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Type I interferon induced by DNA of nontypeable *Haemophilus influenza* modulates inflammatory cytokine profile to promote susceptibility to this bacterium

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ABSTRACT

Type I interferon (IFN) is indispensable for antiviral immunity, but its role in bacterial infections is controversial and not fully described. Nontypeable *Haemophilus influenzae* (NTHi) is one of the most common bacterial pathogens in patients with chronic obstructive pulmonary disease (COPD). NTHi-DNA activates type I IFN production in macrophages, but the function of type I IFN in host-pathogen interactions, in the context of NTHi infection, is still unclear. Here, we showed that type I IFN, induced by NTHi-DNA, restrained bacterial killing *in vitro* and promoted COPD development *in vivo* in response to NTHi. Mice deficient for type I IFN receptor (IFNAR) exhibited improved resistance to NTHi infection. Moreover, similar to exogenous IFN-β, NTHi-DNA-induced type I IFN increased the production of IL-6, IL-1β, IL-12 and CXCL10 *via* p38 MAPK activation. Our findings demonstrated that NTHi-DNA-induced type I IFN signaling played a negative role in host defense against NTHi infection and identified potential targets for future therapeutic management of COPD.

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi), a gram-negative coccobacillus and an exclusively human commensal lacking a polysaccharide capsule, colonizes the upper respiratory tract of most healthy adults [1]. NTHi can spread to the lower respiratory tract, leading to pneumonia, bronchiectasis, and chronic obstructive pulmonary disease (COPD), which results in substantial morbidity and mortality [2–4]. NTHi infections are responsible for 25–80% of exacerbations of COPD with *H. influenzae* isolation [5]. The interplay between NTHi and the host immune system is crucial for the survival of the pathogen and the outcome of patients with COPD. However, this interaction is poorly characterized at the mechanistic level. Moreover, the understanding of the mechanism of interaction between NTHi and the host innate immune response is limited.

In response to host defense mechanisms, NTHi could release highly antigenic proteins into the airways, such as outer membrane protein P6, lipooligosaccharide, and peptidoglycan fragments, resulting in airway inflammation [6]. Hardison RL et al. revealed that microevolution in response to nutrient limitation leads to expansion of the intracellular bacterial community and persistence in a murine model of NTHi-induced otitis media (OM), through decreased activity of 3′,5′-cyclic adenosine monophosphate phosphodiesterase [7]. It is increasingly recognized that escape from innate responses is not the only strategy adopted by NTHi as, on the contrary, host innate immune responses may be actively exploited by NTHi in specific cases. In a murine COPD model, the anti-inflammatory activity of regulatory T cells is suppressed by NTHi-mediated downregulation of Foxp3, promoting the progression of acute exacerbation of COPD [8]. Extracellular DNA (eDNA) in NTHi biofilms binds to human β-defensin-3 (hBD-3) to abrogate the biological activity of this innate immune effector and diminish its antimicrobial activity [9]. But it is not desirable to disavow the existence of other undiscovered innate immune response and associated immunoregulatory mechanism. However, the possibility of other mechanism of innate immune response to NTHi infection cannot be excluded.

It is widely recognized that type I IFN (IFN-I), as an innate immune signaling factor, is able to coordinate immune responses by regulating

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cellular autoimmune and inflammatory events, as well as immune cell communication. IFN-I activates the downstream type I IFN receptor (IFNAR). Although type I IFN mediates host defense against viral infections, recent compelling evidence indicates that type I IFN-mediated responses are also involved in antibacterial immunity [10]. Over a decade of studies has demonstrated an ambivalent role for type I IFN in bacterial infections, including both protective and deleterious effects, depending on the specific pathogen. A strong type I IFN response induced by *Listeria monocytogenes* was found to inhibit the expression of IL-17A and suppress neutrophil recruitment [11], and to promote host susceptibility to infection by induction of anti-inflammatory IL-10 [12], as also demonstrated in mice deficient for either IFNAR or IRF3 [13]. Further, interferon-β aggravates infection by *S. typhimurium*, *a* gram-negative intracellular pathogen, by weakening the ability of the host to initiate a complete immune response. Moreover, mice deficient in IFNAR1 and IFN-β exhibit enhanced survival and resistance to *S. typhimurium* infection, in association with increased expression of the proinflammatory cytokine IL-1β, and of the neutrophil chemoattractants, CXCL1 and CXCL2 [14]. Paradoxically, IFN-I also promotes host resistance to infections caused by *Streptococcus pneumoniae* which may cause life-threatening pneumonia. It was observed that treatment with exogenous IFN-β in mice reduced the transmigration of bacteria from the lung to the blood, whereas IFNAR1 −/− mice exhibited lower bacterial survival and enhanced bacteremia during *S. pneumoniae* infection [15,16]. In the case of *Streptococcus pyogenes* infection, IFNα/β-mediated signaling enhanced host resistance by restraining the expression of the IL-1β gene [17]. These reports indicated that the effect of type I IFN on host defense against bacterial infection involves the regulation of inflammatory response.

Previous studies have shown that NTHi can be internalized by host cells [18]. Moreover, we found that internalized NTHi can release its own DNA, which in turn may induce the expression of type I IFN through the activation of STING signaling [19]. However, the exact role of NTHi-DNA-induced IFN-I in host defense against NTHi infection remains to be characterized. Here we explored the role of NTHi-DNA-induced IFN-I in antibacterial immunity by investigating inflammatory response.

In this study, using a mouse model of COPD, we show that NTHi-DNA-induced IFN-I played a detrimental role in host defense against NTHi infection, both *in vitro* and *in vivo*. Furthermore, by controlling the p38 MAPK signaling pathway, IFN-I functioned as a key regulator of the expression of both pro- and anti-inflammatory cytokine genes. Taken together, our findings provide hints on new possible therapeutic approaches for tackling NTHi infection in COPD patients.

2. Materials and methods

2.1. Cells and mice

The THP-1 cell line was cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Prior to treatment, THP-1 cells were seeded in culture plates and differentiated with PMA (100 ng/ml) for 24 h. Primary peritoneal macrophages (PEMs) were obtained as described previously [20]. Six- to eight-week-old wild-type (WT) C57BL/6 and IFNAR-deficient mice, obtained from the Laboratory Animal Center of Chongqing Medical University, Chongqing, China, were intraperitoneally injected with 1 ml of sterile liquid paraffin. After 4–7 days, PEMs were extracted by peritoneal lavage using 15 ml of sterile PBS. PEMs were plated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Barrington, USA).

2.2. Bacterial strain, growth and cell counting

Nontypeable *Haemophilus influenzae* (NTHi) strain 49,247 was seeded on chocolate agar plates and grown in 5 ml of brain heart infusion broth (BHI, Rishui, Qingdao, China), containing NAD (2 mg/ml) and Hemin (0.2 mg/ml), at 37 °C in 5% CO2 until the optical density at 600 nm reached 0.5 (OD_{600} = 0.5). The *Streptococcus pneumonia* (*S.pn*) standard strain (NCTC 7466, serotype 2) was purchased from the National Collection of Type Cultures (London, United Kingdom) and was grown in C plus Y medium at 37 °C in 5% CO2 until the optical density at 600 nm equaled 0.5. The number of bacteria was calculated by bacterial plate counts. To assess the phagocytosis of macrophages, after pretreatment with *S.pn* DNA, NTHi-DNA or IFN-β overnight and infection with NTHi or *S.pn* at a MOI of 100 for 30 min, macrophages were washed three times with PBS, incubated for additional 30 min with culture medium containing gentamicin (200 μg/ml), and then lysed for 15 min in 100 μl of deionized water. To evaluate macrophage killing, PEMs were incubated with antibiotic-free medium for an additional 1 h after gentamicin treatment, and then lysed for 15 min in 100 μl of deionized water. The lysate was plated onto chocolate plates by serial dilutions and the plates were incubated at 37 °C in 5% CO_2 for 24 h before CFU counting.

2.3. DNA purification and NTHi infection into cells

For DNA transfection *in vitro*, genomic DNA was extracted from NTHi or *S.pn* by a bacterial DNA isolation kit (OMEGA, USA) and stored at −20 °C. For NTHi-DNA transfection *in vivo,* genomic DNA was purified from NTHi by Sangon Biotech (Shanghai, China). Cells were transfected with NTHi DNA (1.5 μg/ml) by Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) for 5 h following the manufacturer's directions, and subsequently infected with NTHi for various times.

2.4. In vivo NTHi infection into mice

The murine COPD model was constructed as reported by Vernooy et al. [21]. In brief, 6–8 weeks-old C57BL/6 mice were challenged with LPS (Sigma, USA) twice a week for a period of 4 weeks by intratracheal instillation (15 μg/instillation/mouse). After each intratracheal treatment, the mice were kept in an upright position for 10 min to allow for sufficient spreading of the fluid in the lungs. To obtain a murine model of NTHi infection, WT, IFNAR−/−, and COPD mice were instilled intratracheally with NTHi-DNA (50 μg) in a complex with Dotap (Sigma, USA) or with recombinant IFN-β (4000 U, PBL, USA) 24 h before intranasal infection with NTHi (1×10^8 CFU). Mice were euthanized and lungs were collected at 24 h post challenge. For survival experiments, mice were intraperitoneally injected with 100 μg of NTHi-DNA in complex with Dotap or with 4000 U of rIFN-β daily after infection, and monitored for 7 days.

