

**Investigation into the Role of the Innate
Immune System in Pancreatic β -cell Physiology
& Dysfunction**

A thesis submitted for the degree of Ph. D.

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Declaration

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ABSTRACT

Type 2 diabetes mellitus (T2D) is a metabolic disorder of fuel homeostasis characterised by hyperglycaemia and altered lipid metabolism caused by islet β -cells being unable to secrete adequate insulin in response to varying degrees of overnutrition, inactivity, obesity, and insulin resistance. β -cell dysfunction is the hallmark pathophysiological step in the progression of this disease. The worldwide prevalence rates of T2D are rapidly increasing owing to rising levels of obesity and sedentary lifestyles. Studies now point to T2D as being a disorder of the innate immune system as it is highly correlated with obesity and low grade inflammation. Circulating levels of proinflammatory cytokines are used as indicators for diagnosis of T2D. Toll-like receptors (TLRs) are pattern-recognition receptors that play a crucial role in the innate immune system, which detects the presence and the nature of pathogenic microbial infection, and thus provides the first line of host defence. In order to assess the effects of activation of the innate immune system on β -cell function we set up an *in vitro* model where we studied the effects of TLR activation on the insulin secreting β -cell line MIN6. Activation of MIN6 cells with TLR ligands altered not only insulin secretion and synthesis, but also the inflammatory status of the cell. Interestingly TLR4 the receptor for bacterial LPS, and TLR5 the receptor for bacterial flagellin had very different effects on MIN6 cells, with TLR5 activation seeming to play a protective role. Furthermore in order to explore how β -cells interact with macrophage, whose infiltration of the pancreas is a hallmark of T2D, we used an *in vitro* model where we conditioned J774A.1 macrophage with MIN6 β -cell supernatants prior to activation. Conditioned macrophages acquired a regulatory phenotype and were less responsive to TLR activation with decreased production of pro-inflammatory cytokines, chemokines and immune cell surface markers, whilst maintaining their ability to phagocytose. This effect was transient and normal phenotype returned once the macrophages were removed from the β -cell supernatants thus showing that these cells secrete mediators involved in this response. As T2D and low grade inflammation are inextricably linked we used an *in vivo* model of DSS-induced colitis to induce inflammation in the gut and saw increased numbers of macrophage in the pancreas of diseased animals.

Our findings show that TLR activation has not only the ability to alter insulin signalling in MIN6 β -cells, but also their inflammatory status. Of note is the role of TLR5 activation in protecting β -cells. Furthermore we have shown that β -cells can produce factors that change and shape macrophage response, this ability is possibly altered in the setting of T2D. Finally we have provided a mechanistic link between low grade inflammation in the gut and macrophage infiltration of the pancreas.

ABBREVIATIONS

AP-1	activator protein 1
APC	allophycocyanin
BMMØ	bone marrow-derived macrophage
BSA	bovine serum albumin
CD	cluster of differentiation
DAMP	damage associated molecular pattern
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
DSS	dextran sulphate sodium
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
IκB	inhibitor of kappa B
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase

MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MØ	macrophage
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	phycoerythrin
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PRR	pattern recognition receptor
qPCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
SSC	side scatter
T2D	type 2 diabetes
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β

PUBLICATIONS

TLR5 plays a protective role in the insulin secreting pancreatic β -cell line MIN6

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Soluble factors from colonic epithelial cells contribute to gut homeostasis by modulating macrophage phenotype (2014)

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PRESENTATIONS

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Fiona Mc Evoy, Harry Holthofer and Christine E. Loscher

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Chapter 1

General Introduction

1 Introduction

Type 2 Diabetes (T2D) is a multifactorial metabolic disorder characterised by chronic hyper-glycaemia, insulin resistance and a relative insulin secretion deficit. The estimated worldwide prevalence of diabetes was 285 million (6.4%) in 2010, and this value is predicted to rise to around 439 million (7.7%) by 2030 (Shaw et al. 2010). Type 2 Diabetes is the predominant form and accounts for at least 90% of the cases (González et al. 2009). This increase in T2D is inextricably linked to changes towards a western lifestyle (high-energy diets with reduced physical activity) in developing countries and the rise in prevalence of overweight and obesity (Colagiuri 2010). Insulin resistance affects the action of insulin in each of its major target tissues leading to increased circulating fatty acids and the hyper-glycaemia of diabetes. Although obesity often leads to insulin resistance, only a subset of obese, genetically susceptible insulin resistant individuals progress to type-2 diabetes (Kahn et al. 2006). In both animal models and humans with T2D the triggering factor is β -cell failure, which involves a decrease in β -cell mass and deterioration of key β -cell functions such as glucose-stimulated insulin secretion (GSIS). The leading hypothesized mechanisms to explain insulin resistance and islet β -cell dysfunction in T2D have been oxidative stress, endoplasmic reticulum stress, amyloid deposition in the pancreas, ectopic lipid deposition in the muscle, liver and pancreas and lipotoxicity and glucotoxicity. All of these stresses can be caused by over-nutrition, and each of these cellular stresses is also thought to be exacerbated by or associated with inflammation (Donath & Shoelson 2011) giving fuel to the concept that activation of innate immunity may underlie the pathophysiology of T2D.

The immune system is a collection of highly regulated processes designed to promote protective immunity against insults from pathogenic organisms. These highly regulated processes (both adaptive and innate immune systems) encompass both stimulatory and regulatory pathways aimed at turning on and off appropriate responses designed to resolve the infection without causing long-term damage to the host. Regulatory mechanisms have evolved to down-regulate and control the immune response and tissue inflammation, however inflammation sometimes fails to subside and this unresolved inflammation may become chronic. Recently, many notable discoveries have provided evidence to support the concept of immune system involvement in obesity and the development of T2D. Cross-sectional and prospective studies have described elevated circulating levels of acute-phase proteins (such as C-reactive protein (CRP), fibrinogen, haptoglobin) and sialic acid as well as cytokines and chemokines in patients with T2D (Pickup et al. 1997; Herder et al. 2005). Furthermore, elevated levels of IL-1 β , IL-6 and CRP are predictive of T2D (Spranger et al. 2003; Pradhan et al. 2001). Not only have proinflammatory cytokines been shown to have the ability to promote insulin resistance by interfering with insulin signalling (Hotamisligil et al. 1994), they have also been shown to have a direct detrimental effect on β -cell function and viability (Wachlin et al. 2003). Tissue inflammation has also been detected in the islets of patients with T2D, along with increased cytokines and chemokines (Ehnes et al. 2007). Of note, patients with T2D and every animal model of T2D investigated to date, display immune cell infiltration of the islets (Ehnes et al. 2007). Islet tissue sections from patients with T2D also show fibrosis which is found in conjunction with amyloid deposits and this also argues for an inflammatory response in islets as fibrosis is a hallmark symptom of chronic inflammation. A recent report shows that human islet amyloid polypeptide

(IAPP) which itself has been linked to the pathogenesis of T2D induces the secretion of IL-1 β by bone marrow-derived macrophages, suggesting that IAPP may contribute to islet inflammation (Masters et al. 2010). Further evidence for roles of inflammation in T2D comes from clinical trials using either small molecule anti-inflammatory approaches or biological agents that target specific proinflammatory cytokine pathways to improve parameters of glucose control, such as glycated haemoglobin levels (HbA1c). The most promising approaches thus far include the selective blockade of IL-1-Receptor 1 activation with either IL-1R antagonists or specific antibodies (Dinarello et al. 2012), and inhibition of the NF- κ B pathway with salicylate derivatives such as salicylate (Fleischman et al. 2008). Both approaches seem to lower blood glucose levels and improve β -cell secretory function and insulin sensitivity as well as reducing evidence of systemic inflammation. These proof-of-concept studies validate the potential approach of targeting of inflammatory mediators as a treatment for T2D and also support a causative role for the immune system and inflammation in the pathogenesis of this disease.

1.2 The Pancreas

The pancreas is an organ in the digestive and endocrine system of vertebrates. The mature pancreas is composed of morphologically and functionally distinct endocrine and exocrine components. The exocrine region is formed by acinar and ductal cells, making up to 95–99% of the pancreas. They produce digestive enzymes such as bile, and electrolytes, which help in nutrient digestion and absorption in the gastrointestinal tract. The endocrine cells form aggregates scattered throughout the exocrine pancreas in the form of islets of Langerhans (Morrison 1937). The

endoderm-derived islets are oval in shape, highly vascularized by an extensive endothelial network and innervated by sympathetic, parasympathetic and sensory nerves (Ahrén 2000).

The islets contain five principle endocrine cell types defined by the hormone they secrete. These include insulin-producing β -cells, glucagon-producing α -cells, somatostatin-producing δ -cells, ghrelin-producing ϵ -cells and pancreatic polypeptide-producing PP-cells (Brissova et al. 2005). In rodents, each islet is made up of 70–80% β -cells, 5% δ -cells and 15–20% α -cells or PP-cells in the dorsal or ventral pancreas respectively (Elayat et al. 1995). However, studies on human islets have shown that this proportion of β -cells and non- β -cells is different from that in rodents, and that β -cells constitute approximately 55% of the islet cell mass and α -cells up to 35% (Brissova et al. 2005). The secretion of islet hormones is highly regulated and collectively maintains glucose homeostasis through actions on many peripheral tissues such as liver, muscle and adipose tissue. Non endocrine cells are also part of an islet. These include nerves, dendritic cells, macrophages, fibroblasts and vascular cells such as endothelial cells (Eberhard & Lammert 2009).

1.3 Insulin

Pancreatic β -cells in the islet of Langerhans are the sole source of insulin, the main hormonal regulator of glycaemia, thus the β -cell is a metabolic hub connecting nutrient metabolism and the endocrine system. Insulin is a small protein of approximately 5.8 kDa, the monomeric structure of which was first discovered by x-ray crystallography in 1926 (Scott 1934). The monomeric insulin consists of the 21 amino acid residue “A” chain and 30 amino acid residue “B” chain. The two chains

are bound together in their quaternary structure by two disulfide bridges. The secondary structure of both chains is mostly alpha helical. Insulin is created as a single peptide chain, which is looped around, bonded to itself by covalent disulfide bonds between cysteine residues (Brange & Langkjoer 1993). In addition, a third disulfide bridge between two amino acids in the “A” chain helps stabilize its tertiary structure (Brange & Langkjoer 1993). Humans have a single copy of the insulin gene, but rodents have two non-allelic insulin genes (insulin I and II). They differ in their number of introns and chromosomal locations (Soares et al. 1985).

1.3.1 Insulin Biosynthesis

The secreted insulin consists of 51 amino acids with a molecular weight of 5.8 kDa however the *insulin* gene encodes a 110 amino acid precursor known as preproinsulin. Cytosolic ribonucleoprotein signal recognition particles (SRP) facilitate preproinsulin translocation across the rough endoplasmic reticulum (ER) membrane into the lumen. This process occurs via the peptide-conducting channel (Chan et al. 1976), where the signal peptide from preproinsulin is cleaved by a signal peptidase to yield proinsulin (Patzelt et al. 1978). Proinsulin then undergoes folding and formation of three disulfide bonds (Huang & Arvan 1995), the folded proinsulin is then transported from the ER to the Golgi apparatus where proinsulin enters immature secretory vesicles and is cleaved to yield insulin and C-peptide. Insulin and C-peptide are then stored in these secretory granules together with islet amyloid polypeptide and less abundant β -cell secretory products (Steiner 2008). Although controlled by multiple factors, glucose metabolism is the most important physiological event that stimulates insulin gene transcription and mRNA translation

(Steiner 2008). Insulin biosynthesis is regulated at both transcriptional and translational levels. In a mouse β -cell there are approximately 13,000 insulin granules (Leiter et al. 1979) however this content is known to be highly dynamic. Insulin accumulates in the presence of nutrients and decreases in response to nutrient depletion. The ability of β -cells to quickly respond to cellular signals is due to transcriptional regulation. A number of sequence elements within the promoter region of the insulin gene, named A, C, E, Z, and CRE elements determine the localization of insulin within β -cells and also serve as binding sites for several β -cell transcription factors to regulate insulin gene expression (Hay & Docherty 2006). An example of one such transcription is pancreatic duodenal homeobox 1 (PDX-1) which binds to the A element. Not only is it a key regulator of insulin gene expression it also plays a central role in pancreatic β -cell development, function and survival (Fujimoto & Polonsky 2009). Complete deficiency of PDX-1 is associated with pancreatic agenesis (Jonsson et al. 1994) and partial deficiency leads to severe β -cell dysfunction, and increases β -cell death and diabetes both in rodent and human (Stoffers et al. 1998; Hani et al. 1999; W M Macfarlane et al. 1999). Chronic hyperglycaemia and dyslipidaemia, which are major features of T2D, cause β -cell dysfunction via reduced PDX-1 expression (W M Macfarlane et al. 1999).

1.3.2 Regulation of Insulin Secretion

Insulin is a crucial hormone required for normal metabolism. In healthy subjects, insulin release is exquisitely exact in order to meet the metabolic demand. Specifically, β -cells sense changes in plasma glucose concentration and respond by releasing corresponding amounts of insulin.

1.3.2.1 Carbohydrate Metabolism and Insulin Secretion

Insulin can respond to many nutrients in the blood circulation, including glucose, other monosaccharides, amino acids, and fatty acids, however glucose is evolutionarily the primary stimuli for insulin release, because it is a principal food component and can accumulate immediately after food ingestion and thus will be the focus of this section. The β -cell glucose transporter (GLUT1 (SCL2A1) in humans and GLUT2 in rodents) senses the glucose concentration and transports it into the β -cell (De Vos et al. 1995; McCulloch et al. 2011; Rorsman & Braun 2013). The K_m of these GLUT1 and GLUT2 transporters for glucose is high (6 and 11 mM respectively), indicating that they are only active at high extracellular glucose, as observed in postprandial conditions (Newsholme et al. 2014). Following uptake glucose is phosphorylated into glucose-6-phosphate by the high K_m glucokinase (GK) which constitutes the flux determining step for glycolysis (Nilsson et al. 1996; Newgard & McGarry 1995). Unlike other hexokinases, GK is not inhibited by its product glucose-6-phosphate and maintains high glycolytic flux in the presence of elevated glucose, coupling carbohydrate sensing to insulin secretion in the β -cell (Newsholme et al. 2014). Alterations in the activity of important glycolytic enzymes such as GK can modulate GSIS, and thus can lead to impaired glucose metabolism and insulin secretion (Gloyn et al. 2005; Osbak et al. 2009).

Following glucose uptake, glycolytic degradation to pyruvate generates ATP which is an important stimulus-secretion coupling factor. Pyruvate sits at a critical branching point in glucose metabolism in β -cells as it can be metabolized in the cytosol by lactate dehydrogenase (LDH) or enter mitochondria to be metabolized by pyruvate dehydrogenase (PDH) or pyruvate carboxylase (PC) (Komatsu et al. 2013). As pancreatic β -cells express low levels of LDH it is not thought to play a role in

GSIS (Sekine et al. 1994; Patterson et al. 2014). PC facilitates the production of oxaloacetate from pyruvate. Oxaloacetate is then converted to malate by mitochondrial malate dehydrogenase and enters the cytosol from the mitochondria. Malic enzyme 1 (ME1) regenerates pyruvate from malate while simultaneously creating NADPH and increasing ATP levels. Pyruvate can then re-enter the mitochondria to continue the process, generating more NADH and increasing ATP levels (Jitrapakdee et al. 2010; Newsholme et al. 2014). The metabolism of pyruvate by pyruvate dehydrogenase leads to the generation of acetyl CoA which can then condense with oxaloacetate to form citrate, activating the tricarboxylic acid (TCA) cycle allowing it to be transferred from the mitochondria to the cytosol via the citrate carrier. Citrate is then converted back to acetyl CoA and oxaloacetate and ME1 generates NADPH and ATP as described above (Newsholme et al. 2014; Maechler 2013). Unsurprisingly, studies have shown that siRNA knockdown of PC in murine models reduces β -cell GSIS and proliferation (Patterson et al. 2014; Hasan et al. 2008) while enhanced expression increases GSIS (Xu et al. 2008).

The increased production of ATP increases the intracellular ratio of ATP/ADP (Ghosh et al. 1991). ATP binds ATP sensitive K^+ channels (K_{ATP}) channels on the β -cell membrane, probably concomitant with phosphorylation of K_{ATP} channel, closes the channels. As the K_{ATP} channel is the primary determinant of the membrane potential of the β -cells, closure of these channels results in depolarization of the β -cell membrane (Tarasov et al. 2004). When the β -cell membrane potential reaches -30 to -40 mV, the voltage-sensitive calcium channels open leading to an influx of calcium ions. This increase of intracellular calcium ions triggers the exocytosis of insulin granules (Ohara-Imaizumi & Nagamatsu 2006). In addition, increased Ca^{2+} then activates voltage-sensitive K^+ channels, thereby restoring the resting potential to

-70 to -60 mV and increasing the concentration of cytoplasmic free calcium (Ohara-Imaizumi & Nagamatsu 2006). Inagaki *et al* first identified the K_{ATP} channel in β -cells as a tetra-octamer composed of four sulfonylurea receptor 1 (SUR1) and inwardly rectifying K^+ channel 6.2 subunits (Kir6.2) (Inagaki et al. 1995; Inagaki et al. 1997). SUR1 is the target of insulin secretagogues, such as sulfonylurea (SU) and glinide used in the treatment of T2D, such that the channel is closed on binding of SU or glinide to the SUR1, enhancing insulin secretion. An acute elevation of intracellular Ca^{2+} facilitates fusion of a ready releasable pool of insulin granules and the plasma membrane leading to an increased rate of exocytosis. This reaction is mediated by the assembly of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Rorsman & Renström 2003).

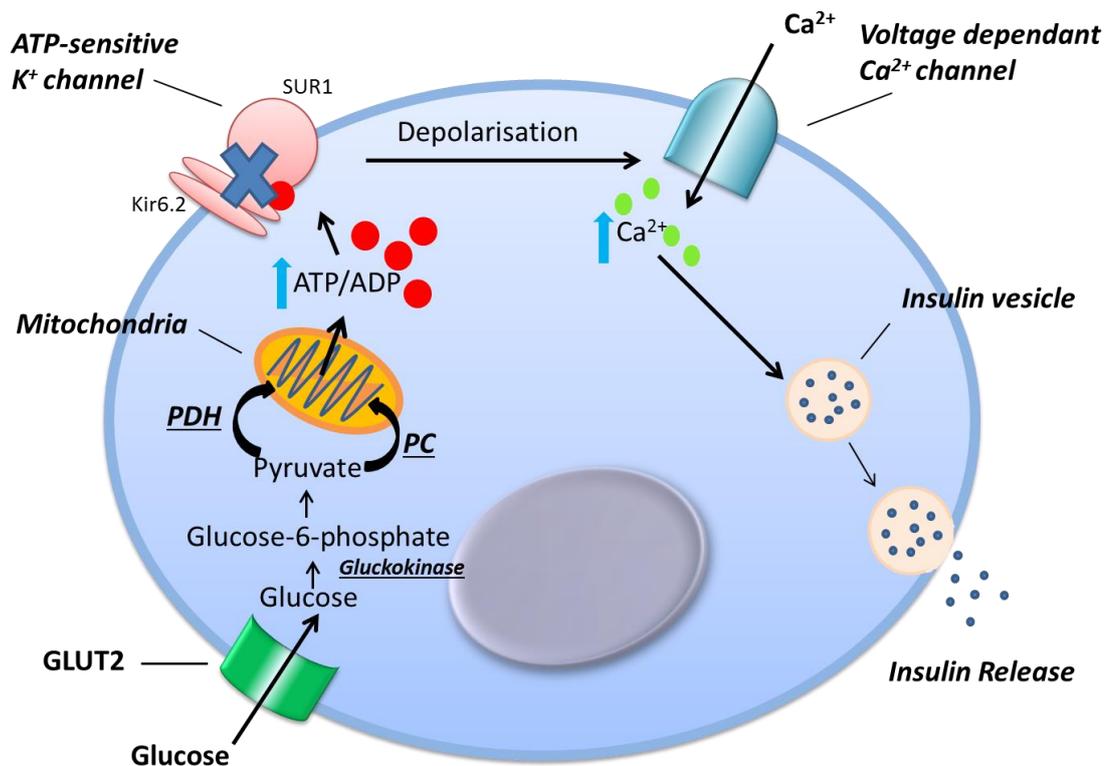


Figure 1.1 Mechanism of glucose stimulated insulin secretion from pancreatic β -cells.

1.3.2.2 Biphasic Insulin Release and K_{ATP} Channel Independent Glucose Action

Insulin secretion occurs in pulsatile fashion in sync with Ca^{2+} influxes during two major phases, and is termed “biphasic.” First-phase insulin secretion occurs within 5–10 min following β -cell stimulation. Second-phase insulin secretion is less robust than the first phase, but can be sustained for several hours if elevated blood-glucose levels persist (Henquin, Nenquin, et al. 2006; Henquin, Dufrane, et al. 2006; Curry et al. 1968). These two phases of secretion are thought to use separate pools of insulin-containing granules. First-phase secretion appears to arise from plasma membrane-pre-docked granules, termed the “readily releasable pool” (RRP), while second-phase secretion is believed to involve release from a granule pool deeper within the cell, the “storage-granule pool,” which presumably replenishes the RRP (Barg et al. 2002). The triggering mechanism of K_{ATP} -dependant GSIS is responsible for the first phase of the insulin secretory response, however the second more sustained phase of insulin release is dependent on metabolic stimulus-secretion coupling as it was shown when K_{ATP} channels were prevented from closing by the addition of diazoxide, in the presence of glucose insulin release was still possible (Gembal et al. 1992; Sato et al. 1992). Although molecule(s) responsible for this so termed K_{ATP} -independent mechanism of GSIS have yet to be identified, anaplerotic metabolism of pyruvate by PC and subsequent efflux of the TCA cycle intermediates are considered key events (Newsholme et al. 2014; Komatsu et al. 2013; Brun et al. 1996)

1.3.2.3 The Role of Gastrointestinal Hormones in Insulin Secretion

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) are gastrointestinal hormones called incretins, which robustly enhance nutrient-induced insulin secretion (Drucker 2006). The effect was first observed when insulin secretion increased more after oral glucose administration than after intravenous infusion of an equivalent glucose dose, while maintaining stable plasma glucose levels (Holst & Gromada 2004). Incretins bind to G protein-coupled receptors on the β -cell membrane and increase cAMP levels. When the β -cells are exposed to a stimulatory concentration of glucose, cAMP further elevates GSIS (Holst & Gromada 2004). Incretin action is resistant to diazoxide, and therefore it is independent of K_{ATP} channel closure (Yajima et al. 1999). cAMP is thought to enhance GSIS through protein kinase A (PKA)-dependent and -independent mechanisms (Seino & Shibasaki 2005). cAMP leads to an increase in the size of RRP in a glucose concentration-dependent manner. This finding strongly indicates that incretins prime the β -cells in the presence of stimulatory ambient glucose concentration. The discovery of the insulintropic effect of these hormones has led to their evaluation as therapies for improved insulin secretion in T2D with promising results to date (Puddu et al. 2013; Erlich et al. 2013). Along with their use as potential therapeutics, the action of these hormones has been shown to be improved following bariatric surgery, which has the ability to induce rapid resolution of T2D, prior to any significant weight loss further supporting a positive role for these hormones in insulin action (Colagiuri 2010; Sala et al. 2014).

1.3.3 Insulin Signalling

Insulin signalling is mediated by a complex, highly integrated network that controls several processes. In the presence of insulin, the insulin receptor (IR) phosphorylates insulin receptor substrate proteins (IRS proteins) that are linked to the activation of two main signalling pathways: the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin, and the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation (Taniguchi et al. 2006). These pathways and the effectors involved will be discussed in further detail later.

1.3.4 Insulin Function

The biological effects of insulin on the integration of fuel metabolism are primarily the uptake of glucose and fatty acids into liver, adipose tissue and muscle and to promote the storage of these nutrients in the form of glycogen, lipids and protein respectively.

1.3.4.1 Glucose storage and uptake

Once in the blood, insulin controls glucose homeostasis by stimulating the uptake of glucose into skeletal muscle, and to a lesser extent the liver and adipose tissue. In skeletal muscle, heart muscle and adipose tissue, this uptake is facilitated by the glucose transporter GLUT4 (Bryant et al. 2002). Once glucose enters the cells it is phosphorylated by hexokinase to glucose-6-phosphate. Glucose-6-phosphate then undergoes glycolysis for energy production or it is stored as glycogen. In addition to

promoting glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis (Saltiel & Kahn 2001).

1.3.4.2 Protein synthesis

Insulin can stimulate protein synthesis in many types of cells and tissues, including human and rat muscles, and many types of cells in culture (Garlick 2005; Proud & Denton 1997). This involves two major kinds of effects: the rapid activation of existing components of the translational apparatus and the longer term increase in the capacity of the cell or tissue for protein synthesis, which includes an increase in ribosome number.

1.3.4.3 Lipid Metabolism

Insulin also promotes the synthesis of lipids, and inhibits their degradation (Saltiel & Kahn 2001). In adipocytes, glucose is stored primarily as lipid, owing to increased uptake of glucose and activation of lipid synthetic enzymes, including pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase. Insulin also profoundly inhibits lipolysis in adipocytes, primarily through inhibition of the enzyme hormone-sensitive lipase (Anthonsen et al. 1998).

1.3.4.4 Autocrine action on β -cell physiology and function

Along with its actions in target tissues, insulin has also been shown to have an autocrine action on β -cell physiology and function (Leibiger et al. 2008). Unlike all other cell types the pancreatic β -cell is unique in that it continuously secretes insulin; basal secretion under nonstimulatory conditions and increased secretion under stimulatory conditions such as high glucose. Although historically insulin was exclusively thought to exert a negative effect on insulin signalling in the β -cell (Best

& Haist 1941), recent data has shown that it plays a positive role in several cellular processes that include regulation of gene transcription, translation, proliferation and β -cell survival (Leibiger et al. 2008). The presence of the IR has been documented in β -cell lines of different origin (HIT-T15, INS1, MIN6) as well as in primary mouse and human β -cells (Hribal et al. 2003; Leibiger et al. 2001; Muller et al. 2006). Knockout of the IR in β -cells of the β IRKO mice, and ablation of IRS2 signalling in β -cells results in an age dependant decrease in β -cell mass (Kulkarni et al. 1999; Cantley et al. 2007). Exposure of islets from β IRKO mice to elevated glucose concentrations or exogenous insulin does not result in the upregulation of endogenous insulin gene transcription, suggesting binding of insulin to the IR is crucial for this process (Kulkarni et al. 1999). Furthermore gene expression profiling of MIN6 cells has shown that most genes that seem to be regulated by glucose were in fact regulated by secreted insulin (Ohsugi et al. 2004; Ohsugi et al. 2005). This data and others point to the pivotal role for autocrine insulin signalling in the β -cell.

1.4 Type 2 Diabetes

As mentioned previously Type 2 Diabetes is a multifactorial metabolic disorder. This disease is often associated with obesity and develops when chronic overnutrition conspires with genetic susceptibility to cause impaired insulin signalling, also dubbed insulin resistance, as well as a relative insulin deficiency of non-autoimmune aetiology. This contrasts with Type 1 diabetes, which is caused by the complete absence of insulin secondary to autoimmune destruction of the pancreatic β -cells. Due to the importance of this hormone in maintaining whole body fuel homeostasis, compromised insulin secretion and action therefore leads to multiple metabolic

abnormalities. These include hyperglycaemia due to impaired insulin stimulated glucose uptake and uncontrolled hepatic glucose production, and dyslipidemia, which includes perturbed homeostasis of fatty acids, triglycerides and lipoproteins. These chronic increases in circulating glucose and lipid levels can further impair insulin secretion and action and cause other forms of tissue damage thus termed glucotoxicity and lipotoxicity. A number of clinical data points support insulin resistance as a critically important etiological factor in Type 2 diabetes. T2D and insulin resistance are highly correlated; more than 80% of individuals with T2D also manifest insulin resistance (Gerich 1999). Insulin resistance is also common in obese individuals who have not yet developed T2D (Balkau et al. 2002). Insulin resistance can in turn cause β -cell dysfunction via the mechanism of β -cell exhaustion such that increased secretory demand, due to peripheral tissue resistance to insulin signalling results in continual β -cell hyperstimulation and eventual failure (Kahn 2001). While T2D is indeed strongly correlated with insulin resistance, insulin resistance is not as strongly correlated with T2D. That is to say prevalence rates of insulin resistance in various populations are often far greater than those of T2D, large majorities of individuals with insulin resistance do not go on to develop the disease (Bonora et al. 1998; Zavaroni et al. 1999). A clinical assessment of β -cell function and insulin resistance in 100 glucose tolerant volunteers – 50 first-degree relatives of patients with T2D and 50 controls noted significant reductions in insulin secretion in the first-degree relatives versus controls (Pimenta et al. 1995). Neither group differed in insulin sensitivity, but the first-degree relatives showed a clear decrement in β -cell response. Not only may β -cell dysfunction pathogenically precede insulin resistance, but there is also some evidence that the former may contribute to the latter, loss of

pulsatile insulin release, a β -cell secretory dysfunction associated with T2D has been associated with development of insulin resistance (Kahn 2001).

1.4.1 β -cell dysfunction in Type 2 Diabetes

Chronic insulin resistance will progress to T2D when the β -cells are unable to secrete adequate amounts of insulin to compensate for decreased insulin sensitivity which is largely due to insulin secretory dysfunction and loss of functional β -cells (Marchetti et al. 2004). Individuals with T2D always manifest increased β -cell apoptosis and reduced β -cell mass (Sakuraba et al. 2002; Marchetti et al. 2004). Although obesity is associated with T2D, most obese individuals do not develop the disease due to enhanced function on β -cells or expansion of β -cell mass compensates and thus maintains normal blood glucose levels (Flier et al. 2001). This enhanced functionality involves increased nutrient signals that stimulate increased growth factor signalling in β -cells. Increased nutrient load in the gut can enhance GLP-1 production leading to anti-apoptotic and growth promoting effects on β -cells (van Citters et al. 2002; Drucker 2006). In susceptible individuals the β -cells lose their ability to compensate and cell dysfunction ensues. Generally, diagnosis of T2D is associated with approximately a 50% reduction in islet function and this is thought to manifest itself at least 10 years prior to disease diagnosis (Holman 1998). Obese non-diabetic humans show increased β -cell volume in islets while obese and non-obese diabetic individuals with T2D show at least a 40% reduction in β -cell volume compared to non-diabetic controls (Butler et al. 2003). As discussed above the β -cell is a target for insulin action, with insulin effects on transcription, translation, cell proliferation, cell size, and β -cell apoptosis (Leibiger et al. 2008) As a consequence,

mechanisms leading to insulin resistance in the classical insulin-target tissues, namely liver, muscle and fat, should also affect β -cell function and survival, i.e., insulin resistance is also a feature of the pancreatic β -cell. An increasing body of data documents that mechanisms that lead to insulin resistance in the classical insulin-target tissues also cause β -cell insulin resistance coupled with β -cell dysfunction and apoptosis (D'Alessandris et al. 2004; Marchetti et al. 2004; Okada et al. 2007; Solinas et al. 2006). These data suggest that β -cell insulin resistance may add to the deterioration of β -cell function and therefore accelerate the progression of the disease. For these reasons the factors that lead to a change in β -cell function (decreased insulin expression and secretion), mass and insulin resistance are central to the pathology of T2D.

1.5 Inflammatory mechanisms contributing to β -cell dysfunction

Multiple mechanisms underlie defective insulin secretion and responses in T2D. These include glucotoxicity, lipotoxicity, oxidative stress, endoplasmic reticulum (ER) stress, alterations of the gut microbiota, and the formation of amyloid deposits in the islets (Bensellam et al. 2012; Lee et al. 1994; Laybutt et al. 2007; Evans et al. 2002; Westermark et al. 2011; Carvalho & Saad 2013). The relative contribution of each of these mechanisms remains unclear. They likely all participate in the pathology of the disease, with inter-individual differences depending on genetic background, nutrition, physical activity, the use of antibiotics and other

environmental factors. Interestingly, all of these mechanisms are associated with inflammatory responses.

1.5.1 Glucotoxicity, Oxidative Stress, ER stress and Lipotoxicity.

Understandably, excessive glucose will lead to an increase in β -cell glucose metabolism and oxidative phosphorylation. Elevated electron transport chain activity is known to promote increased superoxide (O_2^-) anion leakage from the mitochondria and may cause oxidative cellular damage (Newsholme et al. 2007). Furthermore high glucose levels induce NOX activity via NADPH production from the metabolism of glucose to pentose, and through the TCA cycle, both leading to increased O_2^- output (Newsholme et al. 2007). β -cells are particularly vulnerable to ROS generation as they express relatively low levels of antioxidant enzymes such as catalase and glutathione (Gehrmann et al. 2010). For this reason the detrimental combination of increased ROS generation coupled with low antioxidant enzyme production can result in oxidative damage to DNA, proteins and lipids thereby promoting mitochondrial mediated apoptosis. Further to this ROS also have the capability of activating cellular stress pathways, such as NF κ B (Shimada et al. 2012) leading to further negative effects for the β -cell. Excessive glucose can also lead to increased intracellular calcium levels enhancing mitochondrial O_2^- production, but also deplete ER Ca^{2+} thereby promoting activation of the ER stress mediated cell death pathway (Laybutt et al. 2007) along with the unfolded protein response (UPR). Accumulation of unfolded proteins within the lumen causes the initiation of an acute phase inflammatory response (Bensellam et al. 2012) and can ultimately promote cell death by the activation of caspase enzymes (Szegezdi et al. 2006).

Lipid accumulation often termed lipotoxicity within the ER can also mediate ER stress in β -cells. As mentioned previously obesity is a primary risk factor associated with T2D. The process by which free fatty acids accumulate within the ER is not entirely understood, however it has been shown that palmitic acid has the ability to deplete ER Ca^{2+} levels and augment ER morphology and integrity which may lead to activation of ER stress by the mechanisms mentioned above (Cnop 2008).

1.5.2 Cytokines and Chemokines

Cytokines and chemokines, small signalling proteins secreted primarily by immune cells, activate inter- and intracellular signalling during immune responses. Cytokine expression plays a fundamental role in the development and function of the immune system. Many different cell types secrete chemokines, most often to attract immune cells to the site of infection or injury during innate and adaptive immune responses (Luster 1998). The effect of a cytokine release depends on the activated cell type expressing the specific cytokine receptor. Cytokines and their receptors participate in a diverse array of functions beyond innate and adaptive immunity including inflammation, immune cell differentiation, angiogenesis, tumorigenesis, development, neurobiology, and viral pathogenesis (Cohen & Cohen 1996). Dysregulation of cytokine expression is a cause of immunological and inflammatory diseases as well as other disease states (Tracey & Cerami 1993; Finkel et al. 1992; Rogler & Andus 1998).

1.5.2.1 Cytokines in β -cell dysfunction

Many studies have been conducted in order to investigate the relationship between various inflammatory mediators and T2D and have found abnormally high levels of various cytokines, plasminogen activator inhibitor, chemokines and acute phase proteins in type 2 diabetic patients (Herder et al. 2006; Spranger et al. 2003). Indeed high circulating levels of IL-1 β , IL-6 and CRP can be the main predictive indicators for progression of T2D (Spranger et al. 2003; Pradhan et al. 2001). Numerous proinflammatory cytokines such as IL-1 β , TNF- α and IL-6, that are released from adipose tissue macrophage during obesity have been shown to induce inflammation in pancreatic islets (Zhao et al. 2006). The earliest evidence for an inflammatory process in pancreatic islets arose from the observation that hyperglycaemia induces β -cell apoptosis (Donath et al. 1999). By examining the underlying mechanism, it was shown that high glucose concentrations induce the expression of the proapoptotic receptor FAS on β -cells (Maedler et al. 2001), which is further upregulated by glucose induced IL-1 β production in β -cells (Maedler et al. 2002). Therefore IL-1 β and FAS contribute to both the glucose-induced impairment of β -cell secretory function and apoptosis. Besides glucose induced stimulation of IL-1 β , expression of IL-1 β in pancreatic β -cells is also regulated by free fatty acids (FFAs) that are also increased in obesity. FFAs stimulate the production of IL-1 β and IL-1 dependant proinflammatory cytokines including IL-6 (Böni-Schnetzler et al. 2008; Böni-Schnetzler et al. 2009). Deposition of amyloid in islets, a hallmark of T2D also has been shown to contribute to β -cell IL-1 β production (Masters et al. 2010).

1.5.2.2 Cytokine induced insulin resistance

Other than IL-1 β , both TNF- α and IL-6 also provide a link between inflammation, insulin resistance and obesity. Both of these cytokines have been shown to play a role in driving insulin resistance in peripheral tissues and β -cells (Hotamisligil et al. 1994; Pradhan et al. 2001). Engagement of the TNF receptor by TNF- α induces inhibitory phosphorylation of serine residues of IRS1 and activates IKK β /NF- κ B and JNK pathways, two major intracellular regulators of insulin resistance (Tilg & Moschen 2008). IL-6 can induce SOCS1 and SOCS3 that link IRS proteins to ubiquitin-mediated degradation which also promotes insulin resistance (Ueki et al. 2004). IL-1 β has been shown to reduce IRS1 expression via ERK1/2 and can also activate the IKK β /NF- κ B pathway to promote insulin resistance (Tilg & Moschen 2008).

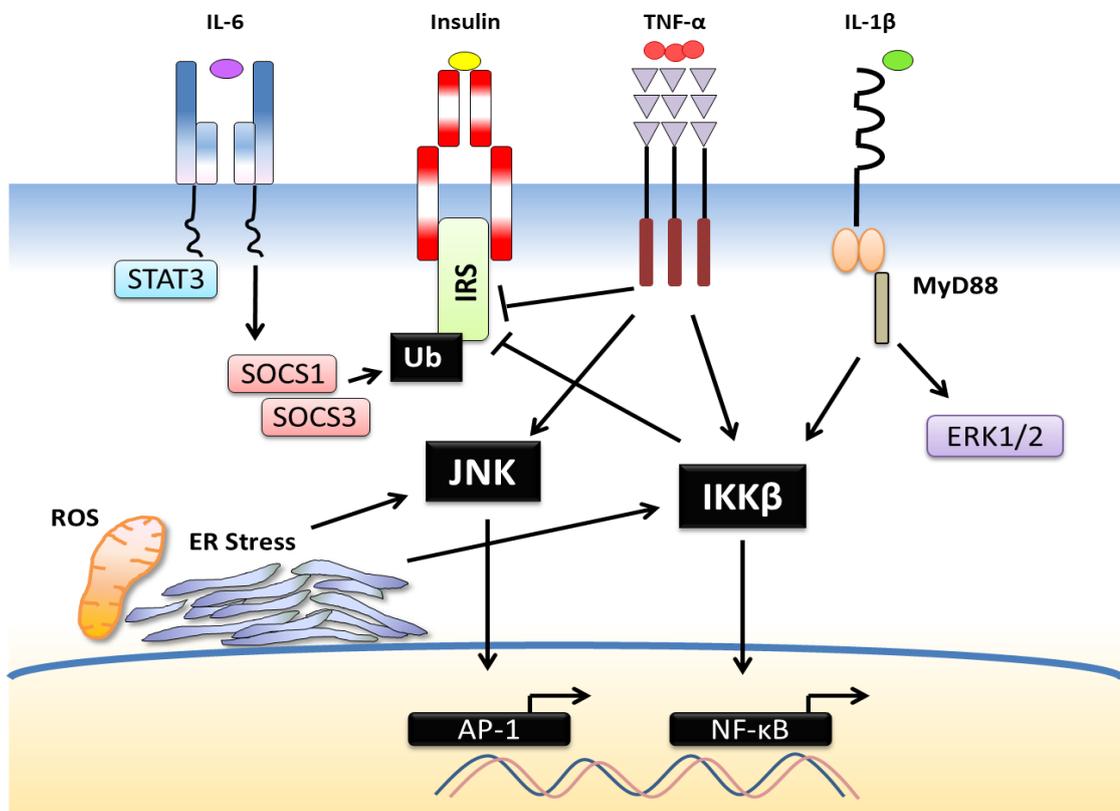


Figure 1.2 Regulation of Insulin Resistance by Cytokines.

1.5.2.3 Chemokines in β -cell dysfunction

Islet cells can also produce a wide range of chemokines in the context of T2D. *In vitro* treatment of islets with high concentrations of glucose and the saturated fatty acid palmitate increases the production of several biologically active chemotactic factors (CXC chemokine ligand 8 (CXCL8) and MCP-1 in human islets; CXCL1 in mouse islets) (Ehse et al. 2007; Boni-Schnetzler et al. 2009). Islets isolated from rodent models of T2D (Goto–Kakizaki rats, high-fat fed mice and Zucker rats) also show increased production of various chemokines, including CXCL1, MCP-1 and MIP-1 α (J. A. Ehse et al. 2009). These findings for humans is supported by studies that have shown upregulation of various chemokines in laser captured nearly pure β -cells from patients with T2D (Donath et al. 2010). The precise functions of the various chemokines remain to be clarified; however, they have a crucial role in tissue infiltration by immune cells in T2D.

1.5.3 Toll-Like Receptors

Toll-like receptors (TLRs) are pattern-recognition receptors that play a crucial role in the innate immune system, which detects the presence and the nature of pathogenic microbial infection, and thus provides the first line of host defence. TLRs, of which 12 members have been identified to date in mammals, comprise a family of type I transmembrane receptors, which are characterized by an extracellular leucine rich repeat (LRR) domain and an intracellular Toll/IL1 receptor (TIR) domain, which shows homology with that of the IL-1 receptor (Kumar et al. 2009; Kawai & Akira 2009). In mammals, ligand recognition by TLRs leads to the recruitment of various TIR domain-containing signalling adaptors such as myeloid differentiation factor

(MyD)88 and TIR domain-containing adaptor inducing interferons (TRIF) which categorize the TLR signalling pathway into MyD88-dependent and TRIF-dependent pathways (Kawai & Akira 2009). This recruitment of adaptors triggers a signalling cascade and ultimately the activation of transcription factors such as NF- κ B and MAP kinases such as p38, JNKs and extracellular signal-regulated kinase (ERK)1/2. These transcription factors induce transcription of proinflammatory cytokines and type I interferons (Kawai & Akira 2007).

1.5.3.1 Role of TLR2 and TLR4 in the β -cell

As T2D and obesity is associated with increased circulating levels of FFA's, it is unsurprising that the majority of research to date has focused on a role for TLR4 and TLR2 in β -cell dysfunction. TLR2 forms heterodimers with TLR1 or TLR6 for ligand recognition and its prototypical ligands are bacterial lipopeptides, while TLR4 forms homodimers and is the classical receptor for bacterial lipopolysaccharides (LPS) (Kawai & Akira 2009). Both TLR2- and TLR4-activating pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) are increased in the circulation of recently diagnosed T2D patients (Dasu et al. 2010) and endotoxemia is a characteristic of humans and rodents with T2D (Cani et al. 2007) implicating these receptors in the pathophysiology of T2D. Expression of these TLRs in islets has been investigated at the mRNA and protein levels in various studies. TLRs 2 and 4 were readily detectable in mouse, rat and human islets (Wen et al. 2004; Amyot et al. 2012) and studies have reported TLR2 and TLR4 mRNA expression in rat INS-1 and BRIN-BD11 and mouse MIN6 β -cell lines (Eguchi et al. 2011; Lee 2011; Kiely et al. 2009). TLR4 and CD14 (which acts as a co-receptor for

bacterial LPS) mRNA and protein expression levels have also been found in rat and human β -cells (Vives-Pi et al. 2003; Garay-malpartida et al. 2011). There is little reported about the effects of TLR2 signalling in β -cells on islet inflammation and β -cell function. In contrast TLR4 activation has consistently been reported to inhibit chronic insulin secretion from the rat islets, (Osterbye et al. 2010) the rat BRIN-BD11 β -cell line, (Kiely et al. 2009) and purified human β -cells (Garay-malpartida et al. 2011). Some studies have also reported that LPS can reduce acute glucose-stimulated insulin secretion in the rat and mouse islets (Vives-Pi et al. 2003; Amyot et al. 2012) likely via reductions in insulin content due to inhibition of insulin gene transcription (Amyot et al. 2012). However, effects of TLR4 signalling on β -cell apoptosis are less consistent with the variable results, possibly owing to the use of different cell lines, mouse models and islets versus purified β -cells (Lee 2011; Kiely et al. 2009; Garay-malpartida et al. 2011; Amyot et al. 2012). Amyot *et al* however have shown that LPS impairs insulin gene expression through activation of NF- κ B as LPS inhibition of gene expression in rat islets was prevented by inhibition of this pathway (Amyot et al. 2012). In addition to gastrointestinal (GI)-derived bacterial TLR2 and TLR4 PAMPs and systemic TLR2 and TLR4 DAMPs, studies have suggested the presence of islet tissue-derived TLR2 and TLR4 ligands. Necrotic β -cell debris has been shown to activate macrophages and dendritic cells via TLR2 (H. S. Kim et al. 2007) Although not investigated as of yet, β -cells undergoing secondary necrosis may also be present in T2D. Recent work on islet amyloid polypeptide also indicates a role for TLR2 in IAPP-induced islet inflammation (Westwell-Roper et al. 2012).

1.5.3.2 Insights from knockout studies

Along with studies showing the direct effects of TLR2 and TLR4 on β -cell function, selective knockout studies have also pointed to a role for these receptors in β -cell physiology and T2D. Both TLR2 and TLR4 deficient mice are protected from insulin resistance and β -cell dysfunction induced by a high-fat diet (Ehnes et al. 2010; Himes & Smith 2010; Li et al. 2013). And in human studies polymorphisms in the TLR4 gene have been found to be protective against the development of T2D (Jiang et al. 2013). This is interesting as it seems to be in direct contrast to observed effects in mice deficient in TLR5 the receptor for bacterial flagellin. Mice deficient in this receptor actually display increased hallmark signs of the metabolic syndrome including hyperlipidemia, hypertension, insulin resistance, and increased adiposity (Vijay-Kumar et al. 2010). Furthermore a nonsense polymorphism in the TLR5 gene in humans actually pre-disposed individuals to the development of T2D (Al-Daghri et al. 2013). This shows that along with their role in β -cell inflammation, some TLRs may also play a homeostatic role in the β -cell. This is further supported by studies investigating TLR adaptor molecules. Mice deficient in the adaptor MyD88, show increased susceptibility to β -cell apoptosis following low dose streptozotocin treatment (Bollyky et al. 2009) and TRIF^{-/-} mice display hyperglycaemia associated with β -cell dysfunction (Hutton et al. 2010).

1.5.4 Islet associated Macrophage

Macrophages are innate immune cells derived from monocytes, which in turn arise from myeloid precursor cells in the bone marrow. Macrophages have many important roles in the innate and adaptive immune response, as well as in tissue

homeostasis. In T1D, immune cells such as T cells and macrophages accumulate within islets, where they produce various cytokines, reactive oxygen species (ROS) and Fas ligand, thereby contributing to β -cell damage (Bending et al. 2012). Within this setting, numerous macrophages are recruited to islets, where their actions have pathogenic effects. However, even under normal physiological conditions macrophages are also constitutively present within islets, where they play important roles during islet development and contribute to the maintenance of islet homeostasis. The presence of macrophages within islets was first reported thirty years ago (Hume et al. 1984). Macrophages are present in mouse islets during embryonic development and *op/op* mice which lack macrophages in many tissues, due to a mutation in colony stimulating factor-1 (CSF-1) including islets, show diminished β -cell mass (Banaei-Bouchareb et al. 2004). The precise mechanism by which macrophages support islet development is not clear, but cytokines produced by macrophages may be involved, as low concentrations of IL-1 β are known to stimulate β -cell proliferation (Maedler et al. 2006).

1.5.4.1 Macrophage polarity in T2D islets

These findings suggest that macrophage may play an important homeostatic role in islets and β -cell physiology however recent studies have shown that they also make a crucial contribution to islet pathology in T2D. Immunohistochemical analysis of pancreas sections from patients with T2D, C57BL/6 mice fed a high-fat diet, *db/db* mice and Goto-Kakizaki (GK) rats all showed elevated numbers of macrophages within islets (Ehnes et al. 2007). In addition, high glucose or palmitate has been shown to induce secretion of chemokines from islets, which promoted monocyte and neutrophil migration (Ehnes et al. 2009; Donath et al. 2010; Boni-Schnetzler et al. 2009). This suggests that the type 2 diabetic milieu may induce chemokine

production and promote macrophage infiltration into pancreatic islets. *In vitro* studies have shown that Th1 cytokines, alone or in concert with microbial products, elicit classical M1 activation of macrophages, while Th2 cytokines (IL-4 and IL-13) elicit an alternative form of activation designated M2 (Gordon & Taylor 2005; Mosser & Edwards 2008). While classically activated M1-type macrophages play a central role in host defence by secreting proinflammatory cytokines and ROS, activated M2-type macrophages promote wound healing and may also modulate immune responses. Eguchi *et al* have reported two sub-populations of macrophage within islets: CD11b⁺Ly-6C⁺ and CD11b⁺Ly-6C⁻ macrophages (Eguchi et al. 2011). They found that under basal conditions, islet-resident macrophages were largely CD11b⁺Ly-6C⁻ cells which exhibit an M2-type phenotype. Fractions of these M2-type cells were not altered in db/db mice, as compared with control db/+ mice however the number of CD11b⁺Ly-6C⁺ macrophages was specifically increased in the T2D models. These cells express inflammatory cytokines, including IL-1 β and TNF- α , and exhibit an M1-type phenotype. Thus macrophage polarity appears to be shifted towards M1 in T2D islets.

1.5.4.2 Macrophage contribution to β -cell dysfunction

TLR activation in macrophages induces NF- κ B and IFR (interferon regulatory factor) driven cytokine induction and ligands activating these receptors may induce pro-inflammatory M1 macrophage in the islets in T2D. Added to this the T2D milieu may induce production of chemokines that promote macrophage infiltration into pancreatic islets (Donath et al. 2010). Inflammasomes are multi-protein complexes important for the maturation and secretion of IL-1 β . To induce IL-1 β secretion, two stimuli are required: the first induces the expression of pro-IL-1 β protein, while the second activates inflammasomes, which in turn activate caspase I to cleave pro-IL-

1 β , yielding mature IL-1 β (Latz 2010). It was recently reported that one of the first stimuli in islets of T2D is minimally modified low density lipoprotein (LDL), which activates TLR4 signalling in macrophages, priming them to process IL-1 β . The second stimulus was identified as IAPP, which is secreted from β -cells in response to high glucose (Masters et al. 2010). A soluble oligomer, IAPP, triggers activation of the NLRP3 inflammasome and induces IL-1 β secretion from the islet macrophages. Thus the interaction between β -cells and macrophages is important for inflammasome activation within islets (Masters et al. 2010). As IL-1 β and other pro-inflammatory cytokines that are secreted by M1 type macrophage have been shown to be detrimental to β -cell function and insulin resistance this provides a mechanistic link between islet macrophage and β -cell dysfunction in the context of T2D (see Figure 1.3). Indeed in a mouse model using infusion of palmitate to induce β -cell dysfunction, increased chemokines and M1 type macrophage were found in the islets and blocking this recruitment of macrophage using clodronate liposomes suppressed β -cell dysfunction (Eguchi et al. 2011). The relationship between macrophage and β -cells in the context of islet inflammation certainly warrants further investigation.

