J Infect Chemother 23 (2017) 51–55

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

TNF-α inhibits the growth of *Legionella pneumophila* in airway epithelial cells by inducing apoptosis

Yasuhide Kawamoto, Yoshitomo Morinaga*, Yumiko Kimura, Norihito Kaku, Kosuke Kosai, Naoki Uno, Hiroo Hasegawa, Katsunori Yanagihara

Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 851-2128, Japan

ARTICLE INFO

Article history:
Received 21 June 2016
Received in revised form
21 September 2016
Accepted 28 September 2016
Available online 17 November 2016

Keywords:
Anti-TNF-α therapy
Cell death
Caspase 3
Caspase 7
Lung epithelial cells

ABSTRACT

Background: TNF-α plays an important role in the pathogenesis of *Legionella pneumophila* (Lp)-induced pneumonia. Patients undergoing anti-TNF-α therapy are at an increased risk of Lp infection. Lp infects both phagocytic and non-phagocytic cells such as airway epithelial cells; however, the role of TNF-α in airway epithelial cells is unknown.

Methods: Human airway epithelial cell line NCI-H292 was infected with Lp NUL1 strain. After infection, both intracellular growth of Lp and cell death were evaluated after treating the cells with or without TNF-α. Apoptosis was examined by performing activated caspase-3/7 staining and by using a pan-caspase inhibitor.

Results: Lp infected and replicated in NCI-H292 cells in a time-dependent manner, and TNF-α treatment of Lp-infected NCI-H292 cells inhibited Lp replication. Inhibitory effects of TNF-α on Lp replication were suppressed after treatment with a TNF-α-neutralizing antibody. Lp infection increased extracellular lactate dehydrogenase levels and decreased the number of living cells. Increased number of Lp-infected NCI-H292 cells showed caspase-3/7 activation, indicating they underwent apoptosis. TNF-α treatment inhibited Lp replication by increasing the apoptosis of NCI-H292 cells.

Conclusions: Thus, our results suggested that airway epithelial cells were involved in the pathogenesis of Lp infection and that TNF-α played a protective role by inhibiting the intracellular replication of Lp and by increasing the apoptosis of Lp-infected airway epithelial cells. However, Lp infection should be investigated further in patients undergoing anti-TNF-α therapy who develop pneumonia.

1. Introduction

Legionella pneumophila (Lp) infection is characterized by life-threatening pneumonia that is caused by the inhalation of contaminated aerosols. Despite the availability of appropriate antibiotics, Lp infection can become severe in a short period and often requires intensive care. Therefore, it is important to determine mechanisms underlying the pathogenesis of Lp infection to develop better strategies for its prevention and treatment.

Although Lp mainly infects and replicates in alveolar macrophages, it also infects and replicates in non-phagocytic cells such as lung epithelial cells [1–3]. Because airway epithelial cells and alveolar macrophages come in contact with inhaled pathogens, they may be involved in the pathogenesis of Lp infection. Previously, we reported that lung epithelial cells produced mucins in response to Lp infection and that this response could exert protective effects against Lp infection [3]. However, information on the relationship between Lp and lung epithelial cells is scarce and the role of these cells in Lp infection is unclear.

Cell death is suggested to be involved in the pathogenesis of Lp infection. Lp-infected macrophages undergo cell death through apoptosis [4,5], pyroptosis [6], and programmed necrosis [7]. Proinflammatory mediators, including IL-1β and high-mobility group box 1, released by dying cells induce inflammation. Therefore, Lp-induced cell death is an important mechanism underlying severe inflammation in patients with Lp infection. However, information on Lp-induced cell death of lung epithelial cells is unavailable.

