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Regulation of Nontypeable Haemophilus influenzae-induced Host Innate Immunity in Lung Epithelium

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Regulation of Nontypeable *Haemophilus influenzae*-induced Host Innate
Immunity in Lung Epithelium

by

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

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ABSTRACT

The host's innate immune response begins with the identification of molecules known as pathogen-associated molecular patterns by a series of germline-encoded pattern-recognition receptors. Retinoic acid-inducible gene I (RIG-I) is one such innate immune receptor that detects viral RNA in the cytosol of cells and is crucial to mount an effective antiviral immune response in the course of viral infections. RIG-I with the adaptor protein mitochondrial antiviral signaling protein (MAVS) leads to the activation of the transcription factors including interferon-regulatory factor 3 (IRF3), IRF7 and nuclear factor- κ B (NF- κ B), which induce type I and III interferon (IFN), and proinflammatory cytokines. Many studies have demonstrated the crucial role of RIG-I in viral infections such as influenza A virus (IAV) and respiratory syncytial virus (RSV) infections. However, as airway viral infection is frequently followed by bacterial co-infections, the role of RIG-I and how it is regulated in airway epithelium inflammation in mixed viral and bacterial infection remains undefined. Despite the well-known role of viruses in bacterial co-infections, the impact of bacterial infections on viral co-infections remains largely unknown. Therefore, understanding the mechanism of airway bacterial pathogen Nontypeable *Haemophilus influenzae* (NTHi)-induced RIG-I will help to understand the complex inflammatory signaling pathway and further contribute to the development of new therapies.

In the present study, we showed that NTHi induced RIG-I upregulation in airway epithelial cells. We also demonstrated that NTHi induced RIG-I upregulation by activation of TNF receptor associated factor 6 (TRAF6)-inhibitor of nuclear factor- κ B kinase subunit β (IKK β)-p65 pathway. Interestingly, interleukin-1 receptor-associated kinase M (IRAK-M) negatively regulated NTHi-induced up-regulation of RIG-I via inhibiting the IKK β -p65

pathway and inflammation. We also confirmed that IKK β activation is sufficient for RIG-I up-regulation. We showed that p65 phosphorylation at S276 and S536 residues is likely critical for RIG-I up-regulation. We also investigated the role of histone deacetylases (HDACs) as a positive regulator for NTHi-induced RIG-I up-regulation. Along with NTHi-induced RIG-I upregulation, we also confirmed the functional significance of RIG-I by confirming the CXCL10 expression mediated via RIG-I. Thus, our study provides new insights into the regulation of NTHi-induced RIG-I upregulation, which may contribute to the development of a new therapeutic strategy for controlling inflammation in mixed airway infections.

INDEX WORDS: RIG-I, RIG-I like receptors, Innate immune response, IRAK-M, NTHi, upper respiratory infections, Nontypeable *Haemophilus influenzae*, Negative regulation, Positive regulation

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2023

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DEDICATION

I dedicate this work to my Indian and the US family, who always inspire me to be the best version of myself. I am fortunate to have them as family; my words are not enough to express my gratitude to them.

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LIST OF ABBREVIATIONS

5' diphosphate (5' pp)

5' triphosphate (5' ppp)

5S ribosomal RNA pseudogene 141 (RNA5SP141)

Adenosine triphosphate (ATP)

Airway epithelial cell (AEC)

All-trans retinoic acid (ATRA)

Brain heart infusion (BHI)

Caspase activation and recruitment domains (CARDs)

Carboxy terminal domains (CTD)

Chronic obstructive pulmonary disease (COPD)

C-X-C motif chemokine ligand 10 (CXCL10)

Dengue virus (DV)

Dimethyl sulfoxide (DMSO)

Double stranded RNA (dsRNA)

Haemophilus influenzae (Hi)

Haemophilus influenzae serotype B (Hib)

Hepatitis C virus (HCV)

Herpes simplex virus (HSV)

Histone deacetylases (HDAC)

IFN regulatory factor 3 (IRF3)

IFN regulatory factor 7 (IRF7)

IkappaB (IκB)

Influenza A virus (IAV)

Inhibitor of nuclear factor kappa-B kinase subunit beta (IKK β)

Inhibitor of nuclear factor- κ B kinase subunit alpha (IKK α)

Inhibitor of nuclear factor- κ B kinase subunit epsilon (IKK ϵ)

Interferon (IFN)

Interferon-stimulated genes (ISG)

Interleukin-1 receptor-associated kinase 3 (IRAK-M)

Janus kinase (JAK)

Knockdown (KD)

Knockout (KO)

Laboratory of genetics, and physiology 2 (LGP2)

Measles virus (MeV)

Melanoma differentiation-associated protein 5 (MDA5)

Mitochondrial antiviral signaling protein (MAVS)

Mitochondrial ribosomal Protein 18 (MRPL18)

Mitochondrial-associated endoplasmic reticulum membrane (MAM)

Multiplicity of infection (MOI)

NF- κ B essential modulator (NEMO)

Nontypeable *Haemophilus influenzae* (NTHi)

Nuclear factor-kappa B (NF- κ B)

Nuclear factor-kappa B p65 (NF- κ B p65)

Otitis media (OM)

Pathogen-associated molecular patterns (PAMP)

Pattern-recognition receptors (PRR)

Phosphate-buffered saline (PBS)

Quantitative PCR (Q-PCR)

Reoviruses (RV)

Respiratory syncytial virus (RSV)

Retinoic acid-inducible gene-I (RIG-I)

RIG-I-like receptor (RLR)

Sendai virus (SV)

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Signal transducer and activator of transcription (STAT)

Standard deviation (SD)

Suberoylanilide hydroxamic acid (SAHA)

TAK1-binding protein 2/3 (TAB2/3)

TANK binding kinase 1 (TBK1)

Thiosulfate sulfotransferase (TST)

TNF receptor associated factor 6 (TRAF6)

Transforming growth factor beta-activated kinase 1 (TAK1)

Upper respiratory tract (URT)

West Nile virus (WNV)

Wild type (WT)

Zika Virus (ZV)

1. INTRODUCTION

Innate immunity is the first line of host defense against pathogens. The innate antiviral immune response begins with the identification of evolutionarily conserved structures known as pathogen-associated molecular patterns (PAMPs) by a series of germline encoded pattern-recognition receptors (PRR). The host must mount defensive and protective responses if PAMPs are detected quickly and efficiently via PRRs. One very important PRR is Retinoic acid-inducible gene-I (RIG-I), which is essential for initiating antiviral and inflammatory responses to stop viral replication in response to RNA structures unique to the virus. RIG-I is found in the cytoplasm of somatic cells and well-expressed in epithelial cells, which tend to be the initial target of pathogen contact with the host (Coch et al., 2017; Le Goffic et al., 2007; Rehwinkel & Gack, 2020; Thoresen et al., 2021; Wang et al., 2007; Zhang et al., 2016). RIG-I was first observed to be increased in acute promyelocytic leukemia, NB4 cells induced by All-trans retinoic acid (ATRA). The RIG-I like receptors (RLRs) family consists of RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics, and physiology 2 (LGP2). RLRs are the principal cytoplasmic viral RNA sensors that induce type I IFN responses after viral infection (Coch et al., 2017; Rehwinkel & Gack, 2020; Thoresen et al., 2021; Wang et al., 2007; Zhang et al., 2016).

Numerous studies have shown that RIG-I identifies viral RNA of several major groups of viruses, including the positive-strand hepatitis C virus (HCV), coronaviruses, and a wide variety of negative-sense RNA viruses, such as orthomyxoviruses, rhabdoviruses, bunyaviruses, and paramyxoviruses (Rehwinkel & Gack, 2020; Thoresen et al., 2021). RIG-I can detect commonly occurring viruses including influenza A virus

(IAV), respiratory syncytial virus (RSV), sendai virus (SV), west Nile virus (WNV), reoviruses (RV), and dengue virus (DV). Additionally, many viral groups exploit the RIG-I receptor to circumvent the host's defensive response (Kao et al., 2018; Rehwinkel & Gack, 2020). Many studies have demonstrated that the RIG-I KO in mouse model causes embryonic death due to liver degeneration. In addition, a separate study demonstrated that RIG-I KO animals generated by deletion of 6.4-kb fragment that contains exons 4 to 8, these exons encode caspase activation and recruitment domains (CARD) and RNA helicase domain of RIG-I, were alive but had spontaneous colitis (Wang et al., 2007). Knockout studies in mice and mouse-derived cells further established that RIG-I is essential for antiviral defense and type I interferon induction in virus infections (Rehwinkel & Gack, 2020; Thoresen et al., 2021; Wang et al., 2007). All RLRs have carboxy-terminal and central helicase domains (CTD). Together, these two domains can recognize immunostimulatory RNAs. RIG-I and MDA5 also feature two CARDs at their amino termini, which drive downstream signal transduction. Although LGP2 lacks the CARDs, it is commonly accepted that it controls RIG-I and MDA5 (Rehwinkel & Gack, 2020; Thoresen et al., 2021; Wang et al., 2007).

1.1 Signaling pathway of RIG-I activation

RIG-I is typically considered a cytoplasmic PRR. However, recent study demonstrated that RIG-I may potentially localize to the cell nucleus and other subcellular organelles (Liu et al., 2018). RIG-I is essential for identifying viral RNA that carries a 5' triphosphate (5' ppp) or 5' diphosphate (5' pp) signature (Coch et al., 2017; Yiliu Liu et al., 2016; Zhu et al., 2017). RIG-I is found in resting form in uninfected cells, with the repressor domain enclosing the RNA-binding and helicase domains. Resting RIG-I

CARDs are also folded over one another, with these interactions controlling RIG-I signaling activity. The helicase domain of RIG-I tightly encircles the RNA in a C-clamp-like manner when it is attached to the RNA ligand. The helical components of RIG-I helicase are thought to attach to the carboxy terminal domains (CTD) and cause conformational changes in RIG-I that open the CARDs up for signaling. According to the current model, RIG-I attaches to RNA monomerically. However, RIG-I oligomers maintained and nucleated by non-degradative K63-polyubiquitin chains are needed for RIG-I to signal through its CARDs (Onomoto et al., 2021; Rehwinkel & Gack, 2020; Thoresen et al., 2021). Following RNA binding and oligomerization, RIG-I CARD domain interacts with the adaptor protein mitochondrial antiviral signaling protein (MAVS) to activate the TANK binding kinase 1 (TBK1)-IKK ϵ complex and IKK α -IKK β complex activates the transcription factor interferon IFN regulatory factor 3 (IRF3), IRF7 and Nuclear factor-kappa B (NF- κ B) (Coch et al., 2017; Le Goffic et al., 2007; Yiliu Liu et al., 2016; Zhu et al., 2017).

Once in the nucleus, these transcription factors coordinate the expression of type I and III IFN and proinflammatory cytokines. IFN- α/β binds to their corresponding receptors and activates Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway to induce the transcription of Interferon-stimulated genes (ISG) (Yiliu Liu et al., 2016; Zhu et al., 2017). The ISG products are essential effectors in limiting pathogen replication, promoting acute proinflammatory immune response, and helping to activate the adaptive immune system further (Zhu et al., 2017).

1.2 Molecular and structural requirements for RIG-I activation

Many studies have been conducted since the discovery of RIG-I to determine the molecular and structural prerequisites for RIG-I activation by immunostimulatory RNAs. RIG-I is generally universally expressed in nucleated cells without displaying tissue-specific expression patterns, indicating a function in the global surveillance of viral infections. RIG-I is inactive in the absence of infection, assuming an autoinhibited conformation as it patrols the cell. The receptor is typically found in the cytoplasm but also the nucleus and particular subcellular compartments, such as mitochondria, microsomes, and membranes connected to the mitochondria. RIG-I continuously samples the RNAs it encounters while patrolling these compartments, dynamically binding and releasing them as it looks for viral RNA targets. RIG-I has a relatively high affinity for various RNAs, and it has evolved active strategies to become selectively triggered exclusively by viral binding RNAs containing specific molecular determinants (Brisse & Ly, 2019; Onomoto et al., 2021; Thoresen et al., 2021).

RIG-I monitors RNA molecules' 5' ends for molecular determinants. The first critical development was finding that RNA molecules with a ppp group at their 5' ends activate RIG-I. It has also been demonstrated that the RIG-I CTD's positively charged surface is essential for detecting the 5'-ppp signal of substrate dsRNA. Following structural studies of the RIG-I CTD and helicase domain with substrate RNA, it was discovered that both wrap around dsRNA, with the CTD covering the end. The RIG-I helicase domain makes interactions with the base-paired region of the RNA. The helicase domain possesses ATPase activity, and the rate of RIG-I dissociation from RNA is determined by ATP hydrolysis. When RIG-I interacts with its dsRNA substrate, it undergoes an intramolecular conformational shift and the release of N-terminal CARDS, allowing the RIG-I CARD to

link with the MAVS CARD on the mitochondria. Simultaneously, RIG-I translocase activity allows it to travel along the RNA substrate, resulting in the development of filamentous oligomers from the aggregation of many RIG-I molecules on RNA. Consequently, several RIG-I proteins' CARDS oligomerize and open up to connect with MAVS, leading to IFN signaling downstream (Brisse & Ly, 2019; Onomoto et al., 2021; Rehwinkel & Gack, 2020; Thoresen et al., 2021).

1.3 Intracellular localization of RIG-I and MAVS

RIG-I is evenly expressed in the cytoplasm in the inactivated state. Since MAVS is expressed on mitochondrial outer membranes, the filamentous aggregates containing activated RIG-I/MDA5 are found adjacent to mitochondria. MAVS is also found in Mitochondrial-associated endoplasmic reticulum membrane (MAM) and peroxisomes, which regulate RLR-mediated signaling. Peroxisomal MAVS is primarily involved in the induction of type III IFN. Furthermore, a recent study using high-resolution assays found that microsomal fractions, such as endoplasmic reticulum membranes, but not mitochondria, play essential roles in RLR-mediated signaling. Nuclear-localized RIG-I is needed for detecting IAV, which replicates in the nucleus. Nevertheless, the mechanism by which it stimulates MAVS-mediated signaling is unknown. Further research is needed to understand RLR intracellular localization (Brisse & Ly, 2019; Onomoto et al., 2021).

1.4 RIG-I agonists

The most prevalent and specific commercially available RIG-I agonists are 5'-pppdsRNA and 5'-pppssRNA, often known as 3pdsRNA and 3pssRNA, respectively. Several viral RNAs exhibit characteristics of RIG-I agonists, including an unmethylated 5' end that carries a PPP group produced from the ribonucleoside triphosphate used by viral

polymerases to initiate genome replication. The IAV is arguably the best-studied example of this, and investigation by several groups has demonstrated that RIG-I can identify IAV genomes in infected cells. More recently, it was discovered that RIG-I is also activated by short aberrant viral RNAs made by the IAV polymerase; these RNAs, like full-length genomes, have a 5'-ppp group and form a duplex structure. RIG-I can also detect infections with some positive-sense RNA viruses in addition to negative-sense RNA virus infections. For instance, these infections include identifying developing viral genomes with 5'-ppp groups before capping, such as those of the Flaviviridae family viruses' DV and ZV. It has also been suggested that RIG-I can be activated by viral RNAs that lack the typical characteristics of RIG-I agonists. For instance, RIG-I has been demonstrated to be activated by a stretch of uridine-rich DNA in the HCV genome that is distant from the 5' ends (Brisse & Ly, 2019; Rehwinkel & Gack, 2020; Thoresen et al., 2021).

1.5 Activation of RIG-I by host RNAs

Recent study has shown that virus infection can also result in host cellular RNAs, rather than viral RNAs, activating RIG-I. The purpose of these experiments was to explain previous findings that cells infected with herpesviruses, a dsDNA virus, generate type I interferon and proinflammatory cytokine responses that are partially dependent on RLRs. The interaction of cellular non-coding RNAs with RIG-I was discovered using RIG-I immunoprecipitation from cells infected with Herpes simplex virus (HSV) type 1 or Kaposi's sarcoma-associated herpesvirus, followed by high-throughput sequencing of co-purifying RNAs. RIG-I immunoprecipitations for HSV-1 were most highly enriched for 5S rRNA pseudogene transcripts, especially 5S ribosomal RNA pseudogene 141 (RNA5SP141). Unlike the parental 5S rRNA, the biological activities of these 5S rRNA

pseudogene transcripts with 5'-ppp groups and self-complementary sections largely remain unknown. These cellular non-coding RNAs are revealed for RIG-I recognition in two ways by HSV-1 infection. First, HSV-1-infected cells mislocalize 5S rRNA pseudogene transcripts to the cytoplasm. The mRNAs that encode the particular binding proteins of RNA5SP141, thiosulfate sulfotransferase (TST) and mitochondrial ribosomal Protein 18 (MRPL18) are downregulated due to HSV-1-induced host shutoff. When small interfering RNAs or antisense oligonucleotides silenced RNA5SP141, cytokine responses to HSV-1 infection and Epstein-Barr virus reactivation were reduced. Notably, RNA5SP141 is required partly for the type I IFN response to IAV, which replicates in the cell nucleus. This finding suggests that both viral and cellular RNA5SP141 play a role in the full activation of RIG-I during IAV infection (Brisse & Ly, 2019; Y. Liu et al., 2016; Rehwinkel & Gack, 2020).

1.6 Knowledge gap between RIG-I and bacterial infections

Despite RIG-I's well-studied involvement in viral infections, how RIG-I is regulated in bacterial infections still needs to be explored. A few reports indicate that some food-borne bacteria, like *Listeria monocytogenes* and *Salmonella typhimurium*, are identified by RIG-I (Abdullah et al., 2012; Johnson et al., 2020; Schmolke et al., 2014); however, it still needs to be determined whether bacteria use RIG-I to elicit innate immune proinflammatory changes and how bacteria regulate RIG-I expression. Most common airway viruses such as IAV, RSV, and Rhinovirus frequently use RIG-I to evade the innate immune response, making it a desiring target to explore in airway infections. It is well known that upper respiratory tract viral infections commonly precede bacterial coinfections (Beadling & Slifka, 2004; Zhang et al., 2020). Nontypeable *Haemophilus*

influenzae (NTHi) is a bacterium, well known to coexist with viral counterparts in bacteria-viral mixed infection. NTHi greatly contributes to the chronic and recurrent nature of airway infections by creating biofilms at the site of infection, resistant to being eliminated by either the host immune system or antibiotics (Zhang et al., 2020). NTHi contributes significantly to the hyperactive airway inflammation that occurs with aggravated chronic obstructive pulmonary disease (COPD), pneumonia, and asthma. Not only NTHi worsen lung pathologies, but it is also one of the leading causes of otitis media (OM), which is characterized by inflammation and pain in the middle ear, potentially leading to hearing loss, especially if NTHi infection persists to be chronic. The commonality of OM can be assessed by the fact that OM affects 80% of children by their third birthday (Li et al., 2013; Liu et al., 2018; Rehwinkel & Gack, 2020; Zhang et al., 2020).

NTHi is a Gram-negative, unencapsulated, opportunistic coccobacillus bacterium colonized in the nasopharynx microbiome (Rehwinkel & Gack, 2020). The unencapsulated Hi strains are designated as 'Nontypeable' because they do not produce a positive serum serotype. The extensive use of the conjugate vaccines for pneumococcal and *Haemophilus influenzae* serotype B (Hib) has increased the prevalence of NTHi infections. Recent research shows that invasive Hib species have been eliminated in regions employing vaccination, and NTHi is now the most prevalent commensal HI strain detected in children and adults (Bohn et al.; Zhang et al., 2020). Further research is required to understand the molecular mechanisms behind exacerbated and prolonged inflammation in NTHi infections. Antibiotics like amoxicillin-clavulanate combination or macrolide antibiotics are the most frequently used treatments for NTHi infection; however, with the rise of antibiotic-resistant strains, it is increasingly vital to comprehend NTHi-

induced host innate immune response in order to develop better therapeutics (Kobayashi et al., 2002).

NTHi is known to cause, exacerbate and worsen the progression of lung diseases such as COPD, asthma, and pneumonia. Epidemiologically, over 1 billion people worldwide are affected by COPD, asthma, and pneumonia, with the number growing annually (Andrews et al., 2015; Coch et al., 2017; Knight et al., 2012; Konduru et al., 2016; Li et al., 2013; Quaderi & Hurst, 2018; Ruuskanen et al., 2011). COPD is represented as any combination of pulmonary diseases, such as pulmonary emphysema and chronic bronchitis. Concurrently it results in a chronic productive cough, poorly reversible airflow obstruction, and alveolar parenchymal destruction, punctuated by episodic acute exacerbations of COPD that lead to hospitalization. Exacerbations of COPD are also associated with the presence of bacteria inside airway epithelial cells (Knight et al., 2012; Quaderi & Hurst, 2018; Weeks et al., 2021; Zhang et al., 2016).