2.5. Lung histological analysis

Lungs from mice infected with NTHi for 24 h were isolated and fixed with 4% paraformaldehyde. Then, lungs were embedded in paraffin and 6-μm sections were cut. Lung sections were stained with hematoxylin and eosin (H&E; Sigma-Aldrich).

2.6. Fluorescence microscopy analysis

PEMs were seeded on coverslips in a 12-well plate and infected with inactivated NTHi labeled with GFP (MOI = 100) for 30 min at 70 °C after NTHi-DNA and IFN-β treatment. PEMs were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100, and their nuclei were stained with DAPI (Santa Cruz Biotechnology, USA) for 10 min. Subsequently, an appropriate fluorescence quencher was added, and the coverslips were mounted on slides. The images were captured under a fluorescence microscope (ECLIPSE Nikon, Japan). The phagocytic index was used to express the level of phagocytosis and calculated as the average value of GFP-NTHi uptake per cell multiplied by the percentage of phagocytes involved in phagocytosis.

2.7. Cytokine analysis with ELISA and Immunoblotting

After pretreatment with NTHi-DNA or IFN-β, the cells were infected with NTHi (MOI = 200) for 12 h, and the cell culture supernatants were collected and frozen at −80 °C. The cell culture supernatants and the lung homogenates were thawed to measure the expression level of IL-1β and IL-6, IL-12p70, IL-10, and CXCL10 using ELISA kits (Biolegend, USA). PEMs were lysed with RIPA buffer (Beyotime, China) supplemented with PMSF, phosphatase inhibitor (BioTools, USA) (100:10:1). Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). The membranes were blocked with 5% skim milk for 2 h at room temperature, followed by incubation with the primary antibodies (anti-mouse GAPDH or anti-mouse phospho-p38 MAPK, Cell Signaling Technology, USA) overnight at 4 °C. Then, the membranes were incubated for 1 h at room temperature with an HRP-conjugated secondary antibody. The target bands were visualized using HRP substrate (Millipore, USA) and the Enhanced Chemiluminescence system (GE Healthcare, Little Chalfont, UK).

2.8. Quantitative real time-PCR (qPCR)

Total RNA (1 μg) was extracted from mouse lungs and cells using RNAiso plus (TaKaRa), according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed using PrimeScript™ RT reagent kit (TaKaRa). All real-time PCR were processed using the TB Green Premix Ex Taq™ II. The expression of each mRNA was calculated relative to GAPDH. The specific primers used are shown in Table 1.

2.9. Statistical analysis

Each experiment was repeated at least three times and data were expressed as the mean ± SD. Statistical analysis was carried out using the GraphPad Prism 5 software. Unpaired Student's *t*-test was used to analyze the comparison between two independent samples and one-way ANOVA was employed to analyze the differences between multiple groups. Survival curves were compared by a log-rank test. $p < 0.05$ was considered to be statistically significant.

Table 1
The sequences of PCR primers.

Gene	Orientation	Sequence
Mouse-*Gapdh*	Sense	5′-CGGAGTCAACGGATTTGGTC-3′
	Anti-sense	5′-GACAAGCTTCCCGTTCTCAG-3′
Mouse-*Il-1β*	Sense	5′-GAGCACCTTCTTTTCCTTCATCTT-3′
	Anti-sense	5′-TCACACACCAGCAGGTTATCATC-3′
Mouse-*Il-6*	Sense	5′-AGAGGATACCACTCCCAACAGAC-3′
	Anti-sense	5′- AGTGCATCATCGTTGTTCATACAA-3′
Mouse-*Il-12p40*	Sense	5′-TCCCTGCAGGGTCCGATCCT-3′
	Anti-sense	5′-CCTGGCTCTGCGGGCATTTA-3′
Mouse-*Cxcl10*	Sense	5′-CCTGCCCACGTGTTGAGAT-3′
	Anti-sense	5′-TGTGGTCTTAGATTCCGGATTC-3′
Mouse-*Il-10*	Sense	5′-GCTCTTACTGACTGGCATGAG-3′
	Anti-sense	5′-CGCAGCTCTAGGAGCATGTG-3′
Mouse-*Nlrp3*	Sense	5′-ACTGAAGCACCTGCTCTGCAAC-3′
	Anti-sense	5′-AACCAATGCGAGATCCTGACAAC-3′
Mouse-*Nos2*	Sense	5′-AGCACTTTGGGTGACCACCAGGA-3′
	Anti-sense	5′-AGCTAAGTATTAGAGCGGCGGCA-3′
Mouse-*Arg1*	Sense	5′-TGACATCAACACTCCCCTGACAAC-3′
	Anti-sense	5′-GCCTTTTCTTCCTTCCCAGCAG-3′
Human-*Gapdh*	Sense	5′-GAAGGGCTCATGACCACAGT-3′
	Anti-sense	5′-GGATGCAGGGATGATGTTCT-3′
Human-*Il-1β*	Sense	5′-CTGAAAGCTCTCCACCTCCA-3′
	Anti-sense	5′-TCATCTTTCAACACGCAGGA-3′
Human-*Il-6*	Sense	5′-GAACTCCTTCTCCACAAGCG-3′
	Anti-sense	5′-ATCTTCTCCTGGGGGTACTGG-3′
Human-*Il-12p35*	Sense	5′-GCTCCAGAAGGCCAGACAAA-3′
	Anti-sense	5′-GGCCAGGCAACTCCCATTAG-3′
Human-*Cxcl10*	Sense	5′-TGGCATTCAAGGAGTACCTC-3′
	Anti-sense	5′-TTGTAGCAATGATCTCAACAC-3′
Human-*Il-10*	Sense	5′-TCAGGGTGGCGACTCTAT-3′
	Anti-sense	5′-TGGGCTTCTTTCTAAATCGTTC-3′

3. Results

3.1. NTHi-DNA-induced IFN-I plays a negative role in host defense against NTHi infection in vitro and in vivo

We previously demonstrated that NTHi DNA activates the cGAS-STING pathway, thus triggering type I IFN production in macrophages [19]. To evaluate the role of NTHi-DNA-induced IFN-I *in vitro*, primary peritoneal macrophages (PEMs) from C57BL/6 mice were pre-treated with NTHi-DNA overnight, and subsequently infected with NTHi, followed by treatment with gentamicin 30 min post infection. The phagocytosis and killing ability of PEMs were assessed by bacterial plate count. Interestingly, priming of PEMs with NTHi-DNA led to a significant decrease in the number of intracellular bacteria compared to media-primed macrophages, confirming that NTHi-DNA-induced IFN-I blunted the phagocytic ability of macrophages (Fig. 1A). Consistently, PEMs killing ability was also impaired by NTHi-DNA pretreatment prior to infection, resulting in higher bacterial survival (Fig. 1A). In line with the results obtained with NTHi-DNA pretreatment, macrophage priming with IFN-β resulted in a significant decrease in both phagocytosis and killing ability during NTHi infection (Fig. 1A).

To further evaluate the specific role of the type I interferon signaling pathway in response to NTHi infection, primary peritoneal macrophages from IFNAR−/− mice were infected with NTHi. As anticipated, compared to wild type PEMs, PEMs from IFNAR−/− mice exhibited a higher NTHi uptake after treatment with gentamicin and a stronger bacterial clearance after additional 1 h incubation post gentamicin treatment, regardless of NTH-DNA or IFN-β priming (Fig. 1B), suggesting that defective IFN-I signaling contributed to enhanced NTHi phagocytosis and bacterial killing abilities by macrophages. Next, we performed a fluorescence phagocytosis assay. Priming of WT PEMs with NTHi-DNA or IFN-β resulted in a significant reduction in uptake of FITC-NTHi, but an increase in IFNAR−/− PEMs (Fig. 1C). The rate of phagocytosis was expressed as phagocytic index (Fig. 1D).

Streptococcus pneumoniae (*S.pn*) can also cause acute exacerbations of COPD [22] and *S.pn* DNA is able to induce the production of type I IFN [16]. Therefore, we pretreated PEMs with NTHi-DNA or *S.pn* DNA to study the effects of type I IFN induced by either of these DNAs on phagocytosis and killing ability of host cells. Compared with media-pretreated PEMs, PEMs pretreated with NTHi-DNA or *S.pn* DNA showed less uptake of NTHi (Fig. 1E) and more NTHi survival (Fig. 1F) upon infection with NTHi. These data indicate that type I IFN induced by both bacterial derived DNAs significantly reduced phagocytosis and killing of NTHi by PEMs. In contrast, when infected with *S.pn*, NTHi-DNA or *S.pn* DNA pretreatment enhanced the phagocytosis (Fig. 1E) and killing (Fig. 1F) of *S.pn* by PEMs, and was beneficial to macrophages against *S.pn* infection, which is consistent with the effect of type I IFN reported by other researchers [15]. However, our results demonstrate that NTHi-DNA or *S.pn* DNA-induced type I IFN is detrimental to host defense against NTHi infection.

