

ABSTRACT

OYEGUNWA, AKINBOLADE OLUKOREDE. Inhibition of Microbially-induced Inflammation by the Naturally Occurring Plant Compounds Tetra-O-methyl nordihydroguaiaretic acid (terameprocol) and Berberine. (Under the direction of Dr. Scott Laster).

The innate immune system is the first line of defense against invading pathogens. Recognition of pathogen associated molecular patterns (PAMPs) is mediated by germline encoded pattern recognition receptors (PRRs) expressed on both immune and non-immune cells. Activation of PRRs by pathogen associated patterns (PAMPs) results in recruitment and activation of immune cells such as macrophages and neutrophils. Subsequent production of inflammatory mediators, including cytokines, chemokines and lipid mediators by activated immune cells leads to various localized and systemic reactions that facilitate elimination of invading pathogens. Although, the inflammatory response is beneficial, excessive and chronic production of inflammatory mediators can also cause extensive damage to healthy host tissues and promote development of chronic inflammatory disorders. Current drug therapies for limiting microbially-induced inflammation are generally effective, however, they have been associated with adverse side effects. In addition, certain segments of the population have been shown to be unresponsive to these treatments. For these reasons, our laboratory has focused on testing and identifying new compounds that can be used to limit microbially-induced inflammation. We have recently reported that two naturally occurring plant compounds tetra-O-methyl nordihydroguaiaretic acid (terameprocol) and berberine are strong inhibitors of inflammatory cytokines and lipid mediators induced by both influenza A virus and

bacterial lipopolysaccharide (LPS). Based on these findings, the goal of our current studies is to elucidate the mechanism underlying terameprocol (TMP) and berberine-mediated suppression of cytokine production.

Our first study investigated the mechanism by which TMP inhibits production of TNF- α and MCP-1 in LPS-stimulated RAW 264.7 cells. Findings presented in chapter 2 reveal that TMP strongly inhibited NF- κ B-dependent transcription in TLR-3, -4, and -8 over-expressing HEK293 cells after stimulation with LPS, Poly (I:C) or Resiquimod (R-848). Mechanistic studies designed to determine how TMP modulates NF- κ B activation demonstrated that phosphorylation of I κ B- α and translocation of NF- κ B into the nucleus were unaltered by TMP. However, further analysis using ChIP assays demonstrated that TMP suppressed LPS-induced TNF- α and MCP-1 production by abrogating RelA binding to the promoters of these genes. Taken together, findings in chapter 2 suggest that TMP could be a useful therapeutic in limiting microbially-induced inflammation and treating RelA-mediated disorders.

In chapter three, we investigated the mechanism by which berberine suppresses influenza A virus (IAV)-induced production of TNF- α in RAW 264.7 cells. Our results indicate that berberine does not affect the accumulation of viral RNAs, which serve as stimulants for activation of PRRs. Immunoblot analysis also revealed that berberine does not prevent IAV-induced activation of mitogen activated protein kinases (MAPK) p38 and ERK1/2 or the transcription factor NF- κ B. Furthermore, we have demonstrated that the synthesis and cell surface levels of TNF- α were unimpaired by berberine. Findings in chapter 3 indicate that berberine is acting extracellularly to cause the loss of TNF- α from culture supernatants of influenza A-

infected cells. Furthermore, we show that this effect occurs in cultures infected with IAV but does not occur in cells stimulated with LPS. Overall, findings presented in chapter 3 demonstrate that berberine is a strong suppressor of IAV-induced cytokine production. However, additional mechanistic studies are needed to determine how berberine inhibits production of TNF- α in this system.

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Inhibition of Microbially-induced Inflammation by the Naturally Occurring Plant
Compounds Tetra-O-methyl nordihydroguaiaretic acid (terameprocol)
and Berberine

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Microbiology

Raleigh, North Carolina

2012

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DEDICATION

This work is dedicated to my loving mother, Mrs. Patience Abiola Oyegunwa (June 20th 1960 - December 16th 2006). You have been my sole inspiration for completing this work. Your sacrifices, courage, kindness and devotion to your family will always be cherished.

BIOGRAPHY

Akinbolade Olukorede Oyegunwa was born in Benin City Nigeria to Mr and Mrs Olapade Oyegunwa. He attended the University of North Carolina at Charlotte from 2000-2004 where he obtained a Bachelor of Science in Cellular Biology. Upon graduation he enrolled at North Carolina State University (NCSU) where he received a professional Science Master's in Microbial Biotechnology. Following completion of this degree he enrolled in the Microbiology doctoral program at NCSU. His research focuses on attenuation of microbial-induced inflammatory response by naturally occurring plant compounds.

ACKNOWLEDGMENTS

I will like to express my sincere gratitude to Dr. Scott Laster for his superior mentorship and friendship. The many lessons that you have taught me will be invaluable as I continue my career as a professional scientist. I will also like to thank Drs. Frank Scholle, Mike Sikes, Ian Petty and Sue Tonkonogy for serving on my graduate committee. Your support, insights and guidance have been greatly appreciated. I will like to express my deepest gratitude to Drs. Frank Scholle and Mike Sikes; a significant portion of this work could not have been completed without their intellectual and financial support.

Lastly, I will like to express my profound appreciation to Dr. Chad Cecil, Rush Killens, Dr. Jason Wilson, Dr. Paola Florez, Dr. Mindy Kitrell, Clayton Morrison, Farah Alayli and Kristen Crook for their friendship and support during completion of this work.

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Chapter 1. Literature Review

1. Introduction

The innate immune system is essential for protection against invading pathogens. Vast networks of highly specialized tissues, professional innate- and non-immune cells constitute an effective host defense by limiting the systemic dissemination of microbial compounds. [1-3].

Innate immune recognition by phagocytes [neutrophils, monocytes, macrophages, myeloid dendritic cells (DCs)], innate lymphocytes [natural killer (NK) cells, NKT cells, $\gamma\delta$ T cells, plasmacytoid DCs] and non-immune cells such as epithelial and endothelial cells is mediated by germline-encoded pattern recognition receptors (PRRs). PRRs expressed on innate and non-immune cells recognize a broad range of evolutionarily conserved pathogen-associated molecular patterns (PAMPs) [4, 5], formed from proteins, carbohydrates, lipids and nucleic acids from viruses, fungi, bacteria, protozoa and other pathogens [6, 7]. Several classes of PRRs have been identified; these families include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I and NOD-like receptors (NLRs). Upon encountering their targets on the cell surface or within intracellular compartments, the bloodstream or tissue fluids, PRRs initiate complex signaling cascades via recruitment of cellular adaptor molecules [6, 7]. This leads to

production of pro-inflammatory mediators including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6. Collectively, these cytokines stimulate production of acute phase proteins by hepatocytes and activate smooth muscle and endothelial cells as well as leukocytes [8-11]. Inflammatory mediators also increase the rate of vasodilation and increase vascular permeability, thereby promoting the migration of immune system cells and delivery of essential serum components to infection sites [8]. Furthermore, inflammatory cytokines have also been shown to exert various effects on the central nervous system (CNS). To this end, IL-1 β has been shown to initiate fever by modulating the activities of the hypothalamic thermoregulatory center. Additionally, IL-1 β and TNF- α have also been shown to stimulate the production of PGE₂ and several hypothalamic and pituitary peptides from hypothalamic vascular organs [8, 12]. Cooperatively, these actions facilitate elimination of invading pathogens and promote return to homeostasis [5, 9, 13, 14]. Even though these actions are beneficial to the host, excessive and protracted production of inflammatory cytokines can also be detrimental. Proteases produced during the inflammatory response have been shown to cause extensive damage to healthy host tissues and bones [15]. Additionally, cytokines and chemokines released during this process have been shown to induce toxic shock syndrome and promote the development of chronic inflammatory disorders, as well as cardiovascular diseases [15]. As a result, therapeutic agents that limit the production of these molecules have been used to treat various inflammatory disorders. Although, these therapies are generally effective, they have been associated with

the development of adverse clinical side effects. Additionally, certain segments of the population have been shown to be unresponsive to these treatments [16, 17]. For these reasons, it is imperative to identify safer efficacious remedies that can be used to attenuate the inflammatory response. Naturally occurring plant products are lead compounds in drug screening and design and have consistently supplied pharmaceutical industry pipelines for several years. The minimal side effects and numerous pharmacological activities associated with these compounds make them important drug candidates [18, 19].

This literature review describes cells and receptors (TLRs and RLRs) that initiate and coordinate microbially-induced acute inflammation. The various molecules produced during this process and their roles during the acute inflammatory response are also discussed with emphasis on pro-inflammatory cytokines. In addition, this review describes various disorders and clinical conditions associated with excessive and chronic production of pro-inflammatory cytokines. The effective of current therapies for limiting production of inflammatory cytokines and for ameliorating inflammatory disorders are also reviewed. Lastly, synthetic and natural pharmaceuticals of plant origin and their usefulness in attenuating the inflammatory response and treating inflammatory disorders are also discussed in detail.

2. Microbially-induced inflammation: A brief overview

Acute inflammation is the immediate and early response of the innate immune system to invading pathogens. Professional immune system cells such as dendritic cells, mast cells, neutrophils, macrophages, eosinophils and NK cells rapidly identify invading pathogens and initiate the inflammatory response. At the site of infection, epithelial and endothelial cells can also participate in initiation of the inflammatory response [20]. Soluble factors produced by professional and non-immune cells during this process such as inflammatory lipid metabolites, nitric oxide, cytokines and chemokines act in concert to increase vasodilation, vascular permeability and expression of cellular adhesion molecules. Collectively, these actions facilitate recruitment of immune system cells such as polymorphonuclear neutrophils (PMNs) and macrophages, as well as delivery of essential serum components to the site of infection [8-10, 13]. In addition, inflammatory mediators produced during the inflammatory response have been shown to induce various systemic effects. For instance inflammatory cytokines such as TNF- α and IL-6 have been shown to enhance the production of acute phase proteins by hepatocytes. These cytokines also act on the hypothalamus to induce fever and the release of PGE₂ from hypothalamic vascular organs [8]. Following the termination of the inflammatory response, cytokines coordinate wound healing and tissue repair by inducing production of collagenase, gelatinase and proteoglycanase from fibroblasts, synoviocytes and chondrocytes [21, 22]. Overall, the inflammatory response plays

an essential role in eliminating invading pathogens and promoting return to homeostasis [23].

Chronic inflammation is the response to unresolved acute inflammation or persistent exposure to infection. Persistence of pathogens during the chronic inflammatory response amplifies influx of immune cells such as mast cells, lymphocytes and eosinophils to infected tissues for destruction of invading microorganisms. The robust production of inflammatory mediators such as cytokines, chemokines and lipid mediators at the site of infection [24], ultimately leads to destruction of healthy host tissues and development of chronic inflammatory disorders [24].

3. Activation of the inflammatory response

3.1 Cellular receptors and activation of the inflammatory response

Recognition of foreign agents and the ensuing activation of the inflammatory response are mediated by various PRRs. PRRs are broadly expressed on various cells including polymorphonuclear phagocytes (PMN), monocytes/macrophages, dendritic cells and natural killer cells. The extensive network of PRRs present in different cellular compartments such as plasma membrane, endosomes and cytoplasm as well as extracellularly in body fluids ensures maximum protection from

systemic dissemination of microbial compounds [25-28]. TLRs 1, 2, 4, 5 and 6 are expressed on the plasma membrane and recognize a wide array of microbial products. TLRs 3, 7, 8 and 9 are expressed within intracellular compartments; these TLRs readily detect pathogen-associated nucleic acids of bacterial and viral origin [29].

Recognition of PAMPs in the cytoplasm is mediated by NOD-like receptors (NLRs) and RIG-I-Like receptors (RLRs), which consist of retinoic acid inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5). NOD1 (Nucleotide-binding oligomerization domain protein 1) and NOD2 recognize a host of bacterial agents including peptidoglycan-derived peptides, γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), while RIG-1 and MDA-5 detect viral nucleic acids that are present in the cytoplasm [30-33]. PAMPs are also readily detected by several PRRs present in body fluids including lipopolysaccharide (LPS) binding protein (LBP), complement activating MBL, pentraxin family members serum amyloid protein (SAP), pentraxin 3 (PTX3), C reactive protein (CRP), and complement components (C3 and C1q) [30-33].

Upon ligand-induced activation, PRRs play essential roles in host protection by activating key cellular signal transduction pathways that result in production of various inflammatory mediators [34]. The discussion in this review will be limited to TLRs and RLRs.

3.2 Toll-like receptors (TLR)

TLRs are evolutionarily conserved pattern recognition receptors; in fact, their presence is reported in organisms of the phylum Porifera, the sponges [35]. Toll was first discovered in 1996 as a *Drosophila* transmembrane protein with key immunological functions. These findings revealed the critical roles of Toll in recognition of fungal pathogens as well as embryo dorsal-ventral polarity formation [36, 37]. Since these discoveries, 10 human and 13 murine TLRs that recognize a wide array of microbial products from bacteria, viruses, fungi and parasites have been identified [38, 39].

Most classes of TLRs were originally discovered on professional innate immune cells such as PMNs, monocytes/macrophages, dendritic cells, NK cells, mast cells as well as lymphocytes (T and B cells). Non-immune cells including endothelial cells, epithelial cells, skin keratinocytes and fibroblasts [40] also express TLRs. Furthermore, the expression of TLRs in various tissues such as heart, lungs and brain is required for effective host antimicrobial responses. For example, TLR expression in the CNS has been shown to limit the spread of herpes simplex virus (HSV-1) [41].

TLR2 dimerizes with either TLR1 or TLR6 to detect peptidoglycan from Gram-positive bacteria such as mycoplasma or bacterial lipopeptides from mycobacteria [42-44]. TLR4 is the main mammalian PRR that mediates recognition of LPS from Gram-negative bacteria [45]. LPS in the serum is bound by LPS binding protein (LBP) and transported to CD14, a GPI-linked cell surface protein at the plasma

membrane [46]. Exactly how TLR4 recognizes CD14-LPS complexes is still incompletely understood, however, it is now clear that association of TLR4 with a co-receptor, myeloid differentiation factor 2 (MD2), which is expressed on the cell surface, is requisite for TLR4-mediated recognition of CD14-LPS complexes [47, 48]. Upon binding its ligand, TLR4 initiates signal transduction pathways from the cell surface and then translocates to the endosome where it further amplifies the inflammatory response by recruiting a different set of cellular adaptor molecules [34].

Nucleic acid binding TLRs 3, 7, 8 and 9 can detect viral nucleic acids or nucleic acid derivatives and small molecules mimetic of purine nucleotides. These TLRs are mostly expressed and function within intracellular compartments such as the endoplasmic reticulum (ER) as well as acidified compartments of the endosome and lysosome [34, 49]. TLR3 detects double stranded RNA (dsRNA) from dsRNA viruses and dsRNA generated during replication of DNA and RNA viruses [50]. Synthetic analogs of dsRNA such as polyinosinic:polycytidylic acid poly(I:C) have also been shown to activate TLR3 [51]. TLR7 and TLR8 are phylogenetically and functionally related proteins that are both expressed within intracellular compartments of the endosome and lysosome. Guanosine- and uridine-rich ssRNAs of human immunodeficiency virus (HIV) and influenza virus origins, virus-associated polyU RNAs and certain siRNAs have been identified as natural ligands of TLR7 [52-55]. TLR7 can also be activated by imidazoquinoline derivatives such as resiquimod (R-848) and guanine analogs such as loxoribine [55]. TLR8 recognizes ssRNA derived from HIV and synthetic compounds such as R-848, albeit, mice deficient in

TLR8 have been shown to effectively respond to these molecules. These findings suggest that there might be a functional redundancy between TLR7 and TLR8 [56]. TLR9 is expressed within the intracellular compartments of myeloid cells, T cells, B cells, NK cells, endothelial cells, epithelial cells and keratinocytes. TLR9 is a potent activator of the immune system in response to viral and bacterial CpG unmethylated DNA [55]. Ligand recognition by the TLR ectodomain induces a series of conformational changes that result in receptor dimerization. This extracellular crosslinking of the ectodomains engenders rearrangement of the cytoplasmic and TIR domains. Rearranged TLR domains serve as a scaffold for recruiting cellular adapters and kinases that coordinate downstream activation of key signaling pathways and transcription factors [55, 57].

3.3 Retinoic acid inducible gene-1-like receptors (RLRs)

Recognition of PAMPs in the cytoplasm is mediated by retinoic acid inducible gene-1-like receptors (RLRs) such as RIG-I and MDA-5. RLRs are structurally composed of two N-terminal caspase recruitment domains (CARD), which are required for ligand-induced signal transduction through interactions with other CARD-containing proteins [34, 58]. A large concentration of basic amino acids at the C-terminal creates an effective ligand-binding loop for 5'-triphosphates and 5'-monophosphate found on genomic RNA or replication intermediates of dsRNA and ssRNA viruses as well as dsRNA generated as replication intermediates. It is believed that the ability of these PRRs to bind 5'-phosphorylated RNA evolved in the

innate immune system as a way to discriminate between self and non-self [59]. RIG-1 and MDA-5 have been shown to activate signal transduction pathways in response to several virus families including flaviviruses, paramyxoviruses, orthomyxoviruses, rhabdoviruses and picornaviruses. Ligand-induced dimerization of RLRs also engenders conformational changes within the RLR-CARD domains that lead to activation of downstream signaling events. This rearrangement facilitates the recruitment of CARD-containing cellular signaling molecules to the RLR-CARD domain, resulting in the activation of multiple signal transduction pathways. Although some signaling molecules and kinases are shared between the RLR and TLR pathways, it appears that RLRs initiate signal transduction by utilizing distinct sets of adaptor molecules [7].

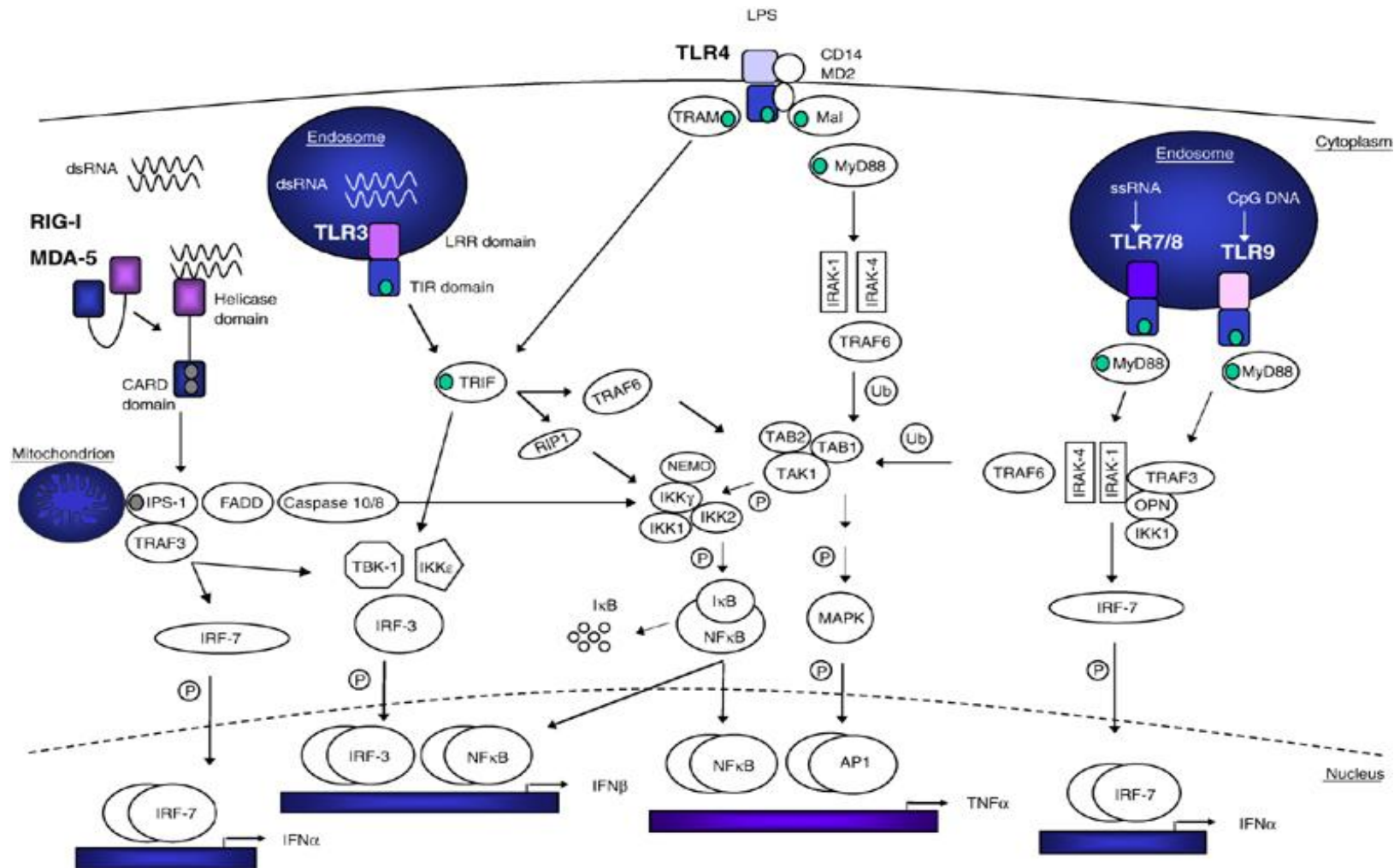
3.4 Signaling pathways activated by TLRs and RLRs

Ligand-induced activation of TLRs and RLRs results in activation of mitogen activated protein kinase (MAPK) pathways and key transcription factors such as nuclear factor- κ B (NF- κ B), activating protein-1 (AP-1) and interferon regulatory factors (IRFs). These molecules have been shown to coordinate the subsequent transcriptional activation of various inflammatory genes including, type 1 interferons, chemokines and cytokines [55, 57]. NF- κ B proteins are activated by a wide array of microbial products including, bacteria components, viruses, viral proteins, dsRNA, ssRNA and microbial DNA. Rapid activation of NF- κ B ensures a timely response to

invading pathogens via transcriptional activation of a large repertoire of genes involved in immune signaling and modulation [60]. Inducible I κ B- α phosphorylation at serine 32 and 36 is the earliest event in the common activation pathway of NF- κ B [61]. Several deletion studies have demonstrated that mutation of these sites abolishes inducible NF- κ B activation in response to diverse stimuli [61]. Phosphorylation of I κ B- α leads to the F-box/WD40 E3RSIB/-TrCP-Skp1-Cullin-F-box (SCF)-typeE3-mediated polyubiquitination of I κ B- α at lysine residues 21 and 22 [62], reviewed in [63, 64]. This event is necessary for the 26S proteasomal degradation of I κ B α and the subsequent translocation of NF- κ B into the nucleus where it initiates the transcriptional activation of several inflammatory mediators by occupying its cognate motifs on the DNA [63, 64].

