Resistance of Melligen cells to pro-inflammatory cytokines involved in beta cell death

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Doctoral Philosophy Science
2010

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

JANET LAWANDI

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Presentations

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Simpson A.M, Tao C, Lawandi J, Swan MA, O'Brien BA, Williams P. (2009) Physiological insulin secretion from a human insulin-secreting liver cell line: Melligen cells. Proc. Aust. Diabetes Society, Melbourne A381, p. 200-1.

Abstract

It has been shown that stable transfection of insulin cDNA into the human liver cell line Huh7 resulted in synthesis, storage and regulated release of insulin to a glucose stimulus (Huh7ins cells). However, Huh7ins cells responded to glucose at sub-physiological levels (2.5mM) and in order to maintain normoglycaemia, insulin secretion in response to physiological levels of glucose is required. Consequently, the Huh7ins cells were further transfected with the human glucokinase gene to correct the skewed insulin secretory profile. The resulting cell line (Melligen) responds to glucose in the 4-5mM physiological range.

If Melligen cells are to be used clinically to correct patient blood glucose concentrations, they need to be resistant to the toxic effects of pro-inflammatory cytokines responsible for the immune-mediated destruction of beta cells. Pro-inflammatory cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) play a major role in beta cell elimination during diabetes development. The aim of the present study was to determine if Melligen cells were resistant to the toxic effects of these cytokines.

Cells were exposed to IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for up to 10 days. Cell viability was measured using the Annexin V/propidium iodide (PI), PI and MTT cell viability assays. Insulin storage and chronic secretion were measured by radioimmunoassay. Acute insulin secretion was also determined by static incubations with increasing concentrations of 0, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5 and 20mM glucose. Nitric oxide levels were assayed by the Griess reaction. The glucose-responsive beta cell line, MIN-6, was used as a positive control throughout. Expression of the cytokine receptors IFNR1, IFNR2, IL1R1, IL1R2, TNFR1 and TNFR2 was determined in human pancreatic islet cells and the liver cell lines cultured in the absence of the cytokine treatment by RT-PCR. The quantitative analysis of the inhibitors of NF-κB (IκB-α, IκB-β IκB-ε) and NF-κB downstream effectors, iNOS, MCP-1 and Fas, was determined by real time RT-PCR in cytokine treated Huh7ins and Melligen cells with islet cells used as a positive control. Microarray analysis was used to determine which gene networks were being induced after 1h or 24h cytokine treatment of Melligen cells. Melligen cells were also tested for suitability for implantation. They were encapsulated (AustriaNova, Singapore) and insulin secretion

and glucose responsiveness were determined. The cells were also treated with a single or double dose of 100μM or 20mM STZ to determine susceptibility to this beta cell toxin.

The viability of MIN-6 cells was reduced after 2 days of culture with cytokines ($P < 0.05$). In contrast, the viability of Huh7, Huh7ins and Melligen cells was unaffected by cytokine treatment over 10 days. In addition to this, flow cytometry results corroborated the fact that there was no apoptotic cell death in these cell lines. The triple cytokines did not diminish chronic insulin secretion, storage or the glucose-responsiveness of Melligen cells even after 10 days of co-culture. After exposure to cytokines MIN-6 cells also produced higher levels of nitric oxide as compared to Huh7ins cells ($P<0.05$). Expression of cytokine receptors IFNR1, IFNR2, IL1R1, IL1R2 and TNFR1 was detected in pancreatic islet cells and all liver cell lines. However, there was an absence of cytokine receptor TNFR2 expression in the liver cell lines. Real time RT-PCR results revealed down-regulation of the inhibitors of NF-κB and Fas in Huh7ins and Melligen cells, which is in contrast to the molecular mechanism seen in islet cells. The liver cell lines expressed very low levels of iNOS and there was no expression of MCP-1 detected by RT-PCR. Microarray analysis also revealed a network of genes up-regulated by NF-κB. Encapsulation of Melligen cells did not affect insulin function of the cells and the double dose 100μM STZ treatment did not affect the cells ($P>0.05$).

In summary, Huh7ins and Melligen cells were more resistant to the toxic effects of pro-inflammatory cytokines as compared to pancreatic beta cells most likely by NF-κB dependent pathways. These findings suggest that insulin-secreting hepatocytes will be less susceptible to destruction by the autoimmune process that eliminates beta cells in Type 1 diabetes development.

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CHAPTER 1: Exploring the Autoimmune Response in Type 1 Diabetes and Possible Therapies for the Disease

1.1 Diabetes mellitus

Diabetes mellitus is a chronic metabolic condition characterised by an inability to regulate glucose homeostasis, resulting in hyperglycaemia (WHO, 1999). While there are several types of diabetes, each with diverse aetiology, the two most prevalent are Type 1 (insulin-dependent or juvenile-onset) and Type 2 (noninsulin-dependent or mature-onset) (CDC, 2002).

Nationally, about 1.5 million Australians had diagnosed diabetes in December 2007. Based on the trends over the past 13 years, the latest data confirms earlier predictions that around 4.2 million Australians will have diagnosable diabetes by 2013 (Pieris-Caldwell et al., 2008). Diabetes affects approximately 246 million people worldwide (Dunstan et al., 2006). As the sixth leading cause of death in Australia over 10,000 Australian deaths were attributable to diabetes in 2004-05 (ABS, 2006). In 2004-05 the estimated direct health care costs of diabetes in Australia were $989 million. Type 1 diabetes affects over 150 000 Australians. Type 1 diabetes is the most common chronic childhood disease in developed nations (Pieris-Caldwell et al., 2008). Clearly, diabetes carries an enormous public health and social burden.

The underlying defect in both Type 1 and Type 2 diabetes is dysregulated blood glucose homeostasis resulting in hyperglycaemia. Therefore, these syndromes share many complications caused by hyperglycaemia (Tapp et al., 2004). However, a fasting glucose plasma level consistently over 7.0mM, ketoacidosis (elevated ketones), extreme thirst, sudden weight loss and blurred vision typically characterise Type 1 diabetes (WHO, 1999).

Whereas Type 1 diabetes is marked by a severe deficiency or absence of insulin, Type 2 diabetes is caused by insufficient insulin production by beta cells, inefficient utilisation by peripheral tissues and/or resistance to the actions of insulin (Mahler & Adler, 1999). Traditionally, Type 2 diabetes develops later in life when a genetic disposition coupled with increased weight gain precipitate insulin resistance. However, more recent studies show that Type 2 diabetes is increasingly diagnosed in children and its incidence in this demographic is correlated with rising obesity rates. The treatment encompasses weight-loss, oral hypoglycaemic agents and, in some instances, exogenous insulin therapy (Colagiuri et al., 2002).

Type 1 diabetes is classified into two sub-classes, Type 1A and 1B. Type 1A diabetes results from an autoimmune process that destroys the insulin secreting beta cells in the pancreatic islets of Langerhans (Pipeleers et al., 2001). Combinations of diverse genetic and environmental factors work in concert to initiate the autoimmune reaction against beta cells, however the factors that initiate autoimmunity are yet to be fully elucidated (Mathis et al., 2001). In contrast, Type 1B diabetes is defined as severe loss of insulin secretion that is not immune-mediated and does not have known genetic aetiology (Gianani & Eisenbarth, 2005). Since the main focus of this thesis will be on Type 1A diabetes it will be hereinafter referred to as Type 1 diabetes.

Despite the multifactorial nature of Type 1 diabetes, pro-inflammatory cytokines, secreted by classically activated macrophages and T helper 1 (Th1) T cells, have been found to play an important role in beta cell destruction. These include interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), and tumour necrosis factor alpha (TNF-α), which are secreted by immune cells infiltrating the islets (Wachlin et al., 2003). Immune cells include autoreactive T-cells that selectively destroy insulin producing beta cells. Macrophages and dendritic cells also play a major role. Besides their role as antigen-presenting cells, macrophages and dendritic cells may be a source of oxygen radicals or other soluble cytotoxic mediators. This complex interaction between the activated lymphocytes, cytokines, and macrophages leads to the destruction of beta cells (Figure 1.1) (Mandrup-Poulsen, 2001) (see Section 1.3 for further discussion).

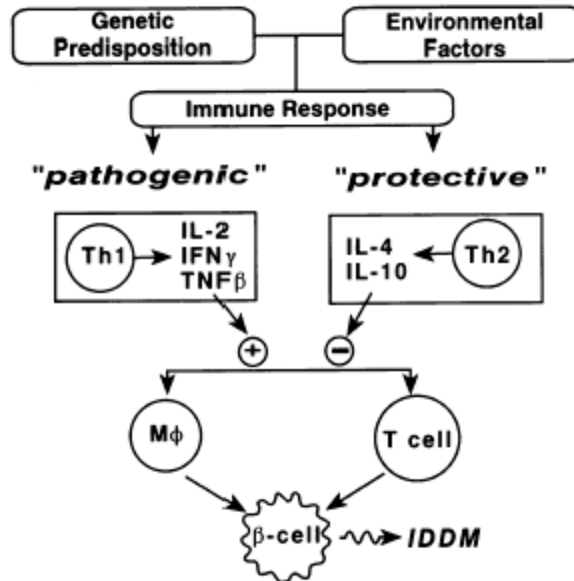


Figure 1.1: Diagrammatic representation of the pathogenesis of Type 1 Diabetes.
A multitude of genetic and environmental factors influence the initiation and perpetuation of the immune response, either tolerance or autoimmunity, that leads to pancreatic beta cell protection or destruction, respectively. Cytokine production by cytotoxic T cells and other immune cells play a central role in the destruction of beta cells and hence are central to pathogenesis (adapted from Rabinovitch & Suarez-Pinzon, 1998).

1.1.1 Treatment

Ensuing beta cell death in Type 1 diabetes results in an absolute insulin deficiency and hyperglycaemia that must be controlled by exogenous insulin therapy. The most common method for the administration of bolus doses of insulin is via subcutaneous injection or infusion via an insulin pump.

Even with the introduction of new analogues and novel administration routes, exogenous insulin does not provide Type 1 diabetics with the minute-to-minute response to glucose that is normally supplied by the glucose-stimulated secretion from functional beta cells. Further complications arise when tight control increases the risk of hypoglycaemia and loose control increases the risk of hyperglycaemia. Exogenous insulin therapy only temporarily restores the patient's ability to regulate blood glucose levels. Long-term control of Type 1 diabetes by exogenous insulin leads to

fluctuations in blood glucose levels predisposing patients to many complications associated with hypoglycaemia and hyperglycaemia (Atkinson & Eisenbarth, 2001).

1.1.2 Complications due to hyperglycaemia

Glycaemic control of Type 1 diabetes using exogenous insulin therapy increases morbidity and mortality by giving rise to several complications. A large body of evidence from both *in vivo* and *in vitro* experiments support three major pathways of glucose metabolism in the development of both micro- and macro-vascular complications in diabetes mellitus (King et al., 1996). These include hyperactivity in sorbitol-aldose reductase pathway, increased oxidative stress and increase in non-enzymatic glycation of proteins and lipids with irreversible formation and deposit of reactive advanced glycation end-products (AGEs), known as the Maillard reaction (Brownlee, 2001; Cameron et al., 2001).

Macrovascular complications (disease of the large blood vessels) include coronary heart disease, stroke and peripheral vascular disease. Microvascular complications (disease of the small blood vessels) include retinopathy, nephropathy, and neuropathy (Lee et al., 2001). In Australia, 15.4% of people with diabetes suffer from retinopathy and the incidence increases dramatically with the duration of the disease (NCMD, 2002). Patients with diabetes are also significantly more likely to develop glaucoma and other retinopathies, a major cause of blindness, than people without diabetes (Klein & Klein, 1995).

Diabetic nephropathy results from high blood glucose levels damaging the blood-filtering capillaries in the kidney. This allows large quantities of albumin into the urine. After the onset of end-stage renal disease, waste products cannot be removed from the body, and dialysis or a kidney transplant is required to sustain life (Russ, 2001). Diabetic nephropathy is the second most common cause of end-stage renal disease in Australia with 11.2% of patients being treated for, or suffering from kidney disease (Dunstan et al., 2001).

Diabetic neuropathy, which causes damage to peripheral nerves or to autonomic nerves, is a result of chronically high blood glucose levels, which affect metabolism within nerves and accumulation of toxins which damage the nerve structure and

function. This causes pain, digestive problems, muscle weakness, non-healing ulcers and lower extremity amputation, and is associated with increased mortality (Feldman, 2003). Table 1.1 shows the incidence of chronic diabetic complications in Australia.

Table 1.1: Frequency of Diabetic Complications in Australia (1999-2000 with a 2004-2005 follow-up)

Complication	Rate among people without diabetes (%)	Rate among people with diabetes (%)
Cardiovascular disease		
Heart attack	3	11
Stroke	2	15.3
Eye diseases		
Retinopathy	UN†	15.4
Cataracts*	<1.65	9.9
Glaucoma*	<0.8	3.2
Blindness*	<0.98	4.9
Kidney disease	1.0	30
Neuropathy	UN†	24.2
Foot Ulcer	UN†	19.4
Amputation*	<0.525	2.1

* 2005 AusDiab; †: statistic unknown

1.1.3 Complications due to hypoglycaemia

The clinical syndrome of hypoglycaemia unawareness (loss of warning symptoms of developing hypoglycaemia, which previously allowed the patient to recognise and abort the episode by eating) is the limiting factor in the glycaemic management of Type 1 diabetes. It causes recurrent physical and psychosocial morbidity, and some mortality, in patients with Type 1 diabetes. Type 1 diabetic patients in good glycaemic control (HbA1c= 7.2%) experience 3-8 episodes of hypoglycaemia per week and intensive insulin therapy produces a threefold increase in the incidence of severe hypoglycaemia (American Diabetes Association, 1997).

1.2 Aetiology of Type 1 diabetes

Susceptibility to Type 1 diabetes is attributable to a combination of multiple genetic factors and environmental factors that trigger autoimmunity. Other stages involved in the aetiology of Type 1 diabetes include activation of autoimmunity and the progressive loss of insulin secretion that eventually leads to overt diabetes and insulin

dependence. Transgenic mouse modeling (Mellanby et al., 2008; Nakayama et al., 2005) has provided direct support that it is the peptide-binding activity of the HLA class II molecules in antigen-presenting cells (APCs) for T lymphocyte peptide recognition that is the main mechanism of action of the DR and DQ molecules in T1D etiology. This is by far the major determinant of disease in the genome (Cucca et al., 2001; Zhang et al., 2008). In humans and mice, susceptibility and resistance to T1D has been mapped to particular polymorphic peptide-binding pockets of the DQ molecule, pocket 9, and of DR, pockets 1 and 4 (and their mouse orthologs, IA and IE, respectively) (Cucca et al., 2001; Suri et al., 2005). It remains a goal to identify molecules that modulate the function of these pockets and could be therapeutic.

1.2.1 Genetic factors

Familial studies support a role for susceptibility genes in Type 1 diabetes development with more closely related individuals exhibiting increased disease incidence. However, studies of monozygotic twins indicate that over 50% are pair-wise concordant for Type 1 diabetes (Redondo et al., 2008). This suggests that there is either acquired post-conceptional genetic discordance, or trigger(s) of beta cell autoimmunity by putative environmental factor(s) (Knip, 2003).

1.2.1.1 MHC susceptibility genes

The categorization of Type 1 diabetes as an autoimmune disease is not ambiguous, with the presence of the predisposing HLA class II haplotypes, DRB1*04-DQB1*0302 and DRB1*03, in at least 90% of cases (Todd, 2010). The presence of autoantibodies and, more recently, autoreactive, anti-islet antigen-specific T cells (Skowera et al., 2008) in the circulation of prediabetic individuals and of newly diagnosed and established cases are also observed (Figure 1.2). Recent fine mapping of the extended MHC region, 8 Mb of chromosome 6p21, which was made possible by the development of high-throughput, dense, and genome-wide single nucleotide polymorphism (SNP) genotyping (referred to as genome-wide association studies, GWAS), has confirmed that the major susceptibility, and resistance, to Type 1 diabetes does indeed map to the HLA class II region of the MHC (Howson et al., 2009; Nejentsev et al., 2007). Class II molecules in APCs bind peptides from the currently identified autoantigens, preproinsulin, insulinoma-associated antigen 2 (I-A2), glutamic acid decarboxylase (GAD), and zinc transporter (ZnT8) (Wenzlau et

al., 2008), and present these to $CD4^+$ T cell antigen receptors (TCRs) in the thymus and in the periphery, for example, in pancreatic lymph nodes and within the islets themselves (von Herrath, 2009). $CD4^+$ T cells provide help to $CD8^+$ cytotoxic T cells, which are the widely accepted most important killer of human islet beta cells in T1D autoimmunity (Skowera et al., 2008; Willcox et al., 2009) (Section1.3).

In a study by the Diabetes Genetics Consortium (McKinnon et al., 2009), univariate analysis yielded strong associations with T1D susceptibility that were dominated by SNPs in the class II HLA-DR/DQ region but extending across the MHC. Similar effects were frequently observed across SNPs within multiple genes, sometimes spanning hundreds of kilobases. SNPs within a region at the telomeric end of the class II gene HLA-DRA yielded significant associations with and without adjustment for carriage of the predictive DR3, DR4, DR2 and DR7 HLA haplotypes, and remained highly prominent in a secondary analysis that was restricted to 66 families where at least one of the affected siblings carried neither the DR3 nor DR4 haplotype. Susceptibility loci HLA typing estimates the risk of developing Type 1 diabetes in various populations, however, the efficiency of the markers will be highly dependent on the population studied (Kawabata et al., 2000). There are multiple candidate genes within the MHC, and for some autoimmune disorders, alleles of MIC-A strongly influence disease risk (e.g. Addison’s disease) (Gambelunghe et al., 1999).

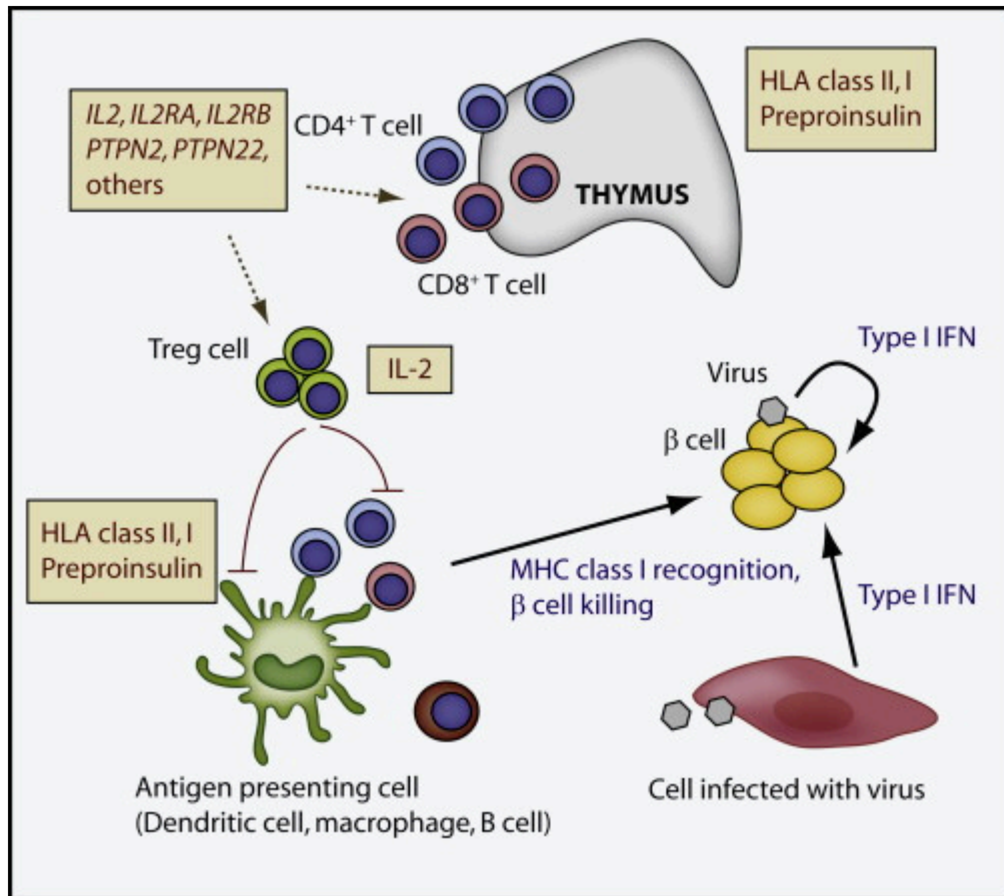


Figure 1.2: A Model for the Pathogenesis of Type 1 Diabetes Based on Genetic Etiological Studies in Humans.

Shown are genes (e.g. those that encode MHC HLA class II and class I antigen-presentation molecules, preproinsulin (INS) in the thymus, CTLA-4 in Treg cells, cytokines) believed to be causal in the process of Type I diabetes. Also shown are influences of viral infections and type 1 interferon (IFN) production and effects; autoimmune repertoire development in the thymus; and the main immune cell types, $CD4^+$ and $CD8^+$ T cell subsets, T regulatory (Treg) cells, and B cells and various other antigen-presenting cells, interact to kill pancreatic beta cells (Todd, 2010).

1.2.1.2 Non-MHC susceptibility genes

Other characterised diabetes susceptibility loci outside the MHC region have also been identified. Co-stimulatory molecules of CD28, CTLA4, and ICOS are expressed on cell surfaces and provide regulatory signals for T-cell activation. Not in childhood-onset, but the adult-onset diabetes, which does not have the high-risk HLA alleles is associated with a polymorphism in exon 1 of CTLA4 (Park, 2004). Other non-MHC

susceptibility genes include the insulin gene on chromosome 11 (chromosome 11p15), chromosome 11q, chromosome 6q and chromosome 18 these include VNTR (variable number tandem repeat) insulin alleles (Vafiadis et al., 1997; Pugliese et al., 1997) and SUMO4 (Guo et al., 2004). There are two additional genetic loci commonly associated with a relative risk for Type 1 diabetes, *INS* and *PTPN22* (Concannon et al., 2009) (Figure 1.3). Allelic variation at *INS*, the gene encoding proinsulin, confers differential susceptibility to Type 1 diabetes. Variation in *INS* is categorized by polymorphisms in a VNTR element, associated with an odds ratio for Type 1 diabetes susceptibility of 2.2 in *INS VNTR I* subjects relative to the heterozygous or "protective" *INS VNTR III* genotypes (Bennett et al., 1995). The *INS VNTR III* genotype is associated with high expression of thymic proinsulin in thymic medullary epithelial cells, suggesting a correlation between antigen level and negative selection of high-avidity autoreactive T cells, consistent with a prominent role for central tolerance in protection from insulin autoimmunity (Pugliese et al., 1997; Vafiadis et al., 1997).

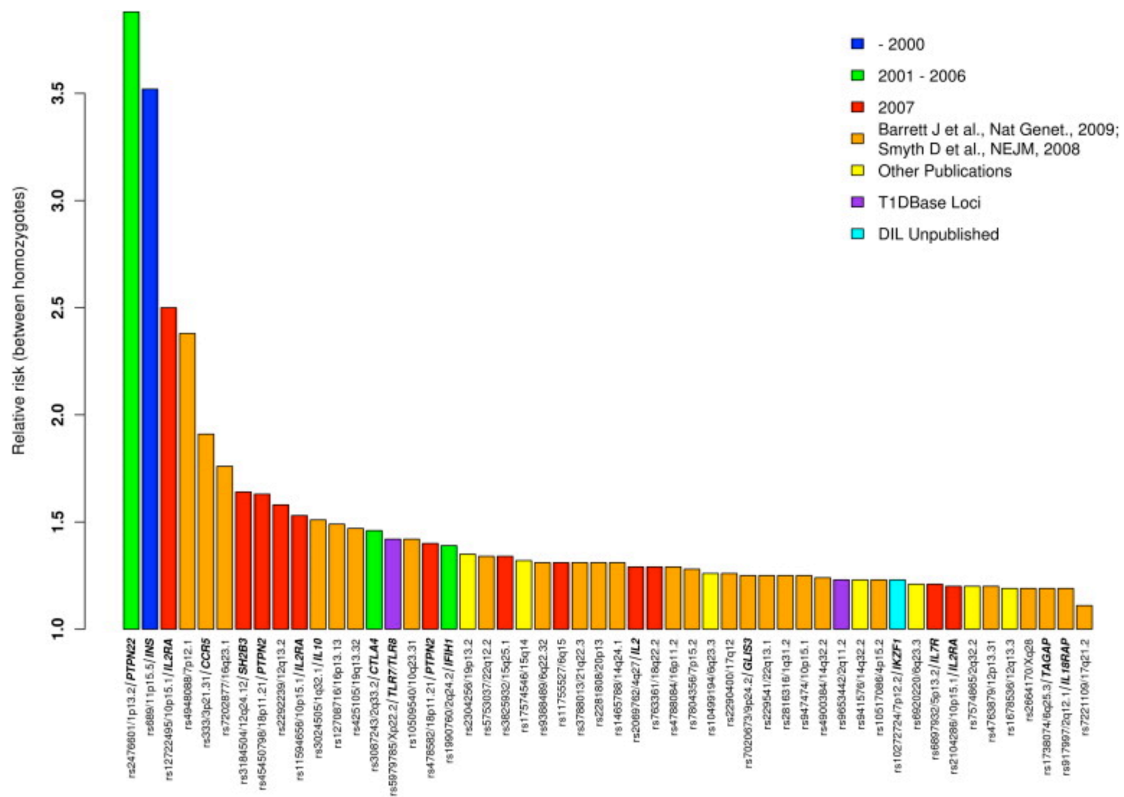


Figure 1.3: Currently mapped Type 1 non-HLA diabetes loci.

Candidate causal genes where known current Type 1 diabetes loci exist, their chromosome location, the most disease-associated SNP and, for 15 of them, the most likely causal gene in the locus, as supported by current functional evidence, e.g., IL2RA (Dendrou et al., 2009)

1.2.2 Environmental factors

Studies in both humans and animal models of Type 1 diabetes have revealed a multitude of environmental factors that may either promote or protect against diabetes. Putative environmental factors implicated in the pathogenesis of Type 1 diabetes include viral infections, dietary factors, toxins, standard of hygiene, vaccinations, psychosocial factors, geographical location and seasonal influences (Knip, 2003). According to a European study, sporadic cases comprise 83-98% of all children with newly diagnosed diabetes; therefore the precise role of specific environmental factors is yet to be elucidated (Yoon, 1991).

Due to the extensive yet silent preclinical period of Type 1 diabetes (Figure 1.4), environmental studies can only show that specific factors are correlative rather than

being causative to the development of diabetes. For instance, Type 1 diabetes has been observed in 10-20% of people who were infected with congenital rubella syndrome with a latent period of 5-25 years (Akerblom et al., 1998). Currently the main candidate for a viral trigger of human diabetes is the group of enteroviruses and more specifically poliovirus (Tauriainen et al., 2010). Enterovirus infections are frequent among children and adolescents causing aseptic meningitis, rash, paralysis and respiratory infections. The virus can spread to various organs including the pancreas (Gianani & Eisenbarth, 2005). Some observations suggest that the presence of autoantibodies against Cocksackie B virus increases the risk of the manifestation of overt diabetes in genetically susceptible individuals (Helfand et al., 1995). Enterovirus can cause beta cell damage by two main mechanisms. They may infect beta cells and destroy them directly (Ahmad & Abraham, 1982) or they may induce an autoimmune response against beta cells, thus mimicking the situation observed in the pancreas of patients affected by Type 1 diabetes (Roivainen et al., 2000). A possible preventative vaccine could be used against the diabetogenic enterovirus strains.

Mumps, measles, cytomegalovirus and retroviruses have also been identified as viruses involved in the onset of Type 1 diabetes. In epidemiological studies, peaks in the incidence of childhood Type 1 diabetes have been observed between 2 and 4 years after mumps epidemics (Hyoty et al., 1993). However, evidence is strongest for the role of enteroviruses as a trigger (Akerblom et al., 2002).

Studies are underway to determine whether dietary factors contribute to the development of Type 1 diabetes. Initial epidemiologic reports suggest that cow's milk in the diet of young infants may contribute to the disease. About a two-fold risk of Type 1 diabetes has been associated with breast-feeding duration shorter than three to four months or the early exposure to cow's milk formula before the age of two to three months (Gerstein, 1994). Increased T cell immunity to beta casein has been reported in patients with newly diagnosed Type 1 diabetes (Cavallo et al., 1996) but also in their unaffected relatives (Ellis et al., 1998). This indicates that a genetic failure in the regulation of the immune response to this dietary protein may be related to Type 1 diabetes. The exposure to cow's milk protein as such does not cause diabetes, but the pathogenic mechanisms may be related to the regulation of oral

tolerance. Also environmental factors that either modifies the normal gut flora or causes changes in the gut cytokine environment may interact with the disease process (Vaarala, 1999). Other studies implicate that early digestion of cereals may increase development of anti-islet autoantibodies (Ziegler et al., 2003). However, double-blinded, randomised dietary intervention trials are required before dietary recommendations are changed in infants.

Experiments in non-obese diabetic mice (NOD) have shown that supplementation with the active form of vitamin D or its analogue prevents insulitis or the development of autoimmune diabetes (Mathieu et al., 1994). Cod liver oil administration during pregnancy was associated with a decreased risk of Type 1 diabetes in the offspring (Stene et al., 2000). Although great advances have been made in this area more studies are required in more than one population to confirm these dietary associations.

In humans, the incidence of anti-islet autoimmunity includes the presence of autoantibodies to insulin, GAD-65 (glutamic acid decarboxylase), or IA-2 (islet antigen) (Bingly et al., 1994). The activation of these autoantibodies can be used to assess the development of Type 1 diabetes. Positively testing for two or more of these autoantibodies correlates with higher risk of diabetes development (Maclaren et al., 1999). Historically, the prediction of Type 1 diabetes relied on the detection of islet cell antibodies (ICAs) in the serum of first-degree relatives of patients with autoimmune diabetes (Verge et al., 1998).

1.3 Pathogenesis of Type 1 diabetes

Type 1 diabetes is considered as an autoimmune disease in which T lymphocytes, macrophages and dendritic cells infiltrate the pancreatic islets and destroy the insulin-producing beta cell population (Csorba et al., 2010). NOD mice have been shown to be a good model for observing the phases of the pathogenesis of Type 1 diabetes. Autoimmune destruction of beta cells in the NOD mouse model can be divided into three distinct phases (Atkinson & Eisenbarth, 2001). In phase one, antigen presenting cells (APCs; classically activated macrophages and dendritic cells [DCs]) accumulate within the islets, prior to observable inflammation. In phase two, autoreactive T cells are activated and expanded, initially in the draining pancreatic lymph nodes (PLN), and then subsequently in the pancreas itself. At this time, lymphocytic infiltrates,

containing T and B cells as well as macrophages, DCs and NK cells, are evident. Finally, in phase three, $CD8^+$ T cells and inflammatory scavenger macrophages invade the islets and utilize immune-mediated effector mechanisms to cause the final wave of beta cell destruction that precipitates clinical disease (Atkinson & Eisenbarth, 2001; Bluestone et al., 2010). During activation of autoimmunity there is normal insulin release and it is only during the later widespread silent preclinical phase that immune cell infiltration into the islets (insulitis) destroy beta cell populations and essential insulin release is lost (Figure 1.4) (Atkinson & Eisenbarth, 2001).

Type 1 diabetes spontaneously appears, mostly in female NOD mice, by 12-16 weeks of age and is preceded by quite a long phase of asymptomatic 'prediabetes' characterized by quite a long phase of asymptomatic insulitis (Figure 1.4) starting as early as 3 weeks of age. Initially, the insulitis is not aggressive and the T cell infiltrate is composed of mononuclear cells, including $CD4^+$ and $CD8^+$ lymphocytes, confined at the periphery of the islets (peripheral insulitis). By the time the mice are 13 weeks of age, T cells invade the islets and initiate the destruction of insulin-producing beta cells (invasive insulitis), leading to the advent of hyperglycaemia when approximately 90% of the beta cell mass is destroyed by the time symptoms become apparent. Aside from showing Type 1 diabetes, NOD mice exhibit a number of other autoimmune polyendocrine manifestations, notably, sialitis and thyroiditis (You et al., 2008).

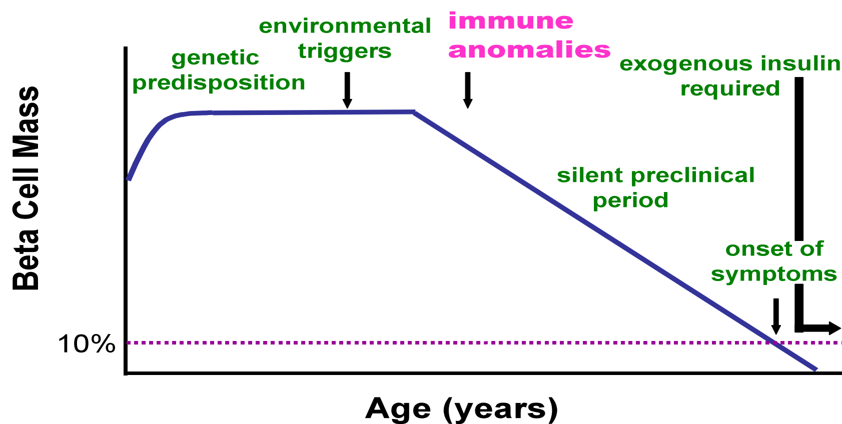


Figure 1.4: Beta cell death- the underlying cause of Type 1 diabetes.

Genetic and environmental factors work in concert to initiate autoimmune destruction of beta cells. During silent preclinical period, which can range from months to years, beta cells are continually being destroyed. The onset of symptoms occurs when there are only residual amounts of beta cells present (about 10%). (Modified from Eisenbarth, 1986).

Cytokines have become the major agents in studies on the mechanisms of beta cell death (Mandrup-Poulsen, 1995; Rabinovitch, 1998; Kim & Lee, 2009). Their use was initiated after the observation that the addition of interleukin-1 (IL-1β) to isolated rat islets leads to loss of beta cell function and damage to beta cells (Mandrup-Poulsen, 1995). The cytokines released by the infiltrating mononuclear cells like activated macrophages and T cells (Rabinovitch, 1998) play an important role in autoimmune destruction of the beta cells, which are known to be more toxic to the beta cells than the other endocrine cell types in the islets of Langerhans (Nielsen et al., 1999). In investigating the pathogenesis of Type 1 diabetes studies are largely based on animal models of human Type 1 diabetes, namely non-obese diabetic (NOD) mice and bio-breeding (BB) rats (Kikutani & Makino, 1992; Mordes et al., 2007; Bluestone et al., 2010). NOD mice have been shown to have a deficit in the function of T regulatory cells, particularly as they age (Gregori et al., 2003). Gregg et al (2004) have also shown that there is some decline in T regulatory function in NOD mice associated with a loss of membrane-bound TGF-β from the surface of $CD4^{+}CD25^{+}$ T cells. In

1995, Sakaguchi and colleagues showed that inoculation of T cell-deficient (nude) BALB/c mice, also used as a model in immune related diseases, with $CD4^+$ T cells that had been depleted of $CD25^+$ cells caused a variety of autoimmune diseases in the recipients, including thyroiditis, gastritis, insulitis, polyarthritis and glomerulonephritis (Sakaguchi et al., 1995). Further to this, inoculation of mice with $CD4^+CD25^+$ cells following three-day thymectomy prevents the development of autoimmune disease that is normally induced following the procedure (Asano et al., 1996).

Insulin and other beta cell antigens have been proposed as putative auto-antigens triggering this autoimmune attack. Autoimmune $CD8^+$ T-cells responding to a low-affinity insulin B-chain peptide have been identified in the onset of Type 1 diabetes (Wong et al., 2009). Additionally, the N-terminal insulin A-chain region has also emerged as a hot spot for $CD4^+$ T cell clones (Mannering et al., 2009) with a human $CD4^+$ T cell epitope that spans the C-peptide-insulin A-chain junction of proinsulin, identified as being more frequent in children with recent onset (Kent et al., 2005; Mannering et al., 2005). By screening a pancreatic islet cDNA library, Wong et al. (1999) identified the auto-antigen recognized by highly pathogenic $CD8^+$ T cells in the NOD mouse. The peptide recognized by the cells was in the same region of the insulin B chain as the epitope recognized by previously isolated pathogenic $CD4^+$ T cells. Further evidence suggesting insulin as an auto-antigen in Type 1 diabetes, is a study involving NOD mice lacking native insulin genes and carrying a mutated proinsulin transgene. These mice did not develop insulin autoantibodies, insulitis, or diabetes (Nakayama et al., 2005). Other putative auto-antigens in Type 1 diabetes include glutamic acid decarboxylase, and islet antigen 2, which have been found to correlate with insulitis in NOD mice (Tisch et al., 1993; Kaufman et al., 1993).

1.3.1 Autoimmune-mediated beta cell signalling

The beta cell population is maintained by a balance between beta cell death (by apoptosis or necrosis) and beta cell generation (by replication and neogenesis). Apoptosis represents programmed cell death, involving a cascade of cysteine protease (caspase) activation and eventually the activation of a caspase-activated DNAse (CAD) (Kay et al., 2000). Necrosis, in contrast, is associated with the injury and disruption of the plasma membrane. As opposed to the cell lysis characteristic, seen in

necrosis, membrane integrity is maintained throughout the apoptotic process. Importantly, during cell death by apoptosis, molecules that are exposed on the cell surface will stimulate their rapid removal by macrophages in an immunologically benign manner (O'Brien et al., 2002). As apoptotic cells undergo phagocytosis before membrane rupture occurs, the intracellular content is contained within the dying cell, thereby avoiding the initiation of an immune response (Cohen, 1994). The signals inducing apoptosis are complex and varied and identical signals can induce differentiation and proliferation, depending on conditions and cell type (Hale et al., 1996). In Type 1 diabetes the rate of beta cell death is increased due to the destruction of beta cells by auto-reactive immune cells (Mandrup-Poulsen, 2001).

The destruction of beta cells during the pathogenesis of Type 1 diabetes is multi factorial. Destruction of beta cells mainly involves complex networks of cytokines, autoreactive cytotoxic ($CD8^+$) T cells and MHC class I induction on beta cells mediated by infectious agents (von Herrath et al., 1994; Oldstone et al., 1991; Ohashi et al., 1991). Specifically, cytotoxic $CD8^+$ T cells and macrophage products such as NO and cytokines play a major role in beta cell destruction (Walter & Santamaria, 2005). Recruited macrophages are stimulated by IFN-γ to produce IL-1β and TNF-α, which in synergy with IFN-γ lead to beta cell toxicity via beta cell specific induction of inducible nitric oxide synthase (iNOS) and apoptosis activating pathways. In addition to this IL-1 leads to beta cell Fas expression, rendering the beta cells susceptible to lysis by Th1 and cytotoxic T-cells expressing FasL (Nerup et al., 1994).

The auto-reactive T cell responses are directed against islet beta cells in a cascade of immune/inflammatory processes within the islets also known as insulitis, which culminate in the destruction of beta cells largely by cytokine secretions (Thomas & Kay, 2000). Th1 cells secrete IL-2, IFN-γ, and TNF-β, whereas Th2 (T helper 2 subset of T cells; antigen activated) cells secrete IL-4 and IL-10 to stimulate antibody production (Romagnani, 1992). Type 1 diabetes has been described as a Th1 autoimmune disease as the cytokine profile of immune cells is skewed this direction (Figure 1.1).

The apoptotic cascade leading to deletion of beta cells may be provoked by several stimuli including intracellular events such as metabolic imbalance, cell cycle

perturbation, or DNA damage, and extracellular factors such as activation of TNF, TNF-related apoptosis-inducing ligand (TRAIL) and Fas death receptors, withdrawal of growth factors, free radical damage (nitric oxide (NO) and reactive oxygen species (ROS)), hormone effects, and inflammatory mediators such as cytokines (that destroy beta cells both directly or indirectly) (Mandrup-Poulsen, 2001).

Pro-inflammatory cytokines may be directly cytotoxic to beta cells or may act indirectly to up regulate Fas receptor and sensitise for FasL-Fas-induced apoptosis (Augstein, 2004). Results from a study by Augstein et al., 2004 underline the contribution of the caspase-3 pathway to Fas-induced beta cell death. They found that FasL-mediated activation of caspase-3 was additive to IL-1β and IFN-γ induced caspase-3 activation in the *in vitro* beta cell model of mouse insulinoma NIT-1 cells, which was also observed in NOD mouse islets. Consistent with these results, a similar 2-fold increase in caspase-3 activation has been reported in response to exposure of rat islets to IL-1β, IFN-γ and TNF-α.

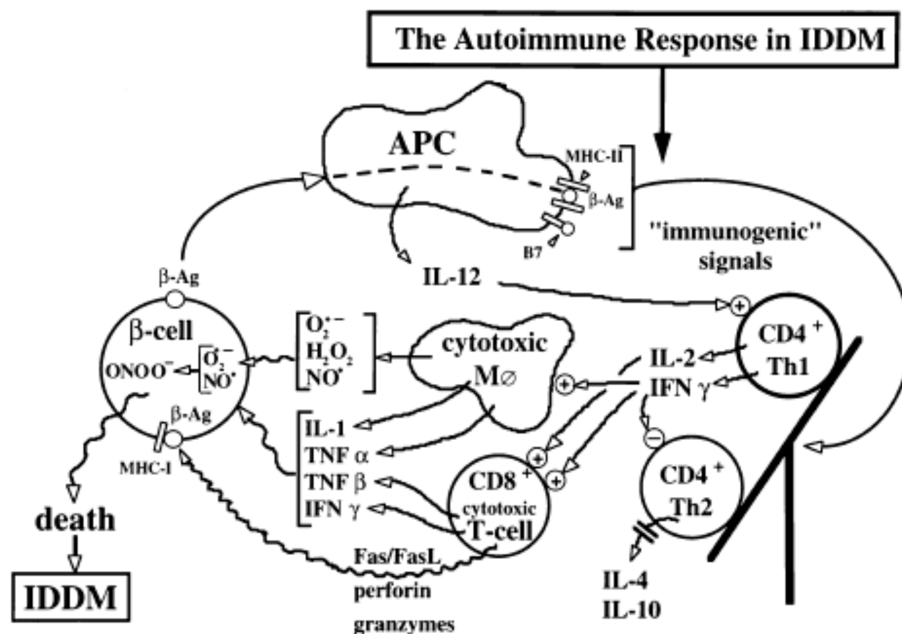


Figure 1.5: During the pathogenesis of Type 1 diabetes auto-antigens to beta cell antigens are generated.

After beta cell components are processed by antigen presenting cells (APC) such as dendritic cells, auto-antigens are presented in the pancreatic lymph nodes to naïve auto-reactive T cells resulting in their activation. These T cells home to the pancreas and begin destroying beta cells. The beta cell directed immune response involves: i) direct killing of beta cells by cytotoxic T cells against beta cells and ii) indirect mechanisms such as cytokine secretion by macrophages and cytotoxic T cells. (Adapted from Rabinowitch & Suarez-Pinzon, 1998).

Defining the signalling components of apoptosis present in beta cells is important because it could provide insights into potential pathogenic mechanisms and might lead to the development of pharmacological interventions for the treatment of Type 1 diabetes.

1.3.1.1 Cytokines

Cytokines are soluble proteins produced and released by individual cells (mostly cells of the immune system) for the purpose of transmitting distinct messages of activation, inhibition, chemoattraction and apoptosis. A message is received through a specific

interaction of the cytokine with its corresponding receptor located on the plasma membrane of the target cell. Individually, IL-1, TNF-α, TNF-β, and INF-γ have been shown to inhibit insulin synthesis and secretion by beta cells (Table 1.2). Cells may subsequently recover after the cytokine is removed (cytostatic effect). However, when added in combination, these cytokines destroy the beta cells (cytocidal effect) (Rabinovitch & Suarez-Pinzon, 1998).

Table 1.2: Effects of the major cytokines involved in the pro-inflammatory response to beta cells (adapted from Thomas & Kay, 2000)

Cytokine	**Source**	**Effect on Beta Cell**
IL-1β	Activated macrophages	Inhibits glucose-stimulated insulin secretion; induces DNA damage; induces cell death by iNOS up-regulation and NO production
IFN-γ	T cells and natural killer cells	Regulation of MHC molecule expression; macrophage activation; apoptosis; Up-regulation of adhesion molecules (promote homing of leucocytes to islets); directly toxic to beta cells
TNF-α	Activated macrophages and T cells	Adhesion; induction of class I and II MHC expression on islet cells; activation of antigen presenting cells and T cells; directly induce beta cell apoptosis.

IL-12 is a potent stimulant of Th1 cells and notably induces the cytokine IFN-γ in a pro-inflammatory response (Scott, 1993). As shown in Figure 1.1, IL-10 exerts anti-inflammatory effects by inhibiting the production of IL-12 and preventing cell death. In contrast, pro-inflammatory macrophage cytokines (IL-1, IL-6, IL-8, TNF-α)

mediate an inhibitory effect on islet function and induce islet destruction involving expression of iNOS and increased production of NO as production of IL-1 increases (Rabinovitch & Suarez-Pinzon, 1998). While the cytokine milieu involved in the development of Type 1 diabetes is complex, IL-1, TNF-α, TNF-β, and INF-γ are thought to play the key roles in beta cell destruction.

1.3.1.1.1 Interleukin-one beta

IL-1β has been found to induce suppression of various islet functions such as glucose metabolism, proinsulin biosynthesis and insulin secretion (Mandrup-Poulsen et al., 1986). Corbett et al., 1991 demonstrated that IL-1β induces the formation of the cytokine inducible isoform of nitric oxide synthase (iNOs) by pancreatic islets, and confirmed that NO mediates IL-1β-induced inhibition of glucose-stimulated insulin secretion by functioning as an effector molecule. This study suggests a possible signalling role for NO by the constitutive pathway during glucose-stimulated insulin secretion, and an effector role of NO by the cytokine inducible pathway that mediates the toxic effects of IL-β on islet function. The mechanism by which IL-1β inhibits glucose-stimulated insulin secretion by islets is believed to be the result of impairment in mitochondrial function. Sandler et al. (1989) have shown that pre-treatment of islets with IL-1β results in an inhibition of glucose oxidation to CO_2, which was demonstrated to reflect impairment in mitochondrial function. Furthermore, IL-1β-induced iron-dinitrosyl complex formation by islets suggests that at least one effect of NO is the destruction of iron-sulfur containing enzymes (Corbett et al., 1991). These results suggest that the cellular mechanism by which IL-1β inhibits insulin secretion may result from the destruction of iron containing enzymes resulting in impairment in mitochondrial function.

Since functional IL-1 receptors (IL-1R) are present on pancreatic beta cells (Labow et al., 1997; Glaccum et al., 1997), IL-1R-deficient mice have been studied to examine whether IL-1 action plays a role in diabetes development in the NOD mouse and other models of diabetes. Results from this study showed that IL-1R deficiency slows progression to diabetes but on its own does not prevent diabetes (Thomas et al., 2004). It has been suggested that binding to type I IL-1 receptor is a prerequisite for the IL-1β action on pancreatic cells, with a possible contribution of the type II

receptor (Eizirik et al., 1991). The average number of receptors per cell (RIN-m5F) was calculated to be 7300 on adherent cell layer (Vassiliadis & Soteriadou, 1997).

In a study by Ezirik et al., (1993) the effects of IL-1β on the function of human pancreatic islets and rat islets were compared. They reported that IL-1β, in the concentrations that lead to functional inhibition in rat islets, had stimulatory effects on human pancreatic islets. Their data showed that in human islets IL-1β does not lead to any significant increase in islet nitrite production, suggesting that the different responses of human and rat islets to rIL-1β are explained by the lack of IL-1β-induced activation of iNOS in human islets. In the absence of NO production, the initial stimulatory effects of the cytokine will predominate and will not be followed by any major inhibition of beta cell function.

Although genetic or pharmacological abrogation of IL-1 action has been shown to reduce disease incidence in animal models of Type 1 diabetes mellitus (T1DM), clinical trials have been started to study the feasibility, safety and efficacy of IL-1 therapy in patients with Type 1 diabetes (Mandrup-Poulsen et al., 2010). The AIDA study is currently being undertaken where IL-1R antagonist Anakinra has been administered into 80 patients with T1DM, which has recently been shown to improve beta-cell function in patients with type 2 diabetes (Pickersgill & Mandrup-Poulsen, 2009).

1.3.1.1.2 Tumor necrosis factor alpha and TRAIL

The TNF superfamily of receptors and ligands play an important role in immune surveillance and tolerance. TRAIL, a 40kDa type II transmembrane protein, signals apoptosis through a cytoplasmic death domain via a caspase-dependent pathway (Aggarwal et al., 2003). TRAIL shows structural and functional similarities to FasL, including the use of FADD as an adaptor molecule. The unique feature of TRAIL with respect to CD95L and TNF-α is considered to be its ability to induce apoptosis of most tumor cells without displaying toxic effects on most normal cells and tissues *in vitro* (Wiley et al., 1995). Unlike FasL or TNF-α, which have one or two receptors, TRAIL interacts with five different receptors, demonstrating the complexity of this ligand (Pan et al., 1997).

A study by Ou et al., (2002) showed that TRAIL mRNA is expressed in normal human primary islet cells and in human pancreatic beta cell lines CM and HP62 (none of these beta cells retained insulin function in general culture conditions). These results also showed that the TRAIL death pathway is functional in human beta cells and is likely to be mediated through TRAIL-R1 and –R2 (a decoy receptor).

While TNF-α is one of the important death effector molecules, most primary or immortalised cells are not susceptible to apoptosis by TNF-α alone (Beg et al., 1996). Studies have implicated NF-κB as an important player in the protection of target cells against TNF-α-induced apoptosis (Manna et al., 1999). Targeted disruption of TNF receptor 1 has been shown to abrogate the development of spontaneous diabetes in NOD mice. However, it has also been shown that TNF receptor 1 knockout does not completely inhibit diabetes after adoptive transfer of wild-type lymphocytes, although it does delay diabetes onset (Lee et al., 2004). These results suggest a role for TNF-α in pancreatic beta cell apoptosis of NOD mice; however, it also implies the existence of other effectors in beta cell death. In contrast to the results from these studies, indicating a role for TNF-α in the development of autoimmune diabetes, other studies have shown the opposite effect of TNF-α in NOD mice (Jacob et al., 1990; Yang et al., 1994), reflecting the complexity of the pathogenesis and diverse roles of cytokines involved in the progression to Type 1 diabetes.

1.3.1.1.3 Interferon gamma

Interferon gamma alone or when combined in high concentrations with other cytokines reduces the phosphorylation of extracellular signal-regulated kinase (ERK) substrates. IFN-γ signalling involves association of homodimeric IFN-γ with two Type 1 integral membrane proteins IFNGR1 (IFN-γ receptor subunit 1) each connected to a Janus kinase 1 (JAK1). This leads to recruitment of two IFNGR2 (IFN-γ receptor subunit 2) each associated with a JAK2 and resulting in activation of the JAKs by autophosphorylation and transphosphorylation. The signal transducer and activator of transcription 1 (STAT1) is then recruited to two binding sites in the receptor complex. The receptor-associated STATs are subsequently phosphorylated

by the receptor-bound JAKs leading to dissociation and dimerisation of the STATS (Stark et al., 1998; Ihle, 1995; Hoey & Schindler, 1998).

Suppressor of cytokine signalling-1 (SOCS1) is an intracellular molecule that blocks signalling by cytokines that use the JAK/STAT pathway, including type I and II IFNs, as well as other signalling pathways (Yoshimura et al., 2007). *In vitro*, islets that over-express SOCS1 (RIP-SOCS1) are resistant to both the toxicity and effects of cytokines on gene expression, particularly IFNs. *In vivo*, transgenic NOD mice with beta cells expressing SOCS1 also have reduced incidence of diabetes (Chong et al., 2004; Flodstrom-Tullberg et al., 2003).

IFN-γ inhibits the production of Th2 cytokines, IL-4 and IL-10, which in turn inhibit Th1 cytokine production. Moreover, correlation studies have detected IFN-γ in lymphocytes infiltrating islets in humans with recent-onset Type 1 diabetes (Foulis et al., 1991) pointing to the predominant involvement of IFN-γ in the death of beta cells.

1.3.1.2 Cytokine synergism

A combination of IFN-γ and TNF-α, but not either cytokine alone, has been shown to induce classical apoptosis in murine insulinoma cells MIN6N8 and pancreatic islet cells. This was indicated by Hoechst staining, DNA ploidy assay, electron microscopy, and DNA fragmentation pattern in a study by Lee et al. (2004). Transfection of phophorylation-defective STAT1 abrogated islet cell apoptosis by IFN-γ and TNF-α combination, suggesting that STAT1 phosphorylation plays a critical role in mediating IFN-γ-induced TNF-α susceptibility. STAT1 and IRF-1 (interferon regulatory factor 1) were expressed in pancreatic islets of diabetic NOD mice and colocalised with apoptotic cells (Suk et al., 2001). While these *in vitro* studies suggest a possibility that IFN-γ and TNF-α synergism is responsible for beta cell apoptosis in Type 1 diabetes and illustrate a novel signal transduction in IFN-γ and TNF-α synergism, further *in vivo* studies are warranted to prove this model.

Besides IFN-γ and TNF-α synergism, another candidate for cytokine synergism in the development to Type 1 diabetes is IL-1β and IFN-γ combination as shown in studies on rat islet cells (Oyadomari et al., 2001). NO produced by IL-1β and IFN-γ

synergism induced the depletion of endoplasmic reticulum (ER) Ca^{2+} and ER stress. This is also in agreement with other studies that report the important role of NO as the death effector in pancreatic beta cell death (Mandrup-Poulsen et al., 1996). However, these data are inconsistent with *in vivo* studies that suggest a minor effect of IL-1 blockade on the development of diabetes in NOD mice (Schott et al., 2004). *In vivo*, multiple stimuli including membranous/soluble forms of cytokines and other death effector molecules coexist at different concentrations depending on the stage of development of Type 1 diabetes.

1.3.1.3 Fas receptors

Cytokines have the potential to kill beta cells, or up-regulate Fas on beta cells, and increase their susceptibility to FasL. The main inflammatory cells involved in the onset of Type 1 diabetes in both humans and NOD mice include macrophages, dendritic cells (DC), B cells, $CD4^{+}$ and $CD8^{+}$ T cells (Matthis et al., 2001). There are two main pathways by which auto-reactive T cells induce beta cell death in Type 1 diabetes (Figure 1.6). In the perforin pathway the auto-reactive T cells release granules and apoptosis is caused by the direct effects of perforin and granzymes on the beta cell (Kay et al., 2000). It has been shown, however, that NOD mice lacking perforin have islet infiltrates, but a greatly reduced incidence of diabetes (Kagi et al., 1997). An indirect pro-apoptotic effect of cytokines is the up-regulation and surface expression of the Fas receptor in beta cells. This increases the susceptibility of beta cells to apoptosis mediated by the FasL expressed on islet-infiltrating beta cell specific cytotoxic ($CD8^{+}$) T-cells and neighbouring beta cells (Eizirik & Mandrup-Poulsen, 2001). Kang et al. (1997) showed that FasL expression in the islets of Langerhans targeted beta cells for rapid destruction by cytotoxic T cells, while the lack of Fas expression in pre-diabetic non-obese diabetic (NOD) mice prevented spontaneous diabetes in this animal model showing the importance of Fas in triggering Type 1 diabetes. *In vivo* studies involving NODlpr/lpr mice (NOD mice with a mutation in the Fas gene) have been used to investigate the role of Fas and to further demonstrate the importance of Fas mediated beta cell death in the development of diabetes.

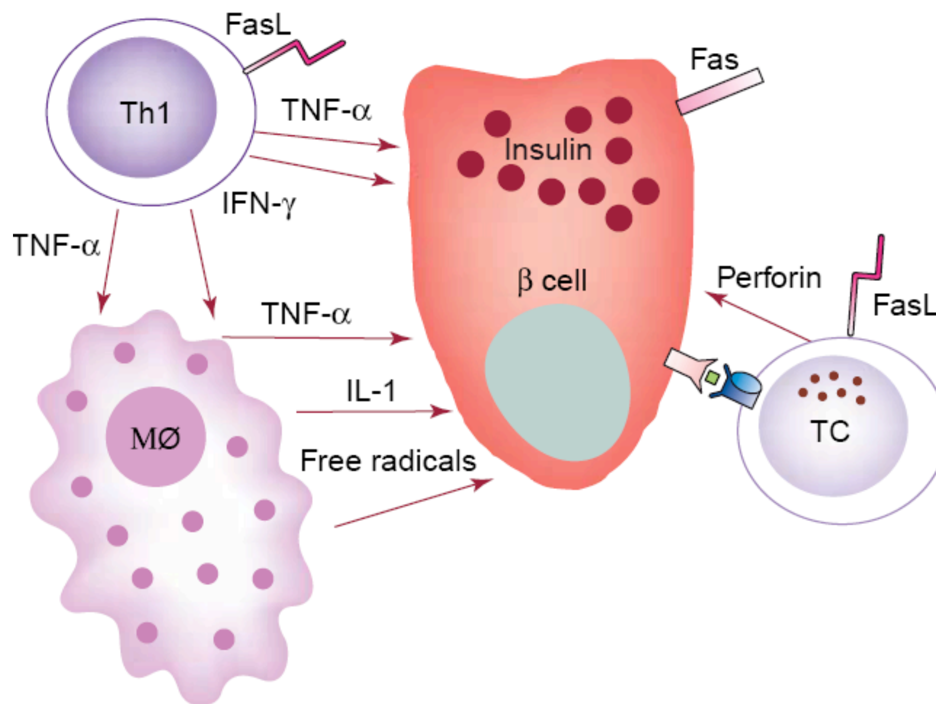


Figure 1.6: The activated T cells migrate to the pancreas and eventually cause beta cell death.

T cells (TC) might recognise the islet directly through antigen presented on MHC Class I. In this case, killing might result by production of perforin or by Fas-FasL interaction. CD4$^+$ T cells recognise islet antigen on macrophages or DCs in close proximity, and TH1 effector cells could then kill in one of several ways: Fas-FasL interaction, or by production of cytokines such as IFN-γ and TNF-α. These cytokines might act directly on the beta cell by causing it to up-regulate 'death mediators' such as iNOS, or indirectly through macrophages. Macrophages activated by T-cell-derived TNF-α and IFN-γ might kill beta cells through production of IL-1, TNF-α and free radicals (adapted from Cooke et al., 2004).

The lpr phenotype (naturally occurring mutants that bear a deletion of the Fas gene) initiates several changes in the immune system of mice, this includes increased FasL expression on many cell types, which might cause deletion of wild type lymphocytes introduced into lpr mice (Allison & Strasser, 1998). Although Kim et al. (1999) found that Fas was not involved in the development of diabetes in NODlpr mice, the same group found that the neutralization of Fas-FasL interactions did not prevent cyclophosphamide-accelerated diabetes. A study by Itoh et al. (1997) also showed that the NODlpr/lpr mice did not develop diabetes or insulitis. These results suggest

that Fas-mediated cytotoxicity is critical to initiate beta cell autoimmunity in NOD mice. The Fas-FasL system might be required in an initial step of autoimmune beta cell destruction leading to Type 1 diabetes. It is possible that the absence of diabetes in NODlpr mice is the result of their abnormal immune system, rather than a specific defect in the interaction between T cells and beta cells. Clarification of the role of Fas in NOD mice with mutations of Fas and its signalling pathways in beta cells has been investigated in several studies (Allison et al., 2005; Savinov et al., 2003; Apostolou et al., 2003). In agreement are the studies by both Itoh et al. (1997) and Chervonsky et al. (1997), which found that the induction of Fas expression on beta cells and then their subsequent destruction constitutes the main pathogenic mechanism of autoimmune Type 1 diabetes.

Cytokine-induced expression of the Fas receptor sensitises beta cells to FasL-induced apoptosis. Studies with well-established models of apoptosis demonstrate that triggering of Fas by FasL rapidly induces cell death by recruitment of the adaptor protein Fas-associated death domain (dominant-negative mutant) (dnFADD), formation of the death-inducing signalling complex (DISC) and activation of the caspase cascade (Curtin & Cotter, 2003). Activated caspase 8 can then process downstream effector caspases, such as caspase 3, leading to cell death. Studies in NOD mice have shown that the down regulation of dnFADD has a protective effect, implicating Fas signalling as a contributing, albeit minor, mechanism of beta cell death. In addition, *in vitro* data suggest that dnFADD may interact with the IL-1R pathway as well (Allison et al., 2005). However studies on pancreatic beta cells have also shown that FasL is not the main death effector in the apoptosis of these cells. FasL was found to have no effect on the islet cell viability *in vitro* (Lee, 2001) consistent with *in vivo* findings by the same group of researchers (Lee et al., 1999).

Angstetra et al. (2009), examined the role of cytokines in beta cell death by testing if the over-expression of SOCS1 in beta cells (model described in Section 1.3.1.1.3), or deficiency of cytokine receptors IFN-γR or IL-1R, would decrease $CD4^{+}$-dependent diabetes. Perforin deficient RIP-SOCS1 mice have also been used to examine if the residual diabetes was due to the effects of cytokines on beta cells (McKenzie et al., 2009). These findings showed that Fas-deficiency, SOCS-1 overexpression and

blockade of IFN-γ and TNF-α all protect beta cells from residual cytotoxicity of perforin-deficient CTL by blocking Fas upregulation. This study further indicated that wild-type CTL destroy antigen-expressing islets via a perforin-dependent mechanism. However, in the absence of perforin, the Fas/FasL pathway provides an alternative mechanism dependent on islet cell Fas upregulation by cytokines IFN-γ and TNF-α. These studies strongly support the important role of cytokines in beta cell death.

1.3.1.4 Caspases

During beta cell death by the two main pathways involving Fas and perforin and by means of an enzyme cascade intracellular proteins are cleaved (Thornberry & Lazebnik, 1998; Shi, 2002). Caspases, a set of proteases, cleave proteins after an aspartate residue, specificity residing in a four-residue sequence upstream of the cleavage site which, for the main effector caspase, caspase-3, is DEVD [z-D(Ome)E(Ome)VD(Ome)] (Alnemri et al., 1996). Caspases are activated after the engagement of death receptors (such as Fas) on the cell surface (Strasser et al., 2000) or by stress (NO production) or intracellular damage (Cory & Adams, 2002). Targets of these proteins are not completely known, but their activation leads to the cleavage of DNA into nucleoside-sized fragments, a late event characterising apoptotic cell death. A member of the caspase family, caspase-3, has been identified as a key mediator of apoptosis of mammalian cells (Thomas & Kay, 2000).

Caspase-3 plays a pivotal role in the apoptotic death of beta cells during the development of Type 1 diabetes. Studies using Caspase-3 knockout mice were shown to be protected from developing diabetes in a multiple-low-dose streptozotocin autoimmune diabetes model; furthermore, there was an absence of lymphocyte infiltration within the vicinity of the pancreatic islets in these models (Liadis et al., 2005). Techniques used to assess apoptosis in isolated islets of Langerhans include TUNEL (based on DNA fragmentation detecting late events of programmed cell death), annexin V staining and caspase-3 measurement (for early detection of apoptosis) (Cattan et al., 2001).

1.3.1.5 Free radical damage

Insulin-secreting beta cells are subject to injury from oxidative stress. Formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide, hydroxyl radicals, and the concomitant generation of NO have been implicated in beta cell dysfunction or cell death caused by autoimmune attack and actions of cytokines in Type 1 diabetes. Compared with many other cell types, the beta cell may be uniquely at high risk of oxidative damage and has an increased sensitivity for apoptosis (Mandrup-Poulsen, 2003; Robertson et al., 2003). Reports have identified oxygen or nitrogen free radicals as mediators of cytokine-induced islet beta cell destruction (Mandrup-Poulsen et al., 1990). Several pathways have been suggested to be involved in NO mediated beta cell death. Among these, NO inactivates Krebs cycle aconitase by nitrosylation of Fe-S groups thereby preventing glucose oxidation and ATP generation, leading to cell death by necrosis (Welsh et al., 1991).

Among the free radicals, NO has attracted special attention (Eizirik et al., 1997). Expression of the inducible form of nitric oxide synthase (iNOS) has been observed in different autoimmune diseases (Cook et al., 1996), and iNOS is expressed by islet cells and/or invading macrophages in the insulitis present in diabetes-prone BB rats and NOD mice (Kleeman et al., 1993; Rothe et al., 1994). Moreover, iNOS mRNA expression is detected in both rodent and human pancreatic islets exposed *in vitro* to cytokines (Eizirik et al., 1997). Transgenic expression of iNOS in beta cells induces beta cell destruction and diabetes (Takamura et al., 1998), whereas lack of iNOS expression (iNOS knockout mice) prevents diabetes induced by streptozotocin (Flodstrom et al., 1999).

Although studies by Stassi et al., (1997) and Giannoukakis et al., (1999) show that IL-1β alone induces NO production, Fas upregulation, and functional suppression of human islets, several other studies on human islet preparations reported induction of iNOS expression, NO production, and beta cell functional suppression in human islets when these cells were exposed to combinations of two or three cytokines (IL-1β and IFN-γ or IL-1β, IFN-γ and TNF-α) but not when they were cultured in the presence of IL-1β alone (Liu et al., 2000).

Perforin, Fas, Caspase-3, TNF-α, IL-1, IFN-γ, NO, and their combinations have been implicated in the destruction of pancreatic beta cells. Thus these factors are likely to play a pivotal role in the elimination of beta cells both during the development of Type 1 diabetes and after the transplantation of beta cells into diabetic patients.

1.3.2 Intrinsic damage and gene networks

Although functional impairments of beta cells are induced shortly after exposure to cytokines, apoptosis is detected only after several days of their co-culture with IL-1β, TNF-α and IFN-γ (Liu et al., 2000). These findings indicate that an active process is taking place at the beta cell level, protective and destructive mechanisms where the destructive effects eventually prevail leading to beta cell death and Type 1 diabetes. Several studies suggest that the transcription factor NF-κB is an important cellular signal in initiating the cascade of events culminating in beta cell death (Cnop et al., 2005; Baker et al., 2001). NF-κB has been shown to regulate the expression of numerous genes that play important roles in cellular stress responses, cell growth, survival, and apoptosis (Karin et al., 2000; Karin et al., 2002). As such, the specificity and temporal control of gene expression by NF-κB are of crucial physiological significance (Figure 1.7).

The process of T-cell recruitment to the pancreatic islets involves local production of chemokines by specialized APCs, such as the resident dendritic cells and macrophages, endothelial cells and the beta cells themselves (Rotondi et al., 2007). The cytokines TNF-α and IFN-γ induce the release of chemokines by resident macrophages and epithelial cells. Transgenic expression of the chemokine macrophage chemoattractant protein-1 (MCP-1) under the control of the rat insulin promoter has been shown to induce monocyte infiltration in pancreatic islets (Grewal et al., 1997). The expression of MCP-1 was found to be up-regulated in IL-1β induced human and rat islets, and islets isolated from pre-diabetic NOD mice (Chen et al., 1999; Chen et al., 2001). Piemonti et al., (2002) also showed that human islet cell death was caused by MCP-1. *In vivo* footprinting in murine fibroblasts showed that TNF-α induces site occupancy in both proximal and distal regions of the MCP-1 promoter (Ping et al, 1996). However, IFN-γ does not stimulate MCP-1 expression in rat beta cells or human pancreatic islets (Chen at al., 2001), suggesting that regulation

of this gene in beta cells differs from other cell types and is mostly dependent on IL-1β. Kutlu et al., (2003) using gel-shift assays determined that NF-κB is involved in the induced activity of the MCP-1 promoter *in vitro*. *In vivo* binding of NF-κB to the enhancer region in the rat MCP-1 promoter was confirmed by chromatin immunoprecipitation (ChIP) assay, followed by real-time RT-PCR quantification. To show that NF-κB translocation to the nucleus is necessary for MCP-1 mRNA induction in response to cytokines, an adenovirus expressing a non-degradable form of IκB or the NF-κB inhibitor pyrrolidine dithiocarbamate was used to inhibit the translocation.

Gurzov et al. (2008) demonstrated that JunB counteracts cytokine-induced ER stress and apoptosis and might therefore be part of defense mechanisms triggered by beta cells during exposure to pro-inflammatory cytokines. Modulating JunB could represent a novel strategy to ameliorate beta cell resistance to immune damage.

In islets, cytokine ligand-ligand receptor interaction up-regulates production of the free radical nitric oxide (NO) by increased expression of an inducible form of nitric oxide synthase (Corbett et al., 1991; Southern et al., 1990). This activation of the iNOS gene occurs in part by iNOS promoter binding by the transcription factor NF-κB (Flodstrom et al., 1996; Kwon et al., 1995).

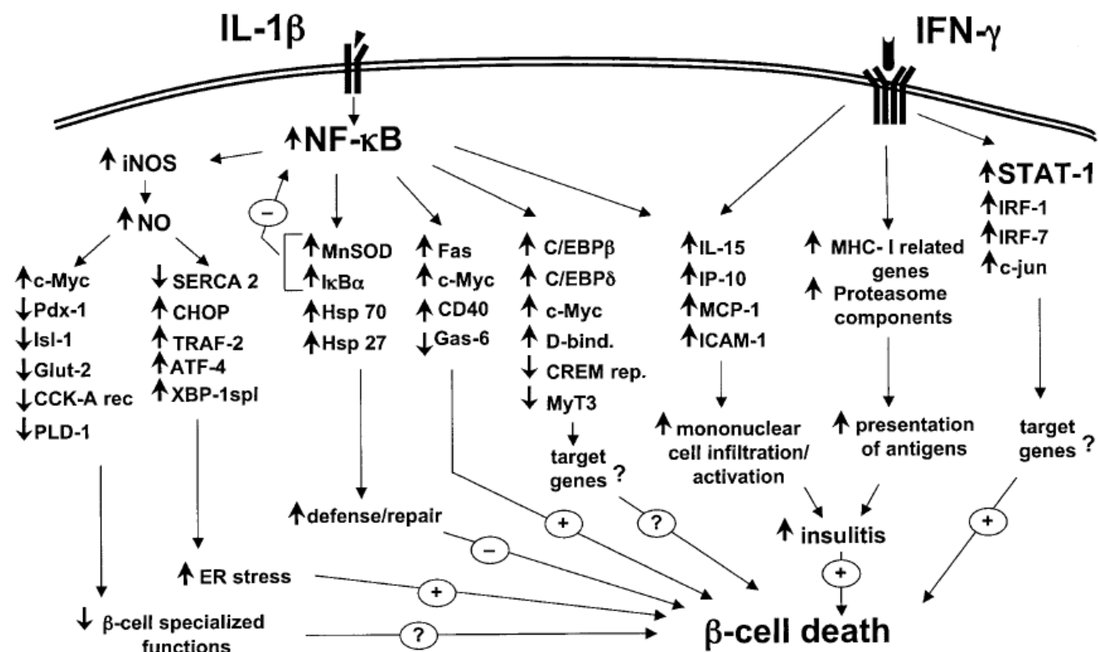


Figure 1.7: The transcription factor and gene networks putatively involved in the cytokine-promoted beta cell decision to undergo apoptosis.

The transcription factors NF-κB and STAT-1 are the main regulators of the pathways triggered by IL-1β and IFN-γ, respectively (Adapted from Cnop et al., 2005).

