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A study on the immunoregulatory properties of camel milk derived lipids

(Camelus dromedarius)

By

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BSc . MSc

A thesis submitted in partial fulfilment of the requirements of Cardiff Metropolitan University for the degree of Doctor of Philosophy (PhD) in the discipline of Biomedical Science.

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The research contained within this thesis was undertaken under the auspices of

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This work has not previously been accepted in substance for any other degree and is not currently submitted in candidature for any degree.

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Abstract

Camel (*Camelus dromedarius*) milk is believed to have beneficial effects in inflammatory diseases such as diabetes, however, there are few studies reporting the immunoregulatory properties of the milk's lipids. This study aimed to extract the lipid component from camel milk and investigate its ability to regulate macrophage inflammatory responses using the human macrophage derived cell-line, dTHP-1. Omani camel milk lipid was over 95% triglyceride (TG) with major saturated fatty acids (SFAs) identified as palmitic acid (35.28%), myristic acid (14.46%), stearic acid (7.40 %); and unsaturated (USFAs) oleic acid (19.31%) and palmitoleic acid (14.00%). These fatty acids were identified as fatty acid methyl esters and analysed by Gas Chromatography – Mass Spectrometer (GC-MS). The camel milk's total lipids (TL) total free fatty acids (TFAs) and unsaturated free fatty acids (USFAs) significantly reduced glycated protein (gBSA) proinflammatory cytokine secretion (TNF-α, IL-1β, IL-18 and IL-6) by dTHP-1 cells. In addition the lipids down regulated gene expression of markers of the proinflammatory M1 macrophage phenotype CD86 but in contrast the lipids significantly enhanced markers of the M2 phenotype (CD206, CD163, Dectin-1, IL-1Ra, and IL-10. TL also enhanced the translocation of the p50/p50 homodimers of the nuclear transcription factor NF-κB, at the expense of p50/p65 heterodimer translocation. The lipids significantly down regulated the expression and activation of the inflammasome NLRP3/caspase-1/ASC assembly formation. Finally all the lipid componets up-regulated significantly expression of the Ten-Eleven Translocation-2 (TET-2) transcription regulator, linking their actions to this recently discovered regulator of the NLRP3 inflammasome. This study reports evidence that camel milk lipids are *in-vitro* highly immunoregulatory in macrophages and their consumption may be beneficial in inflammatory diseases such as diabetes.

"Do they not look at the camels, how they are created?"

(Holy Quran, Al-Ghaashiyah 88:17)

This thesis is dedicated to my parents

My dear father Hamdan Salim Mohammed Al-Nasseri

And to my dear mother Moza Rashed Salim Al-Nasseri

Who both passed away before sharing with me these moments and whom inspired me in all my life, and gave their love and kindness to me and to my brothers and sisters

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And to my sweetest daughters, Thank you all for your love and patience

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Abbreviations

Δ	Delta (change)
AGEs	Advanced glycosylation End Products
ANOVA	Analysis of variance
AP-1	Activator protein-1
Arg	Arginine
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
Atherosclerotic lesion	Stable atherosclerotic plaques, rich in extracellular matrix and smooth muscle cells.
Bis-Tris	Bis-(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
Bovine milk	Cow's milk
BSA	Bovine serum albumin
Ca	Calcium
Camelus dromedarius	One-humped camel
Caspase-1	Interleukin-1 (IL-1 beta)converting enzyme (ICE)
CD	Cluster of differentiation
CD14	Cluster of differentiation 14
CD163	Cluster of differentiation 163
CD206	Cluster of differentiation 206
CD36	Cluster of differentiation 36
CD86	Cluster of differentiation 86
Cl	Chloride
CT	Threshold cycle
DAMPs	Danger-associated molecular pattern
Dectin-1	C-type lectin domain family 7 member A
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dTHP-1	Differentiated human monocytic cell line THP-1
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FABP4	Fatty acid binding protein 4
FAs	Fatty acids
FCS	Foetal calf serum
gBSA	Glycated Bovine serum albumin
GC-MS	Gas chromatography–mass spectrometry
Glu	Glutamic acid
Gly	Glycine
HDL	High density lipoprotein
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule 1
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IKK	Inhibitor of NF-κB Kinase
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-18	Interleukin-18
IL-1Ra	Interleukin-1 Receptor Antagonist
IL-1β	Interleukin- 1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IRF5	Interferon regulatory factor 5
IκBα, IκBβ	Inhibitors of NF-κB
K	Potassium
LCFAs	Long-chain fatty acids

LDL	Low-density lipoprotein
Leukocytes	White blood cell
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene (88)
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor-kappa B
NF-kB (p50/p65)	p50 and p65 subunits of NF-kappa B
NLRP3	Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing-3
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid having two or more carbon-carbon double bonds in its carbon chain
qRT-PCR	Quantitative- reverse transcription- polymerase chain reaction
RAGE	Receptor for AGE
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase - polymerase chain reaction
SCFAs	Short-chain fatty acids with two to six carbon atoms
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes mellitus
TFAs	Total free fatty acids
TGF-β	Transforming Growth Factor-β
THP-1	Human leukaemia monocytic cell line
TL	Total lipids
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor- alpha
Tris	Tris-(hydroxymethyl)aminomethane
TT	Tribulus terrestris
USFAs	Usaturated free fatty acids
v/v	Volume/ Volume
VC	Vehicle Control
VCAM-1	Vascular cell adhesion molecule 1
w/v	Weight/Volume

CHAPTER 1

General introduction

1.1. Camel domestication

Camels have played an important part in the daily life and prosperity of the Bedouin people, who inhabit desert regions. The camel has played a major role in the Bedouin's ability to survive in the particularly harsh, hot and arid climate of the deserts of Africa, Arabia and Asia. Camels are among the animals that are of economic value for the Bedouins who live in the Arabian Peninsula and in pastoralists of East Africa (Al-Awadi and Al-Judaibi, 2014; Mochabo *et al*., 2006).

Archaeologists discovered the remains of camels from the early bronze age at site of Ra's al-Hadd (HD-6) in the Sultanate of Oman, that has been dated to between 2890-2580 B.C, and is the oldest evidence of camels in the Sultanate. (Curci *et al.,* 2014). In another publications (Mohamed, 2006; Peters, 1997), it was reported that; the camel was domesticated in the Arabian Peninsula, possibly as early as the 4th millennium B.C.

In the south region of Salalah of Oman (Dhufar), local tribes tamed the camel, and mainly used it for consuming its milk (ElMahi, 2011; Bulliet, 1975). Camels have also played an important part in the lives of traders travelling with their goods and the transportation of different trading items and cultural dialogue between Arabian Peninsula, the Sahara and the Levant to the Far East and Asia, and northern Arabia at the trading routes (Burger, 2016).

1.1.2. Camel physiology

Camels are even-toed ungulates in the genus *Camelus* that has on its back a distinctive fatty hump. These humps act as fat stores and minimize the insulating effect of fat distributed over the whole body, helping camels survive in hot climates. When this fat is metabolized, it yields more than one gram of water for every gram of fat processed, releasing energy (Fahmy, 2015). Camels have a series of physiological adaptations that allow them to withstand long periods of time without any external source of water. For

examples; the camel's kidney has a strong capacity of water reabsorption and a faculty to eliminate very concentrated urine (3200 mOsm); and characterized by a long loop of henle, and a well-developed medulla (the ratio medulla: cortex is about 4:1). During thirst, the kidneys reduce water losses both by decreasing the glomerular filtration rate and by increasing the tubular re-absorption of water (Gebreyohanes and Assen, 2017). The dromedary camel can drink only once every 10 days even under very hot conditions, and can lose up to 30% of its body mass due to dehydration. Another physiological feature of camel its red blood cells shape, unlike other mammals, camel red blood cells are oval rather than biconcave in shape (Figure 1.1) (Gaughan, 2011), this facilitates the flow of red blood cells during dehydration and withstanding extraordinary osmotic variation without rupturing when drinking large amounts of water as a 600/kg camel can drink 200/litres of water in three mins (National Geographic Society, 2012; Eitan *et al*., 1976). The number of camels are estimated at about 23.9 million worldwide, with 89% being the one-humped dromedary (*Camelus dromedarius*) and 11% the two-humped bactrian (*Camelus bactrianus*) that is found in the cold deserts of Asia (Sharma and Singh, 2014). The average life expectancy for a camel has been reported as 25 to 30 years, or 40 to 50 years depending on the environment and their living conditions (Fahmy, 2015). Camels have their first calf typically at the age 6 or 7 years, and they can have between 8-10 calves during their life with pregnancy lasting 13 months. Depending on management and environmental conditions the average lactation length in camels is 12 months, but can range from as little as 9 to as long as 18 months (Khan and Iqbal, 2001). After delivery, the young are not separated from the mother, however, if that happens, the mother stops producing milk (Brezovecki *et al*., 2015). Camels graze on all desert plants, with the main forage that is eaten from trees and shrubs of species such as; *Acacia, Atriplex, Indigofera, Dispera*, and *Tribulus*. *Tribulus Terrestris* (*TT*) is a small leafy plant that camels graze

on, its extract can present in the camel's milk and this plant is recommended as a therapy for infertility, impotence, and low libido and has been used as a traditional ancient medicine in Greece, China and India (Pokrywka *et al*., 2014). All of this plant parts are useful in cases of kidney stones, high cholesterol, hypertension, and as a diuretic (Chhatre *et al*., 2014). Currently, *TT* is mainly marketed as a testosterone booster, and as enhancers for performance in athletes (Pokrywka *et al*., 2014). The highest content of moisture, electrolytes and oxalates can be found in *Acacia, Salsola and Atriplex* (Figure 1.2), and these are the preferred diet plants to camel (Yagil, 1982). The foraging of plants may be one of the reasons of the salty taste of camel milk attributed to *Atriplex* and *Acacia* plants that have a high salt content of chloride (Singh *et al*., 2017).

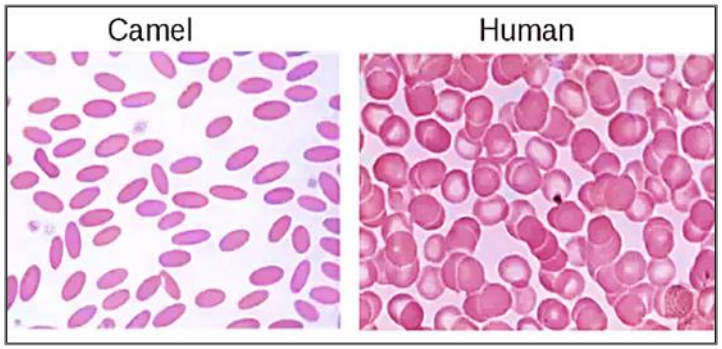


Figure 1.1. Camel red blood cells and human red blood cells. In camel red blood cells are oval rather than circular as in human red blood cells, this facilitates the flow of red blood cells in camel during dehydration, and withstanding extraordinary osmotic variation without rupturing when drinking large amounts of water during rehydration. Retrieved From: https://www.quora.com/why do camels have nucleated red blood cells).

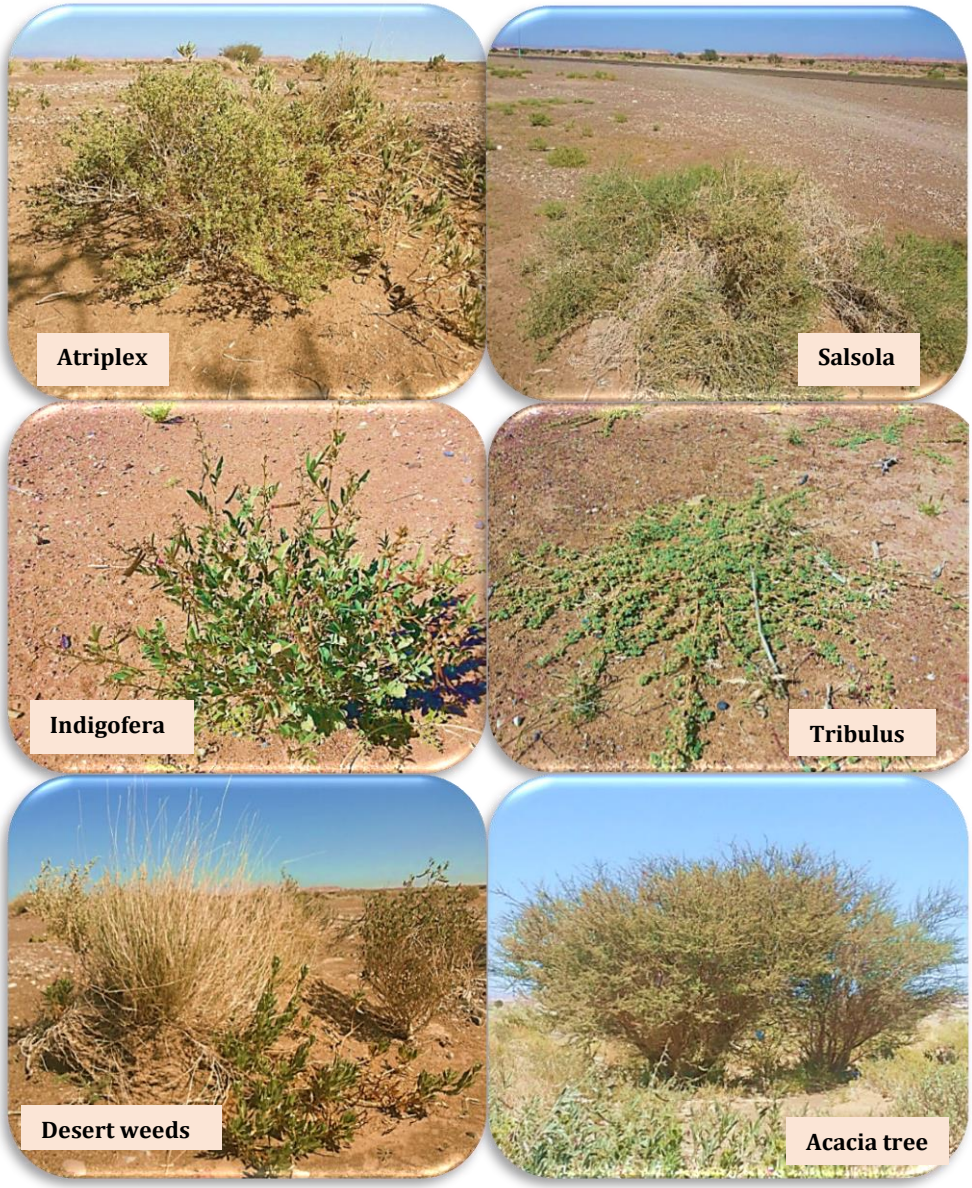


Figure 1.2. Typical desert plants that are part of camel's diet. The highest content of moisture, electrolytes and oxalates can be found in (*Acacia, Salsola and Atriplex*) which are an important elements needed during the dry season for the camels and their owners. Consequently, these elements are present in the camel milk. Pictures supplied by and used with permission: Sanad Hamad Hamdan Al-Nasseri. Tanam village, Ebri, Al-Dhahirah Region, Sultanate of Oman, 2018.

1.1.3. Composition of camel milk

Milk is important in the human diet as a source of various nutrients and it has been suggested can play a role in the management or prevention of several diseases (Kergoat *et al*., 1992). Camels are adapted to live in the desert in highly challenging conditions, with a lack of water and high temperatures and only modest quantities of food available. However, camels are capable of providing milk with high contents of water (Table 1.1), and nutrition to feed their calves and owners, with daily yields of between 3 to 10 kg during the lactation period (Gizachew *et al*., 2014). It was reported by Yagil and Etzion (1980), that there is a 4.3 to 1.1% decrease in fat content in camel milk when produced by thirsty camel, with an increase in water content. The concentrations of Sodium (Na), Potassium (K), and Chloride (Cl) in milk increased, with Calcium (Ca) and Magnesium (Mg) concentration decreased.

Composition %	Range %	Reference
Fat	1.2 - 6.4	Zibaee ,2015
Ash	0.35 - 0.95	Nikkhah, 2011
Protein	2.5 - 4.5	Khan and Iqbal, 2001
Non-fat solids	8.9 -14.3	Nikkhah, 2011
Lactose	2.9 - 4.12	Khaskheli *et al*.,2005
Water	85.7- 91.2	Yagil and Etzion, 1980

Table 1.1. The major components of camel milk during normal living conditions. The main constituent of camel milk as for all milks, is water.

Camel milk composition varies as compared to the milk of other mammals, with camel milk possessing a smooth white appearance, due to its fat containing a large proportion of polyunsaturated fatty acid (PUFA), These fatty acids (FAs) having two or more carbon-carbon double bonds in their carbon chain and are totally homogenized within the milk (Yadav *et al*., 2015). However, the foamy enrichment of camel milk may be attributed to its feeding on desert shrubs and herbs (Kumar *et al*., 2016). Camel milk contains a reduced amount of short-chain fatty acids (SCFAs) as compared to that found in bovine milk (Table 1.2), (Barłowska *et al*., 2011).

Fatty acids	Camel milk (%)	Cow milk (%)
***C4:0**	0.34	3.84
***C6:0**	0.29	2.28
***C8:0**	0.27	1.69
***C18:3**	0.51	0.25
MUFA	33.03	25.56
SFAs	64.86	71.24

Table 1.2. Difference in fatty acids profile of camel milk and cow milk (% of total fatty acids). *Saturated short-chain fatty acids (SCFAs) in camel milk are reduced than cow milk, meanwhile, the percentage of mono-unsaturated fatty acids (MUFAs) are increased in camel milk. Camel milk rich with *Poly-unsaturated fatty acid (PUFA) linolenic acid (C18:3). Adapted from (Barłowska *et al*., 2011).

However, Yoganandi *et al*., (2015), in their study compared dromedary camel milk composition collected from three different districts in India, with cow and buffalo milk. They reported; the average fat content in camel milk was in districts 1and 2 (4.43%), district 3 (2.90%), in cow (4.68%), and in buffalo (6.38%). The protein content in camel, cow and buffalo was similar being 2.66%, 3.32% and 3.87% respectively. Also

they showed that camel milk contains higher chloride (0.25%) than cow (0.11%), and buffalo (0.12%), (Table 1.3).

Milk composition			
Camel	**Lipid (%)**	**Protein (%)**	**Chloride (%)**
District 1	4.43	2.66	0.20
District 2	4.43	2.66	0.20
District 3	2.90	2.66	0.25
Cow	4.68	3.32	0.11
Buffalo	6.38	3.87	0.12

Table 1.3. Variation in camel, cow and buffalo milk composition (camel milk in three Indian districts). Camel milk contains lower protein and higher chlorine content as compared with cow and buffalo milk. Adapted from (Yoganandi *et al*., 2015).

Camel milk is rich with desert plant derived nutrients and minerals including; Mn (Manganese), Cu (Copper), Fe (Iron), Na (Sodium), K (Potassium), and Zn (Zinc), the concentrations of these minerals associated with seasonal variations and by improved grazing of camel on fresh grasses and herbs which are stimulated by the winter rains, other factors affecting the concentrations of these minerals are breeds differences , and water intake in winter or summer (Singh *et al*., 2017; Haddadin *et al*., 2008). Desert plants are reported as having medicinal benefits among the Bedouins, which may be reflected in camel milk and its potential therapeutic characteristics, as having anti-hypertensive, anti-diabetic and anti-carcinogenic properties (Sharma and Singh, 2014). Camel milk also demonstrates antioxidant properties and reduces the activity of free radicals and reactive oxygen species (Alabdulkarim, 2012). This antioxidant

activity may be due to the milk being rich in vitamin C, at a level that is three times that found in cow milk (Rasheed, 2017; Nikkhah, 2011).

In the following studies, authors have reported a health benefits attributed to the various components of the milk. An investigation carried out to evaluate anticancer activity of several milks (donkey, sheep, goat, cow and mare) including camel milk and their casein and whey proteins against human breast cancer MCF7 cell line, results demonstrated strong anti-tumour cytotoxic activities on growth inhibition of MCF7 cells (Shariatikia *et al*., 2017). An *in vivo* anti-tumour potential of camel's milk against hepatocellular carcinoma was reported in Wistar rats and furthermore, an improvement in the side effects (nephrotoxicity, neurotoxicity, ototoxicity, nausea and vomiting) of the anti-cancer agent (cisplatin) a chemotherapy drug, treated the rats with cisplatin beside the camel milk treatment. Camel milk managed to decrease hepatocarcinogenesis in Wistar rats in the presence of cisplatin (El Miniawy *et al*., 2017).

Camel milk lactoferrin is a multifunctional glycoprotein, known for its therapeutic potential as anti-bacterial, anti-inflammatory and anti-cancer remedy (Gader and Alhaider, 2016). The potential biological activities of camel milk lactoferrin (iron-binding protein of milk), was found to inhibit the proliferation of colon cancer cells HCT-116 *in vitro*, in a dose and time dependent manner at concentrations of 3mg/mL and 5mg/mL (Habib *et al*., 2013). The evidence of the health benefits of camel milk was recently systematically reviewed, on the clinical effectiveness and value of camel milk in a range of diseases , including diabetes, autism, cancer, various infections, heavy metal toxicity, colitis, and alcohol-induced toxicity, that included data from 430 studies (Mihic *et al*., 2016). Currently, there is insufficient available evidence, camel milk should not replace standard therapies for any indication in humans. In addition, although camel milk has been widely characterized concerning casein and whey

proteins (Saadaoui *et al*., 2013), little work has been undertaken on the potential role of the milk's lipids in regulating diseases such as T2D and atherosclerosis.

1.1.4. Anti-diabetic benefits of camel milk in human and animal studies

Although most studies (Al-Ayadhi and Elamin, 2013; Hamad *et al*., 2011; Sboui *et al*., 2010; Agrawal *et al*., 2009; Shabo *et al*.,2005) using both human and animal models do show a clinical benefit when camel milk was used as an intervention or as part of the diet, no specific mechanism or clear role for the milk was shown. Camel milk's anti-diabetic characteristics has been proposed as it being due to it contains 'insulin-like' small molecule substances which is encapsulated in nanoparticles (lipid vesicles) at a high concentration of approximately 52 micro unit/mL insulin (Agrawal *et al*.,2005). Being coated in lipid vesicles makes it possible for insulin like peptide to pass through the stomach and enter into circulation (Malik *et al*., 2012). Several studies have linked the consumption of camel milk with potential benefits in regulating diabetes (Ejtahed *et al*., 2015; Al Kanhal, 2010; Sboui *et al*., 2010; Mohamad *et al*., 2009). In a randomised controlled study undertaken in patients with type 1 diabetes, the effect of camel milk on glycemic control was evaluated. Patients were divided into two groups; group 1 receiving usual care (diet, exercise and insulin) and group 2 received 500mL camel milk with usual care for 3 months. The after 3 months and at beginning of the study, glycated haemoglobin A1c (HbA1c), lipid profile, plasma insulin and c-peptide was evaluated. There were significant improvements in fasting blood sugar and HbA1c levels (115.66 ± 7.17 to 100 ± 16.2; $p< 0.002$) and a significant 30% reduction in doses of insulin requirement in 92% of patients of group 2 receiving 500mL camel milk compared to group1 (Agarwal *et al*., 2003). Studies using animal models and in humans, both demonstrate that, the consumption of camel milk is effective in

regulating diabetes. For instance, in a study utilising a rat model of diabetes (Mansour *et al*., 2017) camel milk supplementation regulated serum biochemical measurements that altered after diabetes induction including a number of molecules associated with insulin secretion and production. In a review of *in vivo* study Shori (2015), reported a study on alloxan-induced diabetic dogs fed with a supplement of 500mL camel milk for 5 weeks. This supplementation resulted in a significant decrease in the level of blood glucose from 10.88 ± 0.55mmol/L to 5.77 ± 0.44mmol/L. Dietary supplementation with camel milk and its whey protein, demonstrated an enhanced range of beneficial inflammatory responses, including restoring the levels of oxidative stress and inflammatory cytokines (Ebaid *et al*., 2011) during wound healing, in diabetic albino rats. These effects were also observed in streptozotocin (a chemical that is particularly toxic to the insulin-producing β-cells of the pancreas in mammals) (Khan *et al*., 2013). Apart from enhanced wound closure a significant modulation in a number of regulatory and inflammatory cytokine, interleukins IL-4, IL-10, IL-1β, IL-6, and tumour necrosis factor-α (TNF-α) levels were observed in the whey protein treated animals (Badr *et al*., 2017). In a similar study (Badr, 2013) demonstrated beneficial wound repair effects in diabetic mice that included enhanced expression of the potent angiogenic regulator transforming growth factor-β (TGF-β).

Studies undertaken in India, in the Raica community of North-west Rajasthan, who consume large quantities of camel milk, report a zero prevalence of diabetes, as compared to a prevalence of 5.5% in non-Raica communities, who did not consume camel milk (Agrawal *et al*., 2011; Agrawal *et al*; 2007). Clearly, other factors including genetics and life-style, could be responsible for these differences, however, this data is suggestive of a potential link between camel milk consumption and diabetes prevention. At the same time, it has been found that the daily intake of insulin was reduced in type I diabetic patients in the non-Raica subjects with diabetes, when they

consumed camel milk (Agrawal *et al*., 2011; Agrawal *et al*; 2007). In type 1 diabetes, one of the complications facing those patients is microvascular nephropathy, which is related to the level of microalbuminuria present in their urine. When diabetics with microalbuminuria consumed camel milk for 6 months, a significant reduction in the level of microalbuminuria was reported (119.48 ± 1.68 to 22.52 ±2.68) with a concomitant decline in insulin doses (Agrawal *et al*., 2009). For reasons such as this, it has been proposed that camel milk consumption can act as a natural remedy, inexpensive treatment for chronic inflammatory diseases such as diabetes, inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease (Arab *et al*.,2014) and chronic hepatitis B (Saltanat *et al*., 2009).

1.1.5. Dairy products

Consumption of regular fat dairy products and in particular cholesterol therein, is known among the public to contribute in raising saturated fatty acids (SFAs) which are known to be involved in the etiology of cardiovascular disease (CVD) (Briggs *et al*., 2017). This in turn affects blood lipid concentrations and other cardiometabolic disease risk factors, such as blood pressure and inflammation markers (Lordan *et al*., 2018). United States and Canadian dietary guidelines recommend to consume low-fat dairy products as alternative to regular-fat products (Drouin-Chartier *et al*., 2016; Mozaffarian, 2016; Kalergis *et al*., 2013; Kris-Etherton *et al*., 2009; Kris-Etherton *et al*.,2007). However, conflicting research findings in relation to dairy products and their negative impact role in health, has generated considerable misunderstanding and confusion for the general public. This is in part due to the role of advocacy groups with their own agenda which not necessarily based on any evidenced based studies (Odia *et al*., 2015). In a randomized controlled trial (RCT) and a meta-analyses studies,

suggested that there is no risk effects of dairy intake, regardless of the content of dairy fat, on cardiometabolic variable, such as lipid risk factors, including blood pressure, systemic inflammation, and insulin resistance(Benatar *et al*.,2013). However, SFAs are safe if consumed as part of a multi meal, which contains complex foods such as milk, cheese, yogurt, or other total dairy food or low fat dairy, cheese and yogurt (Drouin-Chartier *et al*., 2016). There is no effect of dairy intake on blood pressure (BP) or vascular function, low density lipoprotein (LDL), and high density lipoprotein (HDL) known as good cholesterol in healthy individuals (Chiu *et al*., 2016; Abdullah *et al*., 2015). Most of the study results related to consumption of high/low fat dairy products, with no effect on inflammatory biomarkers concentration in the blood such as adiponectin, IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP1), and TNF-α (Labonte *et al*.,2014; Jones *et al*., 2013; Van Loan *et al*., 2011).

1.2. Fatty acids and fatty acids classification

The Western diet contains 30% to 40% of calories obtained from the consumption of oils, margarines, dairy products, and various meats that are sources for triglycerides (TG) (Ros, 2000). These dietary lipids contain mainly FAs, the major structural component of TG mass that contain 3 FAs (Figure 1.3). Other dietary animal derived products contain a substantial level of cholesterol. Other dietary derived fats can stimulate significantly serum cholesterol level and, thereby, impact and act as a risk of atherosclerosis and other related complications (Badimon and Vilahur, 2012; Laposata, 1995). FAs are considered as large group of lipids, which are essential in the maintenance and growth of health in humans and other mammals (Leonard *et al*., 2004). Camel milk, contains a number of long chain fatty acids (LCFAs) (92–99%), of which unsaturated fatty acids (USFAs) concentration can range from 35–50%. These

camel milk components have been reported as being able to reduce serum lipids in humans and potentially play a role in regulating cardiovascular diseases (Singh *et al*., 2017), including coronary heart disease (CHD) and stroke. These diseases are also linked to cardiovascular risk factors such as diabetes, high blood pressure and obesity (Reddy and Katan, 2004).

FAs consist of carbon chains with a methyl group (CH_3) at one end and a carboxyl group (COOH) at the other. The carbon chain may be saturated 'filled' with hydrogen with straight hydrocarbon chains, or it may contain one or more carbon–carbon double bonds, which can occur in different positions and are defined as monounsaturated fatty acids (MUFA) and polyunsaturated (PUFA) (Rustan and Drevon, 2001). In PUFA, the number, position of cis hydrogen atoms on either side of the double bond are oriented in the same direction. Trans fatty acids have the same straight chain, three-dimensional structure as saturated fatty acids. State of the double bond have dramatic effects on cell function as well as membrane fluidity (Esmaeili *et al*., 2015; Rustan and Drevon, 2001; Laposata, 1995).

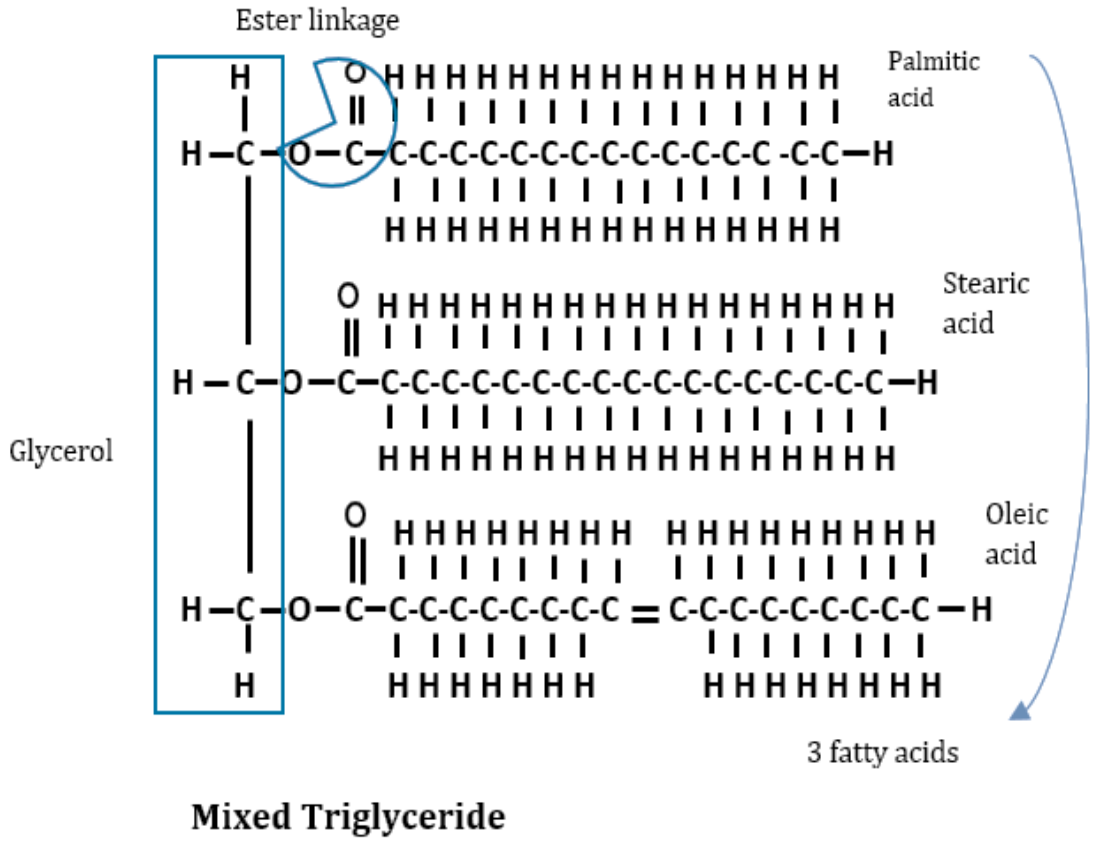


Figure 1.3. Structure of fatty acids and a mixed triglyceride. Formation of a triglyceride by esterification of glycerin with palmitic acid, stearic acid, and oleic acid which usually are the most abundant fatty acids in food. An ester bond (O-C=O) links a glycerol backbone to three fatty acids. Modified from (Campbell and Farrell, 2006).

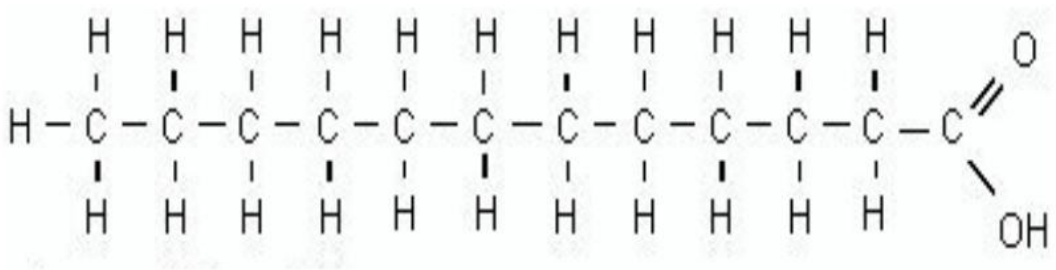
1.2.1. Saturated fatty acids

Saturated fatty acids (SFAs) have a built in unbranched chain (Figure 1.4), with no double bonds. Examples of SFAs include palmitic acid (C16:0) and stearic acid (C18:0). SFAs are commonly obtained from animal sources (fatty meats, eggs and dairy products) (De Souza *et al.*, 2015). Vegetable products can also act as a source for SFAs with coconut and palm oils possessing high levels of SFAs. Replacement of saturated fatty acids with monounsaturated and polyunsaturated fatty acids, decreases the

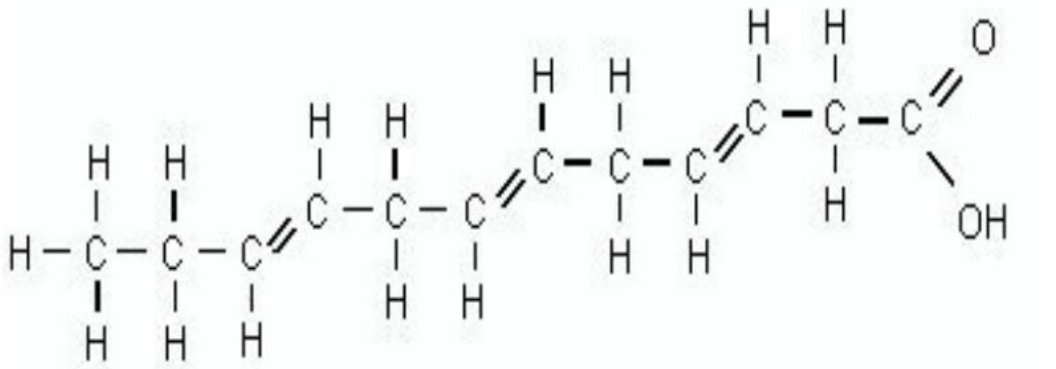
plasma concentration of LDL cholesterol. The mechanism for these effects may be increased uptake of LDL particles from the circulation by the liver. (Rustan and Drevon, 2001).

1.2.2. Monounsaturated fatty acids

Monounsaturated fatty acids (MUFAs) contain only one double bond; if the double bond the two hydrogens are on the same side of the molecule as each other this cis configuration is what gives the molecule a curved structure (Tvrzicka *et al*., 2011). Oleic acid has 18 carbon atoms and one double bond in the n-9 position with the first carbon at the methyl position (C18:1 n-9). MUFAs can be found in high concentrations in vegetable oils including peanut, canola and olive oil (Zevenbergen *et al*., 2009) and also present due to desaturation of SFA. Fatty acid desaturase appears in all organisms for example; bacteria, fungus, plants, animals and humans. Four desaturases occur in humans, delta-9 (Δ^9) desaturase, delta-6 (Δ^6) desaturase, delta-5 (Δ^5) desaturase, and delta-4 (Δ^4) desaturase (Los and Murata, 1998). This desaturation of stearic and palmitic acid requires enzymes including Δ9-Desaturase (Stearoyl-CoA desaturase 1, SCD-1) that converts them to MUFA (Guillou *et al*., 2010). In addition, Δ6-Desaturase (D6D) and Δ5-desaturase (D5D) are involved in the metabolism of linoleic and α-linolenic acid to polyunsaturated metabolites. MUFAs lowers LDL cholesterol and total cholesterol while at the same time increasing the production of the 'good' cholesterol, high density lipoproteins (HDL) cholesterol (Legrand-Poels *et al*., 2014).



Saturated C12 fatty acid



Unsaturated C12 fatty acid

Figure 1.4. Saturated and unsaturated C12 fatty acids. The saturated fatty acids are linear with the unsaturated being branched. The conformations of the unsaturated fatty acid lies in the double bonds between some of the carbon atoms. These double bonds can lead to distortion of the fatty acids, being linear unsaturated fatty acid molecules can be tightly packed at the molecular level forming for themselves a relatively solid and rigid biochemical complex in the blood.

1.2.3. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are made up of 18 carbons or more in length with no less than two double bonds and can be classified into two classes, n-3 and n-6, i.e. (LA) linoleic acid (C18:2 n-6) and (ALA)α-linolenic acid (C18:3 n-3). LA and ALA are known to be essential for humans as they are necessary for biological processes (Simopoulos, 1999). Essential fatty acids cannot be synthesized, mammals lack the

ability to introduce double bonds in fatty acids beyond carbon 9 and 10, due to a lack of the necessary fatty acid desaturase enzymes (Cook and McMaster, 2002). Therefore, essential fatty acids must be obtained from the diet and synthesized from dietary essential fatty acids.

There is a great deal of variation in the sources and bioactivities between the two classes of PUFAs the n-3s and the n-6s (Catalá, 2013). The n-3s, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are found mainly in seafood and are linked with various beneficial effects including cancer regulation, cardiovascular disease prevention, and improved cognitive ability reviewed by Xu and Qian (2014). The n-6s, in particular a derivative arachidonic acid, are much more abundant in our daily diet and are generally associated with adverse effects on the human body, including cancer promotion (Huerta-Yépez, *et al*., 2016). For instance, the high intake of n-6s was found to correlate with a risk of breast, prostate, and colon cancer (Al-Taan *et al*., 2013). Most double bonds in PUFAs are in the cis configuration. PUFAs are present in many species of nuts and vegetable oils, sesame, sunflower, and soybean, as well as in fatty fish (Esmaeili *et al*; 2015; Laposata, 1995).

1.2.4. Short chain fatty acids

Propionate, acetate and butyrate are short chain organic fatty acids (SCFAs) with only 1-6 carbon (Soldavini and Kaunitz, 2013). These FAs are the major products of bacterial fermentation of undigested dietary fibre and resistant starch in the colon. SCFAs can be active as nutrients and signalling molecules and can put on some potential therapeutic effects. SCFA also modulate inflammation and can affect several leukocyte functions. They suppress production of pro-inflammatory mediators such as TNF-α, and IL-6 (Vinolo *et al*., 2011). Butyrate SCFA present in the colon, and can

enhance the release of the anti-inflammatory cytokine IL-10 in gastrointestinal diseases and obesity (Huang *et al*., 2017).

