RESEARCH ARTICLE

Influenza A virus inhibits influenza virus replication by inducing IL-37

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Backgrounds: The influenza virus is one of the major pathogens that seriously affect human health. It can cause a strong immune response and trigger a series of complications. Interleukin 37 (IL-37) is a newly discovered cytokine that plays an important regulatory role in infection and immunity. To date, there have been few studies on the correlation between influenza virus infection and IL-37.

Methods: Serum levels of IL-37 in 115 patients with influenza A virus (IAV) infection and 102 healthy subjects were measured by an enzyme-linked immunosorbent assay (ELISA). Real-time quantitative PCR (RT-qPCR) was used to detect differences in IL-37 expression in peripheral blood mononuclear cells (PBMCs) between IAV patients and healthy subjects. IL-37 expression was measured in A549 cells and PBMCs infected with IAV H3N2 using ELISA and RT-qPCR. After the H3N2-infected A549 cells were treated with human IL-37, the concentration of viral RNA was determined using RT-qPCR, and the titer of influenza virus was determined by a hemagglutination test.

Results: The IL-37 levels in the sera and PBMCs of patients infected with IAV were higher than those of healthy subjects. The expression of IL-37 mRNA and protein in IAV-infected A549 cells and PBMCs was upregulated, and IL-37 protein was able to inhibit the replication of IAV RNA.

Conclusion: IAV-induced IL-37 expression inhibits IAV replication.

KEYWORDS

influenza A virus, interleukin 37, replication

1 | INTRODUCTION

Influenza A virus (IAV) is an RNA virus of the orthomyxoviridae family. It can cause acute upper respiratory tract infection, and it spreads rapidly through the air, leading to frequent regional and seasonal pandemics throughout the world. Due to its high infectiousness and rapid spread, IAV infection can cause a local or global epidemic outbreak in a short period of time, leading to catastrophic consequences.^{1,2} IAV infection can activate the host immune system and promote the expression of chemokines, proinflammatory cytokines, and other immunoregulatory cytokines.^{3,4} Interleukin-37 (IL-37) is a newly discovered cytokine in the interleukin-1 (IL-1) family,

which plays an immunosuppressive role in multiple human tissues and cell types. The human IL-37 gene is located on chromosome 2, encodes a gene product with a total length of 3 kb, and generates five different subtypes (IL-37a to IL-37e) through alternative splicing.^{5,6} IL-37 is implicated in a variety of inflammatory and immune diseases, and it plays an important role in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), atherosclerosis (AS), and psoriasis. IL-37 expression is closely related to disease activity and severity.⁷⁻¹⁰ In addition, IL-37 is involved in the development and progression of certain tumors and is associated with disease prognosis.¹¹⁻¹³ In this study, we demonstrated that IAV can induce the expression of IL-37 at clinically detectable levels in serum and cells. We also

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confirmed that IL-37 can inhibit the replication of IAV and is an important antiviral gene in the host immune response.

2 | MATERIALS AND METHODS

2.1 | Study subjects

A total of 115 patients with a clinical diagnosis of IAV infection were recruited from Renmin Hospital of Wuhan University, including 55 men and 50 women, with a mean age of 18.5 ± 15.7 years. One hundred and two healthy subjects were used as the control group, including 53 men and 49 women, with a mean age of 18.9 ± 16.1 years. IAV infection was diagnosed according to symptoms, clinical laboratory tests, and an immunofluorescence assay for IAV. None of the included patients were infected with respiratory syncytial virus (RSV), adenovirus (AdV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), *Mycoplasma pneumoniae* (MP), or *Chlamydia pneumoniae* (CP), and none received any related medication treatment. Written informed consent was obtained from each participant.

2.2 | Cell culture

Human lung adenocarcinoma A549 cells were cultured in F12K medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin and were incubated at 37°C and 5% CO₂.

