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Abstract

Background/purpose: Severe infection with influenza A(H1N1)pdm09 virus is characterized by acute lung injury. The limited efficacy of anti-viral drugs indicates an urgent need for additional therapies. We have previously reported that neutralization of gamma interferon (IFN-γ) could significantly rescue the thymic atrophy induced by severe influenza A(H1N1)pdm09 infection in BALB/c mice. A deeper investigation was conducted into the influence of neutralizing IFN-γ to the BALB/c mice weight, survival rate, and lung injury.

Methods: The BALB/c mice was infected with severe influenza A(H1N1)pdm09. Monoclonal antibodies against IFN-γ were injected into the abdominal cavities of the mice. After neutralization of IFN-γ occurred in mice infected by severe \ influenza A(H1N1)pdm09, observing the influence of neutralizing IFN-γ to the BALB/c mice weight, survival rate, lung injury.

Result: Our results here showed that anti-IFN-γ therapy alleviated the acute lung injury in this mouse model. Neutralization of IFN-γ led to a significant reduction in the lung microvascular leak and the cellular infiltrate in the lung tissue, and also improved the outcome in mice mortality. Several pro-inflammatory cytokines, including interleukin (IL)-1α, tumor necrosis factor (TNF)-α and granulocyte-colony stimulating factor (G-CSF) in the bronchoalveolar lavage fluid (BALF), and the chemokines including G-CSF, monocyte chemoattractant protein-1 (MCP-1) in serum samples were found to be significantly reduced after anti-IFN-γ treatment.

Conclusion: These results suggested that IFN-γ plays an important role in acute lung injury induced by severe influenza A(H1N1)pdm09 infection, and monoclonal antibodies against IFN-γ could be useful as a potential therapeutic remedy for future influenza pandemics.

KEYWORDS:

H1N1

IFN-γ

lung injury

Introduction

During the spring of 2009, the emergence of a pandemic strain of influenza A (H1N1) was originally seen in Mexico[1]. Pandemic novel swine-origin influenza A (H1N1) virus (S-OIV) contains a combination of genes from influenza viruses previously known to circulate in pigs, birds, and humans[2]. It can cause severe pneumonia and even acute respiratory distress syndrome (ARDS), which is reported to be the predominant cause of death.

The immune mechanism by which influenza A virus infection induces the tissue injury to the host is complicated. Some evidence indicates that the main cause of serious illness and even death is the occurrence of excessive immune response and aggressive inflammatory response in some patients[3-7]. After the influenza virus invades the respiratory tract epithelium, immune response of the host is triggered by the release of cytokines and chemokines, which results in the infiltration of abundant macrophages and neutrophilic granulocytes and the activation of a large number of T cells, followed by the excessive production of inflammatory cytokines, which is called a cytokine storm. This is known as one of the major factors inducing serious illness and even death, from the Spanish influenza[8] in 1918 to influenza A(H1N1) virus[9] in 2009 to H5N1 avian influenza[10] and H7N9 avian influenza[11]. Previous clinical reports have indicated that inflammatory response, including overproduction of cytokines and chemokines, was involved in the pathology of severe 2009 pandemic influenza[12]. Our previous results showed that severe influenza A(H1N1)pdm09 infection induces thymic atrophy through activating innate CD8(+)CD44(hi) T cells by upregulating interferon (IFN)-γ[13]. Therefore, we studied the role of IFN-γ in acute lung injury induced by severe influenza A(H1N1)pdm09 infection. The concurrent implications were caused by neutralizing IFN-γ in the process of severe influenza A(H1N1)pdm09 treatment.

Materials and Methods

Mice, virus, and infection

Female 4-week-old, specific pathogen-free BALB/c mice were obtained from the Institute of Laboratory Animal Sciences (Beijing, China). The influenza A virus strain A/California/07/2009 (H1N1v) was used in this study. Mice were anesthetized and inoculated intranasally with virus (10^6 $TCID_{50}$ in 50 µl) as described in our previous report. Live-mouse experiments and live-virus experiments were performed in Biosafety Level 3 facilities following governmental and institutional guidelines. At the defined time points, the lung, peripheral blood and BALF were analyzed as described below. All animal experimental protocol were evaluated and approved by the Institute of Animal Use and Care Committee of the Institute of Laboratory Animal Science of Peking Union Medical College (ILAS-PL-2012-009).