In contrast, pneumonia is a common acute respiratory infection that damages the lungs' alveoli and distal bronchial tree. Children, the elderly, and people with chronic respiratory diseases are at the most significant risk. In adults, NTHi is a well-known cause of pneumonia (Ruuskanen et al., 2011; Slack, 2015; Torres et al., 2021).

Recent research has revealed that NTHi is one of the most prevalent bacteria isolated from asthmatic patients and may be crucial to developing and worsening asthma (Balaci et al., 2007; Zhang et al., 2020). Asthma affects more than 300 million individuals worldwide. Because inhaled corticosteroids are so widely used, the death rate of asthma has considerably dropped globally in recent decades. However, the number of new asthma cases continues to rise. By 2025, the Global Initiative for Asthma estimates that

400 million individuals globally will have asthma (Balaci et al., 2007; Zhang et al., 2020). The prevalence of NTHi is rising every year consequence of the widespread use of the conjugate vaccination against HI type b (Balaci et al., 2007; Zhang et al., 2020). A recent study reported the involvement of the RIG-I inflammasome in respiratory viral infections, such as rhinovirus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the initiation and progression of asthma (Radzikowska et al., 2021).

One of the first physical barriers NTHi encounter in the lungs is airway epithelial cells (AECs). In addition to supporting normal airway function, AECs play a significant role in developing host innate immune responses to antigens, making them essential airway anatomical and physiological integrity regulators, including inflammatory response (Andrews et al., 2015; Kim, 2022). AECs have a crucial role in creating a local inhibitory microenvironment, which inhibits the adaptive immune response and antigen-presenting cells. These reactions may be compromised in people with airway illness, making them more vulnerable to infections that trigger an inflammatory response and worsen the disease. This necessitates that the duration and severity of the inflammatory response induced by epithelial cells be tightly controlled. Hence, AECs are critical for maintaining immunological homeostasis (Charo & Ransohoff, 2006; Nathan & Ding, 2010).

These NTHi-induced disorders are characterized by significant epithelial damage and airway remodeling attributed to chronic inflammation and excessive mucus secretion. Injuries to the epithelium can result in cell death, tight junction disruption, loss of epithelial integrity, and tight junction disruption. The development and progression of chronic inflammation are significantly influenced by respiratory bacterial infections, such as NTHi (Andrews et al., 2015; Komatsu et al., 2020; Li et al., 2013; Torres et al., 2021). Even

though inflammation is essential for eliminating these pathogens, too much of it can cause tissue damage, as is evident in several chronic inflammatory conditions (Andrews et al., 2015; Konduru et al., 2016; Segel et al., 2011; Shuto et al., 2001; Susuki-Miyata et al., 2015). In order to maintain inflammation homeostasis in epithelial cells, inflammatory signaling pathways must be tightly regulated, and addressing these pathways would improve our comprehension of host immune responses (Charo & Ransohoff, 2006; Komatsu et al., 2020; Konduru et al., 2016; Shuto et al., 2001).

1.7 We hypothesize that NTHi may regulate RIG-I expression in airway infections

Earlier clinical studies demonstrated the significance of RLR receptors in upper respiratory infections and COPD exacerbations, where tonsils, adenoids, nasal polyps, and healthy nasal mucosa exhibit mRNA expression of RIG-I, MDA-5, and LGP-2 (Bogefors et al., 2011; Kim et al., 2010). Corresponding proteins are mainly found in tonsillar and adenoid lymphocytes and the surface epithelium. RIG-I is also expressed by cultured airway epithelial cells, producing cytokines in response to RIG-I activation. Additionally, untreated polyps had much higher RIG-I mRNA levels than healthy nasal mucosa. RIG-I mRNA expression was also found to be changed in the OM patient population (Bogefors et al., 2011; Kim et al., 2010). Furthermore, NTHi has been shown to live and thrive alongside common upper respiratory viruses like IAV and RSV active infections. Subsequently, later causes numerous modifications in the host epithelium that promote NTHi adhesion, colonization, decreased expression of antimicrobial peptides, increased expression of eukaryotic cell surface proteins that act as ligands for bacterial adhesins, dysregulated nutritional immunity, and other adverse effects (Mokrzan et al., 2020).

Although RIG-I is explored widely in viral infections, it still needs to be uncovered how RIG-I is regulated. It also needs to be determined whether bacteria, such as NTHi, that significantly impact the airways, coexist with other pathogens, and are known to induce chronic inflammation also play a role in regulating RIG-I. Hence, it is still unclear how RIG-I is regulated in airway epithelial cell inflammation in response to NTHi infection. Thus, comprehending the mechanism of NTHi-induced RIG-I regulation in airway epithelial cells would aid in understanding the complicated, inflammatory pathways caused by NTHi and eventually leads to the development of new therapeutics in NTHi-induced infections.

In the present study, we explored the role of NTHi-induced RIG-I regulation in airway epithelial cells. We showed that NTHi-induced RIG-I upregulation was mediated via activation of the TNF receptor associated factor 6 (TRAF6)-IKK β -p65 pathway. It is interesting to note that interleukin-1 receptor-associated kinase 3 (IRAK-M) negatively regulates NTHi-induced up-regulation of RIG-I and C-X-C motif chemokine ligand 10 (CXCL10) via targeting TRAF6-IKK β -p65 pathway. We also demonstrated that IKK β activation is not only necessary but also sufficient to induce RIG-I upregulation. We also showed that NF- κ B p65 phosphorylation at S276 and S536 residues might be critical for RIG-I up-regulation. Previous studies showed that histone deacetylases (HDACs) activate IKKs and NF- κ B p65 (Ashburner et al., 2001; Gatla et al., 2017). Thus, we decided to determine the role of HDACs in NTHi-induced RIG-I upregulation. We demonstrated that HDACs may positively regulate NTHi-induced RIG-I and CXCL10 upregulation. Finally, we confirmed the significance of RIG-I in the regulation of CXCL10. Our study provides new insights into how bacterium NTHi induces RIG-I upregulation,

which may open up a new avenue for the management of airway inflammation in mixed infection.

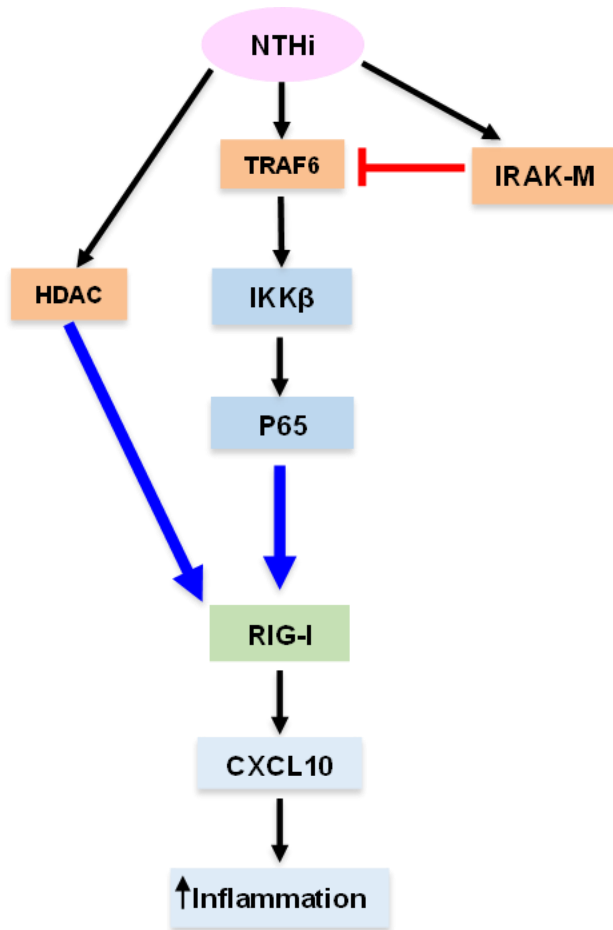


Figure 1: Model diagram: NTHi induces RIG-I upregulation

2. MATERIAL AND METHODS

2.1 Cell Culture

Human bronchial epithelial BEAS-2B cells were cultured in RPMI 1640 medium (Gibco), supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Gibco). IRAK-M KD BEAS-2B cells were established using CRISPR-Cas9 systems. To establish IRAK-M KD BEAS-2B cells, IRAK-M CRISPR/Cas9 KO plasmid (sc-403219-KO-2, Santa Cruz Biotechnology), IRAK-M HDR plasmid (sc-403219-HDR-2, Santa Cruz Biotechnology) and Cre vector (sc-418923, Santa Cruz Biotechnology) were used following the manufacturers' instruction. All cells were maintained in a humidified environment of 5% CO₂ at 37°C.

2.2 Bacterial Strain and Culture Conditions

Clinical isolate of NTHi strain 12 was used in this study (Susuki-Miyata et al., 2015). NTHi was grown overnight on chocolate agar plates in 5% CO₂ at 37°C and inoculated in BHI broth supplemented with 3.5 g/ml NAD and hemin. After overnight incubation, NTHi was then subcultured into fresh BHI broth and the log phase NTHi, monitored by measurement of optical density value, was washed and suspended in PBS for *in vitro* experiments. Unless otherwise specified, the cells were stimulated with NTHi at a MOI of 100 for 6 hours or as indicated.

2.3 Reagents and Antibodies

The IKK β inhibitor IKK-2 inhibitor IV was purchased from Calbiochem. SAHA was purchased from Cayman Chemical. TRAF6 inhibitor (C25-140) was purchased from MedChemExpress. RIG-I agonist 5'ppp-dsRNA was purchased from InvivoGen. 5'ppp-dsRNA control was also purchased from InvivoGen and used as a negative control

for 5'ppp-dsRNA. 5'ppp-dsRNA RIG-I agonist and negative control were dissolved in sterile endotoxin-free water. Dimethyl sulfoxide (DMSO) was used to reconstitute each inhibitor before diluting with RPMI 1640 medium to a final DMSO concentration of 0.1%. As a control, DMSO in RPMI 1640 was used. Unless otherwise stated, cells were treated with 1 μ M IKK β inhibitor, 1 μ M SAHA or 20 μ M TRAF6 inhibitor at a final volume of 500 μ L per well of 12 well plates. Cells were treated with or without IKK β inhibitor or SAHA for 1 hour before stimulation with NTHi, or TRAF6 inhibitor for 2 hours before NTHi stimulation. Unless otherwise stated, cells were transfected with 100 ng of the 5'ppp-dsRNA for 48 hr using Lipofectamine RNAiMAX (Thermo Fisher Scientific) in the final volume of 500 μ L per well of 12 well plates.

Antibodies against IRAK-M (#2355) were purchased from Prosci. Antibodies against RIG-I (sc-376845), p65 (sc-8008), and β -actin (sc-47778) were purchased from Santa Cruz Biotechnology. Antibodies against p65 S276A (ab183559) and p65 S536A (ab76302) were purchased from Abcam. Anti-rabbit HRP-linked antibodies (#7074) and anti-mouse HRP-linked antibodies (#7076) were purchased from Cell Signaling Technology. Goat anti-mouse IgG Alexa Fluor 488 (A-11029) was purchased from Thermo Fisher Scientific.

2.4 siRNA-Mediated Knockdown

Human siRNA oligonucleotides for RIG-I (L-012511-00-0005), p65 (L-003533-00-0005), and control siRNA (D-001206-14-05) were purchased from Dharmacon. Human siRNA oligonucleotides for IRAK-M (SR323378B) and control siRNA (SR30004) were purchased from OriGene. BEAS-2B cells were transfected with siRNA around 60-70% confluence using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the

manufacturer's protocol. Cells were stimulated with NTHi or 5'ppp-dsRNA after 24 hr siRNA transfection.

2.5 RNA isolation and Real-time Q-PCR analysis

According to the manufacturer's protocol, total RNA was isolated with TRIzol reagent (Life Technologies). Reverse transcription reaction was performed using PrimeScript reverse transcription Master Mix (Takara Bio). For Q-PCR analysis, PCR amplifications were performed with Fast SYBR Green Master Mix (Applied Biosystems). PCR reactions were performed in triplicate containing 2X Universal Master Mix, 1 μ L of template cDNA, and 400 nM primers in a final volume of 12.5 μ L, and they were analyzed in a 96-well optical reaction plate (USA Scientific). Reactions were amplified and quantified by using a StepOnePlus Real-Time PCR machine and StepOne software (v2.3) (Applied Biosystems). The relative quantities of mRNAs were obtained by using the comparative Ct method and normalized to human Cyclophilin as an endogenous control.

The primer sequences for RIG-I, CXCL10, TNF- α , and Cyclophilin are as follows:

Human RIG-I: forward 5'-GGACGTGGCAAACAAATCAG-3' and reverse 5'-GCAATGTCAATGCCTTCATCA-3';

Human CXCL10: forward 5'-GAAATTATTCCTGCAAGCCAATTTTG-3' and reverse 5'-CCCTTCTTTTTCATTGTAGCAATG-3';

Human TNF- α : forward 5'-CCCAGGCAGTCAGATCATCTT-3' and reverse 5'-AGCTGCCCCTCAGCTTGA-3';

Human Cyclophilin: forward 5'-CGGGTCCTGGCATCTTGT-3' and reverse 5'-GCAGATGAAAACTGGGAACCA-3'.

2.6 Plasmid constructs transfection

The expression plasmids including p65 WT, mutant p65 (p65 S276A and p65 S536A), constitutively active forms of IKK β (IKK β -CA, S177E/S181E) were described [10, 48]. An empty vector pcDNA3.1/myc-His(-) was used as a control and was also added where necessary to ensure an equivalent amount of input DNA. All plasmid constructs transfections were carried out in triplicate by using TransIT-LT1 reagent (Mirus) following the manufacturer's instruction.

2.7 Western Blot Analysis

Whole-cell extracts were recovered with protein lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF) with freshly added 1 mM PMSF, 1 mM Na₃VO₄ and protease inhibitor cocktail (Sigma-Aldrich). Cell extracts were incubated on ice for 30 minutes and centrifuged at 12,000 g for 30 minutes to remove cell debris. Whole-cell lysates were separated in 8-10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBS-T). After washing three times with TBS-T, the membrane was incubated in a 1:2000 to 1:4000 dilution of a primary antibody in 5% BSA-TBS-T overnight at 4 °C. After washing three times with TBS-T, the membrane was incubated in a 1:5000 dilution of corresponding secondary HRP-conjugated IgG in 5% non-fat dry milk-TBS-T for 1 hour at room temperature. After washing three times with TBS-T, the proteins were visualized using Amersham ECL Prime Detection Reagent (GE Healthcare Life Sciences) and images were obtained by ChemiDoc XRS+ system (Bio-Rad). For presentational

purposes, images have been cropped. Contrast and brightness adjustments were made equally to all images. These adjustments do not obscure or eliminate any information.

2.8 Immunofluorescence

For immunofluorescence, cells were cultured on a coverslip placed in a 12-well plate. After stimulation with NTHi, the cells were washed with PBS and fixed with 4% paraformaldehyde solution. Then, the cells were permeabilized with 0.1% Triton X-100 and incubated with antibody against RIG-I overnight at 4°C. Goat anti-mouse IgG Alexa Fluor 488 was used as secondary antibody. Nuclei were counterstained with mounting medium containing DAPI. Images of stained cells were recorded with a BZ-X710 fluorescence microscope (Keyence).

2.9 Statistical Analysis

All experiments were reproduced at least three times with consistent results. Data were shown as mean \pm SD. Unpaired two-tailed Student's t-test assessed statistical analysis, and $P < 0.05$ was considered statistically significant.

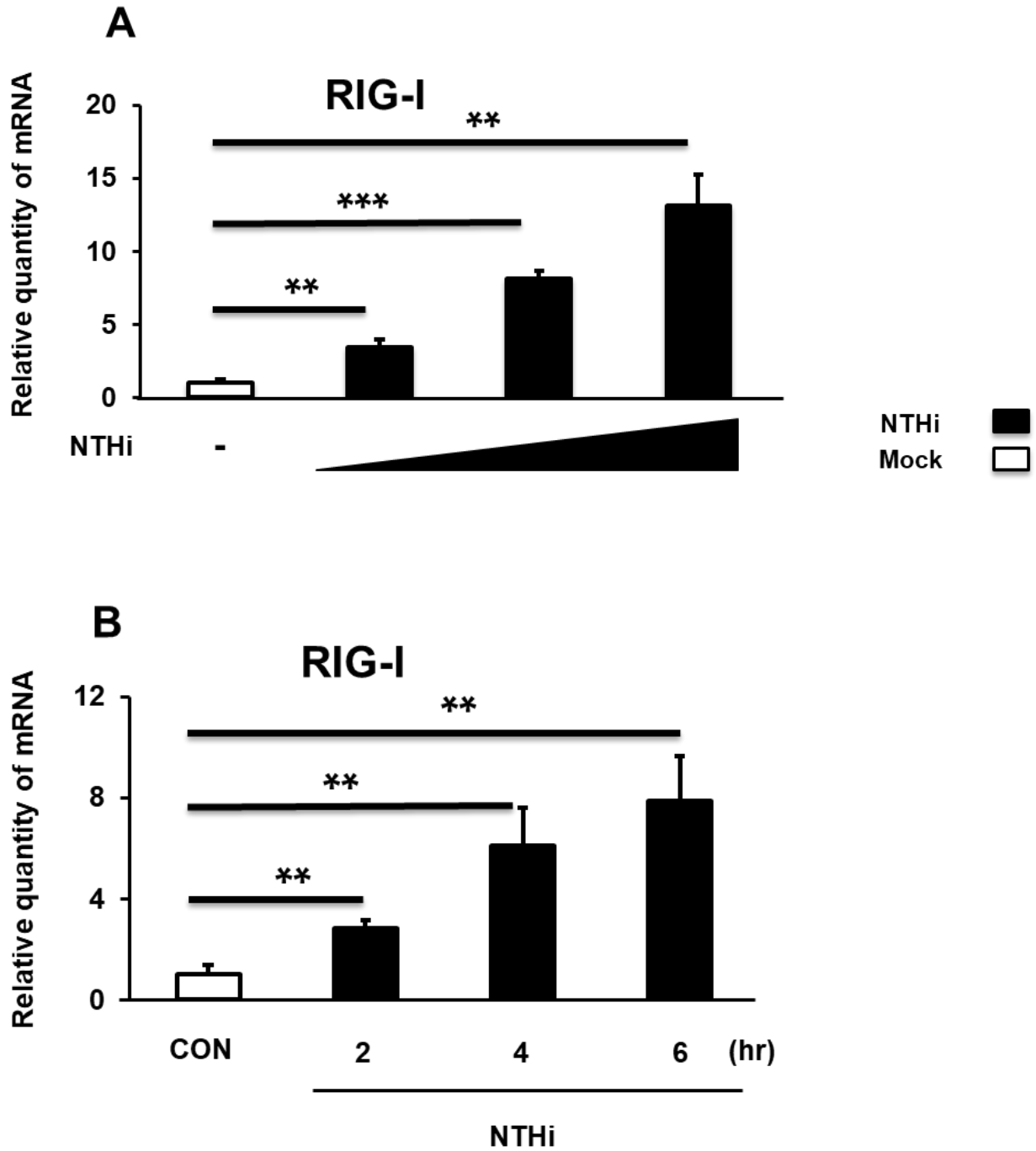
3. RESULTS

3.1 NTHi up-regulates RIG-I expression in human bronchial epithelial cells in vitro

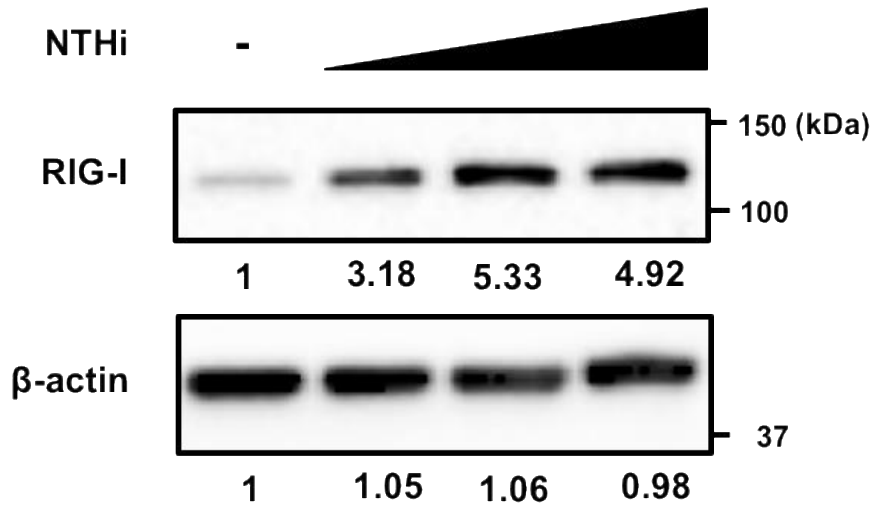
Previous clinical studies have revealed that RLR increases in infected airways (Bogefors et al., 2011; Kim et al., 2010). In many cases, viral infections of the URT, including IAV, RSV, and other common viral illnesses, precede bacterial coinfections (Beadling & Slifka, 2004; Feldman & Anderson, 2021). The host epithelium undergoes numerous changes due to URT virus infection, some of which favor bacterial adhesion and colonization. Previous research has demonstrated that mice lacking RLR signaling pathways are immunodeficient, prone to colitis, and extremely sensitive to RNA viruses (Kandasamy et al., 2016; Wang et al., 2007; Zhang et al., 2020).

