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인플루엔자 바이러스 (H1N1) 감염에 대한 알로페론과 자나미비르의 효과적인 항바이러스 효능에 관한 연구

A study on effective antiviral activity of Alloferon and Zanamivir against influenza A virus (H1N1) infection

2023년 2월

서울대학교 대학원

의학과 해부 및 세포생물학 전공

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알로페론과 자나미비르의 효과적인 항바이러스 효능에 관한 연구

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A study on effective antiviral activity of Alloferon and Zanamivir against influenza A virus (H1N1) infection

By

Dahae Lee

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Science in Medicine (Anatomy and Cell Biology) at Seoul National University College of Medicine

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ABSTRACT

A study on effective antiviral activity of Alloferon and Zanamivir against influenza A virus (H1N1) infection

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Along with studies related to vaccination, studies related to the development of therapeutic agents to treat viral infection are very important. Zanamivir is developed as an inhibitor of neuraminidase, a key molecule during influenza virus infection. It is administered through inhalation for the treatment of infection by influenza A virus/H1N1, but it has relatively lower efficacy than other drug, oseltamivir. Alloferon is an immunomodulatory substance developed as a therapeutic agent for herpes simplex virus (HSV) infection. It also has an effective antiviral effect to various viruses including influenza virus, human papillomavirus (HPV) and hepatitis B virus (HBV). Therefore, in this study, it was examined whether alloferon could be used to enhance the antiviral activity of zanamivir against influenza A virus/H1N1

infection.

First, it was confirmed that the combined treatment of alloferon and zanamivir effectively inhibits not only replication of influenza A virus/H1N1 in Madine-Darby canine kidney cell line (MDCK) and A549, human lung epithelial cell line, but also the production of IL-6 and MIP-1α by influenza A virus/H1N1 infection. It was mediated by the inhibition of the activation of p38MAPK and c-*Jun*, which are known to be activated by influenza A virus/H1N1 infection. In animal experiment using C57BL/6 mice treated with alloferon and zanamivir after influenza A virus/H1N1 infection, it was found that the survival of mice increased through 1) the inhibition of viral replication, 2) the inhibition of IL-6 and MIP-1α production and 3) the suppression of inflammatory responses in the lung. Interestingly, the suppression of inflammatory cell infiltration, especially macrophages, was found. It is generally known that vitamin C has an antiviral effect on infection caused by influenza A virus/H1N1. Therefore, the same experiment as in C57BL/6 wild type mice was performed in *gulo* knock out mice, which can not synthesize vitamin C *in vivo* as in human due to the lack of the L-gulono-γ-lactone oxidase (*gulo*) gene. As a result, the same results as in wild type mice that synthesize vitamin C *in vivo* were observed. It suggests that vitamin C itself may not have an antiviral effect against influenza A virus/H1N1 infection. Taken together, alloferon effectively increases the antiviral efficacy

of zanamivir and combined treatment of alloferon and zanamivir could be a useful treatment for influenza A virus/H1N1 infection.

Keywords: Alloferon, Zanamivir, Influenza virus A/H1N1, Viral replication, Lung inflammation

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LIST OF ABBREVIATIONS

IAV: influenza A viruses

MDCK: Madin-Darby Canine Kidney cell

TNF-α: tumor necrosis factor-α

RPM: rotations per minute

Gulo: L-gulono-γ-lactone oxidase

H&E: Hematoxylin and eosin

PFU: plaque forming unit

MOI: multiplicity of infection

TPCK: tosyl phenylalanyl chloromethyl ketone

RBC: red blood cell

BALF: bronchoalveolar lavage fluid

MAPK: mitogen activated protein kinase

JNK: c-*Jun* N-terminal kinase

HA: hemagglutinin

NA: neuraminidase

HAU: hemagglutinating units

EDTA: ethylene-diamine-tetraacetic acid

BCA: bicinchoninic acid

SDS: sodium dodecyl sulfate

ELISA: enzyme-linked immunosorbent assay

HRP: horseradish peroxidase

RNA: ribonucleic acid

DNA: deoxyribo nucleic acid

RT-PCR: reverse transcription polymerase chain reaction

SARS: severe acute respiratory syndrom

AMV: avian myeloblastosis virus

TCID50: median tissue culture infectious dose

DEPC: diethyl pyrocarbonate

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

IL: interleukin

PBS: phosphate buffered saline

PBS-T: PBS containing 0.1% Tween 20

PFA: paraformaldehyde

INTRODUCTION

Flu is a seasonal infectious disease that causes a significant number of deaths worldwide every year, and it is caused by influenza virus infection (1-3). The influenza virus, a single-stranded RNA virus belong to Orthomyxoviridae, is classified into four types: Type A, Type B, Type C, and Type D (3-5). Of these, the H1N1 and H3N2 subtypes (both included in influenza A viruses) are major causes of acute severe respiratory diseases in human (6, 7). Influenza A viruses poses a threat to human and animal health due to their high transmission rates, leading to high mortality and morbidity. In addition to humans, the virus is able to cause acute respiratory infections in birds and pigs (8, 9). There are two types of antigens on the surface of influenza A virus: hemagglutinin (HA) and neuraminidase (NA) (5, 10, 11). HA plays an important role during infection of host cells, and viruses use NA to infect other cells after the end of viral RNA transcription (12, 13). To date, the therapeutic agents, such as oseltamivir and zanamivir are developed for treatment of influenza A virus infection through the inhibition on the function of NA (10, 14-16).

Alloferon is a naturally occurring immunomodulatory peptide found in the

blood and lymph nodes of blow flies (*Calliphora vicina*) infected by bacteria (17, 18). It consists of 13 amino acids: HGVSGHGQHGVHG (19). Recent studies show that it controls acute inflammatory responses in epithelial cells in the skin and cornea, as well as chronic inflammatory responses involved in rheumatoid arthritis and asthma (20-23). It also controls herpes simplex virus infection and its recurrent infection (17, 24, 25). The antiviral and antitumor efficacy appears to be mediated via increased activity of human NK cells and the production of interferons (21, 23, 25, 26). In fact, clinical studies of the antiviral effects of alloferon against HCV and influenza virus are being conducted in Russia at this moment (27).

Globally, more than 600,000 people die each year by influenza A virus infection, especially when the seasons change (28). Because influenza A virus is also able to infect animals, it is necessary to effectively prevent transmission between animals and humans, through the development of effective drugs and treatments. Amantadine and rimantadine have been developed before development of oseltamivir and zanamivir and used as antiviral drugs to treat the infection by H1N1 and H3N2 (29-31). Even though amantadine and rimantadine were used to prevent influenza A virus infection, they are not used currently due to the rapid occurrence of resistance to them (10, 14, 28, 32, 33). For this reason, oseltamivir and zanamivir, which

bind to the NA receptor in host cells to prevent releasing viruses from infected cells, are currently the most frequent antiviral drug for the treatment of influenza A virus infection (11, 34-36). However, oseltamivir can cause nausea, vomiting, headache, and gastrointestinal disorders, as well as psychological disorders such as delirium, convulsions, and hallucinations, especially in adolescents (29, 31, 37, 38). In addition, drug resistance develops faster than for zanamivir (39-42). Because zanamivir is inhaled into the nasal cavity, most of the drug is delivered to the lungs, resulting in fewer systemic side effects than oseltamivir. However, the absorption rate is lower than that of oseltamivir and so the drug tends to be less effective (40, 43, 44).

L-ascorbic acid (Vitamin C) is an essential antioxidant that scavenges free radicals and is crucial for physiological processes such as hormone production, collagen synthesis, immune potentiation, and defence to virus infection (45-47). It is known that vitamin C plays a key role in preventing the common cold and limiting lung pathology after influenza virus infection (45). Also, vitamin C inhibits cortisol synthesis, which decreases the susceptibility of mice to influenza viral infection (48-50). It has previously been reported that vitamin C induces antiviral immune responses at the earliest stages of influenza virus H3N2 infection, through an increase in interferon-α/β production (47, 51). Most of the mammals, except human and

some of non-human primates, synthesize vitamin C from glucose in their livers. Because human and some of the non-human primates lack the enzyme L-gulono-γ-lactone oxidase (*Gulo*), which is essential for synthesis of the vitamin C (52). Without supplementation of vitamin C for 2 weeks, the plasma vitamin C concentration of *gulo* (-/-) mice decreased by 10-15% (53). Therefore, it is necessary that therapeutic agents against influenza virus infection is examined in *gulo* (-/-) mice to elucidate the effects of vitamin C on virus infection.

Recently, with the increase in acute inflammatory respiratory diseases caused by respiratory infectious viruses, including Severe Acute Respiratory Syndrom (SARS-CoV2) and influenza virus, not only vaccine development, but also treatment development is becoming increasingly important (54, 55). Therefore, the aim of this study is to examine whether the combination of zanamivir and alloferon can overcome the limitations of conventional antiviral drugs currently being developed for the treatment of influenza A virus/H1N1 infection.

MATERIALS AND METHODS

Cell culture The human lung epithelial cell line A549 was maintained in RPMI 1640 (Welgene, Daegu, Korea), Madin-Darby canine kidney cell line, MDCK and human airway epithelial cell line, Calu-3 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene). Both media contained 10% heat-inactivated fetal bovine serum (Gibco, Queensland, Australia) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Welgene) at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of influenza A virus/H1N1 The influenza A/Puerto Rico/8/1934 was generously provided by Nam Hyuk Cho (Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul, Korea). All animal experiments with influenza A virus were performed in an Animal Biosafety Level 2 facility at Seoul National University College of Medicine. Influenza A/H1N1 viruses were propagated in MDCK cells cultured in DMEM (Welgene) supplemented with tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (2 μg/mL; Sigma-Aldrich, St. Loise, MO, USA), harvested at 72 h after infection. And then

virus titer for infection was determined through plaque forming unit (PFU) assay.

Plaque forming unit (PFU) assay MDCK (5.0×10^5/well) and A549 (4.0×10^5/well) were seeded onto 6-well plates at 37°C in a 5% CO_2 atmosphere. After overnight culture, cells were washed twice with 1× PBS buffer and treated with the serial 10-fold dilutions of influenza virus/H1N1 in serum-free medium containing only 1% penicillin streptomycin (Welgene). The plates were incubated for 1 h at 37°C with constant agitation to induce virus infection. And then washed with 1× PBS to remove virus and overlaid with 2 mL of agar medium comprising 2× DMEM (Welgene), 2 μg/mL TPCK-treated trypsin, and 1.6% agar (Lonza Bioscience, Morrisville, NC, USA). Plates were then incubated for 3 days at 37°C under 5% CO_2 and 95% relative humidity. The agar-covered monolayers were fixed with 4% paraformaldehyde (Daejung, Seoul, Korea). After removing the agar, the fixed cells were stained with 0.5% crystal violet solution (Sigma-Aldrich) and plaques were counted.

Western blot analysis Western blotting was used to examine expression of proteins involved in signaling pathways for alloferon and/or zanamivir.

Protein lysate was obtained from MDCK, A549 and lung tissue of mice with or without H1N1 infection (MOI 0.01) in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) for 12 and 24 h by using lysis buffer containing 50 mM Tris-HCL (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktails (Sigma-Aldrich). BCA assay was used to quantify the protein concentration. The proteins (20 μg/sample) were resolved in a 10% polyacrylamide-SDS gel at 100 V for 2 h and then transferred to nitrocellulose membranes. Blocking was performed for 1 h at room temperature with 5% non-fat milk in PBS. The blocked membrane was incubated at 4°C overnight with antibodies specific for p38MAPK (1:1000; Cell Signaling; Boston, MA, USA), c-*Jun* (1:1000; Cell Signaling), p42/44MAPK (1:1000; Cell Signaling), p-p38MAPK (1:1000; Cell Signaling), p-c-*Jun* (1:1000; Cell Signaling), p-p42/44MAPK (1:1000; Cell Signaling) or/and β-actin (1:5000; Santa Cruz Biotechnology; Santa Cruz, CA, USA). After washing three times (5 min each) with 0.1% PBST, the membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000; Cell Signaling Technology) to detect p38MAPK, c-*Jun*, p42/44MAPK, p-p38MAPK, p-p42/44MAPK and p-c-*Jun*, or with HRP-conjugated anti-mouse IgG (1:10,000; Cell Signaling) to detect β-actin. An

electrochemical luminescence detection system (Thermo Scientific, Wilmington, DE, USA) was used to visualize the immunoreactive proteins after three washes (5 min each). Image J software (NIH) was used to analyze the density of the bands and normalize the phospho form to the total form.

Quantitative real time polymerase chain reaction (qRT-PCR) analysis To examine the expression of alloferon and/or zanamivir, total RNA was extracted using Trizol Reagent (Invitrogen, Grand Island, NY, USA). Reverse transcription of RNA samples into cDNAs was performed using oligo (dT) primers and AMV reverse transcriptase (iNtRON; Daejeon, Korea). One microgram of total RNA was combined with 5 mM $MgCl_2$, 1 mM of each dNTP, 1 μL of RT buffer, 1 U/mL RNasin, 15 U/mL reverse transcriptase, and DEPC treated water up to a total volume of 20 μL. The samples were incubated for 10 min at room temperature, followed by incubation for 1 h at 42°C, 5 min at 72°C, followed by another 5 min on ice. Following addition of 180 μL of DEPC treated water, samples were stored at −70°C. Each reaction was run in a quadrant for each sample, and an analysis of the results was performed using the Rotor-Gene SYBR Green PCR kit (Qiagen, Hilden, Germany) and Rotor-Gene Q 2plex-real-time RT PCR instrument (Qiagen). Amplification was performed using SYBR Green master mix (Qiagen). Each

cycle of PCR was monitored by measuring the fluorescence produced by binding of SYBR Green dye to dsDNA. The following primers were used: H1; Forward 5'-AGC AAA AGC AGG GGA AAA TAA AA-3'; Reverse 5'-CAC GAG GAC TTC TTT CCC TTT ATC AT-3'; and GAPDH; Forward 5'-GGT GGT CCA GGG TTT CTT A-3'; Reverse 5'-GTT GTC TCC TGC GAC TTC A-3'. Calculation of differences in the amount of H1N1 was performed using the ΔΔCT method ($2^{-\Delta\Delta Ct}$).

Enzyme-linked immunosorbent assay (ELISA) MDCK (1×10^5/well) and A549 (2×10^5/well) were seeded into 6-well plates for overnight and then treated with alloferon and/or zanamivir after H1N1 infection, and were allowed to grow to confluence for 48 or 72 h. And cell supernatants were collected and stored at −80°C before measured using ELISA kits. In the case of lysates of mouse lung tissues, BALF and serum (sampling method is described in *Materials and Methods 10*) were stored at −80°C prior to preparation for BioLegend's ELISA MAX™ Standard Set Kits. The concentration of IL-6, MIP-1α, IL-1β, IFN-λ, TNF-α and IL-10 in cells, serum and BALF was measured ELISA kits according to manufacturer's protocol (BioLegend; San Diego, CA, USA) based on Avidin-Biotin reaction. After addition of stop solution to each well. When positive wells turned from

blue to yellow, the relative absorbance was measured at 450 nm using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA).

