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UNDERSTANDING THE MECHANISMS OF ANTI-INFLAMMATORY ACTIVITIES OF CRYPTOLEPINE

BY

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THIS THESIS IS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY, UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A DOCTOR OF PHILOSPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

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DECLARATION

I, Ahmed Rufai Abdulrahman, do hereby declare that, this report is the product of my own research and that other people's work referenced in it, have been duly acknowledged. The research was carried out under the supervision of Dr. Kwadwo Asamoah Kusi and Dr. Jonathan P. Adjimani.

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DEDICATION

I dedicate this work to the service of the Almighty Allah.

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I give absolute thanks and praises to the Almighty Allah for His blessings, mercies, protection and guidance throughout this project. Certainly, without His interventions, I could not have reached this stage of the work. I also thank my parents and other family members for their unflinching support, love and care. I acknowledge my supervisors for their supervision and contributions to the work. I am grateful to my colleagues for the good discussions and ideas we shared throughout the PhD programme. I also acknowledge the support I received from the graduate interns and postdoctoral fellows at WACCBIP when I was doing some of the laboratory analyses. Finally, I acknowledge WACCBIP for funding the project.

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LIST OF ABBREVIATIONS

AEG 1	Astrocyte Elevated Gene-1
AKIP 1	A-Interacting Protein 1
APC	Antigen Presenting Cells
ARE	AU-Rich Elements
ATP	Adenosine Triphosphate
CLRs	C-Type Lectin Receptors
COPD	Chronic Obstructive Pulmonary Disease
CsyA	Cyclosporine A
DAMPS	Damage-Associated Molecular Patterns
DHMEQ	Dehydroxymethylepoxyquinomicin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
FBS	Foetal Bovine Serum
GRO	Growth-related Oncogenes
H3-Me3K4	Tri-Methylated at Lysine 4
IBD	Inflammatory Bowel's Disease
IFNs	Interferons
IL-1β	Interleukin-1Beta
IL-1RA	IL-1 Receptor Antagonist
IL-6	Interleukin-6
ING 4	Inhibitor of Growth 4
IRAK	IL-1R-Associated Kinase
IRF	Interferon Regulatory Factor
JNK	c-Jun N-terminal Kinase
LFA-1	Lymphocyte Function–Associated Antigen
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeats

Mal	MyD88-adaptor-like
MS	Multiple Sclerosis
MSK1	Mitogen- And Stress-Activated Protein Kinase-1
MTT	3-(4, 5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NAP1	NAK-associated Protein 1
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor-κ B
NHEM	Normal Human Epidermal Melanocytes
NLRs	NOD-Like Receptors
NLS	Nuclear Localization Signal
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PAMPS	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PDCs	Plasmacytoid Dendritic Cells
PGE2	Prostaglandin E2
PHD	Plant Homeodomain
PKA	Protein Kinase A
PRR	Pattern Recognition Receptors
RA	Rheumatoid Arthritis
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
RHD	Rel Homology Domain
RIG	Retinoic acid-Inducible Gene 1
RIP	Receptor interacting Protein 1
RLRs	RIG-I-Like Receptors
ROS	Reactive Oxygen Species
RPS3	Ribosomal Protein S3
SARM	Sterile-alpha and Armadillo Motif-containing Protein
SEAP	Secreted Embryonic Alkaline Phosphatase
SLE	Systemic Lupus Erythematosus

SOC-1	Suppressor of Cytokine Signaling 1
TAB1	TAK1 -Binding Protein 1
TADs	Transcription Activation Domains
TAK1	Transforming Growth Factor-Activated Protein Kinase 1
TCR	T cell Receptor
TFBS	Transcription Factor Binding Sites
TIR	Toll/Interleukin-1 (IL-1) Receptor Homology
TIRAP	TIR Domain-containing Adaptor Protein
TLRs	Toll-Like Receptors
TNFR1	TNF Receptor 1
TNF-α	Tumour Necrosis Factor-alpha
TRAF6	Tumour Necrosis Factor Receptor-Associated Factor 6
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR Domain-Containing Adaptor Inducing IFN-β
TPA	12-O-Tetradecanoyl Phorbol-13-Acetate
WAT	White Adipose Tissue

ABSTRACT

Although inflammation coordinates host immunity against infectious pathogens and alarmins, its dysregulation results in severe pathological conditions. Dysregulated inflammatory responses come about as a result of many factors including hyperactivity of pro-inflammatory signaling pathways. Hyperactivity of the Toll like receptor (TLR) - nuclear factor kappa B (NF-κB) signaling pathway is reported to be associated with many inflammatory diseases, which makes the pathway a good therapeutic target. Although cryptolepine, an alkaloid obtained from *Cryptolepis sanguinolenta*, has been demonstrated to possess anti-inflammatory properties *in vivo*, its mechanisms of action are not fully understood. This study sought to determine the anti-inflammatory effects of cryptolepine and its underlying mechanisms of action in a murine macrophage cell line (RAW Blue cells) stably transfected with secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of a promoter inducible by NF-κB transcription factor. The cytotoxic effect of cryptolepine on the cells was determined by 3-(4, 5Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The activity of the TLR1/TLR2-NF-κB signaling pathway was induced with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (0.5 - 1 μM) for 24 hours. Afterwards, culture supernatants were harvested and the activity of the pathway measured by assessing the levels of SEAP using Quanti-Blue assay. In addition, the effect of cryptolepine on the transcript levels of *Tnf-α, Il-6, Il-1β, Il-23, Ccl2, Cxcl2, Ikbkb, Nfkb1, Rela, Tlr2 and Tlr1* were assessed by RT-qPCR and the levels of TNF-α, IL-6, IL-1β, IL-23, MIP-2α and MCP-1 determined using multiplex ELISA technique. Compared to untreated cells, cryptolepine-treated cells showed a dose-dependent decline in viability with 1 μM being the maximum non-toxic concentration of cryptolepine.

Cryptolepine (0.5 – 1 μM) also dose-dependently inhibited the TLR1/TLR2-NF-κB signaling pathway activity. Additionally, the transcript levels of *Tnf-α, Il-6, Il-1β, Il-23, Mip-2α, Mcp1,Tlr1, Tlr2, Rela, Ikkβ*

and *Nf-κb1* were all attenuated by cryptolepine. Cryptolepine also suppressed the protein levels of TNF-α, IL-6, IL-23, MCP-1 but not IL-1β and MIP-2α. In summary, the results from this study demonstrated that cryptolepine inhibited the activity of the TLR1/TLR2 signaling pathway, attenuated the transcript levels of key signaling molecules and suppressed the levels of key pro-inflammatory cytokines and chemokines, which suggest that cryptolepine may be a good anti-inflammatory therapeutic agent.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Although inflammation is crucial in coordinating host immunity against infectious pathogens and endogenous alarmins, its dysregulation causes collateral damage to host tissues resulting in severe pathological conditions (Tisoncik *et al.,* 2012).

Inflammatory responses are triggered by immune cells upon sensing of endogenous or exogenous stimuli using receptors such as the Toll-like receptors (TLRs). One of such key TLRs is the TLR1/TLR2 heterodimeric receptor which senses triacyl lipoprotein on bacteria (Takeda & Akira, 2005). When TLR1/TLR2 is activated by an appropriate ligand, an intracellular signaling cascade is induced and ultimately the transcription factor nuclear factor- kappa B (NF-κB) gets activated. In mammals, five related members of the family of NF-κB exist; RelA (p65), RelB, cRel as well as the breakdown products of NF-κB1 (p105) and NF-κB2 (p100); p50 and p52 respectively (Gilmore, 2006). The family members can homodimerize or heterodimerize with p50-p65 being the most predominantly active heterodimer (Tak & Firestein, 2001).

NF-κB is normally found inactivated through its association with the inhibitor of κB (IκB) (Hayden *et al.,* 2006). When the TLR1/TLR2 is stimulated, IκB kinase (IKK) complex gets activated by the ensuing signaling cascade which in turn phosphorylates IκB thereby activating it for proteolysis (Häcker & Karin, 2006). This makes NF-κB free to translocate into the nucleus to induce transcription of its target genes (Häcker & Karin, 2006).

NF-κB has a broad-range transcriptional activity. It regulates transcription of hundreds of genes which include cytokines, chemokines, cell cycle regulators, receptors like TLR1 and TLR2, signaling molecules

like IKKβ, RelA and NF-κB1 (p105), among others (Yang *et al.*, 2016). Cytokines including tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin- 1beta (IL-1β) and interleukin-23 (IL-23) are crucial in orchestration of inflammatory responses (Arango Duque and Descoteaux, 2014; Croxford *et al.*, 2014). Also, chemokines including macrophage chemoattractant protein-1 (MCP-1) (referred to as C-C motif ligand 2 (CCL2)) and macrophage inflammatory protein-2 alpha (MIP-2α) (referred to as C-X-C motif ligand 2 (CXCL2)) play major roles in inflammation by attracting macrophages, neutrophils and hematopoietic stem cells to sites of inflammation (Amanzada *et al.*, 2014; Pelus and Fukuda, 2006).

To prevent overexpression of the NF-κB-regulated genes which may cause inflammation to spiral out of regulation, NF-κB transcriptional activity is regulated in several ways. However, in chronic inflammatory conditions, NF-κB is found to be hyperactivated due to some defects in the regulatory mechanisms of the signaling pathway (Aksentijevich & Zhou, 2017; Peng et al., 2010). This results in overexpression of the products of many genes regulated by NF-κB including TNF-α, IL-6, IL-1β, IL-23, MCP-1, IKKβ and TLR2 which are important in the pathogenesis of many inflammatory conditions (Yang *et al.*, 2016; Feldmann *et al.*, 1996; Aupperle *et al.*, 1999; Pallone and Monteleone, 2001; Nishimoto and Kishimoto, 2004; Croxford *et al.*, 2014; Wu *et al.*, 2015).

Thus, the NF-κB signaling pathway serves as an important therapeutic target for inflammatory conditions associated with hyperactivity of the transcription factor and overexpression of its target genes. Several studies with various compounds that target the NF-κB signaling pathway have shown positive outcomes in terms of suppression of the levels of pro-inflammatory mediators and inhibition of inflammation. However, clinical use of many of the compounds that have successfully become drugs for inflammatory diseases is hampered by their adverse side effects. Particularly, anti-inflammatory drugs like corticosteroids proved to be unsatisfactory largely because of their global suppression of host's immune response that diminished the host's general

ability to control infection (Singanayagam *et al.*, 2018). Also, studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs), which are used to manage inflammatory conditions, cause adverse side effects including peptic ulcers (Dinarello, 2010; Tseng & Wolfe, 2000). The afore-mentioned challenges with the existing anti-inflammatory drugs therefore highlight the need for novel therapies with not only potent anti-inflammatory properties but also minimal side effects.

Cryptolepine is an indoloquinoline alkaloid which is present in large amounts in the root of *Cryptolepsis sanguinolenta*. Ethnomedicinal studies reported the use of the plant for treatment of several conditions in West Africa (Mshana, 2000). Other studies have also reported on the antimicrobial effects of cryptolepine (Kirimuhuzya *et al.*, 2012; Paulo *et al.*, 2000).

A study by Olajide *et al* (2009) showed that cryptolepine exerted anti-inflammatory property *in vivo* by inhibiting carrageenan-induced rat paw oedema. Another study showed that, synthetic cryptolepine hydrochloride, was able to suppress the level of nitric oxide produced in lipopolysaccharide stimulated macrophages (Olajide *et al.*, 2007). These reports add weight to the potential effect of cryptolepine on suppressing inflammation by inhibiting signaling pathways that mediate inflammatory processes.

It was against this backdrop that this study was designed to further explore the mechanisms by which cryptolepine suppresses inflammation and determine its specific molecular targets. The outcome of this study can shape strategies for development of novel analogues of cryptolepine with potent anti-inflammatory therapeutic properties and minimal side effects. Data generated from these investigations will also add to the body of knowledge on therapeutic targets of anti-inflammatory agents.

1.2 Problem statement and justification

Chronic inflammatory diseases are among the factors that impact negatively on the socioeconomic lives of patients. The diseases have high costs for treatment and care, cause life-long debilitating illness and increased mortality (Straub & Schradin, 2016). In addition, chronic inflammatory diseases result in low productivity of patients at work and affect their general quality of life (Jacobs *et al.*, 2011). Also, anti-inflammatory drugs such as cyclooxygenase 2 (COX-2) inhibitors and non-steroidal anti-inflammatory drugs (NSAIDs), have been shown to pose severe health challenges. NSAIDs, at the effective drug concentrations, are known to cause severe gastrointestinal toxicities including causing bleeding and perforations (Dinarello, 2010; Tseng & Wolfe, 2000). Although, COX-2 inhibitors posed minimal gastrointestinal toxicities, their usage is hampered by other health challenges including risk of myocardial infarction and other vascular diseases (Gislason *et al.*, 2006). These challenges, therefore, warrant the need for new anti-inflammatory drugs with potent activities and minimal toxicity.

Although cryptolepine has been shown in *in vivo* studies to possess anti-inflammatory activities, the underlying mechanisms of its actions are not fully understood (Olajide *et al.*, 2009). Furthermore, given that several other studies have reported on some level of toxicity of cryptolepine in some cell lines (Ansah and Gooderham (2002); Pal *et al.*, (2017)), it is imperative to determine the optimal non-toxic concentration of cryptolepine with potent anti-inflammatory activity in inflammatory cells like macrophages which play central roles in inflammation. This study will, therefore, determine the optimal nontoxic concentration of cryptolepine, explore and shed more light on its molecular targets of anti-inflammatory actions in mouse macrophages. Results of this study will provide evidence supporting the potential usage of cryptolepine in

clinical trials as a candidate anti-inflammatory drug. Findings from this study will also help in the design of novel analogues of cryptolepine with possible higher efficacy and lower toxicity.

1.3 Hypotheses:

1. Cryptolepine suppresses the activity of the TLR1/TLR2-NF-κB signaling pathway.
2. Cryptolepine downregulates the expression of NF-κB target genes in the TLR1/TLR2-NF-κB signaling pathway.

1.4 General aim:

To determine the molecular mechanisms of anti-inflammatory action of cryptolepine.

1.5 Specific objectives:

1. To determine the cytotoxic effect of cryptolepine *in vitro* by MTT assay.
2. To determine the effect of cryptolepine on the general activity of the TLR1/TLR2-NF-κB signaling pathway *in vitro* by a reporter gene assay.
3. To determine the effect of cryptolepine on expression of TNF-α, IL-6, IL-1β, IL-23, MIP-2α, MCP-1, IKKβ, NFκB1, RelA, TLR2 and TLR1 genes *in vitro* by RT-qPCR.
4. To determine the effect of cryptolepine on the protein levels of TNF-α, IL-6, IL-1β, IL-23, MIP-2α and MCP-1 *in vitro* by multiplex ELISA.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Inflammation

Inflammation is defined as a pervasive but regulated immune response to disrupted tissue homeostasis that involves release of soluble plasma proteins, recruitment of leukocytes and fluid, clearance of damage and resolution (Medzhitov, 2008; Ashley *et al.*, 2012).

Although most inflammatory responses are triggered as a result of infections by microbial pathogens, physical tissue damage and contact with foreign particles can also activate inflammation (Medzhitov, 2008). Since both infections and wound cause damage to cells and tissues, it is understandable why they trigger similar inflammatory responses (Bianchi, 2007). It is therefore probable that inflammation evolved as a general adaptation for handling damaged or malfunctioning tissues (Matzinger, 2002).

The mechanism that underlies classical inflammatory process involves four main stages; (1) recognition of infection or damage and activation of signaling pathways, (2) production of proinflammatory mediators and recruitment of inflammatory cells (3) clearance of threat and (4) resolution of inflammation (D'Elia *et al.*, 2013).

2.1.2 Types of Inflammatory Cells

Recognition of infection or damage is the first step of the inflammatory process. It is carried out by the innate immune cells which detect a broad range of molecular structures mostly found on pathogens but foreign to mammals, referred to as pathogen associated molecular patterns (PAMPS). The cells also detect endogenous molecules, termed as damage-associated molecular patterns (DAMPS), which signal damage or necrosis (Medzhitov, 2008). Although macrophages,

granulocytes and dendritic cells (DCs) are the main cells that coordinate innate immune responses, others including epithelial cells and endothelial cells also serve significant roles (Takeuchi & Akira, 2010).

2.1.3 The central roles of macrophages in inflammatory responses

Macrophages are multipurpose large leukocytes that serve important functions in all stages of inflammation (Delavary *et al.*, 2011). They are found in many tissues where they act as sentinels against infectious agents and tissue damage (Laskin, *et al.*, 2011).

Majority of macrophages develop from monocytes that develop from precursor stem cells as a result of exposure to granulocyte macrophage colony stimulating factor (GMCSF) (Sieweke & Allen, 2013). Upon differentiating into monocytes, the cells remain in the bone marrow for less than 24 hours after which they migrate into the bloodstream and pass throughout the body (Sieweke & Allen, 2013). Some of the monocytes cross the walls of the capillaries into connective tissue and differentiate into macrophages through cellular processes that include increase in size, increase in number and complexity of organelles and increase in phagocytic capacity (Sieweke & Allen, 2013). However, it is worth noting that, this contribution of monocytes to the macrophage population is only minimal. Recent studies have shown that most tissue resident macrophages do not develop from bloodstream monocytes but are rather derived from embryonic progenitors before birth. The embryonic precursors are able to maintain the macrophage pool in adulthood through self-renewal (Sieweke & Allen, 2013).

Macrophages play essential roles in all stages of inflammation. They are mostly at rest in all tissues but act early against any foreign invader. Upon sensing damage, either caused by an invading pathogen or its product, macrophages become activated and secrete many pro-inflammatory

mediators (Laskin *et al.*, 2011). Aside from the secretion of the pro-inflammatory mediators, the activated macrophages also carry out rapid phagocytosis of the foreign agents, engulf and clear apoptotic cells (Laskin *et al.*, 2011)

2.1.4 Detection of damage and inflammatory signaling mechanisms

Macrophages and all the other cells of the innate immunity detect PAMPs and DAMPs using their germline-encoded pattern recognition receptors (PRRs) (Akira *et al.*, 2006). These receptors recognize molecules such as glycoproteins, proteoglycans, nucleic acid motifs and lipopolysaccharides that are commonly found on many pathogens and play crucial roles in their survival or pathogenecity (Jensen & Thomsen, 2012).

Upon sensing PAMPs, the PRRs, with the exception of some NLRs, activate intracellular signaling (Akira *et al.*, 2006). The genes expressed result in the production of many molecules such as cytokines, immunoreceptors, cell adhesion molecules, immunoreceptors, antimicrobial proteins, type I interferons (IFNs) and protein modulators of PRR signaling (Akira *et al.*, 2006; Takeuchi and Akira, 2010). The patterns of genes expressed differ from one PRR to the other.

2.1.5 Roles of TLRs as PRRs

The TLR family is a well-characterized PRR family that detects extracellular microbial invaders as well as intracellular microbes in lysosomes and endosomes (Akira *et al.*, 2006). The TLRs characterized by a transmembrane or luminal ligand binding domain consisting of N-terminal leucine-rich repeats (LRR) motifs and a cytoplasmic Toll/interleukin-1 (IL-1) receptor homology (TIR) domain that relays intracellular signal (O'Neill & Bowie, 2007). Humans contain ten TLRs with each TLR capable of recognizing distinct molecular patterns from microbes and self-components (Akira *et al.*, 2006). Unlike TLR3, TLR7, TLR8, TLR9 and TLR10 which are known

to be localized in endolysosomes, TLR1, TLR2, TLR4, TLR5 and TLR6 are localized on the plasma membrane as transmemberane receptors (Figure 2.1) (Akira *et al.*, 2006). The TLRs have also been categorized into sub-groups according to the PAMPs they recognize; those that recognize lipids including TLR1, TLR2, TLR4 and TLR6 and those that detect nucleic acids including TLR3, TLR7, TLR8 and TLR9 (Takeuchi & Akira, 2010).

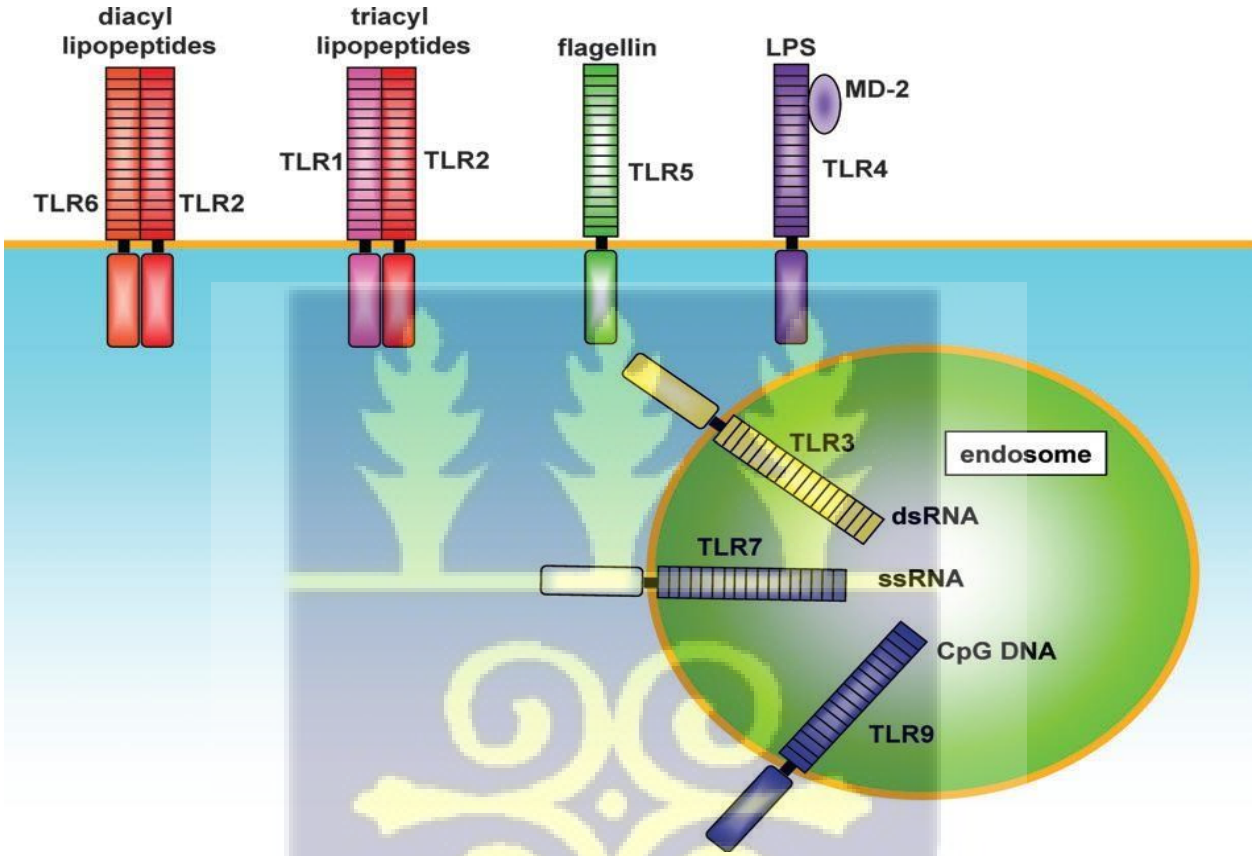

Fig. 2.1: Localization of TLRs and the types of molecules they recognize. TLR1, TLR2, TLR3, TLR5 and TLR6 are localized on the plasma membrane whereas TLR3, TLR7 and TLR9 are found on the endosome. TLR1, TLR2, TLR4 and TLR6 recognize various kinds of microbial lipids whiles TLR3, TLR7 and TLR9 recognize viral dsRNA, ssRNA and CpG DNA respectively. Source: Takeda and Akira (2005)

With respect to those that recognize lipids, TLR1 cooperates with TLR2 in recognizing triacyl lipopeptides present on bacteria whereas TLR6 cooperates with TLR2 in sensing diacyl lipopeptides found mainly on bacteria and viruses (Takeda & Akira, 2005). In contrast, TLR3 detects viral-derived double-stranded (ds) RNA and TLR9 is implicated in recognition of DNA found in the genetic make-up of bacteria, viruses and protozoa (Takeuchi & Akira, 2010). TLR5 is the receptor for flagellin present on bacteria whereas TLR4 recognizes lipopolysaccharide (LPS) on viruses and bacteria (Takeuchi and Akira, 2010). Binding of the relevant PAMPs to the TLRs leads to intracellular downstream signaling that ultimately results in production of inflammatory cytokines and antimicrobial response (Takeuchi & Akira, 2010).

