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RESEARCH ARTICLE

Immune Response of Infected Lung Cells to Nanofluid Containing Carbon Nanotubes and Antibiotic

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ABSTRACT

Background: *Klebsiella pneumoniae* (*K.p*) is one of the pathogens causing hospital-acquired infections, which has been prioritized by the WHO due to its increased resistance to a variety of antibiotics. Cytokines are pro-inflammatory markers and their secretion in macrophages changes in pulmonary pneumoniae. Carbon nanotubes (CNTs), as novel approaches in drug delivery, despite their pros, may induce acute inflammation in animal lungs.

Objective(s): The present study aimed to evaluate the effects of functionalized multi walled carbon nanotubes and ciprofloxacin (*f*-MWCNTs+cip) combination on the immune responses, by cytokine assessment in the treated THP1-derived macrophages and the A549 cell line infected with resistant and ATCC 700,603 *K.p* strains.

Methods: Nanofluid containing *f*-MWCNTs were prepared using ultrasonic method. THP1 cells were differentiated to macrophages and were infected with resistant and ATCC 700,603 *K.p*. The infected THP1–derived macrophages and A549 cells were treated with *f*-MWCNT+cip. ELISA and real-time PCR assays were performed to assess the TNF-α and IL-1β cytokines in the THP1-derived macrophages and A549 cell line before and after the treatments.

Results: The level of TNF-α and IL-1β cytokines after exposure to the *f*-MWCNT+cip were upregulated significantly, in the resistant and ATCC *K.p* strains, from infected THP1–derived macrophages and the A549 cell line in comparison with control.

Conclusions: Our findings showed that *f*-MWCNTs noticeably upregulated the expression of *IL-1β* and *TNF-α* genes in comparison with controls. Combination of *f*-MWCNTs+ciprofloxacin induced inflammatory response in both infected THP1-derived macrophages and A549 cell lines compared to the ciprofloxacin treatment.

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fINTRODUCTION

Klebsiella pneumoniae (*K.p*), as an opportunistic pathogen, is the second leading cause of hospital-acquired infections (HAIs). The rate of HAIs in Iran varies from 1.9 to 25%, of which, 26.4% are caused by pneumonia; whereas, the occurrence rate is 5-10% in developed countries (1). Currently, the combination of antibiotics, including third- to fifth-generation cephalosporins, is prescribed against *K.p*, to address the multidrug resistance (MDR), albeit in many cases *K.p* shows increasing resistance to the conventional treatments (3). Due to their ability to effectively escape a variety of antibiotic treatments, the World Health Organization (WHO) prioritized the need for new drugs or methods against them (2). Fluoroquinolones are a family of drugs against *K.p* that the resistance to them is

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increasing dramatically (4). Nanotechnology has shown a remarkable impression in nanomedicine, specifically in drug delivery. Nanomaterials are materials whose dimensions range between 1-100 nm and are effectively applied in a wide range of science including biomedicine (5). The effectiveness of various nanomaterials against pathogens has been approved by numerous studies so far (6-8). Carbon nanotubes (CNTs), as a group of nanomaterials, are one or several one-atom-thick carbon sheets that are rolled to form single walled CNTs (SWCNTs) and multi walled CNTs (MWCNTs), respectively. CNTs are the modern tools employed in target-specific drug delivery, diagnosis, tissue regeneration, wound dressing, and their antibacterial activity has been reported (9-12). According to the findings of our previous study, the combination of ciprofloxacin or cefepime with *f*-MWCNTs was effective in counteracting the *K.p* resistance to these antibiotics (13,14). On the other hand, exposure to the CNTs induces the immune response activation leading to inflammation in lung cells (14). Alveolar macrophages can detect and destroy pathogens through phagocytosis and secrete proinflammatory factors in response to them.