Animals C57BL/6 wild-type and L-glunolactone-γ-oxidase (*Gulo*) (-/-) mice (C57BL/6 background) were housed in pathogen-free conditions at the Seoul National University College of Medicine. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-200319-2). Eight-weeks-old mice (n=30/group) were used for the experiments. Vitamin C (3.3 grams per liter) was given to *gulo* (-/-) mice in drinking water to prevent the death of the mice due to a vitamin C deficiency. Influenza A virus /H1N1 (32 HAU/50 μl) was administered intranasally to mice under isoflurane anesthesia. Alloferon (0.5 μg) and/or zanamivir (35 μg) were also intranasally administered daily, followed by intranasal administration of H1N1. Symptoms and mortality were monitored daily for 8 days. Over the course of 8 days, body weight was measured daily, and survival rate along with clinical signs; 1) changes in activity/mobility, 2) behavior changes with slow movement. Mice with weight loss exceeding 20-30%, or that seemed to be clearly terminal, were euthanized in accordance with animal ethics committee guidelines.

Hemagglutination (HA) assay HA assay was performed using virus-containing culture medium to measure virus replication. Virus supernatants in 96-well U-bottom plates were serially diluted in saline. Then, each well was filled with 50 μL of a 0.5% human red blood cell (RBC) suspension. After that, the plates were placed at room temperature for 30 min. As soon as the incubation period was complete, agglutination of RBCs in each well was determined. The concentration of mouse administration according to the HA assay was dilution of 1:32, 32 HAU/50 μl.

Measurement of TCID50 In order to determine viral titers, the lungs of mice were harvested after virus inoculation on days 4 and 6. Lung tissues were homogenized in DMEM medium to achieve 10-fold serially diluted suspensions of tissue homogenates and were titrated of MDCK on 96-well culture plates. Titers were calculated using the Spearman-Karber method ($-(x0 - d/2 + d \sum ri/ni)$) and expressed as $\log_{10}TCID_{50}$/g lung tissue. Based on these experiments, the average values were obtained.

Collection of serum and bronchoalveolar lavage fluid (BALF) Blood samples were collected from the retro-orbital plexus of each animal using

heparinized capillaries and immediately centrifuged at 600 g for 10 minutes at 4°C to obtain serum and stored at –80°C. BALF was prepared with PBS containing 1% bovine serum albumin and 0.1% NaN3 (blocking solution) through lavage the trachea and lungs (56). After centrifuging the BAL fluids for 10 min at 600 g, the supernatant was stored at –80°C and resuspended in 300 μL of PBS for cytospin preparation, then the composition of infiltrated immune cells into the lung upon influenza A virus/H1N1 was determined after Wright's Giemsa (SIGMA) staining.

Histological analysis Lung tissues were harvested and fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Fixed tissues were embedded in paraffin prior to cutting of 4 μm sections. Sections were deparaffinized and hydrated, and then stained with hematoxylin and eosin prior to examine under a light microscope. Masson's Trichrome staining was performed to determine fibrotic changes in the lung using a VitroView^{TM} Masson's Trichrome Stain Kit (VitroView^{TM}, Hanam, Korea); Weigert's iron hematoxylin, Biebrich Scarlet-Acid Fuchsin, and Aniline Blue Stain solutions were applied to deparaffinized sections pretreated with Bouin's fluid at 56°C for 1 h. Finally, 1% acetic acid solution was used to remove non-specific staining. All analysis were carried out using Masson's Trichrome staining image analyzer,

Celleste (Thermo Scientific).

Statistical analysis Data are presented as the mean ± SD. Unpaired t-tests were used to compare data between two groups. Statistical analysis was performed using GraphPad Software Prism version 6.01 (GraphPad Software, La Jolla, CA, USA).

Results

Alloferon increases the antiviral effect of zanamivir against influenza A virus/H1N1 infection.

First, the effect of alloferon and/or zanamivir on replication of H1N1 was examined in Madin-Darby canine kidney (MDCK), A549 and Calu-3 by real-time RT-PCR. As shown in Figure 1-3, when alloferon is combined with zanamivir, viral replication was remarkably suppressed in MDCK (Figure 1), A549 (Figure 2) and Calu-3 (Figure 3) respectively. The combination of alloferon and zanamivir suppresses the combination of alloferon and zanamivir, A549 showed a greater suppression of the combination treatment than MDCK and Calu-3.

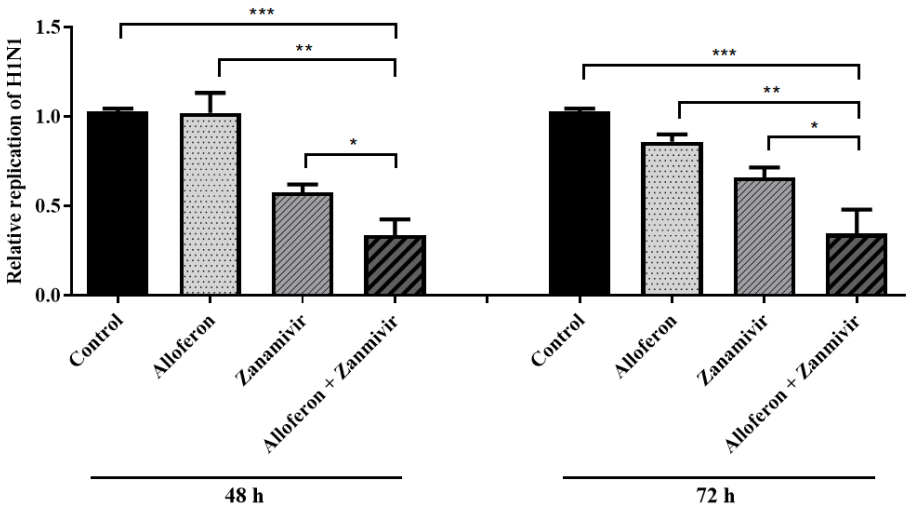

Figure 1. Alloferon increases the antiviral efficacy of zanamivir in MDCK infected by influenza A virus/H1N1. Quantitative real-time RT-PCR analysis of influenza virus replication in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) was performed. Cells were infected with influenza A virus (MOI 0.01), total RNA was extracted and cDNA was made. And then real-time RT-PCR was performed using primers specific for H1N1. The amount of virus in treated cells is shown relative to that in control cells (cells infected with virus but not treated with alloferon and/or zanamivir). The replication is normalized with GAPDH. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$

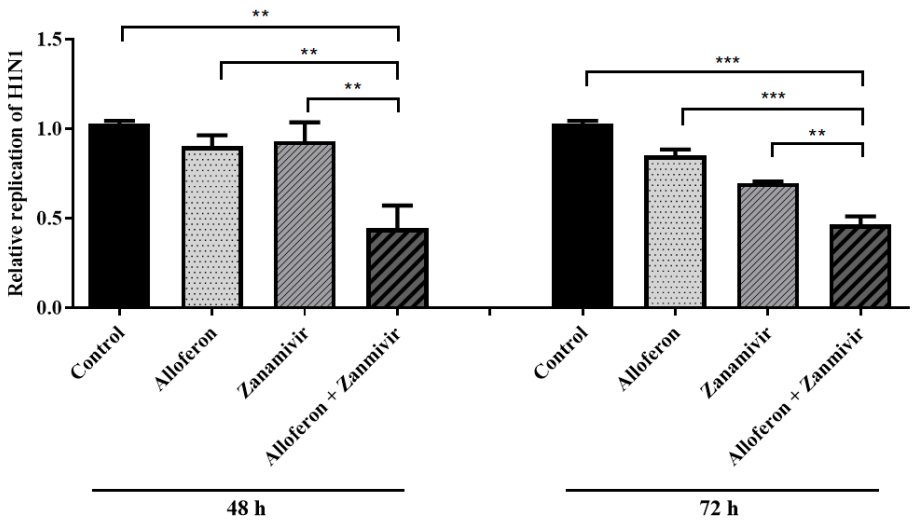

Figure 2. Alloferon increases the antiviral efficacy of zanamivir in A549 infected by influenza A virus/H1N1. Quantitative real-time RT-PCR analysis of influenza virus replication in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) was performed. Cells were infected with influenza A virus (MOI 0.01), total RNA was extracted and cDNA was made. And then real-time RT-PCR was performed using primers specific for H1N1. The amount of virus in treated cells is shown relative to that in control cells (cells infected with virus but not treated with alloferon and/or zanamivir). The replication is normalized with GAPDH. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$

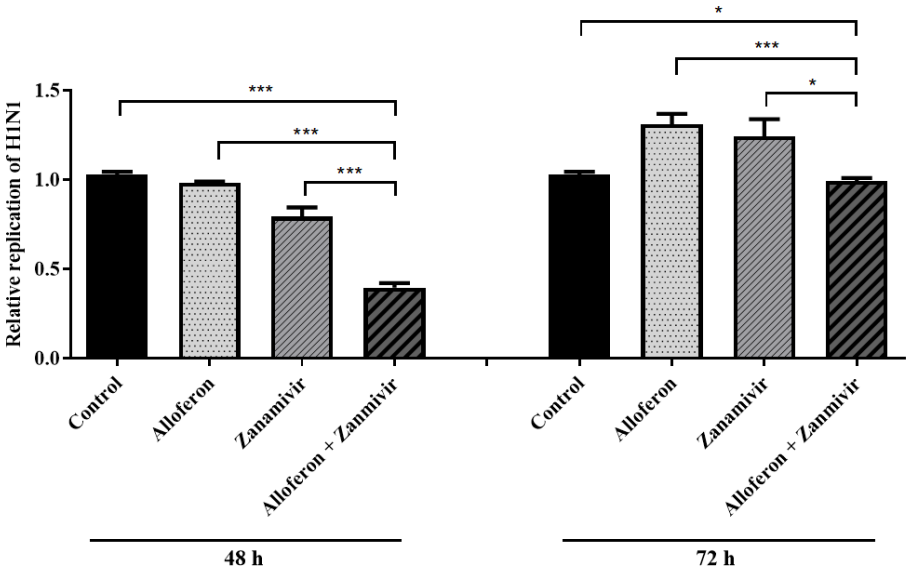

Figure 3. Alloferon increases the antiviral efficacy of zanamivir in Calu-3 infected by influenza A virus/H1N1. Quantitative real-time RT-PCR analysis of influenza virus replication in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) was performed. Cells were infected with influenza A virus (MOI 0.01), total RNA was extracted and cDNA was made. And then real-time RT-PCR was performed using primers specific for H1N1. The amount of virus in treated cells is shown relative to that in control cells (cells infected with virus but not treated with alloferon and/or zanamivir). The replication is normalized with GAPDH. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$

Combined treatment of alloferon and zanamivir suppresses production of IL-6 and MIP-1α induced by influenza A virus/H1N1 infection.

Influenza A virus infection induces IL-6 and MIP-1α, which play a role in development of inflammation in the lung. Therefore, it was determined whether combined treatment of alloferon and zanamivir effectively suppresses IL-6 and MIP-1α production after H1N1 infection. It was found that production of IL-6 from MDCK increased at 72 h after infection with H1N1. And it was effectively suppressed in MDCK and A549, by combined treatment of alloferon and zanamivir (Figure 4 and 5). However, there was no definite suppressive effect in Calu-3 by combined treatment (Figure 6). As shown in Figure 7 and 8, the production of MIP-1α in MDCK was increased at 48 h after infection and at 72 h in A549, and it was also suppressed by combined teatment of alloferon and zanamivir. In the case of Calu-3 (Figure 9), MIP-1α production was not highly suppressed by combined treatment, shown in other two cell lines.

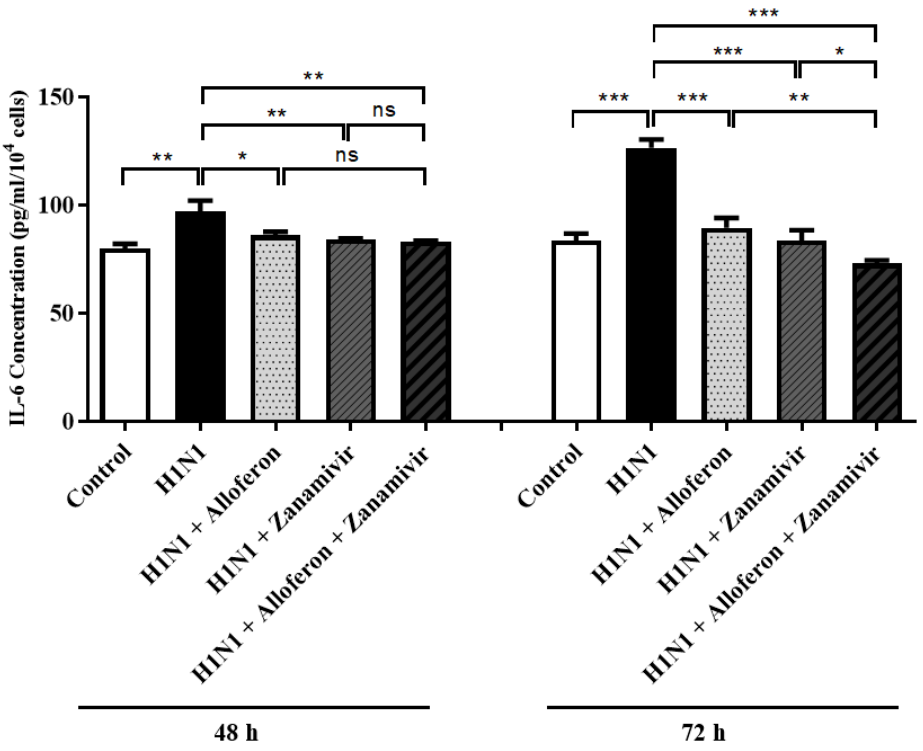

Figure 4. Combined treatment of alloferon and zanamivir suppresses the production of IL-6 by influenza A virus/H1N1 infection in MDCK. At 48 and 72 h after H1N1 infection, culture supernatant of MDCK was collected and the concentration of IL-6 in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) were measured by ELISA. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$

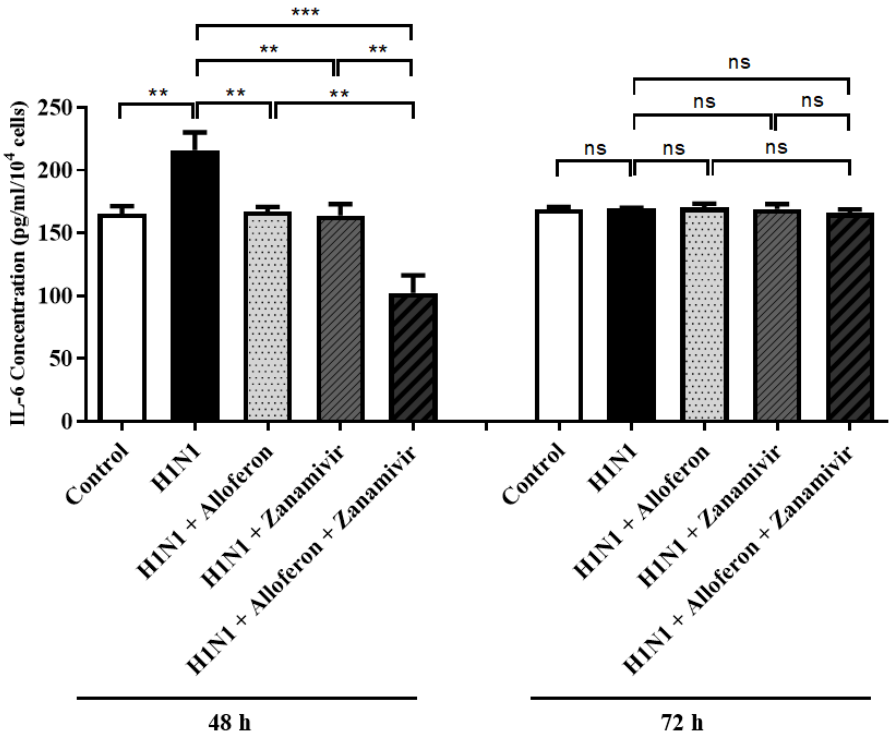

Figure 5. Combined treatment of alloferon and zanamivir suppresses the production of IL-6 by influenza A virus/H1N1 infection in A549. At 48 and 72 h after H1N1 infection, culture supernatant of A549 was collected and the concentration of IL-6 in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) were measured by ELISA. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, NS; no significant.