We sought to determine whether NTHi affects RIG-I expression in human airway epithelial cells. Human bronchial epithelial BEAS-2B cells were stimulated with NTHi, and then RIG-I mRNA expression was measured by real-time quantitative PCR (Q-PCR). Interestingly, NTHi induced RIG-I up-regulation at mRNA level in BEAS-2B cells in a dose- (**Fig. 2A**) and time-dependent (**Fig. 2B**) manner. To determine whether up-regulation of RIG-I mRNA is accompanied by increased RIG-I protein, western blot analysis was carried out with RIG-I-specific antibody. As shown in **Fig. 2C-2F**, NTHi-induced up-regulation of RIG-I was also observed in a dose- and time-dependent manner in BEAS-2B cells. Immunofluorescent staining studies were consistent with these findings showing RIG-I up-regulation in BEAS-2B 6hr and 12hr after stimulation with NTHi (**Fig. 2G and 2H**). Also, as shown in **Fig. 2G and 2H**, 100% of the cells demonstrated RIG-I expression from the NTHi stimulated groups compared to 76% RIG-I expression seen in the mock group, with relatively much lower intensity. Taken together, these data

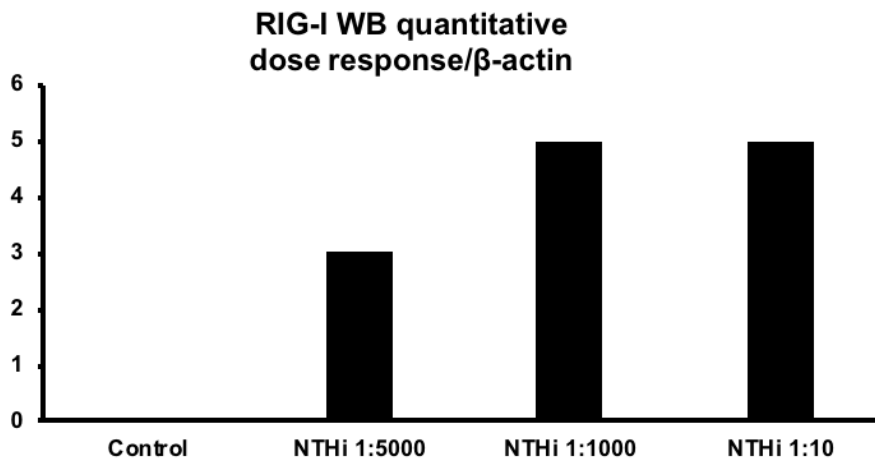
demonstrate that NTHi up-regulates RIG-I expression at both mRNA and protein levels *in vitro*.

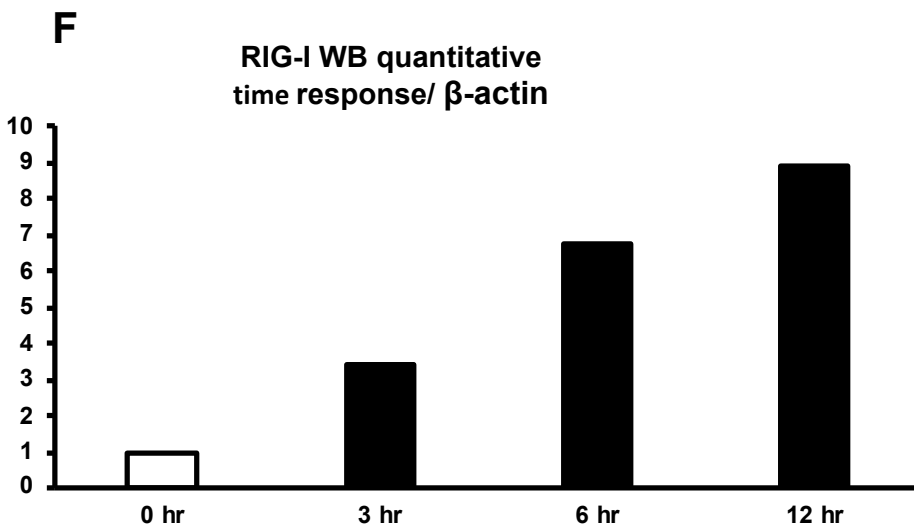
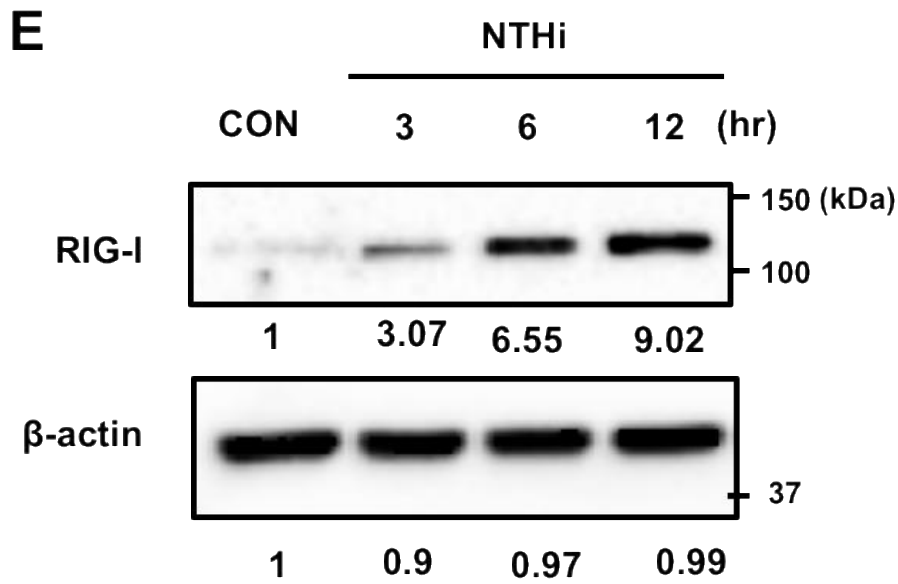


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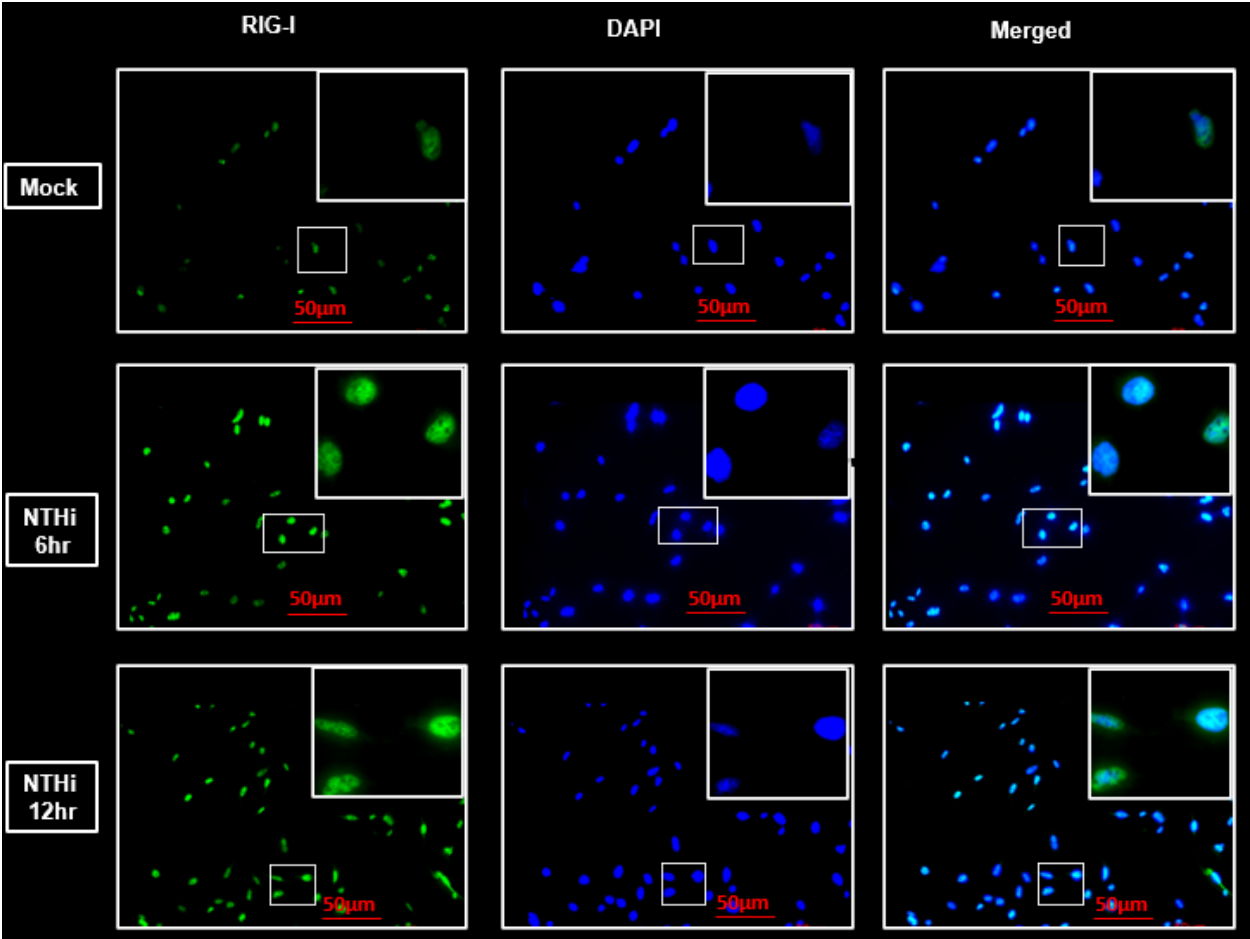


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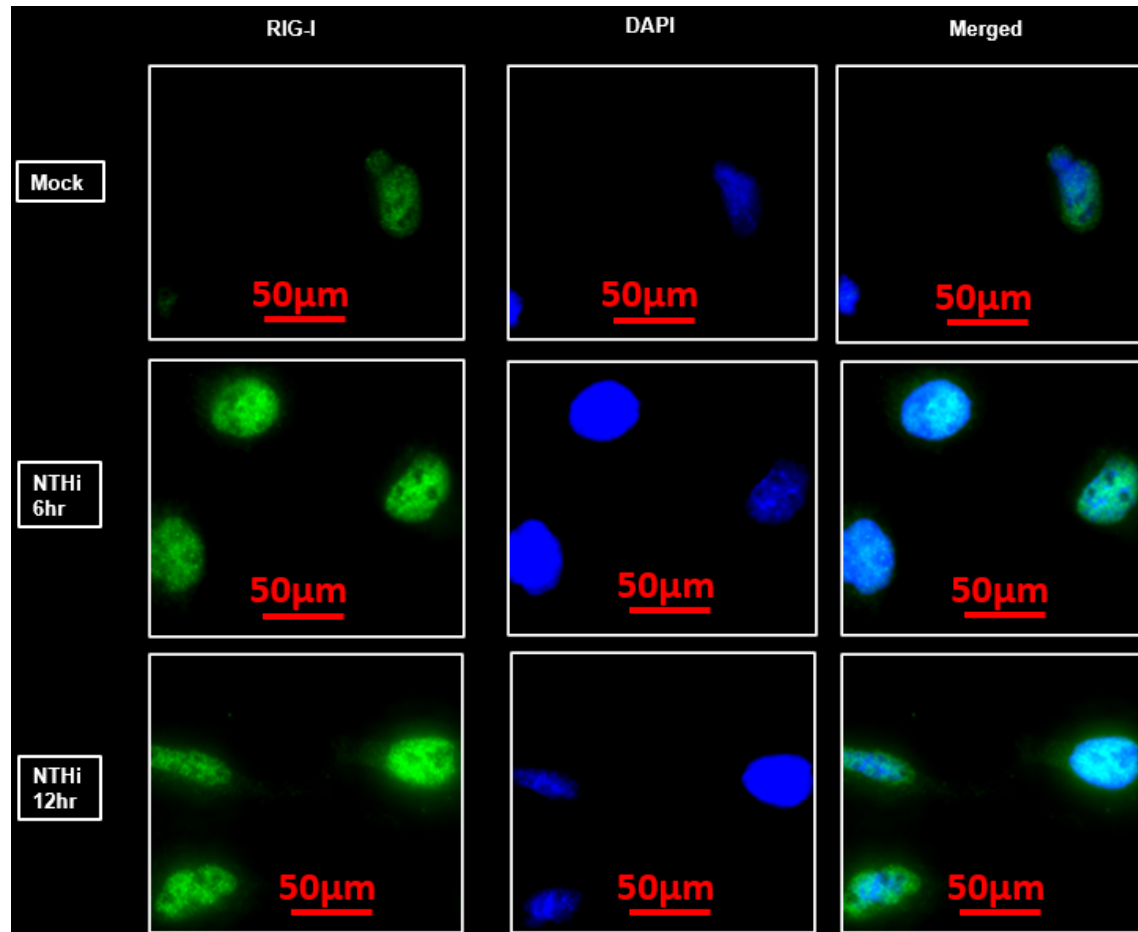


Figure 2. NTHi induces RIG-I up-regulation in a dose- and time-dependent manner in vitro

(A) BEAS-2B cells were stimulated with NTHi (MOI of 0.2, 1, 100) for 6hr, and RIG-I mRNA expression was measured by Q-PCR. (B) BEAS-2B cells were stimulated with NTHi (MOI of 100) for 2, 4, or 6 h, and RIG-I mRNA expression was measured by Q-PCR. (C,D) BEAS-2B cells were stimulated with NTHi (MOI of 0.2, 1, 100) for 6hr, and RIG-I, β -actin protein levels were visualized by Western Blotting. (E,F) BEAS-2B cells were stimulated with NTHi (MOI of 100) for 3, 6, or 12hr, and RIG-I, β -actin protein levels were visualized by Western Blotting. (G and H) BEAS-2B cells were stimulated with NTHi (MOI of 100) for 6 and 12 hr, and RIG-I was visualized by immunofluorescence. Magnification: 200x and 400x. Scale bar: 50 μ m. Data are Mean \pm SD (n=3). **, $p < 0.01$; ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments.

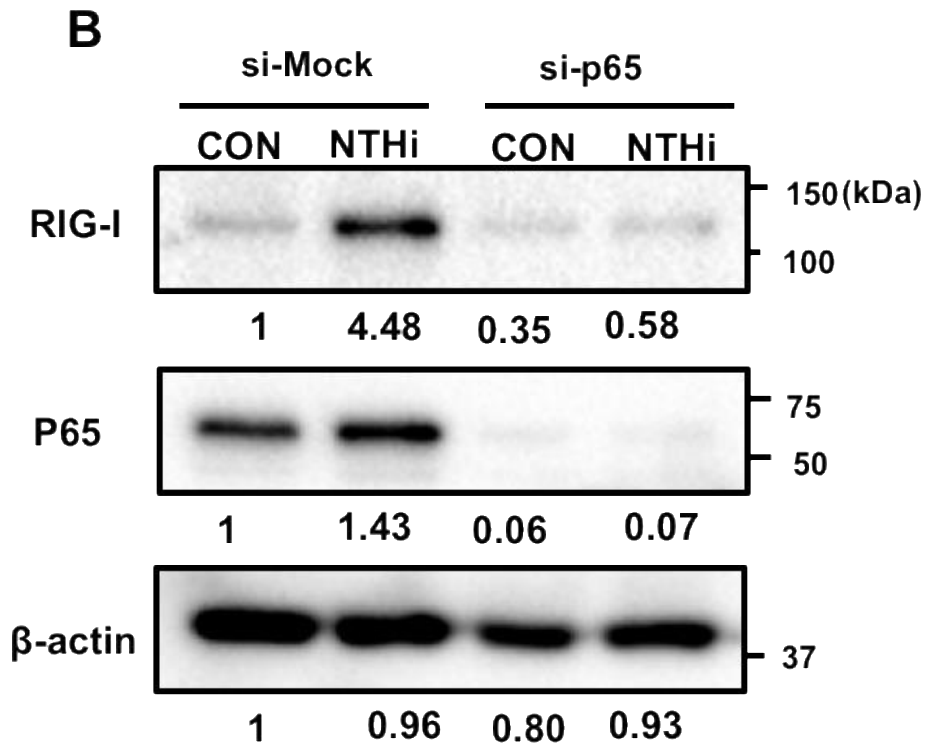
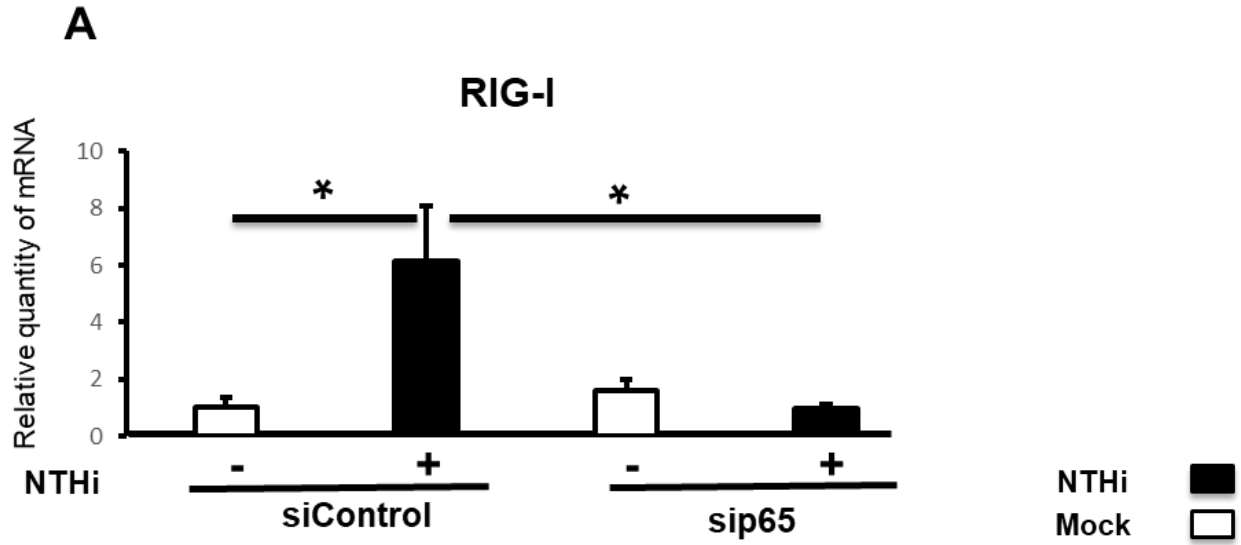
3.2 NF- κ B p65 positively regulates NTHi-induced up-regulation of RIG-I