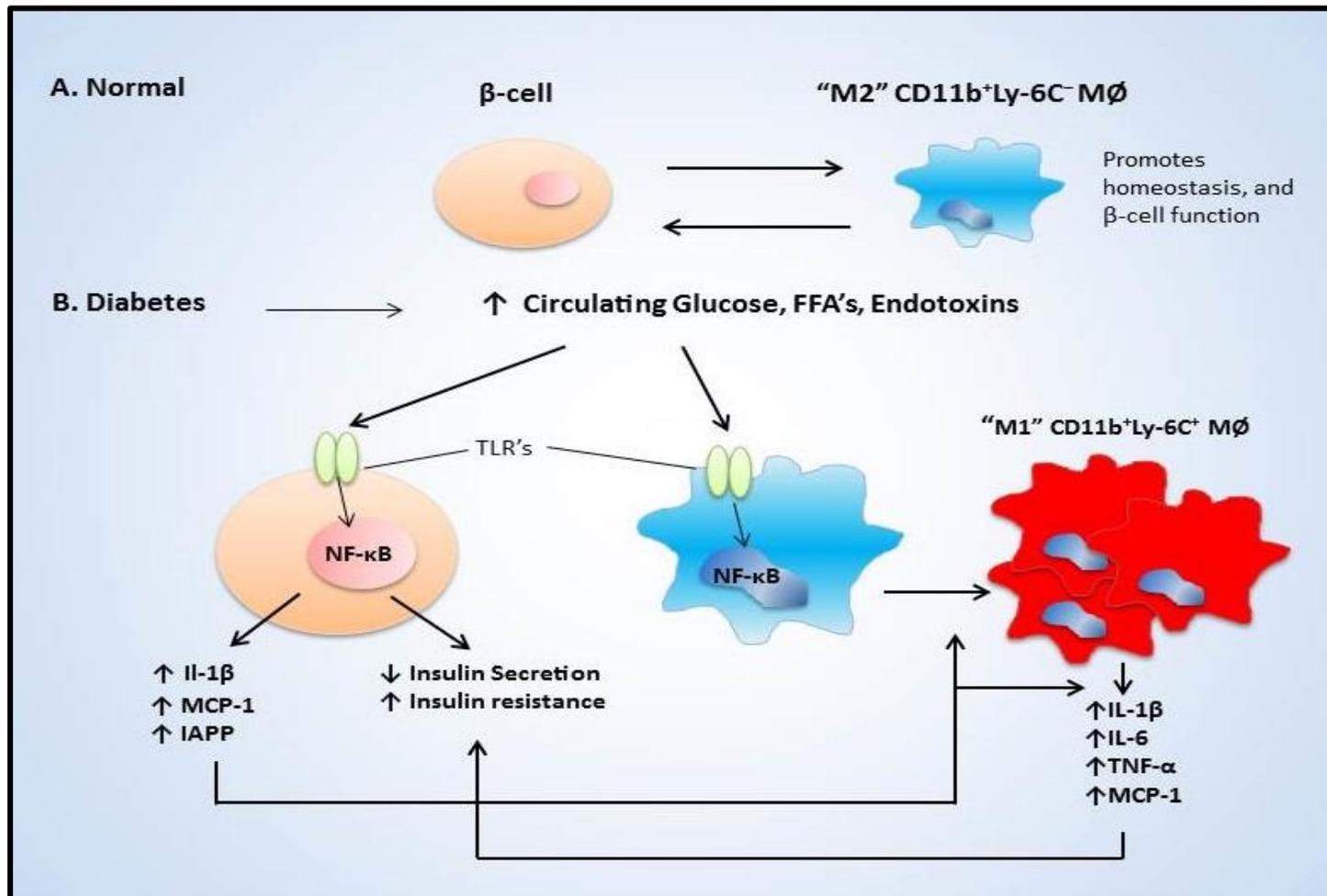


Figure 1.3 Contribution of macrophage to β -cell dysfunction during Type 2 Diabetes.

1.6 Aims and Objectives

There is a growing body of evidence supporting a role for activation of the innate immune system and low grade inflammation in the development and pathogenesis of T2D. Dysfunction of the β -cells, when they can no longer compensate for insulin resistance by increasing their insulin output, is the critical step when insulin resistant individuals develop overt T2D. Recent work has focused on a role for the innate immune system in β -cell physiology and dysfunction. Toll-like receptor ligands both exogenous and endogenous have been shown to be increased in T2D. We wanted to investigate the effect of activation of these receptors on β -cell physiology and function. As is the case with many tissues throughout the body, islets contain resident macrophage, and increased numbers of islet macrophage are associated with and contribute towards type 2 diabetic pathology. We hypothesised that crosstalk between these two cell types must occur, and perhaps is dysregulated during the progression to T2D. We also hypothesised that as T2D and β -cell dysfunction is linked with low grade inflammation, and altered gut homeostasis can contribute to the pathogenesis of T2D, perhaps gut inflammation may provide a source of the infiltrating macrophages seen in diabetic islets.

To test these hypotheses the aims of this thesis were to

- Investigate the effect of TLR activation on pancreatic β -cells *in vitro* using the MIN6 insulin secreting pancreatic β -cell line.
- To examine the crosstalk that occurs between pancreatic β -cells and macrophage with the use of the MIN6 β -cell line and the J774A.1 macrophage cell line.
- To investigate a possible link between gut inflammation and macrophage infiltration of the pancreas with the use of the DSS induced model of colitis.

Chapter 2

Materials and Methods

2.1 Materials

CELL CULTURE MATERIALS/REAGENTS

Material	Source
Foetal Calf Serum	Gibco
DMEM	Sigma-Aldrich
DMEM-Glutamax	Gibco
RPMI-1640	Gibco
PBS	Gibco
Tissue culture flasks T-75cm ²	Nunc
Tissue culture flask T-25cm ²	Nunc
Sterile Petri dishes	Nunc
6, 24 & 96 well tissue culture plates	Nunc
96 well round bottomed plate	Starstedt
Glucose solution	Sigma-Aldrich
Trypan blue (0.4% w/v)	Sigma-Aldrich
Trypsin/EDTA	Sigma-Aldrich
Penicillin Streptomycin/Glutamine	Gibco
CellTiter 96™ Aqueous One Solution	Promega
rmIL-4	R&D Systems
Streptozotocin	Sigma-Aldrich
PAM ₃ CSK ₄	Invivogen
LPS	Invivogen
Flagellin	Invivogen

Griess Reagent	Promega
Exosome Free-FBS	Strattech
rmIL-27p28	R&D Systems
Transwell® Plates	Analab

Table 2.1 Cell Culture Materials. All cell culture materials/reagents used and corresponding sources.

ELISA REAGENTS

Material	Source
96-well microtiter plate	Nunc
TMB	BD
Tween 20	Sigma-Aldrich
Bovine Serum Albumin	Sigma-Aldrich
Sodium Azide (NaN ₃)	Sigma-Aldrich
ELISA DuoSet kits	R&D Systems
Insulin ELISA kit	Mercodia
DPBS	Gibco

Table 2.2 ELISA Reagents. All ELISA reagents used and corresponding sources

FLOW CYTOMETRY REAGENTS

Material	Source
FACS Flow	BD
FACS RINSE	BD

FACS Clean	BD
37% (v/v) paraformaldehyde	Sigma-Aldrich
Sodium Azide	Sigma-Aldrich
EDTA	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Anti-F4/80 antibody	Biolegend
Anti-CD86 antibody	BD
Anti-CD80 antibody	BD
Anti-TLR2 antibody	eBioscience
Anti-TLR4 antibody	eBioscience
Anti-TLR5 antibody	Abcam
Anti-MHCII antibody	eBioscience
Anti-CD40 antibody	BD
Anti-CCR5 antibody	BD
Anti-CD124 antibody	BD
Anti-CD14 antibody	BD
Anti-PDX1 antibody	R&D systems
Anti-Insulin Receptor antibody	R&D systems
DCFDA	Abcam
CompBeads	BD
Fluorescent Latex Beads	Sigma-Aldrich

Table 2.3 Flow Cytometry Reagents. All flow cytometry reagents used and corresponding sources.

RNA ISOLATION AND cDNA SYNTHESIS

Material	Source
Nucleospin RNA II Columns	Macherey-Nagel
DEPC treated water	Invitrogen
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Molecular grade ethanol	Sigma-Aldrich

Table 2.4 RNA isolation and cDNA synthesis materials. All reagents used for RNA isolation and cDNA synthesis and corresponding sources.

qPCR REAGENTS

Material	Source
Taqman [®] Universal Mastermix	Applied Biosystems
SYBR [®] Green Maternix	Roche
Microamp [®] Optical Adhesive film	Applied Biosystems
Microamp [®] Optical 96-well plate	Applied Biosystems
IAPP PrimeTime qPCR Assay	IDT
FAM3B PrimeTime qPCR Assay	IDT
INS1 PrimeTime qPCR Assay	IDT
INS2 PrimeTime qPCR Assay	IDT
B2M PrimeTime qPCR Assay	IDT
IRS1 PrimeTime qPCR Primers	IDT
IRS2 PrimeTime qPCR Primers	IDT
NOS2 PrimeTime qPCR Primers	IDT
IL-1 β PrimeTime qPCR Primers	IDT

ARG1 PrimeTime qPCR Primers	IDT
MRC1 PrimeTime qPCR Primers	IDT
TLR4 PrimeTime qPCR Primers	IDT
TLR5 PrimeTime qPCR Primers	IDT
TNF- α PrimeTime qPCR Primers	IDT
IL-6 PrimeTime qPCR Primers	IDT
GUSB PrimeTime qPCR Primers	IDT
Actin PrimeTime qPCR Primers	IDT
IFN- λ PrimeTime qPCR Primers	IDT
IL-17 PrimeTime qPCR Primers	IDT

Table 2.5 Quantitative PCR Reagents. All qPCR reagents used and corresponding sources.

GEL ELECTROPHORESIS REAGENTS

Material	Source
Agarose	Thermo-Fisher Scientific
SYBR [®] Safe DNA Gel Stain	Applied Biosystems
Gene-ruler 100 bp plus DNA ladder	Thermo-Fisher Scientific
DNA loading Buffer	Fermentas

Table 2.6 Gel Electrophoresis Reagents. All gel electrophoresis reagents used and corresponding sources.

IMMUNOHISTOCHEMISTRY & IMMUNOCYTOCHEMISTRY REAGENTS

Material	Source
Acetone	Sigma-Aldrich
HCL	Sigma-Aldrich
Sodium Bicarbonate	Sigma-Aldrich
Ethanol	Merck
Harris Haematoxylin	Sigma-Aldrich
Eosin	Sigma-Aldrich
Histo-clear	National Diagnostics
Histobond microscope slides	RA Lamb
OCT solution	Tissue-Tek
HRP-DAB Cell & Tissue Staining Kit	R&D Systems
Paraffin wax	VWR
Formalin	Sigma
Xylene	Sigma
Anti-F4/80 antibody	Abcam
Glycine	Sigma-Aldrich
Fish-Gelatin	Sigma-Aldrich
DAKO fluorescent mounting medium	DAKO
Anti-PDX1 antibody	Abcam
DPX mounting medium	Sigma-Aldrich

Table 2.7 Immunohistochemistry & Immunocytochemistry Reagents. All immunohistochemistry reagents used and corresponding sources.

2.2 Cell Culture

All cell culture was carried out in a class II laminar airflow unit (Holten 2010 – ThermoElectron Corporation, OH, USA). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all the items used in the cabinet. The cabinet was cleaned each week with industrial detergents (Virkon, Antec. International; TEGO, TH. Goldschmidt Ltd). Cell cultures were maintained in a 37°C incubator with 5% CO₂ and 95% humidified air (Model 381 -Thermo Electron Corporation OH USA). Cells were grown in complete DMEM (Sigma-Aldrich) or DMEM-glutamax (Biosciences) supplemented with 10% FBS and 2% penicillin - streptomycin/glutamine. FCS was heat-inactivated (56°C for 30 min) to inactivate complement and aliquoted for storage at -20 °C. Supplemented medium was stored at 4 °C for no longer than one month.

2.2.1 Transgenic C57BL/6 mouse insulinoma cell line (MIN6).

The MIN6 cell line (P15-30) was maintained in complete DMEM-glutamax substituted with 25 mM glucose (Sigma-Aldrich) in vented 75 cm² flasks. Cell monolayers were passaged at confluency of ~80% (every 3-4 days). To subculture cells were washed once with warm PBS (Biosciences). Cells were detached by adding 2ml of warm 0.25% trypsin, 0.02% EDTA solution (Sigma-Aldrich). Trypsin/EDTA solution was left on the cells at 37°C until the cells detached (~3-4 mins). Fresh culture medium was then added to deactivate the trypsin/EDTA. Cells

were centrifuged at 1000 rpm for 5 min, resuspended in fresh media and subcultured in a ratio 1:4, in fresh 75 cm² flasks

2.2.2 Murine monocyte-derived macrophage cell line, J774A.1

The J774A.1 cell line was purchased from the European Collection of Cell Cultures (ECACC) and maintained in complete DMEM in 75 cm² flasks. Cell monolayers were passaged at a confluency of 80% (every 3 to 4 days). Cells were detached using a cell scraper and transferred into a 50 ml falcon. Cells were centrifuged at 250 g for 5 min and resuspended in complete DMEM. For subculture, cells were plated at 1×10^6 /25 ml DMEM in a fresh 75 cm² flask.

2.2.3 Cell counting

Cell counting and viability determinations were carried out using the trypan-blue (Sigma-Aldrich) dye exclusion technique. This test is based on the ability of viable cells to actively exclude dye as a result of having an intact cell membrane. Dead cells are unable to exclude the dye and appear blue when viewed under a microscope. 100 µl of cell suspension was mixed with 150 µl PBS and 250 µl of trypan blue solution. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass cover slip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically. An average per corner grid was calculated.

A viable cell count was determined using the following formula:

$$\text{Cell/ml} = N \times 5 \times 10^4$$

Where, N = average cell number counted, 5 = dilution factor, and 10^4 = constant.

2.2.4 Preparation of cell stocks

Cryoprotective medium or freezing medium was prepared in complete culture medium containing 20% dimethylsulfoxide (DMSO; Sigma-Aldrich) and filter sterilised using a 0.22 μm filter and syringe. The freezing medium was then placed on ice until used. Appropriate number of cryogenic vials (Nalgene®, Lennox) were labelled with the cell line, passage no, date, and placed on ice. At this point cells were collected and counted as previously described. . The supernatant from the centrifuged cells were removed and the pellet was re-suspended in 1 ml of fresh media. This solution (20% DMSO) was slowly added drop wise to the cell suspension to give a final concentration to 10% of DMSO, and a final cell concentration of 5×10^6 cells/ml. Cryovials were placed in a Nalgene® Mr. Frosty freezing container. The Mr. Frosty container was filled with isopropanol and placed in a -80°C freezer. Mr. Frosty freezing container provided slow preservation of cells at a rate of $1^\circ\text{C}/\text{min}$. After 2 hr vials were transferred to liquid nitrogen or -80°C for long term storage.

2.2.5 Retrieval of Frozen stocks

Cryovials were carefully removed from liquid nitrogen or -80°C freezer and quickly thawed in a 37°C water bath. Thawed cells were transferred to 9 ml room-temperature DMEM on ice and carefully resuspended. Cells were spun at 1200 rpm

for 5 min to remove excess DMSO. Following this, supernatant was discarded and cells resuspended in 10 ml warm DMEM, and left overnight to adhere in a 25 cm² vented flask. The next day cells were collected as previously described and resuspended in fresh media to remove any excess DMSO and transferred to a 75 cm² flask in 25 ml media.

2.2.6 Glucose-Stimulated Insulin secretion from MIN6 cells

MIN6 cells were plated at a density of 1 x10⁶ cells/ml and left to reach confluency. Media was then removed and cells were washed with PBS and left to equilibrate for 30 mins in Krebs-Ringer Buffer (KRB) containing 25 mM HEPES, 0.1% BSA and 2 mM glucose at 37°C and 5% CO₂. Following Equilibration cells were incubated for 1hr in KRB-BSA containing various concentrations of glucose (0 – 20 mM) at 37°C and 5% CO₂. The assay was terminated by placing the plate on ice, the media was removed, centrifuged at 1000 rpm for 5 mins to remove any debris and supernatant was analysed for insulin content by ELISA.

2.2.7 Measurement of total MIN6 insulin content

MIN6 cells were plated at a density of 1 x10⁶ cells/ml and left to reach confluency. Cells were washed in PBS and were lysed in ice-cold acid/ethanol solution (HCl 1.5% [vol./vol.], ethanol 75% [vol./vol.] and H₂O 23.5% [vol./vol.]) cells were then disrupted by sonication. Protein was quantified and normalised using the BCA assay (Thermo Fisher Scientific, Rockford, IL, USA) and insulin content was measured using a mouse insulin ELISA (Mercodia, AB, Uppsala, Sweden).

2.2.8 Conditioning experiments

MIN6 cells were grown until they were confluent after which fresh media was added to cells and left for a further 24hr. This media was then removed and centrifuged at 1000 rpm for 5 mins to remove any cell debris. This media is here on referred to as “conditioned media”. In order to assess the effect of the conditioned media on macrophage function J774A.1 macrophage were plated at a concentration of 1×10^6 cells/ml in either normal DMEM or MIN6 conditioned media. Cells were then left to rest for 24hr prior to activation with TLR ligands and assessment of various parameters.

2.3 Flow Cytometry

2.3.1 Basic principles of flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyses multiple physical characteristics of single cells, as they flow in a fluid stream through a beam of light. The properties measured can include a particles relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

The fluidics system transports particles in a stream to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate

detectors. The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

When a particle or a cell passes through a laser beam, laser light is scattered in all directions. Light that scatters axial to the laser beam is known as forward scatter (FSC); light that scatters perpendicular to the laser beam is known as side scatter (SSC). FSC and SSC are related to certain physical properties of cells. FSC is proportional to the cell-surface area or size, while SSC is proportional to cell granularity or internal complexity.

Fluorescent probes can also be added to the cell for detecting specific molecules in or on the cell. These probes are typically antibodies to cellular antigens with a covalently attached fluorochrome. A fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence. The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle.

2.3.2 Cell surface staining

To carry out cell surface staining cells were washed once with PBS and then removed from tissue culture plates with a cell scraper. Cells were then placed in 96 well round bottomed plates at a concentration of ~ 250,000 cells per well. Cells were then blocked with an equal amount of foetal bovine serum for 15 mins at room

temperature to prevent binding of non-specific antibodies. The plate was then centrifuged at 2000rpm to pellet cells and cells were washed twice with FACS buffer (2% FBS, 0.05% NaN₃/PBS). Cells were then incubated with appropriate fluorochrome-conjugated antibodies for 30min at 4°C protected from light. Cells were then washed twice with FACS buffer to remove the unbound antibodies and were analysed using a FACSAria I (BD). All the flow cytometry data was analysed using FlowJo software (Treestar).

2.3.3 Intracellular Staining

In order to stain intracellular antigens, the cell must be permeabilised to allow binding of the antibody to the intracellular antigen. Depending on the specific location of the intracellular antigen, different methods of permeabilisation may be carried out. Cells were washed once with PBS and then removed from tissue culture plates with a cell scraper. Cells were then placed in 96-well round bottomed plates at a concentration of ~250,000 cells per well. Cells were then blocked with an equal amount of foetal bovine serum for 15 mins at room temperature to prevent binding of non-specific antibodies. The plate was then centrifuged at 2000rpm to pellet cells and cells were washed twice with FACS buffer (2% FBS, 0.05% NaN₃/PBS). Cells were then fixed in 4% paraformaldehyde (PAF)/PBS for 15 mins at room temperature. Cells were then permeabilised in either 0.5% Tween/PBS or 0.5% Triton/PBS depending on antigen of interest, for 15 mins at room temperature. Cells were then washed twice in buffer containing detergent. Cells were incubated with fluorescently conjugated antibodies for 30 mins at 4°C. Antibodies were diluted in permeabilisation buffer to ensure cells remained permeable. Cells were then washed

twice with FACS buffer containing detergent to remove the unbound antibodies and were analysed using a FACSAria I (BD). All the flow cytometry data was analysed using FlowJo software (Treestar).

2.3.4 Measurement of Phagocytosis

J774A.1 macrophages were cultured in 6-well plates at a concentration of 5×10^5 cells in a total volume of 2ml/well. To assess phagocytosis, macrophages were incubated with 1 μm fluorescent latex beads (Sigma-Aldrich), at a concentration of 20 beads per cell, for 2hr at 37°C. Cells were then washed with PBS/2% FCS and the uptake of beads ($\lambda_{\text{ex}} \sim 470 \text{ nm}$; $\lambda_{\text{em}} \sim 505 \text{ nm}$) was measured by flow cytometry. For the analysis of phagocytosis after stimulation, cells were treated with Pam3CSK4 1 $\mu\text{g/ml}$, LPS 100 ng/ml & Flagellin 5 $\mu\text{g/ml}$ for 24hr prior the addition of latex beads.

2.3.5 Measurement of reactive oxygen species (ROS) production

To measure the intracellular ROS production, 0.5×10^6 cells/ml were incubated with 20 μM of the cell permeant reagent 2',7' -dichlorofluorescein diacetate (DCFDA; Abcam) for 30 min at 37°C in the dark, according to manufacturer's instructions. DCFDA is a fluorogenic dye that measures hydroxyl, peroxy and other ROS activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' -dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission

spectra of 495nm and 529nm respectively. The fluorescence intensity of DCF was measured by flow cytometry.

2.4 Enzyme-linked immune-sorbent assay (ELISA)

2.4.1 Basic principles of ELISA

ELISA is a sensitive and reproducible method used to measure antigen concentration in an unknown sample. In all our studies we used a sandwich ELISA (or an adaptation of) to measure the amount of cytokines, chemokines and insulin in cell supernatants. To detect the antigen, the wells of 96-well microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and an antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by incubation. The detection antibody is usually conjugated with biotin which then allows for the detection reagent (streptavidin-HRP) to bind to it. The streptavidin and biotin bind with high affinity and the amount of horseradish-peroxidase (HRP) is proportional to the amount of target antigen in the sample. Substrate solution is then added (tetramethylbenzidine, TMB) and the HRP enzyme catalyses the oxidation of the TMB into a coloured product. The reaction is allowed to progress for a defined period after which the reaction is stopped by altering the pH of the system. Stop solution is used to terminate the enzyme substrate reaction for ELISA applications after attaining the desired colour intensity which is an indication of analyte level. The optical density (O.D.) of the colour is measured on a microplate reader. For each

set of samples, a standard curve is generated and the values of unknown samples are calculated from the standard curve.

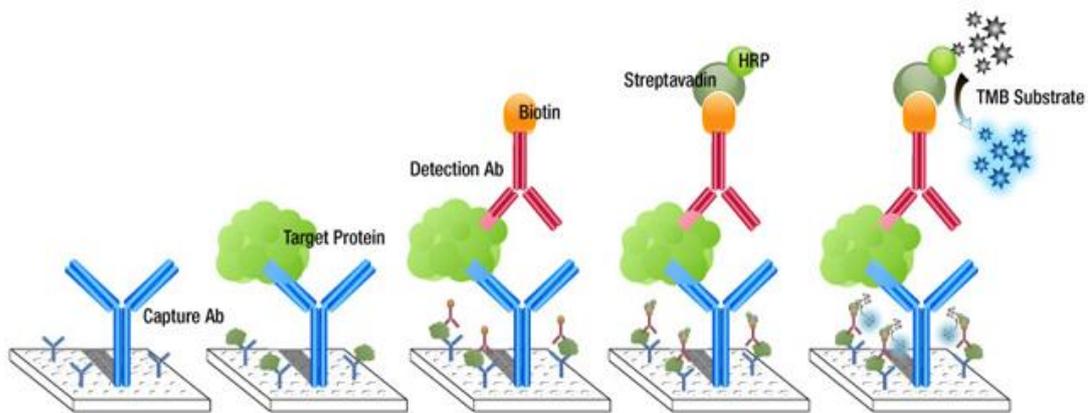


Figure 2.1 Schematic representation of sandwich ELISA. Schematic adapted from ELISA Kit Technology Principals on www.epitomics.com.

2.4.2 Cytokine ELISA

The concentration of cytokines IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, IL-27p28 and TNF- α were examined in cell using ELISA DuoSet kits from R&D Systems in accordance with the manufacturers' instructions. Briefly, 100 μ l/well of capture antibody (diluted to the appropriate concentration in PBS) was added to an ELISA plate (Nunc) and incubated overnight at room temperature. Plates were then washed three times by immersion in Wash Buffer (PBS + 0.05% Tween-20) and blocked by adding 300 μ l of Blocking buffer (1% (w/v) BSA/PBS) for a minimum of 1hr at room temperature. The washing step was repeated and 100 μ l of samples and standards (diluted to appropriate concentrations) were added per well, in triplicate. Plates were then incubated at 4°C overnight. The plates were then washed

again and 100 µl of the biotinylated detection antibody was added to each well and incubated for 2hr at room temperature. Plates were washed and 100 µl of Streptavidin-HRP (R&D) was then added to each well. Plates were incubated for 20 min at RT. Plates were washed for a final time and 100 µl of TMB (BD Biosciences) was added to each well and plates were left in dark for 20 min or until the blue colour develops. Colour development was stopped by adding 50 µl of 1M sulphuric acid (Sigma-Aldrich) to the wells. The optical density was determined at 450nm, using a VersaMax™ microplate reader (Molecular Devices). The average co-efficient of variation obtained with this kit was lower than 0.2 using the following calculation $\text{Std.Dev of samples}/\text{Mean} * 100$.

2.4.3 IL-1β and IFN-λ ELISA

The method above was followed with two deviations:

Blocking buffer used was 1% (w/v) BSA/PBS + 0.05% NaN₃. The reagent diluent was 0.1% (w/v) BSA/TBS + 0.05% Tween.

2.4.4 Chemokine ELISA

The concentration of chemokines MIP-1α, MIP-2, MCP-1 and RANTES in cell supernatants was determined using ELISA Duoset kits from R&D Systems in accordance with the manufacturers' instructions. The protocol is as described in the last section 2.4.2. Prior to analysis, supernatants were diluted 1 in 1000. The average co-efficient of variation obtained with this kit was lower than 0.2 using the following calculation $\text{Std.Dev of samples}/\text{Mean} * 100$.

2.4.5 Insulin ELISA

The concentration of insulin in cell supernatants was determined using a mouse insulin ELISA kit from Merckodia in accordance with the manufacturers' instructions. This kit is also based on the "sandwich" ELISA method; however the plates come pre-coated with capture and detection antibodies. Briefly 10 µl of sample was added to each well in along with 100 µl of an enzyme-conjugate solution (Merckodia). The plate was sealed and left on a plate shaker for 2hr after which the plate was washed 5 times with wash buffer (Merckodia). 200 µl of substrate TMB (Merckodia) was then added to each well and left for 15 mins or until blue colour develops. 50 µl of stop solution (Merckodia) was then added to each well, the plate was briefly shaken and the optical density was determined at 450 nm, using a VersaMax™ microplate reader (Molecular Devices). Depending on the number of cells used in the original experiment, supernatants were subjected to a dilution of 1 in 30 or 1 in 60 prior to analysis. The average co-efficient of variation obtained with this kit was lower than 0.2 using the following calculation $\text{Std.Dev of samples/Mean} * 100$.

2.5 MTS Viability Assay

Cell viability was determined by the MTS assay (Promega). The CellTiter 96® AQueous Assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bio-reduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan product at 490 nm can be measured directly from 96-well assay plates without additional

processing. Briefly, 100 μl cell suspension (containing 1×10^6 cells/ml) was plated in a 96-well plate. At the end of each experiment the cell proliferation reagent MTS (20 μl) was added to each well, and the cells were incubated at 37°C for 1hr. $A_{490\text{nm}}$ was measured using a VERSA Amax microplate reader (Molecular Devices, CA, USA). Results are expressed as a percentage of control cells.

2.6 Measurement of Nitric Oxide (NO) Formation

NO levels were detected in the cell supernatants by measuring the production of nitrite (NO^{2-}) which is a stable breakdown product of NO. The production of nitrite was measured using the Griess Reagent System (Promega). The Griess Reagent system uses sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED), under acidic (phosphoric acid) conditions, which form the coloured azo-compound in the presence of nitrite. The intensity of the colour is proportional to the concentration of nitrite in the sample. The nitrite concentration is then determined using sodium nitrite as a standard. Briefly, 50 μl of samples and standards were added to the 96-well plate in triplicate. 50 μl of the Sulfanilamide Solution (Promega) was then added to each well and plate was incubated for 10 min at RT, protected from light. 50 μl of the NED solution (Promega) was added to all wells and incubated for another 10 min at RT, protected from light. A purple/magenta colour develops and the absorbance was measured at 543nm using a VersaMax™ microplate reader (Molecular Devices).

2.7 Macrophage chemotaxis assay

The transwell migration assay also known as the Boyden or modified Boyden chamber assay [Figure 2.2] is a method of measuring cell chemotaxis towards a stimulus/or invasion through a substrate. Supernatants were taken from MIN6 cells after 24hr incubation in the presence or absence of TLR ligands Pam (1 ug/ml), LPS (100 ng/ml) and Flagellin (5 ug/ml), centrifuged at 1000rpm for 5 min to remove any cell debris and placed in the bottom wells of a transwell[®] plate (Analab Ltd, Rathfarnham, Dublin, Ireland). 250 µl of a suspension of J774 Macrophage containing 0.5×10^6 cells was then placed in each insert after which the plate was left at 37°C and 5% CO₂ for 6hr. The inserts were then carefully removed and the supernatants collected from the bottom wells. The numbers of migrated cells were then counted on a BD FACS Aria.

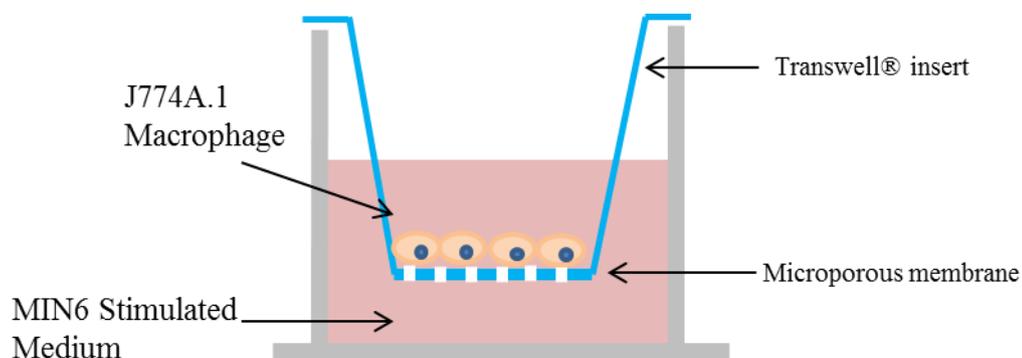


Figure 2.2 Diagrammatic representation of Chemotaxis Transwell[®] Assay.

2.8 Confocal Microscopy

In order to assess the effect that glucose has on PDX-1 localisation in MIN6 cells, cells were plated at a density of 1×10^6 cells/ml on coverslips contained in 6-well quiescence of insulin signalling. The cells were left overnight to attach. Following this the media was replaced with fresh low glucose (0.5 mM) or high glucose (20 mM) media for 20 mins. Cells were washed with PBS and immediately fixed by immersion in ice-cold acetone for 1 min. Cells were then allowed to air-dry, coverslip edges were sealed with a hydrophobic pen. Cells were then washed for 5 mins in PBS. To prevent non-specific binding cells were then blocked for 1hr at RT using blocking buffer (100mM Glycine and 1.2% fish gelatine in PBS). Cells were then incubated with PDX1 antibody in blocking buffer overnight at 4°C. Cells were then washed for 3 x 5 min in PBS and incubated with an appropriate secondary antibody for 1hr in the dark. Cells were washed once more with PBS, stained for 10 mins with DAPI for nuclear visualisation and washed again. Coverslips were then mounted on slides using DAKO fluorescent mounting medium and sealed using clear nail varnish.

2.9 RNA Analysis

2.9.1 Total RNA isolation

2.9.1.1 RNA isolation from MIN6 and J774A.1 cell lines

Cells (both MIN6 and J774) were plated at a density of 1×10^6 cells/ml. Cells were then treated under various conditions. At the completion of the experiment cells were washed once with PBS, RA1 + β -ME was added to wells and cells were scraped using the insert of a 1ml syringe. The cells were then disrupted by passing up and down through a 19.5 g needle and syringe. Total RNA was then isolated using a Nucleospin RNA II kit (Macherey-Nagel) according to manufacturer's instructions. Briefly, tissue was once again disrupted by passing up and down through a 19.5 g needle. The viscosity of the lysate was cleared by filtration through a NucleoSpin[®] filter and centrifuging for 1min at 11,000 x g. 70% ethanol was added to the homogenised lysate to adjust RNA binding conditions. The NucleoSpin[®] RNA II column was then used to bind RNA, with the addition of Membrane Desalting Buffer to remove salt and improve the efficiency of rDNase digestion. DNA was digested using a DNase reaction mixture at room temperature for 15 min. After 15 min Buffer RA2 and RA3 were added to inactivate rDNase and wash the column. The NucleoSpin[®] RNA II column was then placed into a nuclease free collection tube and RNA was eluted in RNase-free H₂O. The concentration of RNA was then measured using the Nanodrop[®] ND-1000 (Thermo-Fisher Scientific). The purity of the RNA was determined using 260nm and 280nm absorption wavelengths. All the RNA used in this study had an A280/A260 ratio between 1.8 and 2.1.

2.9.1.2 Colonic RNA isolation

Colonic tissue (20-30 mg) was homogenised in Lysis buffer RA1 (Macherey-Nagel) containing β -mercaptoethanol (β -ME) using the Qiagen TissueLyser LT for 5 min at 50 Hz. Total RNA was then isolated using a Nucleospin RNA II kit (Macherey-Nagel) according to manufacturer's instructions (see section 2.9.1.1)

2.9.2 cDNA synthesis

Complementary DNA (cDNA) is generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). This kit contains a master mix of dNTP, random primers and the enzyme reverse transcriptase. Reverse transcriptase synthesises single strand cDNA using the RNA strands as a template. This is then used as the template for the subsequent qPCR experiments. 1 μ g (colon) and 2 μ g (cell lines) of RNA was used in each cDNA synthesis reaction. The settings in **Table 2.1** are used in the reaction on a PTC-200 PCR thermal cycler (MJ Research).

	Step 1	Step 2	Step 3	Step 4
Temperature ($^{\circ}$ C)	25	37	85	4
Time (min)	10	120	5	∞

Table 2.8 Thermal cycling conditions

2.9.3 Basic principles of quantitative real time PCR (qPCR)

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences within a DNA or cDNA template can be copied, or “amplified”, many thousand- to a millionfold. In traditional (endpoint) PCR, detection and quantitation of the amplified sequence are performed at the end of the reaction after the last PCR cycle, and involve post-PCR analysis such as gel electrophoresis and image analysis. The advantage of qPCR is that the amount of PCR product is measured at each cycle. This ability to monitor the reaction during its exponential phase enables users to determine the initial amount of target with great precision. In real-time PCR, the amount of DNA is measured after each cycle by the use of fluorescent markers that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of PCR product generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA (dsDNA)- binding dyes, or dye molecules attached to PCR primers or probes. These fluorescent reagents can be sequence specific or non-sequence specific. In this thesis both types of reagents were used, Taqman[®] assay and SYBR[®] green dye.

2.9.3.1 SYBR[®] green dye

SYBR[®] green is a fluorescent dye that binds to the minor groove of double-stranded DNA. SYBR[®] green, an intercalating dye is a non-sequence-specific fluorescent dyes that exhibits a large increase in fluorescence emission when it intercalates into double-stranded DNA. During PCR, the primers amplify the target sequence and multiple molecules of the dye are inserted between bases of the double-stranded

product, causing fluorescence. SYBR[®] green dye is non-sequence specific, which means it can bind to any double-stranded sequence such as primer-dimer artefacts or non-target sequence. To assess the specificity of the SYBR[®] green assay we performed the dissociation analysis for each product. The dissociation step was added after the final PCR cycle. If there is only one product present in the tube it should have one specific melting temperature, T_m. The PCR software transforms the melt profile into a peak. If there is more than one peak present that usually signals the amplification of non-target products. Presence of one pure peak shows that one target amplified, but to further evaluate this the products were also run on a gel [appendix B].

2.9.3.2 Taqman[®] assay

TaqMan[®] probes require a pair of PCR primers in addition to a probe with both a reporter and a quencher dye attached. The probe is designed to bind to the sequence amplified by the primers. During qPCR, the probe is cleaved by the 5' nuclease activity of the Taq DNA polymerase; this releases the reporter dye and generates a fluorescent signal that increases with each cycle. Because all three components (2 primers and 1 probe) must hybridize to the target, this method allows detection of the PCR product with greater specificity and higher accuracy.

2.9.4 qPCR protocol

PCR was prepared in triplicate for each sample by adding cDNA, master mix and RNase free water (Invitrogen). In experiments with Taqman[®] primer/probes

Taqman® Gene Expression Mastermix (Applied Biosystems) was used. In other experiments SYBR Green Mastermix (Roche) was used. All the primers and primer/probes were ordered from IDT and are listed in **Table 2.5** and Appendix C. ROX dye was used as a passive reference to normalise the well to well differences that may occur due to artefacts such as pipetting errors or instrument limitations. Samples were added to a 96-well reaction plate (Applied Biosystems) and sealed with optically clear film (Applied Biosystems). Plates were centrifuged for 1 min at 1000 x g and run on the ABI Prism 7500 (Applied Biosystems) or Lightcycler® 96 system (Roche) under the conditions shown in **Table 2.9** The results were analysed using the ABI Prism sequence detection software (Applied Biosystems), the Lightcycler® 96 software (Roche) and Excel Software (Microsoft).

	Step 1	Step 2	Step 3a	Step 3b
Temp (°C)	50	95	95	60
Time	2min	10min	15sec*	15sec*
*Repeat for 40 cycles				

Table 2.9 Thermal cycling conditions.

2.9.5 PCR data analysis

For gene expression analysis we used a relative quantification method in which the gene levels are expressed as a fold difference between a sample and a calibrator (such as untreated/control cells). Samples were first normalised to an endogenous control which is a gene that maintains consistent expression levels despite treatment. Several endogenous controls were tested and the most suitable one was selected. For MIN6 cells this was B2M, for J774A.1 macrophage Actin and for murine colonic

tissue GUSB [see **Appendix**]. Normalised samples were then compared to the calibrator (control) using $\Delta\Delta Ct$ method:

$$\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ Calibrator}$$

Where $\Delta Ct \text{ sample} = Ct \text{ sample} - Ct \text{ endogenous control}$ and $\Delta Ct \text{ calibrator} = Ct \text{ calibrator} - Ct \text{ endogenous control}$.

2.9.6 DNA product analysis by gel electrophoresis

Gel electrophoresis was used to check DNA products following qPCR [see **appendix**]. Samples were run on a 2% agarose gel. The gel was prepared by dissolving 2 g of agarose (Thermo-Fisher Scientific) in 100 ml 1X TAE buffer and heating to boiling point. After cooling, 10 μ l of SYBR[®] safe DNA gel stain (Applied Biosystems) was added to the gel mix before pouring into a gel mould and setting the well comb. DNA samples were mixed with loading buffer (Fermentas) and loaded straight onto the gel, together with GeneRuler 100 bp DNA ladder (Thermo-Fisher Scientific). Gels were run for 1hr in 1X TAE buffer at 100 V and visualised using the G-box imaging system (Syngene).

2.10 Primary cell isolation

2.10.1 Mice

BALB/c mice, 6-8 weeks old, were purchased from Charles River. Mice were kept under specific pathogen-free conditions at the Bioresource Unit, Faculty of Health

and Science, Dublin City University, Ireland. All mice were maintained according to the guidelines of the Irish Department of Children and Health.

2.10.2 Isolation and differentiation of bone marrow-derived macrophages

The bone marrow was flushed out of the femurs and tibias of adult mice. Cells were then centrifuged at 250 x g for 5min and resuspended in complete RPMI (Gibco) supplemented with 10% FBS (Gibco) and 2% penicillin streptomycin/glutamine (Gibco). 50 ng/ml M-CSF (eBioscience) was added to generate macrophages (Sweet & Hume 2003). Cells were plated on petri dishes (4 petri dishes/mouse; 10 ml/petri dish) and placed in a CO₂ incubator at 37°C. After 3 days, 10 ml of fresh media with 50 ng/ml M-CSF was added to each plate. At day 6 non-adherent cells were removed by washing with RPMI and the adherent cells were displaced using a cell scraper (Sarstedt) and collected for further experiments. The purity of harvested bone marrow-derived macrophages was assessed by flow cytometry and was typically >95% F4/80+.

2.11 Dextran sulfate sodium (DSS) induced model of colitis

Dextran sodium sulphate (DSS) model of colitis is one of the most widely used chemically induced models of inflammatory bowel disease. It is directly toxic to the colonic epithelial cells in the basal crypts and induces an acute inflammatory response (Kullmann et al. 2001).

10 BALB/c female mice were housed at the Biological Resource Unit (BRU), at Dublin City University. DSS was administered to mice in drinking water. DSS was

prepared fresh every day in normal tap water at a final concentration of 5%. Mice were administered DSS for 5 days followed by 2 days on water alone and were sacrificed for sample and tissue processing on day 7. Mice were split into two groups for this study.

1. Control

No DSS was administered to the control mice, animals were sacrificed for sample and tissue processing on day 7.

2. DSS

Mice were administered DSS for 5 days (day 0 – day 4) followed by 2 days on water and sacrificed for sample and tissue processing on day 7.

The clinical symptoms recorded in DSS-treated and the Control mouse were body weight and fur texture/posture which were measured every day. These were used to generate a daily disease activity index (DDAI) as described below.

Score	0	1	2	3
Stool Consistency	Normal, well-formed pellets	Changed formed pellets	Loose stool	Diarrhoea
Fur texture/ Posture	Smooth/ hunched	Not Mildly hunched	scruffy Very scruffy	Very hunched

Table 2.10 Daily Disease Activity Index (DDAI).

At the end point of each group the length & weight of each colon was also measured and used as an indication of colitis in the mouse model. Sections of distal colon were collected for tissue homogenisation and RNA purification.

2.11.1. Tissue Sectioning - Pancreas

The mice were euthanized and pancreas was removed within 10 mins of death. The pancreas was then cut in two – the tail section was infused multiple times with RA1 buffer (Macherey-Nagel) until inflation was observed and then flash frozen in liquid nitrogen. The head of the pancreas (part closest to the intestine) was placed in a tissue cassette, flattened between two sponges and left in formalin overnight at RT to fix. Following overnight fixation the tissue was then put in a TP1020 processor (Leica Biosystems) for paraffin tissue processing. Briefly the steps included 1Hr in 70% EtOH, 1hr in 96% EtOH, 1hr in 100% EtOH followed by 2hr in 100% EtOH. This ensures adequate dehydration of the tissue. The program continued with 1hr in xylene, 2hr in xylene to remove alcohol before paraffin embedding, and finally 3 x 1hr in paraffin. The tissue is then fixed in small paraffin blocks and left to set. 0.5 µm sections were cut from the blocks and put onto slides. The slides were left to dry and then baked at 50°C for at least 40 mins.

2.11.2 Tissue Sectioning – Colon

Tissue sections of 0.5 µm were removed from the distal part of the washed colon and submitted to the paraffin tissue processing program outlines in section 2.11.1.

2.11.3 Haematoxylin and Eosin staining

Prior to staining slides had to be de-paraffinised, this was done by immersion in HistoClear (National Diagnostics) for 2 x 10 mins. This was followed by re-hydration of tissue which includes 2 x 5 mins in 100% EtOH, 2 mins in 95% EtOH and 2 mins in 75% EtOH. This allows for gradual re-hydration of the tissue. Slides were stained with Harris haematoxylin (Sigma-Aldrich) for 10 mins and washed again under running tap water for 5 min. The slides were differentiated in 1% acid/alcohol for 30 s 3 times and then washed under a tap for 1 min. After washing slides were placed in 0.1% sodium bicarbonate (Sigma-Aldrich) for 1 min followed by washing under a tap for 5 min. The slides were then rinsed in 95% ethanol (Merck) for 10 dips before counterstaining with Eosin (Sigma-Aldrich) for 1 min by dipping up and down. Finally the slides were dehydrated again by dipping in 75% ethanol for 3 min, 95% ethanol for 3 min (x2), followed by 100% ethanol for 3 min and 3 min in HistoClear. Slides were then mounted with mounting medium (DPX) and the cover slips pushed firmly to remove bubbles.

2.11.4 HRP-DAB-F4/80 staining Pancreas

F4/80 was visualised in the pancreas using the HRP-DAB SYSTEM (R&D Systems) according to the manufacturer's protocols. Detection is based on the formation of the Avidin-Biotin Complex (ABC) with primary antibodies that react with tissue antigens under study. Visualization is based on enzymatic conversion of a chromogenic substrate 3,3' Diaminobenzidine (DAB) into a colored brown precipitate by horseradish peroxidase (HRP) at the sites of antigen localization, which can then be viewed using brightfield microscopy. Briefly slides were first de-

parrafinised as outlined in section 2.11.3. Samples were then covered with 1-3 drops of the Peroxidase Blocking Reagent for 5 mins. Samples were then rinsed and washed in buffer (PBS) for 5 mins. Samples were then incubated with 1-3 drops of Serum Blocking Reagent G for 15 mins. Buffer was drained off and carefully wiped from slide. Sample was then incubated with Avidin blocking reagent for 15 mins and washed with buffer prior to 15 min incubation with Biotin Blocking Buffer. Samples were rinsed with buffer and incubated with anti F4/80 antibody overnight at 4°C. The next day samples were was for 3 x 15 mins in buffer prior to incubation with Biotinylated Secondary Antibody 60 mins. Samples were once again washed for 3 x 15 mins with buffer prior to incubation for 30 mins with HSS-HRP. Samples were washed with buffer for 3 x 2 mins and incubated with freshly prepared DAB-Chromogen solution for 15 mins. Samples were rinsed with distilled water and counterstained with Harris Haematoxylin by dipping for 30 seconds. Samples were rinsed with tap water for 5 mins to remove excess stain, left to air dry and were then mounted with mounting medium (DPX) and the cover slips pushed firmly to remove bubbles.

2.12 Exosome Fractionation and Isolation

In order to ascertain if the effects of MIN6 conditioning on macrophage function are due to something secreted in exosomes we aimed to isolate different fractions from MIN6 conditioned medium based on their size. In order to do this MIN6 cells were cultured in complete medium containing exosome free- FBS (Strattech). As FBS is known to contain a lot of exosomes this helped us ensure that exosomes isolated were indeed from the MIN6 cells and not FBS associated exosomes. Once cells had

reached confluency fresh media was added for a further 24hr. This media was then taken, spun down at 1000 rpm for 5 mins to remove any cell debris and stored at -80°C for further processing. The media (a total of 75 ml) was subjected to hydrostatic dialysis through a homemade device made of a dialysis membrane tube with a molecular weight cut off (MWCO) of 1,000 kDa (Spectrum Laboratories) connected to a separating funnel and the other end sealed by a clip. The hydrostatic pressure of the media in the funnel is strong enough to push the solvent and all the analytes below the MWCO through the mesh of the dialysis membrane made of cellulose ester. After the concentration of the media (5-8 ml) the funnel was refilled with 200 ml of PBS to wash away what was left until the volume reached 8 ml. The retained fraction was then spun at 20,000g calculated at maximum radius 105 mm in an ultra JA-20 fixed angle rotor (clearing factor or k factor = 770) (Beckman Coulter)-centrifuge for 1hr at 4°C using a Beckman JA26 centrifuge (Beckman Coulter). The pellet was resuspended in 1ml PBS. The supernatant was then taken and centrifuged at 200,000g calculated at the maximum radius 91.9 mm of 70 Ti fixed angle rotor (k factor = 44) (Beckman Coulter) using a Beckman XL-80 ultracentrifuge (Beckman Coulter) for 2hr at 4°C. The pellet formed was resuspended in 1vml PBS. The supernatants and all resuspended pellets were then stored at -80°C. After the first step anything in the flow through below 1,000 kDa (HDb) would not have been collected in the column. The flow through therefore was taken and subjected to another hydrostatic dialysis step using a dialysis membrane of 300 kDa in size MWCO (Spectrum Laboratories) and repeated the steps described above including the differential centrifugation protocol. Both the pellet and the supernatant were kept and all fractions were stored at -80°C until further use. Prior to

use the fractions were UV irradiated for 15 mins and then resuspended in 50 ml complete DMEM.

2.13 Statistical Analysis

Results are presented as mean \pm standard error of the mean (SEM) and groups were compared using an unpaired Student's t-test or for multiple groups, a one-way ANOVA followed by a Newman-Keuls post-hoc test. All data were analysed using Prism Software (GraphPad Software, Inc.). Values of less than $p < 0.05$ were considered statistically significant.

Chapter 3

Characterisation of the insulin secreting cell line MIN6 and the effects of TLR ligation on insulin signalling.

3.1 Introduction

β -cells, which are located in islets of Langerhans in the pancreas are the sole producers of insulin and thus are critical regulators of metabolism. Insulin is synthesized as preproinsulin and processed to proinsulin. Proinsulin is then converted to insulin and C-peptide and stored in secretory granules awaiting release on demand. β -cells release insulin in response to a number of stimuli, glucose being the primary inducer however they also respond to signals from amino acids, some hormones and free fatty acids to name a few (Fu et al. 2013). Thus, the β -cell is a metabolic hub in the body, connecting nutrient metabolism and the endocrine system.

MIN6 cells originate from a transgenic C57Bl/6 mouse insulinoma expressing an insulin-promoter/t-antigen construct (Miyazaki et al. 1990). MIN6 cells produce insulin and have morphological characteristics of pancreatic beta cells. MIN6 cells exhibit glucose stimulated insulin secretion (GSIS) comparable with cultured normal mouse islet cells (Miyazaki et al. 1990) and are considered as an appropriate model for investigating the mechanism of GSIS. Glucose metabolism and the glucose concentration-dependence of insulin secretion from MIN6 cells closely resemble those in normal beta cells, and this cell line is thus a useful tool for studying GSIS in normal pancreatic beta cells (Ishihara et al. 1993).

Toll-like receptors (TLRs) are pattern-recognition receptors and play a crucial role in the innate immune system, which detects the presence and the nature of pathogenic microbial infection, and thus provides the first line of host defence (Takeda et al. 2003).

Ligand recognition by TLRs leads to the recruitment of various TIR domain-containing signalling adaptors such as myeloid differentiation factor (MyD)88, TIR domain-containing adapter protein (TIRAP), TIR domain-containing adaptor inducing interferon (TRIF) and TRIF-related adaptor molecule (TRAM), which categorize the TLR signalling pathway into MyD88-dependent and TRIF-dependent pathways (Kumar et al. 2009). MyD88 is an essential signalling adaptor for most TLRs (Kawai & Akira 2009). This recruitment of adaptors triggers a signalling cascade and ultimately the activation of transcription factors such as NF- κ B. Moreover, MAP kinases such as p38, JNKs and extracellular signal-regulated kinase (ERK)1/2, which subsequently activate the transcription factor AP-1, are also activated. These transcription factors induce transcription of inflammatory cytokines and type I interferons. This proinflammatory reaction is fundamental for the initiation of an innate immune response and subsequent adaptive responses but can also damage tissues if inflammation persists too long.

TLRs can also interact with endogenous ligands generated at sites of injury and endogenous ligands such as oxLDL (oxidized LDL), HSPs (heat shock proteins) 60 and 70, fibrinogen, fibronectin and free-fatty acids (FFAs) (Wagner 2006; Yu et al. 2010). It is thought that TLRs play a role not only in host defence but also in maintenance of homeostasis of cells and or tissue. Indeed recent evidence has implicated the TLR adaptor protein Myd88 as playing a homeostatic role in pancreatic β -cell function (Bollyky et al. 2009). Myd88 $-/-$ mice are normoglycemic however have reduced β cell volume and islet size in comparison to C57BL/6 controls and suffer enhanced β -cell apoptosis following low-dose streptozotocin (STZ) treatment. This suggests that Myd88 signalling and certain TLRs may mediate a homeostatic effect on β -cells primarily in the setting of injury. Findings like these

taken together with the fact that Type 2 diabetes is now being closely associated with low grade levels of inflammation throughout the body and within the pancreas itself (Marselli et al. 2013; J. a Ehses et al. 2009; Donath et al. 2013; Yin et al. 2014; Dasu et al. 2012) make Toll-like receptors and their function within the β -cell an attractive study.

The aim of this study was to characterise the properties of the insulin secreting cell β -cell line MIN6 and assess the effects that activation with TLR ligands had on their properties. This was carried out by assessing a number of parameters including cell viability, insulin secretion and content, insulin gene expression and cell surface marker expression.

3.2 Results

3.2.1 Characterization of a Murine Insulinoma cell line, MIN6

The insulin secreting Min6 cell line is described in the literature as a good model for insulin producing pancreatic beta cells (Ishihara et al. 1993) . We first needed to establish the optimal glucose stimulated insulin secretion conditions for this cell line.

3.2.1.1 Glucose stimulation of Min6 cells results in a dose dependant insulin secretion response

MIN6 cells were grown in 24 well plates and left to reach confluency. Prior to stimulation cells were left in low glucose (2mM) Krebs-Ringer Buffer for 1hr for quiescence of insulin secretion. Cells were then stimulated with various

concentrations of glucose for 1hr (0, 2, 5, 10, 20 and 40mM). This conditioned medium was then analysed for insulin content by ELISA (Mercoxia AB).

Glucose stimulation resulted in a dose dependant increase in insulin secretion [Figure 3.1]. Insulin secretion peaked at 20mM glucose stimulation and decreased with 40mM glucose stimulation. This was most likely due to the harmful effect high concentrations of glucose have on MIN6 cells.

3.2.1.2 Min6 cells display greatest glucose dose response when cultured in 25mM glucose

There was variation in the literature regarding methods of culturing MIN6 cells therefore we tested their glucose responsiveness after long term culture in DMEM-glutamax containing various concentrations of glucose.

To examine whether the amount of glucose in the MIN6 culture medium affected cell viability the cells were plated on 96 well plates in media containing different amounts of glucose (5.6, 11 and 25mM). After 24 and 48hr the cell viability was assessed using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. **Figure 3.2** shows that the amount of glucose in the culture medium does not affect cell viability.

To assess the glucose-stimulated insulin response following culture in media containing different glucose concentrations (5.6, 11 and 25mM) cells were grown in 24 well plates and left to reach confluency. Prior to stimulation cells were left in low glucose (2mM) Krebs-Ringer Buffer for 1hr for quiescence of insulin secretion.

Cells were then stimulated with various concentrations (2, 10 20 and 40mM) of glucose for 1hr. Conditioned medium was then analysed for insulin content by ELISA (Mercoia AB). Cells grown in culture medium containing 25mM glucose showed the greatest glucose stimulated insulin secretion response [**Figure 3.3**]. This amount of glucose was added to culture medium for MIN6 cells in all subsequent experiments unless stated otherwise.