2.3 | Isolation of human peripheral blood mononuclear cells (PBMCs) and viral infection

Human PBMCs were isolated using lymphocyte separation solution and density gradient centrifugation as described previously.¹⁴ The isolated PBMCs were washed twice with phosphate-buffered saline (PBS) and then cultured in Roswell Park Memorial Institute (RPMI) 1640 medium at 37°C and 5% CO₂. The cells were then infected with IAV A/HongKong/498/97 H3N2 provided by the China Center at 37°C in a 5% CO₂ incubator. ELISA was used to measure IL-27 levels in the supernatant 24 hours postinfection.

2.4 | Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol[®], and cDNA was synthesized by M-MLV reverse transcription at 37°C. Fluorescence RT-qPCR was performed for IL-37 using a SYBR[®] GreenER qPCR mix kit with GAPDH as the internal reference. The IL-37 primers were as follows: upstream primer - 5'TAC TGG TCC TGG ACT CTG GG3', downstream primer - 5'ACT CCC CTT TAG AGA CCC CC3'.

2.5 | Measurement of virus replication

A strain of IAV A/HongKong/498/97 H3N2 was provided by the China Center for Type Culture Collection. A549 was infected with

A/Hong Kong/498/97 (H3N2) at 1 multiplicity of infection (MOI). The influenza virus titer was determined by a hemagglutination titer test. The RNA content of the virus was determined by fluorescence quantitative PCR, as described previously.¹⁵

2.6 | IL-37 measurement

The levels of IL-37 in the cell supernatant and serum were measured using a human IL-37 ELISA kit.¹⁶ The procedure was performed according to the instructions. Each experiment was repeated three times.

2.7 | Statistical analysis

All data are presented as the mean $\pm$ standard deviation ($\bar{X} \pm s$). SPSS20.0 statistical software was used for the statistical analysis. The comparison between the means was performed using the Student's *t* test, and differences with $P < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | The serum level of IL-37 in the patients with IAV was increased

ELISA was used to determine the IL-37 expression levels in the serum samples of 115 patients with IAV infection within 48 hours after diagnosis and 102 healthy controls. The results showed that the serum IL-37 content was 324.4 ± 43.17 pg/mL in the patients with IAV infection and 129.2 ± 19.23 pg/mL in the healthy control group, with a statistically significant difference ($P < 0.05$) between the two groups (Figure 1).

3.2 | The level of IL-37 in the PBMCs of the patients with IAV was increased

PBMCs were isolated from the blood samples of 30 IAV patients and 30 healthy subjects. RT-qPCR was used to determine the IL-37

FIGURE 1 Comparison of the serum levels of IL-37 in IAV patients and healthy controls; * $P < 0.05$

FIGURE 2 Comparison of the expression levels of IL-37 mRNA in the PBMCs of IAV patients and healthy controls. The total RNA of PBMCs was extracted from IAV patients ($n = 30$) and healthy subjects ($n = 30$) using TRIzol[®], and IL-37 mRNA expression was measured by RT-qPCR; * $P < 0.05$

mRNA expression in PBMCs. The results showed that the IL-37 mRNA expression in the PBMCs of the IAV patients was upregulated (Figure 2), which is consistent with the results of the serum test.

3.3 | IAV upregulates IL-37 expression at the cell level

The IL-37 mRNA expression was determined using RT-PCR and the content of IL-37 in the supernatant of cells was determined using ELISA after the A549 cells and PBMCs were infected with 1 MOI IAV A/Hong Kong/498/97 H3N2. The results showed that the expression of IL-37 in A549 cells and PBMCs was upregulated after the IAV infection (Figure 3).

3.4 | IL-37 protein inhibits IAV replication

A549 cells were pretreated with 50 ng/mL recombinant human IL-37 (R&D System, Minneapolis, MN) for 12 hours and then infected with H3N2 for 3 hours. The cells were harvested, and the expression levels of the three different forms of IAV were measured (mRNA, cRNA, and vRNA).^{15,17} The results showed that the expression levels of the viral mRNA, cRNA, and vRNA were significantly inhibited compared to those of the control group (Figure 4A). Furthermore, the virus titers at different time points (12, 24, 36, and 48 hours) were measured using the hemagglutination titer test.^{15,17} As time progressed in the IL-37-pretreated A549 cells, the progeny virus

