# Expression of TNF-α at mRNA level in Thp-1 cells exposed to LPS and HSP60: Possible impact on diabetic vascular inflammation

A thesis

submitted partial fulfilment

of the requirements for the degree

of

**Master of Science (Research) in Biological Sciences**

at

**The University of Waikato**

by

**Afreen Azfar**


THE UNIVERSITY OF
WAIKATO
*Te Whare Wānanga o Waikato*

**2018**

# Abstract

Diabetes mellitus is a chronic disease characterized by hyperglycaemia. It occurs when the β cells of the pancreas produce either low levels of insulin or no insulin at all. The chronic hyperglycaemic condition of diabetes is associated with the dysfunction, long-term damage and failure of organs such as the heart and blood vessels. Diabetes is closely linked to the prevalence of cardiovascular diseases, which is one of the highest causes of death worldwide. In particular, the hyperglycaemic and chronic inflammatory state of diabetes has been linked with atherogenesis, which is when fatty deposits build up in the arteries and they become inflamed. It still remains unknown how diabetes contributes to atherosclerosis.

Heat Shock Protein 60 (HSP60) is an important endogenous inflammatory mediator elevated in diabetic patients. HSP60 can be released from various cell types, including monocyte cells, which are the most common cell type found in atherosclerotic plaques. The mechanism behind how HSP60 triggers endothelial cell inflammation remains unknown, but has been hypothesised to be similar to that of a Toll like receptor 4 ligand, lipopolysaccharide (LPS). Understanding the molecular mechanism of HSP60 could give insights into the pathological, cellular and molecular basis of vascular inflammation in diabetes as well as other vascular diseases such as cardiovascular disease.

It is hypothesized that monocyte-derived HSP60 has a role in causing inflammation in the diabetic vasculature through the expression of pro-inflammatory cytokines such as TNF-α, which could contribute to atherosclerosis. In this study a human monocyte cell line (Thp-1) was used to mimic monocyte behaviour. First a growth curve was established, which indicated when the cells were in their exponential phase. This enabled RNA to be extracted during the period when cells were growing the fastest.

Thp-1 cells were then exposed to LPS for 6, 12, and 24 hours to determine the expression of TNF-α. After conducting RT-PCR, a 12 hour time point

was selected. Since inconsistent results were obtained using this semi-quantitative analysis, qPCR was carried out for the rest of the project.

Thp-1 cells were then exposed to 50ng/mL and 100ng/mL of LPS for 12 hours. After conducting and analysing qPCR using the delta-delta Ct method and the Pfaffl method, a ~2 fold increase was seen in the expression of a pro-inflammatory cytokine, TNF-α. These results however were not significant (determined by a Student's T test).

In order to obtain a higher level of TNF-α expression, this experiment was repeated with 10ng/mL and 50ng/mL of LPS for 4 hours, based on previous published research. These results were analysed using the delta-delta Ct method and the Pfaffl method. The results were not what was expected; only an insignificant <1 fold increase was seen in the samples.

Thp-1 cells were also exposed to 500ng/mL and 1500ng/mL of HSP60 for 4 hours in order to see a TNF-α induction. HSP60 has been hypothesised to work through the same mechanism as LPS to trigger TNF-α , leading to an inflammatory response. However again, no significant increase in expression was seen (as determined by a Student's T test).

The inability to detect a significant up-regulation of the expression of TNF-α at mRNA level with LPS (positive control) and HSP60 in Thp-1 cells was surprising as this is well documented in the literature. This could possibly have been due to the high passage number of the Thp-1 cell cultures used in this study; mycoplasma contamination or stability of the LPS used. The study therefore needs to be repeated with cells of a lower passage number and cells checked for mycoplasma contamination to establish appropriate time frames of exposure and concentrations of LPS and HSP60 required to see a TNF-α induction. This could then be followed by experiments using receptor antagonists, such as TAK-242, (a TLR4 antagonist) to determine if TNF-α expression levels would give an indication of whether or not HSP60 was using the same pathway as LPS, and if it indeed played a part in causing diabetic vascular inflammation.

# Acknowledgements

I would like to thank my supervisor Dr Ryan Martinus for his support during this project, and for reading all of my thesis drafts no matter how late I sent them.

I would also like to express my gratitude to Dr Steve Bird and Dr Gregory Jacobson, without whom I would not have completed this thesis. Their support to me was invaluable and got me through the days I spent confused wondering whether or not I was capable of completing this thesis. I would also like to thank Dr Sari Karppinen and Olivia Patty, the two most wonderful technicians who helped me get through the lab work and didn't make me feel bad for all my silly questions.

I also want to thank Usha Chandra, who made life in the lab a whole lot nicer, and with whom I was able to complain and share all my thesis worries with. I also want to thank Emily Grout and Andrew Howard, who made sure I was staying on top of my thesis work and always provided me with help and support and lots of stress-free adventures.

I want to thank one of the most talented people I know, Sarah Ather. Although I have said this far too many times already, she helped me believe in myself and did not give up on me even when I did.

Lastly, the biggest thank you and love goes to my parents, without whom I would not be where I am today. I can't put into words how much their support has carried me through, and I will be forever grateful for all the effort they have put in not only to help me complete my thesis, but to help and support to me to become this capable. I really would not have been able to complete my thesis if I did not have such a wonderful support system.

# Table of Contents

# List of Figures

# List of Tables

# 1 Literature Review

## 1.1 Statistics

An estimated 451 million people worldwide have Diabetes Mellitus as of 2017, with type 2 diabetes being the most common metabolic disease in the world (Cho et al., 2018, Lowell and Shulman, 2005, Marett, 2014). This increasingly common disease is one of the leading causes of death worldwide (World Health Organisation, 2016), and the number of patients continues to increase every day. According to the International Diabetes Federation, by 2035 there will be an estimated 500 million diabetic patients worldwide. In 2012 1.5 million deaths were caused by diabetes and an additional 2.2 million caused by an increased blood glucose level, which lead to an increase in the risk of cardiovascular diseases (World Health Organisation, 2016). Type 2 diabetic patients have a 2-3 times higher risk of cardiovascular disease, which accounts for 50-80% of deaths in diabetic patients (World Health Organisation, 2011). People with diabetes are at risk of developing various life-threatening health problems, which results in higher medical care costs, reduced quality of life, and an increased mortality rate.

Diabetes is increasing rapidly in low and middle income countries (World Health Organisation, 2016). The estimated global healthcare expenditure for diabetes is estimated to be around USD 727 billion, and is set to increase over the coming years (Cho et al., 2018). In New Zealand alone around 257,000 people are diagnosed with diabetes and another 100,000 have undiagnosed diabetes (with type II being the most common form). Maori and Pacific islanders are three times as likely to get diabetes as other New Zealanders. South Asians are also more prone to develop this disease. New Zealand, like the rest of the world, sees an increase in the number of diabetic patients and the largest increase is seen in type 2 diabetes related to obesity (Ministry of Health, 2018). This increase can be correlated to the changes towards a sedentary lifestyle that has come about due to rapid changes and urbanisation. Diabetes and its link with cardiovascular

diseases is the main cause of death in New Zealand (Ministry of Health, 2018). Cardiovascular diseases are the main cause of death worldwide.

## 1.2 Diabetes Mellitus definition

Diabetes mellitus is a chronic disease characterized by hyperglycaemia (high blood sugar) (Polonsky, 2012). Diabetes occurs when the hormone insulin is not produced at the regular rate (3.9-7.8mmol/L) (Diabetes Self-Management, 2017). Insulin is responsible for regulating glucose in the bloodstream. In a healthy body, glucose consumed from food is broken down into ATP and used as energy. However, if enough insulin is not produced, or if the insulin present is not active, it results in an excess of glucose in the bloodstream (World Health Organisation, 2016). The prolonged hyperglycaemic condition creates a range of metabolic disorders that can cause generalised vascular damage that can affect the eyes, heart, kidneys and nerves (Cho et al., 2018). There are 4 types of diabetes; type 1, type 2, gestational diabetes (common during pregnancy), and diabetes due to causes such as diseases of the exocrine pancreas. The most common types of diabetes are type 1 and type 2 (American Diabetes Association, 2012).

Type 1 diabetes has a strong genetic basis and occurs due to destruction of the pancreatic beta cell, which produces insulin. This leads to less insulin being produced, and therefore glucose is not regulated in the body. Type 2 diabetes, the most common form, deals with a decrease in the production of insulin as well as a decrease in insulin's ability to take up glucose (Olokoba et al., 2012). This depends on the interplay between skeletal muscle responsiveness to glucose, and glucose stimulated insulin production via the pancreas (Lowell and Shulman, 2005). Although it has some genetic basis, it is seen commonly in people who are overweight and who have a sedentary lifestyle with high fat and high calorie diets, which is becoming increasingly common today (Polonsky, 2012). It is also becoming clear that type 2 diabetes is associated with progressive destruction of pancreatic beta-islet cells thorough autoimmune processes linked to inflammation (Juwono and Martinus, 2016). Not only does diabetes lead to

a decreased quality of life, it has also been named a precursor to cardiovascular disease, the leading cause of death worldwide.

## 1.3 Diagnosis

The World Health Organisation's conditions for diagnosing diabetes are summarised in the table below. Pre-diabetes is an intermediate and prolonged stage that leads to type II diabetes. Through altering diet and lifestyle, the progression to diabetes can be slowed down (Inzucchi, 2012).

**Table 1.1 Diabetes diagnosis tests**

| Test | Prediabetes / Impaired glucose regulation | Diabetes |
|---|---|---|
| Fasting plasma glucose | 110-125 mg/dl | ≥126 ml/dl |
| 2 Hr plasma glucose (Oral Glucose Tolerance Test) | 140-199mg/dl | ≥200mg/dl |
| Glycated hemoglobin | - | ≥6.5% |
| Random plasma glucose | - | ≥ 200 mg/dl |

A fasting plasma glucose test requires that an individual fasts for 8-10 hours, followed by a blood glucose test. The OGTT requires a blood test to be taken in the morning before any consumption of food. Then the individual consumes a fluid with 75g of glucose, and after 2 hours glucose is measured in the blood again (Diabetes New Zealand, 2016). These are the two most common tests used for diagnosing diabetes (World Health Organisation, 2011). Another more recent test is measuring the glycated haemoglobin in the blood. This is a measure of the glucose in the blood over the past 2-3 months. An increase in glycated haemoglobin correlates to some of the complications in diabetes such as nephropathy, retinopathy and neuropathy (Stolar, 2010).

## 1.4 Symptoms

Some of the symptoms hyperglycaemia induces are excessive thirst, frequent urination, weight loss and blurred vision. Diabetic patients are also highly susceptible to certain infections such as yeast infections (American

Diabetes Association, 2014). Long term complications include both microvascular and macrovascular problems (King and Grant, 2016), such as retinopathy and autonomic neuropathy (which can lead to cardiovascular symptoms). The chronic hyperglycaemic condition of diabetics is also associated with dysfunction, long-term damage and failure of organs such as the kidneys, eyes, nerves, heart and blood vessels (Inzucchi et al., 2010). Diabetic patients have an increased rate of atherosclerotic cardiovascular disease (American Diabetes Association, 2014), however, the actual cause for this remains unknown (Shah and Brownlee, 2016). This with be discussed further in Chapter 1.7 and onwards.

In addition to medical symptoms, diabetes can bring about a lower quality of life and a large economic loss to the patients as well as to health systems and national economies (World Health Organisation, 2016). WHO has named diabetes as one of the four priority non-communicable diseases, including cardiovascular disease, cancer and chronic respiratory diseases (World Health Organisation, 2016). With the burden of diabetes increasing, it has become essential to understand this disease and its complications in order to reduce or slow down the rate of diagnosis in the world today.

## 1.5 The effects of hyperglycemia

A prolonged hyperglycaemic condition in the body can lead to ill effects on the body such as the over production of free radicals, endothelial dysfunction and inflammation. These can result in other disorders such as renal disease and cardiovascular diseases. The effects of hyperglycemia will be explained in this section.

### 1.5.1 Oxidative Stress

Reactive oxygen species (ROS) highly are reactive molecules that are generated through normal cell metabolic processes and environmental factors such as cigarettes smoke. They alter cell structures such as proteins and nucleic acids by damaging them. Regulating the amount of ROS produced is necessary to ensure cell viability, activation, proliferation and function. This is done through enzymatic and non-enzymatic antioxidants

that are able to block the damaging effects of ROS. In some pathological conditions including diabetes and atherosclerosis, the antioxidant systems are overwhelmed, either due to the excess of ROS produced or the pathological condition itself. This shift in which there are more harmful oxidants present in an organism than antioxidants is termed 'oxidative stress' (Birben et al., 2012).

ROS are produced by enzymes that use oxygen as an electron acceptor, including NADPH oxidase, NOS and xanthine oxidase. These three enzymes are responsible for the majority of ROS in the vascular wall. ROS are chemically instable and highly reactive. They act as secondary messengers in the production of inflammatory mediators and regulate redox sensitive genes, such as the NFκβ gene. They affect various cell processes such as apoptosis, hypertrophy, extracellular matrix modification and mitosis (Gomes, 2013).

When there is an excess of glucose in the bloodstream, different pathways become activated for metabolism. These include the polyol and protein kinase C (PKC) pathways which are not only related to the complications of diabetes, but also trigger an increase in ROS production. In normal conditions, around 3% of glucose goes through the polyol pathway. This is when glucose is converted to sorbitol via NADPH oxidation, followed by the conversion of sorbitol to fructose via NAD+ reduction. However, in hyperglycaemic conditions almost 30% of the glucose passes through the polyol pathway (Funk et al., 2012). This results in a loss of NADPH and glutathione, which increases the sensitivity to oxidative stress. Furthermore, when sorbitol is metabolised to fructose via sorbitol dehydrogenase, the ratio of NADH / NAD+ increases, resulting in the *de novo* synthesis of diacylglycerol (DAG) (Rolo and Palmeira, 2006). Increased amount of DAG also activates the PKC pathway, which leads to many diabetic complications such as capillary and vascular occlusion via increased pro-inflammatory gene expression through the expression of NF-κβ. This pathway also leads to an increased amount of NADPH oxidases, leading to an further increase in the production of ROS (Brownlee, 2001).

The formation of advanced glycation end products (AGEs) also increase oxidative stress in diabetic patients. AGEs are formed when glucose reacts with a free amino group, which rearranges to form a product that accumulates on proteins. This starts off the process of advanced glycation and the resulting products are able to modify protein function and produce ROS (Rolo and Palmeira, 2006).

Under normal conditions, once glucose is released into the bloodstream, it is taken into the cytoplasm of cells and converted into pyruvate thorough glycolysis. It then moves to the mitochondria and goes through the citric acid cycle (TCA), generating NADH. These substrates move through the redox carriers in the ETC, complex I, III and IV, to lastly to molecular oxygen, where it is converted to water. However, during this process incomplete reduced forms of oxygen are produced which leak from the mitochondria. In normal conditions only 0.1% of the oxygen leaks to generate ROS. The generation is primarily dependant on the redox state of the ETC; as electrons transfer through the chain, a proton / voltage gradient in generated. This goes on to generate energy in the form of ATP.

In hyperglycaemic conditions when there is an increased generation of electron transfer donors such as NADH and FADH2, there is an increased influx into the ETC. This hyperpolarises the proton gradient, leading to an accumulation of electrons. This then drives the generation of ROS (Rolo and Palmeira, 2006). The mitochondrial hyperoxide ion may initiate events that lead to the activation of the NF-κβ pathway and increases the production of inflammatory cytokines (Gomes, 2013). The NF-κβ pathway will be discussed further in Chapter 15.

These processes lead to an imbalance in ROS and the antioxidant defence, resulting in an overall increased oxidative state. In normal conditions an increase in ROS results in an increased expression of antioxidant enzymes, such as Cu-Zn superoxide dismutase, catalase and glutathione peroxidase. This is not seen in diabetic conditions; in type 1 diabetic patients, hyperglycaemia indicted increased lipid peroxidation, however the antioxidant enzyme was not induced, showing that although that although

the oxidative stress had increased, diabetic individuals were unable to generate a stronger antioxidant response (Gomes, 2013).

### 1.5.2 Hyperglycaemia and endothelial dysfunction

There are many ways in which hyperglycaemia can cause endothelial dysfunction, several of them being the activation of certain metabolic pathways. The activation of these pathways are most likely the result of oxidative stress through the generation of ROS in the mitochondria (Van den Oever et al., 2010).

As explained in Chapter 1.5.1, hyperglycaemia can induce the protein kinase C pathway (PKC) and this has several effects on vascular function. These include dysregulation of vascular permeability in smooth muscle, dysregulation of blood flow and base membrane thickening (Van den Oever et al., 2010).

AGEs can accumulate in plasma and tissue and proteins modified by these products cause increased blood flow, increased capillary permeability and vasodilation. AGEs have the ability to increase endothelial permeability and decrease arterial elasticity. Collagen modified by AGE prevents normal matrix formation and cross-linking. AGEs in the bloodstream are detoxified by enzymes, however when they are not eliminated by the kidneys they can generate new AGEs and react with plasma and tissue components, leading to the deterioration of tissues (Van den Oever et al., 2010).

### 1.5.3 Hyperglycemia and inflammation

As explained earlier, it has become increasingly clear that diabetes is linked to inflammation and the progressive destruction of beta islet cells through autoimmune processes. Autoimmunity can be seen in a large number of pathological conditions including diabetes. Autoimmunity is a complex multifactorial process that results in the loss of self-tolerance and a chronic excess reactivity of B and T cells, resulting in danger signals being released in the body. Danger signals being released results in the production of inflammatory cytokines, leading to inflammation (Itariu and Stulnig, 2014).

The mitochondria play a key role in secreting insulin from the B-islet cells, once glucose has been detected in the bloodstream. A protein called Heat Shock Protein 60 (HSP60) is released from the mitochondria when under stress or when the mitochondria is impaired. This protein is able to activate a signalling cascade through the activation of Toll-Like Receptors, leading to the production of pro-inflammatory cytokines, such as TNF-α and IL-1 and IL-6. TNF-α is also involved in insulin resistance and therefore propagates the condition of diabetes, as well as causing inflammation (Gomes, 2013; Juwono et al., 2016). This pathway will be discussed in detail in Chapter 1.8.

## 1.6 Heat Shock Proteins (HSPs)

As their name suggests, HSPs were first discovered by chance when they were expressed due to increased temperature (Grundtman et al., 2011). HSPs are highly conserved proteins in both prokaryotic and eukaryotic cells. They are involved in the maintenance of many cellular proteins, such as transporting, intracellular folding and unfolding of proteins (Wick et al., 2014). Under normal conditions, HSPs are responsible for assisting organisms during protein folding and refolding (Xu et al., 2012). Each type of HSP have different roles in the process of protein folding and unfolding, and overall they work to maintain protein structure and function (Xu et al., 2012). HSPs are categorised based on their size, ranging from small HSPs to HSP110 (5 kDa to over 100kDa) (Grundtman et al., 2011). They can be classified into six major families that are present in different cellular compartments, summarised in the table below. When cells are under physiological stress, such as high temperatures or hyperglycaemia, the expression of these proteins are increased (Wick et al., 2014). While they may be relatively temperature stable, many proteins are sensitive to sudden environmental changes and will begin to unfold and deform under stress. Since organisms must try to survive in a diverse range of conditions, HSPs act as a protecting mechanism for many cellular proteins (Xu et al., 2012).

**Table 1.2 Heat Shock Protein families size and locations (Adapted from Itoh et al., 2002)**

| Family | Size | Localisation |
|---|---|---|
| sHsps | 12 | Cytoplasm and nucleus |
| Hsp40 | 43 | Cytoplasm and nucleus |
| HSP60 | 60 | Mitochondria and cytoplasm |
| Hsp70 | 70 | Cytoplasm, nucleus and ER |
| Hsp90 | 90 | Cytoplasm and ER |
| Hsp110 | 110 | Cytoplasm |

### 1.6.1 HSP60

HSP60 is a highly conserved ring shaped mitochondrial stress protein. Within bacterial species there is 95% homology of HSP60 at the DNA and protein level, and up to 72% homology between human and bacterial species (Grundtman et al., 2011, Juwono and Martinus, 2016). It works in an ATP-dependent manner, encapsulating non-native proteins and aiding them with folding into the correct shape (Xu et al., 2012). This protein is expressed in the mitochondria and during stressful conditions can be translocated to the cytosol and cell surface to be exposed to the extracellular environment (blood plasma) (Grundtman et al., 2011).

The most studied member of the HSP60 family is the GroEL protein found in *E. Coli*, which aids in protein folding. It works together with its co-factor GroES, also known as Hsp10, in an ATP dependent manner to assist with protein folding (Hansen et al., 2003). A GroEL monomer contains 3 domains; Apical, Intermediate and Equatorial. 14 of these identical subunits assemble into two heptameric rings stacked back to back. GroES has 7 identical subunits that form a heptameric ring. There are two main conformational states of GroEL-GroES, with ATP being converted between the states. First GroEL binds a non-native polypeptide to its centre through hydrophobic contacts, and then expands and shields itself from the external area, allowing the polypeptide to fold (Xu et al., 1997).

## 1.7 Cardiovascular diseases: Major complication in diabetics

The leading cause of death in diabetic patients are cardiovascular diseases (International Diabetes Federation, 2018). Cardiovascular disease is a collective term for the diseases of the blood and heart vessels. They include microvascular diseases such as renal disease, various neuropathies, and macrovascular diseases, mainly covering atherosclerotic incidents such as myocardial infarction and strokes (Brownlee, 2001). Diabetic patients have a 2-4 fold increase in the risk of cardiovascular dieases compared to healthy individuals. After diagnosis, their prognosis is significantly worse than that of cardiovascular disease patients without diabetes (King and Grant, 2016).

The causes behind these diseases include high blood pressure, high blood glucose levels or a poor dietary intake. In an unhealthy lifestyle many young people lead today, the prevalence of both diabetes and cardiovascular diseases is set to increase. Cardiovascular diseases are usually identified and diagnosed after a patient has had a stroke, showing that these diseases go undetected until medical treatment becomes necessary, not giving the patient a warning or a chance to improve their lifestyle (American Heart Association, 2016). However, it has become clear that people diagnosed with diabetes can reduce or slow down the onset of this disease, which in turn would help to slow down the onset of cardiovascular diseases.

A link between diabetes and cardiovascular disease has been recognized for decades (Funk et al., 2012). It becomes important to understand the pathological basis of this link in order to allow us to create any possible protective measures against them. The hyperglycaemic condition in diabetes has been proven to contribute to atherosclerosis. The exact details of how this occurs remains unknown, however there has been much speculation, including the production of free radicals and the overexpression of HSP60.

### 1.7.1 Atherosclerosis

Atherosclerosis is responsible for over 80% of deaths and 75% hospitalizations in diabetic patients (Basa and Garber, 2001). Atherosclerosis is a condition that occurs when materials such as cholesterol, lipids and inflammatory cells are deposited in the endothelium of arteries in the heart (King and Grant, 2016). The arterial wall is part of the circulatory system and is remodelled continually when exposed to stressors including toxins and hypercholesterolemia. Stimuli like these are able to create a change in blood pressure and damage the vessel wall, which leads to arterial stiffness and starts the onset of atherosclerosis (Xu et al., 2012). This leads to plaques forming, which can cause myocardial infarction, stroke, unstable angina and lower limb ischemia (King and Grant, 2016). Atherosclerosis leads to the diagnosis of various other cardiovascular diseases, as it begins to block off the arteries.

### 1.7.2 The possible effect of HSP60 in Cardiovascular diseases

HSP60 is the only heat shock protein that shows a direct link to atherosclerosis through experimental and clinical trials (Wick et al., 2014). The movement of this protein to the cell surface has been recognised as a stress response and correlates strongly with the development of cardiovascular disease (Grundtman et al., 2011). Several researches have been conducted that support that HSP60 has a big role in the development of atherosclerosis which will be further explained below.

A study carried out by Yuan et al in 2011 revealed that diabetic patients have elevated levels of HSP60 in saliva and serum, which could be a contributing factor to the development of atherosclerosis and plaque formation (Yuan et al., 2011). In Type 2 diabetic patients, salivary HSP60 was found to be 4 fold higher and serum HSP60 16 fold higher than non-diabetics (Juwono and Martinus, 2016). In a recent paper published by Martinus and Goldsbury in 2017, it was clearly demonstrated that when Thp-1 cells were grown in the presence of glucose 25mM glucose, HSP60 protein levels increased by 3 fold compared to Thp-1 cells grown in 5mM glucose.

Xiao et al. proposed that HSP60 was involved in activating pro-inflammatory processes (discussed below), which is a key factor in atherogenesis (Xiao et al., 2005). HSP60 is able to activate both the innate and adaptive immune system (Schoneveld et al., 2008). It is also able to act as a ligand for innate immune receptors and is recognized as an antigen by adaptive immune receptors (Juwono and Martinus, 2016). Humans acquire immunity against bacterial HSP60 through infection or vaccination, however this defence can increase the risk of cross-reactivity with HSP60 produced by stressed endothelial cells, such as those of the arteries under hyperglycaemic conditions (Wick et al., 2014). It has been shown that when endothelial cells are exposed to stress, HSP60 is released into the supernatant. HSP60 is then able to activate Toll-like receptors (TLRs) and trigger a cascade of reactions that lead to an inflamed state (Tian et al., 2013).

## 1.8 Toll-Like Receptors (TLRs)

The innate immune system contains a group of Pattern Recognition Receptors (PRRs) called TLRs that sense the presence and type of an invading pathogen. They initiate a rapid antimicrobial response and allow the development of adaptive immunity specific for the detected pathogen (Makkouk and Abdelnoor, 2009).