Neutralization of IFN-γ in vivo

For neutralizing endogenous IFN-γ, mice were either intraperitoneally injected with the rat anti-mouse IFN-γ monoclonal antibody (mAb; R4-6A2; eBioscience) or with phosphate-buffered saline (PBS) as a control. Antibody (0.25 mg per mouse) was administered starting on day 1 before virus infection.

Survival rate and body weight changes

Female 4-week-old BALB/c mice of the IFN-γ neutralization and PBS control groups were treated intranasally with 10^6 $TCID_{50}$ in 50 µl of virus diluent. The survival percentages and body weights in each group of ten mice were monitored daily for 14 days. Survival data were analyzed by Kaplan-Meier survival analysis using GraphPad Prism 5 software.

Histological examination

Lung tissues were fixed in 10% neutral-buffered formalin for 24 h and subsequently embedded in paraffin. Lung sections (4–6 µm) from non-infected and 3-days post-infection (dpi) mice were deparaffinized and hydrated using xylene and an alcohol gradient. The sections were then stained with hematoxylin and eosin (H&E) for the assessment of general histopathology.

Virus titrations

The homogenized lung tissues were collected from 6 mice on day 3 after inoculation, and virus titrations were performed by end-point titration in Madin-Darby canine kidney (MDCK) cells as described previously.

Analysis of BALF

The lungs were lavaged with 2 ml of PBS. The BALF was centrifuged 3000 rpm at 4°C for 10 minutes, and the supernatant was collected and stored at -80 °C for further analysis.

Measurements of lactate dehydrogenase (LDH) in BALF

The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) was

used to measure LDH in BALF according to the manufacturer's guidelines.

Cytokine and chemokine measurements in BALF and serum samples

For cytokine and chemokine measurements, the *BALF and serum samples* were processed using the Bio-Plex Pro Mouse Cytokine 23-Plex panel arrays (Bio-Rad Laboratories) and detected using the Bio-Plex Protein Array System (Bio-Rad Laboratories) according to the manufacturer's instructions.

Statistical analysis

The data were analyzed using GraphPad Software (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA, USA) and are presented as the mean ± standard deviation (SD). Statistically significant differences were assessed using an unpaired two-tailed Student's *t*-test. *P* values$<$0.05 were considered statistically significant.

Result

Neutralization of IFN-γ improved symptoms in the BALB/c mice infected by severe influenza A(H1N1)pdm09

Our previous experiments showed that severe influenza A(H1N1)pdm09 infection aggravated acute lung injury and led to progressive thymic atrophy. Innate CD8(+)CD44(hi) single-positive (SP) thymocytes secreting a large amount of IFN-γ resulted in thymocyte apoptosis. We conducted a deeper investigation into the influence of neutralizing IFN-γ to the BALB/c mice weight, survival rate, and lung injury.

The day before infection of BALB/c mice with severe influenza A(H1N1)pdm09, monoclonal antibodies against IFN-γ were injected into the abdominal cavities of the mice. Three days after infection, compared with the non-infected and influenza virus-infected groups, neutralization of IFN-γ led to a significant reduction of IFN-γ in the BALF (Figure 1A). The results showed that anti-IFN-γ could significantly reduce the expression of IFN-γ in the abdominal cavities of the mice. Of ten influenza virus-infected mice, all were fatal after the first six days of infection. However, the survival rates of the anti-IFN-γ group were 20% (Figure 2A). Additionally, the anti-IFN-γ group began the restoration of weight after the first eight days of infection (Figure 2B). The above results showed that neutralization of IFN-γ partially improved symptoms in the BALB/c mice infected by severe influenza A(H1N1)pdm09 and survival rates.

