Polyadenylation as a target for anti-inflammatory drugs

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Declaration

Except where acknowledged in the text, I declare that this dissertation is my own work and is based on research that was undertaken by me in the School of Pharmacy, Faculty of Science, University of Nottingham.

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Abstract

Polyadenylation is a universal step in the maturation of all eukaryotic mRNAs except histone mRNAs. Despite being universal, previous experiments have implicated it in the regulation of inflammatory response. An inflammatory system using RAW 264.7 murine macrophage cells was established with bacterial lipopolysaccharide (LPS) used as a stimulus.

The inflammatory response is a tightly regulated reaction. It involves both signals that initiate and maintain inflammation and signals that shut the process down. An imbalance between the two signals leaves inflammation unchecked, resulting in cellular and tissue damage. Macrophages are widely-distributed innate immune cells and considered as a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes, and tissue resident macrophages. Stimulation of macrophages with LPS results in the production of various cytokines (e.g., TNF-α and IL-1β) and inflammatory effectors (e.g., prostanoids, leukotrienes, and nitric oxide). NF-κB is the master regulator of the inflammatory response in macrophages. Upon phosphorylation and subsequent degradation of its inhibitor, IκBα, the liberated NF-κB dimer translocates to the nucleus and switches on the transcription of genes controlling inflammation, immunity, cell proliferation and apoptosis. PI3K/Akt/mTOR signal transduction pathway has been implicated in the regulation of both innate and adaptive immunity.

Cordycepin is one of the major bioactive substances produced by *Cordyceps militaris*, a parasitic fungus used in the traditional Chinese medicine. It is an adenosine analogue that lacks the 3’ hydroxyl group, and therefore functions as a chain

terminator if phosphorylated and incorporated into a growing polyadenylation reaction. Cordycepin was shown to possess an anti-inflammatory effect both in cell culture and in animal models of inflammation associated-disorders.

The role of cordycepin in regulating the expression of inflammatory genes in macrophages, essential transcription factors as well as its involvement in PI3K/Akt/mTOR signal transduction pathway were studied. Cordycepin was shown to have fast anti-inflammatory effect and it is likely to be primary in nature. Cordycepin was also shown to repress the inflammatory gene expression at the transcriptional level. It was found to interfere with NF-κB signalling. It reduced the nuclear localization of p65 in macrophages, but this effect was not IκBα degradation-dependent. Furthermore, cordycepin has shown inhibitory effect on PI3K/Akt/mTOR signal transduction pathway by reducing the phosphorylation of both Akt and 4EBP, and increasing the phosphorylation of AMPK. It was also found to affect the subcellular localization of the key components of this signalling pathway. PI3K/Akt pathway was addressed to be an upstream regulator of NF-κB pathway.

Depleting polyadenylation factors (Cpsf4 and Wdr33) in macrophages was associated with repression of the LPS-induced inflammatory gene expression, reduction in the nuclear localization of p65 and inhibition of PI3K/Akt signalling. This suggests that polyadenylation factors is an upstream regulator of NF-κB pathway and PI3K signal transduction pathway. Thus, cordycepin produces its anti-inflammatory effect in macrophages through is effect on polyadenylation. Furthermore, LPS and cordycepin treatment affected the localization of polyadenylation factors, suggesting that polyadenylation machinery is implicated in the inflammatory response.

Inhibition of either import of cordycepin into cells or phosphorylation of cordycepin was sufficient to abolish its anti-inflammatory effects. It was found that there is no bioavailable cordycepin in rat's plasma after oral administration (Lee 2019), 3'deoxyinosine was considered an inactive metabolite. Our results showed that 3'deoxyinosine has anti-inflammatory effect as it repressed the relative mRNA level of the assessed genes (Tnf, Il1b, Acod1 and Ticam) in macrophages.

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List of abbreviations

4EBP	**eukaryotic translation initiation factor 4E-binding protein**
A	adenine
ADA	adenosine deaminase
ADK	adenosine kinase
AMPK	AMP-activated protein kinase
AMPK	AMP-activated serine/threonine protein kinase
APA	alternative polyadenylation
C	cytosine
CD	cluster of differentiation
cDNA	complementary DNA
CF	cleavage factor
COL10a1	Collagen type X alpha 1 chain
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding protein
CstF	cleavage stimulating factor
DAMP	disease-associated molecular pattern
DNA	deoxy ribonucleic acid

DUSP	Dual specificity phosphatase
DWNN	domain with no name
eIF4E	eukaryotic translation initiation factor 4 E
eIF4G	eukaryotic translation initiation factor 4 G
Fip1	factor interacting with papola and CPSF1
G	guanine
GC	glucocorticoid
hFip1	human Fip1
IgM	immunoglobulin M
IL1B	interleukin 1 beta
Inos2	inducible nitric oxide synthetase
IRAK	IL-1 receptor-associated kinase
LPS	lipopolysaccharide
miRNA	microRNA
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation factor 88

NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization signal
OA	osteoarthritis
PABP	Poly (A) binding protein
PABP	poly (A) binding protein
Paip	PABP-interacting proteins
PAMP	pathogen-associated molecular pattern
PAN	poly(A) Specific Ribonuclease Subunit Homolog
PAP	Poly (A) poymerase
Papola (PAPα)	poly (A) polymerase alpha
Papolg (PAPγ)	poly (A) polymerase gamma
PARN	Poly (A)-Specific Ribonuclease
Pcf11	cleavage and polyadenylation factor subunit
PDK1	protein3-phosphoinositide-dependent protein kinase-1
Peli1	Pelino-1
PI3K	Phosphoinositide 3-kinases
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B

RBBP6	retinoblastoma-binding protein 6
RNA	ribonucleic acid
SG	stress granule
SGK	serine/threonine-protein kinases
Star-PAP	speckle targeted PIPKIa regulated poly(A) polymerase
T	thymine
TAK1	transforming growth factor beta-activated kinase 1
TF	transcription factor
TIA-1	T cell intracellular antigen-1
TLR	Toll like receptor
TNF-α	tumour necrosis factor-alpha
TRAF	TNF receptor associated factor
TUT	Terminal Uridylyl Transferase
U	Uracil
UTR	untranslated region
UTR	untranslated region
VEGF	vascular endothelial growth factor
VSV	vesicular stomatitis virus

VSV	vesicular stomatitis virus
WDR33	WD repeat domain 33
ZF	Zinc finger

1. Introduction

It is well established that gene expression is regulated at multiple levels, and cells need to integrate and coordinate different layers of control to implement the information in the genome. Gene transcription has received the most attention, both through traditional studies and via recent genome-wide approaches such as expression profiling, location analyses of transcription factors and global chromatin remodelling. However, post-transcriptional regulation, including the processing, export, localization, turnover and translation of messenger ribonucleic acid (mRNAs), adds substantial complexity to the control of gene expression (Mata 2005). In this introduction, I will discuss the role of regulating 3' mRNA end processing in fine-tuning the expression of inflammatory genes in macrophages and the involvement of 3' processing regulation in controlling signal transduction pathways and important transcription factors will be discussed. Cleavage and polyadenylation is an essential step in the 3' processing of most eukaryotic mRNAs. This reaction involves two nuclear steps which are the cleavage of the pre-mRNA and then the addition of a poly (A) tail to the 3'untranslated region (UTR). The process is governed by *cis*-acting elements which are located around the cleavage site in the nascent mRNA and trans-acting factors that bind to them (Tian 2012). In spite of the simple composition of the poly (A) tail, it has a critical impact on the mRNA life cycle. First, it helps in transporting the fully processed mRNA from the nucleus to the cytoplasm. Furthermore, it enhances the translational efficiency. Finally, polyadenylation has a central role in regulating the quality control and turnover of mRNA (Jalkanen 2015). The regulation of 3' UTR formation has an important impact on many biological processes such as blood coagulation and innate immunity (Gehring 2001 and Peterson 2011). In addition, misregulation in mRNA 3′ end processing has been associated with pathological consequences, including cancer (Hollerer 2012).

Inflammation is a beneficial response to tissue injury and infection that aims to restore the normal tissue function. Proinflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions (Zhang 2007 and Janssen 2012).

Medicinal mushrooms produce bio-metabolites which are used or studied to treat a wide variety of medical ailments. Cordycepin, an adenosine analogue, is a polyadenylation inhibitor derived from the caterpillar fungi prized in Far Eastern traditional medicine. Cordycepin is reported to have anti-inflammatory properties (among many others), and this has been shown in cell culture systems as well as animal disease models (Kondrashov 2012, Yang 2015, Kim 2011, Rottenberg 2005, Kim 2006, Shin 2009 and Li 2016). Briefly, this project aimed to gain an insight into the mechanism of action of cordycepin and whether it produces its anti-inflammatory effect through inhibition of polyadenylation or if it does this through other targets. My project is part of a larger study that aims to investigate polyadenylation as a novel therapeutic target in osteoarthritis.

1.1 Polyadenylation

1.1.1 Nuclear polyadenylation

Biochemical studies have shown that there are sequences embedded in the 3' end of pre-mRNAs, *cis*-elements, to control cleavage and polyadenylation. Nearly every known mRNA contains a polyadenylation signal sequence, the hexanucleotide AAUAAA, located 10-30 nucleotide upstream of the respective poly (A) site. AAUAAA is found in 90% of all sequenced polyadenylation sites and is one of the most highly conserved sequence elements known. Most of the 10% of mRNAs that do not have an exact match to the consensus differ by only a single substitution (an A → U conversion at the second position is the most common variant).

Two distinct types of upstream U-rich elements have been identified. They are located either upstream of the poly A signal or between the poly (A) signal and the cleavage site. In addition, an upstream G-rich element was reported to be significantly associated with frequently used poly (A) sites, albeit with a lower frequency of occurrence than the U-rich elements. Downstream of the poly (A) site, 20-40 nucleotides, GU-rich and U-rich elements are located. In contrast to AAUAAA, this GU-rich element is found in ∼70% of mammalian pre-mRNAs and is considerably more variable in sequence composition (Diana 1979 and Guo 2011). Poly (A) sites also contain distal downstream G-rich elements, typically >30 bases from the poly A site which contain runs of guanines that are interrupted by A or U. Finally, a putative C-rich element in the distal downstream region, 40 to 100 nucleotides from the poly (A) site, was reported by a bioinformatics analysis (Tian 2012).

The above mentioned *cis*-regulatory elements were shown to be binding sites for *trans*-acting factors to govern 3' UTR maturation reaction. By combining glycerol gradient sedimentation and RNA tag-based affinity purification, Shi et al have purified the human cleavage and polyadenylation machinery and determined its protein composition. They found that, remarkably, this complex consists of ~85 proteins **(Figure 1.1)**. In addition to known polyadenylation factors, they identified new factors which have an essential role in the 3' mRNA processing and over 50 proteins that mediate crosstalk with other cellular processes such as splicing **(Table 1.1)** (Shi 2009). They proposed a new model for the 3' polyadenylation machinery, in which cleavage and polyadenylation specificity factor (CPSF), cleavage stimulating factor (CstF) and cleavage factor (CF) Im form the "core" 3' processing complex at a 1:2:2 stoichiometry and CF IIm dynamically associates with the "core" complex. Poly (A) polymerase-α (PAPα, Papola) or PAPγ (Papolg) is recruited to the complex at a later stage. Cleavage and polyadenylation specificity factor (CPSF) is a multi-subunit complex comprising of CPSF160 (CPSF1), CPSF100 (CPSF2), CPSF73 (CPSF3) and CPSF30 (CPSF4). It specifically recognizes the poly (A) signal (AAUAAA) element through its CPSF1 subunit. Cleavage stimulating factor (CstF) complex comprises of CstF77 (CSTF3), CstF64 (CSTF2) and CstF50 (CSTF1) subunits. CstF binds to the downstream G/U-rich sequence via CstF64. Cleavage factor (CF) Im complex [CF Im 68 (CPSF6), CF Im 59 and CF Im 25 (CPSF5)] aids with the recognition of the poly (A) signals by the specific processing factor and it plays a role in the additional RNA binding. The function of the CF IIm complex (Pcf11) in 3' mRNA processing

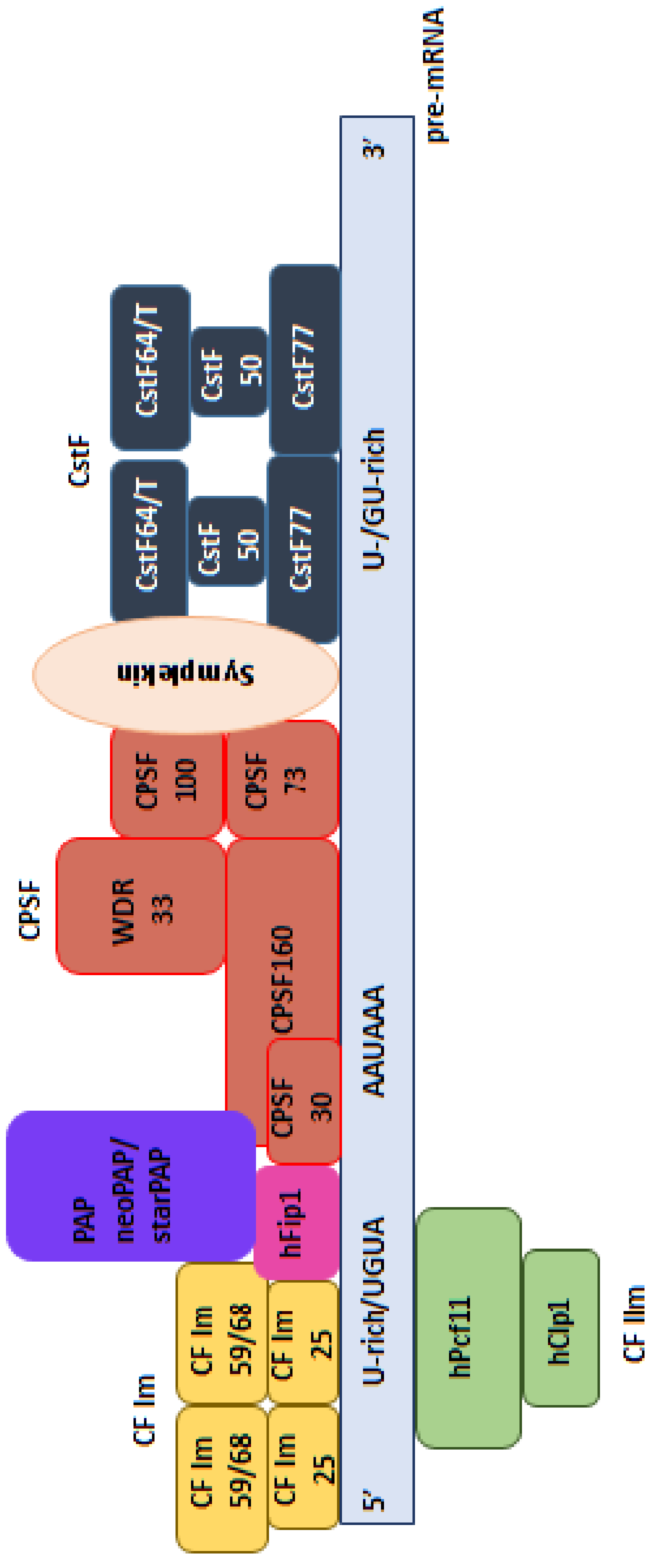


Figure 1.1. A schematic model for the mammalian mRNA 3' processing complex. This model shows CPSF, CstF, and CF Im as the "core" mRNA 3' processing complex. CF IIm, PAP, and other mRNA 3' processing factors such as Rbbp6 are transiently associated with the core complex.

in mammalian cells remains poorly understood (Shi 2009). Symplekin is a nuclear protein that functions in the regulation of polyadenylation and promotes gene expression. It is thought to serve as a scaffold for recruiting regulatory factors to the polyadenylation complex (Kolev 2005). Within the polyadenylation machinery, 3' UTR mRNA processing takes place into two steps. First the endonucleolytic cleavage is completed with the aid of CPSF3 and then the addition of poly (A) tail by poly (A) polymerase ensues (Zhao 1999).

Structural studies recently shed the light on the mechanism of interaction between the *cis*-regulatory element and *trans*-acting proteins. Sun et al. carried out a cryo-electron microscopy to illuminate the molecular mechanism of how poly (A) signals are recognized by the processing factors. They found that a quaternary complex consisting of CPSF1, WD repeat domain 33 (WDR33), CPSF4, and an AAUAAA is formed. CPSF4 recognizes A1 and A2 nucleotides and this is primarily mediated by its zinc finger 2 (ZF2). While the A4 and A5 bases are recognized by ZF3. CPSF4 RNA-binding activity is essential for the entire CPSF complex to interact with RNA. Interestingly, the U3 and A6 bases form an intramolecular Hoogsteen base pair and directly interacts with WDR33. CPSF1 functions as an essential scaffold and pre-organizes CPSF4 and WDR33 for high-affinity binding to AAUAAA (Sun 2017).

Schonemann et al. have shown that only four of the seven putative subunits that constitute CPSF (CPSF1, CPSF4, hFip1, and WDR33) together with recombinant poly (A) polymerase are essential to carry out the AAUAAA-dependent polyadenylation reaction *in vitro*. Genome-wide analysis showed that the CPSF subunit that recognises the polyadenylation signal is WDR33. It has shown that WDR33 and CPSF4 mediate the RNA binding, held together by CPSF1, which is essential for recognising the correct RNA sequence (Schonemann 2014, Shi 2009). Combining cross-linking-coupled mass spectrometry and crystal structure of CPSF1-WDR33 and biochemical assays, Clerici et al. also characterised the molecular architecture of the CPSF complex. They showed that, in addition to the zinc finger domain (ZF2 and ZF3) in CPSF30, an N-terminal lysine/arginine rich motif in WDR33 synergize together to recognize AAUAAA in a sequence specific manner. AAUAAA motif binding occurs at the subunit interface of CPSF1 and WDR33 in close proximity to the WDR33 N-terminus, where the N-terminal lysine/arginine-rich motif of WDR33 and the ZF2 and ZF3 domains of CPSF4 act synergistically to recognize the AAUAAA motif in a sequence-specific manner (**Figure 1.2**). This enables the CPSF complex to bind the AAUAAA motif with subnanomolar affinity and remarkable specificity (Clerici 2017).

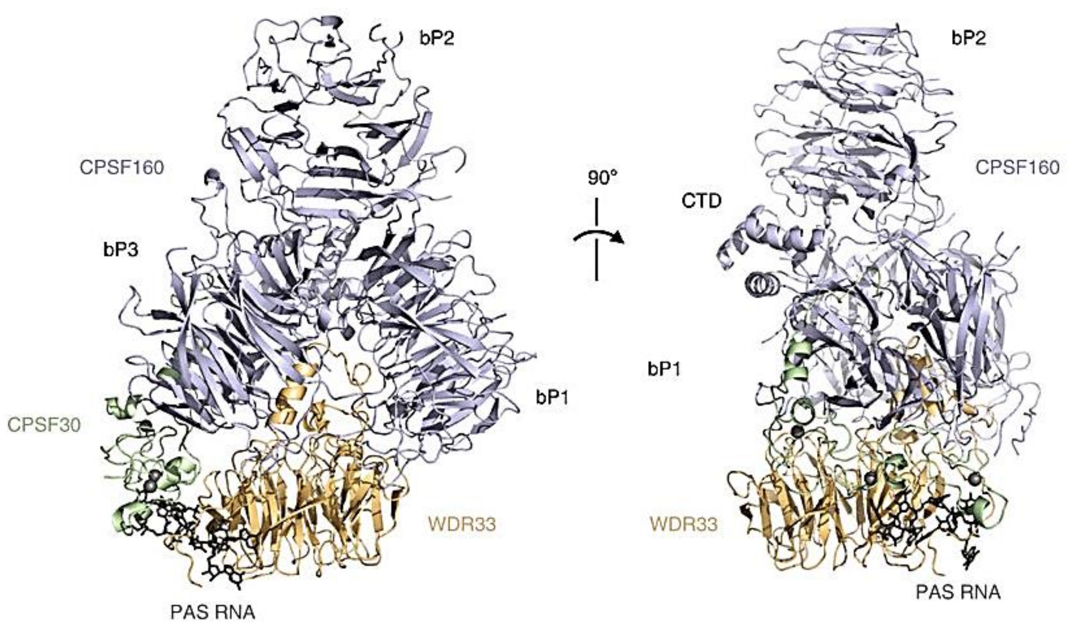


Figure 1.2. Crystal structure of CPSF1-WDR33 complex (Clerici 2018)**.**

CFIm also plays a central role in poly (A) site recognition. It binds to UGUA motifs, which are typically located upstream of the AAUAAA hexamer (Shi 2015). Sequence-specific RNA binding of CFIm can function as the fundamental determinant for the recognition of a human poly A signal in the absence of the A(A/U)UAAA hexamer. Moreover, it has been shown that CFIm can direct sequence-specific, A(A/U)UAAA-independent polyadenylation reaction, through recruiting the CPSF subunit hFip1 and poly A polymerase to the 3'UTR of the RNA substrate. Chromatin immunoprecipitation assays confirmed that CFIm is recruited to the transcription site together with CPSF and CstF at the early stages of transcription (Venkataraman 2005).

Researchers have shown that cleavage stimulation factor is a heterotrimer necessary for the first step of polyadenylation, endonucleolytic cleavage, and it plays an important role in determining the efficiency of polyadenylation. Although a considerable amount is known about the RNA binding properties of CstF, the protein-protein interactions required for its assembly and function are poorly understood. They therefore first identified regions of the CstF subunits, CstF77, CstF64, and CstF50, required for interaction with each other. Unexpectedly, small regions of two of the subunits participate in multiple interactions. In CstF77, a proline-rich domain is necessary not only for binding both other subunits but also for self-association, an interaction consistent with genetic studies in Drosophila. In CstF64, a small region, highly conserved in metazoa, is responsible for interactions with two proteins, CstF77 and symplekin (Takagaki 1999).

Poly (A) polymerase (PAP) is the enzyme that has a central role in the addition of the poly (A) tail at the 3' mRNA terminus and probably for the elongation of poly (A) chains. The coupling of the two 3'-end processing reactions, cleavage and polyadenylation, appears to be another potential function of the poly A polymerase (Terns 1989). There are at least three reported canonical PAP which are PAPα, PAPβ and PAPγ (neoPAP). Canonical PAPs are known to control the nuclear co-transcriptional polyadenylation. The three canonical PAPs are proposed to have arisin from a common PAP by gene duplication. Star-PAP (Speckle Targeted PIPKIa Regulated Poly (A) Polymerase) is a nuclear non-canonical PAP. It is also known as TUT1 (Terminal Uridylyl Transferase 1). It has been found that TUT1 polyadenylates specific mRNAs that have key role in important cellular processes such as oxidative stress response and apoptosis. PAPD5 and PAPD7 are involved in nuclear surveillance of a wide range of nuclear target RNAs. These two non-canonical PAPs polyadenylate specific aberrant rRNA precursors easing their degradation (Laishram 2014 and Ustyantsev 2016). At least six isoforms of poly (A) polymerase alpha (PAPαI-VI) can arise from a single alternatively spliced transcript. Three of them are enzymatically active: PAP I, PAP II, and PAP IV. PAP II is 740 amino acids in length and it is commonly isolated from cDNA libraries. Its catalytic region is followed by a ∼20-residue primer binding domain that also encompasses one of two nuclear localization signal (NLS) sequences, both of which are essential for nuclear localization. The second NLS lies ∼140 amino acids downstream of the first. Both of the NLS sequences encompass a Serine/Threonine-rich domain which is thought to perform regulatory functions. Three short forms of PAP encode proteins that terminate prior to the first NLS and appear to arise from competition between polyadenylation and splicing (Colgan 1997).

It has been shown that RBBP6 (retinoblastoma-binding protein 6), is a component of the cleavage and polyadenylation complex. RBBP6 interacts with the cleavage and polyadenylation complex unusual ubiquitin-like domain, DWNN (‘‘domain with no name’’). This protein is necessary for the cleavage and polyadenylation reaction and it is also expressed as a single domain (isoform 3). Importantly, it has been identified that isoform3 is down-regulated in several cancers. It also competes with the full length RBBP6 for binding to the core machinery, thereby inhibiting 3’ processing and thus gene expression. Genome-wide analyses following alterations in cellular RBBP6 levels affect both alternative polyadenylation and abundance of certain mRNAs, especially those with AU-rich 3’ untranslated regions such as c-Fos and c-Jun, and increased usage of distal poly (A) sites (Giammartino 2014).

Protein name	Yeast homolog
Known polyadenylation factors	
CPSF complex	
CPSF160 (CPSF1)	SFT1
CPSF100 (CPSF2)	β-CASP
CPSF73 (CPSF3)	β-CASP
CPSF30 (CPSF4)	YTH1
hFip1	FIP
CstF complex	
CstF77 (CSTF3)	RNA14
CstF64 (CSTF2)	RNA15
CstF50 (CSTF1)	
CF Im complex	
CF Im 68 (CPSF6)	
CF Im 59 (NUDT21)	
Cf Im 25 (CPSF5)	
CF IIm complex	
Pcf11	Pcf11
Other Known Polyadenylation Factors	
Symplekin	PTA1
PAPOLG	PAP
PABPC1	PAB1
PABPC4	

PABPN1	
Additional Homologs of Yeast Polyadenylation Factors	
WDR33	PFS2
RBBP6	MPE1
CstF64 tau (CSTF2T)	RRM
PP1 alpha	Phosphatase
PP1 beta	Phosphatase
Splicing factors	
U2AF65	MUD2

Table 1.1. Human Pre-mRNA 3' Processing Complex. Adapted with modification from Shi 2009.

1.1.2 Cytoplasmic poly (A) tail binding protein and cytoplasmic polyadenylation

Cytoplasmic poly (A) binding proteins (PABPs) regulate mRNA stability and translation. They are predominantly localized in the cytoplasm, however PABPs also shuttle to the nucleus. It has been found that RNA is a major determinant of the localization of PABP (Afonina 1998). In normally proliferating mammalian cells a significant portion of PABPC1, and also PABPC4, is associated with actively translating mRNAs and thus they are restricted in the cytoplasm and are unavailable for transporting to the nuclear compartment. Unbound PABP molecules are available to be associated with α-importins and can be imported to the nucleus. For instance, when in the nucleus, PABP1 has been shown to bind pre-mRNAs alongside the predominantly nuclear and functionally distinct poly (A) binding protein, PABPN1, which binds nascent poly (A) tails as they are added. Therefore exported mRNAs likely leave the nucleus with a mixed population of poly (A)-binding proteins, comprised of a majority of PABPN1 and a minority of cytoplasmic PABPs, coating their poly (A) tails (Burgess 2012). The latter do not contribute to mRNA export but may potentially prime mRNAs for efficient translation. Once the mRNA reaches the cytoplasm, PABPN1 is exchanged for cytoplasmic PABPs in a translation-dependent process (Hosoda 2006).

The cytoplasmic poly (A) binding proteins are known to directly enhance translation by promoting mRNA circularization through simultaneous interactions with eIF4G and the 3′ poly (A) tail. PABP activity is regulated by the PABP-interacting proteins (Paip) family. This family consists of several proteins which are known by their ability to bind with other proteins to regulate translational activities and other cellular processes including mRNA stability and export. The well-studied members of this family are Paip1, a translational stimulator, and Paip2A and Paip2B, two translational inhibitors (Derry 2006).

Cytoplasmic polyadenylation is the elongation of the poly (A) tail of an mRNA after it has been exported into the cytoplasm. Cytoplasmic polyadenylation was first found in germ cells and embryonic development across a number of different species, and is known to play crucial roles in these processes (Mishima 2016, Sartain 2011 and Luitjens 2000). Cytoplasmic polyadenylation was characterized in Xenopus oocytes, and all well studied examples of regulatory elements and factors are known from this system. However, this process was also extensively studied in the C. elegans germ line, as well as in neuronal plasticity and mitosis in somatic cells (Charlesworth 2014). In both vertebrates and invertebrates, the expression of several maternal mRNAs is regulated by cytoplasmic polyadenylation. The earliest stages of animal development are driven by maternally supplied mRNAs because transcription from the zygotic genome is quiescent immediately after fertilization. Subsequently, the zygotic genome is activated and a subset of maternal mRNAs is degraded. Cytoplasmic polyadenylation is a key process that serves to unmask specific translationally repressed mRNAs and enables them to be translated. In addition to its well-established role in oogenesis, cytoplasmic polyadenylation regulates many biological processes ranging from cell cycle control and cancer to learning and memory (Ivshina 2014).

Classical studies in Xenopus oocyte identified two *cis*-acting sequences in the 3’ UTR of substrate mRNAs: the conserved polyadenylation hexanucleotide, mainly consisting of AAUAAA or AUUAAA and also required for nuclear polyadenylation, and the less conserved cytoplasmic polyadenylation element (CPE), with the general structure UUUUUA1–3U (Villaba 2011).

The best characterized cytoplasmic poly (A) polymerase, PAPD4 (Gld-2) was first discovered in *C. elegans* and its enzymatic activity was confirmed in mammalian orthologues. It has been found that GLD-2 primarily mediated stability of many mRNAs that are translationally repressed. Furthermore, GLD-2 strongly promotes bulk polyadenylation. However, GLD-2 seems to play little role in translational efficiency. A second cytoplasmic poly (A) polymerase, GLD-4, mildly stimulates bulk polyadenylation and does not affect general mRNA stability. However, GLD-4 is primarily required for efficient polysome formation and general mRNA translation (Nousch 2014).

Cytoplasmic polyadenylation-induced translation is mediated by interaction of 3’ UTR *cis*-acting cytoplasmic polyadenylation element (CPE) with cytoplasmic polyadenylation element binding protein (CPEB), an mRNA binding protein. CPEB also interacts with other three *trans*-acting factors including GLD2, a poly (A) polymerase; PARN (Poly (A)-Specific Ribonuclease), a deadenylating enzyme, and Maskin, which also associates with eIF4E, the cap-binding factor. Initial CPEB-induced translational repression has been proposed to occur when Maskin binds to eIF4E, thus impeding the formation of the eIF4E-eIF4G complex. This interaction is crucial to recruit the 40S ribosomal unit to the 5’ end of the mRNA. Since Maskin can tether to CPE, only CPE-containing RNAs are repressed. CPEB-induced translational stimulation occurs when the CPEB is phosphorylated. This phosphorylation dislodges PARN from the ribonucleoprotein complex. Thereafter, GLD2 adds the poly a tail to the mRNA by default which is then bound to the poly (A) binding protein (PABP). Then PABP forms a complex with eIF4G. The formation of this complex expels Maskin from eIF4E. The newly elongated poly(A) tail is then bound by poly(A) binding protein (PABP), which also binds eIF4G; the PABP/eIF4G complex displaces Maskin from eIF4E, allowing for formation of the initiation complex on the 5’ cap structure (Alexandrov 2012 and Ivshina 2014).

It has been found that all the key factors that are involved in cytoplasmic polyadenylation in *Xenopus* (CPEB, CPSF, PAP, IAK1/Eg2) are also stored in the cytoplasm of mouse oocytes. Upon the induction of oocyte maturation, the Aurora A kinase (IAK1/Eg2) is most likely activated. This kinase phosphorylates CPEB on Ser174 phosphorylation site, an event that is crucial for cytoplasmic polyadenylation. Blocking the activity of Aurora A impedes meiotic progression. Furthermore, blocking the phosphorylation of CPEB by Aurora A inhibits cytoplasmic polyadenylation (Hodgman 2001). Thus Aurora A and CPEB are essential components for cytoplasmic polyadenylation in oocytes. This phosphorylation event has been reported to stimulate an interaction between CPEB and CPSF. CPEB interacts directly with CPSF (160 KDa subunit) and does not need RNA tethering and occurs through the 160 kDa subunit of CPSF (Mendez 2000). Thus, the molecular function of Aurora A-mediated phosphorylation of CPEB is to recruit CPSF into an active cytoplasmic polyadenylation complex.

The spatial arrangement of CPEs in the 3’ untranslated region (3’ UTR) of an mRNA is important to determine the translational fate. CPEs-mediated translational repression requires a cluster of CPE, at least 2, in close proximity (less than 50 nucleotides apart), regardless of their position in the 3’ UTR. This might suggest that the translational repression is mediated by a heterodimer of Maskin and two CPEBs and not CPEB-Maskin heterodimer. For robust CPE-mediated cytoplasmic polyadenylation and translation, a CPE normally has to be within 100 nucleotides of the hexanucleotide and optimally within 25 nucleotides. Cytoplasmic polyadenylation mediated by weak CPEs benefits from the presence of a second CPE in close proximity and/or the presence of the RNA recognition element UGUANUAU (Pique 2008).

1.1.3 Mitochondrial polyadenylation

As mentioned earlier, poly (A) tail has been suggested as an important player for translational regulation as well as for mRNA stability in mammalian mRNAs (Park 2016). In bacteria, chloroplasts, and plant mitochondria, polyadenylation has a different function. It is a transient feature which promotes RNA degradation. Surprisingly, in spite of their prokaryotic origin, human mitochondrial mRNAs possess stable poly (A) tails in their 3’UTR, akin to the nucleus-encoded mRNAs. However, it has been also found that human mitochondria retain truncated and transiently polyadenylated transcripts in addition to stable poly (A) tails. Thus, bacterial and organellar poly (A) -dependent RNA degradation mechanism operates in human mitochondria (Slomovic 2005).

In mammals, mitochondrial RNAs are produced as polycistronic RNAs that are then processed by endoribonucleases to yield separate, intronless mRNAs. For several mitochondrial mRNAs, this cleavage leaves a 3’ end with an incomplete stop codon, with either a terminal UA or just U which is completed by poly- or oligo-adenylation (Anderson 1981). The role of polyadenylation is still not well understood in mammalian mitochondria, although it has been suggested to complete the translational stop signal on several mitochondrial mature mRNAs. It has been shown that polyadenylation does not have any regulatory effect on the mRNA stability or translational efficiency. However, it might be involved in the maturation of certain mitochondrial tRNAs. Therefore, it has been concluded that in addition to the role in completing translational stop signals, mitochondrial polyadenylation protects the 3’ termini of tRNAs from degradation (Bratic 2016). Mitochondrial polyadenylation is catalysed by the non-canonical poly (A) polymerase (ncPAP) PAPD1 (mtPAP, TUTase1) in mammals. This enzyme is able to function on its own, without the need for an RNA-binding domain or an RNA-binding protein cofactor (Chang 2012). It has been found that there are two kinds of adenylated mitochondrial transcripts: polyadenylated and oligoadenylated. The length of the oligo (A) tail is not affected by the depletion of mitochondrial poly (A) polymerase (PAPD1) suggesting that there may be a separate mitochondrial apparatus that can produce the oligo (A) tails (Chang 2012 and Wilson 2014).

1.1.4 Biological importance and role of the poly(A) tail

Through binding the poly (A) tail, poly (A) binding proteins (PABPs) perform a wide variety of biologically important functions. Nuclear PABPs are necessary for the synthesis of the poly (A) tail, regulating its ultimate length by controlling the interaction between CPSF complex and poly (A) polymerase. It has been reported that nuclear PABP is important to restrict CPSF binding to the AAUAAA sequence and to permit the stimulation of poly (A) polymerase by AAUAAA-bound CPSF to be maintained throughout the elongation reaction (Kuhn 2009). Association with PABP is also a requirement for some mRNAs to be exported from the nucleus to the translational pool (Cheknova 2003). In the cytoplasm, the PABPC family of proteins facilitates the formation of the 'closed loop' structure of the messenger ribonucleoprotein particle that is crucial for additional PABP activities which promote translation initiation and termination, recycling of ribosomes, and stability of the mRNA (Rissland 2017). In contrast, PABPN1 can enhance the decay of some hyperadenylated mRNAs that are not efficiently exported from the nucleus. In the cytoplasm, mRNAs are generally bound by the cytoplasmic poly (A) binding protein, PABPC1, on the poly (A) tail, while the 5' cap is bound by eIF4E, as part of the cap binding complex, comprising eIF4E, eIF4G, and eIF4A (Mangus 2003).

The poly (A) tail plays a large role in mRNA stability. Transcriptional control mechanisms chart the course of transient mRNA changes, such as in the inflammatory response by synthesizing mRNAs encoding proteins that promote or inhibit inflammation. Inflammatory mRNAs are generally short-lived, so turning off their synthesis rapidly stops or changes the direction of inflammation. Post-transcriptional mechanisms that modify mRNA stability and/or translation provide a rapid and flexible control of this process and are particularly important in coordinating the initiation and resolution of inflammation. Most eukaryotic mRNA degradation is thought to require shortening of the poly (A) tail (deadenylation) by deadenylase complexes (PAN2-PAN3, Ccr4-Not) to some critical length, whereupon the mRNA then becomes susceptible either to decapping (i.e. hydrolytic removal of the mRNA 5' cap structure) followed by 5'-3' exonucleolytic decay (Chen 2011), or exosomal decay in the 3'-5' direction. Some mRNAs contain regulatory *cis* elements that increase deadenylation and decay rates. AU-rich elements are a well-studied example of such sequences, and are found in many mRNAs encoding growth factors and inflammatory mediators (Anderson 2010, Stumpo 2010 and Khabar 2010). The AU-rich sequence UUAUUUAU, for example, is bound by tristetraprolin (TTP/ZFP36) in TNF mRNA and mediates instability (Stoecklin 2004 and Mahtani 2001). This sequence also appears to play a role in TNF translational repression (Han 1990 and Kontoyiannis 1999).

However, poly (A) tail size has not been found to correlate with ribosome binding. Rather, data suggested that a very short or no poly(A) tail was detrimental for translation, but beyond a critical length of ~25 nucleotides, further increases in poly(A) tail size had no bearing on translation. High throughput analysis in Arabidopsis showed a modest negative correlation between poly (A) tail length and mRNA half-life (Kappel 2015). Data yielded from PAL-seq, a similar technique to TAIL-seq, found that tail lengths were coupled to translational efficiency in early zebrafish and frog embryos. However, this strong coupling diminished at gastrulation and was absent in non-embryonic samples, indicating a rapid developmental switch in the nature of translational control. This switch complements an earlier switch to zygotic transcriptional control and explains why the predominant effect of microRNA-mediated deadenylation concurrently shifts from translational repression to mRNA destabilization (Subtelny 2014). Another similar recently published technique, PAT-seq, found little or no correlation between mean poly (A) tail length for a given mRNA and its read count or protein abundance (Harrison 2015). Using an in vitro mRNA decay system, it was investigated how poly (A) tail and its associated poly (A)-binding protein affect mRNA stability. The model was proposed to explain how the stability of an mRNA could be affected by the stability of its poly (A)-PABP complex. It has been indicated that an important function of poly (A) tail, in conjunction with its binding protein, is to protect polyadenylated mRNAs from indiscriminate destruction by cellular nucleases (Bernstein 1989). Thus, the poly (A) tail and PABP, together, have a protective role, and it could be the case that the closed-loop complex reduces accessibility of the mRNA ends to decay machinery. However, the relationship between PABP and mRNA turnover is more complex than that, as it can also promote deadenylation and therefore decay, via TOB1 recruitment which is considered one of the key anti-proliferative protiens (Ezzeddine 2012). In summary, the poly (A) tail, through interaction with PABP, is important for mature mRNA nuclear export, translation, and lifetime.

1.1.5 Alternative polyadenylation

Alternative polyadenylation (APA) is a widespread mechanism of gene regulation that generates distinct 3′ ends in transcripts made by RNA polymerase II. APA is tissue specific and globally regulated in various conditions, such as cell proliferation and differentiation, and in response to extracellular cues. APA occurs in 3′ untranslated regions (3′ UTRs) leading to the production of mRNA isoforms with different stability and translation efficiency. Alternative polyadenylation can also affect the localisation of the encoded protein. It is often coupled with splicing and can lead to the production of distinct protein isoforms. It can also function by repressing gene expression (Tian 2016). The majority of APA examples utilize alternative poly (A) sites located within the terminal exon proximally downstream of the stop codon (tandem APA). As a result, while the protein-coding sequence is unaltered, the regulatory elements in the distal 3’ UTR that might reduce mRNA stability or impair translation efficiency can be removed, including AU-rich elements and microRNA (miRNA)-binding sites. A small percentage of APA sites can be located within internal introns/exons (splicing APA) and are coupled with alternative splicing that produce mRNA isoforms encoding distinct proteins (Xia 2014).

Cleavage factor (CF) Im regulates global alternative polyadenylation by specifically binding and activating enhancer-containing poly (A) sites. Importantly, the CFIm activator functions are mediated by the arginine-serine repeat (RS) domains of CFIm68/59 (CPSF6/ NUDT21), which bind specifically to an RS-like region in the CPSF subunit Fip1, and this interaction is inhibited by CFIm68/59 hyper-phosphorylation. CFIm is an UGUA enhancer-dependent activator of mRNA 3’ processing. In a subset of mRNAs, the enrichment of UGUA enhancers at the distal poly (A) sites leads to higher CFIm recruitment and, in turn, specific activation of these sites. CFIm depletion will cause decreased activities of the distal poly (A) sits in these mRNAs while the proximal sites are less affected, thus resulting in a net shift to proximal poly (A) sites. For mRNAs in which UGUA enhancers are distributed similarly at alternative poly (A) sites, changes in CFIm levels would affect these sites to a similar degree, thus their overall profiles are unaffected (Zhu 2018).

During B-cell differentiation, upregulation of CstF64 results in a switch from distal to proximal poly (A) site selection, resulting in conversion of immunoglobulin M (IgM) heavy chain from membrane-bound to secreted form. This was shown to reflect a greater affinity of the purified CstF complex for the distal GU-rich downstream element relative to the corresponding promoterproximal site, leading to a model in which the stronger, high-affinity site is utilized under conditions of limiting CstF. While at high concentrations of CstF, the first site encountered during transcription, i.e., the proximal site, is preferentially used (Takagaki 1998).

In genome-wide studies, the expression of most 3' pre-mRNA processing factors varies in correlation with APA changes. It was found that the expression of most of the core polyadenylation factors, including CstF and CPSF subunits was upregulated during generation of induced Pluripotent Stem (iPS) cells derived from different cell types, correlating with general shortening of the 3'UTR. However, the expression of the same factors was reduced in differentiated embryonic tissues where longer 3'UTRs were observed (Ji 2009). Furthermore, it has been revealed that genes encoding several 3' processing factors were upregulated in cancer cells and correlated with shorter 3'UTRs (Mayr 2009).

Studies have shed light on the importance of APA in human diseases such as cancer, but its clinical significance to tumorigenesis is only beginning to be appreciated. Both proliferating cells and transformed cells have been shown to favour expression of shortened 3' UTRs through APA, leading to activation of several proto-oncogenes, such as cyclin D1. Collectively, these observations imply that truncation of the 3’ UTRs may serve as prognostic biomarkers. While compelling, these studies were highly limited to either a low number of genes or a small sample size (Mayr 2009).

Researchers have examined how the size of 3’ UTR is changed in response to inflammatory stimuli in macrophages. In genome wide analysis, that 3’ UTR length shortens gradually in response to vesicular stomatitis virus (VSV) infection in macrophages. Genes with alternative polyadenylation (APA) and mRNA abundance change are enriched in immune-related categories such as the Toll-like receptor and apoptosis-related signalling pathways. The expression of 3’ UTR processing factors is down-regulated upon VSV infection. When the core 3’ end processing factors are depleted, viral replication is affected (Jia 2017). It has been shown that T-cell activation, neuronal activity, development and several human diseases including viral infections all include APA. Differential PA site usage of Ferritin, light polypeptide (FTL), Peroxiredoxin-1 (PRDX1) and vesicle-associated membrane protein (VAPA) results in transcription of mRNA isoforms with distinct sets of miRNA and protein binding sites that influence processing, localization, stability, and translation of the respective mRNA. APA of these candidate genes consequently harbours the potential to modulate the host cell response to infection (Grunz 2015).

Shell et al. have found that activation of RAW264.7 murine-like macrophages with lipopolysaccharide (LPS) caused an increase in proximal poly (A) site selection within a reporter mini-gene containing two linked poly (A) sites that occurred concomitant with the increase in CstF64 expression. In addition, they showed that forced overexpression of the CstF64 protein also induced alternative poly (A) site selection on the reporter minigene (Shell 2005). Altogether, these aforementioned findings clearly demonstrate that APA changes can accompany many biological processes and probably play important roles, thus it could be a potential target for new anti-cancer or anti-inflammatory medicines.

1.2 Macrophages and inflammation

Although it is vital to restore tissue homeostasis following infection or tissue injury, inflammation can also cause collateral damage. As such, the inflammatory response is tightly regulated. This involves both signals that initiate and maintain inflammation and signals that shut the process down. An imbalance between the two signals can leave inflammation unchecked, resulting in cellular and tissue damage (Shin 2009). Recently, basic transcriptional mechanisms that regulate inflammatory response in macrophages have been clarified on a genome scale. The regulatory logic of distinct groups of inflammatory genes can be explained to some extent by identifiable sequence-encoded features of their chromatin organization, which impacts on transcription factor (e.g. NF-κB, FOXP3, IRF, and STAT families) accessibility and imposes different requirements for gene activation (Smale 2014). In addition to transcriptional regulatory mechanisms, epigenetic regulation, including DNA methylation and covalent histone modifications, are suggested to play a pivotal role in the regulation of inflammatory genes (Bayarsaihan 2011). Macrophages are widely-distributed innate immune cells and considered as a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes, and tissue resident macrophages. From the bone marrow, monocytes can migrate into the blood, and from there into various tissues where they differentiate into macrophages. In inflammation, macrophages have three major functions; antigen presentation, phagocytosis, and immunomodulation through production of cytokines, chemokines and growth factors. Macrophages have been emerging as a heterogeneous group of cells. They are highly plastic and can adopt distinct activation states in response to the diverse biological cues (e.g. growth factors, cytokines, pathogens, O_2, damage-associated molecular patterns, etc.) which they sense in the tissue environment (Fujiwara 2005).

Response to inflammatory stimuli and maintenance of homeostasis necessitate strict control of genes involved in stimulation or repression of the inflammatory response in macrophages, as well as genes involved in determining their polarization state. Such control occurs through epigenetic regulation of macrophage function that renders inflammatory or polarizing gene promoters accessible to transcriptional complexes. Thus, histone acetylation and methylation have an essential role in regulating the expression of both inflammatiory and anti-inflammatory genes and determining their activation state too (Daskalaki 2018).

Pathogen-associated molecular pattern (PAMPs) are molecules derived from microorganisms that are recognized by pattern recognition receptor (PRR) expressed by innate immune cells as well as many epithelial cells. The major PAMPs are microbial nucleic acids (DNA, single-stranded RNA or double-stranded RNA), lipoprotein and bacterial cell wall components such as LPS. PRRs can also recognize damage-associated molecular pattern (DAMPs) which are endogenous molecules. By activating PRRs and downstream inflammatory signalling pathways, DAMPs alert the body of trauma, ischemia, and tissue damage, either in the absence or in the presence of infection, driving tissue repair. DAMPs are normally localized within the nucleus, cytoplasm, exosomes, the extracellular matrix, or in plasma but are recognized by PRRs only when released into the intra-cellular space. For example when they are secreted or released from damaged cells where TLR4 on the surface can detect them. They could be either proteins such as S100 protein and heat shock protein or non-protein components for example ATP, uric acid, heparin sulphate, RNA, and DNA (Tang 2012).

LPS is an integral component of the outer membrane of gram-negative bacteria, a potent activator of macrophages and a causative agent of endotoxic shock. Structurally, LPS is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A, which is responsible for its biologic activity. Stimulation of macrophages with LPS results in the production of various cytokines (e.g., TNF-α and IL-1β) and inflammatory effectors (e.g., prostanoids, leukotrienes, and nitric oxide). Cluster of differentiation 14 (CD14) is a glycosylphosphatidyl inositol (GPI)–anchored cell surface glycoprotein that functions as a binding receptor for LPS. When LPS is in the bloodstream, it is immediately captured by LPS-binding protein (LBP), one of the acute-phase proteins produced in the liver. The complex formed binds to CD14 expressed on the cell surface of macrophages and leukocytes. In CD14-negative cells (e.g., endothelial cells and fibroblasts), the soluble form of CD14 present in serum can functionally replace membrane-bound CD14 (Akira 2003).

Toll-like receptors (TLRs) are a major family of pattern recognition receptors that play a critical role in innate and adaptive immune responses. There are 10 TLRs discovered in humans and 12 in mice, and the ligands for most of these receptors have been identified. TLRs can be classified into two main classes depending on their cellular location. TLR1, TLR2, TLR4, TLR5 and TLR6 are localized on the cell surface, while TLR3, TLR7, TLR8 and TLR9 are found in the endosomes (Troutman 2012).

The secreted glycoprotein, MD-2, acts as an extracellular adaptor protein in the activation of TLR4 by LPS and is essential for LPS signalling to occur. It has been found that LPS does indeed bind to MD-2, which in turn associates with TLR4 via the extracellular leucine-rich repeats of TLR4, inducing TLR4 homodimerization and downstream signalling. A cooperation of a number adaptor proteins is needed in order to switch on the LPS-stimulated TLR4 signal transduction pathway (**Figure 1.3**). These adaptors include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also known as TIRAP), TIR-containing adaptor molecule (TRIF, also known as TICAM-1), and TRIF-related adaptor molecule (TRAM, also known as TICAM-2) (Palsson 2004).

MyD88 is the canonical adaptor for inflammatory signalling pathways implicated in signalling downstream of members of the Toll-like receptor (TLR) and interleukin-1 (IL-1) receptor families. MyD88 links IL-1 receptor (IL-1R) or TLR family members to IL-1R-associated kinase (IRAK) family kinases via homotypic protein-protein interaction forming a structure termed myddosome (Deguine 2014).

