

Lipids derived from Camel milk regulate NLRP3 inflammasome subunit-dependent inflammatory responses in human macrophages

Raya Hamdan Al-Nasseri^{1,2}, Huda Al-Ruqashi³, Jamal Al-Sabahi³, Ahmed Al-Harrasi², Ara Kanekanian¹, Yaj Banerjee⁴, Keith Morris^{1*}, Nurudeen Hassan⁵

¹Cardiff School of Health Sciences, Cardiff Metropolitan University, Cardiff. CF5 2YB, UK; ²Royal Court Affairs, Directorate of veterinary services, Muscat, Sultanate of Oman; ³College of Agricultural and Marine Sciences, Sultan Qaboos University, Sultanate of Oman; ⁴Department of Basic Medical Sciences, Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Academic Medical Center, Dubai Health Care City (DHCC), Dubai- 505055; ⁵Ortho Clinical Diagnostics, Pencoed, CF35 5PZ, UK.

Corresponding Author: Nurudeen Hassan, Ortho Clinical Diagnostics, Pencoed, CF35 5PZ, UK.

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ABSTRACT

Background: Camel milk is widely used for its reported anti-diabetic and health promoting effects. Lipids derived from the milk have also been shown to exhibit potent anti-inflammatory effects. The mechanism through which these lipids and constituent fatty acids exert these effects remains elusive. The aim of this study was to investigate the effect of camel milk on glycated protein-mediated macrophage inflammation.

Methods: To determine the effect of Total Lipids (TL) and Total Fatty Acids (TFA) derived from camel milk on an *in vitro* model of diabetic inflammation, differentiated THP-1 (dTHP-1) cells stimulated with glycated serum albumin (gBSA) was employed. Cells were pre-treated with TL or TFA before challenging cells with gBSA.

Results: Gas Chromatography-Mass Spectrometry (GC-MS) analysis found that TL was 96% triacylglycerol (TAG) while the TFA comprised 65% saturated and 35% unsaturated fatty acids. Both TL and TFA significantly ($p < 0.05$) decreased gBSA-induced secretion of pro-inflammatory cytokines (Tumour necrosis factor-(TNF)- α , Interleukin-(IL)-1 β /18). TL also demonstrated the ability to regulate the expression of p50/p65 sub-units of Nuclear Factor-kappa B (NF- κ B), while concomitantly increasing the expression of regulatory cytokines IL-10, IL-1 Receptor Antagonist (IL-1Ra) and Cluster of Differentiation 163 (CD163)-shifting cells towards an M2 macrophage phenotype. Additionally, we found that TL significantly regulated the expression of Nucleotide-

binding oligomerization domain-like receptor family pyrin domain containing-3 (NLRP3) inflammasome subunit and its regulator; Ten-Eleven Translocation-2 (TET-2).

Conclusion: This paper demonstrates the ability of camel milk lipids to regulate gBSA-induced macrophage inflammation *in vitro*, by modulating the expression of key inflammatory regulators such NF- κ B and NLRP3 inflammasome subunit.

Keywords: Camel milk lipids, Macrophages, NF- κ B, NLRP3-inflammasome, TET-2

INTRODUCTION

Camel milk is traditionally consumed as fresh or in the form of naturally fermented products [1,2], and has been proposed to possess a range of therapeutic benefits, such as anti-hypertensive and anti-diabetic properties [3]. However, there remains substantial uncertainty as to precise mechanisms associated with the benefits of consuming camel milk. Diabetes may be aided with regular consumption of camel milk which has been reported to reduce patients' need for insulin [4] and current studies suggest that camel insulin and other peptides can be encapsulated in lipid vesicles that enhance their passage through the stomach and entry into the circulation [5]. Camel milk has also been shown to enhance wound repair in diabetic mice via the restoration of normal levels of the anti-inflammatory cytokine IL-10, while reducing levels of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-18 and IL-6 [6].

The fatty acid composition of camel milk varies as a property of its source. For instance, in Saudi camels, the saturated fatty acid content is 65% [7], while camel milk from Jordanian breeds was reported to have equal amounts of saturated and unsaturated fatty acids [8,9]. Studies in relation to the health benefits of dietary lipids have generated contradictory data [10-12], however, the health-promoting benefits of camel milk has been attributed to the presence of oleic acid, conjugated linoleic acid, omega-3 fatty acids, short and medium chain fatty acids and other bioactive compounds such as vitamins and minerals [12]. It is known that dietary saturated lipids enhance the expression of pro-inflammatory cytokines [13], however, camel milk is also unique concerning its fatty acid profile as it contains 6 to 8 times less short chain fatty acids compared to milk from cows, goats, sheep, and buffalo [14].

Macrophages are heterogeneous cells that play a pivotal role in the development and progression of diabetes through their capacity to switch into a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype [15]. Stimuli associated with infection such as lipopolysaccharide (LPS) [16] and diabetes-related inflammation, glycated serum albumin [17] shift macrophages to an M1 phenotype. A role for lipids in several mechanisms that control macrophage lipid content and even inflammation by regulating M1/M2 macrophage responses would be expected.

The NLRP3 inflammasome is a multiprotein complex formed by the activation of pattern recognition receptors by danger-/pathogen- associated molecular patterns (DAMPs/PAMPs), which then recruits ASC and procaspase-1 to activate the complex. The NLRP3 complex then cleaves the pro-inflammatory cytokines pro-IL-1 β and pro-IL-18 to their active and secretable forms-IL-1 β and IL-18 [18]. The NLRP3 inflammasome has been linked to several diseases such as diabetes, atherosclerosis and metabolic syndrome [19]. The ten-eleven translocation-2 (TET-2) is an important and key regulator of the NLRP3 inflammasome and IL-1 β secretion [20]. Landén

et al. linked the activity of TET-2 to resolution of inflammation in human macrophages [21]. Also, we recently linked the anti-inflammatory effects of a dietary component, Vicenin-2 to the expression of TET-2 in human macrophages [22]. This study aimed to investigate the ability of camel milk lipids to regulate the NLRP3 subunit of the inflammasome complex and TET-2.

MATERIALS AND METHODS

Ethical approval

The ethical approval for this study was obtained from Cardiff Metropolitan University ethics committee (ethical approval no. 7293).