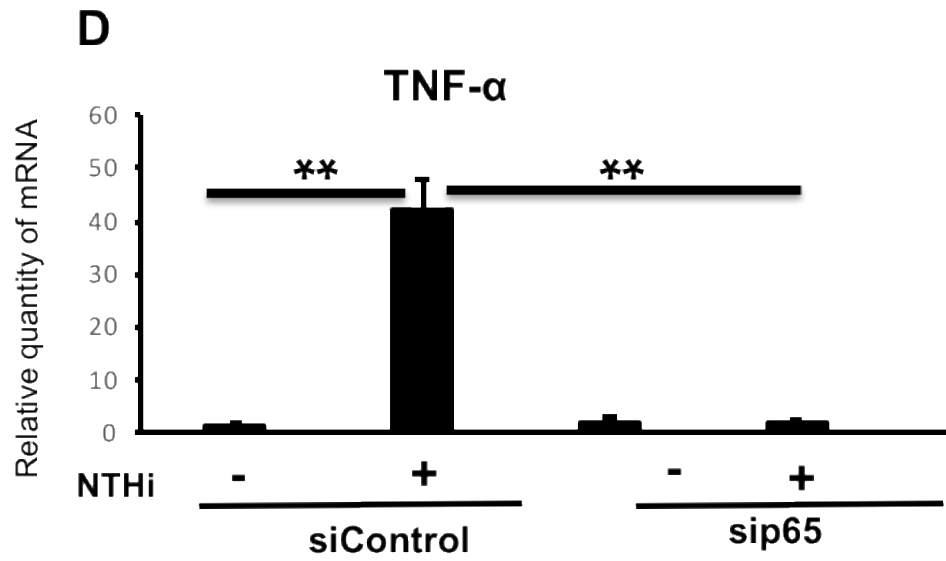
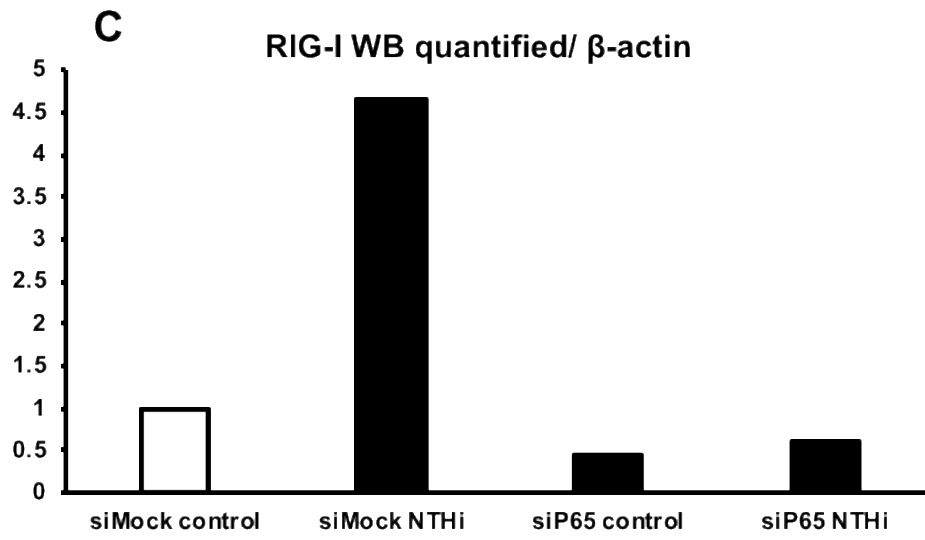
The NF- κ B is a dimeric transcription factor consisting of the p50 and p65 subunits. In a resting cell, NF- κ B stays in the cytoplasm, but it moves into the nucleus in response to various stimuli, such as bacterial infections. The inhibitory subunit I κ B, which keeps NF- κ B in the cytoplasm, regulates NF- κ B activation. NF- κ B activation needs the sequential phosphorylation, ubiquitination, and degradation of I κ B, as well as the subsequent exposure of a nuclear-localization signal on the NF- κ B protein. NF- κ B has been demonstrated to have an essential role in regulating the expression of several genes encoding cytokines, chemokines, and other mediators involved in inflammatory responses. Previous research has shown that NTHi activates NF- κ B p65, which contributes to the activation of innate immune responses. The NF- κ B pathway controls proinflammatory cytokine activation in response to stimuli. NTHi and endogenous factors like TNF- α are shown to work together to activate NF- κ B (Bonizzi & Karin, 2004; Oeckinghaus & Ghosh, 2009; Shuto et al., 2001; Watanabe et al., 2004; Xu et al., 2011).

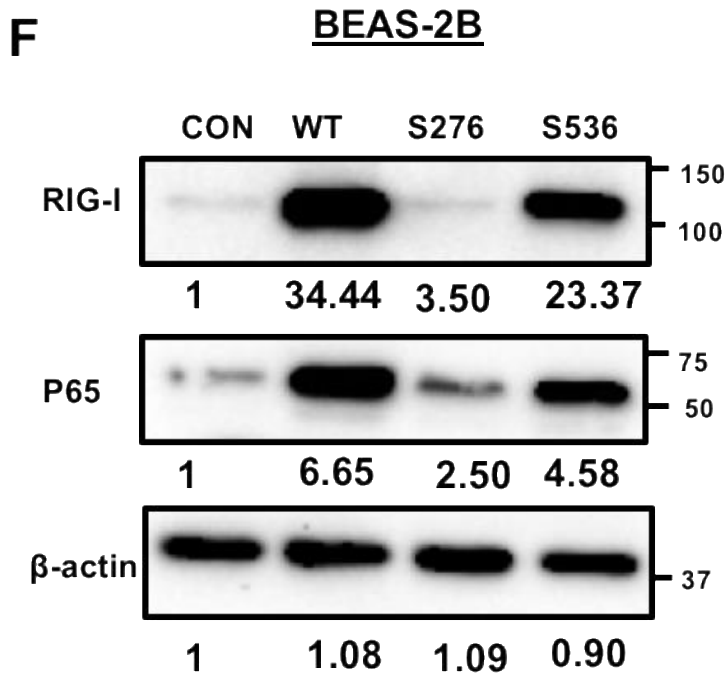
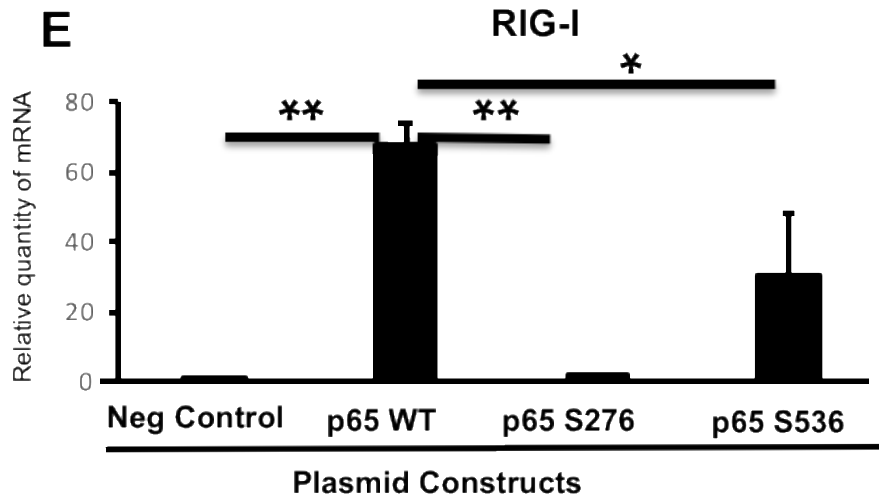
Thus, we sought to determine the role of NF- κ B p65 in NTHi-induced RIG-I upregulation. Interestingly, knockdown of p65 inhibited NTHi-induced up-regulation of RIG-I expression at mRNA and protein levels (**Fig. 3A, 3B and 3C**). In addition, NTHi-induced TNF- α mRNA expressions were evaluated and were also reduced on p65 knockdown in BEAS-2B cells (**Fig. 3D**). These data demonstrated that p65 acts as a positive regulator of NTHi-induced RIG-I up-regulation and inflammation in airway epithelial cells.

NF- κ B signaling pathways have been shown to play a critical role in controlling inflammatory responses. Phosphorylation at multiple residues of p65 has been shown to

regulate various functions of p65, such as DNA binding and transcriptional activities (Lee et al., 2016; Susuki-Miyata et al., 2015; Xu et al., 2011). However, the role of p65 phosphorylation in NTHi-induced RIG-I upregulation remains largely unknown. To evaluate the role of p65 phosphorylation at key residues including serine 276 (S276) and serine 536 (S536) in NTHi-induced RIG-I upregulation, we determined the effect of p65 WT and phosphorylation-deficient mutant (S276A, S536A) on NTHi-induced RIG-I upregulation assessed by Q-PCR and western blot analysis. As shown in **Fig. 3E, 3F and 3G**, RIG-I expression at mRNA and protein levels was decreased in S276A- and S536A-transfected cells compared with p65 WT-transfected cells. Consistent with above result, the expression of proinflammatory mediator TNF- α was also decreased in S276A- and S536A-transfected cells compared with p65 WT-transfected cells (**Fig. 3H**). However, impact of S276A mutation on RIG-I and TNF- α expression is evidently much stronger than S536A mutation as shown in **Fig. 3E, 3F, 3H**. Taken together, these results suggest that phosphorylation of p65 at Ser276 and Ser536 might be critical for RIG-I expression and inflammation.







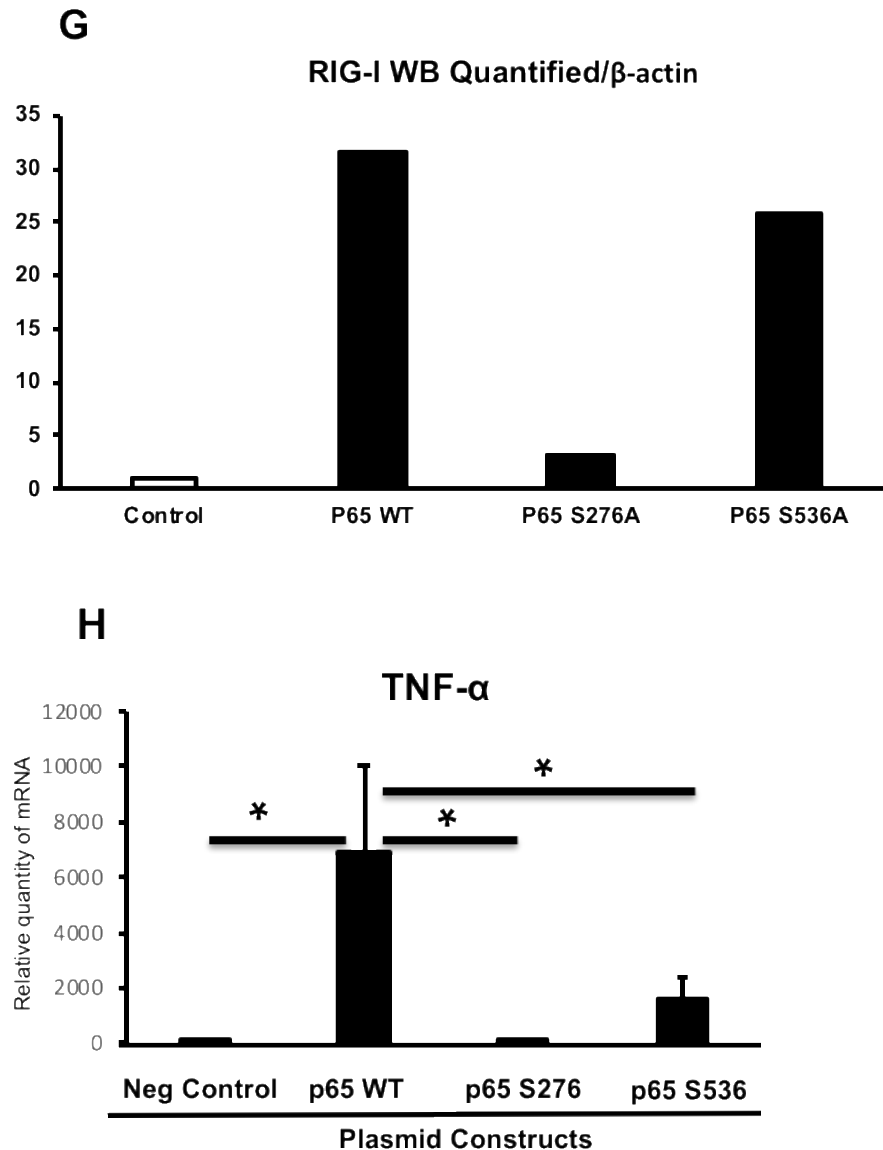


Figure 3. NF- κ B p65 positively regulates NTHi-induced up-regulation of RIG-I
(A) BEAS-2B cells transfected with control siRNA or p65 siRNA were stimulated with NTHi (MOI of 100) for 6hr, and RIG-I mRNA expression was measured by Q-PCR. **(B,C)** BEAS-2B cells transfected with control siRNA or p65 siRNA were stimulated with NTHi (MOI of 100) for 6hr, and RIG-I, p65, β -actin proteins were visualized by Western Blotting. **(D)** BEAS-2B cells transfected with control siRNA or p65 siRNA were stimulated with NTHi (MOI of 100) for 6hr, and TNF- α mRNA expression was measured by Q-PCR. **(E-H)** BEAS-2B cells transfected with p65 WT, p65 S276A, p65 S536A or negative control. **(E)** After 24hr, RIG-I mRNA expression was measured by Q-PCR. **(F,G)** After 24hr, RIG-I, p65, β -actin proteins were visualized by Western Blotting. **(H)** After 24hr, TNF- α mRNA expression was measured by Q-PCR. Data are Mean \pm SD (n=3). *, $p < 0.05$; **, $p < 0.01$, t-test. Data are representative of three or more independent experiments. sip65, p65 siRNA.

3.3 IKK β positively regulates NTHi-induced up-regulation of RIG-I

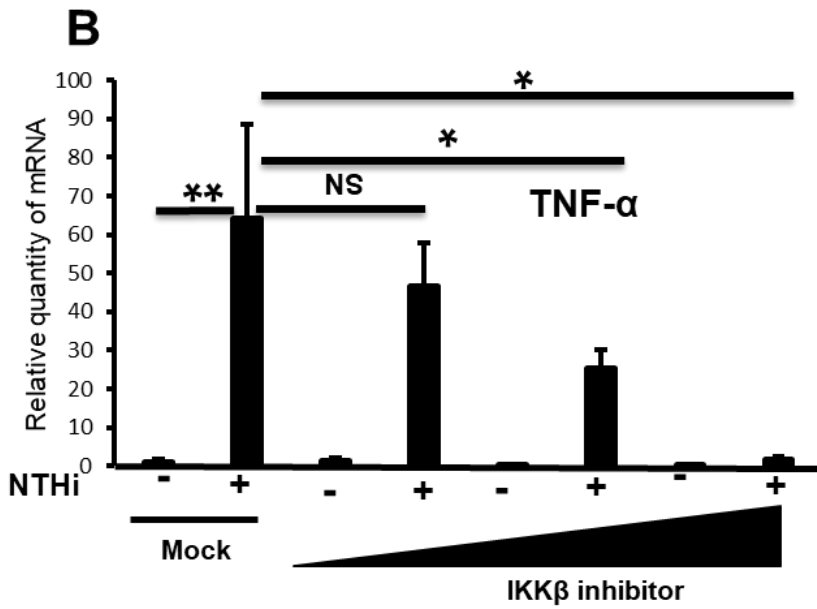
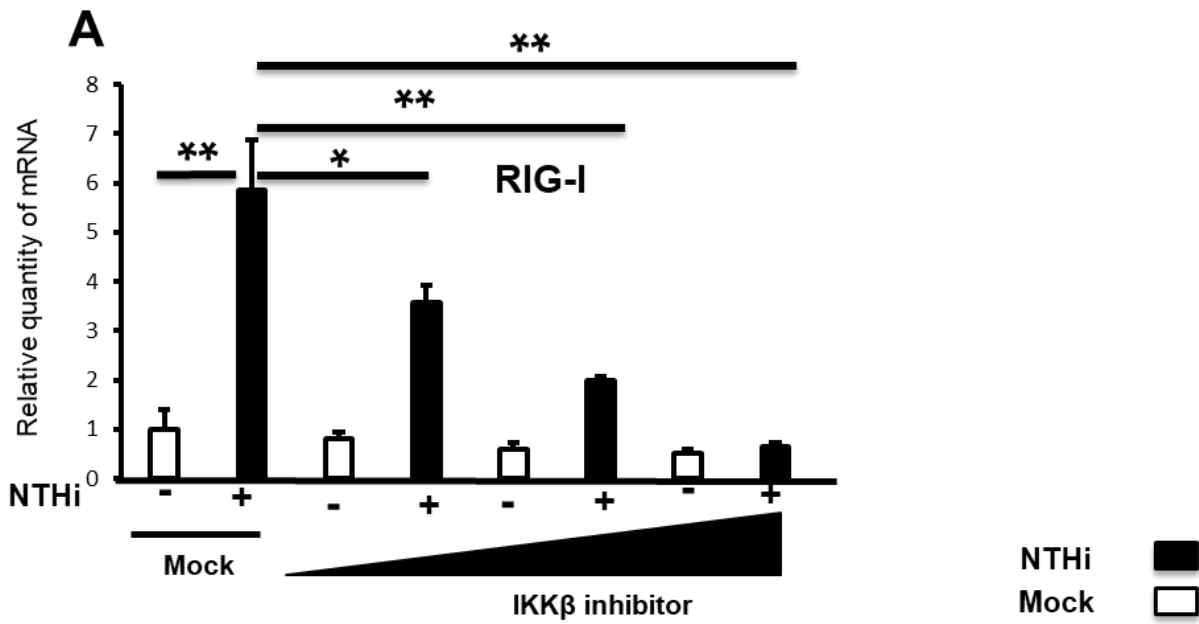
IKKs regulate NTHi-mediated NF- κ B activation. This kinase complex enzyme is vital in communicating the cellular response to inflammation to NF- κ B and functions upstream of the NF- κ B signal transduction cascade. The IKK complex is made up of two catalytic subunits known as IKK α (IKK1) and IKK β (IKK2), as well as a non-catalytic regulatory component known as NF- κ B essential modulator (NEMO) or IKK γ . Many different combinations of the IKK complex subunits, such as homodimers of IKK α or IKK β that are associated with or independent from NEMO, have been proposed; nonetheless, the majority of biochemical data suggest that the majority of cellular IKKs complexes have a core of IKK α -IKK β -NEMO in the ratio of 1:1:2. IKK activity must be activated by phosphorylating either IKK α or IKK β on two specific serine residues found in the T loop of the catalytic domain of each kinase (Solt & May, 2008).

Despite their striking similarities, a succession of exquisite genetic investigations demonstrated that IKK α and IKK β perform separate roles within the NF- κ B signaling paradigm. In this context, fast and transitory TNF-induced I κ B α degradation occurs via a pathway involving IKK β and NEMO. The failure of IKK β deficient mice to produce an NF- κ B-dependent anti-apoptotic response caused them to die during development from severe TNF-induced hepatocyte apoptosis. This phenotype was also seen in animals deficient in the NF- κ B subunit p65, establishing a genetic link between NEMO, IKK β , and p65 in a shared signaling cascade. The classical NF- κ B signaling pathway is now described as IKK β -dependent I κ B phosphorylation and degradation releasing NF- κ B complexes exemplified by the ubiquitous p50-p65 heterodimer. The classical pathway controls the vast majority of NF- κ B-activated genes, including those producing

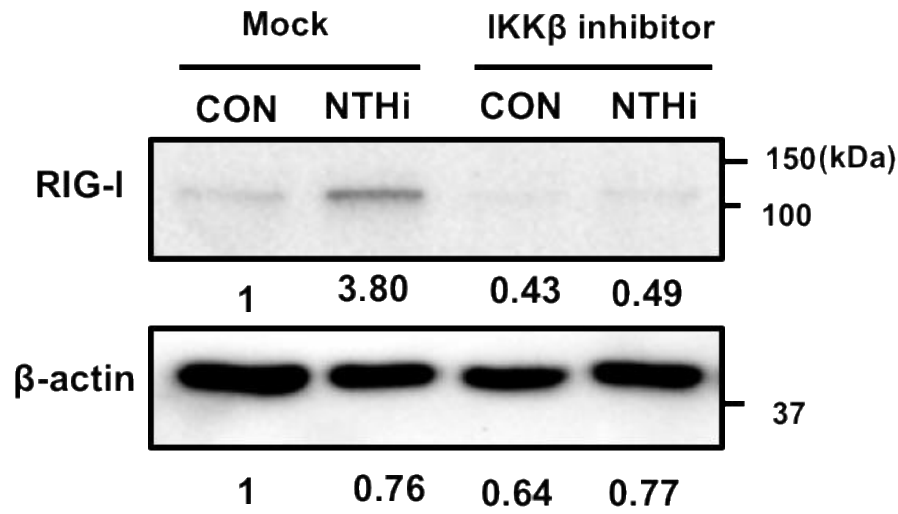
proinflammatory and immunomodulatory cytokines (such as TNF- α) and chemokines (such as CXCL10). Furthermore, disrupting the IKK α locus did not eliminate IKK activation by proinflammatory stimuli and resulted in only a minor reduction in nuclear factor NF- κ B activation (Andrews et al., 2015; Konduru et al., 2016; Li et al., 1999; Shuto et al., 2001; Solt & May, 2008).