1.4 NF-κB

NF-κB is a ubiquitous and well-characterised protein. It maintains a pivotal role in controlling cell signalling in the body under certain physiological and pathological conditions in response to a large number of different stimuli. There are five related mammalian gene products that participate in NF-κB functions: RelA/p65, cRel, RelB, p50 and p52. NF-κB/Rel proteins exist as homodimers or heterodimers, but the predominant species in most cell types is the p65:50 heterodimer. NF-κB/Rel proteins share a highly conserved 300-aa-long N-terminal Rel homology domain, which is responsible for DNA binding, dimerization, and association with the inhibitor of NF-κB (IκB) proteins (Ghosh et al., 1998).

In resting cells, most of the NF-κB/Rel dimers are bound to three major IκB isoforms IκB-α, IκB-β and IκB-ε in the cytoplasm, preventing their nuclear translocation and DNA association. Signals such as cytokine-receptor binding at the cell surface

activate specific IκB kinase complexes (Karin, et al., 1999), which phosphorylates the N-terminal domain of the IκBs, tagging them for polyubiquitination. Upon ubiquitination, the IκB proteins are rapidly degraded by the proteosome, thereby freeing NF-κB, which then enters the nucleus, binds to DNA and activates transcription (Melloul, 2008).

The regulation of NF-κB activity has been implicated in beta cell impairment. NF-κB activity is increased in rat insulinoma cells (RIN) in culture in response to IL-1β, whereas cytokines were also shown to promote NF-κB activity in human and rodent islets *in vitro* (Darville & Eizirik, 1998). The different isoforms of IκB play distinct and complementary roles in the regulation of specific NF-κB dimers, resulting in both quantitative and qualitative changes in gene expression (Hoffmann et al., 2003). These characteristics vary in a particular cell type exposed to different stimuli. In a study by Baker et al. (2001), they attempted to preserve beta cell viability by disrupting one potential intracellular signal for apoptosis, namely the cellular production of NO and thus cytokine-induced cell death by inhibiting NF-κB activation. This was done by transfecting MIN-6 beta cell line with a gene encoding for mutant IκB lacking the normal phosphorylation sites and the MIN-6 cells became protected from the cytotoxicity of the cytokine cocktail.

Several research groups aimed to determine the final effector cytokine or cytokine combinations involved in beta cell death. The studies examining the cytokines and combinations of cytokines outlined in Table 1.4 were specifically performed to determine the effects of the cytokines on NF-κB activation.

Table 1.3: Studies determining the effects of cytokines and cytokine cocktails on beta cell NF-κB activation.

Author	Cytokine/s studied	Effect on NF-κB activity	Model used
Giannoukakis, et al., 2000	IL-1β	Protection of islet cells from effects of cytokine by IκB repressor	Human islet cells
Dupraz, et al., 2000	IL-1β	IL-1β induces NF-κB-dependent NO production and apoptosis in beta cells and MyD88 inhibits this. ALSO Cytokine–induced beta cell secretory dysfunctions are due to the action of IFN-γ not IL-1β.	Immortalised transgenic mouse βTc-Tet cells (CDM3D cells)
Heimberg, et al., 2001	Il-1β + IFN-γ	NF-κB activation has pro-apoptotic function determined in beta cells expressing IκB Cytokines were changed every three days for 6 days.	Purified rat beta cells
Ortis, et al., 2006	IL-1β + TNF-α	Cytokine-induced NF-κB activation in insulin-producing cells is more rapid, marked and sustained than in fibroblasts. Correlates with more pronounced activation of downstream genes and pro-apoptotic outcome.	INS-1E cells
Cardozo, et al., 2001	IL-1β/ IL-1β+IFN-γ	Microarray analysis showed cytokine induced NF-κB mostly pro-apoptotic in beta cells. Also blocked using IκB super repressor.	Purified rat beta cells
Chang, et al., 2003	TNF-α/ IFN-γ+TNF-α	Protective role of NF-κB activation against cytokine-mediated beta cell death. Used IκB superrepressor. Cytokine synergism did not affect TNF-α induced NF-κB activation although affected MTT viability	MIN-6N8 Mouse islet cells

		significantly. Contrary to reports implicating NF-κB as a mediator of islet cell death.	
Baker, et al., 2001	IL-1β+TNF-α+IFN-γ	Inhibition of NF-κB and NO production by dominant negative inhibitor of NF-κB is cytoprotective.	MIN-6
Thomas, et al., 2006	TNF-α	NIT-1 defective in activation of NF-κB as result of deficient RelA activity. RelA overexpression protects NIT-1 from apoptosis. Blocking NF-κB would make beta cells sensitive to cytokines. Contrary to the studies performed by Eizirik and his group.	NIT-1 cells Primary mouse islet cells

In the case of pancreatic islet cells, most reports show a pro-apoptotic role of NF-κB (Sekine et al., 2000; Chen et al., 2000). In a study by Lee et al., 2004, the role of NF-κB was studied in both MIN-6N8 cells and pancreatic islets using the IFN-γ/TNF-α synergism model. TNF-α was shown to induce IκB degradation and p65 translocation from cytoplasm to nuclei in MIN6N8 insulinoma cells. Using an Electrophoretic mobility shift assay (EMSA) it was also shown that NF-κB DNA-binding nuclear complex in insulinoma cells was activated by TNF-α, comprising both p65 and p50 subunits.

Specific inhibition of NF-κB activation by transfection or adenoviral transduction of IκB 'superrepressor' also sensitised insulinoma cells to TNF-α-induced apoptosis. These results suggest the protective role of NF-κB activation against pancreatic beta cell death by TNF-α. However, since these results regarding NF-κB were obtained mostly from MIN6N8 insulinoma cells using IFN-γ/TNF-α synergism model, further studies using other cell types should be carried out to elucidate the bona fide role NF-κB in islet cell death of Type 1 diabetes.

To further investigate the role of beta cell NF-κB in autoimmune diabetes *in vivo*, Kim et al. (2007) produced transgenic mice expressing a nondegradable form of

IkappaBalpha in pancreatic beta cells (RIP-mIκBα mice). Beta cells in these mice were more susceptible to killing by TNF-α plus IFN-γ but more resistant to IL-1β plus IFN-γ than normal beta cells. Similar results were obtained with beta cells lacking IκB kinase beta, a protein kinase required for NF-κB activation. Inhibition of beta cell NF-κB accelerated the development of autoimmune diabetes in nonobese diabetic mice but had no effect on glucose tolerance or serum insulin in C57BL/6 mice, precluding a nonphysiological effect of transgene expression (Kim et al., 2007).

1.5 Possible cures for Type 1 diabetes

The current treatment of Type 1 diabetes relies on insulin supplementation coupled with close monitoring of blood glucose concentrations. Relatively few patients, however, are capable of complying with the strict regimens required for achieving normal blood glucose levels with insulin injection therapy (Atkinson & Eisenbarth, 2001). Strategies focused on altering the underlying disease process in Type 1 diabetes address intervention shortly after diagnosis in order to arrest the destruction of the beta cell and to preserve residual beta cell function as long as possible (Gianani & Eisenbarth, 2005). The other strategy in protecting beta cell mass is to identify individuals at risk for the development of diabetes and to halt the immune process before it leads to overt clinical disease. Other approaches focus on developing sources of beta cell mass. This includes islet transplantation as a means of beta cell replacement and regeneration of the beta cell by embryonic stem cell therapy (Aguoay-Mazzucato & Bonner-Weir, 2010).

1.5.1 Beta cell protection

If therapeutic prevention is to be used in curing Type1 diabetes then it is an essential requirement for the clinical trials of diabetes prevention to be able to identify individuals at risk for progression to diabetes. Depending on whether the therapy involves primary or secondary intervention, diabetics would need to be identified either preceding or proceeding the onset of ‘immune anomalies’ (Gianani & Eisenbarth, 2005). Since it is possible to identify stages of diabetes by means of islet autoantibody screenings and insulin release measures, it may be possible to design immunotherapy trials targeting subjects in different phases of Type 1 diabetes development.

Primary intervention is the prevention of diabetes in subjects who do not have any signs or symptoms of the disease (and are thus islet-antibody negative). However, preventative therapy would be reserved for those with increased genetic risk based on HLA genotypes (Lambert et al., 2004). A major concern even in high-genetic risk group is that the intervention may trigger anti-islet autoimmunity where none would have initially existed (Sadeharju et al., 2003). This could lead to disease exacerbation. Secondary prevention of Type 1 diabetes is aimed at ameliorating the disease after its clinical onset by preventing further beta cell destruction. The most efficient current measure of the intervention at the onset of Type 1 diabetes is the continual secretion of C-peptide (Palmer et al., 2004).

1.5.1.1 Immune suppression

Intervention during the silent preclinical period has been attempted with both nicotinamide and cyclosporine. Nicotinamide may protect beta cells against the cytolytic activity of streptozotocin *in vivo* by leading to a rapid increase in intracellular levels of NAD through inhibition of the metabolism of NAD (Elliot et al., 1993). Nicotinamide may also prevent the beta cell cytotoxic effect of macrophages by inhibiting the action of cytokine generated NO radicals (Knip, 1998). Although nicotinamide delays diabetes in the NOD mouse nicotinamide could neither prevent nor delay progression to Type 1 diabetes (Reddy et al., 1990). Results from the European Diabetes Prevention Trial showed that through a randomised, double-blind placebo control trial high dose nicotinamide did not significantly affect the risk of developing overt diabetes compared to those treated with placebo (European Nicotinamide Diabetes Intervention Trial Group, 2004). The Deutsche Nicotinamide Intervention Study similarly examined the effect of high doses of nicotinamide in children at high risk for Type 1 diabetes but did not observe a major delay in the development of the disease (Lampeter et al., 1998).

Cyclosporine is a lipophilic polypeptide that inhibits the transcription of IL-2 and several other cytokines, especially in T helper lymphocytes. It has been used effectively in the prophylaxis and treatment of transplant rejection (Faulds et al., 1993). Enthusiasm about the efficacy of cyclosporine in Type 1 diabetes intervention and prevention has been counterbalanced by concern about its severe side effects.

Cyclosporine therapy was associated with neuropathy, hirsutism, gingival hyperplasia, and nephrotoxicity (Wysocki et al., 1983). Most importantly, studies using cyclosporine did show that immunosuppression initially halted or slowed the disease process, which confirmed that Type 1 diabetes was a T-cell immune-mediated process (Mahon et al., 1993). In a study by Assan et al., 2002 it was shown that although patients with islet autoimmunity receiving cyclosporine had a delay in onset of Type 1 diabetes, the drug did not completely prevent glucose intolerance.

1.5.1.2 Immunologic tolerization

Clinical researchers have attempted to induce peripheral tolerance by presenting potential antigens via a novel route. Mucosal autoantigen administration includes oral, intranasal, and aerosol inhalation (Singh & Palmer, 2005). It is believed that large oral doses of antigen may induce passive tolerance, whereby antigen-specific lymphocytes undergo apoptosis and functional inactivation on the other hand smaller serial doses of oral antigen can lead to active tolerance (Hanninen, 2000). Through bystander suppression of effector cells of even different specificities, regulatory lymphocytes can down modulate effector cells. Potential Type 1 diabetes autoantigens include insulin, the 65kDa isoform of GAD, and protein tyrosine phosphatase-related proteins, such as IA2 (Knip, 1998).

In 1991, Zhang et al. reported that, in NOD mice, insulin administration delayed the onset, reduced the incidence of diabetes, and decreased the severity of lymphocyte infiltration of pancreatic islets. In subsequent experiments, oral insulin appeared to shift the pattern of cytokine production from a Th1 to Th2 immune response (Hancock et al., 1995). However, there was a lack of efficacy and this was partially explained by an inadequate daily dose and the possible need for an adjuvant carrier to enhance the ability of insulin to induce tolerance (Diabetes Prevention Trial, 2002). The rationale for the use of parenteral insulin to alter the course of the Type 1 diabetes process is diverse and can be used as an immune modulator. Boerschamm et al. (2010) report the success of this therapy, and show that it could potentially induce tolerance.

More recently, a clinical trail, Pre-POINT, has looked at the mucosal administration of insulin and aims to identify an optimal insulin dose and route of application (orally

or intranasally) that is well tolerated and can induce a protective immune response to insulin for additional use in a phase II/III primary prevention trial in children at risk (Achenbach et al., 2008).

1.5.1.3 Anti-CD3$^+$ and anti-CD4$^+$

Genetically designed antibodies or monoclonal antibodies that are specifically directed against factors believed to trigger the disease process are being investigated. These include anti-CD3$^+$ and anti-CD4$^+$ (Chatenoud, 2005). Use of anti-CD3 antibody can skew the cytokine balance from a Th1 to a Th2 response (Raz et al., 2005). The use of an anti-CD3$^+$ monoclonal antibody usually suppresses immune response by transient T-cell depletion and antigenic modulation of the CD3$^+$-T cell receptor complex. Anti-CD3$^+$ antibodies helped maintain residual insulin or even improve insulin production in nine out of 12 patients over the course of a year (Herold et al., 2005). Minor acute side effects linked to a still persisting cytokine release, although limited, were observed after the first infusions. Approaches taken to minimise toxicity associated with classic anti-CD3$^+$ monoclonal antibodies (i.e. the cytokine release syndrome and development of human anti-murine anti-bodies) include the development of dimeric Fc receptor (FcR) nonbinding anti-CD3$^+$ antibody hOKT3. This new antibody delivers an activation signal to T cells that is quantitatively and qualitatively different from OKT3- it induces IL-4 and IL-10-producing Th2 cells while suppressing IFN-producing Th1 cells (Herold & Taylor, 2003).

While these antibody-based approaches are being tested, it is likely that prevailing gene and cell therapies can overcome the safety and negative effects associated with the antibody approach (Atkinson & Eisenbarth, 2001). Most of the immune-suppressive strategies have the major obstacle of clinical applicability of these therapies, being the requirement for heavy immune-suppression, to preserve the beta cell mass remaining at diagnosis. So regardless, beta cell replacement will be required.

1.5.2 Beta cell replacement

Permanent correction of Type 1 diabetes by pancreas or islet transplantation is restricted by the availability of donor tissue. Xenotransplantation may offer hope as a source of large amounts of islets. However, difficulties persist in the prevention of

transplant rejection and the requirement for lifelong immunosuppressive therapy, to prevent recurring autoimmune damage to the transplanted tissue (Remuzzi et al., 1994). Bioartificial pancreases have been trialed in animals resulting in good control of glucose levels in fasted animals, in the absence of any immunosuppression, but have not displayed successful glycaemic control in response to a meal or an intravenous glucose challenge (Calafiore, 1998).

1.5.2.1 Islet transplantation

The physical replacement of the beta cell mass constitutes the rationale for which islet transplantation, as opposed to whole pancreas transplantation, was originally proposed (Lacy, 1982). It was recently demonstrated that islet cell transplants could be performed with greater chances of success.

Researchers in Edmonton were the first to develop human islet transplantation into a reliable, clinical therapy. Transplantation using the Edmonton Protocol involves selection of an appropriate type of patient, use of an effective immunosuppressive regimen, isolation of appropriate numbers of viable islets and transplanting these into sufficient numbers into the recipient (Shapiro et al., 2006). The preparation of islets for transplantation involves islet isolation by ductal perfusion with cold, purified collagenase, digestion and purification of the islets in xenoprotein-free medium, and transplantation immediately by means of percutaneous transhepatic portal embolization. A follow-up study revealed that after one year of receiving the islet transplant 80% remained exogenous-insulin independent, however, after the second year this was reduced to 70% and recently a follow up study showed that only 10% of the recipients were no longer in need of insulin therapy (Ryan et al., 2005). Pancreatic islets lose their capacity to maintain insulin function once transplanted into the recipient this is known as islet exhaustion. In a more recent follow-up study on the Edmonton protocol, there was a high prevalence of ovarian cysts in premenopausal women (70.5%) receiving Sirolimus and Tacrolimus after clinical islet transplantation (Alfadhli et al., 2009).

The constraints under which islet transplantation is clinically possible are still too numerous to allow the broad application of this procedure to permanently cure the disease (Azzi et al., 2010). The immunosuppressive drug regimen necessary to protect

islets from a recurrent autoimmune response and allorejection may, over time, irreversibly damage kidney function, while the process of islet isolation itself, even though drastically improved during the last few years, damages transplantable islets and, consequently, two or three donors are required in order to obtain the minimal cell mass sufficient for transplantation into a single recipient (Ryan et al., 2001).

Successful islet transplantation in the absence of long-term immunosuppression may be possible by the microencapsulation of islets of Langerhans in an alginate-based capsule (Schneider et al., 2001). However, some studies have shown that microencapsulation using alginate/poly-L-lysine/alginate capsules does not protect islets against the detrimental effects of IL-1β and TNF-α (King et al., 2000). This data also indicates that microencapsulated islets may be slightly functionally suppressed, with insulin release and glucose oxidation being affected but not insulin synthesis. The development of glucocorticoid-free immunospressive therapies and improved methods for islet isolation are also being investigated. Finding a method to prevent the autoimmune destruction of beta cells may be helpful in extending the survival of grafts after islet transplantation.

The immune response to beta cells has been shown to involve the regulation of multiple classes of genes. *In vivo* and *in vitro* studies suggest that these genes include anti-apoptosis genes (Rabinovitch et al., 1999; Dupraz et al., 1999), genes encoding anti-oxidant proteins (Efrat et al., 1995), and those encoding proteins interfering with cytokine receptor signal transduction pathways (Dupraz et al., 2000; Chong et al., 2004) or antigen presentation on the cell surface (Von Herrath et al., 1997). In addition, expression of inhibitory cytokines, which can be secreted from the cells and locally suppress the function of the cells of the immune system, has also been shown to be effective in beta cell protection (Gallichan et al., 1998; Moritani et al., 1998). However, modifying a beta cells' genetic profile could lead to unknown effects.

1.5.2.2 Xenotransplantation

With the limited availability of human cadaveric donor tissue, xenotransplantation may be considered as an alternative approach to human islet transplantation. Porcine islets offer the best choice for humans as donor tissue based on a number of factors that include availability, size and number of islets in the pancreas and cost (Weir et

al., 1997). Nonhuman primate recipients of porcine donor organs have exhibited xenoreactive antibody-mediated hyperacute rejection (Lin et al., 1998; Cotterell et al., 1995). A dominant xenoepitope has been defined in porcine tissues as an α-galactosyl oligosaccharide that is synthesised by a galactosyltransferase present in all pig tissues but only in the gut-inhabiting flora of humans (Sandrin et al., 1993). Complement-mediated lysis of porcine xenografts occurs within minutes by the classical pathway, culminating in the formation of the membrane attack complex. Depletion of the circulating xenoreactive antibodies has been shown to significantly prolong xenograft survival in pig-to-baboon renal transplantation (Sandrin et al., 1997).

Unlike vascularised solid organs, xenogenic islets do not undergo hyperacute rejection but do eventually succumb to delayed xenograft rejection (Roos et al., 2002). Although this type of rejection is xenoantigen induced, it does not appear to be α-galactosyl xenoepitope-dependent, despite an increase in anti-α-galactosyl antibodies in patients receiving porcine foetal islet cell clusters (Mirenda et al., 1997). There continues to be an ongoing debate as to which is the more appropriate porcine tissue to use foetal/newborn islet like cell clusters (ICC) or adult porcine islets. Adult islet isolation remains a technically difficult procedure and the ability of foetal tissue to develop, and ultimately secrete insulin in a regulated manner remains a concern (O'Connell, 2002). Foetal islet tissue secretes the hormone pancreatic icosapeptide with a structure that is species specific. This has allowed the development of an enzyme-linked immunosorbent assay (ELISA) designed to monitor the fate of foetal porcine ICC for the first few weeks after transplantation (Amaratunga et al, 2001; Tuch et al., 2001). Isolation of adult pig islets has also experienced significant improvements (Brandhorst et al., 1999). The development of enzymes for pancreatic dissociation, such as liberase, and the demonstration that transplantation of large numbers of adult pig islets can control blood glucose in diabetic baboons (albeit for short periods) (O'Neil et al., 2001; Swanson et al., 2001) was hindered by the fact that liberase was produced in medium containing bovine brain extract.

Although there are several immunosuppressive strategies that successfully suppress the alloimmune response, the T-cell mediated xenoimmune response has proven more resistant to immunosuppressive therapy (O'Connell et al., 2000). This is because of

the greater molecular incompatibility between donor and recipient, which provides greater potential for innate immune activation even in responses mediated predominantly by T cells (Yi & O'Connell, 2002). One approach to escaping immune surveillance has been to use encapsulation as a procedure to protect islets from the rejection response. Numerous membranes have been proposed and although encapsulation isolates the islets from leucocytes, immune activation still occurs because soluble antigens derived from the xenograft gain access to the immune system (Gill et al., 2001; Gray, 2001). The technological aspects of encapsulation require more investigation to solve problems of compatibility and swelling; however, despite these caveats there has been a report of encapsulated porcine islets normalising blood sugars in a diabetic baboon (Kendall et al., 2001). Although islet xenografts can survive for a long time in rodents, it is much more difficult to achieve this in primates and it has been shown that even when the islets are not in direct contact with the circulation a robust xenograft rejection response is evident (Badet et al., 2001). Although complete xenogenic tolerance may never become possible, gene therapy-mediated accommodation of xenogenic islets and immunomodulation required in preventing acute and chronic rejection needs to be further investigated.

Porcine endogenous retrovirus (PERV) and multiple copies of PERV proviruses are present in the genomic DNA of all pig cells (Herring et al., 2001). Therefore, the removal of all PERV-related genomic elements via selective breeding or knockout technologies will not be possible (Patience et al., 2001). Persisting ethical and bio-safety issues still hinder global support for xenotransplantation and the risks associated with transmission of donor-resident micro-organisms and viruses and their effect on human health are yet to be elucidated.

1.5.3 Beta cell regeneration by embryonic stem cell therapy

The demonstration of the existence of tissue-specific adult stem cells has had a great impact on stem cell biology and its application in clinical medicine. Their existence has revolutionized the implications for the treatment of many degenerative diseases characterized by either the loss or malfunction of discrete cell types. However, successful utilization of this opportunity requires sufficient manipulation of stem cell differentiation and isolation (Trucco, 2005).

The limited success in generation of abundant and functional beta cells from pancreatic sources has prompted attempts to differentiate insulin-producing cells from embryonic stem cells as well as progenitor or mature cells from non-pancreatic tissues. There are various mechanisms believed to be involved in the homeostasis and regenerative repair of the beta cell. Early studies suggest that adult pancreatic endocrine cells belong to a class of tissues that could be maintained by the self-duplication of differentiated cells (Tsubouchi et al., 1987). More recent immunohistochemical observations suggest that it is the adult pancreatic stem or progenitor cell that is the origin of the beta cell and these reside in the epithelium of pancreatic ducts, inside islets or in the bone marrow (Bonner-Weir & Sharma, 2002; Zajicek et al., 1990; Zulewski et al, 2001; Calne et al., 2010). Others have suggested that beta cells form in the adult by transdifferentiation of pancreatic acinar cells or splenocytes (Lipsett & Finegood, 2002; Kodama et al., 2003).

Methods designed to distinguish stem-cell-derived beta cells from the progeny of pre-existing beta cells have shown that pre-existing beta cells, rather than pluripotent stem cells, are the major source of new beta cells during adult life (Dor et al., 2004). These results suggest that terminally differentiated beta cells retain a significant proliferative capacity *in vivo* and cast doubt on the idea that adult stem cells have a significant role in beta cell replenishment. Immortalisation of the beta cell or differentiation of non-beta cells *in vivo* that simulate the function of a beta cell could potentially provide a solution to this problem- production of large amounts of beta cells (Aguayo-Mazzucato & Bonner-Weir, 2010).

Human and mouse embryonic stem cells are capable of spontaneous differentiation into insulin-producing cells (Soria et al., 2000; Assady et al., 2001; Calne et al., 2010). In addition, they can be induced to preferentially differentiate into insulin-containing cells by changing the composition of the culture medium (Lumelsky et al., 2001; Segev et al., 2004) and express dominant transcription factor genes such as Pax4 (Blyszczuk et al, 2003), *Pdx-1* (Miyazaki et al., 2004) and neurogenin-3 (Dominguez-Bendala et al., 2005; Treff et al., 2006) which are involved in pancreas development. However, these cells express low levels of insulin and lack key beta-cell properties such as regulated insulin secretion in response to physiological secretagogues. In addition, these results have been challenged by findings that the

insulin content may result from uptake from the culture medium, rather than synthesis (Rajagopal et al., 2003; Hansson et al., 2004; Sipione et al., 2004). Additional considerations involved in the use of embryonic stem cells are the risk of uncontrolled proliferation of residual undifferentiated cells, as well as the ethical controversy surrounding their utilisation.

Recent development in human induced pluripotent stem cells (iPS) allows researchers to obtain pluripotent stem cells without the controversial use of embryos. They also avoid the issue of graft-versus host disease and immune rejection unlike embryonic stem cells because they are derived entirely from the patient (Yu et al., 2007).

1.6 Cellular and molecular biology of the pancreatic beta cell

Islets of Langerhans in the pancreas are clusters of endocrine tissue containing four types of cells. In order of abundance, they are the beta cells, which secrete insulin; alpha cells, which secrete glucagon; delta cells, which secrete somatostatin, and gamma cells, also known as pancreatic polypeptide cells, which secrete a polypeptide of unknown function (Muller et al., 2007).

1.6.1 Structure and biosynthesis of insulin

The hormone secreted by the beta cell, insulin, is composed of two peptide chains, A and B, linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain of 30 amino acids (Blundell et al., 1972). Although the amino acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, including the positions of the three disulfide bonds, both ends of the A chain and the C-terminal residues of the B chain (Figure 1.8). These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among species, and insulin from one animal is very likely biologically active in other species. Indeed, pig insulin has been widely used to treat human patients (porcine differs from human insulin by a single amino acid).

Insulin is synthesized in significant quantities only in beta cells in the pancreas. The insulin mRNA is translated as a single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum

generates proinsulin (Docherty et. al., 1982). Proinsulin is then processed into mature insulin by two proteolytic reactions in the secretory granules. Firstly, by the prohormone convertases PC1 and PC2; and secondly, by carboxypeptidase H (Yanagita et al., 1992). Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide, thereby generating the mature form of insulin. Insulin and free C peptide are packaged in the Golgi into secretory granules, which accumulate in the cytoplasm. When the beta cell is appropriately stimulated, insulin is secreted from the cell by exocytosis and diffuses into islet capillary blood. C peptide is also secreted into blood, but has no known biological activity.

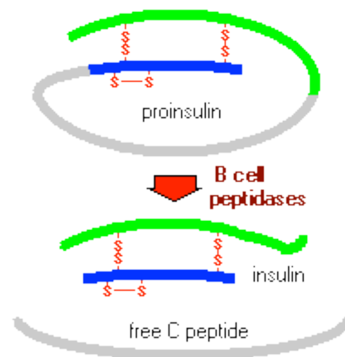


Figure 1.8: Cleavage of proinsulin to produce mature insulin.

Insulin is composed of two peptide chains, A 21 amino acids (blue strand) and B 30 amino acids (green strand), linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. C-peptide is released into the blood when mature insulin is processed from proinsulin by the action of beta cell peptidases.

1.6.2 Control of insulin secretion

Insulin controls the transport of glucose, the body's main energy source, from the bloodstream into cells. In a normal pancreas, insulin is secreted from the mature beta cells in response to elevated blood glucose levels (Orci et al., 1988). Glucose enters the cell by facilitated diffusion and via specific transport proteins such as GLUT2, which are usually Na^+-dependent (Figure 1.9). Metabolism of glucose by glycolysis, alanine via transamination to pyruvate, and leucine to acetyl-CoA will result in subsequent oxidation in the TCA cycle. This results in an increase in the ATP/ADP ratio, closure of ATP-sensitive K^+ channels, membrane depolarisation, opening of voltage activated Ca^{2+} channels, Ca^{2+} influx, a rise in $[Ca^{2+}]_i$ and activation of the exocytotic machinery. The opening of Ca^{2+} channels is intermittent, oscillating with the membrane potential therefore resulting in oscillations of $[Ca^{2+}]_i$ (Santos et al., 1991, Gilon & Henquin 1992) that in turn trigger oscillations of insulin secretion (Gilon et al., 1993). Attenuation in oscillating $[Ca^{2+}]_i$ concentration, for example by exposure to a high extracellular concentration of glucose (27mM) for extended periods (e.g. 48h), results in attenuation of normal patterns of insulin secretion (Bjorklund et al., 2000). In the case of Type 1 diabetes, an absolute insulin deficiency

prevents the entry of glucose into the cells. Consequently, glucose accumulates in the blood, leading to hyperglycaemia- the hallmark of diabetes.

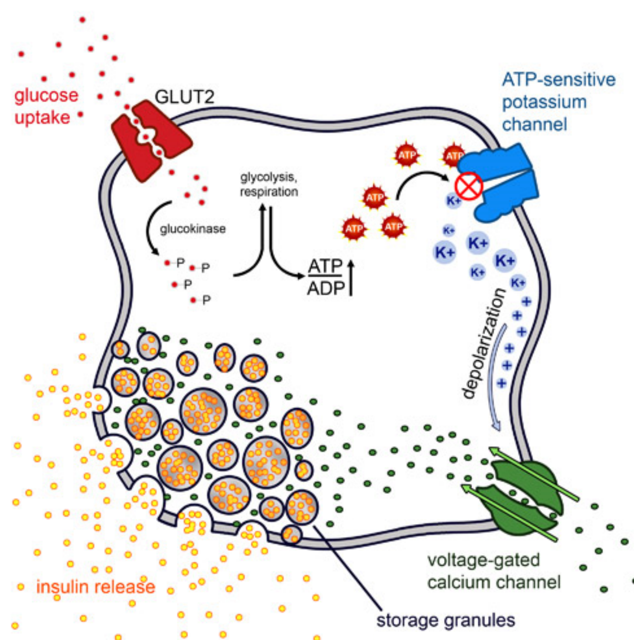


Figure 1.9: The beta cell- glucose metabolism and insulin secretion.

Glucose enters the cell via GLUT2 glucose transporter. Then by phosphorylation and the TCA cycle ATP/ADP increases thereby closing potassium channels and in turn causing depolarisation. Depolarisation of the cell causes an influx in calcium via the calcium channels and activation of the exocytotic machinery (Beta Cell Biology Consortium, 2004).

A large body of evidence suggests that GK is the flux-controlling enzyme for glycolysis in beta cells and, as such, serves as the sensing apparatus for metabolic signalling (Meglasson et al., 1986). The low-affinity glucose phosphorylating enzyme glucokinase (GK, hexokinase type IV) couples changes in the millimolar glucose concentration range to glucose metabolism in pancreatic beta cells and hepatocytes (Meglasson et al., 1986). In beta cells, glucokinase is the glucose sensor and serves as the signal-recognition enzyme for the initiation of insulin secretion (Tiedge & Lenzen, 1991). The phosphorylation of glucose within beta cells is tightly coupled to insulin secretion. The unique kinetics of GK underlie the ability of these cells to sense and respond to fluctuations in the plasma glucose concentration and because of this, GK is considered the glucose-sensor of the beta cell (Meglasson & Matschinsky, 1984).

Modelling of changes in GK activity, as well as studies of the effects of graded increases in the enzyme, have established that glucose phosphorylation is the key point of control for glycolytic flux in the beta cell (Liang et al., 1992; Wang & Iynedjian, 1997). Consequently, even small changes in GK activity can be physiologically significant, since they directly affect the threshold for glucose-stimulated insulin secretion. Rodent beta cells express two specialised proteins that have been considered as candidates for the glucose sensor: the facilitated glucose transporter isoform, GLUT2, and the low affinity glucose-phosphorylating enzyme, GK. Glucokinase is unique among mammalian hexokinases in having a K_m for glucose in the physiological (8-10mM) glucose range (Trus et al., 1981). The high capacity glucose transporter, GLUT2, is also unique in having a high K_m (~17mM) for glucose (Thorens, 1996) and is believed to allow the rapid equilibration of glucose across the plasma membrane (Sweet & Matschinsky, 1997).

1.6.3 Regulation of insulin gene transcription

Insulin, synthesised by the beta cells of the pancreatic islets, is of major physiological importance in metabolic homeostasis. Insulin is regulated at several levels, from gene transcription to insulin secretion. Deletion and mutational analyses of various insulin promoter regions, conducted in transfected cell lines and in transgenic mice, led to the identification of cis-acting regulatory sequences necessary for specific gene expression (Odagiri et al., 1996). Regulated insulin gene transcription relies on the interaction of sequence motifs in the promoter with a number of ubiquitous and islet specific transcription factors. These interactions determine the positive and negative regulation of insulin gene expression and its inducibility by physiological stimuli. The binding of proteins to regulatory elements has led to the identification and cloning of putative insulin transcription factors. The characterisations of such factors (and their genes) contribute to the understanding of insulin gene expression, endocrine pancreas development and beta cell differentiation (Melloul et al., 2004).

1.6.4 Transcription factors

Transcription factors play an important role during pancreatic development ensuring normal differentiation of the islet endocrine cells. In mature beta cells, expression of specific transcription factors is essential in maintaining normal beta cell morphogenesis and function.

Pancreatic duodenal homebox-1 (*Pdx-1*) functions both in the early commitment of the primitive gut to pancreatic fate and in the maturation of beta cells (Edlund, 2001). It is expressed in adult beta cells, regulates the transcription of the insulin gene, the beta cell glucose transporter, GLUT2, glucokinase, and Nkx 6.1. *Pdx-1* is considered to be the main regulator involved in pancreatic development and beta cell differentiation. It has a dual role as an inducer of the endocrine lineage from ductal epithelial cells and in the maturation of beta cells (Hill & Duvillie, 2000).

Pdx-1 may be directly activated by the transcription factors Neuro-D, Hnf-1α, and Hnf-3β (Figure 1.10). Pancreatic agenesis occurs in *Pdx-1* null mutant mice (Jonsson et al., 1994). A characteristic feature of *Pdx-1* is the highly conserved homeodomain, a 61 amino acid domain. *Pdx-1* links glucose metabolism to the regulation of insulin gene transcription. Glucose has been shown to activate *Pdx-1* through an insulin-dependent cell-signalling pathway involving phosphatidylinositol 3-kinase, and the stimulation of this pathway leads to phophorylation and activation of a cytoplasmic form of *Pdx-1* that translocates to the nucleus. *Pdx-1* regulates the expression of the GLUT2 transporters in a doseage-dependent manner. Thus, lowered *Pdx-1* expression or activity resulting in impaired expression of both GLUT2 and insulin could cause hyperglycaemia. *Pdx-1* expression is required in mature beta cells to maintain hormone production, GLUT2 expression, and euglycaemia (Ahlgren et al., 1998). In rodents, reduced expression of *Pdx-1* results in impaired insulin secretion and glucose intolerance (Brissova et al., 2002; Kushner et al., 2002). In humans, individuals carrying the mutations in the *Pdx-1* gene are predisposed to the development of diabetes (Leibowitz et al., 2001)

Neurogenic differentiation 1 or BETA2 is a beta cell type restricted basic helix-loop-helix transcription factor expressed in all endocrine cells (Naya et al., 1995). It is expressed in the mouse pancreatic bud with Nkx 6.1 which is specific to fetal cell differentiation and function. In investigating the importance of *NeuroD1* in pancreatic development, studies in homozygous null mutants of the *NeuroD1* gene showed a marked reduction in β, α, and δ cells in newborn mice (Naya et al., 1997). Targeted

disruption of the Nkx 6.1 gene, in mice, results in inhibition of beta cell formation (Sander, et al., 1998).

The paired domain homeobox gene, Pax 4, is expressed in the developing pancreas. Pax 4 is essential for proper beta cell development and especially for their maturation and maintenance. Pax 4 has been identified only as a regulator of endocrine development (Dohrmann et al., 2000). Pax 6, which is highly related to Pax 4, is also required for normal endocrine pancreatic development, and double null mutants for both Pax 4 and Pax 6 fail to produce any mature pancreatic endocrine cells (Fujitani et al., 1999), suggesting that these two factors together are required for endocrine cell differentiation.

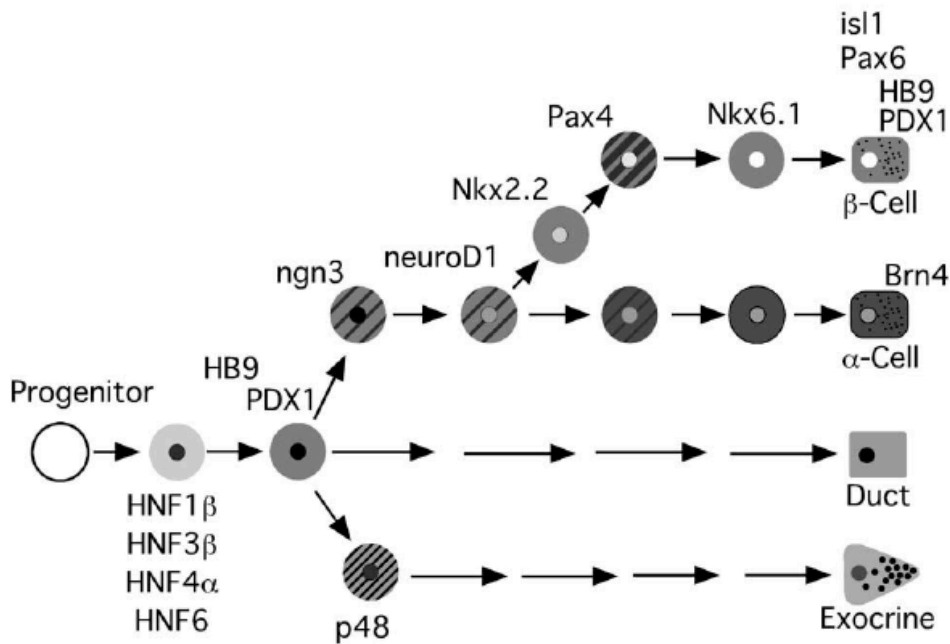


Figure 1.10: Model of the role of islet transcription factors in endocrine differentiation in the developing pancreas.

The proposed position of each transcription factor is based on its timing of expression and the functional role of each transcription factor in the processes (adapted from Hill & Duvillie, 2000).

1.7 Gene therapy

Gene therapy is broadly defined as an intervention in which the genome of a patient is purposely altered to ameliorate a pathophysiological condition (Selden et al., 1987). Gene therapy can be subdivided into germ-line and somatic-cell therapy. Germ-line therapy carries several ethical and practical obstacles, including the possibility that the genes may cause adverse changes in future generations (Rabino, 2003). In contrast to germ-line therapy, somatic-cell therapy only affects the person given the therapy. The new gene would not enter the gene pool. For this and other related reasons, somatic-cell gene therapy is currently being investigated as a putative cure of certain disorders in humans (Bailey et al., 1999).

Desirable features of gene-delivery systems include the full characterisation of the transfected cells before implantation into the patient. Additionally efficient delivery and regulated expression of the gene and the ability of transfecting cells that are derived from different tissues would be pivotal in providing a successful therapeutic regime (Bailey et al., 1999). The ability to reimplant transfected cells into different anatomical locations needs to be further investigated before use in the clinic (Lipes et al., 1997). Gene-delivery systems also need to be able to detect, monitor and modulate the function of the transfected cells after implantation and destroy or inactivate implanted cells if necessary. These delivery systems need to have significant therapeutic benefit without subjecting the patient or population to undue risk before they can be widely implemented (Rabino, 2003).

Early studies in gene therapy focused on the replacement of a defective gene with a normal copy of that gene in patients with monogenetic orders. Examples include replacement of the transmembrane regulator gene in patients with cystic fibrosis (Knowles et al., 1995), the replacement of the dystrophin gene in the muscle of patients with Duchenne muscular dystrophy (Morgan, 1994), and the replacement of the low-density lipoprotein (LDL) receptor gene in the liver of patients with familial hypercholesterolaemia (Grossman et al., 1994). Successful trials in humans include patients with adenosine deaminase deficiency (Blaese et al., 1995). Unlike these disorders, Type 1 diabetes is not caused by a single genetic defect.

1.7.1 Gene therapy and diabetes mellitus

Paradoxically, the cell type of choice for the gene therapy of Type 1 diabetes is not the beta cell for several reasons. Firstly, beta cells are not readily accessible even in normal people and are greatly reduced in number or totally absent from the pancreata of persons with Type 1 diabetes. Secondly, beta cells carry a multitude of autoantigens that are subject to autoimmune attack in Type 1 diabetes (Mandrup-Poulsen, 2003).

1.7.1.1 Immunoregulatory gene therapy

With the aim of preserving islets from immunologic damage, approaches using gene therapy include tolerance induction, modification of antigen presentation and regulating cytokines (Figure 1.11). Clinical trials in patients with autoimmune disorders such as advanced rheumatoid arthritis using cDNA encoding IL-1 receptor antagonist transferred into their knuckle joints show promising results (Evans et al., 2000).

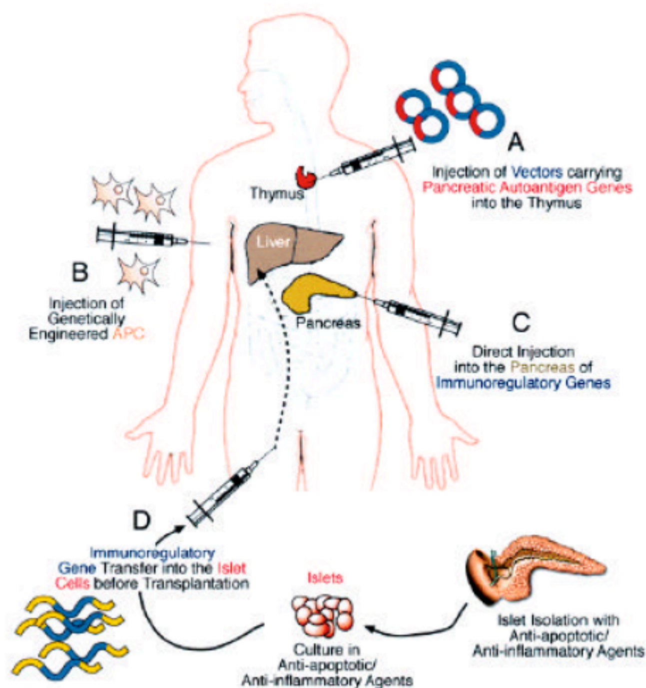


Figure 1.11: Blocking the immunological attack against the islets in genetically at-risk individuals or against transplanted allogenic islets with gene therapy.

***A)** tolerance induction to autoantigens **B)** APC genetically engineered to produce autoantigens and molecules that interfere with their ability to co-stimulate autoreactive T-cells. These APC can be injected to render autoreactive T-cells anergic or apoptotic **C)** protection of islets in situ by locally produced immunosuppressive molecules expressed in and around the islets after administration of vectors D) islets can be infected with vectors carrying genes that facilitate islet transplantation, targeting different pathways in auto- and alloimmunity. In addition, donor pancreas can be perfused with anti-inflammatory chemical agents (iNOs inhibitors), as well as infected with vectors encoding anti-apoptotic and antioxidant molecules to promote survival and functionality of islets during and after isolation. Potential genes include* IκB*, MnSOD, thioredoxin, and catalase. (Adapted from Giannoukakis et al., 1999)*

The collagenase-based isolation procedure of primary islet cells can affect their viability and engraftment potential. Involvement of the immune system of the recipient may reduce the chances of islet graft survival. The addition of anti-inflammatory compounds, such as cyclocoxygenase-2 (COX-2), XIAP and iNOS inhibitors (Koliopanos et al., 2001; Barshes et al., 2005), to the digestive enzyme

cocktail that is used to perfuse the pancreas during the isolation process could be valuable. Alternatively or in addition such compounds could be added to the cell culture immediately after islet isolation (Figure 1.10). Although significant gene-based protection of the transplanted islets has been achieved in various animal models, as outlined earlier, there is still concern that gene products able to prevent autoimmunity recurrence may not be sufficient to completely protect allogenic transplanted islets. Additional pathways other than those already considered may also be involved, and could be candidates for genetic manipulation (Table 1.4) (Giannoukakis et al., 1999).

Table 1.4: Candidate molecules to facilitate islet transplantation and survival (Giannoukakis et al., 1999)

Interactions/reactions/processes	Potential modulator
Oxidant stress	MnSOD, catalase, glutathione peroxidase, thioredoxin
Tryptophan catabolism	IDO
Apoptosis induction	Bcl-2, Bcl-XL, crmA, dominant-negative FADD, TRADD, I-FLICE, A20
IL-1/IL-1R	IRAP, soluble IL-1-IR-Ig, IL-1-IIR-Ig
TNF-α/TNFR-1	Soluble TNFR-1-Ig
Chemokine effects	Soluble receptors, chemokine binding proteins
Neutrophil activation	Soluble CD18, ICAM-1, LFA-3
Adhesion molecules	Soluble chimeric immunoglobulin proteins: LFA-3Ig, CD48, ICAM-1, ICAM-2, VCAM, selectins
NK cells	MHC class I peptides, soluble HLA-G
Fas/FasL	Soluble FasIg, dominant-negative Fas
Complement-mediated destruction	Soluble CR1-receptor, CD59, DAF, MCP-1

1.7.1.2 Strategies for beta cell replacement

Substantial progress has been made with the use of immortalised beta cell lines, which emulate the secretory activity of normal islet beta cells, however, such cells require immunoisolation or some degree of immunosuppression unless their presentation of antigens can be appropriately modified or the defence mechanisms of the beta cell improved. An alternative strategy would be to genetically engineer non-beta cells to secrete insulin as a function of plasma glucose concentration.

1.7.1.2.1 Immortalizing the beta cell

Given the shortage of donor pancreata, alternative sources of islets have been sought. Differentiation and expansion of embryonic and pancreatic stem cells and expansion of differentiated beta cells *in vitro* is limited and the expansion of primary beta cells by growth factors is also limited by the senescence of the cells (Halvorsen et al., 2000). Immortalisation of purified beta cells by the addition of genes such as TERT (Telomerase Reverse Transcriptase) could allow the cells to grow indefinitely; however, immortalised beta cell lines established in this way lose their differentiated function (de la Tour et al., 2001). Establishing a human pancreatic beta cell line that is functionally equivalent to primary beta cells yielding large amounts of cells may be a viable alternative for transplantation.

A study of glucose-responsive beta cell lines, such as MIN-6 (Miyazaki et al., 1990), linked normal glucose responsiveness to adequate GLUT-2 expression. RIN cells, also a rodent beta cell line, displayed glucose-stimulated insulin release, however, passaging these cells resulted in the loss of insulin secretion (Ferber, 1994). It is likely however, as these cell lines are of beta cell origin, they would be susceptible to recurrent autoimmune attack. Transplantation of immortalised beta cells in humans would also require micro-encapsulation; to avoid immune surveillance and the potential risk of tumour development in the event that the micro-encapsulation device leaked would always be present (Mitanchez et al., 1997). Additionally, non-protected cells would be expected to be quickly rejected by the immune system since they are a murine beta cell line. Most importantly, murine beta cell lines also secrete rodent insulin, which would not be optimal or even safe for regulating blood glucose in humans.

Transforming human primary beta cells with immortalizing genes for expansion and then excising the immortalizing genes has been shown to remove tumorigenic potential without loss of beta cell function. Reversibly immortalised pancreatic beta cell clone NAKT-15 showed insulin secretion in response to glucose stimulation. These cells also expressed proteins characteristic of beta cells, Isl-1, Pax-6, Nkx 6.1, *Pdx-1* and prohormone convertases PC 1/3 and PC2 (Narushima et al., 2005). However, the insulin content and amount of secreted insulin was lower than in normal human islets.

Some of these problems associated with replacing beta cell function may be overcome by engineering, from the patient's own cells, an 'artificial beta cell': a non-islet cell capable of synthesizing, storing and secreting mature insulin in response to metabolic signals (Table 1.5). This approach may reverse diabetes without the need for immunosuppression (Simpson et al., 1997).

1.7.1.2.2 Engineering non-beta Cells

The process of conversion of one differentiated cell type to another is called transdifferentiation. Transcription factors are also known to promote differentiation of non-endocrine cells to an insulin-producing cell type. The search for cell types that are capable of being engineered to process and regulate insulin secretion began with cell types that are specifically adapted to protein secretion (Table 1.5). As islet-specific antigens are not expressed in non-beta cells, genetically engineered non-beta cells may evade immune surveillance and subsequent autoimmune destruction in Type 1 diabetic patients. Additionally, the utilisation of insulin-secreting non-beta cells could solve the problem of an insufficient supply of islets for transplantation. Cultured cells engineered *ex vivo* to secrete insulin for subsequent implantation into Type 1 diabetic patients may be a viable approach to curing Type 1 diabetes.

Table 1.5: Shortcomings of various non-beta cells used to express and release insulin

Cell Line/type	**Author**	**Type of Insulin Trasfected/ Secreted**	**Shortcomings**
NIH 3T3/ Fibroblast	Kasten-Jolly et al., 1997	Low levels of proinsulin secretion (unprocessed insulin).	Reversal of diabetes, depending on site of implantation. Unable to store insulin.
AtT20/ Neuroendocrine	Hughes et al., 1992	PC1 and PC2 processed mature insulin.	No GLUT-2 transporter. Other hormones and peptides secreted (ACTH), however, insulin could be secreted and stored.
Rat Myoblast	Simonson et al., 1996	Transfection of human proinsulin cDNA	Not glucose responsive.
HepG2ins/g/ Hepatocyte	Simpson et al., 1995; Simpson et al., 1997	Transfection of insulin and GLUT2 cDNA	Constitutive release not endogenous, unable to reverse diabetes due to loss of GLUT2 expression. However, cells could synthesise, store and release insulin in a regulated manner.
Rat Adipocyte	Ito et al., 2005	Secretion of mature insulin after insertion of furin cleavable proinsulin cDNA	Requires a very large number of cells for slight improvement of diabetes.

1.7.1.2.2.1 Transdifferentiated tissue i*n vivo*

Use of gene therapy vectors in the transfer of the proinsulin gene *in vivo* into diabetic animal models has been made possible by means of lentiviral, retrovirus, adenovirus, and adeno-associated virus vectors. Non-viral insulin gene transfection *in vivo* has also been reported using cationic liposomes and naked DNA methods (Morishita et al., 2000; Goldfine et al., 1997). Target tissues tested include muscle, haematopoietic stem cells, fibroblasts, gut K cells and exocrine glands of the gastrointestinal tract (Bartlett et al., 1997; Bochan et al., 1999; Falqui et al., 1999; Cheung et al., 2000; Goldfine et al., 1997). However, these cells do not contain storage granules and hence could not release insulin via regulated pathways. Therefore they could not efficiently mimic the glucose-stimulated insulin secretion of normal beta cells.

In particular, hepatocytes originally derived from the same endodermal origin as pancreatic cells have been successfully used in a number of studies (Sapir et al., 2005; Ber et al., 2003; Zalzman et al., 2003; Ferber et al., 2000; Karasik et al., 2005). Transiently expressing adenoviral vectors have been employed to deliver *Pdx-1* and *NeuroD*/betacellulin (Ferber et al., 2000; Ber et al., 2003; Kojima et al., 2003) to streptozotocin (STZ)-diabetic mice, which have resulted in liver to pancreas transdifferentiation of isolated human liver cells. Ferber et al. (2000), transferred the rat *Pdx-1* gene to the livers of STZ-induced diabetic mice using an adenovirus-mediated gene delivery system, demonstrating that hepatic production of *Pdx-1* results in the expression of the mouse insulin gene 1 and 2 in the liver (Ferber et al., 2000). As a result, diabetic mice treated with the *Pdx-1* vector exhibited near normoglycaemia following one week of hepatic *Pdx-1* production. In contrast, STZ-induced diabetic mice treated with a control vector showed persistent hyperglycaemia. This study showed that hepatic *Pdx-1* production has an effect of recapitulating the process of beta cell differentiation in the liver, converting a fraction of hepatocytes to a cell type that is able to synthesize and release insulin. Although the underlying mechanism has not been elucidated, there is evidence to suggest that *Pdx-1* induced transdifferentiation did not take place in mature hepatocytes, but most likely occurred in the pluripotent progenitor cells of the liver, since transduction of primary hepatocytes by the *Pdx-1* vector failed to induce transdifferentiation *in vitro*.

Hepatic insulin gene therapy (HIGT) has been shown to diminish liver glycogen despite insulin responsive transcriptional effects in diabetic CD-1 mice (Zhang et al., 2009). HIGT produces near-normal glycemia in diabetic rats stimulates glycogen storage. However, the effect of HIGT on hepatic glycogen metabolism *in vivo* is unknown. After administration of an adenoviral vector capable of inducing glucose responsive insulin production from hepatocytes, circulating hormones, cytokines, hepatic gene expression and hepatic glycogen content in diabetic CD-1 mice receiving intravenous streptozotocin were evaluated. Non-diabetic mice and diabetic mice treated with empty adenovirus served as controls. Results from this study showed that peripheral concentrations of human insulin in HIGT mice were less than concentrations of mouse insulin among controls. However, expression of insulin responsive genes in HIGT livers indicated a significant intra-hepatic insulin effect, with expression changes reflecting appropriate responses to fed-fasting transitions.

Ren et al., (2007), achieved long-term transgene expression in quiescent hepatocytes *in vitro*. Blood glucose was normalised for 500 days making this the first successful study using a lentiviral vector. This procedure also resulted in the expression of genes encoding several beta cell transcription factors such as *Pdx-1*, *NeuroD1*. Hepatic insulin storage in granules was also observed in the transfected cells pointing to the initiation of secretory granule biogenesis. Importantly, liver function tests remained normal and pancreatic exocrine transdifferentiation did not occur.

Achieving glucose-dependent insulin release continues to limit the clinical application of these approaches. Another important criteria besides the physiologically regulated expression of the insulin gene is the processing of proinsulin into biologically active insulin (Mitanchez et al., 1998). Thule et al. (2000) constructed a family of glucose and insulin sensitive promoters that function in primary cultured hepatocytes. When transfected with human proinsulin cDNA cleavable by convertase furin, primary rat hepatocytes secreted fully processed, immuno-reactive and biologically active human insulin. Other *in vivo* studies involved engineered human insulin produced by a transgene efficiently activating the expression of the reporter gene under the control of various gene promoters such as PEPCK (phosphoenolpyruvate carboxykinase) and L-PK (liver-type pyruvate kinase) (Valera et al., 1994; Mitanchez et al., 1998). However, in none of these systems does transcriptional control efficiently reproduce

the immediate secretion of insulin seen from the secretory granules of beta cells. This is mainly due to the fact that the transfection of insulin, using the promoters PEPCK and L-PK, into the livers of these mice did not initiate secretory granule biogenesis. Another hypothesis to the development of hypoglycaemia in mice in these studies is that the insulin produced by the liver may rapidly interact with its receptor in the hepatocyte membranes and be internalized and degradated. The liver is highly responsive to insulin and has a high number of receptors. In addition, as the liver probably does not inherently contain specific endoproteases for the cleavage of the C-peptide, like the pancreatic PC1/3 and PC2, it is probably unable to complete the processing of the proinsulin molecule, which is less effective than the hormone (Valera et al., 1994).

Several studies have suggested that the expression of GLUT2 is required for conferring glucose-sensing capabilities into non-beta cell lines (Hughes et al., 1992; Motoyoshi et al., 1998). In addition, it has been observed that GLUT2 and GK are coexpressed not only in beta cells and hepatocytes but also in glucose-responsive neurons in the hypothalamus and the gut (Schuit et al., 2001), further suggesting that GLUT2 may be an important component of the glucose-sensing apparatus in non-islet cells. It has been suggested that engineering glucose-sensing components into cells or cell lines may simulate the performance of normal islet beta cells (Clark et al., 1997).

1.7.1.2.2.2 *Ex vivo* engineered non-beta cells

The ultimate gene therapy 'cure' for Type 1 diabetes may be the direct delivery of the insulin gene to a patient's own cells. However, the only possible vehicles available at present to deliver genes directly to the primary cells of an individual's body is by the use of viral vectors; either retroviral, lentiviral or adenoviral. There is currently concern in the community regarding the transfer of unwanted viral material and other safety concerns associated with the use of such viral vector systems. Consequently, the development of a rigorously tested artificial beta cell line, which can be packaged in an encapsulation system, provides a more attractive alternative to directly delivering genes to body tissues using viral vectors.

Cultured pituicytes, capable of processing proinsulin to insulin, may be suitable candidates for gene therapy in Type 1 diabetes, however, few studies have examined

the performance of transfected cells after implantation into diabetic or non-diabetic animal models. The rationale behind this engineering strategy is that regulated hormone secretion is a phenotype of a variety of endocrine and neuroendocrine cells, including beta cells and pituitary cells, and that these cells utilize a conserved set of proteins and mechanisms. For example, these cell types contain secretory granules that not only serve as a reservoir for hormones, but also interact with signal transduction pathways during the final steps of exocytosis (Tooze, 1991).

Moore et al. (1983), reported results obtained using a non-islet neuroendocrine cell line derived from adrenocorticotrophic (ACTH)-secreting cells, the AtT20 cell line, which was engineered by stable transfection with a human proinsulin cDNA controlled by a viral promoter. These cells contained secretory granules and expressed the pro-convertases, PC1 and PC2 (Smeekens et al., 1991). Therefore, the AtT20-ins cells released mature insulin polypeptide. A similar mechanism of glucose control mediated by Ca^{2+} influx and secretagogues in beta cells was reported in the AtT20-ins cells (Luini et al., 1985). However, as the cells lacked the GLUT2 transporter, they did not exhibit glucose-stimulated insulin release, glucose dependent potentiation of non-glucose secretagogues, and a glucose-sensitive increase in insulin content, all of which feature in normal islet beta cells (Hughes et al., 1992). Before using neuroendocrine cells, ACTH hormones and possibly other peptides should be monitored for production levels to prevent hormonal disorders such as Cushing's syndrome (Hughes et al., 1992). The therapeutical potential of neuroendocrine cells is also limited as the anterior pituitary tissue is not easily accessible.

Treating spontaneously-diabetic NOD mice with allogenic, proinsulin-secreting NIH 3T3 fibroblasts stably-transfected under the control of an inducible metallothionein promoter successfully reversed the hyperglycaemic state. However, the site of implantation of the transfected cells appeared to affect the efficiency of the reversal (Kasten-Jolly et al., 1997). A tighter mechanism by which to regulate the production of proinsulin in a dynamic fashion in these cells also needs to be further developed.

1.7.1.2.2.3 *Ex vivo* engineered hepatocytes

Hepatocytes, which are intimately involved in glucose synthesis, storage and homeostasis possess the same glucose-sensing apparatus as the beta cell (GLUT2 and

glucokinase (GK)) as both cells come from the same embryonic tissue (Seldon et al., 1987). Moreover, many liver-specific genes are controlled at physiological glucose concentrations. The location of the liver is somewhat privileged for controlling glucose homeostasis, as the portal vein carries glucose and other products absorbed after feeding. In liver, GK has long been thought to be essential for the unique metabolic functions of this tissue. Studies in humans, animal models and isolated hepatocytes have established that hepatic GK exerts a very strong influence on glucose utilisation and glycogen synthesis. Even small changes in the expression of GK in transgenic mice lead to measurable impact on the blood glucose concentration. Moreover, complementary studies in primary hepatocytes have shown that GK overexpression elevates intracellular G6P (Seoane et al., 1996), which triggers an increase in both glycolysis and glycogen synthesis (Aiston et al., 1999).

Although early studies show that FAO hepatoma cells do not possess secretory granules and therefore no storage compartment for secretory protein or release of insulin via the constitutive pathway (a process that is unregulated and unresponsive to the individual's second-to-second metabolic needs) (Vollenweider et al., 1992), FAO cells were not found to express beta cell transcription factors such as Neuro-D, Ngn3 and *Pdx-1* (unpublished results from our laboratory). In comparison, both HEPG2ins/g and Huh7ins hepatoma cells possess *NeuroD* (Tuch et al., 2003). It is highly likely that the presence of *NeuroD* allowed the cells to store insulin and the regulated insulin response to glucose was related to the exocytotic process.

Kojima et al. (2003) also showed that the induction of *NeuroD*-betacellulin and insulin in cells by gene therapy, resulted in insulin granules that secrete insulin by regulated exocytosis as seen in normal pancreatic beta cells. However, betacellulin has been linked to tumour-formation and would not be appropriate to use in human gene therapy (Kopp et al., 2003).

A study by Simpson et al. (1995) revealed that hepatoma cells (HEPG2) transfected with human cDNA insulin (HEPG2ins cells) resulted in the synthesis, storage (in structures resembling the secretory granules of beta cells) and chronic release of insulin via the constitutive and regulated pathways (Simpson et al., 1995). In contrast, the rat liver cell lines FAO (Vollenweider et al., 1992) and H4-II-E (Short et al.,

1998) did not display storage or regulated secretion of insulin as a product of differentiation. Regulated release of insulin in HEP G2ins cells was not demonstrated with the physiological stimulus of glucose and this was linked to the presence of GLUT1 only and the absence of GLUT2. When transfected with GLUT2, HEPG2ins/g exhibited glucose-stimulated insulin secretion (Simpson et al., 1997). This cell line has been shown to respond to glucose by K_{ATP} channels identical to those seen in pancreatic beta cells (Liu et al., 2003). Despite these beta cell-like characteristics, HEPG2ins/g cells were unable to reverse diabetes in an animal model since the GLUT2 expression was not maintained *in vivo* (Simpson et al., 1997).

Transfection of the insulin gene into the neoplastic Huh7 cells created insulin secreting human liver cells. These cells which have endogenous expression of GLUT2 and GK protein also possess granules that secrete insulin rapidly in a regulated manner in response to glucose stimulus and at only small amounts basally (0.3pmol/10^6 cells/24 h) (Tuch et al., 2003). Some disadvantages of the Huh7ins cells include some release of insulin via the constitutive pathway and this together with unrestricted proliferation results in hypoglycaemia. Some of the pancreatic features of Huh7ins cells may have been expressed as a result of ontogenetic regression of the neoplastic liver cell from which the line was derived. These characteristics include expression of both enzymes PC1 and PC2 in Huh7ins (also seen in normal beta cells) to cleave proinsulin to the bioactive diarginyl insulin (Tuch et al., 2003) and the expression of *NeuroD*.

Lutherborrow et al. (2010) has more recently shown that the Huh7ins cells have a larger number of beta cell transcription factors than are found in islets, which may be pivotal in the initiation and maturation stages of secretory granule biogenesis. Microarray data in this study, based on differences between Huh7 and Huh7ins cells, revealed no change in the expression of any known genes involved in secretory granule biogenesis, although genes possibly involved in the regulated secretion of insulin were identified. Huh7 and Huh7ins were found to express several important beta cell transcription factor mRNAs. Huh7 and Huh7ins cells both express various beta cell genes, and while none of these factors have a recognized effect on secretory granule biogenesis, the expression of *NeuroD* in liver cells *in vivo* have been shown to result in the formation of secretory granules that contained insulin (Kojima et al.,

2003). Overexpression of *NeuroD* in a human fetal epithelial cell line induced a neuron-like morphology and upregulated the expression of several genes present in the exocytotic machinery (Ishizuka et al., 2007). Where Tuch et al. (2003) have previously described the presence of beta cell markers GLUT2, glucokinase, PC1 and PC2, and the pancreatic transcription factor *NeuroD* in Huh7ins cells, Lutherborrow et al. (2009) reported the presence of other transcription factors HNF 1β, -3β, *Pdx-1*, Pax 4 and Pax 6 and *NeuroD*, and variable expression of Ngn 3, Nkx6.1 and HNF4α in the Huh7 cells. It is therefore possible that the acquisition of secretory granules and regulated insulin secretion in the Huh7ins cells, may have been aided by the expression of pancreatic and beta cell proteins already present in the parental Huh7 cells. Hence, genetically engineering these cells could provide the basal secretion of insulin needed in Type 1 diabetes to allow better control of metabolism to potentially prevent the complications of diabetes.

1.7.2 Development of an artificial beta cell line that is glucose responsive in the 4-5mM range: Melligen cells

Whilst the artificial beta cell line Huh7ins has been shown to normalise blood glucose levels in mice, the animals eventually became hypoglycaemic. This occurs because secretion of insulin by Huh7ins cells in response to glucose commences at 2.5 mM glucose, as compared to between 4 and 5 mM glucose for a normal beta cell. This has been the main stumbling block for developing the clinical applications of Huh7ins cells. Consequently, we have recently implemented further molecular strategies to correct the skewed insulin secretory profile of Huh7ins cells. The resulting cell line (Melligen cells) responds to glucose in the 4-5mM physiological range.

It is well documented that pancreatic beta cells and insulinoma cell lines (such as rodent INS-1 and MIN-6 cells) that respond to glucose in the physiological range, express GLUT2 and GK as their predominant glucose transporter and glucose phosphorylating enzyme, respectively. By contrast, insulinoma cell lines; such as RIN 1046-38 cells (Clark et al., 1997) and insulin-secreting foreign cell types such as AtT20ins cells (Hughes et al., 1992) that do not respond to glucose in the physiological range express low or undetectable levels of GLUT2 and glucokinase. Tiedge et al. (2000) experienced some success with rat-derived RINm5F cells.

However, these rodent cells will be of little use for human applications. The half-maximally effective glucose concentration for the RIN5F-GK (glucokinase transfected) cells was calculated to be 2.6mM, somewhat lower than for normal beta cells. Findings such as these have raised doubts about whether it is possible to generate an insulin-secreting cell with physiological glucose responsiveness.

Other work has shown that the overexpression of GLUT2 alone, or in combination with glucokinase, successfully reduces the glucose responsiveness of artificial beta cells (Hohmeier et al., 1997; Clark et al., 1997). However, the ratio of glucokinase:hexokinase expression is crucial for the maintenance of physiological glucose secretion. It is apparent that an imbalance in the glucokinase:hexokinase ratio in favour of hexokinase will result in enhanced glycolytic flux at low glucose levels and consequently increased sensitivity of the glucose-stimulated insulin secretory response. Accumulation of 2-deoxyglucose-6-phosphate in cells inhibits hexokinase, but has little effect on glucokinase activity. Incubation of RIN insulinoma cells with 2-deoxyglucose before the assay of glucose-responsiveness caused a shift in the glucose concentration required for a maximal response, from 50 μM in untreated cells to 5 mM in pretreated cells (Newgard, 1994). Additionally, overexpression of antisense hexokinase I in cells resulted in a 75% reduction in hexokinase I levels and a shift in the glucose dose-response curve, similar to that observed after incubation with 2-deoxyglucose (Newgard, 1994). Whilst it has been established that Huh7ins cells express glucokinase and levels of GLUT 2 comparable to normal beta cells, the glucokinase:hexokinase ratio is markedly in favour of hexokinase.

In an attempt to increase the expression of glucokinase relative to hexokinase, Huh7ins cells were stably engineered by Dr. Chang Tao (Postdoctoral Fellow) to overexpress human islet glucokinase (Huh7ins cells endogenously express the liver form of glucokinase). The presence of islet glucokinase has been verified by both PCR and Western blot analysis. As with the parent cell line (Lutherborrow et al., 2009), Melligen cells also express GLUT2 (unpublished data).

After exposure of Melligen cells to increasing concentrations of glucose from 1.5 to 5 mM, a dose-response curve for insulin secretion was generated and compared with that for the parent Huh7ins cells. Figure 1.12 illustrates that while glucose

responsiveness commenced at 2.5 mM in Huh7ins cells, it began in the physiological range (between 4 and 5 mM) in Melligen cells. Levels of stored insulin were not altered in Melligen cells as compared to Huh7ins cells. Further experiments determined the exact concentration of glucose at which Melligen cells commenced glucose-responsive insulin secretion to be 4.2 mM. Preliminary data also show that the Melligen cells are able to reverse diabetes in an animal model. The provisional patent on this cell line has been submitted (Patent: PCT AU 2007904310 "Genetically modified cells and uses thereof" AM Simpson, C Tao). The Melligen cells thus represent a notable step towards the engineering of a glucose-responsive artificial beta cell for insulin replacement therapy. Additionally, Melligen cells provide excellent starting material for determining the requirements for optimal glucose sensing in surrogate beta cells.

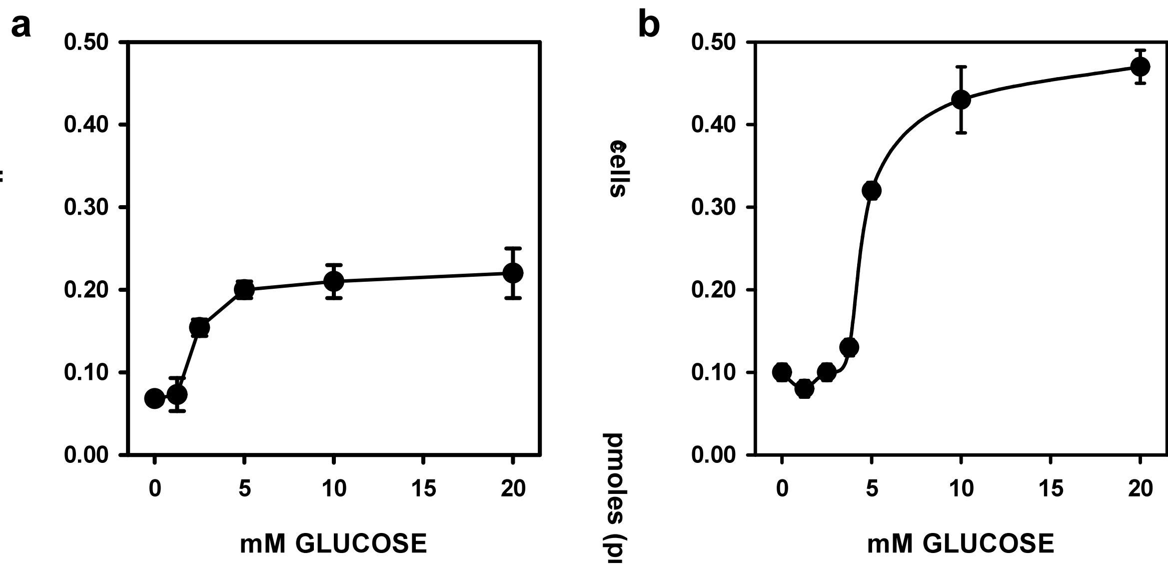


Figure 1.12: Glucose responsiveness of Huh7ins and Melligen cells in the millimolar range.

*Insulin secretion from **A)** Huh7ins and **B)** Melligen cells in response to increasing concentrations of glucose: 1.5-20 mM. Results are expressed as mean ± SE; n= 3 independent experiments.*

1.8 Overcoming immunological rejection

The beta cell antigens, which are targeted by the autoimmune response, remain poorly characterised (Section 1.3). If transplanted insulin-producing Huh7ins and Melligen cells express some or all of these antigens they are likely to be exposed to recurring autoimmunity. Immune exclusion is achieved by separating allogeneic or xenogeneic cells from the host by semipermeable membranes that allow only small molecules, such as glucose, insulin, and nutrients, to pass through (Murua et al., 2008). Immune lymphocytes and immunoglobulins are excluded by the membrane and are unable to cause destruction to the cells (Jen et al., 1996). The genetically modified cells are immobilised to express the ectopic insulin *in vivo* without the modification of the host's genome.

1.8.1 Development in microencapsulation technologies

Encapsulation of cells in a membrane prior to implantation holds potential for controlling the adverse immune response that may be generated against the transplanted cells, by physically isolating the cells from the host's immune system. If successful, encapsulation eliminates or minimizes the adverse effects of immunosuppressive therapy and permits the use of xenogeneic cells. Ideally, the capsule membrane holds permselective properties, so that the passage of nutrients, growth factors, and the therapeutic product secreted by the cells occur readily across the membrane, but mediators of the immune system do not penetrate the membrane (Figure 1.12). The major types of immunoisolation devices include intravascular arteriovenous shunts, diffusion chambers of tubular or planar geometry, and microcapsules. Additionally, totally biocompatible materials that do not interfere with cell function must be employed.