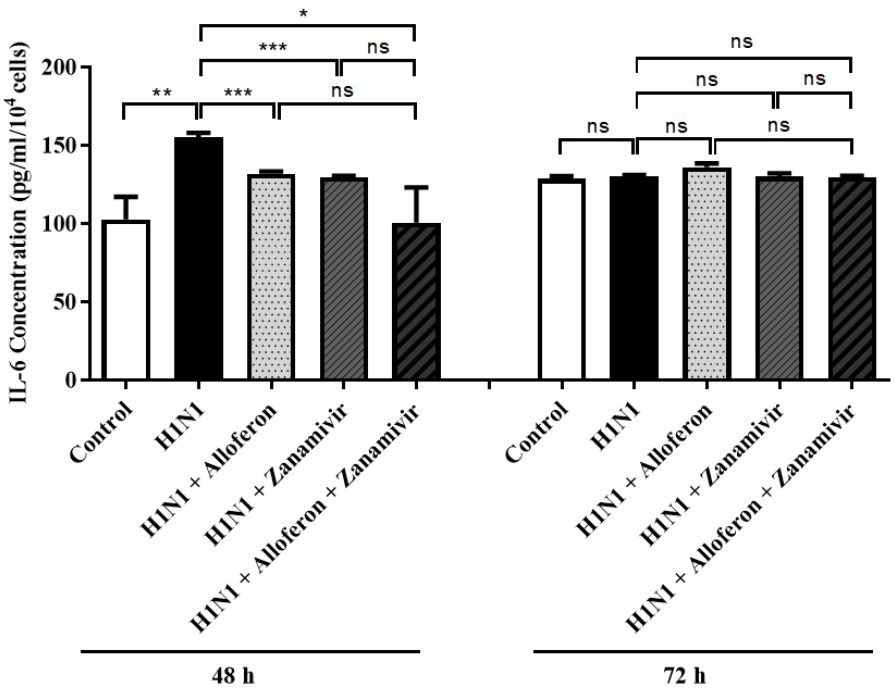

Figure 6. Combined treatment of alloferon and zanamivir suppresses the production of IL-6 by influenza A virus/H1N1 infection in Calu-3. At 48 and 72 h after H1N1 infection, culture supernatant of Calu-3 was collected and the concentration of IL-6 in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) were measured by ELISA. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, NS; no significant.

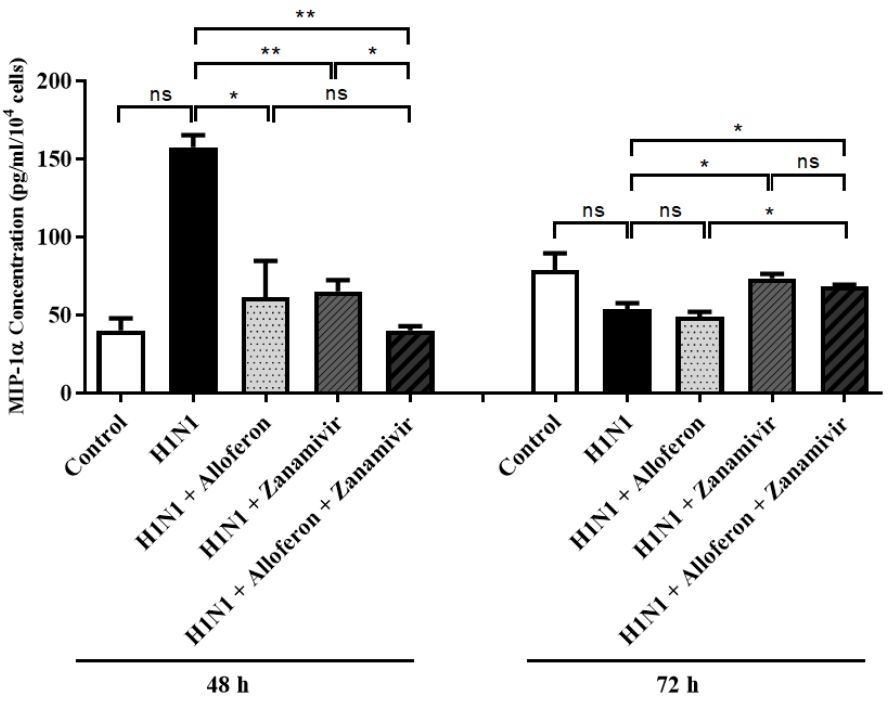

Figure 7. Combined treatment of alloferon and zanamivir suppresses the production of MIP-1α by influenza A virus/H1N1 infection in MDCK. At 48 and 72 h after H1N1 infection, culture supernatant of MDCK was collected and the concentration of MIP-1α in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) were measured by ELISA. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, NS; no significant.

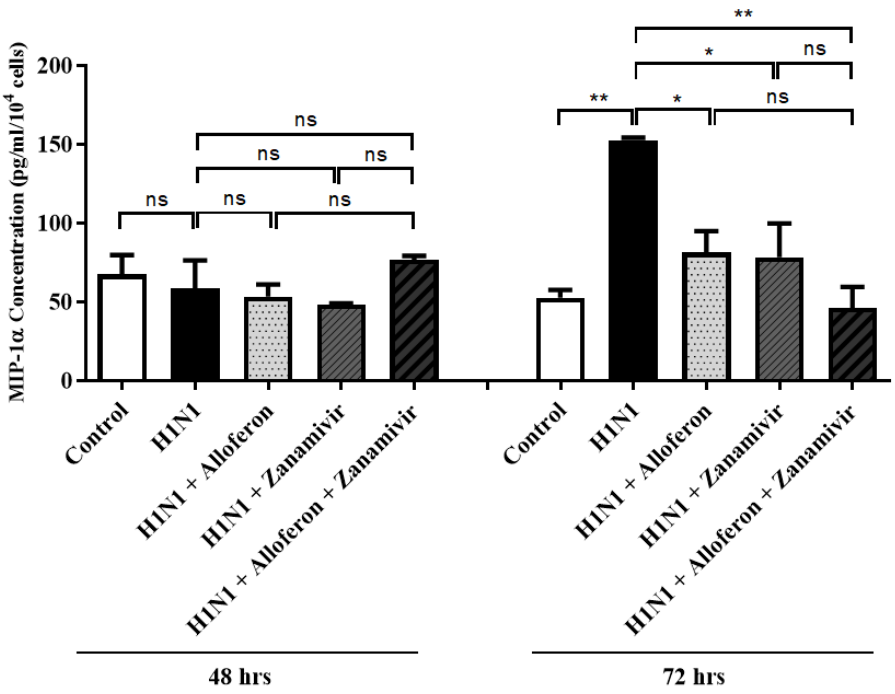

Figure 8. Combined treatment of alloferon and zanamivir suppresses the production of MIP-1α by influenza A virus/H1N1 infection in A549. At 48 and 72 h after H1N1 infection, culture supernatant of A549 was collected and the concentration of MIP-1α in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) were measured by ELISA. Results are representative of more than three independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, NS; no significant.

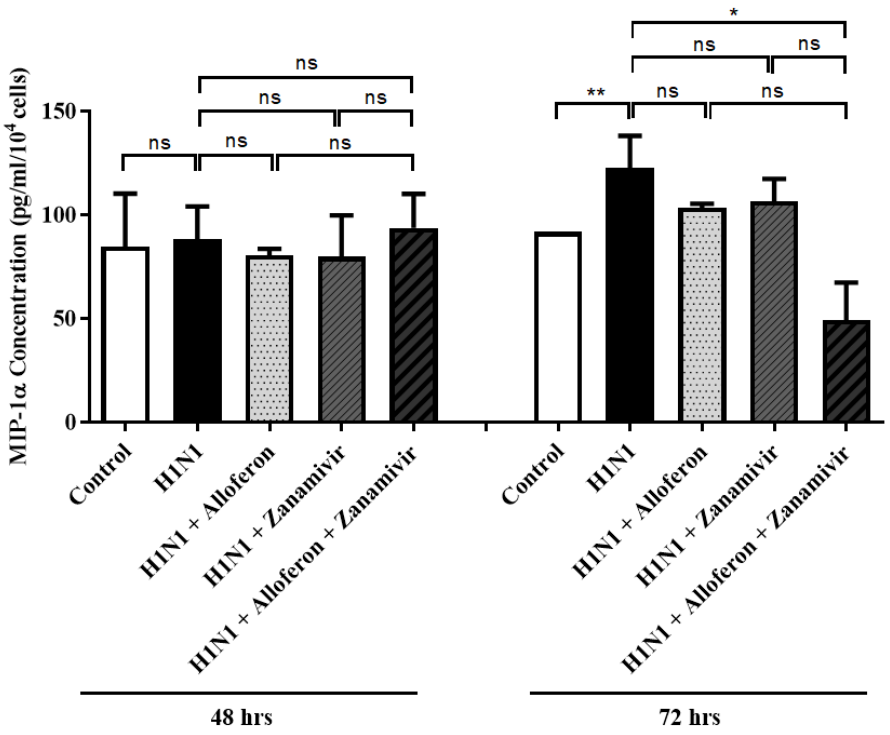

Figure 9. Combined treatment of alloferon and zanamivir suppresses the production of MIP-1α by influenza A virus/H1N1 infection in Calu-3. At 48 and 72 h after H1N1 infection, culture supernatant of Calu-3 was collected and the concentration of MIP-1α in the presence or absence of alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) were measured by ELISA. Results are representative of more than three independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

p38MAPK and JNK play an important role in the antiviral effects of combined treatment of alloferon and zanamivir.

To examine the signaling pathways involved in the antiviral effects of combined treatment of alloferon and zanamivir, a study was conducted to determine whether alloferon (0.5 μg/mL) and/or zanamivir (35 μg/mL) activated p38MAPK and *Jun*-amino-terminal kinase (JNK) in virus-infected cells. As shown in Figure 10, the phosphorylation of p38MAPK in MDCK was remarkably increased after H1N1 infection, and it was effectively suppressed by combined treatment. In case of c-*Jun*, it was also increased by virus infection, but suppressed by combined treatment (Figure 11). It has also been observed the activation of p38MAPK and c-*Jun* was suppressed by combined treatment of alloferon and zanamivir in A549 (Figure 12 and 13).

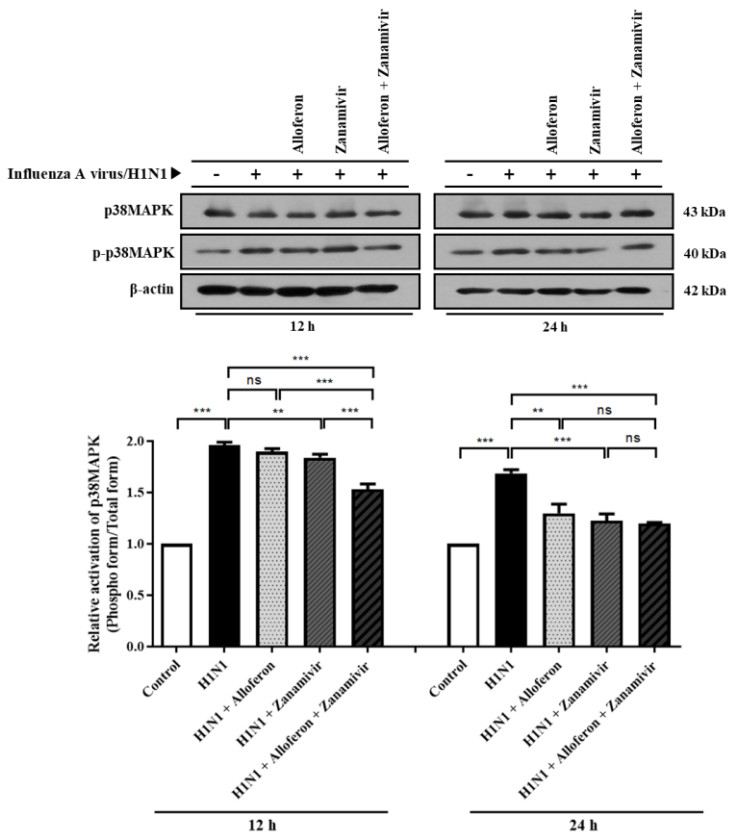

Figure 10. Combined treatment of alloferon and zanamivir suppresses the activation of p38MAPK by influenza A virus/H1N1 infection in MDCK. MDCK were cultured in the presence or absence of alloferon and/or zanamivir for 12 and 24 h after virus infection. Cell lysates were prepared, and western blot analysis performed to detect changing on the activation of p38MAPK. Results are representative of more than three independent experiments. (A) Western blot analysis (B) After measuring the values of phospho form and total form by using of Image J software, the relative activation is calculated by normalization the phospho form to total form. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

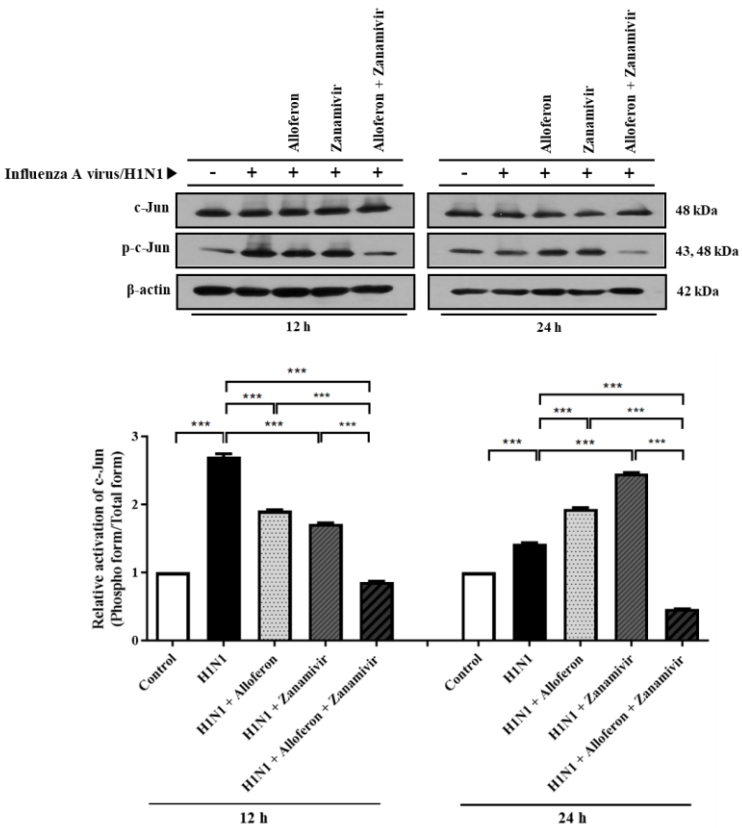

Figure 11. Combined treatment of alloferon and zanamivir suppresses the activation of JNK by influenza A virus/H1N1 infection in MDCK. MDCK were cultured in the presence or absence of alloferon and/or zanamivir for 12 and 24 h after virus infection. Cell lysates were prepared, and western blot analysis performed to detect changing on the activation of c-*Jun*. Results are representative of more than three independent experiments. (A) Western blot analysis (B) After measuring the values of phospho form and total form by using of Image J software, the relative activation is calculated by normalization the phospho form to total form. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