TLRs were first discovered in the fruit fly (Matsunaga et al., 2011) and were found mainly to be involved with the body development of the fly. Later they were discovered in mammals, however it was noted they had no role in development, but played an important part of the innate immune system. TLRs are a group of highly conserved glycoproteins located intracellularly or on the surface of many cell types, such as B cells, monocytes, regulatory T cells, respiratory epithelial and endothelial cells. They recognise Pathogen Associated Molecular Patterns (PAMPs), which are sets of specific pathogen components shared by groups of (invading) microorganisms (Makkouk and Abdelnoor, 2009). PAMPs act as exogenous ligands for TLRs, and when they come into contact with specific TLRs, they sets of a signalling cascade. In humans 10 TLRs have been identified (Tian et al.,

2013) and each become activated by different PAMPs (Makkouk and Abdelnoor, 2009; Juwono and Martinus, 2016). TLR 1, 2, 4, 5, 6, and 10 are located on the cell surface and the rest intracellularly. TLRs contain two significant domains; a ~ 25 tandem leucine-rich repeat motif which is the external antigen recognition domain, and a cytoplasmic domain made up of 200 amino acids called the Toll interleukin-1 resistance (TIR) domain. These two domains are attached to one another by a transmembrane helix, which is sometimes referred to as the third domain (Patra and Choi, 2016) (Makkouk and Abdelnoor, 2009).

**Table 1.3 Toll-Like receptors location and ligands (Adapted from Makkouk and Abdelnoor, 2009)**

| TLR | Location | Exogenous PAMPs |
|---|---|---|
| 1, 2, 6 | Cell surface | Lipopeptides |
| 4 | Cell surface | LPS |
| 3 | Intracellular | dsRNA |
| 7, 8 | Intracellular | ssRNA |
| 9 | Intracellular | Non-methylates CpG DNA |
| 5 | Cell surface | Flagellin |

TLRs sense the invasion of microorganisms through PAMPs and respond by triggering a range of defence mechanisms (Juwono and Martinus, 2016, Makkouk and Abdelnoor, 2009). TLRs can recognize their endogenous ligands, as well as proteins that are released due to cellular stress (Wong and Wen, 2008) such as HSP60. TLR2 and TLR4, which are both upregulated in diabetic patients, can be activated by recognising PAMPs on their ligands such as HSP60, HSP70, endotoxins (LPS) and AGEs. Once activated, TLRs homodimerize or heterodimerize, and recruit adaptor proteins (Patra and Choi, 2016). This activates one of two distinct pathways that are dependent on either the adaptor proteins myeloid differentiation factor 88 (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adaptor protein inducing interferon (IFN)-β-mediated transcription factor (Trif). This

leads to the activation of nuclear factor-kb and IFN regulatory factors, ultimately leading to the production of inflammatory cytokines such as IL-b, IL-8 and TNF-α (Tian et al., 2013) (Juwono and Martinus, 2016). All TLRs signal using the MyD88-dependant pathway except for TLR3. TLR4 uses the Trif pathway to a certain extent once activated, however TLR2 does not (Tian et al., 2013).

A study carried out by Devaraj et al (2009), and Dasu et al (2010) demonstrated that diabetic patients have an increase in circulating TLR levels, in particular TLR2 and TLR4. They also have an increase in HSP60, which TLR2 and TLR4 can use as a ligand (Tsan and Gao, 2004). Due to the increase of expression in TLR2 and TLR4, an increase in inflammation was observed. This was mediated by the NF-kb pathway. Dasu et al. also found increased concentrations of pro-inflammatory mediators such as IL-1B, IL-6, IL-8, MCP-1 and TNF-α.

### 1.8.1 TLR Receptor agonists and antagonists

TLR agonists are small molecular mimics of the natural ligands of TLR receptors. The aim of these agonists is to ultimately lead to the production of cytokines by stimulating signal transduction pathways. Mimics of natural microbial ligands have been created that have enhanced pharmacokinetic and pharmacodynamic properties compared to the natural ligands. Some agonists used so far include imiquimod and resiquimod, which are able to activate TLR7 as they mimic its ligands. TLR7 agonists have been used to target disorders caused by papillomavirus. Another agonist is LPS for TLR4. LPS is an outer membrane glycolipid constituent of Gram negative bacteria. It has three portions; a hydrophobic lipid A component, a hydrophilic core, and an O-antigen, which are all covalently linked. LPS however can't be used medically as it is toxic, but it has been found to stimulate TLR4, which is useful for research purposes (Makkouk and Abdelnoor, 2009). As explained above, TLR2 and TLR4 both go through the MyD88 pathway and ultimately lead to the activation of pro-inflammatory cytokines including TNF-α.

TLR antagonists are usually structural analogs of agonists and can bind to the TLR receptors, but they do lead to signal transduction as they are not able to induce the structural rearrangement required. This means that the usual signal transduction pathway is not activated and therefore no inflammatory cytokines are produced (such as TNF-α) (Makkouk and Abdelnoor, 2009, Patra and Choi, 2016). To date antagonists developed to inhibit or reduce TLR signalling include aptamers, oligonucleotides, peptides, antibodies, proteins and small molecules. TLR antagonists can target the different domains of the TLR receptor itself, or can work in an indirect way, such as by interacting with receptor agonist and reducing their ability to activate their receptors (Patra and Choi, 2016).

## 1.9 Importance of monocytes

Monocytes are leukocytes derived from bone marrow progenitor cells. They were one of the first cells to be identified in atherosclerotic plaques (Moore and Tabas, 2011) (Woollard and Geissmann, 2010). Monocytes are recruited to areas dense with ApoB-LPs due to cytokines released from endothelial cells (Moore and Tabas, 2011). An accumulation of apolipprotein B containing lipoproteins (ApoB-LPs) has been identified as an initiating step in atherosclerosis, and blood-borne monocytes releasing HSP60 could be contributing to the inflammatory response. As inflammation increases, monocytes travel from the blood to lymphoid and non-lymphoid tissues and into the intima and sub intima. It is assumed that when they come into contact with fatty deposits, they undergo activation and begin to accumulate in the lesion where inflammation occurs. Here they are able to phagocytose toxic molecules as well as other cells, and are able to differentiate into inflammatory DCs, macrophages, or foam cells. (Woollard and Geissmann, 2010). This aids in the process of forming plaques, commonly called fatty streaks, inside the intima. Fatty streaks indicate the beginning of atherosclerotic plaques (Woollard and Geissmann, 2010). For the process of atherosclerosis to continue, it is required that cells are continually recruited to the area. Through studies that use labelled monocytes and radioactive tracers, it has been shown that the monocyte number in the aorta is consistent with the surface area of the plaque

(Woollard and Geissmann, 2010), indicating the build-up of monocytes over time.

Under some experimental conditions, some monocytes have been seen to emigrate from the plaques back into the bloodstream. With these differences it becomes apparent that there may be subsets of monocytes with different functions. They can be distinguished partly by the different surface markers they express. A subset of monocytes has been found to turn into macrophages, while another set reside in the luminal side of the endothelium of small blood vessels during inflammation (in the brain, arteries in the dermis and mesentery). These types both express different surface markers, However, it is important to note that blood monocytes have been seen in the atherosclerotic plaques, and their role in this condition has not properly been elucidated (Woollard and Geissmann, 2010).

In this research the Thp-1 cell line was used as an imitation for monocytes in the serum of diabetic patients. The thp-1 cell line originates from a 1 year old acute monocyte leukaemia patient (Tsuchiya et al., 1980). Most of the studies so far have been conducted in mice, however human blood monocytes show similarities to the subsets of monocytes in mice(Woollard and Geissmann, 2010).

### 1.9.1 Macrophages

Monocytes can differentiate into macrophages using differentiation factors including macrophage colony-stimulating factor (M-CSF) (Moore and Tabas, 2011). In early atherosclerotic plaques, a large percentage of monocytes become macrophages or dendritic cell like. Like monocytes, within the macrophages there are subsets in which some have been suggested to be involved in repair, while others in pro-inflammatory processes (Moore and Tabas, 2011).

These cells have many pattern recognition receptors, such as the scavenger receptors, which are known to connect the innate and adaptive immune response in the process of atherosclerosis. In particular, CD36, a

B scavenger receptor increases macrophage spreading, which inhibits their migration and essentially traps them in the arterial intima. Scavenger receptors are another important area that need to be elucidated concerning atherosclerosis (Woollard and Geissmann, 2010).

Interestingly, although monocytes are recruited into the plaque, monocytes derived macrophages in the plaque may secrete apoB-LP binding proteoglycan. This may have a major role in LP retention in the plaque, which increases inflammation, promoting atherogenesis (Moore and Tabas, 2011).

## 1.10 Project: Expression of TNF-α at mRNA level in Thp-1 cells exposed to LPS and HSP60: Possible impact on diabetic vascular inflammation

It has been observed that HSP60 is present in high levels in the serum of diabetic patients. This may initiate pro-inflammatory cytokines in certain cell types through the interaction of TLR2 and TLR4 receptors, leading to non-resolved vascular endothelial cell inflammation (Juwono and Martinus, 2016). Since the prevalence of diabetes is increasing worldwide and this inflammation can lead to atherosclerosis, it is important to understand the connection between these two diseases in order to see if certain pathways can be blocked to slow down or reduce diabetic atherosclerosis. Research done around this topic so far suggests that HSP60 has a significant role in the development of diabetic atherosclerosis and this could provide a key missing link between diabetes and vascular inflammation.

## 1.11 Aims and objectives

It is known that LPS induction of Thp-1 cells lead to an increased expression of the pro-inflammatory cytokine TNF-α through the interaction of TLR4. It has also been shown through research that HSP60, which is elevated in diabetic patients, may also be using TLR4 to induce a TNF-α response. Recently research has also emerged showing that HSP60 may also be able to trigger TLR2. HSP60 triggering the TLRs may be leading to an inflamed state in the arteries of the heart, contributing to the atherogenesis and the

development of cardiovascular diseases. It becomes of interest to understand the molecular mechanism of HSP60 to see whether this protein is causing diabetic vascular inflammation. The objectives of this project are described below.

**Objective 1:** Establishing a Thp-1 cell growth curve and optimising RNA extraction

This was achieved by growing Thp-1 (Human monocyte cell line) cells in RMPI growth media supplemented with fetal bovine serum and penstrep. Growth curves were carried out to determine the best time RNA should be extracted. RNA was extracted using the Trizol method. Quantity of RNA was determined by testing the sample using a nanodrop machine (Nanodrop2000,Thermo Fisher). Quality of the RNA was determined by running formaldehyde and agarose gels.

**Objective 2:** Quantifying a TNF-α response from thp-1 cells

This objective was achieved by exposing Thp-1 cells to LPS, which is a ligand for TLR4 and can induce TNF-α induction. RNA was extracted and cDNA was synthesised. PCR for TNF-α and the house keeping gene GAPDH were carried out. The PCR products were visualised on a gel and the band intensities were compared using Thermo Fisher software on the iBright imaging system (Thermo Fisher) for conducting semi-quantitative analysis.

**Objective 3:** Real Time PCR analysis of Thp-1 cells induced with LPS

This objective was met by exposing Thp-1 cells to LPS for different time periods. RNA was extracted and cDNA was synthesised. Real time PCR for TNF-α and the house keeping gene GAPDH were carried out and the results analysed.

**Objective 4:** Real Time PCR analysis of Thp-1 cells induced with extracellular Hsp60 to generate a TNF-α response

Extracellular Hsp60 was exposed to the Thp-1 cells in order to trigger the TNF-α response. This was done by exposing different concentrations of HSP60 to Thp-1 cells for a certain time period. TNF-α induction was measured through qPCR and analysed.

# 2 Materials and Methods

All methods were carried out at the University of Waikato, in either the E3.11, E3.13 (PC2 lab), or C.2.03. All experiments were carried out aseptically in areas cleaned with 70% ethanol. RNA work was done is designated areas specific for RNA work only.

This chapter contains the general methods that were carried out repeatedly throughout this project. Individual experimental details are written in the methods section of each chapter.

## 2.1 Common solution preparations

**Table 2.1 Common solutions and compositions**

| Solutions | Composition |
|---|---|
| 0.1% DEPC water | 100ul of DEPC in 1L of MQ water |
| MOPS buffer (10x) | 0.4 M MOPS – 8.37g<br>0.1M Sodium Acetate – 0.82g<br>Made up to 100mls with DEPC water, pH adjusted to 7 using NaOH |
| RNA Loading Dye | 95% formamide<br>0.025% SDS<br>0.025% bromophenol blue<br>0.025% xylene cyanol FF<br>0.025% ethidium bromide<br>0.5mM EDTA |
| TAE buffer (50x) | Tris base – 121g<br>Glacial acetic acid – 28.55mL<br>0.5M disodium EDTA- 50mL<br>Tris was first dissolved in 300mL of DEPC water, then disodium EDTA and glacial acetic acid was added |
| THP-1 cell media | Gibco RPMI media with 10% fetal bovine serum and penstrep |
| Tris-HCl buffer | Tris – 121.4g<br>HCl<br>Tris was dissolved in 1L of MQ water. The pH was adjusted to 7.0 using concentrated HCl |
| Tris-HCl with NaCl buffer | 25mLs of Tris-HCl with 25 mLs of NaCl |

## 2.2 Thp-1 Cell culture

Frozen Thp-1 cells were obtained from the -80°C cell culture inventory in the Department of Biological Sciences at the University of Waikato. These cells were grown in RPMI media (Gibco) with 10% FBS and 500ul Penstrep (Gibco) at 10,000U/mL. These cells were kept in standard incubator conditions; 37°C, 5% $CO_2$ in a humidified incubator. The cells were split every four days and topped up with fresh media to keep them healthy and growing.

## 2.3 RNA extractions for THP-1 cells

This RNA extraction method was used on all cell lines. After obtaining a cell count of ~ $10^5$ cells, the cells were centrifuged for 2000 rcf for 7 min to form pellets. After removing the media layer, .5mL of Trizol was added and the cell pellet was resuspended. This was left for 15 min, allowing the cells to lyse. 0.1mL of chloroform was added and the tubes were shaken vigorously for 20 seconds. This was left for three min, and then centrifuged at 12,000 rcf for 15 min at 4°C. The layers were then separated; the clear upper aqueous phase was put into a separate 1.5mL RNAse/DNAse free Eppendorf tube. 0.5mL of isopropyl alcohol was added to this, and it was left for 10 min at room temperature, followed by 20 min at -20°C, to allow RNA to precipitate. This was then centrifuged at 12,000 rcf for 10 min at 4°C. Regardless of whether a pellet was seen, the supernatant was removed and 1mL of 75% alcohol was added to was the precipitate. This was then centrifuged at 7600 rcf for 5 min at 4°C. The pellet was then air dried in the fume hood for 15 min. The pellet was resuspended in 20µl of DEPC water and stored at -80°C for further use.

## 2.4 Nanodrop readings

After RNA had been extracted, 2uL of the sample was tested on the Nanodrop2000 (Thermo Fisher) machine to determine the quantity and quality of the RNA. Absorbance at 280nm is used as an indicator of the purity of nucleic acid samples. A 260/280 ratio of ~ 2.0 is accepted as pure

for RNA (Thermo Scientific, 2012). If the value is significantly lower (<1.8), it indicates contamination by residual phenol or reagents used in the extraction process. If the concentration of RNA extracted is very low (~ 10ng/μL), the absorbance ratio cannot be determined accurately (Thermo Scientific, 2012).

## 2.5 cDNA synthesis

The qScript XLT Supermix cDNA synthesis kit was used according to the manufacturer's directions. Each reaction contained up to 1 μg of RNA, 2 μl of the supermix and DEPC water to reach a total volume of 10μl. This was run in PTC-200 Peltier Thermal Cycler at 25°C for 5 minutes, 42°C for 60 minutes, 85°C for 5 minutes and held at 4°C. After synthesis, cDNA was diluted 1:5 and 1:10 with DEPC water, which resulted in clear PCR product bands after PCR.

### 2.5.1 Primers

Based on previous research, the primer pairs specific for GAPDH and TNF-α were ordered from Sigma-Aldrich. These primers were supplied at 100uM. They were diluted down to 10uM (80uLs of DEPC water, 10uM forward primer and 10uL of reverse primer). The sequences and locations they bind are shown below in Figure 4.3 and 4.4. These primers were verified with the online database at GenBank to ensure that they matched the human genes.

**Table 2.2 Primer sequences and product sizes of GAPDH and TNF-α**

| Gene | Primer Sequence | Product size (pb) |
|---|---|---|
| GAPDH forward | ACC ACA GTC CAT GCC ATC AC | 452 |
| GAPDH reverse | TCC ACC ACC CTG TTG CTG TA | |
| TNF-α forward | CAG AGG GAA GAG TTC CCC AG | 325 |
| TNF-α reverse | CCT TGG TCT GGT AGG AGA CG | |

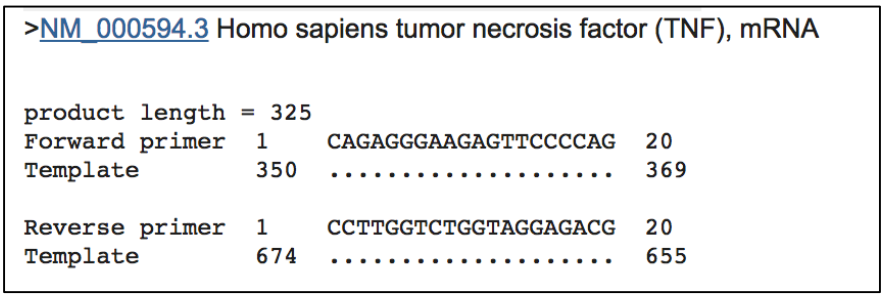


**Figure 2.1 TNF-α forward and reverse primers showing position on TNF-α gene and final product length of 325 bp.**

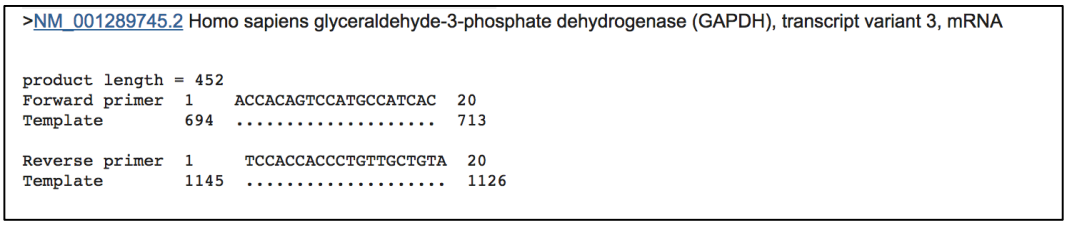


**Figure 2.2 GAPDH forward and reverse primers showing position on GAPDH gene and final product length of 452 bp.**

## 2.6 2% Agarose gel electrophoresis

The quality and quantity of the RNA was assessed using the Nanodrop2000 (Thermo Fisher) as well as running the extracted RNA on a 2% agarose gel. The PCR bands were also visualised using 2% agarose gels. This was prepared by using 2g of agarose and 100mls of 1x TAE buffer. For staining, 2ul of EtBr was added to the molten gel. A 100bp ladder (SolisBiodyne) was run alongside the samples to judge size of products. The gel was run for approximately 1 hour at 70-80V, and then viewed under UV light.

# 3 Optimising Thp-1 cell culture and RNA extraction methods

## 3.1 Introduction

Diabetes Mellitus is associated with high levels of mitochondrial stress protein HSP60 in the bloodstream. HSP60 is released from many cell types and is known to activate pro-inflammatory responses in target cells. This leads to the release of pro-inflammatory cytokines such as TNF-α (Chapter 1.7.2). This upregulation initiated by HSP60 has been hypothesised to cause inflammation in diabetic patients, which can eventually lead to the formation of atherosclerotic plaques. It is known that one of the first cells and most common cells to be found in these plaques are blood borne monocytes. These cells are also able to differentiate into macrophages or foam cells in the plaque (Chapter 1.9).

In this project the expression of TNF-α was to be measured in monocyte cells they after were exposed to LPS (a trigger that is well documented to lead to the expression of TNF-α), and HSP60. The cells used throughout this project were Thp-1 cells. Thp-1 cells are a monocytic cell line originally derived from a leukaemia patient (Chapter 1.9). These cells were used to mimic monocyte behaviour in the bloodstream of diabetic patients.

The first task of this project was to establish a growth curve for Thp-1 cells. This enabled subsequent RNA extractions to be undertaken during the exponential phase of growth, maximising the amount of RNA extracted. Then RNA was extracted and assessed for quantity, quality and integrity.

## 3.2 Methods

### 3.2.1 Growth Curve

Thp-1 cells were seeded at $10^5$ cells (in approximately 2 mLs) into each well of a 6 well plate and incubated in a humidified $CO_2$ incubator at 37°C (standard incubator conditions for cell growth). Cells from 2 wells were harvested every 24h and cell counts were done using trypan blue. An equal amount of cells and trypan blue solution was pipetted onto the haemocytometer and viewed under a phase contrast microscope (Nikon eclipse TS100). The haemocytometer has several grid areas. Cells in the 4 outer 4 by 4 squares, and the middle square were counted. The volume in each of these areas can be represented as 1mm x 1mm x 0.1 mm = $0.1mm^3$. The total volume of the squares in which cells are counted is $0.1mm^3$ x 5 = $0.5mm^3$. As 1mL volume = $1000mm^{3,}$ the volume counted would be 1/2000 of 1 ml ($1000mm^3$ / .5 $mm^3$ = 2000). Therefore, the total cell number from 5 squares was multiplied by 2000 to give a final cells per mL count. Cells that took up the dye and appeared dark blue under the microscope were recognised as dead cells, and were not counted when determining cell number.

### 3.2.2 RNA extraction

RNA extraction was carried out using the TRIzol method as described in Chapter 2.3. 2uL of the samples were then tested on the Nanodrop, with an expected 260/280 ratio of ~2, as described in Chapter 2.4.

### 3.2.3 Visualising RNA using Formaldehyde gels

1g of agarose was added to 72ml of DEPC water. This was heated in a microwave until boiling, and agarose was fully dissolved. After cooling to approximately 60°C, the flask was transferred to a fumehood where 10x MOPS buffer and 18ml of 73% formaldehyde were added. RNA samples were run at varying concentrations (1800ng, 1350ng, 675ng, 225ng) on the gel with 10x MOPS buffer for approximately 1 hour and 20 minutes and the visualised under UV using the Omega Lum G Imaging system (Apelgen).

### 3.2.4 Visualising RNA using Agarose gels

This method is described in Chapter 2.6 and was used throughout the rest of the project.

## 3.3 Results

### 3.3.1 Phase Contrast Images of Thp-1 cells

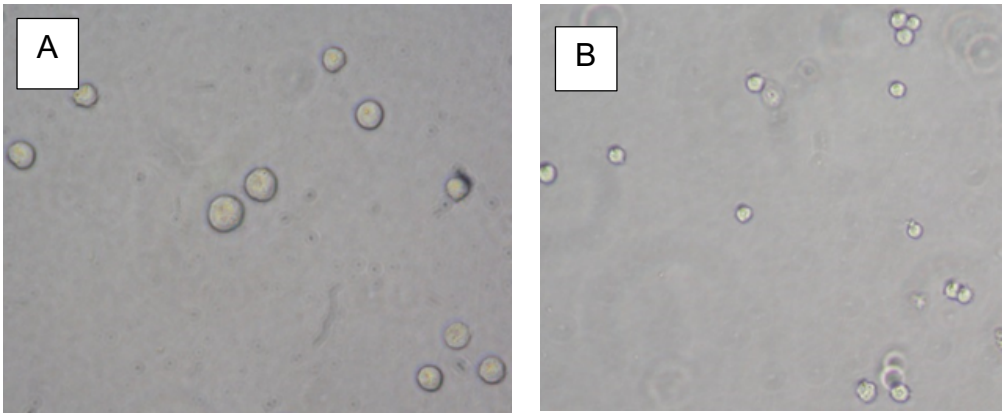


**Figure 3.1 Thp-1 cells growing in standard conditions (RMPI supplemented with FBS and Penstrep). A: 40x magnification, B: 10x magnification.**

The cells seen above show healthy Thp-1 cells, as the membranes are intact and well defined. They appear to be circular in shape and often grow in little clusters as seen in the second figure. They appear like this before they split off and continue growing.

### 3.3.2 Growth Curve

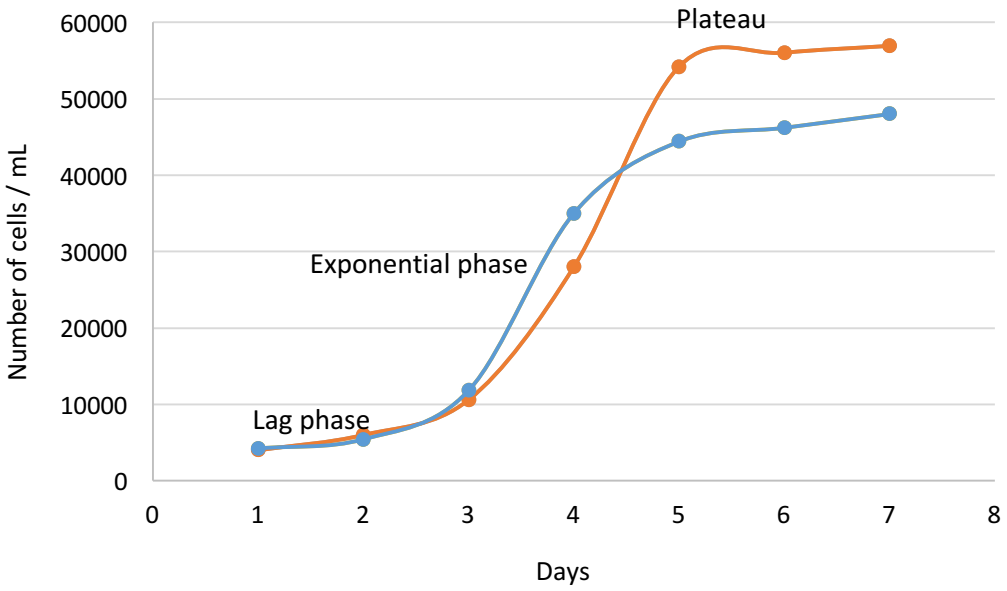


**Figure 3.2 Growth curve for Thp-1 cells over the period of 7 days.**

The growth curves show a lag phase straight after cells have been seeded at approximately 5000 cells per well in a 6-well plate, showing that at this point the cells have just begun to slowly divide. This is followed by the exponential phase of growth around day 3 when there are ~10,000 cells growing at its fastest rate. Around day 5 the growth curve begins to plateau, showing that cell growth has significantly slowed down. RNA should be extracted around day 3-5 as this is when the cells are growing the fastest by using the environment around them for nutrients, before reaching the stationary phase when the nutrients have been exhausted.