In a study by Park et al., 2005, functionalized gels with a putative cell-binding (-Arg-Gly-Asp-) (RGD) domain were designed in an effort to regulate mammalian cell behaviour in cells entrapped with gel. Adhesion molecules composed of Gly-Arg-Gly-Asp-Ser (GRGDS) peptides and cell recognition ligands were inculcated into thermo-reversible hydrogel composed of *N*-isopropylacrylamide, with a small amount of succinyl poly(ethylene glycol) (PEG) acrylate (MW 2000) used as a biomimetic extracellular matrix (ECM). The GRGDS-containing p(NiPAAm-co-PEG) copolymer gel was studied *in vitro* for its ability to promote cell spreading and to increase the viability of cells by introducing PEG spacers. Hydrogel lacking the adhesion molecules proved to be a poor ECM for adhesion, permitting only a 20% spread of the seeded cells after 10 days. Improvements to this material include increased sensitivity to physiologically relevant glucose concentrations, and increased stability.

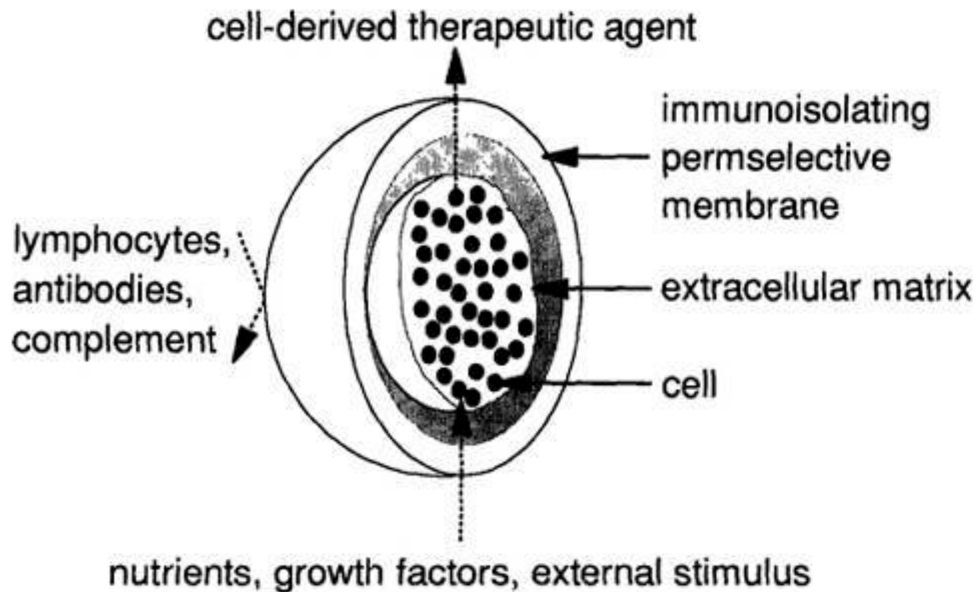


***Figure 1.13: Schematic drawing of a model microcapsule**.*
Cells are encapsulated with or without an extracellular matrix in a permselective membrane, which provides isolation from the mediators of the immune system, but allows passage of nutrients, growth factors, and the therapeutic product secreted by the cells (adapted from Lahooti & Sefton, 1998).

The concept of protecting transplanted islets by immunoisolation in a semi-permeable membrane has been tested using both macrocapsules (Lacy et al., 1991) either intravascular or extravascular or microcapsules (Sun et al., 1996). However, while cell encapsulation proved successful with other cell types, the specific problems associated with islet cell encapsulation precluded significant progress in animal models of diabetes. The major obstacle has been poor cell survival caused by hypoxia, due to the high metabolic rate of beta cells, and by small immune effector molecules, such as cytokines and free radicals, which can penetrate the capsule. In NOD mice immunosuppressants were required (Weber et al., 1997). In addition, the kinetics of secretagogue equilibration across the membrane was too slow for the rapid insulin secretory response required of functional beta cells. However, recent studies with alginate/polyamino acid microcapsules suggested that these obstacles could be overcome (Duvivier-Kali et al., 2001; Luca et al., 2003).

The objective of a study performed by Cheng et al. (2005) consisted of using genetically modified cells as an insulin source and of regulating insulin release by incorporating a glucose-responsive material, which acts as a control barrier for insulin in a cell-material hybrid device. Experiments were performed with insulinoma βTC cells, HepG2 hepatomas, and C2C12 myoblasts, the latter two genetically modified to constitutively secrete insulin. The control barrier consisted of concanavalin A-based glucose responsive material, which forms a gel at low and a sol at high glucose concentrations. Their results demonstrated that the device released insulin at a higher rate in response to glucose challenges. In contrast, a device containing an inert hydrogel instead of glucose-responsive material released insulin at an essentially constant rate, irrespective of the surrounding glucose concentration. It was concluded that the continuous or slowly responsive insulin secretion dynamics from these cells could not provide physiologic glucose regulation in patients. However, if a suitable material is designed to allow the total amount of insulin produced by the cells in response to rising glucose levels there is a possibility that hyperglycaemic episodes can be reversed using the encapsulated Melligen cells.

1.9 Aims

While the mechanisms of beta cell destruction are not completely understood, beta cell apoptosis mediated by both T lymphocytes and macrophages is known to play a major role. Pro-inflammatory cytokines are thought to be the ultimate effectors of beta cell death through the inhibition of glucose-induced insulin secretion and the stimulation of apoptosis through nitric oxide (NO) induction in the islet. The cell death receptor, Fas (CD95), has also been postulated to play a role in autoimmune beta cell damage.

With the exception of insulin, islet-specific autoantigens are not expressed in engineered artificial beta cells. Ultimately, it may be possible to engineer a patient's own hepatocytes *ex vivo* or *in vivo* to become insulin-secreting cells. Such cells may escape immune surveillance and subsequent autoimmune destruction in Type 1 diabetic patients. However, the limited supply of human liver tissue for experimental purposes and the difficulties associated with the maintenance and transfection of primary hepatocytes *in vitro* currently preclude the generation of artificial beta cells from primary hepatocytes.

If liver cells are to be a suitable treatment of Type 1 diabetes, they need to be resistant to the autoimmune process, which initially caused the destruction of the pancreatic beta cells. Melligen cells, with an encapsulation system, may provide a good model to determine the potential of gene therapy in Type 1 diabetes with further development. Also as a working model of insulin-secreting liver cells they provide excellent candidates to test the effects of the autoimmune process. Melligen cells will also provide a useful cellular model to elucidate the minimal molecular machinery required to generate a surrogate beta cell.

The specific aims for this thesis include:

1- To determine the effects of pro-inflammatory cytokines IFN-γ, TNF-α and IL-1β on the viability, insulin secretion and insulin storage capacity of Melligen cells *in vitro*
2- To analyse whether apoptosis of Melligen cells can be induced by the pro-inflammatory cytokine cocktail and if NF-κB, iNOS, Fas and MCP-1 are involved in the cascade of altered gene expression events in the cell after treatment
3- To derive gene expression profiles for cytokine treated and untreated Melligen cells over 1h and 24h after treatment using microarray analysis.
4- To determine the effects of STZ on viability, insulin secretion, insulin storage and GLUT2 expression in HepG2, HepG2ins, HepG2ins/g and Huh7, Huh7ins and Melligen cells *in vitro*.
5- To perform insulin studies on Melligen cells that have been encapsulated *in vitro* by another laboratory (AustriaNova, Singapore).

CHAPTER 2: Materials and Methods

2.1 Cell culture and cytokine treatment

2.1.1 Maintenance and passaging of cell lines

Seven cell lines were used: i) MIN-6 murine insulinoma cells (Miyazaki et al., 1990), ii) Huh7 human hepatoma cells (Nakabayashi et al., 1982) iii) Huh7ins cells developed by transfecting the parent cell line Huh7 with the cytomegalovirus (RcCMV) plasmid containing recombined human insulin cDNA (Tuch et al., 2003) iv) Melligen cells generated by the further genetic modification of Huh7ins cells through transfection with the human islet glucokinase gene (Patent: PCT AU 2007904310 “Genetically modified cells and uses thereof” AM Simpson, C Tao) v) HepG2 human hepatoma cells vi) HepG2ins cells developed by transfecting the parent cell line HepG2 with human insulin cDNA (Simpson et al., 1995) and vii) HepG2ins/g cells generated by further genetic modification of the HepG2ins cells through transfection with the human GLUT2 glucose transporter gene (Simpson et al., 1997).

MIN-6 cells were grown in monolayer cultures and were maintained in Dulbecco’s Modification of Eagles Medium (DMEM) (Trace Biosciences, Australia) containing 25mM glucose (MP, Australia) and 32mM sodium bicarbonate (Sigma, USA) supplemented with 5μL/L β-mercaptoethanol (Sigma, USA) plus penicillin streptomycin sulphate (PS) (60μg/ml) (GIBCO, USA) and 15% heat-inactivated foetal calf serum (FCS) (Trace Biosciences, Australia) at 37°C/ 5% CO_2.

Huh7, Huh7ins and Melligen cells were maintained as monolayer cultures in DMEM containing 25mM glucose and 32mM sodium bicarbonate plus 10% FCS and 60μg/ml PS at 37°C/ 5% CO_2. In addition, 550μg/mL of eukaryocidal antibiotic G418 (GIBCO, Australia) was added during culture to ensure positive selection of transfectants. Puromycin antibiotic (GIBCO, Australia) was used for selecting Melligen cells as they were transformed by a vector that expressed puromycin-N-acetyl-transferase.

Cells were subcultured at 80% confluency. Usually, MIN-6 cells were subcultured every 6 to 7 days, while Huh7, Huh7ins and Melligen cells were subcultured every 3 to 4 days. Media was aspirated and the cells were washed twice with sterile phosphate buffered saline (PBS; 0.14M NaCl, 2.66mM KCl, 6mM $Na_2HPO_4.2H_2O$, 1.75mM KH_2PO_4) (Sigma, USA) and then trypsin/ethylenediamine tetraacetic acid (EDTA) (500mg/mL trypsin (Sigma, USA), 0.54mM EDTA) (Sigma, USA), 5.6mM D-glucose, 5.33mM KCL, 0.14M NaCl, 7mM NaHCO3, 2mg/L phenol red) was added to the cell monolayer, which was then incubated for several minutes at 37°C until the cells detached from the tissue culture flask as observed by microscopy. The cells were then resuspended in appropriate media. Cells were counted using a haemocytometer (Improved Neubauer Counting Chamber, Germany) and seeded at the desired density.

2.1.2 Preparation of cytokine stock solutions

Cytokines were prepared according to the method of Tabiin et al., (2001). Murine interferon gamma (mIFN-γ) was used to treat MIN-6 cells while human IFN-γ (hIFN-γ) was used to treat Huh7, Huh7ins and Melligen cells because, unlike the other cytokines used, the action of IFN-γ is species-specific (Ternell & Green, 1993). Recombinant human IFN-γ (hIFN-γ) (Biosource, USA) was prepared by dissolving 100μg of hIFN-γ in 5mL of sterile 10mM acetic acid containing 0.1% bovine serum albumin (BSA) to make a stock solution of 20μg/mL. Recombinant murine IFN-γ (mIFN-γ) (Biosource, USA) was prepared by dissolving 330μg in 8.33mL of sterile PBS to give a stock solution of 39.6μg/mL. Recombinant human TNF-α (hTNF-α) (Biosource, USA) was prepared by dissolving 10μg of hTNF-α in 1mL of sterile PBS with 0.1% BSA to give a stock solution of 10μg/mL. Recombinant human IL-1β (hIL-1β) (Biosource, USA) was prepared by dissolving 5μg of lyophilised IL-1β in 5mL of sterile PBS with 0.1% BSA to make a stock solution of 1000ng/mL.

2.1.2.1 Cytokine treatment of cells

Cells were harvested from tissue culture flasks and plated at the appropriate cell densities for the assay being performed (Table 2.1). Cells were allowed to adhere overnight and were then treated with either fresh media (control cells) or media containing the following concentrations of cytokines IFN-γ (384ng/mL), TNF-α

(10ng/mL) and IL-1β (2ng/mL) (treated cells). Media and cytokines were replaced daily.

2.1.3 Streptozotocin treatment of cells

Streptozotocin (STZ; 20mM) (Sigma, USA), was always freshly prepared by dissolving 0.21μg of lyophilised STZ in 10mL of sterile DMEM. The 20mM stock was further diluted in DMEM to obtain 100μM STZ. Cells were allowed to adhere overnight and were then treated with either fresh media (control cells) or media containing 100μM or 20mM of STZ. STZ or media alone was changed either once or twice daily. Cell viability was determined by the MTT assay (Section 2.2) and insulin secretion and glucose responsiveness by radioimmunoassay (RIA) (Section 2.3.2). Trypan blue exclusion (Section 2.2.2) was also used to assess the viability of the cells. The effect of the STZ treatment on GLUT2 expression was also determined (Section 2.5).

Table 2.1: Seeding densities for assays used to determine the effects of cytokines on insulin-secreting and parental cell lines.

Assay	**Well Plate Used for Culture**	**Huh7/Huh7ins/Melligen cells/well**	**MIN-6 cells/well**
MTT Cell Viability Assay	96	$1x10^{3}$	$2x10^{3}$
PI Stain	---	$2x10^{6}$	$2x10^{6}$
Annexin V/PI Live/Dead Assay	---	$2x10^{6}$	$2x10^{6}$
Griess Reaction	24	$2x10^{5}$	$2x10^{5}$
Insulin Secretion and Storage	12	$5x10^{3}$	$2x10^{4}$
Glucose Responsiveness	6	$1x10^{4}$	---
	12	---	$2x10^{4}$

2.2 Viability assays

It should be noted that other vital dyes are available and fluoresce only after uptake by viable cells such as Calcein-AM. Like the MTT assay, this would be a direct method

of assessing viability compared to trypan blue exclusion (an indirect method of assessing viability).

2.2.1 MTT assay

The MTT assay measures mitochondrial activity as an indication of cell viability. The reduction of MTT (3-4,5-dimethylthiazol-2,5 diphenyl tetrabromide, a yellow tetrazole) is primarily due to glycolytic activity within the cell and is dependent upon the presence of NADH and NADPH. In the assay, MTT is reduced by mitochondrial dehydrogenase in viable cells to form a purple crystal, formazan, and optical density is recorded by a microplate reader at 570nm (Janjic & Wollheim, 1992). MTT (Sigma, USA) was dissolved in PBS to give a 5.0mg/mL stock solution that was filter (0.2μm) sterilised. Preliminary experiments (Section 3.2) determined optimal seeding densities to ensure that cells reached confluence by the final day of the experiment (day 10 for cytokine treated cells and day 4 for STZ treated cells). This prevented monolayers becoming over-confluent, thereby reducing the amount of mitochondrial dehydrogenase released and compromising the validity of results obtained.

Exponentially growing cell lines were plated (five replicates per treatment per time point) in flat-bottomed 96-well plates (NUNC, Roskilde, Denmark), in accordance with the seeding densities detailed in Table 2.1, and incubated overnight at 37°C/ 5% CO_2 to allow cells to adhere. Freshly prepared cytokines in media were added daily to the treated cells, and media only was added to control cells, throughout the experimental period, to give a final volume of 100μL/well. Cells were assayed for viability on days 1, 2, 3, 6, 8 and 10. To each well, 20μL of MTT was added and the plate was incubated for 4h to allow colour development. The media was then aspirated from the wells and 100μL of dimethylsulfoxide (DMSO) (Sigma, USA) was added to lyse cells, thereby releasing formazan. The plate was incubated for 15 minutes to allow the formazan to equilibrate in the DMSO. The absorbance of each well was read at 570 nm single fixed wavelength with the Bio-Tek spectral scanner Synergy HT. The KC4™ Software (Bio-Tek® Instruments Inc., USA) was used for the set-up and read process. Results were corrected for blanks (wells containing media alone) and cell viability was calculated using the following calculation:

$$\% \text{ Cell Viability} = \left(\frac{\text{mean absorbance from replicate test wells}}{\text{mean absorbance from replicate control wells}} \right) \times 100$$

Results were expressed as the mean percentage cell viability ± standard error. Viability of control cells was arbitrarily set at 100% and viability percentages for treated cells were corrected accordingly. Therefore, if the average absorbance of the treated cells was higher than the average absorbance of the untreated cells then the percentage cell viability was greater than 100%, albeit not significantly.

2.2.2 Trypan blue exclusion

The vital dye, trypan blue, was used to determine cell viability. Cells were washed with PBS and harvested by trypsinisation. Approximately 200 µL of cell suspension was placed in an eppendorf tube and an equal volume of 0.4% (w/v in 1mL) Trypan blue was added and the cells were gently mixed. The tube was then left to stand for 5 minutes at room temperature and then 10 µl of cells were placed in a haemocytometer and numbers of viable (unstained) and dead (stained) cells were quantified.

To calculate numbers of viable cells, the mean of unstained cells in each quadrant of the haemocytometer were counted, and multiplied by 2×10^4 to calculate the number of cells/mL. The percentage of viable cells was calculated as the number of viable cells divided by the total number of non-viable and viable cells. Results were expressed as the mean percentage cell viability ± standard error.

2.2.3 AnnexinV/ propidium iodide staining

Cells were treated with cytokines for 48h and samples were collected at 24h and 48h. After cells were trypsinised, approximately 2×10^6 cells were collected by centrifugation at 7500g (eppendorf, Germany) for 5min. Cells were then washed with PBS followed by re-suspension in Annexin-V Binding Buffer (Biocource, USA). To 100µL of cell suspension, 5uL of Alexa Fluor 488 Annexin-V (Biosource, USA) and 100µL of 1mg/mL propidium iodide (PI) (Sigma, USA) was added. Cells were incubated at room temperature for 15min. and a further 400uL of annexin V binding buffer was added. Samples were kept on ice and analysed by flow cytometry using

FACScan (Becton Dickinson, USA). Data was analysed using Cellquest software (Becton Dickinson, USA).

Additionally, for DNA staining of cells, PI (Sigma, Aldrich, USA) only was used to determine apoptotic cell populations exhibiting cells in the subG1 phase of cell cycle. Cells were treated with cytokines for 48h and samples were collected at 24h and 48h. Cells were harvested and washed with PBS once and then cell pellets were fixed for 1h in ice-cold ethanol. Fixed cells were then washed with PBS collected by centrifugation , and resuspended in PBS at a cell concentration of $2x10^6$/mL. RNase A (Sigma, USA) was added to degrade cellular RNA and 100μL of 1mg/mL PI was added. Samples were kept on ice and analysed by flow cytometry using FACScan (Becton Dickinson, USA). Data was analysed using Cellquest software (Becton Dickinson, USA). Apoptotic cells were identified by their hypochromic, sub-diploid staining profiles. ModFit was used to analyse cell cycle patterns. Experiments were repeated three times.

2.3 Nitric oxide determination

The conventional Griess reaction provides an indirect measure of nitric oxide (NO) release from cells by assaying nitrite concentration in the media. The means of measuring nitric oxide formation is to assay nitrite (NO_2^-), which is one of two primary, stable and non-volatile breakdown products of NO. This assay relies on a diazotisation reaction (Green et al., 1982). The modified Griess reaction enhances the detection of lower concentrations of NO (Marzinzig et al., 1997) and was used in this project.

MIN-6, Huh7, Huh7ins and Melligen cells were co-incubated with cytokines (IFN-γ [384ng/mL], TNF-α [10ng/mL] and IL-1β [2ng/mL]) in triplicate at appropriate seeding densities (Table 2.1). Control cells were plated with media only in triplicate. Cytokines and media were changed daily for 2 days.

A working nitrite solution was prepared by diluting 0.1M nitrite standard to 0.1mM in media. In a 96 well plate, 50μL of the diluted nitrite solution was dispensed to produce six serial 2-fold dilutions in triplicate wells of a 96 well plate to generate a

nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56μM). Nitrite solution was not added to one set of triplicate wells to provide a blank reference that contained 50μL of media alone. Next, 50μL of each experimental sample was added to the sample wells in triplicate. Fifty microlitres of dapsone (Sigma-Aldrich, USA) solution (1% 4,4'-Diaminodiphenyl sulfone in 5% hydrochloric acid) was added to all experimental samples and wells containing the dilution series for the nitrite standard curve. The plate was incubated for 5 to 10min at room temperature, protected from light. Fifty microlitres of NED (Sigma-Aldrich, USA) solution (0.1% N-1-napthylethylenediamine dihydrochloride in water) was then added to each well and the plate was incubated for 5-10min at room temperature, protected from light. The absorbance was measured at 540nm within 30min. using a Bio-Tek spectral scanner Synergy HT. The KC4™ Software (Bio-Tek® Instruments Inc., USA) was used to analyse data. The experiment was repeated four times.

2.4 Chronic insulin secretion and storage

Cells were seeded (at densities specified in Table 2.1) into a 12-well plate. Cells were allowed to adhere overnight. Insulin secretion was measured on days 1, 2, 3, 5, 8, 10 and 12. The triple cytokine combination of IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) was added and thereafter replaced daily for cells in the treated wells (six replicates) and fresh media was added to the control wells (six replicates). The experiment was repeated three times.

Chronic insulin secretion was determined by collecting media from each plate daily and pooling these samples. For example, the sample for day 3 was pooled with samples taken on days 1 and 2. The volume of media removed from each well daily was recorded and stored at -20°C for subsequent RIAs to measure chronic insulin secretion.

Insulin storage was determined for the same cells for which chronic insulin secretion was measured. The cell monolayer was rinsed with PBS and cells were collected by trypsinisation. Cell suspensions were centrifuged at 7500g (Sorvall RT7 centrifuge) for 5min. (5000g for 5min for MIN-6 cells). To cell pellets, fresh media was added (one of the replicates was retained to determine cell numbers). The cells were then

centrifuged again and the supernatant was discarded. This procedure ensured the complete removal of trypsin. Acid ethanol was then added (0.18M HCl in 70% ethanol) in amounts varying between 60μL and 300μL (depending on the size of the cell pellet) to lyse cells, thereby releasing stored insulin. An extraction period of 48 h at 4°C was used and then the insulin concentration of the sample was determined by an insulin RIA (Section 2.4.2).

2.4.1 Measurement of glucose responsiveness

Cells were plated according to the seeding densities outlined in Table 2.1 in 6-well plates, however, MIN-6 cells were plated in 12 well plates since the amount of insulin they secrete is much higher than that secreted by the insulin-secreting liver cell lines. The cells were allowed to adhere overnight at 37˚C/ 5% CO_2. Triple cytokine combinations of IFN-γ, TNF-α and IL-1β (Section 2.1.2) were added and thereafter replaced daily in the wells containing cytokine-treated cells. Six replicates were plated for untreated cells with glucose stimulation, untreated cells without glucose stimulation, cytokine treated cells with glucose stimulation and cytokine treated cells without glucose stimulation. The experiment was repeated three times.

After 10 days of culture, with cytokine and medium changed daily, cells were washed with basal medium (0.14M NaCl, 2.7mM KCl, 1.5mM KH_2HPO_4, 10mM Na_2HPO_4, 0.9mM $CaCl_2$, 0.5mM $MgCl_2$, 27mM $NaHCO_3$, 20mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid) HEPES, 2g/L BSA and 1.5mM D-Glucose, pH 7.4). Cells were incubated in 1mL of basal medium for 2h. After each hour the media was collected and labelled basal 1 and basal 2, respectively. Glucose was then added to determine insulin secretion, in response to elevated glucose levels, by MIN-6, Huh7ins and Melligen cells. Cells were stimulated for 1h with medium containing 0, 1, 2, 2.5, 3, 3.5, 4, 4.5 and 5mM glucose for measuring insulin secretion in response to glucose concentrations in the millimolar range or 20mM glucose for acute insulin secretion. Cells incubated with basal medium alone throughout the experiment provided controls for each cell line. A final basal incubation, basal 3, was performed to determine if insulin secretion returned to basal levels after removal of the glucose stimulus, as would be expected if glucose responsiveness was regulated. The samples

were assayed by RIA (Section 2.4.2). Cell counts were also performed using the trypan blue exclusion assay (Section 2.2.2).

2.4.2 Insulin radioimmunoassay (RIA)

The RIA assay buffer (1% BSA-RIA buffer) consisted of 0.05M EDTA, 0.14M NaCl, 0.01M PO_4 ($Na_2HPO_4.7H_2O/NaH_2PO_4.H_2O$), 0.01% NaH_3 at pH 7.6 and 1% BSA (Sigma, USA). Human insulin standard solution (1000μU/mL) was prepared from lyophilised human insulin (Sigma, USA) using RIA buffer (without BSA) and was stored at -80°C until use. Standards were prepared immediately prior to use by diluting the stock solution with 1% BSA-RIA buffer and performing serial dilutions to obtain concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125μU/mL. Low (12.5μU/mL), medium (50μU/mL) and high (150μU/mL) controls of insulin were also prepared.

A rat insulin standard solution of 100μg/mL (a gift from Professor B.Tuch, Department of Endocrinology, Prince of Wales Hospital, Sydney) was diluted in RIA buffer (without BSA) to provide a stock solution of 200ng/mL that was aliquoted and stored at -80˚C. The standards used in the assay were prepared immediately prior to use by serial dilutions with 1% BSA-RIA buffer to obtain concentrations of 32, 16, 8, 4, 2, 1, 0.5 and 0.25ng/mL. Rat insulin controls were prepared from the 200ng/mL rat insulin standard by diluting in 1% BSA-RIA buffer and 150μL aliquots were stored at -80˚C. A low control of rat insulin was prepared at 2ng/mL, a medium control at 8ng/mL, and a high control at 24ng/mL. Primary anti-insulin antibody was prepared by dissolving lyophilised antibody (a gift from Ms. Robyn Baume, Department of Endocrinology, Prince of Wales Hospital, Sydney) in 1% BSA-RIA buffer and was stored as 12mL aliquots at -20°C until use. This antibody detects both fully processed insulin and its precursor proinsulin. For preparation of the tracer, 200μL aliquots of stock ^{125}I labelled insulin (a gift from Dr Paul Williams, Department of Medicine, University of Sydney) were diluted with 1% BSA-RIA buffer to obtain a working tracer solution of approximately 12 000 counts per minute (cpm) per 50μL aliquot which was stored at 4°C.

Tubes were labelled as detailed in Table 2.2 with triplicate total count (TC) and standard tubes and duplicate control and sample tubes. Fifty microlitres of standards, controls, and samples were added to the appropriate tubes. Non-specific binding tubes (NSB) contained 100μL of the RIA buffer with 1% BSA. To all tubes, excluding NSB and TC tubes, 50μL of primary (anti-insulin) antibody was added, the tube was vortexed and then incubated for 4h at 4˚C. After the incubation period, 50μL of ^{125}I labelled insulin tracer was added to each tube and the contents were mixed by vortexing. The samples were then covered and incubated overnight at 4˚C. The next day, 50μL of 1.5% gammaglobulins (Sigma, USA), prepared in RIA buffer without BSA, and 1mL 15% v/v polyethylene glycol (PEG, Sigma, USA) in dH_2O was added to each tube, excluding TC tubes. All tubes were vortexed and then incubated for 15 min at 4˚C. Tubes were then centrifuged for 20 min at 10000g (Sorvall RT7 centrifuge) at 4˚C. Tubes were placed into dump racks and supernatants were discarded. The tubes were drained for approximately 5 min. before reading radioactivity in a gamma counter (WALLAC RIACalc 1470 WIZARD™). Each RIA was repeated in duplicate.

Table 2.2: Radioimmunoassay (RIA) procedure for the measurement of insulin (Lowy et al., 1971)

Standards	**Sample Volume**	**RIA Buffer +1%BSA**	**1° Antibody (anti-insulin antibody)**	**Tracer (I^{125} insulin)**	**2° Antibody (1.5% γ-globulin)**	**PEG**
Total counts	-	-	-	50	-	-
Non-specific binding	-	100	-	50	50	1
Blank (B_0)	-	50	50	50	50	1
6.25 μU insulin/mL	50	-	50	50	50	1
12.5	50	-	50	50	50	1
25	50	-	50	50	50	1
50	50	-	50	50	50	1
100	50	-	50	50	50	1
200	50	-	50	50	50	1
400	50	-	50	50	50	1
Insulin controls						
Low (12.5 μU/mL)	50	-	50	50	50	1
Medium (50 μU/mL)	50	-	50	50	50	1
High (150μU/mL)	50	-	50	50	50	1
Samples	50	-	50	50	50	1

2.5 Gene expression studies

2.5.1 RNA isolation

Prior to commencing the RNA isolation procedure, all bench surfaces were cleaned with RNase Zap™ (Invitrogen, USA). Flasks containing Huh7, Huh7ins, Melligen and human pancreatic islet cells were washed with 3mL of PBS (37°C). Cell monolayers were incubated with 7.5mL of TRIZOL™ (Invitrogen, USA) for 5min at room temperature and then 1.5mL of chloroform was added, the flasks of cells were shaken vigorously for 15s and then incubated for 2-3min at room temperature. Cells were transferred to fresh eppendorf tubes and were centrifuged (12 000g at 2-8°C for 15min) and then the upper aqueous RNA-containing phase was transferred to fresh tubes. Into each tube, containing RNA, 1mL isopropyl alcohol was added and the tube was incubated at room temperature for 10min. Samples were centrifuged (12 000g at 2-8°C for 10min) and the supernatant was discarded. To each tube, 1mL of 75% ethanol was added, and the contents were mixed by vortexing. The tubes were centrifuged (7 500g at 2-8°C for 5min) and the supernatants were discarded while the pellets were inverted to air dry. Pellets were then resuspended in 13-14µL H_2O, spun down, and placed into a 60°C water bath for 10min. The concentration of RNA was measured using a BioRad SmartSpec 3000 at a wavelength of 260 nm. Samples were then kept on ice and 0.5µL RNase inhibitor was added to 10µL aliquots, which were stored at -80°C until required for cDNA synthesis.

2.5.2 Reverse transcription

Complimentary DNA (cDNA) was synthesized from RNA by reverse transcription using 5µg RNA aliquots. The total reaction volume was 40µL and contained 8µL of 5x RT Buffer, 4µL of 10mM dNTP Mix, 3µL of 500µg/mL Oligo-d T Primer (random primer), 1µL of 40 Units/µL RNase inhibitor, 1.5µL of 10 Units/µL Omniscript AMV Reverse Transcriptase (Promega, USA). The volume of water added to the tube varied according to the amount of template used. This was done to ensure that 5µg of template RNA was added to the mixture. All reagents were centrifuged briefly to ensure that all reaction components were located at the base of the tube. Tubes were incubated for 1h at 37°C within the PCR machine and at 1h the temperature was elevated to 99°C for 1 minute. Tubes were then immediately placed

on ice. The concentration and purity of cDNA synthesised were determined by using the NanoDrop (NanoDrop® ND-1000 Spectrophotometer).

2.5.3 Primer sequences for cytokine receptors

Primer 3 (Whitehead Institute and Howard Hughes Medical Institute, USA) was the software program used to design the primers. The optimal length of PCR primers chosen was between 18-22 bp. The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer was chosen to be between 40-60%. Presence of hairpins at the 3' end were avoided when designing the primers. Optimally a 3' end self dimer with a ΔG of -5 kcal/mol and an internal self dimer with a ΔG of -6 kcal/mol was tolerated generally. Additonally, a 3' end cross dimer with a ΔG of -5 kcal/mol and an internal cross dimer with a ΔG of -6 kcal/mol was tolerated generally. Repeats in the sequences were also avoided for example ATATATAT. For qPCR, the target length was closer to 100 bp and for standard PCR, it was near 500 bp.

Two sets of primers were generally designed for each amplicon to test for amplification efficiency. Primers listed in Table 2.3 were adapted from previously published sequences.

Table 2.3: Primer sets for cytokine receptors

Gene	Amplicon Length (bp)	Sequence	Tm (°C)	Annealing Temperature (°C)	Reference
IFNGR1	594	F 5'-GTC CTC AGT GCC TAC ACC AAC TAA-3'; R 5'-CCA CAC ATG TAA GAC TCC TCC TGC-3'	65.2 67.8	56	Allione et al., 1999
IFNGR2	339	F 5'-GCA AGA TTC GCC TGT ACA ACG CA-3'; R 5'-GTC ACC TCA ATC TTT TCT GGA GGC-3'	71.9 67.9	56	Allione et al., 1999
IL1BR1	300	F 5'-ACA CAT GGT ATA GAT GCA GC-3'; R 5'-TTC CAA GAC CTC AGG CAA GA-3'	57.5 64.8	56	Lee et al., 2002
IL1BR2	392	F 5'-GGA GAA GAA GAG ACA CGG AT-3'; R 5'-TCA GGA CAC AGC GGT AAT AG-3'	59.8 60.3	56	Lee et al., 2002
TNFAR1	481	F 5'- CCT CAA TGG GAC CGT GCA CCT CT- 3'; R 5'-AGC CGC AAA GTT GGG ACA GTC AC-3'	74.1 72.2	61.9	Lee et al., 2002
TNFAR2	450	F 5'- CTC ACT TGC CTG CCG ATA AGG-3'; R 5'-CGA AAG GCA CAT TCC TCC TTG-3'	67.8 67.7	50	Lee et al., 2002
β-ACTIN	285	F 5'- TCA TGA AGT GTG ACG TTG ACA TCC GT-3' R 5'- CTT AGA AGC ATT TGC GGT GCA CGA TG-3'	62.2 63.5	56	Promega, USA

2.5.4 RT PCR

Temperature gradients were performed on each primer set to determine optimal annealing temperature. This ensured good PCR product yield with minimum false amplicon production. Cycling conditions varied for each primer set depending on their annealing temperatures (Table 2.3). DNA was amplified in a 25µL reaction mixture containing Master Mix (Promega, USA) 2x- 12.5µL; Primer 1 (forward) 10x- 2.5µL; Primer 2 (reverse) 10x- 2.5µL (Sigma, USA); dH_2O- 4.5µL; cDNA- 2.0µL. Contents were briefly centrifuged. All PCR experiments included negative controls in which either H_2O or RNA replaced cDNA template.

The following PCR cycle parameters were used for PCR using the thermocycler (Master Cycler Gradient eppendorf, Germany): 1 cycle of 95°C, 5min; 95°C, 40s; 56°C, 35s; 72°C, 40s then 40 cycles of denaturation 95°C, 40s; annealing 56°C, 35s and elongation 72°C, 40s); followed by a final extension at 72°C for 10min.

Once amplification was completed, agarose gel (1.5%) electrophoresis was used to verify amplicon size and purity. The gels contained 0.5µg/mL ethidium bromide dissolved in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA). A 50bp DNA Step Ladder (Promega) was used for fragment size comparison. Gels were observed under ultraviolet light using the Gel-Dock System (Biorad) transilluminator and the image captured using a Kodak digital camera and software.

2.5.5 Real time quantitative RT-PCR

For real-time RT-PCR analysis, primer matrices were designed to establish optimum reactions. The optimum concentration for each primer set, forward and reverse, was found to be 10pmol/reaction.

Real-time PCR was carried out in a total volume of 25µl. The reaction mix contained 1X SYBR® GreenER™ qPCR SuperMix Universal (hot start Taq DNA polymerase, SYBR® GreenER™ fluorescent dye, $MgCl_2$ dNTPs (with dUTP instead of dTTP), UDG and stabilizers), 20pmol of each forward and reverse primer and a minimum of 9.25pg to a maximum of 1ng of cDNA. A negative control that contained dH_2O instead of cDNA was included with each qRT-PCR experiment. qRT-PCR was

carried out using an Eppendorf Mastercycler EP *realplex* (Eppendorf, Hamburg, Germany). PCR was initiated with an incubation for uracil N-glycosylase (50°C, 2min) followed by an initial DNA denaturation (95°C, 5min), and then 34 cycles of denaturation (95°C, 40sec), primer annealing (58°C, 40sec) and extension (72°C, 40sec). This was followed by a dissociation step or melt curve analysis (95°C for 15sec, 60°C for 1min and 95°C for 15sec). The heating rate in the dissociation step was controlled at a rate of 0.1^{o}C/sec. On completion, the amplification plots were analysed for amplification efficiency and the dissociation curves were analysed to determine melting temperatures of PCR products.

The ΔCt was determined by the Livak Comparative Ct Method ($2^{-\Delta\Delta Ct}$) for Relative Quantification:

1. Normalize C(t): $\Delta C(t) = C(t)_{target} - C(t)_{hskg}$
2. Calculate $\Delta C(t)_{Mean}$: $\Delta C(t)_{Mean}$ = Mean $\Delta C(t)$ of replicates
3. Normalize to calibrator: $\Delta\Delta C(t) = \Delta C(t)_{Sample\ Mean} - \Delta C(t)_{Calibrator\ Mean}$

   (For calibrator, $\Delta\Delta C(t) = 0$)
4. Fold difference: $2^{-\Delta\Delta C(t)}$ = Normalized fold difference

   (For calibrator, $2^{-\Delta\Delta C(t)} = 1$)

2.5.5.1 Primer sequences used in quantitative RT-PCR

To determine NF-κB inhibition and its downstream effects by qRT-PCR (Section 4.3), Huh7ins and Melligen cells were cultured and treated with and without the cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 4h and mRNA was extracted. Table 2.4 shows the nucleotide sequence of PCR primers used for NF-κB inhibition and its downstream effects. The housekeeping gene used was β-actin: F 5'- TCA TGA AGT GTG ACG TTG ACA TCC GT-3' and R 5'- CTT AGA AGC ATT TGC GGT GCA CGA TG-3' (Promega, USA).

Melligen cells were cultured and treated with and without the cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 1h and 24h and mRNA was extracted. Table 2.4 shows the nucleotide sequence of PCR primers used to determine cell death genes and their effects by qRT-PCR.

HepG2, HepG2ins, HepG2ins/g and Huh7, Huh7ins and Melligen cells were cultured and treated with and without the 100μM STZ treatment for 0 and 4 days and mRNA was extracted. The nucleotide sequence of the human GLUT2 PCR primer used to determine GLUT2 expression by quantitative real time RT-PCR is listed in Table 2.4.

Table 2.4: Oligonucleotide primer pairs used to detect NF-kappaB inhibitors and cascade genes and microarray analysis follow-up genes involved in cell death pathways chosen for further analysis by real time RT-PCR

Gene	Amplicon Length (bp)	Sequence	Tm (°C)	Annealing Temperature (°C)
IκB-α	202	F 5'-CAC TCC ATC CTG AAG GCT ACC AAC-3' R 5'-CAC ACT TCA ACA GGA GTG ACA CCA G-3'	68.6 68.9	57
IκB-β	169	F 5'- GTA CTC CCG ACA CCA ACC AT-3' R 5'-CGG ACC ATC TCC ACA TCT TT-3'	63.6 63.8	57
IκB-ε	157	F 5'-GGG CAT CTC ATC CAC TCT GT-3' R 5'-GCT TCA GTC GGT ACA CAG CA-3'	64.1 64.1	57
Fas	210	F 5'-CAC AGA CCA CCT GCT TCT GA-3' R 5'-TCC GTC GTG GAG TAA CAG TG-3'	64.2 63.8	57
MCP-1	173	F 5'-CCC CAG TCA CCT GCT GTT AT-3' R 5'-CAT GGA ATC CTG AAC CCA CT-3'	63.9 63.7	57
iNOS	195	F 5'-GGA GCC AGC TCT GCA TTA TC-3' R 5'-TTT TGT CTC CAA GGG ACC AG- 3'	63.7 63.9	57
BIRC3	156	F 5'-TGT GGG TAA CAG TGA TGA TG-3' R 5'-TGA ACT TGA CGG ATG AAC TC-3'	55 56	63
CASP1	71	F 5'-TGT TCC TGT GAT GTG GAG GA-3' R 5'-TCT TTC AGT GGT GGG CAT CT-3'	60 61	63
IRF1	137	F 5'-CAA ATC CCG GGG CTC ATC TGG-3' R 5'-CTG GCT CCT TTT CCC CTG CTT TGT-3'	70 69	63
SOD2	225	F 5'-GGA AGC CAT CAA ACG TGA CT-3 R 5'-ACA CAT CAA TCC CCA GCA GT-3'	47 47	63
STAT1	82	F 5'-CCC AGCA ATG CCC TGA TTA ATG-3' R 5'-CTG CAG CTG ATC CAA GCA AG-3'	62 62	63
GLUT2	184	F5'-TTG GTG TGA TCA ATG CAC CT-3' R 5'-GCT GCC ACA GTC TCT TCC TC-3'	54 50	62

2.6 Transcriptomics analysis

Melligen cells were cultured and treated with the cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) or media alone for control samples, for either 1h or 24h and RNA was isolated (Section 2.6.1). The samples were purified using the RNeasy Kit (Qiagen, USA), according to the manufacturer's instructions. Quality and quantity of isolated RNA was analysed by clear separation of bands by electrophoresis of agarose gel and NanoDrop (NanoDrop® ND-1000 Spectrophotometer) respectively. Preparation of cRNA, hybridization, and scanning of the Human Geneome ST 1.0 arrays were performed according to the manufacturer's protocol (Affymetrix, USA). The Microarray procedure was performed at the Clive and Vera Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Australia) where labelling reactions, hybridisation procedures, reading of the signals and normalisation were performed.

Obtained data was imported into GeneSpring 6.1 software for analysis (GeneSpring 6.1, Silicon Genetics, Redwood City, CA). The fold changes were analyzed by filtering the dataset using P-value < 0.01 and a signal-to-noise ratio > 2 for use in ANOVA statistical analysis. Data analysis was performed using Partek Genomics Suite 6.2 software (Partek Inc, USA). The software includes algorithms that determine whether a gene is absent or present and whether the expression level of a gene in an experimental sample is significantly increased or decreased relative to a control sample. In order to detect artefacts present in each array, the surface image was analysed. A zoom-in tool was used to identify white or yellow marks on the chips suggesting probe sets that were not amplified due to an error on the platform or hybridisation error. Several of these were found at the centre of the chip, indicating that these probe sets were not amplified. However, since this array is designed so that the probes are spaced across the array and not clustered, a small area of white/yellow squares on the chip will not have an impact on the data and will not affect the final result since there are so many probes per gene and so no result was lost.

Additional filtering (minimum 2-fold change) was applied to extract genes, which were further analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). Those genes with known gene symbols and their

corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset but may not be an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes.

The network identified was then presented as a graph indicating the molecular relationships between genes/gene products. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node color indicated the degree of up- or down-regulation. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. The node shapes denote enzymes, phosphatases, kinases, peptidases, G-protein coupled receptor, transmembrane receptor, cytokines, growth factor, ion channel, transporter, translation factor, nuclear receptor, transcription factor and other.

Canonical pathways analysis identified the pathways, from the IPA library of canonical pathways, which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (2) a P value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

2.7 Microencapsulating Huh7, Huh7ins and Melligen cells

The encapsulation procedure and sample collection were performed by AustriaNova, Singapore. The samples for glucose responsiveness and insulin secretion were sent to our laboratory on dry ice. Insulin determination by RIA (Section 2.4.2) was performed in our laboratory (Section 6.2.1.1). The Alamar Blue® Assay (Invitrogen,

USA) was used to determine the viability and proliferation rate of the Huh7, Huh7ins and Melligen cells (Section 6.2.1) (AustriaNova, Singapore).

2.8 Statistical analysis

Data are represented as mean values ± standard error (SE). Comparisons between two groups were analysed using Student's t-test. Comparisons between two or more variables were analysed using ANOVA (SPSS 17.0.0, SPSS Inc. 2008). P values less than 0.05 were considered to be statistically significant.

CHAPTER 3: Determining the Effects of Pro-inflammatory Cytokines on Cell Viability and Insulin Function in Melligen cells

3.1 Introduction

It is postulated that cytokines can play a crucial role in the development of autoimmune Type 1 diabetes. In the pathogenesis of Type 1 Diabetes, the pro-inflammatory cytokines IFN-γ, TNF-α and IL-1β dominate the autoimmune response that ultimately destroys insulin secreting beta cells (Kim & Lee, 2009). Type 1 diabetes is associated with a T lymphocyte and monocytic infiltration of the pancreatic islets (insulitis), and T cells directed against intrinsic beta cell antigens have been isolated (Csorba et al., 2010). Observations in several Type 1 diabetes animal models have shown that the first immune cells to infiltrate the islets are macrophages and dendritic cells, which are capable of releasing cytokines that can induce beta cell damage, these include IFN-γ, TNF-α and IL-1β (Yoon & Jun, 2005). However, *in vivo* there are several other cytokines that are most likely to be present simultaneously, and additive or synergistic actions of the cytokines are envisaged (See Section 1.3 for T cell review and cytokine models).

Insulin and other beta cell antigens have been proposed as putative auto-antigens triggering this autoimmune attack. Evidence suggesting insulin as an auto-antigen in Type 1 diabetes has been shown in a study involving NOD mice lacking native insulin genes and carrying a mutated proinsulin transgene (Nakayama et al., 2005). These mice did not develop insulin autoantibodies, insulitis, or diabetes. Other putative auto-antigens in Type 1 diabetes include glutamic acid decarboxylase, and islet antigen 2, which have been found to correlate with insulitis in NOD mice (Tisch et al., 1993; Kaufman et al., 1993; Bingley et al., 1994). Studies on NOD and NOR mice have demonstrated that B- and T-lymphocytes, that are specific for beta cell auto-antigens, play a key role in disease development and that CD4+ and CD25+ T-cells are

required to adoptively transfer diabetes (Ott et al., 2005). Thus the interaction between activated macrophages and T-cells, together with cytokines secreted from these immunocytes, act synergistically to destroy beta cells, resulting in the development of autoimmune Type 1 diabetes.

One of the well-known effects of pro-inflammatory cytokines on beta cell lines, such as INS-1E and NIT-1, and rat islets, is the inhibition of insulin mRNA expression, total protein and pro-insulin biosynthesis, and decreased insulin release (Mandrup-Poulsen, 1996; D'Hertog et al., 2007; Andersson et al., 2001; Zumsteg et al., 2000). Exposure of rat islets to exogenous IL-1β for 24h induces a transient decrease in glucose-stimulated insulin release and insulin storage and in combination with IFN-γ and TNF-α there was an even further decrease in insulin storage (Wachlin et al., 2003). Exposure of neonatal porcine islets to IFN-γ, IL-1β and TNF-α for 48h has also been shown to significantly decrease insulin content but not secretion or glucose-responsiveness (Harb et al., 2007). However, the response of human islets following cytokine treatment has been less predictable than that in animal models. It has been shown that IL-1β on its own, applied for 48h, can stimulate (Eizirik et al., 1993) or inhibit (Vara et al., 1994) insulin secretion from human islets. However, TNF-α and IFN-γ inhibited secretion from 2 to 6 days when applied to human or rat islets, singly (Kawahara & Kenney, 1991) or in combination (Hadjivassiliou et al., 1998).

The persistence of immunological memory is a major hurdle for attempts to cure Type I diabetes by replacing beta cell populations, as these beta cells will be once again destroyed largely through the release of pro-inflammatory cytokines by immune cells. The only way to prevent the reoccurrence of autoimmune reactions against transplanted beta cells is to maintain the recipient on intensive immunosuppressive therapy which carries with it adverse side effects (Tan et al., 2006). Therefore, an alternative strategy for replacing beta cells in diabetic patients would be to engineer a non-beta cell for implantation, to provide a source of physiologically regulated insulin secretion from cells that lack other important beta cell and islet auto-antigens to avoid immune surveillance. Also such genetically engineered cells can offer a solution to the cell availability problem in finding a pancreatic substitute for the treatment of Type 1 diabetes (Bagley et al., 2008). To examine the use of engineered non-beta

cells as an alternative solution, it is vital to determine if the use of these engineered non-beta cells in the delivery of insulin is affected by the autoimmune response described above.

Previous studies using this approach have involved the use of both neuroendocrine cells and non-endocrine cells, including pituitary cells (Hughes et al., 1992), fibroblasts (Falqui et al., 1999), gut K cells (Cheung et al., 2000), Vero (African green monkey kidney cells) cells (O'Driscoll et al., 2002), human lung or kidney epithelial cells (Tatake et al., 2007) and hepatocytes (Wu et al., 2003). These cells have been genetically engineered to secrete recombinant insulin constitutively or under transcriptional regulation. However, an artificial beta cell must possess beta-cell-characteristics necessary for insulin secretion in a glucose-regulated manner and that would be considered safe to implant into humans. Hepatocytes are known to play an important role in glucose metabolism and in the synthesis and storage of proteins in the liver making hepatocytes a more suitable candidate for production of an artificial beta cell. The anatomical location and role in drug and xenobiotic detoxification, suggests that the liver is confronted almost continuously with factors, such as lipopolysaccharide, that induce pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1β (Trautwein et al., 1996). In a cascade of events, the cytokines can up-regulate genes in hepatocytes leading to either apoptosis or a mainly regenerative and proliferative outcome (Diehl, 2000). Experiments have confirmed that the treatment of liver cell lines, such as Huh7 and HepG2, with pro-inflammatory cytokines leads to the up-regulation of several putative survival factors such as NF-κB and JNK, as well as a number of mitochondrial proteins, including Bfl-1, Bcl-xL, UCP-2, BH-3 (Fabregat, 2009; Mott & Gores, 2007), and iNOS (Rai et al., 1998), that may allow hepatocytes to survive apoptotic and oxidative stress.

In our laboratory, the liver cell line Huh7 was transfected with the insulin gene. The resulting Huh7ins cells were shown to synthesize, store and secrete insulin in response to a glucose stimulus. Whilst Huh7ins were shown to be glucose responsive, they responded to sub-physiological levels of glucose (2.5mM), and the mice eventually became hypoglycaemic. To correct this problem, the cells were further engineered (into Melligen cells) to respond to more physiological levels of glucose

(4.5mM). Although the resulting Melligen cells have desirable characteristics as surrogate beta cells, to be a viable treatment for Type 1 diabetes, they must withstand the effects of cytokines.

Since the liver cell lines Huh7ins and Melligen have been transfected with the insulin gene, absence of the other auto-antigens possessed by the beta cell may not render these cells susceptible to the autoimmune attack seen in Type 1 Diabetes. Further to this, if Melligen cells are to be suitable candidates as artificial beta cells they must continue to be glucose-responsive and store and secrete insulin in the pro-inflammatory cytokine milieu. Therefore, the effects of IFN-γ, TNF-α and IL-1β on viability, insulin secretion, storage and glucose-responsiveness of Huh7ins and Melligen cells were determined.

3.2 Characterisation of MIN-6, Huh7, Huh7ins and Melligen cell growth in DMEM

To establish the growth kinetics of each of the cell lines (MIN-6, Huh7, Huh7ins and Melligen) used in this study, cell numbers were quantified over varying periods of time. An initial seeding density of $1x10^4$cells/mL in six well plates was used, based on previous experiments in our laboratory, and growth curves were generated (Figure 3.1). Huh7, Huh7ins and Melligen cells approached exponential growth by day 8, when the cell concentration was approximately $1x10^6$cells/well. In contrast, MIN-6 cells reached a cell concentration of $5x10^5$cells/mL at a similar stage of exponential growth (Figure 3.1A). It was also found that the highest recorded cell number for the MIN-6 cells ($1x10^6$cells/mL) was only reached at day 8 and cell numbers then remained stationary until day 10. As MIN-6 and Huh7 cells are of murine pancreatic and human liver origin, respectively, each cell line exhibited distinct growth kinetics. These cell growth characteristics were used to determine the time course for experiments in which cytokines were co-incubated with cell lines. Since MIN-6 cells reached the log growth phase at a later time point, this cell line was plated at a 2-fold higher seeding density when run in parallel experiments with Huh7 and Huh7ins to ensure that the cells were in log phase when used in the cytokine treatment experiments, which were performed over several days. This experiment provided proof of principle that different initial seeding densities resulted in log phase growth at the time of experimentation.

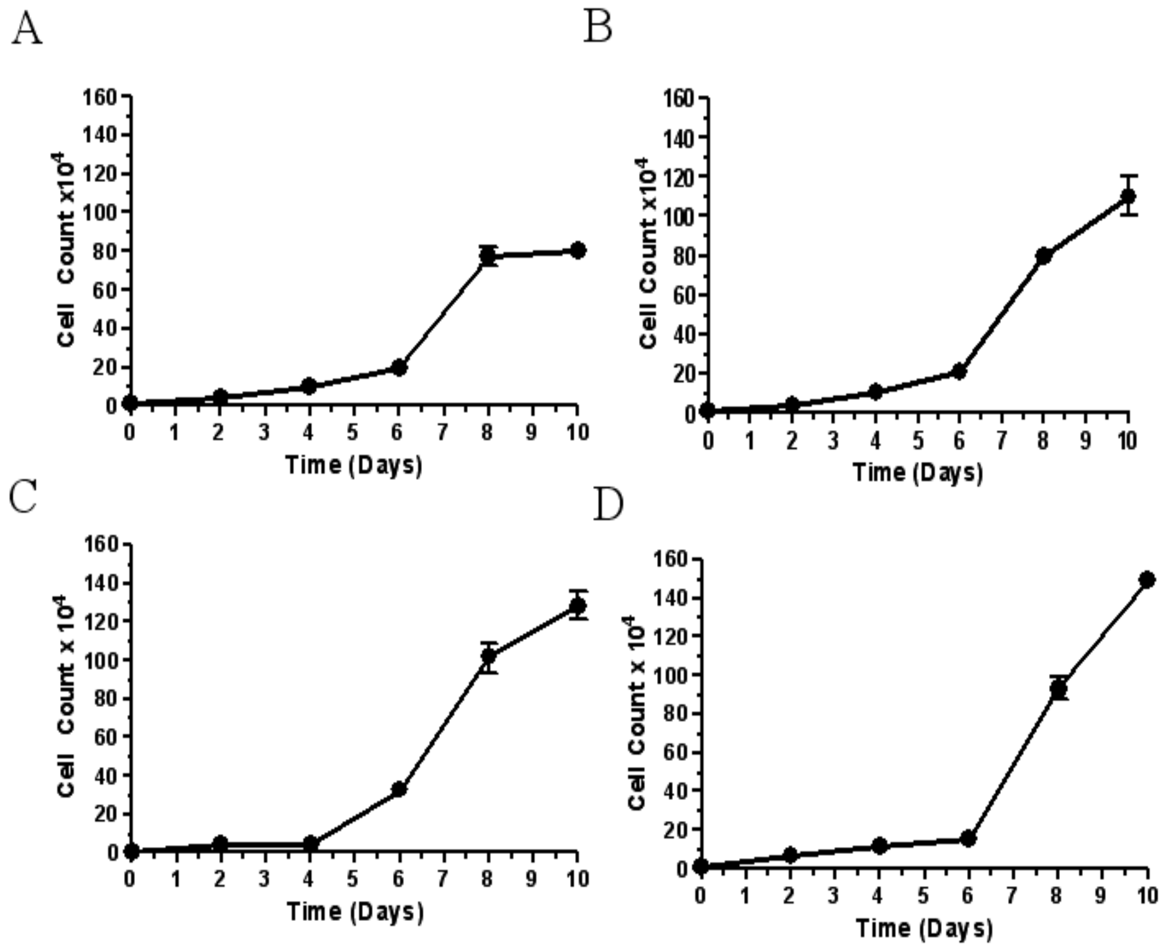


Figure 3.1: Growth kinetics of MIN-6, Huh7, Huh7ins and Melligen cells.

Cells were initially seeded at a density of $1x10^4$*cells/mL, for liver cell lines, and* $2x10^4$*cells/mL, for MIN-6 cells, into six well plates. Cell counts were obtained using the trypan blue exclusion method every second day throughout the period studied A) MIN-6 B) Huh7 C) Huh7ins and D) Melligen cells. Results are expressed as mean ± SE, n=4 independent experiments.*

The growth kinetics of the untreated MIN-6 cells and the liver cell lines, Huh7, Huh7ins and Melligen cells was determined (Figure 3.2) using the MTT assay as a measure of cell viability based on mitochondrial activity. The liver cell lines were seeded at an initial density of $1x10^3$cells/well and the MIN-6 cells at $2x10^3$cells/well, into 96-well plates and cultured for 10 days.

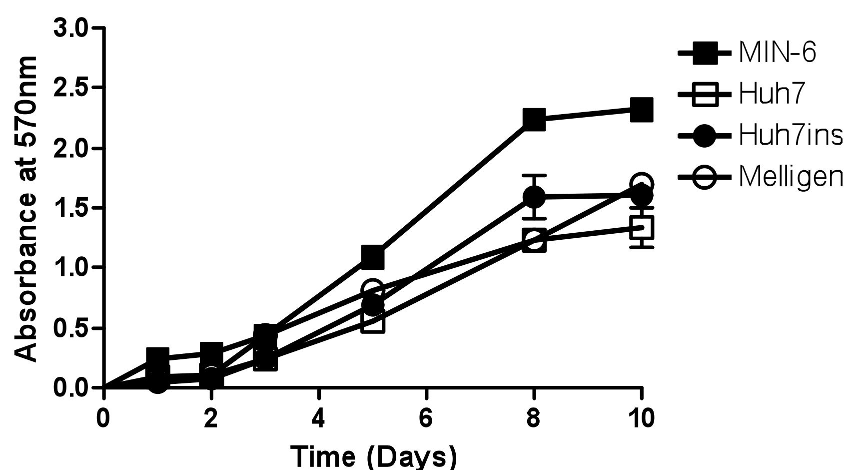


Figure 3.2: The viability of MIN-6, Huh7, Huh7ins and Melligen cells.

The MTT assay was performed to ensure that exponential growth was detected by the assay over 10 days. Results are expressed as mean ± SE, n=5 independent experiments.

The results obtained from this preliminary experiment showed that the seeding density chosen approached an absorbance value of 1.5 for the liver cell lines and 2.5 for the MIN-6 cells over the 10-day period with about half of this absorbance seen at day 5 (midpoint of the experimental timeline). The initial seeding density used was optimal since subsequent experiments to assess the cytotoxic effect of cytokines were to be conducted for between six and ten days (Section 3.3). These results also showed that the exponential growth of the cells was reflected by the mitochondrial activity of the cells. This was an important indication that the measurement of mitochondrial activity, using the MTT assay did reflect the viability of the cells used in this experiment and could be used in subsequent cytokine treatment experiments as to determine viable cells. From the growth kinetic results seen in Figure 3.1, the

absorbance values obtained from the MTT assay performed on all untreated cell lines in Figure 3.2 and previous studies performed in our lab on these cells, it was determined that MIN-6 cells would be plated at twice the seeding density of the liver cell lines.

3.3 Determination of cytokine concentrations

Initial experiments used serial dilutions of single cytokine concentrations of IFN-γ, TNF-α and IL-1β. The effective dose required to destroy half the cell population (ED_{50}) indicated on the respective data sheet for each cytokine constituted the most dilute concentration used. These initial titration experiments were necessary as each ED_{50} value provided on the data sheets applies to a specific application and a specific cell line. Additionally, different preparations of cytokines exhibit differing potencies. IFN was serially diluted from 19.2ng/mL to 0.15ng/mL, TNF from 5.00ng/mL to 0.04ng/mL and IL-1 from 0.50ng/mL to 0.004ng/mL.

Results from the MTT assay showed that co-incubation of MIN-6 cells with the single cytokines at the concentrations used did not reduce the viability of the cells as compared to the untreated cells, even after 10 days of co-incubation (data not shown). A literature search revealed that using the pro-inflammatory cytokine cocktail at the following concentrations induces beta cell death: IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL). Tabiin et al. (2001) investigated this cytokine treatment over 14 days on the rodent insulinoma cell line, NIT-1, and the insulin secreting human hepatocyte cell line, HEPG2 ins/g. Souza et al. (2004) also determined that the same cytokine cocktail with a lower concentration of IFN-γ (1.4ng/mL), 6ng/mL IL-1β and 9.3ng/mL TNF-α was sufficient to induce beta cell death genes in RINm5F cells after 6h. However, it must be noted that rat islet cells are susceptible to lower concentrations of cytokine treatment (almost half) when studied *in vitro* (Wachlin et al., 2003) compared to human islets (Rabinovitch et al., 1999; Hadjivassiliou et al., 1998). Consequently, the cytokines were further titrated in combination and at higher and lower concentrations to the concentrations reported in the literature to ensure MIN-6 cells reached lowest viability by day 10.

Media and cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) singly and in combination were changed once every two days and cells were assayed for viability over a six-day period. However, at the initial seeding density used ($2x10^3$ cells/well), approximately 48 ± 4% of the cytokine cocktail treated MIN-6 cells were still viable at the final day of the experiment (Figure 3.3A). The observation period was therefore increased to 8 days and the media and cytokines were changed daily. Under these conditions, by day 8, only 37 ± 4% of MIN-6 cells were viable as compared to 100% for untreated cells (*P*=0.0045) (Figure 3.3B).

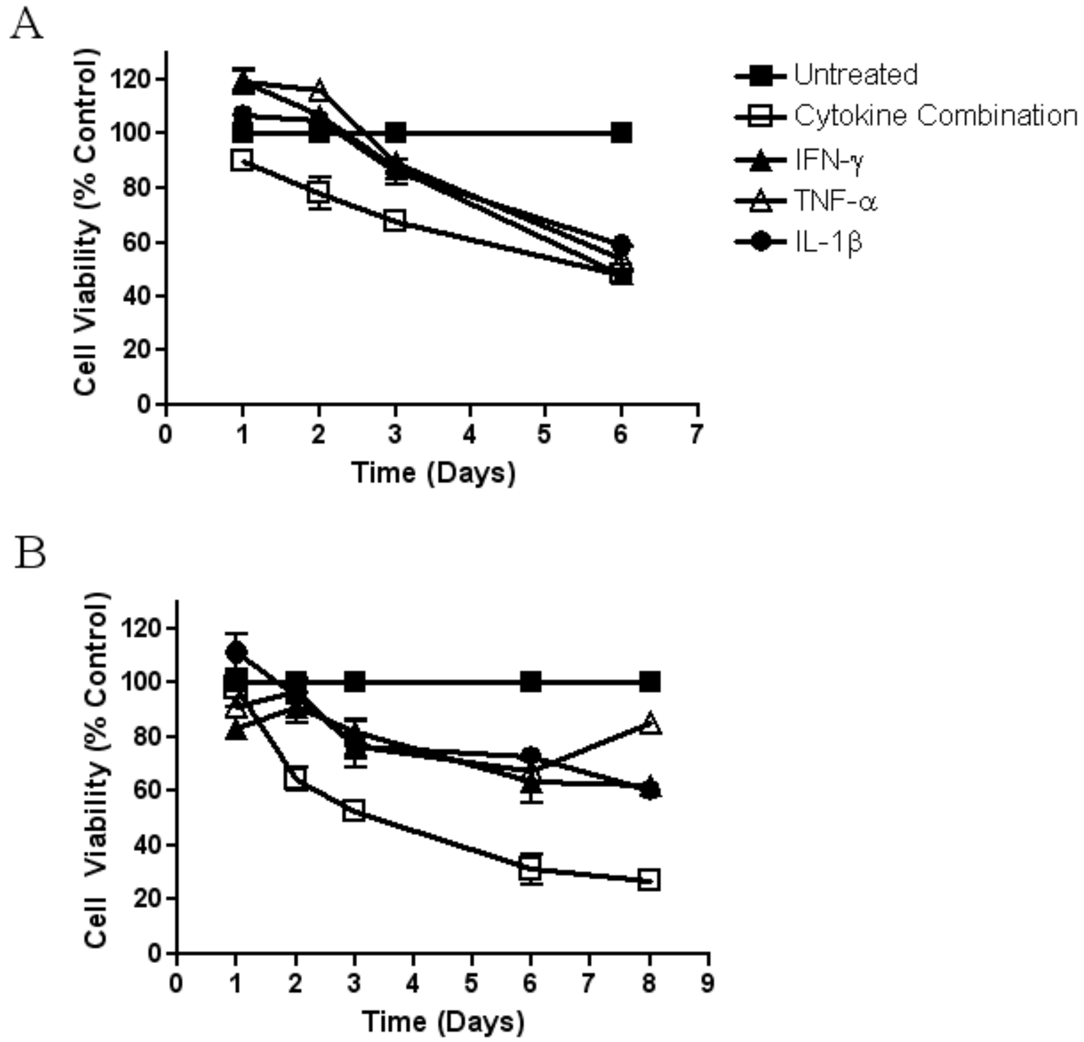


Figure 3.3: The effect of cytokines on MIN-6 cells, singly and in combination.

MIN-6 cells were incubated with the pro-inflammatory cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) in combination and individually A) over 6 days replacing the cytokines and media once every 2 days and B) over 8 days with the cytokines and media changed daily. Cell viability was determined by the MTT assay Control cells were incubated in media alone. Results are expressed as mean ± SE, n=3 independent experiments.

Since the MIN-6 cells were to be used as a beta cell control model in these experiments the cytokine treatment chosen was required to have a full effect on the cells. As it was preferable to obtain the lowest percentage of viable cells by the final day of the experiment and to precisely determine the kinetics of cytokine induced death, the triple cytokine combination concentration was further titrated by adding the cytokines at twice IFN-γ (768ng/mL), TNF-α (20ng/mL) and IL-1β (4ng/mL) (Figure 3.4A) and half the triple cytokine concentrations IFN-γ (192ng/mL), TNF-α (5ng/mL) and IL-1β (1ng/mL) (Figure 3.4B) previously used (Figure 3.3A and B). The results showed that at twice the concentration (Figure 3.4A) 26 ± 3% cells remained viable and at half the concentration almost 47 ± 2% remained viable by day 8, indicating that doubling the concentration produced a greater toxic effect on the cells.

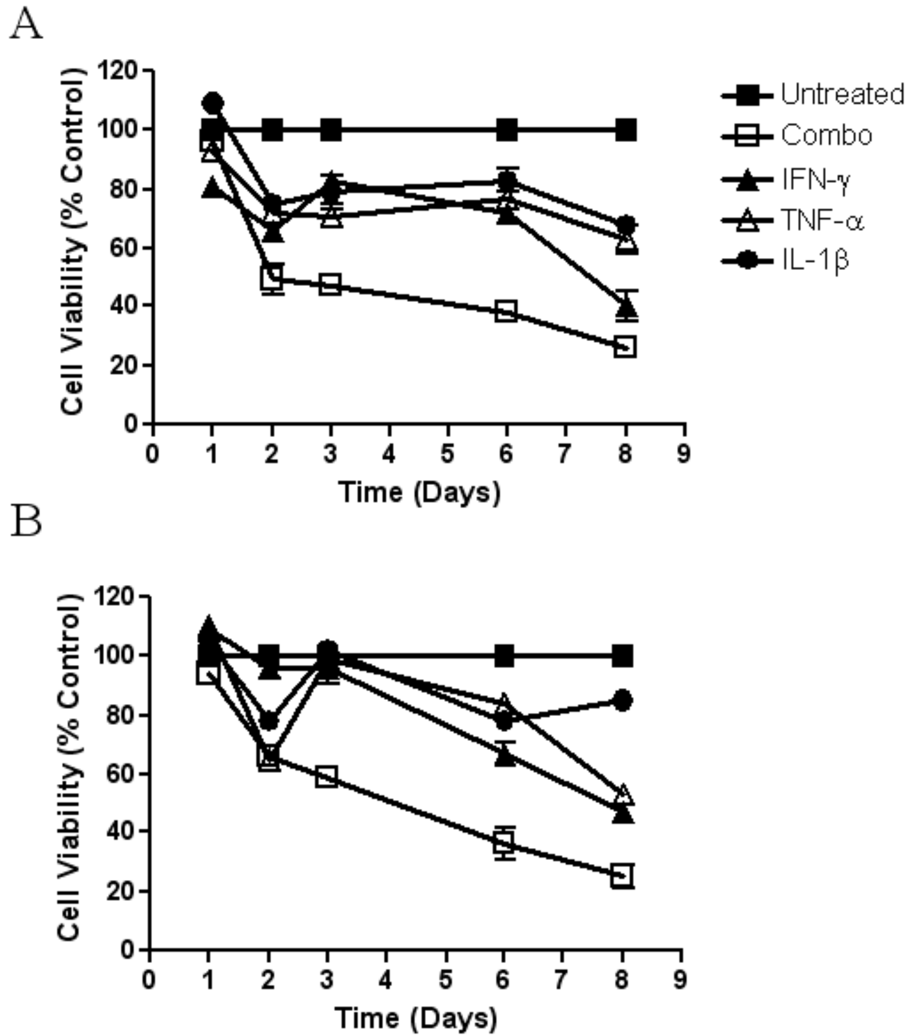


Figure 3.4: The effect of adding double and half the initial cytokine concentration to MIN-6 cells.

To obtain maximum cytotoxicity, MIN-6 cells were treated with cytokine concentrations titrated over 8 days A) at twice (IFN-γ (768ng/mL), TNF-α (20ng/mL) and IL-1β (4ng/mL)) and B) at half (IFN-γ (192ng/mL), TNF-α (5ng/mL) and IL-1β (1ng/mL)) the initial concentrations used. Cell viability was determined by the MTT assay Results are expressed as mean ± SE, n=3 independent experiments.

To determine if changing the cytokines and media daily and extending the experimental time to 10 days would have a greater toxic effect on the cells, the cytokines were added at one, two and five times the initial concentration (Figure 3.5A, B and C).

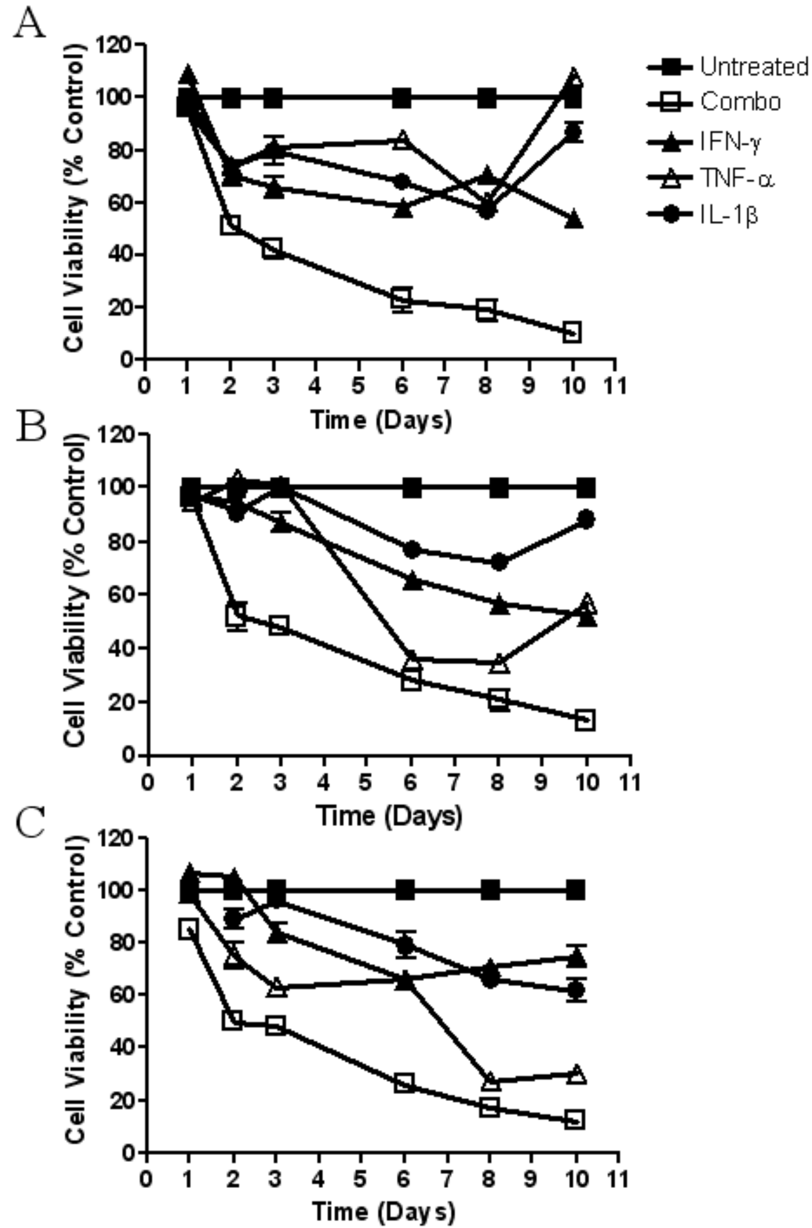


Figure 3.5: The effect of increasing cytokine concentrations on MIN-6 cells over 10 days.