Cytokines are immune modulators that are secreted by the innate immune cells to regulate the immune system responses in confronting an infection (25). Upon exposure of macrophage to the pathogens, an uncontrolled and excessive release of proinflammatory cytokines, called "cytokine storm", is induced by the innate immune system. On the other hand, pro-inflammatory cytokines secretion by alveolar macrophages in CNT-exposed lungs operate the inflammation. Interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) cytokines are inflammatory markers, whose secretion in macrophages is altered in pulmonary pneumoniae (16). IL-1β is essential for host immunity against *K.p* infection in the lung (16). The mechanism of the acute inflammation, induced by CNT, is through three elucidated pathways (Fig.4). MWCNTs enhance the expression of TNF-α, IL-1β, IL-6 genes through activating NF-κB pathway in mouse RAW264.7 macrophages. The second mechanism is the IL-1R signaling pathway which is induced by IL-1α and IL-1β and leads to CNT-induced inflammation. The last pathway is CNT-triggered NLRP3 inflammasome signaling that transform pro-IL-1β to active IL-1β (28).

Studies have shown that, after *f*-MWCNTs treatment of several tumor cell lines, high percentages of necrosis and apoptosis occurred (17). The clinical *K.p* strains have dissimilar mechanisms for escaping the immune system,

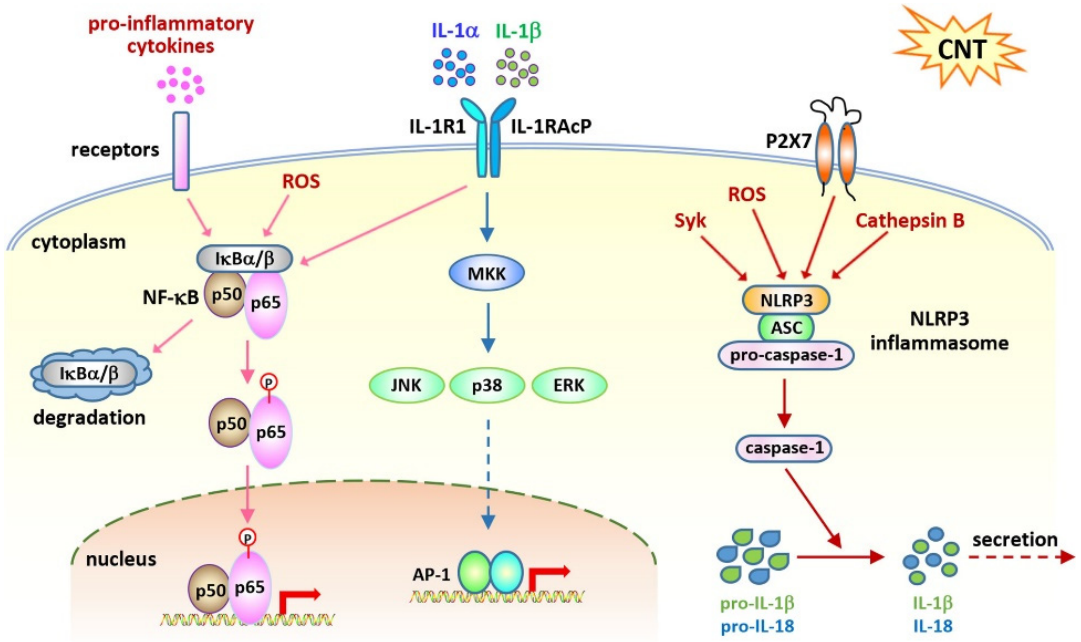

Fig. 1. Carbon nanotubes (CNTs)-induced acute inflammation occurs through three main pathways. (1) NF-κB signaling leading to TNF-α, IL-1α, and IL-1β expression. (2) IL-1R signaling is activated by IL-1α and IL-1β and promotes inflammation in the lung. (3) NLRP3 inflammasome signaling that IL-1β and IL-18 maturation is triggered by this pathway (29).