MyD88-dependent signalling is CD14- dependent only at low LPS concentrations, whereas activation of the TIR-domain–containing adaptor-inducing IFN-β (TRIF) pathway requires CD14 at all LPS concentrations, leading to internalization of the Toll-like receptor 4 (TLR4) complex into endosomes whereupon TRIF is recruited. Using alternative TLR4 agonists, or macrophages rendered tolerant to LPS, TLR4 complex internalization can be dissociated from CD14 and TRIF-dependent signalling. In response to LPS, CD14 contributes to the formation of a TLR4/ MD2 complex dimer that, in turn, promotes endocytosis and IRF3 activation (Rajaiah 2015).

TLR4 is unique among TLRs because it can signal through two distinct pathways, MyD88-dependent and MyD88-independent pathways. MyD88-dependent signalling induces pro-inflammatory cytokines through activation of central inflammatory transcription factors such as NF-κB. On the other hand, MyD88-independent signalling induces type I IFN through interferon regulatory transcription factors (IRFs) (Yamamoto 2003). Furthermore, it was found that those signalling events are spatially separated. It has been suggested that TLR4 first induces TIRAP-MyD88 signalling at the plasma membrane and is then endocytosed and activates TRAM-TRIF signalling from early endosomes (Hughes 2008). In that specific case, receptor internalization induces a switch from MyD88 to TRIF signalling because of a change of adaptors, from TIRAP to the other TIR-containing adaptor TRAM (Yamamoto 2003).

MyD88 interacts with IRAK (IL-1 receptor-associated kinase) family members forming a complex called myddosome. The IRAK family includes IRAK1, IRAK2, IRAK4 and IRAK-M. IRAK4 is initially activated, which in turn phosphorylates and activates IRAK1. However, IRAK-M lacks kinase activity and functions as a negative regulator of TLR signalling by inhibiting the dissociation of the myddosome. The function of IRAK2 is not completely understood. After IRAK4 and IRAK1 have been sequentially phosphorylated, they dissociate from MyD88 and interact with TRAF6 (TNF receptor associated factor). TRAF6 is a RING domain E3 ubiquitin ligase, it promotes Lys63-linked polyubiquitination of target proteins, including TRAF6 itself. Ubiquitinated TRAF6 subsequently recruits a protein kinase complex including TAK1 (transforming growth factor-b-activated kinase-1) and TABs (TAK1 binding proteins) (TAB1, TAB2 and TAB3) (Kawai 2007). TAK1, but not TAB family members, is required for the phosphorylation and activation of the IKKβ (Inhibitor of IκB kinase) which is considered the upstream activator of the canonical NF-κB signalling pathway (**Figure 1.3**). Furthermore, it has been indicated that cells lacking TAK1 complex do not respond to inflammatory stimuli such as TNF-α, IL1β or LPS, suggesting a central role for TAK1 in mediating the inflammatory response (Shim 2005). The IKK kinase complex is considered as a core element in the TLR signalling. It is essentially made of two kinases (IKKα and IKKβ) and a regulatory subunit, IKKɤ. Phosphorylation of IKKβ is a key regulating step in the inflammatory response, as phosphorylated IKKβ in turn phosphorylates IκBα (Inhibitor of κB-α) (Israel 2010). Phosphorylation of IκBα on two conserved serines (S32 and S36) in the N-terminal regulatory domain of IkBs eases the recognition by the E3 ligase complex and subsequent polyubiquitination. Ubiquitination of IκBα mediates its 26S proteasomal degradation. The enzymes that catalyses the ubiquitination of phospho-IkB are constitutively active. Hence, the only step that determines the fate of IkB is the phosphorylation of the two serine residues in the IkB molecule (Karin 1999). It has been convincingly demonstrated that phosphorylation of IkB proteins is fundamental for the canonical NF-κB pathway. Upon IκB degradation, the liberated NF-κB dimer translocates to the nucleus and switches on the transcription of genes controlling inflammation, immunity, cell proliferation and apoptosis (**Table 1.2**) (Viatour 2005).

1.2.1. Inflammation and NF-κB

In vertebrates, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) refers to a family of related transcription factors that homo- or hetero- dimerize to form more than a dozen transcription factors. This family consists mainly of five members: p50 (p105), p52 (p100), c-Rel, RelA/p65 and RelB which are encoded by the Nfkb1, Nfkb2, Rel, Rela and Relb genes. These proteins share an approximately 300 residue long homologous domain near their N termini. This domain, called the Rel Homology Domain (RHD), is responsible for DNA binding, dimerization, inhibitor binding and nuclear localization (Hoffmann 2006). Two distinct nuclear factor κB signalling pathways have been explored; the canonical pathway that mediates inflammatory responses, and the non-canonical pathway that is responsible for immune cell differentiation and maturation. The former is dependent on the NF-κB essential modulator (NEMO, IKKɣ), the latter is independent of it (Shih 2011).

Both p105 and p100 are synthesized as large precursor proteins, which are partially processed by the proteasome to produce NF-κB subunits p50 and p52, respectively. Both precursors also contain C-terminal ankyrin repeats, so they serve as both NF-κB subunit precursors and IκB proteins. Phosphorylation plays a key role in the regulation of p100 and p105 processing by the proteasome. IKKα phosphorylates p100 on a C-terminal degron with sequence homology with Lys 22 of IkBa, leading to either degradation or processing to p52. Phosphorylation of p105 Ser 923/927 leads to polyubiquitination at multiple lysine residues, and 26S proteasomal degradation of the entire protein (Hayden 2012). It has been found that p105 interacts with other non-NF-κB signalling pathways. P105 and IKKβ together have been found to regulate tumour progression locus-2 (TPL-2), an upstream kinase in the Erk1/2 pathway. IKKβ-induced degradation of p105 leads to the release and activation of TPL-2, rendering IKKβ and p105 critical for both NF-kB and MAPK activation (Beinke 2004).

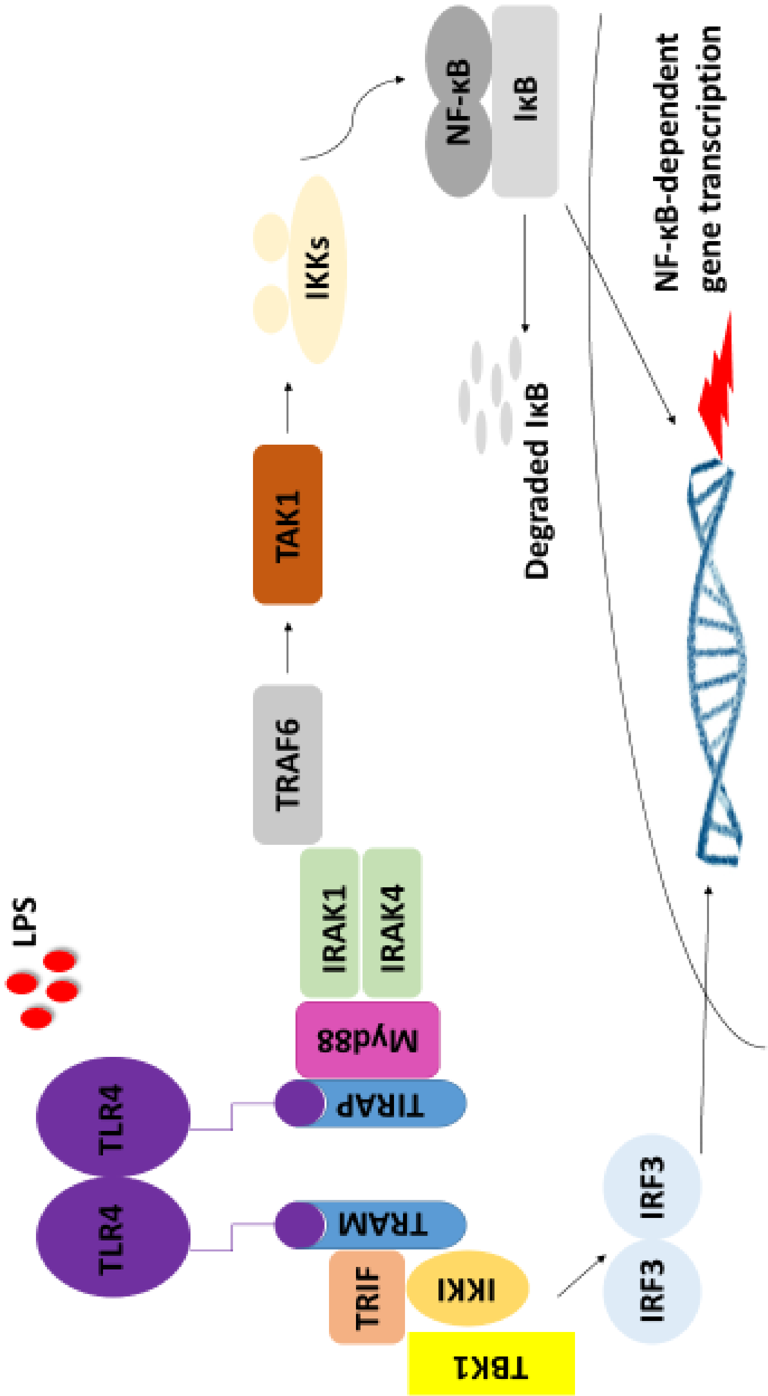


Figure 1.3. A schematic model of TLR4 signalling through the MyD88 pathway. This model shows the consequences of stimulating Toll-like receptor 4 by lipopolysaccharide and activation of canonical NF-κB signalling pathway. Upon ligand binding, TLR4 dimerizes and recruits downstream adaptor molecules such as MyD88, TRIF and TRAM. The activated MyD88 then activates IRAK4, TRAF6, TAK1, and IKK complexes. The cytoplasmic NF-κB complex is maintained in the inactive state by IκB, which is in turn degraded by proteasomes, resulting in the translocation of NF-κB into the nucleus.

Phosphorylation of NF-κB has been found to modulate its ability to induce or repress transcription of its target genes **(Table 1.3)**. It has been shown that p65 is also phosphorylated during the phosphorylation and degradation of IkBs. IKKs were considered as a possible p65 kinases in the cytokine-induced NF-kB activation. It was found that IKKs phosphorylate the p65 subunit on Ser-536 in the COOH-terminal transactivation domain (Sakurai 1999). In contrast to other p65 phosphorylation sites, which positively regulate NF-κB function, Maski et al. identified phosphorylation on threonine 505 of p65 subunit as a negative regulator of NF-κB activity. It has been indicated that RelA T505 phosphorylation normally acts to suppress NF-κB activity and thus oncogenicity. However, reduction in p65 T505 phosphorylation, for example during carcinogenesis, leads to enhanced oncogenic capability of NF-κB and thus development of malignant and metastatic cancer cells (Maski 2011). Furthermore, it has been found that TNF-α induces the phosphorylation of RelA at Thr435. Mutational analysis of this site revealed gene-specific effects on transcription. For instance, mutation at Thr435 site influences the ability of RelA to bind to the Cxcl2 promoter. It also enhances the acetylation status of histone tails at this promoter that can be attributed to the elevated level of HDAC1 (histone deacetylase 1) binding to the promoter. TNF-α stimulation results in decreased HDAC1 interaction with RelA which was later found to be due to Thr435 phosphorylation (O'Shea 2010, Ashburner 2001).

Other post-translational modifications of NF-κB, including acetylation and methylation, have central roles in regulating the mechanism and determining the duration and extent of NF-κB nuclear transcriptional output. Acetylation or methylation at different lysine residues of RelA have emerged to modulate divergent functions of NF-κB, including DNA binding and transcription activity, protein stability, and its interaction with various NF-κB modulators (Chen 2015,). For instance, it has been found that the acetylation of lysine 310 of RelA has a role in regulating the Set9-mediated methylation of lysines 314 and 315, which is essential for the ubiquitination and subsequent degradation of chromatin-associated RelA. Inhibiting the acetylation of lysine 310, either by the deacetylase SIRT1 or by mutating lysine 310 to arginine, enhances methylation. Conversely, enhancing the acetylation of lysine 310 by depleting SIRT1 or by replacing lysine 310 with acetyl-mimetic glutamine inhibits methylation by inhibiting the effective assembly of RelA and Set9. Accordingly, the stability of promoter-associated RelA is enhanced by reducing its ubiquitination-mediated degradation, and thus promoting the transcriptional activity of NF-κB (Yang 2010). Among the factors that play a pivotal role in post translational acetylation of P65 are CBP/p300 and TGF-β. CBP/p300 acetyltransferase has been found to play a key role in p65 acetylation. Moreover, it was found that p65-CBP binding increased after LPS stimulation; this process is critical for NF-κB enhanceosome assembly. Acetylation of p65 subunit at lysine 221 by p300/CBP is essential for enhancing the DNA-binding activity, NF-κB activation and Tnf and Il1b transcriptional output (Ishinaga 2007 and Hwang 2013).

Gene	Function	Reference
CCL15/Leukotactin/ SCYA15	Chemokine for cell attraction	Shin 2005
CXCL 11	Chemokine ligand for CXCR3	Tensen 1999
Fractalkine	TNF-induced chemokine	Ahn 2004
Gro-1	Growth regulated oncogene; chemokine	Wood 1995
IFN-g	Interferon, critical for innate and adaptive immunity	Sica 1997
IL-1b	Interleukin-1b, regulation of immune cells	Hiscott 1993
MCP-1/JE	Macrophage chemotactic protein, beta Chemokine	Ueda 1997
TNFalpha	Tumor necrosis factor alpha, regulation of immune cells	Collart 1990
VEGI	Vascular endothelial growth inhibitor	Xiao 2005

Table 1.2. Immune genes that have a kB site in their promoters and have clearly been shown to be controlled by NF-kB.

Post translational modification: phosphorylation		
Site	**Enzyme involved**	**P65 activity**
Serine 276	PKAc	Increased
Serine 536	IKKs, ribosomal subunit kinase-1 (RSK1), and TANK binding kinase (TBK1)	Increased
Serine 468	GSK3β, IKKβ and IKKε	Decreased
Serine 311	PKCζ	Increased
Serine 529	Casein kinase 2 (CK2)	Increased
Threonine 435	Unknown	Decreased
Threonine 505	Chk1	Decreased
Post translational modification: acetylation		
Lysine 122	p300/CBP and PCAF	Reduces RelA binding to the κB enhancer
Lysine 123	p300/CBP and PCAF	Reduces RelA binding to the κB enhancer
Lysine 218	p300/CBP	Impairs NF-κB association with IκBα
Lysine 221	p300/CBP	Enhances the DNA binding of NF-κB
Lysine 310	p300/CBP	Required for full transcriptional activity of NF-κB
Lysine 314	p300/CBP	Differentially regulates the expression of specific sets of NF-κB target genes in response to TNF-α stimulation
Lysine 315	p300/CBP	Differentially regulates the expression of specific

		sets of NF-κB target genes in response to TNF-α stimulation
Post translational modification: methylation		
Lysine 314	Set9	Induces the ubiquitination and degradation of promoter-bound RelA
Lysine 315	Set9	Induces the ubiquitination and degradation of promoter-bound RelA
Lysine 37	Set9	Stabilizes the binding of NF-κB to its enhancers

Table 1.3. Post translational modification of P65. Phosphorylayion, acetylation and methylation of P65 subunit at different residues and the effect of these modifications on its transcriptional activity (adapted with modification from Huang 2010).

1.2.2. Inflammation and osteoarthritis

Osteoarthritis (OA) is a common age-related degenerative joint disease and is considered the most prevalent type of arthritis. This disorder is characterized by degradation of cartilage, formation of new connective tissue on the joint surface and at the joint margins (osteophyte) and synovial inflammation (Nakashima 2012, Zheng 2012). Historically, it has been considered a ''wear and tear'' disorder. Later, it was discovered that cytokines and prostaglandins lead to the production of matrix metalloproteinases (MMPs) in cartilage that are responsible for breaking down the extracellular matrix. This discovery established the ''inflammatory theory'' and considered inflammation as the main etiological feature in OA (Berenbaum 2013). Inflammatory stimuli initiate a cascade of events, including the release of proinflammatory mediators by chondrocytes, leading to complex biochemical and mechanical interaction with other biological mediators to induce osteoarthritis and promote hyperalgesia **(Figure 1.4)**. Inflammatory mediators play a key role in both cartilage degradation and sensitization of nociceptors. For instance, IL-1B and TNF-α demonstrate potent bioactivities in inhibiting extracellular matrix (ECM) synthesis and promoting cartilage breakdown.

This results from repressing the expression of essential ECM components such as aggrecan and collagen type II in chondrocytes, and stimulating a wide variety of proteolytic enzymes such as matrix metalloproteinases (MMP-1 and MMP-13) and a disintegrin and metalloproteinase with thrombospondin motifs-4 and -5 (ADAMTS-4 and ADAMTS-5) in both chondrocytes and synovial fibroblasts (Lee 2013).

It has also been found that TLRs are upregulated in the chondrocytes of osteoarthritic patients. TLR-2 and TLR-4 ligands such as low molecular-weight hyaluronic acid, fibronectin and tenascin-C have been found in OA synovial fluid. These factors can induce catabolic responses in chondrocytes and/or inflammatory responses in synoviocytes. Eventually, these signals lead to the overexpression of inflammatory soluble mediators such as prostaglandins, chemokines and cytokines when a certain threshold is reached suggesting that inflammatory mediators play a central role in the initiation and perpetuation of the OA process (Berenbaum 2013).

1.3 PI3K/Akt and mTOR signal transduction pathways

Life is the delicate balance of different cellular events orchestrated in a highly regulated and coordinated manner, such as cell cycle progression, survival, apoptosis, cell motility and gene expression. These cellular events are the intricate outcome of signalling pathways that operate in time and space (Kolch 2015). Among them, phosphoinositide signalling, is initiated by the generation of phosphorylated phosphatidylinositol lipid moieties. The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signal transduction pathway plays an essential role in cellular growth and survival in both physiological and pathological aspects. These two pathways are tightly interconnected so they are mostly considered as one pathway and called PI3k/Akt/mTOR signalling pathway (Datta 1999). PI3K phosphorylates PI(4,5)P2 into the second messenger PI(3,4,5)P3 in a few seconds leading to the recruitment of protein kinase B (PKB)/Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane and the initiation of downstream signal transduction pathways (PI3K/Akt/mTORC1). Although PI(4,5)P2 is 10-100 fold more abundant that PI(3,4,5)P3, it is unable to recruit Akt and PDK1 (Thapa 2016).

Akt kinases belong to the AGC kinase family which includes AMP/GMP kinases and protein kinase C. They consist of three conserved domains: an N-terminal PH domain, a central kinase CAT domain, and a C-terminal extension (EXT) containing a regulatory hydrophobic motif (HM). AKT kinases are activated by phosphorylation at a Threonine residue in the activation T loop (T308 in AKT1, T309 in AKT2 and T305 in AKT3) and a Serine residue in the C-terminal HM (S473 in AKT1, S474 in AKT2 and

S472 in AKT3). Phosphorylation of AKT1 at Thr308 results in 100-fold increase in the enzymatic activity. However, serine 473 phosphorylation enhances the kinase activity by another 10-fold. Collectively, phosphorylations at both sites augment AKT kinase activity by 1000-fold (Kumra 2005).

Mammalian target of rapamycin is a major effector of cellular growth and proliferation by regulating translational efficiency. It is evolutionarily conserved from yeast to human. In normal cells, multiple positive and negative regulators modulate mTOR activity at various levels (Hay 2004). Positive regulators include growth factors and their receptors, such as insulin-like growth factor-1 (IGF-1) and its cognate receptor IGFR-1, members of the human epidermal growth factor receptor (HER) family and associated ligands, and vascular endothelial growth factor receptors (VEGFRs) and their ligands, which transmit signals to mTOR through the PI3K-Akt. Negative regulators of mTOR activity include phosphatase and tensin homolog (PTEN) that inhibits signalling by dephosphorylating PI(3,4,5)P3, and tuberous sclerosis complex (TSC) 1 (hamartin) and TSC2 (tuberin), which inhibits GTP exchange on RHEB. Phosphorylation of TSC2 by Akt releases its inhibitory effect on mTOR upregulating mTOR activity through GTP-RHEB (Hay 2004).

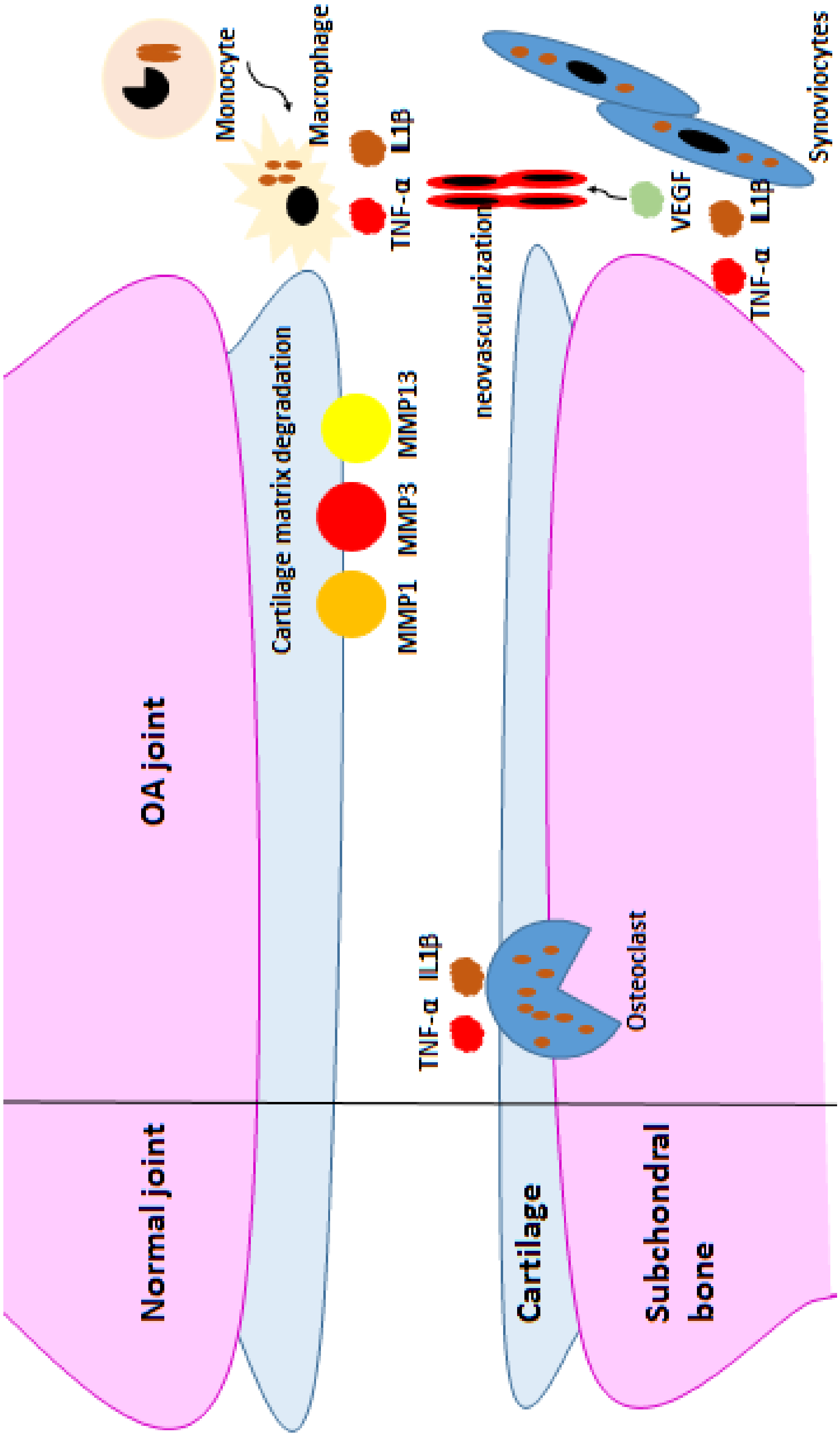


Figure 1.4. A simplified model of the pathophysiologic mechanisms involved in the development and progression of osteoarthritis. This model shows the main pathological findings in osteoarthritis: cartilage matrix degeneration, synovitis and bone deformity.

Another negative regulator, LKB1, is in an energy-sensing pathway upstream of TSC. Furthermore, LKB1 serves to activate AMP-activated protein kinase (AMPK), and AMPK serves to negatively regulate mTOR (Shaw 2004). Mammalian target of rapamycin activity is carried out by two distinct complexes: mTORC1 and mTORC2. The two complexes are similar in structure but have distinct functions. The mTORC1 complex is made up of mTOR, mLST8, and raptor. The mTORC2 complex is composed of mTOR, mLST8 and rictor. Rapamycin binds to the raptor component of mTORC1 but not to rictor, thus mTORC1 represents the target of the first-generation mTOR inhibitors. It also activates S6K and inactivates 4EBP1, leading to protein translation and cell growth. On the other hand, mTORC2 is less sensitive to rapamycin and its role in normal cell function and oncogenesis has not been well clarified. However, it is known to activate Akt, thereby promoting cell proliferation and survival (Hay 2004, Memmott 2009 and Pal 2015).

The AMP-activated serine/threonine protein kinase (AMPK) is a sensor of cellular energy status found in all eukaryotes that is activated under conditions of low intracellular ATP following stresses such as nutrient deprivation or hypoxia. AMPK is considered a major regulator of mTOR signal transduction pathway. Interestingly, like AMPK, the mTOR serine/threonine kinase plays key roles not only in growth control and cell proliferation but also in metabolism. Furthermore, it has been found that mTORC1 is more sensitive to AMPK regulation than mTORC2. AMPK directly phosphorylates at least two proteins to induce rapid suppression of mTORC1 activity, the TSC2 tumour suppressor and the critical mTORC1 binding subunit raptor (Shaw 2009).

1.3.1 Signal transduction pathways and inflammation

It is now evident that mTOR has an important role in the modulation of both innate and adaptive immunity. mTOR regulates diverse functions of professional antigen-presenting cells, such as dendritic cells (DCs), and has important roles in the activation of conventional T cells and the function and proliferation of regulatory T cells (Thomson 2009). mTOR senses intra- and extracellular nutrients, growth factors (GM-CSF), cytokines (e.g., interleukin [IL]-4 and IL-15) and pathogen-associated molecular patterns (e.g., Toll-like receptor 4 [TLR4] agonists) to bioenergetically regulate and optimize effector functions (e.g., proliferation and cytokine expression) of immune cells (e.g., macrophages) (Weichhart 2015).

It has been revealed that mTOR downstream of Akt controls NF-κB activity in PTEN-null/inactive prostate cancer cells via interaction with and stimulation of IKK. The mTOR-associated protein Raptor is required for the ability of Akt to induce NF-κB activity. Correspondingly, the mTOR inhibitor rapamycin is shown to suppress IKK activity through a mechanism that may involve dissociation of Raptor from mTOR (Dan 2008). It has also been suggested that the stimulation of NFκB by Akt is dependent on the phosphorylation of p65 at S534, mediated by IKK (IκB kinase) α and β. Akt phosphorylates IKKα on T23, and this phosphorylation event is a prerequisite for the phosphorylation of p65 at S534 by IKKα and β. It has been demonstrated that IKK complex has two separate functions in NF-κB activation in cells with constitutive Akt activity: the phosphorylation and consequent degradation of IκB and the phosphorylation of p65 (Bai 2009).

2-methyl-pyran-4-one-3-O-b-D-29,39,49, 69-tetra-Oacetyl glucopyranoside (MPTAG) is a compound which is known by its ability to inhibit the TNF-α-induced nuclear translocation of NF-κB. It has been observed that MPTAG does not directly inhibit IKK-β but prevents TNF-a-induced activation of IKK-β by blocking its association with Akt and thereby inhibits NF-kB activation. Interestingly, it was revealed that the inhibitory effect of MPTAG on Akt and NF-kB activation was independent of PI-3K and dependent on PKA (Balwani 2012). Moreover, it has also been reported that a subset of NF-κB-dependent genes require Akt for optimal upregulation during T cell activation. The selective effects of Akt were manifest at the level of mRNA transcription and p65/RelA binding to upstream promoters, and appear to be due to altered formation of the Carma1/Bcl10 complex. The pro-inflammatory cytokine TNF-α was found to be particularly sensitive to Akt inhibition or knock-down in primary human blood T cells and a murine model of rheumatoid arthritis (Cheng 2011). Recent studies suggest that mTOR plays a vital role in cartilage growth and development and in altering the articular cartilage homeostasis as well as contributing to the process of cartilage degeneration associated with osteoarthritis in which inflammation is thought to be a major cause. Both pharmacological inhibition and genetic deletion of mTOR have been shown to reduce the severity of OA in preclinical mouse models (Pal 2015).

Signal transduction pathways regulate gene expression in part by modulating the stability of specific mRNAs. For example, the mitogen-activated protein kinase (MAPK) p38 pathway mediates stabilization of tumour necrosis factor-alpha (TNF-α) mRNA in myeloid cells stimulated with bacterial lipopolysaccharide (LPS). The zinc finger protein tristetraprolin (TTP) is expressed in response to LPS and regulates the stability of TNF-α mRNA. It has been shown that stimulation of RAW264.7 mouse macrophages with LPS induces the binding of TTP to the TNF-α 3' untranslated region. The p38 pathway is required for the induction of TNF-α RNA-binding activity and for the expression of TTP protein and mRNA. Following stimulation with LPS, TTP is expressed in multiple, differentially phosphorylated forms. So it is evident that phosphorylation of TTP is mediated by the p38-regulated kinase MAPKAPK2 (MAPK-activated protein kinase 2). A direct link between a specific signal transduction pathway and a specific RNA-binding protein has been established, both of which are known to regulate TNF- gene expression at a posttranscriptional level (Mahtani 2001).

Although AMPK plays well-established roles in the modulation of energy balance, recent studies have shown that AMPK activation has potent anti-inflammatory effects. It has been shown that AMPK has a novel role in modulating the phagocytic activity of macrophages, neutrophils, and, presumably, of other phagocytic cell populations. The ability of AMPK activation to enhance phagocytosis may be important in host defense through increasing uptake and clearance of bacteria and other microorganisms, and may also contribute to the resolution of inflammation by phagocytosis of dead cells, resulting in a more effective removal of apoptotic neutrophils and other cell populations from inflammatory foci (Bae 2011). The outcomes of investigating PI3K/Akt/mTOR signalling pathway modulation both *in vitro* and *in vivo* inflammatory models support the use of inhibitors of this pathway as a potential and promising therapeutic target for inflammatory diseases.

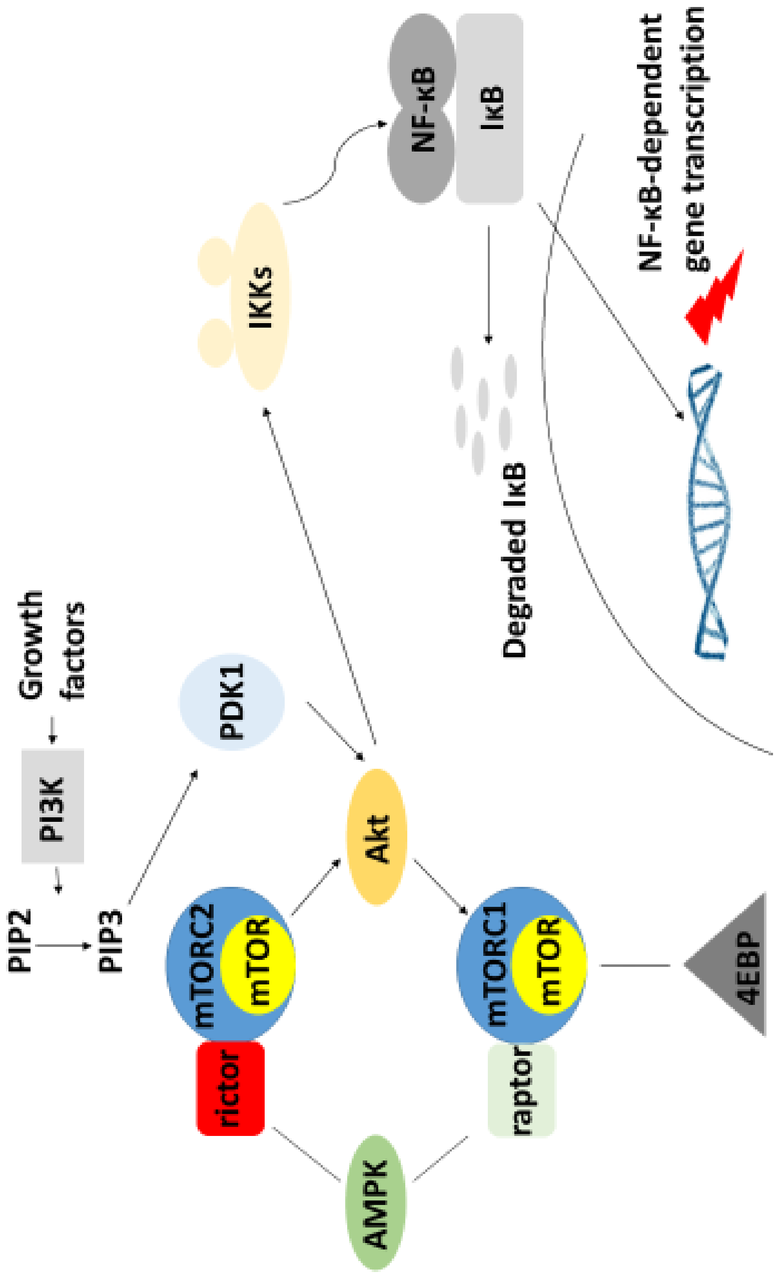


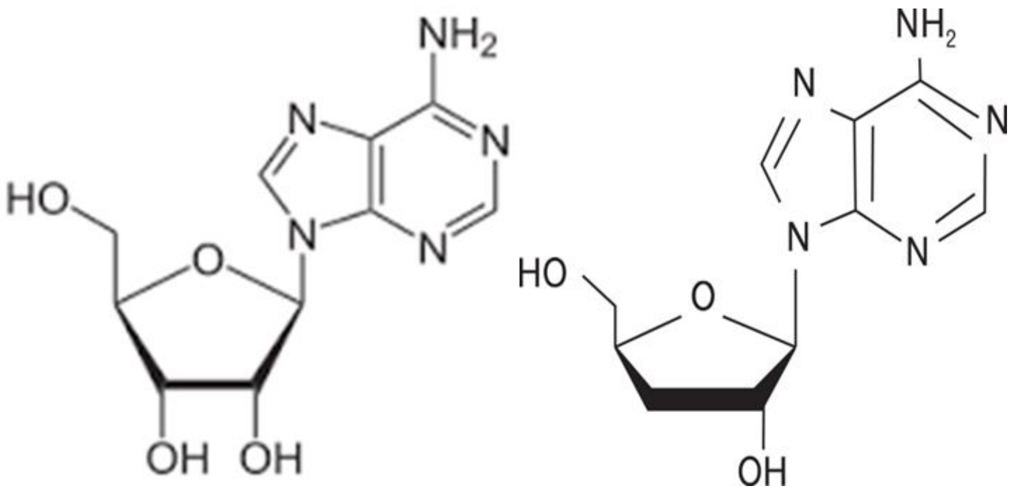
Figure 1.5. A schematic model of the PI3K/Akt and mTOR signalling pathways. This model shows the main components of the PI3K (Phosphoinositide 3-kinases) /Akt, mTOR and AMPK signalling. It also shows the interaction of this signalling pathway with NF-κB activity. PI3K is activated by growth factors. PIP2 is converted by PI3K into PIP3. This leads to activation of PDK1 which phosphorylates Akt1 to partially activate it. Full activation is dependent on an additional phosphorylation by mTORC2. Akt1 phosphorylates and activates mTORC1 which phosphorylates 4EBP and inactivates it. AMPK inhibits mTORC1 activation. It also shows the activation of IKK complex by Akt.

1.4 Cordycepin

Cordycepin (3'-deoxyadenosine) is one of the major bioactive substances produced by *Cordyceps militaris*, a parasitic fungus used in traditional Chinese medicine. It is reported to possess a wide range of biological effects, including anti-thrombotic, anti-cancer, anti-viral, anti-fungal and anti-inflammatory activities. It is an adenosine analogue that lacks the 3' hydroxyl group (**Figure 1.6**), and therefore functions as a chain terminator if phosphorylated and incorporated into a growing polynucleotide chain (Jeong 2010, Sugar 1998, Ng 2005 and Lee 2010). All commercially available cordycepin preparations are isolated from cultivated *Cordyceps militaris* and are only 98-99 % pure, with variable contaminants (Ortori, Kim, Barrett and de Moor, unpublished results). This is a complicating factor in determining the effects of cordycepin, making it imperative to summarise the effects observed with different batches.

1.4.1 Cordycepin and polyadenylation

As shown above cordycepin is an adenosine analogue, so it was originally thought to be used by RNA polymerase instead of ATP leading to transcription termination. It was suggested that RNA polymerase II has higher sensitivity to 3-deoxyadenosine than RNA polymerase I and that ribosomal RNA synthesis is indeed affected (Desrosiers 1976 and Rose 1977). This differential sensitivity toward cordycepin is recently suggested to be mediated by its effect on signal transduction pathways, as we will see later. However, as it was shown in Hela cells, cordycepin does not affect the transcription of pre-mRNAs (heterogenous RNAs) or the cytoplasmic transport of messenger RNAs (Penman 1970 and Rose 1977). More recently, cordycepin did not affect the transcription of housekeeping genes in air-way smooth muscle cells (Kondrashov 2012). These data indicate that cordycepin does not terminate mRNA transcription in intact cells.



Adenosine

3'deoxyadenosine (cordycepin)

Figure 1.6 Chemical structure of cordycepin. The only difference between the structure of adenosine and cordycepin is the absence of the 3' hydroxyl group in cordycepin. This property would make it a chain terminator, if incorporated into a growing polynucleotide strand.

Cordycepin has also been reported to be an inhibitor of phosphoribosylphosphate amidotransferase and ribose phosphate phosphokinase. Both of these enzymes are involved in purine biosynthesis. Thus, there is a possibility that cordycepin might cause a partial inhibition of the synthesis of endogenous purines (Rottman 1964 and Tyrsted 1968).

However, the best-researched enzymatic reaction inhibited by cordycepin triphosphate is polyadenylation reaction, which occurs as a rapid, initial addition of 100-200 adenylate residues to the 3'UTR of mRNAs. Subsequently, a slower chain extension (6-8 bases) of the poly (A) tail seems to occur both in the nucleus and in the cytoplasm. Although the effect on polyadenylation well-documented, it is entirely possible that cordycepin has multiple mechanisms of action. It has been found that the initial polyadenylation reaction can be specifically inhibited by the drug cordycepin in cell culture, presumably by its conversion to the triphosphate. However, cordycepin has little effect on the slower poly (A) extension reaction or on the formation of mRNA precursor molecules, but it can inhibit rRNA synthesis, as discussed above (Rose 1977).

Cordycepin has been widely used as a polyadenylation inhibitor to study 3'mRNA processing. The use of cordycepin triphosphate for *in vitro* polyadenylation reactions, has brought new insight into the cleavage and polyadenylation of mRNAs. It helped with the identification of a complex that recognizes the poly (A) signal (AAUAAA) to start cleavage and polyadenylation reaction (Ryner 1987 and Zarkower 1986). This complex is transient in nature, but it becomes more stable when associated with cleaved mRNA with cordycepin-terminated tail. Other studies also showed that non-hydrolyzable ATP analogues help in increasing the stability of this complex (Zarkower 1987, Zhang 1987 and Ryner 1987).

It has been demonstrated that cordycepin reduces the poly (A) tail length of a subset of mRNAs in NIH3T3 cells. These mRNAs are likely to represent unstable mRNAs that need constant co-transcriptional polyadenylation to maintain their levels (Wong 2010).

However, it was not clear how cordycepin could specifically affect gene expression by inhibiting cleavage and polyadenylation reaction. Cordycepin triphosphate terminates the poly (A) tail and arrests the cleavage complex in nuclear extract (Ryner 1987, Zarkower 1987). In addition, the pre-mRNA cleavage was reduced. Cordycepin also affects newly synthesized mRNAs in air smooth muscle cells and a reduction in the efficiency of mRNA cleavage and transcription termination is observed (Kondrashov 2012).

I hypothesised a number of mechanisms to explain the anti-inflammatory effect of cordycepin in macrophages. Cordycepin-terminated RNAs may be sequestering the 3’ processing factors, thereby inhibiting further proper processing of other mRNAs. For genes being induced (e.g. inflammatory genes in response to an inflammatory stimulus), mRNA production could be hampered by a limited availability of cleavage and polyadenylation factors if they have high processing factor requirements. Such requirements could either be due to suboptimal *cis* elements around the poly (A) site; or simply because transcription becomes so fast that very many factors are required to process the large number of nascent transcripts. This hypothesis is further supported by the finding that cordycepin caused transcription termination defects in cells – a sign of decreased pre-mRNA cleavage efficiency (Luo 2006, West 2008 and Richard 2009 and Kondrashov 2012).

mRNA processing steps are closely coupled to one another, Mapendano et al. has suggested that transcription and mRNA maturation are interdependent events. They demonstrated that 3’ end formation stimulates transcription initiation and suggested that coordinated recycling of factors from a gene terminator back to the promoter is essential for sustaining continued transcription. They also showed that disruption of 3’ end processing and transcription termination do indeed trigger decreased levels of gene-associated RNAPII and promoter-associated TATA-binding protein (TBP) and transcription factor IIB (TFIIB). As seen above, cordycepin inhibits the proper processing of 3’ UTR of mRNAs and thus could inhibit the induction of any genes for which rapid transcriptional increase is required (e.g. inflammatory genes in response to a pro-inflammatory stimulus (Kondrashov et al. 2012)).

Degradation of mRNAs plays an essential role in modulation of gene expression and in quality control of mRNA biogenesis. Nearly all major mRNA decay pathways in eukaryotes are initiated by deadenylation, which is often the rate-limiting step for mRNA degradation. Many inflammatory mediators have unstable mRNAs due, in part, to the presence of AU-rich elements in their 3’ UTR, which recruit deadenylation complexes (Stumpo 2010). Therefore, if cordycepin inhibits polyadenylation (thus removing the rate-limiting step of deadenylation), it could be hypothesised that intrinsically unstable mRNAs (like those produced from inflammatory genes) would be particularly sensitive. This could help in explaining the specific effect of cordycepin on the induction of inflammatory mRNAs by cytokines in air-way smooth muscle cells without affecting the expression of housekeeping genes (Kondrashov 2012).

1.4.2 Cordycepin and adenosine receptors

The nucleoside adenosine is a potent physiologic and pharmacologic regulator that is produced by cells in response to stress by breakdown of adenosine triphosphate (ATP). ATP is broken down both intracellularly and extracellularly to generate adenosine. Intracellular adenosine is exported from cells via equilibrative nucleoside transporters or during apoptosis or necrosis (Hasko 2013). Adenosine receptors (ARs) comprise a group of G protein-coupled receptors (GPCRs) which mediate the physiological actions of adenosine. To date, four AR subtypes have been cloned and identified in different tissues, namely the A1, A2A, A2B and A3ARs (Sheth 2014). It has been found that cordycepin inhibits neointimal formation in rat aortic smooth muscle cells (RASMCs). This effect was attributed to its inhibitory effect on the platelet-derived growth factor-BB (PDGF-BB)-induced migration and proliferation via interfering with adenosine receptor-mediated nitric oxide synthetase (NOS) pathway (Won 2009). Researchers have also suggested agonistic effect of cordycepin on adenosine A3 receptor as a mechanism for its anti-tumour effect in mouse melanoma and lung carcinoma cells (Nakamura 2006).

It has been found that cordycepin has rapid and robust antidepressant effects in mice. Cordycepin is an adenosine analogue, which is a neurotransmitter in the nervous system. It has been shown that adenosine-derived drugs can be transported from blood to the brain via an adenosine transporter across the blood-brain barrier. It was hypothesized that cordycepin may produce this effect through the adenosine A3 receptor to regulate the AMPA receptor function which is common mechanism for the treatment of mood disorders (Li 2016 and Li 2001). Recently, adenosine and its receptors (A1R and A2AR) are implicated to have a major role in learning and memory. Studies found that cordycepin has nootropic effect by improving short term spatial memory which is attributed to the modulation of A2AR in the hippocampus subregions to control the adaptive processes of memory performance (Gao 2018).

Kadomatsu et al. suggested a mechanism to explain the proapoptotic effect of cordycepin in the rat renal tubular epithelial cell line. They thought that this effect is produced via increasing the sensitization of the cell to the TNF-α-induced apoptosis. They also showed that cordycepin has suppressive effect on NF-κB which could be mediated by inducing the eIF2a–mTORC1 pathway. They suggested adenosine transporters and adenosine A3 receptors as an underlying mechanism for NF-κB activation (Kadomatsu 2012). These findings contradict many others which showed an inhibitory effect of cordycepin on mTOR signalling pathway (**see section 1.4.4**).

Moreover, in air-way smooth muscle cells, the results were at odds with the above findings. They revealed that inhibition of the adenosine transporters or the adenosine kinase completely abrogate the effect of cordycepin on the induction of inflammatory mRNAs by TNF-α. This indicates that cordycepin has to enter the cell using the adenosine transporter and be phosphorylated by adenosine kinase to affect the inflammatory response (Kondrashov 2012). Other studies have also revealed that cordycepin acts intracellularly and as a nucleotide, rather than a nucleoside (Wong 2010 and Imamura 2010).

1.4.3 Cordycepin and the inflammatory response

The outcome of an inflammatory response depends upon the coordinated regulation of a variety of both pro-inflammatory and anti-inflammatory cytokines and other proteins. Regulation of these inflammatory mediators can occur at multiple levels, including transcription, mRNA translation, post-translational modifications, and mRNA degradation. Post-transcriptional regulation has been shown to play an important role in controlling the expression of these mediators, allowing for normal initiation and resolution of the inflammatory response (Stumpo 2010).

A wide variety of studies have shown that cordycepin has an anti-inflammatory effect both in cell culture and in animal models of inflammation associated-disorders (Kim 2011, Jeong 2010b, Shin 2009, Li 2016, Kondrashov 2012 and Shin 2009). Some of these studies correlate the anti-inflammatory effect of cordycepin to its status as a polyadenylation inhibitor while others show that this compound interferes with important mediators in the inflammatory cascade such as NF-κB.

Heme oxygenase-1 (HO-1) is an enzyme having anti-inflammatory, anti-oxidative, anti-proliferative and anti-apoptotic effects. It has also regulatory effects on immune system by controlling cell death and survival. In LPS-stimulated macrophages, cordycepin reduces the gene expression of cytokines through upregulation of HO-1 (Qing 2017). In the same inflammatory system, it has also been shown that cordycepin represses the production of nitric oxide (NO) which is one of the inflammatory mediators in macrophages. They also showed that inhibition of adenosine kinase and adenosine transporter abrogates the anti-inflammatory effect of cordycepin in macrophages. This suggested that cordycepin need to be transported inside the cell and phosphorylated to exert its anti-inflammatory effect (Imamura 2015). Kim et al. also showed that cordycepin reduced the production of NO and the expression of iNOS due to inhibition of both NF-κB nuclear transcription output and MAPK activation (Kim 2005). In BV2 microglia, it was found that cordycepin attenuates the production of inflammatory mediators (PEG2, TNF-α and IL1β) induced in response to LPS stimulation (Jeong 2010).

Studying the intracellular mechanism behind the anti-inflammatory activity of this compound revealed that it attenuates the activity of NF-κB through inhibiting the DNA binding and transcriptional activities of p65 subunit, proteasomal degradation of IκBα or the IKKs-mediated activation of NF-κ. Moreover, the results showed that cordycepin inhibits the phosphorylation of Akt, ERK-1/2, JNK and P38 kinase (Ren 2012, Choi 2014 and Jeong 2010).

In airway smooth muscle cells, cordycepin was found to specifically inhibit the induction of several inflammatory mRNAs by cytokines without affecting the expression of their precursor mRNAs, indicating a post-transcriptional regulation. Although it had effects at the transcriptional level for some inflammatory genes, no effect on the nuclear translocation of NF- κB was observed in these cells (Kondrashov 2012).

Poly (ADP-ribose) polymerase (PARP) inhibitors have a profound effect in fighting not only cancer, but inflammatory diseases as well. It has been reported that cordycepin inhibits PARP-1 with a rather potent efficacy. The ability of cordycepin to inhibit PARP-1 was proposed to be behind its anti-cancer and anti-inflammatory effect (Kim 2011 and Shin 2009). As the number of reports indicating an anti-PARP activity of cordycepin is low, it is possible that this is linked to a specific impurity in a particular cordycepin batch, rather than a property of cordycepin itself.

In a model of intervertebral disc degeneration (IDD), cordycepin was found to diminish the LPS-induced production of matrix metalloproteinases (MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5). Thus, cordycepin could be a potential therapeutic option for low back pain (Li 2016). In a mouse model of LPS-mediated bone loss, the effect of *Cordyceps militaris* extract (CME) on bone loss was investigated. Micro computed tomography (CT) of the femurs showed that CME significantly reverses the decline in bone mass induced by LPS. Moreover, CME represses the mRNA expression of osteoclastogenesis-related genes (Kim 2015). Thus cordycepin and perhaps other *C. militaris* derived molecules may provide a potential therapeutic approach for inflammation-associated disorders.

1.4.4 Cordycepin and signal transduction pathways

It has been found by many groups that cordycepin inhibits Akt/PKB and activates AMPK (Kim 2006, Takahashi 2012 and Wong 2010). Since the former is a positive regulator of mTOR pathway and the latter is a negative regulator of the same signalling pathway, cordycepin is suggested to be an inhibitor of the mTOR signal transduction pathway. It was for instance found that cordycepin reduces the accumulation of lipids in adipocytes. This was attributed to both decrease lipogenesis and increase lipolysis. The underlying mechanism was suggested to be through intervention with mTORC1-mediated adipogenic pathway, especially via targeting upstream kinases Akt and AMPK (Takahashi 2012). It has also been observed that cordycepin reduces total protein synthesis and this effect strongly correlates with its inhibitory effect on mTOR signalling pathway in NIH3T3 cells. Additionally, it was found to reduce the phosphorylation of 4EBP and Akt and increasing the phosphorylation of AMPK, suggesting that cordycepin exerts its effect through inhibition mTOR signalling pathway (Wong 2010).