Neutralization of IFN-γ could relieve lung bleeding and the infiltration of inflammatory cells

The lung bleeding and infiltration of inflammatory cells are the main features of lung injury. Therefore, we detected whether neutralization of IFN-γ could relieve the above symptoms. After the first three days of infection, the most prominent feature of the anti-IFN-γ group was relieving the bleeding and lesions in BALF (Figure 3A). Compared with the influenza virus-infected group, lung tissue sections stained by HE indicated that neutralization of IFN-γ reduced the infiltration of inflammatory cells, the fluid exudation in bronchial lumen, and the bleeding (Figure 3B). The LDH activity reflects cell damage and necrosis. In this study, we detected the LDH activity of BALF in the non-infected, influenza virus-infected, and anti-IFN-γ groups, respectively. The results showed that the activity of LDH in the anti-IFN-γ group was lower than that in the influenza virus-infected group, presenting a significant difference ($P<0.05$). However, the activity of LDH in the anti-IFN-γ group was higher than that in the non-infected group (Figure 3C). The above results indicated that neutralization of IFN-γ ameliorated acute lung injury in mice infected with severe influenza A (H1N1) pdm09. IFN-γ was one of the factors that mediated acute lung injury in mice. The virus titers of the influenza virus was detected by using lung homogenates. There was no difference in the virus titers capacity between the influenza virus-infected and anti-IFN-γ groups (Figure 3D).

The results showed that neutralization of IFN-γ had nearly no influence on the virus replication of infection sites.

Neutralization of IFN-γ led to a significant reduction of granulocyte-colony stimulating factor (G-CSF) and monocyte chemoattractant protein-1 (MCP-1) in peripheral blood

The increasing number of cytokines and chemokines may result in the overproduction of neutrophilic granulocytes and monocytes[14]. Excessive inflammatory response can lead to lung injury and more severe illness, as the influenza virus violates the lung. It has previously been reported that hyper cytokinemia is involved in the pathology of the severe 2009 pandemic influenza[9]. In an effort to further clarify the cause of lung injury, we profiled 20 cytokines and chemokines from the serum samples of mice. The results showed that the G-CSF concentration of peripheral blood in the influenza virus-infected group (2400±534.5 pg/ml) was significantly higher than that in the anti-IFN-γ group (1083±216.9 pg/ml) (Figure 4A). Compared with the anti-IFN-γ group (555.4±54.53 pg/ml), the MCP-1 concentration of peripheral blood notably increased in the influenza virus-infected group (855.3±125.1 pg/ml) (Figure 4B). There was no difference between the influenza virus-infected group and the anti-IFN-γ group when detecting the other cytokines in the peripheral blood (Figure 4C).

Neutralization of IFN-γ led to a significant reduction of G-CSF in BALF

The concentration of 20 cytokines and chemokines were also detected in BALF. The results showed that the G-CSF of BALF in the influenza virus-infected group (1,566±51.61 pg/ml) was significantly higher than that in the anti-IFN-γ group (1,304±54.45 pg/ml). However, no difference in the MCP-1 concentration of both groups was found. Compared with the influenza virus-infected group (182.1±7.193 pg/ml), the concentration of TNF-α (inflammatory cytokines) was reduced in the anti-IFN-γ group (153.9±7.229 pg/ml). Compared with the influenza virus-infected group, the anti-IFN-γ group exhibited significant reduction of cytokines, such as IL-1α, IL-2, IL-13, eotaxin, and keratinocyte chemoattractant (KC), but showed an increase of cytokines, including IL-5, IL-12 (p40), MIP-1β (Figure 5A). However, there were no differences in any of the cytokines in either group (Figure 5B).

Above all, after neutralization of IFN-γ occurred in mice infected by severe \ influenza A(H1N1)pdm09, the reduction of lung bleeding and inflammatory cell infiltration was observed, while some kinds of pro-inflammatory cytokines and chemokines, such as G-CSF, were decreased in both peripheral blood and BALF.