including regulation of bactericidal modifiers and the host cell death. NLRC4 inflammasome is a substantial factor in the *K.p*-mediated inflammatory response in infected macrophages, whose activation commonly ends in programmed immune cell death by a caspase-1 dependent pathway. While a *K.p* strain induced high IL-1β secretion, causing macrophage apoptosis, in another strain, inflammasome activation was inhibited due to the effect of IL-10. Therefore, the expression of IL-1β was downregulated, and the pathogen could escape the immune system and be released. (16, 18). Overall, MWCNTs can stimulate macrophages to produce the proinflammatory cytokine, tumor necrosis factor-α (TNF-α), by activating the NF-κB signaling pathway and inducing IL-1β secretion through procaspase-1 conversion to caspase-1, which results in the activation of the NLRP3 inflammasome (19). Given that TNF-α is a ligand for the NF-κB pathway, the release of it is independent of the inflammasome and leads to a proinflammatory cascade (20). The *f*-MWCNTs in vivo induces the secretion of TNF-α and IL-1β from immune cells. This study aimed to evaluate the effects of *f*- MWCNTs in combination with ciprofloxacin on the innate immune system. For this purpose, secretion and expression of TNF-α and IL-1β in THP1-derived macrophages and alveolar epithelial cancer cell (A549) lines, infected with *K.p* and treated with *f*-MWCNTs+ ciprofloxacin was assessed.

METHODS

f-MWCNTs

were purchased from the US research company (USA). Characterization tests including transmission electron microscope (TEM) ، energy dispersive X-ray (EDX) analyzers with scanning electron microscope (SEM), thermogravimetric analysis (TGA) and X-ray diffraction (XRD) were performed by the company. In the next step, by adding some compounds, nanofluid was prepared using ultrasonic method. Characterization results and nanofluid preparation method were described in detail in our previous articles (14, 15). The next stages of the research were continued in the following order.

Cell lines proliferation, differentiation, seeding

In the present study, two human cell lines, the lung monocyte cell line THP1 and the lung cancer A549, were provided by the cell bank of Pasteur institute of Iran. The THP1 cell line was proliferated in RPMI-1640 medium (Roswell Park Memorial Institute) (Biosera, France), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen/Strep, 10 mg/mL of penicillin, and 10,000 µg/mL of streptomycin). The A549 cell line was proliferated using DMEM (Dulbecco's Modified Eagle Medium, Biosera, France). The proliferated cells were washed by phosphate-buffered saline (PBS) followed by seeding in 6 well-plates (500,000 cells per well). For the A549 cells removal from the culture flask, 5 mL of sterile and filtered trypsin-EDTA (ethylene diamine tetra acetic acid) solution (5.0 g porcine trypsin and 2 g EDTA; Sigma Aldrich, Germany) was used. To induce THP1 differentiation into a macrophage, 50 ng /µL of phorbol 12-myristate 13-acetate (PMA) was added to each well (21).

Infection and treatment of macrophages and A549 cell line

The cells were infected with 0.5 McFarland standard solution (1.5×10^8 CFU/mL) at the multiplicity of infection (MOI) of 1:10 (1cell:10 bacteria) (22). After 4 hours of incubation at 37°C, the extracellular bacteria were washed twice with PBS solution. For treatment, considering the MIC (1 µL of antibiotic per 100 µL), the appropriate volume of the nanofluid containing *f*-MWCNTs and antibiotic were added to each well; relevant culture medium including 10% FBS was added to reach the volume of 2 ml; then the 6-well plates were incubated overnight at 37 °C. The supernatant was separated from the cell pellet by centrifugation.

RNA extraction, cDNA synthesis and real-time- PCR

The expression of cytokine genes in response to *f*-MWCNTs+ ciprofloxacin was evaluated. The RNA was extracted from the pellet, according to RiboEX kit instructions (GeneAll Biotechnology, South Korea). Pars Tous kit (Iran) was used for cDNA synthesis. Since *β-actin* gene expression in the infected peripheral blood mononuclear cells (PBMCs) has been reported stable, it was assayed as a housekeeping gene to evaluate the cytokine gene expression (24, 25). The real time-PCR procedure was performed, following the instruction enclosed in the SYBR Green 2X master mix kit (Yekta Tajhiz, Iran). The primers were designed with Gene Runner software that the sequence of which is given in the table below (Table 1).