MIN-6 cells were incubated with the cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) and media was changed daily. Cell viability was determined by MTT assay at various time points throughout the 10-day period. The cytokine concentrations used were A) IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) B) at twice this concentration (IFN-γ (768ng/mL), TNF-α (20ng/mL) and IL-1β (4ng/mL)) and C) 5 times more than the initial concentrations (IFN-γ (1920ng/mL), TNF-α (50ng/mL) and IL-1β (10ng/mL)). Results are expressed as mean ± SE, n=3 independent experiments.

After extending the experimental time period to 10 days and changing the cytokine treatment [IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL)] daily, by day 10 approximately 10 ± 3% of the cells remained viable compared to 100% viability of the control cells (*P*=0.0065) (Figure 3.5A). Application of the single cytokines to the MIN-6 cells did not produce the same toxic effect as the triple cytokine combination, however, the viability was significantly affected at all three concentrations of single cytokine treatments (one, two and five times) seen from day 2 onwards ($P<0.05$) (Figure 3.5A, B and C) except on day 10 at concentration one times with single cytokine treatment TNF-α in Figure 3.5A (*P*=0.2).

At twice the cytokine combination concentration (IFN-γ (768ng/mL), TNF-α (20ng/mL) and IL-1β (4ng/mL)) 13 ± 3% of cytokine treated MIN-6 cells remained viable compared to 100% for untreated cells by day 10 (*P*=0.0011) (Figure 3.5B) even after the concentrations were increased by up to five times the initial concentrations (Figure 3.5C) 12 ± 3% remained viable. These experiments indicated that increasing cytokine concentrations beyond the initial concentration did not further reduce cell viability when changed daily over 10 days.

In summary, after titrating the cytokine concentrations on the MIN-6 cell line it was determined that the following concentrations would be suitable for future experiments over 10 days changed daily because they were most toxic to the pancreatic beta cell line when replenished daily in combination IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL).

3.3.1 Viability of liver cell lines Huh7, Huh7ins and Melligen are not affected by the cytokine treatment

After establishing the optimised cytokine treatment (Section 3.3) to reduce the viability of MIN-6 cells, these experimental parameters were applied to Huh7 and Huh7ins and Melligen cells. This was done to determine if the cytokines would have the same detrimental effect on liver cell lines that have been engineered to have a beta-cell-like phenotype i.e., after the stable transfection of the insulin gene alone (Huh7ins cells) or with both the insulin and glucokinase genes (Melligen cells). Whilst the action of IFN-γ is species specific, IL-1β and TNF-α are effective on beta

cells obtained from different species (Ternell & Green, 1993). Hence, hIFN-γ was used on subsequent experiments involving the human liver cell lines.

A significant difference in the susceptibility of MIN-6 cells and the liver cell lines to cytokine-induced toxicity was observed from day 2 ($P<0.05$) (Figures 3.6A, B, C and D). By day 2, MIN-6, Huh7, Huh7ins and Melligen cells that were treated with cytokines had viabilities of 44 ± 5%, 100 ± 2%, 99 ± 3% and 101 ± 7% respectively. Hence, the liver cell lines were more resistant to the toxic effects of the cytokines. The results showed that the untreated MIN-6 cells remained viable over the 10 days of the experiment. Treatment of MIN-6 cells with the triple cytokines for 10 days caused a significant decrease in cell viability (24 ± 2%) when compared to untreated cells, which maintained 100 % viability ($P<0.005$) (Figure 3.4 A).

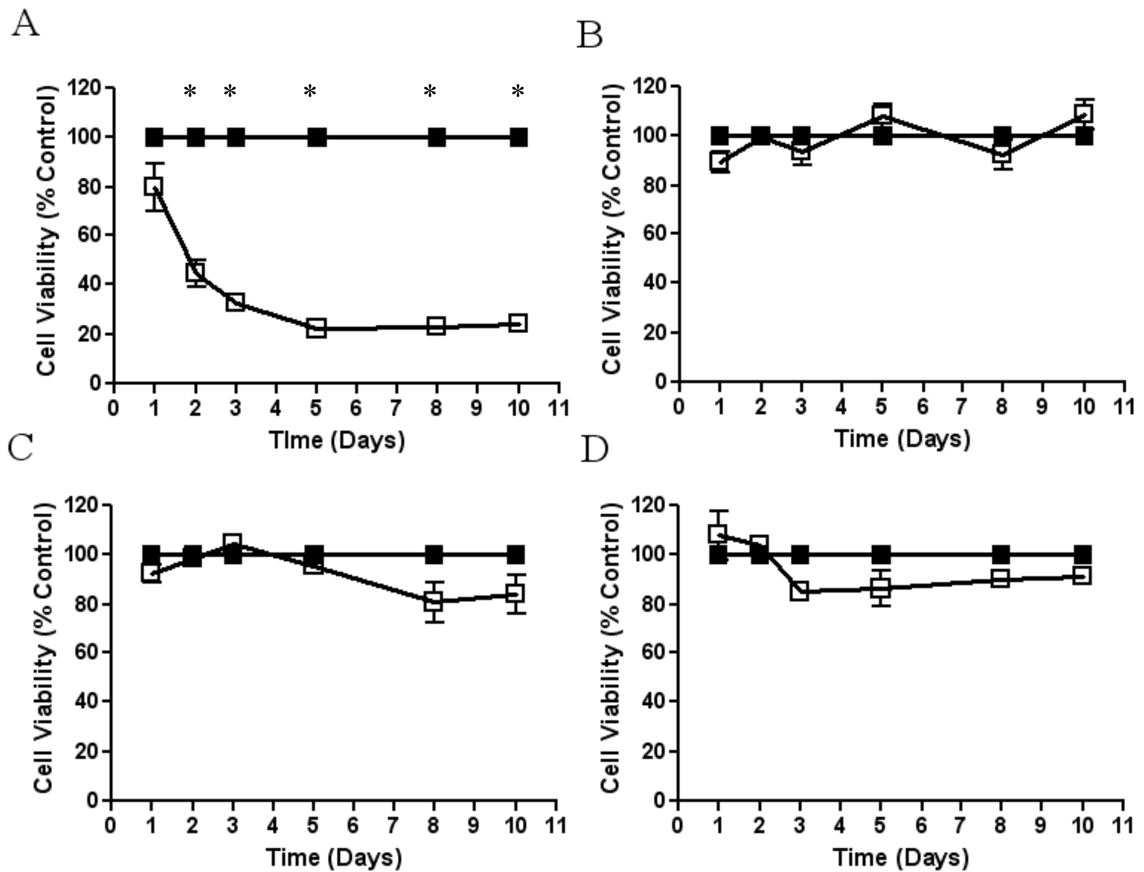


Figure 3.6: Determining the effects of the cytokine treatment on Huh7, Huh7ins and Melligen cells.

*The four cell lines (including MIN-6) were incubated with the cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) with cytokines changed daily over a 10-day period (unfilled squares) and without the cytokine treatment (filled squares). Cell viability was determined by the MTT assay. A) MIN-6 B) Huh7 C) Huh7ins D) Melligen. Results are expressed as mean ± SE, n=4 independent experiments. *P<0.05 for cytokine-treated versus untreated cells.*

When exposed to the cytokine combination for 10 days, Huh7 cells showed no significant decrease in cell viability compared to the control Huh7 cells. Experiments using Huh7ins and Melligen cells also showed that there was no significant decrease in mitochondrial activity after exposure to the triple cytokines over 10 days. These results indicate that the presence of the insulin gene and the glucokinase gene do not alter the susceptibility of the liver cell lines to the pro-inflammatory cytokine treatment.

3.3.2 Liver cell lines maintain cell morphology over 10 days cytokine treatment

In Figures 3.7A, B and C, the untreated MIN-6 cells appeared to have intact cell membranes and remained attached to the plate in colonies growing as monolayers. By day 10, MIN-6 cells were confluent. In contrast, cytokine-treated MIN-6 cells started to degenerate with ruptured membranes causing cells to detach after 6 days (Figure 3.7E). Complete degeneration with cell debris scattered between remaining cells was seen on day 10 (Figure 3.7F).

Co-incubation of Huh7ins and Melligen cells with the cocktail of pro-inflammatory cytokines did not induce morphological changes consistent with cell death after day 1 (Figure 3.8D) or day 6 (Figure 3.8E). At both these time-points Huh7ins and Melligen cells were attached and of uniform size and proliferation was not reduced. By day 10, the cytokine-treated Huh7ins and Melligen cells (Figure 3.8F) were not different in appearance or cell count when compared to the untreated cells (Figure 3.8C). The remaining treated cells appeared intact, attached and there was an absence of cell debris as observed for the cytokine-treated MIN-6 cells. The morphology of both the MIN-6 and liver cell lines corroborates the results obtained from the MTT cell viability assay, which indicated that liver cell lines were more resistant to the toxic effects of the pro-inflammatory cytokines.

A D

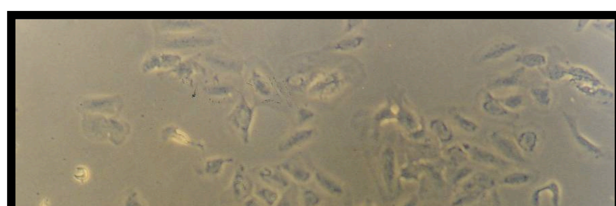
B E

C F

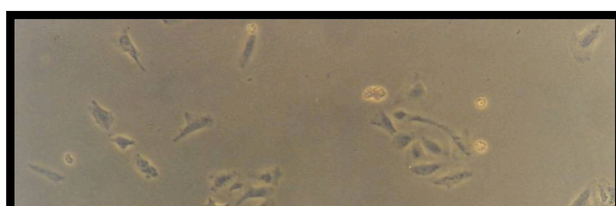
Figure 3.7: The effect of cytokines on the morphology of MIN-6 cells.

Cell morphology of untreated MIN-6 cells following A) 1 day, B) 6 days, and C) 10 days of incubation without cytokines. The cell morphology of MIN-6 cells following cytokine treatment with IFN-γ, TNF-α and IL-1β can be seen in D) 1 day, E) 6 days F) 10 days. (200x magnification).

A



E



B

F

C

G

Figure 3.8: The effect of cytokines on the morphology of Huh7ins cells.

Cell morphology of untreated Huh7ins cells following A) 1 day, B) 6 days, and C) 10 days. The cell morphology of Huh7ins cells incubated with the cytokines IFN-γ, TNF-α and IL-1β following the same time points D) 1 day, E) 6 days, and F) 10 days. (100x magnification). Morphology of the Melligen cells was the same as the Huh7ins cells.

3.4 The effect of cytokines on insulin secretion, storage and glucose responsiveness

Since it has been shown that pro-inflammatory cytokines impair insulin function in beta cell models, the aim of the following section was to determine the effects of the pro-inflammatory cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) on insulin function- insulin secretion, insulin storage and glucose responsiveness in the two insulin secreting liver cell lines, Huh7ins and Melligen cells.

3.4.1 Chronic insulin secretion of Huh7ins and Melligen cells is not affected by the pro-inflammatory cytokine cocktail

For the effects of cytokines on chronic insulin secretion media was collected every day for 10 days with cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) changed daily. Insulin concentration was determined by radioimmunoassay (RIA) (See Chapter 2 Section 2.3.3). To determine insulin storage, the cells were treated for 10 days and insulin was extracted using acid ethanol. Viable cells were counted using trypan blue exclusion. For the determination of glucose responsiveness after 10 days of cytokine exposure, acute insulin secretion to 20 mM glucose was measured by static incubation experiments and insulin was quantified by the RIA method.

Cytokine-treated MIN-6 cells secreted significantly less insulin (7 ± 3pmol/well) than untreated MIN-6 cells (39 ± 2pmol/well) at day 1 (P=0.012) and throughout the entire period studied (Figure 3.9A). Insulin levels for cytokine-treated MIN-6 cells at day 10 represented the total amount of insulin secreted over the entire 10 days. In contrast, Huh7ins cells co-incubated with cytokines secreted 0.37 ± 0.03pmol/well of insulin that was not significantly different to amounts of insulin (P=0.357) secreted by the untreated Huh7ins cells (0.40 ± 0.02pmol/well) by day 10 (Figure 3.9B). Similarly there was no significant difference at day 10 between the untreated Melligen cells, secreting 0.15 ± 0.01pmol/well, and treated Melligen cells, secreting 0.14 ± 0.02pmol/well (P= 0.496) (Figure 3.9C). However, it should be noted that the amount of insulin secreted by the Huh7ins cells by day 10 was nearly four-fold the amount secreted by the Melligen cells at day 10.

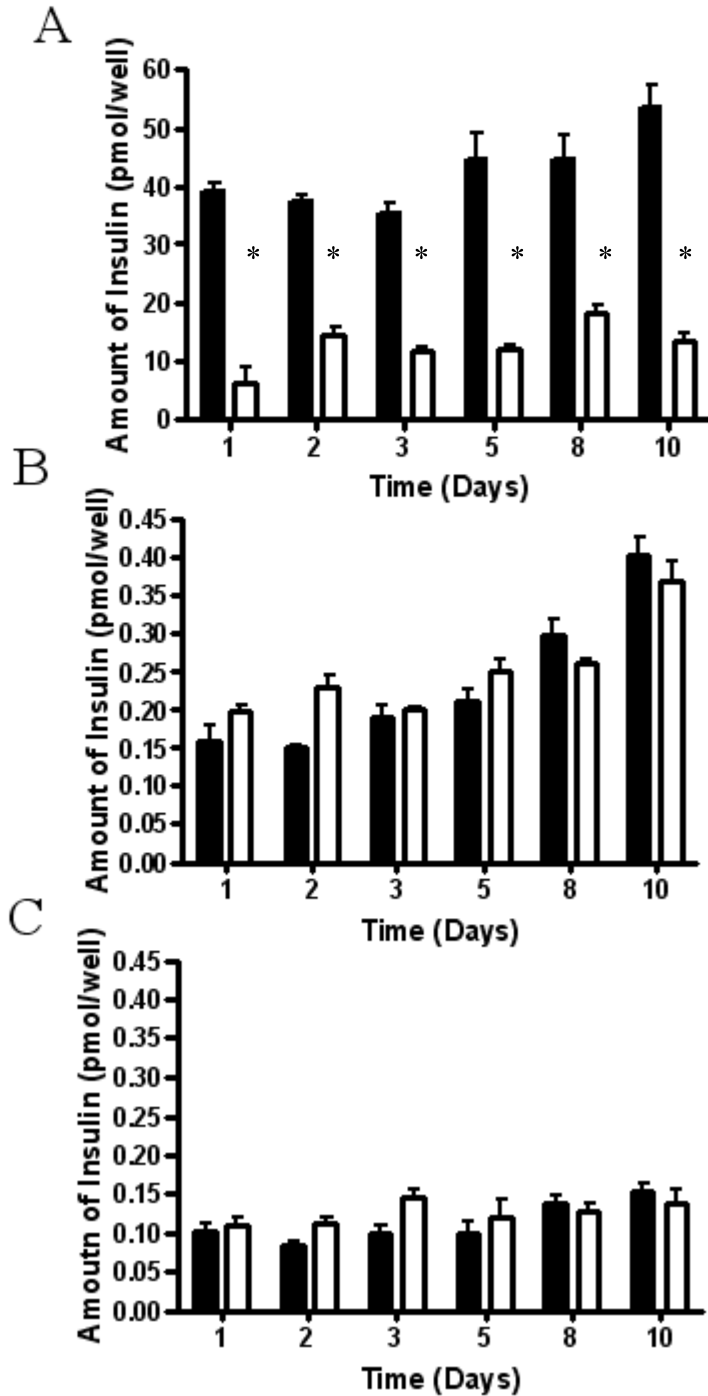


Figure 3.9: The effect of cytokine treatment on MIN-6, Huh7ins and Melligen chronic insulin secretion.

*Total chronic insulin secretion after incubation with (unfilled bars) and without (filled bars) the cytokine cocktail IFN-γ, TNF-α and IL-1β for 1, 2, 3, 5, 8 and 10 days A) MIN-6 cells B) Huh7ins cells C) Melligen cells. Results are expressed as mean ± SE, n=3 independent experiments. *P<0.05 for cytokine-treated versus untreated cells.*

Throughout the duration of this study, trypan blue exclusion studies were performed (Figure 3.9.1A) to assess cell viability showing treated MIN-6 cell viability does not increase over time compared to the untreated cells. Significant differences in untreated and treated MIN-6 cells start at day 2 (P=0.03) onwards. In addition, there is no difference seen between the untreated and treated Huh7ins and Melligen cells (Figure 3.10B and C). The cell samples used for the Trypan blue exclusion method were obtained from the cells cultured for insulin secretion and storage experiments.

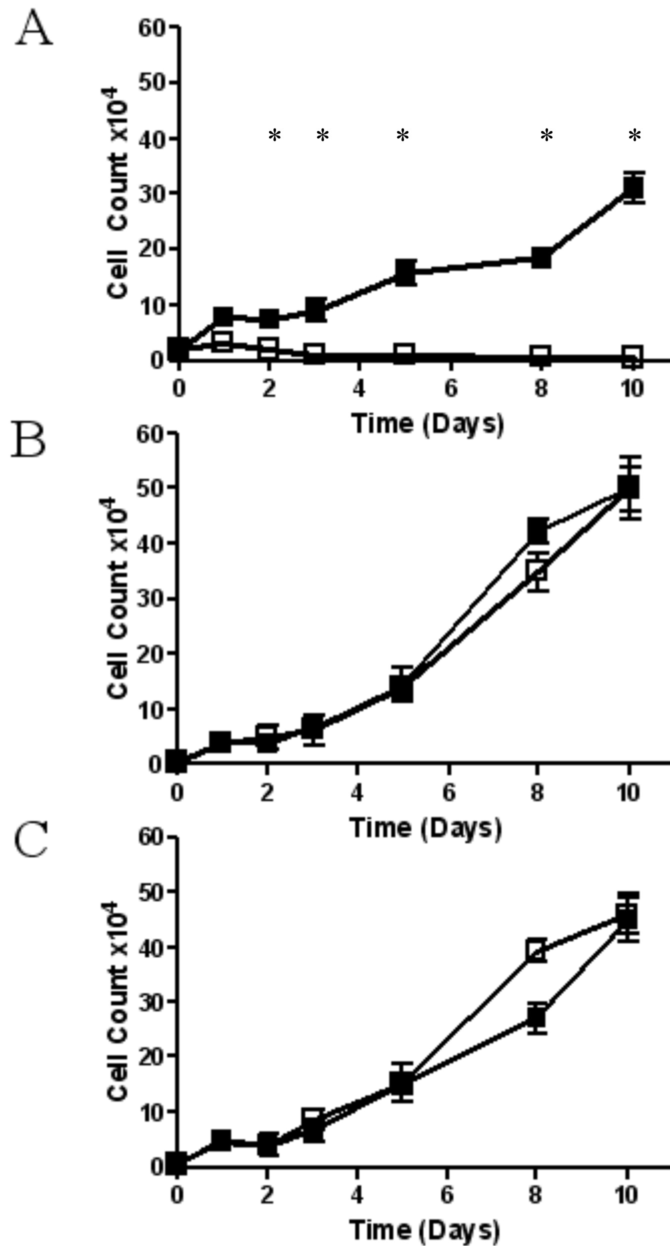


Figure 3.10: Cell counts determined by Trypan blue exclusion method for treated and untreated MIN-6, Huh7ins and Melligen cells.

*Trypan blue exclusion studies were performed on cytokine treated (unfilled squares) and untreated (filled squares) A) MIN-6 B) Huh7ins and C) Melligen cells over a 10-day period. Results are expressed as mean ± SE, n=3 independent experiments. * P<0.05 for cytokine-treated versus untreated cells.*

3.4.2 Insulin storage of Huh7ins and Melligen cells remain unaffected by the pro-inflammatory cytokine cocktail

MIN-6, Huh7ins and Melligen cells were treated over 10 days with the cytokine cocktail, IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL). Stored insulin was extracted using the acid ethanol method (Chapter 2 Section 2.3) and amounts of insulin determined by RIA on days 1, 2, 3, 5, 8, and 10. Statistically significant results were determined by ANOVA. MIN-6 cells were significantly affected by the cytokine treatment at day 2 (P=0.0017), with cells storing 52.6 ± 0.97pmol/well insulin in the untreated sample compared to 20.2 ± 2.24 pmol/well for treated cells. In contrast, Huh7ins and Melligen cells retained their ability to store insulin over the entire 10 days without significant difference between the treated and untreated cells at day 10 (P=0.257 and 0.82 respectively).

From Figure 3.11A it can be seen that insulin storage per well steadily increased in untreated MIN-6 cells over the 10 days of the experiment as the cells proliferated. After exposure of MIN-6 cells to the cytokine cocktail over 10 days, insulin content was significantly diminished after day 1 compared to the control MIN-6 cells (*P*=0.017). The difference between the treated and untreated MIN-6 cells continued to be significant throughout the remaining days of the experiment with untreated cells and treated cells reaching 81.9 ± 0.5 pmol/well and 21.2 ± 1.7 pmol/well stored insulin by day 10, respectively. In contrast, Huh7ins and Melligen cells treated with the cytokine combination did not show a significant difference in insulin storage compared to the untreated Huh7ins and Melligen cells, respectively.

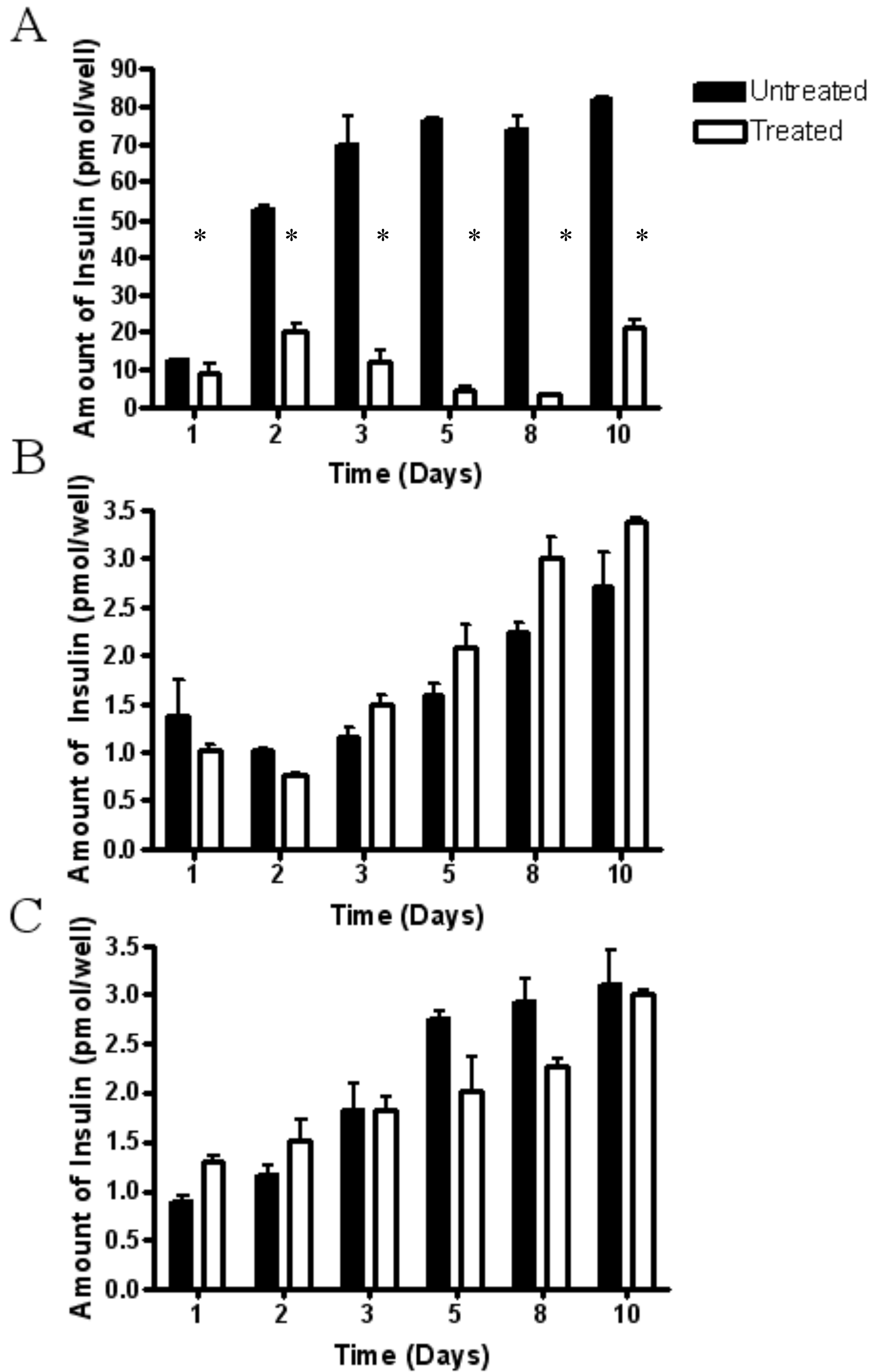


Figure 3.11: The effect of cytokine treatment on MIN-6, Huh7ins and Melligen insulin storage.

*Insulin storage in A) MIN-6 cells B) Huh7ins cells and C) Melligen cells after incubation with (unfilled bars) and without (filled bars) the cytokine mixture IFN-γ, TNF-α and IL-1β for 1, 2, 3, 5, 8 and 10 days. Results are expressed as mean ± SE, n=3 independent experiments. *P<0.05 for cytokine-treated versus untreated cells.*

3.4.3 Glucose responsiveness of Huh7ins and Melligen cells remain unaffected by the pro-inflammatory cytokine cocktail

After 10 days of cytokine treatment, MIN-6 cells showed a significant decrease in insulin response to a glucose stimulus when compared to the control MIN-6 cells (P=0.0009). Insulin secretion of untreated MIN-6 cells in response to 20mM glucose increased more than 5-fold over 1h when compared to basal levels (4 ± 2pmol/well/h) (*P*=0.0002) (Figure 3.12A). The treated MIN-6 cells did not release significantly higher levels of insulin to the glucose stimulus compared to basal levels (*P*=0.60) (Figure 3.12A). This observation is likely attributable to these cells exhibiting reduced viability after 10 days of co-incubation with cytokines, whereas control cells continued in the log growth phase as indicated by trypan blue exclusion (Figure 3.10A). By day 10, cell counts were $3x10^6$ cells/well for untreated MIN-6 cells and $3x10^3$ cells/well for treated MIN-6 cells. Therefore, at day 10 there were more control cells and hence greater insulin storage.

The effect of the pro-inflammatory cytokines on glucose-responsiveness was also determined for the Huh7ins and Melligen cells. Untreated Huh7ins and Melligen cells gave a 5-fold increase in insulin secretion when stimulated with 20mM glucose, with return to basal levels of insulin secretion (0.13 ± 0.014pmol/well/h) upon removal of the glucose stimulus (Figure 3.12B and C). Huh7ins and Melligen cells incubated with cytokines for 10 days showed a 4.5-fold increase in insulin secretion upon the 20mM glucose stimulus and a return to basal levels of secretion (0.06 ± 0.006pmol/well/h) within 1h after stimulation (Figure 3.12B and C). The amount of insulin secreted by the treated Huh7ins and Melligen cells during the stimulus was not significantly different to the results obtained for the untreated Huh7ins and Melligen cells, respectively. This indicates that Huh7ins and Melligen cells retain the ability to respond to a glucose stimulus even after 10 days of cytokine treatment. Control basal samples collected during the course of the experiment showed no significant increase in amounts of insulin (Figure 3.13).

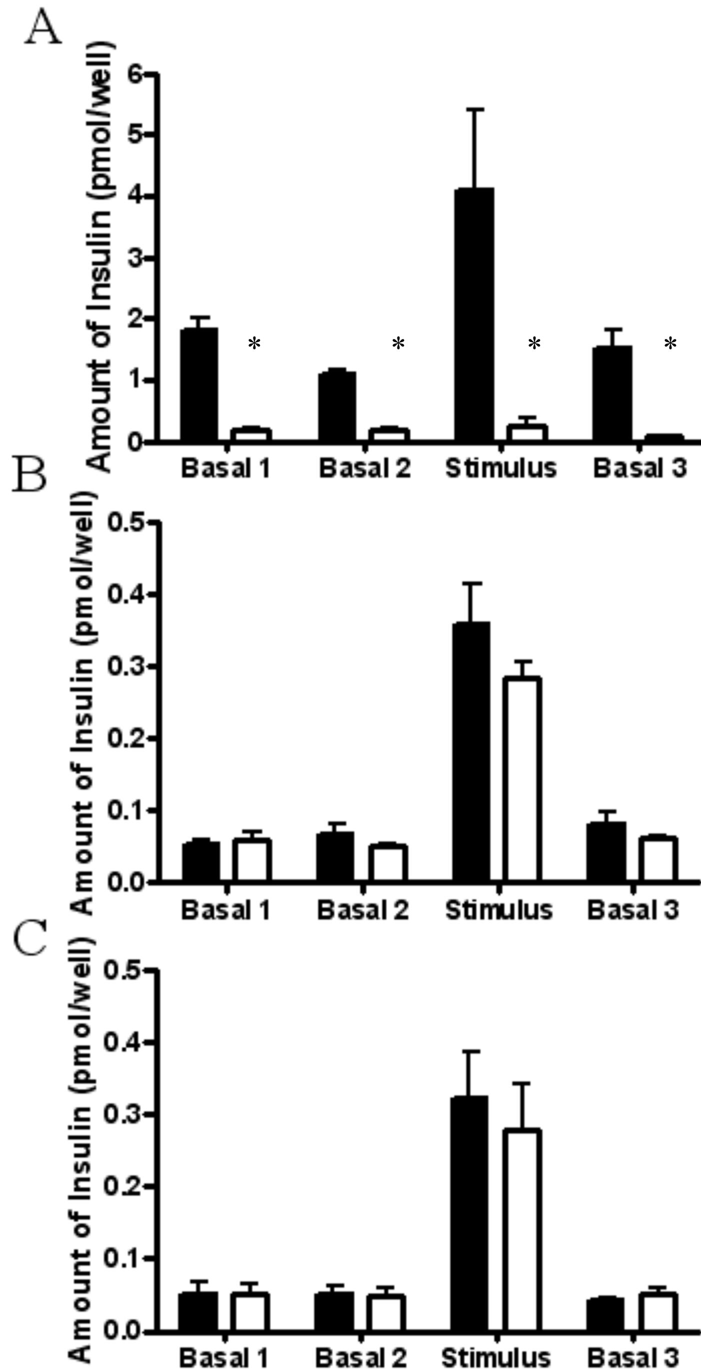


Figure 3.12: The effect of cytokine treatment on glucose responsiveness in MIN-6, Huh7ins and Melligen cells.

*Glucose responsiveness was determined using 20mM glucose stimulus with (unfilled bars) and without (filled bars) cytokine treatment. A) MIN-6 cells B) Huh7ins cells and C) Melligen cells after 10 days incubation with the cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL). Results are expressed as mean ± SE, n=3 independent experiments. * P<0.05 for cytokine-treated versus untreated cells.*

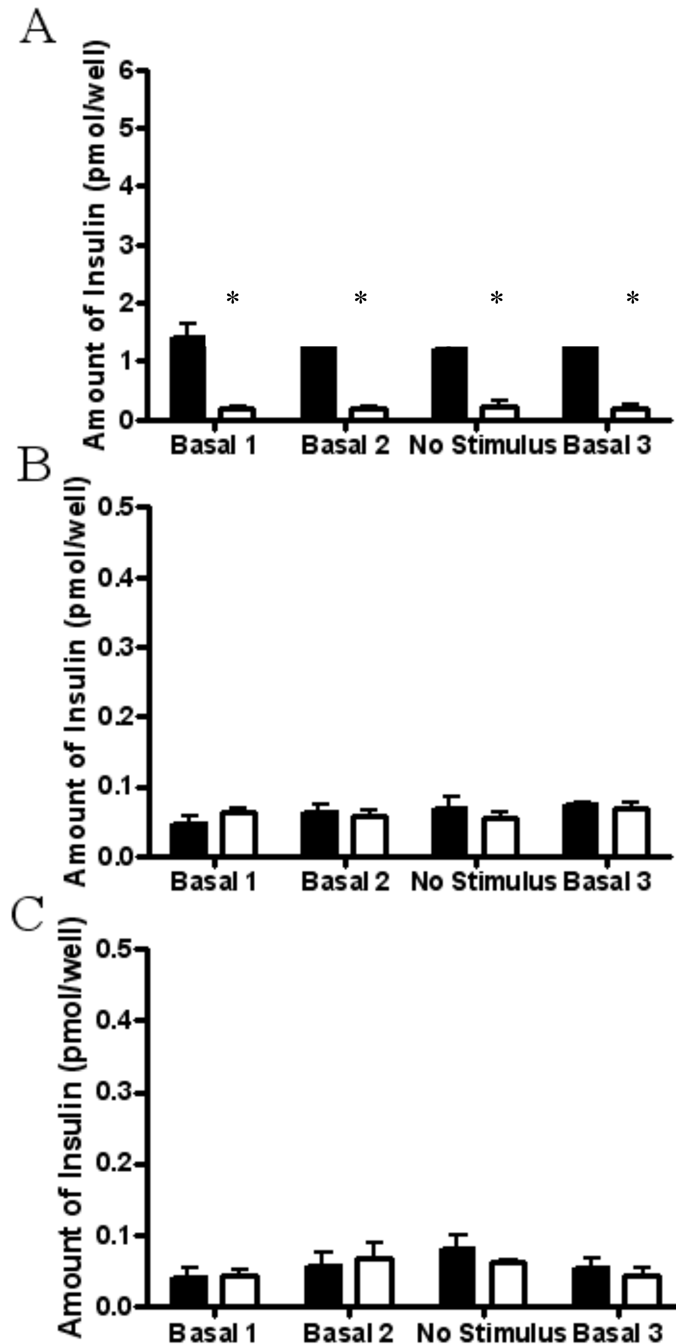


Figure 3.13: Unstimulated control MIN-6, Huh7ins and Melligen cells for basal readings.

*Glucose Responsiveness parallel basal readings without using 20mM glucose stimulus (negative control) with (unfilled bars) and without (filled bars) cytokine treatment. A) MIN-6 cells B) Huh7ins cells and C) Melligen cells after 10 days incubation with the cytokines IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL). Results are expressed as mean ± SE, n=3 independent experiments. * P<0.05 for cytokine-treated versus untreated cells.*

To determine if the response of the Huh7ins and Melligen cells to millimolar amounts of glucose remains unaffected by the cytokine treatment, Huh7ins and Melligen cells were cultured for 10 days with and without cytokines. At day 10, the cells were stimulated with increasing concentrations of glucose in basal medium. Huh7ins cells secreted increased amounts of insulin in response to 2.5mM glucose (Figure 3.14A) and Melligen cells at 4.5mM glucose (Figure 3.14B). In both cell lines there was no significant difference observed between cytokine treated and untreated cells at any glucose concentration ($P>0.05$).

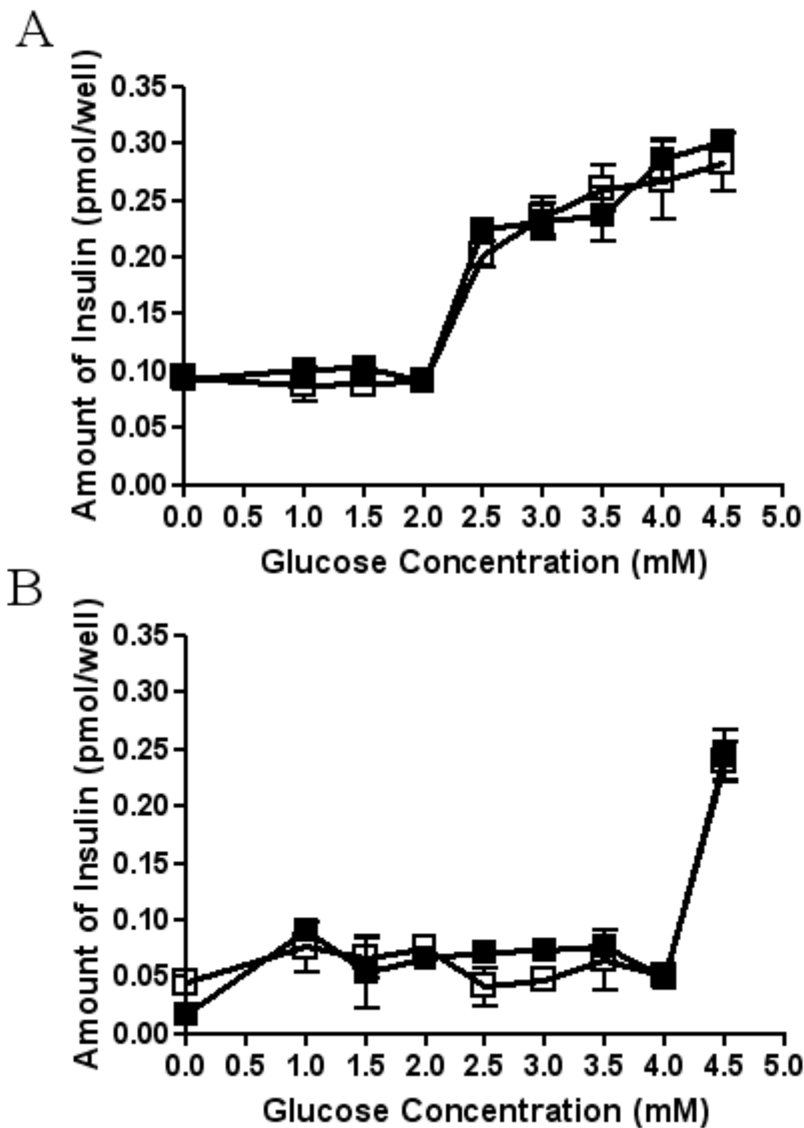


Figure 3.14: The effect of cytokines on the glucose responsiveness of Huh7ins and Melligen cells in the millimolar range.

10-day cytokine treatment did not affect A) Huh7ins and B) Melligen cell responsiveness to glucose in the millimolar range with (unfilled bars) and without (filled bars) the cytokine mixture IFN-γ, TNF-α and IL-1β. Melligen cells secrete insulin in response to 4.5mM glucose (the physiological range) and Huh7ins cells to 2.5mM glucose. Results are expressed as mean ± SE, n=3 independent experiments.

3.5 Discussion

From this study it has been established that Melligen cells are resistant to the effects of the cytokine cocktail over the 10-day period studied. The chronic insulin secretion and storage of MIN-6 (control pancreatic beta cell line) cells were significantly affected by the cytokine treatment at day 1 ($P<0.05$) and glucose responsiveness was diminished after the 10 days of treatment ($P<0.05$). Huh7ins and Melligen cells in the insulin secretion, storage and glucose responsive experiments showed no significant difference compared to the untreated cells after exposure to the cytokines. Cell counts performed on all three hepatocyte cell lines using trypan blue exclusion confirmed that both cytokine treated and untreated cell counts were similar even after 10 days. Glucose responsive data showed that the control cells responded to the 20mM glucose stimulus, confirming that MIN-6 cells constituted an appropriate glucose responsive beta cell model.

A major cue for beta cell death during the development of Type I diabetes occurs when the cytokine balance is skewed in favour of pro-inflammatory cytokines. Testament to these studies spontaneous animal models of autoimmune diabetes have shown that increased levels of anti-inflammatory cytokines (either through transgenic expression or exogenous delivery) preserve beta cell mass and prevent diabetes development (Goudy et al., 2003; Park et al., 2003; Choi et al., 2004).

In vitro, TNF-α only fails to inhibit insulin secretion in rat and human beta cells, although some studies report inhibition in murine cells. However, TNF-α in combination with IL-1 has an additive inhibitory effect in rat beta cells, which probably reflects a potentiated inhibition of the glucose metabolism Mandrup-Poulsen et al., 1986; Eizirik, 1993). It should be noted that the effects of IL-1 on the human pancreatic beta cells may differ to those outlined for the rat or mouse beta cells. Using combinations of cytokines, both beta cell inhibitory actions and cytotoxic actions have been reported regularly (Wachlin et al., 2003; D'Hertog et al., 2007; McKenzie et al., 2008). Thus several cytokine concentrations seem to act in an additive or synergistic manner. In particular the presence of IFN-γ appears to contribute to such an effect. One important observation is that combinations of cytokines can be inhibitory and even cytotoxic to human beta cells (Eizirik et al., 1993; Corbett et al., 1993;

Rabinovitch et al., 1998). Of the individual cytokines, TNF-α is one of the most important death effector molecules, however, most primary or immortalised cells are not susceptible to apoptosis by TNF-α alone because of concomitant activation of the anti-apoptotic process by TNF-α (Beg et al., 1996). In a study by Lee et al., (2004) a combination of IFN-γ and TNF-α, but not either cytokine alone, induced apoptosis of murine insulinoma and pancreatic islet cells as indicated by Hoechst staining, DNA ploidy assay, electron microscopy, and DNA fragmentation pattern. The results of the current study confirm previous investigations using alternative murine pancreatic beta cells that were exposed to similar cytokine combinations and found that insulin content was decreased and glucose-stimulated insulin release was inhibited (Wachlin et al., 2003). Wu et al. (2001) reported that glucose-stimulated insulin release of MIN-6 cells was not suppressed by the individual cytokines, however, significant suppression of glucose-stimulated insulin release occurred when cells were exposed to all three pro-inflammatory cytokines. Tabiin et al. (2001) found that cell viability, insulin storage and insulin secretion of NIT-1 cells were adversely affected by incubation with the triple cytokines at similar concentrations to those used in the current study.

The toxic effects of the autoimmune cytokines on MIN-6 cells have also been investigated in several other studies. These studies have indicated that similar to beta cells in type I diabetes, MIN-6 cells also express major histocompatibility complex II antigen expression in response to cytokine treatment, thereby increasing their susceptibility to immune attack (Cucca et al., 2003; Kim et al., 2002; Ishihara et al., 1993). Consistent with these observations, in the current study the use of the triple cytokine mixture led to a greater reduction in the viability of MIN-6 cells. Using single cytokines did have a significantly marked effect on the MIN-6 cells from day 2 onwards, however, the cytokine cocktail had the most toxic effect (Figure 3.5). In contrast to the reduced viability of MIN-6 cells observed after 2 days co-culture with cytokines, Huh7ins and Melligen cells were resistant to the toxic effects of the pro-inflammatory cytokines even after 10 days co-incubation. Likewise the parent cell line, Huh7, was resistant to the toxic effects of the cytokines. Thus, the expression of insulin by Huh7ins cells does not render them susceptible to cytokine cytotoxicity. Insulin secreting liver cells such as Huh7ins and Melligen may exhibit increased resistance to cytokine treatment, as they are not of beta cell origin. These results

suggest that the resistance to pro-inflammatory cytokine-induced damage may be inherent to liver cells.

The results of this study corroborate previous findings that liver cell lines transfected with the insulin gene are less susceptible to the toxic effects of pro-inflammatory cytokines that decrease the viability and function of both primary beta cells and beta cell lines. For instance, Tabiin et al. (2001) found that the autoimmune cytokines, IFN-γ, TNF-α and IL-1β, did not affect the viability or glucose-induced insulin secretion of genetically engineered hepatocytes, HEPG2ins/g cells, after co-incubation for up to 6 days. However, similar to the current study the presence of insulin in the HEPG2ins/g cells did not increase their susceptibility to cytokine attack. Lipes et al. (1996) generated transgenic nonobese diabetic (NOD) mice (a spontaneous model of autoimmune diabetes) in which insulin expression was targeted to proopiomelanocortin-expressing pituitary cells. The proopiomelanocortin-expressing intermediate lobe pituitary cells efficiently secreted fully processed, mature insulin via a regulated secretory pathway, similar to islet beta cells. Importantly, they found that the insulin-secreting surrogate cells were unaffected by the autoimmune process even after the destruction of beta cells in the inflamed pancreatic islet of the NOD mice. The absence of autoimmune infiltration in intermediate lobe pituitary cells engineered to secrete insulin may have been attributable, in part, to the immune privileged nature of pituitary cells. However, being of pituitary origin these cells have the disadvantage of also secreting adenocorticotropin as a major endogenous secretory product *in vivo* (Lipes et al., 1996). In contrast, being non-endocrine in nature, insulin secreting Huh7ins liver cells, that have been shown to be resistant to the toxicity of the cytokines in the current study, may be more suitable candidates for surrogate beta cells.

Also a preliminary study by Tabiin et al. (2002) has addressed the problem of autoimmune attack of insulin-secreting liver cells by MHC class I molecules in an animal model. Non-obese diabetic mice, which express insulin in the liver, were made using the PEPCK promoter to drive mouse insulin. The mice were found to express insulin and release it in response to stimuli. However, no infiltrate of immune cells was found indicating that insulin expressing liver cells are not subject to autoimmune

attack. These findings provide encouraging *in vivo* evidence of the potential of surrogate cells for gene therapy in Type 1 diabetes.

Zhang et al. (2009) administered an adenoviral vector capable of inducing glucose responsive insulin production from hepatocytes in diabetic STZ CD-1 mice. After evaluating circulating cytokines it was found that elevated TNF-α may have played a role in diminishing insulin stimulation of glycogen synthesis. The TNF-α elevations were specifically associated with insulin transgene delivery, rather than adenoviral exposure, because the values in mice treated with empty vector remained similar to control mice. Although insulin has been implicated as an auto-antigen in Type 1 diabetes (Mannering et al., 2009), these insulin secreting liver cells were resistant to the autoimmune processes. Although these findings suggest that insulin is not a pathogenic target of autoimmunity in NOD mice, the requirements for any protein, including insulin, to be recognised as an auto-antigen in Type 1 diabetes is unknown. Wong et al (2009) have evidence to the contrary and have shown that insulin stimulates $CD8^+$ T cells.

The responses of human islets following cytokine treatment are less predictable than those of rat. IL1-β on its own, applied for 48h, has been shown to stimulate (Eizirik et al., 1993) and inhibit (Vara et al., 1994) insulin secretion from human islets, but when applied for a period of 6 days, either was without effect or inhibited secretion. TNF-α or IFN-γ inhibited secretion when applied for 2 to 6 days individually or in combination on human islets (Kawahara et al., 1991; Soldevila et al., 1991), as did IL-1β and TNF-α when used for 2 or 4 days. A combination of the three cytokines was used here and was found to have an inhibitory effect on the insulin secretion, storage and glucose-responsiveness of MIN-6 cells taking effect after day 1 and 2 but this effect was not observed in the Huh7ins and Melligen cells. Differential sensitivity of rat versus human islets to inhibitory and cytotoxic effects of cytokines or chemical treatment has been reported (Eizirik et al., 1993; Eizirik et al., 1994). Although human islets have been shown to be more resistant than rat islets to alkylating agents and superoxide, they have been shown to be equally affected by nitric oxide donors (Eizirik et al., 1994; Eizirik et al., 1996). Corbett et al. (1993) reported that the cytokine combination stimulated secretion at 24h, while higher doses inhibited

secretion. So it may be possible that exposure of the insulin secreting liver cell lines to extremely high concentrations of cytokines could inhibit insulin secretion, however this may also be related to general cell toxicity (necrosis), and not associated with autoimmune attack (apoptosis).

Retaining viability in the presence of pro-inflammatory cytokines, while necessary is not sufficient; surrogate beta cells must continue to synthesise and secrete insulin in a regulated manner. Treatment of Huh7ins and Melligen cells with the triple cytokines for 10 days did not cause a decrease in insulin content and chronic secretion. In contrast, similarly treated MIN-6 cells secreted and stored a decreased amount of insulin by day 1 whereas untreated MIN-6 cells stored and secreted insulin over the 10-day period.

When islet beta cells were exposed to the triple cytokine treatment the total insulin output of the islets in response to both low and high glucose stimulation was significant (Wachlin et al., 2003). However, it has been suggested that the synergistic action of all three cytokines caused injury of islet cell membrane integrity and that this could account for insulin leakage and for the total insulin output of the islets in response to both low and high glucose. This may also explain the presence of insulin in the later days of the experiments even when the MTT assay confirmed lower MIN-6 cell viability. This proposal is supported by morphological examination of cytokine-treated MIN-6 cells, which indicated significant cell lysis.

Beta cells by their nature are susceptible to autoimmune cytokine attack due to the low levels of superoxide dismutase and other free radical scavenging enzymes such as glutathione peroxidase and thioredoxin that protect from cytokine-induced stresses (Tiedge et al., 1997). One possible explanation for the resistance of the liver cell line is the presence of a protective mechanism in the cells including increased levels of antioxidative enzymes such as catalase (Tiedge et al., 1997). Lortz et al. (2000) showed that over-expression of oxidative enzymes in RINm5F cells (a rodent insulinoma cell line) conferred protection to cytokine-mediated toxicity. Tabiin et al. (2001) showed that cytokine treated insulin-secreting liver cells (HEPG2ins/g cells) expressed higher levels of these oxidative enzymes compared to the pancreatic beta cell line (NIT-1). An investigation of the gene expression of anti-apoptotic genes and

anti-oxidative enzymes in Melligen cells would be beneficial to determine if the protective mechanisms involved in cytokine resistance are related to up-regulated defence mechanisms in the liver cell lines.

In a study by Vadrot et al., (2006) various readouts of apoptosis were analysed in the following liver cell lines Hep3B, Chang-Liver, HepG2 and Huh7 and the cells were incubated with or without IFN-γ (100U/mL) for 1-48h. This dose was chosen after preliminary experiments in which higher doses (1000-5000U/mL) were tested without supplemental effect. Similar results were obtained in this study when titrating the cytokines at five times and ten times the cytokine concentration in that a further decrease in MIN-6 cell viability was not observed. After incubation with IFN-γ for 48h, Vadrot et al (2006) in examining the differences between various liver cell lines, showed that only Hep3B and Chang-Liver cells had numerous detached cells in the supernatant, with a decrease in adherent cells in the layer and characteristic nuclear alterations of apoptosis after DAPI staining. This group did not observe apoptosis after IFN-γ treatment in either HepG2 or Huh7 cells, hence supporting the cell viability results obtained in this study.

Results obtained from the chronic insulin secretion experiment performed on Melligen cells (Figure 3.9C) showed that although there was no difference between treated and untreated cells over 10 days, both cell populations (treated and untreated) secreted lower amounts of insulin compared to Huh7ins cells. Huh7ins cells showed increased chronic insulin secretion with increased numbers of cells as compared to the Melligen cell line. Furthermore, insulin storage increased with cell count in both Melligen and Huh7ins cells over the entire 10 days. A contributing factor to this trend may be the lower levels of constitutive insulin secreted by the Melligen cells in the DMEM, which may be the consequence of the insertion of the islet GK gene. However, insulin storage results between the cell lines remain the same. Another contributing factor to the lower levels of insulin detected in the Melligen cells compared to the Huh7ins cells could be insulin degradation. Since the liver is the primary site of insulin degradation Huh7ins cells have been found to degrade the insulin they secrete (Tuch et al., 2003) and Melligen cells may be degrading more insulin than the Huh7ins cells, hence causing a decrease in the amount of insulin detected in the chronic insulin secretion experiments.

Beta cells possess several characteristics that increase their susceptibility to cytokine-induced toxicity. Presumably, many of these characteristics may be absent in Huh7ins and Melligen cells. Beta cells carry many auto-antigens (Eisenbarth et al., 2002) whereas the transformed liver cells carry only one putative auto-antigen, insulin. Since insulin auto-antigens are expressed on the surface of pancreatic beta cells in Type I diabetics (Ziegler et al., 1999), there is the possibility of activating an autoimmune response upon transplantation of insulin-producing cells. However, the current study suggests that insulin-expressing Huh7ins cells may withstand cytokine-mediated damage. Huh7ins cells may carry the advantage of only expressing insulin and not the other auto-antigens of native beta cells (Colman et al., 2000). Additionally, since the viabilities of Huh7, Huh7ins and Melligen cells in the presence of cytokines are similar, this suggests that it is something inherent to the liver cell that enables it to resist cytokine attack and the expression of insulin in these liver cells does not adversely affect viability.

3.6 Conclusion

Huh7ins and Melligen cells have been shown to be resistant to proinflammtory cytokines and are particularly suited for delivering insulin *in vitro*. These cells are highly resistant to the cytokine cocktail, which is otherwise toxic to the beta cells. Therefore, they should be resistant to damage or destruction by pro-inflammatory cytokines *in vivo*, however, because other destructive immune mechanisms also operate *in vivo*, futher experiments in animal models are warranted. It needs to be determined, however, if these cells, even if non-autologous do not trigger the normal immune response when transplanted. Other experiments which could be performed include testing whether insulin autoantigens are expressed at the cell surface of Melligen cells.

CHAPTER 4: Investigation of the Molecular Mechanisms that Render Huh7ins and Melligen Cells Immune from Cytokine-Induced Cell Death

4.1 Introduction

A promising strategy to cure Type1 diabetes is to replace the beta cell mass that has been destroyed by the autoimmune process, which would restore regulated endogenous insulin secretion. The restoration of beta cell mass is currently most commonly accomplished through the transplantation of pancreas or islets from allogeneic donors. However, current islet-transplant protocols require large numbers of islets (two or more donors for each recipient and often more than one transplant is required to maintain long-term normoglycaemia) to reverse diabetes (Shapiro et al., 2000). A relatively large mass of islets must be transplanted to overcome islet loss due to hypoxic conditions, which follow islet delivery while the graft becomes vascularised. Additionally, to prevent host immunity leading to rejection of the allograft and the recurrence of auto-immunity, immunosuppressive drugs must be taken by the patient, however, most are cytotoxic to beta cells (Sato et al., 2003). Thus, several gene therapy based approaches, have been explored, using multiple tissue types (see Section 1.6.1.2.2 for review in an attempt to i) generate a sufficient mass of cells to produce, store and release insulin in response to physiologic stimuli and ii) protect the insulin-secreting cells from recurrent autoimmunity.

Mature hepatocytes are glucose-sensitive and share similarities in gene expression with mature beta cells. In addition, hepatocytes are a primary target of insulin action, which is delivered from the pancreas through the portal vein (Efrat, 2008). Although, hepatocytes are not equipped with a regulated secretory pathway, they have the glucose sensing apparatus responsible for the regulation of insulin in the beta cell. It is believed that Melligen cells have a regulated secretory pathway, which is associated with the pancreatic transdifferentiation and secretory granule biogenesis (see Section

1.7). In Chapter 3 it was determined that the Melligen cells were resistant to the toxic effects of pro-inflammatory cytokines that are involved in the process of eliminating beta cell mass. Further, Melligen cells continued to store and secrete insulin in response to physiological levels of glucose even after cytokine exposure. Melligen cells have the ability to reverse diabetes and resist the autoimmune responses that normally destroy pancreatic beta cells (Simpson et al., 2010), therefore, it needs to be determined if the cells are protected from the apoptotic mode of cell death caused by the pro-inflammatory cytokine treatment, which plays a role in beta cell death.

During the development of Type 1 diabetes, immune cells, notably macrophages and T cells, infiltrate the islets (insulitis) (Eizirik et al., 2009). As insulitis progresses, macrophages become classically activated and secrete significant amounts of pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ, which play an important role in islet beta cell apoptosis (Rabinovitch, 1993; Mandrup-Poulsen, 1996). IL-1β alone, or in combination with IFN-γ, has been shown to induce the expression of an inducible form of iNOS, leading to increased levels of NO and increased production of Th1 cytokines (Borjesson et al., 2006; Corbett et al., 1993; Jorns et al., 2005; Mendez et al., 2004). Pancreatic beta cells express constitutive and inducible nitric oxide synthases (cNOS and iNOS), the enzymes that synthesise NO from L-arginine (Eizirik et al., 1992; Schmidt et al., 1992). Elevated expression levels of iNOS have been associated with the development of several autoimmune diseases (Kroncke et al., 1995; Cook & Cattell, 1996). Additionally, iNOS is expressed by islet cells and/or infiltrating macrophages in the insulitic lesion in rodent models of Type 1 diabetes, such as the diabetes-prone BioBreeding (BB) rat and NOD mouse (Kleeman at al., 1993; Rothe et al., 1994; Rabinovitch et al., 1996). The activation of iNOS and the accumulation of NO represent a central mechanism by which beta cells are destroyed after cytokine exposure (Delaney et al., 1996; Southern et al., 1990). The increased susceptibility of beta cells to the detrimental effects of NO, as compared to many other cell types, is attributable, in part, to their poor antioxidant defense status, given that they express low levels of antioxidant enzymes involved in metabolizing reactive oxygen species (Roma et al., 2009).

In vitro studies have demonstrated that AdV-mediated delivery of the gene encoding IL-1 receptor antagonist (IL-1Rα) into human islets protects against IL-1β-induced NO formation and inhibition of Fas-dependent beta cell apoptosis (Giannoukakis et al., 1999). The utility of this approach has been confirmed by *in vivo* studies, in which the overproduction of IL-1Rα in transduced islet grafts leads to increased replication of the islet graft mass and a reduction in the number of islets required to achieve normoglycaemia after syngeneic islet transplantation in streptozotocin-induced diabetic Lewis rats (Tellez et al., 2005; Tellez et al., 2007). Graft function was also prolonged in non-obese diabetic-severe combined immunodeficiency (NOD-*Scid*) mice transplanted with islets modified to express IL-1Rα (Bertera et al., 2004). However, islets deficient in the ability to express functional iNOS are still susceptible to the development of insulitis and do not extend the period of normoglycaemia post-transplantation, as compared to islets producing iNOS (Borjesson et al., 2006). This suggests that, in addition to the down-regulation of iNOS, IL-1β antagonists reduce inflammation through other pathways. To further highlight the varying detrimental effects of cytokines on beta cells, studies using the beta cell line INS-1E, have confirmed that iNOS gene silencing did not totally abrogate the apoptotic effects of the pro-inflammatory cytokine cocktail treatment *in vitro* (De Paula et al., 2007). Targeting the inflammatory cytokine TNF-α using AdV-mediated transfer of a gene encoding a soluble type 1 TNF receptor decoy can similarly prevent beta cell apoptosis (Machen et al., 2004) (See Section 1.3.1.1 for cytokine receptor models).

In beta cells, IL-1β, alone or in combination with IFN-γ, has also been shown to induce NF-κB nuclear translocation (Darville & Eizirik, 1998). NF-κB has been shown to regulate the promoter activity of several genes whose expression has been modified following exposure to cytokines. These are the genes coding for iNOS (De Paula et al., 2007; Kwon et al., 1995), the pro-apoptotic cell surface receptor Fas that interacts with Fas ligand to induce apoptosis (Darville et al., 2001; Cnop et al., 2005), chemokine MCP-1, which can attract mononuclear cells to the site of insulitis (Kutlu et al., 2004), and the free radical scavenger, MnSOD (Steiner et al., 1997) that may participate in beta cell defense. NF-κB is ubiquitously expressed in cells, however its activity is suppressed by the binding of inhibitors of NF-κB (IκB) proteins. IκBα, IκBβ and IκBε are the major regulatory IκB proteins in mammalian cells (Ghosh et

al., 1998). Cytokines induce NF-κB activation in both human (Giannoukakis et al., 2000) and rodent islets (Heimberg et al., 2001) after *in vitro* co-incubation. NF-κB activation in insulin-producing beta cells such as RIN and INS-1E cells is more rapid, marked and sustained than in other cell types, such as fibroblasts, which correlates with a more pronounced activation of downstream genes and a pro-apoptotic outcome (Ortis et al., 2006; Lee et al., 2009). Inhibiting the expression of NF-κB in RIN cells has also been shown to play a cytoprotective role in the beta cell (McCabe et al., 2006). Thus, the specificity and temporal control of gene expression by NF-κB are of crucial physiological significance in insulin secreting cells (Figure 1.5).

The liver is a multifunctional organ that plays an essential role in metabolism, biosynthesis, excretion, secretion and detoxification (Malhi et al., 2006). These processes also impart vulnerability of hepatocytes to anoxia, increase susceptibility to noxious insults, and create a demand for cell replacement after tissue loss. Blocking of the NF-κB pathway in TNF-α-stimulated hepatocytes results in a shift towards apoptosis. This implies the existence of NF-κB-regulated anti-apoptotic genes, such as c-IAP (cellular inhibitor of apoptosis protein) (Xu et al., 1998; Bradham et al., 1998; Schwabe et al., 2001; Schoemaker et al., 2002). Resistance to Fas-induced apoptosis *in vitro* by mouse hepatocytes may be due to NF-κB activation during cell culture, since Fas ligation *in vivo* induces significant hepatocyte apoptosis (Ogasawara et al., 1993). However, the response to Fas ligation is also species dependent with rat hepatocytes being relatively insensitive and cultured human hepatocytes more sensitive even in the absence of NF-κB inhibition (Galle et al., 1995; Schulze-Bergkamen et al., 2003). Since the insulin-secreting liver cell lines, Melligen and Huh7ins, are derived from the parent Huh7, it was necessary to determine if these genetically modified cell lines initiated the signaling pathways reported for liver cells in response to the cytokine treatment. Such data would indicate if the insulin secreting liver cell lines constitute a putatively more attractive alternative in the search for an insulin source, given that pancreatic beta cells initiate apoptotic pathways in response to pro-inflammatory cytokine treatment (Giannoukakis et al., 1999), while liver cell lines follow anti-apoptotic pathways (Fabregat, 2009; Mott & Gores, 2007).

The aims of this study were to elucidate the molecular mechanisms that render Melligen cells resistant to cytokine induced death and to determine if common mechanisms exist after cytokine treatment in beta cells and the insulin-secreting liver cells.

4.2 Cytokine receptors are expressed at RNA level in liver cell lines

To establish if the resistance of the liver cell lines, Huh7ins and Melligen, to the pro-inflammatory cytokine cocktail (Chapter 3) was due to the absence, or decreased expression, of the cytokine receptors, RT-PCR was performed using cDNA generated from RNA isolated from human primary islets (positive control), Huh7 (parent cell line), Huh7ins (Huh7 transfected with the insulin gene) and Melligen (Huh7ins transfected with the glucokinase gene) cells to determine expression of IFNGR1, IFNGR2, IL1BR1, IL1BR2, TNFAR1 and TNFAR2 (see Chapter 2). Beta actin was used as a house-keeping gene (data not shown). Expression of cytokine receptors IFNGR1, IFNGR2, IL1BR1, IL1BR2 and TNFAR1 at the mRNA level was confirmed in all cells (Figure 4.1).

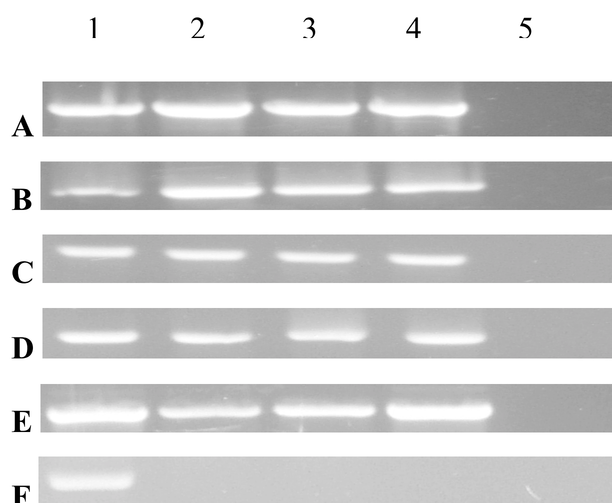


Figure 4.1: Cytokine receptor expression in islet cells and liver cell lines by RT-PCR.

*RT-PCR was performed to determine expression of cytokine receptors **A**)IFNR1, **B**)IFNR2, **C**)IL1R1, **D**)IL1R2, **E**)TNFR1 and **F**)TNFR2 cytokine receptors. Lanes contain amplicon after RT-PCR for: **1-**Panceatic Islet Cells (positive control) **2-**Huh7 Cells **3-**Huh7ins Cells **4-**Melligen Cells **5-**Negative control containing no cDNA template. 40 cycles were used in the PCR program.*

4.2.1 Apoptosis of insulin-secreting human liver cells is not induced by pro-inflammatory cytokines

Since the liver cell lines were found to express the cytokine receptors for the pro-inflammatory cytokines, TNF-α, IL-1β and IFN-γ, the ability of the liver cell lines to resist cytokine-mediated death likely resided in the initiation/inhibition of intracellular pathways regulating apoptosis. In autoimmune diabetes, beta cell death is initiated by a cascade of events ultimately leading to apoptosis. Therefore, cytokine treated Huh7ins and Melligen cells were examined for their susceptibility to apoptosis.

Analysis of Annexin V binding was performed using the MIN-6 cells at 24h, with and without the cytokine cocktail treatment (Figure 4.2A i and ii). After 24h of cytokine treatment, MIN-6 cells were used to gate for early and late apoptotic cells. Successful staining of the 24h cytokine treated MIN-6 cells showed 19.84 ± 1.2% early apoptotic, 15.4 ± 2% late apoptotic and 5.82 ± 0.78% necrotic cells. The viable cell population was significantly decreased after cytokine treatment of MIN-6 cells as compared to the untreated cells, determined as 60.96 ± 2.9% and 77.19 ± 4.3% respectively (P= 0.035). Statistical analysis also showed that the early and late apoptotic populations were higher in the treated cells as compared to the untreated cells, however, there was no difference between treated and untreated cells with respect to the proportion of necrotic cells (P= 0.009, 0.04 and 0.39 respectively).

As a positive control for these experiments, a known apoptosis-inducing drug, Norcantharidin (5μg/mL), was co-incubated with the cells, as previously described (Chen et al., 2002). After 1h co-incubation with Norcantharidin, Huh7ins cells showed 7.6 ± 1.2% early and 10.04 ± 0.9% late apoptotic populations. Melligen cells similarly showed 3.85 ± 0.56% early and 13.56 ± 1.34% late apoptotic populations (Figure 4.2A iii and iv). These results revealed that an apoptotic population was induced, as expected, by the Norcantharidin treatment and, as such, this was included as a positive control in subsequent experiments analyzing cytokine-induced apoptosis in insulin-secreting liver cell lines.

It was hypothesized that since the Huh7ins and Melligen cells have been engineered to adopt a more beta cell like phenotype, the cells may have an increased

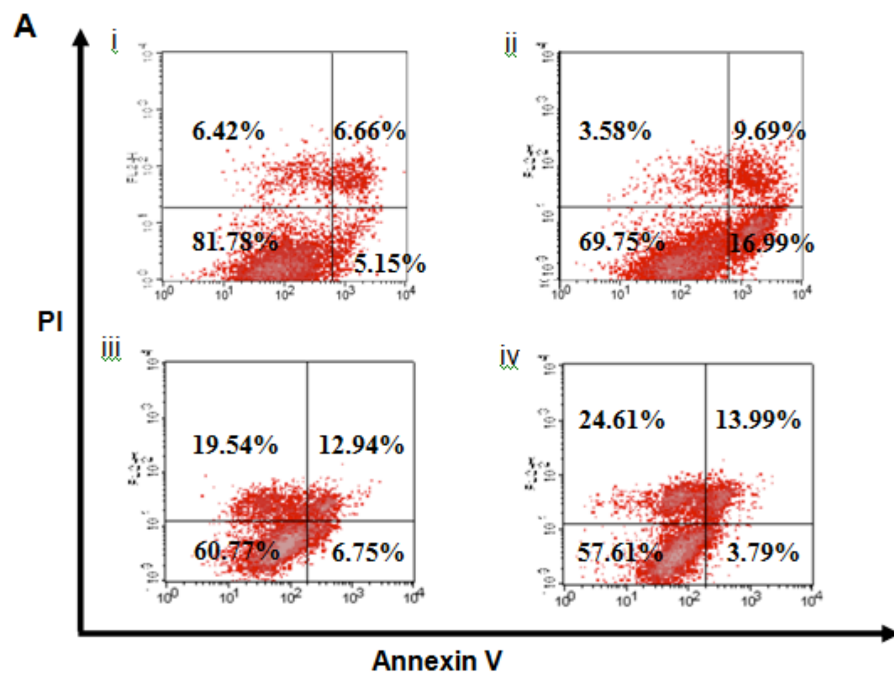
susceptibility to factors that destroy beta cells, namely pro-inflammatory cytokines. To investigate the mode of cell death induced by proinflammatory cytokines after co-incubation with Melligen cells, which express the human islet glucokinase gene, Huh7ins (without the human islet glucokinase gene) cells were treated with the cytokine cocktail for 24h and stained with Annexin V/PI.

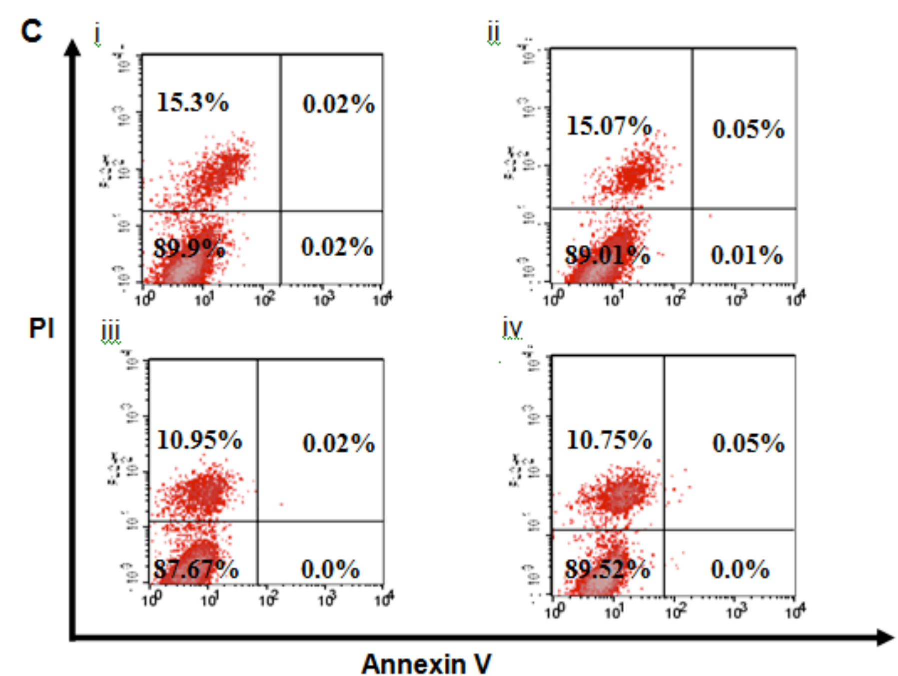
Huh7ins cells treated with the cytokine cocktail for 24h showed 0.06 ± 0.01% late apoptotic, 0.03 ± 0.02% early apoptotic and 22.6 ± 1.3% necrotic. The respective control Huh7ins populations at 24h showed no significant differences to the treated Huh7ins cells 0.04 ± 0.02%, 0.03 ± 0.02% and 23.9 ± 1.7% (*P*= 0.3, 0.8 and 0.6 respectively). Similarly at 48h, Huh7ins cells treated with the cytokine cocktail showed 0.08 ± 0.04% late apoptotic, 0.03 ± 0.01% early apoptotic and 17.3 ± 1.8% necrotic. The respective control Huh7ins populations at 48h showed 0.03 ± 0.02%, 0.03 ± 0.03% and 22 ± 1.8% (*P*= 0.3, 1 and 0.14, respectively). There was no significant difference between the viable population at 24h for treated cells (81.6 ± 4.9%) and control cells (81.9 ± 7%) (*P*= 0.97). There was also no difference between the treated (82.4 ± 1.8%) and untreated (83.7 ± 6.05%) viable cell populations at 48h cytokine treatment (*P*=0.85).