Discussion

The main result of this study of well-infected mice with acute lung injury secondary to A/California/07/2009 (H1N1v) infection was that neutralization of IFN-γ can alleviate acute lung injury in mice. Monoclonal antibodies against IFN-γ were actually associated with significantly better outcome and a lower risk of mortality (Figure 2B). This positive effect was apparent when detecting lung bleeding and the infiltration of inflammatory cells (Figure 3A, 3B). Given the positive effect of using anti-IFN-γ in acute lung injury associated with influenza A(H1N1)pdm09, our results are of potential importance for clinical practice.

The article, published in Nature-Biotechnology in 2009, indicated that H1N1 influenza A virus bound to not only the α-2,6 sialic acid receptors on the respiratory epithelium of the nasal cavity and throat but also the α-2,3 sialic acid receptor in alveolar epithelial cells of deep lung. In contrast, seasonal influenza virus could only bind to the α-2,6 sialic acid receptors on the epithelial cells of the upper respiratory tract, but do not have the ability to bind to the α-2,3 sialic acid receptors on the epithelial cells of the lower respiratory tract[15]. The lung tissue of BABL/c mice could express both receptors, which resulted in the H1N1 influenza A virus invading the lung and inducing immune injury. Our previous study showed that influenza A(H1N1)pdm09 can be translocated to the thymus via dendritic cells (DCs) and subsequently activate innate $CD8^+$ T cells to swiftly initiate proliferation and secrete high quantities of IFN-γ, which mediates thymocyte apoptosis and thymic atrophy[13].

The existence of activated innate $CD8^+$ T cells in the thymus is crucial and might lead to organ destruction via secreting large amounts of IFN-γ[16-18]. Neutralization of IFN-γ could significantly rescue the atrophy and the depletion of double-positive (DP) thymocytes[13]. There is no evidence to suggest that IFN-γ has a protective or damaging function on mice infected by H2N2 influenza virus[19]. However, neutralization of IFN-γ could relieve inflammatory cellular infiltration in the lung tissue of mice infected with the H3N1 influenza virus[20]. IFN-γ gene knockout mice indicated that IFN-γ had a protective effect against re-infection of influenza virus[21]. The results of a pulmonary pathological slide study of patients who had died from severe A(H1N1) influenza virus suggested that there was a significant increase in the number of $CD8^+$ T cells and the expression of TLR-3, IFN-γ, and granzyme B[22]. The increasing expression of IFN-γ occurred in the peripheral blood of severe flu cases, but the increasing expression of IFN-γ was not found in both light flu cases and normal controls[23]. The function of IFN-γ in the lung injury caused by severe flu remained ambiguous. Our results here showed that anti-IFN-γ therapy alleviated not only the thymus injury but also the lung injury in this mouse model and also improved the outcome in mouse mortality. No significant changes were found in the influence of virus replication. Neutralization of IFN-γ led to a significant reduction in lung bleeding and the cellular

infiltration of the lung tissue, and inflammatory cytokines, such as TNF-α, decreased. IFN-γ was initially confirmed by our laboratory as one of the factors inducing the lung injury of mice with severe influenza A(H1N1)pdm09 .

G-CSF plays a role in maintaining and regulating the number and function of neutrophilic granulocytes and was produced by endotoxins, TNF-α, and IFN-γ to activate the monocyte and macrophage. The partial animal experiments showed that G-CSF could enhance the function of neutrophil granulocytes and reduce their apoptosis[24]. Some studies have also found that G-CSF could induce cells generating superoxide anion free radicals, while improving the phagocytic ability of neutrophilic granulocytes to increase cytotoxicity[25]. Our research showed that lung bleeding and the infiltration of inflammatory cells was reduced after neutralization of IFN-γ in mice infected by severe influenza A(H1N1)pdm09, while G-CSF had a significant reduction in both peripheral blood and BALF, the remaining IFN-γ may make use of monocyte and macrophage activation to produce G-CSF, and neutrophilic granulocytes showed an increase in aggregation and activation. All of that resulted in much more serious lung injury.

