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Modulation of nontypeable Haemophilus influenza-induced inflammation in the pathogenesis of otitis media by curcumin

Anuhya S. Konduru

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MODULATION OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE*-INDUCED INFLAMMATION IN THE PATHOGENESIS OF OTITIS MEDIA BY CURCUMIN

by

ANUHYA SHARMA KONDURU

Under the direction of Dr. Jian-Dong Li, M.D., Ph.D

ABSTRACT

Otitis media (OM) is the most common childhood bacterial infection, and leading cause of conductive hearing loss. Nontypeable *Haemophilus influenzae* (NTHi) is a major bacterial pathogen causing OM. During infection, epithelial cells act as the first line of defense by secreting numerous pro-inflammatory mediators including C-X-C motif chemokine ligand 5 (CXCL5) and mucin 5AC (MUC5AC). While appropriate inflammatory responses are critical for the containment and removal of the invading pathogen, excess inflammation can lead to tissue damage, impaired mucociliary clearance and be detrimental to the host. Therefore, inflammatory responses must be tightly regulated. Current therapies for OM are ineffective due to the emergence of antibiotic-resistant NTHi strains and risk of side effects with prolonged use of immunosuppressant drugs. Therefore, therapeutic strategies that increase the levels of endogenous negative regulators of inflammation while leaving the positive pathways intact are gaining prominence. Thus, understanding the underlying molecular mechanisms regulating inflammation is critical for developing effective therapeutic strategies.
Despite the importance of CXCL5 chemokine in mediating inflammation, the signaling cascade mediating its up-regulation in OM remains largely unknown. Here we show that NTHi up-regulates CXCL5 expression by activating IKKβ-IκBα and p38 MAPK pathways via NF-κB nuclear translocation-dependent and -independent mechanism in middle ear epithelial cells. We also show that MKP-1 is a negative regulator of NTHi-induced CXCL5 expression. We further demonstrated the translational significance of these findings by reporting for the first time that curcumin, derived from Curcuma longa plant suppressed CXCL5 expression by direct inhibition of IKKβ phosphorylation, and inhibition of p38 MAPK via induction of negative regulator, MKP-1. Next, we show that curcumin also suppressed NTHi-induced MUC5AC expression via up-regulation of MKP-1, demonstrating for the first time the efficacy and pleiotropic anti-inflammatory action of curcumin to suppress NTHi-induced inflammatory responses in OM model. Finally, we demonstrate for the first time that MKP-1 protein undergoes lysine 63 (K63)-linked polyubiquitination, suggesting the importance of post-translational modification on MKP-1 activity. We also show that curcumin enhanced K63-linked polyubiquitination of MKP-1. Since K63-linked polyubiquitination mediates non-degradative molecular functions such as protein-protein interactions, protein trafficking and regulation of signal transduction events, our findings suggest a new mechanism of action of curcumin on MKP-1. Taken together our study demonstrates the therapeutic potential of curcumin in treating NTHi-induced OM by modulating the expression and activity of negative regulator MKP-1.

INDEX WORDS: Otitis media, Inflammation, Nontypeable Haemophilus influenzae, CXCL5, MUC5AC, Curcumin, MKP-1, Negative regulator, Post-translational modifications, Ubiquitination, K63-linked polyubiquitination
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ANUHYA SHARMA KONDURU

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In the College of Arts and Sciences
Georgia State University
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ANUHYA SHARMA KONDURU

Committee Chair: Jian-Dong Li

Committee: Zhi-Ren Liu

Sang-Moo Kang

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2016
DEDICATION

This work is dedicated to my mom Deepika Konduru, for her love, support, patience and prayers throughout my life. None of my accomplishments would have been possible if it weren’t for her countless sacrifices made and hardships endured to provide for us. Thank you, Mom!
ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my family, without them, none of this would have been possible. I am forever grateful for my mom’s support through every endeavor I chose to pursue and for instilling in me at a very young age the importance of education. My dad Nagendra and brother Anmol’s understanding, motivation, encouragement and support were instrumental in me pushing through during the final year of grad school. I would also like to thank my friend Saraswati & her family for being my family here in Atlanta. No words are enough to express my heartfelt gratitude to her for being my support system, for lending a patient ear, for giving the push when I needed, for feeding me, for being my shopping guru and for being the ‘big sister’ I never had. In short, the last five years would not have been easy without her.

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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A549</td>
<td>Human lung epithelial cell</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CXCL5</td>
<td>C-X-C chemokine 5</td>
</tr>
<tr>
<td>DUSP</td>
<td>Dual-specificity phosphatase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>HMEEC</td>
<td>Human middle ear epithelial cell</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of kappa B kinase</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor-associated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K48</td>
<td>Lysine 48</td>
</tr>
<tr>
<td>K63</td>
<td>Lysine 63</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MKP-1</td>
<td>MAPK phosphatase-1</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Mucin 5AC</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-B</td>
</tr>
<tr>
<td>NTHi</td>
<td>Nontypeable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>OM</td>
<td>Otitis media</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β- activated kinase 1</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll–interleukin 1 (IL-1) receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor-necrosis factor-receptor-associated factor 6</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Nontypeable Haemophilus influenzae

*Haemophilus influenzae* is a rod-shaped, non-motile, Gram-negative aerobic bacterium colonized in the nasopharynx of nearly 80% of the humans [1]. Unlike many other bacterial species, it is fastidious and requires both X-factor (hemin) and V-factor (nicotinamide adenine dinucleotide) for growth and proliferation [2]. *Haemophilus influenzae* are classified based on the composition of the polysaccharide capsule around the bacterium. Six encapsulated serological type strains (a-f) have been identified to date. These strains are associated with systemic diseases, such as bacteremia, meningitis [2]. The availability of a polysaccharide-protein conjugate vaccine against the most virulent *Haemophilus influenzae* type b strain has nearly eliminated its associated diseases in developed countries [1].

Additionally, many strains lacking the polysaccharide capsule, termed nontypeable *Hemophilus influenzae* (NTHi) exist. While these strains are less virulent compared to encapsulated strains, the absence of the polysaccharide capsule makes it difficult to develop an effective vaccine [1, 3]. Respiratory tract infections associated with NTHi are one of the leading causes of morbidity and mortality in both developed and developing nations. NTHi is mainly identified as a respiratory pathogen and has been shown to predominantly target airway epithelium cells [2]. *In vitro* studies suggest that NTHi preferentially adheres to respiratory epithelial cells that either lack cilia or are structurally damaged. Many outer membrane proteins (OMPs) of NTHi can bind to the sialic acid-containing oligosaccharides of mucin in the nasopharynx, and initiate its colonization. NTHi evades host immune system due to type I IgA1 protease that cleaves the host’s lysosome-associated membrane protein 1 [4]. NTHi does not possess any secretion system, which suggests its need to manipulate host pathways via
alternative mechanisms to invade and colonize the host [5]. Lack of reliance on any single mechanism of attachment and its ability to respond rapidly to host defense mechanisms by antigenic variation of OMPs, lipooligosaccharide (LOS) proteins, and enzymes make it harder to prevent, contain and treat NTHi infections.

1.2 Otitis Media

Otitis media (OM) is the most common childhood bacterial infection [6, 7] with more than 700 million ear infections in the US each year. OM frequently leads to conductive hearing loss, affecting children during the crucial period of speech and language development [8, 9]. By age 3, nearly 90% of children have suffered from an episode of OM, resulting in approximately 30 million doctor visits each year and 5 billion dollars in patient care. NTHi represents the cause of almost one-third episodes of OM. *Streptococcus pneumoniae* and *Moraxella catarrhalis* represent other bacterial causes of OM.

OM occurrences are often preceded by a viral upper respiratory tract infection, which can cause swelling and congestion of the Eustachian tube [10]. This change in physiology not only results in the inability to equilibrate the pressure in between the nasopharynx and middle ear cavity but also leads to a decrease in the drainage of middle ear secretions. The collection of fluid in the middle ear cavity provides an ideal environment for the overgrowth of bacteria, such as NTHi. Children are particularly susceptible to OM due to the decreased length of the Eustachian tube as well as the insufficient function of its muscular opening [11]. Since the advent of pneumococcal vaccines (PCV7 and PCV13) NTHi-related OM has become most prevalent [12].
1.3 Pathogenesis of bacterial OM

Innate immunity is the dominant player in clearing middle ear infections. In response to antigenic stimuli, the middle ear triggers a rapid immune response of IgG in animal models, which leads to transudation of serum and leukocyte infiltration from the peripheral blood into the tympanic cavity. Adaptive immunity seems to be poorly sensitized by the invading pathogens at the middle ear side [13]. Therefore deficiencies in the components of innate immunity could lead to an increase in incidence, severity, and duration of OM [14]. While appropriate immune responses are critical for the containment and removal of the pathogens, excess inflammation can be detrimental to the host by causing tissue damage and perpetuating a never-ending cycle of immune responses [15-17]. OM is characterized by the presence of mucus overproduction, excess inflammation and impaired bacterial clearance in the middle ear.

1.4 Inflammation

Inflammation is the primary protective physiological response of the body against tissue injury, irritation, and infection from pathogens like bacteria, virus and the presence of foreign substances. It is triggered by the body’s first line of defense – innate immune system. The body responds to several of these stress stimuli by mounting an immune response, which involves the recruitment of leukocytes like macrophages, neutrophils in response to secreted cytokines and chemokines [18]. Inflammation is a double-edged sword characterized by five cardinal signs - swelling, redness, heat, pain and loss of function [19]. Following an injury or infection, the body initiates the healing/pathogen clearance via short-term acute inflammatory responses. Innate immunity is the first line of defense protecting the host until the sensitization required for eliciting adaptive immune responses in generated [20].
1.5 Pattern-recognition receptors in innate immunity

The innate immune system recognizes conserved structures on pathogens; called pathogen-associated molecular patterns (PAMPs), through various pattern recognition receptors (PRRs) expressed on many immune and non-immune cells [20, 21]. Of the different classes of PRRs, Toll-like receptors (TLRs) have been widely studied for their role in triggering immune responses against invading pathogens. TLRs are type I transmembrane proteins with leucine-rich repeats in the ectodomain which recognizes PAMPs; a transmembrane domain; and intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain that propagates downstream signal transduction. To date, ten functional TLRs have been identified in humans. PAMPs recognized by TLRs include lipopolysaccharides (LPS), lipids, lipoproteins, peptidoglycans, proteins, and nucleic acids from bacteria, viruses, fungi and parasites [21]. TLRs can be categorized into two subgroups depending on their cellular localization. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surfaces, while TLR7, TLR8, and TLR9 are expressing in the intracellular vesicles such as endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes [22].

Upon recognition of corresponding PAMPs, TLRs recruits the TIR domain-containing adaptor protein myeloid differentiation factor 88 (MyD88). MyD88 then recruits and activates IL-1 receptor-associated kinases (IRAKs), which further lead to the recruitment and activation of tumor necrosis factor-receptor-associated factor 6 (TRAF6). IRAK-TRAF6 complex dissociates from the receptor bound complex and further interacts with transforming growth factor-β-activated kinase 1 (TAK1). Activation of TAK1 leads to initiation of further downstream signaling pathways. Phosphorylation and activation of inhibitor of kappa B kinases (IKKs) is one of the major pathways contributing to mounting an immune response. Phosphorylation and
proteasomal degradation of IκBα, by IKK, is necessary for activation of transcription factor nuclear factor kappa B (NF-κB). Additionally, activation of mitogen-activated protein kinases (MAPKs), p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) also occurs. These pathways act independently or in concert resulting in nuclear translocation of transcription factors, which in turn regulates the expression of pro-inflammatory mediators [22].

1.6 Toll-like receptors in NTHi-induced OM

NTHi contains several PAMPs that are recognized by many TLRs. Peptidoglycan and peptidoglycan-associated proteins such as P6 (an OMP) are recognized by TLR2 [23]. TLR2 usually forms heterodimers with TLR1 or TLR6. TLR2-TLR1 heterodimer recognizes triacetylated lipopeptides, typical of Gram-negative bacteria, while TLR2-TLR6 heterodimer recognized diacetylated lipopeptides, characteristic of Gram-positive bacteria [24, 25]. LOS can activate immune responses via both TLR2 and TLR4 [26]. Injection of peptidoglycan or LPS into chinchilla ear has shown to cause mucosal inflammation, neutrophil infiltration, edema, and abnormalities in middle ear pressure and macrophage infiltration into the sub-epithelial space [27]. While the immune responses to TLR ligands have been under study for decades, there is increasing evidence suggesting the role of TLRs itself in the pathogenesis of OM.

NTHi induces TLR2 expression in middle ear epithelial cell lines. TLR2 activation leads to the expression of pro-inflammatory mediators. NTHi infection has been shown to induce expression of inflammatory mediators such as interleukin (IL)-1β, IL-8, CXCL5, and MUC5AC via TLR2 in middle ear epithelial cells [23, 28, 29]. Additionally, the appropriate functioning of TLRs is necessary for resolution of inflammatory responses. TLR deficiencies have been shown to cause significant abnormalities in recovery from infections [14]. Polymorphisms in TLRs and their co-receptor CD14 have been reported to increase susceptibility to OM in children [30].
1.7 Current Treatment and Challenges

Treatment for OM can range in severity, from managing pain via oral analgesics and allowing the infection to subside independently to the use of antibiotics to actively combat the bacteria. The standard method of antibiotic treatment is the use of amoxicillin, beta-lactam [31]. However, since over 80% of NTHi strains are resistant to amoxicillin, the next regimen of treatment involves antibiotics such as trimethoprim, a dihydrofolate reductase inhibitor, and fluoroquinolones; inhibitors of DNA gyrase and topoisomerase [31, 32]. Decongestants, antihistamines, glucocorticoids have not found to be effective. Persistent, recurrent and chronic forms of OM affecting nearly 15% of the children poses a challenge regarding disease management. Prophylactic usage of antibiotics has minimal effect of resolving chronic OM and has led to the emergence of multi-drug resistant bacterial strains. In severe cases, tympanostomy tubes are placed to drain the accumulation of fluid in the middle ear [33]. Prolonged usage of these treatments poses severe risks due to serious side effects, weakened immune system and otorrhea (discharge from ear). Development of NTHi vaccine remains a challenge due to the high genetic diversity of NTHi strains and high antigenic variability of surface-exposed antigens [34, 35]. Therefore there is an urgent need for developing alternate therapeutic strategies for treating OM, based on full understanding of the molecular signaling pathways leading to its pathogenesis.

1.8 Inflammatory mediators in OM

1.8.1 C-X-C chemokine CXCL5

CXCL5/ENA-78 (epithelial neutrophil-activating peptide 78) of the Glu-Leu-Arg (ELR) motif-containing C-X-C chemokine family is critical for recruiting neutrophils in response to bacterial infections [36]. Epithelial-cell derived CXCL5 is vital in polymorphonuclear leukocytes
(PMN)-driven destructive inflammatory responses in *Mycobacterium tuberculosis*-induced pulmonary tuberculosis. *Cxcl5*−/− mice were found to be resistant to fatal tuberculosis [37]. The role of CXCL5 in neutrophil trafficking in lipopolysaccharide (LPS)-induced lung inflammation in mice has been reported [38]. A recent study demonstrated that LPS-induced deregulated CXCL5 expression resulted in exaggerated neutrophil-mediated inflammation in pulmonary bronchiolar cells [39]. CXCL5 is also involved in angiogenesis, tumor growth, and metastasis [40], with CXCL5 overexpression leading to poor survival in cancer patients [41]. Affymetrix chip analysis on mouse genome revealed marked up-regulation of CXCL5 expression by NTHi in the middle ear of mouse [42]. Middle ear effusion samples from patients with acute and chronic OM have shown the presence of viable NTHi trapped within neutrophil extracellular traps, which continued to elicit inflammatory responses [43, 44]. While the appropriate neutrophil response is critical for the removal of the invading pathogen, excess inflammation can lead to tissue damage and perpetuate inflammation leading to detrimental effects, as seen across several inflammatory pathologies including OM [15-17, 45]. Thus, these findings suggest that therapeutic strategies to control aberrant CXCL5 production are of utmost importance for modulating inflammation in OM.