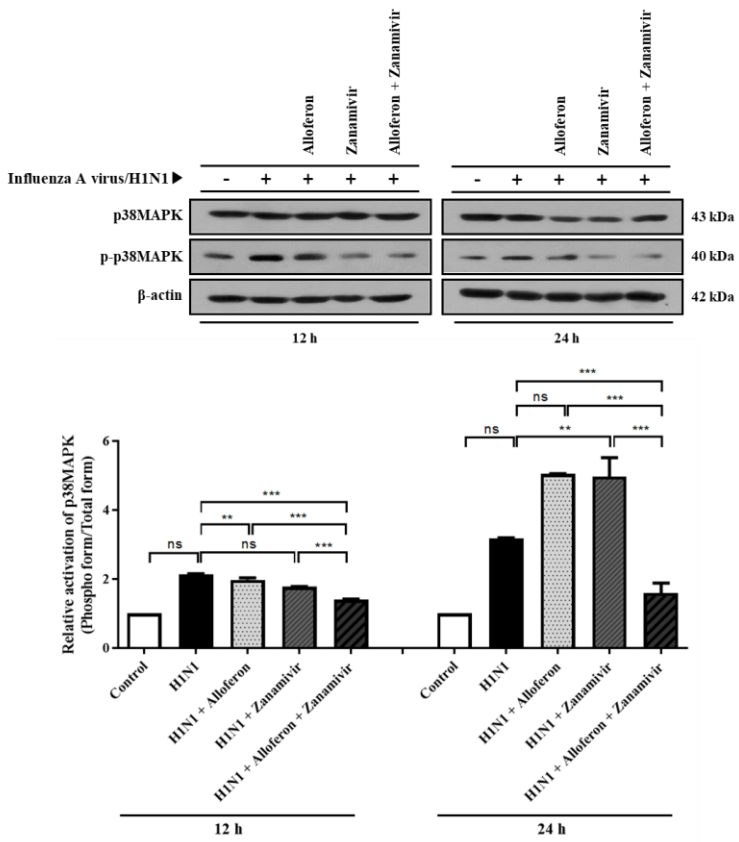

Figure 12. Combined treatment of alloferon and zanamivir suppresses the activation of p38MAPK by influenza A virus/H1N1 infection in A549. A549 were cultured in the presence or absence of alloferon and/or zanamivir for 12 and 24 h after virus infection. Cell lysates were prepared, and western blot analysis was performed to examine changing on the activation of p38MAPK. Results are representative of more than three independent experiments. (A) Western Blot analysis (B) After measuring the values of phospho form and total form by using of Image J software, the relative activation is calculated by normalization the phospho form to total form. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

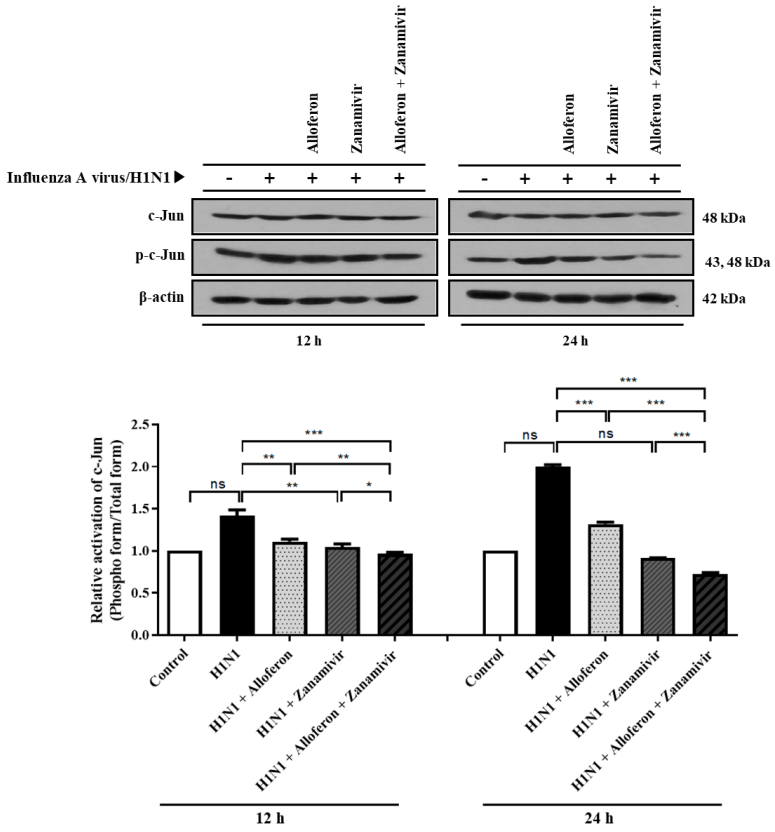

Figure 13. Combined treatment of alloferon and zanamivir suppresses the activation of JNK by influenza A virus/H1N1 infection in A549. A549 were cultured in the presence or absence of alloferon and/or zanamivir for 12 and 24 h after virus infection. Cell lysates were prepared, and western blot analysis was performed to examine changing on the activation of c-*Jun*. Results are representative of more than three independent experiments. (A) Western Blot analysis (B) After measuring the values of phospho form and total form by using of Image J software, the relative activation is calculated by normalization the phospho form to total form. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

Combined treatment of alloferon and zanamivir inhibits viral replication and increases survival of mice infected with influenza A virus/H1N1.

Based on the *in vitro* results in MDCK and A549, the antiviral effects of alloferon and/or zanamivir were examined in mice infected with influenza A virus/H1N1. Virus titer for intranasal inoculation was determined through HA assay. As shown in Figure 14B, it was 32 HAU. On Day 4 after virus infection, the weight of virus-infected mice without treatment of alloferon and/or zanamivir decreased, but it was effectively prevented by combined treatment of alloferon and zanamivir (Figure 15A). In addition, the survival rate of infected mice was decreased from 3 days after virus infection, but it was remarkably prevented by combined treatment of alloferon and zanamivir (Figure 15B). To examine whether combined treatment of alloferon and/or zanamivir affects viral replication in the lung, $TCID_{50}$ value in lung tissue was determined. Virus replication was decreased by the treatment of alloferon and zanamivir, respectively. However, combined teatment of alloferon and zanamivir more effectively suppressed viral replication in the lung of virus-infected mice (Figure 16A and B).

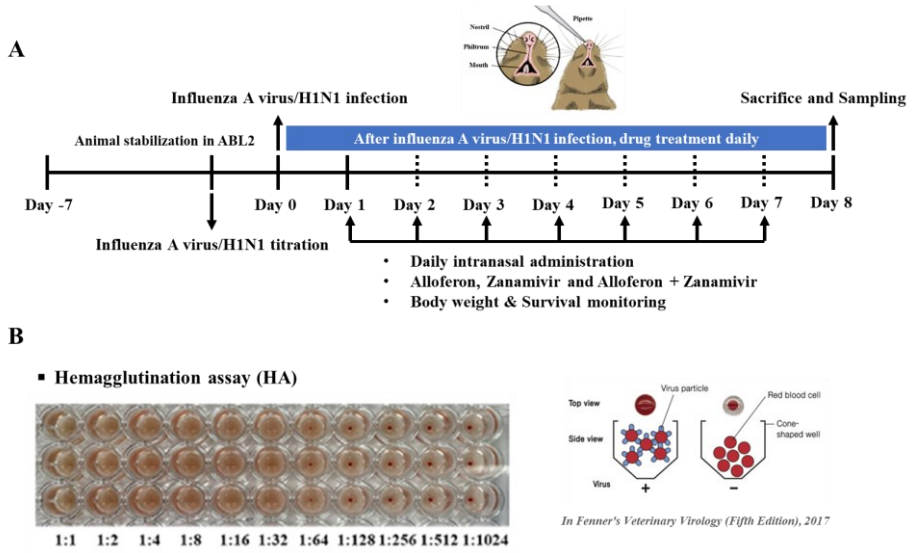

Figure 14. Determination of viral titer by hemagglutination (HA) assay for intranasal administration. Mice were intranasally inoculated with influenza A/Puerto Rico/8/1934 virus. (A) At 24 h after influenza A virus/H1N1 infection, mice (n=30/group) received a daily dose of alloferon (0.5 μg) and/or zanamivir (35 μg) intranasally administrated per daily for 7 days. (B) Optimal titer of influenza A virus/H1N1 (32 HAU/50 μl) for infection was determined by HA assay.

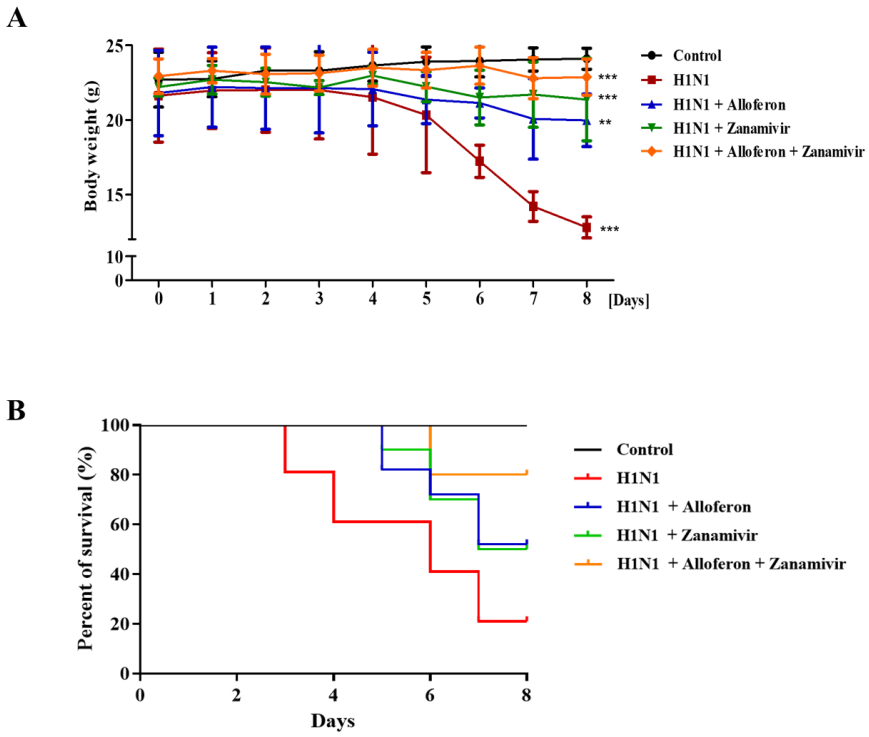

Figure 15. Combined treatment of alloferon and zanamivir increases the survival of mice infected with influenza A virus/H1N1. (A) The body weight of mice (n=30/group) infected with H1N1 was monitored while administering alloferon (0.5 μg) and/or zanamivir (35 μg) for 8 days from 24 h after virus infection (32 HAU/50 μl). (B) After virus infection, changes in mouse survival rate were monitored for 8 days along with changes in weight accroding to the administration of alloferon (0.5 μg) and/or zanamivir (35 μg). The experimental group is 1) uninfected control, 2) virus only, 3) virus + alloferon (0.5 μg), 4) virus + zanamivir (35 μg), 5) virus + alloferon (0.5 μg) + zanamivir (35 μg).

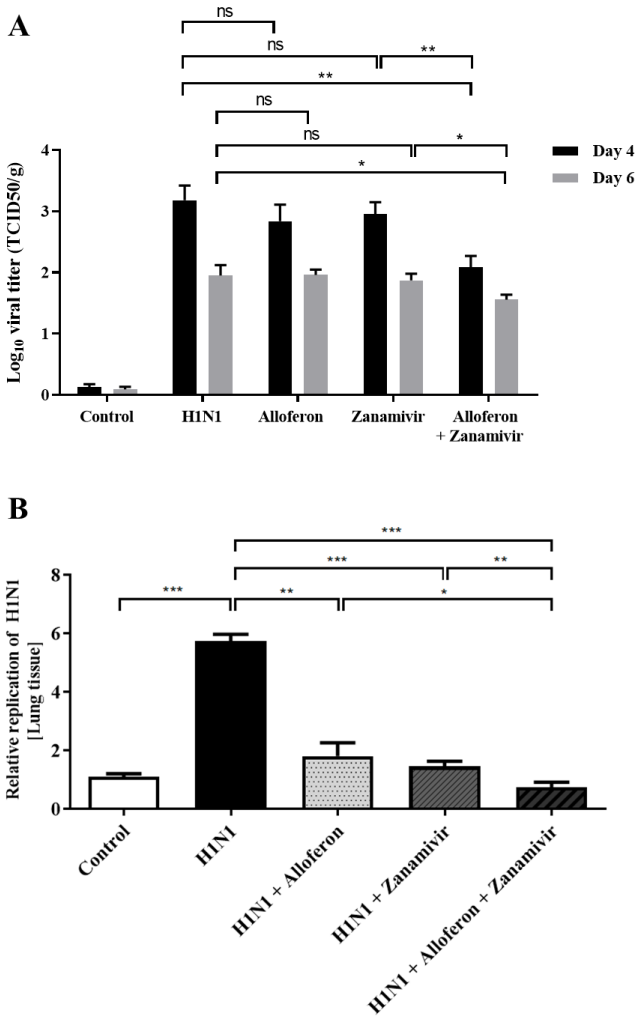

Figure 16. Combined treatment of alloferon and zanamivir suppresses viral replication *in vivo*. At 24 h after virus infection, mice (n=30/group) were intranasally received an alloferon (0.5 μg) and/or zanamivir (35 μg) every day for 8 days. (A) At day 4 and 6, the virus lung load in the presence or absence of alloferon and/or zanamivir was determined with the tissue culture infectious dose 50 ($TCID_{50}$) by titrating lung tissue homogenate in cell culture. (B) At day 8, total RNA was extracted from lung tissues and viral replication was analyzed

by real-time RT-PCR. A quantitative real-time PCR analysis for influenza viral replication is normalized to GAPDH. A group that is not infected with the virus is referred to as Control. Results are representative of seven independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$

Combined treatment of alloferon and zanamivir suppresses the production of IL-6 and MIP-1α induced by influenza A virus/H1N1 infection.