* Corresponding author. Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Fax: +81 95 819 7422.
E-mail address: y-morina@nagasaki-u.ac.jp (Y. Morinaga).

http://dx.doi.org/10.1016/j.jiac.2016.09.010
1341-321X/

TNF-α, a proinflammatory cytokine, is rapidly released by various immune cells upon pathogen recognition. TNF-α exerts protective effects against invasive pathogens, especially intracellular pathogens, and inhibits the growth of Lp in macrophages [8]. However, TNF-α is also involved in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease and is a therapeutic target for treating these diseases because it is a major cause of inflammation. Therefore, TNF-α inhibitors that provide significant benefits such as reduction of disease activity and improvement of symptoms are used for treating patients with the above diseases. However, patients treated with TNF-α inhibitors are at an increased risk of bacterial, fungal, and viral infections [9,10] because important initial responses to invasive pathogens may be suppressed in these patients. Lp is a pathogen of those risk-elevated infection and the U.S. Food and Drug Administration added Lp into the emerging pathogen list in the patients under the anti-TNF-α therapy [11]. Fatal cases of Lp infection have been observed in patients undergoing anti-TNF-α therapy [12]. Moreover, incidence of Lp-induced pneumonia is high in patients undergoing anti-TNF-α therapy [13].

TNF-α inhibits intracellular bacterial proliferation in and cell death of Lp-infected macrophages. However, the role of TNF-α in lung epithelial cells is unknown. Therefore, we examined the effect of TNF-α on bacterial growth in and cell death of lung epithelial cells in the present study.

2. Materials and methods

2.1. Bacterial strain

Lp NUL1 strain (serogroup 1), which was clinically isolated from the sputum of a patient with *L. pneumophila* pneumonia, was used in this study [3,7]. The bacteria were stored at −80 °C in a Microbank system (Pro-Lab Diagnostics, Ontario, Canada) until further use. The bacteria were cultured on Wadowsky–Yee–Okuda agar plates (Eiken Kagaku, Tokyo, Japan) for 3 days. Next, a single colony was subcultured in 3 mL buffered yeast extract (BYE) broth for 12 h at 37 °C under constant shaking. The bacterial suspension was then transferred into a fresh BYE broth and was cultured for 24 h under the same conditions. After these processes, bacteria reach post-exponential phase.

2.2. Cell line and Lp infection

Human airway epithelial cell line NCI-H292 (ATCC CRL-1848) and monocyte/macrophage cell line THP-1 (ATCC TIB202) were maintained in a continuous culture in RPMI 1640 medium (Thermo Fischer Scientific, Waltham, MA) supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. After cells reached confluence in 96-well plates, NUL1 was added at a multiplicity of infection (MOI) of 50. After Lp NUL1 infection, the plate was centrifuged at 1000 × *g* for 5 min and the cells were incubated further for 2 h. Gentamicin (100 μg/mL) was added to the culture medium after 2 h of incubation with Lp NUL1 to kill extracellular bacteria, and the plate was washed twice with a fresh medium. Bacterial growth was determined by lysing the cells in distilled water. Serial dilutions of cell lysate obtained were quantitatively inoculated on buffered charcoal yeast extract agar plates, and the number of colonies formed was counted at 72 h after incubation at 37 °C.

2.3. Treatment with TNF-α and TNF-α-neutralizing antibody

Lp-infected NCI-H292 cells were treated with recombinant TNF-α (R&D Systems, Inc., Minneapolis, USA). For inhibition study, For inhibition study, Lp-infected cells were coincubated with TNF-α and TNF-α-neutralizing antibody (Hycult Biotech, Frontstraat, Netherlands). TNF-α level in the cell culture supernatant was measured using TNF-α Quantikine ELISA kit (R&D system), according to the manufacturer's instructions.

2.4. Cytotoxicity assay

Cell death was evaluated by measuring lactate dehydrogenase (LDH) levels by using a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. To determine cytotoxicity, the value of samples from 1% Triton-X-treated cells was defined as 100% cell death.

2.5. Cell death evaluation

Cells were stained with 0.2% trypan blue, and positively and negatively stained cells were counted. Cells showing trypan blue positivity under a light microscope were considered as dead cells. Activity of caspase-3/7 was determined using CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fischer Scientific), according to manufacturer's instructions. After staining, the cells were observed under light and fluorescence microscopes, and cells stained in green were considered as apoptotic cells. Apoptosis was analyzed by incubating the cells with 10 μM zVAD-fmk (zVAD).