### 1.8.2 MUC5AC mucin

Mucin glycoproteins are a major component of mucus secretions in the middle ear, trachea, digestive and reproductive tracts. Mucus production represents a protective innate defense mechanism to protect and lubricate the epithelium and trap invading pathogens for removal by the mucociliary clearance system [46]. However, in chronic infections, excess mucin impairs the mucociliary clearance system, resulting in mucus accumulation and defective function of the mucus-lined epithelial tracts. Of the ~24 mucin genes identified till date
MUC5AC mucin has been shown to play a critical role in the pathogenesis of upper respiratory tract infections including OM [47, 48]. In patients with OM, increased mucus effusion into the ear’s tympanic cavity impairs the movement of the eardrum and middle ear bones and leads to hearing problems. A higher concentration of mucin in the middle ear effusion has been shown to correlate with the extent of hearing impairment [9, 49]. While mucin up-regulation is an important innate defense response of the host to contain infections in the middle ear, excess mucin can lead to impaired mucociliary clearance and hearing loss [47]. Therefore mucin expression must be tightly controlled.

1.9 Negative Regulation of NTHi-induced TLR signaling pathways

While mounting an appropriate inflammatory response against invading microbial pathogens is critical for the survival of the host, deregulated overactive inflammatory responses are detrimental to the host. Therefore, TLR signaling pathways must be tightly regulated to maintain immune balance by negative regulation. Negative regulation of TLRs has been reported to occur via numerous mechanisms at multiple levels of the signal transduction.

1.9.1 Mechanism of negative regulation of TLR signaling

The negative regulatory mechanisms can be broadly categorized into three groups.

i. Degradation of signal proteins. Degradation of signal transduction components is one of the principal mechanisms to inhibit any signaling pathway. The addition of a 76-amino acid containing ubiquitin molecule to a protein tags the protein for degradation by the 26S proteasome. Triad domain containing protein 3 (Triad3A) is an E3 ubiquitin ligase that enhances the ubiquitination of TLRs. Suppressor of cytokine signaling (SOCS-1) induces polyubiquitination of TIR domain-containing MyD88-adaptor like protein (MAL). In addition to proteasomal degradation, protein degradation by lysosomes is also known to
contribute to negative regulation of TLR signaling. Tripartite-motif protein 30α (Trim 30α) is a negative regulator of TLR-mediated NF-κB activation by targeting TAK1 binding protein (TAB) TAB2 and TAB3 for lysosomal protein degradation [50, 51].

ii. Transcriptional regulation. Recruitment of histone deacetylase (HDAC) to the promoter regions of proinflammatory cytokines, represses transcription of these cytokines by blocking access to the transcriptional machinery. Cyclic AMP-dependent transcription factor (ATF3) recruits HDAC1 to the promoter regions of IL-12p40, IL-6, and TNF-α genes, thereby repressing their transcription. Non-coding RNAs, including microRNAs (miRNAs), have emerged as fine tuners of TLR signaling by targeting mRNAs of pro-inflammatory cytokines for miRNA-mediated gene silencing by promoting mRNA degradation [51].

iii. Dissociation of adaptor proteins. TLRs can identify different pathogens and activate appropriate signaling pathways. The specificity of the activated pathway depends on the TIR domain-containing adaptor proteins such as MyD88, MAL, TIR domain-containing adaptor protein-inducing IFN-β (TRIF), TRAM being recruited to the TLR. Sterile alpha and Armadillo motif containing protein (SARM) is a TIR domain-containing protein is a negative regulator of TRIF-dependent TLR signaling. SARM competes with TRIF to associate with TLR. IFN-regulatory factor (IRF) family of transcription factors; IRF-5 and IRF-7 interact with MyD88 to induce expression of proinflammatory mediators. IRF-4 competes with IRF-5 and IRF-7 to bind with MyD88. IRF-4 binding to MyD88 suppresses cytokine expression. A splicing isoform of MyD88, MyD88s is a negative regulator of TLR signaling. MyD88s is recruited to TLR via its TIR-domain. However, due to the lack of death domain (DD) in MyD88s, subsequent recruitment of IRAK-4 and activation of IRAK-1 fails to occur, thereby blocking the transmission of activating stimuli [50]. Post-translational modifications (PTMs)
such as phosphorylation, acetylation, ubiquitination, SUMOylation also play critical roles in signal transduction by regulating the interactions between adaptor proteins [51]. Upon TLR stimulation, activated IRAKs interact with TNF-receptor association factor (TRAF) 6 resulting in polyubiquitination (ubiquitin protein conjugated at lysine 63 (K63)), that lead to phosphorylation and activation of the IKK and MAPKs. Tumor suppressor cylindromatosis (CYLD) is a deubiquitinase that cleaves K63 linked polyubiquitin chains, on TRAF2, TRAF6, TRAF7 and IKKγ, thereby suppressing TLR signal transduction [52]. MAPK activation is mainly regulated by MAPK phosphatases (MKPs), comprising of 10 dual-specificity phosphatases (DUSPs) that dephosphorylate p38, ERK, JNK MAPKs [53]. MKP-1−/− macrophages produce excess amounts of pro-inflammatory mediators. MKP-1 deficiency increases the susceptibility of mice to endotoxic shock [54]. Aberrant innate immune responses in MKP-1−/− mice highlight the importance of MKP-1 in the negative regulation of TLR signaling [55].

### 1.9.2 MKP-1

MKP-1 belongs to a subfamily of the larger DUSP family, which specifically dephosphorylates the threonine and tyrosine residues within the signature motif T-X-Y present in the activation loop of MAPKs. MKP-1 consists of an N-terminal non-catalytic domain and a C-terminal catalytic domain. MKP-1 binds to its substrate MAPK via the kinase interacting motif (KIM) present within the N-terminal domain [56].

### 1.9.3 MKP-1 regulation

#### 1.9.3.1 Transcriptional Regulation

MKP-1 expression is induced at both mRNA and protein by many mitogens and stress mediators via TLR activation [57]. An increase in MKP-1 levels correlates with a decrease in
p38 and JNK signaling pathways. TLR induces MKP-1 expression via recruiting both MyD88 and TRIF adaptor proteins [57]. p38, ERK, and JNK MAPKs have been shown to mediate TLR-induced MKP-1 expression. Additionally, several immunosuppressive agents such as glucocorticoids, phosphodiesterase (PDE) inhibitors, vinpocetine, resveratrol mediate their inhibitory effects via MKP-1 [58-61]. These agents inhibit inflammation by inducing MKP-1 expression, which would, in turn, inhibit MAPK activation, thereby suppressing inflammation. Anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and IL-10 have also been shown to increase MKP-1 expression [62, 63]. Thus, MKP-1 up-regulation seems to be a major mechanism for negative regulation of TLR signaling.

1.9.3.2 Post-translational modifications of MKP-1

In addition to transcriptional up-regulation of MKP-1 expression, PTMs that increase the stability and activity of MKP-1 and its affinity to MAPK substrates have been widely reported.

Phosphorylation. Transient activation of ERK leads to phosphorylation of MKP-1 at the C-terminal Ser359, and Ser364 increasing its stability without altering its phosphatase activity [64]. However, prolonged activation of ERK results in phosphorylation of Ser296 and Ser323 residues, promoting MKP-1 interaction with E3 ubiquitin ligase Skp-cullin-F-box, which targets MKP-1 for proteasomal degradation [65, 66].

Oxidation. Reactive oxygen species (ROS)-induced oxidation of Cys258 residue in the C-terminal of MKP-1 resulted in a decrease in phosphatase activity. This phenomenon was observed in TNF-α induced sustained activation of JNK in NF-κB deficient cells. MKP-1 inactivation leads to prolonged activation of JNK, leading to apoptosis. Thus MKP-1 oxidation is detrimental to the survival of the host [64].
**Acetylation.** MKP-1 has been reported to undergo acetylation at Lys57 within the KIM, in response to TLR stimulation. Acetylation of MKP-1 did not affect its intrinsic phosphatase activity. However, acetylated MKP-1 demonstrated increased affinity to p38 MAPK substrate, resulting in an indirect increase in MKP-1 activity [67]. Thus, MKP-1 acetylation inhibits TLR signaling.

1.10 Curcumin - an alternative therapeutic strategy for regulating inflammation in OM

1.10.1 Phytochemicals as potential anti-inflammatory drugs

Current anti-inflammatory therapies act via suppressing the positive signaling pathways involved in the production of inflammatory mediators. However, prolonged use of these drugs could have severe side effects because these pathways are also involved in mediating physiological responses. Therefore, therapeutic strategies that increase the levels of endogenous negative regulators of inflammation while leaving the positive pathways intact are gaining prominence [51]. In this regard, plant-based phytochemicals are gaining prominence for their minimal side effects even with prolonged usage. We previously showed that vinpocetine an alkaloid extracted from the periwinkle plant inhibits TNF-α and LPS induced up-regulation of proinflammatory mediators via inhibition of NF-κB signaling [68]. We also demonstrated that vinpocetine also suppressed mucus overproduction by up-regulating the negative regulator MKP-1, thereby inhibiting MAPK activation [59]. Vinpocetine is currently available as a dietary supplement. Another study from our lab showed that resveratrol, a non-flavonoid polyphenolic compound found in berries and grapes has anti-inflammatory properties. Resveratrol suppressed NTHi-induced inflammation by up-regulating the negative regulator MyD88s, which would in turn inhibit TLR signaling. Resveratrol is also available as a daily supplement [58].
1.10.2 Curcumin

Curcumin, a polyphenol derived from the rhizome of *Curcuma longa plant* was long-used for its medicinal properties [69]. Curcumin has been widely reported to have anti-inflammatory, anti-oxidant, anti-microbial, anti-diabetic, anti-carcinogenic anti-tumorigenic, anti-amyloidogenic effects. Due to its tolerability and non-toxicity at high doses, curcumin could be used for prolonged periods without any side effects [69]. Completed clinical trials revealed promising therapeutic effects of curcumin in patients with cancer, inflammatory bowel disease, irritable bowel disease, rheumatoid arthritis, Alzheimer’s disease, and diabetes [70-76]. More clinical trials evaluating the efficacy of curcumin on a broad range of inflammatory conditions are currently underway. Despite it’s widely known potent anti-inflammatory properties; the effect of curcumin on NTHi-induced inflammatory responses remains to be understood.

1.11 Significance

NTHi represents the cause of approximately one-third episodes of OM. Current treatments for bacterial OM rely on the systemic use of antibiotics, which often leads to the emergence of multi-drug resistant bacterial strains. Development of NTHi vaccine remains a challenge due to the high genetic diversity of NTHi strains and high antigenic variability of surface-exposed antigens. Therefore, there is an urgent need for developing alternate therapeutic strategies for treating NTHi infections. Additionally current anti-inflammatory therapies act via suppressing the positive signaling pathways involved in the production of inflammatory mediators. However, prolonged use of these drugs could have severe side effects because these pathways are also involved in mediating physiological responses. Therefore, therapeutic strategies that increase the levels of endogenous negative regulators of inflammation while leaving the positive pathways intact are gaining prominence. Thus, identifying the underlying
molecular mechanisms leading to up-regulation of inflammation is critical in the quest for novel therapeutic strategies with increased specificity and reduced side effects. Though curcumin has been long known as an anti-inflammatory agent, its effect on modulating NTHi-induced inflammatory responses in OM remains to be evaluated.

We performed this study to test the hypothesis that curcumin suppresses NTHi-induced expression of inflammatory mediators: chemokine CXCL5 and mucin MUC5AC in OM model. We tested this hypothesis in the following chapters. In chapter 2 we, (i) identified the molecular mechanism involved in the up-regulation of NTHi-induced CXCL5 chemokine expression (ii) demonstrated the inhibitory effect of curcumin on CXCL5 expression and (iii) identified the negative regulator MKP-1 as the key signaling molecule mediating the inhibitory effect of curcumin on CXCL5 expression. In chapter 3, we extended the findings observed from the CXCL5 studies to examine the effect of curcumin on MUC5AC mucin expression. In chapter 4, we further evaluated the effect of curcumin on promoting MKP-1 activity, by demonstrating that curcumin enhanced the post-translational K63-linked polyubiquitination of MKP-1, which could in turn enhance MKP-1 activity. Thus the insights from this study will help develop alternative therapies for treating NTHi-induced OM.

1.12 References


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2 CURCUMIN SUPPRESSES NTHI-INDUCED CXCL5 EXPRESSION VIA INHIBITION OF POSITIVE IKKB PATHWAY AND UP-REGULATION OF NEGATIVE MKP-1 PATHWAY

Publication: Anuhya S Konduru, Byung-Cheol Lee, Jian-Dong Li*
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2.1 Abstract

Otitis media (OM) is the most common childhood bacterial infection, and leading cause of conductive hearing loss. Nontypeable Haemophilus influenzae (NTHi) is a major bacterial pathogen for OM. OM characterized by the presence of overactive inflammatory responses is due to the aberrant production of inflammatory mediators including C-X-C motif chemokine ligand 5 (CXCL5). The molecular mechanism underlying induction of CXCL5 by NTHi is unknown. Here we show that the NTHi up-regulates CXCL5 expression by activating IKKβ-IκBα and p38 MAPK pathways via NF-κB nuclear translocation-dependent and -independent mechanism in middle ear epithelial cells. Current therapies for OM are ineffective due to the emergence of antibiotic-resistant NTHi strains and risk of side effects with prolonged use of immunosuppressant drugs. In this study, we show that curcumin, derived from Curcuma longa plant, long known for its medicinal properties, inhibited NTHi-induced CXCL5 expression in vitro and in vivo. Curcumin suppressed CXCL5 expression by direct inhibition of IKKβ phosphorylation, and inhibition of p38 MAPK via induction of negative regulator MKP-1. Thus, identification of curcumin as a potential therapeutic for treating OM is of particular translational significance due to the attractiveness of targeting overactive inflammation without significant adverse effects.
2.2 Introduction

Otitis media (OM) is the most common childhood bacterial infection [1] with 700 million occurrences globally each year [2]. OM frequently leads to conductive hearing loss, affecting children during the crucial period of speech and language development [3]. The gram-negative bacillus Nontypeable *Haemophilus influenzae* (NTHi) represents the cause of approximately one-third episodes of OM. Current treatments for OM rely on the systemic use of antibiotics, which has led to the emergence of multi-drug resistant bacterial strains [4, 5]. Therefore, there is an urgent need for developing alternate therapeutic strategies for treating OM.

OM is characterized by the presence of excess inflammation in the middle ear [1]. During infection, epithelial cells act as the first line of defense by secreting numerous pro-inflammatory mediators including chemokines. Chemokines mainly act by recruiting neutrophils to the site of infection. While the appropriate neutrophil response is critical for the removal of the invading pathogen, excess inflammation can lead to tissue damage and perpetuate inflammation leading to detrimental effects, as seen across several inflammatory pathologies including OM [6-9]. Thus, tight regulation of inflammation is necessary.