2.1.6 Signaling pathways through TLRs

Depending on the cell type involved, binding of PAMPs to TLRs activates signaling pathways resulting in unique gene expression patterns. There are some differences among the molecules recruited in downstream signaling cascades activated by the individual TLRs (Akira, *et al.*, 2006). Five main adaptor molecules exist which are normally associated with signaling through the TLRs; MyD88, TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM) (Takeuchi & Akira, 2010).

Depending on the type of the molecule used, the pathways are grouped into two; MyD88 dependent pathway and MyD88-independent (TRIF) pathway.

2.1.7 Signaling through the pathway that uses MYD88

All the TLRs but TLR3 require MyD88 for their downstream signaling (Takeuchi & Akira, 2010). Also, the types of genes expressed through the pathway differ from one set of TLRs to another. In

response to TLR2, TLR4 and TLR5 stimulation, MyD88 drives expression of inflammatory genes whereas in TLR7- and TLR9-induced signaling, which occur primarily in plasmacytoid dendritic cells (pDCs), MyD88 mediates release of type I IFN (Mogensen, 2009). In the signaling pathway via TLR4, another protein TIRAP/Mal is used for bridging between the particular TLR and MyD88 (Takeuchi & Akira, 2010). Binding of an appropriate PAMP to a particular TLR results in recruitment of IL-1R-associated kinase (IRAK)-4 which activates IRAK1 and IRAK2 (Suzuki *et al*., 2002). After its activation, IRAK1 undergoes autophosphorylation at several sites and binds to tumor necrosis factor receptor-associated factor 6 (TRAF6), which in turn activates transforming growth factor-activated protein kinase 1 (TAK1) (Xia et al., 2009). TAK1 belongs to the MAPKKK family (Kanayama *et al*., 2004). Activation of TAK1 serves as a critical junction that leads to stimulation of two distinct pathways; IKK complex-NF-κB pathway and the MAPK pathway that results in AP-1 activation (Kawasaki & Kawai, 2014).

Activated TAK1 in turn activates IKK-α, IKK-β and IKK-γ (Kawasaki & Kawai, 2014). In the resting state, NF-κB is bound by IκB (Figure 2.2). The activated IKK complex phosphorylates IκB making it separate from NF-κB (Häcker & Karin, 2006). The free NF-κB then migrates into the nucleus (Figure 2.2) to induce gene expression of pro-inflammatory mediators (Häcker & Karin, 2006).

In the second pathway, TAK1 activates the MAPK pathway by phosphorylating MAPK kinase (MKK) family members, such as MKK4, MKK6 and MKK7 (Chang & Karin, 2001). Phosphorylated MKK3 and MKK6 (also called SKK) phosphorylate and activate p38 MAPK. These two pathways ultimately trigger the activation of AP-1, another transcription factor that induces expression of cytokine genes (Chang & Karin, 2001).

Signaling through TLR7 and TLR9, which occurs in pDCs, a subset of dendritic cells which produce large amount of type I IFN as an antiviral response is also MyD88-dependent (Kawai and Akira, 2006). Upon viral stimulation, TLR7 and TLR9 associate with MyD88 which in turn binds IRAK-1, IRAK-4, TRAF3, TRAF6, IKK-α, and the transcription factor interferon regulatory factor 7(IRF7) to form a complex (Takeuchi & Akira, 2010). In this complex, IRAK-1 and or IKK-α phosphorylate IRF7 which migrates into the nucleus to induce type I IFN gene expression (Kawai & Akira, 2006). In pDCs, stimulation of TLR7 and TLR9 results in activation of IRF1 but not IRF7. The activated IRF1 translocates into the nucleus to activate gene induction of IFN (Negishi *et al.*, 2006; Schmitz *et al.*, 2007).

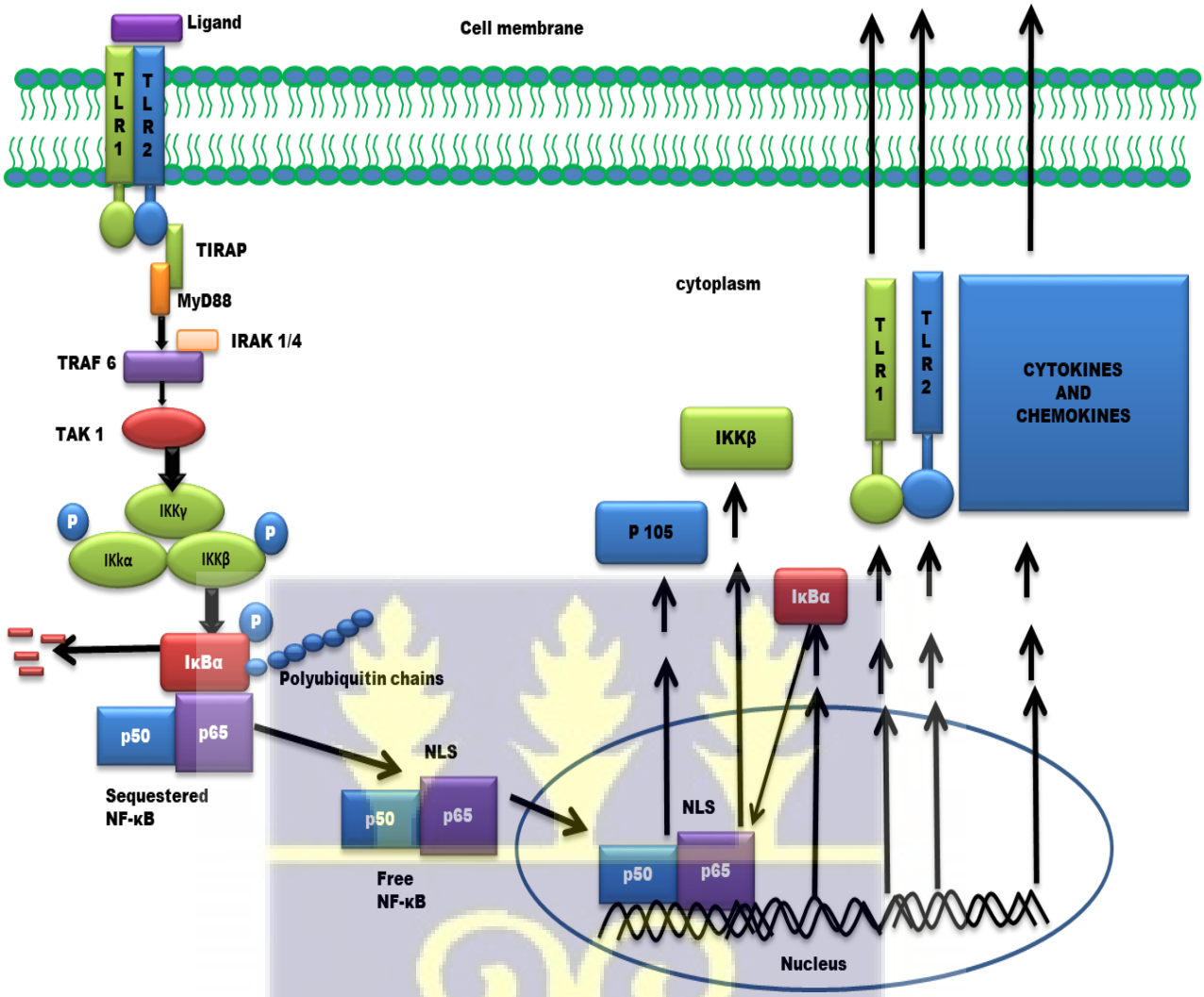

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Fig. 2.2: Model mechanism of activation of the TLR1/TLR2 signaling pathway. In unstimulated state, NF-κB is sequestered in the cytoplasm by its inhibitor IκBα. Upon binding of an appropriate ligand, such as Pam3CSK4, to the TLR1-TLR2 heterodimeric receptor, the pathway gets activated. The activated receptor recruits the adaptor molecules TIRAP and MyD88, whereby the latter activates IRAK4. The actions of IRAK4 finally lead to the activation of TRAF6 which in turn activates TAK1. Activated TAK1 phosphorylates IKKβ which becomes activated to phosphorylate IκBα resulting in its polyubiquitination and subsequent degradation, thereby freeing NF-κB to translocate into the nucleus and induce transcription of its target genes. NF-κB induces expression of pro-inflammatory cytokines, chemokines, TLR1, TLR2, IKKβ, p105 as well as its cytoplasmic inhibitor, IκBα. Newly synthesized IκBα enters the nucleus, binds to NF-κB, dislodges it from the DNA and exports it back into the cytoplasm thereby terminating the transcriptional activity of NF-κB. Adapted from Takeda and Akira (2005) and modified.

2.1.8 Signaling through pathway that uses TRIF

This pathway is used by TLR3 and TLR4. TLR3 recognizes viral dsRNA. This results in expression of IFN (type I) and pro-inflammatory cytokines .When TLR3 is activated by dsRNA, it attracts TRIF which in turn associates with TRAF3 and TRAF6, the latter two proteins leading to polarization of the signal via two different routes which activate IRF3 and AP-1, respectively (Takeuchi & Akira, 2010). To activate IRF3, TRAF3 forms a complex with some other proteins which are required for TBK1/IKK-I activation (Guo & Cheng, 2007; Ryzhakov & Randow, 2007; Sasai *et al*., 2006). Activated IKK-I then phosphorylate IRF3 which migrates into the nucleus to activate IFN (type I) gene expression (Mogensen, 2009). In the route that activates AP-1, another protein, receptor interacting protein 1 (RIP1) is polyubiquinated to associate with TRAF6, which has an E3 ubiquitin ligase activity (Takeuchi and Akira, 2010). The downstream activities from TRAF6 are similar to what happen in the MyD88-dependent pathway explained above.

2.1.9 The role of NF-κB signaling pathway in inflammation

NF-κB was initially found in B lymphocytes to regulate the expression of the kappa light-chain gene (Sen & Baltimore, 1986). Subsequently, further research showed that it is ubiquitous in mammalian cells (Oeckinghaus & Ghosh, 2009).

In mammalian cells, proteins including RelA, NF-κB1 (p105/p50), NF-κB2 (p100/p52), Rel B and c-Rel are closely related to NF-κB (Fig. 2.3). They function by forming distinct homo and heterodimers. Each of the proteins is made up of Rel Homology domain (RHD) at its N-terminus (Rothwarf & Karin, 1999). The RHD is used by the proteins for forming dimers, nuclear translocation, binding to DNA and binding to IκB (Chen & Greene, 2004). Among all the proteins, only RelA, c-Rel and RelB possess structures known as transcription activation domains (TADs) at their C-terminal regions (Ghosh *et al.,* 1998). The TADs are responsible for binding specific DNA sequences in the NF-κB binding sites known as κB elements, to promote gene transcription

(Ghosh *et al.*, 1998). The other two family members; p50 and p52 lack the TADs and so their homodimers are incapable of activating gene transcription (May & Ghosh, 1997). They are known to rather act as transcriptional repressors. However, when they form heterodimers with the TAD-containing proteins, they become transcriptional activators. The p50 and p52 are breakdown products of p105 and p100 respectively (Silverman & Maniatis, 2001).

The predominant form of the transcription factor is the p65-p50 heterodimer (Silverman & Maniatis, 2001). The NF-κB proteins also contain a nuclear localization signal (NLS) sequence which is required by the transcription factor for translocation into the nucleus (Chen & Greene, 2004).

NF-κB is normally found in inactive state through its interaction with any of the typical IκB family members; IκBα, IκBβ, IκBγ, IκBε, p105 and p100. Two additional members, namely Bcl3 and IκBζ are expressed upon stimulation (Hayden & Ghosh, 2004). When the cells are stimulated, the signaling cascade eventually culminates into the IKKβ-mediated phosphorylation of IκBα which then becomes tagged for proteolytic degradation by the 26S proteosome thereby freeing NF-κB to migrate into the nucleus (Karin & Ben-Neriah, 2000).

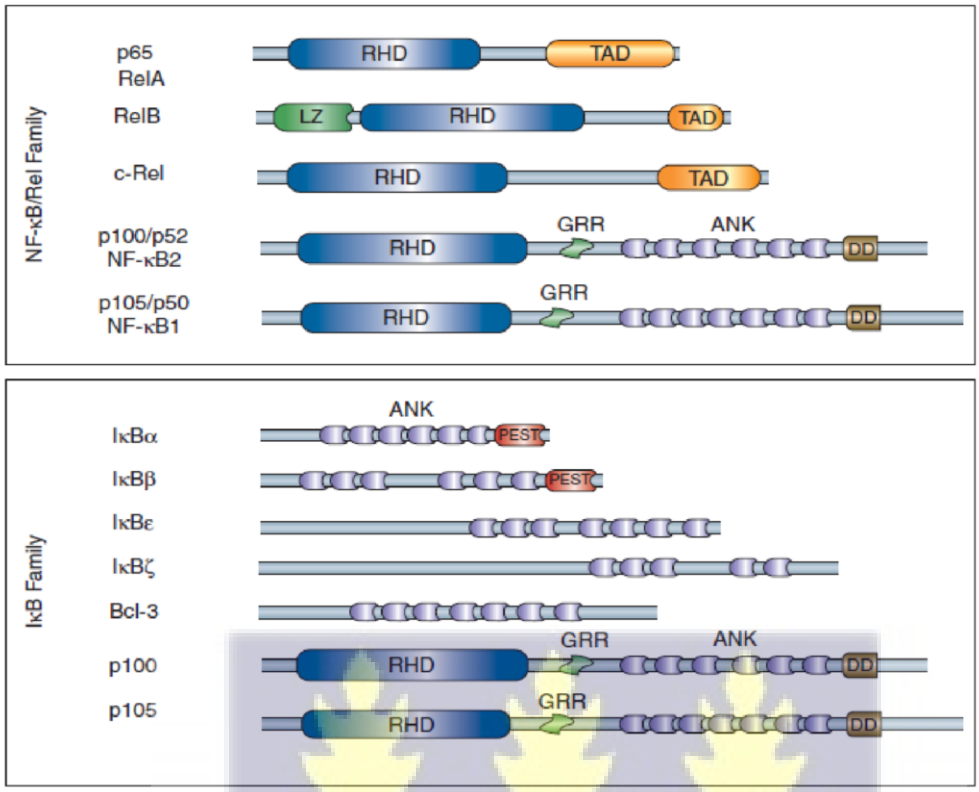

Fig. 2.3: NF-κB and IκB protein family members. The top figure shows the NF-κB family containing RHD, a characteristic feature of all the members of that family. In addition to RHD, RelA, c-Rel and RelB also contain TAD at their c-terminal regions. The bottom figure shows the IκB proteins containing their characteristic ankyrin repeats (ANK). The other domains in the proteins are: leucine-zipper (LZ), coiled-coil (CC); glycine-rich region (GRR); death domain (DD);proline-, glutamic acid-, serine-, and threonine-rich region (PEST). Source: Oeckinghaus and Ghosh (2009).

2.2.0 Nuclear activities of NF-κB

When NF-κB moves into the nucleus, it attaches to a consensus DNA sequence found in the regulatory elements of its target genes, known as the κ-B binding site 5'-GGGRNWYYCC-3' (where R represents any purine, N is any nucleotide, Y stands for any pyrimidine and W is adenine or thymidine) (Hoffmann *et al.*, 2006). This, however, is not enough to elicit gene transcription. In fact, many other factors contribute to the transcriptional activity of NF-κB, including nuclear proteins, coregulators, general transcription factors and chromatin modifying complexes (O'shea & Perkins, 2008).

There is strong evidence from a number of genome wide analyses that give indication to the presence of other non-Rel proteins as integral components of the NF-κB complex. An example is ribosomal protein S3 (RPS3) (Figure 2.4), which was found in certain NF-κB complexes and was shown to play an essential role in recruiting Rel A (p65) to promoters of specific gene *(*Wan *et al*., 2007). Proteomic analysis indicated that RPS3 associates with p65 (Wan *et al*., 2007). Furthermore, RPS3 is believed to be a very important subunit of NF-κB complex and not a coactivator in that, it is found in resting cells associated with the p65-p50-IκB complex and upon induction with diverse stimuli, it moves across the nuclear membrane and binds to κB sequences in many NF-κB target genes (Wan *et al*., 2007).

Examples of the nuclear proteins are members of the highly conserved Akirin protein family, which are nuclear proteins that contribute to the NF-κB-mediated gene transcription in mice and drosophila (Goto *et al*., 2007). In Drosophila, the NF-κB ortholog, Relish, requires Akirin for induction of responses against Gram-negative bacteria (Goto *et al*., 2007). Akirin is also present in both human and mouse genomes as two homologues (Goto *et al*., 2007). Goto and colleagues

(2007) further observed that although Akirin-1 knockout mice showed no apparent phenotype, Akirin-2 knockout mice were embryonic lethal. The role of Akirin-2 was further confirmed in Akirin-2-deficient mouse embryonic fibroblasts which failed to upregulate expression of IL-6 upon induction by several ligands (Goto *et al*., 2007). Although the precise role played by Akirin-2 in the NF-κB-mediated gene transcription is still unclear, it is thought that it binds to a molecule that in turn associates with NF-κB, and this impacts on interaction with DNA *(Goto et al*., 2007).

There are other nuclear proteins that contribute to the NF-κB-mediated gene transcription and this include protein kinase A-interacting protein 1 (AKIP1) which keeps Rel A (p65) in the nucleus and promotes its phosphorylation (Gao *et al.*, 2008). Essentially, AKIP1 strengthens the assembly of Rel A with the coactivator, CBP/p300, thereby enhancing target DNA binding by NF-κB.

2.2.1 The roles of chromatin modulation and co-regulators in gene transcription regulated by NF-κB

In order for transcription to take place, the chromatin microenvironment, which poses a steric barrier to DNA binding by NF-κB, has to be altered to allow easy access to the DNA (Bhatt & Ghosh, 2014). The DNA of eukaryotic organisms is highly condensed by its wrapping around histone proteins to form a DNA: histone complex that is termed as the nucleosome, the core repeating unit that forms the chromatin. This strategy allows for large genomes to be compacted into a nuclear volume of a relatively smaller size. Each nucleosome is formed by a complex of 147 bp of DNA that wraps around an octamer.

The extent to which chromatin fibres are compacted plays a major role in making cognate binding sites accessible to DNA-binding proteins (Bhatt & Ghosh, 2014). Thus, modifications of chromatin to ease accessibility of cognate binding sites to DNA-binding proteins are critical

processes for transcription. Accordingly, the tails of histone proteins in chromatin undergo several post-translational modifications that in effect alter chromatin biology. The two most well studied histone modifications that influence transcription are lysine acetylation and methylation (Bhatt & Ghosh, 2014). Histone acetyltransferases such as the p300/CBP complex are the enzymes add acetyl groups to lysine residues on histone proteins during active transcription (Bhatt & Ghosh, 2014). In contrast, removal of the acetyl groups is catalysed by histone deacetylases. Lysine methylation has been shown to activate or repress transcription, based on the specific lysine residue that is methylated (Bhatt & Ghosh, 2014). For instance, H3-Me3K4, a trimethylated histone 3 at lysine 4, is abundant at promoters of active genes where there is active transcription while H3K9, histone 3 methylated at lysine 9, is associated with repression of transcription (Bannister & Kouzarides, 2011).

A number of co-regulators of the nuclear NF-κB signaling have been identified. An example of co-regulator acting as an activator is astrocyte elevated gene-1 (AEG-1), a cytoplasmic protein which upon TNF-α stimulation, was observed to interact with p65 and CBP, thus bridging them with the transcriptional machinery complex at the IL-8 gene promoter (Sarkar *et al*., 2008).

Another protein, inhibitor of growth 4 (ING4), which belongs to the ING tumour suppressor family, including ING 1, ING2, ING3 and ING5 (Gong *et al*., 2005; He *et al*., 2004), was found to co-repress the transactivation of NF-κB in malignant glioma tissues (Nozell *et al*., 2008). Structural studies have shown that, the ING proteins harbour a plant homeodomain (PHD) at their C-termini (He *et al*., 2004), which they use for recognition and binding to H3-Me3K4, a protein that is associated with transcriptionally active DNA (Martin *et al*., 2006). In the study on glioma tissues, Nozell and colleagues (2008) found that ING4 interacts with NF-κB at the

promoters of NF-κB-regulated genes and that this interaction does not abrogate binding to DNA or transactivation. Instead, interaction of ING4 with NF-κB was found to reduce phosphorylation of p65 and recruitment of p300. The changes in NF-κB-mediated activities caused by ING-4 are thought to lower expressions of genes regulated by NF-κB. To confirm the co-repressive role of ING-4 on expressions of NF-κB-regulated genes, Nozell and colleagues (2008) used a knock-down approach to show that reductions in the levels of ING4-protien correlated with increased expressions of *COX-2* and *MMP-9.* In a related study, ING-4-null mice were shown to be hypersensitive to LPS stimulation, which shows that ING-4 plays a negative regulatory role in innate immunity (Coles *et al.*, 2010). Furthermore, ING-4 was shown to reduce the levels of some cytokines in mice stimulated with LPS. The negative regulatory role of ING-4 on the production of the select cytokines was shown to be mediated by its enhancement of Rel A activation of I-κBα promoter.

