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Synergistic Activation of NF- κ B by Nontypeable *H. influenzae* and *S. pneumoniae* Is Mediated by CK2, IKK β -I κ B α and p38 MAPK

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Abstract

In review of the past studies on NF- κ B regulation, most of them have focused on investigating how NF- κ B is activated by a single inducer at a time. Given the fact that, in mixed bacterial infections *in vivo*, multiple inflammation inducers, including both Nontypeable *Haemophilus influenzae* (NTHi) and *S. pneumoniae*, are present simultaneously, a key issue that has yet to be addressed is whether NTHi and *S. pneumoniae* simultaneously activate NF- κ B and the subsequent inflammatory response in a synergistic manner. Here, we show that NTHi and *S. pneumoniae* synergistically induce NF- κ B-dependent inflammatory response via activation of multiple signaling pathways *in vitro* and *in vivo*. The classical IKK β -I κ B α and p38 MAPK pathways are involved in synergistic activation of NF- κ B via two distinct mechanisms, p65 nuclear translocation-dependent and independent mechanisms. Moreover, casein kinase 2 (CK2) is involved in synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. These studies bring new insights into the molecular mechanisms underlying the NF- κ B-dependent inflammatory response in polymicrobial infections and may lead to development of novel therapeutic strategies for modulating inflammation in mixed infections for patients with otitis media and chronic obstructive pulmonary diseases.

Keywords

Synergistic activation; NF- κ B; CK2; Polymicrobial infection

Gram-negative bacterium Nontypeable *Haemophilus influenzae* (NTHi) and Gram-positive bacterium *Streptococcus pneumoniae* (*S. pneumoniae*) are important human pathogens [1,2].

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In children, they cause otitis media (OM), the most common childhood infection and the leading cause of conductive hearing loss (3), while in adults, they exacerbate chronic obstructive pulmonary diseases (COPD), the fourth leading cause of death in the United States [1,2]. Although a majority of OM or COPD is mainly associated with a single bacterial pathogen, there is a growing body of evidence that a portion of patients diagnosed with OM or COPD have mixed infections of NTHi and *S. pneumoniae* [4,5]. Like most other bacterial infections, both OM and COPD are also characterized by inflammation, which is mainly mediated by inflammatory cytokines and chemokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-8 (IL-8) [6,7]. Among a variety of transcription regulators, nuclear factor κ B (NF- κ B) has been shown to play a critical role in regulating the expression of large numbers of genes encoding cytokines, chemokines and other mediators involved in inflammatory responses [8,9].

Over the past two decades, tremendous efforts have been made toward understanding how NF- κ B is activated by a variety of inducers including bacteria, virus and cytokines. However, in review of the past studies on NF- κ B regulation, most of them have focused on investigating how NF- κ B is activated by a single inducer at a time. Given the fact that, in mixed infections *in vivo*, multiple inflammation inducers, including both NTHi and *S. pneumoniae*, are present simultaneously, a key issue that has yet to be addressed is whether NTHi and *S. pneumoniae* simultaneously activate NF- κ B and the subsequent inflammatory response in a synergistic manner.

In the present study, we report that NTHi and *S. pneumoniae* synergistically induce NF- κ B-dependent inflammatory response via multiple signaling pathways *in vitro* and *in vivo*. The classical IKK β -I κ B α and MAPK p38 signaling pathways are involved in synergistic activation of NF- κ B via p65 nuclear translocation-dependent and -independent mechanisms. Moreover, CK2 is involved in synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. These studies bring novel insights into the molecular mechanisms underlying the synergistic activation of NF- κ B in polymicrobial infections and may help to identify novel therapeutic targets for treating patients with otitis media and chronic obstructive pulmonary diseases.

MATERIALS AND METHODS

Reagents

CK2 inhibitor, SB203580, and MG132 were purchased from Calbiochem (La Jolla, CA)

Bacterial Strains and Culture Condition

NTHi strain 12 and *S. pneumoniae* strain 6B were used in this study. Bacteria were grown on chocolate agar at 37°C in an atmosphere of 5% CO₂. NTHi crude extracts were used as described [10]. For making *S. pneumoniae* crude extracts, *S. pneumoniae* were harvested from a plate of chocolate agar after overnight incubation and incubated in 100 ml of Todd Hewitt broth and yeast extracts. After overnight incubation, *S. pneumoniae* was centrifuged at 10,000 $\times g$ for 10 min, and the supernatant was discarded. The resulting pellet of *S. pneumoniae* were suspended in 10 ml of phosphate-buffered saline and sonicated. Subsequently, the lysates were collected and stored at -70°C.