3.2.1.3 Glucose stimulation induces PDX-1 translocation to the nucleus in MIN6 cells

One of the mechanisms whereby glucose stimulates insulin gene transcription in pancreatic β -cells involves activation of the homeodomain transcription factor PDX1 (pancreatic/duodenal homeobox-1) via a stress-activated pathway involving stress-activated protein kinase 2 (SAPK2) (Elrick & Docherty 2001). Glucose stimulates translocation of PDX1 from the cytoplasm to the nucleus in pancreatic β –cells (Wendy M Macfarlane et al. 1999). To investigate if this occurs in the MIN6 cell line, cells were grown overnight on coverslips in low glucose media (0.5mM). Cells were then stimulated with low (0.5mM) or high (20mM) glucose for 20 minutes, and subsequently fixed and stained with antibodies for PDX-1 and DAPI (for nuclear localisation). Stimulation of MIN6 cells with 20mM glucose induces translocation of PDX-1 from the cytoplasm to the nucleus [**Figure 3.4**]. This does not occur when cells are in low glucose [**Figure 3.4**].

3.2.2 TLR ligation of MIN6 cells does not significantly affect cell viability

Given that we wanted to examine the effects of TLR ligands on MIN6 cells, we first assessed their effect on MIN6 cell viability. MIN6 cells were plated on a 96 well plate and left to reach confluency. Following this cells were stimulated with a panel of TLR ligands (Pam3CSK4 0.25-1 μ g/ml, LPS 25-100ng/ml & Flagellin 1-5 μ g/ml) for 24hr after which the cell viability was assessed using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. These TLR ligand concentrations have previously been shown to elicit an inflammatory response from immune cells. As the conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells the quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. No significant decrease in cell viability was seen comparing control to TLR treated cells [Figure 3.5].

3.2.3 The effects of TLR ligation on MIN6 insulin secretion

3.2.3.1 TLR stimulation alters the normal glucose stimulated insulin secretion profile of MIN6 cells

MIN6 cells were plated on 6 well plates and left to grow to confluency. Cells were then activated with TLR ligands (Pam3CSK4 1 μ g/ml, LPS 100ng/ml & Flagellin 5 μ g/ml). Following 24hr stimulation, the media was removed and the cells were left in low glucose (2mM) Krebs-Ringer Buffer for 1hr for quiescence of insulin secretion and were then stimulated with various concentrations of glucose (0 –

40mM) for 1hr. Conditioned medium was then analysed for insulin content by ELISA (MercoDIA AB). Under normal circumstances MIN6 cells show a typical dose dependant secretion of insulin following glucose stimulation [Figure 3.6A]. Following 24hr stimulation with all TLR ligands, Pam (1µg/ml), LPS (100 ng/ml) and Flagellin (5µg/ml), MIN6 cells basal levels of insulin secretion were significantly increased versus control cells [Figure 3.6B] ($p < 0.05$). This was in stark contrast to the significant decrease in stimulated insulin secretion observed at 20mM following TLR ligation [Figure 3.6D], with Flagellin ($p < 0.01$) causing a more profound effect than LPS ($p < 0.05$). Both Pam ($p < 0.001$) and LPS ($p < 0.001$) stimulation resulted in a very significant decrease in total insulin content [Figure 3.6E] whereas Flagellin ($p < 0.05$) caused a slight increase in total insulin content [Figure 3.6E].

3.2.3.2 Expression levels of INS1 and INS2 genes following 24h TLR ligation

Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam 1µg/ml, , LPS- 100ng/ml & Flagellin- 5µg/ml) for 24 hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The levels of INS1 and INS2 mRNA were quantified using qPCR. The expression levels were normalised to B2M levels and the gene expression is shown as a fold change relative to the control. The only significant change found in gene expression was a decrease

in INS2 [Figure 3.7] following stimulation with flagellin ($p < 0.001$). No other results achieved significance.

3.2.3.3 Expression levels of IRS1 and IRS2 following 24h TLR ligation

Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam $1 \mu\text{g/ml}$, LPS 100ng/ml & Flagellin $5 \mu\text{g/ml}$) for 24 hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The levels of IRS1 and IRS2 mRNA were quantified using qPCR. The expression levels were normalised to B2M levels and the gene expression is shown as a fold change relative to the control. As a general trend stimulation with all TLR ligands seemed to decrease IRS1 expression (all be it insignificantly) and increase IRS2 expression Pam ($p < 0.01$) Flagellin ($p < 0.05$) and LPS (n/s) [Figure 3.8].

3.2.3.4 TLR ligation alters Insulin Receptor expression on MIN6 cells

As insulin itself can act in an autocrine manner on the β -cell through the insulin receptor (IR) and knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in Type 2 Diabetes (Kulkarni et al. 1999) we examined the expression of the β -subunit of the IR following 24 h stimulation with TLR ligands. Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands (Pam $1 \mu\text{g/ml}$, LPS

100ng/ml & Flagellin 5 μ g/ml). Cells were then analysed for cell surface expression of the β subunit of the IR. Cell-surface expression of the IR- β subunit, as examined by flow cytometry was decreased following stimulation with both LPS and Flagellin [Figure 3.9], however in contrast to this stimulation with Pam resulted in an increased surface expression of the IR- β .

3.2.4 Optimisation of permeabilisation technique for MIN6 cells

Detection of intracellular antigens requires a cell permeabilisation step prior to staining. Depending on the location of the specific antigen of interest the optimum method used for permeabilisation can differ greatly. As we intended to look at a number of intracellular antigens it was important to figure out the best method of permeabilisation for each antigen. In order to do this MIN6 cells were fixed by a number of methods and subsequently stained with antibodies for intracellular antigens Insulin (APC), TLR5 (FITC) and PDX-1 (PE). Cells were fixed prior to permeabilisation with triton (A), tween (B) or Saponin (C) [Figure 3.10]. Figure 3.10 shows that the FSC of cells can vary greatly following different methods of permeabilisation. As our data showed greatest expression levels when cells were permeabilised with tween when looking at PDX-1 and saponin for TLR5 these were used for all future experiments unless stated otherwise.

3.2.4 TLR ligation alters PDX-1 expression on MIN6 cells

PDX-1 (Pancreatic and duodenal homeobox 1), also known as insulin promoter factor 1, is a transcription factor necessary for pancreatic development, β -cell

maturation and the maintenance of β -cell function. Islet β cell-specific transcription of the insulin gene is mediated through the binding of the islet-enriched PDX-1, BETA2, and MafA transcription factors to conserved 5'-flanking region regulatory elements (Le Lay & Stein 2006). Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands (Pam 1 μ g/ml, LPS 100ng/ml & Flagellin 5 μ g/ml). Cells were then analysed for intracellular expression of PDX-1 by flow cytometry. Stimulation with Pam, LPS and Flagellin all resulted in a minor but consistent decrease in the expression levels of PDX-1 [**Figure 3.11**].

3.2.5 TLR stimulation does not affect Reactive Oxygen Species production from MIN6 cells

Pancreatic β -cells exposed to conditions such as hyperglycaemia produce reactive oxygen species (ROS) which in turn can suppress glucose-induced insulin secretion (Sakai et al. 2003). Toll-like receptor activation has long been known to induce ROS generation (Gill et al. 2010) therefore we sought to investigate the effect of TLR activation of MIN6 cells on ROS production. ROS levels were measured using the cell permeant reagent 2',7' – dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxy and other ROS activity within the cell. MIN6 cells were plated and stimulated with TLR ligands as previously described. The fluorescent signal relative to intracellular ROS generation was then measured by flow cytometry and compared between control and treated cells. TLR stimulation of MIN6 cells did not result in any significant change in ROS production [**Figure 3.12 A & B**]. In **Figure 3.12A** histograms representative of ROS fluorescence are off-set

to easily allow visualisation. In **Figure 3.12B** the mean fluorescence intensity (MFI) from n=3 samples/treatment was graphed and assessed for significance by unpaired t-test.

3.2.6 Effect of TLR stimulation on MIN6 surface marker expression

Pancreatic β -cells have been shown to express certain toll-like receptors and co-stimulatory molecules (Lee et al. 2012; Yin et al. 2014). We used flow cytometry to confirm expression of the molecules on MIN6 cells in control conditions and following TLR stimulation. For all markers investigated cell surface staining was used apart from for TLR5 in which case the antibody used recognizes an intracellular epitope in the cytoplasmic domain of TLR5. Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml & Flagellin $5\mu\text{g/ml}$). Following this cells were stained with fluorescently labelled antibodies against TLR2, TLR4, TLR5, CD14, MHCII, CD40, CD80 & CD86. Histograms show surface marker expression of unstimulated control cells (black line) compared to TLR-ligand stimulated cells (dashed line). Filled histograms represent fluorescence of unstained cells. MIN6 cells were found to express surface markers TLR2, TLR4, TLR5, CD14 MHCII and CD40 however no or minimal expression of both CD80 and CD86 was observed [**Figure 3.13**]. TLR stimulation of MIN6 cells with Pam, LPS and flagellin failed to illicit a significant change in expression of these surface markers [**Figures 3.13, 3.14 & 3.15**] except in the case of flagellin which caused a slight increase in expression of MHCII, TLR2 and CD40 [**Figure 3.15**].

Expression on TLR4 and TLR5 on MIN6 cells was also confirmed by qPCR. Again cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands as previously described. RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The levels of TLR4 and TLR5 mRNA were quantified using qPCR. The expression levels were normalised to B2M levels and the gene expression is shown as a fold change relative to the control. The only significant change found in gene expression was a decrease TLR4 ($p < 0.01$) and TLR5 ($P < 0.01$) [Figure 3.16] following stimulation with flagellin. No other results achieved significance.

3.2.7 Effect of TLR stimulation on expression levels of IAPP and FAM3B

Two molecules closely associated with islet inflammation and β cell failure in the context of diabetes are islet amyloid polypeptide (IAPP) and pancreatic-derived factor (PANDER or FAM3B). Both have been linked with islet dysfunction and or death (Cao et al. 2003; Wang et al. 2011; Montane et al. 2012). Expression levels of these molecules were measured by qPCR. Again cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands as previously described. RNA was extracted as previously described and equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The levels of IAPP and FAM3B mRNA were quantified using qPCR. The expression levels were normalised to B2M levels and the gene expression is shown as a fold change relative to the control. IAPP expression levels

remain unchanged apart from following Pam stimulation [**Figure 3.17A**] where a significant decrease in expression was detected ($P < 0.05$). FAM3B expression levels also remain unchanged apart from after Flagellin stimulation [**Figure 3.17B**] where a significant decrease in expression was detected ($P < 0.05$).

3.3 Figures

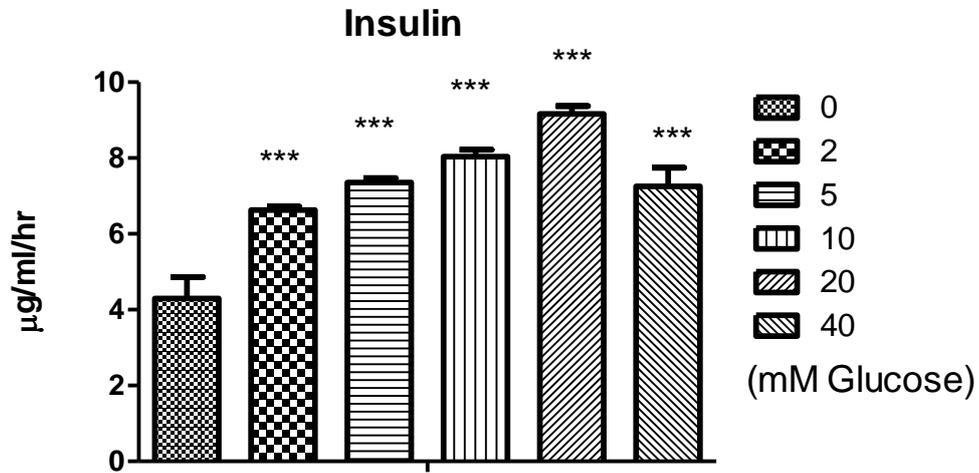


Figure 3.1 Glucose Stimulated Insulin Secretion from MIN6 cells. Cells were seeded at 1×10^6 cells/ml in a 24 well plate and left to grow until confluent. Cells were then washed twice in warm KRB-BSA and left in KRB-BSA containing 2mM Glucose for 1hr. Following this cells were stimulated with varying concentrations of Glucose in KRB-BSA (0-40mM). Supernatants were then analysed for insulin content using a mouse insulin ELISA (Merckodia AB, Upsalla, Sweeden). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. *** $p < 0.001$ as determined by one way ANOVA followed by post-hoc Newman-Keuls analysis.

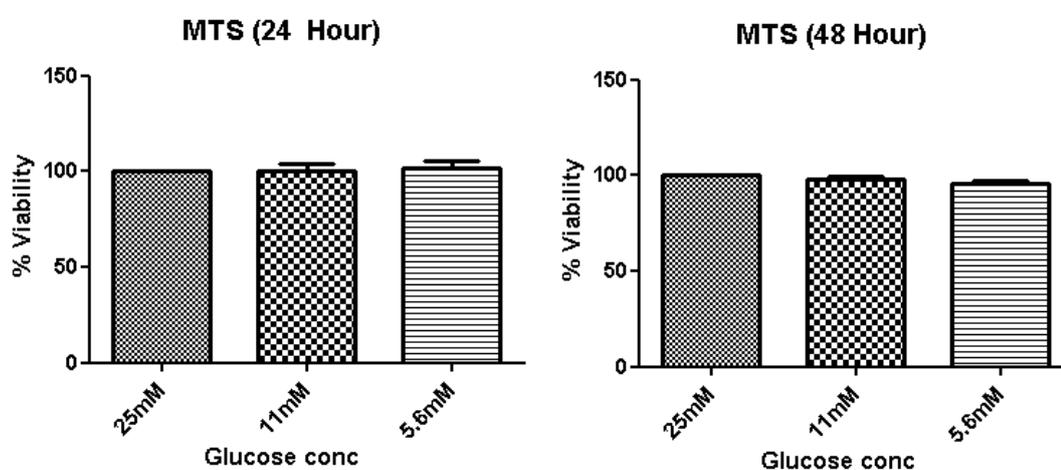


Figure 3.2 Culture of MIN6 cells in different concentrations of Glucose does not affect cell viability. Cells were plated at a concentration of 1×10^6 cells/ml on a 96 well plate in DMEM-Glutamax containing either 5.6, 11 or 25mM glucose and left for 24 and 48hr. Following this 20 μ l of the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO₂ for 1hr. The plate was then read at a wavelength of 450nm. Results are expressed as a percentage of the control (100%). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments.

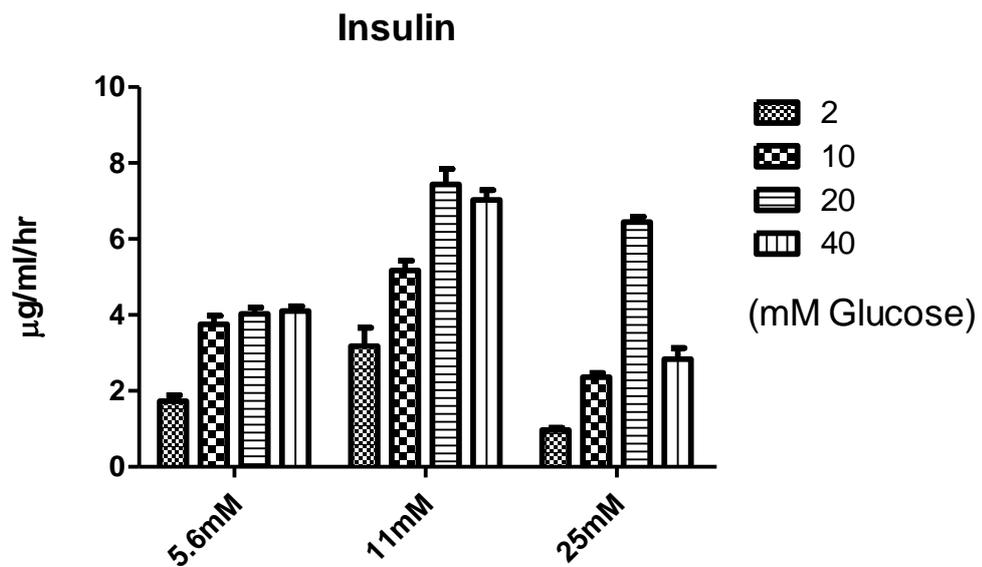


Figure 3.3 MIN6 Cells Cultured in 25mM Glucose show Greatest Dose Response to Glucose Stimulation. Cells were seeded at 1×10^6 cells per ml in a 24 well plate and left to grow until confluent in media containing either 5.6, 11 or 25mM glucose. Fresh media was added for 24hr. Cells were then washed twice in warm KRB-BSA and left in KRB-BSA containing 2mM Glucose for 1hr. Following this cells were stimulated with varying concentrations of Glucose in KRB-BSA (2 – 40mM). Supernatants were then analysed for insulin content using a mouse insulin ELISA (Mercodia AB, Upsalla, Sweden).

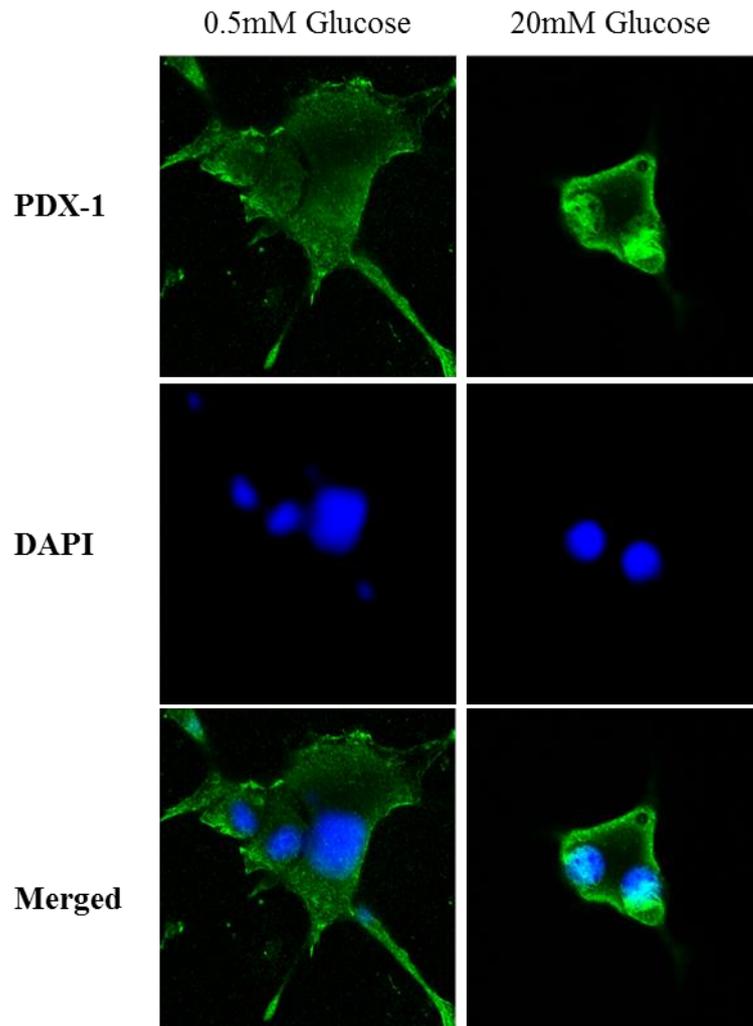


Figure 3.4 Glucose induces PDX-1 translocation to the nucleus in MIN6 cells. Cells (1×10^6 / ml) were left overnight to attach to coverslips in low glucose (0.5mM) media. Cells were then stimulated for 20 mins with 0.5 & 20 mM glucose prior to acetone fixation and subsequent staining with PDX-1 (green) and DAPI (blue) antibodies.

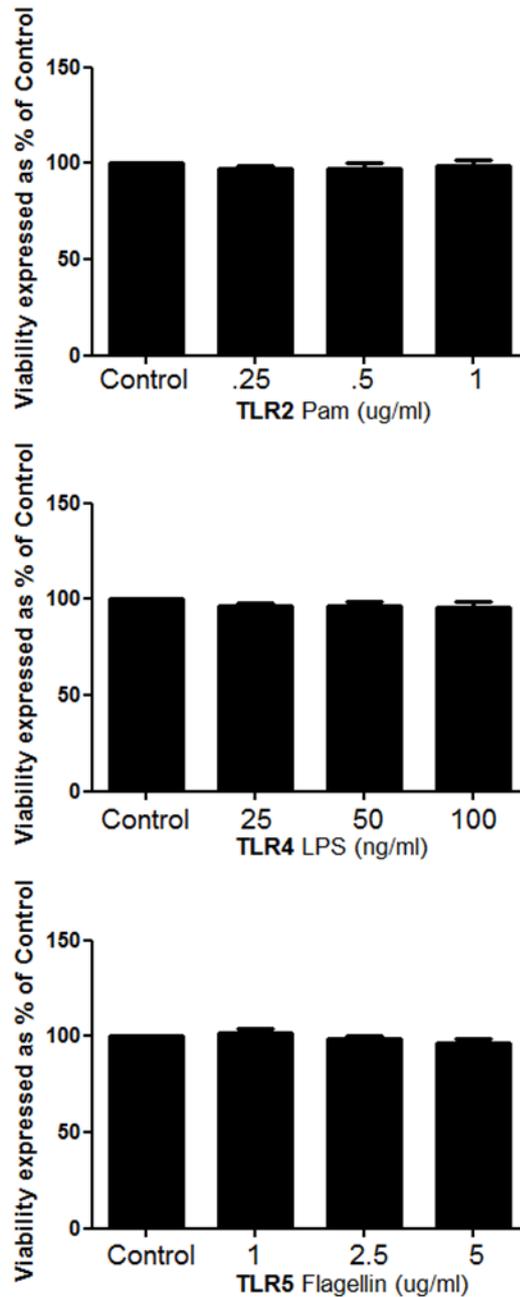


Figure 3.5 TLR Ligation of MIN6 cells do not significantly affect cell viability. Cells were seeded at 1×10^6 cells per ml in a 96 well plate and left to grow overnight prior to 24hr stimulation with various TLR Ligands (PAM 0.25 - $1\mu\text{g/ml}$, LPS 25 - 100ng/ml & Flagellin 1 - $5\mu\text{g/ml}$). Following this $20\mu\text{l}$ of the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO_2 for 1hr. The plate was then read at a wavelength of 450nm. Results are expressed as a percentage of the control viability (100%). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments.

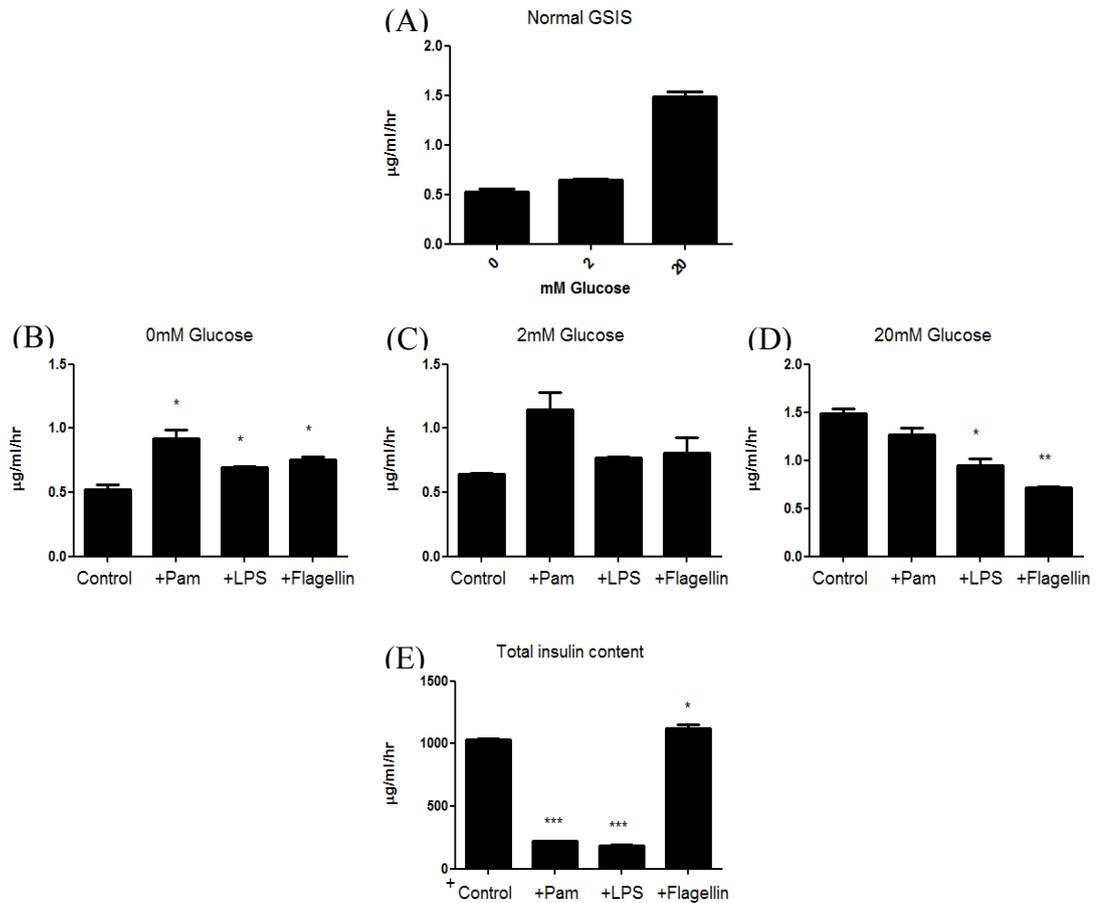


Figure 3.6 TLR stimulation alters the normal glucose stimulated insulin secretion profile of MIN6 cells. Cells were seeded at 1×10^6 cells/ml on a 24 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml , Flagellin $5\mu\text{g/ml}$) for 24hr. Cells were then washed twice in warm KRB-BSA and left in KRB-BSA containing 2mM Glucose for 1hr. Following this cells were stimulated with varying concentrations of Glucose in KRB-BSA (0-40mM) for 1hr. Supernatants were then analysed for insulin content using a mouse insulin ELISA (Merckodia AB, Upsalla, Sweden). Total insulin was extracted from cells using ice cold acid/ethanol. Supernatants were then analysed for insulin content using a mouse insulin ELISA (Merckodia AB, Upsalla, Sweden). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by unpaired t-test.

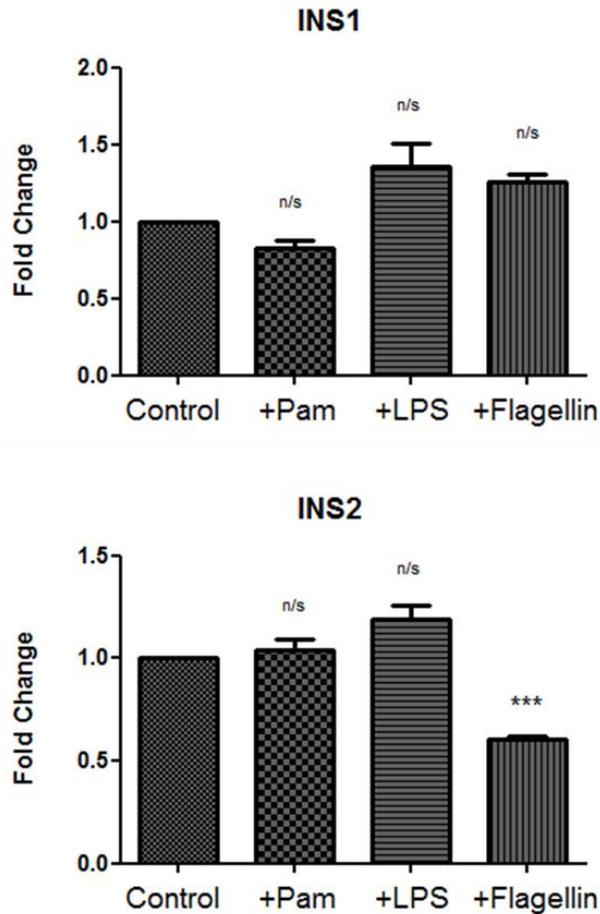


Figure 3.7 Expression levels of INS1 & INS2 following 24hr TLR ligation. The expression of INS1 and INS2 was measured by qPCR. Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam $1 \mu\text{g/ml}$, LPS 100ng/ml , Flagellin $5 \mu\text{g/ml}$) for 24hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for INS1 & INS2 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, B2M. The mean relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Results are mean \pm SEM. *** $P < 0.001$ vs. control, determined by unpaired *t*-test

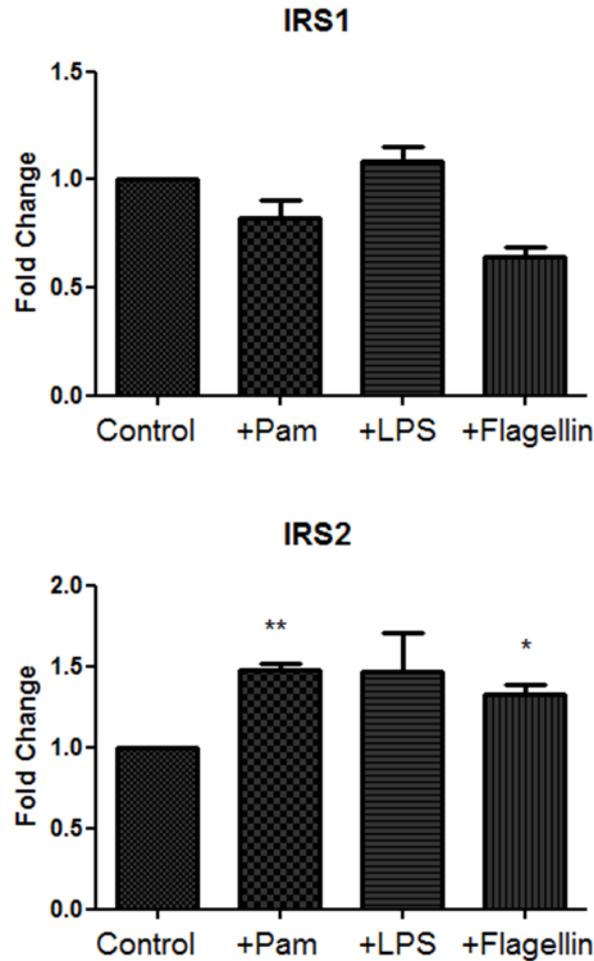


Figure 3.8 Expression levels of IRS1 & IRS2 following 24hr TLR ligation. The expression of IRS1 and IRS2 was measured by qPCR. Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml , Flagellin $5\mu\text{g/ml}$) for 24hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for IRS1 & IRS2 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, B2M. The mean relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Results are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. control, determined by unpaired *t*-test

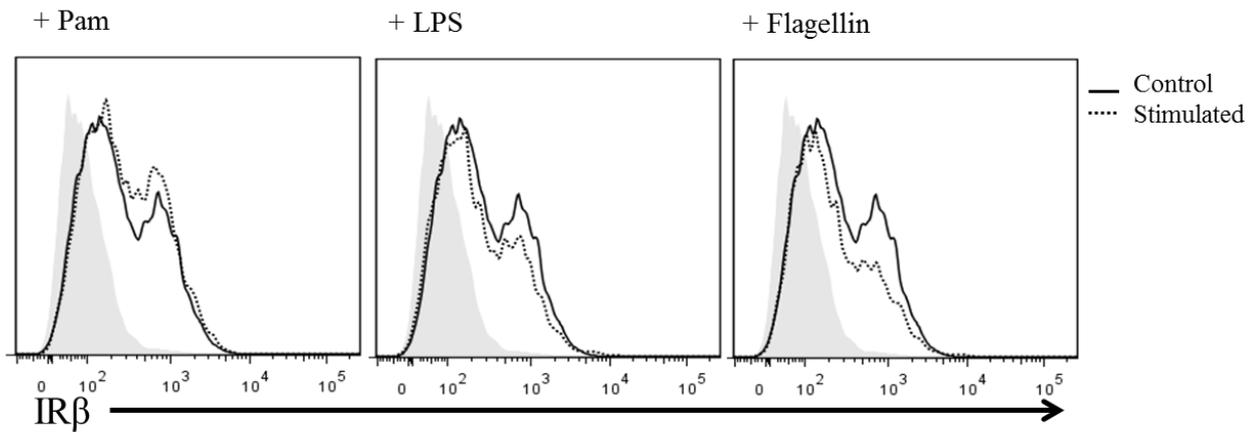


Figure 3.9 TLR ligation alters Insulin Receptor expression on MIN6 cells. Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml , Flagellin $5\mu\text{g/ml}$). Cells were then analysed for cell surface expression of the β subunit of the IR by flow cytometry. Histograms show IR- β expression on control (black line) and TLR stimulated (dashed line) cells. Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

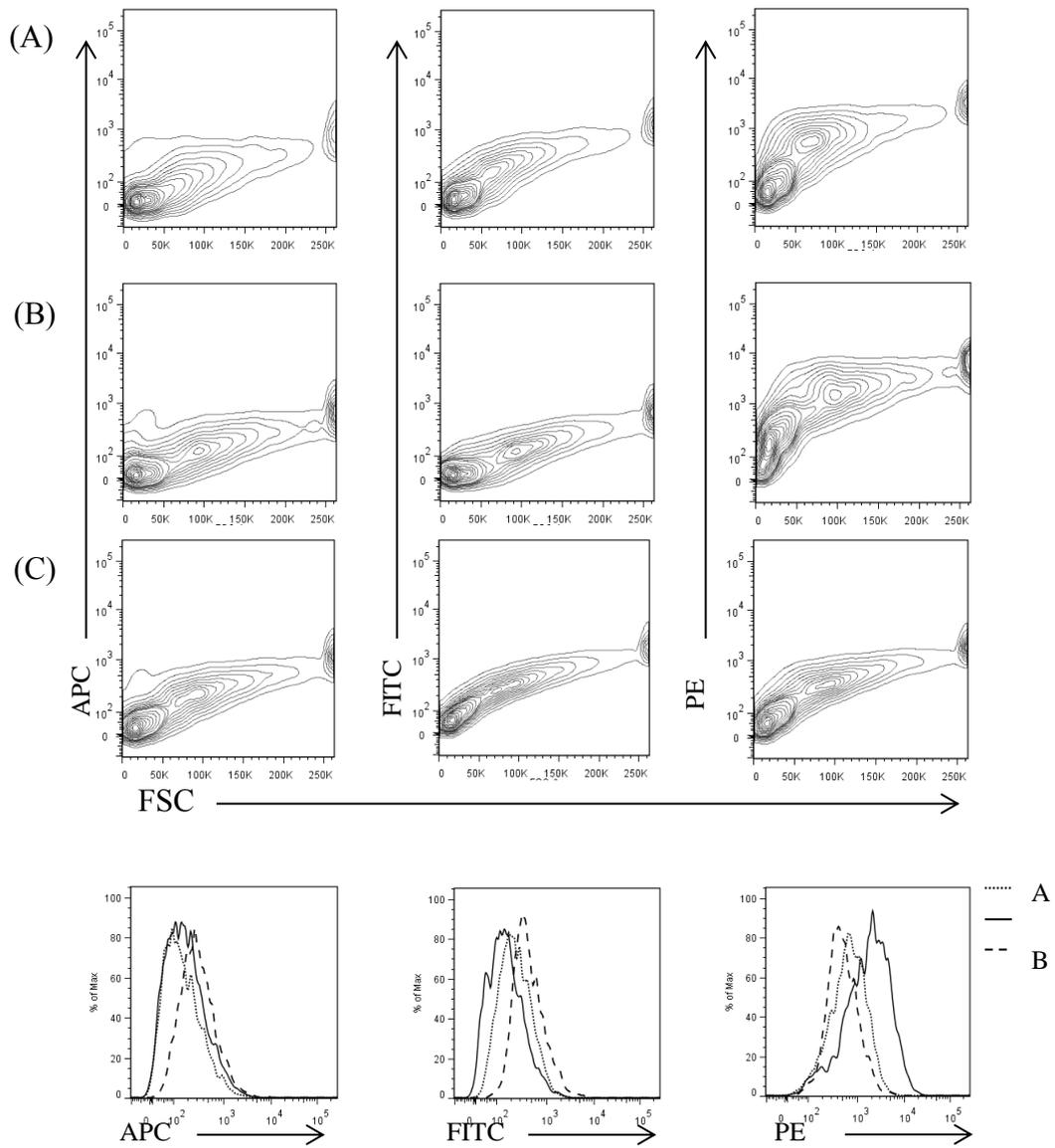


Figure 3.10 Optimisation of permeabilisation technique for MIN6 cells. Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency. Cells were then collected and permeabilised with a number of different methods (A) Triton, (B) Tween & (C) Saponin. Following this cells were stained with antibodies to Insulin (APC), TLR5 (FITC) & PDX-1 (PE) and analysed by flow cytometry on the BD FACS Aria.

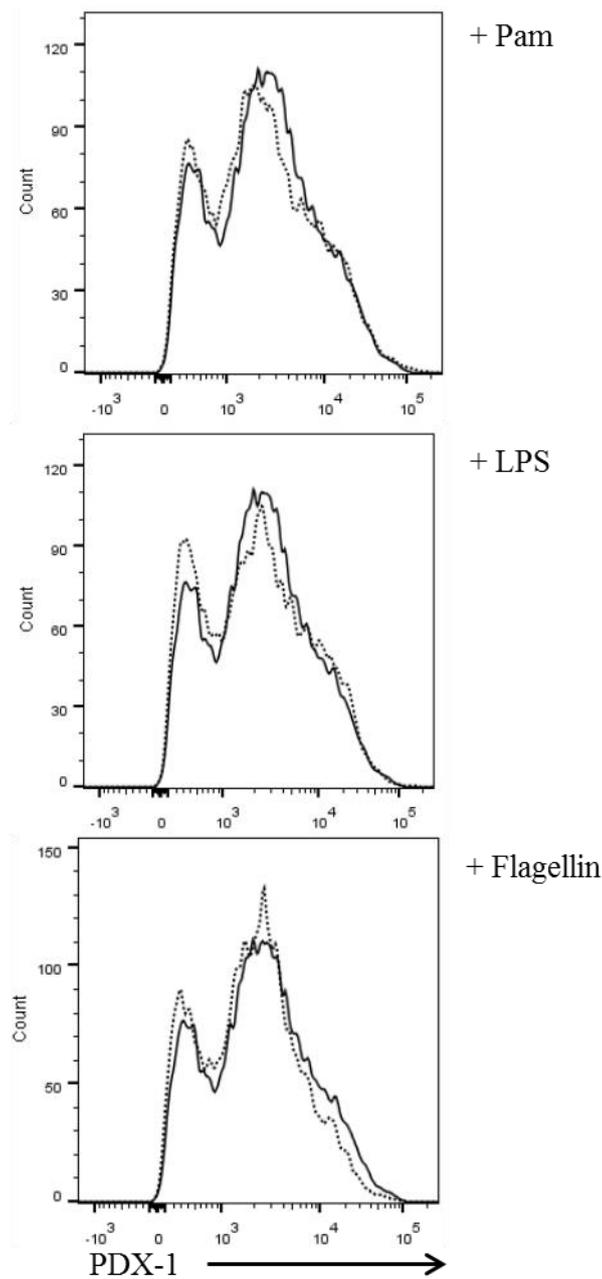


Figure 3.11 TLR ligation alters PDX-1 expression on MIN6 cells. Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands Pam ($1\mu\text{g/ml}$), LPS (100ng/ml) & Flagellin ($5\mu\text{g/ml}$). Cells were then analysed for expression of PDX-1 by flow cytometry. Histograms show PDX-1 expression on control (black line) and stimulated (dashed line) cells. Data are representative of three independent experiments

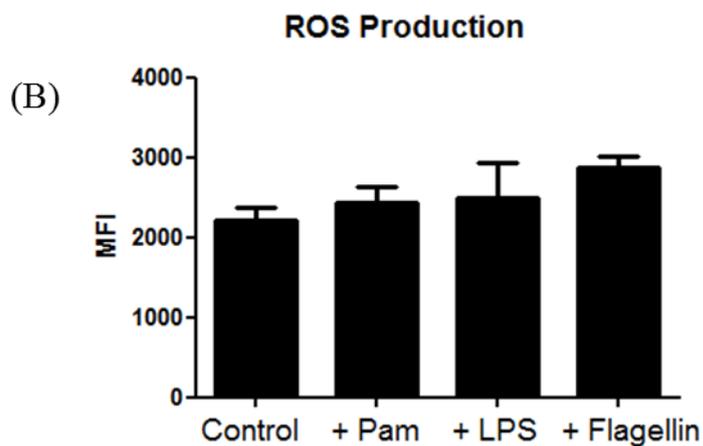
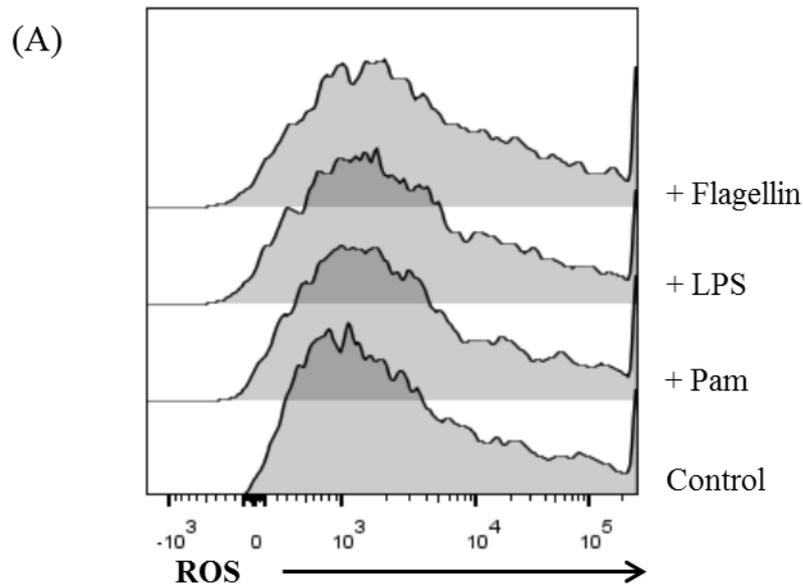


Figure 3.12 TLR stimulation does not affect Reactive Oxygen Species production from MIN6 cells. . Cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands Pam ($1\mu\text{g/ml}$), LPS (100ng/ml) & Flagellin ($5\mu\text{g/ml}$). To measure the production of reactive oxygen species, cell were labelled with DCFDA (Abcam), according to manufacturer's instructions, and the fluorescent signal relative to intracellular ROS generation was then measured by flow cytometry. In figure (A) Histograms represent fluorescence of DCFDA, histograms are offset to allow for easy comparison of multiple parameters. In Figure (B) MFI from 3 independent experiments is collated to analyse significance. Data are representative of three independent experiments.

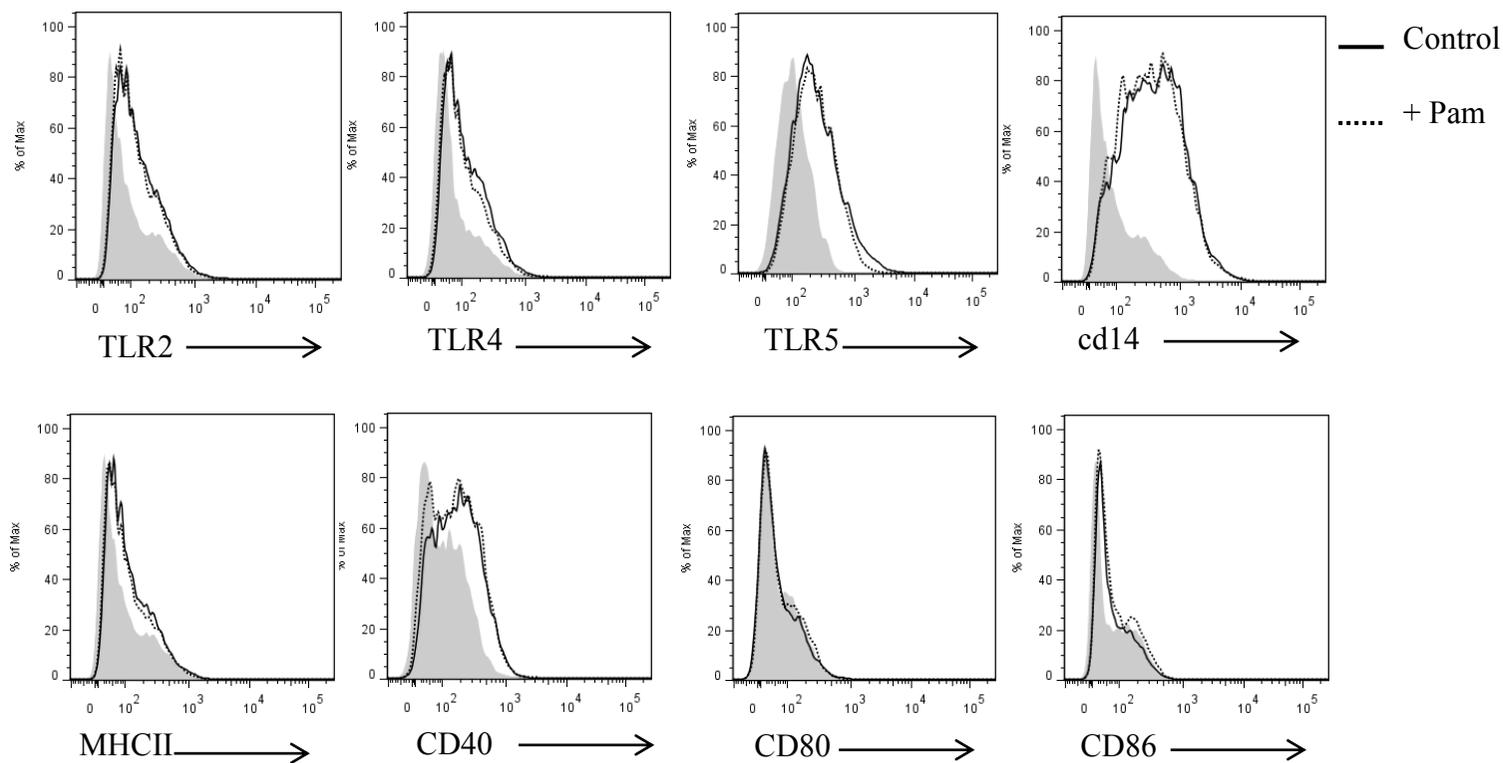


Figure 3.13 Expression of cell surface markers on MIN6 cells in response to stimulation with Pam. MIN6 cells were stimulated with PAM3CSK4 (1 μ g/ml) or medium alone as a control. After 24hr cells were stained with fluorescently labelled antibodies and the expression of surface markers was measured by flow cytometry. Histograms show surface marker expression of unstimulated control cells (black line) compared to TLR-ligand stimulated cells (dashed line). Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

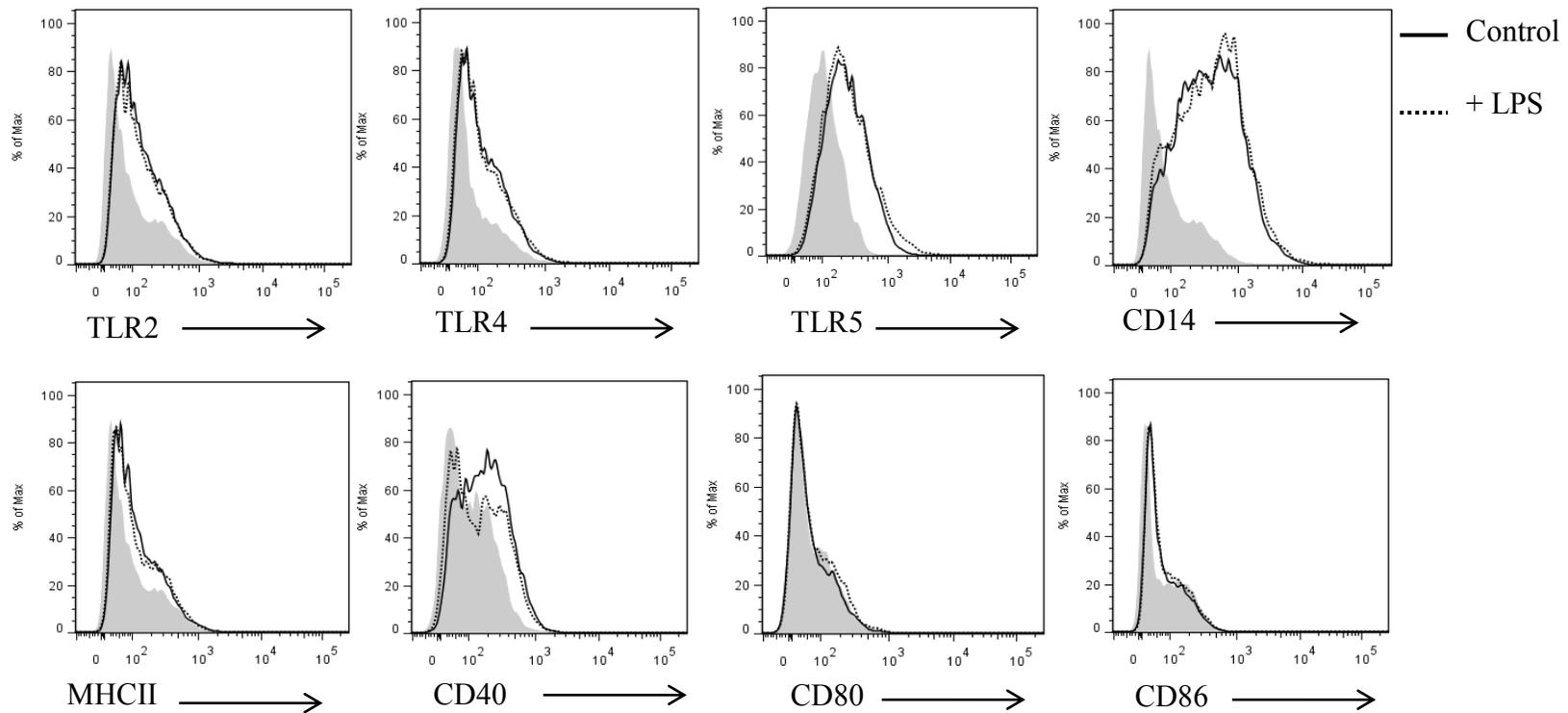


Figure 3.14 Expression of cell surface markers on MIN6 cells in response to stimulation with LPS. MIN6 cells were stimulated with LPS (100ng/ml) or medium alone as a control. After 24hr cells were stained with fluorescently labelled antibodies and the expression of surface markers was measured by flow cytometry. Histograms show surface marker expression of unstimulated control cells (black line) compared to TLR-ligand stimulated cells (dashed line). Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

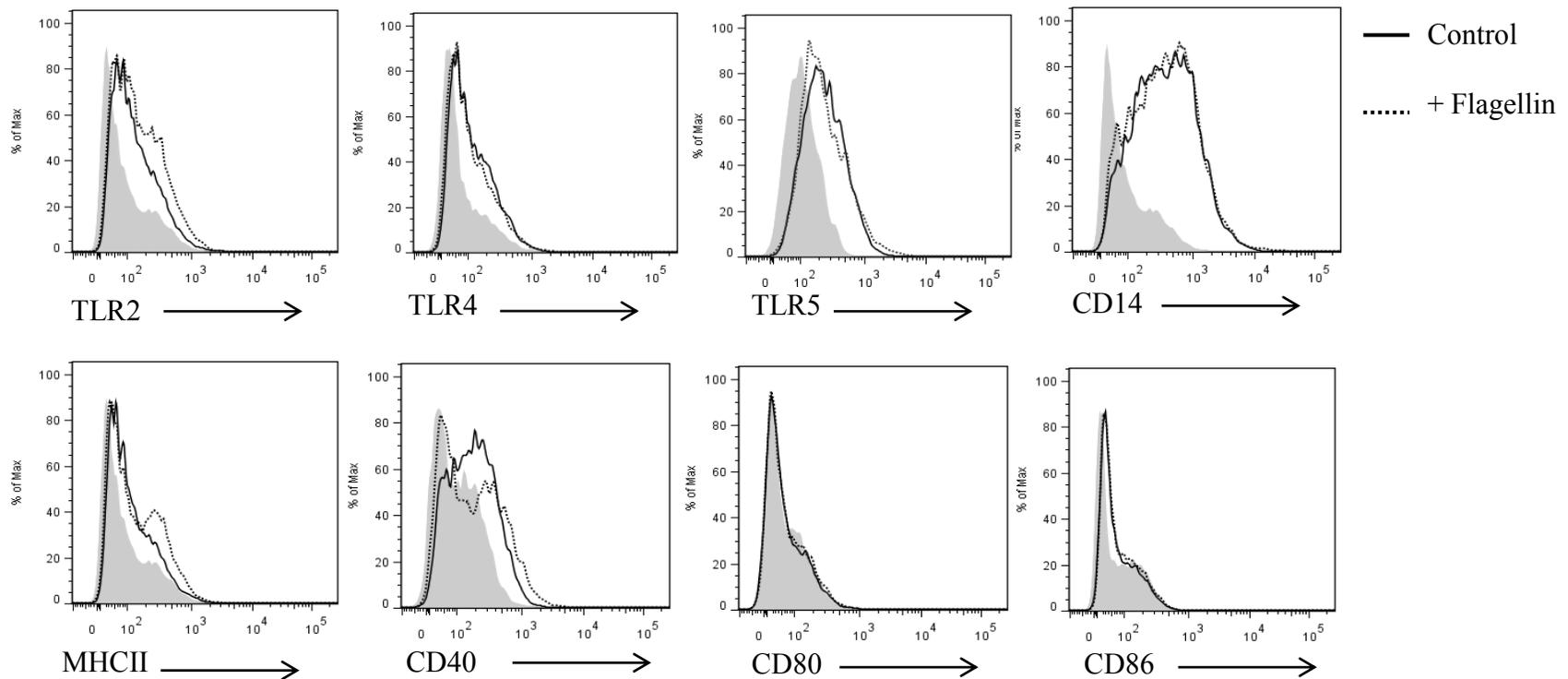


Figure 3.15 Expression of cell surface markers on MIN6 cells in response to stimulation with Flagellin. MIN6 cells were stimulated with Flagellin (5 μ g/ml) or medium alone as a control. After 24hr cells were stained with fluorescently labelled antibodies and the expression of surface markers was measured by flow cytometry. Histograms show surface marker expression of unstimulated control cells (black line) compared to TLR-ligand stimulated cells (dashed line). Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

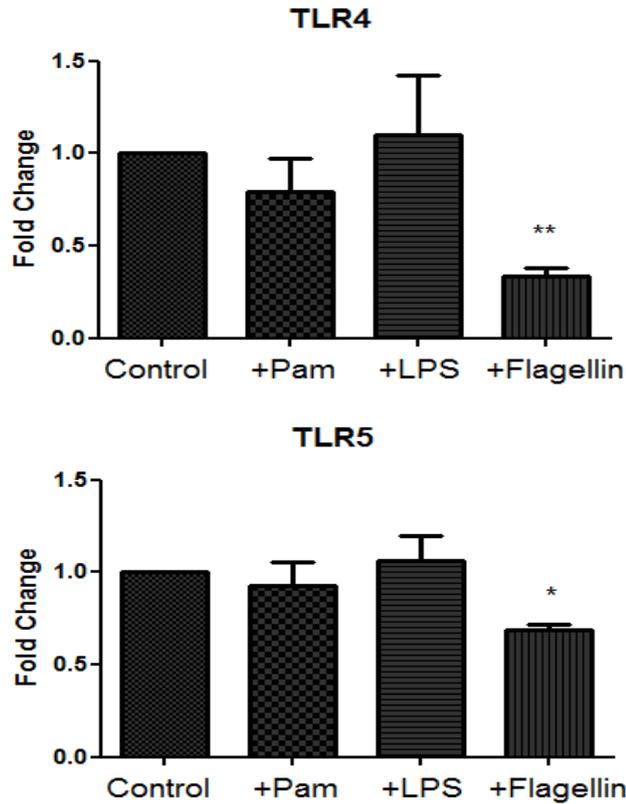


Figure 3.16 Expression levels of TLR4 & TLR5 following 24h TLR ligation. The expression of TLR4 and TLR5 was measured by qPCR. Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml , Flagellin $5\mu\text{g/ml}$) for 24hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for TLR4 & TLR5 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, B2M. The mean relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Results are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, vs. control, determined by unpaired *t*-test

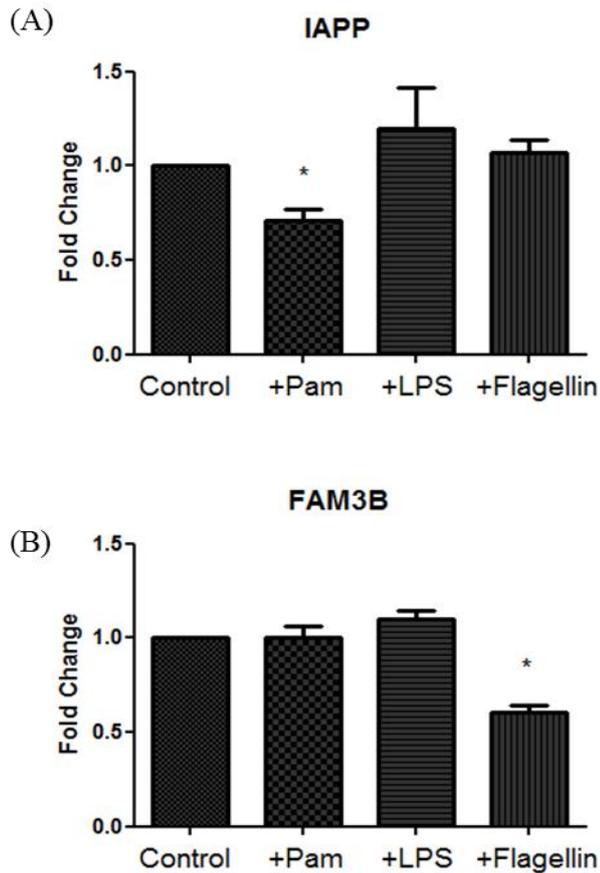


Figure 3.17 Expression levels of IAPP & FAM3B following 24h TLR ligation. The expression of IAPP and FAM3B was measured by qPCR. Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml , Flagellin $5\mu\text{g/ml}$) for 24hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for TLR4 (A) & TLR5 (B) (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, B2M. The mean relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Results are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, vs. control, determined by unpaired *t*-test

3.4 Discussion

MIN6 cells originate from a transgenic C57Bl/6 mouse insulinoma expressing an insulin-promoter/t-antigen construct (Miyazaki et al. 1990). They produce insulin and have morphological characteristics of pancreatic beta cells. MIN6 cells exhibit GSIS comparable with cultured normal mouse islet cells (Miyazaki et al. 1990) and are considered as an appropriate model for investigating the mechanism of glucose stimulated insulin secretion (GSIS). Glucose metabolism and the glucose concentration-dependence of insulin secretion from MIN6 cells closely resemble those in normal beta cells, and this cell line is thus a useful tool for studying GSIS in normal pancreatic beta cells (Ishihara et al. 1993). We have shown that MIN6 cells secrete insulin in a dose dependant manner following glucose stimulation. Although the increase seen in insulin secretion following glucose stimulation is not as great as that seen using either isolated islets or indeed *in vivo* the fact that glucose stimulation induced a reproducible insulin response still provided us with the possibility to study GSIS in an *in vitro* system. Glucose activation also induced PDX-1 translocation to the nucleus. Taken together these findings suggest that we were working with optimal culturing conditions for this cell line to promote insulin sensitivity.