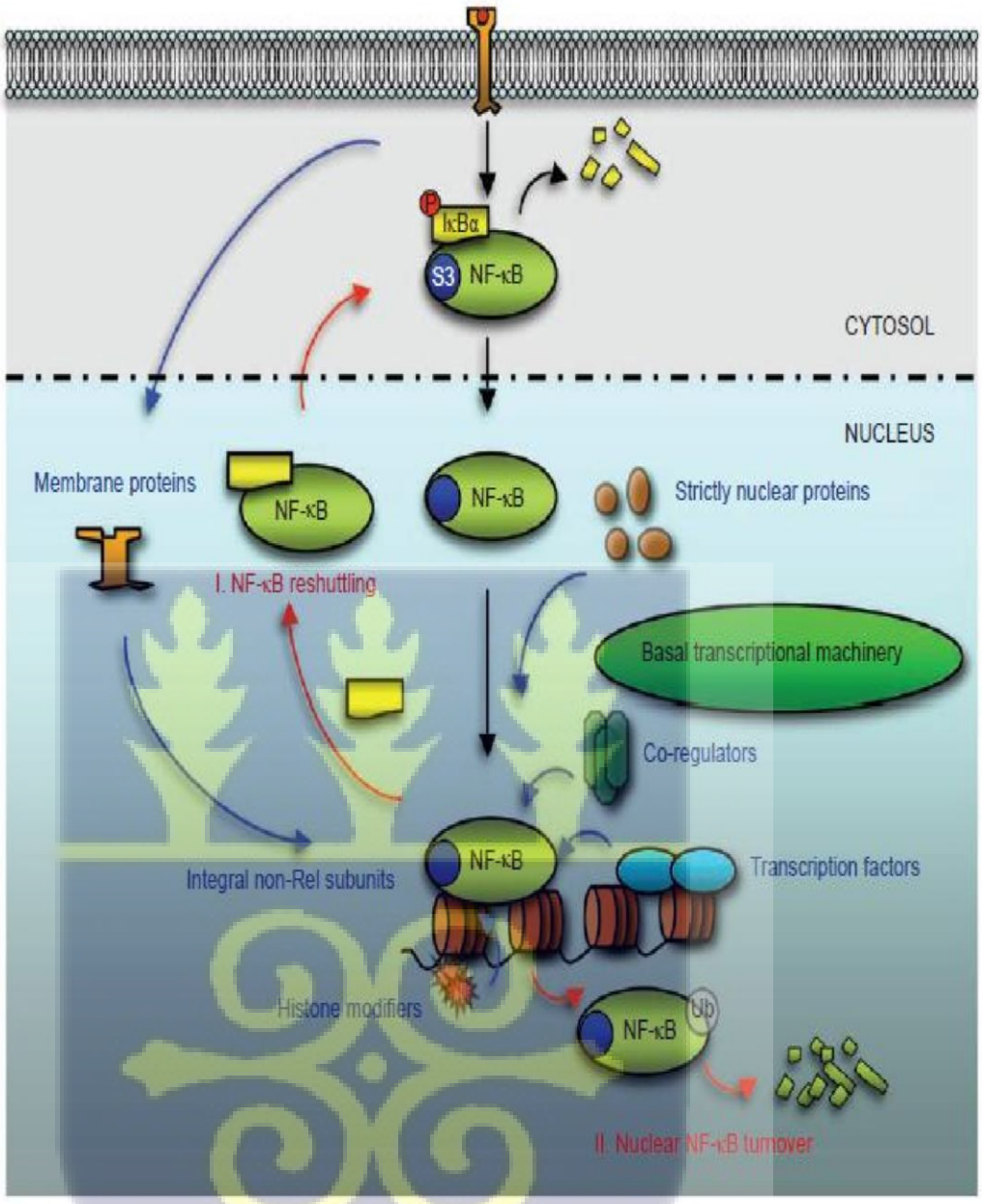

Fig. 2.4: NF-κB regulation in the nucleus. Inside the nucleus, NF-κB binds to DNA and induces gene transcription. NF-κB transcriptional activity is regulated by many factors including NF-κB – associated non-Rel subunits such as RPS3 (S3), nucleus-restricted proteins, chromatin modifiers and other transcription factors. Two mechanisms of termination of NF-κB transactivation, shown in red, are transport of NF-κB to the cytoplasm by I κB and proteolytic degradation by ubiquitination. Source: Wan *et al.*, (2007).

2.2.2 The roles of post-translational modifications in NF-κB-mediated gene transcription

Many studies have shown that post-translational modifications of the subunits of NF-κB play critical roles in NF-κB-mediated gene transcription. Particularly, post-translational modifications of the Rel A subunit, including phosphorylation and acetylation are said to play major roles in the transactivation of NF-κB and have been studied extensively.

RelA phosphorylation at Serine-529 and Serine-536 has been shown to induce its transactivation (Vermeulen, *et al.*, 2003; Viatour *et al.*, 2005). Phosphorylation of RelA within the TA2 subdomain at Ser-468, has been shown to be catalyzed by various kinases including IKKε and described as both RelA activating and repressing (Buss *et al.*, 2004). Upon stimulation of cells, RelA is phosphorylated at Ser-279 making it preferentially bind to CBP/p300 to form a RelA-CBP/p300 complex which in turn dislodges the p50-HDAC1 complex from the DNA and activate NF-κB-mediated gene transcription (Zhong *et al.*, 2002).

RelA also undergoes acetylation at many residues which result in differential effects on its activation. Acetylation of RelA increases its DNA binding and together with acetylation at Lys218, prevents export of NF-κB to the cytoplasm by inhibiting RelA association with newly synthesized IκBα (Chen *et al.*, 2002). Acetylation at Lys-310 induces transactivation of RelA (Chen *et al.*, 2002).

2.2.3 Genes regulated by NF-κB

NF-κB is a powerful transcription factor that regulates transcriptions of hundreds of genes that play significant roles in inflammation as well as other physiological activities such as immune response, cell survival and proliferation (Yang *et al.*, 2016). The various genes whose expressions are regulated by NF-κB are shown in Figure 2.5

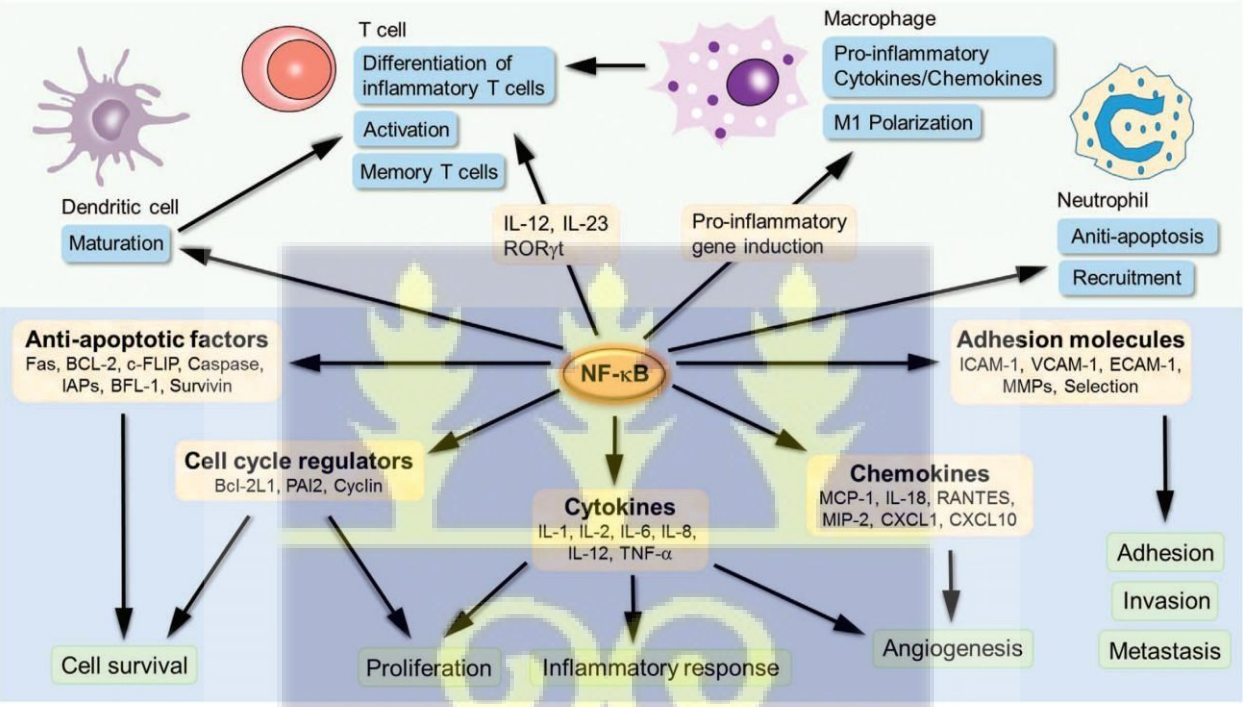

Fig. 2.5: Genes regulated by NF-κB. Activated NF-κB induces expression of its target genes which then mediate various cellular processes including inflammation, immune responses, angiogenesis, cell survival and proliferation. Source: Liu *et al* (2017).

In inflammatory conditions, stimulation of inflammatory cells, mainly macrophages, leads to activation of NF-κB which induces expression of pro-inflammatory mediators such as cytokines, chemokines, leukotrienes and immune receptors (Tak & Firestein, 2001). Interestingly, NF-κB also activates transcription of the gene of its cytoplasmic negative regulator IκBα which when

expressed, shuttles into the nucleus to stop NF-κB from further induction of transcription (Hayden & Ghosh, 2004).

Many studies have shown that transcription of genes regulated by NF-κB follows a pattern whereby some genes are transcribed shortly after its translocation into the nucleus whereas others take a few to several hours for their transcription to occur (Zhou *et al.*, 2003; Tian *et al.*, 2005). According to Tian and colleagues (2005), this transcriptional pattern has led to the categorization of the target genes into 'Early', 'Middle' and 'Late' genes which peak 1, 3 and 6 hours respectively, post stimulation with TNF-α (Tian et al., 2005)(Tian *et al*., 2005).

In a related study, Zhou *et al* (2003) showed that stimulation of Hela cells with TNF-α produced a pattern of gene expression where a number of NF-κB-regulated genes were significantly upregulated thirty (30) minutes after stimulation whereas others were upregulated only after four (4) hours of stimulation. Zhou *et al* (2003) noted that some of the genes that were upregulated at 30 minutes of stimulation, including cytokines such as IL-6 and GRO-1, had their expressions declining sharply at 4 hours of stimulation. Expression of other genes including A20, IκBα and COX-2 also peaked at 30 minutes but showed only a little decline at 4 hours. In contrast, expression of other genes including MCP-1 and PGES increased slowly and peaked at 4 hours.

Although many other studies have reported similar segregation in timing of induction of genes regulated by NF-κB, the mechanism that is responsible for this observation is not fully understood. There are some hypotheses that have been proposed to explain the phenomenon. For example Iwanaszko and colleagues (2012) suggested that the transcriptional dynamics may be due to the number and nature of transcription factor binding sites (TFBS) in the promoters of the early genes compared to those in the late genes. Their study found that, the 'Early' genes contained

significantly higher NF-κB binding sites in evolutionarily conserved domains compared to the 'Late' genes, suggesting that the rapid expression of the 'Early' genes may be attributed to enhanced gene transcription as a result of NF-κB loading. Another factor is the presence of AU-rich elements (ARE) in 3′UTR of the transcript of the genes. 'Early' genes were found containing more ARE sequences in the 3′UTR of their mRNAs than the 'Late' genes (Iwanaszko *et al.*, 2012).

2.2.4 Termination of NF-κB signaling

Because of the large number of target genes whose induction is mediated by NF-κB and the wide range of immunological and physiologic impacts they exert, control of the pathway activity is necessary to prevent overexpression of the genes which can have deleterious health consequences. A number of mechanisms by which the cell terminates the NF-κB response exist including the negative feedback regulation mediated by the newly synthesized IκBα.

The IκBα-mediated control regulation is the best studied mode for termination of the NF-κBmediated transcription. When activated NF-κB migrates into the nucleus, one of the early genes it mediates their transcription is the *NFKBIA* gene. The gene encodes the IκBα protein (Hayden & Ghosh, 2004). When produced, IκBα migrates into the nucleus, binds to NF-κB, dislodges it from the DNA and exports it back into the cytoplasm thereby terminating the NF-κB activation (Hayden & Ghosh, 2004).

Termination of NF-κB response by post-translational modifications includes phosphorylation, acetylation, nitrosylation, oxidation, alkylation and nitration of key residues on the subunits. For example, RelA turnover has been shown to be increased when it is phosphorylated at Ser-536 by IKKα (Lawrence & Karin, 2005). Another NF-κB subunit, Rel B has also been demonstrated to undergo degradative turnover when phosphorylated at Thr-84/Ser-552, and this occurs under T cell receptor (TCR) induction (Marienfeld *et al.*, 2001). Acetylation of RelA at lysine-rich residues including Lys-122 and

Lys-123 was said to decrease its binding affinity to target DNA (Kiernan *et al*., 2003). A study by Mathews and colleagues (1996) has shown that posttranslational modification by nitrosylation involves nitric-oxide-mediated S-nitrosylation of Cys62 residue of p50 which inhibits its affinity to DNA. Furthermore, modifications of c-Rel by Cys-27 oxidation and alkylation inhibit its phosphorylation and this affects its DNA-binding capacity (Glineur *et al.*, 2000). Finally, Park *et al.*, (2005) also demonstrated that, RelA undergoes nitration at Tyr-66 and Tyr-152 leading to its separation from p50, enhancing its interaction with IκBα and subsequent export to the cytoplasm.

Another way by which the NF-κB-mediated transcription is terminated is via polyubiquitination and subsequent proteosomal proteolysis of p65. Ryo *et al.*, (2003) reported that the ubiquitin ligase, suppressor of cell signalling 1(SOC-1) mediates such p65 proteolysis.

2.2.5. Roles of NF-κB and its regulated mediators in inflammatory diseases

Regulation of the activity of NF-κB is essential in keeping NF-κB signaling in a controlled manner and preventing its dysregulation. Dysregulated NF-κB signaling resulting from hyperactivated NF-κB or loss-of-function of its negative regulators is reported to cause many diseases including cancers and insulin resistance (Wong & Tergaonkar, 2009). Particularly, many studies have reported on chronic NF-κB activity in inflammatory diseases including, sepsis, inflammatory bowel's disease (IBD), asthma, arthritis, gastritis and atherosclerosis.

In arthritis, NF-κB has been shown in many studies using both murine models and humans samples, to be a key player in the inflammatory process and disease progression. For instance, in a murine model of collagen induced arthritis, NF-κB binding was found to be highly activated in synovial tissues (Han *et al.*, 1998). For instance, NF-κB was found to be highly activated in rat models of arthritis (Miagkov *et al.*, 1998). Furthermore, in an experimentally induced arthritis study

with mice which were knockouts for NF-κB subunits p50 or c-Rel, it was shown that absence of c-Rel had no effect on one of the disease models whereas p50 deficiency completely inhibited humoral response and made mice resistant to the induced arthritis. This confirms that the p50 subunit, presumably in dimeric association with p65, plays a key role in the underlying inflammatory process in arthritis (Campbell *et al.*, 2000). In many studies on human patients of rheumatoid arthritis (RA), NF-κB was found to be highly activated in synovial tissues (Asahara *et al.*, 1995; Marok *et al.*, 1996), indicating the major contribution of the transcription factor in the pathogenesis of the disease.

Several studies have demonstrated the crucial roles that NF-κB plays in the pathogenesis and pathophysiology of IBD. For instance, studies by Rogler *et al.*, (1998) and Schreiber *et al* (1998) found NF-κB to be constitutively activated in inflamed colonic tissue samples of IBD patients. NF-κB was also found to be highly expressed and activated in mucosal macrophages of IBD patients, and the severity of the inflammatory condition correlating significantly with the level of NF-κB activation (Rogler *et al.*, 1998). This increased activation of NF-κB in mucosal macrophages has been shown to be linked with higher production of pro-inflammatory cytokines, which cause extensive damage to mucosal tissue (Pallone & Monteleone, 2001). Furthermore, genetic mutations and polymorphism in some key molecules in the NF-κB signaling pathway have been shown to be associated with IBD. For instance, mutation in *NFKB1*, a gene that encodes p105 which has an IκB-like regulatory activity on NF-κB, has been linked with IBD and mice carrying such mutation have been reported to develop IBD-like intestinal inflammation (Chan *et al.*, 2009; Kaustio *et al.*, 2017).

NF-κB is also reported to play major roles in other inflammatory diseases such as multiple sclerosis (MS) and atherosclerosis. A number of genome-wide association studies have suggested a link between NF-κB and MS by identifying key molecules in the NF-κB signaling pathway such as RelA, IκBα and IκB-ζ as candidates of susceptibility (Hussman *et al.*, 2016; Miterski *et al.*,

2002). Other studies using mouse model of MS have shown that transgenic expression of IκB-α-dn, a non-degradable form of IκB-α, in astrocytes, inhibits NF-κB resulting in suppression of levels of pro-inflammatory cytokines and reducing severity of the disease.

Additionally, dysregulated activity of the transcription factor is implicated in the pathogenesis of many inflammation-associated human cancers including lymphomas, leukaemias and solid tumours (Rayet & Gelinas, 1999).

2.2.6 The roles of some NF-κB-regulated pro-inflammatory cytokines in inflammation and inflammatory diseases

Tumour Necrosis Factor (TNF)

This is a cytokine that is also referred to as cachetin. It is a glycoprotein with 185 amino acid residues. The cytokine was initially identified as a factor responsible for induction of necrosis in certain tumours (Carswell *et al*., 1975). TNF is one of the first cytokines produced upon detection of a pathogen (Beutler, 1999). It has a multi-organ effect and is among the cytokines that cause septic shock (Arango Duque & Descoteaux, 2014). It also stimulates the release of corticotropic releasing hormone and suppresses appetite (Arango Duque & Descoteaux, 2014). In inflammatory responses, TNF plays essential roles including induction of vasodilation, expression of adhesion molecules and regulation of chemokine release (Arango Duque & Descoteaux, 2014). These processes help to attract other inflammatory cells to the site of inflammation. Particularly, TNF together with IL-1, has been shown to be able to stimulate secretion of CXCL1, CXCL2 and CXCL5, which are chemokines that attract neutrophils (Griffin *et al*., 2012).

As a major contributor of the inflammatory response, hypersecretion of this cytokine is found in inflammatory diseases and cancers. For example, Pallone and Monteleone (2001) reported that

TNF was highly produced by mucosal macrophages of IBD patients and the cytokine was partly responsible for causing mucosal tissue damage due to its mediation in upregulation of expression of tissue-damaging matrix metalloproteinases.

TNF has been shown to be present in synovial biopsies of rheumatoid arthritis patients, and in various arthritis models, its inhibition has been demonstrated to suppress disease severity (Keffer *et al.*, 1991; Feldmann *et al.*, 1996). Furthermore, the key biological effects of TNF including activation of leukocyte and endothelial cells, angiogenesis and sensitization of pain receptors, are known to form part of the crucial processes in the pathogenesis of rheumatoid arthritis (McInnes and Schett, 2007). Anti- TNF therapy has been reported to show positive clinical outcomes in close to 70% of rheumatoid arthritis patients, by resulting in quick decline in plasma levels of acute phase proteins and interleukin -6, decreased leukocyte movement and deactivation of endothelial cells (Maini & Taylor, 2000). Aside from its established pro-inflammatory roles in rheumatoid arthritis, TNF also contributes to the tissue destructive activities that are associated with the disease, by driving the formation of osteoclasts and generating osteoclast precursors (Ritchlin *et al.*, 2003; Li *et al.*, 2004).

Interleukin-1 Beta (IL-1β)

This cytokine belongs to the IL-1 family that is made up of eleven members. It is also one of the early cytokines that is produced in response to infection, stress and lesions (Arango Duque &

Descoteaux, 2014). IL-1β expression is stimulated mostly by microbial molecules and by its own stimulation (Dinarello, 2010). Aside from that, IL-1β plays other roles including induction of expression of adhesion molecules and CD4 T cells differentiation (Ben-Sasson *et al*., 2009).

IL-1β production is elevated in IBD patients and this was found to induce excessive production of matrix metalloproteinases resulting in mucosal tissue damage (Pallone & Monteleone, 2001). IL-1β is present in the synovium of rheumatoid arthritis patients indicating a key role of the cytokine.

Interleukin-6 (IL-6)

IL-6 affects many biological processes including inflammation, immunity and metabolism (Arango Duque & Descoteaux, 2014). It is also a fever-promoting cytokine. It also acts on the liver, in concert with IL-1, to produce acute phase proteins (Dinarello, 2009; Kishimoto, 2010). It was reported to be elevated in Crohn's disease and rheumatoid arthritis (Nishimoto & Kishimoto, 2004).

Interleukin -12 (IL-12)

This is a cytokine that is made up of two hetreodimers p35 and p40 subunits (Arango Duque & Descoteaux, 2014). It plays vital roles in immune defense against infectious agents and cancer. It also stimulates T helper 1 (Th1) and thus promotes cell-mediated immunity (Arango Duque & Descoteaux, 2014). Furthermore, it induces production of IFN-gamma production by cooperating with other pro-inflammatory cytokines (Wang *et al.*, 2000).

Interleukin-23 (IL-23)

Similar to IL-12, IL-23 also induces IFN-gamma production and activation of T cells. It plays important roles in psoriasis and schizophrenia (Croxford *et al.*, 2014).

2.2.7 The roles of some NF-κB-regulated pro-inflammatory chemokines in inflammation and inflammatory diseases

Chemokines are a family of small (8-12 kDa) proteins that have the capacity to attract and guide immune cells to inflammatory sites (Arango Duque & Descoteaux, 2014). The movement of the immune cells in the direction of the chemokines is known as chemotaxis. During inflammatory responses, leukocytes travelling through the blood stream making immune surveillance are induced by chemokines to express integrins (Constantin *et al.*, 2000). The integrins halt the rolling movement of the leukocytes along the endothelium and make them pass through pores in the endothelium to reach inflammatory sites (Constantin *et al.*, 2000). Aside from chemotaxis, chemokines play many other physiological roles including haematopoiesis, differentiation and angiogenesis (Moser *et al.*, 2004).

Chemokines have a characteristic feature of 3 to 4 cysteine residues in their structures. Depending on where the cysteine residues are positioned, chemokines are grouped into four families. Those whose first two cysteines are separated by a variable amino acid belong to the CX-C subfamily. The second subfamily, C-C, comprise of those with cysteine residues positioned adjacent to each other. The third subfamily known as the C subfamily, consist of those with only one cysteine residue in the conserved position. The fourth subfamily, designated CX3C, is made of chemokines which contain 3 varying amino acids positioned in between the 2 N-terminal cysteine residues (Bazan *et al.*, 1997). The following are some of the macrophage-derived chemokines:

Interleukin-8

Interleukin-8 is a member of the CXC chemokine subfamily and thus referred to as CXCL8 (Hall, *et al.*, 1999). It is a critical pro-inflammatory mediator that potently chemoattracts neutrophils and

stimulates them to degranulate and undergo morphological changes (Gouwy *et al.*, 2004; Starckx *et al.*, 2002). It also chemoattracts other immune cells such as basophils, eosinophils, lymphocytes and monocytes at inflammatory sites (Miller *et al.*, 1992). Due to its potent chemoattraction of neutrophils and several other leukocytes, IL-8 is implicated in inflammatory diseases including Crohn's disease and psoriasis, and cancer (Damme *et al.*, 2004; Gijsbers *et al.*, 2004).

CXCL1 and CXCL2 (MIP-2α)

These are of the major chemokines that help to recruit neutrophils and hematopoietic stem cells to sites of inflammation (Moser *et al.*, 2004; Pelus & Fukuda, 2006). They belong to the C-X-C family of chemokines. They are described as murine homologues of human growth-related oncogenes (GRO) chemokines which include IL8 and GRO *α, β* and γ. Both CXCL1 and CXCL2 promote angiogenesis and development of tumours such as melanomas (Addison *et al.*, 2000).