1.3. Metabolism of fatty acids

1.3.1. Fatty acids uptake

Most human dietary milk lipids consists of triglycerides (TG), which is composed of more than 400 different fatty acids, and consumed as as liquid milk, cream, butter, ghee, cheese and ice cream (Kontkanen *et al*., 2011). Digestion of these milk lipids proceeds in the small intestine by the breakdown and absorption of the digested free fatty acids (FFA) and monoacylglycerols, FFA are re-esterified to triglycerides in mucosa cells of the intestina (Figure1.5), and transported to the circulation via lymphatic vessels (Rustan and Drevon,2001). Briefly, the breakdown of milk TG involves gastric lipase secreted from the gastric mucosal cells that hydrolyzes fatty acids from the *sn*-3 position to release diacylglycerols (Mu and Høy, 2004). Gastric lipase accounts for 10% or more of dietary TG hydrolysis but varies with the composition of the milk (Reviewed by Innis, 2011). Fat digestion continues with the enzyme pancreatic lipase which is specific for TG at their *sn*-1, 3 ester linkages on the triglyceride molecule and this completes the TG hydrolysis to give monoacylglycerol and unesterified fatty acids (Kuksis, 2000). The absorption of the unesterified fatty acids varies on their physical and chemical properties, particularly their phase transition temperatures, which influence fatty acid partitioning into mixed micelles or through the formation of insoluble hydrated soaps. At the pH of the intestine, unesterified long-chain SFA have an increased tendency to form insoluble hydrated fatty acid soaps and these are subsequently lost in the stools (Innis, 2011).

Lipoprotein lipase on the blood vessel wall breakdown the lipoprotein linked triglycerides to free fatty acids, which in turn are taken up by mucosal cells. Meanwhile,

the extra fatty acids esterified into phospholipids, fused to triglyceride and accumulate in cytoplasmic lipid droplets, they are highly hydrophobic, and stored as triglycerides within cells (Laposata, 1995). FFA enter cells by simple diffusion, and can also enter through the plasma membrane with the aid of four different transporter proteins. These are Caveolins, FA transport proteins (FATP1-6), FA translocases (FAT, CD36), and FA binding proteins (FABPs) inside the cells (Glatz, 2015). Activation of Acyl-CoA by FFA facilitate their transportation to mitochondria or peroxisomes for β-oxidation through Acyl-CoA-binding protein (ACBP), resulting in the formation of energy as ATP and heat. FFA also can be transported to endoplasmic reticulum (ER) for esterification to different classes of lipid. These FFA involved in transcription factors that regulate gene expression and signal molecules (eicosanoids) (Rustan and Drevon, 2001).

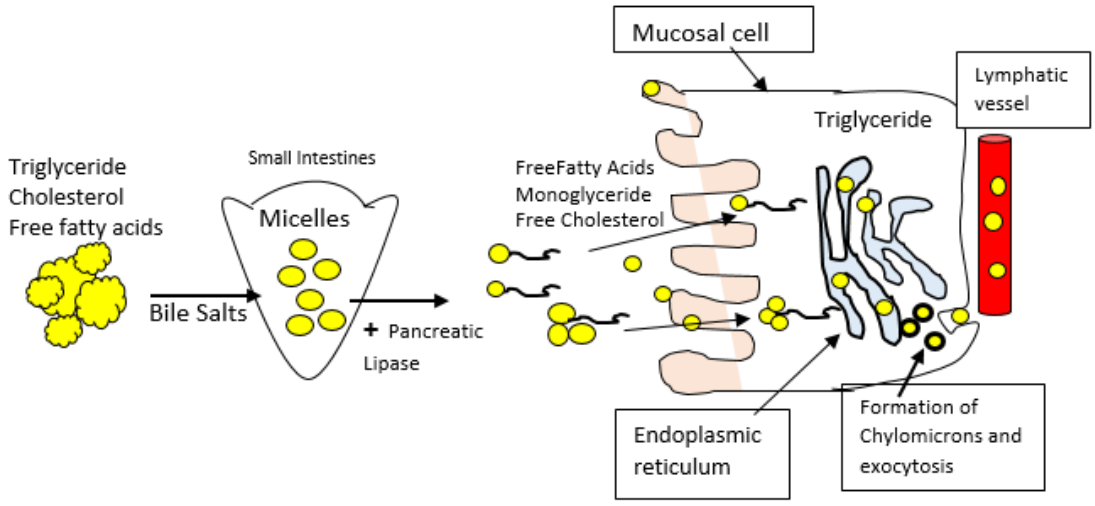


Figure 1.5. Schematic representation of lipids breakdown in the intestine. Triglyceride, Cholesterol and free fatty acids breakdown in the small intestine, absorption of the digested free fatty acids (FFA), monoglyceride and the free cholesterol by mucosal cells. Thereafter, the FFAs are re-esterified to triglycerides and transported to the circulation via lymphatic vessels.

1.3.2. Fatty acids binding proteins

Fatty acids binding proteins (FABPs) are a family of 14–15-kDa proteins, known as intracellular lipid chaperones, which control lipid movement in cells. FABPs can reversibly bind with high affinity and wide-ranging selectivity with hydrophobic ligands such as eicosanoids (hormones), saturated and unsaturated long-chain fatty acids, and other lipids. FABPs are found in all species including rodents and humans (Furuhashi *et al*., 2014). Cytoplasmic proteomic analysis of FABP4 and FABP5, was detected in cell supernatants derived from differentiated THP-1 macrophages. These proteins modulate intracellular lipid metabolism and transport fatty acids between cellular compartments. These proteins have been proposed to act as biomarkers for atherosclerosis related diseases (Fach *et al*., 2004).

1.3.3. Fatty acids synthesis and esterification in human tissues

Fatty acyl-CoA synthetase is an enzyme present in nearly all cells, whose function is to convert fatty acids to their corresponding fatty acyl-CoAs. Malonyl-CoA has 3-carbon fragments and is carboxylated from acetyl-CoA by the rate limiting enzyme acetyl-CoA carboxylase, this enzyme limits the rate of fatty acid synthesis (Harwood *et al*., 2003). Fatty acids gain 2 carbons with each condensation cycle from Malonyl-CoA and the third carbon is lost as CO_2. Esterification of free fatty acids (FFA) shown in (Figure 1.6) in to phospholipids, triglycerides, and cholesteryl esters facilitated with fatty acyl-CoA in presence of acyltransferase enzymes in the endoplasmic reticulum (ER). In turn, acyl-CoA-cholesterol acyltransferase is the enzyme that assists the formation of cholesteryl esters from fatty acyl-CoA and cholesterol in cells (Laposata, 1995). FFA are further elongated by the enzymes (elongases) and allocated to the membrane ER. These FFA can participate as substrates for other FA desaturases and facilitate the

production of new double bond FA's (Tvrzicka *et al*., 2011). Elongation and desaturation of of (n-3) α-linolenic acid (ALA, C18:3 n-3) to docosahexaenoic acid (DHA, C22:6 n-3), and (n-6) linoleic acid (LA, C18:2 n-6) to arachidonic acid (AA, C20:4 n-6) are all catalysed in the ER and peroxisomes (Patterson *et al*., 2012). However, these pathways are slow in human tissues, and depends on the availability of the essential fatty acids through dietary intake (Bradbury, 2011).

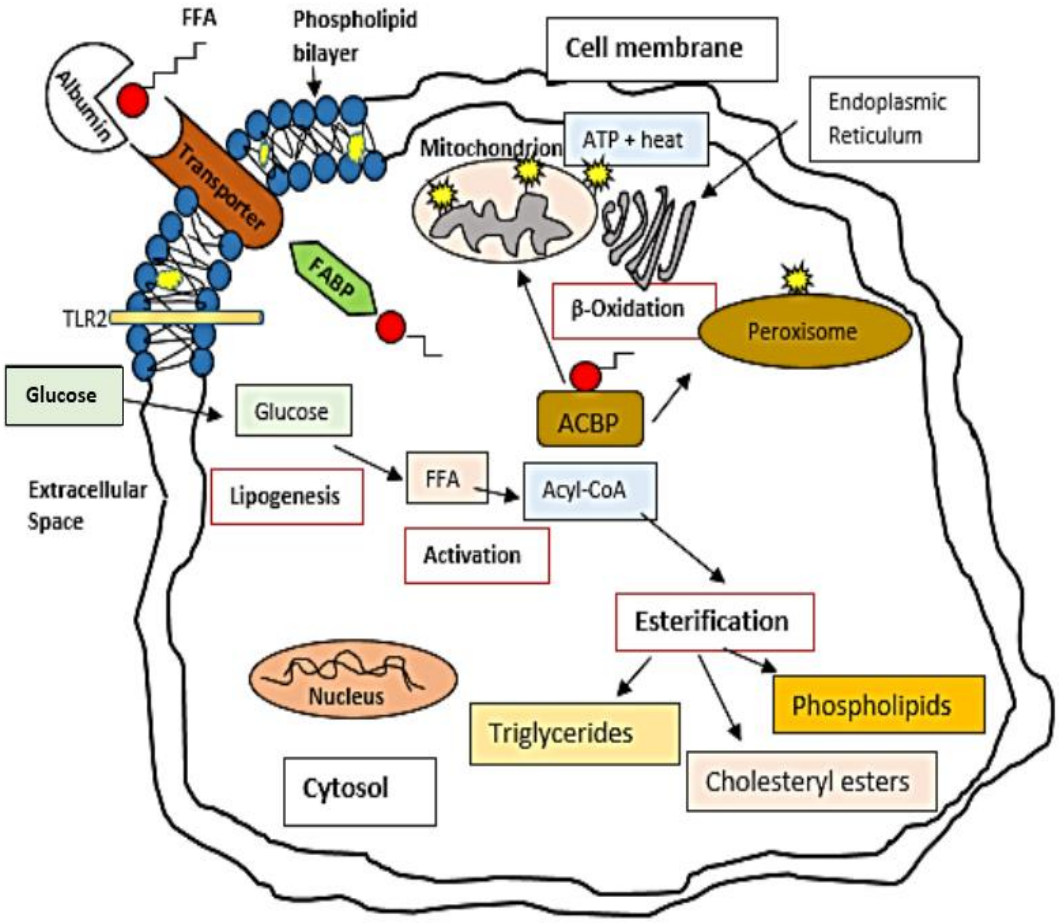


Figure 1.6. Schematic representation of fatty acids metabolism. Free fatty acids (FFA) enter the cells by diffusion, and through the plasma membrane with the aid of various transporter proteins such as: fatty acid (FA) transport proteins (FATP1-6) and FA binding proteins (FABPs) that aid fatty acid transportation within the cell. Activation of Acyl-CoA by FFA facilitate their transportation to mitochondria or peroxisomes for β-oxidation thru Acyl-CoA-binding protein (ACBP), resulting in formation of energy as ATP and heat. FFA also can be transported to endoplasmic reticulum for esterification to different classes of lipids. The FFA bind to transcription factors that regulate gene expression and produce signalling molecules including FFA derived eicosanoids.

1.4. Inflammation

Inflammation is the body's immediate response to damage to its tissues and cells by viral and bacterial pathogens, noxious stimuli such as chemicals, or physical injury (Punchard *et al*., 2004). Acute inflammation is seen as a response that is typically rapid and short-lived. This response involves the infiltration of immune cells such as leukocytes to damaged or infected tissues, removing of the stimulus and initiating stimuli that including bacterial and viral componets and eventual tissue repair (Weiss, 2002). The physical signs and response of inflammation include; pain, heat, redness, and swelling, the cellular responses can include a range of inflammatory meditors such as cytokines and vasoactive lipids (Turner *et al*., 2014). The inflammatory response can be brief and rapid during its severe acute phase and is essential for dealing with exogenous threats such as bacteria and viral infrctions and tissue damage (Ricciotti and FitzGerald, 2011). Chronic inflammation differs from acute inflammation by being much more prolonged, poorly regulated (Cekici *et al*., 2014) and a maladaptive response that involves active inflammation, tissue destruction and attempts at tissue repair. This chronic and persistent inflammation is a major feature of many chronic human diseases, including atherosclerosis, cancer, arthritis and neuroinflammatory disorders (Ji *et al*., 2014). It is regarded that, the development of acute inflammation its initiation and regulation and cellular pathways are far better understood than that of the chronic form (Serhan *et al*., 2011).

An organism can react against infection or tissue injury by generating an inflammatory response, this will activate the migration of macrophages cells and also neutrophils towards the site of injury or infection (Janeway *et al*., 2005).

The mechanisms protecting the host from invaders such as bacteria, or repair of damaged tissues, involves pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-18, and also their regulation by anti-inflammatory cytokines such as IL-10. Extracellular

matrix proteins such as proteoglycans, fibrous proteins including collagen, elastin, and fibronectin will also be released (Koziel and Potempa, 2013). Lipid derived metabolites including proinflammatory eicosanoids (leukotrienes), prostaglandins (thromboxanes), and the enzymes involved in controlling and production of these mediators will be involved. However, immune cells, including neutrophils, resident and recruited macrophages are responsible in controlling these inflammatory reactions by turning off the production of inflammatory mediators, or reducing the accumulation of inflammatory cells, and removing dead cells remains (Gomez-Munoz *et al*., 2016).

1.5. Macrophages phenotype activation and polarization

Macrophages are phagocytic cells that release proinflammatory cytokines, and exist within almost all tissues. These cells release antimicrobial peptides, attract other immune cells to infected tissues and communicate with other cells by secreting chemokines molecules (Silva, 2010). The analysis of adipose tissue for the presence of macrophages in their contents was positively detected, the number of macrophages in adipose tissue increase in obesity, and contribute in the inflammatory pathways that are triggered in adipose tissues of obese individuals (Weisberg *et al*., 2003). Proinflammatory cytokines TNF-α and IL-6 detected in adipose tissue, however, TNF-α and IL-6 involved in hypertriglyceridemia and increased serum free fatty acids (FFA) levels, related to obesity (Xu *et al*., 2003; Zhang *et al*., 2002). Gene expression of monocyte chemoattractant protein -1 (MCP-1) is crucial, its expression in high level will lead to pathological effect of hyperinsulinemia and obesity, including type 2 diabetes (T2D) and adipocyte dedifferentiation (Sartipy and Loskutoff, 2003). Obesity in humans is associated with many risk factors such as myocardial infarction, stroke, hypertension, dyslipidaemia, and several cancers including those of the oesophagus, colon rectum, liver, gall-bladder, pancreas, kidney, non-Hodgkin lymphoma, and

multiple myeloma (Pi-Sunyer, 2009; Hubert *et al*., 1983). Weight control is associated with the reduction of obesity's complications, however, high saturated fat diet induced obesity alters adipose tissue macrophages (ATMs) from an anti-inflammatory M2 polarized to an M1 proinflammatory state and involves in insulin resistance, and effect the secretion of anti-inflammatory cytokine IL-10 (Lumeng *et al*., 2007). Therefore, an unbalanced diet with lack of exercise will lead to high pathological incidence of obesity, hepatosteatosis, insulin resistance and atherosclerosis, which is known as the metabolic syndrome. Consequences' in rising diseases, like diabetes mellitus and premature death (Grundy *et al*., 2004).

1.6. Obesity

Adipose tissue is a specific tissue that supports, joins and protect organs, and is one of the body's method of generating heat and acts as an energy store and source when required. The content of the adipose tissue consists of triglycerides. An abnormal increase in the mass of the adipose tissue among obese individuals affects the endocrine and metabolic functions of the tissues. Production of fatty acids, hormones, and proinflammatory molecules such as TNF-α, IL-6, increased in obesity, and increases its complications (Bastard *et al*., 2006; Weisberg *et al*., 2003).

1.7. Diabetes mellitus

Diabetes mellitus (DM) is a lifelong, progressive metabolic disorder characterized by abnormally high blood glucose levels, resulting from low insulin secretion and/or increased insulin resistance, resulting in body's defeat to maintain a normal condition of glucose homeostasis (Röder *et al*., 2016).The long term inconsistency of diabetes

contributes to its status as a leading cause of premature illness and fatality world wide (Tabish, 2007). The number of adults and older teens with diabetes in the UK has more than doubled over the past 20 years, with 3.7 million people aged 17 or older now known to be living with the disease (Diabetes, U.K, 2017). Its complications are associated with cardiovascular disorders and atherosclerosis, which are the main concern of the researchers worldwide (De Rosa *et al*., 2018). Nowdays, management factors involved in diabetes treatment include diet, insulin, oral drugs, and exercise, with a high demand for these resources as the disease has a large inflammatory component and complications that results in huge and increasing effort from health services, physicians and the patients themselves. Interest in finding alternative therapies from natural dietary products available at low cost for the prevention of diabetes and its complications are in demand (Gader and Alhaider, 2016).

1.7.1. Type 2 diabetes

Type 2 diabetes (T2D) is chronic metabolic disorder that is characterized by a glucose level insulin resistance and reduced level of insulin. 200 million individuals worldwide (Yang and Zhao, 2011) suffer from T2D. This number is projected to increase by 54% to more than 54.9 million Americans between 2015 and 2030 (Rowley *et al*., 2017). Type 2 diabetes primarily occurs as a result of adiposity, physical inactivity, and insulin resistance (Gress *et al*., 2000). Some people have a stronger genetic risk than others to this disorder (Gerich, 1998). Common symptoms include increased thirst, urine production and although the disease is often associated with weight loss (Ramachandran, 2014). Long term complications from high blood sugar include cardiovascular disease, stroke, and atherosclerosis that can result in blindness and renal failure (Soumya and Srilatha, 2011). Other type of diabetes are type 1

diabetes or insulin-dependent diabetes mellitus because of the clinical need for insulin (Notkins and Lernmark, 2001). In diabetes mellitus type 1 there is a lower total level of insulin to control blood glucose due to β-cell destruction, usually leading to absolute insulin deficiency (American Diabetes Association, 2014). Another type of diabetes detected during pregnancy, gestational diabetes mellitus (GDM) defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Although most cases resolve with delivery (American Diabetes Association, 2014; Bellamy *et al*., 2009). All the diseases symptoms are associated with reduced blood flow in the limbs which may lead to amputations. Diabetes can be diagnosed by blood tests such as fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) glycated haemoglobin (A1C) (American Diabetes Association, 2010; International Expert Committee, 2009). Type 1 diabetes requires insulin to control it, diet, and exercise. However, type 2 diabetics require insulin or oral medication to lower blood sugar, if diet and exercise alone fail to lower blood glucose, then a medical supervision needed from a team of doctor, nutritionist, and health educator or nurse working together. For both types of diabetes the best control would be to balance the glucose and the insulin in the blood by regulating diet, activity, and if needed taking medication (Asif, 2014). In type 2 diabetes (T2D), a decrease in pancreatic β-cells mass affects insulin production-an important hormone in controlling the glucose circulatory level. Controlling β-cells mass depends on their mechanism of survival, replication and hypertrophy. A member of a family of proteins that are involved in controlling growth and survival of β-cells is the insulin receptor substrate 2 (IRS-2) protein, inhibition of this protein IRS-2 pathway is critical in balancing the β-cells mass and insulin resistance (Rhodes, 2005). Diabetes development involves a range of pathogenic factors, starting with β-cells of the pancreas autoimmune destruction which end in deficiency of insulin action on

target tissues, to abnormalities in carbohydrate, fat, and protein metabolism (American Diabetes Association, 2010).

1.8. Macrophages metabolic regulation

Monocyte–macrophage lineage cells are present on all vertebrate species and play critical roles in homeostasis, wound healing, and immune responses and provide a first line of defense against pathogens, and undergo molecular reprogramming in response to microbial, environmental, and immune derived signals (Edholm, *et al*., 2017). These cells are highly plastic with polarized populations that vary in terms of effector functions, expression of surface receptor, and cytokine production (Davis *et al*., 2013). In *vitro* macrophages are activated toward an M1 functional program by bacterial lipopolysaccharides (LPS) and by cytokines TNF-α or IFN-γ, and produce nitric oxide (NO) or reactive oxygen intermediates (ROI) and inflammatory cytokines IL-1β, TNF-α, and IL-6 (Italiani and Boraschi, 2014). M2 type responses can be amplified by IL-4, IL-10, or IL-13 (Wang *et al*., 2014). M2 macrophages generate anti-inflammatory IL-10 and upregulate Dectin-1, CD206 mannose receptor, scavenger receptor A, scavenger receptor B-1, CD163 (Martinez *et al*., 2009). Meanwhile, M1 macrophages produce pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-12 (Sica and Mantovani, 2012). M2 macrophages can undergo further polarization to a range of M2 subtypes. Thes subtypes include, M2a (induced by exposure to IL-4 and IL-13) and M2b (induced by combined exposure to immune complexes and TLR or IL-1R agonists) whereas M2c macrophages (induced by IL-10, TGF-β) are more related to suppression of immune responses and tissue remodelling (Mantovani *et al*., 2004).

Håversen *et al*., (2009) concluded in their study, that the long-chain SFA palmitate, stearate and myristate, induce proinflammatory cytokines secretion and mRNA expression of TNF-α and IL-1β in differentiated THP-1 human macrophages cells via pathways involving metabolism of the SFA including synthesis of ceramide and, activation of the kinases p38 and JNK, and of the transcription factor c-Jun. However, in T2D this might enhance the activation of macrophages in atherosclerotic plaque formation and induce inflammation (Ito and Ikeda, 2003). Meanwhile, shorter chain SFA, myristate and laurate, or linoleate did not induce proinflammatory cytokine secretion. M2 phenotype resident macrophages are detected in healthy adipose tissues from lean animals and humans, under normal diet conditions. M2 or "alternatively activated" macrophages express anti-inflammatory genes of cytokines like IL-10, arginase 1, and protect adipocytes from TNF-α induced insulin resistance (Lauterbach and Wunderlich, 2017). In disparity, a high fat diet, leading to obesity; will increase the infiltration of M1 macrophages and gather around the apoptotic fat cells forming a shape like a crown, and express proinflammatory cytokines genes such as, TNF-α, iNOS, and IL-1β, contributing to insulin resistance (Lumeng *et al*., 2007). Signals generated from fading hypertrophy adipocytes attract macrophages to recruit to the site of inflammation and reinstate homoeostasis (Dalmas *et al*., 2011). Meanwhile, dysfunctional fat cells release SFA and further activate the recruited macrophages thus intensifying the inflammatory response. Dead fat cells and lipid droplets taken up by macrophages are the cause for stromal vascular fraction in obesity (Weisberg *et al*., 2006). Secretion of monocyte chemoattractant proteins (MCPs) to the extracellular matrix (ECM) during inflammation increases, which recruit more macrophages and prevent adipocyte insulin signalling (Xu *et al*., 2003). Accumulation of high levels of low-density lipoprotein (LDL), in sub endothelial matrix, recruit more monocytes/macrophages in to the site, they bind the locally oxidized (oxLDL) through

their receptor scavenger receptor A (SR-A) (Kzhyshkowska *et al*., 2012). Foam cells generated from macrophages carrier-lipid, and a long with the releasing of inflammatory molecules, these cells become necrotic cell and die leaving behind vicious cycle of chronic inflammation (Baker *et al*., 2011). Subsequently, smooth muscle cells travel to the site of lesion and participate in the fibrotic plaque formation and rupture (Moore, and Tabas. 2011).

1.8.1. Advanced glycation end products

Diabetes is a very complex and multifactorial metabolic disease, with associated hyperglycaemia, produces intracellular oxidant stress leading to cell failure, vascular dysfunction and insulin resistance (Nowotny *et al*., 2015). Advanced glycation end products (AGEs), are heterogeneous fluorescent derivatives and irreversibly cross-linked products. AGEs generated from their nonenzymatic glycation reaction of glucose and other reducing sugars with the amino groups of plasma proteins such as (albumin, fibrinogen, globulins and collagen), to form reversible Schiff bases and, then, Amadori compounds (Figure 1.7). Collagen, AGE cross-links are responsible for stiffening of the extracellular matrix (ECM) and often involved in organ and vessel dysfunction like in atherosclerotic lesion formation (Figure 1.8). Glucose also reacts with lipids to form different types of AGEs. Accumulation of AGEs in diabetes facilitate its complication such as, retinopathy, renal failure, and neuropathy, cardiomyopathy with some other diseases such as rheumatoid arthritis, osteoporosis and aging. This accumulation of AGEs will affect the nature of the proteins function by altering molecular conformation, modifying the enzymatic activity, and distracting the receptor functioning (Singh *et al*., 2014; Ramasamy *et al*., 2005; Tanaka *et al*., 2000). In the present, AGEs can be generated even in the absence of diabetes; AGEs can be significantly accelerated with

processed food, with dry heat, ionization or irradiation (Vlassara and Uribarri, 2014). In the food industry AGEs are added to enhance the flavour and consumption. Home cooking by simple methods of heat and dehydration also can accelerate AGEs formation (Sharma *et al*., 2015). Smoking tobacco, little physical activity also are ways in introducing AGEs (Vlassara and Uribarri, 2014). Expression of AGE receptors (RAGE) are up-regulated with the generation of AGEs and with the stage of diabetes complication. RAGE receptors are expressed in several cell types including smooth muscle cells, monocytes, macrophages, endothelial cells, podocytes, astrocytes and microglia (Yan *et al*., 2009), and belong to the immunoglobulin superfamily of cell surface molecules, induce cellular oxidant stress, and activate the transcription factor nuclear factor-κB (NF-κB) (Yamagishi and Matsui, 2010; Tanaka *et al*., 2000).

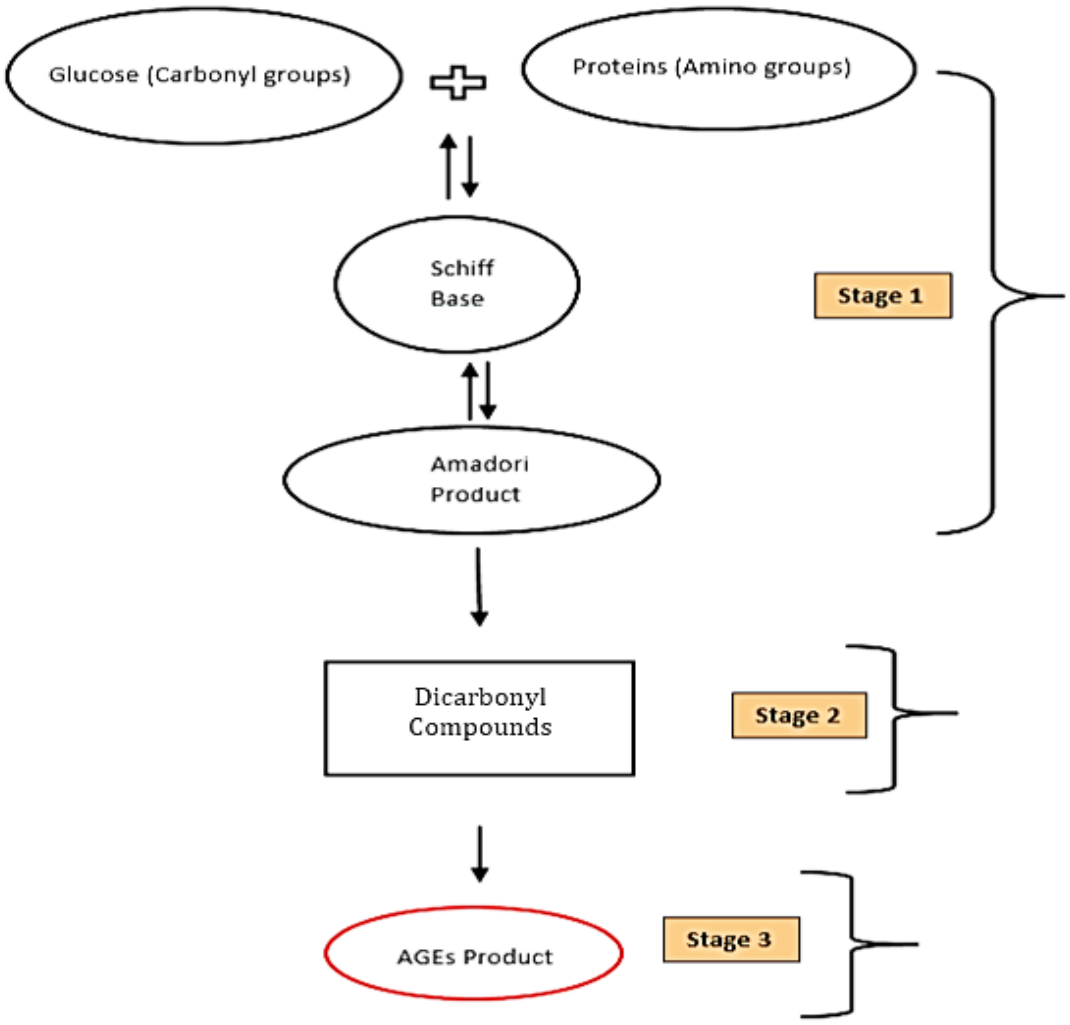


Figure 1.7. Formation of advanced glycation end products. In **stage 1**, the sugars (glucose, fructose, pentoses, and galactose) or any other sugars react nonenzymatically (Maillard reaction) with amino groups of proteins to form unstable - reversible Schiff bases compounds and, then, Amadori compounds. In **stage 2**, the Amadori product degrades to a variety of reactive dicarbonyl compounds such as glyoxal, MGO, and deoxyglucosones via dehydration, oxidation and other chemical reactions. In **stage 3**, these early glycation products undergo further complex reactions to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed advanced glycation end products (AGE). AGEs are formed through oxidation, dehydration and cyclization reactions. Adapted from (Singh *et al*., 2014).

1.9. Inflammatory pathways

Nuclear factor-κB (NF-κB), is expressed in almost all cell types and tissues, and its specific binding sites are present in the promoters/enhancers of a large number of genes (Wan and Lenardo, 2009; Oeckinghaus and Ghosh, 2009). Facilitate responses to a variety of external stimuli, and thus is an essential key element in several physiological and pathological processes (Oeckinghaus and Ghosh, 2009). NF-κB can be activated with different stimuli, like viruses or bacterial products, or inflammatory cytokines (Pires *et al*., 2018). NF-κB can be activated with physiological or physical stimuli such as ischemia and hyperosmotic shock, high fat diet, oxidative stresses, and with UV or radiation (Aggarwal *et al*., 2006). This activation facilitated through different receptors like Toll-like receptors (TLRs), cytokines receptors (Baeuerle and Henkel, 1994). The NF-κB transcription factor family includes five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2) that combined with each other to form distinct transcriptionally active homodimers or heterodimers complexes (Barkett and Gilmore, 1999). NF-κB situated in the cytoplasm in its inactive state is bound to an inhibitory protein of the IkB family. IkB proteins include IkBα, IkBβ, IkBε, IkBγ, p105, p100, and Bcl-3, plus others. Activation of IkB kinase complex (IKK) in conjunction with numerous signalling pathways, results in phosphorylation of IkBα and its degradation by the proteasome (Hoesel and Schmid, 2013). The liberated Rel/ NF-κB complex can then enter the nucleus and bind to DNA and control transcription (Cadera *et al*., 2009). In inflammation linked β-cell dysfunction and insulin resistance, dietary SFA such as palmitic acid unlike USFAs (polyunsaturated ω–3), (Figure 1.9) activate proinflammatory signalling pathways by TLR4 and or TLR2 which in turn activate NF-κB and gene transcription factors of inflammatory cytokines such as TNF-α, and IL-1β (Shi *et al*., 2006).

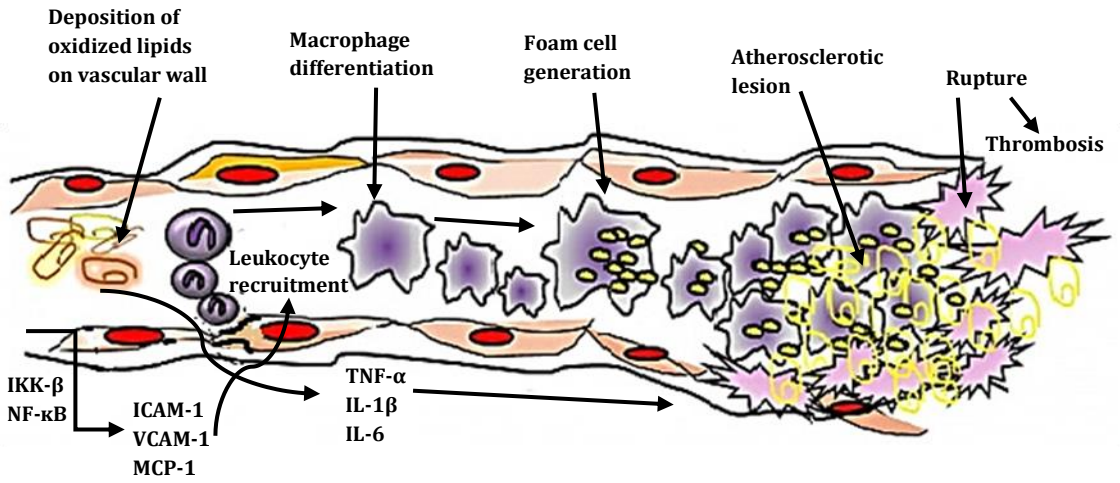


Figure 1.8. Schematic representation of atherosclerotic lesion formation. Binding of AGEs to the transmembrane receptor (RAGE) results in protein synthesis–dependent sustained activation of NF-κB. In vascular endothelia, deposition of oxidized lipoproteins on vascular wall, result in expression of adhesion molecules ICAM-1, VCAM-1, MCP-1, and leukocyte derived cytokines. Circulating monocytes migrate and are retained at the site of inflammation and differentiate into cytokine-producing (TNF-α, IL-1β, IL-6) macrophage cells, these in turn take-up lipids on the vascular wall and puffiness into foam cells that nucleate the atherosclerotic plaque. Proinflammatory signalling generated through NF-kB that controls transcription of DNA, cytokine production and cell survival. Continuation of exposure of cells to inflammatory, infectious, or other stressful stimuli such as in diabetes and its complications, generating AGEs and its receptors RAGEs, enhancing rapid phosphorylation and degradation of inhibitors IκBs proteins (IkBα, IkBβ) release NF-kB (p50/p65) from the cytoplasm into the nucleus which is sequestered in the cytoplasm by inhibitors IkBs.

1.10. Inflammasome

The interleukin 1 family cytokines are important activators and mediators of inflammation in many diseases including atherosclerosis and diabetes (Lukens *et al*., 2012). One of the main inflammatory mediators that contributes to a wide range of vascular and metabolic disorders with IL-1β are involved in both central and systemic inflammatory mechanisms (Brough and Denes , 2015; Heneka *et al*., 2015; Murray *et al*., 2013; Najjar *et al*., 2013; Smith *et al*., 2013; Newton and Dixit, 2012; Dinarello, 2011; Rock and Kono, 2008). Blockade of IL-1β actions is markedly protective against the development of T2D in animal models (Murray *et al*., 2015; Denes *et al*., 2011). In addition IL-18 is another inflammatory cytokine whose involvement in acute and chronic inflammatory conditions has been observed (Lenart *et al*., 2016). Both IL-1β and IL-18 production are regulated by caspase-1 activation and both are produced as inactive pre-forms. Inflammasomes are complex multimeric-proteins, induced in several cell types including macrophages by inflammatory stimuli that process inactive precursors of IL-1β and IL-18 into mature secretable cytokines (Guo *et al*., 2015). Inflammasomes are composed of a sensor molecule, adaptor proteins and proinflammatory caspases (Figure 1.9). The sensor molecule is a pattern recognition receptor (PRR) that senses pathogens, or damage associated molecular patterns (DAMPs), which are markers of cell damage stress/injury (Xiang and Fan, 2010). Several inflammasomes include NLRP1 (Abulafia *et al*., 2009; De Rivero Vaccari *et al*., 2009; De Rivero Vaccari *et al*., 2008), NLRP3 (Ito *et al*., 2015; Yang *et al*., 2014; Fann *et al*., 2013), NLRC4 (Denes *et al*., 2015), and AIM2 (Adamczak *et al*., 2014). The endogenous ligand for NLRP1 is unknown but NLRP3 (NLR family pyrin domain containing-3) senses a diverse array of stimuli such as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns or (DAMPs) (Guo *et al*., 2015). Upon DAMP sensing NLRP3 and ASC (apoptosis-associated speck-like protein

containing a caspase recruitment domain) oligomerises to form a large inflammasome speck within the cell based on a prion like aggregation (Cai *et al*., 2014; Lu *et al*., 2014). Pro-caspase-1 reacts with this complex becomes activated and converts pro-IL-1β and pro-IL-18 to their mature active secretable forms (Lopez-Castejon and Brough, 2011). Once formed these inflammasome specks can also become extracellular and propagate further inflammatory responses (Baroja-Mazo *et al*., 2014; Franklin *et al*., 2014). A link between the NLRP3 inflammasome and atherosclerosis as an inflammatory lipid based disease has been considered as crystalline cholesterol and oxidized low-density lipoprotein (ox-LDL) which are present in atherosclerotic plaques; have been shown to activate NLRP3 inflammasome (Awad *et al*., 2017; Lenart *et al*., 2016).

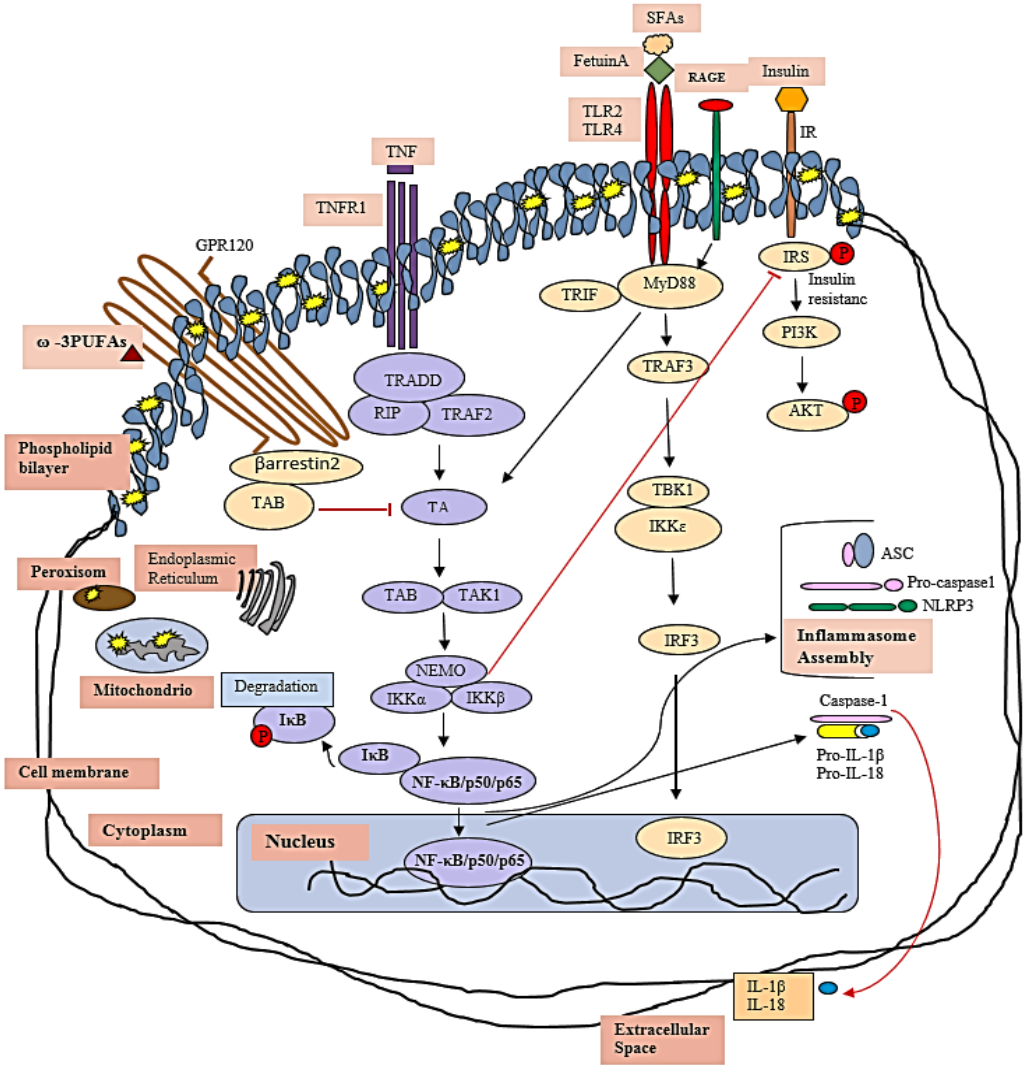


Figure 1.9. Schematic representation of inflammatory signalling pathway and inflammasome formation. Saturated fatty acid **(**SFAs), Insulin receptor (IR), omega 3 polyunsaturated fatty acids (ω-3 PUFAs), tumour necrosis factor (TNF) TNF receptor-1(R1),Toll like receptors 2/4 (TLR2/TLR4), G protein-coupled receptors (GPR120) and Advaned glycated endproducts(AGEs), binding of these ligands to their specific receptors on the cell membrane activate nuclear factor NF-κB-IκB-Kinase (IKK) complex phosphorylation (P), and translocation of NF-κB/p50/p65 to the nucleus, which activates specific inflammatory cytokines (IL-1β, and IL-18) and the components of inflammasome complex formation (ASC, Pro-caspase-1, NLRP3) resulting in release of active caspase-1 ,which then convert pro-IL-1β and pro-IL-18 to their active mature state.