Sample processing and standard lipids

All camels used in this study (*Camelus dromedarius*) were obtained from the Royal Corps farm (Muscat, Sultanate of Oman). Early morning camel milk samples were collected from 7 healthy lactating camels (240-300 days post-partum). All camels were aged 9 to 11 years with (1-2 calves). Milk samples were filtered through a sieve, pooled and stored at -80°C until use. Standard lipids and saturated triacylglycerol were obtained from Sigma-Aldrich (U.K).

Camel milk Total lipid (TL) extraction

The Bligh and Dyer standard method as adapted from [23] was employed for total lipid extraction from camel milk. In brief, samples of camel milk (5.0g) were extracted with 20:10 ml methanol:chloroform (Sigma Aldrich, UK). The mixture was centrifuged, and the bottom organic layer was collected. The solvent was evaporated using a rotary evaporator, then further dried in an oven (100°C), and placed in a desiccator chamber for further drying.

Camel milk total free fatty (TFAs) acid extraction from camel milk TL

Total fatty acids (TFAs) were extracted from total camel milk lipids (T.L) according to the adapted method from [24,25]. Accurately, 0.1g of total lipids were weighed and transferred to a 100ml round bottom flask. 4ml sodium hydroxide (NaOH) was added and refluxed at 100°C for 30 minutes. Heptane (C₇H₁₆) was added drop-wise until a volume of 4ml was added drop-wise and boiled for 1 minute. The lower layer of the mixture was collected on ice and treated with HCL and heptane. The top layer containing the fatty acids was collected and added to anhydrous sodium sulphate (Na₂SO₄) to remove excess water. The solvent was further dried under nitrogen.

Preparation and analysis of fatty acid methyl esters

Fatty acid methyl esters (FAME) were prepared as described by [26]. In brief, 0.1g of total lipids (TL) extracted from camel milk was refluxed using a mixture of methanol and sodium hydroxide. Boron trifluoride (ACROS, US) and heptane (BDH, England) were added and further heated. The clear upper heptane layer was collected for further analysis. Analysis was performed on a Perkin Elmer Clarus 600 GC System, fitted with a SP-2560 Supelco capillary column (100 m × 0.250 mm inner dimension × 0.2µm film thickness) coupled to a Perkin Elmer Clarus 600C Mass-Spectrometer (Perkin Elmer, UK).

Cell culture

The human monocytic cell line, THP-1 cells (ATCC, UK) were maintained and cultured in RPMI 1640 (Gibco, UK) supplemented with 10% Foetal Calf Serum (FCS; Lab-tech International), 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin (100IU/ml), streptomycin (100µg/ml). Cells were cultured in an incubator at 37°C in the presence of 5% CO₂. Following treatment with phorbol myristate acetate (PMA) THP-1 cells differentiate into macrophage-like cells (dTHP-1) that show characteristics of primary monocyte-derived macrophages [27]. To differentiate cells, THP-1 (seeded at 6 x 10⁵ cells/ml) were treated with 5nM of PMA for 48 hours. The cells were left in fresh media for 48 hours as a wash-out period before treatments.

Immunoassay

The cell-free supernatants were collected from treated cells and stored at -20°C. Pro-inflammatory cytokines: TNF-α, IL-1β, IL-6, IL-18 and fatty acid binding protein 4FABP4 were measured using enzyme-linked immunosorbent assay (ELISA). All ELISA kits were obtained from R&D Systems (Oxford, UK) and carried out according to manufacturer's guidelines.

Quantitative Real Time-PCR (qRT-PCR)

Trizol™ reagent (Ambion, UK) was used to isolate RNA from the cell monolayer. Complementary DNA (cDNA) was synthesized using High Capacity cDNA RT Kit (Applied Biosystems, UK). Gene expression was determined using Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, UK). The comparative CT method ($2^{-\Delta\Delta CT}$) was used to calculate relative gene expression. Gene expression was determined using Taqman methodology. Pre-designed probes for target genes; *TET-2* (Hs00758658_m1), *IL-1Ra* (Hs00991010_m1), *NLRP3* (Hs00918082_m1), *IL-10* (Hs00961619_m1), *Dectin-1* (Hs00224028_m1) and housekeeping genes; *GAPDH* (Hs02758991_g1) and *GUSB* (Hs99999908_m1), were obtained from Thermo-fisher, UK. Gene expression of target genes was normalized against the housekeeping genes and untreated samples were used as experimental controls.

NF-κB proteome profile and activation and Caspase-Glo®1 Inflammasome Assay

The Proteome Profiler Human NF-κB Pathway array kit (Cat # ARY029, R&D SYSTEMS®) was employed to study the effect of treatments on protein expression of a panel of proteins involved in NF-κB signalling. Active Motif® extraction kit (Cat #40010 and #40410) was used for nuclear and cytoplasmic protein extraction according to manufacturer's guidelines. BCA Protein Assay Kit (Cat #7780, Cell signaling Technology, UK) coupled with Tecan Infinite® 200 plate reader (Tecan AG, Switzerland) were used to quantify the protein concentration. To determine the level of protein expression of the major subunits of NF-κB complex (p50 and p65) within the nuclear extract, TransAM® NF-κB Family Transcription factor assay kit (Cat. # 43296) was used. The level of Caspase-1 was measured using Caspase-Glo® 1 inflammasome assay (Cat # G9952) in whole cell lysate.

Western blotting

To extract total proteins, the cell culture media was removed from wells and Radio-Immunoprecipitation Assay (RIPA) Buffer (ThermoFisher, UK) was added to wells and placed on ice for 5 min. The cell lysate was centrifuged at 14000g for 15 min at 4 °C. Protein concentration

was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher, UK). A total of 25µg total protein was separated on 10% Bis-Tris NuPAGE® gels (Invitrogen Ltd, UK) and transferred to a nitrocellulose membrane using the iBlot® dry blotting system (Invitrogen Ltd., UK). The membrane was blocked with 5% Marvel milk powder for 1 hour then incubated with the primary antibody overnight at 4°C overnight at the dilution of 1:1000. The membrane was washed and incubated with HRP-conjugated secondary antibody (Cell Signalling, UK) at a dilution of 1:5000 for 1 hour. Subsequently, the membrane was visualised with ECL HRP-substrate (Amersham™, UK) and exposed to Hyperfilm™ ECL film (Sigma Aldrich, UK). ImageJ® software was used for densitometry analysis of immunoblots. Primary antibodies: anti-TET2 (MABE462), anti-NLRP3 (ABF23) and β-Actin (MABT825) were obtained from Merck Millipore, Watford, UK.