CCL5

CCL5 recruits dendritic cells, T cells, basophils and eosinophils to sites of inflammation (Donlon *et al.*, 1990). It belongs to the CCL family of chemokines. CCL5 shares similarity with CXCL1 and 2 in terms of promotion of tumourigenesis and metastasis (Addison *et al.*, 2000).

Concerning its role in inflammation-related diseases, Wu *et al.* (2007) reported that CCL5 is overexpressed in murine obesity-related white adipose tissue (WAT). The study further shows accumulation of T cells in the obese WAT and found that to be associated with the overexpression of CCL5 as well as its receptor CCR5. This implies that increased expression of CCL5 may contribute to the dysregulated inflammatory condition in obese individuates that may lead to atherosclerosis.

2.2.8. Therapeutic targets in the NF-κB signaling pathway

The NF-κB signaling pathway is a multi-step pathway that is a made up of a number of discrete points of regulation. Each of these points of regulation is critical for the activation of the pathway and, therefore, serves as an important therapeutic target.

IκBα phosphorylation mediated by IKKβ is one of the major steps of the pathway regulation. IκBα phosphorylation leads to its breakdown which renders the sequestered NF-κB free to go across the nuclear membrane the activate gene transcription (Karin & Ben-Neriah, 2000). Inhibition of IKK enzymatic activity, therefore, leads to blockade of the subsequent steps in the pathway. There are a number of anti-inflammatory drugs that inhibit phosphorylation of IκBα or its proteosomal degradation. For example, aspirin and sodium salicylate which serve as anti-inflammatory drugs for several chronic inflammatory conditions, act partly by suppressing IKK enzymatic activity through inhibition of its interaction with adenosine triphosphate (ATP) (Yin *et al.*, 1998). A report by Pierce *et al.* (1996) indicated that salicylate inhibited phosphorylation of IκBα resulting in downregulation of expression of adhesion molecules in TNF- α stimulated endothelial cells.

Aside from inhibition of IκBα phosphorylation, inhibition of ubiquitination and/or the subsequent proteasome-dependent degradation of the cytosolic IκBα is also an important therapeutic target. For instance, cyclosporine A (CsyA) an immunosuppressive agent has been demonstrated in LPS-stimulated murine macrophages, to inhibit the activity of the proteasome thereby keeping IκBα in the ubiquitinated form and preventing it from undergoing degradation (Frantz *et al.*, 1994). In a related study, CysA was also shown to inhibit phorbol ester and ionomycin-induced IκBα degradation in Jurkat cells and human primary T lymphocytes respectively (Meyer *et al.*, 1997).

The next step in the pathway which serves as a good therapeutic target is the point at which NFκB translocates into the nucleus (Fig. 2.6). Many studies have shown that inhibition of this step

suppresses the NF-κB-mediated gene transcription. An example is the study by Karki and colleagues (2013) which showed that magnolol inhibited NF-κB translocatioin in cells of vascular smooth muscle.

Furthermore, a sizeable number of therapeutic agents that inhibit transcription mediated by NFκB are known to do so by inhibiting the nuclear processes the transcription factor undergoes including DNA binding, transactivation or posttranslational modifications. For instance, dexamethasone has been shown to inhibit p65 transactivation in mouse endothelial fibroblasts (De Bosscher *et al*., 1997). Also, mesalamine, a derivative of aminosalicylate was reported to inhibit p65-phosphorylation in IL-1-stimulated cells (Egan *et al*., 1999). NF-κB DNA binding is inhibited by dehydroxymethylepoxyquinomicin (DHMEQ) (Funakoshi *et al*., 2012; Matsumoto *et al*., 2000). Finally, a very key therapeutic mechanism of action of some anti-inflammatory agents, including glucocorticoids such as dexamethasone, involves upregulating the NF-κB-mediated expression of IκBα (Scheinman *et al*., 1995; Auphan *et al*., 1995). High expression of IκBα prevents the transcription factor from further induction of gene expression.

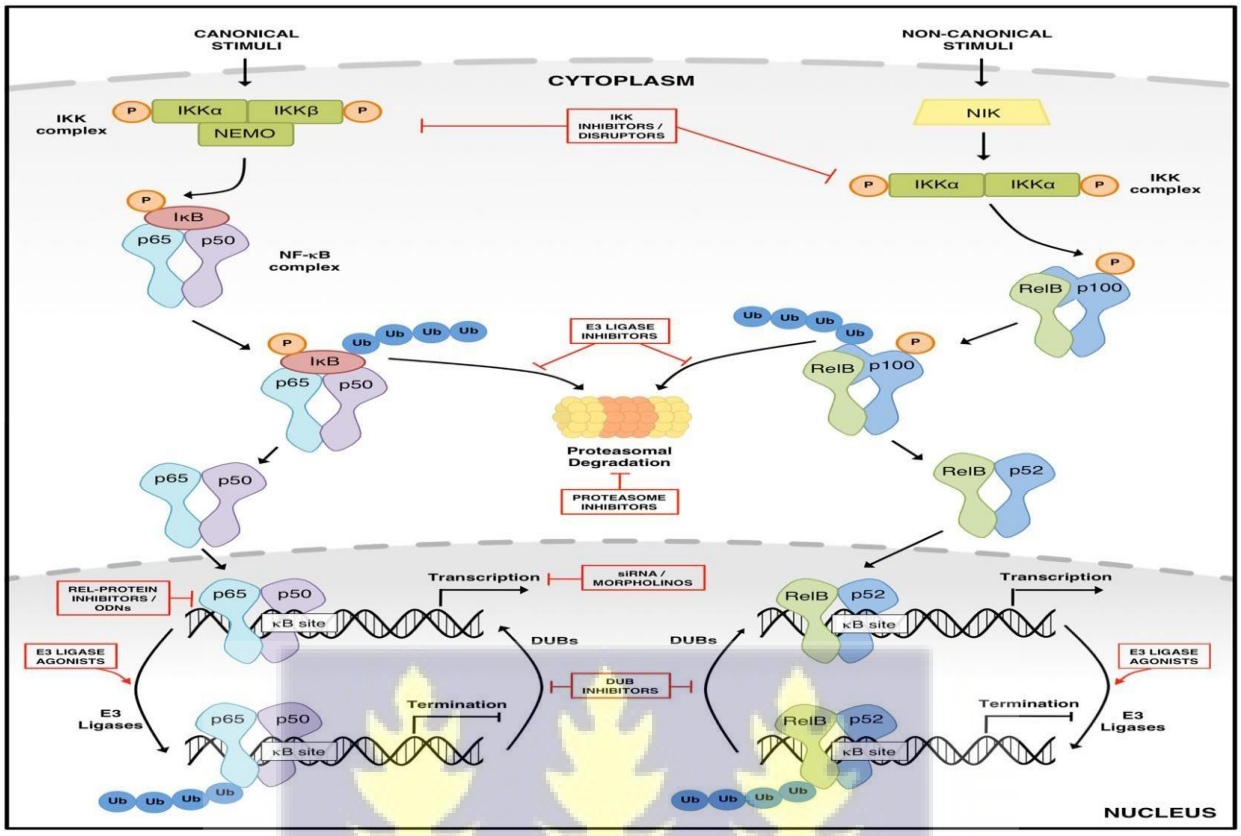

Fig. 2.6: The NFκB signaling pathways showing potential therapeutic targets. Activation of the canonical and the non-canonical pathways by various stimuli leads to cascades of signaling events with each key step, including phosphorylation by IKK, ubiquitination by E3 ligases, proteosomal degradation, NFκB DNA binding and transactivation, serving as a target of inhibition for therapeutic agents. Other potential inhibitors are the E3 Ligase agonists, DUB inhibitors and SiRNA/Morpholines. Source: Herrington *et al.*, (2015).

2.2.9 Sources of Cryptolepine

Cryptolepine (1, 5-methyl-10H-indolo [3, 2-b] quinoline) is an indoloquinoline alkaloid shown to possess many medicinal properties. Cryptolepine was initially found in *Cryptolepis triangularis* (Boye & Ampofo, 1983). Cryptolepine is also present in large quantity in the root of *Cryptolepis sanguinolenta*.

2.3.0 Isolation and purification of cryptolepine

Cryptolepine can be isolated from *Cryptolepis sanguinolenta* by the procedure described by Wright *et al* (1996). Briefly, the root of the plant is dried in open air and then pulverized. The powdered product is then moistened with ammonia and dissolved in chloroform which helps to extract the alkaloid. The concentrated chloroform extract is chromatographed over aluminium oxide and eluted using chloroform: methanol solvent mixture in a 95:5 ratio. The purified cryptolepine is crystallized as the hydrochloride. The identity of the crystallized cryptolepine is determined by spectroscopic techniques such as Mass Spectroscopy, Nuclear Magnetic Resonance (NMR), Ultraviolet-Visible (UV) and Infrared.

2.3.1 Physical and chemical properties of cryptolepine

Chemically, cryptolepine is a weak base that has four fused rings (Fig. 2.7) and a molecular weight of 268.5 g/mole. In terms of physical appearance, cryptolepine hydrochloride salt is yellowish in colour and has a melting point close to 268^{o}C (Bierer *et al*., 1998).

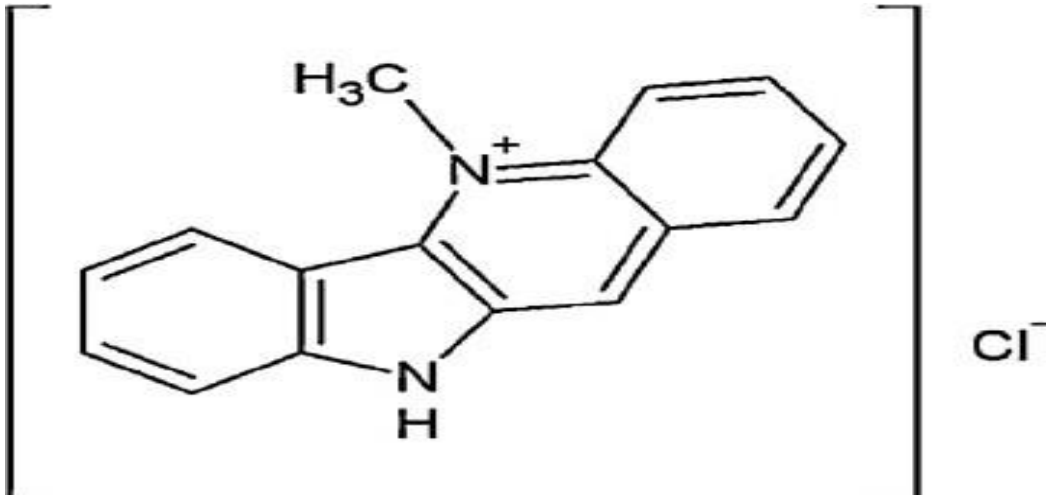

Fig. 2.7: Chemical structure of cryptolepine.

2.3.2 Anti-inflammatory activities of cryptolepine

Cryptolepis sanguinolenta is used in traditional medicine to manage several ailments including fever which indicates the potential anti-inflammatory property of some components of the plant. Cryptolepine, a bioactive compound of this plant, has been reported in many studies to possess potent anti-inflammatory activities.

Olajide *et al* (2007) found in their study that synthetic cryptolepine-hydrochloride suppressed the level of nitric oxide in LPS-stimulated mouse macrophages. Another study by Olajide *et al* (2013) further elaborated the anti-inflammatory activity of cryptolepine by showing that, the alkaloid was able to suppress the levels of pro-inflammatory proteins in rat primary microglial cells stimulated with lipopolysaccharide (LPS). The report also indicated that anti-inflammatory activity of cryptolepine was due to its inhibition of translocation of NF-κB and inhibition of P38 MAPK phosphorylation.

Aside from the *in vitro* studies, there are some prominent *in vivo* studies that have demonstrated significant anti-inflammatory activities of cryptolepine in murine models. For example in a study

by Olajide and colleagues (2009), cryptolepine was shown to inhibit rat paw oedema and microsvascular permeability in a dose-related fashion. The same study also demonstrated that, cryptolepine (10-40 mg/kg i.p.) showed analgesic activity by inhibiting acetic acid-induced writhing in mice.

2.3.3 Cytotoxicity of cryptolepine

Although *Cryptolepis sanguinolenta* and its alkaloid cryptolepine are widely used in traditional medicine in West and Central Africa, they have been shown to also have cytotoxic effects on several human and other mammalian cell lines (Charles Ansah & Gooderham, 2002). Cryptolepine was also shown to induce embryotoxic effect in zebrafish embryo *ex vivo* (Mensah, *et al.*, 2019). Findings by Bonjean *et al* (1998) and Lisgarten *et al* (2002) indicated that the cytotoxic properties of cryptolepine are mediated by its DNA-intercalating activity, inhibition of activity of the nuclear ubiquitous enzyme topoisomerase II and inhibition of DNA synthesis (Bonjean *et al*., 1998; Lisgarten *et al.*, 2002). Another report by Ansah and Gooderham (2005) showed that, treatment of human lymphoblastoid cell line (MCL-5) and hepG2 cells with cryptolepine markedly reduced cell viability and elevated the level of reactive oxygen species (ROS). Thus, apart from its DNA-intercalating effect and inhibition of topoisomerase II, cryptolepine is also a potent producer of ROS which are known to have considerable damaging effects on cellular membranes and biological macromolecules.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design and Site

An *in vitro* interventional experimental design was employed in this study. The experiments of the study were conducted at the Department of Clinical Microbiology, KNUST and the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

3.2 Cell Culture

The RAW-Blue™ cell line (Invivogen, Toulouse, France), which is a mouse macrophage reporter cell line derived from RAW 264.7 macrophages, was used in this study. The RAW-Blue™ cell line was chosen for this study because it is a macrophage cell line and macrophages are known to express toll-like receptors, play central roles in activation and orchestration of inflammatory responses and release pro-inflammatory mediators, which make them suitable for this study. Furthermore, the RAW-Blue™ cell line was chosen for this study because the cells are stably transfected with the gene of secreted embryonic alkaline phosphatase (SEAP) under the transcriptional control of NF- κB. This makes it convenient to study the activity of the TLR1/TLR2-NF-κB signaling pathway using reporter gene assays with SEAP as the marker.

The cells were cultured in T-25 and T-75 tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM) containing High-Glucose, L-Glutamine and sodium pyruvate supplemented with 10% volume by volume (v/v) heat-inactivated Foetal Bovine Serum (FBS) (ScienCell, Carlsbad, CA,

USA), 100 mg/ml Normocin™, 200 mg/ml Zeocin™ (Invivogen, Toulouse, France) and 50 µg/ml Penicillin-Streptomycin (Amresco, Leicestershire, England) (this mixture was hereafter referred to as the growth medium) and maintained in a humidified incubator at 37 ^{o}C and 5% CO_2. The

growth medium in the culture flask was routinely checked and replaced whenever nutrients were found to be depleted, usually indicated by a change in medium colour from pink to yellow. The cells were sub-cultured routinely by scraping whenever they reached 80% confluence.

3.2.1 Passaging of cells

To passage the cells, the spent growth medium was aspirated and discarded and a pre-warmed Phosphate Buffered Saline (PBS) (Hampshire, England) was used to gently wash the monolayer of cells and to remove detached dead cells and cellular debris. This was followed by addition of 2 mL of growth medium and gentle scraping of the cells with a sterile scraper. After scraping all the cells and detaching them from the surface, the flask was placed vertically on the surface of the hood to make the cells gently drain to the bottom of the flask. Additional volume of the growth medium was then used to wash down all the cells on the surface of the flask and make them drain to the bottom. At this stage, the cell suspension was aspirated and transferred into a 15 mL Falcon tube and then centrifuged to pellet the cells. This was followed by aspiration and discarding of the supernatant and the cell pellet re-suspended in 1 mL of freshly warmed growth medium to be used for cell counting.

3.2.2 Cell counting

The cells were counted using the Neubauer haemocytometer and thereafter seeded at the right cell density in the appropriate plates for the assay. An aliquot of the re-suspended cell pellet after centrifugation was used to count the cells for subsequent assays. Firstly, the cell suspension was mixed thoroughly and twenty (20) µL were transferred into an Eppendorf tube and diluted with eighty (80) µL of 0.4% Trypan Blue (Sigma Aldrich, Taufkirchen, Germany) to obtain a 1:5 dilution ratio. A cover slip was placed on the haemocytometer to cover the two counting chambers of the haemocytometer after which 10 µL of the Trypan Blue-cell suspension were taken and

applied to the two vertically opposite sides of the cover slip. The haemocytometer was subsequently placed on an inverted Nikon microscope (x10 objective) and using a cell counter, live cells in the squares of each large quadrant were counted. The average of the two counts was determined. The total number of cells per mL of the cell suspension was calculated as shown below:

Number of cells per mL = Average number of cells counted in the two chambers X dilution factor X 10^4

3.3 Cytotoxicity assays of cryptolepine and triptolide

Cryptolepine (in natural powdered form) was a generous donation from Dr Kwasi B. Mensah of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology.

Cryptolepine at 1 mg was dissolved in sterile distilled water. Afterwards, the solution was subsequently sterile-filtered, aliquoted into Eppendorf tubes, covered with aluminium foil and stored at -20 ^oC until the time of its usage to reduce frequent freeze-thaw cycles and exposure to light.

The RAW-Blue cells previously cultured under conditions described in section 3.1 were seeded at a density of 20,000 cells per well in a 96-well plate. The cells in the 96-well plates were again cultured under the same condition described in section 3.1 until they reached 80% confluence at which point they were exposed to treatment with increasing concentrations of cryptolepine (0, 1, 2.5, 5, 10 and 20 µM) in duplicate wells for 24, 48 and 72 hours. At each time point, cytotoxicity of cryptolepine was assessed by the (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Sigma Aldrich, Taufkirchen, Germany) assay following the kit manufacturer's guidelines. The principle of the MTT assay is that, live metabolically active cells are able to change the yellow coloured MTT reagent into a violet coloured formazan as a result of the enzymatic activity of their mitochondrial reductase enzymes. At each of the 24, 48 and 72 hour incubation periods, 15 µL of the yellow-coloured MTT reagent were applied to treated and untreated cells

and incubated for 2 hours. Thereafter, the purple coloured crystals formed were dissolved with 100 µL of isopropanol and the absorbance read at 570 nm. Three (3) independent experiments were conducted in duplicate wells of the 96-well plate. Cell viability was evaluated as the ratio between cryptolepine-treated cells and untreated cells and the maximum non-toxic concentrations of cryptolepine corresponding to the treated wells with the highest percentage of cell viability was chosen for the subsequent experiments.

Triptolide (Invivogen, Toulouse, France), used as a positive control in this study, is a well-known potent inhibitor of the NF-κB signaling pathway (Liu *et al.*, 2000). One (1) milligram of triptolide was dissolved in 280 µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich, Taufkirchen, Germany) to obtain a concentration of 10 mM. The solution was further diluted to a 10 µM stock concentration which was then aliquoted into many Eppendorf tubes, wrapped with aluminium foil and stored at -20 ^{o}C until ready to be used. To assess the cytotoxicity of triptolide, the RAW Blue cells were seeded at a density of 20,000 cells per well in a 96-well plate and incubated under conditions previously described under section 3.1. At 80% confluence, the cells were treated with increasing concentrations of triptolide (0, 10, 20, 40 and 80 nM) for 24, 48 and 72 hours, and at each time point, cytotoxicity was determined by MTT assay as explained above. Two independent experiments were carried out in duplicate wells of the 96-well plate. Cell viability was evaluated as the ratio between triptolide-treated cells and non-treated cells and the maximum non-toxic concentrations of triptolide corresponding to the treated wells with the highest percentage of cell viability was chosen for the subsequent experiments.

3.4 Stimulation of RAW Blue cells with Pam3CSK4

The RAW-Blue cells are mouse macrophage reporter cell lines that have been stably transfected with the gene of secreted embryonic alkaline phosphatase (SEAP) under the transcriptional control

of NF- κB. When NF-κB is activated it induces expression of SEAP which serves as the reporter or marker of activity of the pathway. To test whether the NF-κB signaling pathway could be activated in the RAW-Blue cells and measured by a reporter gene assay, the cells were seeded in a 96-well plate at a density of 100,000 cells per well and the plate incubated for 24 hours under conditions described in section 3.1. After the incubation period, Pam3CSK4 (50 - 400 ng/mL) (Invivogen, Toulouse, France), a synthetic triacylated lipopeptide that specifically binds to the TLR1-TLR2 heterodimeric receptor and activates the TLR1/TLR2-NF-κB signaling pathway, was used to stimulate the cells for 24 hours as recommended by the manufacturer. After the incubation period, the levels of expressed SEAP in the wells were measured by Quanti-Blue assay following the kit manufacturer's instruction. The Quanti-Blue assay is an assay that indirectly measures the levels of SEAP in a solution. When SEAP is expressed and released in solution it catalyzes the conversion of the Quanti-Blue reagent (Invivogen, Toulouse, France) from its original bluish colour to a final pinkish coloured solution whose absorbance is determined using a spectrophotometer. The absorbance reading is proportional to the activity of the NF-κB pathway. Briefly, 50 µL of the supernatant from each well were taken, mixed with 150 µL of the Quanti-Blue reagent (Invivogen, Toulouse, France) in a flat-bottom 96-well plate and the plate incubated for 1 hour 15 minutes at 37^{o}C as recommended by the manufacturer. After the incubation period, the absorbance was read at 655 nm using an iMark Microplate reader (Bio-Rad, Hercules, CA, USA). Two independent experiments were carried out, each in duplicate wells.

3.5 The effect of cryptolepine on the activity of the TLR1/TLR2-NF-κB signaling pathway in RAW Blue cells

The effect of cryptolepine on the activity of Pam3CSK4-induced TLR1/TLR2-NF-κB signaling pathway was assessed to determine its anti-inflammatory action. Briefly, the RAW-Blue cells were

seeded in 96-well plate at a density of 100,000 cells per well and the plate incubated for 24 hours under conditions described in section 3.1. After the incubation period, 100 ng/mL of Pam3CSK4, as recommended by the manufacturer (Invivogen, Toulouse, France), was used to stimulate the cells in the absence or presence of cryptolepine (0.5 and 1 μM) for 24 hours. Triptolide, at its determined maximum non-toxic concentration, 15 nM, was also included in the experiment as a positive control. Thereafter, the levels of expressed SEAP in the wells were measured by Quanti-Blue assay as described previously.

3.6 Induction of expression of three NF-κB-regulated genes by Pam3CSK4

Having tested the ability of Pam3CSK4 to induce expression of a reporter gene (*SEAP*) under the transcriptional control of NF-κB, this assay was conducted to test whether Pam3CSK4 could also induce expression of a selected group of actual NF-κB-regulated genes. This could help to confirm the ability of the agonist to cause a significant rise in expression before the effect of cryptolepine on the expression could be tested.