Table 1. Sequence of cytokines primers and the *β-actin* housekeeping gene

Gene Name	Primers	Product Length (bp)
β-actin	Forward:5- AGACGCAGGATGGCATGGG-3 Reverse: 5-GAGACCTTCAACACCCCAGCC-3	161
Il1-β	Forward: TCACCTCTCCTACTCACTTAA Reverse: GCGGTTGCTCATCAGAAT	103
TNF-α	Forward: GGTATCCTTGATGCTTGTGT Reverse: ATCTGGAGGAAGCGGTAG	105

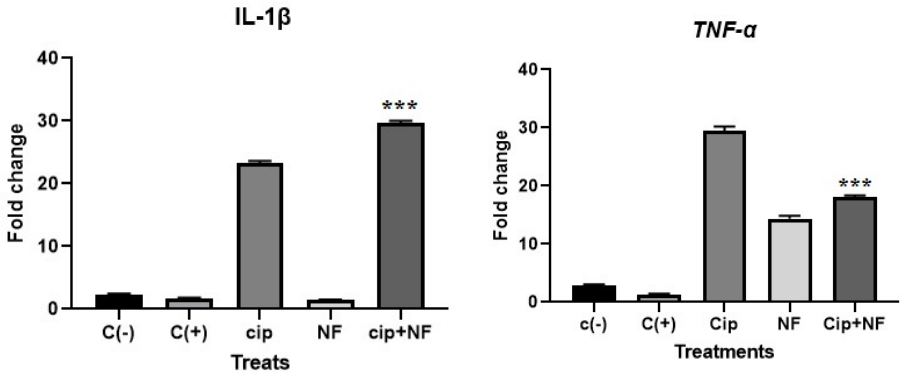

Fig. 2. Expression of *TNF-α and IL-1β* genes in *f*-MWCNTs+ciprofloxacin treated macrophages infected with resistant *K. pneumoniae* strain. Treatment states from left to right respectively: uninfected THP1-derived macrophage (negative control), infected THP1-derived macrophages with resistant strain and untreated (positive control), 8 µg/ml antibiotic, 400 µg/ml *f*-MWCNT, 400 µg/ml *f*-MWCNT with 8 µg/ml ciprofloxacin.

ELISA

The supernatants were filtered so that the *f*-MWCNT would not interfere with the ELISA reading. The enzyme-linked immunosorbent assay (ELISA) was performed on both infected THP1-derived macrophages and A549 cells, to assess the TNF-α and IL-1β cytokine secretion from immune cells in response to ciprofloxacin and *f*-MWCNTs treatment (Human IL-1β Catalog Number DLB50, Human TNF-α Catalog Numbers: DY210). The ELISA reading was performed by Dana3200 ELISA reader device. Following that, the light absorption of each well was measured by a spectrophotometer, with 450 nm as the main wavelength.

Statistical analysis

The data of the ELISA reader of the studied groups were collected in GraphPad Prism 7.0 and was analyzed. The expression levels were also normalized. To determine significance, the one-way ANOVA was used.

RESULTS

Characterization tests of *f*-MWCNTs and procedure of nanofluid containing *f*-MWCNTs preparation were mentioned in detail in our previous articles. (14, 15).

IL1-β and TNF-α expression analysis

According to the ELISA data analysis, in *f*-MWCNTs+cip treated THP1-derived macrophages, the expression of both *IL1-β* and *TNF-α* cytokine genes upregulated significantly compared to the positive control. *TNF-α* gene expression level downregulated in macrophages treated with *f*-MWCNTs+cip compared with the ciprofloxacin alone, in contrast, *IL-1β* gene expression level upregulated significantly ($P< 0.0001$) (Fig. 1).