Recent studies suggest that mTOR plays a vital role in cartilage growth and development and in altering the articular cartilage homeostasis as well as contributing to the process of cartilage degeneration associated with OA. Both pharmacological inhibition and genetic deletion of mTOR have been shown to reduce the severity of OA in preclinical mouse models (Pal 2015). Inflammation could inhibit the proliferation and cell cycle of rat chondrocytes and reduce the autophagy rate. Inhibition of PI3K/AKT/mTOR signalling pathway could promote the autophagy of articular chondrocytes and attenuate inflammation response in rats with OA (Xue 2017). Autophagy is an essential cellular homeostasis mechanism whereby cellular organelles and macromolecules are recycled to maintain cellular metabolism. Autophagy is reported to exert cytoprotective effects for articular cartilage, and osteoarthritis is associated with decreased autophagy (Jeon 2016). It has been suggested that rapamycin, at least in part by autophagy activation, reduces the severity of experimental OA. Pharmacological activation of autophagy may be an effective therapeutic approach for OA (Carames 2012). It has been demonstrated that the intraarticular injection of rapamycin reduces mTOR expression, which leads to a delay in cartilage degradation after surgically inducing joint instability. Activation of LC3 (an autophagy marker) in the OA-induced chondrocytes as well as reduction in vascular endothelial growth factor (VEGF), collagen type X alpha 1 chain (COL10A1), and matrix metalloproteinase 13 (MMP13) expression was also observed in the rapamycin-treated knees. These observations suggest that local intra-articular injection of rapamycin may represent a strategy to prevent the development of articular cartilage damage (Takayama 2012).

1.4.5 Adenosine metabolism

Adenosine deaminase (ADA) is an enzyme involved in purine metabolism which catalyses the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively **(Figure 1.7)**.

$AMP + H_2O \rightarrow IMP + NH_3$

Inosine and deoxyinosine are then further degraded to uric acid or returned to the pool of purine (Cristalli 2001). Although the above reaction is considered irreversible, IMP might be re-aminated into AMP by the catalysis of adenylosuccinate synthetase and adenylosuccinate lyase (Meyer 1980).

IMP + aspartate + GTP

$\rightarrow$ adenylosuccinate + GDP + P_i

adenylosuccinate $\rightarrow$ AMP + fumarate

Since ADA has an essential role in cellular proliferation and differentiation, ADA inhibitors have been characterised to be potential candidates for antibiotics, anticancer drugs, and anti-viral drugs. However, most of these compounds are highly toxic. Currently, only one effective ADA inhibitor is in use for the treatment of leukemia, pentostatin (Lee 2008).

3'-Deoxyinosine is reaminated by Leishmania promastigote to give 3'-deoxyinosine-5'-monophosphate, 3'-deoxy-adenosine (cordycepin)-5'-mono, di-, and triphosphates. This means that Leishmania can aminate the 6-position of 3'-deoxyinosine-5'-monophosphate, thereby converting it into a highly toxic compound (Wataya 1984). This metabolic conversion illustrates the parasite-selective toxicity of 3'-deoxyinosine (Wataya 1984).

It has been found that ADA is responsible for the deamination of 3’ deoxyadenosine (cordycepin). Diminishing ADA activity by either potent ADA inhibitor such as naringin, 2’ deoxycofrmycin or pentostatin or depleting its gene results in potentiation cordycepin’s activity (Li 2014 and Rodman 1997).

Adenosine kinase (ADK) is considered an essential regulator of the extracellular as well as the intracellular level of adenosine as shown in animal models of epilepsy and ischemia. Furthermore, ADK functions in phosphorylating other nucleosides and their analogues including bio-metabolites with well-known biological activities (Park 2013). The ADA, ADK and potential reamination enzymes are likely to play a significant role in the biodistribution and metabolism of cordycepin in whole animals and cells.

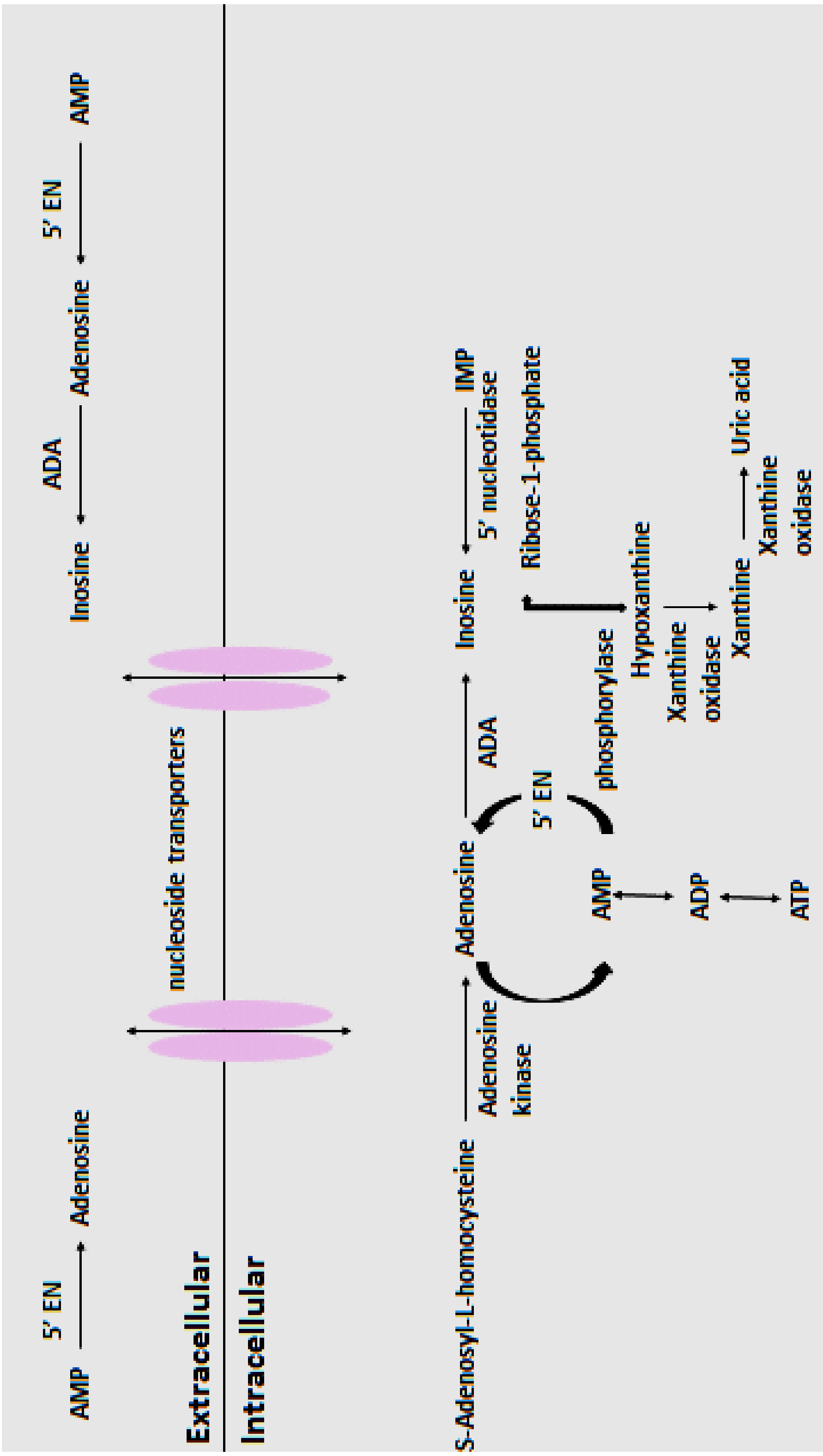


Figure 1.7. A schematic diagram shows the metabolic pathway of adenosine and inosine.

1.5 Project aims

There were two main aim for my PhD project (1) to investigate the mechanism by which cordycepin produces its anti-inflammatory action, and (2) to research the interconnection between polyadenylation and inflammation. Most of the work was done in inflammatory system established by stimulating RAW264.7 murine-like macrophages with LPS. The cells were treated with cordycepin either one hour before or 10 minutes after LPS stimulation.

Previous work, done by Raj Gandhi, confirmed the anti-inflammatory effect of cordycepin in this inflammatory system and showed that cordycepin produces identical anti-inflammatory effect when it is added before or after LPS as indicated above. Also, he showed the effect of cordycepin on the length of poly (A) of inflammatory transcripts. My project's aim was directed to gain insight into the intracellular mechanism of action and test the effect on multiple potential targets for example NF-κB activation and PI3K/Akt and mTOR signalling pathways.

Based on previous work done in air-way smooth muscle cells, it was found that depletion polyadenylation factors is associated with repressing inflammatory gene expression induced by TNF-α. In RAW264.7 cells, knocking down polyadenylation factors also repressed the LPS-stimulated inflammatory gene expression. To further investigate the mechanism, the effect of depletion those factors on NF-κB activation and PI3K/Akt and mTOR signal transduction pathways have been investigated.

Overall, the results suggested that cordycepin reduces the activation of NF-κB and has an inhibitory effect on mTOR signalling pathway. Furthermore, my data suggest that these effect are predominantly mediated by inhibition of polyadenylation.

2. Materials and methods

2.1 Cell culture and reagents

RAW264.7 cells (ATCC® TIB-71™) were maintained in Dulbecco's Modified Eagle Medium (Merck) with 10% foetal bovine serum (Merck) in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The cells were split when their confluency was about 80%. All the experiments were done at a passage number range from 20-40. Phosphate-buffered saline (PBS, BR0014G) was purchased from Thermo Fisher Scientific.

2.2 Stimulation of RAW264.7 cells with LPS and cordycepin treatment

RAW 264.7 cells were seeded into 15 cm plates (TPP, cat no 93150) 10 cm plates (TPP, cat no 93100), 6 cm plates (TPP, cat no 93060), 6-well plates (TPP, cat no 92006), 12-well plates (TPP, cat no 92012) or 8-well chamber (ibidi cat no 80826). Seeding densities ranged from 2×10^3-6×10^4 cells/cm2.

Lipopolysaccharide (LPS) was used (Merck cat no L6761) to stimulate inflammatory response in RAW264.7 cells. It was used at a final concentration of 1µg/ml. The duration of treatment is indicated in the legends.

Cordycepin was bought from Merck, Tocris or Carbosynth. If cordycepin treatment was required as well, it was dissolved in dimethylsulfoxide (DMSO) at 20mM and the cells were treated at a final concentration of 20µM (unless otherwise indicated). It was added 1 hr before or 10 min after LPS depending on the experiment.

In all experiments, the cells were seeded in the first day with 10% FBS media. After 24 hr, the media was aspirated and the cells were washed with PBS. Then, 0.5% serum media was added for 24 hr before treatment.

2.3 Treatment with bioactive compounds

RAW264.7 macrophage cells were treated with a variety of bioactive compounds. They were added at different concentrations one hour before stimulation with LPS. PI3K inhibitor LY294002 (cat no L9908) was purchased from Merck. MEK inhibitor PD98059 was purchased from Cell Signalling Technology (cat no 9900). mTOR inhibitor Torin1 (cat no 4247) and AMPK activator A769662 (cat no 3336) were purchased from Tocris Bioscience. AKT inhibitor MK2206 was purchased from Source BioScience (cat no ABE4221). Rapamycin (cat no A10782) was purchased from AdooQ Bioscience. Compound C (cat no ab120843) was bought from abcam. Adenosine (Merck, cat no A4036) was added at the same time and concentration as cordycepin. 3'-deoxyinosine (CAS: 13146-72-0) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The adenosine transporter inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBTI) (Merck, cat no N127) and the adenosine kinase inhibitor 5-Iodotubericidin (ITu) (Merck, cat no I100) were added 15 minutes prior to cordycepin treatment. **Table 2.1** shows the official names and the final concentrations of the bioactive compounds used to treat RAW264.7 macrophage-like cell line. The solvents were chosen according to the manufacturer's instruction.

Compound	Official name	Final concentration	Mechanism
PI3K inhibitor	LY294002	100μM	competitor inhibitor of the ATP binding site of the enzyme
Akt inhibitor	MK-2206	10 μM	highly selective inhibitor of Akt 1/2/3
AMPK activator	A-769662	20 μM	Allosteric activator
Compound C	Dorsomorphin	50 μM	selective and reversible AMP-kinase inhibitor
MEK inhibitor	PD98059	30 μM	Allosteric inhibitor
Rapamycin	Rapamycin	20 nM	Allosteric inhibitor
Torin1	Torine1	500 nM	mTOR 1 and 2 complexes inhibitor
Adenosine	Adenosine	20 μM	Purine nucleoside
3'deoxyinosine	3'deoxyinosine	20 or 200 μM	Cordycepin metabolite
NBTI	S-(4-Nitrobenzyl)-6-thioinosine	10μM	Adenosine transporter inhibitor
ITU	5-Iodotubericidin	100nM	Adenosine kinase inhibitor

Table 2.1. The concentrations of the bioactive compounds used to treat RAW264.7 macrophage-like cell line in this project.

2.4 RNA isolation, reverse transcription and quantitative real time polymerase chain reaction (qPCR)

The RNA extraction was performed following the protocol of the manufacturer (Promega, ReliaPrep™ RNA Cell Miniprep System), except DNase treatment was done for 1 hour rather than 15 minutes. RNA concentration was determined with NanoDrop 1000 (Thermo Scientific). In case of precious RNA samples or high demand for RNA quality, RNA isolated from above was analysed with an agarose gel to check relative integrity. To make up the gel, agarose was dissolved in 1x TBE buffer (1.08% (w/v) Tris-base, 0.55% (w/v) boric acid, 2% (v/v) EDTA pH 8.0 and make up with deionised water) to maintain a concentration of 1% (w/v). This gel solution was then autoclaved to reduce RNase activity. SYBR safe (Invitrogen, S33102) was added to autoclaved gel solution at the ratio of 1:10,000 and mixed well. Warm gel solution was then added to the gel tray and bubbles were removed with a pipette tip. Up to 2 µL of RNA sample was mixed with 10 µL loading buffer (0.25% (w/v) bromophenol blue dye and xylene cyanol dye, 10% (v/v) 1M Tris-HCl pH 7.5-8.0, 90% (v/v) deionised formamide), followed by heating at 70°C for 3 mins. Heated RNA samples were loaded on the gel and run at 4V/cm2 till around 3 cm from the end. Pictures were taken using ImageQuant LAS4000 (GE Healthcare Life Sciences) or BIO-RAD Gel Doc Universal Hood II.

Then, the cDNA was synthesized using superscript III (Invitrogen, cat NO. 18080-044) according to the protocol recommended by the manufacturer. For each reverse transcription sample, 500 ng of RNA was used. Furthermore, 60 ng random hexamers, 1 µl of 10 mM dNTPs (supplied separately from Thermo), 5×First Strand

Buffer (4µl) (Invitrogen) and 100 mM DTT (1µl) (Invitrogen) were used to synthesize the cDNA which was diluted five times with water after incubating the reaction mixture with 0.5 µl superscript III for 1 hr at 50 °C. The relative mRNA levels were measured by RTqPCR (Qiagen Rotor-Gene Q qPCR machine) using Go Taq qPCR master mix and specific primers **(Table 2.2)**. The primers were made by Merck according to our designs and diluted to a concentration of 20 mM. For each sample, the qPCR mix was prepared as follow: 0.5 µl of each primer (forward and reverse), 2 µl of water and 5 µl of the dye and then 2 µl of cDNA were added. The experimental levels are normalized to GAPDH and calculated relative to the mean value for each gene. All the data were analysed using $2^{-\Delta\Delta C_T}$ method (Schmittgen 2008).

To design primers, various methods were used for different types of primers. For primers designed for mature mRNAs, NCBI Primer-Blast was used to choose primers based on the mRNA sequence from NCBI. To specifically detect mature mRNAs, one of the primers must span an exon-exon junction. For primers designed for pre-mRNAs, UCSC Genome Browser was used. Primer3 (software) was used to select suitable primers from sequences at exon-intron junctions. Pre-mRNA primers were chosen according to the principle that the forward primer is located within the exon and the reverse primer is located within the intron, or vice versa. Candidate primers were then examined again by NCBI Primer-Blast, to make sure no off-target products are predicted. For all primers designing, product size was restricted to between 150 nt and 250 nt, with melting temperatures higher than 58°C but lower than 60°C. GC% was optimised to be more than 40% and less than 60%. Scores for self-complementarity and self-3'- complementarity should be as low as possible. Primer specificity was determined by analysing the melt curve and running PCR products on an agarose gel. Presence of multiple peaks on the melt curve or multiple bands when running the PCR products on an agarose gel would indicate that the primers are non-specific and unsuitable for use, and would need to be redesigned.

Gene	Sequence of forward primer	Sequence of reverse primer
Gpdh	AAGAAGGTGGTGAAGCAGGC	ATCGAAGGTGGAAGAGTGGG
Rpl28	TACAGCACGGAGCCAAATAA	ACGGTCTTGCGGTGAATTAG
Tnf	CTATGGCCCAGACCCTCACA	CCACTTGGTGGTTTGCTACGA
Il1β	AGATGAAGGGCTGCTTCCAAA	GGAAGGTCCACGGGAAAGAC
Irg1	ACTCCTGAGCCAGTTACCCT	CTGTGACAGACTTGAGCATCAT
Cxcl2	TGAACAAAGGCAAGGCTAACTG	ACATCAGGTACGATCCAGGC
Ticam	GTTACATAGCTTGCTGGGCC	CGTTACACTCCACCAACAGC
Peli1	TTTTCTGAACACACGTGCCC	TATGTCCCTCTGTGGCTTGG
Tak1	AGACCACACAGTACTCCACC	GATTCTGCTTCTGTGGAGCG
Traf6	GTCTTAAGGCTTCCAGGTGC	AGAACTGCTTGCCTTTCAGC
Sgk1	GTGAAAGTCGGAGGGTTTGG	ATGAACGCTAACCCCTCTCC
Myc	AACCGCTCCACATACAGTCC	TGGAGGAGACATGGTGAACC
Dusp4	AAACCACTCTATCCCCAGCC	TTGGTCACTTTTGCAGCTGG
Inos2	TTGTAATCTGAGGGCTGGCA	AGACCCTCTGTGAGAATGGC
Trib1	AACATAGCCCGGTTCCAAGA	TCAGATCCGAGTGCCCTTTT
Wdr33	AGGTGGGTGGCCAATCATTA	CCACCCGGTACTCAAGGAAT
Cpsf4	ACTCTGCATGACCCCAATGA	ACCCGGAGAGTCATTTGTGT
Tnf (unspliced)	ACACTGACTCAATCCTCCCC	AGCCTTGTCCCTTGAAGAGA

Table 2.2. The sequence of forward and reverse primers used for RTqPCR.

2.5 Western blotting analysis

Three million RAW264.7 cells were seeded in a P15 plate with 20 ml. Medium. The day after, the medium was aspirated and replaced with 0.5% FBS containing medium. After 24hr, the cells were treated as indicated for each experiment and the medium was aspirated and the cells were washed with chilled PBS. On ice, scrapers are used to detach the cells. Then, the cells ware centrifuged at 10000 for 1 min at 4°C. After that, the cells were re-suspended in 0.5 ml cold phosphate buffer sulphate (PBS) and centrifuged as above. The RAW264.7 cells were lysed using lysis buffer containing (0.5 % Igepal, 0.5% deoxycholate, 0.05% SDS, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM PMSF and make up with PBS). The protein extracts were quantified using Bradford protein assay.

Approximately, 30-50 µg of protein was loaded on an SDS-PAGE gel to run for 4 hr and were transferred to a Polyvinylidene fluoride (PVDF) membrane for 2 hr after wetting it with methanol and then with blotting buffer (5.8 g Tris base, 2.9 g glycine, 0.37 g SDS (or 3.7 ml 10%), water to 800 ml, 200 ml methanol) . Blotting occured at 0.8 mA per square cm of blotting stack. 5% milk powder in TBST (10 ml 1M Tris/HCl, pH 8, 30 ml 5M NaCl, 0.5 ml Tween 20, water to 1L) was used as a blocking agent for a period of 1 hr. Then, the blots were incubated with primary antibody overnight (**Table 2.3**) followed by a 1hr incubation with the corresponding secondary antibody. The antibodies were diluted according to the manufacturer protocol.

Immunoreactivity was detected using chemiluminescence solutions (GE Healthcare). Western blot images were not reassembled after cuts between the vertical lanes. The blots were cut horizontally so to make it easier to probe for various antibodies simultaneously on the same blot. Each continuous image represents a single exposure.

2.6 Immunocytochemistry

The cells were seeded in 8-well chamber (ibidi) at a density of 7000 cells/chamber with 300 µl of media. The chambers were placed in the incubator overnight. The medium was replaced with 0.5% FBS medium. The day after, the cells were treated as indicated in each experiment and then the medium was removed and the cells were fixed with 4% paraformaldehyde (PFA) in PBS. The cells were permeabilized using 0.1% Triton×100 in PBS. The cells were incubated with 3% bovine serum albumin (BSA) for 1 hr before probing them with the primary antibody overnight (**Table 2.3**). In the next day, the cells were incubated with the suitable secondary antibody for 1 hr in a dark place wrapping the chamber with foil. The primary and secondary antibodies were diluted according to the manufacturer protocol in 3% BSA. For nuclear stain, DRAQ5 was used which is diluted according to the manufacturer protocol in PBS. The cells were incubated with DRAQ5 for 5 min. All the in between washes were done carefully with PBS three times for 1 min. Finally, the analysis was done by confocal microscopy using Zeiss LSM510. All images quantification was performed using imagej as follows:

-Open LSM files in imagej.

-Duplicate files (Ctrl D).

-Open the channels needed (Image-duplicate-OK), untick hyperstack.

-Segment Nuclei (Process Filters - Gaussian Blur - Sigma Radius 2 - ok).

-Image-Adjust Threshold-apply-ok.

-Edit Selection-create selection.

-Edit Selection-Restore selection.

-Analyse-Set Measurements-Area.

Antibody	Manufacturer and cat no	Species
P65	CST #8242	Rabbit
IκBα	CST#4814	Mouse
pAkt Ser473	CST #4051 and #3787	Rabbit
pAkt Thr308	CST #13038	Rabbit
Akt	CST #75692	Rabbit
pAMPK Thr 172	CST #2535	Rabbit
AMPK	CST #4150	Rabbit
p4EBP Thr37/46	CST #9459	Rabbit
4EBP	CST #9452	Rabbit
mTOR	CST #2983	Rabbit
TIA-1	Abcam ab40693	Mouse
pACC	CST #11818	Rabbit
pTSC2	#23402	Rabbit
pERK Thr202/Tyr204	CST #4370	Rabbit
CPSF4	Protein tech 15023-1-AP	Rabbit
WDR33	Santa cruz sc-374466	Mouse
Vinculin	CST #13901	Mouse
Symplekin	CST #13071	Mouse
β-actin	CST #3700	Mouse
α-tubulin	CST #3873	Mouse
Lamin	CST #4777	Mouse
ɣ-tubulin	CST #5886	Rabbit

CD14	Bioscience	Rabbit
CD68	Bioscience	Mouse
CD11b	Bioscience	Rabbit

Table 2.3. The primary antibodies used in all immunoblotting and immunofluorescence experiments.

2.7 Fractionation of cytoplasmic and nuclear fractions

To separate the cytoplasmic and nuclear fraction in the RAW264.7 cells, the cells pellet was dissolved in buffer A (Hepes 10mM, KCl 10mM, EDTA 0.1M, EGTA 0.1M, DTT 1mM, PMSF 1 mM and water to 20ml). Then 25 µl of 10% NP-40 (Igepal) was added and left on ice for 5 min. To collect the nuclei, centrifugation 16000g for 1 min was done. The supernatant cytoplasmic fraction was collected and the nuclear pellet was washed with buffer A three times and then dissolved with lysis buffer (0.5 % Igepal, 0.5% deoxycholate, 0.05% SDS, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF and make up with PBS).

2.8 siRNA knockdown

RAW264.7 macrophages were seeded in 6-well plate or 12-well plate and incubated for 24 hours. The next day, the siRNA transfection mixture was made in a total volume of 300 µl of optimum medium for each siRNA to be tested. For all the experiment lipofectamine RNAiMAX transfection reagent was used, as follows:

siRNA mixture		Lipofectamine mixture	
Optimem	145 µl	Optimem	142.5 µl
siRNA	5 µl	Lipofectamine	7.5 µl

The above components need to be mixed gently by slow vortexing for 10 seconds then the mixture was incubated at room temperature for 5-10 minutes to allow transfection complex to form between siRNA and lipofectamine. Meanwhile, the medium in each well was replaced with 1.7 ml of fresh pre-warmed 10 FBS-containing medium. Then, 300 µl of the transfection mixture was added to the appropriate well followed by gentle swirl to mix the mixture with the medium. The plate was incubated for another 24 hours. On the third day, the siRNA mixture was made as mentioned previously and incubated for 10 minutes. Meanwhile, the medium was removed and the cells were washed with PBS. Then, 1.7 ml of 0.5% FBS-containing medium was added to each well followed by 300 µl of the siRNA mixture to the appropriate well and incubated for 24 hours. The day after, the cells were stimulated with LPS for 60 minutes and then harvested for RNA isolation technique or for preparation of lysate for immunoblotting. The cells were also fixed and stained for immunocytochemistry experiments.

Lipofectamine RNAiMAX transfection reagent (cat no 13778150) was purchased from Thermo Fisher Scientific. SMARTpool: ON-TARGETplus Wdr33 siRNA (cat no L-051645-01-0005 5) and SMARTpool: ON-TARGETplus Cpsf4 siRNA (cat no L-052851-01-0005) were purchased from Dharmacon.

2.9 Human blood-derived monocytes (primary cell work)

We were covered under the ethics for "Isolation and purification of peripheral blood leukocytes for proliferation and other immunological studies" – reviewed and approved by the University of Nottingham Medical School Ethics Committee (BT/11/2005).

2.9.1 Isolation of PBMCs from leukocyte cone

The leukocyte cones were collected and transported from the blood bank in the Queen's Medical Centre. The cones were washed with IMS before being transferred inside the hood. The content of each cone was transferred to 75 cm^2 flash, then the blood was made up to 75 ml with HBSS. 20 ml of lymphoprep was added to 50 ml Falcon tube (three tubes), then the blood was added very slowly into these three tubes (25 ml in each) and centrifuged at 400×g for 30 minutes at 20˚C without brake and using swinging bucket rotor. The peripheral blood mononuclear cells (PBMCs) in the thin white interphase were carefully isolated using 10 ml pipette and pooled in a 50 ml Falcon tube. Then, the tube was filled with Buffer 1 (according to Miltenyi cat no 130-096-537). Centrifugation at 300×g for 10 minutes at 20˚C was done and then the supernatant was removed carefully. The pellet was resuspended in 50 ml HBSS and centrifuged 200×g for 10 minutes at 20˚C and the supernatant was discarded (repeated twice). If the pellet was red, 1 ml of RBCs lysis buffer was added and diluted to 20 ml using Buffer 1. Centrifugation at 250-500×g for 7 minutes was done. The supernatant layer was decanted and if the pellet was still red, the RBCs lysis buffer step could be repeated again. The cells were declumped using cell strainer and the pellet was dissolved in the appropriate amount of HBSS to get a cell density of 1×10^8 cells/ml.

2.9.2 The purification of monocytes from PBMCs

The purification was done according to the protocol provided by Miltenyi Biotec (cat no 130-096-537). The purity of the enriched monocytes was evaluated by flow cytometry using the anti-CD14 antibody. The monocytes were then differentiated into macrophages.

2.9.3 Differentiation of human blood-derived monocytes into macrophages

Purified monocytes were seeded on 10 cm plate at a density of 1×10^6 cells/ml in medium (RPMI+L-Glutamine+5% FBS+1:1000 0f 100 μg/ml M-CSF). The cells were incubated at 37°C and 5% CO2 for 4 days. To harvest the cells, the medium was discarded and the cells were washed twice with LPS-free PBS. Then 2 ml of medium (RPMI + 3% FBS + penicillin/streptomycin) was added and the cells were detached using a cell lifter and collected in a 50 ml Falcon tube followed by a centrifugation at 1500 rpm for 5 minutes and the supernatant was discarded carefully as the pellet was slightly loose. The pellet was resuspended using medium (RPMI + 3% FBS + penicillin/streptomycin). The cells were counted using trypan blue and haemocytometer.

2.9.4 Staining of cell surface and intracellular proteins for FACS analysis

The cells suspension was centrifuged (1500 rpm for 5 minutes) and the cells pellet was washed using PBS and then the supernatant was discarded. The cells were resuspended in 0.5 ml of FACS buffer (2% BSA + 2mM EDTA + 0.02% NaN3 + PBS). 3×10^5 cells were transferred to U-bottom 96-well plate and spinned 1800 rpm for 2 minutes and the supernatant was discarded. Then, the antibody solution was added and incubated 20-30 minutes at 4°C in the dark. The cells were washed with FACS buffer and the medium was discarded (repeated twice). Analyse by FACS. The cells could be fixed using 4% PFA and kept in 4°C for later FACS analysis. If the target protein is intracellular, a permeabilization step is required. This was achieved by adding 0.5% saponin to the FACS buffer.

3. The anti-inflammatory effect of cordycepin

Inflammation is a response of the innate immune system to infection and tissue injury. The term refers to a complex biological process whose purpose is to defend the host by eliminating invading pathogens, repairing damaged tissues, and restoring homeostasis. During the early stage of inflammation, circulating monocytes leave the bloodstream and migrate into tissues where, following conditioning by local growth factors and pro-inflammatory cytokines, they differentiate into macrophages (Shi 2014). I will focus specifically on macrophages, since studies have found this cell type to be sensitive to cordycepin (Kim 2006, Shin 2009).

Lipopolysaccharide (LPS) is an archetypal pathogen-associated molecular patterns found on the outer membrane of gram-negative bacteria. It triggers the innate immune system into action through activation of Toll-like receptor 4 (TLR4). LPS-induced activation of macrophages results in the production of pro-inflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukines-1beta (IL-1β) (Jack 1997, Meng 1997 and Fang 2018). These two prototypic inflammatory cytokines have been implicated in the pathological consequences of many inflammatory disease (Leal 2013, Munoz 2008 and Wojdasiewicz 2014).

In innate immunity, Myeloid differentiation primary response 88 (MyD88) is a common adaptor for nearly all TLRs. It interacts with IL-1-receptor-associated kinase (IRAK) 1,2 and 4. IRAK 1 and IRAK2 subunits can then interact with TNF receptor-associated factor 6 (TRAF6) and induce TRAF6 dimerization, which triggers the activation of its E3 ligase activity and activates transforming growth factor beta-activated kinase 1 (TAK1). TAK1 has a critical role in the MyD88 signalling network. It also plays a role in activating the canonical IκB kinase (IKK) complex, and mitogen-activated protein (MAP) kinase kinases (MKKs). Pelino-1 plays an important role in this signalling pathway. It mediates the interaction between TAK1 and IKK (Strickson 2017 and Medvedev 2015).

Because of the central role of this pathway in controlling the inflammatory response, I tested the effect of cordycepin on the expression of its mediators at the mRNA level. In addition, I assessed the effect of cordycepin on the expression of other gene encoding proteins that link signal transduction pathways with inflammatory reactions including Dusp4, Myc, Sgk1 and Inos2. Dual specificity phosphatase 4 (DUSP4) is a negative regulator of the mitogen-activated protein kinase (MAPK) superfamily, specifically extracellular-regulated kinase (ERK) activity, which has a role in cellular proliferation and survival (Baglia 2014). The proto-oncogene, MYC, lies at the crossroads of many growth promoting signal transduction pathways including MAPK. It has been found that Myc drives tumour growth through its ability to regulate the tumour microenvironment particularly the immune response, inflammation and angiogenesis (Kortlever 2017). It has been found that SMAD7 plays an essential role in some inflammatory diseases such as inflammatory bowel disease. Studies have shown that inhibiting SMAD7 using antisense oligonucleotide-based pharmaceutical compound is considered a promising therapeutic approach in inflammatory diseases (Monteleone 2012). Serum and Glucocorticoid Regulated Kinase 1 (SGK1), an Akt kinase homolog, plays a key role in regulating neutrophil survival signalling, and thus may prove a valuable therapeutic target for the treatment of inflammatory diseases (Burgon 2014). It has been found that inducible nitric oxide synthetase (Inos2) plays a major role in the pathogenesis of osteoarthritis. Moreover, inhibition of this mediator either genetically of pharmacologically restore normal joint function (Berg 1999).

A number of studies have shown that cordycepin has anti-inflammatory effects in cell culture as well as animal models. In particular, these studies have shown the late anti-inflammatory effect of cordycepin by treating the cells for 24 hours. However, the activity of cordycepin at earlier stages has never been investigated (Kim 2011, Jeong 2010, Shin 2009 and Li 2016). Here, I shall establish the inflammatory system first and then offer new insight into the early effect of cordycepin on the inflammatory gene expression in RAW264.7 murine macrophages. Unfortunately, a large number of these studies have utilized high doses of cordycepin. In this study, I attempted to focus primarily on low micromolar concentrations to avoid overwhelming intracellular molecular processes, given the nucleotide-like structure of the drug.

3.1 Establishing the inflammatory system

To establish the inflammatory system and observe the expression profile of inflammatory genes over time, RAW264.7 cells were stimulated with different inflammatory stimuli (TNF, IL1B and LPS) for (0, 30 and 60 min) and RTqPCR was done on samples from the time course. Over one hour time period, the expression of Tnf mRNA was not significantly increased upon stimulation with TNF and IL1B. However, a significant increase in the magnitude of Tnf expression was observed after 30 min treatment with LPS, that remained elevated for the entire time course, up to 60 minutes **(Figure 3.1)**. This suggests that LPS is the best inflammatory stimuli for RAW264.7 cells and will be used to carry out our experiments to study the anti-inflammatory effect of cordycepin.

3.2 Cordycepin reduces inflammatory transcription in macrophages

Our lab group previously observed anti-inflammatory effects of cordycepin in airway smooth muscle cells (Kondrashov 2012). To assess whether these effects were also present in macrophages, I incubated RAW 264.7 cells with DMSO or cordycepin prior to stimulating the cells with LPS to provoke an inflammatory response. Expression of inflammatory genes was then measured by RT-qPCR at a number of time points over a course of 2 hours. Inflammatory gene expression of assessed genes (Tnf, Il1b, Acod1 and Cxcl2) was found to be repressed by cordycepin (**Figure 3.2**), while housekeeping gene expression (Hprt and Gapdh) was unaffected.

In order to assess how fast the effects of cordycepin were, cells were treated with cordycepin 10 minutes after LPS stimulation (**Figure 3.3**). The repressive effects of cordycepin for cells pretreated with cordycepin and cells treated with cordycepin after LPS addition were identical. Cordycepin’s observed effects are clearly very fast, and are thus likely to be primary in nature.

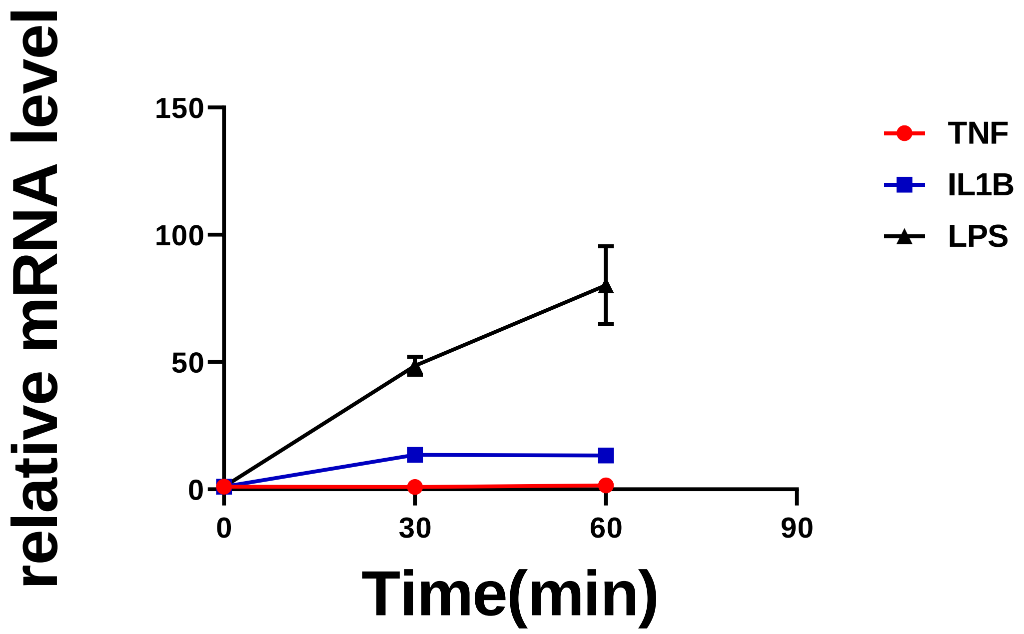
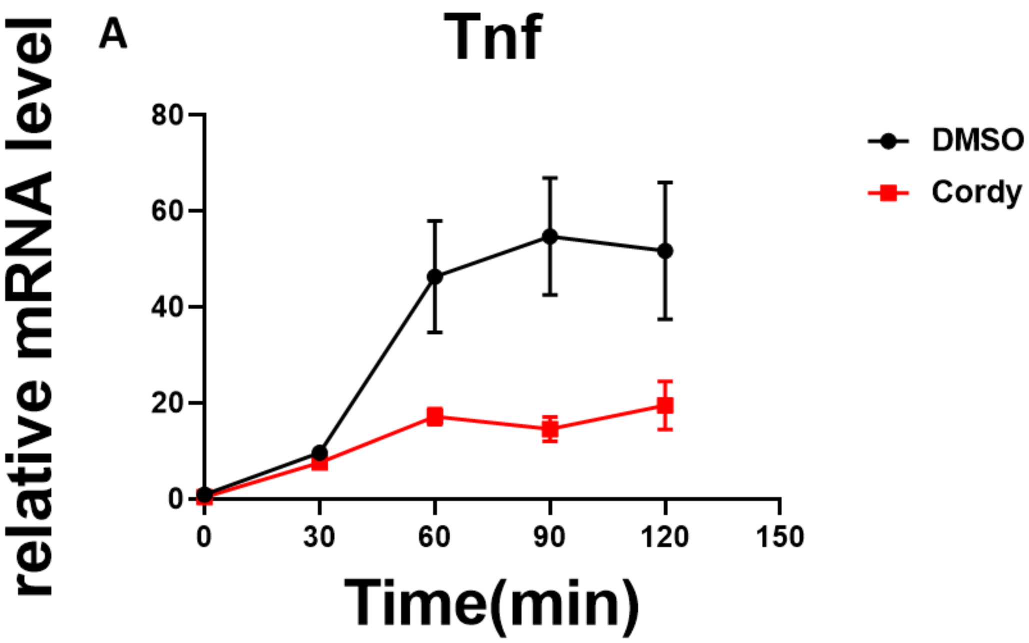
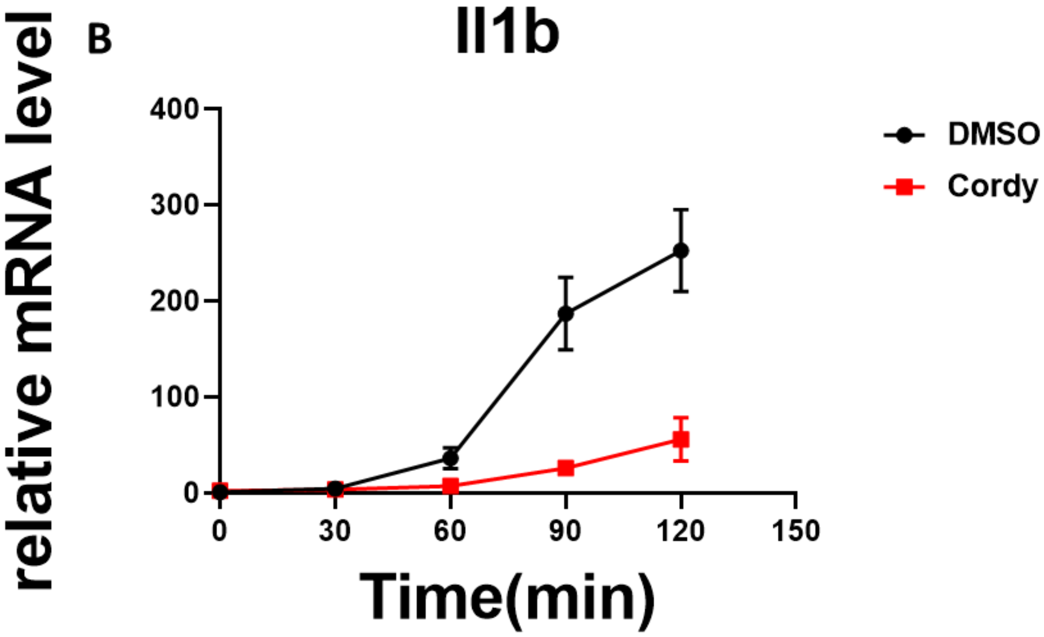
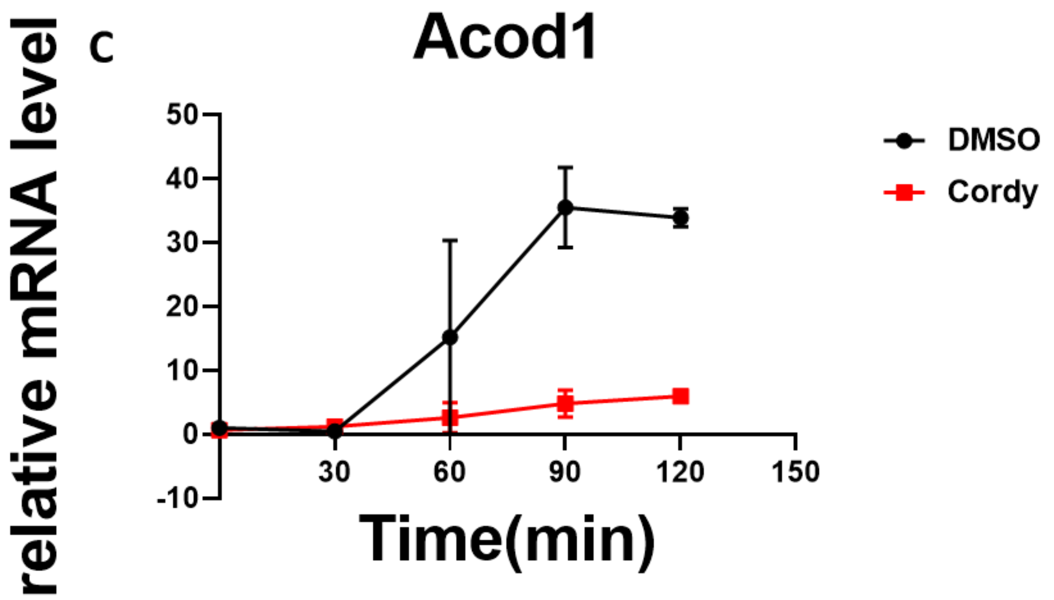
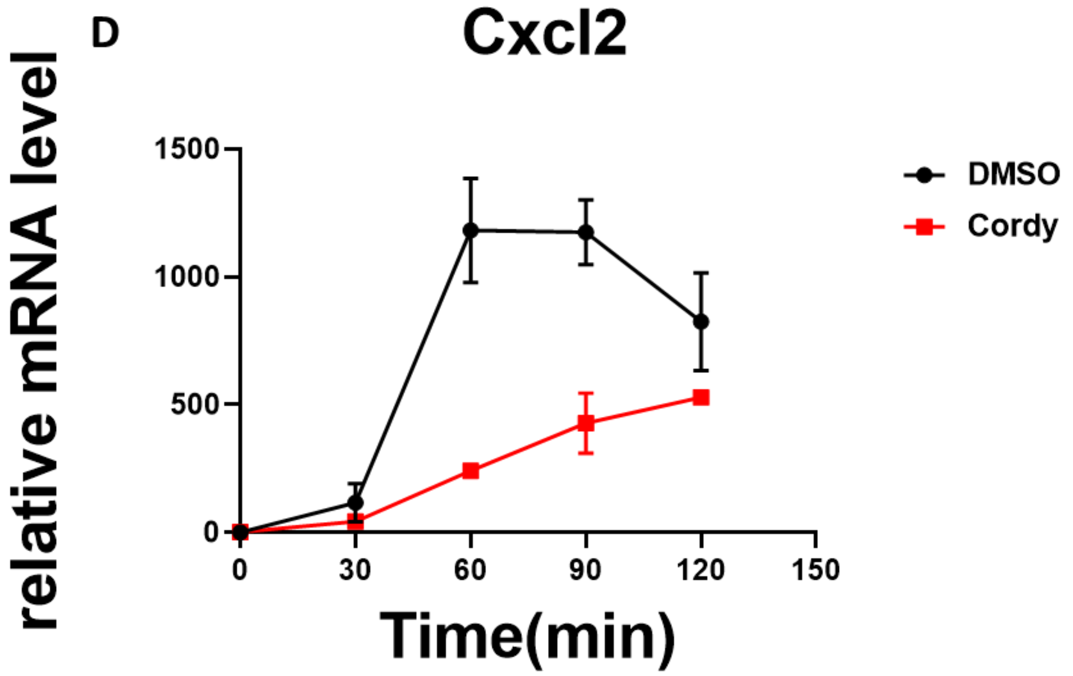


Figure 3.1. LPS induces inflammatory gene expression in macrophages. RAW264.7 cells were stimulated with TNF, IL1B or LPS for 0, 30 or 60 minutes. RTqPCR was performed, error bars represent standard deviations across 3 technical replicates.