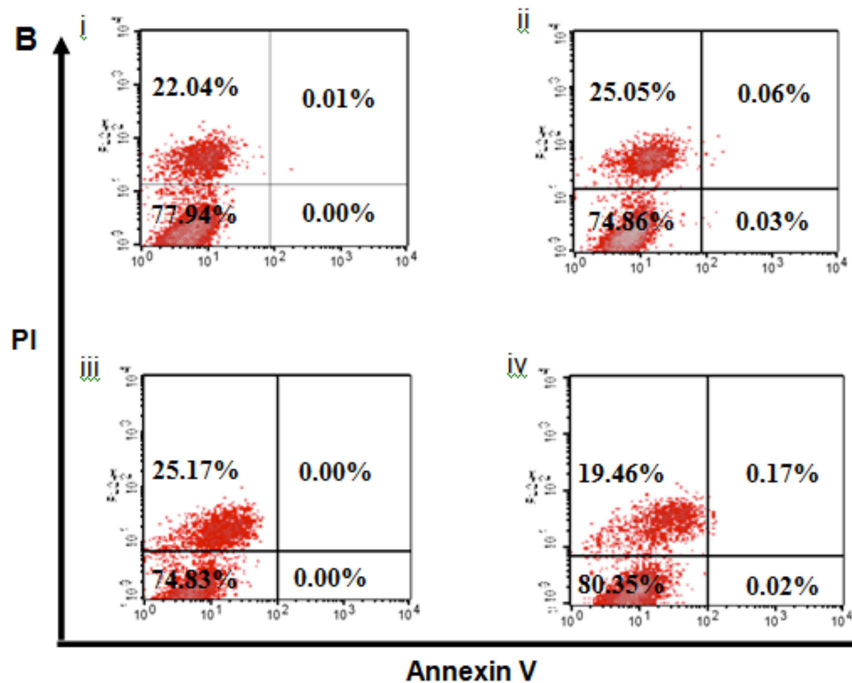
Staining Melligen cells at the 24h time-point showed similar results to those obtained with the Huh7ins cells (Figure 4.2B; Figure 4.2C). Treated Melligen cells showed 0.07 ± 0.05% early apoptotic, 0.05 ± 0.04% late apoptotic and 15.07 ± 2.7% necrotic and respective control cells showed 0.02 ± 0.02%, 0.02 ± 0.003%, and 15.3 ± 1.5% with no significant difference detected at 24h between the cytokine treated and untreated populations (*P*= 0.3, 0.46 and 0.94 respectively). At 24h, the percentage of viable cells in the treated (83.9 ± 3.1%) and untreated (89.9 ± 4%) cells were also not significantly different (*P*= 0.28). Similarly at 48h, Melligen cells treated with the cytokine cocktail showed 0.0 ± 0.003% early apoptotic, 0.05 ± 0.03% late apoptotic and 9.4 ± 0.9% necrotic cell populations. The respective control Melligen cell populations at 48h showed 0.007 ± 0.007%, 0.1 ± 0.08% and 11 ± 1.3% (*P*= 0.68, 0.6 and 0.4). There was no significant difference between the cells in the viable population at 48h for treated cells (89.5 ± 0.3%) and control cells (87.7 ± 3.7%) *P*= 0.64.

The results obtained from these experiments suggested that the cytokine treatment had no effect on the viability of the insulin secreting liver cell lines, Huh7ins and Melligen. However, after pro-inflammatory cytokine treatment, the Melligen cells had significantly less necrotic cells than the Huh7ins cells, suggesting that the addition of the human islet glucokinase gene may induce changes at the molecular level in response to the cytokine treatment. It is also worth noting that the Huh7ins cells have a significant baseline level of necrosis (23.9 ± 1.7%), which was not significantly increased after cytokine treatment. In contrast, Melligen cells had a lower baseline of necrotic cells (15.3 ± 1.5%), which was also not significantly different after cytokine treatment and the MIN-6 cells also showed a lower baseline of necrotic cell populations (4.6 ± 0.98%). However, necrotic populations in the untreated parent Huh7 cells is consistent with other findings in the literature using Annexin V/PI stained Huh7 cells (Fan et al., 2009).

Figure 4.2D shows that data represented as percentage cell death. These figures reveal the very low levels of apoptosis seen in the Huh7ins and Melligen cells in a bar graph.


A
i
6.42%
6.66%
81.78%
5.15%
ii
3.58%
9.69%
69.75%
16.99%
iii
19.54%
12.94%
60.77%
6.75%
iv
24.61%
13.99%
57.61%
3.79%
PI
Annexin V


C
i
15.3%
0.02%
89.9%
0.02%
ii
15.07%
0.05%
89.01%
0.01%
iii
10.95%
0.02%
87.67%
0.0%
iv
10.75%
0.05%
89.52%
0.0%
PI
Annexin V


B
i
22.04%
0.01%
77.94%
0.00%
ii
25.05%
0.06%
74.86%
0.03%
iii
25.17%
0.00%
74.83%
0.00%
iv
19.46%
0.17%
80.35%
0.02%
PI
Annexin V

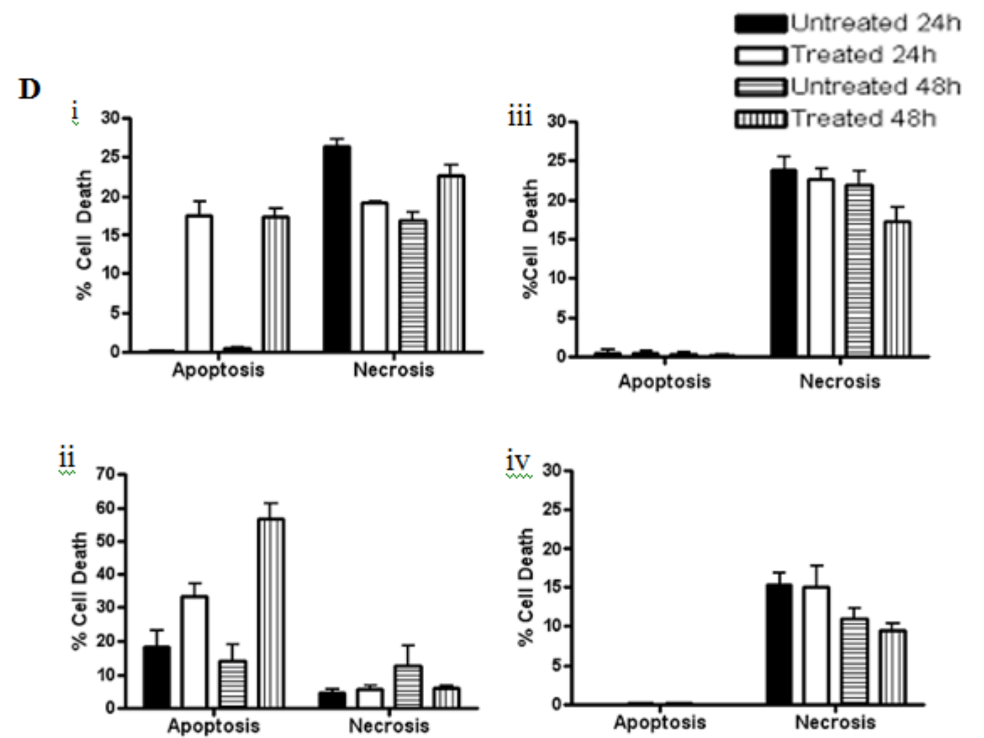

D
Untreated 24h
Treated 24h
Untreated 48h
Treated 48h
i
% Cell Death
Apoptosis
Necrosis
ii
% Cell Death
Apoptosis
Necrosis
iii
%Cell Death
Apoptosis
Necrosis
iv
% Cell Death
Apoptosis
Necrosis

Figure 4.2: Annexin V/PI staining of insulin secreting liver cell lines of Huh7ins and Melligen.

A) *Positive controls for AnnexinV/PI analysis. MIN-6 cells were treated for 24h with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL)* ***i)*** *untreated and* ***ii)*** *treated. Identifying apoptotic populations in the insulin-secreting liver cell lines using Norcantharidin. Insulin secreting liver cell lines were treated with 5μg/mL Norcantharidin for 1h.* ***iii)*** *Huh7ins and* ***iv)*** *Melligen treated cells. Representative diagram of n=3 independent experiments*

B) *AnnexinV/PI stained Huh7ins cells. Huh7ins cells were treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 24h* ***i)*** *untreated and* ***ii)*** *treated and 48h* ***iii)*** *untreated and* ***iv)*** *treated. Representative diagram of n=3 independent experiments*

C) *AnnexinV/PI stained Melligen cells. Melligen cells were treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 24h* ***i)*** *untreated and* ***ii)*** *treated and 48h* ***iii)*** *untreated and* ***iv)*** *treated. Representative diagram of n=3 independent experiments*

Lower left quadrant indicates viable population, lower right early apoptotic, upper right late apoptotic and upper left necrotic. Results are representative plots of three independent experiments.

D) *Apoptotic effect of cytokines determined as percentage cell death. Control Norcantharidin treated cells* ***i)****Huh7ins and Melligen (results obtained from Figure 4.2A iii) and iv)). Cytokine treated* ***ii)*** *MIN-6* ***iii)*** *Huh7ins and* ***iv)*** *Melligen cells (results obtained from Figures 4.2A, B and C respectively). The apoptotic (represented as a total of early and late apoptotic cells) and necrotic cells are indicated as a percentage of gated cells. Results expressed as mean ± SE, n=3 independent experiments*

4.2.2 Cell cycle arrest is not induced in insulin-secreting human liver cells after treatment with pro-inflammatory cytokines

Since the viable cell number was not decreased in the insulin-secreting liver cell lines after pro-inflammatory cytokine treatment at 24h and 48h, according to the apoptotic studies in section 4.2.1, further studies were conducted to characterise the effects of the cytokines on the insulin secreting liver cell lines. Rather than inducing cell death, pro-inflammatory cytokine treatment may have induced an arrest in cell cycle progression without progression to apoptosis (Wisdom et al, 1999; Karin, 2006). To further investigate the effect of cytokines on cell death, cell cycle analysis of Huh7ins and Melligen cells using PI-only staining was performed.

To determine cell cycle progression in the insulin secreting liver cell lines and to confirm the observed lack of an apoptotic population using the Annexin V/PI stain, the Norcantharidin treatment was repeated using the PI stain. These results showed that by inducing apoptosis in the Huh7ins (Figure 4.3A i) and Melligen (Figure 4.3A ii) cell lines there is a prominent sub-G1 (6.94 ± 0.57% and 7.81 ± 0.86% respectively) indicative of apoptosis in cell cycle arrest, G1/G0 (40.65 ± 0.9% and 40.02 ± 2.9% respectively), S (8.5 ± 0.88% and 11.7 ± 1.9% respectively) and G2 (11.96 ± 0.54% and 11.7 ± 0.53% respectively) peak. Cell Quest software was used to quantify peaks in the Norcantharidin experiments. The sub-G1 phase was significantly increased after Norcantharidin treatment of Huh7ins cells as compared to the untreated cells (P= 0.0005). Statistical analysis also showed that the sub-G1 phase was significantly higher in the treated Melligen cells as compared to the untreated cells (P= 0.00087). Subsequent experiments using the cytokine treatment were analysed using ModFit analysis software to determine cell cycle phases G1, S and G2. Sub-G1 peaks were analysed using Cell Quest.

The insulin secreting liver cell lines were incubated with the cytokine cocktail for 48h and analysed at 24h and 48h after cytokine treatment. To examine whether the cytokine cocktail treatment induced apoptosis or cell-cycle arrest in Huh7ins and Melligen cells, the DNA content in these cells was analysed using flow cytometry after propidium iodide staining. The cytokine treatment did not significantly affect the number of Huh7ins cells in the sub-G1 phase of the cell-cycle, representing apoptotic

cells, at either 24h [0.21 ± 0.05% control and 0.24 ± 0.01% treated (*P*= 0.73)] or 48h [0.54 ± 0.75% control and 0.56 ± 0.05% treated (*P*= 0.81)] (Figure 4.3B). In addition, there was no increase in the number of cells in the G0/G1 phase (Figure 4.3D i) in the cells treated (56.61 ± 0.83%) or untreated (54.98 ± 0.6%) (*P*= 0.19) at 24h or at 48h with treated 56.13 ± 1.6% compared to control 62.22 ± 3.1% (*P*= 0.16). These results suggest that cytokines do not reduce the number of viable cells through inducing apoptosis and cell cycle arrest at G0/G1 phase in the insulin-secreting hepatoma cell Huh7ins. There were also no significant differences observed between the treated and untreated Huh7ins cells at 24h or 48h in the S or G2 phases of cell cycle (P>0.05) (Figure 4.3D i).

The cytokine treatment did not significantly affect the number of Melligen cells in the sub-G1 phase of the cell-cycle, representing apoptotic cells, at 24h [0.35 ± 0.03% control and 0.3 ± 0.03% treated (*P*= 0.94)] or 48h [0.44 ± 0.23% control and 0.31 ± 0.07% treated (*P*= 0.64)] (Figure 4.3C). In addition, there was no increase in the number of cells in the G0/G1 phase in the cells treated (55.78 ± 1.12%) or untreated (58.5 ± 3.3%) (*P*= 0.34) at 24h or at 48h with treated 62.83 ± 2.29% compared to control 55.78 ± 1.12% (*P*= 0.06). Once again these results suggest that cytokine treatment did not reduce the number of viable cells through inducing apoptosis and cell cycle arrest at the G0/G1 phase in insulin-secreting Melligen cells. There were also no significant differences observed between the treated and untreated Melligen cells at 24h or 48h in the S or G2 phases of cell cycle (P>0.05) (Figure 4.3D ii). These results support earlier findings in Section 4.2.1 showing negligible apoptosis in the cytokine treated Huh7ins and Melligen cells.

A

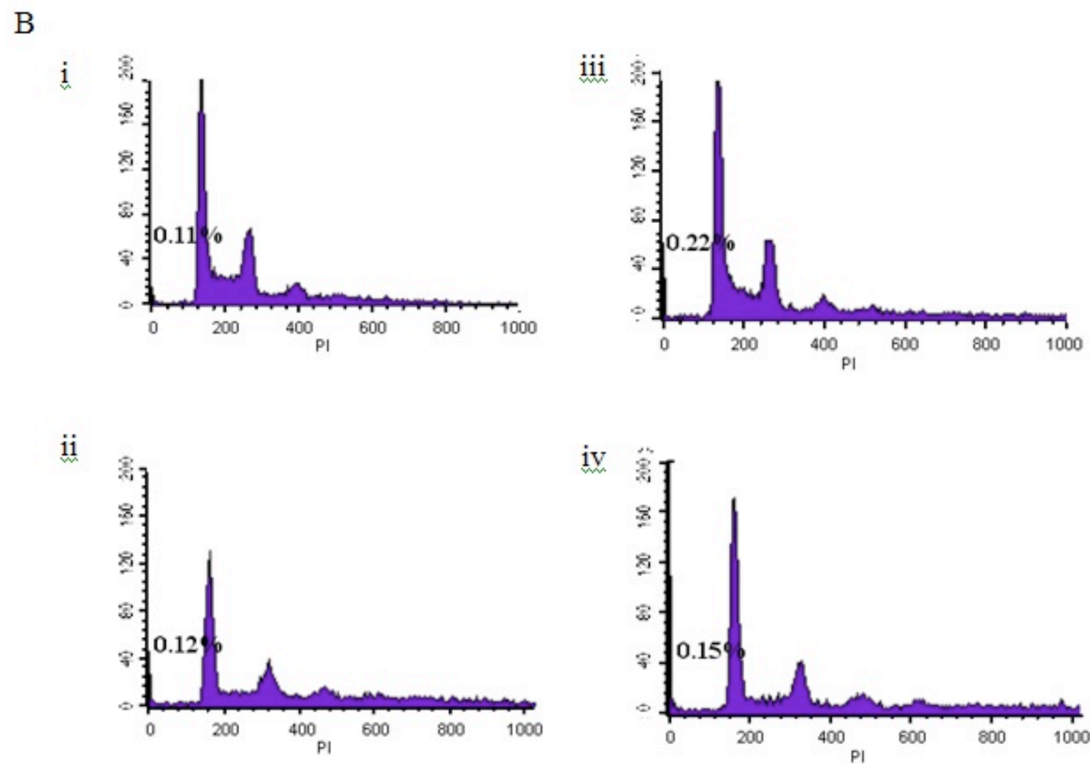
i
39.37%
12.01%
7.95%
9.92%

ii
38.08%
11.53%
7.26%
10.23%

C

i
0.31%

iii
0.37%



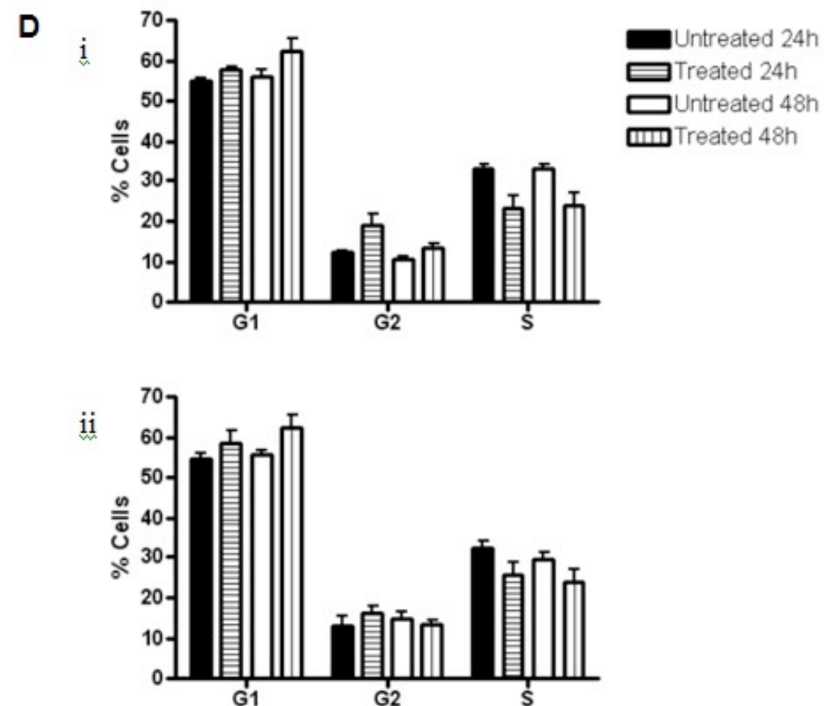


Figure 4.3: PI staining of insulin secreting liver cell lines Huh7ins and Melligen

A) *Positive control for cell cycle arrest in the insulin-secreting liver cell lines using Norcantharidin. Insulin secreting liver cell lines were treated with 5μg/mL Norcantharidin for 1h* ***i)*** *Huh7ins and* ***ii)*** *Melligen treated cells. Representative diagram of n=3 independent experiments*

B) *Cell cycle arrest determined in PI stained Huh7ins cells. Huh7ins cells were treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 24h* ***i)*** *untreated and* ***ii)*** *treated and 48h* ***iii)*** *untreated and* ***iv)*** *treated. Area under curve measured for sub-G1 phase only. Representative diagram of n=3 independent experiments*

C) *Cell cycle arrest determined in PI stained Melligen cells. Melligen cells were treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 24h* ***i)*** *untreated and* ***ii)*** *treated and 48h* ***iii)*** *untreated and* ***iv)*** *treated. Area under curve measured for Sub-G1 phase only. Representative diagram of n=3 independent experiments*

D) *ModFit analysis of cell cycle arrest determined in PI stained insulin-secreting cells. ModFit analysis was performed on samples taken at 24h and 48h to determine G1, G2 and S phases in cell cycle arrest after cytokine treatment* ***i)*** *Huh7ins and* ***ii)*** *Melligen. Results expressed as mean ± SE, n=3 independent experiments*

4.3 NF-κB Signaling Pathways

Apoptosis studies, described in the preceding section of this chapter, identified necrotic populations that were not significantly different in the cytokine treated and control liver cell lines. Unlike the pancreatic beta cell line, MIN-6, which was used to identify apoptotic populations using AnnexinV/PI staining, the insulin secreting liver cell lines Huh7ins and Melligen exhibited negligible apoptotic cell populations after cytokine treatment. The transcription factor NF-κB, known to mediate cytokine-induced beta cell apoptosis, paradoxically has mostly anti-apoptotic effects in other cell types (Schwabe et al., 2001; Schoemaker et al., 2002, Ortis et al., 2006). In non-stimulated cells, NF-κB is sequestered in the cytoplasm where it is associated with the IκB inhibitory molecules (Gilmore, 1999). Following activation, through the TNF receptor, distinct signaling cascades are activated that converge upon formation of the IκB kinase (IKK) complex. The trimeric IKK complex phosphorylates IκBα, leading to its degradation by the proteasome, thereby allowing NF-κB to translocate to the nucleus, where it functions as a transcription factor and activates target genes. Beta cell exposure to IL-1β leads to degradation of IκBα and nuclear translocation of NF-κB (Eizirik et al., 1996). Whereas on one hand, NF-κB is important for the activation of pro-inflammatory genes, it is also essential for the resolution of inflammation and protection from apoptosis.

The cellular actions of NF-κB depend on the type, the nature and duration of the stimulus, the periodicity, and the degree of activity of the particular dimers involved (Ortis et al., 2006; Nelson et al., 2004). Several studies observing the effect of cytokines on liver cell lines and primary liver cells have pointed to the presence of increased levels of anti-oxidative proteins and anti-apoptotic signalling pathways, which have been found to render the cells resistant to the cytotoxic effects of cytokines (Schoemaker et al., 2002; Wullaert et al., 2007). However primarily, NF-κB plays a pivotal role in triggering the cascade of events involved in protecting the liver cells against toxins and other insults. To determine how the pro-inflammatory cytokines, IFN-γ, TNF-α and IL-1β, exert biological effects on the insulin secreting liver cell lines, molecular pathways leading to the activation of transcription factor NF-κB were investigated. It was hypothesized that the downstream effects will be blocked or that they will be different from that of the beta cell. The rationale for the

selection of the time-points used in this experiment was that NF-κB is activated by proinflammatory cytokine, IL-1β, after 10-30min in insulin-producing cells (de Mello et al., 1996), while an increase in downstream target genes such as iNOS, Fas and MCP-1 is induced after 4h and cell death is detected only after a 24 hour exposure to the cytokines IL-1β and TNF-α (Ortis, et al., 2006).

4.3.1 IκB genes are expressed in cytokine-induced insulin-secreting human hepatoma cell

After treating the cells for 4h with the pro-inflammatory cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL), mRNA was extracted from the liver cell lines, Huh7, Huh7ins and Melligen, and islet cells were used as a positive control. RT-PCR was used to determine if a particular gene is expressed in the cell line. Beta actin was used as a house-keeping gene to confirm that equivalent amounts of cDNA were being compared (data not shown).

Molecular expression of the inhibitors of NF-κB- IκBα, IκBβ and IκBε at their respective fragment sizes, 202, 169 and 157bp was detected in all cell lines with and without the cytokine treatment (Figure 4.4). Subsequent analysis by real-time RT-PCR was performed to reveal quantitative expression of the genes in the Melligen cells only, since this is the cell line whose insulin responsive characteristics most closely reflected those of the beta cell.

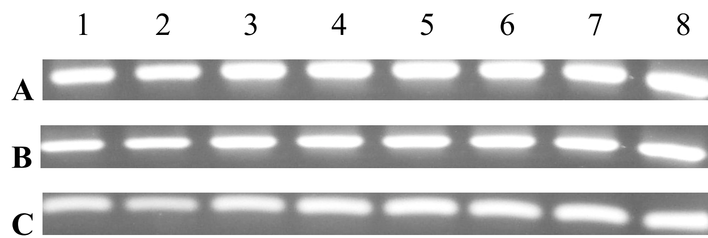


Figure 4.4: RT-PCR was performed using primers for IκBα, IκBβ and IκBε.
*Lanes contain amplicon after RT-PCR for: 1-Panceatic islet cells (positive control) untreated 2- islet cells treated 3-Huh7 cells untreated 4-Huh7 cells treated 5-Huh7ins cells untreated 6-Huh7ins cells treated 7-Melligen cells untreated 8-Melligen cells treated. Results show bands for **A)**IκBα, **B)**IκBβ and **C)**IκBε. 40 cycles were used in the PCR program.*

Following from the RT-PCR results, which showed that the liver cell lines Huh7, Huh7ins and Melligen with and without the cytokine treatment expressed genes for the IκBα, IκBβ and IκBε (Figure 4.4), it was determined that further studies using real-time RT-PCR were required to establish if these genes were being up- or down-regulated by the cytokine treatment. From Figure 4.5 it can be seen that there is a down-regulation of the inhibitors of NF-κB in the cytokine-treated Melligen cells. From the three inhibitors examined the inhibitor which was significantly reduced after cytokine treatment was IκBε which was down regulated by 0.55 ± 0.005 fold ($P<0.05$). IκBα and IκBβ were also significantly down regulated but by only 0.57 ± 0.0125 fold and 0.66 ± 0.098 fold respectively ($P<0.05$). These results imply that the down-regulation of NF-κB inhibitors may be allowing for the activation and translocation for NF-κB from the cytoplasm into the nucleus.

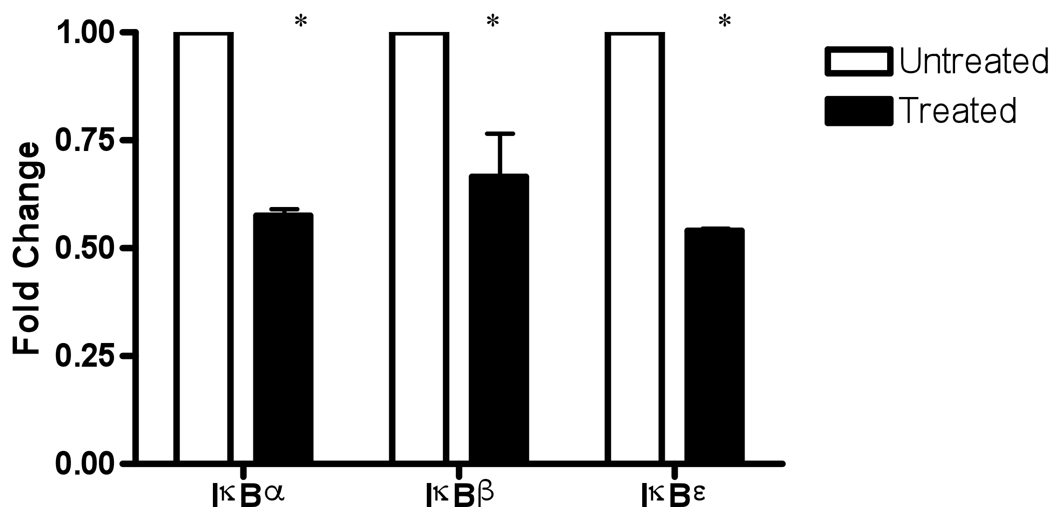


Figure 4.5: Inhibitors of NF-κB observed as fold changes in treated Melligen cells following Real Time-RTPCR.

*Melligen cells were treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 4h. Real-time RT-PCR was performed with specific primers for IκBα, IκBβ and IκBε corrected by Beta Actin expression. The data are expressed as fold variation of the respective controls (considered as 1). Results expressed as mean ± SE n=3, independent experiments. * P<0.05 for cytokine treated verses untreated cells.*

4.3.2 NF-κB target genes, iNOS and Fas, are expressed in Melligen cells

To investigate the downstream effects of NF-κB signaling, cytokine-induced gene expression in Melligen cells was evaluated. After treating the cells for 4h with the pro-inflammatory cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL), mRNA was extracted from the liver cell lines, Huh7, Huh7ins and Melligen, and human pancreatic islet cells were used as a positive control.

Densitometry studies performed on the amplicons obtained in Figure 4.6, to obtain semi-quantitative values, revealed an up-regulation of Fas in the cytokine-treated positive control human islet cells compared to the untreated cells (3.3 fold up-regulation). Beta actin was used as a house-keeping gene to confirm that equivalent amounts of cDNA were being compared (data not shown). The cytokine treated Huh7, Huh7ins and Melligen cells showed a 1.0, 1.1 and 1.1 fold increase respectively indicating no or only very slight changes in gene expression. To further investigate

these findings, Melligen cell cDNA was further analysed by real-time RT-PCR. The real-time RT-PCR data showed that the cytokine treatment was in fact inducing a 0.5±0.019 fold down-regulation in the Fas gene (Figure 4.7A and B). The densitometry studies are therefore not considered to be an accurate method of determining gene regulation in this instance.

There was a clear up-regulation of iNOS gene expression in the cytokine treated islet cells (2.2 fold up-regulation) (Figure 4.8). RT-PCR results also showed that the gene expression of iNOS in the treated Huh7, Huh7ins and Melligen cells was down regulated or remained the same (0.8, 0.98 and 1.0 fold change respectively) after the 4h cytokine treatment. Following cytokine exposure, the relative abundance of iNOS in the cDNA of Melligen cells (optical density corrected per beta actin abundance) remained unchanged. To confirm these findings, real-time RT-PCR Ct charts showed late but distinct amplification of the iNOS fragment in untreated Melligen cells (Figure 4.8A) and weak amplification of iNOS in the treated Melligen cells (Figure 4.8B). Additionally, expression of the housekeeping gene beta actin was not affected by exposure to the cytokines (Figure 4.8A and B). Melt curve analysis for iNOS further confirmed the formation of a single product at very low amplification in the cytokine treated Melligen cell. Therefore, gene expression determined as fold change could not be calculated for the iNOS gene from the Ct values obtained.

NF-κB target gene MCP-1 was also found to be up-regulated in the cytokine treated human islet cells (1.4 fold up-regulated) (Figure 4.6). Molecular expression of this gene was not detected in any of the liver cell lines and was not further investigated.

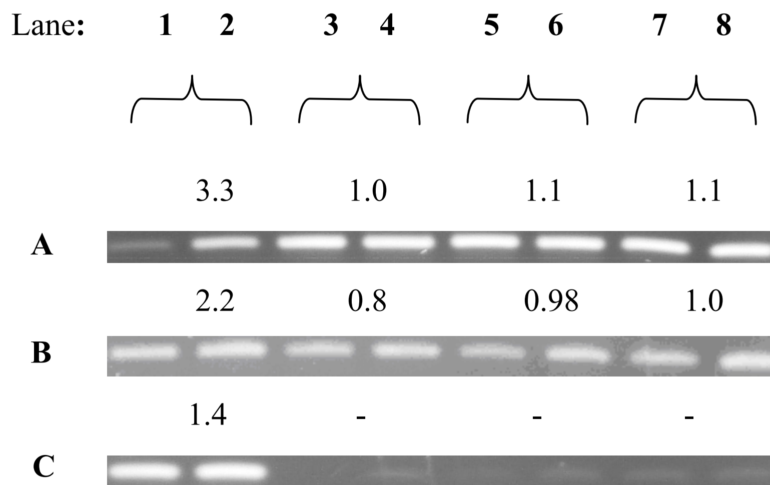
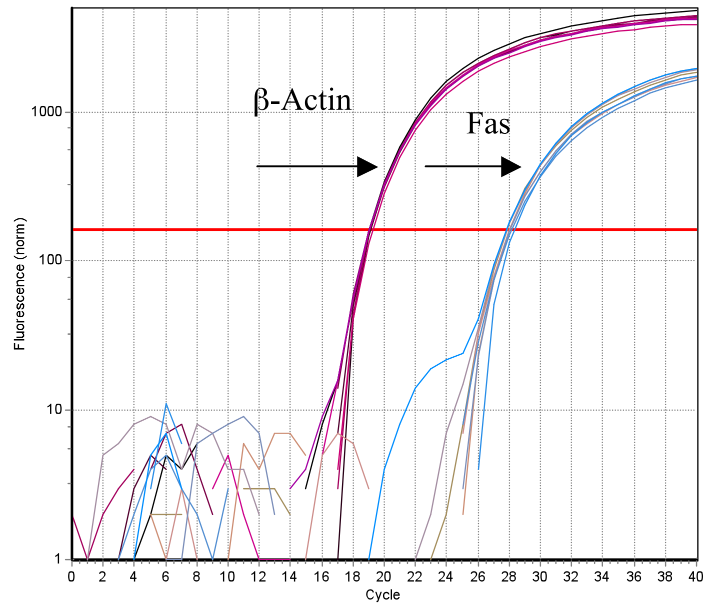


Figure 4.6: Expression levels of NF-κB target genes detected using RT-PCR in treated islet cells and liver cell lines determined as fold changes.

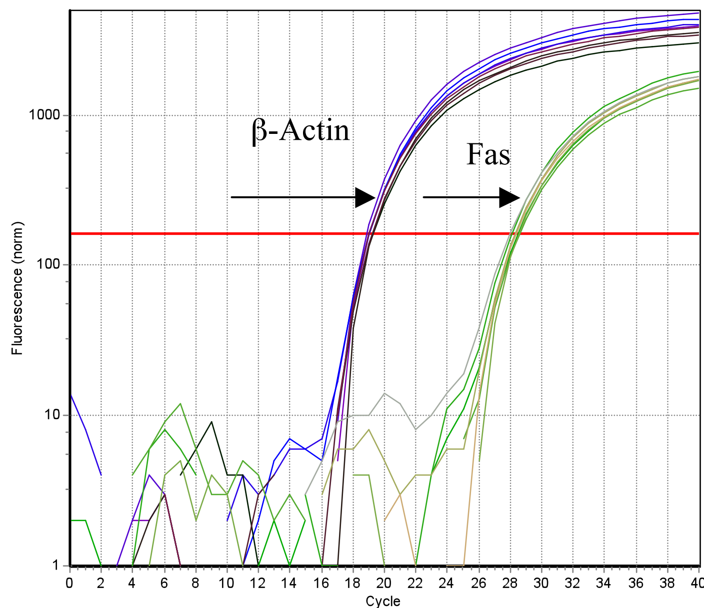
*Cells were exposed to IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 4h and RT-PCR was performed using primers for **A**)Fas, **B**)iNOS and **C**)MCP-1. Lanes contain amplicon after RT-PCR for: **1**-Panceatic islet cells untreated **2**-Pancreatic islet cells treated **3**-Huh7 cells untreated **4**-Huh7 cells treated **5**-Huh7ins cells untreated **6**-Huh7ins cells treated 7-Melligen cells untreated **8**-Melligen cells treated. 40 cycles were used in the PCR program.*

A



Threshold: 162 (Adjusted manually)
Baseline settings: automatic, Drift correction OFF

B

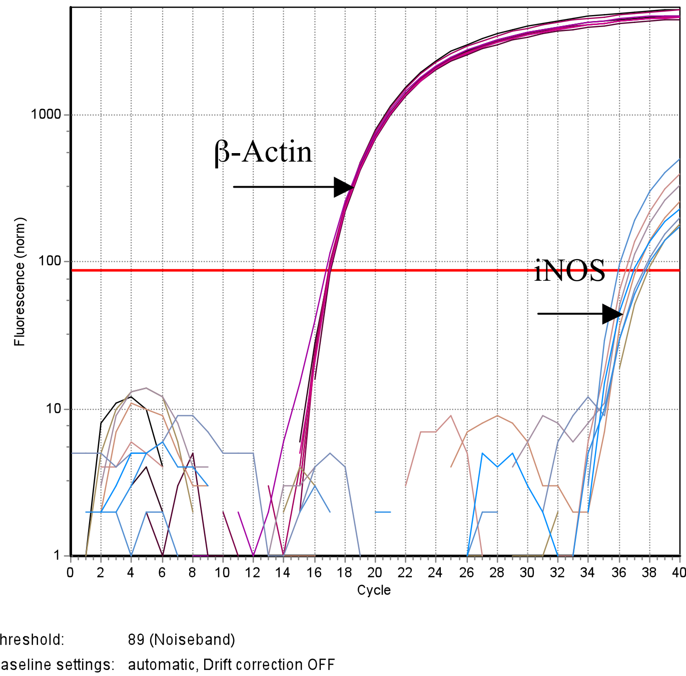


Threshold: 162 (Adjusted manually)
Baseline settings: automatic, Drift correction OFF

Figure 4.7: Cytokines induce expression of NF-kB target gene Fas in Melligen cells.

*Melligen cells were exposed to IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 4h. mRNA was extracted and real-time RT-PCR performed for **A**) untreated and **B**) treated. Beta Actin used as housekeeping gene. Eight replicates presented.*

A



B

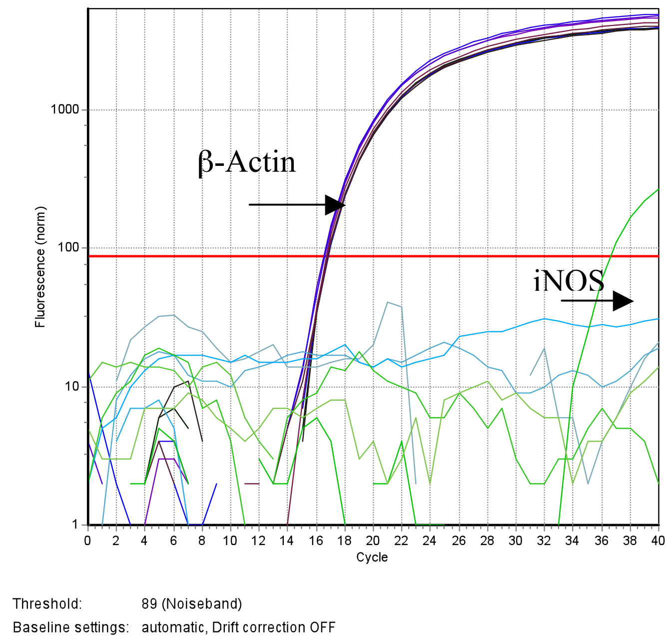


Figure 4.8: Cytokines induce weak expression of NF-kB target gene iNOS in Melligen cells.

*Melligen cells were exposed to IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 4h. mRNA was extracted and real-time RT-PCR performed for **A)** untreated and **B)** treated. Beta Actin used as housekeeping gene. Eight replicates presented.*

4.4 Nitric oxide levels are not increased after cytokine treatment of liver cell lines

In combination with the densitometry studies analysing iNOS, the real-time RT-PCR data showed that the iNOS gene expression in the treated Melligen cells was low (densitometry study showed a 1 fold change). While this analysis showed that iNOS was expressed at the level of RNA it was next determined if nitric oxide was involved in the response of the liver cell lines to pro-inflammatory cytokine treatment. The modified Griess reaction was used to determine nitric oxide levels, and hence iNOS activity. The iNOS enzymatic activity was estimated by measurements of nitrite in tissue culture medium (a stable product of NO oxidation) accumulation (Green et al., 1982), during a 48h exposure to cytokines, IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL). Nitrite concentration was determined in triplicate within a concentration range that corresponded to the linear portion of the standard curve.

Nitrite released into the culture media of cytokine treated and untreated MIN-6 cells were determined at 24h and 48h (Figure 4.9A). The treated MIN-6 cells generated a significantly greater amount of nitrite compared to the untreated MIN-6 cells at 24h (10.31 ± 1.55μM and 8.65 ± 0.39μM, respectively; P=0.008) and at 48h (17.9 ± 1.2μM and 9.7 ± 0.6μM, respectively P=0.003).

The cytokine treatment did not have a significant effect on any of the liver cell lines. The levels of NO in the untreated and treated Huh7 cells at 24h [8.9±0.1μM and 8.56±0.01μM, respectively (P=0.3)] (Figure 4.9B) and 48h [8.7±0.13μM and 8.7±0.08μM, respectively (P=0.8)] were not significantly different. Huh7ins cells (Figure 4.9C) showed similar levels of NO production at 24h and 48h as the Huh7 cells. Untreated and treated Huh7 cells at 24h [8.7±0.1μM and treated cells 8.64±0.1μM, respectively (P=0.5)] and 48h [8.56±0.09μM and 8.72±0.1μM, respectively (P=0.6)] were not significantly different. Treated and untreated Melligen cells (Figure 4.0D) also did not exhibit an increase in NO production at either 24h [8.72±0.067μM and 8.46±0.07μM respectively, (P=0.21)] or 48h [(8.6±0.058μM and 9.02±0.189μM, respectively (P=0.07)].

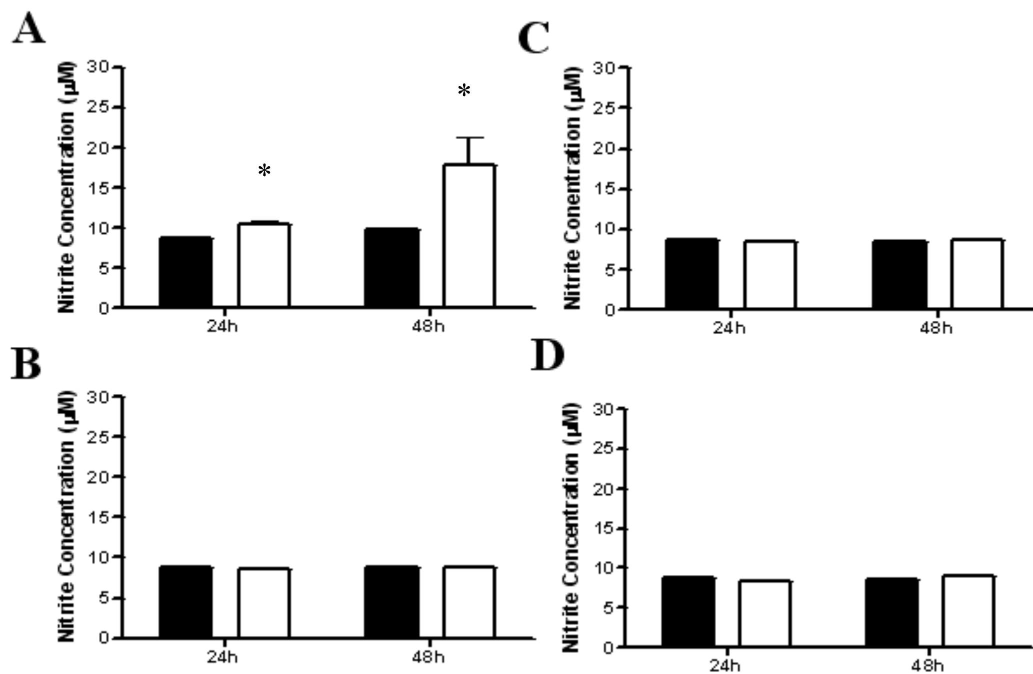


Figure 4.9: Nitrite determination in MIN-6, Huh7, Huh7ins and Melligen cells.
Nitrite production in cytokine treated (unfilled) and untreated (filled) ***A****) MIN-6* ***B****) Huh7* ***C****) Huh7ins and* ***D****) Melligen cells as determined by Modified Griess reaction after 24h and 48h. Results expressed as mean ± SE, n=5 independent experiments. *P<0.05 for cytokine-treated versus untreated cells.*

4.5 Discussion

If surrogate beta cells are to be a viable alternative for beta cell replacement in Type 1 diabetes, they must withstand the effects of pro-inflammatory cytokines. Pro-inflammatory cytokines have been suggested to play a role in both the rejection of islet allografts as well as in the pathogenesis of Type 1 diabetes (Diamond & Gill, 2000; Robson et al., 2005; Satoh et al., 2007). The results from this study suggest that the cytokine treatment has no affect on the viability of the liver cell lines and also suggests that anti-apoptotic pathways may exist to make these cells resistant to the toxic effects of the cytokines. Expression of cytokine receptors IFNGR1, IFNGR2, IL1BR1, IL1BR2 and TNFAR1 at the mRNA level was confirmed in all liver cell lines (Figure 4.1), indicating that the pro-inflammatory cytokines were able to bind to and signal via their cognate receptors. The results obtained in this study also showed that the insulin secreting liver cell line, Melligen, expressed activated NF-κB, which was up-regulated after 4h of cytokine treatment. The real-time RT-PCR results also indicated a down-regulation of the inhibitors of NF-κB, IκBα, IκBβ and IκBε, further pointing to the activation of NF-κB in the cells. Decreased expression levels of both Fas and iNOS were also found. According to other studies, human hepatocellular carcinoma cells including the parent cell line of the Melligen cells, Huh7, constitutively express, at the protein level, high levels of NF-κB (Kaufmann & Earnshaw, 2000; Herr & Debatin, 2001; Chiao et al., 2002, Guo et al., 2005). These results suggest that the Melligen cells have maintained the cytokine signaling cascade of the parent cell line.

Pro-inflammatory cytokines stimulate apoptosis through the up-regulation of genes that have been shown to play roles in diabetes development. Several studies suggest that the transcription factor NF-κB is an important cellular signal in initiating the cascade of events culminating in beta cell death. Most reports have described a pro-apoptotic role for NF-κB in pancreatic beta cell death (Heimberg et al., 2001; Giannoukakis et al., 2000; Sekine et al., 2000; Han et al., 2001; Kim & Lee, 2009). In contrast an anti-apoptotic function for NF-κB has been reported in most other cell types (Beg & Baltimore, 1996; Wang et al., 1996; Li et al., 1999; Kim et al., 2005). Beg and Baltimore (1996) found that there was a pivotal role for activated NF-κB in the resistance of mouse fibroblasts and macrophages to the treatment of TNF-α

induced through TNFR1. Ortis et al. (2006) similarly discovered that fibroblasts, 208F, were being protected against the effects of the pro-inflammatory cytokines (TNF-α and IL-1β) by the activation of NF-κB. The same group performed further i*n vitro* studies on beta cells revealing that the molecular pathways involved in the cytokine induced apoptotic death, constitute the up-regulation of the transcription factor NF-κB and a cascade of signals including Fas, iNOS and MCP-1 (Ortis et al., 2006). The difference between the effect of NF-κB in beta and non-beta cells could be due to the sensitivity of pancreatic beta cells to the pro-inflammatory cytokines but also may reflect the involvement of different death effectors and NF-κB activators.

It has been shown that NF-κB activation by TNF family members serves an anti-apoptotic function and that NF-κB activity is essential for prevention of TNF-α-induced death (Beg & Baltimore, 1996). Chang et al. (2003) found an anti-apoptotic role for NF-κB in murine insulinoma cells and Kim et al. (2006), found that the inhibition of NF-κB activation in beta cells accelerated the development of Type 1 diabetes in NOD mice, but these results are contradicted by the pro-apoptotic role of NF-κB in cultured primary islet cells or rat insulinoma cells (Heimberg et al., 2001; Chen et al., 2000; Giannoukaki et al., 2000). These differences could be explained by the different species of beta cells whether primary or cell line, or by the fact that the results obtained *in vitro* are not mirrored *in vivo*. By performing Annexin V/PI studies and cell cycle analysis it was confirmed that the Melligen cells followed a non-apoptotic mode of death. The Huh7ins and Melligen cells had a higher baseline of necrosis, revealed by the untreated cells, compared to the beta cells but by their nature being derived from a human hepatocellular carcinoma cell, it is expected that the hepatoma cell death pattern is mainly through a mode of oncotic necrosis when subjected to mitochondrial injury (Manjo & Joris, 1995).

The Melligen cells also had a smaller population of necrotic cells compared to the Huh7ins cells, which may be attributable to the presence of the human islet glucokinase gene. In support of these results Kim et al. (2005) found that during apoptosis glucokinase gene expression is decreased in high glucose-treated MIN6N8 cells due to the reduction in ATP levels indicating that the expression of glucokinase may protect the cells from apoptosis. In hepatocytes, phosphorylated Bad promotes

interactions between hexokinase and the voltage-dependent anion channel (VDAC) necessary for pumping newly synthesized ATP from the mitochondria (Majewski et al., 2004). Interactions between hexokinase and mitochondrial VDAC inhibit apoptosis by preventing the channel from binding to Bax and releasing cytochrome C. Hence, as hexokinase is regulated by the level of glucose metabolism, the interactions between Bax and VDAC may be responsible for the cytokine induced protection from apoptosis. However, cell cycle analysis using the ModFit model revealed no significant differences in the viable population sizes between the cell lines at 24h or 48h.

Further supporting the role of anti-apoptosis in the Huh7 cell line, and other hepatocellular carcinoma cell lines (HCC) such as HepG2 cells, induced by pro-inflammatory cytokines, Legrand et al. (2004) determined that IFN-α (1000U/mL) did not not alter cell cycle in these cells as determined by PI stain analysed by flow cytometry. Flow cytometry profiles obtained in the study by Legrand et al. (2004) for the Huh7 cells revealed no sub-G1 peak as was determined in the Huh7ins and Melligen cells in the current study. Similarly, apoptosis was not detected in the Huh7 cells after the IFN-α treatment as was also found in the Huh7ins and Melligen cells, however, the IFN-α signalling pathway was functional in the liver cell lines through gamma-activated sequence and interferon-stimulated regulatory element. A moderate anti-proliferative effect was found after treatment of the cells. This group suggests that the preventive role of IFN-α on HCC cannot be explained by an apoptotic and/or an anti-proliferative effect, but possibly by its action on several specific nuclear sequences that protect liver cells from transformation. Experiments following-up this possibility are included in Chapter 5 of this thesis where transcriptomics show an up-regulaion of genes related to a network of protective mechanisms after treatment with IFN-γ, TNF-α and IL-1β.

Since the expression of cytokine receptors IFNR1, IFNR2, IL1R1, IL1R2 and TNFR1 was observed at the mRNA level, the reduced susceptibility to cytokine-induced killing displayed by Melligen cells is not likely to be attributable to the absence of receptors for the cytokines. There is evidence to suggest that the molecular expression of cytokine receptors translates to the protein expression and function of these

receptors (Gao et al., 2008; Secher et al., 2009; Bellehumeur et al., 2009). Although TNFAR2 expression was found to be present in the islet cells it was absent in the liver cell lines, TNF-α can ligate TNFAR1, reflective of the redundancy of cytokine networks (Bernhart & Peter, 2003; Wicovsky et al., 2009). The majority of the biological effects of TNF are mediated by TNFAR1 triggering, which can initiate signalling pathways leading to the activation of the transcription factor NF-κB (Van Antwerp et al., 1996 Conzelmann et al., 2002) and TNFAR2, an accessory receptor. Therefore, the presence of TNFAR1 expression and absence of TNFAR2 expression in the liver cell lines indicates that TNF-α is still able to induce the cascade signalling response. It is also possible that TNFAR2 expression and function is up regulated after treatment of the liver cell lines with both IFN-γ and TNF-α as demonstrated by Wang et al. (2006) in intestinal epithelia. These results showed that IFN-γ (10ng/mL) primes intestinal epithelia to respond to TNF-α (2.5ng/mL) by inducing TNFAR2 up-regulation, which in turn mediates the TNF-induced myosin light chain kinase-dependent barrier dysfunction. The data further suggest that epithelial TNFAR2, and not TNFAR1, blockade may be a novel approach to restore barrier function in intestinal disease such as Crohns' (Wang et al., 2006).

The initiation of beta cell damage in rats by IL-1β has been shown to require signalling via the high affinity IL-1 receptor, mRNA transcription and *de novo* protein synthesis, and diminished mitochondrial function (Helqvist et al., 1989; Hammonds et al., 1990). In a study by Vadrot et al. (2006), STAT1 nuclear translocation was investigated in four liver cell lines including Huh7 cells. It was determined that plasma membrane IFN-γ receptors IFNGR1 and IFNGR2 are functional in these cell lines and that the first step of IFN-γ signalling can be initiated (See Chapter 5 for STAT1 signalling in Melligen cells). Okada et al., (2007) also showed that hepatocellular carcinoma cells show resistance to the anti-proliferative effect of IFN-γ, due mainly to the down regulation of IFNGR2, even though IFNGR1, the domain that includes the binding site of IFN-γ, is stably expressed. However, since both IFNGR1 and IFNGR2 were expressed in the liver cell lines, IFN-γ should have bound to these receptors, thereby initiating a response at the molecular level.

Contrary to the low expression levels of iNOS observed in the Melligen cells, the expression of iNOS mRNA has been detected in both rodent and human pancreatic islets exposed to cytokines *in vitro* (Eizirik & Pavlovic, 1997). Transgenic expression of iNOS in beta cells has been shown to stimulate their destruction and the development of diabetes (Takamura et al., 1998). In rodents, islet exposure to IL-1β or IL-1β and IFN-γ induces expression of iNOS, and subsequent formation of NO (Holohan et al., 2008; Kleeman et al., 1993; Kolb & Kolb-Bachofen, 1992). In previous studies it has been observed that inhibition of iNOS or a deletion of the iNOS gene is protective in animal models of Type 1 diabetes (Floström et al., 1999). Furthermore, iNOS deficiency in islet allografts have been shown to be more resistant to destruction in the transplantation model (Börjesson et al., 2006). Therefore the observed weak expression of the iNOS gene in the cytokine treated Melligen cells, determined by Real Time RT-PCR, and the low levels of NO after cytokine treatment compared to the MIN-6 cells, are desirable characteristics if these cells are to be ultimately used to reverse autoimmune diabetes.

Cytokine-induced nitric oxide production has been proposed to participate in the inhibition of insulin secretion in human islets (Corbett et al., 1993). Nitric oxide is believed to be an effector molecule that mediates cytokine-induced destruction and dysfunction of pancreatic beta cells (Andersson et al., 2001; Cetkovic-Cvlje & Eizrik, 1994; Corbett & McDaniel, 1995). After 48h, MIN-6 cells treated with the cytokine cocktail produced greater levels of NO compared to the untreated cells. Andersson et al. (2001) showed that expression of inducible nitric oxide synthase (iNOS) and subsequent NO formation induced by IL-1β or (IL-1β and IFN-γ) for 48h may impair islet insulin function in rodent islets. Furthermore, inhibition of iNOS or a deletion of the iNOS gene confers protection against cytokine-induced beta cell suppression. Similar to the results obtained in the current study, Wu et al. (2001) observed that the greatest production of nitrite in MIN-6 cells occurred in the presence of all three cytokines with maximum production at 48h. It has also been shown that the pro-inflammatory cytokines, IFN-γ, TNF-α and IL-1β inhibit insulin synthesis and secretion and induce apoptosis via nitric oxide free radicals in mouse, rat, and human beta cells and islets *in vivo* and *in vitro* (Zumsteg et al., 2000; Suarez-Pinzon et al., 1999).

Nakata et al. (1999) examined the effects of NO production by MIN-6 cells in response to cytokine treatment. They reported that *in vitro* exposure to cytokines induced the NO producing pathway in the mouse beta cells to trigger Ca^{2+}-dependent apoptosis of beta cells involving protease activation. In a study by Laffranchi et al. (1995) the consequent production of NO by islet beta cells was shown to be the major cause of the inhibition of insulin release and ultimate destruction of islet beta cells. IFN-γ plays a central role in iNOS expression, with subsequent synthesis of nitric oxide (Ko et al., 2008). Some results have shown that iNOS elicits or inhibits apoptosis in liver cells (Moreau, 2002). NO can induce resistance to apoptosis through stimulation of the chaperone protein HSP70, which is known to be anti-apoptotic (Kim et al., 1997).

In contrast to MIN-6 cells, Huh7, Huh7ins and Melligen cells did not produce increased levels of NO at either 24h or 48h. Despite the low expression levels of iNOS in the cytokine treated Melligen cells after 4h, there was no detectable change in the levels of NO produced at the protein level by the cells after 48h. Similarly, Chang et al. (2004) identified down-regulated iNOS gene expression in cytokine treated rat hepatocytes, but a negative feedback mechanism of NO production and a reduction in NO levels overall. Therefore, a negative feedback mechanism may explain the unchanged level of NO production in the cytokine treated liver cell lines. In a study by Kim et al. (1997) the effects of NO were determined in relation to caspase-3 activity. These data indicated that NO prevented apoptosis in hepatocytes by either directly or indirectly inhibiting caspase-3-like activation.

In Type 1 diabetes, cytotoxic T cells kill the target cells through one of two pathways. In the perforin pathway, the direct effects of perforin and granzymes on the target cell cause death. In the Fas pathway, a T-cell membrane ligand (FasL) binds a target cell surface receptor (Fas) that induces apoptosis when ligated (Kagi et al., 1995). An indirect pro-apoptotic effect of cytokines is the up-regulation and surface expression of the Fas receptor on beta cells, increasing the susceptibility of beta cells to apoptosis mediated by the Fas ligand expressed on islet-infiltrating auto-reactive cytotoxic ($CD8^+$) T-cells or added in vitro to mouse islets (Darwiche et al., 2003; McKenzie et al., 2008). As well as having a pivotal role in the canonical death receptor pathway,

the deletion of Fas from the beta cell *in vivo* has also been shown to enhance insulin secretion (Choi et al., 2009).

In this current study although Fas gene expression was down-regulated in cytokine-treated Melligen cells, as determined by Real Time RT-PCR, the cell viability and insulin secretion of the treated cells (see Chapter 3) remained similar to the untreated cells. These results may indicate the presence of an anti-apoptotic mechanism. It has been suggested that NF- κB activation may protect hepatocytes from TNF-α-mediated apoptosis and mediate hepatocyte proliferation (Plumpe et al., 2000; Van Antwerp et al., 1996). There are also some molecules that exist whose role is to protect cells from undergoing apoptosis these include anti-apoptotic proteins such as Bcl2, viral inhibitors such as FLIP (FLICE/caspase-8–inhibitory protein) and antioxidants such as MnSOD. In a study by Schoemaker et al. (2003), they reported that primary hepatocytes, when stressed in the form of cytokine stimulation, similar to the cytokine treatment employed in the current study, induced two anti-apoptotic proteins, A1/bfl 1 (prosurvival Bcl-2 homologue A1) and BIRC3 protecting the hepatocytes from cell death. XIAP has also been shown to protect liver cells from Fas/FasL killing.

Numerous cells in the liver have been shown to express Fas and/or FasL, and the Fas/FasL system plays a major role in the pathogenesis of many liver diseases. However, many Fas-expressing cells including hepatocytes are resistant to Fas-induced apoptosis in culture, suggesting that cellular factors exist that inhibit Fas signalling which includes activation of NF-κB (Hatano et al., 2001). Furthermore, inhibition of the transcription factor NF-κB makes cells sensitive to apoptosis induced by TNFα. This leads to the theory that NF-κB induces expression of genes, which normally protect cells from apoptosis induced by TNF-α (Baichwal & Baeuerle, 1997).

Macrophages are among the first cell types to infiltrate the islets and they play a major role in the destruction of beta cells in Type 1 diabetes (Kolb-Bachofen et al., 1989). Results from a study by Cardozo et al., 2001 suggested that beta cells exposed to IL-1β and/or IFN-γ express several chemokines, cytokines, and adhesion molecules that may potentially contribute to the homing, adhesion, and activation of mononuclear cells in the course of insulitis. Piemonti et al. (2002) determined that

human islets were able to attract monocyte/macrophages through the production and secretion of biologically active MCP-1. Data on clinical islet transplantations in patients with Type 1 diabetes also suggested a relevant role of MCP-1 secreted by the islets (Piemonti et al., 2002). Since MCP-1 has been shown to attract mononuclear cells, recent data indicate that IL-1β also induces MCP-1 mRNA and protein expression in human islets, and that the chemokine is present in pancreatic islets of prediabetic NOD mice (Chen et al., 2001). In liver cells, however, MCP-1 has been shown as a modulator in liver cell injury. In a study by Czaja et al. (1994), MCP-1 gene expression was found in non-diseased liver and at greatly increased levels in livers from patients with fulminant hepatic failure supporting a role for MCP-1 in the modulation of liver injury. Hence, the resistance of our insulin secreting liver cell lines to the cytotoxic effects of cytokines may be attributable, in part, to reduced expression of MCP-1. This feature will likely be advantageous as the cells may be afforded protection against autoimmune mediators produced by mononuclear cells.

It has been shown that over-expression of antioxidant enzymes (Lortz et al, 2000) or the use of inhibitors of nitric oxide production (Hadjivaailiou et al., 1998) protects insulin-producing cells from the harmful effects of certain cytokine or free radical combinations. It is possible that some intrinsic properties of the hepatic cell lines, such as increased oxidative enzymes Catalase, MnSOD (Chapter 5 microarray data confirms up regulation of this gene in cytokine treated Melligen cells) and Gpx, reduced expression of stimulatory and adhesion molecules, or diminished susceptibility to cytokine-mediated damage, allow the cells to evade the initiation of immune responses and/or susceptibility to destruction. Tabiin et al. (2004) determined that transgenic NOD mice that produce (pro)insulin, driven by the PEPCK promoter, in their liver do not develop cellular infiltration of their liver when autoimmune destruction of pancreatic beta cells occurs, indicating that the presence of the insulin gene in the liver cells does not illicit an immune response. Testament to the findings in this chapter, liver cells have the ability to evade the attack otherwise shown to destroy beta cells. Melligen cells may therefore be a suitable source of insulin-secreting cells for the treatment of Type 1 diabetes.

4.6 Conclusion

In conclusion these results show that the resistance of insulin-secreting liver cell lines to the detrimental effects of pro-inflammatory cytokines is likely not attributable to decreased expression of their cognate receptors. Unlike pancreatic beta cells, pro-inflammatory cytokine treatment is unable to induce apoptosis in the insulin-secreting liver cell lines. It is further a possibility that the minor role of apoptosis is induced by the action of the pro-inflammatory cytokines on several specific molecular mechanisms that protect the liver cells from this cytotoxicity. Further studies on the molecular machinery involved in this cytokine resistance are described in Chapter 5.

CHAPTER 5: Cytokine-Induced Gene Expression in Melligen Cells

5.1 Introduction

Hepatocytes are known to play an important role in glucose metabolism and in the synthesis and storage of proteins in the liver. These attributes make hepatocytes attractive candidates for the production of an artificial beta cell. When the insulin gene is introduced into hepatocytes, such that they express the gene, under certain circumstances, they have the ability to store and secrete insulin and respond to glucose both *in vitro* and *in vivo* (Muniappan & Ozcan, 2007; Tuch et al., 2001). In our laboratory, the insulin-secreting liver cell line Melligen, has also been shown to store and secrete insulin, and respond to glucose in the physiological range (Chapter 3). Although the surrogate beta cell was shown to be resistant to the cytokine-induced apoptotic events otherwise known to destroy beta cells (Chapter 4), to be therapeutically viable in the treatment of Type 1 diabetes, Melligen cells must also possess molecular machinery to enable the cells to be resistant to the other effects of cytokine-induced toxicity such as the decreased expression of beta cell transcription factors (Cardozo et al., 2001). Additionally, in the event of an autoimmune response towards the cells, minimal effect on glucose metabolism and other protein transport functions is not only desirable, but also essential if the cells are to be used to reverse diabetes.

Human liver cells can be transdifferentiated into insulin-producing cells by the insertion of insulin and other beta cell transcription factors (Simpson et al., 1997; Zalzman et al., 2005). One of the complications, however, in generating transdifferentiated cells for cell-replacement therapy is the continued presence of specific beta cell genes that are involved in the autoimmune destruction of the beta cells (see Chapter 1). It has been shown that human fetal liver

cells can be induced, by ectopic expression of Pdx1, to produce and store mature insulin in significant amounts, release it in response to physiological glucose levels, and replace beta-cell function in immuno-deficient mice made diabetic by STZ injection (Zalzman et al., 2003). However, the modified cells expressed multiple beta-cell genes and genes found in other islet cells and exocrine pancreas. They also continued to express a number of hepatic genes. Zhang et al., (2009) determined that there was loss of liver glycogen in diabetic CD-1 mice after hepatic insulin gene therapy, using an adenoviral vector, showing that there may be loss of normal liver function. This was probably due, however, to vector-induced cytokine effects rather than the presence of the beta cell auto-antigen insulin. Manipulation of the human fetal liver cells has been shown to further promote the differentiation of these cells towards the beta-cell phenotype, as judged by gene expression and insulin content (Tabiin et al., 2001). If Melligen cells express some or all of these antigens they are likely to be exposed to recurring autoimmunity when transplanted, suggesting that the response of the Melligen cells to the cytokines, at a molecular level, may be similar to the beta cell's apoptotic cascade signaling response to cytokines. However, it is also possible that the beta cell antigens are not induced by genetic engineering events and so the Melligen cells will not express them but rather maintain their cytokine resistant parent hepatocyte phenotype.

Microarray data in a study by Lutherborrow et al. (2009), based on differences between Huh7 and Huh7ins cells, revealed no change in the expression of any known genes involved in secretory granule biogenesis, although genes possibly involved in the regulated secretion of insulin were identified. Huh7 and Huh7ins were found to express several important beta cell transcription factor mRNAs. Huh7 and Huh7ins cells both express various beta cell genes, and while none of these factors have a recognized effect on secretory granule biogenesis, the expression of *NeuroD* in liver

cells *in vivo* have been shown to result in the formation of secretory granules that contained insulin (Kojima et al., 2003). Unpublished data in our laboratory revealed that the Melligen cells also express some of these transcription factors and this includes *Pdx-1*, Ngn3, *NeuroD*, Nkx2.2, Nkx6.1 and Pax 6. In establishing the extent of transdifferentiation, PC1/3 and PC2 were also found in the Melligen cells, however, unlike the study by Lutherborrow et al. 2009, Nkx6.1 was not found in the Huh7 or Huh7ins cells in our laboratory.

One of the ways in which beta cells are killed is by pro-inflammatory cytokines. The main cause of destruction remains unclear (Jahromi & Eisenbarth, 2007; Daneman, 2006). The autoimmune destruction of the pancreatic beta cells by proinflammatory cytokines IFN-γ, TNF-α and IL-1β regulate the expression of a number of immune-related genes, as determined by microarray analyses. In primary rat and human islets and insulin producing cell lines the expression of cytokine-induced genes that are affected include major histocompatibility complex proteins, cytokines, chemokines and cytotoxic enzymes, many of which are regulated by the transcription factor, NF-κB (Liuwantara et al., 2006; Cardozo et al., 2001, Sarkar et al., 2009 Rasschaert et al., 2003; Kutlu et al., 2003; Ortis et al., 2008). Microarray technology has indicated that the deleterious effects of cytokines upon insulin-producing cells results from a shift in the gene expression profile that is responsible for the up-regulation of apoptosis promoting genes. Overall the expression of several hundred genes are affected by cytokines in insulin-producing cell lines and primary beta cells. Among these, are genes classified under beta cell metabolism, transcription factors and apoptosis. In addition to the up regulation of known pro-apoptotic genes, such as iNOS (Montolio et al., 2007), caspase-1 (CASP1) (Karlsen et al., 2000), cyclooxygenase (COX-2) (Corbett et al., 1993), and MCP-1 (Johansson et al., 2003), which are potentially related to insulitis and beta cell damage, others such as MnSOD (SOD2) and catalase (Chen et al., 2005), heat shock protein (HSP-70) (Burkat et al., 2008), BIRC3 (Sarkar et al., 2009) and A20 (TNFAIP3) (Liuwantara et al., 2006), are probably part of beta cell defence mechanisms and none of these proteins are responsible for cytokine-induced apoptosis on their own.

Beta cell death is potentiated several fold when pro-inflammatory cytokines are used in combination (Figure 5.1). Additionally, pro-apoptotic genes such as Bid and Bcl-2 are up regulated in beta cells in response to cytokines, Fas/FasL and perforin (McKenzie et al., 2008). IFN-γ binding to its receptor induces oligomerization and the cytoplasmic recruitment of JAK1 and JAK2 in beta cells (Gysemans et al., 2008). Once activated by transphosphorylation, JAK1 and JAK2 recruit STAT-1 and trigger its activation by phosphorylation. STAT-1 then homodimerizes and migrates to the nucleus where it regulates the expression of genes containing γ-activated sequence (GAS) elements in their promoter, such as caspases, FAS and iNOS (Suarez-Pinzon et al., 1999; Stassi et al., 1997). In addition to the STAT-1 pathway, the JAKs also activate a member of the MAPK family, namely the extracellular signal-regulated kinase (ERK) (Krasilnikov et al., 2003). STAT1 and IRF1 (interferon regulatory factor 1) have been shown to play a critical role in the signal transduction of cytokine synergism (IFN-γ and TNF-α), which has also been found to be pertinent to the *in vivo* beta cell death and diabetes in NOD mice (Suk et al., 2001). On the other hand, IL-1β has a minor pro-apoptotic effect in rodent beta cells when used singly (Suk et al., 2001; Delaney et al., 1997; Campbell et al., 1988).