Flow cytometry

Following treatments, cells were harvesting cells using pre-chilled 1mM EDTA in PBS, then washed by centrifugation at $250 \times g$ at 4°C before reconstitution in FACS buffer (3% BSA in PBS). 20µl of conjugated antibodies was added to cells in FACS buffer for 20 minutes on ice. The antibody was washed off by centrifugation and the cell pellet was reconstituted in 500µl of FACS buffer for analysis. Cytomics FC500MPL (Beckman-Coulter) flow cytometer was used to analyse samples, using a minimum of 5000 events as a threshold.

Statistical analysis

All data are presented as mean \pm standard deviation. Depending on the study design, One-way analysis of variance (ANOVA) or two-way ANOVA was conducted for within group comparisons with Tukey's and Bonferroni's *post hoc* tests. Graphpad Prism® (Version 7) was used for all statistical analysis. Statistically significant differences between treatments and/or controls are denoted as (*) for $p < 0.05$. Higher levels of significance were reported as (**) $p < 0.01$ and (***) $p < 0.001$ and non-significant results were denoted as NS. The number of experimental repeats, denoted as 'n', represents the technical replicates.

RESULTS

Initially, we determined the lipid composition of the milk of seven lactating camels using Thin Layer Chromatography (TLC) (data not shown) and the TL extracted represented approximately 3.0 % of the total milk constituents. This analysis supported a previous finding that over 95% of the lipids present in camel milk are triacylglycerols [7]. To further characterise the lipid content of camel milk, the extracted lipids were derivatised to fatty acids methyl esters (FAME). The predominant saturated fatty acids were Palmitic acid (C16:0), Myristic acid (C14:0) and Stearic acid (C18:0) and unsaturated fatty acids were Oleic acid (C18:1, *cis*9), Palmitoleic acid (C16:1, *cis*-9), 11-Octadecenoic acid (C18:1n-7). The saturated component comprised 61% of the total fatty acids (Table 1).

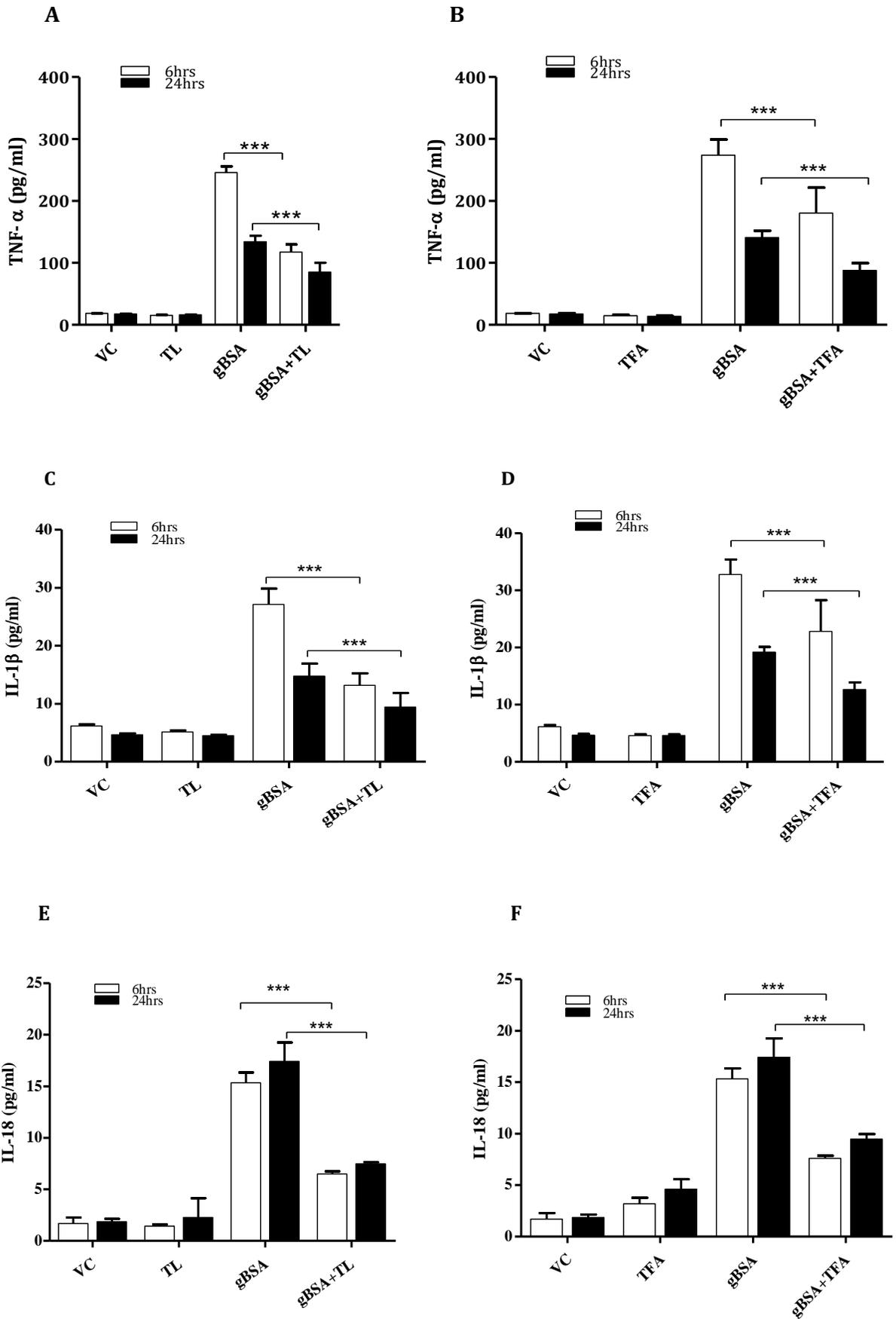
TL and TFA regulate gBSA-induced inflammatory cytokine release and FABP4 secretion