We next sought to determine the role of IKK β , the key positive regulator of NF- κ B-dependent inflammation, in NTHi-induced RIG-I upregulation and inflammation. As shown in **Fig. 4A and 4B**, IKK β inhibitor significantly suppressed NTHi-induced up-regulation of RIG-I and TNF- α mRNA in a dose-dependent manner. Consistent with this result, IKK β inhibitor significantly suppressed NTHi-induced up-regulation of RIG-I protein (**Fig. 4C, 4D**). These data demonstrated that IKK β acts as a positive regulator of NTHi-induced RIG-I up-regulation and inflammation in airway epithelial cells.

We next sought to determine if activation of IKK β is not only required but also sufficient for RIG-I up-regulation. We examined the effects of the constitutively active form of IKK β (IKK β -CA) on RIG-I expression in BEAS-2B cells. As shown in **Fig. 4E**, IKK β -CA induced RIG-I up-regulation in a dose-dependent manner. Thus, these data demonstrate that activation of IKK β is sufficient for RIG-I up-regulation (**Fig. 4F**).

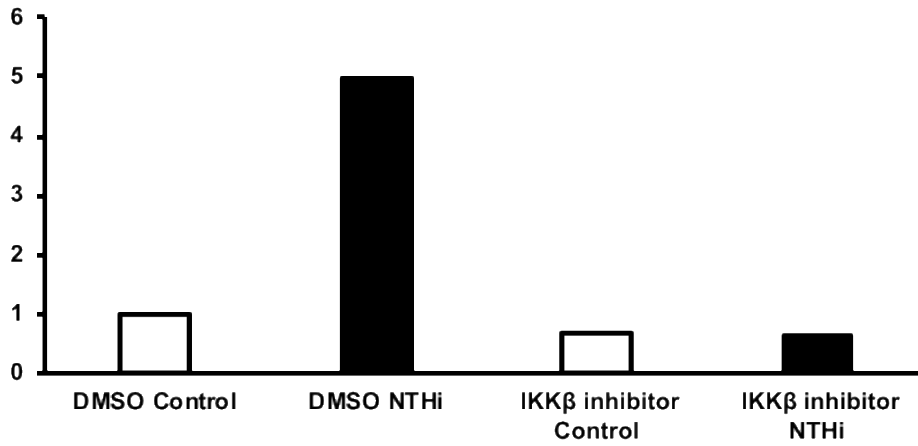


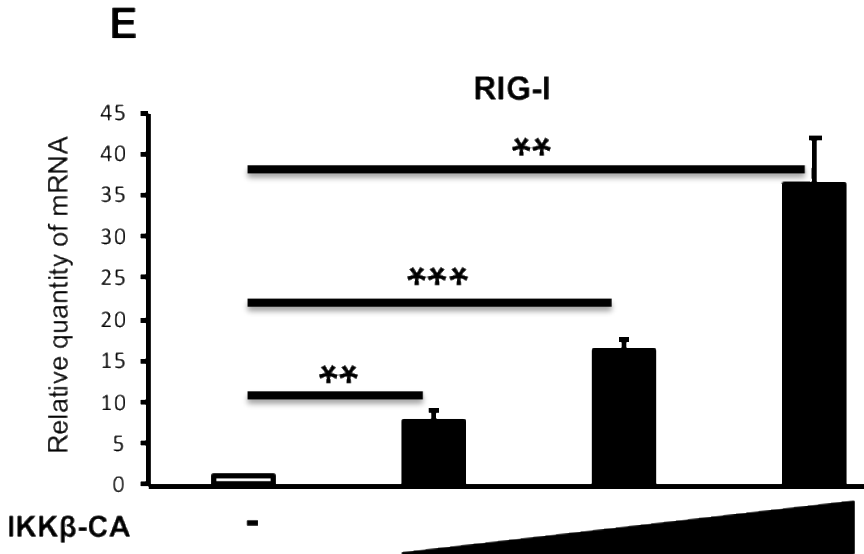
C



D

RIG-I Quantified WB/ β -actin





F

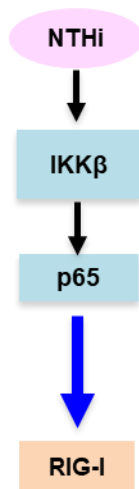


Figure 4. IKKβ positively regulates NTHi-induced up-regulation of RIG-I

(A-B) BEAS-2B cells were pretreated with IKKβ inhibitor (10 nM, 100 nM, 1 μM) for 1 hr, followed by NTHi stimulation (MOI of 100) for 6hr, and (A) RIG-I and (B) TNF-α mRNA expression were measured by Q-PCR. (C,D) BEAS-2B cells were pretreated with IKKβ inhibitor 1 μM for 1 hr, followed by NTHi stimulation (MOI of 100) for 6hr, and RIG-I and β-actin proteins were visualized by Western Blotting. (E) RIG-I mRNA expression was measured in BEAS-2B cells transfected with IKKβ-CA (0.05 μg, 0.1 μg, 0.2 μg) or empty vector for 40 hr. (F) Schematic diagram. Data are Mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments. IKKβ-CA, the constitutively active form of IKKβ; NS, not significant.

3.4 TRAF6 positively regulates NTHi-induced up-regulation of RIG-I

Pattern recognition receptors activate several downstream molecules, such as TRAF6, IKK β , and NF- κ B, to produce proinflammatory cytokines. TRAF6 is a signal transducer in the NF- κ B pathway that regulates the activation of NF- κ B and IKKs in response to proinflammatory cytokines. TRAF6 is known to bridge signaling by the TNFR and TLRs; it is known to activate TAK1-binding protein 2/3 (TAB2/3) and recruit the kinase transforming growth factor beta-activated kinase 1 (TAK1), which in turn phosphorylate IKKs. These events lead to the activation of the transcription factors NF- κ B. TRAF6 activity is often coordinated with other TRAFs, such as TRAF2/5 and TRAF3 in some TLR complexes. TRAF3-TRAF6 coordination may be necessary for the activation of the IRF family of transcription factors (Deng et al., 2000; Gupta et al., 2010; Lee et al., 2016; Oeckinghaus & Ghosh, 2009; Shi & Sun, 2018; Xu et al., 2011).

Previous studies demonstrated that NTHi induces IKK β -NF- κ B-mediated inflammatory responses via activation of TRAF6 (Lee et al., 2016). Thus, we hypothesized that TRAF6 may play a critical role in NTHi-induced RIG-I upregulation. We first determined the effect of TRAF6 inhibitor on NTHi-induced RIG-I mRNA expression by performing Q-PCR analysis. As shown in **Fig. 5A**, TRAF6 inhibitor significantly suppressed NTHi-induced RIG-I up-regulation. Thus, our data demonstrate that TRAF6 is a positive regulator for NTHi-induced RIG-I upregulation.

Taken together, our data suggest that TRAF6-IKK β -p65 acts as a positive regulator of NTHi-induced RIG-I up-regulation and inflammation in airway epithelial cells (**Fig. 5B**).

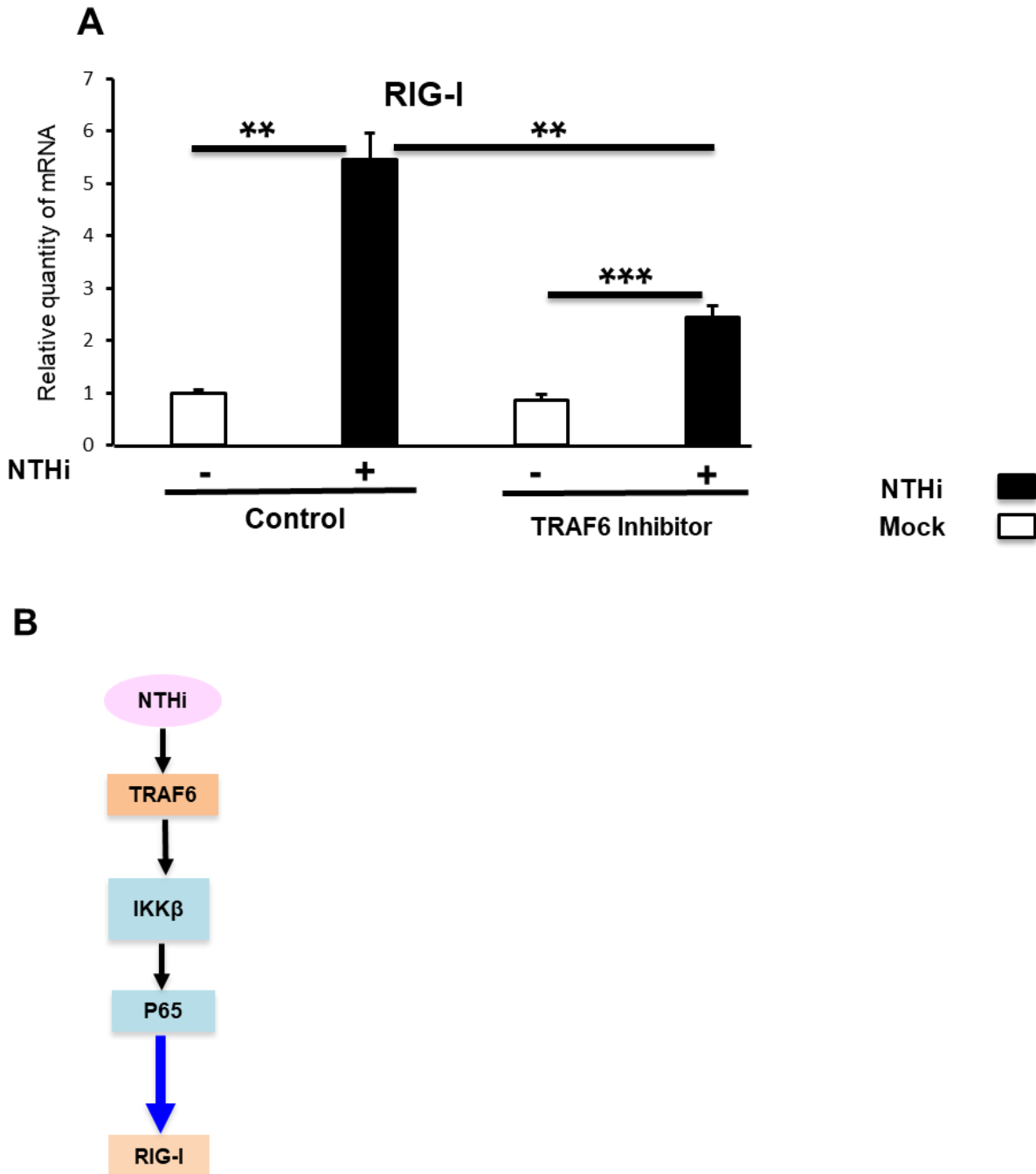


Figure 5. TRAF6 positively regulates NTHi-induced up-regulation of RIG-I

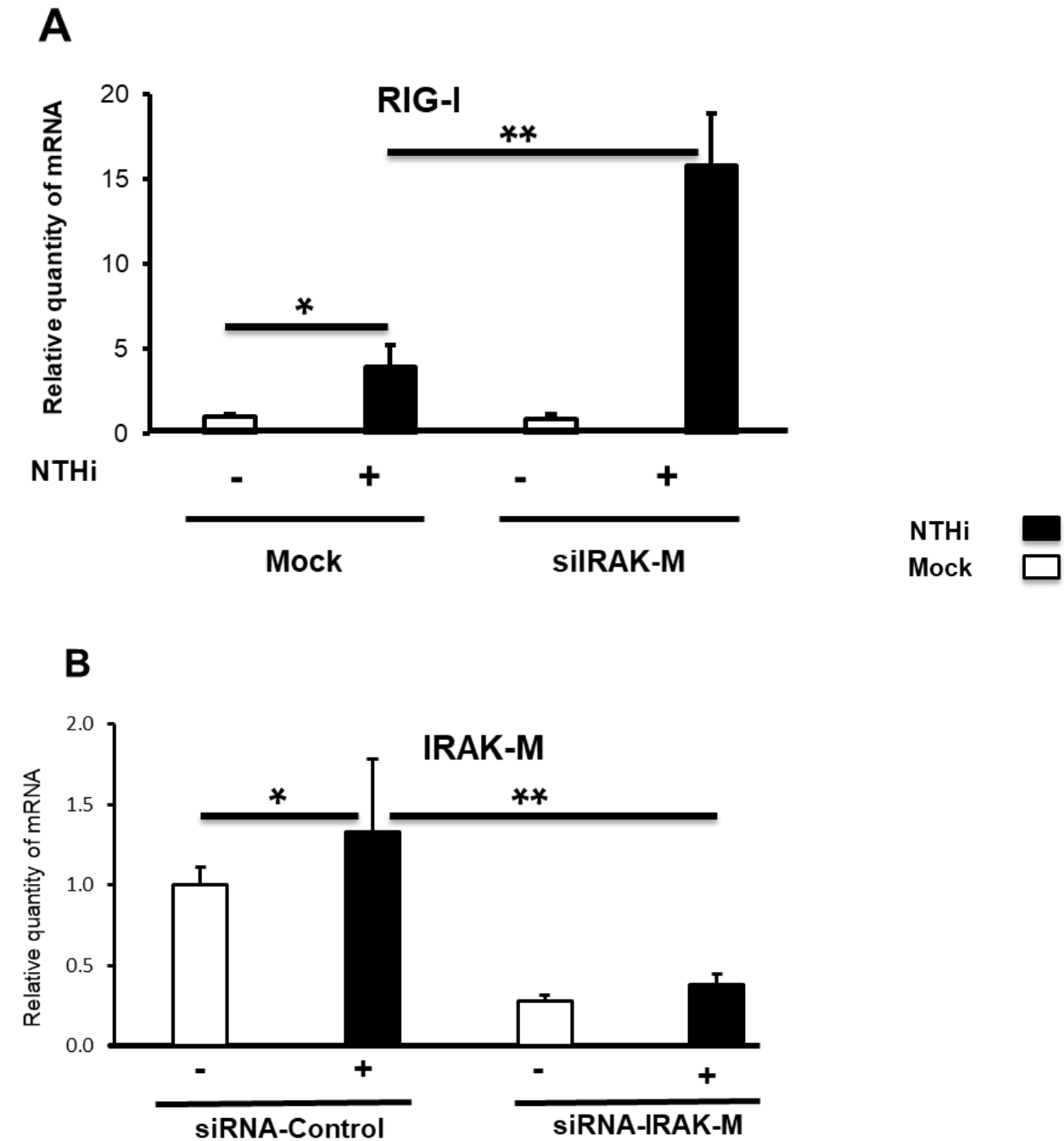
(A) BEAS-2B cells were pretreated with TRAF6 inhibitor (20 μ M) for 2 hr, followed by NTHi stimulation (MOI of 100) for 6 hrs, and mRNA expression of RIG-I was measured by Q-PCR. (B) Schematic diagram. Data are Mean \pm SD (n=3). **, $p < 0.01$; ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments.

3.5 IRAK-M acts as a negative regulator for NTHi-induced RIG-I upregulation

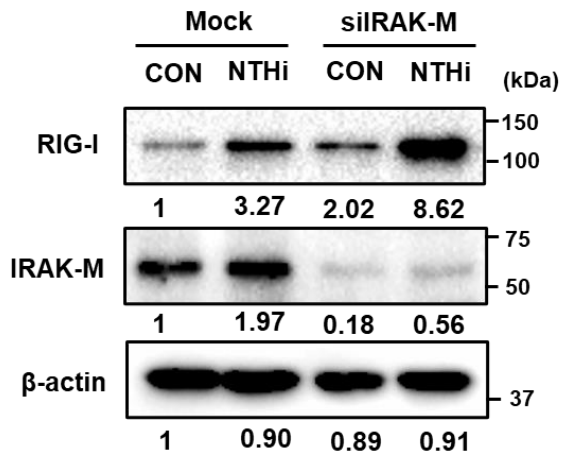
Negative feedback regulators of inflammation have been suggested to play critical roles in tightly regulating inflammatory responses to preserve homeostasis (Balaci et al., 2007; Hubbard & Moore, 2010; Miyata et al., 2015). IRAK3, also known as IRAK-M is one of the most critical negative feedback regulators of TLR family signaling via inhibition of MyD88 and IRAK1/4 activation. IRAK-M is a member of the IRAK family which consists of three conserved domains. It lacks a crucial aspartate in its kinase domain, which prevents it from having any kinase activity. Thus, IRAK-M has been identified as a competitor for IRAK1 in its interactions with MyD88 and TRAF6. IRAK-M-deficient animals exhibited increased inflammatory response in several models. Recent research has shown that IRAK-M is expressed in airway epithelial cells. Several inflammatory stimuli have been demonstrated to induce IRAK-M expression, which suppresses inflammation in a negative feedback way in multiple cell types, including epithelial cells (Bennett & Starczynowski, 2022; Hubbard & Moore, 2010; Jiang et al., 2017; Miyata et al., 2015). Since IRAK-M is a critical inducible negative regulator for NTHi-induced inflammatory responses, it is unclear if IRAK-M affects NTHi-induced RIG-I up-regulation.

We sought to determine the role of IRAK-M in NTHi-induced RIG-I up-regulation by depleting IRAK-M using IRAK-M siRNA. Interestingly, IRAK-M knockdown by siRNA markedly enhanced NTHi-induced up-regulation of RIG-I expression at both mRNA and protein levels in BEAS-2B cells (**Fig. 6A-6D**). To further determine the role of IRAK-M in NTHi-induced RIG-I up-regulation, we established IRAK-M knockdown (IRAK-M KD) BEAS-2B cells using CRISPR-Cas9 systems. As shown in **Fig. 6E & 6F**, NTHi-induced RIG-I up-regulation was significantly enhanced in IRAK-M KD BEAS-2B cells. Taken

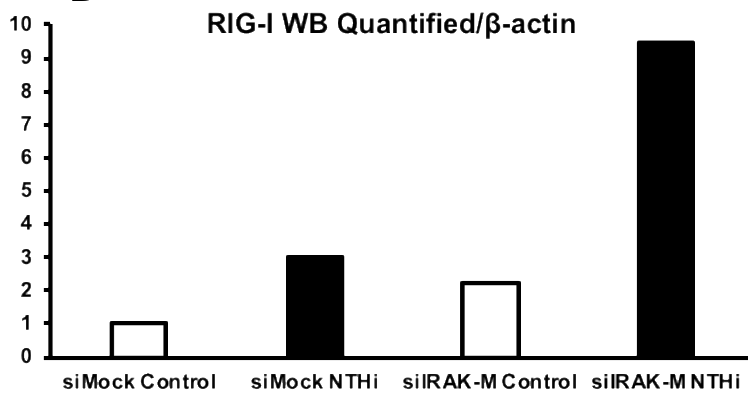
together, these data suggest that IRAK-M acts as a negative regulator for NTHi-induced RIG-I upregulation (**Fig. 6G**).