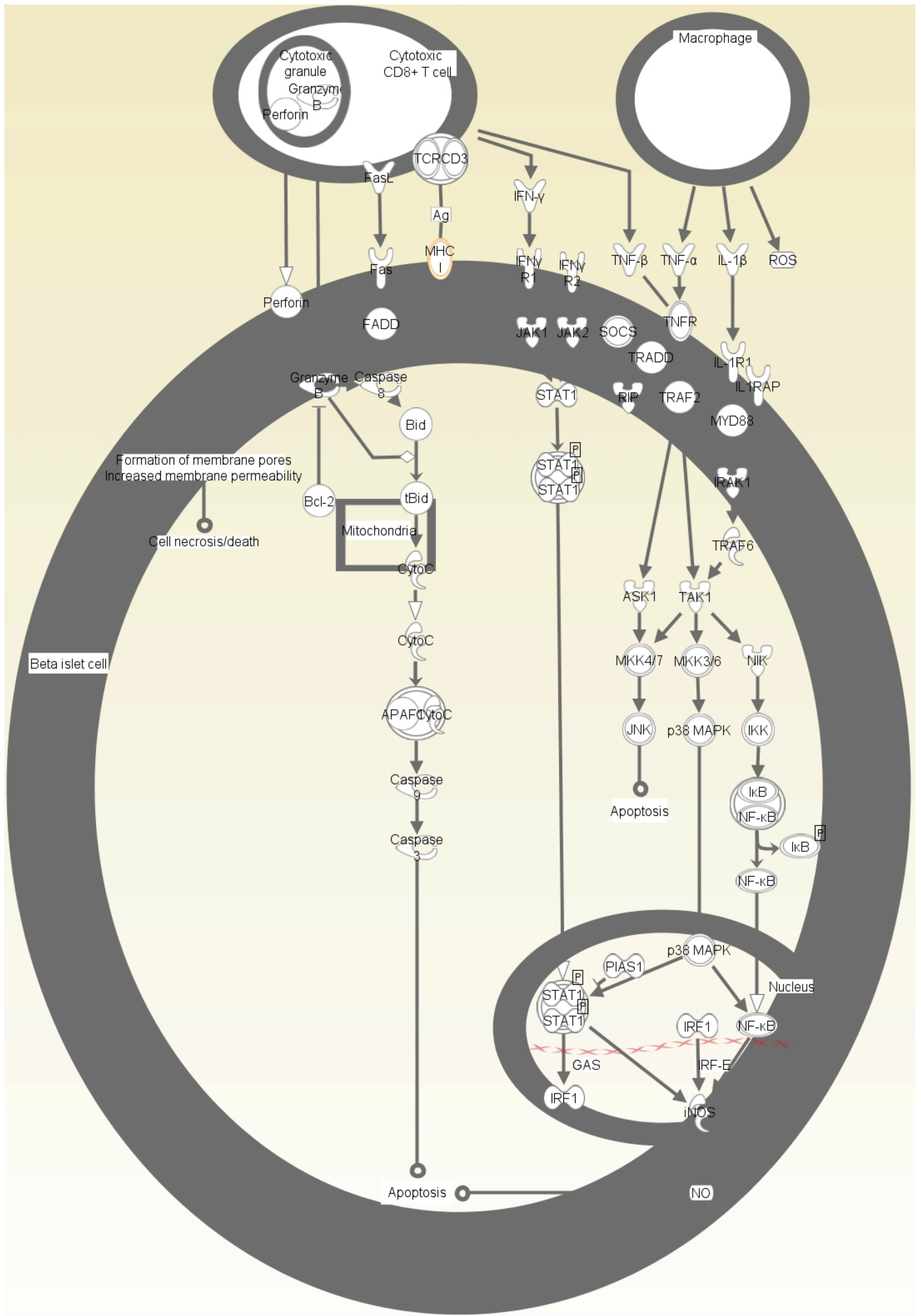

Macrophage
Cytotoxic granule
Granzyme B
Perforin
Cytotoxic CD8+ T cell
TCRCD3
FasL
Ag
MHC I
IFN-γ
IFNγ R1
IFNγ R2
TNF-β
TNF-α
IL-1β
ROS
Fas
Perforin
FADD
JAK1
JAK2
SOCS
TNFR
TRADD
IL-1R1
IL1RAP
Granzyme B
Caspase 8
STAT1
RIP
TRAF2
MYD88
Bid
Formation of membrane pores
Increased membrane permeability
STAT1
STAT1
P
P
IRAK1
Bcl-2
tBid
Mitochondria
Cell necrosis/death
TRAF6
CytoC
ASK1
TAK1
CytoC
Beta islet cell
MKK4/7
MKK3/6
NIK
APAF1
CytoC
JNK
p38 MAPK
IKK
Caspase 9
Apoptosis
IκB
NF-κB
Caspase 3
IκB
P
NF-κB
p38 MAPK
PIAS1
Nucleus
STAT1
STAT1
P
P
IRF1
NF-κB
GAS
IRF-E
IRF1
iNOS
Apoptosis
NO

Figure 5.1: Type 1 diabetes canonical pathway

Three main pathways are involved in the execution of apoptosis in the beta cell: I) Fas/FasL or perforin/granzyme initiate a cascade of caspase activation through Bid and Bcl-2 which lead directly to beta cell death by the final effector of apoptosis, caspase 3. II) Docking of IFN-γ to its receptors IFNγ-R1 and IFNγ-R2 leading to the phosphorylation of STAT1 by JAK1 and JAK2. The phosphorylated STAT1 complex is activated upon entering the nucleus and binds to DNA on the GAS site inducing IRF-1 and iNOS. The formation of NO then leads to death by apoptosis. III) The docking of TNF-α and IL-1β to their receptors initiates a cascade of events leading to complex formation and nuclear activation of NF-κB and eventually apoptotic death by NO. In addition to this, the activation of ASK1 and MKK4/7 lead to the increased expression of JNK, which induces apoptosis. (Ingenuity Pathway Analysis Software, USA).

Cytokines also synergise to induce iNOS, leading to NO production (Ko et al., 2008; Corbett et al., 1992). The iNOS promoter contains two binding sites for NF-κB, which are activated by IL-1β and TNF-α, and a binding site for STAT-1, which is activated by IFN-γ. Combinations of cytokines, but not any one of these cytokines alone, induce iNOS expression in human islets (Eizirik et al., 1994). High concentrations of NO provoke DNA damage, activating p53, which then induces poly-ADP-ribose-polymerase (PARP) (Kim & Kim, 2007). PARP activation may trigger cell death due to ATP depletion secondary to NAD^+ consumption in the reaction to DNA repair (Welsh et al., 1991). In line with this, almost 50% of the cytokine-induced genes in insulin producing cells, as observed by microarray analysis, are NO dependent (Kutlu et al., 2003) (see Figure 1.5 for some of these related genes). IFN-γ/TNF-α synergism involving STAT1/IRF1 pathways (Figure 5.1) have also been found to play a critical role as the final effector in the development of diabetes *in vivo* (Suk et al., 2001).

Because pancreatic beta cells contain very low levels of antioxidant enzymes (Lenzen et al., 1996; Roma et al., 2009), their enhanced sensitivity to oxidative stress is considered an important component of the toxic action of cytokines (Eizirik et al., 1994). Similarly over-expression of antioxidant enzymes in insulinoma cells results in significantly increased resistance to cytokine-mediated toxicity (Azevedo-Martins et al., 2003). Transgenic mice that exhibit beta cell targeted over-expression of the MnSOD enzyme were also shown to be resistant to autoimmune and STZ-induced diabetes (Hotta et al., 1998). In developing a surrogate beta cell, having inherent mechanisms that render the liver cells resistant to the apoptotic effects of an autoimmune attack, and the effects of cytokines on insulin function, could present as a significant advantage in treating Type 1 diabetes. The Melligen cell fate following immune-mediated damage will depend on the intricate pattern of dozens of genes up or down regulated in parallel and/or sequentially. A microarray analysis was utilized to clarify the pattern of gene expression in Melligen cells exposed to the proapoptotic cytokines, IL-1β, IFN-γ and TNF-α.

Similar to the beta cell, primary hepatocytes have the ability to follow the pathway to the trimerisation of the TNF-α type I receptor (TNFR-1) by TNF-α resulting in docking of adaptor proteins, such as Fas-associated protein, with death domain (FADD), TNFR-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2) and the serine/threonine kinase receptor interacting protein (RIP). This then leads to the recruitment of FADD to TNFR-1-bound TRADD resulting in the activation of caspase-8 followed by the activation of effector caspases, in particular caspase-3, resulting in apoptosis (Xu et al., 1998; Bradham et al., 1998). However, unlike the beta cell, the NF-κB survival pathway is initiated in hepatocytes by the activation of NF-κB-inducing kinase (NIK). This protein activates an IκB kinase (IKK) complex, which results in the specific phosphorylation and proteasomal degradation of the inhibitors of NF-κB, IκBα, IκBβ and IκBε. The release of NF-κB is followed by its migration to the nucleus, where it activates the transcription of NF-κB-responsive genes (Schwabe et al., 2001; Wang et al., 1998). Studies have shown that blocking the NF-κB pathway in TNF-α-stimulated hepatocytes results in a shift towards apoptosis (Xu et al., 1998; Iimuro et al., 1998). This implies the existence of NF-κB-regulated anti-apoptotic genes in hepatocytes and it is important to determine if such an anti-apoptotic pathway still exists in the transdifferentiated Melligen cells, since beta cells possess a cytokine-induced apoptotic pathway.

The results obtained in Section 3.3 showed that Melligen cells exposed to cytokines for 10 days have no significant impairment in insulin function. It was also found that Melligen cell culture in the presence of IL-1β, IFN-γ and TNF-α does not lead to apoptosis after 24h or 48h as indicated by AnnexinV/PI studies (Chapter 4). However, it has been shown that 4h cytokine treatment is sufficient to down regulate genes involved in the NF-κB signalling pathway indicating an effect on gene regulation. In Chapter 4, particular focus was on Melligen cells generating an inducible anti-apoptotic response contrary to that in beta cells after exposure to proinflammatory cytokines. Little is known about the genetic control of apoptosis and metabolism in the insulin secreting liver cell line, Melligen. However, having found that Melligen cells do have a resistance or inducible protective response to pro-inflammatory cytokines, it is important to now examine the molecular basis of this response.

The Th-1 proinflammatory cytokines used in this study were chosen based on their established roles in inducing beta cell apoptosis and metabolic dysfunction both *in vivo* and *in vitro* (Grey et al., 1999; Arnush et al., 1998; Cetkovic & Eizirik et al., 1994; Thomas et al., 2004). According to Sarkar et al., (2009) and Liuwantara et al. (2006), studies performed on primary human islet cells showed maximal stimulation of TNFAIP3 (A20) at 1h treatment with the cytokines TNF-α and IL-1β. Further treatment for 6h and 24h, as shown by Cardozo et al. (2001), confirmed the up and down regulation of several functional genes including those involved in metabolic function, such as GLUT2 and glucokinase. Therefore, the time-points in this study were chosen in agreement with previous work indicating that early and relevant changes of the gene expression profile in insulin secreting cells were particularly evident 1h and 24h after cytokine exposure.

With regard to the toxic effects of cytokines on beta cells, the aim of this study was to determine the extent of the transdifferentiation of Melligen cells in their response to the pro-inflammatory cytokine cocktail IFN-γ, TNF-α and IL-1β. The extent of the transdifferentiation of Melligen cells was determined by the use of microarray to study the general pattern of cytokine induced gene expression in these cells. Finally, this chapter will address the loss of the hepatocyte phenotype by comparing the effects of the cytokine treatment on selected genes involved in the signalling pathway of the parent liver cell line, Huh7, and Melligen cells.

5.2 Preliminary analysis indicates cytokine effect in Melligen cells is greater at 24h than at 1h

The Melligen cells were treated with and without the cytokine cocktail IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 1h and 24h. After extracting RNA and hybridising to the gene array whole transcript coverage was analysed. Each of the 28,869 genes was represented on the Affymetrix ST 1.0 Human Genome Array by approximately 26 probes per gene spread across the full length of the gene. In order to capture variance in the dataset, Principle Components Analysis (PCA) mapping was employed. This analysis was used to reduce dimensionality of the data and summarise the most important parts of the dataset.

Exposure of the Melligen cells to cytokines resulted in an altered gene expression pattern at both 1h and 24h of the cytokine treatment. Using the Partek Genomic Suite, PCA mapping of the Gene Array showed that the control and treated samples are more similar at the 1h time point compared to the 24h time point. The PCA map also revealed a cluster pattern showing that the duplicate chips for treated and untreated samples at the 1h and 24h time points were on the same plane and could be encompassed by an ellipse (Figure 5.2).

PCA Mapping (64.9%)

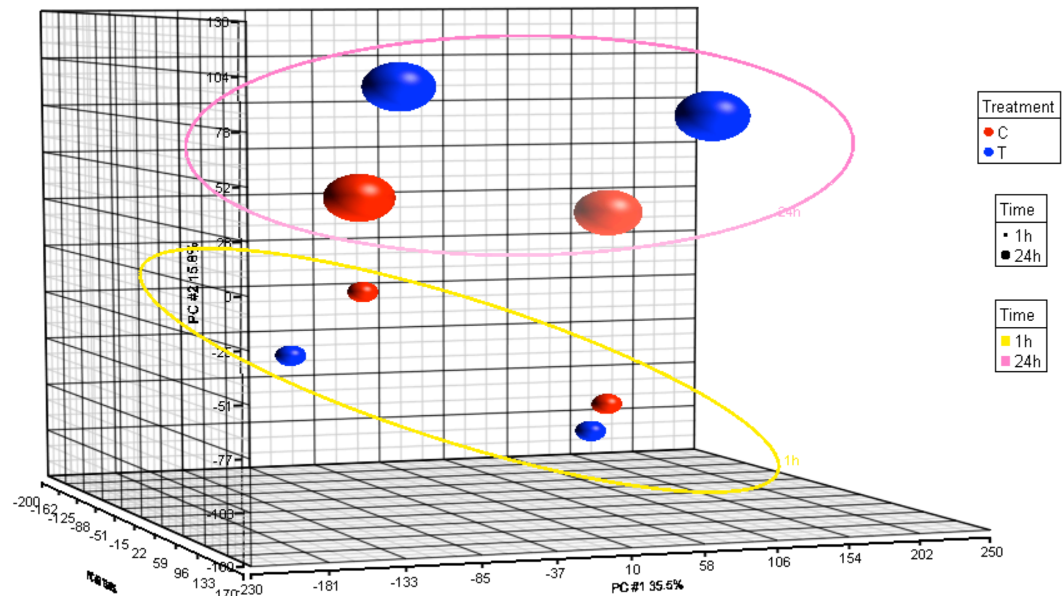


Figure 5.2: PCA mapping of Melligen cell microarray dataset at 1h and 24h cytokine treatment.

A histogram showing the distribution of the intensities for each of the samples (in duplicate) for 1h untreated, 1h treated, 24h untreated and 24h treated showed that all of the samples follow the same distribution pattern indicating that there are no obvious outliers in the data.

Melligen cells were treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 1h and 24h. Untreated cells used as a control. Variation in high dimensional microarray data was visualised in a PCA map. Plots show PC#1, PC#2 and PC#3 for a three dimensional plot with the greatest source of variation highlighted by an ellipse around sample chips at 1h and one at 24h. Data represents duplicate chips for each sample.

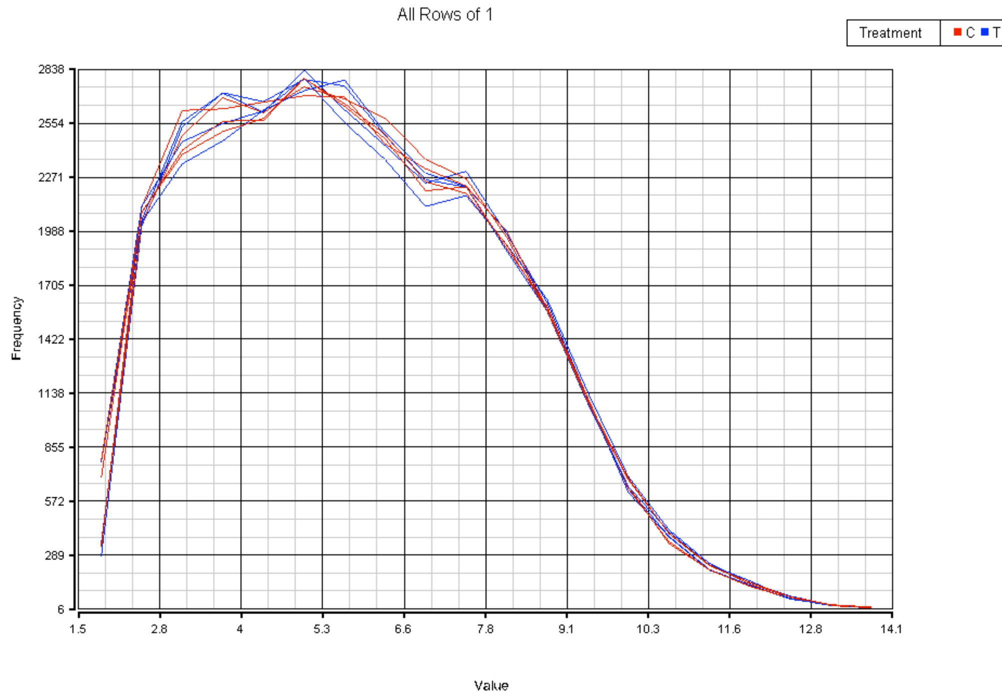


Figure 5.3: Intensity distribution in histogram plot of Melligen cell microarray dataset at 1h and 24h cytokine treatment.

Melligen cells were treated with and without IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 1h and 24h. All eight samples follow the same distribution pattern with no obvious outliers. Data represents duplicate chips for each sample. (The intensity of the probes are graphed on the x-axis and the frequency of the probe intensity on the y-axis).

5.2.1 Identification of differentially expressed genes in 1h cytokine treated Melligen cells

In order to identify differentially expressed genes the ANOVA was used to generate a list of genes whose expression is significantly different (P<0.05) between the untreated and cytokine treated Melligen cells. A difference of greater than twofold was considered significant.

After 1h exposure of Melligen cells to the cytokines, 18 genes were to be differentially expressed by twofold or greater, and with a P-value less than 0.05, are presented in Table 5.1 (only 1 gene was down-regulated and 17 up regulated). The up-regulated genes included genes from the chemokine (C-X-C motif) ligand family, CXCL1 (P= 0.01), CXCL2 (P= 0.002), CXCL3 (P= 7.71×10^{-5}) and CXCL10. The

expression of CXCL10 was found to be up-regulated by the greatest level with a 19 fold up regulation (*P*= 0.0001). Interferon regulatory factor 1 (IRF1), induced by IFN-γ, was also significantly up-regulated (P= 1.57x10^{-5}). Genes rapidly induced by IL-1β included TIFA (P= 0.014) and PTX3 (P= 0.0008). Putative NF-κB target genes up-regulated in the Melligen cells at 1h cytokine treatment included baculoviral inhibitor of apoptosis protein repeat-containing 3 (BIRC3) (*P*= 0.014), tumor necrosis factor, alpha-induced protein 3 (TNFAIP3) with the highest significance level at 1h (P= 8.35x10^{-7}), NUAK2 (*P*= 0.005), mitogen-activated protein kinase kinase kinase 8 (MAP3K8) inducing the nuclear production of NF-κB (*P*=0.00097) and TRAF interacting protein with forkhead-associated domain (TIFA) (*P*= 0.014). The only gene down-regulated at 1h cytokine treatment compared to 1h untreated cells (Figure 5.5), was NFKBIA (also known as IκBα) (P= 2.54x10^{-5}). This result is consistent with the real time RT-PCR result obtained in Chapter 4.

Table 5.1: Genes differentially expressed in Melligen cells after 1h cytokine treatment.

Data are for genes differentially expressed greater than twofold. The genes are categorised for pathway involvement taking into account the P-values in the significance of differential gene expression). -, decreased compared to respective control (Melligen cells not exposed to cytokines for 1h). P-values <0.05

Gene Assignment	Protein	Fold Change
Growth factors; chemokines; interleukins:		
CXCL1	Chemokine (C-X-C motif) ligand 1	2.58
CXCL2	Chemokine (C-X-C motif) ligand 2	4.57
CXCL3	Chemokine (C-X-C motif) ligand 3	2.11
CXCL10	Chemokine (C-X-C motif) ligand 10	19.70
IL8	Interleukin 8	3.05
Interferon signalling pathway:		
IRF1	Interferon regulatory factor 1	8.96
Interleukin signalling pathway:		
PTX3	Pentraxin-related protein	4.10
TIFA	TRAF-interacting protein with forkhead-associated domain	2.47
Tumor necrosis factor signalling pathway:		
BIRC3	Baculoviral IAP repeat-containing 3 (cIAP2)	3.18
NUAK2	NUAK family, SNF1-like kinase, 2	2.40
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3 (A20)	4.69
NF-κB nuclear activation:		
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	2.54
NFKBIA	Nuclear factor of light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα)	-2.53
Various:		
APOL6	Apolipoprotein 6	2.32
CSF1	Colony stimulating factor 1 (macrophage)	2.13
ICAM1	Intercellular adhesion molecule-1	2.28
TAP1	Transporter 1, ATP-binding cassette, sub-family B	2.61
UBD	Ubiquitin D	2.75

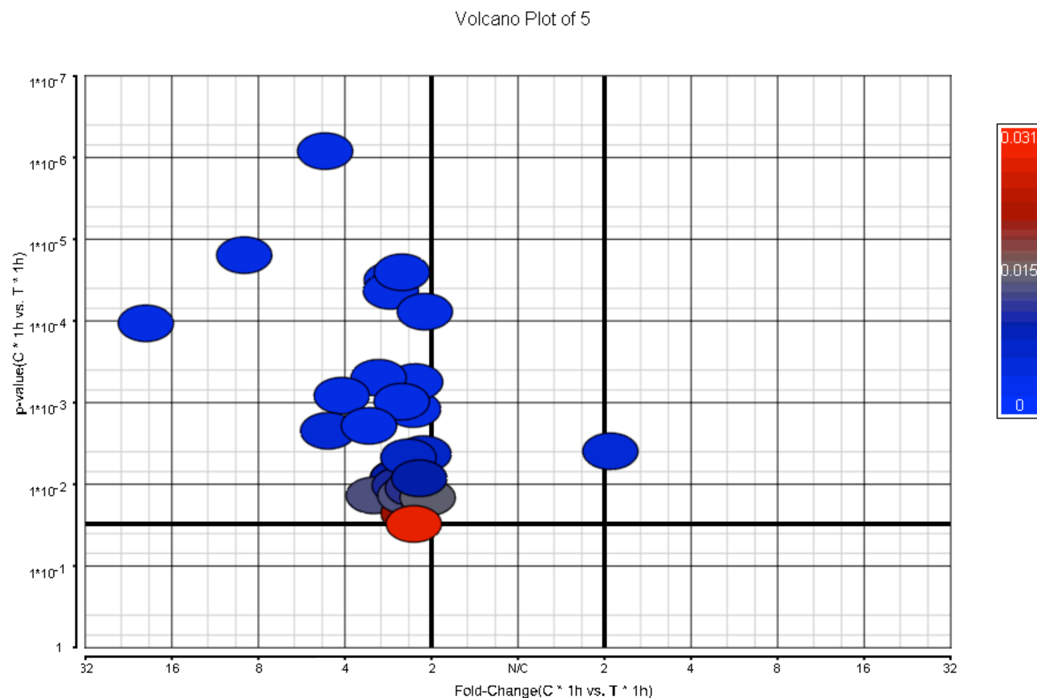


Figure 5.4: Volcano plot for 1h cytokine treated Melligen cells.

Showing the number of genes up- and down-regulated in the cells exposed to IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 1h. Red indicates P-values close to 0.05 and blue indicates P-values close to 0.

5.2.2 Identification of differentially expressed genes in 24h cytokine treated Melligen cells

RNA transcript levels for different genes in the Melligen cells were also determined after 24h of cytokine treatment, with untreated cells used as a control. Exposure of the Melligen cells to the same combination of cytokines for 24h significantly modified the expression of 148 genes. The listed 148 genes identified are presented in Table 5.2. The large majority of the modified genes (140 of 148) were up regulated upon cytokine treatment and mainly represented genes known to be inducible by IFN-γ, TNF-α and IL-1β. This group included some of the following: for IFN-γ related genes- myxovirus resistance 1 (MX1) (*P*= 0.00012), 2',5'-oligoadenylate synthetase 1 (OAS1) (*P*= 0.0036), the signal transducer and activator of transcription 1 (STAT1) (*P*= 0.00036), interferon alpha inducible protein 6 (IFI6) (*P*= 2.34×10^{-5}), interferon-induced protein with tetratricopeptide (IFIT2) (*P*= 0.00026), lectin galactoside-

binding soluble 3 binding protein (LGALS3BP) (*P*= 0.01) and the interferon inducible guanylate binding proteins (GBP) 1, 2, 3, 4, 5 and 7. For TNF-α related genes BIRC3 (*P*= 0.002), TNFAIP 2/3 (*P*= 0.014 and 5.88×10^{-6} respectively), indoleamine 2,3 dioxygenase 1 (IDO1) (*P*= 0.0076) and optineurin (OPTN) (*P*= 1.93×10^{-5}) were up-regulated. For IL-1β related genes- caspase 1 (CASP1) (*P*= 0.00064), IL7, 8 and 32, interleukin 2, receptor gamma (IL2RG) (*P*= 0.00018), interleukin 6 signal transducer (IL6ST) (*P*= 0.00096). Some IFN-α induced genes were induced, even though the cells were treated with IFN-γ, suggesting either IFN-α itself may have been induced, or there is overlap of the effects if these cytokines on liver cells.

A total of eight genes were down-regulated in Melligen cells treated with cytokines for 24h, villin 1 (VIL1) (*P*= 0.0001), sodium channel nonvoltage-gated 1 alpha (SCNN1A) (*P*= 0.00014), amino acid/proton antiporter SCL38A3 (*P*= 0.0001), the enzyme for condensing acetyl-CoA HMGCS2 (*P*= 0.0018), from the sulphotransferase family SULT2A1 (*P*= 0.0066), retinol binding protein 2 (RBP2) (*P*= 0.011), chromosome 9 open reading frame 45 (C9ORF45) (*P*= 0.0018) and scaffold protein PDZK1 (*P*= 0.0065).

The genes most up regulated in the cytokine treated Melligen cells include guanylate binding protein 1 (GBP1) and CXCL 9 and 10 (19.2, 20.2 and 33.06 fold respectively). However, the most significantly up regulated genes were HLA-E, TNFAIP3 and UBD (*P*= 8.38×10^{-8}; $.88 \times 10^{-6}$ and 8.20×10^{-7} respectively). Genes for MHC antigen presentation were also up regulated including HLA-A, B, C, E, F and J. Three ubiquitin activating enzymes (UBE and UBA7) and poly (ADP-ribose) polymerase family (PARP9 and PARP14), tripartite motif containing 31 (TRIM 31) and apolipoprotein 1 and 6 (APOL 1 and 6) were also up regulated by the cytokine treatment.

The induction of putative Melligen cell defense/repair, cellular degradation and turnover genes was more evident after 24h exposure to IFN-γ, TNF-α and IL-1β. This group includes SOD2, and cathepsins O and S (CTSO and CTSS). Cellular function

and integrity pathways including ion transport and lipid proteins were also affected by the cytokine treatment.

Table 5.2: Genes differentially expressed in Melligen cells after 24h cytokine treatment.

Data are for genes differentially expressed greater than twofold. The genes are categorised for pathway involvement taking into account the P-values in the significance of differential gene expression). -, decreased compared to respective control (Melligen cells not exposed to cytokines for 24h). P-values <0.05

Gene Assignment	*Protein*	*Fold Change*
Growth factors; chemokines; interleukins:		
CXCL1	Chemokine (C-X-C motif) ligand 1	2.36
CXCL2	Chemokine (C-X-C motif) ligand 2	2.24
CXCL5	Chemokine (C-X-C motif) ligand 5	2.82
CXCL9	Chemokine (C-X-C motif) ligand 9	20.22
CXCL10	Chemokine (C-X-C motif) ligand 10	33.06
CXCL11	Chemokine (C-X-C motif) ligand 11	4.65
IL7	Interleukin 7	2.03
IL8	Interleukin 8	3.28
IL32	Interleukin 32	4.81
IL2RG	Interleukin 2 receptor, gamma	2.73
IL6ST	Interleukin 6 signal transducer	2.00
Interferon-inducible proteins:		
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.17
GBP1	Guanylate binding protein 1, interferon-inducible	19.20
GBP2	Guanylate binding protein 2, interferon-inducible	6.43
GBP3	Guanylate binding protein 3, interferon-inducible	4.78
GBP4	Guanylate binding protein 4, interferon-inducible	13.55
GBP5	Guanylate binding protein 5, interferon-inducible	9.58
GBP7	Guanylate binding protein 7, interferon-inducible	2.44
IFI6	Interferon, alpha-inducible protein 6	7.67
IFI30	Interferon, alpha-inducible protein 30	3.36
IFI35	Interferon, alpha-inducible protein 35	4.34
IFI44	Interferon, alpha-inducible protein 44	2.19
IFIH1	Interferon induced with helicase C domain 1	3.82
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	2.66
IFIT3	Interferon induced protein with tetratricopeptide repeats 3	6.74

IFIT5	Interferon induced protein with tetratricopeptide repeats 5	2.10
IFITM1	Interferon induced transmembrane protein 1	2.07
IFITM3	Interferon induced transmembrane protein 3	2.21
LGALS3BP	Lectin galactoside-binding, soluble, 3 binding protein	2.90
MX1	Myxovirus (influenza virus) resistance 1	4.80
OAS1	2'-5'-Oligoadenylate synthetase 1	3.023
OAS3	2'-5'-Oligoadenylate synthetase 3	3.69
PLSCR1	Phospholipid scramblase 1	2.15
PSMB8	Proteasmone (prosome, macropain) subunit beta type 8	9.19
PSMB9	Proteasmone (prosome, macropain) subunit beta type 9	15.66
PSMB10	Proteasmone (prosome, macropain) subunit beta type 10	3.35
PSME1	Proteasmone (prosome, macropain) activator subunit 1	2.65
PSME2	Proteasmone (prosome, macropain) activator subunit 2	2.95
RTP4	Receptor transporter protein 4	3.21
SP110	SP110 nuclear body protein (IFI41)	3.02
Interferon signalling pathway:		
IRF1	Interferon regulatory factor 1	5.91
IRF9	Interferon regulatory factor 9	3.83
NMI	N-myc (and STAT) interactor	4.19
STAT1	Signal transducer and activator of transcription 1	4.58
STAT2	Signal transducer and activator of transcription 2	2.07
TAAR3	Trace amine associated receptor 3	4.14
Tumor necrosis factor signalling pathway:		
BIRC3	Baculoviral IAP repeat-containing 3 (cIAP2)	6.61
IDO1	Indoleamine 2,3-dioxygenase 1	3.21
OPTN	Optineurin	2.91
TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	2.03
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3 (A20)	2.58
Interleukin signalling pathway:		
CARD16	Caspase recruitment domain family, member 16 (COP)	2.80
CASP1	Caspase 1	9.12
MHC antigen presentation:		
B2M	Beta-2-microglobulin	3.06
HLA-A	Major histocompatibility complex, class I, A	5.13

HLA-B	Major histocompatibility complex, class I, B	6.11
HLA-C	Major histocompatibility complex, class I, C	4.74
HLA-E	Major histocompatibility complex, class I, E	6.00
HLA-F	Major histocompatibility complex, class I, F	2.83
HLA-G	Major histocompatibility complex, class I, G	4.07
HLA-J	Major histocompatibility complex, class I, J	3.72
HLA-DOB	Major histocompatibility complex, class II, DO beta	3.40
LGMN	Legumain	2.57
Toll like receptor signalling:		
TLR3	Toll-like receptor 3	2.84
TRAFD1	TRAF-type zinc finger domain containing 1 (FLN29)	2.48
Cathepsins:		
CTSO	Cathepsin O	2.08
CTSS	Cathepsin S	9.00
NF-κB nuclear production:		
IKBKE	I kappa-B kinase epsilon	2.38
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	2.19
RELB	Reticuloendotheliosis viral oncogene homolog B	2.05
Ion transport:		
CP	Ceruloplasmin	2.93
PDZK1	PDZ domain containing 1	-2.45
RCN1	Reticulocalbin 1, EF-hand calcium binding domain	2.64
SCNN1A	Sodium channel, nonvoltage-gated 1 alpha	-2.28
SLC6A14	Solute carrier family 6 (amino acid transporter), member 14	3.29
SLC15A3	Solute carrier family 15, member 3 (OCTP)	2.67
SLC38A3	Solute carrier family 38, member 3 (NAT1)	-2.96
TMEM140	Transmembrane protein 140	2.12
Complement activation:		
C4A	Complement component 4A	6.30
CFB	Complement factor B	2.71
CFH	Complement factor H	3.06
C1S	Complement component 1, s sub component	14.20
C1R	Complement component 1, r sub component	9.46
DDX60L	DEAD box polypeptide 60-like	2.17

SERPING1	Serpin peptidase inhibitor, clade G (C1-inhibitor), member 1	3.07
Ubiquitination pathway:		
DTX3L	Deltex 3-like (Rhysin)	2.98
ERAP1	Endoplasmic reticulum aminopeptidase 1	3.29
ERAP2	Endoplasmic reticulum aminopeptidase 2	2.19
GPR109A	G protein-coupled receptor 109A	2.04
GPR109B	G protein-coupled receptor 109B	2.64
LBP	Lipopolysaccharide binding protein	2.44
NUB1	Negative regulator of ubiquitin-like proteins 1	2.09
PARP9	Poly (ADP-ribose) polymerase family, member 9	4.35
PARP14	Poly (ADP-ribose) polymerase family, member 14	5.45
TAP1	Transporter 1, ATP-binding cassette, sub-family B	19.06
TAP2	Transporter 2, ATP-binding cassette, sub-family B	5.18
TAPBP	TAP binding protein (Tapasin)	3.13
TAPBPL	TAP binding protein-like	2.93
TRIM21	Tripartite motif-containing 21	2.76
TRIM22	Tripartite motif-containing 22	2.29
TRIM31	Tripartite motif-containing 31	4.00
UBA7	Ubiquitin-like modifier activating enzyme 7	3.92
UBD	Ubiquitin D	12.57
UBE2L6	Ubiquitin-conjugating enzyme E2L 6	6.09
Lipid proteins:		
APOL1	Apolipoprotein L, 1	6.87
APOL6	Apolipoprotein L, 6	7.27
LCN1L1	Lipocalin 1-like1	2.33
LRP2	Low density lipoprotein-related protein 2 (Megalin)	2.83
PLA1A	Phospholipase A1 member A	3.12
PLA2G2A	Phospholipase A2, group IIA	5.97
Anti-oxidants		
SOD2	Superoxide dismutase 2, mitochondrial (MnSOD)	3.043
Various:		
ACSL5	Acyl-CoA synthetase long-chain family member 5 (ACS2)	6.22
ADAM23	ADAM metallopeptidase domain 23	2.26
ALPK1	Alpha-kinase 1	2.35
BST2	Bone marrow stromal cell antigen 2	9.04
BTN3A1	Butyrophilin, sub family 3, member A1	3.13
BTN3A2	Butyrophilin, sub family 3, member A2	3.09
BTN3A3	Butyrophilin, sub family 3, member A3	3.60
C9orf45	Chromosome 9 open reading frame 45	-2.48
CD38	CD38 molecule	3.94

CD47	CD47 molecule (integrin associated protein)	2.02
CD274	CD274 molecule	2.25
CPA2	Carboxypeptidase A2 (Pancreatic)	2.99
DDX60	DEAD box polypeptide 60	8.86
EPSTI1	Epithelial stromal interaction 1 (breast)	8.42
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	-2.52
ICAM1	Intercellular adhesion molecule 1	3.68
KIAA1618	KIAA1618	2.02
LAMC2	Laminin, gamma 2	2.02
LAP3	Leucine aminopeptidase 3	2.22
LOC93432	Maltase glucoamylase-like pseudogene	2.39
MLKL	Mixed lineage domain-like	2.58
MUC13	Mucin 13, cell surface associated	2.28
NAMPT	Nicotinamide phosphoribosyltransferase (Visfatin)	2.01
NFE2L3	Nuclear factor (erythroid-derived 2)-like 3	2.11
NLRC5	NLR family, CARD domain containing 5	2.73
PIK3AP1	Phosphoinositide-3-kinase adaptor protein 1	2.01
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	3.75
RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	14.17
RBP2	Retinol binding protein 2, cellular (CRBP2)	-2.07
RFTN1	Raftlin, lipid raft linker 1	2.25
SERPINA7	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	3.64
SQRDL	Sulphide quinone reductase-like	2.32
SULT2A1	Sulphotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	-2.10
VIL1	Villin 1	-2.40
VNN2	Vanin 2	2.33
VNN3	Vanin 3	2.35

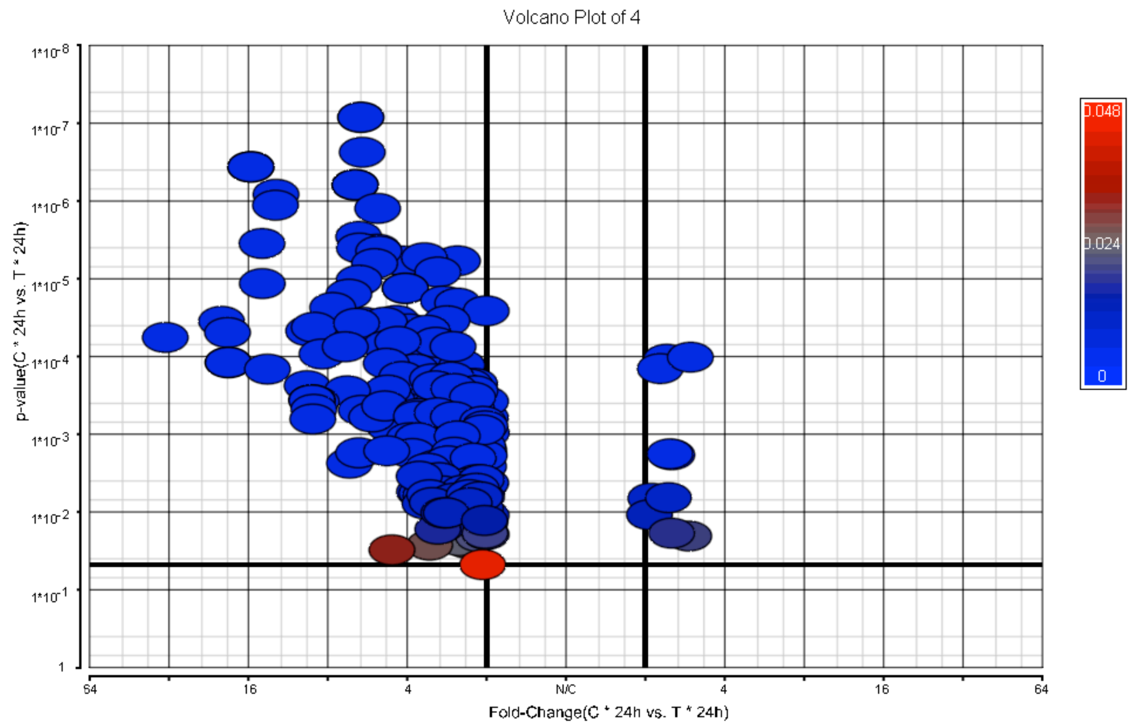


Figure 5.5: Volcano plot for 24h cytokine treated Melligen cells.

Showing the number of genes up- and down-regulated in the cells exposed to IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 24h. Red indicates P-values close to 0.05 and blue indicates P-values close to 0.

Of the six genes down-regulated in Chapter 4 (IκBα, IκBβ, IκBε, Fas, iNOS and MCP-1), none were detected as modified by cytokines in the present analysis at 24h. Iκbα was only differentially expressed at 1h, however genes IKBKE and RELB, involved in the formation and nuclear activation of the NF-κB complex and NFKB2, also differentially expressed, were significantly up-regulated at 24h (2.38, 2.05 and 2.19 fold; P= 0.0004, 0.0026 and 0.0091 respectively). This indicates that Iκbβ and Iκbε, MCP-1, iNOS and Fas, which were present in the array, had fold changes too low to be detectable by the microarray analysis. Real time quantitative RT-PCR analysis is also more sensitive in detecting changes in gene expression compared to microarray.

To determine the overlapping genes that were differentially expressed at both 1h and 24h, a Venn diagram was produced using Partek Genomic Suite software (Figure 5.7). This diagram revealed that 11 genes overlapped at 1h and 24h cytokine treatment with P<0.05 and with fold changes greater than twofold. These up-regulated genes included TAP1, UBD, ICAM1, IL-8, APOL6, CXCL1, CXCL2, CXCL10, TNFAIP3, BIRC3 and IRF1.

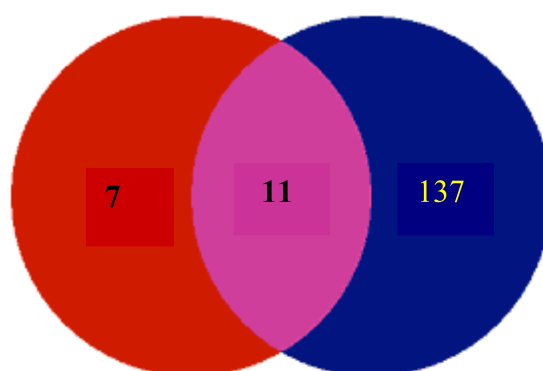


Figure 5.6: Venn diagram for the detection of overlapping genes differentially expressed in Melligen cells at 1h and 24h.

Eleven genes overlap with the number of genes differentially expressed at 1h encompassed in the red circle and the number of genes differentially expressed at 24h encompassed in the blue circle. Diagram is for genes with P< 0.05 and with fold changes greater than twofold.

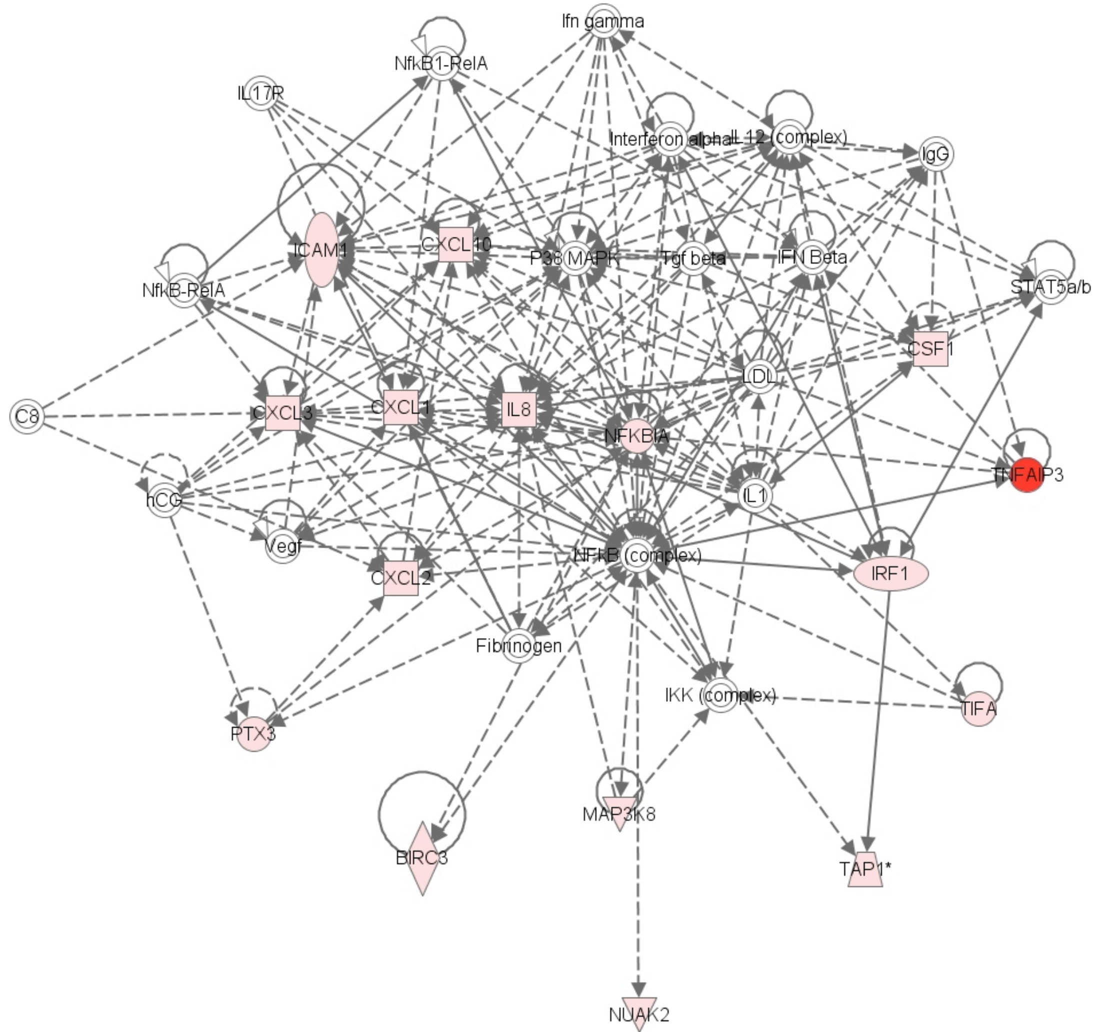
5.3 Melligen cells have an inducible anti-apoptotic response

After deriving gene lists for the differentially expressed genes at 1h (Table 5.1) and 24h (Table 5.2) with $P<0.05$ and fold change greater than twofold, the molecular effectors and signal transduction in cytokine treated insulin-secreting Melligen cells were elucidated. The gene lists were further scrutinized according to genetic networks, gene ontology, biological processes and molecular function with the aid of Ingenuity Pathways Analysis software (IPA) (see Chapter 2). The subsequent networks and gene groups established by IPA were then used to determine suitable candidate genes for verification by real time quantitative RT-PCR.

The highest scored relevance network selected at 1h was a network involving 16 of the 18 differentially expressed genes on the list (relevance score= 46) (Figure 5.7A) and a second network involving only 3 of the 18 genes differentially expressed at 1h (relevance score= 3) (Figure 5.7B). The network with the highest relevance score, Figure 5.7A, consisted of a known cluster of genes related to cellular movement and immune cell trafficking. This included genes with direct actions on IRF-1, TNFAIP3 and BIRC3, which are involved in the formation of the NF-κB complex. For the 24h treatment, five networks were established with the list of genes differentially expressed at 24h. These were allocated with relevance scores of 45, 43, 30, 30 and 28 respectively. In the highest scoring network at 24h they included clusters of genes directly related to IRF-1 (solid lines in Figure 5.8A) for cell-mediated immune responses, antigen presentation/cell-to-cell signaling and interaction, immunological disease/dermatological disease and conditions, antigen presentation/humoral immune response and antigen presentation/cell-mediated immune response respectively. The second highest scored relevance network selected at 24h (relevance score 43) was a network involving several direct links (solid lines) to STAT1 and indirect links (broken lines) to other interferon inducible genes (Figure 5.8B). The second network involved 26 of the 148 genes differentially expressed at 24h (relevance score= 43). Only the top two networks with the highest relevance score were chosen for further investigation in this chapter.

A

Network 1 : inGENUITY 1h - 2009-05-20 05:40 PM : inGENUITY 1h.xls : inGENUITY 1h - 2009-05-20 05:40 PM



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B

Network 2 : inGENUITY 1h - 2009-05-20 05:40 PM : inGENUITY 1h.xls : inGENUITY 1h - 2009-05-20 05:40 PM

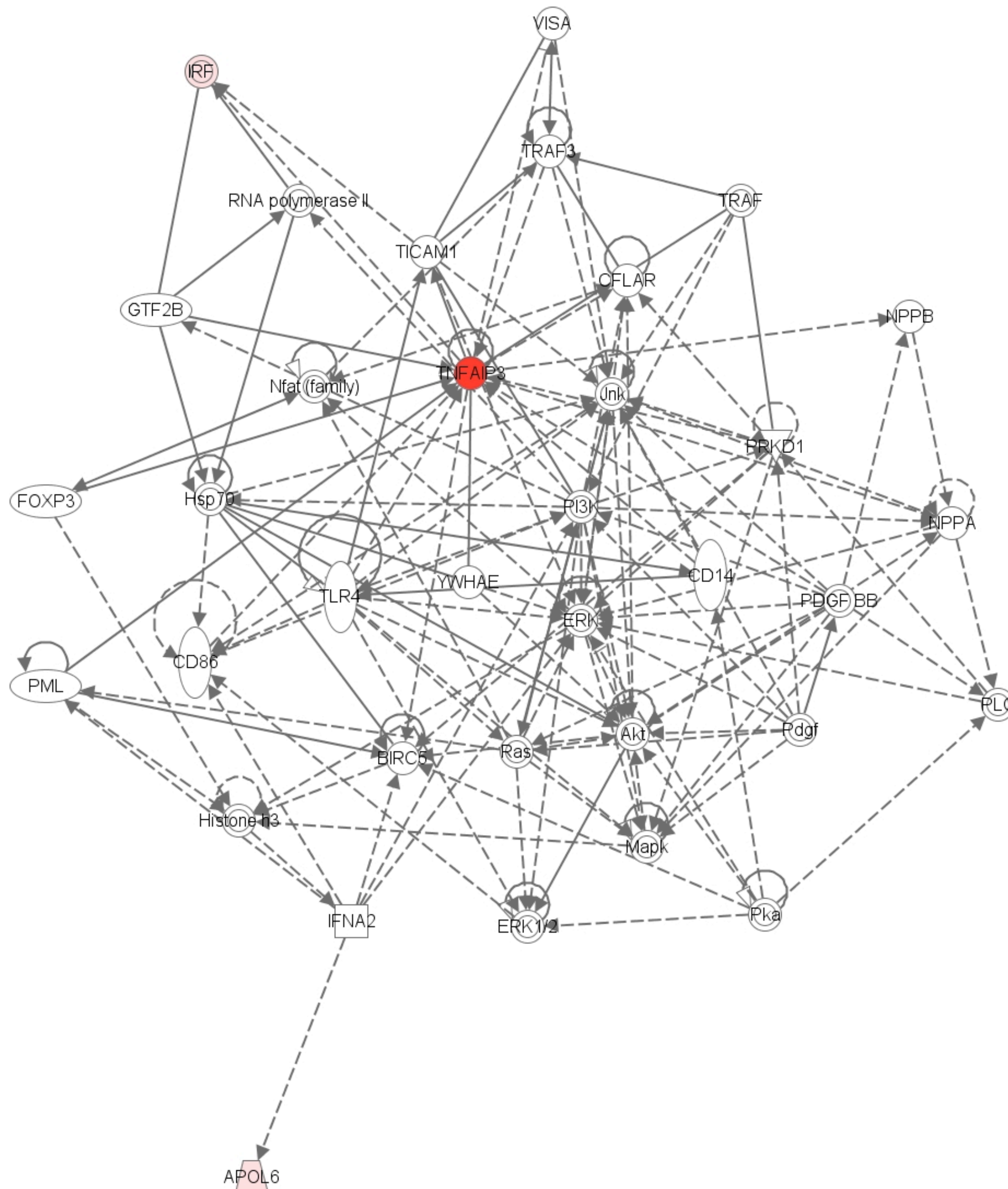

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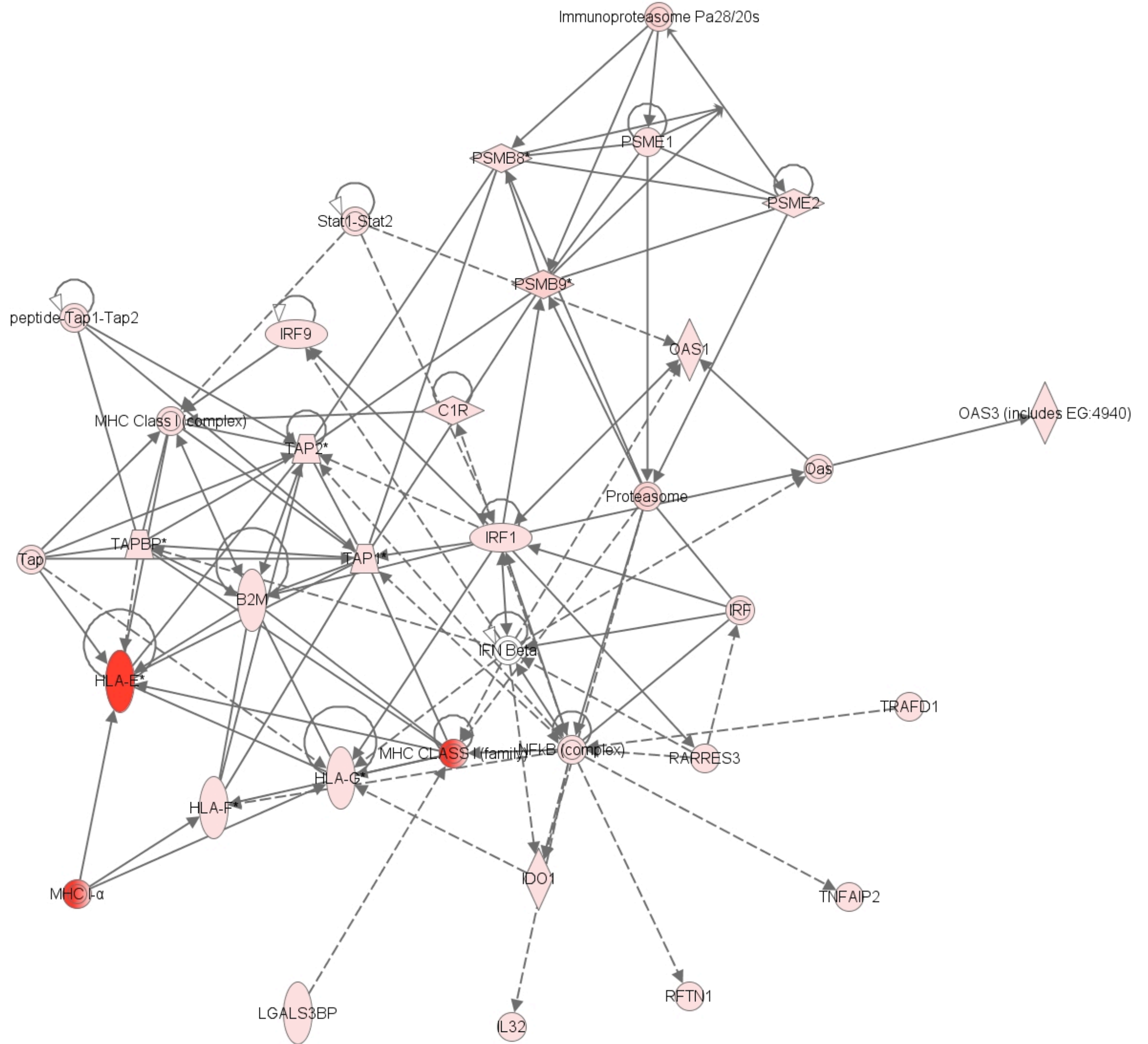
Figure 5.7: Pathway analysis of genes differentially expressed by Melligen cells after 1h cytokine treatment.

*A total of 18 genes (Table 5.1) were used to construct a network. IPA mapped the set of genes into two networks with relevance scores. The two highest scoring networks are reported as individual networks **A**) relevance score 36 and **B**) relevance score 3. The score indicates the degree of relevance of a network to the molecules in the input data set, which takes into account the number of network-eligible genes and the size of the network. The brighter the color of the gene, the higher the fold changes. *Multiple Probe Set identifiers in the array data set file map to a single gene.*

A



B

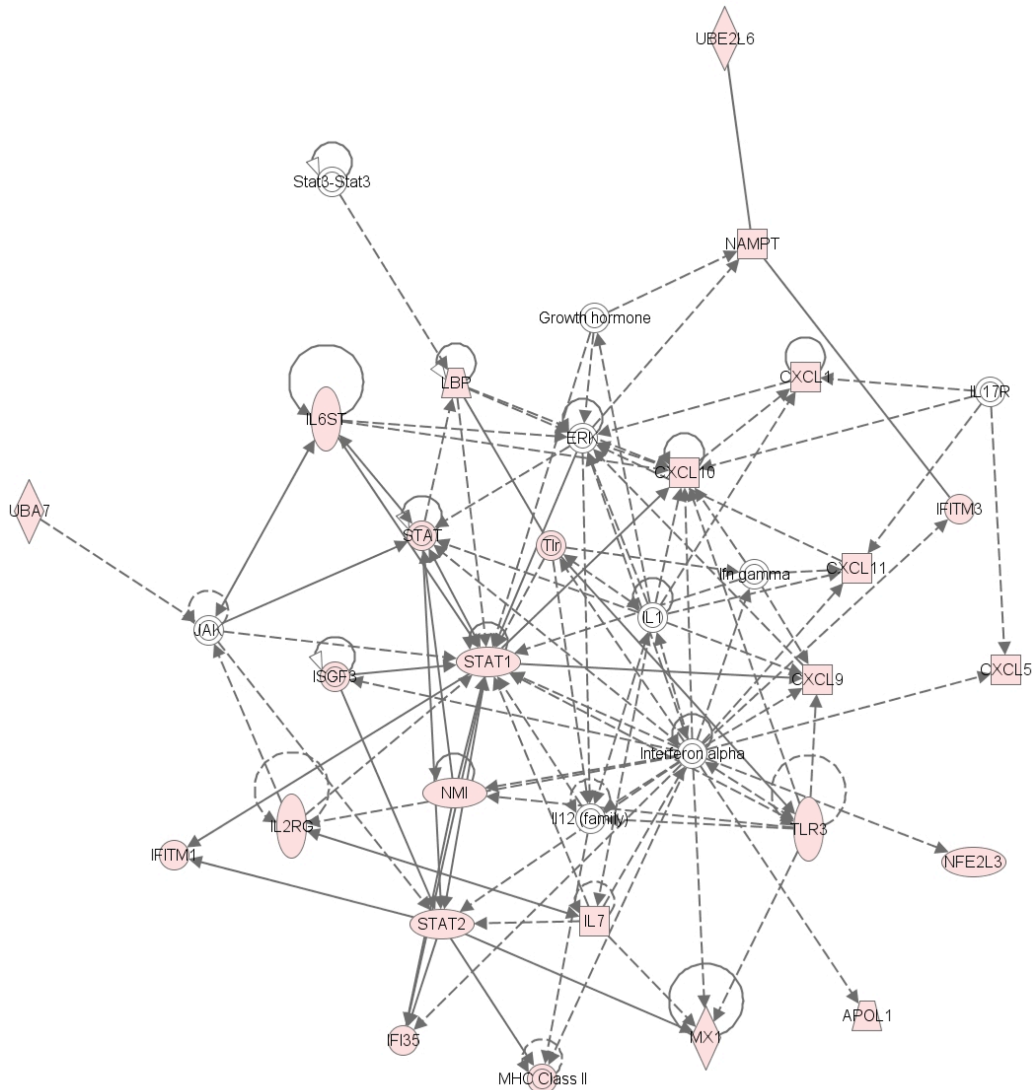


Figure 5.8: Pathway analysis of genes differentially expressed by Melligen cells after 24h cytokine treatment.

A total of 148 genes (Table 5.2) were used to construct a netwok. IPA mapped the set of genes into five networks with relevance scores. The two highest scoring networks are reported as individual networks ***A****) relevance score 45 and* ***B****) relevance score 43. The score indicates the degree of relevance of a network to the molecules in the input data set, which takes into account the number of network-eligible genes and the size of the network. The brighter the color of the gene, the higher the fold changes. *Multiple Probe Set identifiers in the array data set file map to a single gene.*

The top canonical pathways induced at 1h cytokine treatment included hepatic fibrosis/hepatic stellate cell activation (P= 3.74x10^{-6}; ratio 4/135 (0.03)) and CD40 signaling transduction (P= 2.13x10^{-5}; ratio 3/70 (0.043)) (Figure 5.9A). The top toxicological list included hepatic fibrosis (P= 7.62x10^{-7}; ratio 4/85 (0.047)), NF-κB signaling (P= 1.02x10^{-4}; ratio 3/112 (0.027)) and anti-apoptosis (P= 3.75x10^{-4}; ratio 2/32 (0.062)) (Figure 5.9B). The top biological functions identified in the differentially expressed genes with the highest recorded significance level at 1h cytokine treatment included inflammatory response (P= 6.61x10^{-15}-2.71x10^{-3}; 12 molecules involved in this function), cellular movement (P= 6.61x10^{-15}-2.71x10^{-3}; 10 molecules involved in this function), cellular growth and proliferation (P= 6.17x10^{-10}-2.71x10^{-3}; 12 molecules involved in this function) (Figure 5.9C).

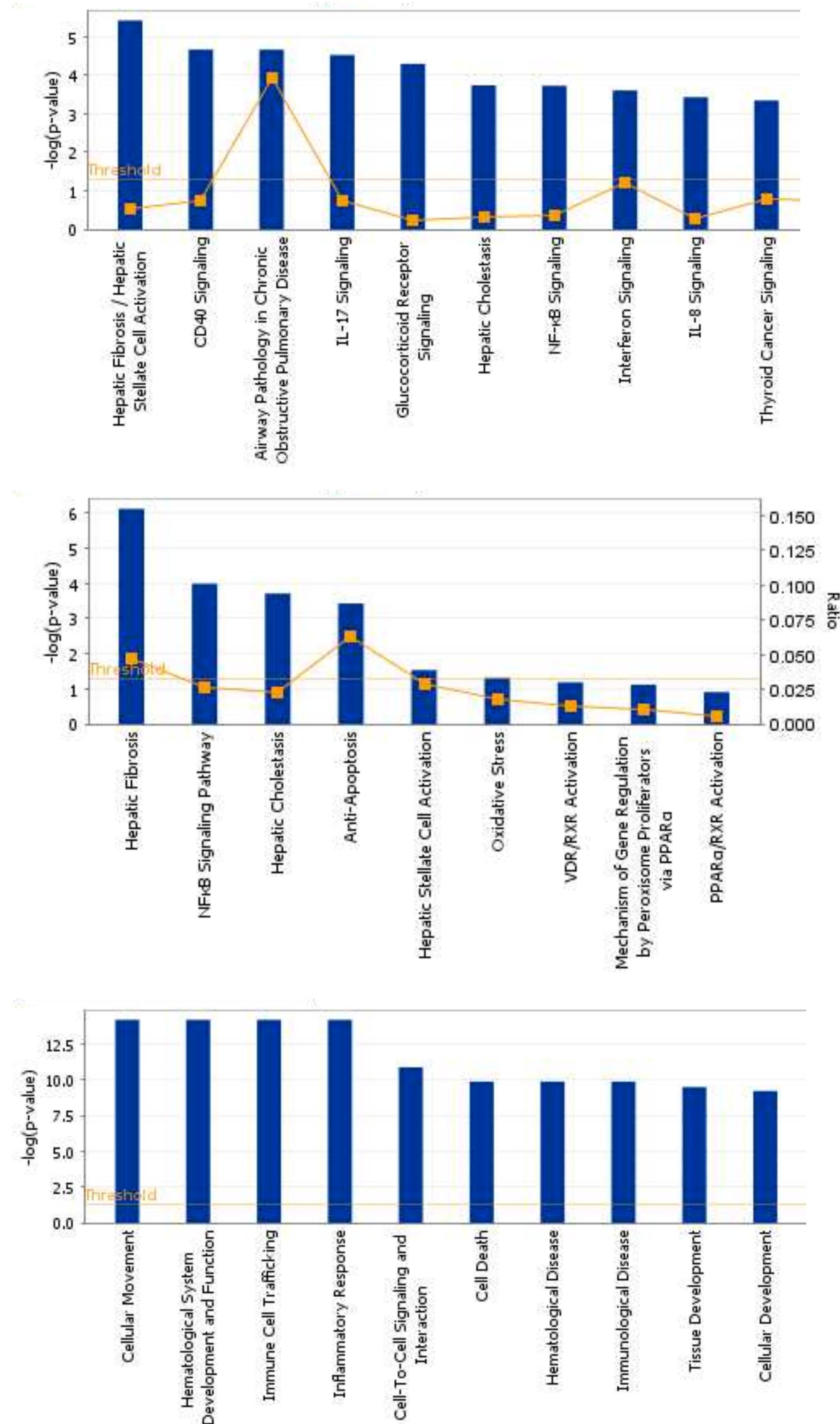


Figure 5.9: Biological processes and molecular functions for genes differentially expressed at 1h in Melligen cells.

The top 10 ***A)*** *canonical pathways* ***B)*** *listed pathways and* ***C)*** *functions in Melligen cells treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 1h. Blue bars represent –log(p-value) for the significance of the pathway or function in the list of*

differentially expressed genes and orange points represent the ratio of the number of genes differentially expressed in the current experiment over the total number of genes involved in the pathway known to represent these pathways and functions.

The top canonical pathways induced at 24h cytokine treatment included interferon signaling (P= $3.21x10^{-16}$; ratio 11/29 (0.379)) and antigen presentation pathway (P= $5.59x10^{-12}$; ratio 9/39 (0.231)) (Figure 5.10A). The top toxicological list included positive acute phase response proteins (P= $1.49x10^{-4}$; ratio 4/32 (0.125)), hepatic cholestasis (P= $1.53x10^{-4}$; ratio 7/135 (0.052)), NF-κB signaling (P= $2.67x10^{-3}$; ratio 5/112 (0.045)) and oxidative stress response (P= $1.26x10^{-2}$; ratio 3/57 (0.053)) (Figure 5.11B). It is worthy to note that hepatotoxicty was identified as being in the top toxicological function list and that liver necrosis/cell death was a pathway significantly affected by the 24h cytokine treatment (2.98x10-2-3.76x10-2; 3 molecules involved in this function). The top biological functions identified in the differentially expressed genes with the highest recorded significance level at 24h cytokine treatment included inflammatory response (P= $2.76x10^{-21}$-$8.51x10^{-3}$; 55 molecules involved in this function), antigen presentation (P= $2.76x10^{-21}$-$8.51x10^{-3}$; 54 molecules involved in this function), cell mediated immune response (P= $2.76x10^{-21}$-$8.51x10^{-3}$; 59 molecules involved in this function) (Figure 5.10C).

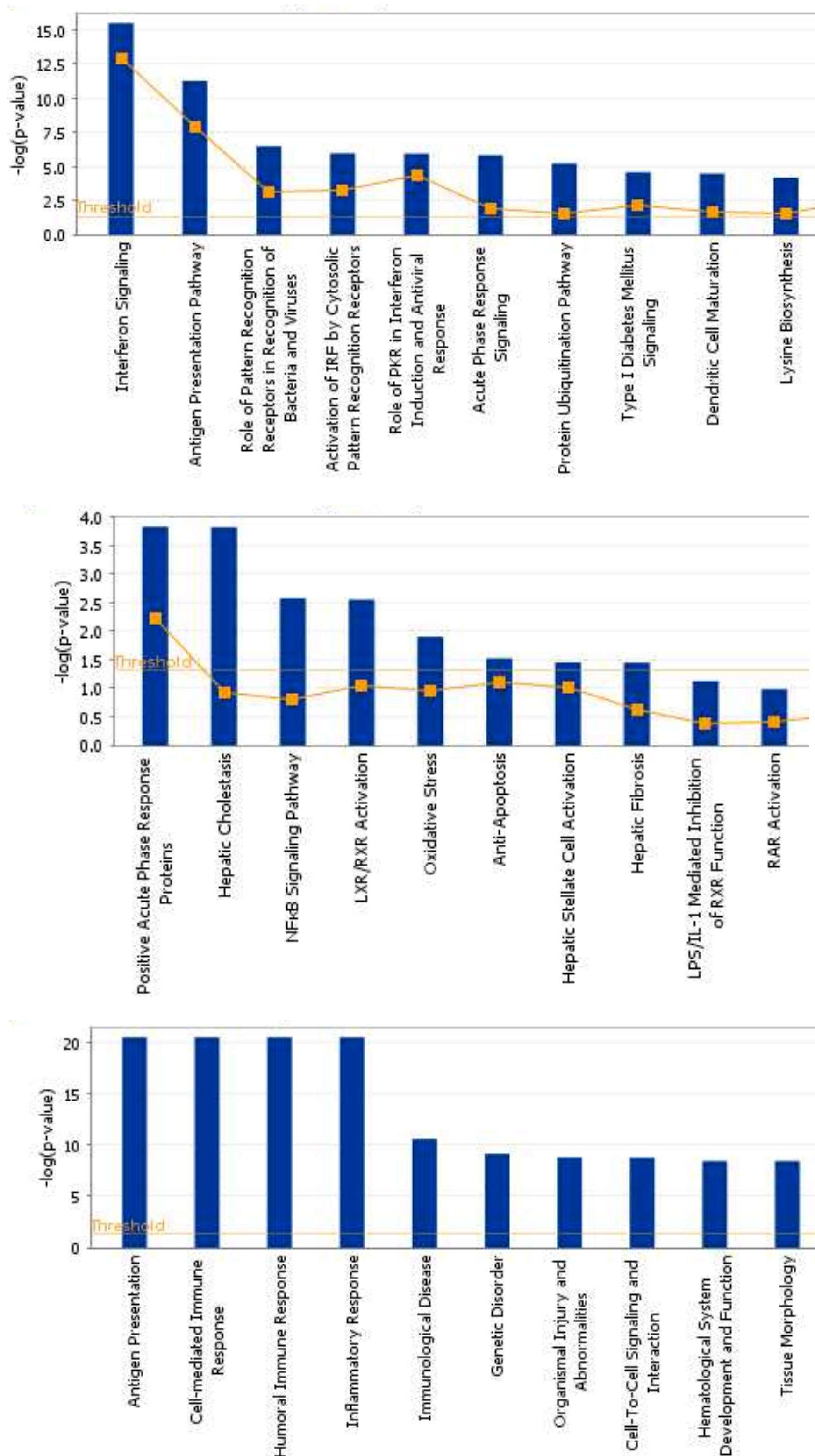


Figure 5.10: Biological processes and molecular functions for genes differentially expressed at 24h in Melligen cells.

The top 10 ***A)*** *canonical pathways* ***B)*** *listed pathways and* ***C)*** *functions in Melligen cells treated with IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) for 24h.*

Blue bars represent –log(p-value) for the significance of the pathway or function in the list of differentially expressed genes and orange points represent the ratio of the number of genes differentially expressed in the current experiment over the total number of genes involved in the pathway known to represent these pathways and functions.

5.3 Verification of microarray data by real time quantitative RT-PCR

To further validate the results of the microarray analysis, five genes were selected for confirmation by real time quantitative RT-PCR. These were genes not previously found to be differentially expressed in the parental cell line Huh7 treated with cytokines, as determined in the literature. These genes were also involved in the highest scoring networks established by IPA in the previous Section 5.4 and are of particular interest in the pro-inflammatory cytokine treatment of beta cells (see Chapter 1).

Real-time quantitative RT-PCR analysis was performed on five of the genes detected by the Gene Array to be up regulated by more than twofold and with a significance level of $P<0.05$. The five genes, BIRC3, CASP1, IRF1, SOD2 and STAT1, were selected from the cascades identified in the previous section and based on their potential involvement in the resistance of these cells to cytokine toxicity. As defined in the introduction Section 5.1, the genes selected have also been shown to play pivotal roles in the effects and signalling pathways regulated by IFN-γ, TNF-α and IL-1β singly and in combination in the Type 1 diabetes canonical pathway (Figure 5.1). IRF1 and BIRC3 were also up regulated at both 1h and 24h and were included in the list of overlapping genes.

The results obtained by real time quantitative RT-PCR confirmed the up-regulation of BIRC3, CASP1, IRF1, SOD2 and STAT1 by the 24h cytokine treatment of Melligen cells. Following normalisation with beta actin mRNA levels, there was an up regulation of BIRC3 by 6.6 fold and this was the highest recorded fold change in gene expression of the five genes selected. It was found that CASP1, IRF1, SOD2 and STAT1 were up regulated by 2.7, 3, 4.4 and 3 fold respectively (Table 5.3). To determine if the absence of the insulin and glucokinase gene expression in the liver

cell line regulates the five confirmatory genes to the same level, Huh7 cells were treated with the cytokine cocktail [IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL)] for 24h. mRNA was extracted and used to determine quantitative expression analysis by real time RT-PCR. The results showed that BIRC3 and CASP1 were down regulated by 1.64 and 1.76 fold respectively, STAT1 was up regulated by 1.53 fold and IRF1 and SOD2 gene expression remained unchanged (fold change close to 1) after cytokine treatment (Table 5.4).

Table5.3: Real-time quantitative RT-PCR results of the gene expression changes detected by microarray analysis as modified by cytokines in Melligen cells.

Genes	**Ratios Obtained by Real-Time qRT-PCR**	**Ratios Obtained by Microarray**
BIRC3	6.6 (up-regulated)	2.4 (up-regulated)
CASP1	2.7 (up-regulated)	2.3 (up-regulated)
IRF1	3.0 (up-regulated)	3.0 (up-regulated)
SOD2	4.4 (up-regulated)	2.1 (up-regulated)
STAT1	3.0 (up-regulated)	2.4 (up-regulated)

Table 5.4: Real-time qRT-PCR results of gene expression as modified by cytokines in Huh7cells.

Genes	**Ratios Obtained by Real-Time PCR**
BIRC3	1.6 (down-regulated)
CASP1	1.8 (down-regulated)
IRF1	1.2 (up-regulated)
SOD2	1.1 (up-regulated)
STAT1	1.5 (up-regulated)

5.4 Discussion

In order to develop a better understanding of the effects of cytokine treatment on Melligen cells, oligonucleotide microarrays were utilised for a systematic quantitative analysis of IFN-γ, TNF-α and IL-1β inducible genes in the insulin secreting liver cell line, Melligen. Previous chapters in this thesis observed resistance of the Melligen cells to the cytokine treatment. The treated cells remained viable over 10 days of cytokine treatment and continued to secrete and store insulin and responded to glucose levels similar to the untreated cells. Here, 148 genes were differentially expressed and classed as being regulated by the cytokine cocktail IFN-γ, TNF-α and IL-1β after 24h of treatment and only 18 genes after 1h treatment. Further to this, on the basis of the results obtained in this study, a novel regulatory pathway that might operate in Melligen cells exposed to pro-inflammatory cytokines is proposed.