In this chapter we set out to characterise MIN6 cells and assess the effects of TLRs on these cells, given the reports that pancreatic beta cells express TLRs. In our study we have confirmed that the MIN6 cells express receptors TLR2 and TLR4 and also provide novel data that they express TLR5. Activation of these receptors on MIN6 cells did not result in a significant decrease in cell viability. This is similar to findings from (Kiely et al. 2008) who describe no effect on cell viability following

24 hour stimulation with LPS (0-1000ng/ml) of the BRIN-BD11 β -cell line. Others (Garay-malpartida et al. 2011) have described a reduction in cell viability of isolated human islets following exposure to LPS (50ng/ml) however this was after 48 hour incubation in comparison to our 24 hour incubation so this may account for the differences observed. Another study (Weile et al. 2011a) also found no visible or measurable signs of death in cultured rat islets following 24h exposure to flagellin (50ng/ml).

A primary factor in the pathogenesis of type 2 diabetes is the inability of the β -cell to sustain the increased demand for insulin secretion in response to decreased insulin sensitivity seen in organs such as the liver, skeletal muscle and adipose tissue. The regulation of insulin secretion from the β -cell is complex and the molecular defects underlying the relative failure of the β -cell in type 2 diabetes are still unclear. One of the characteristic features of this defect is a failure of the β -cell to respond to a glucose stimulus while retaining its response to other secretagogues such as amino acids (Muio & Newgard 2008). Here we have shown that MIN6 cells in resting state actually increase their insulin output following stimulation with Pam, LPS and Flagellin however their ability to respond to glucose (20mM) is altered with a decrease seen in GSIS following TLR stimulation particularly with LPS and Flagellin. This is similar to findings from Vives-Pi et al who report that LPS enhanced insulin secretion from rat islets at low glucose concentrations (5.5mM) while reducing secretion at higher glucose concentrations (16.7mM) (Vives-Pi et al. 2003). The same can be said for Flagellin, with Weile *et al* finding that basal insulin secretion was increased, however glucose stimulated insulin secretion was decreased in rat islets following TLR5 activation (Weile et al. 2011a). This suggests that

activation of these TLRs on the β -cell is in some way inhibiting glucose stimulated insulin secretion from the cell. Although all three TLR ligands similarly effect glucose stimulated insulin secretion, they differentially alter intracellular insulin content with Pam and LPS significantly reducing and Flagellin significantly increasing total insulin. In the case of LPS similar findings have been reported elsewhere (Garay-malpartida et al. 2011; Amyot et al. 2012) in the case of both isolated islets and MIN6 β -cells, however Kiely *et al* reported no change in insulin content in the clonal rat BRIN-BD11 cell line. Slight variations in the MIN6 and BRIN-BD11 cell lines may account for the differences observed. The fact Pam and LPS decreased and Flagellin increased insulin content suggests that the observed effect of ligation on glucose stimulated insulin secretion occurs independent of an effect on intracellular insulin stores. Furthermore the novel observation that Flagellin activation actually resulted in an increased intracellular insulin concentration shows that it is most likely activating a different downstream signalling pathway than that of LPS. Whereas secretion of insulin is similarly affected by all three ligands, Pam and LPS appear to be having a negative impact on insulin translation and synthesis. Flagellin on the other hand appears to be having a positive effect on insulin translation with an increase in intracellular insulin stores observed.

Most animals have only a single copy of the insulin gene, but rodents have two non-allelic insulin genes (INS1 & INS2). They differ in their number of introns and chromosomal locations (Soares et al. 1985). Although no great effect of TLR ligand activation was seen on insulin gene expression with only Flagellin ($p < 0.05$) causing a decrease in INS2 expression, a more profound effect of TLR ligation can be seen

on the expression of the IRS proteins with Pam and Flagellin increasing IRS2 expression levels. Insulin receptor substrate (IRS) proteins mediate a variety of the metabolic and growth-promoting actions of insulin and IGF-1. After phosphorylation by activated receptors, these intracellular signalling molecules recruit various downstream effector pathways including phosphatidylinositol 3-kinase and Grb2 (Burks & White 2001). Ablation of the IRS-2 gene in mice results in a phenotype with characteristics of type 2 diabetes; IRS-2-deficient animals present defects in both insulin action and insulin production and reduced β -cell mass (Withers et al. 1998). In direct contrast to the reduced β -cell mass caused by deletion of IRS-2, disruption of the gene for IRS-1 enhances β -cell mass in mice; IRS1^{-/-} mice have a twofold increase in pancreatic β cells (Brüning et al. 1997). The absence of IRS1 in islets appears to also convey functional abnormalities in β -cell physiology. IRS1^{-/-} β -cells display a reduced response to both glucose and arginine (Kulkarni et al. 1999). With this in mind it is interesting to note that TLR ligation seemed to cause somewhat of a decrease in expression, particularly with Flagellin (although not significantly) in IRS1 expression. If this is the case, it ties in nicely with the observed reduction in GSIS from MIN6 cells following TLR ligation. The increase seen in IRS2 expression following TLR2 and TLR5 ligation could perhaps serve as a protective mechanism in the β -cell. IRS2 is also known to play an anti-apoptotic role in the β -cell (Withers et al. 1998) where increased numbers of apoptotic cells were found to be present within the β -cells of IRS2^{-/-} mice compared with wild-type animals. In the setting of activation of innate immunity and perhaps inflammation, shifting of the balance from IRS1 to IRS2 may therefore provide the β -cell with some level of protection. This “shift” is most apparent when cells are activated with Flagellin as a decrease is seen in IRS1 mRNA expression with a concurrent increase

in IRS2 expression levels. Once again this suggests a potential protective role for TLR5 in β -cells.

There are other ways in which the ligands may be affecting GSIS. Insulin is known to act on the β -cell in an autocrine and paracrine fashion through the transmembrane insulin receptor (IR) (Okada et al. 2007). Mice lacking the β -cell insulin receptor show a loss of insulin secretion in response to glucose, but not to arginine (Kulkarni et al. 1999), and depletion of the IR with siRNA in murine insulinoma cells leads to inhibition of glucose stimulated insulin secretion (Da Silva Xavier et al. 2004). These studies and others (Okada et al. 2007; Lee et al. 2012; Ohsugi et al. 2005) highlight a role for the β -cell IR in glucose stimulated insulin secretion, however its role remains undefined. Here we have shown that stimulation of MIN6 cells with TLR ligands (LPS and Flagellin) leads to a significant reduction in the cell surface expression of the β -subunit of the insulin receptor. This may account for the reduction observed in glucose stimulated insulin secretion following TLR stimulation. Both the insulin receptor and Toll-like receptors share common downstream signalling pathways (Fresno et al. 2011). It is possible that engagement of TLRs on β -cells results in the activation of MAP-kinases that interfere with insulin receptor substrate downstream of the insulin receptor leading to a compromised insulin secretion. One possible scenario in which TLR activation of β -cells could be relevant is in the case of sepsis in which there are increased levels of circulating TLR ligands. GSIS suppression may be relevant in this scenario to ensure a sufficient supply of glucose to important tissues such as the brain, and to phagocytic and reparative cells (Mizock 1995). This correlates with a general switch

from primarily insulin-dependent glucose uptake to primarily non-insulin dependent uptake during sepsis (Lang & Dobrescu 1991).

Pancreatic β -cells have the ability to produce reactive oxygen species (ROS) in response to certain stimuli such as high glucose levels and palmitate (Ježek et al. 2012) which in turn can affect insulin secretion (Sakai et al. 2003). TLR activation has been shown to induce ROS production in a number of cell types (Gill et al. 2010) however we did not find any increase in production of ROS from MIN6 cells following TLR activation. Pancreatic β -cells are also known to produce islet amyloid polypeptide (IAPP) and pancreatic derived factor (PANDER or FAM3B) in times of stress (Cao et al. 2003; Wang et al. 2011; Cai et al. 2011). Amyloid forms within pancreatic islets in type 2 diabetes from aggregates of the β -cell peptide IAPP. These aggregates are toxic to β -cells, inducing β -cell death and dysfunction, as well as inciting islet inflammation (Westermarck et al. 2011). PANDER is a cytokine-like protein that is highly expressed in pancreatic islets. *In vitro*, recombinant PANDER pre-treatment or PANDER overexpression significantly promotes apoptosis of α and β cells of mouse, rat, and human islets in a dose- and time-dependent manner (Cao et al. 2003; Cao et al. 2005). Overexpression of PANDER in mouse islets or the β -cell line β TC6 also represses glucose-stimulated insulin secretion (Yang et al. 2005). Treatment of MIN6 cells with TLR ligands did not increase expression levels of IAPP or FAM3B. In fact treatment with Pam significantly reduced expression of IAPP and treatment with Flagellin significantly reduced mRNA expression of FAM3B. Although we did not look at the protein levels, therefore post-translational modifications cannot be taken into account; Xiang *et al* while using the fatty acid palmitate to induce FAM3B expression in the β -cell line β TC6 reported findings

whereby treatment of β TC6 cells with palmitate significantly increased FAM3B mRNA and protein levels, and that the mRNA expression pattern was consistent with the protein expression pattern (Xiang et al. 2012). They also report that treatment of the cells with a JNK-specific inhibitor reduced the palmitate induced PANDER protein expression. This is a novel finding that TLR activation actually works in a way to reduce mRNA expression of proteins that can be harmful to the β -cell, once again highlighting a potential protective role for TLR5 in the β -cell. Also as there is no increase in mRNA expression levels of FAM3B, it is unlikely that it is contributing to the observed decrease in GSIS seen following TLR activation in MIN6 cells.

We also assessed the cell surface expression of a variety of immune surface markers on MIN6 cells in both control and TLR stimulated conditions and demonstrated that Min6 cells express TLR2, TLR4, TLR5, CD14, CD40, MHCII Our findings are similar to those of Garay-Malpartida *et al* who showed TLR4 and CD14 expression in murine islets and MIN6 cells, and observed a 4.5 and 7.6 fold increase in both TLR4 and CD14 mRNA levels respectively in response to LPS (50ng/ml) stimulation (Garay-malpartida et al. 2011). Contrary to their findings we did not see a significant increase in expression levels of these surface markers following stimulation, however this may be accounted for by the fact that they used a 48 hour time point as opposed to a 24 hour time point, and they looked at the expression at a transcriptional level where as we used flow cytometry. TLR2 (Vives-Pi et al. 2003) and TLR5 (Weile et al. 2011a) expression has been described in mouse islet cells, but to our knowledge had not been published in MIN6 cells as of yet. The only increase seen in surface expression was that of TLR2, MHCII and CD40 following exposure to Flagellin. As β -cells are not immune-cells per-se, changes in surface

marker expression may be minimal and therefore hard to detect flow cytometrically. Interestingly when examined at the level of mRNA, flagellin stimulation resulted in a significant decrease in expression levels of both TLR4 and TLR5. This once again may be a protective mechanism within the cell whereby upon initial activation of this TLR leads to down regulation of other TLRs on the cell to limit propagation of an inflammatory response.

To summarise we have shown that activation of TLR ligands on MIN6 cells has the ability to alter their insulin secretion in response to glucose, their insulin content, and insulin receptor expression as well as a number of other parameters without significantly affecting cell viability. Of note is that although all TLR ligands similarly affected MIN6 GSIS, they differentially affected MIN6 insulin content and INS and IRS expression levels among other things. This suggests that the TLR ligands are working in different pathways within the cell. Also worthy of mention is the fact that of all ligands used TLR5 seemed to have the most profound effect on MIN6 cells. It was the only ligand to increase MIN6 insulin content, decrease INS2 expression and it induced a “shift” from IRS1 to IRS2 expression. It also caused a decrease in FAM3B, TLR4 and TLR5 mRNA expression levels. To date the majority of research has focused on the role of TLR4 (and to a lesser extent TLR2) in the β -cell, however our work provides novel data on the important role of TLR5 in the β -cell. This is further emphasised by the fact that recent work has shown that mice lacking TLR5 develop hallmark features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity (Vijaykumar et al. 2010). Other reports have shown that glucose activation of mouse islet cells induced an increase in TLR5 mRNA expression levels (Weile et al. 2011a).

Taken together this highlights TLR5 as a novel player in β -cell physiology and it certainly warrants further investigation. In the next chapter we will further investigate the effect of TLR activation of MIN6 cells on secretion of inflammatory mediators. We will also address the crosstalk that exists between these cells and macrophage, which have been shown to infiltrate the islet in the setting of type 2 diabetes (Ehse et al. 2007).

Chapter 4

Inflammatory properties of MIN6 cells and investigation of their crosstalk with J774A.1 macrophage

4.1 Introduction

Cytokines and chemokines, small signalling proteins secreted primarily by immune cells, activate inter- and intracellular signalling during immune responses. Cytokine expression plays a fundamental role in the development and function of the immune system. Many different cell types secrete chemokines, most often to attract immune cells to the site of infection or injury during innate and adaptive immune responses. The effect of a cytokine release depends on the activated cell type expressing the specific cytokine receptor. Cytokines and their receptors participate in a diverse array of functions beyond innate and adaptive immunity including inflammation, immune cell differentiation, angiogenesis, tumorigenesis, development, neurobiology, and viral pathogenesis. Dysregulation of cytokine expression is a cause of immunological and inflammatory diseases as well as other disease states.

Typical cytokines thus far associated with diabetes and β -cell death are IL-1 β , IFN- γ and TNF- α . IL-1 β and/or TNF- α plus IFN- γ have been shown to induce β -cell apoptosis via the activation of β -cell gene networks under the control of the transcription factors NF- κ B and STAT-1 (Cnop et al. 2005; Wachlin et al. 2003). Additionally it has been shown that oligomers of islet amyloid polypeptide (IAPP) triggered the NLRP3 inflammasome and generated mature IL-1 β in pancreatic islets (Hull et al. 2009). Other mediators such as high glucose and FFA's have also been shown to induce islet cytokine secretion (Boni-Schnetzler et al. 2009; Yin et al. 2014; Böni-Schnetzler et al. 2008; Donath et al. 2010)

Cytokines and chemokines both have the ability to activate macrophages. Macrophages are key innate immune effector cells best known for their role as professional phagocytes. Macrophages play a crucial role in innate and adaptive immunity in response to microorganisms and are major mediators of the inflammatory response. During infection, macrophage effector functions are critical for the elimination of pathogens; however uncontrolled inflammatory responses can induce injury of the tissue environment and must be repressed to allow the process of repair. Two major macrophage populations have been characterised *in vitro*. Classically activated or type I macrophages, induced in particular by IFN γ (Dalton *et al* 1993), display a proinflammatory profile whereas alternatively activated or type II macrophages, induced by Th-2 cytokines, express anti-inflammatory and tissue repair properties (Gordon 2003). Tissue macrophages respond to changes in the local environment by changing their polarization status, and, thus, the M1 and M2 classifications are oversimplifications of the more dynamic and varied polarization states of macrophages that can be observed *in vivo*.

To date the majority of research has focused on the role of the adipose tissue macrophages (ATM) in the pathogenesis of obesity, insulin resistance and type 2 diabetes. The discovery that adipose tissue from obese mice and humans is infiltrated with increased numbers of macrophages provided a major mechanistic advancement into understanding how obesity propagates inflammation (Weisberg *et al.* 2003). These ATMs are a major source of proinflammatory cytokines, which can function in a paracrine and autocrine fashion to cause decreased insulin sensitivity. Activation of these tissue macrophages leads to a release of various chemokines, which in turn recruit additional macrophages, setting up a feed-forward process that further

increases ATM content and propagates the chronic inflammatory state (Kanda et al. 2006). It was relatively recently that the first study demonstrating increased islet macrophage infiltration in patients with type 2 diabetes (Ehnes et al. 2007) was published. Increased islet-associated immune cells were also observed in a variety of animal models of this disease including the GK (Goto-Kakizaki) rat (Homo-Delarche et al. 2006) the high-fat-diet fed and *db/db* mouse (Ehnes et al. 2007) and the Cohen diabetic rat (Weksler-zangen et al. 2008). Whether or not the presence of macrophage is causative or a symptom of type 2 diabetes islet pathology remains unclear.

In the last chapter we characterised the MIN6 β -cell line. Our aim now is to investigate the effect of TLR ligation on MIN6 cytokine and chemokine secretion and explore what role they may play in the function of the β -cell. Secondly given that we found that activation of MIN6 cells with TLRs increased chemokine production, and these supernatants were capable of inducing macrophage migration our objective was to investigate the crosstalk that exists between the pancreatic β -cell line MIN6 and the macrophage cell line J774A.1 This was examined by culturing J774A.1 macrophages in MIN6 conditioned media and assessing their viability, surface marker expression, cytokine and chemokine secretion and subsequent response to activation by a panel of TLR ligands.

4.2 Results

4.2.1 Effect of TLR stimulation on MIN6 cytokine and chemokine secretion

TLR stimulation has long been understood to be an inducer of both cytokine and chemokine secretion in numerous cell types. To investigate the effect of TLR stimulation of MIN6 cytokine and chemokine production cells were plated at a concentration of 1×10^6 cells per ml and allowed to grow to confluency prior to 24hr stimulation with ligands (Pam $1\mu\text{g/ml}$, LPS 100ng/ml & Flagellin $5\mu\text{g/ml}$). Following this supernatant was removed, spun down in a centrifuge to remove any cell debris and analysed for cytokine and chemokine content by ELISA (R&D systems, Minneapolis, MN, USA). There was no detectable levels of the cytokines IL- 1β , IL-2, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, TNF- α and IFN- γ . Under control conditions MIN6 cells secrete the cytokines IL-4 & IL-27p28 and chemokines Mip-1, Mip-2, MCP-1 & RANTES [Figure 4.1].

Stimulation with Pam did not alter IL-4 secretion but did cause a significant increase in levels of IL-27p28 ($P<0.05$), Mip-1 ($P<0.001$), Mip-2 ($P<0.001$), MCP-1 ($P<0.05$) and RANTES ($P<0.05$) [Figure 4.1]. Stimulation with LPS resulted in a significant decrease in IL-4 secretion ($p<0.001$) and a significant increase in secretion of IL-27p28 ($p<0.01$), Mip-1 ($p<0.05$), Mip-2 ($p<0.001$), MCP-1 ($p<0.01$) and RANTES ($p<0.001$) [Figure 4.1]. Stimulation with flagellin also resulted in a significant decrease in IL-4 secretion ($p<0.001$) and a significant increase in secretion of IL-27p28 ($p<0.001$), Mip-1 ($p<0.001$), Mip-2 ($p<0.001$), MCP-1 ($p<0.05$) and RANTES ($p<0.01$) [Figure 4.1].

Although no detectable levels of IL-1 β , TNF- α and IFN- γ secretion were found, as these cytokines are known to induce β -cell death (Wachlin et al. 2003) we assessed their expression at mRNA level. Stimulation with Flagellin did cause a significant increase in both IL-1 β (p<0.01) and TNF- α (p<0.001) mRNA expression levels [Figure 4.4]. With IL-1 β only a half a fold increase is seen however with TNF- α 30 fold increase. No significant change in IFN- γ expression was observed.

4.2.2 Min6 cells express the IL-4 receptor and stimulation with IL-4 results in increased PDX-1 expression

As we found that TLR stimulation resulted in the decreased secretion of interleukin 4 from MIN6 cells, along with altered glucose stimulated insulin secretion and intracellular content, we thought it pertinent to investigate the effects of IL-4 on Min6 cells. We first checked for expression of the IL-4 receptor (also known as CD124) by flow cytometry. To do this, cells were plated as previously described and stained with a fluorescently labelled antibody to the IL-4 receptor. **Figure 4.5A** shows expression of the IL-4 receptor on MIN6 cells when compared to unstained cells. In order to assess the effects of IL-4 on PDX-1 expression, MIN6 cells were stimulated with 10ng/ml of recombinant IL-4 for 24 hr. Cells were then fixed, permeabilised and stained with a fluorescently labelled PDX-1 antibody. Cells treated with recombinant IL-4 showed higher expression levels of PDX-1 [Figure 4.5B]. Histograms show surface marker expression of unstimulated control cells (black line) compared to IL-4 stimulated cells (dashed line). Filled histograms represent fluorescence of unstained cells.

4.2.3 Pre-incubation with Flagellin and IL-27 provides partial protection against Streptozotocin induced cell death in MIN6 cells

As we found that MIN6 cells secrete IL-27p28 in response to TLR ligation [Figures 4.1 - 4.3] we wanted to see if perhaps IL-27p28 played a protective role in MIN6 cells. Streptozotocin (STZ) is a known inducer of cell death in pancreatic β -cells. As LPS and Flagellin were shown to be the strongest inducers of IL-27 secretion cells were pre-incubated (for 24hr) with either IL-27p28 (10ng/ml), LPS (100ng/ml) or Flagellin (5 μ g/ml) prior to 24hr stimulation with STZ (2.5mM). 20 μ l of the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO₂ for 1 Hour. The plate was then read at a wavelength of 490nm. Results are expressed as a percentage of control cell viability. Both IL-27p28 (p<0.05) and Flagellin (p<0.01) significantly reduced cell death when compared to cells treated with STZ alone [Figure 3.21]. Pre-incubation with LPS did not significantly alter cell viability.

4.2.4 TLR stimulated MIN6 secretions induce J774A.1 macrophage migration

Type 2 diabetes is associated with an increased number of islet associated macrophages (Richardson et al. 2009; Ehses et al. 2007). As we saw that stimulation of MIN6 cells with TLR ligands had the ability to elicit secretion of chemokines [Figures 4.1 – 4.3] we wanted to investigate whether these secretions could elicit a chemotactic response. MIN6 cells were plated and stimulated as previously described. Following stimulation supernatants were removed and spun down to remove any cell debris. Supernatants were then transferred to the bottom of a trans-well plate. Microporous inserts containing 5 x 10⁵ J774A.1 macrophage were then

placed in each well and left for 6hr after which time the inserts were removed and the number of migrated cells were counted using the BD FACS ARIA. **Figure 4.7** shows that supernatants from all treatments increased J774A.1 migration, with LPS ($p<0.05$) and Flagellin ($P<0.01$) reaching significance.

4.2.5 Effect of MIN6 conditioning on J774A.1 macrophage in control conditions

4.2.5.1 Culture of J774A.1 Macrophage in MIN6 conditioned medium does not affect cell viability

Given that we wanted to examine the effect that MIN6 conditioned media had on the inflammatory profile of J774 macrophages it was first essential to investigate whether culture in this conditioned media affected the viability of J774 macrophages.

MIN6 cells were cultured in DMEM-glutamax for 24hr, after which the conditioned media was removed and spun down to remove any cell debris. J774 macrophages were then plated in 96 well plates in either MIN6 conditioned media or unconditioned DMEM as a control. The viability of the cells was then assessed using the MTS assay, 24 and 48hr later.

Culture of J774 macrophage in MIN6 conditioned media for either 24 or 48hr did not significantly alter cell viability [**Figure 4.8**].

4.2.5.2 MIN6 conditioned media does not have a strong effect of J774A.1 surface marker expression

J774A.1 macrophage were plated at a concentration of 1×10^6 cells/ml in 6 well plates in either MIN6 conditioned media or unconditioned DMEM-glutamax (control). The cells were left for 24hr prior to analysis of surface marker expression by flow cytometry. Cells were stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CCR5, CD80, CD86 and CD40. Histograms show surface marker expression of control cells (black line) compared to MIN6 conditioned cells (dashed line). Filled histograms represent fluorescence of unstained cells. As would be expected of a macrophage cell line J774A.1 show clear expression levels of all surface markers tested for [Figure 4.9]. Conditioning with MIN6 secretions resulted in a slight decrease in expression levels of surface markers TLR2, MHCII, CCR5 and CD40.

4.2.5.3 Cytokine and chemokine secretion from MIN6 conditioned J774A.1

J774A.1 macrophage were plated at a concentration of 1×10^6 cells/ml in 6 well plates in either MIN6 conditioned media or unconditioned DMEM-glutamax (control). The cells were left for 24hr, the media was then removed and spun down to remove any cell debris and analysed for cytokine and chemokine content by ELISA (R&D). Conditioning of macrophage did not result in any significant changes in cytokine secretion [Figure 4.10]. Conditioned macrophage show no difference in chemokines MIP-1 and MIP-2 secretion, however display a significant increase in MCP-1 ($p < 0.01$) and a significant decrease in RANTES ($p < 0.01$) secretion when compared to control macrophage [Figure 4.11].

4.2.6 Effect of MIN6 conditioning on macrophage response to TLR ligation

To better assess the effect of MIN6 conditioning on macrophage phenotype we next investigated how conditioned macrophage respond to TLR ligation compared to control macrophage.

4.2.6.1 Conditioned macrophage show altered surface marker expression following TLR ligation

J774 macrophage were then plated at a concentration of 1×10^6 cells/ml in 6 well plates in either MIN6 conditioned media or unconditioned DMEM-glutamax (control). The cells were then left to rest for 24hr prior to 24hr stimulation with either PGN (5 μ g/ml), LPS (100ng/ml) or Flagellin (5 μ g/ml). Cells were stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CCR5, CD80, CD86 and CD40. Histograms show surface marker expression of stimulated cells (black line) compared to stimulated MIN6 conditioned cells (dashed line). Grey line represents unstimulated control cells and filled histograms represent fluorescence of unstained cells. Note for CCR5 and CD40 histograms are off-set as there are two populations and it allows for easier visualisation. J774.A1 macrophages respond to TLR ligation (TLR2, TLR4 & TLR5) with an increase in cell surface marker expression [**Figure 4.12, 4.15 and 4.18**]. In almost all cases conditioning of macrophages prior to TLR stimulation results in a decreased surface marker expression response [**Figure 4.12, 4.15 and 4.18**]. Exceptions to this rule are in the case of Flagellin stimulated macrophage where conditioning fails to decrease surface marker expression of TLR4, MHCII, CD80 and CD86 and CD40 expression is actually increased [**Figure 4.18**].

4.2.6.2 Conditioned macrophage show altered cytokine response following TLR ligation

J774 macrophage were plated at a concentration of 1×10^6 cells/ml in 6 well plates in either MIN6 conditioned media or unconditioned DMEM-glutamax (control). The cells were then left to rest for 24hr prior to 24hr stimulation with either PGN (5 μ g/ml), LPS (100ng/ml) or Flagellin (5 μ g/ml). The media was then removed and spun down to remove any cell debris and analysed for cytokine content by ELISA (R&D). TLR activation of J774A.1 macrophage resulted in increased secretion of cytokines [Figures 4.13, 4.16 and 4.19]. Exceptions to this are once again only in the case of activation with flagellin which did not elicit an IL-6 or IL-27 response [Figure 4.19]. As a general rule preconditioning of macrophages with MIN6 media prior to activation with TLR ligands resulted in significant decreases in cytokine secretion. Upon activation with PGN, preconditioned macrophage show decreased secretion of IL-6 ($p < 0.001$), IL-12p40 ($p < 0.001$), IL-23 ($p < 0.001$), TNF- α ($p < 0.001$) and IL-27 levels remain unchanged in stimulated versus preconditioned stimulated cells [Figure 4.13]. Upon activation with LPS preconditioned macrophage show decreased secretion of IL-6 ($p < 0.001$), IL-12p40 ($p < 0.001$), IL-27p28 ($p < 0.05$), IL-23 and TNF- α levels remain unchanged in stimulated versus preconditioned stimulated cells [Figure 4.16]. Upon activation with flagellin preconditioned macrophage show decreased secretion of IL-12p40 ($p < 0.001$), IL-23 ($p < 0.05$), there is no change to TNF- α levels in stimulated versus preconditioned stimulated cells and there is a slight increase in IL-27 levels although it did not reach significance [Figure 4.19]. Data is summarised in Table 4.1.

	+ PGN	+ LPS	+ Flagellin
IL-6	↓***	↓***	↔
IL-12p40	↓***	↓***	↓***
IL-23	↓***	↔	↓**
IL-27	↔	↓**	↔
TNF-α	↓***	↔	↔
↓ = decrease in secretion ↔ = no change in secretion **p<0.01, ***p<0.001 as determined by one way ANOVA			

Table 4.1 Summary of conditioning effect on macrophage cytokine secretion following TLR activation.

4.2.6.3 Conditioned macrophage show altered chemokine response following TLR ligation

J774 macrophage were plated at a concentration of 1×10^6 cells/ml in 6 well plates in either MIN6 conditioned media or unconditioned DMEM-glutamax (control). The cells were then left to rest for 24hr prior to 24hr stimulation with either PGN (5 μ g/ml), LPS (100ng/ml) or Flagellin (5 μ g/ml). The media was then removed and spun down to remove any cell debris and analysed for chemokine content by ELISA (R&D). TLR activation of J774A.1 macrophage resulted in increased secretion of all chemokines tested for [Figures 4.14, 4.17 and 4.20]. Upon activation with PGN, preconditioned macrophage show decreased secretion of MIP-1 (p<0.001), MIP-2 (p<0.001), MCP-1 (p<0.01) and RANTES (p<0.01) [Figure 4.14]. Upon activation with LPS, preconditioned macrophage show decreased secretion of RANTES (P<0.001), no change in secretion of MIP-1 or MIP-2 and enhanced secretion of MCP-1 (p<0.001) [Figure 4.17]. Upon activation with flagellin, preconditioned

macrophage show decreased secretion of MIP-1 ($p<0.001$) and RANTES ($P<0.001$) and no significant change in secretion of MIP-2 or MCP-1, however the trend appears to be increased [Figure 4.20]. Data is summarised in Table 4.2.

	+ PGN	+ LPS	+ Flagellin
MIP-1	↓***	↔	↓***
MIP-2	↓***	↔	↔
MCP-1	↓**	↑***	↔
RANTES	↓**	↓***	↓***
↓ = decrease in secretion ↔ = no change in secretion ↑ = increase in secretion ** $p<0.01$, *** $p<0.001$ as determined by one way ANOVA			

Table 4.2 Summary of conditioning effect on macrophage chemokine secretion following TLR activation.

4.2.7 Conditioned macrophage display phagocytotic ability comparable to control cells

Macrophages are often referred to as “professional phagocytes” in so far as one of their key roles in the body is to phagocytose pathogens and cell debris which initiates the innate immune response, which in turn orchestrates the adaptive response (Aderem & Underhill 1999). It has been reported that macrophage from patients with T2D have impaired phagocytic capability (Lecube et al. 2011). For this reason we thought it pertinent to assess phagocytotic ability of conditioned macrophage compared to control. To do this J774A.1 macrophages were cultured in the presence of conditioned or control media for 24hr before we assessed their ability to phagocytose. When the phagocytosis was measured in response to stimuli, TLR

ligands were added for 24hr following 24hr pre-conditioning. To measure phagocytosis, fluorescent latex beads were added to the culture and macrophages were left to phagocytose for 3hr. Cells were then washed and the uptake of beads was measured flow cytometrically. Cells that ingested the beads display green fluorescence which can be measured in the FITC channel of the flow cytometer and expressed as a percentage of phagocytosing (FITC⁺) cells. Macrophages do increase their phagocytosis following activation with TLR ligands, however only minimally [Figure 4.21]. Pre-conditioning macrophage prior to activation does not appear to affect phagocytosis [Figure 4.21].

4.2.8 Conditioned macrophages produce lower levels of reactive oxygen species (ROS) in a steady state and in response to stimulation

Classically activated macrophages produce large quantities of inflammatory mediators such as reactive oxygen species (ROS) in response to bacterial endotoxins (Gill et al. 2010). ROS levels were measured using the cell permeant reagent 2',7' – dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxy and other ROS activity within the cell. J774A.1 macrophages were conditioned as previously described and the fluorescent signal relative to intracellular ROS generation was then measured by flow cytometry and compared between unconditioned (black histograms) and conditioned cells (dashed histograms). Conditioned cells showed decreased production of ROS in both a steady state and following stimulation with both PGN and LPS [Figure 4.22].

4.2.9 Conditioned macrophage show increased “M2” genes and decreased “M1” genes

As mentioned previously two major macrophage populations have been characterised *in vitro*. Classically activated or type I macrophages, induced in particular by IFN γ , display a proinflammatory profile whereas alternatively activated or type II macrophages, induced by Th-2 cytokines, express anti-inflammatory and tissue repair properties (Gordon 2003). One method to identify activation status of macrophage is to look at expression levels of certain genes. To do this J774A.1 macrophage were plated and conditioned as previously described. Following 24hr conditioning, cells were either stimulated with PBS (control) or LPS (100ng/ml) for a further 24 Hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The levels of IL-1 β , NOS2, ARG1 and MRC1 were quantified using qPCR. The expression levels were normalised to actin levels and the gene expression is shown as a fold change relative to the control. MIN6 conditioned macrophage show decreased expression of the “M1” genes IL-1 β and NOS2 in both steady state (IL-1 β p<0.05, NOS2 p<0.001) and following stimulation with LPS (IL-1 β p<0.01, NOS2 p<0.001) [Figure 4.23]. Concurrently conditioned macrophage show an increase in typical “M2” genes ARG1 and MRC1 both in steady state (ARG1 p<0.001, MRC1 p<0.05) and also following stimulation with LPS (ARG1 p<0.001, MRC1 p<0.05) [Figure 4.24].

4.2.10 Conditioning macrophage alters IRS2 expression but not IRS1 expression

As T2D has been shown to impair IRS2 mediated PI3K activity in primary macrophage (Connor et al. 2007a) we thought to investigate the effect of stimulation with LPS had on IRS expression levels in both control and conditioned macrophage. J774A.1 macrophage were plated and conditioned as previously described. Following 24hr conditioning cells were either stimulated with PBS (control) or LPS (100ng/ml) for a further 24hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The levels of IRS1 and IRS2 were quantified using qPCR. The expression levels were normalised to actin levels and the gene expression is shown as a fold change relative to the control. Stimulation of J774 macrophage with LPS caused an increase in IRS1 expression in both conditioned ($p<0.01$) and control cells ($p<0.01$) [Figure 4.25A]. Stimulation with LPS also caused an increase in IRS2 expression ($p<0.05$) however this increase is blocked in conditioned macrophage ($p<0.05$) [Figure 4.25B]. This decrease in IRS2 expression is seen in both steady state and LPS treated macrophage.

4.3 Figures

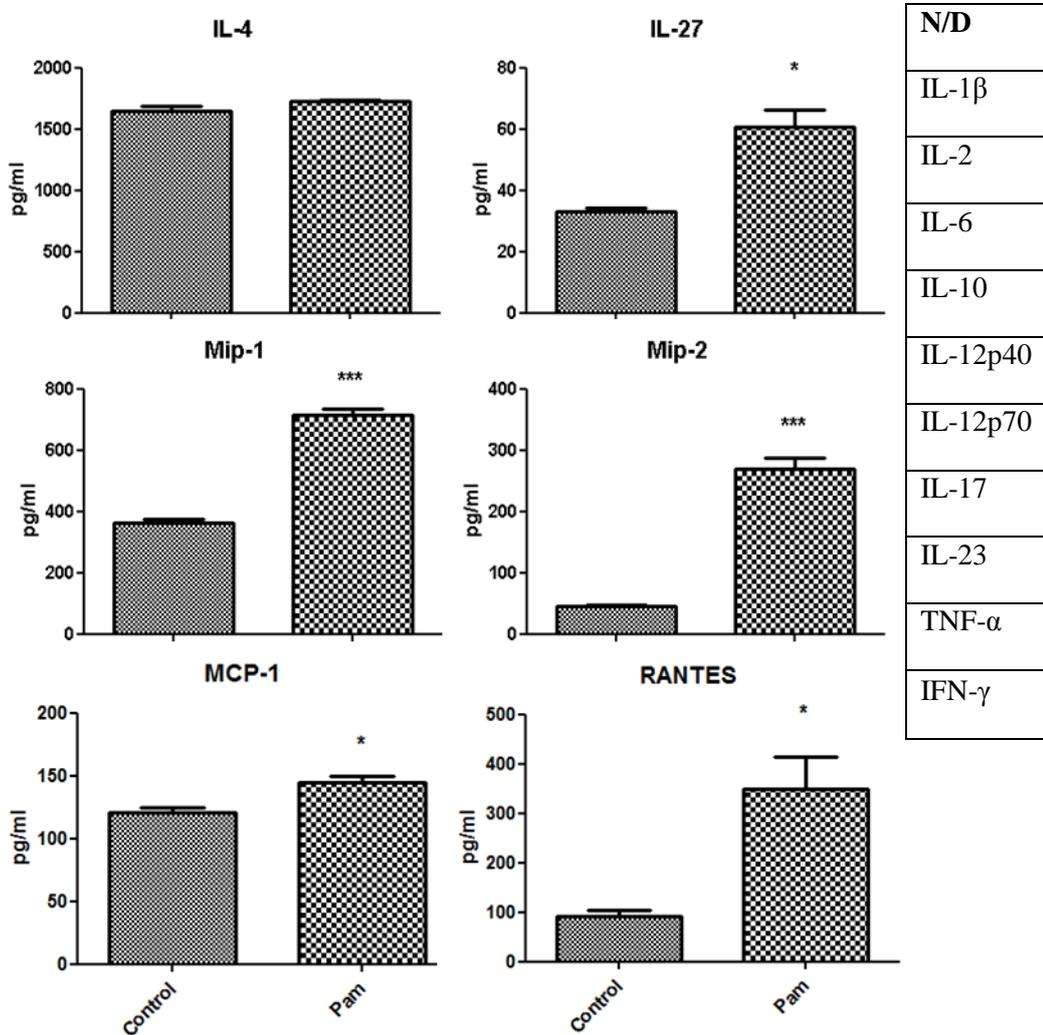


Figure 4.1 Stimulation with Pam alters the Cytokine and Chemokine Profile of MIN6 Cells. Cells were seeded at 1×10^6 cells per ml in a 6 well plate and were allowed to grow to confluency prior to stimulation for 24hr with Pam ($1\mu\text{g/ml}$) in fresh media. Following stimulation supernatants were removed and were analysed by ELISA (R&D systems, Minneapolis, MN, USA). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by unpaired t-test.

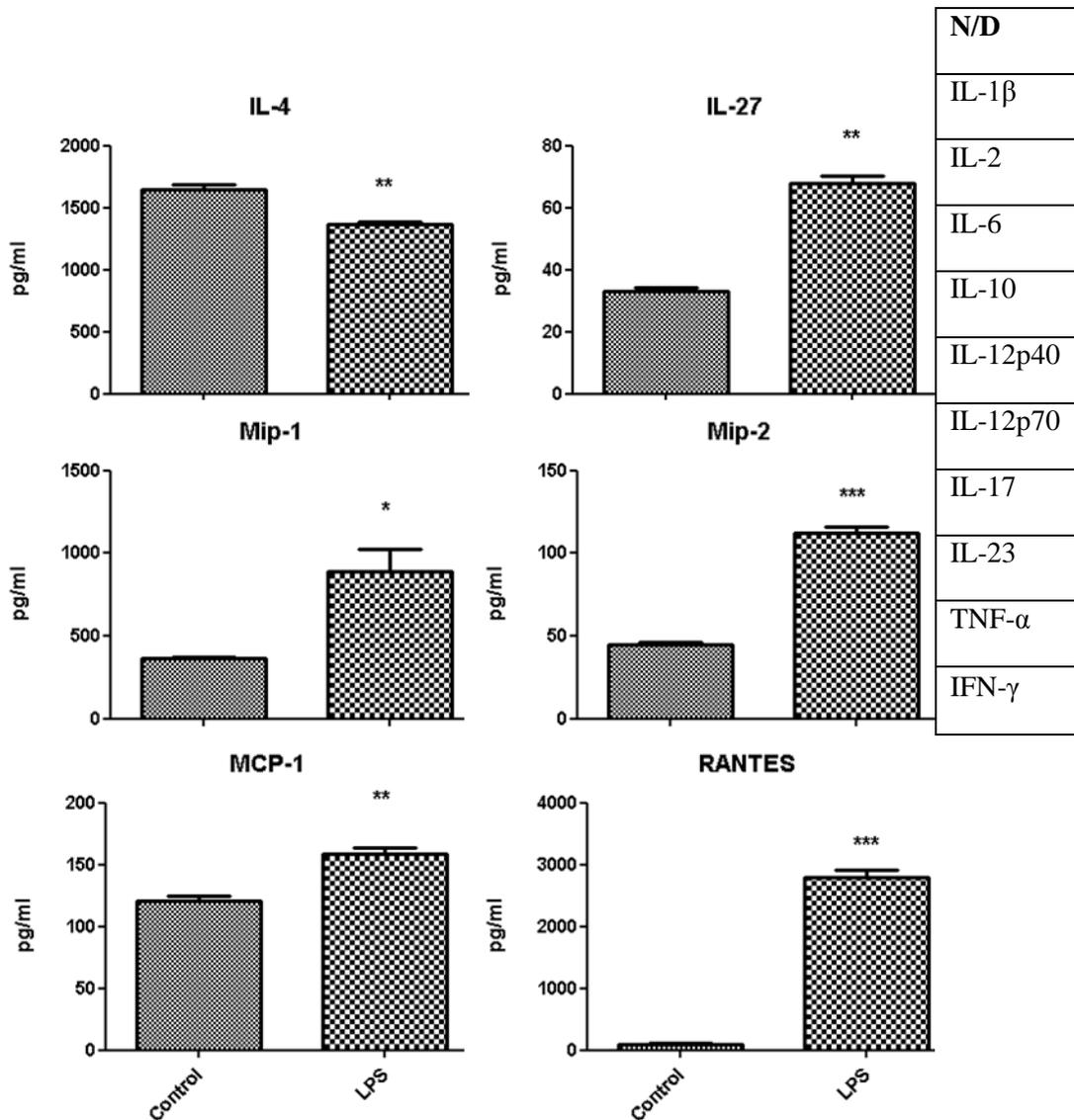


Figure 4.2 Stimulation with LPS alters the Cytokine and Chemokine Profile of MIN6 Cells. Cells were seeded at 1×10^6 cells per ml in a 6 well plate and were allowed to grow to confluency prior to stimulation for 24hr with LPS (100ng/ml) in fresh media. Following stimulation supernatants were removed and analysed by ELISA (R&D systems, Minneapolis, MN, USA). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by unpaired t-test.

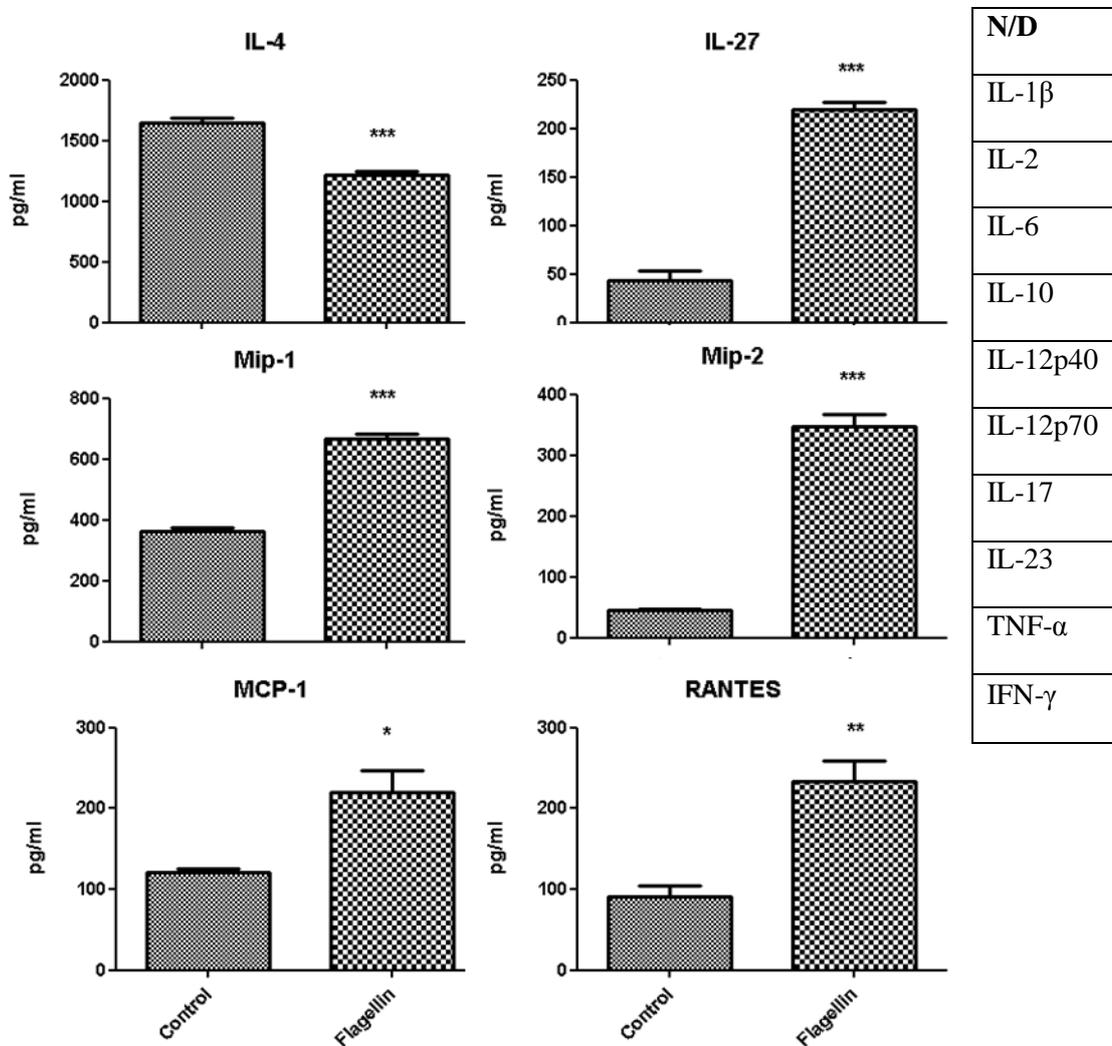


Figure 4.3 Stimulation with Flagellin alters the Cytokine and Chemokine Profile of MIN6 Cells. Cells were seeded at 1×10^6 cells per ml in a 6 well plate and were allowed to grow to confluency prior to stimulation for 24hr with Flagellin ($5\mu\text{g/ml}$) in fresh media. Following stimulation supernatants were removed and were analysed by ELISA (R&D systems, Minneapolis, MN, USA). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by unpaired t-test.