Both NTHi-DNA and *S.pn* DNA can induce type I IFN production by host cells, but the effects on the outcomes of the two bacterial infections are different. In the presence of *S.pn* infection, *S.pn* DNA-activated type I IFN response enhances the phagocytosis and killing of host cells, which is the host's strategy against bacterial infection. However, in the presence of NTHi infection, the type I IFN response elicited by NTHi-DNA may be exploited by bacteria to evade host immune clearance, which is detrimental to the outcome of NTHi infection.

To ascertain the role of NTHi-DNA-induced IFN-I during COPD, we sought to establish a chronic pulmonary inflammation murine model by repeated intratracheal instillation of lipopolysaccharide (LPS) [23,24]. Substantial thickening of the alveolar walls, leukocyte infiltration in peribronchial areas, and lung dysfunction were observed in LPS-treated

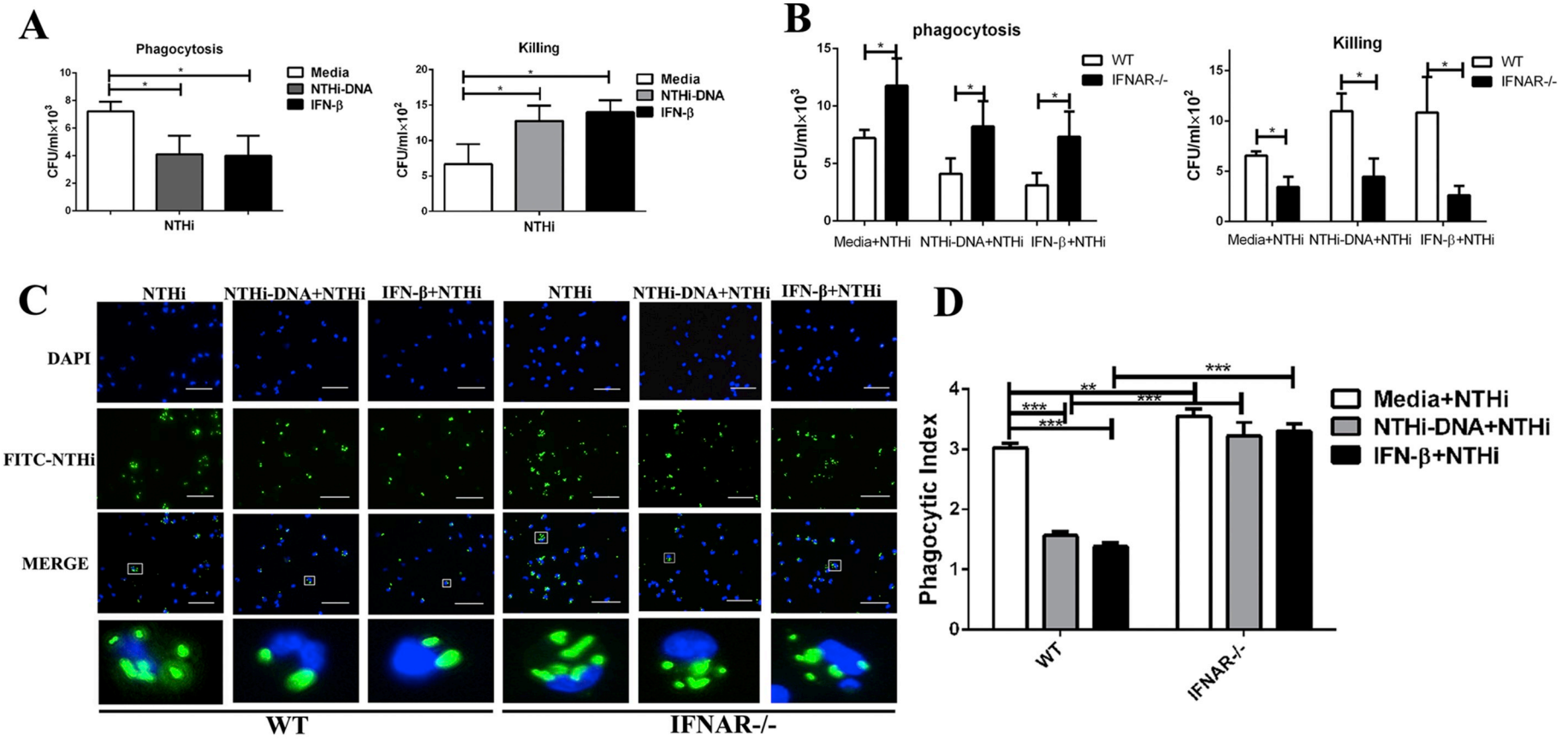

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Fig. 1. NTHi-DNA-induced IFN-I attenuates phagocytosis and killing of NTHi.
(A,B) Wild type or IFNAR−/− PEMs were pre-incubated with media alone, NTHi-DNA (1.5 μg/ml) or IFN-β (100 U/ml) overnight, respectively, and subsequently infected with NTHi at an moi of 100 for 30 min. Bacterial plate count was used to detect the phagocytic capacity and intracellular bacterial killing of macrophages. (C, D) Wild type or IFNAR−/− PEMs were treated as in (A) and the phagocytic ability of PEMs was detected by fluorescence assay. Green signals represent FITC-NTHi and blue signals represent nuclei. Bars = 50 μm. (D) Quantification of the fluorescence assay. (E, F) PEMs were pre-incubated with media alone, 1.5 μg/ml NTHi-DNA or 1.5 μg/ml *S.pn* DNA overnight, and subsequently infected with NTHi or *S.pn* at an moi of 100 for 30 min. Bacterial plate count was used to detect the phagocytic and killing ability of macrophages. Data are shown as mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(COPD) mice (Fig. S1 and Table S1). COPD mice were intranasally infected with NTHi and the lethality was monitored for 7 days. NTHi-DNA or IFN-β pretreatment resulted in significantly decreased survival (Fig. 2A) and in pronounced weight loss (Fig. 2B) compared to PBS controls, demonstrating the potentially detrimental effect of NTHi-DNA-induced IFN-I in COPD progression, when combined with NTHi infection. Analysis of lung sections from mice pretreated with NTHi DNA or IFN-β, followed by NTHi infection, showed that leukocytic infiltrates increased compared to mice only infected with NTHi (Fig. 2C). Notably, mice only pretreated with NTHi DNA exhibited increased infiltration of inflammatory cells compared to PBS controls, further suggesting that NTHi-DNA may contribute to the activation of inflammatory responses.

In addition, we employed WT and IFNAR−/− mice to directly verify whether type I IFN signaling affected the outcome of NTHi infection. The changes in body weight of WT and IFNAR−/− mice were monitored after intratracheal infection with 1×10^8 CFU of NTHi. WT mice pretreated with NTHi-DNA showed a significantly more pronounced weight loss, upon NTHi infection, compared to NTHi-infected WT mice (Fig. 2D), demonstrating the harmful effect of NTHi-DNA-induced IFN-I *in vivo*. Furthermore, the IFNAR−/− mice lost less weight in response to NTHi infection over a 7-day period when compared to WT mice, independently of NTHi-DNA pretreatment (Fig. 2D), suggesting that IFNAR deficiency was beneficial to host defense against NTHi infection. In addition, H&*E*-stained lung sections showed more severe bronchopneumonia in WT compared to IFNAR −/− mice, both with and without NTHi-DNA pretreatment (Fig. 2E).

Collectively, these data suggested that NTHi-DNA-induced IFN-I negatively affected host defense against NTHi infection in a model of COPD, and the presence of a functional type I IFN signaling pathway promoted susceptibility to NTHi infection.

NOS2 and Arg1 have been reported to be markers for pro-inflammatory and anti-inflammatory response, respectively [25]. Therefore, their mRNA levels were tested in relation to NTHi-DNA priming and/or bacterial infection. We observed that PEMs priming with NTHi-DNA or IFN-β, prior to NTHi infection, increased Arg1 expression by 2–3 folds, and NOS2 expression by 60–200 folds, compared with PEMs only infected with NTHi (Fig. 2F). These effects of NTHi-DNA were suggestive of pro-inflammatory response.