### 3.3.3 RNA quality and quantity

**Table 3.1 RNA concentration and 260/280 ratio of control Thp-1 cells**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control sample A | 201.6 | 2.09 |
| Control sample B | 454.7 | 1.94 |

The table above shows the concentrations of the RNA extracted were 201.6ng/mL and 454.7ng/mL. This initial experiment was carried out two times as seen above (N=2). The RNA concentration and 260/280 ratio was considered sufficient. Ideally the 260/280 ratio is expected to be ~2, which is seen in both samples.

### 3.3.4 RNA integrity

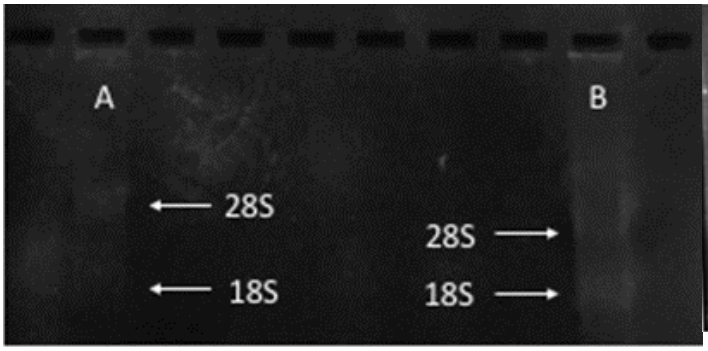


**Figure 3.3 Formaldehyde gel run for 1 hour and 20 min at 80V with sample A (600µg/mL RNA) and B (1350 µg/mL RNA).**

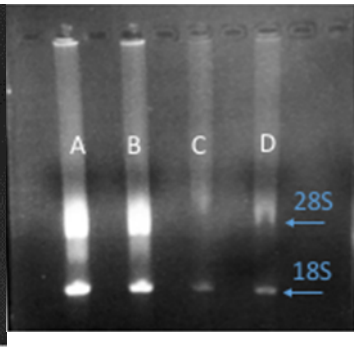


**Figure 3.4 2% Agarose gel run for 1 hour and 20 minutes at 70 V with sample B. Lanes shown A: 1800µg RNA. B: 1350µg RNA, C: 675µg RNA D: 225µg RNA.**

RNA integrity refers to the tertiary structure of the RNA as it travels through the gel. If the structure is altered, the bands appear to be smudged on the gel. There are two distinct RNA bands that can be visible on a gel; 28S and 18S (with the numbers referring to their sizes). Initially RNA extracted from control cell (cells grown in growth media supplemented with 10% FBS and Penstrep (Gibco) under standard incubator conditions) were run on both a formaldehyde gel and a 2% agarose gel to assess which would be more suitable for this project. A formaldehyde gel is commonly used for visualising RNA as the formaldehyde acts as a denaturing agent, allowing the two RNA bands, 28S and 18S to separate and become clearly visible on the gel. Formaldehyde can also act as an RNAse inhibitor, which helps to maintain the RNA during handling (Mansour and Pestov, 2013). However, as seen in Figure 3.3, in the formaldehyde gel the two RNA bands are difficult to see.

This may have been because of the quality of the gel made, or because the amount of RNA loaded was not enough to show on the gel. In Figure 3.4, a 2% agarose gel was loaded with RNA at different concentrations (1800ng, 1350ng, 675ng, 225ng). This was done to assess how much RNA (in ng/mL) should be loaded in order to see clear bands, as well as to assess the integrity of the RNA. Even at a very low concentration of ~200ng of RNA, bands are visible.

## 3.4 Discussion

The objectives of the experiments detailed in this chapter were to generate a growth curve for thp-1 cells, and become familiarised with the RNA extraction process in order to extract sufficient and useable RNA in the following experiments. THP-1 cells were cultured under standard growth conditions in a humidified $CO_2$ incubator at 37°C. Generating a growth curve gives insight into the phases of growth in a cell culture. A cell culture generally goes through phases called a lag phase and a logarithmic phase, after which it will go into plateau and reach a stationary phase. The lag phase occurs at the beginning as the cells slowly start to grow. The exponential phase is when the cells are duplicating and this is the best time to extract RNA as the cells are actively growing and using the environment around them to aid growth. When the cells growth begins to plateau, it indicates that the media has been exhausted (van Meerloo et al., 2011). Monitoring the viable cell number (using Typan blue staining) every 24h during a 5 day growth study enabled the construction of a growth curve for THP-1 cells. When cells were seeded at 5000 cells per mL they remained in a lag phase for 2 days, exponential phase during ~ 2 days and reached stationary phase after 4 days. Repeating this growth study gave similar responses (Figure 3.2).

The TRIzol method was used to extract RNA. TRIzol reagent is a solution containing phenol and guanidine isothiocyanate. When the cells and cellular components lyse, TRIzol reagent maintains the integrity of the RNA. After TRIzol reagent has been added and the samples have been incubated, chloroform is added and the samples are spun in the centrifuge. This allows

two layers to be formed; an upper aqueous phase and lower organic phase. The upper phase contains RNA and is separated from the organic layer and precipitated with isopropanol. The lower organic phase contains DNA and proteins (which can be precipitated with ethanol to yield DNA, and precipitated further to yield proteins) (Simms et al., 1993). This method resulted in RNA with a 260/280 ratio between 1.9-2.10, as well as clear bands that can be seen in Figure 3.5. If the ratio was well below 1.8, it would indicate contamination by the reagents used, leaving behind guanidine or residual phenol in the extracted samples (Thermo Scientific, 2012). This showed that the TRIzol method was successful and that the RNA for the coming experiments can be extracted using this method. Although quality is measured using the 260/280 ratio through the Nanodrop, it is important to note that DNA is also able to absorb around 260nm. This means that although RNA is present, it may be contaminated with DNA. To further assess the RNA samples by looking at their integrity, they were run on gels.

To assess the integrity of the bands, RNA could be run on either a formaldehyde gel or an agarose gel. Initially a formaldehyde gel was used to test a sample of RNA. A 2% agarose gel was also made to test a sample of RNA and this RNA was loaded at different concentrations (1800µg, 1350µg, 675µg, 225µg) on the gel. The formaldehyde gel not only required the use of more chemicals, but was also a lot more time consuming to make than a 2% agarose gel. The purpose of running the RNA samples down a gel was to see the two bands at 28S and 18S to assess the integrity of them, and this was able to be done sufficiently on an agarose gel. Therefore, only agarose gels were used in the following experiments.

Although the 260/280 ratio values (1.9-2.10) around the recommended values (1.8-2.0), some contamination may be seen in the agarose gel (Figure 3.4) lanes A and B. These lanes were all loaded with the same sample, but lanes A and B were loaded with a higher concentration of RNA (1800ng and 1350ng) than lanes C and D (675ng and 225ng). Although RNA is visible in all four bands, slight genomic DNA contamination may be seen in the lanes loaded with more RNA. This could go on to affect any

further experiments done. Ideally, the RNA samples could have gone through a DNase treatment to remove any DNA contamination.

Thus RNA purity and integrity were considered good after extraction took place in the exponential phase of growth. The next chapter discusses RNA extraction once the Thp-1 cells are stimulated with LPS, and semi-quantitative analysis of the expression of the pro-inflammatory cytokine TNF-α.

# 4 Thp-1 cells induced with LPS to determine TNF-α expression at mRNA level through semi-quantitative PCR

## 4.1 Introduction

LPS, an endotoxin derived from the cell wall of Gram negative bacteria, is a known trigger for TLR4 that leads to the production of the pro-inflammatory cytokine TNF-α. It is also suggested that the mitochondrial stress protein HSP60 triggers inflammation in the same way as LPS, by acting through TLR4, however some studies suggest that TLR2 may also play a role in this process, ultimately leading to the production of TNF-α (Kilmartin and Reen, 2004).

In this part of the project, a preliminary experiment was carried out before the Thp-1 cells were incubated with HSP60. Thp-1 cells (as described in Chapter 1.9) were incubated with 50ng/mL, 100ng/mL, and 200ng/mL of LPS for 6, 12 and 24 hours. RNA was extracted and cDNA was synthesised, followed by PCR. In order to do semi-quantitative analysis, the number of PCR cycles had to be run within the exponential amplification range. Once the optimum number of cycles were decided, semi-quantitative analysis was conducted for TNF-α, using GAPDH as the housekeeping gene.

## 4.2 Methods

### 4.2.1 Thp-1 cells stimulated with LPS for 6, 12, and 24 hours.

Thp-1 cells were seeded at 3 x $10^5$ cells (in approximately 2 mLs) into each well of a 6 well plate. The plate was set up as shown in the diagram below; each plate had a control well with Thp-1 cells and PBS, a well with Thp-1 cells with 50ng/mL LPS, a well with Thp-1 cells with 100ng/mL LPS, and a well with Thp-1 cells with 200ng/mL LPS. This was incubated in a humidified $CO_2$ incubator at 37°C (standard incubator conditions) for cell growth) for 6, 12, and 24 hours, followed by RNA extraction.

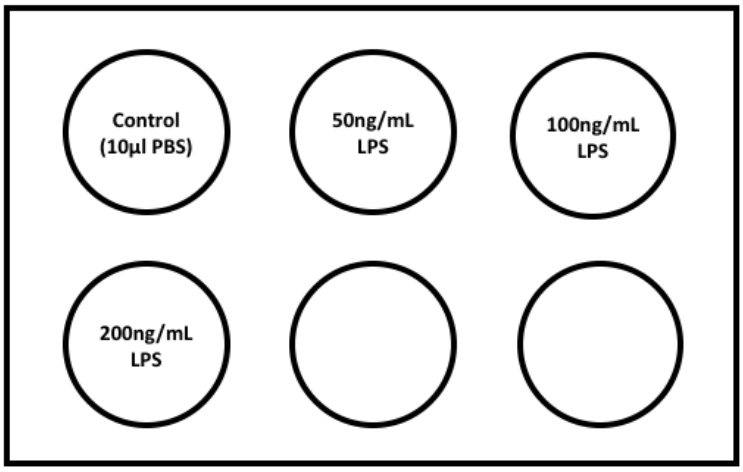


**Figure 4.1 Set up of Thp-1 cells with LPS at different concentrations in a 6 well plate for 6, 12, and 24 hours.**

### 4.2.2 Thp-1 cells stimulated with LPS for 12 hours only

After obtaining results from Thp-1 cells seeded with different concentrations of LPS for 6, 12, and 24 hours (RNA concentration and 260/280 ratio shown in appendix Table A1, A2 and A3 and TNF-α PCR products shown below in Figure 4.5), a 12 hour time frame was selected. This was based on Figure 4.5, where bands are present for most of the 12 hour samples. Thp-1 cells were seeded at 3 x $10^5$ cells (in approximately 2 mLs) into each well of a 6 well plate. The plate was set up as shown in the diagram below; each plate had two control wells with Thp-1 cells and PBS, 2 wells with Thp-1 cells with 50ng/mL LPS, and 2 wells with Thp-1 cells with 100ng/mL LPS. This was incubated in a humidified 5% $CO_2$ incubator at 37°C (standard incubator conditions for cell growth) for 12 hours, followed by RNA extraction.

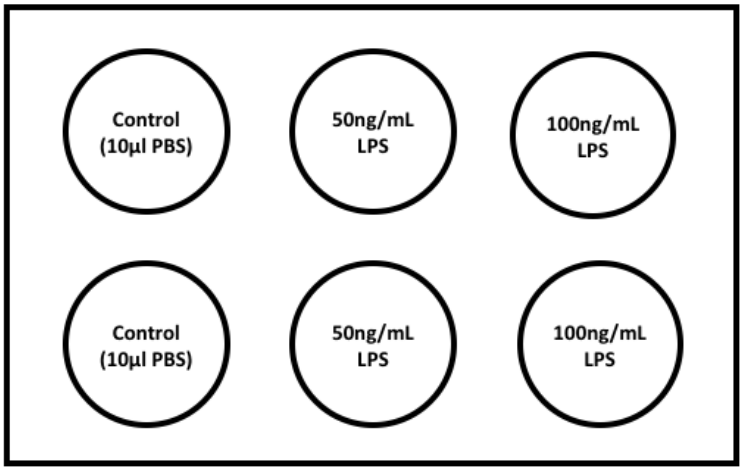


**Figure 4.2 Set up of Thp-1 cells with LPS at different concentrations in a 6 well plate for 12 hours.**

### 4.2.3 RNA extraction

The TRIzol method was carried out for RNA extraction as described in Chapter 2.3.

### 4.2.4 cDNA synthesis and dilution

cDNA synthesis was done using the qScript XLT cDNA SuperMix following manufacturer's directions, as described in Chapter 2.5. This was initially used in the PCR reactions without dilution. This resulted in no PCR product bands on the 2% agarose gels. Therefore, the cDNA was diluted 1:5 and 1:10 with DEPC water, which resulted in clear PCR product bands after PCR.

### 4.2.5 PCR optimization

#### 4.2.5.1 PCRs for GAPDH and TNF-α (Made up master mix)

PCR was performed specifically for GAPDH and TNF-α. Each reaction mix consisted of 36.6uL DEPC water, 5uL 10x PCR buffer containing 25mM $MgCl_2$ (Roche), 5uL sample cDNA, 1uL 10mM dNTP mixture (Roche), 2uL of each forward and reverse primers at 10mM, and 0.4uL Taq polymerase (Roche).

The PTC-200 Peltier Thermal Cycler was used to carry out the PCR reactions as shown below (Table 4.1). These 25-30 cycles were run for each PCR reaction

**Table 4.1 PCR cycle conditions using the made up master mix**

| Operation | Temperature | Time |
|---|---|---|
| Initial activation | 95°C | 4 min |
| Denaturation | 95°C | 1 min |
| Annealing | GAPDH: 63°C<br>TNF-α: 60°C | 1 min |
| Elongation | 72°C | 1 min |
| Final Extension | 72°C | 10 min |

**Table 4.2 Primer sequences, product sizes and annealing temperatures**

| Gene | Primer Sequence | Product size (bp) | Annealing temp °C |
|---|---|---|---|
| GAPDH forward | ACC ACA GTC CAT GCC ATC AC | 452 | 64 |
| GAPDH reverse | TCC ACC ACC CTG TTG CTG TA | | |
| TNF-α forward | CAG AGG GAA GAG TTC CCC AG | 325 | 60 |
| TNF-α reverse | CCT TGG TCT GGT AGG AGA CG | | |

#### 4.2.5.2 PCRs for GAPDH and TNF-α using HOT FIREPol® Blend Master Mix

HOT FIREPol® Blend Master Mix was used for these PCR reactions. This mix was used according to manufacturer's directions; with 15 minutes of initial activation time at 95°C.

**Table 4.3 PCR reaction components.**

| Component | Volume |
|---|---|
| HOT FirePol Blend Master Mix Ready to Load | 4uL |
| 10uM forward and reverse primer | 0.5ul |
| DNA template | 1uL |
| DEPC water | Up to 20uL |

**Table 4.4 PCR cycle conditions using the HOT FIREPol® Blend Master Mix.**

| Operation | Temperature | Time |
|---|---|---|
| Initial Activation | 95°C | 15 min |
| Denaturation | 95°C | 20 s |
| Annealing | GAPDH: 63°C<br>TNF-α: 60°C | 30s |
| Elongation | 72°C | 1 min |
| Final extension | 72°C | 10 min |

#### 4.2.5.3 Semi-quantitative PCR

##### *4.2.5.3.1 Cycle number and time alterations*

In order to be able to quantify the PCR products through semi-quantitative analysis, PCR needed to be carried out and stopped in the middle of the exponential growth phase (Marone et al., 2001). Once it reaches the plateau stage, it indicates that all the cDNA has been converted to the PCR product and the bands appear to be thick and bright for all of the samples, not allowing to determine the quantity based on band intensity, as they have reached saturation.

#### *4.2.5.3.2 Analysis of results*

2% agarose gels were visualised on the iBright Imaging system (Thermo Fisher Scientific). Using the analysis tool, the bands were auto-selected based on the colour difference between the background and the bands. A report was printed with the band intensity details (as shown in appendix, Table A4, A5 and A6).

The method used to analyse was described in a paper by (Mitchell and Iadarola, 2010). First, the averages were worked out between the same samples in one experiment (for example, the two controls in experiment 1A, etc). Next the averages were worked out between all the experiments (for example, the average from the control averages from experiment 1A, 1B and 1C). From here, the fold difference was calculated by dividing the sample (50ng/mL and 100ng/mL of LPS) average by the control average. The standard deviation and standard error was calculated.

### 4.2.6 Statistical analysis

ANOVA and the Student's T-test was carried out on Microsoft Excel to determine the significance of the results. For the T-test a two tailed distribution was used and it was performed using 2 samples with unequal variants.

## 4.3 Results

### 4.3.1 RNA quality and quantity of Thp-1 cells exposed to LPS for 12 hours

RNA quality and quantity was measured on the Nanodrop2000 (Thermo Fisher). The concentrations and 260/280 ratios are shown in the three tables below as the experiment was repeated three times. The concentrations of the samples are measures based on their absorbance potential (Thermo Fisher Scientific, 2016). The tables shown are for the 12 hour time point as described in methods (Chapter 4.2.2), as this was selected for further analysis. The RNA quantity and quality details for the 6, 12 and 24 hour time points as described in Chapter 4.2.1 are in the

appendix (Table A1, A2 and A3. As seen in the Tables 4.5, 4.6 and 4.7, the RNA concentrations were high enough to work with, and 260/280 ratio were all between 1.80-1.91. These samples were all considered sufficient and were used to create cDNA.

**Table 4.5 RNA concentrations and 260/280 ratios of Thp-1 cells induced with LPS for 12 hours (Experiment 1A).**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control A | 325.3 | 1.84 |
| Control B | 234.9 | 1.88 |
| 50ng/mL A | 123.3 | 1.82 |
| 50ng/mL B | 153.1 | 1.80 |
| 100ng/mL A | 150.2 | 1.90 |
| 100 ng/mL B | 63.1 | 1.86 |

**Table 4.6 RNA concentrations and 260/280 ratios of Thp-1 cells induced with LPS for 12 hours (Experiment 1B).**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control A | 319.5 | 1.86 |
| Control B | 224.1 | 1.82 |
| 50ng/mL A | 110.4 | 1.83 |
| 50ng/mL B | 112.0 | 1.83 |
| 100ng/mL A | 173.7 | 1.91 |
| 100 ng/mL B | 353.5 | 1.90 |

**Table 4.7 RNA concentrations and 260/280 ratios of Thp-1 cells induced with LPS for 12 hours (Experiment 1C).**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control A | 55.6 | 1.89 |
| Control B | 92.0 | 1.88 |
| 50ng/mL A | 114.6 | 1.81 |
| 50ng/mL B | 346.0 | 1.81 |
| 100ng/mL A | 69.1 | 1.80 |
| 100 ng/mL B | 168.1 | 1.83 |

### 4.3.2 RNA integrity

RNA integrity has been described in Chapter 3.3.4. 2% Agarose gels were used to visualise the two RNA bands (28S and 18S) of the RNA extracted from Thp-1 cells following the LPS treatment for 12 hours. These can be seen in Figure 4.3 below. The top band is 28S and the bottom 18S. The top band, 28S appears thicker than the bottom band, 18S, due to its larger size. The bands shown appear sharp, which indicates that the RNA is still in folded into its tertiary structure.

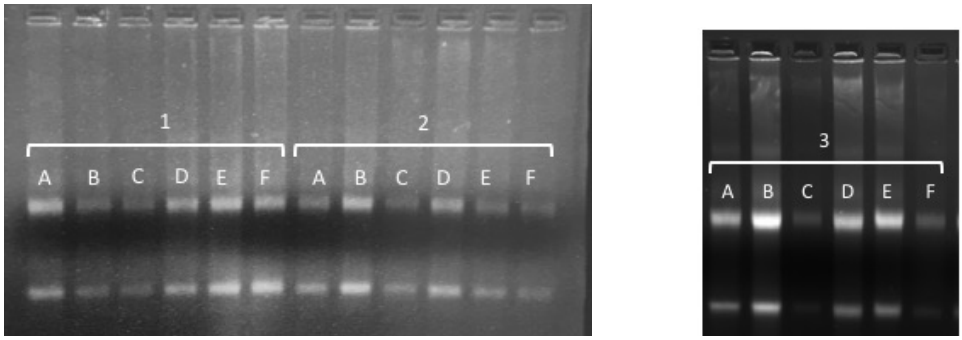


**Figure 4.3 RNA from LPS induced cells for 12 hours run on a 2% agarose gel for 1 hour. A and B: Control with LPS, C and D: 50ng/mL LPS, E and F: 100ng/mL LPS.**

### 4.3.3 Different amounts of cDNA and changes in annealing temperature

When the cDNA was used without diluting it down and directly after it had been synthesised, no bands were seen on the gel. However, with a 1:5 and 1:10 dilution with DEPC water, clear bands are seen on the gel (Figure 4.4). The samples with a 1:5 dilution of cDNA show thicker bands as excepted (since the cDNA was added at a higher concentration that 1:10 dilution).

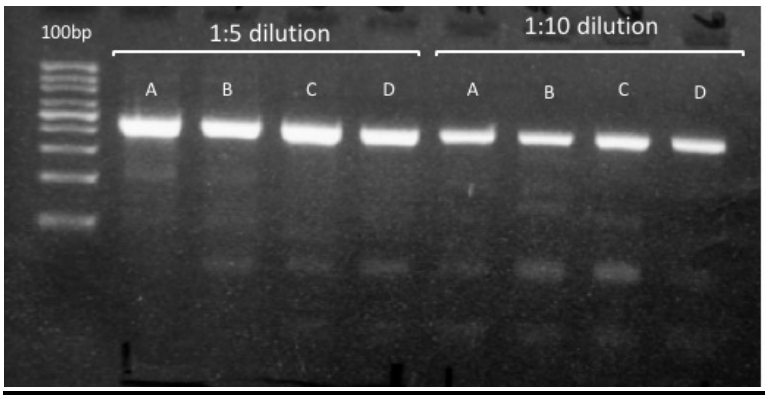


**Figure 4.5 PCR for GAPDH using different concentrations of cDNA from Thp-1 cells induced with LPS for 12 hours run on a 2% agarose gel for 1 hour. A: Control with PBS, B: 50ng/mL LPS, C: 100ng/mL LPS, D: 200ng/mL LPS.**

### 4.3.4 Different concentrations and time periods of LPS

The 12 hour time period showed thick bands (Figure 4.5) for all three of the concentrations, as did the 24 hour time point. Based on these results, a 12 hour time point was taken for the rest of the experiments, as well as the 50ng/mL and 100ng/mL concentrations for LPS.

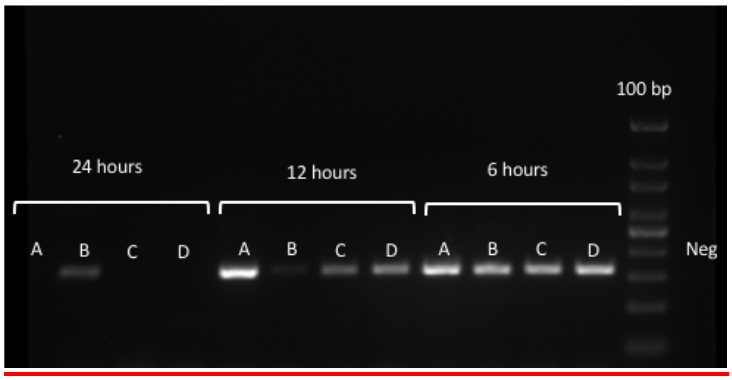


**Figure 4.6 2% agarose gel run for 1 hour showing PCR products of TNF-α expression From left, A: Control with PBS, B: 50ng/mL, C: 100ng/mL, D: 200ng/mL at 24 hours, 12 hours and 6 hours.**

### 4.3.5 Semi-quantitative optimisation

#### 4.3.5.1 GAPDH

At 36 and 30 PCR cycles the GAPDH PCR product bands appeared to be the same thickness (figures not shown). At 25 cycles, only a very slight to no change can be seen in the thickness of the bands (Figure 4.6). This

shows that the final number of amplicons are approximately the same, which corresponds to the amount of cDNA that was added to the PCR mix. Therefore, PCR was done at 18 cycles, as seen in Figure 4.7. Here the bands appear very weak and differences can be seen in them.