Cell Culture

Human epithelial cell lines HeLa, A549 and HMEEC-1 and primary airway epithelial NHBE cells were maintained as described [7,10,11] and used for all experiments unless otherwise indicated. All mouse embryonic fibroblast (MEF) cells were maintained as

described [12]. Wild type (WT), IKK $\alpha^{-/-}$, and IKK $\beta^{-/-}$ MEFs were kindly provided by Dr. I. Verma.

Real-time Quantitative PCR Analysis of TNF- α , IL-1 β , and IL-8

TRIzol® Reagent (Invitrogen) by following the manufacturer's instruction. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. Briefly, the reverse transcription reaction was performed for 60 min at 37°C, followed by 60 min at 42°C by using oligo(dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix for human TNF- α , IL-1 β and IL-8 as described previously [12].

Plasmids, Transfection, and Luciferase Activity Assays

Expression plasmids I κ B α (S32/36A), IKK α (K44M), IKK β (K49A), fp38 α (AF) and fp38 β 2(AF) have been described previously [7,10]. The reporter construct NF- κ B luc was generated as described [10]. It contains three copies of the NF- κ B site from IL-2 receptor promoter by using following oligonucleotides: 5'-T C G A G A C G G C A G G G A A T C T C C C T C T C C G - 3' and 3' - CTGCCGTCCCCTTAGAGGGAGAGGCAGCT-5'. All transient transfections were carried out in triplicate using a TransIT-LT1 reagent from Mirus (Madison, WI) following the manufacturer's instructions. At 40 h after starting the transfection, cells were pretreated with or without chemical inhibitors including CK2 inhibitor, MG132 and SB203580 for 1 h. NTHi or *S. pneumoniae* were then added to the cells for 5 h before cell lysis for luciferase assay. Luciferase activity was normalized with β -galactosidase activity.

Western Blot Analysis

Western blot analysis was performed as described [11]. Antibodies against phospho-p38, p38, phospho-I κ B α , total I κ B α , phospho-p65 (S536) and phospho-p65 (S276) were purchased from Cell Signaling Technology. Antibodies against p65 and TFIIB were purchased from Santa Cruz Biotechnology. Antibody against β -actin was purchased from Sigma. Antibody against CK2 was purchased from BD biosciences.

Immunofluorescent Staining

Immunofluorescent staining was performed as described [7,10 and Supplementary Material].

Nuclear Protein Extraction and Electrophoretic Mobility-Shift Assay (EMSA) and Super Shift Assay

Nuclear protein extraction and EMSA were performed as described [13 and Supplementary Material].

RNA-mediated Interference

RNA-mediated interference for down-regulating CK2 expression was done using SiRNA-CK2 β (Dharmacon) as described [11,12 and Supplementary Material].

CK2 Kinase Assay

(Supplementary Material).

Mouse and Animal Experiments

BALB/c mice were purchased from Charles River Laboratories, and all animal experiments were approved by the Institutional Animal Care and Use Committee at House Ear Institutes and University of Rochester. Under the anesthesia, mice were intratracheally inoculated with

S. pneumoniae (1.25×10^7 CFU), NTHi (3×10^7 CFU), or *S. pneumoniae* with NTHi for 3h, saline was inoculated as control. Broncho-alveolar lavage (BAL) was performed by cannulating the trachea with sterilized PBS, and cells from BAL fluid was stained with Wright-Giemsa stain after cytocentrifuge. For cytokine mRNA expression analysis, total RNA was extracted from whole lung tissues of mice inoculated with *S. pneumoniae*, NTHi, or *S. pneumoniae* with NTHi for 3 hours, and real-time quantitative PCR (Q-PCR) was performed as described above. For CK2 inhibition experiment *in vivo*, CKII inhibitor (5mg/kg) was inoculated intraperitoneally 2 hours prior to bacterial inoculation.