3.2. NTHi-DNA-induced IFN-I promotes inflammatory cytokine response to NTHi in macrophages

Since the expression of the pro-inflammatory marker NOS2 was significantly increased in macrophages primed with either NTHi DNA or IFN-β, we examined the production of inflammatory cytokines following NTHi DNA-induced IFN-I production during infection. PEMs were pre-treated with NTHi-DNA for 5 h to induce IFN-I, and then infected with NTHi. The expression of IL-6, IL-1β, and IL-12 was detectable following the sole priming with NTHi DNA (Fig. 3A and B), indicating that the stimulation of STING signaling under these conditions may be sufficient to induce the production of these cytokines. Previous studies showed that STING was activated by bacterial DNA for partial production of the inflammatory cytokines IL-6, IL-1β, TNF-α, CXCL10 [25] and IL-12 [26]. C-X-C motif chemokine 10 (CXCL10) is an inflammatory gene and a target of IFN-I, which may explain the absence of differences between the NTHi-DNA and NTHi-DNA plus NTHi groups (Fig. 3A and B). Remarkably, we observed that expression of the pro-inflammatory cytokines IL-6, IL-1β, IL-12p70 (composed of p35 and p40 chains), and CXCL10 was significantly higher in NTHi-DNA-pretreated compared to non-pretreated macrophages post infection, both at the mRNA and protein levels (Fig. 3A and B). Similarly, NTHi-DNA pretreatment significantly increased the secretion of IL-6 and CXCL10 by NTHi-infected RAW264.7 cells, but it did not modify the release of IL-1β and IL-12p40 (Fig. S2). No significant differences were observed in the induction of CXCL1, CXCL2, and TNF-α (Fig. S2). Additionally, we verified the effects of NTHi-DNA pretreatment on the production of IL-10, an immunosuppressive cytokine that is essential for immune homeostasis [27]. Pretreatment with NTHi-DNA significantly enhanced IL-10 production in NTHi–infected macrophages at both mRNA and protein levels (Fig. 3A and B), indicating that the increase in expression of IL-10 may be accompanied by a concomitant increase in the levels of pro-inflammatory cytokines. A previous study reported that the delicate balance between IL-12 and IL-10 is breached, and this could lead to immunologic disorders and pathogenesis [28].

To verify whether NTHi DNA-mediated cytokine upregulation in murine macrophages was generalizable to humans, we used a human macrophage-like cell line, THP-1. We observed that pretreatment with NTHi-DNA increased the transcription of the pro-inflammatory cytokines IL-6, IL-1β, IL-12p35, and CXCL10, as well as that of anti-inflammatory IL-10, as assessed by qPCR analysis in NTHi-infected macrophages (Fig. 3C).

These results suggested that NTHi-DNA-induced IFN-I could cause an excessive production of inflammatory cytokines during NTHi infection.

3.3. Exogenous addition of IFN-I enhances the expression of inflammatory cytokines upon NTHi infection in macrophages

To further characterize the role of type I interferon in inflammatory response, WT mouse peritoneal macrophages were primed with murine recombinant IFN-β for 60 min prior to infection with NTHi. Notably, IFN-β administration enhanced the subsequent NTHi-induced production of IFN-β mRNA (Fig. 4A), in accordance with previous findings showing that exposure of macrophages to IFN-β increased IFN-β expression in response to *Salmonella Typhimurium* infection [14]. Inflammatory cytokine induction in PEMs was also analyzed by qPCR at 6 h post NTHi infection. In agreement with our previous results, exposure of PEMs to IFN-β dramatically enhanced the expression of the inflammatory cytokines IL-6, IL-1β, IL-12p40, and CXCL10 (Fig. 4B). Consistently, the protein levels of IL-6, IL-1β, IL-12p70, and CXCL10 were significantly higher in the IFN-β-pretreated PEMs at 12 h post-infection compared to those in control macrophages, as assessed by ELISA (Fig. 4C). No significant effects of IFN-β pretreatment were observed on CXCL1, CXCL2, or TNF-α expression (Fig. S3A). Prior exposure to IFN-β markedly increased the secretion of IL-6 and CXCL10 by NTHi-infected RAW264.7 cells (Fig. S3B).

Furthermore, pretreatment with IFN-β significantly elevated IL-10 mRNA and protein production by NTHi–infected PEMs (Fig. 4B and C), suggesting a direct influence of IFN-I on inflammatory response. Similar effects were detected in THP-1 cells, in which IFN-β markedly upregulated IL-6, IL-1β, IL-12p35, and CXCL10 mRNA, as shown by qPCR (Fig. 4D).

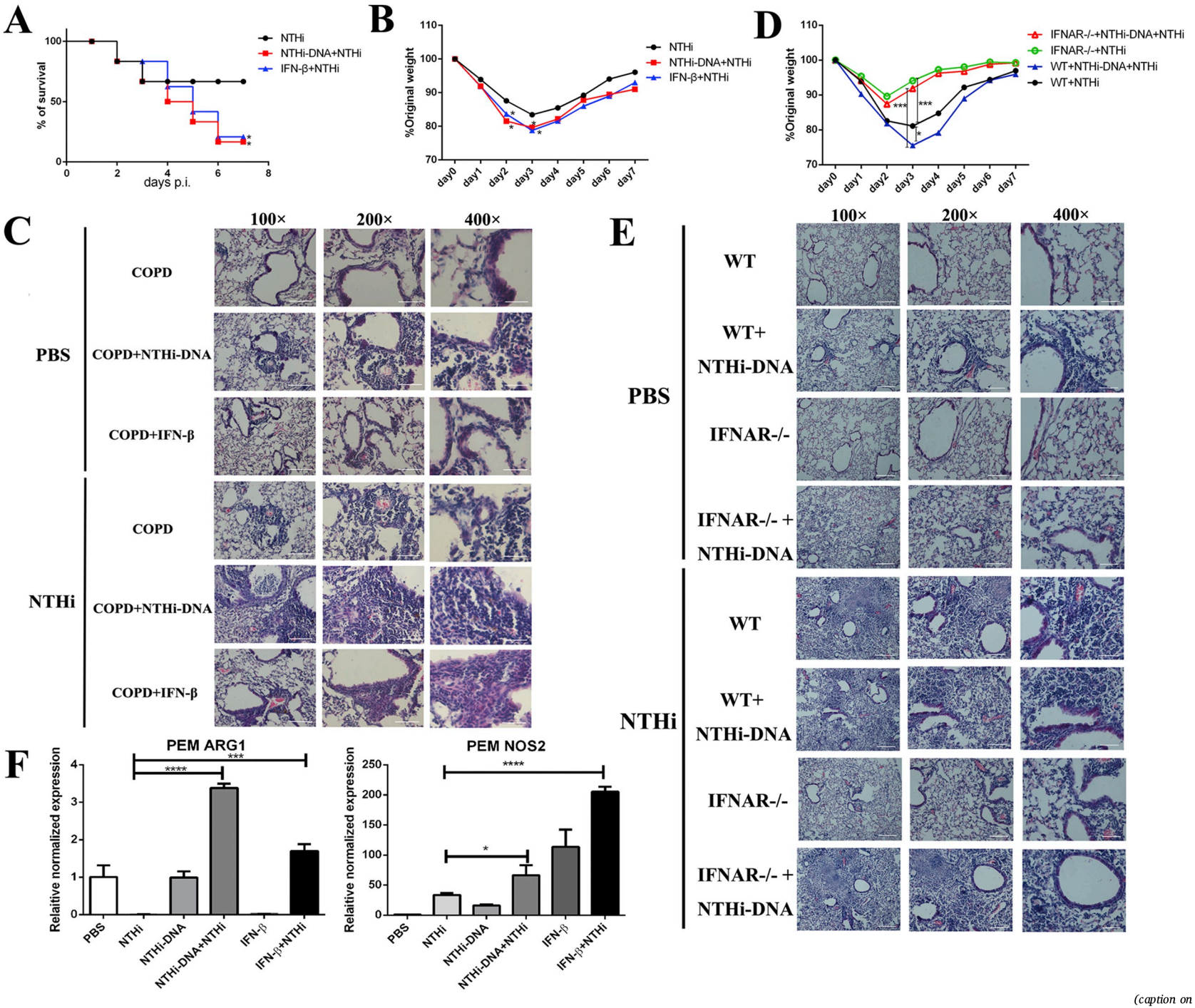

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Fig. 2. NTHi-DNA-induced IFN-I aggravates pulmonary inflammatory response during NTHi infection *in vivo*.
Wild type, mimic COPD mice (n = 6–8 mice) were infected intranasally with NTHi (1×10^8 CFU) at 24 h after intratracheally instillation with Dotap, 50 μg of NTHi-DNA in complex with Dotap or with murine IFN-β. (A, B) Survival and body weight was monitored for 7 days. Kaplan-Meier survival curves are shown. Statistical evaluation: Log-rank (Mantel-Cox) test. (C) Representative hematoxylin and eosin section from infected wild type, COPD mice showing peribronchial inflammatory infiltration 24 h p.i. Bars = 200 μm (left panel), 100 μm (mid panel), 50 μm (right panel). (D, E) WT and IFNAR−/− mice (n = 5–10 mice) were infected intratracheally with NTHi (1×10^8 CFU) at 24 h after intratracheally instillation with Dotap or 50 μg of NTHi-DNA in complex with Dotap. Body weight was monitored for 7 days (D). HE staining is used to detect pathological morphology of the lungs 24 h post infection. Bars = 200 μm (left panel), 100 μm (mid panel), 50 μm (right panel) (E). (F) Wild type PEMs was pre-incubated with media alone, NTHi-DNA (1.5 μg/ml) or IFN-β (100 U/ml) for 5 h, and infected with NTHi at an moi of 200. Total RNA was harvested and expression of indicated mRNA was analyzed by qPCR. Data are shown as mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

These observations collectively showed that type I IFN could significantly reinforce the inflammatory response to NTHi.