CXCL5/ENA-78 (epithelial neutrophil-activating peptide 78) of the Glu-Leu-Arg (ELR) motif-containing C-X-C chemokine family is critical for recruiting neutrophils in response to bacterial infections [10]. Epithelial-cell derived CXCL5 is vital in polymorphonuclear leukocytes (PMN)-driven destructive inflammatory responses in *Mycobacterium tuberculosis*-induced pulmonary tuberculosis. *Cxcl5*−/− mice were found to be resistant to fatal tuberculosis [11]. The role of CXCL5 in neutrophil trafficking in lipopolysaccharide (LPS)-induced lung inflammation in mice has been reported [12]. A recent study demonstrated that LPS-induced deregulated CXCL5 expression resulted in exaggerated neutrophil-mediated inflammation in pulmonary
bronchiolar cells [13]. CXCL5 is also involved in angiogenesis, tumor growth, and metastasis [14], with CXCL5 overexpression leading to poor survival in cancer patients [15]. Affymetrix chip analysis on mouse genome revealed marked up-regulation of CXCL5 expression by NTHi in the middle ear of mouse [16]. Middle ear effusion samples from patients with acute and chronic OM have shown the presence of viable NTHi trapped within neutrophil extracellular traps, which continued to elicit inflammatory responses [17, 18]. Thus, these findings suggest that therapeutic strategies to control aberrant CXCL5 production are of utmost importance for modulating inflammation.

Current anti-inflammatory therapies act via suppressing the positive signaling pathways involved in the production of inflammatory mediators. However, prolonged use of these drugs could have severe side effects because these pathways are also involved in mediating physiological responses. Therefore, therapeutic strategies that increase the levels of endogenous negative regulators of inflammation while leaving the positive pathways intact are gaining prominence [19]. Despite the importance of CXCL5 in mediating inflammation, the molecular mechanisms underlying the up-regulation of CXCL5 production in OM remains largely unknown. Therefore, understanding the mechanism of NTHi-induced CXCL5 regulation in OM will help develop new therapies.

Curcumin, a yellow pigment, common spice derived from the rhizome of *Curcuma longa* plant was long-used for its medicinal properties [20]. Curcumin has been widely reported to have anti-inflammatory, anti-oxidant, anti-microbial, anti-diabetic, anti-carcinogenic anti-tumorigenic, anti-amyloidogenic effects. Due to its tolerability and non-toxicity at high doses, curcumin could be used for prolonged periods without any side effects [20]. Completed clinical trials revealed promising therapeutic effects of curcumin in patients with cancer, inflammatory bowel disease,
irritable bowel disease, rheumatoid arthritis, Alzheimer’s disease, and diabetes [21-27]. More clinical trials evaluating the efficacy of curcumin on a broad range of inflammatory conditions are currently underway. Despite its widely known potent anti-inflammatory properties, the effect of curcumin on NTHi-induced inflammatory responses, especially CXCL5 expression remains to be evaluated.

In the present study, we investigated the underlying molecular mechanism of NTHi-induced CXCL5 expression. We show that NTHi up-regulates CXCL5 expression by activating IKKβ-IκBα and p38 MAPK pathways. Interestingly both pathways mediated CXCL5 expression in an NF-κB-nuclear translocation-dependent and -independent manner, respectively. Also, we show that curcumin suppresses NTHi-induced CXCL5 expression in middle ear epithelial cells. Curcumin suppressed CXCL5 expression by direct inhibition of IKKβ phosphorylation and inhibition of p38 MAPK via induction of negative regulator MKP-1. Thus, our study provides novel insights into the regulation of CXCL5 chemokine and identifies curcumin as a potential therapeutic for treating OM.

2.3 Materials and Methods

2.3.1 Reagents and antibodies

IKKβ inhibitor IV and MG-132 were purchased from EMD Millipore. CAPE, SB203580 were purchased from Enzo Life Sciences. Curcumin was purchased from Sigma. Antibodies for p-IKKα/β (#2697), p-p38 (#9211), p38 (#9212), anti-rabbit HRP-linked antibody (#7074) and anti-mouse HRP-linked antibody (#7076) were purchased from Cell Signaling Technology. Antibodies for IKKα/β (sc-7607), p65 (sc-8008), MKP-1 (sc-370), α-tubulin (sc-69969), c-Myc (sc-40), anti-mouse FITC-conjugated antibody (sc-2010), anti-rabbit Rhodamine-conjugated antibody (sc-2091) were purchased from Santa Cruz Biotechnology.
2.3.2 **Cell culture**

All media described below were supplemented with 10% fetal bovine serum and 100U/ml penicillin and 100 µg/ml streptomycin (Gibco). Human middle ear epithelial cells (HMEECs) were maintained in DMEM (Cellgro) supplemented with BEGM SingleQuots (Lonza). Lung epithelial A549 cells were maintained in F-12K medium (Gibco). Human cervical epithelial HeLa cells were maintained in DMEM (Cellgro). Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere.

2.3.3 **Bacterial strains and culture conditions**

Clinical isolates of NTHi strains 12, 2627, 9274 were used for this study [28, 29]. NTHi was grown on chocolate agar plate in 5% CO₂ atmosphere for 16 h, followed by overnight culture in brain heart infusion (BHI) broth supplemented with 3.5 µg/ml NAD and 10 µg/ml hemoglobin (BD Biosciences). Subsequently, bacteria were subcultured in 5 ml fresh BHI broth and the growth was monitored by measurement of optical density (OD). Log phase bacteria were harvested, washed and re-suspended in DMEM for *in vitro* experiments and isotonic saline for *in vivo* experiments. For all *in vitro* experiments the cells were stimulated with NTHi at a multiplicity of infection (MOI) of 50, with an exception for dose-dependent experiment. Cells were stimulated with NTHi for 5 h, or otherwise as indicated. For inhibition study, cells were pretreated with the respective inhibitor for 1 h prior to NTHi stimulation. For post-treatment studies cells were treated with curcumin 1 h after NTHi stimulation.

2.3.4 **Plasmids, transfection and luciferase assay**

The expression plasmids, for dominant negative (DN) forms of TLR2, TLR4, TRAF6, MyD88, IKKα (K44M), IKKβ (K49A), p38α (fp38α (AF)), p38β (fp38β2 (AF)), trans dominant
IκBα (S32A/S36A), constitutively active form of IKKβ (IKKβ-CA, S176E/S180E), p65 have been described previously [31, 59]. NF-κB luciferase reporter vector (pGL4.32) was purchased from Promega. Myc-MKP-1 overexpression plasmid was subcloned from previously described pSG5 – MKP-1 plasmid [53]. MKP-1 sequence was amplified using the primers 5’-GGTCTCGAGCGATGGTCATGGAAGTGG-3’ and 5’-GGTGGATCCTCCGCAGCTGGGAGAGGT-3’ and inserted into the XhoI and BamHI sites of pcDNA3.1/mycHis(-) vector. All transient transfections were performed using TransIT-LT-2020 transfection reagent (Mirus) according to the manufacturer’s protocol. Cells were assayed 48 h after transfection. Empty vector was transfected as a control. pSV-β-Galactosidase vector was used as a control for luciferase assay. Luciferase activity and β-Galactosidase activity was measured using Luciferase Assay System (Promega) and β-Galactosidase Enzyme Assay system (Promega). NF-κB luciferase activity was normalized with respect to β-Galactosidase activity.

2.3.5 RNA-mediated interference

Human siRNA (Control, D001810-10; IKKβ, L003503-00; p65, L003533-00; TAK1, M003790-06-0005) were purchased from GE Health care. Cells were transfected with 20 nM siRNA using DharmaFECT-4 (Thermo Scientific) according to manufacturer’s protocol. Human pSUPER-shMKP-1 knockdown construct was previously described [60]. shMKP-1 was transfected using TransIT-LT-2020 transfection reagent (Mirus) according to manufacturer’s protocol. Cells were assayed 48 h after transfection.

2.3.6 Real-time quantitative PCR (Q-PCR) analysis

Total RNA was isolated with TRIzol reagent (Life Technologies) according to manufacturer’s protocol. Reverse transcription reaction was performed with 1μg RNA using
TaqMan reverse transcription reagents (Applied Biosystems) according to manufacturer’s protocol. Quantitative PCR was performed using Fast SYBR Green Master Mix. PCR reactions containing 2X universal master mix, 1 µL template cDNA, 500 nM primers in a final volume of 12.5 µL, were amplified and quantified using StepOnePlus Real-Time PCR system (Applied Biosystems). Relative quantities of mRNAs were obtained using the comparative Ct method and were normalized to human Cyclophilin or mouse glyceraldehyde-3-phosphate (GAPDH); serving as an endogenous control. Human (h) and mouse (m) primer sequences are as follow:
hCXCL5 5’-GTGGTAGCCTCCCTGAAGAAC-3’ and 5’-TCCTTGTTCACCCTGCAA-3’;  
hTAK1 5’-GCAACAGAGGTGTCCGAC-3’ and 5’-CAGACATGCAGCACTCAT-3’;  
mCXCL5 5’-GCTGGCATTTCTGTTGCTGTTC-3’ and 5’-GGCAGCTTCAGCATGCT-3’.  
hMKP-1, hCyclophilin and mGAPDH primer sequences were previously described [60].

2.3.7 Enzyme-linked immunosorbenent assay (ELISA)

Cells were stimulated with NTHi for 12h. Culture media was harvested and centrifuged at 12,000 x g for 10 min to precipitate cell debris. Culture supernatants were assayed using human ENA78/ CXCL5 ELISA kit (Sigma) according to manufacturer’s protocol. OD was measured using Benchmark Plus microplate spectrophotometer. A standard curve showing the relationship between concentration and OD was generated for CXCL5 protein standards. CXCL5 protein concentration in culture supernatants was determined by interpolating from the standard curve.

2.3.8 Western Blot Analysis

Following NTHi stimulation, whole cell extracts were recovered with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, supplemented with 1 mM PMSF, 1 mM Na3VO4 and protease inhibitor cocktail). Cell extracts were incubated on ice for 30 min and centrifuged at 12,000 x g for 30 min
to precipitate cell debris. Supernatants were separated on 10% SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with blocking buffer (TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk). After 3 washes with TBS-T, the membrane was incubated overnight with primary antibodies at 1 : 1,000 – 1 : 2,000 dilutions in antibody dilution buffer (TBS-T containing 5% BSA) at 4 °C. After 3 washes with TBS-T, the membrane was incubated with corresponding secondary antibody at 1 : 5,000 dilution in blocking buffer for 1 h. After 3 washes with TBS-T, the proteins were visualized using Amersham ECL Prime Detection Reagent (GE Healthcare). Images have been cropped for presentation. Full-size images are presented in Supplementary Figs. 1–4.

2.3.9 Mice and Animal Experiments

C57BL/6 mice were purchased from Jackson Laboratories. Anesthetized mice were trans-tympanically inoculated with NTHi at a concentration of 5 x 10^7 CFU per mouse. Saline was inoculated as control. For inhibition studies, mice were injected intraperitoneally (i.p) with curcumin (50 mg/kg) 1 h prior or 1 h after NTHi inoculation. For mRNA analysis, mice were sacrificed 6 h post-NTHi inoculation. Total RNA was extracted from the dissected mice middle ear. For PMN analysis, mice were sacrificed 9 h post-NTHi inoculation. Middle ear effusions from mice were harvested with 10µl saline (×3). Following cytocentrifugation cells were stained with Diff-Quik stain kit (Siemens) according to manufacturer’s protocol. Images were recorded with light microscopy system (AxioVert 40 CFL, AxioCam MRC and AxioVision LE Image system, Carl Zeiss). All animal studies were carried out in accordance with the guidelines of, and were approved by, The Institutional Animal Care and Use Committee at Georgia State University.
2.3.10 Immunofluorescence staining

HMEECs were grown on 18 mm round glass coverslips (VWR). Cells were fixed in 4% paraformaldehyde. Cells were then incubated with primary antibodies at 1 : 100 − 1 : 400 dilutions. Primary antibody was detected with FITC or Rhodamine-conjugated secondary antibody. The coverslips were mounted onto glass slides using VECTASHIELD HardSet Antifade mounting Medium with DAPI (Vector). Images were recorded with fluorescence microscopy system (AxioVert 40 CFL, AxioCam MRC and AxioVision LE Image system, Carl Zeiss).

2.3.11 Statistical analysis

All experiments were repeated in at least three independent experiments. Data are shown as mean ± standard deviation (s.d.). The difference in means was assessed with unpaired student’s t-test for data with two conditions ($k = 2$), ANOVA (with Tukey’s post-hoc) for data with more than two conditions ($k > 2$), using SPSS 22 statistics software (IBM). *$p < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 NTHi induces CXCL5 expression in middle ear epithelial cells in vitro and in vivo.

Epithelial cells act as the first line of defense against injurious stimuli by mediating inflammatory responses. We sought to determine if NTHi induces CXCL5 expression in human middle ear epithelial cells (HMEECs). NTHi induced CXCL5 mRNA expression in a dose- (Fig. 2.1A) and time-dependent (Fig. 2.1B) manner in HMEECs. NTHi induced up-regulation of CXCL5 protein expression in HMEECs as quantified by ELISA (Fig. 2.1C). Effect of NTHi on CXCL5 expression was also confirmed in human airway epithelial BEAS-2B cells, human lung epithelial A549 cells and human cervical epithelial HeLa cells, suggesting the generalizability of
this phenomenon to multiple epithelial cells (Fig. 2.1D). We further explored the generalizability of NTHi-induced CXCL5 expression by employing two additional commonly used clinical NTHi strains 2627 and 9274 known to cause OM [28, 29]. NTHi strains 2627 and 9274 also induced CXCL5 mRNA expression at levels comparable to that of NTHi strain 12 (used throughout the study) in HMEECs (Fig. 2.1E). Consistent with the in vitro findings, NTHi also induced up-regulation of CXCL5 mRNA in mouse middle ear (Fig. 2.1F).

2.4.2 TLR2-MyD88-TRAF6-TAK1 signaling axis is required for NTHi-induced CXCL5 expression.

Toll-like receptors (TLRs) are cell surface receptors that play a critical role in mounting early innate immune responses against invading pathogens. TLRs recognize conserved motifs known as pathogen-associated microbial patterns (PAMPs), expressed on microbial pathogens and initiate signaling cascades leading to the production of pro-inflammatory mediators. To date, at least 11 human TLRs have been identified. Among them, TLR2 is known to recognize lipopolysaccharide (LPS), characteristic of Gram-negative bacteria. Based on our previous finding that TLR2 mediated NTHi-induced pro-inflammatory signaling cascades, we sought to determine its role in CXCL5 chemokine production. HMEECs were transfected with TLR dominant-negative mutants TLR2-DN and TLR4-DN. Overexpression of TLR2-DN significantly decreased NTHi-induced CXCL5 mRNA expression, whereas TLR4-DN had no significant effect on CXCL5 expression (Fig. 2.2A). Next, we sought to determine the signaling molecules downstream of TLR2, involved in mediating CXCL5 expression. Following recognition of NTHi by TLR2, myeloid differentiation factor 88 (MyD88) adaptor protein is recruited to the receptor. MyD88 then recruits IL-1 receptor-associated kinases (IRAKs), which further lead to the recruitment and activation of tumor-necrosis factor-receptor-
associated factor 6 (TRAF6). IRAK-TRAF6 complex dissociates from the receptor bound complex and further interacts with transforming growth factor-β-activated kinase 1 (TAK1). Activation of TAK1 leads to initiation of further downstream signaling pathways, resulting in nuclear translocation of transcription factors, and in turn regulates the expression of pro-inflammatory mediators. To determine the involvement of MyD88 and TRAF6 in CXCL5 expression, HMEECs were transfected with dominant-negative mutants MyD88-DN and TRAF6-DN. Overexpression of MyD88-DN and TRAF6-DN significantly suppressed NTHi-induced CXCL5 expression (Fig. 2.2B). Depletion of endogenous TAK1 with TAK1 siRNA also decreased CXCL5 mRNA expression (Fig. 2.2C). TAK1 siRNA knockdown efficiency was confirmed by Q-PCR. Therefore, these results suggest that TLR2-MyD88-TRAF6-TAK1 signaling axis is required for NTHi-induced CXCL5 expression.