In conclusion, neutralization of IFN-γ could improve the survival rate of mice infected by severe influenza A(H1N1)pdm09 and reduce the lung injury. The results provide a new approach for treating the lung injury caused by severe influenza A(H1N1)pdm09.

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Conflict of interest

There is no conflict of interest.

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Figure legends

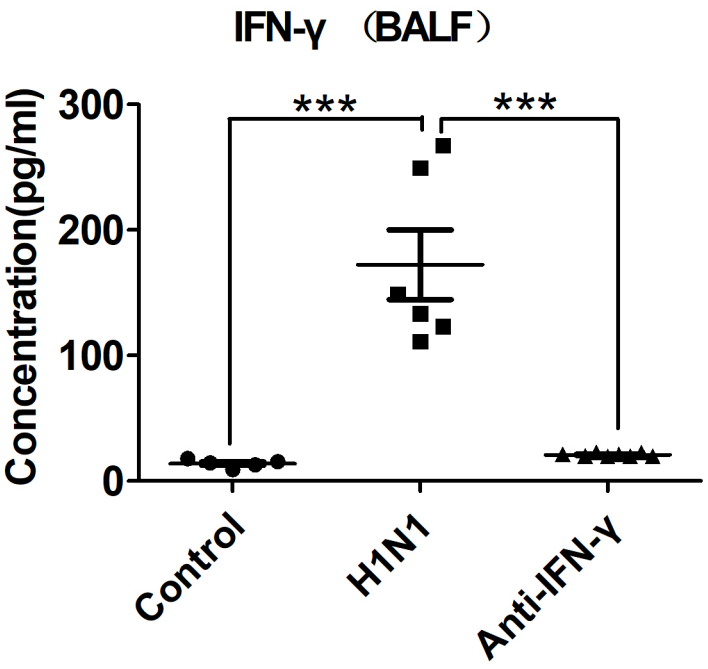
Figure 1. Assessment of IFN-γ levels in BALF. The concentrations of certain associated cytokines in the BALF were determined using the Bio-Plex Mouse Cytokines 23-Plex panel. The data are presented as the mean±SD (n=8), Significant differences from the non-infected control were revealed by an unpaired two-tailed t-test. ***P<0.001.