3.4. Production of inflammatory cytokines in response to NTHi is impaired in the absence of IFN-I signaling in vitro

To appraise the importance of type I IFN signaling during NTHi infection, we pre-incubated PEMs from WT or IFNAR−/− mice with 1.5 μg/ml NTHi-DNA, followed by infection with NTHi, and analyzed the expression of various cytokines and chemokines by qPCR and ELISA. Notably, the lack of a functional type I IFN signaling pathway led to a defect in IFN-β secretion by NTHi-infected macrophages (Fig. 5A). Transcript and protein levels of the pro-inflammatory cytokines IL-6, IL-1β, IL-12, and CXCL10 were significantly induced by NTHi infection in WT but not IFNAR−/− PEMs, independently of NTHi-DNA pretreatment (Fig. 5B and C). No significant differences in the production of CXCL1, CXCL2, or TNF-α were observed (Fig. S4). Consistently, the expression of the anti-inflammatory cytokine IL-10 was significantly decreased in PEMs from IFNAR−/− mice (Fig. 5B and C).

3.5. NTHi-DNA-induced IFN-I promotes pulmonary inflammatory cytokine production in response to NTHi in vivo

To investigate the role for NTHi-DNA-induced IFN-I in a model of disease *in vivo*, COPD mice were intratracheally instilled with NTHi-DNA (50 μg), and then intranasally infected with 1×10^8 CFU of NTHi. We observed that NTHi-DNA pretreatment alone resulted in expression of the cytokines IL-6, IL-1β, IL-12p70, and CXCL10 in COPD mice (Fig. 6A and B). In agreement with our *in vitro* results, NTHi-DNA pretreatment further stimulated the expression of the pro-inflammatory cytokines IL-6, IL-1β, IL-12, as well as of the chemokine CXCL10, in the lung of NTHi-infected COPD mice, compared to model mice only receiving NTHi infection. (Fig. 6A and B). IL-12p70 and CXCL10 levels in the lung of COPD mice were not significantly affected by NTHi-DNA priming (Fig. 6B). This analysis indicated that pro-inflammatory cytokine expression could partially depend on bacterial DNA sensing *via* the STING pathway.

To in-depth examine the role of IFN-I in pulmonary immunity, COPD mice were intratracheally administered with exogenous IFN-β, and subsequently intranasally infected with NTHi. We observed a significant increase in the expression of IL-6, IL-1β, IL-12, and CXCL10 in lung tissues of mice exposed to IFN-β prior to NTHi infection as compared to those only infected with NTHi (Fig. 6C). Next, we employed WT and IFNAR−/− mice to further establish the role of IFN-I signaling. The expression of IL-6, IL-1β, IL-12p40, and CXCL10 was induced by NTHi infection only in WT mice, whereas it was reduced in NTHi-infected IFNAR−/− mice, regardless of the presence of NTHi-DNA pretreatment (Fig. 6D).

Altogether, these results demonstrated that NTHi-DNA-induced IFN-I led to the upregulation of specific pro-inflammatory cytokines in NTHi-infected animals. This may account for the more severe inflammation observed by pathological examination in the lungs of COPD mice.

3.6. P38 MAPK activation is related to augmented inflammatory response mediated by NTHi-DNA-induced IFN-I

It has been demonstrated that type I IFN signaling can upregulate the expression of p38 MAPK and NF-κB during infection by *Franciscella tularensis* and *Acinetobacter baumannii* [29,30]. To investigate the mechanism by which NTHi-DNA-induced IFN-I regulates the production of inflammatory cytokines, we analyzed the level of phosphorylated p38 MAPK (P-p38) and NF-κB (P-p65) by immunoblot analysis in NTHi-infected macrophages with or without prior exposure to NTHi-DNA or IFN-β.

At 30 min post-infection, we observed that the priming of NTHi-infected PEMs with NTHi-DNA or IFN-β enhanced the level of P-p38 (Fig. 7A). However, no effects were detected on NF-κB activation (data not shown). To further validate this result, we pretreated PEMs with the p38 MAPK inhibitor SB203580. Notably, the increase in the level of the cytokines IL-6, IL-1β, IL-12p70, and CXCL10, induced by NTHi DNA or IFN-β priming followed by NTHi infection, was prevented by pretreatment with SB203580 (Fig. 7B).

Collectively, these findings indicated that the NTHi-DNA-induced IFN-I mediated enhancement of inflammatory cytokines was dependent on p38 MAPK activation.

4. Discussion

Lower airway infection by NTHi is responsible for approximately half of bacterial exacerbations of COPD. AECOPD caused by NTHi infection is serious, and disease progression is complex and heterogeneous. Here, we investigated the exact role of NTHi-DNA induced IFN-I signaling in macrophages and COPD by using a chronic pulmonary inflammation murine model. NTHi-DNA-induced IFN-I signaling played a harmful role in successful host defense against NTHi infection by modulation of inflammatory response and p38 MAPK signal is relative to the up-regulation of inflammatory cytokines.

The effect of type I IFN on the outcome of various bacterial infections is complex and unpredictable. Over the past few decades, numerous studies have revealed a pivotal role for type I IFNs in the regulation of host defense against bacterial infections, distinct from their effects on viral infections [31]. Importantly, at present we are unable to predict whether type I IFN would be protective or harmful based on the biological characteristics of pathogens. In the present work, NTHi-DNA-induced IFN-I impaired PEM resistance to NTHi infection by attenuating the phagocytic and killing abilities of macrophages. By using PEMs from IFN-I receptor knockout mice, we demonstrated that these macrophagic abilities were enhanced in the absence of IFN-I signaling in NTHi-infected mice. This effect is at odds with the study by Kaplan et al., which indicated that IFN-β enhances phagocyte killing of *S. aureus* in BMDCs [32]. On the other hand, our *in vivo* evidence suggests that NTHi-DNA-induced IFN-I markedly exacerbated COPD upon NTHi infection. Other previous findings indicated that impaired type I IFN signaling associates with a decreased risk of tuberculosis in humans [33], and our experiments with IFNAR−/− mice showed that type I IFN signaling increased the susceptibility to NTHi. Type I IFN expression seems to be detrimental also to the clearance of other bacterial