To do so, Pam3CSK4 was used to stimulate RAW-Blue cells for 24 hours and induction of expression of the genes of TLR2, NF-κB1 (p105) and IκBα, all inducible by NF-κB1, was determined. TLR2 is a component of the dimeric receptor of the signaling pathway, NF-κB1 is the precursor of a subunit (p50) of NF-κB and IκBα is the main negative regulator of NF-κB. The primers of the genes of TLR2, NF-κB1, IκBα and β-actin (endogenous control) used for the RT-qPCR were purchased from Biomers in Germany **(Table 3.1).**

Table 3.1: Sequences of primers

Gene	Primer Sequence (5'-…….-3')
tlr2	Forward: AAGGCATTAAGTCTCCGGAATTATC Reverse: TCGCTTAAGTGAAGAGTCAGGTGAT
nf-κb1	Forward: GAAATTCCTGATCCAGACAAAAAC Reverse: ATCACTTCAATGGCCTCTGTGTAG
IκBα	Forward: GAAGCCGCTGACCATGGAA Reverse: GATCACAGCCAAGTGGAGTGGA
β-actin	Forward: TCACCCACACTGTGCCCATCTACGA Reverse: GGATGCCACAGGATTCCATACCCA

Briefly, the RAW-Blue cells were cultured as previously described and then stimulated with 100 ng/mL of Pam3CSK4 (Invivogen, Toulouse, France) for 24 hours. Thereafter, the plates were placed on ice and 1 mL of sterile ice-cold 1 x PBS was used to wash the cells three times. Thereafter, the plates were placed on ice and one mL of sterile ice-cold 1 x PBS was used to wash the cells three times. The cells were then harvested in 1 mL of 1 x ice-cold PBS and transferred to labelled RNase/DNase free Eppendorf tubes and subsequently centrifuged at 500 x g for ten minutes at 4^{o}C. The supernatants were discarded and the cell pellet used for RNA extraction.

3.6.1 RNA extraction, DNase treatment and elution of pure RNA

The extraction of RNA from the cells was performed using SV RNA Isolation System (Promega, Madison, WI, USA) following the kit manufacturer's instruction. Briefly, to each tube containing the cells, RNA lysis buffer (175 µL) was added and mixed thoroughly. After that RNA Dilution

Buffer (350 µL) was added and mixed thoroughly. This was followed by warming of the tube at 70°C for three minutes and then centrifugation at 12,000 x g for ten minutes at 25°C. The cleared lysate was transferred to a fresh microcentrifuge tube and 200 µL of 95% ethanol was added and mixed thoroughly. It was then transferred to a spin column assembly and centrifuged at 12,000 x g for one minute. The supernatant was discarded and RNA wash solution (600 µL) was added to the spin column assembly and centrifuged at 12,000 x g for another one minute. The supernatant was discarded and the pelleted extract taken through DNAse treatment. DNase treatment was carried out on the extract to remove left over DNA in the samples in order to have pure RNA completely free of DNA. Briefly, a cocktail of reagents including Yellow Core Buffer (40 µL), 0.09 M $MnCl_2$ (5 µL) and DNase I (5 µL) known as DNase incubation mix was prepared in a sterile tube. The components were mixed gently and 50 µL of the mixture transferred to the tube containing the extract. This was then incubated for fifteen minutes at 25°C and after that DNase Stop Solution (200 µL) was added to stop the DNase enzymatic activity. The tubes were centrifuged at 12000 x g for one minute to separate the digested DNA from the remaining RNA extract. The extracts remaining in the spin basket were taken through two steps of washing to remove all impurities. An RNA wash solution (600 µL) was added to the extract and centrifuged at 12,000 x g for one minute. The washing step was repeated with RNA wash solution (250 µL).

Finally, nuclease-free water (100 µL) was added to the extract and the tube centrifuged at 12,000 x g for one minute to elute the RNA. The purity and quantity of the total RNA were determined using a Nanodrop One spectrometer (Thermofisher Scientific Inc.). The pure RNA was stored at -80°C until ready for analysis.

3.6.2 Quantification of relative gene expression levels (using the delta delta C_T ($\Delta\Delta C_T$) method)

The RT-qPCR reactions were run on a QuantStudio 5 qPCR machine using Luna Universal One-Step RT-qPCR kit (New England Biolabs, United Kingdom) following the manufacturer's instructions. All the reactions were conducted in duplicate wells and negative controls were included. The $\Delta\Delta C_T$ and relative quantification values were computed using the QuantStudio design and analysis software (Applied Biosystems Inc.).

3.7 The effect of cryptolepine on transcript levels of some key NF-κB-regulated genes

The effect of cryptolepine on transcript levels of some key NF-κB-regulated genes including *Il1β, Il-6, Tnf-α, Il-23, Ccl2, Cxcl2, Ikbkb, Nfkb1, Rela, Tlr2 and Tlr1* which code for the cytokines, IL-1β, IL-6, TNF-α and IL-23, the chemokines MCP-1 and MIP-2α, the signaling molecules, IKKβ, NFκB1 and RelA, and the receptors TLR2 and TLR1 respectively, were determined by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using Taqman Array Mouse NF-κB pathway standard 96-well plates (Thermofisher Scientific, Paisley, Scotland). The plates contained primers and probes of the various genes embedded in wells specifically designated for the genes and the endogenous control. The endogenous control used was 18S rRNA. Briefly, the RAW Blue cells were cultured as previously described and then stimulated with 100 ng/mL of Pam3CSK4 (Invivogen, Toulouse, France) in the absence or presence of cryptolepine (1 µM) for 1 hour. Thereafter, the cells were taken through the same procedures for RNA extraction, DNase treatment and elution of pure RNA as explained above.

3.7.1 Quantification of relative gene expression levels using the delta delta C_T ($\Delta\Delta C_T$) method

The RT-qPCR reactions were run on a QuantStudio 5 qPCR machine (Applied Biosystems Inc.) using the Taqman Array Mouse NF-κB pathway standard 96-well plates (Thermofisher Scientific, Paisley, Scotland) and the TaqMan® RNA-to-C_T™ 1-Step kit (Applied Biosystems Inc.) following the kit manufacturers' instructions. The assay IDs and symbols of the target genes assessed are listed in table 3.2. All the reactions were conducted in duplicate wells and negative controls were included. The $\Delta\Delta C_T$ and relative quantification values were computed using the QuantStudio design and analysis software (Applied Biosystems Inc.).

Table 3.2: Symbols and assay IDs of target genes

The primers and probes of the selected genes of the Taqman Array mouse NF-κB pathway, with the below-listed IDs, were designed by Thermofisher Scientific, Paisley, Scotland.

Gene Symbol	Assay ID
*18s rRna***	Hs99999901_s1 18s rRNA
Il1bI	Mm00434228_m1 Il1b
Il6	Mm00446190_m1 Il6
Tnf	Mm00443258_m1 Tnf
Il23	Mm00518984_m1 Il23a
Ccl2	Mm00441242_m1 Ccl2
Cxcl2	Mm00436450_m1 Cxcl2
Nfkb1	Mm00476361_m1 Nfkb1
Rela	Mm00501346_m1 Rela
Ikbkb	Mm01222247_m1 Ikbkb
Tlr2	Mm00442346_m1 Tlr2
Tlr1	Mm00446095_m1 Tlr1

** Endogenous control

3.8 The effect of cryptolepine on the production of pro-inflammatory cytokines and chemokines

Having determined the effect of cryptolepine on the mRNA levels of the pro-inflammatory cytokines and chemokines, the next task was to determine the effect of the alkaloid on their protein levels to see whether

the anti-inflammatory mechanisms of action of cryptolepine involve interference at both transcriptional and translational stages of expression of its target genes. Since cytokines and chemokines are critical pro-inflammatory mediators with some of them such as IL-1β and TNF-α, acting also as NF-κB activators via paracrine and autocrine loops, potential suppression of their protein levels by cryptolepine will reveal more details about its potent anti-inflammatory mechanism of action. Briefly, the RAW Blue cells were cultured in 6-well plates as previously described and then stimulated with Pam3CSK4 and treated with cryptolepine as explained previously. After the incubation period, the culture supernatants (200 µL) from each well were aspirated and placed in labelled sterile Eppendorf tubes. The tubes were centrifuged at 1,500 rpm for ten minutes at 4 °C. Afterwards, the supernatants were aliquoted into new sterile Eppendorf tubes and kept at -80 °C. The cytokine and chemokine analyses were performed using the Magnetic Luminex Assay Mouse Premixed Mutli-Analyte Kit (R & D systems, Minneapolis, MN, USA) following the kit manufacturer's instructions.

3.8.1 Sample and reagent preparation for cytokine and chemokine analyses

Briefly, the supernatants were clarified through centrifugation at 1,500 rpm for five minutes followed by a 2-fold dilution using Calibrator Diluent RD6-52. Thereafter, the other reagents were placed on the working bench and allowed to warm up to ambient temperature before analysis. To prepare wash buffer (500 mL), Wash Buffer Concentrate (20 mL) was added to distilled water (480 mL). To prepare the standards for the assay each of the two unique Standard Cocktails (A and B) provided was mixed with the Calibrator Diluent RD6-52 to form a 10X concentrate. Subsequently, each of the two standards (100 µL) was mixed with Calibrator Diluent RD6-52 (800 µL) to form 1 mL of a mixture referred to as Standard 1. Using Standard 1 as the highest concentrated standard, serial dilutions were made into 5 other Falcon tubes. Calibrator Diluent RD6-52 was used as the blank.

Antibody Cocktail, Microparticle Cocktail and Streptavidin-PE were each prepared first by centrifuging their vials for thirty seconds at 1000 x g and then gently vortexing the vial without inverting it. Microparticle Cocktail and Biotin Antibody Cocktail were subsequently diluted by mixing 250 µL of each with Assay Diluent RD1W (2.5 mL) in labelled Falcon tubes. The tube containing the Microparticle was wrapped with aluminium foil to protect it from light.

Streptavidin-PE concentrate (110 µL) was diluted by mixing it with the wash buffer (2.65 mL).

3.8.2 Assay Procedure

The assay was run on the xMAP Technology platform (Luminex Corporation, Austin, TX, USA) following the kit manufacturer's instructions (R & D systems, Minneapolis, MN, USA).Using a paper containing the layout of the plate as a guide, standard (50 µL) or sample (50 µL) was added into its designated well. Afterwards, the diluted Microparticle Cocktail (50 µL) was added to each well of the microplate after resuspending it using a vortexer. The microplate was then properly covered with a sealer and placed on a horizontal orbital microplate shaker set at 800 rpm for a two-hour incubation at room temperature. After the incubation period, the plate was washed three times by filling each well with the wash buffer (100 µL). After the washing step, the diluted Biotin-Antibody Cocktail (50 µL) was added to each well and the microplate covered and incubated on a horizontal orbital microplate shaker as stated above. After the incubation period, the plate was washed as described above followed by addition of diluted Streptavidin-PE (50 µL) to each well and another incubation for 2 hours. Afterwards, the microplate was again taken through three steps of washing. The microparticles were then resuspended by adding wash buffer (100 µL) to each well and the plate incubated for 2 minutes on the shaker set at 800 ± 50 rpm. The microplate was immediately inserted in the Luminex® analyser and read. The result was exported to Excel and analyzed statistically.

3.9 Statistical analysis

Results obtained from each experiment were entered into Excel sheets where means and standard deviations were calculated. Statistical analyses were done on GraphPad Prism version 8.0 (GraphPad Software Inc., USA). Analysis of statistical significance of differences between results of experimental groups was done using ANOVA followed by Tukey's or Dunnett's post hoc test. Differences between experimental groups were considered statistically significant when *P* values were < 0.05.

CHAPTER FOUR

4.0 RESULTS

4.1 Cytotoxicity of cryptolepine and triptolide on RAW Blue cells

Macrophages are innate immune cells that initiate and orchestrate inflammatory responses by using their PRRs such as the TLRs to detect microbial surface molecules. Due to this pivotal role macrophages play in inflammatory responses, this study was conducted in a murine macrophage cell line, RAW Blue cells. Prior to testing the anti-inflammatory effects of cryptolepine on the cells, the effects of cryptolepine and triptolide (positive control) on cell viability were assessed by MTT assays.

The results of the MTT assay showed that, compared to untreated cells in which cell viability was 100%, the cryptolepine-treated cells demonstrated a dose-dependent decline in cell viability (Fig.4.1.1). The maximum non-toxic concentration was found to be 1 μM at which the mean percentage viability of the treated cells was 92%. At that concentration, no significant difference ($P = 0.72$) was observed between the viability of the cryptolepine-treated cells and that of the untreated cells. Also, there was no significant difference ($P = 0.82$) in the viability of the cells over time after treatment with 1 μM cryptolepine. Triptolide-treated cells also demonstrated a dose-dependent decline in cell viability (Fig.4.1.2). The maximum non-toxic concentration was observed to be 15 nM at which the mean percentage cell viability was 88%. At that concentration, no significant difference ($P = 0.09$) was observed between the viability of the triptolide-treated cells and that of the untreated cells. There was no significant difference ($P = 0.98$) in the viability of the cells over time after treatment with triptolide.

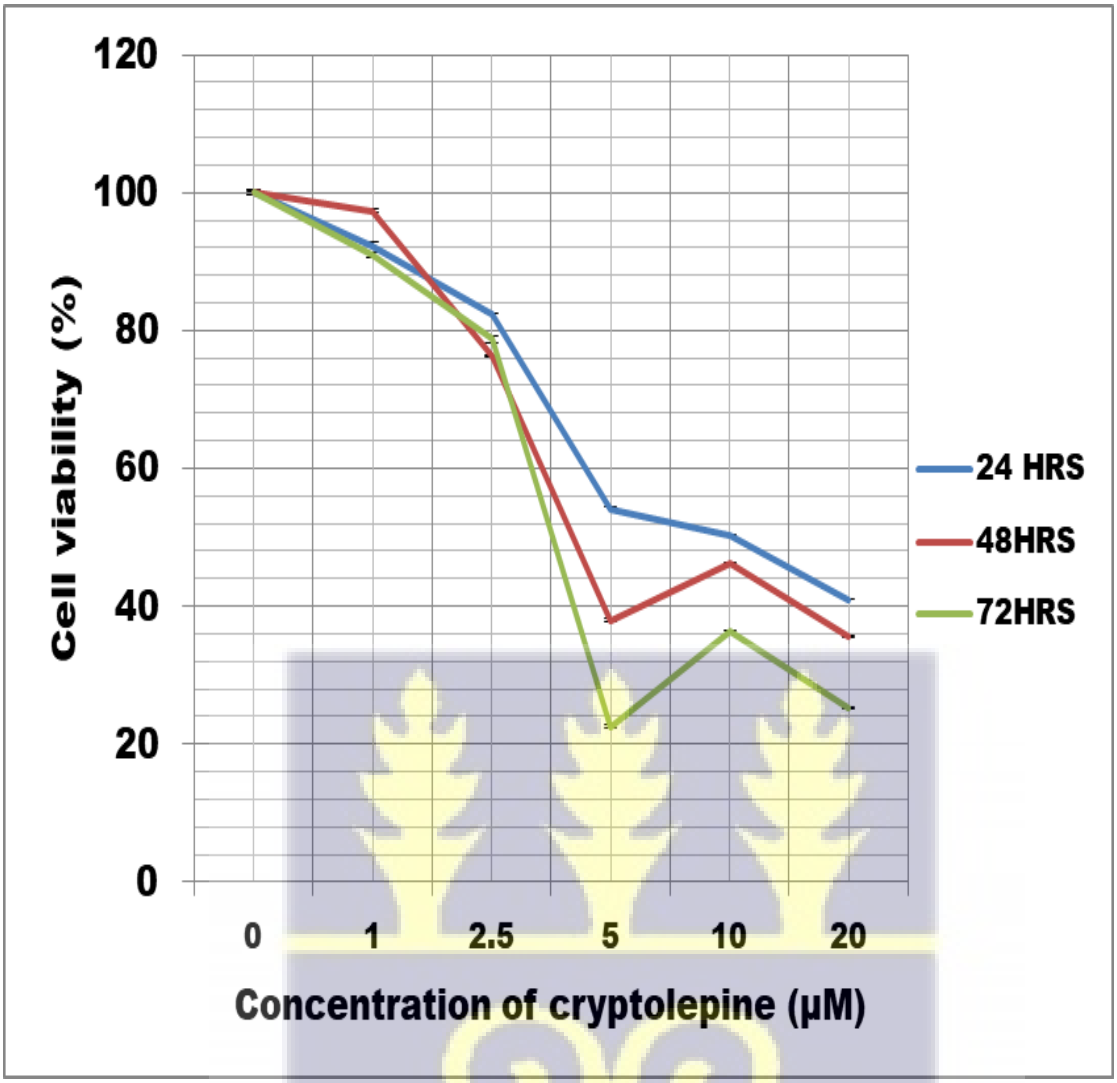

Fig. 4. 1. 1: Cytotoxicity of cryptolepine on RAW Blue cells. The cells were plated and then treated with increasing concentrations of cryptolepine (0, 1, 2.5, 5, 10 and 20 µM) for 24, 48 and 72 hours. Cytotoxicity of cryptolepine was assessed by MTT assay at each time point. Cell viability was evaluated as the ratio between cryptolepine-treated cells and untreated cells. The results represent mean percentage cell viability and standard deviations of three independent experiments each conducted in duplicate wells. P = 0.82 for 1 µM cryptolepine-treated group at 24 hours in comparsion with 1 µM cryptolepine-treated group at 48 and 72 hours as determined by one-way analysis of variance (ANOVA).

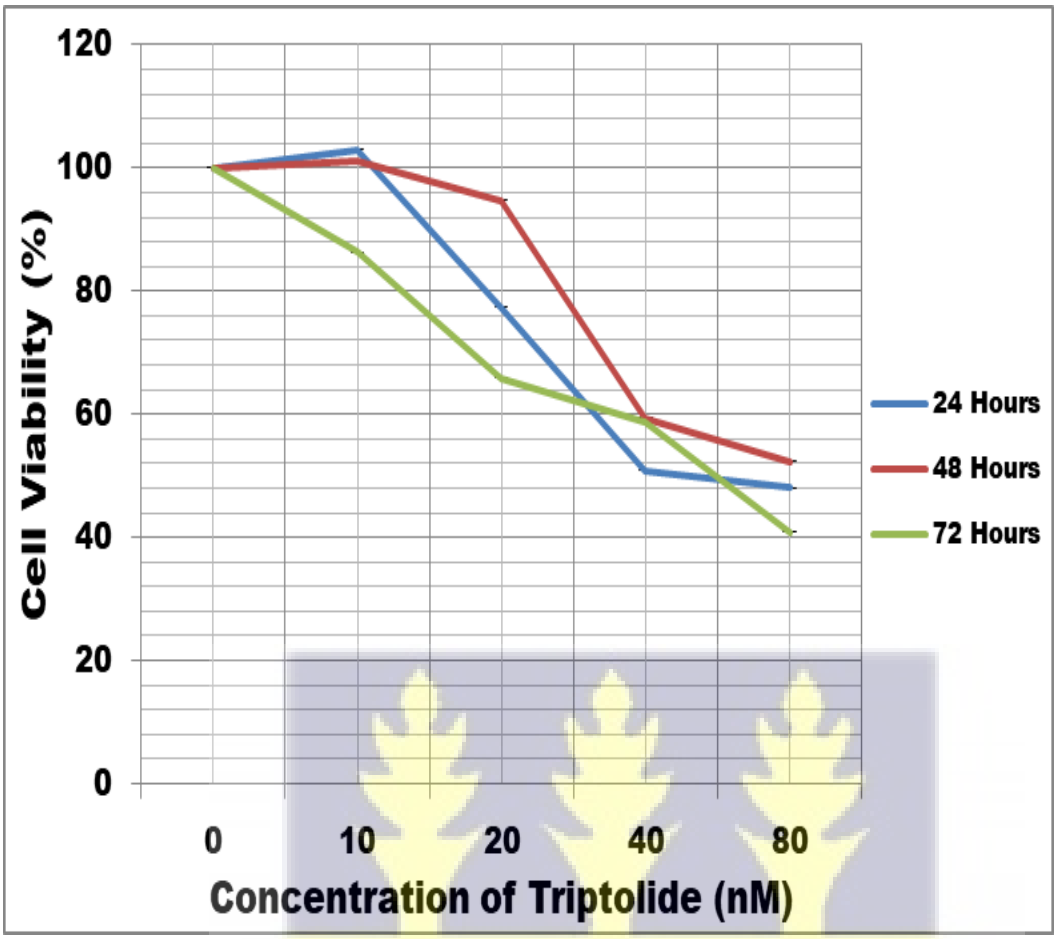

Fig. 4.1. 2: Cytotoxicity of Triptolide on RAW Blue cells. The cells were plated and then treated with increasing concentrations of triptolide (0, 10, 20, 40 and 80 nM) for 24, 48 and 72 hours. Cytotoxicity of triptolide was assessed by MTT assay at each time point. Cell viability was evaluated as the ratio between triptolide-treated cells and untreated cells. The results represent mean percentage cell viability of two independent experiments each conducted in duplicate wells. P = 0.98 for 15 nM triptolide-treated group at 24 hours in comparsion with 15 nM triptolide-treated group at 48 and 72 hours as determined by one-way ANOVA.

4.2 PAM3CSK4 induced dose-dependent NF-κB signaling pathway activation

The NF-κB signaling pathway can be activated through many TLRs including the TLR1/TLR2 heterodimeric receptor. To determine whether the activity of the NF-κB signaling pathway could be stimulated in the RAW-Blue cells and assessed by a reporter gene assay, the cells were stimulated with Pam3CSK4, a synthetic triacylated lipopeptide that specifically binds to the TLR1/TLR2 heterodimeric receptor. The results showed that, stimulation of the cells with increasing concentration of Pam3CSK4 (50 – 400 ng/mL) resulted in dose-dependent increase in pathway activity relative to unstimulated cells (Fig. 4.2). In the Pam3CSK4-stimulated cells, significant increase in pathway activity relative to the negative control was observed at only 200 ng/mL ($P = 0.04$) and 400 ng/mL ($P = 0.01$).

