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BASIC SCIENCE ARTICLE

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Granulocyte-macrophage colony-stimulating factor suppresses induction of type I interferon in infants with severe pneumonia

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BACKGROUND: The underlying mechanisms for infantile bronchopneumonia development remain unknown.
METHODS: Peripheral blood mononuclear cell (PBMCs) and serum derived from severe and mild infantile bronchopneumonia were obtained, and the expression of various molecules was detected with enzyme-linked immunosorbent assay and quantitative PCR. Such molecules were also detected in granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced bone marrow-derived NFκB2$^{-/-}$ dendritic cells (DCs) or NIK SMI1 (NF-κB-inducing kinase inhibitor) administrated DCs.
RESULTS: The relative mRNA expression levels of type I interferons (IFNs) (*IFN-α4*, *IFN-β*), Th17 cell-associated markers (interleukin-17A, retinoic-acid-receptor-related orphan nuclear receptor gamma, and *GM-CSF*), and non-canonical NF-κB member (*NFκB2*) were significantly up-regulated in PBMCs and DCs derived from infantile bronchopneumonia compared with healthy controls. However, compared with Th17 cell-associated markers and non-canonical NF-κB molecules, the expression of IFN-α4 and IFN-β was significantly inhibited in severe infantile bronchopneumonia compared with mild infantile bronchopneumonia. The relative protein expression of the above molecules also showed a similar expression pattern in the PBMCs or serum. NF-κB2 knockout or NIK SMI1 administration could reverse the diminished expression of IFN-β in GM-CSF-induced bone marrow-derived DCs.
CONCLUSIONS: GM-CSF-dependent non-canonical NF-κB pathway-mediated inhibition of type I IFNs production in DCs contributes to the development of severe bronchopneumonia in infant.

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IMPACT:

- Granulocyte-macrophage colony-stimulating factor-dependent non-canonical NF-κB pathway-mediated inhibition of type I IFNs production in dendritic cells is critical for the development of infantile bronchopneumonia.
- Our findings reveal a possible mechanism underlying the development of severe infantile bronchopneumonia.
- The results could provide therapeutic molecular target for the treatment of such disease.

INTRODUCTION

As the most common form of pneumonia with suppurative and localized inflammation induced by viral and/or bacterial infection in patches around bronchi, bronchopneumonia is the leading cause of death in under-five children and accounts for 85% of respiratory system diseases in under-two children.[1,2] Due to under-developed respiratory anatomies and immune systems, susceptible infants are accompanied by high fever and febrile convulsions, leading to respiratory and heart failure.[3,4] Antibiotics and antiviral chemicals are mainly utilized in the clinic. Due to the severe adverse reactions and overuse of antibiotics, the efficacy of such treatment is decreased.[5] Therefore, it is urgent to seek more effective therapeutic strategies for infantile bronchopneumonia.

Interferon (IFN) is a multifunctional cytokine family, which can be classified into three main types: I (α or β), II (γ), and III (λ).[6–8] Type I IFNs may be produced immediately after bacterial, viral, and fungal infection to enhance or dampen the innate and adaptive immune reaction depending on the context.[9,10] This ambiguous contribution of type I IFNs to infantile bronchopneumonia is still incompletely understood. Dendritic cells (DCs) are considered as the highest IFN producers, and the primary function of type I IFNs could be attributed to DCs. The role of DC-derived type I IFNs in severe infantile bronchopneumonia is unknown.

In this study, we aimed to explore the effects of T helper type 17 (Th17)-associated granulocyte-macrophage colony-stimulating factor (GM-CSF) on the induction of type I IFNs and decipher the underlying mechanisms.