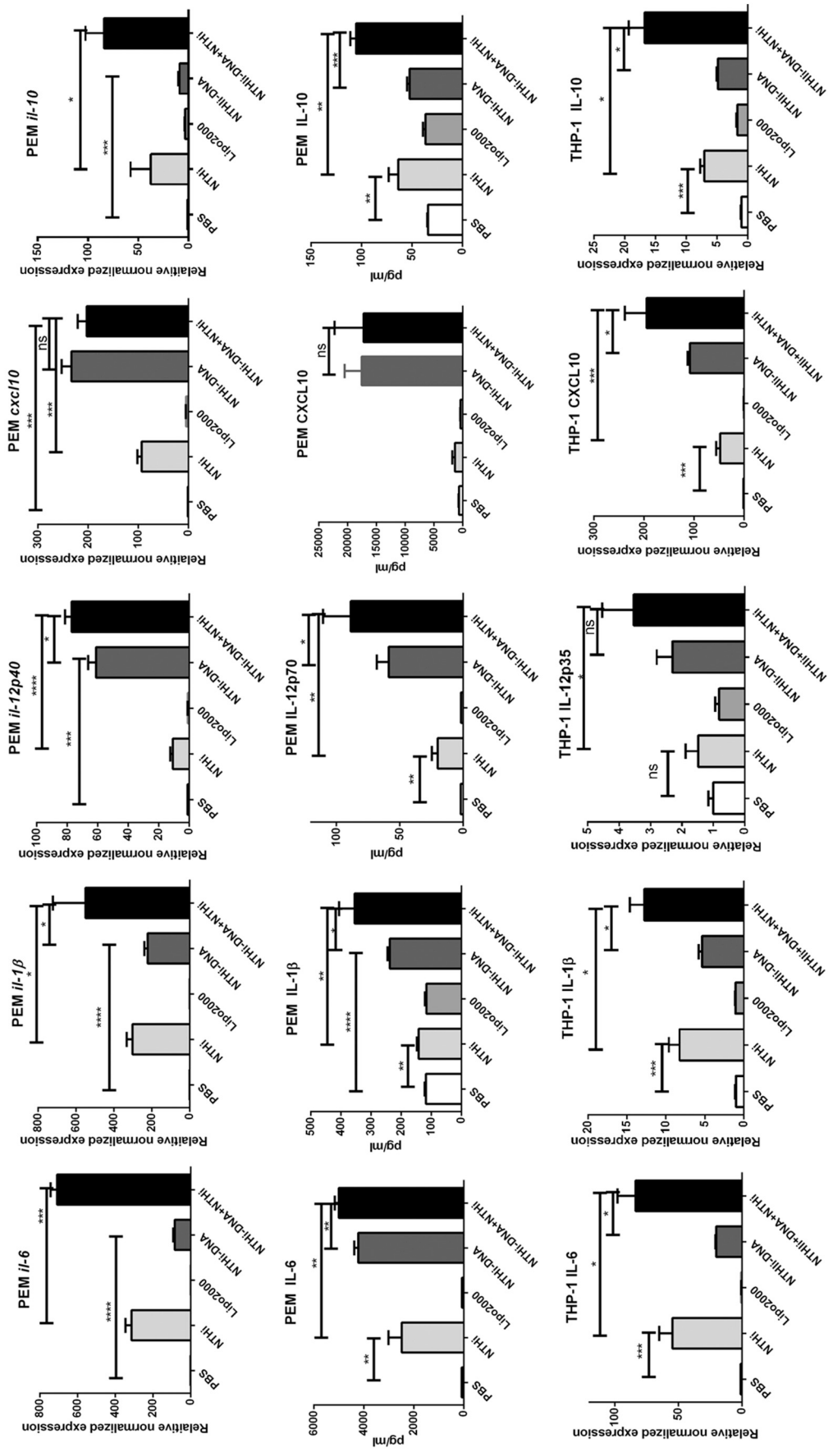

Fig. 3. NTHi-DNA-induced IFN-I up-regulate the expression of inflammatory cytokines after NTHi infection in macrophages.
Wild type PEMs was pre-incubated with media alone or 1.5 μg/ml NTHi-DNA for 5 h, and infected with NTHi at an moi of 200. (A) Total RNA was harvested p.i. 6 h and expression of inflammatory cytokines mRNA was analyzed by qPCR. (B) Culture supernatants were harvested 12 h after infection to measure inflammatory cytokines by ELISA assay. The Lipo2000 group served as a control group. (C) Human-derived THP-1 cells line were differentiated for 24 h in 100 ng/ml PMA prior to incubation cells line with media alone or 1.5 μg/ml NTHi-DNA for 5 h, gene expression of inflammatory cytokines was measured by qPCR 6 h after NTHi infection at an moi of 200. Data are shown as mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

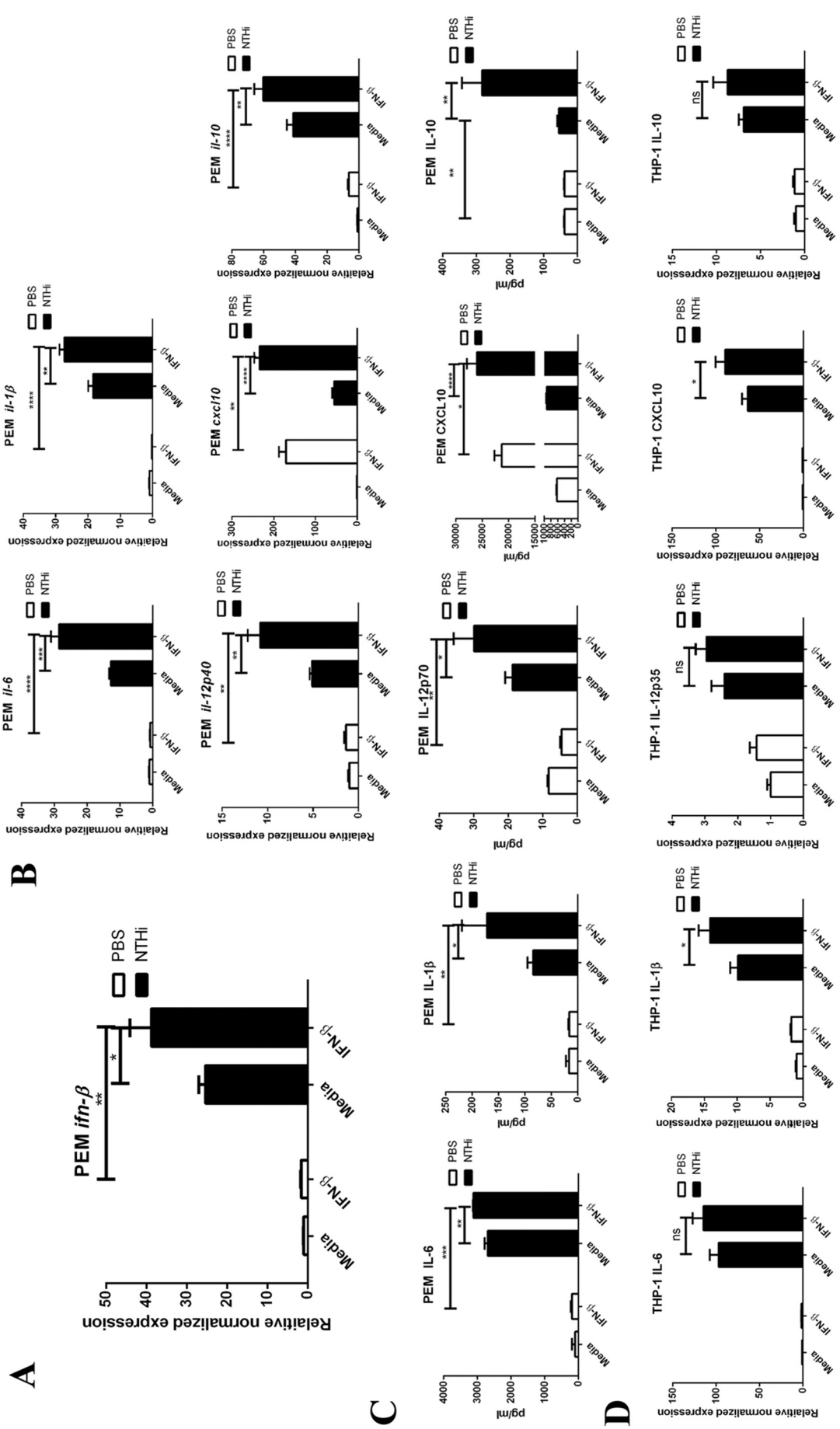

Fig. 4. Inflammatory cytokine profiles responses to NTHi infection of macrophages are promoted by exogenous IFN-β.
Wild type PEMs were pre-treated for 1 h with media alone or IFN-β (100 U/ml) and subsequently infected with NTHi at an moi of 200. (A, B) Total RNA was extracted and qPCR was performed to measure inflammatory cytokines expression. (C) Culture supernatants were harvested 12 h after infection to measure inflammatory cytokines by ELISA assay. (D) THP-1 cells line was infected with NTHi for 6 h prior to incubating with media alone or IFN-β (100 U/ml) for 1 h, and RNA was harvested and the transcription level of inflammatory cytokines was analyzed by qPCR. Data are shown as mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

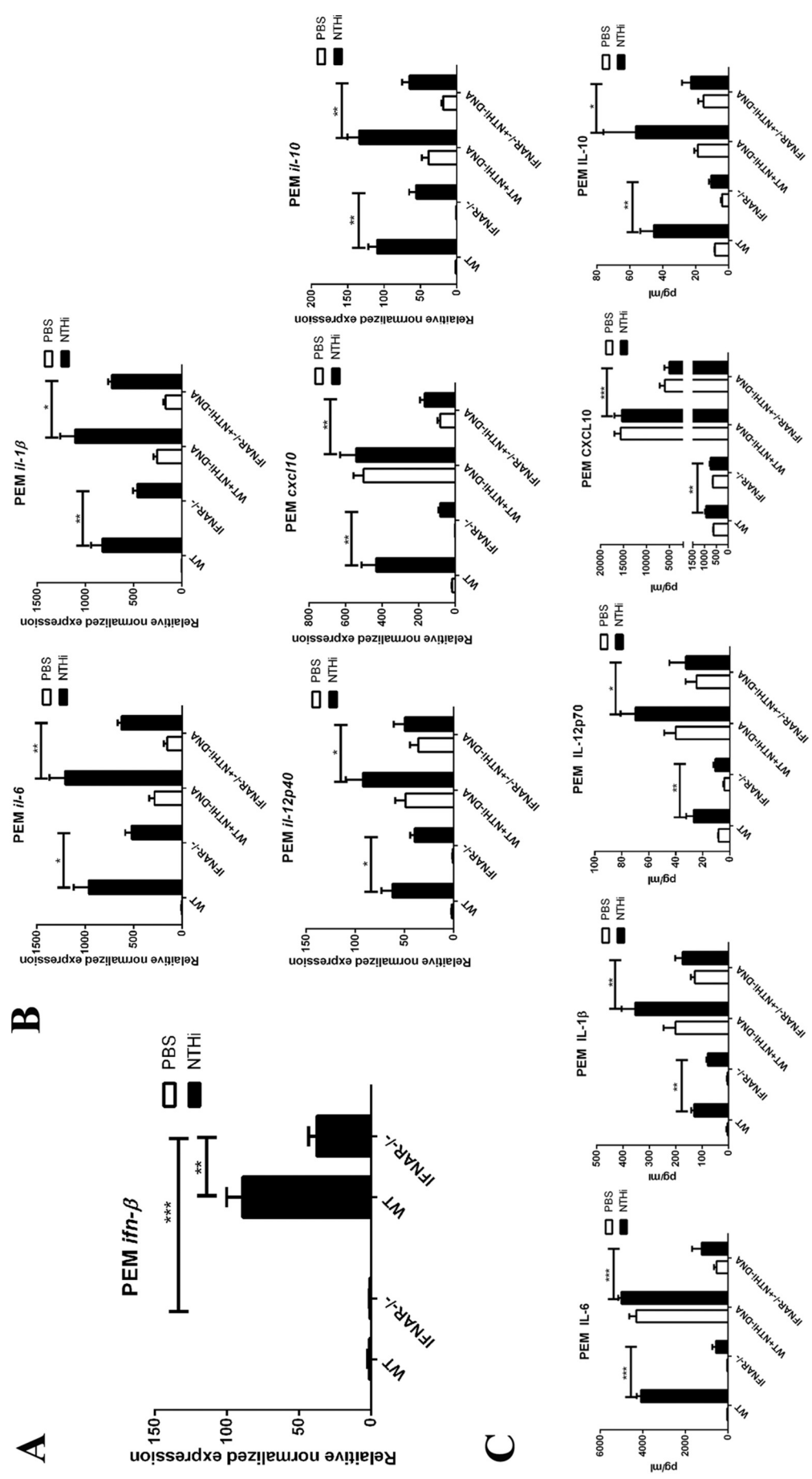

Fig. 5. Inflammatory cytokine expression is reduced following NTHi infection of IFNAR-deficient macrophages.
(A, B) Wild type or IFNAR −/− PEMs were pre-incubated with media alone or 1.5 μg/ml NTHi-DNA for 5 h, and subsequently infected with NTHi at an moi of 200 for 6 h and RNA harvested for analysis by qPCR. (C) Culture supernatants were harvested 12 h after treatment for analysis by ELISA assay. Data are shown as mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

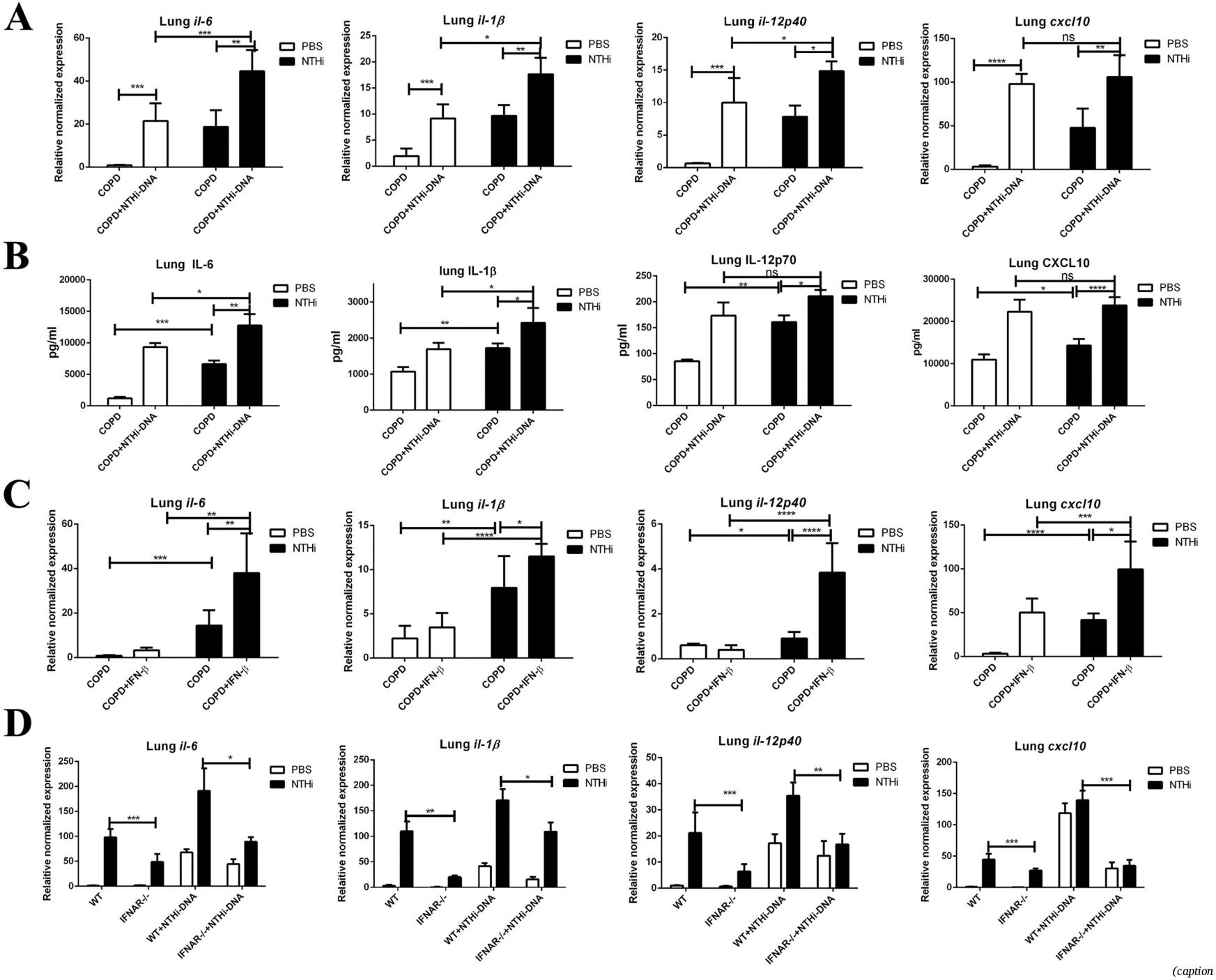

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Fig. 6. NTHi-DNA-induced IFN-I increases inflammatory cytokine profile in a disease model of NTHi infection.
Mimic COPD mice were infected intranasally with PBS or NTHi (1×10^8 CFU) after intratracheally instillation with Dotap, NTHi-DNA (50 μg) in complex with Dotap (A, B) or murine IFN-β (4000 U) (C). (D) WT and IFNAR−/− mice were infected intratracheally with NTHi (1×10^8 CFU) after intratracheally instillation with Dotap or NTHi-DNA (50 μg) in complex with Dotap. Expression of inflammatory cytokines in the lung collected 24 h post infection was determined using qPCR (A, C, D) and ELISA (B). Data are shown as mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

infections. Consistent with our results, Storek et al. reported that its absence in melanoma 2 (AIM2) was beneficial to host defense, and deletion of the cGAS-STING axis or IFNAR1 rendered the host resistant to infection by *F. novicida* [34,35], *Listeria monocytogenes*, *Salmonella enterica*, and *Mycobacterium leprae* [13,14,36]. In contrast, the immune regulation exerted by IFN-I was found to be advantageous in several bacterial infection models. Watanabe et al. indicated that type I IFN, induced by NOD1 sensing of *Helicobacter pylori* peptidoglycan, contributes to decreased susceptibility to infection *in vivo* [37]. In addition, type I IFN also plays a protective role in host defense against other bacteria, including L. *pneumophila*, *K. pneumoniae*, and *S. pneumoniae* [15,38,39]. The discrepancy in the role of type I IFN may be mostly due to differences between bacteria species. Most likely, this field will benefit from more in-depth and comprehensive analysis of type I interferon response during different bacterial infections.

Type I IFN has been reported to be a powerful immunomodulator in host antibacterial response, as it can control the balance between pro-inflammatory and anti-inflammatory factors [10]. However, the function of these factors in host defense against NTHi infection is not well characterized. The present study indicated that NTHi-DNA-induced IFN-I signaling promoted inflammatory responses by enhancing the expression of the pro-inflammatory cytokine IL-6, IL-1β, IL-12, and of the chemokine CXCL10, thus aggravating macrophage infection and COPD disease following NTHi infection. In addition, we observed an accompanied increase in the levels of the anti-inflammatory cytokine IL-10, which is known to be induced by type I IFN and has a suppressive impact on both innate and adaptive immunity [40–42]. In light of the enhanced pro-inflammatory response, we hypothesized that increase in IL-10 level plays a role in the balance and coordination of the inflammatory response. Our discovery was unexpected because IFN I-induced upregulation of anti-inflammatory IL-10 was reported to decrease the production of pro-inflammatory cytokines and to inhibit bacterial killing during infection by *Salmonella enterica* and *M. tuberculosis* [14,40]. By contrast, our data showed that the effect of IFN-I signaling, mainly pro-inflammatory, was detrimental to host antibacterial immunity, both in isolated macrophages and in a mouse model of COPD infection. No significant effects of type I IFN signaling were observed on the expression of CXCL1, CXCL2, and TNFα, which was different from a previous study, in which IFN-I-induced suppression of CXCL1 and CXCL2 reduced neutrophil responses and contributed to the control of bacterial outgrowth [43]. Moreover, TNF-α directly promoted bacterial control in an influenza–*Streptococcus pneumoniae* coinfection model [44].