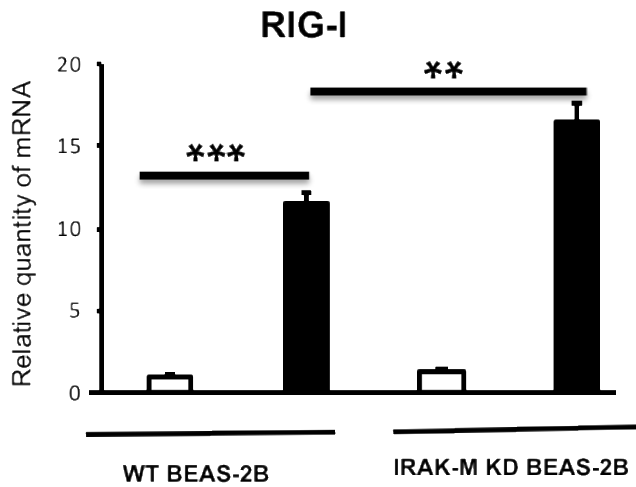
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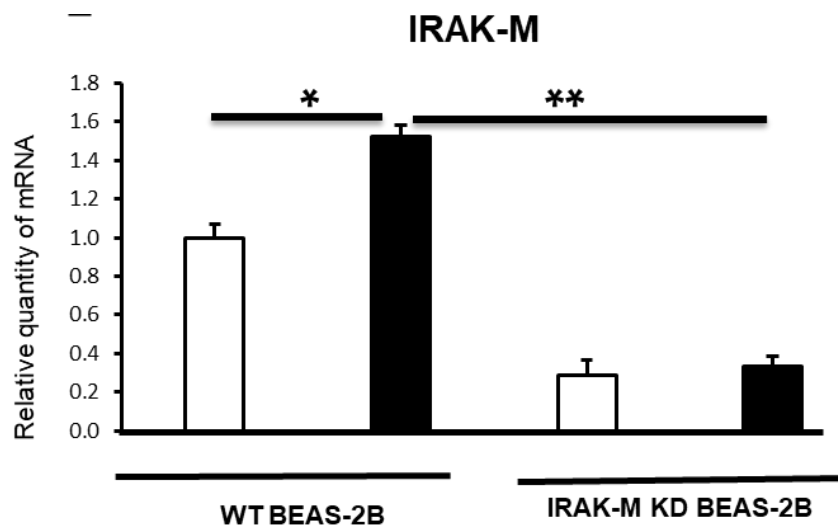
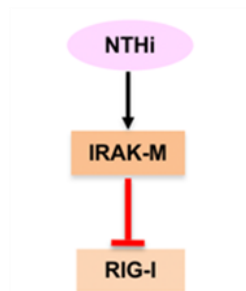
F**G**

Figure 6. IRAK-M acts as a negative regulator for NTHi-induced upregulation of RIG-I

(A-D) BEAS-2B cells transfected with control siRNA or IRAK-M siRNA were stimulated with NTHi (MOI of 100) for 6hr. (A) RIG-I and (B) IRAK-M mRNA expressions were measured by Q-PCR. (C,D) RIG-I, IRAK-M and β -actin proteins were visualized by Western Blotting. (E-F) BEAS-2B WT and IRAK-M knockdown BEAS-2B cells were stimulated with NTHi (MOI of 100) for 6 hr. (D) RIG-I and (E) IRAK-M mRNA expressions were measured by Q-PCR. (G) Schematic diagram. Data are Mean \pm SD (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments. siIRAK-M, IRAK-M siRNA; IRAK-M KD, IRAK-M knockdown.

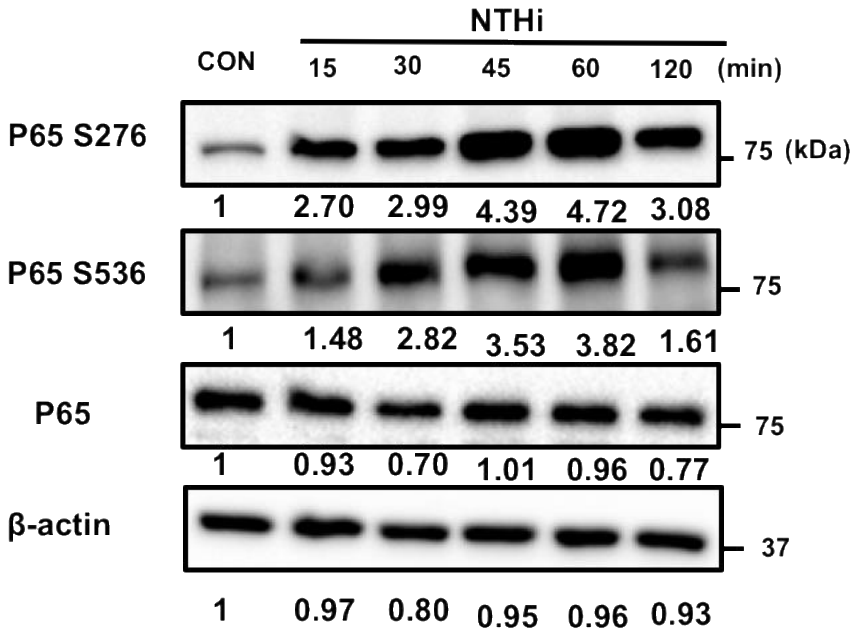
3.6 IRAK-M negatively regulates NTHi-induced up-regulation of RIG-I via inhibition of IKK β -NF- κ B p65 signaling pathway

We next sought to determine how IRAK-M negatively regulates NTHi-induced RIG-I up-regulation. Previous studies have been shown that IRAK-M suppressed TLR-mediated activation of IKK β -NF- κ B signaling pathway (Kobayashi et al., 2002). Thus, we first sought to determine if IRAK-M inhibits NTHi-induced activation of NF- κ B p65 (phosphorylation of p65 at serine 276 and serine 536 residues). As shown in **Fig. 7A-7C**, NTHi induced phosphorylation of p65 at serine 276 (S276) and serine 536 (S536) residues in a time-dependent manner. Interestingly, IRAK-M knockdown by IRAK-M siRNA significantly enhanced NTHi-induced phosphorylation of p65 at S276 and S536 residues (**Fig. 7D-7F**). These data suggest that IRAK-M negative regulates NTHi-induced activation of p65, the key positive regulator for NTHi-induced RIG-I up-regulation.

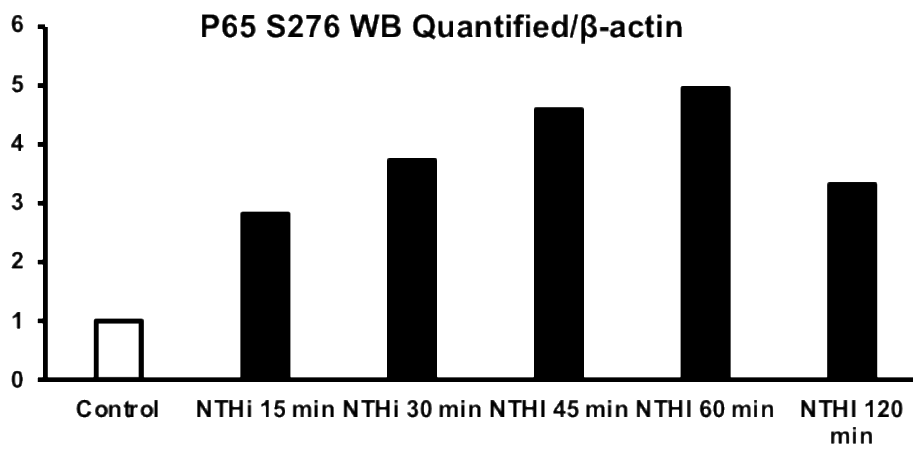
We next determined if IRAK-M negatively regulates NTHi-induced RIG-I up-regulation via targeting IKK β , the key positive regulator for NTHi-induced RIG-I up-regulation. To test our hypothesis, we determined the effect of IKK β inhibitor on NTHi-induced RIG-I up-regulation in IRAK-M-depleted BEAS-2B cells. Interestingly, IKK β inhibitor significantly suppressed IRAK-M siRNA-mediated enhancement of RIG-I mRNA and protein up-regulation induced by NTHi in BEAS-2B cells (**Fig. 7G-7I**). These data demonstrate that IRAK-M negatively regulates NTHi-induced RIG-I up-regulation via inhibition of IKK β -NF- κ B p65 signaling pathway (**Fig. 7J**).

BEAS-2B

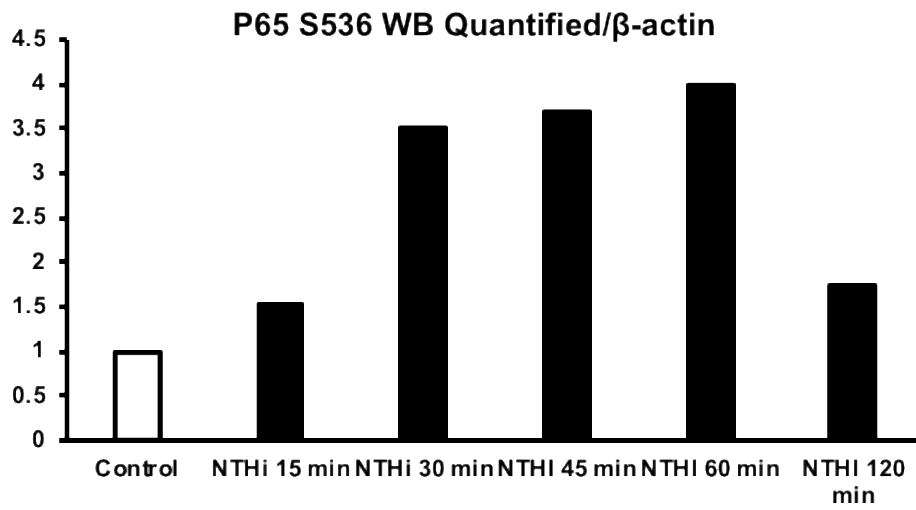
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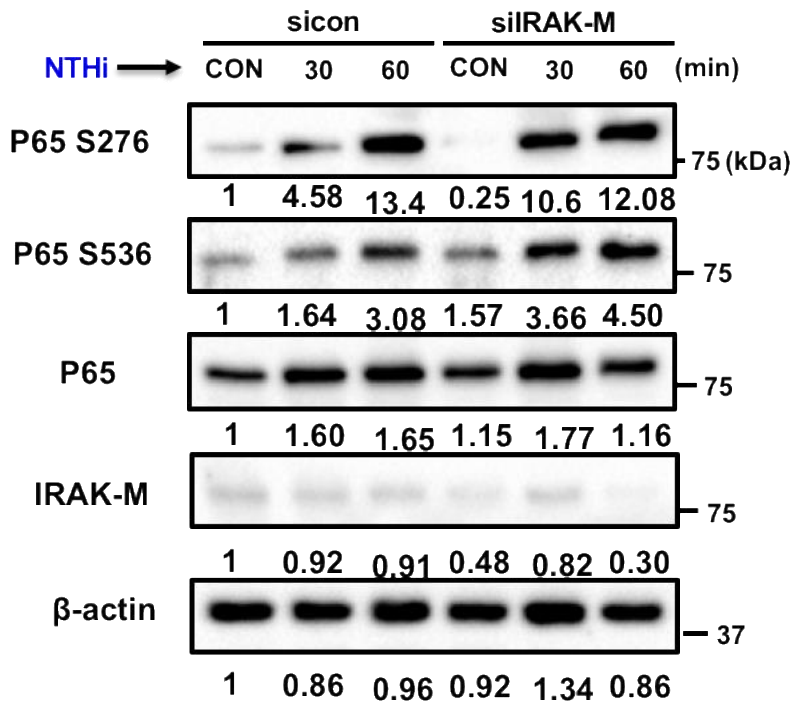


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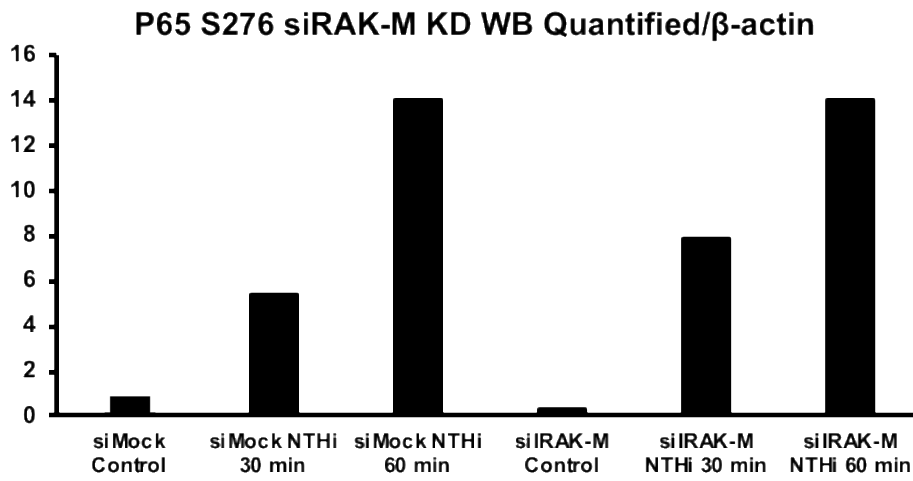


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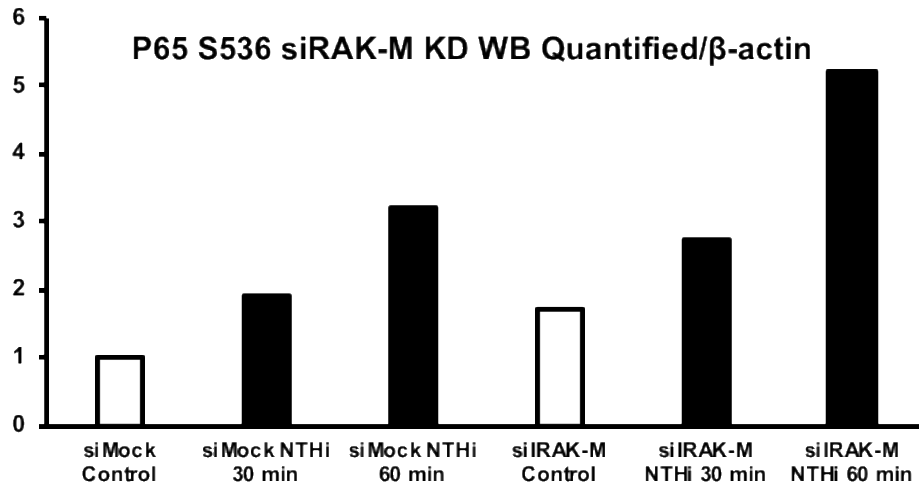
BEAS-2B

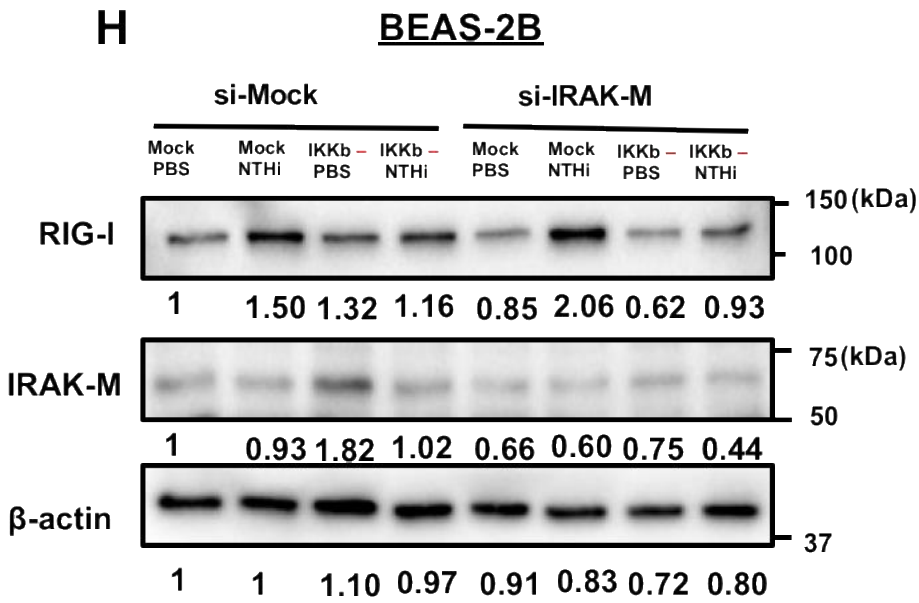
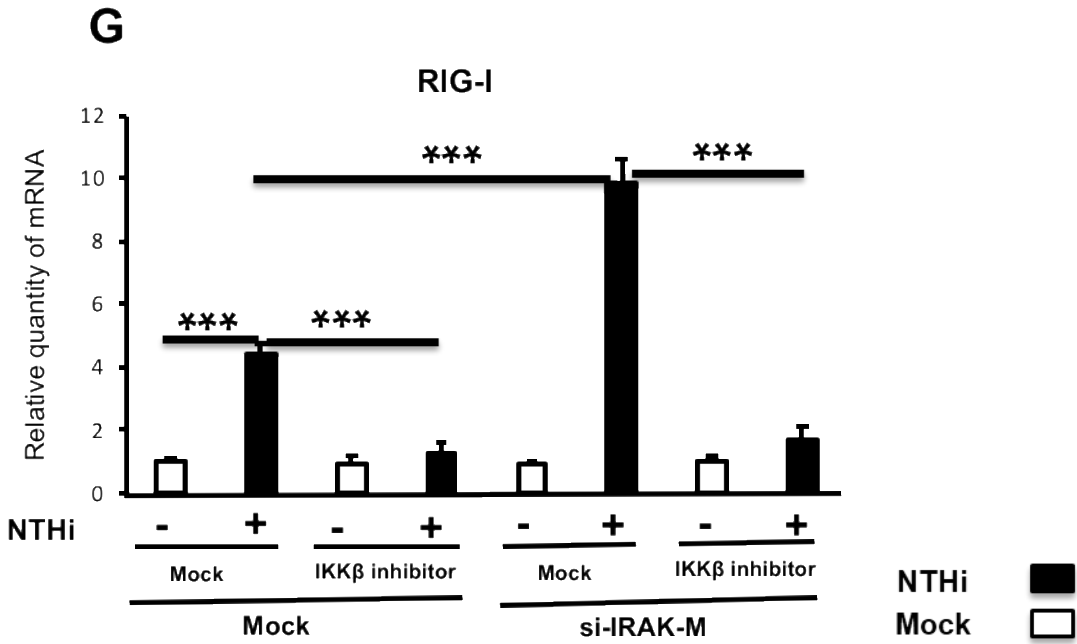