The binding of advanced glycated end products (AGEs) such as gBSA to the cognate receptor, RAGE, on the surface of macrophages and subsequent production of pro-inflammatory cytokines is a crucial event in the development of metabolic complications of type 2 diabetes such as atherosclerosis and cardiovascular disease [28]. To investigate if extracted camel milk lipids could

regulate gBSA-induced inflammatory cytokine secretion, dTHP1 cells were pre-treated with lipids for 1 hour and then cells were stimulated with 500 μ g/ml gBSA for 6 and 24 hours. We initially demonstrated that 20 μ g/ml of TL and TFA did not significantly reduce cell viability of dTHP-1 cells up to 48 hours of incubation (data not shown). Pre-incubating cells with 20 μ g/ml of TL and TFA significantly reduced the secretion of TNF- α , IL-1 β and IL-18 at 6 and 24 hours (Figure 1A-F), and IL-6 at 24 hours (Figure 1G-H). IL-6 was only detected at 24 hours. FABP4 is an adipokine associated with metabolic disease and insulin resistance [29], and is upregulated by AGEs, leading to cholesterol triacylglycerol accumulation in dTHP-1 cells [30]. FABP4 is secreted by adipocytes and macrophages and elevated circulating FABP4 levels is associated with insulin resistance and atherosclerosis [31]. Both TL and TFA significantly ($p < 0.05$) blunted gBSA-induced secretion of FABP4 in dTHP-1 cells at both 6 and 24 hours (Figure 1I).

Fatty acid methyl ester	Composition %
Saturated	
Palmitic acid (C16:0)	35.3
Myristic acid (C14:0)	14.5
Stearic acid (C18:0)	7.4
Pentadecanoic acid (C15:0)	1.0
Lauric acid (C12:0)	1.0
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	
Oleic acid (C18:1, cis-9)	19.3
Palmitoleic acid (C16:1, cis-9)	14.0
Vaccenic acid: 11-Octadecenoic acid (C18:1n-7)	1.7
Myristoleic acid (C14:1n9c)	1.4
Heptadecenoic acid (C17:1, cis-10)	1.011
7(Z)-Hexadecenoic acid (16:1 trans-9)	1.027
Arachidonic acid (C20:4n6)	0.411
12,15-Octadecadienoic acid (C18:2)	0.240
11,14-Eicosadienoic acid (C20:2) n6	0.211

Table 1: Major Fatty acid methyl esters (FAMES) present in Omani camel milk total lipids. FAMES were extracted using Bligh and Dyer method [23], from pooled camel milk (n=7)



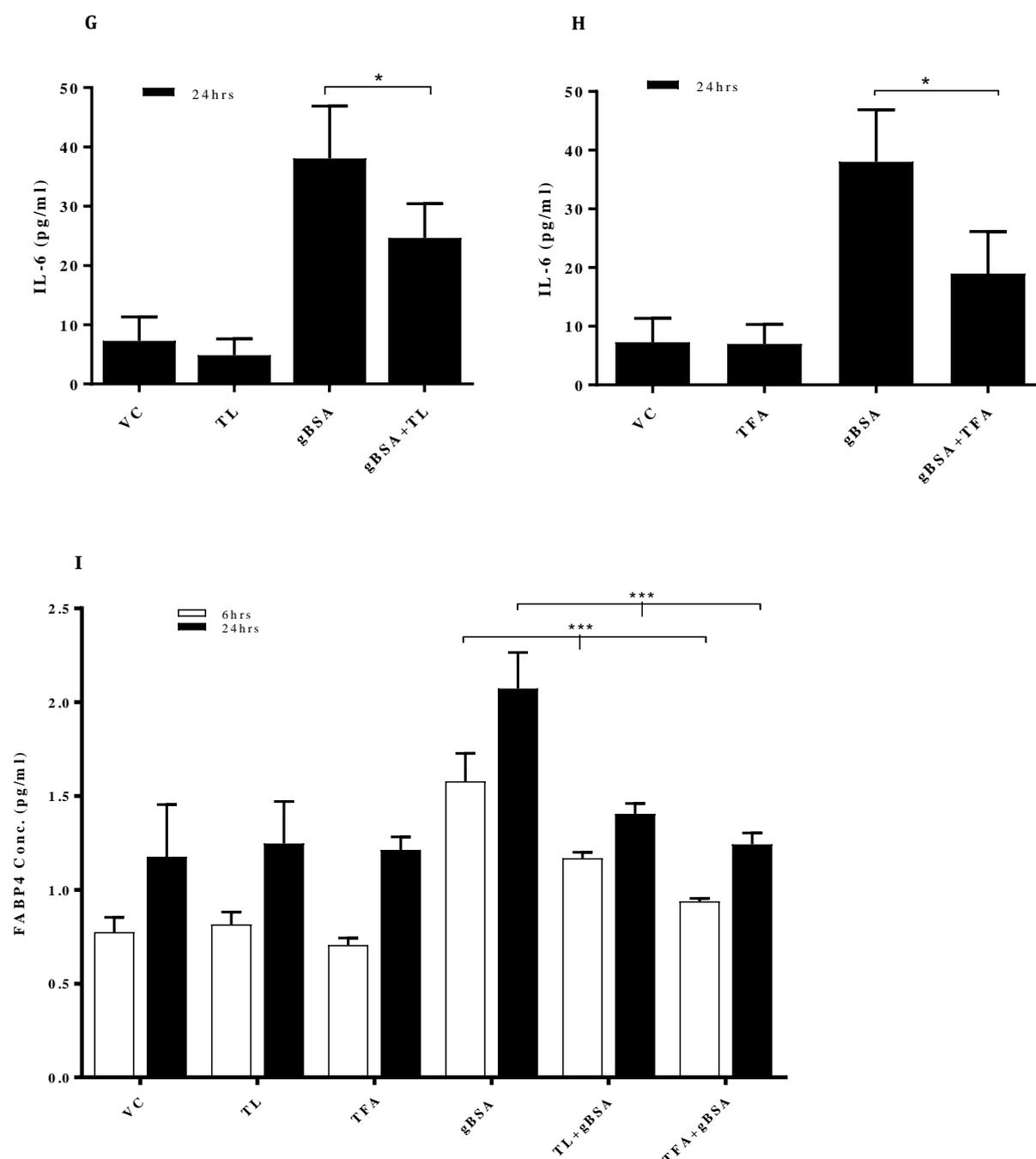


Figure 1. The effect of TL and TFA on gBSA-induced pro-inflammatory cytokines and FABP4. dTHP-1 cells were pre-treated with 20µg/ml of TL, TFA or VC (0.01% DMSO) for 1 hour. Cells were then stimulated with 500µg/ml gBSA for 6 or 24 hours. The secretion of TNF-α, IL-1β, IL-18, IL-6 and FABP4 was measured in cell-free supernatants using ELISA. Data represents mean ± SD (n=3).