METHODS AND MATERIALS

Infantile bronchopneumonia

Hospitalized 30 infants with bronchopneumonia in the Daqing Oilfield General Hospital were enrolled. The infants who need to be delivered to the intensive care unit (ICU) with respiratory failure were defined as severe infantile bronchopneumonia, and the other infants who needed to be observed in the hospital were defined as mild infantile bronchopneumonia. Clinical and demographic information was collected and chest

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radiography or chest computed tomography imaging was conducted to confirm the diagnosis. Thirty age-matched healthy controls were randomly selected on the routine exam without infection within 4 weeks. Peripheral blood mononuclear cells (PBMCs) were obtained with the MACSprep PBMC Isolation Kit, and BD Vacutainer™ Venous Blood Collection Tubes were applied to obtain the serum following the manufacturer's advice. This study was approved by the Institutional Ethical Committee of Daqing Oilfield General Hospital. Written consent was obtained from all the guardians related to the participant.

Mice

C57BL/6 mice (8 weeks old) and Nfkb2$^{tm1a(EUCOMM)Hmgu}$ mice with B6 background were obtained from GemPharmatech (Nanjing, China). Animal studies were approved by the institutional animal care and use committee of Daqing Oilfield General Hospital.

GM-CSF-induced bone marrow-derived DC generation

Flushed bone marrow cells derived from the femur and tibia of Nfkb2$^{tm1a(EUCOMM)Hmgu}$ mice or C57BL/6 mice were further purified by gradient centrifugation with Lymphocyte Separation Medium (Sigma-Aldrich, St. Louis, MO). The enriched mono-nucleated cells were cultured in RPMI 1640 media (10% fetal bovine serum, 10 ng/ml GM-CSF, and 10 ng/ml interleukin (IL)-4, Invitrogen, Waltham, MA). Fresh media containing the floating cells was replaced on day 3, and half the amount of media was replaced on day 6, then GM-CSF-induced bone-marrow-derived DCs were obtained for further experiments on day 7.

Quantitative polymerase chain reaction (qPCR) assay

RNeasy Mini Kit (Qiagen, Valencia, CA) was applied to extract total RNA from human PBMCs and mice bone marrow-derived DCs following the manufacturer's instructions, which was further reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen). The amplification of double-stranded DNA was assayed with Brilliant II SYBR® Green qPCR Mastermix (Agilent Technologies, Santa Clara, CA). The amplification procedure was set as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min on Qiagen's Rotor-Gene 6000 cycler. The relative mRNA expression was quantified with the comparative ΔCT method and normalized to *Actb* (β-actin) expression. The sequences of primers were as following: human *IL17A* forward, CGGACTGTGATGGTCAACCTGA, human *IL17A* reverse, GCACTTTGCCTCCCAGATCACA; human RORγt (*RORC*) forward, GAGGAAGTGACTGGCTACCAGA, human RORγt (*RORC*) reverse, GCACAATCTGGTCATTCTGGCAG; human *GM-CSF* forward, GGAGCATGTGAATGCCATCCAG, human *GM-CSF* reverse, CTGGAGGTCAAACATTTCTGAGAT; human nuclear factor (NF)-κB Inducing Kinase NIK (*MAP3K14*) forward, GGAATACCTCCACTCACGAAGG, human *MAP3K14* reverse, CTGTGAGCAAGGACTTTCCCAG; human p52/p100 (*NFκB2*) forward, GGCAGACCAGTGTCATTGAGCA, human p52/p100 reverse, CAGCAGAAAGCTCACCACACTC, human *RELB* forward, TGTGGTGAGGATCTGCTTCCAG, human *RELB* reverse, TCGGCAAATCCGCAGCTCTGAT; mouse *MAP3K14* forward, CAAGCAGACAGGCTTCCAGTGT, mouse *MAP3K14* reverse, GCCTTCTCTCACAGCTCCATAG; mouse p52/p100 forward, TGCTGATGGCACAGGACGAGAA, mouse p52/p100 reverse, GTTGATGACGCCGAGGTACTGA; mouse *RELB* forward, GTTCTTGGACCACTTCCTGCCT, mouse *RELB* reverse, TAGGCAAAGCCATCGTCCAGGA; mouse *Ifn-α4* forward, GCAATGACCTCCATCAGCAGCT, mouse *Ifn-α4* reverse, GTGGAAGTATGTCCTCACAGCC; mouse *Ifn-β1* forward, GCCTTTGCCATCCAAGAGATGC, mouse *Ifn-β1* reverse, ACACTGTCTGCTGGTGGAGTTC.