1.11. THP-1 cells

THP-1 cells are an immortalized monocyte-like cell line, derived from the peripheral blood of a childhood case of acute monocytic leukaemia classified as M5 subtype (Bosshart and Heinzelmann, 2016; Qin *et al*., 2012). Since their establishment in Japan (Tsuchiya *et al*., 1980) nearly 40 years ago, THP-1 cells have become a widely used cell line for studying the characteristics of monocytes and macrophages in health and inflammatory diseases such as atherosclerosis (Bosshart and Heinzelmann, 2016). However, given that THP-1 cells are a cell line derived from malignant acute monocytic leukaemia, the level to which THP-1 cells can imitate monocytes and macrophages in tissues such as the vasculature is not entirely understood. In addition their ability to represent primary peripheral blood monocytes or tissue macrophages is limited. For instance THP-1 monocytes express reduced levels of the lipopolysaccharide (LPS) receptor CD14 and hence are less responsive to challenge by LPS than primary monocytes (Hu *et al*., 2016). THP-1 cells can be differentiated to macrophage like cells using phorbol myrisitae acetate at a concentration of as little as 5ng/mL (Park *et al*., 2007) as opposed to the original use of a much higher concentration of 400-500ng/mL. This higher concentration was itself a potentially inflammatory stimulus and by using 5ng/mL concentration, differentiated macrophages respond well to a secondary stimuli such as gBSA, without being overwhelmed by undesirable gene upregulation induced by PMA (Park *et al*., 2007). A concentration of 5ng/mL added to THP-1 cells induced adherence of the cells (a feature of macrophages rather than monocytes) and increased cytoplasmic volume as compared to monocytes (Daigneault *at al*., 2010). Furthermore, increased expression of both CD14 and the scavenger receptor CD36 was also observed. DNA-bindable NF-κB which is not detectable in the cytoplasm of undifferentiated THP-1 cells is highly detectable in the cytoplasm post-PMA differentiation, CD36 was also observed. PMA treated THP-1 cells demonstrated

substantial constitutive and inducible IL-1β and TNF-α production following TLR2 or TLR4 ligand stimulation (Vaure and Liu, 2014). In the context of this study glycated end-products induce proinflammatory cytokine secretion in PMA differentiated dTHP-1 cells and express the receptor for AGEs (RAGE) (Wang *et al*., 2017; Kumar *et al*., 2013).

PMA treated THP-1 cells have been shown to be capable of polarization from an undifferentiated THP-1 macrophages classified as M0 (Genin *et al*., 2015; Chanput *et al*., 2013). These M0 cells on stimulation with IFNγ and LPS and which are known to induce macrophage polarization *in vivo* and *in vitro* into the M1 and M2 phenotype, whereas IL-4 and IL-13 induce the M2 macrophage phenotype as demonstrated by a range of M1 (LPS and IFNγ) and M2 marker genes (IL-4 and IL-13) stimulated M0. However, M2 chemokine and their receptor genes were only slightly up-regulated which might be due to the complexity of the secondary cell-cell interaction of the chemokine system. Therefore, they can be used as a new macrophage polarizing model to estimate the polarizing/switching ability of test food compounds. The time taken to induce polarization to the M2 phenotype has (Shiratori *et al*., 2017) been shown to be greater than for LPS/IFNγ induction of M1 a point that should be borne in mind when studying the action of various stimuli on M0 THP-1 cells. In addition the M2 subtype can show enhanced sensitivity to various lipid components and in particular ox-LDL (Isa *et al*., 2011).

1.12. Thesis aims and objectives

1.12.1. Rationale

There is considerable interest in the role of dietary lipids in regulating inflammatory diseases. This thesis will investigate a mechanism by which camel milk lipids could regulate the pathogeneses of diseases such as type 2 Diabetes (T2D), cardiovascular disease, atherosclerosis and Alzheimer's disease. These diseases are major public health problems world wide. This study will enhance our ability to use traditional, non-pharmaceutical dietary components to prevent major diseases such as T2D, for which there is an urgent world wide need.

1.12.2. Hypothesis

There is evidence now available, that various dietary lipids including those found in milk have immuneregulatory properties. This study aims to investigate the hypothesis that the anti-inflammatory and immuneregulatory effects characteristic of camel milk is derived from its lipids, which can regulate the inflammatory response of cultured human monocyte derived macrophages *in vitro*.

1.12.3. Overall aim

To investigate the cellular and molecular mechanisms of Omani camel milk derived extracted lipids and its anti-inflammatory effects in human monocyte derived macrophages. Macrophages cells selected as a model of inflammatory processes in this investigation *in vitro*, these cells play a critical role in pathogenesis of major chronic inflammatory diseases such as diabetes and cardiovascular disease.

1.12.4. Specific objectives

Aim 1. To undertake a detailed analysis of the fatty acid components of camel milk.

Objectives

1. To extract total camel lipids (TL) from 7 pooled camel milk and characterized their fatty acid components as their fatty acids methyl ester (FAME) using Gas Chromatography- Mass Spectrometer (GC-MS).
2. To separate the other fractions of camel milk lipids, total free fatty acids (TFAs) from un-saturated free fatty acids (USFAs) by Urea Complexation Fraction method.

Aim 2. To investigate the roles of these three fractions fatty acids (TL, TFAs, USFAs) in regulating-remodelling the inflammatory response and immunoregulatory effects on cultured human monocyte/macrophage cell line THP-1, which differentiated to (dTHP-1) macrophage cells.

Objectives

1. To investigate the effect of camel milk lipids on the viability of cultured human monocyte/macrophage cell line THP-1 differentiated (dTHP-1) cells.
2. To investigate camel milk lipids reduce the expression of proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-18 in glycated bovine serum albumin (gBSA) treated dTHP-1 cells.

Aim 3. To investigate the ability of camel milk lipids in the polarization of human monocyte/macrophage cell line dTHP-1 towards macrophages phenotypes M1 or M2.

Objective

1. To investigate the ability of camel milk lipids in regulating the mRNA expression of M1 (markers CD86) andM2 type (markers CD163, CD206) macrophages using reverse transcription polymerase chain reaction (RT-PCR).

Aim 4. To investigate the ability of these camel milk lipids in regulating the transcription nuclear factors NF-κB activation and its cleavage with its subunits proteins (p50/p65), linked with inflammasome formation.

Objectives

1. To investigate the ability of camel milk lipids to regulate the activation of NF-κB-subunits p50 /p65.
2. To investigate the ability of camel milk lipids in the activation of pro-caspase-1 protein responsible in converting the inflammatory cytokines pro-IL-1β and pro-IL-18 to their active forms IL-1β and IL-18.
3. To investigate the ability of camel milk lipids in the activation of 3 proteins (pro-caspase-1, NLRP3, ASC), important in formation of the inflammasome complex assembly.

CHAPTER 2

General materials and methods

2.1. General chemicals, reagents and materials lists

Chemicals	Suppliers
Methanol G (MeOH)	Sigma Aldrich
Chloroform ($CHCL_3$) Sodium hydroxide 0.5M(NaOH) Heptane (C_7H_{16}) Hexane (C_6H_{14}) Sodium chloride (NaCl) Sodium Sulphate anhydrous purified (Na_2SO_4) Hydrochloric acid (HCL) Phosphate Buffer Saline (PBS) Petroleum ether (C_6H_{14}) Diethyl ether ($C_4H_{10}O$) 25% Ammonia solution (NH_3) Formaldehyde **(**CH_2O)	BDH , UK
Urea (CH_4N_2O)	Pharmacia Biotech, Sweden
Boron Tri-fluoride (BF_3)	ACROS-US
Phorbol myristate acetate (PMA) Glycated Bovine Serum Albumin (gBSA) Dimethyl Sulfoxide (DMSO) Methanol (MeOH) Isopropanol **(**C_3H_8O)	Sigma Aldrich,UK

Table 2.1. List of general chemicals used and materials.

Reagents	Supplier
Foetal Calf Serum (FCS)	Labtech
RPMI 1640	Gibco (Life technologies)
Non-essential amino acids (10X) Sodium pyruvate Penicillin (100IU/mL), Streptomycin(100µg/mL)	Thermofisher Scientific
Trypan Blue	Sigma Aldrich, UK
Oil Red O	Sigma Aldrich, UK
Human recombinant IL-10 Human recombinant IL-4	Peprotech R&D Labsystem,UK

Table 2.2. Tissue culture reagents used. All reagents were obtained from UK-based companies, or designated distributors.

2.2. Camel milk collection

Camel milk samples were collected during early morning hand milking from 7 healthy lactating camels (240-300 days post-partum), aged 9 to 11 years with 1or 2 calves each. These camels were kept in Royal Corps farm (Muscat-Sultanate of Oman). Milk samples were transported in cooled carrier box to the Sultan Qaboos University, College of Agriculture, filtered through a sieve pore size (25-100 mm), pooled and stored at -80˚C.

2.3. Camel milk total lipids extraction using the Bligh and Dyer standard method

Samples of the pooled camel milk 5.0g were extracted by adding a mixture of 20mL methanol and 10mL chloroform in a 250mL conical flask and shaken for 2mins. An

extra 10mL chloroform was added to each sample and the samples further shaken for 2mins. 18mL of distilled water (D.W) was added to the samples and shaken for 2mins, transferred to a 50mL centrifuge tube and centrifuged for 10mins, at 2000rpm, at room temperature RT. The lower layer chloroform extracts were collected with a Pasteur pipette and placed in a pear-shaped flask (Manirakiza *et al.,* 2001). The solvents were evaporated in rotary evaporator (R-215, BUCHI), connected to; a cooling system at 2˚C, vapour temperature set at 27˚C, rotation at 60rpm, vacuum controller 235mbar and water bath at 60˚C. The lipid samples then dried in a 100˚C oven for 1hr and placed overnight at RT to solidify in a desiccator chamber containing silica pellets to absorb residual moisture (Figure 2.1). The lipids were weighed (flask weight + lipids) – (flask weight and the lipids' weights were calculated in (g), and defined as percentage of the total lipids (TL) and stored under Nitrogen at (- 80˚C) for further analysis.

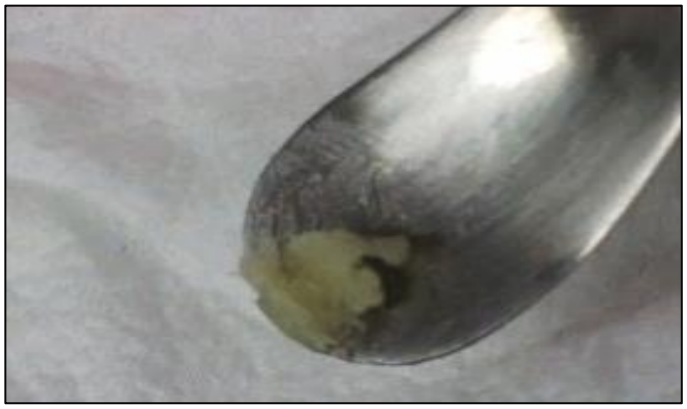


Figure 2.1. Camel milk total lipids as observed post-extraction on a spatula. The lipids appear as a cream coloured emulsion.

2.4. Camel milk total lipids extraction using Roese - Gottlieb method

Camel milk 1g mixed with 1mL 25% ammonia solution (ammonia in water) were vortexed for 2mins, then 7.5mL methanol was added and vortexed for 2mins. 17mL diethyl ether was added and the mixture were shaken. 17mL petroleum ether was added and shaken strongly for extraction the upper layer was transferred to a tarred beaker. Concentration of the lipids was performed with rotary evaporator, and further drying was done at 104°C for 1hr. Any solvent remaining in the sample was removed by placing the flask containing the lipid extract overnight inside a desiccator, with silica pellets at room temperature, (Manirakiza *et al.,* 2001). Lipid weight was calculated in (g), and defined as 100% or the total lipids (TL) and stored under liquid nitrogen at (-80˚C) for further analysis.

2.5. Thin layer chromatography analysis of total lipids

Thin-layer Chromatography (TLC) is classically used for separation and identification of individual lipids (Fuchs *et al*., 2011). To identify the lipids present in camel milk TL a screening with TLC of the TL, extracted using the Bligh & Dyer standard method was undertaken. Respectively; a standard of triglyceride mix 100mg (Sigma-Aldrich), dissolved in 0.5mL solvent and was prepared working solution standard (10µl + 80µl solvent). 10mg of camel milk TL extract was dissolved in 1mL of solvent contain 2:1 (chloroform 40mL: methanol 20mL). The mixture were spotted at 15µl on a line on the silica plate (K6 silica Gel 60A, size 20x20cm, layer thickness 250um) apart. The plate was developed for 1hr with a mobile phase consisting of hexane 80mL: diethy Ether 20mL: formic acid 2mL. Standard lipids and camel TL were visualized by exposure to Iodine vapour for 10mins.

2.6. Preparation of fatty acid methyl esters of total lipids

The determination of fatty acid methyl esters (FAMEs) by GC-MS is among the most commonplace analysis techniques in lipid research BF3-Methanol is a convenient method for the derivatization of fatty acids (Salimon *et al*., 2014). Esterification chemistry requires the reaction of a carboxylic acid with an alcohol in the presence of an acid catalyst. BF3-Methanol is a methanol-catalyst that, when used in excess with heating, rapidy and quantitatively converts carboxylic acids to their methyl esters. Most protocols for preparation of methyl esters with BF3- Methanol require the separation of the methyl ester from the reaction mixture after extraction followed by evaporation of the solvent, this method is suitable for derivatizing higher boiling carboxylic acids.

The method for the production of FAME using boron trifluoride is based on Horwitz and Latimer (2000). Accurately 0.1g of TL were weighed and placed in a 250mL round flask, anti-bumping granules (BDH, UK) were added to the lipids; 4mL of methanolic sodium hydroxide solution mixture of sodium hydroxide 2g (0.5MNaOH, BDH , UK) in 100mL methanol (MeOH - Sigma Aldrich) were added. The flask was connected to a condenser joined with water-cooled, reflux with 20-30/cm jacket and standard taper inner joint. The flask was secured with a holder and heated at ≈100°C-300°C. Anti-bumping granules were added to the lipids, 4mL of sodium hydroxide (0.5M NaOH) added and refluxed, boiled for 7mins until lipid globules disappear. 5mL of boron trifluoride was added dropwise and boiled for 2/mins. In addition, 4mL heptane a non-polar solvent was added dropwise and boiled for a further 1min. Samples were removed and the mixture allowed to cool and 15mL of saturated NaCL solution added to the mixture and mixed thoroughly. The upper heptane layer was transferred to a test tube with screw cap, and the solution shaken for 1min, and the solution allowed to stand for several mins. The top clear heptane layer was collected in another tube, to

which anhydrous Na_2SO_4 was added to remove excess water from the heptane containing the FAMEs. In a GC-MS vial, 1mL of heptane containing the FAMEs was added and analysed using Gas Chromatography-Mass Spectrometry. To confirm the peaks identity, camel milk FAMEs was complemented (spiked) with internal standard (methyl tricosanoate acid), and based on the retention times, which is the time taken for a solute to pass through a chromatography column, the presence of standard and the camel milk FAMEs was confirmed.

2.7. Extraction of camel milk total free fatty acids from camel milk total lipids

Accurately, 0.1g of TL were weighed and transferred to a 100mL round bottom flask. Lipids were saponified with 4mL sodium hydroxide (0.5MNaOH) and the heated (Reflux) at 100˚C for 30mins. Heptane 4mL was added drop by drop and boiled for 1 min. The heating stopped, the mixture allowed to cool at RT. The lower layer of the mixture collected in a test tube and the tube placed on ice to which dropwise cold 2M HCL was added to reach a pH 1-3 using a pH meter. A further 4mL of heptane was added and the upper layer separated in another test tube. An extra 4mL heptane and 15mL of NaCl added and mixed (Hashim and Salimon, 2015; Wanasundara and Shahidi, 1999). The top layer of heptane containing TFAs were collected in another test tube, and dried over anhydrous Na_2SO_4 to remove excess water, filtered through filter paper in a tube and further dried under nitrogen (N_2) to obtain dry residue and stored at -80°C.

2.8. Extraction of camel milk free total fatty acids and free unsaturated fatty acids with urea

There are three main factors that affect the percentage of final product after urea extraction, they are the urea-to-fatty acid-ratio, crystallization temperature and crystallization time. These factors need to be controlled to yield a good amount of unsaturated free fatty acids (USFAs) and saturated free fatty acids (TFAs) with the desired purity of fatty acids of interest (Fei *et al*., 2011). In this study, urea complexation was a focus to increase the content of fatty acids, particularly unsaturated oleic acid (C18:1) and saturated palmitic acid (C16:0) from camel milk (FFAs). In this experiment, a mixture of 100mg camel milk free fatty acids were weighed and added to 1g urea and 3mL methanol. The mixture was heated in a water bath at 65˚C with stirring until the mixture turned a clear homogeneous solution. The mixture was incubated at room temperature overnight to allow the urea to crystallize, then refrigerated overnight for 24hrs at -10˚C. The crystals formed (urea complex fraction - UCF) were separated from the liquid filtrate (non urea complex fraction – NUCF-methanol) by fast vacuumed filtration using a Buchner funnel (Wanasundara and Shahidi, 1999). The free fatty acids present in urea complex fraction (UCF - crystal) and non-urea complex fraction (NUCF-filtrate-methanol) were separated. The urea crystals were collected in a test tube, to which 2mL of distilled water and 1mL of heptane was added, mixed and shaken strongly. Two layers formed, the upper heptane layer was collected in a test tube with screw cap contained total free fatty acids (TFAs) including palmitic acid, and dried over anhydrous Na_2SO_4. The solvent was further dried in a stream of nitrogen gas (Mendes *et al*., 2007). The filtrate (methanol) contains the concentrated total unsaturated free fatty acids (USFAs), collected in a test tube with screw cap and 2mL distilled water and 1mL heptane added, mixed and shaken. An upper layer containing the unsaturated fatty acids and concentrated with oleic acid

was collected in small test tube with screw cap, and dried over anhydrous Na_2SO_4, and subjected to nitrogen (N_2) to obtain dry residue and stored at -80°C.

2.9. Gas chromatography- mass spectrometer fatty acid methyl esters analysis

Analysis of camel milk derived FAMEs was carried out at the Sultan Qaboos University, Muscat - Sultanate of Oman. Gas Chromatography- Mass Spectrometer (GC-MS) analysis was performed on a Perkin Elmer Clarus 600 GC System, fitted with a SP-2560 Supelco® capillary column (100/mm × 0.250/mm inner dimension × 0.2µm film thickness) coupled to a Perkin Elmer Clarus 600C Mass-Spectrometer. Ultra-high purity helium (99.99%) from Air products (UK) was used as carrier gas at a constant flow of 1.0/mL/min. The injection temperature, transfer line and ion source temperatures were all at 250°C. The ionizing energy 70 Electron Voltage (EV). Electron multiplier voltage (EMV) was obtained through auto-tune. All data was collected for a full-scan mass spectra within the scan range 40-550 atomic mass unit (amu). The injected sample volume was 1µl with a split ratio of 50:1. The oven temperature program was 80°C (5 mins) and accelerated at a rate of 4°C /min to 240°C and held for 15mins. The unknown FAMEs, were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition) and further confirmed with Supelco 37 component FAME mixture (cat.# 47885-U).

2.10. Reconstitution of lipid extracts for cells treatment

Before treating the cells with lipids, the extracts were reconstituted in RPMI media and 0.1% Dimethyl sulphoxide (DMSO), to the required concentration in accordance with the experimental design.

2.11. Cell culture

2.11.1. THP-1 cell culture (The human monocyte cell line THP-1)

THP-1 cells were obtained from American Type Culture Cultures (ATCC®, TIB202™). THP-1 cells are human promyelocytic cells, isolated originally from a one-year old child with acute myelocytic leukaemia (Qin, 2012). THP-1 cells were maintained in supplemented RPMI 1640 (Gibco, UK), 10% heat inactivated Foetal Calf Serum (FCS) (Lab-tech International), 1% sodium pyruvate (Gibco, UK), 1% non-essential amino acids (Gibco, UK), and 1% penicillin (100IU/mL), streptomycin(100µg/mL). THP-1 cells were grown in suspension (Takashiba *et al.*, 1999) (Figure 3.5. A).

THP-1 cells were sub-cultured before reaching 80% confluence of about (1.5×10^6 cells/mL) and were used between passages 7-25. Cell splitting and sub culturing are important to make sure that the cells are not forming clumps and float individually in the media suspension. Cells were monitored, counted and their viability determined by transferring 200ul cells to a sterile bijoux bottle then the aliquot were used to perform a cell count. Both sides of the counting chamber of the haemocytometer were filled with cell suspension (approx. 10ul each side), viewed under an inverted microscope at 20x magnification and total cell count performed.

2.11.2. THP-1 cells viability using Trypan Blue™

Cells were counted before seeded of cells using the Trypan Blue™ (Promega, UK) exclusion assay to determine the cell viability to achieve a uniform level of viable cells during all the experiments. Trypan Blue™ dye is a traditional method used to assess the percentage of cells viability, and that involves manual staining THP-1 cell suspension with trypan blue dye in a 1:1 ratio and incubated at room temperature for 5 mins and the use of haemocytometer for counting both viable (unstained) and non-viable (stained) when observed under a microscope (magnification 100x). This results in the ability to easily differentiate between viable and nonviable cells, since the first are unstained (live), small, and round, while the latter are stained and swollen (dead).

2.11.3. Differentiation of THP-1 cells

To achieve differentiated cells that have the same makeup of macrophages by using THP-1 monocytes cells, the cells are exposed to a non-toxic concentration of phorbol-12-myristate-13-acetate (PMA). For the differentiation process, cells were seeded at the density of (3-6 x 10^5 cells/mL) in supplemented RPMI media containing PMA for 48hrs in a humidified incubator maintained at 37°C, 5% CO_2. Thereafter, non-attached cells were removed by aspiration and the media was replaced with fresh media for an additional 48hrs before commencing any further experiments on the differentiated cells (Figure 3.5. B), for all experiments, this cells density of (3-6 x 10^5 cells/mL) were used. The concentration of PMA to use for the differentiation of THP-1 cells 5ng/mL was selected as the optimum concentration for use in all subsequent experiments in this study, and was optimised by (Hassan, 2017).

2.11.3.1. Oil Red O stain

THP-1 cells were maintained as detailed in section 2.11.3 in this chapter, and cultured in 6 well plates. The cells were treated with camel milk TL at concentrations of 20µg/mL and 100µg/mL, then incubated for 1hr and old media removed. Cells were washed (1X) with sterile PBS (1mL/well) prior to staining and fixed in 10% formaldehyde (v/v in PBS) for 15mins at RT, and washed (1X) with distilled water (dH_2O). A working solution of Oil Red O (Sigma, UK) stain was freshly prepared from 0.5% Oil Red O stock solution (w/v) in isopropanol, diluted 3:2 (v/v) with dH_2O and allowed to stand for 15mins, before filtration. Cells were stained with Oil Red O stain working solution 500µl/well, at RT for 15mins. The stain was removed and excess stain washed (1X) with 60% isopropanol (v/v in PBS), followed wash by PBS (2X). The cells were photographed with (Nikon U-200 attached to an inverted Leica microscope) at 200x, magnification using View Finder™ software (version 3.0.1., Better Light Inc., USA) (Figure 3.7).

2.11.4. Determination of dTHP-1 cell viability with Celltiter-blue® assay

To determine the effect of the various camel milk lipids extract on dTHP-1 cell viability, the Celltiter-blue® assay (Promega, UK) was employed. This assay is based on the ability of viable (live) cells to reduce the non- fluorescent blue dye *Resazurin* to a pink fluorescent product *Resorufin,* which emits fluorescence at 590nm ($560_{ex}/590_{em}$). dTHP-1 cells were seeded into tissue-culture sterile 96 well round bottomed polystyrene microtitre plates (Corning Costar Ltd, NY, and USA) and treated with the compounds to be tested. dTHP-1 Cells were grown to a confluence of 80-90% in a 96 well microtitre plate. The cells were incubated with a range of concentrations of the extracted camel milk TL (1µl, 10µl, 20µl, 50µl, 100µl, and 200µl), for 6hrs, 24hrs and 48hrs. The media, which includes the supernatant was aspirated, and replaced with

100µl of fresh media and 20µL of the Celltiter-blue® reagent added to each well in the microtitre plates. CellTiter Blue® (Promega) assay was performed as described by (Lo *et al*., 2008). The plates were incubated for 2hrs at 37°C to allow cells to react with the dye and the absorbance was measured at 560nm, excitation and 590nm emissions using a fluorescent plate reader (Tecan infinite M200, Mannedorf, Switzerland).

2.11.5. dTHP-1 cells treatment with standard lipids

In order to investigate the effect of standard lipids beside the camel milk lipids,on inflammatory cytokines secretion of TNF-α and IL-1β, dTHP-1 cells were treated with standard lipids, unsaturated oleic acid and saturated palmitic acid at concentration of 10µg/mL and 20µg/mL, individually or combined with 500µg/mL glycated BSA, and incubated for 6hrs. Standard ELISA used to determine in culture medium supernatant of dTHP-1 cells the protein concentrations of inflammatory cytokines TNF-α and IL-1β.

2.11.6. dTHP-1 cells treated with camel milk total lipids and stimulated with lipopolysaccharide or glycated bovine serum albumin

dTHP-1 cells were treated with camel milk TL extract at concentrations of 10µg/mL or 20µg/mL and pre-incubated for 1hr. To induce an inflammatory response in the cells the old media was aspirated, and replaced with a fresh media containing glycated bovine serum albumin (gBSA) 500µg/mL, or with 100ng/mL lipopolysaccharide (LPS). Both stimuli used alone or combined with camel milk TL, and incubated for 6hrs in CO2 (5%) incubator at 37˚C. The supernatant was collected and stored at (-20C˚) for analysis with standard ELISA to detect secreted inflammatory cytokine TNF-α.

2.11.7. Human peripheral blood mononuclear cells

Volunteer blood donors were recruited by the Welsh Blood Service. Human peripheral blood mononuclear cells (PBMCs) were obtained from a pooled buffy coat sample of five healthy donors. Ethical approval was obtained from the Cardiff School of Health Sciences Research Ethics Panel (ethics number: 7758). To isolate the PBMCs, 10mL of buffy coat were diluted 1:1 with PBS and separated by density centrifugation using Lymphoprep™ (Stem Cell Technologies, UK) according to manufacturer's instruction. PBMCs were cultured in RPMI-1640 culture media supplemented with 10% FCS and 1% penicillin/streptomycin for 24hrs to allow cell adherence in plastic culture plates. The non-adherent cells were washed and removed with pre-warmed fresh media and the adherent cells stimulated with 5ng/mL PMA in RPMI media and incubated for 48hrs. The cells were further rested in fresh media for another 48hrs before conducting experiments. PBMCs Pre-incubated for 1hr with (20µg/mL) of camel milk TL, then stimulated with (500µg/mL) gBSA for 6 and 24hrs. In this study ELISA used to determine in culture medium supernatant of PBMCs the protein concentrations of inflammatory cytokines TNF-α, IL-1β, at 6hrs and 24hrs, and IL-18 detected only at 24hrs.

2.12. Detection of cytokine secretion using enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISAs) are applied in different areas of biomedical research and discovery as well as for diagnostic purposes. ELISA has a wide dynamic range for cytokine detection, e.g. IL-1β (3.6-300pg), IL-18 (15.6-1000pg) (De Jager *et al*., 2003). In this study ELISA used to determine the protein concentrations of inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-18, secreted in culture medium supernatant of dTHP-1 cells, which were pre-treated with camel milk TL or free TFAs, or free USFAs at concentration of (20μg/mL), the lipids were used individually or combined with gBSA (500μg/mL), and incubated for 6 and 24hrs. In order to create a model of inflammation the cells were stimulated with gBSA or LPS according to the experimental design, and seeded in 24 well culture plates at the density of 6 x 10^5 cells/mL. Cell supernatants were collected and stored at -20˚C. For the purpose of normalisation, cell counts were conducted before seeding of cells using the Trypan Blue™ (Promega, UK) exclusion assay. DuoSet® ELISA kits were used for specific cytokines detection. All kits were obtained from R&D systems (Abingdon, UK): Human TNF-α (Cat. DY210), Human IL-1β (Cat. DY201), Human Total IL-18 (Cat. DY318), IL-6 (Cat. DY206), and used according to the protocols outlined by manufacturer. The concentration of the cytokines produced by the cells was determined by using a linear standard curve.

2.13. Flow cytometeric analysis of surface markers of polarization for dTHP-1 cells treated with camel milk total lipids

Flow cytometry was used to investigate the expression of surface markers of M1 and M2 polarization, including CD86 and CD163 in dTHP-1 cells treated with camel milk TL. THP-1 cells were maintained in RPMI, as detailed in section 2.11.1. THP-1 cell viability monitored using Trypan Blue® dye exclusion method. THP-1 monocyte cells were differentiated as detailed in section 2.11.3 and the cells were incubated with (20µg/mL) of TL alone or combined with (500µg/mL) gBSA for 24 and 72hrs. Cells were harvested by adding 1mM EDTA in PBS solution and detached from the well/plate by gentle scraping and centrifuged at 300xg for 10mins at 4˚C. The supernatant was removed and the cells reconstituted in 100µl FACS buffer (3% BSA in 100mL PBS). Specific anti- CD86 and CD163 antibodies conjugated to fluorophores (20µl) were added to cells and re-suspended in FACS buffer for 20mins on ice, (Table 2.3). The antibodies were washed in PBS and centrifugation at 300xg for 10mins at 4˚C. Supernatants were removed and the cell pellets reconstituted in 500µl FACS buffer for analysis using Cytomics FC500MPL flow cytometer (Beckman Coulter, Buckinghamshire, UK).

Antibody (Human)	Conjugated fluorophore	Dilutions used**	Isotype Control (Mouse)	Dilutions used**	Supplier
CD86	PE	1:20	IgG2b	1:5	Biolegend Ltd, UK
CD163	APC	1:20	IgG1	1:5	

Table 2.3. Antibodies used for flow cytometric analysis. Antibodies and mouse isotype antibodies were all purchased as conjugated antibodies. **Dilutions refer to volume ratios of antibody (Ab) to FACS buffer used (e.g. 1:20=5µl Ab in 100µl FACS buffer).

2.14. Real-time polymerase chain reaction analysis

To determine the effect of different treatments and stimuli on gene expression of selected gene markers, real-time reverse transcriptase – polymerase chain reaction (RT-PCR) was applied.

2.14.1. Background

RT-PCR also called quantitative PCR (qPCR), and widely used for its advantages of high sensitivity, its specific detection and quantification of nucleic acid targets. Throughout thermal cycling, fluorescent dyes are used to label PCR products. During the exponential phase of the fast reaction real-time PCR instruments measure the fluorescent signal accumulation and quantify precisely the PCR products and analyse the data.

2.14.2. Ribonucleic acid extraction and quantification

To isolate total ribonucleic acid (RNA) from cell pellets TRIzol® reagent (Cat.15596-026, Thermo-Fisher Scientific, UK) was used. TRIzol (or TRI Reagent) is a monophasic solution that contains phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein, guanidinium isothiocyanate is an RNAse inhibitor (Rio *et al*., 2010).

dTHP-1 cells were seeded in 12 well tissue culture plates at the density of 6 x 10^5 cells/mL and pre-treated with (20µg/mL) camel milk derived lipids TL,or TFAs, or USFAs for 1hr then stimulated with (500µg/mL) gBSA and incubated for 6 and 24hrs. The culture media was and 500µl of TRIzol® reagent added to the adherent cell

monolayer to produce a viscous homogenate according to manufacturer's guidelines and the samples stored at -80˚C. TRIzol® reagent is highly effective in inhibition of RNase activity; while simultaneously maintaining the integrity of RNA during homogenization. RNA was extracted by using a phenol/chloroform extraction. In brief, chloroform was added to the cell homogenate resulting in an organic and aqueous phase. To precipitate the RNA from the aqueous phase, isopropanol was added, resulting in a very small pellet attached to the inner surface of the eppendorf tubes. To make sure that RNA extracts are clean and don't contain any contamination of protein or phenol (Taylor *et al.*, 2010); the RNA pellets were washed with 75% v/v ethanol and air-dried under a laminar flow cabinet and resuspended in nuclease-free water. To determine the concentration (ng/µl) and purity of RNA NanoDrop® ND-1000 spectrophotometer (Thermo-Fisher Scientific, UK) was used. Purity of the RNA was assessed using the A_{260}/A_{280} and A_{260}/A_{280} absorbance ratios that give an estimate of protein and polysaccharide/phenol contamination in the RNA suspension (Ouyang *et al.*, 2014). Pure RNA has an A_{260}/A_{280} ratio of 1.8-2.2 and an A_{260}/A_{280} ratio of >2.0.

2.15. Complementary deoxyribonucleic acid conversion

RNA was converted to complementary deoxyribonucleic acid (cDNA) using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK) according to manufacturer's guidelines. RNA concentrations were adjusted accordingly to produce 250ng/µl total cDNA at a volume reaction of 20µl (10µl RNA samples + 10µl TaqMan® master mix). To produce cDNA, random primers, dNTP mix, MultiScribe™ Reverse Transcriptase and buffer were added to the RNA samples and placed in a thermal cycler (Applied Biosystems, UK), (Table 2.4). cDNA samples were then stored at -20˚C until use.

Steps	Step1	Step2	Step3	Step4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Table 2.4. The thermal cycle set conditions. This table present the steps of setting the thermal cycle temperature and time for RNA conversion to complementary DNA (cDNA).

2.15.1. Polymerase chain reaction efficiency

To determine the PCR amplification efficiency of the genes analysed, a range of dilutions of cDNA was prepared in the PCR reaction to obtain C_T values. The PCR efficiency (E) was calculated through the semi-log regression plot of the log (cDNA concentration) plotted against the C_T values. An efficiency range of 100 ± 10% was used during the experiments. The PCR equation efficiency is given by the equation:

$E = (10^{-1/m} - 1) \times 100$,

Where m=slope of the linear plot.

2.16. Gene expression analysis

Gene expression was determined by Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems; Warrington, UK). Relative gene expression was calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$). To calculate the relative expression of genes of interest (GOI) the C_T comparative method was applied (Livak and Schmittgen, 2001). To obtain the fold change in (GOI) expression relative to a housekeeping gene (HKG, e.g. GAPDH) using the following formula:

Fold change = $2^{-\Delta\Delta C_T}$

Where $\Delta C_T = C_T$ (GOI) – C_T (HKG)

And $\Delta\Delta C_T = \Delta C_T$ (Sample) - ΔC_T (Control).

2.17. Real-time polymerase chain reaction polarization and immunomodulatory markers in dTHP-1 cells

RT- PCR experiments were undertaken to determine if the various camel milk derived lipids could regulate the expression of markers of inflammatory cytokines, macrophage polarization and immunomodulatory markers in dTHP-1 cells. Single stranded cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kits Protocol (Applied Biosystems-USA). With predesigned primers and probes obtained from (Applied Biosystems) the following genes expression were investigated, (Table 2.5).

Gene	Spanning exon	Amplicon Length	ID	References
IL-10	1-2	98	Hs00961619_m1	Travers *et al.*, 2015
CD86	5-6	104	Hs01567026_m1	Ohlsson *et al.*, 2016
CD36	4-5	83	Hs00354519_m1	Biziota *et al.*, 2016
CD163	6-7	72	Hs00174705_m1	Morandi *et al.*, 2015
CD206	4-5	82	Hs00267207_m1	Sharma *et al.*, 2015
NLRP3	6-7	85	Hs00366461_m1	Bostanci *et al.*, 2011
Dectin-1	1-2	72	Hs00224028_m1	Stopinšek *et al.*, 2016
IL-1Ra	8-9	97	Hs00991010_m1	Nieman *et al.*, 2006
TET2	2-3	62	Hs00758658_m1	Valentini *et al.*, 2016
GAPDH	6-7	93	Hs02758991_g1	Brennan *et al.*, 2015
GUSB	11-12	81	Hs99999908_m1	Imberti *et al.*, 2015

Table 2.5. TaqMan® Primers and probes. Predesigned primers and probes used in RT- PCR to determine markers of dTHP-1 cells, and normalized with housekeeping genes GAPDH and GUSB.

All genes expression were normalized with housekeeping genes GAPDH and GUSB, and was significantly determined with TaqMan-Real-Time PCR reagents which was performed according to the (TaqMan Fast Universal PCR Master Mix, 2X), No AmpErase UNG user guide (life technology- Applied Biosystems- USA), (Table 2.6).

PCR reaction mix component	Volume per 20µl reaction(µl)
20X TaqMan® Gene expression Assay	1
2X TaqMan® fast Gene expression master mix	10
cDNA (100ng/µl to 1.5 ng/µl)	4
RNase-free water	5

Table 2.6. Reaction mix components for TaqMan® Gene expression assay.

2.18. Active Motif® method for cytoplasmic and nuclear cells lysates extraction from dTHP-1 cells treated with camel milk lipids

Active Motif® kit (version D3, Cat #: 40010 and 40410, US) was used for nuclear and cytoplasmic cells lysates extraction according to manufacturer's protocol guidelines. This method has been developed for the preparation of cytoplasmic, nuclear, and whole-cell fractions extracts from various cell and tissue types, fresh or frozen extracts of cells or tissue can be used along with this kit. To achieve the above the following experiment was conducted. In 6 well cell culture plates (Corning®Costar®) with a growth area of 9.5cm^2, surface area 21cm^2, 2mL of the THP-1 cells in suspension media RPMI were seeded in each well at a confluence of 1.5 x 10^6 cells /mL, and differentiated as described in section 2.11.3 by adding PMA at concentration of 5ng/mL of cell. Old media was replaced with fresh media before starting the experiments. dTHP-1 cells

were pre-treated with camel milk TL (20µg/mL), then the supernatant media aspirated away and replaced with a fresh RPMI media containing gBSA at a concentration of (500µg/mL), and incubated at (30mins, 1hr, 2hrs, 4hrs, 6hrs and 24hrs). DMSO (0.1µl/mL) was used as a vehicle control.