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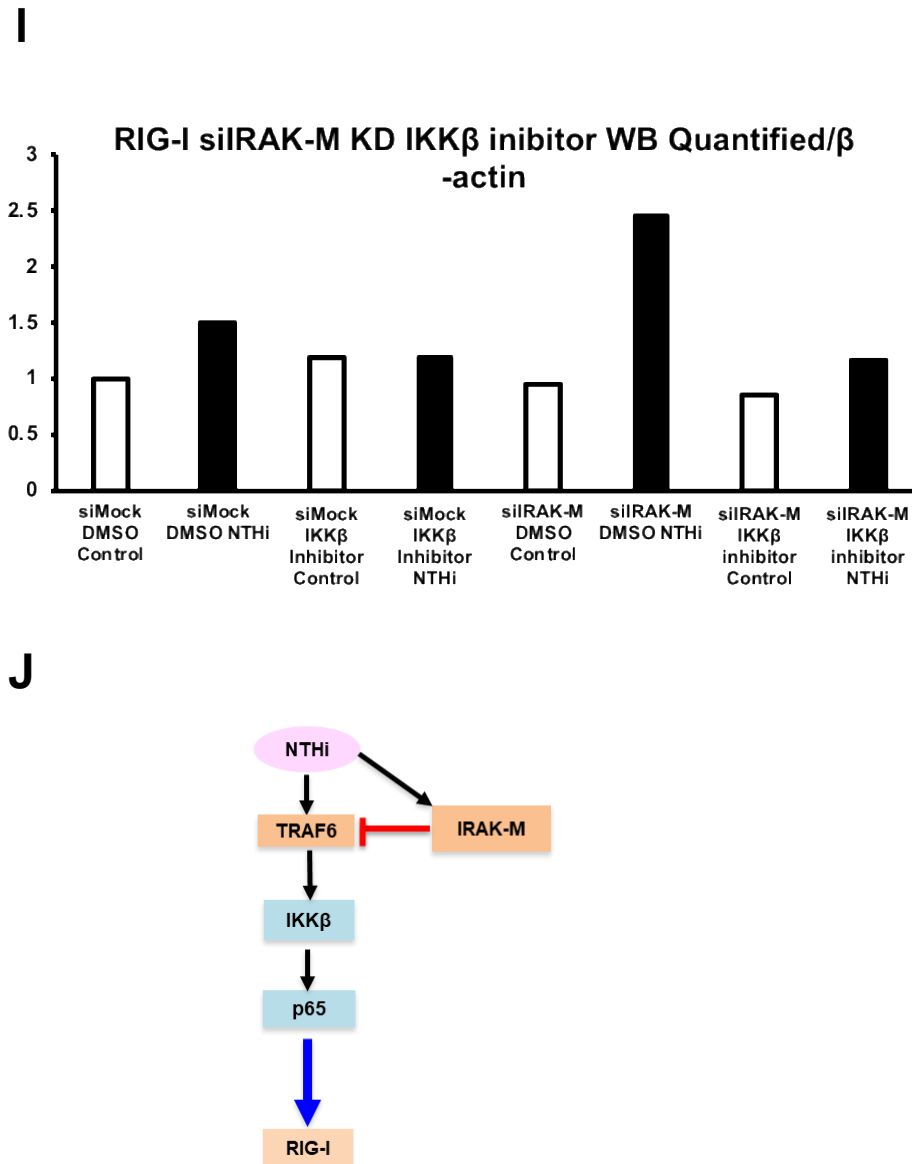
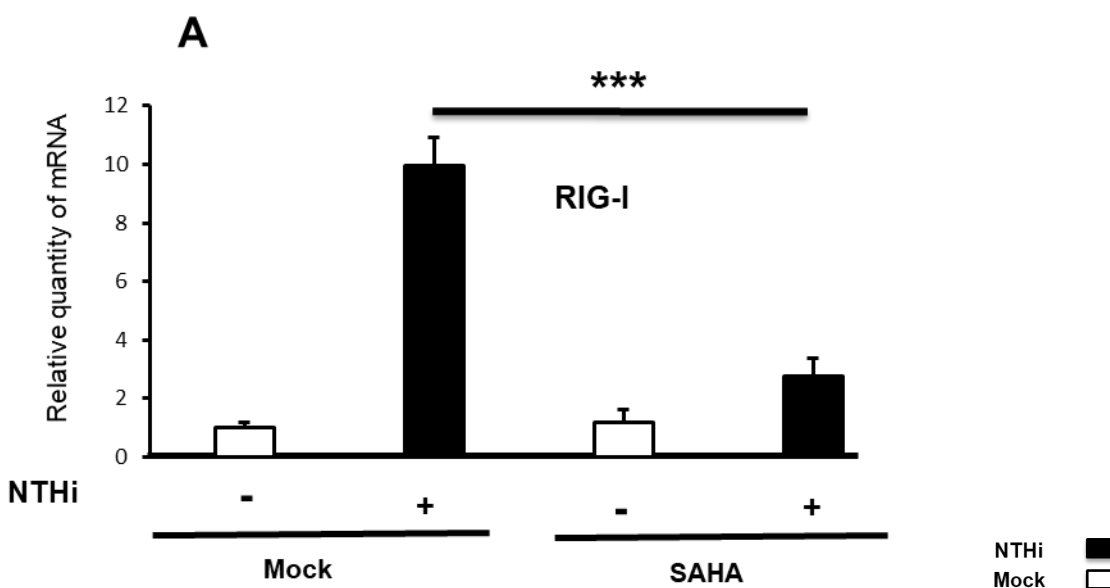


Figure 7. IRAK-M negatively regulates NTHi-induced up-regulation of RIG-I via inhibition of IKKβ-NF-κB p65 pathway

(A-C) BEAS-2B cells were stimulated with NTHi (MOI of 100) for 15, 30, 45, 60, 120 minutes. p65 S276, p65 S536, total p65 and β-actin proteins were visualized by Western Blotting. (D-F) BEAS-2B cells transfected with control siRNA or IRAK-M siRNA were stimulated with NTHi (MOI of 100) for 30 and 60 minutes. p65 S276, p65 S536, total p65, IRAK-M and β-actin proteins were visualized by Western Blotting. (G-I) BEAS-2B cells transfected with control siRNA or IRAK-M siRNA were pretreated with IKKβ inhibitor (1 μM) for 1 hr, followed by stimulation with NTHi (MOI of 100) for 6hr. (G) RIG-I mRNA expression was measured by Q-PCR. (H-I) RIG-I, IRAK-M and β-actin proteins were visualized by Western Blotting. (J) Schematic diagram. Data are Mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments. siRAK-M, IRAK-M siRNA.

3.7 HDAC acts as a positive regulator for NTHi-induced RIG-I up-regulation

HDACs are a class of enzymes that play a crucial role in the epigenetic regulation of gene expression by removing acetyl groups from histone and non-histone proteins and are frequently dysregulated in cancer cells (Gatla et al., 2017; Seto & Yoshida, 2014; Shi et al., 2017). Recent studies have demonstrated that HDAC is involved in IKK-mediated NF- κ B p65 activation (Asare et al., 2020; Ashburner et al., 2001; Gatla et al., 2017). In addition, HDAC inhibitors have been shown to inhibit IKK β -CA-mediated NF- κ B activation and induction of proinflammatory cytokines (Penzo et al., 2009). Thus, we next sought to determine whether HDAC is involved in NTHi-induced RIG-I up-regulation. Interestingly, HDAC inhibitor SAHA significantly suppressed NTHi-induced RIG-I up-regulation (**Fig. 8A**). These data suggest that HDAC act as a positive regulator for NTHi-induced RIG-I up-regulation in airway epithelial cells (**Fig. 8B**). Our future studies will focus on identifying how HDAC tightly regulates NTHi-induced RIG-I up-regulation and inflammation.



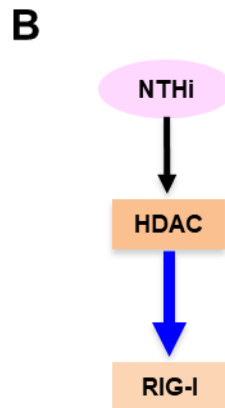


Figure 8. HDAC acts as a positive regulator for NTHi-induced RIG-I up-regulation

(A) BEAS-2B cells pretreated with SAHA (1 μ M) for 1 hr were stimulated with NTHi (MOI of 100) for 6 hr. RIG-I mRNA expression was measured by Q-PCR. (B) Schematic diagram. Data are Mean \pm SD (n=3). ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments.

3.8 Role of RIG-I upregulation in proinflammatory responses

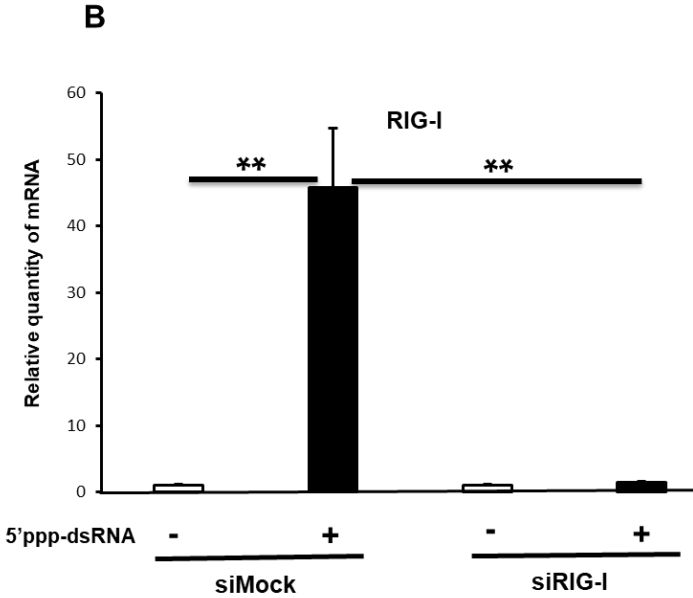
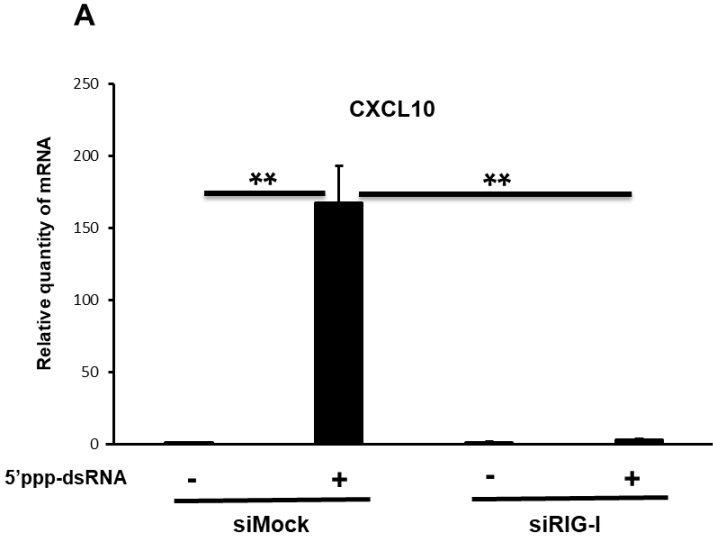
It is reported that invasive bacteria or proinflammatory cytokines promote the production of chemokines such as CXCL10 by epithelial cells that play a pivotal role in regulating inflammation (Brownell et al., 2014; Kawaguchi et al., 2009). Furthermore, CXCL10 express in viral infections such as coronaviruses, HCV, ZV, IAV, Measles virus (MeV) suggesting an essential role for this chemokine in host anti-viral response (Brownell et al., 2014; Elemam et al., 2022). CXCL10 production is directly related to RIG-I, MAVS, IRF3 activations; hence RIG-I is essential for virus and bacteria-induced CXCL10 up-regulation (Brownell et al., 2014; Elemam et al., 2022).

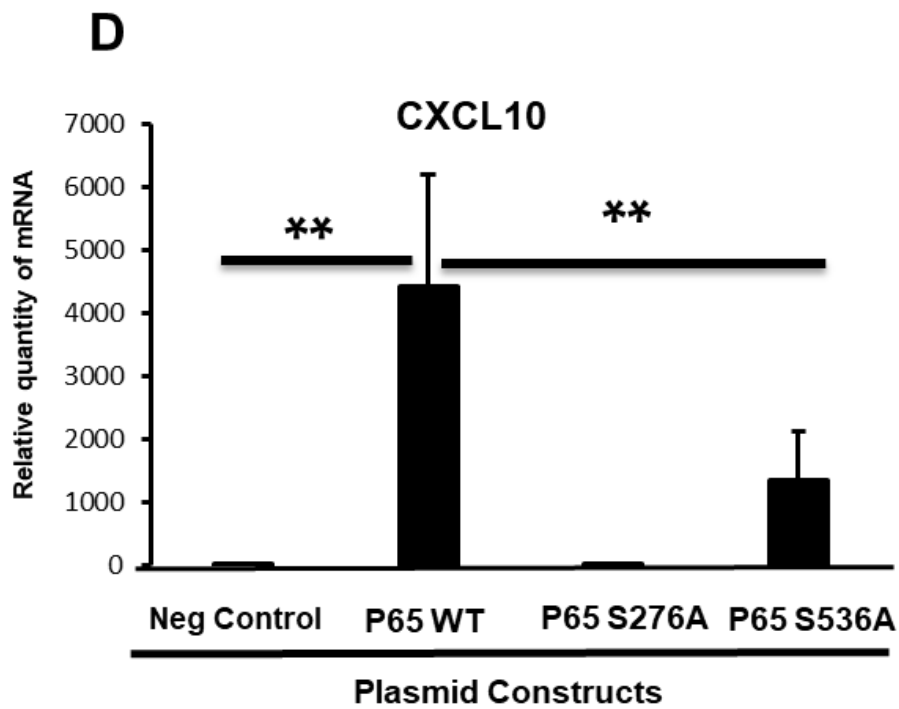
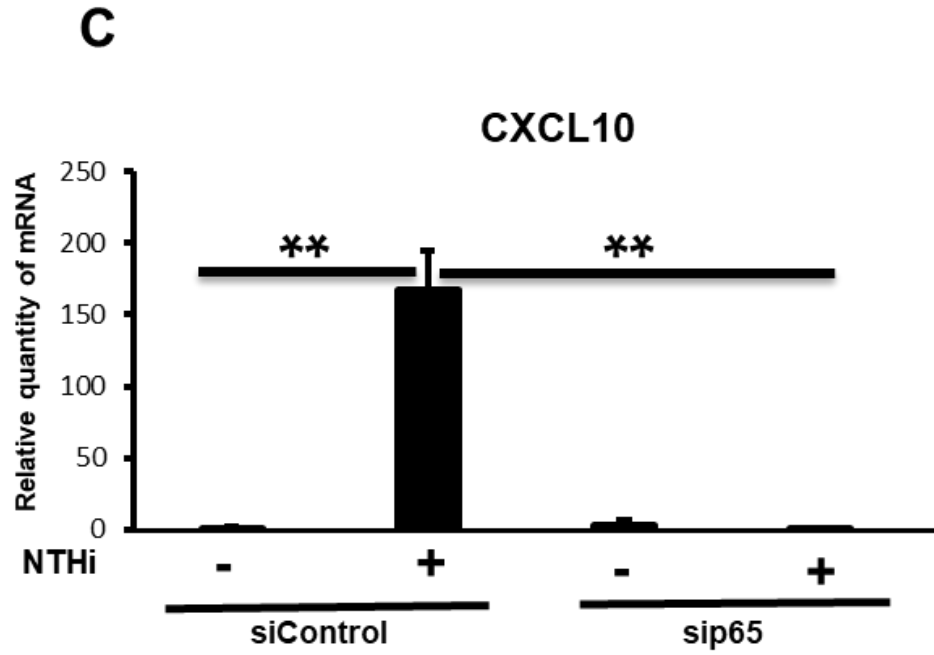
To confirm if RIG-I agonist (5'ppp-dsRNA) induces CXCL10 up-regulation via RIG-I-dependent manner in airway epithelial cells, BEAS-2B cells transfected with RIG-I siRNA were stimulated with 5'ppp-dsRNA. As shown in **Fig. 9A & 9B**, 5'ppp-dsRNA-induced CXCL10 up-regulation was significantly decreased in RIG-I-depleted BEAS-2B cells. These data suggest that RIG-I agonist induces CXCL10 up-regulation via RIG-I dependent manner in BEAS-2B cells.

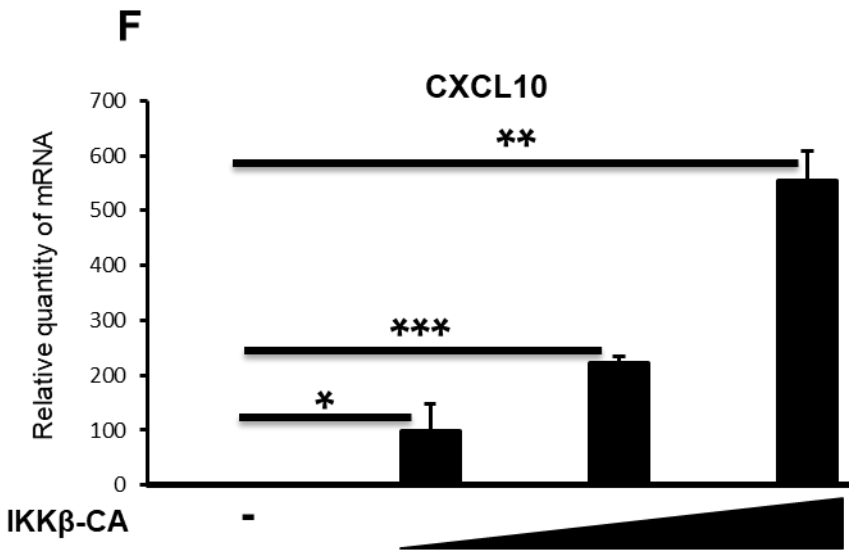
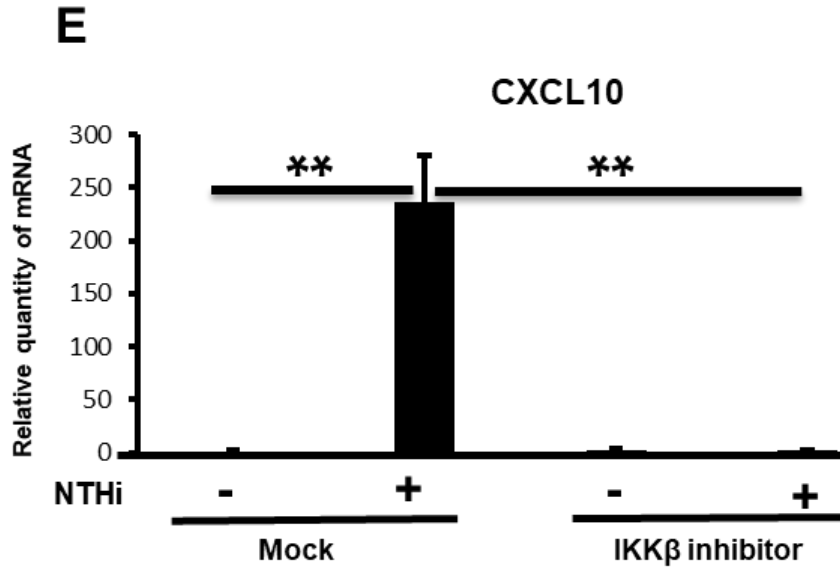
We next sought to determine if NTHi induces CXCL10 up-regulation via activation of -TRAF6-IKK β -NF- κ B p65 signaling pathway. We first determine the effect of p65 siRNA on NTHi-induced CXCL10 up-regulation in BEAS-2B cells. As shown in **Fig. 9C**, p65 knockdown by siRNA significantly suppressed NTHi-induced CXCL10 up-regulation. To evaluate the role of p65 phosphorylation at key residues including serine 276 (S276) and serine 536 (S536) in NTHi-induced CXCL10 up-regulation, we determined the effect of p65 WT and phosphorylation-deficient mutant (S276A, S536A) on NTHi-induced CXCL10 up-regulation assessed by Q-PCR. As shown in **Fig. 9D**, CXCL10 mRNA expression was

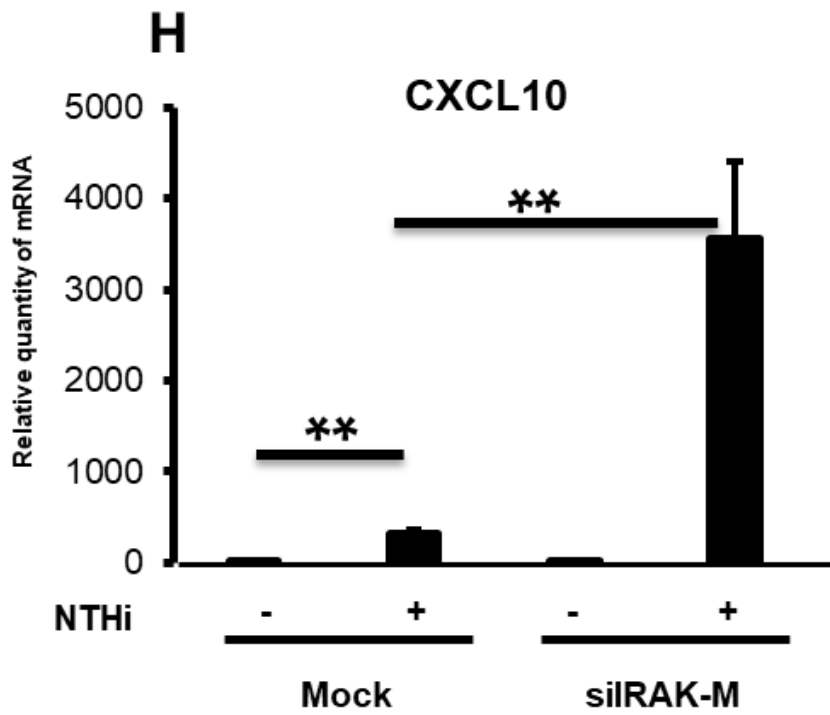
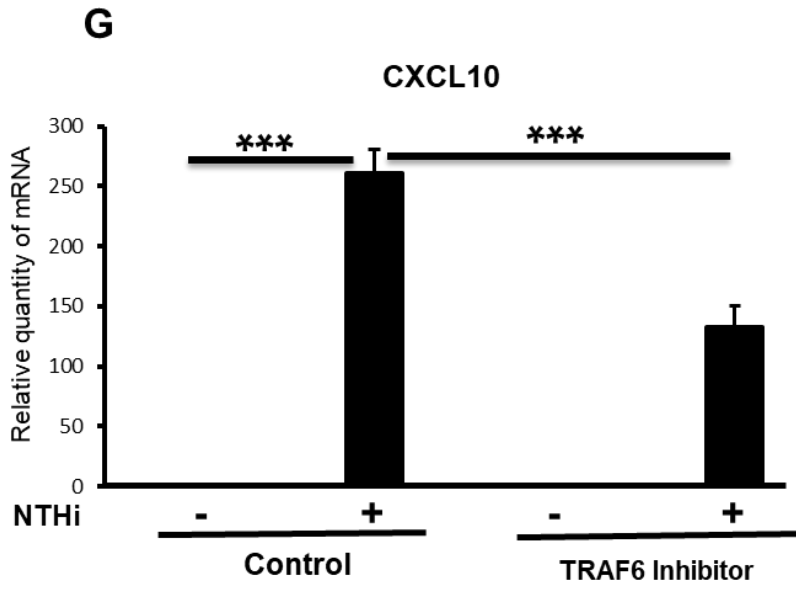
decreased in S276A- and S536A-transfected cells compared with p65 WT-transfected cells. In addition, IKK β inhibitor significantly suppressed NTHi-induced up-regulation of CXCL10 (**Fig. 9E**). Moreover, IKK β -CA induced CXCL10 up-regulation in a dose-dependent manner (**Fig. 9F**). As shown in **Fig. 9G**, TRAF6 inhibitor significantly suppressed NTHi-induced CXCL10 up-regulation. These data demonstrated that NTHi induces CXCL10 up-regulation via activation of TRAF6-IKK β -NF- κ B signaling pathways.