ELISA results analysis

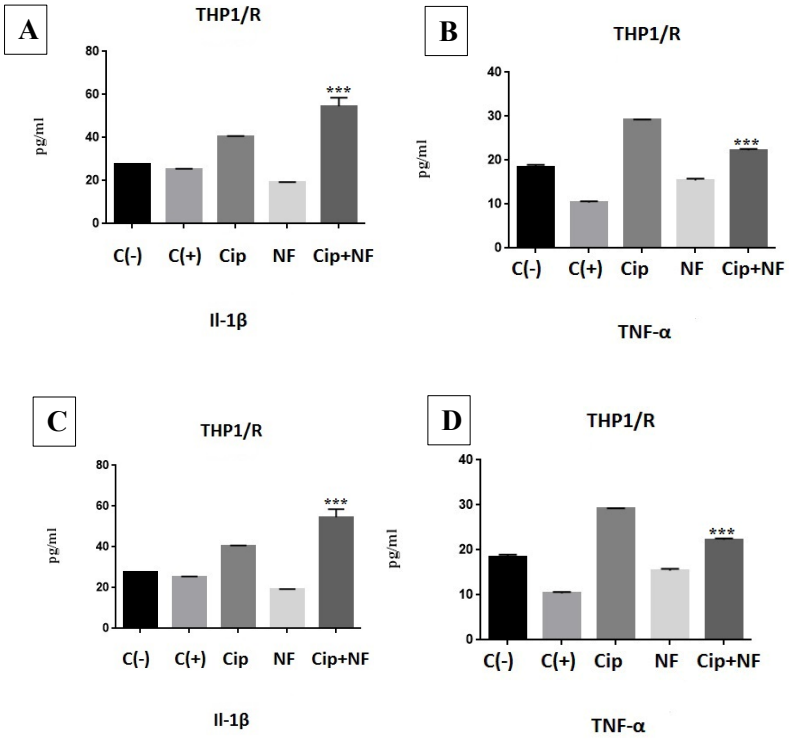

Fig. 3. Secretion of Il-1β and TNF-α in the THP1-derived macrophages infected with the resistant and ATCC strains and treated with *f*–MWCNTs+ ciprofloxacin. Columns from left to right respectively: uninfected macrophage (negative control), macrophage infected with untreated *K. pneumoniae* strain (positive control), infected macrophage treated with antibiotic, infected macrophage treated with *f*-MWCNT, infected macrophage treated with *f*-MWCNT + ciprofloxacin.

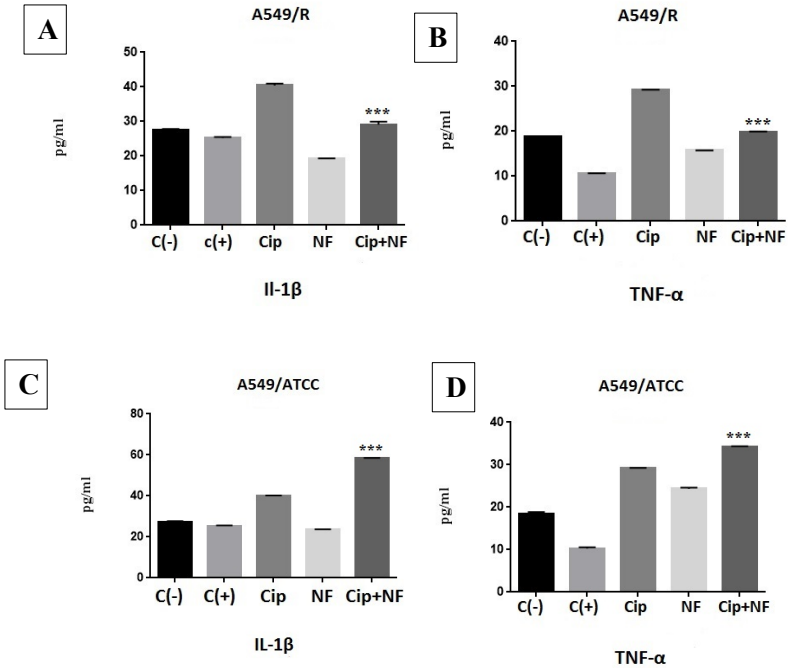

Fig. 4. Secretion of Il-1β and TNF-α in A549 cell line infected with the resistant and ATCC strains and treated with *f*–MWCNTs+ ciprofloxacin. Columns from left to right respectively: uninfected A549 cells (negative control), infected A549 cells with *K. pneumoniae* strain (positive control), A549 cells infected and treated with antibiotic, infected A549 cells treated with *f*-MWCNTs, infected A549 cells treated with *f*-MWCNT + ciprofloxacin.

For the THP1-derived macrophages infected with the resistant strain and treated with a combination of *f*-MWCNT and ciprofloxacin, the Il-1β secretion was upregulated, compared to the ciprofloxacin alone and control groups ($P < 0.0001$). The TNF-α secretion decreased compared to the alone antibiotic and increased compared to the positive control ($P < 0.0001$) (Fig. 2). For the THP1-derived macrophages infected with the ATCC strain and treated with 100 µg/mL of *f*-MWCNT and 2 µg/mL of ciprofloxacin, the secretion of both TNF-α and IL-1β increased significantly compared to the antibiotic alone and positive control groups ($P < 0.0001$) (Fig. 2).

For the A549 cells infected with resistant strain and treated with *f*-MWCNTs+cip, both TNF-α and IL-1β secretion showed an increase compared to the positive control and decreased in comparison with the ciprofloxacin treated cells. For the ATCC-infected A549 cells treated with *f*-MWCNTs+cip, secretion of both TNF-α and IL-β showed a significant increase compared to the ciprofloxacin treated A549 cells and positive control (Fig. 3).

CONCLUSIONS

This study aimed to investigate the level of proinflammatory cytokines in THP1-derived macrophages and A549 cell lines infected with resistant and ATCC *K.p* strains and treated with *f*-MWCNTs+ cip. In the present study, as the diagrams show, exposure to the ciprofloxacin significantly upregulated the cytokines at both transcriptional and translational levels ($P < 0.0001$) (Figs. 1-3). In contrast, by the effect of *f*-MWCNTs +ciprofloxacin, the expression and secretion of cytokines in infected THP1-derived macrophages were downregulated. These findings revealed that the combination of *f*-MWCNTs+cip resulted in a milder inflammatory response, compared to the ciprofloxacin alone. The cytokines alteration profiles were the same at both the transcription (mRNA) and translation (secretion) levels in the studied cell lines. The findings of the present study showed that, for the THP1-derived macrophages infected with the resistant *K.p*, the secretion of IL-1β was upregulated in response to the *f*-MWCNTs+ cip, while the secretion of TNF-α was downregulated (Fig.2). Similar to the literature, our findings revealed that the secretion profiles of cytokines in cells treated with different bacterial strains are not necessarily the same. In THP1-derived macrophages and A549 cells infected with the resistant *K.p* strain, TNF-α secretion was downregulated in response to the *f*-MWCNTs+cip compared to the ciprofloxacin alone, while, in both cell lines treated with ATCC strain, it was upregulated (Fig. 2, Fig. 3). Also, the secretion level of IL-1β decreased in the A549 cells infected with the resistant strain due to the effect of *f*-MWCNTs+ cip compared to the ciprofloxacin alone, while it increased in cells infected with the ATCC strain (Figs. 2,3). Our findings were consistent with the study of Zhang *et al.* as *f*-MWCNTs significantly increased the expression of *IL-1β* and *TNF-α* genes in comparison with the control group (26). Due to the variety of signaling pathways in immune cells, the defense mechanism of macrophages may be different under the influence of different strains and treatments. After all, the immune response of treated THP1-derived macrophages and A549 cell line was altered in comparison with the untreated cells. To the best of our knowledge, this is the first study that evaluated the secretion of cytokines in infected macrophages treated with nanofluid containing *f*-MWCNTs+ ciprofloxacin.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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