2.18.1. Protein quantification

For protein quantification of the cytoplasmic and nuclear extract was collected from dTHP-1 cells treated with camel lipids as stated in section 2.18, the BCA Protein Assay Kit (Cat # 7780, Cell signalling Technology™) was used. This assay principle is based on the production of an intense purple colour in the presence of Cu^{1+} that reacted with bicinchoninic acid. The reaction of protein with an alkaline solution resulting in reduction of Cu^{2+} to Cu^{1+}. The colour produced from this reaction is related to the protein concentration as determined by the absorbance reading at 562nm (product datasheet, Cell signalling Technology™). The assay was carried out according to manufacturer's guidelines. Briefly, a seven-point serial dilution was performed ranged from 125-2000µg/mL with the BSA standard 2mg/mL (component # 11623) provided in the kit. A working reagent was prepared (A+B; 50:1) from Reagent A (component # 11621) was added to reagent B (component # 11622) provided in the kit. The standards and samples were added to a 96 well microtiter plate and working reagent was added to each well, incubated at 37°C for 30mins. After, the plate was left to cool at room temperature RT for 5mins and the absorbance was measured at 562nm using a Tecan Infinite®200 plate reader (Tecan AG, Switzerland).The standard curve was used to determine the protein concentration of the samples.

2.18.2. Human NF-κB Pathway Array Kit (Proteome Profiler™)

Proteome Profiler™ (Human NF-κB Pathway array kit, Cat # ARY029, R&D SYSTEMS® biotechne®brand, USA) was used to detect the levels of proteins involved in the phosphorylation of the diverse signalling proteins involved in induction and activation of the transcription factor NF-κB pathway in dTHP-1 cells treated with camel milk lipids. Cytoplasmic cells lysates extracted as in section 2.18, and quantified as in section 2.18.1 according to manufacturer's guidelines. dTHP-1 cells pre-treated with camel milk TL (20µg/mL) and incubated for 1hr, , then stimulated with gBSA (500µg/mL) and incubated for 2hrs. The cytoplasmic cells lysates collected at 2hrs, and used in this study. The assay principle depends on a spotted capture and control antibodies on a nitrocellulose membrane. The membrane blocked with buffer 3/6 and incubated for 1hr at RT. The buffer aspirated, then; the cytoplasmic lysates mixed with array buffer 1 and incubated overnight. The membrane was washed 3X with wash buffer, followed by adding a detection antibody cocktail and incubation for 1hr, then washing. Streptavidin-HRP was added and incubated for 30mins, then washed 3X with wash buffer. The membrane was placed in a plastic sheet and Chemi Reagent mix added incubated for 1min, then squeezed out any excess Chemi Reagent Mix. The membrane placed in an autoradiography film cassette, and exposed to x-ray film for 5mins. The visible spots containing the proteins can be analysed using transmission-mode scanner and image analysis software (Array procedure sheet, R&DSystems®, USA). (For more details see Appendices A.3).

2.18.3. Detection of NF-κB p50/p65 subunit activation using (Active Motif® TransAM® NF-κB) Kit in cytoplasmic and nuclear extracts of dTHP-1 cells

Active Motif® TransAM® NF-κB Family Transcription Factor Assay kit (version C2, Cat # 43296) was used to detect transcription factor activation in nuclear and cytoplasmic cells lysates extract according to manufacturer's guidelines. This method is an ELISA-based kit which detects transcription factor activation of NF-κB subunits p50/p65 in a 96 well plate coated with oligonucleotide which has a site for the active NF-κB subunits to bind with. By adding a secondary antibody which is conjugated to horseradish peroxidase (HRP) will result in a colorimetric development that can be detected and reading with spectrophotometry (product datasheet, Active Motif®).

The cytoplasmic and nuclear extract, extracted in section 2.18 and stored at -80˚C, and quantified for protein concentration as in section 2.18.1, was used for this assay, the protocol recommend to use protein concentration from 2-20/µg. First step was to add 30µl complete binding buffer to each well and a concentration of 10/µg of lysate for each nuclear and cytoplasmic extract added and mixed with lysis buffer at a volume of 20µl. To the blank wells, only complete lysis buffer was added. The plates were incubated for 1hr at RT, washed with wash buffer (3X). 100µl of primary antibody of NF-κB/p50 or NF-κB/p65 was added to the appropriate plate, then incubated for 1hr at RT, and washed (3X) with with wash buffer. Secondary HRP-conjugated antibody (100µl) of diluted for all wells in plates, covered and incubated at RT for 1hr. Then washed with wash buffer (4X). Finally 100µl of developing solution was added to all the wells, and incubated for 5mins at RT in the dark. When the colour turns blue the stop solution was added forming a yellow colour. The absorbance was measured at 450nm with a reference wavelength of 655nm using a Tecan Infinite®200 plate reader (Tecan AG, Switzerland) and a standard curve used to determine the NF-κB/p50 and

NF-κB/p65 subunits activation. The level of Caspase-1 was measured using Caspase-Glo®1 inflammasome assay (Cat # G9952) according to manufacturer's guidelines, in dTHP-1 cells treated with camel milk TL, TFAs, USFAs at a concentration of (20µl/ml) alone or combined with gBSA (500µg/ml). The luminescent signal of caspase-1 was measured in dTHP-1 cells at 1hr, 2hrs and 4hrs, using a Tecan Infinite® 200 plate reader.

2.19. Western blotting

2.19.1. Background

Western blot is used in immuno-genetics and other molecular biology applications to detect specific proteins from a mixture of proteins extracted from cells or tissues, proteins involved in activation or cell signalling. This method was applied to detect proteins by autoradiography. This technique also called immunoblotting, because an antibody is used to specifically detect its antigen (Towbin *et al*., 1979). The principle of the technique based on separation of a mixture of proteins, which separated based on their molecular weight, and thus by type, over gel electrophoresis. Then transferred to a membrane producing a band for each protein. The protein of interest on the membrane is then incubated with specific labels antibodies, detected by developing the film. The unbound antibody is removed with wash buffer (Mahmood and Yang. 2012).

2.19.2. Protein extraction and quantification

dTHP-1 cells were treated with TL (20µg/ml) or stimulated with gBSA (500µg/ml), and the protein extracted as in section 2.18 of this chapter by using Active Motif® kit (version D3, Cat #: 40010 and 40410, US) and was used for nuclear and cytoplasmic

cells lysates extraction according to manufacturer's protocol guidelines. The Protein Quantification was carried with the BCA Protein Assay Kit (Cat # 7780, Cell signalling Technology™) as in section 2.18.1 of this chapter.

2.19.3. SDS-Polyacrylamide gel electrophoresis

dTHP-1 cells incubated for 1hr with camel milk TL (20µg/mL), and stimulated with (500µg /mL) gBSA or combined with gBSA. The 3T3-L1 adipocytes cell lysate used as control for expression FABP4, were kindly provided by Dr Katherine Connolly. 10µg of total protein from each sample and control were used. All samples were loaded into individual wells of Bis-Tris gels 10% (Novex™, Thermo Scientific). The samples were equilibrated to the same concentration by adding 4X LDS NuPage® loading buffer (Invitrogen Ltd, UK), and 10X Dithiothreitol and RIPA buffer. To denature the proteins, samples were heated in heating block to 90°C for 5mins. The gels were placed in mini-Cell tank (XCell-SureLock™, Invitrogen Ltd, UK), the tank filled with running buffer (MOPS) 3-(N-morpholino) propane sulphonic acid (Invitrogen Ltd, UK). The gel run for 50mins at 200V and 100mA with electrophoresis. Pre-stained protein standards (NovexTM, Thermo Scientific) were used to estimate molecular weights. Resolved proteins were transferred to nitrocellulose membranes using the iBlot® dry blotting system (Invitrogen Ltd, UK), according to manufacturer's guidelines at voltage of 20V for 7mins.

2.19.4. Protein immunoblotting

Antibodies used in this study to investigate the presence of fatty acid binding protein 4 (FABP4). Primary antibody FABP4 (0.099mg/mL) (Abcam, ab92501), horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG (GE Healthcare). Immunoblotting was carried out according to manufacturer's guidelines. In brief, the membrane was washed in 1X with Tris-buffered saline (TBS) containing 0.1% v/v Tween-20 (TBS-T) and blocked for 1hr with blocking buffer (5% Marvel dried milk, Premier foods, Ltd, London, UK) in TBS-T for 1hr at room temperature RT. Next, the membrane incubated with primary antibody (FABP4) diluted 1:1000 in blocking buffer for 12-16hrs at 4°C, with gentle agitation. Membrane was washed with 1X Tris-buffered saline(TBS-T) and incubated with secondary antibody horseradish peroxidase diluted 1:1000 (HRP)-conjugated secondary antibody (goat anti-rabbit IgG, GE healthcare) in blocking buffer for 1hr, at RT with shaking. According to manufacturer's guidelines the proteins bands are visualised by incubating the membrane with ECL HRP-substrate (Amersham™, UK), and exposed to Hyperfilm™ ECL film (Sigma Aldrich, UK). To develop the film, 1X developer and fixer solutions (Sigma Aldrich, UK) was used. The bands detected by western blot indicate the expression of FABP4 in dTHP-1 cells cytoplasm extract.

2.20. Quantikine® ELISA Human FABP4 Immunoassay

Quantikine®ELISA, Catalog Number DFBP40, used for the quantitative determination of human fatty acid binding protein 4 (FABP4) concentrations in cell culture supernatants, and in cell lysates. The Quantikine® ELISA Human FABP4 immunoassay was performed according to the manufacturer's protocol. Briefly, this study was conducted to determine FABP4 in dTHP-1 cells; in cytoplasmic cell lysates, treated with

camel milk TL at concentration of (20µg/mL) and stimulated with (500µg/mL) gBSA which collected previously in section 2.18 at incubation time points (30mins, 1hr, 2hrs, and 6hrs). Also FABP4 detected in dTHP-1 cell culture supernatants, the cells treated with camel milk extracts TL, TFAs, USFAs at concentration of (20µg/mL) and stimulated with (500µg/mL) gBSA and incubated at different time (6hrs, and 24hrs).

2.20.1. Principle of the assay

The mechanism of this assay based on quantitative sandwich enzyme immunoassay technique. The microplate pre-coated with monoclonal antibody specific for human FABP4. A 100µL of assay diluent RD1-89 was added to each well. Then followed by adding a 50µL of standards and samples into the wells. All assays were performed in duplicate. FABP4 present in the samples will bind to the immobilized antibody. The plate was incubated for 2hrs at room temperature. Following, the plate was washed three times for a total of four washes with wash buffer (400µL) using a squirt bottle, any remaining wash buffer was removed by aspirating or decanting and inverting the plate and blot it against clean paper towels. A 200µL of Human FABP4 Conjugate was added to the wells to each well and the plate covered with adhesive strip and incubated for 2hrs at room temperature. After the incubation, the plate was washed as mentioned above. A 200µL of substrate solution was added to each well and incubated for 30mins at room temperature and protected from light. The colour will develop in proportion to the amount of FABP4 bound in the initial step. Finally, a 50µL of stop solution was added to each well. The colour in the wells should change from blue to yellow, and the optical density of the colour is measured of each well within 30mins, using a microplate reader set to 450/nm and a standard curve was used to determine the protein concentration of the samples.

2.21. Statistical analysis

The data presented as the mean ± standard deviation. Depending on the study design, One-way analysis of variance (ANOVA) or two-way ANOVA was conducted for within group comparisons. The statistical package, Graphpad Prism® (Version 5) was used for all statistical analysis. Statistically significant differences between treatments/groups are denoted as * for $p<0.05$, unless otherwise stated. Higher levels of significance were reported as: $p<0.01$ (**); $p<0.001$ (***). The number of experimental repeats denoted as 'n' represents the technical replicates in individual experiments. Dunnett's and Tukey's post hoc tests were used to determine differences between treatments and controls and between different treatments, respectively.

CHAPTER 3

Effect of camel milk derived lipids on dTHP-1 cells gBSA induced inflammatory responses

3.1. Introduction

As previously described in chapter 1. Camel milk has been regarded as having health benefits throughout the history of the Arab world, especially among those people who live in the desert and who depend for their livelihood on camels (Agrawal *et al*., 2005). Milk and milk products are nutritious foods containing numerous essential nutrients, but in Western society, the consumption of milk has decreased partly due to claimed and perceived negative health effects (Haug *et al*., 2007). However, milk's content of oleic acid, conjugated linoleic acid, omega-3 fatty acids, short- and medium chain fatty acids, vitamins, minerals and bioactive compounds may promote positive health effects as it has been suggested that ingesting full-fat milk or fermented milk might be favourable for glycaemic regulation (Haug *et al*., 2007). The fatty acid composition of camel milk varies as a property of the countries where camels live. Camel milk has been reported from Jordanian locations camels to have an equal ratio of saturated fatty acids and unsaturated fatty acid, (Ereifej *et al*., 2011) but these ratios varies with camel species (Farah *et al*., 1989). Of the lipids present, triglycerides accounted for 96% of the total lipids in milk (Lindmark Månsson, 2008). Triglycerides of camel milk contained saturated fatty acids 66.1% and unsaturated fatty acids 30.5% (MS Gorban and Izzeldin, 2001). Dominant fatty acids found in camel milk have been reported palmitic and oleic acid (Attia *et al*., 2000). Studies in relation to the health benefits of dietary lipids have generated contradictory data over many years (Simopoulos, 2016; Malik *et al*., 2012). More than half of bovine milk fatty acids are saturated, accounting to about 19g/L whole milk (Haug *et al.,* 2007). Given the huge amount of interest in milk lipids, there remains very few studies on the health benefits of camel milk. There is a belief in the Middle East that camel milk can prevent the development of diseases such as T2D, if consumed regularly (Malik *et al.,* 2012). Camel milk intake is highly recommended for children having lactase-deficiency and who are allergic to cow milk,

this has been proposed due to the camel milk composition of low fat, non-allergenic proteins and digestible lactose (Shabo *et al.,* 2005).

Currently there is a large demand for alternative treatments and natural products world wide, with on-going research being undertaken to discover the mechanisms through which these products regulate disease. Camel milk has been studied as a treatment and prevention of range of disorders, including inflammatory diseases such as diabetes and allergic disorders (Al-Ayadhi *et al*., 2015). Diabetes prevention and control may be aided with regular consumption of camel milk which, will reduce patients need for insulin. However, much of the evidence of its health benefits are anecdotal and many of the health benefits of camel milk may actually be due to other life style factors of which increased levels of exercise and other dietary levels and components (Mohamad *et al.,* 2009).

Macrophages are heterogeneous cells, they display multiple functions, and regarded as a major source of proinflammatory cytokines secretion in inflammatory diseases; such as diabetes (Murray and Wynn, 2011; Pertyńska-Marczewska *et al*., 2004). In diabetic subjects with high blood sugar (Vlassara and Palace, 2002), complications such as vascular dysfunction, retinopathy, atherosclerosis and hypertension will appear; due to non-enzymatic reaction of sugars and glucose with lipids, proteins and nucleotides (Murthy *et al*., 2003; Baynes, 2001; Narayana Murthy and Sun, 2000).These complications are all related to the hyperglycaemia, which is the accumulation of advanced glycation end products (AGEs) in diabetic's microvasculature (Singh *et al*., 2002).

To study the effect of extracted lipids from camel milk (TL, USFAs, and TFAs) on proinflammatory cytokine secretion, and on cell viability, this study used glycated bovine serum albumin (gBSA) (Pertyńska-Marczewska *et al*., 2004) as an inflammatory stimulus, and dTHP-1 macrophages cells derived from human acute monocytic

leukaemia THP-1 cells. These cells can be phenotypically switched by a wide range of environmental factors such as lipopolysaccharide (LPS) or gBSA in to the proinflammatory M1 which produce inflammatory cytokines such as tumour necrosis factor TNF-α, IL-1β, IL-6, and IL-18, or can be switched to anti-inflammatory M2 macrophages which secretes anti-inflammatory and immunoregulatory cytokines and receptors including IL-10, Dectin-1, IL1Ra. dTHP-1 cells activation status is thought to reflect a beneficial or detrimental role in various diseases (Vogel *et al.,* 2014; Ribatti, 2013; Fujiwara and Kobayashi, 2005). M2 polarization is further amplified by regulatory cytokines such as IL-4 and IL-10 (Mantovani *et al*., 2004). Other types of M2 subsets can be identified depending on the type of stimuli that is used to induce polarization from M0 to M2a, M2b or M2c which are induced by IL-4/IL-13 (Chistiakov *et al.,* 2015). SFAs and USFAs in the diet and their relation with atherosclerosis development are unclear, and recognised to effect macrophage function and lipids and cholesterol content. The SFAs increase the stress regulators within the endoplasmic reticulum (ER) an effect that can lead to apoptosis (Ramírez and Claret, 2015). The presence of USFAs reduce these effects through preventing most SFAs-mediated deleterious effects and that leading to a decrease the cholesterol efflux. Considering the multiple effect of fatty acids on intracellular signalling pathways such as IKK/NF-κB ,PKC-θ, IKKα, and JNK (Shi *et al*., 2006). The role of dietary fat on several mechanisms, relay on controlling macrophage lipids content and inflammation by regulating M1/M2 macrophage responses (Alfonso *et al.,* 2014). The exact mechanism and action of camel milk derived lipids in regulating inflammation has not yet fully investigated hence, this study will explore the mechanisms through which the camel milk derived lipids regulate gBSA and LPS induced inflammation in dTHP-1 macrophages.

3.2. Aims

1. To extract and characterize the camel milk total lipids (TL) from 7 pooled camel milk and analyse for their triglycerides (TG) using thin layer chromatography (TLC) and to characterize the TL fatty acid methyl esters (FAMEs) using GC-MS.
2. To investigate if a higher concentration of unsaturated fatty acids (USFAs) and free total fatty acids (TFAs) could be achieved, by urea crystallization fraction method and characterize their FAMEs using GC-MS.
3. To investigate the effect of camel milk TL on the viability of cultured human monocyte/macrophage dTHP-1 cells.
4. To investigate the effect of standard fatty acids beside the camel milk TL, free USFAs, and free TFAs in regulating a range of proinflammatory and anti-inflammatory response of gBSA treated dTHP-1 cells and confirm any modulatory effects are observed in primary human mononuclear cells PBMCs.

3.3. Results

3.3.1. Extraction and characterization of camel milk total lipids

The initial part of this study was to extract the camel milk TL of whole camel milk. The Bligh and Dyer method was used as a standard method to determine TL from milk such as camel milk (Smedes and Askland, 1999). The analysis of fatty acids containing compounds requires their hydrolysis or saponification, the separation of the non-acidic constituents (when necessary) and the liberation of the acids from the mixture. This method requires a lower solvent ratio 10% (v/v) MeOH in CHCL3 and as methanol (MeOH) and chloroform (CHCL3) are appropriate solvents for TL extraction, including the neutral and polar lipids. Before gas-chromotography, it is necessary to prepare non-reactive derivatives of fatty acids as methyl esters, which are more volatile than the free acid components. Acylated lipids are transformed by a transesterification reaction by which the glycerol moiety is displaced by another alcohol in acidic conditions using BF_3. The results of chromatographic analysis using GC-MS analysis and chromatogram are presented in (Table 3.1) and in (Figure 3.1).

This extraction contained fatty acids saturated (60.69%), and unsaturated fatty acids (39.30%). These results are similar to the concentration of lipids extracted from camel milk reported by MS Gorban and Izzeldin, (2001). The predominant SFAs are palmitic acid (35.28%), myristic acid (14.46%) and stearic acid (7.40%), and the predominant USFAs were oleic acid (19.31%), and palmitoleic acid (14.00%). In addition the fatty acids results represents a lipid yield of approximately 3.0 % of the total milk.

Compound name (FAME)	Composition%
Saturated fatty acids	
Palmitic acid (C16:0)	35.28
Myristic acid (C14:0)	14.46
Stearic acid (C18:0)	7.400
Pentadecanoic acid (C15:0)	1.023
Lauric acid (C12:0)	1.033
Tridecanoic acid (C13:0)	0.352
Margaric acid (C17:0)	0.501
Arachidic acid (C20:0)	0.141
Capric acid (C10:0)	0.132
Heneicosanoic acid (C21:0)	0.374
Un-Saturated fatty acids	
Oleic acid (C18:1,cis9)	19.31
Palmitoleic acid (C16:1, cis-9)	14.00
11-Octadecenoic acid (C18:1n-7) (vaccenic acid)	1.721
Myristoleic acid (C14:1n9c)	1.400
Heptadecenoic acid (C17:1, cis-10)	1.011
7(Z)-Hexadecenoic acid (16:1 trans-9)	1.000
Arachidonic acid (C20:4n6)	0.411
12,15-Octadecadienoic acid (C18:2)	0.240
11,14-Eicosadienoic acid (C20:2)n6	0.211

Table 3.1. Camel milk total fatty acids compounds presented as % of total camel milk lipids. Fatty acids extracted from Omani camel milk (n=7 pooled camel milk) using the Bligh and Dyer method, the saturated lipid components was approximately 61% as compared to the unsaturated 39%.

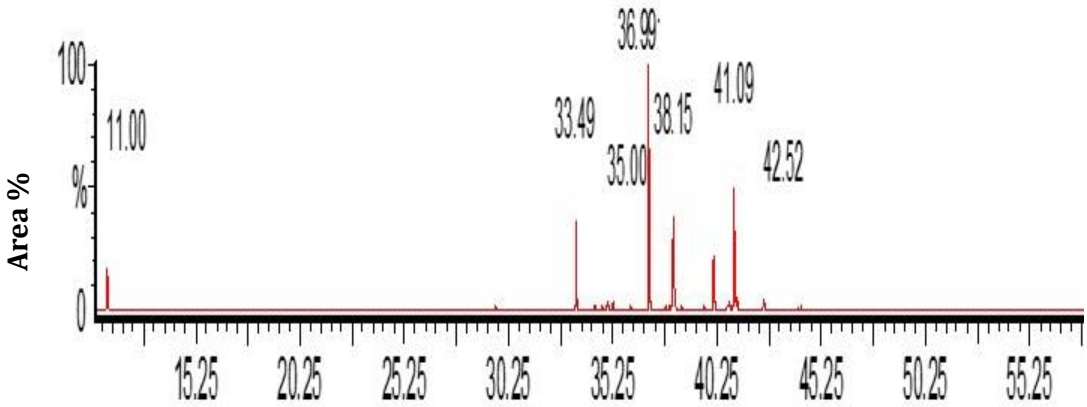


Figure 3.1. A typical GC-MS chromatograph. The fatty acids methyl ester (FAMEs) of the camel milk lipids extracted by Bligh and Dyer Standard (BDS) method. Each peak represents a single fatty acid species. In this chromatograph at 36:99 mins, identifies the fatty acid methyl ester of palmitic acid (C16:0).

3.3.2. Camel milk total lipids extraction using Roese - Gottlieb method

In addition to the Bligh and Dyer method an alternative extraction method and further analysis for the fatty acids was undertaken. This extraction carried by the Roese-Gottlieb method demonstrated very similar results to that observed with the Bligh-Dyer methods as the GC-MS chromatography profile of the fatty acids is presented in (Table 3.1). Both these methods suggested similar concentration range of the saturated and unsaturated fatty acids present in camel milk, (Table 3. 2).

Method	Saturated fatty acids	Composition %	Un-Saturated fatty acids	Composition %
Bligh-Dyer	Myristic acid (C14:0) Palmitic acid (C16:0) Stearic acid (C18:0)	14.46 35.285 7.599	Palmitoleic acid (C16:1) Oleic acid (C18:1)	13.658 18.997
Roese-Gottlieb	Myristic acid (C14:0) Palmitic acid (C16:0) Stearic acid (C18:0)	13.902 37.150 8.367	Oleic acid (C18:1)	19.033

Table 3.2. Camel milk total fatty acids compounds presented as % of total camel milk lipids. Two methods were used for total camel lipids extraction: Bligh and Dyer Standard, and Roese – Gottlieb. All these methods show substantial camel milk lipids extraction, but method Bligh - Dyer were used for its beneficial and reasonable recovery of extracts.

3.3.3. Thin layer chromatography analysis of camel milk triglyceride lipids extract

Thin layer chromatography (TLC) is a simple, fast and in-expensive method used to separate complex compounds according to their polarity by using different solvent system as a mobile phases on a silica gel plate, and visualized by different staining or by exposure to compounds vapours like Iodine (Fuchs *et al*., 2011). In (Figure 3.2), two samples of camel milk lipids extract analysed by TLC as their total lipids and visualized by exposure to Iodine vapours for 10mins. This analysis would suggest that the only identifiable components of camel milk TL were triglycerides. This data is similar to that previously reported by MS Gorban and Izzeldin, (2001).

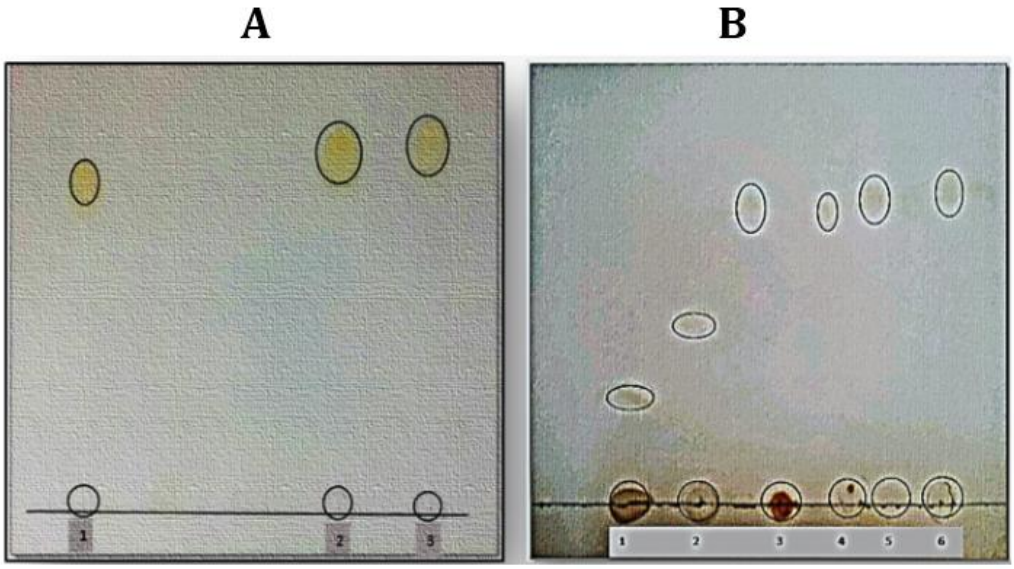


Figure 3.2. (A, B) Typical thin-layer chromatography of standard lipids and camel milk lipids extract. A (1) Standard triglyceride 100mg, (2, 3) Triglyceride of camel milk lipids extract 10mg. **B** (1) Cholesterol standard 5mg, (2) α-D-palmitoyl-Phosphatidyl-Choline standard, (3) Palmitic acid standard 10mg (4) Standard triglyceride 100mg, (5-6) camel milk lipids extract 10mg. Standard lipid and camel milk lipids were visualized by exposure to Iodine vapors for 10mins.

3.3.4. Camel milk free unsaturated fatty acids and total free fatty acids concentrated by the urea crystallization fraction method

To investigate if the unsaturated fatty acids (USFAs) lipids could be extracted separately from the total free fatty acids (TFAs) so that their immunomodulatory effects could be investigated separately from the TL in later experiments. To investigate if the USFAs and TFAs could be extracted separately, further extraction and analysis of these fatty acids was undertaken by GC-MS after urea complex crystallization fractionation (Fei *et al*., 2010). Upon crystallization, TFAs form complexes with urea rapidly as trans- fatty acids with their formation being less efficient with increasing numbers of double bonds or in the presence of branched chains. This procedure is frequently applied to obtain a concentrate of polyunsaturated or branched-chain fatty acids. The data for this analysis and the GC-MS chromatograms are presented in (Figures 3.3, 3.4) and (Tables 3.3, 3.4).

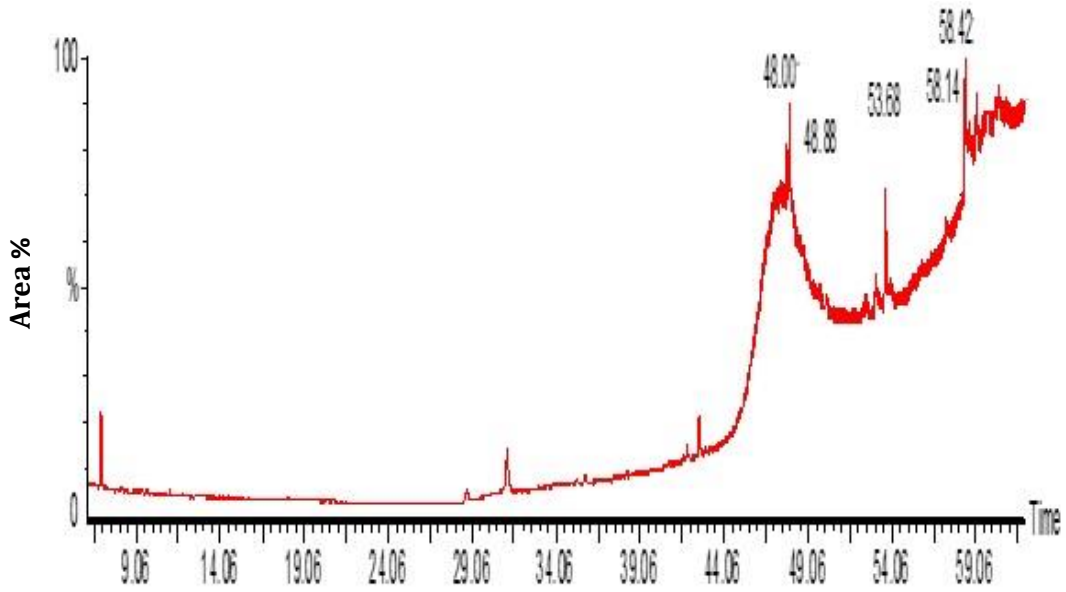


Figure 3.3. Representative GC-MS chromatograph for the unsaturated free fatty acids methyl ester (USFAMEs). Extracted with urea crystallization method, the extracts collected from the urea filtrate by fast vacuumed filtration using a Buchner funnel, incubated for 24hrs and refrigerated at -10˚C. The filtrate (methanol) contains the concentrated 81.22% unsaturated free fatty acid oleic acid (C18:1n9).

As compared with the TL urea crysatllization produced a mixture that was over 90% unsaturated lipids with the two major components being oleic and linoleic acid. Palmitic acid was the only substantial saturated component at less than 3% of total free fatty acids. This component of predominantly free unsaturated fatty acids were labelled USFA when used in this investigation.

Compound name (FAME)	**Composition%**
Oleic acid (C18:1n9)	81.22
Linoleic acid (C18:2)	11.11
Palmitic acid (C16:0)	2.791
Palmitoleic acid (C16:1)	1.344
Myristic acid (C14:0)	0.856
11-Octadecenoic acid (vaccenic acid) (C18:1n-7)	0.345
Caprylic acid (C8:0)	0.206
Heptacosanoic acid (C27:0)	0.173
Palmitic acid (C16:0)	0.130

Table 3.3. Unsaturated free fatty acids (USFAs) in camel milk identified by GC-MS. The greatest proportion of unsaturated consisted of oleic acid (C18:1) 81.22%, and linoleic acid (C18:2) 11.11%. These fatty acids were collected from non-urea complex fraction (filtrate phase) after incubation for 24hrs at -10˚C.

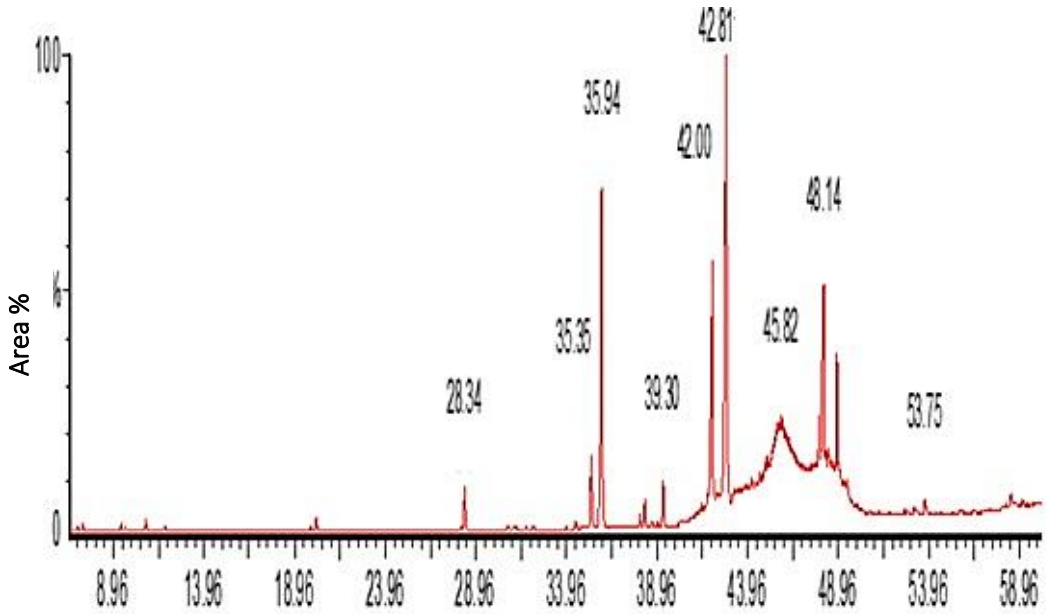


Figure 3.4. Representative GC-MS chromatograph for the free total fatty acids (TFAMEs). Enriched with saturated extract of free total fatty acids (TFA) extracted with urea crystallization and collected from the urea crystal method, incubated for 24hrs and refrigerated at -10˚C.

The residue from this extraction was expected to contain SFAs as its major components. However as shown in (Table 3.4), a substantial (>35%) of USFAs were also present in this extract. Regardless, it was decided to continue using this extract that was a mixture of free saturated and unsaturated fatty acids for further studies and was labeled Total Free Fatty acids (TFAs).

Compound name (FAME)	Composition%
Palmitic acid (C16:0)	34.11
Myristic acid (C14:0)	22.31
Oleic Acid(C18:1)	20.10
Palmitoleic acid (C16:1)	14.11
Lauric acid (C12:0)	2.810
Pentadecanoic acid (C15:0)	2.150
Heneicosanoic acid (C21:0)	1.023
Pentadecanoic acid (C15:0)	0.511
Capric acid (C10:0)	0.450
Tridecanoic acid (C13:0)	0.210

Table 3.4. GC-MS result of free total fatty acids (TFAs) compounds separated by urea method. Fatty acids were collected from urea complex fraction (crystal phase) after incubation for 24hrs at -10˚C. Enriched with saturated palmitic acid (C16:0) at concentration of 34.11%. A substantial (>35%) of USFAs were also present in this extract.

3.4. dTHP-1 cells morphological characteristics

THP-1 monocytes have been shown to be differentiated into macrophage like cells by exposing them to the phorbol-12-myristate-13-acetate (PMA) (Genin *et al*., 2015) the concentration of PMA used for the cells differentiation was 5ng/mL and selected as the optimum concentration for use in all subsequent experiments. (Figure 3.5) demonstrates the microscopical features of THP-1 monocytes and dTHP-1 cells before and after PMA treatment. The cells post-differentiation became adherent and showed substantial morphological changes, macrophages take on a more elongated shape, with increased cytoplasmic volume (Sa *et al*., 2014). Treating THP-1 monocytes with PMA, activates protein kinase C (PKC), and induces a greater degree of differentiation in their expression of surface markers such as CD36, (Figure 3.6) which is associated with macrophage differentiation (Daigneault *et al*., 2010).

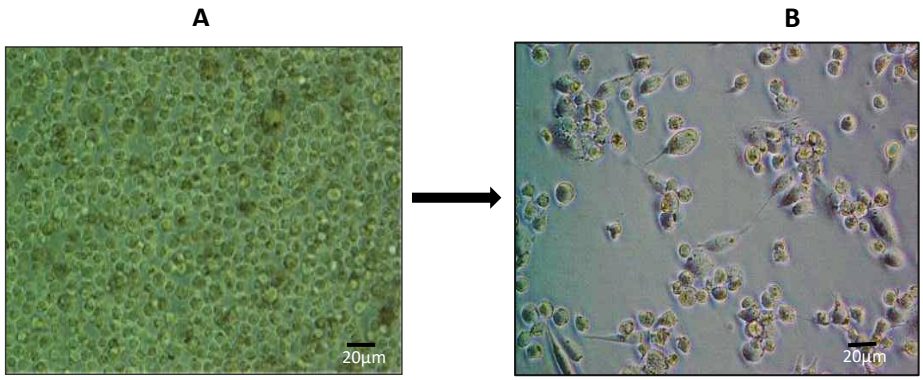


Figure 3.5. (A) Monocytes THP-1 cells undifferentiated, (B) Differentiated dTHP-1 cells. Macrophages (+PMA) incubation for 48hrs. Magnified 200x with Nikon U-200 attached to an inverted Leica microscope (Leica Microsystems UK Ltd., UK).

The cells in (Figure 3.5.B) demonstrated significantly enhanced CD36 expression as compared to those in (Figure 3.5.A). When CD36 mRNA was analysed by RT-PCR, hence, in conjunction with the microscopical features reported, this data would support the ability of 5ng/mL PMA to convert THP-1 cells to differentiate into macrophage-like dTHP-1 cells.

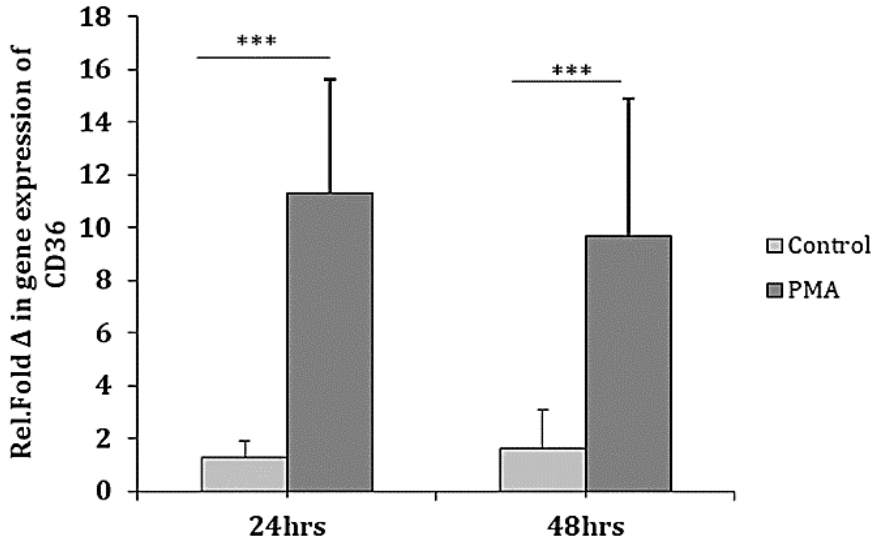


Figure 3.6. CD36 mRNA expression in THP-1 cells and in dTHP-1 cells. The expression of CD36 mRNA in (control) THP-1 cells and in differnetiated (dTHP-1) cells at 24 and 48hrs post-treatment with PMA (5ng/ml). A significant increase in the gene-expression of CD36 mRNA was ($p<0.001$) observed when cells were treated with PMA. Results are reported relative to control THP-1 cells. The gene expression was normalised against GAPDH. Difference between treatments was analysed by Two-way ANOVA with Bonferroni post-tests. Data presented as mean ± SD. [*** $p<0.001$, n=3].

3.4.1. The CellTiter-Blue® Cell Viability Assay used to determine the effect of extracted camel milk total lipids on cell viability of dTHP-1 cells

dTHP-1 cells were treated with a range concentrations of camel milk TL (1µl, 10µl, 20µl, 50µl, 100µl, and 200µl), and incubated for 6, 24 and 48hrs. The effect of lipids on cell viability was determined using the CellTiter-Blue® Cell Assay. The CellTiter-Blue® Cell Viability Assay is a fluorescent method for monitoring cell viability and is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Non-viable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal (Figure 3.7 A, B, C). A small but significant increase in cell viability was observed when cells treated with camel milk TL at concentration of 20µg/mL at 48hrs. However, at 6 and 24hrs the camel milk TL significantly did not affect the cells viability. Based on this observation and the ability of different concentrations of camel milk TL which significantly did not affect the dTHP-1 cells viability, it was decided to select the 10µg/mL or 20µg/mL as doses of treatments in the subsequent experiments. Furthermore, it was decided that the major time points in which the cells would be incubated with lipids would be 6 and 24hrs and hence, the increase in viability was unlikely to be a factor in the results obtained. Therefore, it was decided to select the CellTiter-Blue® Cell Viability Assay to determine the cell viability of dTHP-1 cells in all subsequent experiments.