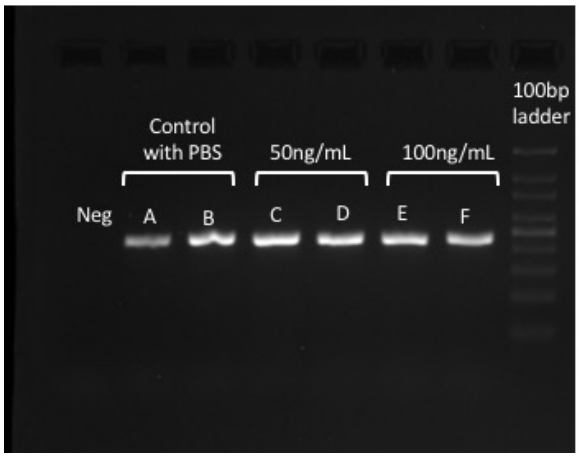


**Figure 4.7 2% agarose gel run for 1 hour showing PCR products of GAPDH at 25 cycles. From left Neg: Negative control, A and B: control with PBS, C and D: 50ng/mL LPS, E and F: 2 100ng/mL LPS.**

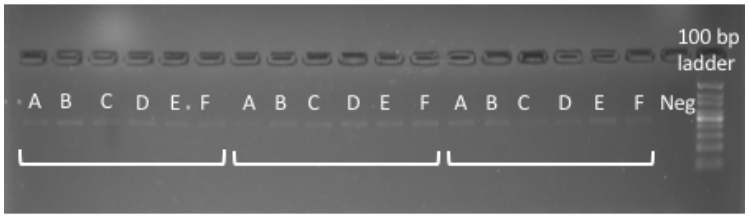


**Figure 4.8 2% agarose gel run for 1 hour showing PCR products of GAPDH at 18 cycles, with Thp-1 cells induced with LPS for 12 hours. A and B: Controls, C and DL 50 ng/mL LPS, E and F: 100 ng/mL, Neg: Negative control.**

#### 4.3.5.2 TNF-α

At 36 cycles there are no differences in the intensity of the bands (Figure 4.8). At 30 cycles, there are slight differences in intensity of the bands (Figure 4.9). When bought down to 25 cycles, no amplicons show up in the gel (Figure 4.10). 30 cycles was picked as an ideal cycle number to run the samples at for semi-quantitative analysis.

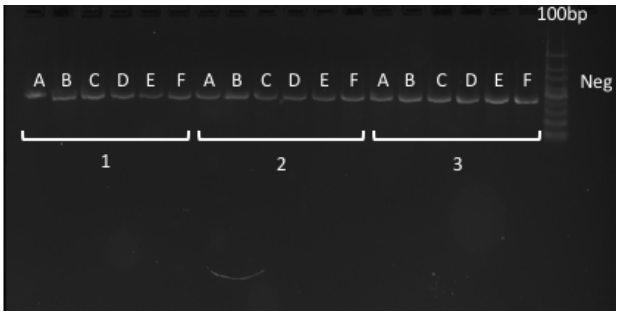


**Figure 4.9 2% agarose gel run for 1 hour showing PCR products of TNF-α at 36 cycles with Thp-1 cells incubated with LPS for 12 hours. A and B: Control with PBS, C and D: 50ng/mL LPS, E and F: 100 ng/mL LPS. Neg: Negative control. Experiment repeated 3 times.**

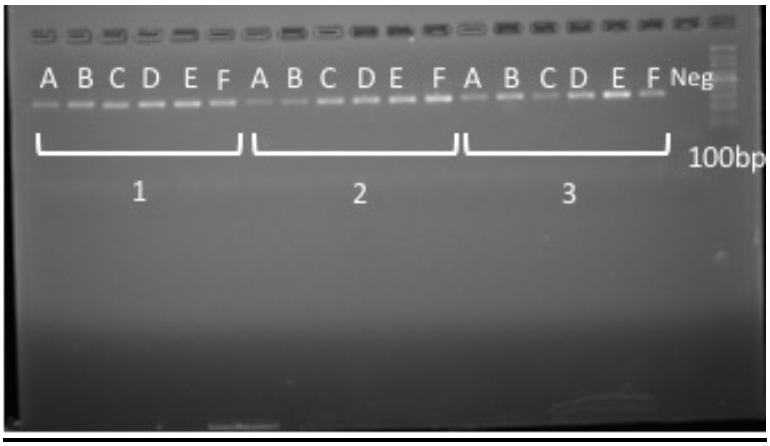


**Figure 4.10 2% agarose gel run for 1 hour showing PCR products of TNF-α at 30 cycles with Thp-1 cells incubated with LPS for 12 hours. A and B: Control with PBS, C and D: 50ng/mL LPS, E and F: 100 ng/mL LPS. Neg: Negative control. Experiment repeated 3 times.**

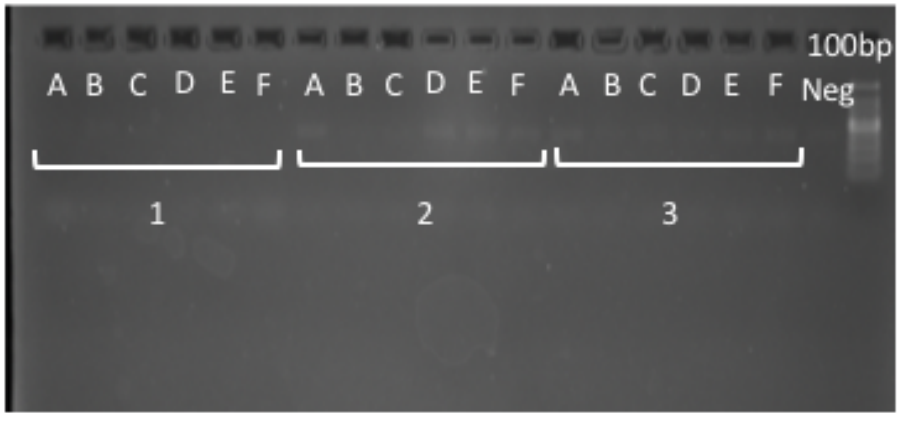


**Figure 4.11 2% agarose gel run for 1 hour showing PCR products of TNF-α at 25 cycles, with Thp-1 cells induced with LPS for 12 hours. A and B: Controls, C and D: 50 ng/mL LPS, E and F: 100 ng/mL, Neg: Negative control.**

#### 4.3.5.3 Semi-quantitative Analysis

TNF-α at 30 cycles and GAPDH at 25 cycles were selected as the final cycle numbers for the PCR reactions for semi-quantitative analysis. This is because at this number it was clear that the bands were not saturated, and differed slightly based on the expression levels of the gene. This allowed for the quantification of the expression of TNF-α relative to the housekeeping gene, GAPDH.

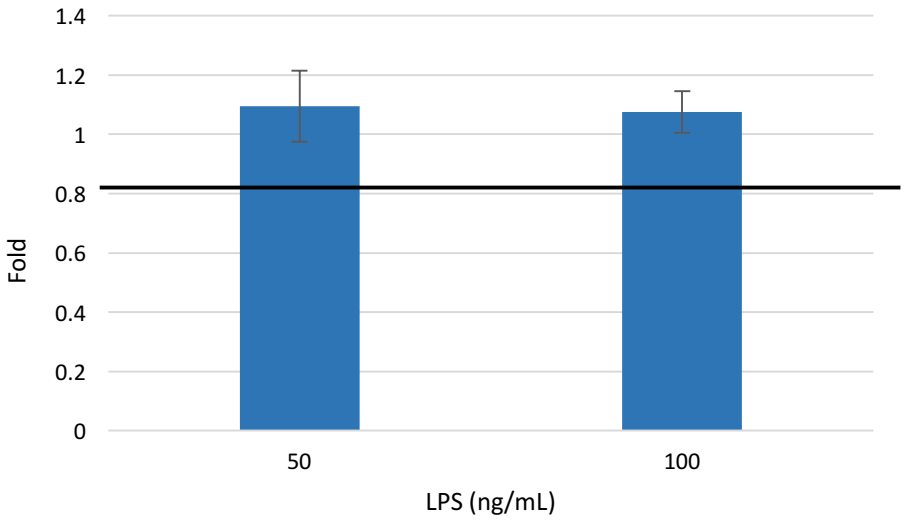


**Figure 4.12 Fold change of TNF-α expression of Thp-1 cells exposed to LPS for 12 hours. Black line represents control sample cells (N=3).**

The graph above shows that there was a very little fold change of less than 0.5 (Figure 4.11). This is statistically insignificant ($p>0.05$).

## 4.4 Discussion

There were two objectives of this portion of the study; to determine an appropriate time frame and concentration for Thp-1 cells to be incubated with LPS in order to generate a TNF-α response, and to optimise the number of cycles in PCR in order for the reactions to be stopped in the exponential phase to do semi-quantitative analysis.

Initially in the cDNA synthesis process, 1uL of cDNA that was synthesised from the qScript mix was added to the PCR reaction. No bands were seen on the 2% agarose gels when the PCR products were run down. One reason for this could be that an excess of cDNA from the original cDNA mix created may have a very high concentration. This means that when it's added to the PCR reaction, it may bind all the primers, not allowing PCR to take place correctly (Biosynthesis, 2015). When the cDNA was diluted down 1:5 and 1:10 (as seen in Figure 4.4), bands were seen in the gel.

Initially a PCR mix was created using reagents stored in a -20°C freezer in the PC1 lab. After several PCR unsuccessful attempts using these reagents, the Hot Fire Pol mix was used. The initial reagents used were dated from 2005, and may have become inactive during this time as they were used by many students and would have gone through many thawing and freezing cycles.

As seen in Figure 4.5, the 24 hour time period was too long and no TNF-α induction was seen on the agarose gel. This may be because TNF-α had returned to its basal levels at the end of 24 hours, as seen in the research conducted by Schilberger et al. At 12 hours all bands were seen except for the 50ng/mL sample. At 6 hours all the bands were seen. In order to be able to incubate and extract RNA within working hours, the 12 hour time point was selected and concentrations of 50ng/mL and 100ng/mL were selected.

The expression of TNF-α was compared with the housekeeping gene GAPDH, or glyceraldehyde-3-phosphate dehydrogenase. GAPDH is widely used for comparing gene expression data. It is a gene coding an enzyme that catalyses the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the process of glycolysis. As different tissues in the body will have different requirements, GAPDH expression varies considerably between them (Barber et al., 2005).

On the other hand, TNF-α expression increases when it has been induced due to cellular stress. Cytokines such as TNF-α, are glycoproteins secreted mainly by immunological cells (T-cells, macrophages, neutrophils) in response to disease or tissue damage (Leung and Cahill, 2010). Without a stressor, there is little to no expression of this gene at mRNA level (Vila-del Sol et al., 2008). In a study carried out by Szlosarek et al. in 2006, they found that TNF-α expression was detected in qPCR for normal epithelial cells, however was not seen when conventional PCR was done (Szlosarek et al., 2006). However, the control samples in these experiments (10uL of PBS added to Thp-1 cells only) do show an expression of TNF-α when conventional PCR was done. The cells may have been under stress prior to treating them with LPS, as there are bands visible in the control samples. Although the cells were kept in standard incubator conditions in a PC2 lab as required, any error in this may have resulted in the cells becoming slightly stressed, which may have triggered an inflammatory response.

Another reason a TNF-α response may have been seen in the control samples is because of mycoplasma contamination. Mycoplasma contamination is one of the most common contamination concerns in cell cultures. Mycoplasmas are a group of prokaryotic microorganisms that do not have a cell wall. They are also one of the smallest self-replicating organisms known, and cannot be detected using a microscope. Cell cultures in early passage have been found to have less contamination (1-5%) compared to cells with higher passage numbers (15-35%). Although an antibiotic (penstrep) was used, mycoplasmas are resistant to most antibiotic, including penstrep (Drexler and Uphoff, 2002). Mycoplasma are able affect cell culture in a myriad of ways. They can alter the expression of hundreds of genes, alter protein, RNA and DNA synthesis, alter cell metabolism, change cell membrane composition, and alter cell morphology. Mycoplasma contamination can be detected reliably through PCR (Drexler, 2002; Miller, 2003). The primers used are designed based on the highly conserved regions of genes that are present across all mycoplasma (Drexler and Uphoff, 2002).

TNF-α expression was very difficult to see at 25 cycles as shown in Figure 4.6, however GAPDH expression was seen in as little as 18 cycles (Figure 4.7). These results show that the basal level of expression of GAPDH is higher than the expression of TNF-α. This may be due to the fact that GAPDH is always expressed as it is required for glycolysis, a process that allows the breakdown of glucose for ATP (Tristan et al., 2011), however TNF-α generally requires a cell stressor.

In an ideal situation, given more time and resources, it has been suggested that more than one housekeeping gene should have been used for a more accurate quantification of TNF-α expression. Although it is inherently believed that the housekeeping gene's expression remains constant in the samples being used, the expression may change depending on the circumstances or treatments Barber et al., 2005; Silver et al., 2006). In a study carried out by Baber et al (2005) it was found that GAPDH expression varied significantly between different tissue, but was very similar in the same tissue. As explained above, GAPDH plays a role in glycolysis. The expression of GAPDH would vary depending on a tissue's needs. For example, a higher GAPDH expression level was seen in skeletal muscle and many brain regions as they require more energy (Barber et al., 2005). Since comparisons between TNF-α and GAPDH were made using the same cell line and samples, this would be considered a viable result.

The number of cells used was decided on the basis of getting a good RNA concentration. However, this may have had an impact on the sensitivity to LPS. If the cell concentration was too high and more LPS was needed to trigger a reaction, no significant difference would have been seen.

The amount of TNF-α released from Thp-1 cells induced with LPS was done using semi-quantitative PCR analysis, which looks at the intensity of the PCR product bands when they are run out on a gel, and compared to a housekeeping gene. Incubating Thp-1 cells with LPS for 12 hours indicated that there was no significant increase in TNF-α production. In a study carried out by Schildberger et al, a peak of TNF-α was observed at 4 hours when

10ng/mL of LPS was used on 106 cells, tapering off until around 24 hours (Schildberger et al., 2013). With concentrations at 50ng/mL and 100ng/mL and a time period of 12 hours, it is highly possible that the peak was reached not only quicker, but that over the 12 hours will also have decayed and would be difficult to determine from a PCR reaction. This relates to the fact that TNF-α mRNA is very short lived, and has a shorter stability as it has a very short poly(A) tail (Mijatovic et al., 2000). This is discussed further in Chapter 6.4. Therefore, after looking into this paper, 10ng/mL and 50ng/mL of LPS was used for a time period of 4 hours before extracting RNA.

Another issue that was faced was optimising the PCR cycles for GAPDH in order to stop the amplification in the exponential stage. In Figure 4.6, the bands appear to almost be saturated. The number of cycles was changed to 18, as seen in Figure 4.7. However, the machine was unable to pick up the bands in this picture, and GAPDH at 25 cycles was used. This may have caused the results to change as the change could have been more accurately determined using bands that appeared less saturated. Therefore, it was proposed that Real Time PCR / qPCR, should be done instead, as seen in the following chapter.

# 5 Thp-1 cells induced with LPS to determine TNF-α expression through qPCR.

## 5.1 Introduction

Based on the previous experiment, it was found that there was no significant TNF-α expression when semi-quantitative analysis was carried out on Thp-1 cells exposed to LPS for 12 hours (as seen in Figure 4.5). It was noted that there may have been systematic error introduced through the frequent handling of nucleic acids. The machine was also not able to pick up the lower levels of PCR product bands on the agarose gels, which made it difficult to analyse. Therefore, qPCR was suggested as another method to determine relative gene expression.

qPCR is a common and modern alternative to semi-quantitative PCR that measures DNA as it is being synthesised over time. It does this by using a fluorescent probe (such as SYBR green or SYTO82) that binds to double stranded DNA as soon as it is synthesised. The qPCR machine is able to measure the fluorescence and detect the amount of DNA synthesis in each reaction (Pfaffl, 2001). Threshold cycle, or Ct, is when the fluorescence in the reaction rises above background fluorescence. After the PCR is complete, the fluorescence of the end product is proportional to the amount of DNA synthesised.

Using the same samples used for semi-quantitative analysis as mentioned in Chapter 4.31 (Table 4.5, 4.6 and 4.7), qPCR was carried out and results were analysed using the delta-delta Ct method as well as the Pfaffl method. The differences in the methods were explained and the Pfaffl method was considered as the more accurate measurement.

## 5.2 Methods

The RNA used in this experiment was the same RNA extracted and described in Chapter 4. The details of its quality, quantity and integrity can be seen in Chapter 4.3.1 and 4.3.2. The same cDNA was used to set up the qPCR reactions, as explained below.

### 5.2.1 qPCR

#### 5.2.1.1 Reaction set up

qPCR was carried out on a MIC qPCR instrument (BioMolecular systems, Australia). Each reaction contained 2.5 $MgCl_2$, 200uM dNTPs, 1uM forward and reverse primer, HotFirePol Taq polymerase, 2uM SYTO82 (Life Technologies), and 1uL of diluted cDNA. Each reaction had a final volume of 10uL. The thermocycling conditions are described in the table below. The PCR reactions were run for 40 cycles.

**Table 5.1 qPCR cycle conditions**

| Gene | Initial denaturation | Denaturation | Annealing | Extension | Fluorescence acquisition |
|---|---|---|---|---|---|
| GAPDH | 95°C for 15 minutes | 94°C for 12 seconds | 60°C for 10 seconds | 72°C for 18 seconds | 80°C for 10 seconds |
| TNF-α | 95°C for 15 minutes | 94°C for 12 seconds | 60°C for 10 seconds | 72°C for 18 seconds | 80°C for 10 seconds |

#### 5.2.1.2 Analysis

Analysis of qPCR was done using the delta-delta Ct method and the Pfaffl method. These two methods allow for a calculation of relative gene expression between samples. The main difference between the two methods is that the delta-delta Ct method does not account for any differences in the efficiency of cycles, whereas the Pfaffl method takes in these differences (Pfaffl, 2001). Both methods were used for analysis.

#### 5.2.1.2.1 Delta-delta Ct method

The delta-delta Ct method presumes that the real time amplification efficiency values of the reference and target gene are identical and at their optimum value (E=2). However, in reality the efficiencies vary slightly and can be recorded by the qPCR systems. This method therefore is only applicable for making quick estimations of the expression ratios. The equations used are stated below, where Ct is threshold cycle (which is when the fluorescence picked up is above the background fluorescence) (Pfaffl, 2001). GOI: gene of interest, HKG: housekeeping gene (GAPDH),

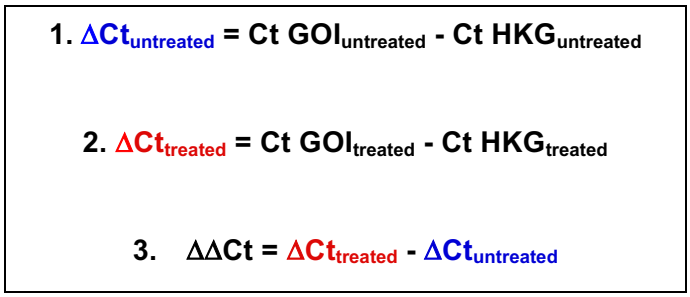


**Figure 5.1 Equations used for the delta-delta Ct method to determine gene expression ratios between samples. Ct: Threshold cycle, GOI: gene of interest, HKG: housekeeping gene (GAPDH).**

#### 5.2.1.2.2 Pfaffl method

The Pfaffl method is more reliable as it takes in the real time amplification efficiency values of the reference and target gene for each sample (Pfaffl, 2001). The equations used to determine relative gene expression are shown below.

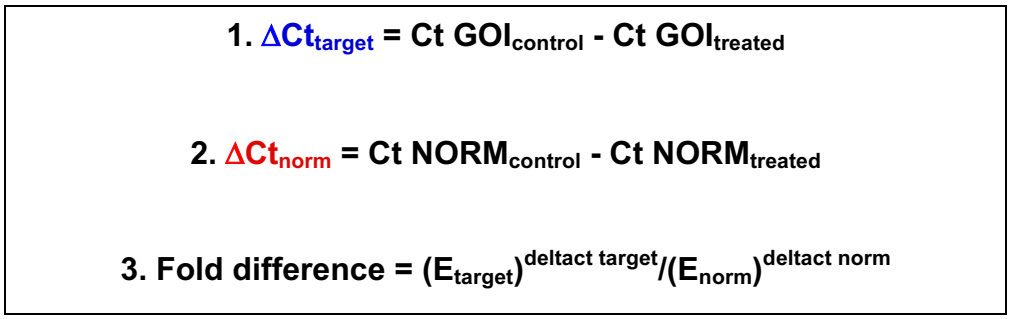


**Figure 5.2 Equations used for the Pfaffl method to determine gene expression ratios between samples. GOI: gene of interest, NORM: Housekeeping gene, GAPDH, Ct: Threshold cycle.**

### 5.2.2 T Test for statistical analysis

ANOVA and the Student's T-test was carried out on Microsoft Excel to determine the significance of the results. For the T-test a two tailed distribution was used and it was performed using 2 samples with unequal variants.

## 5.3 Results

### 5.3.1 Delta-delta Ct Method

The equations used to determine Δctuntreated, Δcttreated ΔΔCt and fold difference are shown in Figure 5.1 The tables below (Table 5.2, 5.3, 5.4, and 5.5) show these results based on the threshold cycle values obtained from the qPCR instrument.

**Table 5.2 Analysis of first experiment with Thp-1 cells incubated with LPS at 12 hours using the Delta-delta Ct method.**

| Tube | Ct average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF-α C | 26.67 | 7.85 | | | |
| TNF-α 50 | 25.79 | | 6.43 | -1.42 | 2.68 |
| TNF-α 100 | 25.88 | | 6.41 | -1.44 | 2.72 |
| GAPDH 0 | 18.82 | | | | |
| GAPDH 50 | 19.35 | | | | |
| GAPDH 100 | 19.47 | | | | |

**Table 5.3 Analysis of second experiment with Thp-1 cells incubated with LPS at 12 hours using the Delta-delta Ct method.**

| Tube | Ct average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF-α C | 27.61 | 7.50 | | | |
| TNF-α 50 | 25.48 | | 6.49 | -1.01 | 2.02 |
| TNF-α 100 | 25.18 | | 6.26 | -1.24 | 2.36 |
| GAPDH 0 | 20.11 | | | | |
| GAPDH 50 | 18.99 | | | | |
| GAPDH 100 | 18.91 | | | | |

**Table 5.4 Analysis of third experiment with Thp-1 cells exposed to LPS for 12 hours using the Delta-delta Ct method.**

| Tube | Ct average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF-α C | 26.29 | 7.40 | | | |
| TNF-α 50 | 26.46 | | 6.22 | -1.18 | 2.26 |
| TNF-α 100 | 25.46 | | 6.11 | -1.28 | 2.43 |
| GAPDH 0 | 18.89 | | | | |
| GAPDH 50 | 20.24 | | | | |
| GAPDH 100 | 19.34 | | | | |

**Table 5.5 Mean fold difference of TNF-α expression relative to HKG GAPDH in Thp-1 cells exposed to LPS for 12 hours using the Delta-delta Ct method.**

| LPS (ng/mL) | Fold d. in Ex. 1 | Fold d. in Ex 2. | Fold d. in Ex 3 | Mean | SD | SE |
|---|---|---|---|---|---|---|
| 50 | 2.68 | 2.02 | 2.26 | 2.32 | 0.33 | 0.19 |
| 100 | 2.72 | 2.36 | 2.43 | 2.50 | 0.19 | 0.11 |

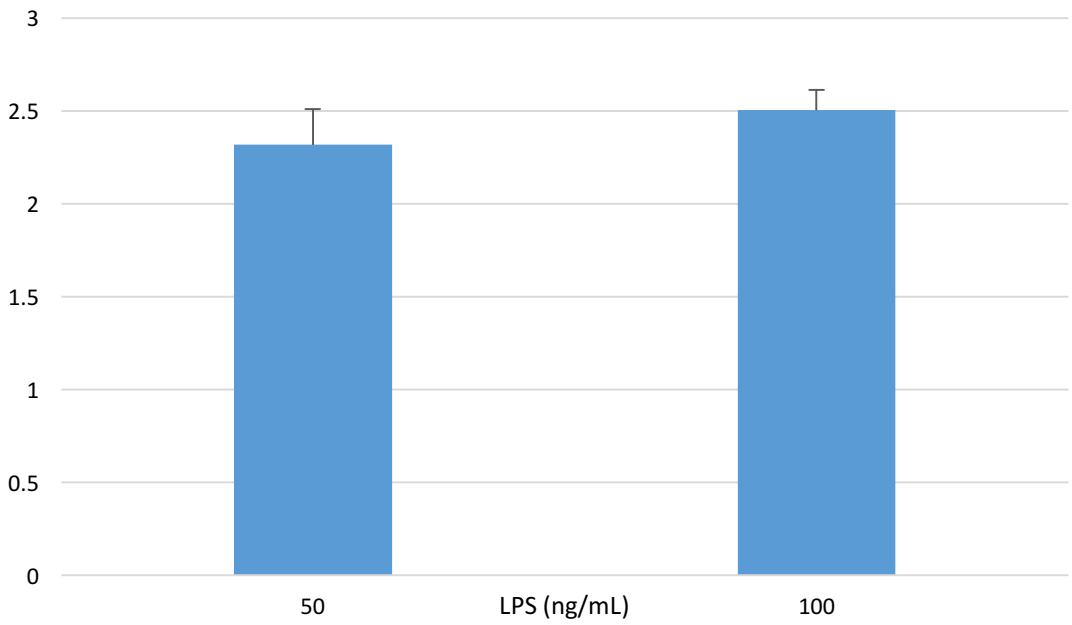


**Figure 5.3 Fold change of TNF-α expression of Thp-1 cells exposed to LPS for 12 hours using the delta-delta method for qPCR analysis (N=3).**

Using the delta-delta method for analysis, a 2.32 fold increase is seen in the expression of TNF-α when cells are exposed to 50ng/mL of LPS for 12

hours. There is only a small and insignificant (p>0.05) increase of 2.50 fold compared to the 2.32 fold increase when the concentration of LPS is increased from 50ng/mL to 100ng/mL. ANOVA and the Student's T test were carried out and results were found to be insignificant (p >0.05).