Activation of MAPK pathways such as those of extracellular signal-regulated kinases (ERK)1/2, C-jun N-terminal kinases (JNK) and p38 during TLR/RLR signaling has also been shown to play a role in the production of inflammatory mediators. For instance, signaling by p38 and JNK is requisite for the selective activation of different subunits of the transcription factor AP-1 [65]. Other transcription factors activated during TLR and RLR signaling include the IRF family members. This group has been shown to play a key role in the upregulation of type 1 interferons [57], in fact; IRF3 has been identified as the master regulator of interferon signaling through a process that requires the activity of the two I κ B kinase-related kinases IKK β and TBK1 [66, 67]. Interferon produced in this manner also activates IRF7, which acts to further amplify interferon production [68, 69]. Furthermore, other

IRF family members such as IRF5 have also been shown to be vital for the induction of pro-inflammatory cytokines including IL-6, IL-12 and TNF- α in response to multiple TLR ligands [70].



Adapted from Thompson *et al*, Immunology and Cell Biology. (2007) **85**, 435-445

Figure 1. Activation of TLR and RLR signaling. TLR3 and TLR4 signal via MyD88-independent pathway using TRIF to activate IRF3 and NF-κB. TLR4, TLRs7/8 and TLR9 signal through MyD88 to activate NF-κB, AP1 and IRF7. The RLRs, RIG-I and MDA both signal via IFN-β promoters stimulation (IPS)-1 to activate IRF-7, IRF-3 and NF-κB.

4. Regulation of cytokine production

4.1 Transcriptional regulation of cytokine production

Activation of PRRs results in the production of various inflammatory molecules, including cytokines, chemokines, and lipid mediators [8-11]. The discussion in this literature review will be limited to inflammatory cytokines. Activated transcription factors initiate the transcription of cytokine genes by recruiting and acting in concert with various transcriptional coactivators [71-73]. This phenomenon is highly coordinated and regulated by various cellular proteins that facilitate chromatin re-modeling and ensure accessibility of transcription factors to their cognate motifs on the DNA [74, 75]. Additionally, the transactivating potential of activated transcription factors can be augmented or decreased via cellular modifications such as phosphorylation and acetylation. These activities have been shown to either potentiate or diminish expression of various inflammatory cytokines [76] and [77, 78]. For instance, phosphorylation and acetylation of the NF- κ B subunit p65 on different residues has been shown to significantly augment p65-mediated cytokine production [79].

4.2 Post-transcriptional regulation of cytokine production

Following transcriptional activation of cytokine genes, additional control of cytokine production can be achieved by post-transcriptional mechanisms that control

mRNA stability and degradation. Tristetraprolin (TTP) has been identified as an RNA-binding protein that plays a central role in the post-transcriptional regulation of cytokine mRNAs. To this end, it has been demonstrated that TTP promotes transcript degradation by binding to AU-rich elements (ARE) present in the 3' untranslated region of target mRNA [80]. This process has been shown to be dependent on the association of TTP with various components of the basic RNA decay machinery [81]. TTP has been shown to regulate the transcript levels of various inflammatory mediators including TNF- α [82], GM-CSF (granulocyte macrophage colony-stimulating factor) [83], IL-2 [84], IL-3 [82], IL-6 [85], c-jun [86], c-fos [81], Ier3 (immediate early response 3) [87] and IL-10 [88]. Other ARE binding proteins such as TIA-1, TIAR, CUGBP2, HuR, AUF1, AUF2, BRF1, BRF2, FXR1P and KSRP have also been shown to either associate with TTP or promote degradation of cytokine mRNA independently [89]. Additionally, it has been reported that MicroRNAs (MiRNAs) also play a role in the post-transcriptional regulation of cytokine genes [90]. MiR-16 has been shown to regulate the levels of TNF- α and COX-2 mRNA possibly by directly binding to MiRNA and ARE sites present in the 3'UTR [91]. It has also been proposed that MiRNAs may indirectly regulate stability of cytokine mRNAs by modulating the expression of ARE-components and machinery [90].

4.3 Regulation of cytokine secretion

Vesicular transport of cytokines to the extracellular milieu is coordinated by a broad range of molecular families including members of the *ras*-related superfamily of guanosine triphosphates (GTPases), the Rab and Rho proteins, actin microtubule motors, lysosomal trafficking molecules such as *lys1* and a family of intracellular membrane receptors known as SNAP (soluble NSF attachment protein) receptors (SNARES) [92-96]. Collectively, these proteins function as vital cellular transport machineries to promote trafficking and release of cytokines into the micro-tissue environment, by organizing and facilitating the interaction of carrier vesicles with cellular membranes [97].

Granulocytes such as mast cells, eosinophils and neutrophils secrete cytokines through regulated exocytosis. This process involves the constitutive synthesis and packaging of cytokines or cytokine precursors into secretory vesicles and granules for storage in the Golgi. Following ligand-induced receptor dimerization, direct signaling to secretory vesicles or granules mediates Golgi-dependent trafficking and release of preformed cytokines [98-100]. Conversely, macrophages release cytokines into the tissue environment via constitutive exocytosis. This process is dependent on ligand-receptor-mediated signaling events, which result in the transcription of cytokine mRNAs in the nucleus. Following translation, transport of cytokine proteins to the extracellular milieu is mediated by tubulovesicular structures called recycling endosomes (RE). RE containing newly

synthesized cytokine proteins interact with the cellular transport machinery for delivery of vesicular contents to the extracellular environment [101-103].

Cytokine secretion can also proceed via ER-Golgi-independent pathways. The underlying molecular details of this process are incompletely understood. However, recent investigations that have shed light on the molecular mechanisms that control IL-1 β secretion could help improve our current understanding of these events. Following transcriptional up-regulation and translation, pro-IL-1 β is made in the cytoplasm because it lacks a hydrophobic signal sequence. After cytoplasmic processing of pro-IL-1 β to IL-1 β by caspase-1, mature IL-1 β species are released into the extracellular environment through the use of membrane transporters [104, 105]. Other cytokines such as migration inhibitory factor (MIF) are also made in the cytoplasm and chaperoned to the plasma membrane for secretion by ABC transporters [106].

5. Inflammation and disease development

5.1 Microbially-induced inflammation and disease development

Inflammatory mediators produced during the inflammatory response can cause extensive damage to healthy host tissue and lead to the development of chronic inflammatory disorders [107]. Although, several inflammatory mediators

have been implicated in causing damage to host tissues, this discussion will only focus on cytokine-mediated disorders.

Microbially-induced inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8 and GM-CSF have all been shown to exacerbate disease and cause damage to healthy host tissues when produced chronically or in excessive quantities [108-110] and [111, 112]. To this end, staphylococcal enterotoxins have been shown to cause leukocyte accumulation at extravascular sites [113] and lethal shock [114] by inducing production of TNF- α . Furthermore, bacterial lipopolysaccharide (LPS) has also been shown to induce septic shock syndrome through mass activation of mononuclear cells and stimulation of inflammatory cytokines such as TNF- α , IL-1 β and IL-6. This robust production of inflammatory cytokines has been shown to cause diminished myocardial contractility, impaired perfusion, disseminated intravascular coagulation (DIC), as well as fatalities resulting from multiple organ failure [115-117]. Elevated levels of the pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β have also been shown to contribute to acute lung injury and damage of healthy host tissues in endotoxemic rats [118]. In addition IL-6, TNF- α and IL-1 β produced in response to influenza virus infections have been shown to cause vascular hyperpermeability and multiple organ failure [119]. Other studies have also demonstrated that influenza virus-induced IL-15 production promotes CD8⁺ T cell-mediated acute pneumonia and damage to lung tissue [120]. TNF- α production in response to rhinovirus (RV) infections has also been shown to play a role in exacerbation of airway disease and destruction of healthy host tissues [121].

Several studies have also linked microbial-induced inflammation to the pathophysiology of inflammatory and autoimmune disorders, including multiple sclerosis, diabetes, rheumatoid arthritis, adult respiratory distress syndrome, systemic lupus erythematosus, and inflammatory bowel disease (IBD) [122]. Microbially-induced activation of TLR pathways has also been linked to the development and progression of various chronic disorders and cardiovascular diseases including atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus, asthma, acute respiratory distress syndrome, coronary heart disease and psoriasis [123]. Long term activation of macrophages and chronic production of cytokines such as TNF- α , IL-10 and TGF- β in response to infectious agents have also been shown to play a role in the immunopathology of cryptoglandular and Crohn's anorectal fistulas [124]. Researchers have also shown that infectious agents that trigger CNS inflammation may play a role in the development and exacerbation of Alzheimer's disease [125]. Recent evidence also suggests that cytokines produced during the inflammatory response contribute to the development of several myelomas by acting as a growth factor for hematological malignancies [126-128]. As a result therapies that limit the production of inflammatory cytokines have been used to treat symptoms of these various disorders.

TNF- α is considered a key proinflammatory cytokine because of its potent immunomodulatory and pleiotropic activities [129]. TNF- α has been implicated in the pathophysiology of numerous inflammatory-related disorders, autoimmune diseases

and malignancies [130, 131]. In fact several studies have demonstrated that anti-TNF- α therapies mitigate structural damage to healthy host tissues as well as various symptoms associated with inflammatory disorders [132-136]. These experiments also revealed that therapies that limit production of TNF- α lead to the down-regulation of other inflammatory mediators such as IL-1 β , GM-CSF, IL-6 and IL-8 [132-136]. Taken together, these findings suggest that TNF- α plays a critical role in regulating the expression of various cytokines and inflammatory mediators that have been linked to the development of chronic inflammatory disorders and autoimmune diseases. As a result, TNF- α has become a key therapeutic target for treatment of these disorders. Since TNF- α is required for proper functioning of the host immune response, therapies that target TNF- α tend to be a delicate balancing act that must not sacrifice immune surveillance capabilities that are requisite for protection from various infections and malignancies.

5.2 Antibody based anti-TNF- α therapies

Currently, there are 5 FDA-approved anti-TNF- α therapies, which include monoclonal anti-TNF- α -neutralizing antibodies (infliximab, golimumab, certolizumab pegol, adalimumab) as well as a chimeric TNF- α -receptor mimetic (etanercept/enbrel). Most of these therapies antagonize TNF- α activity by binding and neutralizing secreted TNF- α molecules [137]. Infliximab is a chimeric IgG1k monoclonal antibody of high TNF- α neutralizing capacity that is specific for human TNF- α [138]. It is composed of human constant and murine variable regions.

Infliximab is widely used in the treatment of Crohn's disease, rheumatoid arthritis [139] and inflammatory bowel diseases [140]. Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75kDa TNFR linked to the Fc-portion of human IgG1. Etanercept was designed as a TNF- α -decoy receptor, which binds TNF- α molecules to prevent dimerization of TNF- α with its cognate cellular receptors [119]. It is widely used in treatment of psoriatic arthritis [141] and ankylosing spondylitis [142]. Adalimumab is the first fully human monoclonal antibody against TNF- α to gain FDA approval. It is a recombinant human IgG1 monoclonal antibody specific for human TNF- α . Structurally, Adalimumab is comprised of human-derived heavy and light chain variable regions as well as a human-derived IgG1k constant region. It is widely used in the treatment of inflammatory disorders such as rheumatoid arthritis, psoriatic arthritis ankylosing spondylitis, Crohn's disease and ulcerative colitis [143]. Golimumab is a human IgG1k monoclonal antibody that exhibits multiple glycoforms. It was created by using genetically engineered mice immunized with human TNF- α resulting in an antibody with human derived variable and constant regions. It is also routinely used in the treatment of rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis [144-146]. Certolizumab pegol is a recombinant humanized antibody Fab' fragment conjugated to a 40kDa polyethylene glycol molecule. Polyethylene glycol is unable to cross the placenta; therefore, certolizumab pegol is considered a safer alternative for pregnant women that are in need of anti-TNF- α therapies [147]. It is also used for treating rheumatoid arthritis [148] and Crohn's disease[149].

5.3 Efficacy and toxicity of anti-TNF- α therapies

Anti-TNF- α therapies have been successfully used to treat a variety of inflammatory disorders. These therapies have been shown to ameliorate structural damage to host tissues and mitigate other symptoms associated with these disorders [150]. Additionally, anti-TNF- α therapies have been shown to suppress production of inflammatory cytokines stimulated by TNF- α [150]. However, these treatments are contraindicated in certain patients who develop leukocytoclastic vasculitis (LCV) [151]. In addition, a few reports have shown that certain segments of the population become refractory and unresponsive to these therapies following long-term use [152, 153]. Although anti-TNF- α therapies are generally effective they have been linked to the development of adverse side effects and clinical conditions including severe pneumonia, meningitis, sepsis, histoplasmosis, aspergillosis, candidiasis and pneumocystis [154-156]. These conditions probably arise as a result of the immunosuppressive effects of anti-TNF- α therapies. According to FDA reports, other adverse side effects including the development of various malignancies and severe neurological disorders, production of autoantibodies, heart failure as well as fatalities have also been associated with anti-TNF- α therapies [154-156]. However, the occurrences of adverse effects associated with these therapies have been decreased with improved patient selection and administration of optimal doses [155].

5.4 Other cytokine based therapies for controlling inflammation

Other immunomodulatory cytokines have also been used to control excessive inflammation [157]. IL-10 and IL-11 have both been tested in rheumatoid arthritis

disease models; these studies revealed that these cytokines failed to confer any significant clinical improvement as compared to placebo [158-160]. IFN- β based therapies such as anovex and rebif have been shown to reduce production of inflammatory mediators. IFN- β therapies have been shown to mitigate symptoms associated with multiple sclerosis during placebo-controlled trials. In fact, a variety of IFN- β based therapies are now routinely used in the treatment of multiple sclerosis [161-163].

5.5 NF- κ B as a therapeutic target

Activation of NF- κ B is essential for transcription of the TNF- α gene [164] as well as those of other inflammatory mediators, cytokines, chemokines and cellular adhesion molecules [63, 64]. Additionally, NF- κ B has also been shown to regulate TNF- α -mediated signaling events [165]. For these reasons, NF- κ B has become a therapeutic target for the treatment of several acute and chronic inflammatory disorders. In fact, the feasibility and efficacy of NF- κ B targeted therapies have been demonstrated in several inflammatory disease models [166]. NF- κ B decoy oligonucleotides have been shown to ameliorate streptococcal cell wall and collagen induced arthritis (CIA) in several disease models [167-170]. Other therapies that target NF- κ B activation have also been shown to attenuate adjuvant-induced arthritis [166]. Researchers have also demonstrated that disruption of p65 activity mitigates many symptoms associated with various inflammatory bowel disease models induced by 2, 4, 6-trinitrobenzene sulfonic acid [171]. Current therapies that target

NF- κ B also include glucocorticoids and antioxidants; glucocorticoids are effective inhibitors of NF- κ B, however, they pose severe metabolic and endocrine side effects when administered systemically [172]. Several antioxidants including, vitamins C and E, as well as acetylcysteines that inhibit the activity of NF- κ B have also been identified [172, 173]. However, these compounds are relatively weak and ineffective. Small molecule compounds including, sulfasalazine and its salicylate moiety 5-aminosalicylic acid, have also been shown to inhibit NF- κ B activity. These drugs function by preventing I κ B- α degradation and the subsequent translocation of NF- κ B into the nucleus. Other small molecule compounds such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can also block NF- κ B activity by competitively inhibiting IKK β activity. However, these compounds have been shown to lack specificity and require extremely high doses to achieve effective NF- κ B inhibition [172, 174]. Furthermore, the high incidence of cardiovascular side effects associated with NSAIDs has made them unattractive candidates for anti-NF- κ B therapies [175, 176]. Lastly, naturally occurring small molecule compounds have also been used for anti-NF- κ B therapies; for instance, glitoxin, which is derived from aspergillus, has been identified as a potent inhibitor of NF- κ B [177], albeit, its toxicity and efficacy in any inflammatory disease model remains to be clarified.

6. Naturally occurring compounds and inflammation

6.1 Medicinal plants

Plants have been routinely used in traditional medicine for centuries to treat a variety of disorders. Natural plant products provide a steady pipeline for the identification and development of pharmaceuticals due to the vast structural diversity of their active compounds [178, 179]. Natural products and herbal medicines constitute a multibillion-dollar industry; over 50 percent of new chemical entities (NCEs) with pharmaceutical potential are of plant origin. The FDA also estimates that more than 60 percent of approved and pre-clinical drug application candidates are either natural products or compounds related to them [178, 179]. *In vivo* and *in vitro* clinical studies have revealed that hundreds of plant compounds show promise in the areas of antimicrobial [180, 181], anti-inflammatory [182, 183] and anticancer [184] drug development. Various natural compounds with medicinal value have been identified [185]. A large proportion of these compounds have been shown to modulate intracellular signaling pathways and expression of inflammatory genes as well as antigen-induced cellular and humoral effector responses [185]. As a result, these compounds have been successfully tested in several inflammatory disease models, including cerebral ischemia [186], asthma, rheumatoid arthritis [187, 188], cancers [189] and septic shock [190]. This literature review focuses on two naturally occurring plant compounds terameprocol and berberine.

6.2 Terameprocol: Background and history

The cresote bush *Larrea tridentata* is indigenous to the Sonoran deserts of Mexico and the American southwest. The Pima, Yai, Maricopa and Seri tribes have used extracts and preparation of this plant to treat a wide variety of disorders [191]. The leaves have been used in a bath for chickenpox and decoctions made from boiled leaves are used as a poultice for skin sores. The leaves can also be used to make a tea (chaparral tea) that is used to treat various disorders including cancer, venerea disease, tuberculosis, colds and rheumatism. High doses of *L. tridentata* have been shown to cause hepatic necrosis, although damage is reversible when *L. tridentata* is withdrawn from diet [192, 193]. The medicinal effects of *L. tridentata* have been attributed to the presence of the phenolic compound nordihydroguaiaretic acid (NDGA) [191], a lipophilic anti-oxidant that has been used as a preservative in fats and oil. NDGA has been shown to inhibit 5-lipoxygenase activity *in vitro* [194, 195] and several reports have also demonstrated its ability to inhibit neutrophil production of leukotriene (LT) B₄, [196, 197] degranulation [196, 197], phagocytosis [198] and the respiratory burst [198]. Furthermore, NDGA has been shown to affect levels of intracellular calcium [199, 200] and to exert effects on mitochondria [201, 202] and the Golgi complex [203-205]. NDGA has also been shown to display anti-tumor effects [206] and prevent apoptosis induced by either TNF- α [207-210] or CD95 ligand [211, 212].

L. tridentata leaves also contain several other polyphenolic compounds that are derivatives of NDGA. One such compound is 3-O-methyl NDGA, which differs

from NDGA by substitution of one of the four hydroxyl groups found on NDGA for a methyl group [213]. 3-O-methyl NDGA has been shown to inhibit replication of a number of strains of HIV and prevent both basal transcription and Tat-regulated transactivation *in vitro* [213]. These effects have been linked to the ability of 3-O-methyl NDGA to interfere with the binding of the transcription factor Sp1 to the long terminal repeats of HIV, an effect that was not noted with NDGA itself [213].

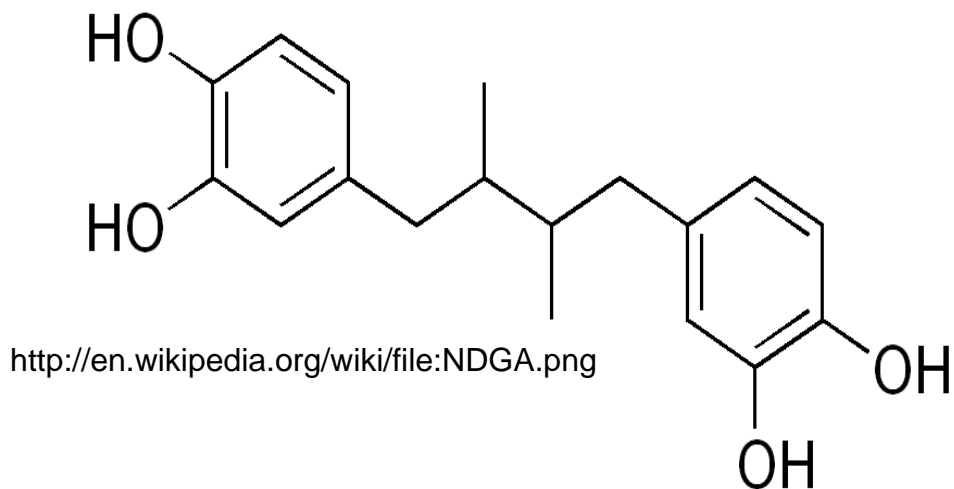


Figure 2. Structure of NDGA. NDGA is a naturally occurring compound found in the leaves of *L. tridentata*

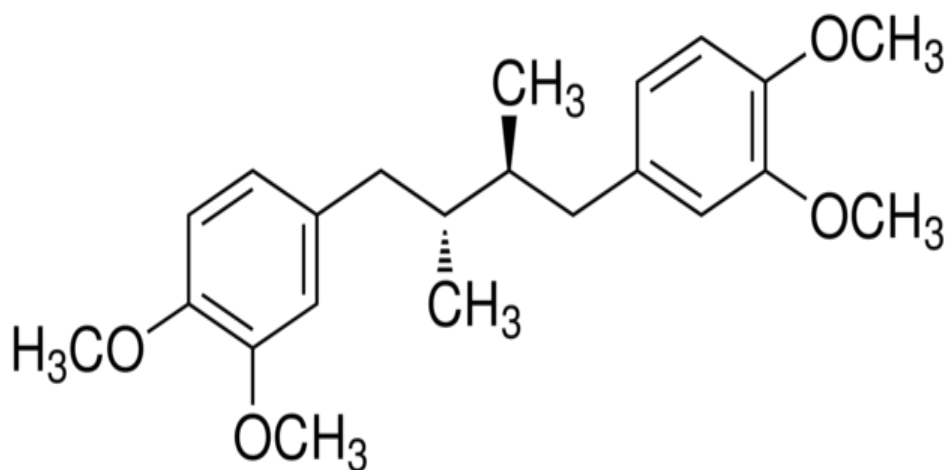
6.3 Antiviral activities of terameprocol

As a result of the findings described above, various methylated forms of NDGA were tested for their effects on HIV Tat-mediated transactivation in COS cells. These studies revealed that TMP displayed higher anti-HIV activity in

comparison to mono-, di-, and tri-methylated NDGA [214]. Since these findings, other reports have also confirmed the antiviral activities of TMP. Experiments using Vero cells have shown that TMP inhibits replication of the herpes simplex virus (HSV) by downregulating the expression of the immediate early gene product α -ICP4 [215]. Pollara *et al* [216], have also demonstrated that TMP potently inhibits the growth of both cowpox and vaccinia virus in a variety of cell lines. This study revealed that TMP inhibited the spread of viral particles from cell to cell by reducing the number of actin tails present at the surface of poxvirus-infected cells. Furthermore, Craigo *et al* [217], have demonstrated that TMP inhibits transcription from the early promoter p97 of human papilloma virus.