### 2.4.3 Activation of IKKβ-IκBα signaling pathway is required for NTHi-induced CXCL5 expression.

Previous studies have shown that IKKβ signaling axis is crucial for NTHi-induced inflammatory responses [30]. Therefore, we examined the role of IKKβ in up-regulation of CXCL5. We first confirmed that NTHi activates IKKβ in HMEECs. IKKα/β phosphorylation was observed at 15 minutes, followed by a peak at 30 minutes, and declined after that (Fig. 2.3A). To determine if IKKβ is required for NTHi-induced CXCL5 expression, we used multiple approaches. IKKβ inhibitor significantly suppressed NTHi-induced CXCL5 mRNA expression in a dose-dependent manner (Fig. 2.3B). To identify the major IKK isoform involved in NTHi-induced CXCL5 regulation, HMEECs were transfected with IKK dominant-negative (DN) mutants IKKα-DN and IKKβ-DN. Overexpression of IKKβ-DN significantly decreased NTHi-induced CXCL5 mRNA expression, whereas IKKα-DN had no significant effect (Fig. 2.3C).
Consistent with this result, depletion of endogenous IKKβ with IKKβ siRNA also decreased CXCL5 mRNA expression (Fig. 2.3D). IKKβ siRNA knockdown efficiency was confirmed by Western blot. To further confirm that activated IKKβ induces CXCL5 expression, HMEECs were transfected with constitutively active (CA) form IKKβ-CA. Overexpression of IKKβ-CA markedly induced CXCL5 expression in a dose-dependent manner (Fig. 2.3E). Phosphorylation and proteasomal degradation of IκBα, by IKKβ, are required for signal transduction [31]. Overexpression of a trans-dominant mutant form of IκBα (IκBα-DN) suppressed NTHi-induced CXCL5 expression (Fig. 2.3F). To further confirm that IκBα degradation is essential for NTHi-induced CXCL5 up-regulation, we used MG-132 proteasome inhibitor. MG-132 significantly suppressed NTHi-induced CXCL5 mRNA expression in a dose-dependent manner (Fig. 2.3G). Thus, these results suggest that IKKβ-IκBα signaling pathway is required for CXCL5 induction by NTHi.

2.4.4 Activation of p38 signaling is required for NTHi-induced CXCL5 expression.

NTHi has been shown to mediate inflammatory responses via activation of p38 MAPK signaling axis in addition to activation of IKKβ-IκBα pathway [31]. Therefore, we examined the role of p38 in up-regulation of CXCL5. We first confirmed that NTHi activates p38 MAPK in HMEECs. p38 phosphorylation was observed at 15 minutes, followed by a peak at 30 minutes, and declined after that (Fig. 2.3H). To determine if p38 MAPK activation is essential for NTHi-induced CXCL5 expression, multiple approaches were used. SB203580, a specific inhibitor of p38 activation, significantly suppressed NTHi-induced CXCL5 mRNA expression in a dose-dependent manner (Fig. 2.3I). To identify the major p38 isoform involved in NTHi-induced CXCL5 regulation, HMEECs were transfected with p38 DN mutants p38α-DN and p38β-DN.
Over-expression of either or both p38 α, β DN forms significantly decreased NTHi-induced CXCL5 expression (Fig. 2.3J), consistent with SB203580 data. Together, these data suggest that p38 pathway is required for induction of CXCL5.

2.4.5 **IKKβ-IκBα and p38 signaling axes mediate CXCL5 induction via p65 nuclear translocation-dependent and -independent mechanism, respectively.**

Next, we sought to determine how IKKβ-IκBα and p38 signaling pathways induce CXCL5 expression. Co-treatment with IKKβ inhibitor and SB203580 synergistically suppressed CXCL5 expression at mRNA (Fig. 2.4A) and protein (Fig. 2.4B) levels. As NF-κB is a known major transcription factor for pro-inflammatory mediators [32], we investigated its involvement in NTHi-induced CXCL5 expression. Nuclear translocation of NF-κB is critical for its activity [32]. NTHi induces nuclear translocation of p65, the major subunit of NF-κB complex [31]. To determine that p65 nuclear translocation and activation is required for NTHi-induced CXCL5 expression, we used multiple approaches. Pretreatment with caffeic acid phenyl ester (CAPE), a specific inhibitor of NF-κB nuclear translocation (independent of IκBα degradation), completely abrogated NTHi-induced CXCL5 expression (Fig. 2.4C). Pretreatment with CAPE markedly diminished NTHi-induced NF-κB promoter-driven luciferase activity (Fig. 2.4D). Depletion of endogenous p65, with p65 siRNA, decreased CXCL5 mRNA expression (Fig. 2.4E). p65 siRNA knockdown efficiency was confirmed by Western blot. Overexpression of p65 further enhanced NTHi-induced CXCL5 expression (Fig. 2.4F). Therefore, these results suggest that NTHi induces CXCL5 transcription via activation of NF-κB, specifically by p65 subunit.

To determine whether p38 MAPK induces CXCL5 expression via p65, HMEECs transfected with p65 were pre-treated with SB203580, prior to NTHi stimulation. SB203580 decreased CXCL5 expression in p65-transfected cells (Fig. 2.4G). We further confirmed the
requirement of p38 for NF-κB activation by multiple approaches. Pre-treatment with SB203580 markedly decreased NTHi-induced NF-κB promoter-driven luciferase activity (Fig. 2.4H). Consistent with this result, over-expression of either or both p38α and p38β DN forms decreased NTHi-induced NF-κB promoter activity (Fig. 2.4I). These results suggest that p38 MAPK also mediates NTHi-induced CXCL5 expression in a p65 dependent mechanism.

Since nuclear translocation of p65 is imperative for NF-κB-driven gene expression [32], we determined if p38 up-regulates CXCL5 expression via facilitating nuclear translocation of p65 by immunofluorescence staining. CAPE markedly inhibited nuclear translocation of p65, whereas SB203580 did not show any significant effect (Fig. 2.4J). Taken together these results suggest that p38 mediates CXCL5 expression via a mechanism independent of the nuclear translocation of p65. Therefore, these results suggest that IKKβ and p38 signaling pathways mediate NTHi-induced CXCL5 up-regulation via activation of p65 in a p65-nuclear translocation-dependent and -independent mechanism, respectively.

2.4.6 Curcumin suppresses NTHi-induced CXCL5 expression in vitro and in vivo.

Having identified the molecular mechanisms underlying NTHi-induced up-regulation of CXCL5, we next sought to explore the translational significance of these findings. Because curcumin, a promising anti-inflammatory agent, has previously been shown to inhibit NF-κB [33, 34], we first determined if curcumin inhibits NTHi-induced up-regulation of CXCL5. Curcumin pre-treatment inhibited CXCL5 mRNA expression in a dose-dependent manner (Fig. 2.5A). Curcumin's inhibitory effect on CXCL5 protein levels was also confirmed (Fig. 2.5B). Additionally, the inhibitory effect of curcumin on CXCL5 mRNA expression was also observed in HMEECs stimulated with other common clinical NTHi strains 2627 and 9274 (Fig. 2.5C), thereby suggesting the generalizability to more OM-causing NTHi strains. Consistent with in
*in vitro* findings, curcumin pre-treatment inhibited CXCL5 mRNA expression in the middle ear of mice inoculated with NTHi (Fig. 2.5D). These data suggest that curcumin inhibits NTHi-induced CXCL5 expression in middle ear epithelial cells *in vitro* and *in vivo*.

Next, we sought to determine the therapeutic relevance of the inhibitory effect of curcumin in NTHi-induced OM model. Thus, we evaluated the effect of administering curcumin post-NTHi infection that resembles a clinically relevant setting. Administering curcumin post-NTHi infection significantly suppressed NTHi-induced CXCL5 mRNA expression *in vitro* (Fig. 2.5E) and *in vivo* (Fig. 2.5F). Curcumin suppressed CXCL5 expression to the same extent under both pre-NTHi and post-NTHi infection conditions. Since CXCL5 is a neutrophil chemoattractant, we further evaluated the effect of curcumin on PMN recruitment in response to NTHi infection in a mouse model of OM. Consistent with the above results curcumin (pre-NTHi and post-NTHi infection) inhibited PMN infiltration as assessed by PMN staining of middle ear effusion from mice (Fig. 2.5G). Thus, these data suggest that curcumin is a potential therapeutic for treating NTHi-induced inflammation as seen in OM.

### 2.4.7 Curcumin suppresses NTHi-induced CXCL5 expression via inhibition of IKKβ and p38 pathways.

Next, we sought to determine the mechanism by which curcumin inhibits CXCL5 expression. Since activation of IKKβ-IκBα and p38 signaling pathways has been shown to be involved in NTHi-induced CXCL5 expression, we assessed the effect of curcumin on these pathways. Curcumin abrogated NTHi-induced IKKα/β phosphorylation (Fig. 2.6A). Moreover, curcumin inhibited IKKβ-CA-induced CXCL5 expression (Fig. 2.6B). Curcumin reduced NTHi-induced p38 phosphorylation (Fig. 2.6C). We also confirmed the inhibitory effect of curcumin on NTHi-induced p38 phosphorylation by immunofluorescence staining (Fig. 2.6D). Next, we
determined the effect of curcumin on p65 nuclear translocation and NF-κB-driven luciferase promoter activity. Consistent with the above findings, curcumin suppressed NTHi-induced p65 nuclear translocation (Fig. 2.6E) and NF-κB luciferase activity (Fig. 2.6F). Thus, these data suggest that curcumin inhibits NTHi-induced CXCL5 expression via inhibition of both IKKβ-IκBα and p38 signaling pathways.

2.4.8 Curcumin suppresses CXCL5 expression via up-regulation of negative regulator MKP-1.

MKP-1, a member of a class of dual specificity phosphatases collectively termed MAPK phosphatases, has been shown to be a key negative regulator of inflammatory responses via dephosphorylation and inactivation of MAPKs, including p38 [35, 36]. Since we identified the requirement of p38 MAPK activation in NTHi-induced CXCL5 expression, we determined the role of MKP-1 in CXCL5 regulation. Overexpression of MKP-1 suppressed NTHi-induced CXCL5 expression (Fig. 2.7A). Depletion of endogenous MKP-1 with MKP-1 shRNA enhanced CXCL5 mRNA expression (Fig. 2.7B). MKP-1 shRNA knockdown efficiency was confirmed by Q-PCR. These data suggest that MKP-1 is a negative regulator of NTHi-induced CXCL5 induction.

To further determine if MKP-1 acts as a negative regulator of CXCL5 induction via inactivation of p38, we evaluated the effect of MKP-1 on p38 phosphorylation. Overexpression of MKP-1 reduced NTHi-induced p38 phosphorylation (Fig. 2.7C). In contrast, depletion of MKP-1 enhanced p38 phosphorylation (Fig. 2.7D). These data suggest that MKP-1 negatively regulates NTHi-induced CXCL5 expression by targeting p38 MAPK.

Since curcumin and MKP-1 were identified to suppress activation of p38, we sought to determine if curcumin up-regulates MKP-1 expression. Curcumin markedly enhanced NTHi-
induced MKP-1 expression at mRNA (Fig. 2.7E) and protein (Fig. 2.7F) levels. These data suggest the curcumin increases NTHi-induced expression of negative regulator MKP-1.

Having shown that curcumin inhibits NTHi-induced CXCL5 via inhibition of p38 and up-regulation of MKP-1 expression, we sought to determine if the inhibitory effect of curcumin on p38 is dependent on the up-regulation of MKP-1. Depletion of MKP-1 with shMKP-1 rendered curcumin treatment ineffective in inhibiting NTHi-induced CXCL5 expression (Fig. 2.7G). Additionally, curcumin no longer suppressed NTHi-induced p38 phosphorylation in the absence of MKP-1 (Fig. 2.7H). These data suggest that curcumin inhibits NTHi-induced activation of p38 via up-regulating MKP-1. Thus, our results suggest that curcumin inhibits NTHi-induced CXCL5 expression via MKP-1-dependent inhibition of p38 MAPK.

2.5 Discussion

Inflammatory responses are essential for the containment, removal of the invading pathogens and recovery of the host. However, excess inflammation can be detrimental to the host as seen in OM [6-9, 37, 38]. Therefore, tight regulation of the intensity and duration of inflammatory responses is necessary. In the present study, we show that curcumin inhibits CXCL5 chemokine up-regulation in NTHi-induced OM model, in vitro and in vivo. We found that NTHi up-regulated CXCL5 expression by activating IKKβ-IκBα and p38 MAPK pathways via NF-κB nuclear translocation-dependent and -independent mechanism. Curcumin not only inhibited the positive IKKβ pathway but also up-regulated the expression of MKP-1, a key negative regulator of p38 MAPK, thereby suppressing CXCL5 expression by dual action. Thus, the current study provides novel insights into the molecular mechanism underlying the tight regulation of neutrophil attractant chemokine CXCL5 in the pathogenesis of NTHi-induced OM and also demonstrates the potential of curcumin as a novel therapeutic for treating OM (Fig. 2.8).
NF-κB was found to be the major transcription factor regulating NTHi-induced CXCL5 expression. In our study, both IKKβ-IκBα and p38 MAPK pathways were found to act via p65 subunit to regulate NF-κB transcriptional activity, albeit through different mechanisms to induce CXCL5 expression. Under resting conditions NF-κB is present in the cytoplasm bound to IκBα. Upon activation of upstream signaling pathways, NF-κB dissociates from IκBα and translocates to the nucleus to regulate gene expression. CAPE, an inhibitor of NF-κB nuclear translocation, inhibited p65 nuclear translocation and suppressed NF-κB transcriptional activity that in turn suppressed CXCL5 expression. Interestingly p38 MAPK inhibitor SB203580 failed to inhibit p65 nuclear translocation but suppressed NF-κB transcriptional activity and CXCL5 expression. These findings suggest that p38 MAPK regulates NF-κB transcriptional activity itself but not p65 nuclear translocation. Previous studies have reported that post-translational modifications such as phosphorylation and acetylation of p65 are critical for promoting its DNA binding and interaction with transcriptional machinery to regulate gene expression. p38 MAPK was found to regulate the acetylation status of p65 but not its phosphorylation. In response to activating stimuli, p38 was found to phosphorylate the transcriptional coactivator p300 (a histone acetyltransferase). Phosphorylated p300 binds to and acetylates K310 residue on p65. Acetylation of K310 was shown to enhance p65 transcriptional activity [39]. Therefore it likely that p38 MAPK mediates NTHi-induced CXCL5 expression in a similar manner by increasing NF-κB transcriptional activity, independent of p65 nuclear translocation.

OM, a leading cause of conductive hearing loss in children, is caused by NTHi [1]. OM is characterized by the presence of excessive inflammation in the middle ear [3, 40]. Current therapies for OM involve the use of analgesics and antipyretics for symptomatic treatment [41]. Though these medications are effective during certain stages of the disease, prolonged usage
poses the risk of serious side effects due to unknown “off-targets” and weakened immune system. Decongestants, antihistamines, and corticosteroids have not been effective in treating OM [42]. Prophylactic use of antibiotics has rendered over 80% of the NTHi strains drug-resistant [4, 5]. Also, development of vaccines against NTHi remains a challenge due to the high genetic diversity of NTHi strains and high antigenic variability of surface-exposed antigens [43, 44]. Thus, there is an urgent need for developing alternative therapeutics for OM with increased efficiency and safety. Therefore, identifying the underlying molecular mechanisms leading to up-regulation of inflammation is critical for the development of novel therapeutic strategies with increased specificity and reduced side effects. Interestingly, in the current study we provide evidence that curcumin inhibits NTHi-induced CXCL5 chemokine expression in middle ear epithelial cells. Interestingly, both pre-infection and post-infection treatment with curcumin not only inhibited NTHi-induced CXCL5 up-regulation but also suppressed PMN infiltration into the middle ear in a mouse model of OM. Curcumin treatment’s efficacy in inhibiting CXCL5 expression and PMN recruitment post-NTHi infection is of particular clinical significance. Recently chemokines and chemokine receptors are increasingly considered as targets for developing new drugs to control inflammation [45]. Our finding that curcumin suppresses NTHi-induced CXCL5 chemokine expression is of particular relevance in the current scheme of identifying chemokine-drug combinations to treat inflammation. Thus, curcumin could be repurposed as a new therapeutic for treating OM.