Immunoblot assay

The bone marrow-derived DCs and PBMCs were lysed with the radioimmunoprecipitation solution, and the supernatants (50 μg) were separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, which were further blocked with 5% non-fat dry milk and incubated with the primary antibodies specific for NIK (A-12, Santa Cruz, Dallas, TX), Lamin B (8D1, Santa Cruz), RelB (D-4, Santa Cruz), GAPDH (6C5, Santa Cruz), and p100/p52 (TB4, NCI) at a 1:1000 dilution at 4 °C overnight, and then incubated in peroxidase-conjugated secondary antibody (Sigma-Aldrich) at a 1:1000 dilution for 1 h at room temperature. The signal was developed with an ECL system (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom), and the relative intensity was normalized with GAPDH expression with NIH-Image J1.51p 22.

Enzyme-linked immunosorbent assay (ELISA) assay

The serum or supernatant concentrations of IL-17A and GM-CSF were detected with commercial ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. All standards and samples were measured with a SpectraMax M5 microplate reader at a wavelength of 450 nm.

Statistical analysis

Statistical analysis was performed with Graph-Pad Prism 6.01. Data are presented as mean ± SEM. One-way analysis of variance with a multiple comparison test was performed to reveal the statistical significance between groups. A p value <0.05 was considered statistically significant.

RESULTS

Inhibited type I IFN inductions in PBMCs of severe infantile bronchopneumonia

We collected 30 healthy infants and 30 pneumonia infants with different clinical severity and detected the relative expression of type I IFNs and multiple chemokines in PBMCs. The results showed that the relative expression of type I IFNs (*IFN-α4* and *IFN-β*) and chemokines (*ICAM1*, *VCAM1*, and *MMP9*) were significantly up-regulated in infants with pneumonia compared with healthy controls (Fig. 1a, b). Correspondingly, the expression levels of *ICAM1*, *VCAM1*, and *MMP9* were highly expressed in severe infantile bronchopneumonia compared with mild infantile bronchopneumonia (Fig. 1b). In contrast, compared with mild infants, these two type I IFN expression levels were significantly inhibited in severe infants (Fig. 1a, $p < 0.001$). These results indicated that type I IFN production was inhibited in severe infantile bronchopneumonia compared with mild infantile bronchopneumonia.

Up-regulated Th17-related markers in severe infantile bronchopneumonia

In order to explore the down-regulation of type I IFNs in infants with severe bronchopneumonia, the relative expression of Th17-related markers was detected. The molecular markers related to Th17 cells, such as *IL-17A*, *RORγt*, and *GM-CSF*, were significantly up-regulated in PBMCs of infants with bronchopneumonia compared with the healthy group. It was further found that such up-regulation was observed in severe infantile bronchopneumonia compared with mild infantile bronchopneumonia (Fig. 2a). Up-regulated IL-17A and GM-CSF concentration was also observed in the serum of infants with severe bronchopneumonia compared with infants with mild bronchopneumonia (Fig. 2b). These results indicated that up-regulated Th17-related markers or Th17 cells might contribute to the progress of infantile bronchopneumonia.

GM-CSF promotes the activation of non-canonical NF-κB

To study whether GM-CSF from Th17 could play a role in this process, we first detected the relative expression of non-classical NF-κB signaling pathway molecules. The results showed that the relative mRNA expression of p52/p100 increased significantly in PBMC of infants with severe bronchopneumonia compared with healthy or mild infants, while the mRNA levels of *MAP3K14* and *RELB* did not change significantly (Fig. 3a). The protein levels of non-classical NF-κB family molecules p52 and RELB in the nucleus of PBMC in infants with severe pneumonia increased significantly compared with healthy or mild infants (Fig. 3b). Similarly, GM-CSF-induced, bone-marrow-derived DCs showed up-regulated *NFκB2* expression compared with bone marrow cells, while there was no significant change in *MAP3K14* and *RELB* expression (Fig. 3c). The protein level of p52 and RelB also showed up-regulation in both the cytoplasmic (Fig. 3d) and nuclear (Fig. 3e), and up-regulated *MAP3K14* expression could also be observed in the cytoplasm.