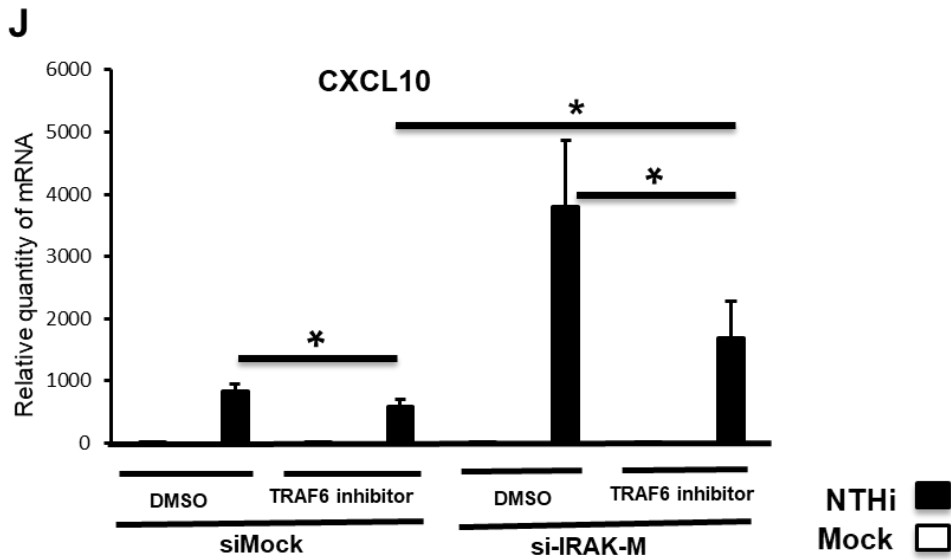
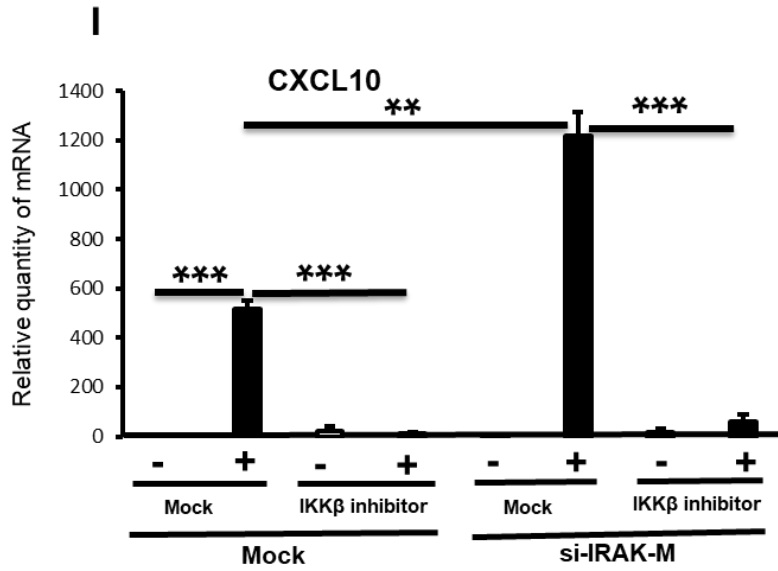
We next sought to determine the role of IRAK-M in NTHi-induced CXCL10 up-regulation by depleting IRAK-M using IRAK-M siRNA. Interestingly, IRAK-M knockdown by siRNA markedly enhanced NTHi-induced up-regulation of CXCL10 expression in BEAS-2B cells (**Fig. 9H**). We next determined if IRAK-M negatively regulates NTHi-induced RIG-I up-regulation via targeting TRAF6-IKK β , the key positive regulators for NTHi-induced RIG-I up-regulation. To test our hypothesis, we determined the effect of IKK β inhibitor and TRAF6 inhibitor on NTHi-induced CXCL10 up-regulation in IRAK-M-depleted BEAS-2B cells. Interestingly, IKK β inhibitor and TRAF6 inhibitor significantly suppressed IRAK-M siRNA-mediated enhancement of CXCL10 up-regulation induced by NTHi in BEAS-2B cells (**Fig. (9I & 9J)**). These data demonstrate that IRAK-M negatively regulates NTHi-induced CXCL10 up-regulation via inhibition of TRAF6-IKK β -NF- κ B p65 signaling pathway (**Fig. 9K**).











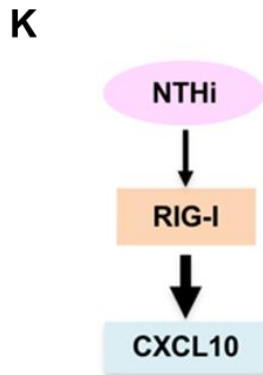


Figure 9. Role of RIG-I upregulation in proinflammatory responses

(A-B) BEAS-2B cells transfected with control siRNA or RIG-I siRNA were stimulated with 5'ppp-dsRNA (100 ng/mL) for 48 hours. (A) CXCL10 and (B) RIG-I mRNA expressions were measured by Q-PCR. (C) BEAS-2B cells transfected with control siRNA or p65 siRNA were stimulated with NTHi (MOI of 100) for 6hr. CXCL10 mRNA expressions were measured by Q-PCR. (D) BEAS-2B cells were transfected with p65 WT, p65 S276A, p65 S536A or negative control. After 24hr, CXCL10 mRNA expression was measured by Q-PCR. (E) BEAS-2B cells pretreated with IKK β inhibitor (1 μ M) for 1 hr were stimulated with NTHi (MOI of 100) for 6hr. CXCL10 mRNA expression was measured by Q-PCR. (F) BEAS-2B cells were transfected with IKK β -CA (0.05 μ g, 0.1 μ g or 0.2 μ g) or empty vector. After 40hr, CXCL10 mRNA expression was measured by Q-PCR. (G) BEAS-2B cells pretreated with TRAF6 inhibitor (20 μ M) for 2 hr were stimulated with NTHi (MOI of 100) for 6 hrs. CXCL10 mRNA expression was measured by Q-PCR. (H) BEAS-2B cells transfected with control siRNA or IRAK-M siRNA were stimulated with NTHi (MOI of 100) for 6hr. CXCL10 mRNA expression was measured by Q-PCR. (I) BEAS-2B cells transfected with control siRNA or IRAK-M were pretreated with IKK β inhibitor (1 μ M) for 1 hr, followed by stimulation with NTHi (MOI of 100) for 6hr. CXCL10 mRNA expression was measured by Q-PCR. (J) BEAS-2B cells transfected with control siRNA or IRAK-M siRNA were pretreated with TRAF6 inhibitor (20 μ M) for 2 hr, followed by stimulation with NTHi (MOI of 100) for 6hr. CXCL10 mRNA expression was measured by Q-PCR. (K) Schematic diagram. Data are Mean \pm SD (n=3) *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, t-test. Data are representative of three or more independent experiments. siRIG-I, RIG-I siRNA; sip65, p65 siRNA; IKK β -CA, the constitutively active form of IKK β ; siIRAK-M, IRAK-M.

4. OVERALL SIGNIFICANCE AND DISCUSSION

The regulatory mechanisms of innate immunity are sophisticated and complex. RIG-I is an important PRR that senses viral infection and activates antiviral and proinflammatory cytokine defenses, limiting viral replication and increasing infection resistance (Coch et al., 2017; Le Goffic et al., 2007; Zhu et al., 2017). Recent work has also shown that RIG-I directly inhibits viral replication independent of antiviral signaling (Kell & Gale, 2015; Radzikowska et al., 2021). RIG-I role in inflammation evolved as a mechanism to prevent the viral spread and induce a robust proinflammatory immune response, thus linking innate antiviral immunity with inflammatory signaling and responses that control virus infection (Bogefors et al., 2011; Rehwinkel & Gack, 2020; Zhu et al., 2017). Even though acute inflammation is essential for eliminating these pathogens, an overactive inflammatory response can cause tissue damage, as seen in several chronic inflammatory conditions (Charo & Ransohoff, 2006; Nathan & Ding, 2010; Segel et al., 2011). Respiratory viral infections frequently contribute to the development of bacterial co-infection, especially in immunocompromised patients. NTHi is known to coexist with viral counterparts in bacteria-viral upper respiratory co-infections (Beadling & Slifka, 2004; Feldman & Anderson, 2021). In this study, we demonstrated that NTHi stimulation in airway epithelial cells leads to up-regulation of RIG-I in a time and dose-dependent manner, which validates earlier clinical studies where RLR receptors were implicated in inflamed upper respiratory tract tissues (Bogefors et al., 2011; Kim et al., 2010; Zhang et al., 2016). Earlier studies have established that RIG-I is a central regulator of viral-induced expression of antiviral cytokines in human lung epithelial cells (Coch et al., 2017; Kandasamy et al., 2016; Kim, 2022; Le Goffic et al., 2007; Marx et al., 2022);

however, it is still unexplored if the virus and bacterial co-infection utilize RIG-I or bacteria altered RIG-I expression in the progression of viral-bacterial co-infections. Here, our data indicate RIG-I's significant role in NTHi-stimulated airway epithelium. Also, airways epithelial cells are critical regulators of immune homeostasis as they are the first cells to encounter an invading pathogen (Kim, 2022; Le Goffic et al., 2007). Therefore, these data also exhibit the critical role that airway epithelial cells play in the tight regulation of inflammation and immune homeostasis. However, 2D cell culture is a monolayer of cells, above phenomenon can be further explored using organoids and 3D cell culture, as data from 3D cell culture mimic more in tune with in vivo models relatively than 2D cell culture (Jensen & Teng, 2020).

It is well known that RIG-I is localized in the cytoplasm in resting stage (Kell & Gale, 2015) It would be interesting to further investigate where RIG-I is localized at subcellular level using confocal imaging upon NTHi stimulation .

We also investigated the signaling pathways that regulate NTHi-induced RIG-I expression. NF- κ B is a prominent family of transcription factors that control the expression of many inflammatory mediators. Two key upstream molecules participating in the regulation of NF- κ B canonical pathway activation are the IKK β and TRAF6 (Bonizzi & Karin, 2004; Oeckinghaus & Ghosh, 2009). We found that NTHi-induced upregulation of RIG-I downstream signaling in airway epithelium is positively regulated via the TRAF6-IKK β -p65 pathway, which highlighted the importance of the canonical NF- κ B pathway in NTHi-induced RIG-I signaling upregulation. Many studies have confirmed that the degradation of I κ B α and nuclear translocation of NF- κ B are insufficient to promote maximal NF- κ B transcriptional activity. Instead, the NF- κ B complex must undergo

additional post-translational modification involving site-specific phosphorylation (Bonizzi & Karin, 2004; Li et al., 1999; Oeckinghaus & Ghosh, 2009). The p65 subunit of NF- κ B is a principal phosphorylation target by various kinases. Ser 276 and Ser 536 are the most physiologically inducible phosphorylation sites for p65 seen during NTHi infection (Susuki-Miyata et al., 2015). The phosphorylation of Ser 276 is mediated by catalytic protein kinase A subunit or mitogen- and stress-activated protein kinase 1; in addition, Serine 536 is phosphorylated by the IKK complex (Arun et al., 2009; Yang et al., 2003). Here, we demonstrated that NF- κ B P65 phosphorylation at sites Ser276 and Ser536 might be critical for NTHi-induced RIG-I and downstream cytokines and chemokines such as TNF- α and CXCL10. Our study also demonstrated that activation of IKK β is not only required for NTHi-induced RIG-I upregulation but also sufficient for up-regulating RIG-I downstream signaling.

It is imperative to keep inflammatory pathways tightly regulated, making negative feedback regulators of inflammation a key regulator in tight control of inflammation homeostasis (Lee et al., 2016; Miyata et al., 2015). Earlier studies have shown that IRAK-M KO mice presented with significantly higher IFN- γ concentration and increased percentage of Th1 cells in the lung tissue than wild-type mice, which is in tune with clinical findings where the expression of IRAK-M was upregulated in peripheral blood leukocytes in asthmatic children, and high mRNA expression of IRAK-M in sputum was a marker for frequent exacerbations of asthma (Balaci et al., 2007; Bennett & Starczynowski, 2022; Jiang et al., 2017; Yansheng Liu et al., 2016).

Here, we have shown that IRAK-M acts as a negative regulator of NTHi-induced RIG-I upregulation and downstream signaling. In further exploration, we found IRAK-M

inhibits NTHi-induced RIG-I downstream signaling upregulation via the TRAF6-IKK β -p65 pathway. Therefore, this data demonstrates the significant role that IRAK-M plays in the tight regulation of NTHi-induced inflammation in airway epithelial cells.

Collectively, these data identify the positive and negative regulatory signaling pathways that control NTHi-induced RIG-I expression in airway epithelial cells.

It is well known that RIG-I triggers type I and III IFN via IRF-3,7 activation (Le Goffic et al., 2007; Rehwinkel & Gack, 2020; Wang et al., 2007); In the present study, we confirmed that RIG-I induced CXCL10 expression to initiate and progress innate immunity. It is known that CXCL10 plays a pivotal role in regulating inflammation in epithelial cells (Brownell et al., 2014). Here we show that RIG-I mediates CXCL10 mRNA expression, and CXCL10 expression is significantly abolished by the knockdown of RIG-I, which confirms that RIG-I functionally employs chemokine CXCL10 to induce an innate immune response and antiviral effects. We further showed that RIG-I-mediated CXCL10 expression was positively controlled by the TRAF6-IKK β -p65 pathway, which emphasized the importance of the NF- κ B canonical pathway in regulating CXCL10 via RIG-I. Also, RIG-I-mediated CXCL10 expression was negatively controlled by IRAK-M. Interestingly, IRAK-M acts as a negative regulator to RIG-I-mediated CXCL10 via the TRAF6-IKK β -p65 pathway. Hence, our data confirmed that CXCL10 expression is mediated via RIG-I and shows the functional significance of RIG-I.

It is reported earlier that HDACs activate IKKs and p65 (Ashburner et al., 2001; Gatla et al., 2017). Also, HDAC inhibitors have been shown to inhibit IKK β -CA-mediated NF- κ B activation and induction of proinflammatory cytokines (Penzo et al., 2009). We also found that HDACs act as a positive regulator in NTHi-induced RIG-I and RIG-I

downstream chemokine CXCL10 mRNA expression. However, the role of specific HDACs needs to be explored in NTHi-induced RIG-I downstream signaling. As HDACs activate IKKs and p65, hence it also complicates exploring the role of HDACs' interaction with a negative regulator such as IRAK-M in NTHi-induced RIG-I signaling by blocking HDAC non-specifically by using commercially available HDAC inhibitors. Most commonly available HDAC inhibitors are relatively nonspecific to HDAC subtypes (Komatsu et al., 2006; Shi et al., 2017); hence to explore the role of specific HDAC in NTHi-induced inflammation, specific HDAC subtype knockdown using siRNA should be performed.

Besides, molecular regulation of NTHi, clinically, it is well known that NTHi co-infects with other respiratory viruses, such as IAV, RSV, and Rhinoviruses (Beadling & Slifka, 2004; Feldman & Anderson, 2021), hence NTHi co-infection with common URT viruses, can be tried to check whether viral-bacterial co-infection affects RIG-I signaling and if they alter host antiviral response and animal survival rate. The effect of virus infection on bacterial co-infection is well studied. In contrast, the effect of bacterial infection on viral co-infection remains less clear and thus needs to be further explored. This study points in the direction that deciphering the signaling pathways that control the regulatory molecules of inflammation is critical for developing effective therapeutics in NTHi-induced inflammation.

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6. VITAE

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Summary

- A highly motivated biomedical professional with 10+ years of instructing and research experience in innate immunity, chemotherapy, systemic pharmacology, physiology as demonstrated by 5 publications
- Pioneered placement of antibiotics prescription policy in perioperative cases at the institutional level and 1 presentation at national conference by analyzing perioperative data in Cholecystectomy with 4 Physicians
- Communicated and built relationships with 100 HCPs and KOLs to monitor compliance, reporting and management of new ADR, resulting in creating an ADR reporting training program at the institutional level and 1 publication

Work Experience

Project management and Innovation:

Gained as a Graduate research assistant at Georgia State University (GSU):

- Initiating discussions with 10+ academic KOLs to design and perform scientific experiments in host innate immune response to upper respiratory infections

- Created weekly scientific presentations and spearheaded project to advance therapeutic research focused on the inflammation in NTHi and viral agonist induced respiratory infections
- Analyzed data on projects involving bacterial and viral agonist induced inflammation in upper respiratory infections, to expand immunology knowledge

Leadership and Subject matter expertise:

Gained as an assistant Professor and Instructor at Punjab Institute of Medical Sciences, India:

- Coordinated departmental activities and prompted faculty development through weekly seminars, resulting in an active research environment and 3 research publications
- Managed student attrition and retention by analyzing feedback from periodic student meeting and heading the development and editing of student curriculum
- Trained, mentored, graded 450+ medical students on clinical, systemic, basic pharmacology, and clinical case presentations, leading to a 90% success rate in their graduation to medical residencies and doctoral programs

Partnership, Compliance and Cost mitigation:

Gained as a graduate research assistant at GSU, Biology and IBMS:

- Coordinated, collaborated, consolidated project to advance therapeutic research involving how oxidative stress affects the metabolic, apoptotic and autophagy genes in yeast experimental model concluded into thesis research

- Maintained up-to-date record on cybersecurity, security awareness, hazardous waste material safety, biomedical waste safety, pathogen safety via in-person and virtual training
- Effectively reduced cost with an alternative plan for experiment while ensuring project integrity to support master thesis
- Managed data backup system for published works and unpublished data and supported Biology Core facility at the GSU, by optimization, maintenance in *Flow Cytometry, Western Blot apparatus, PCR thermal cyclers* etc.

Academic Qualification

Georgia State University, Atlanta, GA

- PhD Candidate in Translational Biomedical Sciences with focus on bacteria induced host innate immunity, signaling transduction, graduating in 04/2023
- Two MS; Medical pharmacology, Manipal University, India and Molecular biology, GSU

Technical skills

Cardiac Puncture	Analgesic tests:	Western Blot
Oral Gavage	Tail Flick Method,	Flow Cytometry
Intra-peritoneal Injection	Hot Plate method	Microarray data analysis
Force swim test	DNA extraction	Cell culture
Tail suspension test	RNA extraction	
Electrical induced seizures	Electrophoresis	
PTZ induced seizures	Quantitative PCR	

Publications

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Accolades and Achievements

- Presented a research paper on title "Rationality of Perioperative Antibiotic Prophylaxis in Elective Laparoscopic Cholecystectomy" at a national conference at the Subharti medical college, India
- Certification in Project management from Walden University in 12/2021
- Certification and knowledge on ethical obligation in pharmacological research at a workshop on pharmacovigilance and Ethics in Clinical Trials in 2011 at Manipal University
- Certification in situ drug designing from IBI Biosolutions Pvt. Ltd, Chandigarh, India in 2009
- Volunteered as a Hostel manager at PIMS, India from 2011 to 2015
- Volunteered as a Project coordinator for a wellness initiative 2022 to 2023
- Nature explorer, volunteered as a yoga instructor