One of the well-known effects of pro-inflammatory cytokines in beta cells is the inhibition of insulin mRNA expression, total protein and pro-insulin biosynthesis, and decreased insulin release (Mandrup-Poulsen, 1996; Eizirik & Pavlovic, 1997; Harb et al., 2007). In this study, there was an observed up regulation after 1h and 24h exposure to cytokines of several genes related to antigen presentation such as the HLA group of genes and cell-mediated immune response including the interferon, tumor necrosis factor and interleukin signalling pathways but not protein synthesis, modification, or secretion, which may explain the results obtained in Chapter 3, unchanged insulin secretion, storage and glucose responsiveness in Huh7ins and Melligen cells. Moreover, there were no modifications in the expression of transcription factors including *Pdx-1*, C/EBPβ and Isl-1 as determined in the microarray, which contributes to a decrease in insulin mRNA expression (Lu et al., 1997, Nielson et al., 2004). Canonical pathways analysis in the cytokine treated Melligen cells did, however, reveal that the NF-κB pathway was highly up regulated at both 1h and 24h treatment. Other pathways of interest include the anti-apoptotic pathway regulated at 1h and oxidative stress pathway at 24h involved in the protection of the Melligen cells against the cytokine treatment.

The extent of the transdifferentiation of the Melligen cells into beta cells, by the insertion of the insulin and human islet glucokinase genes, did not alter the cytokine

induced glucose metabolic effects otherwise seen in beta cells. A microarray study examining cytokine treated primary rat beta cells by Cardozo et al., (2001) showed that the most frequent changes were observed in beta cell metabolism, by IL-1β + IFN-γ for 6h, IL-1β + IFN-γ for 24h, and IL-1β alone for 24h with 19, 20, and 20% of all differentially expressed genes induced, respectively. IL-1β + IFN-γ induced an early suppression of beta cell metabolism, with a decrease in ~80% of the 18 modified genes after 6h exposure. This suppression was maintained at 24h with nearly 70% of the metabolism related genes inhibited by the combination of cytokines or IL-1β alone. These alterations occurred in genes related to the metabolism of carbohydrates, amino acids, lipids, and ATP production. The cytokines also decreased the expression of mRNAs for GLUT2 and glucokinase, whereas they increased expression of GLUT1 representing an adaptive/compensatory mechanism for the decrease in GLUT2 expression. Further to this, the same group showed that the gene microarray study corroborated proteomic studies (mass spectrometry) on rodent islet cells (Cardozo et al., 2003) showing that the differential gene expression is translated to the protein level. These findings are also supported by several *in vivo* studies examining the expression of GLUT2 in mouse models (Clark et al., 1997; Thorens et al., 2000). Although several genes encoding lipid proteins, such as APOL1 and PLA2G2A, were up regulated in Melligen cells after the pro-inflammatory cytokine treatment, these are most likely involved in the liver cell injury compensatory mechanism (Thompson et al., 2003; Law et al., 2006) rather than a contributing effect to insulin and glucose metabolic dysfunction as seen in beta cells.

A more recent microarray study by Sarkar et al., (2009) performed on human pancreatic islets also showed metabolic perturbations after treating the islets with IFN-γ, TNF-α and IL-1β. These changes included those involved in glucose transport and responsiveness, GLUT2 and glucokinase. However, in addition to the up regulation of pro-apoptotic genes, seen in the study by Cardozo et al., (2003), there was an up regulation of anti-apoptotic genes including BIRC3 and TNFAIP3 in the study by Sarkar et al., (2009). In the present study, metabolic mechanisms involving GLUT2 and glucokinase molecular expression were not affected in the Melligen cells at neither 1h nor 24h, suggesting that these cells may be protected against the defects in glucose metabolism that cytokines otherwise cause in primary rat and human beta

cells at these particular time points. Additionally, the canonical pathway involving anti-apoptotic genes was considered significantly up regulated in the Melligen cells at both 1h and 24h rendering the cells resistant to the cytokines over a greater time period compared to the immediate anti-apoptotic response observed in cytokine treated primary and beta cell lines (Ortis et al., 2008). Ortis et al. (2008) further determined that the INS-1E beta cell line treated with IL-1β, compared to TNF-α alone, induced a higher rate of expression of NF-κB target genes putatively involved in beta cell dysfunction and death and a stronger activation of the IKK complex, leading to an earlier translocation of NF-κB to the nucleus. On the other hand, the anti-apoptotic effect observed in the Melligen cells was prolonged and was induced at both 1h and 24h after the cytokine cocktail treatment.

In acute liver failure or acute viral hepatitis, hepatocytes are exposed to high levels of a variety of cytokines. These cytokines, in particular TNF-α, activate both cell survival and apoptotic pathways in primary rat hepatocytes (Schoemaker et al., 2002). Recent data suggest that members of the Inhibitor of Apoptosis (IAP) family may represent these genes. In primary rat hepatocytes treated with IFN-γ, TNF-α and IL-1β, it has been demonstrated that the expression of members of the IAP family is regulated by NF-κB. This family includes BIRC3, cIAP1, X chromosome-linked IAP (XIAP), Survivin11 and Livin12 (Hofer-Warbinek et al., 2000; Stehlik et al., 1998; Kasof & Gomes, 2001). These proteins have been reported to directly bind and inhibit the activation of caspase-3, -7 and –9. As determined by a microarray on HepG2 cells, with TNF-α treatment for 6h, BIRC3 was induced by the cytokine in an NF-κB-dependent manner and the over-expression of BIRC3 was shown to inhibit apoptosis in this liver cell line (Liu et al., 2005). Supporting the prevalent hepatocyte phenotype maintained by the Melligen cells, the results from this study showed an increased expression in BIRC3 at both 1h and 24h cytokine treatment indicating protection against cytokine induced apoptosis.

NUAK2 is normally induced by TNF-α via NF-κB. The up-regulation of NUAK2 in the Melligen cells at 24h treatment further indicates that the cells are being protected from Fas-mediated apoptosis and is required for the increased motility and invasiveness of Fas-activated tumor cells (Legembre et al., 2004). The anti-apoptotic

Bcl-2 family member A1/Bfl-1 and the pro-apoptotic members Bak and Bid are also induced by cytokines in rat hepatocytes and are NF-κB-dependent (Gu et al., 2005; Gross et al., 1999). However, these anti-apoptotic genes were not detected in the cytokine treated Melligen cells. A reason for this is that immortalized cell lines have a dramatically different gene expression profile than primary hepatocytes (Boess et al., 2003). It can therefore be suggested that Melligen cells have the advantage of being a liver cell line and not only possess a liver cell phenotype but also certain characteristics of tumor cells.

Grunnet et al., (2009) demonstrated that the intrinsic mitochondrial apoptotic pathway contributed significantly to cytokine-induced beta cell death and suggested a functional role of calcineurin-mediated Bad Ser136 dephosphorylation and Bax activity in cytokine-induced apoptosis. Bcl-X has also been shown to play an important role in the apoptotic cascade in cytokine induced beta cell death. Transduction of RIN-r cells with Ad-Bcl-X blocked both iNOS and cytokine-mediated mitochondrial changes and subsequent apoptosis, downstream of nitric oxide (Holohan et al., 2008). McKenzie et al. (2008) also demonstrated that Bid is essential for death receptor-induced apoptosis of islets, similar to its demonstrated role in hepatocytes. If this study indicates that blocking Bid activity may be useful for protection of islets from immune-mediated attack and possibly also in other pathological states in which beta-cells are destroyed, then the Melligen cells may have an inherent advantage in being protected against an autoimmune attack since the expression of Bid is unchanged after cytokine treatment. However, it must be noted that Bid needs to be activated by proteolytic cleavage before it can play a role in apoptosis. Although cytokine-induced beta cell death increases mostly after 2-6 days (Delaney et al., 1997; Pavlovic et al., 2000), it cannot be excluded that an array analysis performed after 48h of cytokine exposure would detect modifications in the classic pro- and anti-apoptotic genes in the Melligen cells.

From the toll-like receptor family, TLR 3 was also up regulated in the Melligen cells. This has also been identified to be up regulated in human pancreatic islets exposed to IFN-γ critical for beta cell survival and protects the cells against Cocksakie virus (Hultcrantz et al., 2007) (Section 1.2.2.1) indicating an additional desirable characteristic in the Melligen cells. It is also worthy to note that of the top

toxicological functions regulated in the Melligen cells, liver necrosis was listed as having a significant role in the cytokine induced response at 24h treatment only. This may indicate that although the necrosis inducing genes differentially expressed in the Melligen cells at 24h were up-regulated, there are many more other anti-apoptotic and anti-oxidative genes significantly up-regulated (Figures 5.19 and 5.10) in the cells that outweigh the cell death genes and play a role in protecting the cells from this insult. This result therefore corroborates the findings in Chapter 4 where there was no significant increase in necrotic populations observed between the treated and untreated cells. Studies confirm this result in Huh7 cells where they have been found resistant to the effects of the pro-inflammatory cytokine IFN-γ by the up regulation of TNFAIP3 even though cell death molecules such as TNF-related apoptosis-inducing ligand (TRAIL) and other interferon inducing genes are regulated (Girard et al., 2002). Huh7 cells inserted with a hepatitis C virus construct have also been shown to inhibit and stimulate the apoptotic process simultaneously with the expression of apoptotic genes such as LGALS1 and APPD at the same time as the expression of anti-apoptotic gene PRKCZ (Dou et al., 2006). The results from the current study point to a simultaneous activation of both apoptotic and anti-apoptotic signalling cascades in response to the stress of pro-inflammatory cytokines. This is confirmatory of the predominant liver cell phenotype in the Melligen cells.

The fact that a high proportion of the genes identified in this study were originally described as IFN-inducible genes may reflect, in part, the role of IFN-γ in the resistance of Melligen cells to the pro-inflammatory cytokines. However, it may also be a reflection of the major efforts devoted to characterising IFN-γ-induced genes (Boehm et al., 1997; De Veer et al., 2001), many of which are also induced by LPS or other inflammatory cytokines. This would then be the result of the TNF-α and IL-1β cytokine synergism. Gene expression studies have been performed on the parent liver cell line Huh7 treated with cytokine concentrations similar to those used in this study (Girard, et al., 2002; Geiss, et al., 2003). Approximately 27 genes and/or proteins have been previously described as modified by IFN-γ in this cell line. They mainly include immune related genes such as the antigen presenting HLA group and IFN inducible pathway genes such as TNFAIP3 and MX1 similar to those seen in the Melligen cells. However, in the current study, the real time quantitative RT-PCR data

for the treated Huh7 and Melligen cells at 24h showed that for the five genes selected, BIRC3, CASP1, IRF1, SOD2 and STAT1, only IRF-1, SOD2 and STAT1 were marginally up regulated in the Huh7 cells. Only BIRC3 and CASP1 were both down regulated. These results suggest that the presence of the insulin and human islet glucokinase genes in the Huh7 cells may have altered the cytokine-induced expression of genes since these genes were not found to be induced in the cytokine treated parent cell line.

It is also a significant finding that the 'Type 1 Diabetes Mellitus' canonical pathway, as identified by the IPA software, was determined as a pathway being significantly involved in the dataset for the 24h cytokine treated Melligen cells (Figure 5.10A). Although the Melligen cell phenotype has been changed to become more beta cell like, the late response to the cytokine cocktail in this study was not beta cell like according to the findings of Liuwantara et al., (2006). After 1h cytokine treatment, Melligen cells exhibited an increase in TNFAIP3 expression (4.7 fold), which was also found to be up regulated after 24h cytokine treatment (2.6 fold). In the study by Liuwantara et al., (2006), using the cell death CHIP, the gene TNFAIP3 was increased by approximately 10 fold from islets isolated from NOD and BALB/c mice treated with IL-1 for 1h whereas 1h TNF treatment induced an average increase of 4 fold in the NOD and BALB/c mice. Thus these results show that TNFIAP3 is both a cytokine-inducible immediate early and late response gene in Melligen cells, which is not consistent with its regulation as an early response gene in beta cells.

It should also be noted that a number of executioner caspases are up-regulated in beta cells, including CASP1 (Karlsen et al., 2000) (Section 1.3.1.4). Although CASP1 was up-regulated at 24h in the Melligen cells, the inhibitor of CASP1, CARD16 (Hong & Jung, 2002) was also up-regulated pointing to a possible inhibition of the inflammatory actions by CASP1. It has been reported that caspase-1 is negligible in the apoptosis of primary hepatocytes (Jones et al., 1998; Zheng et al., 1998). Consequently, it is unlikely that caspase-1 plays an important role in the apoptosis of Melligen cells but may be an inflammatory response. Furthermore, the expression of apoptotic executioners, the members of the caspase-3 family, were not affected in this study further supporting the negligible apoptotic population reported in Chapter 4 (Section 4.2.1).

Cytokines are known to induce oxidative injury to cells and beta cells are particularly sensitive to damage induced by the pro-inflammatory cytokines, due at least in part to their low expression levels of cytoprotective enzymes (Tiedge et al., 1997, Souza et al., 2008). In the current study, the Melligen cells have been shown to be capable of expressing genes that allow for the repair/restoration of damage caused by the cytokines. Up regulation of the gene VNN3, related to oxidative stress and acute phase reaction, represented the Melligen cell response to oxidative injury (Berruyer et al., 2004). This indicates that the Melligen cell is susceptible to oxidative injury. Genes related to complement and chemokines were thus activated following oxidative injury to the cell. Contrary to beta cells, however, involved in the Melligen cells' repair and restoration signalling are cytoprotective genes MX1 (Myxovirus resistance 1) and SOD2 (superoxide dismutase 2) (Delhalle et al., 2002; Epperly et al., 2003). In the study by Cardozo et al., (2001), primary human cultured beta cells treated for 24h with IL-1β and IFN-γ, SOD2 increased by 2.4 fold. In contrast however, studies performed on BALB/c and NOD mice did not demonstrate increased levels of SOD2 greater than 2 fold (1.3 fold) (Liuwantara et al., 2006). In the microarray performed in this study on the Melligen cells, cytokine treatment increased levels of MX1 by 4.8 fold and the confirmatory real time RT-PCR results showed an up regulation of SOD2 by almost 4.4 fold. These results indicate that the Melligen cells highly express SOD2 in response to the proinflammtory cytokine cocktail, which is inherent to liver cells (Czaja et al., 1994) as a stress response but otherwise not seen in the beta cell.

NF-κB activation is an essential component of the cytokine signalling pathway responsible for several genes found to be up regulated in the current array such as group II PLA_2 gene regulation (Walker et al., 2003) and Poly (ADP-ribose) polymerase (PARP) (Oliver, et al., 1999). Souza et al., (2008) also observed the important role of pro-inflammatory and anti-inflammatory cytokines on NF-κB in beta cells pointing to increased cell death via the NF-κB pathway when exposed to pro-inflammatory cytokines for 6h. There was an observed expression of several cytokine-modified genes that are putative target genes for NF-κB common in Melligen cells and beta cells as indicated by the most significantly affected pathways at both 1h and 24h (Figure 5.10 and 5.11. See Figure 5.8A for NF-κB network genes

at 1h). These genes increased in expression are IRF1 and STAT1. Furthermore, the direct interactions identified in this network shows that strongly up-regulated IRF1, TAP and the HLA group of molecules were also up regulated at 24h cytokine treatment and this places STAT1 and NF-κB as central transcription factors in the process of cytokine-induced Melligen cell gene expression.

In the liver, STAT1 is mainly activated by IFN-α/β and IFN-γ (Darnell et al., 1994). Data from STAT1 knockout mice suggest that STAT1 plays a key role in antiviral defence, inflammation, and injury in the liver (Meraz et al., 1996). Signal mediators were further traced downstream of STAT1 induction in the Melligen cells. Among the genes acting downstream of STAT1 activation, it was found that IRF1 was induced at 1h and 24h after cytokine cocktail treatment. Recent studies indicate that blocking STAT1, the main signalling pathway of IFN-γ, prevents multiple-low dose STZ-induced diabetes by an action at the beta cell level (Gysemans et al., 2005; Callewaert et al., 2007). These findings together with the results from the current study suggest that the up regulation of IRF-1 in the Melligen cells may be implicated in the survival of the cells when implanted *in vivo*.

The pro-inflammatory cytokines used in this study induced several genes common to antigen presentation in both beta cells and liver cells. This was mostly an effect of IFN-γ, because Cardozo et al. (2001) showed that IL-1β alone induced only two of these genes, whereas a combination of IL-1β and IFN-γ induced 14 genes. Other studies also indicate that IFN-γ is the main inducer of MHC class I mRNA and protein in rat and human islet cells (Pavlovic et al., 1997). The microarray study by Cardozo et al. (2001) also showed that IL-1β and IFN-γ up regulated several components of the 'machinery' for MHC class I antigen presentation, including mRNAs for several MHC-I- related components, proteasome subunits, and both TAP1 and TAP2. There was an observed up regulation in several molecules involved in peptide presentation including TAP1 and TAP2, which were up regulated by over 4 fold. This may imply that TAP1 and TAP 2 are participating in the process of intracellular trafficking after cytokine exposure, a phenomenon of potential relevance for the expression of chemoattractants by beta cells in response to the pro-inflammatory stimulus (Rasschaert et al., 2003).

5.5 Conclusion

Similar to beta cells, Melligen cells have been shown to have an immediate and late inducible anti-apoptotic response to the cytokine cocktail as indicated by the results obtained after 1h and 24h treatment. After 24h cytokine treatment of the Melligen cells, defence/repair genes were up regulated to protect the cells against cytokine toxicity. These repair/defence genes include SOD2, and MX1. Under pro-inflammatory conditions, the insulin-secreting Melligen cell survival is shown to be dependent on NF-κB activation and the anti-apoptotic genes such as BIRC3 and TNFAIP3 contribute significantly to this protection. Furthermore, the Gene Array established that glucose metabolism in the Melligen cells was not affected by the cytokine treatment at either 1h or 24h treatment indicating desirable cell function notwithstanding the presence of an autoimmune attack. Although the Melligen cells have become more beta cell like, they respond to cytokine treatment more as liver cells and not as beta cells since they have the ability to inhibit apoptotic function based on their gene expression profile. Comparing the five candidate genes by real time quantitative RT-PCR results revealed that the presence of the beta cell autoantigen (insulin) and the presence of glucokinase do affect the Melligen cells at a molecular level in response to proinflammatory cytokines. Incomplete transdifferentiation could prevent the generated insulin-producing cells from being attacked by a destructive autoimmune response in Type 1 diabetics. Further *in vivo* studies are needed to confirm this, however micro-encapsulation technology will be required to both ensure the cells are contained at the site of administration, because of their tumorigenic nature, and to further protect them from a hostile immune response if they are used *in vivo* for gene therapy.

CHAPTER 6: Susceptibility of Melligen Cells and Other Modified Liver Cell Lines to the Effect of the Beta Cell Toxin STZ

6.1 Introduction

Melligen cells could be used in the reversal of diabetes in STZ-diabetic mice if encapsulated prior to transplantation. In this technique, cells are surrounded by a semi permeable membrane that allows free exchange of oxygen, nutrients, and metabolites while excluding the passage of cells and high molecular weight substances such as immunocytes, antibodies, and complement factors in the order of 50kD (Kühtreiber & Lanza, 1999). However, since it is likely that, *in vivo*, pro-inflammatory cytokines are released near the microencapsulated liver cells, and they are sufficiently small enough to enter the capsules, it was therefore important to establish the resistance of Melligen cells to pro-inflammatory cytokines *in vitro* as determined in previous chapters.

Immune exclusion devices containing insulin-secreting cells are designed to provide glycaemic control through transplantation without immunosuppression (Efrat, 2008). However, because some of the immunological mechanisms that cause or regulate allograft rejection are different from those that affect autoimmunity, immunosuppression targeted and tailored to mitigate allograft rejection might not necessarily reverse or suppress autoimmunity (Li et al., 1998; Salomon & Bluestone, 2001; Makhlouf et al., 2002). It is therefore desirable if Melligen cells are resistant to beta cell toxins, in addition to pro-inflammatory cytokines, to prevent damage to the cells.

Encapsulation has been shown to allow successful transplantation of cells such as hepatocytes and fibroblasts without the loss of physiological function and without immunosuppression for up to two months (Josephs et al., 1999; Kahn et al., 1995; Takebe et al., 1996). Kahn et al. (1995) encapsulated primary rat hepatocytes in alginic acid poly-*l*-lysine membrane and transplanted them intraperitoneally in rabbits. The capsules were found intact even after 60 days of transplantation. The retrieval was 75% with 80% of viable cells displaying normal functional capacity to

produce urea. This study showed that the microencapsulation technique is effective in maintaining functional capacity and can provide a physical barrier between the host's immune system and immobilized cells.

Hunt et al. (2010) have shown that fibroblasts (3T3) encapsulated at a density of 7.5×10^5cells/mL in both 2% and 5% w/v alginate remain viable for at least 60 days. Rheological analysis was used to study how the mechanical properties exhibited by alginate hydrogel changed during 28 days *in vitro* culture. Fibroblasts were shown to increase the rate of degradation during the first 7 days when compared with acellular samples in both 2% and 5% w/v gels, but after 28 days both acellular and cell-encapsulating samples retained disc-shaped morphologies and gel-like spectra. The results demonstrate that although at an early stage cells influence the mechanical properties of encapsulating alginate, over a longer period of culture, the hydrogels retain sufficient mechanical integrity to exhibit gel-like properties (Hunt et al., 2010).

The concept of protecting transplanted islets by immunoisolation in a semi-permeable membrane has also been tested using microcapsules. Lanza et al. (1995) reported that uncoated alginate microspheres (800-900μm in diameter) containing porcine or bovine islets reversed hyperglycaemia in STZ-diabetic C57 mice for at least 30 days and 43 days respectively, without any immunosuppression, while non-encapsulated grafts survived less than 4-days. Sun et al. (1996) also showed that porcine pancreatic islets could be microencapsulated in alginate-polylysine-alginate capsules and transplanted intraperitoneally into spontaneously diabetic monkeys. After one, two, or three transplants of $3\text{-}7 \times 10^4$ islets per recipient, the monkeys became insulin independent for periods ranging from 120 to 804 days with fasting blood glucose levels in the normoglycemic range. Glucose clearance rates in the transplant recipients were also significantly higher than before the graft administration and the insulin secretion during glucose tolerance tests was significantly higher compared with pre-transplant tests. Additionally, three months after restoration of glycaemia, the capsules were found intact (Sun et al., 1996). However, while cell encapsulation proved successful with other cell types such as liver cells and fibroblasts, the specific problems associated islet cell encapsulation include poor cell survival caused by hypoxia, due to the high metabolic rate of beta cells, and by small immune effectors

such as cytokines and free radicals, which can penetrate the capsule (Tai et al., 1995; de Groot et al., 2003).

Of advantage in the engineering of a surrogate beta cell, hepatocytes express the high-capacity glucose transporter GLUT2 (Permutt et al., 1989) and the glucose phosphorylation enzyme glucokinase (Wilson, 1997), which comprise the key elements of the "glucose sensing system" that regulates insulin secretion from pancreatic beta cells in response to small external nutrient changes. Similar to the HepG2ins/g cells (Simpson et al., 1997) (Chapter 1), the Huh7ins (Tuch et al., 2003) cells were also genetically modified in an attempt to achieve a response to more physiological levels of glucose. With the insertion of the human islet glucokinase gene into the Huh7ins cells, the resulting cell line, Melligen, was found to respond to glucose in the 4-5mM physiological range (unpublished data; see Figure 1.11). HepG2ins/g cells did not reverse diabetes in mice (Simpson et al., 1997) and Huh7ins cells are responsive to sub-physiological levels of glucose (Tuch et al., 2003).

The antibiotic streptozotocin (STZ) is a nitrosamine compound, which has been widely used to induce chemical diabetes in rodents. Upon uptake STZ induces DNA strand breaks and subsequent increased activity of poly-ADPribose synthetase results in NAD depletion and beta cell death. When delivered in multiple low doses (MLDS; 5 daily doses of 40mg/kg) to susceptible strains of mice, STZ induces autoimmune diabetes, characterized by insulitis and subsequent destruction of islet beta cells within 5-7 days (Like & Rossini, 1976; Kolb-Bachofen et al., 1988; Papaccio et al., 1991). Although the underlying mechanisms of autoimmunity induction are yet to be elucidated, they likely involve changes to suppressor T cell populations. In contrast, when STZ is given in a single high-dose (SHDS; 200mg/kg body weight), it rapidly destroys islet beta cells (within 4h) by a direct cytotoxic action (Yamamoto et al., 1981). A controversial hypothesis has also been postulated regarding the action of this compound when given in multiple low doses because, in addition to the immune cell-mediated mechanism, a synergistic direct cytotoxic action seems also to occur (Papaccio et al., 1992).

STZ and alloxan are toxic because they fragment nuclear DNA through an effect of accumulation of superoxide or hydroxyl radicals (Yamamoto et al., 1981). The use of

STZ to generate altered forms of native proteins is distinctly different from the development of spontaneous IDDM in NOD mice, wherein autoimmunity entails a presumed loss of tolerance to native self-antigens. Studies indicate that beta cell surface proteins altered by the glucopyransoe ring of STZ may indeed be immunogenic *in vivo* (Ihm et al., 1990). *In vitro* studies show that STZ depletes NAD levels so the effect here is likely to be different to immuno-modulation effects seen in some humans and NOD mice (Elliot et al., 1993). It is believed that STZ, is taken up by the beta cells through GLUT2 where it decomposes intracellularly, causing damage to DNA directly, by alkylation, and indirectly via generation of nitric oxide, resulting in beta cell death (Wang & Gleichmann, 1998). High affinity for STZ uptake into rodent pancreatic beta cells is mediated via GLUT2 transporters. Resistance to the toxic effects of these agents increases the potential for the use of transfected liver cells in reversing diabetes.

If insulin secreting liver cell lines are to be an option in reversing Type 1 diabetes, it would be desirable if the cells were resistant to the induction of intracellular stress. DNA strand breaks, and apoptosis in the case of MLDS, is analogous to the mechanism of action of many beta cell cytotoxins including cytokines (Wang & Gleichman, 1998). It follows that expression levels of GLUT2 in these modified liver cell lines will modulate STZ uptake and action and putatively the increased levels of GLUT2 expression may render these genetically modified cells more susceptible to the toxic effects of STZ.

Since the Melligen cells will be encapsulated and implanted into STZ model mice in future studies, the aim of this chapter is to firstly establish the effect of the encapsulation technology on cell viability, insulin secretion and glucose responsiveness in the Huh7, Huh7ins and Melligen cells. Secondly, the effect of STZ treatment, in single dose and double dose, on the liver cell lines HepG2, HepG2ins, HepG2ins/g, Huh7, Huh7ins and Melligen. It will also be determined whether these results corroborate with the amount of GLUT2 expression found in these cells. Since the Melligen cells are the most beta cell like, it is imperative that the resilience of the more beta cell-like cells against known beta cell stressors is determined.

6.2 Insulin secreting liver cell lines remain viable in microcapsules

The success of encapsulation is initially dependent on the number of viable cells that are entrapped within the capsule, on an absolute basis or compared to the theoretical number of cells fed to each capsule- the encapsulation efficiency (Lanza et al., 1995). In order to transplant the Melligen cells into an animal model, the Melligen cells must be encapsulated for the reasons highlighted in Section 6.1 (the composition of the capsule used in these experiments is commercial-in-confidence). After expansion and selection of the cells, Huh7, Huh7ins and Melligen, were encapsulated in sodium cellulose sulphate (Figure 6.1) (encapsulation procedure performed and images taken by AustriaNova, Singapore)

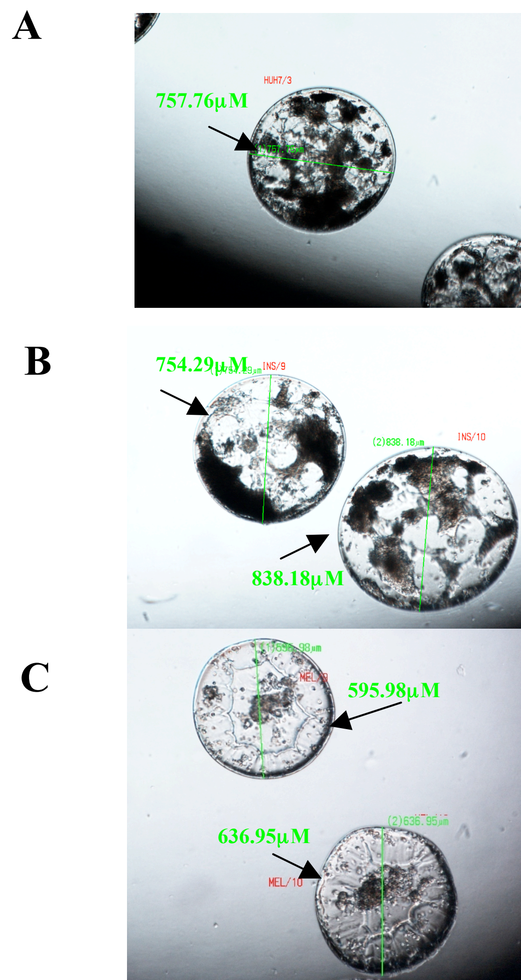


Figure 6.1: Brightfield image of the encapsulated liver cell lines.

*Brightfield images of encapsulated **A)** Huh7 **B)** Huh7ins and **C)** Melligen cells were taken. The diameter of two capsules is indicated. Magnification x100 (encapsulation procedure and images taken by AustriaNova, Singapore).*

To assess the viability and proliferation rate of the cells in the microcapsule, AlamarBlue® assays were conducted every 3-4 days (Figure 6.2). The Huh7, Huh7ins and Melligen cells were found to be viable inside the capsules with increasing metabolic activity indicating proliferation of the cells over the observed period of 110 days (assay was conducted by AustriaNova, Singapore). However, Melligen cell viability did lag over the first 25 days compared to the Huh7 and Huh7ins cells and cell viability decreased after day 72. This is because there were more cells seeded than what was recorded at day 0 as a result of miscalculating cell number. It was noted that the media had become acidic (yellow) by the end of the experiment. The results were recorded as relative fluorescence units (RFU).

These results are preliminary and need to be repeated in order to perform statistical analysis, especially since there was a technical error in the results obtained for the Melligen cell viability.

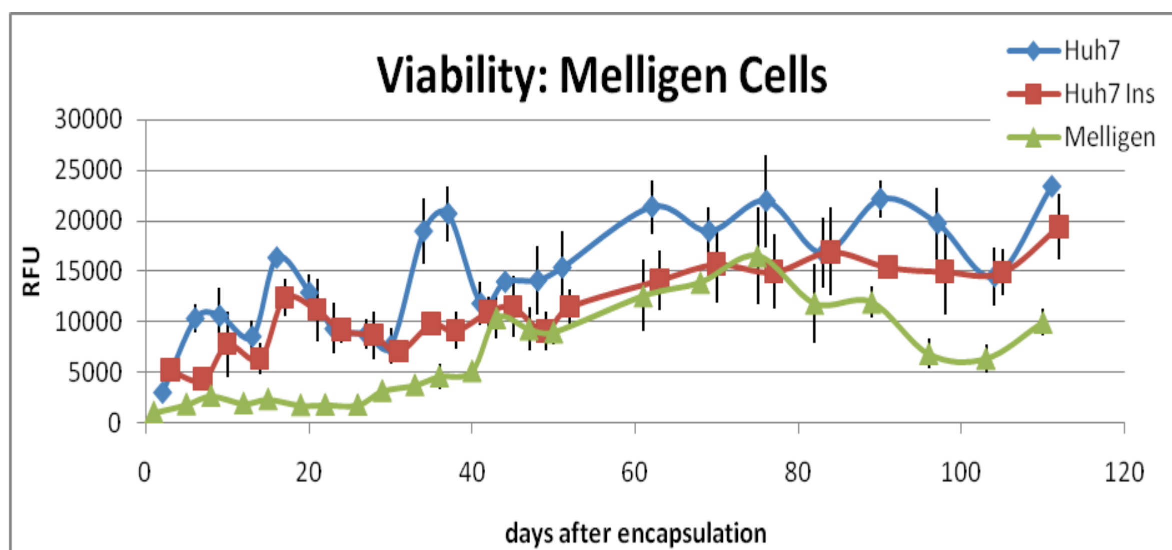


Figure 6.2: Time course analysis of the metabolic activity of encapsulated Huh7, Huh7ins and Melligen cells.

Huh7, Huh7ins and Melligen cells were initially seeded into 96-well plates and metabolic activity was obtained using the AlamarBlue® method every 3-4 days throughout the period studied. Results are expressed as mean ± SE, n=4 replicate wells (AustriaNova, Singapore).

6.2.1 Insulin function is maintained in Huh7ins and Melligen cells after encapsulation

For each cell line, based on their metabolic activity, as determined by the AlamarBlue® assay, the determined amount of $5x10^6$ capsules was seeded into a 12-well plate (AustriaNova, Singapore). For the determination of glucose responsiveness after 24h post-encapsulation, acute insulin secretion to 20mM glucose was measured by static incubation experiments (AustriaNova, Singapore) and insulin was quantified by the RIA method (Section 2.3.3). The results clearly show that the encapsulated cells firstly secrete insulin and secondly react to changes in the glucose content of the medium.

Encapsulated Huh7ins cells gave a 5-fold increase in insulin secretion when stimulated with 20mM glucose (0.44 ± 0.016pmol/well/h), with return to basal levels of insulin secretion (0.095 ± 0.017pmol/well/h) upon removal of the glucose stimulus (Figure 6.3A). Encapsulated Melligen cells also gave a 5-fold increase in insulin secretion when stimulated with 20mM glucose (0.2 ± 0.036pmol/well/h), with return to basal levels of insulin secretion (0.04 ± 0.008pmol/well/h) upon removal of the glucose stimulus (Figure 6.3C). Non-encapsulated Huh7ins cells also showed a 5-fold increase in insulin secretion upon the 20mM glucose stimulus (0.36 ± 0.06pmol/well/h) and a return to basal levels of secretion (0.08 ± 0.02pmol/well/h and 0.06 ± 0.006pmol/well/h) within 1h after stimulation (see Section 3.4.3). Non-encapsulated Melligen cells showed a 5-fold increase in insulin secretion upon the 20mM glucose stimulus (0.32 ± 0.062pmol/well/h) and a return to basal levels of secretion (0.063 ± 0.013pmol/well/h) within 1h after stimulation (see Section 3.4.3). The fold-change in insulin secreted by the non-encapsulated (as determined in Chapter 3) and encapsulated Huh7ins and Melligen cells during the stimulus was the same.

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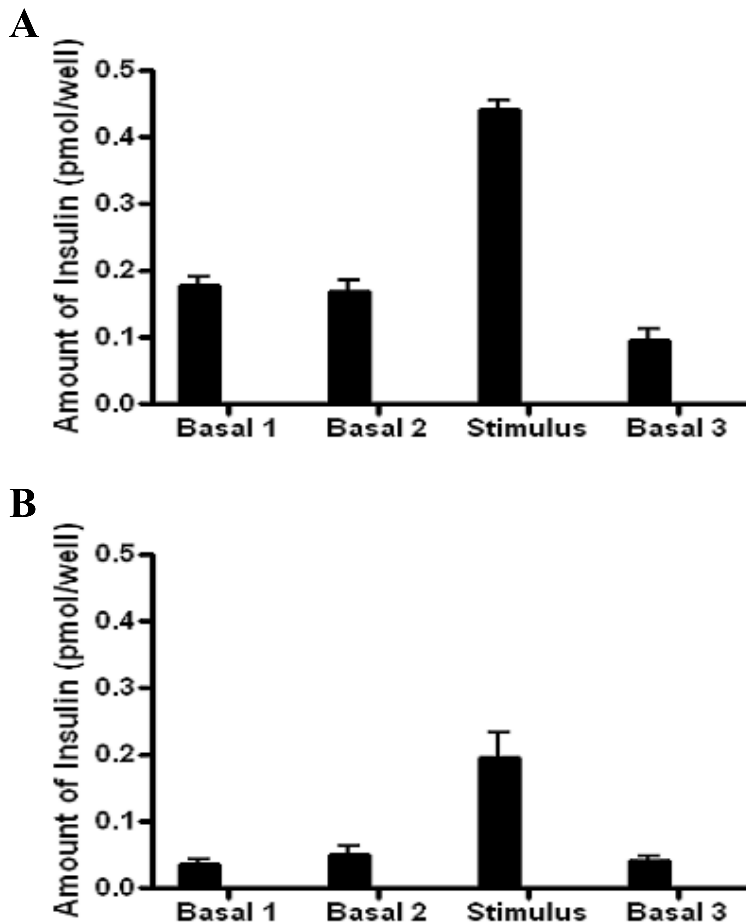

Figure 6.3: Glucose responsiveness of encapsulated insulin secreting liver cell lines.

A*)Huh7ins and* ***B****)Melligen cells after static stimulation. Glucose responsiveness was determined using 20mM glucose stimulus (*AustriaNova, Singapore*) and the samples assayed by RIA. Results are expressed as mean ± SE, n=2 independent experiments.*

Levels of chronic insulin secretion for encapsulated Huh7ins and Melligen cells determined by RIA, after 48h post-encapsulation, showed that the amount of insulin secreted after 24h by the Huh7ins cells was significantly higher than that secreted by the Melligen cells (0.37 ± 0.05pmol/well and 0.1 ± 0.03pmol/well, respectively) (*P*= 0.0099). After 48h post-encapsulation Huh7ins and Melligen cells showed that the amount of insulin secreted by the Huh7ins cells was again significantly higher than that secreted by the Melligen cells (0.44 ± 0.08pmol/well and 0.11 ± 0.04pmol/well, respectively) (*P*= 0.02) (Figure 6.4). Consistently, these results were also observed in

Section 3.4.1 where the chronic insulin secretion of non-encapsulated Huh7ins cells is higher than that observed in the Melligen cells at the cell count of $5x10^6$ cells ($P<0.05$). Therefore, the encapsulation technology has not altered the amount of insulin secreted by the cells. However, the results obtained from this study are preliminary and the encapsulation procedure would need to be repeated in order to obtain statistically sound results.

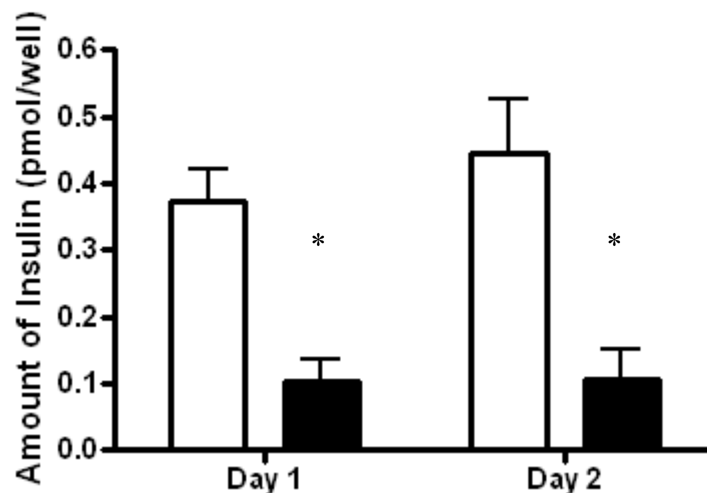

Figure 6.4: Determination of insulin content in media of encapsulated Huh7ins and Melligen cells after 48-hour secretion.

Huh7ins (unfilled) and Melligen (filled) cells after chronic insulin secretion. $5x10^6$ *capsules were seeded and media containing insulin secreted by the cells was collected over 48h (AustriaNova, Singapore). The insulin samples were assayed by RIA. Results are expressed as mean ± SE, n=2 independent experiments * P<0.05 for encapsulated Huh7ins versus encapsulated Melligen cells.*

6.3 Optimisation of streptozotocin treatment

It is well established that STZ is toxic to beta cells *in vivo* and induces diabetes in mice (Like & Rossini, 1976; Kolb-Bachofen et al., 1988; Papaccio et al., 1991). To determine if the presence of the GLUT2 glucose transporter in a liver cell line has an effect on the resistance of cells to STZ, the HepG2 (the parent cell line), HepG2ins and HepG2ins/g cells (Simpson et al., 1997) were used in addition to the Huh7, Huh7ins and Melligen cells otherwise used in this study. The cells were treated over 4 days. Two concentrations of STZ, 100μM and 20mM, were applied to the cells and the MTT assay was used to determine cell viability. The choice of these concentrations was based on experiments performed by Tuch et al. (2000) on STZ-induced HepG2ins/g cells *in vitro*. The beta cell line, MIN-6, was used as a positive control throughout the current experiments.

Results from the MTT assay showed that co-incubation of MIN-6 cells with the single dose of STZ, at the concentrations 100 μM and 20mM, did not reduce cell viability below 50% (72.2% ± 8.2% and 59.3% ± 2.7%, respectively), as compared to the untreated cells (for which viability was normalized to 100%), even after 4 days of co-incubation (Figure 6.5A and B). The only significant difference observed at the single dose 100μM (Figure 6.5A), was that HepG2ins viability was higher than MIN-6 cells, but only on day 4 (P= 0.038). Treatment of the HepG2, HepG2ins, and HepG2ins/g cells with a single dose of 100μM STZ showed no decrease in viability as compared to untreated cells by day 4 (111.8% ± 3.2%, 118% ± 3.1% and 80.8% ± 9.3%, respectively) (Figure 6.5A). When treated with a single dose of 20mM STZ, the liver cell lines HepG2, HepG2ins and HepG2ins/g cells showed a decrease in viability by day 4 (93% ± 9.7%, 69.8% ± 10.7 % and 82% ± 9.5%, respectively). At the single dose of 20mM all the cell lines followed the same pattern without significant differences observed between the HepG2, HepG2ins, HepG2ins/g or MIN-6 cell lines on any of the days (P>0.05), although there was a significant difference observed between the days for all the cells lines (viability decreasing with time) (P<0.05).

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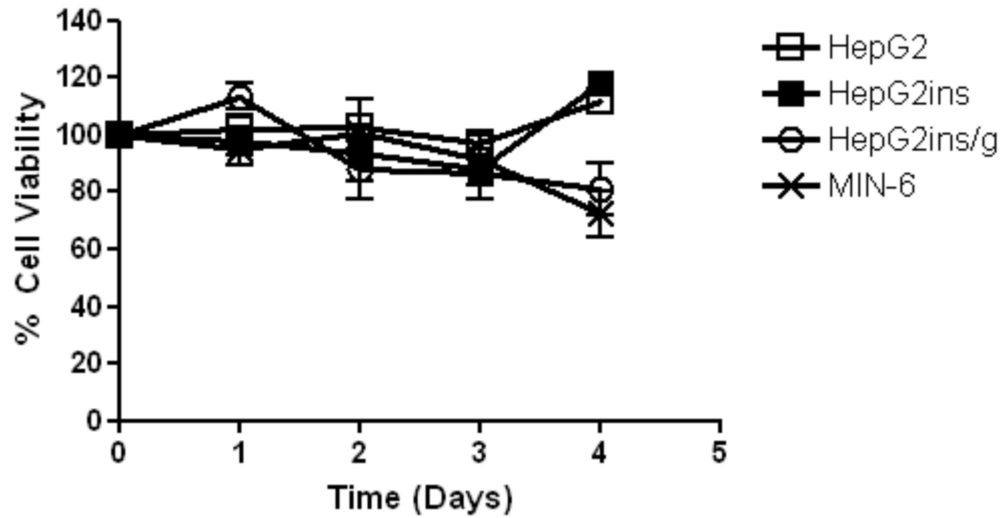

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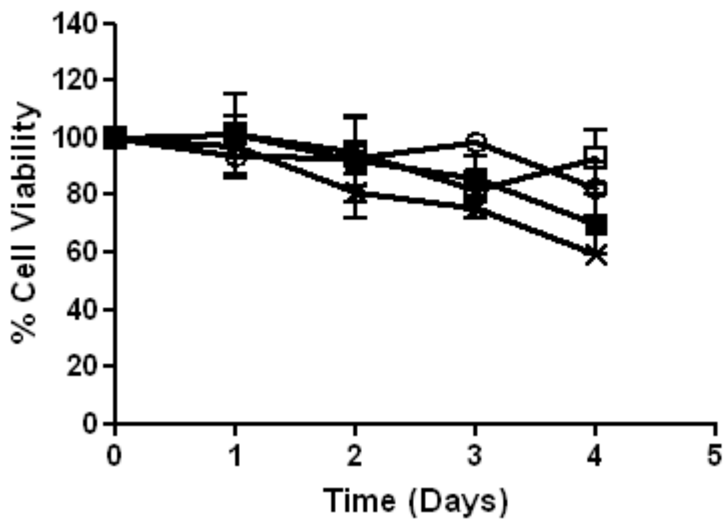

Figure 6.5: The effect of a single dose STZ on the viability of HepG2, HepG2ins, HepG2ins/g and MIN-6 cells.

*Cells were treated with STZ at concentrations **A**) 100µM and **B**) 20mM over 4 days. Results are expressed as mean ± SE, n=3 independent experiments.*

When Huh7, Huh7ins and Melligen cells were treated with a single dose of 100μM STZ, the lowest number of viable was seen in the Huh7 cells (80.8% ± 1.9%) (Figure 6.6A). Exposing the Huh7, Huh7ins and Melligen cells to the single dose of 20mM STZ treatment for four days showed that the viability was below 100% at day 4 (80.83% ± 2%, 95.76% ± 8.4% and 89.2% ± 5.5% for the cell lines respectively. At single dose 20mM all the cell lines followed the same pattern without significant differences in cell viability observed between the Huh7, Huh7ins, Melligen or MIN-6 cell lines on any of the days ($P>0.05$), although there was a significant difference observed between the days for all the cells lines (viability decreasing with time) ($P<0.05$). This effect was also observed in the HepG2, HepG2ins and HepG2ins/g cells exposed to the single dose 20mM STZ (Figure 6.6B). However, the cell viability of all cell lines in the data set did plateau from day 3 onwards (Figure 6.6B).

Moreover, the viability of the MIN-6 cells was only reduced to 60% by day 4 of the higher single dose of 20mM STZ treatment (Figure 6.5B), the cells were further subjected to the double dose of STZ at both concentrations according to the multiple dose STZ treatment model (Section 6.1). Consequently, the STZ treatment was added at a double dose 8h apart at the higher, 20mM, and lower, 100μM concentrations to ensure MIN-6 cells reached lowest viability by day 4. Since the MIN-6 cell line is the most readily available beta cell model in this study, it is imperative that the cell viability is abrogated, as this is what occurs in the STZ-diabetic mouse model. According to the treatment that is most toxic to the beta cell lines, it will be determined if the insulin secreting liver cell lines can retain viability after the same treatment.

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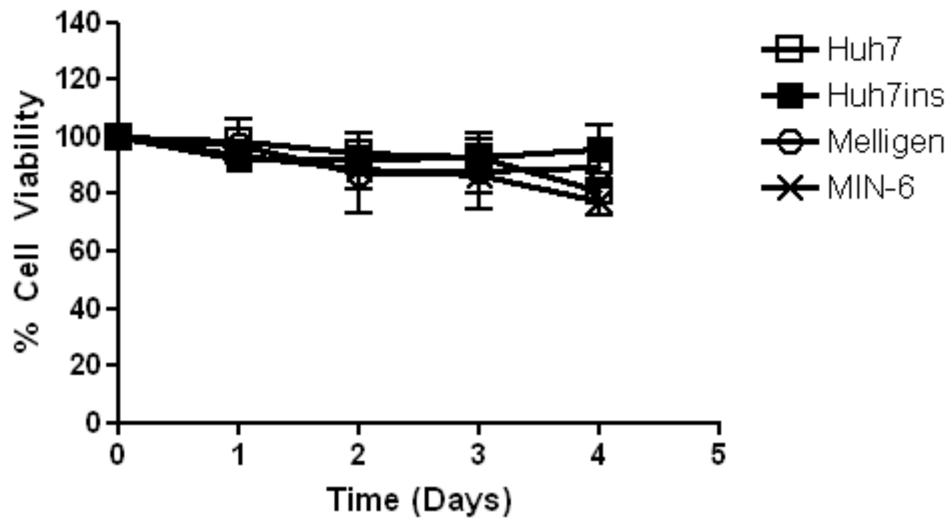

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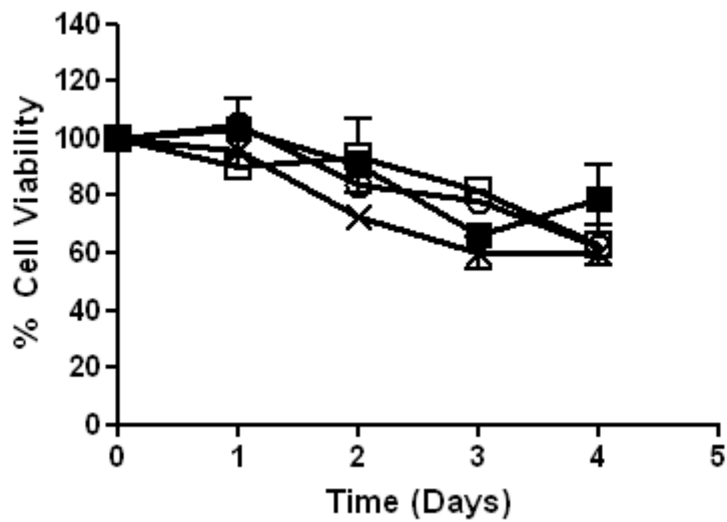

Figure 6.6: The effect of a single dose STZ on the viability of Huh7, Huh7ins, Melligen and MIN-6 cells.

*Cells were treated with SZ at concentrations **A**) 100µM and **B**) 20mM over 4 days. Results are expressed as mean ± SE, n=3 independent experiments.*

After treating the MIN-6 cells with the double dose of 100μM and 20mM STZ, these results showed a significant decrease in viability (*P*<0.05) over the four days compared to the single dose treatment. Notably, with the double dose of 20mM STZ there was a significant difference observed between the HepG2ins and MIN-6 cells, but only on day 1 (*P*= 0.015). From day 2 onwards the HepG2ins cell viability was the same as that of the MIN-6 cells. The double dose of 20mM STZ also caused the MIN-6 cells to follow a significant decrease in viability over days 1, 2, 3 and 4 (88.3% ± 2.8%, 61% ± 3%, 56.2% ± 1.2% and 37.1% ± 2.8% respectively) with significant differences observed between the days (*P*<0.05). Additionally, the double dose of 100μM STZ reduced the viability of the MIN-6 cells to 45.1% ± 6.5% by day 4 (Figure 6.7A).

To further establish the effect of the double dose treatment on the liver cell lines, the HepG2, HepG2ins and HepG2ins/g cells were incubated with the double dose 100μM and 20mM STZ treatments for 4 days with each dose added 8h apart. By day 4 of the double dose 100μM STZ treatment, the HepG2, HepG2ins and HepG2ins/g cells reached viabilities of 92.6% ± 1.4%, 75% ± 0.78% and 46.8% ± 6.8% respectively (Figure 6.7A). HepG2 viability was significantly higher than that seen in the HepG2ins/g viability from day 2 onwards (*P*<0.05) and the HepG2 cell viability was also significantly higher that that of the MIN-6 cells from day 2 onwards (*P*<0.05). However, there were no significant differences observed between the HepG2ins and HepG2ins/g cells or the HepG2 and HepG2ins cells over the 4 days of treatment (P<0.05).

The double dose 20mM STZ treatment reduced the cell viabilities to 47.5% ± 2.6%, 44.2% ± 2.5% and 34.2% ± 4.5% respectively (Figure 6.7B). There were no significant differences observed between the cell lines at this treatment (*P*>0.05). At low dose STZ the data indicates that HepG2ins/g cells shows the greatest decrease in cell viability after the double dose treatment (*P*<0.05). Therefore, the modification of the HepG2ins cells with the insertion of the GLUT2 gene does render the cells susceptible to the effects of STZ compared to the parent HepG2 cells and the control MIN-6 cells.

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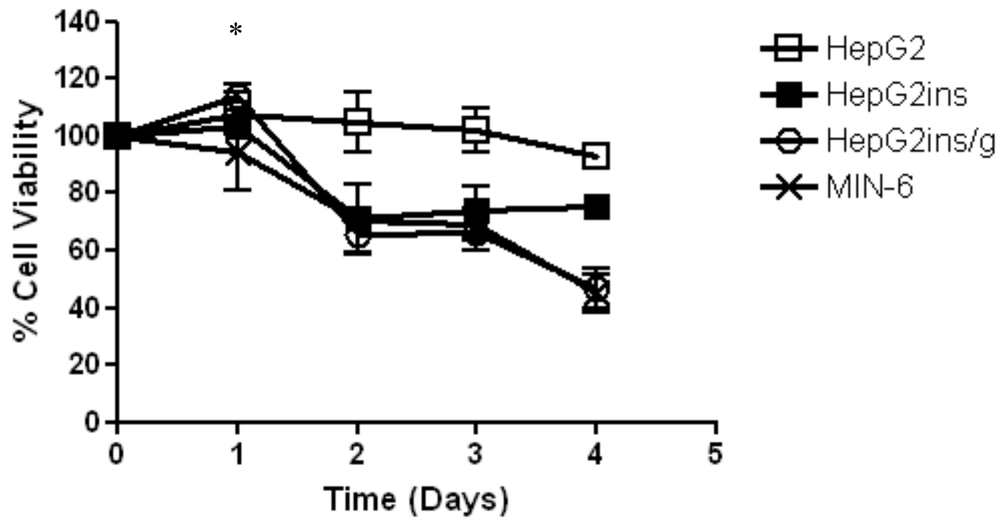

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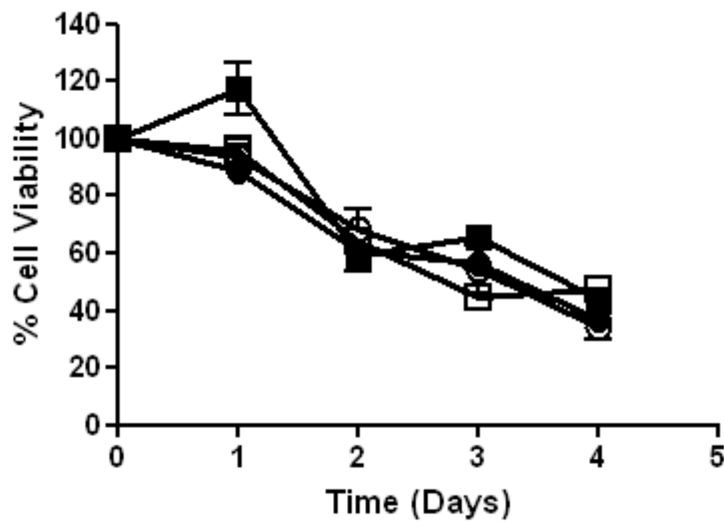

Figure 6.7: The effect of a double dose STZ on the viability of HepG2, HepG2ins, HepG2ins/g and MIN-6 cells.

*Cells were treated with STZ at concentrations **A**) 100μM and **B**) 20mM over 4 days. Results are expressed as mean ± SE, n=3 independent experiments. * P<0.05 for STZ treated versus untreated cells.*

Apparent changes in cell viability were also recorded in the Huh7, Huh7ins and Melligen cells after the double dose of 100μM and 20mM STZ treatment. By day 4 of the double dose 100μM STZ treatment, the Huh7, Huh7ins and Melligen cells reached viabilities of 81% ± 4.4%, 97.4% ± 4.3% and 82.5% ± 1.3% (Figure 6.8A). There were no significant differences observed between the liver cell lines at this treatment ($P>0.05$). Exposing the Huh7, Huh7ins and Melligen cells to the double dose of 20mM STZ treatment for four days showed that the greatest loss of viability occurred at day 4 (79.6% ± 10.7%, 77% ± 5% and 63% ± 5% respectively). There were also no significant differences observed between the liver cell lines at this treatment ($P>0.05$), however, the sharp decline in cell viability seen in the MIN-6, HepG2, HepG2ins and HepG2ins/g cells exposed to the double dose 20mM STZ (Figure 6.7B) over the 4 days was also seen in the Huh7, Huh7ins and Melligen cells (Figure 6.8B). Significant differences were observed from day 2 onwards between the MIN-6 cells and the Huh7, Huh7ins and Melligen cells ($P<0.05$) for both the double dose 100μM and 20mM STZ treatments.

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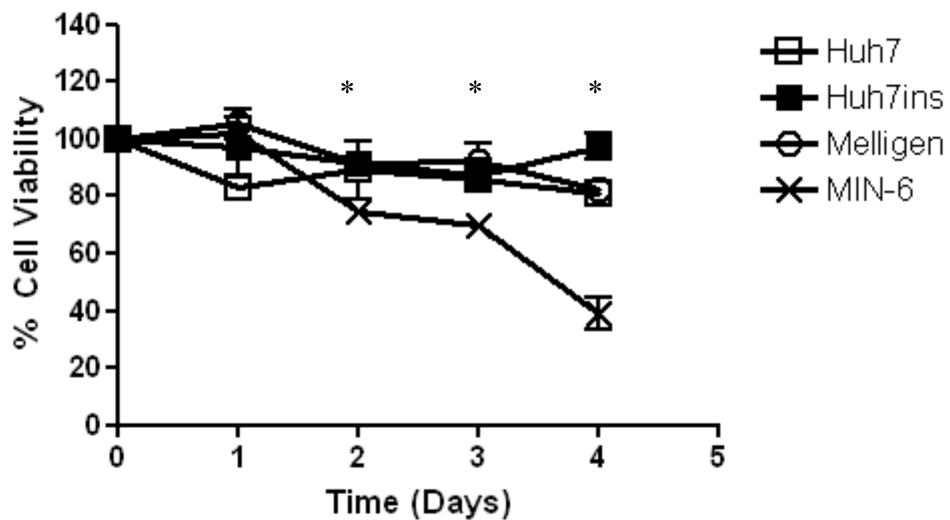

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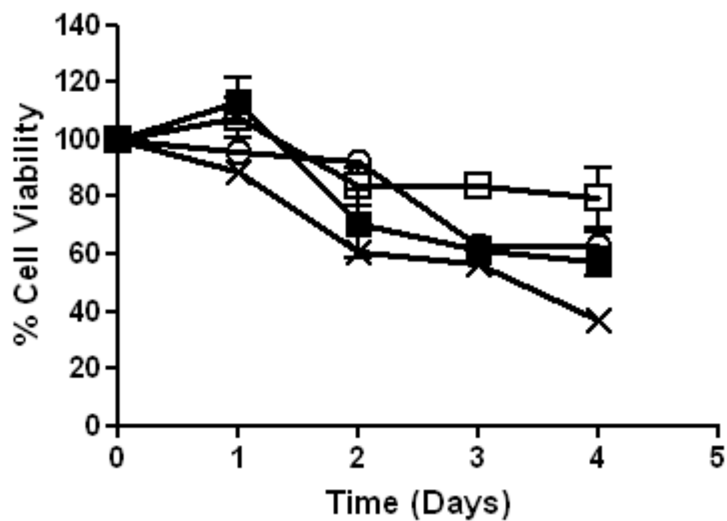


Figure 6.8: The effect of a double dose STZ on the viability of Huh7, Huh7ins, Melligen and MIN-6 cells.

*Cells were treated with STZ at concentrations **A)** 100μM and **B)** 20mM over 4 days. Results are expressed as mean ± SE, n=3 independent experiments. * P<0.05 for STZ treated MIN-6 cells versus STZ treated Huh7, Huh7ins and Melligen cells.*

Since the results obtained in Figures 6.5, 6.6, 6.7 and 6.8 revealed that the double dose 100μM STZ treatment (Figure 6.5A and 6.6A) induced more significant trends in cell viability over 4 days in the control MIN-6 cells and greater variability within the data set of the liver cell lines, this treatment was used in subsequent experiments. These results show that the treatment significantly affected MIN-6 cells and confirms that MIN-6 cells serve as a good control under these experimental conditions. The MTT cell viability results also showed that the Huh7, Huh7ins and Melligen cells were not affected by the double dose of 100μM STZ on any day over the 4 days.

In summary, the results showed that the double dose of low concentration STZ (100μM) and the double dose of the high concentration STZ (20mM) produced greater decrease in cell viability over 4 days. However, the double dose 20mM treatment was most toxic to all the liver cell lines in this study, which followed the same trend as MIN-6 cell viability and will not be further investigated. Since the HepG2ins/g cells were significantly affected by the double dose 100μM STZ treatment at day 2 of the experiment further experimentation needed to be conducted in order to determine if these cells behaved differently to the other cell lines as a consequence of the level of GLUT2 expression in the cells.

6.3.1 A significant difference in GLUT2 expression is observed in the HepG2ins/g and Melligen cells after 4 days of double dose 100μM STZ treatment

Real time quantitative RT-PCR analysis was performed on the six liver cell lines, HepG2, HepG2ins, HepG2ins/g, Huh7, Huh7ins and Melligen. GLUT2 has been shown to play pivotal roles in the toxicity of STZ in the beta cell in the development of Type 1 diabetes in STZ induced mice. The presence of the GLUT2 glucose transporter facilitates STZ entry into the cell, rendering the cells susceptible to STZ toxicity. Therefore, real time quantitative RT-PCR was used to determine the level of expression of GLUT2 in the liver cell lines. Time points day 0 and day 4 were chosen to show if the effect of the double dose 100μM STZ treatment had a significant effect on the expression levels of GLUT2 in the liver cell lines. At time point day 0 it was determined how much the contribution of fold change was due to the difference between day 0 and day 4. Results at time point day 0 showed that the HepG2,

HepG2ins and HepG2ins/g expressed GLUT2 at levels represented by the following fold changes up-regulated by 2.13 ± 0.3, 1.69 ± 0.044 and 3.58 ± 0.021, respectively (Figure 6.9). Statistical analysis showed that HepG2ins/g cells expressed significantly higher levels of GLUT2 than the parent cell line HepG2 (P= 0.009) and the insulin secreting HepG2ins cells (P= 0.000006). However, the difference observed between the GLUT2 expression in the HepG2 and the HepG2ins cells was not significant (P= 0.24).

Huh7, Huh7ins and Melligen cells expressed levels of the GLUT2 gene as determined by the fold changes up-regulated by 2.14 ± 0.24, 1.74 ± 0.014 and 3.6 ± 0.04, respectively (Figure 6.9). Statistical analysis revealed that at day 0 the difference observed between the Huh7 and the Melligen cells was significantly different (P= 0.004) and the difference between the Huh7ins and the Melligen cells was also significantly different (P= 0.0000017). However, the difference between the Huh7 and the Huh7ins cells was not significantly different (P= 0.17). These results show that the insertion of the insulin gene into the Huh7 cells (Huh7ins) did not change the GLUT2 expression, however, a second transfection with the human islet glucokinase gene (Melligen cells) increased GLUT2 expression at day 0. Similarly, the HepG2 transfected with the insulin gene (HepG2ins) did not change the GLUT2 expression profile, whereas a second transfection with GLUT2 (HepG2ins/g) did increase GLUT2 expression at day 0. These results show that the more a cell line diverges from its parent cell line, by transfection of genes characteristic to the beta cell, the cell becomes endowed with a higher level of GLUT2 expression.

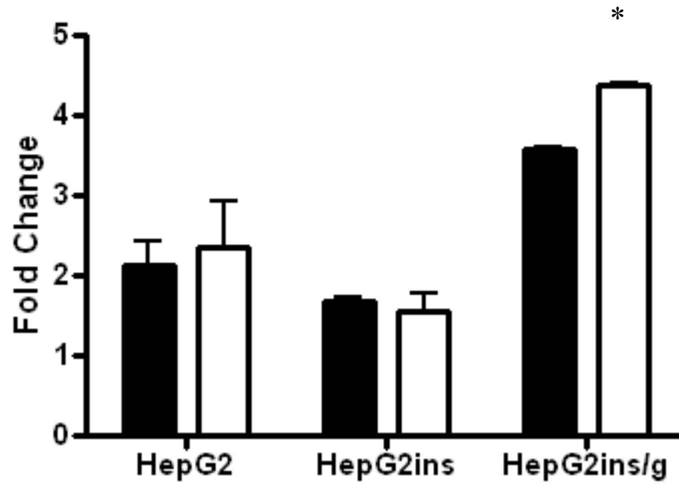
To determine if the absence of the insulin and over expressed GLUT2 gene expression in the liver cell line HepG2 regulates the GLUT2 expression to the same level, the cells were treated with the double dose 100μM STZ treatment for 4 days. mRNA was extracted and used to determine quantitative expression analysis by real time quantitative RT-PCR. The results showed that HepG2, HepG2ins and HepG2ins/g expressed an up-regulation of GLUT2 gene expression after STZ treatment by 2.36 ± 0.56, 1.54 ± 0.23 and 4.4 ± 0.015 fold changes respectively (Figure 6.9A). The highest recorded fold change in gene expression of the six liver cell lines used in the study was found in the STZ treated HepG2ins/g cells at day 4

and this was also significantly higher than the GLUT2 expression in HepG2ins/g cells recorded at day 0 (*P*= 0.02).

Following normalisation with beta actin mRNA levels, there was an up-regulation of GLUT2 by 2.09 ± 0.31 fold in the Huh7 cells at day 4 which was not significantly different at day 0 (*P*= 0.27). It was found that GLUT2 levels in the Huh7ins and Melligen cells were also up-regulated by 1.8 ± 0.18 and 1.93 ± 0.26 fold respectively (Figure 6.9B). However, only the Melligen cells showed a significant decrease in GLUT2 expression after the treatment (*P*= 0.0004). Figures 6.10 and 6.11 show that the Ct values at day 0 were higher in the Huh7, Huh7ins and Melligen cells compared to the HepG2, HepG2ins and HepG2ins/g respectively.

In summary, the HepG2ins/g expressed significantly more GLUT2 than the HepG2 and HepG2ins cells. The Melligen cells also expressed significantly more GLUT2 than the Huh7 and Huh7ins cells before treatment. The results also showed that by the fourth day of the double dose 100μM STZ treatment the highest recorded up-regulation of GLUT2 was in the HepG2ins/g cells compared to the other cell lines. Further to this the Melligen cells showed a significant decrease in GLUT2 expression after the STZ treatment.

A



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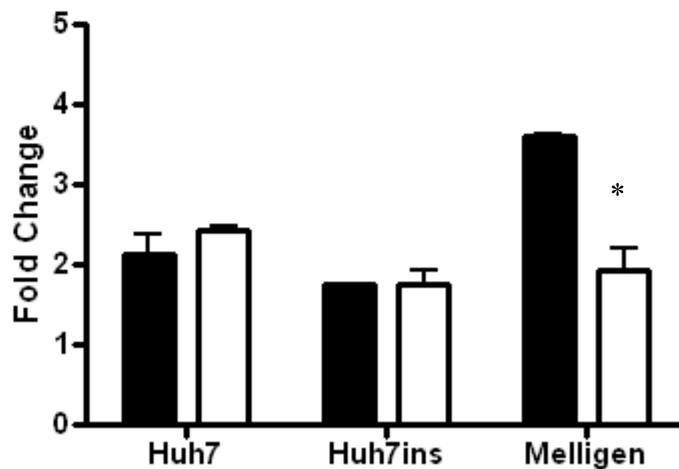

Figure 6.9: GLUT2 mRNA expression after double dose 100µM STZ treatment for 0 and 4 days.

Values observed as fold changes in STZ treated liver cell lines following quantitative real time RT-PCR. Real time RT-PCR was performed with specific primers for GLUT2 corrected by beta actin expression ***A****) HepG2, HepG2ins and HepG2ins/g and* ***B****) Huh7, Huh7ins and Melligen cells (day 0 filled and day 4 unfilled). The data are expressed as fold variation of the respective untreated control (considered as 1). Results expressed as mean ± SE, n=3 independent experiments. * P<0.05 for differences between day 0 and day 4.*

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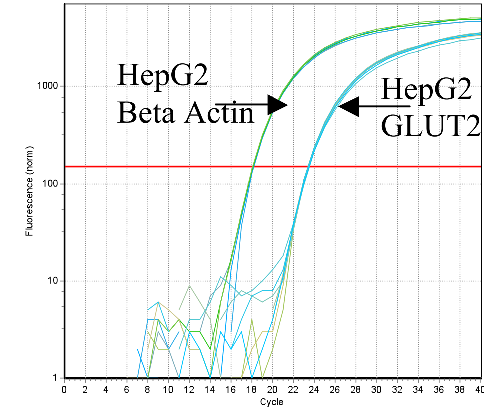

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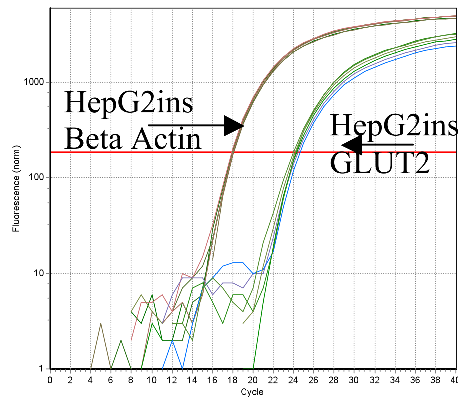

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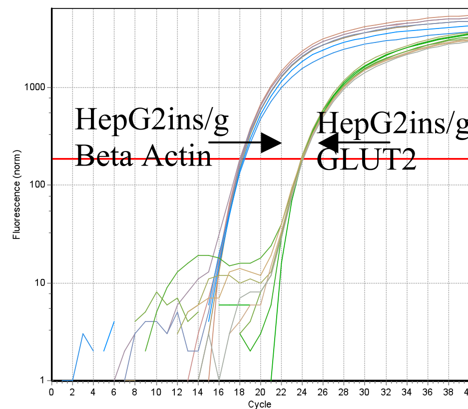

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Figure 6.10: Amplification plots for GLUT2 expression in liver cell lines at day 0. *mRNA was extracted and real-time RT-PCR performed for **A)** HepG2 **B)** HepG2ins **C)**HepG2ins/g Beta Actin used as housekeeping gene. Eight replicates presented.*

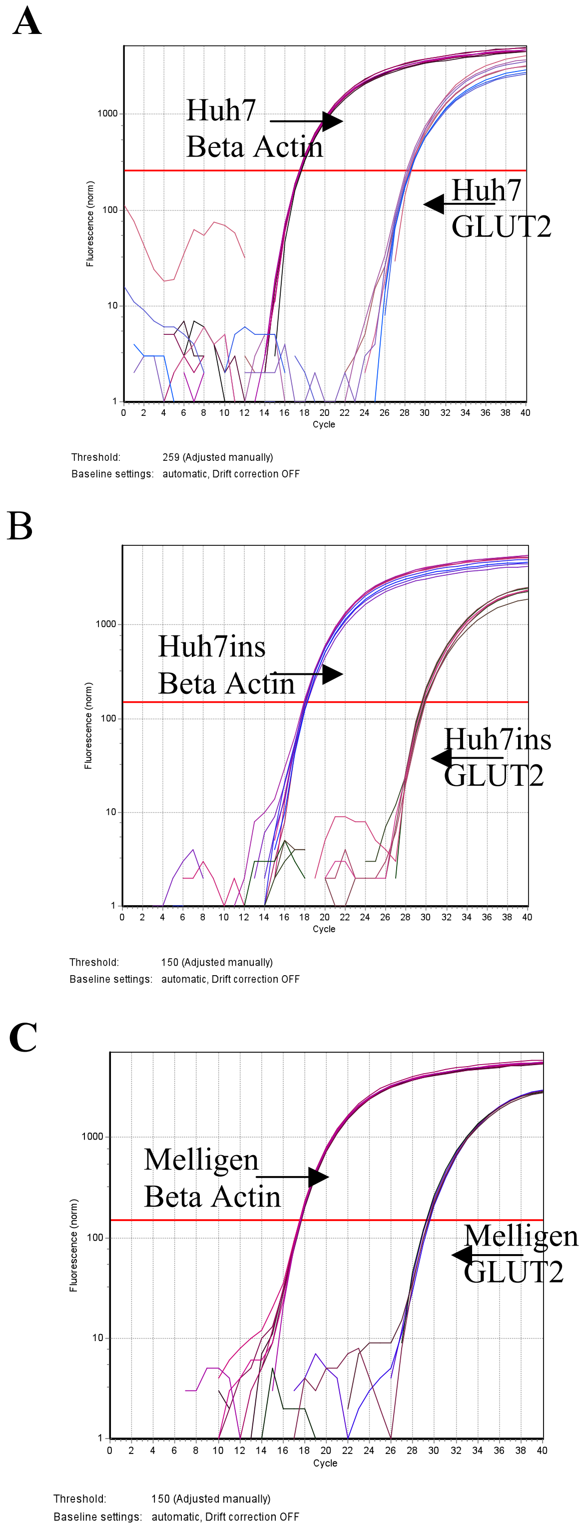

Figure 6.11: Amplification plots for GLUT2 expression in liver cell lines at day 0. *mRNA was extracted and real-time RT-PCR performed for* ***A****) Huh7* ***B****) Huh7ins and* ***C****) Melligen cells. Beta Actin used as housekeeping gene. Eight replicates presented.*

6.3.2 The effect of STZ on insulin secretion and glucose responsiveness

Since it has been shown that STZ impairs beta cell function both *in vitro* and *in vivo*, the effects of the double dose (2x100μM) STZ treatment (see section 2.1.3) on insulin function- insulin secretion and glucose responsiveness in the four insulin secreting liver cell lines, HepG2ins, HepG2ins/g, Huh7ins and Melligen were determined. MIN-6 cells were used as a positive control throughout.