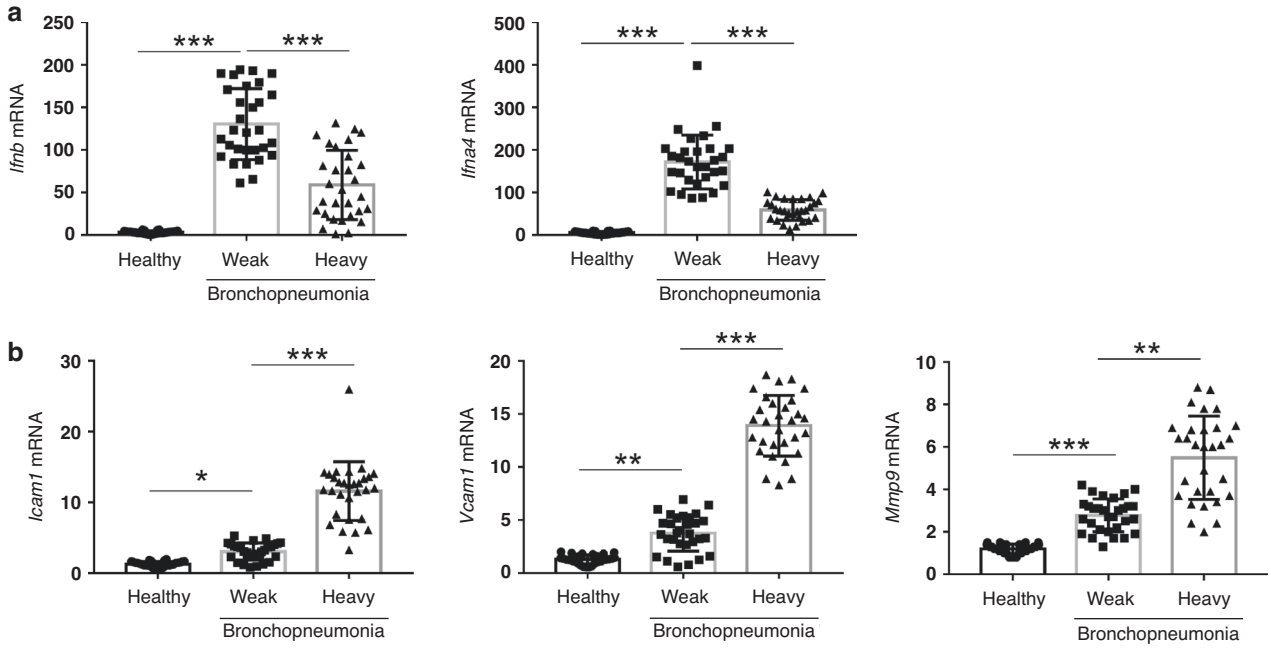

Fig. 1 The inductions of type I interferons (IFNs) were significantly inhibited in peripheral blood mononuclear cells (PBMCs) from infants with heavy bronchopneumonia. a The relative mRNA levels of IFN-α4 and IFN-β1 in the PBMCs from the infants with bronchopneumonia or healthy controls were assessed by quantitative PCR (qPCR) ($n = 30$). **b** The relative mRNA levels of intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion protein 1 (VCAM1), and matrix metallopeptidase 9 (MMP-9) in the PBMCs of the infants with bronchopneumonia were detected by qPCR ($n = 30$). The data are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