Curcumin is a nutraceutical that has been in use in South Asian countries for many centuries owing to its medicinal properties [20]. Curcumin can interact with a myriad of signaling molecules including transcription factors, protein kinases, growth factors, receptors, adhesion molecules, pro-inflammatory cytokines [46], thus explaining its pleiotropic therapeutic
potential against a wide range of diseases. Curcumin does not present a dose-limiting toxicity, making it suitable for prolonged usage. Completed clinical trials reported usage of curcumin dosage ranging from 0.045 to 8 g/day. Currently, 38 clinical trials evaluating the efficacy of curcumin at a dosage ranging from 0.18 to 8 g/day for treating pathologies such as Alzheimer’s disease, diabetes, kidney disease, Crohn’s disease, cancer are underway [47]. United States Food and Drug Administration classified curcumin as GRAS (generally recognized as safe), warranting its use as a supplement. In the current study, we identified a novel role of curcumin in suppressing CXCL5 chemokine production. Co-administration of curcumin along with piperine, docetaxel, soy isoflavones, bioperine, lactoferrin, mesalamine in clinical trials [48-52], suggest the possibility of customizing curcumin-based therapies to maximize its therapeutic efficiency. Since bioavailability of curcumin is a challenge [47], further studies combining the use of adjuvants, lipids, nanoparticles are needed to elucidate further the potency of curcumin in treating OM.

Another relevant finding of biological significance in the current study is the dual acting mechanism of curcumin in inhibiting CXCL5 expression. Curcumin inhibited NTHi-induced IKKβ phosphorylation, thereby suppressing CXCL5 up-regulation. Moreover, we found that curcumin also inhibits NTHi-induced CXCL5 expression via MKP-1-dependent suppression of p38 MAPK. In the absence of MKP-1, curcumin failed to suppress CXCL5 expression. Due to the importance of p38 in maintaining homeostasis, up-regulation of negative regulator MKP-1 by curcumin could play an important role in controlling the over-active immune responses with minimal side effects. This finding is of particular translational significance due to the attractiveness of targeting overactive inflammation via induction of negative-regulators [19].
We previously demonstrated that dexamethasone glucocorticoid inhibits p38 MAPK via up-regulation of MKP-1 [53]. Glucocorticoids owing to their potent immunosuppressive and anti-inflammatory effects have been in use for treating a gamut of diseases such as asthma, allergies, skin disorders, multiple sclerosis, immune disorders and cancer. However, prolonged usage has been reported to cause severe, sometimes irreversible side effects such as osteoporosis, endocrine and metabolic disorders, behavioral and cognitive changes, gastrointestinal tract complications, uveitis and weakened immune system [54]. Numerous studies over the past years have demonstrated curcumin’s efficacy in resolving pathologies was similar to that of dexamethasone [55, 56]. No evidence of side effects with low to moderate consumption of curcumin exists. With a higher curcumin dosage 12 g/day, mild symptoms such as diarrhea, low blood sugar, abdominal pain, and indigestion have been reported [47]. Additionally, curcumin has been reported to aid in overcoming the side effects of glucocorticoid usage [57]. Curcumin is also effective against oxidative stress, characteristic of many inflammatory conditions. Curcumin supplementation could be an effective disease preventive strategy due to its immunomodulatory activity [58]. Thus, curcumin fits the bill for an alternative therapeutic with minimal side effects.

In conclusion, our study demonstrates for the first time that curcumin is a potent inhibitor of CXCL5 chemokine, which could, in turn, suppress inflammation. Further studies promoting curcumin bioavailability may provide means to develop therapies to modulate inflammation more stringently without adverse effects. Development of drug delivery systems in the form of a topical ointment and ear drops could be of clinical significance in treating OM. The findings of this study may have applications in a broader context to other pathologies including chronic obstructive pulmonary disease, tuberculosis, cancer and Alzheimer’s disease.
2.6 References


Figure 2.1 NTHi up-regulates CXCL5 expression in middle ear epithelial cells in vitro and in vivo

(A) HMEECs were stimulated with NTHi (MOI of 25, 50 or 250) for 5 h, and CXCL5 mRNA expression was measured by Q-PCR. (B) HMEECs were stimulated with NTHi (MOI of 50) for 1, 3 or 5 h, and CXCL5 mRNA expression was measured by Q-PCR. (C) HMEECs were stimulated with NTHi for 12 h, and CXCL5 protein levels in cell culture supernatants was measured by ELISA. (D) Airway epithelial BEAS-2B cells, lung epithelial A549 cells and cervical epithelial HeLa cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured by Q-PCR. (E) HMEECs were stimulated with NTHi strains 12, 2627 or 9274 for 5 h, and CXCL5 mRNA expression was measured by Q-PCR. (F) Mice were trans-tympanically inoculated with NTHi (6 x 10^7 CFU) for 6 h, and CXCL5 mRNA expression in middle ear was measured by Q-PCR. Data are mean ± s.d. (n=3). A, B, E; *p < 0.05, ANOVA (Tukey’s post-hoc). C, D, F; *p < 0.05, t-test. Data are representative of three or more independent experiments.
Figure 2.2 TLR2-MyD88-TRAF6-TAK1 signaling axis is required for NTHi-induced CXCL5 expression

(A-B) HMEECs were transfected with Mock, TLR2-DN, TLR4-DN, MyD88-DN or TRAF6-DN plasmid. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. (C) HMEECs were transfected with control siRNA or TAK1 siRNA. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. Knockdown of TAK1 by siRNA was confirmed by Q-PCR. Data are mean ± s.d. (n=3). A, B; *p < 0.05, ANOVA (Tukey’s post-hoc). C; *p < 0.05, t-test. Data are representative of three or more independent experiments.
Figure 2.3 Activation of IKKβ-IκBα and p38 signaling pathways are required for NTHi-induced CXCL5 expression

(A) HMEECs were stimulated with NTHi for various time intervals as indicated in the figure. Phospho-IKKα/β, total IKKα/β protein levels were visualized by western blot. (B) HMEECs were pre-treated with IKKβ inhibitor (0.25, 0.5 or 1.0 µM) for 1 h, followed by stimulation with NTHi for 5 h, and CXCL5 mRNA expression was measured. (C) HMEECs were transfected with Mock, IKKα-DN or IKKβ-DN plasmid. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. (D) HMEECs were transfected with control siRNA or IKKβ siRNA. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. Knockdown
of IKKβ protein by siRNA was confirmed by western blot. (E) HMEECs were transfected with Mock, IKKβ - CA (0.25, 0.5 or 1 µg) plasmid. CXCL5 mRNA expression was measured. (F) HMEECs were transfected with Mock or IκBα (S32A/S36A) plasmid. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. (G) HMEECs were pre-treated with MG-132 (5, 10 or 20 µM) for 1 h, followed by stimulation with NTHi for 5 h, and CXCL5 mRNA expression was measured. (H) HMEECs were stimulated with NTHi for various time intervals as indicated in the figure. Phospho-p38, total p38 protein levels were visualized by western blot analysis. (I) HMEECs were pre-treated with SB203580 (5, 10 or 20 µM) for 1 h, followed by stimulation with NTHi for 5 h, and CXCL5 mRNA expression was measured. (J) HMEECs were transfected with Mock, p38α - DN, p38β - DN or both (p38α - DN and p38β - DN) plasmids. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. Data are mean ± s.d. (n=3). B, C, E, G, I, J; *p < 0.05, ANOVA (Tukey’s post-hoc). D, F; *p < 0.05, t-test. n.s., not significant. Data are representative of three or more independent experiments.
Figure 2.4 IKKβ-IKBα and p38 signaling axes mediate CXCL5 induction via p65 nuclear translocation–dependent and –independent mechanism, respectively

(A-B) HMEECs were treated with IKKβ inhibitor (0.5 µM), SB203580 (10 µM) or both for 1 h, followed by stimulation with NTHi for (a) 5 h, and CXCL5 mRNA expression was measured, (b) 12 h, and CXCL5 protein levels in cell culture supernatants was measured by ELISA. (C) HMEECs were pre-treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h, and CXCL5 mRNA expression was measured.

(D) Relative luciferase activity of NF-κB-Luc was measured in HMEECs treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.

(E) Relative luciferase activity of NF-κB-Luc and relative quantity of mRNA for CXCL5 were measured in HMEECs pre-treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.

(F) Relative luciferase activity of NF-κB-Luc was measured in HMEECs treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.

(G) Relative luciferase activity of NF-κB-Luc and relative quantity of mRNA for CXCL5 were measured in HMEECs pre-treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.

(H) Relative luciferase activity of NF-κB-Luc was measured in HMEECs treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.

(I) Relative luciferase activity of NF-κB-Luc was measured in HMEECs treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.

(J) Immunofluorescence images of HMEECs treated with CAPE (5, 10 or 25 µM) for 1 h, followed by stimulation with NTHi for 5 h.
µg/ml) for 1 h, followed by stimulation with NTHi for 5 h, and CXCL5 mRNA expression was measured. (D) HMEECs were transfected with NF-κB luciferase vector. Cells were pre-treated with CAPE (25 µg/ml) for 1 h, followed by NTHi stimulation for 5 h. NF-κB promoter activity was measured by luciferase assay. (E) HMEECs were transfected with control siRNA or p65 siRNA. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. Knockdown of p65 protein by siRNA was confirmed by western blot. (F-G) HMEECs were transfected with Mock or p65. Cells were (F) stimulated with NTHi for 5 h, (G) pre-treated with SB203580 (20 µM) for 1 h, followed by stimulation with NTHi for 5 h; and CXCL5 mRNA expression was measured. (H-I) HMEECs were transfected with (H) NF-κB luciferase vector alone, (I) NF-κB luciferase vector and Mock, p38α-DN, p38β-DN or both (p38α-DN and p38β-DN) plasmids. Cells were (H) pre-treated with SB203580 (20 µM) for 1 h and (H,I) stimulated with NTHi for 5 h. NF-κB promoter activity was measured by luciferase assay. (J) HMEECs were pre-treated with CAPE (25 µg/ml) or SB203580 (20 µM) for 1 h, followed by NTHi stimulation for 1 h. p65 translocation was visualized by immunofluorescence by FITC staining. DAPI, nuclear stain. Magnification: 400×. Data are mean ± s.d. (n=3). A-C, F, G, I; *p < 0.05, ANOVA (Tukey’s post-hoc). D, E, H; *p < 0.05, t-test. Data are representative of three or more independent experiments.
Figure 2.5 Curcumin suppresses NTHi-induced CXCL5 expression in vitro and in vivo

(A) HMEECs were pre-treated with curcumin (10, 20 or 50 µM) for 1 h, followed by stimulation with NTHi for 5 h, and CXCL5 mRNA expression was measured. (B) HMEECs were pre-treated with curcumin (20 µM) for 1 h, followed by stimulation with NTHi for 12 h, and CXCL5 protein levels in cell culture supernatants was measured by ELISA. (C) HMEECs were pre-treated with curcumin (20 µM) for 1 h, followed by stimulation with NTHi strains 12, 2627 or 9274 for 5 h, and CXCL5 mRNA expression was measured. (D) Mice were pretreated with curcumin (50 mg/kg) (i.p) for 1 h, followed by trans-tympanic inoculation with NTHi (5 x 10^7 CFU) for 6 h, CXCL5 mRNA expression in dissected middle ear was measured. (E) HMEECs were pre-treated with curcumin (20 µM) 1 h prior NTHi stimulation or post-treated with curcumin (20 µM) 1 h after NTHi stimulation. 5 h after NTHi stimulation CXCL5 mRNA expression was measured. (F) Mice were pre-treated with curcumin (50 mg/kg) (i.p) for 1 h, followed by trans-tympanic inoculation with NTHi or post-treated with curcumin (50 mg/kg) (i.p) 1 h after NTHi inoculation. 6 h after NTHi inoculation CXCL5 mRNA expression in dissected middle ear was measured. (G) Mice were pre-treated with curcumin (50 mg/kg) (i.p) for 1 h, followed by trans-tympanic inoculation with NTHi or post-
treated with curcumin (50 mg/kg) (i.p) 1 h after NTHi inoculation. Middle ear effusion was harvested 9 h after NTHi inoculation. Following cytocentrifugation, cells were stained with Diff-Quik staining kit. n.d., not detected. Magnification: 400X. PMN cell count in middle ear effusion was determined using a hemocytometer under the microscope. n.d., not detected. Data are mean ± s.d. (n=3). A, E-G; *p < 0.05, ANOVA (Tukey’s post-hoc). B-D; *p < 0.05, t-test. n.s., not significant. Data are representative of three or more independent experiments.
Figure 2.6 Curcumin suppresses NTHi-induced CXCL5 expression via inhibition of IKKβ and p38 pathways

(A) HMEECs were treated with curcumin (20 μM) for 1 h, followed by stimulation with NTHi for various time intervals as indicated in the figure. Phospho-IKKβ, total IKKβ protein levels were visualized by western blot. (B) HMEECs were transfected with Mock or IKKβ-CA plasmid. Cells were treated with curcumin (20 μM) for 1 h, and CXCL5 mRNA expression was measured. (C) HMEECs were treated with curcumin (20 μM) for 1 h, followed by stimulation with NTHi for various time intervals as indicated in the figure. Phospho-p38, total p38 protein levels were visualized by western blot. (D-E) HMEECs were pre-treated with curcumin (20 μM) for 1 h, followed by NTHi stimulation for (D) 30 min, (E) 1 h. (D) Phospho-p38, total p38 protein levels (Rhodamine stain), (E) p65 translocation (FITC stain) was visualized by immunofluorescence. DAPI, nuclear stain. Magnification: 400×. (F) HMEECs were transfected with NF-κB luciferase vector. Cells were pre-treated with curcumin (20 μM) for 1 h, followed by stimulated with NTHi for 5 h. NF-κB promoter activity was measured by luciferase assay. Data are mean ± s.d. (n=3). B, F; *p < 0.05, t-test. Data are representative of three or more independent experiments.
Figure 2.7 Curcumin suppresses NTHi-induced CXCL5 expression via up-regulation of negative regulator MKP-1

(A-B) HMEECs were transfected with (A) Mock or myc-MKP-1, (B) Mock or MKP-1 shRNA. Cells were stimulated with NTHi for 5 h, and CXCL5 mRNA expression was measured. Knockdown of MKP-1 protein by
shRNA was confirmed by Q-PCR. (C, D) HMEECs were transfected with (C) Mock or myc-MKP-1 plasmid, (D) Mock or shMKP-1 shRNA. Cells were stimulated with NTHi for times indicated. Phospho-p38, total p38, MKP-1 protein levels were visualized by western blot. (E) HMEECs were pre-treated with curcumin (20 µM) for 1 h, followed by stimulation with NTHi for 1 h, and MKP-1 mRNA expression was measured. (F) HMEECs were pre-treated with curcumin (20 µM) for 1 h, followed by stimulation with NTHi for various time intervals as indicated in the figure. MKP-1 and α-tubulin protein levels were visualized by western blot analysis. (G) HMEECs were transfected with Mock or MKP-1 shRNA. Cells were pre-treated with curcumin (20 µM) for 1 h, followed by NTHi stimulation for 5 h, and CXCL5 mRNA expression was measured. (H) HMEECs were transfected with Mock or MKP-1 shRNA. Cells were pre-treated with curcumin (20 µM) for 1 h, followed by NTHi stimulation for 30 min. Phospho-p38, total p38 protein levels were visualized by western blot. Data are mean ± s.d. (n=3). A, B, E; *p < 0.05, t-test. G; *p < 0.05, ANOVA (Tukey’s post-hoc). n.s., not significant. Data are representative of three or more independent experiments.
Figure 2.8 Schematic representation of NTHi-induced CXCL5 expression and curcumin-mediated suppression of CXCL5.
3 CURCUMIN INHIBITS NTHI-INDUCED MUC5AC MUCIN OVERPRODUCTION IN OTITIS MEDIA VIA UP-REGULATION OF MAPK PHOSPHATASE MKP-1

Anuhya S Konduru, Byung-Cheol Lee, Jian-Dong Li
Under review for publication

3.1 Abstract

Otitis media (OM) characterized by the presence of mucus overproduction and excess inflammation in the middle ear is the most common childhood infection. Nontypeable Haemophilus influenzae (NTHi) pathogen is responsible for approximately one-third episodes of bacteria-caused OM. Current treatments for bacterial OM rely on the systemic use of antibiotics, which often leads to the emergence of multi-drug resistant bacterial strains. Therefore, there is an immediate need for developing alternative therapies strategies for controlling mucus overproduction in OM. MUC5AC mucin has been shown to play a critical role in the pathogenesis of OM. Here we show that curcumin derived from Curcuma longa plant is a potent inhibitor of NTHi-induced MUC5AC mucin expression in middle ear epithelial cells. Curcumin inhibited MUC5AC expression by suppressing activation of p38 MAPK by up-regulating MAPK phosphatase MKP-1. Thus, our study identified curcumin as a potential therapeutic for inhibiting mucin overproduction in OM by up-regulating MKP-1 a known negative regulator of inflammation.