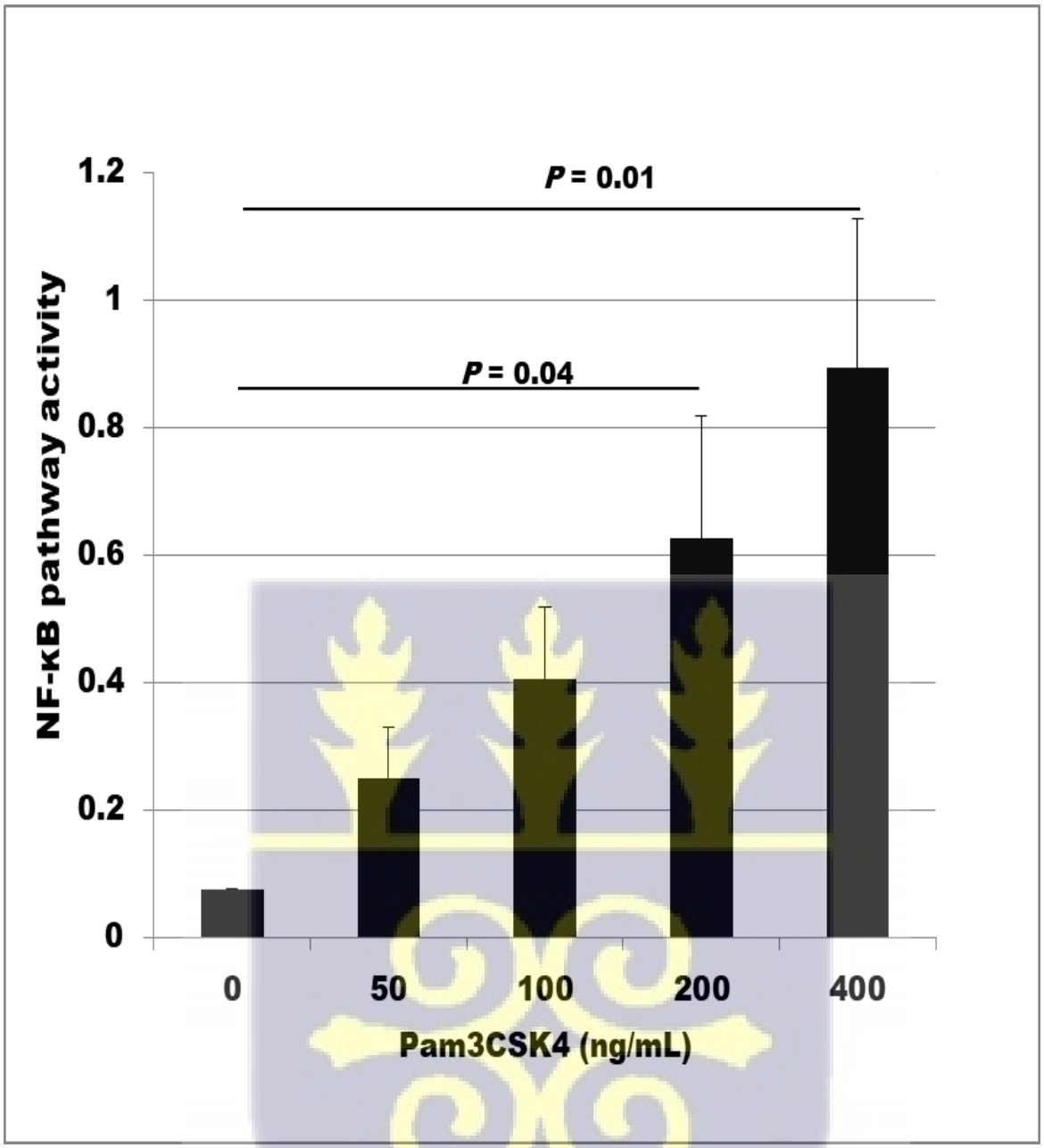

Fig. 4.2: Activation of NF-κB pathway by Pam3CSK4 in RAW Blue cells. The cells were stimulated with Pam3CSK4 (50 - 400 ng/mL for 24 hours. After the incubation, supernatants were harvested and the NF-κB signaling pathway activity assessed by evaluating the level of secreted embryonic alkaline phosphatase (SEAP) by Quanti Blue assay. The results are presented as means and standard deviations for two independent experiments each carried out in triplicate wells. Data were analyzed by multiple comparisons of treatments vs. control as determined by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc test*. $P < 0.05$.

4.3 Cryptolepine suppressed Pam3CSK4-induced TLR1/TRL2-NF-κB signaling pathway activity in RAW Blue cells

Hyper-activation of the TLR-NF-κB pathway is reported to be associated with inflammatory diseases (Tak & Firestein, 2001). To determine the effect of cryptolepine on the activity of the TLR1/TLR2-NF-κB pathway, Pam3CSK4, a synthetic tricylated lipopeptide that specifically binds to the TLR2-TLR1 heterodimer was used as an agonist to stimulate RAW Blue cells in the absence or presence of cryptolepine and the levels of SEAP measured by Quanti-Blue assay. The results of the Quanti-Blue assay showed that, the NF-κB pathway activity in the Pam3CSK4stimulated cells was significantly ($P < 0.0001$) increased to about five-fold relative to the activity in the unstimulated cells (negative control). However, treatment with cryptolepine markedly suppressed the activity of the NF-κB pathway in a dose-dependent fashion (Fig. 4.3). Significant suppression was observed in the presence of cryptolepine at 0.5 µM ($P = 0.01$) as well as at 1 µM ($P = 0.001$). The suppressive effect of 1 µM cryptolepine was found to be significantly ($P = 0.03$) different from that of 0.5 µM cryptolepine. Also, significant ($P = 0.0003$) suppression of the activity of the pathway was recorded following treatment with triptolide. About 29.8% suppression was recorded following treatment with 1 µM cryptolepine compared to 35% suppression following treatment with 15nM triptolide.

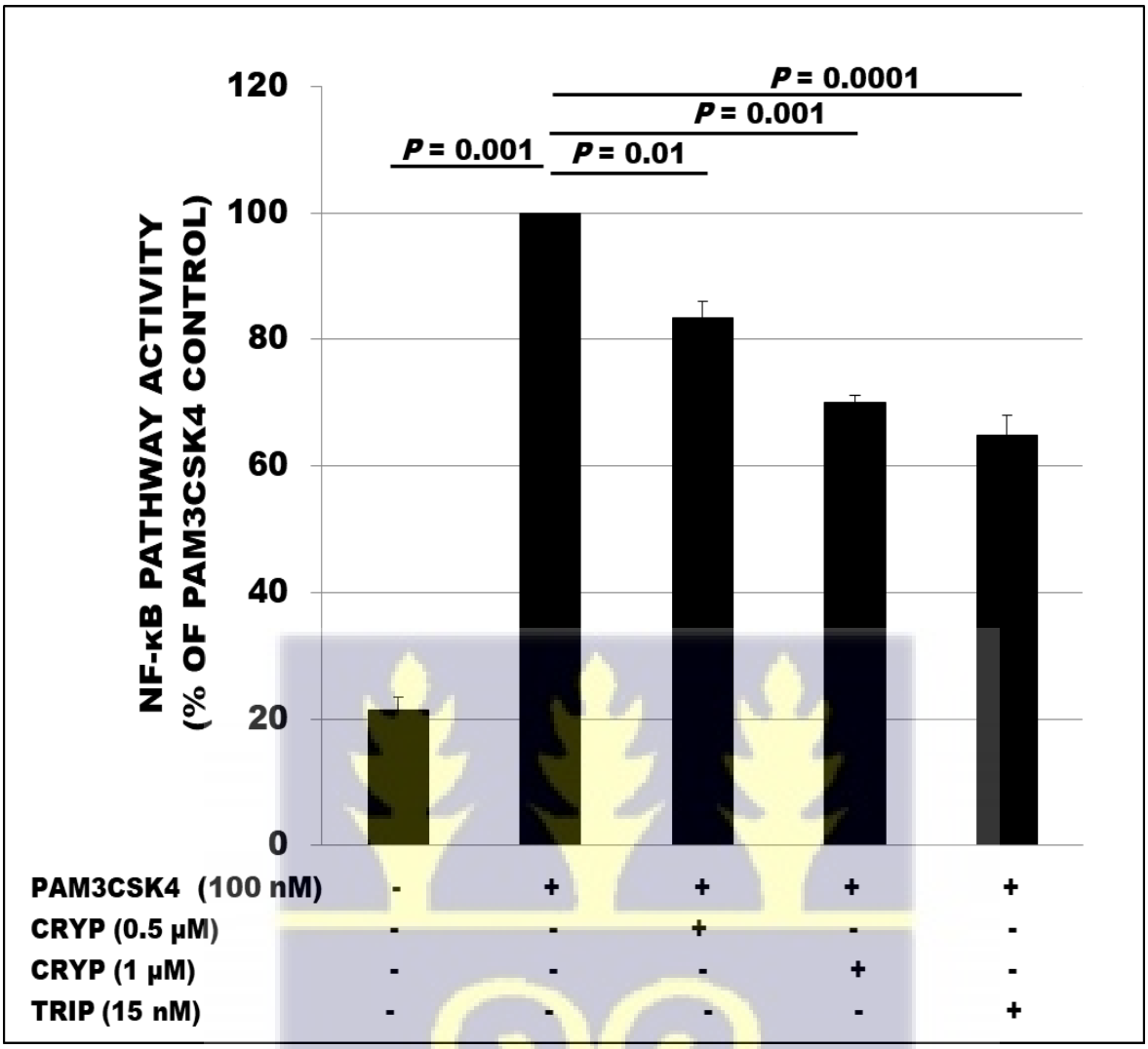

Figure 4.3: Cryptolepine suppressed Pam3CSK4-induced TLR1/TLR2-NF-κB activity in RAW Blue cells. The cells were stimulated with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (CRYP) (0.5 and 1 μM) for 24 hours. Triptolide (TRIP) was used as a positive control. At 24-hours post incubation, supernatants were collected and the activity of the NF-κB pathway assessed by evaluating the expression levels of secreted embryonic alkaline phosphatase (SEAP) by Quanti Blue assay. The results are expressed as percentage (%) by normalizing the value of each sample to the value of Pam3CSK4-stimulated cells. All values are presented as means and standard deviations for two independent experiments each conducted in triplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$.

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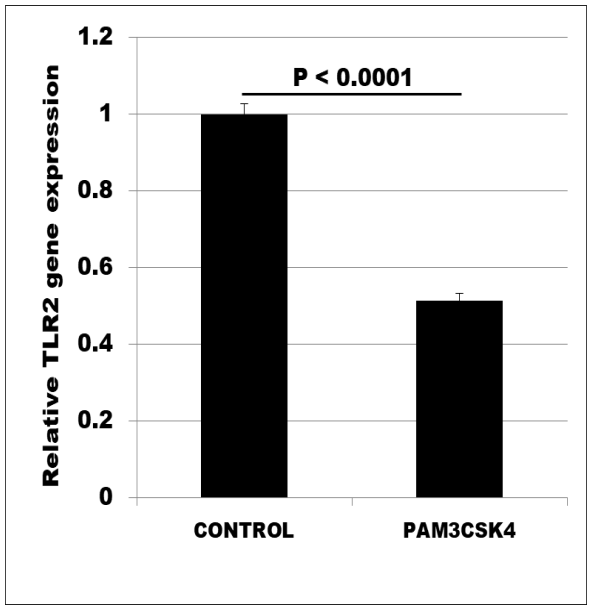
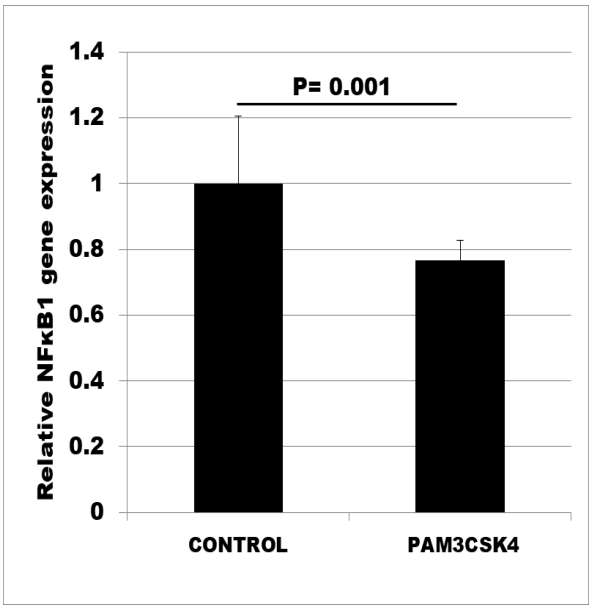
4.4 Pam3CSK4 induced differential effects on expression of selected NF-κB-regulated genes

Having shown that Pam3CSK4 was capable of inducing the expression of a reporter gene (gene of SEAP) inducible by NF-κB, it was necessary to determine the level of induction of expression of a selected group of actual NF-κB-regulated genes by Pam3CSK4. This was to confirm the ability of the agonist to induce a significant rise in expression of the genes before the effect of cryptolepine could be tested.

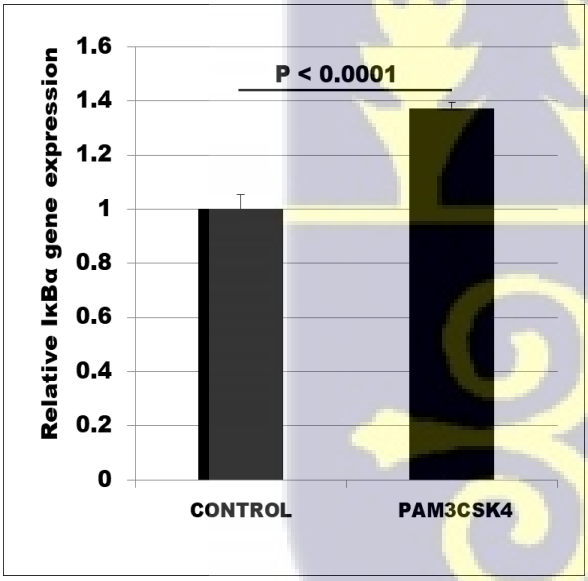
To do that, Pam3CSK4 was used to stimulate RAW Blue cells for 24 hours and then RNA was isolated and assayed by RT-qPCR for expression of the genes TLR2, NF-κB1 (p105) and IκBα, the main negative regulator of NF-κB. The results showed that, after stimulation for 24 hours, the transcript levels of TLR2 ($P < 0.0001$) (Fig.4.4A) and NF-κB1 ($P = 0.001$) (Fig.4.4B) were significantly down-regulated in Pam3CSK4-stimulated cells relative to control. This is in sharp contrast to the result of IκBα (Fig. 4.4C), where it shows that Pam3CSK4-stimulated cells had significantly ($p < 0.001$) higher levels of expression of the gene relative to un-stimulated cells.



A



B



C

Figure 4.4: Pam3CSK4 induced differential effects on expression levels of TLR2, NF-κB1 and IκBα in Raw Blue cells. The cells were stimulated with Pam3CSK4 (100ng/mL) for 24 hours. After the incubation period, RNA was isolated and assayed by RT-qPCR for expression of the genes of (A) TLR2, (B) NF-κB1and (C) IκBα. Data is a representative of two independent experiments each conducted in duplicate wells. All values are presented as means and standard deviations. $^{*}P < 0.05$ in comparison with control.

4.5 Cryptolepine attenuated Pam3CSK4-induced transcript levels of key NF-κB -regulated genes

The transcription factor NF-κB is known to regulate transcription of numerous genes including pro-inflammatory mediators, receptors such as TLRs, signaling molecules such as NFκB1 (p105), IKKβ and Rel A, among others. From the observation that cryptolepine inhibited the activity of the TLR1/TLR2-NF-κB signaling pathway (Fig. 4.3), it was hypothesized that the effect would translate into inhibition of expression of actual NF-κB-regulated genes including pro-inflammatory cytokines, chemokines, signaling molecules and toll-like receptors. RT-qPCR was used to determine the effect of cryptolepine (1 µM) on expression of IL-1β, IL-6, TNF-α, IL-23, MCP-1, MIP-2α, IKKβ, NFκB1, RelA, TLR2 and TLR1 genes, which play key roles in inflammation and inflammatory diseases.

The results showed that, relative to unstimulated cells, stimulation of the cells with Pam3CSK4 alone upregulated the expression of all the genes studied. However, in the presence of cryptolepine, the transcript level of each of the genes was markedly attenuated. Precisely, cryptolepine significantly attenuated the transcript levels of the cytokines IL-1β (81.1 %, $P < 0.0001$), IL-6 (85.9%, $P = 0.001$), TNF-α (81.3%, $P < 0.0001$) and IL-23 (87.4%, $P = 0.001$) (Fig.4.5.1). Cryptolepine also markedly reduced the transcript levels of the chemokines MCP-1 (86.6%, $P = 0.0001$) and MIP-2α (81.6%, $P < 0.0001$) (Fig. 4.5.2).

It also significantly attenuated the transcript levels of the signaling molecules, NFκB1 (81.8%, $P < 0.0001$), RelA (83.2%, $P = 0.0001$) and IKKβ (79.4%, $P < 0.0001$) (Fig. 4.5.3) and the transcript levels of the receptors TLR1 (85.8%, $P < 0.0001$) and TLR2 (85.6%, $P < 0.0001$) (Fig. 4.5.4).

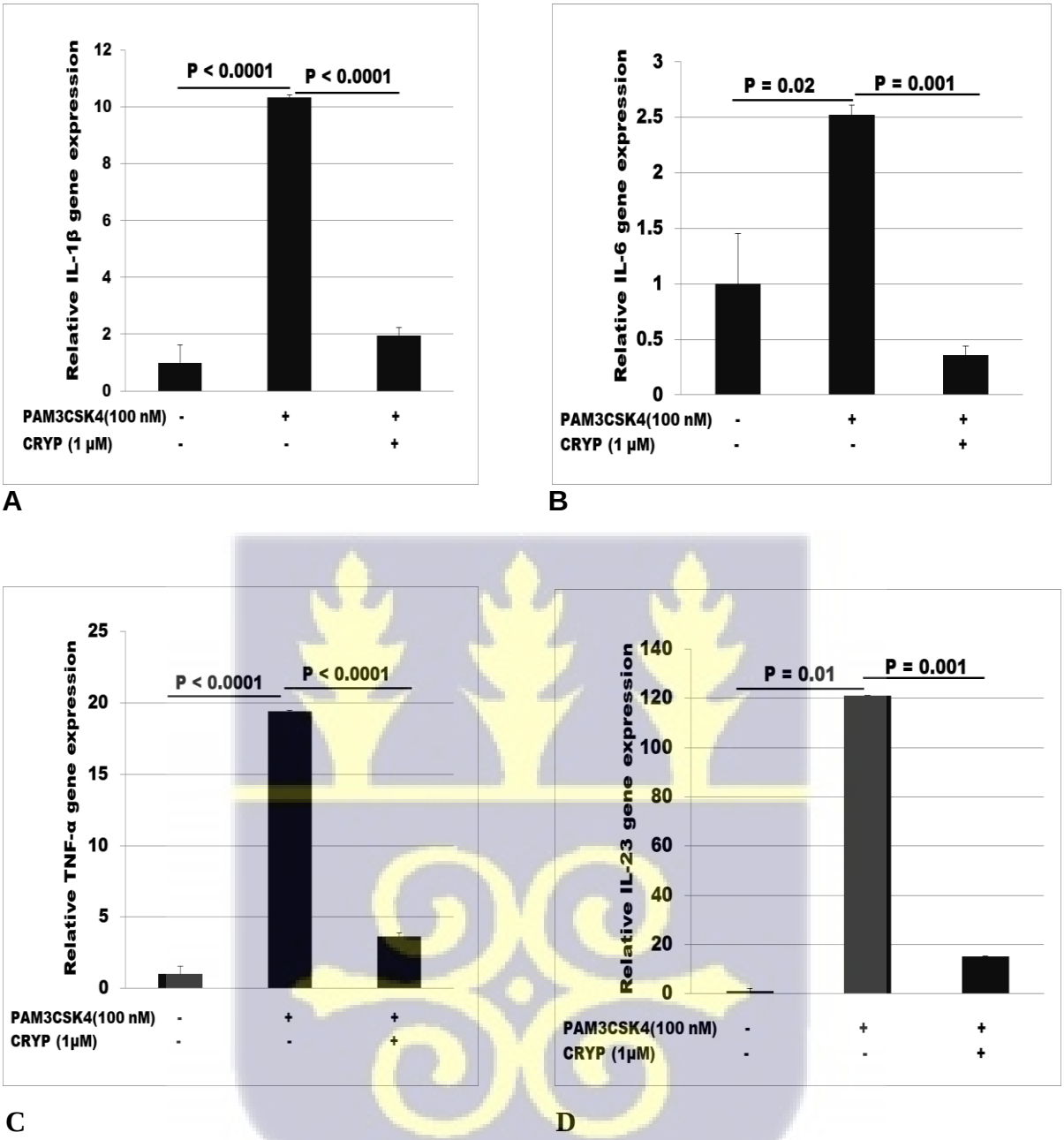
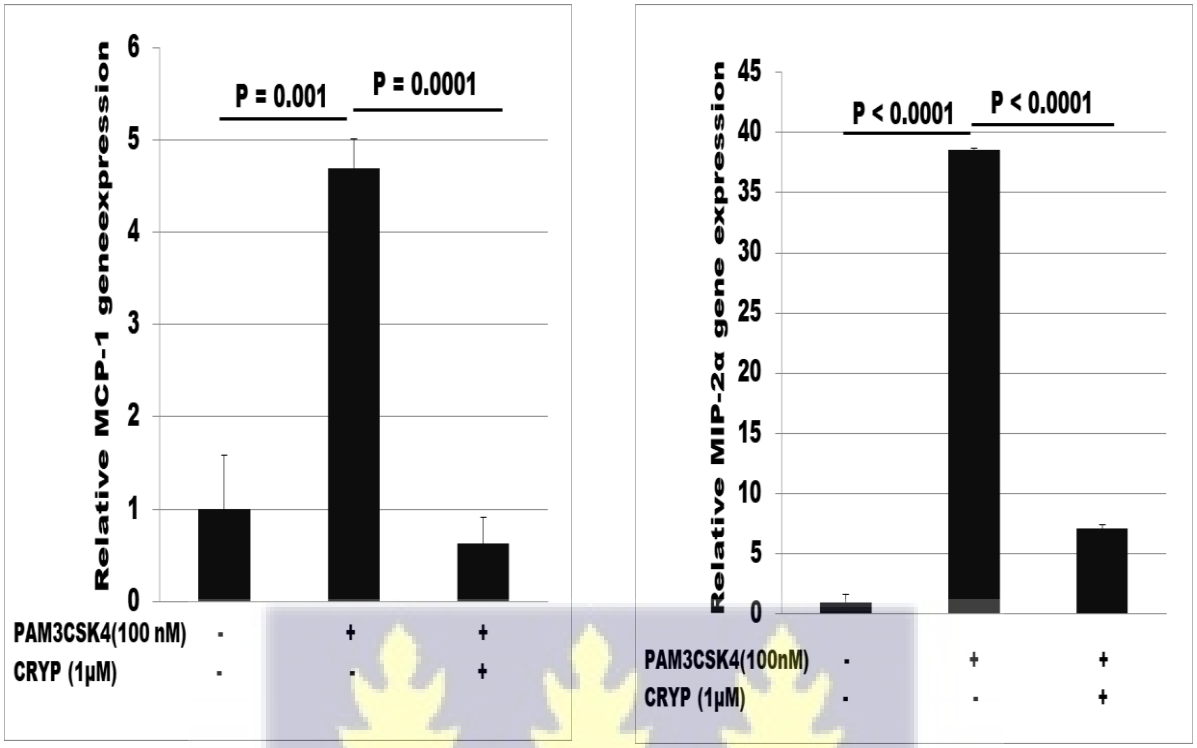


Fig. 4.5.1: Cryptolepine suppressed the transcript levels of pro-inflammatory cytokines in Pam3CSK4-induced RAW Blue cells. The cells were stimulated with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (1 µM) for 1 hour. After the incubation period, RNA was isolated and assayed by RT-qPCR for expression of **(A)** IL-1, **(B)** IL-6 and **(C)** TNF-α and **(D)** IL-23 genes. 18S rRNA was used as an endogenous control. Data are a representative of two independent experiments each conducted in duplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$**.**



A **B**

Fig. 4.5.2: Cryptolepine suppressed the transcript levels of chemokines in Pam3CSK4induced RAW Blue cells. The cells were stimulated with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (1 μM) for 1 hour. After the incubation period, RNA was isolated and assayed by RT-qPCR for expression of **(A)** MCP-1 and **(B)** MIP-2α genes. 18S rRNA was used as an endogenous control. All values are presented as means and standard deviations for experiment conducted in duplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$.