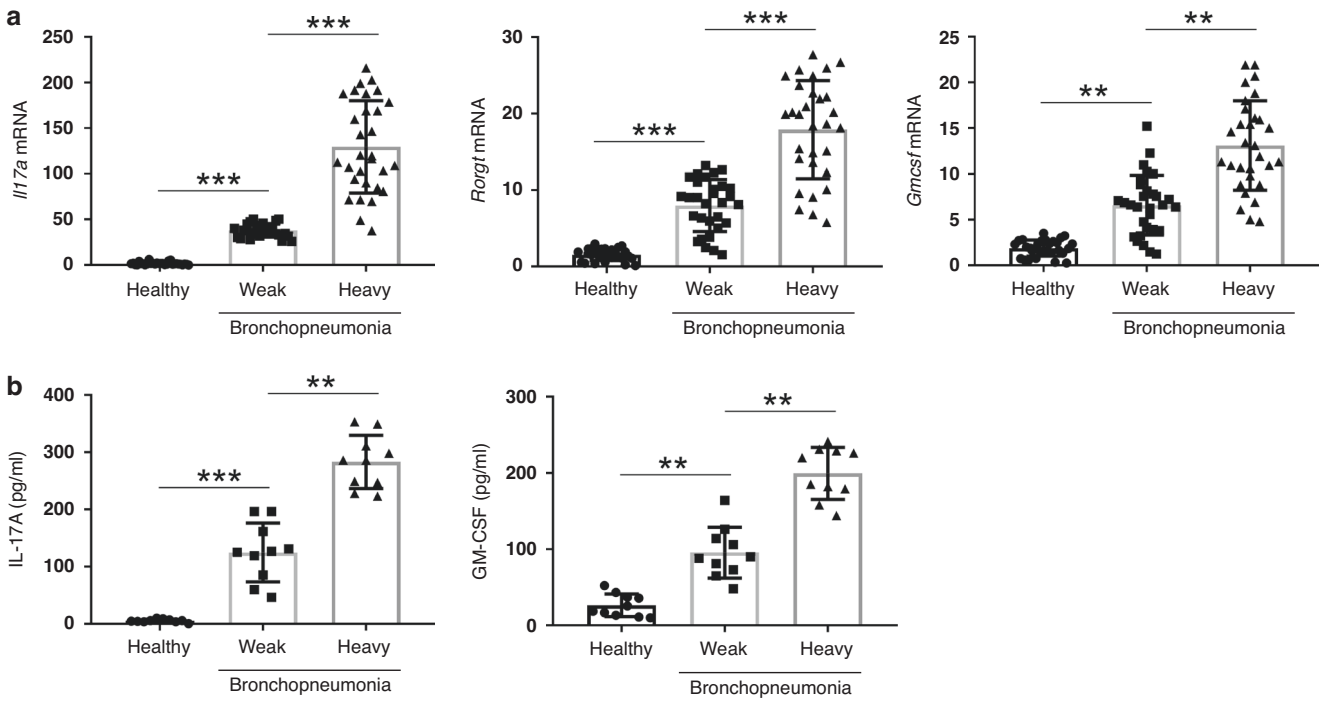

Fig. 2 The infants with heavy bronchopneumonia displayed an increased expression of Th17-related genes. a The relative mRNA levels of interleukin (IL)-17A, RAR-related orphan receptor C (RORC), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the PBMCs of infants with bronchopneumonia or healthy controls were assessed by qPCR ($n = 30$). **b** ELISA assay was used for evaluating the protein levels of IL-17A and GM-CSF in the serum of infants with bronchopneumonia. The data are expressed as mean ± SEM. **$p < 0.01$; ***$p < 0.005$.

These results indicated that non-canonical NF-κB pathway molecules were up-regulated in the PBMCs of infants with severe bronchopneumonia, and GM-CSF could significantly induce the activation of non-classical NF-κB pathway in bone marrow-derived DCs.

GM-CSF suppresses the induction of type I IFNs via the activation of non-canonical NF-κB pathway

We then directly detected the effect of GM-CSF on poly I:C-induced type I IFN production. As expected, poly I:C could

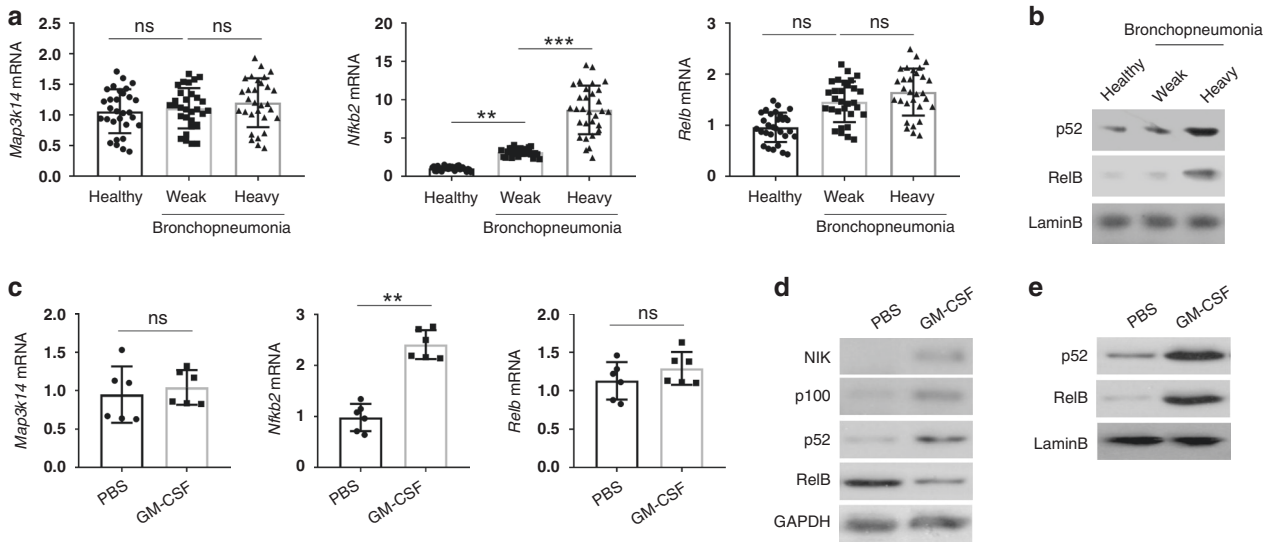

Fig. 3 GM-CSF promoted the activation of non-canonical nuclear factor-κB (NF-κB). a The relative mRNA levels of non-canonical NF-κB members in the PBMCs from the infants with bronchopneumonia or healthy controls were assessed by qPCR ($n = 30$). **b** IB analysis of non-canonical NF-κB signal in the nuclear extract of PBMCs from the infants with bronchopneumonia or healthy controls. **c** Bone marrow cells were cultured with GM-CSF (20 ng/ml) for 7 days. The expression levels of non-canonical NF-κB members were determined by qPCR. **d**, **e** IB analysis of the indicated total proteins in cytoplasmic (**d**) and nuclear (**e**) extracts of bone marrow cells treated with GM-CSF (20 ng/ml) for 7 days. The data are expressed as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.005$.