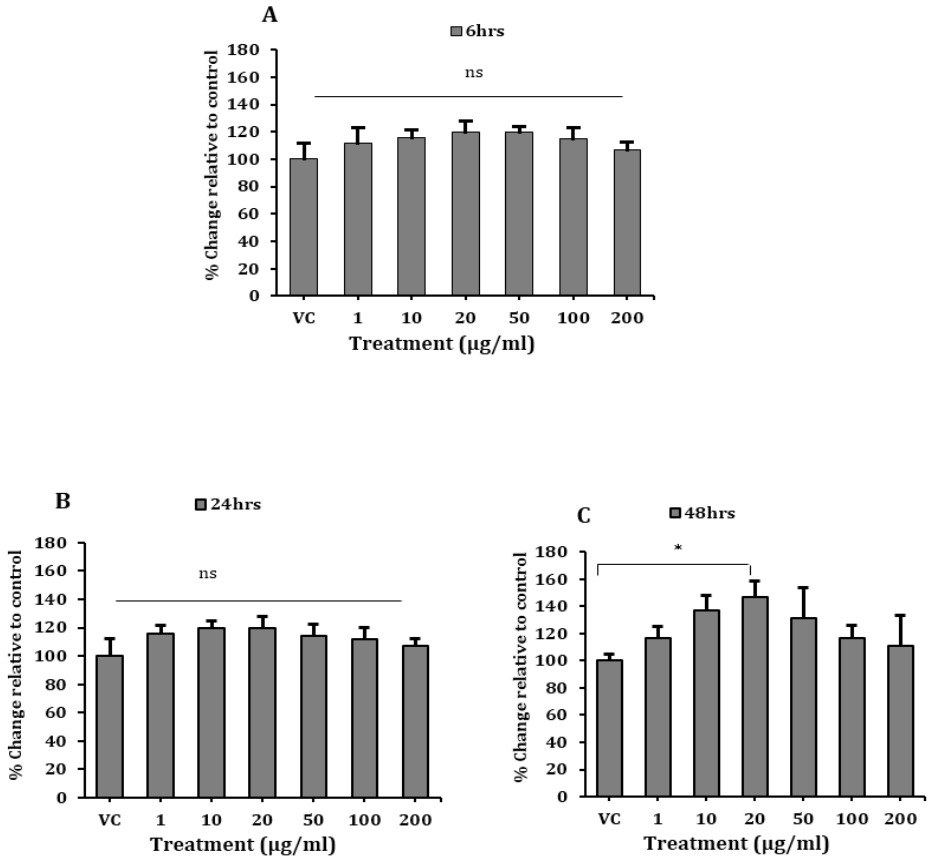


Figure 3.7. Effect of camel milk total lipids on dTHP-1 cells viability. Cells viability as determined using CellTiter-Blue® assay. dTHP-1 cells were treated with VC control (0.1% DMSO) or camel milk TL at concentrations (1, 10, 20, 50,100,200 µg/mL). (**A**) 6hrs, (**B**) 24hrs no significance difference observed within cell viability. (**C**) 48hrs a significant increase in cell viability was observed ($p<0.05$) within cells treated with (20µg/mL). Data presented as mean ± SD. Differences between treatments was analysed by Two-way ANOVA with Bonferroni post-tests. [* denotes $p<0.05$; ns: Not significant; n=3].

3.4.2. Oil Red O stain for the detection of total lipids uptake in dTHP-1 cells

Oil Red O is a fat soluble diazo dye used for the staining of triglycerides and neutral lipids. Oil Red O been used for visually determining lipid uptake by cells including macrophages and THP-1 cells in several studies including in the uptake of fatty acids (Hua *et al*., 2015; Xu *et al*., 2010; Kao *et al*., 2009). To investigate if the triglycerides present in camel milk TL, were observable in dTHP-1 cells after incubation in RPMI media, cells were incubated for 1hr with camel milk TL at 20µg/mL and 100µg/mL and visulalised the lipids after staining with Oil Red O stain (Figure 3.8).

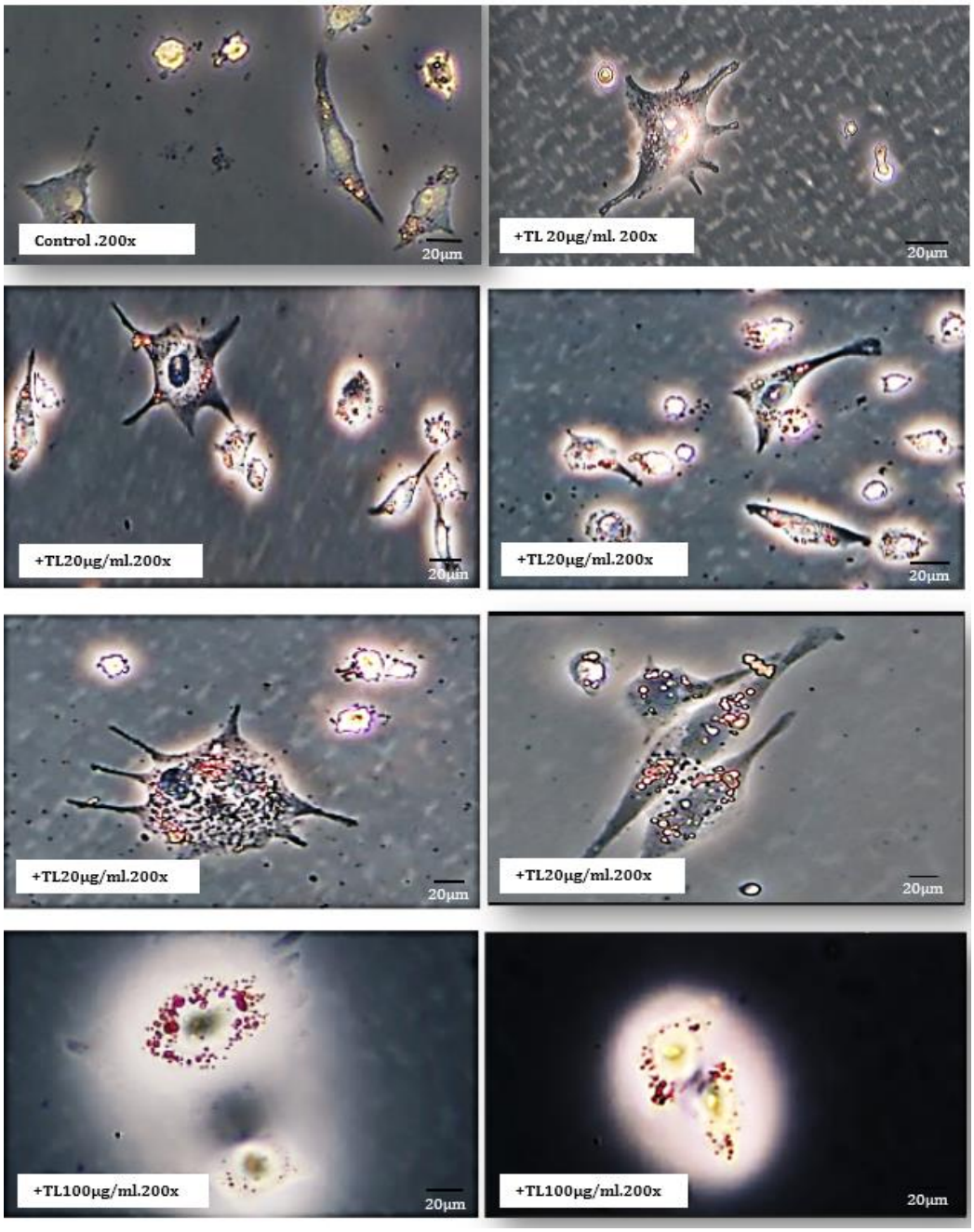


Figure 3.8. Oil Red O stain demonstrates lipids uptake by dTHP-1 (1 x 10^6/mL) treated with camel milk total lipids. After incubation with the TL (20µg/mL) and (100µg/mL), lipids were observable in cells. Stained with Oil Red O stain working solution 500µl/well, and incubated at room temperature (RT) for 15mins. Figures demonstrates that dTHP-1 cells were capable in taking up the camel TL. Magnification 200x with Nikon U-200 attached to an inverted Leica microscope. (Control: dTHP-1 cells untreated with TL).

3.5. Lipopolysaccharide or glycated bovine serum albumin induced TNF-α secretion and the anti-inflammatory effect of camel milk total lipids on both stimuli

T2D is a disease leading to high glucose concentrations within tissues that react with tissue and plasma proteins, such as albumin leading to the production of glycated proteins (Zhang *et al*., 2008). These glycated protein-products can lead to macrophage inflammation, itself leading to cytokine secretion that are mediators of the disease and several complications of T2D, such as nephropathy, retinopathy, atherosclerosis and coronary artery disease (Arasteh *et al*., 2014; Rosenson *et al*., 2011; Fowler, 2008).

This *in vitro* investigation, investigated the ability of camel milk lipids in regulating inflammatory cytokines secretion from dTHP-1 cells. dTHP-1 cells were stimulated with two inflammatory stimuli, gBSA and LPS and the results analysed in (Figure 3.9). To confirm whether the effect of camel milk TL on pro-inflammatory cytokines associated with gBSA or LPS and if its specific gBSA or LPS, dTHP-1 cells were stimulated with gBSA or LPS in the presence or absence of TL with two concentrations 10µg/mL and 20µg/mL. The level of TNF-α secreted was determined in cell free supernatants using standard enzyme-linked immunosorbent assay (ELISA). High secretion of TNF-α from dTHP-1 cells treated with gBSA. This change can be observed with the gBSA vs LPS results analysis, gBSA: 101.765 ± 5.801 pg/mL vs LPS: 18.695 ± 0.522 pg/mL in (Figure 3.9). The results demonstrate gBSA-induced very significant ($p<0.001$) TNF-α secretion in dTHP-1 cells at the time point investigated. Therefore, it was decided to select gBSA to stimulate an inflammatory response in dTHP-1 cells model in all subsequent experiments.

The results in (Figure 3.9) demonstrate as well the effect of camel milk TL at concentrations of 10µg/mL and 20µg/mL, on the gBSA induced TNF-α secretion in dTHP-1 cells, treated with gBSA alone or when gBSA combined with camel milk TL at only 6hrs. The analysis demonstrates a significant reduction ($p<0.001$) in the level of TNF-α secretion. In the presence of gBSA alone which was: 101.765 ± 5.801 pg/mL vs gBSA+ TL10: 65.679 ± 6.986. The level of TNF-α secretion was reduced significantly when gBSA combined with TL 10µg/ml. At a higher concentrations 20µg/ml of camel milk TL treatment, the camel milk TL were further able to significantly reduce ($p<0.001$) the gBSA induced TNF-α secretion; gBSA alone: 101.765 ± 5.801 pg/mL vs gBSA+TL20: 57.942 ± 4.155.

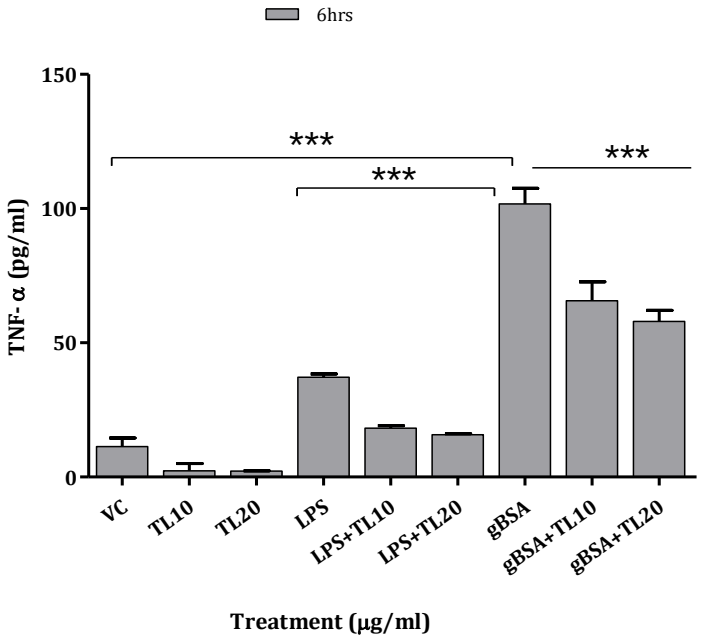


Figure 3.9. Camel milk total lipids regulate gBSA and LPS induced TNF-α secretion. dTHP-1 cells were pre-incubated for 1hr with (10µg/mL) and (20µg/mL) of camel milk TL, then stimulated with (500µg/mL) gBSA or (100ng/mL) LPS for 6hrs. Data presented as mean ± SD relative to VC (0.1% DMSO). Differences between treatments and control were determined by Two-way ANOVA with Bonferroni post-tests. [*** $p<0.001$; n=3].

3.6. Human FABP4 detection with western blot and with immunoassay in dTHP-1 cells treated with camel milk total lipids, total fatty acids, and unsaturated fatty acids

Fatty acid binding protein 4 (FABP4)/ (aP2) is a highly homologous denoting protein, and its sequence structure similar is to myelin P2 protein (Smathers and Petersen, 2011). FABP4 expression is identified in macrophages, mainly upon inflammatory stimulation (Hotamisligil and Bernlohr, 2015), and detected in cell supernatants derived from dTHP-1 cells (Furuhashi *et al*., 2014). Moreover, binding affinities of FABP to different ligands, FABP4 was exceptional among FABPs for its high attraction for saturated and unsaturated fatty acids, palmitate, stearate, oleate, linoleate, linolenate, and arachidonate (Friedman *et al*., 2006; Richieri *et al*., 1994). The ability of macrophages and adipocytes to accumulate lipids will increase the expression of receptors such as CD36 and cytokines such as IL-1β and TNF-α, in these cells (Bouhlel *et al*., 2007; Pelton *et al*., 1999). Oil Red O staining demonstrated uptake of lipids by dTHP-1 cells, however, it was decided to determine if FABP4 a regulator of lipid transportation and uptake within cells such as macrophages, was modulated by lipid treatment (Furuhashi *et al*., 2016).

3.7. Western blot analysis confirm of fatty acid binding protein 4 in cytoplasmic extract of dTHP-1 cells

The western blot in (Appendices A.2) confirm the presence of fatty acid binding protein 4 (FABP4) in dTHP-1 cells cytoplasmic extract. The dTHP-1 cells treated with TL (20µg/mL) or with inflammatory stimulator gBSA (500µg/mL) alone or combined with gBSA and incubated for 1hr. dTHP-1 cells cytoplasmic extract collected as in section 2.18. The results show that FABP4 present in the dTHP-1 cells cytoplasmic

extract in all three treatments with the lower level of expression was detected when the dTHP-1 cells treated with gBSA+TL for 1hr.

3.8. Quantikine® ELISA Human FABP4 immunoassay analysis of localization of fatty acid binding protein 4 in cytoplasmic extract of dTHP-1 cells

Western blot analysis demonstrated that fatty acid binding protein 4 (FABP4) was detected in dTHP-1 cells cytoplasmic extract, treated with camel milk TL (Appendices A.2), following this detection, a Quantikine® ELISA Human FABP4 immunoassay kit was undertaken in order to confirm localization of FABP4 in cytoplasmic extract of dTHP-1 cells treated with TL or gBSA or TL + gBSA, and incubated for 30mins, 1hr, 2hrs, and 6hrs. The results confirm the presence of FABP4 in cytoplasmic dTHP-1 cells extracts and confirm that; the lower level of expression was detected at 1hr incubation of the dTHP-1 cells treated with gBSA+TL (Figure 3.10).

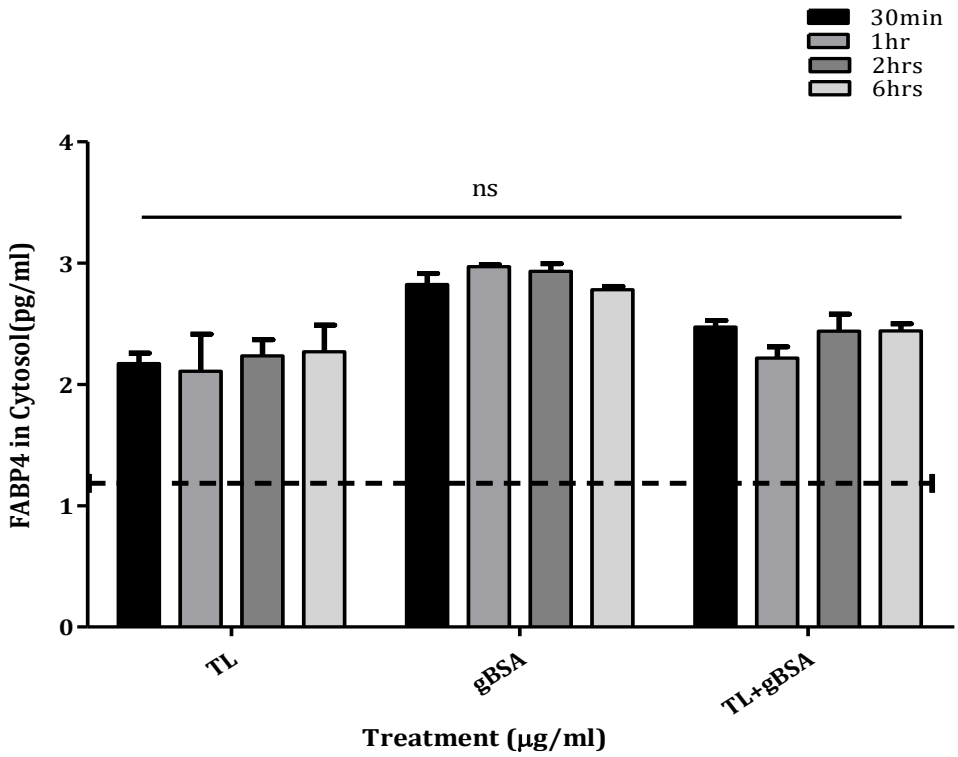


Figure 3.10. Secretion of fatty acid binding protein 4 in cytoplasmic (cytosol) extract of dTHP-1 cells. Human FABP4 immunoassay results confirm the presence of FABP4 in cytosolic extract of dTHP-1 cells, FABP4 reduced at 1hr when cells treated with TL+gBSA.This result suggests the localization of FABP4 in the cytosol of the dTHP-1 cells and supports the western blot presented in (Appendices A.2). In this analysis cells incubated for 30mins, 1hr, 2hrs, and 6hrs with (20µg/mL) camel milk TL, and stimulated with (500µg /mL) gBSA. Data presented as mean ± SD relative to VC (0.1% DMSO), difference between treatments and control were determined by Two-way ANOVA with Bonferroni post-tests. [ns: Not significant; n=3]. Dotted line represents the VC.

3.9. Quantikine® ELISA Human FABP4 immunoassay analysis and detection of fatty acid binding protein 4 in dTHP-1 cell supernatants

dTHP-1 cells incubated with different extracts of camel milk TL,TFAs, and USFAs alone or combined with gBSA and incubated for 6 and 24hrs. Results show a significant secretion of fatty acid binding protein 4 (FABP4) in the supernatant among all three extracts of camel milk lipids. The results were are presented in (Figure 3.11). In general, the stimulatory effect of gBSA was significantly reduced when gBSA was combined with camel milk lipids different extracts at both time points of incubation 6 and 24hrs.

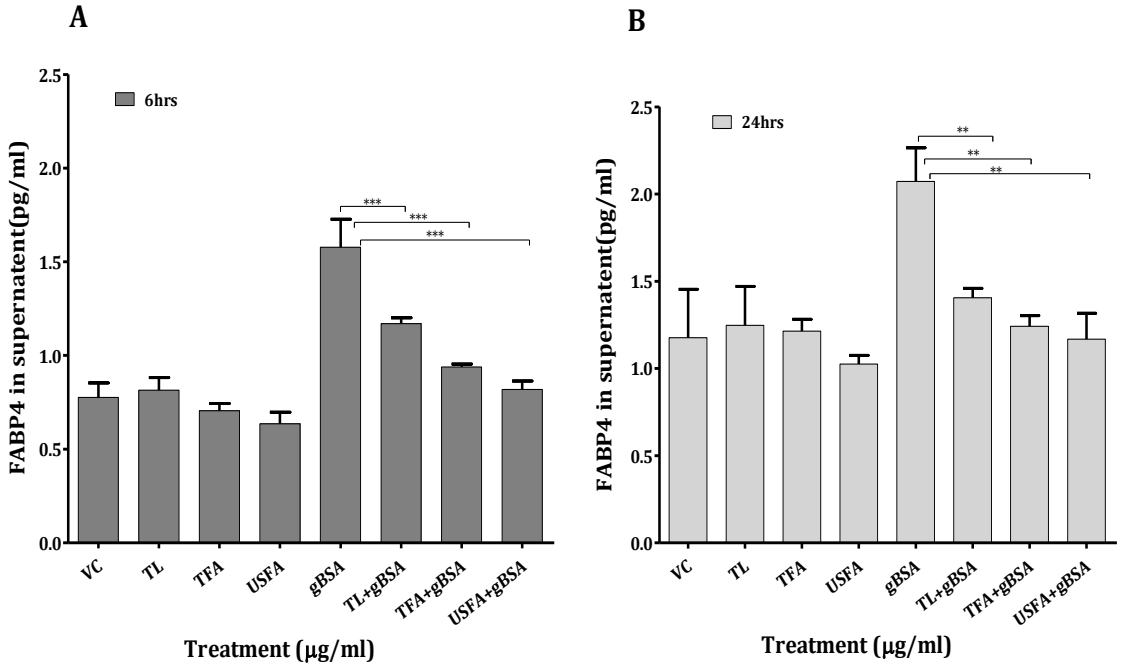


Figure 3.11. gBSA induced secretion of fatty acid binding protein 4 in dTHP-1 cells is regulated by camel milk lipids. Fatty acid binding protein 4 (FABP4) secretion at (**A**) 6hrs, and (**B**) 24hrs, in supernatant of dTHP-1 cells treated with camel milk lipids extracts. Cells were incubated for 6 and 24hrs with (20µg/mL) camel milk TL, TFA, USFA and stimulated with (500µg/mL) gBSA. Data presented as mean ± SD relative to VC (0.1%DMSO), difference between treatments and control was determined by Two-way ANOVA with Bonferroni post-tests. [** $p<0.01$ and *** $p<0.001$; n=3].

3.10. The effect of standard fatty acids on gBSA induced TNF-α and IL-1β secretion

dTHP-1 cells are a well validated human macrophage model, and upon stimulation with a proinflammatory agent, dTHP-1 cells are known to release a wide range of proinflammatory and immunoregulatory cytokines (Chareonsirisuthigul *et al*., 2007). To understand the regulatory effect of camel lipids on dTHP-1 cells the standard fatty acids unsaturated oleic acid and saturated species palmitic acid were studied besides the camel milk lipids in assessing their beneficial effect on secretion of the proinflammatory cytokine TNF-α. In (Figure 3.12), dTHP-1 cells were incubated for 6hrs with standard palmitic acid and oleic acid alone or combined with 500µg/mL glycated BSA. The investigation shows, that there is a significant reduction ($p<0.001$) of TNF-α secretion in both concentrations of unsaturated oleic acid when cells treated with gBSA: 24.40±3.980 pg/mL vs gBSA+OA20: 11.65±1.440 pg/mL as demonstrated in (Figure 3.12.A). However, treatments with saturated palmitic acid increased the secretion level of TNF-α significantly ($p<0.05$) in both concentrations used.

The differences on the effect between the unsaturated oleic acid and saturated palmitic acid on inflammatory responses has been previously reported and has been linked to their ability to regulate NF-κB (Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells) (Zhao *et al*., 2005).

In the same manner, IL-1β levels were assessed and presented in (Figure 3.12.B) its secretion significantly decreased when the dTHP-1 cells were incubated for 6hrs with 20µg/mL of oleic acid unsaturated fatty acid or when combined with gBSA. The analysis demonstrates the treatment with gBSA: 22.500± 3.950 pg/mL vs gBSA+OA20: 3.279± 2.746 pg/mL, show a reduction in the level of secretion ($p<0.001$). However; treatment with saturated palmitic acid increased the IL-1β cytokine secretion in cells ($p<0.05$), significantly.

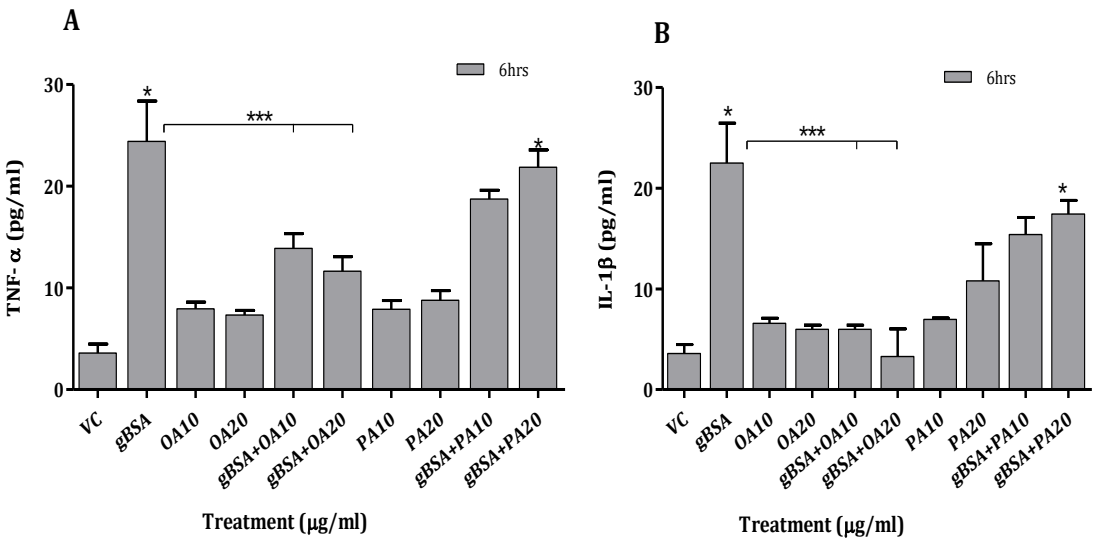


Figure 3.12. Standard saturared and unsaturated fatty acids modulate gBSA induced (A) TNF-α and (B) IL-1β secretion. dTHP-1 cells were pre-treated for 1hr with palmitic acid and oleic acid (10μg/mL) and (20μg/mL) and the cells stimulated with gBSA (500μg/mL) for 6hrs, and cytokines secretion determined. Unsaturated oleic acid significantly decreased ($p<0.001$) the secretion of TNF-α, and saturated palmitic acid significantly ($p<0.05$) enhanced TNF-α secretion after gBSA treatment compared to gBSA alone. Saturated palmitic acid significantly increased the IL-1β secretion in cells ($p<0.05$) after gBSA treatment compared to gBSA alone. The unsaturated oleic acid significantly decreased ($p<0.001$) the secretion of IL-1β cytokine when used alone or when combined with gBSA compared to gBSA alone. Data presented as mean ± SD relative to VC (0.1% DMSO). Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [* denotes $p<0.05$, and *** $p<0.001$; n=3].

3.11. Effect of camel milk derived lipids on proinflammatory cytokines release in dTHP-1 cells

Since this study hypothesized that camel milk lipids could regulate the inflammatory response of human macrophages; proinflammatory cytokines, TNF-α, IL-1β, IL-18 and IL-6 were investigated. dTHP-1 cells were pre-treated with total camel milk lipids TL which extracted with Bligh and Dyer standard method, or TFAs, or USFAs at concentration of 20μg/mL, the lipids were used individually or combined with gBSA 500μg/mL, and incubated for 6 and 24hrs.

3.11.1. Quantification of TNF-α using ELISA

TNF-α, is a proinflammatory cytokine, produced by activated macrophages (Barkett and Gilmore, 1999). TNF-α level of secretion was investigated in this study in the dTHP-1 cells treated with camel milk lipids extracts TL, TFA, and USFA, alone or combined with gBSA. This investigation showed a significant reduction in the level of TNF-α secretion when cells treated with gBSA alone at 6hrs: 245.759 ± 17.419 pg/mL vs gBSA+TL: 117.5185 pg/mL. There was further significant reduction in level of TNF-α secretion ($p<0.001$) at 24hrs, (Figure 3.13.A). Further treatment with USFA or TFA alone or combined with gBSA compared with gBSA alone showed a significant reduction effect in TNF-α secretion ($p<0.001$) at both 6 and at 24hrs, (Figure 3.13.B).

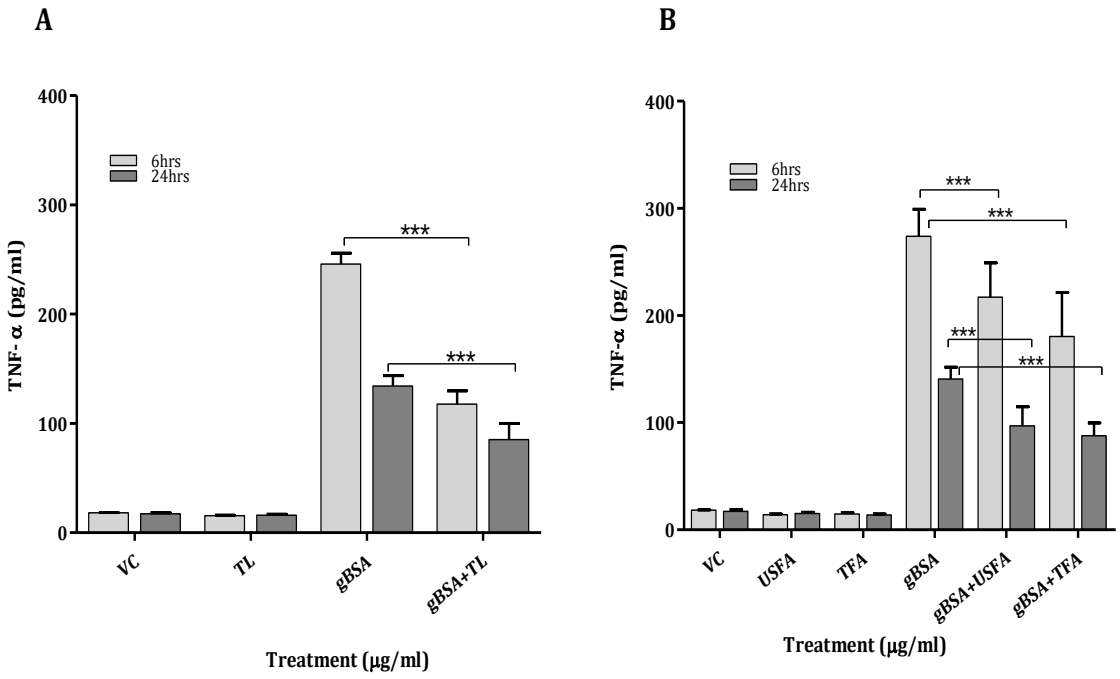


Figure 3.13. Camel milk derived lipids down-regulate gBSA induced TNF-α secretion. dTHP-1 cells were pre-incubated for 1hr with (20µg/mL) of **(A**) TL, **(B)**TFA, and USFA, then stimulated with (500µg/mL) gBSA for 6 and 24hrs. Data presented as mean ± SD relative to VC (0.1% DMSO). A significant reduction ($p<0.001$) in TNF-α secretion at 6 and 24hrs when the cells treated with camel milk lipids extracts alone or when combined with gBSA. Difference between treatments was determined by Two-way ANOVA with Bonferroni post-tests. [*** $p<0.001$; n=3].

3.11.2. Quantification of IL-1β using ELISA

Stimulation of macrophages by a diverse range of stimuli, such as LPS and gBSA, will activate the macrophages to polarize towards M1 type, resulting in cytotoxic and tissue-damage proinflammatory functions, in which proinflammatory cytokines are produced such as IL-1β and IL-18 (Wang *et al*., 2014). In this investigation dTHP-1 cells were incubated with camel milk TL, TFA, USFA alone or combined with gBSA for 6 and 24hrs, the effect of camel milk lipids on level of IL-1β secretion was detected in (Figure 3.14. A, B). The amount of IL-1β secreted by the cells at 24hrs was substantially lower than that observed at 6hrs. However, incubating the cells for 6hrs with gBSA alone: 27.111 ± 2.7337 pg/mL vs gBSA+TL: 13.185± 2.0555 pg/mL, resulted in significant ($p<0.001$) reduction in the level of IL-1β secretion. The same significant ($p<0.001$) reduction was observed in treatments of cells with TFA and USFA combined with gBSA compared to gBSA alone and incubated for 6 and 24hrs.

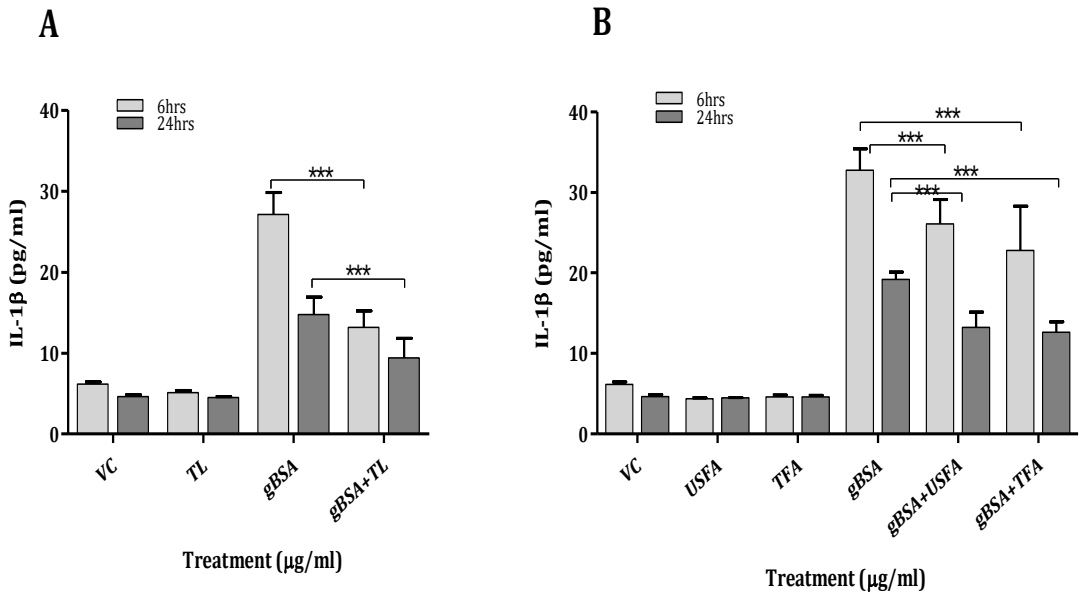


Figure 3.14. Camel milk derived lipids down-regulate gBSA induced IL-1β secretion. dTHP-1 cells were pre-incubated for 1hr with (20µg/mL) of **(A)** TL**, (B)**TFA, USFA, or VC (0.1% DMSO), then stimulated with (500µg/mL) gBSA for 6 and 24hrs. A significant reduction ($p<0.001$) in IL-1β secretion at 6 and 24hrs when the cells treated with camel milk lipids extracts alone or when combined with gBSA. The amount of IL-1β secreted by the cells at 24hrs was substantially lower than that observed at 6hrs. Data presented as mean ± SD relative to VC (0.1% DMSO). Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [*** $p<0.001$; n=3].

3.11.3. Quantification of IL-18 using ELISA

Obesity mediated by proinflammatory cytokines such as IL-18 can lead to other complications like diabetes and cardiovascular disease; this effect can be controlled by controlling the secretion of proinflammatory cytokines with a combination of diet and physical activity (Esposito *et al*., 2003). In this investigation, camel milk TL, TFA, USFA when administrated to inflammatory dTHP-1 cells alone or combined with gBSA, for 6 and 24hrs, resulted in a significant change in the level of IL-18 secretion ($p<0.001$) for all three extracts of lipids TL, TFA, USFA, (Figure 3.15. A, B). For instance, analysis was observed at 6hrs in presence of gBSA: 15.333± 0.9944pg/mL vs gBSA+TL: (6.4962± 0.2311pg/mL, the reduction in level of IL-18 secretion was significant ($p<0.001$), the same reduction was observed at 24hrs and when cells treated with camel TFA and USFA combined with gBSA.

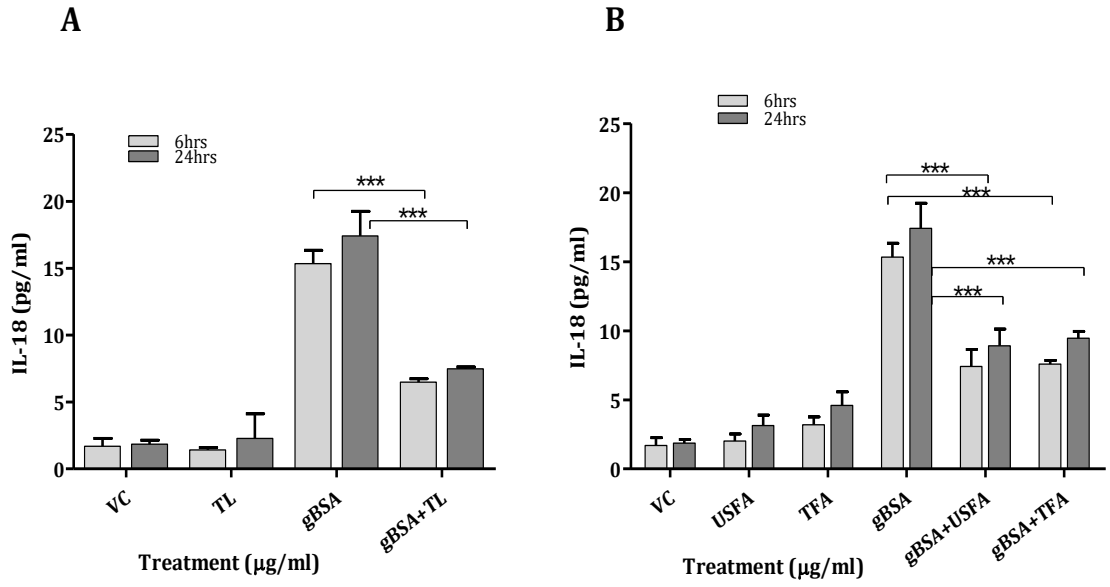


Figure 3.15. Camel milk derived lipids down-regulate gBSA induced IL-18 secretion. dTHP-1 cells were pre-incubated for 1hr with (20µg/mL) of camel milk **(A)** TL, **(B)**TFA, USFA, then stimulated with (500µg/mL) gBSA for 6 and 24hrs. Data presented as mean ± SD relative to VC (0.1% DMSO). Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [*** $p<0.001$; n=3].

3.11.4. Qunatification of IL-6 using ELISA

Earlier studies have verified a high level of proinflammatory cytokines TNF-α and IL-6 in T2D patients (Esposito *et al*., 2002). In this study, at 24hrs incubation of dTHP-1 cells with camel milk TL, TFA, USFA or gBSA combined with lipids, the effect of camel milk lipids on level of IL-6 secretion was detected, (Figure 3.16. A, B).The analysis was observed at 24hrs incubation, at 6hrs IL-6 not detected. A reduction in the cytokine level of secretion was observed when the cells treated with TL, TFA and USFA or combined with gBSA.