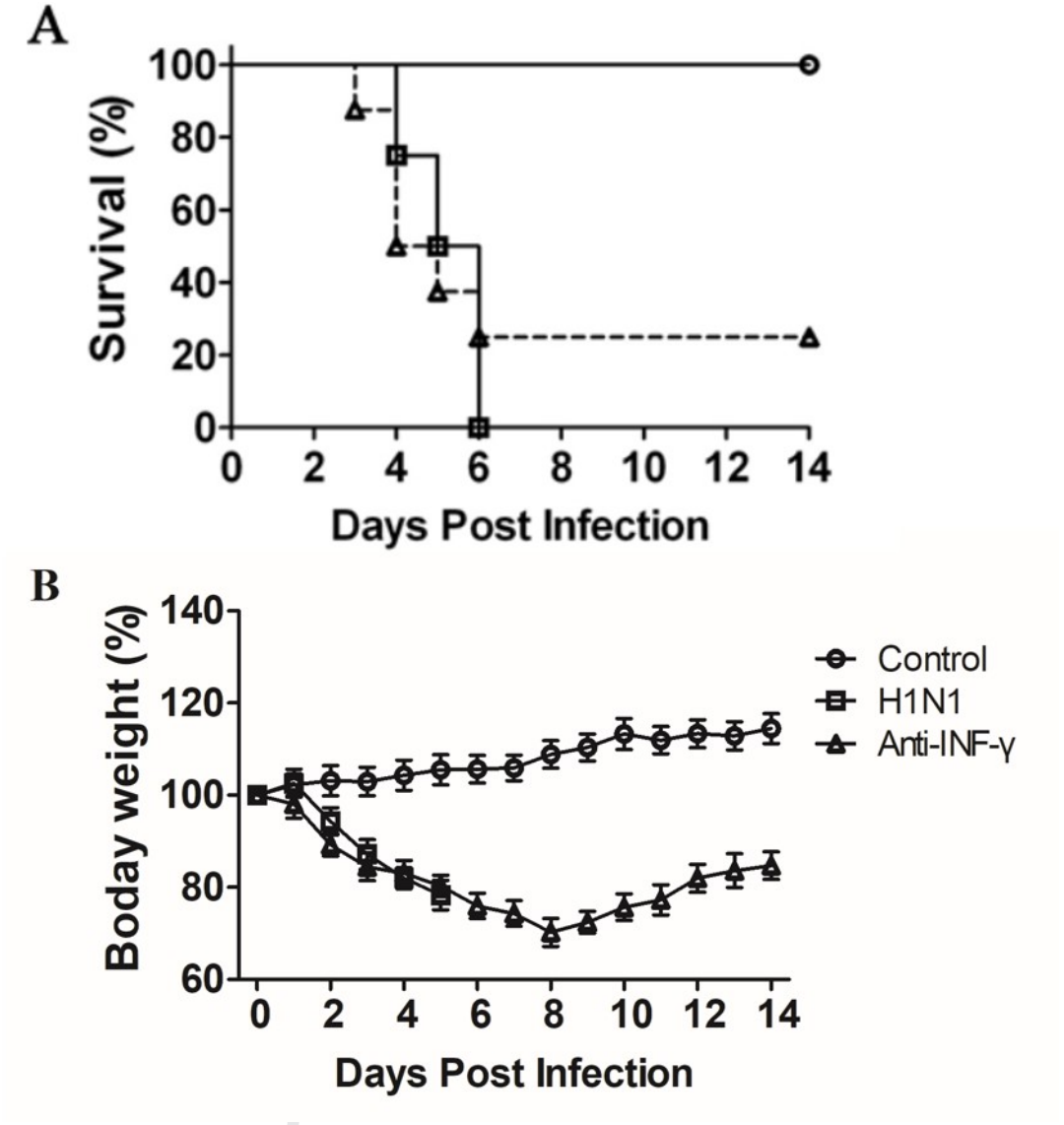
Figure 2. The influence of neutralizing IFN-γ to the BALB/c mice infected by severe influenza A (H1N1) pdm09. A: Survival rate and B: changes in body weight of non-infected, influenza virus-infected, and anti- IFN-γ mice (n=10).

Figure 3. Neutralization of IFN-γ ameliorated acute lung injury in mice. A: BALF of 3 non-infected, influenza virus-infected and anti-IFN-γ mice at 3 days post-infection (dpi). B: hematoxylin and eosin staining showing lung histology of BALB/c mice in response to H1N1 influenza A virus infection at 3 dpi (original magnification, ×100). C: Detection of LDH activity in the BALF at 3 dpi. The data are presented as the mean±SD (n=3). D: Viral RNA loads of influenza virus-infected and anti-IFN-γ mice (n=3). Significant differences from the non-infected control were revealed by an unpaired two-tailed t-test. *P<0.05; ***P<0.001.

Figure 4. Assessment of cytokine levels in the serum of three non-infected, influenza virus-infected and anti-IFN-γ mice at 3 dpi. A: Neutralization of IFN-γ led to a significant reduction of G-CSF and B: MCP-1 in the serum at 3 dpi. C: The concentrations of certain associated cytokines in the serum were determined using the Bio-Plex Mouse Cytokines 23-Plex panel and Th178-Plex panel arrays. The data are presented as the mean ± SD (n=3). Significant differences from the non-infected control were revealed by an unpaired two-tailed t-test. *P<0.05; **P<0.01; ***P<0.001.

Figure 5. Assessment of cytokine levels in the BALF of three non-infected, influenza virus-infected and anti-IFN-γ mice at 3 dpi. A: Significant and B: no-significant reduction of cytokine levels in the BALF after neutralization of IFN-γ at 3 dpi. The concentrations of certain associated cytokines in the serum were determined using the Bio-Plex Mouse Cytokines 23-Plex panel and Th178-Plex panel arrays. The data are presented as the mean ± SD (n=3). Significant differences from the non-infected control were revealed by an unpaired two-tailed t-test. *P<0.05; **P<0.01; ***P<0.001.