It is already shown that production of IL-6 and MIP-1α in MDCK and A549 is suppressed by combined treatmnet of alloferon and/or zanamivir (Figure 4,5,7 and 8). In vivo production of IL-6 and MIP-1α upon combined treatment of alloferon and/or zanamivir was examined by using of serum and BALF from H1N1 infected mice. As shown in Figure 17A and 18A, the concentration of IL-6 and MIP-1α in BALF were decreased by combined treatment, but there was no remarkable change in seurm (Figure 17B and 18B). In addition, the production of TNF-α, IL-1β, IFN-λ and IL-10, which is generally changed by virus infection was also examined in serum and BALF. Even though they were decreased by the treatment of alloferon and zanamivir respectively, but there were no remarkable changes by combined treatment of alloferon or zanamivir (Figure 19 and 20).

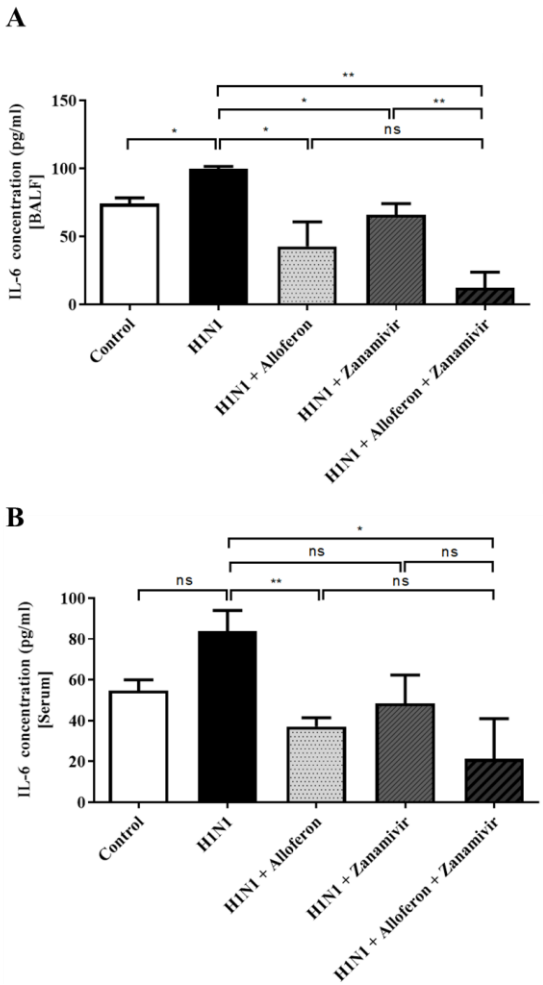

Figure 17. Combined treatment of alloferon and zanamivir suppresses production of IL-6 by influenza A virus/H1N1 infection in mice. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, BALF (A) and serum (B) were collected. The concentration of IL-6 were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, NS; no significant.

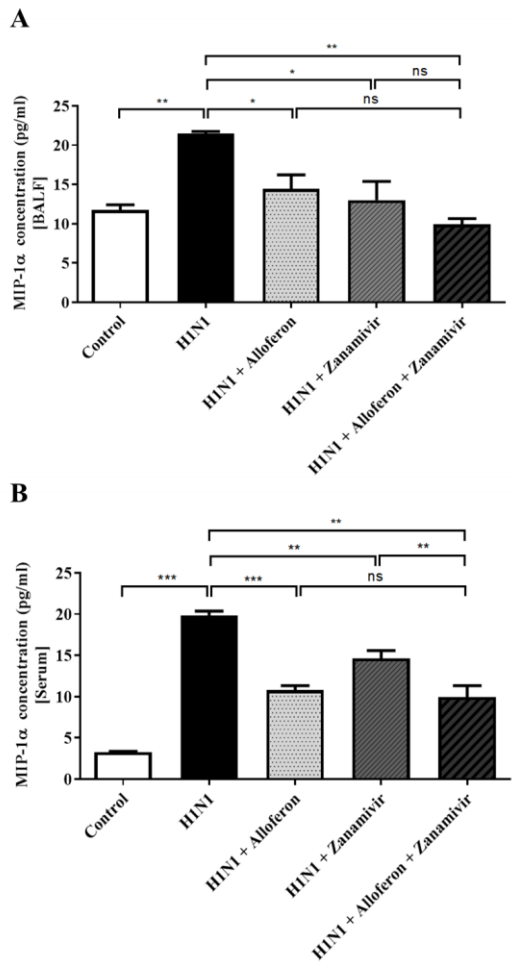

Figure 18. Combined treatment of alloferon and zanamivir suppresses production of MIP-1α by influenza A virus/H1N1 infection in mice. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, BALF (A) and serum (B) were collected. The concentration of MIP-1α were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

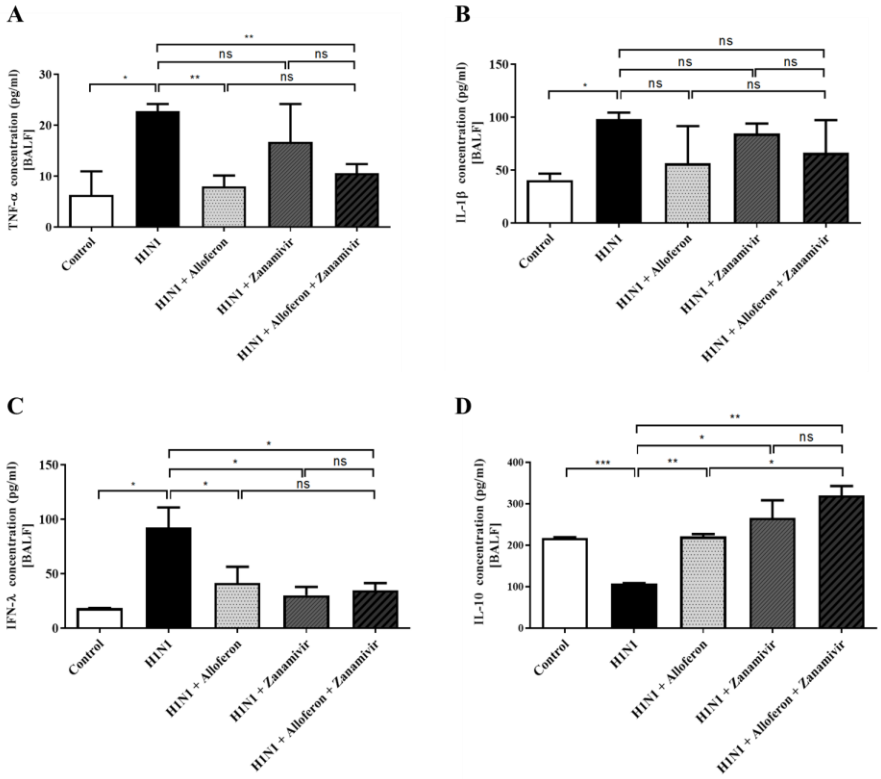

Figure 19. Combined treatment of alloferon and zanamivir does not remarkably suppress the production of TNF-α, IL-1β, IFN-λ and IL-10 by influenza A virus/H1N1 infection in mice; BALF. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, BALF were collected. The concentration of TNF-α (A) IL-1β (B) IFN-λ (C) and IL-10 (D) were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

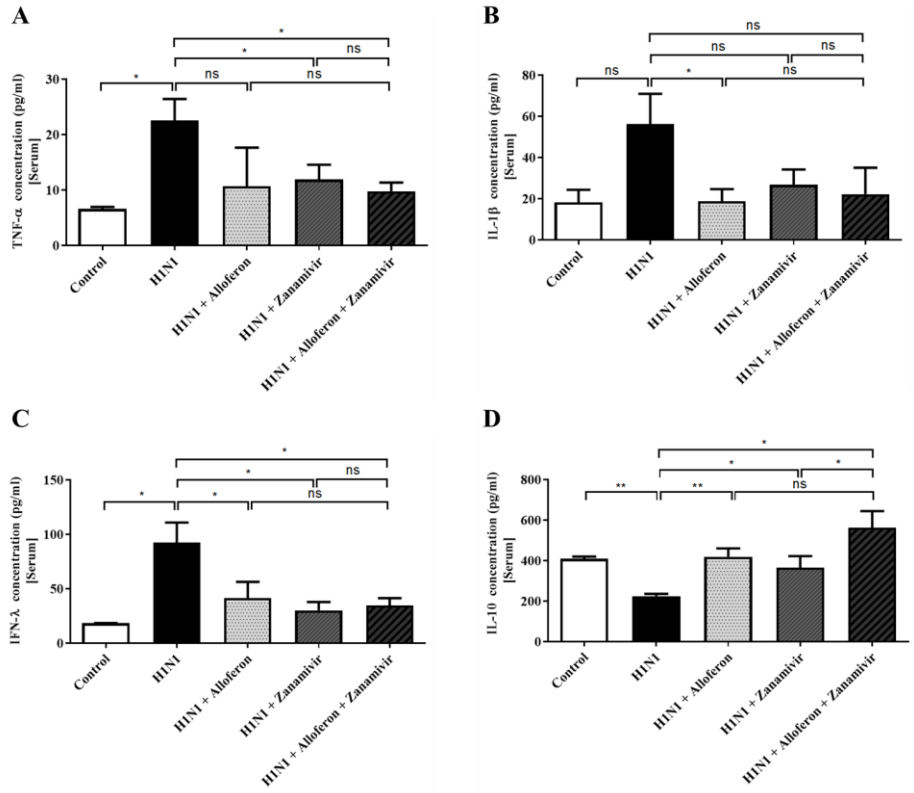

Figure 20. Combined treatment of alloferon and zanamivir does not remarkably suppress the production of TNF-α, IL-1β, IFN-λ and IL-10 by influenza A virus/H1N1 infection in mice; Serum. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, serum were collected. The concentration of TNF-α (A) IL-1β (B) IFN-λ (C) and IL-10 (D) were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

Combined treatment of alloferon and zanamivir suppresses the infiltration of inflammatory cells to the lung in mice by influenza A virus/H1N1 infection.

Since combined treatment effectively suppressed H1N1 induced production of IL-6 and MIP-1α in BALF (Figure 17 and 18), the number of inflammatory cells in the BALF was examined. The results were as expected, the number of inflammatory cells was definitely decrease by combined treatment (Figure 21). Among the various types of inflammatory cells, it was observed that the number of neutrophils and macrophages decrease, especially the number of macrophages were decreased significantly.

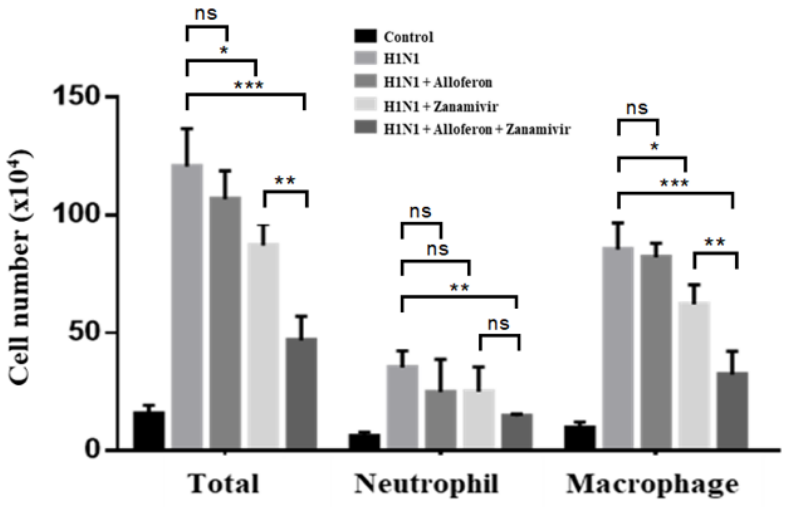

Figure 21. Combined treatment of alloferon and zanamivir decreases the number of inflammatory cells in BALF from influenza A virus/H1N1 infected mice. Cells in BALF were collected by flushing the lungs with ice-cold PBS containing EDTA (0.03%). And then, centrifugation and resuspended in 300 μl of PBS for cytospin preparation. The number of infiltrating inflammatory cells in the BALF were counted after Wright's Giemsa staining. Results are representative of seven independent experiments. *$p < 0.1$. **$p < 0.01$, ***$p < 0.001$, NS; no significant.

Combined treatment of alloferon and zanamivir effectively prevents severe lung inflammation in influenza A virus/H1N1 infected mice.

Lung tissues from mice were harvested and subjected to histological examination to determine pathological change caused by H1N1 infection and compared with the changes upon combined treatment of alloferon and zanamivir. As shown in Figure 22A, extensive inflammatory lesions were found in the lungs of mice infected with H1N1. However, these pathological changes were effectively prevented by treatment of alloferon and/or zanamivir, especially by the combination of alloferon and zanamivir. Even though inflammation was prevented by alloferon and zanamivir respectively, the number of damaged alveoli was lower by combined treatment than the treatment of alloferon or zanamivir, respectively (Figure 22B). Further analysis by Masson's Trichrome staining was performed to examine fibrotic changes in lung tissue caused by H1N1 infection and compare the changes in the lung from the mice treated with alloferon and zanamivir. There was severe fibrosis in the lungs caused by H1N1, and it was effectively prevented by combined treatment of alloferon and zanamivir (Figure 23).

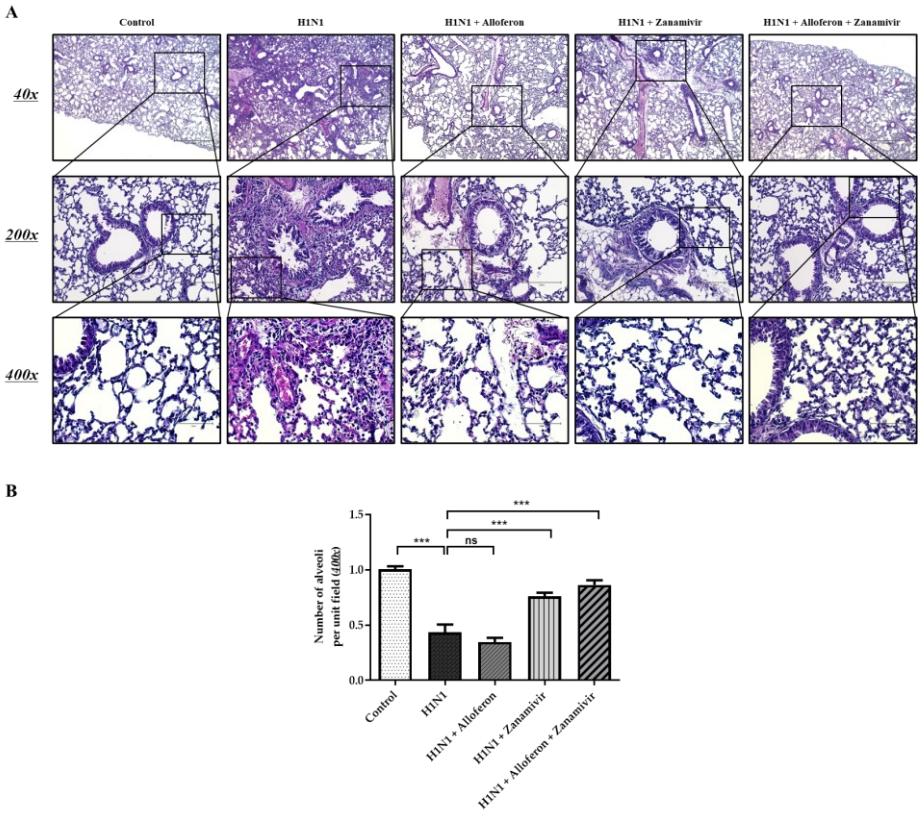

Figure 22. Combined treatment of alloferon and zanamivir effectively prevents damage of alveoli in mice induced by influenza A virus/H1N1 infection. (A) Lung tissues were collected from mice and embedded in paraffin. Tissues were sectioned (4 μm thick) and stained with hematoxylin and eosin. (B) The numbers of normal and damaged alveoli were counted in unit field and compared. Result is presented as mean ±SD after three independent counting. *p < 0.1. **p < 0.01, ***p < 0.001, NS; no significant.