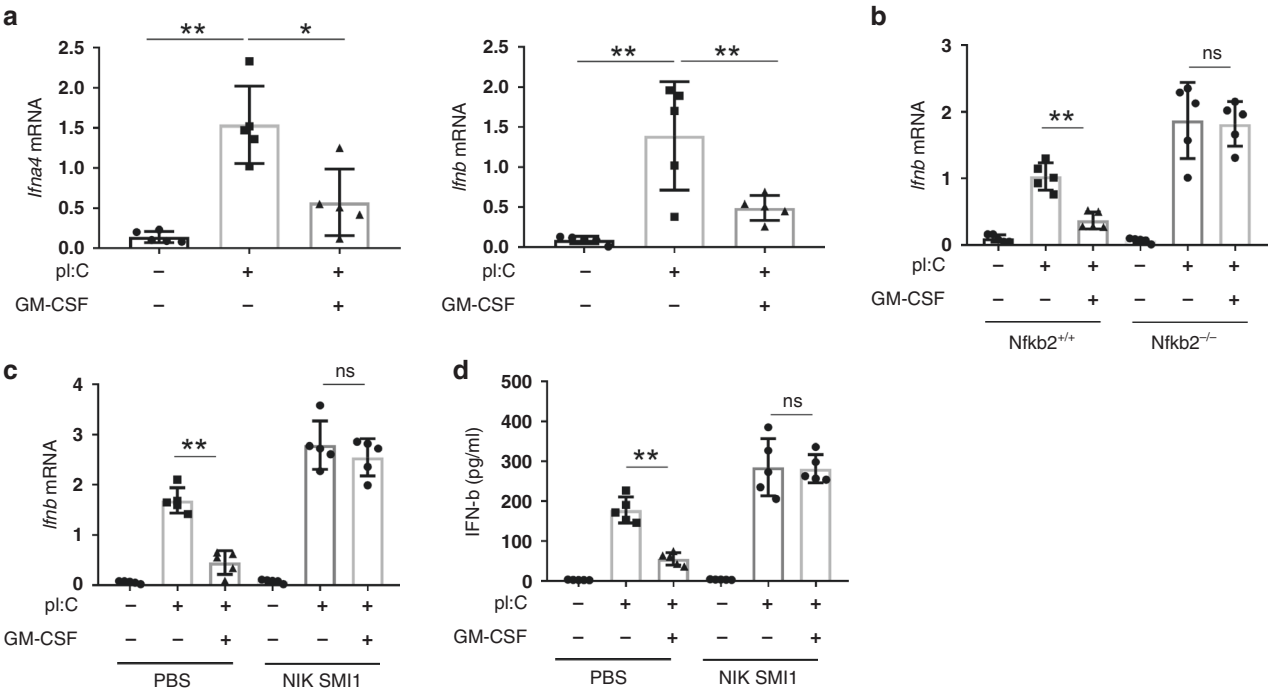

Fig. 4 GM-CSF suppressed the induction of type I interferon via activating the non-canonical NF-κB pathway. a Murine bone marrow-derived dendritic cells were incubated in GM-CSF (20 ng/ml) for 24 h and stimulated with poly I:C (20 μg/ml) as indicated. The expression levels of Ifna4 and Ifnb1 were determined by qPCR. "b" NfκB2$^{+/+}$ and Nfκb2$^{-/-}$ bone marrow-derived dendritic cells pretreated with GM-CSF (20 ng/ml) for 24 h and stimulated with poly I:C (20 μg/ml) for 6 h. The expression of IFN-β1 was determined by qPCR. **c**, **d** WT bone marrow-derived dendritic cells were pretreated with NIK SMI1 (0.5 nM) for 24 h and stimulated with poly I:C (20 μg/ml) for 6 h. The expression of Ifnb1 was determined by qPCR (**c**) and ELISA (**d**). Actin was utilized as a reference gene. Data are presented as mean ± SEM values. $^{*}p < 0.05$; $^{**}p < 0.01$.

significantly influence the production of IFN-α4 and IFN-β, and such effects could be inhibited by the administration of GM-CSF (Fig. 4a). While such inhibition was diminished in the NFκB2 knockout bone marrow-derived DCs (Fig. 4b), all these results indicated GM-CSF-dependent NFκB2-mediated type I IFN production inhibition. In order to verify whether a non-classical NF-κB inhibitor can restore the inhibited expression of type I IFNs in infants with severe pneumonia, we treated GM-CSF-induced DCs with NIK inhibitor, NIK SMI1, to detect the expression of type I IFNs induced by poly I:C. The data showed that inhibiting NIK activity can significantly restore the inhibited mRNA expression (Fig. 4c) and protein expression (Fig. 4d) of IFN-β caused by GM-CSF

administration. These data demonstrated that GM-CSF suppressed the induction of type I IFNs via the activation of the non-canonical NF-κB pathway, which may contribute to the diminished expression of type I IFNs in severe infantile bronchopneumonia.

DISCUSSION

In this investigation, we find that type I IFNs are significantly up-regulated in PBMCs of infants with bronchopneumonia compared with healthy infants. Such up-regulation is diminished in severe bronchopneumonia when compared with mild bronchopneumonia. Up-regulated Th17 cell-associated markers, such as *IL-17A*, *RORγt*, and *GM-CSF*, are correlated with the severity of bronchopneumonia. GM-CSF promotes the activation of non-canonical NF-κB, which could down-regulate the expression of type I IFNs. Up-regulated *ICAM1*, *VCAM1*, and *MMP9* expression indicate increased cell adhesion or lymphocyte exudation during inflammation. These results suggest that the differential regulation of Th17-associated marker expression and type I IFN production in bronchopneumonia and Th17 cell-associated GM-CSF may contribute to the down-regulation of type I IFNs through non-canonical NF-κB activation.

Bronchopneumonia is mainly caused by bacterial, viral, or fungal infection, and Th17 cells are reported to mediate the immunophysiological reaction and immunopathological damage depending on the reaction intensity,[11,12] which are the key source of GM-CSF-producing cells.[13,14] On the other hand, GM-CSF is vital for the proinflammatory functions of Th17 cells, which can promote IL-23-driven pathogenic Th17 cell induction with the deterioration of pulmonary injury.[15]

Type I IFNs are essential cytokines produced by macrophages or DCs and have profound regulatory effects on their properties.[16,17] It is worth noting that type I IFN-producing plasmacytoid DC loss is the hallmark of HIV infection, resulting in susceptibility to an opportunistic *pneumocystis* pneumonia infection,[18,19] which indicates the necessity of type I IFNs to counter pneumonia. Type I IFN signaling may promote the maintenance of epithelial barrier and epithelial integrity to reduce the migration of the pathogen from the alveoli to the lung parenchyma during *Streptococcus. pneumoniae* infection.[20,21] These results demonstrate that type I IFNs have a protective effect on pneumonia infection.

Toll-like receptor signaling sequentially activates adapters and kinases, such as IκB Kinase, IL-1 receptor-associated kinase, and tumor necrosis factor receptor-associated factors families, to activate transcription factors of IFN regulatory factor (IRF)−3, IRF-7, and NF-κB to promote the production of type I IFNs.[22–24] This investigation deciphered a novel GM-CSF-mediated type I IFN regulation mechanism, which will pave a new way for the type I IFN research.

Some limitations should be indicated here. Defining pneumonia in critically ill infants or children is hard, and validating diagnostic criteria is lacking.[25,26] In the present study, the critical need to be delivered to ICU is utilized to discriminate the infants into wild and severe bronchopneumonia. The consensus of experts based on guidelines from published literature, professional organizations, and governmental agencies should be constructed. The mechanism mediated by GM-CSF to activate the non-canonical NF-κB signal pathway to inhibit the type I IFNs should be analyzed with more detailed analysis, such as the cellular source of GM-CSF and the interaction between GM-CSF and NF-κB. The animal model to mimic the infection of bronchopneumonia should be established to confirm the treatment benefit of type I IFNs or GM-CSF inhibition.

All in all, this study indicates that GM-CSF derived from Th17 cells may suppress type I IFN induction in infants with severe bronchopneumonia through the activation of the non-canonical NF-κB signal pathway, and GM-CSF may be considered as a novel treatment option in the future.

In conclusion, GM-CSF-dependent non-canonical NF-κB activation-mediated diminish of type I IFN production may contribute to severe infantile bronchopneumonia.

DATA AVAILABILITY

Data could be obtained upon reasonable request to the corresponding author.

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AUTHOR CONTRIBUTIONS

L.X. designed and supervised the study. Z.L. and H.W. performed experiments and analyzed data. Z.L. wrote and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Protocol of this research was approved by the Ethics Committee of Daqing Oilfield General Hospital.

ADDITIONAL INFORMATION

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