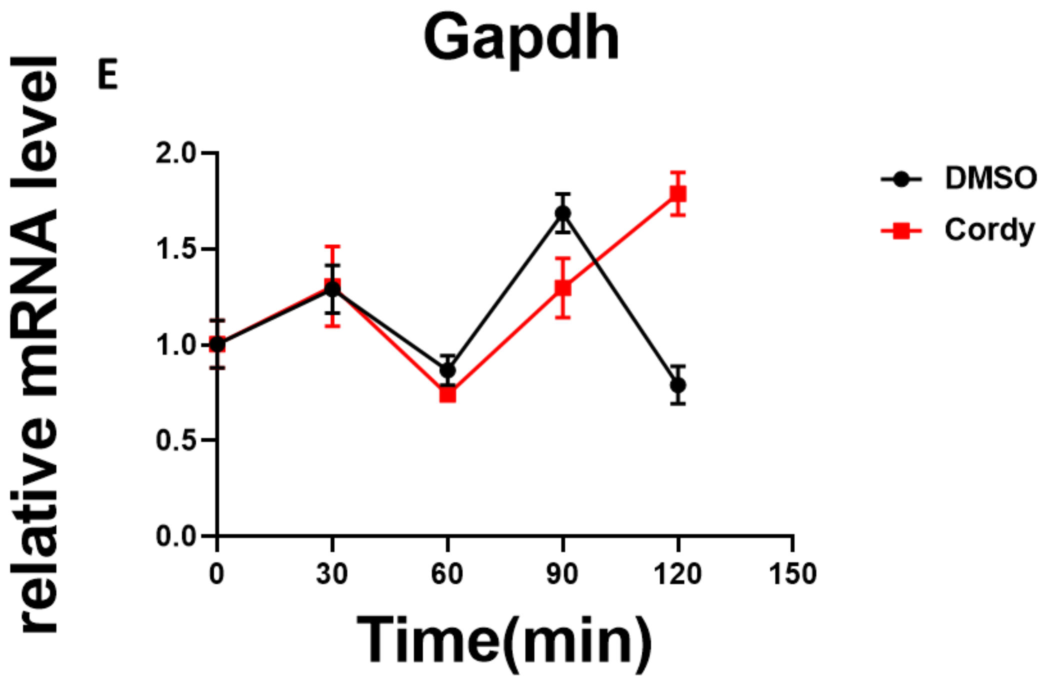

A
Tnf
relative mRNA level
Time(min)
DMSO
Cordy
0
20
40
60
80
0
30
60
90
120
150


B
Il1b
relative mRNA level
Time(min)
DMSO
Cordy
0
100
200
300
400
0
30
60
90
120
150


C
Acod1
relative mRNA level
50
40
30
20
10
0
-10
30
60
90
120
150
Time(min)
DMSO
Cordy


D
Cxcl2
relative mRNA level
1500
1000
500
0
0
30
60
90
120
150
Time(min)
DMSO
Cordy

E



F

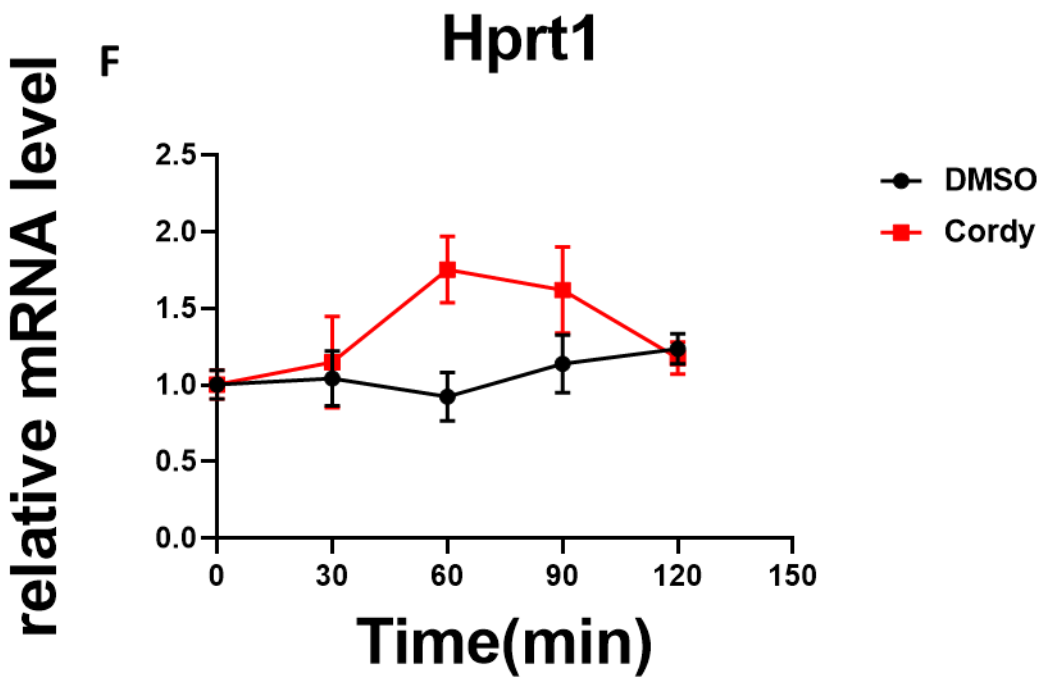
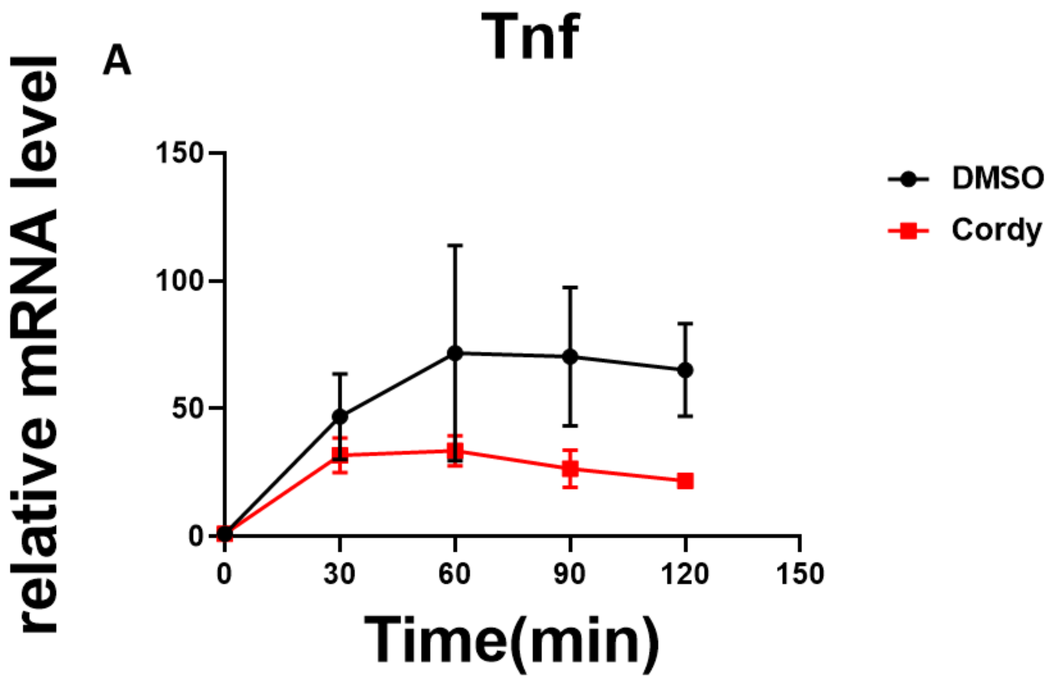
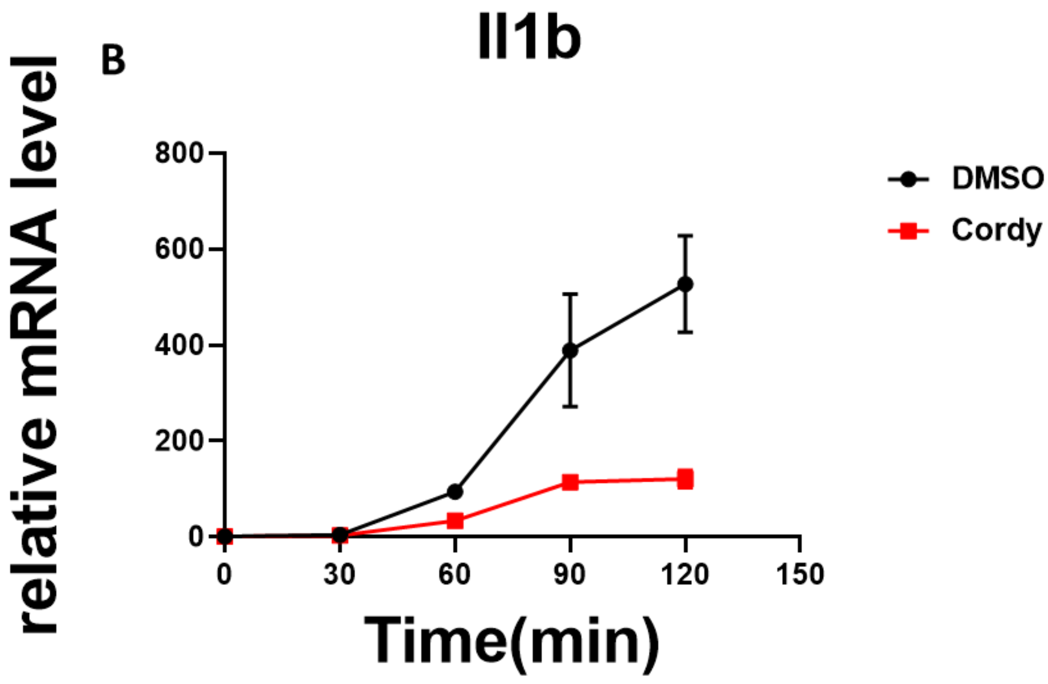
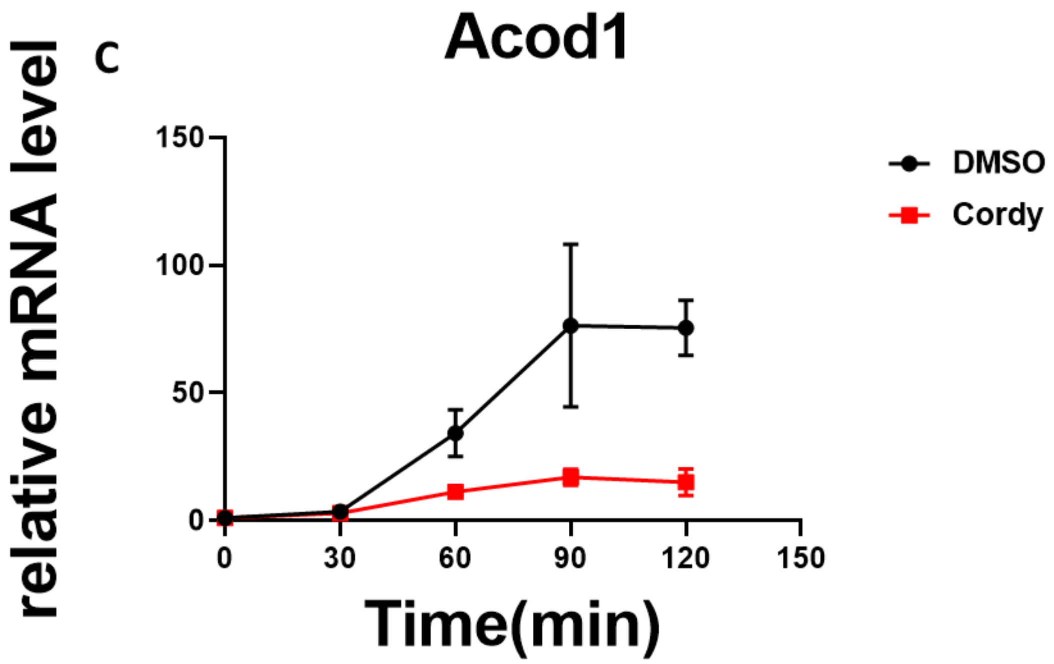
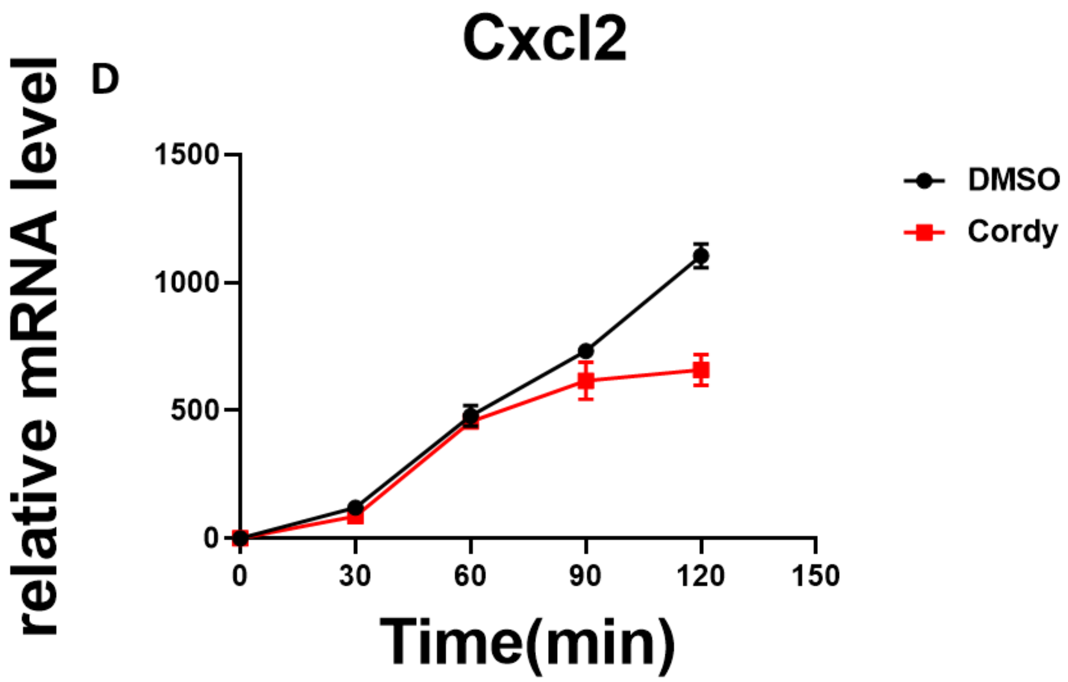


Figure 3.2. Cordycepin represses inflammatory gene expression. RAW 264.7 cells were treated with DMSO or 20 µM cordycepin for an hour (DMSO and Cordy respectively) prior to LPS addition. RTqPCR was performed, error bars represent standard deviations across 3 independent replicates except for Gapdh and Hprt1, for which they represent standard deviations across 3 technical replicates. **A.** Tnf, **B.** Il1b, **C.** Acod1, **D.** Cxcl2, **E.** Gapdh and **F.** Hprt1.


A
Tnf
relative mRNA level
150
100
50
0
0
30
60
90
120
150
Time(min)
DMSO
Cordy


B
Il1b
relative mRNA level
800
600
400
200
0
0
30
60
90
120
150
Time(min)
DMSO
Cordy


C
Acod1
relative mRNA level
150
100
50
0
0
30
60
90
120
150
Time(min)
DMSO
Cordy


D
Cxcl2
relative mRNA level
1500
1000
500
0
0
30
60
90
120
150
Time(min)
DMSO
Cordy

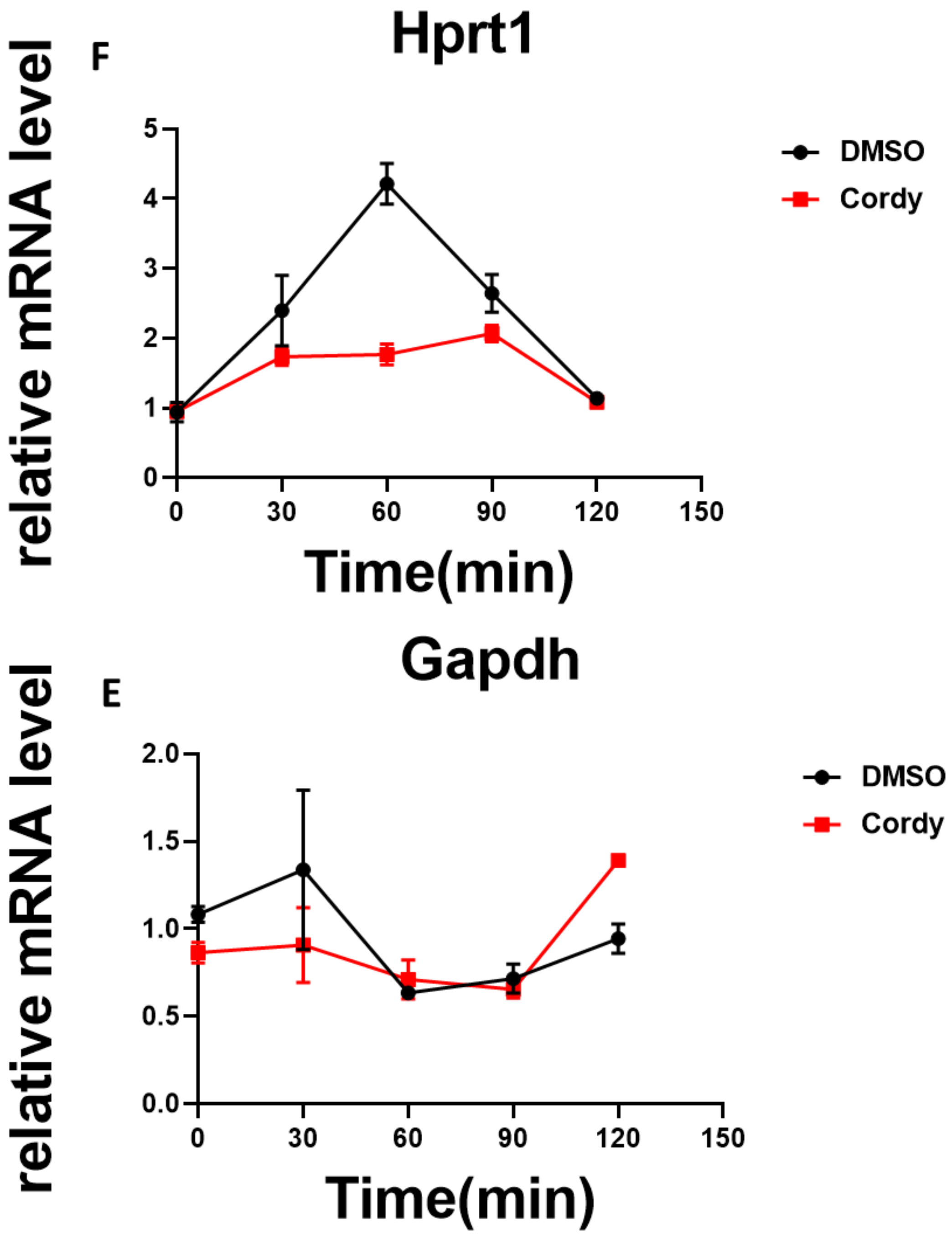


Figure 3.3. Administering cordycepin 10 minutes after LPS causes the same repression as a 1 hour pre-treatment. RAW264.7 were stimulated with LPS for the indicated durations. DMSO or 20μM cordycepin (DMSO and cordy respectively) were added 10 minutes after LPS. RTqPCR was performed, error bars represent standard deviations across 3 independent replicates except for Gapdh and Hprt1, for which they represent standard deviations across 3 technical replicates. **A.** Tnf, **B.** Il1b, **C.** Acod1, **D.** Cxcl2, **E.** Gapdh and **F.** Hprt1.

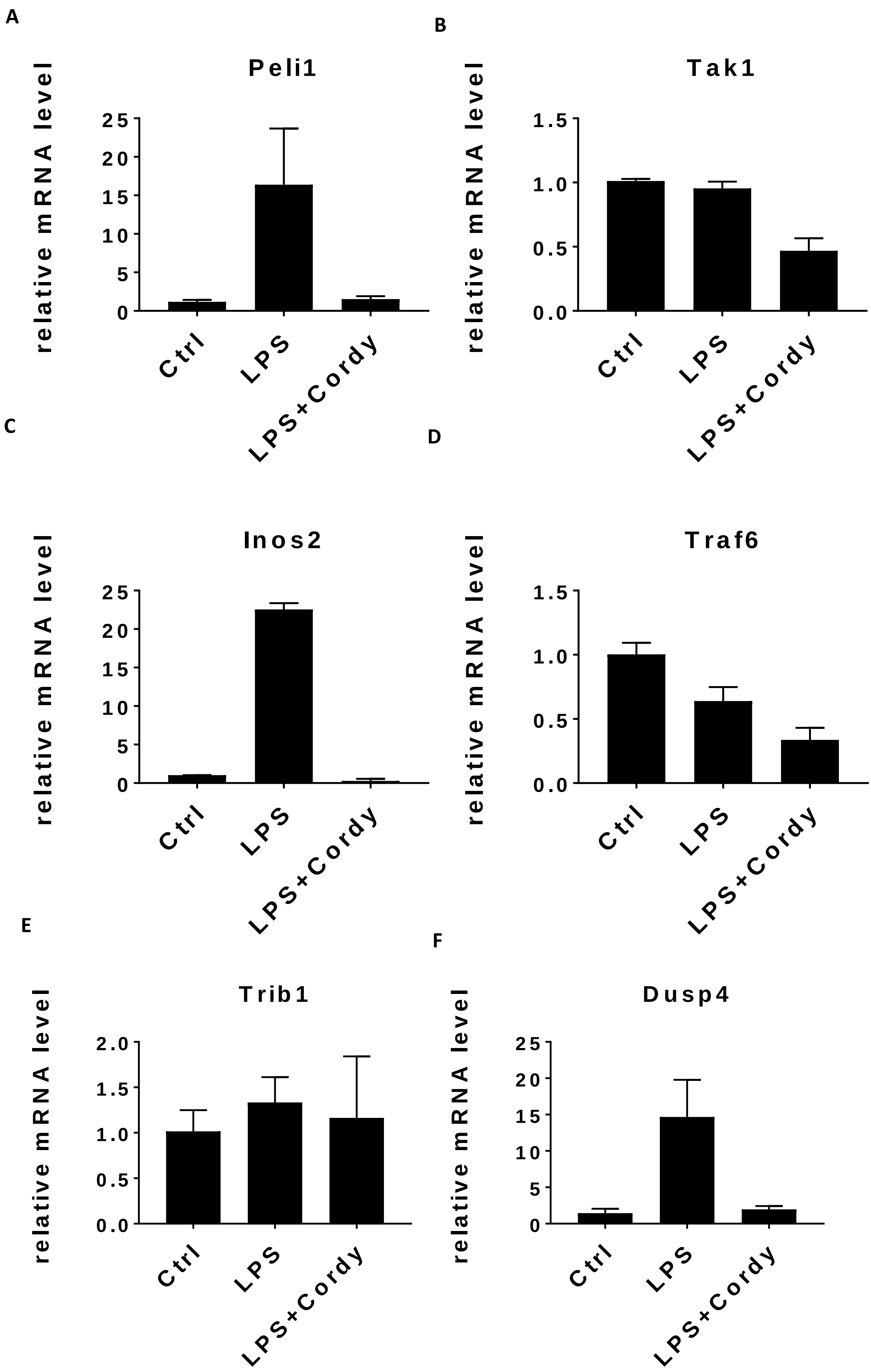
3.3 The effect of cordycepin on genes encoding proteins involved in Toll-like receptor pathway and signal transduction

To assess the effect of cordycepin at the long term, I looked at its effect on the expression of genes encoding proteins involved in the regulation of TLR pathway and signal transduction. Some of these genes were chosen on the basis of a previous microarray experiment done by Raj Gandhi. Performing gene ontology analysis on the list of genes most strongly downregulated in the LPS with cordycepin treatment compared to LPS alone revealed that immune and inflammatory gene clusters are the most significantly enriched (Raj Gandhi).

The cells were treated with DMSO (control) or cordycepin before stimulating them with LPS and the expression of these genes was measured by RTqPCR. The expression of the genes assessed is significantly repressed by cordycepin. This could indicate that cordycepin has also secondary anti-inflammatory effects which affect the mRNA level of some signal transduction mediators which are known for their ability to regulate inflammation.

3.4. Cordycepin reduces the differentiation of human blood-derived monocytes into macrophages

To extrapolate our results to human macrophages and to assess the effect of cordycepin on the earlier stages of inflammation, I investigated the effect of cordycepin on the differentiation of human blood- derived monocytes into macrophages. Monocytes were isolated from peripheral human blood using negative


A
Peli1
relative mRNA level
25
20
15
10
5
0
Ctrl
LPS
LPS+Cordy
B
Tak1
relative mRNA level
1.5
1.0
0.5
0.0
Ctrl
LPS
LPS+Cordy
C
Inos2
relative mRNA level
25
20
15
10
5
0
Ctrl
LPS
LPS+Cordy
D
Traf6
relative mRNA level
1.5
1.0
0.5
0.0
Ctrl
LPS
LPS+Cordy
E
Trib1
relative mRNA level
2.0
1.5
1.0
0.5
0.0
Ctrl
LPS
LPS+Cordy
F
Dusp4
relative mRNA level
25
20
15
10
5
0
Ctrl
LPS
LPS+Cordy

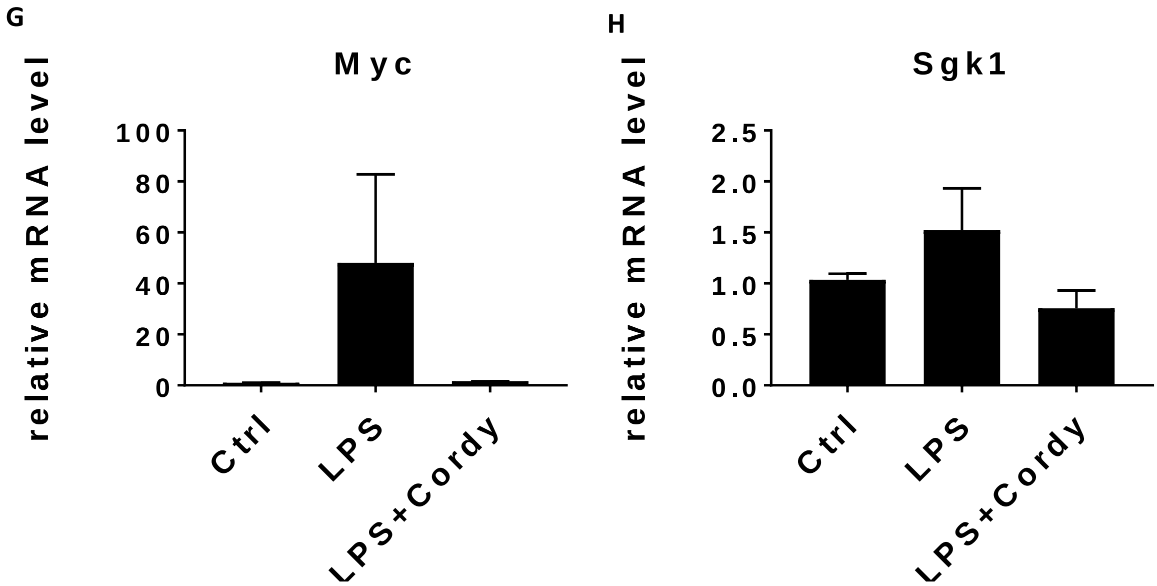


Figure 3.4. Cordycepin has a repressive effect on genes encoding proteins involved in Toll-like receptor pathway and signal transduction. RAW 264.7 cells were treated with DMSO or 20 µM cordycepin for an hour prior to LPS addition. RTqPCR was performed. Error bars represent standard deviation across two independent replicates. **A.** Peli1, **B.** Tak1, **C.** Inos2, **D.** Traf6, **E.** Trib1, **F.** Dusp4, **G.** Myc and **H.** Sgk1.

selection technique and their purity was assessed by measuring the expression of CD14 (as indicated by the green color, other colors represent other cell populatios) **(Figure 3.5)**. Thereafter, the human monocytes were differentiated into macrophages using macrophage-colony stimulating factor (M-CSF). The differentiation had been done in the presence or absence of cordycepin in order to assess the effect of cordycepin on this early stage of inflammatory response which could mimic the recruitment of monocytes to the site of injury where they differentiate into macrophages to elicit the inflammatory response. To this end, flow cytometry had been done to measure the expression of CD11b and CD68 which are macrophages maturation markers **(Figure 3.6)**. The data showed that in the presence of cordycepin the expression of CD11b and CD68 were reduced. This could indicate that cordycepin affects the differentiation of human blood-derived monocytes into macrophages.

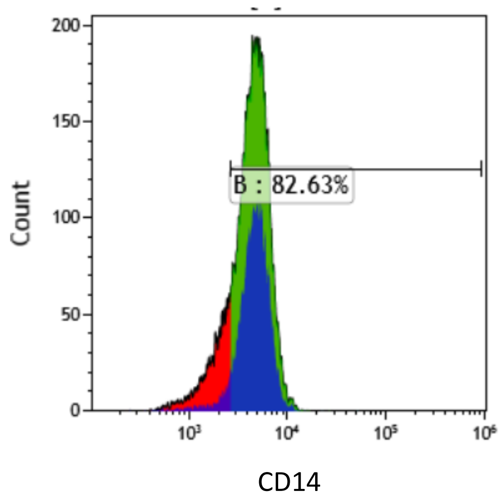


Figure 3.5. The purity of human blood-derived monocytes. Monocytes were isolated from blood and flow cytometry was used to measure the expression of CD14.

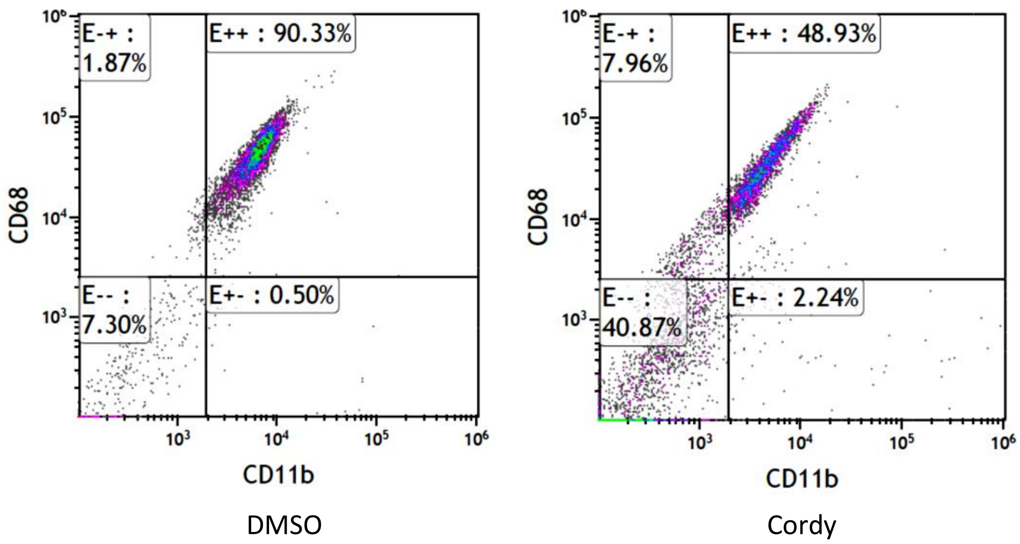


Figure 3.6. Cordycepin affects the differentiation of human blood-derived monocytes into macrophages. Monocytes were incubated with M-CSF for 5 days in the presence or absence of 10 μM cordycepin. CD11b and CD68 expression were measured by flow cytometry.

Conclusion

A number of studies have shown that cordycepin has anti-inflammatory effects in cells (Kim 2011, Jeong 2010, Moon 2009, Li 2016 and Kondrashov 2012). In addition, the antiinflammatory effect of this compound has beeb indicated in a number of animal models with inflammatory disease, including lung injury, cerebral ischemia and asthma (Yang 2015, Chen2012, Kim 2011 and Cheng 2011). Data in this chapter demonstrated the anti-inflammatory effects of cordycepin in RAW 264.7 cells. A working inflammatory model was established in the RAW 264.7 cells. Although primary macrophages are considered the ideal choice for being more representative of true macrophage biology, the RAW 264.7 cell line has been used because the cell lines offer greater reproducibility across different types of experiments. The RAW264.7 cells were treated with three different inflammatory stimuli (LPS, TNF-α and IL1β) for 0, 30 and 60 minutes. LPS is the strongest inflammatory stimulus as measured by levels of mature mRNA of inflammatory genes **(Figure 3.1)**. Accordingly, LPS will be used in all our experiments to trigger the inflammatory response in RAW264.7 murine macrophages. Cordycepin decreased the expression of the assessed inflammatory genes. It produced identical effect when it was added either one hour before LPS or 10 minutes after LPS stimulation **(Figure 3.2 and Figure 3.3)**. This indicates that the anti-inflammatory effect of cordycepin is fast and primary in nature. Furthermore, cordycepin repressed the mRNA level of genes encoding proteins that have a role in Toll-like receptor activation including Traf6, Tak1 and Peli1. Other genes that encode protein that have a role in linking signal transduction pathways with inflammation were also repressed such as Inos2, Dusp4, Myc, Smad7 and Sgk1 **(Figure 3.4)**. This could indicate that, beside its primary effect, cordycepin might have a secondary anti-inflammatory activity in macrophages. Many mRNAs involved in inflammation are inherently unstable due to the presence of regulatory *cis* elements in their 3' UTRs, a well-studied example of such elements is the AU-rich elements (AREs) (Anderson 2010, Stumpo 2010 and Hao 2009). ARE functions, at least in part, to recruit the deadenylase machinery via trans-acting factors that removes the poly A tail. This is often regarded as the first and rate-limiting step of mRNA decay (Chen 2011), and so a model can be conceived wherein the time taken to remove the poly A tail represents a delay before the mRNA can be degraded. Thus, regulation of poly (A) tail size could be particularly important for those mRNAs that are inherently unstable (like inflammatory mRNAs) and thus depend on the delay to exist long enough to be translated.

Then I investigated the effect of cordycepin on the differentiation of human blood-derived monocytes into macrophages. The results showed that this compound reduces the number of CD11b- CD68-double positive macrophages compared with cells differentiated in the absence of cordycepin **(Figure 3.6)**. The results of this experiment suggest that cordycepin may also affect the early stage of inflammatory response by reducing the maturation of inflammatory cells. However, only one replicate was done, thus two more biological replicates are still needed to confirm this finding. To summarize, cordycepin produces a fast anti-inflammatory effect in macrophages and this is likely due to a direct effect on inflammatory gene expression, with secondary longer term effects on the expression of key components of the TLR signalling pathway.

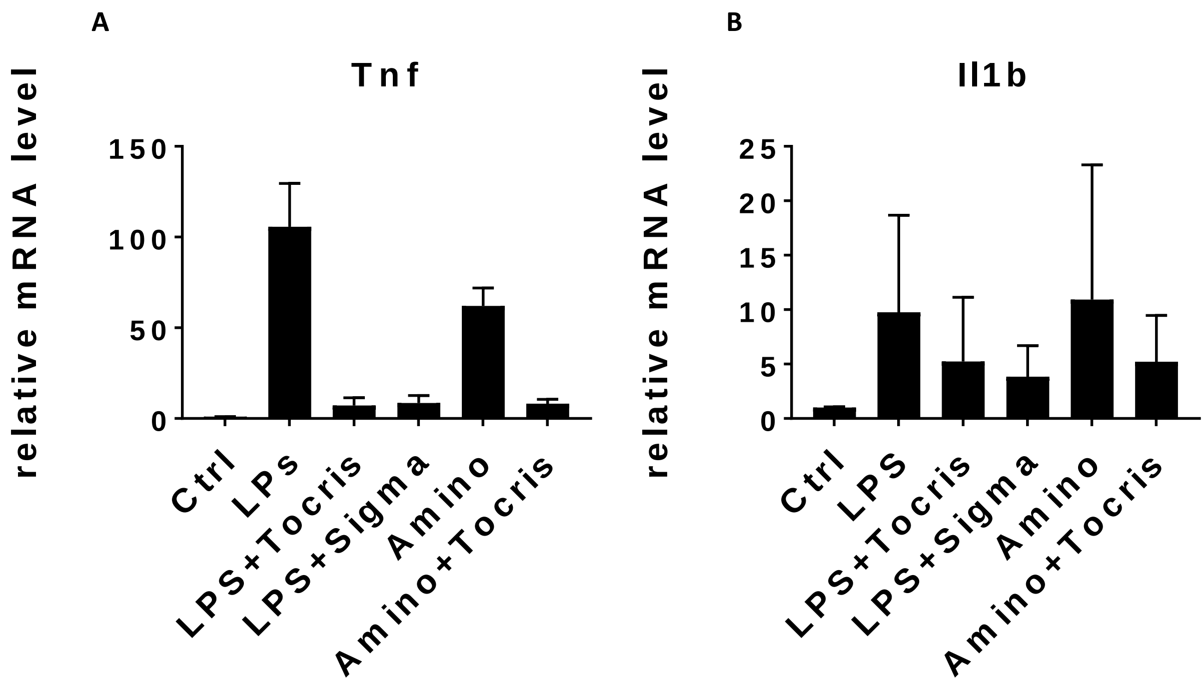
4. Cordycepin metabolism and other nucleosides

Because of the structural similarity between adenosine and cordycepin, I hypothesized that cordycepin might act as an adenosine receptor agonist in an attempt to identify the underlying mechanism of action of cordycepin. It has been found that adenosine has an anti-inflammatory activity and this activity is attributed mainly to its activation of the adenosine receptor. Three types of adenosine receptors have been recognized which are A1, A2, and A3. A2 is further subdivided into A2a and A2b. It has been reported that the anti-inflammatory effect of cordycepin is mainly A2 receptor-dependent (Imamura 2015, Hu 2013, Li 2018 and Gao 2018). However, studies done by our group have reported that cordycepin works intracellularly in both airway smooth muscle cells and NIH3T3 cell line (Kondrashov 2012 and Wong 2010). In this chapter, I aimed to answer the question whether cordycepin produces its anti-inflammatory effect in RAW264.7 cells as an adenosine receptor agonist or whether it has another target.

Animal studies done by our group showed that cordycepin reduces pain and improves disease pathogenesis in osteoarthritic animal models (Ashraf 2019). However, in spite of the significant activity, no cordycepin was detected in blood or plasma of the animals used in the pharmacokinetic studies (Jong Bong Lee, Cornelia de Moor and Pavel Gershkovich, unpublished data). I hypothesized that cordycepin is converted into an intermediate metabolite that is responsible for its therapeutic effect in the osteoarthritic animal models. Looking at the nucleoside metabolism pathways in the literature, I hypothesized that this active metabolite could be 3’deoxyinosine (Wataya 1984, Sugar 1998). To confirm this hypothesis, I investigated the effect of this compound on the inflammatory system to see whether it has comparable anti-inflammatory effect to cordycepin or not.

4.1 Comparing the anti-inflammatory effect of cordycepin from different sources

To compare the cellular effects of cordycepin produced by different commercial sources and assess the impact of different types of contaminants on its biological effect, we bought cordycepin from Merck and Tocris which are both derived from natural sources (*Cordyeceps militaris*), as well as aminocordycepin produced by Carbosynth. MS and MS/MS data showed that Merck cordycepin is contaminated with 2% aminocordycepin while Tocris cordycepin is contaminated with 2% adenosine (Catherine Ortori and David Barrett, unpublished results). In addition, the mass spectrometry showed that the aminocordycepin is significantly contaminated with at least 2 other adenine-containing compounds. The plan was to compare the effect of Merck cordycepin, Tocris cordycepin, Carbosynth aminocordycepin, and 50:1 mixture of the last two by RTqPCR. RAW264.7 cells were pre-treated with cordycepin for an hour and then stimulated with LPS for another one hour in order to produce a pronounced inflammatory response. I looked at the fold change of genes under different conditions: DMSO, aminocordycepin, Merck cordycepin, Tocris cordycepin and aminocordycepin plus Tocris cordycepin (50:1). The results indicated that both sources were able to replicate the repressive effects of cordycepin on inflammatory gene expression in RAW 264.7 cells while aminocordycepin did not affect them. Furthermore, it did not affect the Tocris cordycepin when they were in 50:1 mixture **(Figure 4.1)**. Thus, these results suggested that the compound that downregulates the inflammatory genes is the cordycepin and not any of the contaminants. Then I looked at the anti-inflammatory effect of cordycepin from Carbosynth, against the three previous compounds. The results **(Figure 4.2)** showed that the effect of Carbosynth cordycepin is comparable to those from Merck and Tocris. This finding further strengthens our conclusion that the active ingredient in all cordycepin sources is the 3' deoxyadenosine and not any of the contaminants. Moreover, non-stimulated macrophages were treated with cordycepin only and microarray was done. Results showed that cordycepin does not induce inflammatory gene expression in this system (Raj Gandhi thesis 2017).



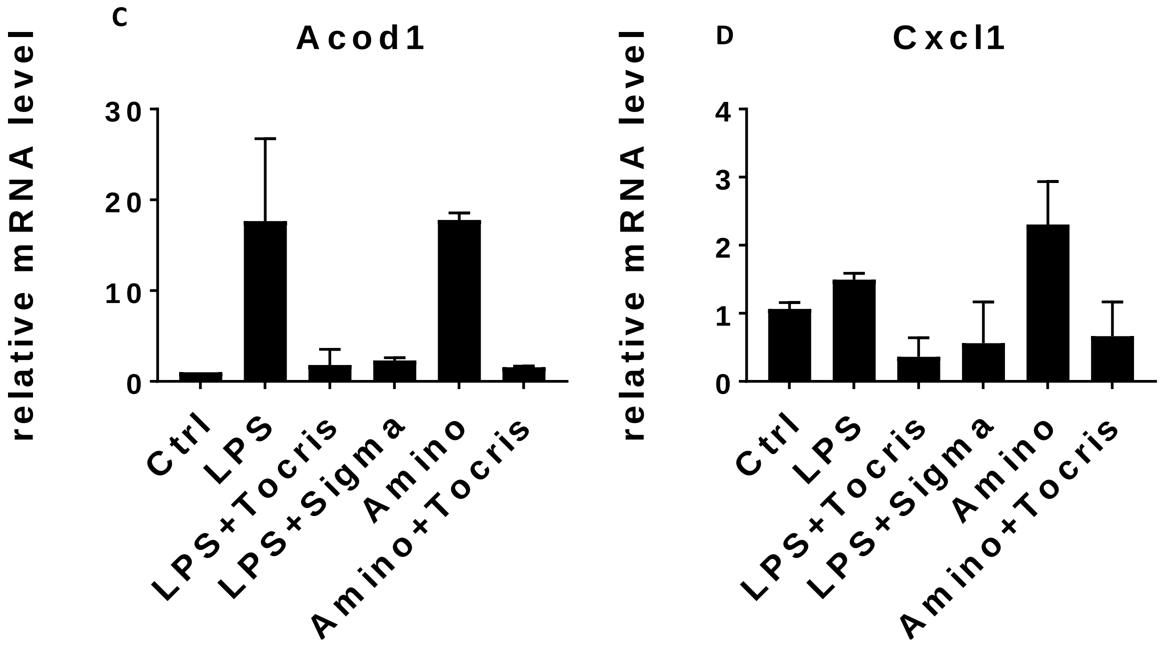


Figure 4.1. Comparing the cellular effect of cordycepin from different commercial sources. RAW 264.7 cells were treated with DMSO or 20 µM cordycepin for an hour prior to LPS addition. RTqPCR was performed, error bars represent standard deviations across 3 independent replicates. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, p values were obtained using Dunnett's test. **A.** Tnf, **B.** Il1b, **C.** Acod1 and **D.** Cxcl1.

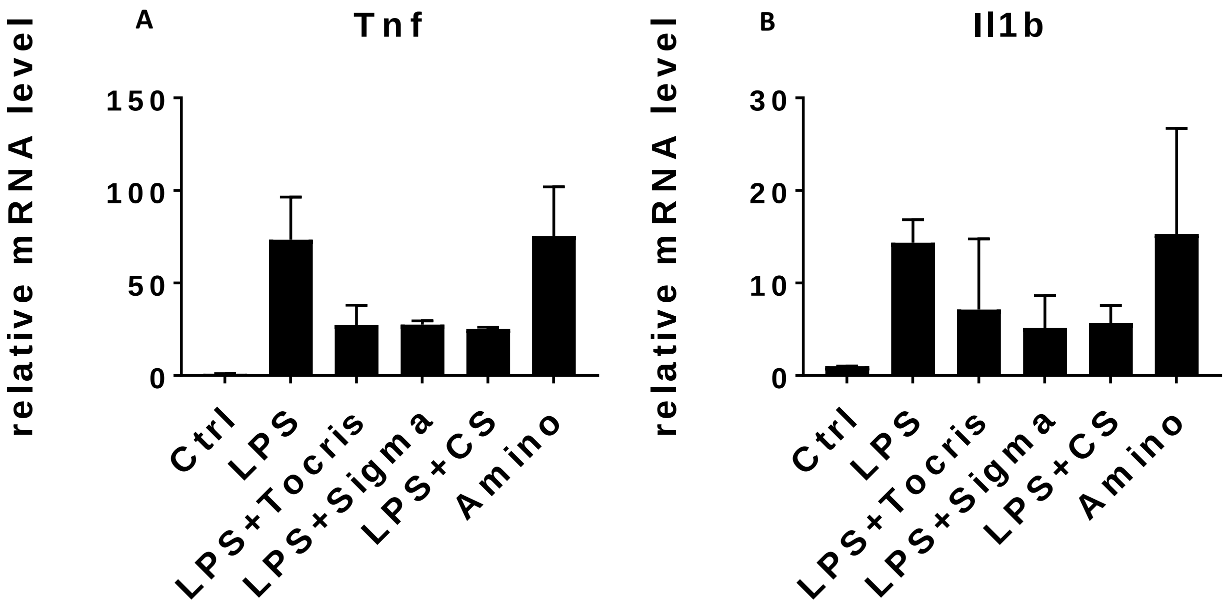


Figure 4.2. Comparing the cellular effect of Carbosynth cordycepin against cordycepin from Merck and Tocris. RAW 264.7 cells were treated with DMSO or 20 µM cordycepin for an hour prior to LPS addition. RTqPCR was performed, error bars represent standard deviations across 2 independent replicates. **A.** Tnf and **B.** Il1b.

4.2 Cordycepin triphosphate is the active metabolite

Stimulation of adenosine receptors with adenosine is known to cause anti-inflammatory effects in macrophages (Hasko 2013), and it has been reported that other cordycepin's biological effects are also mediated through these receptors (Takahashi 2012, Kadomatsu 2012 and Nakamura 2006). As an adenosine analogue, it is conceivable that cordycepin can act extracellularly as an adenosine receptor agonist. Our group has previously shown that inhibiting cordycepin import into the cell, by using an inhibitor of the adenosine transporter, abrogates its anti-inflammatory effects in airway smooth muscle cells (Kondrashov 2012).

Work in the Centre for Bioanalytical Science (Nottingham) showed that cordycepin itself is short-lived intracellularly, while it persists as cordycepin triphosphate in tissue culture cells and liver (Wahyu Utami and David Barrett, unpublished data). Moreover, inhibition of the phosphorylation of cordycepin through inhibition of adenosine kinase also abrogates its anti-inflammatory effects (Kondrashov 2012).

To address the question of whether cordycepin can act extracellularly, possibly through adenosine receptors, or whether it must be imported and phosphorylated, as is the case of airway smooth muscle cells, I performed similar experiments. First, I investigated the effect of adenosine alone or in combination with cordycepin on the inflammatory gene expression. The results showed that adenosine represses the expression of all the assessed genes (Il1b, Acod1 and Ticam), but it does not affect Tnf expression **(Figure 4.3)**. To answer the second part of the question ''whether it must be imported and phosphorylated'', RAW 264.7 cells were incubated with NBTI (adenosine transport inhibitor) or ITu (adenosine kinase inhibitor) or nothing (control) prior to addition of LPS together with DMSO, adenosine, or cordycepin. As it had been the case with airway smooth muscle cells, it was found that NBTI or ITu treatment completely abolished cordycepin’s repressive effects on inflammatory gene expression in RAW 264.7 cells (**Figure 4.4**). These data indicate that cordycepin must be imported and phosphorylated to exert its anti-inflammatory effects in macrophges, and does not act extracellularly.

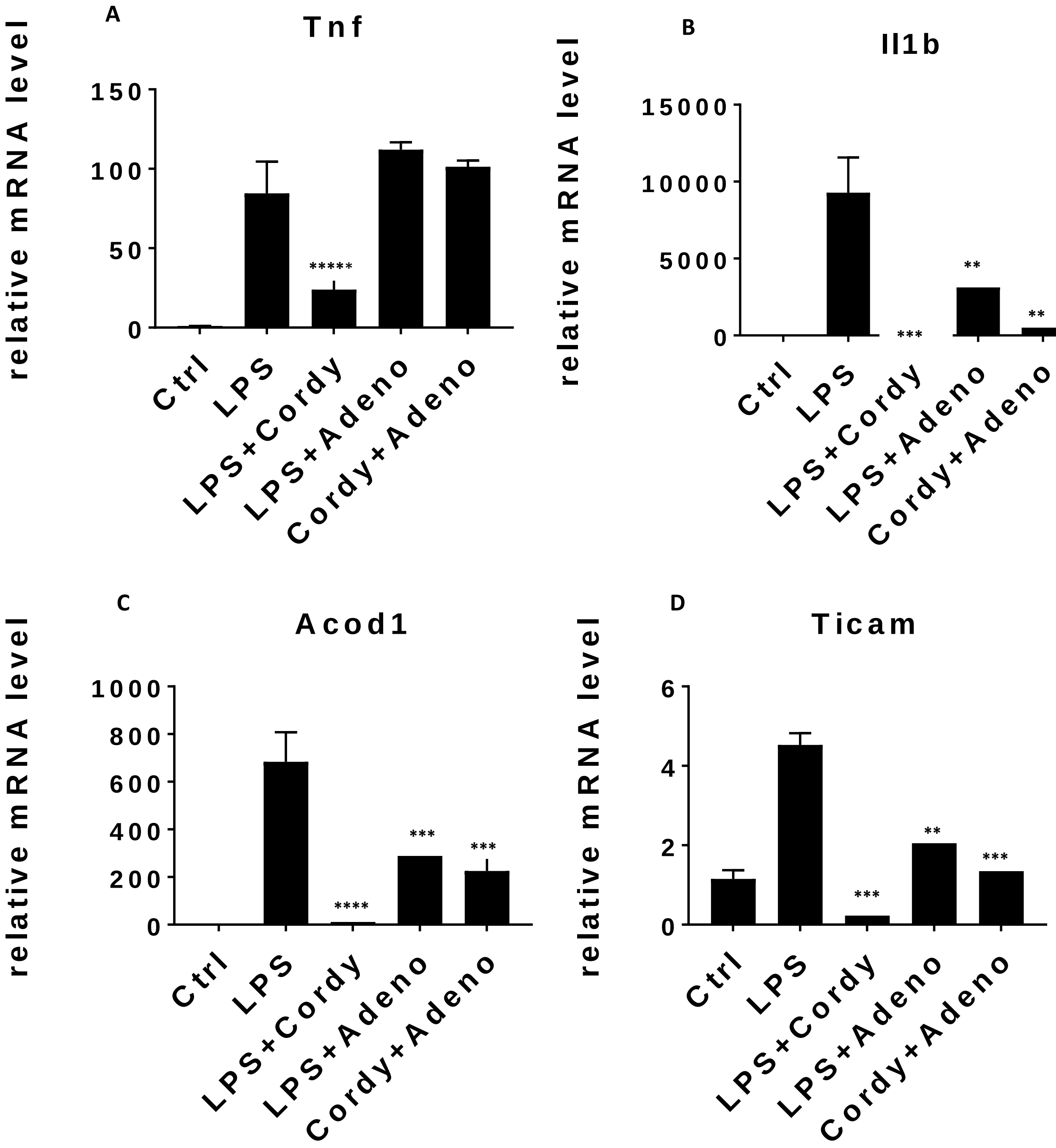
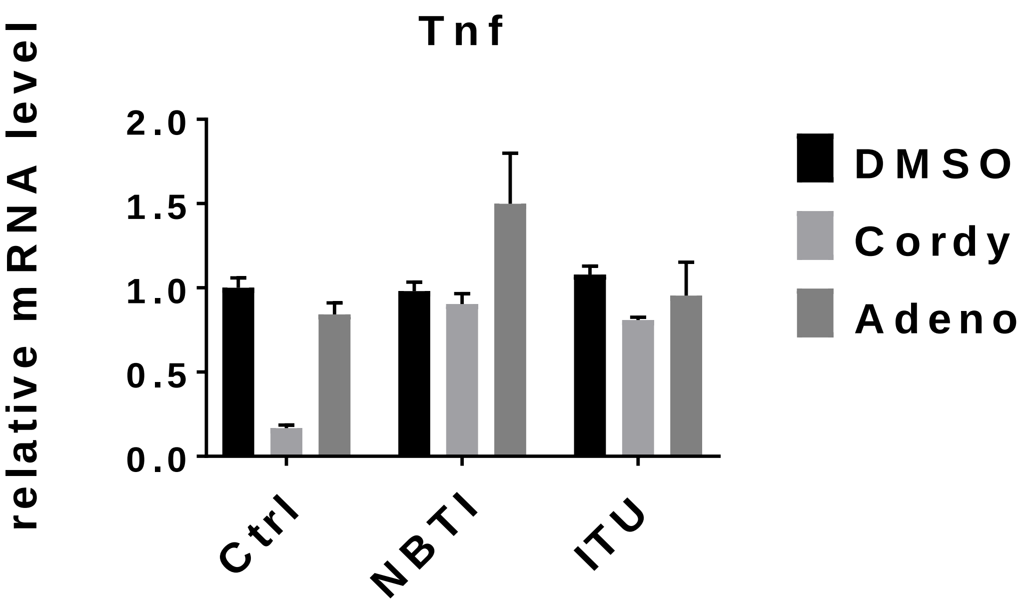
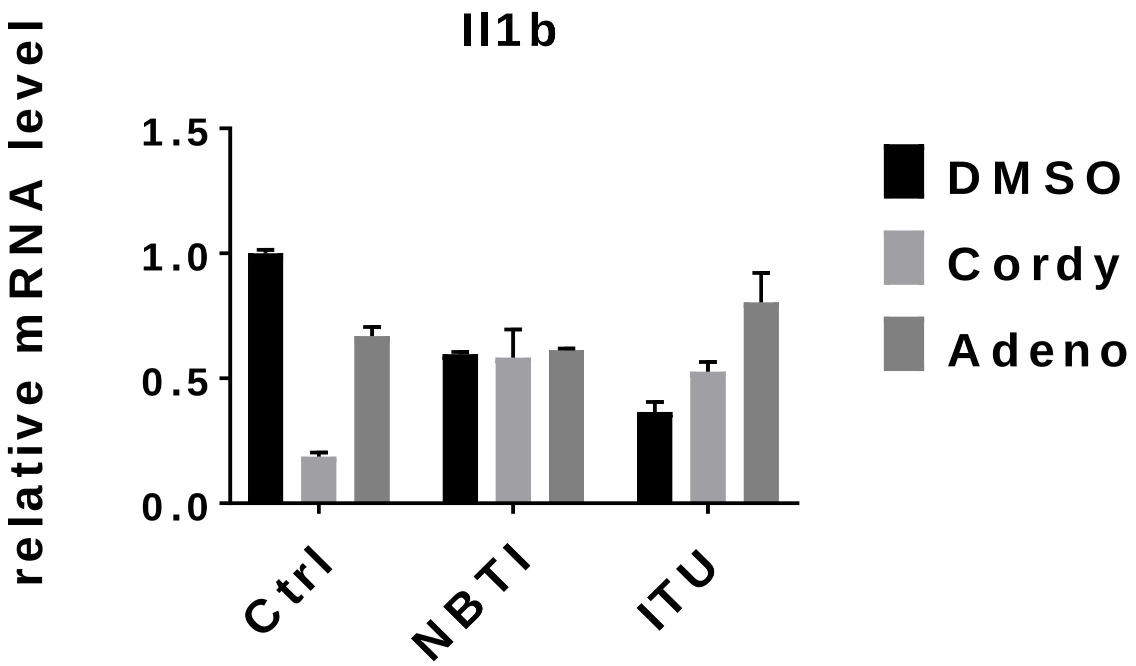


Figure 4.3. Adenosine represses the expression of some inflammatory genes. RAW 264.7 cells were treated with DMSO, 20 µM cordycepin, 20 µM adenosine or 20µM cordycepin and 20 µM adenosine for an hour prior to LPS addition. RTqPCR was performed, error bars represent standard deviations across 3 independent replicates. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, p values were obtained using Dunnett's test. **A.** Tnf, **B.** Il1b, **C.** Acod1 and **D.** Ticam1.