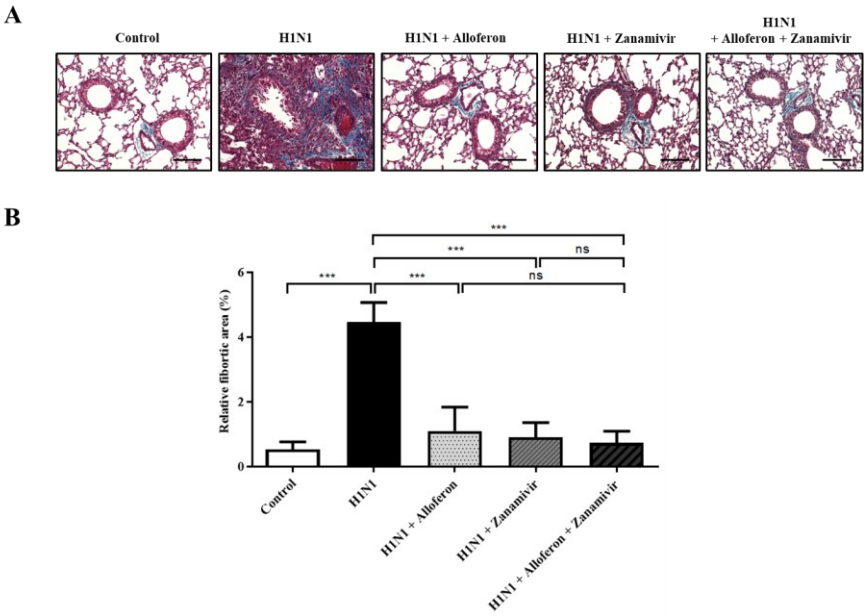

Figure 23. Combined treatment of alloferon and zanamivir effectively prevents lung fibrosis induced by influenza A virus/H1N1 infection. Lung tissues were collected from mice and embedded in paraffin. Tissues were sectioned (4 μm thick) and (A) stained by Masson's Trichrome method to examine fibrotic changes. (B) Analysis of the stained fibrotic area (blue) using celleste. Results are representative of seven independent experiments. (Scale bar, 100 μm) *p < 0.1. **p < 0.01, ***p < 0.001, NS; no significant.

p38MAPK, JNK and p42/44MAPK play an important role in the antiviral effects of combined treatment of alloferon and zanamivir *in vivo*.

As it is previously shown in Figure 10,11,12 and 13, combined treatment of alloferon and zanamivir suppresses H1N1-induced activation of the p38MAPK and JNK signaling pathways in MDCK and A549. Therefore, it was also examined whether combined treatment of alloferon and zanamivir also suppresses the activation of p38MAPK, JNK and p42/44MAPK *in vivo* (Figure 24). The same as in the result in MDCK and A549, p38MAPK and JNK were activated by H1N1 infection, but it was effectively suppressed by combined treatment of alloferon and zanamivir. Unlike the result of the *in vitro* study, the activation of p42/44MAPK was also suppressed by combined treatment *in vivo*.

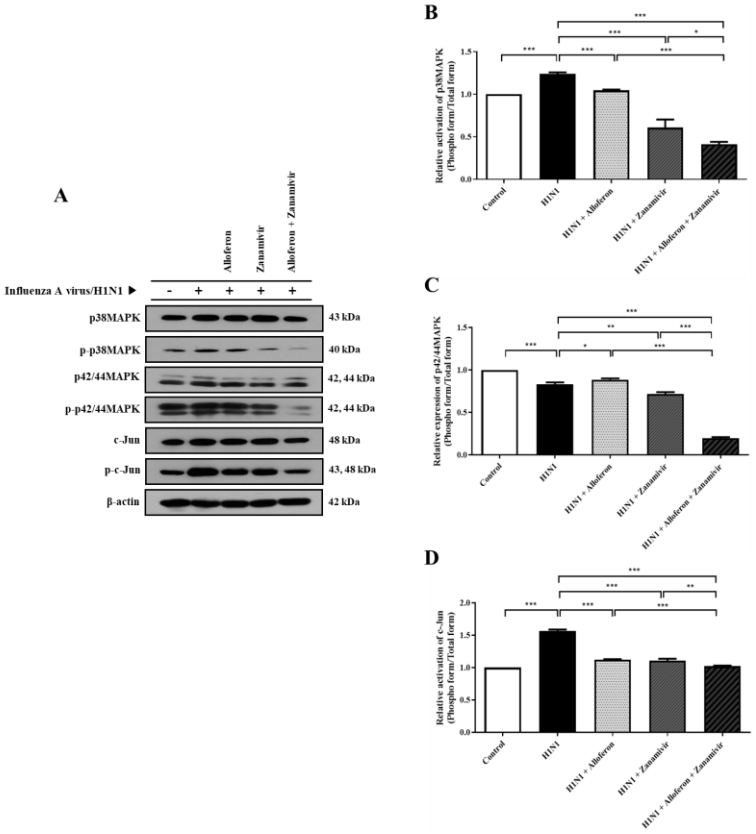

Figure 24. Combined treatment of alloferon and zanamivir inhibits the activation of p38MAPK, JNK and p42/44MAPK by influenza A virus/H1N1 infection. (A) Lung tissues were collected and lysates were prepared in RIPA buffer. And then western blot analysis was performed to examine the change on the activation of p38MAPK, JNK and p42/44MAPK. Results are representative of seven independent experiments. (B, C and D) After measuring the values of phospho form and total form by using of Image J software, the relative activation is calculated by normalization the phospho form to total form. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

Combined treatment of alloferon and zanamivir increases survival of *gulo* (-/-) mice infected with influenza A virus/H1N1.

It is widely known that vitamin C has antiviral effect, especially an influenza virus infection. It is generally known that mice produces more than 6 grams of vitamin C per day without its supplementation through diet. So, antiviral effects of combined treatment of alloferon and zanamivir was examined in *gulo* (-/-) mice after ceasing vitamin C supplementation for two weeks before virus infection to exclude the role of vitamin C on antiviral effect by combined treatment. At 5 days after virus infection, the wight loss in *gulo* (-/-) mice was observed, but it was effectively prevented by combined treatment of alloferon and zanamivir (Figure 25A). In the case of survival rate, as in weight change, it was remarkably decreased at 5 days after exposure to virus, but it was maintained with combined treatment (Figure 25B). To clarify the correlation between survival rate and virus replication, the replication of H1N1 in the lungs of *gulo* (-/-) mice with or without combined treatment was examined by using real-time RT-PCR. As in the result using wild type, combined treatment of alloferon and zanamivir effectively, suppressed viral replication in *gulo* (-/-) mice as well (Figure 26).

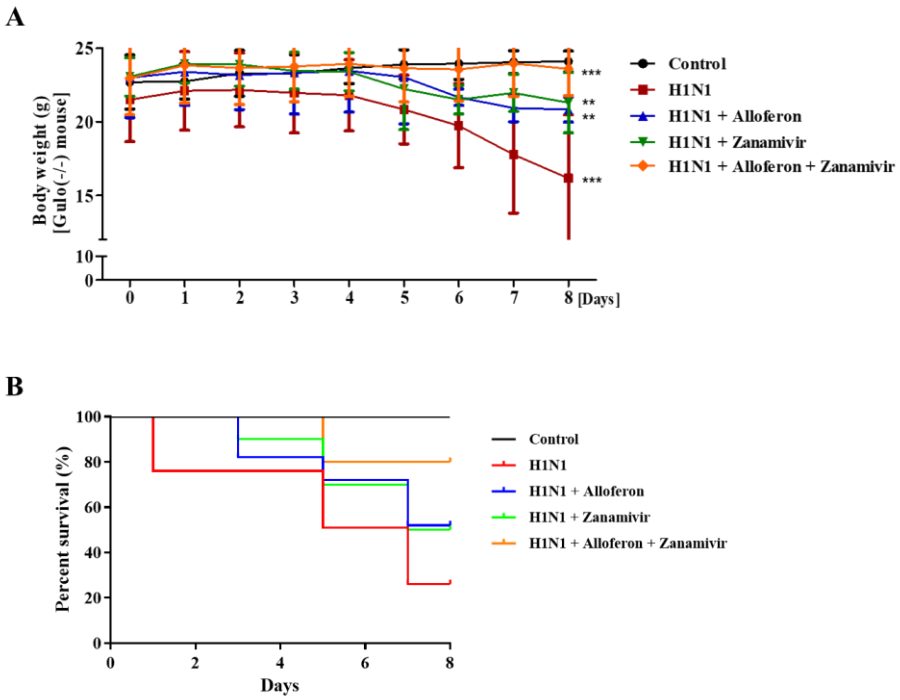

Figure 25. Combined treatment of alloferon and zanamivir increases the survival of *gulo* (-/-) mice infected with influenza A virus/H1N1. After two weeks vitamin C depleted, (A) the body weight of mice (n=30/group) infected with H1N1 was monitored while administering alloferon (0.5 μg) and/or zanamivir (35 μg) for 8 days from 24 h after virus infection (32 HAU/50 μl). (B) After virus infection, changes in mouse survival rate were monitored for 8 days along with changes in weight accroding to the administration of alloferon (0.5 μg) and/or zanamivir (35 μg). The experimental group is 1) uninfected control, 2) virus only, 3) virus + alloferon (0.5 μg), 4) virus + zanamivir (35 μg), 5) virus + alloferon (0.5 μg) + zanamivir (35 μg).

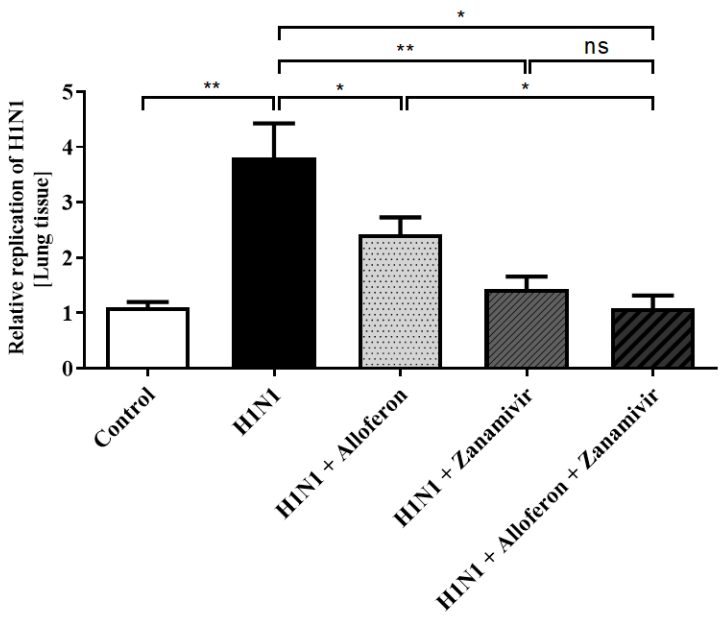

Figure 26. Combined treatment of alloferon and zanamivir suppresses viral replication in the lung of *gulo* (-/-) mice. At day 8, total RNA was extracted from lung tissues and viral replication was analyzed by real-time RT-PCR. A quantitative real-time PCR analysis for influenza viral replication is normalized to GAPDH. A group that is not infected with the virus is referred to as Control. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant

Combined treatment of alloferon and zanamivir suppresses the production of IL-6 and MIP-1α induced by influenza A virus/H1N1 infection in *gulo* (-/-) mice.

As shown in Figure 17 and 18, the production of IL-6 and MIP-1α in BALF from wild type mice was increased by H1N1 infection and suppressed by combined treatment. Next, the concentration of IL-6 and MIP-1α in BALF and serum from *gulo* (-/-) mice infected with influenza A virus/H1N1 upon combined treatment was examined. The same as result in wild type mice, the production of IL-6 and MIP-1α were suppressed in BALF, but there was no remarkable change in seurm (Figure 27 and 28). In addition, the production of TNF-α, IL-1β, IFN-λ and IL-10, which is generally changed by virus infection was also examined in serum and BALF. Even though they were decreased by the treatment of alloferon and zanamivir respectively, but there were no remarkable changes by combined treatment of alloferon or zanamivir (Figure 29 and 30).

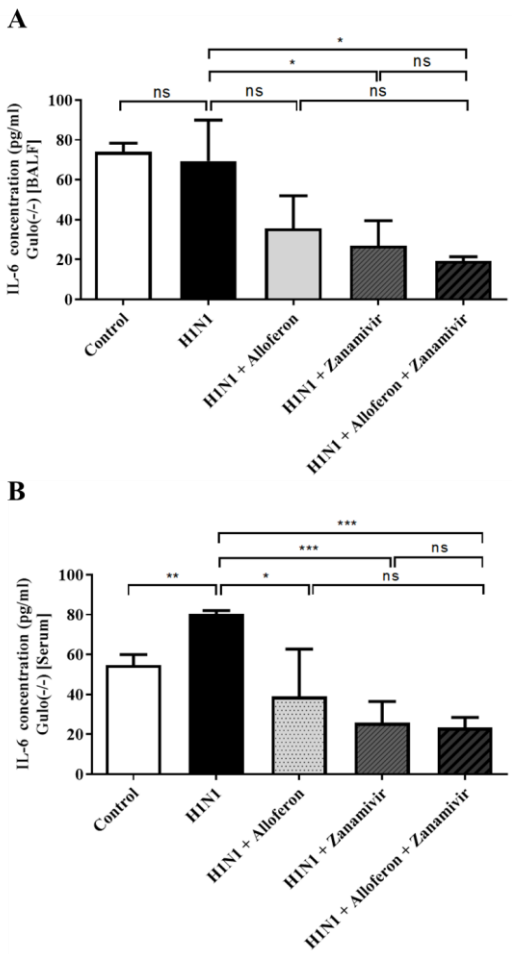

Figure 27. Combined treatment of alloferon and zanamivir suppresses production of IL-6 by influenza A virus/H1N1 infection in *gulo* (-/-) mice. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, BALF (A) and serum (B) were collected. The concentration of IL-6 were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