TL enhances expression of M2 phenotype markers.

After demonstrating that both TL and TFA exhibit a similar pattern of anti-inflammatory activity, the rest of work was focussed on TL. We investigated the effect of TL on the surface expression of M1: CD86 (Figure 2A) and M2 markers: CD163, IL-10, Dectin-1 and IL-1Ra (Figure 2B and 2C). TL treatment alone enhanced the expression of M2 markers CD163, IL-10, IL-1Ra and Dectin-1 (Figure 2B-C). Pre-treatment of the cells with TL before challenge with gBSA enhances M2 marker expression.

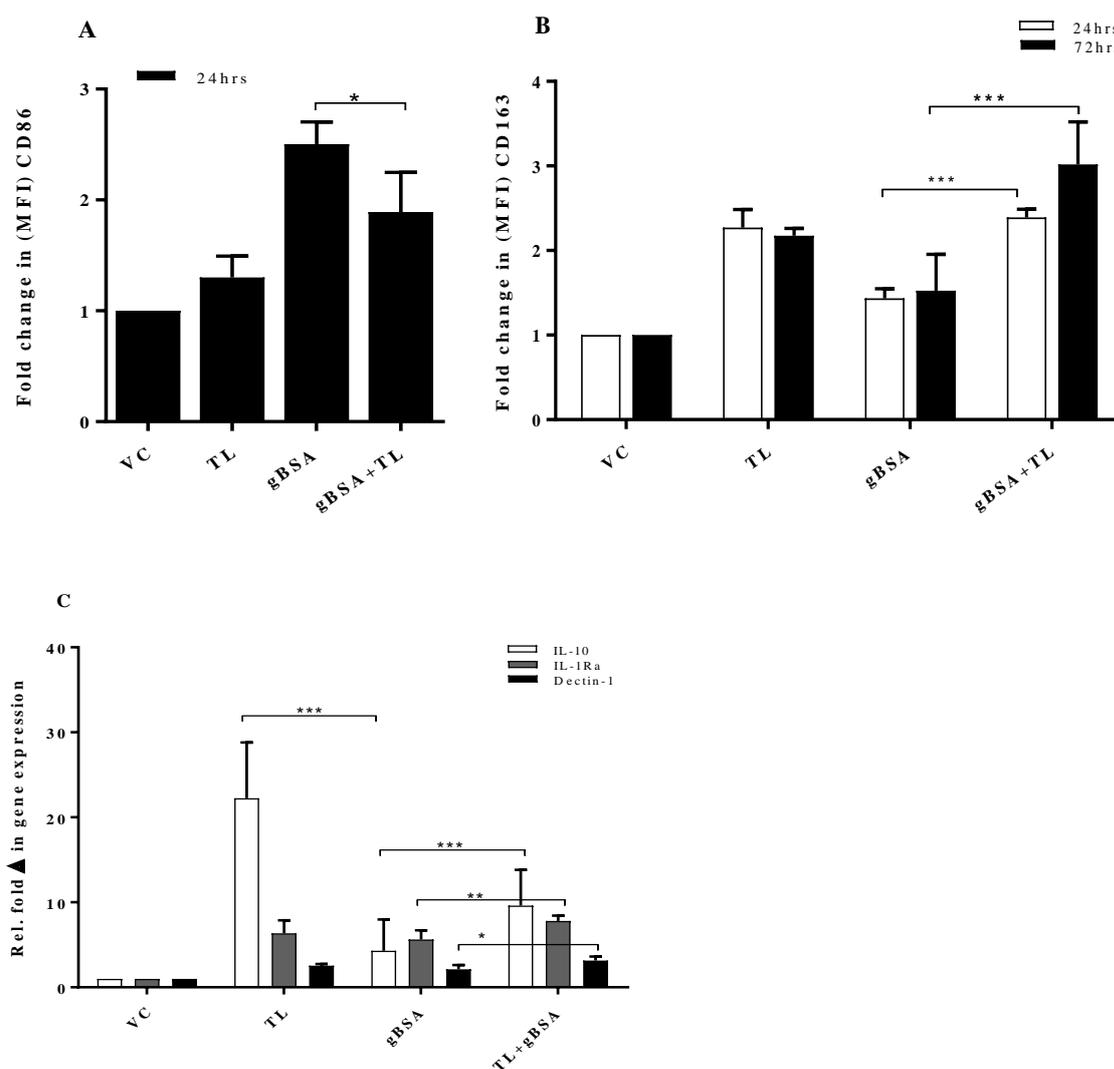


Figure 2. TL modulates the expression of CD86 and M2 markers of macrophage polarization (CD163, IL-10, IL-1Ra and Dectin-1). dTHP-1 cells were pre-treated with (20µg/ml TL) for 1 hour, then stimulated with 500µg/ml gBSA. The effect of TL and/or gBSA on surface expression of A) CD86 and B) CD163 was determined by flow cytometry. C) Following treatments, the gene expression of *IL-10*, *IL-1Ra*, and *Dectin-1* was also determine by RT-PCR. Data represents mean ± S.D (n=3).

TL modulates the expression of p50/p65, Caspase-1, NLRP3 and TET-2.

The induction of inflammatory cytokine secretion is associated with activation of the transcription factor NF-κB, through heterodimerisation of its p50 and p65 subunits [32]. In contrast, the secretion of the anti-inflammatory cytokine IL-10 and M2 polarization is associated with enhanced p50 homodimerisation [33]. Therefore, we determined the action of TL on the cellular localisation of the p50 and p65 subunits through assessing their expression in the nuclear extract of dTHP-1 cells (Figure 3A). Treatment of cells with TL alone significantly increased nuclear expression of p50, whereas p65 remained unchanged. Conversely, gBSA induced a greater increase in the nuclear expression of p65. Further, pre-treatment of cells with TL before gBSA stimulation significantly reduced the nuclear expression of p65.