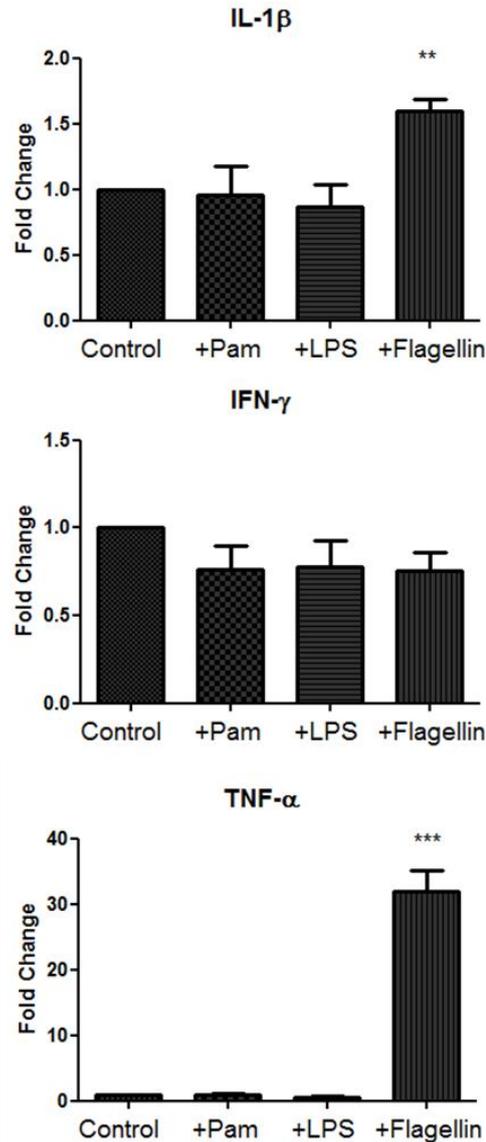


Figure 4.4 Expression levels of IL-1 β , IL-6 and TNF- α following 24h TLR ligation. The expression of the above genes was measured by qPCR. Cells were seeded at 1×10^6 cells/ml on a 6 well plate and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (Pam 1 μ g/ml, LPS 100ng/ml, Flagellin 5 μ g/ml) for 24hr. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for IL-1 β , IL-6 and TNF- α (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, B2M. The mean relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Results are mean \pm SEM. **P<0.01, ***P<0.001 vs. control, determined by unpaired *t*-test

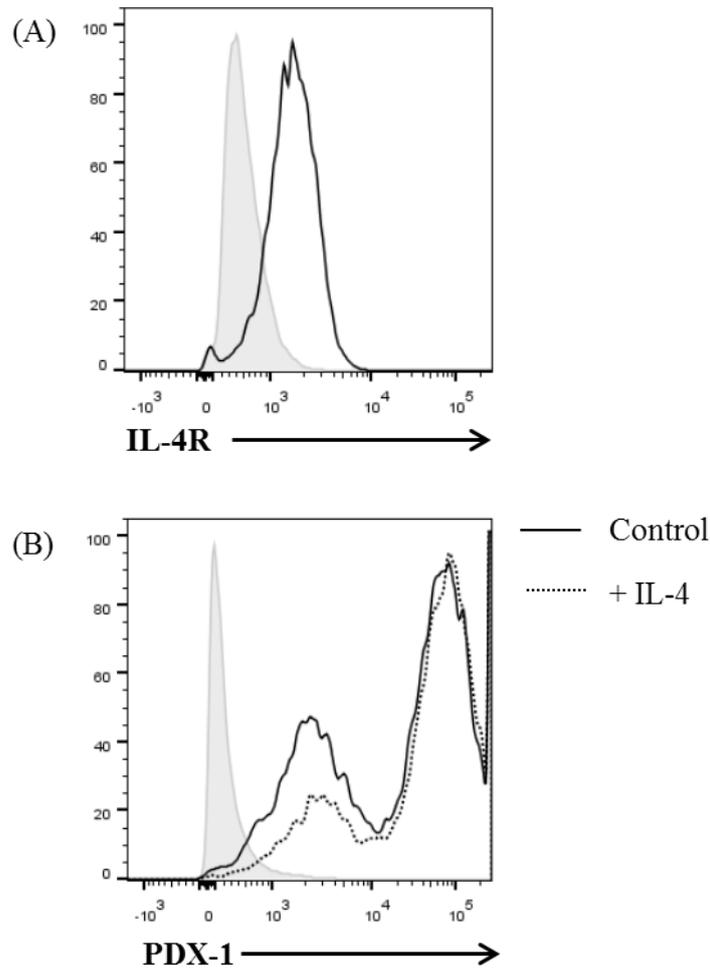


Figure 4.5 MIN6 cells express the IL-4 receptor and increase PDX-1 expression in response to IL-4 stimulation. Cells were seeded at a concentration of 1×10^6 cells per ml and left to grow to confluency. In (A) cells were stained with a fluorescently labelled antibody to the IL-4R (CD124) (BD) and its expression was measured by flow cytometry. Histograms show surface marker expression of control cells (black line) compared unstained cells (grey histogram). In (B) cells were stimulated for 24 Hr with 10ng/ml of recombinant IL-4 (R&D). Following this cells were permeabilised and stained with a fluorescently labelled antibody to PDX-1. Histograms show surface marker expression of unstimulated control cells (black line) compared to IL-4 stimulated cells (dashed line). Filled histograms represent fluorescence of unstained cells.

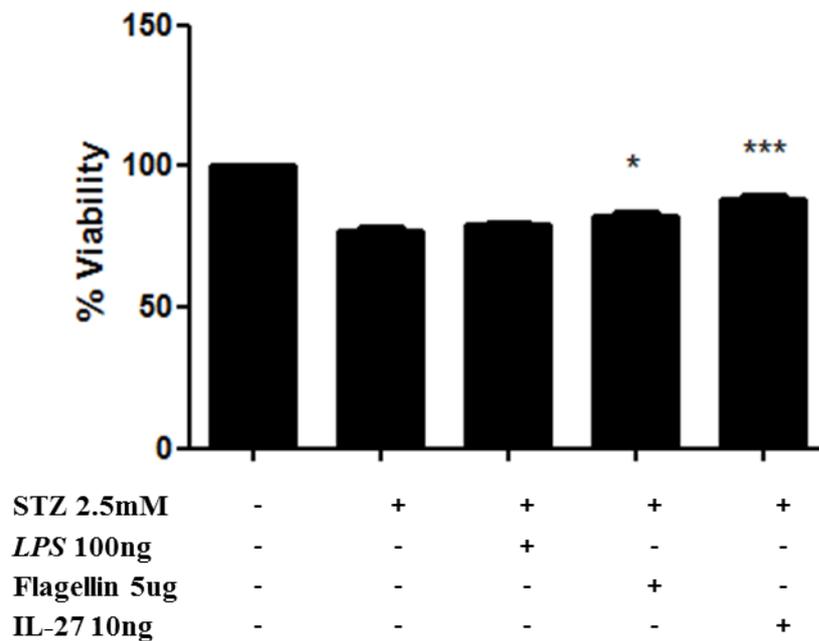


Figure 4.6 Pre-incubation with Flagellin and IL-27, but not LPS provide partial protection against Streptozotocin induced MIN6 cell death. Cells were seeded at a concentration of 1×10^6 cells per ml and left to grow to confluency after which fresh media was added and the cells were stimulated with TLR ligands (LPS 100ng/ml & Flagellin 5 μ g/ml) and IL-27 (10ng/ml) for 24hr. Following this cells were stimulated with 2.5mM STZ for a further 24 hours. 20ul of the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO₂ for 1 Hour. The plate was then read at a wavelength of 490nm. Results are expressed as a percentage of control cell viability. Data are represented as mean \pm SEM of three replicates and are representative of three independent experiments. *P<0.05, ***p<0.01 as determined by one way ANOVA with Newman Keuls post-hoc test.

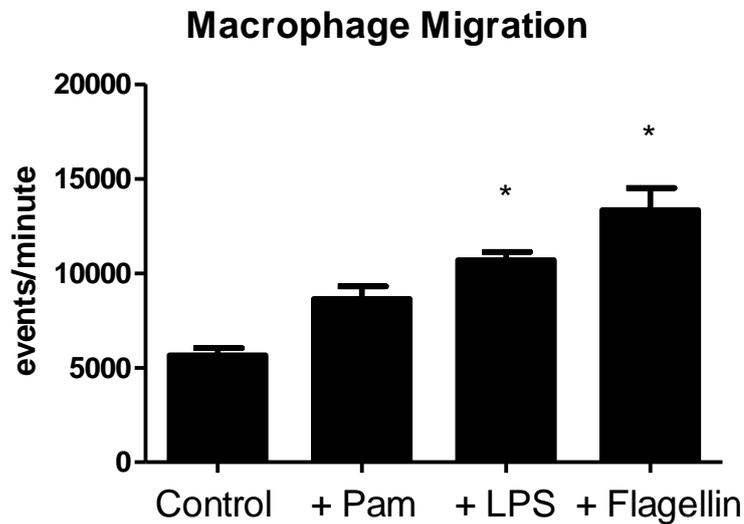


Figure 4.7 TLR stimulated secretions induce J7741.A macrophage migration. MIN6 cells were seeded at 1×10^6 cells per ml and were allowed to grow to confluency prior to stimulation for 24hr with TLR ligands (Pam $1\mu\text{g/ml}$, , LPS- 100ng/ml , Flagellin- $5\mu\text{g/ml}$) in fresh media. Following stimulation supernatants were removed and spun down to remove any cell debris. Supernatants were then transferred to the bottom of a trans-well plate. Microporous inserts containing 5×10^5 J774A.1 MØ were then placed in each well and left for 6 hours after which time the inserts were removed and the number of migrated cells were counted. Data are represented as mean \pm SEM of three replicates and are representative of three independent experiments. * $P < 0.05$ as determined by unpaired t-test.

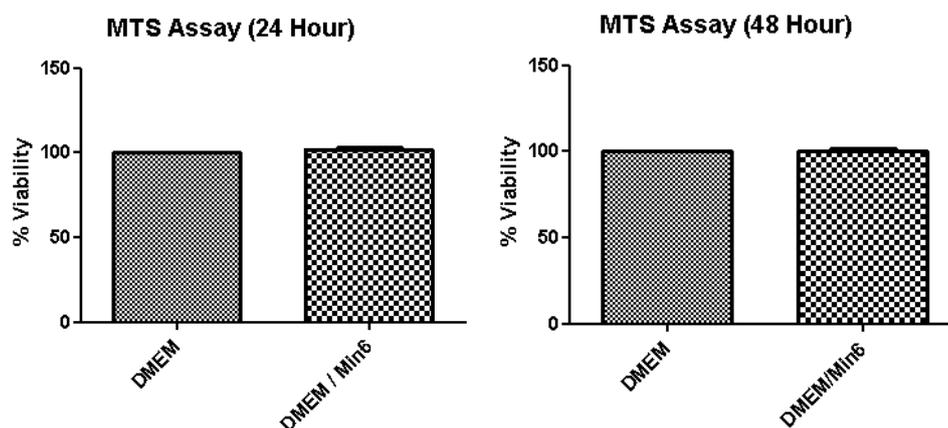


Figure 4.8 Culturing J774 Macrophage in MIN6 conditioned medium does not affect cell viability. MIN6 cells were cultured in DMEM-glutamax for 24hr. The conditioned media was then removed and transferred to J774A.1 macrophages for 24 and 48hr. Following this 20 μ l of the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO₂ for 1 Hour. The plate was then read at a wavelength of 450nm. Results are expressed as a percentage of the control viability (100%). Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments.

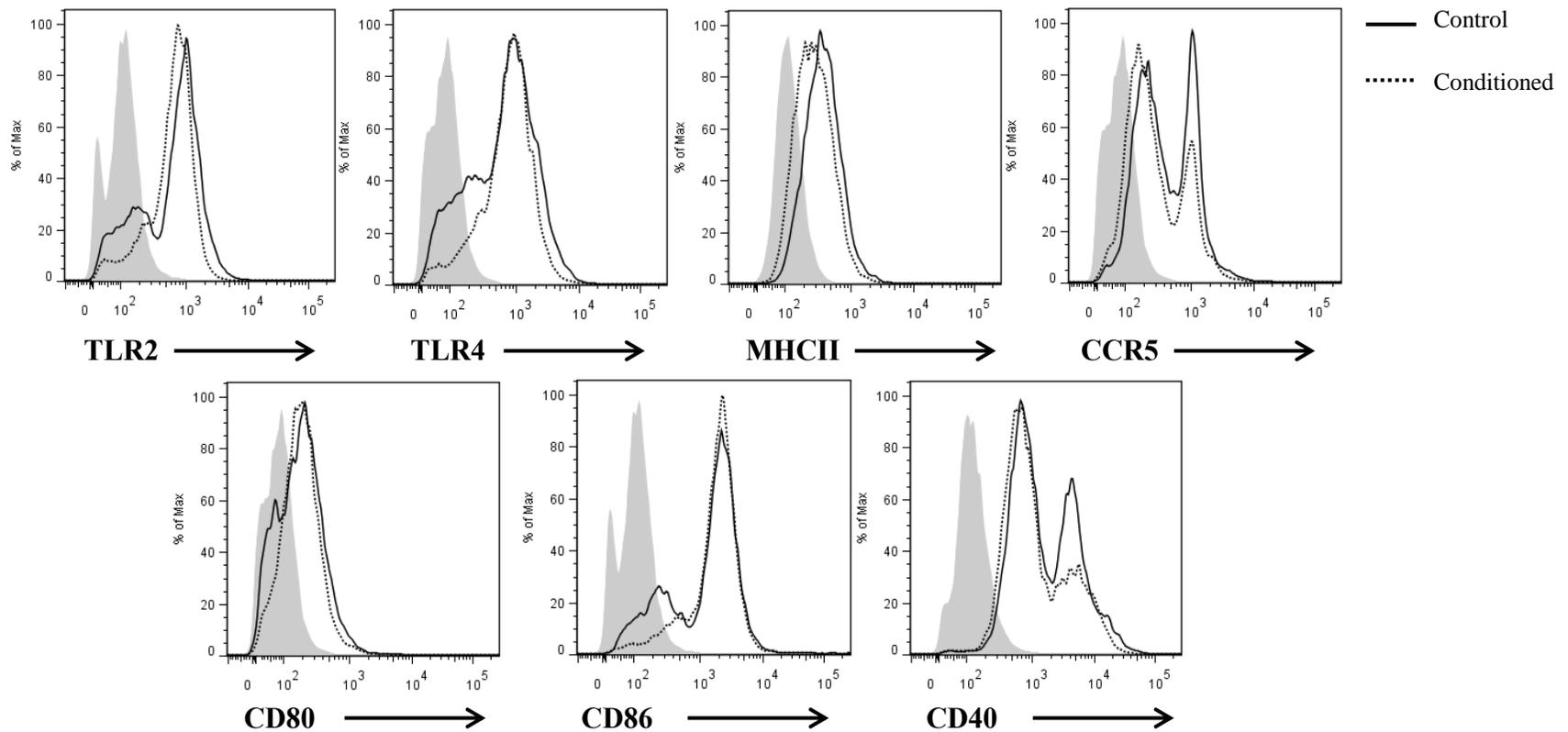


Figure 4.9 Expression of surface markers on MIN6 conditioned macrophages. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Following this cells were stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CCR5, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dashed line) compared to unconditioned control cells (black line). Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

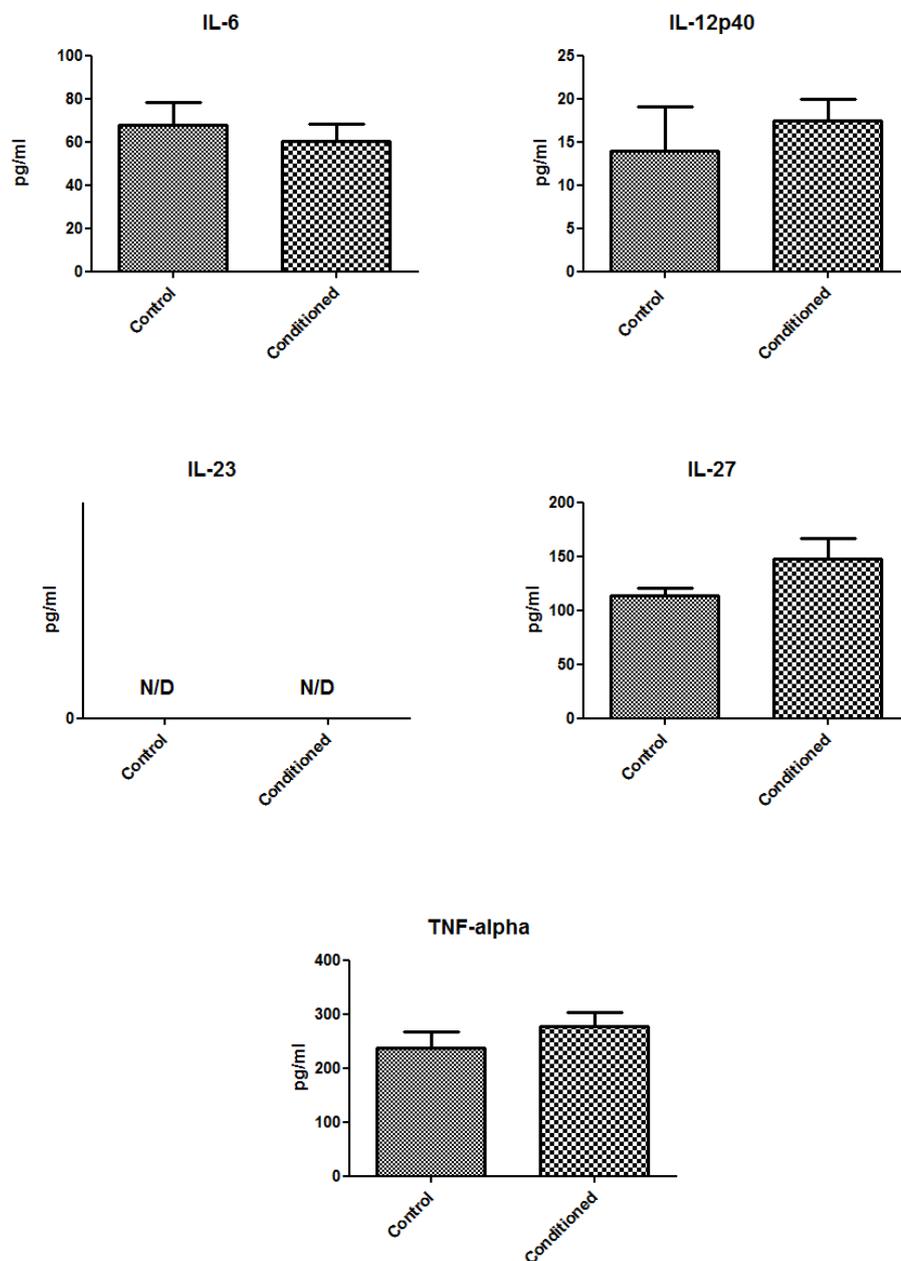


Figure 4.10 Cytokine secretion from MIN6 conditioned macrophages. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments.

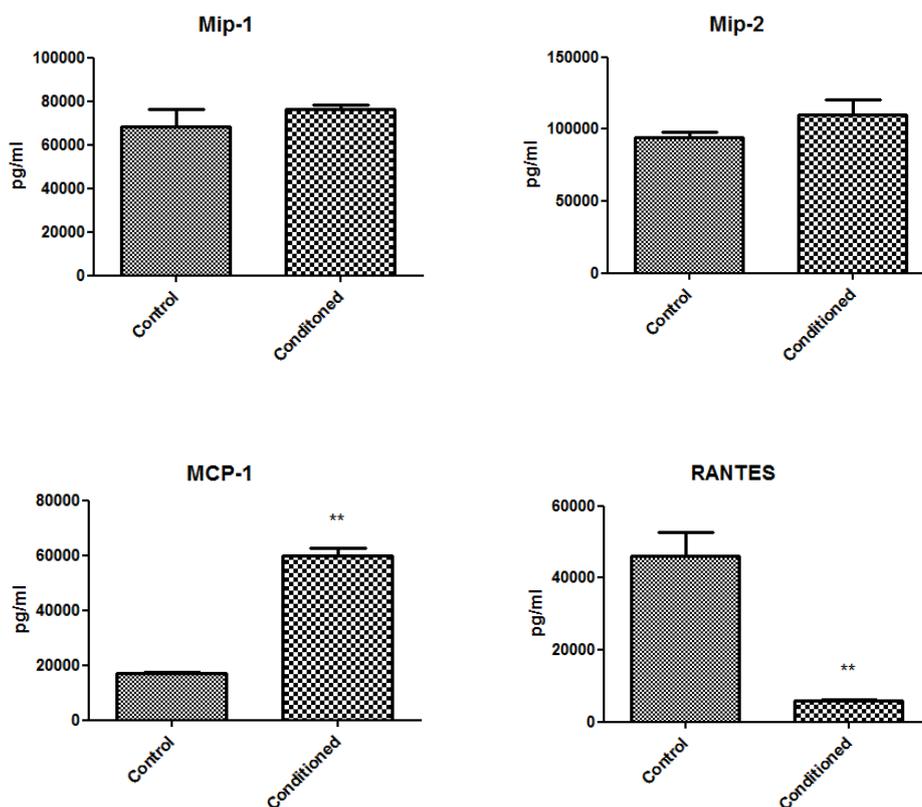


Figure 4.11 Chemokine secretion from MIN6 conditioned macrophages. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Following this supernatants were collected and chemokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. **P<0.01 by unpaired *t*-test.

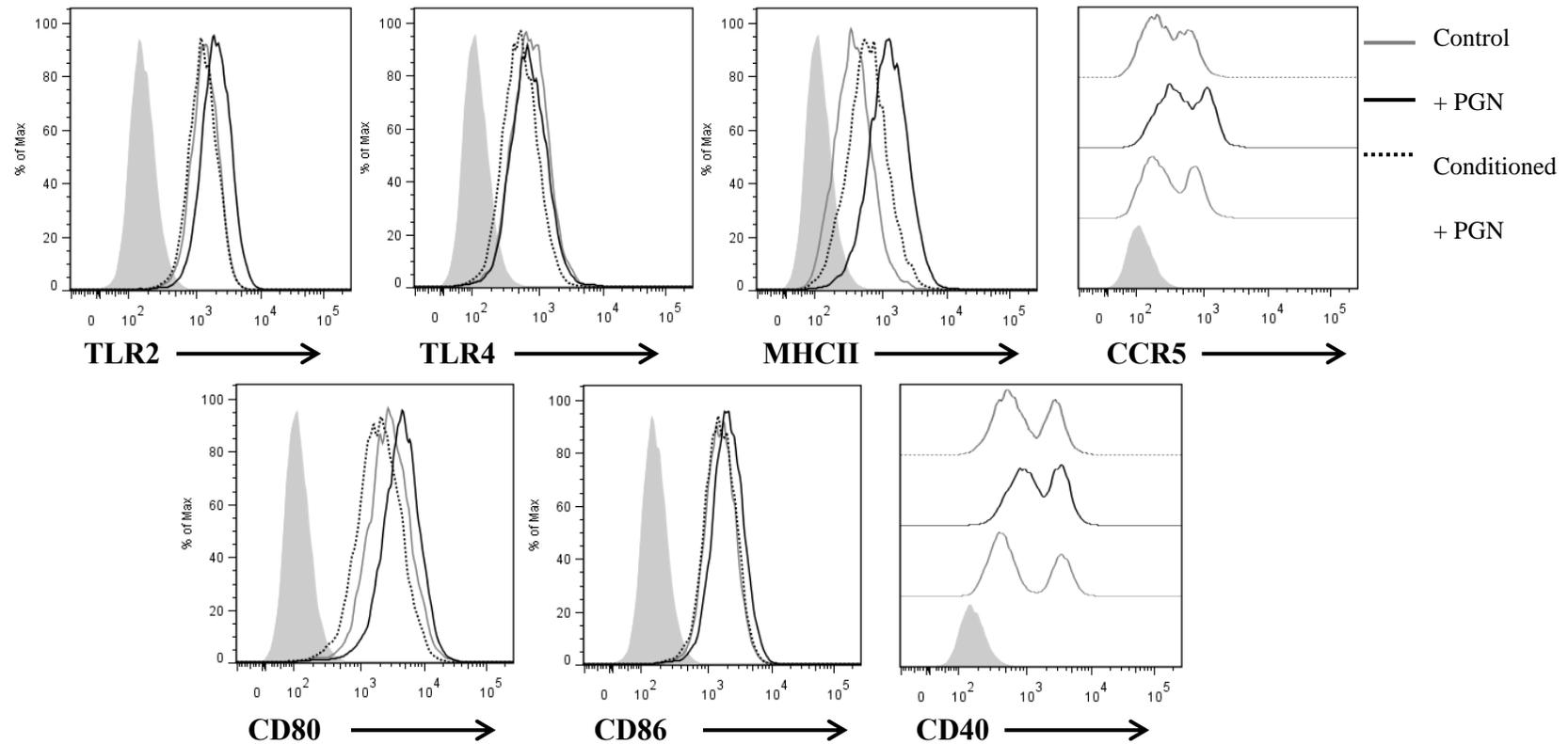


Figure 4.12 Expression of surface markers on MIN6 conditioned macrophages following TLR2 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Cells were then stimulated with PGN (5 μ g/ml) for a further 24hr. Cells were then stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CCR5, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR2 stimulation. Grey line represents the expression on unstimulated controls. Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

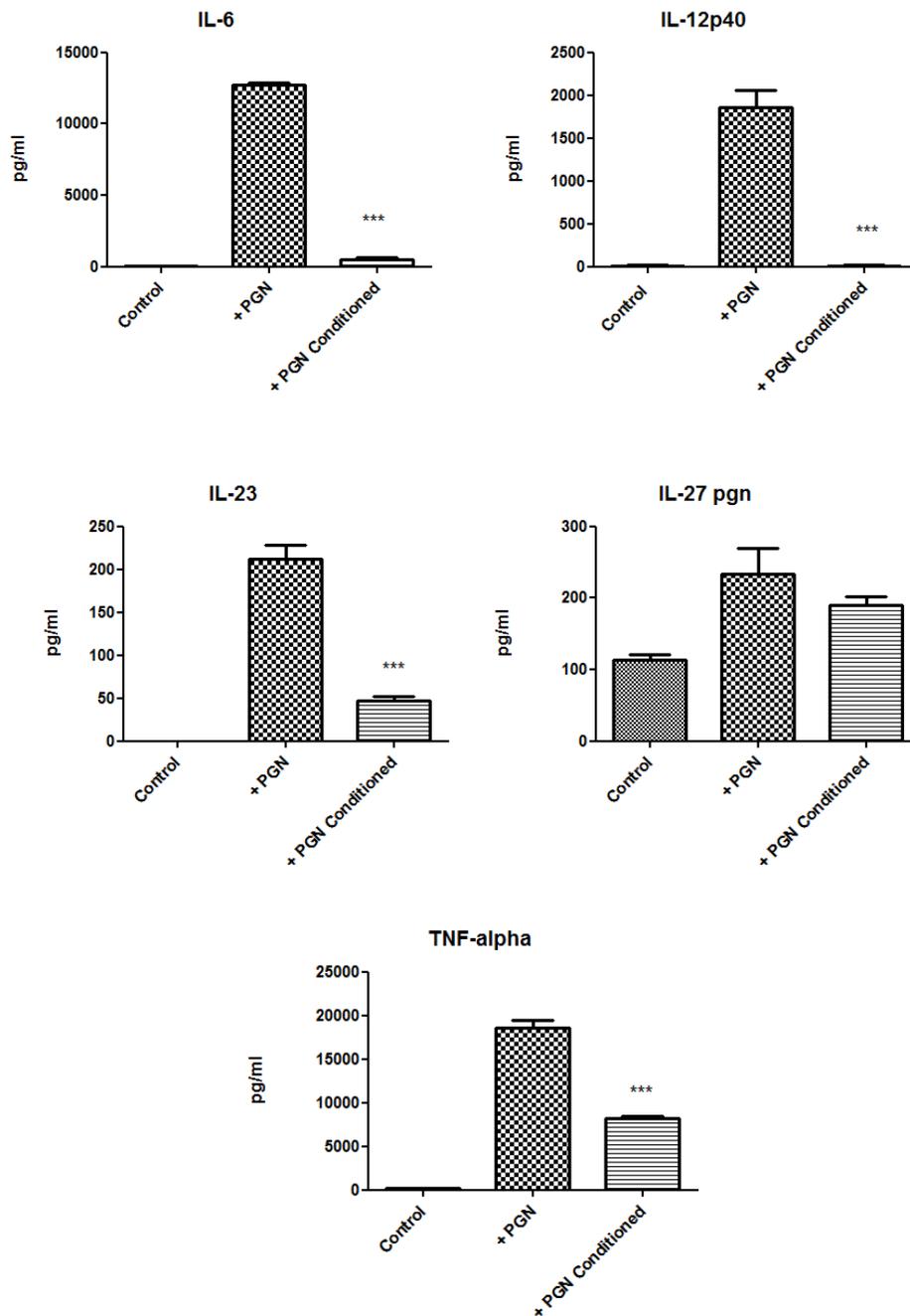


Figure 4.13 Cytokine secretion from MIN6 conditioned macrophages following TLR2 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Fresh media was added to cells followed by 24hr stimulation with PGN (5µg/ml). Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean ± SEM of three replicates and are representative of three independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA followed by Newman-Keuls analysis. ***P<0.01, ***P<0.001 vs. unconditioned PGN-stimulated cells.

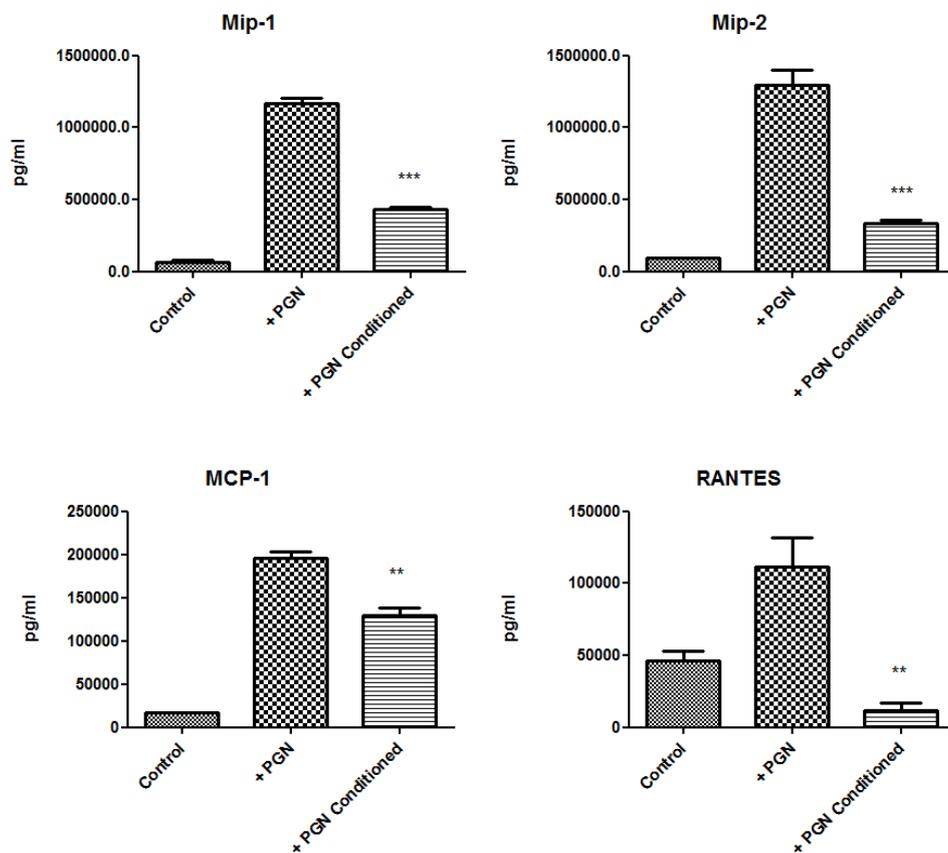


Figure 4.14 Chemokine secretion from MIN6 conditioned macrophages following TLR2 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Fresh media was added to cells followed by 24hr stimulation with PGN (5 μ g/ml). Following this supernatants were collected and chemokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA followed by Newman-Keuls analysis. **P<0.01, ***P<0.01 vs. unconditioned PGN-stimulated cells.

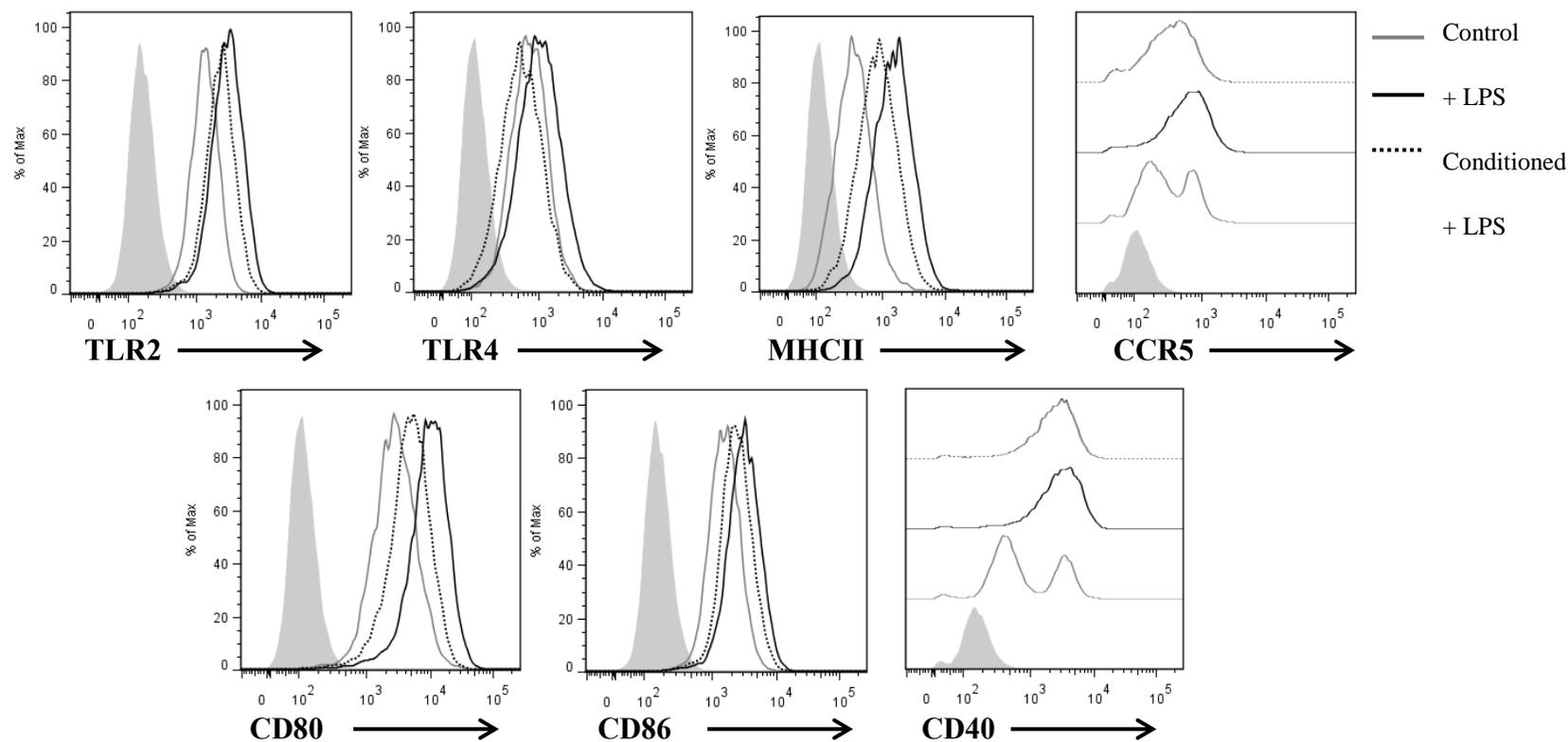


Figure 4.15 Expression of surface markers on MIN6 conditioned macrophages following TLR4 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CCR5, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls. Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

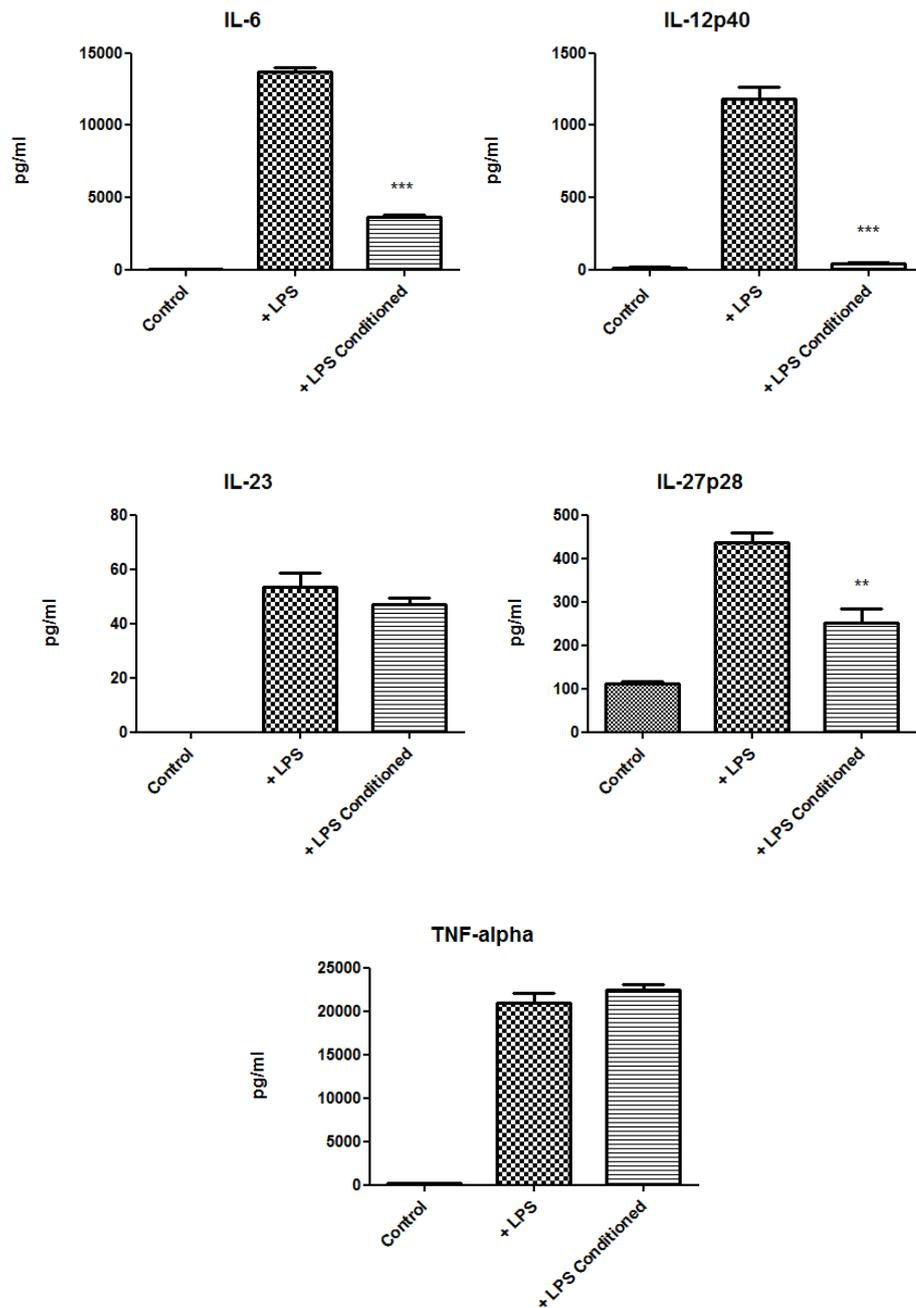


Figure 4.16 Cytokine secretion from MIN6 conditioned macrophages following TLR4 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Fresh media was added to cells followed by 24hr stimulation with LPS (100ng/ml). Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA followed by Newman-Keuls analysis. **P<0.01, ***P<0.001 vs. unconditioned LPS-stimulated cells.

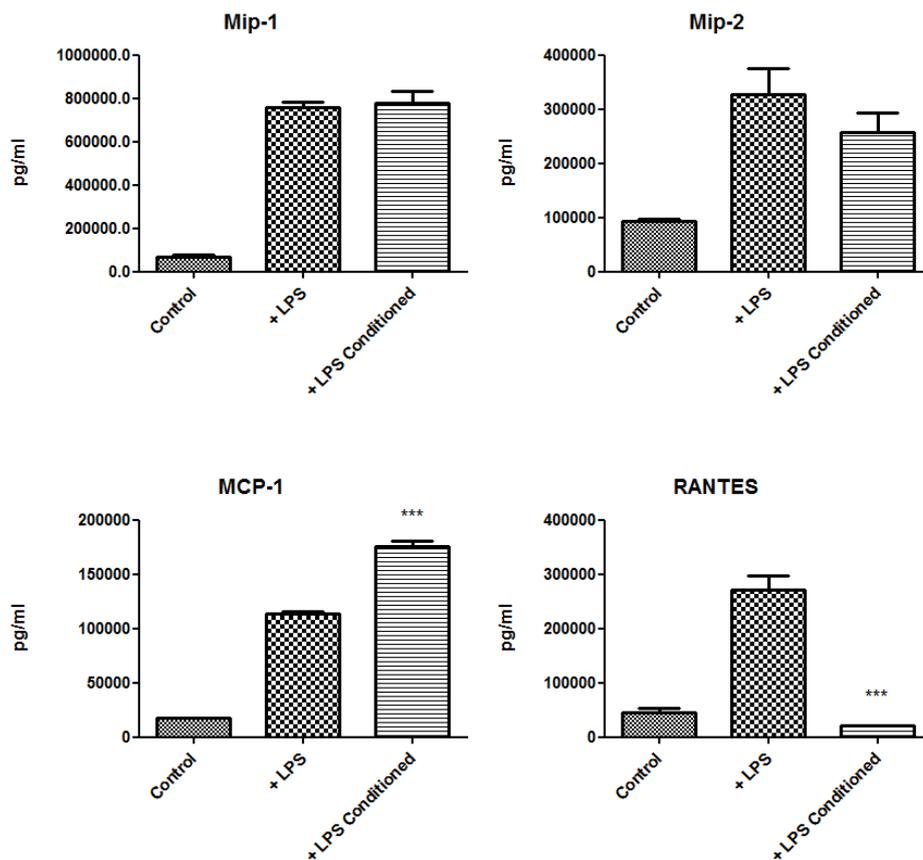


Figure 4.17 Chemokine secretion from MIN6 conditioned macrophages following TLR4 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Fresh media was added to cells followed by 24hr stimulation with LPS (100ng/ml). Following this supernatants were collected and chemokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA followed by Newman-Keuls analysis. *** $P < 0.001$ vs. unconditioned LPS-stimulated cells.

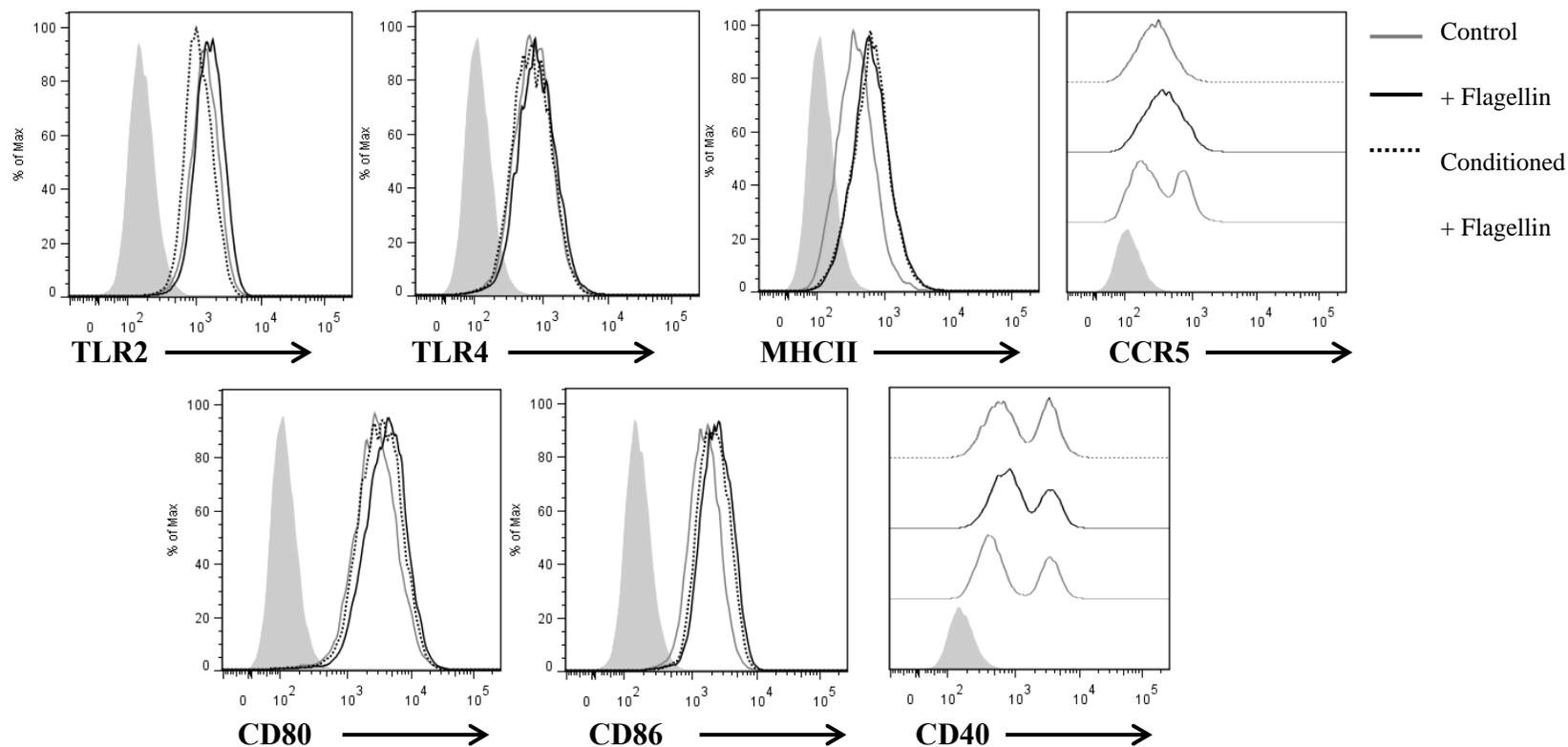


Figure 4.18 Expression of surface markers on MIN6 conditioned macrophages following TLR5 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Cells were then stimulated with Flagellin (5 μ g/ml) for a further 24hr. Cells were then stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CCR5, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR5 stimulation. Grey line represents the expression on unstimulated controls. Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

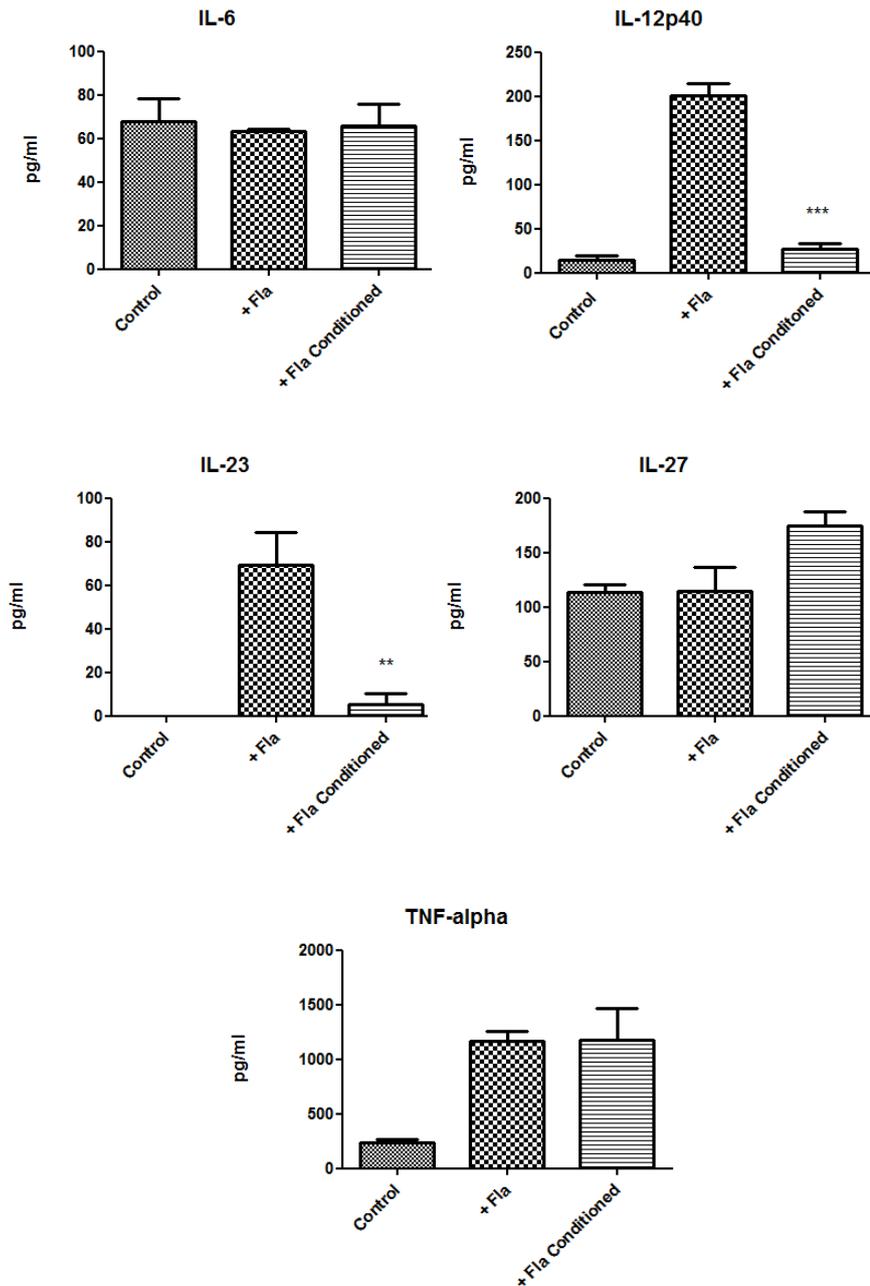


Figure 4.19 Cytokine secretion from MIN6 conditioned macrophages following TLR5 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Fresh media was added to cells followed by 24hr stimulation with Flagellin (5µg/ml). Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean ± SEM of three replicates and are representative of three independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA followed by Newman-Keuls analysis. **P<0.01, ***P<0.001 vs. unconditioned Flagellin-stimulated cells.