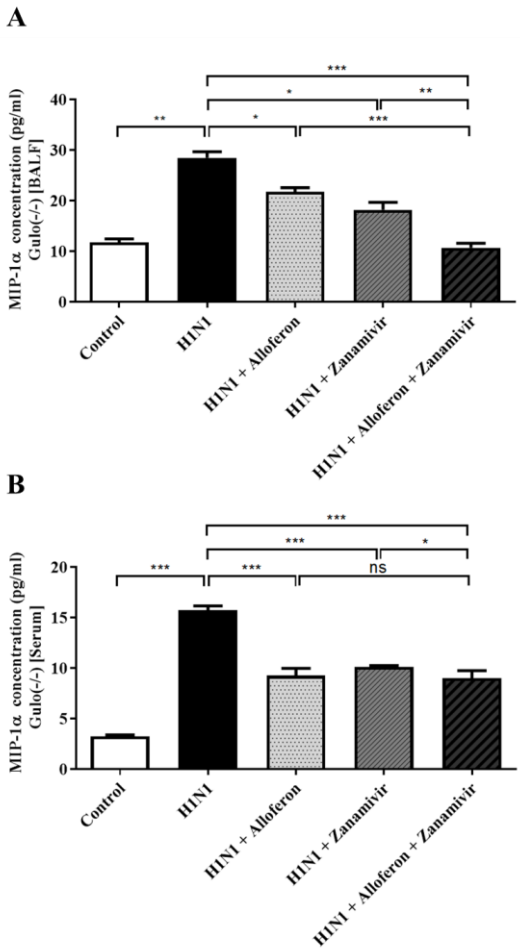

Figure 28. Combined treatment of alloferon and zanamivir suppresses production of MIP-1α by influenza A virus/H1N1 infection in *gulo* (-/-) mice. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, BALF (A) and serum (B) were collected. The concentration of MIP-1α were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

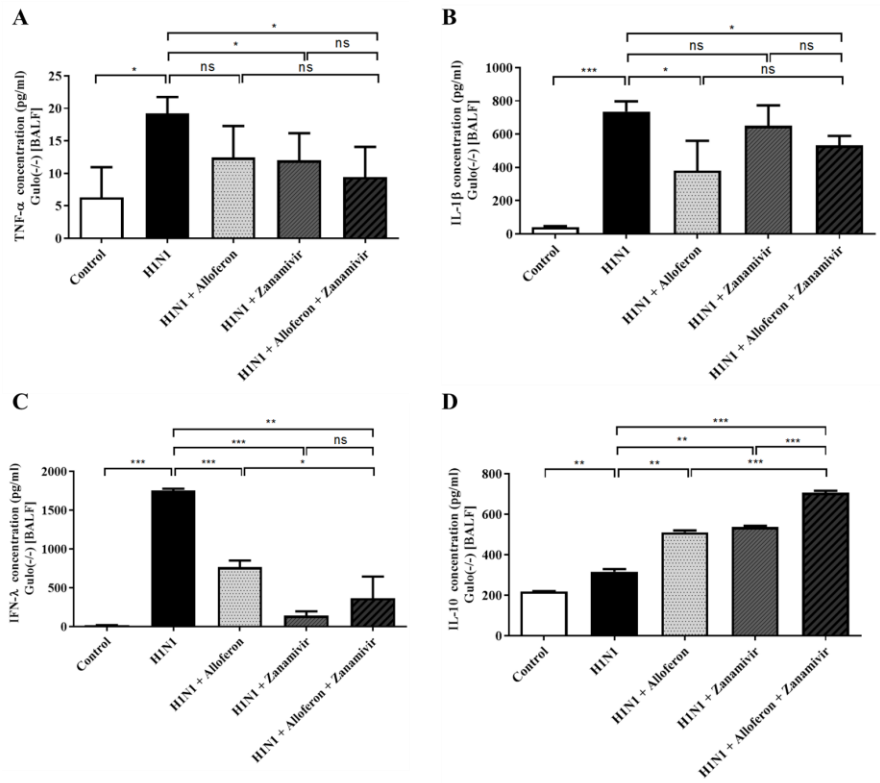

Figure 29. Combined treatment of alloferon and zanamivir does not remarkably suppress the production of TNF-α, IL-1β, IFN-λ and IL-10 by influenza A virus/H1N1 infection in *gulo* (-/-) mice; BALF. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, BALF were collected. The concentration of TNF-α (A) IL-1β (B) IFN-λ (C) and IL-10 (D) were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

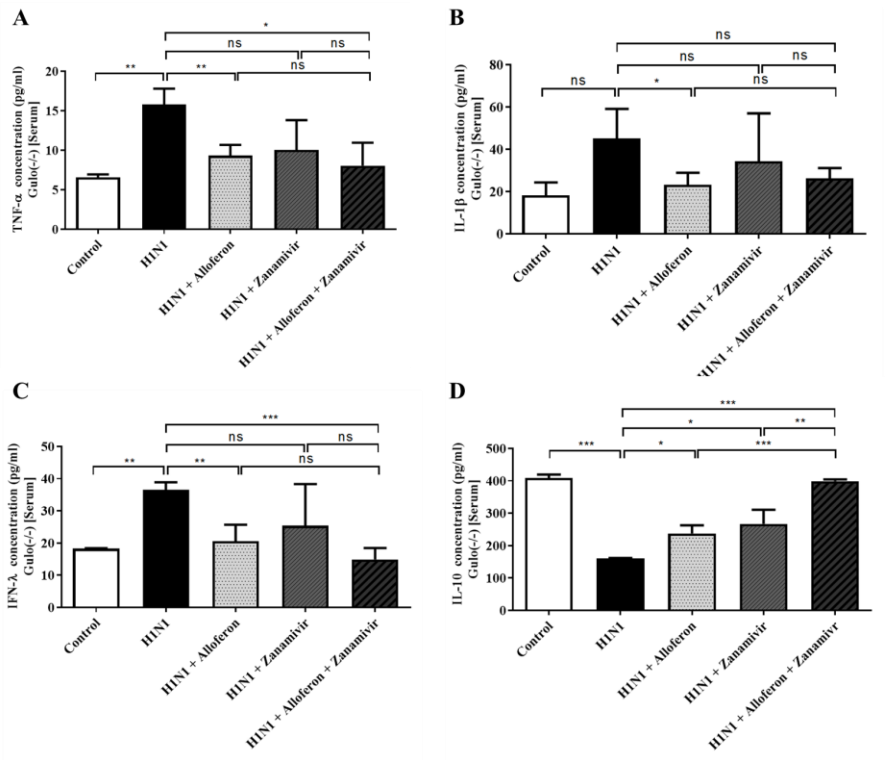

Figure 30. Combined treatment of alloferon and zanamivir does not remarkably suppress the production of TNF-α, IL-1β, IFN-λ and IL-10 by influenza A virus/H1N1 infection in *gulo* (-/-) mice; serum. At day 8 after virus infection in the presence or absence of alloferon and/or zanamivir, serum were collected. The concentration of TNF-α (A) IL-1β (B) IFN-λ (C) and IL-10 (D) were measured by ELISA. Plates were read on a microplate reader at 450 nm. Results are representative of seven independent experiments. *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

Combined treatment of alloferon and zanamivir prevents the severe inflammation in the lungs of influenza A virus/H1N1 infected *gulo* (-/-) mice.

Lung tissues from mice were harvested and subjected to histological examination to determine pathological change caused by H1N1 infection and compared with the changes upon combined treatment of alloferon and zanamivir. As shown in Figure 31A, extensive inflammatory lesions were found in the lungs of mice infected with influenza A virus/H1N1. However, these pathological changes were effectively prevented by treatment of alloferon and/or zanamivir, especially by the combination of alloferon and zanamivir. Even though inflammation was prevented by alloferon and zanamivir respectively, the number of damaged alveoli was lower by combined treatment than the treatment of alloferon or zanamivir, respectively (Figure 31B). Further analysis by Masson's Trichrome staining was performed to examine fibrotic changes in lung tissue caused by H1N1 infection and compare the changes in the lung from the mice treated with alloferon and zanamivir. There was severe fibrosis in the lungs caused by H1N1, and it was effectively prevented by combined treatment of alloferon and zanamivir (Figure 32).

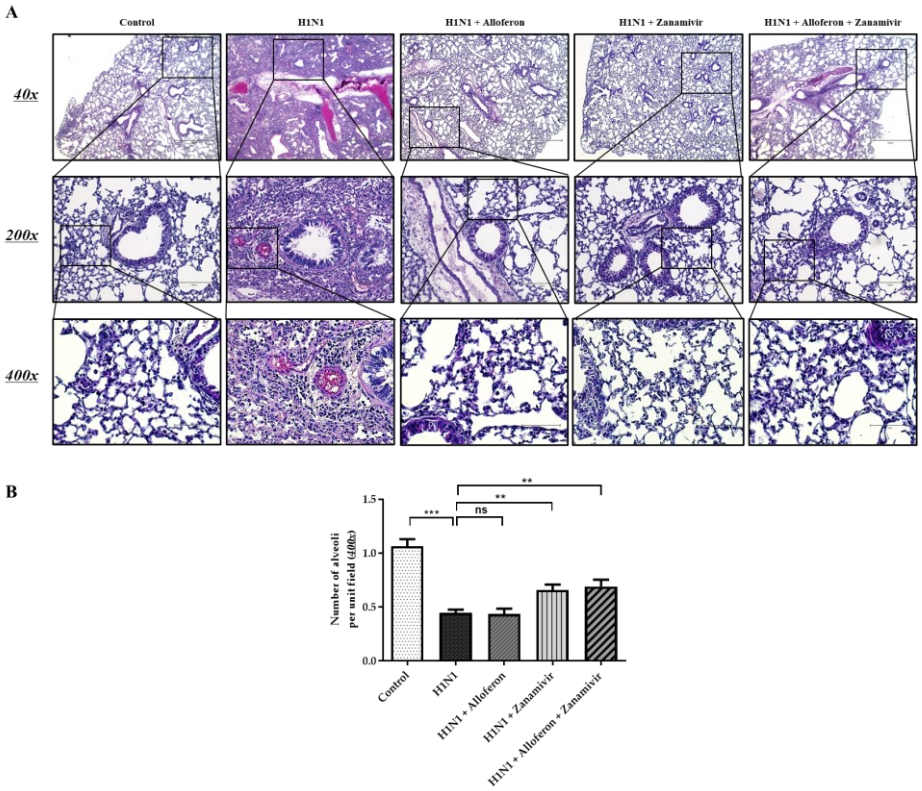

Figure 31. Combined treatment of alloferon and zanamivir effectively prevents damage of alveoli in *gulo* (-/-) mice lung induced by influenza A virus/H1N1 infection. (A) Lung tissues were collected from mice and embedded in paraffin. Tissues were sectioned (4 μm thick) and stained of hematoxylin and eosin. (B) The numbers of normal and damaged alveoli were counted in unit field and compared. Result is presented as mean ±SD after three independent counting. *p < 0.1. **p < 0.01, ***p < 0.001, NS; no significant.

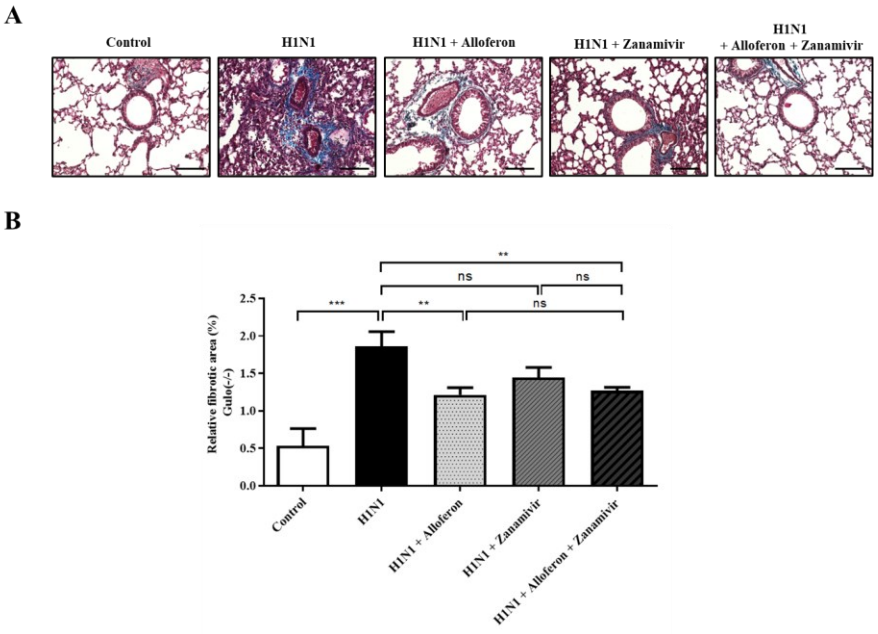

Figure 32. Combined treatment of alloferon and zanamivir effectively prevents lung fibrosis induced by influenza A virus/H1N1 infection in *gulo* (-/-) mice. Lung tissues were collected from mice and embedded in paraffin. Tissues were sectioned (4 μm thick) and (A) stained by Masson's Trichrome method to examine fibrotic changes. (B) Analysis of the stained fibrotic area (blue) using celleste. Results are representative of seven independent experiments. (Scale bar, 100 μm) *p < 0.1. **p < 0.01, ***p < 0.001, NS; no significant.

p38MAPK, JNK and p42/44MAPK play an important role in the antiviral effects of combined treatment of alloferon and zanamivir in the *gulo* (-/-) mice.

Combined treatment of alloferon and zanamivir suppresses H1N1-induced activation of the p38MAPK, JNK and p42/44MAPK signaling pathways in wild type mice (Figure 24). Therfore, it is also examined whether the activation of p38MAPK, JNK and p42/44MAPK by H1N1 infection is suppressed by combined treatment in *gulo* (-/-) mice. The same as in the result in wild type mice, p38MAPK, JNK and p42/44MAPK were activated by H1N1 infection, but it was effectively suppressed by combined treatment of alloferon and zanamivir (Figure 33).

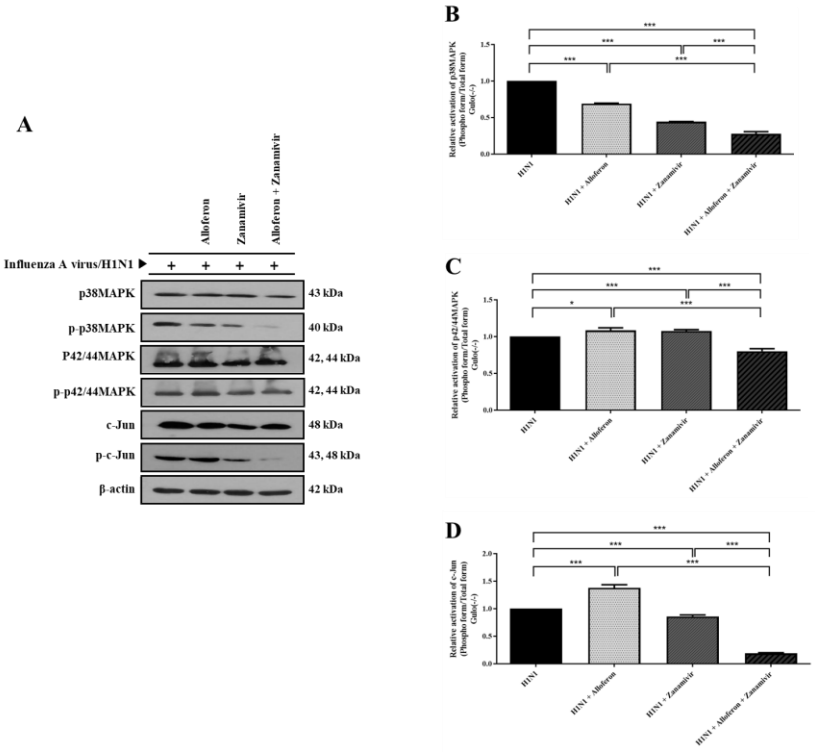

Figure 33. Combined treatment of alloferon and zanamivir inhibits the activation of p38MAPK, JNK and p42/44MAPK in *gulo*(-/-) mice by influenza A virus/H1N1 infection. (A) Lung tissues were collected after sacrifice, lysates were prepared in RIPA buffer, and then western blot analysis was performed to examine the activation of p38MAPK, JNK and p42/44MAPK. Results are representative of seven independent experiments. After measuring the values of phospho form and total form by using of Image J software, the relative activation is calculated by normalization the phospho form to total form (B, C and D). *p < 0.1, **p < 0.01, ***p < 0.001, NS; no significant.