### 5.3.2 Pfaffl Method

The equations used to determine Δcttarget Δctnorm and fold difference are shown in Figure 5.2. The tables below (Table 5.6, 5.7, 5.8 and 5.9) show these results based on the threshold cycle values obtained from the qPCR instrument.

**Table 5.6 Analysis of first experiment with Thp-1 cells incubated with LPS at 12 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF-α C | 26.67 | 1.84 | | | |
| TNF-α 50 | 25.79 | 1.84 | 0.89 | | 2.39 |
| TNF-α 100 | 25.88 | 1.86 | 0.79 | | 2.46 |
| GAPDH C | 18.82 | 1.87 | | | |
| GAPDH 50 | 19.35 | 1.87 | | -0.53 | |
| GAPDH 100 | 19.47 | 1.88 | | -0.65 | |

**Table 5.7 Analysis of second experiment with Thp-1 cells incubated with LPS at 12 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF-α C | 27.61 | 1.81 | | | |
| TNF-α 50 | 25.48 | 1.85 | 2.13 | | 1.85 |
| TNF-α 100 | 25.18 | 1.85 | 2.44 | | 2.10 |
| GAPDH C | 20.11 | 1.88 | | | |
| GAPDH 50 | 18.99 | 1.85 | | 1.12 | |
| GAPDH 100 | 18.91 | 1.87 | | 1.20 | |

**Table 5.8 Analysis of third experiment with Thp-1 cells incubated with LPS at 12 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF-α C | 26.29 | 1.84 | | | |
| TNF-α 50 | 26.46 | 1.85 | -0.16 | | 2.04 |
| TNF-α 100 | 25.46 | 1.86 | 0.84 | | 2.21 |
| GAPDH C | 18.89 | 1.87 | | | |
| GAPDH 50 | 20.24 | 1.84 | | -1.34 | |
| GAPDH 100 | 19.34 | 1.84 | | -0.45 | |

**Table 5.9 Mean fold difference of TNF-α expression relative to HKG GAPDH in Thp-1 cells exposed to LPS for 12 hours using the Pfaffl method.**

| Sample | Fold d. in Ex. 1 | Fold d. in Ex. 2 | Fold d. in Ex. 3 | mean | sd | se |
|---|---|---|---|---|---|---|
| 50 | 2.39 | 1.85 | 2.04 | 2.10 | 0.28 | 0.16 |
| 100 | 2.46 | 2.10 | 2.21 | 2.26 | 0.19 | 0.11 |

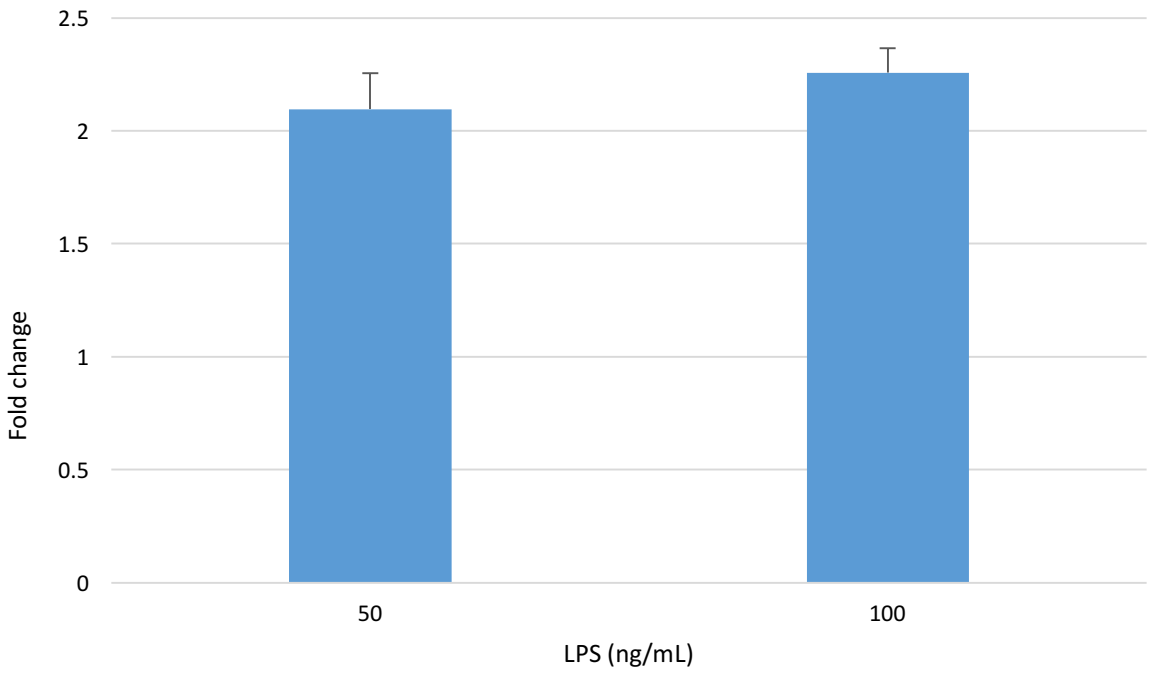


**Figure 5.4 Fold change of TNF-α expression of Thp-1 cells exposed to LPS for 12 hours using the Pfaffl method for qPCR analysis (N=3).**

The overall fold change is slightly lower when using the Pfaffl method for analysis. A 2.10 fold increase is seen in Thp-1 cells exposed to 50ng/mL of

LPS, and a 2.26 increase is seen is Thp-1 cells exposed to 100ng/mL LPS. As seen in the delta-delta Ct method, the fold difference between cells exposed to 50ng/mL and 100ng/mL LPS is insignificant, as determined by ANOVA and the Student's T-test ($p>0.05$).

## 5.4 Discussion

The objective of this portion of the study was to more accurately determine the expression of TNF-α when Thp-1 cells were incubated with LPS for 12 hours. This was done through qPCR, a technique that allows the measure of gene expression by calculating the amount of double stranded DNA being synthesised over time. This was originally done using semi-quantitative analysis, however the results obtained did not seem accurately determined due to human error as well as the machine not being able to pick up lighter PCR product bands on the agarose gels.

The results obtained from both experiments were very different. In semi quantitative analysis, both the 50ng/mL and 100ng/mL LPS samples had a fold change of ~1.1. However, using qPCR analysis methods, the fold change is over 2 using both methods of analysis. Although these results are insignificant ($p>0.05$), overall the qPCR change seems more accurate as it minimises human error by being able to record threshold cycles and amplification as it occurs on a machine, while in semi-quantitative, there is a lot more handling of the RNA and pipetting errors that could occur, which would affect results.

The Ct average for TNF-α was 26.9, and the Ct for GAPDH was 19.34. A smaller Ct number indicates that there is a high level of mRNA transcripts of that gene, as it is rises above background fluorescence earlier, showing that more product has been created with fewer cycles of PCR. As expected, GAPDH has a lower Ct than TNF-α, as GAPDH is expressed as a result of the process of glycolysis, whereas TNF-α is a response to cellular stress.

The results from the Delta-delta Ct method indicate a .2 higher fold change than the Pfaffl method. This is because as stated earlier, the Delta-delta Ct

method does not take in the efficiency of each sample. Instead it presumes the value of 2 (holding the theoretical view that each time DNA goes through a cycle, it doubles). In reality the cycle efficiencies vary, as seen in (appendix). Accounting for this in the equation makes it more accurate. Using the Pfaffl method it is seen that for the 50ng/mL sample a 2.32 fold increase was seen, and for the 100ng/mL samples a 2.50 fold increase was seen.

Using PCR gives an indication of TNF-α expression at the mRNA level. It is important to note that although an increase is seen in the mRNA level, it may not indicate an increase in TNF-α at the protein level. A ~2 fold increase may not be enough to lead to protein translation, and may not lead to the production of TNF-α protein, thus no inflammatory response would be seen. In order to test for TNF-α at the protein level, techniques such as western blotting should be carried out.

In a study carried out by Schildberger et al, a peak of TNF-α was observed at protein level at 4 hours when 10ng/mL of LPS was used on $10^6$ cells, tapering off until around 24 hours (Schildberger et al., 2013). In this experiment, a very different cell number and concentration was used. With concentrations at 50ng/mL and 100ng/mL and a time period of 12 hours, it is highly possible that the peak was reached not only earlier (as the concentration of LPS is higher), but that over the 12 hours the expression of TNF-α would have slowed down or returned to basal levels. As the 2 fold increase seen is insignificant, after looking into this paper, a higher fold increase may be obtained using 10ng/mL of LPS with a time period of 4 hours. Therefore, a 10ng/mL and 50ng/mL of LPS was used for a time period of 4 hours before extracting RNA, to see if the TNF-α expression could be seen with a greater fold difference compared to GAPDH levels.

# 6 Thp-1 cells induced with LPS for 4 hours to determine TNF-α expression through qPCR

## 6.1 Introduction

Previous research shows that in a time course of Thp-1 cells incubated with 10ng/mL of LPS, a peak of TNF-α expression is seen at 4 hours (Schildberger et al., 2013). Therefore, in the previous experiment conducted, the time point of 12 hours may have been too long for a significant higher expression (over 2 fold) of TNF-α to be seen. It was also noted that semi-quantitative results were not able to be accurately determined. Therefore, qPCR was used for the rest of the project.

In this portion of the study, the objective was to expose Thp-1 cells to 10ng/mL and 50ng/mL of LPS for 4 hours only. A higher fold difference was expected to be seen between the control samples and LPS induced samples, in accordance with literature (Schildberger et al., 2013).

## 6.2 Methods

### 6.2.1 Thp-1 cells incubated with LPS for 4 hours

$3 \times 10^5$ Thp-1 cells were harvested from the stock and 2mLs into 6 wells on a 6 well plate. Each well had 2mLs of $3 \times 10^5$ cells. The plate was set up as shown in the diagram. The plate was placed in the incubator under standard incubator conditions for 4 hours, and then RNA was extracted as described below. This was repeated 3 times in order to do statistical analysis.

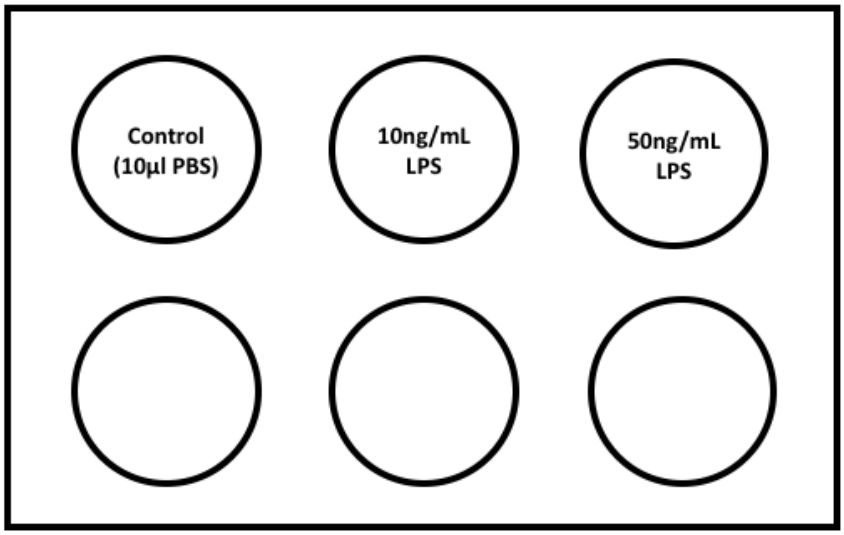


**Figure 6.1 Set up of 6-well plate for Thp-1 cells incubated with different concentrations of LPS for 4 hours**

### 6.2.2 RNA extraction, cDNA synthesis and qPCR

RNA extraction, nanodrop readings, cDNA synthesis and agarose gels were carried out as described in Chapters 2.3, 2.4, 2.5 and 2.6 respectively. qPCR was carried out as described in Chapter 5.2.1.1, and analysed as described in Chapter 5.2.1.2.

### 6.2.3 T Test for statistical analysis

ANOVA and a Student's T-test was carried out using the same method described in Chapter 4.2.6

## 6.3 Results

### 6.3.1 RNA

#### 6.3.1.1 RNA quantity and quality

**Table 6.1 RNA concentrations and 260/280 ratios of Thp-1 cells exposed to LPS for 4 hours.**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control | 264.9 | 1.91 |
| 10ng/mL | 187.2 | 1.89 |
| 50ng/mL | 371.6 | 1.96 |

**Table 6.2 Second repeat of RNA concentrations and 260/280 ratios of Thp-1 cells exposed to LPS for 4 hours.**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control | 584.4 | 1.95 |
| 10ng/mL | 378.5 | 1.95 |
| 50ng/mL | 215.5 | 1.94 |

**Table 6.3 Third repeat of RNA concentrations and 260/280 ratios of Thp-1 cells exposed to LPS for 4 hours.**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control | 215.5 | 1.98 |
| 10ng/mL | 156.1 | 1.98 |
| 50ng/mL | 277.5 | 1.93 |

The RNA extracted all had a concentration greater than180ng/mL, and this was enough to continue with cDNA synthesis. The 260/280 ratio were all between 1.89-1.98. As an ideal 260/280 ratio is considered to be ~2, these values were considered sufficient.

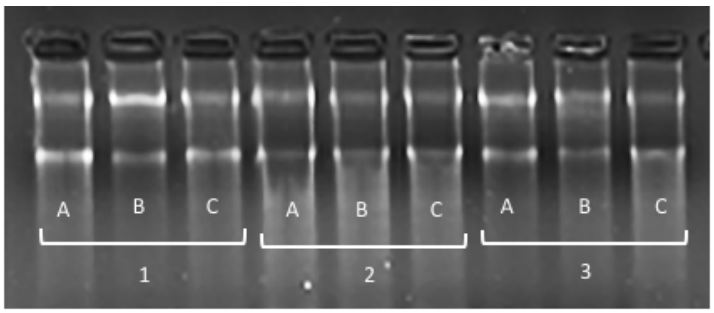


**Figure 6.2 RNA from Thp-1 cells induced with LPS for 4 hours. A: Control cells (with PBS), B: 10ng/mL LPS, C: 50ng/mL LPS. Experiment repeated 3 times.**

RNA from all three experiments was run on a 2% agarose gel with 1xTAE buffer for 1 hour, as seen in Figure 6.2. The two RNA bands (28S and 18S) are seen clearly on the gel for each sample. Ideally, the top 28S band should be twice as thick as the bottom 18S band. However, as the 260/280 ratios were ~2, and the two bands were clear on the gel, this RNA was used for the rest of this experiment.

## 6.3.2 qPCR analysis

### 6.3.2.1 Delta Delta Ct method

This is described in detail in Chapter 5.2.1.2.1

**Table 6.4 Analysis of first experiment with Thp-1 cells incubated with LPS at 4 hours using the Delta-delta Ct method.**

| Tube | Ct Average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF-α 0 | 33.13 | 1.73 | | | |
| TNF-α 10 | 31.18 | | 5.75 | 4.02 | 0.061556144 |
| TNF-α 50 | 31.11 | | 3.93 | 2.20 | 0.217686238 |
| GAPDH 0 | 31.40 | | | | |
| GAPDH 10 | 25.44 | | | | |
| GAPDH 50 | 27.18 | | | | |

**Table 6.5 Analysis of second repeat experiment with Thp-1 cells incubated with LPS at 4 hours using the Delta-delta Ct method.**

| Tube | Ct Average | ∆ctuntreated | ∆cttreated | ∆∆Ct | Fold difference |
|---|---|---|---|---|---|
| TNF-α 0 | 30.50 | 2.32 | | | |
| TNF-α 10 | 29.94 | | 2.14 | -0.17 | 1.127063644 |
| TNF-α 50 | 30.94 | | 1.51 | -0.81 | 1.748982974 |
| GAPDH 0 | 28.18 | | | | |
| GAPDH 10 | 27.80 | | | | |
| GAPDH 50 | 29.43 | | | | |

**Table 6.6 Analysis of third repeat experiment with Thp-1 cells incubated with LPS at 4 hours using the Delta-delta Ct method.**

| Tube | Ct Average | ∆ctuntreated | ∆cttreated | ∆∆Ct | Fold difference |
|---|---|---|---|---|---|
| TNF-α 0 | 31.25 | 5.52 | | | |
| TNF-α 10 | 30.81 | | 7.52 | 2.00 | 0.249709364 |
| TNF-α 50 | 31.53 | | 6.03 | 0.51 | 0.704011205 |
| GAPDH 0 | 25.73 | | | | |
| GAPDH 10 | 23.29 | | | | |
| GAPDH 50 | 25.50 | | | | |

**Table 6.7 Mean fold difference of TNF-α expression relative to HKG GAPDH in Thp-1 cells exposed to LPS for 4 hours using the delta-delta Ct method.**

| Sample | Fold Av ex 1 | Fold av ex 2 | Fold av. ex 3 | Mean | SD | SE |
|---|---|---|---|---|---|---|
| 10ng/mL LPS | 0.06 | 1.13 | 0.25 | 0.48 | 0.57 | 0.33 |
| 50ng/mL LPS | 0.22 | 1.75 | 0.70 | 0.89 | 0.78 | 0.45 |

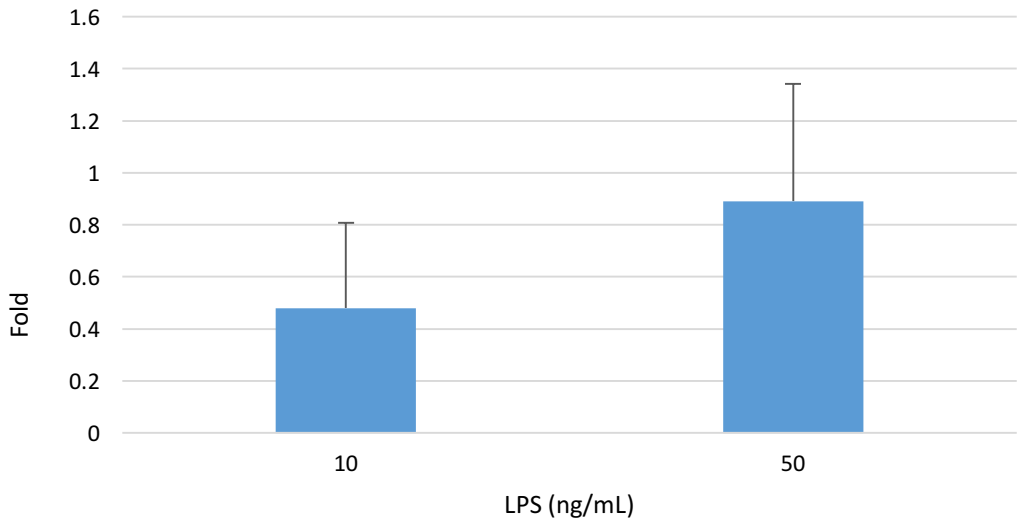


**Figure 6.3 Fold change of TNF-α expression of Thp-1 cells exposed to LPS for 4 hours using the delta-delta method for qPCR analysis (N=3)**

The delta-delta Ct method was used for analysis. When Thp-1 cells were exposed to 10ng/mL LPS for 4 hours, a fold increase of 1.13 was observed. When exposed to 50ng/mL, a 1.75 fold increase was observed. The difference between the samples was insignificant, as seen in a Student's T test ($p>0.05$).

### 6.3.2.2 Pfaffl method

This is explained in detail in Chapter 5.2.1.2.2

**Table 6.8 Analysis of first experiment with Thp-1 cells incubated with LPS at 4 hours using the Pfaffl method.**

| Tube | Ct Average | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF-α 0 | 33.13 | 1.73 | | | |
| TNF-α 10 | 31.18 | 1.81 | 1.95 | | 0.050741989 |
| TNF-α 50 | 31.11 | 1.80 | 2.02 | | 0.175965315 |
| GAPDH 0 | 31.40 | 2.00 | | | |
| GAPDH 10 | 25.44 | 2.00 | | 5.97 | |
| GAPDH 50 | 27.18 | 2.00 | | 4.22 | |

**Table 6.9 Analysis of second repeat experiment with Thp-1 cells incubated with LPS at 4 hours using the Pfaffl method**

| Tube | Ct Average | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF-α 0 | 30.50 | 1.89 | | | |
| TNF-α 10 | 29.94 | 1.97 | 0.55 | | 1.117594808 |
| TNF-α 50 | 30.94 | 1.86 | -0.44 | | 1.807601073 |
| GAPDH 0 | 28.18 | 2.00 | | | |
| GAPDH 10 | 27.80 | 2.00 | | 0.38 | |
| GAPDH 50 | 29.43 | 2.00 | | -1.25 | |

**Table 6.10 Analysis of third repeat experiment with Thp-1 cells incubated with LPS at 4 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF-α 0 | 31.25 | 1.80 | | | |
| TNF-α 10 | 30.81 | 1.81 | 0.44 | | 0.239376721 |
| TNF-α 50 | 31.53 | 1.82 | -0.28 | | 0.723041936 |
| GAPDH 0 | 25.73 | 2.00 | | | |
| GAPDH 10 | 23.29 | 2.00 | | 2.44 | |
| GAPDH 50 | 25.50 | 2.00 | | 0.23 | |

**Table 6.11 Mean fold difference of TNF-α expression relative to HKG GAPDH in Thp-1 cells exposed to LPS for 4 hours using the Pfaffl method.**

| Sample | Fold Av ex 1 | Fold av ex 2 | Fold av. ex 3 | Mean | SD | SE |
|---|---|---|---|---|---|---|
| 10ng/mL LPS | 0.05 | 1.12 | 0.24 | 0.47 | 0.57 | 0.33 |
| 50ng/mL LPS | 0.18 | 1.81 | 0.72 | 0.90 | 0.83 | 0.48 |

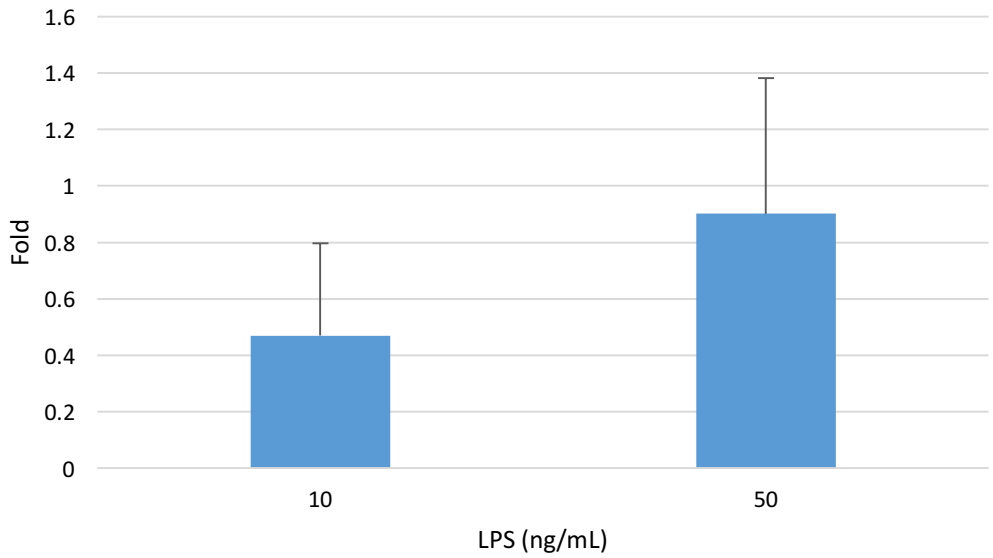


**Figure 6.4 Fold change of TNF-α expression of Thp-1 cells exposed to LPS for 4 hours using the Pfaffl method for qPCR analysis (N=3)**

When using the Pfaffl method, only a 0.47 fold increase in TNF-α expression is seen when exposed to 10ng/mL of LPS, and a 0.90 fold increase seen when exposed to 50ng/mL of LPS. These values, though already low, are even lower than the ones obtained from the delta-delta analysis, and were not significant ($p>0.05$).

## 6.4 Discussion

The objectives of this chapter were to determine TNF-α expression levels when 3 x $10^5$ Thp-1 cells were incubated with 10ng/mL and 50ng/mL LPS for 4 hours. qPCR was continued to be used as it provided more accurate and publishable results. The concentrations (10ng/mL and 50ng/mL of LPS) and time point (4 hours) was selected based on a study by Schildberger et al. in 2013, in which the highest TNF-α expression seen in Thp-1 cells exposed to LPS at 10ng/mL was at 4 hours.

The results from the qPCR delta-delta analysis showed a fold increase of for 10ng/mL of LPS, and for a 50ng/mL increase. Using the Pfaffl method, the fold increases were even lower; for 10ng/mL LPS a fold increase was observed, and for 50ng/mL LPS a fold increase was observed. Although an increase is seen between the 10ng/mL and 50ng/mL as expected (though

insignificant), it is interesting to observe that the experiment carried out in chapter 4, with Thp-1 cells being incubated with LPS for 12 hours, has a higher response. This is because in the Schildberger experiment, the peak of TNF-α expression was seen at 4 hours using 10uL of LPS. In the experiment undertaken in the lab, only 3 x $10^5$ cells were used. This was because initially this was a good amount of cells to use in order to extract RNA of a sufficient concentration. It was also difficult to obtain a large quantity of cells at such a concentration of $10^6$ cells. However when comparing this number with $10^6$, it should be seen that since there were less cells exposed to a higher amount of LPS, the overall increase would be expected to be higher. There may be a variety of reasons for why this was not seen.

Firstly, the cells used had been passaged well over 30 times before they were exposed to LPS. Although the cells were checked under the microscope to see if they were healthy cells, it is possible that having passaged them so many times, the gene expression levels may have been altered or reduced over time. This could be due to clonal selection. Thp-1 cells are an immortalized cell line and can be passaged up to 25 times without any changed in cell sensitivity and activity (Chanput et al., 2014). It would have been more reliable and accurate if the cells used were recently purchased and new. This could explain the experimental results in this chapter compared to the results seen in the Schildberger et al. 2013 paper.

Given enough time and resources, it would be best to incubate Thp-1 cells for a variety of concentrations and time points as was done in Chapter 4.3.4, and then to do qPCR analysis on them to look at the different TNF-α expression levels. This would give a good indication of where the peak expression of TNF-α was with the cells being used, and it would account for any differences that would have occurred because factors such as passage number.

LPS in the lab was stored in plastic Eppendorf tubes. At a concentration of lower than lower than 0.1mg/mL, it has been recommended that LPS be stored in glass tubes/containers as it is able to bind to the plastic. Also,

repeated freeze thaw cycles are not recommended. The LPS used was from a made up stock in the -20 freezer of the E3.11 lab at the University of Waikato. This LPS had been used my students before being used for this experiment and would have undergone several freeze thaw cycles. Being kept in a plastic tube may have reduced the concentration of LPS slightly, as it is able to bind to the plastic as well as some kinds of glass. This is especially prominent when LPS is stored at concentrations lower than 0.1mg/mL (Merck, 2018).

It would have made the experiment more reliable if a duplicate of each sample was done in all of the three experiments, as it was done in Chapter 4. However due to a time shortage and availability of resources, only 1 sample at each concentration was used for each of the three experiments. If a larger sample size was taken, this would help to reduce the large standard errors in the experiments.

It is widely agreed upon that low quality RNA can compromise gene expression results (Fleige et al., 2006). It becomes important to isolate a high quality of RNA from many samples. Looking back to Tables 6.1, 6.2 and 6.3, the 260/280 ratio of all samples are between 1.89-1.98. In Figure 6.2, the RNA shows clear 28S and 18S bands. However, it is possible that some genomic DNA is present in the samples, as seen by the light smears below the 18S bands. Given more time and resources, the RNA extracted could have been put through a DNAse treatment.

Not only is mRNA very unstable (especially long pieces of mRNA), it is possible that through frequent handling RNAses may be introduced (Fleige et al., 2006). It has been reported that TNF-α mRNA has a very short half-life. Although TNF-α is produced at its maximum levels seen when it is exposed to LPS, it's mRNA remains short lived. The poly A tail added to mRNA helps to keep the mRNA stable. It has been shown that TNF-α mRNA has very short homogenous poly(A) tails compared to GAPDH mRNA, which has a long pol(A) tail from ~70 to ~220 nucleotides. (Mijatovic et al., 2000).

Although the results were insignificant, a greater fold change was seen when LPS was incubated with  LPS for 12 hours. Therefore it would be suitable for the next experiment to be incubating Thp-1 cells with  HSP60 for 12 hours. However due to time constraints, a 4 hour HSP60 experiment has been set up and conducted before the analysis of the  results in this chapter. This will be presented in the following chapter.

# 7 Thp-1 cells induced with HSP60 for 4 hours to determine TNF-α expression through qPCR

## 7.1 Introduction

HSP60 is a protein known to be released upon cell stress, such as increased temperatures and high glucose environments (Chapter 1.6). It is the only HSP that shows a direct link to atherosclerosis through experimental and clinical trials (Wick et al., 2014). The movement of this protein to the cell surface has been recognised as a stress response and correlates strongly with the development of cardiovascular disease (Grundtman et al., 2011).

HSP60 has been suggested to activate TLR4, similar to the way LPS does and may trigger a signalling cascade that ultimately leads to the release of pro-inflammatory cytokines such as TNF-α. More recent studies show that HSP60 may also be able to activate TLR2 (Kilmartin and Reen, 2004). In this portion of the study, Thp-1 cells were incubated with different concentrations of HSP60 (500ng/mL and 1500ng/mL based on HSP60 levels found in diabetic patients by Yuan et al., 2011) for 4 hours to determine an increase in TNF-α expression at mRNA level could be seen.

## 7.2 Methods

### 7.2.1 Endotoxin removal from human HSP60 grown in *E.Coli*

The human HSP60 used in this project was a recombinant protein expressed in *E.coli*. Endotoxin was removed from HSP60 using the Pierce High Capacity Endotoxin Removal kit using the manufacturer's directions. First the spin column was bought to room temperature. The column's bottom closure was twisted off and the cap loosened. It was placed in a collection tube and spun at 500 x g for 1 minute in a centrifuge to remove the storage solution, which was discarded. A bottom plug was added to the column and

the cap was removed. To regenerate, 2mL 0.2 NaOH was added and the cap was replaced. The column was inverted several times to suspend the resin in the solution and this was incubated overnight at room temperature. The next day the cap was loosened and bottom plug was removed. The column was placed in a collection tube and centrifuged at 500 x g for 1 minute, and the solution in the collection tube was discarded. After removing the cap and inserting a bottom plug, 2mL of 2M NaCl was added. The cap was tightened and the column was inverted several times to resuspend the resin in solution. The cap was loosened and bottom plug removed, and the column was placed in a collection tube and spun at 500 x g for 1 minute. The solution in the collection tube was discarded. The cap was removed and plug inserted, and 2 mL of endotoxin-free ultra-pure water was added. The cap was tightened and the column inverted several times, resuspending the resin. After loosening the cap and removing the plug, the column was put inside a collection tube and spun for 500 x g for 1 minute. The solution in the collection tube was discarded. The cap was removed and plug inserted, and 2 mL of endotoxin free buffer was added. The column was inverted several times to resuspend the resin. The plug was removed and cap loosened, and the column was placed in a collection tube and spun at 500 x g for 1 minute. The solution was discarded. This was repeated twice more with the endotoxin buffer. Then the plug was inserted and HSP60 was added to the column. After tightening the cap, the column was left with gentle end over end mixing at room temperature for four hours. Then the cap was loosened, plug removed, and column placed in a collection tube. This was spun at 500 x g for 1 minute to collect endotoxin-free HSP60.

### 7.2.2 Thp-1 cells Incubated with HSP60

Thp-1 cells were seeded at 3 x 105 cells (in approximately 2mLs) into 5 wells of a 6-well plate. The plate was set up as shown in the diagram below. In the first well, 10uL of PBS was added as a control. The next well had 500ng/mL of HSP60 added, followed by 1500ng/mL HSP60, 500ng/mL of untreated (endotoxin not removed) HSP60, and lastly 1500ng/mL untreated HSP60. This was incubated in a humidified $CO_2$ incubator at 37°C

(standard incubator conditions for cell growth) for 4 hours, followed by RNA extraction.

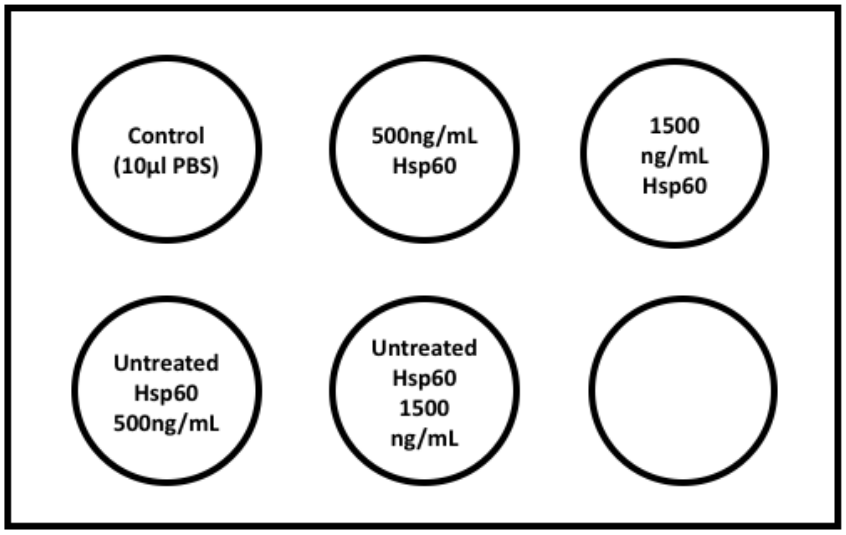


**Figure 7.1 Set up of 6 well plate with Thp-1 cells and different concentrations of HSP60**

### 7.2.3 RNA extraction, cDNA synthesis and qPCR

RNA extraction, nanodrop readings, cDNA synthesis and agarose gels were carried out as described in Chapters 2.3, 2.4, 2.5 and 2.6 respectively. qPCR was carried out and analysed as described in Chapter 5.2.1.

### 7.2.4 Statistical analysis

ANOVA and the Student's T-test was carried out on Microsoft Excel to determine the significance of the results. For the T-test a two tailed distribution was used and it was performed using 2 samples with unequal variants.

## 7.3 Results

### 7.3.1 RNA

#### 7.3.1.1 Quantity and quality

The tables (Table 7.1, 7.2, 7.3) present the concentrations and 260/280 ratios of the RNA extracted from Thp-1 cells incubated with HSP60 for 4 hours. The concentrations were all suitable for cDNA synthesis. The

260/280 ratios, range from 1.75-1.98, with 1 sample 1.68. Although this value is considered low compared to threshold values, however, these samples were used in this experiment, as there were 2 repeats of this experiment (as shown in Table 7.1 and 7.2).

**Table 7.1 RNA concentrations and 260/280 ratios of Thp-1 cells exposed to HSP60 for 4 hours.**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control | 256.8 | 1.93 |
| 500ng/mL endotoxin removed HSP60 | 174.2 | 1.81 |
| 1500ng/mL endotoxin removed HSP60 | 108.2 | 1.87 |
| 500ng/mL untreated HSP60 | 237.2 | 1.90 |
| 1500ng/mL untreated HSP60 | 239.4 | 1.88 |

**Table 7.2 Repeat of RNA concentrations and 260/280 ratios of Thp-1 cells exposed to HSP60 for 4 hours.**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control | 199.6 | 1.82 |
| 500ng/mL endotoxin removed HSP60 | 271.9 | 1.87 |
| 1500ng/mL endotoxin removed HSP60 | 298.0 | 1.88 |
| 500ng/mL untreated HSP60 | 223.8 | 1.89 |
| 1500ng/mL untreated HSP60 | 147.4 | 1.82 |

**Table 7.3 Repeat of RNA concentrations and 260/280 ratios of Thp-1 cells exposed to HSP60 for 4 hours.**

| Sample | RNA concentration (µg/mL) | 260/280 |
|---|---|---|
| Control | 168.8 | 1.84 |
| 500ng/mL endotoxin removed HSP60 | 301.6 | 1.85 |
| 1500ng/mL endotoxin removed HSP60 | 219.3 | 1.75 |
| 500ng/mL untreated HSP60 | 78.7 | 1.76 |
| 1500ng/mL untreated HSP60 | 98.2 | 1.68 |

#### 7.3.1.2 RNA Integrity

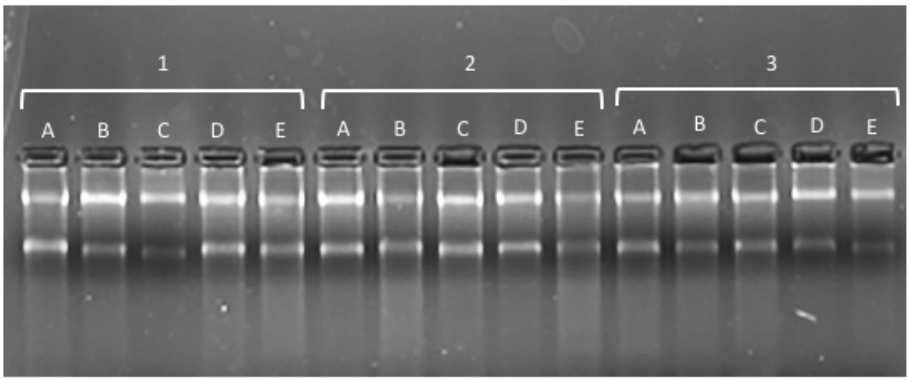


**Figure 7.2 RNA from Thp-1 cells induced with HSP60 for 4 hours. A: Control with PBS, B: .5µg/mL HSP60, C: 1.5µg/mL HSP60, D: .5µg/mL HSP60 (with endotoxin), E: 1.5µg/mL HSP60 (with endotoxin).**

The RNA was run on a 2% agarose gel for 1 hour at 70V. Figure 7.2 shows RNA extracted from Thp-1 cells incubated with HSP60 for 4 hours. The figure shows 2 clear bands, the top band being 28S RNA subunit, and the bottom one 18S RNA. As expected, the 28S band appears thicker than the bottom 18S band due to its larger size.

### 7.3.2 qPCR analysis

#### 7.3.2.1 Delta-delta Ct Method

The three tables (Table 7.4, 7.5, 7.6) show the average Ct of the experimental duplicates. Following this, using the delta-delta Ct equations as shown in Figure 5.1 the $\Delta$ctuntreated, $\Delta Ct_{treated,}$ $\Delta\Delta$Ct and fold difference

was worked out. Table 7.7 shows the average Ct of the samples between all 3 experiments, with the fold difference worked out as mean.

**Table 7.4 Analysis of first experiment with Thp-1 cells incubated with HSP60 for 4 hours using the delta-delta Ct method.**

| Tube | Ct average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF C | 25.43 | 6.80 | | | |
| TNF 500 | 30.40 | | 4.40 | -2.40 | 5.27 |
| TNF 1500 | 40.00 | | 10.37 | 3.57 | 0.08 |
| TNF U500 | 27.80 | | 7.26 | 0.46 | 0.73 |
| TNF U1500 | 30.66 | | 11.17 | 4.37 | 0.05 |
| GAPDH C | 18.63 | | | | |
| GAPDH 500 | 26.00 | | | | |
| GAPDH 1500 | 29.63 | | | | |
| GAPDH U500 | 20.54 | | | | |
| GAPDH U1500 | 19.49 | | | | |

**Table 7.5 Analysis of second repeat experiment with Thp-1 cells incubated with HSP60 for 4 hours using the delta-delta Ct method.**

| Tube | Ct average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF C | 28.11 | 4.65 | | | |
| TNF 500 | 32.02 | | 8.72 | 4.07 | 0.06 |
| TNF 1500 | 40.00 | | 14.87 | 10.22 | 0.00 |
| TNF U500 | 28.22 | | 8.19 | 3.54 | 0.09 |
| TNF U1500 | 29.91 | | 7.67 | 3.02 | 0.12 |
| GAPDH C | 23.46 | | | | |
| GAPDH 500 | 23.30 | | | | |
| GAPDH 1500 | 25.13 | | | | |
| GAPDH U500 | 20.03 | | | | |
| GAPDH U1500 | 22.24 | | | | |

**Table 7.6 Analysis of first experiment with Thp-1 cells incubated with HSP60 for 4 hours using the delta-delta Ct method.**

| Tube | Ct average | Δctuntreated | Δcttreated | ΔΔCt | Fold difference |
|---|---|---|---|---|---|
| TNF C | 27.78 | 7.11 | | | |
| TNF 500 | 31.43 | | 4.84 | -2.27 | 4.83 |
| TNF 1500 | 31.42 | | 5.92 | -1.19 | 2.28 |
| TNF U500 | 27.96 | | 6.26 | -0.85 | 1.80 |
| TNF U1500 | 32.39 | | 9.61 | 2.50 | 0.18 |
| GAPDH C | 20.67 | | | | |
| GAPDH 500 | 26.59 | | | | |
| GAPDH 1500 | 25.49 | | | | |
| GAPDH U500 | 21.70 | | | | |
| GAPDH U1500 | 22.77 | | | | |

**Table 7.7 Mean fold difference of TNF-α expression relative to HKG GAPDH in Thp-1 cells exposed to HSP60 for 4 hours using the delta-delta Ct method.**

| Sample | Fold Av ex 1 | Fold av ex 2 | Fold av. ex 3 | Mean | SD | SE |
|---|---|---|---|---|---|---|
| 500.00 | 5.27 | 0.06 | 4.83 | 3.39 | 2.89 | 1.67 |
| 1500.00 | 0.08 | 0.00 | 2.28 | 0.79 | 1.29 | 0.75 |
| U500 | 0.73 | 0.09 | 1.80 | 0.87 | 0.87 | 0.50 |
| U1500 | 0.05 | 0.12 | 0.18 | 0.12 | 0.06 | 0.04 |

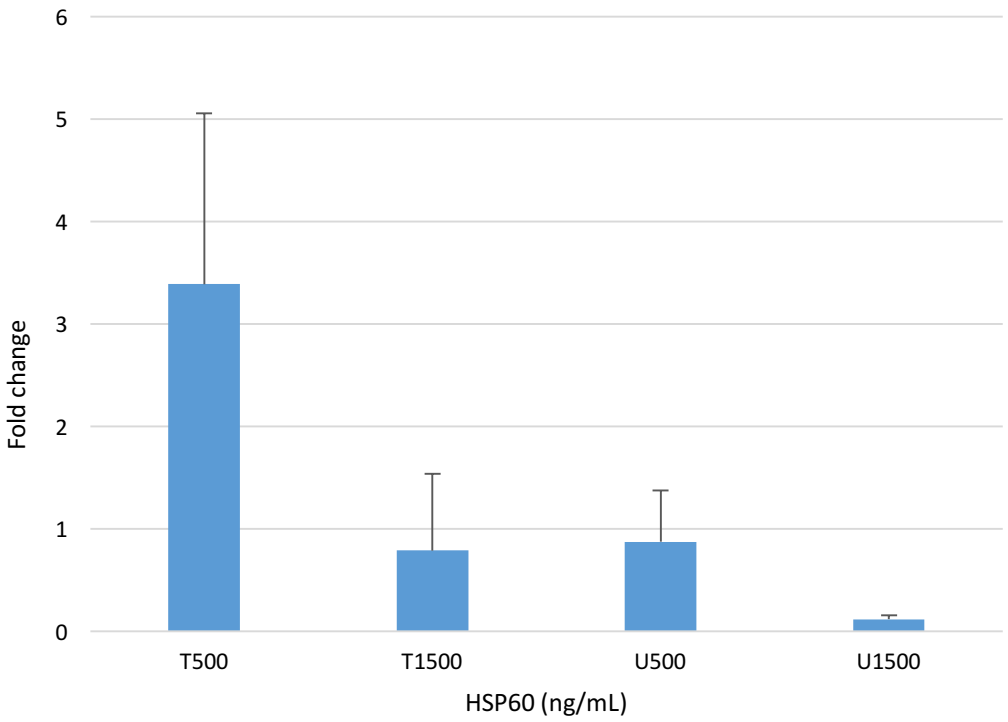


**Figure 7.3 Fold change of TNF-α expression of Thp-1 cells exposed to HSP60 for 4 hours using the delta-delta method for qPCR analysis (N=3).**

The graph indicates that the highest fold change of 3.39 was seen in the Thp-1 cells incubated with 500ng/mL of HSP60 for 4 hours. The rest of the samples had a fold change of less than 1, with the untreated 500ng/mL HSP60 samples having a 0.87 fold change, the 1500ng/mL HSP60 samples with a .79 fold change and untreated 1500ng/mL HSP60 with a 0.12 fold change. These results were statistically insignificant.

### 7.3.2.2 Pfaffl method

The three tables (Table 7.8, 7.9, 7.10) show the average Ct of the samples. Δcttarget, Δctnorm, and fold difference was worked out using the Pfaffl equations shown in Figure 5.2. Table 7.11 shows the average Ct of the samples between all 3 experiments, with the fold difference worked out.

**Table 7.8 Analysis of first experiment with Thp-1 cells incubated with HSP60 for 4 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF C | 25.43 | 1.92 | | | |
| TNF 500 | 30.40 | 1.92 | -4.97 | | 4.25 |
| TNF 1500 | 40.00 | 1.92 | -14.57 | | 0.09 |
| TNF U500 | 27.80 | 1.89 | -2.36 | | 0.75 |
| TNF U1500 | 30.66 | 1.87 | -5.23 | | 0.07 |
| GAPDH C | 18.63 | 1.90 | | | |
| GAPDH 500 | 26.00 | 1.89 | | -7.37 | |
| GAPDH 1500 | 29.63 | 1.91 | | -11.00 | |
| GAPDH U500 | 20.54 | 1.90 | | -1.91 | |
| GAPDH U1500 | 19.49 | 1.90 | | -0.86 | |

**Table 7.9 Analysis of second repeat experiment with Thp-1 cells incubated with HSP60 for 4 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF C | 28.11 | 1.91 | | | |
| TNF 500 | 32.02 | 1.84 | -3.92 | | 0.08 |
| TNF 1500 | 40.00 | 1.84 | -11.89 | | 0.00 |
| TNF U500 | 28.22 | 1.90 | -0.12 | | 0.10 |
| TNF U1500 | 29.91 | 1.94 | -1.81 | | 0.14 |
| GAPDH C | 23.46 | 1.90 | | | |
| GAPDH 500 | 23.30 | 1.90 | | 0.15 | |
| GAPDH 1500 | 25.13 | 1.94 | | -1.68 | |
| GAPDH U500 | 20.03 | 1.89 | | 3.43 | |
| GAPDH U1500 | 22.24 | 1.88 | | 1.21 | |

**Table 7.10 Analysis of third repeat experiment with Thp-1 cells incubated with HSP60 for 4 hours using the Pfaffl method.**

| Tube | Ct value | Efficiency | Δcttarget | Δctnorm | Fold difference |
|---|---|---|---|---|---|
| TNF C | 27.78 | 1.92 | | | |
| TNF 500 | 31.43 | 1.91 | -3.65 | | 4.64 |
| TNF 1500 | 31.42 | 1.90 | -3.64 | | 2.00 |
| TNF U500 | 27.96 | 1.82 | -0.18 | | 1.74 |
| TNF U1500 | 32.39 | 1.88 | -4.61 | | 0.22 |
| GAPDH C | 20.67 | 1.88 | | | |
| GAPDH 500 | 26.59 | 1.93 | | -5.92 | |
| GAPDH 1500 | 25.49 | 1.87 | | -4.82 | |
| GAPDH U500 | 21.70 | 1.90 | | -1.03 | |
| GAPDH U1500 | 22.77 | 1.94 | | -2.11 | |

**Table 7.11 Mean fold difference of TNF-α expression relative to HKG GAPDH in Thp-1 cells exposed to HSP60 for 4 hours using the Pfaffl method.**

| Sample | Fold Av ex 1 | Fold av ex 2 | Fold av. ex 3 | Mean | SD | SE |
|---|---|---|---|---|---|---|
| C500 | 4.25 | 0.08 | 4.64 | 2.99 | 2.53 | 1.46 |
| C1500 | 0.09 | 0.00 | 2.00 | 0.70 | 1.13 | 0.65 |
| U500 | 0.75 | 0.10 | 1.74 | 0.86 | 0.82 | 0.48 |
| U1500 | 0.07 | 0.14 | 0.22 | 0.14 | 0.08 | 0.04 |

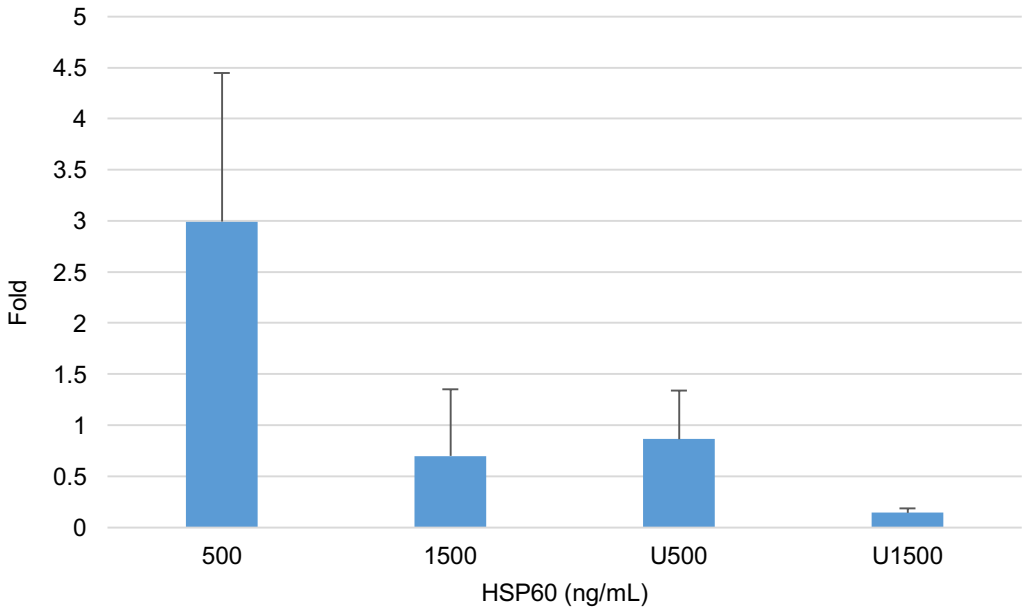


**Figure 7.4 Fold change of TNF-α expression of Thp-1 cells exposed to HSP60 for 4 hours using the Pfaffl method for qPCR analysis (N=3).**

The Pfaffl method gave slightly lower results than the delta delta Ct method. Like the delta delta Ct method, the highest fold difference (2.99) was seen in the Thp-1 cells incubated with 500ng/mL of HSP60 for 4 hours. This was followed by the untreated 5000ng/mL HSP60 (0.86), 15oong/mL HSP60 (0.70) and lastly, untreated 1500ng/mL HSP60 (0.14). These results were statistically insignificant.

## 7.4 Discussion

The objective of this chapter was to see how much TNF-α was being expressed at mRNA level when incubated with different concentrations of HSP60. The concentrations of HSP60 used were 500ng/mL and 1500ng/mL. These were chosen based on the amount of HSP60 found in the serum of type 2 diabetic patients (28 -1043ng/mL) (Yuan et al., 2011).

The HSP60 was expressed in the Gram negative bacteria called *Escherichia coli.* LPS is found in the outer wall of all Gram negative bacteria. Therefore, any recombinant protein expressed in *E. coli* will have LPS (endotoxin) contamination. LPS, as previously explained is a ligand for TLR4 and is able to set off a signalling cascade that leads to the production

of inflammatory cytokines. Therefore, in order to determine if the HSP60 was triggering the TNF-α response, the LPS needed to be removed. This was done using a high capacity endotoxin removal column as explain in Chapter 7.2.1. In the results it was expected that both the samples of HSP60 that did not go through the spin column (untreated or U) would show a heightened expression of TNF-α. However, both the untreated samples show a lower expression than the treated samples. One reason for this may be that the spin column method did not work efficiently. This could have been tested through the Lumulus Amebocyte Lysate (LAL) test. This test uses the blood of a horseshoe crab, *Limulus Polyphemus*, which clots up when it comes into contact with Gram negative bacteria, revealing the presence of LPS (Novitsky, 1994). There are kits that use this method and detect the presence of LPS by colour change or through gelation (GenScript, 2018).

Interestingly, it was seen that the highest fold increase in the results was with 500ng/mL of HSP60 at 3-fold. At 1500ng/mL of HSP60, only a ~ 1 fold change was seen. This may be because at a high concentration of 1500ng/mL the TNF-α response occurs much quicker in the cells. This means that the TNF-α mRNA would be transcribed faster, and since the TNF-α mRNA is short lived, as discussed in Chapter 6.4, the mRNA may have already been degraded at the end of 4 hours. Ideally a time course could be carried out, where HSP60 at 500ng/mL were incubated with Thp-1 cells for 2, 4, 6, 8, 10 hours etc. This would give a good indication of where the mRNA of TNF-α is being expressed the highest. TNF-α should also be measured at the protein level, as it is at the protein level that it would have an inflammatory response on the target cells. This could be done through ELISA.

In a study carried out by Ueki et al. in 2002, 10μg/mL of HSP60 was incubated with Thp-1 cells for 12 hours, and revealed an increase of TNF-α production through ELISA (~110pg/mL) as compared to control (5pg/mL). Only human HSP60 was used, as bacterial HSP60 (or GroEL/GroES) did not induce TNF-α in Thp-1 cells (Ueki et al., 2002). Looking at literature, it seems that a more appropriate time frame for HSP60 induction of Thp-1

cells would have been 12 hours. However the concentration used in this experiment (500ng/mL and 1500ng/mL) was essential, as it represented the amount of HSP60 found in the serum of diabetic patients (30-1000ng/mL) (Yuan et al., 2011). It was thought that a TNF-α expression would be seen a lot earlier since the concentration used was very high compared to the 10ng/mL of LPS in 2 x $10^6$ cells/mL used in Ueki et al study. Also, it has been indicated through research that LPS and HSP60 used very similar methods of triggering a TNF-α response, so 4 hours was picked as an appropriate time frame. Unfortunately, due to time constraints, the 4 hour LPS experiment and 4 hour HSP60 experiment were set up around the same time, not giving enough time in between to analyse results. If the results had been analysed before setting up the HSP60 experiment, a 12 hour time frame may have been selected, as the highest TNF-α response with LPS is seen with 100ng/mL of LPS for 12 hours (Figure 5.4).

Initial research indicated that HSP60 activated monocytes and macrophages through a complex consisting TLR4, but studies indicate that TLR2 is also able to respond to HSP60 (Kilmartin and Reen, 2004). However, more research tends to be based around the TLR4 activation of HSP60. It was discovered by Xu et al. in 2001 that aortic root lesions of high fat diet-fed mice expressed TLR4 mRNA expression higher than control mice, and that TLR4 mRNA expression could be upregulated when human monocyte derived macrophages were exposed to LDL particles (as seen in atherosclerotic plaques) (Xu et al., 2001). It has been generally indicated through literature that HSP60 may use TLR4 to trigger an inflammatory response that contributes to atherogenesis. To prove this, the next step in this research would to generate a TNF-α response curve using different time points to determine the highest point of TNF-α expression (as mentioned earlier). Then the Thp-1 cells could be incubated with TAK-242, an antagonist TLR4. This would be followed by exposing the Thp-1 cells with HSP60 for the ideal time frame determined by the TNF-α expression curve. If it is found that TNF-α has been markedly reduced, it would indicate that HSP60 was using TLR4 to mediate an inflammatory response. It is interesting to note that exposure to TNF-α can also lead to a higher

expression of HSP60 (Ferm et al., 1992). This creates a cycle that may lead to a long-term inflammatory state, propagating atherosclerosis.

# 8 Concluding remarks and Future Directions

The question of how diabetes mellitus is linked to cardiovascular diseases has been asked for decades, yet a molecular mechanism linking the two diseases is yet to be found. This project investigated the expression of a pro-inflammatory cytokine, TNF-α when Thp-1 cells were exposed to LPS (a known trigger to TNF-α ) and HSP60 (a pro-inflammatory mediator elevated in diabetic patients). Thp-1 cells were used to mimic monocytes, the most abundant cell type found in atherosclerotic plaques.

Thp-1 cells were incubated with 50ng/mL, 100ng/mL and 200ng/mL of LPS for 6, 12, and 24 hour periods. Based on the results shown in Chapter 4.3.4, a time period of 12 hours was initially selected for the rest of the experiments. The concentrations of LPS used were 50ng/mL and 100ng/mL. RNA was extracted using the TRIzol method, and cDNA was synthesised. Initially semi-quantitative analysis was done on these samples, however after human error and the lengthy process of determining cycle numbers, qPCR was selected as a more appropriate method. qPCR was carried out for TNF-α and GAPDH, and after analysis a 2 fold increase was seen. However after doing a Student's T test, these results were considered to be statistically not-significant. Thp-1 cells were then incubated with 10ng/mL and 50ng/mL of LPS for 4 hours (based of research values), however the results obtained during this study was once again shown to be not significant.

Despite the positive control not working (given the documented evidence in the literature which has shown LPS to be a potent inducer of TNF-α expression), the effect of exposing Thp-1 cells to clinically relevant levels of HSP60 was investigated. Thp-1 cells were incubated with 500ng/mL and 1500ng/mL of human HSP60 for 4 hours. After qPCR analysis, a ~3 fold increase was seen in the samples of Thp-1 cells incubated with 500ng/mL of HSP60. The results were not statistically significant. However, since a

small increase was seen, the experiment could have been repeated with more samples, which would reduce the errors made in the experiments and may have shown an increase in the expression of TNF-α.

In a recent paper published by Martinus and Goldsbury in 2017, it was clearly demonstrated that when Thp-1 cells were grown in the presence of glucose 25mM glucose, HSP60 levels increased by 3 fold compared to Thp-1 cells grown in 5mM glucose (Martinus & Goldsbury, 2017). It was also demonstrated that when a human endothelial cell line was incubated with 25mM glucose conditioned media (containing HSP60), an increase of TNF-α at the protein level was observed. However when the HSP60 was immune-depleted from the media, a significantly lower TNF-α expression was seen (Martinus and Goldsbury, 2017).

Although the exact mechanism has not been demonstrated, literature does point towards HSP60 playing a role in the inflammatory condition in diabetes, which leads to the development of an inflamed vascular state, stimulating atherogenesis. Given that HSP60 (released from monocytes grown under hyperglycaemic conditions) was capable of triggering the release of TNF-α (from endothelial cells) may be an important factor causing vascular inflammation, the next step would be to investigate ways to repress the expression of HSP60. This could be done at mRNA level by microRNA approaches (Shan et al., 2010). MicroRNAs are small single stranded RNAs that are primarily involved in negatively regulating gene expression. They can act as silencers by selective base pairing and reduce target gene expression by inhibiting translation initiation or degrading the mRNA (Betel et al., 2008). By stopping the mRNA of HSP60, it would be ensured that the protein was not synthesised and it would not be able to activate TLRs

Another approach could also be to target the receptor complex and signal transduction pathways responsible for the HSP60 mediated induction of TNF-α. Current research is not able to show exactly what TLR HSP60 uses to trigger an inflammatory response in target cells. When significant increase in TNF-α expression was seen by incubating Thp-1 cells with human HSP60, the next step in this project could be to use an antagonist

for TLR4, the receptor that HSP60 has been hypothesised to use the most. One such antagonist is TAK-242. TAK-242 is a cyclohexane derivative. This novel small molecule compound binds to TLR4 and is able to stop signal transduction, therefore stopping the production of cytokines. TLR4 is well documented to be involved in the production of pro-inflammatory cytokines. The activation or suppression of TLR4 has been seen to have an effect on the development and progression of many inflammatory diseases. TAK-242 is able to disrupt the interaction between TLR4 with its adaptor molecules TIRAP and TRAM by binding directly via Cys747 in the TIR domain of TLR4. When TAK-242 was used on the human embryonic kidney cell line HEK293, it bound specifically to TLR4, limiting the expression of inflammatory cytokines such as TNF-α. This small-molecule-specific inhibitor is a good target for treating inflammatory diseases (Matsunaga et al., 2011).

An interesting study also showed that the addition of purified recombinant human HSP10 was able to lower TNF-α expression in RAW cells when triggered by LPS. It was suggested that HSP10 reacted with HSP60, and inhibited it from activating TLRs (Johnson et al., 2005). HSP60 also acts as a chaperonin for folding proteins, and there are a few HSP60 inhibitors that reduce its folding ability, such as mizoribine (a purine nucleotide analog isolated from fungi) (Itoh et al., 1999). Another inhibitor is a small molecule inhibitor, a pyrazolo-pyrimidine derivative EC3016. This inhibitor interacts with the ATP binding pocket of HSP60 and inhibits it's ATPase activity, and overall reduce its ability to fold proteins (Chapman et al., 2009). However it is not certain that these inhibitors would have an effect on the other properties of HSP60, such as its ability to activate TLRs.

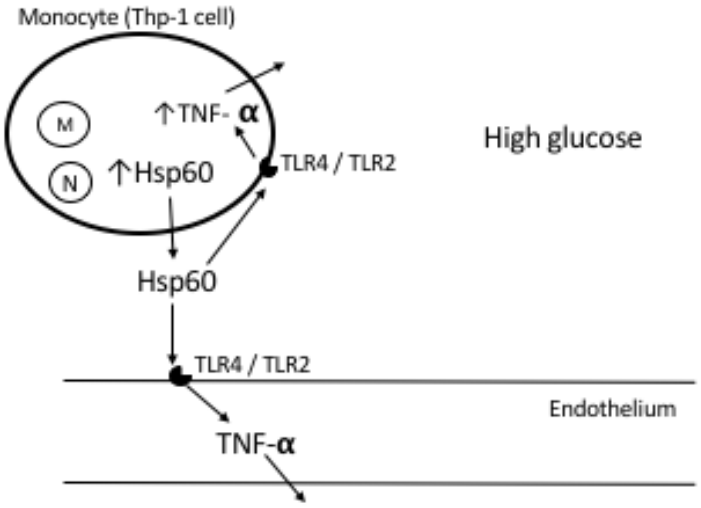


**Figure 8.1 HSP60 release from monocytes under high glucose conditions leading to non-resolved endothelial inflammation.**

As shown in the diagram below, an elevated level of HSP60 in the bloodstream can interact with TLRs of the endothelium, and trigger the release of pro-inflammatory cytokines. Although this research limits itself to the expression of TNF-α, other pro-inflammatory cytokines such as interleukins can also be tested for. Interestingly, as monocytes themselves have TLRs, an autocrine effect may also be seen, in which HSP60 released from the monocyte cells may interact with the TLRs on the cells themselves. This would enhance TNF-α production and lead to non-resolved vascular inflammation.

Although this project was only able to show insignificant increases of TNF-a expression at the mRNA level when incubated with LPS and HSP60, it is important to note that the incubation of Thp-1 cells with LPS and HSP60 has been well documented in literature. The next step in this area of research would be to focus on what receptor HSP60 is using to trigger a pro-inflammatory response. Using microRNA and receptor antagonists could be a good start to elucidating the pathway of HSP60 and understanding its role in diabetic vascular inflammation.

# References


AMERICAN DIABETES ASSOCIATION 2012. Standards of Medical Care in Diabetes—2012. *Diabetes Care,* 35**,** S11-S63.

BARBER, R. D., HARMER, D. W., COLEMAN, R. A. & CLARK, B. J. 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiological Genomics,* 21**,** 389-395.

BASA, A. L. P. & GARBER, A. J. 2001. Cardiovascular Disease and Diabetes: Modifying Risk Factors Other Than Glucose Control. *The Ochsner Journal,* 3**,** 132-137.

BETEL, D., WILSON, M., GABOW, A., MARKS, D. S. & SANDER, C. 2008. The microRNA. org resource: targets and expression. *Nucleic acids research,* 36**,** D149-D153.

BIOSYNTHESIS. 2015. *Ten Things That can Kill Your PCR* [Online]. Available: http://www.biosyn.com/TEW/Ten-Things-That-Can-Kill-Your-PCR.aspx [Accessed June 2018].

BIRBEN, E., SAHINER, U. M., SACKESEN, C., ERZURUM, S. & KALAYCI, O. 2012. Oxidative Stress and Antioxidant Defense. *The World Allergy Organization journal,* 5**,** 9-19.

BROWNLEE, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature,* 414**,** 813.

CHANPUT, W., MES, J. J. & WICHERS, H. J. 2014. THP-1 cell line: an in vitro cell model for immune modulation approach. *International immunopharmacology,* 23**,** 37-45.

CHAPMAN, E., FARR, G. W., FURTAK, K. & HORWICH, A. L. 2009. A small molecule inhibitor selective for a variant ATP-binding site of the chaperonin GroEL. *Bioorganic & medicinal chemistry letters,* 19**,** 811-813.

CHO, N. H., SHAW, J. E., KARURANGA, S., HUANG, Y., DA ROCHA FERNANDES, J. D., OHLROGGE, A. W. & MALANDA, B. 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice,* 138**,** 271-281.

DASU, M. R., DEVARAJ, S., PARK, S. & JIALAL, I. 2010. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. *Diabetes Care,* 33**,** 861-8.

DEVARAJ, S., DASU, M. R., PARK, S. H. & JIALAL, I. 2009. Increased levels of ligands of Toll-like receptors 2 and 4 in type 1 diabetes. *Diabetologia,* 52**,** 1665-8.

DREXLER, H. G. & UPHOFF, C. C. 2002. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. *Cytotechnology,* 39**,** 75-90.

FERM, M. T., SÖDERSTRÖM, K., JINDAL, S., GRÖNBERG, A., IVANYI, J., YOUNG, R. & KIESSLING, R. 1992. Induction of human HSP60 expression in monocytic cell lines. *International immunology,* 4**,** 305-311.

FLEIGE, S., WALF, V., HUCH, S., PRGOMET, C., SEHM, J. & PFAFFL, M. W. 2006. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnology letters,* 28**,** 1601-1613.

FUNK, S. D., YURDAGUL, A., JR. & ORR, A. W. 2012. Hyperglycemia and endothelial dysfunction in atherosclerosis: lessons from type 1 diabetes. *Int J Vasc Med,* 2012**,** 569654.

GENSCRIPT. 2018. *Endotoxin Assay Kits* [Online]. Available: https://www.genscript.com/endotoxin_assay_kits.html [Accessed June 2018].

GOMES, M. D. B. 2013. Impact of diabetes on cardiovascular disease: an update. *International journal of hypertension,* 2013.

GRUNDTMAN, C., KREUTMAYER, S. B., ALMANZAR, G., WICK, M. C. & WICK, G. 2011. Heat shock protein 60 and immune inflammatory responses in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology,* 31**,** 960-968.

HANSEN, J. J., BROSS, P., WESTERGAARD, M., NIELSEN, M., EIBERG, H., BØRGLUM, A. D., MOGENSEN, J., KRISTIANSEN, K., BOLUND, L. & GREGERSEN, N. 2003. Genomic structure of the human mitochondrial chaperonin genes: HSP60 and HSP10 are localised head to head on chromosome 2 separated by a bidirectional promoter. *Human genetics,* 112**,** 71-77.

INZUCCHI, S. E. 2012. Diagnosis of Diabetes. *New England Journal of Medicine,* 367**,** 542-550.

ITARIU, B. K. & STULNIG, T. M. 2014. Autoimmune aspects of type 2 diabetes mellitus-a mini-review. *Gerontology,* 60**,** 189-196.

ITOH, H., KOMATSUDA, A., WAKUI, H., MIURA, A. B. & TASHIMA, Y. 1999. Mammalian HSP60 is a major target for an immunosuppressant mizoribine. *Journal of Biological Chemistry,* 274**,** 35147-35151.

JOHNSON, B. J., LE, T. T., DOBBIN, C. A., BANOVIC, T., HOWARD, C. B., FLORES, F. D. M. L., VANAGS, D., NAYLOR, D. J., HILL, G. R. & SUHRBIER, A. 2005. Heat shock protein 10 inhibits lipopolysaccharide-induced inflammatory mediator production. *Journal of Biological Chemistry,* 280**,** 4037-4047.

JUWONO, J. & MARTINUS, R. D. 2016. Does HSP60 Provide a Link between Mitochondrial Stress and Inflammation in Diabetes Mellitus? *Journal of Diabetes Research,* 2016**,** 6.

KILMARTIN, B. & REEN, D. J. 2004. HSP60 induces self‐tolerance to repeated HSP60 stimulation and cross‐tolerance to other pro‐inflammatory stimuli. *European journal of immunology,* 34**,** 2041-2051.

KING, R. & GRANT, P. 2016. Diabetes and cardiovascular disease: pathophysiology of a life-threatening epidemic. *Herz,* 41**,** 184-192.

LEUNG, L. & CAHILL, C. M. 2010. TNF-α and neuropathic pain - a review. *Journal of Neuroinflammation,* 7**,** 27.

LOWELL, B. B. & SHULMAN, G. I. 2005. Mitochondrial dysfunction and type 2 diabetes. *Science,* 307**,** 384-387.

MAKKOUK, A. & ABDELNOOR, A. M. 2009. The potential use of Toll-like receptor (TLR) agonists and antagonists as prophylactic and/or therapeutic agents. *Immunopharmacol Immunotoxicol,* 31**,** 331-8.

MANSOUR, F. H. & PESTOV, D. G. 2013. Separation of long RNA by agarose–formaldehyde gel electrophoresis. *Analytical biochemistry,* 441**,** 18-20.

MARETT, A. 2014. *Obesity and diabetes in New Zealand* [Online]. Available: https://www.parliament.nz/en/pb/research-papers/document/00PLLawRP2014041/obesity-and-diabetes-in-new-zealand [Accessed June 2018].

MARONE, M., MOZZETTI, S., RITIS, D., PIERELLI, L. & SCAMBIA, G. 2001. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. *Biological procedures online,* 3**,** 19.

MARTINUS, R. D. & GOLDSBURY, J. 2017. Endothelial TNF-α induction by HSP60 secreted from THP-1 monocytes exposed to hyperglycaemic conditions. *Cell Stress and Chaperones***,** 1-7.

MATSUNAGA, N., TSUCHIMORI, N., MATSUMOTO, T. & II, M. 2011. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Molecular pharmacology,* 79**,** 34-41.

MERCK. 2018. *Lipopolysaccharides* [Online]. Available: https://www.sigmaaldrich.com/technical-documents/protocols/biology/lipopolysaccharides.html [Accessed June 2018].

MIJATOVIC, T., HOUZET, L., DEFRANCE, P., DROOGMANS, L., HUEZ, G. & KRUYS, V. 2000. Tumor necrosis factor‐$\alpha$ mRNA remains

unstable and hypoadenylated upon stimulation of macrophages by lipopolysaccharides. *The FEBS Journal,* 267**,** 6004-6012.

MILLER, C. J., KASSEM, H. S., PEPPER, S. D., HEY, Y., WARD, T. H. & MARGISON, G. P. 2003. Mycoplasma infection significantly alters microarray gene expression profiles. *BioTechniques,* 35**,** 812-815.

MINISTRY OF HEALTH. 2018. *Diabetes* [Online]. Available: https://www.health.govt.nz/your-health/conditions-and-treatments/diseases-and-illnesses/diabetes [Accessed June 2018].

MITCHELL, K. & IADAROLA, M. J. 2010. RT-PCR Analysis of Pain Genes: Use of Gel-Based RT-PCR for Studying Induced and Tissue-Enriched Gene Expression. *Methods in molecular biology (Clifton, N.J.),* 617**,** 279-295.

MOORE, KATHRYN J. & TABAS, I. 2011. Macrophages in the Pathogenesis of Atherosclerosis. *Cell,* 145**,** 341-355.

NOVITSKY, T. 1994. Limulus amebocyte lysate (LAL) detection of endotoxin in human blood. *Journal of Endotoxin Research,* 1**,** 253-263.

OLOKOBA, A. B., OBATERU, O. A. & OLOKOBA, L. B. 2012. Type 2 diabetes mellitus: a review of current trends. *Oman medical journal,* 27**,** 269.

PATRA, M. C. & CHOI, S. 2016. Recent progress in the development of Toll-like receptor (TLR) antagonists. *Expert opinion on therapeutic patents,* 26**,** 719-730.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research,* 29**,** e45-e45.

POLONSKY, K. S. 2012. The Past 200 Years in Diabetes. *New England Journal of Medicine,* 367**,** 1332-1340.

ROLO, A. P. & PALMEIRA, C. M. 2006. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicology and applied pharmacology,* 212**,** 167-178.

SCHILDBERGER, A., ROSSMANITH, E., EICHHORN, T., STRASSL, K. & WEBER, V. 2013. Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. *Mediators of inflammation,* 2013.

SHAH, M. S. & BROWNLEE, M. 2016. Molecular and cellular mechanisms of cardiovascular disorders in diabetes. *Circulation research,* 118**,** 1808-1829.

SHAN, Z.-X., LIN, Q.-X., DENG, C.-Y., ZHU, J.-N., MAI, L.-P., LIU, J.-L., FU, Y.-H., LIU, X.-Y., LI, Y.-X. & ZHANG, Y.-Y. 2010. miR‐1/miR‐

206 regulate HSP60 expression contributing to glucose‐mediated apoptosis in cardiomyocytes. *FEBS letters,* 584**,** 3592-3600.

SILVER, N., BEST, S., JIANG, J. & THEIN, S. L. 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC molecular biology,* 7**,** 33.

SIMMS, D., CIZDZIEL, P. E. & CHOMCZYNSKI, P. 1993. TRIzol: A new reagent for optimal single-step isolation of RNA. *Focus,* 15**,** 532-535.

STOLAR, M. 2010. Glycemic control and complications in type 2 diabetes mellitus. *The American journal of medicine,* 123**,** S3-S11.

SZLOSAREK, P. W., GRIMSHAW, M. J., KULBE, H., WILSON, J. L., WILBANKS, G. D., BURKE, F. & BALKWILL, F. R. 2006. Expression and regulation of tumor necrosis factor α in normal and malignant ovarian epithelium. *Molecular Cancer Therapeutics,* 5**,** 382.

THERMO SCIENTIFIC. 2012. *Interpretation of Nucleic Acid 260/280 Ratios: Technical Bulletin T123* [Online]. Available: http://tools.thermofisher.com/content/sfs/brochures/T123-NanoDrop-Lite-Interpretation-of-Nucleic-Acid-260-280-Ratios.pdf [Accessed June 2018].

TIAN, J., GUO, X., LIU, X.-M., LIU, L., WENG, Q.-F., DONG, S.-J., KNOWLTON, A. A., YUAN, W.-J. & LIN, L. 2013. Extracellular HSP60 induces inflammation through activating and up-regulating TLRs in cardiomyocytes. *Cardiovascular Research,* 98**,** 391-401.

TRISTAN, C., SHAHANI, N., SEDLAK, T. W. & SAWA, A. 2011. The diverse functions of GAPDH: views from different subcellular compartments. *Cellular signalling,* 23**,** 317-323.

TSAN, M. F. & GAO, B. 2004. Endogenous ligands of Toll-like receptors. *J Leukoc Biol,* 76**,** 514-9.

TSUCHIYA, S., YAMABE, M., YAMAGUCHI, Y., KOBAYASHI, Y., KONNO, T. & TADA, K. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP‐1). *International journal of cancer,* 26**,** 171-176.

UEKI, K., TABETA, K., YOSHIE, H. & YAMAZAKI, K. 2002. Self‐heat shock protein 60 induces tumour necrosis factor‐$\alpha$ in monocyte‐derived macrophage: possible role in chronic inflammatory periodontal disease. *Clinical & Experimental Immunology,* 127**,** 72-77.

VAN DEN OEVER, I. A., RATERMAN, H. G., NURMOHAMED, M. T. & SIMSEK, S. 2010. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. *Mediators of inflammation,* 2010.

VAN MEERLOO, J., KASPERS, G. J. & CLOOS, J. 2011. Cell sensitivity assays: the MTT assay. *Cancer cell culture.* Springer.

VILA-DEL SOL, V., PUNZÓN, C. & FRESNO, M. 2008. IFN-γ-induced TNF-α expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages. *The Journal of Immunology,* 181**,** 4461-4470.