Keywords: Nontypeable Haemophilus influenzae, mucin, MUC5AC, otitis media, curcumin
3.2 Introduction

Mucin glycoproteins are a major component of mucus secretions in the middle ear, trachea, digestive and reproductive tracts. Mucus production represents a protective innate defense mechanism to protect and lubricate the epithelium and trap invading pathogens for removal by the mucociliary clearance system [1]. However, in chronic infections, excess mucin impairs the mucociliary clearance system, resulting in mucus accumulation and poor function of the mucus-lined epithelial tracts. Of the ~24 mucin genes identified till date MUC5AC mucin has been shown to play a critical role in the pathogenesis of upper respiratory tract infections including otitis media (OM). OM is characteristic of the presence of mucus overproduction and excess inflammation in the middle ear [2, 3]. In patients with OM, increased mucus effusion into the ear’s tympanic cavity impairs the movement of the eardrum and middle ear bones and leads to hearing problems. A higher concentration of mucin in the middle ear effusion has been shown to correlate with the extent of hearing impairment [4, 5]. While mucin up-regulation is an important innate defense response of the host to infections in the middle ear, excess mucin can lead to impaired mucociliary clearance and conductive hearing loss [2]. Therefore, mucin expression must be tightly controlled.

Nontypeable *Haemophilus influenzae* (NTHi) represents the cause of approximately one-third episodes of OM. Current treatments for bacterial OM rely on the systemic use of antibiotics, which often leads to the emergence of multi-drug resistant bacterial strains [6, 7]. Development of NTHi vaccine remains a challenge due to the high genetic diversity of NTHi strains and high antigenic variability of surface-exposed antigens [8, 9]. Therefore, there is an urgent need for developing alternate therapeutic strategies for treating NTHi infections. Previous studies have shown that NTHi up-regulates MUC5AC transcription via Toll-like receptor (TLR)-
dependent activation of p38 MAPK and transcription factor AP-1 [10]. Due to the involvement of p38 MAPK in multiple cellular processes, therapies inhibiting it can have detrimental effects in the long-term. Thus identification of novel therapeutic strategies with minimal side effects is strongly desired.

Curcumin, a yellow pigment derived from the rhizome Curcuma longa, is reported to possess a broad range of pharmacological effects, including antioxidant, anti-tumor, anti-inflammatory, anti-microbial and anti-diabetic properties [11]. Curcumin does not present a dose-limiting toxicity, thereby potentiating long-term usage with minimal side effects [12]. Curcumin is classified as “generally recognized as safe (GRAS)” by the United States Food and Drug Administration. Despite its pleiotropic effects on a multitude of diseases, poor bioavailability presents a major limitation for curcumin usage [12]. We recently reported the inhibitory effect of curcumin on NTHi-induced neutrophil recruitment in a mouse model of OM [13]. However, the effect of curcumin on regulating MUC5AC mucin, a major contributor of OM pathology, remains to be evaluated.

In this study, we demonstrate that curcumin inhibits NTHi-induced MUC5AC expression \textit{in vitro} and \textit{in vivo} in middle ear epithelial cells. Curcumin inhibited MUC5AC expression via inhibition of p38 MAPK via induction of negative regulator, MKP-1. Thus, our study provides evidence for the anti-inflammatory potential of curcumin in treating NTHi-induced OM by suppressing MUC5AC mucin overproduction.

3.3 Materials and methods

3.3.1 Reagents and antibodies

Curcumin was purchased from Sigma. SB203580 was purchased from Enzo Life Sciences. Antibodies for MUC5AC (sc-21701) and MKP-1 (sc-370) were purchased from Santa
Cruz Biotechnology. Anti-mouse HRP-linked antibody (#7076) was purchased from Cell Signaling Technology. Anti-mouse Alexa 488-conjugated antibody (A11029), anti-rabbit Alexa 488-conjugated antibody (A21206) were purchased from Life Technologies.

### 3.3.2 Cell culture

Human middle ear epithelial cells (HMEECs) were grown and maintained in DMEM (Cellgro) supplemented with BEGM SingleQuots (Lonza), 10% fetal bovine serum and 100U/ml penicillin and 100 µg/ml streptomycin (Gibco) in a humidified 5% CO2 atmosphere at 37 °C.

### 3.3.3 Bacterial strains and culture conditions

Clinical isolates of NTHi strains 12, 2627, 9274 were used for this study [14, 15]. NTHi was prepared as described previously [13]. For in vitro experiments NTHi re-suspended in DMEM and used at a multiplicity of infection (MOI) of 50. For in vivo experiments, NTHi was re-suspended in isotonic saline and used at a concentration of 5 x 10^7 CFU per mouse.

### 3.3.4 Plasmids, transfection and luciferase assay

The expression plasmids, for constitutively active (CA) forms of MKK3 (MKK3b (E)), MKK6 (MKK6b (E)), dominant negative forms of p38α (fp38α (AF)), p38β (fp38β2 (AF)) have been described previously [16]. MUC5AC-Luc luciferase reporter vector, AP-1 mutants of MUC5AC-Luc luciferase reporter vectors have been described previously [17]. Myc-MKP-1 overexpression plasmid has been described previously [13]. All transient transfections were performed using TransIT-LT-2020 transfection reagent (Mirus) according to the manufacturer’s protocol. Cells were assayed 48 h after transfection. Empty vector was transfected as a control. pRL-Renilla luciferase vector was from Promega Luciferase activity was measured using Dual-
Luciferase Reporter Assay System (Promega). MUC5AC luciferase activity was normalized to Renilla activity.

### 3.3.5 RNA-mediated interference

The human pSUPER-shMKP-1 knockdown construct as described previously [18] was transfected using TransIT-LT-2020 transfection reagent (Mirus) according to manufacturer’s protocol. Cells were assayed 48 h after transfection.

### 3.3.6 Real-time quantitative PCR (Q-PCR) analysis

Total RNA was extracted with TRIzol reagent, according to manufacturer's protocol (Life Technologies). TaqMan reverse transcription reagents were used to perform Reverse transcription reaction (Applied Biosystems). Real-time quantitative PCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems) and amplified, quantified with StepOnePlus Real-Time PCR system (Applied Biosystems). Relative quantities of mRNAs were calculated using the comparative Ct method and were normalized to control; human Cyclophilin or mouse glyceraldehyde-3-phosphate (GAPDH). Human (h) and mouse (m) primer sequences for hMUC5AC, hMKP-1, hCyclophilin, mMUC5AC, mGAPDH primer sequences were previously described [18].

### 3.3.7 Enzyme-linked immunosorbent assay (ELISA)

HMEECs were stimulated with NTHi for 12h. Cell-culture media was harvested and centrifuged at 12,000 x g for 10 min to precipitate cell debris. Supernatants were assayed by direct ELISA method as described previously [19]. OD was measured using Benchmark Plus microplate spectrophotometer. MUC5AC protein concentration in the supernatant was determined by normalizing to the control group.
3.3.8 Immunofluorescence staining

Cells were grown on 18 mm round glass coverslips (VWR). Following NTHi stimulation, cells were fixed with 4% paraformaldehyde, followed by incubation with primary antibodies at 1:100 – 1:400 dilutions. Alexa-conjugated secondary antibody was used to detect the primary antibody. The coverslips were mounted onto glass slides using VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector). Images were recorded with fluorescence microscopy system (BZ-X710 Microscope and BZ-X Viewer, BZ-X Analyzer imaging system, Keyence).

3.3.9 Mice and Animal Experiments

C57BL/6 mice (Jackson Laboratories) were employed. Anesthetized mice were inoculated with NTHi via the trans-tympanic route. For inhibition studies, mice were injected intraperitoneally (i.p) with curcumin (50 mg/kg) 1 h prior or 1 h post-NTHi inoculation. Total RNA was extracted from the dissected mice middle ear. All animal experiments were carried out following the guidelines approved by The Institutional Animal Care and Use Committee at Georgia State University.

3.3.10 Statistical analysis

All experiments were repeated at least three independent times. Data are represented as mean ± standard deviation (s.d.). Statistical significance was assessed with unpaired student's t-test for data with two conditions ($k = 2$), ANOVA (followed by Tukey’s post hoc) for data with more than two conditions ($k > 2$), using SPSS 22 statistics software (IBM). *$p < 0.05$ was considered statistically significant.
3.4 Results and Discussion

3.4.1 Curcumin suppresses NTHi-induced MUC5AC expression in middle ear epithelial cells in vitro and in vivo.

Curcumin has been reported to suppress neutrophil migration, an early inflammatory response to NTHi infection in OM model [13]. To further evaluate the anti-inflammatory potential of curcumin in treating OM, we determined the effect of curcumin on MUC5AC mucin expression, a major contributor of OM pathogenesis. Curcumin pre-treatment inhibited NTHi-induced MUC5AC mRNA expression in a dose-dependent manner in HMEECs (Fig. 3.1A). Curcumin also inhibited MUC5AC mRNA expression in HMEECs stimulated with other common OM-causing NTHi strains 2627 and 9274 (Fig. 3.1B), suggesting the generalizability of the inhibitory effect of curcumin on MUC5AC mucin. We next determined if curcumin inhibits MUC5AC transcription by determining the activity of MUC5AC promoter-driven luciferase vector. Curcumin suppressed NTHi-induced MUC5AC transcription (Fig. 3.1C). The inhibitory effect of curcumin on MUC5AC protein levels was also confirmed by ELISA (Fig. 3.1D) and by immunofluorescence staining (Fig. 3.1E). Consistent with in vitro findings, curcumin pre-treatment inhibited MUC5AC mRNA expression in the middle ear of mice inoculated with NTHi (Fig. 3.1F). Therefore, these data suggest that curcumin inhibits NTHi-induced MUC5AC mucin expression in middle ear epithelial cells in vitro and in vivo.

3.4.2 Curcumin suppresses NTHi-induced MUC5AC expression via inhibition of p38 MAPK.

Next, we sought to determine the mechanism by which curcumin inhibits MUC5AC expression. p38 MAPK signaling pathway has been shown to be critical in mediating NTHi-induced inflammatory responses. We previously reported that NTHi up-regulates MUC5AC expression via activation of p38 MAPK [10]. In a recent study, we showed that curcumin inhibits
NTHi-induced p38 phosphorylation [13]. Thus, we determined if curcumin inhibits MUC5AC expression via suppression of p38 activation. MAPK kinases MKK3 and MKK6 are upstream activators of p38 MAPK. MKK3 activates p38α only, whereas MKK6 activates both p38α and p38β isoforms. Overexpression of constitutively active (CA) forms of MKK3 (MKK3-CA) or MKK6 (MKK6-CA) markedly induced MUC5AC expression, while curcumin suppressed MKK3-CA or MKK6-CA-induced MUC5AC expression (Fig. 3.2A). To determine if curcumin inhibits MUC5AC expression via suppression of p38, we used multiple approaches. Pretreatment with curcumin or SB203580 (a specific inhibitor of p38 activation) alone markedly suppressed NTHi-induced MUC5AC expression in HMEECs. However, co-treatment of curcumin along with SB203580 did not further suppress MUC5AC expression (Fig. 3.2B). Consistent with the p38 inhibitor data, curcumin failed to suppress NTHi-induced MUC5AC expression further in HMEECs transfected with dominant negative (DN) mutant forms of p38; p38a-DN and p38b-DN (Fig. 3.2C).

AP-1 is one of the major transcription factors downstream of p38 MAPK. Sequence analysis of MUC5AC promoter region revealed the presence of two AP-1 transcription factor binding sites located between base pairs -3576/-3570 and -3535/-3529. Selective mutagenesis of these sites showed that the distal AP-1 site (-3576/-3570) is necessary for up-regulation of MUC5AC transcription, while the proximal AP-1 site (3535/-3529) is involved in the negative regulation of MUC5AC transcription [17]. Therefore to further determine which one of these AP-1 sites is involved in curcumin-mediated inhibition of MUC5AC transcription, we employed wild-type MUC5AC promoter, distal AP-1 mutant MUC5AC promoter and proximal AP-1 mutant MUC5AC promoter containing luciferase vectors. Curcumin pre-treatment suppressed NTHi-induced transcription of both wild-type and proximal AP-1 mutant MUC5AC promoter
constructs, while curcumin did not further inhibit transcription of distal AP-1 mutant MUC5AC promoter (Fig. 3.2D). These findings suggest that curcumin inhibits MUC5AC transcription via inhibiting the positive AP-1 (distal site) pathway. Together these data suggest that curcumin suppresses NTHi-induced MUC5AC expression via inhibition of MKK3/6-dependent activation of p38 MAPK.

3.4.3 *Curcumin inhibits NTHi-induced MUC5AC expression via up-regulation of MKP-1 phosphatase.*

MAP Kinase Phosphatase-1 (MKP-1) has been shown to be a key negative regulator of inflammation via dephosphorylation of MAPKs. We previously reported that MKP-1 is a negative regulator of NTHi-induced MUC5AC expression via inhibition of p38 MAPK [20]. We previously demonstrated that curcumin markedly enhanced NTHi-induced MKP-1 expression at mRNA and protein levels. The same study showed that curcumin suppressed p38 MAPK phosphorylation via up-regulation of MKP-1 expression [13]. Therefore, it is likely that curcumin suppresses NTHi-induced MUC5AC expression via a similar mechanism. We first confirmed that curcumin increases NTHi-induced MKP-1 expression by immunofluorescence staining (Fig. 3.3A). Next, we confirmed the role of MKP-1 in MUC5AC regulation. MKP-1 overexpression suppressed NTHi-induced MUC5AC expression (Fig. 3.3B). Knockdown of endogenous MKP-1 with MKP-1 shRNA enhanced MUC5AC expression (Fig. 3.3C). Since curcumin up-regulates MKP-1 expression and MKP-1 is a negative regulator of MUC5AC expression, we sought to determine the role of MKP-1 in curcumin-mediated MUC5AC suppression. Depletion of MKP-1 with shMKP-1 rendered curcumin treatment ineffective in inhibiting NTHi-induced MUC5AC mRNA expression (Fig. 3.3D). Together, these data suggest
that curcumin inhibits NTHi-induced MUC5AC expression via an MKP-1 dependent mechanism.