The secretion of IL-1β and IL-18 requires the formation of an NLRP3 inflammasome complex that results in the release of mature active Caspase-1, which in turn cleaves the precursor forms of IL-1β and IL-18 (pro-IL-1β and pro-IL-18) that are released as mature, active cytokines [34]. To determine the effect of TL on NLRP-3 inflammasome, we investigated the effect of TL on Caspase-1 activity, NLRP3 subunit expression and NLRP3

regulator, TET-2. We demonstrated that TL was capable of down-regulating gBSA-induced Caspase-1 activity (Figure 3B). NLRP3 gene expression was reduced significantly in the presence of TL alone compared with gBSA treatment at 24 hours. Pre-treatment of cells with TL before gBSA treatment induced a decrease in NLRP3 gene expression (Figure 3C). To determine whether the effect of TL on NLRP3 and TET-2 as observed at mRNA level are reflected at protein level, Western blot analysis was conducted. TL treatment alone significantly increased TET-2 protein expression in dTHP-1 cells, and decreased gBSA-induced NLRP3 protein expression (Figure 3D-E).

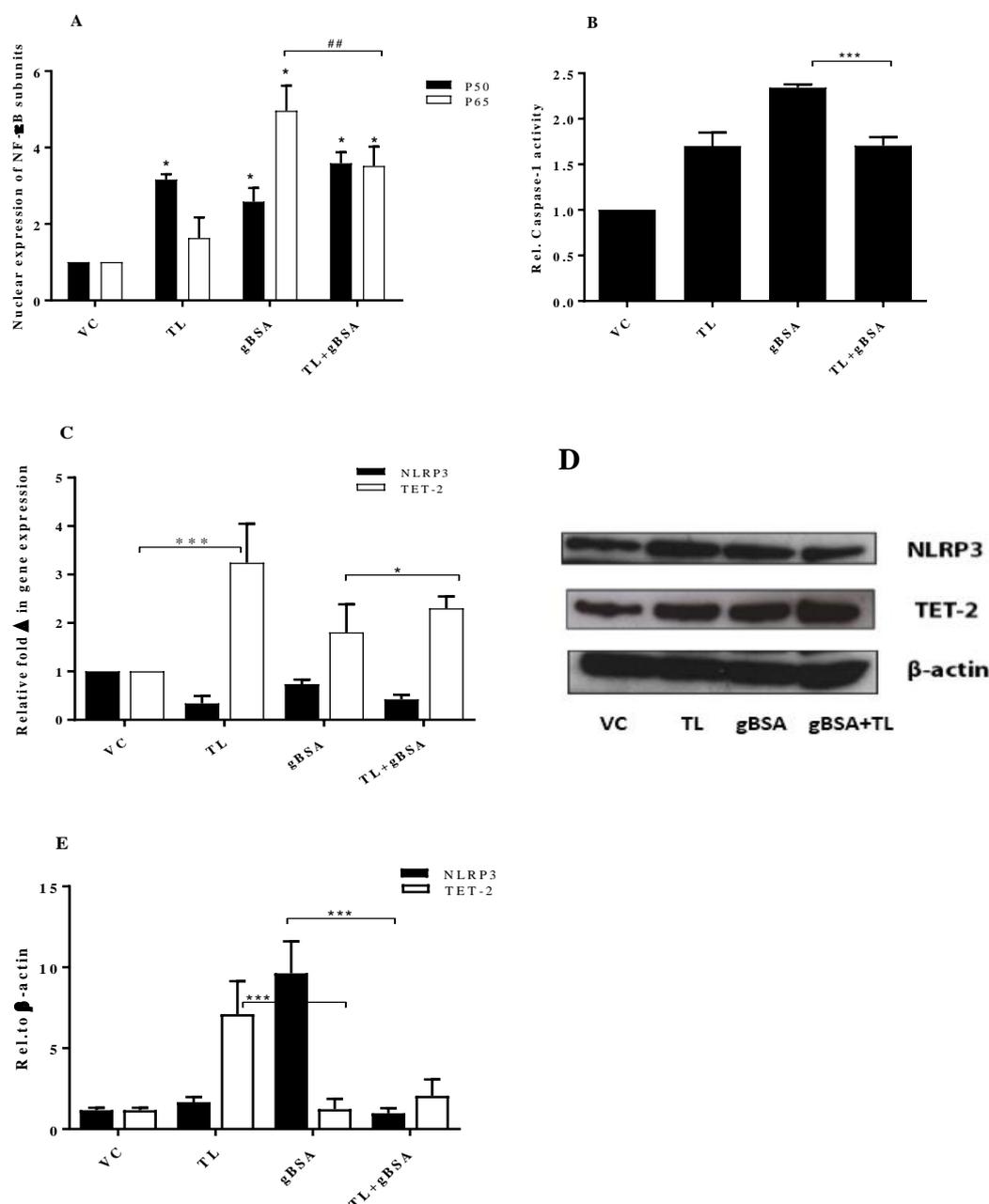


Figure 3. Effect of TL on the expression of p50/p65, Caspase-1 activity and NLRP3 and TET-2 expression. dTHP-1 cells were pre-treated with 20µg/ml TL or VC for 1 hour then stimulated with 500µg/ml gBSA. A) the nuclear protein extract was isolated for expression analysis of p50/p65 subunits of NF-κB 24 hours post-stimulation. B) Caspase-1 activity was determined in the whole cell lysate at 120 minutes following stimulation with gBSA. C) mRNA expression of *NLRP3* and TET-2 at 24 hours post-stimulation was determined using qRT-PCR. D-E) Immunoblots and densitometric analysis of NLRP3 and TET-2 expression. The data in E was normalized versus VC. Data represents mean ± SD (n=3). Where * and # are used to denote statistical significance, * indicates significance between control and treatment groups, while # represents significance between treatment groups.

Standard triacylglycerols with saturated fatty acids do not significantly reduce IL-1 β or NLRP3 inflammasome in dTHP-1 cells.

The fatty acid component of TL, a triacylglycerol mixture, used in this investigation were a mixture of saturated and unsaturated fatty acids. To elucidate if the presence of this mixed species of fatty acids played any role in the activity of TL, we investigated the actions of standard, commercially available saturated triacylglycerols on macrophage mediated inflammation. When dTHP-1 cells were incubated with standard triacylglycerol (TAG) mixture composed of exclusively saturated fatty acids, no significant reduction in gBSA-induced secretion of IL-1 β protein was observed (Figure 4A). Unlike the camel milk TL, the standard TAG mixture did not have an effect on NLRP3 protein levels as determined by Western blot analysis (Figure 4B). This data suggests that the anti-inflammatory effects observed with TL, and TFA, are associated with TL being composed of both saturated and unsaturated fatty acids.