WICK, G., JAKIC, B., BUSZKO, M., WICK, M. C. & GRUNDTMAN, C. 2014. The role of heat shock proteins in atherosclerosis. *Nature Reviews Cardiology,* 11**,** 516.

WONG, F. S. & WEN, L. 2008. Toll-Like Receptors and Diabetes. *Annals of the New York Academy of Sciences,* 1150**,** 123-132.

WOOLLARD, K. J. & GEISSMANN, F. 2010. Monocytes in atherosclerosis: subsets and functions. *Nature Reviews Cardiology,* 7**,** 77.

WORLD HEALTH ORGANISATION. 2016. *Global Report on Diabetes* [Online]. Available: http://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf;jsessionid=95A9A9AB51F3224C985270E14CED90B2?sequence=1 [Accessed June 2018].

XIAO, Q., MANDAL, K., SCHETT, G., MAYR, M., WICK, G., OBERHOLLENZER, F., WILLEIT, J., KIECHL, S. & XU, Q. 2005. Association of serum-soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study. *Stroke,* 36**,** 2571-6.

XU, Q., METZLER, B., JAHANGIRI, M. & MANDAL, K. 2012. Molecular chaperones and heat shock proteins in atherosclerosis. *Am J Physiol Heart Circ Physiol,* 302**,** H506-14.

XU, X. H., SHAH, P. K., FAURE, E., EQUILS, O., THOMAS, L., FISHBEIN, M. C., LUTHRINGER, D., XU, X.-P., RAJAVASHISTH, T. B. & YANO, J. 2001. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation,* 104**,** 3103-3108.

XU, Z., HORWICH, A. L. & SIGLER, P. B. 1997. The crystal structure of the asymmetric GroEL–GroES–(ADP) 7 chaperonin complex. *Nature,* 388**,** 741.

YUAN, J., DUNN, P. & MARTINUS, R. D. 2011. Detection of HSP60 in saliva and serum from type 2 diabetic and non-diabetic control subjects. *Cell Stress Chaperones,* 16**,** 689-93.

# Appendix

**Table A1. Concentration and 260/280 ratio of RNA extracted from Thp-1 cells exposed to LPS for 6 hours.**

| Sample (LPS ng/mL) | Concentration | 260/280 |
|---|---|---|
| C | 226.7 | 1.87 |
| 50 | 117.3 | 1.90 |
| 100 | 62.7 | 1.55 |
| 200 | 281.2 | 1.93 |

**Table A2. Concentration and 260/280 ratio of RNA extracted from Thp-1 cells exposed to LPS for 12 hours.**

| Sample (LPS ng/mL) | Concentration | 260/280 |
|---|---|---|
| C | 181.9 | 1.89 |
| 50 | 384.4 | 2.00 |
| 100 | 93.0 | 1.95 |
| 200 | 291.0 | 2.02 |

**Table A3. Concentration and 260/280 ratio of RNA extracted from Thp-1 cells exposed to LPS for 24 hours.**

| Sample (LPS ng/mL) | Concentration | 260/280 |
|---|---|---|
| C | 141.5 | 1.77 |
| 50 | 127.2 | 2.01 |
| 100 | 220.2 | 1.98 |
| 200 | 323.1 | 1.89 |

# PCR band density data (from iBright Imaging system software)

**Table A4. Cell density data for Thp-1 cells incubated with LPS for 12 hours (Ex. 1).**

| Sample | Volume | Area | Density | Median | Local Cor. Vol. | Local Cor. Density | Rf |
|---|---|---|---|---|---|---|---|
| GAPDH C1 | 1.29E+07 | 585 | 21,983 | 21,461.00 | 4.57E+06 | 7,811 | 0.156 |
| TNF-α C1 | 8.15E+06 | 468 | 17,407 | 17,555.00 | 1.92E+06 | 4,100 | 0.629 |
| GAPDH C2 | 1.90E+07 | 585 | 32,420 | 31,685.00 | 9.35E+06 | 15,986 | 0.156 |
| TNF-α C2 | 1.05E+07 | 468 | 22,529 | 22,649.00 | 3.68E+06 | 7,869 | 0.629 |
| GAPDH 50 1 | 1.45E+07 | 468 | 30,922 | 31,385.00 | 6.46E+06 | 13,805 | 0.155 |
| TNF-α 50 1 | 1.40E+07 | 532 | 26,330 | 25,588.00 | 5.34E+06 | 10,042 | 0.630 |
| GAPDH 50 2 | 1.50E+07 | 468 | 31,934 | 32,205.00 | 6.63E+06 | 14,167 | 0.155 |
| TNF-α 50 2 | 1.50E+07 | 546 | 27,511 | 26,917.00 | 5.66E+06 | 10,363 | 0.630 |
| GAPDH 100 1 | 1.84E+07 | 546 | 33,666 | 33,032.00 | 8.45E+06 | 15,466 | 0.1542 |
| TNF-α 100 1 | 1.20E+07 | 456 | 26,238 | 25,588.00 | 4.45E+06 | 9,757 | 0.6303 |
| GAPDH 100 2 | 1.56E+07 | 570 | 27,336 | 25,654.00 | 6.07E+06 | 10,641 | 0.1517 |
| TNF-α 100 2 | 1.25E+07 | 546 | 22,820 | 22,358.00 | 3.68E+06 | 6,732 | 0.629 |

**Table A5. Cell density data for Thp-1 cells incubated with LPS for 12 hours (Ex. 2).**

| Sample | Volume | Area | Density | Median | Local Cor. Vol. | Local Cor. Density | Rf |
|---|---|---|---|---|---|---|---|
| GAPDH C1 | 1.21E+07 | 585 | 20,629 | 18,852.00 | 2.69E+06 | 4,591 | 0.150 |
| TNF-α C1 | 8.25E+06 | 456 | 18,097 | 18,199.00 | 1.07E+06 | 2,355 | 0.629 |
| GAPDH C2 | 1.84E+07 | 507 | 36,333 | 35,597.00 | 9.48E+06 | 18,695 | 0.151 |
| TNF-α C2 | 1.02E+07 | 492 | 20,665 | 20,870.00 | 2.24E+06 | 4,559 | 0.629 |
| GAPDH 50 1 | 1.69E+07 | 507 | 33,402 | 32,978.00 | 7.99E+06 | 15,757 | 0.151 |
| TNF-α 50 1 | 1.31E+07 | 546 | 23,995 | 23,203.00 | 4.17E+06 | 7,634 | 0.630 |
| GAPDH 50 2 | 1.62E+07 | 585 | 27,691 | 25,095.00 | 6.44E+06 | 11,003 | 0.150 |
| TNF-α 50 2 | 1.35E+07 | 574 | 23,574 | 22,538.00 | 4.18E+06 | 7,285 | 0.630 |
| GAPDH 100 1 | 1.75E+07 | 507 | 34,590 | 33,288.00 | 8.58E+06 | 16,915 | 0.151 |
| TNF-α 100 1 | 1.44E+07 | 532 | 27,073 | 26,660.00 | 5.58E+06 | 10,491 | 0.630 |
| GAPDH 100 2 | 1.63E+07 | 507 | 32,163 | 31,655.00 | 7.55E+06 | 14,891 | 0.151 |
| TNF-α 100 2 | 1.35E+07 | 532 | 25,293 | 24,206.00 | 4.54E+06 | 8,538 | 0.630 |

**Table A6. Cell density data for Thp-1 cells incubated with LPS for 12 hours Ex. 3.**

| Sample | Volume | Area | Density | Median | Local Cor. Vol. | Local Cor. Density | Rf |
|---|---|---|---|---|---|---|---|
| GAPDH C 1 | 1.84E+07 | 585 | 31,421 | 29,843.00 | 8.37E+06 | 14,305 | 0.150 |
| TNF-α C1 | 9.91E+06 | 468 | 21,180 | 21,155.00 | 2.48E+06 | 5,291 | 0.629 |
| GAPDH C 2 | 1.76E+07 | 570 | 30,943 | 27,468.00 | 7.62E+06 | 13,359 | 0.150 |
| TNF-α C2 | 1.13E+07 | 468 | 24,069 | 23,919.00 | 3.84E+06 | 8,214 | 0.629 |
| GAPDH 50 1 | 1.28E+07 | 468 | 27,438 | 26,681.00 | 5.17E+06 | 11,046 | 0.150 |
| TNF-α 50 1 | 1.00E+07 | 546 | 18,374 | 17,995.00 | 1.70E+06 | 3,109 | 0.628 |
| GAPDH 50 2 | 1.54E+07 | 570 | 27,072 | 24,480.00 | 6.17E+06 | 10,826 | 0.150 |
| TNF-α 50 2 | 1.26E+07 | 546 | 23,059 | 22,772.00 | 4.08E+06 | 7,472 | 0.628 |
| GAPDH 100 1 | 1.66E+07 | 507 | 32,753 | 31,333.00 | 8.24E+06 | 16,253 | 0.151 |
| TNF-α 100 1 | 1.18E+07 | 546 | 21,641 | 20,445.00 | 3.73E+06 | 6,832 | 0.628 |
| GAPDH 100 2 | 1.14E+07 | 456 | 25,058 | 24,053.00 | 4.25E+06 | 9,308 | 0.150 |
| TNF-α 100 2 | 9.99E+06 | 456 | 21,911 | 22,011.00 | 3.46E+06 | 7,590 | 0.628 |

# qPCR Data

This data was collected when qPCRs were carried out for each experiment using the MIC qPCR instrument (BioMolecular systems, Australia).

**Table A7. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for GAPDH (Ex. 1).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 1 | AC | 19.38932874 | 0.856199114 | 0.99827 |
| 2 | AC | 19.28082443 | 0.869697435 | 0.99861 |
| 3 | AC1 | 18.21937992 | 0.893314714 | 0.99885 |
| 4 | AC1 | 18.38813664 | 0.863695635 | 0.99808 |
| 5 | A50 | 19.09060523 | 0.882732495 | 0.99864 |
| 6 | A50 | 19.29035527 | 0.865977506 | 0.99861 |
| 7 | A50.1 | 19.46365385 | 0.864866407 | 0.9984 |
| 8 | A50.1 | 19.56651863 | 0.860698356 | 0.99842 |
| 9 | A100 | 18.96113961 | 0.905359825 | 0.99819 |
| 10 | A100 | 19.05844951 | 0.891051964 | 0.99864 |
| 11 | A100.1 | 19.6089853 | 0.851736061 | 0.99832 |
| 12 | A100.1 | 20.24370233 | 0.876615459 | 0.99879 |

**Table A8. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for GAPDH (Ex. 2).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 13 | BC | 21.13708234 | 0.860925907 | 0.99855 |
| 14 | BC | 21.03970005 | 0.880621358 | 0.99868 |
| 15 | BC1 | 19.21756066 | 0.862309655 | 0.99847 |
| 16 | BC1 | 19.04794929 | 0.897936314 | 0.99872 |
| 17 | B50 | 18.87079887 | 0.839166636 | 0.99885 |
| 18 | B50 | 18.84447968 | 0.832599856 | 0.99873 |
| 19 | B50.1 | 19.11773941 | 0.869910476 | 0.9985 |
| 20 | B50.1 | 19.12239565 | 0.87546126 | 0.99857 |
| 21 | B100 | 18.85097339 | 0.843803127 | 0.9988 |
| 22 | B100 | 18.93859087 | 0.904759156 | 0.99807 |
| 23 | B100.1 | 18.74705058 | 0.857370838 | 0.99861 |
| 24 | B100.1 | 19.10689858 | 0.872063054 | 0.99856 |

**TABLE A9. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for GAPDH (Ex 3).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 25 | CC | 19.0957157 | 0.886946098 | 0.99864 |
| 26 | CC | 19.17977337 | 0.860372003 | 0.99832 |
| 27 | CC1 | 18.6011912 | 0.864653335 | 0.99836 |
| 28 | CC1 | 18.70167474 | 0.855278736 | 0.99837 |
| 29 | C50 | 20.61954675 | 0.845811581 | 0.99835 |
| 30 | C50 | 20.58677819 | 0.841449597 | 0.99825 |
| 31 | C50.1 | 19.95175002 | 0.825996094 | 0.99876 |
| 32 | C50.1 | 19.78202007 | 0.842117442 | 0.9988 |
| 33 | C100 | 18.77844804 | 0.858031477 | 0.99857 |
| 34 | C100 | 18.82558295 | 0.845946809 | 0.99883 |
| 35 | C100.1 | 19.71245932 | 0.851111365 | 0.99876 |
| 36 | C100.1 | 20.05060425 | 0.794243017 | 0.99824 |

**Table A10 qPCR data for Thp-1 cells stimulated with LPS for 12 hours for GAPDH negative controls.**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 37 | NEG | -1 | -1 | |
| 38 | NEG | -1 | -1 | |

**Table A11. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for TNF-α (Ex. 1).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 1 | AC | 27.23560763 | 0.849697306 | 0.99875 |
| 2 | AC | 27.02209039 | 0.840220628 | 0.99884 |
| 3 | AC1 | 26.27259717 | 0.827118186 | 0.99847 |
| 4 | AC1 | 26.16518709 | 0.835267828 | 0.99851 |
| 5 | A50 | 25.41561441 | 0.842942708 | 0.99875 |
| 6 | A50 | 25.27480351 | 0.849753163 | 0.99869 |
| 7 | A50.1 | 26.15415285 | 0.839140718 | 0.99859 |
| 8 | A50.1 | 26.30358754 | 0.815716595 | 0.9983 |
| 9 | A100 | 25.33532382 | 0.848252957 | 0.99875 |
| 10 | A100 | 25.72119567 | 0.863494153 | 0.99883 |
| 11 | A100.1 | 26.80771342 | 0.85984002 | 0.99897 |
| 12 | A100.1 | 25.65712084 | 0.861624829 | 0.9988 |

**Table A12. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for TNF-α (Ex. 2).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 13 | BC | 28.13878152 | 0.77522857 | 0.99759 |
| 14 | BC | 27.86799259 | 0.799287955 | 0.99833 |
| 15 | BC1 | 27.22537607 | 0.827179469 | 0.99834 |
| 16 | BC1 | 27.22075255 | 0.829957475 | 0.99849 |
| 17 | B50 | 25.19575221 | 0.849399805 | 0.99865 |
| 18 | B50 | 25.60514595 | 0.824119817 | 0.99901 |
| 19 | B50.1 | 25.43486942 | 0.846040635 | 0.99881 |
| 20 | B50.1 | 25.67736071 | 0.860708733 | 0.99876 |
| 21 | B100 | 25.25658555 | 0.841298214 | 0.99754 |
| 22 | B100 | 25.28690221 | 0.84154523 | 0.99842 |
| 23 | B100.1 | 25.00716776 | 0.855942948 | 0.99882 |
| 24 | B100.1 | 25.15167363 | 0.843299029 | 0.99869 |

**Table A13. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for TNF-α (Ex. 3).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 25 | CC | 26.56900842 | 0.820132286 | 0.99894 |
| 26 | CC | 26.64092804 | 0.87104907 | 0.99891 |
| 27 | CC1 | 25.94338175 | 0.845113542 | 0.99873 |
| 28 | CC1 | 26.01040514 | 0.843318185 | 0.99873 |
| 29 | C50 | 27.05323984 | 0.854337253 | 0.99896 |
| 30 | C50 | 27.12461142 | 0.847855141 | 0.99885 |
| 31 | C50.1 | 25.84379139 | 0.857573 | 0.99885 |
| 32 | C50.1 | 25.80178085 | 0.853952039 | 0.99891 |
| 33 | C100 | 25.08844893 | 0.850581664 | 0.99882 |
| 34 | C100 | 25.22112663 | 0.846462271 | 0.99859 |
| 35 | C100.1 | 26.01527169 | 0.857909355 | 0.99892 |
| 36 | C100.1 | 25.49539992 | 0.889853253 | 0.99845 |

**Table A14. qPCR data for Thp-1 cells stimulated with LPS for 12 hours for TNF-α negative controls.**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 37 | NEG | -1 | -1 | |
| 38 | NEG | -1 | -1 | |

**Table A15. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for GAPDH (Ex. 1).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| **1** | E1 con | 31.66842411 | 0.989747288 | 0.99927 |
| 2 | con | 31.14100642 | 1.025981643 | 0.99918 |
| 3 | 10 | 25.51918274 | 1.159156427 | 0.99837 |
| 4 | 10 | 25.35373566 | 1.042758007 | 0.99921 |
| 5 | 50 | 27.31661331 | 1.038015067 | 0.99911 |
| 6 | 50 | 27.0505123 | 1.067813985 | 0.9988 |

**Table A16. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for GAPDH (Ex. 2).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 7 | e2 con | 28.10514166 | 1.074944764 | 0.99877 |
| 8 | con | 28.25332881 | 1.038306735 | 0.99904 |
| 9 | 10 | 27.77982907 | 1.095399625 | 0.99824 |
| 10 | 10 | 27.82262828 | 1.085218651 | 0.99857 |
| 11 | 50 | 29.55989162 | 1.014939725 | 0.99939 |
| 12 | 50 | 29.29790118 | 1.026252455 | 0.99929 |

**Table A17. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for GAPDH (Ex. 3).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 13 | 3e con | 26.09897467 | 1.062308492 | 0.99889 |
| 14 | con | 25.35857867 | 1.044015775 | 0.99917 |
| 15 | 10 | 23.49089712 | 1.18587482 | 0.99823 |
| 16 | 10 | 23.09305019 | 1.127635148 | 0.99796 |
| 17 | 50 | 25.54667439 | 1.187977927 | 0.9982 |
| 18 | 50 | 25.45766932 | 1.157945941 | 0.99842 |

**Table A18. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for GAPDH negative control.**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 19 | neg | -1 | -1 | |

**Table A19. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for TNF-α (Ex. 1).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| **20** | E1 con TNF-α | 35.06862591 | 0.614640146 | 0.9871 |
| 21 | con TNF-α | 31.19164465 | 0.837029968 | 0.99733 |
| 22 | 10 TNF-α | 31.04683334 | 0.79969027 | 0.99506 |
| 23 | 10 TNF-α | 31.32083176 | 0.822327728 | 0.99664 |
| 24 | 50 TNF-α | 31.22181432 | 0.764645728 | 0.99554 |
| 25 | 50 TNF-α | 30.9955071 | 0.835738519 | 0.99788 |

**Table A20. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for TNF-α (Ex. 2).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 26 | e2 TNF-α | 30.98145978 | 0.842370063 | 0.99777 |
| 27 | con TNF-α | 30.0092559 | 0.930471866 | 0.99897 |
| 28 | 10 TNF-α | 29.38431458 | 1.024069115 | 0.99906 |
| 29 | 10 TNF-α | 30.50525001 | 0.915103561 | 0.99844 |
| 30 | 50 TNF-α | 30.25220243 | 0.91864061 | 0.99878 |
| 31 | 50 TNF-α | 31.62480309 | 0.794592856 | 0.99748 |

**Table A21. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for TNF-α (Ex. 3).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 32 | 3e con TNF-α | 31.12001615 | 0.794720785 | 0.99665 |
| 33 | con TNF-α | 31.37621845 | 0.807608009 | 0.99531 |
| 34 | 10 TNF-α | 31.14693632 | 0.799694499 | 0.99663 |
| 35 | 10 TNF-α | 30.47904859 | 0.83009539 | 0.99661 |
| 36 | 50 TNF-α | 31.51073571 | 0.825293308 | 0.997 |
| 37 | 50 TNF-α | 31.54494867 | 0.810910142 | 0.99676 |

**Table A22. qPCR data for Thp-1 cells stimulated with LPS for 4 hours for TNF-α negative control.**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 38 | neg | 30.78079295 | 0.847089331 | 0.99827 |

**Table A23. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for GAPDH (Ex. 1).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 1 | B1 Control | 18.69087635 | 0.893804824 | 0.99994 |
| 2 | B1 Control | 18.57205422 | 0.913582121 | 0.99992 |
| 3 | B1 500ng | 26.28813319 | 0.895436693 | 0.99997 |
| 4 | B1 500ng | 25.70616598 | 0.878339556 | 0.99996 |
| 5 | B1 1500ng | 29.62530421 | 0.910393458 | 0.99999 |
| 6 | B1 1500ng | 29.6395002 | 0.90506872 | 0.99995 |
| 7 | B1 U 500ng | 20.54322155 | 0.890644261 | 0.99999 |
| 8 | B1 U 500ng | 20.53334545 | 0.90332767 | 0.99999 |
| 9 | B1 U 1500ng | 19.43411722 | 0.903879442 | 0.99999 |
| 10 | B1 U 1500ng | 19.54103313 | 0.892420492 | 0.99999 |

**Table A24. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for GAPDH (Ex. 2).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 11 | B2 Control | 23.42913901 | 0.89746006 | 0.99999 |
| 12 | B2 Control | 23.48130515 | 0.907602063 | 0.99996 |
| 13 | B2 500ng | 23.29998009 | 0.912458895 | 0.99993 |
| 14 | B2 500ng | 23.30395455 | 0.89722406 | 0.99992 |
| 15 | B2 1500ng | 25.22026601 | 0.906334536 | 0.99997 |
| 16 | B2 1500ng | 25.04800754 | 0.969011559 | 0.99994 |
| 17 | B2 U 500ng | 20.24403607 | 0.893014071 | 0.99997 |
| 18 | B2 U 500ng | 19.81529475 | 0.89480579 | 0.99996 |
| 19 | B2 U 1500ng | 22.21683444 | 0.876711377 | 0.99999 |
| 20 | B2 U 1500ng | 22.26592869 | 0.878444517 | 0.99999 |

**Table A25 qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for GAPDH (Ex. 3).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 21 | B3 Control | 20.84149876 | 0.86744179 | 0.99999 |
| 22 | B3 Control | 20.49658408 | 0.882827569 | 0.99998 |
| 23 | B3 500ng | 26.57491811 | 0.934667683 | 0.9999 |
| 24 | B3 500ng | 26.59913546 | 0.929367088 | 0.99992 |
| 25 | B3 1500ng | 25.66749081 | 0.873938128 | 0.99998 |
| 26 | B3 1500ng | 25.32048788 | 0.86665606 | 0.99892 |
| 27 | B3 U 500ng | 21.56846103 | 0.901213725 | 0.99998 |
| 28 | B3 U 500ng | 21.82937885 | 0.900035751 | 0.99996 |
| 29 | B3 U 1500ng | 22.68531981 | 0.939741559 | 0.99993 |
| 30 | B3 U 1500ng | 22.86421808 | 0.93318024 | 0.99993 |

**Table A26. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for GAPDH negative controls.**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 31 | neg | -1 | -1 | |
| 32 | neg | -1 | -1 | |

**Table A27. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for TNF-α (Ex. 1).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 1 | B1 Control | 25.23825063 | 0.934950474 | 0.99996 |
| 2 | B1 Control | 25.62831079 | 0.904541313 | 0.99997 |
| 3 | B1 500ng | 30.17121317 | 0.899820033 | 0.99991 |
| 4 | B1 500ng | 30.63056519 | 0.932111241 | 0.99988 |
| 5 | B1 1500ng | | | |
| 6 | B1 1500ng | | | |
| 7 | B1 U 500ng | 28.0070355 | 0.883778086 | 1 |
| 8 | B1 U 500ng | 27.5855153 | 0.906156841 | 0.99998 |
| 9 | B1 U 1500ng | 30.29722605 | 0.886174054 | 0.99998 |
| 10 | B1 U 1500ng | 31.0251914 | 0.848738681 | 0.99988 |

**Table A28. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for TNF-α (Ex. 2).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 11 | B2 Control | 28.1253283 | 0.911861792 | 0.99991 |
| 12 | B2 Control | 28.08500316 | 0.916738616 | 0.99994 |
| 13 | B2 500ng | 30.97542075 | 0.88275932 | 0.99991 |
| 14 | B2 500ng | 33.06648151 | 0.787388971 | 0.9988 |
| 15 | B2 1500ng | 8.791426482 | 0.039146744 | 0.9302 |
| 16 | B2 1500ng | | | |
| 17 | B2 U 500ng | 28.24608311 | 0.882056009 | 1 |
| 18 | B2 U 500ng | 28.19534939 | 0.921334052 | 0.99999 |
| 19 | B2 U 1500ng | 30.25479583 | 0.959399391 | 0.99996 |
| 20 | B2 U 1500ng | 29.56594905 | 0.917521891 | 0.99988 |

**Table A29. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for TNF-α (Ex. 3).**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 21 | B3 Control | 27.67190262 | 0.916666826 | 0.99992 |
| 22 | B3 Control | 27.89064927 | 0.925072331 | 0.99992 |
| 23 | B3 500ng | 31.01995223 | 0.915356066 | 0.99992 |
| 24 | B3 500ng | 31.83425541 | 0.907715262 | 0.99962 |
| 25 | B3 1500ng | 31.18259736 | 0.894062627 | 0.9999 |
| 26 | B3 1500ng | 31.65002215 | 0.900140132 | 0.99984 |
| 27 | B3 U 500ng | 27.87551662 | 0.773970155 | 0.99941 |
| 28 | B3 U 500ng | 28.04288115 | 0.869938694 | 0.99989 |
| 29 | B3 U 1500ng | 32.23579271 | 0.863371171 | 0.99946 |
| 30 | B3 U 1500ng | 32.54315002 | 0.902493634 | 0.99911 |

**Table A30. qPCR data for Thp-1 cells stimulated with HSP60 for 4 hours for TNF-α negative controls.**

| Well | Sample | Cq | Efficiency | $R^2$ |
|---|---|---|---|---|
| 31 | neg | -1 | -1 | |
| 32 | neg | -1 | -1 | |