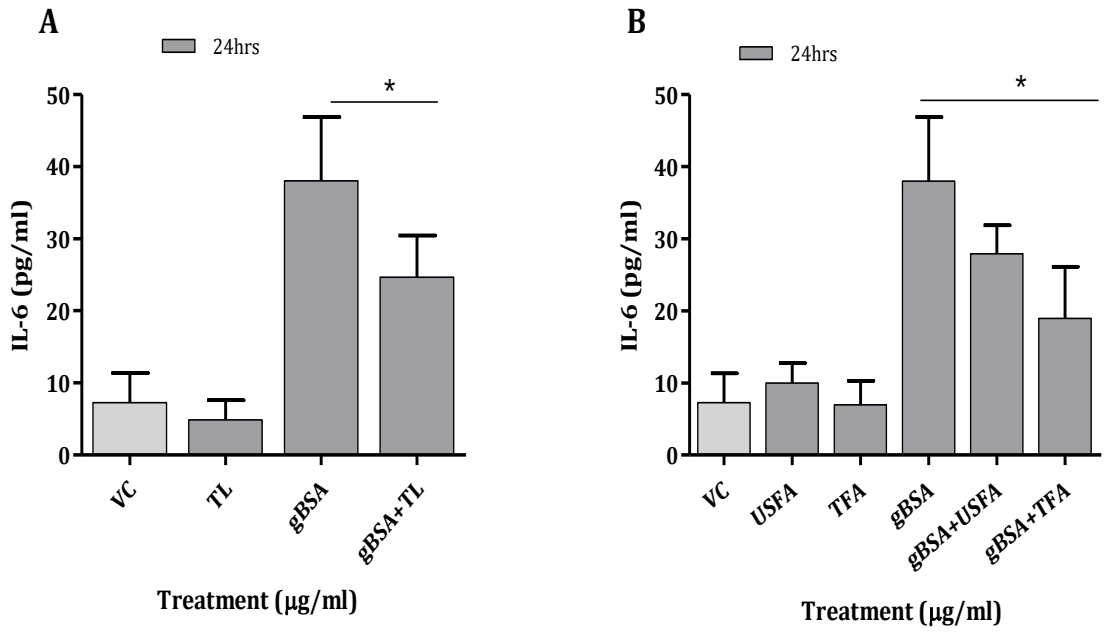


Figure 3.16. Camel milk derived lipids down-regulate gBSA induced IL-6 secretion. dTHP-1 cells were pre-incubated for 1hr with (20µg/mL) of camel milk **(A)** TL, **(B)** USFA, TFA, then stimulated with (500µg/mL) gBSA for 6 and 24hrs. Data presented as mean ± SD relative to VC (0.1% DMSO). Difference between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [*$p<0.05$, n=3].

3.12. Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs), retaining a single, round nucleus and are collected from the peripheral blood from middle layer called the buffy coat after centrifugation (Zhang and Huang, 2012). Subsequent experiments used PBMCs as a human *in vitro* model to investigate the effects of camel milk lipids on, a model of a human *in vitro* in which PBMCs are stimulated with camel milk TL and gBSA in a similar way to dTHP-1 cells. Monocytes were isolated from PBMCs cells and differentiated to macrophages by treating them with 5ng/mL PMA. Cell morphology was examined microscopically to confirm macrophage-like features.Treatment of PBMCs with gBSA resulted in increased expression of inflammatory cytokines TNF-α, IL-1β at 6hrs and 24hrs and IL-18 detected at 24hrs only. gBSA combined with camel milk TL was able significantly to decrease ($p<0.001$) the secretion of TNF-α, also IL-1β significantly decreased ($p<0.01$), both cytokines decreased at 6hrs and 24hrs. However, IL-18 significantly decreased ($p<0.05$) only at 24hrs, (Figure 3.17).

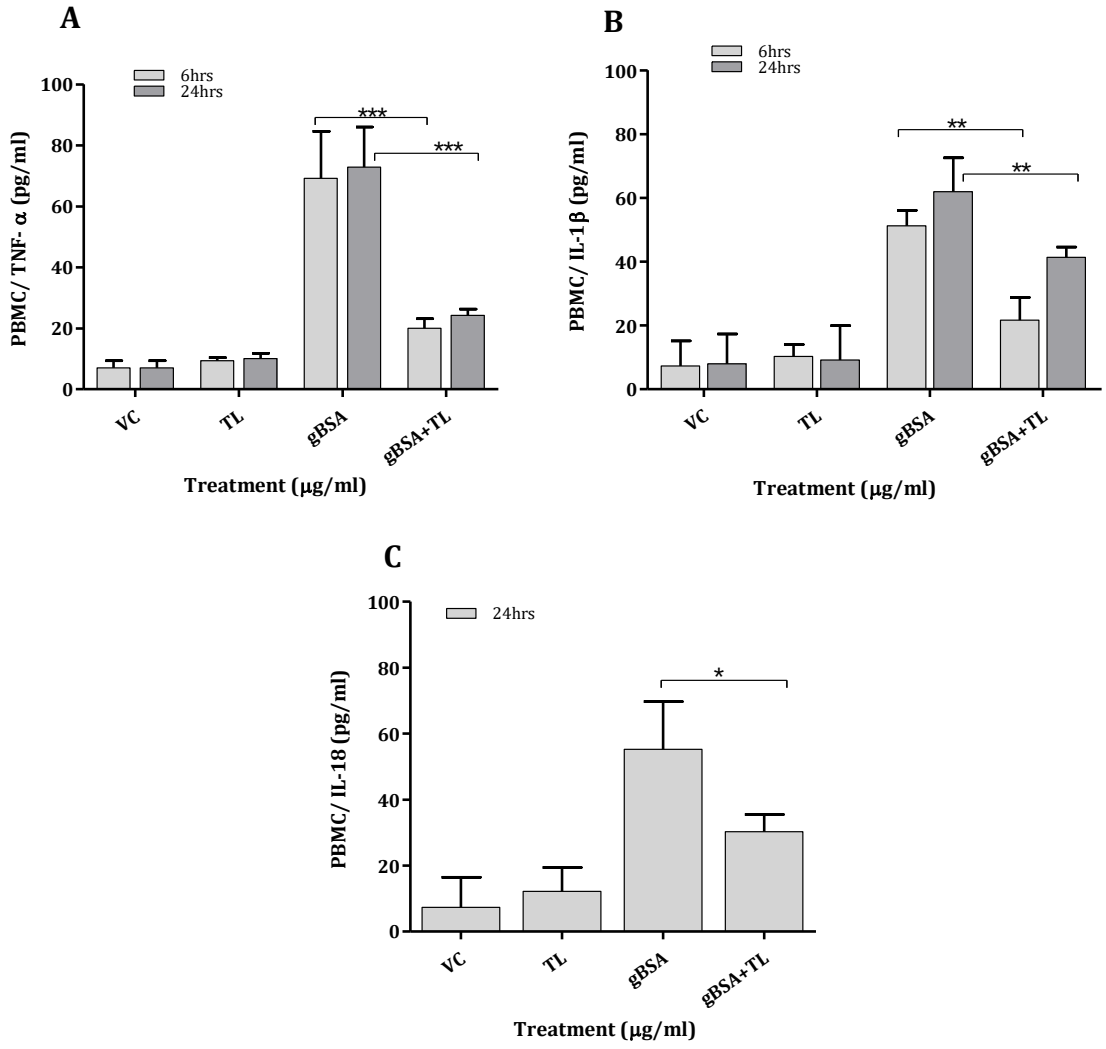


Figure 3.17. Camel milk total lipid down-regulates gBSA induced (A) TNF-α, (B) IL-1β, (C) IL-18 secretion in PBMCs. Differentiated PBMCs were pre-incubated for 1hr with (20µg/mL) of camel milk TL, then stimulated with (500µg/mL) gBSA for 6hrs or 24hrs. Data presented as mean ± SD relative to VC (0.1% DMSO). Difference between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [* denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$; n=3].

3.13. Discussion

This study confirmed that the major lipid component of camel milk was in triglyceride (TG) form with a relatively small proportion of other lipids. Major fatty acids of this TG from GC-MS acids profile demonstrated, that the saturated lipid components were approximately 61% as compared to the unsaturated percentage of 39% of these fatty acids, palmitic acid and myristic acid were the major saturated and oleic and palmitoleic acid unsaturated .

Historically SFAs have been regarded as an important risk factor in the development of cardiovascular disease (Briggs *et al*., 2017). The consumption of SFAs is reported to increase low-density lipoprotein (LDL) cholesterol, a strong risk factor for cardiovascular disease (Mensink & World Health Organization, 2016). The American Heart Association and American College of Cardiology (AHA/ACC) guidelines on lifestyle management to reduce cardiovascular risk recommended reducing SFA intake to lower plasma LDL cholesterol (Eckel *et al*., 2014) and strongly support the view that dietary saturated lipid intake should be restricted. However, there currently remains considerable confusion as to how saturated fats could be replaced by other dietary components including unsaturated lipids or carbohydrates and the effect that such replacements will have on health, lipid metabolism and cardiovascular health (Mozaffarian, 2016). Some lipid sources of SFAs such as coconut oil however have shown positive health results, and controversy remains about the type of lipids consumed in the diet (Lawrence, 2013; Assunçao *et al*., 2009). To add further confusion other factors in the process of lipid ingestion, proposed in the prevention of chronic inflammatory diseases, requires improved understanding (Silva Figueiredo *et al*., 2017). Dietary SFAs have been implicated in inducing a range of inflammatory responses in macrophages, including significant inflammatory cytokine secretion,

regulation of macrophage polarization and activation of the p65 component (Enos *et al*., 2014; Enos *et al*., 2013) of NF-κB. These effects were also associated with enhanced markers of insulin resistance in mice (Silva Figueiredo *et al*., 2017).

This study focussed on the role of camel milk lipids in the regulation of the inflammatory response of macrophage cells that have a major role in the development of inflammatory disease such as atherosclerosis. The major part of this study used dTHP-1 cells that although possessing a number of limitations, have been used for many years and well validated in their inflammatory responses. Type 2 diabetes (T2D) is a highly inflammatory disease and is a highly significant mediator of atherosclerosis and cardiovascular disease (Huang, 2009), this study used gBSA, the glycated form of albumin that is present in and is a highly inflammatory agent in T2D. In addition gBSA, induces inflammatory cytokine secretion through the receptor RAGE and the NF-κB pathway in dTHP-1 cells (Figure 1.9). All classes of lipids used in this study including the complete TG form of the lipids were found to significantly reduce gBSA induced inflammatory cytokine secretion in dTHP-1 cells. All the cytokines investigated are known to play a role in the pathogenesis of diverse inflammatory diseases including T2D and cardiovascular disease (Guest *et al*., 2008). Consequently, it can be proposed that the camel milk lipids could act in a beneficial way in reducing the inflammation associated with chronic inflammatory diseases. Given that dTHP-1 cells are a monocytic leukaemia cell line, and although these are human cells, any data derived from such cells is limited by features of such cells that include mutations that make the data generated not directly transferrable to *in vivo* actions. Therefore, the data in (Figure 3.17) in primary PBMC cells is additional evidence that the action of the total lipids is not specific to an immortalised cell line such as dTHP-1 cells. Clearly, further studies are required to confirm that the anti-inflammatory actions of these lipids is

transferable to other macrophages or specific tissue macrophages such as those present in adipose tissues.

It can be argued that, the use of total lipids derived from camel milk in the triglyceride (TGs) form is not appropriate as dietary lipids as present in camel milk are not what human macrophages would interact with in tissues. The absorption and metabolism of TGs is complex and dietary TGs present in camel milk will be acted upon by pancreatic lipase, bile salts, and colipase protein co-enzyme, this protein required for optimal enzyme activity of pancreatic lipase (Lowe, 1994). Colipase secreted by the pancreas in an inactive form (procolipase) which is activated in the intestinal lumen by trypsin, which function co-operatively to ensure the efficiency of lipid digestion (Iqbal and Hussain, 2009) and leading to absorption of free fatty acids and monoglycerides, the two digestive products of lipids. This study did not investigate the range of enzymes that cells such as macrophages have available for both uptake and metabolism of lipids. However, the images in (Figure 3.8) suggest, using lipid staining, that these TGs are absorbed and internalised by the cells and that the cell viability data are not (at the concentrations utilised) deleterious to the cells. Some studies have demonstrated lipotoxic (Aronis *et al*., 2008) effects in J774.2 macrophages associated with TGs. This present study however, would not support this finding.

It is possible that the fatty acids present in these TGs can become available to tissue macrophages such as those present in adipose tissue, where much of the inflammation in T2D is observed (Cildir *et al*., 2013). It can be argued that the final potential status of these fatty acids within adipose cells supports a beneficial effect for camel milk derived lipids. A paper that demonstrates that fatty acids released from adipose cell lipolysis do not induce an inflammatory response in THP-1 macrophages (Caspar-Bauguil *et al*., 2015) supports this argument.

This study used mainly, gBSA as the agent for inducing an inflammatory response in dTHP-1 cells. The actions of gBSA as an AGE are mediated by binding to its receptor RAGE. This study did not determine the specific signalling pathways and ability of the lipids to bind to modulate expression or signalling pathways that involve RAGE and/or CD14 TLRs. The camel milk TL were able to significantly suppress the actions of LPS (Figure 3.9), whose proinflammatory activity involve binding to its receptor CD14 and then interaction with members of the Toll-like receptors including TLR2/4. This data would suggest that the actions of these lipids are not specific to the AGE-RAGE binding or LPS binding. However, both AGE-RAGE and LPS-CD14-TLR binding result in the complex activation of the nuclear receptor NF-κB (Wang *et al*., 2017).The actions of these lipids on this important nuclear transcription factor will be explored in chapter 5.

The analysis of the fatty acids extracted from the camel milk TG, also supports an anti-inflammatory action of these camel milk derived lipids that mirrors that seen in the TGs. This part of the study was limited by a failure to specifically extract the saturated component of the fatty acids. However, it was possible to concentrate the USFAs component. The studies on the fatty acid component of camel milk, demonstrated that even in the presence of a major percentage of SFAs retained a significant ability to suppress inflammatory cytokine secretion by gBSA (Figures 3.13, 3.14, 3.15, 3.16, 3.17). Interestingly, unsaturated oleic acid was able to significantly reduce TNF-α and IL-1β secretion (Figure 3.12) when used in its pure form or when combined with gBSA compared to gBSA alone. The actions of the unsaturated oleic acid appeared to be substantially greater than that of saturated palmitic acid. This result would suggest that the anti-inflammatory cytokine actions of the highly complex mixture of the camel milk lipids, is not specific to the unsaturated or saturated lipids.

The anti-inflammatory actions of these fatty acids were maintained when the major USFAs were extracted at the expense of the saturated components. This would support the generally favourable actions of USFAs that previously reported (Lee *et al*., 2004). However, although the unsaturated components was not removed, when the mixture of USFAs and SFAs were added to the cells, the action of this mixture was similar to that of the USFAs. This is interesting as it suggests that a dietary mixture of fatty acids that is highly saturated, as present in many dairy and in this case camel products, do not induce proinflammatory cytokine secretion in human macrophage like cells. This data is supported by a major paper by (L'homme *et al*., 2013) and is explored further in later chapters.

3.13.1. Conclusion and limitations

This study demonstrated a highly significant anti-inflammatory cytokine action of both the TGs component and the fatty acids extracted from camel milk. The study was limited by the data being entirely *in vitro* and used in the main dTHP-1 cells. It is necessary for these studies to be taken further with *in vivo* work. The work presented in this chapter acts as a basis for such studies. This study was not able to specify if an individual fatty acid present in the camel milk were responsible for these actions or if some minor components played a significant role in these anti-inflammatory activities. However, the results would suggest that the TL, including the saturated lipids are anti-inflammatory. This chapter also demonstrates that the actions of the lipids are not specific to the gBSA pathway with the data generated using LPS. However, it is highly likely that the lipids act in such a way that the transcription factor NF-κB is regulated and/or the signalling pathways that regulate its actions. Also, the work in this chapter is specific to inflammatory cytokines and the actions of anti-inflammatory cytokine is

also necessary. Also the role of these lipids in regulating other inflmmatory mediators in particular prostaglandins and other products of the cyclooxygenase enzymes should be investigated in future. It is becoming very apparent that interactions between adipocytes and immune cells are key to the integrated control of adipose tissue inflammation and lipid metabolism in obesity, but little is known about the non-inflammatory functions of adipose tissues and the interaction of macrophages with adipose cells. This is a limitation of this study and requires further work.

CHAPTER 4

The ability of camel milk lipids to regulate polarization of dTHP-1 cells

4.1. Introduction

This chapter will focus on extracted camel milk lipids and their potential to regulate dTHP-1 phenotypical changes, in particular the ability of monocytic/macrophage cells to show plasticity and their ability to polarize towards the anti-inflammatory M2 phenotype at the expense of the M1 phenotype (Chinetti-Gbaguidi and Staels, 2011). Macrophages are known to be a heterogeneous population of cells, with a major role in the innate immune response, particularly in inflammation and infection (Sridharan *et al*., 2015). Inflammation induces, blood monocytes to migrate into inflamed tissues where they differentiate into tissue macrophages (Gordon, 2007). Importantly, macrophages are highly plastic cells, which allows them to change their phenotype in response to a wide range of physiological and inflammatory stimuli (Genin *et al*., 2015). The two major polarization states associated with macrophages have been referred to as the classically activated type 1 (M1) and the alternatively activated type 2 (M2) (Mantovani *et al*., 2002). However, these two macrophage phenotypes are extremes of a continuum of functional states. Macrophages are classically activated through interaction with components such as LPS and the cytokines IFN-γ or TNF-α (Mantovani *et al*., 2002). M1 macrophages demonstrate increased synthesis and secretion of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-18, and also enhanced levels of reactive oxygen and nitrogen species (Wu *et al*., 2017). M2 macrophage polarization can be induced by the T helper 2 cells (Th2) cytokines IL-4, IL-13, IL-10, Toll-like receptor 4 (TLR4), and IL-1Ra (Genin *et al*., 2015). Alternatively activated macrophages produce reduced levels of proinflammatory cytokines, but they secrete anti-inflammatory cytokines in particular IL-10 (Wang *et al*., 2014). They are also characterized by the expression of several receptors such as the mannose receptor CD206 (or MRC1), CD163, Dectin-1, IL-1Ra (Cassol *et al*., 2010).

In chapter 3, it was demonstrated that, camel milk lipids were capable of significantly reducing secretion of proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-18 in gBSA treated dTHP-1 cells. A reduction in the secretion of inflammatory cytokines in the presence of inflammatory stimuli such as gBSA, is a key feature of the M2 macrophage phenotype (Hassan *et al*., 2015). This chapter will investigate if the lipids' ability to regulate macrophage inflammation is also associated with M2 phenotypic changes in dTHP-1 cells. dTHP-1 cells are readily polarizable (Jin *et al*., 2015) to M1 and various M2 phenotypes (Martinez *et al*., 2008). The M1 phenotype of macrophages can cause tissue damage when their activity is sustained as can occur in T2D, by production of proinflammatory cytokines and other inflammatory mediators and are involved in the development of inflammatory derived complications of the disease including the atherosclerosis and cardiovascular disease (Mantovani *et al*., 2013). In contrast the M2 phenotype produce a range of anti-inflammatory cytokines such as IL-10, Dectin-1, and IL-1Ra, which react against inflammatory responses caused by proinflammatory cytokines (Stoger *et al*., 2012).

To evaluate the amount of change at gene level of expression, real-time quantitative polymerase chain reaction (qPCR) was applied for its sensitivity and its ability in quantify the differences in levels of gene expression (Reim *et al*., 2013). THP-1 cells have previously been shown to be polarizable to the M1 (using IFN-Ɣ) and M2 phenotype using IL-4 and IL-13, and in a recent paper a natural product Vicenin-2 was shown to regulate dTHP-1 polarization through its ability to upregulate IL-10 production (Hassan *et al*., 2018). This data also suggests that the action of IL-10 was suggestive of dTHP-1 cells being shifted towards an M2b phenotype. Hence, given that the lipids extracted from camel milk had shown an ability to reduce gBSA mediated inflammatory responses, it was hypothesised that these anti-inflammatory effects

would be associated with a phenotypic change in dTHP-1 cells and this hypothesis would be investigated by the following aims.

4.2. Aims

1. To determine RNA purity and concentration with NanoDrop® analysis and to convert RNA to complementary DNA (cDNA) using high capacity cDNA reverse transcription kit (Applied Biosystems, UK) and validate the PCR amplification efficiency of target gene of interest and reference genes.
2. To investigate the ability of camel milk lipids to polarize dTHP-1 cells through their expression of markers of polarization for M1 type (CD86) and M2 type (CD163, and CD206) and the level of expression of the anti-inflammatory cytokine IL-10, Dectin-1 and IL-1Ra that are markers of M2 cells.

4.3. Results

4.3.1. NanoDrop® for RNA purity and concentration

The purity and consistency of RNA is regarded as an important issue in real time RT-qPCR experimental RNA analysis. Pure RNA has an A_{260}/A_{280} ratio of 1.8-2.2, (Figure 4.1), (Riedmaier *et al*., 2010). TRIzol™ Reagent sustains the integrity of the RNA due to highly active inhibition of RNase activity whilste disturbing the cells and dissolving cell constituents during sample homogenization. RNA was separated from Trizol® by adding chloroform.

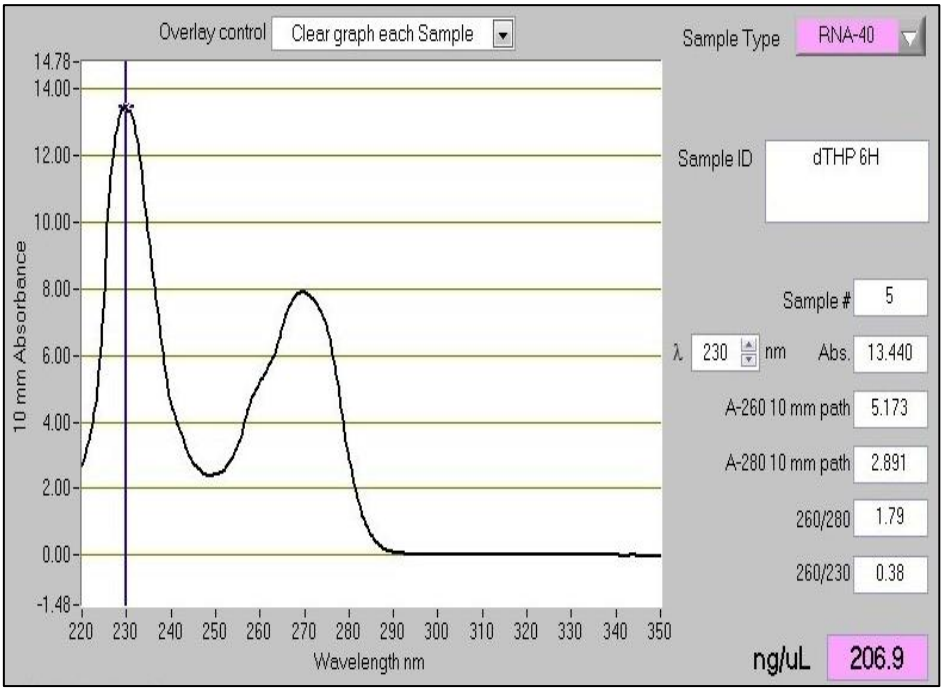


Figure 4.1. Typical spectral NanoDrop pattern for RNA measurement of purity with NanoDrop® ND-1000 Spectrophotometer. The ratio of absorbance at A_{260} nm / A_{280} nm is used to assess the purity of RNA, this figure showing a ratio of >1.8 measured in dTHP-1 cells (control) and is generally accepted as "pure" for RNA.

4.3.2. PCR amplification of relative efficiency of target gene of interest and reference gene (housekeeping genes) validation

To determine effectiveness of reference gene efficiency, and efficiency of gene of interest, cDNA concentration was optimised by a doubling dilution of cDNA concentrations of 250ng/µl to 1ng/µl and quantified to the housekeeping gene transcripts glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as in Thellin *et al*., (1999). Real-time PCR reaction mix was prepared as in (Table 2.6) in method section 2.17. Housekeeping genes that have been chosen as a reference gene, must be constant and stable in there expression secure. Another feature to be chosen as a housekeeping gene is that their genes synthesis are essential for cell survival and takes place in all types of nucleated cells and are not affected by the experimental conditions. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH = GAPDH) mainly functions as an enzyme in the glycolytic pathway and is frequently used as housekeeping gene. The 'minimum information for the publication of quantitative real-time PCR experiments' (MIQE) guidelines was applied in conducting qPCR experiments in this study (Taylor *et al*., 2010). The gene expression in all the samples were normalized to reference housekeeping gene GAPDH, chosen for its level of constant expression among diverse tissues of an organism, and not being affected by experimental conditions (Reim *et al*., 2013). The different dilutions of the cDNA of housekeeping genes are displayed in (Figure 4.2) (A) for GAPDH and (B) for β-glucuronidase (GUSB). The gene of interest samples relative efficiency plot of Log input (Total RNA in ng) versus the ΔCt of target genes (CD86) and gene (NLRP3) with reference gene (GAPDH) presented in (Figure 4.3).

To confirm the suitability of reference gene; the efficiencies of reference genes and target gene must be approximately equal, therefore, a relative efficiency plot was produced for each target gene and the absolute value of the slope of linear curve should be <0.1. The examples presented defines a value of the slope less than 0.1.

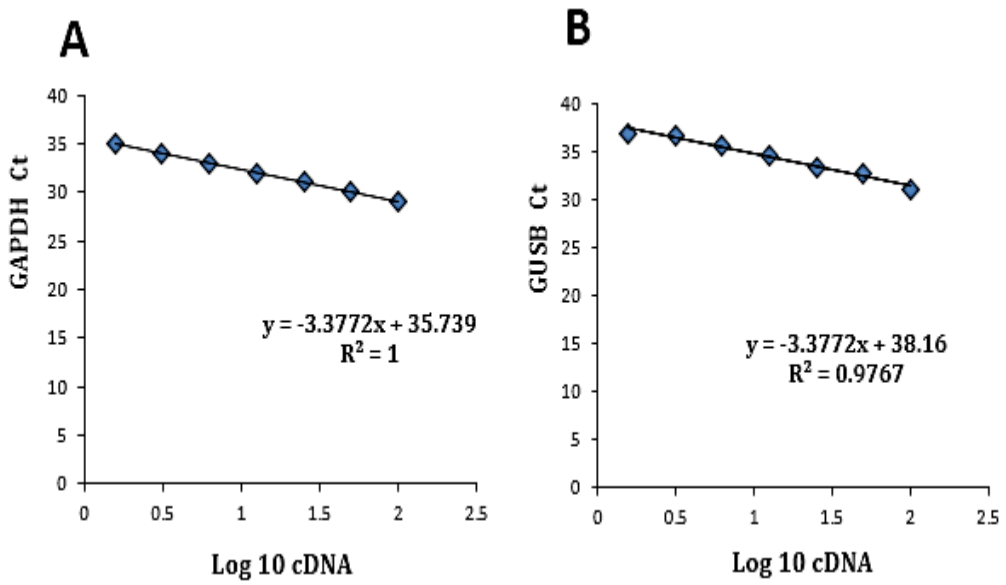


Figure 4.2. Validation of efficiency of the qPCR for the housekeeping genes. The different dilutions of the cDNA are displayed in the graph for housekeeping genes **(A)** GAPDH and **(B)** GUSB. Ct was then plotted against its corresponding log10 RNA value. GAPDH was chosen to be the housekeeping gene in all the experimental studies for its reliable significant results.

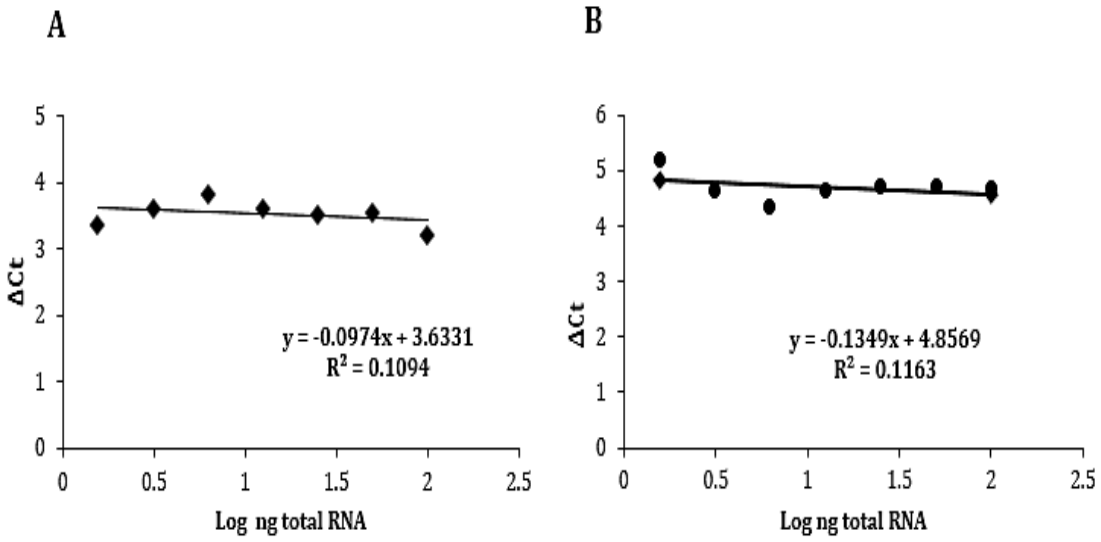


Figure 4.3. Sample relative efficiency. **(A)** A plot of the log input total RNA (ng) versus the ΔCt of target gene (CD86) and reference gene (GAPDH). **(B)** A plot of Log input amount total RNA (ng) versus the ΔCt of target gene (NLRP3) and reference gene (GAPDH). Efficiencies of target and reference are approximately equal, and the absolute value of the slope of log input amount vs ΔCt should be < 0.1. The examples in Figure 4.3 (A, B), demonstrates a value of the slope less than 0.1.

The efficiency graphs presented Figures 4.2 and 4.3 confirms that the PCR reactions for the genes represented are undertaken under optimised conditions for their analysis.

4.3.3. Expression of M1 and M2 marker of polarization CD86 and CD163 in dTHP-1 cells treated with camel milk total lipids

A reduction in the ability of macrophages to secrete inflammatory cytokines can be associated with polarization of macrophages towards the M2 phenotype. dTHP-1 cells treated with camel milk TL, as studied in chapter 3 demonstrates a significant ability of dTHP-1 cells to suppress gBSA induced inflammatory cytokine secretion when treated with camel milk TL.

24hrs was chosen to determine surface expression of CD86 and CD163 as it is known that surface expression of the protein requires processing of the receptors and its transfer to the surface of the cell.

Initially gBSA induces expression of CD86 M1 surface marker as would be expected but camel milk TL was able to significantly regulate the expression of CD86 in dTHP-1 cells treated with gBSA. The surface marker's expression was significantly reduced following treatment with camel milk TL ($p<0.05$) in dTHP-1 cells incubated for 24hrs only and stimulated with gBSA combined with TL, (Figure 4.4).

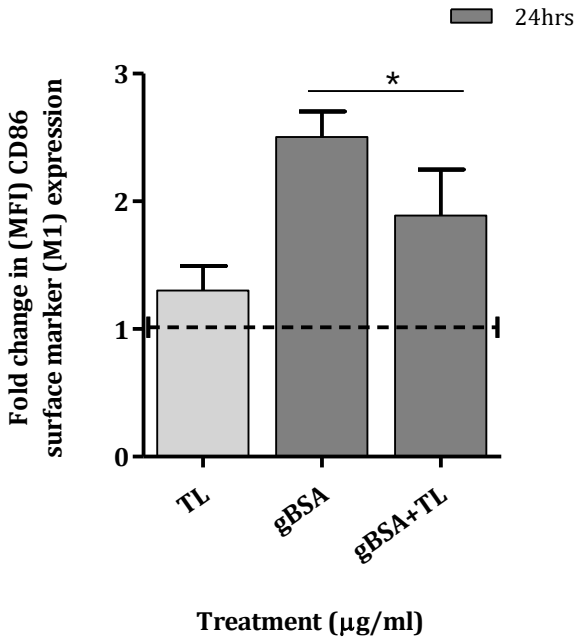


Figure 4.4. Camel milk TL down-regulate the surface expression of the M1 marker CD86. dTHP-1 cells were pre-treated with TL (20µg/mL) for 1hr and then stimulated with gBSA (500µg/mL), for 24hrs. CD86 expression was significantly down-regulated when cells were treated with camel milk TL. CD86 expression was determined using flow cytometry (MFI: Mean, or Median, Fluorescence Intensity). Data presented as mean ± SD. CD86 reported relative to VC (0.1%DMSO). Difference between treatments was determined by Two-way ANOVA with Bonferroni post-tests. [*$p<0.05$; n=3]. Dotted lines represent the VC.

However, results shown in (Figure 4.5) also demonstrates that, there was a significant fold change expression ($p<0.001$) of the M2 surface marker CD163 in dTHP-1 cells treated with camel milk TL or gBSA+TL after incubation the cells for 24hrs and 72hrs. dTHP-1 cells treated with gBSA+TL20 at 72hrs : 2.883±0.586 µg/mL fold vs gBSA: 1.523±0.095 µg/mL fold and compared with camel milk TL20 alone: 2.173±0.087 µg/mL fold. Treatment of the cells with camel milk 20µg/mL combined with gBSA, significantly increased M2 surface marker CD163 expression within longer incubation time at 72hrs ($p<0.001$).

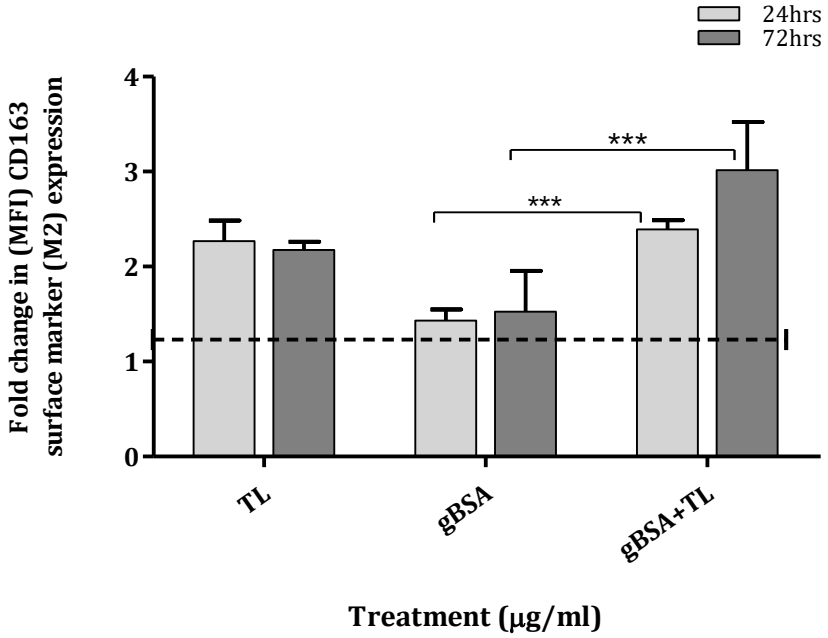


Figure 4.5. Camel milk TL enhances expression of M2 surface marker CD163. dTHP-1 cells were pre-treated with camel milk TL (20µg/mL) for 1hr and then stimulated with gBSA (500µg/mL), for 24hrs and 72hrs. The level of surface expression CD163 was determined using flow cytometry. CD163 reported relative to VC (0.1% DMSO). Data presented as mean ± SD. Difference between treatments was determined by Two-way ANOVA with Bonferroni post-test. [*** $p<0.001$; n=3]. Dotted lines represent the VC.

4.3.4. Expressions of M2 markers IL-1Ra and Dectin-1 in dTHP-1 cells treated with camel milk total lipids

The Anti-inflammatory phenotype M2 macrophages are regarded as beneficial in reducing inflammation and express greater levels of the cytokine receptors IL-1Ra and Dectin-1 (Stoger *et al.*, 2012).

dTHP-1 cells treated with camel milk TL (20µg/mL), alone or combined with gBSA resulted in a significant ($p<0.001$) increase in gene expressions of M2 phenotype macrophages markers IL-1Ra and Dectin-1. When the dTHP-1 cells stimulated with inflammatory gBSA alone, resulted in a significant ($p<0.001$) increase in gene expressions of M2 markers IL-1Ra and Dectin-1 (Figure 4.6).

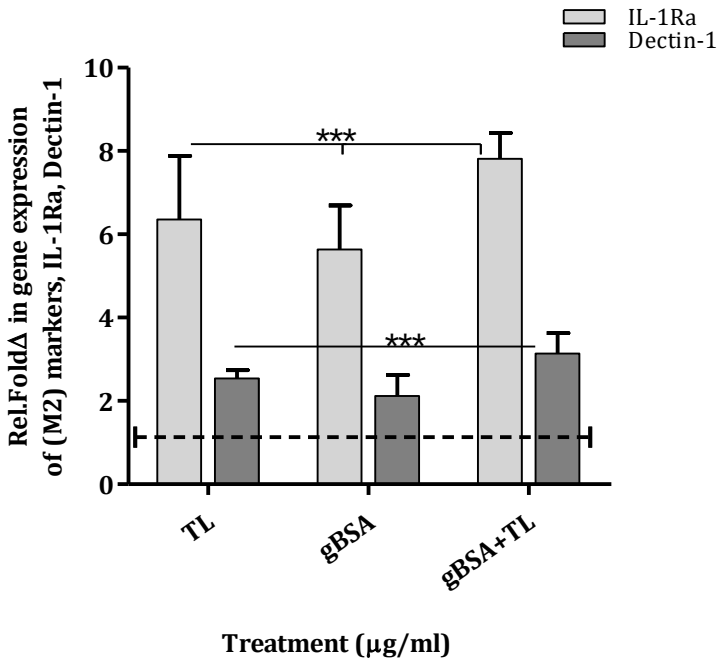


Figure 4.6. Camel milk TL enhances M2 marker IL-1Ra and Dectin-1 gene expression. dTHP-1 cells were pre-treated with (20µg/mL) TL for 1hr and then stimulated with (500µg/mL) gBSA for 24hrs. A significant ($p<0.001$) change in expressions of the mRNA of IL-1Ra was observed in cells treated with camel milk TL alone or when combined with gBSA. Gene expression was determined using RT-PCR. All results are reported relative to VC (0.1%DMSO). The gene expression was normalised against GAPDH. Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. Data presented as mean ± SD. [*** $p<0.001$; n=3]. Dotted lines represent the VC.

4.3.5. Camel milk total lipids enhance the expression of the anti-inflammatory cytokine IL-10

The immunoregulatory cytokine IL-10 regulates the secretion of several inflammatory cytokines including TNF-α and IL-10 (Kessler *et al*., 2017). Their expression is enhanced and is a feature of an M2 phenotype. There was a significant increase in the gene-expression of mRNA IL-10 ($p<0.01$) at only 6hrs, when dTHP-1 cells treated with, camel milk TL alone: 26.98±12.22 μg/mL fold, or when lipids combined with gBSA+TL: 9.612±4.203 μg/mL fold, expression was significant ($p<0.05$) suggestive of camel milk TL enhance the polarization of M2 macrophages phenotype (Figure 4.7).