It is well established that downstream effectors of toll-like receptors (TLRs), such as p38 MAPK and NF-κB, are major contributors to inflammation, and are important for IFN-I-mediated activation of inflammatory cytokines during infection by *F. tularensis* [29,45]. In the current study, we found elevated level of P-p38 MAPK, but no effects on NF-κB phosphorylation in NTHi-DNA- or IFN-β-pretreated macrophages after NTHi infection. Moreover, the expression of the cytokines IL-6, IL-1β, IL-12p70, and CXCL10 was suppressed by the p38 MAPK inhibitor SB203580 in macrophages from mice that had been primed with NTHi-DNA or IFN-β and subsequently infected with NTHi. However, Punturieri and colleagues found that co-stimulation with the NTHi-derived outer membrane proteins PCP and IFN-β induces the activation of STAT1 and IRF-1 without affecting MAPK expression [46]. This discrepancy is likely due to differences intrinsic to whole bacteria and single bacterial protein used. Notably, we employed a more complex whole bacteria than single bacterial protein, which is a suitable model for studying infectious diseases. Although we found p38 MAPK to be involved in NTHi-DNA-induced IFN-I-mediated inflammation following NTHi infection, the contribution of other signaling molecules cannot be excluded. Previous studies showed that STING is responsible for the production of both pro- and anti-inflammatory cytokines [47,48]. Our data showed that NTHi-DNA alone was able to activate p38 MAPK and inflammatory cytokine expression, suggesting that, in addition to NTHi-DNA-induced IFN-I, STING also had an impact on the secretion of pro-inflammatory cytokines. However, Kang Chen et al. demonstrated that STING signaling, but not type I IFN, suppressed inflammatory cytokine production by inhibiting NF-κB activation, thereby enhancing *Pseudomonas aeruginosa* killing [45]. Nonetheless, further research is needed to clarify the roles of bacterial DNA, STING, and inflammatory cytokines in host defense against bacterial infection.

Intracellular bacteria such as *Mycobacterium tuberculosis(M.tb)* and dsDNA viruses such as Hepatitis B virus (HBV) can induce type I IFN response. *M.tb*-derived DNA and *c*-di-AMP induce type I IFN response *via* the STING/TBK1/IRF3 pathway, whereas *M.tb* RNA initiates the response *via* the RIG-I/MAVS/TBK1/IRF7 signaling pathway [49–52]. HBV genome-derived dsDNA also triggers type I IFN production *via* the cGAS-STING signaling pathway. We previously reported that NTHi-DNA induces the production of type I IFN (IFN-β and IFN-α) *via* the cGAS-STING signaling pathway [19], which is similar to that seen with *M.tb* and HBV. No other findings have been made so far, but there may be other signaling pathways to recognize NTHi-DNA or secondary metabolites to induce type I IFN response. This warrants further clarification.

Type I IFN is both beneficial and harmful to the host against pathogen infections. During infection with the intracellular bacteria *Brucella abortus* or the dsDNA virus HBV, the STING-dependent type I IFN pathway upregulates inflammatory cytokine gene expression to confer host protection [25,53]. In contrast, type I IFN inhibits production of the protective cytokines TNF-α, and IL-12 and attenuates IFN-γ–mediated bacterial growth inhibition and killing in *M.tb* infected-macrophages [40,54]. Here, our results demonstrate that NTHi-DNA-induced type I IFN promotes host susceptibility to NTHi through upregulating the production of IL-6, IL-1β, IL-12, and CXCL10. Therefore, it is unique that NTHi-DNA will mediate a type of IFN-I production different from that seen with other intracellular bacteria/ds DNA viruses.

It has been reported that the pattern of autoantibody reactivities in patients may correlate with COPD disease status [55]. Patients with systemic lupus erythematosus exhibit symptoms of type I interferonopathy, including tissue inflammation and elevated anti-DNA antibody titers, and have a significantly higher risk of developing COPD compared with the control population [56]. This suggests that the sustained exposure to abnormal DNA may lead to high risk of COPD.

In summary, our report highlights a detrimental role of NTHi-DNA-induced IFN-I signaling in host defense against NTHi infection, leading to excessive inflammation. This study expanded our previous research on the relationships between NTHi-DNA-induced IFN-I and the host immune system. Moreover, we present clear evidence that interfering with this interaction may allow to control the outcome of NTHi infection in COPD. Restoration of effective bacterial clearance and control over inflammatory responses could substantially advance the clinical management of COPD.

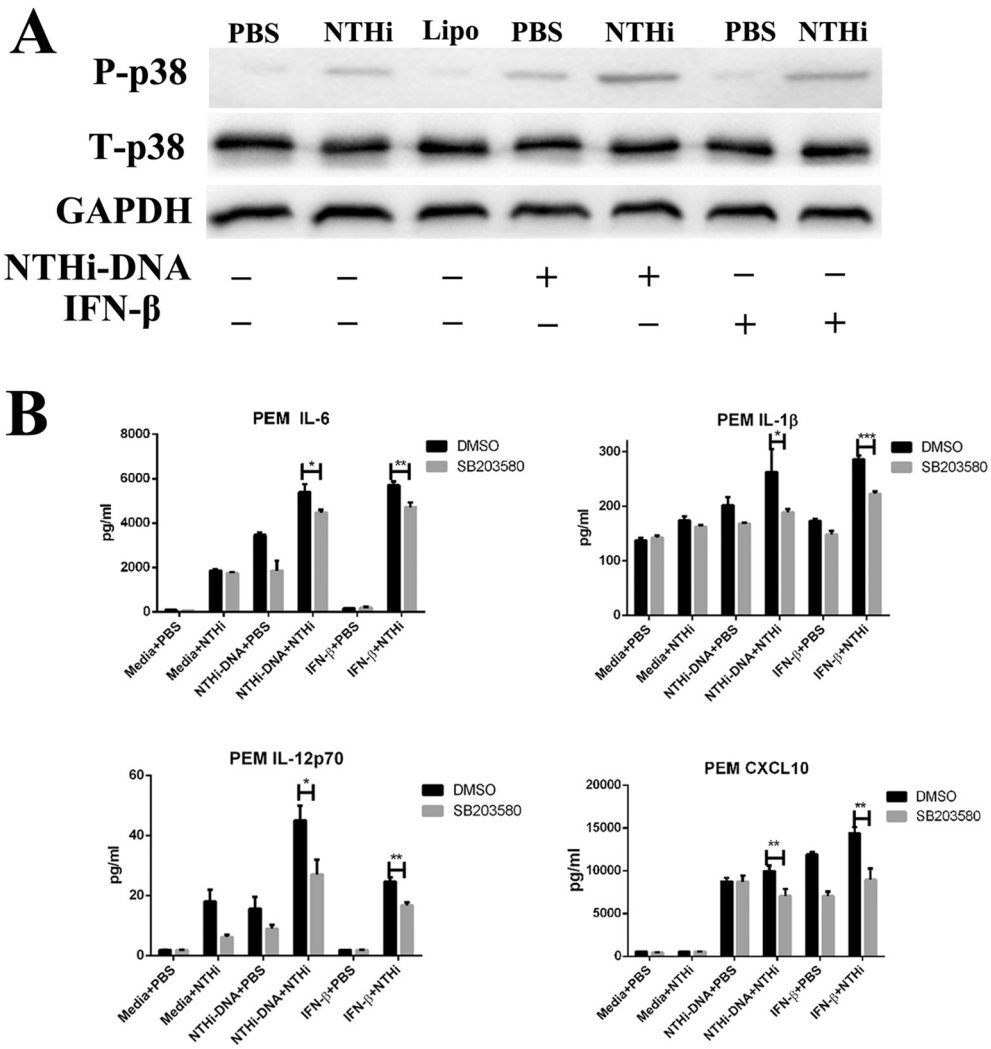

Fig. 7. NTHi-DNA-induced IFN-I mediated-inflammatory cytokine profile involve in the activation of p38 MAPK signal pathway.
Wild type PEMs was pre-incubated with media alone, 1.5 μg/ml NTHi-DNA or IFN-β for 5 h, and infected with NTHi at an moi of 200. (A) The protein level of P-p38 was analyzed by western blot 30 min post infection. (B) Inflammatory cytokines in culture supernatants pretreatment with P38 inhibitor SB203580 (20 μM) before addition of NTHi-DNA or IFN-β were measured by ELISA assay 12 h post infection. Data are shown as mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

Ethics statement

Animal experiment in this research were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chongqing Medical University and were authorized by the Animal Ethics Committee of Chongqing Medical University.

Author contribution statement

Conceived and designed the experiments: SHY, YBY and WCX. Performed the experiments: SHY, YG and HYL. Analyzed the data: SHY, JW and XMZ. Wrote the manuscript: SHY, XXH and HW. Reviewed and edited the manuscript: YBY, HW.

Declaration of Competing Interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105710.

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