2.6. Statistical analysis

All data are expressed as mean ± standard deviation (SD). One-way analysis of variance was used to determine statistically significant differences among the groups. Dunnett's test was used to confirm the observed differences by comparing with the control group. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. TNF-α inhibits the intracellular growth of Lp in airway epithelial cells

Bacterial count in Lp-infected NCI-H292 cells was measured (Fig. 1A). Bacterial count was 2.93 ± 0.04 $\log_{10}$ cfu/mL at 0 h and increased to 6.31 ± 0.02 $\log_{10}$ cfu/mL at 48 h after incubation with Lp in a time-dependent manner ($p < 0.01$ compared with bacterial count at 0 h). We also confirmed that Lp did not replicate in the culture medium alone (data not shown).

Effects of TNF-α on Lp growth were examined at 24 and 48 h. At 24 h, no significant differences were observed in bacterial count in cells treated with TNF-α (0.1–50 ng/mL) and control cells (data not shown). However, at 48 h, the bacterial count in cells treated with TNF-α decreased in a dose-dependent manner, with significant decreases observed with TNF-α concentrations of 10 and 50 ng/mL (Fig. 1B). Effect of TNF-α on bacterial growth was inhibited after treatment with the TNF-α-neutralizing antibody in a dose-dependent manner (5.62 ± 0.4 $\log_{10}$ cfu/mL vs. 6.56 ± 0.11 $\log_{10}$ cfu/mL for cells treated with 50 ng/mL TNF-α plus 1000 ng/mL anti-TNF-α antibody vs. cells treated with 50 ng/mL TNF-α alone, $p < 0.01$; Fig. 1C). Direct effect of TNF-α on bacterial growth was determined by incubating Lp with various concentrations of TNF-α in the BYE broth. However, no differences were observed in bacterial count after treatment with different concentrations of TNF-α (data not shown). To exclude the effect of endogenous TNF-α produced by NCI-H292 cells, we measured TNF-α levels in the culture supernatant at 48 h and found that the level of endogenous TNF-α after Lp infection was below the concentration of exogenous TNF-α used (70.81 pg/mL).

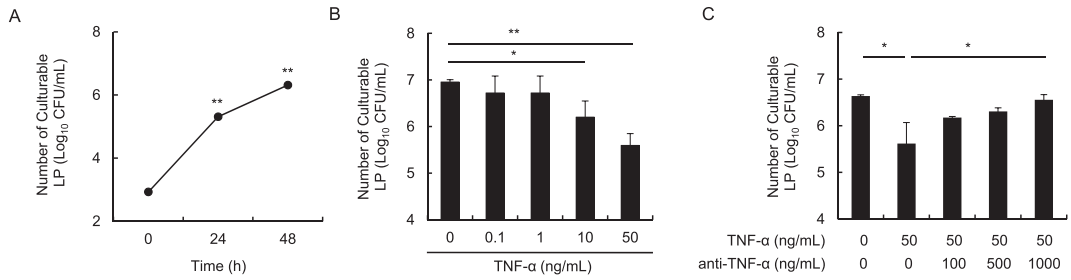

Fig. 1. TNF-α inhibits Lp replication in airway epithelial cells. (A) Bacterial count in NCI-H292 cells infected with Lp at an MOI of 50. (B) Bacterial count in Lp-infected NCI-H292 cells at 48 h after treatment with the indicated concentrations of TNF-α. (C) Bacterial count in Lp-infected NCI-H292 cells at 48 h after treatment with TNF-α and TNF-α-neutralizing antibody. Error bars represent mean ± SD; *p < 0.05 and **p < 0.01.