6.4 Antiinflammatory activities of terameprocol

Most importantly, TMP has been shown to be a potent anti-inflammatory agent. Recent studies conducted by Eads *et al.* [218], showed that TMP strongly inhibited the production of PGE₂ from LPS-stimulated RAW 264.7 cells and normal peritoneal macrophages. These effects were attributed to the down-regulation of COX-2 mRNA and protein levels [218]. These experiments also revealed that TMP suppressed protein and mRNA levels of the pro-inflammatory cytokines TNF- α and MCP-1 in LPS-stimulated RAW 264.7 cells. Furthermore, *in vivo* experiments showed that TMP suppressed overall levels of TNF- α and MCP-1 in the serum of C57BL6/J mice that were challenged by intraperitoneal (*i.p*) injection with LPS [218].



<http://ars.sciencedirect.com/content/image/1-s2.0-S0090825807006452-gr1.sml>

Figure 3. Structure of terameprocol. Terameprocol is a non-ionic hydrophobic small molecule, semi-synthetic tetra-methylated derivative of nordihydroguaiaretic acid (NDGA) with a molecular weight of 358.5 g/mol.

6.5 Other activities of terameprocol

The anticancer and tumoricidal activities of TMP are also well documented. To this extent, TMP has been shown to induce growth arrest and cellular apoptosis in C3 transformed cells by downregulating the expression of cyclin-dependent kinase 1 (Cdc2) and survivin [219]. TMP has also been shown to arrest the proliferation of C3, C33a, CEM-T4 and TC-1 cell lines at the G2 stage of the cell cycle [220]. These studies revealed that when injected intratumorally in a C3-cell-induced C57BL/6 mouse tumor model, TMP strongly suppressed tumor growth and this correlated with a decrease in tumor cell Cdc2 expression [220]. Systemic administration of TMP has also been shown to suppress the growth of human hepatocellular carcinoma, prostate carcinoma, colorectal carcinoma and breast

carcinomas as well as erythroleukemia xenograft tumors in nude mice [221]. As a result of these findings, TMP has been tested in phase I/II clinical trials for the treatment of glioma, cervical dysplasia, head and neck squamous cell carcinoma, acute myeloid leukemia and various hematological cancers (clinicaltrials.gov). TMP has also been tested as a topical therapeutic vaginal ointment for limiting the spread of HIV, HPV and HSV [222]. Results of early phase clinical trials indicate that TMP could be a useful therapeutic for treating cancers and sexually transmitted diseases (www.clinicaltrials.gov). These studies also revealed that TMP is well tolerated *in vivo* when administered at a dose of 1700mg/day [223].

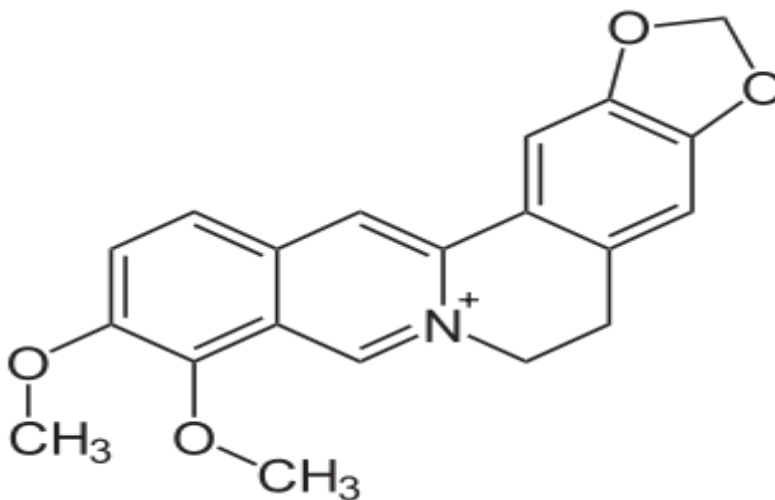
6.6 Terameprocol's mechanism of action

The antiviral and anticancer effects of TMP have been linked to its ability to inhibit the activity of the transcription factor Sp1. Experiments using electrophoretic mobility shift assays (EMSA) have demonstrated that TMP prevents the binding of Sp1 to its cognate sites [215]. These studies revealed that TMP competes with Sp1 for specific Sp1-binding domains within gene promoter regions. A number of preclinical studies have shown that TMP prevented the proliferation of HIV, HSV and HPV by deactivating viral promoters that are dependent on Sp1-transactivation [224]. Additionally, inhibition of Cdc2 and survivin mRNA levels by TMP correlated with abrogation of Sp1-dependent transcriptional activation [219]. Unsurprisingly, the anticancer activity of TMP has also been linked to its ability to inhibit Sp1-mediated transactivation [225].

7. Berberine

7.1 Background and history of berberine

A number of plants such as *Rhizoma coptidis*, goldenseal, *Berberis vulgaris*, *Coptis chinensis* and *Mahonia aquifolium* have been used as medicinal herbs in traditional Chinese and Indian medicines. These herbs have been used to treat a variety of disorders, which include, oriental sores, diarrhea, breast cancer, leukemia, hepatoma, pancreatic cancer, skin and eye infections, as well as upper respiratory disorders [226-229]. The therapeutic effects of these herbs have been attributed to the presence of berberine, an isoquinoline alkaloid of the protoberberine type. In fact, several independent studies have demonstrated that berberine displays a broad range of pharmacological activities.



<http://en.wikipedia.org/wiki/file:berberin.svg>

Figure 4. Structure of berberine. Berberine is an isoquinoline alkaloid found in plants such as *Rhizoma coptidis*, goldenseal, *Berberis vulgaris*, *Coptis chinensis* and *Mahonia aquifolium*

7.2 The anti-inflammatory activities of berberine

The anti-inflammatory effects of berberine have been researched extensively; these studies have revealed that berberine displays potent anti-inflammatory activities. Berberine has been shown to concurrently increase the survival of LPS-treated mice and mitigate histological damage to the lungs by suppressing the levels of TNF- α , IFN- γ and NO [230]. Jeong *et al.* [231], have also demonstrated that berberine suppressed mRNA levels of TNF- α , IL-6, MCP-1, inducible nitric oxide synthase (iNOS) and matrix metalloproteinase 9 in LPS-stimulated RAW 264.7 cells. Along these lines, other studies have revealed that berberine inhibits LPS-induced NO production in RAW 264.7 cells [232]. Furthermore, experiments by Zha *et al.* [233], have shown that berberine inhibits HIV protease inhibitor-induced production of TNF- α and IL-6 in J774A.1 macrophages. Berberine has also been shown to attenuate clinical severity of experimental autoimmune encephalomyelitis (EAE) by reducing blood brain barrier dysfunction and downregulating expression of matrix metalloproteinase (MMP)-9 in the cerebrospinal fluid (CSF) and brain of EAE mice [234]. Other groups have since confirmed these findings; Qin *et al.* [235], have demonstrated that berberine ameliorates EAE by inhibiting the differentiation as well as function of Th1 and Th17 cells. Additionally, these studies revealed that berberine decreased mRNA levels of IL-6 and various costimulatory molecules [235]. Berberine has also been shown to inhibit pulmonary inflammation; experiments by Lee *et al.* [236], showed that berberine suppressed the production of TNF- α and IL-1 β from HFL1 lung fibroblasts stimulated with LPS, TPA or H₂O₂. Additional studies

have revealed that berberine inhibited IL-8 production in colonic epithelial cells [237] and ameliorated 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in C3H/HeN mice by preventing lipid peroxidation as well as production of TNF- α , IL-6 and IL-1 β [237, 238]. It has also been demonstrated that berberine suppresses production of TNF- α in LPS-stimulated rat cardiomyocyte cells [239]. Furthermore, berberine has been shown to inhibit LPS-induced acute inflammatory response in broiler chickens by preventing production of IL-6 [240]. Other studies have also demonstrated that berberine downregulates mRNA levels of proinflammatory mediators such as COX-2, NO, TNF- α , IL-6 and GM-CSF in B16F-10 melanoma cells [241].

Although berberine displays a broad range of anti-inflammatory activities, it appears that the various effects could be cell and/or ligand dependent. Along these lines, berberine has been shown to enhance the expression of iNOS in leishmania-infected peritoneal macrophages from BALB/C mice [242]. Contrary to these findings, Jeong *et al.* [231], have reported that berberine inhibits iNOS production in LPS-stimulated RAW 264.7 cells. These studies also revealed that berberine downregulated the expression of iNOS in LPS-stimulated RAW 264.7 cells, peritoneal macrophages, and adipose tissue of obese *db/db* mice [231]. Saha *et al.* [242], have published that berberine augments IL-12 production in leishmania-infected peritoneal macrophages from BALB/C mice. Kang *et al.* [243], have since confirmed these findings by demonstrating that berberine also increases expression and production of IL-12p40 in LPS-stimulated splenic macrophages from DBA/2

mice. Conversely, Wang *et al.* [230], have reported that berberine did not affect IL-12 production in LPS-treated Kunming strain mice.

Effects of berberine on activation of MAPK pathways also appear to be cell type and/or ligand dependent. For example, berberine has been shown to activate p38 in LPS-stimulated splenic macrophages [243] and leishmania-infected peritoneal macrophages [242]. However, other studies have shown that berberine suppressed the activation of p38 in LPS-stimulated RAW 264.7 cells and peritoneal macrophages [231], as well as in α -CD3/28 stimulated NOD CD4⁺ cells [244]. Conversely, findings by Guo *et al.* [245], have demonstrated that berberine did not affect the activation of p38 following LPS-stimulation in human peripheral blood monocytes (PBMCs). In support of these findings, other reports have shown that berberine did not inhibit p38 activation in LPS- or IFN- γ -stimulated BV-2 microglial cells [246, 247].

Several studies have also examined the effects of berberine on activation of the ERK MAPK pathway. Some of these studies have revealed that berberine prevents ERK1/2 activation in leishmania-infected peritoneal macrophages [242] as well as LPS-stimulated RAW 264.7 cells and peritoneal macrophages [231]. Conversely, berberine has been shown to enhance ERK1/2 activation in α -CD3/28 stimulated CD4⁺ cells from NOD diabetic mice [244]. Recently, Choi *et al.* [247] demonstrated that berberine inhibits expression of inflammatory molecules in 3T3-L1 adipocytes without suppressing the activation of ERK1/2. A number of experiments have also revealed that berberine differentially regulates activation of the JNK MAPK

pathway. Studies by Guo *et al.* [245] have shown that berberine did not affect JNK activation in LPS–stimulated human PBMC as well as LPS or IFN- γ stimulated BV-2 microglial cells [246]. However, Cui *et al.* [244], have demonstrated that berberine inhibits JNK activation in α -CD3/28-stimulated CD4⁺ cells from NOD mice. Conversely, findings by Lee *et al.* [248], revealed that berberine increases JNK activation in HepG2 hepatocytes. Taken together, it appears that the anti-inflammatory activities of berberine are cell and/or ligand specific.

7.3 Other activities of berberine

Experiments have demonstrated that berberine is a potent antimicrobial drug. In fact, berberine has been shown to inhibit the growth of certain bacteria [249-252] and several viruses including human cytomegalovirus [253] as well as herpes simplex virus [254]. Berberine has also been used to successfully treat various disorders including diabetes [255], hypercholesterolemia [256-258], Alzheimer's disease [255, 259, 260] and a host of cardiovascular diseases [261-263] in mice and rat disease models. The anticancer activities of berberine have also been well documented. Berberine has been shown to induce death in human hepatoma cells *in vitro* by downregulating CD147 [264]. A number of *in vivo* studies using rats have confirmed the anti-proliferative and anti-cancer activities of berberine [265-267].

7.4 Berberine's mechanism of action

Berberine has been shown to modulate gene expression by inhibiting activation of NF- κ B [241, 268, 269] and AP-1 [269, 270]. These studies revealed that berberine blocks NF- κ B activity by inhibiting activation of IKK- α [268, 271]. Additional studies indicate that berberine can inhibit the activation of other transcription factors such as CREB and ATF-2 [241]. Furthermore, the anticancer [272], antidiabetic [273], anti-inflammatory [231, 246, 274] and hypolipidemic [263, 275] activities of berberine have all been linked to activation of the adenosine monophosphate protein kinase (AMPK) pathway. Berberine-mediated activation of AMPK has been shown to play a role in glucose uptake [276, 277] and fatty acid metabolism [278, 279]. A few studies (using circular dichroism) have demonstrated that berberine binds to DNA and RNA [280, 281]. It is likely that this could be one of the mechanisms by which berberine modulates gene expression. Experiments by Wang *et al.* [282], recently showed that berberine globally downregulated mRNA expression by preventing the association of the TATA binding protein and the TATA box. Experiments by Zha *et al.* [233], have also shown that berberine blocks the function of the mRNA stabilizing protein HuR. Taken together, these findings suggest that berberine can modulate gene expression through diverse mechanisms.

8. Current study

Microbially-induced inflammation promotes eradication of invading pathogens and return to homeostasis. However, acute and chronic inflammatory responses can also be damaging to host. In fact, development of chronic inflammatory disorders and autoimmune diseases has been attributed to activities of inflammatory mediators such as cytokines and chemokines produced in response to infectious agents. Current therapies for controlling excessive cytokine production are effective, however, they have been associated with various clinical side effects. Additionally, these treatments are contraindicated in certain segments of the population. For these reasons, it is imperative to discover safer efficacious compounds for limiting the inflammatory response. Medicinal plants have been used successfully for centuries to treat a variety of disorders with little to no side effects. The pharmacological activities of these herbs have been attributed to presence of naturally occurring compounds. We have previously tested two plant compounds terameprocol and berberine for their effects on microbially-induced inflammation. These experiments revealed that berberine and terameprocol are potent inhibitors of virus and LPS-induced inflammation respectively. The work described in this dissertation focuses on elucidating the mechanisms by which these compounds suppress the production of key inflammatory mediators.

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Chapter 2. Tetra-O-methyl nordihydroguaiaretic acid (Terameprocol) inhibits the NF- κ B-dependent transcription of TNF- α and MCP-1/CCL2 genes by preventing RelA from binding its cognate sites on DNA (*Published in J. Inflammation: Dec 2010*).

1. Abstract

Tetra-O-methyl nordihydroguaiaretic acid, also known as terameprocol (TMP), is a naturally occurring phenolic compound found in the resin of the creosote bush. We have shown previously that TMP will suppress production of certain inflammatory cytokines, chemokines and lipids from macrophages following stimulation with LPS or infection with H1N1 influenza virus. In this study our goal was to elucidate the mechanism underlying TMP-mediated suppression of cytokine and chemokine production. We have focused our investigations on the response to LPS and the NF- κ B protein RelA, a transcription factor whose activity is critical to LPS-responsiveness. Reporter assays using an NF- κ B response element in TLR-3, -4, and -8 over-expressing cells, revealed that TMP caused strong inhibition of NF- κ B-dependent transcription. Experiments also revealed that the phosphorylation of I κ B- α and the translocation of NF- κ B into the nucleus was unaltered by TMP. Further analysis using ChIP assays demonstrated that TMP abrogated RelA binding to its cognate motifs on the promoters of TNF- α , MCP-1/CCL2, and RANTES/CCL5 genes. However, experiments using electrophoretic mobility shift assays (EMSAs) demonstrated that TMP could not directly prevent binding of RelA to naked DNA templates *in vitro*. Taken together, our results suggest that TMP is acting indirectly to

inhibit NF- κ B-dependent transcription by abrogating RelA binding to its cognate motifs on the TNF- α , MCP-1 and RANTES promoters.

2. Introduction

The NF- κ B proteins are sequence-specific transcription factors that play critical roles in the immune system. NF- κ B proteins regulate the expression of cytokines, chemokines, growth factors, and inflammatory enzymes in response to activation of T-cell, B-cell, Toll/IL-1R, and TNF- α receptors [1, 2]. The NF- κ B family of proteins is characterized by the presence of a conserved 300 amino acid Rel Homology Domain (RHD) which controls dimerization, DNA binding, and association with the inhibitory I κ B proteins [3]. The five members of the mammalian NF- κ B family; RelA (p65), RelB, c-Rel, NF- κ B1 (p50) and NF- κ B2 (p52) are present in unstimulated cells as homo- or heterodimers bound to inhibitory I κ B proteins. This association prevents NF- κ B proteins from translocating to the nucleus, thereby maintaining an inactive state [4]. In response to inflammatory stimuli such as TNF- α , IL-1, or LPS, multiple signaling pathways are activated resulting in the phosphorylation of I κ B- α [5, 6]. Subsequent poly-ubiquitination and proteosomal degradation of I κ B- α permits the translocation of NF- κ B proteins into the nucleus where transcription is activated [7, 8]. NF- κ B dimers exhibit variable binding affinities for consensus κ B binding sites. These proteins also differ in their ability to initiate

transcription; RelA, RelB and c-Rel have been shown to have potent trans-activating domains, while NF- κ B proteins that lack transactivating domains such as p50 and p52 have been shown to mediate transcriptional repression [3]. Activated NF- κ B proteins can be inhibited by newly synthesized I κ B proteins which cause re-export back to the cytosol [9].

Extracts of the Creosote bush, *Larrea tridentata*, found in deserts of the Southwestern United States and Northern Mexico, have been used for centuries by indigenous peoples to treat inflammatory disorders. Many of the medicinal effects of *L. tridentata* have been ascribed to the polyphenolic compound nordihydroguaiaretic acid (NDGA) [10]. In addition, *L. tridentata* also contains polyphenolic compounds with modifications to the backbone structure of NDGA [11]. A number of these compounds have been examined for their antiviral activity. For example, an analysis of eight methylated forms of NDGA for their ability to inhibit HIV replication revealed that tetra-O-methyl NDGA, also known as terameprocol (TMP), displayed the highest level of activity. Mechanistic studies suggest that TMP mediates this effect by inhibiting HIV Tat-mediated transactivation [12]. TMP has also been shown to block the replication of herpes simplex virus *in vitro* and this effect has been attributed to the drug's ability to block the binding of the transcription factor Sp1 to viral DNA, which is required for virus replication [13].

Based on these reports, we have recently evaluated the efficacy of TMP as an anti-inflammatory agent. We reasoned that since inflammation is heavily dependent on *de novo* transcription, TMP might be a useful therapeutic compound. We found

that TMP exerted a range of effects on various inflammatory cytokines, chemokines and lipid mediators both *in vivo* and *in vitro* following treatment with LPS or infection with H1N1 influenza A virus strain PR/8/34 [14]. TMP strongly inhibited the production of TNF- α , MCP-1/CCL2, G-CSF, and several prostaglandins, while modestly inhibiting the production of IL-6 and MIP-1 α /CCL3. Since the NF- κ B RelA protein has been reported to regulate the expression of several of these genes [15-18], we have focused our current studies on how TMP modulates RelA activation and occupancy at its cognate DNA binding motifs. We report that TMP did not affect the cytoplasmic activation and nuclear localization of RelA in RAW 264.7 cells following treatment with LPS. However, reporter assays revealed strong inhibition of NF- κ B-dependent transcription. Chromatin immunoprecipitation (ChIP) assays revealed that TMP abrogated the LPS-induced binding of RelA at the TNF- α , MCP-1/CCL2, and RANTES/CCL5 promoters despite its inability to block NF- κ B association with electrophoretic mobility shift assay (EMSA) probes *in vitro*. We conclude, therefore, that TMP acts indirectly to inhibit the binding of RelA to the promoters of certain key pro-inflammatory cytokine and chemokine genes. Taken together our data suggest that TMP could be useful for the treatment of inflammatory disorders where NF- κ B RelA-dependent transcription plays a pathogenic role.

3. Methods

3.1 Cells and media

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modification of minimal essential medium (DMEM) with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate with 10% FCS. Media and supplements were obtained from Sigma-Aldrich, St. Louis, MO and Cellgro, Manassas, VA. FCS was obtained from Atlanta Biologicals, Atlanta, GA and Cellgro. Constitutive TLR3(293/TLR3-YFP), TLR8(293/TLR8) (InvivoGen, San Diego, CA) and TLR4(293/TLR4-YFP/MD2) (a gift from D. Golenbock) expressing HEK293 cells were grown in DMEM supplemented with 10% FCS, 1% antibiotics, 20 µg/ml gentamicin at 37°C. Stable expression of TLRs was maintained with the addition of 10 µg/ml blasticidin for (293/TLR3) and (293/TLR8) cells, and 400 µg/ml of G418 (Geneticin) for (293/TLR4) cells.