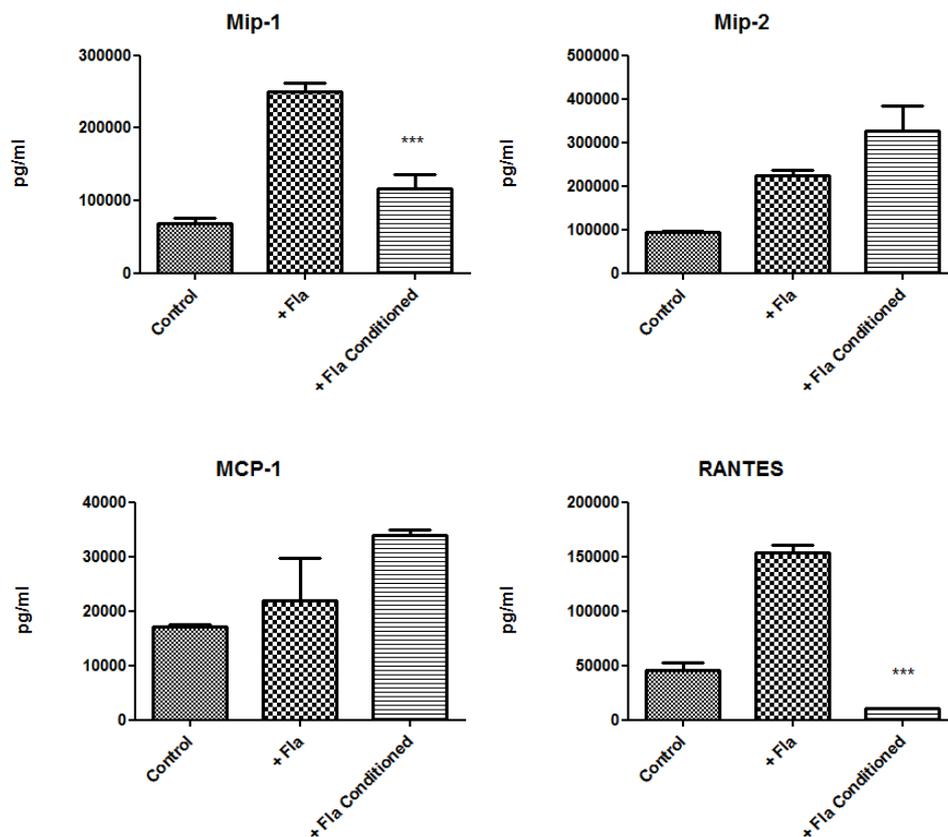


Figure 4.20 Chemokine secretion from MIN6 conditioned macrophages following TLR5 ligation. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Fresh media was added to cells followed by 24hr stimulation with Flagellin (5 μ g/ml). Following this supernatants were collected and chemokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. Data are presented as mean \pm SEM of three replicates and are representative of three independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA followed by Newman-Keuls analysis. ***P<0.001 vs. unconditioned Flagellin-stimulated cells

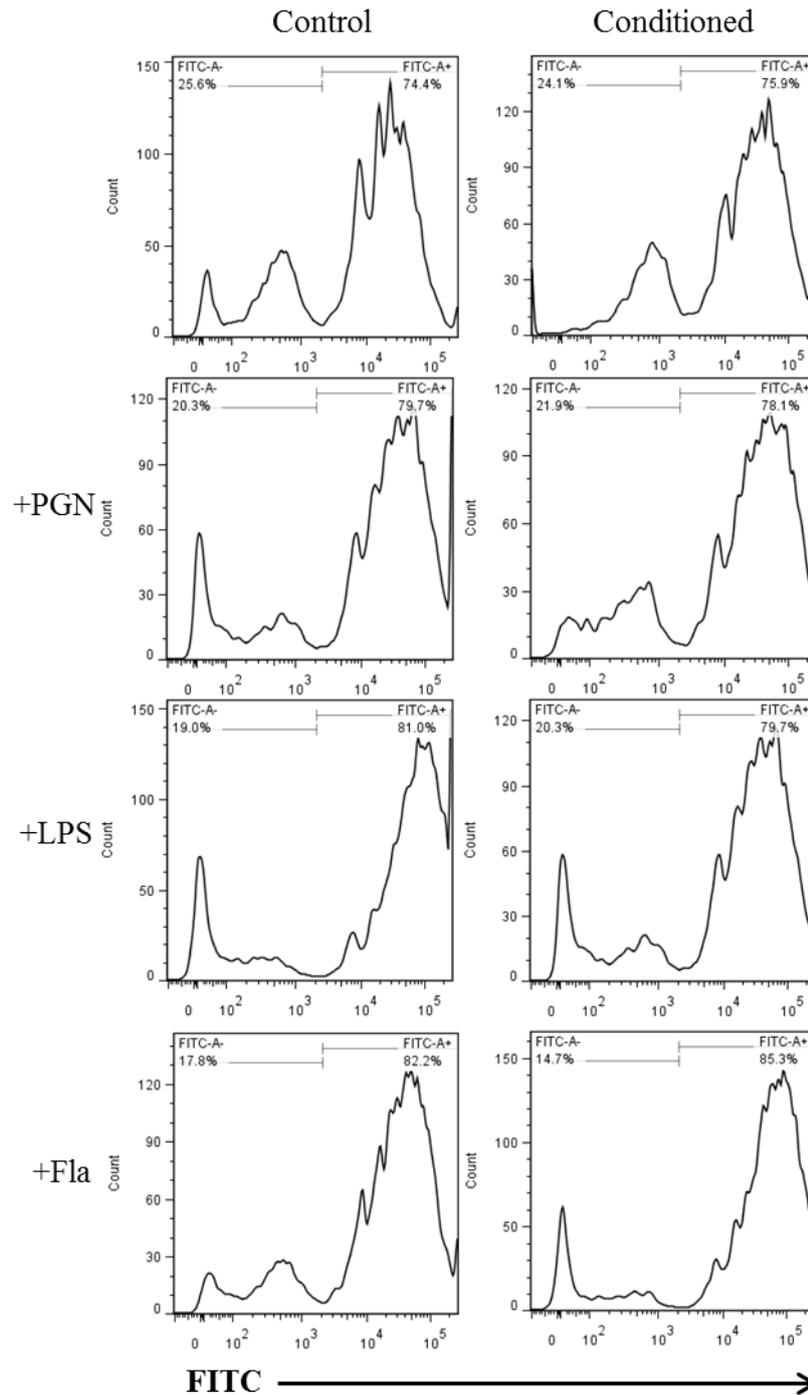


Figure 4.21 Conditioned and control macrophage display comparable phagocytotic activity. J774A.1 macrophages were incubated with normal media or MIN6 conditioned media 24hr. Pre-conditioned cells were stimulated with PGN (5 μ g/ml) LPS (100ng/ml) & Flagellin (5 μ g/ml) for 24hr. To assess phagocytic ability of cells, 1 μ m of fluorescent latex beads (Sigma) were added to the culture and macrophages were left to phagocytose for 2hr. Cells were then washed and analysed by flow cytometry for the uptake of beads. Histograms show the percentages of cells that contain beads (FITC+) or do not contain beads (FITC-).0

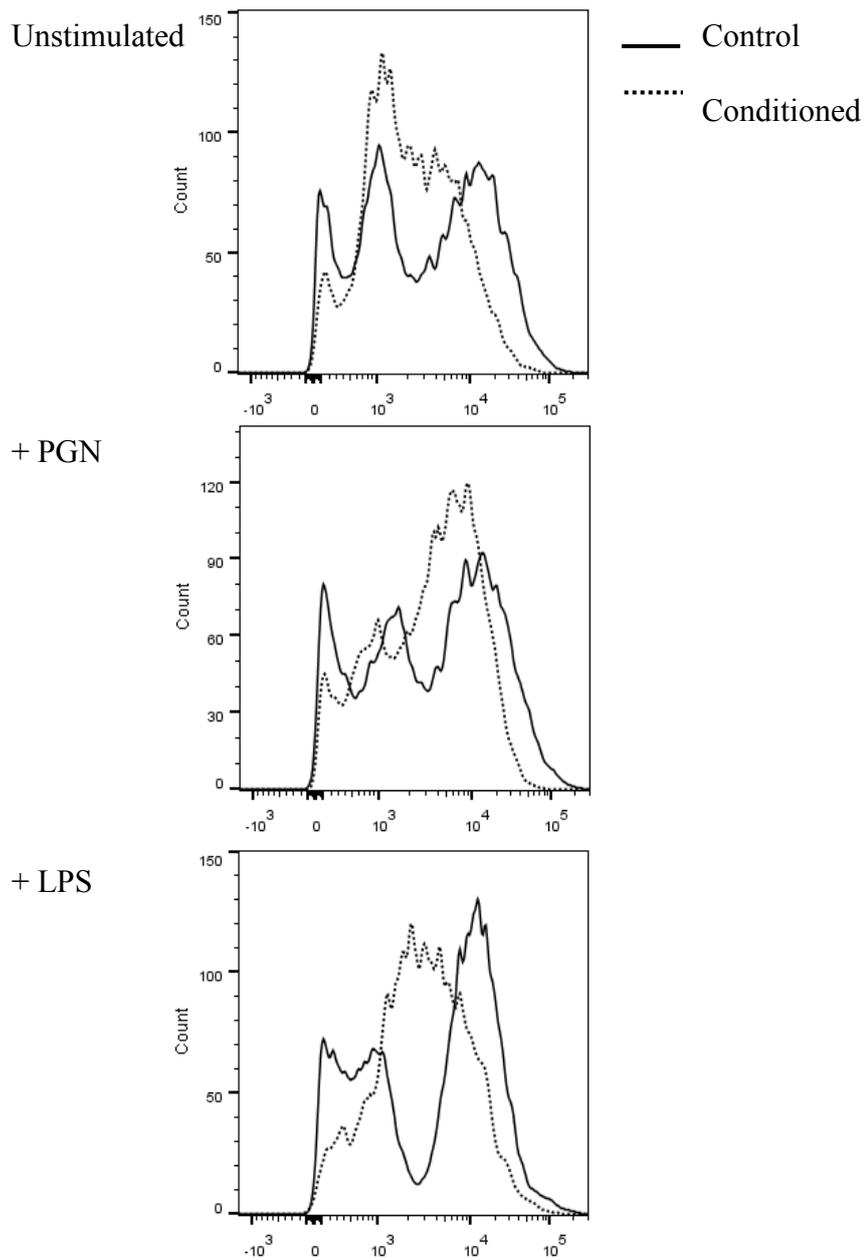


Figure 4.22 Conditioned macrophages produce lower levels of reactive oxygen species (ROS) in a steady state and in response to PGN & LPS stimulation. J774A.1 macrophages were incubated with control media or MIN6 supernatants for 24hr. Cells were stimulated with PGN (5 μ g/ml) & LPS (100ng/ml) for 24hr. To measure the production of reactive oxygen species, cell were labelled with DCFDA (Abcam), according to manufacturer's instructions, and the fluorescent signal relative to intracellular ROS generation was then measured by flow cytometry and compared between unconditioned cells (black line) and conditioned cells (dashed line). Data are representative of three independent experiments.

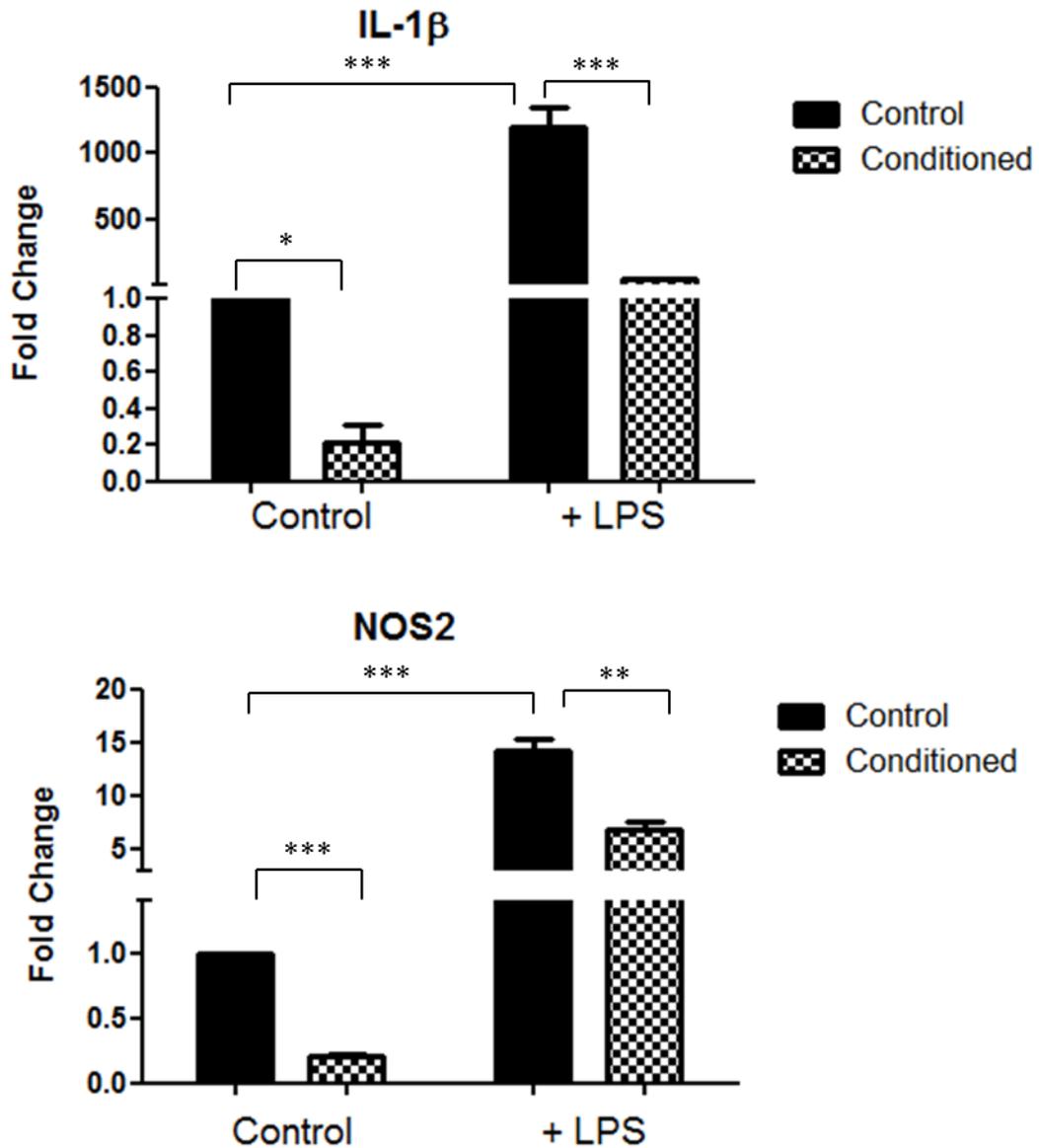


Figure 4.23 Conditioned macrophage display decreased IL1 β & NOS2 activity in steady state and following stimulation with LPS. The expression of IL1 β and NOS2 was measured by qPCR. J774A.1 macrophages were cultured in MIN6 conditioned or control media for 24hr prior to 24hr stimulation with LPS 100ng/ml. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for IL1 β & NOS2 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, actin. The mean relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Results are mean \pm SEM. **P<0.01, ***P<0.001, vs. control, determined by unpaired *t*-test

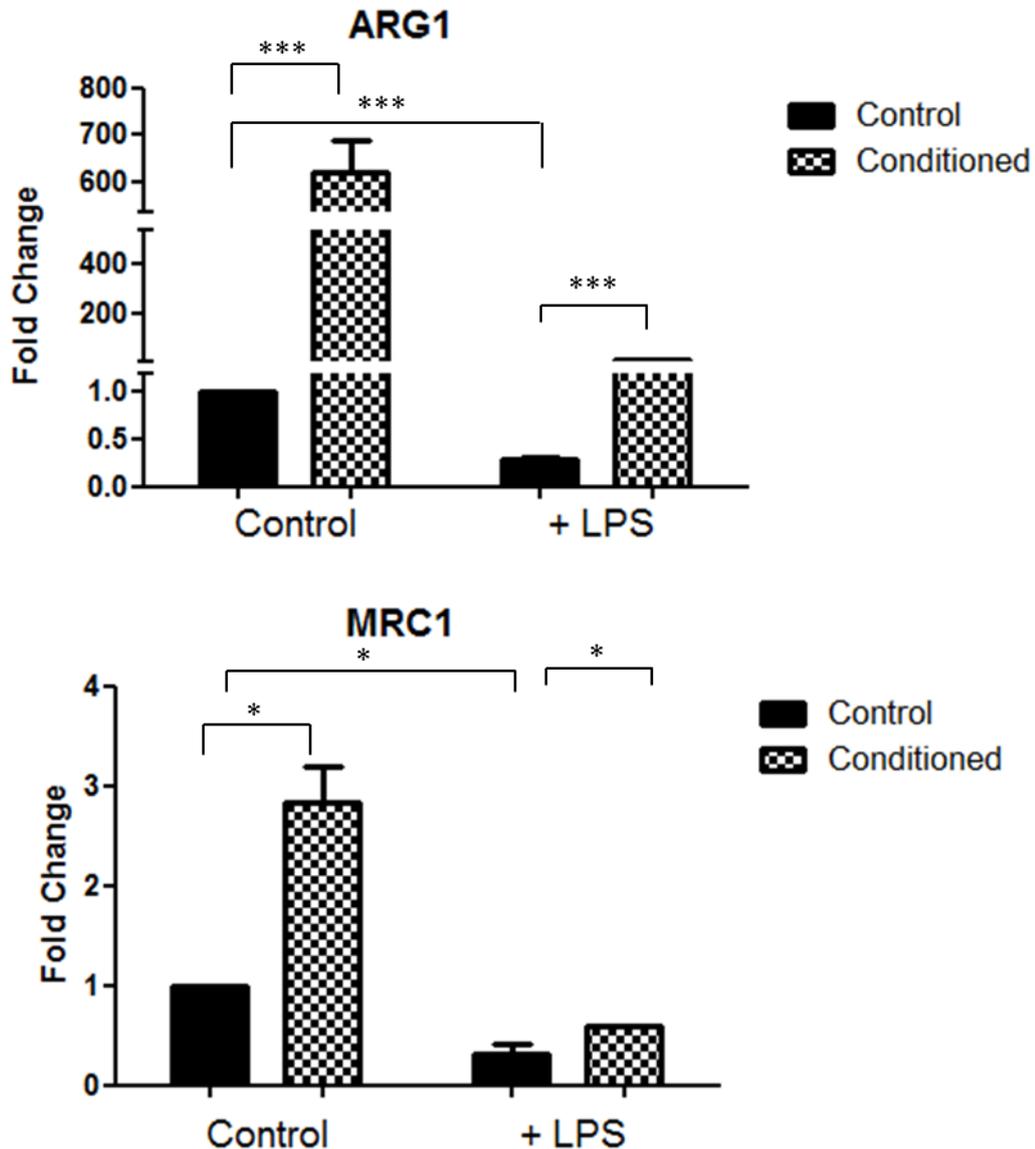


Figure 4.24 Conditioned macrophage display increased ARG1 & MRC1 activity in steady state and following stimulation with LPS. The expression of ARG1 and MRC1 was measured by qPCR. J774A.1 macrophages were cultured in MIN6 conditioned or control media for 24hr prior to 24hr stimulation with LPS 100ng/ml. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for ARG1 & MRC1 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, actin. The mean relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Results are mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, vs. control, determined by unpaired t -test.

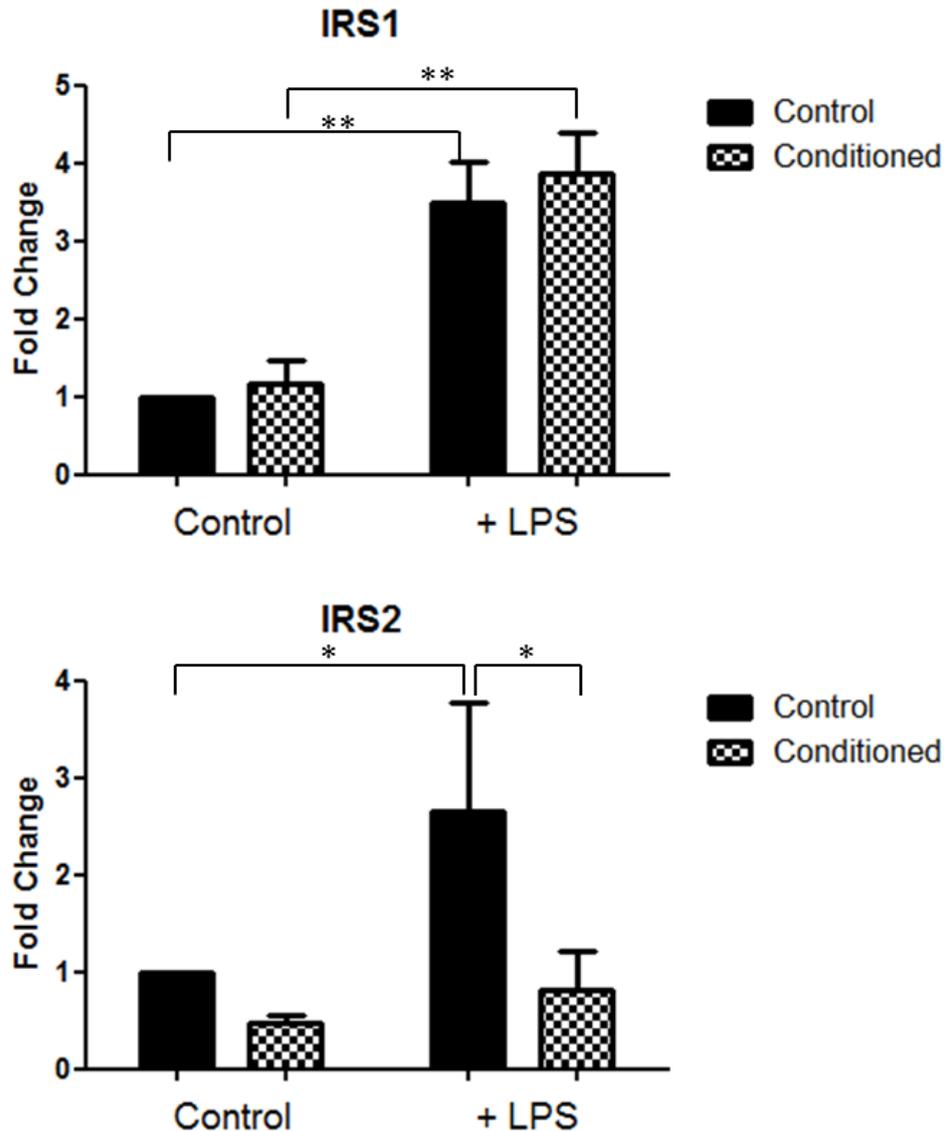


Figure 4.25 LPS stimulated macrophage show increased IRS1 and IRS2 expression and IRS2 expression in decreased by conditioning. The expression of IRS1 and IRS2 was measured by qPCR. J774A.1 macrophages were cultured in MIN6 conditioned or control media for 24hr prior to 24hr stimulation with LPS 100ng/ml. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for IRS1 and IRS2 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, actin. The mean relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Results are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, vs. control, determined by unpaired *t*-test.

4.4 Discussion

Of all cytokines we assessed in MIN6 cells we only detected that they secreted IL-4 and IL-27p28 in a resting state. Under control conditions MIN6 cells are seen to secrete high levels of the cytokine IL-4. IL-4 is a pleiotropic cytokine that exerts its effects via activation of the transcription factor Signal Transducer and Activator of Transcription (STAT) 6 (Takeda et al. 1996). The majority of research has focused on its functions in the immune system which include Th2 differentiation of helper T cells, activation of B cells to release IgE, and stimulation of alternative macrophage activation (Gordon 2003). In the context of the pancreatic β -cells and diabetes, genetic studies have revealed a significant association between IL-4 promoter polymorphisms and T2D (Ho et al. 2010). In recent times IL-4 has been associated with regulation of glucose metabolism and insulin sensitivity in major metabolic organs including liver, fat and muscle (Ricardo-gonzalez et al. 2010). Disruption of STAT6 decreases insulin action and enhances a peroxisome proliferator-activated receptor α (PPAR α) driven program of oxidative metabolism (Ricardo-gonzalez et al. 2010). Conversely, activation of STAT6 by IL-4 improves insulin action by inhibiting the PPAR α -regulated program of nutrient catabolism and attenuating adipose tissue inflammation. These findings have identified an unexpected molecular link between the immune system and nutrient metabolism. Taking this in consideration we have shown that TLR stimulation results in a significant decrease in production of IL-4 from MIN6 β -cells in the case of TLR4, and TLR5. This suggests perhaps a role for IL-4 in the islet as an insulin sensitizing agent and TLR stimulation may decrease this affect. Indeed Gaddy *et al* reported that a single

treatment with double-stranded adeno-associated virus serotype 8 (dsAAV8) vectors expressing IL-4 under regulation of the insulin promoter enhanced β -cell proliferation and survival *in vivo*, significantly delaying diabetes progression in NOD mice (Gaddy et al. 2012). This is in line with our own findings which show that treatment of MIN6 β -cells with recombinant IL-4 causes an increase in the transcription factor PDX-1 which is essential for pancreatic development and for the maintenance of β -cell function. Another role for IL-4 is the alternative activation of macrophages (Gordon 2003). This decrease in IL-4 production seen upon TLR stimulation may result in an alteration in the phenotype of and resident islet or infiltrating macrophages which in turn could affect the function of the β -cell.

Under control conditions there is a low level of secretion of the cytokine IL-27p28 from Min6 cells. IL-27 is a hetero-dimeric cytokine of the IL-6 and IL-12 family composed of the IL-27p28 and EBI3 subunits. IL-27p28 and EBI3 are produced primarily by antigen-presenting cells after stimulation by microbial products or inflammatory mediators (Hunter & Kastelein 2012). IL-27 was initially described as a proinflammatory cytokine that promoted T helper (Th)1 responses (Pflanz et al. 2002). Subsequent studies in multiple models of infectious and autoimmune disease have confirmed an anti-inflammatory role for IL-27 in Th1, Th2 and Th17 responses (Stumhofer & Hunter 2008) and recent work has shown that IL-27 can induce T cells to produce the anti-inflammatory cytokine IL-10 (Awasthi et al. 2007), thus IL-27 can exert both inflammatory and anti-inflammatory effects. As low amounts of IL-27 are secreted from MIN6 β -cells under control conditions we postulate that it may play an anti-inflammatory regulatory role in the islet. Upon activation of TLR2, TLR4 and TLR5 in MIN6 cells we observed an increase in the production of IL-27.

Recently Fujimoto *et al* have shown that the administration of recombinant IL-27 decreased blood glucose levels, immune cell infiltration into islets, and IL-1 β mRNA expression in the pancreas and increased islet proinsulin levels in mice treated with streptozotocin (Fujimoto et al. 2011). Our findings that MIN6 cells secrete IL-27p28 and TLR5 stimulation in particular increases this secretion may help explain their findings and may point to a protective role for IL-27 in islets. This may represent a novel therapeutic approach for β -cell protection in diabetes. Indeed we found that when MIN6 cells were preconditioned with IL-27 and Flagellin (strongest inducer of IL-27 in these cells) STZ induced cell death was decreased when compared with control STZ treated and LPS preconditioned STZ treated cells.

As mentioned previously IL-1 β , TNF- α and IFN- γ are cytokines that are known to induce β -cell death and dysfunction (Wachlin et al. 2003). Although no detectable secretion levels of these cytokines were found in control or stimulated conditions from MIN6 cells. Flagellin stimulation of MIN6 cells resulted in a significant increase in mRNA expression levels of IL-1 β and TNF- α . The mechanisms by which IL-1 β may contribute to the pathogenesis of diabetes are controversial. Islet cells demonstrate autocrine increases in IL-1 β when exposed to IL-1 β or glucose (Zeender et al. 2004; Maedler et al. 2002). It is possible that IL-1 β acts in concert with other cytokines to cause islet cell death. For example, whereas no apoptosis was observed in nondiabetic human islet cells incubated with either IL1 β or IFN- γ alone, exposure to both cytokines simultaneously resulted in increased apoptosis as a result of death protein 5/harikari activation (Gurzov et al. 2009), which in turn is associated with an NF κ B-regulated increase in expression of the p53 upregulated modulator of apoptosis (Makeeva et al. 2006). Although *in vitro* experiments suggest that IL-1 β is

detrimental to insulin secretion, mouse studies from the 1980s suggest that IL-1 β is protective against chemically induced diabetes development. IL-1 β injected into alloxan-treated mice decreased glucose levels within 30 min. In streptozotocin-treated mice the decrease in glucose levels was more modest and required multiple IL-1 β injections, whereas there was improvement in diabetic db/db mice with one injection of IL-1 β (del Rey & Besedovsky 1989). As flagellin is the only ligand that results in an increase in IL-1 β mRNA expression, and we have shown that activation of MIN6 cells with flagellin prior to STZ treatment provides partial protection we postulate that IL-1 β could be acting in a protective mechanism in this case. The same could be said for the increase seen in TNF- α expression levels following flagellin stimulation. TNF- α has an extremely complex role in the immune system. It is a regulatory cytokine that co-ordinates communication between immune cells and controls many of their functions, such as activation, apoptosis, survival, proliferation and differentiation (Parameswaran & Patial 2010). Exposure of cells to TNF- α can result in activation of a caspase cascade leading to apoptosis. However, binding of TNF- α can also lead to activation of major transcription factors, AP-1 and NF- κ B, that in turn induce the genes to suppress apoptosis (Bradley 2008). TNF- α has been shown to cause β -cell death in MIN6 cells, however only when used in conjunction with IFN- γ (Suk et al. 2001) and we did not find increased expression of IFN- γ following stimulation of MIN6 cells. Once again as Flagellin is the only activator of TNF- α in MIN6 cells, and as flagellin seems to act in a protective role in that setting activation of TNF- α could be playing an anti-apoptotic role. In line with the previous chapter this data once again highlights a potential protective role for TLR5 in the β -cell.

TLR2, TLR4 and TLR5 activation of MIN6 cells also resulted in a significant increase in the production of the chemokines MIP-1 α (CCL3), MIP-2 α (CXCL2), MCP-1 (CCL2) and RANTES (CCL5). This suggests that TLR activation results in increased chemotactic properties of MIN6 cells. This is supported by the fact that we have shown that supernatants from TLR stimulated MIN6 cells have the ability to induce macrophage migration. Increased levels of chemokines have also been associated with type 2 diabetes (Ehse et al. 2007; Herder et al. 2006). Immunohistochemical analysis of pancreas sections from patients with T2D, C57BL/6 mice fed a high-fat diet, db/db mice and Goto-Kakizaki (GK) rats all showed elevated numbers of macrophages within islets (Ehse et al. 2007). Of note is the observation that increased numbers of macrophages were detectable very early in high-fat-fed mouse islets, before the onset of diabetes and possible early infiltration of macrophages may be beneficial to islet function (Ehse et al. 2007). Increased circulating levels of MCP-1 are increased in obesity, associated with insulin resistance and predict development of T2D (Herder et al. 2006). Recent findings by Cai *et al* also have shown that MCP-1 up-regulates amylin expression in murine pancreatic β -cells and Min6 cells (Cai et al. 2011). Islet amyloid deposition is a characteristic pathological feature of the pancreas in type 2 diabetes patients. Therefore the ability of TLR activation of β -cells to induce macrophage migration may be protective in the short term, however throughout disease progression macrophages may become constitutively activated and play a role accelerating pancreatic islet dysfunction and death. Further work needs to be carried out to investigate the polarity of migrating macrophages induced following TLR stimulation.

As MIN6 media (both control and stimulated) had the ability to induce J774A.1 macrophage migration we next sought to investigate the effect that this media had on macrophage function. In a steady state, conditioning of J774A.1 macrophage with MIN6 media results in a slight decrease in some surface markers with no change to cytokine secretion. Conditioning of J774 macrophage with MIN6 media seems to confer a certain level of anti-inflammatory protection against TLR ligand stimulation. As a general trend we saw a reduction in the secretion of pro-inflammatory cytokines and chemokines from conditioned cells in comparison to control cells with a few exceptions. As it is known that pro-inflammatory cytokines can cause β -cell dysfunction and death, and interfere with insulin signalling (Donath et al. 2010) it is likely that there are measures in place in the islet to tightly regulate these processes, and crosstalk between the β -cell and macrophages may be key in this. We know that Min6 β -cells secrete IL-4 and IL-27p28 therefore it is probable that these cytokines play a role in the crosstalk between these two cell types.

IL-4 is a known inducer of alternative macrophage activation (Gordon 2003). From an immunological stand-point these macrophages play prominent roles in host responses to parasitic infections that stimulate the production of Th2 cytokines IL-4 and IL-13, however there is an increasing body of evidence that alternatively activated macrophages play important roles in the maintenance of tissue homeostasis and wound repair (Gordon 2003). For this reason, the driving of an alternative activation phenotype may be beneficial for resident or infiltrating macrophages in the islet, and perhaps is not maintained during T2D leading to classically activated macrophages secreting pro-inflammatory cytokines in the environment of the islet causing damage to the β -cells. Consistent with our data IL-4 (Fiorentino et al. 1991) and IL-27 (Hölscher et al. 2005) have been shown to inhibit production of pro-

inflammatory cytokines in macrophages such as TNF- α , IL-6 and IL-12 and alternatively activated macrophages have been shown to have increased responsiveness to the suppressive effects of IL-27 (Rückerl et al. 2006). A further link to the importance of IL-4 for macrophages in the context of T2D comes from a study which shown macrophages isolated from obese/diabetic db/db mice have impaired IRS-2-mediated PI3K activity and constitutively overexpress suppressor of cytokine signaling (SOCS)-3, which impairs IL-4-dependent production of IL-1RA, an important anti-inflammatory function (Connor et al. 2007b). In terms of macrophage activation status our own studies have shown that conditioning of macrophage results in a decrease in “M1” type genes IL-1 β and NOS2 and an increase in “M2” type genes ARG1 and MRC1 in both control and LPS stimulated cells. Once again, as this was studied at an mRNA level and not at the protein level we cannot say for certain that processes such as post-translational modification and activation of caspases are not influencing our results to be certain of this protein work would have to be carried out. However, in similar studies reporting a shift from an M1 to M2 phenotype in mouse bone-marrow derived macrophage Cho *et al* reported a similar decrease in NOS2 expression with a concurrent increase in ARG1 expression. The authors found that both protein levels and mRNA levels correlated with each other (Cho et al. 2014) leading us to believe that if investigated we would see similar results in our cells.

The effect of MIN6 conditioned media on chemokine secretion from J774 macrophage does not seem as straight-forward however, a dampened down effect can be seen in some cases. In the case of un-stimulated cells, the levels of MIP-1 and MIP-2 secretion are similar, RANTES secretion is decreased whereas MCP-1

secretion is increased in conditioned versus control cells. RANTES is the only chemokine that we found to be consistently down-regulated in both control and stimulated states. RANTES is involved in direct antimicrobial activity by inducing NO in macrophages, however RANTES can have detrimental effects via the recruitment of immune cells such as T cells, dendritic cells, eosinophils, NK cells, mast cells, and basophils to sites of inflammation and infection that enhance inflammatory processes such as arthritis, atopic dermatitis and colitis (Villalta et al. 1998). For this reason the dampening down of the response in both control and stimulated cells may be beneficial for the β -cells to exert a measure of control over the inflammatory environment in the islet. In the case of MCP-1, in un-stimulated conditions, levels are increased in conditioned cells versus control cells and this is also the case in the majority of TLR stimulated conditioned cells vs control cells. As MIN6 media alone had the ability to induce MCP-1 secretion from J774 macrophage we postulate that this effect is potentiated in conditioned cells upon TLR stimulation. MCP-1 has been shown to induces amylin expression in mouse islets and MIN6 β -cells (Cai et al. 2011) and is associated with insulin resistance therefore secretion of MCP-1 by macrophage may pose a detrimental effect to the β -cell. However in our own studies we did not find an increase in IAPP mRNA levels following TLR stimulation. Although dampening of the immune response is beneficial in some environments, some level of response needs to be mounted to fight off infection; therefore the preservation of MCP-1, MIP-1 and MIP-2 secretion in conditioned macrophages may be important for a normal immune response. MIP-1 and MIP-2 have also been implicated in playing homeostatic roles in some cell types and environments (Rollins 1997), and this may be another reason that their expression is not dramatically altered in response to MIN6 conditioning.

Molecules expressed on the cell surface of macrophage are a functional interest because they determine the ability the cell to interact with pathogens, and with other cell types, to generate an appropriate innate and acquired immune response. In general activation of macrophage with TLR ligands results in an increase in many cell surface markers required to mount an immune response. In resting conditions, J774 macrophage cultured in MIN6 media show a slight decrease in cell surface expression of TLR2, TLR4, MHCII, CCR5 and CD40 when compared with control cells. This once again lends itself to the idea of the MIN6 media exerting an overall anti-inflammatory action on the macrophages. This idea is carried through by the fact that although MIN6 conditioned J774 macrophage respond to TLR stimulation by up-regulation of their cell surface markers, the response is severely blunted in comparison to control cells. In many cases the response to TLR stimulation from MIN6 conditioned J774 macrophage is 50% of that of control cells. As before this is most likely a protective mechanism as hyper-responsive macrophages in the environment of the islet could lead to uncontrolled inflammation which would be detrimental to β -cell function and survival. Another key finding pointing to MIN6 conditioning of macrophage resulting in a hypo-responsive phenotype is the decrease seen in ROS production in conditioned macrophage in both a steady state and following stimulation. Although ROS production is decreased, their phagocytotic ability – the hallmark of macrophage function is not affected. This is of note as in type 2 diabetes and metabolic syndrome macrophages have been shown to display some altered immune and inflammatory activities. For example, these macrophages have decreased phagocytosis of microbes (Plotkin et al. 1996).

In this chapter we have demonstrated that activation of MIN6 cells with TLR ligands has the ability to alter their immune response by altering both cytokine and chemokine secretion and cell surface marker expression. Some of the mediators involved, most notably IL-27p28 whose secretion is induced most strongly upon flagellin stimulation, appear to play a protective role in the β -cell. For this reason we postulate that TLRs may exist on β -cells not only for immune mediated defence but they may also play a homeostatic role. This is further supported by that fact that mice deficient in the TLR adaptor protein MyD88 were more susceptible to HFD- induced diabetes (Hosoi et al. 2010). We have also shown that MIN6 cells are capable of interacting with J774 macrophage. MIN6 secretions have the ability to induce macrophage migration, and induce an overall anti-inflammatory phenotype in the macrophage. In the next chapter we will further confirm the influence of MIN6 cells on macrophage phenotype and then explore whether the factors that mediate this effect are present in MIN6 β -cell exosomes.

Chapter 5

Investigation of mediators involved in β -cell – M \emptyset crosstalk

5.1 Introduction

In the previous chapter we have shown the development of an anti-inflammatory phenotype in macrophages, induced by MIN6 β -cell secretions. These macrophage display low production of pro-inflammatory cytokines and other pro-inflammatory mediators, a reduced response to stimuli and a decrease in proinflammatory “M1” genes and increased “M2” genes. With this in mind we sought to investigate what may be mediating this response.

The 2013 Nobel Prize in Physiology or Medicine was jointly awarded to three scientists for their discovery of vesicle transport in cells, a clear indication of the importance of vesicles in physiology and their applied potential in diagnostics and therapy. Almost all cells release different types of membrane microvesicle and nanovesicle, which have a variety of important physiological effects. Exosomes, classed as nanovesicles, are between 30–100 nm in diameter. The protein content of different types of extracellular vesicle mostly reflects that of the parent cells and vesicles are enriched in certain molecules, including adhesion molecules, membrane trafficking molecules, cytoskeleton molecules, heat shock proteins, cytoplasmic enzymes, signal transduction proteins, cytokines, chemokines, proteinases and cell-specific antigens (Robbins & Morelli 2014; Raposo & Stoorvogel 2013). It is thought that extracellular vesicles have important roles in intercellular communication, both locally and systemically, as they have the ability to transfer their contents, including proteins, lipids and RNAs, between cells (Ratajczak et al. 2006; Valadi et al. 2007). Recent evidence has pointed to a role for exosomes from

both immune and non-immune cells in numerous immunological processes (Robbins & Morelli 2014). Of particular interest to us is the observation that cell derived exosomes have been shown to have immunosuppressive effects in a number of different models (Wieckowski et al. 2009; S. H. Kim et al. 2007; van Koppen et al. 2012). With this in mind we sought to investigate whether the extracellular vesicles isolated from MIN6 culture medium, were mediating the effect on macrophage function.

We have shown that there is crosstalk between beta cells and macrophage and there is evidence in the literature to demonstrate that infiltration of macrophage into the pancreas may play a role in the development of type 2 diabetes (Westwell-Roper et al. 2014; Richardson et al. 2009; Eguchi & Manabe 2013; Ehses et al. 2008; Ehses et al. 2007). Furthermore, studies have shown that inflammation in the gut may have the ability to influence insulin signalling and T2D. This has been particularly evident from two lines of investigation. Firstly, studies have demonstrated that after bariatric surgery, used to correct obesity which is linked to low grade inflammation (Donath et al. 2013) , there is an almost immediate recovery from type 2 diabetes following the anatomical change which impacts on the connection between the gut and the pancreas (Rubino et al. 2006; Buchwald et al. 2009; Schauer et al. 2003; Dixon et al. 2008). Furthermore numerous studies have linked altered gut microbiota and inflammation with T2D and metabolic syndrome (Chassaing & Gewirtz 2014; Carvalho & Saad 2013; Ding et al. 2010). These studies clearly outline important signalling mechanisms between the gut in the context of inflammation, obesity and T2D. Therefore, we decided to explore whether inflammation in the gut has the

ability to influence infiltration of macrophage into the pancreas. To do this we used the mouse model of DSS-induced colitis.

5.2 Results

5.2.1 Conditioned macrophage return to normal phenotype once removed from MIN6 media

To be sure that the effect on macrophage phenotype was arising from conditioning with MIN6 media, we looked at how they responded when they were returned to normal media and re-stimulated. To do this J774 macrophage were plated at a concentration of 1×10^6 cells/ml in 6 well plates in either MIN6 conditioned media or unconditioned DMEM-glutamax (control). The cells were then left to rest for 24hr prior to 24hr stimulation with LPS (100ng/ml). Media was then removed and cells were washed and normal (unconditioned) DMEM was added to all cells. Cells were then left to rest for 6hr prior to reactivation with LPS (100ng/ml) for 24 hours. Cells were stained with fluorescently labelled antibodies for CD40, CD80, CD86, MHCII, TLR2 and TLR4. Histograms show surface marker expression of stimulated cells (black line) compared to stimulated (previously conditioned) cells (dashed line). Filled histograms represent fluorescence of unstained cells. **Figure 5.1** clearly shows that surface marker expression on previously conditioned cells returns to expression levels similar to that of non-conditioned cells once the MIN6 media has been removed.

5.2.1 Pre-conditioning macrophage with recombinant IL-4 does not mimic effects of MIN6 conditioning

As we had previously shown that MIN6 cells constitutively secrete IL-4 [Figure 3.17] and as this is a known inducer of alternative macrophage activation (Gordon 2003), we sought to investigate whether preconditioning with recombinant IL-4 would have similar effects to that of MIN6 conditioning of macrophage surface marker expression. In order to do this, J774A.1 macrophage were plated at a concentration of 1×10^6 cells per ml in 6 well plates. 2000pg/ml of recombinant IL-4 (R&D) was added to the cells to mimic the amount of IL-4 secreted from MIN6 cells (~1500 pg/ml). Cells were then left for 24hr prior to surface expression analysis by flow cytometry. Cells were stained with fluorescently labelled antibodies for CD40, CD80, CD86, MHCII, TLR2 and TLR4. Histograms show surface marker expression of stimulated cells (black line) compared to IL-4 conditioned, stimulated cells (dashed line). Filled histograms represent fluorescence of control cells. Pre-conditioning cells with 2000 pg/ml of IL-4 for 24hr does not mimic the effect of conditioning with MIN6 supernatants [Figure 5.2]. Conditioning cells with IL-4 prior to activation with LPS did result in lower surface marker expression of CD40 and CD80. However CD86 and TLR2 remain the same and MHCII and TLR4 expression is actually higher in IL-4 treated cells than in control cells [Figure 5.2].

5.2.3 Expression of surface markers on MIN6 conditioned bone marrow derived macrophage

In order to assess the effect of MIN6 conditioning in an “*ex vivo*” situation, bone marrow cells were isolated from the femurs and tibias of BALB/c mice and

differentiated into BMMØ *in vitro* in the presence of the macrophage colony-stimulating factor (eBioscience) for 6 days. The macrophage colony-stimulating factor (M-CSF) is a lineage-specific growth factor responsible for differentiation and proliferation of myeloid progenitors into cells of macrophage lineage (Sweet & Hume 2003). Cells were then plated at a concentration of 1×10^6 cells per ml in 6 well plates in either control media or MIN6 conditioned media. Cells were then left to rest for 24hr prior to 24hr stimulation with or without LPS (100ng/ml). Cells were then stained with fluorescently labelled antibodies for CD40, CD80, CD86, MHCII, TLR2 and TLR4. Histograms show surface marker expression of stimulated cells (black line) compared to MIN6 conditioned, stimulated cells (dashed line). Grey line represents unstimulated control cells and filled histograms represent fluorescence of unstained cells. Conditioning of BMMØ in MIN6 supernatants for 24hr prior to activation with LPS results in decreased expression of cell surface markers when compared to control LPS treated cells [Figure 5.3]. This decrease can be seen in the case of all surface markers examined with the exception of CD40 in which conditioned cells show slightly higher expression compared to control LPS treated cells [Figure 5.3].

5.2.4 MIN6 exosome isolation and fractionation

Extracellular vesicles, including exosomes, are small membrane vesicles derived from multivesicular bodies or from the plasma membrane. Most, if not all, cell types release extracellular vesicles, which then enter the bodily fluids. These vesicles contain a subset of proteins, lipids and nucleic acids that are derived from the parent cell. It is thought that extracellular vesicles have important roles in intercellular

communication, both locally and systemically, as they transfer their contents, including proteins, lipids and RNAs, between cells. As cell culture medium is known to be rich in vesicles, we thought to investigate whether or not these fractions had the capability to alter macrophage function. In order to do this, vesicles were isolated from MIN6 conditioned medium and fractioned according to their size.

Name	Size	ug Protein
FR1	↑ 1000 kDa P20,000g	290.5ug
FR2	↑ 1000 kDa P200,000g	1135.8ug
FR3	↑ 1000kDa SN 200,000g	745.7ug
FR4	↑ 300 kDa SN 200,000g	3929.2ug
FR4	↑ 300 kDa P200,000g	407.5 ug
P = Pellet, FR = Fraction, SN = Supernatant ↑=above size		

Table 5.1 MIN6 exosome isolation.

5.2.4.1 Culturing MIN6 cells in exosome-free FBS does not affect cell viability

As FBS is known to contain a lot of vesicles, it was necessary to culture the MIN6 cells in exosome-free FBS to make sure that anything isolated was indeed from the cells themselves. Cells were grown to confluency in DMEM supplemented with either normal FBS or exosome free FBS. Cells were then plated in 96 well plates at a concentration of 1×10^6 cells/ml and left for 24hr. The viability of the cells was then assessed using the MTS assay. Culturing MIN6 cells in exosome-free FBS did not significantly alter cell viability when compared to normal media [**Figure 5.4 A**].

5.2.4.2 Culturing J774 cells in media containing resuspended exosome fractions does not alter cell viability

Once MIN6 vesicle fractions were isolated they were stored at -80°C until further use. The fractions were isolated from a total of 75mls of conditioned media. Each fraction was then resuspended in 50ml of complete DMEM. To assess the effect of fractions on viability, J774 macrophage were plated at a concentration of 1×10^6 cells/ml in 96 well plates in normal media and media containing 1 of 5 fractions isolated (from here on in referred to as FR1- FR5). Cells were left for 24hr and viability was assessed using the MTS assay. None of the 5 fractions tested had a significant effect on cell viability when compared to control cells [Figure 5.4 B].

5.2.3 Effect of vesicle fractions on J774 macrophage response to TLR ligation

5.2.3.1 J774 cell surface markers

In chapter 4 we showed that conditioning J774 macrophage with MIN6 conditioned medium prior to activation with LPS resulted in an over-all dampening down of the immune response to this stimulus. In order to ascertain whether or not this effect may be caused by mediators released in exosomes, J774 macrophage were cultured in DMEM containing resuspended vesicle fractions from MIN6 conditioned medium for 24hr prior to activation with LPS (100ng/ml) for a further 24hr. Cells were stained with fluorescently labelled antibodies for TLR2, TLR4, CD40, CD80 and CD86. Histograms show surface marker expression of stimulated cells (black line) compared to stimulated MIN6 conditioned cells (dashed line). Grey line represents unstimulated control cells. TLR2 expression is decreased following stimulation with

LPS in cells cultured with FR2, FR4 & FR5 [Figures 5.5 B, D & E] in comparison to non-conditioned LPS treated cells. Cells cultured with FR1 and FR 3 did not show decreased TLR2 expression when compared to non-conditioned LPS treated cells [Figures 5.5 A & C]. TLR4 expression is decreased following LPS activation in cells cultured with all fractions [Figures 5.6 A – E] in comparison to control LPS treated cells. CD40 expression remains unchanged in fraction treated vs control cells [Figure 5.7 A – D] following stimulation with the exception of FR5 treated cells where a decrease in expression is seen [Figure 5.7 E]. Treatment with all fractions resulted in a decrease in CD80 expression following LPS stimulation [Figure 5.8 A-C & D] with the exception of FR4 [Figure 5.8 D] where no change is observed in control vs fraction treated LPS stimulated cells. Finally no change to CD86 expression was observed for any fraction [Figure 5.9 A – D]. All data is summarised in Table 5.2.

	TLR2	TLR4	CD40	CD80	CD86
FR1	↔	↓	↔	↓	↔
FR2	↓	↓	↔	↓	↔
FR3	↔	↓	↔	↓	↔
FR4	↓	↓	↔	↔	↔
FR5	↓	↓	↓	↓	↔
↔ = no change in expression ↓=decrease in expression vs control LPS treated J774					

Table 5.2 Summary of surface marker expression of J774 cultured with MIN6 vesicle fractions following LPS stimulation

5.2.3.2 J774 cytokine secretion

We next assessed the effects of the exosome fractions on MIN⁶ induced macrophage cytokine and chemokine secretion. J774 macrophage were cultured in DMEM containing resuspended vesicle fractions from MIN6 conditioned medium for 24hr prior to activation with LPS (100ng/ml) for a further 24hr. Supernatants were then analysed for cytokines IL-6, IL-12p40, IL-23, IL-27p28 and TNF- α secretion by ELISA (R&D). IL-6 secretion is decreased in J774 macrophage conditioned with FR1 (p<0.001), FR2 (p<0.05), FR4 (p<0.001) and FR5 (p<0.01) [**Figures 5.10 A, B, D & E**] following LPS stimulation in comparison to unconditioned LPS stimulated cells. However conditioning with FR3 actually resulted in increased IL-6 secretion compared to control LPS stimulated cells [**Figure 5.10 C**]. IL-12p40 secretion is decreased following LPS stimulation in macrophage conditioned with FR1 (p<0.01), FR3 (p<0.05), FR4 (p<0.001) and FR5 (p<0.01) and remains unchanged with FR2 [**Figures 5.11 A-E**]. IL-23 secretion is decreased in macrophage conditioned with FR2 (p<0.001), FR3 (p<0.05) and FR4 (p<0.001) and remains unchanged with FR1 and FR5 [**Figures 5.12 A-E**]. IL-27p28 secretion is increased following LPS stimulation in macrophage conditioned with all fractions [**Figures 5.13 B-E**] (p<0.01, p<0.01, & p<0.01 respectively) apart from FR1 (p<0.01) [**Figure 5.13 A**] where a decrease is observed in comparison to control LPS treated macrophage. Finally TNF- α secretion is decreased following conditioning with FR1 (p<0.05), FR2 (p<0.001) and FR3 (p<0.05) whereas no change in secretion levels is observed with FR4 and FR5 [**Figure 5.14 A-E**]. Data is summarised in **Table 2.3**.

	IL-6	IL-12	IL-23	IL-27p28	TNF- α
FR1	↓	↓	↔	↓	↓
FR2	↓	↔	↓	↑	↓
FR3	↑	↓	↓	↑	↓
FR4	↓	↓	↓	↑	↔
FR5	↓	↓	↔	↑	↔
↓decrease in secretion ↑=increase in secretion ↔+ no change in secretion vs control LPS treated J774					

Table 2.3 Summary of cytokine secretion from J774 cultured with MIN6 vesicle fractions following LPS stimulation

5.2.4 DSS-induced model of Colitis

As recent evidence had linked changes in gut homeostasis as playing a possible role in type 2 diabetes we decided to investigate whether or not the DSS-model of colitis, which causes inflammation in the intestine, had any effect on the pancreas.

5.2.4.1 Clinical assessment of DSS-induced colitis

The DSS model was carried out Biological Resource Unit (BRU), at Dublin City University as described in materials and methods (2.11). To assess the development of the disease, mice were weighed and scored (every day) for daily disease activity index (DDAI) based on stool composition, fur texture and posture. No significant difference in weight was observed in control vs DSS mice throughout the 7 day period [Figure 5.15 A]. The DDAI however showed disease progression. No disease activity was observed in the control mice, while there was an increase in the disease activity scores in DSS treated mice as soon as day 1 which peaked on day 6 [Figure

5.15B]. At each end point, colons were removed, measured and weighed. The weight and length of the colon are useful indicators of disease progression as the cell infiltration and inflammation increase the weight of the colon and also shrink the colon length (Okayasu et al. 1990). As we can see in **Figure 5.15 C** colons from the DSS treated mice were significantly ($p < 0.05$) shorter than control. Also, there was what appears to be an increase in colon weight in DSS treated mice [**Figure 15.D**] however it did not achieve significance.

Small sections of distal colon (0.5cm) were removed for histology and stained with haematoxylin and eosin (H&E), as described in Materials and methods section 2.11.3. The H&E staining of control shows a healthy colon with crypts and goblet cells present [**Figure 5.16**] In the DSS treated colon we can see a visible reduction in goblet cells, together with loss of crypts and infiltration of inflammatory cells to the mucosa [**Figure 5.16**].

5.2.4.2 Cytokine expression in Colon in DSS-induced colitis

RNA was isolated from colonic tissue from healthy control and DSS treated mice as described in materials and methods section 2.9.1.2. Complementary DNA (cDNA) was then generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA) as described in materials and methods section 2.9.2. The levels of IL-1 β , IFN- γ , TNF- α , IL-6 and IL-17 mRNA were quantified using qPCR. The expression levels were normalised to GusB levels and the gene expression is shown as a fold change relative to the control. It appears as if IL-1 β and IFN- γ expression is increased in DSS colon however this fails to achieve significance [**Figure 5.17 A&B**]. Both TNF- α and IL-6 expression are increased in

DSS colon compared to control healthy colon with a 5 fold increase seen in TNF- α levels ($p < 0.05$) and a 10 fold increase in IL-6 levels ($p < 0.05$) [Figure 5.17 C&D]. No expression of IL-17 was found in either control or DSS colon [Figure 5.17 E].

5.2.4.3 TLR expression in Colon in DSS- induced colitis

The role that TLRs play in the DSS colitis model is unclear, however it has been shown that mice deficient in the TLR adaptor molecule MyD88 develop severe intestinal inflammation during DSS-induced colitis (Araki et al. 2005). With this in mind the levels of TLR4 and TLR5 mRNA in both healthy and DSS treated colon were quantified using qPCR. The expression levels were normalised to GusB levels and the gene expression is shown as a fold change relative to the control. No significant difference in TLR4 expression between control and diseased colon was found, although a slight increase is observed [Figure 5.18A]. On the other hand TLR5 expression is significantly decreased ($p < 0.01$) in diseased colon compared to healthy controls [Figure 5.18B].