3.4.4 Post-infection administration of curcumin inhibits MUC5AC expression in middle ear epithelial cells in vitro and in vivo.

Next, we sought to evaluate the therapeutic relevance of the inhibitory effect of curcumin on MUC5AC mucin in an NTHi-induced OM setting. Thus, we evaluated the effect of injecting curcumin post-NTHi infection, which resembles a clinically relevant context. Curcumin administration post-NTHi infection significantly suppressed NTHi-induced MUC5AC mRNA expression in vitro (Fig. 3.4A) and in vivo (Fig. 3.4B). Curcumin suppressed MUC5AC expression to the same extent under both pre-NTHi and post-NTHi infection conditions. Thus, these data suggest that curcumin is a potential therapeutic for treating NTHi-induced MUC5AC mucin overproduction as seen in OM.

In summary, our study demonstrates that curcumin inhibits NTHi-induced MUC5AC expression in vitro and in vivo. Curcumin suppressed NTHi-induced MUC5AC expression by inhibition of p38MAPK via up-regulation of MKP-1 (Fig. 3.4C). Curcumin treatment post-NTHi infection also inhibited MUC5AC expression in a mouse model of OM, suggesting the clinical relevance of our findings. Thus, our study reports for the first time the efficacy of curcumin in treating NTHi-induced MUC5AC mucin overproduction. Further studies targeted towards increasing the bioavailability and development of ototopical drug delivery systems will be of clinical significance in treating OM. The insights of this study may have broader applications in the context of other chronic inflammatory conditions.
3.5 References


Figure 3.1 Curcumin suppresses NTHi-induced MUC5AC expression in middle ear epithelial cells in vitro and in vivo

(A) HMEECs were pre-treated with 10, 20 or 50 µM curcumin for 1 h, followed by NTHi for 5 h, and MUC5AC mRNA expression was measured. (B) HMEECs were pre-treated with 20 µM curcumin for 1 h, followed by NTHi strains 12, 2627 or 9274 for 5 h, and MUC5AC mRNA expression was measured. (C) HMEECs were transfected with MUC5AC promoter-driven luciferase vector. Cells were pre-treated with 20 µM curcumin for 1 h, followed by stimulation with NTHi for 5 h. MUC5AC promoter-driven luciferase activity was measured. (D) HMEECs were pre-treated with 20 µM curcumin for 1 h, followed by stimulation with NTHi for 12 h, and MUC5AC protein levels in cell culture supernatants was measured by ELISA. (E) HMEECs were pre-treated with 20 µM curcumin for 1 h; followed by NTHi stimulation for 12 h. MUC5AC protein (Alexa 488) was visualized by immunofluorescence staining. DAPI, nuclear stain. Magnification: 400×. (F) Mice were administered with 50 mg/kg (i.p) curcumin 1 h prior trans-tympanic inoculation of NTHi. 6 h after NTHi inoculation MUC5AC mRNA expression in dissected middle ear was measured. Data are mean ± s.d. (n=3). A; *p < 0.05, ANOVA (Tukey's posthoc). B-D, F; *p < 0.05, t-test. Data are representative of three or more independent experiments.
Figure 3.2 Curcumin suppresses NTHi-induced MUC5AC expression via inhibition of p38 MAPK

(A) HMEECs were transfected with Mock, MKK3 - CA or MKK6 - CA plasmids. Cells were treated with 20 µM curcumin for 1 h, and MUC5AC mRNA expression was measured. (B) HMEECs were pre-treated with 20 µM curcumin and 10 µM SB203580 for 1 h, followed by stimulation with NTHi for 5 h, and MUC5AC mRNA expression was measured. (C) HMEECs were transfected with Mock, p38α - DN, or p38β - DN plasmids. Cells were pre-treated with curcumin (20 µM) for 1 h, followed by NTHi stimulation for 5 h and MUC5AC mRNA expression was measured. (D) HMEECs were transfected with wild-type MUC5AC promoter, distal AP-1 mutant MUC5AC promoter or proximal AP-1 mutant MUC5AC promoter containing luciferase vectors. Cells were pre-treated with 20 µM curcumin for 1 h, followed by NTHi stimulation for 5 h. MUC5AC promoter-driven luciferase activity was measured. Data are mean ± s.d. (n=3). B-D; *p < 0.05, ANOVA (Tukey’s post hoc). Data are representative of three or more independent experiments.
Figure 3.3 Curcumin inhibits NTHi-induced MUC5AC expression via up-regulation of MKP-1 phosphatase

(A) HMEECs were pre-treated with curcumin (20 μM) for 1 h; followed by NTHi stimulation for 1 h. MKP-1 protein (Alexa 488) was visualized by immunofluorescence staining. DAPI, nuclear stain. Magnification: 400×. (B, C) HMEECs were transfected with (B) Mock or myc-MKP-1, (C) Mock or MKP-1 shRNA. Cells were stimulated with NTHi for 5 h, and MUC5AC mRNA expression was measured. MKP-1 knockdown was confirmed by Q-PCR. (D) HMEECs were transfected with Mock or MKP-1 shRNA plasmid. Cells were pre-treated with 20 μM curcumin for 1 h, followed by NTHi stimulation for 5 h, and MUC5AC mRNA expression was measured. B, C; *p < 0.05, t-test. D; *p < 0.05, ANOVA (Tukey’s post hoc). n.s., not significant. Data are representative of three or more independent experiments.
Figure 3.4 Post-infection administration of curcumin inhibits MUC5AC expression in middle ear epithelial cells in vitro and in vivo

(A) HMEECs were pre-treated with curcumin (20 μM) 1 h prior NTHi stimulation or post-treated with curcumin (20 μM) 1 h after NTHi stimulation. 5 h after NTHi stimulation MUC5AC mRNA expression was measured. (B) Mice were administered with 50 mg/kg (i.p) curcumin 1 h prior trans-tympanic inoculation of NTHi or were post-administered with 50 mg/kg (i.p) curcumin 1 h post-NTHi inoculation. 6 h after NTHi inoculation MUC5AC mRNA expression in dissected middle ear was measured. (C) Schematic representation illustrating that curcumin suppresses NTHi-induced MUC5AC expression via MKP-1-dependent inhibition of p38 MAPK. A, B; *p < 0.05, ANOVA (Tukey’s post hoc). n.s., not significant. Data are representative of three or more independent experiments.
4 CURCUMIN SUPPRESSES NTHI-INDUCED INFLAMMATION BY PROMOTING K63-LINKED POLYUBIQUITINATION OF MKP-1

Anuhya S Konduru, Shingo Matsuyama, Jian-Dong Li*
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4.1 Introduction

Protein ubiquitination is one of the important post-translational modification (PTM) in the control of many biological processes including immune responses. Ubiquitin (Ub), a 76 amino acid protein is covalently attached to other proteins via an isopeptide bond formed between the glycine residue in its C-terminal and the -amino group of the lysine (K) residue on its substrate. Ubiquitination is carried out by the sequential activity of three enzymes. Ub-activating enzyme (E1) catalyzes the formation of a thioester bond between the cysteine residue in its active center and the C-terminal glycine on the Ub molecule. Activated Ub gets transferred to an Ub-conjugating enzyme (E2) via a similar thioester bond formation. These two steps are ATP-dependent. Next, an Ub-ligase (E3) binds to both E2 and the substrate protein and facilitates the transfer of Ub from E2 to the internal lysine residue on the substrate. In addition to monoubiquitination, a protein can also undergo polyubiquitination. Formation of polyubiquitination chains follows the same pattern with the ubiquitin’s C-terminal glycine (Gly76) conjugated to the internal lysine residues within the ubiquitin protein. The presence of 7 internal lysine residues (K6, K11, K27, K29, K44, K48 and K63) contributes to the diversity of the ubiquitination chains. While different Ub chain types adopt different conformations that promote interactions with linkage-specific Ub-binding domains, the physiological roles of the Ub linkages (with an exception to K48 and K63-linked chains), remains to be understood [1, 2].
The presence of K48-linked Ub chains on substrate proteins has been long identified to be responsible for recognition and degradation by the proteasome [3]. The Ub-tagged substrate protein is degraded by the proteasome, while the free Ub is released [4]. In contrast K63-linked Ub chains exert a non-degradative effect on the proteins as seen in cell signaling cascades. The presence of K63-linked Ub chains on the proteins alters the function of the proteins in a reversible manner. K63-linked Ub chains can alter the interaction of the substrate protein with other proteins. K63-linked Ub has been widely known to render the proteins as scaffold/adaptors molecules that serve by promoting protein-protein interactions and thereby enhances the activity of the signaling cascade [1, 5]. MKP-1 protein has been shown to undergo various PTMs such as phosphorylation, acetylation, oxidation and K48-linked ubiquitination in response to stimuli [6-9]. However, the molecular mechanism of K63-linked polyubiquitination of MKP-1 remains largely unknown.

In this study, we demonstrate for the first time that NTHi-induces K63-linked polyubiquitination of MKP-1 in vitro and that the anti-inflammatory compound curcumin enhances K63-linked polyubiquitination, which could, in turn, promote MKP-1 activity.

4.2 Methods and Materials

4.2.1 Cell culture

Human middle ear epithelial cells (HMEECs) were maintained in DMEM (Cellgro) supplemented with BEGM SingleQuots (Lonza), 10% fetal bovine serum and 100U/ml penicillin and 100 µg/ml streptomycin (Gibco). Cells were cultured at 37 °C in 5% CO₂ atmosphere.

4.3 Reagents and antibodies

Curcumin was purchased from Sigma. Antibodies for c-Myc (sc-40), HA (sc-805) and, α-tubulin (sc-69969), IgG (sc-3877) and Protein G Plus agarose beads (sc-2002) were purchased
from Santa Cruz Biotechnology. Antibodies for anti-mouse HRP-linked antibody (#7076) and anti-rabbit HRP-linked antibody (#7074) were purchased from Cell Signaling Technology.

4.3.1 Bacterial strains and culture conditions

Clinical isolates of NTHi strain 12 was used for this study [10, 11]. NTHi was prepared as described previously [12]. NTHi re-suspended in DMEM was used at a multiplicity of infection (MOI) of 50 for time as indicated. For evaluating the effect of curcumin on protein ubiquitination, cells were pre-treated with curcumin for 1 h prior to NTHi stimulation.

4.3.2 Plasmids and transient transfection

Myc-MKP-1 expression plasmid has been described previously. Expression plasmids pRK5-HA-WT Ub (ubiquitin with all lysine (K) residues intact), pRK5-HA-K48 (ubiquitin with only K48 residue), pRK5-HA-K63 (ubiquitin with only K63 residue) were purchased from Addgene. All transient transfections were performed using TransIT-LT-2020 transfection reagent (Mirus) according to the manufacturer’s protocol. Cells were assayed 48 h after transfection.

4.3.3 Ubiquitination Assay

Cells were washed, harvested and collected in 1ml PBS, followed by centrifugation at 6000 \( \times g \) for 2 min. The “cell lysate” pellet was dissolved in 100\( \mu l \) denaturation buffer containing 20 mM HEPES, 150 mM NaCl, 1 % SDS, 5 % glycerol, 10 mM \( \beta \)-mercaptoethanol, 10 mM NEM (\( N \)-ethylmaleimide), 1 mM Na\( _3 \)VO\( _4 \) and protease inhibitor cocktail and boiled for 10min to denature the noncovalent interactions. Samples were then diluted in 900\( \mu l \) dilution buffer containing 20 mM HEPES, 150 mM NaCl, 2.2 mM EDTA, 1.1 % Triton-X, 5 % glycerol, 10 mM NEM (\( N \)-ethylmaleimide), 1 mM Na\( _3 \)VO\( _4 \) and protease inhibitor cocktail. Diluted samples were used for immunoprecipitation and western blot analysis.
4.3.4 Immunoprecipitation

Cell lysates were incubated with 1µg of corresponding primary antibody overnight at 4°C, followed by incubation with Protein G Plus agarose beads for 2 h. The beads were washed for 4 times, followed by suspension in sample loading buffer.

4.3.5 Western Blot Analysis

Immunoprecipitates and cell lysates were separated on 10% SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with blocking buffer (TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk). After 3 washes with TBS-T, the membrane was incubated overnight with primary antibodies at 1 : 1,000 – 1 : 2,000 dilutions in antibody dilution buffer (TBS-T containing 5% BSA) at 4 °C. After 3 washes with TBS-T, the membrane was incubated with corresponding secondary antibody at 1 : 5,000 dilution in blocking buffer for 1 h. After 3 washes with TBS-T, the proteins were visualized using Amersham ECL Prime Detection Reagent (GE Healthcare).

4.4 Results

4.4.1 NTHi induces K63-linked polyubiquitination of MKP-1

Among all the major post-translational modifications, ubiquitination of the host’s signal transduction molecules is an important regulatory mechanism to ensure tight control of the immune responses [5]. Since MKP-1 is a major negative regulator of inflammation, we initially sought to determine if MKP-1 undergoes polyubiquitination in response to NTHi stimuli. NTHi induced polyubiquitination of MKP-1 in a time-dependent manner in HMEECs transfected with Myc-MKP-1 and HA-WT Ub overexpression vectors (Fig. 4.1A). To further identify the nature of the polyubiquitination chains on MKP-1, we employed HA-K48 Ub and HA-K63 Ub expression vectors. NTHi predominantly induced K63-linked polyubiquitination of Myc-MKP-1
compared to K48-linked polyubiquitination (Fig. 4.1B). Taken together these data demonstrate for the first time that MKP-1 protein undergoes K63-linked polyubiquitination in response to NTHi infection.

4.4.2 Curcumin enhances NTHi-induced K63 polyubiquitination of MKP-1

Curcumin has been previously reported to enhance the mRNA and protein expression of the negative regulator MKP-1 [12]. However, its role in regulating MKP-1 protein activity is unknown. Curcumin pre-treatment enhanced NTHi-induced polyubiquitination of MKP-1 in HMEECs transfected with Myc-MKP-1 and HA-WT Ub in a time-dependent manner (Fig. 4.2A). Since NTHi predominantly induced K63-linked polyubiquitination of MKP-1, we evaluated the effect of curcumin on K63-linked polyubiquitination of MKP-1. Consistent with the WT polyubiquitination data, curcumin pre-treatment enhanced NTHi-induced K63-linked polyubiquitination of MKP-1 (Fig. 4.2B). Since K63-linked polyubiquitination has been widely reported to regulate the activity of the target proteins, these data suggest that curcumin not only up-regulates MKP-1 expression, but also regulates its activity by enhancing K63-linked polyubiquitination.

4.5 Discussion

Despite the significant role of MKP-1 in modulating immune responses, the mechanisms underlying its activity are unclear. Several immunosuppressive agents such as glucocorticoids, phosphodiesterase (PDE) inhibitors, vinpocetine, resveratrol, and curcumin mediate their inhibitory effects via MKP-1 [12-16]. Therefore, understanding the mechanism of action of these drugs on regulating MKP-1 activity is critical for developing alternative therapeutics. Here, we show that NTHi induces K63-linked polyubiquitination of MKP-1. We also demonstrate that curcumin enhances NTHi-induced K63-polyubiquitination of MKP-1 (Fig. 4.3).
The importance of K63 Ub linkages in mounting an appropriate immune response is widely known [17]. However, the role of K63 polyubiquitination in negatively regulating immune responses is not entirely understood. We show for the first time that MKP-1, a negative regulator of p38 MAPK undergoes K63-linked polyubiquitination. This finding is of particular significance due to the increasing importance of modulating PTMs of signaling proteins in regulating immune responses.