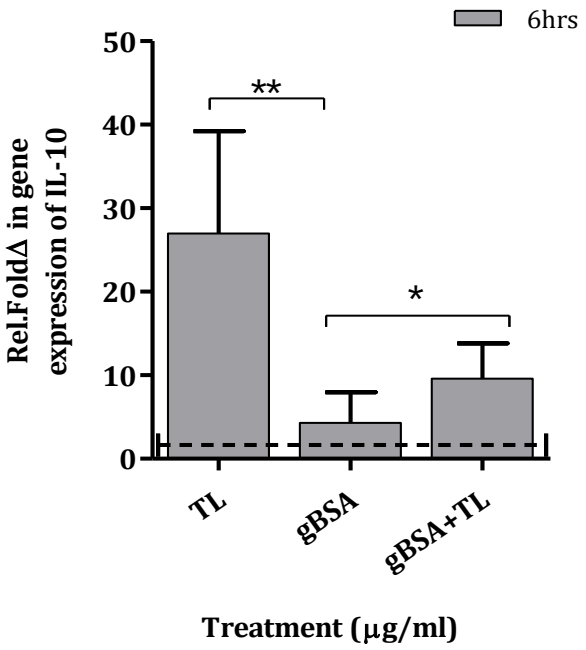


Figure 4.7. Camel milk TL enhances the expression of gBSA-induced IL-10 gene expression. dTHP-1 cells were pre-treated with (20µg/mL) camel milk TL for 1hr and then stimulated with (500µg/mL) gBSA for 6hrs. IL-10 mRNA was determined using RT-PCR. A significant increase in the gene-expression of mRNA IL-10 was ($p<0.01$) observed when cells were treated with TL. Results are reported relative to VC (0.1% DMSO). The gene expression was normalised against GAPDH. Difference between treatments was analysed by Two-way ANOVA with Bonferroni post-tests. Data presented as mean ± SD. [* denotes $p<0.05$, ** $p<0.01$, n=3]. Dotted lines represent the VC.

4.3.6. Gene expression of M2 markers following treatment with camel milk total lipids

dTHP-1 cells were pre-treated with camel milk TL (20μg/mL) and then stimulated with gBSA, the expression of mRNA M2 marker associated genes was analysed using RT-PCR. In addition cells incubated for 6hrs and treated with TL alone or stimulated with IL-10, and IL-4 which are both M2 inducers, and a combination of IL-10 or IL-4 with TL. There was a significant induction of gene expression of markers of M2 macrophages CD163, CD206, and CD86 (M2b) sub type marker (Martinez and Gordon, 2014; Martinez *et al*., 2008) (Figures 4.8., 4.9, 4.10). The significant induction of CD86 mRNA (M2b subset marker) together with an increased in IL-10 (Figure 4.7), is suggestive that camel milk lipids can induce a potentially beneficial M2b sub-type of dTHP-1 cells. Macrophages M2b subtype mRNA CD86 was significantly expressed when dTHP-1 cells treated with camel milk TL combined with M2 inducers IL-10 and IL-4. When cells stimulated with IL-10+TL: 1.597±0.908 μg/mL fold vs IL-10: 1.203±0.597 μg/mL fold (Figure 4.8.A). In the same manner, when cells stimulated with IL-4 an M2 type macrophages inducer, mRNA CD86 was significantly expressed in presence of IL-4 alone: 1.718±1.517 μg/mL fold vs IL-4+TL : 2.961±1.122 μg/mL fold (Figure 4.8.B).

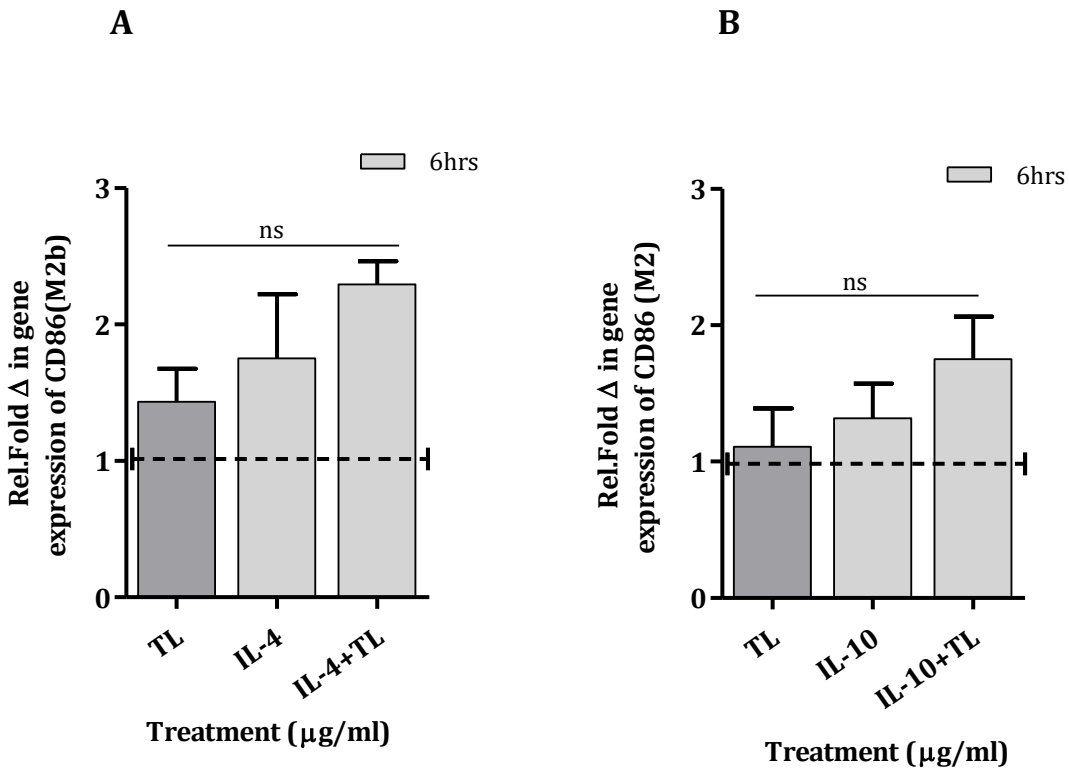


Figure 4.8. Camel milk TL, IL-4, and IL-10 effect on CD86 (M2b) gene expression. dTHP-1 cells pre-treated with (20μg/mL) camel milk TL for 1hr then stimulated with the cytokines, **(A)** IL-4 and **(B)** IL-10 for 6hrs. The significant expression of CD86 mRNA (M2b subset marker) together with an increased in IL-10 is suggestive that camel milk lipids can induce a potentially beneficial M2b sub-type of dTHP-1 cells. mRNA was determined using RT-PCR. Data presented as mean ± SD and all results are reported relative to VC (0.1%DMSO). The gene expression was normalised against GAPDH. Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [ns: Not significant; n=3]. Dotted lines represent the VC.

The gene expression of the M2 marker of polarization CD163 was detected with RT-PCR. dTHP-1 cells stimulated with M2 inducers IL-4 and IL-10 and incubated for 6hrs. In presence of IL-4 alone the CD163 expression was reduced when cells treated with camel milk TL alone or when IL-4 combined with camel milk TL, the gene expression was increased (Figure 4.9.A). On the other hand, when dTHP-1 cells stimulated with IL-10 M2 inducer alone or combined with camel milk TL there was a significant expression of mRNA CD163. Stimulation with IL-10 increase the expression of CD163 marker of M2 macrophages, IL-10 alone: 2.481±0.331 µg/mL fold and when combined with TL the expression was high IL-10+TL: 2.904±0.386 µg/mL fold (Figure 4.9.B).

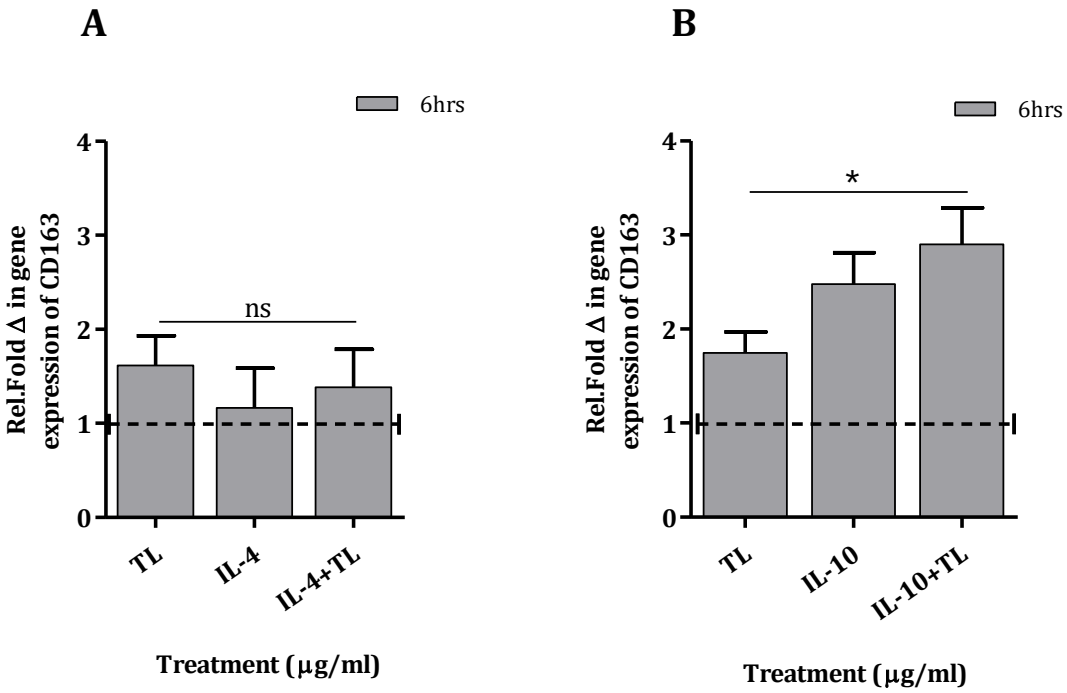


Figure 4.9. Camel milk TL, IL-4, and IL-10 regulates CD163 gene expression. dTHP-1 cells pre-treated with (20µg/mL) camel milk TL for 1hr then stimulated for 6hrs with M2 inducers, **(A)** IL-4 or **(B)** IL-10. There was a significant expression of CD163 mRNA M2 marker. mRNA was determined using RT-PCR. Data presented as mean ± SD. All results are reported relative to VC (0.1%DMSO). The gene expression was normalised against GAPDH. Difference between treatments was determined by Two-way ANOVA with Bonferroni post-tests. [* denotes $p<0.05$, ns: Not significant; n=3]. Dotted lines represent the VC.

dTHP-1 cells were stimulated with M2 inducers IL-4 and IL-10 and incubated for 6hrs with substantial relative changes in mRNA of CD206 was detected with RT-PCR analysis. In stimulating the cells with IL-4 alone: 6.75±3.318 µg/mL fold vs IL-4+TL: 6.179±3.232 µg/mL fold, there was a significant expression ($p<0.05$), in treatment with camel milk TL (Figure 4.10.A). However, when cells stimulated with M2 polarization inducer IL-10 combined with TL, IL-10+TL, there was a significant increase in level of mRNA CD206 expression ($p<0.05$) M2 marker (Figure 4.10.B).

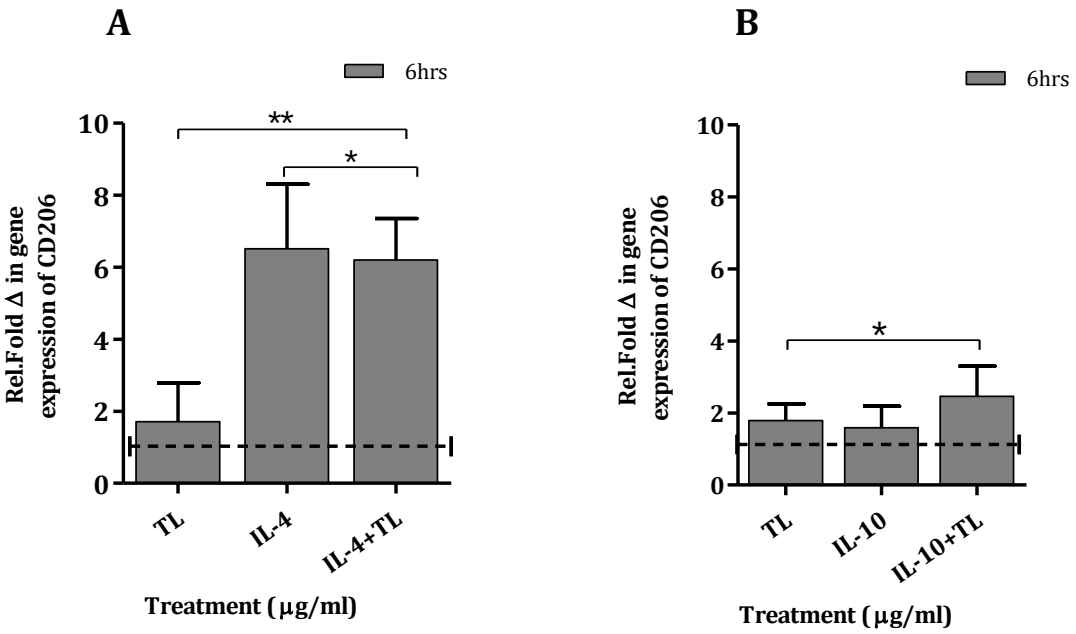


Figure 4.10. Camel milk TL, IL- 4, and IL-10 regulate CD206 gene expression. dTHP-1 cells pre-treated with (20µg/mL) TL for 1hr then stimulated for 6hrs with, **(A)** IL-4 and **(B)** IL-10. A significant increase in expression of CD206 mRNA M2 marker in treatment with camel milk TL. mRNA was determined using RT-PCR. Data presented as mean ± SD. All results are reported relative to VC (0.1%DMSO). The gene expression was normalised against GAPDH. Difference between treatments was determined by Two-way ANOVA with Bonferroni post-tests. [* denotes $p<0.05$, ** $p<0.01$; n=3]. Dotted lines represent the VC.

4.4. Discussion

The data presented here demonstrate a highly significant effect of camel milk TL in regulating a range of markers of M1 and M2 polarization of dTHP-1 cells. The results would strongly suggest that the various forms of lipids extracted from camel milk are in the presence of an AGE (gBSA) capable of increasing a range of markers associated with the M2 macrophage phenotype. The results of this chapter are in agreement with many of those presented in chapter 3, in supporting an anti-inflammatory action of camel milk TL in dTHP-1 cells. This action of camel milk TL was confirmed at both the protein level and at the gene or mRNA level. Interestingly, the M2 phenotype is regarded as a continuum, rather than a single specific entity and M2 cells have been further sub-categorized into M2a, M2b, M2c and M2d based upon gene expression profiles (Arora *et al*., 2017).

A number of cytokines are involved in the regulation of polarization in monocytes and macrophages and IL-4 in particular is known to induce M2 polarization (Mantovani *et al*., 2004). Interestingly, gBSA as an inflammatory mediator would be expected to demonstrate a clear ability to induce M1 polarization. The data presented here in many ways is indicative of the complexity of the actions of proinflammatory agents and anti-inflammatory agents in macrophage physiology. To resolve and regulate inflammatory events, proinflammatory agents such as gBSA and LPS (Figure 3.9), will induce their own inflammatory events. This is clearly seen in the actions of LPS and gBSA in inducing both proinflammatory TNF-α, and IL-1β but later inducing IL-10. IL-10's primary role is in regulating the secretion of these inflammatory cytokines through suppressing NF-κB proinflammatory activity. (Figures 4.6 and 4.7) make this point clearly since gBSA itself induced the expression of a range anti-inflammatory agents,

but throughout it can also be observed that TL significantly enhances the production of anti-inflammatory agents and the expression of M2 phenotypic markers.

The action of camel milk TL on M2 on the expression of CD163 clearly supports an ability to enhance the expression of the M2 phenotype. However, the story in relation to CD86 is less clear as it has been described as an M1 marker (Dalmas *et al*., 2011). In contrast, it has been reported that CD86 a marker of the M2b sub-phenotype (Martinez and Gordon.2014). In this study the lipids were able to significantly enhance the actions of IL-10, when cells were treated by the cytokines IL-4 and IL-10, then it is probable that the actions of camel milk TL is suggestive of its ability to induce an M2b like phenotype in camel milk TL treated dTHP-1 cells (Figure 4.8). In addition, this effect is mediated by the action of these lipids alone as well as in coinjunction with exogenous IL-10 (Figure 4.7).

It is interesting to note that the high concentrations of lipids used throughout this study, did suggest a possibility of lipotoxicity having a role in the results. Although there was no evidence that these lipids at the concentrations used had any effects on cell viability, it is useful to observe that, the induction of the M2 phenotype has been shown to protect cells from lipotoxicity and this may be the case for the cells treated by these camel milk lipids (Dai *et al*., 2017). In contrast, M2 cells can demonstrate higher sensitivity to oxLDL induced lipotoxicity through their effects on the unfolded protein response (Isa *et al*., 2011). This aspect of lipotoxicity is necessary further work in the study of these camel milk derived lipids.

The peroxisome proliferator receptors (PPARs) are a group of lipid sensors and nuclear transcription factors that have a potent regulatory role in inflammation. PPARγ has been shown to improve insulin sensitivity and also control M2 activation in macrophages both *in vitro* and in human studies (Stienstra *et al*., 2008; Odegaard *et al*.,

2007). It is possible that the lipids used in this investigation of camel milk lipids, may well be activating or regulating the PPARs to induce their anti-inflammatory and macrophage polarization activity. To study these transcription factors would require the use of reporter gene assays that are very challenging in dTHP-1 cells. Alternatively, PPAR specific inhibitors of the receptors could be investigated (Yang *et al*., 2016).

4.4.1. Conclusion and limitations

The results of this chapter contains a number of limitations limited by use of mainly THP-1 cells for investigation of camel milk lipids. Only camel milk TL used in this investigation due to insufficient availability of extracts of USFAs and TFAs. However, camel milk TL were able to confirm the action of camel milk TL in the polarization of dTHP-1 cells by up-regulating the gene of M1 and M2 markers. The camel milk TL components identified by GC-MS did not specify whether the effects of TL on dTHP-1 polarization mediated by an individual lipid compound or as a combined action of all the components of the compounds present in the whole extracts. This an area of future investigation. A number of the markers of polarization investigated in this study are proteins expressed on the surface of the cells. These include CD86, CD163 and CD206. Of the ones studies, only CD86 and CD163 surface expression was reported here, the others were only reported at the mRNA/gene expression level. Future studies, would require that changes in these markers are all confirmed by their expression on the surface of these cells.

CHAPTER 5

The regulation of the NF-κB pathway and NLRP3 inflammasome by camel milk lipids

5.1. Introduction

The anti-inflammatory effects of camel milk lipids was discussed in chapter 3 and chapter 4 of this thesis and highlights the ability to enhance the polarization of dTHP-1 cells to macrophage phenotypes, in particular an M2b phenotype. A transcription factor that is a critical regulator of the inflammatory response in many cell types is NF-κB and its activity reduceds in M2 cells (Sica *et al*., 2008). NF-κB plays a multifaceted role in the inflammation that includes not only the induction of inflammatory cytokines but the induction of immunoregulators (Cao *et al*., 2006). NF-κB is a family of proteins and activation occurs due to the dimerization and translocation from the cytosol of various members of the NF-κB/Rel family of transcription factors. Heterodimerization of p65 activates proinflammatory responses, but IL-10 synthesis is associated with a single NF-κB family member p50.

This chapter will investigate the ability of camel milk derived lipids to regulate the signalling of proteins phosphorylation involved in transcription nuclear factor KappaB (NF-κB p50/p65) signalling pathway (Figure 5.1). NLRP3 inflammasome complex formation and regulation of its activation, with pro-caspase-1 activation will be investigated. In addition given the role of TET-2 in NLRP3 inflammasome regulation, also will be investigated if these lipids modulated TET-2 mRNA gene expression along with the modulating and regulating the inflammatory cytokines investigated in chapter 3.

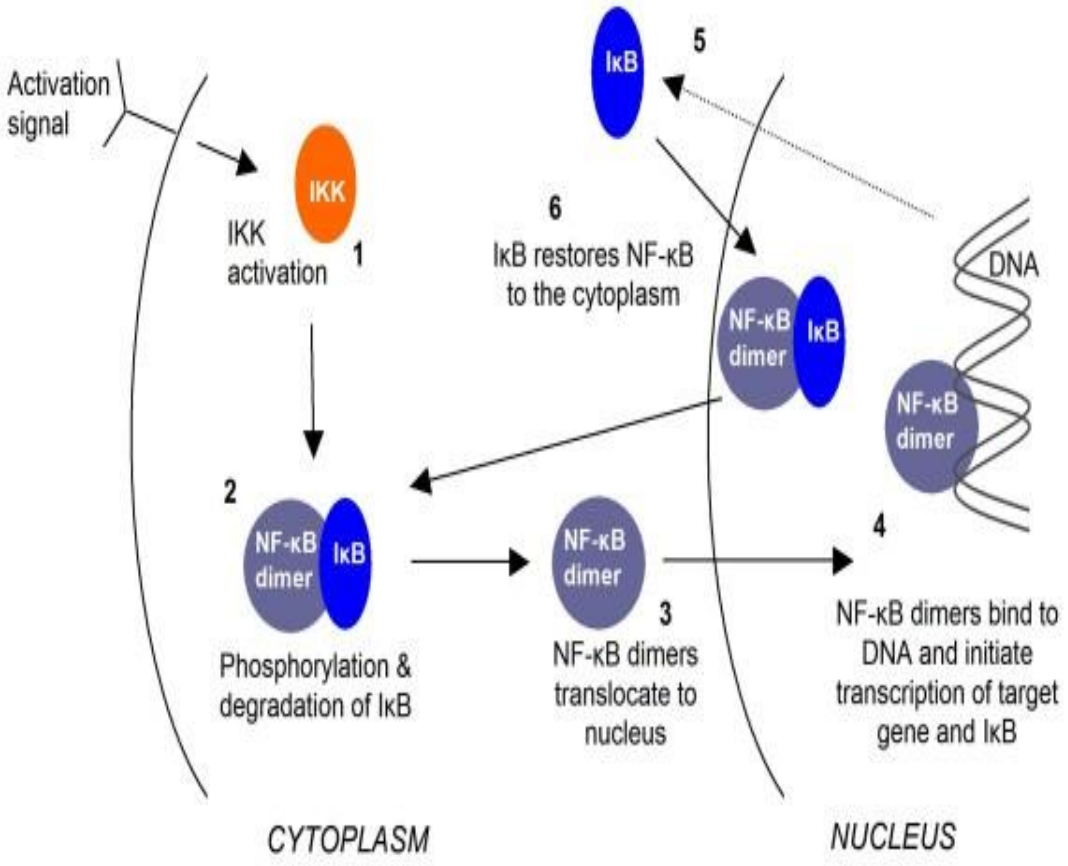


Figure 5.1. The NF-κB inflammatory pathway. Inflammatory signals such as the binding of AGE to RAGE induces IKK activation (**1**) that leads to phosphorylation of p50 or p65 monomers in the cytoplasm (**2**) that leads to dimerization of p50 and p65 (**3**) and results in translocation of these dimers and binding to gene specific portions of DNA (**4**) that itself results in further activation through IκB. (**5** and **6**). In contrast to p50/p65 hetrodimers leading to activation of inflammatory signals, p50/p50 homodimerization results in activation of anti-inflammatory signalling through IL-10. Taken from (Patel *et al*., 2011).

There has been increasing interest in the role of the inflammasomes and in their immunoregulatory properties. An inflammasome is a high molecular weight complex and consists of the NLRP3, the ASC (PYCARD) adaptor, and caspase-1. Caspase-1 activates cytokines of the IL-1 family IL-1β, and IL-18 (Schroder and Tschopp. 2010). IL-1β secretion by the NLRP3 inflammasome is triggered by high extracellular glucose in β-cells. Among the NLRs, several members including NLRP1, NLRP2 and NLRP3, are able to form a multimeric inflammasome complex. These complexes contain a nucleotide-binding oligomerization domain-like receptor (NLR) Of the NLR inflammasome complexes, the NLRP3 inflammasome has been the most widely characterized and is a crucial signalling node that controls the maturation of two proinflammatory interleukin IL-1 family cytokines: IL-1β and IL-18 (Jo *et al*., 2016). These two cytokines were significantly regulated by the lipids extracted by camel milk used in this study.

The inflammasomes are activated by a wide range of signals, including pathogen-derived such as LPS and serve to alert the cell to stress or insult (Camell *et al*., 2015) induced by DAMPs. In relation to this study, components of nutrition can induce inflammasome activity including metabolites (glucose and fatty acids) or byproducts of metabolites (cholesterol, ceramide, uric acid). DAMPs are commonly elevated during chronic nutrient excess as seen during obesity, which can lead to chronic inflammation.

5.2. Aims

1. To investigate the ability of camel milk derived lipids to regulate the phosphorylation of proteins associated with the NF-κB signalling pathway (MYD88, IKKβ, and IKKα) in dTHP-1 cells.
2. To investigate the ability of camel milk derived lipids to regulate the dimerization and translocation of NF-κB p50/p65 in nuclear and cytoplasm extracts in dTHP-1 cells.
3. To investigate the ability of camel milk derived lipids in reducing the activation of inflammasome protein NLRP3 in dTHP-1 cells and to investigate formation of inflammasome complex assembly compartment contains proteins responsible in converting the pro-IL-1β and pro-IL-18 to their active forms.
4. To investigate the ability of camel milk derived lipids in activation of TET-2.

5.3. Results

5.3.1. Proteomic profiling of NF-κB/p50/p65 pathway phosphorylated proteins

Human NF-κB Pathway Array Kit (Proteome Profiler™), was used as stated in section 2.18.2. (For more details see Appendices A.3),this kit used to detect the expression of proteins phosphorylated during the signalling pathway that leads to NF-κB/p50/p65 translocation in dTHP-1 cells treated with camel milk TL (Figures 5.2 A,B). Given that NF-κB is a major regulator of inflammatory cytokine synthesis , the significant effect of camel milk derived lipids on dTHP-1 suggested that regulation of the NF-κB signalling pathway was likely, in addition proteins involved in formation and activation of the NLRP3 inflammasome were likely to have been regulated by the lipids used in this study.

Extracellular membrane receptors such as cytokine receptors, and other intermembrane receptors involved in NF-κB/p50/p65 protein phosphorylation are presented in (Figure 5.2.A). In this analysis an increase in expression of TNF receptor associated factor (TRAF2) which interacts with the cIAP1 also known as (BIRC2-Baculoviral IAP Repeat-Containing protein 2) and cIAP2/BIRC3 (BIRC3-Baculoviral IAP Repeat-Containing protein 3), and functions as a mediator of the anti-apoptotic signals from TNF receptors (TNF-R). On the other hand, there was a decrease in cytosol membrane receptor MYD88 protein that provide instruction for making proteins involved in signalling within immune cells. Cytosol membrane receptor MYD88 transfer signals from Toll-like receptors (TLRs) surface membrane and M1 macrophages proinflammatory cytokine interleukin-1 (IL-1β) receptors, which are important in early immune response to foreign invaders such as bacteria (Hirayama *et al*., 2017). There was a decrease in interleukin IL-18 receptor (IL-18R) that binds IL-18 an inflammatory cytokine of M1 type macrophages. However, camel milk TL combined with gBSA was able to decrease other receptors on dTHP-1 cells such as

TRAIL R1/R2 known as (Death receptors) for TRAIL which can signal death and induces apoptosis. A reduction in tumour necrosis factor receptors superfamily (TNFRSF, 1A, 1B, 16) members that binds (TNF-α) inflammatory cytokine of M1 type macrophages was also detected. Results are duplicates only and no statistical analysis performed. Data represent (n=2; ± the range).

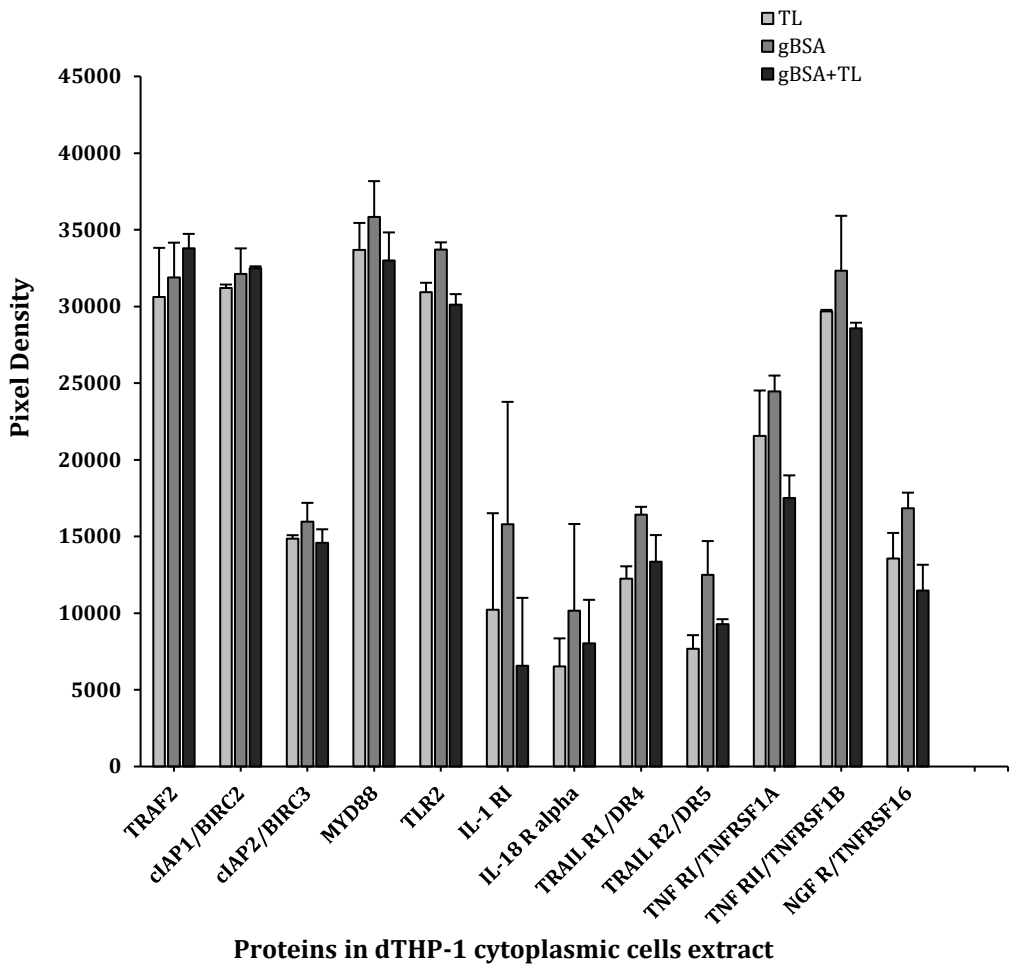


Figure 5.2. (A) Human NF-κB Pathway Array Kit (Proteome Profiler™) of proteins involved in phosphorylation in dTHP-1 cells. dTHP-1 cells pre-treated with camel milk TL (20µg/mL) for 1hr and then stimulated with gBSA (500µg/mL) for 2hrs. Data represents expression of NF-κB/p50/p65 phosphorylation of receptors and cytokines involved in NF-κB pathway in cytoplasmic extract of dTHP-1 cells such as (MYD88, TLR2, IL-1 β, TNF-α, IL-18) were reduced. TRAIL R1/R2 signal proteins for death and apoptosis also reduced. Anti-apoptotic protein mediators (TRAF2, BIRC2, and BIRC3) were increased. Results are duplicates only and no statistical analysis performed. Data represent (n=2; ± the range).

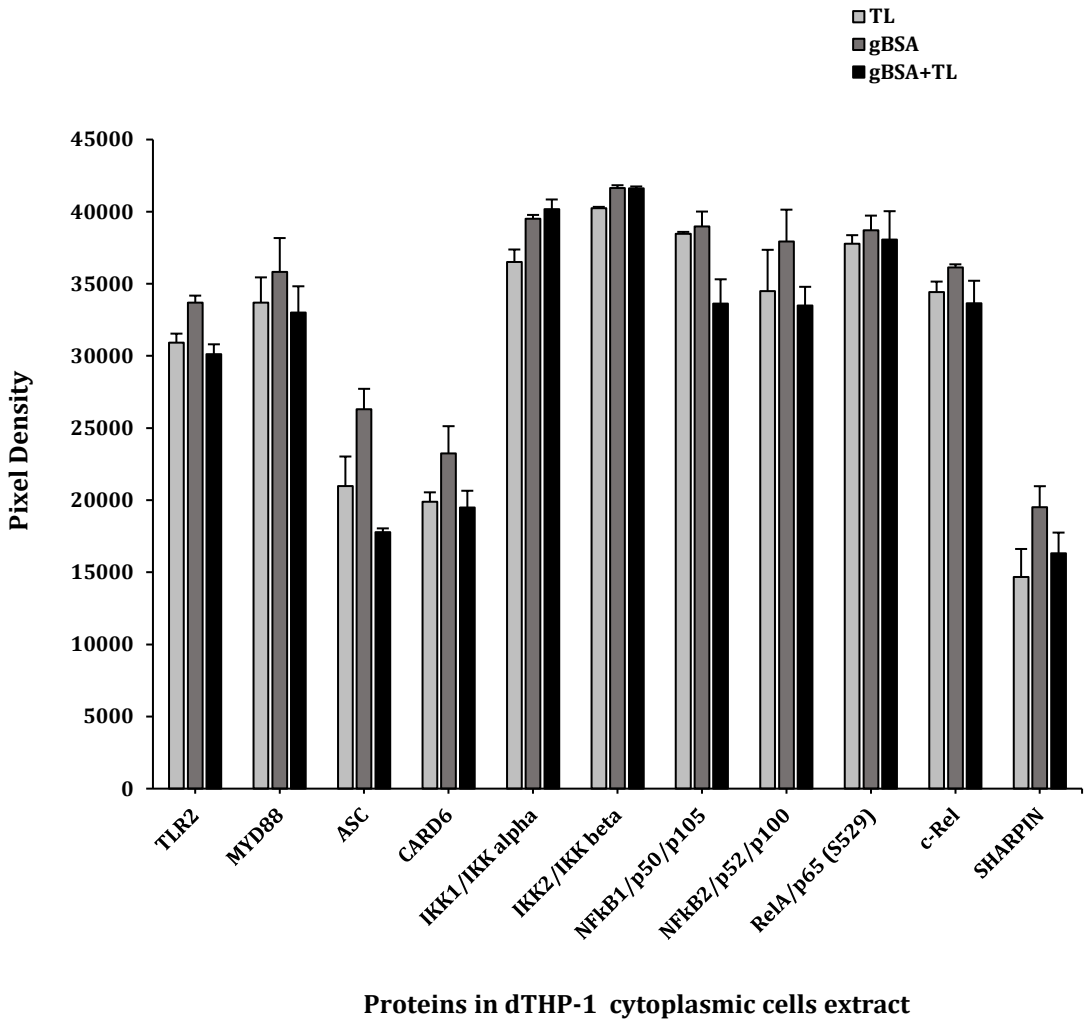


Figure 5.2. (B) Protein array of the effect of camel milk TL on protein phosphorylation in NF-κB/p50/p65 pathway in dTHP-1 cells. Cells pre-treated with TL (20µg/mL) for 1hr and then stimulated with gBSA (500µg/mL) for 2hrs. Data represents protein expression in cytoplasmic extract of dTHP-1 cells treated with TL alone or with gBSA alone or with gBSA + TL, for 2hrs. Human NF-κB Pathway Array Kit was used to detect the proteins phosphorylation. Results are duplicates only and no statistical analysis performed. Data represent (n=2; ± the range).

5.3.2. Treatment of dTHP-1 cells with camel milk total lipids induces increased NF-κB p50 homo-dimerization

The induction of inflammatory cytokine secretion is associated with activation of the transcription nuclear factor NF-κB through heterodimerisation of the p50 and p65 subunits (Collins and Cybulsky. 2001). In contrast, the secretion of the anti-inflammatory cytokine IL-10 and M2 polarization as presented in chapter 4 is associated with enhanced p50 homodimerisation (Porta *et al*., 2009). The action of camel milk TL on NF-κB activity was determined in both in the nucleus and cytoplasm using an activity-based immunoassay (Figure 5.3. A, B).

In the presence of gBSA there was a significant increase in NF-κB /p65 activity in dTHP-1 nucleus which was significantly reduced ($p<0.01$) when dTHP-1 cells where treated with gBSA combined with camel milk TL and incubated for 1hr or 2hrs (Figure 5.3. A) conversely, a significant increase ($p<0.01$) in the p50 subunit was observed in dTHP-1 cells nucleus extract, after incubation for 1hr and treatment cells with camel milk TL: 0.949±0.042 vs gBSA+TL: 1.076±0.088 (Figure 5.3.B).

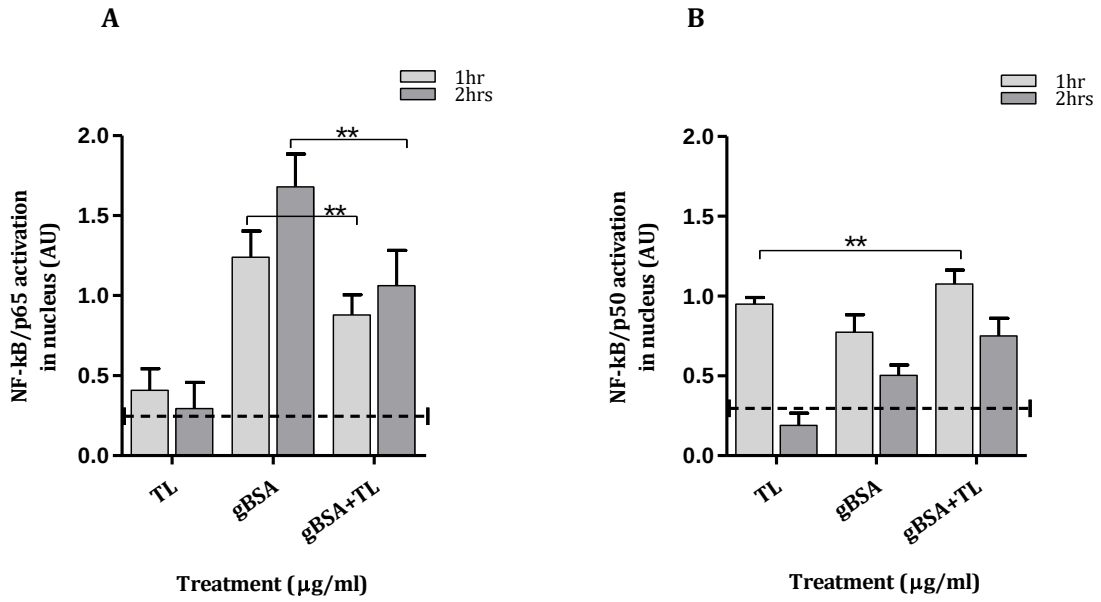


Figure 5.3. (A) NF-κB /p65 and (B) NF-κB /p50 activation in nuclear extract of dTHP-1 cells treated with camel milk TL. dTHP-1 cells were pre-treated with (20µg/mL) camel milk TL for 1hr, then stimulated with (500µg/mL) gBSA for 1hr or 2hrs. The nuclear extracts were collected at each time point. Activation of NF-κB/p65 and NF-κB/p50 was assayed with TransAM® NF-κB family Transcription factor Kit. All results are reported relative to VC (0.1%DMSO). Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [** $p<0.01$]. Data represents mean ± SD (n=4). Dotted lines represent the VC.

5.3.3. Treatment of dTHP-1 cells with camel milk total lipids induces increased NF-κB p50/p50 homo-dimerization and decrease in p65/p50 hetero-dimerization

In the presence of gBSA at 1hr there was a significant increase ($p<0.01$) in NF-κB p65 activity in dTHP-1 cells cytoplasm gBSA: 0.6083±0.0783 which was significantly reduced ($p<0.01$) when dTHP-1 cells treated with camel milk TL: 0.2857±0.0384. (Figure 5.4. A). Expression in p65 subunit at 2hrs in cells cytoplasm was increased significantly ($p<0.05$) when treating the cells with gBSA only: 0.6709±0.0642, comparing that with treating the cells with camel milk TL: 0.4109±0.0405, there was a reduction in p65 subunit expression.