5.2.5 Pancreas histology in DSS-induced Colitis

5.2.5.1 Haematoxylin & Eosin

Sections from the head of the pancreas from both healthy and DSS treated mice were removed, paraffin embedded and stained with Haematoxylin and Eosin. **Figure 5.19** shows sections from both control and DSS treated pancreas. Staining clearly shows pancreatic lobules made up mainly of acinar cells (dark pink). Islets (light pink

circles) can be seen in both sections. No visible morphological difference between control and DSS-treated mice was observed.

5.2.5.2 Macrophage infiltration to pancreas in DSS-induced colitis

During the course of DSS-induced colitis an inflammatory response is occurring throughout the intestine. As the pancreas is a part of the gastro-intestinal system we sought to investigate whether or not inflammation occurring in the intestine could affect the pancreas. In order to do this we stained sections of pancreas from both healthy control and DSS-treated mice with an antibody to the macrophage marker F4/80 using the HRP-DAB system (R&D Systems). **Figure 5.20** shows sections from control (left) and DSS-treated (right) mice. Acinar cells are counter stained a light blue colour. F4/80 positive cells stain a light brown colour. **Figure 5.20** clearly shows a higher number of F4/80 positive cells in the DSS-treated pancreas. This is indicative of macrophage infiltration.

5.3 Figures

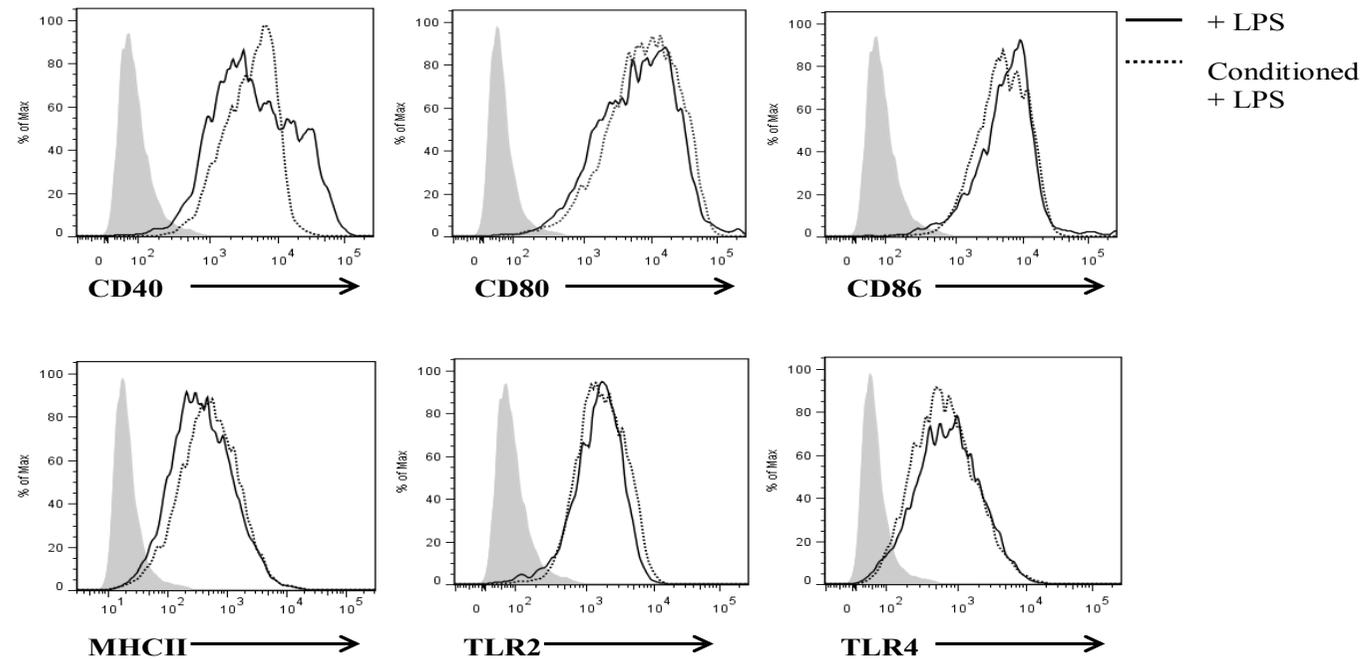


Figure 5.1 Conditioned macrophage returned to control media return to normal phenotype. J774A.1 macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Cells were then stimulated with LPS (100ng/ml) for a 24hr. Cells were then washed twice and returned to normal media, left to rest for 6hr and stimulated once more with LPS (100ng/ml) for 24hr. Cells were then stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Filled histograms represent fluorescence of unstained cells. Data are representative of three independent experiments.

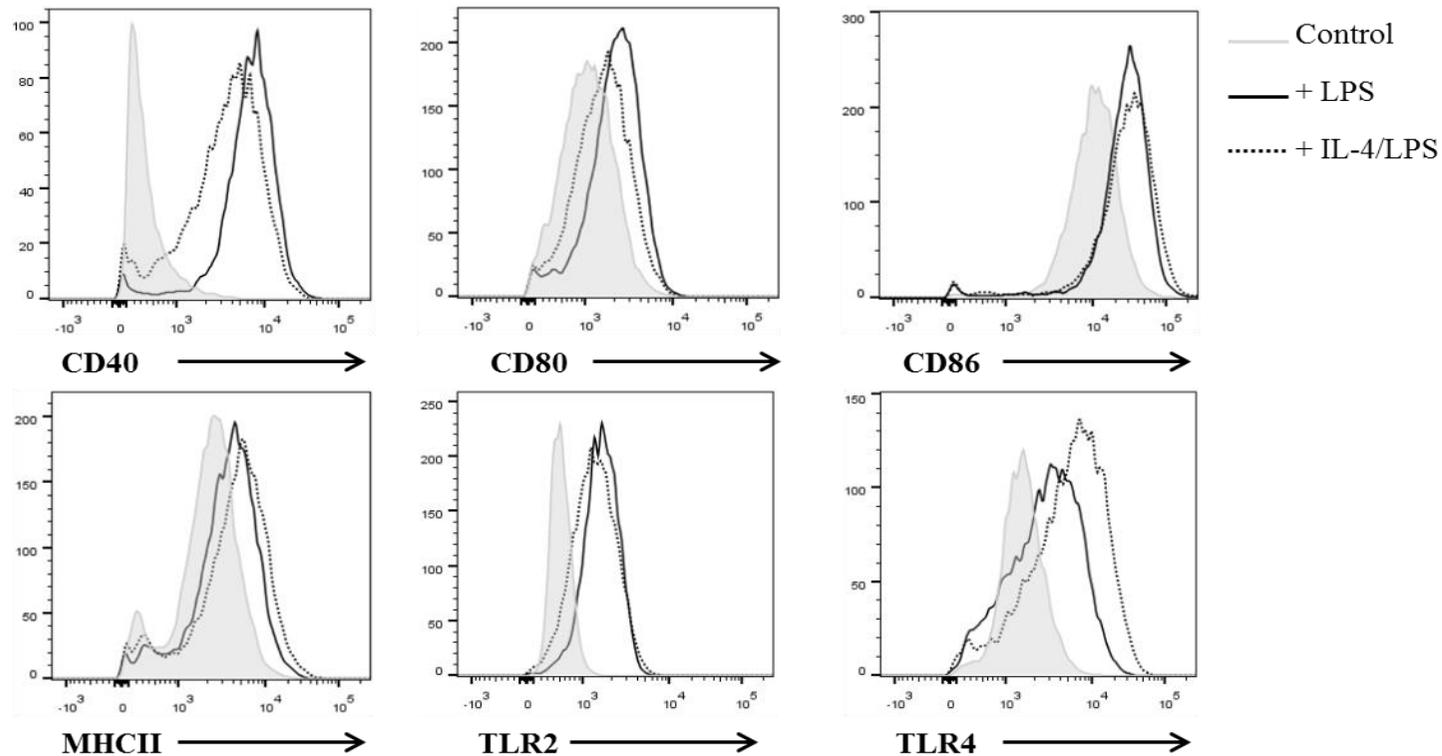


Figure 5.2 Pre-conditioning macrophage with IL-4 does not mimic effects of MIN6 conditioning. J774A.1 macrophages were incubated with normal medium containing $2\mu\text{g/ml}$ of recombinant IL-4 for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of LPS stimulated IL-4 treated cells (dotted line) compared to control cells (black line) following TLR4 stimulation. Filled histograms represent control cells (unstimulated). Data are representative of three independent experiments.

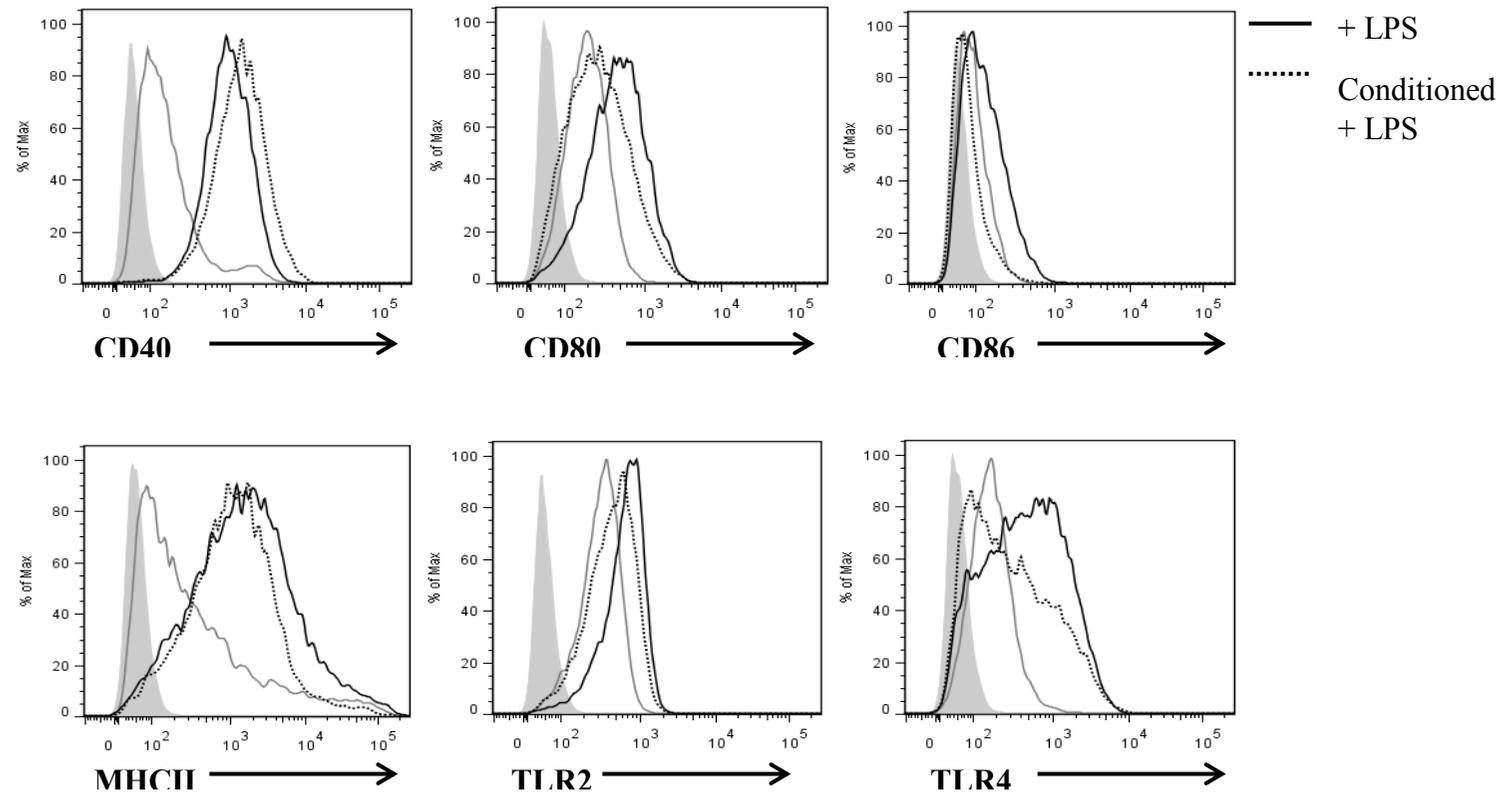


Figure 5.3 Expression of surface markers on MIN6 conditioned bone marrow derived macrophage. Bone marrow-derived macrophages were incubated with normal medium or MIN6 conditioned medium for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with fluorescently labelled antibodies for TLR2, TLR4, MHCII, CD80, CD86 & CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls. Filled histograms represent fluorescence of unstained cells.

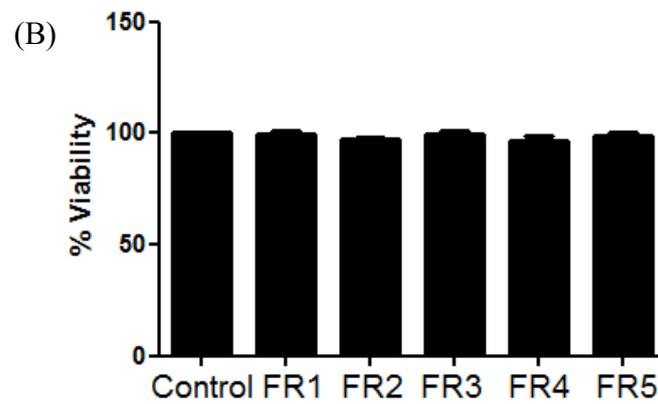
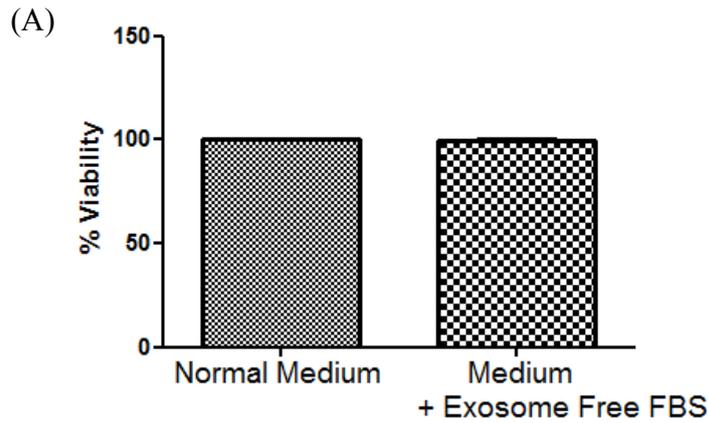


Figure 5.4 Exosome free FBS does not affect MIN6 viability & Conditioning J774 macrophage with media containing MIN6 fractions does not affect viability. (A) MIN6 cells were cultured in normal DMEM and DMEM containing exosome free FBS for 24hr. Following this 20 μ l of the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO₂ for 1hr. The plate was then read at a wavelength of 450nm. Results are expressed as a percentage of the control viability (100%). (B) J774 macrophage were cultured in normal DMEM and DMEM containing 1 of 5 fractions (FR1-FR5) from MIN6 conditioned medium for 24hr. Following this 20 μ l of the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO₂ for 1hr. The plate was then read at a wavelength of 450nm.

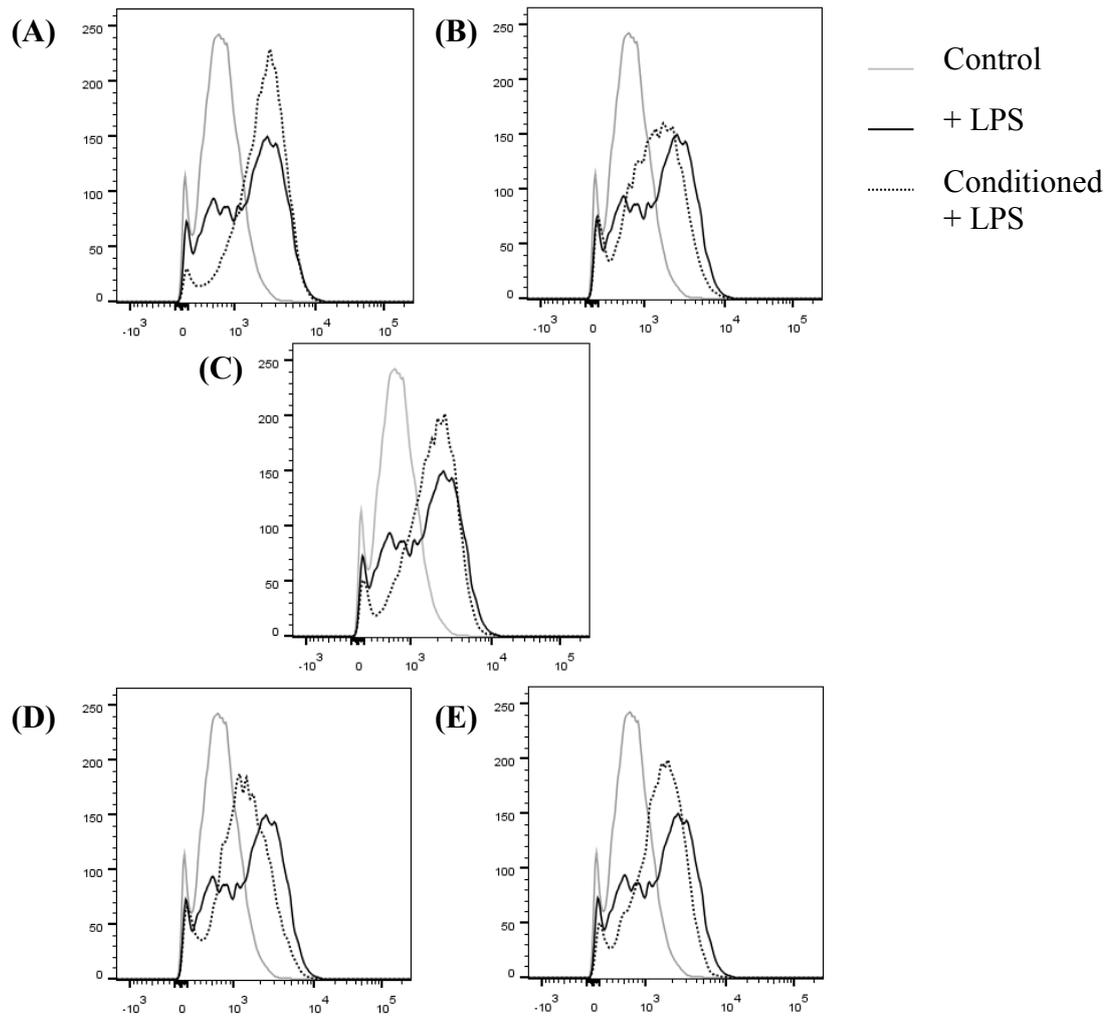


Figure 5.5 TLR2 expression on LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with a fluorescently labelled antibody for TLR2 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls.

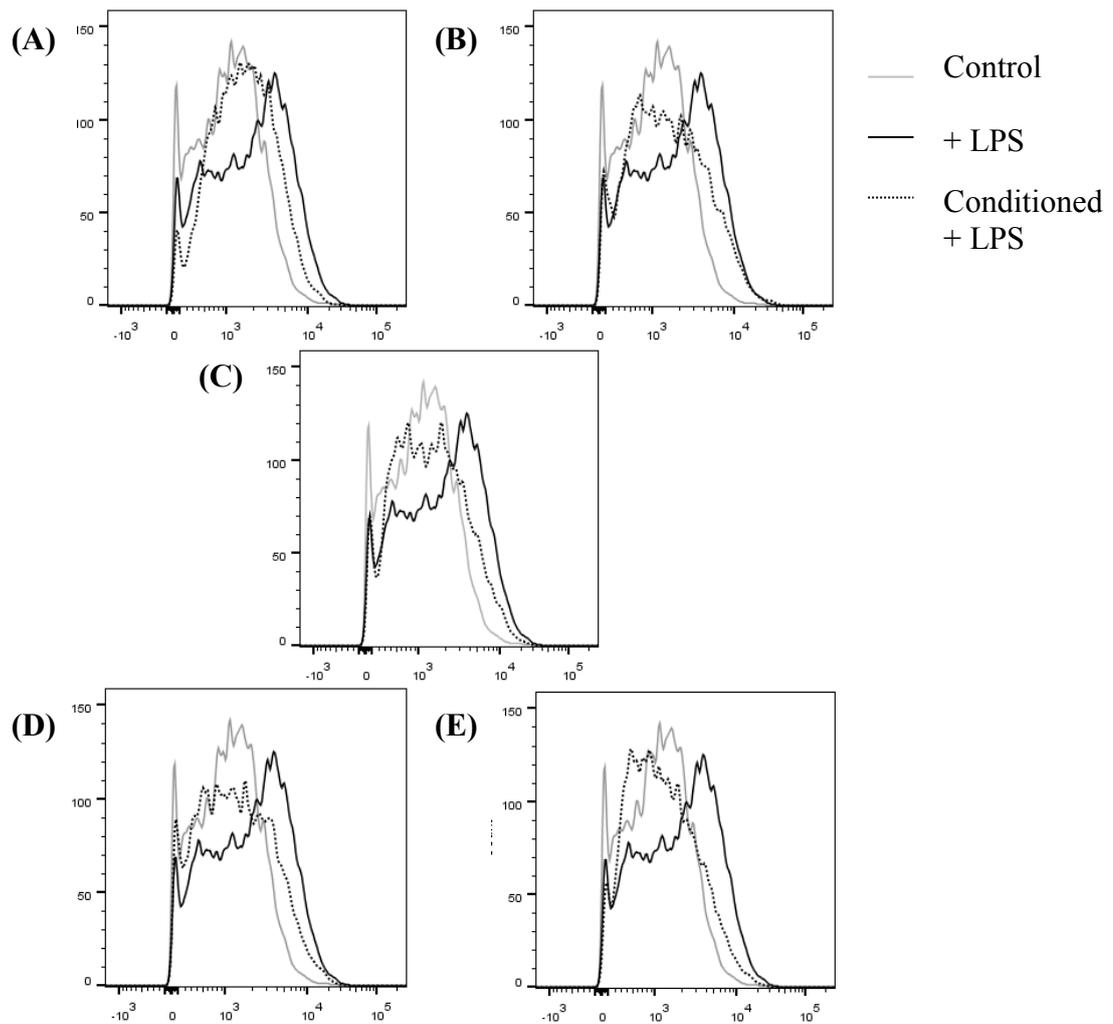


Figure 5.6 TLR4 expression on LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with a fluorescently labelled antibody for TLR4 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls.

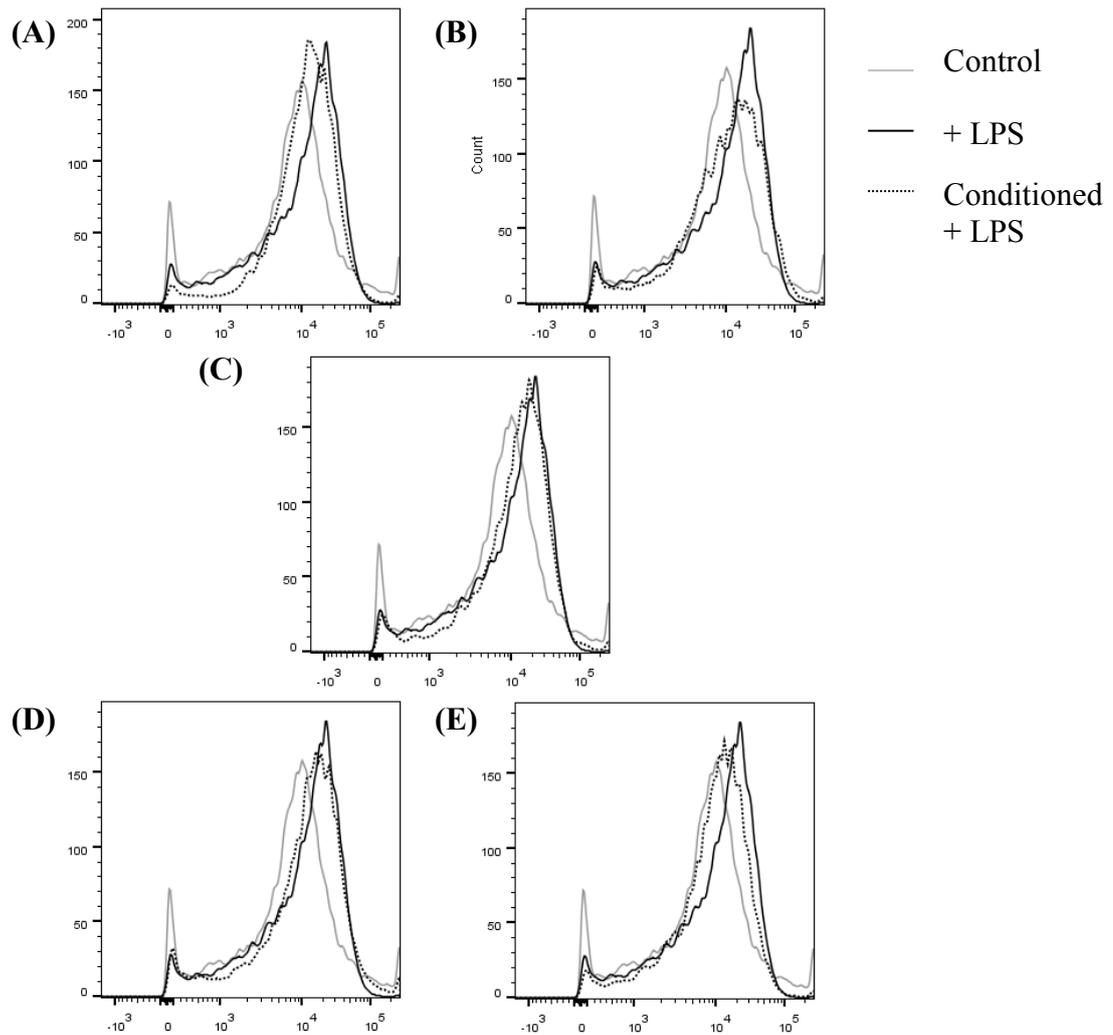


Figure 5.7 CD40 expression on LPS stimulated J774 macrophage following conditioning with fractionated MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with a fluorescently labelled antibody for CD40 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls.

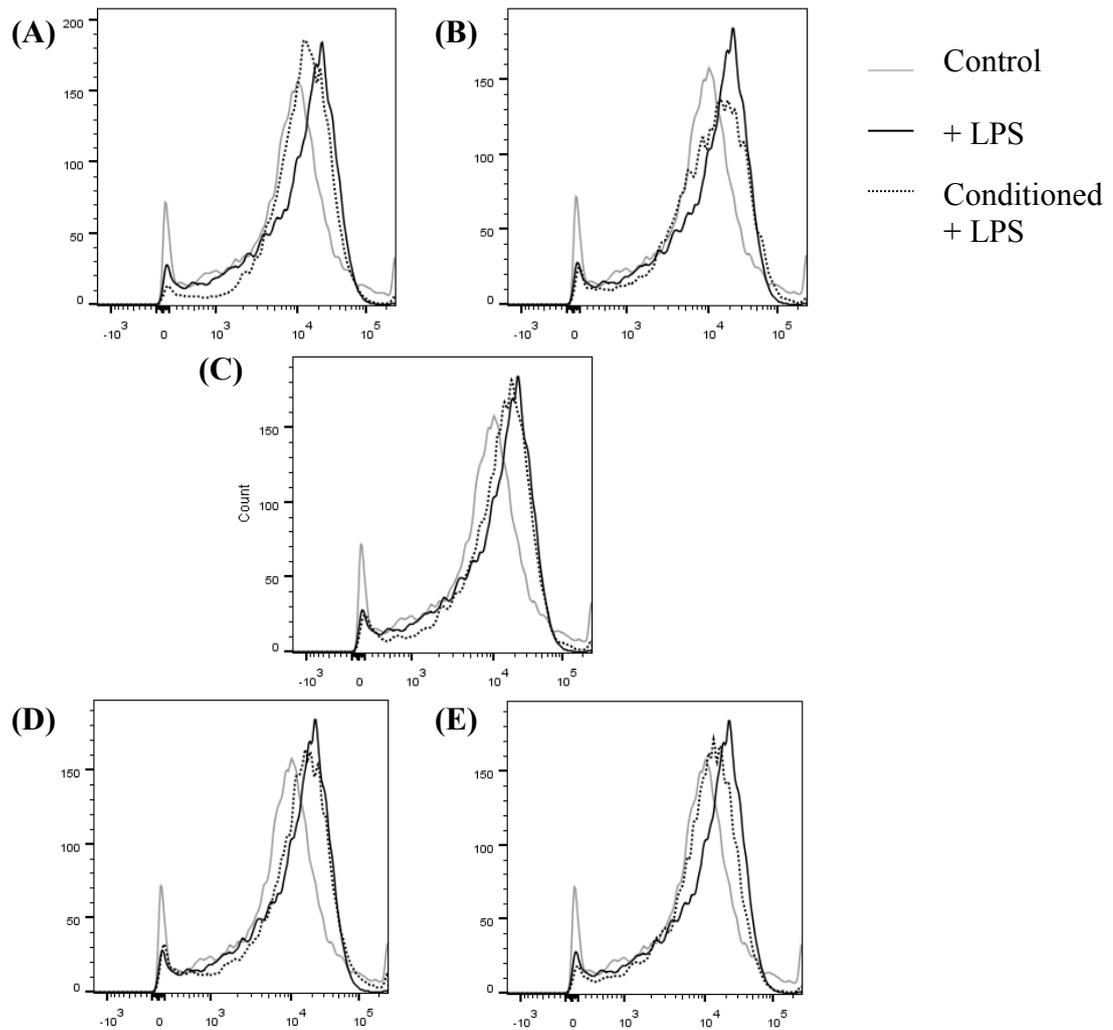


Figure 5.8 CD80 expression on LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with a fluorescently labelled antibody for CD80 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls.

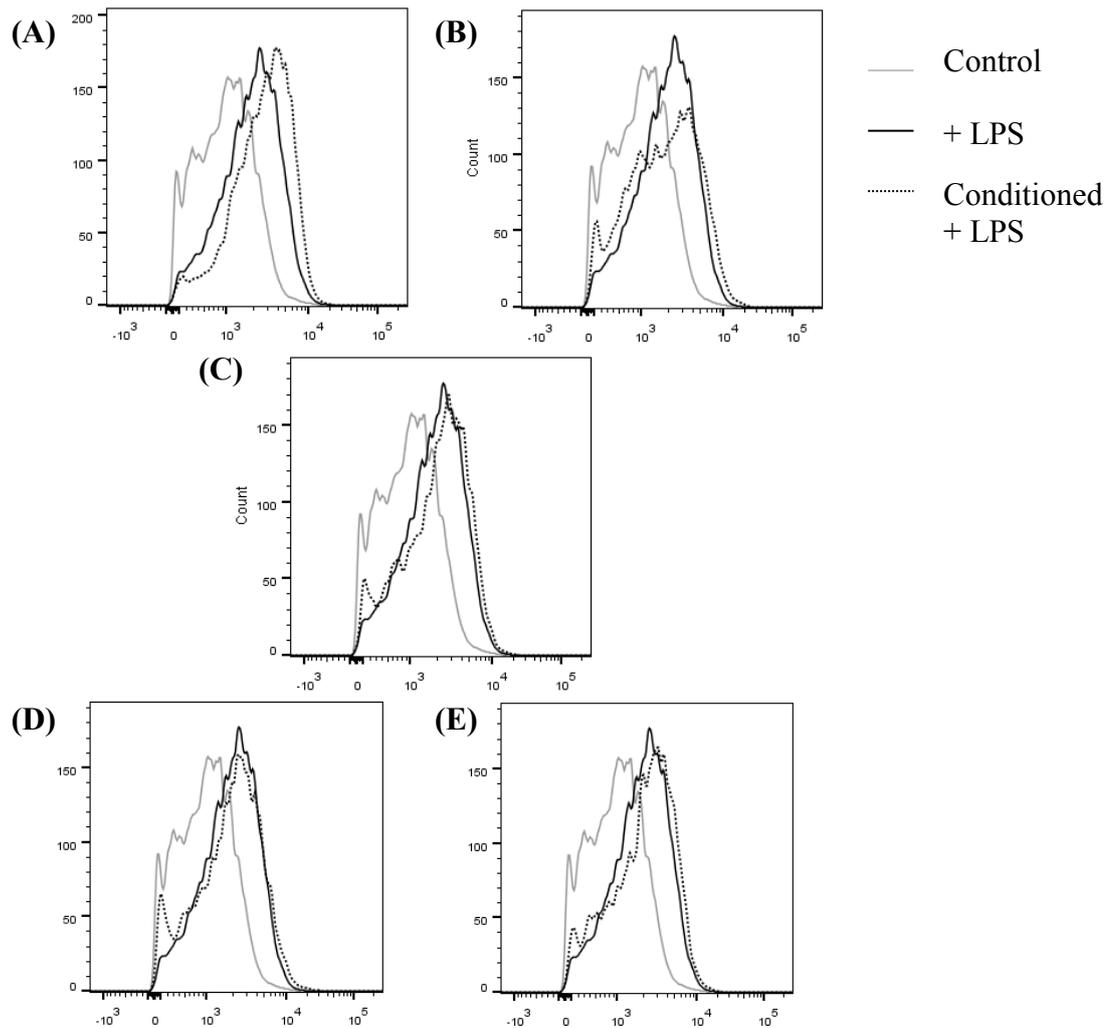


Figure 5.9 CD86 expression on LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Cells were then stained with a fluorescently labelled antibody for CD86 and expression was measured by flow cytometry. Histograms show surface marker expression of conditioned cells (dotted line) compared to unconditioned control cells (black line) following TLR4 stimulation. Grey line represents the expression on unstimulated controls.

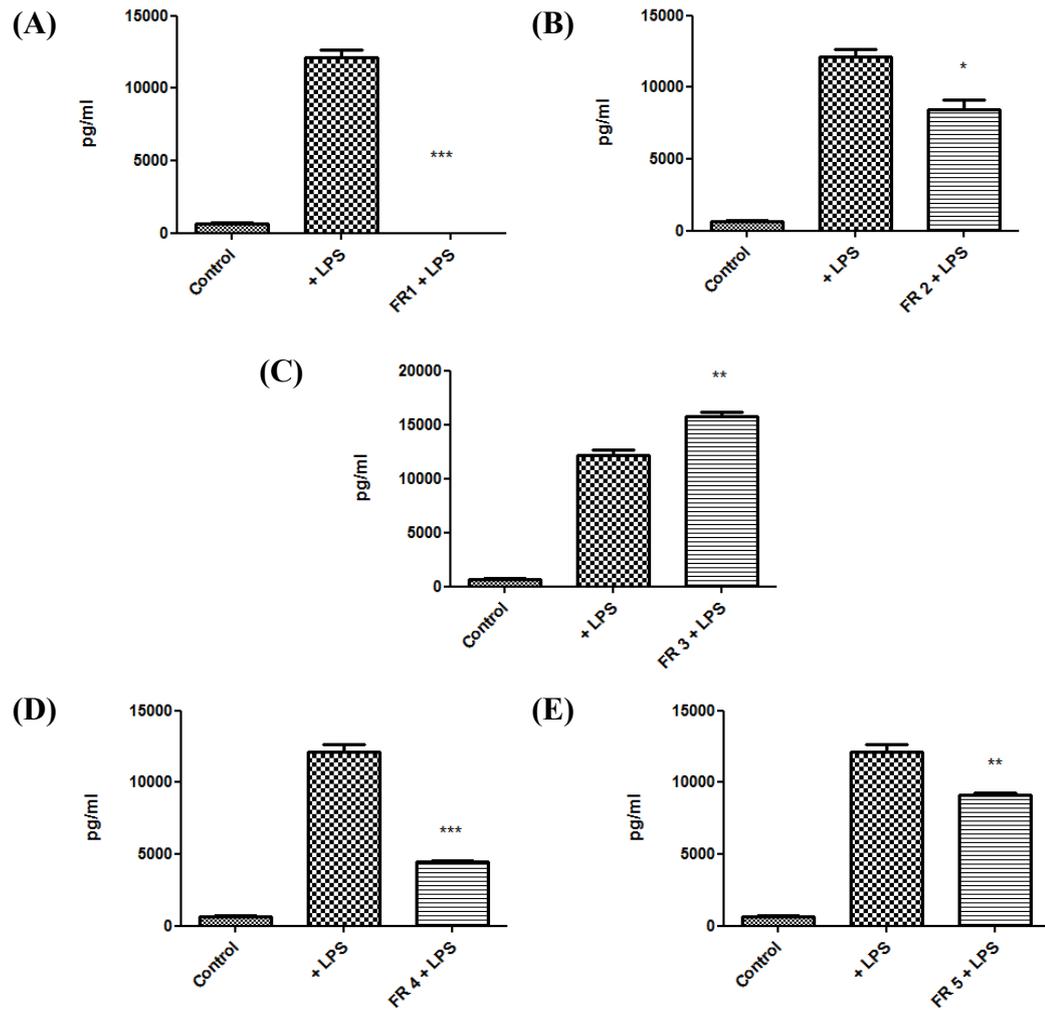


Figure 5.10 IL-6 secretion from LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction. **P<0.01, ***P<0.001 as determined by unpaired *t*-test.

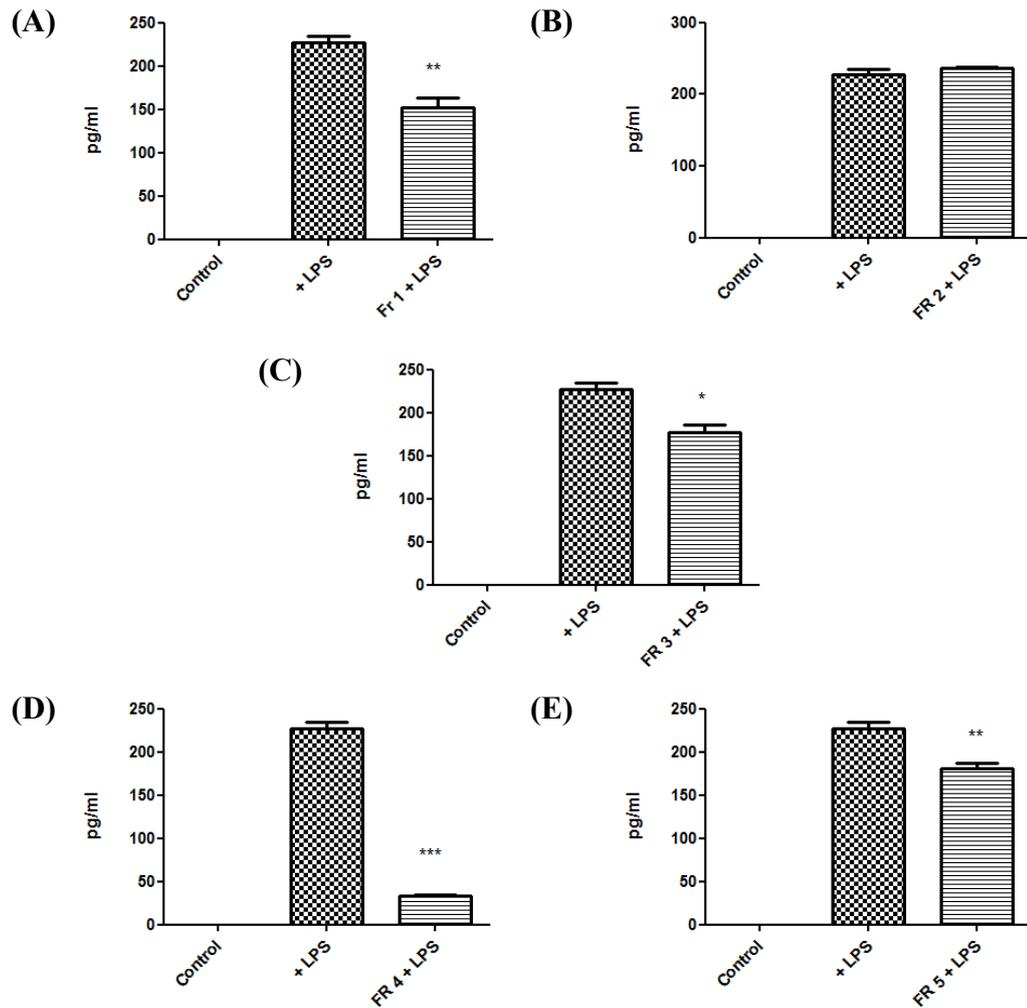


Figure 5.11 IL-12p40 secretion from LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined by unpaired t -test.

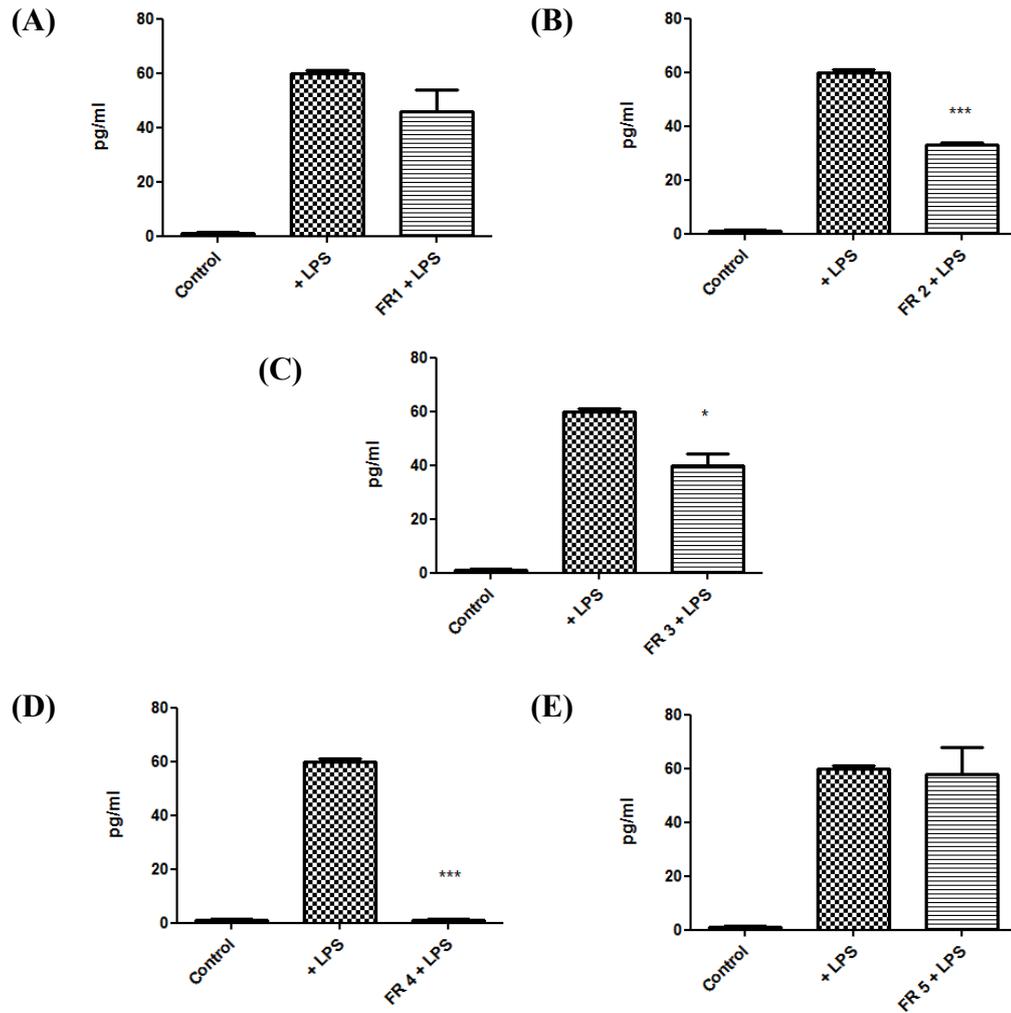


Figure 5.12 IL-23 secretion from LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction.* $P < 0.05$, *** $P < 0.001$ as determined by unpaired *t*-test.

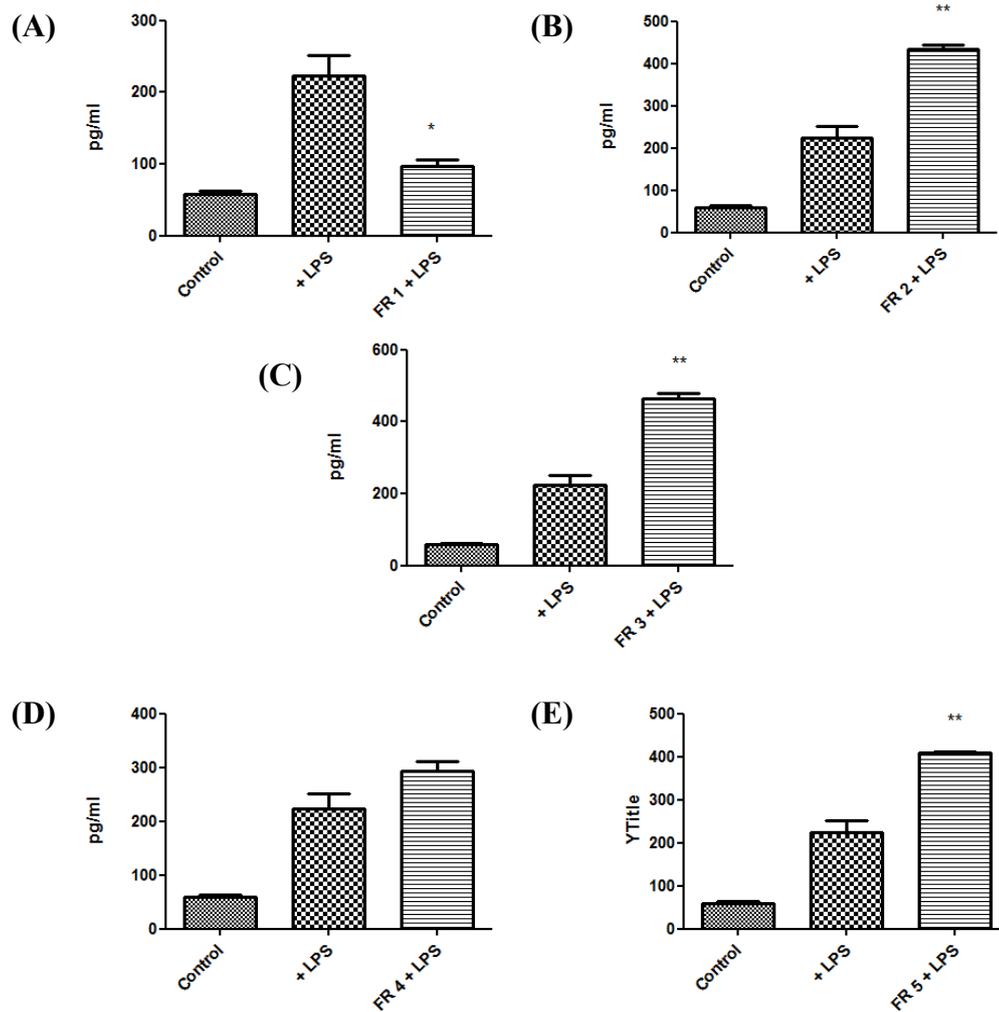


Figure 5.13 IL-27p28 secretion from LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction.*P<0.05, **P<0.01 as determined by unpaired *t*-test.

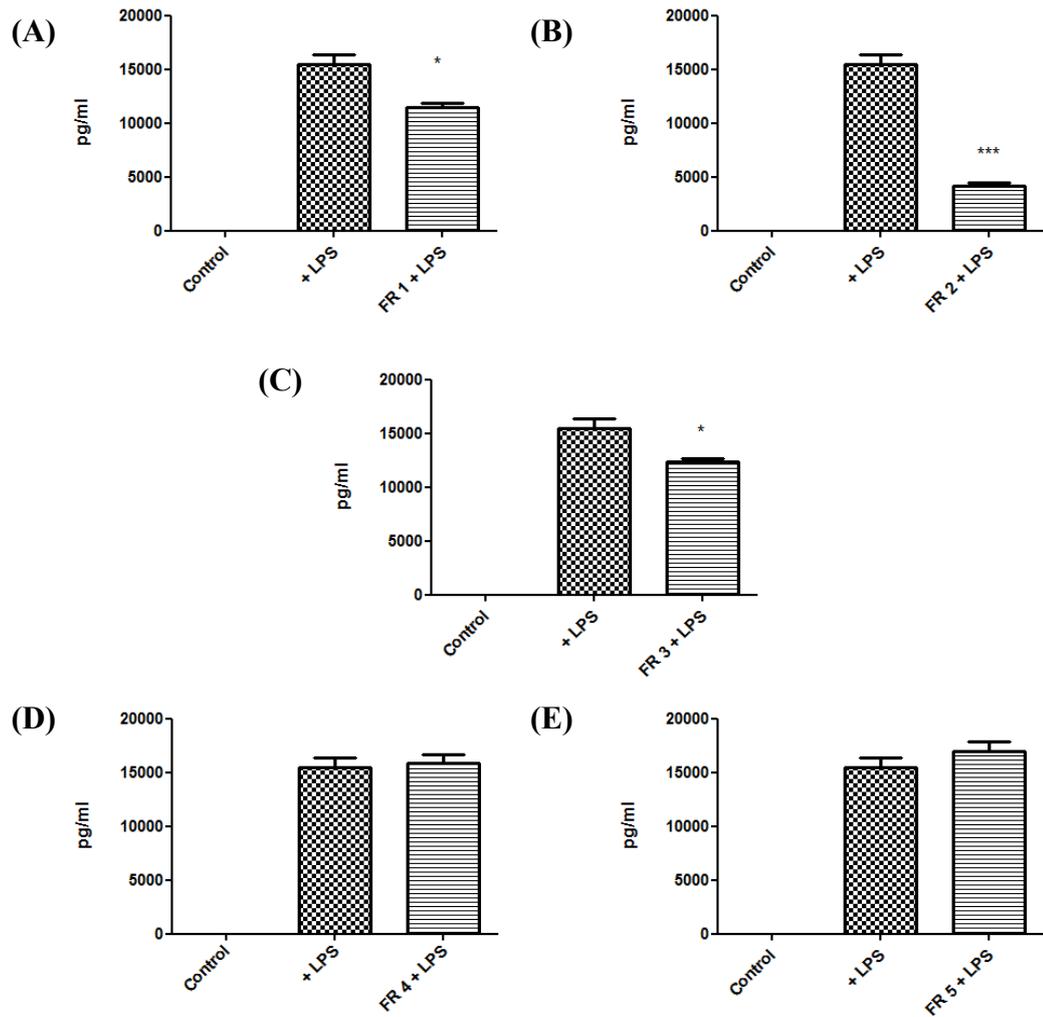


Figure 5.14 TNF- α secretion from LPS stimulated J774 macrophage following conditioning with fractioned MIN6 medium. J774A.1 macrophages were incubated with media containing 1 of 5 fractions from MIN6 conditioned medium (1-5 = A-E) for 24hr. Cells were then stimulated with LPS (100ng/ml) for a further 24hr. Following this supernatants were collected and cytokine secretion was measured by ELISA (R&D) according to manufacturer's instruction.*P<0.05, ***P<0.001 as determined by unpaired *t*-test.