RESULTS

NTHi synergizes with *S. pneumoniae* to induce NF- κ B-dependent inflammatory response *in vitro* and *in vivo*

To determine whether Gram-negative bacterium NTHi synergizes with Gram-positive bacterium *S. pneumoniae* to induce NF- κ B activation and NF- κ B-dependent inflammatory response, we first assessed NF- κ B-dependent transcriptional activity by using NF- κ B-dependent luciferase reporter construct in human epithelial HeLa cells. As shown in **Fig. 1A**, NTHi and *S. pneumoniae* synergistically induced NF- κ B-dependent promoter activity. Similar results were also observed in human airway epithelial cells line A549, middle ear cell line HMEEC-1 and human primary bronchial epithelial NHBE cells (data not shown), suggesting that synergistic activation of NF- κ B by NTHi and *S. pneumoniae* may be generalizable to a variety of human epithelial cells. Consistent with this result, p65, the key subunit of NF- κ B complex, was translocated into the nucleus 15 min after simultaneous treatment with NTHi and *S. pneumoniae*, as assessed by performing Western blot analysis using nuclear protein in HeLa cells (**Fig. 1B**). Similar result was also observed in HeLa cells by performing immunofluorescent staining using antibody against p65 (**Supplementary Fig. 1A**). Moreover, NTHi and *S. pneumoniae* also synergistically increased DNA binding activity of NF- κ B as assessed by performing Electrophoretic Mobility Shift Assay (EMSA) (**Fig. 1C**). Further analysis by super-shift assay revealed that p65 and p50 are the major subunits of NF- κ B complex (data not shown). Because phosphorylation of p65 has been shown to play a critical role in NF- κ B-dependent transcriptional activity [14], we determined whether NTHi and *S. pneumoniae* also synergistically induce phosphorylation of p65. Interestingly, NTHi and *S. pneumoniae* synergistically induced phosphorylation of p65 at S536 and S276 residues (**Fig. 1D & E**). To determine whether NTHi and *S. pneumoniae* also synergistically induce NF- κ B-dependent expression of the key inflammatory mediators, we next determined the effects of NTHi and *S. pneumoniae* on expression of TNF- α , IL-1 β , and IL-8 by performing real-time quantitative PCR (Q-PCR) analysis. As shown in **Fig. 1F**, NTHi and *S. pneumoniae* synergistically induced expression of TNF- α , IL-1 β , and IL-8 in HeLa cells. Similar results were also confirmed in A549, HMEEC-1 and primary NHBE cells (data not shown). Consistent with these *in vitro* results, NTHi and *S. pneumoniae* also synergistically induced expression of TNF- α , IL-1 β , and MIP-2 in the lungs of mice (**Supplementary Fig. 1B**). Moreover NTHi and *S. pneumoniae* also synergistically induced polymorphonuclear neutrophil (PMN) accumulation in broncho-alveolar lavage (BAL) fluids from the lungs of the inoculated mice (**Fig. 1G**). Collectively, these data demonstrate that NTHi and *S. pneumoniae* synergistically induce NF- κ B-dependent inflammatory response *in vitro* and *in vivo*.

IKK β -I κ B α and p38 MAPK signaling pathways are involved in synergistic activation of NF- κ B by NTHi and *S. pneumoniae* via a p65 nuclear translocation-dependent and -independent mechanisms

We next sought to determine if the classical I κ B α kinases (IKKs)-I κ B α signaling pathway is involved in the synergistic activation NF- κ B by NTHi and *S. pneumoniae* [8-9]. As

shown in Fig. 2A, NTHi and *S. pneumoniae* synergistically induced phosphorylation and degradation of I κ B α in HeLa cells. Next, we determined the involvement of I κ B α in this synergistic activation of NF- κ B by assessing the effects of perturbing I κ B α degradation using proteasome inhibitor MG132 and co-expressing transdominant mutant of I κ B α (S32A, S36A). As shown in **Supplementary Fig. 2A & B**, the synergistic activation of NF- κ B was inhibited by both treatments. Next, we sought to determine whether IKK complex is also involved in this synergistic NF- κ B activation. As shown in **Fig. 2B**, co-transfection with a dominant-negative mutant form of IKK β but not IKK α markedly inhibited synergistic NF- κ B activation in HeLa cells. Consistent with these results, synergistic activation of NF- κ B was not observed in IKK β -deficient mouse embryonic fibroblasts (MEFs) as compared with Wild-type (WT) MEFs (**Fig. 2C**). Moreover, perturbing I κ B α signaling using MG132 blocked nuclear translocation of p65 and DNA binding activity of NF- κ B induced by NTHi and *S. pneumoniae* (**Fig. 2D and 2E**). Together, these data indicate that the classical IKK β -I κ B α signaling pathway is involved in synergistic activation of NF- κ B by NTHi and *S. pneumoniae* via a p65 nuclear translocation-dependent mechanism.

Because many cellular stress stimuli can activate both NF- κ B and p38 modules simultaneously [10,15], we explored the possibility that activation of p38 is also involved in this synergistic NF- κ B activation. As shown in **Fig. 2F**, NTHi and *S. pneumoniae* synergistically induced activation of p38 in HeLa cells in a time-dependent manner. We then determined whether activation of p38 is required for synergistic NF- κ B activation by assessing the effects of perturbing p38 signaling using SB203580, a specific inhibitor for p38 and co-expressing dominant-negative mutant forms of p38 α or p38 β . As shown in **Fig. 2G and Supplementary Fig. 2C**, both treatments inhibited the synergistic NF- κ B activation, confirming the requirement of p38 in synergistic activation of NF- κ B. Next, we sought to determine how p38 is involved in mediating this synergistic activation of NF- κ B. Interestingly, SB203580 treatment did not affect I κ B α phosphorylation and degradation, the nuclear translocation of p65 and the DNA binding activity of p65 induced by NTHi and *S. pneumoniae* (**Fig. 2H and 2I**; and **Supplementary Fig. 2D and 2E**). Collectively, our data suggest that MAPK p38 is involved in synergistic activation of NF- κ B by NTHi and *S. pneumoniae* via a p65 nuclear translocation-independent mechanism.

CK2 is required for NTHi- and *S. pneumoniae*-induced synergistic activation of NF- κ B and NF- κ B-dependent inflammatory response *in vitro* and *in vivo*

We sought to determine whether CK2 (formerly known as casein kinase II) is involved in mediating this synergistic activation of NF- κ B [16-17]. As shown in **Fig. 3A**, NTHi and *S. pneumoniae* indeed synergistically induced kinase activity of CK2. Moreover, perturbing CK2 signaling using a specific CK2 inhibitor inhibited the synergistic induction of NF- κ B (**Fig. 3B**). We next sought to confirm the requirement of CK2 by knockdown of CK2 β , the key regulatory subunit of CK2 complex, using CK2 β siRNA. We first confirmed the efficiency of CK2 β siRNA in reducing endogenous CK2 β expression in HeLa cells transfected with CK2 β siRNA or control siRNA. CK2 β protein was markedly reduced by CK2 β siRNA (**Fig. 3C**, upper panel). As expected, CK2 β knockdown inhibited the synergistic induction of NF- κ B (**Fig. 3C**, lower panel). Furthermore, CK2 inhibitor also inhibited synergistic induction of TNF- α , IL-1 β , and IL-8 in HeLa cells (**Fig. 3D**). To further confirm whether NTHi and *S. pneumoniae* also synergistically induce inflammatory response via CK2 *in vivo*, we next determined the effects of CK2 inhibitor on synergistic induction of IL-1 β , and MIP-2 in the lungs of the mice. As shown in **Supplementary Fig. 3**, CK2 inhibitor inhibited the synergistic induction of IL-1 β and MIP-2 in the lungs of the mice. Consistent with this result, CK2 inhibitor also inhibited NTHi- and *S. pneumoniae*-induced synergistic induction of polymorphonuclear neutrophil (PMN) accumulation in broncho-alveolar lavage (BAL) fluids from the lungs of the inoculated mice (**Fig. 3E**).

Collectively, these data demonstrate that NTHi and *S. pneumoniae* synergistically induce NF- κ B-dependent inflammatory response via a CK2-dependent mechanism *in vitro* and *in vivo*.

CK2 mediates synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites

Next we sought to determine how CK2 mediates synergistic induction of NF- κ B. We first determined whether CK2 mediates synergistic induction of NF- κ B via I κ B α -dependent nuclear translocation of NF- κ B. As shown in **Fig. 4A, 4B & Supplementary 4A & 4B**, treatment with CK2 inhibitor did not affect synergistic induction of I κ B α phosphorylation and degradation, the nuclear translocation of p65 and DNA binding activity of NF- κ B in response to both NTHi and *S. pneumoniae*. These unexpected findings thus led us to explore the possibility that CK2 may mediate synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. As shown in **Fig. 4C and 4D**, synergistic induction of phosphorylation of p65 at both Ser536 and Ser276 sites was attenuated by both CK2 inhibitor and CK2 β knockdown using siRNA. To further confirm whether the Ser536 and Ser276 sites of p65 are indeed functionally involved in mediating the synergistic induction of NF- κ B, we assessed the effects of co-expressing the dominant-negative mutant forms of p65 (S536A and S276A) on synergistic induction of NF- κ B. As shown in **Fig. 4E**, co-expressing both mutant forms inhibited the synergistic induction of NF- κ B. Consistent with these results, synergistic induction of NF- κ B was observed only in p65-deficient MEFs that were reconstituted with p65 WT but not with p65-S536A or p65-S276A mutants (**Fig. 4F**). Thus, our data suggest that CK2 mediates synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites.

DISCUSSION

Under *in vivo* situations such as polymicrobial infections, mucosal epithelial surfaces are simultaneously colonized by multiple microbial pathogens, little is known about how NF- κ B-dependent inflammatory response, a hallmark of polymicrobial infection, is induced and regulated by multiple pathogens. Despite recent study demonstrating the synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces [18], the signaling mechanisms underlying the synergistic induction of inflammation remain largely unknown. In the present study, we showed that NTHi and *S. pneumoniae* synergistically induce NF- κ B-dependent inflammatory response via IKK β -I κ B α and p38 signaling pathways. Moreover, CK2 mediates synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites (**Fig. 5**). These studies bring new insights into the molecular mechanisms underlying the synergistic regulation of NF- κ B in polymicrobial infections.

Of particular interest in this study is the direct evidence that CK2 mediates synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. CK2 is a highly conserved and ubiquitous serine/threonine kinase [16-17,19]. Previous studies showed that CK2 is involved in mediating activation of NF- κ B in response to TNF- α and UV radiation [20-21]. While our data confirmed these earlier findings that CK2 is involved in NF- κ B activation, we also provide unique evidence for the first time for the CK2-dependent phosphorylation of p65 at both Ser536 and Ser276 sites in response to polymicrobial infections. Previous study identified serine 529 as the major phosphorylation site of p65 in response to TNF- α treatment. In the present study, we showed that perturbing CK2 signaling using chemical inhibitor or CK2 siRNA greatly inhibited the synergistic induction of p65 phosphorylation at both Ser536 and Ser276 sites. Moreover, the synergistic activation of NF- κ B by NTHi and *S. pneumoniae* was observed

only in p65-deficient MEFs that were reconstituted with p65 WT but not with p65-S536A or p65-S276A mutants. Thus, our data provide direct functional evidence that CK2 mediates synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. It should also be noted that our data do not rule out the possibility that CK2 may mediate the synergistic induction of NF- κ B also via a mechanism dependent on phosphorylation of p65 at Ser529 since Ser529 was constitutively phosphorylated in the cell line used for this study. All of our findings are consistent with the notion that posttranslational modifications, particularly phosphorylation of p65, play critical roles in NF- κ B-dependent transcriptional activity. It should also be noted that no crosstalk was found between CK2 and p38 MAPK signaling pathways in the present study (data not shown), indicating that CK2 and p38 MAPK are involved in mediating the synergistic activation of NF- κ B independently.

Another interesting finding in our study is the synergistic activation of NF- κ B-dependent inflammation in polymicrobial infections. Our finding may have important implications in understanding the molecular basis underlying inflammatory response during bacterial infections under *in vivo* situation. In review of the past studies on NF- κ B-dependent inflammation, most studies focused on studying the activation of NF- κ B by one inducer at a time. While these studies are indeed critical for our understanding of the molecular mechanism underlying inflammation, the information derived from these single-inducer studies may be insufficient for fully understanding how NF- κ B-dependent inflammation is induced and regulated *in vivo* where host mucosal surfaces are simultaneously exposed to multiple inducers including multiple microbial stimuli. Given the marked difference in induction of multiple signaling pathways and the subsequent NF- κ B activation induced by NTHi or *S. pneumoniae* alone vs both, the molecular mechanisms underlying inflammation appear to be more complicated under *in vivo* situation. Thus, taking this difference into consideration would certainly be helpful for develop more feasible and efficient therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
NTHi synergizes with *S. pneumoniae* to induce NF-κB-dependent inflammatory response *in vitro* and *in vivo*.

(A) NTHi and *S. pneumoniae* synergistically induced NF-κB-dependent promoter activity in HeLa cells. Similar results were also observed in human airway A549, middle ear HMEEC-1 and primary airway NHBE cells. (B) Synergistic induction of nuclear translocation of NF-κB by NTHi and *S. pneumoniae* was observed in HeLa cells by Western blotting analysis. (C) NTHi and *S. pneumoniae* synergistically induced DNA binding activity of NF-κB as assessed by EMSA assay in HeLa cells. Please note that unlabeled probe was added in the last lane. (D & E) NTHi and *S. pneumoniae* synergistically induced phosphorylation of p65 at both Ser536 and Ser276 sites in HeLa cells. (F) NTHi and *S. pneumoniae* synergistically induced expression of TNF-α, IL-1β, and IL-8 as assessed by

performing real-time quantitative PCR (Q-PCR) analysis. (G) NTHi and *S. pneumoniae* synergistically induced polymorphonuclear neutrophil (PMN) accumulation in broncho-alveolar lavage (BAL) fluids from the lungs of the inoculated mice. Arrows indicate PMN. Values are means $\pm$ SD. Data are representative of three or more independent experiments.

Fig. 2. IKKβ-IκBα and p38 MAPK signaling pathways are involved in synergistic activation of NF-κB by NTHi and *S. pneumoniae* via p65 nuclear translocation-dependent and -independent mechanisms.

(A) Synergistic induction of phosphorylation and degradation of IκBα was observed in HeLa cells treated with both NTHi and *S. pneumoniae*. (B) Co-transfection with a dominant-negative mutant form of IKKβ but not IKKα markedly inhibited synergistic NF-κB activation in HeLa cells. (C) Synergistic activation of NF-κB by NTHi and *S. pneumoniae* was not observed in IKKβ-deficient mouse embryonic fibroblasts (MEFs) as compared with

Wild-type (WT) MEFs. **(D & E)** Perturbing I κ B α signaling using MG132 blocked nuclear translocation of p65 and DNA binding activity of NF- κ B induced by NTHi and *S. pneumoniae* in HeLa cells. **(F)** Synergistic induction of p38 phosphorylation was observed in HeLa cells treated with both NTHi and *S. pneumoniae*. **(G)** Co-expression of a dominant-negative mutant form of p38 α or p38 β abrogated the synergistic activation of NF- κ B. Pretreatment of HeLa cells with SB203580 did not alter the synergistic induction of nuclear translocation of p65 **(H)** and DNA binding activity of NF- κ B **(I)**. Please note that unlabeled probe was added in the last lane (E & I). Values are means $\pm$ SD. Data are representative of three or more independent experiments.

Fig. 3.

CK2 is required for NTHi- and *S. pneumoniae*-induced synergistic activation of NF-κB and NF-κB-dependent inflammatory response *in vitro* and *in vivo*.

(A) NTHi and *S. pneumoniae* synergistically induced CK2 kinase activity. Perturbing CK2 signaling by CK2 specific inhibitor (B) or CK2β knockdown using CK2β siRNA (C) inhibited synergistic induction of NF-κB by NTHi and *S. pneumoniae* in HeLa cells. (D) CK2 specific inhibitor also inhibited up-regulation of TNFα, IL-1β, and IL-8 in HeLa cells. (E) CK2 inhibitor also inhibited NTHi- and *S. pneumoniae*-induced synergistic induction of polymorphonuclear neutrophil (PMN) accumulation in broncho-alveolar lavage (BAL) fluids from the lungs of the inoculated mice. Arrows indicate PMN. Values are means ±SD. Data are representative of three or more independent experiments.

Fig. 4.

CK2 mediates synergistic induction of NF- κ B via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites.

(A) Synergistic induction of I κ B α phosphorylation and degradation remained unaffected in HeLa cells treated with CK2 inhibitor. (B) CK2 inhibitor did not alter the nuclear translocation of p65 in response to both NTHi and *S. pneumoniae*, as assessed by immunostaining with anti-p65 antibody. (C & D) Synergistic induction of phosphorylation of p65 at both Ser536 and Ser276 sites was attenuated by both CK2 inhibitor and CK2 β knockdown using siRNA. (E) Overexpressing the mutant forms p65(S536A) and p65(S276A) inhibited the synergistic induction of NF- κ B. (F) Synergistic induction of NF- κ B was observed only in p65-deficient MEFs that were reconstituted with p65 WT but not with p65-S536A or p65-S276A mutants. Values are means $\pm$ SD (G & H). Data are representative of three or more independent experiments.

Fig. 5.
Schematic representation depicting how NTHi and *S. pneumoniae* synergistically activate NF-κB as well as the subsequent inflammatory responses *in vitro* and *in vivo*.