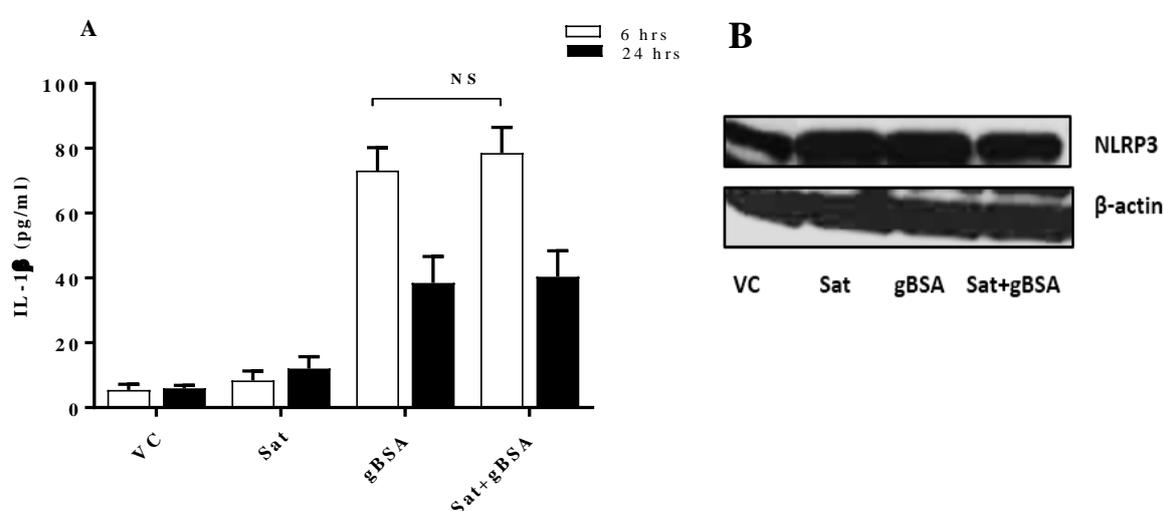


Figure 4. Triacylglycerols with saturated fatty acids do not reduce IL-1 β or NLRP3 inflammasome subunit expression in dTHP-1 cells. dTHP-1 cells were pre-treated with 20 μ g/ml of saturated triacylglycerol or VC for 1 hour then stimulated with 500 μ g/ml gBSA for A) 6 or 24 hours and the secretion of IL-1 β was determined using ELISA. B) The protein expression of NLRP3 and IL-1 β was determined at 24 hours by ELISA and NLRP3 subunit by immunoblots. Data represents mean \pm SD (n=3).

DISCUSSION

Camel milk has been proposed as having numerous health benefits, in particular, it is seen to have anti-diabetic properties. In T2D, macrophages play a major role in the development and progression of the disease through cytokine secretion via the activation of inflammatory regulators such as the NLRP3 inflammasome. This study demonstrates that the total lipid extract of camel milk, comprising of more than 95% triacylglycerols, and its free fatty acid component significantly regulated a range of inflammatory cytokines in gBSA-challenged THP-1 macrophages, and enhanced expression of markers of the anti-inflammatory M2 phenotype (Figure 2). Activation of macrophages into an inflammatory phenotype (M1), plays an important role in the pathogenesis of insulin resistance, T2D and atherosclerosis [35]. Hence, the ability of these lipids to reduce macrophage mediated inflammation, while concomitantly enhancing M2 polarization, suggests that consumption of these lipids through camel milk and its associated products, could beneficially modulate chronic, sterile inflammation in conditions such as T2D.

In this study, the differentiated monocytic cell line, THP-1 (dTHP-1) was employed as a model of macrophages. dTHP-1 cells have been shown in numerous studies to be a valid model of the inflammatory response. These cells also demonstrate phenotypic plasticity *in vitro* as they are transformable into the pro-inflammatory M1 phenotype in the presence of pro-inflammatory stimuli [36], and in the presence of anti-inflammatory cues such as IL-4/ IL-13, dTHP-1 cells demonstrate the characteristics of the M2 phenotype. M2 macrophages are known to be beneficial in preventing the development of insulin resistance/T2D and atherosclerosis, and modulating their inflammatory complications [37].

We also demonstrated that both TL and TFA significantly downregulated the secretion of gBSA-induced FABP4 in these cells (Figure 1I). FABP4 is an important mediator of metabolic and inflammatory pathways in adipocytes and macrophages, and also plays a key role in the development and progression of T2D and its associated complications such as atherosclerosis [38,34]. Regulation of FABP4 could be a therapeutic strategy against metabolic and cardiovascular diseases and this study presents evidence that camel milk lipids could play a beneficial role in the regulation of FABP4.

The glycation of proteins due to hyperglycaemia as found in T2D patients gives rise to AGEs such as gBSA. AGEs bind to their cognate receptor, RAGE, and potently activate NF- κ B—leading to p50/p65 heterodimerization and pro-inflammatory cytokine release. We also present data showing that TL increases homodimerization of the p50 subunits of NF- κ B at the expense of p50/p65 heterodimerization. Homodimerization of the p50 subunit is known to induce IL-10 synthesis [39], a cytokine that is itself a regulator of pro-inflammatory cytokine secretion [40]. We would therefore propose that the anti-inflammatory actions of camel milk lipids are in part mediated by its ability to enhance NF- κ B p50 homodimerization and consequent IL-10 expression.

The actions of TL in regulating IL-1 β and IL-18 secretion (Figure 1C and E), the activity of Caspase-1 (Figure 3B), and the regulator of NLRP3, IL-1Ra (Figure 2C) supports an inflammasome-modulating activity of TL in dTHP-1 cells.