3.2 Chemicals and biological reagents

Unless otherwise indicated, reagents were purchased from Sigma-Aldrich. TMP was supplied by Erimos Pharmaceuticals, Raleigh, NC. DMSO was used as the solvent for TMP in all experiments. The maximum DMSO concentration was 0.1% in all assays. This concentration of DMSO was tested in all assays and did not affect the results. LPS from *Salmonella Minnesota* R595 was purchased from LIST Biological Laboratories, Inc. (Campbell, CA).

3.3 Quantitative RT-PCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. Residual genomic DNA was eliminated using on-column DNase digestion with the RNase-free DNase set (Qiagen) and resulting extracts were resuspended in nuclease free water. Amount and purity of RNA was determined using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). RNA (1 μ g) was denatured and reverse transcription was performed with the Improm II reverse transcription kit (Promega, Madison, WI) in a reaction mix containing random hexamers as primers (50 ng/ μ l) for 60 min at 42°C. The iQTM SYBR Green supermix kit (BioRad, Hercules, CA), was used for Real-time PCR analysis. cDNA was amplified using primers specific for murine GAPDH, TNF- α , MCP-1/CCL2, and RANTES/CCL5 genes. Primer combinations are GAPDH [antisense: 5' ATG TCA GAT CCA CAA CGG ATA GAT 3'; sense: 5' ACT CCC TCA AGA TTG TCA GCA AT 3']; TNF- α [antisense: 5' AGA AGA GGC ACT CCC CCA AAA 3'; sense: 5' CCG AAG TTC AGT AGA CAG AAG AGC G 3']; MCP-1/CCL2 [sense: 5' CAC TAT GCA GGT CTC TGT CAC G 3'; antisense: 5' GAT CTC ACT TGG TTC TGG TCC A 3']; RANTES/CCL5: [sense: 5' CCC CAT ATG GCT CGG ACA CCA 3'; antisense: 5' CTA GCT CAT CTC CAA ATA GTT GAT 3']. All primer pairs were purchased from Integrated DNA Technologies (Coralville, IA). PCR was performed in 96 well plates (Eppendorf AG, Hamburg, Germany). Samples were amplified for a total of 50 cycles, followed by a meltcurve analysis to ensure the specificity of reactions. To generate a standard curve, total

RNA was isolated from the cells and 300–600 bp fragments of the gene of interest were amplified by RT-PCR using cognate primer sets. PCR fragments were gel purified, quantified, and the copy number was calculated. Serial tenfold dilutions were prepared for use as templates to generate standard curves. All samples were normalized to amplified murine GAPDH. GAPDH control was analyzed per plate of experimental gene to avoid plate-to-plate variation. Final RT-PCR data is expressed as the ratio of copy numbers of experimental gene per 10^3 or 10^4 copies of GAPDH for samples performed in duplicates.

3.4 Western blot analysis

After treatments, cell monolayers were washed twice with cold phosphate buffered saline (PBS), solubilized in lysis buffer (50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS) and collected by scraping. The protein concentration for each sample lysate was determined using the Pierce BCA system (Pierce, Rockford, IL). Equal protein samples (25 μ g) were loaded on 12% Tris-Glycine gels and subjected to electrophoresis using the Novex Mini-Cell System (Invitrogen). Following transfer, and blocking, blots were probed with antibodies specific for the phosphorylated serine 32 residue of I κ B- α and total I κ B- α protein (Cell Signaling; Beverly, MA). Bands were visualized using the SuperSignal Chemiluminescent system (Pierce).

3.5 Immunofluorescence

RAW 264.7 cells were seeded onto 8 well chamber slides and stimulated with 1 μ g/ml of LPS or co-stimulated with 1 μ g/ml of LPS and 25 μ M TMP for various amounts of time. To visualize NF- κ B subcellular localization at the end of each treatment period, cells were briefly washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked (2% bovine serum albumin, 5% normal horse serum, and 10 mM glycine in phosphate-buffered saline). The cells were then incubated with a rabbit monoclonal anti-NF- κ B (p65) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by incubation with a goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Southernbiotech, Birmingham, AL). Fluorescence was viewed using a Zeiss Axioskop 2 microscope (Zeiss AG, Oberkochen, DE). Images were captured using a spot camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

3.6 Cytokine Measurements

MCP-1/CCL2 and TNF- α ELISA kits were purchased from R&D Systems (Minneapolis, MN), Assay Designs (Ann Arbor, MI) or eBioscience (San Diego, CA). RAW 264.7 cells were stimulated with 1 μ g/ml of LPS for 24 hrs and supernatants were collected for ELISA assays. In each case, sample values were interpolated from standard curves. Optical density was determined using a PolarStar microplate reader (BMG Labtechnologies, Durham, NC).

3.7 Reporter Assays

Reporter assays were performed using a luciferase gene under the control of an NF- κ B response element (NF- κ B -Luc; Stratagene, Santa Clara, CA). Briefly, the plasmid contains 5 consecutive NF- κ B binding motifs designed from a consensus sequence cloned into a PGL3 vector. 100 ng each of NF- κ B-Luc and pCMV beta (β -Gal) (Clontech) and 300 ng of pcDNA6 (Invitrogen) were cotransfected into 293/TLR3, 293/TLR4-YFP/MD2 and 293/TLR8 cells using the TransIT-LT1 transfection reagent (Mirus, Madison, WI). pcDNA6 was used to keep the overall DNA concentration at a total of 500 ng which has proven itself suitable for reporter assay in this system. At 24 h post-transfection, cells were either treated for 4 hours with 20 μ g/ml poly(I:C) (pIC; Calbiochem, Gibbstown, NJ), 1 μ g/ml LPS or 1 μ g/ml resiquimod (R-848; Axxora, San Diego, CA) alone, or co-treated with 25 μ M TMP. Following treatment, cells were lysed in reporter lysis buffer (Promega, Madison, WI) containing 0.1% Triton X-100 and assayed for Luc and β -Gal activities using a Promega Luc assay system and an ONPG (*o*-nitrophenyl- β -D-galactopyranoside)-based β -Gal assay. β -Gal activity was used to normalize the Luc data for all experiments. All data are expressed as relative light units/mU of β -Gal activity.

3.8 Chromatin immunoprecipitation (ChIP) assays

4.5×10^7 RAW 264.7 cells were stimulated with 1 μ g/ml LPS or co-treated with 1 μ g/ml LPS and 25 μ M TMP for 4 hours and chromatin was isolated by methods

previously described [19]. Briefly, after treatments, cells were harvested and nucleoprotein complexes were crosslinked with formaldehyde (1% final) with shaking for 10 min at room temperature, followed by incubation with glycine (125 mM final) for an additional 5 min. Cells were pelleted, washed and resuspended in 500 μ l lysis buffer (10 mM Tris-HCl, pH7.5, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40) supplemented with 1 mM PMSF and 1X Protease Inhibitor Cocktail (PIC, Roche). Nuclei were pelleted and resuspended in Micrococcal nuclease buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 1 mM CaCl₂, 4% NP-40) supplemented with 1 mM PMSF and 1X PIC, and chromatin was sheared with the addition of 10U MNase for 7 min at 37 °C. Digestion was stopped with the addition of EDTA (10 mM final), and the resultant chromatin was stored at - 80 °C. Shearing was confirmed by electrophoresis and >80% of the DNA was in fragments <400 bp.

Using magnetic capture, Protein A and G-coupled Dynabeads (Invitrogen) (5 μ l each/IP) were washed 2X (100 μ l/IP) in RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% NaDeoxycholate and sheared salmon sperm DNA (0.5 mg/ml). Beads were conjugated with 1–5 μ g antibody for 1 h at 4 °C in RIPA buffer supplemented with 1 mM PMSF and 1X PIC. Conjugated antibody:bead complexes were washed 3X in RIPA buffer as described above, and protein-DNA complexes were immunoprecipitated for 2 h at 4 °C with rotation in RIPA buffer (100 μ l) supplemented with 1 mM PMSF, 1X PIC and chromatin (10⁵ cell equivalents). Following IP, beads were successively washed 4X in RIPA buffer and 2X in TE 8.0,

and protein–DNA complexes were eluted in 100 mM NaHCO₃ by gentle vortexing for 15 min at room temp. Supernatants were recovered and crosslinks were reversed in NaCl (100 mM final) together with matched input samples by heating at 95 °C for 15 min. Proteins were removed using Proteinase K (10 µg/ml final) for 1 h at 45 °C and DNA was purified using Qiaquick nucleotide removal columns (Qiagen) according to the manufacturer's instructions.

3.9 CHIP Q-PCR and data analysis

For realtime PCR, bound (3 µl) and input samples were amplified in a MyIQ thermal cycler (Bio-RAD) using 1X SensiMix *Plus* (Quantace, London, UK) and primers specific for the RelA binding sites at the TNF- α , MCP-1/CCL2 and RANTES/CCL5 promoters. TNF- α : [sense: 5' TCTCAAGCTGCTCTGCCTTC 3'; antisense: 5' CACCAGGATTCTGTGGCAAT 3']; RANTES/CCL5:[sense:5' TGGAGGGCAGTTAGAGGCAGAG3';antisense:5'AGCCAGGGTAGCAGAGGAAGT G 3']; MCP-1/CCL2: [sense: 5' ATTCTTCCCTCTTTCCCCCCCC 3'; antisense:5'TCCGCTGAGTAAGTGCAGAGCC 3'] Cycling parameters for 20 µl reactions were 95 °C 10 min, followed by 50 cycles of 95 °C, 20 s; 60 °C, 30 s; 72 °C, 30 s, for all genes listed. Fold enrichment in the bound fractions relative to input was calculated as previously described [20], and the average enrichment for triplicate amplifications was reported.

3.10 Electrophoretic mobility shift assays (EMSA)

RAW 264.7 nuclear extracts and radioactive probes were prepared and EMSA reactions performed as previously described [21]. Sequences of wildtype and mutant oligonucleotide EMSA probes include: wildtype TNF- α κ B3 sense (5'-AACAGGGGGCTTTCC-3') and antisense (5'-AGGAGGGAAAGCCCC-3'), and mutant TNF- α κ B3 sense (5'-AACAGGGGGCTGAGCCTC-3') and antisense (5'-GAGGCTCAGCCCCCTGTT-3').

3.11 Statistical Analysis

All graphs and statistical analyses were produced using Prism software (GraphPad Software Inc., La Jolla CA).

4. Results

4.1 TMP acts early to inhibit synthesis of TNF- α and MCP-1/CCL2 mRNAs

We have previously shown that TMP inhibits the LPS-induced production of TNF- α and MCP-1/CCL2 from RAW 264.7 macrophage-like cells [14]. Representative experiments illustrating this effect are shown in Figs. 1A and B. Typically, following a 24 h treatment with 1 μ g/ml LPS in the presence of 25 μ M TMP, levels of TNF- α and MCP-1/CCL2 are suppressed by 40 and 80%,

respectively. Previously we found that the TMP-mediated reduction in these protein levels correlated with effects on accumulation of the specific mRNAs, leading us to speculate that TMP could interfere with transcription [14]. However, because regulation of cytokine and chemokine mRNA can be complex, we sought direct evidence for an early effect of TMP on mRNA synthesis. As shown in Fig. 1C, the effect of TMP on the synthesis of TNF- α mRNA was evident early and maintained throughout the 8 h experiment [14] consistent with an effect on the transcriptional activation of the TNF- α gene. The rapid rise and fall in levels of TNF- α mRNA following treatment with LPS is typical and has been attributed to the action of various transcription factors [22, 23] followed by tristetrapolin (TTP)-mediated mRNA degradation [24, 25]. As shown in Fig. 1D, an early effect of TMP on the synthesis of MCP-1/CCL2 mRNA was also noted; results that are again consistent with an effect of TMP on transcriptional activation. In this case, however, we also observed a reduction in steady state levels of MCP-1/CCL2 mRNA in the presence of TMP (Fig. 1D). This effect was selective for MCP-1/CCL2 mRNA; TMP did not alter TNF- α mRNA expression kinetics.

4.2 TMP inhibits NF- κ B dependent reporter activity

NF- κ B proteins, primarily RelA/NF- κ B1 heterodimers, have been reported to play a key role in the transcriptional activation of cytokine genes after LPS stimulation [26]. Therefore, we hypothesized, that the inhibitory effects of TMP on

the transcription of TNF- α and MCP-1/CCL2 mRNAs might stem from the effect of the drug on the activity of NF- κ B proteins. To test this hypothesis, we performed reporter assays with cells expressing an NF- κ B response element. HEK293 cells co-expressing TLR4 and MD2 (a co-receptor needed for TLR4 signaling) (HEK293/TLR4-YFP/MD2) were stimulated with 1 μ g/ml of LPS or 1 μ g/ml of LPS and 25 μ M of TMP for a period of 4 hours and cell lysates were analyzed for NF- κ B dependent luciferase activity. As shown in Fig. 2A, LPS stimulation strongly increased NF- κ B dependent reporter activity approximately 7 fold. This effect was inhibited by TMP by approximately 60%, a result consistent with the hypothesis that TMP inhibits the activity of NF- κ B. This effect was dose dependent with a concentration of 12.5 μ M TMP inhibiting NF- κ B reporter activity by 35% (data not shown). It should also be noted that western blots with a TLR-4 specific Ab did not reveal an effect of TMP on the expression of TLR-4 following transfection (data not shown).

The possibility that TMP was affecting the activity of LPS and/or its receptor, as opposed to NF- κ B-dependent transcription, was examined by testing the effects of TMP on TLR-3 and TLR-8-mediated activation of NF- κ B [27, 28]. The natural ligands for TLR-3 and TLR-8 are double and single stranded RNA, respectively. In these experiments we used the artificial ligands poly(I:C) for TLR-3 and resiquimod (R-848) for TLR-8. HEK293/TLR3 and HEK293/TLR8 cells were stimulated with either 20 μ g/ml poly(I:C) or 1 μ g/ml R-848, respectively. As with LPS, we found that

TMP blocked both poly(I:C)- and R-848-induced, NF- κ B-dependent reporter activity (Figs. 2B and C, respectively). Taken together these data suggest that TMP mediates a broad, receptor-independent, inhibitory effect on NF- κ B-dependent transcription.

4.3 TMP inhibits RelA binding to its cognate motifs *in vivo*

ChIP assays were used next to confirm this hypothesis and to gain insight into the mechanism of NF- κ B inhibition. Furthermore, with these assays we could examine RelA activity specifically since this is the major NF- κ B protein responsible for cytokine and chemokine transcription following LPS stimulation [5]. RAW 264.7 cells were treated with LPS and/or TMP, the resulting nucleo-protein complexes were cross-linked, and RelA specific antibodies were used to precipitate RelA:DNA complexes. DNA was subsequently purified and analyzed by quantitative RT-PCR using primers specific for the NF- κ B binding sites on the TNF- α , MCP-1/CCL2, and RANTES/CCL5 promoters. RANTES/CCL5 was included since its promoter does contain NF- κ B binding sites, although previous studies showed that its expression was not blocked by TMP. As shown in Fig. 3, treatment with LPS strongly enhanced the binding of RelA to each promoter, an effect that was completely blocked by treatment with TMP. We conclude, therefore, that TMP prevents NF- κ B-dependent transcription by preventing RelA from binding to its cognate motifs on the DNA *in vivo*.

4.4 TMP does not directly inhibit RelA:DNA binding

Loss of RelA binding at the TNF- α promoter in our CHIP analyses suggests that TMP either directly inhibits RelA:DNA binding or acts indirectly to alter assembly of the TNF- α promoter nucleoprotein complex. To determine if TMP competitively impairs RelA:DNA binding, we tested the ability of NF- κ B nuclear proteins to bind radiolabeled ds oligonucleotides of cognate κ B sites on the TNF- α promoter by EMSA (Fig.4). LPS treatment of RAW 264.7 cells induced high levels of nuclear protein binding to a radiolabeled probe of the κ B3 site (-311 relative to the TNF- α transcription start site) (Fig. 4A, compare lanes 1 and 2). Binding was readily competed by unlabeled wildtype κ B3 probe (Fig. 4A, lane 3), whereas a 3-base substitution in the probe abolished competition (lane 4). The ability of anti-p65 antibody to specifically supershift the upper nucleoprotein complex (lane 5) confirms the identity of this band and recapitulates recent findings in LPS-treated RAW 264.7 cells [29]. In contrast to our *in vivo* CHIP analyses, addition of 25 μ M TMP during LPS induction of RAW 264.7 cultures had no apparent impact on NF- κ B binding at either the κ B3 (Fig. 4B, lane 3) or κ B2 sites of TNF- α (data not shown). Likewise, NF κ B binding was unaffected when nuclear extracts from LPS-treated RAW 264.7 cells were pre-incubated with varying concentrations of TMP prior to addition of the radiolabeled DNA probe (Fig. 4B, lanes 4-6), suggesting that TMP does not directly inhibit NF κ B binding to DNA.

4.5 TMP does not inhibit the nuclear translocation of NF- κ B RelA

Antibody to RelA was used in immunofluorescence experiments to determine whether TMP blocked the nuclear translocation of RelA. As shown in Figs. 5A-C, LPS treatment of RAW 264.7 cells caused strong nuclear translocation of RelA; twenty min. after treatment with LPS was initiated virtually all cells displayed nuclear RelA (Fig. 5B). At later time points nuclear staining became more diffuse but overall staining intensity in the nuclear region of the cells remained relatively constant (Fig. 5C and G). As shown in Figs. 5D-G, TMP did not affect this process. Nuclear staining was evident in virtually all cells 20 min. after treatment with LPS was initiated and signals remained high at subsequent time points. TMP also failed to affect the translocation of RelA in C3HA mouse fibroblasts and NTERA-2 neuronal cells following treatment with LPS (data not shown). Together, these results suggest that TMP does not interfere with signaling to, and movement of RelA into the nucleus following treatment with LPS.

4.6 TMP does not affect the phosphorylation of I κ B- α

Finally, to confirm this hypothesis we examined the effects of TMP on the LPS-induced phosphorylation of I κ B- α , the final step in the signaling cascade, which results in dissociation of the RelA/p50 heterodimer from I κ B- α , permitting nuclear translocation of RelA/p50 [30]. As shown in Fig. 6A, we found that LPS stimulation induced phosphorylation of I κ B- α within 10 mins and that levels of phospho-I κ B- α remained relatively constant for up to 4 hours. Note that levels of total I κ B- α drop

below levels of detection at the 10 min. time point (Fig. 6A). According to the antibody manufacturer, this occurs because phosphorylation of I κ B- α is complete and this modification blocks the binding of the total I κ B- α antibody. Detection of total I κ B- α at later time points represents newly synthesized, non-phosphorylated molecules. As shown in Fig. 6B, the pattern of I κ B- α phosphorylation did not change in the presence of TMP. Small changes were noted from experiment to experiment however none of these effects were significant (Fig. 6C). We conclude, therefore, that TMP is not interfering with signaling pathways that result in the activation and translocation of NF- κ B. It should also be noted that TMP did not affect the resynthesis of total I κ B- α , which is dependent on RelA ([31]), indicating that TMP does not inhibit the RelA dependent transcription of I κ B- α .

5. Discussion

Previously we showed that TMP could inhibit the expression of a number of cytokines and chemokines following stimulation with LPS [14]. The production of TNF- α , MCP-1/CCL2, and G-CSF were most strongly inhibited and we hypothesized that these effects might stem from effects on NF- κ B RelA, which is thought to play a key role in the activation of these genes. The results of reporter and ChIP assays confirmed this hypothesis. We found strong inhibition of NF- κ B-dependent transcriptional activation and loading of RelA to the promoters of several genes.

Based on these results, a series of experiments was performed in an attempt to understand the molecular mechanism underlying this activity of TMP.

One hypothesis we considered was a direct inhibitory effect of TMP on the interaction between RelA and its cognate sites on the DNA. TMP could be acting on RelA itself, binding to conserved motifs present in the amino terminus RHD thereby preventing RelA from recognizing its DNA binding site. Alternatively, TMP could be interacting with the DNA, preventing RelA from occupying its binding sites. In support of this hypothesis, Chen et al., [13] have shown that TMP can bind the HSV ICP4 promoter and prevent Sp1 binding. Additionally, compounds with structures similar to TMP; 3'-O-methyl NDGA [13, 32] and tetra-O-glycyl-NDGA [33] have been shown to bind DNA and prevent Sp1 binding. The recent finding that Sp1 can directly bind to certain NF- κ B sites on the DNA [34] also supported this hypothesis and raised the possibility that it is the same activity of TMP that is responsible for both RelA and Sp1 inhibition of binding. However, the results of our EMSA experiments did not support this hypothesis. TMP did not interfere with the ability of RelA to bind its cognate site when TMP was incubated with cells prior to nuclear extract preparation. Similarly, TMP did not inhibit RelA:DNA binding when it was added *in vitro* to the nuclear extracts and DNA. We conclude, therefore, TMP is working indirectly, upstream of DNA binding in the NF- κ B pathway to prevent RelA from loading its promoter following LPS stimulation.

We next considered the hypothesis that TMP inhibits the signaling pathway that results in RelA translocation into the nucleus. TLR3/8 transcription was blocked

more effectively than was TLR4. Since both TLR3 and 8 are localized to endosomal compartment this difference could suggest an effect of TMP on endocytosis. However, the phosphorylation of I κ B- α and nuclear translocation of RelA were not altered following treatment with TMP suggesting that TMP is affecting additional regulatory systems. The results of our experiments also showed that, in the presence of TMP, I κ B- α was resynthesized normally after treatment with LPS. Transcription of I κ B- α is dependent on RelA [31] suggesting that the effect of TMP is selective for only certain RelA:promoter interactions. Phosphorylation of RelA is a mechanism that has been shown to confer selectivity for certain promoters. For example, phosphorylation at Ser²⁷⁶ has been shown to be critical for transcription of IL-8 and GRO β /CXCL2 but not I κ B- α [35]. RelA which is phosphorylated at this site interacts with positive transcription elongation factor b (PTEF-b), which is required for IL-8 and GRO β /CXCL2 transcription but not I κ B- α [35]. Similarly, phosphorylation at Ser³¹¹ has been shown to regulate the interaction of RelA with other transcriptional coactivators such as cyclic AMP-responsive element binding protein/p300 and RNA polymerase II [36-38] while acetylation of RelA is also known to be a molecular switch that regulates its activity [39]. Clearly future experiments with TMP will need to evaluate its effects on the post-translational modification of RelA.