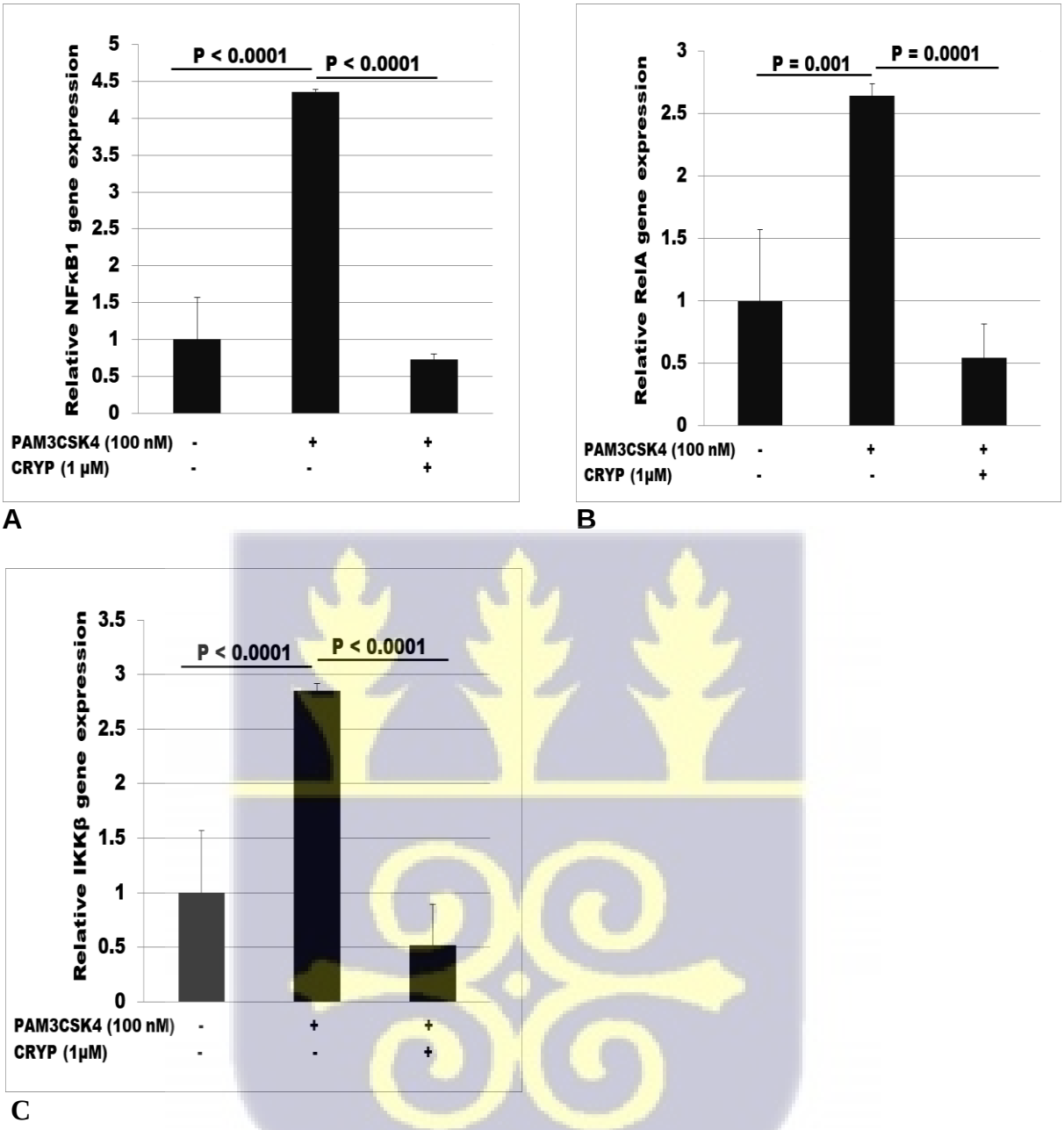
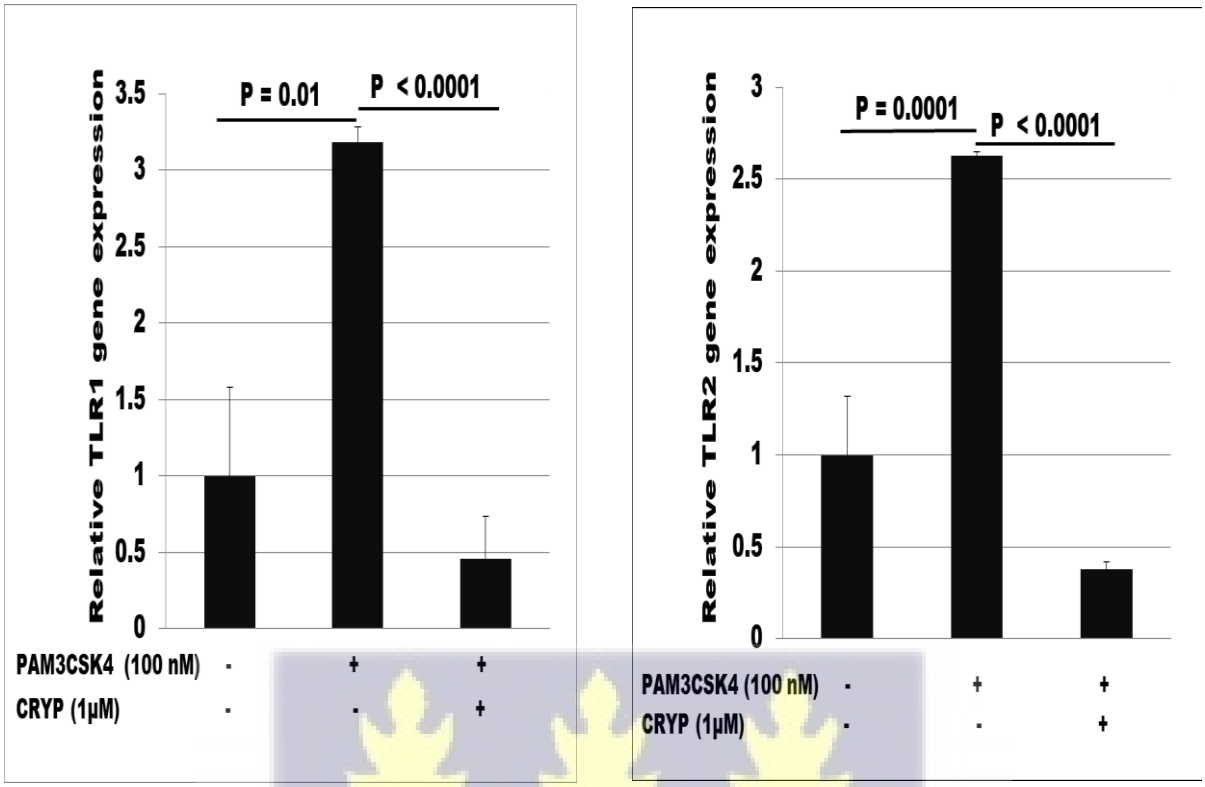


Fig. 4.5.3: Cryptolepine suppressed the transcript levels of key NF-κB pathway signaling molecules in Pam3CSK4-induced RAW Blue cells. The cells were stimulated with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (1 µM) for 1 hour. After the incubation period, RNA was isolated and assayed by RT-qPCR for expression of **(A)** NF-κB1, **(B)** RelA, and **(C)** IKKβ genes. 18S rRNA was used as an endogenous control. All values are presented as means and standard deviations for experiment conducted in duplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$.



A **B**

Fig. 4.5.4: **Cryptolepine suppressed the transcript levels of TLR1 and TLR2 in Pam3CSK4-induced RAW Blue cells**. The cells were stimulated with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (1 µM) for 1 hour. After the incubation period, RNA was isolated and assayed by RT-qPCR for expression of **(A)** TLR1 and **(B)** TLR2 genes. 18S rRNA was used as an endogenous control. All values are presented as means and standard deviations for experiment conducted in duplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$.

4.6 Cryptolepine suppressed Pam3CSK4-induced production of pro-inflammatory cytokines

Excessive production of pro-inflammatory cytokines is a hallmark of many inflammatory diseases. Having shown in section 4.5 that cryptolepine attenuated the mRNA levels of the pro-inflammatory cytokines and chemokines, multiplex ELISA was used to determine whether cryptolepine would have similar effect on their protein levels as well, given that proteins are the functional end products of genes and that transcript levels of genes do not always correlate with protein levels (Liu *et al.*, 2016). The results showed that the levels of the cytokines were markedly elevated in the Pam3CSK4-stimulated cells relative to unstimulated cells. However, in the presence of cryptolepine (1 µM), the levels of all the cytokines but IL-1β, were markedly suppressed. Precisely, cryptolepine significantly suppressed the levels of IL-6 (79.8%, P = 0.02), TNF-α (6.4%, P = 0.02) and IL-23 (71.4%, P = 0.02) but showed no significant effect on the level of IL-1β (P= 0.59) (Fig.4.6). Comparatively, triptolide, significantly suppressed the levels of IL-6 (67%, P = 0.02), TNF-α (6.4%, P = 0.02) and IL-23 (61.7%, P = 0.02) but showed no significant suppression of the level of IL-1β (P = 0.80).

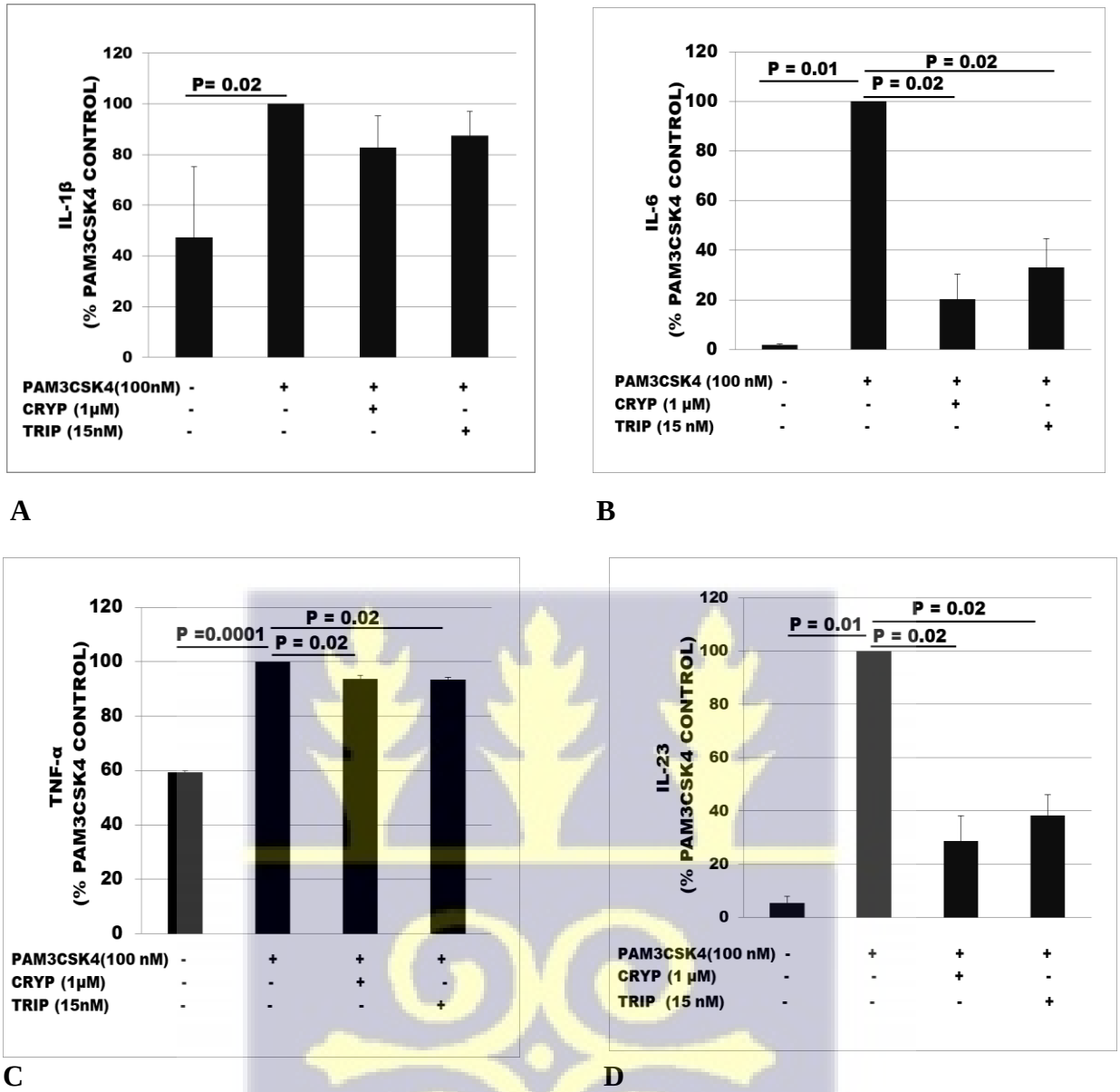
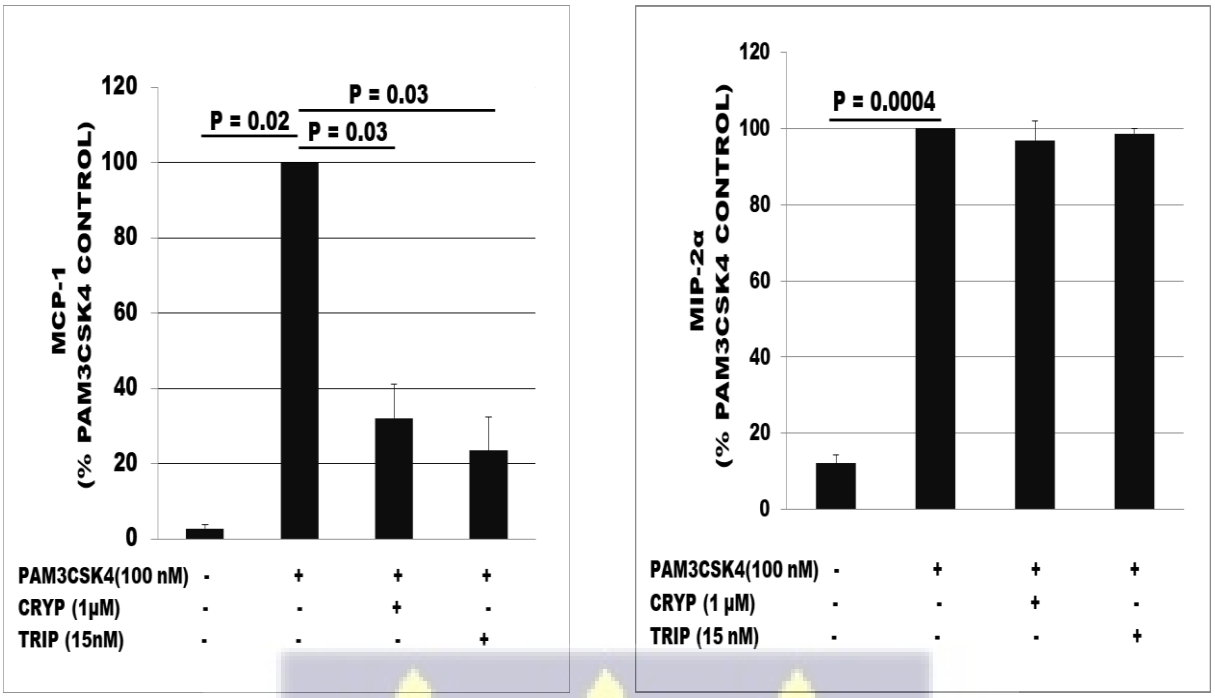


Fig.4.6: Cryptolepine suppressed the levels of key pro-inflammatory cytokines in Pam3CSK4-induced RAW Blue cells. The cells were stimulated with or without Pam3CSK4 (100 ng/mL) in the presence or absence of cryptolepine (1 μM) for 24 hours. Triptolide was used as a positive control. At 24 hours post incubation, supernatants were collected and the levels of **(A)** IL1-β, **(B)** IL-6, **(C)** TNF-α and **(D)** IL-23 measured by multiplex ELISA. The results are expressed as percentage (%) by normalizing the value of each sample to the value of Pam3CSK4-stimulated cells. All values are presented as means and standard deviations for two independent experiments each conducted in duplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$.

4.7 Cryptolepine suppressed Pam3CSK4-induced production of a C-C chemokine but not a C-X-C chemokine

Chemokines are a special group of cytokines that induce chemotaxis. They primarily attract immune and inflammatory cells to inflammatory sites. Having observed that the transcript levels of MCP-1, a C-C chemokine and MIP-2α, a C-X-C chemokine, were attenuated by cryptolepine (Fig. 4.5.2A and 4.5.2B), the effect of the alkaloid was subsequently tested on the protein levels of the chemokines. The results show that the levels of the chemokines were markedly elevated in the Pam3CSK4-stimulated cells relative to unstimulated cells (Fig.4.7). In the presence of cryptolepine (1 µM), the level of MCP-1 was significantly suppressed (67.9%, P = 0.03) (Fig. 4.7A). Comparatively, triptolide, significantly suppressed the level of MCP-1 (76.5%., P = 0.03). In contrast to the result of MCP-1, there was no significant suppression in the level of MIP-2α (Fig. 4.7B) by cryptolepine (P = 0.45) and triptolide (P = 0.90).



A **B**

Fig. 4. 7: Cryptolepine suppressed the level of MCP-1 but not MIP-2α in Pam3CSK4induced RAW Blue cells. The cells were stimulated with Pam3CSK4 (100 ng/mL) in the absence or presence of cryptolepine (1 μM) for 24 hours. Triptolide was used as a positive control. At 24 hours post incubation, supernatants were collected and the levels of **(A)** MCP-1 and **(B)** MIP-2α measured by multiplex ELISA. The results are expressed as percentage (%) by normalizing the value of each sample to the value of Pam3CSK4-stimulated cells. All values are presented as means and standard deviations for two independent experiments each conducted in duplicate wells. Data were analyzed by one-way ANOVA of the differences within treatments followed by Tukey's *post hoc* test. $P < 0.05$.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

Cytotoxicity of Cryptolepine

The use of many anti-inflammatory drugs for management of chronic inflammatory conditions is fraught with many challenges, key among which is toxicity, thus underscoring the need for development of novel and safer anti-inflammatory agents (Henry & McGettigan, 2003). In the present study, the anti-inflammatory effect of cryptolepine was determined by first determining its cytotoxicity to know the maximum concentration of the compound with minimum toxicity to be used for the assays. The result indicates that, cryptolepine, at 1 µM showed no significant cytotoxicity whereas beyond that concentration, cytotoxicity was high. This makes 1 µM the maximum non-toxic concentration of cryptolepine that was used in testing for its anti-inflammatory effects.

Several other studies have demonstrated similar cytotoxic property of cryptolepine in different cell lines. For example, Ansah and Gooderham (2002) showed that cryptolepine dose-dependently reduced the viability of Chinese hamster lung fibroblast, which is similar to the pattern of cytotoxicity demonstrated in this study. Furthermore, consistent with the outcome of the present study, the result of Ansah and Gooderham (2002) also showed that 1 µM was the maximum concentration with the minimal cytotoxic effect. Another study by Pal *et al* (2017) also showed that cryptolepine at 1 µM showed no significant cytotoxic effect on normal human epidermal melanocytes (NHEM). Together, these reports show the tolerability of the three different cell lines to the 1 µM concentration and suggest the safety of that concentration for *in*

vitro studies and the possibility of using that concentration as a basis for extrapolating the dosage to be used in *in vivo* studies and possible future clinical trials.

Studies have suggested the mechanism underlying the cytotoxic effect of cryptolepine to be through its inhibition of activity of topoisomerase II, inhibition of DNA synthesis and arrest of cell cycle in G2/M phases (Bonjean *et al*., 1998; Lisgarten *et al*., 2002).

Effect of cryptolepine on the activity of the TLR1/TLR2-NF-κB signaling pathway

The TLR1/TLR2-NF-κB signaling pathway is one of the numerous pathways that culminate in activation of NF-κB, making it a good pathway for the study of effects of anti-inflammatory compounds. The effect of cryptolepine on the activity of the TLR1/TLR2-NF-κB signaling pathway was investigated after determining its maximum non-toxic concentration to be 1µM. The result shows that cryptolepine markedly suppressed the expression of SEAP, which was used as a reporter gene inducible by the promoter of NF-κB to serve as a marker of the activity of the pathway. Importantly, the time course of NF-κB activation differs from one stimulus to the other with cytokine stimuli such as TNFα or IL-1β causing rapid and transient NF-κB activation whereas stimuli acting through the TLRs result in slower and more sustained NF-κB activation (Werner *et al.*, 2005; Vallabhapurapu and Karin, 2009). Specifically, Damien *et al.* (2017) also showed that, NF-κB activation via Pam3CSK4-mediated stimulation of TLR2 is slow and sustained. This suggests that, since cryptolepine has shown significant inhibitory effect on Pam3CSK4-induced activity of the TLR1/TLR2-NF-κB signaling pathway, the alkaloid may inhibit inflammation in diseases associated with sustained TLR2-mediated NF-κB activation.

Dysregulated NF-κB signaling resulting from hyper-activated NF-κB is reported to cause many diseases including cancers and insulin resistance (Wong & Tergaonkar, 2009). Therefore,

suppression of the activity of the TLR1/TLR2-NF-κB signaling pathway by cryptolepine as shown in this study suggests that the effect may translate into the suppression of expression of the actual NF-κB -regulated genes with pro-inflammatory activities and hence may result in suppression of inflammation. Particularly, suppression of expression of pro-inflammatory cytokines and chemokines will reduce the rate of recruitment of inflammatory cells and also minimize the risk associated with hyper-inflammation. Additionally, suppression of the activity of the TLR1/TLR2-NF-κB signaling pathway may lead to inhibition of NF-κB -mediated expression of proteases such as the matrix metalloproteinases which cause extensive damage to synovial tissues of rheumatoid arthritis patients (Shlopov *et al*., 1997). Indeed, previous studies have showed that NF-κB was highly activated in rat models of arthritis (Miagkov *et al*., 1998) as well as in synovial tissues of human arthritis patients (Asahara *et al*., 1995; Marok *et al*., 1996). Other studies by Rogler *et al.* (1998) and Schreiber *et al* (1998) also found NF-κB to be constitutively activated in inflamed colonic tissue samples of IBD patients. These reports add to the body of evidence indicating that hyperactivity of NF-κB is central to the pathogenesis of the afore-mentioned diseases which implies that compounds shown to have significant inhibitory effect on activation of NF-κB, as demonstrated for cryptolepine in this study, may serve as good therapeutic agents for the diseases.

Effect of cryptolepine on expression of NF-κB -regulated genes

NF-κB is known to induce transcription of many genes including cytokines, chemokines, signaling molecules, negative regulators and some Toll-like receptors (Yang *et al.*, 2016). Having shown above that, cryptolepine suppressed the general signaling activity of the TLR1/TLR2-NF-κB pathway, it was hypothesized that, consequently cryptolepine will inhibit the expression of eleven key NF-κB-regulated genes that play crucial roles in activation of the NF-κB signaling pathway and are associated with many inflammatory diseases.

Furthermore, having observed in Fig. 4.4 that the 24-hour period of stimulation for induction of expression of the selected group of the NF-κB-regulated genes was inappropriate for achieving a significantly high level of expression of the two main NF-κB-regulated genes chosen (TLR2 and NF-κB1), a relatively shorter period of stimulation, 1 hour, was chosen for induction of their expression by Pam3CSK4. Induction of a significantly high level of expression of a target gene is necessary for allowing a test of effects of an intervention compound on the expression of the gene.

The results indicate that, the transcript levels of *Il-1β, Il-6, Tnf-α, Il-23, Ccl2, Cxcl2, Ikbkb, Nfkb1, Rela, Tlr2 and Tlr1* were significantly up-regulated by Pam3CSK4. However, treatment with cryptolepine significantly attenuated the transcript levels of all the genes, consistent with the inhibitory effect of cryptolepine on the activity of the TLR2/TRL1-NF-κB signaling pathway explained above. This confirms that the inhibitory effect of cryptolepine on the activity of the TLR1/TLR2-NF-κB signaling pathway, truly translates into suppression of expression of actual NF-κB-regulated genes. Suppression of the transcript levels of the genes suggests that cryptolepine interfered at a particular point in the steps of NF-κB activity; either at its point of separation from IκBα, nuclear translocation or the final stage of its induction of gene transcription in the nucleus. Although not confirmed in this study, a previous study had ruled out any effect of cryptolepine on the pre-nuclear stage of NF-κB activation by showing that cryptolepine demonstrated no effect on the cytoplasmic degradation of IκBα or nuclear translocation of NF-κB (Olajide *et al.*, 2007). A plausible explanation for cryptolepine's ability to directly inhibit transcription of NF-κB target genes is its DNA intercalating capacity. A study by Lisgarten *et al.* (2002) demonstrated through competition dialysis assays that cryptolepine intercalates in the DNA by binding CG-rich sequences containing nonalternating CC sites. It is established that DNA intercalating molecules are capable of occupying the major and/or the minor grooves of the DNA helix thereby blocking

or limiting the ability of the DNA binding domains of transcription factors to access DNA sequences and activate transcription of their target genes (Lambert *et al.*, 2018). This assertion is corroborated by the findings of Olajide *et al* (2007) which revealed that cryptolepine inhibited NF-κB activation by blocking DNA binding of the transcription factor which consequently reduced its ability to transcribe its target genes. This might explain why cryptolepine significantly attenuated the transcript levels of all the genes assessed in this study.

Specifically, cryptolepine markedly attenuated the expression levels of *Tlr2* and *Tlr1*. The TLR1/TLR2 heterodimeric receptor detects a wide range of microbial surface molecules (Takeda & Akira, 2005). Binding of ligands such as Pam3CSK4 to the receptor induces intracellular signaling that culminates in the production of many pro-inflammatory mediators, which make the receptor very important in inflammatory responses. Suppression of transcript levels of *Tlr2* and *Tlr1* by cryptolepine suggests that it may further reduce the protein levels of the receptors and consequently reduce their cell surface expressions. This may consequently dampen inflammatory responses associated with TLR1/TLR2 stimulation especially in inflammatory diseases associated with over-expression of the receptors. Indeed a previous study has shown that, *Tlr2* is highly expressed in systemic lupus erythematosus (SLE), which suggests the critical role that it plays in the pathogenesis of the disease (Wu *et al.*, 2015). Thus, suppression of expression of *Tlr2* by cryptolepine shown in this study suggests that the alkaloid may be of good therapeutic value in SLE.

The result also showed that cryptolepine attenuated the transcript level of the gene of IKKβ, a critical kinase which activates the cytoplasmic NF-κB inhibitor, IκBα, leading to its breakdown (Karin & Ben-Neriah, 2000). The catalytic activity of IKKβ is thus the main downstream trigger of NF-κB activation which makes IKKβ an extremely important therapeutic target in the signaling

pathway**.** Therefore, direct inhibition of the catalytic activity of IKKβ or suppression of its expression which reduces its cytolplasmic concentration may significantly decrease the rate of activation of NF-κB and consequently attenuate inflammation. A previous study has shown that, well- known anti-inflammatory drugs like aspirin and salicylate exert their anti-inflammatory activities partly due to inhibition of the activity of IKKβ (Yin *et al.,* 1998). Although there is no data from this study to confirm the suppressive effect of cryptolepine on IKKβ at the protein level, reduction of its transcript level by cryptolepine indicates that the alkaloid interferes with the NF-κB-mediated transcription of the IKKβ gene, possibly due to its DNA intercalation as explained above. Suppression of the transcript level of *Ikbkb* by cryptolepine observed in this study also indicates that the possible suppressive effect of the alkaloid on the protein level may confirm it to be of good therapeutic value in inflammatory diseases associated with overexpression of IKKβ. Evidence has shown that high level of IKKβ is associated with inflammatory diseases including RA (Aupperle *et al*., 1999), indicating the significant role the protein plays in the pathogenesis of the disease and also suggest a possible therapeutic value of compounds that inhibit the expression of the protein. This study is the first to show the suppressive effect of cryptolepine on the transcript level of *Ikkβ*.

Cryptolepine suppressed the expression of the genes of NF-κB1and RelA which are also key molecules in the pathway downstream IKKβ**.** NF-κB1 is the precursor protein that undergoes proteolysis to produce p50, a protein that dimerizes with p65 (RelA) to form the classical NF-κB heterodimer. This may suggest that increased expression of *Nf-κb1* and *Rela* could contribute to elevation of the cytoplasmic pool of NF-κB and this may increase its activation and subsequent expression of pro-inflammatory mediators. Therefore, suppression of the expression of *Nf-κb1* and

Rela by cryptolepine may lead to reduction in the cytoplasmic pool of NF-κB and this may consequently attenuate the rate of activation of NF-κB.

Consistent with the suppression of the activity of the TLR1/TLR2- NF-κB signaling pathway, cryptolepine also suppressed the expression of genes of key pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, IL-23, MIP-2α, MCP-1 and protein levels of TNF-α, IL-6 and IL-23 which are known to play significant roles in the orchestration of inflammatory responses. Specifically, TNF-α is known to play a central role in the induction of expression of adhesion molecules and inflammatory mediators (Rubio-Perez & Morillas-Ruiz, 2012). Furthermore, there is evidence to suggest that these cytokines are major contributors to the pathogenesis of a number of inflammatory diseases. For instance, TNF-α is reported to be elevated in IBD and RA (Feldmann *et al.*, 1996; Pallone and Monteleone, 2001). IL-6 is implicated in the pathogenesis of Crohn's disease and RA while IL23 is reported to play a role in psoriasis and schizophrenia (Nishimoto and Kishimoto, 2004; Croxford *et al.*, 2014). Therefore, suppression of the transcript and protein levels of these cytokines by cryptolepine, observed in this study, suggests that the alkaloid may be of good therapeutic value in these diseases. It is worth noting that, NF-κB-mediated transcription of *Tnf-α* leads to the release of TNF-α protein which also by autocrine and paracrine positive feedback loops binds to the TNF receptor 1 (TNFR1) to induce further activation of NF-κB that leads to production of more TNF-α and other pro-inflammatory mediators (Beg & Baltimore, 1996; Coward *et al.*, 2002; Wu *et al.*, 1993). Production of TNF-α therefore leads to a continuous cycle of NF-κB activation with each cycle increasing the level of the cytokine. Therefore, suppression of the production of TNF-α is of great therapeutic value because it will lead to attenuation of secondary NF-κB activation induced by the autocrine and paracrine actions of the cytokine. Suppression of both the transcript and proteins levels of TNF-α demonstrated for

cryptolepine in this study suggests that the alkaloid may attenuate the TNF-α-mediated positive feedback loop and this may significantly attenuate inflammation. Suppression of the transcript and protein levels of the pro-inflammatory cytokines by cryptolepine is consistent with the findings of Olajide *et al* (2013) which showed that cryptolepine suppressed the expression of major pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β in LPS-stimulated cells. Thus, despite the difference between the agonist (Pam3CSK4, which acts via the TLR1/TLR2- NF-κB pathway) used in this study and the one used by Olajide *et al* (2013) (LPS, which acts via the TLR4- NF-κB pathway), the suppressive effect of cryptolepine on production of the major pro-inflammatory cytokines from the two pathways follows a similar fashion. This suggests that the suppressive effects of cryptolepine on downstream activity of NF-κB and expression of its target genes are not affected by the type of toll-like receptor activated upstream.

Aside from the cytokines, cryptolepine also suppressed the transcript and protein levels of the chemokines, MCP-1 and MIP-2α, albeit the suppression in the protein level of the latter was not significant. Chemokines are major players in inflammation as they are chemoattractant molecules that induce cellular chemotaxis and guide immune cells to inflammatory sites. Specifically, MCP-1, a C-C chemokine, attracts monocytes whereas MIP-2, a C-X-C chemokine, is known to recruit neutrophils and hematopoietic stem cells to sites of inflammation (Moser *et al*., 2004; Pelus & Fukuda, 2006). Previous studies have also shown that MCP-1 is highly produced in synovial fluid of arthritis patients and in chronic obstructive pulmonary disease (COPD) patients (Barnes, 2004; Szekanecz *et al*., 2010). Therefore, suppression of the level of MCP-1 shown by cryptolepine suggests that the alkaloid may attenuate the release of MCP-1 and inhibit recruitment of monocytes to the site of inflammation.

In summary, the results of this study suggest that cryptolepine exerted its anti-inflammatory activities by modulating the NF-κB signaling pathway. Based on the observations from this study, the possible mechanism of action of cryptolepine in inflammation is proposed in the scheme below (Fig. 5.1)

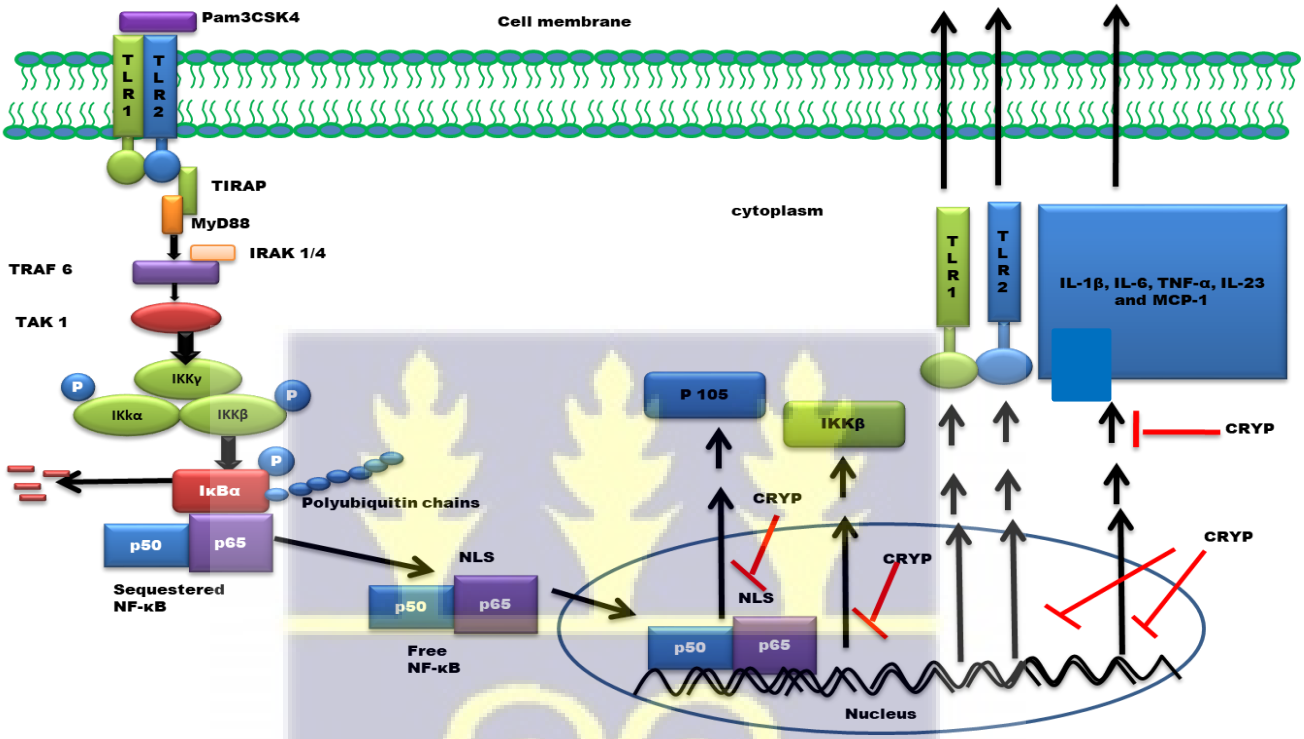


Fig. 5. 1: Model mechanism of cryptolepine-mediated inhibition of inflammation via the TLR1/TLR2-NFκB signaling pathway in Pam3CSK4-stimulated RAW Blue cells. NF-κB is normally sequestered in the cytoplasm through association with IκBα. After binding of Pam3CSK4 to the TLR1-TLR2 heterodimeric receptor, the pathway gets activated. The activated receptor would recruit the adaptor molecules such as TIRAP and MyD88, which would lead to the activation of IRAK4. The actions of IRAK4 would lead to the activation of TRAF6 which would in turn activate TAK1. Activated TAK1 would phosphorylate IKKβ which would then become activated to phosphorylate IκBα resulting in its polyubiquitination and subsequent degradation, thereby freeing NF-κB to translocate into the nucleus and induce transcription of its target genes. In the nucleus NF-κB would induce the expression of pro-inflammatory cytokines, chemokines, TLR1, TLR2, IKKβ and p105. Cryptolepine (CRYP) would inhibit the pathway activity by suppressing the transcription of the genes of IL-1β, IL-6, TNF-α, IL-23, MCP-1, IKKβ, p105, RelA, TLR2 and TLR1. Cryptolepine would also attenuate the protein levels of IL6, TNF-α, IL-23 and MCP-1.

5.2 CONCLUSIONS

The present study has shown that the maximum non-toxic concentration of cryptolepine on RAW Blue cell line is 1 µM. The study also provides evidence that cryptolepine inhibited inflammation by inhibiting the activity of the TLR1/TLR2-NF-κB signaling pathway. The study has also shown that cryptolepine attenuated the expression of the genes of TNF-α, IL-6, IL-1β, IL-23, MIP-2α, MCP-1, IKKβ, NFκB1, RelA, TLR2 and TLR1 and the protein levels of IL6, TNF-α , IL-23 and MCP-1 which are key pro-inflammatory cytokines. The results therefore indicate multiple therapeutic targets of the alkaloid along the NF-κB signaling pathway. These findings suggest that cryptolepine may be a good therapeutic agent for inflammatory diseases associated with over-expression of the afore-mentioned pro-inflammatory mediators and signaling molecules.

5.3 RECOMMENDATIONS

There is the need for further experiments to confirm the inhibitory effect of cryptolepine on the protein levels of the signaling molecules including IKKβ, NF-κB1 and RelA as well as those of the receptors, TRL2 and TLR1.

It is also imperative to compare the effects of cryptolepine with its analogues to determine which one has the lowest cytotoxic effects and the highest inhibitory effects on the activity of the TLR1/TLR2-NF-κB signaling pathway and expressions of pro-inflammatory mediators and signaling molecules.

Lastly, as this study was carried out in murine macrophages, there is the need to repeat it in human inflammatory cells such as macrophages, neutrophils and epithelial cells which play very important roles in many human inflammatory diseases. This will augment the evidence supporting the anti-inflammatory activities of cryptolepine.

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APPENDICES

Appendix A

Table 1: Chemicals and reagents used

Chemical /Reagent	Composition	Manufacturer/ Supplier
Complete Dulbecco's modified Eagle's medium (DMEM)	Glucose, L-Glutamine, Sodium Pyruvate and Foetal Bovine Serum	ScienCell, USA
Normocin™	Normocin	Invivogen, France
Zeocin™	Zeocin	Invivogen, France
Penicillin-Streptomycin	Penicillin and streptomycin	Amreso, United Kingdom
Pam3CSK4	Tricylated lipopeptide	Invivogen, France
Quanti-Blue	Unknown	Invivogen, France
Cryptolepine	Natural cryptolepine dissolved in distilled water	Faculty of Pharmacy, KNUST
Triptolide	Triptolide dissolved in dimethyl sulfoxide	Invivogen, France
Trypan Blue solution	Trypan blue dye prepared in 0.081% sodium chloride and 0.06% potassium phosphate	Sigma Aldrich, Germany
Dimethyl sulfoxide (DMSO)	Dimethyl sulfoxide	Sigma Aldrich, Germany
Phosphate Buffered Saline	NaCl, Phosphate and KCl.	Oxoid limited, UK
Nuclease-Free Water	Water	Promega, USA

Appendix B

Table 1: Buffers and solutions used

Buffer	Composition	Manufacturer/Supplier
RNA Lysis Buffer	4 M guanidine isothiocyanate 0.01 M Tris (pH 7.5) 0.97% βmercaptoethanol	Promega, USA
Yellow Core Buffer	0.0225 M Tris (pH 7.5) 1.125 M NaCl 0.0025% yellow dye (w/v)	Promega, USA
DNase incubation mix	40 µL Yellow Core Buffer, 5 µL 0.09 M $MnCl_2$ and 5 µL of DNase I enzyme	Promega, USA
DNase Stop Solution	2 M guanidine isothiocyanate, 4 mM Tris-HCl (pH 7.5) and 57% ethanol	Promega, USA
RNA Wash Solution	60 mM potassium acetate, 10 mM Tris-HCl (pH 7.5 at 25°C) and 60% ethanol	Promega, USA
PBS buffer, 10X (per liter)	11.5 g Na2HPO4 2 g KH2PO4 80 g NaCl 2 g KCl	Promega, USA
Mouse Standard Cocktails A and B	Lyophilized recombinant mouse biomarkers in a buffered protein base with preservatives	R & D systems, USA
Mouse Magnetic Premixed Microparticle Cocktail	0.6 mL of a concentrated microparticle cocktail with preservatives	R & D systems, USA
Mouse Premixed Biotin-Ab Cocktail	0.6 mL of a concentrated biotinylated antibody cocktail with preservatives	R & D systems, USA
Streptavidin-PE Concentrate	0.250 mL of a concentrated streptavidin-phycoerythrin conjugate with preservatives	R & D systems, USA
Assay Diluent RD1W	11 mL of a buffered protein base with preservatives	R & D systems, USA

Calibrator Diluent RD6-52	21 mL of a buffered protein base with preservatives.	R & D systems, USA
Wash Buffer Concentrate	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative	R & D systems, USA

Appendix C:

Table 1: Antibodies

Antibody	Dilution	Supplier
Mouse Magnetic Premixed Microparticle Cocktail	1:11	R & D systems, USA
Mouse Premixed Biotin-Ab Cocktail	1:11	R & D systems, USA

Appendix D:

Table 1: RT-qPCR cycle conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLE
Reverse Transcription	55°C	10 minutes	1
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	10 seconds	40
Extension	60°C	60 seconds	40
Melt Curve	95°C	1 minute	1

Table 2: Taqman RT-qPCR reaction set-up

REACTION COMPONENT	VOLUME (µL) / REACTION
Taqman RT Enzyme Mix (40X)	0.5
Taqman RT-PCR Mix (2X)	10
Taqman Gene Expression Assay (20X)	1
Template RNA	1.5
Nuclease-free water	7
Total Volume	20

Appendix E: Supplementary figure

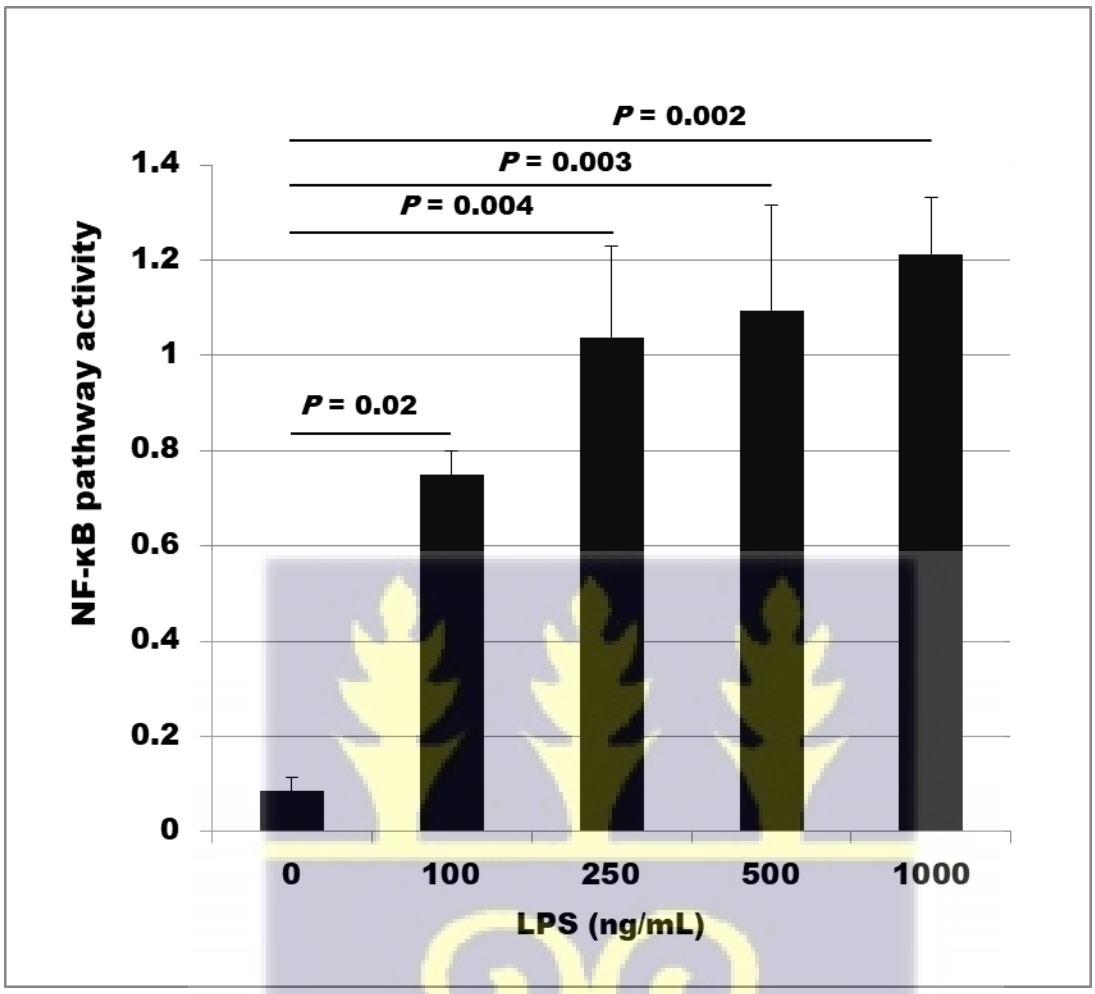

Figure S1: Induction of NF-κB pathway activity by LPS in RAW Blue cells. The cells were stimulated with LPS (100 - 1000 ng/mL) for 24 hours. At 24-hours post incubation, supernatants were collected and the activity of the NF-κB signaling pathway assessed by evaluating the expression levels of secreted embryonic alkaline phosphatase (SEAP) by Quanti Blue assay. The result is presented as means and standard deviations for two independent experiments each conducted in triplicate wells. Data were analyzed by multiple comparisons of treatments vs. control as determined by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc test*. $P < 0.05$