Consumption of TAGs results, through the action of proteolytic enzymes/lipases in the gut, in their breakdown into free fatty acids and triglycerides. The TAGs are reformed and stored in adipose tissue. Therefore, it is conceivable that TAGs, in the form of TL could be made bioavailable and interact with the adipose tissue macrophages. Large numbers of macrophages accumulate by different mechanisms in obese adipose tissue and considerable cross-talk exists between macrophages in adipocytes [41]. In particular, in T2D and metabolic disorders, there is dysregulation and enhanced release of triacylglycerols [42]. Hence, increased levels of TAG with the fatty acid components associated with camel milk may beneficially regulate glycosylated protein-mediated inflammation, as found in T2D.

Our study also demonstrated that the fatty acid components, present in the total lipids, were themselves able to regulate the inflammatory response in a similar fashion to TL. This observation is important as the principal fatty acids present were predominantly saturated, in particular, palmitic acid—that has previously been identified as being pro-inflammatory [43]. Given that, we attributed the potent anti-inflammatory actions of TL to the presence of a mixture of saturated and unsaturated FAs. Our observations herein are supported by a landmark study by L'homme *et al.* [44], who demonstrated that a mixture of saturated and unsaturated FAs including palmitic and oleic acid downregulated a range of inflammatory responses in a NLRP3-dependent manner. The data from Figure 4, showing that saturated TAGs alone did not affect IL-1 β secretion or NLRP3 protein expression further supports our observation in this

study. Considering the central role of the NLRP3 inflammasome in metabolic diseases, including T2D, this finding in relation to camel milk lipid intake and regulation of disease are highly relevant and important.

Fuster *et al.* [45] demonstrated that macrophages produced increased levels of IL-1 β following TET-2 knock-down. They further confirmed that this increase was mediated by NLRP3 activity. The fact that these camel milk derived lipids increased TET-2 expression suggests that this increase may be associated with its anti-inflammatory/inflammasome-regulatory actions. We hereby argue that the observed increase in IL-10, IL-1Ra and TET-2 by an inflammatory mediator, gBSA and further increase observed when cells were treated with both TL and gBSA is part of the regulatory response in macrophages as previously described by Zhang *et al.* [46]. A recently published paper has linked the glucose regulatory enzyme, AMPK to the function of TET-2 [47]. This further underscores the importance of the findings from this study and highlights other areas of further research. For example, the role of a lipid sensor and transcription factor, Peroxisome Proliferator Activated Receptors (PPARs).

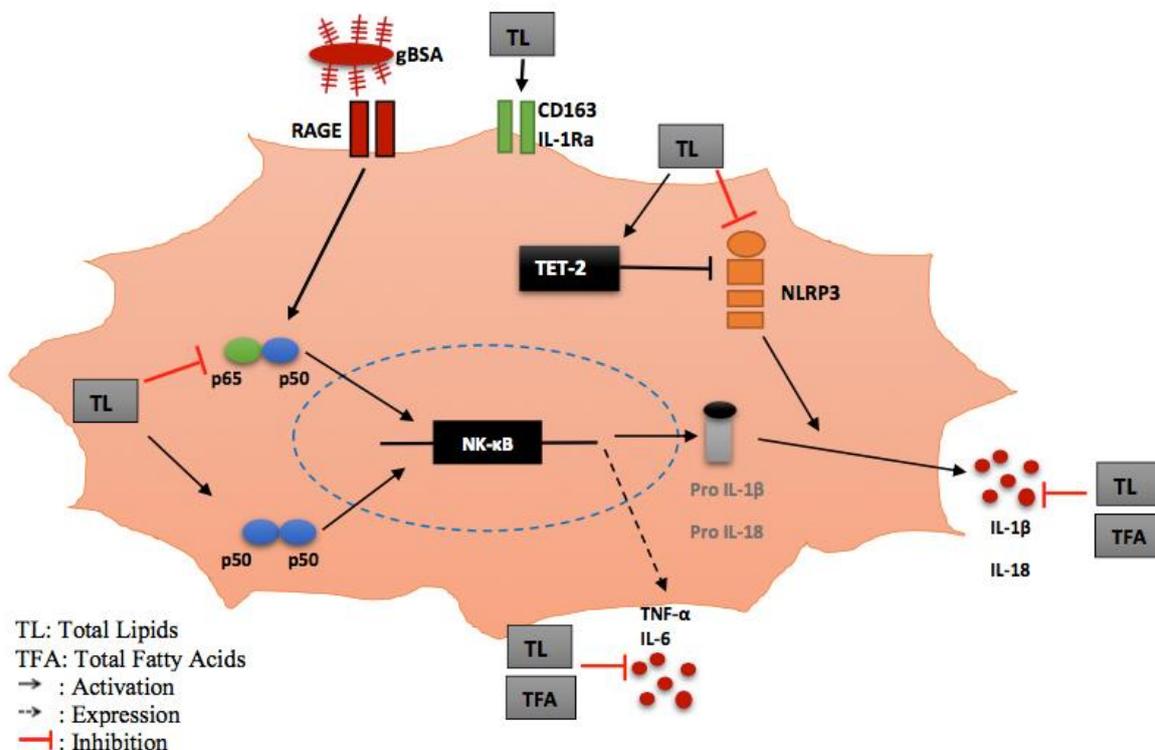


Figure 5. Proposed mechanistic model of the immunoregulatory effects of camel-milk derived total lipids and fatty acids in human macrophages.

CONCLUSION

This paper demonstrates the ability of camel milk lipids to regulate gBSA-induced macrophage inflammation *in-vitro* through modulating the expression of key inflammatory regulators such NF- κ B and NLRP3, while increasing the expression of anti-inflammatory markers/regulators (IL-10 and IL-1Ra and TET-2). Although the precise mechanism by which these lipids mediate their activity remains to be confirmed, data presented in this paper sheds light on the potential mechanism through which camel milk lipids can be beneficial in diabetes and other inflammatory conditions. However, further work should be undertaken in order to confirm the direct effect of these lipids on the function of NLRP3 and its related genes.

List of Abbreviations: NF- κ B, Nuclear Factor- κ B; NLRP3, Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing-3; gBSA, glycated bovine serum albumin; TET-2, Ten-eleven translocator-2; TNF, Tumour necrosis factor, IL, Interleukin, CD, Cluster of differentiation; PMA, Phorbol myristate Acetate, FAME, Fatty acid methyl esters, TL, Total lipids; TFA, Total fatty acids; FABP4, Fatty acid binding protein 4; LPS, Lipopolysaccharide.

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