The range of inhibitory effects seen with TMP with different cytokines and chemokines may arise from the differential requirements of these genes for the various modified forms of RelA as discussed above. Alternatively, the variation

might stem from the degree to which NF- κ B RelA is required for transcription of each gene. For example, several groups have reported that transcriptional activation of the TNF- α and MCP-1/CCL2 genes is strongly dependent on the trans-activating activities of NF- κ B RelA [17, 40], likely explaining the strong inhibition of these molecules by TMP. Similarly, inhibition of NF- κ B RelA binding might explain the strong inhibition of G-CSF production by TMP we noted previously [14]. NF- κ B binding sites have been shown to be present at the G-CSF promoter (CSF box) [41] and nuclear factors have been shown to associate with these sequences. In contrast, TMP only weakly inhibited production of IL-6, MIP-1 α /CCL3, and RANTES/CCL5 [14]. It is possible that for these genes, although NF- κ B sites are present in their promoters, their transcription in RAW 264.7 cells treated with LPS is not predominantly dependent on NF- κ B. Transcription of IL-6, for example, can be entirely dependent on NF-IL-6 (C/EBP β) [42]. Similarly, while the MIP-1 α /CCL3 LPS response element does contain an NF- κ B c-rel binding site it also contains four C/EBP family binding sites [43]. For RANTES/CCL5, although Fessele, et. al. [44] reported that NF- κ B is essential for LPS-induced transcription in mono mac 6 cells [44] Shin et. al. [45] observed that NF- κ B is not required for its LPS-induced transcription in RAW 264.7 cells [46] (the cells we used in our investigation). In agreement, our ChIP assays showed complete inhibition of RelA binding to the RANTES/CCL5 promoter, while at the same time levels of RANTES/CCL5 mRNA and protein were not blocked by TMP [14].

In addition to the effects we noted on NF- κ B, in our experiments we also noted an effect of TMP on the steady state levels of MCP-1/CCL2 mRNA (Fig. 1D). To our knowledge, post-transcriptional regulation of MCP-1/CCL2 mRNA has not been reported. It is possible, that the effects of TMP may be related to the normal regulation of this mRNA. If levels of TTP-mediated degradation are normally low, they may be masked by the high levels of LPS-induced MCP-1/CCL2 transcription and only revealed when transcription is effectively blocked by TMP. In support of this hypothesis, MCP-1/CCL2 mRNA does contain the TTP AUUUA recognition site in its 3' untranslated region. It is also possible that TMP could be modifying TTP or the 3' untranslated region to enhance rates of degradation. If so, then one might also predict enhanced rates of TNF- α message degradation, which did not occur.

In summary, we have examined the effects of TMP on NF- κ B activation, translocation and binding. We report that TMP inhibited NF- κ B-dependent transcription and NF- κ B RelA binding at the promoters of TNF- α , MCP-1/CCL2, and RANTES/CCL5. Since NF- κ B RelA-dependent transcription is critical to numerous inflammatory and pathological responses, TMP might be useful to treat a variety of disorders. The safety of TMP has been established in several clinical trials, and testing for efficacy in inflammation should begin immediately.

This work was published in the Journal of Inflammation (London). 2010 Dec 7;7:59. Sikes, ML, Scolle, F, Wilson, JR and Laster, SM are also listed as authors on this manuscript.

Figure 1: TMP inhibits TNF- α and MCP-1/CCL-2 protein and mRNA.

RAW 264.7 cells were either stimulated with 1 μ g/ml of LPS or 1 μ g/ml of LPS and 25 μ M TMP. Following 24 h of treatment, supernatants were collected and levels of TNF- α (A) and MCP-1/CCL2 (B) were determined by ELISA. To assess the effects of TMP on the transcription of TNF- α (C) and MCP-1/CCL2 (D) genes, RNA was prepared from RAW 264.7 cells stimulated with 1 μ g/ml of LPS or 1 μ g/ml of LPS and 25 μ M TMP for the indicated time periods. Quantitative RT-PCR was used to analyze the levels of TNF- α and MCP-1/CCL2 mRNA. Asterisks indicate significant differences between treatments with LPS and LPS and TMP ($p < 0.05$, T-test).

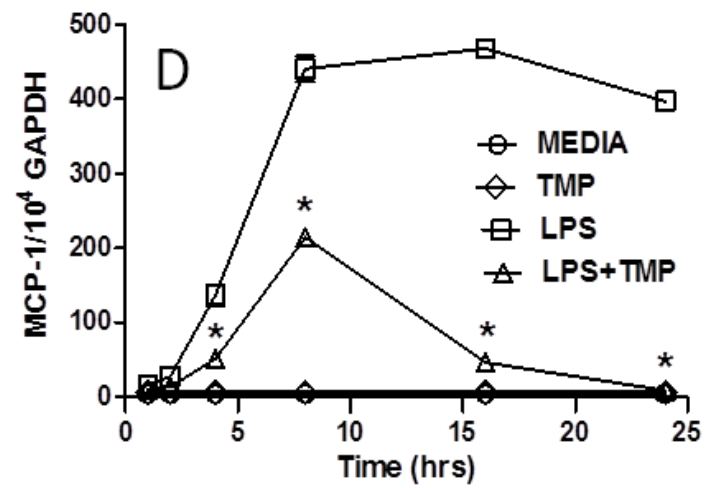
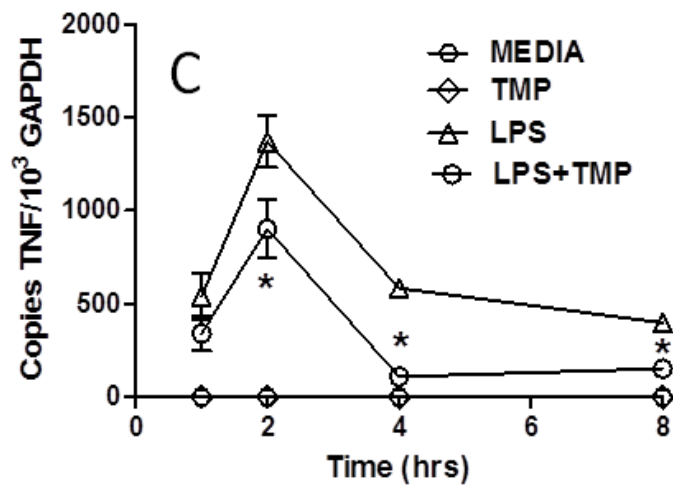
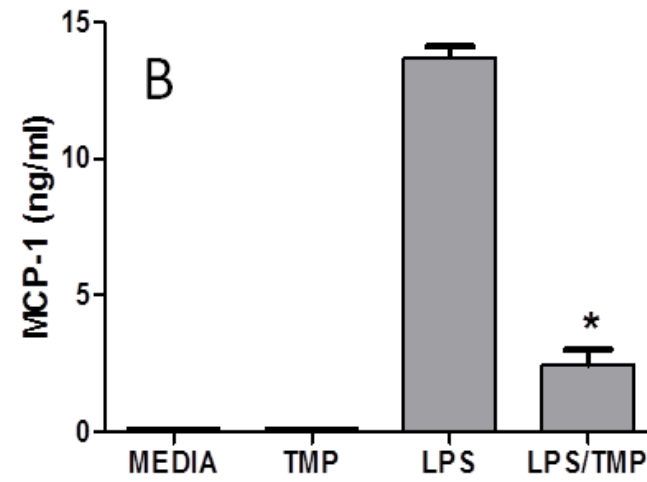
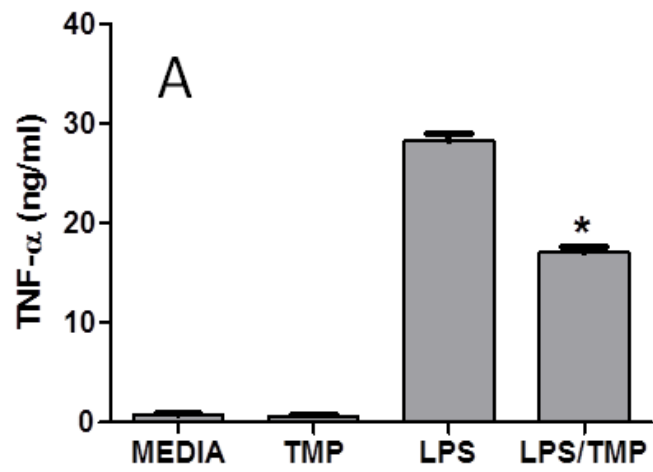


Figure 2: TMP represses NF- κ B dependent reporter activity.

The effect of TMP on LPS induced TLR4 signaling was evaluated by reporter analysis. HEK293/TLR4-YFP/MD2 cells were co-transfected with NF- κ B-Luc and β -gal control plasmids then, after 4 hours of treatment, luciferase activity was measured in cell lysates (A). To analyze the effects of TMP on other TLR family members HEK293/TLR3 (B) and HEK293/TLR8 (C) cells were co-transfected as above, treated for four hours with 10 μ g/ml poly(IC) (B) or 1 μ g/ml R-848 (C) and/or 25 μ M TMP, and luciferase activity determined in cell lysates. Each experiment was performed at least 3 times and representative experiments are shown. Asterisks indicate significant differences between ligand treatments and ligand treatments with TMP ($p < 0.05$, T-test).

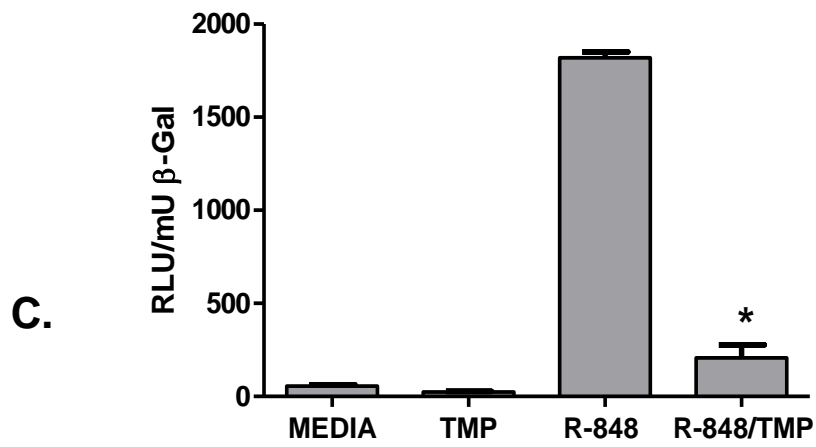
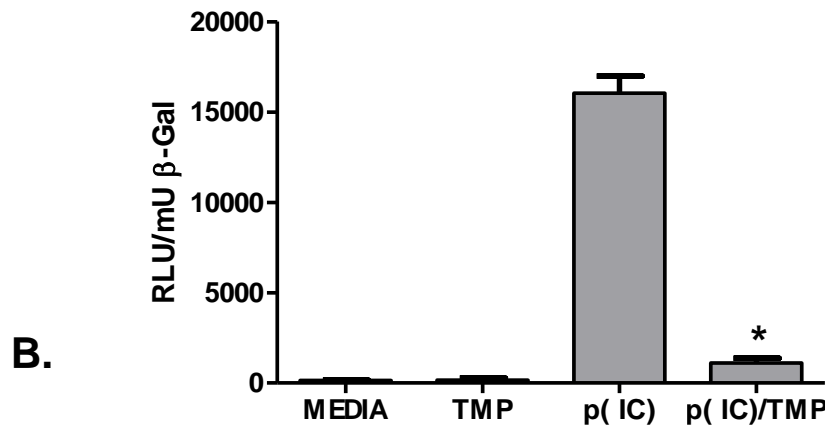
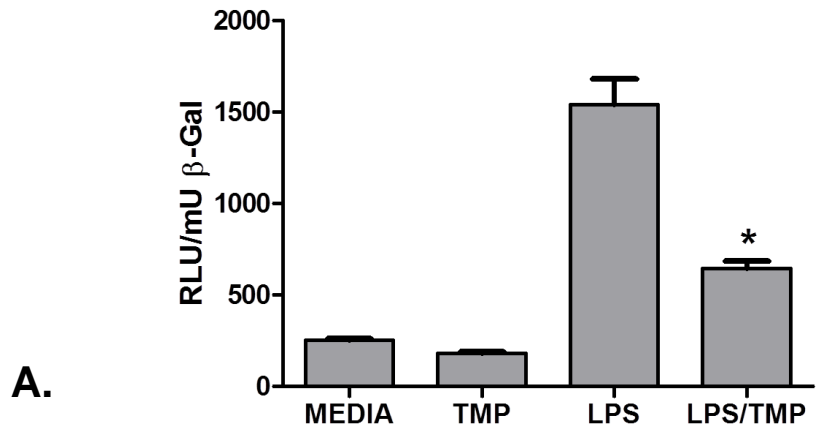
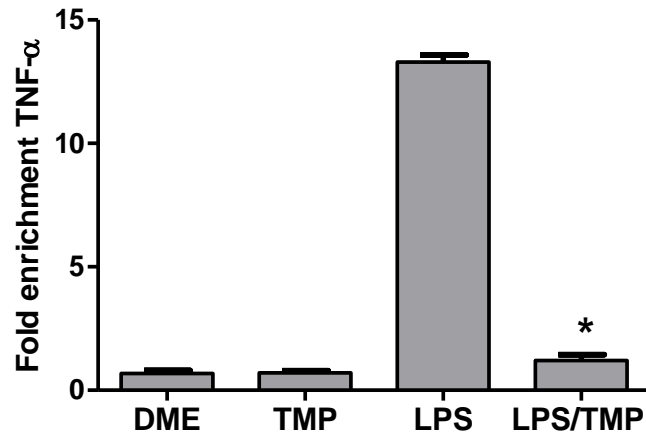


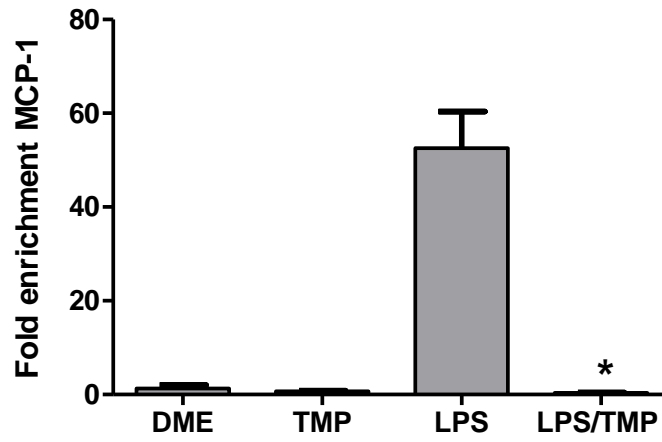
Figure 3: TMP inhibits RelA DNA binding

RAW 264.7 cells were either stimulated with 1µg/ml of LPS or 1µg/ml of LPS and 25µM TMP for 4 hours. Following treatment, protein:DNA complexes were cross-linked, and RelA binding at the TNF- α (A), MCP-1/CCL2 (B) and RANTES/CCL5 (C) promoters was assessed by chromatin immunoprecipitation. Enrichment was calculated relative to pre-IP input control levels and was normalized to signals obtained with non-specific IgG control antibodies. Data shown are representative of two independent experiments and chromatin preparations. Asterisks indicate significant differences between LPS treatments and LPS treatments with TMP ($p < 0.05$, T-test).

A.



B.



C.

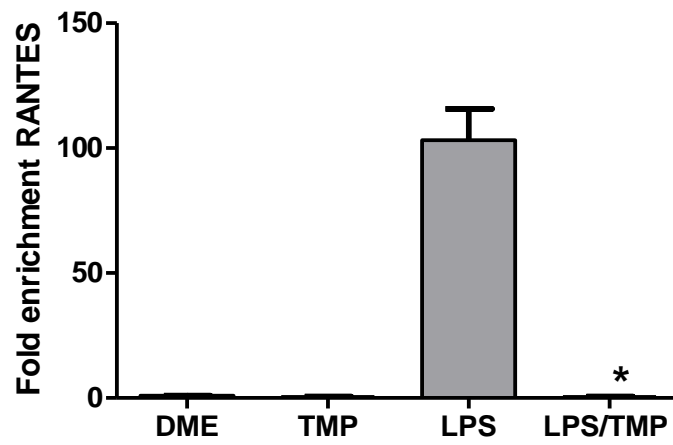


Figure 4: TMP does not impair NF κ B binding *in vitro* to the TNF- α promoter.

(A) Nuclear extracts from untreated (*lane 1*) RAW 264.7 cells or cells stimulated 4 hrs with 1 μ g/ml LPS (*lanes 2-6*) were incubated with a radiolabeled ds oligonucleotide probe to the κ B3 site of the TNF- α promoter. Probes were incubated with nuclear extract alone (*lanes 1 and 2*), in the presence of 100-fold molar excess of unlabeled wt (*lane 3*) or mutant κ B3 competitor oligonucleotides (*lane 4*), or in the presence of the indicated Abs (*lanes 5 and 6*). Specific nucleoprotein (*filled arrows*) and Ab-supershifted complexes (*empty arrows*) are indicated. (B) The impact of TMP on protein binding to TNF- α κ B3 (upper panels) or control NF- γ (bottom panel) was assessed in nuclear extracts from LPS-treated RAW 264.7 cells co-stimulated with TMP (*lane 3*) or upon addition of exogenous TMP to the binding reaction (*lanes 4-6*, 0.25 μ M, 2.5 μ M, and 25 μ M, respectively).

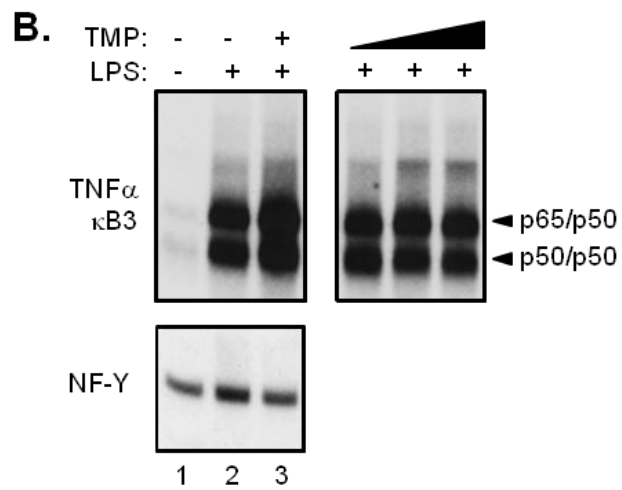
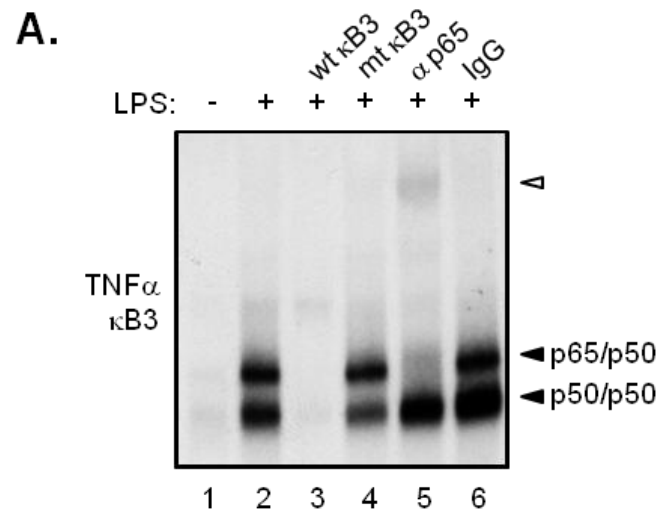


Figure 5: TMP does not prevent nuclear translocation of NF- κ B

Cells were either left untreated (A) or treated with LPS (1 μ g/ml) for 20 (B) or 60 min (C) then stained. In panel D, cells were treated with 25 μ M TMP for 60 min while panels D and F show treatments with LPS and TMP for 20 and 60 min, respectively. Following treatment cells were fixed, permeabilized and stained with anti-RelA Ab and a fluorescein coupled secondary Ab. Representative images from a single experiment are shown in A-F. For G, Photoshop (Adobe) was used to analyze images and determine mean fluorescence intensity for the nuclear region of 120 cells at each time point for each variable (20 cells from two fields from three independent experiments). Treatment with LPS and LPS in combination with TMP did not produce significant differences ($p < 0.05$, T-test).