Meanwhile, the NF-κB p50 subunit activity detected in cytoplasm dTHP-1 cells was significantly reduced ($p<0.05$) within 2hrs after treating the dTHP-1 cells with TL: 0.2454± 0.0188 vs gBSA: 0.4178± 0.0405. Detection of NF-κB p50 subunit activity was not significant ($p>0.05$) at 1hr with the different treatments TL,gBSA,gBSA+TL in the cytoplasm of dTHP-1 cells in (Figure 5.4.B), comparing that with its significant ($p<0.01$) detection p50/p50 homo-dimerization in the nuclear extract of dTHP-1 cells treated with camel milk TL at 1hr in (Figure 5.3.B)

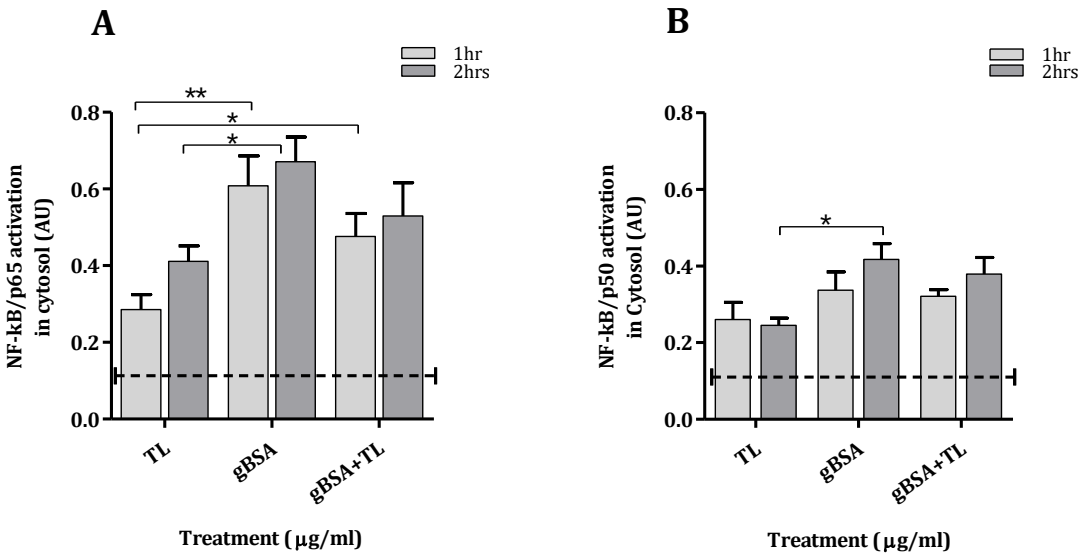


Figure 5.4. Detection of (A) NF-κB/p65 and (B) NF-κB/p50 activation in cytoplasmic extract of dTHP-1 cells treated with camel milk TL. dTHP-1 cells were pre-treated with (20µg/mL) TL for 1hr then stimulated with (500µg/mL) gBSA for 1hr and 2hrs. The cells cytoplasm extracts were collected at each time point. Activation of NF-κB/p65 and NF-κB/p50 was assayed with TransAM® NF-κB family transcription factor Kit. All results are reported relative to VC (0.1% DMSO). Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [* denotes $p<0.05$, ** $p<0.01$]. Data represents mean ± SD (n=4). Dotted lines represent the VC.

5.3.4. Effect of camel milk lipids on NLRP3 inflammasome mRNA expressions in dTHP-1 cells

As previously demonstrated in chapter 3 that the camel milk derived lipids TL, USFAs, and TFAs were able to significantly down-regulate IL-1β and IL-18, two cytokines whose secretion is dependent on caspase -1 activation through the NLRP3 inflammasome complex. Furthermore, the proteomic analysis revealed that protein (SHARPIN) known to have a role in the NLRP3 activity were down-regulated by these lipids (Figure 5.2, B). Hence, it was decided to determine if these lipids could regulate NLRP3 gene expression in dTHP-1 cells challenged with gBSA and the results are presented in (Figure 5.5. A, B).

The formation of the NLRP3 inflammasome assembly (NLRP3 + pro-caspase-1 + soluble protein ASC (Apoptosis-associated Speck-like protein containing a CARD), results in the release of mature active caspase-1, which in turn activates pro-IL-1β and pro-IL-18 to be released as mature active cytokines (Lee *et al*., 2013). dTHP-1 cells stimulated with gBSA which increase the mRNA gene NLRP3 expression, however, this expression was regulated in treatment with camel milk derived lipids extracts at 6 and 24hrs (Figure 5.5. A, B). dTHP-1 cells incubated for 6 and 24hrs, and treated with camel milk TL combined with gBSA show a significant reduction ($p<0.01$) of mRNA gene expression of NLRP3. However, dTHP-1 cells treated with camel milk derived extracts of USFAs or TFAs combined with gBSA, the mRNA gene expression of NLRP3 was down-regulated significantly ($p<0.001$) at 6 and 24hrs.

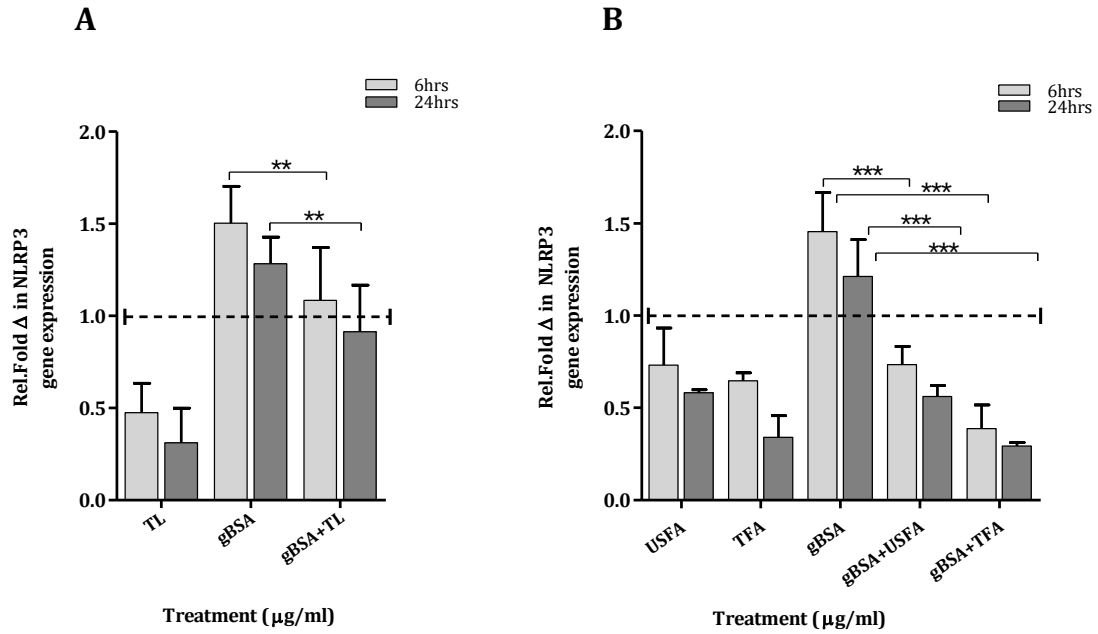


Figure 5.5. Effect of camel milk lipids on NLRP3 mRNA expression. dTHP-1 cells were pre-treated with (20µg/mL) camel milk derived lipids **(A)** TL, (**B)** USFA or TFA for 1hr, then stimulated with (500µg/mL) gBSA for 6 or 24hrs. mRNA gene expression of NLRP3 was determined by RT-PCR**.** All results are reported relative to VC (0.1% DMSO). GAPDH used as a reference gene. Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [** $p<0.01$; ***$p<0.001$]. Data represents mean ± SD (n=4). Dotted lines represent the VC.

5.3.5. The effect of camel milk lipids on caspase-1 activation in dTHP-1 cells

Formation of multiprotein NLRP3 inflammasome assembly induces activation of caspase-1 (Mao *et al*., 2013). In this chapter, activation of NF-κB/p50/p65 subunits signalling pathway (Figure 5.3) and (Figure 5.4), first with a priming signal of gBSA stimulation the dTHP-1 cells surface receptors such as Toll-like receptor (TLRs) or on cytokines receptors detected in (Figure 5.2.A). The second activation signal required a danger-associated molecular pattern (DAMPs) signal that is involved in activating the mRNA gene expression of inflammasome NLRP3, (Figure 5.5. A,B) and its compartments caspase-1, and ASC (Figure 5.2.B), this leads to the caspase-1 cleavage of pro-IL-1β and activating it to its mature IL-1β form which in turn will be secreted to the extracellular stream of macrophages cells. This on the other hand will activate the IL-1β receptors on the cells surface membrane (Esser *et al*., 2014). Activation of IL-1β is associated with T2D inflammatory complication.

For the analysis, dTHP-1 cells were treated with camel milk derived lipids (20µg/mL) alone or combined with gBSA (500µg/mL) and incubated for 6hrs. A significant reduction ($p<0.001$) in caspase-1 released level was measured at 3 reading times (1hr, 2hrs and 4hrs) within cells treated with camel milk derived lipids TL, USFAs, and TFAs. For instance, at 1hr and when cells treated with camel milk TL: 544.333±47.2475 vs gBSA: 742.333±2.08166, the reduction in caspase-1 level was significant ($p<0.001$) and at treatment with gBSA+TL: 545.333±30.5505, there was a significant ($p<0.001$) reduction. dTHP-1 cells treated with USFA, show a significant reduction ($p<0.001$) in the level of caspase-1 released measured within 1hr in culture cells medium after adding the detection reagent Caspase-Glo®1 Inflammasome Assay, (Figure 5.6. A, B).

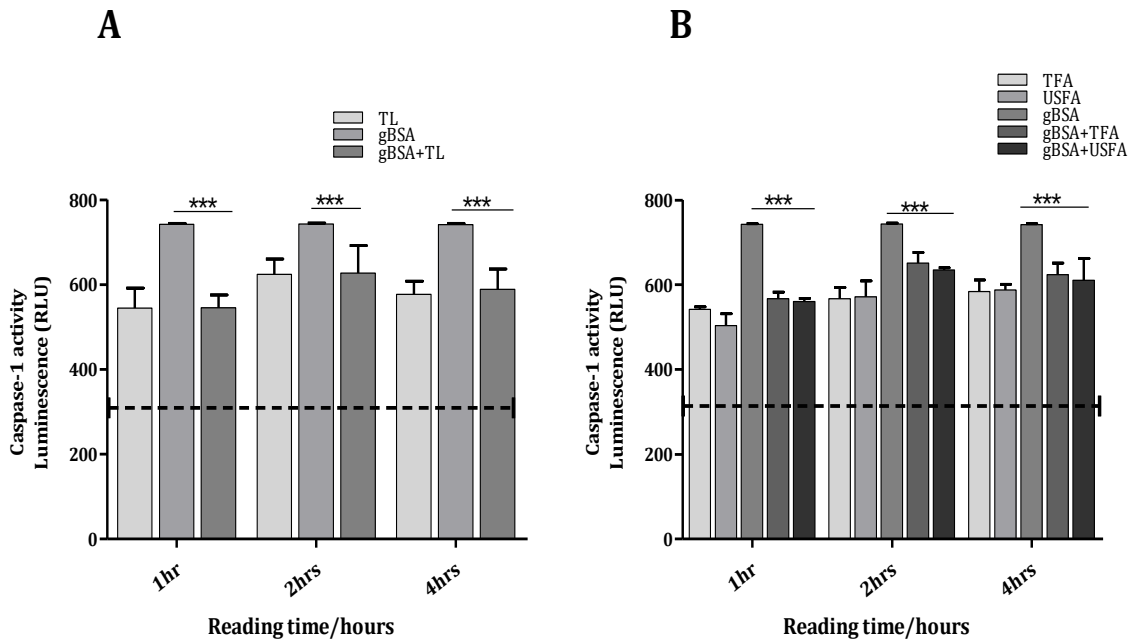


Figure 5.6. Camel milk derived lipids significantly down-regulate caspase-1 activity in dTHP-1 cells. dTHP-1 cells were pre-treated with (20µg/mL) camel milk derived lipids, **(A)** TL, **(B)** TFAs or USFAs, for 1hr, then stimulated with (500µg/mL) gBSA for 6hrs. All results are reported relative to VC (0.1% DMSO). Caspase-Glo®1 Inflammasome Assay kit was used to detect the effect of camel milk derived lipids on released caspase-1 activity level in dTHP-1 cells at 1hr, 2hrs and 4hrs. Differences between treatments were determined by Two-way ANOVA with Bonferroni post-tests. [*** $p<0.001$]. Data represents mean ± SD (n=4). Dotted lines represent VC.

5.3.6. The effect of camel milk lipids on TET-2 mRNA expression in dTHP-1 cells

TET (ten-eleven translocation)-2 is an epigenetic modifier that has recently gained considerable attention in the pathophysiology of atherosclerosis and cardiovascular disease (Wende, 2016). TET-2 also plays an important role in the inflammatory response of macrophages, and loss of this regulator results in the up-regulation of many inflammatory mediators following LPS stimulation (Hassan *et al*., 2018). Deficiency of TET-2 in macrophages increase proinflammatory cytokines (Cull *et al*., 2018; Cull *et al*., 2017), in their study used a primary mouse cell line and demonstrated that mouse Tet-2 mRNA expression reached its highest level at 3hrs, and at 24hrs was the basal levels of expression. It is known that, a mutation in TET-2 starts to appear in the elderly over 65 years. High rates of mortality, diabetes and atherosclerosis considered as risk factors at this stage of aging (Abegunde *et al*., 2017). In a recent paper and using dTHP-1 cells, a dietary flavonoid, was able to regulate TET-2 expression, an action that was associated with a reduction in inflammatory cytokine secretion and reduction in NLRP3 expression (Hassan *et al*., 2018). As camel milk lipids had shown similar characteristics, it was decided to also determine if these anti-inflammatory actions were also associated with TET-2 expression.

Detection the effect of camel milk lipids on mRNA expression of human TET-2 in dTHP-1 cells treated with (20µg/mL) camel milk TL, USFAs or TFAs for 1hr, alone or combined with gBSA (500µg/mL) for 6 or 24hr, revealed that TET-2 gene expression was significant. Data analysis confirmed, at 6hrs incubation with TL 3.243±0.8061 vs gBSA+TL 2.3031±0.2460, TET-2 gene expression was significant ($p<0.05$). When compared with treatment gBSA alone: 1.8063±0.5775 vs gBSA+TL: 2.302928±0.2460, at 6hrs TET-2 gene expression was not significant ($p>0.05$). At 24hrs incubation, dTHP-1 cells treated with gBSA alone: 1.5012±0.4622 vs gBSA+TL: 2.0111±0.5401, TET-2 gene expression was significant ($p<0.01$). dTHP-1 cells treated with camel milk USFA

alone at 6hrs: 1.4581±0.5487 vs gBSA: 0.2843± 0.2771 alone, TET-2 gene expression was significant ($p<0.01$). At 24hrs USFA: 4.5878±0.8511 vs gBSA alone: 0.6221±0.1656, TET-2 gene expression was significant ($p<0.001$). TFA at 24hrs: 2.2322±0.5699 vs gBSA at 24hrs: 0.6221±0.1656, TET-2 mRNA gene expression was significant ($p<0.001$). At 24hrs gBSA alone: 0.6221±0.1656 vs gBSA+TFA: 3.0162±0.2283, TET-2 mRNA gene expression was significant ($p<0.001$) (Figure 5.7. A, B).

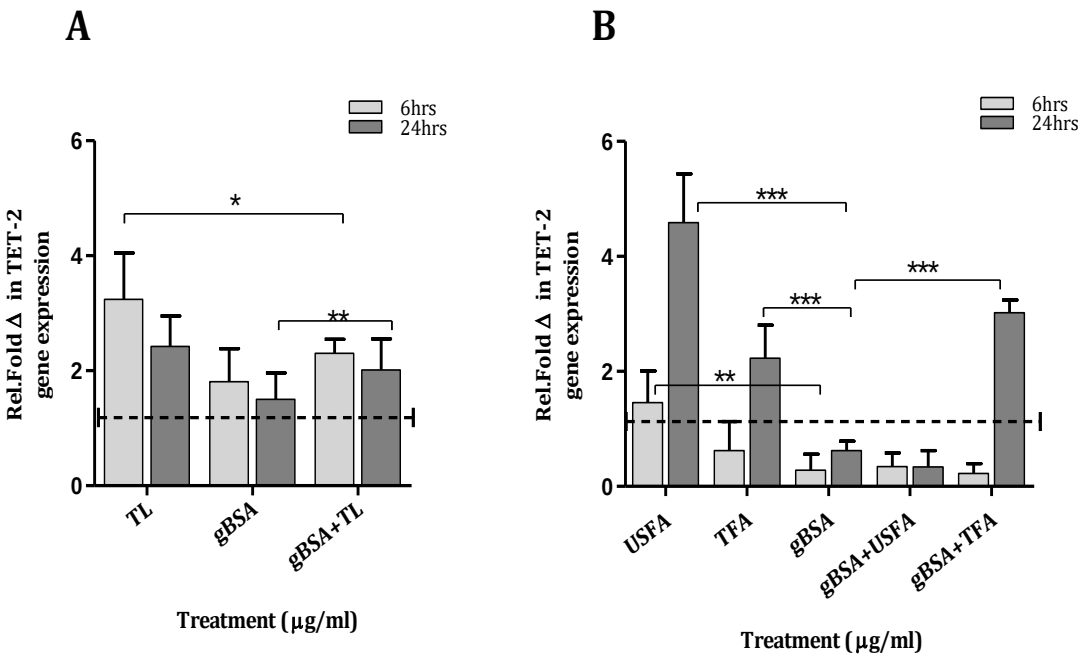


Figure 5.7. Camel milk lipids significantly increase TET-2 mRNA expression in dTHP-1. dTHP-1 cells were pre-treated with (20µg/mL) camel milk lipids **(A)** TL,**(B)** USFAs or TFAs, for 1hr, then stimulated with (500µg/mL) gBSA for 6 or 24hrs. mRNA expression of TET-2 was determined by RT-PCR. All results are reported relative to VC (0.1% DMSO). GAPDH was used as a reference gene. Differences between treatments were determined by Two-way ANOVA with Bonferroni post-test. [* denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$]. Data represents mean ± SD (n=4). Dotted lines represent the VC.

5.4. Discussion

The evidence as to the health benefits of dietary lipids remains unclear. Dietary saturated lipids have been shown to enhance the expression of proinflammatory cytokines and polyunsaturated fatty acids can modulate the downstream effects of inflammatory receptors such as pattern recognition receptors (PRRs); Toll-like receptors (TLRs), and (NODs-NLRP3) nucleotide-binding oligomerization domain proteins (Sui *et al*., 2016). This study provides new evidence of the action of dietary fatty acids (in this case camel milk derived lipids) in modulating NF-κB activation and the NLRP3 are novel result that links these lipids to a newly recognised regulator of the immune response TET-2 (Lee *et al*., 2010).

The proteomic profiles although limited by the availability number of assays and the number of repeat measures that can be undertaken. Nevertheless, the data presented in this proteomic analysis confirmed a range of effects on proteins phosphorylation of the NF-κB pathway and on a number of cytokine receptors. This thesis presents data showing that camel milk TL significantly reduces NF-κB p50/p65 heterodimerization translocation from the cytoplasm to the nucleus (Figure 5.3 and Figure 5.4), a known proinflammatory action but enhanced p50/p50 homodimerization both in the presence of, but also in the absence of TL. This would suggest that pre-treating dTHP-1 cells with camel milk lipids primes the cells towards an anti-inflammatory or M2 phenotype. Homodimerization of p50 subunit known to induce IL-10 synthesis (Driessler *et al*., 2004) a cytokine that is itself a regulator of inflammatory cytokine secretion and an M2 phenotypic marker. We would therefore propose that the anti-inflammatory actions of camel milk TL are in-part mediated by its ability to enhance p50 homodimerization and consequent IL-10 expression.

Throughout this study the camel milk lipids used were a complex mixture of saturated and unsaturated lipids apart from the USFA, that constituted over 90% unsaturated lipids. Based on this make-up of lipids, of interest to this study is data reported in a paper of L'homme *et al* (2013). In their study they demonstrated the anti-inflammatory mechanism of USFAs by preventing the activation of the NLRP3 inflammasome and the secretion IL-1β and importantly, using dTHP-1 cells. They showed that the activation of the NLRP3 that was a feature of saturated lipid palmitic acid and was a potential proinflammatory action of these lipids, was negated in the presence of USFAs. It can be proposed from the data presented in this study, that naturally derived lipids from camel milk that has a substantial saturated component down-regulates NLRP3 inflammasome activity and IL-1β and IL-18 secretion in a similar way the proinflammatory actions of the saturated lipid component of camel milk is entirely blunted by the unsaturated component. This is an area for further study and also specifying the individual or more than one unsaturated component that are responsible for NLRP3 blunting. It also introduces the intriguing possibility that other milk sources that are a complex mixture of saturated and unsaturated lipids may well have similar actions on the inflammasome and this is also an area of further study.

This study revealed for the first time that anti-inflammatory properties of these lipids significantly regulated the expression of TET-2 gene expression. It is known that, mutations in TET-2 are linked with a high rate of in mortality, diabetes, atherosclerosis and aging (Abegunde *et al*., 2017). Fuster *et al*., (2017) demonstrated that TET-2 deficient macrophages increased IL-1β secretion in an NLRP3-dependent manner. Thus, TET-2 regulates the inflammasome in macrophages. Therefore, this study has novel features demonstrating that camel milk TL was capable of increasing TET-2 expression and that this is linked with its anti-inflammatory/inflammasome-regulatory actions. Zhang *et al*., (2015) demonstrated that LPS induces activation of

TET-2 and that this event is critical to the resolution of the inflammatory response in macrophages. Jaiswal *et al*., (2017) and Fuster *et al*., (2017) linked the actions of TET-2 to clonal haematological malignancies and a mutation of TET-2. However, this study confirmed a role for TET-2 in regulating the inflammasome, in dTHP-1 cells that to date have not demonstrated TET-2 mutations.

5.4.1. Conclusion and limitations

The three lipids components significantly regulated gBSA induced NLRP3 gene expression was reduced significantly in the presence of derived camel milk lipids alone or when combined with gBSA as compared with cells stimulated only with gBSA for 6 and 24hrs. The caspase -1 level of expression was reduced when dTHP-1 cells incubated for 6 and 24hrs, and treated with derived camel milk lipids alone, or when combined with gBSA, a significant reduction was observed at measuring the level of caspase-1 at 1hr and 4hrs reading intervals.

Further investigations and studies are required to determine the precise actions of camel milk TL on the NF-κB pathway. These could involve specific inhibition of the proteins that are part of the transcription factor's signalling pathway. Furthermore, the phosphorylation of IKBα is an important step in the activation of p50/p65 heterodimerization and this should be confirmed in camel milk TL treated cells and this is an area for further work. A further limitation and an area for further study, would be to confirm the activity of NF-κB through the use gene reporter activity assays. This work would require transfection of dTHP-1 cells with luciferase reporter plasmid DNA. It should be noted, that dTHP-1 cells have a low transfection efficiency by most methods and hence, this work may require investigating alternative methods to those that were available in this study.

CHAPTER 6

General discussion and conclusions

6.1. General discussion

The overall aim of this study was to investigate the ability of camel milk derived lipids to modulate the immunoregulatory responses of human macrophage like cells. THP-1 cells selected as a model of inflammatory processes in this investigation *in vitro*, these cells play a critical role in pathogenesis of several chronic inflammatory diseases such as T2D and cardiovascular disease.

This study confirmed that the major lipid component of camel milk was in the triglyceride (TG) form, with a less than 5% of other lipids identified apart from the TGs. The major FAs components of these TGs as determined by GC-MS profiling demonstrated that approximately 61% were saturated lipids as compared to the unsaturated 39%. Palmitic acid and myristic acid being the major saturated, and oleic, linoleic, and palmitoleic acid the major unsaturated fatty acid species. Urea crystallization method was also used to enrich the extracted free fatty acids to form an unsaturated enriched fatty acid mixture mainly oleic acid (Table 3.3). An attempt was undertaken to produce a mixture of saturated free total fatty acids, however, a substantial residue of unsaturated free fatty acids remained in the mixture (Table 3.4). This extraction was undertaken to allow a greater understanding of the action of the free fatty acid components as opposed to TGs components. These various components of camel milk were classified as TL, free TFAs and free USFAs.

Ranges of highly significant anti-inflammatory effects were observed in relation to cytokines produced by the action of gBSA by all the lipids in relation to inflammatory and immunoregulatory markers. Interestingly, data was also generated that supported the view that the lipids could enhance markers of M2 polarization of macrophages in particular an M2b like phenotype that THP-1 cells are known to favour. An interesting phenomenon was the fact that the camel milk TGs (a group of lipids that have been

associated with proinflammatory phenomenon) also produced beneficial anti-inflammtory actions. Given that dietary TGs are broken down in the gut prior to absorption, it could be argued that using TL had little *in vivo* usefulness. However, it is known that after absorption of the triglyceride component, these are reattached to the fatty acids to reform TL like component for storage. Thus it is likely that macrophages within for instance, adipose tissue could be in the presence of TL like components, whose make-up is similar to the camel milk TL used here. Further studies are required to determine if the fatty acids present in TL, that could be removed enzymatically, retain their anti-inflammatory actions in the absence of the triglyceride component. However, the later studies undertaken with the camel milk free USFA and free TFA that demonstrated similar actions of these free fatty acids to the TL are highly suggestive of this being the case.

This study was unable to specify the action of the TL, USFA and TFA to any individual lipid component or even to the major lipid components. Interestingly, and given the work undertaken by Lhomme *et al*., (2013), who as in this study was able to demonstrate that a saturated lipid, with a reputation for proinflammation, palmitic acid was not capable of inducing a proinflammatory response, even though it was present in substantial proportion in all of the lipids used. Many of the studies used when investigating dietary lipids in diseases such as atherosclerosis and diabetes have focussed on individual lipid and fatty acids. As a diet invariably contains complex mixtures of lipids, with milk and dairy products being particular examples, the work presented in this thesis would suggest that dietary lipids may have a reduced proinflammatory effect. Further studies including *in vivo* studies are required to confirm this including if similar effects are seen in other milk products from a range of animal sources.

All camel milk lipids were effective in attenuating NLRP3 inflammasome gene expression and caspase-1 activity necessary for the production of mature IL-1β and IL-18. In addition, a significant role to dampen or attenuating the potent biological consequences of inflammatory diseases was also observed, as the lipids enhanced nuclear transcription factor activation of NF-κBp50/p50 dimerization at the expense NF-κB p50/p65. This p50/p50 homodimerization is critical in IL-10 synthesis and is associated with the M2 phenotype of macrophages. This is also suggestive of a broad anti-inflammatory action of in particular TL and given the range of biological processes, in, inflammation, cell growth, survival and development of NF-κB activation, preventing NF-κB signalling has enormous influence in therapeutic applications in cancer and inflammatory diseases (Park and Hong, 2016). The specific mechanism by which the lipids action was mediated is likely to involve a broad range of signalling molecules and that is an area for further study.

The ability of camel milk lipid extracts in preventing the phosphorylation of IkB-α with the increase in nuclear p50 translocation, suggested that the effect of camel milk lipids may be mediated through p50 homodimerization. Camandola *et al*., (1996) reported that a diet containing unsaturated n-6/n-3 fatty acid, such as arachidonic acid (AA), enhances NF-κB /p50 and induces NF-κB /p65, which was proposed as a way to modulate the expression of c-jun and c-fos genes. In the same manner Lytle *et al*., (2017) reported that omega-3(ω3) Polyunsaturated fatty acid DHA is a major bioactive compound accumulate in tissues and is expected to be responsible for many of the beneficial effects of ω3 fatty acids seen *in vivo*. Camel milk lipids extracts reduced the signalling of proteins phosphorylation (MYD88, IKKβ, and IKKα) activation in dTHP-1 cells cytoplasm extract, which involved in nuclear factors KappaB NF-κB-subunits proteins p50/p65 activation (Figure 5.2.B).

As mentioned in chapter 5, the data in relation to TET-2 is novel and firmLy links the actions of the TL to the regulation of the inflammasome and the secretion of two inflammatory cytokine IL-1β and IL- 18. There is currently considerable interest in the roel of IL-1β and its regulation of cardiovascular disease. In a recent review, (Peiro *et al*., 2017) the benefits of its inhibition using very expensive specific antibodies has been highlighted in the cardiovascular complications of diabetes and has been given considerable publicity through the results of the CANTOS trial (Ridker *et al*., 2011). These monoclonal antibody therapies highlight the importance of reducing the activity of IL-1β in cardiovascular and other inflammatory diseases, but are currently very expensive and arguably prohibitively expensive. The combined NLRP3 inflmmasome and TET-2 regulating activity-itself regarded as important in the development of cardiovascular risk (Jaiswal *et al*., 2017) would suggest that the camel milk lipids could play a part in dietary and lifestyle strategies in reducing the complications of diseases such as T2D. The data from this thesis requires further validation through both *in vitro* and *in vivo* studies, but acts as a basis for such work.

This study has several limitations and includes the use of data that is mainly obtained from a cellular model dTHP-1 and limited data from primary mononuclear cells. However, this model has been highly validated in inflammatory disease and its ability to demonstrate the phenotypic characteristics of M2 cells has been well reported (Lin *et al*., 2015). Some data in relation to receptors was obtained from gene expression this was also backed up using a range of markers of M2 (CD163, CD206, IL-1ra, Dectin-1, IL-10) and M1 (CD86, TNF-α, IL-1β, IL-18, IL-6) cells (Wang *et al*., 2014). Further studies would include a range of doses of the lipids, although the dose used did not affect the viability of the cells here. Whether the actions of the lipids was mainly mediated through a single class of lipid or is combined effect was not elucidated. However, the data is suggestive of a combined lipid effect and may also support

previous studies (Robblee *et al*., 2016; Legrand-Poels *et al*., 2014; Lhomme *et al*., 2013) in that the presence of mixed saturated/unsaturated lipids is anti-inflammatory. The importance of the inflammatory response of macrophages in binding to the vascular endothelium would also require co-culture studies with endothelial cells in particular HUVEC cells. The NF-κB work is limited by not using gene-reporter studies that would specifically determine translocation of the transcription factor. The protein array data was itself limited by the work being performed in duplicate only, but was highly useful in demonstrating the role of camel milk TL in the NF-κB pathway protein phosphorylation and in its role in the inflammasome. These are areas of further study as the pathway(s) that the lipids regulate was beyond the scope of this study.

6.2. Conclusion

This study has demonstrated that the camel milk lipids extracts are significantly capable of suppressing the gBSA induced secretion of the inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-18 in dTHP-1 cells and in PBMCs cells. Also, this study verified that the various camel milk lipids investigated, significantly up-regulated the expression of M2 markers alone and also when combined with gBSA. This study also confirmed that camel milk lipids modulated the activation of NF-κB a transcription factor that plays a ubiquitous and critical role inmany cell types inflammatory response. The anti-inflammatory effect of camel milk lipids reported in this thesis are mediated through its ability to suppress NF-κB inhibitory protein (IκB-α) phosphorylation, and increasing homodimerization of p50 subunits of NF-κB, and through mediating the inflammasome NLRP3 complex formation and reduction in its compartments level of expression (Caspase-1, ASC).

This study has generated important evidence for an anti-inflammatory role of the camel milk lipids extracts TL, TFAs, and USFAs at non-toxic levels in dTHP-1 macrophages cells with a critical role in T2D and atherosclerosis. Additionally, this study has identified a novel mechanism through which these camel milk lipids can up-regulate the gBSA induces inflammatory responses with in T2D in a model of dTHP-1 cells *in vitro* through NF-κB/p50/p65 pathways and Toll-like receptors (TLRs), cytokines receptors and lipids receptors on dTHP-1 cell membrane. This study suggests a potential role for the camel milk lipids as a non-pharmacological, safe, and readily available product, in prevention and treatment of major worldwide public health problems, which increasingly affect the health of the world's population.

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Appendices

A.1. Ethical approval for the study

Cardiff Metropolitan University | Prifysgol Metropolitan Caerdydd

Friday, 20 November 2015
cshs/ethics /approved - iterim/

Al-Nasseri, Raya
PhD
Cardiff School of Health Sciences

Dear Applicant

Re: Application for Ethical Approval: The regulation of Macrophage inflammation and polarization by camel milk derived lipids

Ethics Reference Number : 7293

Your ethics application, as shown above, was considered by the Biomedical Sciences Ethics Panel on 19/11/2015

I am pleased to inform you that your application for ethical approval was **APPROVED,** subject to the conditions listed below – *please read carefully.*

Standard Conditions of Approval

- Your Ethics Application has been given a Project Reference number as above. This **MUST** be quoted on all documentation relating to the project (E.g. consent forms, information sheets), together with the full project title.
- All documents must also have the approved University Logo and the Version number in addition to the reference and project title as above.
- A **full Risk Assessment** must be undertaken for this proposal, as appropriate, and be made available to the Committee if requested.
- Any changes in connection to the proposal as approved must be referred to the Panel/Committee for consideration ***without delay quoting your Project Reference Number.*** Changes to the proposed project may have ethical implications and so must be approved.
- Any untoward incident which occurs in connection with this proposal must be reported back to the Panel/Committee ***without delay.***
- If your project involves the use of **samples of human origin,** your approval is given on the condition that you or your supervisor **notify the School** of your intention to work with such material by **completing Part One** of the form entitled *"Notification of Intention to Work with Human Relevant Material or Human Bodily Material"* which **must** be obtained from the PD (Sean Duggan), **BEFORE** any activity on this project is undertaken.

This approval expires on **19/11/2016** . Please set a reminder on your Outlook calendar or equivalent if you need to continue beyond this approval date. It is your responsibility to reapply / request extension if necessary.

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Yours sincerely

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PLEASE RETAIN THIS LETTER FOR REFERENCE

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A.2. Western blot analysis of FABP4

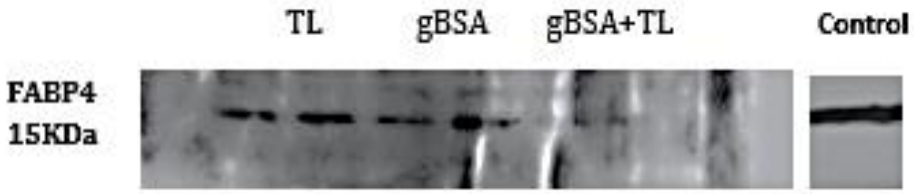

Figure A.2 Western blot analysis of FABP4 in cytoplasmic extract of dTHP-1 cell. Cells incubated for 1hr with camel milk TL (20µg/mL), and stimulated with (500µg /mL) gBSA or combined with gBSA. The bands detected by western blot indicate the expression of FABP4 in dTHP-1 cells cytoplasm extract. Detection of band 15KDa supports FABP4 expression in cells treated with TL or gBSA, and reduced expression in cells treated with gBSA+TL. This result suggests that gBSA expression FABP4 is modulated by TL in dTHP-1 cells. Control is FABP4 detected in adipocyte cells 3T3 cells lysate.FABP4 is a 15KDa protein.

A.3. Human NF-κB pathway array detects multiple proteins

Figure A.3 The human NF-κB pathway array detects multiple proteins in dTHP-1 cells cytoplasmic extract. (1) dTHP-1 macrophage cells pre-treated with camel milk TL (20µg/mL) only and incubated for 1hr, old media aspirated and fresh media was added and incubated for 6hrs. **(2)** dTHP-1 stimulated with gBSA (500µg/mL) only and incubated for 6hrs. **(3)** dTHP-1 macrophage cells pre-treated with camel milk TL (20µg/mL) and incubated for 1hr, old media aspirated and fresh media was added, then stimulated with gBSA (500µg/mL) and incubated for 6hrs. Profiles of mean spot pixel density were created using a transmission-mode scanner image analysis software (5mins exposure to X-ray film).RS=Reference Spots. Each analyte presented with duplicate spots on the nitrocellulose membranes and the numbers above the spots with the letters from A-F on the left side of the membrane (coordinates) can be used to identify each target proteins by referring to a table provided with the human NF-κB pathway array kit. (B1, B2) = (ASC-Apoptosis –associated Speck-like protein containing a Carboxy-terminal CARD), (B5, B6) = (CARD6-Caspase recruitment domain 6), (D1, D2) = (NF-kB/P50 unit), (D11,D12)=(RelA/P65 subunit of NF-kB), (C23,C24) = (MyD88- transfers signals from certain proteins Toll like receptors and interleukin-1 ,IL-1), (E7,E8) = (TLR2- Toll like receptor2), (C3,C4) = (IL-1RI- interleukin-1 receptor), (C7,C8) = (IL-18Rα- interleukin-18 receptor alpha chain), (B17,B18) = (IkBα-Inhibitor of NF-kB transcription factor).

A.4. Publication

Publication Abstract: conferenceseries.com. Clinical Nutrition 2016, December 08-10, 2016 Dubai, UAE, 8th International Conference on Clinical Nutrition.

Al-Nasseri Raya Hamdan Salim et al., J Nutr Food Sci 2016, 6:8 (Suppl)
http://dx.doi.org/10.4172/2155-9600.C1.035

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8th International Conference on

CLINICAL NUTRITION

December 08-10, 2016 Dubai, UAE

The anti-inflammatory properties of lipids extracted from Omani camel milk

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[2]Cardiff Metropolitan University, UK
[3]Sultan Qaboos University, Oman

Introduction: Very little evidence exists to date on the potential health benefits of camel milk derived lipids. Macrophage activation status reflects a beneficial or detrimental role in various diseases, in particular; switching macrophages to an anti-inflammatory M2 phenotype could be important in preventing the development of inflammatory diseases such as atherosclerosis and type-2 diabetes.

Objectives: This study aimed to determine the lipid content and characteristics of fatty acids derived from Omani camel milk and investigate their ability to regulate macrophage inflammatory responses using the human macrophage cells dTHP-1.

Method: Camel milk lipids were converted their fatty acid methyl esters and analyzed by Gas Chromatography-Mass Spectrometry. dTHP-1 cells were pre-treated with the extracted lipids, stimulated with glycated-serum albumin and inflammatory mediators associated with M1 and M2 macrophages determined by ELISA, Real-Time PCR and Flow Cytometry.

Results: Fatty acids in Omani Camel milk included saturated fatty acids (SFAME) myristic acid ME (C14:0), palmitic acid ME (C16:0), stearic acid ME (C18:0), and un-saturated (UNSFAME) palmitoleic acid ME (C16:1), 9-octadecenoic acid ME (E- C18:1 n-9), which were recovered from methylation of total camel lipids. These lipids were able to significantly reduce secretion of two inflammatory cytokines, TNF-α and IL-1β without any reduction in cell-viability. The lipids enhanced the anti-inflammatory cytokine IL-10 and up-regulated expression of the M2 marker CD163.

Conclusion: This study suggests that the lipid component of Omani camel milk significantly reduces macrophage inflammation, an action associated with the switching of macrophages to an anti-inflammatory M2 phenotype.

Biography

Al-Nasseri Raya Hamdan Salim has completed her degree in Biotechnology (1997) from University of Abertay, Dundee, United Kingdom and her MSc in Biomedical Sciences (distinction) from University of Wales Institute Cardiff (UWIC), Wales, UK (2004). Currently she is undertaking PhD at Cardiff Metropolitan University, UK and her aim is to investigate on Omani camel milk lipids and its anti-inflammatory properties, her study is funded by the Royal Court Affairs, Sultanate of Oman. In Oman, she works as a Biotechnologist in the Center of Research and Diagnostic Laboratory, Royal Court Affairs, Directorate of Veterinary Services, Muscat, Sultanate of Oman.

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A.5. Oral Presentation

Cardiff Metropolitan University postgraduate student symposium, Swalec Stadium, Cardiff, UK.

The anti-inflammatory properties of lipids extracted from Omani camel milk (*Camelus Dromedaries*).

May 2015, May 2016, May2017.