6.3.3 The double dose 100μM STZ treatment only affected HepG2ins/g cell chronic insulin secretion on day 4

To determine the effects of STZ on chronic insulin secretion, media was collected every day for 4 days with 100 μM STZ being changed twice daily. Insulin concentration was determined by RIA (Section 2.3.3). Viable cells were quantified using trypan blue exclusion. For the determination of glucose responsiveness after 4 days of STZ exposure, acute insulin secretion to 20 mM glucose was measured by static incubation experiments and insulin was quantified by RIA.

STZ-treated MIN-6 cells secreted significantly less insulin (0.59 ± 0.003pmol/well) than untreated MIN-6 cells (6.44 ± 0.6pmol/well) at day 2 (P=0.0035) and throughout the entire period studied (Figure 6.13C). Insulin levels for STZ-treated MIN-6 cells at day 4 represented the total amount of insulin secreted over the entire 4 days. In contrast, Huh7ins cells co-incubated with STZ secreted 4.0 ± 0.55pmol/well and this level of insulin secretion was not significantly different to amounts secreted by untreated Huh7ins cells (4.4 ± 0.9pmol/well) by day 4 (P=0.67) (Figure 6.13A). Similarly there was no significant difference at day 4 between the amounts of insulin secreted by untreated Melligen cells (0.3 ± 0.12pmol/well), and treated Melligen cells (0.2 ± 0.01pmol/well) (P= 0.43) (Figure 6.13B).

HepG2ins cells co-incubated with STZ secreted 1.03 ± 0.07pmol of insulin per well and this value was not significantly different to the amounts of insulin secreted by untreated HepG2ins cells (1.3 ± 0.43pmol/well) by day 4 (P=0.67) (Figure 6.12A). Unlike the HepG2ins cells there was a significant difference from day 2 onwards between the untreated HepG2ins/g cells (P<0.05), which secreted 0.96 ± 0.23pmol/well and treated HepG2ins/g cells, which secreted 0.31 ± 0.12pmol/well by day 4 (P= 0.039) (Figure 6.12B).

Throughout the duration of this study, trypan blue exclusion studies were performed to assess cell viability showing treated MIN-6 cell viability is affected over time compared to the untreated cells. Statistical analysis showed significant differences between untreated and treated MIN-6 cells starting at day 2 (P=0.02) onwards. In addition, there were no significant differences observed between the untreated and treated HepG2ins, Huh7ins and Melligen cells (P>0.05). However, a decrease in cell viability was observed in the HepG2ins/g cells from day 3 of STZ treatment. The cell samples used for the Trypan blue exclusion method were obtained from the cells cultured for insulin secretion experiments (data not shown).

Time course analysis of the cell viability and insulin secretion data obtained for the liver cell line, HepG2ins/g cells, and the positive control, MIN-6 cells, both showed that significant cell death occurred on the same day or the day after the insulin secretion function was affected in the cells. Therefore, a reduction in insulin secretion is attributable to increased cell death.

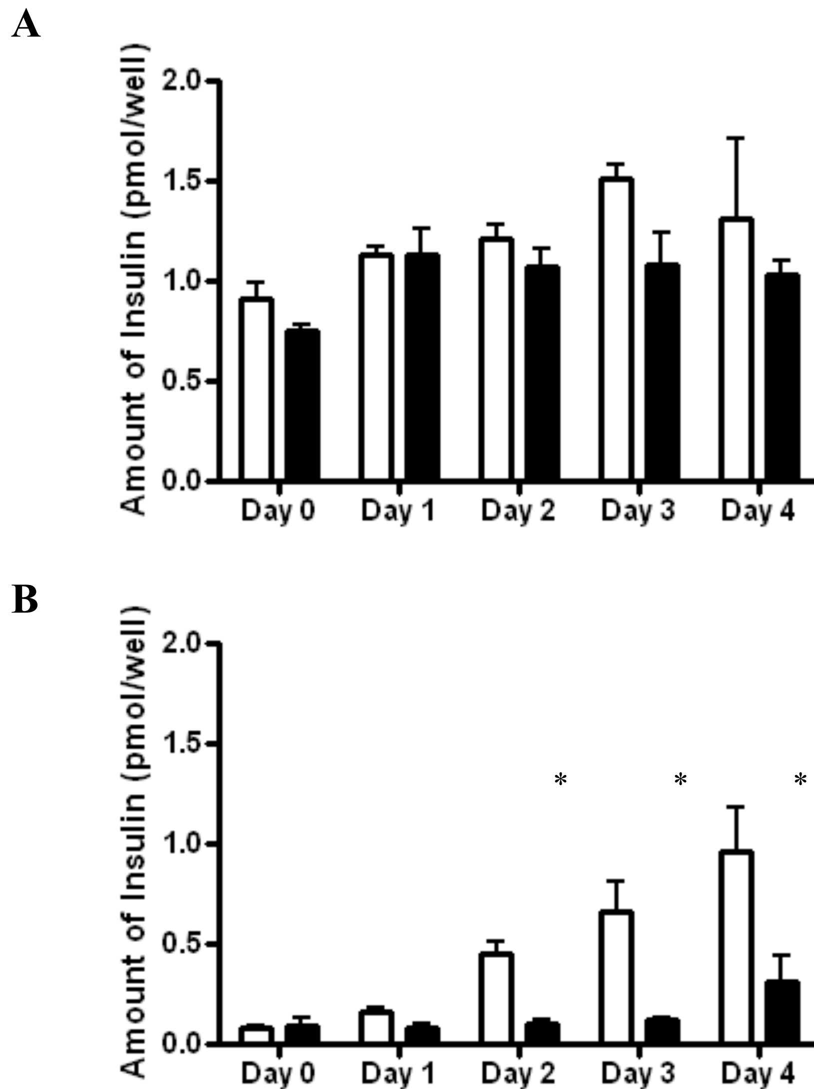

Figure 6.12: The effect of STZ treatment on HepG2ins and HepG2ins/g chronic insulin secretion.

*Total chronic insulin secretion after incubation without (unfilled bars) and with (filled bars) the double dose 100µM STZ treatment for 4 days **A**) HepG2ins **B**) HepG2ins/g. Results are expressed as mean ± SE, n=3 independent experiments *P<0.05 for STZ-treated versus untreated cells.*

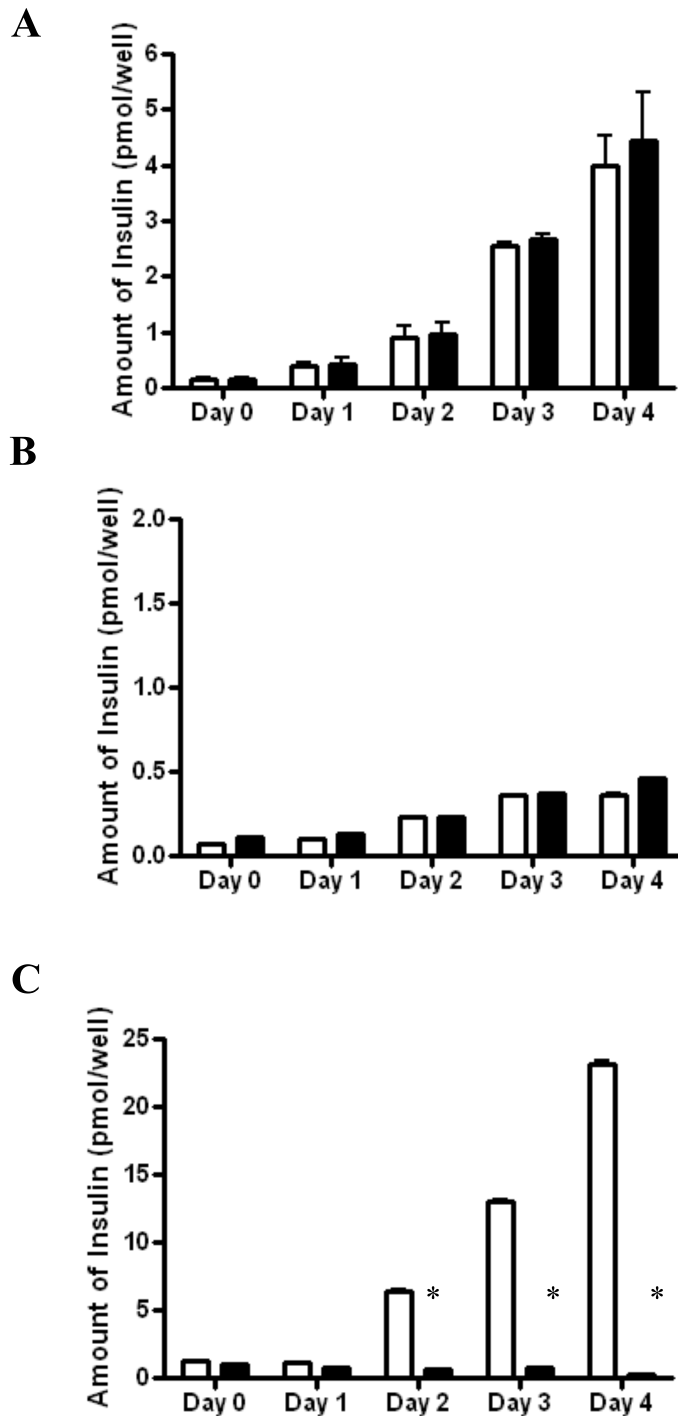

Figure 6.13: The effect of STZ treatment on Huh7ins, Melligen and MIN-6 chronic insulin secretion.

*Total chronic insulin secretion after incubation without (unfilled bars) and with (filled bars) the double dose 100μM STZ treatment for 4 days **A)** Huh7ins **B)** Melligen **C)** MIN-6 cells. Results are expressed as mean ± SE, n=3 independent experiments *P<0.05 for STZ-treated versus untreated cells.*

6.3.4 Glucose responsiveness of HepG2ins/g Cells is affected after treatment with double dose 100μM STZ

After 4 days of double dose 100μM STZ treatment, MIN-6 cells showed a significant decrease in insulin response to a glucose stimulus when compared to the untreated (media alone) MIN-6 cells (P=0.00013). Insulin secretion of untreated MIN-6 cells in response to 20mM glucose increased nearly 4-fold over 1h when compared to basal levels (amount of insulin secreted when stimulated was 3.9 ± 0.43pmol/well/h) (P=0.002) (Figure 6.14A). The treated MIN-6 cells did not release significantly higher levels of insulin to the glucose stimulus compared to basal levels (amount of insulin secreted when stimulated 0.2 ± 0.076pmol/well/h) (P=0.98) (Figure 6.14A). By day 4, cell counts were $3x10^6$ cells/well for untreated MIN-6 cells and $3x10^3$ cells/well for treated MIN-6 cells. It can be deduced from the data presented that MIN-6 cells present in the well after 4 days of double dose 100μM STZ treatment secreted 50 times more insulin per cell compared to the untreated MIN-6 cells.

The effect of the STZ treatment on glucose-responsiveness was determined for the HepG2ins/g cells only since the HepG2 and HepG2ins cells are not glucose responsive. Untreated HepG2ins/g cells gave an almost 4-fold increase in insulin secretion when stimulated with 20mM glucose (4.0 ± 0.52pmol/well/h), with return to basal levels of insulin secretion upon removal of the glucose stimulus (Figure 6.14B). However, a significant difference was observed between the amount of insulin secreted to the glucose stimulus with and without the STZ treatment in the HepG2ins/g cells (P=0.0015). The treatment significantly reduced the amounts of basal insulin secreted by the HepG2ins/g cells (P<0.05).

The effect of the 4-day double dose of 100μM STZ treatment on glucose-responsiveness was also determined for the Huh7ins and Melligen cells. Untreated Huh7ins and Melligen cells gave a 4-fold increase in insulin secretion when stimulated with 20mM glucose (0.37 ± 0.049pmol/well/h and 0.32 ± 0.09pmol/well/h respectively), with return to basal levels of insulin secretion upon removal of the glucose stimulus (Figure 6.15A and B). Huh7ins incubated with STZ for 4 days showed a 4-fold increase, from the basal amount, in insulin secretion upon the 20mM

glucose stimulus (0.36 ± 0.06pmol/well/h for stimulated and 0.10 ± 0.07pmol/well/h for basal levels). Melligen cells also showed a 5-fold increase in insulin secretion after glucose stimulus, even after the STZ treatment (0.34 ± 0.14pmol/well/h for stimulated insulin and 0.06 ± 0.03pmol/well/h for basal levels) and a return to basal levels of secretion within 1h after stimulation (Figure 6.15A and B). The amount of insulin secreted by the treated Huh7ins and Melligen cells during the stimulus was not significantly different to the results obtained for the untreated Huh7ins and Melligen cells, respectively ($P>0.05$). This indicates that Huh7ins and Melligen cells retain the ability to respond to a glucose stimulus even after 4 days of double dose STZ treatment.

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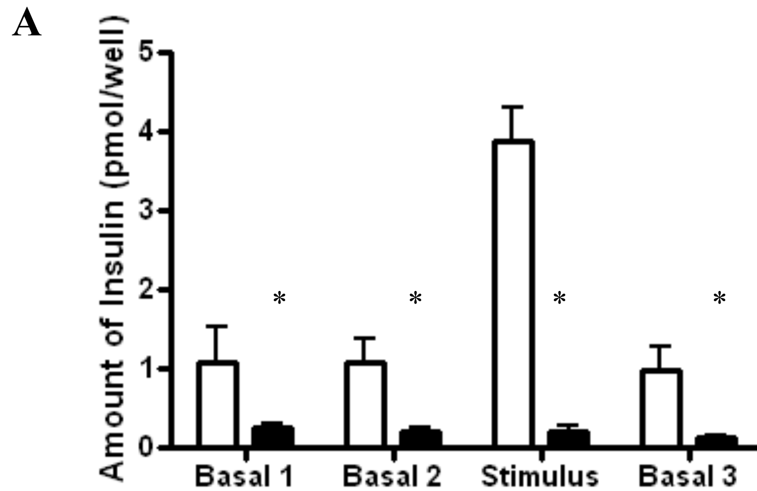

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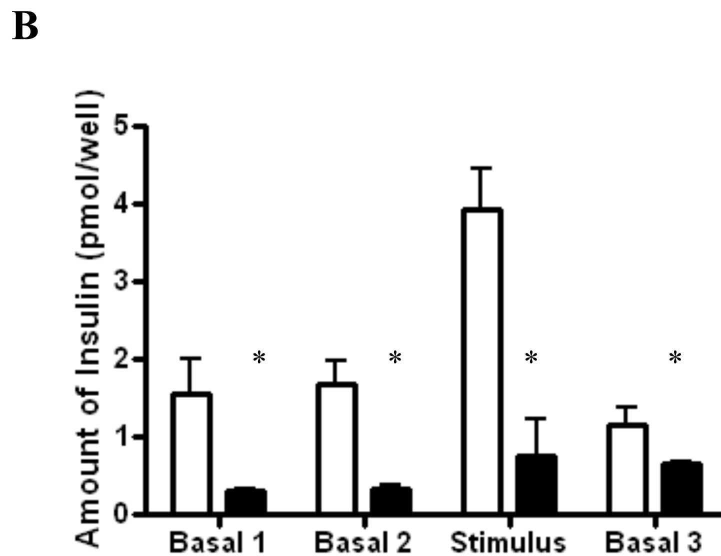

Figure 6.14: The effect of STZ treatment on glucose responsiveness in MIN-6 and HepG2ins/g cells.

Glucose responsiveness was determined using 20mM glucose stimulus without (unfilled bars) and with (filled bars) STZ treatment. ***A****) MIN-6 and* ***B****) HepG2ins/g cells after 4 days incubation with the 100µM STZ treatment. Results are expressed as mean ± SE, n=3 independent experiments * P<0.05 for STZ-treated versus untreated cells.*

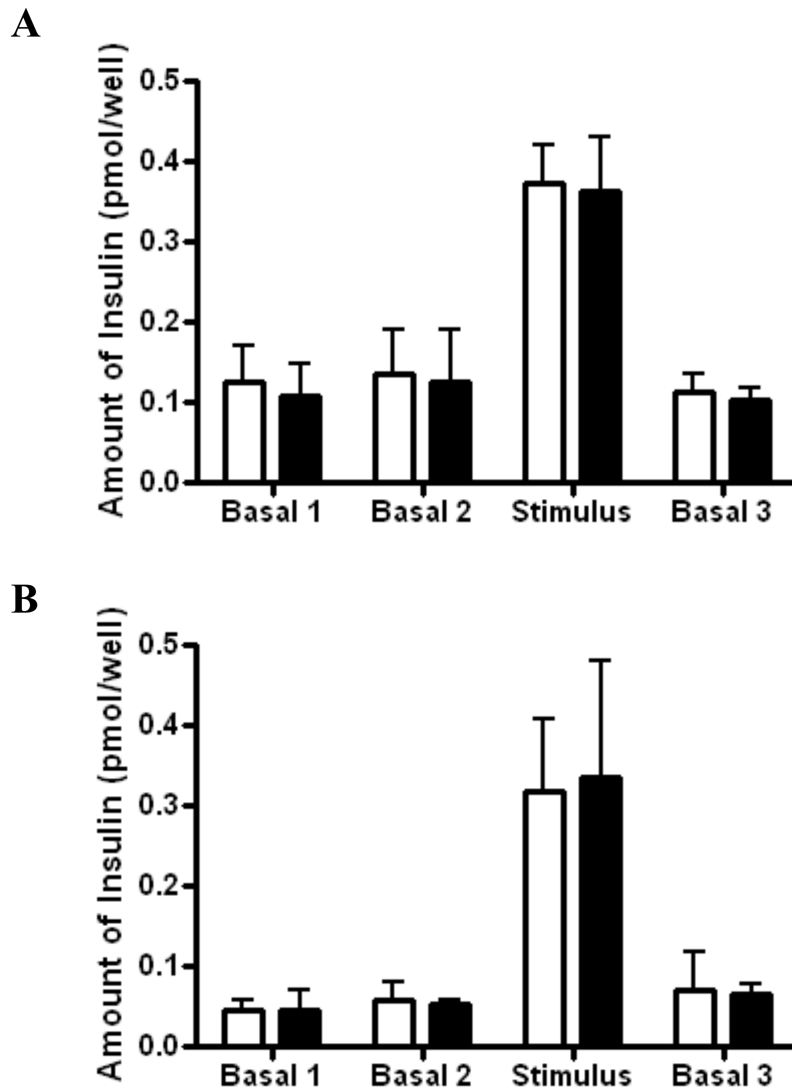

Figure 6.15: The effect of STZ treatment on glucose responsiveness in Huh7ins and Melligen cells.

Glucose responsiveness was determined using 20mM glucose stimulus without (unfilled bars) and with (filled bars) STZ treatment. ***A****) Huh7ins and* ***B****) Melligen cells after 4 days incubation with the 100µM STZ treatment. Results are expressed as mean ± SE, n=3 independent experiments * P<0.05 for STZ-treated versus untreated cells.*

6.4 Discussion

For the ultimate application of encapsulated Melligen cells in the reversal of Type 1 diabetes, the cells must not only survive the encapsulation process and remain viable, but they must also express their differentiated functions when in the microcapsule. The results in the current study showed that, *in vitro,* the Huh7 and Huh7ins cells maintained viability over 110 days after successful encapsulation. However, the seeding density of the encapsulated Melligen cells needs to be further investigated in order to obtain optimal viability. Insulin function in the Huh7ins and Melligen cells also remained unchanged after the encapsulation procedure with the same levels of insulin secreted by the cells in response to a glucose stimulus and at basal levels before and after the encapsulation procedure.

Several studies have been aimed at encapsulating the beta cell in an attempt to restore normal insulin function in Type 1 diabetics (Kühtreiber & Lanza, 1999). Allogeneic pancreas transplantation has been successful, particularly in combination with kidney transplants (Sutherland, 1996). However, it is severely limited by donor availability. Transplantation of allogeneic isolated islets into Type 1 diabetic patients suffers from the same limitation and has shown less promise with the conventional immunosuppression regimens, which cannot avoid the recurring autoimmunity. With the development of novel immunoisolation approaches (Lanza & Chick, 1997) xenogeneic islets, most notably porcine, have been considered as a more readily available source of tissue for transplantation (Sullivan et al., 1991; Lacy et al., 1991; Sun et al., 1996). With the exception of the study by Sun et al. (1996), encapsulation by itself has not been able to offer long-term protection to islets transplanted into an autoimmune environment, such as the NOD mouse, without additional immunosuppressive agents (Weber et al., 1997).

After encapsulating islet grafts, de Groot et al. (2003) determined that the loss of viability was due to necrosis, and only partly due to apoptosis. They examined the grafts after 2 and 5 days and determined that hypoxia was not associated with changes in the Bcl-2/Bax mRNA ratio, but did increase the expression of iNOS and MCP-1 mRNA. The increased mRNA levels were, however, not associated with elevated concentrations of nitrite nor with elevated levels of MCP-1 protein (de Groot et al., 2003). The increased iNOS mRNA levels may imply a role for NO in the completion

of cell death by hypoxia. The increased MCP-1 mRNA levels suggest that encapsulated islets *in vivo* contribute to their own graft failure by attracting cytokine-producing macrophages. However, in contrast to islet cells, the results obtained in Chapter 4 highlight a low expression of iNOS and MCP-1 in the Melligen cells, which indicate desirable characteristics in these surrogate beta cells.

The rate of proliferation of encapsulated cells may be lower than under normal tissue conditions, in part because the environment inside the capsule and the environment in the normal tissue culture dish are different. For cell types such as pancreatic islets, which do not proliferate in culture, the initial encapsulation efficiency must be high to provide adequate cell mass within the capsule (de Groot et al., 2003). However, in encapsulating HepG2 cells with medium augmented with 20% Ficoll-400 (regular capsules), the encapsulation efficiency was 20-25% (Uludag et al., 1994). However, inclusion of the cell immobilization matrix Matrigel® in the capsule core increased the encapsulation efficiency for HepG2 cells to 50% (Babensee et al., 1992). The encapsulation efficiency was also dependent on the sensitivity of the cells to the encapsulation process and on the quality of the encapsulation run, with better-centered capsules having higher efficiencies as more cells were completely enclosed by the polymer wall (Babensee & Sefton, 1999). Prior to transplanting the encapsulated Melligen cells into animal models it will need to be ensured that the Melligen cells are at the highest efficiency. From the results obtained by AustriaNova, Singapore, the viability of the Huh7 and Huh7ins cells were not altered by the encapsulation procedure. Further studies need to be performed on the Melligen cells for optimal results and statistical validity.

The objective of a study performed by Cheng et al. (2005) consisted of using genetically modified cells as an insulin source and of regulating insulin release by incorporating a glucose-responsive material, which acts as a control barrier for insulin in a cell-material hybrid device. Experiments were performed with insulinoma βTC cells, HepG2 hepatomas, and C2C12 myoblasts, the latter two genetically modified to constitutively secrete insulin. The control barrier consisted of concanavalin A-based glucose responsive material, which forms a gel at low and a solid at high glucose concentrations. Their results demonstrated that the device released insulin at a higher

rate in response to glucose challenges. In contrast, a device containing an inert hydrogel instead of glucose-responsive material released insulin at an essentially constant rate, irrespective of the surrounding glucose concentration. It was concluded that the continuous or slowly responsive insulin secretion dynamics from these cells could not provide physiologic glucose regulation in patients. However, if a suitable material is designed to allow the total amount of insulin produced by the cells in response to rising glucose levels there is a possibility that hyperglycaemic episodes can be reversed using the encapsulated Melligen cells.

It has been well established that STZ is taken up by pancreatic beta cells via the glucose transporter GLUT2. A reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ (Schnedl et al., 1994; Thulesen et al., 1997). Wang and Gleichmann (1995, 1998) observed that STZ itself restricts GLUT2 expression *in vivo* and *in vitro* when administered in multiple doses. In a study by Adeghate et al. (2009) processed the pancreas and plasma of rats treated with STZ for morphological and biochemical parameters 1-24 h and 4 weeks after STZ treatment. Marked reduction in body weight was observed as early as 3 h post STZ treatment and hyperglycemia coupled with hypoinsulinaemia appeared in rats 1 h after treatment with STZ. Hyperglycemia, hyperglucagonemia and hypoinsulinemia became permanent 24 h after STZ treatment. The number of insulin-positive cells decreased significantly at 24 h after STZ treatment with a concomitant increase in the number of glucagon-immunoreactive cells. Further to this electron microscopy showed coalescing of beta cell granules 18 h after STZ treatment. If Melligen cells are to be a suitable surrogate beta cell, they need to be tested *in vitro* for their ability to resist beta cell toxins such as cytokines and STZ and be encapsulated for implantation into an STZ-induced diabetes model. These cells also must display normal viability and insulin function when encapsulated.

In this study, the liver cell lines HepG2, HepG2ins and HepG2ins/g and Huh7, Huh7ins and Melligen cells were subjected to up to two iterative STZ exposures at 100μM and 20mM. The results in the study showed that only the HepG2ins/g cells were significantly affected by day 2 of the experiment, which was the first time point detecting a significant change in viability. This result was further grounded by the GLUT2 expression results where these cells showed the greatest fold change in

expression by day 4 of the STZ treatment. The HepG2ins/g cells expressed the highest amount of GLUT2 out of the HepG2, Hep2ins and HepG2ins/g cells and the Huh7ins cells express the lowest amount of GLUT2 out of the Huh7, Huh7ins and Melligen cells at day 0. The Huh7, Huh7ins and Melligen cell lines may possess multiple defense mechanisms (as determined in Chapter 5 after cytokine treatment) providing a wider spectrum of protection of the cells.

STZ impairment of beta cell function has been reported in both *in vivo* and *in vitro* studies. Exposure to STZ, depending on dose, can lead to hyperglycaemia and severe hypoinsulinemia (Flatt et al., 1989) and in beta cells to inhibition of glucokinase, hexokinase and aconitase activites, down-regulation of GLUT2 expression and defective insulin secretion (Tuch et al, 1997; Park et al., 1999; Turk et al., 1993; Strandell et al., 1997). Streptozotocin is similar enough to glucose to be transported into the cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to beta cells, since these cells have relatively high levels of GLUT2. Several studies suggest that GLUT2, in addition to its role in glucose transport, may also have other functions in glucose-stimulated insulin secretion. As a first step in addressing this possibility, Ishihara et al. (1995) engineered MIN6 cells overexpressing human GLUT-2 by transfection with human GLUT-2 cDNA. Stable transformants harboring human GLUT-2 cDNA exhibited an approximately two-fold increase in 3-O-methyl-D-glucose uptake at 0.5 and 15 mM. Glucokinase activity or glucose utilization measured by conversion of [5-3H] glucose to [3H]H_2O was not, however, altered in the MIN6 cells overexpressing human GLUT2. Furthermore, glucose-stimulated insulin secretion was not affected by over-expression of human GLUT2. These results showing that an abundance of GLUT2, therefore, does not correlate with the glucose responsiveness of cells in which glycolysis is regulated at the glucose phosphorylating step. These data suggest that GLUT2 by itself does not have significant functions other than its role in glucose transport in glucose sensing by MIN6 cells.

The results obtained in the current study also support these results. Melligen cells exhibited the lower fold change in GLUT2 expression after STZ treatment compared to the day 0 time point, however, insulin secretion and glucose responsiveness was not altered. On the other hand HepG2ins/g cells displayed a 4.4 ± 0.015 fold up-

regulation in GLUT2 expression by day 4, which was significantly higher than that at day 0, and the insulin secretion and glucose responsiveness was significantly affected. These findings strongly suggest a protective mechanism involved in the Melligen cells that is not present in the HepG2ins/g cells. However, it has also been suggested that JNK is activated by STZ downstream of PARP-1 through inactivation of phosphatases such as MKP, which plays important roles in STZ-induced beta cell death, as determined in NIT-1 cells exposed to 10mM STZ for 1h (Cheon et al., 2010). And together with the expression of GLUT2 beta cells become susceptible to the effects of STZ treatment.

Another explanation for the difference in response to the STZ treatment is that GLUT2 has high capacity but low affinity (high K_m, ca. 5 mM) and this could be the rate-limiting step (Sweet and Matschinsky, 1997). The incubation of isolated pancreatic islets of C57 BL/6 mice with STZ for 30 min resulted in a concentration-dependent gradual loss of beta cell function as determined by basal and D-glucose-stimulated insulin release (Gai et al., 2004). However this study found that STZ did not affect the mRNA expression of GLUT2 and GK, but concentration dependently reduced the GLUT2 protein expression.

The double dose 20mM STZ treatment caused a sharp and uniform decline in viability in all cell lines over the four days. This may be the consequence of necrotic death. In the MLD there is already a necrotic basis for cell death (Section 6.1). So a multiple high dose model seems to have a distinguished mode of death by necrosis that is immediate and anticipated. A literature search revealed that using the STZ treatment at 20mM and 100μM induces beta cell death. Tuch et al. (2000) investigated this STZ treatment over 14 days on the rodent insulinoma cell line, NIT-1, and the insulin secreting human hepatocyte cell line, HEPG2 ins/g. However, Gai et al. (2004) also determined that the same STZ concentration was sufficient to induce beta cell death genes in RINm5F cells after 30min. The conflicting results in the literature may be the consequence of poor STZ storage or the lack of freshly added treatment. In addition to this, Tuch et al. (2000) made the STZ stock in 0.2M citrate and this may have changed the pH *in vitro,* creating variable results.

Over expression of GLUT2 in insulinoma-derived RIN cells significantly enhanced sensitivity of transfected cells toward STZ compared with non-transfected cells (Elsner et al., 2000; Schnedl et al., 1994). Also compared with other alkylating agents that produce similar DNA damage, GLUT2 transfected cells showed an increased sensitivity toward STZ (Schnedl et al., 1994). However, results from other studies using GLUT2 transfected genetically engineered non-beta cells that produce insulin, suggested that other factors such as radical enzymes might be involved (Tuch et al., 1997). STZ-resistant RIN cells became insensitive to both STZ and alloxan and also demonstrated increased cellular insulin content and glucose-induced insulin release (Bloch et al., 2000; Bloch et al., 2005). In addition, GLUT2 expression was decreased suggesting a potential explanation for the STZ resistance in the resistant cells.

In a mathematical model, Sweet and Matchinsky (1997) show that GLUT2 has low affinity using an experiment involving glucose transportation in primary human liver cells and pancreatic islet cells. This study showed that unlike the unidirectional Michaelis Menten kinetics where an increase in affinity necessarily results in an increase in flux, increasing the affinity of a transporter in this system can result in a lowering of the phosphorylation rate. This is because the rate of exit via the transporter is in direct competition with the phosphorylation of glucose. The higher the ratio of transporter affinity relative to the affinity of glucokinase, the higher the fraction of intracellular glucose that will be exported rather than phosphorylated. So the optimal value of K represents a balance between the enhanced rate of uptake that a high affinity favours and the increased reflux out of the cell. Thus the increase in GLUT2 expression in the Huh7ins and Melligen cells may have inhibited the influx of STZ. If the expression of GLUT2 in the HepG2ins/g was at the level where STZ could be transported into the cells, this would have rendered the cells susceptible to the STZ and hence highlight the significance of the level of GLUT2 expression in the transportation of toxins into surrogate beta cells.

A study by Laguens et al. (1980) examined the relationship between diabetes incidence and liver damage in C3H-s mice 21 days after STZ administration (administered using a double dose of STZ). These findings showed that beta cells and hepatocytes have synchronic circadian sensitivity to STZ and that liver damage is present whether the animals became diabetic or not, suggesting the presence of a

different threshold for STZ effect in hepatocytes. Another important action of STZ is to inhibit glucokinase activity and gene expression in beta cells consistent with observed impairment of glucose induced insulin secretion after acute STZ exposure (Park et al., 1999). The transdifferentiation of the Huh7 cells to Melligen cells involves factors involved in beta cell maturation. Studies suggest that cytokine exposed beta cells die because they acquire particular sensitivity to these compounds as a consequence of their maturation program (Nielsen et al., 1999). Hence, the results obtained in the cell viability studies, which suggest a greater susceptibility of the HepG2ins/g cells compared to the HepG2 cells in response to the STZ treatment.

The generation of hydrogen peroxide and NO from STZ has been measured and suggested to increase oxidative and nitrosative stress leading to DNA damage and apoptosis in pancreatic beta cells (Saini et al., 1996; Turk et al., 1996). A variety of reactive oxygen species (ROS) scavengers have been shown to reduce STZ-induced beta cell cytotoxicity by inclusion in cell incubations or by gene overexpression (Flatt et al., 1989; Bast et al., 2002; Chen et al., 2001). Several of which such oxidative species have been found in liver cells (Chapter 4).

To understand the impact of STZ tolerant cell selection on toxin resistance and insulin-secretory function, Liu et al (2007) generated STZ-resistant BRIN-BD11 cells by iterative acute exposure to 20mM STZ. These cells came to exhibit resistance to toxic challenges from STZ, H_2O_2 and ninhydrin. Insulin content and both glucose and arginine stimulated insulin secretion were significantly enhanced in the BRIN-BD11 STZ-resistant cells. In this study it was also determined that SOD activity was decreased, catalase activity was increased and appeared to be important for the ninhydrin and STZ resistance of the cells. The mechanism underlying this effect included upregulation of genes for *Pdx-1*, GLUT2, glucokinase, and insulin as well as enhancement of cellular insulin content. This supports the findings in this experiment that the Huh7, Huh7ins and Melligen cells were protected even though GLUT2 levels were expressed, albeit at lower levels than the HepG2ins/g cells, after the 4-day STZ treatment.

Since STZ is a NO donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced

DNA damage (Kröncke et al., 1995; Morgan et al., 1994). NO has been shown to participate in the cytotoxic effects of STZ in several experiments (Turk et al., 1993; Kröncke et al., 1995). STZ is not a spontaneous NO donor, however, NO is liberated when STZ is metabolized inside cells, but NO synthase is not required for this effect. On the other hand, the lowering of NO concentrations in pancreatic islet cells by inhibition of the inducible form of NO synthase partially counteracted DNA cleavage induced by STZ (Bedoya et al., 1996). However, the results of several experiments provide the evidence that NO is not the only molecule responsible for the cytotoxic effects of STZ. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells (Takasu et al., 1991; Bedoya et al., 1996). Restriction of mitochondrial ATP generation is partially mediated by NO (Szkudelski, 2001). The findings in the current study show that MIN-6 cells are significantly affected by the STZ treatment 2x100 starting from day 2 onwards. This finding corroborates previously reported data by Saini et al. (1996) confirming that low doses (15mM) of STZ produce apoptosis and high doses (30mM) induce necrosis in NIT-1 cells.

A reduction in insulin secretion is attributable to increased cell death in the MIN-6 and HepG2ins/g cell lines. Eizirik et al. (1994) determined the susceptibility of human pancreatic islets to STZ, or alloxan. After 5-8 days in tissue culture, human or rodent islets were exposed for 30 min to STZ (1-3 mM). Glucose (16.7 mM)-induced insulin release by human islets was not impaired after a 30min exposure to STZ at concentrations that inhibited insulin release from rat (30-80% inhibition) or mouse (10-70% inhibition) islets. The viability of human beta cells purified by flow cytometry was not affected by STZ (5 mM), as judged 1 or 4 days after a 10min exposure and subsequent culture. These conditions were cytotoxic for rat beta cells, with 65-95% dead beta cells after 4 days. This group found that human beta cells are resistant to STZ at concentrations that decrease survival and function of rat or mouse beta cells. These marked interspecies differences emphasises the relevance of repair and/or defense mechanisms in beta-cell destruction and raise the possibility that such differences may also be present among individuals of the same species or that increased GLUT2 expression is the factor that contributes to the reduced resistance. Two independent studies also reported very low levels of GLUT2 expression in human islet cells (De Vos et al., 1995; Ferrer et al., 1995).

Despite the fact that some transplantation studies indicate that down regulation of islet GLUT2 is the consequence rather than the cause of the diabetic condition (Ogawa et al., 1992), it can be argued that the observed reduction of GLUT2 in rat beta cells impairs glucose sensing, thereby contributing to the beta-cell failure in diabetes. However, kinetic measurements in rat islets (Hellman et al., 1971) or pure beta cells (Heimber et al., 1993) show that transport can provide a metabolic flux that is two orders of magnitude higher than the actual glycolytic rate in these cells, suggesting that down regulation of GLUT2 in rat beta cells should be at least 95% in order to impair glucose-induced insulin release.

6.5 Conclusion

In conclusion, the Melligen cells are a suitable surrogate beta cell for implantation into STZ-diabetic mice. They are able to remain viable in the microcapsules for 110 days and are responsive to physiological levels of glucose. The cells were also resistant to the effects of STZ compared to the MIN-6 cells. Melligen cell viability was not affected by the double dose 100μM STZ treatment and it was determined that GLUT2 expression was decreased after the treatment. Future studies for the Melligen cells include encapsulation into mouse models and higher animal models such as dogs.

CHAPTER 7: Discussion

7.1 Final Discussion

The pathological hallmark of Type 1 diabetes is the infiltration of immune cells into the pancreatic islets, a process known as insulitis. During insulitis, beta cell destruction is largely mediated by the release of pro-inflammatory cytokines, of which IFN-γ, TNF-α and IL-1β play major roles in beta cell destruction (Eizirik et al., 2009). The persistence of immunological memory is a major hurdle for attempts to cure Type 1 diabetes by replacing beta cell populations, as these beta cells will be once again destroyed largely through the release of the aforementioned pro-inflammatory cytokines. The only way to prevent the reoccurrence of autoimmune reactions against transplanted beta cells, either syngeneic or allogeneic, is to maintain the recipient on intensive, lifelong immunosuppressive therapy, which carries a multitude of adverse side effects (Mitanchez et al., 1997; Gao et al., 2007; Shapiro et al., 2006).

An alternative strategy for replacing beta cells would be to use cell therapy to target insulin expression to non-beta cells, in other words to create a cell that performed the insulin secretory function of beta cells, but withstood the recurrent autoimmune processes (Tisch et al., 1996). This approach carries several advantages, as the 'artificial' beta cells would not carry all of the beta cell antigens, against which immune responses were generated. The surrogate beta cells would need to be endowed with analogous physiological characteristics of beta cells; namely biologically active insulin production, insulin storage, and glucose-dependent insulin secretion. The Huh7ins cells were found to posess some of these characteristics, however, these cells were further modified to secrete insulin in response to glucose in the millimolar range, and are called Melligen cells.

Pancreatic beta cells possess several characteristics that increase their susceptibility to pro-inflammatory cytokine-induced cytotoxicity. Presumably, many of these characteristics may be absent in Melligen cells. Beta cells carry many auto-antigens (Eisenbarth et al., 2002) whereas the transformed liver cells carry only one auto-antigen, albeit a major one, insulin. Since insulin and other auto-antigens are expressed in and on the surface of pancreatic beta cells in Type 1 diabetics (Ziegler et

al., 1999), there is the possibility of activating an autoimmune response upon transplantation of insulin-producing cells. However, the current study suggests that insulin-expressing Huh7ins and Melligen cells withstand cytokine-mediated damage. Huh7ins and Melligen cells may carry the advantage of only expressing insulin and not the other auto-antigens of native beta cells (Colman et al., 2000). Additionally, since the viabilities of Huh7, Huh7ins and Melligen cells in the presence of cytokines are similar, this suggests that it is something inherent to the liver cell that enables it to resist cytokine attack and the expression of insulin in these liver cells does not affect their viability in the presence of a pro-inflammatory milieu.

The current study was conducted to determine the effect of the pro-inflammatory cytokines, IFN-γ, TNF-α and IL-1β, on the viability, glucose-stimulated insulin release and chronic insulin secretion and storage in the insulin secreting liver cell lines, Huh7ins and Melligen. Cytokine-induced changes, such as nitric oxide production, iNOS, MCP-1, Fas and inhibitors of NF-κB gene expression, have been reported to play major roles in beta cell damage during both the development of Type 1 diabetes and destruction of islet transplants (Mandrup-Poulsen, 2003; Ortis et al., 2006). Therefore, it was also determined if such changes were induced in Melligen cells after co-incubation with pro-inflammatory cytokines. In obtaining a more detailed profile of up or down regulated genes and their networks, microarray analysis was conducted on the cytokine treated and untreated Melligen cells after 1h and 24h.

The pancreatic beta cell line, MIN6 was used as a positive control throughout most experiments. This cell line is derived from a transgenic mouse expressing the large T antigen of SV40 in pancreatic beta cells. The cell line behaves in a manner very similar to isolated pancreatic beta cells in terms of glucose-stimulated insulin secretion, glucose utilization, and glucose transport due to the presence of high levels of the glucose transporter, GLUT 2 (Ishihara et al., 1993). In providing an appropriate alternative to primary beta cells, MIN-6 cells overcome the technical problems associated with isolating an adequate number of purified beta cells from intact isolated islets. Hence, the MIN-6 cell line has been widely used in previous studies including transcriptomics, to investigate beta cell function (Wu et al., 2001; Åkerfeldt et al., 2008; Sarkar et al., 2009). Therefore, while ideal positive controls would have

been primary hepatocytes and primary beta cells of human origin, these cells are not readily available.

Initial experiments determined that cytokine concentrations required to reduce cell viability in MIN-6 cells were IFN-γ (384ng/mL), TNF-α (10ng/mL) and IL-1β (2ng/mL) with cytokines changed daily over a 10-day period. After 3 days exposure of MIN-6 cells to combinations of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-1β, cell viability was reduced. The cytokine concentrations used in this study were the same as those used by Tabiin et al. (2001) in an investigation of cytokine effects on the murine pancreatic beta cell line, NIT-1, and the HepG2ins/g liver cell line. The NIT-1 cell line showed a decrease in proliferation and mitochondrial activity by day 1 of the 14-day observation period. However, NIT-1 cells are not glucose responsive and therefore do not constitute an optimal model for the investigation of the effects of cytokines on beta cell viability. Previous studies using MIN-6 cells have shown that incubation for two days with pro-inflammatory cytokines resulted in decreased mitochondrial activity as compared to both untreated cells and cells pre-incubated with anti-inflammatory cytokine, IL-6 (Choi et al., 2004).

In this study, the effect of the co-incubation of MIN-6 cells with individual cytokines, IFN-γ, TNF-α and IL-1β, did not reduce cell viability to the same extent as these cytokines added in a cocktail. This finding is consistent with previous reports that cocktails of pro-inflammatory cytokines are required for toxicity. For example, Seewaldt et al. (2000) showed that exposure of beta cells to a single pro-inflammatory cytokine, such as IFN-γ (1μg/mL), as opposed to the triple cytokine combination (IFN-γ, TNF-α and IL-1β at 1μg/mL each) over 24h, resulted in significant islet cell lysis only in the latter case. This study suggests that the toxic effects of cytokines are synergistic when added at 1μg/mL, which is higher than any of the concentrations used in the current study. Furthermore, Cetkovic-Cvlje and Eizirik (1994) reported that while TNF-α (1000U/mL) can exert a direct toxic effect on mouse islet and RIN cells, cytotoxicity is greatly enhanced by the presence of additional pro-inflammatory cytokines, especially IL-1β (50U/mL) and IFN-γ (1000U/mL). These concentrations are similar to those used in the current study. A multitude of other studies have reported the greater toxic effect elicited by the cytokine combination when compared

to the effect of the single cytokines (Lee et al., 2004; Augstein et al., 2004, Park et al., 2003). Presumably this might be because multiple transcriptional pathways are activated, or because of the synergistic effect of cytokines on gene expression.

Retaining viability in the presence of pro-inflammatory cytokines, while necessary is not sufficient; surrogate beta cells must continue to synthesise and secrete insulin in a regulated manner. Treatment of Huh7ins and Melligen cells with the triple cytokines for 10 days did not cause a significant decrease in insulin content or chronic insulin secretion. In contrast, similarly treated MIN-6 cells secreted a decreased amount of insulin by day 1 and stored lower amounts of insulin by day 2. On the other hand, untreated MIN-6 cells stored and secreted insulin over the 10-day period. The effect of the cytokines on glucose-responsiveness was also determined for the MIN-6, Huh7ins and Melligen cells. Untreated cells were found to give approximately a 5-fold increase in insulin secretion when stimulated with 20mM glucose, with a return to basal levels upon removal of the stimulus. When Huh7ins and Melligen cells were incubated with cytokines for 10 days they showed a 4-fold increase in insulin secretion after the glucose stimulus and a return to basal levels of secretion within 1h after stimulation. This result was not significantly different to the result obtained for the untreated cells on any of the days. These results suggest Huh7ins and Melligen cells retain the ability to respond to a glucose stimulus even after prolonged cytokine treatment and confirm that the expression of the insulin gene in the hepatoma cell line does not increase susceptibility to a cytokine-mediated attack. In contrast cytokine treated MIN-6 cells did not produce a significant amount of insulin to the glucose stimulus when compared to the untreated cells.

The results of the current study confirm previous investigations using pancreatic islets from diabetes-prone BB rats that were exposed to cytokines IL-1β, IFN-γ and TNF-α (10, 500 and 500U/mL, respectively) in combination and found that insulin content was decreased and glucose-stimulated insulin release was inhibited (Wachlin et al., 2003). Wu et al. (2001) reported that glucose-stimulated insulin release from MIN-6 cells was not suppressed by exposure to the individual cytokines IL-1β, TNF-α and IFN-γ at concentrations 50, 1000 and 750U/mL, respectively, however, significant suppression of glucose-stimulated insulin release occurred when cells were exposed to

all three pro-inflammatory cytokines. Tabiin et al. (2001) also found that cell viability, insulin storage and insulin secretion of NIT-1 cells were adversely affected by incubation with the triple cytokines TNF-α, IL-β, IFN-γ at concentrations 1000, 374 (2ng/mL) and 1000U/mL, respectively. The results from this study and the current study indicate that the cytokine concentrations used in the cocktail have inhibitory effects on insulin function in the beta cell model. Since the Huh7ins and Melligen cells could withstand the effects of the cytokines in this regard, it is possible that the cells will continue to maintain insulin function after transplantation in this cytokine milieu.

In contrast to the reduced viability of MIN-6 cells observed after 3 days co-culture with cytokines, Huh7ins and Melligen cells were resistant to the toxic effects of the pro-inflammatory cytokines even after 10 days co-incubation. Likewise the parent cell line, Huh7, was resistant to the toxic effects of the cytokines. Thus, the expression of insulin by Huh7ins and Melligen cells does not render them susceptible to cytokine cytotoxicity. Similarly, when treated with the double dose 100μM STZ treatment, the expression of insulin in the Huh7ins and Melligen cells did not render them susceptible to STZ cytotoxicity. Insulin secreting liver cells, such as Huh7ins and Melligen cells, may exhibit increased resistance to cytokine and STZ treatments, as they are not of beta cell origin. These results suggest that the resistance to pro-inflammatory cytokine-induced damage may be an intrinsic characteristic of liver cells and this was confirmed in the microarray study performed on the Melligen cells.

A considerable amount of microarray data on cytokine-mediated NF-κB-regulated gene expression patterns in beta cells is available from the reports of Eizirik and coworkers (Cardozo et al., 2001; Kutlu et al., 2003; Eizirik et al., 2003). These studies suggest that NF-κB mediated induction of pro-apoptotic genes predominantly mediates beta cell death in Type 1 diabetes. Some of these pro-apoptotic genes could exert delayed indirect effects. For example, NF-κB-dependent inducible nitric oxide synthase generates nitric oxide, which causes beta cell dysfunction and death (Mandrup-Poulsen, 2001). Furthermore, Fas mediated extrinsic pathway of apoptosis interacts with the intrinsic mitochondrial pathway through generation of truncated Bid, which induces the release of cytochrome C from the mitochondria (Luo et al.,

1998). Cytokines could also induce apoptosis through NF-κB-independent pathways including activation of c jun N-terminal kinase (Bonny et al., 2001). Thus, beta cell death induced by cytokines could result from late events triggered by NF-κB-regulated pro-apoptotic genes and NF-κB-independent signalling pathways.

Considering the complex nature of cytokine-mediated signalling, it can be ascertained, on the basis of the array of genes induced, that NF-κB is predominantly involved in anti-apoptotic cascades in Melligen cells. In the microarray study on the Melligen cells, the concentration of cytokines used were comparable to those used in the microarray study performed by Sarkar et al. (2009) on cultured human islets, IL-1β (2 ng/ml; 100 U/ml), IFN-γ (10ng/ml; 200U/ml) and TNF-α (10ng/ml; 1000U/mL) used in combination. In this study the genes CXCL10, BIRC3 and TNFAIP3 were found to be up-regulated by fold-changes similar to those observed in the cytokine treated Melligen cells in the current study. This could either indicate that a particular cascade or pathway is being induced by the pro-inflammatory cytokines common to all cell types or that the Melligen cells are more beta cell like and are following the cytokine inducible cascade inherent to beta cells. A large body of *in vitro* experiments suggests that cytokine-induced NF-κB activation is an important signaling event in triggering beta cell apoptosis (Ortis et al., 2008; Heimberg et al., 2001; Eizirik et al., 2003). NF-κB has been suggested to be pro-apoptotic in beta cells, whereas it is protective in other cell types such as the HT1080V fibrosarcoma cell line and embryonic fibroblasts when induced with TNF (20ng/mL) (Wang et al., 1998). This further supports the finding that Melligen cells are resistant to the cytokines because the cytokines are triggering the NF-κB pathway, inducing an anti-apoptotic response. Apoptosis results using AnnexinV/PI and cell cycle analysis in cytokine treated Huh7ins and Melligen cells further confirms the absence of apoptosis in these cells.

Beta cells by their nature are susceptible to autoimmune cytokine attack due to the low levels of superoxide dismutase (MnSOD) and other free radical scavenging enzymes, such as glutathione peroxidase and thioredoxin that protect from cytokine-induced stresses (Gurgul et al., 2004). One possible explanation for the resistance of the liver cell line is the presence of a protective mechanism in the cells including increased levels of anti-oxidative enzymes such as catalase (Tiedge et al., 1997).

Zwacka et al. (1998) have shown that adenoviral transfection of the liver with MnSOD significantly reduced post-ischemic liver injury implicating O_2^- as an important mediator of hepatocyte ischaemic injury. The microarray data confirmed by qRT-PCR also indicated a 4-fold increase of MnSOD in the cytokine treated Melligen cells indicating that the cells have maintained hepatocyte characteristics which will enable them to be more resistant to insults compared to the beta cells.

The pro-inflammatory cytokine cocktail IFN-γ, TNF-α and IL-1β induced significant apoptosis in the MIN-6 cells, whereas the effect of these cytokines were not significant on the Huh7ins and Melligen cells as determined by AnnexinV/PI stain over 48h. Cytokines induced significant levels of NO in the MIN-6 cells observed after 24h and 48h of cytokine treatment, but did not have a significant impact on Huh7ins or Melligen cells. Alterations in Bcl-2 mRNA and protein levels have been related to apoptosis induced by cytokines either in MIN-6 and RINm5F beta cell lines (Iwahashi et al., 1996). The beta cell line βCT1 is also sensitive to cytokine-induced apoptosis that conversely, is barely detectable in the α-cell line (αTC1), which is characterized by significantly higher Bcl-2 mRNA and protein levels. Moreover, it was further demonstrated in this study that lentivirus-mediated over-expression of Bcl-2 in βTC-tet cells significantly improves their resistance to hypoxia and cytokine-induced apoptosis (Iwahashi et al., 1996). Therefore, the results from this study, showing that the Melligen cells do not follow an apoptotic cascade upon cytokine treatment, may be advantageous when implanting for the reversal of Type 1 diabetes.

The Fas/FasL system has been shown to be of major importance in the homeostasis of an organism especially in regulation of the immune system. In a study by Hatano et al. (2001) apoptosis was induced in primary murine hepatocytes by TNF-α and Fas ligand. However, Fas expressing hepatocytes were found to be resistant to Fas-induced apoptosis in culture, suggesting that cellular factors exist, such as the PI3K/Akt pathway, that inhibit Fas signalling this includes activation of NF-κB (Hatano et al., 2001). Furthermore, inhibition of the transcription factor NF-κB makes cells sensitive to apoptosis induced by TNFα. This leads to the theory that NF-κB induces expression of genes, which normally protect cells from apoptosis induced by TNF-α (Baichwal & Baeuerle, 1997). Results from the current study show that the

down-stream genes of NF-κB, Fas and iNOS, were expressed in the Huh7, Huh7ins and Melligen cells. Where iNOS was weakly expressed in the treated cells lines, this may indicate that the cells are not stimulated to the point of apoptosis. Fas on the other hand was down-regulated in the Melligen cells indicating protection from apoptosis.

Aoudjehane et al., (2007) showed that human recombinant IL-4 induced apoptosis in HepG2 cells and primary human hepatocytes, however, in contrast, Huh7 cells were resistant to apoptosis induced by IL-4. This may be due to the mutated p53 found in the Huh7 cells (Müller et al., 1997) that renders them resistant to cell death effectors such as FasL. This finding shows that although the parent cell line, Huh7, is a hepatocyte, it does not respond to cytokines in the same manner as other liver cells or liver cell lines such as HepG2. Therefore, it is likely that the Melligen cells, derived from the Huh7 cell line, will exhibit a protective response when exposed to other cytokines, such as IL-4, *in vivo*.

Few studies have been conducted to assess the toxic effects of pro-inflammatory cytokines on primary human liver cells. While some studies report that primary hepatocytes are highly susceptible to cytokine-mediated damage the concentrations used were higher than those used on the current experiments and the hepatocytes used were virally infected and involved other additives in the cocktail such as glucose or LPS (Ceppi & Titheradge, 1998; Geller et al., 1993). Both the hepatoma cell lines Huh7, which does not express insulin, and the Huh7ins and Melligen cell lines which express the insulin gene, remained resistant to the effects of the pro-inflammatory cytokines for an increased period of time when compared to the control MIN-6 cell line.

In primary rat hepatocytes, Shoemaker et al. (2002) determined that the cytokine mixture of TNF-α, IL-1β and IFN-γ (20ng/mL, 10ng/mL and 100U/mL) and lipopolysaccharide inhibited NF-κB activation and this resulted in apoptosis as determined by an electrophoretic mobility shift assay. cIAP1 and XIAP expression were not increased by cytokines, however, BIRC3 (IAP2) expression was strongly increased 3h after cytokine addition. Additionally, elevated cIAP2 expression

persisted for at least 12h after cytokine exposure. In agreement with these results, BIRC3 of the IAP family of molecules was up-regulated in the cytokine-treated Melligen cells after 1h and 24h, in the transcriptome analysis and was further confirmed by qRT-PCR. The results of the current study suggest that BIRC3 is up-regulated as soon as 1h after treatment and for as long as 24h. Like the primary rat heaptocytes in the study by Shoemaker et al (2002), the Melligen cells did not up-regulate cIAP1 and XIAP otherwise shown to be up-regulated in the INS-1E beta cell line after treatment with cytokines IL-1β and TNF-α following blocking of NF-κB activation (Ortis et al., 2008). The apoptosis studies performed on the Melligen cells after 24h and 48h of cytokine treatment further established the anti-apoptotic effect for 48h induced in the cells. Negligible amounts of apoptotic cell populations mirror these results obtained in the mircoarray study. Collectively, these data suggest that the Melligen cells may be suitable for implantation without becoming apoptotic due to the autoimmune response.

Few *in vivo* studies have been performed to determine the effect of insulin-secreting non-beta cells on the immune system. Despite producing near normal glycemia, HIGT (hepatic insulin gene therapy) in CD-1 mice diminished hepatic glycogen content in both fasted and fed mice. In addition to this, serum cytokine responses revealed both vector-related (MCP-1 and IL-6) and transgene-specific (resistin and TNF-α) effects (Zhang et al., 2009). Zhang et al (2009) also showed that HIGT produced low circulating concentrations of insulin, but produces significant intra-hepatic effects on gene expression. Despite controlling hyperglycaemia, this group also showed that HIGT exerted insulin effects on hepatic carbohydrate metabolism. Although the precise mechanisms remain to be determined, they may relate to vector-induced cytokine effects. In the current study it has been shown that insulin insulin production per se does not alter cytokine signalling in a liver cell.

In addition to this study, Tabiin et al (2004) showed that insulin expressing hepatocytes were not destroyed in NOD mice (Tabiin et al., 2004). Histological examination of the livers of the diabetic transgenic mice showed no infiltration of the liver by immune cells even though insulitis was observed in the pancreatic sections. This indicates that hepatic insulin production does not cause the development of

tolerance to insulin in these PEPCK-Ins transgenic NOD mice. The lack of infiltration of immune cells in the livers of the diabetic transgenic mice could also suggest that the mouse insulin mRNA transcribing hepatocytes were not targeted or destroyed by the immune system. In addition, as the transgenic mice were sacrificed 4 weeks after the onset of diabetes, this reduced the possibility of delayed destruction of the (pro)insulin expressing hepatocytes. However, it was found that at the low levels of transgene expression, (pro)insulin was not targeted by the immune system in this transgenic PEPCK-Ins mouse model superimposed on the autoimmune diabetes model of the NOD mouse. Similarly, Lipes et al. (1996) reported that when pituitary cells in transgenic NOD mice were made to produce insulin (POMC1), the cells were not targeted by the immune system even though the pancreatic beta cells were. Likewise, no cellular infiltrate was shown when the pituitary cells that produced pro-insulin were taken outside the blood brain barrier and transplanted beneath the renal capsule of NOD mice.

Data by Tabiin et al. (2004) and those of Lipes et al. (1996) suggest that autoimmune destruction of pro-insulin producing cells in the NOD mouse was specific to the islets. It is possible that a critical amount of pro-insulin needs to be produced for an autoimmune effect to be observed. The pro-insulin content of the transgenic liver cells in normoglycaemic mice was much lower than that of a pancreatic beta cell and was also lower than that in the liver of PEPCK-human insulin C57BL/6 transgenic mice produced by Valera et al. (1994). Using induced pluripotent stem (iPS) cells from skin fibroblasts, Alipio et al. (2010) were able to derive surrogate beta cells similar to the endogenous insulin-secreting cells in mice. These cells secreted insulin in response to glucose and corrected a hyperglycemic phenotype in two mouse models of Type 1 and 2 diabetes via an iPS cell transplant for 4 months. Long-term correction of hyperglycaemia was achieved, as determined by blood glucose and hemoglobin A1c levels. Although the immune response to these cells were not considered in this study, the long duration of the study suggests that the cells were not attacked by the immune response. It was reported that on the seventy-third day, one mouse in the Type 2 model developed a tumor. These studies indicate that reversal of diabetes may be possible using insulin delivery from liver cells, however, the complications of *in vivo* transfection has its disadvantages.

Similar to the study by Hughes et al. (1992) on the AtT20ins cells (see Section 1.7.2), pituitary GH3 cells were stably transfected with a furin cleavable human insulin cDNA (InsGH3) (Meoni et al., 2000) and were grafted into STZ-induced diabetic nude mice. The subcutaneous implantation of InsGH3 cells resulted in the progressive reversal of hyperglycaemia and diabetic symptoms, even though the progressive growth of the transplanted cells eventually led to glycaemic levels below the normal mouse range. Compared to pancreatic β-TC3 cells, InsGH3 cells showed *in vitro* a higher rate of replication, an elevated resistance to apoptosis induced by serum deprivation and a 5-day treatment with the pro-inflammatory cytokine cocktail consisting of IL-1 (50U/mL), TNF-α (1000U/mL) and IFN-γ (1000U/mL) (Davalli et al., 2000). Significantly higher anti-apoptotic Bcl-2 protein levels in the InsGH3 cells were associated with the cells resistance to the insults. Moreover, InsGH3 cells were resistant to the 2 and 10mM STZ toxicity that, in contrast, reduced β-TC3 cell viability to 50-60% of controls as demonstrated in the MTT assay (Davalli et al., 2000). In this study it was also shown that the beta cell line did not decrease viability any further at 10mM STZ. These results were also obtained in the current study where the MIN-6 cell viability did not decrease below 50% at 100μM and 20mM STZ even after 4 days and this is why the treatment was added twice daily.

In agreement with the results obtained in the study by Davalli et al. (2000) the Huh7ins and Melligen cells were not susceptible to the STZ or cytokine insults, as determined by the MTT assay. However, the HepG2ins/g cell viability was significantly reduced by the STZ treatment and this susceptibility to STZ was likely dependent upon the high GLUT2 expression levels in the cells, as compared to the parent HepG2ins and HepG2ins and not because of the presence of the autoantigen, insulin. GLUT2 studies on the AtTinsGLUT2.36 cells (parent cell line AtT20) showed that increasing concentrations of glucose produced a concentration-dependent increase in GLUT2 mRNA in the presence of both sub-physiological and physiological glucose concentrations (Davies et al., 1998). The amount of GLUT2 mRNA was increased more than 10-fold in the presence of 5mM glucose compared with baseline (no glucose), however, these levels were determined using densitometry studies and not qRT-PCR which is a more accurate method for quantitation of gene expression. Insulin was released at 1 ng/10^6 cells/3 h. These cells were not destroyed

by the autoimmune system *in vivo* (Davies et al., 1998). The results obtained for the Melligen cells will be important in the clinical setting since STZ is also an environmental factor (described further in Chapter 1). Firstly however, the cells will need to be implanted into STZ-diabetic mice models and this is where their resistance to STZ will be an advantage during implantation.

Despite the ability of many cell types to express an introduced insulin gene, physiologic regulation of insulin secretion presents a number of challenges. Non-beta cells can be engineered to express insulin, but most cells such as myocytes and fibroblasts lack the ability to store and properly process insulin because they do not contain secretory granules with their accompanying hormone-processing enzymes. Furthermore, these cells secrete insulin via the constitutive secretory pathway and thus will secrete some insulin regardless of the extracellular glucose concentrations (Kolodka et al., 1995; Gros et al., 1999; Falqui et al., 1999). Insulin secretion independent of the glucose concentration is a serious limitation of such approaches with non-beta cells (Tabiin et al., 2004, Valera et al., 1994). Thus, most non-beta cells must be engineered to not only sense the extracellular glucose concentration, but also to express a large number of proteins required for regulated secretion via secretory vesicles *in vivo*. The current study has demonstrated that the Melligen cells have the ability to store insulin and secrete it to physiological amounts of glucose. The Melligen cells also retain insulin function even after 10 days of cytokine treatment.

Since beta cells are most likely killed by cytotoxic $CD8^+$ T-cells, that recognize their target through class I HLA molecules presenting short peptides on the cell surface (Roep, 2008; Peakman, 2008), recurrence of the T-cell attack will also need to be considered when implanting into mouse models. As insulin has been identified as a putative auto-antigen in Type 1 diabetes (Eisenbarth & Stegall, 1996) surrogate beta cells such as Melligen cells must be able to maintain regulated insulin production and secretion in the autoimmune environment if they are to ultimately be of therapeutic benefit. Whatever, the cause of the insensitivity of Melligen cells to STZ (Chapter 6), such a feature appears as an additional advantage for transplantation purposes.

Another immunological issue to be considered is allograft rejection. Because they are allogeneic they will need to be encapsulated to prevent transplant rejection. The

decision on the use of autologous versus allogenic cells depends on multiple considerations. The relative vigour of recurring autoimmunity against insulin-producing cells derived from autologous tissues, compared with that against allogenic surrogate beta cells, is unknown (Efrat et al., 2008). Encapsulation of chromaffin cells for chronic pain (Joseph et al., 1994) and genetically engineered immortalised cells for neurodegenerative diseases have been demonstrated experimentally and are being tested clinically (Aebisher et al., 1996; Emerich & Vasconcellos, 2009). The Melligen cells were shown to be successfully encapsulated and continued to store and secrete insulin and remained glucose responsive even after encapsulation for 110 days. These results indicate that the encapsulation technology will successfully allow the transfer of insulin out of the cell without limiting the amount of insulin secreted by the cells.

Encapsulation will also allow the possibility of transplanting the cells into animal models without a xenogeneic reaction. In this technique, cells are surrounded by a permselective synthetic membrane whose pores are suitably sized to allow diffusion of nutrients, neurotransmitters and growth factors, but which restricts the diffusion of the large molecules of the immune system and prevents contact with immunocompetent cells (Kühtreiber & Lanza, 1999). The encapsulation technique therefore allows transplantation of xenogeneic tissue between species as well as retrieval of transplanted cells for further study. This characteristic will be required when reversing diabetes in animal models.

Su et al. (2009) demonstrated that a poly(ethylene glycol)-containing hydrogel network, formed by native chemical ligation and presenting an inhibitory peptide for islet cell surface IL-1 receptor, was able to maintain the viability of encapsulated MIN-6 cells in the presence of a combination of cytokines including IL-1β (5ng/mL), TNF-α (10ng/mL), and INF-γ (25ng/mL) for a maximum of 96h post-encapsulation. In stark contrast, cells encapsulated in unmodified hydrogels were mostly destroyed by cytokines, which diffused into the capsules. At the same time, these peptide-modified hydrogels were able to efficiently protect encapsulated cells against beta-cell specific T-lymphocytes and maintain glucose-stimulated insulin release by the MIN-6 cells. However, after 96 days of encapsulation there was a significant drop in viability and this could have been due to a lack of cell-extracellular matrix interaction (Su et

al., 2009) or hypoxia. With further development, the approach of encapsulating cells and tissues within hydrogels presenting anti-inflammatory agents may represent a new strategy to improve cell and tissue graft function in transplantation and tissue engineering applications. But in the meantime it will be required that an insulin secreting cell is endowed with characteristics that will make it resistant to the effects of cytokines, such as the Melligen cells.

It is a potential problem that the Melligen cells exhibit uncontrolled replication, making them unsuitable for transplantation due to the risk of tumorigenesis. However, containment of cells in microcapsules (as opposed to macrocapsules) has been reported to inhibit cell replication (Bonini et al., 2007). There is also future potential to use conditional gene expression systems allowing reversible regulation, thereby tightly controlling cell numbers. With further improvements in cell encapsulation techniques this could become a realistic therapy for Type I diabetes in the near future.

The concerns about using transformed cells in humans could be addressed by designing ways to eliminate the oncogenes from the cells, for example by using the Cre-loxP DNA recombination, Scribble, Dlg or lgl approach (Hirrlinger et al., 2009; Humbert et al., 2008) and by introduction of suicide mechanisms into the engineered cells, such as the herpes simplex thymidine kinase gene (Ramesh et al., 1998; Bonini et al., 2007), which will allow cell elimination in case of escape from the encapsulation device. Further to this, Huh7ins and Melligen cells have a faster replication rate *in vitro,* compared to the MIN-6 cells. The higher replication rate in the Melligen cells, compared to MIN-6 cells is important because it would allow one to obtain, more rapidly and at lower costs, a large mass of tissue for transplantation in large mammals. Additionally, further studies on the Melligen cells could include the propagation of the cells under selective pressures in tissue culture that could provide an opportunity for increasing their resistance to immune responses by selecting for the most resistant sub populations (Chen et al., 2000), followed by cell encapsulation in semi-permeable membrane devices.

Essentially the Melligen cells are a transformed cell line and while this may actively work against therapeutic applications they are an invaluable resource to elucidate the minimal molecular machinery required to make a surrogate beta cell from a non-beta

cell. Ultimately it would be more desirable if a patient's own primary hepatocyte is engineered to synthesise, store and secrete insulin in a regulated manner while simultaneously resisting the autoimmune attack that eliminated the original beta cell population. The transplant would then be considered syngeneic rather than allogeneic. Despite the limited insulin storage and secretory capacity of Melligen cells compared to beta cells this may not alter the efficiency when implanted. Future *in vivo* studies will determine if the amount of insulin secreted by the cells is sufficient for the reversal of diabetes.

7.2 Conclusion

In conclusion, in the presence of autoimmune cytokines, IFN-γ, TNF-α and IL-1β, MIN-6 cells exhibited diminished viability, insulin secretion, storage and glucose responsiveness. The MIN-6 cells were also susceptible to the STZ treatment. In contrast, the viability, glucose-responsiveness and ability to secrete and store insulin of the Huh7ins and Melligen cells were not adversely affected by pro-inflammatory cytokine treatment or the STZ treatment for an extended period of time. Microarray analysis showed that cytokine treatment for 24h induced several up-regulated genes involved in anti-apoptotic networks in the Melligen cells.

If the recent rapid progress in cell engineering is matched in the coming years by progress in cell protection techniques, one can expect clinical trials in diabetic patients involving an engineered cell replacement strategy in the not too distant future. These cell protection techniques may provide increased protection against the beta cell toxins described in this thesis. The immunological problems are less difficult in Type 2 diabetes, which does not involve autoimmunity. Therefore it is possible that the first clinical trials will be performed in patients with Type 2 diabetes, rather than in those with Type 1 diabetes (Efrat, 2008).

Given the experimental evidence that Melligen cells are able to respond to glucose in the physiological range under the cytokine and STZ treatments, Melligen cells represent ideal surrogate cells for insulin production. With the development of microencapsulation technologies, Melligen cell-mediated insulin production is anticipated to result in tight glucose glycaemic control without the need for insulin injection in Type 1 diabetes. Finally, the results of general resistance of the surrogate

beta cell to beta-cell toxins with differing modes of action offer hope that this cell, or cells created in a similar manner from primary hepatocytes, may be at least partly resistant to the adverse effect of beta-cell toxins involved in autoimmune destruction of the pancreas. This increases the potential of the use of these cells for reversal of diabetes.

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