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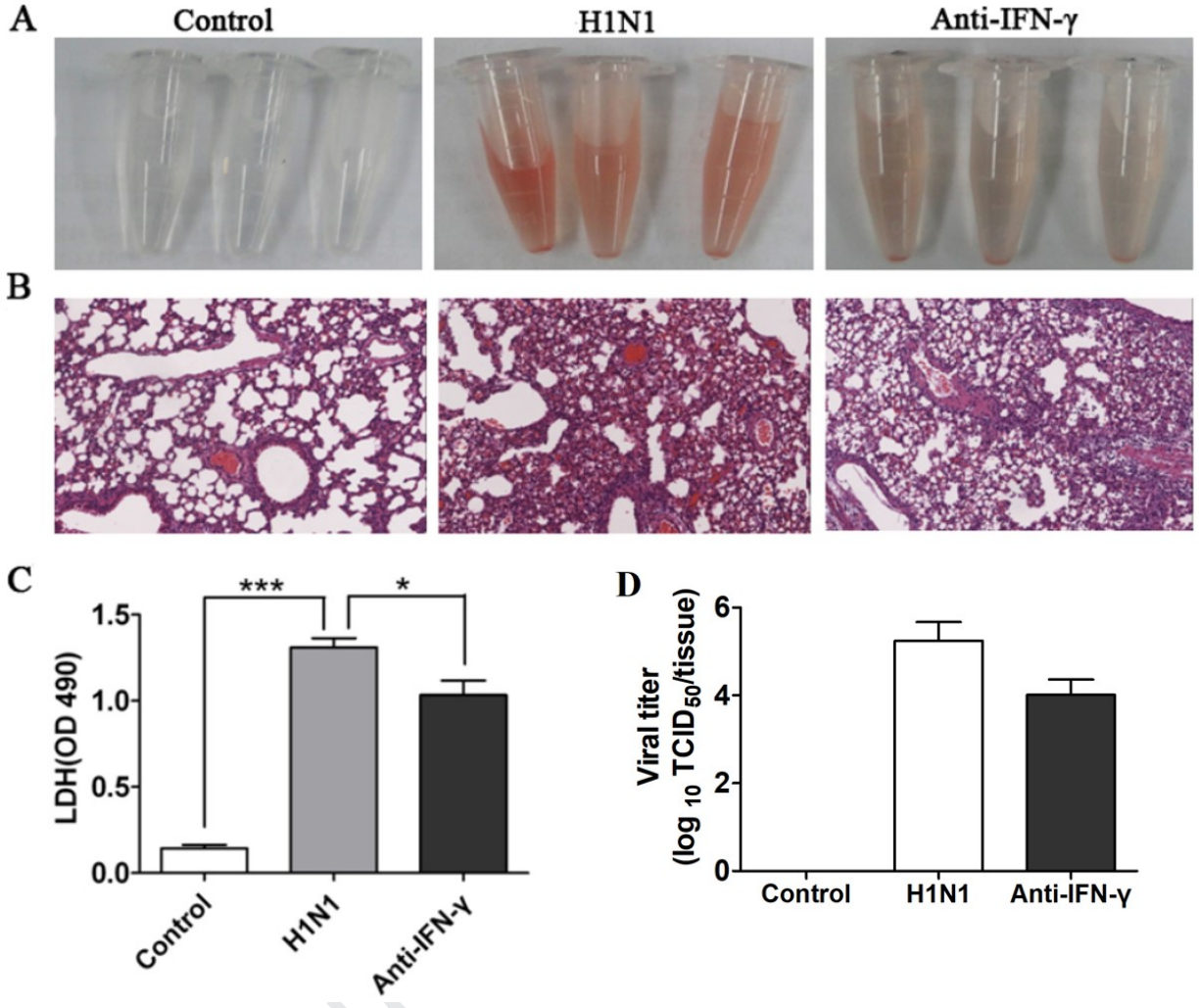
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