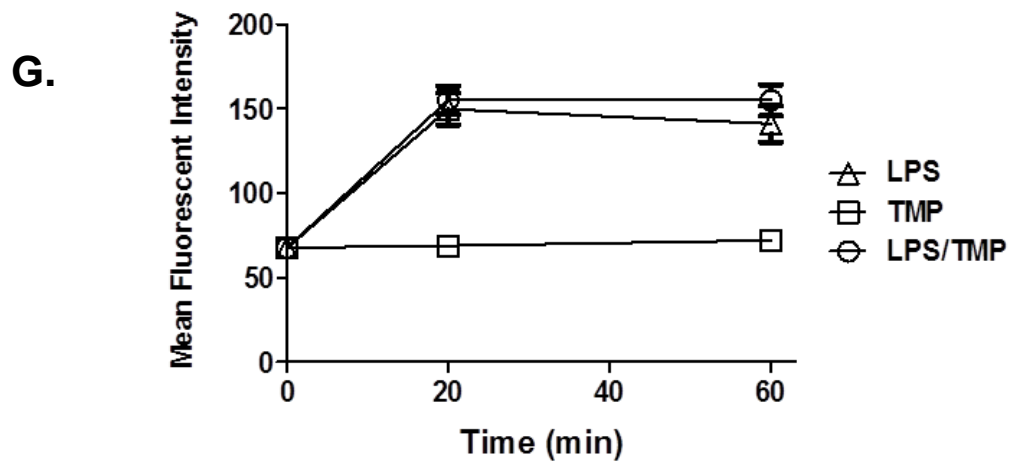
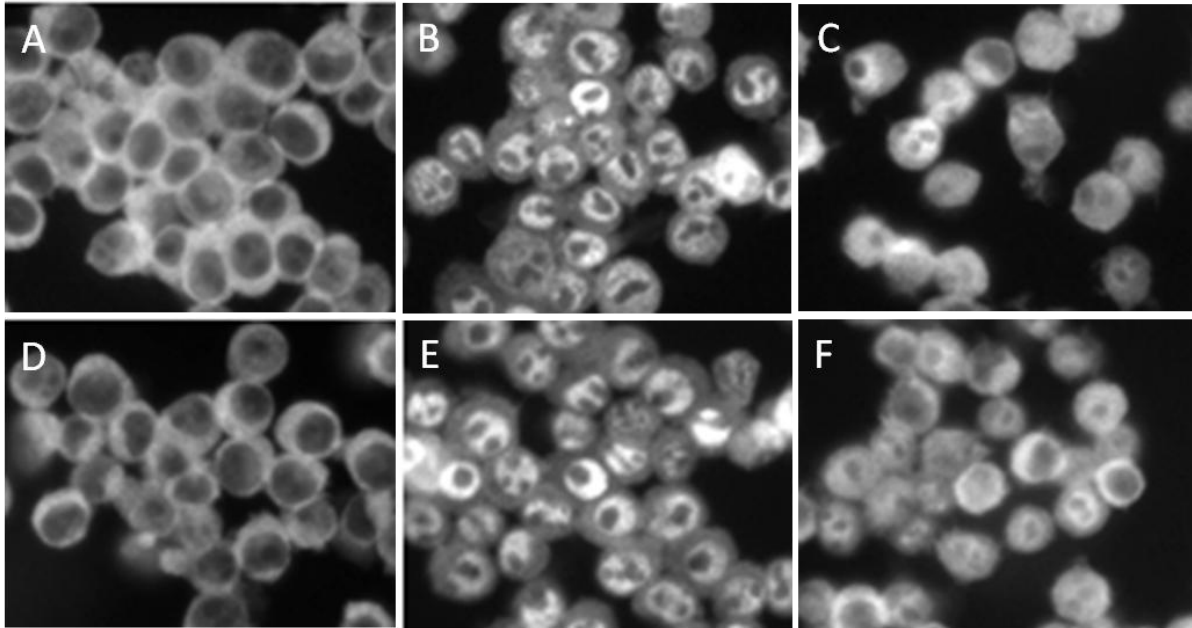
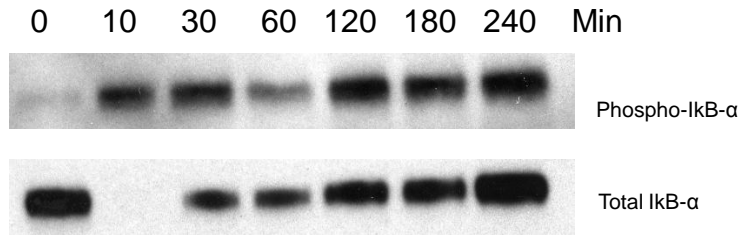


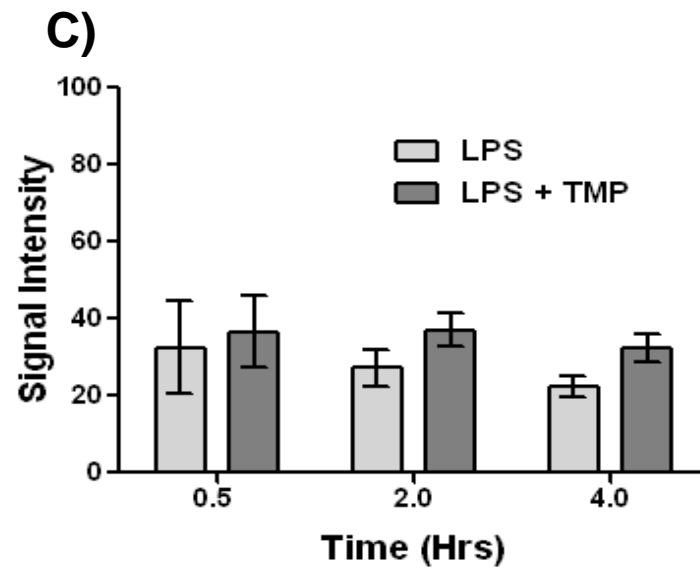
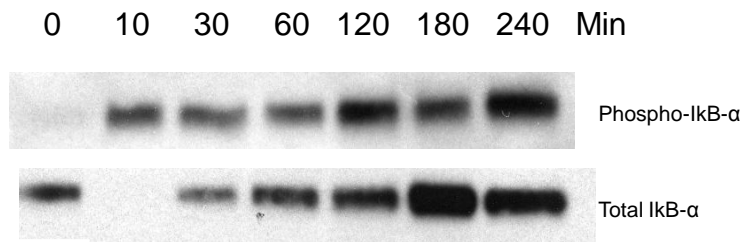
Figure 6: TMP fails to affect I κ B- α phosphorylation

RAW 264.7 cells were stimulated with either 1 μ g/ml of LPS (A) or 1 μ g/ml of LPS and 25 μ M TMP (B) for the indicated times. Lysates were prepared and analyzed by western blot with Abs specific for the phosphorylated serine 32 residue of I κ B- α and total I κ B- α . Representative experiments are shown in panels A and B. For densitometric analysis (C), phospho-I κ B- α blots were scanned and band intensity determined using Photoshop. Values shown are means \pm SEM from three independent experiments. Treatment with LPS and LPS in combination with TMP did not produce significant differences ($p < 0.05$, T-test).

A) LPS



B) LPS + TMP



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Chapter 3. Berberine acts extracellularly to inhibit influenza A-induced cytokine production in RAW 264.7 cells

1. Abstract

We have recently shown that the alkaloid berberine can inhibit the production of TNF- α from influenza A virus-infected macrophages-like cells. In this report we have investigated the mechanism underlying this effect. Our findings indicate that berberine does not affect accumulation of influenza A virus HA vRNAs and cRNAs, which serve as ligands for activation of host PRRs. Immunoblot analysis also revealed that berberine does not prevent activation of signaling kinases p38 or ERK1/2. Activation of the transcription factor NF- κ B and accumulation of the TNF- α mRNA in influenza were also unaffected by berberine. Furthermore, we found that the amounts of intracellular and membrane-associated TNF- α were unaffected by berberine. Our findings indicate that berberine is acting extracellularly to cause the loss of cytokine proteins in culture supernatants of influenza A virus-infected cells. A model is proposed for the activity of berberine that depends on its ability to modulate the activity of influenza A virus-induced cellular proteases.

2. Introduction

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae* and are characterized by segmented negative-stranded RNA genomes [1, 2]. IAVs infect alveolar macrophages as well as epithelial cells of the upper and lower respiratory tract, thereby leading to development of the influenza disease. Influenza is characterized by the manifestation of clinical symptoms and conditions including headaches, fevers, chills [3], sore throat [4], acute respiratory distress syndrome [5] as well as acute necrotizing encephalopathy [6]. Complications from seasonal outbreaks of influenza lead to significant morbidity, particularly among neonates and the elderly. The Centers for Disease Control and Prevention (CDC) currently estimates that approximately 23,000 people die annually in the United States from influenza-related complications (<http://www.cdc.gov/flu>).

Following influenza virus entry and uncoating in endosomal compartments, viral nucleic acids such as vRNAs, cRNAs and mRNAs are detected by a variety of pattern recognition receptors (PRRs) expressed on innate immune cells, including Toll-like receptors (TLR)-3 [7], TLR-7 [8], TLR-8 [9] as well as the RNA helicases, retinoic acid-inducible gene (RIG)-I [7, 10] and melanoma differentiation-associated gene (MDA)-5 [11]. Recognition of viral particles by PRRs results in activation of mitogen activated protein kinase (MAPK) pathways and transcription factors such as NF- κ B, AP-1, as well as the interferon regulatory factor (IRF) family members [11]. Collectively, these molecules coordinate a strong induction of pro-inflammatory

cytokines known as a “cytokine storm”, which has been linked to the destruction of healthy host tissue [12-17] and fatalities associated with IAV infections [1, 18].

Current treatments for influenza involve the use of pharmacological compounds such as amantadine and rimantidine, which limit viral replication by preventing virus uncoating within endosomal compartments [19]. Other anti-viral agents such as zanamivir [20] and oseltamivir, [19] inhibit viral budding by blocking the actions of neuraminidase an essential viral envelope antigen required for release of virus from the cellular apical membrane. According to the CDC, most seasonal strains of IAV are highly resistant to amantadine and rimantidine. In addition, the use of amantadine, rimantidine, zanamivir and oseltamivir have been linked to adverse clinical side effects including insomnia, anxiety and a host of central nervous system (CNS) disorders [21], (www.FDA.gov/drugs). Furthermore, these compounds do not curtail IAV-induced “cytokine storm” which is the main cause of fatalities associated with the disease. Consequently, vaccination has become the dominant approach for controlling IAV infections. However, IAV evolves constantly making it necessary to produce new vaccine strains on a yearly basis. As a result influenza vaccines do not display the near-sterilizing, lifelong immunity associated with most vaccines. Vaccine effectiveness ranges from 70-90% when vaccine and infectious strain are well matched. However, vaccine effectiveness drops to 50% in years when poor matching occurs. Although influenza vaccines are efficacious they are contraindicated in neonates and the elderly, two of the most susceptible groups to

IAV infections [22, 23]. For these reasons, it is imperative to discover new and safer compounds that can be used to treat influenza.

Plants such as Goldenseal (*Hydrastis canadensis*), barberry (*Berberis vulgaris*), coptis (*Coptis chinensis*) and Oregon grape (*Mahonia aquifolium*) have been used for centuries to treat a variety of disorders including skin and eye infections, upper respiratory disorders, diarrhea, and cancer [24]. The medicinal effects of these plants have been attributed to the presence of the isoquinolone alkaloid, berberine [25]. Accordingly, various studies have revealed that berberine is a potent antitumor, antimicrobial and anti-inflammatory drug [26-30]. Berberine has been shown to limit the growth of tumors in certain tumor derived cell lines [31-34]. Berberine has also been shown to inhibit the growth of certain bacteria [27, 30, 35] and several viruses including human cytomegalovirus [36] and herpes simplex virus [37]. Other reports have also demonstrated that berberine suppresses the production of microbially-induced pro-inflammatory molecules, including cytokines, prostaglandins and nitric oxide from macrophages [38-41].

Recently, we investigated the effects of berberine on replication of IAV *in vitro*. These studies revealed that berberine strongly suppressed the growth of influenza virus in both murine and human cell lines. These studies also revealed that berberine inhibited the production of influenza A-induced pro-inflammatory molecules, including TNF- α and prostaglandin E₂ [42] and that this effect appeared separate from the effect on virus replication. Wu *et al.* [43] have also reported inhibition of IAV and cytokine production by berberine *in vivo*, using a mouse model.

In this current report, we have investigated the molecular mechanisms by which berberine inhibits IAV-induced cytokine production. We investigated several areas of the TNF- α production pathway including signal transduction, transcription, and production of the protein itself. Our studies suggest that berberine does not act intracellularly to inhibit production of TNF- α . Preliminary studies indicate that berberine could be acting extracellularly in the culture medium to promote the loss of cytokine proteins by modulating the activity IAV-induced cellular proteases present in culture supernatants.

3. Materials and methods

3.1 Cells, media, reagents and virus infections

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modification of minimal essential medium (DMEM) with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate with 10% FCS. Media and supplements were obtained from Sigma-Aldrich, St. Louis, MO and Cellgro, Manassas, VA. FCS was obtained from Atlanta Biologicals, Atlanta, GA and Cellgro. Berberine was purchased from Sigma-Aldrich and used at a concentration of 25 μ M. Water was used as the solvent for berberine in all experiments. LPS from *Salmonella Minnesota* R595 was purchased from LIST

Biological Laboratories, Inc. (Campbell, CA). All experiments were performed using 1µg/mL of LPS.

Influenza virus strain A/PR/8/34 was originally obtained from the American Type Culture Collection (Manassas, VA). Virus stocks were prepared by infecting MDCK cells at a multiplicity of infection (moi) of 0.001 (1 infectious particle per thousand cells). The virus was added to cells for 30 min in a small volume of serum free virus growth media (DMEM with 4 mM l-glutamine, 4.5 g/L glucose, and 3.0 g/L sodium bicarbonate and supplemented with 0.2% BSA, 2 µg/ml Trypsin-TPCK and 25 mM HEPES buffer) followed by the addition of fresh virus growth media and incubating for 36–48 h or until cells displayed 90+% cytopathic effect (CPE). Cell supernatants were collected, cell debris was removed by centrifugation (1000 rpm for 10 min), aliquots prepared, and stored at – 80 °C. For production of experimental supernatants, viruses were added to cells at high (5) moi following the same protocol. Berberine and/or extracts were added with the virus and supernatants were collected at the indicated time points. VEFSM purchased from Gibco, (Carlsbad, CA) was used for serum free virus infections and supplemented as above.

3.2 Cytokine Measurements

TNF-α, MCP-1 and IL-6 ELISA kits were purchased from eBioscience (San Diego, CA). RAW 264.7 cells were infected with influenza virus strain A/PR/8/34 (H1N1) in the presence or absence of 25µM berberine. Supernatants were collected and cytokine levels were determined by ELISA according to manufacturer's

instructions. In each case, sample values were interpolated from standard curves. Optical density was determined using a PolarStar microplate reader (BMG Labtechnologies, Durham, NC).

3.3 Western blot analysis and SDS-PAGE

After treatments, cell monolayers were washed twice with cold phosphate buffered saline (PBS), solubilized in lysis buffer (50 mM Hepes, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS) and collected by scraping. The protein concentration for each sample lysate was determined using the Pierce BCA system (Pierce, Rockford, IL). Equal protein samples (25 μ g) were loaded on 12% Tris-Glycine gels and subjected to electrophoresis using the Novex Mini-Cell System (Invitrogen). Following transfer and blocking, blots were probed with antibodies specific for TNF- α , phospho-p65, phospho-p38, phospho-p42/44, p42/44, p38, p65 and β -actin. All antibodies were purchased from Cell Signaling (Beverly, MA). Bands were visualized using the SuperSignal Chemiluminescent system (Pierce). For silver staining, supernatants were collected and resolved on a conventional 1.5mm, 12.5% polyacrylamide gel by electrophoresis. Gels were stained using a silver staining kit (GE, Fairfield, CT) according to manufacturer's instructions.

3.4 Quantitative RT-PCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. Residual genomic DNA was eliminated using on-column DNase digestion with the RNase-free DNase set (Qiagen) and resulting extracts were resuspended in nuclease free water. Amount and purity of RNA was determined using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). RNA (1 µg) was denatured and reverse transcription was performed with the Improm II reverse transcription kit (Promega, Madison, WI) in a reaction mix containing random hexamers as primers (50 ng/µl) for 60 min at 42°C. The iQTM SYBR Green supermix kit (BioRad, Hercules, CA), was used for Real-time PCR analysis. cDNA was amplified using primers specific for murine GAPDH and TNF-α genes. Primer combinations are GAPDH [antisense: 5' ATG TCA GAT CCA CAA CGG ATA GAT 3'; sense: 5' ACT CCC TCA AGA TTG TCA GCA AT 3']; TNF-α [antisense: 5' AGA AGA GGC ACT CCC CCA AAA 3'; sense: 5' CCG AAG TTC AGT AGA CAG AAG AGC G 3']. All primer pairs were purchased from Integrated DNA Technologies (Coralville, IA). PCR was performed in 96 well plates (Eppendorf AG, Hamburg, Germany). Samples were amplified for a total of 50 cycles, followed by a meltcurve analysis to ensure the specificity of reactions. GAPDH control was analyzed per plate of experimental gene to avoid plate-to-plate variation. Fold change was determined using the delta-delta CT method for samples performed in triplicates.

3.5 Amplification of HA vRNA and cRNA by RT-PCR

Amplification of HA vRNA and cRNA have been previously described by Uchide *et al* [44]. Briefly, cells infected with influenza virus strain A/PR/8/34 (H1N1) in the absence or presence of 25 μ M berberine were washed with PBS. Total cellular RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. Residual genomic DNA was eliminated using on-column DNase digestion with the RNase-free DNase set (Qiagen) and resulting extracts were resuspended in nuclease free water. cDNA was synthesized from 1 μ g of total cellular RNA using 500 pmol of primers targeting the nucleotide positions 385-404 of the influenza vRNA (Sense primer : 5'-TGA GGG AGC AAT TGA GCT CA-3') and 815-796 of the influenza cRNA (Antisense primer: 5'-TGC CTC AAA TAT TAT TGT GT-3'). For β -actin controls, cDNA was synthesized from 1 μ g of total RNA using oligo dTs (Qiagen, Valencia, CA) as primers. Reverse transcription was performed with the Improm II reverse transcription kit (Promega, Madison, WI) for 60 min at 42°C. One μ l of the synthesized cDNA solution was used in a PCR reaction containing 20mM Tris-HCL, 50mM KCL, 2.5 mM MgCl₂, 0.2 mM each of deoxyribonucleoside triphosphates and 0.5 μ M each of the sense and antisense primers. PCR products were resolved by electrophoresis on 2.0% agarose gel using TBE buffer.

3.6 Quantification of Intracellular TNF- α by ELISA

Cells infected with influenza virus strain A/PR/8/34 (H1N1) in the absence or presence of 25 μ M berberine were washed twice with PBS, solubilized in lysis buffer (50 mM Hepes, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.1% SDS) and collected by scraping. Lysates were centrifuged for 30 minutes at 14,000 RPM, resulting supernatants were collected and levels of TNF- α were determined by ELISA as per manufacturer's instructions.

3.7 Intracellular cytokine staining and flow cytometry

For intracellular cytokine staining, cells infected with IAV in the presence or absence of 25 μ M berberine or Brefeldin A (BioLegend, San Diego, California) were washed twice with PBS and collected by scraping. Cells were either permeabilized or immediately incubated with antibody. For intracellular cytokine staining, permeabilized cells were incubated in Cytofix/Cytoperm buffer (BD Bioscience, Franklin Lakes, New Jersey) at 4°C for 20 minutes. Cells were washed twice with cytofix/cytoperm wash buffer (BD Bioscience) and incubated with an anti-mouse TNF- α antibody conjugated to Alexa-Fluor® 488 (BioLegend) for 30 minutes on ice. For TNF- α surface staining, live unpermeabilized cells were washed twice and collected by scraping. Cells were washed twice in 1% BSA and then incubated with an Alexa-Fluor® 488 conjugated anti-mouse TNF- α for 30 minutes on ice. 10,000-

gated events were collected per sample and analyzed on an Accuri C6 Flow cytometer (BD accuri Cytometers, Ann Arbor, MI) equipped with 488 nm lasers.

3.8. Statistical Analysis

All graphs and statistical analyses were produced using Prism software (GraphPad Software Inc, La Jolla CA).

4. Results

4.1 Berberine suppresses IAV-induced TNF- α protein levels

We have previously reported that berberine suppresses levels of TNF- α protein in RAW 264.7 cells infected with IAV strain PR/8/34 (H1N1) at 12 and 24 hrs [42]. As a first step in defining the mechanism underlying this effect we performed a more complete examination of the effects of berberine throughout a 24 hour infection period. RAW 264.7 cells were infected in the presence or absence of 25 μ M berberine and levels of TNF- α in culture supernatants were measured by ELISA. As shown in Fig. 1, IAV rapidly and continuously induced the release of TNF- α throughout the 24 hr period. In the presence of berberine we found that levels of the TNF- α protein were unaffected at early time points (1, 3 and 6 h) while 35% and 70% percent reductions in levels of TNF- α were measured at 12 and 24 h time

points, respectively (Fig. 1). These results show that the suppression of IAV-induced TNF- α protein levels by berberine, does not occur until later time periods.

4.2 Berberine does not affect the levels of HA vRNAs and cRNAs

To define the mechanism underlying this effect we examined several aspects of the IAV-induced TNF- α production pathway. First, we investigated the effects of berberine on the production of IAV vRNAs and cRNAs, which serve as ligands that activate TNF- α production [45, 46]. RAW 264.7 cells were infected with A/PR/8/34 for the indicated time periods in the presence or absence of 25 μ M berberine. Total RNA was extracted, and viral cDNAs were generated using primers specific for either v- or cRNA that encode the IAV HA protein. Viral cDNAs were amplified by PCR and PCR products were examined to detect of HA v and cRNAs. As shown in Fig. 2, we found that HA v and cRNAs were detectable as early as 1 hr post infection and these levels increased at the 12 hr time point (Fig. 2). In the presence of 25 μ M berberine, we found that levels of HA v and cRNAs remained unchanged (Fig. 2) suggesting that berberine does not inhibit synthesis of the IAV RNAs that activate the innate response.

4.3 Berberine does not affect IAV-induced signaling pathways

Our next set of experiments investigated the effects of berberine on signaling pathways activated by IAVs that lead to transcription of TNF- α . These experiments focused on the activation of MAPK proteins p38 and ERK1/2, as well as the

transcription factor NF- κ B, because of their role in regulating IAV-induced TNF- α production [47-50]. RAW 264.7 cells were infected with IAV in the presence or absence of 25 μ M berberine and activation of p38, ERK1/2 and NF- κ B were evaluated by immunoblot analysis. As expected, we found strong activation of p38, ERK1/2 and NF- κ B (p65) throughout the 24 hr experiment (Fig. 3A). In the presence of 25 μ M berberine these responses were not inhibited suggesting that berberine does not block IAV-induced kinase or transcription factor activation. We also examined the effects of berberine on the expression of the TNF- α mRNA itself. As shown in Fig. 3B, Q-RT-PCR analysis revealed that in IAV-infected cells, levels of TNF- α mRNA peaked an hour after the infection was initiated and then generally declined during the remainder of the experiment. Again, treatment with 25 μ M berberine did not inhibit this response, in fact a small stimulatory effect was noted at most time points. Taken together, these findings indicate that activation of signaling molecules such as p38 and ERK1/2 as well as the transcriptional activation of the TNF- α gene by NF- κ B in influenza A virus-infected cells are unimpaired by berberine.

4.4 Berberine does not affect synthesis of the TNF- α protein

In this set of experiments we examined the effects of berberine on production of the TNF- α protein. TNF- α mRNA is translated on membrane associated ribosomes, transported to the cell surface as an integral membrane protein, and then liberated from the cell surface by the metalloprotease TNF- α converting enzyme

(TACE) [51]. Flow cytometry was used to quantify levels of TNF- α protein at several points in its production pathway. As a positive control, to validate the sensitivity of this technique, we first examined the effects of brefeldin A, a compound known to inhibit protein transport through the ER/Golgi and cause intracellular protein accumulation. As shown in Fig. 4A and B, in the presence of brefeldin A, we were able to measure a threefold increase in levels of intracellular TNF- α in IAV-infected cells. As shown in Fig. 4C and D, berberine did not cause a significant change in levels of intracellular TNF- α , suggesting that it is not affecting synthesis of this protein. This result was confirmed using two additional techniques. As shown in Fig. 5A and 5B, immunoblot and ELISA assays performed with whole cell lysates did not reveal any effect of berberine on levels of intracellular TNF- α .

Flow cytometry with live cells was also used to test whether berberine was causing accumulation of TNF- α on the cell membrane. As shown in Fig. 4E and F, infection IAV caused a strong increase in levels of surface-associated TNF- α . Again, we found that surface levels of TNF- α were unchanged in the presence of berberine suggesting that berberine is not blocking either insertion into, or release of TNF- α from the cell membrane. We conclude, therefore, that berberine inhibits levels of TNF- α by exerting an effect on the protein after it is secreted into the culture medium.

4.5 Berberine inhibits IAV-induced IL-6 and CCL2/MCP-1 protein levels

To determine whether this effect is specific for TNF- α we also tested the effects of berberine on two other IAV-induced molecules, the cytokine IL-6 and the chemokine CCL-2/MCP-1. RAW 264.7 cells were infected with IAV in the presence or absence of 25 μ M berberine and levels of MCP-1 and IL-6 in culture supernatants were measured by ELISA. As shown in Fig. 6A, in control influenza A-infected cells, the pattern of IL-6 production differed from that seen with TNF- α . Levels of IL-6 in culture supernatants peaked at 6 hr, remained elevated until 12 hr, and then decreased during the remainder of the experiment. However, consistent with our findings for TNF- α , berberine did not affect the early phase of this response but did reduce levels of IL-6 at later time points (30, 40, and 85% reductions at the 6, 12, and 24 hr time points, respectively).

IAV also strongly induced production of the chemokine CCL-2/MCP-1 (Fig. 6B), with a pattern similar to that of TNF- α . Levels of CCL-2/MCP-1 increased rapidly during the first hour following infection, then continued to increase steadily throughout the 24 hr experiment (Fig. 6B). Again, berberine treatment did not affect the early phase of this response, however, a strong inhibition of MCP-1 accumulation was observed at later time points (Fig. 6B).

The non-specific effects of berberine on protein accumulation in culture supernatants can also be observed using SDS-PAGE. Culture supernatants were collected, resolved by electrophoresis on 12.5% polyacrylamide gels and proteins were visualized by silver staining. As shown in Fig. 7, lanes 1 and 2, because IAV-

infections are performed in serum free media supplemented with 0.5% BSA, very few proteins are observed in supernatants from either mock- or berberine-treated cells. In contrast, a large number of proteins are observed in supernatants from cells infected with IAV (lanes 4, 6, and 8). Several of these likely originate in the viral inoculum (lane 10) which is a supernatant harvested from infected MDCK cells. On the other hand a number of these proteins do not appear in the inoculum and represent newly synthesized viral and cellular proteins. In the presence of berberine, we found a decrease in the intensity of many of the protein bands present in supernatants from infected cells (Fig. 7, lanes 5, 7, and 9). And in agreement with previous results this effect was most pronounced at 12 and 24 hr time points. Taken together these results suggest that berberine reduced the levels of many proteins in supernatants from IAV-infected cells.

4.6 IAV infection is required for berberine-mediated inhibition of TNF- α accumulation

We also investigated whether berberine can suppress production of TNF- α following stimulation with other PRR-ligands. A set of experiments was performed with berberine comparing its effects on RAW 264.7 cells infected with IAV or treated with LPS. As shown in Figs. 8A and B, while berberine strongly inhibits production of TNF- α from IAV-infected cells it did not block production of TNF- α from cells treated with LPS suggesting that infection with IAV may be a prerequisite for berberine's activity. Complicating this conclusion may be the different media used to

perform the two sets of experiments. Experiments with LPS are performed in media with 10% FCS as opposed to the serum-free media supplemented with BSA used in the virus experiments. Therefore, we tested the effects of berberine on TNF- α production with LPS stimulation in serum-free medium supplemented with BSA. LPS-treatments performed under these conditions induced lower amounts of TNF- α (Fig. 8C) but again berberine did not cause a substantial reduction in the amount of TNF- α indicating that the failure of berberine to inhibit the LPS-induced levels of TNF- α is unrelated to the media used to perform these experiments.

5. Discussion

We have recently reported that berberine will inhibit the growth of IAV and the production of PGE₂ and TNF- α from IAV-infected cells [42]. In this current report, we have focused on elucidating the mechanism underlying the inhibition of TNF- α accumulation. Our findings demonstrate that berberine does not affect ligand-induced activation of PRR signaling pathways that lead to transcription of the TNF- α gene. We also show that synthesis and membrane association of TNF- α are unaffected by berberine. Our findings indicate that berberine is acting extracellularly to cause the loss of TNF- α from culture supernatants. Furthermore, we show that this effect occurs in cultures of RAW 264.7 cells infected with IAV but does not occur in cells stimulated with LPS.

A number of studies have reported modulation of MAPK pathways by berberine [52, 53]. Berberine has been shown to suppress activation of p38 in LPS-stimulated RAW 264.7 cells and peritoneal macrophages [54] as well as α -CD3/28 stimulated NOD CD4+ cells [52]. Furthermore, Jeong *et al.* [53] have reported that berberine prevents ERK1/2 activation in leishmania-infected peritoneal macrophages as well as LPS-stimulated RAW 264.7 cells and peritoneal macrophages [54]. Based upon these findings, we initially hypothesized that inhibition of MAPK signaling by berberine would explain the suppression of TNF- α production. In our experiments, berberine did not exert an inhibitory effect on the activation of these kinases. In agreement with our results, Jones *et al.* [55] did not find an effect of berberine on p38 activation in LPS-stimulated human peripheral blood monocytes (PBMCs). Similarly, berberine did not block p38 activation in LPS- or IFN- γ -stimulated BV-2 microglial cells [56, 57] or activation of ERK1/2 in 3T3-L1 adipocytes [57]. At present it is unclear why different effects on MAP kinases have been reported. However, it is possible that berberine regulates signaling pathways differently in distinct cell types. Alternatively, the effects of berberine on activation of these kinases may also be ligand specific.

Similarly, based on published reports [58-60], we hypothesized that berberine could be inhibiting the activation of NF- κ B and transcription of the TNF- α gene. Berberine has been shown to prevent activation of NF- κ B in kidney cells of alloxan-induced diabetic mice [60] and LPS-stimulated rat mesangial cells [59]. Experiments by Rempiss *et al.* [61] have also demonstrated that millimolar concentrations of

berberine block the activation of NF- κ B in LPS-stimulated RAW 264.7 cells. We have not observed these effects in influenza A-infected RAW 264.7 cells using micromolar concentrations of berberine. In agreement with our findings, a number of investigators have also failed to find an effect of berberine on the activation of NF- κ B. For example, Enk *et al.* [62] have shown that berberine does not prevent activation of NF- κ B in TNF- α -stimulated human keratinocytes. Jeong *et al.* [54] have demonstrated that berberine inhibits accumulation of TNF- α mRNA in LPS-stimulated RAW 264.7 cells. However, we did not observe these inhibitory effects on transcript levels of TNF- α in IAV-infected cells. Again it is unclear why these results vary, however, it is possible that they may stem from cell type and ligand specific effects of berberine.

Limited proteolysis of the viral envelope protein hemagglutinin (HA) is necessary for IAV infectivity and propagation [63-65]. IAV does not code for HA-processing proteases within its genome, therefore, it induces the production of various cellular proteases including trypsins [66-68] and trypsin-type proteases [69, 70] that convert HA₀ into HA₁ and HA₂ [71]. IAV-induced cellular proteases have also been shown to act on various cellular targets [72]. For instance, cellular trypsins, type II transmembrane proteases (TTSPs), plasmin [73] and MMP-9 have been shown to proteolytically degrade proteins in the extracellular matrix [73, 74], cleave cell surface proteins, interact with cell surface and soluble ligands [72], initiate signaling cascades [75], and activate other cellular proteases [76-78]. We hypothesize that berberine is acting in concert with these proteases to induce

cytokine and chemokine degradation in culture supernatants of IAV-infected cells. A model depicting this hypothesis is shown in Fig. 9. It is possible that berberine is binding to cytokine proteins and inducing a conformational change, which increases their susceptibility to IAV-induced proteases (Fig. 9A). Alternatively, berberine could be interacting with influenza-induced proteases to alter their specificity and function thereby resulting in the enhanced targeting of cytokine proteins for degradation (Fig. 9B). In support of this hypothesis, berberine and other alkaloids have been shown to bind the S1 pocket of cellular trypsins and other secreted-type serine proteases [79]. Future studies will use inhibitors that are specific for IAV-induced cellular proteases to test these hypotheses. Additionally, future studies will also use circular dichroism spectra to evaluate the interaction of berberine with specific proteases or cytokines that are present in culture supernatants of IAV-infected cells. In contrast to IAV, LPS treatments have not been shown to induce extensive production of serine proteases. In fact, increased expression of various serine protease inhibitors including alpha 1 proteinase inhibitor have been observed in LPS-stimulated human macrophages [80]. Taken together, these findings could explain why berberine does not inhibit TNF- α production by LPS-stimulated RAW 264.7 cells.

In summary, we have demonstrated that berberine is a broad suppressor of IAV-induced cytokine production, although, the exact mechanism by which berberine suppresses production of these inflammatory mediators is still unclear. Our results indicate that berberine is likely acting extracellularly to cause the loss of cytokines from the culture supernatants of IAV-infected cells. To our knowledge this effect

would be novel and open numerous possibilities for the use of berberine to inhibit inflammation.

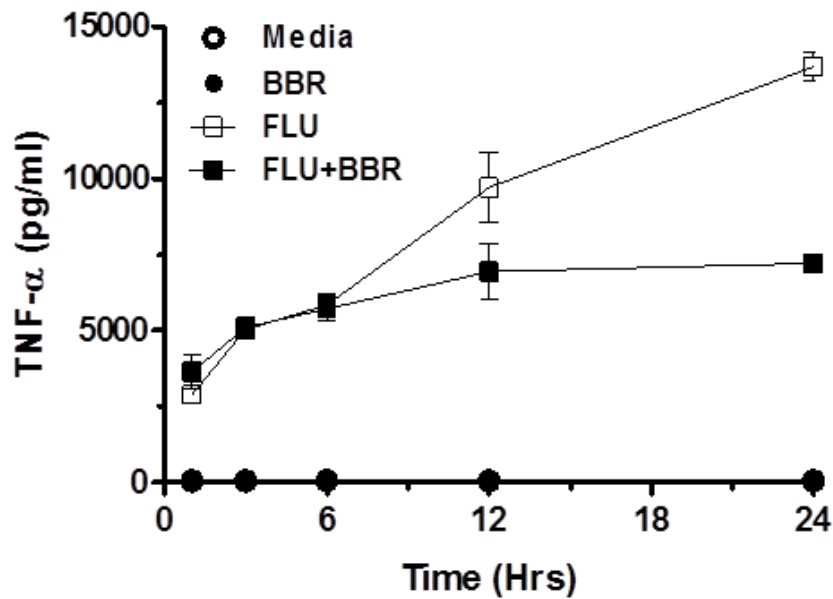


Figure 1: Berberine suppresses IAV-induced TNF- α protein levels

RAW 264.7 cells were infected with influenza A/PR/8/34 (MOI=5) for varying times in the absence or presence of 25 μ M berberine. Supernatants were collected and the levels of TNF- α were determined using a TNF- α ELISA kit. Values shown are means \pm S.E.M of three independent experiments.

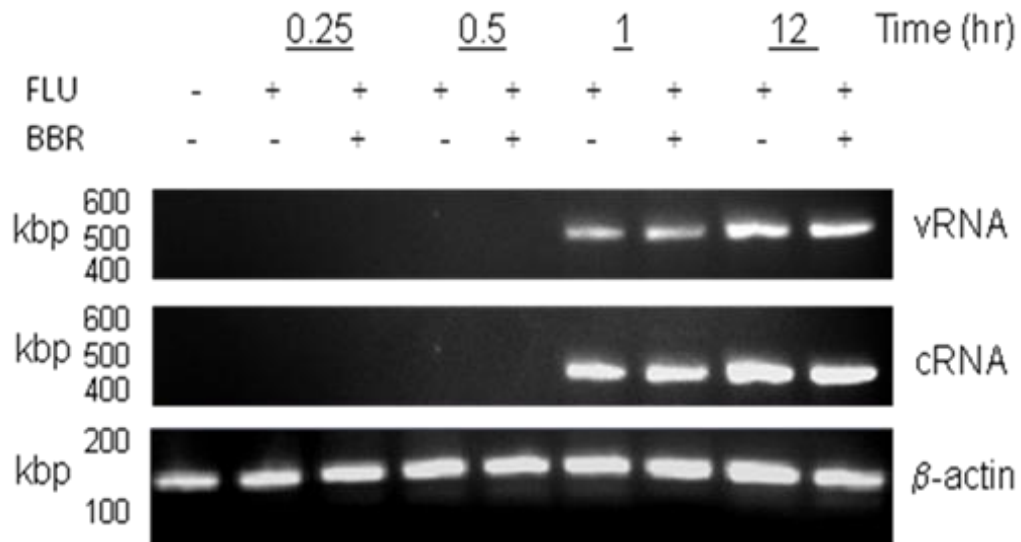


Figure 2: Berberine does not affect levels of HA vRNAs and cRNAs

Effects of berberine on levels of HA v and cRNAs were examined by reverse transcriptase-PCR reactions. RAW 264.7 cells were infected at 5MOI for the indicated times in the absence or presence of 25 μ M berberine. Following cell lysis and RNA extraction, cDNA of influenza's HA v and cRNAs were generated using specific primers as listed in materials and methods. cDNAs were amplified by PCR and PCR products were resolved on a 2% agarose gel. β -actin PCR products were also evaluated for loading accuracy. One representative experiment out of 5 is shown.

Figure 3: Berberine does not affect IAV-mediated signaling events

RAW 264.7 cells were infected with IAV at 5MOI in the absence or presence of 25 μ M berberine for the indicated times. Cell lysates were harvested and levels of phospho-p38 (pp38), phospho-ERK pp42/44), and phospho-p65 (pp65) were determined by immunoblot analysis using rabbit polyclonal antibodies. Blots were re-probed using antibodies to total-p38 (p38), total-ERK (p42/44) and total-p65 (p65) to ensure equal loading of proteins (A). One representative experiment out of 3 is shown in panel A. RAW 264.7 cells were infected with IAV at 5MOI in the presence or absence of 25 μ M berberine for the indicated time periods and levels of TNF- α mRNA were assessed by quantitative-RT-PCR. Values shown are means \pm S.E.M of three independent experiments (B).

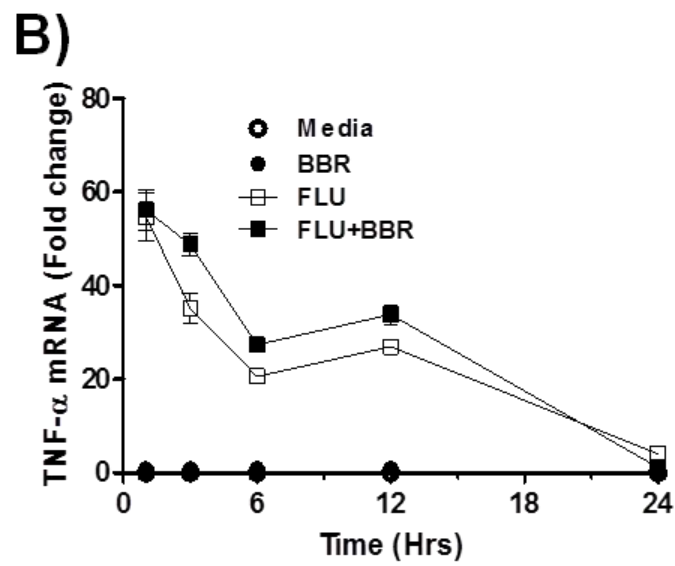
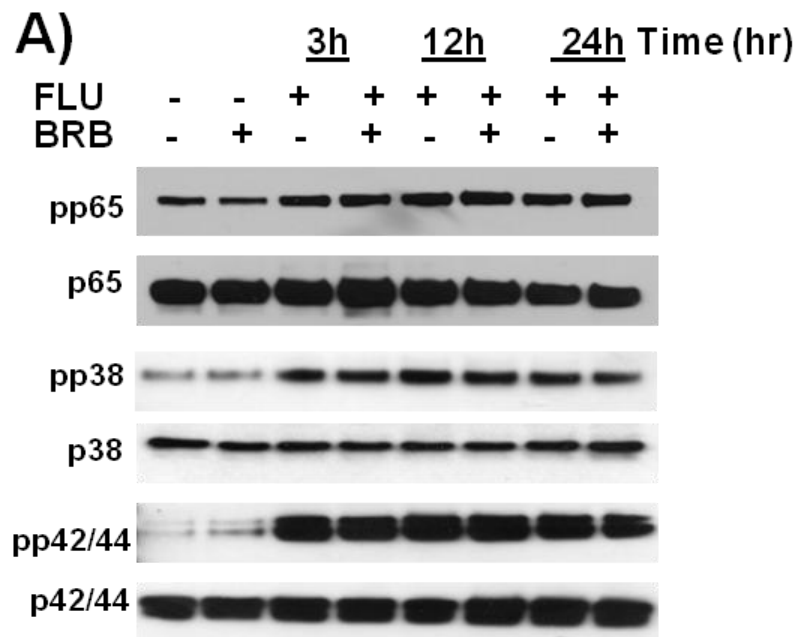


Figure 4: Berberine does not affect synthesis and surface levels of the TNF- α protein

RAW 264.7 cells were infected with IAV at 5MOI for 12 hours. Brefeldin A (BFA) was added at 8 hours post-infection and intracellular cytokine flow cytometry (ICFC) was performed to determine the levels of intracellular TNF- α using an anti-mouse TNF- α antibody conjugated to Alexa-Fluor® 488 (A) and (B). RAW 264.7 cells were infected with IAV for 12 hours in the presence or absence of 25 μ M berberine (BBR). Cells were harvested, permeabilized, and analyzed for levels of intracellular TNF- α by ICFC using an anti-mouse TNF- α antibody conjugated to Alexa-Fluor® 488 (C) and (D). RAW 264.7 cells were infected with IAV for 12 hours in the presence or absence of 25 μ M berberine. Live cells were collected and incubated with anti-mouse TNF- α antibody conjugated to Alexa-Fluor® 488. Surface levels of TNF- α were evaluated by flow cytometry (E) and (F). Representative experiments are shown in panels A, C and E. Values shown in panels B, D and F are means \pm S.E.M of three independent experiments.