Curcumin has been previously reported to suppress NTHi-induced up-regulation of inflammatory mediators such as CXCL5 and MUC5AC mucin via MKP-1. Curcumin up-regulated MKP-1 expression at both mRNA and protein levels. Increased levels of MKP-1, led to inhibition of p38 MAPK activation, resulting in suppression of inflammation [12]. Here we report for the first time a new mechanism of action of curcumin on MKP-1. Curcumin enhanced K63-linked polyubiquitination of MKP-1. K63-linked polyubiquitination mediates non-degradative molecular functions such as protein-protein interactions, protein trafficking and regulation of signal transduction events. Therefore, our finding that curcumin enhances K63-linked polyubiquitination of MKP-1 suggests that curcumin also promotes MKP-1 activity. K63 Ub chains on MKP-1 could increase the affinity of MKP-1 to its substrate p38 MAPK, thereby increasing MKP-1’s phosphatase activity. K63-linked polyubiquitination of MKP-1, could increase MKP-1 protein’s half-life and prolong the duration of immunosuppression. The presence of K63-linked Ub on MKP-1 could pre-empt K48-linked Ub chains from assembling on it, and thereby protecting it from proteasomal degradation.

Further studies identifying the specific lysine residues on MKP-1 undergoing K63-linked polyubiquitination and the E3 ligase(s) involved in promoting K63-linked polyubiquitination are needed. Understanding the specific roles of ubiquitination and the ubiquitination enzymes in
MKP-1 regulation could help identify novel drug targets for counteracting overactive immune responses.

4.6 References


Figure 4.1 NTHi induces K63-linked polyubiquitination of MKP-1

(A) HMEECs were co-transfected with Myc-MKP-1 and HA-WT Ub expression vectors. Cells were stimulated with NTHi for 1, 2, 3 and 4 h. Cell lysates were immunoprecipitated (IP) with c-Myc antibody. Polyubiquitination in the immunoprecipitates was visualized by immunoblotting (IB) using anti-HA antibody. (B) HMEECs were co-transfected with Myc-MKP-1 and HA-K48 Ub or HA-K63 Ub expression vectors. Cells were stimulated with NTHi for 2 and 3 h. Cell lysates were immunoprecipitated (IP) with c-Myc antibody. K48-, K63-linked polyubiquitination in the immunoprecipitates was visualized by immunoblotting (IB) using anti-HA antibody.
Figure 4.2 Curcumin enhances K63-linked polyubiquitination of MKP-1

(A) HMEECs were co-transfected with Myc-MKP-1 and HA-WT Ub expression vectors. Cells were pre-treated with curcumin (20 µM) for 1 h, followed by stimulation with NTHi for 1, 2, 3 and 4 h. Cell lysates were immunoprecipitated (IP) with c-Myc antibody. Polyubiquitination in the immunoprecipitates was visualized by immunoblotting (IB) using anti-HA antibody. (B) HMEECs were co-transfected with Myc-MKP-1 and HA-K63 Ub expression vectors. Cells were pre-treated with curcumin (20 µM) for 1 h, followed by stimulation with NTHi for 2 and 3 h. Cell lysates were immunoprecipitated (IP) with c-Myc antibody. K48-, K63-linked polyubiquitination in the immunoprecipitates was visualized by immunoblotting (IB) using anti-HA antibody.
Figure 4.3 Schematic representation of curcumin mediated up-regulation of K63-linked polyubiquitination of MKP-1
OM is the most common childhood bacterial infection [1, 2] with more than 700 million ear infections in the US each year, resulting in approximately 30 million doctor visits each year and 5 billion dollars in patient care. OM frequently leads to conductive hearing loss, affecting children during the crucial period of speech and language development [3, 4]. NTHi represents the cause of approximately one-third episodes of OM. Current therapies for OM involve the use of analgesics and antipyretics for symptomatic treatment [5]. Though these medications are effective during certain stages of the disease, prolonged usage poses the risk of serious side effects due to unknown “off-targets” and weakened immune system. Decongestants, antihistamines, and corticosteroids have not been effective in treating OM [6]. Prophylactic use of antibiotics has rendered over 80% of the NTHi strains drug-resistant [7, 8]. Also, development of vaccines against NTHi remains a challenge due to the high genetic diversity of NTHi strains and high antigenic variability of surface-exposed antigens [9, 10]. Thus, there is an urgent need for developing alternative therapeutics for OM with increased efficiency and safety.

During infection, epithelial cells act as the first line of defense by secreting numerous pro-inflammatory mediators including chemokines and mucus. Chemokines mainly act by recruiting neutrophils to the site of infection. While the appropriate neutrophil response is critical for the removal of the invading pathogen, excess inflammation can lead to tissue damage and perpetuate inflammation as seen in OM [11-14]. Similarly, mucus production represents a protective innate defense mechanism to protect and lubricate the epithelium and trap invading pathogens for removal by the mucociliary clearance system [15]. However, in chronic infections, excess mucin impairs the mucociliary clearance system, resulting in mucus accumulation and poor function of the mucus-lined epithelial tracts and perpetuates inflammatory responses. While
mucin up-regulation is an important innate defense response of the host to infections in the middle ear, excess mucin can lead to impaired mucociliary clearance and conductive hearing loss as seen in OM [16]. Therefore, tight regulation of the intensity and duration of inflammatory responses is necessary. Thus, understanding the underlying molecular mechanisms leading to up-regulation of inflammatory responses is critical for developing effective therapeutic strategies.

Despite the importance of CXCL5 chemokine in mediating inflammation, the molecular mechanisms underlying its up-regulation in OM remains largely unknown. In Chapter 2 of this work, we showed that NTHi up-regulates CXCL5 expression by activating IKKβ-ικBα and p38 MAPK pathways via NF-κB-nuclear translocation-dependent and -independent mechanism. Since NF-κB plays a central role in regulating immune responses as well as many physiological responses, inhibition of this signaling axis could have detrimental effects. Our finding, that NTHi induces CXCL5 expression via more than one signaling pathway offers the flexibility to modulate CXCL5 expression without completely abrogating its expression.

While mounting an appropriate inflammatory response against invading microbial pathogens is critical for the survival of the host, deregulated overactive inflammatory responses are detrimental to the host in the form of septic shock, lethality from inadequate responses and misdirected responses against host cells resulting in autoimmunity. Multiple proteins targeting the components of TLR-signaling to prevent the deleterious effects of overactive immune responses exist. These negative regulators modulate TLR signaling mainly via transcriptional repression and post-translational modifications. Among the numerous categories of negative regulators, MKP-1, a member of a class of dual specificity phosphatases collectively termed MAPK phosphatases, has been shown to be a key negative regulator of inflammatory responses via dephosphorylation and inactivation of MAPKs, including p38 [17, 18]. Here, in Chapter 2,
we demonstrate that MKP-1 is a negative regulator of NTHi-induced CXCL5 expression. MKP-1 suppressed CXCL5 via targeting p38 MAPK activation, thereby suggesting that up-regulating MKP-1 expression could be a potential therapeutic strategy for regulating overactive inflammatory responses in OM.

We previously demonstrated that dexamethasone glucocorticoid inhibits p38 MAPK via up-regulation of MKP-1 [19]. Glucocorticoids owing to their potent immunosuppressive and anti-inflammatory effects have been in use for treating a gamut of diseases such as asthma, allergies, skin disorders, multiple sclerosis, immune disorders and cancer. However, prolonged usage has been reported to cause severe, sometimes irreversible side effects such as osteoporosis, endocrine and metabolic disorders, behavioral and cognitive changes, gastrointestinal tract complications, uveitis and weakened immune system [20], emphasizing the urgent need to identify safer alternatives to up-regulate negative regulators of inflammation.

In Chapter 2, we provide evidence that curcumin, derived from *Curcuma longa* plant suppressed NTHi-induced CXCL5 expression in mouse model of OM. Curcumin markedly increased expression of negative regulator MKP-1. In the absence of MKP-1, curcumin failed to suppress CXCL5 expression, suggesting that curcumin mediates its inhibitory effect via negative regulator MKP-1. This finding is of particular translational significance due to the attractiveness of targeting overactive inflammation via induction of negative regulators [21]. From a clinical significance point of view, we observed that both pre-infection and post-infection treatment with curcumin not only inhibited NTHi-induced CXCL5 up-regulation but also suppressed PMN infiltration into the middle ear in a mouse model of OM. Curcumin treatment’s efficacy in inhibiting CXCL5 expression and PMN recruitment post-NTHi infection is particularly promising. Recently chemokines and chemokine receptors are increasingly considered as targets
for developing new drugs to control inflammation [22]. Our finding that curcumin suppresses NTHi-induced CXCL5 chemokine expression is of particular relevance in the current scheme of identifying chemokine-drug combinations to treat inflammation.

Having established that anti-inflammatory potential of curcumin in inhibiting PMN-infiltration into the middle ear in a mouse model of OM, we sought to extend this lead to examine the effect of curcumin on MUC5AC mucin expression, a major contributor of OM pathogenesis in Chapter 3. Interestingly, curcumin also suppressed NTHi-induced MUC5AC via up-regulation of MKP-1. Thus, our studies demonstrate for the first time the efficacy and pleiotropic anti-inflammatory action of curcumin by down-regulating expression of CXCL5 chemokine and MUC5AC mucin to suppress NTHi-induced inflammatory responses in OM model.

Immunosuppressive agents such as glucocorticoids, phosphodiesterase (PDE) inhibitors, vinpocetine, resveratrol, and curcumin mediate their inhibitory effects via MKP-1 [19, 23-26]. However, the mechanisms underlying their action are unclear. All proteins encounter some degree of PTMs during their lifetime. Amongst all the PTMs reported so far, protein phosphorylation and protein ubiquitination have emerged as major players in regulation signal transduction in response to environmental stimuli. Recent decades have seen a vast amount of research focused on identifying and characterizing protein kinases and phosphatases in signal transduction. However, protein ubiquitination is gaining prominence as one of the important PTM in the control of immune responses. The presence of 7 internal lysine residues (K6, K11, K27, K29, K44, K48 and K63) with the ubiquitin protein contributes to the diversity of the ubiquitination chains. The presence of K48-linked Ub chains on substrate proteins has been long identified to be responsible for recognition and degradation by the proteasome [27]. In contrast
K63-linked Ub chains exert a non-degradative effect on the proteins as seen in cell signaling cascades. The importance of K63 Ub linkages in mounting an appropriate immune response is widely known [28]. However, the role of K63 polyubiquitination in negatively regulating immune responses is not entirely understood. While MKP-1 protein has been shown to undergo various PTMs such as phosphorylation, acetylation, oxidation and K48-linked ubiquitination in response to stimuli [29-32] no mention about K63-linked polyubiquitination of MKP-1 exists. Therefore, in Chapter 4 we sought to determine K63-linked polyubiquitination status of MKP-1. We showed for the first time that MKP-1, a negative regulator of p38 MAPK undergoes K63-linked polyubiquitination. This finding is of particular significance due to the increasing importance of modulating PTMs of signaling proteins to regulate immune responses.

Curcumin has been previously reported to suppress NTHi-induced inflammation via up-regulating MKP-1 expression at mRNA and protein levels. We next, sought to evaluate if curcumin can also affect K63-linked polyubiquitination of MKP-1. In Chapter 4 we report for the first time a new mechanism of action of curcumin on MKP-1. Curcumin enhanced K63-linked polyubiquitination of MKP-1. K63-linked polyubiquitination mediates non-degradative molecular functions such as protein-protein interactions, protein trafficking and regulation of signal transduction events. Therefore, our finding that curcumin enhances K63-linked polyubiquitination of MKP-1 suggests that curcumin could also promote MKP-1 activity. K63-linked polyubiquitination chain on MKP-1 could increase the affinity of MKP-1 to its substrate p38 MAPK, thereby increasing MKP-1’s phosphatase activity. Additionally, the presence of K63-linked polyubiquitination chains on MKP-1 could pre-empt K48-linked polyubiquitination chains from assembling on it and protect it from proteasomal degradation; extend MKP-1 protein’s half-life and prolong the duration of immunosuppression. It should be noted that these
studies were performed under the presence of exogenously expressed MKP-1 protein. Additionally, this phenomenon needs to be confirmed by evaluating the polyubiquitination status of endogenous MKP-1 protein. The lack of MKP-1 antibody with high specificity hindered this investigation. Further studies identifying the specific lysine residues on MKP-1 undergoing K63-linked polyubiquitination and the E3 ligase(s) involved in promoting K63-linked polyubiquitination are needed. Understanding the specific roles of ubiquitination and the ubiquitination enzymes in MKP-1 regulation could help identify novel drug targets for curcumin to counteract overactive immune responses.

Curcumin is a nutraceutical that has been in use in South Asian countries for many centuries owing to its medicinal properties [33]. Curcumin can interact with a myriad of signaling molecules including transcription factors, protein kinases, growth factors, receptors, adhesion molecules, pro-inflammatory cytokines [34], thus explaining its pleiotropic therapeutic potential against a wide range of diseases. Curcumin does not present a dose-limiting toxicity, making it suitable for prolonged usage. Completed clinical trials reported usage of curcumin dosage ranging from 0.045 to 8 g/day. Currently, 38 clinical trials evaluating the efficacy of curcumin at a dosage ranging from 0.18 to 8 g/day for treating pathologies such as Alzheimer’s disease, diabetes, kidney disease, Crohn’s disease, cancer are underway [35]. Although curcumin holds promise as a safe drug for treating a gamut of conditions, its clinical application is hindered by many limitations. Poor absorption and bioavailability are a major concern. Orally administered curcumin undergoes conjugation and sulfation leading to the formation of curcumin glucuronide and curcumin sulfate in the intestines and liver. Curcumin administered via intravenous (i.v.) or intraperitoneal (i.p.) route undergoes reduction to form tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin. None of these metabolites are biologically active.
The low bioavailability of curcumin is due to its hydrophobic nature. Numerous approaches to increase curcumin bioavailability are currently underway. These include the (i) use of piperine as an adjuvant to protect against glucuronidation, (ii) liposomal curcumin, to increase uptake by cell membranes, (iii) curcumin nanoparticles, (iv) curcumin phospholipid complex and (v) structural analogs of curcumin. Another limitation of curcumin is its poor solubility in aqueous solvents.

United States Food and Drug Administration classified curcumin as GRAS (generally recognized as safe), warranting its use as a supplement. [36]. Curcumin supplementation could be an effective disease preventive strategy due to its immunomodulatory activity [37]. Thus, curcumin's benefits could still be reaped while we wait for more conclusive clinical trials demonstrating its immense potential against multiple pathologies.

To fully evaluate the potential of curcumin as a therapeutic for treating OM in a clinical setting, further studies focusing on the effect of curcumin on bacterial clearance and restoration of hearing loss are needed. Development of drug delivery systems in the form of eardrops and ototopical ointments could be of clinical benefit in treating OM. Our study has only addressed the relationship between curcumin and one particular negative regulator MKP-1. However, it is likely that curcumin mediates its anti-inflammatory actions via other negative regulators of inflammation such as IRAK-M, MyD88s, and CYLD. Moreover, the cross talk between these negative regulators needs to be explored to identify potential targets for suppressing inflammation. Co-administration of curcumin along with other immunosuppressive agents such as resveratrol, dexamethasone, vinpocetine must be evaluated to maximize its therapeutic efficiency. The findings of this work could have applications in broader context to other bacterial infections and pathologies characteristic of chronic inflammation such as chronic obstructive pulmonary disease, cancer, tuberculosis and Alzheimer’s disease. Thus, curcumin meets several
if not all of the requirements of an alternative therapeutic with minimal side effects for treating NTHi-induced OM.

5.1 References