FIGURE 3 Influenza A virus induced the expression of IL-37 at the cellular level. After the A549 cells and PBMCs were infected with 1 MOI IAV for 24 h, A, the expression of IL-37 mRNA in the A549 cells was measured by RT-qPCR; B, IL-37 in the supernatant of the A549 cells was measured using ELISA; C, The expression of IL-37 mRNA in the PBMCs was determined by RT-qPCR; D, IL-37 in the supernatant of the PBMCs cells was measured using ELISA; * $P < 0.05$

FIGURE 4 IL-37 inhibits the replication of IAV. A, After A549 cells were pretreated with 50 ng/mL IL-37 for 12 h, 1 MOI IAV was added to cause infection. Total RNA was extracted after 3 h, followed by evaluation of the NP mRNA, cRNA, and vRNA levels in the A549 cells by RT-qPCR. B, After the A549 cells were pretreated with 50 ng/mL IL-37 for 12 h, 1 MOI IAV was added to cause infection, and the influenza virus titers in the supernatants of A549 cells at different time points (12, 24, 36, and 48 h) were determined by the hemagglutination titer test; * $P < 0.05$

was released into the supernatant of the cell culture after infection with influenza virus was significantly reduced compared to that of the control, demonstrating that the hemagglutination titer could not be increased (Figure 4B) and indicating that IL-37 could significantly inhibit the replication of the influenza virus.

4 | DISCUSSION

IL-37 is an important anti-inflammatory factor and plays an important role in the inflammatory response to infection.¹⁸ Li et al found that IL-37 expression levels in the sera of chronic hepatitis B patients with high viral load were significantly higher than those of uninfected people.¹⁹ However, there has been no reported study of the relationship between IAV and IL-37.

In this study, the relationship between IAV infection and IL-37 was explored for the first time. The IL-37 serum levels in patients with IAV infections were significantly higher than those in the healthy subjects. Consistently, the IL-37 mRNA levels in the PBMCs of patients with IAV infections were higher than those of healthy subjects. Furthermore, the *in vitro* cell experiment confirmed that the expression of IL-37 in IAV-infected A549 cells was upregulated.

Upon infection with viruses, bacteria, parasites, and other pathogenic microorganisms, the body's immune system can clear the foreign body by identifying the invading microorganism and initiating an immune response induced by secreted cytokines. This causes a local or systemic inflammatory response, which plays an important role in the pathogenesis of microbial diseases.²⁰⁻²²

During the process of IAV infection, monocyte/macrophage activation generates a wide variety of cytokines, which play important roles in regulating the immune response and inflammatory response.⁴ Cytokines, components of innate immunity, have an antiviral effect and can assist in the Th1 immune response.^{13,23,24}

During IAV replication, the antisense vRNA serves as a template for the transcription of positive-sense viral mRNAs and cRNA. Viral

mRNA is transcribed to make viral proteins, and the viral genome replicates via positive-stranded cRNA intermediates.²⁵

In our previous study, a protein antibody microarray was used to screen for increased IL-27 expression in IAV-infected patients. IL-27 may act to inhibit the replication of the influenza virus.¹⁵ In addition, recent studies have shown that IL-35 can upregulate IL-35 expression, IL-35 can exert an antiviral effect.²⁶ It has been reported that IL-37 is a natural suppressor of innate inflammatory and immune responses, and its effects can protect mice from LPS-induced shock.⁶ In this study, we confirmed that IL-37 can inhibit the expression of IAV mRNA and protein, but its specific molecular mechanism needs further investigation.

Infection with influenza virus can induce the expression of a variety of interleukins, and these cytokines can produce feedback to inhibit the replication of the virus,^{15,17,26} which may be one of the mechanisms by which host cells protect themselves against influenza virus infection by natural immunity.

In conclusion, our *in vitro* and *in vivo* experiments confirmed that IAV can upregulate the expression of IL-37 and that IL-37 could conversely inhibit the replication of IAV. This lays a foundation for understanding the pathogenesis of IAV and developing antiviral therapies.

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