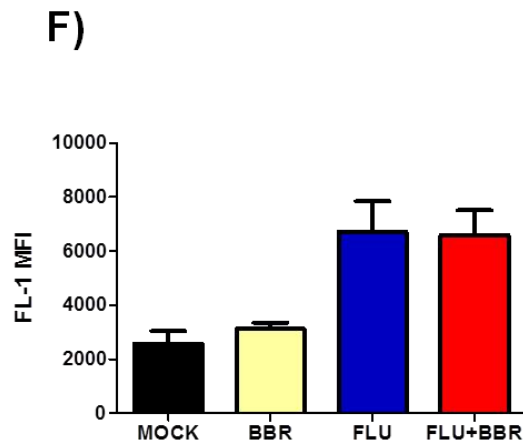
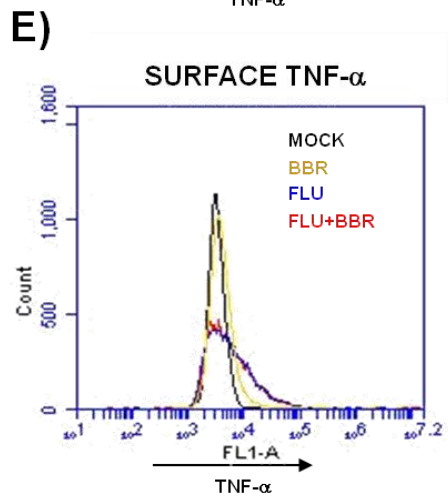
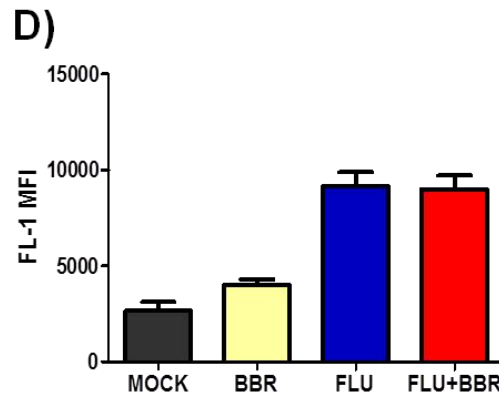
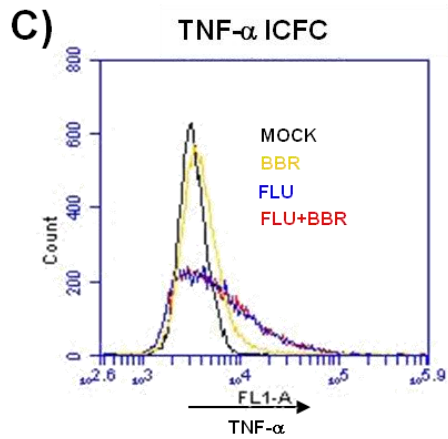
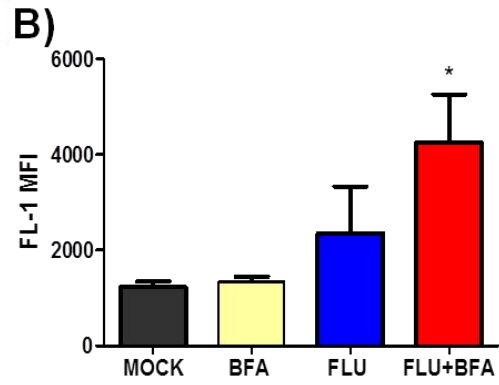
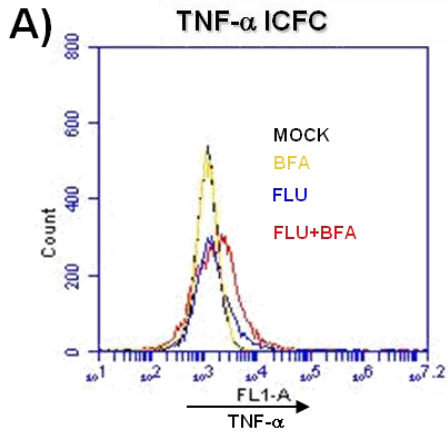
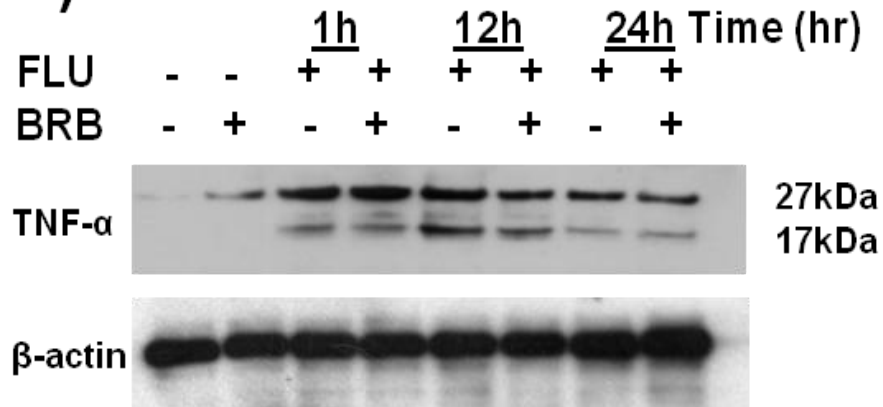


Figure 5: Berberine does not affect the levels of intracellular TNF- α

RAW 264.7 cells were infected with IAV for the indicated time points in the presence or absence of 25 μ M berberine (BBR). Total cell lysates were harvested and analyzed for levels of intracellular and membrane-tethered TNF- α by immunoblot analysis (A) or ELISA (B). Representative experiments are shown for immunoblot analysis and values shown for ELISA are means \pm S.E.M of three independent experiments.

A)



B)

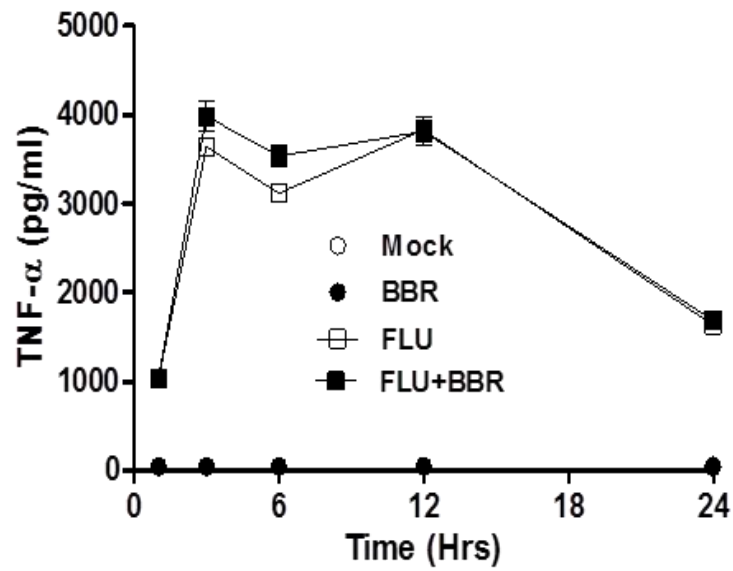


Figure 6. Berberine inhibits IAV-induced IL-6 and CCL2/MCP-1 protein levels

RAW 264.7 cells were infected with influenza IAV at MOI 5 for varying times in the absence or presence of 25 μ M berberine (BBR). Culture supernatants were harvested and the levels of IL-6 (A) and MCP-1 (B) were determined by ELISA. Values shown are means \pm S.E.M of two independent experiments.

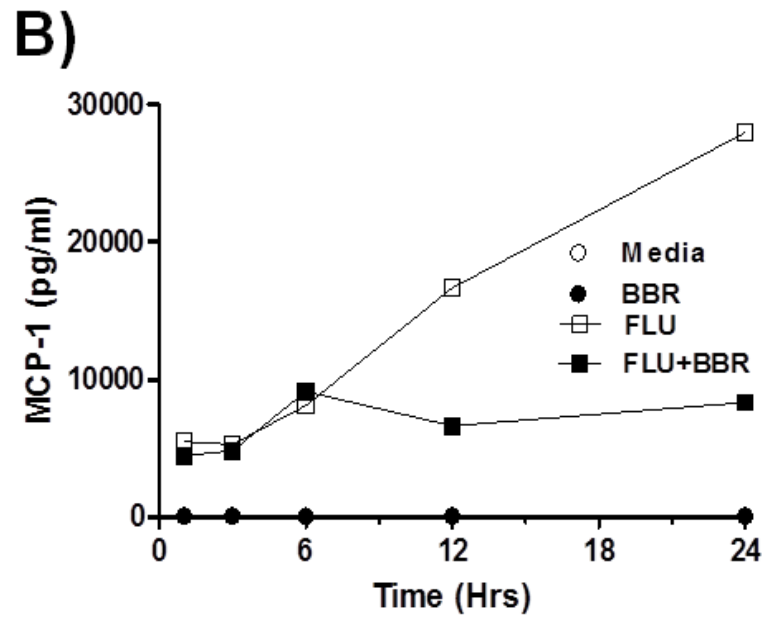
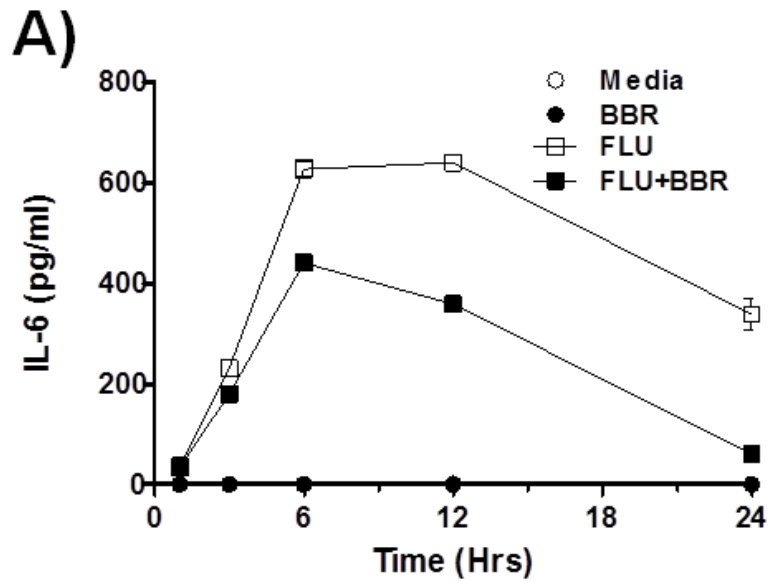


Figure 7. Berberine leads to global reduction in protein levels of culture supernatants from IAV-infected cells

RAW 264.7 cells were infected with IAV alone or IAV and 25 μ M berberine for the indicated time points in low protein VEFSM. Culture supernatants were collected and resolved by electrophoresis on a 12.5% polyacrylamide SDS gel and proteins were detected by silver stain. Representative experiments are shown. Virus inoculum (VI) from MDCK cells used for infection of RAW 264.7 cells was loaded in lane 10 to monitor changes in virus protein levels.

FLU			<u>1</u>		<u>12</u>		<u>24</u>		Time (hr)	
BBR			-	+	-	+	-	+	VI	
LANE	1	2	3	4	5	6	7	8	9	10

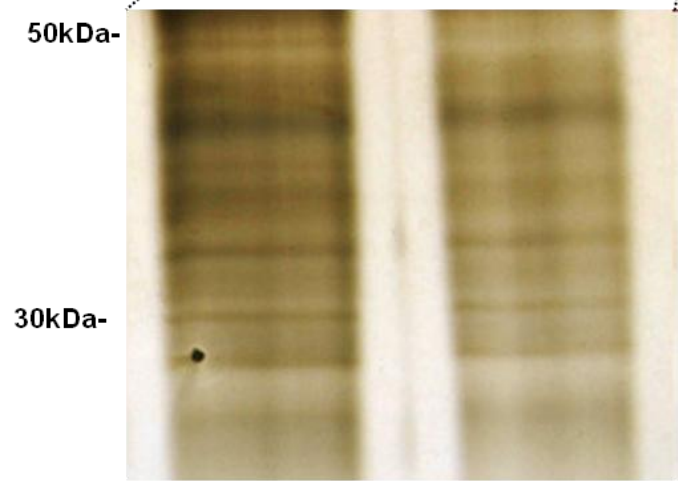
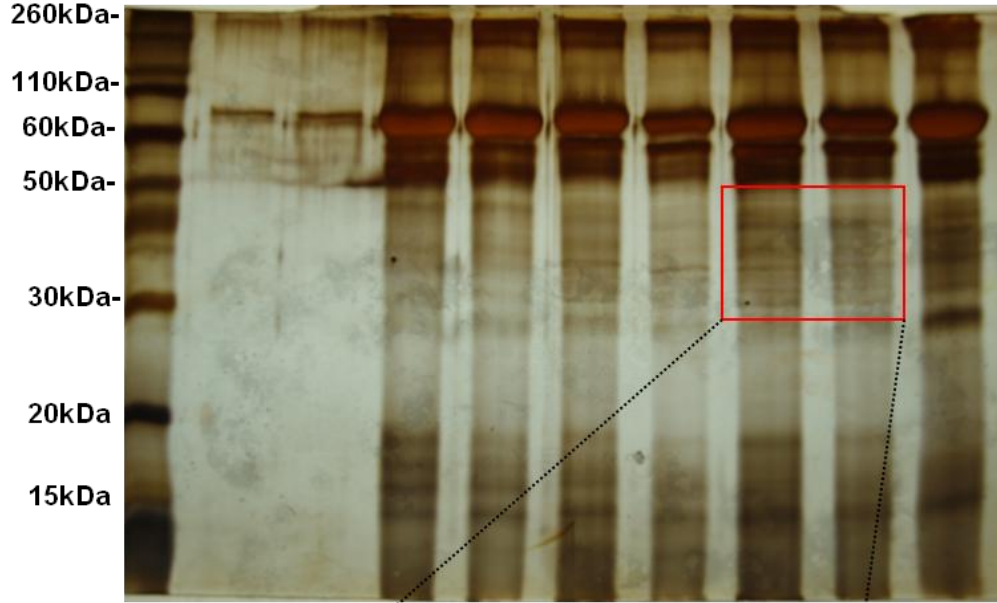
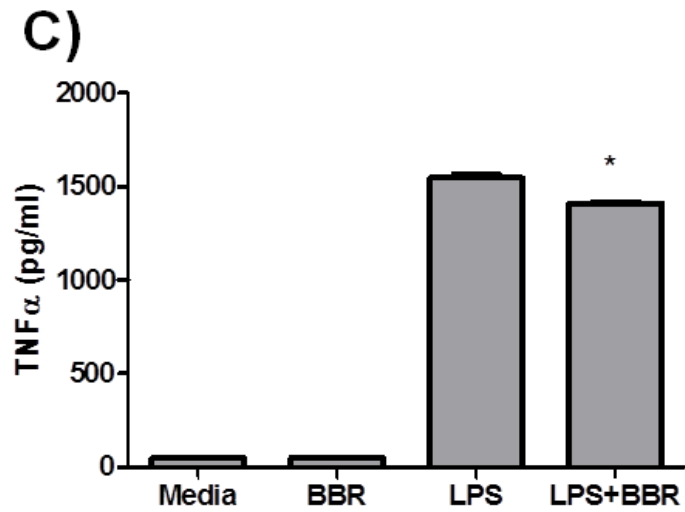
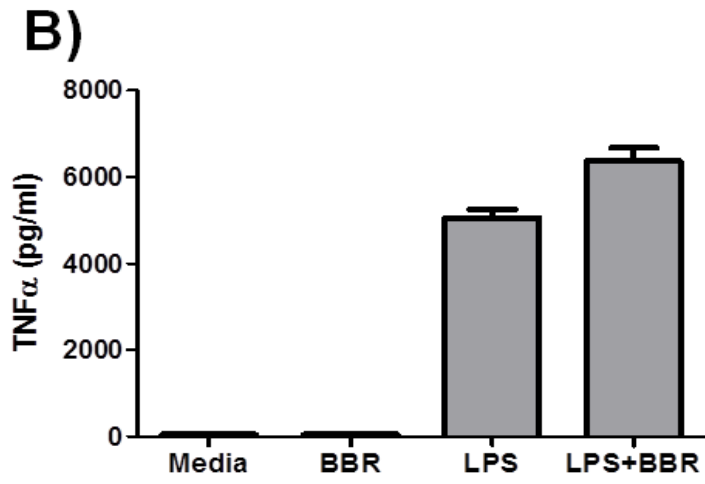
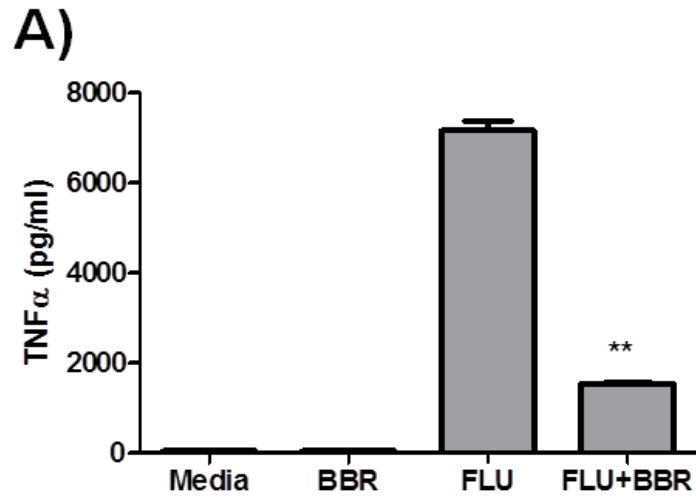


Figure 8. IAV infection is required for berberine-mediated inhibition of TNF- α accumulation

RAW 264.7 cells were infected with IAV MOI 5 for 24 h in the absence or presence of 25 μ M berberine (BBR). Supernatants were collected and the levels of TNF- α were determined using a TNF- α ELISA kit (A). RAW 264.7 cells were stimulated with 1 μ g/ml LPS in either complete serum-rich (B) or serum free virus growth media (C) in the presence or absence of 25 μ M berberine for 24 h. Supernatants were harvested and levels of TNF- α were determined by ELISA. Values shown are means \pm S.E.M of three independent experiments.



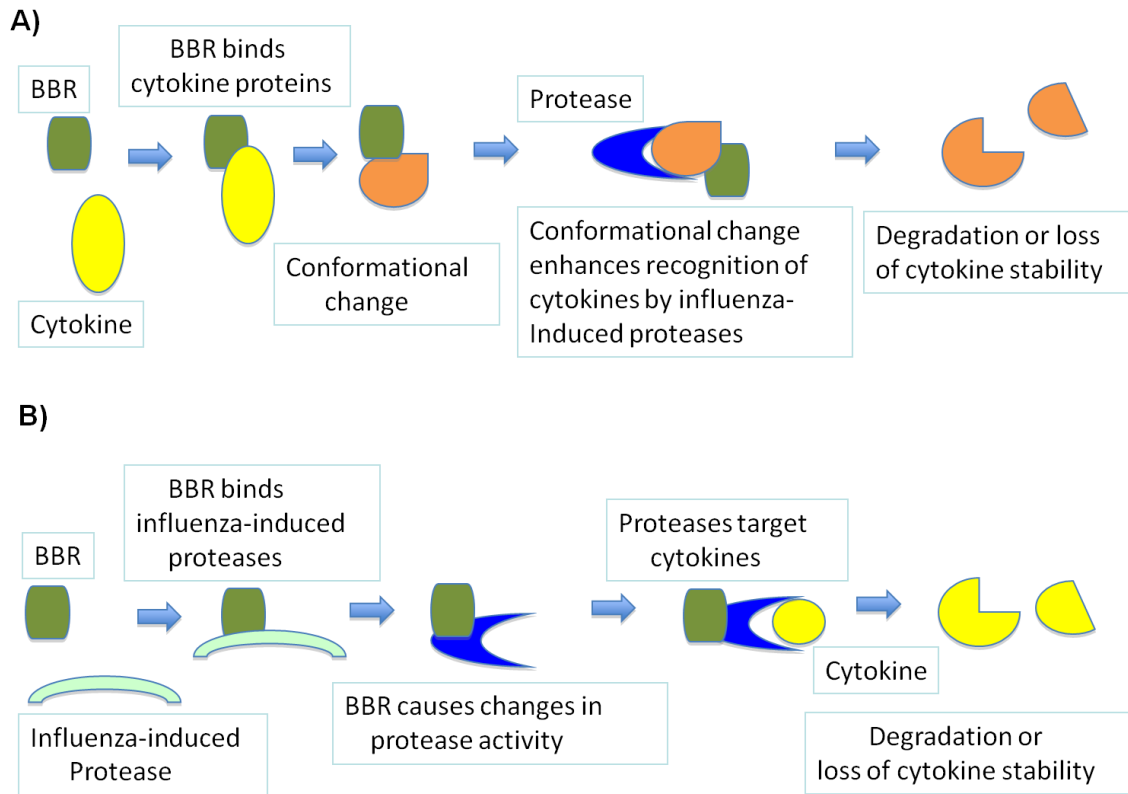


Figure 9. Proposed mechanism of berberine-mediated cytokine inhibition in influenza A-infected RAW 264.7 cells

Berberine could be interacting with inflammatory cytokines to induce a conformational change thereby, enhancing their susceptibility to IAV-induced cellular proteases (A). Alternatively, berberine could be associating with IAV-induced cellular proteases, thereby changing their cellular targets and enhancing their ability to recognize and degrade inflammatory cytokines (B).

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Chapter 4. Summary

1. Summary

We have shown that the naturally occurring compounds tetra-O-methyl nordihydroguaiaretic acid and berberine can reduce the levels of microbially-induced cytokines in RAW 264.7 cells. Currently, the mechanisms of action of these compounds are still incompletely understood. However, it appears that these compounds display a broad range of activity. In the case of TMP, we have demonstrated that it inhibits LPS-induced mRNA levels of inflammatory cytokines by abrogating binding of NF- κ B to its cognate motifs. Interestingly, TMP does not block binding of NF- κ B to linear NF- κ B-consensus oligonucleotides as demonstrated by EMSAs. These results strongly suggest that TMP is an indirect inhibitor of NF- κ B binding to the DNA. It has been demonstrated that recruitment RelA to the promoter region of the MCP-1 gene is dependent upon Sp1 binding to the most proximal promoter [1]. These findings suggest that recruitment of NF- κ B to the promoters of certain genes may be dependent on the activity of Sp1. TMP has previously been shown to prevent binding of Sp1 to its cognate sites, therefore the loss of NF- κ B occupancy at the promoters of TNF- α , MCP-1 and RANTES could be directly related to inhibition of Sp1 binding by TMP. Future experiments should investigate whether TMP also affects binding of Sp1 at the promoters of MCP-1, TNF- α and RANTES. If these experiments reveal loss of Sp1 occupancy at these promoters, additional

experiments should test whether over-expression of Sp1 could rescue NF- κ B binding at its cognate motifs in the presence of TMP. Several unknowns must also be elucidated before TMP can be used to control inflammation in a clinical setting. For instance, the specificity of TMP for various NF- κ B subunits must be determined. Due to the role of NF- κ B in regulating various cellular processes TMP may not be a useful drug for attenuating inflammation if it inhibits the activity of all NF- κ B subunits.

Our results suggest that berberine is acting extracellularly to promote the loss of cytokines from culture supernatants. We hypothesize that berberine modulates the activity of IAV-induced cellular proteases, which results in degradation of cytokines that are present in the culture supernatants. If our hypothesis is confirmed, this will be a novel mechanism of action for a drug. To this end, berberine could be useful for targeting proteins that are present in the extracellular environment. However, the high concentration of proteins in the extracellular milieu could limit the ability of berberine to target specific cytokine proteins *in vivo*. The use of berberine as an anti-inflammatory drug could also be risky because essential extracellular cellular proteins could be targeted for degradation. To address these concerns, the specificity of berberine for various IAV-induced cellular proteases as well as cytokine proteins in the extracellular milieu must be determined. It would also be of interest to determine whether berberine suppresses production of cytokines induced by other viruses. These results could shed more light on berberine's mechanism of action.

In closing, the work described in this dissertation lays the foundation for developing novel anti-inflammatory drugs. Our research suggests that TMP and

berberine could be useful for preventing transcription factor binding to DNA and targeting cytokines in the extracellular milieu, respectively. These findings also demonstrate that naturally occurring plant compounds could serve as alternatives to commonly used anti-inflammatory remedies such as biologics and steroids. However, the effects of these compounds on various cellular processes must be better characterized before they can be used to control inflammation in a clinical setting.

2. References

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