3.2. TNF-α enhances Lp-induced cell death

Next, we determined cell death after Lp infection because the growth of Lp depends on cells. In the absence of TNF-α, Lp-infected cells released significantly levels of LDH in the culture supernatant compared with control cells (9.78% ± 0.18% vs. 56.97% ± 0.71% for control vs. Lp-infected cells, $p < 0.01$; Fig. 2A). Treatment of Lp-infected cells with TNF-α further increased LDH levels in the culture supernatant. However, no difference was observed in LDH levels in the culture supernatant of Lp-uninfected cells treated with or without TNF-α (Fig. 2A). Lp infection decreased the percentage of live cells. However, the percentage of live cells after Lp infection with TNF-α treatment decreased more dramatically than that of live cells not treated with TNF-α (78.66% ± 3.21% vs. 56.66% ± 4.61% for Lp-infected cells not treated with TNF-α vs. Lp-infected cells treated with TNF-α, $p < 0.01$; Fig. 2B). Because cell death is observed in macrophages during Lp infection [14] and TNF-α inhibits Lp growth in macrophages [15], we also examined whether our experimental strain NUL1 can induce cell death in THP-1 cells in conditions with or without TNF-α. Similar to H292 cells, TNF-α enhanced cell death in Lp-infected THP-1 (% live cells at 48 h; 7.33% ± 0.57% in TNF-α-untreated cells vs. 1.66% ± 0.57% in 50 ng/mL TNF-α-treated cells, $p < 0.01$) (Fig. 2C).

3.3. TNF-α induces apoptosis with inhibiting Lp replication

Apoptosis is a caspase-dependent process. Therefore, involvement of apoptosis in TNF-α-induced death of Lp-infected cells was determined by performing microscopic analysis of Lp-infected cells stained with the Caspase-3/7 Green Detection Reagent (Fig. 3A). Activated caspase-3/7-positive cells were more common among Lp-infected cells treated with TNF-α than in Lp-infected cells not treated with TNF-α. Moreover, Lp-uninfected cells did not show caspase-3/7 activation.

To examine the effect of apoptosis on Lp growth, Lp-infected cells were cultured with a pan-caspase inhibitor zVAD. After zVAD treatment, activated caspase-3/7-positive cells, which were induced by Lp-infection and TNF-α treatment, were inhibited (Fig. 3B). Consistently, the percentage of viable cells increased significantly after zVAD treatment (43.66% ± 2.3% vs. 53.33% ± 4.16% for zVAD-untreated vs. zVAD-treated cells, $p < 0.05$; Fig. 3C). Moreover, bacterial count significantly increased in zVAD-treated cells compared with that in zVAD-untreated cells that were treated with TNF-α (6.14 ± 0.11 $\log_{10}$ cfu/mL vs. 5.17 ± 0.08 $\log_{10}$ cfu/mL, $p < 0.01$; Fig. 3D).

4. Discussion

Lp causes community-acquired pneumonia. However, it is also recognized as a re-emerging pathogen in patients undergoing anti-TNF-α therapy. Respiratory tract epithelial cells function as important barriers against airborne infections and are a target of Lp infection. In the present study, we examined the effects of TNF-α on Lp-infected airway epithelial cells.

The relationship between Lp and alveolar macrophages has been mainly discussed in the pathogenesis of Lp infection from the early stage to the severe stage. However, Lp also infects and replicates in non-phagocytic cells. Thus, Lp targets lung epithelial cells as well as macrophages. Particularly, filamentous Lp has been found in the lung tissue of Lp-infected patients and can infect lung epithelial cells *in vitro* study [16,17]. E-cadherin and β1-integrin receptors are required for the attachment of Lp to lung epithelial cells, and activation of these receptors leads to cell membrane rearrangements called “hooks” and “membrane wraps” that promote the internalization of Lp [18]. Similarly, Lp infected and replicated in lung epithelial cells in the present study. Thus, our data suggest that the airway epithelium is involved in the pathogenesis of Lp infection. However, the specific role of airway epithelial cells in Lp infection is still unknown. Therefore, further studies examining various aspects are required to understand the role of airway epithelial cells in Lp infection.

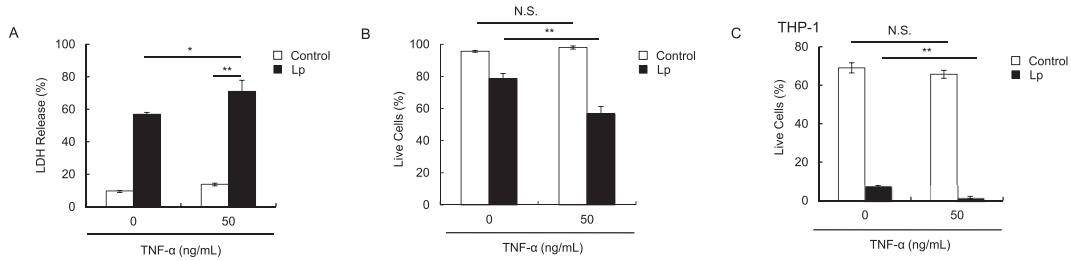

Fig. 2. TNF-α enhances Lp-induced cell death. (A) LDH released from NCI-H292 cells at 48 h after Lp infection. (B) Percentage of live cells in NCI-H292 cells at 48 h after trypan blue staining. (C) Percentage of live cells in THP-1 cells at 48 h after trypan blue staining. Values are expressed as mean ± SD; *p < 0.05; **p < 0.01; N.S., not significant.

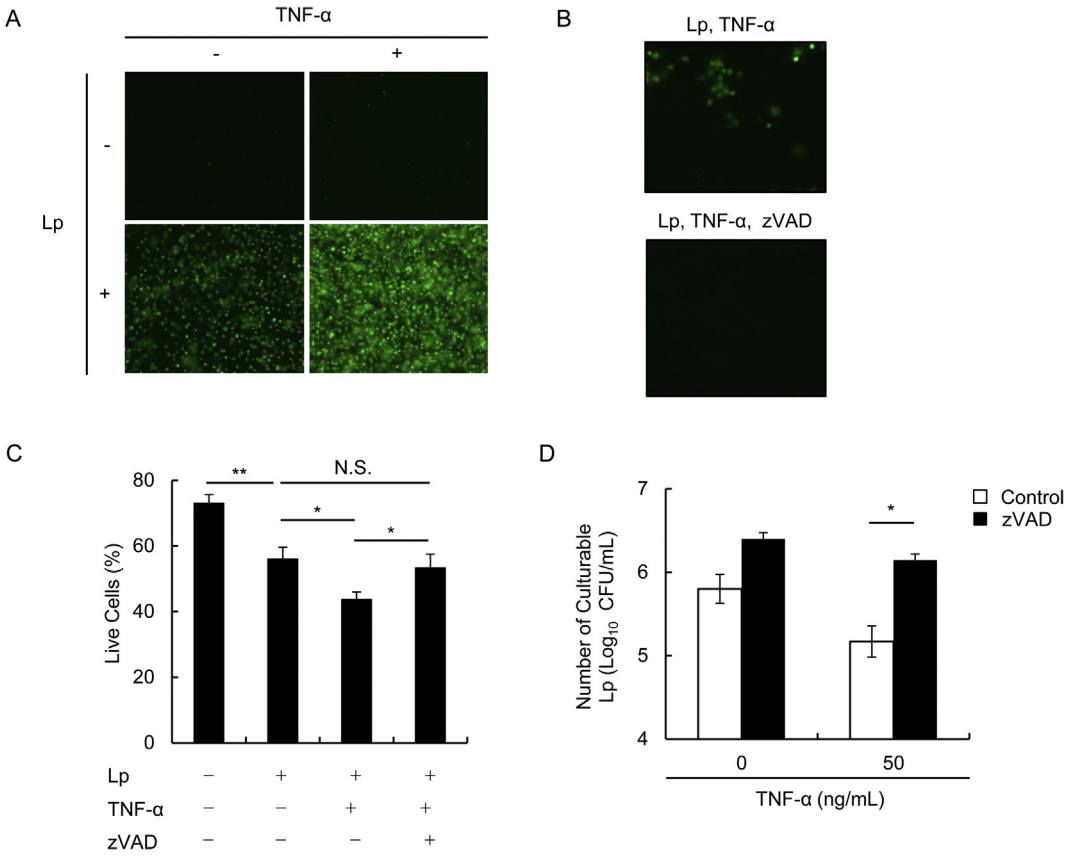

Fig. 3. TNF-α enhances Lp-induced apoptosis with inhibiting Lp growth. (A) Activated caspase-3/7 staining at 48 h after LP infection with or without TNF-α treatment. Green cells indicate activated caspase-3/7-positive NCI-H292 cells. Each image was taken at 1000× magnification. (B) Activated caspase-3/7 staining after zVAD treatment at 48 h. (C) Live cells were determined by performing trypan blue staining at 48 h. (D) Bacterial count after zVAD treatment at 48 h. Values are represented as mean ± SD; *p < 0.05; **p < 0.01; N.S., not significant.

TNF-α induces both apoptotic and necrotic cell death [19]. Caspase-3/7 activation observed in Lp-infected cells in the present study suggested that epithelial cells underwent apoptosis after Lp infection. Moreover, TNF-α treatment increased Lp-induced apoptosis by decreasing bacterial load. The meaning of cell death of airway epithelial cells in respiratory tract infections, particularly those caused by intracellular pathogens, could not be simple. For instance, influenza A virus induces programmed necrosis and apoptosis of airway epithelial cells. Programmed necrosis of the airway epithelium in patients with influenza A virus-induced pneumonia increases disease severity [20]. In contrast, apoptosis of the airway epithelium decreases disease severity by limiting the dissemination of the virus [21]. *Chlamydophila pneumoniae* modulates the apoptotic pathway in epithelial cells and makes them resistant to apoptosis [22], suggesting that *C. pneumoniae* modulates cell death to promote bacterial replication. Therefore, we believe that Lp-induced apoptosis of airway epithelial cells is beneficial for host defense because Lp replicates only in the intracellular milieu. However, structural damage of the airway epithelium may allow Lp to penetrate further into the epithelial tissue.

The Lp-induced cell death was mainly examined in airway epithelial cells but also observed in macrophages. The pathogenesis of Lp infection based on our findings is shown in Fig. 4. Since humans generally have poor anti-Lp immunities such as specific antibodies [23], the cell death might be a ubiquitous cellular phenomenon as a role of inhibiting bacterial growth. Therefore, anti-TNF-α treatment can suppress apoptosis and allow bacterial growth in live cells. This does not conflict with a report about the bacterial strategy which Lp has an ability to inhibit apoptosis until late stage of intracellular replication [14]. In addition, considering the anatomical structure, the airway epithelial cells may serve as delaying of the expansion of pneumonia via bronchi. Thus, the reduced apoptosis by anti-TNF-α treatment can enhance disease severity. However, further studies should be performed to determine the pathological role of apoptosis in Lp infection.

TNF-α inhibits the intracellular replication of Lp in macrophages, and inhibition of TNF-α promotes bacterial growth [15], which is similar to the effects of TNF-α on airway epithelial cells observed in the present study. These effects of TNF-α has been also observed in a Lp infection mouse model [24]. Notably, the suppressive effect of TNF-α on bacterial load was observed at 48 h after Lp infection and not at 24 h after Lp infection in the present and previous studies [15,24], implying that TNF-α affected subsequent replications rather than affecting the initial replication of Lp. These findings also suggested that the effects of TNF-α on bacterial growth were not cell specific and that TNF-α could be an important cytokine for delaying the progression of Lp infection. Thus, decreased TNF-α function could enhance the growth of Lp in a short time after initial exposure and could rapidly lead to life-threatening conditions.

The present study contains some limitations. Because our data was produced by only one strain and a few cell lines, the effect of TNF-α should be examined by other strains and cells to support our findings. This study does not exclude completely the possibility that TNF-α directly inhibits the bacterial growth in live cells. Thus, other approaches will be needed to solve this possibility.

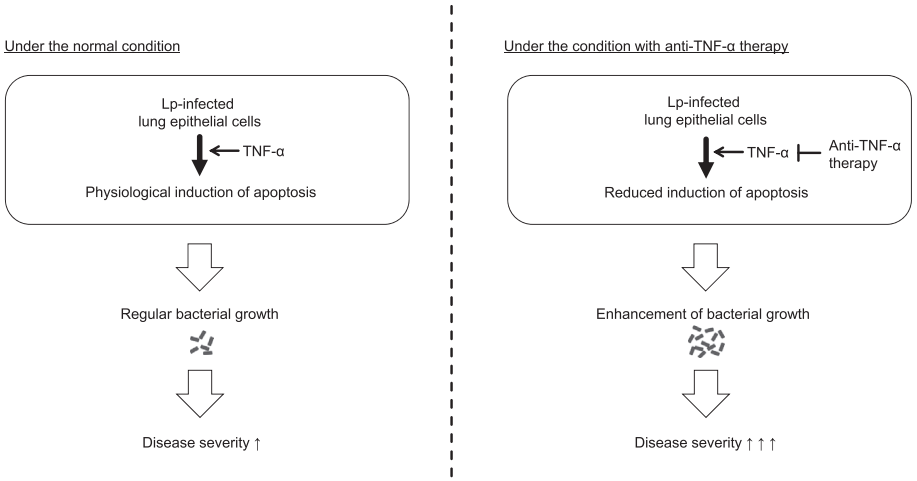

Fig. 4. Schematic illustration of the pathogenesis of Lp infection under the condition with or without anti-TNF-α therapy. Anti-TNF-α therapy can inhibit the TNF-α-induced physiological apoptosis of Lp-infected airway epithelial cells and lead to the enhancement of bacterial growth.

Thus, our results indicate that TNF-α is defensively involved in the pathogenesis of Lp infection in airway epithelial cells as well as alveolar macrophages. Further, our results indicate that TNF-α inhibits Lp replication in infected airway epithelial cells by inducing their apoptosis. Therefore, Lp infection should be investigated in patients undergoing anti-TNF-α therapy who develop severe pneumonia.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This research was partly supported by a Grant-in-Aid for Scientific Research (No. 15K09572 to K.Y.) and Grant-in-Aid for Young Scientists (B) (Nos. 23791137 and 30580360 to Y.M.) from the Japan Society for the Promotion of Science.

References

[1] Newton HJ, Ang DK, van Driel IR, Hartland EL. Molecular pathogenesis of infections caused by *Legionella pneumophila*. Clin Microbiol Rev 2010;23:274–98.
[2] Wagner C, Khan AS, Kamphausen T, Schmausser B, Unal C, Lorenz U, et al. Collagen binding protein mip enables *Legionella pneumophila* to transmigrate through a barrier of NCI-H292 lung epithelial cells and extracellular matrix. Cell Microbiol 2007;9:450–62.
[3] Morinaga Y, Yanagihara K, Araki N, Migiyama Y, Nagaoka K, Harada Y, et al. Live *Legionella pneumophila* induces MUC5AC production by airway epithelial cells independently of intracellular invasion. Can J Microbiol 2012;58:151–7.
[4] Gao L-Y, Kwaik YA. Apoptosis in macrophages and alveolar epithelial cells during early stages of infection by *Legionella pneumophila* and its role in cytopathogenicity. Infect Immun 1999;67:862–70.
[5] Santic M, Asare R, Doric M, Kwaik YA. Host-dependent trigger of caspases and apoptosis by *Legionella pneumophila*. Infect Immun 2007;75:2903–13.
[6] Case CL, Kohler LJ, Lima JB, Strowig T, de Zoete MR, Flavell RA, et al. Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to *Legionella pneumophila*. Proc Natl Acad Sci 2013;110:1851–6.
[7] Morinaga Y, Yanagihara K, Nakamura S, Hasegawa H, Seki M, Izumikawa K, et al. *Legionella pneumophila* induces cathepsin B-dependent necrotic cell death with releasing high mobility group box1 in macrophages. Respir Res 2010;11:158.
[8] Skerrett SJ, Bagby GJ, Schmidt RA, Nelson S. Antibody-mediated depletion of tumor necrosis factor-alpha impairs pulmonary host defenses to *Legionella pneumophila*. J Infect Dis 1997;176:1019–28.
[9] Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. JAMA 2006;295:2275–85.
[10] Grijalva CG, Chen L, Delzell E, Baddley JW, Beukelman T, Winthrop KL, et al. Initiation of tumor necrosis factor-α antagonists and the risk of hospitalization for infection in patients with autoimmune diseases. JAMA 2011;306:2331–9.
[11] Food and Drug Administration. FDA Drug Safety Communication: Drug labels for the tumor necrosis factor-alpha (TNFα) blockers now include warnings about infection with *Legionella* and *Listeria* bacteria. Rockville, MD; 2011. From FDA website [Accessed 7 Sep 2011].
[12] Kaku N, Yanagihara K, Morinaga Y, Sato T, Nakashima M, Sakai T, et al. Detection of *Legionella pneumophila* serogroup 1 in blood cultures from a patient treated with tumor necrosis factor-alpha inhibitor. J Infect Chemother 2013;19:166–70.
[13] Lanternier F, Tubach F, Ravaud P, Salmon D, Dellamonica P, Bretagne S, et al. Incidence and risk factors of *Legionella pneumophila* pneumonia during anti-tumor necrosis factor therapy: a prospective French study. Chest 2013;144:990–8.
[14] Abu-Zant A, Santic M, Molmeret M, Jones S, Helbig J, Abu Kwaik Y. Incomplete activation of macrophage apoptosis during intracellular replication of *Legionella pneumophila*. Infect Immun 2005;73:5339–49.
[15] Skerrett SJ, Martin TR. Roles for tumor necrosis factor alpha and nitric oxide in resistance of rat alveolar macrophages to *Legionella pneumophila*. Infect Immun 1996;64:3236–43.
[16] Boyd JF, Buchanan WM, MacLeod TI, Dunn RI, Weir WP. Pathology of five scottish deaths from pneumonic illnesses acquired in Spain due to Legionnaires' disease agent. J Clin Pathol 1978;31:809–16.
[17] Ogawa M, Takade A, Miyamoto H, Taniguchi H, Yoshida S. Morphological variety of intracellular microcolonies of legionella species in vero cells. Microbiol Immunol 2001;45:557–62.
[18] Prashar A, Bhatia S, Tabatabaeiyazdi Z, Duncan C, Garduno RA, Tang P, et al. Mechanism of invasion of lung epithelial cells by filamentous *Legionella pneumophila*. Cell Microbiol 2012;14:1632–55.
[19] Laster SM, Wood JG, Gooding LR. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 1988;141:2629–34.
[20] Rodrigue-Gervais IG, Labbe K, Dagenais M, Dupaul-Chicoine J, Champagne C, Morizot A, et al. Cellular inhibitor of apoptosis protein ciap2 protects against pulmonary tissue necrosis during influenza virus infection to promote host survival. Cell Host Microbe 2014;15:23–35.
[21] Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza a (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 2002;360:1831–7.
[22] Rajalingam K, Al-Younes H, Muller A, Meyer TF, Szczepek AJ, Rudel T. Epithelial cells infected with *Chlamydophila pneumoniae* (*Chlamydia pneumoniae*) are resistant to apoptosis. Infect Immun 2001;69:7880–8.
[23] Collins MT, Cho SN, Reif JS. Prevalence of antibodies to *Legionella pneumophila* in animal populations. J Clin Microbiol 1982;15:130–6.
[24] Brieland JK, Remick DG, Freeman PT, Hurley MC, Fantone JC, Engleberg NC. In vivo regulation of replicative *Legionella pneumophila* a lung infection by endogenous tumor necrosis factor alpha and nitric oxide. Infect Immun 1995;63:3253–8.