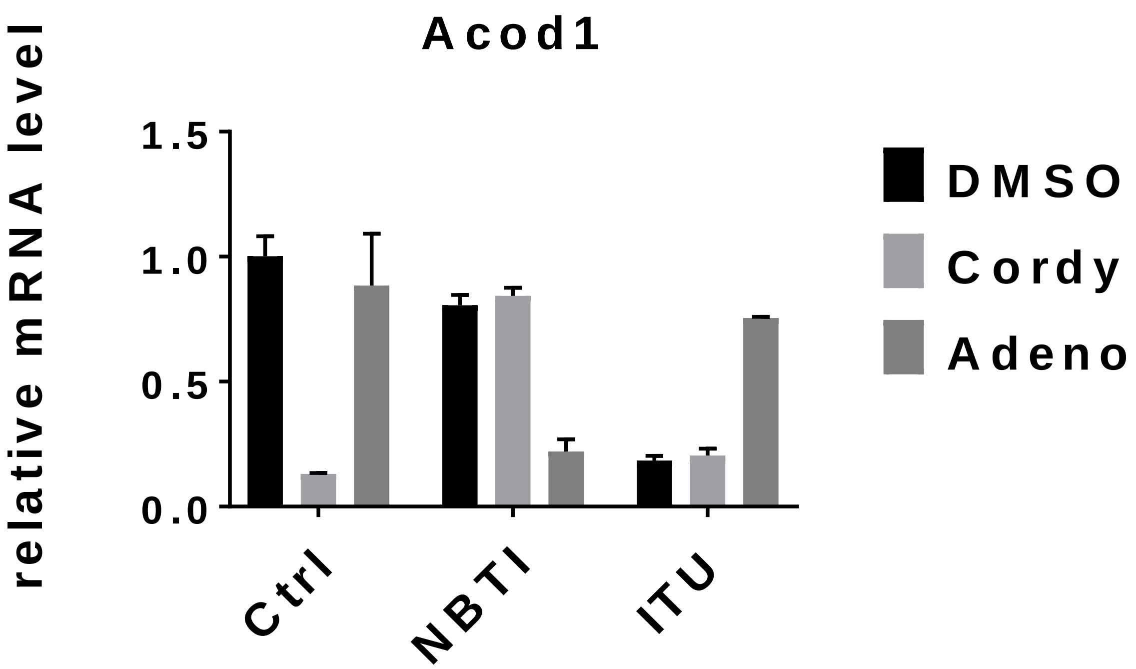
A



B


Il1b
relative mRNA level
1.5
1.0
0.5
0.0
Ctrl
NBTI
ITU
DMSO
Cordy
Adeno

C


Acod1
relative mRNA level
1.5
1.0
0.5
0.0
Ctrl
NBTI
ITU
DMSO
Cordy
Adeno

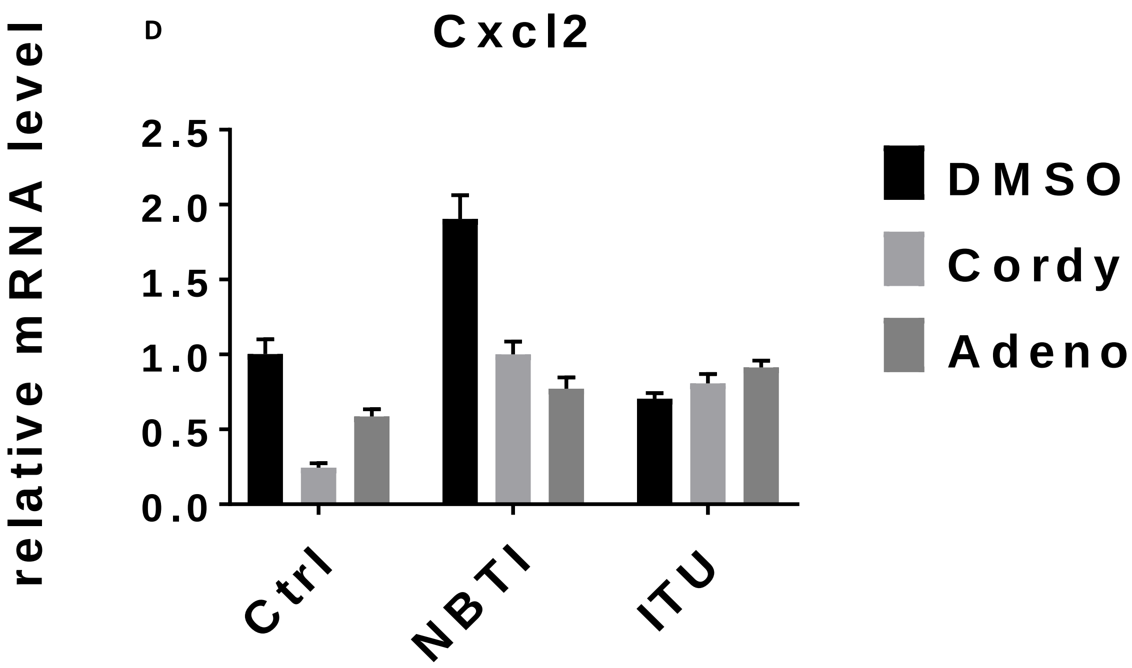
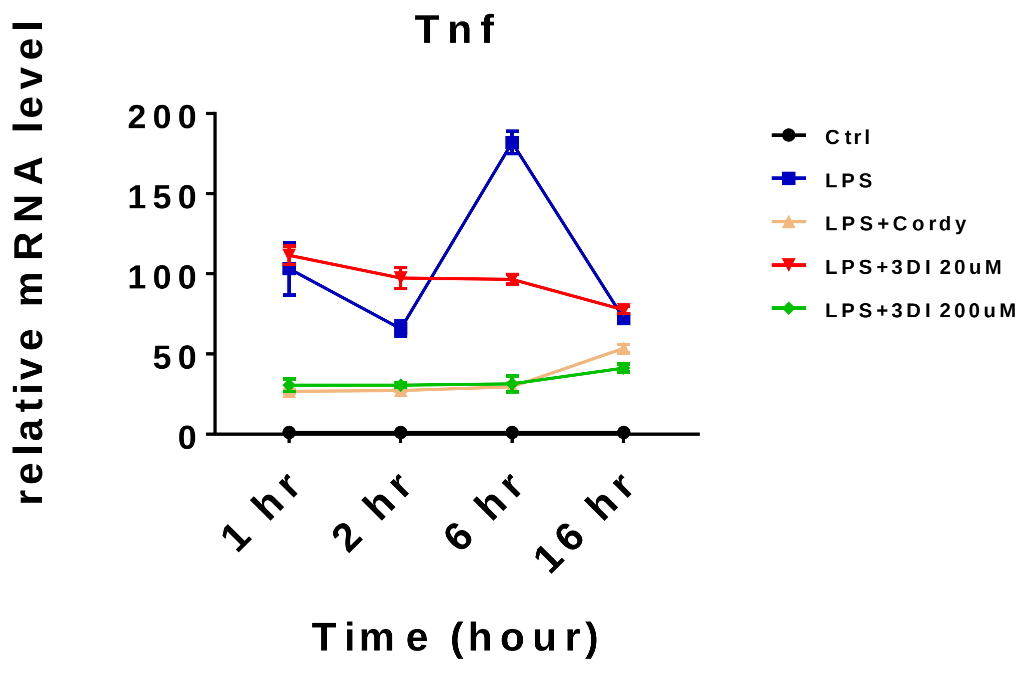


Figure 4.4. Inhibiting import of cordycepin or its phosphorylation abrogates its repression of inflammatory genes. RAW 264.7 cells were treated with nothing (control), 10 µM NBTI, or 100 nM ITu for 15 minutes prior to cordycepin treatment, then stimulated with LPS for an hour. RT-qPCR was performed, error bars represent standard deviations across 3 independent replicates. Two of the replicates were done by Raj Gandhi. **A.** Tnf, **B.** Il1b, **C.** Acod1 and **D.** Cxcl2.

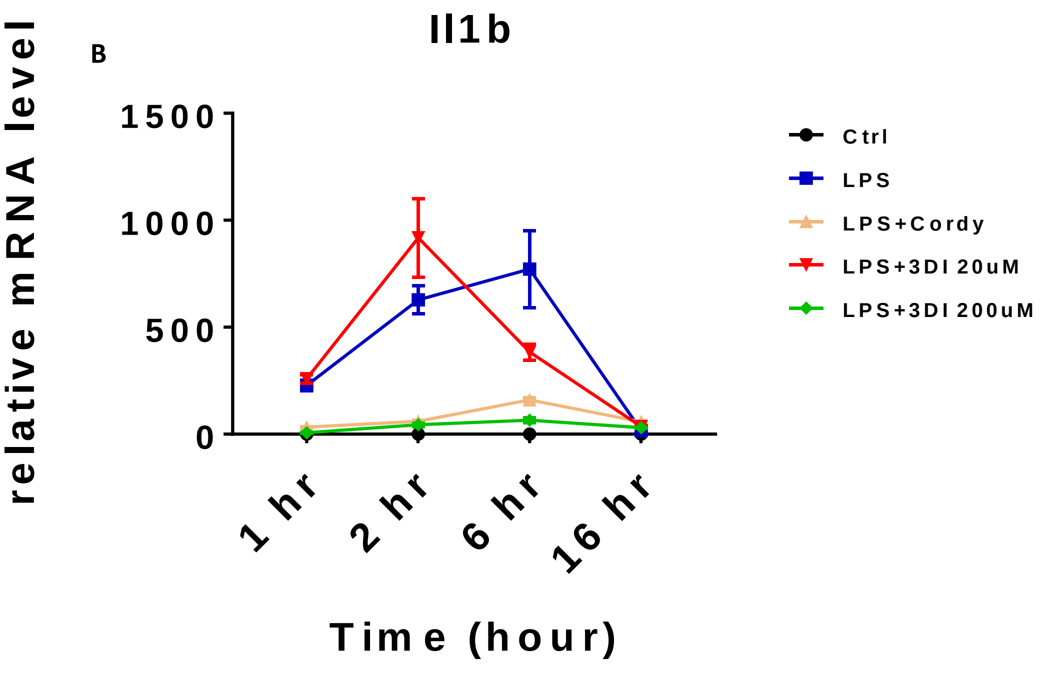
4.3. 3'-deoxyinosine has anti-inflammatory effect

Studying the pharmacokinetic profile of cordycepin in animal models, it has been found that following oral administration of cordycepin, cordycepin was not systemically absorbed but the metabolite 3'-deoxyinosine was. I hypothesized that this metabolite can be eventually metabolized into cordycepin triphosphate to exert the effects. To address the question of whether 3'-deoxyinosine has an anti-inflammatory activity, I treated RAW264.7 cells with this compound at different concentrations for different durations. I found that 3'-deoxyinosine represses the inflammatory gene expression, but at higher doses than cordycepin. It has also slower effect than cordycepin **(Figure 4.5)**. One concentration and one time point of 3'-deoxyinosine treatment were chosen and three biological replicates were confirmed to confirm its repressive effect on the inflammatory gene expression. The data indicate that this compound downregulates the expression of the inflammatory genes **(Figure 4.6)**, but it is slower and less active than cordycepin.

A

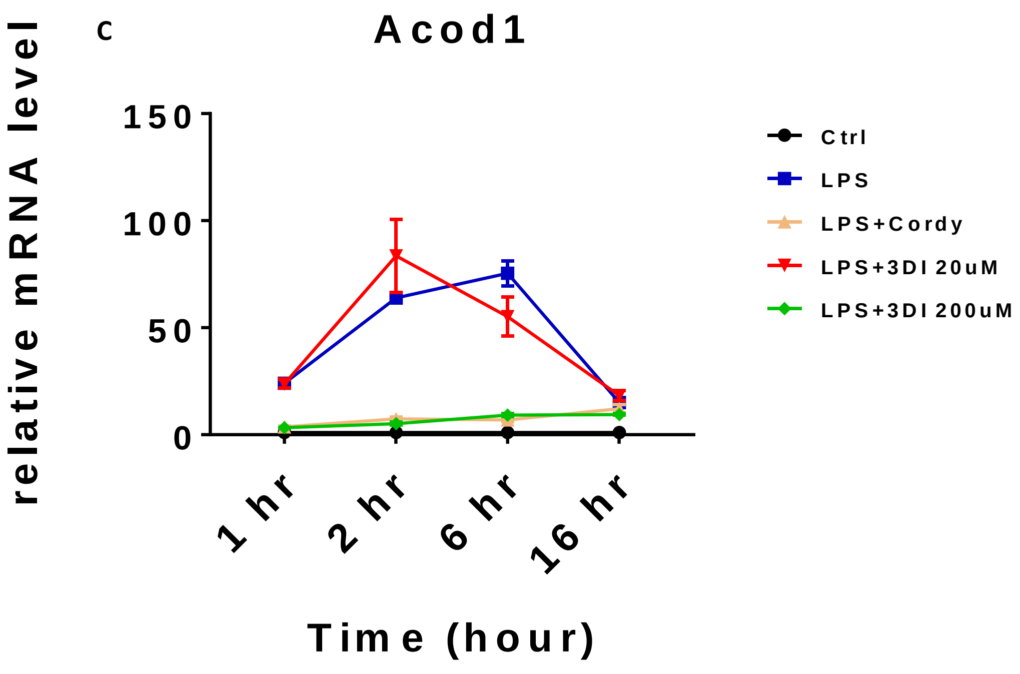


B



Il1b
relative mRNA level
1500
1000
500
0
1 hr
2 hr
6 hr
16 hr
Time (hour)
Ctrl
LPS
LPS+Cordy
LPS+3DI 20uM
LPS+3DI 200uM

C



Acod1
relative mRNA level
150
100
50
0
1 hr
2 hr
6 hr
16 hr
Time (hour)
Ctrl
LPS
LPS+Cordy
LPS+3DI 20uM
LPS+3DI 200uM

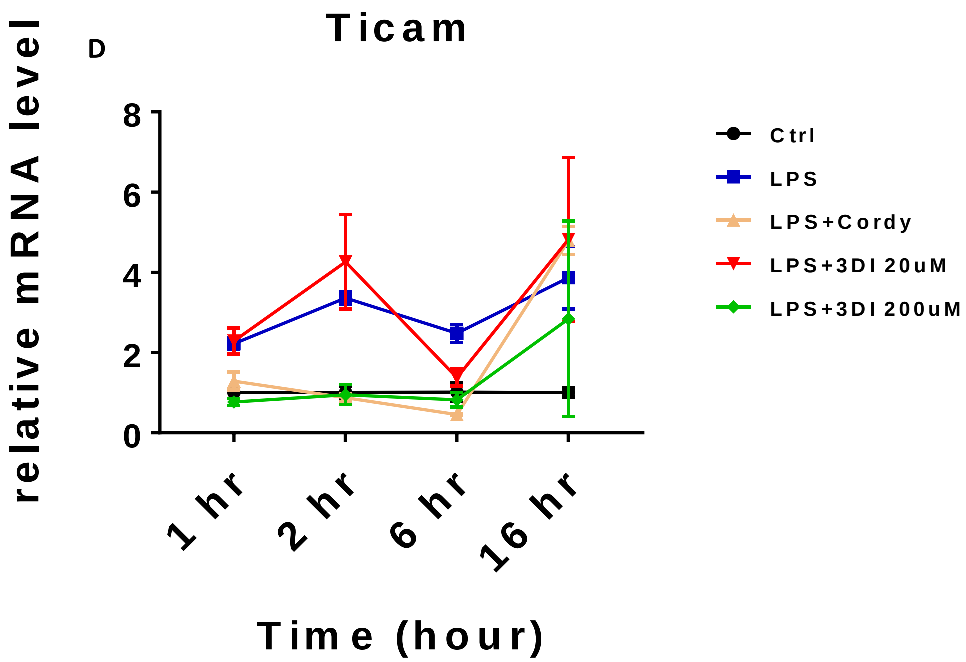


Figure 4.5. 3'-deoxyinosine is less active than cordycepin. RAW264.7 cells were treated with DMSO, Cordycepin (20µM) or 3'-deoxyinosine (20µM and 200µM) for the indicated period prior to LPS stimulation for one hour. qPCR was performed, error bars represent standard deviations across 3 technical replicates. **A.** Tnf, **B.** Il1b, **C.** Acod1 and **D.** Ticam1.

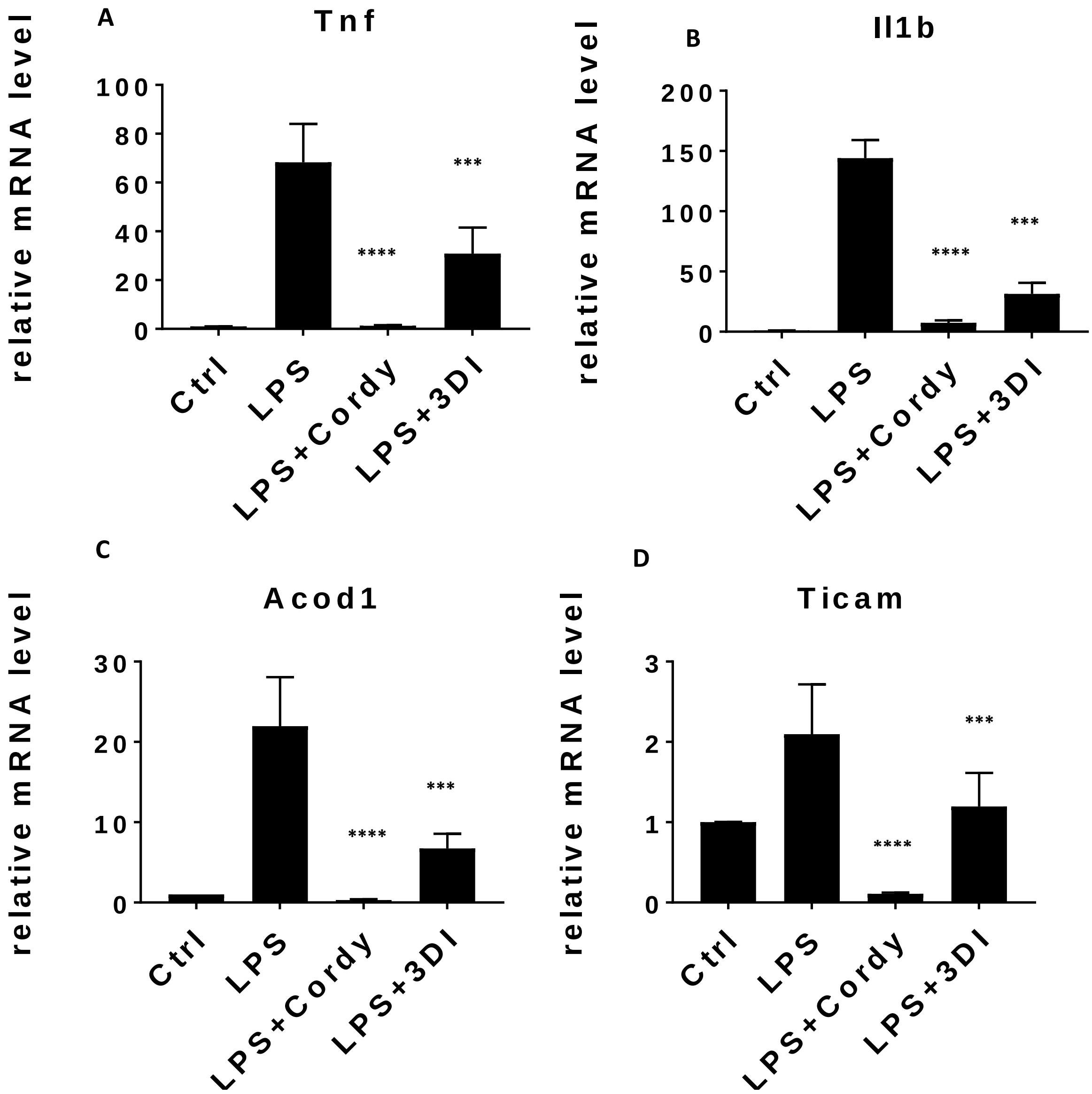


Figure 4.6. 3'-deoxyinosine represses inflammatory gene expression. RAW264.7 cells were treated with DMSO, Cordycepin (20µM) or 3'-deoxyinosine (200µM) for one hour prior to LPS stimulation for one hour. qPCR was performed, error bars represent standard deviations across 3 independent replicates. *** p<0.001, **** p<0.0001, p values were obtained using Dunnett's test. . **A.** Tnf, **B.** Il1b, **C.** Acod1 and **D.** Ticam1.

Conclusion

Cordycepin from different commercial sources contains ~2% impurities. If these impurities are potent, they could either potentiate or mask the activity of cordycepin depending on their nature. To exclude this and to confirm that the anti-inflammatory effect is mainly contributed by the 3'deoxyadenosine, mass spectrometric analysis has been done for cordycepin from three different companies in order to specify the nature and percent of the contaminants (Catherine Otori, unpublished data). Then I assessed the effect of cordycepin from those three companies as well as the effect of the contaminant by its own on the expression of the inflammatory genes in RAW264 murine macrophages. The results demonstrated that cordycepin, but not the contaminants, is responsible for the anti-inflammatory activity **(Figure 4.1 and Figure 4.2)**. The effect of adenosine on the inflammatory gene expression has been tested and the results showed that it represses the relative mRNA level of Il1b, Acod1 and Ticam, but not Tnf, and adenosine reduced the effects of cordycepin for most mRNAs **(Figure 4.3)**. After that, I tested the effect of adenosine kinase inhibitor and adenosine import inhibitor. The results of this experiment demonstrated that cordycepin must be taken up by RAW 264.7 cells via nucleoside transporters followed by its phosphorylation by adenosine kinase in order to produce its anti-inflammatory activity **(Figure 4.4)**. These results demonstrated that cordycepin acts intracellularly as a nucleotide, rather than a nucleoside. Although, these results showed that cordycepin might work as an adenosine receptor agonist, they cannot fully explain cordycepin's activity.

It was found that there is no bioavailable cordycepin in rat's plasma after oral administration (Jong Bong Lee, unpublished data), which is at the odds with many studies showing pharmacological effect of cordycepin after oral administration in animal models (Won 2009, Yoshikawa 2005). 3'deoxyinosine was considered an inactive metabolite, however its potential contribution toward efficacy was investigated. Our results showed that 3'deoxyinosine has anti-inflammatory effect as it represses the relative mRNA level of the assessed genes (Tnf, Il1b, Acod1 and Ticam) **(Figure 4.6)**. However, the RTqPCR data in RAW264.7 cells showed that it is less active than cordycepin as it need more time and higher concentration to produce comparable effect to cordycepin **(Figure 4.5)**. Since cordycepin triphosphate is found to be the active metabolite, the ability of macrophages to generate cordycepin triphosphate from 3'deoxyinosine was investigated. Macrophages were indeed able to generate cordycepin triphosphate from 3'deoxyinosine, although it is 10-fold lower compared to cordycepin triphosphate generated from cordycepin (Jong Bong Lee, unpublished data).

5. The effect of cordycepin on the localization of NF-κB and polyadenylation factors

In the previous chapter, cordycepin triphosphate was shown to probably be the active metabolite of cordycepin **(Figure 4.4)**. It has been found that the highly conserved sequence AAUAAA, found close to the end of mRNAs, forms a transient complex with the cleavage and polyadenylation factors. The complex normally dissociates during or after polyadenylation reaction allowing the release of the cleavage and polyadenylation factors to mediate a second round of 3' pre-mRNA maturation. However, the addition of cordycepin to the 3' terminus of the cleaved pre-mRNAs stabilizes this complex (Zarkower 1987 and Ryner 1987). Moreover, it has been confirmed that cordycepin reduces the size of poly (A) tail of specific pre-mRNAs (Wong 2010). There is some evidence that some of the polyadenylation factors (like CPSF4) may be required for NF-κB dependent gene expression, but this has not been examined in macrophages (Yi 2016). In this chapter, I am going to find out if polyadenylation factors in the complex that is affected by cordycepin are possibly involved in inflammatory gene expression, which would indicate that cordycepin is indeed working as a polyadenylation inhibitor.

The transcription factor NF-κB serves as a master regulator of multiple aspects of innate and adaptive immune functions and as a pivotal mediator of inflammatory responses. NF-κB induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines. NF-κB is composed of a family of related transcription factors; RelA/p65, c-Rel, RelB, p50 and p52. These subunits may hetero- and homo-dimerise to form at least 12 different identified dimers which localize mainly in the cytoplasmic compartment of different types of cells (Liu 2017 and Christian 2016). Inhibitor κB (IκB) proteins are regulators of NF-κB activity. Degradation of IκB is considered as a seminal step in NF-κB activation. They bind tightly to NF-κB dimers and mask the nuclear localization signal (NLS), until stimulus-responsive N-terminal phosphorylation by IκB kinase (IKK) triggers their ubiquitination and subsequent proteasomal degradation. The NLS on NF-κB becomes exposed and the protein translocates to the nucleus, where it turns on transcription and induces specific gene expression (Mathes 2008, Joseph 2012). It has been reported by several groups that cordycepin interferes with NF-κB activity at different levels in this pathway (Zhenghua 2012, Kim 2006). However, it did not have any effect on the activity of this pathway in airway smooth muscle (ASM) cells as reported by our group (Kondrashov 2012). It seems that the effect of cordycepin on the NF-κB pathway is cell-dependent and I aimed to see how it affects the activity of this important transcription factor in RAW264.7 murine macrophages. No clear link between polyadenylation and inflammation has been established. However, it has been recently suggested that CPSF4 could regulate the inflammatory gene expression. This study found that CPSF4 may regulate the inflammatory response by two mechanisms: it could activate NF-κB pathway and/or bind to the promoters of inflammatory genes and enhance their expression (Yi 2016). In ASM cells, we found that inhibition of polyadenylation either genetically or pharmacologically repressed the inflammatory gene expression, but not housekeeping gene expression (Kondrashov 2012). It therefore appears possible that inflammatory genes have specific requirements that make them sensitive to the inhibition of polyadenylation by cordycepin. To establish whether polyadenylation factors play a role in inflammatory gene expression, I planned to knock down some of the important factors in 3’ mRNA processing and assess the effect of their depletion on the inflammatory gene expression and the nuclear localization of NF-κB in RAW264.7 murine macrophages. In the light of the effect of cordycepin on the inflammatory response in our established inflammatory system and the effect of depleting polyadenylation factors on this response, it is important to investigate the effect of cordycepin on the cleavage and polyadenylation processing factors.

5.1 Cordycepin represses inflammatory gene expression at transcriptional level

I have shown that cordycepin represses the inflammatory gene expression in macrophages by measuring the relative level of the mature inflammatory mRNAs. This could be mediated either by the well-established effect of cordycepin on sequestering cleavage and polyadenylation factors (inflammatory mRNAs production are hampered by limited availability of processing factors) or by its effect on transcription factors that have a central role in inflammatory reactions. I started by looking at the effect of cordycepin on the expression of the unspliced inflammatory mRNAs to provide a context for discussing its effect on transcription factors. The previous work on ASM cells indicated that cordycepin did not affect the unspliced mRNA levels of most inflammatory mRNAs assessed (Kondrashov 2012). However, in the RAW 264.7 cells, cordycepin was found to affect unspliced mRNA levels **(Figure 5.1)**. Two key inflammatory genes were assessed (Tnf and Il1b), and the result was observed over three independent replicates.

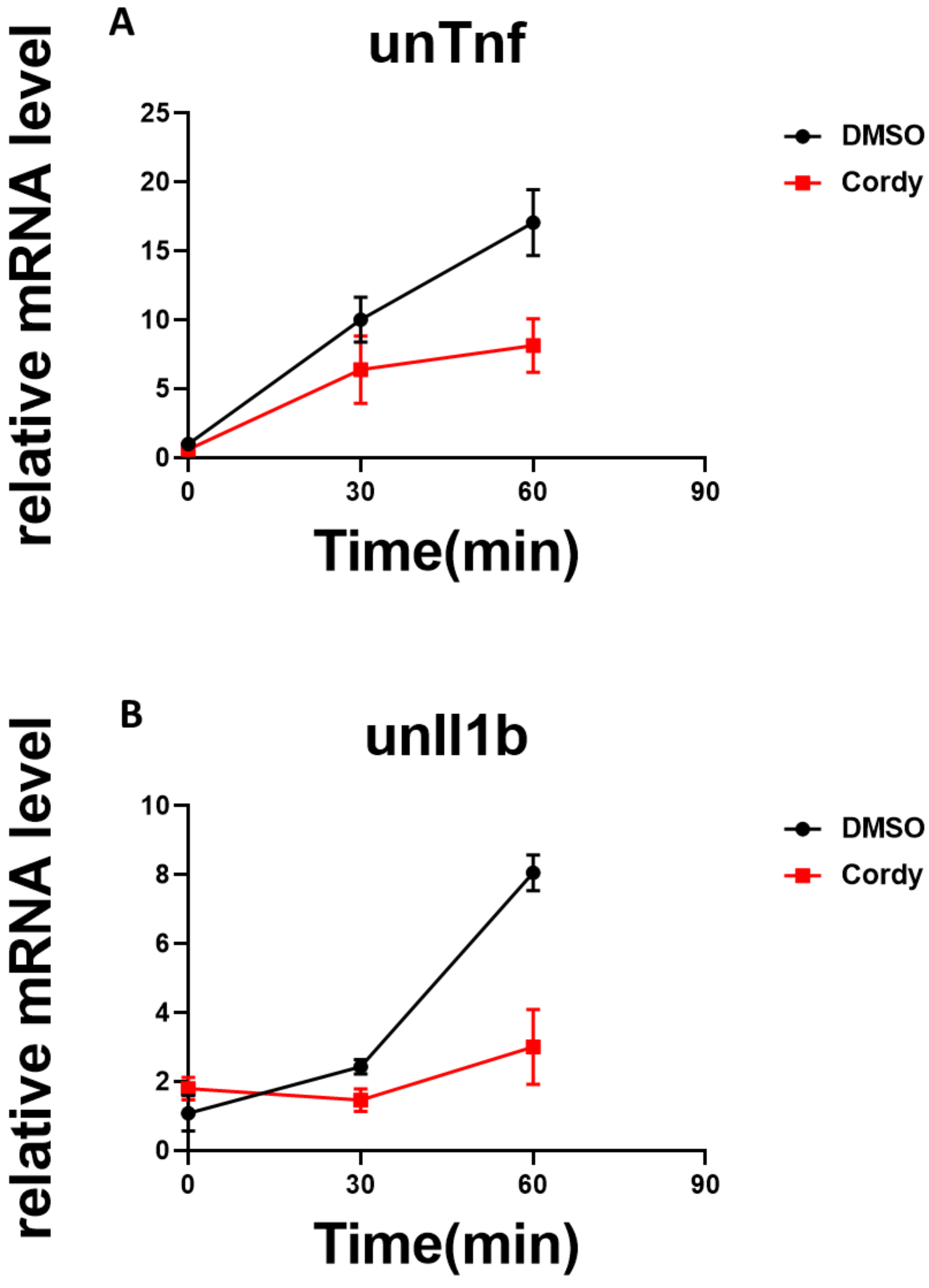


Figure 5.1. Cordycepin represses at the transcriptional level. RAW 264.7 cells were treated with DMSO or 20 µM cordycepin for an hour (DMSO and Cordy respectively) prior to LPS addition. RTqPCR was performed, error bars represent standard deviations across 3 independent replicates. **A.** unTnf and **B.** Il1b.

5.2 Cordycepin affects the nuclear localization of NF-κB (P65)

NF-κB is a family of dimeric transcription factors that regulate the inflammatory response, and includes the ubiquitously expressed p65:p50 heterodimer. The activity of p65-containing dimers is controlled by cytoplasmic sequestration through association with IκB proteins, primarily IκBα. NF-κB activity in macrophages can be triggered by a range of different stimuli, including lipopolysaccharides (Hayes 2016). After knowing the effect of cordycepin on the unspliced mRNA levels, I hypothesised that cordycepin (1) affects the activity of transcription factors that control inflammatory response in macrophages such as NF-κB or (2) cordycepin-terminated RNAs may be sequestering the constituent factors, thereby preventing proper processing of other transcripts. Therefore, for genes being induced (e.g. inflammatory genes in response to LPS) mRNA production could be hampered by a reduced availability of 3' processing factors if they have high processing factor requirements.

To test whether cordycepin produces its anti-inflammatory effect by affecting the NF-κB pathway, I analysed NF-κB by immunofluorescence. The cells were treated with DMSO or cordycepin after stimulating them with LPS. I found that when the cells were treated with LPS, NF-κB (P65 subunit) is mainly localized in the nucleus. However, it is mostly localized in the cytoplasm when the cells were treated with cordycepin after stimulating them with LPS **(Figure 5.2)**. To confirm this finding, I treated the cells with DMSO or cordycepin prior to stimulate them with LPS at different time points. Cytoplasmic/nuclear fractionation was done and western blotting was performed for both fractions to see the effect of cordycepin on the localization of P65. I found that P65 moves out of the cytoplasm in the DMSO treated cells at the 15 minute time point after LPS stimulation. However, in the presence of cordycepin, P65 is in the cytoplasmic fraction at the 30 minute time point although it was noticed that it leaves the cytoplasm after 15 minute of LPS stimulation. The western blotting of the nuclear fraction showed that there is more NF-κB when the cells were treated with DMSO than when they were treated with cordycepin **(Figure 5.3)**. These data indicate that cordycepin reduces the nuclear localization of P65 in RAW264.7 cells and this could be, at least in part, responsible for the anti-inflammatory activity of cordycepin.

To test whether cordycepin affected NF-κB signalling via IκB degradation, an LPS time course was done in RAW 264.7 cells over a 30 minute period. Cells were treated with either DMSO or cordycepin for 1 hour prior to addition of LPS. Western blotting was then done on cell lysates for total IκBα. The DMSO series shows a band for IκBα in unstimulated cells and for a 5 minute LPS treatment **(Figure 5.4)**. This band is almost completely lost, indicating degradation, in the 15 minute time point, and then reappeared in the 30 minute sample. The degradation of IκBα was not at all prevented by cordycepin pre-treatment. These data suggest that cordycepin does not interfere with IκBα degradation. Overall, these results indicate that cordycepin's interference with NF-κB pathway is not IκB-dependent.

5.3 Depleting polyadenylation factors represses the expression of inflammatory genes

In our laboratory, it is confirmed that cordycepin has a pronounced anti-inflammatory effect in macrophages, airway smooth muscle cells and osteoarthritic animal models (Kondrashov 2010, Ashraf 2019). Additionally, cordycepin is known as an efficient chain terminator of polyadenylation in several studies (Rose 1977, Ryner 1987, Zarkower 1986). In an attempt to see whether the inflammatory response in RAW264.7 cells is mediated by 3' processing of the inflammatory transcripts, I did siRNA knockdown for two of the most important polyadenylation factors (CPSF4 and WDR33). Depleting Cpsf4 resulted in repression of the relative mRNA level of Il1b, but not Tnf. However, knocking down Wdr33 repressed the expression of both Tnf and Il1b in the macrophages-like cell line **(Figure 5.5 and 5.6)**. This indicates that polyadenylation is an important regulator of the inflammatory response and it also suggests that cordycepin might produce its anti-inflammatory effect through affecting 3' mRNA processing, with effects on Wdr33 perhaps being stronger.

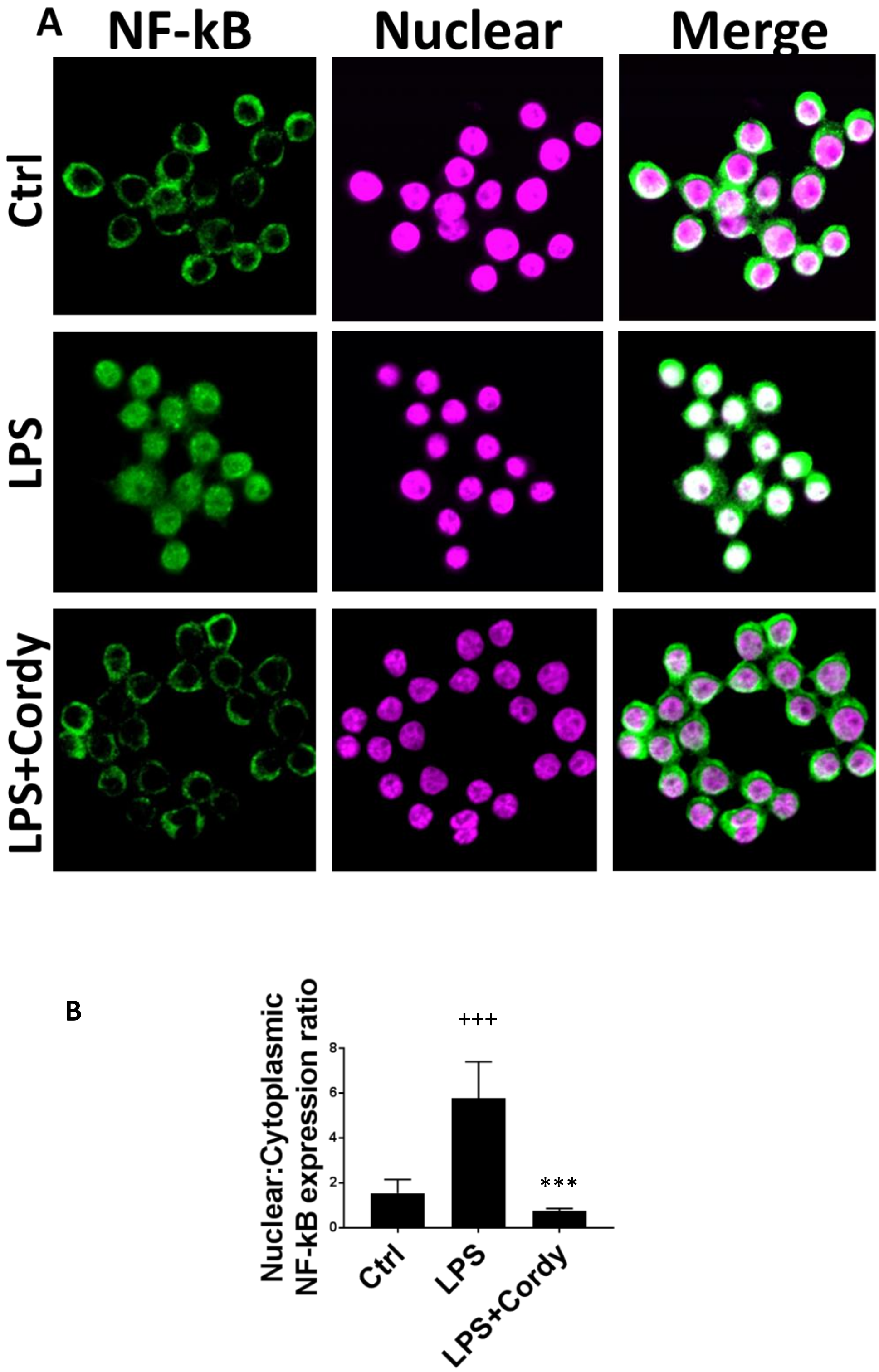


Figure 5.2. A.Cordycepin reduces the nuclear localization of NF-κB (P65). A. RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 minutes. Cells were then fixed and stained. **B. Quantification of nuclear:cytoplasmic ratio of NF-κB**. Error bars represent standard deviation across three independent replicates. (***) $p<0.001$ as compared to LPS, ($^{+++}$) $p<0.001$ as compared to control, p values were obtained using Student's t test. NF-κB is green and DRAQ5 is purple.

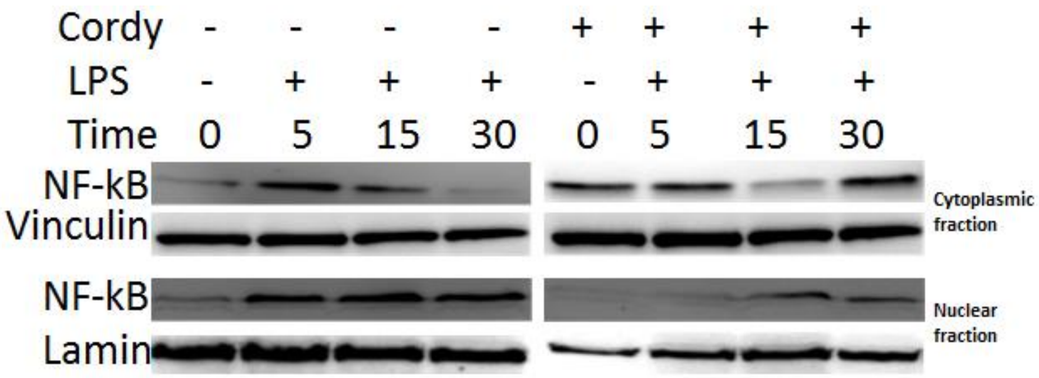


Figure 5.3. Nuclear translocation of P65 is reduced by cordycepin. RAW 264.7 cells were treated with DMSO or cordycepin for an hour prior to LPS addition for the indicated durations. Cells were fractionated (cytoplasmic/nuclear) and Western Blot performed. The image is representative of two independent replicates.

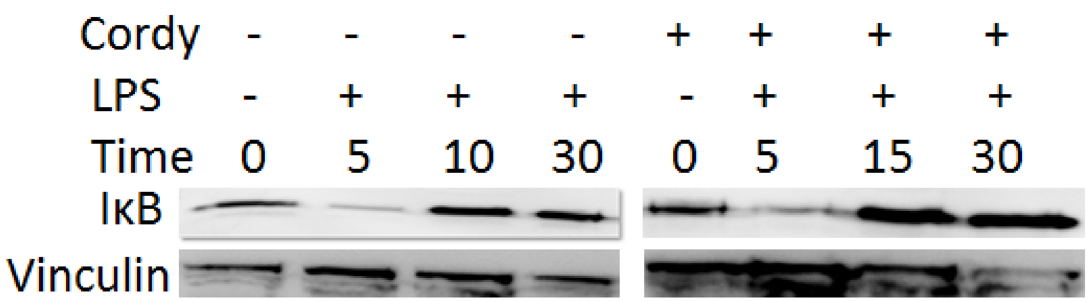


Figure 5.4. IκBα degradation is not prevented by cordycepin. RAW 264.7 cells were treated with DMSO or cordycepin for an hour prior to LPS addition for the indicated durations. Cells were then lysed and Western Blot performed. The image is representative of two independent replicates.

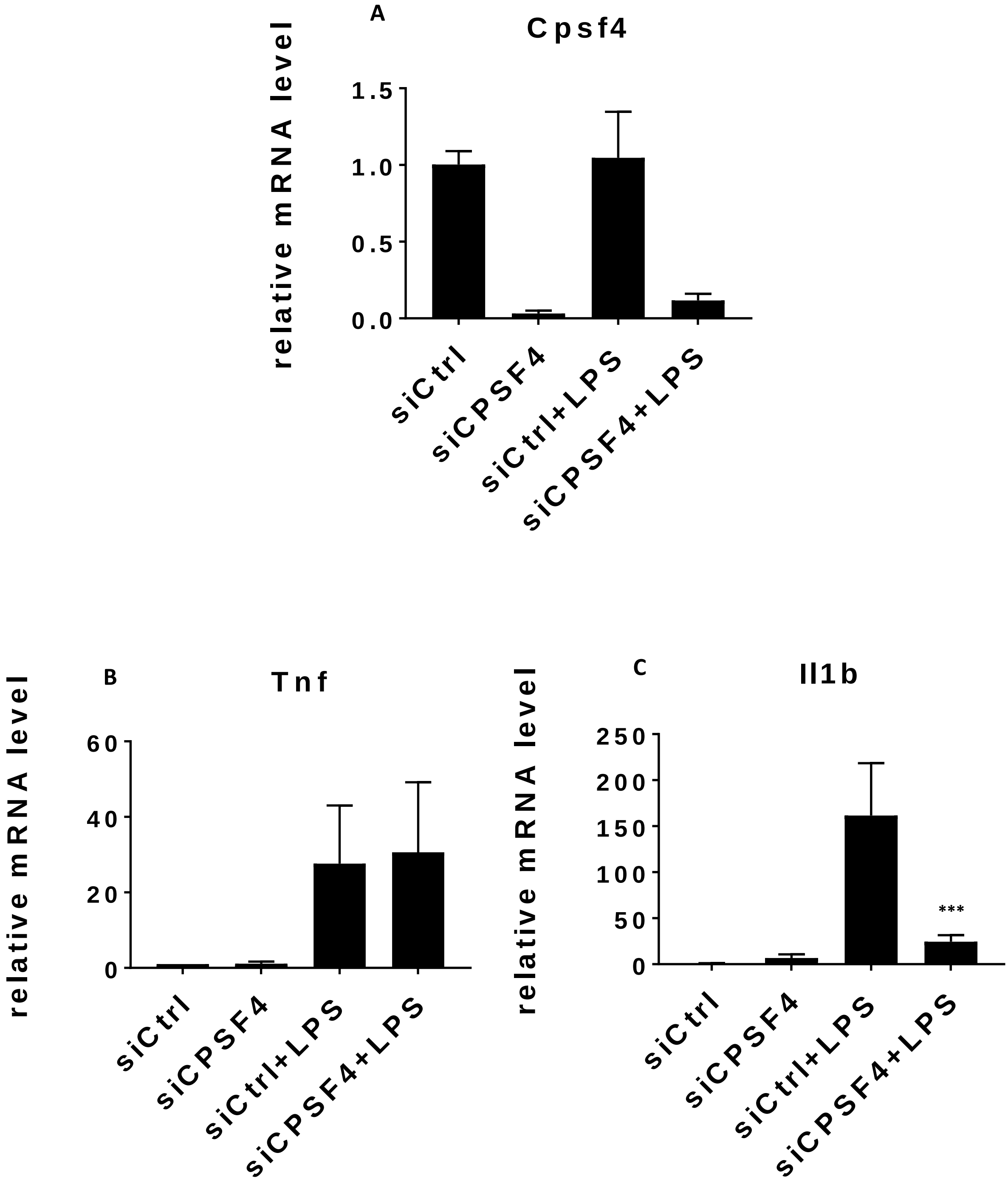


Figure 5.5. Knocking down CPSF4 represses inflammatory gene expression. RAW264.7 cells were treated with control siRNA or siRNA against the indicated mRNA. Cells were treated for 1 h with LPS and RNA was isolated. RT-qPCR for the indicated mRNAs was performed as described. Error bars represent standard deviation across three independent replicates. (***) P ≤ 0.001 compared to siCtrl+LPS, p values were obtained using Student's t test. **A.** Cpsf4, **B.** Tnf and **C.** Il1b.

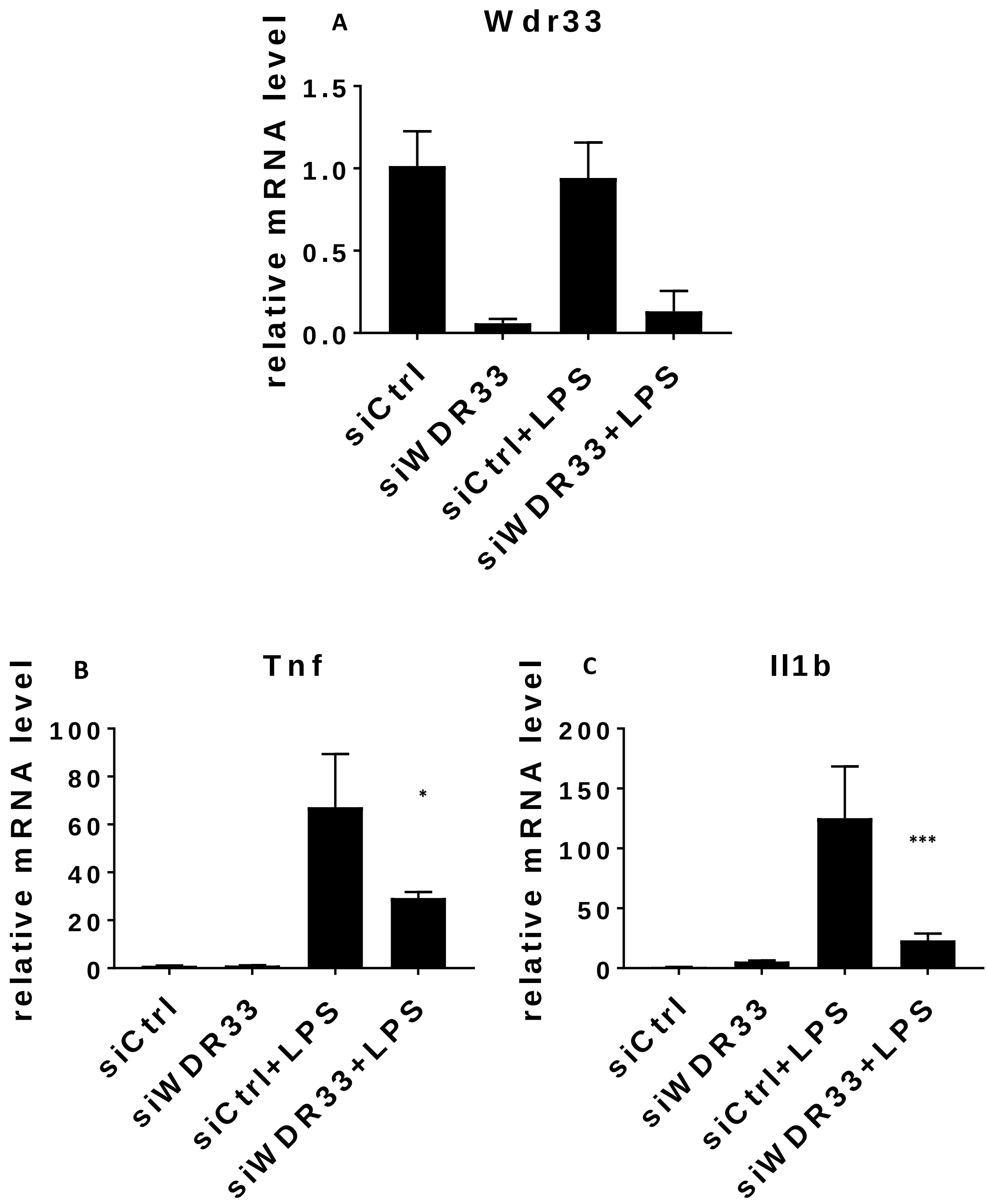
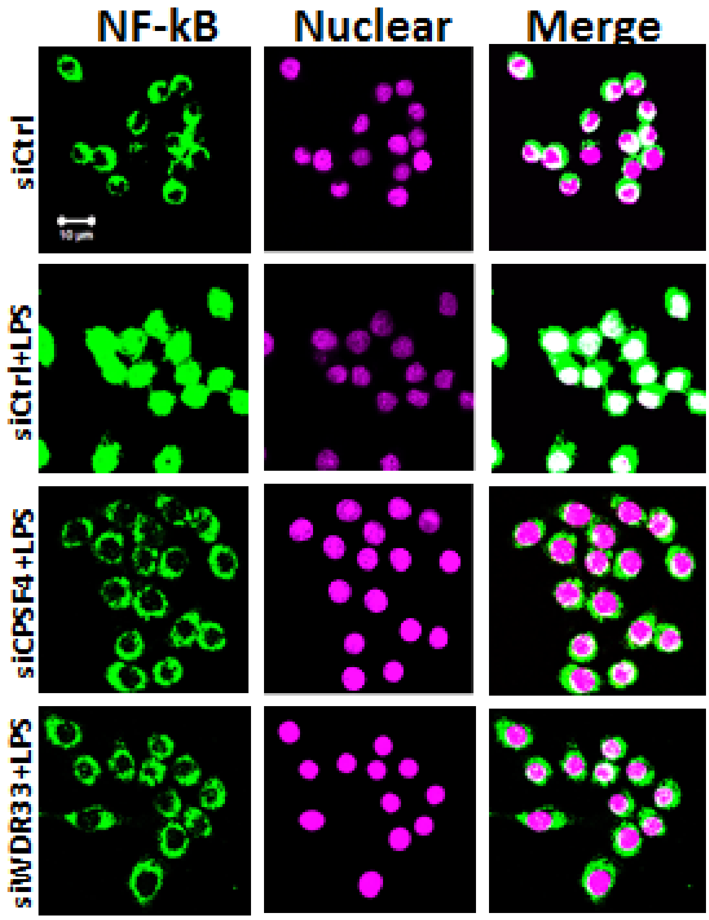


Figure 5.6. Knocking down WDR33 represses inflammatory gene expression. RAW264.7 cells were treated with control siRNA or siRNA against the indicated mRNA. Cells were treated for 1 h with LPS and RNA was isolated. RT-qPCR for the indicated mRNAs was performed as described. Error bars represent standard deviation across three independent replicates. (*) $P \leq 0.05$, (***) $P \leq 0.001$ compared to siCtrl+LPS, p values were obtained using Student's t test. **A.** Wdr33, **B.** Tnf and **C.** Il1b.

5.4 Depleting polyadenylation factors affects the nuclear localization of NK-κB (P65)

After assessing the role of polyadenylation in the inflammatory response in RAW264.7 cells, I aimed to look at the effect of 3' mRNA processing in NF-κB signalling in the same cell line. To this end, I did siRNA knockdown for the previous polyadenylation factors (CPSF4 and WDR33) and then the cells were fixed and stained for p65 to assess the effect of depleting these factors on the localization of P65. The results of this experiment indicated that depletion of each factors reduced the percentage of the NF-κB located in the nuclear compartment. Again, the effect of Wdr33 knockdown on the localization of P65 was stronger than the effect of Cpsf4 knockdown **(Figure 5.7)**. Previously, the results showed that depleting Wdr33 had stronger effect on inflammatory gene expression than depleting Cpsf4 **(Figure 5.5 and Figure 5.6),** as we have seen that WDR33 knock down resulted in repressing the expression of both Tnf and Il1b mRNAs in contrast to CPSF4 knock down that downregulated the relative level of Il1b mRNA only. This could be explained by the less nuclear:cytoplasmic NF-κB expression ratio in case of WDR33 siRNA knockdown than in CPSF knockdown. Altogether, these results indicated that NF-κB signalling is downstream of polyadenylation and it could suggest that the effect of cordycepin on the nuclear localization of P65 is mediated through its effect on the 3'mRNA processing.

A



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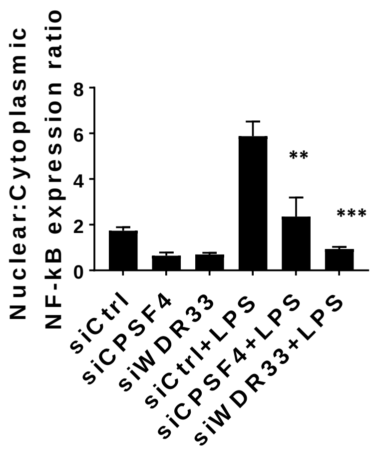


Figure 5.7. A. Knocking down polyadenylation factors reduces the nuclear localization of NF-kB. RAW264.7 cells were treated with control siRNA or siRNA against the indicated mRNA. Cells were treated for one hour with LPS. Cells were then fixed and stained. **B. Quantification of nuclear:cytoplasmic NF-kB expression.** Error bars represent standard deviation across three independent replicates. (**) P ≤ 0.01, (***) P ≤ 0.001 compared to siCtrl+LPS, p values were obtained using Dunnett's test.

5.5 LPS and cordycepin affect the localization of polyadenylation factors

Cordycepin is a well-known polyadenylation inhibitor. It arrests a complex of cleavage and polyadenylation processing factors on the terminated mRNAs in nuclear extracts (Shi 2009). It was confirmed that this compound represses the inflammatory gene expression in a macrophage-like cell line. It downregulates the relative level of both unspliced and mature inflammatory transcripts. In addition, it had the ability to reduce the nuclear localization of NF-κB in LPS-stimulated RAW264.7. On the other hand, depleting polyadenylation factors in the same cells produces similar effect to cordycepin. Knocking down CPSF4 and WDR33 led to repression of the inflammatory gene expression as well as reduction in the nuclear localization of NF-κB. However, we still do not know the mechanism by which polyadenylation regulates inflammatory response. I decided to look at the effect of cordycepin on the localization of the 3' processing factors (CPSF4 and WDR33) in macrophages. Surprisingly, I found that these factors were predominantly located in the cytoplasmic compartment in untreated macrophages. It is generally thought that polyadenylation factors are predominantly nuclear, as this is where most polyadenylation takes place. Even more importantly, LPS stimulation induced nuclear localization of both these factors (CPSF4 and WDR33). Upon cordycepin treatment, these factors were located in the cytoplasmic compartment **(Figure 5.8 and Figure 5.9)**. These data indicate that the localisation of polyadenylation factors is sensitive to inflammatory signalling and that cordycepin causes depletion of the cleavage and polyadenylation factors from the nuclear compartment.

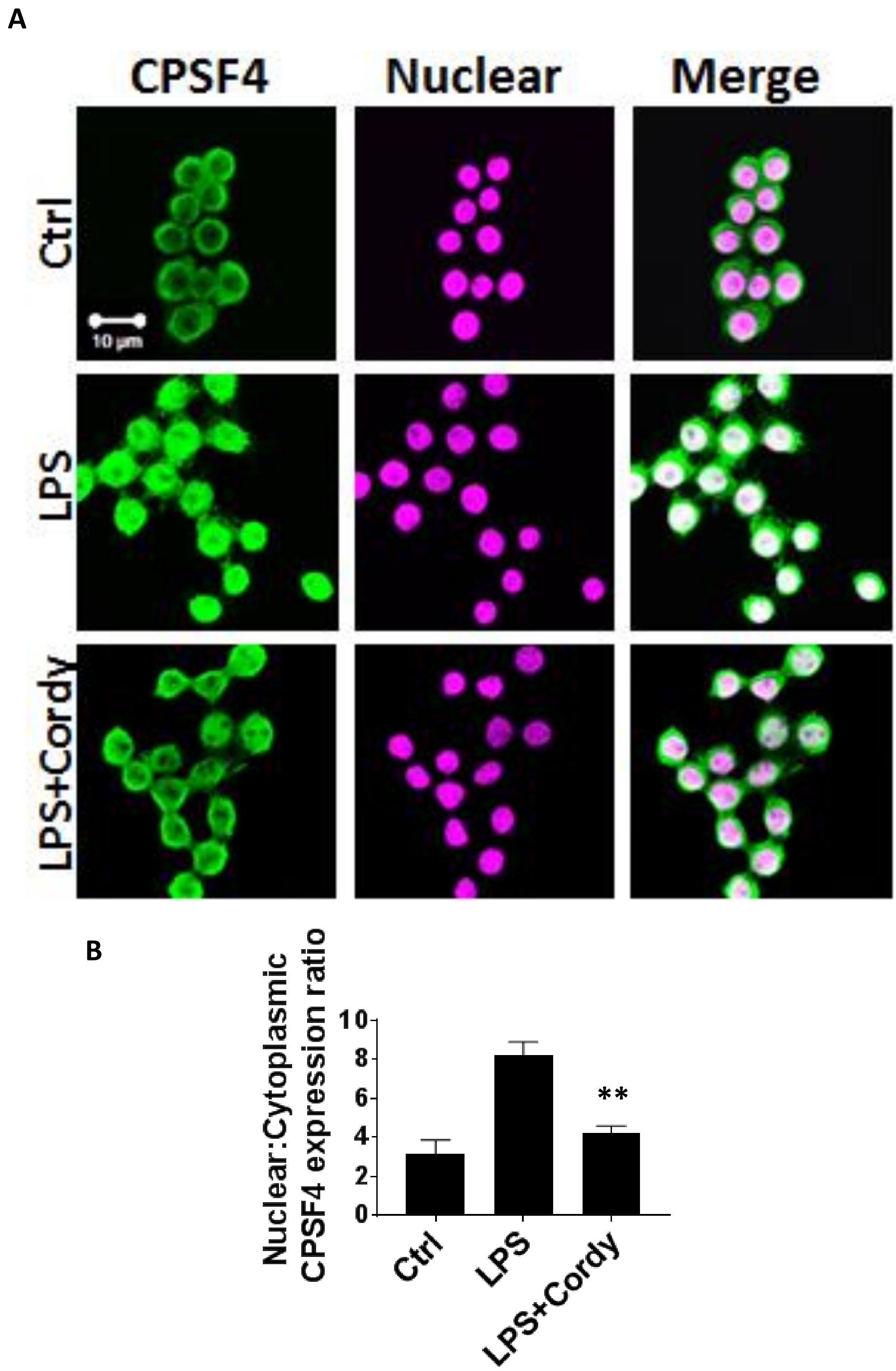


Figure 5.8. A. The localization of CPSF4 is affected by cordycepin. RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 min. Cells were then fixed and stained. **B. Quantification of cytoplasmic:nuclear CPSF4 expression.** Error bars represent standard deviation across three independent replicates. (**) P ≤ 0.001 compared to LPS, p values were obtained using Student's t test.

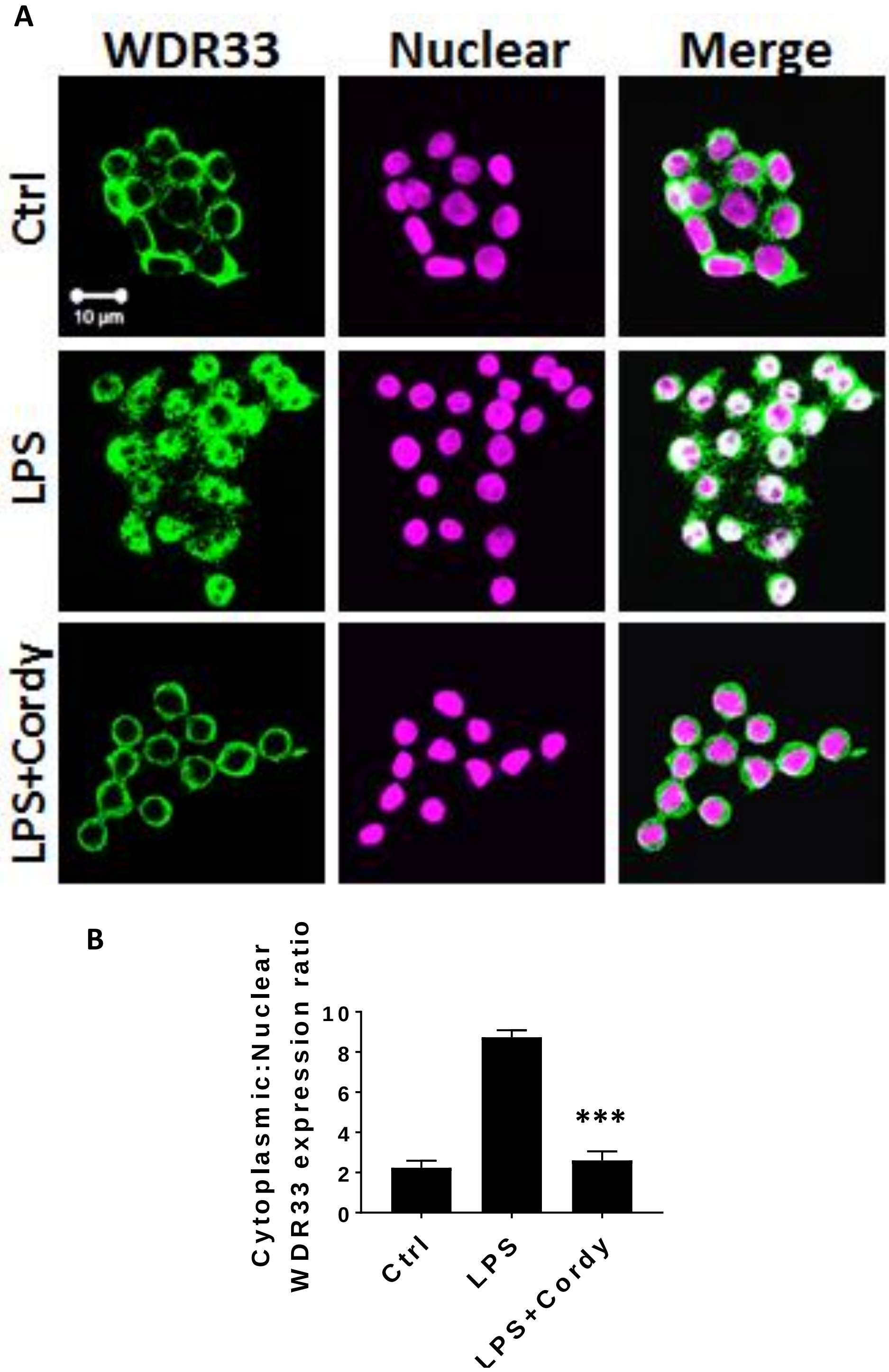


Figure 5.9. A. The localization of WDR33 is affected by cordycepin. RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 min. Cells were then fixed and stained. **B. Quantification of cytoplasmic:nuclear WDR33 expression.** Error bars represent standard deviation across three independent replicates. (***) P ≤ 0.001 compared to LPS, p values were obtained using Student's t test.

Conclusion

In murine macrophage cell line, the nuclear translocation of P65 was prevented by cordycepin (Kim 2006). In HEK 293 cells, high concentrations of this compound abolishes the NF-κB DNA binding and reduces the transcriptional activity (Ren 2012). Recently, a group studied the effect of cordycepin in rat nucleus pulposus (NP). Treatment of NP cells with LPS increased the inflammatory gene expression, which was consequently inhibited by cordycepin treatment (Li 2016). In these cells, phosphorylation of IκBα and p65 was decreased by cordycepin without affecting upstream activators in NF-kB signalling pathway. Data in this chapter offer an insight into the mechanisms by which cordycepin produces its anti-inflamatory effect in a macrophage-like cell line. The fact that unspliced mRNA levels were downregulated **(Figure 5.1),** suggests a transcriptional level of control by cordycepin. Since NF-κB is a master regulator of inflammatory response in macrophages and other cells whose activation is downstream of LPS/TLR4 pathway, I investigated whether cordycepin interferes with the NF-κB pathway as reported by other groups. Immunofluorescence experiment to detect the localization of P65 subunit was done. Immunofluorescence of NF-κB revealed that NF-κB is predominantly cytoplasmic in untreated macrophages, while LPS induces the nuclear localization of NF-κB. The results also showed that cordycepin reduced the LPS-induced nuclear localization of NF-κB **(Figure 5.2)**. To investigate how fast the effect of cordycepin on P65 localization was and to further confirm our finding, the cells were treated with LPS for different durations in the absence and presence of cordycepin and then the cytoplasmic and nuclear fractions were separated to detect the NF-κB in each fraction. Western blotting data showed P65 was cytoplasmic in the presence of cordycepin, while it started to leave the cytoplasm at 10 minute time point after LPS stimulation in the absence of cordycepin. On the other hand, there was less NF-κB in the nuclear fraction in the presence of cordycepin **(Figure 5.3)**. To investigate whether inflammatory signal transduction was affected, I examined the degradation of IκBα after stimulating the cells with LPS for different durations in the presence and absence of cordycepin. The western blotting data showed that cordycepin did not impede the degradation of IκBα **(Figure 5.4)**. Altogether, these data suggested that cordycepin reduces the nuclear localization on NF-κB in macrophage-like cell line, but this interference is not IκBα-dependent. In **figure 5.3**, it appears that P65 leaves the cytoplasm at the 15 minute time point after LPS stimulation in cordycepin-treated cells and comes back at 30 minute time point. This could indicate that cordycepin impedes the DNA-binding ability of this transcription factor rather than inhibiting the canonical IκB-dependent pathway. If this is the case, it might illustrate the effect of cordycepin on the nuclear localization of NF-κB in spite of normal degradation of IκBα. Other possible scenarios to explain the mechanism will be discussed in details in the last chapter. Surprisingly, **figure 5.8** and **5.9** showed that polyadenylation factors are cytoplasmic in uninduced cells and LPS changes the localisation of polyadenylation factors, indicating that polyadenylation may be involved in the inflammatory response. These figures also showed that cordycepin reduces the nuclear localization of the 3' processing factors (CPSF4 and WDR33). This could indicate that the cordycepin arrested polyadenylation factor complex is exported to the cytoplasm. Possibly, this could be explained by the presence of export factors in this specific complex (Shi 2009, supplementary data). As mentioned earlier in this chapter, CPSF4 is required to activate NF-κB pathway and it also binds to the promoters of inflammatory genes and induces their transcription in cancer cell line (Yi 2016). This could characterize a potential link between polyadenylation and inflammatory gene expression and is in agreement with our own siRNA knockdown experiments findings **(Figure 5.5 and Figure 5.6)** which revealed that depletion of CPSF4 and WDR33 in macrophages results in downregulating the inflammatory gene expression and reduction in the nuclear localization of NF-κB. Overall, cordycepin could produce its anti-inflammatory effect through sequestering 3’ mRNA processing factors and thus reducing the nuclear localization of NF-κB.

6. The effect of cordycepin on signal transduction pathways

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signalling pathways are both key regulators of many aspects of cell growth and survival. Indeed, they are so interconnected that, in a certain sense, they could be considered as a single, unique pathway (Porta 2014). The PI3K/Akt pathway is a key regulator of survival during cellular stress. Since inflammation is considered as a stressful environmental factor (Liu 2017), Akt could be a potential therapeutic target for inflammatory diseases. mTOR functions in two supramolecular complexes termed mTORC1 and mTORC2. It responds to and integrates signals initiated by diverse stimuli including Toll-like receptor signalling, growth factors, and nutrient availability (Sinclair 2017). It has been found that the 5'-adenosine monophosphate-activated protein kinase (AMPK) negatively regulates this pathway. AMPK is regulated by intracellular AMP/ ATP ratio. When growth conditions are favourable, mTOR is active. On the other hand, unlike mTOR, AMPK activity is increased during fuel deficiency i.e. when the AMP/ATP ratio is high. The activation of AMPK inhibits mTOR activity (Xu 2012). Thus, this complicated pathway has been regarded as an attractive target for the development of drugs that treat a variety of medical ailments, including cancer and inflammatory diseases. Our laboratory has shown that cordycepin interferes with PI3K/Akt/mTOR signalling pathway in NIH3T3 cells. It produces many of its biological effects as an Akt inhibitor and AMPK activator making it a potential candidate drug for cancer and inflammatory diseases such as asthma and osteoarthritis (Wong 2010). However, it is still not well understood whether cordycepin affects signal transduction pathways through its effect on polyadenylation because there is no established link between polyadenylation reaction and signal transduction pathways. Recently, studies were conducted showing that polyadenylation could be an upstream regulator of signalling pathways, but not in macrophages. CPSF4 is highly expressed in lung adenocarcinoma and it positively correlates with poor prognosis in patients with lung cancer. In addition, depleting CPSF4 by siRNA knockdown results in reduction in cell growth and induction of apoptosis in these cells. Studying the underlying mechanism revealed that these effects were due to modulation of multiple signal transduction pathways. Knockdown of CPSF4 expression by siRNA has a marked inhibitory effect on PI3K/Akt and MAPK signalling pathways. On the other hand, the ectopic expression of CPSF4 had the opposite effects (Chen 2013). In this chapter, I studied the effect of our compound, 3’ deoxyadenosine, on the PI3K/Akt/mTOR pathway in RAW264.7 cells and aimed to find the effect of modulating this pathway on the inflammatory gene expression. Furthermore, I planned to find out whether polyadenylation works upstream of PI3K/Akt/mTOR signal transduction pathway in our inflammatory system.

6.1 The effect of cordycepin on Akt/mTOR pathway

Recently it has emerged that mTOR is an important regulator of the inflammatory response (Gao 2015). In the line with this, our group found that cordycepin has an inhibitory effect on mTOR in NIH3T3 cells through reducing the level of phosphorylated Akt (serine 473) (Wong 2010). I therefore decided to investigate the effect of cordycepin on this pathway in RAW264.7 cells. Western blotting was performed for the phosphorylated and total proteins that play key roles in the mTOR signalling pathway. The results showed that cordycepin reduces the phosphorylation of Akt (on serine 473), but it does not affect the total protein level. Also I found that this compound reduces the phosphorylation of 4EBP. However cordycepin has an opposite effect on the phosphorylation of AMPK. The western blotting showed that cordycepin increased the phosphorylation of AMPKα on threonine 172 **(Figure 6.1)**. Studies have shown that phosphorylation of AMPK on this site accounts for most of the activation by AMPK kinases, although other sites are involved as well (Stein 2000). These data together indicate that cordycepin has an inhibitory effect on the mTOR signal transduction pathway through inhibiting Akt and activating AMPK.

To investigate the mechanism by which cordycepin produces this inhibitory effect on the mTOR pathway, immunofluorescence experiments were done to assess the effect of this compound on the subcellular localization of these proteins **(Figure 6.2)**. The results showed that cordycepin limits the phosphorylated Akt to the cytoplasmic compartment of the LPS-stimulated cells, whereas it was localized in both the nuclear and the cytoplasmic compartment in the cells stimulated with LPS in the absence of cordycepin. Two phosphorylation sites were investigated which are the mTOR phosphorylation site (Ser473) and the PDK1 phosphorylation site (Thr308). The results showed that the effect of cordycepin on the localization of both phosphorylated Akt were identical. However cordycepin does not affect the localization of the total Akt protein. Total Akt is distributed in both cytoplasmic and nuclear compartments in all of the three conditions **(Figure 6.2).** To extrapolate our findings to human macrophages, CD14 positive monocytes were isolated from peripheral human blood and then differentiated them into macrophages in the presence of macrophage-colony stimulating factor (M-CSF). Macrophages were stimulated with LPS either in the presence or the absence of cordycepin. Then the cells were fixed and stained for pAkt (Ser473). The results showed that cordycepin reduced the LPS-induced expression of pAkt in human macrophages **(Figure 6.3)**.

Furthermore, I have checked the effect of cordycepin on the localization of mTOR in the RAW264.7 cells. The immunofluorescence experiments showed that cordycepin affects the subcellular localization of this protein in the macrophage-like cell line. While this protein is located in both compartments in the DMSO and LPS treated cells, it is significantly shifted to cytoplasmic organelles in cordycepin-treated cells. In order to know in which cytoplasmic organelles this protein is specifically located, I did double staining with one of the specific markers of the stress granules (TIA1). The results showed a colocalization between these two proteins. However it is not very clear and needs further inverstigation **(Figure 6.4)**.

The effect of cordycepin on the localization of the negative regulator of this pathway, AMPK, also was tested. Immunofluorescence showed that cordycepin affected the subcellular localization of the phosphorylated form of this protein in the RAW264.7 cells. In the presence of cordycepin, pAMPK was mainly located in the nucleus. However, it was distributed all over the cells in the LPS-stimulated DMSO-treated cells. However, the phosphorylated form of this protein is not highly expressed in the untreated cells. The results showed that the AMPK is activated only in the mitotic cells in the control condition **(Figure 6.5)**. The results also showed that the total AMPK is not changed. It was evenly distributed in both cellular compartments in untreated and LPS-stimulated in the absence and presence of cordycepin **(Figure 6.5)**.

6.2 The effect of modulating Akt/mTOR pathway on the inflammatory gene expression

6.2.1 Akt/mTOR pathway was modulated by using chemical compounds

Having that cordycepin is an Akt inhibitor and AMPK activator with a less well understood effect on MEK signalling in macrophages, modulation of these signalling pathways was studied. Several compounds which are known by their ability to either inhibit or activate key kinases in these pathways were used. AMPK activator (A-769662) is a direct activator of AMPK which mimicks the AMP effect. It has been found that A-769662 increases the phosphorylation of acetyl-CoA carboxylase (ACC) which is a downstream target of AMPK (Göransson 2007). **Figure 6.6** shows that cordycepin, AMPK activator, rapamycin and torin 1 increase phosphorylated ACC, in contrast to Akt inhibitor and MEK inhibitor which reduce the level of phosphorylated ACC.

MK-2206 is a selective Akt inhibitor that binds to Akt protein leading to conformational changes. It has been found that Akt directly phosphorylates tuberous scelerosis complex 2 (TSC2) at multiple sites (Huang 2009 and Ji 2017). Cordycepin, AMPK activator, Akt inhibitor, MEK inhibitor, Rapamycin and torin 1 were all shown to reduce the phosphorylated TSC2, in contrast to LPS which increases the level of pTSC2 (data not shown, more controls need to be done).

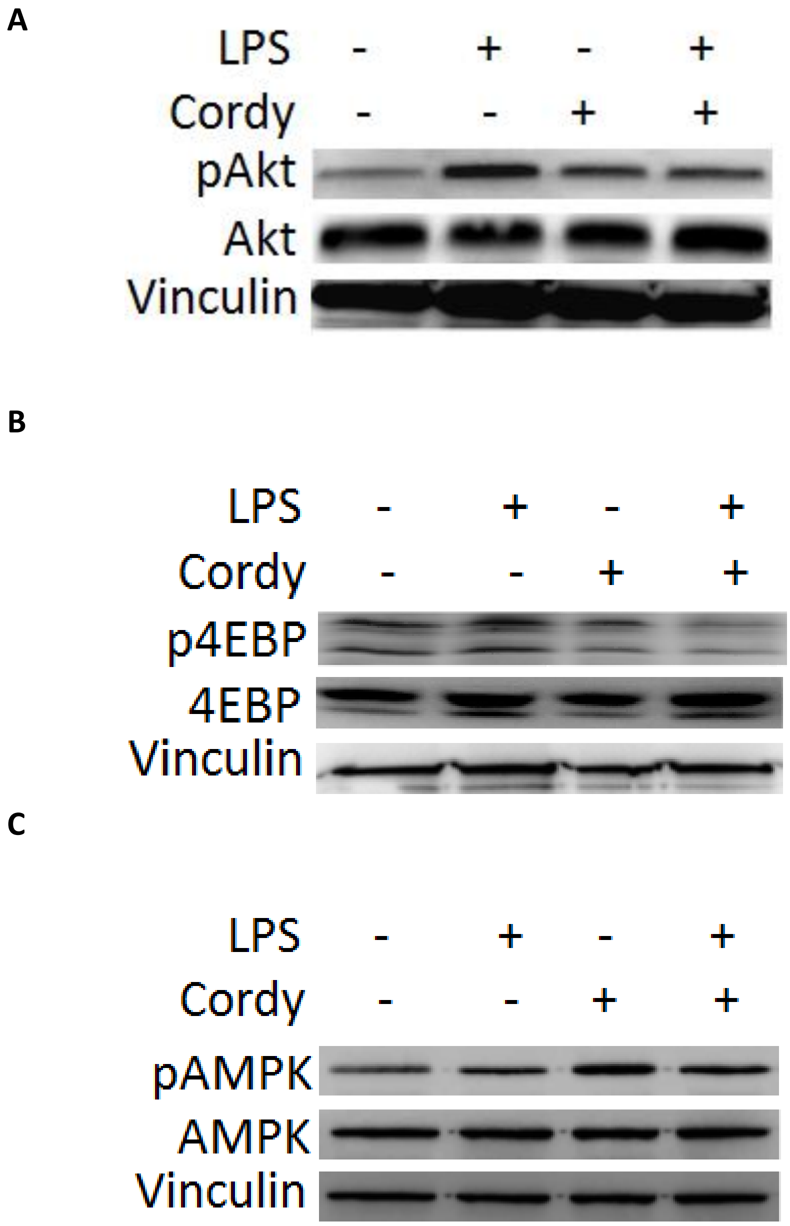
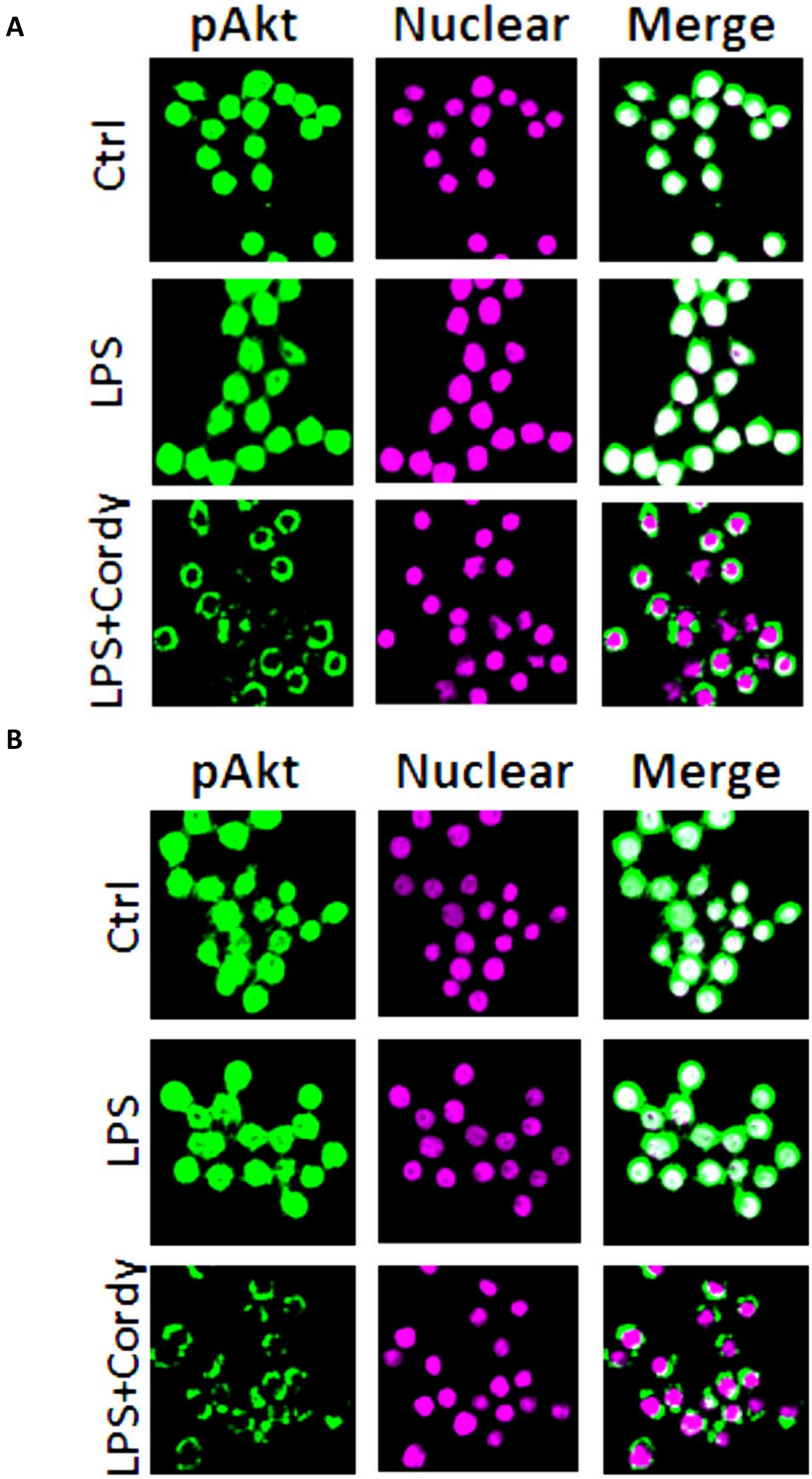


Figure 6.1. Cordycepin has an inhibitory effect on mTOR signal transduction pathway. RAW264.7 cells were treated with DMSO or cordycepin for an hour prior to LPS addition for another one hour. Cells were then lysed and Western Blot performed for **A.** Akt (Ser 473), **B.** 4EBP (Thr 37/46) and **C.** AMPK (Thr 172). The images are representatives of at least two independent replicates.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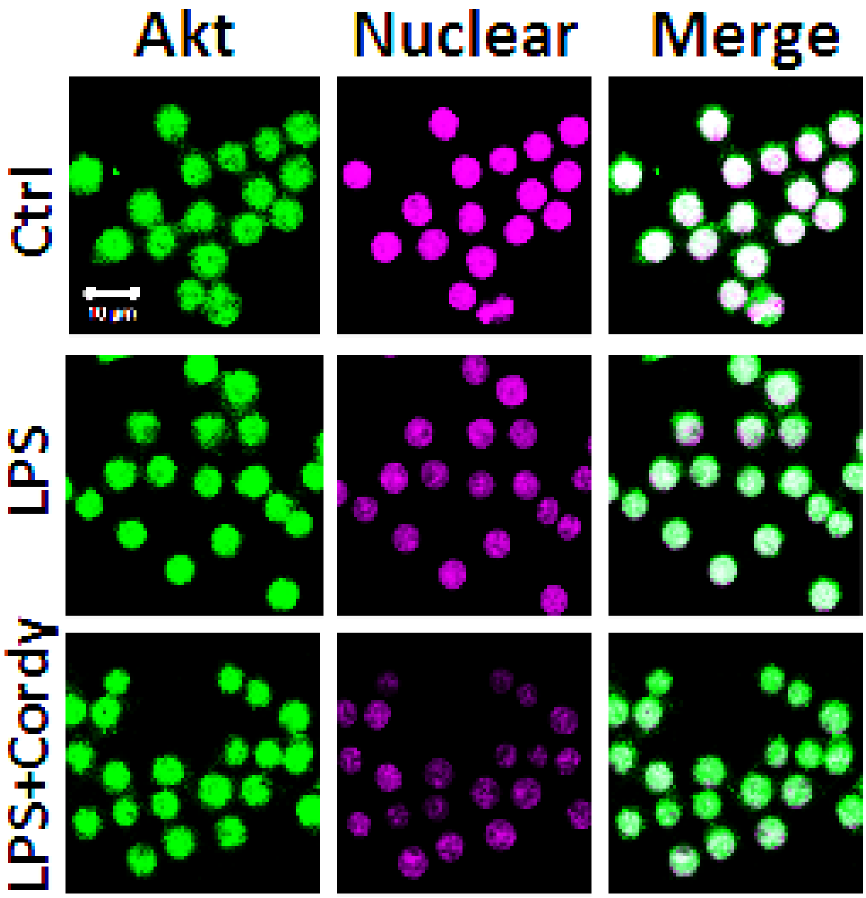

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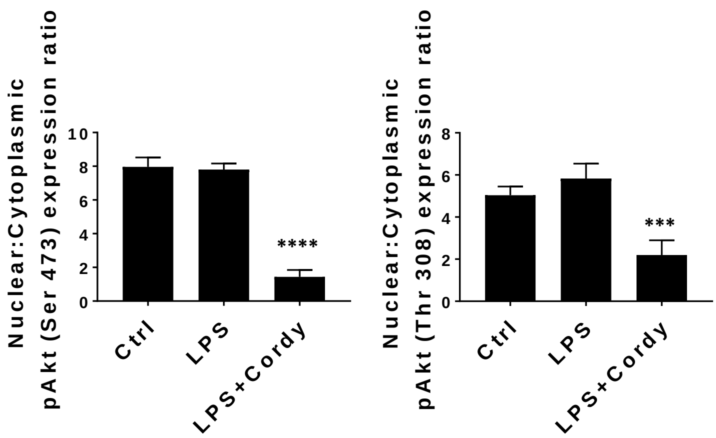


Figure 6.2. Cordycepin affects the localization of phosphorylated Akt. RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 minutes. Cells were then fixed and stained **A.** pAkt Ser473 **B.** pAkt Thr308 and **C.** Akt. **D.** Quantification of nuclear:cytoplasmic ratio for pAkt (Ser 473) and (Thr 308). The images are representatives of three independent replicates for pAkt Ser473 and Thr308, and only one biological replicate for Akt. (***) P ≤ 0.001, (****) P ≤ 0.0001 compared to siCtrl+LPS, p values were obtained using Student's t test.

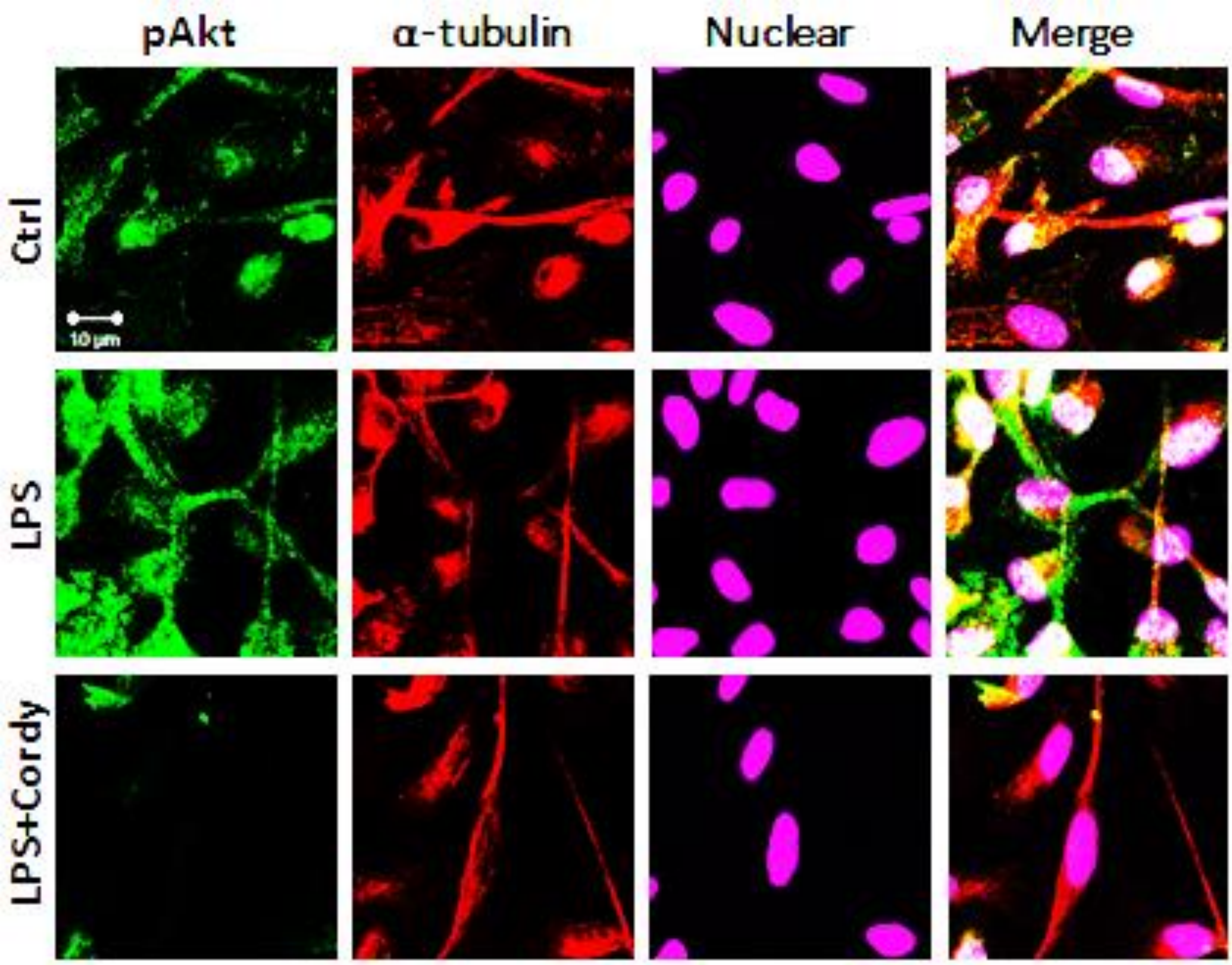


Figure 6.3. Cordycepin reduces the expression of phosphorylated Akt (Ser473). Primary human macrophages were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 minutes. Cells were then fixed and stained for pAkt Ser473. The images are representatives of only one biological replicates.

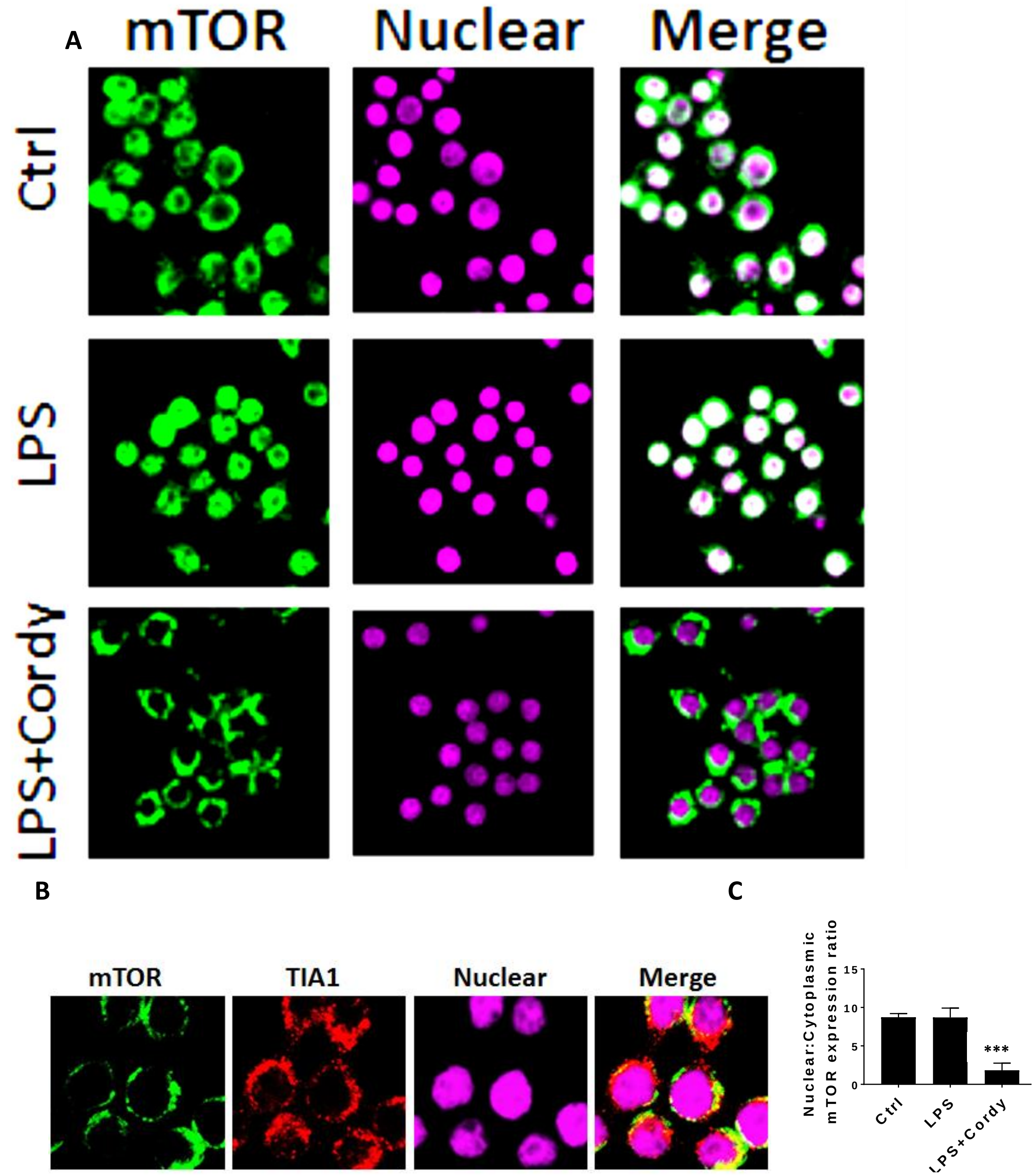
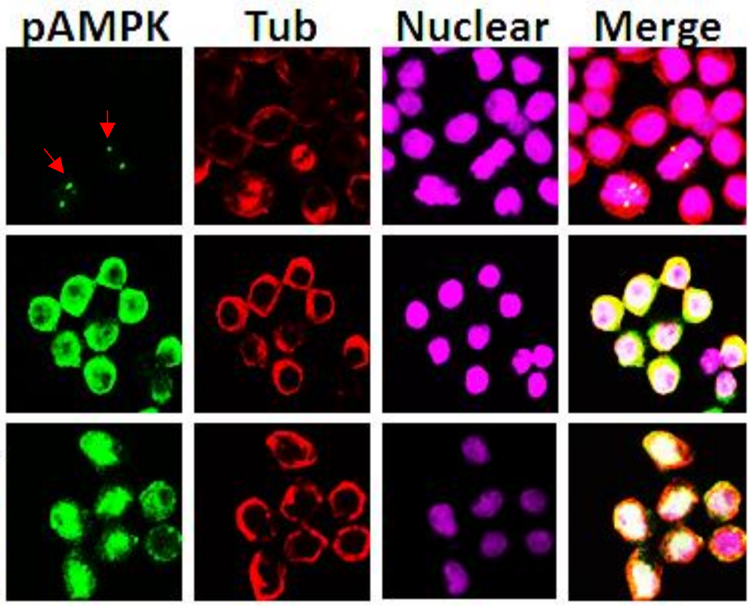


Figure 6.4. Cordycepin affects the localization of mTOR. RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 minutes. Cells were then fixed and stained **A.** mTOR only and **B.** mTOR and TIA1. **C.** Quantification of nuclear:cytoplasmic ratio for mTOR. The images are representative of three independent replicates. (***) P ≤ 0.001 compared to siCtrl+LPS, p values were obtained using Student's t test.

A



B

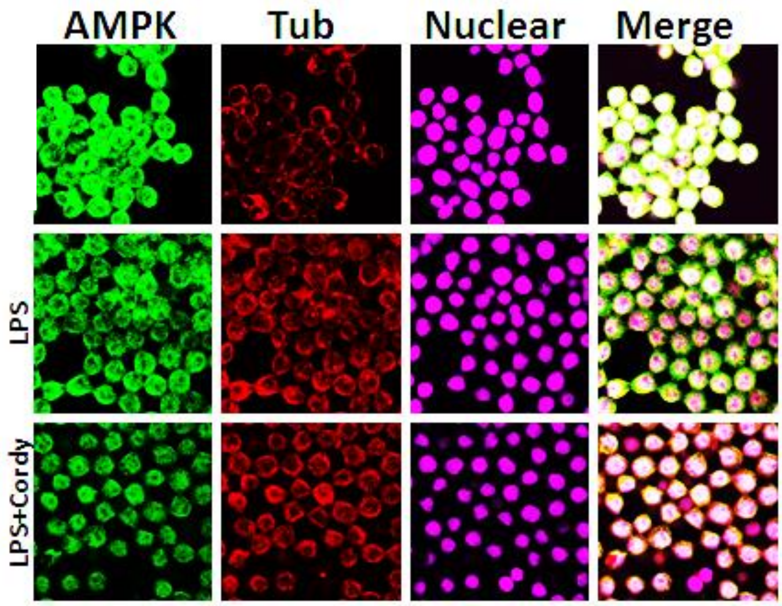


Figure 6.5. Cordycepin affects the localization of phosphorylated AMPK. RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO or 20 µM cordycepin for 50 minutes. Cells were then fixed and stained **A.** pAMPK (Thr 172) and **B.** AMPK. The images are representative of one replicates. Arrows show mitotic cells.

PD98059 is a potent and selective MEK inhibitor. It has been found that PD98059 reduces the phosphorylation of ERK confirming the involvement of MEK in ERK activation (Nijhara 2001). The western blotting below shows that cordycepin, AMPK activator and MEK inhibitor reduce the level of pERK **(Figure 6.6)**.

Rapamycin is a direct mTORC1 inhibitor, however chronic administration of rapamycin in certain cell lines inhibits mTORC2 (Schreiber 2015). Torin 1 is a direct ATP-competitive mTOR inhibitor which inhibits both mTORC1 and mTORC2. S6 kinase (S6K) and the translational inhibitor (4EBP) are well known downstream substrates of mTORC1 (Thoreen 2009). Overall, these results indicate that these compounds work as expected showing their proposed effects on theirdownstream targets. However, more compounds still need to be tested and more loading controls still need to be done to further confirm these findings. Accordingly, these compounds will be used to study the effect of modulating signal transduction pathways on inflammatory gene expression and NF-κB activity pathway.

6.2.2 Comparing the effect of small molecule signal transduction modulators with cordycepin on the inflammatory gene expression

In an attempt to see whether cordycepin produces its anti-inflammatory effect through inhibiting Akt/mTOR pathway, RAW264.7 cells were treated with chemical compounds which are known by their ability to inhibit or activate different kinases in this pathway. Treating the LPS-stimulated cells with Akt inhibitor was associated with downregulation of the relative mRNA level of Il1b, Acod1 and Ticam but not Tnf

(Figure 6.7A). Inhibiting mTOR by using either rapamycin or torin 1 resulted in repression the expression of Il1b, Acod1 and Ticam while they did not repress Tnf expression **(Figure 6.7B and C)**. On the other hand, activating AMPK was also associated with reduction in the relative mRNA level of Il1b, Acod1 and Ticam, but not Tnf. The same results were obtained by inhibiting AMPK signalling. However, Tnf expression was sensitive to the inhibition of AMPK signalling **(Figure 6.7 D)**. **Figure 6.1C** showed that both LPS and cordycepin were increasing the level of phosphorylated AMPK compared to the control. **Figure 6.5A** showed that the expression of pAMPK was more abundant in LPS-stimulated cells both in the presence and absence of cordycepin than in the untreated cells. However, the localization in the subcellular compartment was different. It was distributed evenly in the cellular compartments in the absence of cordycepin. While it was nuclear in cordycepin-treated cells. This unexpected shuttling of pAMPK between cellular compartments in response to different stimuli could explain the response of LPS-stimulated macrophages to AMPK activator and compound C in terms of inflammatory gene expression. Non specific PI3K inhibitor which was used in relatively high concentration resulted in repressing the expression of all the tested inflammatory genes including Tnf, Il1b, Acod1 and Ticam **(Figure 6.7E)**. In a similar way, MEK inhibitor represses the expression of all the tested genes including Tnf **(Figure 6.7F)**. Altogether, these data indicate that inhibiting Akt/mTOR pathway results in downregulation of the inflammatory gene expression and to activation of AMPK, similar to the effects of cordycepin. However, Tnf was resistant to most of these compounds. This suggests that cordycepin exerts it effect, at least in part, through inhibiting Akt/mTOR signal transduction pathway. However, more specific compounds which are approved in clinical trials need to be used to further confirm these findings.

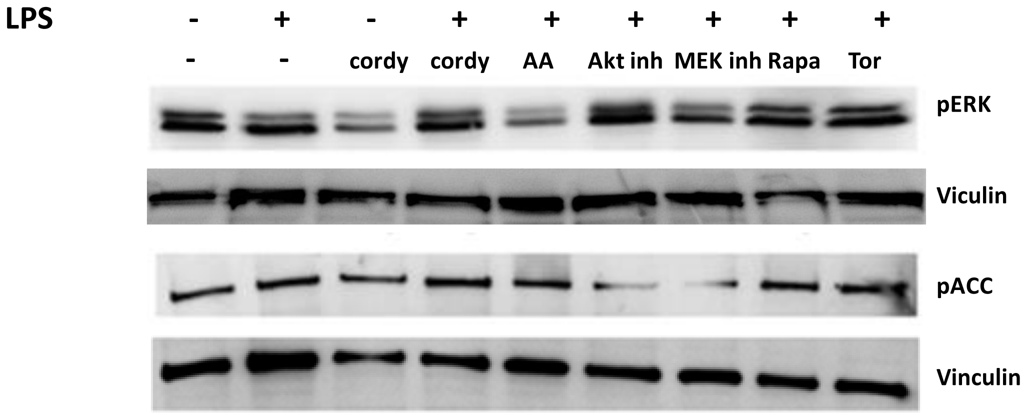
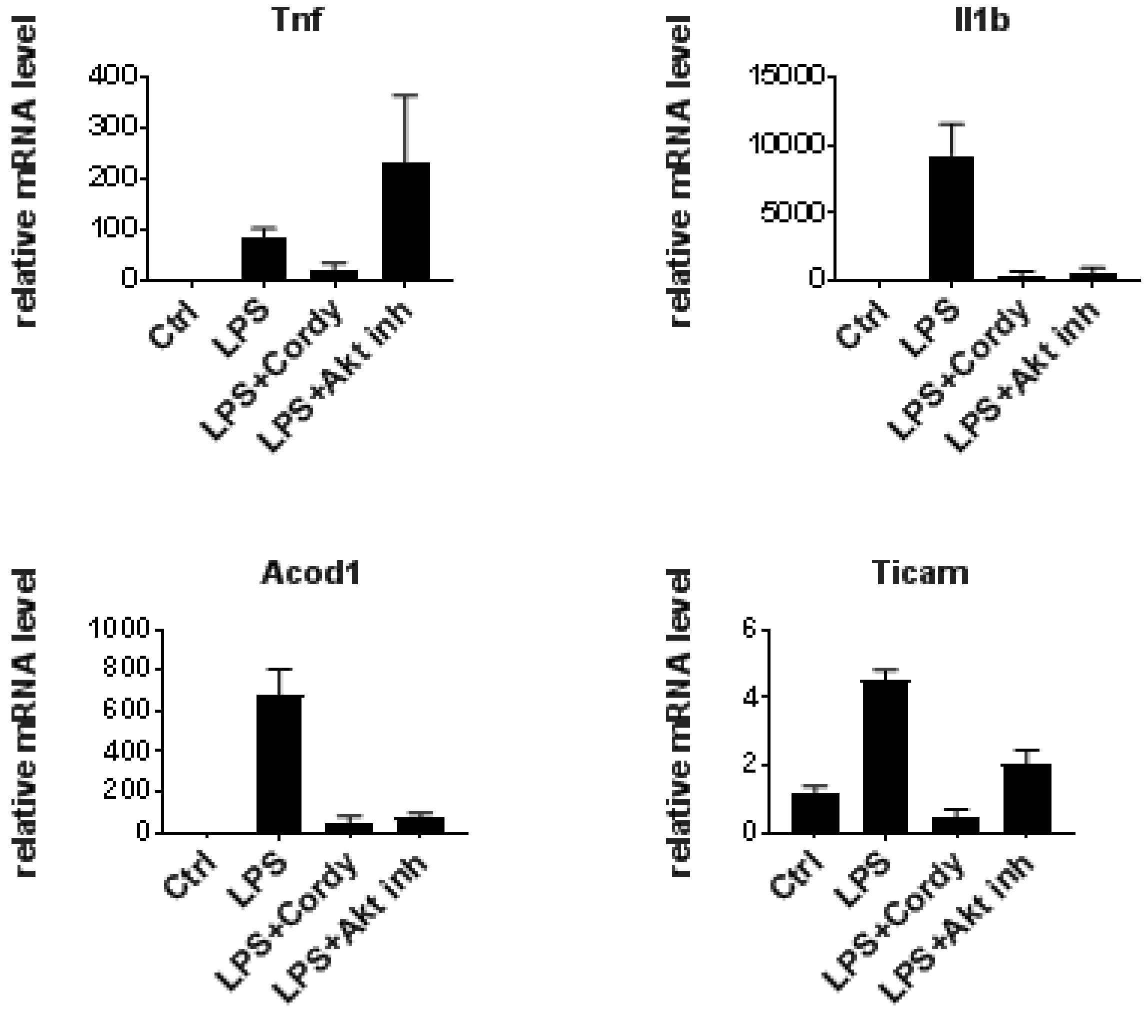
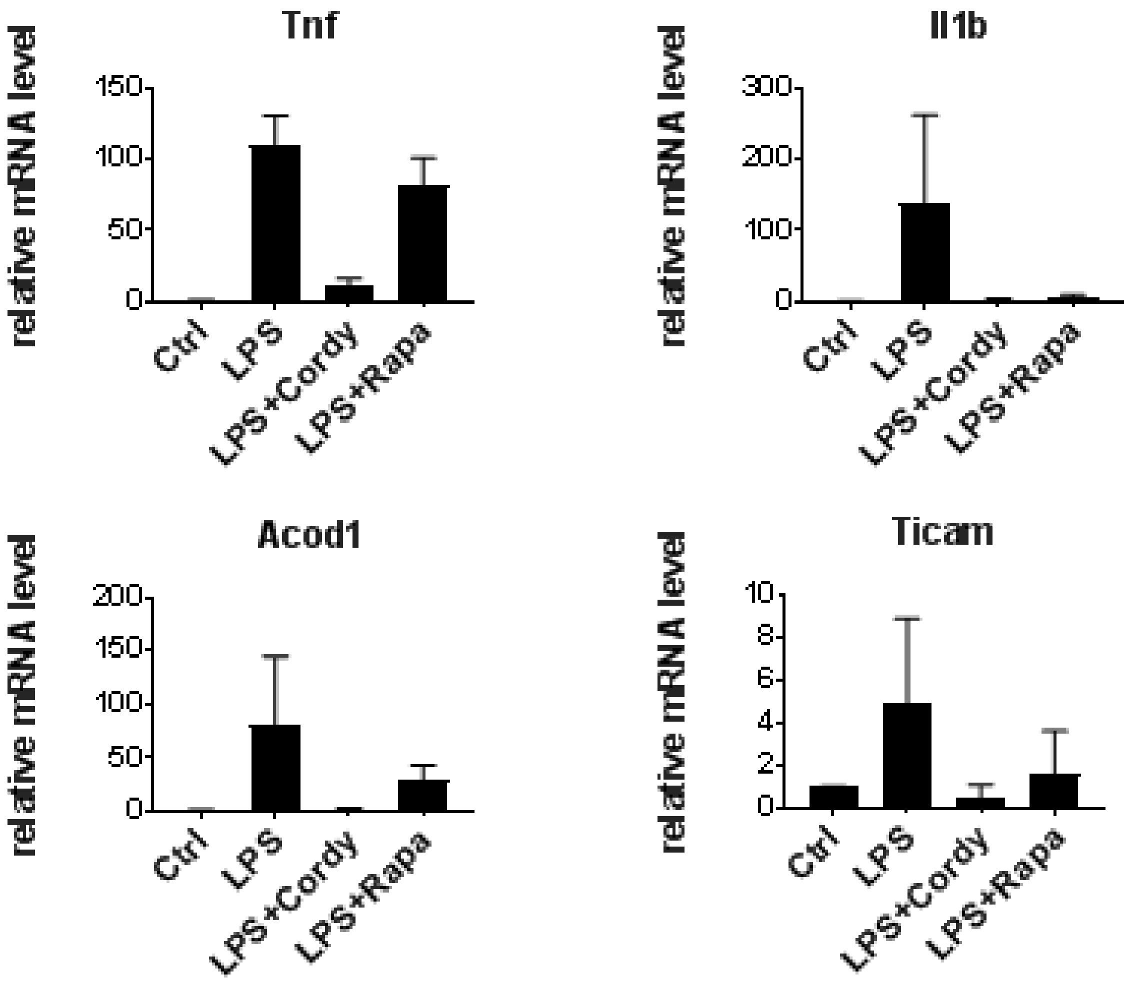


Figure 6.6. The effects of the kinase modulators on the phosphorylation of the downstream targets. RAW 264.7 cells were treated with DMSO, cordycepin or the indicated compound for an hour prior to LPS addition for another one hour. Cells were then lysed and Western Blot performed. The images are representatives of one replicate.

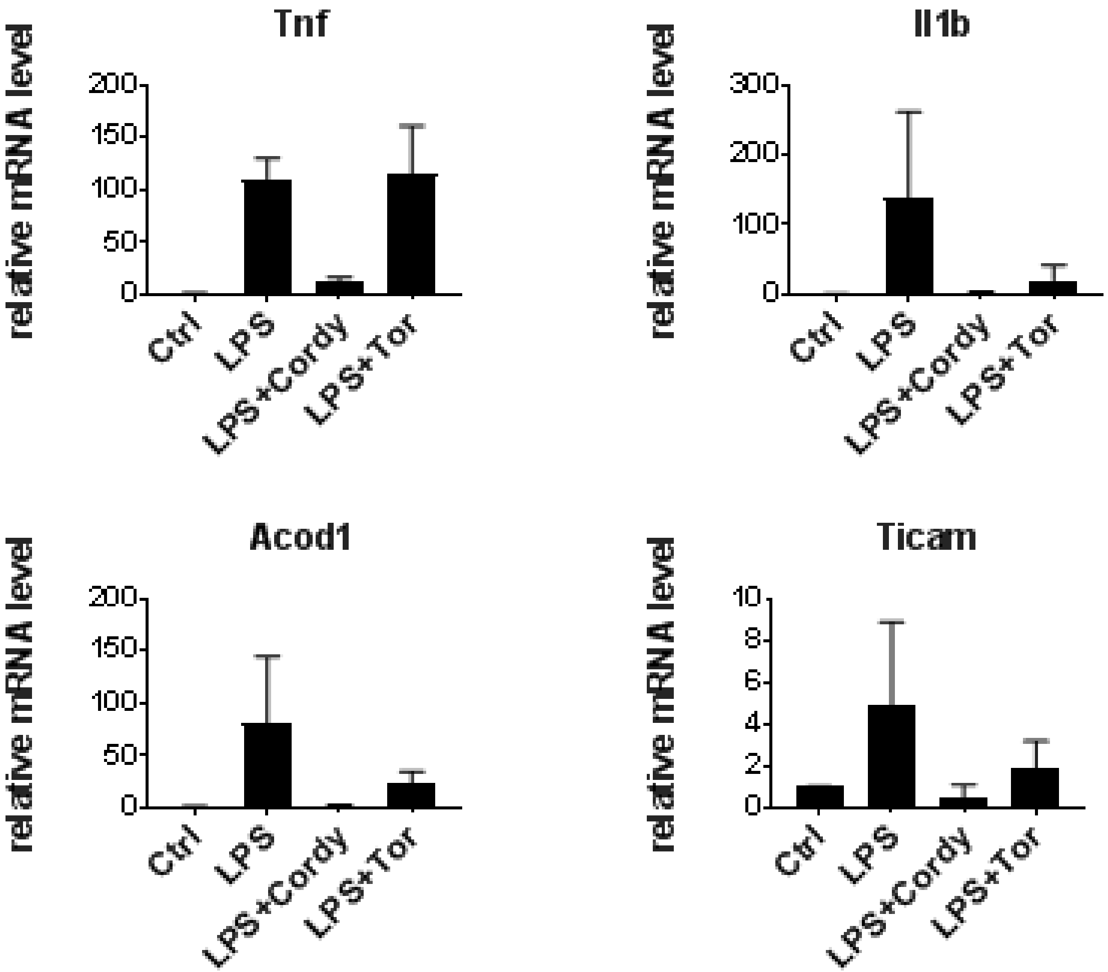
A


Tnf
relative mRNA level
400
300
200
100
0
Ctrl
LPS
LPS+Cordy
LPS+Akt inh
Il1b
relative mRNA level
15000
10000
5000
0
Ctrl
LPS
LPS+Cordy
LPS+Akt inh
Acod1
relative mRNA level
1000
800
600
400
200
0
Ctrl
LPS
LPS+Cordy
LPS+Akt inh
Ticam
relative mRNA level
6
4
2
0
Ctrl
LPS
LPS+Cordy
LPS+Akt inh

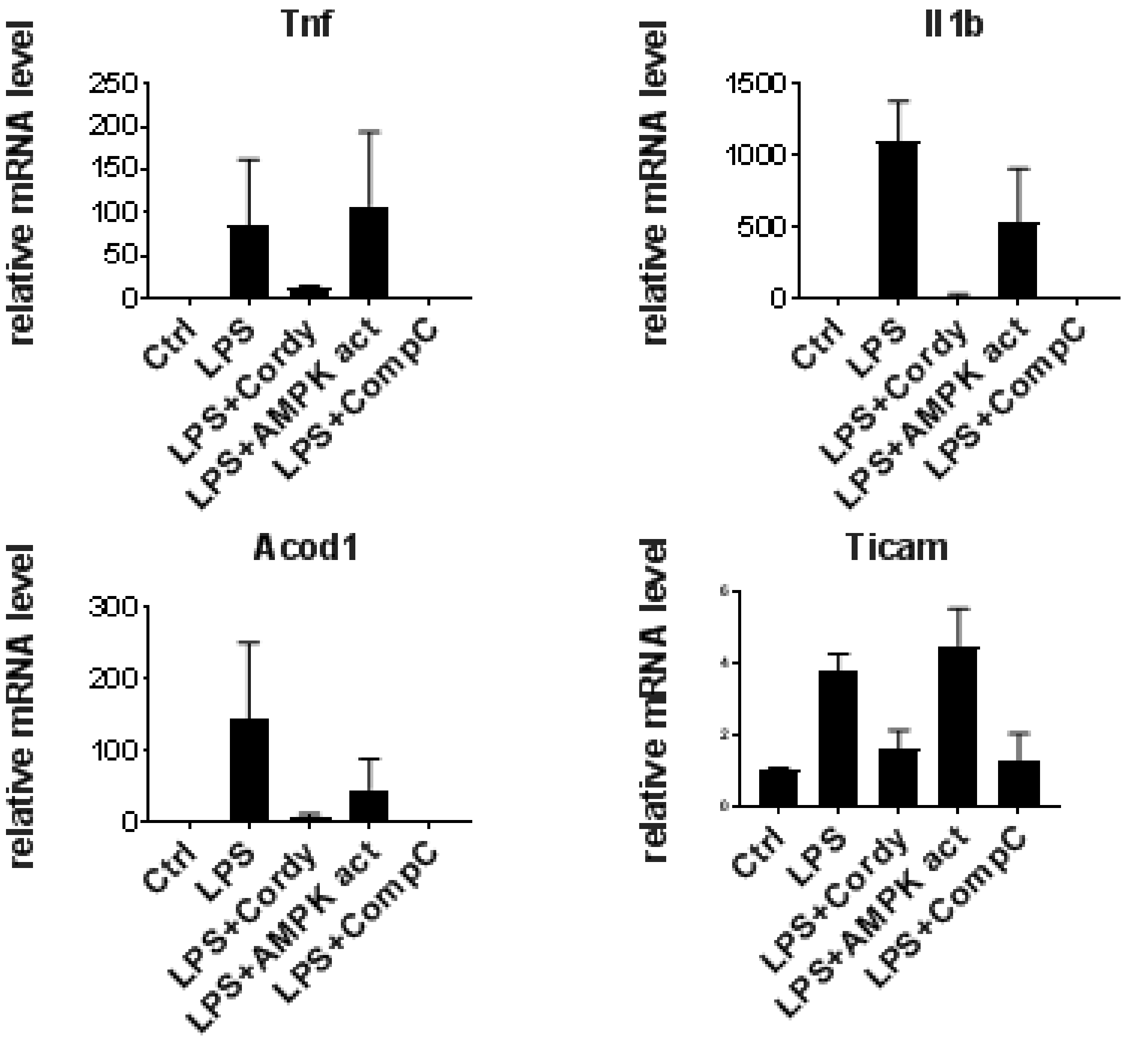
B


Tnf
relative mRNA level
150
100
50
0
Ctrl
LPS
LPS+Cordy
LPS+Rapa
Il1b
relative mRNA level
300
200
100
0
Ctrl
LPS
LPS+Cordy
LPS+Rapa
Acod1
relative mRNA level
200
150
100
50
0
Ctrl
LPS
LPS+Cordy
LPS+Rapa
Ticam
relative mRNA level
10
8
6
4
2
0
Ctrl
LPS
LPS+Cordy
LPS+Rapa

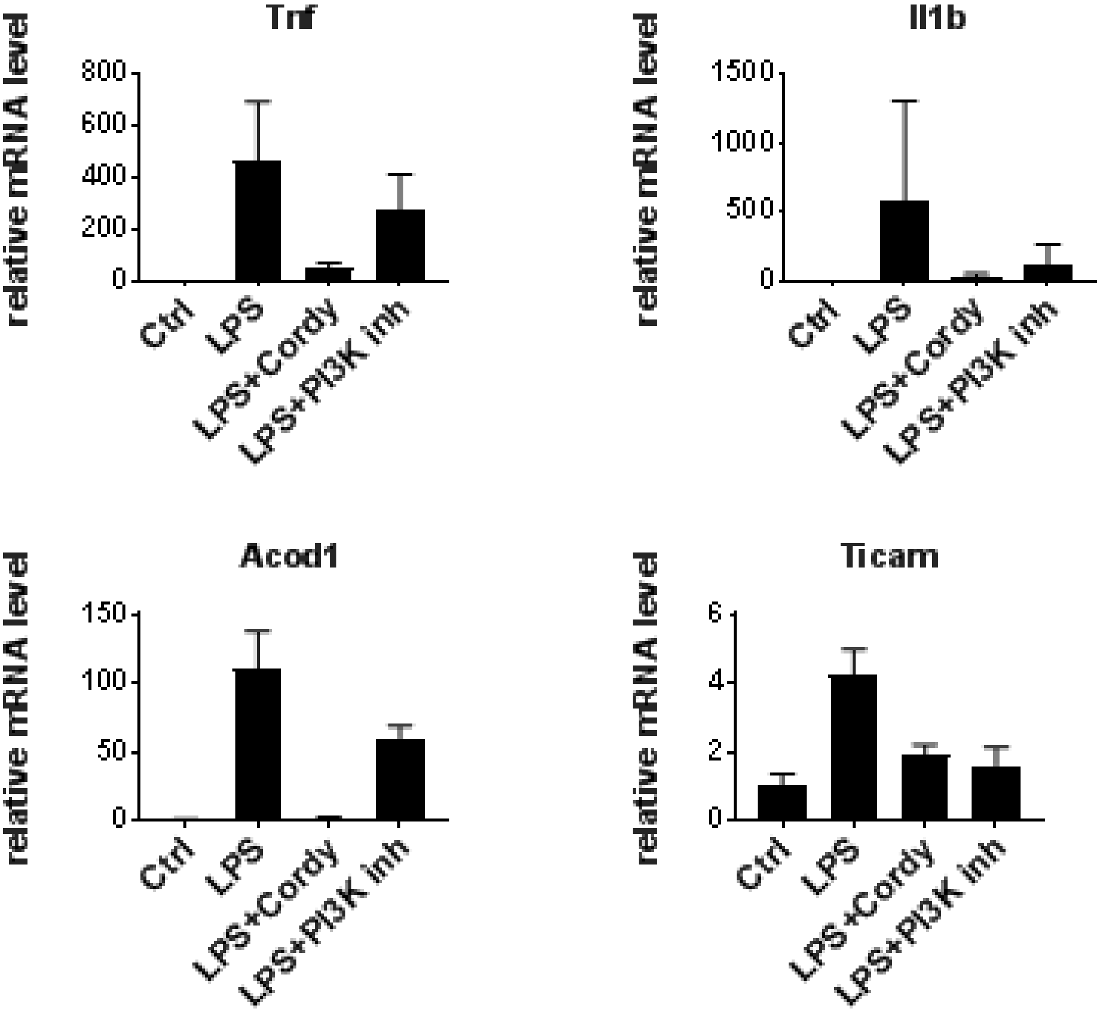
C


Tnf
relative mRNA level
200
150
100
50
0
Ctrl
LPS
LPS+Cordy
LPS+Tor
Il1b
relative mRNA level
300
200
100
0
Ctrl
LPS
LPS+Cordy
LPS+Tor
Acod1
relative mRNA level
200
150
100
50
0
Ctrl
LPS
LPS+Cordy
LPS+Tor
Ticam
relative mRNA level
10
8
6
4
2
0
Ctrl
LPS
LPS+Cordy
LPS+Tor

D


Tnf
relative mRNA level
250
200
150
100
50
0
Ctrl
LPS
LPS+Cordy
LPS+AMPK act
LPS+CompC
Il1b
relative mRNA level
1500
1000
500
0
Ctrl
LPS
LPS+Cordy
LPS+AMPK act
LPS+CompC
Acod1
relative mRNA level
300
200
100
0
Ctrl
LPS
LPS+Cordy
LPS+AMPK act
LPS+CompC
Ticam
relative mRNA level
Ctrl
LPS
LPS+Cordy
LPS+AMPK act
LPS+CompC

E



Tnf
relative mRNA level
800
600
400
200
0
Ctrl
LPS
LPS+Cordy
LPS+PI3K inh
Il1b
relative mRNA level
1500
1000
500
0
Ctrl
LPS
LPS+Cordy
LPS+PI3K inh
Acod1
relative mRNA level
150
100
50
0
Ctrl
LPS
LPS+Cordy
LPS+PI3K inh
Ticam
relative mRNA level
6
4
2
0
Ctrl
LPS
LPS+Cordy
LPS+PI3K inh

F

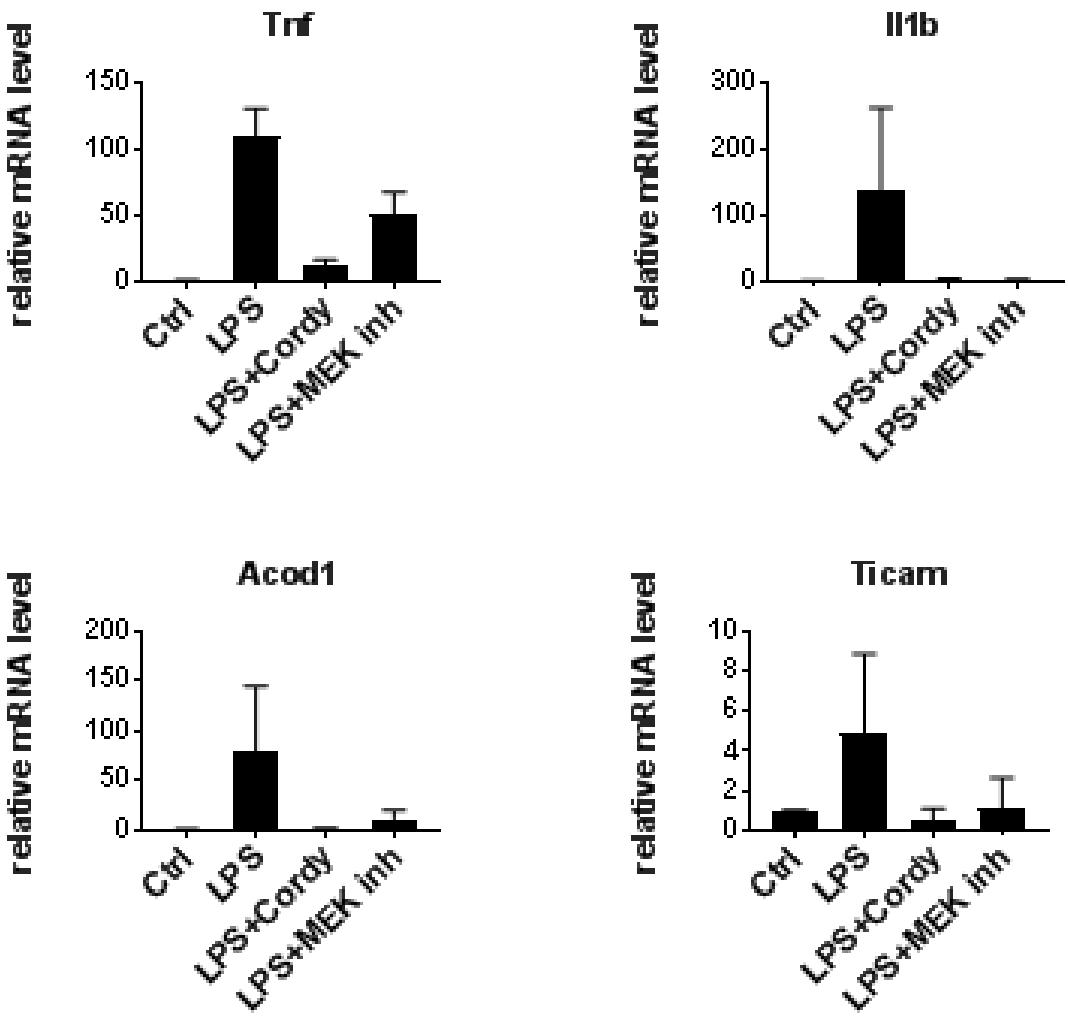
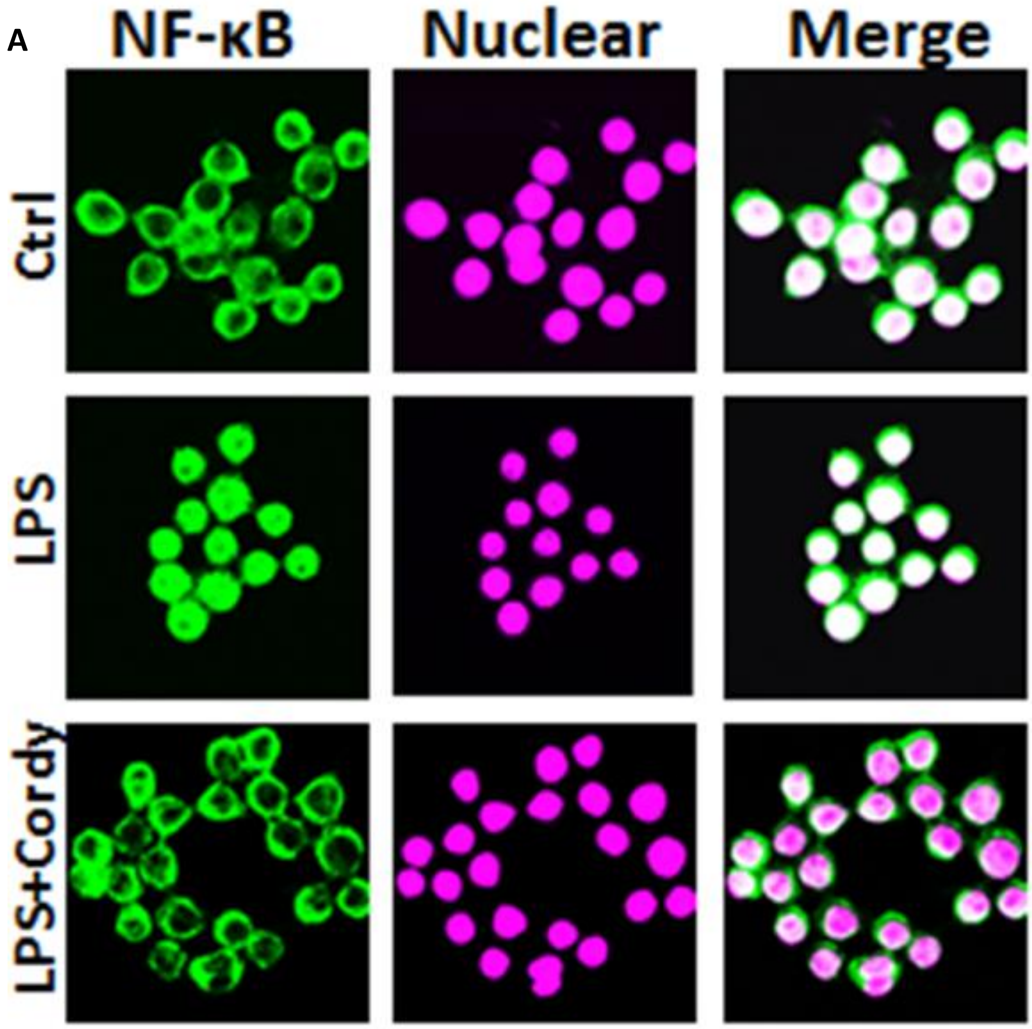
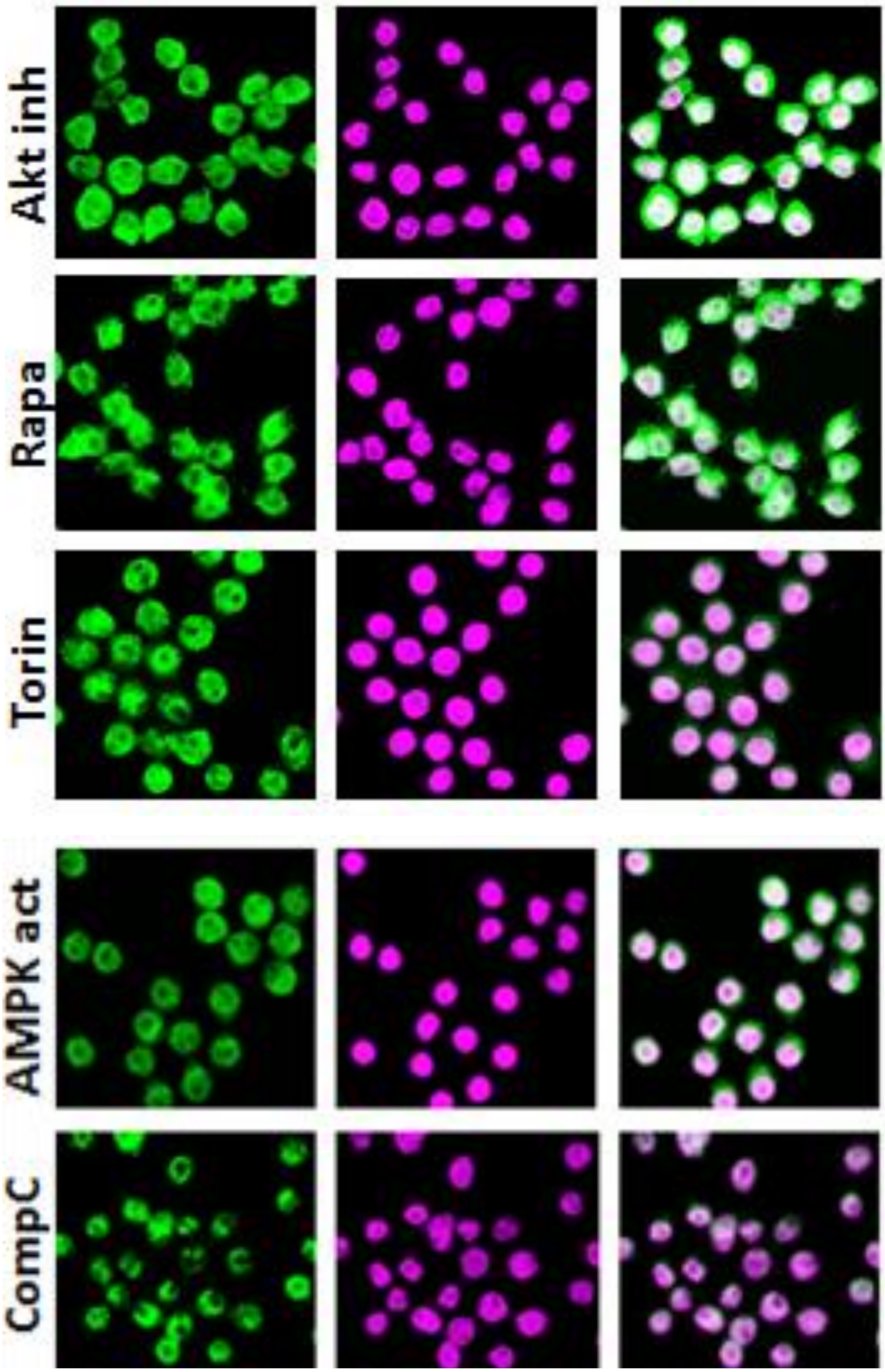


Figure 6.7. Modulating Akt/mTOR signalling pathway represses the inflammatory gene expression. RAW264.7 cells were treated with cordycepin or **A.** Akt inhibitor **B.** Rapamycin **C.** Torin 1 **D.** AMPK activator and compound C. **D.** PI3K inhibitor and **E.** MEK inhibitor for one hour and then stimulated with LPS for another an hour. RTqPCR was performed, error bars represent standard deviations across 3 independent replicates. PI3K inh: 100µM, Akt inh: 10µM, AMPK act: 20µM, Comp C: 50µM, MEK inh: 30 uM, Rapa: 20nM and Tor: 500 nM.

6.3 The effect of modulating Akt/mTOR pathway on the nuclear localization of NF-κB

After knowing that repressing the Akt/mTOR pathway downregulates the inflammatory gene expression (except Tnf), I aimed to determine the mechanism by which these modulators (Akt inhibitor, rapamycin, torin1, AMPK activator and compound C) produce their anti-inflammatory effect. The effect of these compounds on the nuclear localization of NF-κB had been tested to see whether the Akt/mTOR signalling pathway is upstream of NF-κB pathway activation. RAW264.7 cells were treated with these compounds after stimulating them with LPS and immunofluorescence was done to visualize the effect on the nuclear localization of NF-κB. Akt inhibitor and rapamycin reduced the nuclear localization of NF-κB in macrophage-like cell line. However their effect was not as strong as that of cordycepin. On the other hand, the other compounds including torin 1, AMPK activator and compound C did not have any significant effect on the nuclear localization of NF-κB **(Figure 6.8)**. The data show that cordycepin may be partially acting through Akt inhibition, but appears to have additional activity. Furthermore, this could indicate that cordycepin does not produce its effect as a single inhibitor of Akt or mTOR because inhibition of each of these kinases does not affect the NF-kB pathway in a similar way to cordycepin. For a comparison with cordycepin, I tested the effect of these compounds on IκB degradation. After treating the cell with these compounds and stimulating them with LPS for different time points, it was found that IκB started to degrade after 15 minutes of LPS stimulation and was not resynthesized after 30 minutes of LPS stimulation **(Figure 6.9)**, while it was resynthesized in the 30 minute time point when the cells were treated with cordycepin. This could explain why these compounds do not affect the nuclear localization of NF-κB in a similar way to cordycepin. However, this figures lacks a loading control for each condition and it needs to be done to further confirm these findings.




Akt inh
Rapa
Torin
AMPK act
CompC

B

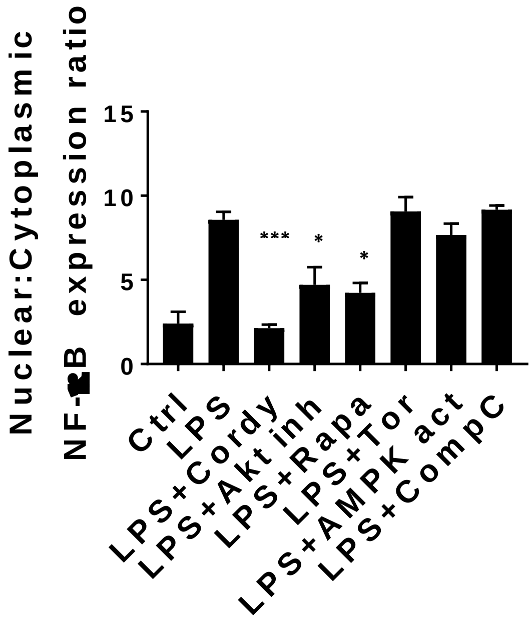


Figure 6.8. A. Comparing the effect of compounds with cordycepin on the nuclear localization of NF-κB (P65). RAW 264.7 cells were stimulated with LPS for 10 min and then treated with DMSO, 20 µM cordycepin or the indicated compound for 50 minutes. Cells were then fixed and stained. **B. Quantification of nuclear:cytoplasmic ratio for NF-κB.** The results are representative of two independent replicates. (*) P ≤ 0.05, (***) P ≤ 0.001 compared to LPS, p values were obtained using Dunnett's test. PI3K inh: 100µM, Akt inh: 10µM, AMPK act: 20µM, Comp C: 50µM, MEK inh: 30 uM, Rapa: 20nM and Tor: 500 nM.

6.4 Depleting polyadenylation factors has an inhibitory effect on Akt/mTOR signalling pathway

In the previous chapter, I showed that depletion of polyadenylation factors also affects NF-κB localisation. To examine if this effect is possibly through inhibition of the on Akt/mTOR pathway, I examined the effect of knocking down the cleavage and polyadenylation factors (CPSF4 and WDR33) in RAW264.7 cells on the phosphorylated level of some kinases in this pathway including Akt, 4EBP and AMPK.

The western blotting results showed that knocking down these polyadenylation factors have a similar effect to cordycepin as both knockdowns reduced the phosphorylated level of both Akt and 4EBP and increased the phosphorylated AMPK **(Figure 6.10 and Figure 6.11)**. Overall, these data suggest that polyadenylation is upstream of the Akt/mTOR pathway and inhibition of polyadenylation either by cordycepin or by knocking down polyadenylation factors has an inhibitory effect on this signal transduction pathway.

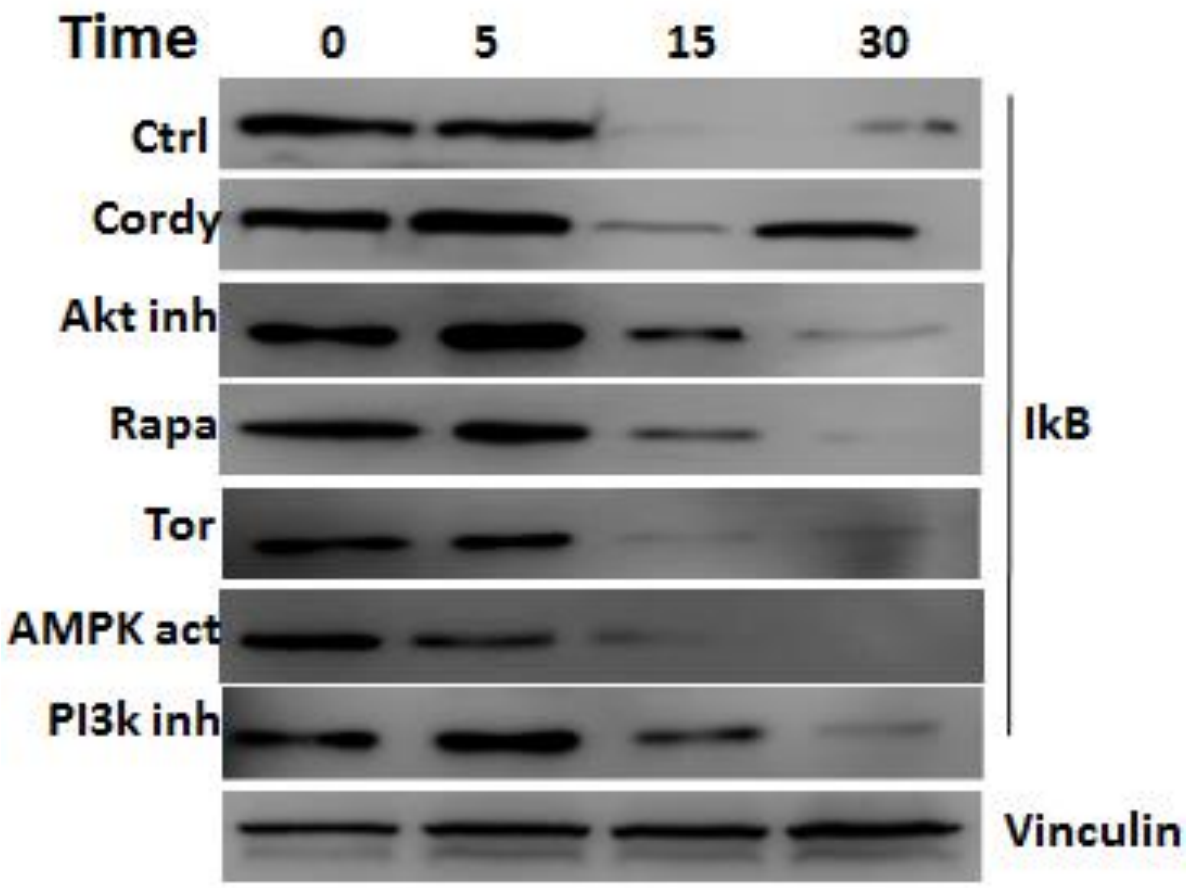


Figure 6.9. Comparing the effect of compounds with cordycepin on IκBα degradation. RAW 264.7 cells were treated with DMSO, cordycepin or the indicated compound for an hour prior to LPS addition for the indicated durations. Cells were then lysed and Western Blot performed. The images are from one to two independent replicates. PI3K inh: 100µM, Akt inh: 10µM, AMPK act: 20µM, Comp C: 50µM, MEK inh: 30 uM, Rapa: 20nM and Tor: 500 nM.

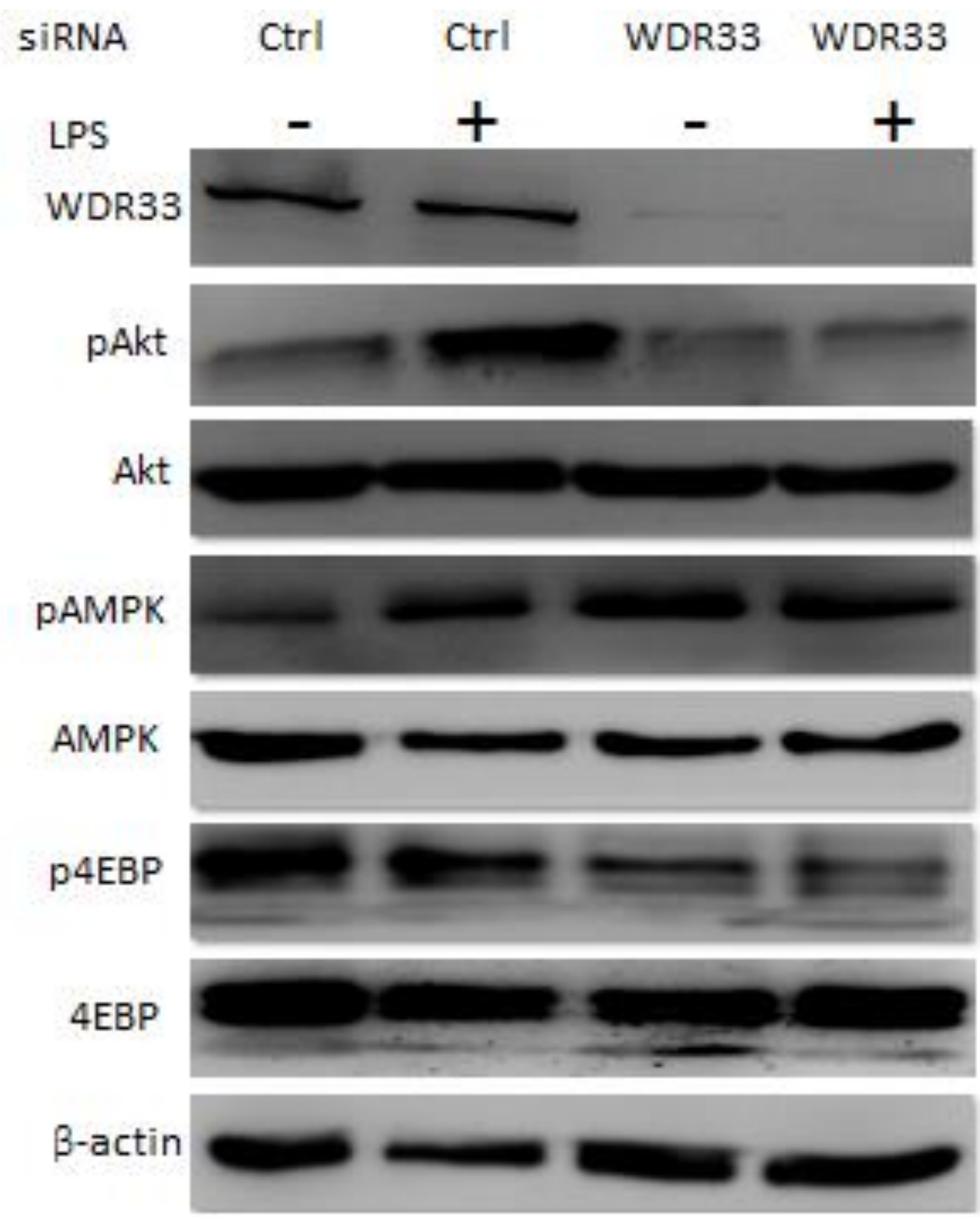


Figure 6.10. Knocking down polyadenylation factor (Wdr33) inhibits PI3K/Akt/mTOR signalling pathway. RAW264.7 cells were treated with control siRNA or siRNA against WDR33. Cells were then lysed and Western Blot performed. The images are representative of two independent replicates.

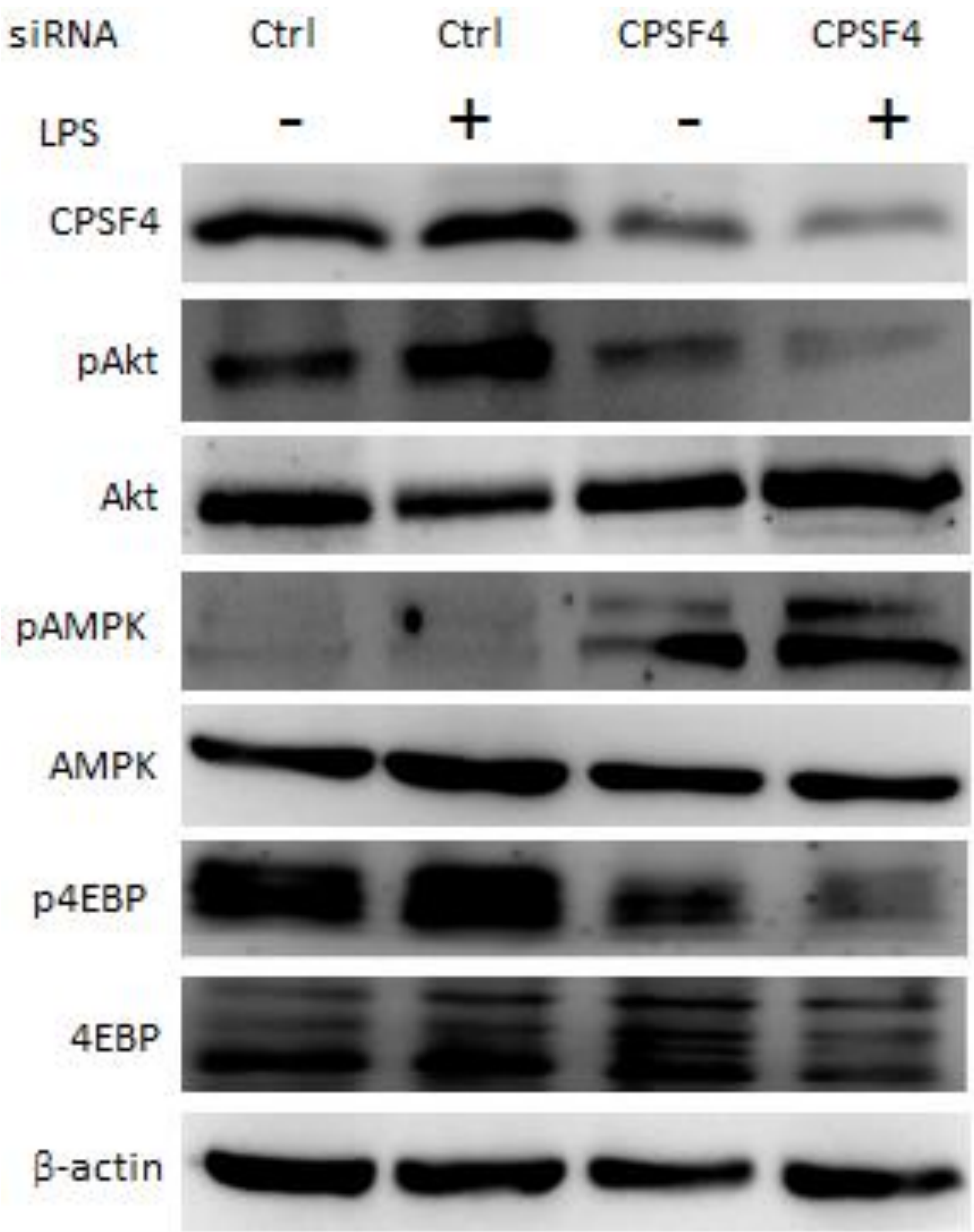


Figure 6.11. Knocking down polyadenylation factor (Cpsf4) inhibits PI3K/Akt/mTOR signalling pathway. RAW264.7 cells were treated with control siRNA or siRNA against WDR33. Cells were then lysed and Western Blot performed. The images are representative of two independent replicates.

Conclusion

This chapter focused on the effect of cordycepin on the PI3K/Akt/mTOR signal transduction pathway and the role of this pathway in mediating the inflammatory response in RAW264.7 macrophage-like cell line. Our results have shown that cordycepin has an inhibitory effect of on the PI3K/Akt/mTOR signal transduction pathway. Western blotting in **Figure 6.1** showed that cordycepin reduces the LPS-induced phosphorylated Akt meaning that it deactivates this kinase. It also showed that it reduces the phosphorylated level of 4EBP meaning that it inhibits mTORC1 as 4EBP is phosphorylated downstream of this mTOR complex, potentially as a consequence of reduced Akt activation. On the other hand cordycepin increases the phosphorylated level of AMPK which means this kinase is activated and expected to inhibit the mTOR pathway since AMPK is considered as a negative regulator for this signalling pathway as I mentioned earlier in this chapter. However, more positive and negative controls for example insulin and torin 1 need to be used to further confirm our results. Furthermore, the results showed that cordycepin affected the localization of pAkt in RAW264.7 cells. The results showed that this protein was distributed in both the nuclear and the cytoplasmic compartments in untreated and LPS-stimulated cells. While it was predominantly cytoplasmic when the LPS-stimulated cells were treated with cordycepin **(Figure 6.2)**. Cordycepin had the same effect on the localization of both pAkt (Ser473) and pAkt (Thr308) which are the mTOR and PDK1 phosphorylation sites respectively. However, it did not show any effect on the localization of total Akt protein. Since Akt can potentially phosphorylate some thousands downstream substrates, it is not surprising that Akt activity can be detected in both cytoplasmic and nuclear compartments on mammalian cells (Rosner 2007). It has been found that Akt is highly expressed in certain types of tumours. The nuclear localization of this kinase is associated with its activation and tumour invasion (Vask0 2004). Using human macrophages to test the effect of cordycepin on the expression of pAkt (Ser473), showed that cordycepin also strongly reduced the LPS-induced phoshorylation of Akt in these primary cells **(Figure 6.3)**. Our immunocytochemistry experiment showed that cordycepin sequestered mTOR in unidentified cytoplasmic compartment of the LPS-stimulated macrophages. Whereas, it was distributed equally in both compartments in untreated and LPS-stimulated cells. Some studies showed that mTOR is sequestered in the stress granules (Heberle 2015), and it might be the case in the inflammatory system as the immunocytochemistry experiment showed **(Figure 6.4)**. It has been found that short and prolonged treatment of HEK293 and NIH3T3 with rapamycin leads to dephosphorylation and cytoplasmic localization of nuclear mTOR subunits (Rosner 2008). Accordingly, the cytoplasmic localization of pAkt (Ser473), pAkt (Thr308) and mTOR could be consistent with the reduction of the level of phosphorylated Akt and phosphorylated 4EBP and further confirm the inhibitory effect of our compound on the PI3K/Akt/mTOR pathway. Immunofluorescence also showed that pAMPK was mainly localized in the nuclear compartment in the presence of cordycepin. However, it was both nuclear and cytoplasmic in untreated and LPS-stimulated macrophages **(Figure 6.5)**. It has been reported that AMPK is localized in the nuclear compartment when it is activated due to stress-independent stimuli (Kodiha 2007). This could further strengthen our finding that cordycepin activates AMPK by increasing the phosphorylated level of this kinase. The effects of modulation of the PI3K/Akt/mTOR signal transduction pathway on the inflammatory gene expression were studied. I used chemical compounds which are known by their ability to activate or inhibit different kinases in this pathway. The compounds that were used in this study are: Akt inhibitor, rapamycin and torin1 which are mTOR inhibitors, AMPK activator, compound C which is AMPK inhibitor, MEK inhibitor and PI3K inhibitor. RTqPCR data showed that most of these compounds repressed the relative mRNA expression of Il1b, Acod1 and Ticam, but not Tnf **(Figure 6.7)**. These data demonstrate that inhibition of this signalling pathway leads to repressing the inflammatory gene expression. Accordingly, cordycepin could produce its anti-inflammatory effect, at least in part, through inhibition of this pathway. The inhibitory effect of cordycepin on PI3K/Akt/mTOR signal transduction pathway does not fully explain the mechanism of action of cordycepin. Inhibition of this pathway by any of the above compound did not repress the relative mRNA level of Tnf. We have seen that Tnf expression was not repressed by adenosine **(Figure 4.3)** or by CPSF siRNA knockdown in the previous chapters **(Figure 5.5)**. This might indicate that Tnf is an early response gene or it is repressed by a different pathway which is sensitive to cordycepin, but not to adenosine, CPSF4 siRNA knockdown or inhibition of PI3K/Akt/mTOR signal transduction pathway. Investigating the effect of the PI3K/Akt/mTOR signalling chemical modulators on the nuclear localization of NF-κB in RAW264.7 marophage-like cells showed that some of these compounds (Akt inhibitor and rapamycin) reduce the nuclear localization of NF-κB, but not as strongly as cordycepin **(Figure 6.8)**. This could explain the weaker effect of these compounds on the inflammatory gene expression compared to cordycepin. The effect of the compounds on the proteasomal degradation of IκBα was also studied. The western blotting results showed that IκBα was degraded after 15 minutes of LPS stimulation in cells treated with all these compounds **(Figure 6.9)**. In addition, the results showed that IκBα was still degraded after 30 minutes of LPS stimulation. In contrast, IκBα was resynthesized after 30 minutes of LPS stimulation in cordycepin-treated cells. This could explain the less significant effect of these compounds on the nuclear localization of NF-κB compared to cordycepin. To characterize a potential link between polyadentlation and PI3K/Akt/mTOR signal transduction pathway, CPSF4 and WDR33 have been depleted and the effect on this pathway has been studied. Western blotting data showed that siRNA knockdown of both CPSF4 and WDR33 was associated with reducing the phosphorylated levels of Akt and 4EBP and increasing the phosphorylated level of AMPK **(Figure 6.10 and Figure 6.11)**. These results revealed that polyadenylation is upstream of PI3K/Akt/mTOR signal transduction pathway as inhibition of polyadenylation by knocking down two of the 3' mRNA processing factors or by cordycepin leads to inhibition of this signalling pathway. Therefore, cordycepin may produce its effect on signal transduction pathway through its effect on polyadenylation factor complexes. Thus, our results provide a rationale for studying polyadenylation as a target for inflammatory diseases.

7. Discussion and concluding remarks

As described in the introduction (**Section 1.4.1**), the effect of cordycepin triphosphate on polyadenylation is well established at the molecular level. The presented study investigated the anti-inflammatory effect of cordycepin in macrophages. Among my key findings, cordycepin was reported to reduce the nuclear localisation of NF-κB and inhibit the PI3K/mTOR signal transduction pathway in macrophages. In this project, I set out to explore the connections between the polyadenylation, NF-κB localisation and signal transduction in order to identify the molecular mechanism of action of cordycepin. Cpsf4 and Wdr33 knockdown remarkably reduced the LPS-induced inflammatory mRNAs, LPS-induced nuclear localization of NF-κB and the activity of PI3K/mTOR/Akt signalling. These data strongly suggest that all the anti-inflammatory effects of cordycepin are mediated through its effects on the polyadenylation machinery. Interestingly, our data suggest that following oral administration cordycepin is delivered to tissues as 3’deoxyinosine, and the latter is re-aminated to generate cordycepin by unknown enzymatic pathway (Jong Bong Lee, unpublished data). Examining the effect of 3’ deoxyinosine in RAW264.7 macrophages, shows that this compound represses the LPS-induced inflammatory gene expression.

7.1 The anti-inflammatory effect of cordycepin

Our group has previously reported that cordycepin has an anti-inflammatory effect in airway smooth muscle cells. This effect was found to be probably mediated by inhibition of polyadenylation (Kondrashov 2012), making this process a putative target for novel anti-inflammatory drugs. A working inflammatory system was established in the RAW264.7 murine-like macrophages and LPS was chosen as an inflammatory stimulus **(Figure 3.1)**. It has been reported that the induced expression of inflammatory genes by LPS stimulation in RAW macrophages is linked to Toll-like receptor 4 signalling-associated transcription factors. Moreover, post-transcriptional mechanisms are also involved. LPS increases the half-lives of Tnf and other inflammatory mRNAs by lengthening their poly (A) tails (Crawford 1997).

In this system, the anti-inflammatory effect of cordycepin was characterized on a genome wide scale (Raj Gandhi, 2017, PhD thesis). TNF-α and IL1β are pro-inflammatory cytokines produces by activated macrophages and they play a central role in inflammation-associated diseases and pain (Zhang 2007). CXCL2 (chemokine) was also found to be involved in the development and progression of neuropathic pain (Zhang 2013). Since synovitis (inflammation of synovia) is one of the characteristic findings in OA and is thought to be the leading cause of OA-associated pain, the expression of Tnf, Il1b and Cxcl2 mRNAs were tested in macrophages. As another inflammatory marker, I also tested immunoresponsive gene 1 (Irg1 or Acod1) which is highly expressed in mammalian macrophages during inflammation and it links cellular metabolism with immune defence (Michelucci 2013). LPS-induced expression of Tnf, Il1b, Acod1 and Cxcl2 were repressed when the macrophages treated with cordycepin one hour before or 10 minutes after LPS stimulation, suggesting that its effects are clearly very fast, and are thus likely to be primary in nature **(Figure 3.2** and **Figure 3.3)**. However, the level of Gapdh and Hprt1 was not affected indicating that cordycepin does, therefore, specifically affect the expression of inflammatory mRNAs and not of control mRNAs, even though both of these classes of mRNAs are known to be subject to polyadenylation **(Figure 3.2** and **Figure 3.3)**. However, the anti-inflamatory effect of cordycepin will need to be checked in primary macrophages.

Despite the accumulating evidence for the anti-inflammatory effect of cordycepin, our understanding of the underlying mechanisms is still limited. I set out to assess the effect of cordycepin on the expression of genes encoding proteins involved in TLR4 pathway and signal transduction. Cordycepin represses the LPS-induced expression of Peli1, Inos2, Dusp4, Myc and Sgk1. It also represses the levels of Tak1, Traf6 and Trib1 expression which were not induced by LPS. This could indicate that cordycepin regulates the inflammatory response in macrophages at the long term as well as its indicated effect on the early stage of inflammatory response. Therefore, these results motivate further testing the effect of cordycepin on important mediators in TLR4 pathway inflammatory response and signal transduction pathways **(chapter 5 and chapter 6)**.

To extrapolate our findings in RAW264.7 murine-like macrophages to human macrophages, human blood-derived monocytes were differentiated into macrophages using M-CSF in the presence and absence of cordycepin. The results **(Figure 3.6)** showed that cordycepin reduces the maturation of these inflammatory cells. This suggests that cordycepin affects the inflammatory response in the early stage, as this model mimics the early stage of inflammatory response when the monocytes are recruited from the blood stream to the site of injury and then differentiated into macrophages. Differentiation of monocyte to macrophage is a major event in the progression of inflammation. Inhibition of this phenomenon forms first line of defence in the prevention and treatment of inflammation-associated disorders (Mohana 2015).

7.2 Cordycepin metabolism and other nucleosides

In **Chapter 4**, I studied the anti-inflammatory effect of cordycepin from different commercial sources. The contaminants in each source were assessed and their contribution to cordycepin's activity was determined. Cordycepin from three different companies (Merck, Tocris and Carbosynth) was sent to MS and MS/MS analysis. It has been found that Merck cordycepin is contaminated with 2% aminocordycepin, Tocris cordycepin was contaminated with 2% adenosine and Carbosynth cordycepin was found to contain contaminants (Catherine Otori, unpublished data). Cordycepin from all sources has comparable anti-inflammatory effect in RAW264.7 macrophages-like cells. The LPS-induced mRNA expression of Tnf, Il1b, Acod1 and Cxcl1 were repressed in response to cordycepin from each of the above sources **(Figure 4.1** and **Figure 4.2)**. In order to characterize the role of the main contaminants (aminocordycepin and adenosine) in producing the anti-inflammatory effect of cordycepin, their effect on inflammatory gene expression was tested **(Figure 4.1** and **Figure 4.3)**. Our results suggest that cordycepin, and not any of the contaminant, produces the anti-inflammatory effect.

Some studies indicated that cordycepin can exert its activity through stimulating adenosine receptor (Nikamura 2006 and Kitamura 2011). To exclude that cordycepin is acting extracellularly, for instance by binding to adenosine receptors, the RAW264.7 macrophages-like cells were pre-incubated with the adenosine import inhibitor nitrobenzylthioinosine (NBTI). As shown in **figure 4.4**, nitrobenzylthioinosine completely blocked the effect of cordycepin on reducing the LPS-induced Tnf, Il1b, Acod1 and Cxcl2. Similarly, iodotubericidin (ITU), an inhibitor of adenosine phosphorylation, also blocked the effect of cordycepin on the inflammatory gene expression. These data show that cordycepin acts intracellularly and as a nucleotide, rather than a nucleoside. Kodrashov et al. showed that these inhibitors completely abrogate the effect of cordycepin on the induction of inflammatory mRNAs in air-way smooth muscle cells (Kondrashov 2012). These results indicate that cordycepin has to enter the cell using the adenosine transporter and be phosphorylated by adenosine kinase to affect the inflammatory response.

Studying the pharmacokinetic properties of cordycepin in animal models showed the absence of 3’ deoxyadenosine in the blood stream after oral administration and rapid unusual elimination phase after intravenous administration. Interestingly, we found that 3’ deoxyinosine, previously considered as an inactive metabolite of cordycepin, is bioavailable after oral administration and exhibited typical plasma concentration-time profile after oral administration. In addition, the results also showed that RAW264.7 macrophages were able to generate cordycepin triphosphate from 3’deoxyinosine, suggesting that 3’deoxyinosine could be a potential active metabolite of cordycepin (Jong Bong Lee, unpublished data) **(Figure 7.1)**. I sought to investigate the anti-inflammatory effect of 3’deoxyinosine in RAW264.7 cells. **Figure 4.6** showed that 3’deoxyinosine represses the LPS-induced relative mRNA expression of inflammatory genes, however, it was less active and less potent than cordycepin **(Figure 4.5)**.

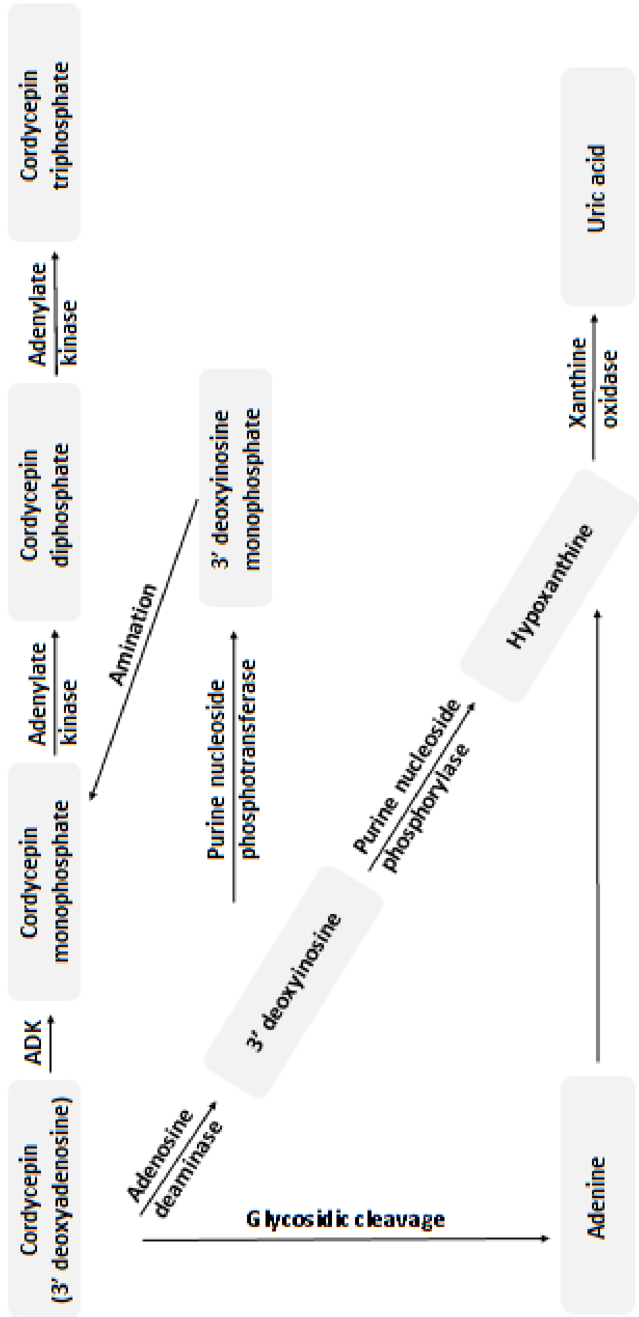


Figure 7.1. The proposed metabolic pathway of cordycepin. This scheme shows the possible reamination of 3’ deoxyinosine monophosphate into 3’deoxyadenosine monophosphate.

7.3 The effect of cordycepin on the localization of NF-κB and polyadenylation factors

The anti-inflammatory effect of cordycepin is well documented, and a range of mechanisms have been proposed to explain its effects. In **chapter 3**, it was confirmed the anti-inflammatory effect of cordycepin in RAW264.7 murine-like macrophages. In **chapter 5**, I focused on investigating the mechanism by which cordycepin represses the LPS-induced inflammatory gene expression. In airway smooth muscle cells, it has been found that the cordycepin-induced reduction in gene expression is entirely post-transcriptional for CXCL1 and CCL2. However, IL8 pre-mRNA levels were severely reduced by cordycepin in these cells (Kondrashov 2012). To determine whether the effect of cordycepin is post-transcriptional in RAW264.7 macrophages, the levels of unspliced premRNA in cordycepin and LPS-treated cells were measured using RT-qPCR. **Figure 5.1** showed that cordycepin repressed the induction of unspliced Tnf and unspliced Il1b premRNAs, suggesting that it has an effect at the transcriptional level.

I hypothesized that cordycepin might affect transcription factors which have a role in the inflammatory transcriptional output. Since NF-κB is considered the master regulator of inflammatory gene expression, the effect of cordycepin on the activation of NF-κB pathway has been tested. **Figure 5.2** showed that P65 subunit is mostly localized in the cytoplasm in DMSO-treated cells, while it is nuclear after one hour of LPS stimulation. However, in cordycepin-treated macrophages, P65 is mainly localizes in the cytoplasmic compartment, indicating that cordycepin reduces the nuclear localization of NF-κB. To investigate the extent and the time at which P65 translocates from and to the cytoplasm, the cytoplasmic and nuclear fraction are separated from each other and western blotting was done for P65 subunit. In the cytoplasmic fraction, P65 has mostly disappeared after 30 minutes of LPS stimulation in DMSO-treated cells while it stayed in the cytoplasmic compartment at the same time point in cordycepin-treated cells. On the other hand, there is less nuclear P65 in the cordycepin-treated cells than DMSO-treated cells **(Figure 5.3)**.

To gain insight into the mechanism by which cordycepin reduces the nuclear localization of NF-κB, its effect on IκB degradation was assessed. The data in **figure 5.4** showed that cordycepin does not affect the proteasomal degradation of IκB, suggesting that the effect of cordycepin on reducing the nuclear localization of NF-κB is not IκB degradation-mediated.

NF-κB regulates the expression of over 500 genes involved in human diseases, and the NF-κB signalling pathway has become a target for pharmacological intervention; however, no NF-κB blocker has been approved for human use. A major challenge is the development of NF-κB inhibitors based on their ability to target specific pathways or cells in different diseases, thereby avoiding undesired side effects (Hwang 2013). However, a previous work done by our laboratory showed that the effect of cordycepin on NF-κB is cell specific. Cordycepin does not affect the TNF-induced nuclear localization of NF-κB in air-way smooth muscle cells. It also does not have any effect on the degradation of IκB in this system (Kondrashov 2012). The western blotting of the cytoplasmic fraction **(Figure 5.3)** showed that P65 disappears from the cytoplasm at 15 minute time point and appears again at 30 minute time point. Although this effect is still not well understood, I hypothesized that cordycepin might affect the binding of this transcription factor to the DNA-binding site. Researchers suggested MafK (Maf protein transcription factor) as an important regulator of NF-κB activation. It regulates the DNA-binding activity of NF-κB by enhancing p65 acetylation and recruiting CBP. MafK may enhance p65-CBP complex formation, thereby increasing p65 acetylation (Hwang 2013). Phosphorylation often precedes other post-translational modifications such as acetylation and ubiquitination. For example, p65 phosphorylation at serine 276 facilitates interaction with CREB-binding protein (CBP)/p300 and diminishes histone deacetylase 1 (HDAC1) binding, leading to p65 acetylation (Pejanovic 2012). Future work is still needed to investigate the effect of cordycepin on the post-translational modifications of p65 and thereby the affinity to the DNA-binding site.

To characterise a potential role of polyadenylation factors in inflammatory gene expression, Wdr33 and Cpsf4 were knocked down using siRNA transfection. This reduced the induction of pro-inflammatory cytokine mRNAs (Tnf and Il1b). The knockdown of Wdr33 had the stronger effect on inflammatory gene expression than Cpsf4 knockdown. As can be seen in **figure 5.5** Cpsf4 knockdown reduced the Il1b relative mRNA expression, while Wdr33 knockdown was sufficient to reduce the expression of both Tnf and Il1b **(Figure 5.6)**. This demonstrates the sensitivity of inflammatory gene expression to the inhibition of polyadenylation. Previous work in our laboratory showed that knocking down Papola was associated with reduction in the inflammatory gene expression in air-way smooth muscle cells (Kondrashov 2012). This effect was not apparent in RAW264.7 cells in my hands (results not shown). It appears likely that different factors are limiting in different cell types and/or the knockdown efficiency and depletion of the protein were not similar in these experiments.

LPS-induced NF-κB nuclear localisation was also reduced when polyadenylation factors were depeleted **(Figure 5.7)**, indicating a similar effect to cordycepin on the nuclear localization of P65 subunit. **Figure 5.7** also shows that Wdr33 knockdown has the stronger effect in reducing the LPS-induced P65 nuclear localization than Cpsf4 knockdown. It is not unlikely that the difference is due to better knockdown of Wdr33 than Cpsf4 as can be seen in **figure 5.5** and **figure 5.6**. It has been found that CPSF4 positively regulates the expression of PTGS2 (COX2), which functions as an inflammatory factor which has high expression in many different kinds of tumours, in both *in vitro* and *in vivo* models. This effect was attributed to the activation NF-κB pathway. CPSF4 was reported to enhance NF-κB-induced PTGS2 expression as a direct transcription factor (Yi 2016). CBP has been shown to regulate the proliferation and apoptosis of cancer cells in synergism with CPSF4 and potentially explained the poor prognosis in patients with highly CPSF4-expressed cancer. CBP recruits, interacts with and acetylates CPSF4 at gene promoter regions to synergistically regulate downstream gene transcription and tumour cell proliferation (Tang 2016). This further strengthens our hypothesis that cordycepin reduces the nuclear localization of NF-κB through affecting its binding ability to the DNA-binding sites. To sum up, these findings indicate that polyadenylation factors are upstream regulator of the NF-κB activation pathway in macrophages.

It has been found that LPS stimulation of RAW264.7 macrophages upregulates the expression of CstF. Genome wide analysis in CstF2 (Cstf64) overexpressing RAW264.7

macrophages cells showed that elevated levels of CstF64 altered the expression of 51 genes, 14 of which showed similar changes in gene expression with LPS stimulation (Shell 2005). I sought to clarify the effect of cordycepin on the expression and localization of 3’ processing factors (CPSF4 and WDR33). The results showed that cordycepin reduces the nuclear localization of these two polyadenylation factors in RAW264.7 murine-like macrophages **(Figure 5.8** and **Figure 5.9)**. Researchers have shown that Symplekin and CPSF2 are co-localized in the cytoplasmic compartment of oocytes. They concluded that polyadenylation factors are involved in 3’ end processing of pre-mRNA in the nucleus as well as in regulating polyadenylation in the cytoplasm (Hofmann 2002). Drosophila symplekin is preferentially localized to histone locus bodies (HLBs) during S-phase in endoreduplicating follicle cells when histone mRNA is synthesized. Symplekin translocates to the cytoplasmic compartment, in addition to the nucleoplasm, at the end of endoreplication. The RNA binding protein, ypsilon schachtel, is the main determinant of Symplekin localization. CPSF3 and a number of mRNAs showed the same pattern of differential localization (Tatomer 2014). However, at present the mechanism by which a reduction of polyadenylation factors in the nucleus affects nuclear localisation of NF-κB is unknown.

Indeed, we are far from having a comprehensive understanding of the regulation of NF-κB activity by the polyadenylation machinery in macrophages. However, I would like to propose several mechanisms that explain my findings:

1. Since the proteomic analysis of the cordycepin arrested cleavage complex reveals that the this complex contains a large number of other proteins including chromatin

modifying proteins (Shi 2009), these could possibly affect the anchoring of NF-κB to the chromatin, leading to a failure of NF-κB retention in the nucleus. IκB is rapidly re-synthesised in murine macrophages, and could contribute to the return of NF-κB to the cytoplasm.

2. Cordycepin might inhibit the post-translational modification of NF-κB by inhibiting the activity of CBP, for example, and thus affecting the binding of NF-κB to its DNA-binding sites. Future work is needed to choose between these hypothesis wich could include proteomic analysis of relevant complexes.

7.4 The effect of cordycepin on signal transduction pathway

A wide range of mechanisms have been proposed to explain the anti-inflammatory effect of cordycepin. Previous findings indicated that cordycepin affects signal transduction pathway. Studies showed that it has inhibitory effects on PI3K/Akt and mTOR pathway (Takahashi 2012, Mayer 2006 and Wong 2010). On the other hand, it has been shown that this compound positively regulates AMPK (Wong 2010). In **chapter 6**, the effect of cordycepin on PI3K/Akt/mTOR signal transduction pathway in RAW264.7 murine macrophages was investigated. The western blotting results showed that cordycepin reduces the phosphorylation form of both Akt and 4EBP, while it increases the phosphorylated level of the negative regulator, AMPK, in RAW264.7 cells **(Figure 6.1)**. The above results confirm the inhibitory effect of cordycepin on PI3K/Akt/mTOR signalling.

There are three Akt isoforms (Akt1, Akt2 and Akt3). All the three isoforms undergo membrane recruitment and then bind to the PIP3 produced by PI3K. This binding leads to the phosphorylation and activation of Akt. The three isoforms contain similar phosphorylation sites: Thr 308 (Akt1)/Thr 309 (Akt2)/Thr 305 (Akt3) and Ser 473 (Akt1)/Ser 474 (Akt2)/Ser 472 (Akt3). The threonine residue is phosphorylated by PDK1, while the serine residue is a target for mTORC2. Phosphorylation at both sites leads to full activation of Akt, however Thr308 p-Akt can phosphorylate some of its substrate without phosphorylation at the serine residue (Martelli 2012).

PI3K and Akt shuttle between the nucleus and cytoplasm upon stimulation with various stimuli. Most of phosphoinositol lipid metabolic enzymes and PI3K signalling machinery occur in the nucleus including PDK1, Akt and PTEN. In addition to nuclear membrane, phosphoinositides also associate with the nuclear speckles thus regulating pre-mRNA splicing, chromatin structure and mRNA export (Okada 2008). Nuclear Akt directly binds Aly (nuclear speckle protein implicated in mRNA export) and phosphorylates it. Depletion of Aly markedly blocks cell cycle progression and reduces cell growth and mRNA export (Okada 2008). Our data suggest that the phosphorylated Akt at both sites (serine 473 and threonine 308) was mostly nuclear when the RAW264.7 macrophages-like cells are stimulated with LPS. However, it was localized in the cytoplasmic compartment when the LPS-stimulated cells were treated with cordycepin as shown in **figure 6.2 A** and **B**. On the other hand, total Akt localization does not change in response to cordycepin treatment **(Figure 6.2 C)**. The above results suggests that either the phosphorylated, but not unphosphorylated, Akt changes its localization in response to different stimuli (LPS only versus LPS plus cordycepin) or Akt is not phosphorylated in the nucleus anymore. In osteoblasts, treatment with different stimulatory growth factors shows a maximal nuclear migration of Akt. This increase in the nuclear Akt level is paralleled by an increase of its enzymatic activity. Conversely, inhibiting PI3K/Akt signalling using non specific PI3K inhibitor (LY294002) blocks the nuclear localization of Akt (Borgatti 2000). This indicates the importance of nuclear localization of Akt for the activation of PI3K/Akt cascade. In parallel, I wanted to assay if cordycepin has the same effect on the localization of Akt in human blood-derived macrophages. However, cordycepin so severely reduced the LPS-induced expression of pAkt (Serine 473) in human macrophages, that it was impossible to study its localisation **(Figure 6.3)**. **)**. This suggest that the primary human cells are even more sensitive to cordycepin than the RAW264.7 mouse macrophage cell line.

The effect of cordycepin on the localization of mTOR was assessed in RAW264.7 cells. Interestingly, the immunofluorescence experiments shows that mTOR is distributed throughout both the nuclear and cytoplasmic compartments in the LPS-stimulated cells. Cordycepin was shown to sequester mTOR in the cytoplasmic compartment. **Figure 6.4** shows that mTOR seems to be localized in cytoplasmic granules in cordycepin-treated macrophages. I hypothesized these cytoplasmic granules could be stress granules. Stress granules (SGs) are cytoplasmic mRNA-protein complexes that form upon the inhibition of translation initiation and promote cell survival in response to environmental insults (Sfakianos 2018). SGs are considered as a key regulators of mTOR activity. Upon stress condition, mTOR is sequestered to the SGs leading to blunting its activity. Moreover, mTOR reactivation is directed through SG disassembly (Takahara 2012). Astrin is known as an essential negative regulator of mTOR signalling. It recruits raptor (mTORC1 component) to the stress granules thereby preventing its hyperactivation (Thedieck 2013). By identifying cordycepin as a negative regulator of mTOR signalling in RAW264.7 macrophages-like cells, I hypothesized that it might sequester mTOR into the stress granules. Immunofluorescence was done using two antibodies one for mTOR and another for a well-known stress granules protein, T cell intracellular antigen-1 (TIA-1). **Figure 6.4 B** shows possible co-localization between mTOR and TIA—1, suggesting that mTOR could be sequestered to the stress granules upon cordycepin treatment in RAW264.7 cells. Lysosomes and Golgi apparatus are other possible cellular organelles that have important role in mTOR signalling (Yao 2017). Further work is still needed to be done to confirm this finding.

Subsequently, the effect on the localization of the phosphorylated and total forms of the negative regulator of PI3K/Akt signalling pathway, AMPK, was assessed. The immunofluorescence experiment **(Figure 6.5 A)** showed that pAMPK is barely present in DMSO-treated macrophage-like cells. Double staining using antibodies against both pAMPK and α-tubulin shows that pAMPK is localized in the centromeres of DMSO-treated cells and does not appear anywhere else in the cellular compartments. In LPS-stimulated cells, pAMPK is evenly distributed throughout the cellular compartments. While in cordycepin-treated cells, pAMPK is mainly localized in the nucleus of RAW264.7 macrophages-like cells. However, this experiment was done only once. More independent replicates are still needed to confirm this finding. Studies showed that AMPK shuttles between the cytoplasm and nucleus by a poorly understood mechanism. Environmental stress regulates the intracellular localization of AMPK, and upon recovery from heat shock or oxidant exposure AMPK accumulates in the nuclei (Kodiha 2007). However, **figure 6.5 B** showed that the intracellular distribution of the total kinase does not change upon exposure of RAW264.7 macrophages cells to different stimuli, suggesting that cordycepin affects the localization of the phosphorylated AMPK only.

In order to test whether cordycepin may produce its anti-inflammatory activity through its effects on signal transduction pathways, modulation of these pathways were investigated. Small molecules which are known by their activity to enhance or reduce the activity of the most important kinases in PI3K/Akt/mTOR signalling were used: Akt inhibitor, mTOR inhibitors (rapamycin and torin1), AMPK activator, AMPK inhibitor (compound C), PI3K inhibitor and MEK inhibitor. The effect of these compounds on the inflammatory gene expression in RAW264.7 cells was assessed. Akt inhibitor and mTOR inhibitors (rapamycin and torin 1) show ability to repress the LPS-induced Il1b, Acod1 and Ticam, but not Tnf **(Figure 6.7 A** and **B)**. While PI3K inhibitor and MEK inhibitor were found to represses the expression of all tested genes including Tnf **(Figure 6.7 E** and **F).** These results suggest that cordycepin could produce its anti-inflammatory effect in RAW264.7 cells, at least in part, through its effect on PI3K/Akt/mTOR signal transduction cascade. However, these results cannot fully explain the effect of cordycepin on the inflammatory gene expression as not all of those compounds has the ability to repress Tnf gene expression. PI3K is highly expressed in immune cells and plays a key role in antigen receptor and cytokine-mediated immune cells development, differentiation and function (Hawkins 2014).

For instance, it has been shown that the PI3K/Akt signalling pathway plays a role in inflammatory skin diseases. Hyperactivation of different components of mTOR signalling was detected in psoriatic skin with differential localization over the epidermis. Akt and mTOR control the differentiation of keratinocytes contributing to the progression of psoriasis. It has also been shown that psoriatic cytokines induce strong mTORC1 activity in the epidermis. Deactivation of mTOR using small molecule inhibitors prevents the cytokine-mediated improper keratinocyte differentiation and restore proper stratification of the psoriatic epidermis (Buerger 2017). These findings suggest the role that PI3K/Akt/mTOR signal transduction pathway plays in inflammatory response and also the effect of its modulation on ablating it. However, at odds with our findings in RAW264.7 macrophages like cells, some studies found that rapamycin and torin 1 prevented the ant-inflammatory effect of glucocorticoids (GCs) in human monocytes and myeloid dendritic cells. GCs could not suppress NF-κB and the expression of proinflammatory cytokines when mTOR was inhibited (Weichhart 2011). It will need to be investigated if a similar effect is observed with cordycepin, as such a drug interaction would be important for informing clinical applications.

Compound C was shown to represses Tnf expression, but we know that cordycepin activates AMPK by increasing the phosphorylated form. Thus, this result does not explain the anti-inflammatory effect of cordycepin in macrophages. On the other hand AMPK activator does repress the mRNA levels of Il1b, Aod1 and Ticam, but not Tnf. Thus, cordycepin could produce its anti-inflammatory effect in macrophages, in part, by activation AMPK. It has been found that mTOR plays a central role in the pathogenesis of osteoarthritis through modulating AMPK. This leads to imbalance between catabolic and anabolic processes leading to loss of cartilage protective mechanism and ultimately cartilage destruction which is one of the main pathological finding in this inflammatory joint disorder. They also showed that chemical and genetic inhibition of mTOR reverse the prognosis of OA through reducing articular cartilage degradation, apoptosis and synovial inflammation (Zhang 2014). It has also been found that AMPK activation plays a key role in reducing the progression of emphysema by reducing inflammatory responses and cellular senescence (Cheng 2017). AMPK activators were shown to attenuate the monocytes to macrophages differentiation and thus reduce the cytokine production (Vasamsetti 2015).

The effect of repressing Akt activation could also be responsible for the anti-inflammatory effects, as Akt regulates transcriptional activity of NF-κB. Akt phosphorylates IKKα on threonine 23. This event is prerequisite for the phosphorylation of p65 at serine 534 by IKKα and β (Bai 2009). Furthermore, IKK is shown to phosphorylate mTOR at serine 1415 downstream of Akt to promote mTORC1 activity, suggesting IKK as an effector of Akt in promoting mTORC1 activity (Dan 2014). In RAW264.7 macrophages-like cells, I assessed the effect of modulation PI3K/Akt/mTOR on NF-κB activation pathway. Only an Akt inhibitor and rapamycin reduced the nuclear localization of NF-κB, however their effect was not as strong as cordycepin. The other compounds including torin 1, AMPK activator and compound C did not affect NF-κB localization in macrophages **(Figure 6.8)**. These results suggest that PI3K/Akt/mTOR signal transduction pathway could be an upstream effector of NF-κB activation in macrophages. Moreover, they suggest that cordycepin reduces the nuclear localization of NF-κB perhaps through its effect on PI3K/Akt/mTOR signal transduction pathway, but it does so more effectively than the other inhibitors tested. Cordycepin could possibly inhibit the Akt-mediated phosphorylation of NF-κB and thus affects its binding to the κB promoter.

In parallel, I wanted to assay whether the above compounds affect the proteasome-mediated degradation of IκBα in macrophages. All the knowm modulators of signal transduction affected the IκBα levels in a different way to cordycepin **(Figure 6.9)**. The IκBα was degraded at 15 minute time point after LPS stimulation and the bands had completely disappeared at 30 minute time point. Thus, the effect of cordycepin on IκBα levels is unique. In cordycepin-treated macrophages, IκBα is resynthesized again after 15 minutes of LPS stimulation. However, the resynthesis of IκBα is attenuated in cells treated with signal transduction modulators. This could be attributed to the effect of these compounds on translational efficiency, as they interfere with mTOR pathway which is considered the key regulator of cellular protein synthesis. I hypothesised that the inhibition of resynthesis of IκBα could explain the weak or lack of effect on the nuclear localization of NF-κB when the macrophages were treated with these compounds.

In chapter 6, my overall aim was to characterize the role of polyadenylation in regulating PI3K/Akt/mTOR signalling in RAW264.7 macrophages-like cell line. To this end, two important polyadenylation factors were knocked down using siRNA. The effect of depleting these factors on the level of key kinases in PI3K/Akt/mTOR signalling was assessed. Interestingly, **figure 6.10** shows that knocking down Cpsf4 in macrophages was associated with reduction in the level of phosphorylated Akt and phosphorylated 4EBP, while the level of phosphorylated AMPK was enhanced. These results indicate that depleting Cpsf4 in macrophages has an inhibitory effect on PI3K/Akt/mTOR signalling cascade. Additionally, another polyadenylation factor was knocked down, Wdr33. Similar to Cpsf4 knock down, Wdr33 depletion resulted in reducing the level of phosphorylated Akt and phosphorylated 4EBP, but the phosphorylated AMPK level was increased (**Figure 6.11**). Thus, depleting Wdr33 in macrophages has inhibitory effect on PI3K/Akt/mTOR signal transduction pathway. Altogether, these results suggest that polyadenylation could be an upstream regulator of PI3K/Akt/mTOR signalling in RAW264.7 macrophages. These findings are consistent with the effect of cordycepin on the expression of the same kinases (**Figure 6.1**). One of the potential future experiments could be studying the effect of knocking down these factors on the localization of these kinases in macrophages, to determine whether the effect is again consistent with cordycepin's effect. Thus, I conclude that cordycepin probably produces its effect on signal transduction through its role as a polyadenylation inhibitor.

Berkovits and Mayr (2015) have discovered the role of alternative polyadenylation in regulating the cellular localization of membrane protein. Their data showed that the localization of a protein can be regulated by the message from which it is transcribed, and that the same amino acid sequence can show multiple intracellular localizations depending on the 3' UTR of the messenger RNA, demonstrating that the information needed for protein targeting is not completely encoded within the coding sequence. They also showed the role of alternative polyadenylation in the functional diversity of proteome without changing their amino acids sequences. For example, CD47 protein has been found to be located in different intracellular compartment depending on whether its transcript has short or long 3' UTR. CD47 protein translated from transcript with short 3' UTR is mainly localized in the endoplasmic reticulum (ER). On the other hand, CD47 transcript with long 3' UTR enables CD47 protein to be highly expressed in the cell surface. However, they found that both short 3' UTR transcripts and long 3' UTR transcripts were localized in the perinuclear ER. It has been found that this differential localization is governed by RNA binding protein, human antigen R (HuR) and SET. The long 3' UTR acts as a scaffold to recruit SET to the translation site where it binds to the newly translated CD47 and traffics it to the cell surface. This finding suggests that usage of alternative 3' UTR isoforms can generate differential localization patterns for functionally diverse proteins. In another study, it has been revealed that two isoforms of Cdc42 gene are differentially localized in neuronal intracellular compartments, at both mRNA and protein level. Using reporter assays and 3' UTR swapping techniques, the essential role of alternative 3' UTRs in governing the localization of alternative CDC42 protein isoforms has been established (Mattioli 2018). A potential hypothesis is therefore that the changes in localisation of for instance mTOR protein are mediated by effects on the polyadenylation of its mRNA.

Overall, our data indicate that polyadenylation is an upstream regulator of the inflammatory response in macrophages. It controls NF-κB activity as well as the expression, and perhaps the localization, of the key kinases in PI3K/Akt/mTOR signal transduction pathway. Thus, cordycepin holds promise as the lead compound for a novel class of anti-inflammatory drugs.

7.5 Concluding remarks and future work

I hypothesised that the cordycepin arrested mRNA cleavage and polyadenylation complex (the cleavage complex). This complex might sequesters co-factors, chromatin modifiers or signal transduction regulators that are required to retain NF-κB in the nucleus. Thus, the mechanistic regulation of NF-κB activity by polyadenylation could be identified. Potential experiments include western blotting of nuclear and cytoplasmic fractions and immunohistochemistry and live imaging of cells expressing fluorescently tagged p65 to characterise how cordycepin affects NF-κB localisation. Knockdown of a larger number of cleavage and polyadenylation factors and poly (A) polymerases could be done to further characterise the requirements of this complex. Determining whether polyadenylation factors knockdown and over expression affects NF-κB translocation and/or its nuclear retention and whether they affect mRNA cleavage and transcript termination could be done.

Our data suggest that cordycepin affects the nuclear localisation of the p65 NF-κB subunit by either inhibiting retention or stimulating export, presumably by changes in interacting proteins. Any cordycepin induced changes in NF-κB modifications or associated factors at the later time points after LPS stimulation can be simply a consequence of the different localisation, rather than the cause. It is therefore important to examine the effect of cordycepin on the complex in nuclear extracts during the brief time that the factor is in the nucleus in both treated and untreated cells. Therefore nuclei from LPS stimulated RAW264.7 cells around the key time point could be isolated and then doing western blot to determine which batches of nuclei contain NF-κB in similar amounts in the presence and absence cordycepin. Firstly, regulatory NF-κB modifications, such as p65 Ser276 and Ser562 phosphorylation and Lys221 acetylation could be examined. Subsequently, immunoprecipitation of p65 complex either with specific antibodies or with a tag could be done to examine the effect of cordycepin on the binding of polyadenylation factors, export factors and transcriptional co-regulators such as p300. Chromatin binding of NF-κB will be examined by qPCR.

Our data showed that PI3K/Akt/mTOR is an upstream regulator of NF-κB activity. To understand the role of signal transduction in NF-κB localization, using small molecules, siRNA knockdown and/or overexpression of key factors could be done. Small molecules as Akt, mTOR, PI3K inhibitors and AMPK activators, knockdown of PTEN as a PI3K activator and overexpression of a constitutively active Rheb as an mTOR activator could be used in order to determine which of these procedures convey resistance to cordycepin treatment. Moreover, the role of more polyadenylation factors in signal transduction could be examined to further characterize the requirement of this complex. For this purpose, knockdown and overexpression of more polyadenylation factors and poly (A) polymerases could be investigated.

In order to identify the main components of the cordycepin arrested polyadenylation complex in cells, immunoprecipitation, western blotting and chromatin immunoprecipitation can be used. This will enable us to determine whether cordycepin does indeed stabilise a complex containing Cpsf4, Wdr33 and other polyadenylation factors, whether this complex is chromatin associated and if it contains known co-factors of NF-κB such as CBP or signal transduction molecules such as mTOR and AMPK. Based on these experiments, a polyadenylation factor could be chosen to express with a Flag tag and proteomic analysis of immunoprecipitates performed. Any chromatin modifying factors and/or signal transduction proteins can then be investigated.

Studying the pharmacokinetic profile of cordycepin, revealed that no cordycepin could be detected in the blood after oral administration (Jong Bong Lee, unpublished data). However, the deaminated product of cordycepin, 3' deoxyinosine, was abundantly available. When tested on RAW264.7 macrophage cells, 3' deoxyinosine also repressed inflammatory gene expression, and the active metabolite of cordycepin, cordycepin triphosphate, was detected in cells. These data suggest that following oral administration cordycepin is delivered to tissues as 3'deoxyinosine and then regenerated by a novel nucleoside rescue pathway, which is likely to be cell type specific. Thus, determining which enzymes mediate the conversion of 3' deoxyinosine to cordycepin triphosphate is really crucial. For this purpose, knock down of candidates in a macrophage cell line and examining the effect on the accumulation of cordycepin triphosphate and correlating the bio-distribution with the known levels of enzymes in tissues could be done. Knocking down adenosine receptor/kinase and investigating the effect on cordycepin's activitiy is also important to role out cordycepin acts through adenosine receptor. I also still need to further investigate mechanism of action of 3' deoxyinosine in the inflammatory system and OA animal models. Another polyadenylation inhibitor could be tested as well. Identifying this unusual metabolic pathway could pave the way to develop this new class of anti-inflammatory drugs.

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