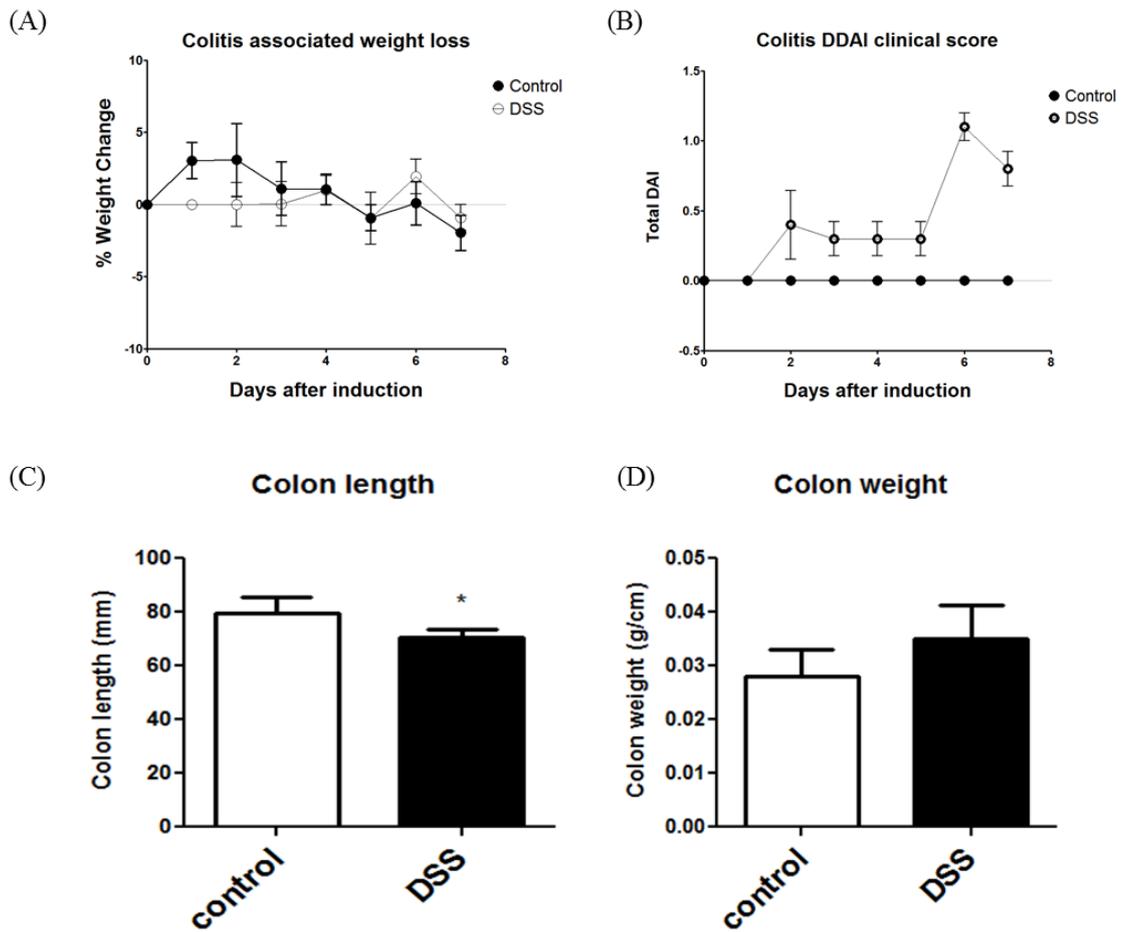
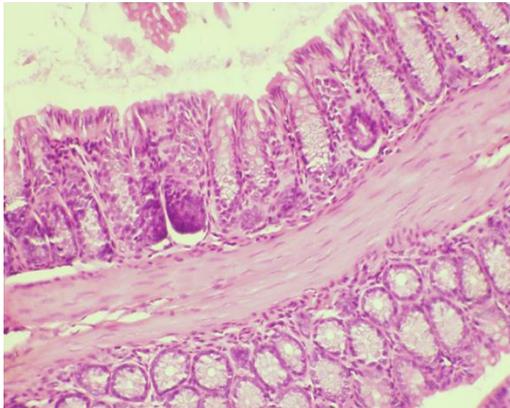


Figure 5.15 Clinical assessment of DSS-induced colitis. Mice were given 5% DSS in drinking water for 5 days, followed by water only. Mice were then sacrificed on day 7. Body weight change was calculated by dividing body weight on the specified day by starting body weight and expressed in percentage (A). Daily disease activity index (DDAI) scores (a combine measure of weight change, stool consistency and fur texture/posture) are reported for each experimental group. A higher score depicts a sicker animal (B). Colons were removed and length (C) and weight (D) measured. Data presented are mean \pm SEM of n=5/early acute; 5/DSS and 5/control group. *P<0.05, by unpaired t-test, compared to control.

Control



DSS

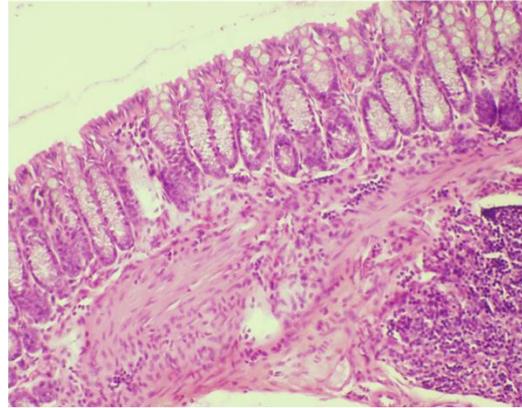


Figure 5.16 Histology of DSS-induced colitis. Sections of a distal colon were removed, paraffin embedded and stained with haematoxylin and eosin. The control is showing a healthy colon, while loss of crypts, the reduction of goblet cells and infiltration of inflammatory cells to the mucosa are visible in the DSS treated colon.

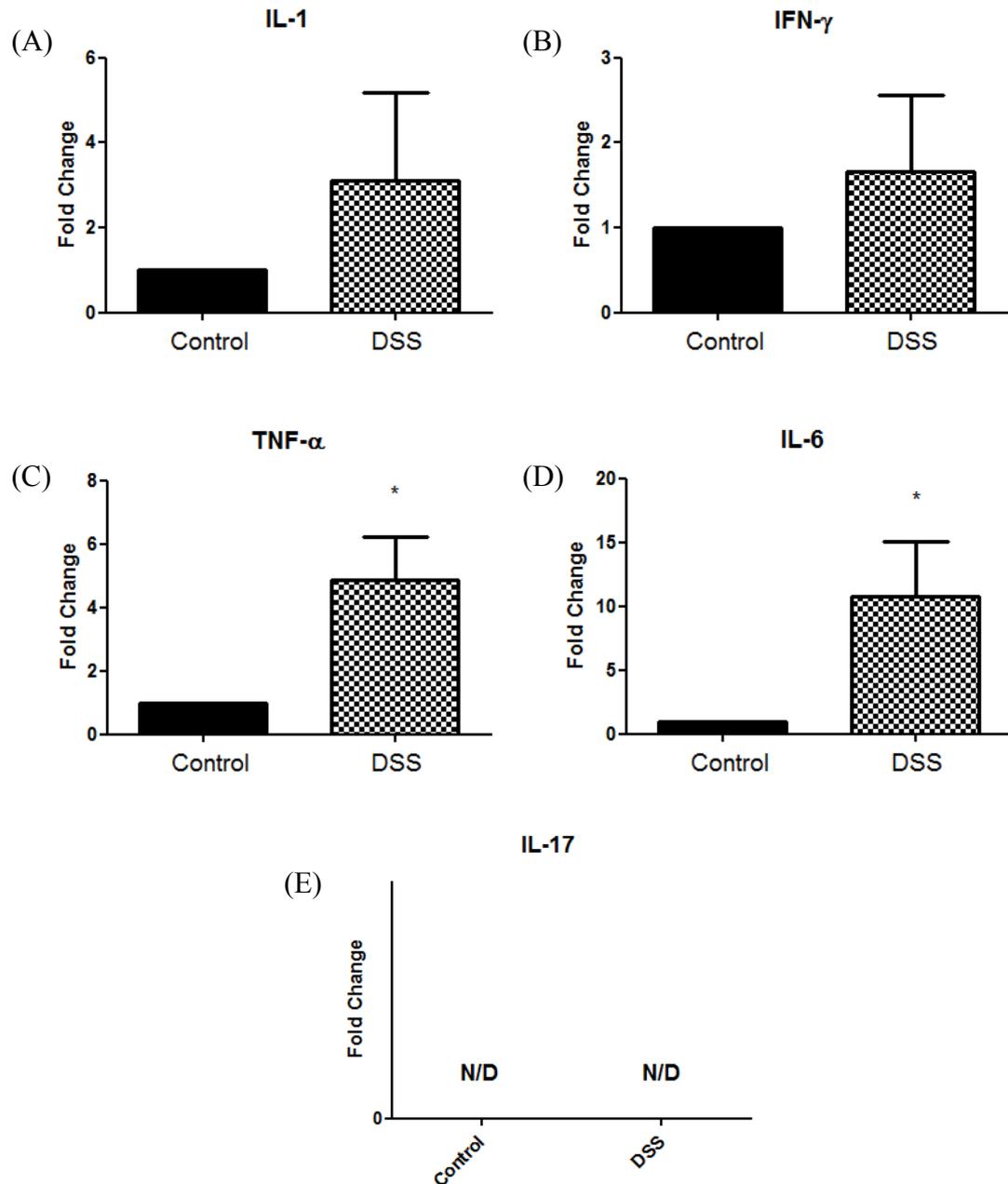


Figure 5.17 Expression of Cytokines in colon in DSS induced colitis. Tissue from each sample was homogenised using the Qiagen TissueLyser LT with stainless steel beads. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for IL-1 β , IFN- λ , TNF- α , IL-17 and IL-6 (all IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, GusB. The mean relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Results are mean \pm SEM of 5 mice/control and 5 mice/DSS * $P < 0.05$, determined by unpaired t-test.

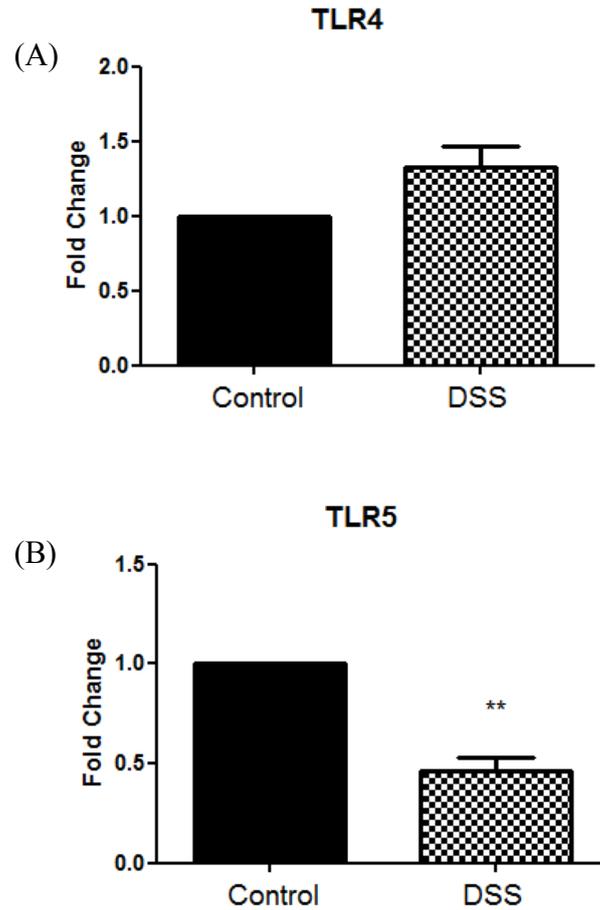


Figure 5.18 Expression of TLR4 and TLR5 in colon in DSS induced colitis. Tissue from each sample was homogenised using the Qiagen TissueLyser LT with stainless steel beads. Following homogenisation, RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel) and quantified on the nanodrop. Equalised amounts of RNA were converted into cDNA using a High Capacity cDNA Mastermix (Roche). The cDNA was mixed with primers for TLR4 and TLR5 (IDT) and analysed on the ABI Prism 7500. cDNA samples were assayed in triplicate and gene expression levels were normalised to endogenous control, GusB. The mean relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Results are mean \pm SEM of 5 mice/control and 5 mice/DSS ** $P < 0.01$, determined by unpaired t-test.

Control

DSS

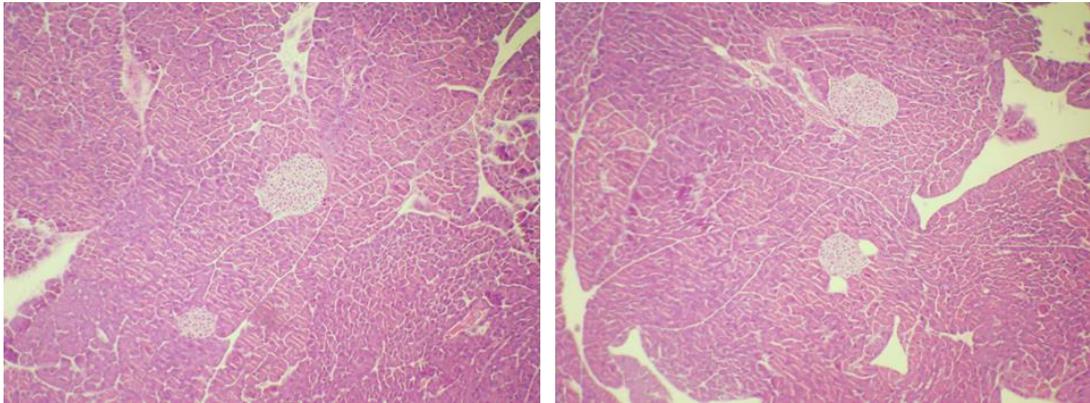
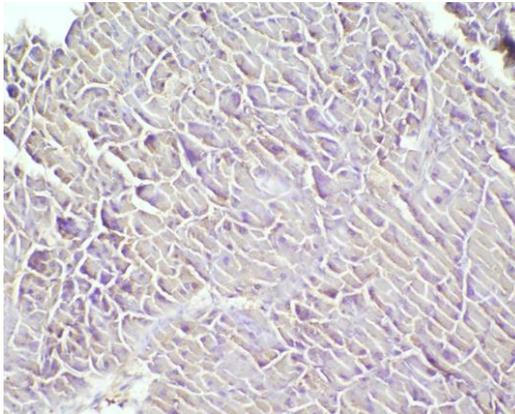


Figure 5.19 Histology of pancreas during DSS-induced colitis. Sections of a proximal pancreas were removed, paraffin embedded and stained with haematoxylin and eosin. Acinar cells appear dark pink and islets are light pink and round in appearance. Images were taken on a light microscope with a camera attachment.

Control



DSS

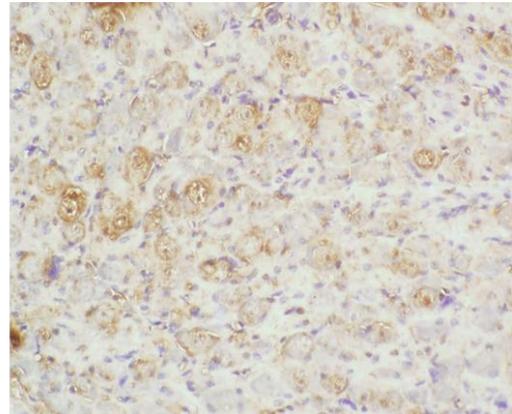


Figure 5.20 F4/80 staining of Pancreas during DSS induced colitis. Sections of proximal pancreas were removed and paraffin embedded. 6um sections were cut and stained for F4/80 using the HRP-DAB SYSTEM (R&D Systems) according to manufacturer's instructions. Slides were then counterstained with Harris Haematoxylin to allow for better contrast. F4/80 positive cells appear brown. Images were taken on a light microscope with a camera attachment.

5.4 Discussion

As we had shown in Chapter 4 that culturing of J774 macrophage with MIN6 conditioned medium exerts an overall “dampening down” of the macrophage immune response we thought it pertinent to investigate what may be causing this effect. We first had to confirm it was indeed the conditioned media that was causing this effect, and this was done by showing that once the J774 macrophage were removed from the MIN6 media they returned to a normal phenotype. When examined in an “*ex-vivo*” situation, MIN6 conditioned medium had a similar effect (decrease in cell-surface marker expression in following LPS stimulation) on bone marrow-derived macrophage. This “hyporesponsive” phenotype we are seeing in the macrophage following conditioning with MIN6 media is akin to the phenotype of certain macrophage observed in the gut (Bain et al. 2013; Bain & Mowat 2011). Mucosal resident macrophage, despite being actively phagocytic fail to produce an immune response to stimuli such as TLRs (Smythies et al. 2005; Smith et al. 2011). Instead they are thought to play a number of crucial homeostatic functions acting as non-inflammatory scavengers of bacteria, as well as assisting maintenance of regulatory T cells and promoting epithelial cell renewal (Hadis et al. 2011; Geissmann et al. 2010). Monocytes, upon entry to the mucosa immediately begin to undergo a process of differentiation into this hyporesponsive mature macrophage phenotype (Bain et al. 2013). This process was thought to be unique to the intestine, however recently a similar developmental process has been described in the skin (Tamoutounour et al. 2013). For this reason we postulate that a similar process may occur to resident macrophage or infiltrating monocytes in the islet, as the bodies only

source of insulin, surely it is a site where constant immune surveillance would be crucial.

Following confirmation, that mediators in the MIN6 media that were causing this “anti-inflammatory” effect we next investigated what they might be. In the intestine it is thought that both IL-10 and TGF- β contribute to the hyporesponsive macrophage phenotype (Bain & Mowat 2014). However a lot remains unclear and it is quite likely that many factors may be needed to explain all aspects of the profile. We found no secretion of IL-10 or TGF- β from MIN6 cells however we have shown that MIN6 cells secrete IL-4. This cytokine is known to be anti-inflammatory to macrophage in some contexts (Takeda et al. 1996; Gordon 2003) we investigated whether this may be causing the observed anti-inflammatory effect of the MIN6 media on the macrophage. However, J774 macrophage conditioned with higher amounts of recombinant IL-4 (2ng/ml) that those observed in MIN6 media (~1.5ng/ml) failed to show the same decrease in cell surface markers following LPS stimulation as those observed in MIN6 conditioned cells. Possibly IL-4 is not involved in this process or may need to work in concert with other mediators to produce this effect.

In addition to the release of soluble mediators, epithelial cells in the gut, which are known to interact with resident macrophage (Peterson & Artis 2014), also release a wide variety of proteins, lipids, mRNAs and microRNAs contained within secreted nanovesicles or exosomes (Mallegol et al. 2007; van Niel et al. 2001). Exosomes, once thought to only be responsible for releasing a cells’ unwanted debris are now under investigation as major players in both local and systemic cell communication

(Harding et al. 2013; Raposo & Stoorvogel 2013) . With this in mind we sought to investigate the effect of MIN6 conditioned medium derived exosomes on macrophage function. As FBS is known to contain a lot of exosomes, to be sure that exosomes isolated were those from MIN6 cells and not FBS, cells were cultured in media supplemented with exosome free FBS. This had no significant effect on cell viability. Subsequent hydrostatic dialysis and centrifugation steps of MIN6 conditioned media resulted in 5 exosome fractions based on their size. As a general rule, when J774 macrophage were conditioned with medium containing resuspended exosome fractions the overall effect seen was a decreased or hyporesponsiveness of the macrophages to LPS stimulation when compared to control cells. This hyporesponsiveness included decreased immune cell surface marker expression and decreased pro-inflammatory cytokine secretion. Similar findings have been reported elsewhere. Oral administration of OVA in mice resulted in the generation of MHC II⁺ vesicles in the serum that could suppress OVA-specific immune responses (Ostman et al. 2005). Other studies have used exosomes from mesenchymal stem cells (MSCs) (van Koppen et al. 2012). MSCs have been shown to confer immunosuppressive effects and have shown promise in clinical trials for Crohn's disease, limb ischaemia and myocardial infarction. However in many of the cases where therapeutic effects have been observed using MSCs the stem cells have not been reported to persist following injection therefore it is probable the main immunosuppressive effect results from paracrine mechanisms mediated by secreted factors, including exosomes (Robbins & Morelli 2014; Caplan & Correa 2011). Indeed in some cases injection of conditioned media from MSC cultures has been confirmed to suppress inflammation as effectively as injection of the stem cells themselves (van Koppen et al. 2012; Caplan & Correa 2011). Other tissues and cells

secrete exosomes bearing immunosuppressive molecules. For instance, placenta-derived vesicles are found in the mother's blood circulation (Taylor et al. 2006) and women delivering at term displayed higher amounts of these vesicles, with higher Fas ligand (FasL)-mediated T-cell inhibiting properties than women delivering preterm. Prostatomes in semen (Tarazona et al. 2011) and placental explants also secrete exosomes that inhibit NK lymphocytes (Hedlund et al. 2009), possibly preventing immune attack of the foetus. Although it has not been reported elsewhere to date, it would make sense for islet cells to secrete mediators that allow it to interact and communicate with macrophage and other immune effectors most importantly during the setting of inflammation as typical pro-inflammatory mediators such as IL-1 β , TNF- α and IFN- λ are known to cause β -cell death (Wachlin et al. 2003).

An interesting observation is that although mostly decreases (or occasionally no change) in secretion of most cytokines is seen following conditioning of macrophage with exosome fractions, all but one fraction causes increased IL-27p28 secretion following stimulation with LPS. As mentioned previously IL-27 is known to have both pro and anti-inflammatory effects depending on the context (Stumhofer & Hunter 2008). In this context, as the increase in IL-27 is concurrent with decreases in other pro-inflammatory cytokines such as IL-6 and IL-12p40, and decreases in cell surface marker expression, we postulate that it may be exerting an anti-inflammatory effect. Fujimoto *et al* have shown that the administration of recombinant IL-27 decreased blood glucose levels and immune cell infiltration into islets in mice treated with STZ (Fujimoto et al. 2011) and we ourselves showed that pre-treatment of MIN6 cells with recombinant IL-27 provided partial protection against STZ induced

cell death. It is therefore most likely beneficial for β -cells to be able to signal to resident or infiltrating macrophage to induce IL-27 secretion. Indeed Kallioliias *et al* have reported that IL-27 has the ability to suppress cultured human macrophages response to the pro-inflammatory cytokines IL-1 β and TNF- α (Kallioliias *et al.* 2010). Although a decrease following LPS stimulation is seen surface marker expression of TLR2, TLR4 and CD80 in most cases following conditioning of macrophage with exosome fractions this is not as robust an effect as we saw when macrophage were conditioned with whole MIN6 conditioned medium. Further to this no change in expression was observed in CD40 and CD86. This suggests that it is likely a number of mediators released in exosomes from MIN6 cells that mediate the effect, as when isolated the impact on macrophage surface marker expression is diminished. Furthermore as this is a technique we are still working on, the efficiency of the purification and quantification procedure remains unknown, and it is likely that ultracentrifugation does not allow for 100% recovery of the vesicles secreted at any given time, and part of the secreted vesicles are inaccessible to purification because they are recaptured by cells rather than released in the culture medium or fluid. Taking into context the complexity of most signalling pathways within the cell, and our results that some fractions had a more profound effect on particular cytokine secretion and surface marker expression than others, it is likely that a combination of a number of mediators secreted by exosomes are responsible for the crosstalk observed between MIN6 cells and J774 macrophage. Further characterisation of the contents of MIN6 exosomes will be carried out in the future.

It is clear from our results so far that MIN6 β -cells have the ability to interact with and affect macrophage phenotype and function. However it is still unclear in what

setting this relationship is pertinent. As mentioned previously immunohistochemical analysis of pancreas sections from patients with T2D, C57BL/6 mice fed a high-fat diet, db/db mice and Goto-Kakizaki (GK) rats all showed elevated numbers of macrophages within islets (Westwell-Roper et al. 2014; Richardson et al. 2009; Eguchi & Manabe 2013; Ehses et al. 2008; Ehses et al. 2007). Of note is the observation that increased numbers of macrophages were detectable very early in high-fat-fed mouse islets, before the onset of diabetes. However the role for islet resident macrophage and what leads to infiltration of additional macrophage still remains unclear. If macrophage infiltrate early on before overt symptoms of diabetes are apparent it is likely that they contribute to the pathogenesis. The pancreas, as part of the gastrointestinal system is likely very influenced by the environment of the gut. This is clear from a number of lines of study including those that demonstrate after bariatric surgery, there is an almost immediate recovery from type 2 diabetes following the anatomical change which impacts on the connection between the gut and the pancreas (Rubino et al. 2006; Buchwald et al. 2009; Schauer et al. 2003; Dixon et al. 2008). Furthermore numerous studies have linked altered gut microbiota and inflammation with T2D and metabolic syndrome (Chassaing & Gewirtz 2014; Carvalho & Saad 2013; Ding et al. 2010). These studies clearly highlight that important signalling systems are in play between the gut and the pancreas.

As we have been investigating the effect of TLRs on MIN6 cells, and the crosstalk between these cells and J774 macrophage we posed the question – would an inflammatory environment in the gut, which is home to the largest compartment of the immune system, have the ability to induce macrophage infiltration into the pancreas? To answer this we used the DSS-induced model of colitis. Dextran sulfate

sodium induced colitis is a well appreciated and widely used model of inflammatory bowel disease. Colitis is induced by addition of DSS to drinking water. DSS is toxic to colonic epithelial cells and induces the break-down of the epithelial barrier function (Poritz et al. 2007) . This allows the entry of microorganisms and antigens from the lumen which then results in activation of immune cells and inflammation. It is characterised by weight loss, blood in the stool, diarrhoea and infiltration of immune cells into the lamina propria and submucosa (Kullmann et al. 2001). In our study, mice were administered DSS for 5 days followed by 2 days on water alone and were sacrificed for sample and tissue processing on day 7. It is clear from our results that a mild form of inflammation was induced in the mice fed DSS. Although there was no significant weight loss observed, there was a significant reduction in colon length, an increase (although not significant) in colon weight and an increased DDAI score that peaked on day 6. This was accompanied by an increased infiltration of inflammatory cells to the mucosa and an increase in mRNA expression of pro-inflammatory cytokines TNF- α and IL-6. What is interesting to note is mRNA expression levels of TLR4 appear to be increased whereas TLR5 expression is significantly decreased. This once again points to TLR5 as playing a protective role not just in the β -cell as previously discussed but in the gut also. Indeed deletion of TLR5 results in the development of spontaneous colitis in mice (Vijay-Kumar et al. 2007) and activation of TLR5 has the ability to protect against *clostridium difficile* induced colitis (Jarchum et al. 2011) . Furthermore as TLR5 deficient mice develop hallmark features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity (Vijay-kumar et al. 2010) this highlights a possible role for TLR5 in the gut- pancreas axis in the context of inflammation and insulin signalling. It remains unclear as to how exactly activation of TLR5 may be

protective, however it has been shown to induce secretory interleukin-1 receptor antagonist (sIL-1Ra) (Carvalho et al. 2011) in both epithelial cells and macrophage.

Although no obvious morphological differences were observed in pancreas sections stained with H&E were observed in DSS treated vs control mice, when stained with an antibody to the macrophage marker F4/80, DSS treated pancreas show increased presence of macrophage. We cannot say definitively the increased number of macrophage seen are a result of infiltration from the intestine to the pancreas however a number of ideas give weight to this as a possibility. Sections were taken from the head of the pancreas, which is the segment of the pancreas that is directly connected to both the duodenum and proximal jejunum. Both the major and minor papilla, which are openings that exist between the pancreas and the duodenum allowing for secretion of bile and other enzymes involved in digestion are located in this area. An increased number of macrophage and inflammatory cells are present during colitis (Kullmann et al. 2001) and colitis is known to result in a breakdown of gut barrier function and integrity (Poritz et al. 2007). Therefore considering increased numbers of macrophage in the gut, breakdown of gut barrier integrity and the close proximity of the pancreas to the duodenum it is probable that the macrophage seen in the pancreas originated in the gut. Although we ourselves used DSS to chemically induce inflammation, a number of factors have the ability to contribute to gut inflammation. The foremost of these is a high-fat diet and obesity which is extensively linked with chronic low-grade inflammation, and T2D (Ding et al. 2010; Ahren & Sorhede Winzell 2004; Carvalho & Saad 2013; Cabré & Domènech 2012; Boni-Schnetzler et al. 2009; Weisberg et al. 2003; Donath et al. 2013; Brestoff & Artis 2013). If during this chronic low-grade inflammation that is

associated with obesity, macrophage had the ability to infiltrate to the pancreas and perhaps the islet, this may provide an explanation for the increased numbers of macrophage seen in mouse models prior to the onset of overt diabetes (Ehse et al. 2007).

To summarise we have shown that MIN6 β -cells release mediators in exosomes that have the capability of affecting macrophage cell surface markers and cytokine secretion. In many cases these exosome fractions induce an almost “hyporesponsive” phenotype in the macrophage with reduced cell surface marker expression and reduced pro-inflammatory cytokine secretion. This indeed provides evidence that important signalling mechanisms exist between the β -cell and macrophage, however this likely involves a number of mediators and unfortunately was not in the scope of this project to further characterise what they were. Secondly we have highlighted a possible means by which macrophage may infiltrate the pancreas during gut inflammation. This is very important due to the fact that both obesity and T2D are intrinsically linked with low-grade inflammation. Perhaps under normal circumstances, any macrophage that do enter are signalled to by the β -cell to enter a “hyporesponsive” state to limit any potential inflammatory damage. However throughout the progression of T2D, the β -cell itself is overworked, trying to compensate for reduced insulin sensitivity by increasing its insulin output, this leads to β -cell exhaustion (Muio & Newgard 2008; Ohsugi et al. 2005) and perhaps an inability of the β -cell to control macrophage function. Further understanding of the complex crosstalk that exists between β -cells and macrophage is therefore crucial, and may provide novel mechanisms for β -cell protection during obesity and diabetes associated inflammation.

Chapter 6

General Discussion

6.1 General Discussion

It is now clear from numerous lines of investigation that Type 2 Diabetes is inextricably linked with chronic low grade inflammation (Donath 2013; Olefsky & Glass 2010; Akash et al. 2013; Fresno et al. 2011; Donath & Shoelson 2011; Cruz et al. 2013). Toll-like receptors (TLRs) are pattern-recognition receptors that play a crucial role in the innate immune system, which detects the presence and the nature of pathogenic microbial infection, and thus provides the first line of host defence. Their activation triggers a signalling cascade and ultimately the activation of transcription factors such as NF κ B and AP-1 (Takeda et al. 2003). These transcription factors induce transcription of inflammatory cytokines and type I interferons (Kumar et al. 2009). This proinflammatory reaction is fundamental for the initiation of an innate immune response and subsequent adaptive responses but can also damage tissues. This is of particular importance when considered in the context of chronic inflammation whereby constitutive activation of these receptors may result in an overt inflammatory reaction. TLRs can also interact with ligands generated sites at of injury (Wagner 2006) and endogenous ligands such as oxLDL (oxidized LDL), HSPs (heat shock proteins) 60 and 70, fibrinogen, fibronectin and free-fatty acids, which are also elevated in diabetes (Wagner 2006; Osterloh & Breloer 2008; Chiu et al. 2009; Tsan & Gao 2004).

To date the majority of research has focused on TLR4 and to a lesser extent TLR2 and their role in the β -cell and T2D (Vives-Pi et al. 2003; Himes & Smith 2010; Yin et al. 2014; Ehses et al. 2010; Kiely et al. 2009; Lee 2011; Garay-malpartida et al.

2011; Liu et al. 2011; Li et al. 2013; Boni-Schnetzler et al. 2009; Amyot et al. 2012). In both cases the TLRs respond to endogenous products from cell death or increased levels of circulating fatty acids and accelerate the progression of the disease through β -cell apoptosis and inhibit islet insulin secretion (H. S. Kim et al. 2007; Schulthess et al. 2009). TLR activation of cells typically results in the initiation of a pro-inflammatory reaction however in this study we have identified a novel role for TLR5, the receptor for bacterial flagellin, in MIN6 β -cell protection. We were surprised when our own investigations showed a much more profound effect on MIN6 β -cell function in general following TLR5 activation, than that of TLR4 or TLR2. Activation of all three receptors had a similar affect in that they increased basal insulin secretion levels but reduced glucose stimulated insulin secretion. However TLR2 and TLR4 activation resulted in a marked decrease in intracellular insulin stores. This suggests that TLR2 and TLR4 have a negative impact on insulin translation and synthesis but TLR5 activation may suppress insulin secretion from the cell. TLR5 activation also resulted in a decrease in IRS1 expression levels. IRS1^{-/-} β -cells display a reduced response to both glucose and arginine (Kulkarni et al. 1999). In the setting of activation of innate immunity and inflammation it may be beneficial for the cell to repress insulin secretion in order to ensure a sufficient supply of glucose to phagocytic and reparative cells. Concurrent with the decrease in IRS1 there is an increase in IRS2 expression following TLR5 activation. Increased IRS2 expression in β -cells may also prove protective in the setting of activation of innate immunity as increased numbers of apoptotic cells were found to be present within the β -cells of IRS2^{-/-} mice compared with wild-type animals (Withers et al. 1998). Aligned with the concept of TLR5 playing a protective role in the β -cell, its activation resulted in decreased mRNA expression of both TLR4 and TLR5 in MIN6

cells, which may help to depress or limit propagation of inflammation. TLR5 activation also decreased expression levels of the PANDER, a cytokine like protein produced in islets that has been shown to induce apoptosis in β -cells from mouse, rat, and human (Cao et al. 2003; Cao et al. 2005). Finally TLR5 activation also proved to be the strongest inducer of IL-27p28 secretion from MIN6 cells, a cytokine with both pro and anti-inflammatory effects (Hunter & Kastelein 2012). We have shown that both flagellin and recombinant IL-27p28 possess the ability to protect against STZ induced MIN6 β -cell death. This is further supported by findings that the administration of recombinant IL-27p28 decreased blood glucose levels, immune cell infiltration into islets, and IL-1 β mRNA expression in the pancreas of mice treated with STZ (Fujimoto et al. 2011). Taken together these findings clearly point to a role for TLR5 induced in β -cell physiology, with an emphasis on a protective role.

The idea of TLR5 playing a role in the β -cell and metabolism in general, although it is novel does not go unfounded. Mice lacking TLR5 display hallmark features of metabolic syndrome including hyperlipidemia, hypertension, insulin resistance, and increased adiposity (Vijay-kumar et al. 2010) and it has been shown that a nonsense polymorphism in TLR5 in humans protects from obesity but pre-disposes to T2D (Al-Daghri et al. 2013). Taken together with the fact that glucose itself has been shown to induce an up-regulation of both TLR5 mRNA and protein levels in isolated mouse islets (Weile et al. 2011b) this suggests TLR5 may be a key player in this metabolic pathway. Although there is no known endogenous ligand to TLR5 the fact that we have shown that activation of this receptor has robust metabolic and inflammatory effect on β -cells, we believe that similar to the way TLR2 and TLR4

respond to activation by fatty acids (Yin et al. 2014) and other endogenous molecules (Yu et al. 2010), there may be an endogenous mediator within this pathway capable of activating TLR5.

Further evidence for a protective and anti-inflammatory role for TLR5 in disease states come from a number of studies. Deletion of TLR5 has been shown to result in spontaneous colitis (a form of inflammatory bowel disease) in mice (Vijay-Kumar et al. 2007) and administration of flagellin prior to dextran sulfate sodium in the DSS induced mouse model of colitis greatly abrogated disease severity with reduction of inflammatory markers and immune cell infiltration in the colon (Vijay-Kumar et al. 2008). These authors also showed that pre-treatment of mice with flagellin provided a measure of protection against lethal doses of *S. typhirium* and γ -irradiation (Vijay-Kumar et al. 2008). Although it is unclear by what mechanism TLR5 activation plays a protective and anti-inflammatory role in these settings it has been shown to induce secretory interleukin-1 receptor antagonist (sIL-1Ra) expression and secretion in intestinal epithelial cells and macrophages (Carvalho et al. 2011). This may provide a mechanistic answer to some of the anti-inflammatory properties observed following TLR5 activation in various models. Taken together these data certainly indicate a role for TLR5 activation in protective and anti-inflammatory functions in a wide variety of disease states. Further work must be carried out to elucidate the role of TLR5 signalling in β -cell physiology and metabolism as understanding of the signalling pathways and mediators involved in this activation may provide insight into novel β -cell therapeutics.

Another aspect of this study was to examine the crosstalk that existed between MIN6 β -cells and J774A.1 macrophage. Macrophages, key innate immune effector cells best known for their role as professional phagocytes play a crucial role in innate and adaptive immunity and are major mediators of the inflammatory response. During infection, macrophage effector functions are critical for the elimination of pathogens; however uncontrolled inflammatory responses can induce injury of the tissue environment and must be repressed to allow the process of repair. Along with their role in the immune system macrophage also have diverse roles in tissue homeostasis, and glucose and lipid metabolism (Murray & Wynn 2011). To date the majority of research has focused on the role of the adipose tissue macrophages in the pathogenesis of obesity, insulin resistance and T2D. The discovery that adipose tissue from obese mice and humans is infiltrated with increased numbers of macrophages provided a major mechanistic advancement into understanding how obesity propagates inflammation (Weisberg et al. 2003). It is now well documented that there is also increased islet macrophage infiltration in patients with T2D (Ehnes et al. 2007). Increased islet-associated immune cells were also observed in a variety of animal models of this disease including the GK (Goto-Kakizaki) rat (Homo-Delarche *et al* 2006), the high-fat-diet fed and *db/db* mouse (Ehnes et al. 2007) and the Cohen diabetic rat (Weksler-Zangen et al. 2008). As we found that MIN6 β -cell secretions had the capability to induce macrophage chemotaxis, we postulated that there must be some form of crosstalk between these two cell types. Indeed we have shown that when J774 macrophages are cultured in the presence of MIN6 conditioned medium, the overall effect is to induce an anti-inflammatory phenotype in the macrophage. Their pro-inflammatory cytokine and chemokine secretion, cell surface marker expression and reactive oxygen species production is decreased

following TLR activation. In other words they lose their ability to respond, or become “inert” to inflammatory stimulus. Decreased cytokine secretion would result in decreased activation of inflammatory signalling cascades that activate master regulators such as NF κ B and AP-1 (Karin & Delhase 2000). These transcription factors induce inflammatory cytokine and type I interferon production (Karin & Delhase 2000). This decrease in cytokine secretion would therefore limit propagation of inflammation. Aside from this it has been shown that pro-inflammatory cytokines can interfere with insulin signalling by phosphorylating IRS proteins downstream of the insulin receptor and thus are linked with the development of insulin resistance (Hotamisligil et al. 1994; Ueki et al. 2004). For these reasons limitation of cytokine secretion in the islet may prove beneficial. An interesting observation was that although not significant, MIN6 conditioned macrophage, stimulated with flagellin appear to have increased IL-27p28 secretion, which we have already shown is protective for β -cells. This once again highlights a potential role for TLR5 and IL-27p28 in β -cell physiology and protection. It appears that in the presence of β -cell mediators, flagellin activated macrophage are driven to produce more IL-27p28 than control activated macrophages. As well as decreased cytokine production there is decreased chemokine production from conditioned macrophage in most cases. This would further limit propagation of inflammation by reducing the signals for macrophage infiltration.

Concurrent with this hyporesponsive phenotype, the pro-inflammatory genes associated with classically activated M1 type macrophage are decreased and the genes associated with alternatively activated M2 type macrophage are increased. While these conditioned macrophage do not represent the typical M2 phenotype (Gordon 2003) as we saw no increase in IL-10 or IL-4 production, the fact that the

environment of the MIN6 β -cells drives the macrophage genetically closer to that of an M2 macrophage is of note and may be beneficial in the context of the islet. Although little work has been done on the activation status of macrophage within islets, in adipose tissue, which is a major contributor to the metabolic setting in T2D, M1 macrophage have been shown to propagate inflammation and drive insulin resistance (Olefsky & Glass 2010). For these reasons we propose that any reduction in M1 type genes in macrophage that are in contact with β -cells may help protect and preserve insulin sensitivity. Aside from this M2 macrophages have been implicated in wound healing, tissue remodelling, and apoptotic cell disposal through phagocytosis (Gordon 2003). Interestingly, although MIN6 conditioned macrophage lose their responsiveness to inflammatory stimuli, they retain their phagocytic ability.

MIN6 β -cell Conditioned macrophages display phenotypic similarities with populations of macrophage found in the gut. The intestine is home to the largest population of macrophage in the body (Hume 2006). This is no surprise as the intestine encounters more antigen than any other part of the body. However, when inflammatory monocytes arrive at the gut, they are shaped by mediators present in the intestine, to undergo a phenotypic transformation that renders them inert to pathogenic stimuli (Bain & Mowat 2011). These macrophage retain their phagocytic capability however ingestion of microbes is not accompanied by the release of pro-inflammatory mediators as is usually the case with other tissue macrophages (Smythies et al. 2005). This is certainly aimed at maintaining gut homeostasis as due to their location the macrophage are likely to come into contact with a host of antigenic material due to resident gut microbiota (Schenk & Mueller 2007). It is

thought that both IL-10 and TGF- β secretion by epithelial cells in the gut contribute to this hyporesponsive macrophage phenotype in the gut (Bain & Mowat 2014) however a lot remains unknown. Although we do not think it likely that macrophage in the pancreas or islets would come into contact with such amounts of antigenic material as those in the gut, we postulate that similar mechanisms in the islet may be in place to drive the phenotype of macrophage to that of a hyporesponsiveness to limit possible inflammation induced damage. As there was no secretion of IL-10 or TGF- β from MIN6 cells, we asked ourselves what could be driving this phenotype. Our first thought was that IL-4, which MIN6 cells secrete, and a known inducer of alternative macrophage activation may be playing a role. However when J774 macrophage were conditioned with higher amounts of IL-4 than those secreted by MIN6 β -cells prior to activation with LPS, we did not see induction of the same phenotype as that observed with conditioned media. Likely due to the fact that there is an increase in M2 genes in the macrophage IL-4 is playing a role, but in together in concert with other mediators. In addition to the release of soluble mediators, epithelial cells in the gut, which are known to interact with resident macrophage and infiltrating monocytes to drive a hyporesponsive phenotype (Peterson & Artis 2014), also release a wide variety of proteins, lipids, mRNAs and microRNAs contained within secreted nanovesicles or exosomes (Mallegol et al. 2007; van Niel et al. 2001). Although first discovered nearly 30 years ago (Johnstone et al. 1987) in recent years there has been a resurgence of interest in these particles. Mainly owing to their ability to profoundly contribute to both local and systemic cell communication (Harding et al. 2013; Raposo & Stoorvogel 2013) as they have the ability to transfer their contents, including proteins, lipids and RNAs, between cells (Ratajczak et al. 2006; Valadi et al. 2007). Given the observation that cell derived

exosomes have been shown to have immunosuppressive effects in a number of different models (Wieckowski et al. 2009; S. H. Kim et al. 2007; van Koppen et al. 2012) we investigated whether or not the observed effect on macrophage phenotype was being driven by mediators carried in exosomes. Exosomes were fractioned according to their size with the intention of identifying a particular fraction with immunosuppressive capability. Interestingly though, all 5 of our exosome fractions exerted anti-inflammatory effects on macrophage function in that they reduced proinflammatory cytokine secretion, and cell surface marker expression. They did not however, do this uniformly for example with FR1 all other cytokines tested for were decreased however IL-23 levels remained unchanged. FR2, 3 and 4 decreased IL-23 levels and with FR5 again IL-23 levels remain unchanged. The same can be said of surface markers with all fractions bar FR2 causing a decrease in TLR2 expression. As we found it difficult to single out one fraction with the most profound immunosuppressive capability we postulate that it is likely a number of mediators secreted in exosomes that contribute to the overall anti-inflammatory effects of MIN6 β -cells. Unfortunately given that we were working with so many fractions, characterisation of their contents was just not in the time frame or scope of this project but it certainly warrants further investigation. What we do understand from our results however is that mediators released in exosomes from MIN6 β -cells are capable of exerting an anti-inflammatory action on macrophage. This is similar to the crosstalk that is seen between epithelial cells and macrophage in the gut. Future work will include characterisation of the contents of these exosomes and may lead to identification of novel immune modulating mechanisms for macrophage and indeed for limiting islet inflammation that is known to drive β -cell dysfunction.

To finalise our study we asked ourselves in what context would this crosstalk that we have described between MIN6 β -cells and macrophage be pertinent. In what setting do β -cells interact with macrophage? As mentioned previously there have been numerous reports linking T2D with macrophage infiltration of islets (Ehse et al. 2007; Richardson et al. 2009) although it is still unknown whether or not macrophage infiltration is causative or a consequence of islet β -cell pathology. It is still unclear also as to the signals that drive macrophage infiltration of islets in the context of T2D. There is also a wealth of evidence linking low grade chronic inflammation with T2D (Donath 2013; Lee et al. 2012; Fresno et al. 2011; Cruz et al. 2013). Inflammation that results from diet induced obesity (Ding et al. 2010), which has the ability to alter the microbial composition of the gut (Everard & Cani 2013; Shen et al. 2013; Carvalho & Saad 2013) which in turn affects the gut's inflammatory status. As T2D, diet induced obesity and inflammation are inextricably linked we asked the question whether or not inflammation in the gut may have the ability to affect macrophage infiltration into the pancreas, thus providing a setting where this cross-talk would be necessary. To address this question we used the well-studied model of dextran sulfate sodium (DSS) induced colitis in mice (Kullmann et al. 2001; Poritz et al. 2007), to assess the effect of gut inflammation on the pancreas. Quality pancreatic RNA is notoriously difficult to isolate due to the presence of large quantities of RNases, DNases and proteases that initiate an autolytic process almost immediately upon harvest. Despite several approaches, including rapid removal of pancreas from the abdominal cavity, homogenizing at cold temperatures and inhibition of contaminating RNases through perfusion of the pancreas we were unable to isolate high-quality RNA. We were therefore unable to look at expression of inflammatory genes in the pancreas during intestinal inflammation. However we

were able to show increased macrophage presence in the pancreas from DSS treated animals compare to control animals. As discussed previously we cannot be certain that these macrophages originated in the intestine but there is compelling evidence in the literature to support this theory. Considering the increased numbers of macrophage in the gut (Kullmann et al. 2001), the breakdown of gut barrier integrity (Poritz et al. 2007) during colitis, and the close proximity of the pancreas to the duodenum it is probable that the macrophage seen in the pancreas originated in the gut. This provides a mechanism by which inflammation in the gut has the ability to affect macrophage infiltration of the pancreas and also addresses the question as to why β -cells possess the ability to alter and control macrophage phenotype.

Our work has highlighted that there may be a novel endogenous molecule within the metabolic signalling pathway that drives TLR5 activation, that may prove protective in the setting of islet dysfunction. This may be novel therapeutic target for islet protection. We have also shown that the islet is an environment similar in ways to the gut that has the ability to influence macrophage phenotype. This is most likely an effect that helps to maintain pancreatic homeostasis and dysregulation of this could be a driving cause in β -cell dysfunction in T2D. Finally we have shown that inflammation in the gut can affect infiltration of macrophage into the pancreas. Taking into consideration the inextricable links between low grade inflammation and T2D, this may be a driving force in β -cell dysfunction that leads to the onset of T2D.

Chapter 7

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Appendices

Appendix A

Buffers

FACS buffer

FCS	2%
NaN ₃	0.05%

Dissolve in PBS

Phosphate buffered saline (PBS)

Na ₂ HPO ₄ x 2H ₂ O	8.0 mM
KH ₂ PO ₄	1.5 mM
NaCl	137 mM
KCl	2.7 mM

Dissolve in dH₂O and pH to 7.4

TAE buffer

Tris	40 mM
Acetic acid	20 mM
EDTA	1 mM

Dissolve in dH₂O

Tris buffered saline (TBS)

NaCl	1.5 M
Trizma Base	0.2 M

Dissolve dH₂O pH to 7.6

TBS-Tween

Add 0.05% Tween-20 to 1x TBS

Krebs Ringer Bicarbonate (KRB)

NaCl 125 mM

KCl 3 mM

MgSO₄ 1.2 mM

CaCl₂ 1.2 mM

NaHCO₃ 22 mM

Na H₂PO₄ 1 mM

Dissolve in dH₂O and pH to 7.4

Appendix B

Primer Sequences

IL-1b

Primer 1 5'-GAC CTG TTC TTT GAA GTT GAC G-3'

Primer 2 5'-CTC TTG TTG ATG TGC TGC TG-3'

IL-6

Primer 1 5'-AGC CAG AGT CCT TCA GAG A-3'

Primer 2 5'-TCC TTA GCC ACT CCT TCT GT-3'

TNF- α

Primer 1 5'-TCT TTG AGA TCC ATG CCG TTG- 3'

Primer 2 5'-AGA CCC TCA CAC TCA GAT CA- 3'

IFN γ

Primer 1 5'-ATG AAC GCT ACA CAC TGC ATC-3'

Primer 2 5'-CCA TCC TTT TGC CAG TTC CTC -3'

IL-17a

Primer 1 5'- AGA CTA CCT CAA CCG TTC CA-3'

Primer 2 5'- GAG CTT CCC AGA TCA CAG AG-3'

TLR4

Primer 1 5'-GAA GCT TGA ATC CCT GCATAG-3'

Primer 2 5'-AGCTCA GAT CTATGT TCTTGGTTG-3'

TLR5

Primer 1 5'-GGA ACATAT GCC AGA CAC ATC T-3'

Primer 2 5'-TGA AGATCA CAC CTATGA GCA AG-3'

INS1

Primer 1 5'-GCC AAA CAG CAA AGT CCA G-3'

Primer 2 5'-GCC ATG TTG AAA CAATGA CCT-3'

INS2

Primer 1 5'-TTT GTC AAG CAG CAC CTT TG-3'

Primer 2 5'-TCC ACC CAG CTC CAGTT-3'

IRS1

Primer 1 5'-CCA GCATCA GCT TCC AGA A-3'

Primer 2 5'-TGT GAATTG TGA AAT AGT TCG AGT C-3'

IRS2

Primer 1 5'-CCT ACA CGC CTATCG CTA GA-3'

Primer 2 5'-GTA CCT GCCTCA CCA AAGTC-3'

IAPP

Primer 1 5'-GCC TCG GAC CACTGA AAG-3'

Primer 2 5'-CACTTC CGT TTG TCC ATC TGA-3'

FAM3B

Primer 1 5'-ACA AGT ATG CCA AGATCT GCT-3'

Primer 2 5'-TCG CTATCA CTT TTC CTG TCT C-3'

ARG1

Primer 1 5'-GAATGG AAG AGT CAGTGT GGT-3'

Primer 2 5'-AGT GTT GAT GTC AGT GTG AGC-3'

NOS2

Primer 1 5'-GACTGA GCT GTT AGA GAC ACT T-3'

Primer 2 5'-CACTTC TGC TCC AAATCC AAC-3'

MRC1

Primer 1 5'-TAT CTC TGT CAT CCCTGT CTC T-3'

Primer 2 5'-CAA GTT GCC GTC TGA ACT GA-3'

ACTB

Primer 1 5'-CGT TGA CAT CCG TAA AGA CCT-3'

Primer 2 5'-CTT GAT CTT CAT GGT GCT AGG AG-3'

Gusb

Primer 1 5'-CCA ATG AGC CTT CCT CTG C-3'

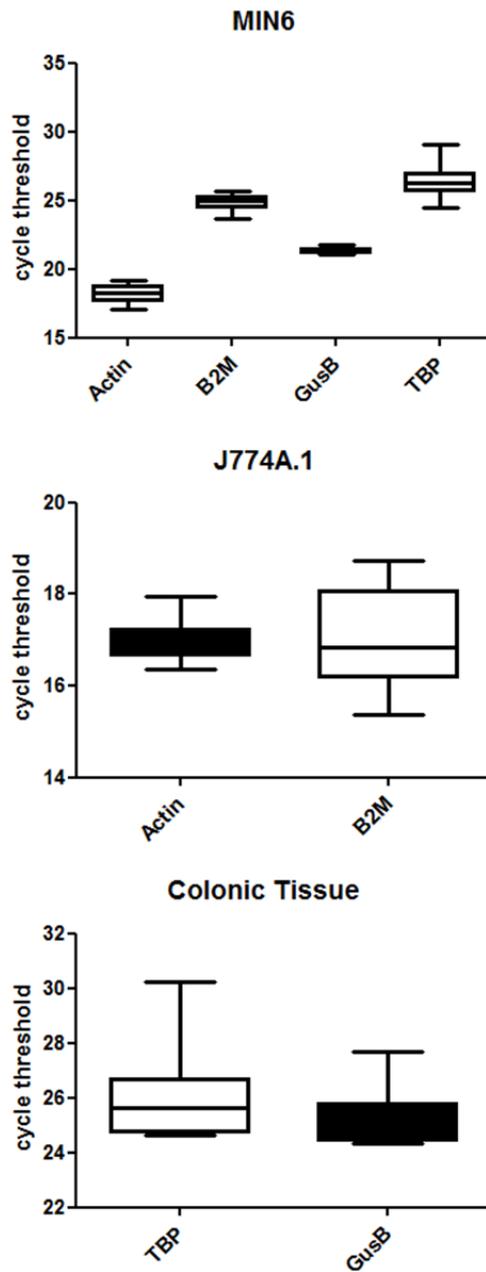
Primer 2 5'-ATC ATA TTT GGC GTT GCT CAC-3'

B2M

Primer 1 5'-GGT CTT TCT GGT GCT TGT CT-3'

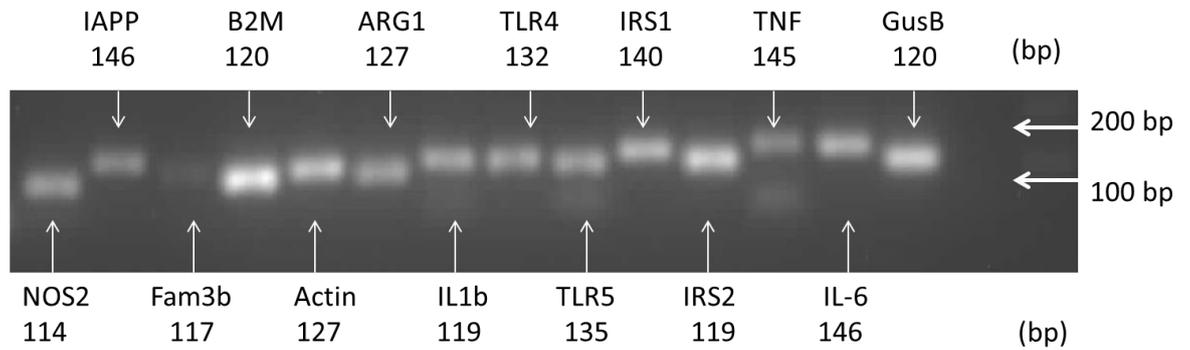
Primer 2 5'-ACG TAG CAG TTC AGT ATG TTC G-3'

Endogenous controls



Endogenous controls testes on MIN6 and J774A.1 cell lines and colonic tissue.

PCR Gel



DNA product analysis by gel electrophoresis DNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene).