was reduced by IL-13 treatment (SW: 46.38±3.5%, SW+IL-13: 27.96±3.3%, $p<0.001$). Importantly, the protection offered by IL-13 was abrogated by STAT6 KD (SW+STAT6 KD: 48.11±3.6%, SW+STAT6 KD+ IL-13: 42.0±2.6% cell death $p>0.05$) (Fig. 5.8).

A similar result was obtained with IL-4 treatment using serum withdrawal to induce cell death. Again, SW induced a significant increase in cell death that was reduced by IL-4 treatment (SW 53.5±1.5%, SW+IL-4 44.0±1.1% cell death; $p<0.01$). The protection offered by IL-4 was completely reversed by the knockdown of STAT6 (SW+KD 58.9±1.8%, SW+KD+IL-4 56.1±2.2% cell death $p>0.05$) (Fig. 5.9).

To determine whether STAT6 was involved in the IL-13 mediated protection from other cytotoxic stimuli, INS-1E cells (with or without STAT6 knockdown) were treated with a pro-inflammatory cytokine cocktail (IL-1β, TNF-α, IFN-γ and IL-6) in the presence or absence of IL-13 and cell viability assessed. As expected, pro-inflammatory cytokine cocktail treatment led to an increase in cell death that was reduced by pre-treatment with IL-13 (Cyt; 92.8±1.3%, Cyt+IL-13; 82.4±1.9%, cell death $p<0.001$). STAT6 knockdown alone had no significant effect relative to the scrambled control (control; 15.5±1.4%, Control+KD; 16.9±2.2% cell death). Knockdown of STAT6 however, reversed the protection offered by IL-13 (cytokines: 92.8±1.3%, cyt+KD+IL-13; 94.1±0.8%, cell death $p>0.05$) (Fig. 5.10).

As expected 250μM palmitic acid (PA) treatment of INS-1E cells for 48h induced a large increase in cell death (PA; 91.37±1.49% cell death), and as observed

previously this was reduced significantly by IL-13 pre-treatment (PA+ IL-13; 75.14±3.33%, cell death $p<0.001$). Strikingly, knockdown (KD) of STAT6 reversed the protection afforded by IL-13 under conditions of palmitic acid treatment (PA+KD+IL-13; 96.16±1.0%, cell death $p<0.001$). Again, in these experiments, knockdown of STAT6 alone did not significantly affect cell viability (control; 9.9±1.6%: KD; 15.19±2.1%, cell death *p>0.05*) Fig. 5.11. Taken together these data support the notion that STAT6 is critical in mediating the protective effects of IL-13 in pancreatic beta cells.

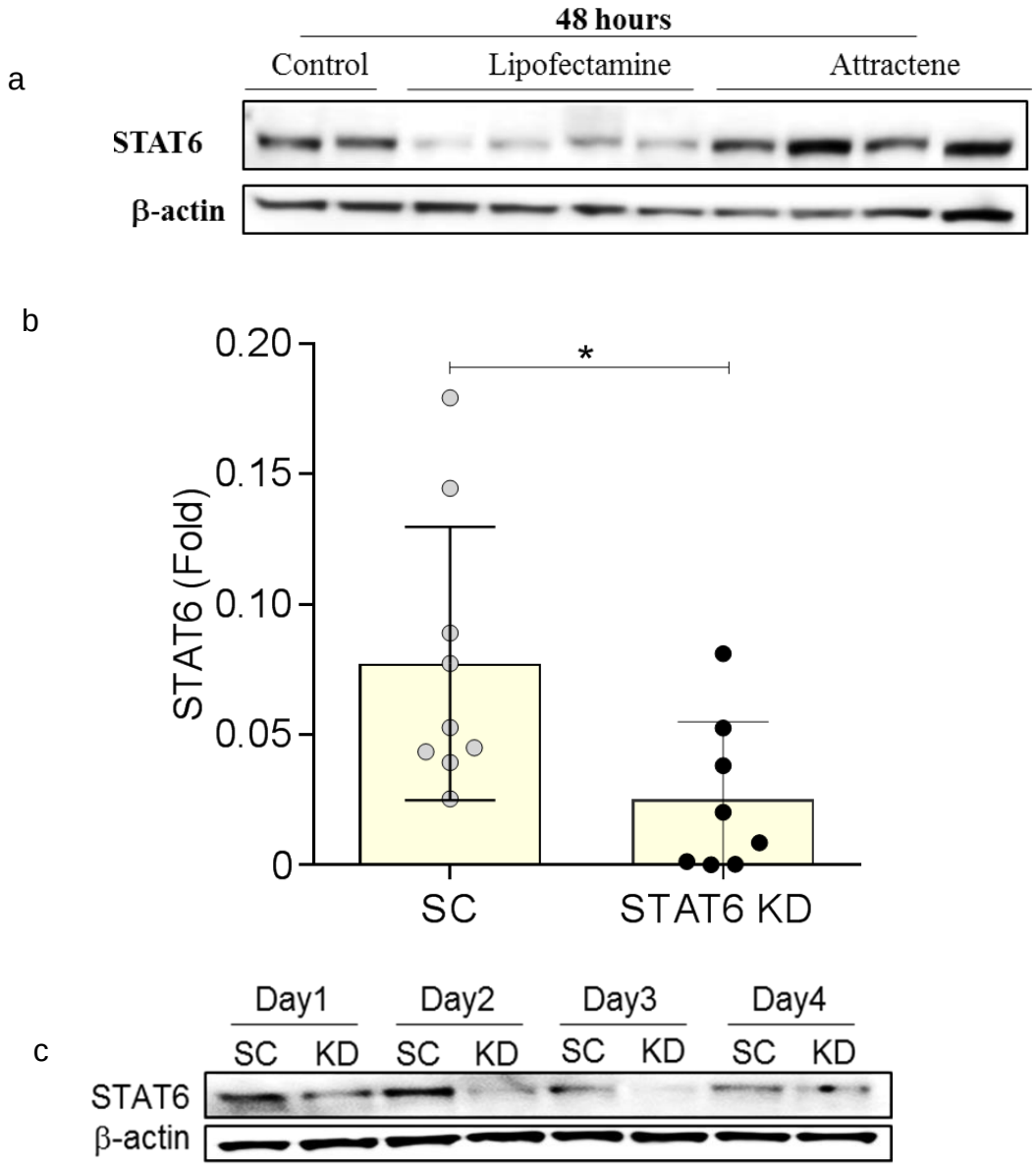


Figure 5. 6: STAT6 was successfully knocked down for four days

INS-1E cells were treated with 10nM of siSTAT6 for **(a-b)** 48h and **(c)** 96h. Cells were washed with PBS, lysed and the supernatant harvested for western blotting. **(a-b)** Western blotting was performed with 20μg of protein and membranes probed with anti-sera against STAT6 and control anti-sera against beta-actin. **(b)** Densitometric analysis with Licor software showed a statistically significant decrease in STAT6 expression.

Western blots results are representative of 3 independent repeats. Data for densitometry analysis represent mean ±SEM of triplicate experiments *p<0.05, determined by student *t*-test.

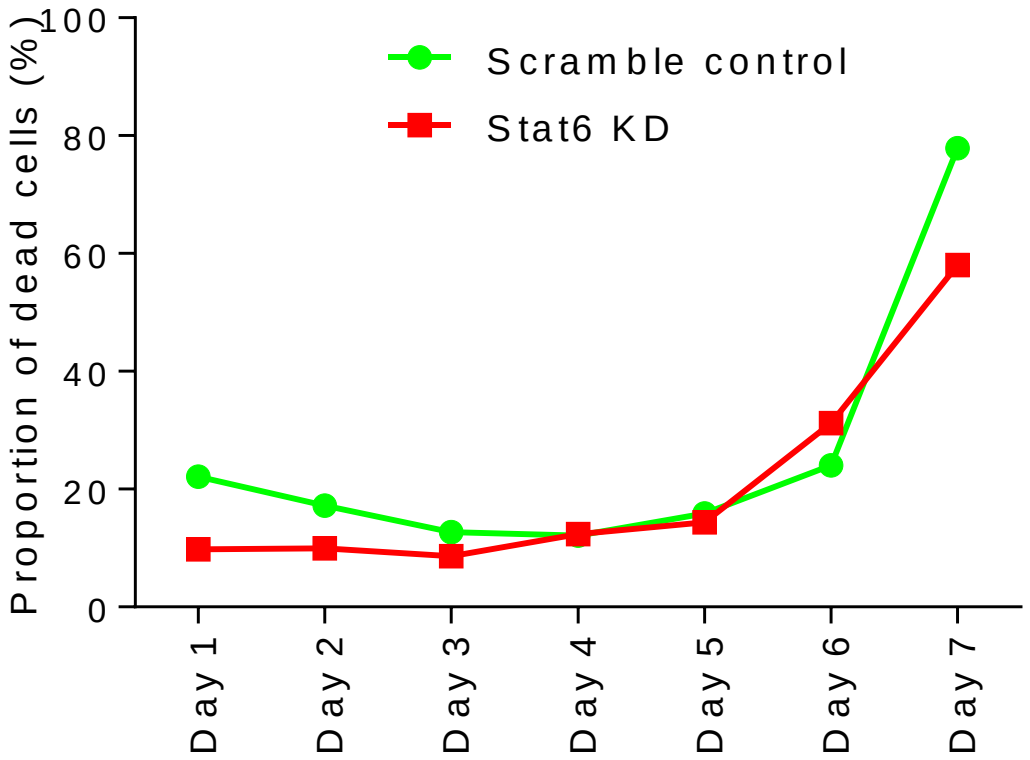


Figure 5. 7: STAT6 knockdown does not induce cell death in beta cells

INS-1E cells were cultured and treated with 10nM of siSTAT6 or scramble siRNA and cells harvested at the end of each day for viability studies using trypan blue staining.

Data represent mean values from two independent experiments

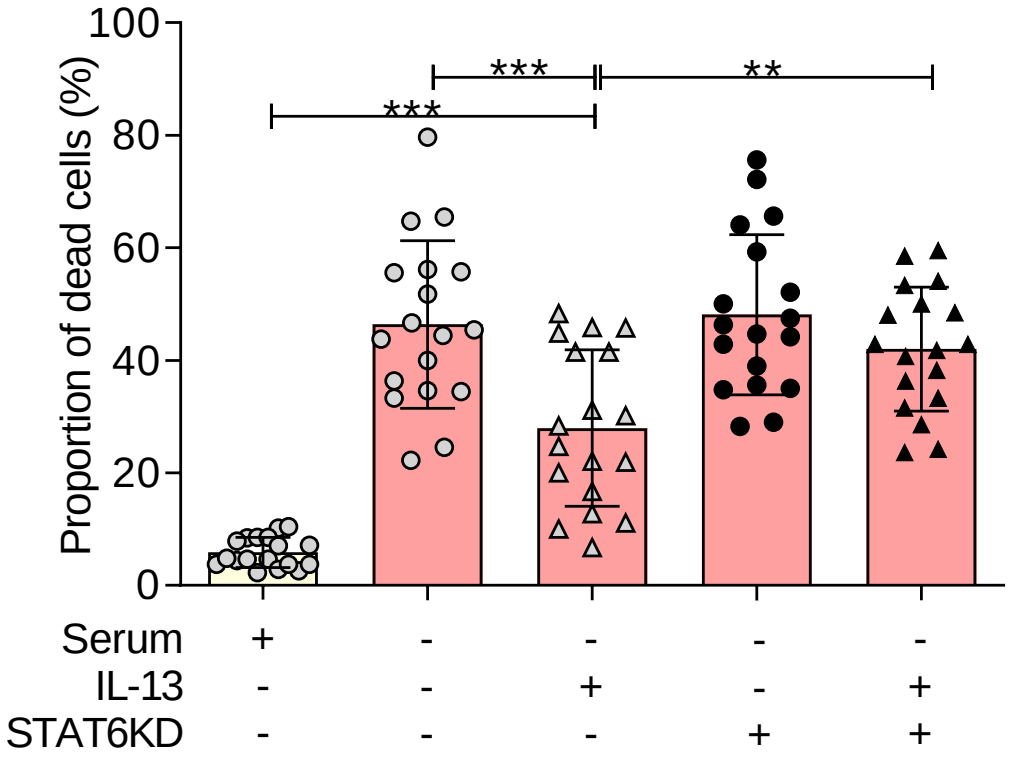


Figure 5. 8: Depletion of STAT6 reverses the partial protective effects of IL-13 on serum withdrawal

INS-1E cells were transfected with siSTAT6 or scrambled siRNA for 24h prior to the addition of 20ng/mL of IL-13 in the absence of serum for 96h. Cells were then harvested and viability assessed by trypan blue staining.

Data represent mean values of 3 independent experiments ±SEM $^{**}p<0.01$, $^{***}p<0.001$, determined by student *t*-test.

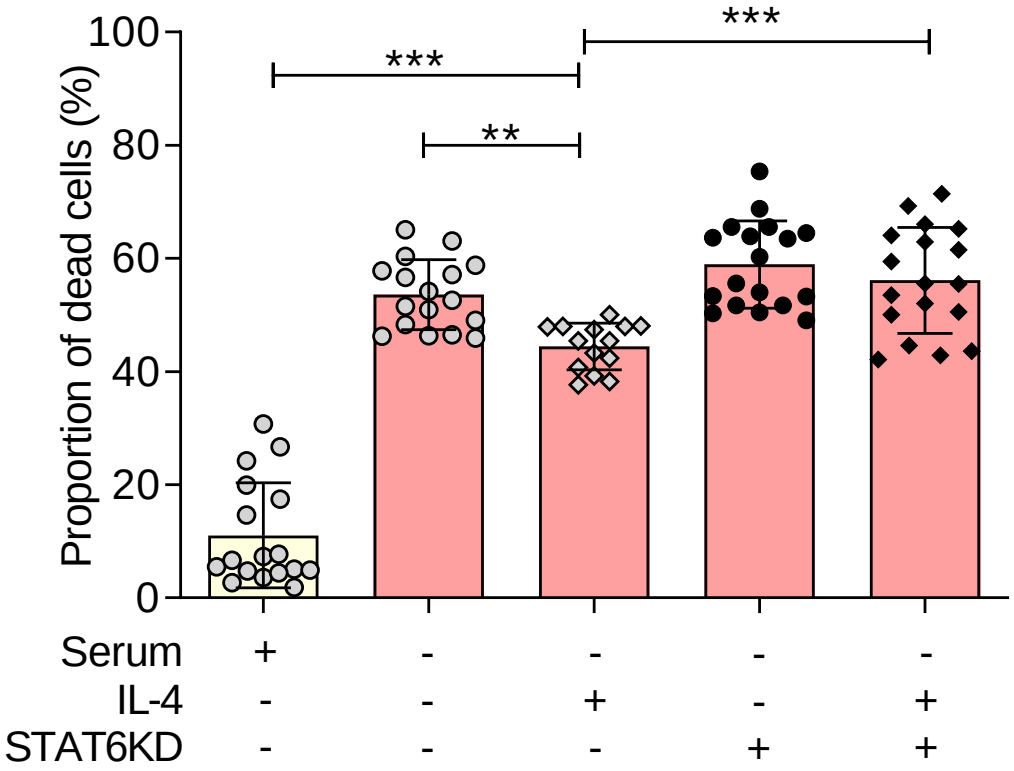


Figure 5. 9: Depletion of STAT6 reverses the partial protective effects of IL-4 on serum withdrawal

INS-1E cells were transfected with siSTAT6 or scrambled siRNA for 24h prior to the addition of 20ng/mL of IL-4 in the absence of serum for 96h. Cells were then harvested and viability assessed by trypan blue staining.

Data represent mean values of 3 independent experiments ±SEM **p<0.01 determined by student *t*-test**.**

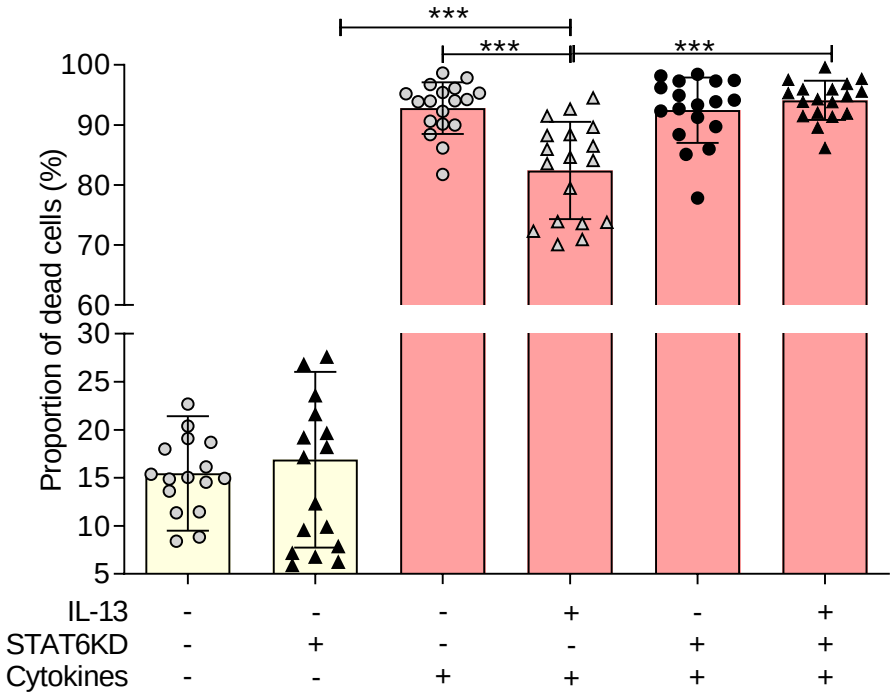


Figure 5. 10: Depletion of STAT6 reverses the partial protective effects of IL-13 on pro-inflammatory cytokines treatment

INS-1E cells were transfected with siSTAT6 or scramble siRNA for 24h prior to the addition of 20ng/mL of IL-13 in the presence or absence of 20ng/mL of pro-inflammatory cytokine cocktail (cytokines) (IL-1β, IL-6, TNF-α & IFN-γ). Cells were then harvested and viability assessed by trypan blue staining.

Data represent mean values of 3 independent experiments ±SEM ***p<0.001 determined by student *t*-test.

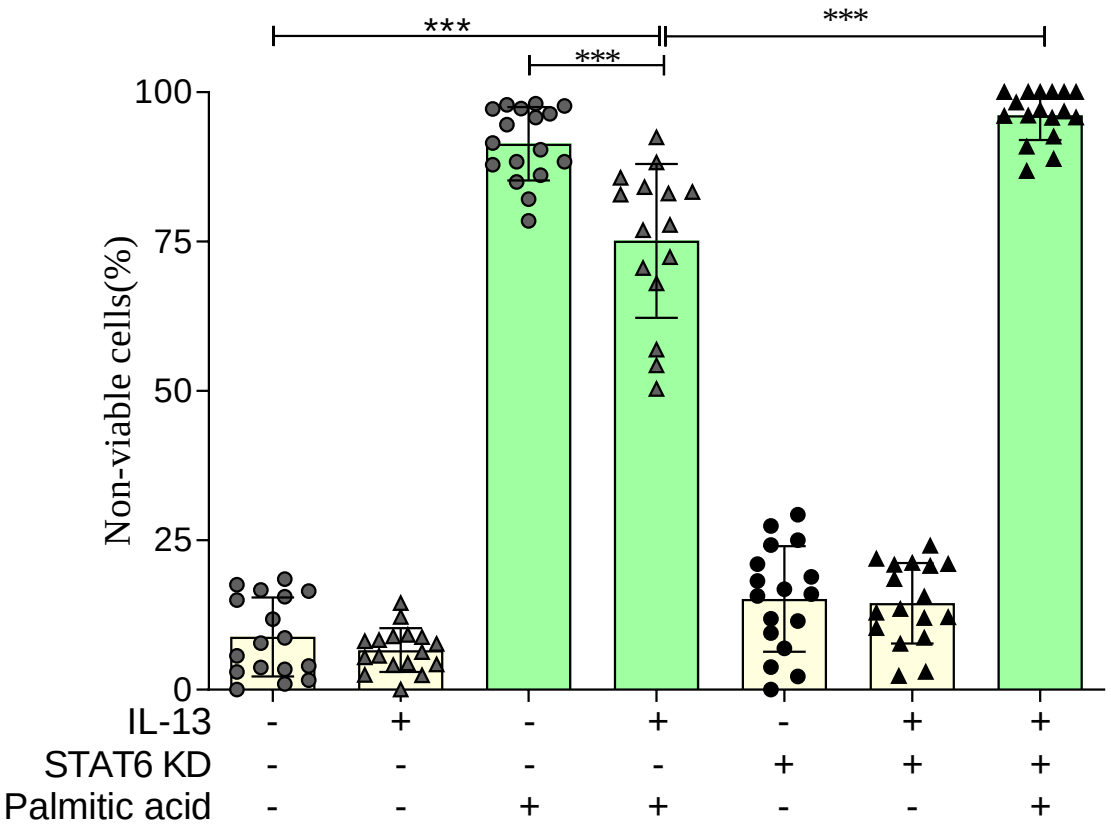


Figure 5. 11: Depletion of STAT6 reverses the partial protective effects of IL-13 on palmitic acid treatment

INS-1E cells were transfected with siSTAT6 or scramble siRNA for 24h prior to the addition of 20ng/mL of IL-13 in the presence or absence of 250μmol/l palmitic acid for 48h. Cells were then harvested and viability assessed by trypan blue staining.

Data represent mean values of 3 independent experiments ±SEM ***p<0.001 determined by student *t*-test.

5.3.2 Knockdown of STAT6 reverses the upregulation of IL-13 responsive genes and gene products

In the previous chapter, it was revealed that IL-13 stimulation of INS-1E cells induced the upregulation of several genes (as illustrated in section 4.3.9). To understand the role of STAT6 in the regulation of these genes, small interference RNA was used to knockdown STAT6, prior to the stimulation of cells with IL-4 and IL-13. Using gene-specific primers, SYBRGreen qPCR confirmed the upregulation of *SIRPα, BCL2L1, SOCS1, MCL-1* and *EPOR* in response to IL-4 and IL-13 stimulation. The fold change was calculated using $2^{-\Delta\Delta Ct}$ formula relative to levels of the housekeeping genes *HPRT1* and *YY1*.

As shown previously (Fig. 4.11a), IL-13 and IL-4 stimulation both significantly increased the expression of *SIRPα.* Importantly, knockdown of STAT6 completely reversed the IL-13 and IL-4 induced increases in *SIRPα* levels (Fig. 5.12a). The expression of *SOCS1* was similarly elevated by IL-13 and IL-4 stimulation, and this effect was again abrogated by *STAT6* knockdown (Fig. 5.12b). Although increases in *MCL-1* mRNA expression did not achieve statistical significance following IL-4 treatment, a significant decrease in *MCL-1* mRNA levels was noted with *STAT6* knockdown when compared to scrambled control. Treatment of cells with IL-13 only slightly increased *MCL-1* mRNA and this was unchanged by *STAT6* knockdown (Fig. 5.12c). *EPOR* was upregulated in response to IL-4 stimulation, but not by IL-13. This upregulation by IL-4 was reversed by *STAT6* knockdown (Fig. 5.12d). IL-4 treatment induced the upregulation of *BCL2L1* and *STAT6* knockdown significantly reduced its expression. IL-13 treatment increased *BCL2L1* mRNA expression, even though this did not attend statistical significance. However, knockdown of *STAT6* significantly reduced *BCL2L1* during IL-13 treatment (Fig. 5.12e). *IFNGR1*

expression was unchanged in these experiments and was not affected by knockdown (Fig. 5.12f). *SMAD1* was reduced under *STAT6* knockdown conditions but was not directly increased by IL-13 or IL-4 (Fig 5.12h). Initially, *B2M* was used as one of the housekeeping genes, but surprisingly it was noticed that its expression was significantly upregulated by knockdown of *STAT6* (Fig 5.12g).

Western blotting analysis were then performed to determine if these gene expression changes led to respective alterations in protein expression. As expected and shown in the previous chapter (Fig. 4.10), SIRPα expression was upregulated by IL-13 and IL-4 stimulation but importantly, this was attenuated by *STAT6* knockdown. Probing the same membranes using antisera raised against BCLXL, the gene product for *BCL2L1,* or *MCL-1* showed an increase in protein levels in response to the anti-inflammatory cytokines (IL-4 and IL-13), an effect which was blocked by *STAT6* knockdown (Fig 5.13) and confirmed by densitometry analysis Fig 5.14 although MCL1 levels did not achieve statistical significance.

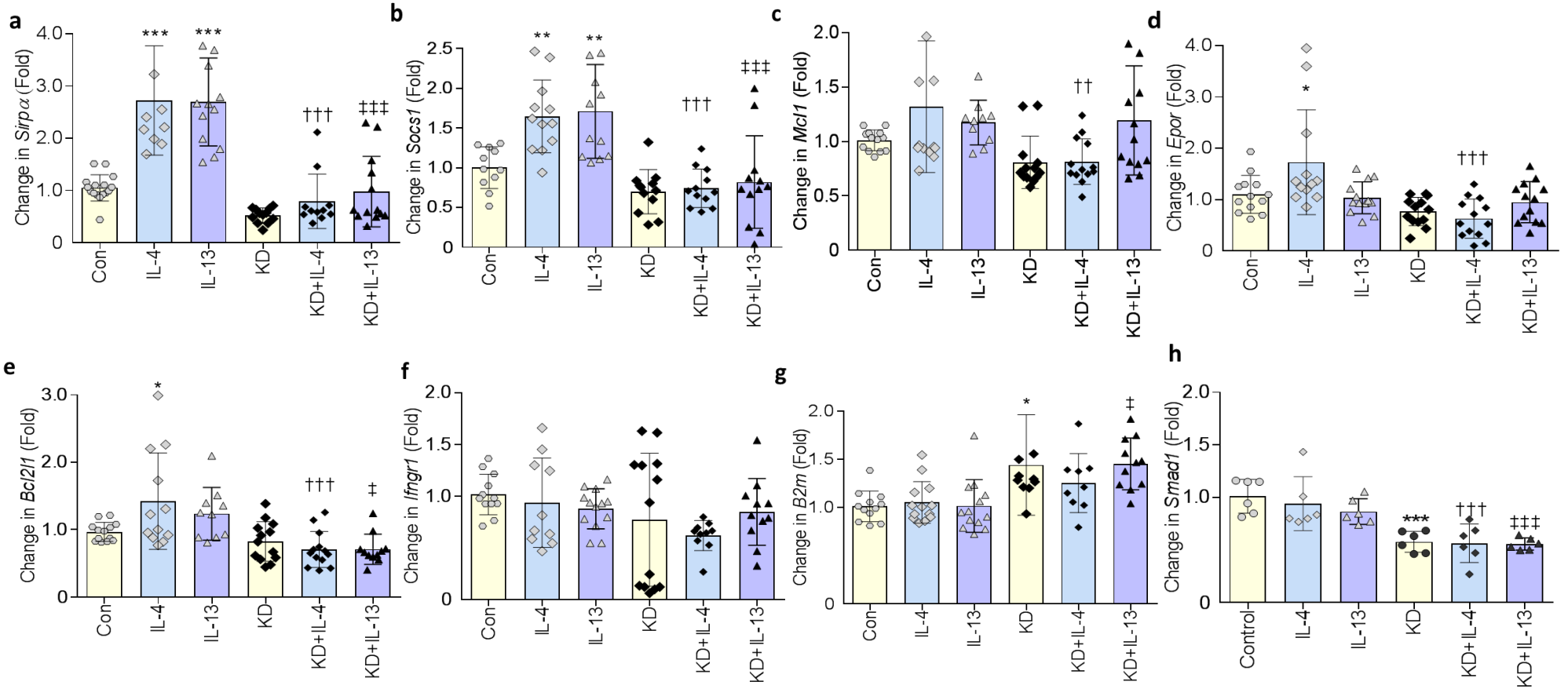


Figure 5. 12: IL-13 and IL-4 stimulation of INS-1E cells induces the upregulation of anti-inflammatory and anti-apoptotic genes

INS-1E cells were stimulated with IL-13 or IL-4 for 48h after *STAT6* knockdown, RNA extracted from treated cells and cDNA synthesize. Specific primers were generated for qRT-PCR analyses of a selection of genes **a.** *Sirpα* **b.** *Socs1* **c**. *Mcl1* **d.** *Epor* **e.***Bcl2l1* **f.** *ifngr1* **g.** *B2m* and **h.** *Smad1* **p*<0.05, ***p*<0.01, ****p*<0.001, relative to the control. † †*p*<0.01, † † †*p*<0.001, relative to IL-4 treatment. ‡*p*<0.05, ‡‡‡*p*<0.001, relative to IL-13 treatment. The significant differences were determined by the student *t*-test.

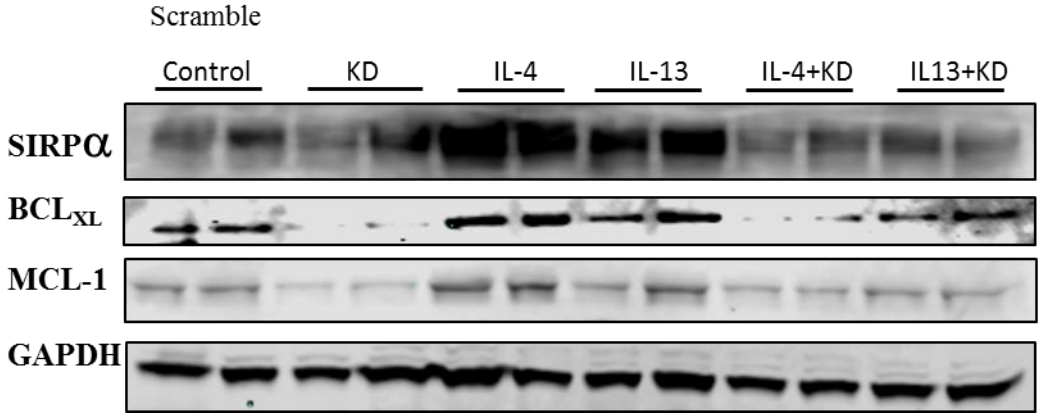


Figure 5. 13: SIRPα, BCLXL, and MCL-1 are upregulated by IL-4 and IL-13 in a STAT6 dependent manner

STAT6 was depleted by siRNA knockdown followed by 20ng/mL of IL-4 or IL-13 for 48h. Cell were lysed, the supernatant collected and western blotting performed. Western blots were probed with anti-sera against SIRPα, BCLXL, MCL-1, and GAPDH.

Data represent western blot results of experiments that were performed 3 independent times.

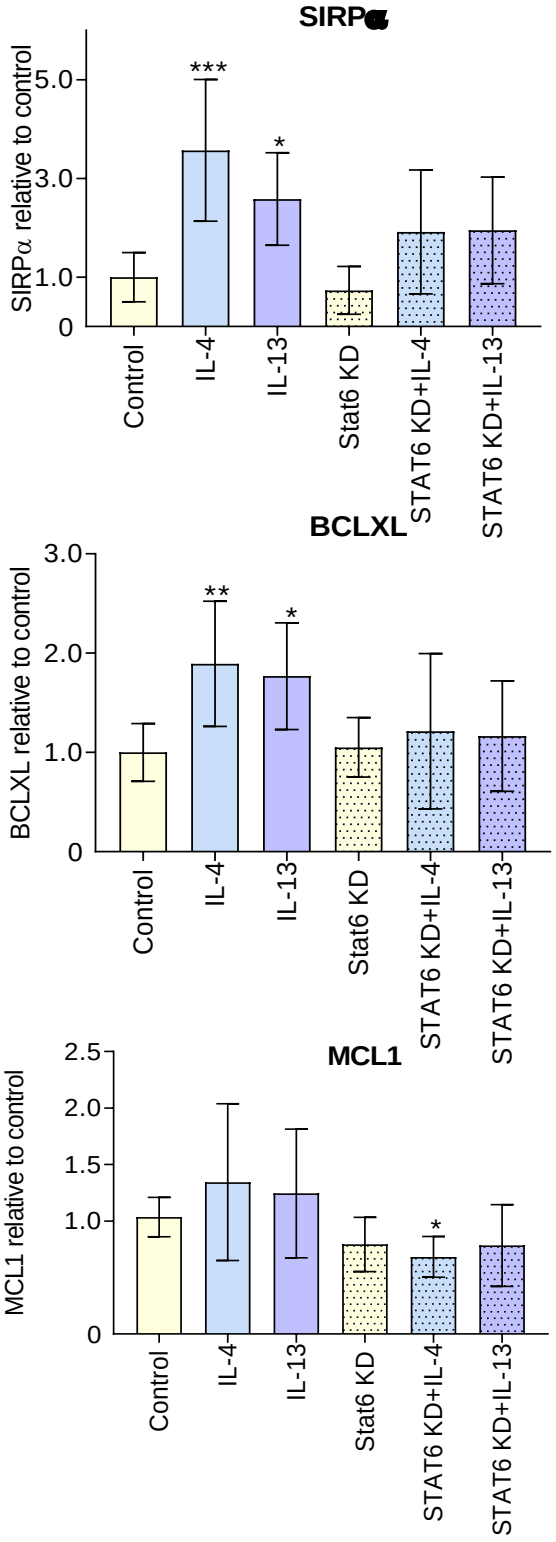


Figure 5. 14: Densitometric analysis on Western blots reveals that SIRPα and BCLXL are upregulated in a STAT6 dependent manner

Western blot densitometry analysis were performed with the Licor software normalised with loading control and expressed relative to the control. Data represent means ±SEM of three independent experiments determined by student t-test

5.3.3 Construction of constitutively active and dominant negative mutant STAT6

In order to obtain constitutively active STAT6, the wild type STAT6 sequence at position 1638-1644 (GTCACT) was changed to GCCGCT hence substituting the amino acids valine (547) and Threonine 548 to alanines in these two positions. For the dominant negative construct, the STAT6 sequence coding for Tyrosine 641 (1920-1923) was changed to phenylalanine. Once SDM was performed and constructs amplified by *E.coli* transformation, plasmids were extracted and sent for sequencing to confirm the mutagenesis.

An online alignment tool (https://www.expasy.org/genomics/sequence_alignment) was used to confirm the changes (Fig. 5.15).

Figure 5. 15: Rat STAT6 cDNA sequence (NC_005106.4) alignment with constitutive active and dominant negative forms

Sequence results from sequencing lab were aligned with wild type STAT6 (WT) **(a)** constitutively active STAT6 (CA), GTC ACT codes for Valine and Threonine were changed to (↑) GCC (↑) GCT which then codes for two alanines enabling STAT6 to be constitutively active. To obtain dominant negative STAT6 (DN), the sequence coding for tyrosine 641 TAT was changed to (↑) TTT to code for phenyalanine.

The successful insertion was confirmed by sequencing of the plasmid and aligning the sequence results with that of the wildtype (Fig 5.15). INS-1E cells were transfected with the plasmid for 24h and then stimulated with IL-13 for 30mins before collecting lysates. A western blot was performed and membranes probed with both phospho-STAT6 and total STAT6 anti-sera to monitor transfection of the plasmid into INS-1E cells (Fig. 5.16). Expression of STAT6 was increased in transfected cells. Treatment of INS-1E cells transfected with various STAT6 mutant with IL-13 for 30min, caused an increase in phosphorylation of STAT6 in cells transfected with (empty vector (EV)), wild type (WT), and constitutively active mutant (CA). Dominant negative STAT6 (DN) had a slight increase in phosphorylation of STAT6 upon IL-13 stimulation (Fig. 5.16).

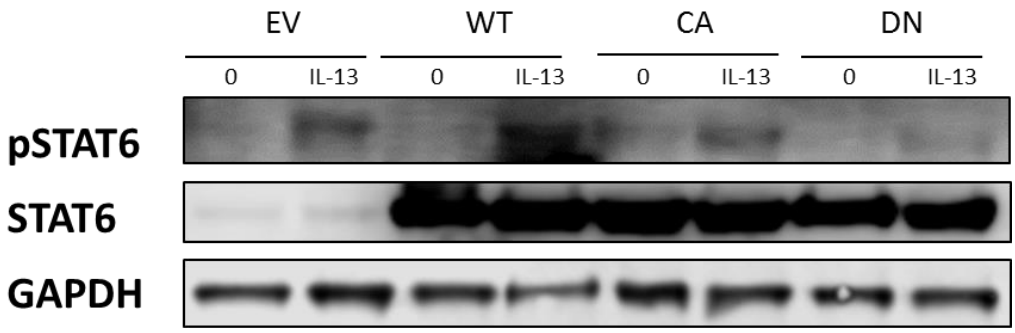


Figure 5. 16: Mutant STAT6 forms are activated by IL-13

INS-1E cells were transfected with a 500ng/mL empty vector (EV), wild type (WT), constitutive active (CA), dominant negative (DN) STAT6 plasmid with Lipofectamine LTX reagent for 24h followed by treatment in the presence and absence of 20ng/mL IL-13 stimulation for 30min. Cells were PBS washed, lysed and the supernatant collected and western blotting performed. Membranes were probed for phospho-STAT6, total STAT6 and a loading control GAPDH.

Data represent western blots of experiments performed twice

5.3.4 Effects of IL-13 on mutant STAT6 variants

Activated STAT6 dimerizes and migrates to the nucleus to transcribe genes by binding to a DNA motif with the consensus sequence $TTCN_{3/4}GAA$; with $N_{3/4}$ representing any three (N3) or four (N4) nucleotides respectively. STAT6 is the only member of the STAT family that has a preference for N4 sites (Li et al., 2016a). The N3 reporter consists of 3 copies of the N3 Gamma Activated Site (GAS) in the promoter (TTCtagGAA) while the N4 reporter contains a 3 “N4” STAT6 binding sites in the promoter (TTCcgagGAA) upstream of a luciferase reporter enzyme (Fig. 5.2).

The dual luciferase assay simultaneously measures the activity of the firefly (reporter) and the amount of *Renilla* luciferase. While the reporter gives us a measure of the activity of the molecule of interest, the *Renilla* is used to normalise the data from the reporter assay. After the measurement of the luciferase activity, the firefly reaction is stopped with simultaneous activation of the *Renilla* luciferase which acts as a control reporter (Sherf et al., 1996). In order to evaluate the functionality of the various STAT6 forms, HEK293 cells were transfected with an expression vector containing WT, CA, or DN STAT6 alongside the N4 luciferase reporter and *Renilla* for 24h before stimulating with IL-4 and IL-13. No activity was observed in the empty vector control even with IL-4 and IL-13 stimulation. Transfection of WT STAT6 did not yield any activity on its own but the addition of IL-4 and IL-13 lead to a nine-fold and 20-fold increase respectively relative to the unstimulated control. Transfection with the CA STAT6 showed a 2.8 fold increase in activity relative to the control, this increased further to 20 fold, and 27 fold with IL-4 and IL-13 stimulation respectively relative to the WT. In contrast, the dominant negative form yielded no activity even after the addition of IL-4 and IL-13. Figure 5.17.

After evaluating our STAT6 variants in HEK293 cells, the INS-1E cells were then transfected with the N4 luciferase reporter and the *Renilla* construct to test this system. We observed a luciferase reporter activity in IL-13 treated cells samples but notice little or no activity with the *Renilla* reporter. In this context, the *Renilla* response was then tested in a separate experiment in INS-1E. The response obtained with the *Renilla* construct in INS-1E cells was dose-dependent with the highest activity realised when 1μg per well of the *Renilla* construct was used (Fig. 5. 18) in contrast to its activity in HEK293 cells which was greatest with 200ng of *Renilla.* The absolute values were also quite different with a much higher activity obtained in the HEK293 cells possibly due to the differences in the cell lines (one being human and the other rat). Because the *Renilla* response was relatively poor in INS-1E cells results were expressed our results relative to the empty vector control and/ or wild type STAT6.

Using the N3 and N4 reporters to study the activity of STAT6 by luciferase assay, INS-1E cells were transfected with the various STAT6 mutants and reporter assays performed as described in section 2.9.4. With the N4 reporter, wild-type (WT) STAT6 did not induce any luciferase activity in the absence of cytokine but the cells responded well to the addition of IL-13 (31.4 fold change relative to the WT unstimulated STAT6). Constitutively active STAT6 (CA) showed an increase in activity in the absence of IL-13 (19.5 fold) and this response was tripled by the addition of IL-13 (80.6 fold relative to WT STAT6). In contrast, the dominant negative STAT6 (DN) showed no activity (Fig 5.19).

When variant forms of STAT6 were co-transfected with the N3 luciferase reporter, only a modest elevation in STAT6 activity was observed in cells transfected with the STAT6WT construct in response to IL-13 (5.2 fold relative to the unstimulated

control). Surprisingly, transfection with STAT6CA alone did not alter transcriptional activity relative to the WT protein (STAT6CA: 1.2 fold), however, activity was increased upon stimulation of these cells with IL-13 (STAT6CA + IL-13: 2.8 fold change). These responses were significantly lower than that of the WT activity with IL-13 stimulation in the N4 reporter. The dominant negative STAT6 showed no activity with the N3 reporter. (Fig. 5.20).

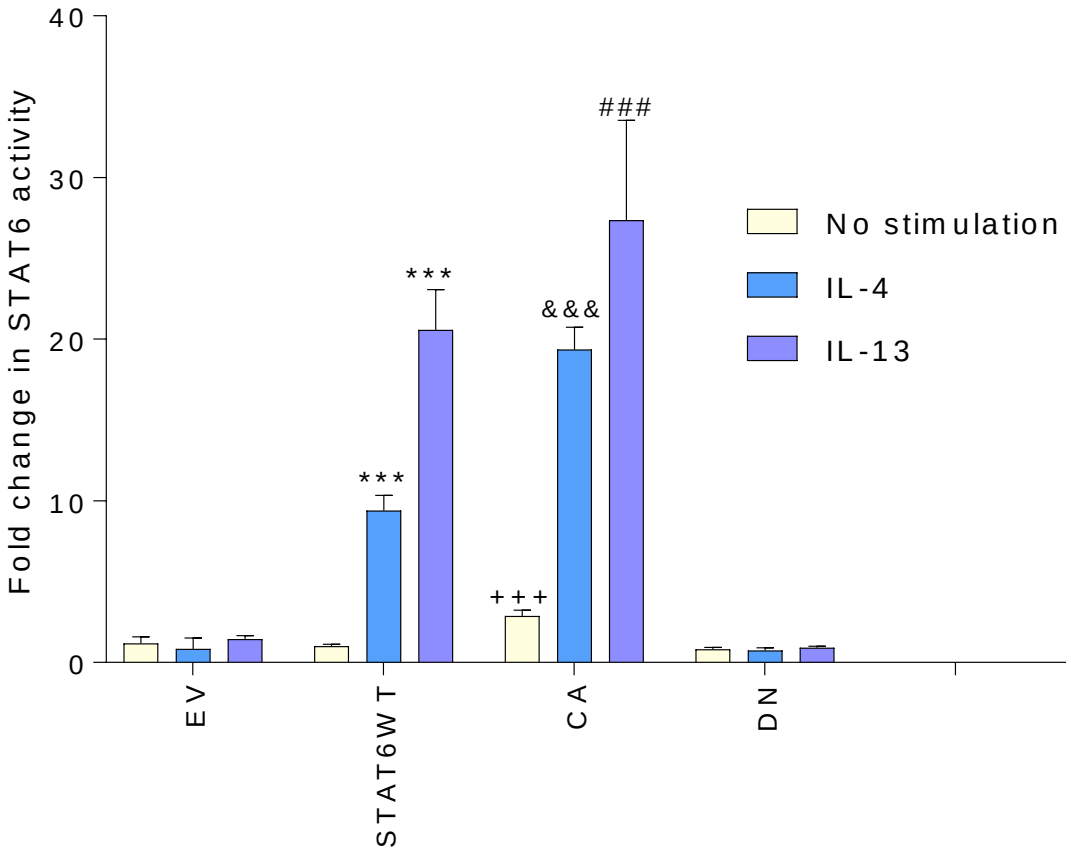


Figure 5. 17: IL-4, IL-13 and STAT6CA induce STAT6 activity as expected.

HEK293 cells were transfected with an empty vector (EV) or STAT6 variants (STAT6WT, STAT6CA, STAT6 DN) with the N4 luciferase reporter and *Renilla* construct for 24h followed by stimulation with 20ng/mL of IL-4 and IL-13 for a further 24h. Cells were washed, lysed and dual luciferase assay performed. Data was normalised to the *Renilla* activity and then to the empty vector and STAT6WT.

Data represent fold change mean values from 3 independent experiments ±SEM. ***p<0.001 relative to STAT6WT: no stimulation, +++p<0.001 relative to STAT6WT: no stimulation, &&&p<0.001 relative to STATWT IL-4, ###p<0.001 relative STAT6WT IL-13, all determined by the student *t*-test.

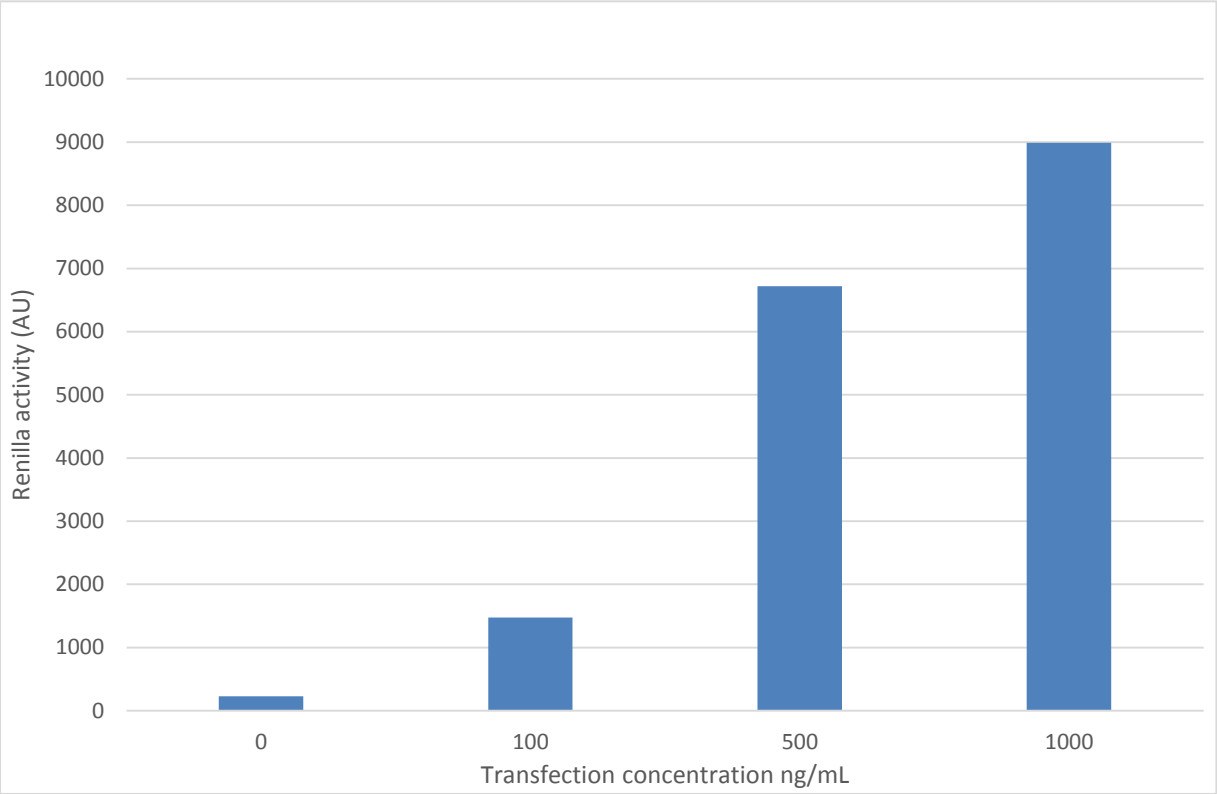


Figure 5. 18: *Renilla* plasmid activity was very low in INS-1E cells

INS-1E cells were transfected with 100ng, 500ng or 1000ng of *Renilla* cDNA construct for 24h. Cells were then harvested and lysed prior to measuring the *Renilla* activity.

Data represents fold change mean of values of experiments performed twice

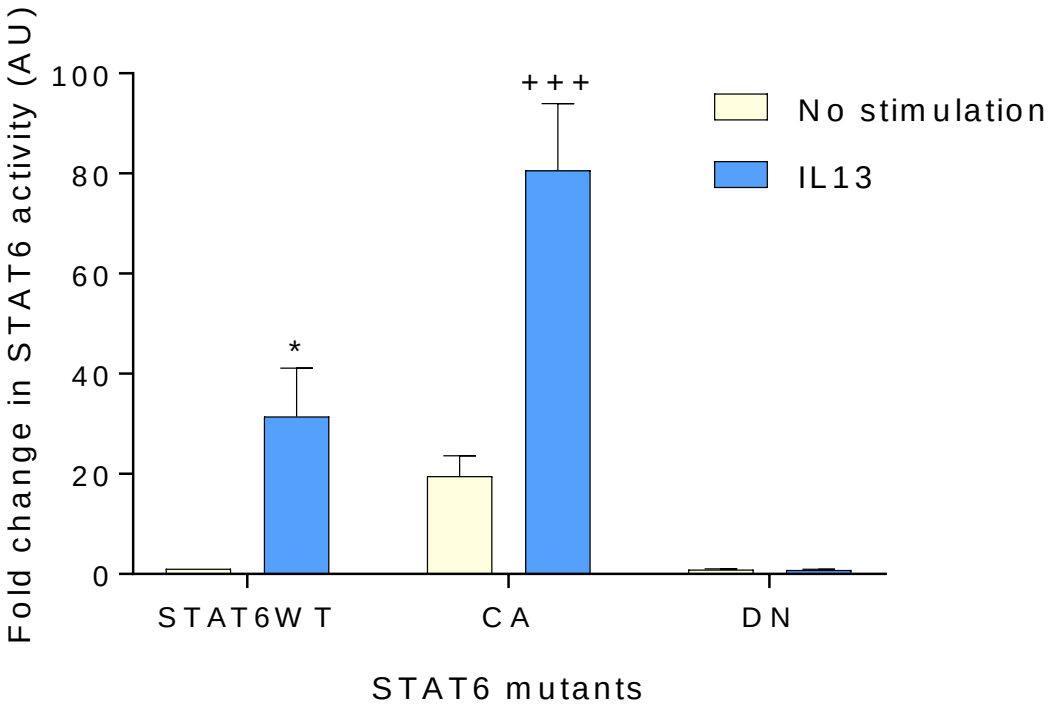


Figure 5. 19: IL-13 and STAT6VT (CA) mutant induce STAT6 activity at the N4 domain

INS-1E cells were transfected with 500ng/mL of STAT6WT, STAT6CA or STAT6DN alongside the N4 STAT6 luciferase reporter construct. Cells were cultured in the presence of (blue bars) or absence (yellow bars) 20ng/mL of IL-13, lysed and a luciferase assay performed with 20μL of lysates.

Data represent fold change mean values of experiments performed twice *$p<0.05$ relative to STAT6WT: no stimulation, +++$p<0.001$ relative to STAT6WT: IL-13, all determined by the student *t*-test.

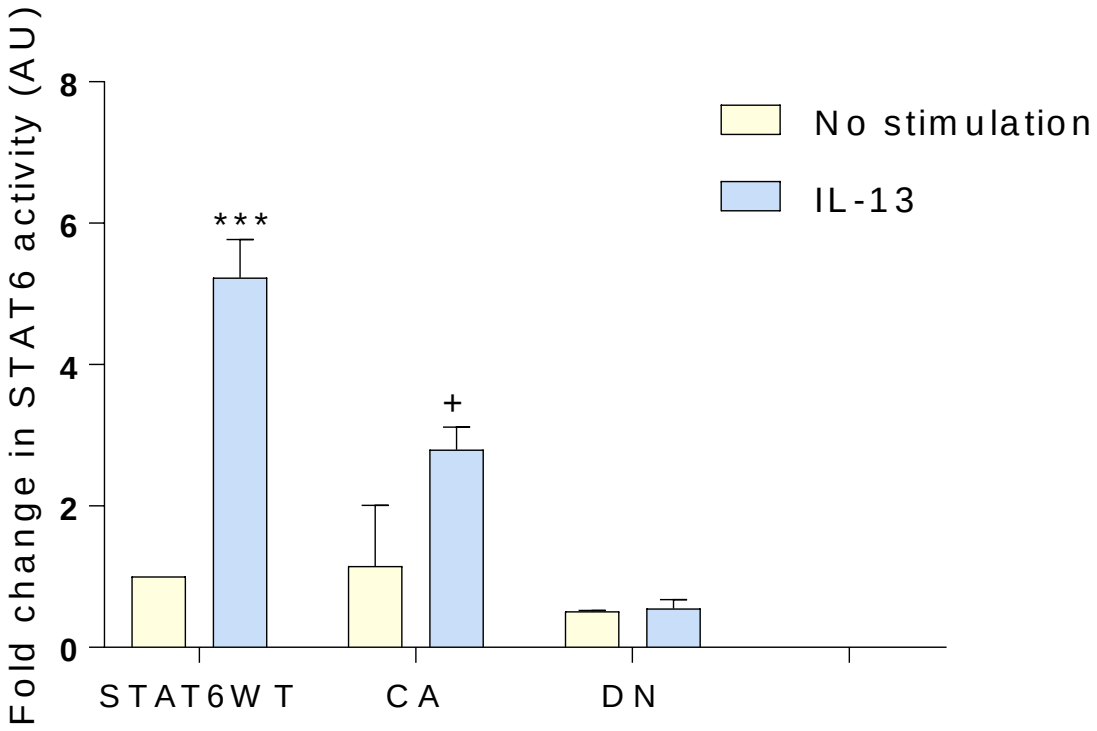


Figure 5. 20: IL-13 induces STAT6 activity but not mutant forms at the N3 domain

INS-1E cells were transfected with 500ng/mL of STAT6WT, STAT6CA or STAT6DN alongside N3 STAT6 luciferase reporter construct. Cells were cultured in the presence (blue bars) or absence (yellow bars) of 20ng/mL of IL-13, lysed and a luciferase assay performed with 20μL of lysates.

Data represent fold change mean values of ±SEM of experiments performed twice. ***p<0.001 relative to STAT6WT, +p<0.05 relative to STAT6WT+IL-13, all determined by the student *t*-test.

5.3.5 STAT6 mutants reduce beta cell death with IL-13 stimulation

Data presented in the preceding chapters has revealed that IL-13 protects beta cells from the cytotoxic effects of pro-inflammatory cytokines (Figs. 3.4-3.5), and in this chapter, the data revealed that this protection is reversed by the knockdown of STAT6 (Fig 5.10). An experiment was designed to assess whether the expression of constitutively active or dominant negative forms of STAT6 could directly impact beta cell viability.

For these experiments, the transfection efficiency of INS-1E cells was first determined by transfecting with a GFP construct then detecting GFP expression using flow cytometry. INS-1E cells showed a transfection efficiency of about 12%. For this reason, experiments performed with INS-1E cells that involved transfection of variants of STAT6 were performed alongside co-transfection of a GFP construct to mark transfected cells for isolation and characterisation in viability experiments based on the co-transfection principle. The co-transfection principle suggests that if two plasmids are co-transfected into cells, there is a positive correlation coefficient of 0.96, which implies that both plasmids are almost equivalently delivered into the recipient cells (Xie et al., 2011). Therefore, GFP expression served as a transfection control.

INS-1E cells were transfected with STAT6WT, STAT6CA, or STAT6DN constructs alongside a GFP reporter with and without IL-13 stimulation and pro-inflammatory cytokines. Viability assays were performed using propidium iodide staining. In the absence of cytokines, there were no significant increases in cell death in the STAT6WT, STATCA when compared to the control. Surprisingly, the DN mutant increased cell death in INS-1E cells when compared to the empty vector controls (EV control), STAT6CA and STAT6WT (EV: 32.48±2.2%, DN:

46.45±2.3%, CA: 30.85±1.6%, WT: 32.21±1.7%, cell death *p<0.05*). Additionally, IL-13 stimulation did not protect from DN mediated cell death (DN: 46.45±2.3, DN+IL-13: 53.92±5.5, cell death p>0.05) or improve viability upon transfection with other STAT6 variants (Fig 5.21).

As previously shown in Chapter 3 and 4, pro-inflammatory cytokines (Pro) induced beta cell death in INS-1E cells (Pro: 86.9±2.2% cell death). Similarly, pro-inflammatory cytokine stimulation of INS-1E cells transfected with variants of STAT6 induced significant cell death (Pro: 86.88±2.2%, Pro+DN: 87.75±1.1%, Pro+CA: 85.48±1.4%, and Pro+WT: 82.4±2.9%, p>0.05). As illustrated in section 3.3.2, IL-13 stimulation protected beta cells from the detrimental effects of pro-inflammatory cytokines in all treatments including STAT6DN (Pro: 86.9±2.2%, Pro+IL-13: 68.3±1.7%, Pro+DN+IL-13: 70.9±2.6%, Pro+WT+IL-13: 67.8±2.4%, and Pro+CA+IL-13: 60.9±2.2%, cell death p>0.01). STAT6CA appeared to have improved beta cell viability after IL-13 treatment compared to the STAT6WT cells treated with pro-inflammatory cytokines although this did not reach significance.

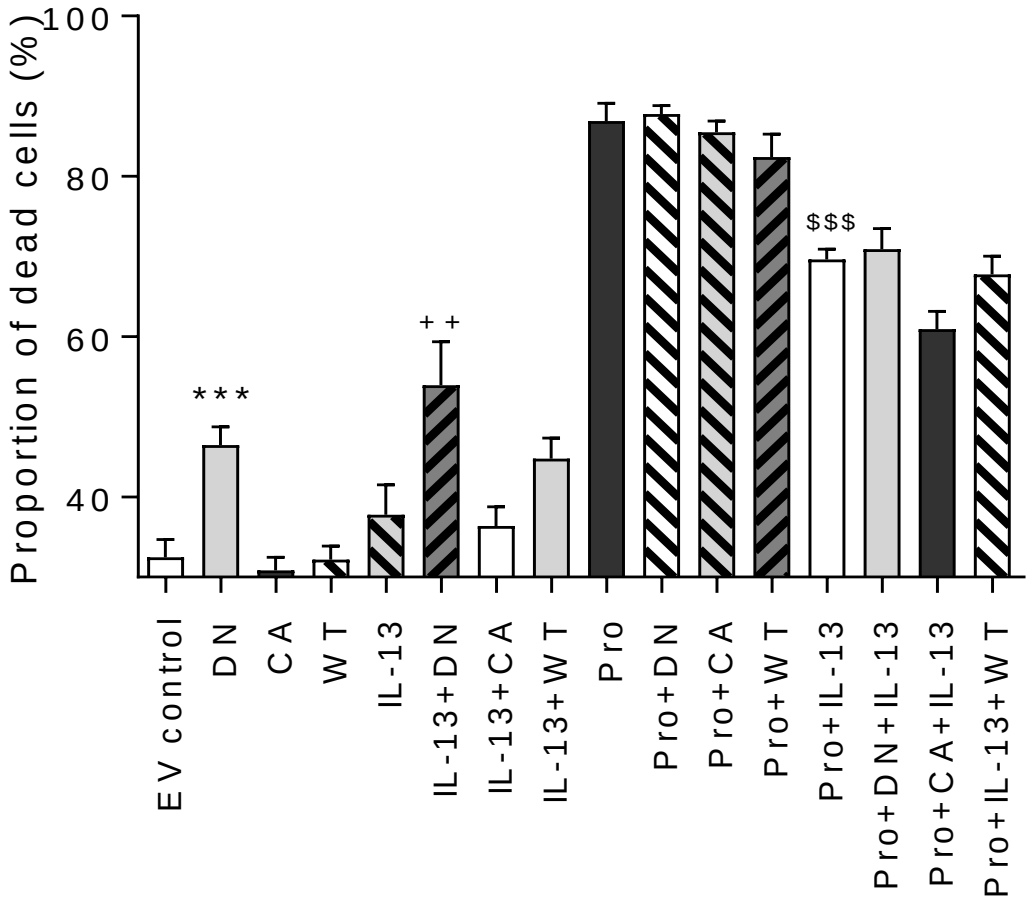


Figure 5. 21: STAT6 mutants reduce beta cells death by Pro-inflammatory cytokines upon IL-13 treatment

INS-1E cells were transfected with various STAT6 mutants and GFP to identify the transfected cells followed by pro-inflammatory cytokines (Pro) (20ng/mL IL-6, IL-1β, IFN-γ, and TNF-α) for 48h in the absence and presence of IL-13. Cells were harvested with the use of trypsin and cell viability assessed by propidium iodide staining.

Data represents mean values ±SEM of three independent experiments. ***p<0.001 relative to EV control, ++p<0.01 relative to EV control, $$$p<0.001 relative to Pro, all determined by the student *t*-test.

5.3.6 STAT6 is lost in cells exposed to pro-inflammatory cytokines and is rescued by IL-13 treatment.

Initial findings using immunohistochemistry suggested that STAT6 is lost from the insulin containing islets of individuals with T1DM when compared to age and sex matched non-diabetes controls (Leslie et al., 2018). It was not clear what caused this change, but we reasoned that it might be due to the release of pro-inflammatory factors from the influent immune cells. In order to test this, INS-1E cells were treated with pro-inflammatory cytokines with and without IL-13. The results revealed a significant loss of STAT6 upon treatment with pro-inflammatory cytokines Fig. 5.22a. Similarly, treatment of INS-1E cells with 250μM palmitate for 48h showed a loss in STAT6 by western blotting (Fig 5.22a). Additionally, INS-1E cells deprived of serum for 96h showed a decrease in STAT6 expression (Fig 5.22b).

Treatment of the cells with IL-13 in combination with pro-inflammatory cytokines revealed a restoration of STAT6 expression (Fig. 5.22c). A similar response occurred in cells incubated in the absence of serum.

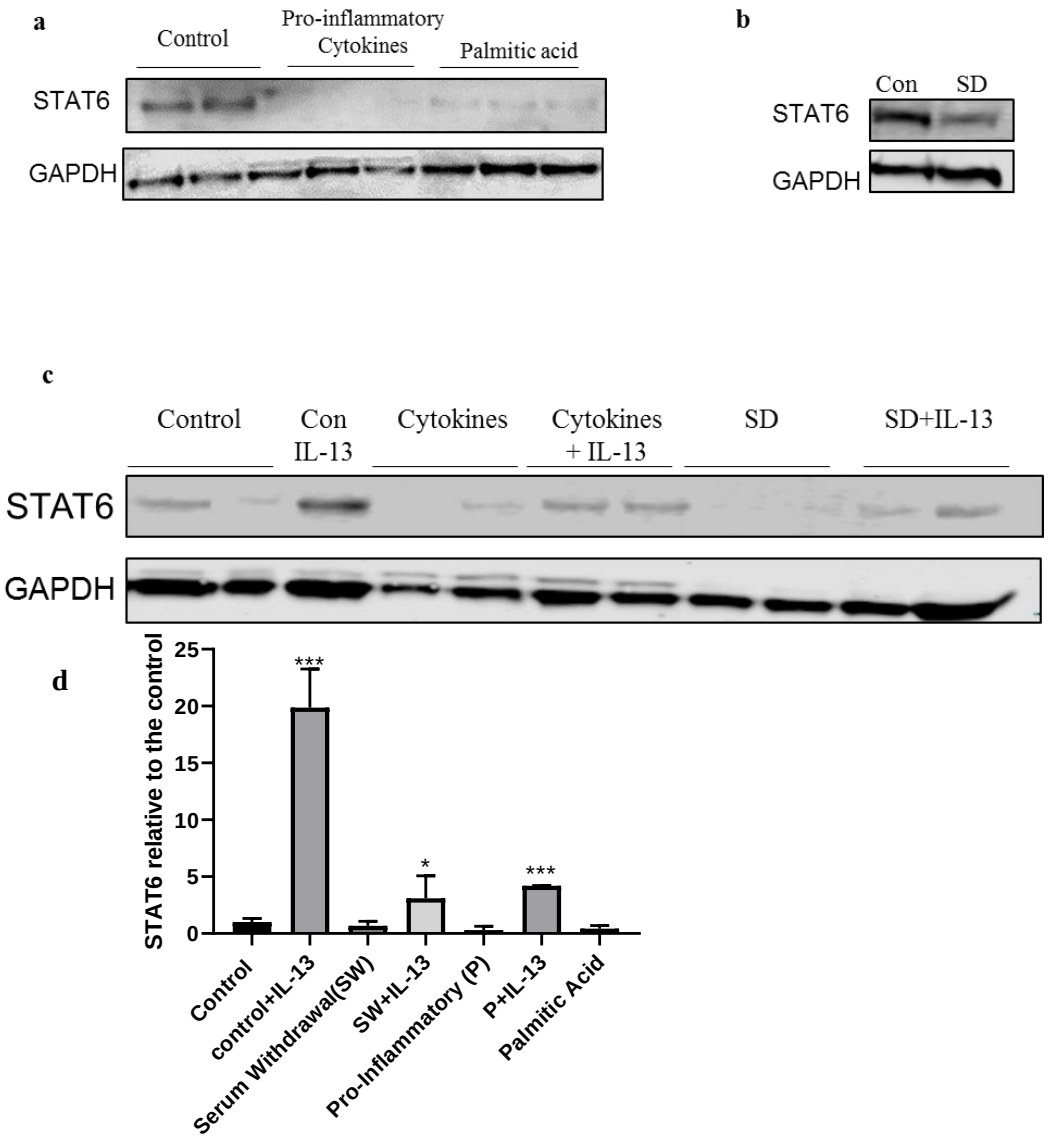


Figure 5. 22 : STAT6 is ablated by cytotoxicity

INS-1E cells were cultured in the presence of **a**. pro-inflammatory cytokines (20ng/mL of each IL-1β, IL-6, TNF-α, and IFN-γ), 250μM palmitic acid or **b.** 96h serum withdrawn conditions **c.** alternatively, cells were cultured with pro-inflammatory cytokines or serum withdrawal conditions in the presence or absence of IL-13. Cell lysates were collected and 10μg used to perform western blotting and membranes probed with antisera against STAT6 and loading control GAPDH. **d**. Densitometric analysis reveal a significant increase in STAT6 relative to respective controls in IL-13 treatments.

Data are representative of experiments performed 3 times independently.

5.4 Discussion

This chapter provides evidence for the importance of STAT6 in mediating the protective effects of IL-13 in pancreatic beta cells. The data suggest that the increased expression of anti-apoptotic proteins, including a consistent increase in SIRPα might be among the mechanisms by which this protection is facilitated. I have shown in this chapter that STAT6 is important in mediating the cytoprotection of the beta cell afforded by IL-13. Additionally, it was shown here that many of the genes upregulated by IL13 and IL-4 are STAT6 modulated.

The results are in agreement with those in chapter 3 and with earlier reports on the protection of beta cells by IL-13 and IL-4 in a cytotoxic milieu (either serum withdrawal, palmitic acid, or pro-inflammatory cytokines) (Kaminski et al., 2010, Russell et al., 2013). In contrast, the data presented in this chapter differ from those of Rutti et al. (2016) who suggest that the protective effects of IL-13 are mediated via a PI3K/Akt signalling route. Although there is activation of the PI3K pathway in response to IL-13, a PI3K inhibitor (wortmannin) did not affect the protection achieved with IL-13 (Russell et al., 2013). The present data suggest that the IL-13 protection primarily works in a STAT6 dependent fashion. Additionally, in a study where STAT6 and IRS2 (shown to be the main activation route of the PI-3K pathway) deficient mice were employed and their lymphocytes cultured overnight in the presence and absence of IL-4, STAT6 was shown to be crucial for IL-4 mediated protection whereas IRS2 was dispensable (Wurster et al., 2002). The role of STAT6 in beta cell has received limited attention in T1DM, but its effect has been documented in other autoimmune diseases. In one such disease (systemic lupus erythematosus), a mouse model was used to illustrate the importance of STAT6. In Lyn (-/-) mice, a lupus like autoimmune model,

depletion of STAT6 (Lyn (-/-) STAT6 (-/-)) exacerbated autoimmunity supporting the role of STAT6 as a protective transcription factor (Lau et al., 2012).

Typically, activated STAT6 dimerises and translocates to the nucleus where it transcribes a specific set of genes (Goenka and Kaplan, 2011). Depleting STAT6 is expected to prevent the upregulation of IL-13/IL-4 induced genes. We identified a number of genes which were upregulated in response to IL-13 in the previous chapter, and now confirm that many of these changes occurred in a STAT6-dependent manner. With the use of small interference RNA molecules, it was shown that anti-apoptotic genes *BCL2L1* and *MCL-1* are STAT6 dependent since knockdown of STAT6 led to a reduced expression of these genes in INS-1E cells (Fig 5.13, 5.14 and 5.15). In immune cells, it is known that IL-4 and IL-13 modulate both BCLXL and MCL-1 expression in a STAT6 dependent manner (Ritz et al., 2008, Steele et al., 2010) but their modulation via STAT6 in pancreatic beta cells has not been previously documented. In one example wherein lymphocytes from STAT6 deficient and relevant control mice were cultured overnight with IL-4, BCLXL expression was significantly upregulated in the wild type controls but diminished in STAT6 deficient lymphocytes (Wurster et al., 2002). Interestingly, loss of STAT6 expression has been observed in tissues from individuals with T1DM (Leslie et al. 2018) and MCL-1 expression is reduced in some islets of individuals with T1DM (Richardson et al., 2013).

The findings in this chapter also revealed that SIRPα expression is regulated in a STAT6 mediated manner in beta cells. This relationship is novel and has not been previously reported in any cell type. It was therefore, important to explore the role of SIRPα in the pancreatic beta cell, and this will be considered further in the next chapter.

It was not surprising that IL-13 and IL-4 upregulate the suppressor of cytokine signalling 1 (SOCS1) in beta cells, since this phenomenon is established in other cell types treated with these cytokines (Hebenstreit et al., 2003, Hebenstreit et al., 2005, Ritz et al., 2008). SOCS1 is a negative regulator of Jak/STAT signalling and is suggested to regulate the pathway by binding to the activation loop of the Jaks (Babon et al., 2006). In this chapter the experiments show that SOCS1 is STAT6 regulated. These findings are in agreement with work by Dickensheets and colleagues (2006) in which monocytes from STAT6 knockout or control animals were stimulated with IL-4 and SOCS1 detected by western blotting. In their experiments, SOCS1 was shown to be STAT6 modulated but also a negative regulator of the Jak/STAT6 pathway when overexpressed (Dickensheets et al., 2006). The role of SOCS1 has been extensively studied in beta cells (Sun et al., 2013, Zaitseva et al., 2009, Barral et al., 2006, Chong et al., 2004) and it may contribute to cytoprotective effect by negatively regulating pro-inflammatory cytokine signalling. In one such study, SOCS1 was shown to inhibit IFN-γ, IL-1β and TNF-α induced beta cell death via the downregulation of caspase 3 activation through the negative regulation of STAT1 activation (Zaitseva et al., 2009). In another study using mice with diabetes that was virally induced, overexpression of SOCS1 reduced the incidence of diabetes by over 90%. In those experiments, it was shown that SOCS1 overexpression rendered beta cells resistant to cytotoxic T lymphocyte attack. It is however, not known whether SOCS1 can impact the protection against non-cytokine forms of cytotoxicity such as serum withdrawal or palmitate.

SMAD1 (SMAD stands for mothers against decapentaplegic) is an intracellular signal transducer of TGF-β receptor. SMAD1 has not been extensively studied in beta cells, but it is known to be expressed in beta cells which agrees with our

qPCR data showing its detection after 23 cycles (Toren-Haritan and Efrat, 2015). So although experiments in beta cells revealed that IL-4 and IL-13 stimulation did not upregulate *SMAD1*, research using hepatic cell lines has reported the upregulation of *SMAD1* by IL-13 in a non-Jak/STAT manner leading to the expression of connective tissue growth factor (Liu et al., 2011). In contrast to these findings, our data show that knockdown of *STAT6* led to a reduced expression of *SMAD1*. Further work will be required better understand the relationship between *SMAD1* and *STAT6* in beta cells.

Initially, *B2M* was recommended as the housekeeping gene for qPCR of STAT6 responsive genes but after performing experiments with STAT6 knockdown it was realised that *B2M* expression may be negatively controlled by STAT6 in beta cells. In these experiments, STAT6 knockdown led to upregulation of *B2M* mRNA suggesting that the transcription factor is important for maintaining the levels of *B2M*. B2M is a protein of low molecular weight (11.8kDa) present in all nucleated cells and is non-covalently linked with MHCI alpha chain (Li et al., 2016b). Due to the changes observed upon STAT6 knockdown I excluded *B2M* as a house keeping gene. Our recently published work suggests that a loss in STAT6 expression occurs in insulin containing islets of T1DM (Leslie et al., 2018). This loss in STAT6 together with evidence of increased in B2M levels in the insulin containing islets of subjects with T1DM (Richardson et al., 2016) resonate with the present findings suggesting that B2M expression maybe negatively controlled by STAT6.

In dual-luciferase reporter assays, STAT6 showed a preference for the N4 GAS domain binding site in beta cells, when compared to the N3 site, this agrees with the work of Li et al. (2016a) who showed by x-ray scattering experiments that

STAT6 possess a key residue (H415) that gives it the selective ability to bind to the N4 site over N3.

The HEK293 cells are an embryonic kidney cell line with a high transfection efficiency (Thomas and Smart, 2005) were transfected with a STAT6 luciferase reporter, and the *Renilla* construct alongside various STAT6 mutants. An increased activity was observed with the constitutively active mutant of approximately 8 fold after IL-13 stimulation; similar to that reported by Daniel and colleagues (Daniel et al., 2000). Also observed, was the inhibition of STAT6 activity by a dominant negative STAT6, results that were also obtained by Daniel et al., 2000 using HEK293 cells (Fig.5.16). In DLR experiments with INS-1E cells, the transfection efficiency of the *Renilla* construct used as a normalisation control was poor, and so the data arising from the INS-1E cells experiments could not be normalised in the typical way. This meant that interpretation of the results was equivocal. The difference in transfection between the INS-1E and HEK293 could be due to the differences in the cell lines (one is a human kidney cell lines and the order is rat beta cell line).

Constitutively active STAT6 acts by virtue of an IL-4 or IL-13 independent phosphorylation of STAT6 due to a conformational changed induced by two amino acids (Fig.5.15). It been suggested that CA STAT6 has a higher binding stability than the wild type is and is more resistant to proteolytic degradation. The dominant negative STAT6 due to that change in the Tyr641 leads to an inability to activate STAT6 with IL-4 or IL-13 and hence no DNA binding activity of STAT6 (Daniel et al., 2000).

INS-1E cells transfected with variants of STAT6 showed an increase in total STAT6 expression (Fig. 5.16) but this did not seem to directly protect the cells

from pro-inflammatory cytokines. Protection was only achieved when IL-13 was used in combination with the STAT6 constructs. Cytoprotective experiments with STAT6 mutants were performed over a period of five days; plasmid transfection for 24h, 48h of IL-13 stimulation and 48h of pro-inflammatory cytokines stimulation followed by PI staining and flow cytometry. Loss of plasmid by cells over the experimental period (5days) in cell division (Middleton and Sugden, 1994) could have been a possible reason for the lack of effect of the dominant negative and other STAT6 variants but this is unlikely given that the flow cytometer gating was performed on transfected cells alone. The mechanism by which IL-13 still protected INS-1E against pro-inflammatory cytokines in STAT6DN transfections remains unclear. One might be tempted to suggest that endogenous STAT6 could have been activated to contribute to this protection. Additionally, it is difficult to say if all GFP positive cells also contained the various STAT6 mutants. Further research with the use of stably transfected cell lines or CRISPR (clustered regularly interspaced short palindromic repeats) modified cell lines will be required to uncover the mechanisms.

Recent findings by our group, suggest that STAT6 is lost from insulin containing beta cells of T1DM individuals although the mechanism by which this occurs remains to be understood (Leslie et al., 2018). The data in this chapter revealed that STAT6 levels might be reduced in response to pro-inflammatory cytokines, serum withdrawal or palmitate although the precise mode by which this occurs remains to be revealed. IFN-γ has been shown to inhibit the phosphorylation and activity of STAT6 by IL-4 treatment in other cell types. This inhibition was achieved by 24h pre-treatment of cells with IFN-γ followed by IL-4 stimulation. STAT6 inhibition was dependent on the concentration of IFN-γ (Heller et al., 2004, Dickensheets et al., 1999). The inhibition of the IL-4 signalling by IFN-γ might lead

to reduced STAT6 expression since it was shown earlier that IL-4 positively regulates STAT6 expression (Fig. 4.14). Additionally, pro-inflammatory cytokines have been reported to upregulate the expression of some noncoding microRNAs (miRs) which negatively regulation the expression of target genes. MiRs achieve this by pairing to the 3'-untranslated region of the target mRNA leading to their degradation or inhibition of gene expression. Two such miRs, miR-155-5p and miR-210, are increased in T1DM, and known to be modulated by inflammatory cytokines (TNF-α, IFN-γ and IL-1β) in beta cells and other cell types (Grieco et al., 2017, Assmann et al., 2017, Huang et al., 2018). These miRs have been reported to suppress STAT6 expression and activity in other cell types (Kopriva et al., 2013, Matsukura et al., 2016, Martinez-Nunez et al., 2011). It is therefore possible that the pro-inflammatory cytokines upregulate these microRNAs leading to loss of STAT6 and hence potentiating beta cell death, but this has yet to be confirmed in beta cells.

In previous experiments as well as in the present work, cytoprotection was achieved by pre-incubation with IL-13 for 48h prior to addition of cytotoxic stimuli. Pre-incubation allows the beta cells to increase the expression of STAT6 and its downstream target genes such as, SOCS-*1*, *SIRP*α and anti-apoptotic proteins MCL-1 and BCLXL before subsequent application of the inflammatory cytokine mixture.

This chapter demonstrates the importance of STAT6 for IL-13 protection since depletion of STAT6 by knockdown completely prevented IL-13 mediated cytoprotection. Furthermore, this work also reveals that many of the IL-13 regulated genes reported in Chapter 4 were controlled in a STAT6-dependent manner. The upregulation of a gene not previously recognised as being

transcriptionally regulated by STAT6 (or IL-13), SIRPα was identified, and its role in beta cell health needs to be investigated further. This will be explored in chapter 6.

Chapter 6.0: Signal Regulatory Protein alpha (SIRPα) in beta cells

6.0 Signal Regulatory Protein alpha (SIRPα)/ CD47 in beta cells

6.1 Introduction

In the earlier chapters, it was revealed that Signal regulatory protein alpha (SIRPα) was strongly upregulated in human and rodent beta cell lines stimulated with IL-13 and IL-4 in a STAT6 dependent manner. However, the impact of SIRPα upregulation on beta cells is unknown.

SIRPα (also known as Src homology 2 domain-containing tyrosine phosphatase substrate 1(SHPS-1), BIT (Brain Ig-like molecule with tyrosine-based activation motifs), P84, MFR (Macrophage fusion receptor) or MyD-1) belongs to the superfamily of immunoglobulins and is predominantly expressed in myeloid and neuronal cells (Oshima et al., 2002). SIRPα is a transmembrane glycoprotein whose extracellular region contains 3 immunoglobulin domains whilst its cytoplasmic tail contains four tyrosine phosphorylation sites that are typically activated following binding to CD47 its cognate ligand (Matozaki et al., 2009) (Fig. 6.1). The phosphorylation of SIRPα can also be stimulated by other mechanisms, such as its association with insulin-like growth factor-1 (IGF1) receptor when activated by IGF1 (Shen et al., 2009). The activation of SIRPα leads to the recruitment of Src homology 2 phosphatases such as SHP1, and SHP2 (Takada et al., 1998) leading to their activation and interaction with Grb2, and SOS Gab1 leading to the recruitment of Ras and its activation. Activated Ras then activates Raf which in turn phosphorylates and activates MEK which then phosphorylates and activates ERK that transcribe genes involved in proliferation or survival (Zhang et al., 2015).

CD47 (also known as integrin associated protein) is the cognate ligand for SIRPα , which is expressed on most somatic cells. Like SIRPα, it is a member of

the Ig superfamily with an extracellular IgV domain, a five transmembrane-spanning domain and a short cytoplasmic tail (Per-Arne, 2013). CD47 is also recognised as a marker of self (or a “don’t eat me” molecule) and is known to inhibit macrophage phagocytosis, promote cell migration, cell-to-cell communication, inhibition of apoptosis, cell proliferation, and downregulation of cytokine production in dendritic cells (Oshima et al., 2002). Importantly, CD47 can signal by either a *trans* (i.e from one cell to another) interaction with SIRPα, together with SIRPγ (another member of the SIRP family lacking the cytoplasmic tail) and thombospodin-1, or in a *cis* interaction where it binds to integrins or SIRPα on the same cell (Soto-Pantoja et al., 2014, Kojima et al., 2016) (Fig 6.1). CD47 and SIRPα are each expressed in rodent islets and it has been proposed that knockout of SIRPα alters insulin secretion although the precise mechanism is not known (Kobayashi et al., 2008).

The role of SIRPα in beta cells has received limited attention but genome wide association studies in diabetic mouse have revealed that the *idd13* locus (chromosome 2*),* correlates with the location of *SIRP*α and is involved disease pathogenesis. Indeed, high-resolution genomic mapping analysis of the six genes in that region, identified *Sirp*α as the causal gene at the *Idd13.2* locus which is crucial for the regulation of insulitis and the susceptibility of NOD mouse to diabetes (Wong et al., 2014). Furthermore, linkage analysis in the Biobreeding rat model of T1DM identified the *Iddm27* locus (orthologous to *Idd13* in mouse) as being important in the control of islet integrity and susceptibility to diabetes (Wallis et al., 2009). The human chromosome 20p13 includes a *SIRP* cluster that encodes *SIRP*α and has been associated with T1DM (Barrett et al., 2009). A report by Takenaka et al. (2007) identified 18 amino acids variations in the

extracellular N-terminal variable region of *SIRPα* between mice susceptible to diabetes (NOD) and mice protected from diabetes (NOR). These changes in amino acids might change the binding dynamics of the SIRPα on T-cells promoting insulitis (Wong et al., 2014). The role of SIRPα in immune cells is established as an inhibitor of phagocytosis when it binds to CD47 on the surface of other cell types (van Bommel et al., 2017, Nuvolone et al., 2013). C57BL/6N mice generated to lack the cytoplasmic region of SIRPα in the islets showed reduced plasma insulin levels, impaired glucose tolerance, and reduced capacity of the beta cells to secrete insulin upon glucose stimulation (Kobayashi et al., 2008). Wong et al., (2014) showed that a SIRPα variant (change in 20 amino acids) expressed by NOD mice drives islet inflammation by binding with higher affinity to CD47 compared to the diabetes resistant strains. Additionally, transfer of this variant SIRPα from NOD mice to myeloid cells of NOD.SCID (without immune system) mice induced diabetes (Wong et al., 2014).

The functional role of SIRPα in beta cells remains an area of limited research but novel data presented in this thesis showing its association with IL-13/IL-4 signalling and the transcription factor STAT6 makes it in an interesting candidate for further study.

This chapter has several objectives;

- To investigate the importance of SIRPα in beta cells. In particular, I aimed to study the role of SIRPα in beta cell viability and protection from cytotoxicity using rodent beta cell lines, human derived beta cells and human islets.

- Additionally, expression of the SIRPα binding ligand CD47 was studied in both human and rodent beta cells.

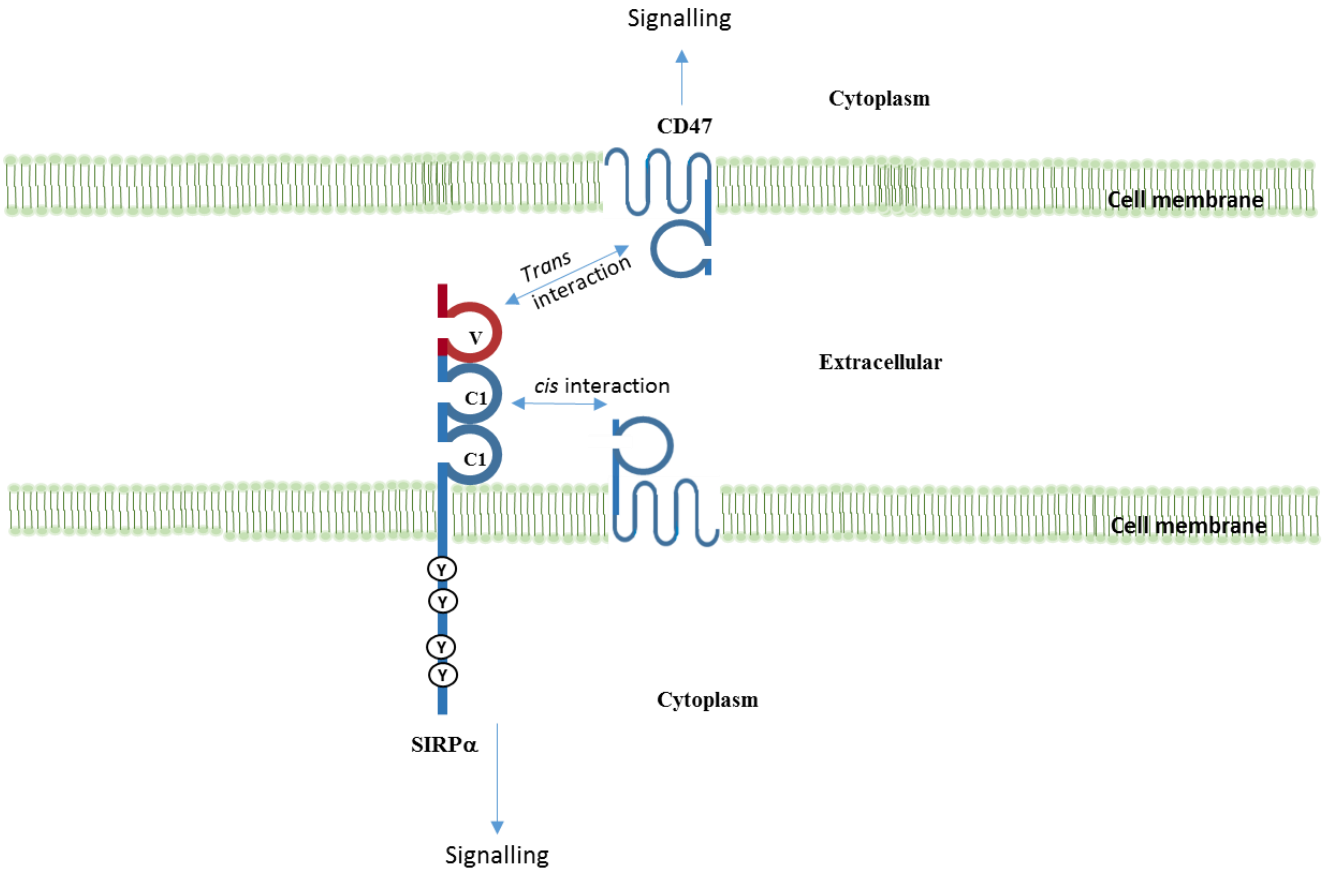


Figure 6. 1: Schematic structure of SIRPα and CD47.

CD47 is the ligand for SIRPα. 'Y' indicate cytoplasmic tyrosine residues which can become phosphorylated upon ligand binding. The arrows indicate the direction of signal after interaction. A *cis* interaction by CD47 and SIRPα occurs on the same cell while a *trans* interaction occurs on different cells

6.2 Materials and methods

6.2.1 Western blotting

Western blotting was performed as described in section 2.7. The antibodies to SIRPα (catalogue number: 13379, 1:1000 5% BSA TBST) and GAPDH (catalogue number: 60004-1, 1:10000 5% milk TBST) were purchased from Cell signalling and Proteintech respectively. Human islets were a kind donation from the Oxford transplant programme.

6.2.2 Transfection studies

Small interference RNA was used to reduce the expression of *SIRPα* in INS-1E cells and was performed as described in section 2.3. Increased *SIRPα* expression was achieved by transfecting INS-1E cells with a pCMV6 vector (catalogue number: RC222380, Origene, USA) containing the human *SIRPα* coding sequence (Fig 6.2). Transfections of these constructs (including relevant controls) were performed with Lipofectamine LTX reagent (Invitrogen) as described in section 2.9.3 and the success of transfection was examined by western blotting.

6.2.3 RT- qPCR

RT-qPCR was performed as described in section 2.6. Primers were commercially obtained from Qiagen, UK.

6.2.4 Viability studies and cell cycle analysis

Viability studies were performed using propidium iodide staining as described in section 2.4, whilst cell cycle analysis was performed as reported in section 2.5.

6.2.5 Immunohistochemistry and immunofluorescent staining

Immunohistochemical and immunofluorescence staining of FFPE human pancreas sections were performed as in section 2.8.2. These samples were obtained from the Exeter Archival Diabetes Biobank (http://foulis.vub.ac.be/), a collection of post-mortem pancreas samples (Table 15). The list of antibodies used and their conditions are described in table 14. Images were captured at the same settings on a fluorescent microscope (Leica Microsystem, UK) and the mean fluorescent intensity (MFI) of the CD47 containing cells was determined using a custom MATLAB script (MATLAB version R2015b) VIOLA (developed by Dr Chilton of the University of Exeter Medical School). The script was used to determine the MFIs of CD47 in regions of interest in the tissue. DAPI (4',6-diamidino-2-phenylindole) was used as a nuclear stain (Dako, UK) at 1:1000 dilution.

Table 14: Antibodies and conditions used in immunostaining

Antibody	Dilution	Source	Catalogue number
CD47	1:1000	RnD systems	AF4670
Insulin	1:300	DAKO	A0564
Glucagon	1:400	Abcam	ab92517
Anti-guinea pig 647	1:400	Invitrogen	A21450
Anti-Goat 555	1:400	Invitrogen	A32814
Anti-Rabbit 488	1:400	Invitrogen	A32732

Table 15: Tissue samples and patient information

Case ID	Case type	Age (Years)	Sex	Duration of Disease
146/66	No Diabetes Control	18	F	
21/89	No Diabetes Control	4	F	
PM34/67	No Diabetes control	41	F	
E556	T1DM	18	M	4months
SC119	T1DM	4	F	2 weeks
E560	T1DM (organ donor)	42	F	18months

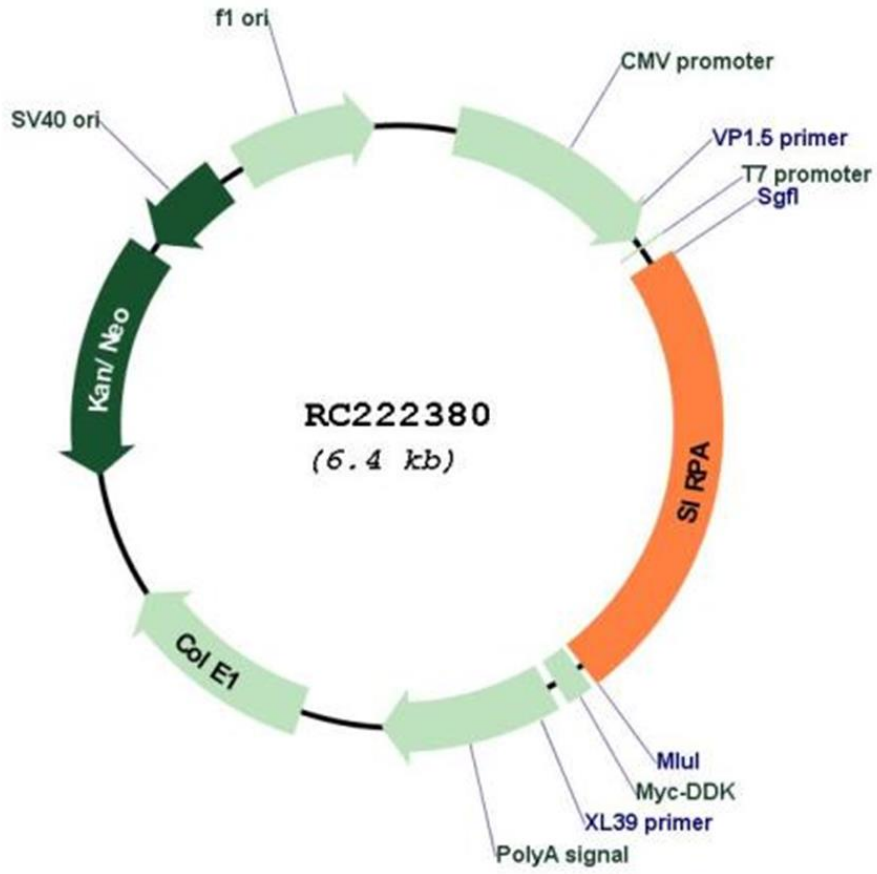


Figure 6. 2 Map of the pCMV6 plasmid containing SIRPα clone

Courtesy:https://www.origene.com/catalog/cdna-clones/expression-plasmids/rc222380/sirp-alpha-sirpa-nm_001040022-human-tagged-orf-clone

6.3 Results

6.3.1 Signal regulatory protein alpha is expressed in human islets and upregulated by IL-13 treatment

In Chapter 4 it was shown by qPCR and western blotting that SIRPα is expressed in the INS-1E and in human EndoC βH1 cells and that its expression was increased in response to IL-13 and IL-4 (Fig. 4.12). The expression of SIRPα in rat beta cells is in agreement with Kobayshi et al. (2008) who showed the expression of SIRPα in rat pancreatic beta cells by immunohistochemistry. It was also important to determine whether a similar pattern could be observed in isolated primary human islets. Therefore, isolated humans islets were lysed and protein extracted for western blotting with an anti-SIRPα antibody (Fig 6.3a). This revealed that SIRPα is expressed in islet cells. A separate preparation of human islets was stimulated with 20ng/mL of IL-13 for 48h prior to western blotting and it was found that SIRPα expression was increased in response to IL-13 stimulation (Fig.6.3b).

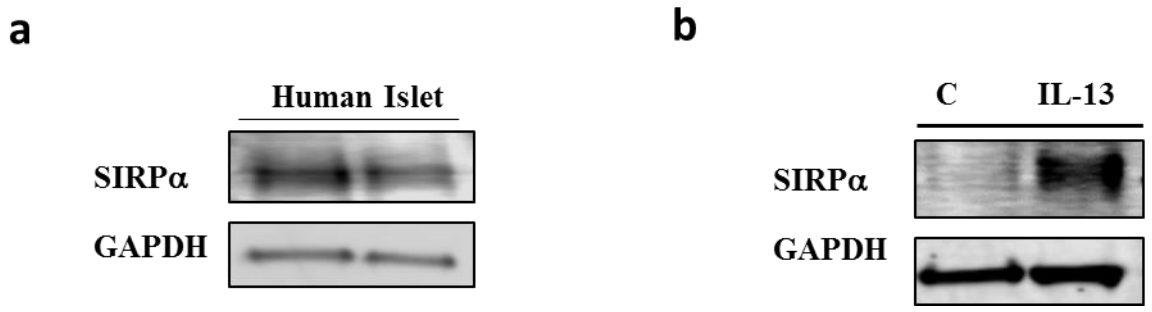


Figure 6. 3: SIRPα is present in human islets and is upregulated by IL-13 stimulation

Human islets from donors were lysed, a western blot performed, and probed for SIRPα and GAPDH as loading control. **a.** unstimulated human islet samples express SIRPα, **b.** stimulated human islet samples with 20ng/mL of IL-13 for 48h prior to lysis for western blotting. IL-13 stimulation upregulated SIRPα expression

Data are representative of two independent islet preparations.

6.3.2 SIRPα knockdown using siRNA induces cell death in INS-1E cells

To assess the impact of SIRPα on beta cell viability, *SIRP*α expression was initially knocked down over a 4 day period in INS-1E cells using specific siRNA molecules, then RNA and protein extracted. Quantitative PCR analysis revealed that *SIRP*α mRNA was successfully depleted over this 4 day period (Fig 6.4a). Western blotting analysis of samples revealed that SIRPα protein levels were depleted by approximately 80% during this period. Importantly, the knockdown did not affect expression of other proteins such as STAT6 or STAT3 (Fig. 6.4b). SIRPα expression was also successfully reduced in EndoC βH1 cells (Fig 6.4c) using siRNA targeting the human sequence. During experiments with INS-1E cells, it was unexpectedly observed at later time-points that there were fewer cells present after knockdown of SIRPα compared to samples transfected with scrambled control siRNA. To examine this phenomenon more closely, cell growth was assessed by manual counting of cells following trypan blue staining. These data confirmed that after three days of SIRPα knockdown there was significantly lower cell numbers in the knockdown treatments compared to the scrambled controls (Day3, control: 83.5±8.6 x10^4cells/mL, SIRPα KD: 51.0±3 x10^4 cells/mL, p<0.05, Day4, control: 137.8±15.2 x10^4cells/mL, SIRPα KD: 84.8±3 x10^4, cells/mL p<0.001) (Fig. 6.7).

To understand whether SIRPα has a role in IL-13 mediated protection of rodent beta cells, it was important to assess whether the depletion of SIRPα expression could alter beta cell viability alone. In initial experiments, cell viability was determined by propidium iodide staining in cells incubated in complete medium but with SIRPα knockdown. This revealed a significant increase in the percentage of dead cells after SIRPα knockdown when compared to the scrambled controls

(Control; 22.5±1.8%, Control + KD; 34.89±2.1%, cell death p<0.001). As expected, serum withdrawal increased cell death and this effect was further potentiated by SIRPα knockdown (serum withdrawal; 49.3±0.9%, SW+KD; 70.24±1.5%, cell death p<0.001) (Fig. 6.6). Under serum withdrawal conditions addition of IL-13 reduced the percentage of dead cells and this was also seen under SIRPα knockdown conditions (SW; 49.3±0.9%, SW+IL-13; 44.4±0.8%, SW+KD; 70.24±1.5%, SW+KD+IL-13; 62.2±2.0% cell death) (Fig 6.6). One possible explanation for this observation is that the levels of SIRPα were still elevated after IL-13 treatment despite the use of siRNA to knockdown the protein. To address this possibility, SIRPα was knocked down in INS-1E cells accompanied by treatment with IL-13. qPCR analysis of these samples revealed that IL-13 treatment reversed the depletion in *SIRPα* levels to at least normal expression levels (Fig 6.5).

Since depletion of *SIRPα* slowed the growth beta cells and increased cell death when compared to cells treated with a scrambled control siRNA, it was important to confirm these findings using a cell cycle analysis protocol. In these experiments, SIRPα was knocked down for four days (Fig. 6.4), and then cell cycle analysis performed as described in section 2.5. The proportion of the cells in SubG0/G1 (apoptotic), G_0/G_1, G_2/M+S (proliferating) was calculated after analysis by flow cytometry. Cell cycle analysis revealed that *SIRPα* knockdown did not alter any phase of the cell cycle, however an increase in the sub G_0/G_1 population of apoptotic cells was observed, consistent with our previous findings (Control Sub G_0G_1: 10.3±0.8% of cells, SIRPα KD Sub G_0G1: 34.5±3.2%, p<0.001, Fig 6.8).

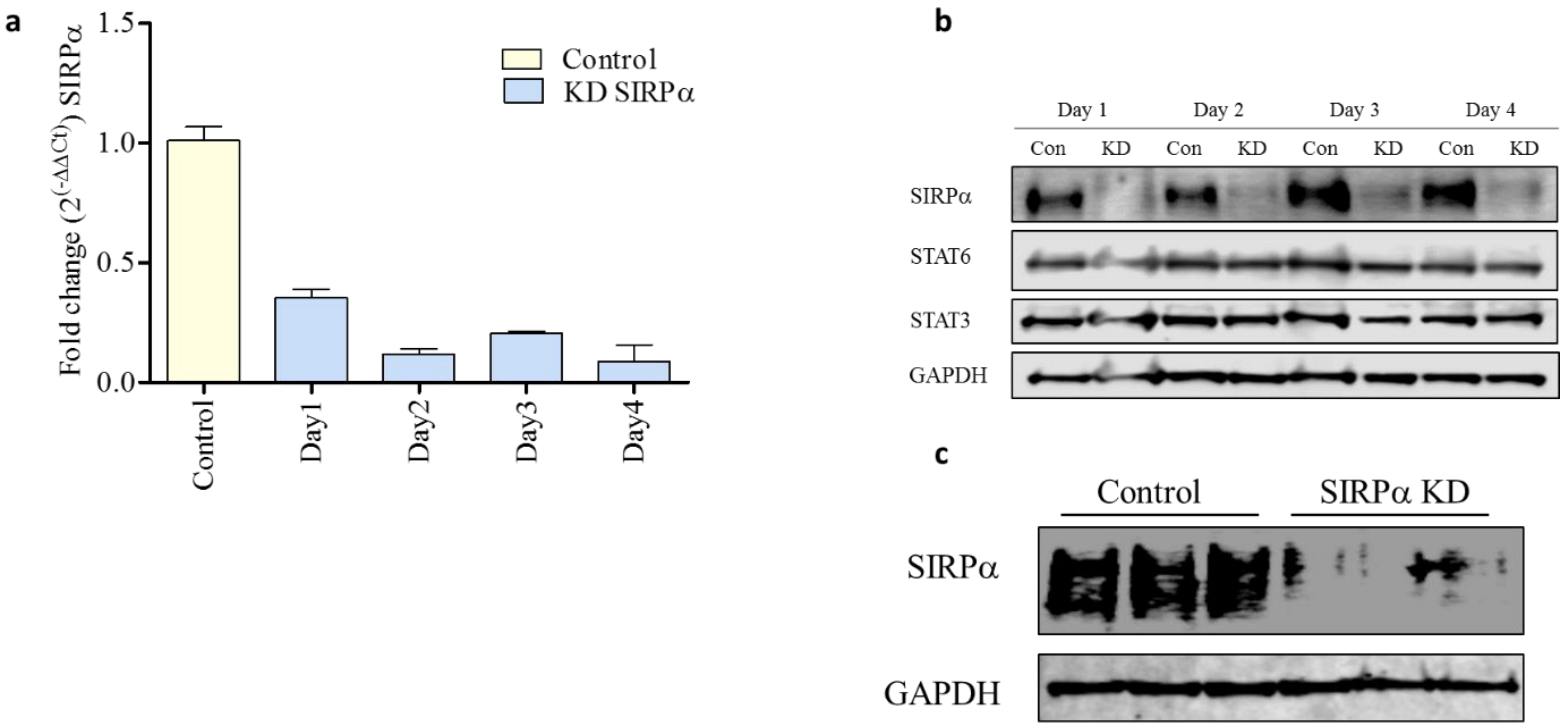


Figure 6. 4: *SIRP*α was successfully knocked down in beta cells

Cells were transfected with 10nM of rat SIRPα siRNA in **a**&**b** INS-1E cells for 96h and **c.** in human EndoC βH1 cells for 48h respectively. **a.** Cells were washed, RNA extracted and RT-qPCR performed or **b** and **c** lysed and western blotting performed, membranes were probed with anti-sera against SIRPα, STAT6, STAT3, and GAPDH.Data represents RT-qPCR results and western blots of experiments performed at least twice independently

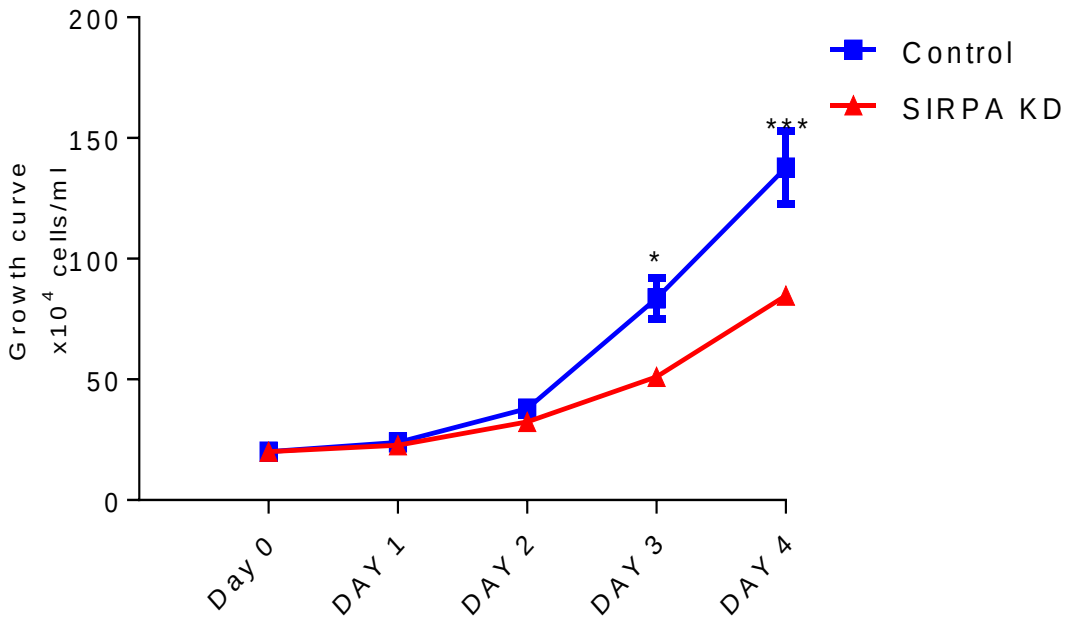


Figure 6. 5: SIRPα knockdown reduces the growth of INS-1E cells

INS-1E cells were cultured and transfected with siRNA SIRPα for 96h and cells were harvested and live cells counted after staining with trypan blue.

Data represents mean values of experiments performed three times ±SEM. *p<0.05, ***p<0.001, determined by student *t*-test.

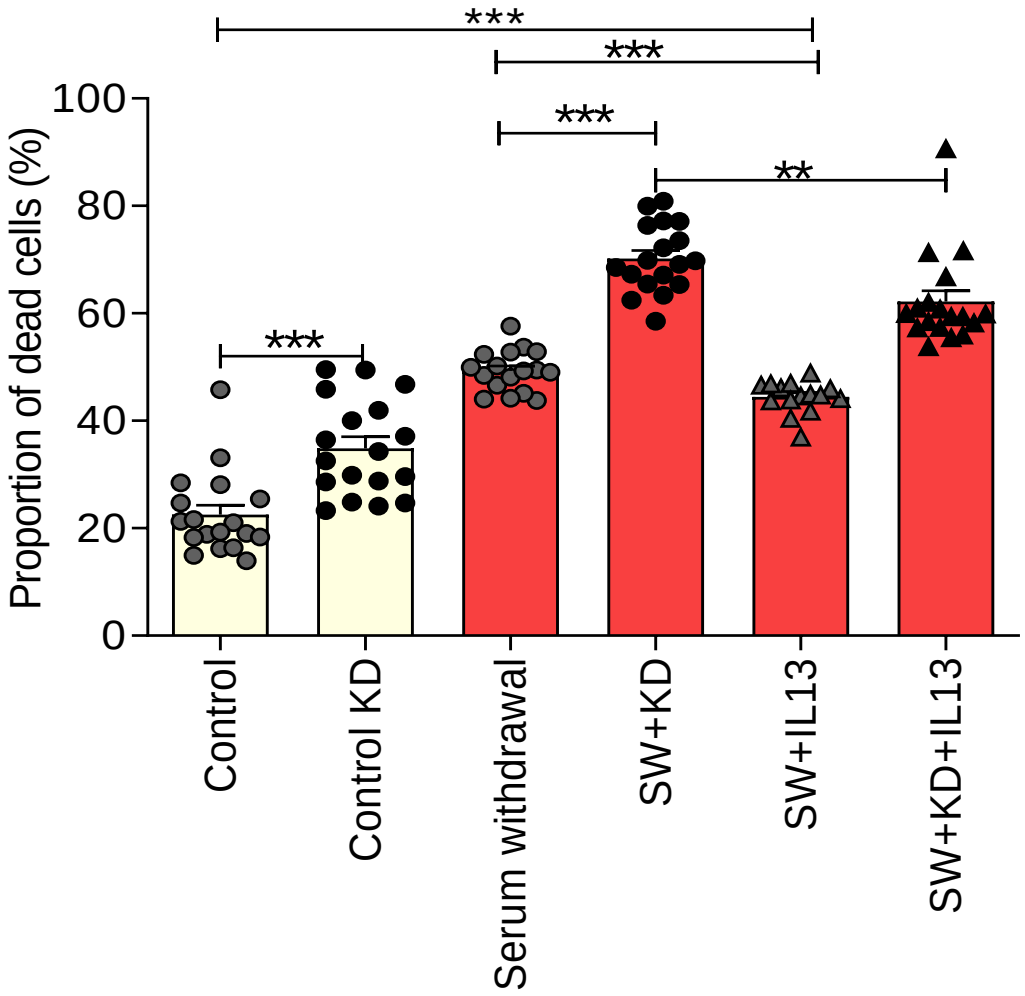


Figure 6. 6: SIRPα knockdown increases cell death in INS-1E cells

INS-1E cells were cultured in serum-withdrawn conditions with and without SIRPα knockdown in the absence and presence of IL-13 for 96h. Cell viability was assessed by flow cytometry after propidium iodide staining.

Data represents mean values of 3 independent experiments ±SEM, ***p<0.001, determined by the student *t*-test.

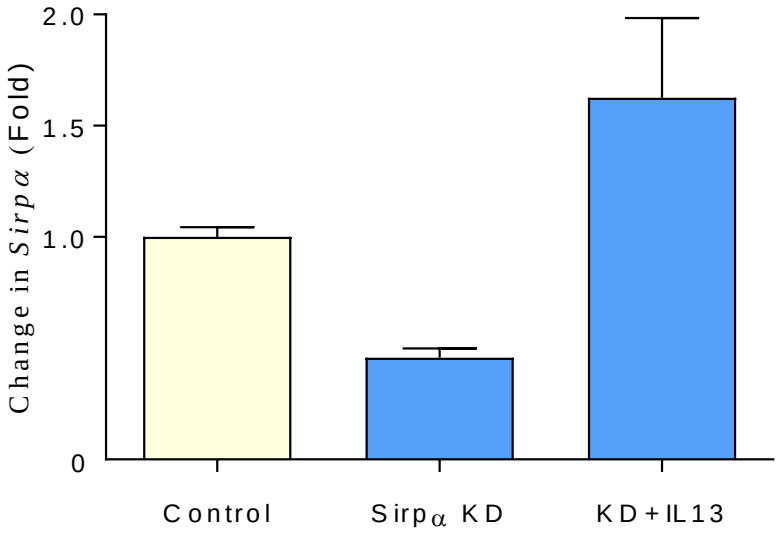


Figure 6. 7: *SIRP*α knockdown is reversed to normal levels by stimulation with IL-13 for 48h

INS-1E cells were depleted of *SIRP*α using small interference RNA followed by IL-13 stimulation. RNA was extracted and cDNA synthesized for qPCR using SYBRGreen.

Data represents mean values of experiments performed twice independently ±SEM.

a.

b.

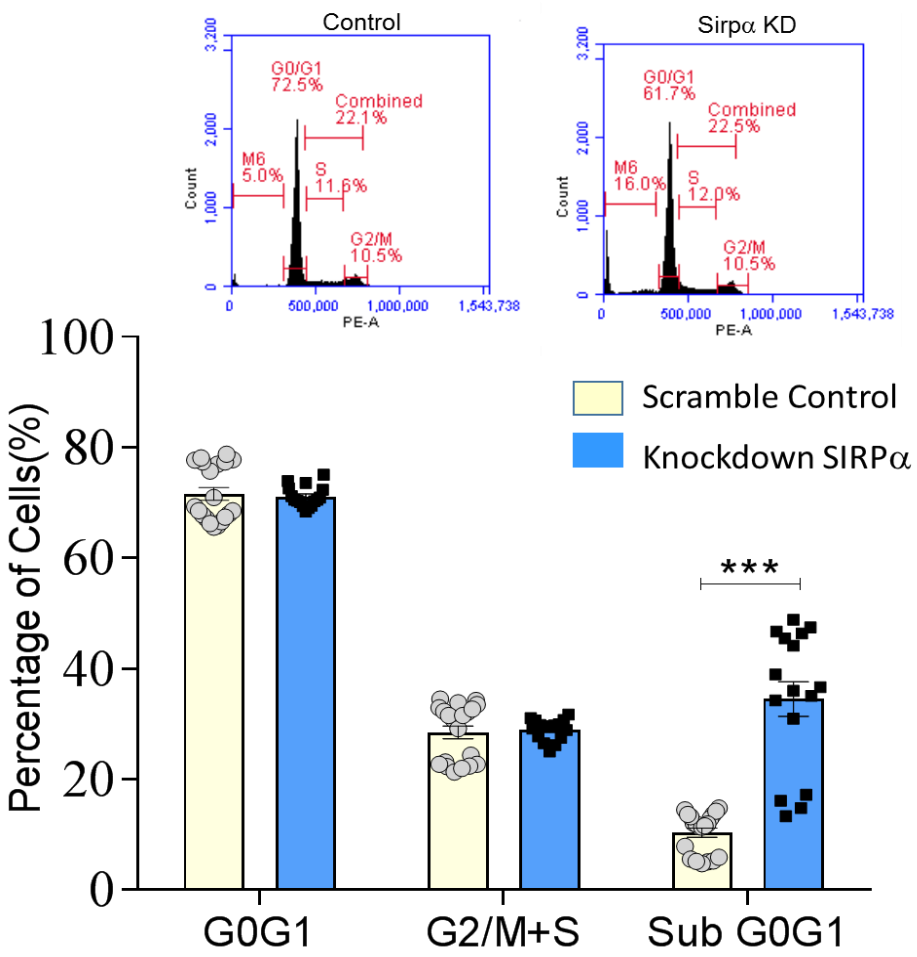


Figure 6. 8: SIRPα knockdown induces an increase in DNA fragmentation in beta cells

Cell cycle analyses were performed on INS-1E cells following SIRPα 48h knockdown. Cells were fixed with 95% ethanol followed by PI staining after treatment with RNAse A and analysed on a flow cytometer. Panel **a**. shows representative plots of control and *SIRPα* cell cycle analysis. There was no significant difference in the G_0G_1 and G_2/M+S (proliferation phase) while the M6 (Sub G0G1) was significantly increased in *SIRPα* knockdown treatment. **b**. is a plot of normalised cell cycle analysis values.

Data represents mean values from three independent experiments ±SEM. ***p<0.001, determined by the student *t*-test.

6.3.3 SIRPα overexpression protects INS-1E beta cells from serum withdrawal induced death

Since *SIRPα* knockdown causes a clear increase in INS-1E cell death, it was assessed whether increasing the expression of *SIRPα* could induce the opposite effect. In these experiments, *SIRPα* was overexpressed under conditions of serum withdrawal using a commercially obtained construct in which *SIRPα* cDNA was placed under the control of a CMV promoter (Origene, UK), and thus stimulated a constitutive expression of the protein (Fig. 6.2). Initially, INS-1E cells were transfected with this construct, and the over-expression of SIRPα was confirmed by western blotting after 24h (Fig. 6.9). Cytotoxicity assays were then performed by transfecting INS-1E cells with the SIRPα plasmid, followed by a 96h period of serum withdrawal. Overexpression of SIRPα in control medium significantly reduced cell death compared to the empty vector transfection (Control: 12.6±0.9%, Control+SIRPα plasmid: 8.5±0.5%, cell death $p<0.001$). As expected serum deprivation enhanced INS-1E cell death (Control: 12.6±0.9%, SW: 51.9±2.0% cell death, $p<0.001$). Importantly, overexpression of SIRPα strongly reduced the level of cell death in INS-1E cells under these conditions (SW: 51.9±0.9%, SW+SIRPα plasmid: 38.0±1.1% cell death, $p<0.001$). Taken together, these data suggest that SIRPα is a novel regulator of beta cell viability.

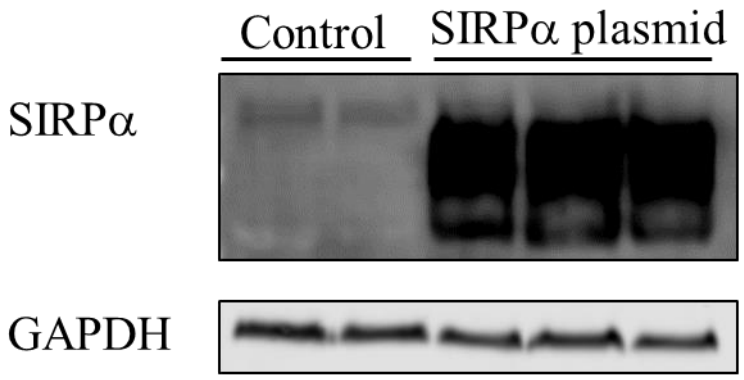


Figure 6. 9: SIRPα was overexpressed in INS-1E cells

INS-1E cells were transfected with the SIRPα clone for 24h with Lipofectamine LTX reagent. Cells were then washed, lysed and western blotting performed. Membranes were probed with anti-sera against SIRPα and GAPDH for loading control.

Data represents results of experiments performed twice independently.

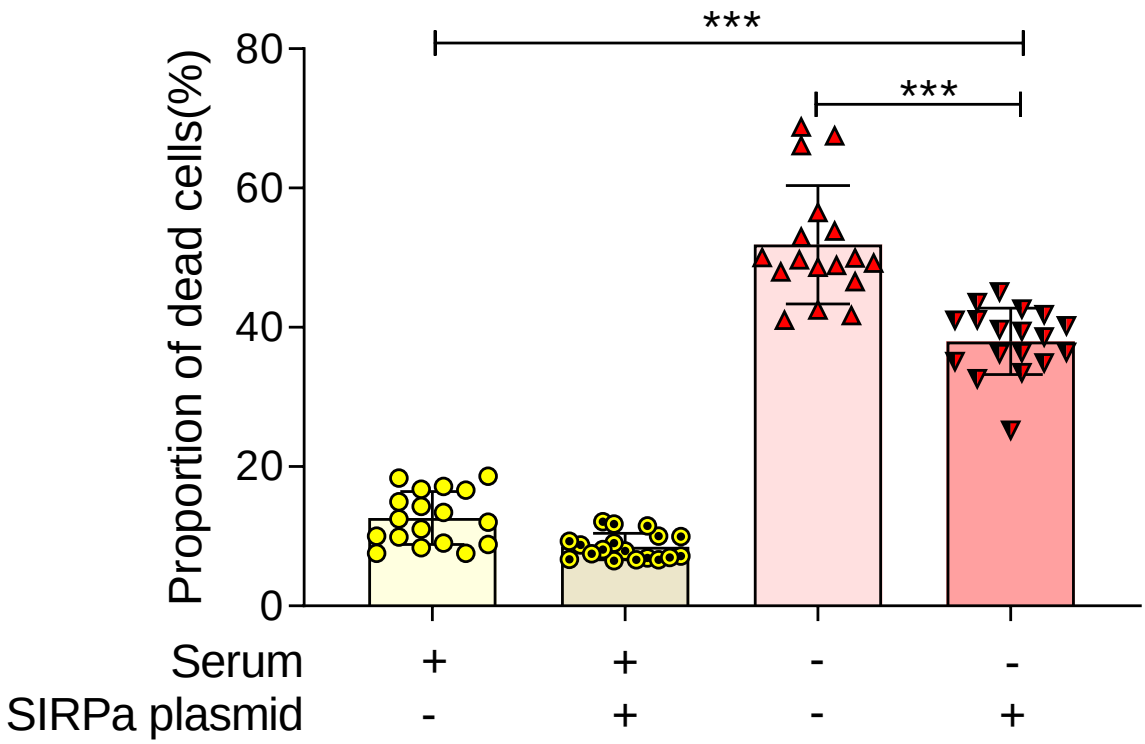


Figure 6. 10: Overexpression of SIRPα protects INS-1E cells from serum withdrawal induced death

INS-1E cells were cultured under serum withdrawn (pink bars) conditions for 96h, either untransfected (grey circles) or after transfection (filled hexagons) with a plasmid encoding SIRPα. Cell viability was assessed by flow cytometry after PI staining.

Data represents mean values of experiments performed 3 times independently ±SEM

***p<0.001, determined by the student *t*-test.

6.3.4 INS-1E and human islets cells express the SIRPα ligand CD47 and this is lost from beta cells of T1DM individuals

SIRPα becomes activated when it binds to its cognate ligand CD47, and this event precipitates the phosphorylation of key tyrosine residues on the cytoplasmic tail of SIRPα (Matozaki et al., 2009). In macrophages, the binding of CD47 to SIRPα prevents phagocytosis, and the system thus functions as a “don’t eat me” signal (Ayi et al., 2016). Since the expression of CD47 has not been examined in pancreatic beta cells we set out to investigate this in beta cell lines, isolated human islets and in human pancreas sections. Western blotting analysis revealed that both rodent beta cells and human islets express CD47 (Fig.6.11).

To study the expression of CD47 in situ within the pancreas, immunohistochemical staining was performed on FFPE human pancreatic sections from non-diabetic individuals using an antiserum raised against CD47. This revealed that CD47 is expressed within the islets of Langerhans, with very little specific staining observed in the exocrine pancreas. CD47 staining was intense in the cytoplasm of the cells, on the plasma membrane but it was not found in the nucleus. Within the islets, the staining of some cells was more intense than others (Fig.6.12). In order to identify which islet cells express CD47, co-immunofluorescence staining of 6 pancreatic sections from 3 individuals with T1DM and 3 age and sex matched non-diabetic controls was performed (Table 15). Insulin was used as a marker of beta cells and glucagon as a marker for alpha cells (Richardson et al., 2016, Arif et al., 2014, Leete et al., 2016, Kobayashi et al., 2008). Using cyan for insulin, red for glucagon and green for CD47 the immunofluorescent staining confirmed our previous observation that CD47 is expressed within the islets of individuals without diabetes. Interestingly, the expression of CD47 was more intense in the beta cells than the alpha cells

within the islets. To quantify this, the mean fluorescence intensity was acquired and processed using a MATLAB script (Alpha; 49.79±1.4, beta; 58.3±1.8 MFI $p<0.001$) (Fig.6.14). Sections from individuals with T1DM were then studied using the same microscope and camera settings as the non-diabetic control sections. As expected, CD47 staining was expressed within the islets, however rather surprisingly, the expression of CD47 in insulin containing beta cells was less intense than that in alpha cells in the sections from individuals with T1DM. The mean fluorescence intensity was evaluated and this confirmed these observations (T1D alpha: 60.36 ± 2.7, T1D beta cells: 49±2.2, $p<0.01$). The mean fluorescence intensity results obtained from non-diabetic controls were then compared with those of T1DM individuals and revealed a reduction in CD47 expression in T1DM donor beta cells (control beta cells; 58.3±1.8, T1D beta; 49±2.2 $p<0.001$). Interestingly, CD47 was upregulated in the alpha cells of individuals with T1D when compared to matched controls (control alpha cells; 49.8±1.4, 60.4±2.7 $p<0.001$).

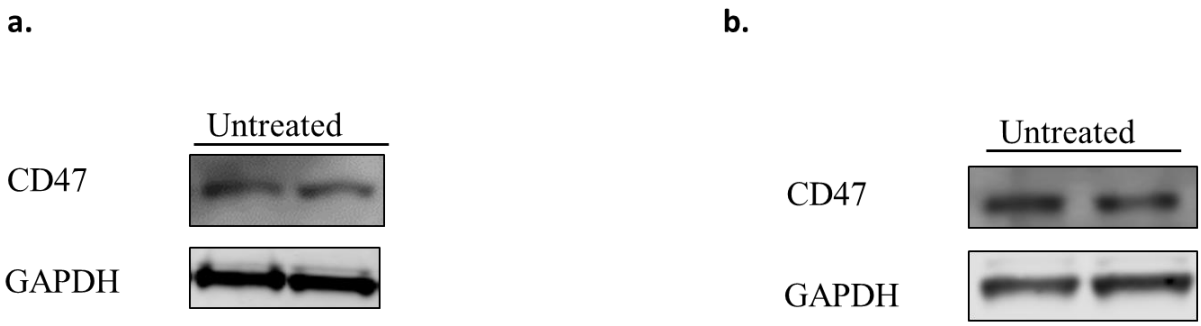


Figure 6. 11: Isolated human islets and rat beta cells express CD47

INS-1E cells **a**. and human islets **b.** were lysed, western blotting performed and probed for CD47 expression with GAPDH as loading control.

Data represents results of experiments performed thrice independently.

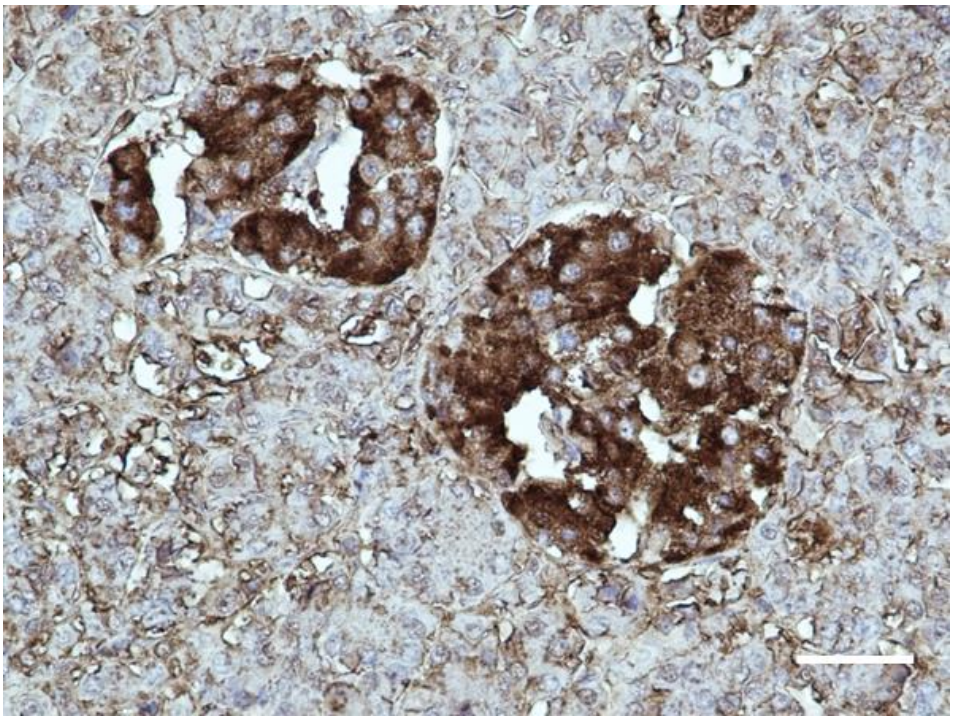


Figure 6. 12: Human islets express CD47

Pancreatic tissue section from an individual without diabetes. Formalin-fixed paraffin embedded pancreas sections were stained for CD47 using an immunoperoxidase approach.

CD47 is highly expressed in the islets as indicated by the intense brown staining. There is some exocrine expression, which on the cell surface membrane.

Scale bar 50μm

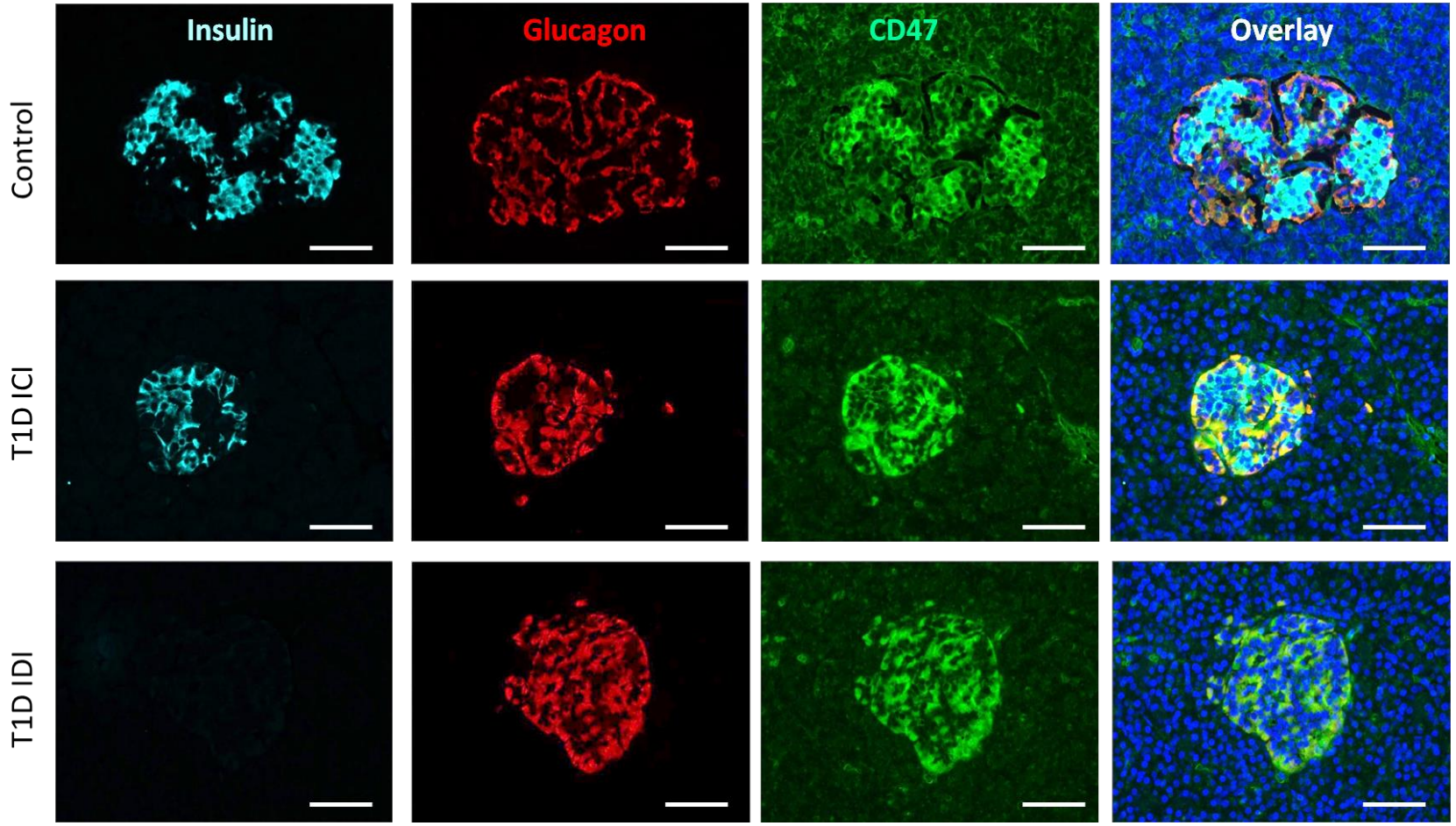


Figure 6. 13: CD47 is expressed in beta cells and is diminished in T1D

Pancreatic tissue section from an individual without diabetes. Formalin-fixed paraffin embedded pancreas sections were stained using co-immunofluoresence approach for insulin (blue), glucagon (red), CD47(green) and the nuclear stain DAPI. In control the case, CD47 is expressed predominantly in the beta cells while in the T1DM case seems to be more expressed in the alpha cells. Scale bar 50μm

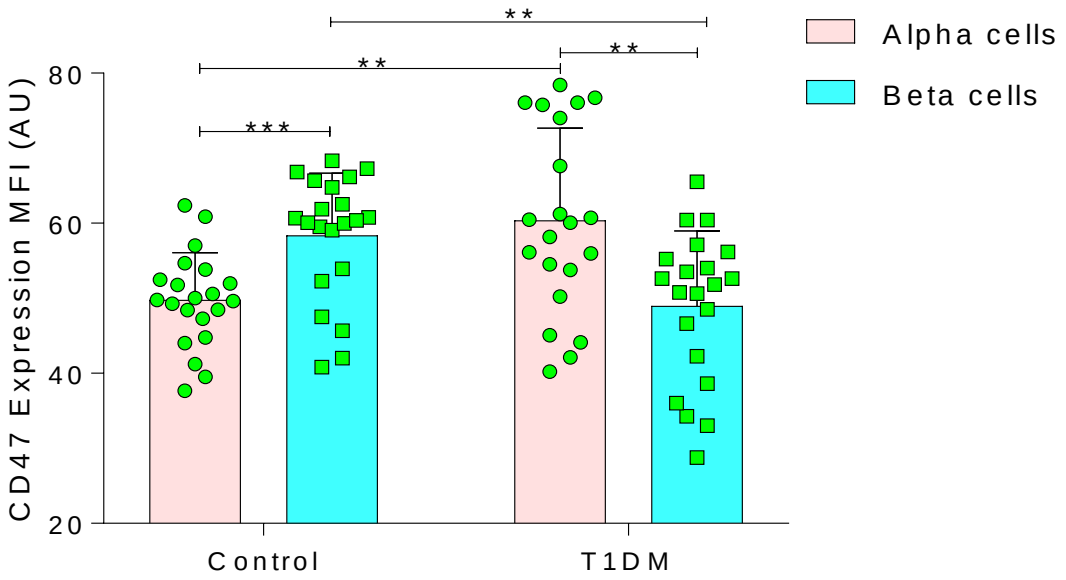


Figure 6. 14: CD47 is reduced in T1DM beta cells and increased in alpha cells

Identical settings were established to collect images and these images were then quantified by measuring the mean fluorescent intensity (MFI) of CD47, insulin, and glucagon in insulin containing islets (ICI). The images were then imported into a Matlab script, VIOLA, to measure CD47 MFI only in cells containing insulin or only in cells containing glucagon. Data was collected from 7 islets/case (3 T1DM individuals and 3 individuals without diabetes). Data represents mean values ±SEM, **p<0.01 in arbitrary units (AU), determined by the student *t*-test.

6.3.5 CD47 is upregulated by IL-13 and IL-4 stimulation

Next we investigated whether stimulation of INS-1E cells with IL-4 or IL-13 affected the levels of *CD47* using RT-qPCR. *CD47* was upregulated 1.5 fold by both cytokines when compared to the unstimulated control. In these experiments, an additional question was addressed in order to establish whether the upregulation was STAT6 dependent. Knock down of *STAT6* alone did not affect *CD47* mRNA levels. However, the addition of IL-13 or IL-4 after *STAT6* knock down did not lead to any increases in mRNA levels of *CD47*, indicating STAT6 knockdown blocks the upregulation of *CD47* by IL-4 and IL-13 stimulation (Fig 6.15).

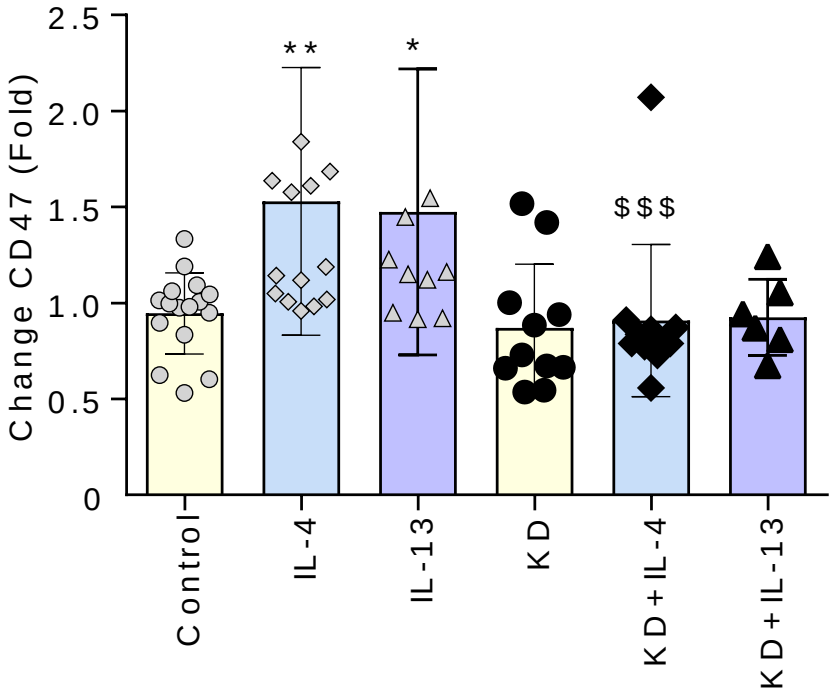


Figure 6. 15: IL-4 and IL-13 upregulated CD47 in INS-1E cells

INS-1E cells were stimulated for 48h with 20ng/mL of IL-4 (◊) or IL-13 (Δ) after 48h small interference STAT6 knockdown(filled shapes). RNA was harvested and cDNA synthesized. qPCR was performed and results analysed.

Data represents qPCR fold change means of experiments performed three times ±SEM *p<0.05, **p<0.01, determined by the student t-test.

6.4 Discussion

6.4.1 SIRPα is important in controlling beta cell viability

It had been established in the previous chapter that STAT6 controls the expression of SIRPα, and here it was shown that this response occurs in rodent and human clonal beta cells, and also in primary human islets. The regulation of SIRPα by STAT6 has not previously been reported in beta cells. These data also revealed that depletion of SIRPα expression results in a significant decrease in beta cell viability in the absence of other cytotoxic stimuli. Cell cycle analysis confirmed an increase in DNA fragmentation in *SIRPα* knockdown cells indicating increased apoptosis. The knockdown of *SIRPα* significantly increased beta cell death when cells were incubated under conditions of serum withdrawal. Conversely, overexpression of SIRPα by transfection, protected beta cells from serum withdrawal-induced cell death. Surprisingly, knockdown of *SIRPα* followed by IL-13 treatment offered cytoprotection in serum withdrawal conditions albeit with lower efficacy. This observation could be explained by the fact that SIRPα lies downstream of STAT6, hence depletion of *SIRPα* by siRNA was reversed by introducing IL-13 and the consequent activation of STAT6 (Fig 6.7).

Cell death attributed to loss of SIRPα has not been studied in beta cells previously, however, similar findings have been reported in neutrophils (Stenberg et al., 2013) and in fibroblastic reticular cells (Saito et al., 2017). Additionally, knockdown of SIRPα in neuronal cells of mice has been shown to induce caspase 3 upregulation and downregulation of BCLXL (Huang et al., 2017), suggesting that, loss of SIRPα might induce caspase 3 activity and loss of anti-apoptotic BCLXL leading to beta cell death.

Taken together, these observations imply that SIRPα might be central to the protection offered by IL-13 in beta cells and more broadly that SIRPα may be a novel regulator of beta cell viability.

6.4.2 The potential mechanism by which SIRPα protects beta cells

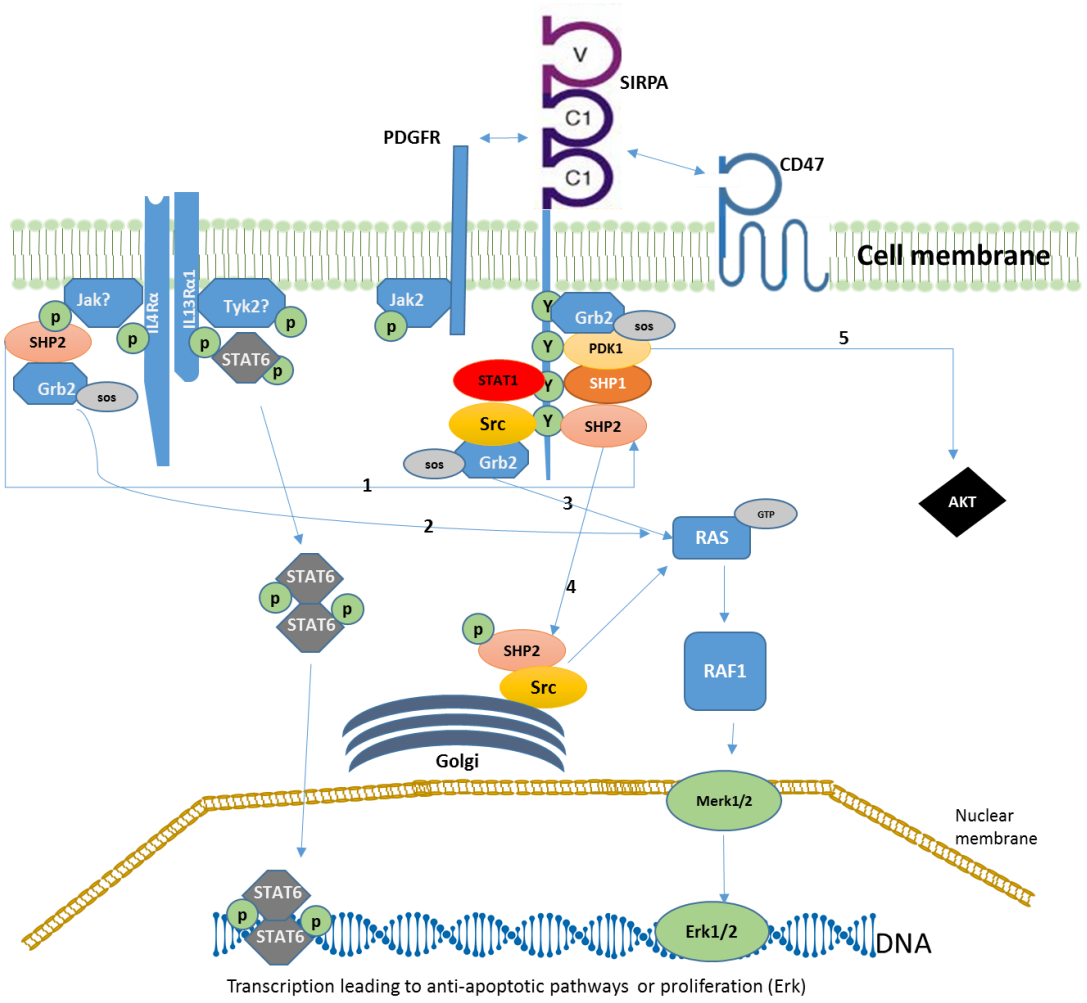
In this present work, it was shown that SIRPα is important for beta cell survival, suggesting that it acts on a signalling cascade to prevent apoptosis. The precise mechanism by which SIRPα reduces apoptosis is still unclear. However, in smooth muscle cells stimulated with IGF, SIRPα recruits PDK1 through Grb2 leading to the activation of the Akt pathway. The activation of Akt leads to the subsequent activation of FOXO1 to prevent it from trans-activating BIM hence inhibiting apoptosis in these cells (Shen et al., 2010). IGF1 has been shown to protect beta cells from apoptosis, and to be important in beta cell proliferation and differentiation (Mallol et al., 2017, George et al., 2002, Maile and Clemmons, 2002). Alternatively, it was also revealed by the same group that SIRPα can recruit STAT1, which is known to transduce apoptotic signals mediated by interferons (Shen et al., 2009). The binding of SIRPα by STAT1 was shown to be independent of SIRPα phosphorylation. It is conceivable that an increase in the level of SIRPα may lead to sequestration of cytoplasmic STAT1, hence depleting the availability of STAT1 for interferon activation and in turn inhibit the propagation of apoptotic signalling. In this regard, it is unclear if IGF can stimulate the activity of SIRPα in beta cells and future experiments could usefully examine this.

Growth hormone activation of Jak2 leads to the phosphorylation of SIRPα in fibroblasts, and this has been shown to enhance its binding to SHP2 suggesting an important role in growth hormone signalling and cell proliferation (Stofega et

al., 1998). This implies that knockdown of *SIRPα* in INS-1E cells may inhibit growth or survival signals initiated by growth factors in culture media. Additionally, beta cells are known to express SHP2 which has been shown in knockout mice studies to be important in the production and secretion of insulin. In that report, the PI3K pathway showed reduced activation in the SHP2 pancreatic knockout mice, when compared to controls. Additionally, there was reduced PDX1, GLUT2 and phospho-FOXO1 activation in SHP2 pancreatic knockout mice(Zhang et al., 2009). PDX1 is important for beta cell identity, GLUT2 for glucose uptake or sensing and FOXO1 to inhibit apoptosis (Fujimoto and Polonsky, 2009).

Grb2 and HSP70 are also reported to bind to SIRPα (Shen et al., 2009) and both are present in human beta cells where they are crucial for beta cell survival (Hügl et al., 1998, Welsh et al., 1995, Rütti et al., 2016). The majority of these studies have been performed in other cell types and the mechanisms by which the growth factors activate SIRPα are still not known. SIRPα has, however, been reported to provide a scaffold for multi-protein complexes essential for the transmission of signals but these need to be studied in the context of beta cells (Timms et al., 1999).

Based on the experiments performed in this chapter using serum withdrawal which induces cell death by depletion of the anti-apoptotic protein BCLXL (Tejedo et al., 2001, Goyeneche et al., 2006), it can be suggested that SIRPα protects from cell death by activating the Akt pathway leading to the activation of FOXO1. FOXO1 activation inhibits the activation BIM hence preventing mitochondrial permeabilisation by the Bak/Bax complex (Fig. 6.16). Future experiments could examine the protection of beta cells from pro-inflammatory cytokines by SIRPα.



1. Sirpα pulls SHP1 and SHP2 from IL-4/IL13 receptor which leads to continuous activation of the Jak/STAT pathway
2. SHP2 could also be activated through Jak2 recruiting Grb2/SOS and activation of the Erk1/2 pathway
3. Sirpα activation recruits Src which recruits Grb2/sos which activates RASgtp leading to the Erk1/2 action
4. SHP2 activation interacts with Src on the golgi leading to Erk1/2 activation via Ras
5. Sirpα recruits PDK1 which activates Akt pathway leading to inhibition of apotosis

Figure 6. 16: Possible mechanisms by which SIRPα protects

STAT6 activation leads to the upregulation of SIRPα, which can be activated by binding to CD47, PDGFR or IGFR leading to the recruitment of either STAT1, Grb2, SHP1, and SHP2. Recruitment of STAT1 could diminish interferon signalling, while Grb2, SHP1 and SHP2 can lead to the activation of the Akt and Erk pathways leading to the upregulation of anti-apoptotic proteins or cell proliferation.

6.4.3 SIRPα and beta cell function

SIRPα has rarely been studied in the pancreatic beta cell, with only a single report examining its expression in these cells (Kobayashi et al., 2008). In that report, the investigators proposed a novel role for SIRPα in the regulation of insulin secretion. They used mice depleted of SIRPα specifically in the pancreas and found effects on insulin secretion in animals fed on high-fat diet when compared to wild type controls. These data suggest that SIRPα plays a role in promoting insulin secretion. It was also shown using immunohistochemical staining that SIRPα was expressed in the islets of Langerhans and within the islets specifically in beta cells alone (Kobayashi et al., 2008) and this differed from our findings. Kobayashi and colleagues, used rat pancreas tissue while our tissues were of human origin and much older than their tissues which could account for the differences (Karlsson and Karlsson, 2011). Additionally, rat tissues were permeabilised with 0.1% triton X overnight at 4^0C after antigen retrieval while our tissue was permeabilised by use of methanol. In muscle cells, SIRPα is able to recruit SHP-2 (which we have shown to be expressed in INS-1E beta cells) (Maile and Clemmons, 2002) and loss of SHP-2 from beta cells causes a decrease in glucose stimulated insulin secretion (Zhang et al., 2009). These data may imply that perhaps SIRPα and SHP2 could interact to positively regulate insulin secretion. This might occur through the maintenance of beta cell identity (PDX1) and by maintaining GLUT2 expression as it is important in glucose sensing (Zhang et al., 2009). This hypothesis has not been tested in beta cells.

6.4.4 CD47 and beta cell survival

The extracellular region of SIRPα binds to its ligand CD47 to induce a bidirectional signal that has been implicated in migration, cell survival and

proliferation (Kobayashi et al., 2008, Matozaki et al., 2009). CD47 is expressed on most somatic cells (Ayi et al., 2016, Bian et al., 2016, Per-Arne, 2013), and here using immunohistochemistry its expression in pancreatic beta cells was revealed. Indeed its expression on these cells was higher than that on other pancreatic cell populations. This result agrees with that of Kobayashi and colleagues in mice (Kobayashi et al., 2008) and with RNA sequencing data from human islets (Segerstolpe et al., 2016). In tissue sections from individuals with T1DM, we noticed an unexpected decrease in the expression of CD47 in beta cells although the mechanism remains unclear.

The novel observation of the reduction in CD47 in beta cells may impact on the recognition of pancreatic beta cells as “self” (Per-Arne, 2013). Loss of CD47 in pancreatic beta cells might make them more vulnerable to immune attack. The loss of CD47 has been linked to increased surface expression of calreticulin and phosphatidylserine suggesting enhanced phagocytic clearance of CD47 depleted cells (Gardai et al., 2005). Additionally, loss of CD47 in TCR transgenic mice has been shown to break immune tolerance leading to autoimmune diabetes (Dugas et al., 2010). In immune cells such as neutrophils, decreased expression of CD47 leads to increased phagocytosis by macrophages (Lawrence et al., 2009), conversely, overexpression decreased neutrophil apoptosis and phagocytosis (Barrera et al., 2017).

Interestingly, as the expression levels of CD47 diminished in T1DM beta cells, expression of this molecule in the alpha cells was increased. One explanation for the diminished CD47 expression in beta cells could be that loss of STAT6 (also observed in sections from individuals with T1DM), leads to the decreased expression. It is not clear why alpha cells in T1DM tissue sections have an

increased expression of CD47 but reports from cancer studies (Cook and Soto-Pantoja, 2017, Jaiswal et al., 2009) suggest that such upregulation enhances the proliferation and protects from immune attacking. This could explain why alpha cells are spared in T1DM and how they evade the immune attack during insulitis (Habener and Stanojevic, 2012). Another intriguing possibility might be a case of beta cells transdifferentiating due to inflammation, changing their phenotype to alpha cells but still retaining CD47 expression (Nordmann et al., 2017). In support of this conclusion, it was observed that not all alpha cells in T1DM have elevated CD47 expression and that the number of alpha cells was generally higher in T1DM islets when compared to relevant controls.

Data arising from qPCR analysis suggest that IL-4 and IL-13 can induce upregulation of *CD47* mRNA in beta cells in a STAT6 dependent manner. Also, the data revealed that knockdown of STAT6 did not directly alter the expression level, suggesting that other factors regulate CD47 expression under basal conditions. Individuals with T1DM have been reported to have reduced levels of IL-4 and IL-13 (Kikodze et al., 2013, Alnek et al., 2015) and STAT6 (Leslie et al., 2018), suggesting that beta cells may not be able to upregulate CD47 during inflammation and that this could lead to enhanced phagocytosis and clearance of CD47 depleted cells (Lawrence et al., 2009).

6.4.5 Conclusion

In summary, SIRPα is an IL-13 regulated gene in beta cell lines and human islets and might play an important role in beta cell survival. SIRPα was shown to be important for beta cell survival as the knockdown of this gene led to significant beta cell loss and its overexpression protected beta cells from cytotoxicity. The mechanism of protection by SIRPα in beta cells remains to be clarified. The

binding of SIRPα to its cognate receptor CD47 may be one of the ways by which this protection is achieved. CD47 expression was significantly decreased in beta cells of sections from individuals with T1DM and this could impact the phagocytosis of these cells. Additionally, the loss in CD47 could lead to upregulation of “eat me” signals such as calreticulin (Gardai et al., 2005).

Chapter 7.0: Discussion

7.0 Discussion

The incidence of type 1 diabetes is increasing in children below the age of 15 years and is predicted to double between 2005 and 2020 (Patterson et al., 2009, Patterson et al., 2018). It is estimated that approximately 35,000 children under the age of 19 live with T1DM in the UK and that the prevalence among children under the age of 14 is about 24.5 per 100,000 (Patterson et al., 2009). The pathophysiology of type 1 diabetes is not completely understood. Our present understanding suggests there is a complex interaction between environmental factors, genetic susceptibility and the immune system (Copenhaver and Hoffman, 2017). GWA studies have identified susceptibility genes that may predispose individuals to T1DM yet only about 33% of high-risk carriers develop the disease, implicating a role for other factors (Steck and Rewers, 2011). Environmental factors implicated include viruses, bacteria, lack of vitamin D, and geographical location (Knip and Simell, 2012).

The involvement of the immune system in T1DM is serologically established by the presence of auto-antibodies, generated against particular islet proteins (Siljander et al., 2009). Immune cell infiltration of the islets of Langerhans has been verified in rare human T1DM pancreatic tissue demonstrates the presence of different immune cell subsets (Arif et al., 2014, Morgan et al., 2014). One route through which these infiltrating immune cells can impact on beta cell function and survival is via the secretion of specific cytokines. There is evidence to suggest that beta cell death occurs as a result of an imbalance between the secretion of pro-inflammatory and anti-inflammatory cytokines (Souza et al., 2008, Berchtold et al., 2016). Work on the detrimental effects of pro-inflammatory cytokines on beta cells has been extensive (Moore et al., 2011, Cetkovic-Cvrlje and Eizirik, 1994, Russell and Morgan, 2014, Pirot et al., 2008, Souza et al., 2008, Collier et

al., 2011) but, the action of “anti-inflammatory” cytokines on pancreatic beta cells has received far less attention. This thesis sought to investigate the actions of two such anti-inflammatory cytokines, IL-13 and IL-4, and examine their role in protecting beta cells from cytotoxicity. The role of the transcription factor STAT6, activated in response to receptor binding of both these cytokines, was studied in detail.

The first part of the thesis investigated the capacity of IL-13 and IL-4 to protect beta cells from cell death induced by exposure to different cytotoxic stimuli (serum withdrawal, palmitic acid, and pro-inflammatory cytokines). The second component explored the signalling pathways stimulated by IL-13 in beta cells and the expression of genes regulated by IL-13. The third part of the work studied the impact of manipulating STAT6 levels in the response to IL-13 either by using small interference RNA to deplete STAT6 expression or by transfecting an expression plasmid to enhance STAT6 levels or to constitutively activate or inhibit STAT6 activation. Finally, the genes upregulated by IL-13 stimulation were examined to better understand the mechanisms of cytoprotection from cytotoxicity in beta cells.

7.1 Overview of IL-13 cytoprotective phenotype

IL-13 was shown to exert a potent protective effect against a range of cytotoxic stimuli in beta cells (pro-inflammatory cytokines, serum withdrawal and palmitate) using rat beta cell lines. IL-13 treatment of human derived beta cell line EndoC βH1 also protected from pro-inflammatory cytokine induced cell death. The precise mode of protection is still not completely known, however, the finding that IL-13 can protect against multiple cytotoxic stimuli suggests that several different anti-apoptotic pathways may be activated. These multiple cytotoxic stimuli

activate apoptotic pathways in beta cells via activation of caspase 3 (serum withdrawal, TNF-α and palmitate), Caspase 8 (TNF-α), Caspase 9 (serum withdrawal), depletion of BCLXL and induction of nitric oxide (IL-1β, IFN-γ) (Cnop et al., 2005, Yang et al., 2015, Tomita, 2017, Eizirik et al., 2009). These results are in accord with previous studies (Kaminski et al., 2007, Kaminski et al., 2010, Russell et al., 2013, Rütti et al., 2016). Furthermore, the protection may be relevant *in vivo* since treatment of NOD mice with IL13 (or IL-4) reduces the incidence and slows the rate of acquisition of diabetes in this mouse model (Zaccone et al., 1999, Cameron et al., 1997, Mi et al., 2004). Importantly, individuals with T1DM have been shown to have defective invariant natural killer T-cells known to secrete IL-13 that can modulate effector T-cells in T1DM (Usero et al., 2016).

7.2 The Jak/STAT6 signalling in the pancreatic beta cell

Given that IL-13 has a protective effect on pancreatic beta cells, it was important to understand the underlying mechanisms by which IL-13 might protect. Typically, IL-13 signals by binding to its cognate receptor, IL-13Rα1 leading to the recruitment of a receptor component IL-4Rα to form a heterodimer. Alternatively IL-13 can also bind to IL-13Rα2 monomer. IL-13 binds to IL-13Rα2 with a higher affinity and although its downstream signalling had initially proved elusive due to its short cytoplasmic tail, evidence now suggests that this monomer could be involved in inducing TGF-β1 production (Fichtner-Feigl et al., 2005). The biology of this second receptor has not been studied in the context of beta cells or T1DM. However, the receptor expression is thought to be very low since RNAseq failed to detect IL-13Rα2 in beta cells of humans (Segerstolpe et al., 2016). The heterodimer formation between IL-4Rα and IL-13Rα1 or between IL-4Rα and γC

brings their associated Jak kinases into close proximity leading to *trans* phosphorylation. The activated Jaks, in turn, phosphorylate specific tyrosine residues in the receptor leading to the recruitment of STAT6 to the phosphorylated docking sites and its subsequent activation (Russell and Morgan, 2014).

It was shown in the present work that INS-1E beta cells express all four Jaks, but that these are activated with differing kinetics following treatment with IL-4 and IL-13. The differing activation kinetics of the Jaks could explain the differences in pathways known to be activated by IL-13 stimulation (pSTAT3, pSTAT6 and PI3K/Akt) (Russell et al., 2013). Importantly, each receptor monomer can recruit up to two different Jaks since they possess Box regions, which are used by the FERM domains in Jaks to bind to receptors (Ferrao and Lupardus, 2017, Usacheva et al., 2002, Noon-Song et al., 2011, Umeshita-Suyama et al., 2000). This work demonstrates that IL-13, as well as IL-4 stimulation of INS-1E cells, led to the phosphorylation of all four Jaks. It will be important in the future to ascertain if a particular Jak or Jaks is recruited to either the IL-4Rα or the IL13Rα1. This could be done either using co-immunoprecipitation assays, to determine which Jak is pulled down with each receptor or site directed mutagenesis by mutating specific residues in either the Box (receptor) or the FERM (Jaks) domains and examining if this alters the outcome of IL-13 or IL-4 treatment. Based on the experiments of Russell et al., (2013), the activation of STAT6 and the protection offered by IL-13 against cytotoxicity were reduced after Jak2 inhibition, implicating Jak2 in the signalling pathway. This correlated well with western blot analysis (Fig 4.9), which suggested that knockdown of Jak2 reduced STAT6 activation in response to IL-13. Other studies suggest that IL-4 requires Jak3 activation to mediate its protection (Kaminski et al., 2010).

In our previous experiments and reports by others, it was shown that both the PI3K/Akt and the Jak/STAT signalling pathways are activated by IL-4 and IL-13 in beta cells (Russell et al., 2013, Kaminski et al., 2010, Rutti et al., 2016). However, using a PI3K/Akt inhibitor wortmannin, it was reported that IL-13 was still able to protect beta cells from serum withdrawal induced cell death (Russell et al., 2013), implying that the Jak/STAT signalling is more likely to be involved in cytoprotection than the PI-3K/Akt pathway, although other investigators have argued the contrary (Rütti et al., 2016). In future experiments, an alternative method to show that the contribution of the PI-3K/Akt to the protection afforded by IL-13 will be to perform a site directed mutagenesis or CRISPR on the IL-4Rα receptor at tyrosine 497 position reported to recruit IRS2 which activates the pathway (Nelms et al., 1999).

The use of a Jak/STAT specific PCR array panel to identify genes modulated during IL-13 stimulation identified a variety of genes with altered expression. A proportion of these were confirmed at both the RNA levels using gene specific primer qPCR assays and at the protein level, by Western blotting. Amongst these genes, the upregulation of signal regulatory protein alpha (*SIRP*α) was demonstrated for the first time. This prompted an investigation into its role in beta cells. Encouragingly, MCL-1 and BCLXL and SOCS-1 were also upregulated at both the RNA and protein level and these have been previously shown to protect beta cells from cell death both *in vitro* and *in vivo* (Allagnat, 2010, Carrington et al., 2009). Additionally, It was shown that IL-13 and IL-4 upregulated the expression of STAT6 itself, implying the presence of a positive feedback loop. Taken together, this implies that pre-incubation of cells with IL-13 upregulates anti-apoptotic proteins such as SOCS1, BCLXL, and MCL-1 and these likely are involved in the cytoprotection observed. Other genes that were upregulated but

not directly studied in this work were *GRB2, JunB, Jun, Crk,* and *SH2B1*. These have also been shown to regulate beta cell health and might contribute to the cytoprotective effects of IL-13 (Gurzov et al., 2008, Chen et al., 2014, Scarim et al., 2003, Hügl et al., 1998, Lee et al., 2004). Further genes implicated in the regulation of inflammation and not directly studied here include *PIAS1* and *PIAS2*. *PIAS1 & 2* are known to inhibit STAT1 and STAT2 signalling by binding to the activated homo or heterodimers of STAT molecules to prevent DNA binding (Liu et al., 2015a). As aberrant STAT1 signalling has been observed in T1DM (Richardson et al., 2016), these molecules may warrant future attention. Figure 7.1 details the current understanding of the IL-4 and IL-13 signalling in pancreatic beta cells.

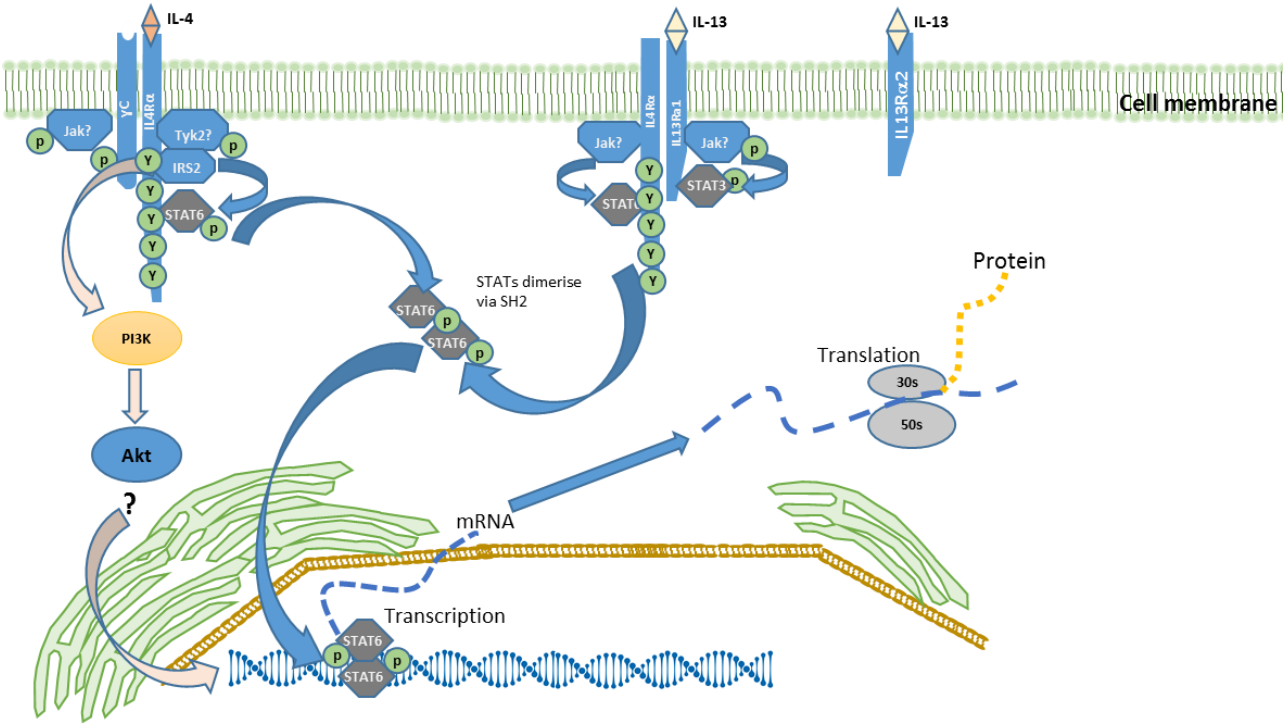


Figure 7. 1: A schematic representation of the present understanding of Jak/STAT6 signalling in beta cells

Stimulation of beta cells with IL-4 or IL-13 recruits receptor components that leads to trans-phosphorylation of Jaks. Activated Jaks then phosphorylate tyrosine residues on the receptor cytoplasmic tail exposing docking sites wherein STAT6 is recruited and activated. Activated STAT6 then migrates to the nucleus where it regulates the expression of genes.

7.3 The role of STAT6 in IL-13 signalling

The core of the thesis was to investigate the role of STAT6 in IL-13 mediated cytoprotection of beta cells. To achieve this, STAT6 expression and activity were either reduced or increased, through the use of small interference RNA or a STAT6 expression plasmid. Somewhat surprisingly, knockdown of STAT6 alone did not affect beta cell viability. Nevertheless, the data reveal that STAT6 facilitates the IL-13 induced protection of beta cells since knockdown of STAT6 attenuated the protective effect of IL-13 following stimulation with pro-inflammatory cytokines, palmitate or serum withdrawal. Knockdown of STAT6 also revealed that many of the genes that were upregulated in response to IL-13 and IL-4 (*SIRPα, SOCS-1, MCL-1* and *BCL2L1*) were modulated by STAT6 confirming the key role of STAT6 in IL-13 mediated protection. Given the results of knockdown studies, alternative validation of these findings using a STAT6 expression vector was performed. Using site-directed mutagenesis, a STAT6 expression vector was used to create a constitutively active and dominant negative form as described by Daniel et al. (2000). These variant forms behaved as expected when assayed using a luciferase reporter system. Their use in viability assays showed subtle protection from pro-inflammatory cytokines by constitutively active STAT6 but this did not achieve statistical significance. It was noticed, however, that the dominant form by itself was detrimental to beta cell survival. The low transfection efficiency of the INS-1E cells was a particular challenge and made it difficult to interpret results arising from the experiments with STAT6 variants. In an attempt to overcome this, co-transfection of the STAT6 variants with a GFP construction was performed. The theory being that the GFP transfected cells are more likely to be transfected with STAT6 (co-transfection principle). However, we cannot guarantee that the identified cells are transfected

with both the GFP and the STAT6 plasmid and the results were not sufficiently clear to draw firm conclusions. In the future, it may be beneficial to generate stably transfected cells or use CRISPR technologies to generate cells containing variant forms of STAT6.

This work also demonstrates that the loss of STAT6 leads to a decline in anti-apoptotic proteins MCL-1 and BCLXL and an increase in the expression of the MHC class I subunit, B2M. These findings align with studies of pancreatic tissue from donors with T1DM in whom B2M expression is increased in insulin containing islets (Richardson et al., 2013, Richardson et al., 2016). Furthermore, immunohistochemical staining of pancreatic sections revealed a decrease in the expression of MCL-1 in certain islet cells in T1DM, when compared to individuals without diabetes (Richardson et al., 2013).

Collectively, this work has demonstrated a role for STAT6 in the protection of rodent and human beta cells against cytotoxic stimuli. It was further shown that the loss of STAT6 can occur following exposure to cytotoxic stimuli and that the use of IL-13 and IL-4 could rescue such loss. Importantly, STAT6 expression is reduced in the islets of individuals with T1DM (Leslie et al., 2018), suggesting that the beta cells in these islets may be more susceptible to death induced by cytokines elaborated by infiltrating immune cells, potentially through reduced expression of anti-apoptotic molecules like MCL1. These cells may also have enhanced visibility to influential immune cells. The loss of STAT6 and its contribution to beta cell health is schematically represented in Figure 7.2.

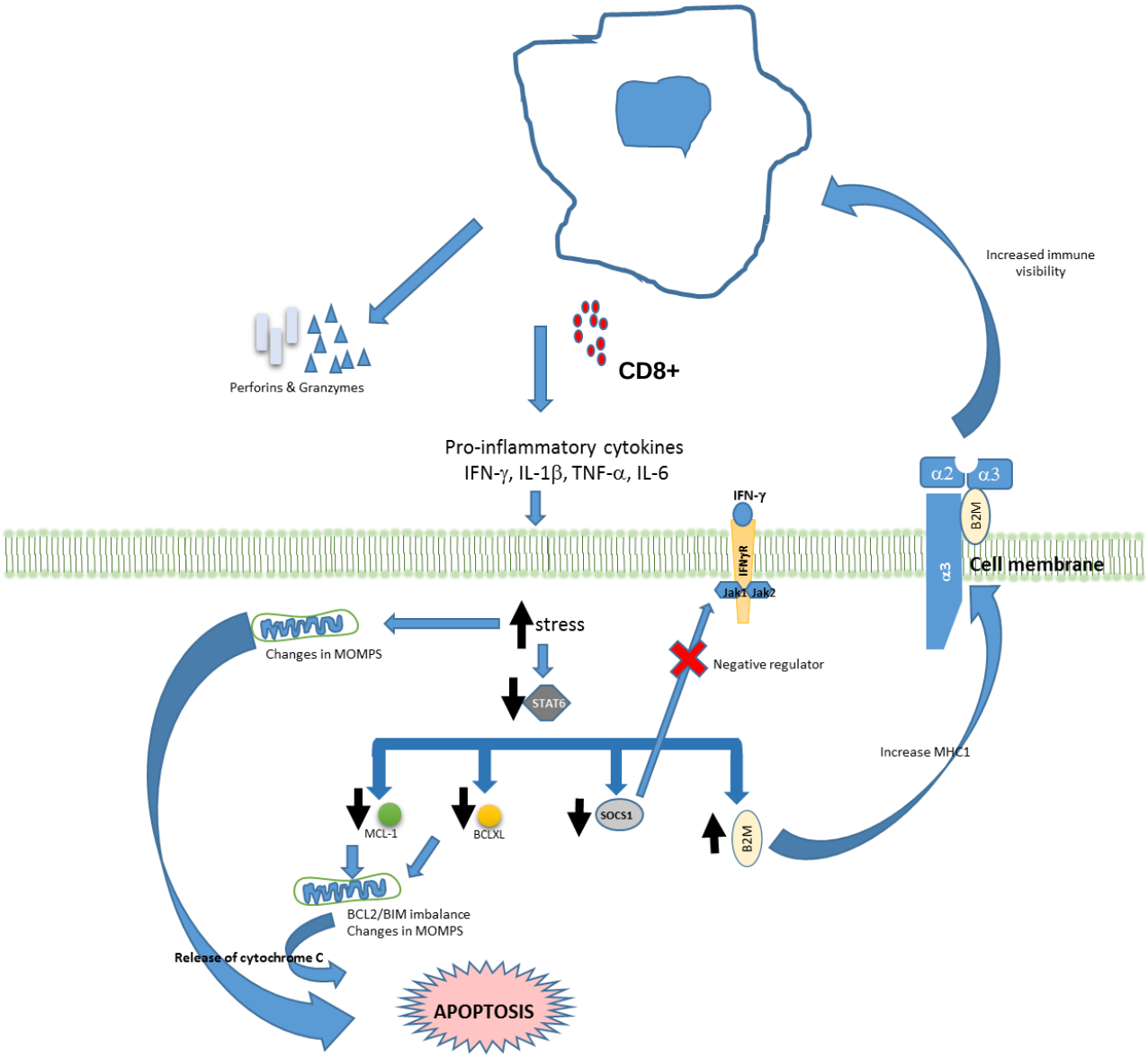


Figure 7. 2: Schematic showing how the Loss of STAT6 could contribute to beta cell loss in T1DM

Under stress, STAT6 expression is reduced, this leads to decreased MCL-1, BCLXL and SOCS1 expression but in contrast leads to increase B2M expression. The decrease in anti-apoptotic proteins enhances apoptosis, while increase in B2M leads to MHC class I expression and hence prompting immune cells which infiltrate and secrete more detrimental cytokines

7.4 The role of SIRPα and its ligand CD47 in beta cells

This work demonstrates for the first time that IL-13 and IL-4 lead to a significant and strong upregulation of SIRPα expression in beta cells. As very little was known about the role of this protein in beta cell survival, additional investigations were carried out. It was shown that SIRPα is expressed in rodent and human beta cell lines and in human islets by western blotting. Strikingly, knockdown of this protein with small interference RNA induced beta cell death directly. SIRPα has only been previously described in a single study in pancreatic islets of rodent models, wherein pancreatic specific knockout affected insulin secretion (Kobayashi et al., 2008) but no information about its impact on beta cell viability was provided. Importantly, the study of SIRPα in other cell systems has revealed that it sequesters a number of binding partners, including Grb2, PDK1, Jak2, STAT1 and SHP2. Intriguingly, all of these molecules have been associated in other studies with the control of beta cell viability (Shen et al., 2009, Zhang et al., 2009, Shen et al., 2010). The increased expression of SIRPα in beta cells exposed to IL-13 or IL-4 may therefore impact on the function of these molecules and subsequent downstream pathways. The ability of SIRPα to bind to these molecules has not yet been studied in beta cells but may be important since an alteration in some of these interactions may facilitate either loss or improvement in cell viability. For example, reduced levels of SIRPα could result in the inhibition of the SHP2, Grb2 and PDK1 mediated activation of the Akt cytoprotection pathway (Shen et al., 2009). Future study of these binding partners in beta cells might shed light on the mechanism of death caused by SIRPα knockdown.

In the present study, a SIRPα expression plasmid was used to artificially increase SIRPα levels and study the impact on apoptosis induced by serum

withdrawal. This revealed that the upregulation of SIRPα might play an important role in the protection of beta cells from cytotoxicity although the precise mechanism of cytoprotection remains to be determined. SIRPα is known to signal by binding to its associated ligand CD47 thereby transmitting signals in a bidirectional manner, that impact upon both phagocytosis and apoptosis pathways in the recipient and donor cells (Oshima et al., 2002). It was shown in the present work that both human and rodent beta cells express CD47, and that CD47 is more abundantly expressed on beta cells compared to alpha cells in donors without diabetes. Surprisingly, the reverse was true in individuals with T1DM, with expression of CD47 being reduced in beta cells and increased in alpha cells.

7.5 Therapeutic potential of the IL-4/IL-13 pathway

The findings arising from this thesis have demonstrated that IL-13 and IL-4 can stimulate anti-apoptotic pathways in beta cells; hence, the Jak/STAT6 presents a potential therapeutic targetable pathway. The administration of IL-4 and IL-13 *in vivo* to reduce the incidence of T1DM has been demonstrated in NOD mice models of the disease and shown to be effective at decreasing the disease incidence (Zaccone et al., 1999, Cameron et al., 1997). The administration of IL-13 to humans has not been reported, and the direct administration of this cytokine would be challenging due to a potential side effect of inducing asthma (Yang et al., 2001). To avoid the direct administration of IL-4 or IL-13, molecules that induce the secretion of either IL-4 or IL-13 could be exploited. One molecule that has been considered in the context of diabetes and which is known to enhance IL-4 synthesis is calcitriol (Vitamin D or 1, 25(OH) $_2D_3$) (Ysmail-Dahlouk et al., 2016). In that study, PBMCs were isolated from individuals with T1DM and non-diabetic controls and treated with calcitriol. In the untreated samples, IL-4

secretion was lower in T1DM and IFN-γ was higher. However, treatment of the PBMCs with calcitriol significantly enhanced IL-4 secretion and STAT6 activation and reversed the production of IFN-γ (Ysmail-Dahlouk et al., 2016). Additionally, the administration a synthetic glycolipid alpha-galactosylceramide (α–GalCer) is a potent activator of invariant NKT-cells that secrete IL-13 could be used, although, the activity of these cells has been reported to be dysregulated in T1DM individuals (Usero et al., 2016, Sullivan and Kronenberg, 2005).

Alternatively, enhancing the activity of some proteins involved in the anti-apoptotic pathway such as STAT6, SIRPα, MCL-1, SOCS1, BCLXL and PIAS1 could serve as potential therapeutic targets. One drug that might be repurposed for the treatment of T1DM which could exploit the pathway is valproic acid, a compound used for the treatment of epilepsy, migraines and bipolar disorder (Chateauvieux et al., 2010). Valproic acid has been shown to increase STAT6 and PIAS1 expression in FBR cell lines (McGarry et al., 2004), and importantly, to reduce beta cell apoptosis in juvenile diabetic rats although the mechanism was attributed to histone deacetylase inhibition (Khan and Jena, 2016).

7.6 Conclusion

Taken together, the data presented in this thesis revealed that loss of STAT6 leads to a reduction in the Jak/STAT6 signalling pathway in beta cell. This does not directly compromise viability but it makes the beta cells more vulnerable to cytotoxicity. In recently published data we show that STAT6 is reduced in T1DM individuals, possibly due to increased pro-inflammatory cytokines secretion (Leslie et al., 2018), highlighting this as a potentially important dysregulated pathway in T1DM.

This loss of STAT6 will cause a reduction in anti-apoptotic proteins such as MCL-1 and BCLXL. STAT6 depletion is also associated with reduced levels of SOCS-1, which is a negative regulator of IFN signalling (Li et al., 2015b, Allagnat et al., 2010, Carrington et al., 2009). Interferon signalling pathways are dysregulated in T1DM (Qaisar et al., 2018) and have been suggested to initiate diabetes in NOD mice (Li et al., 2008). Loss of SOCS1 could contribute to potentiating IFN signalling hence exacerbating the disease. Importantly, in this thesis, I have reported that IL-13 and IL-4 upregulate SIRPα in beta cells and shown that SIRPα is able to protect the cells from cytotoxicity through a still to be defined mechanism. The depletion in STAT6 could imply an eventual loss in SIRPα that would lead to beta cell death.

Finally, the Jak/STAT6 pathway is shown to be critical for maintaining beta cell survival through a variety of mechanisms that involve the regulation of multiple genes, the most differentially expressed being SIRPα whose loss might be deleterious to beta cell survival in T1DM. The updated schematic representation of this pathway is in Fig 7.3.

Taken together, these data implicate STAT6 as an important regulator of beta cell viability.

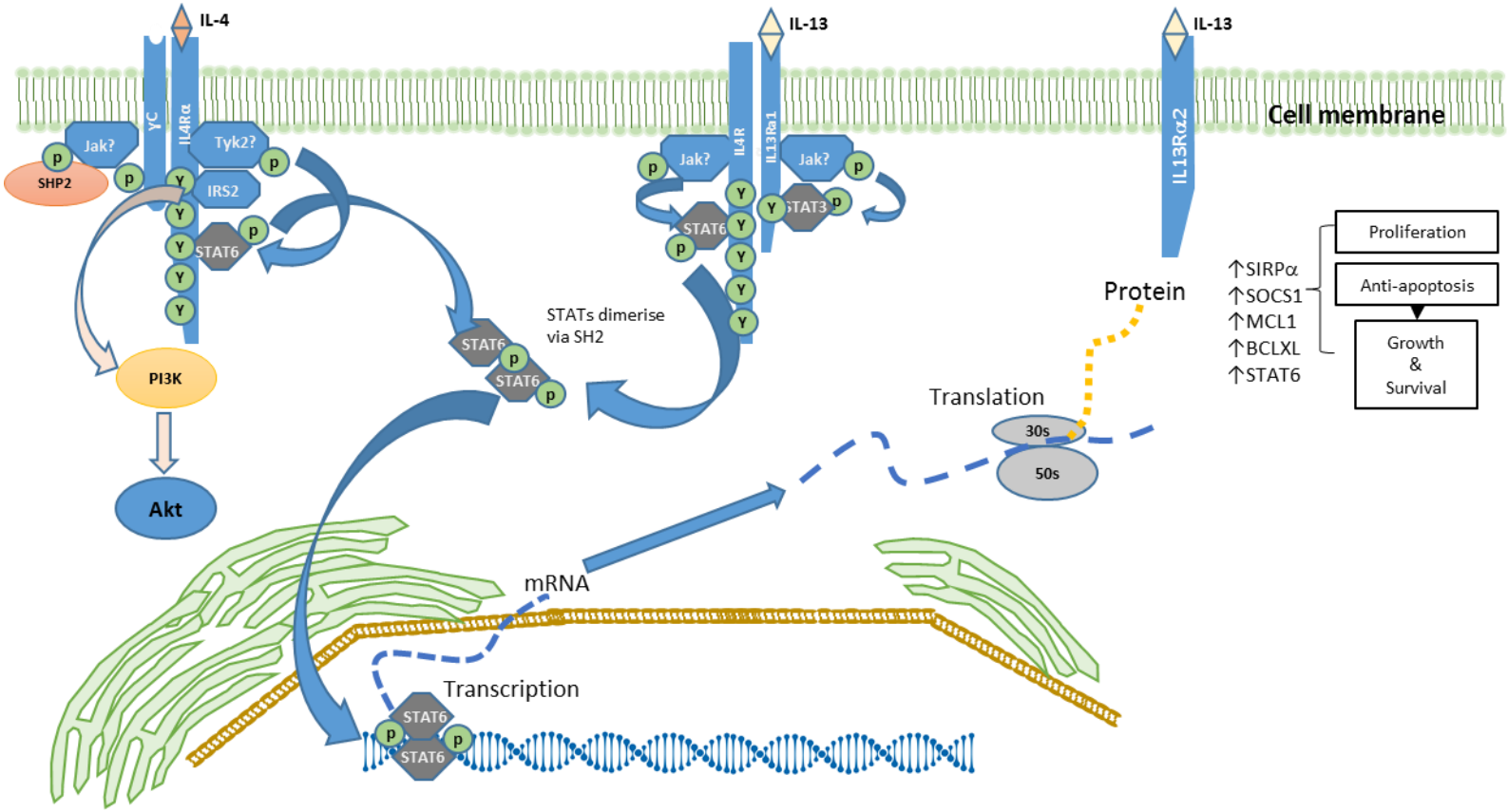


Figure 7. 3: Jak/STAT6 pathway Schematic updated

The pathway illustrates the upregulated genes upon stimulation with IL-13, some of which are anti-apoptotic MCL1, BCLXL, STAT6 and SIRPα. Also upregulated is SOCS1, which negatively regulates STAT molecules.

7.7 Limitation of the study and future works

7.7.1 Limitations

The work presented here has focused on the role of STAT6 in beta cell cytoprotection afforded by IL-4 and IL-13. These cytokines are known to activate in addition to STAT6, both the PI-3K/Akt pathway and STAT3 Russell et al. (2013), which were not studied further.

To study the role of STAT6 in controlling beta cell viability, experiments in which INS-1E cells were transfected with mutated forms of STAT6 with modified activity, did not take into account endogenously expressed STAT6, which could potentially skew or mask the results obtained. To resolve this, cell lines stably expressing these variant forms of STAT6 could be developed by cloning these versions into INS-1E cells using a Flp-In™ T-REx™ system or CRISPR gene editing technology. The Flp-In™ T-REx™ system is used to develop stable cell lines containing the gene of interest under the control of a tetracycline inducible promoter. These cells have a flippase (Flp) recombinase recognition target site at a transcriptionally active locus, which can be targeted to accommodate the gene of interest using a Flp-In™ expression vector (Senkel et al., 2009). While the CRISPR gene editing technology uses helicase and nuclease enzymes on the CRISPR associated system (CAS) protein and a guide RNA (target gene and tracerRNA) to specifically help edit the genome of interest to the desired sequence (Adli, 2018).

The cytoprotective experiments performed in this thesis investigated on INS-1E cells and human derived EndoC βH1 cell lines and it is not known whether these protective effects can occur with human islets.

Immunohistochemical staining of tissue sections with anti-sera against SIRPα did not perform as expected. There was no staining in pancreatic tissue sections when probed with anti-sera against SIRPα. This challenge made it hard to validate the loss of STAT6 seen in tissue (Leslie et al., 2018), with a similar loss in SIRPα. This could be attributed to the type of fixative used in our tissue samples, the antigen retrieval method or the conformation of the protein in tissue, which differs from the linear structures in western blotting (Ivell et al., 2014, Werner et al., 2000).

7.7.2 Future works

Future works arising from the findings in this thesis will include;

- Further assessing the impact of STAT6 in beta cells by generating stable cell lines expressing the various forms of STAT6. This will validate the results obtained in this thesis.
- IL-13 upregulated the expression of SIRPα and in chapter 6 it was shown that knockdown of SIRPα was detrimental to beta cells. Overexpressing SIRPα protected INS-1E cells from serum withdrawal cytotoxicity. Hence, the role of SIRPα in beta cell viability by overexpressing this protein in pro-inflammatory cytokine treatments and measuring cell viability will be investigated. In doing this, the experiments will validate the findings that SIRPα is one of the main mechanisms by which IL-13 protects beta cells from cytotoxicity. Since there is a lot of evidence implicating pro-inflammatory cytokines (Allagnat et al., 2010, Choi et al., 2004, Collier et al., 2011, Fatima et al., 2016, Souza et al., 2008) in beta cell loss, this could serve as a therapeutic target for treatment of T1DM. Additionally, the phosphorylation of tyrosine residues on the cytoplasmic tail of SIRPα is

critical in modulating the activity of the protein will be studied to assess whether phosphorylation of these residues is important in the protective effects of SIRPα in beta cells. SIRPα activated tyrosine residues will be mutated by site directed mutagenesis and the viability experiments performed to determine the impact of each residue on beta cell survival. Also, these tyrosine residues can bind other proteins such as STAT1, SHP1 and SHP2 which will be investigated using co-immunoprecipitation. The role of CD47 in the SIRPα mediated protection will also be investigated by knockdown of CD47 prior to viability experiments with SIRPα.

- Our PCR array detected a range of other genes, which were upregulated in response to IL-13 and IL-4, for example, PIAS1 and SOCS1. However, in this study, the impact of these proteins on beta cells survival and function has not been assessed. Thus additional studies to address whether some of these additional 'hits' might be important in the protective effects of IL-13 in beta cells may well be warranted
- A broad examination of the genes activated by IL-13 will be investigated using RNAseq. The results from RNAseq will be used to validate our findings and enable further research to understand and exploit beta cell protection by IL-13.

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