Discussion

Recently, as acute respiratory infections caused by viruses, including COVID-19, increase, the need to develop vaccines for prevention and treatments for treatment is increasing. Flu is a respiratory infectious disease caused by the influenza virus and has long been a representative respiratory infectious disease that threatens human life, but it can only be prevented by vaccination every year, and treatments are also very limited. The most common influenza viruses that cause Flu are H1N1 and H3N2. And there are two types of antiviral drugs currently approved to treat influenza in humans are the adamantanes (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir, zanamivir, and peramivir) (57-59). However, amantadine and rimantadine resistant mutants have been recovered during pre-clinical studies in mice and clinical studies in children and adults (59, 60). Unlike adamantanes, neuraminidase inhibitors have relatively few appearances of drug-resistant viruses, but depending on the drug, each drug has a serious disadvantage. In case of oseltamivir, its common side effect is nausea, vomiting, diarrhea, abdominal pain, and neuropsychiatric distress (61, 62). Compared to oseltamivir administered orally, zanamivir that is used to

treat influenza A virus infection and administered intranasally has fewer side effects and higher safety, but its biggest disadvantage is that it is relatively less effective than other antiviral drugs used in Flu (14, 16, 57, 63-65). It is thought that through studies on the molecule that can increase the antiviral efficacy of zanamivir and whether the antiviral efficacy increases when it is used with zanamivir can provide the basis for the future development of effective flu treatments.

Alloferon, which is to be used as a molecule that can increase the efficacy of zanamivir in this study, is a molecule known to have anti-cancer and anti-inflammatory function as well as immunomodulatory function (21, 23, 26, 47). It is also known to have antiviral effects against the influenza virus (57, 66). In fact, there is a report that alloferon shows its antiviral efficacy by increasing the production of IFN through intranasal administration (66). Therefore, in this study, it examined whether antiviral efficacy of zanamivir against infection of influenza A virus/H1N1 could be increased through intranasal administration of alloferon along with zanamivir.

First, the antiviral effect of combined treatment of alloferon and zanamivir was examined in MDCK, A549, and Calu-3 at the cellular level. As shown in Figure 1 and 2, the combined treatment of alloferon and zanamivir are more effectively suppressed viral replication than either alloferon or zanamivir

alone. According to the reports by Sergey *et al*, alloferon shows its antiviral activity thtough the production of IFN, but they didn't examine about direct inhibition of virus replication (66). However, it seems that alloferon also has direct antiviral effect through the suppression of viral replication, based on the result in the present study (17, 25). For this reason, it is thought that the combined treatment of alloferon and zanamivir showed more effect antiviral activity against influenza A virus/H1N1 infection. Although both of A549 and Calu-3 are known as a lung adenocarcinoma cell lines, but the experiment is performed with more focusing on A549 because it is derived from type II alveolar epithelial cells and Calu-3 are derived from the submucosal layers of the bronchial airways (67-70).

It is generally known that influenza A virus suppressed Th1 immune response, but enhances Th2 immune response (5, 71, 72). Among the Th2 cytokines, IL-6 can trigger a "cytokine storm" (known as cytokine release syndrome), as well as macrophage activation syndrome (73-75). And MIP-1α (also called CCL3), a chemokine, is not only produced mainly by immune cells, but also produced by non-hematopoietic cells such as osteoblasts, especially inflamed epithelial cells upon stimulation with viruses (76, 77). During the inflammatory process, macrophages are recruited to sites of tissue damage, where they begin the process of regeneration and repair (78). It is

reported that MIP-1α is increased upon viral infection, especially by respiratory viruses, including influenza virus (79). In this study, it is shown that the combined treatment of alloferon and zanamivir effectively suppressed the production of IL-6 and MIP-1α by influenza A virus/H1N1 infection. And it was more effectively than the treatment of alloferon or zanamivir, respectively (Figure 4, 5, 7, and 8). It suggests that the combined treatment of alloferon and zanamivir could regulate the excessive inflammatory responses by influenza A virus/H1N1 infection through the suppression of the production of IL-6 and MIP-1α. Changes in the production of IL-6 and MIP-1α were also confirmed in animal experiments. The production of IL-6 and MIP-1α production according to viral infection and their production after treatment of alloferon and zanamivir were examined by using of BALF and serum from mice after infected with influenza A virus/H1N1, it was confirmed that the production of IL-6 and MIP-1α in BALF was more effectively suppressed than in serum (Figure 17, 18, 27, and 28). Given that the replication and proliferation of influenza viruses are mainly occurred in the lungs, changes in BALF rather than serum are considered to have a significance. This is because, as mentioned earlier, IL-6 exacerbates inflammation in the lungs, and MIP-1α increases inflammatory cell infiltration in the lungs, so changes in BALF separated from lung tissue

are more meaningful than changes in serum. In fact, the numbers of neutrophils and macrophages were increased in BALF of mice infected with influenza A virus/H1N1 (Figure 21), and fibrosis in lung tissue progressed, but it was effectively suppressed by combination therapy of alloferon and zanamivir (Figure 22, 23, 31 and 32). Therefore, the increased production of IL-6 and MIP-1α and effective inhibition of viral proliferation in mice infected with influenza A virus/H1N1 are thought to be able to effectively treat viral replication and proliferation as well as pathological changes caused by viral infection in lung tissue by combined treatment of alloferon and zanamivir.

The optimal amount of alloferon and zanamivir prior to perform an experiment to confirm *in vivo* antiviral effect was determined based on our previous report. The optimal amount of alloferon and zanamivir prior to perform an experiment to confirm *in vivo* antiviral effect was determined based on our previous report. In general, 0.5-2 μg/mL of alloferon has been used for examination of the antiviral efficacy or immune enhancement (17, 24). And the amount of zanamivir is 35 μg/mL based on conversion of the amount administered for the treatment of patients with influenza virus infection into a weight of mice (57, 58). Through the study with the combination of 0.5 and 1 μg/mL of alloferon and 35 μg/mL and 70 μg/mL of

zanamivir, the optimal concentration for the combination of alloferon and zanamivir was determined as 0.5 μg alloferon and 35 μg zanamivir. Although it was not performed in Korea but in Russia, there was no acute toxicity in mice (up to 6000 mg/kg), rat (up to 5000 mg/kg) and dog (up to 20 mg/kg) as well as no embryotoxic or teratogenic effect in rat (up to 15 mg/kg) (80). In addition, no special toxicity was observed in phase 1 clinical studies in which 0.1, 1, 5, and 10 mg of alloferon were administered subcutaneous in 40 healthy people. In a phase 2 clinical study of patients with recurrent genital herpes and acute hepatitis B virus infection, it was observed that it was effective in controlling diseases caused by viruses (39, 66). And since zanamivir is a molecule that has already been secured the safety in humans, it is predicted that the amount of alloferon and zanamivir for combined treatment used in this study will be safe when it is actually applied to the human body, but further investigation on the safety of the two molecules is absolutely needed.

It is reported that p38MAPK activation is closely related to the replication of influenza viruses (81, 82). Indeed, replication of influenza virus is known to be effectively inhibited via suppression of p38MAPK activation (83, 84). In addition, there are several reports that JNK and p42/44MAPK are also involved in the promotion of influenza virus replication (85-89). Therefore,

a study on whether the combined treatment of alloferon and zanamivir could regulate the replication of inflenza A virus/H1N1 and the inhibition of inflammatory response by viral infection thourgh the inhibition of P38MAPK, JNK, and P42/44MAPK activation was performed. As a result of *in vitro* study using cell lines, the combination of zanamivir and alloferon inhibits the activation of p38MAPK and JNK by virus infection (Figure 10,11,12 and 13). And there was no suppressive effect on the activation of p42/44MAPK. In contrast to *in vitro* experiments using cell lines, it was confirmed that P38MAPK, JNK, and P42/44MAPK activated by viral infection were inhibited by the combination treatment of alloferon and zanamivir *in vivo* (Figure 24 and 33). In general, P38MAPK, JNK, and P42/44MAPK are all known to be activated by influenza A virus/H1N1 infection and are involved in the pathogenesis of viral infection. So, results from *in vivo* study using animals are considered more reliable than results *in vitro* study using cell lines. It seems that the reason why *in vitro* study using cell lines differ from *in vivo* study using animals may be that the cell lines used *in vitro* study used lung cancer cell lines and canine kidney cell line, not human primary lung epithelial cell lines. It is, therefore, thought that further investigation using human primary lung epithelial cell line, such as BEAS-2B, also be needed.

In vivo studies on animals were performed using C57BL/6 mice and L-

gulono-γ-lactone oxidase (*gulo*) knockout mice. *Gulo* (-/-) mice is C57BL/6 background and has been produced by deleting the gulono-γ-lactone oxidase gene (47, 50, 53, 90). So, vitamin C should be provided to them in their diets, like human (91). And according to a previous study, the plasma concentration of *gulo* (-/-) mice was decreased by 10-15% after two weeks of vitamin C supplementation and also observed that *gulo* (-/-) mice without vitamin C supplementation for two weeks has been reported a significant decrease in vitamin C levels in most organs (52, 75, 92). Since it is generally known that vitamin C has an antiviral effect on infection caused by influenza virus infection (50). Therefore, in order to exclude the possibility of antiviral efficacy by biosynthetic vitamin C in mice in combination with alloferon and zanamivir, the antiviral effect of *gulo* (-/-) without supplementation of vitamin C for two weeks before viral infection was investigated. As a result, the antiviral effect of the combined treatment of alloferon and zanamivir in *gulo* (-/-) without supplementation of vitamin C was also observed without significant difference from the results in wild type C57BL/6 mice which can biosynthesize vitamin C (Figure 25-33). Therefore, it is thought that vitamin C has no antiviral effect on influenza A virus/H1N1, but further investigation is also needed through adjustment of vitamin C dose and administration period.

Taken together, it has been confirmed that the combination treatment of alloferon and zanamivir against influenza A virus/H1N1 infection has been effective through *in vitro* and *in vivo* experiments. And the results of this study can be summarized as follows: the combined treatment of alloferon and zanamivir are 1) effectively suppressed viral replication 2) inhibiting production of IL-6 and MIP-1α 3) inhibiting virus-mediated activation of p38MAPK and JNK *in vitro* 4) prevented weight loss in mice infected with influenza A virus/H1N1 5) increased survival rates in mice infected with influenza A virus/H1N1 6) inhibiting infiltration of inflammatory cells to the lung in mice infected with influenza A virus/H1N1 7) inhibiting virus-mediated activation of p38MAPK, JNK and p42/44MAPK *in vivo* 8) inhibited inflammatory responses and pulmonary fibrosis as a result of the influenza A virus/H1N1 infection. Taken together, the antiviral effect can be improved by using alloferon as an adjuvant for zanamivir.

Since, drugs administered to the nasal cavity are delivered in the form of nasal spray, it may have fewer side effects, but it has the characteristic that the drug is less effective. In order to overcome these limitations, a formulation study is needed and in progress, which combines alloferon and zanamivir with drug carriers to provide more effective drug delivery to target tissues. Therefore, this study provides a rationale for the development of

therapeutic agents based on the combination of alloferon and zanamivir, which regulate the progression of chronic inflammatory lung diseases, as well as severe acute respiratory syndrome by influenza A virus/H1N1.

REFERENCES

1. Schnitzler SU, Schnitzler P. An update on swine-origin influenza virus A/H1N1: a review. Virus Genes. 2009;39(3):279-92.

2. Hu J, Hu Z, Wei Y, Zhang M, Wang S, Tong Q, et al. Mutations in PB2 and HA are crucial for the increased virulence and transmissibility of H1N1 swine influenza virus in mammalian models. Vet Microbiol. 2022;265:109314.

3. Watanabe T, Kawakami E, Shoemaker JE, Lopes TJ, Matsuoka Y, Tomita Y, et al. Influenza virus-host interactome screen as a platform for antiviral drug development. Cell Host Microbe. 2014;16(6):795-805.

4. Park BJ, Park MS, Lee JM, Song YJ. Specific Detection of Influenza A and B Viruses by CRISPR-Cas12a-Based Assay. Biosensors (Basel). 2021;11(3).

5. Aleebrahim-Dehkordi E, Molavi B, Mokhtari M, Deravi N, Fathi M, Fazel T, et al. T helper type (Th1/Th2) responses to SARS-CoV-2 and influenza A (H1N1) virus: From cytokines produced to immune responses. Transpl Immunol. 2022;70:101495.

6. Shen J, Ma J, Wang Q. Evolutionary trends of A(H1N1) influenza

virus hemagglutinin since 1918. PLoS One. 2009;4(11):e7789.

7. Bai Y, Jones JC, Wong SS, Zanin M. Antivirals Targeting the Surface Glycoproteins of Influenza Virus: Mechanisms of Action and Resistance. Viruses. 2021;13(4).

8. Sempere Borau M, Stertz S. Entry of influenza A virus into host cells - recent progress and remaining challenges. Curr Opin Virol. 2021;48:23-9.

9. Chivukula S, Plitnik T, Tibbitts T, Karve S, Dias A, Zhang D, et al. Development of multivalent mRNA vaccine candidates for seasonal or pandemic influenza. NPJ Vaccines. 2021;6(1):153.

10. Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. J Virol. 2009;83(20):10366-73.

11. Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. Vaccine. 2005;23(50):5751-9.

12. van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol. 2001;39(1):196-200.

13. Youk SS, Leyson CM, Seibert BA, Jadhao S, Perez DR, Suarez DL, et al. Mutations in PB1, NP, HA, and NA Contribute to Increased Virus Fitness of H5N2 Highly Pathogenic Avian Influenza Virus Clade 2.3.4.4 in Chickens. J Virol. 2020.

14. Lv X, Wang P, Li C, Cheng S, Bi Y, Li X. Zanamivir-Cholesterol Conjugate: A Long-Acting Neuraminidase Inhibitor with Potent Efficacy against Drug-Resistant Influenza Viruses. J Med Chem. 2021;64(23):17403-12.

15. Dohna-Schwake C, Schweiger B, Felderhoff-Muser U, Fiedler M, Kaiser GM, Paul A, et al. Severe H1N1 infection in a pediatric liver transplant recipient treated with intravenous zanamivir: efficiency and complications. Transplantation. 2010;90(2):223-4.

16. Jeyaram RA, Anu Radha C. N1 neuraminidase of H5N1 avian influenza A virus complexed with sialic acid and zanamivir - A study by molecular docking and molecular dynamics simulation. J Biomol Struct Dyn. 2021:1-14.

17. Kuczer M, Dziubasik K, Midak-Siewirska A, Zahorska R, Luczak M, Konopinska D. Studies of insect peptides alloferon, Any-GS and their analogues. Synthesis and antiherpes activity. J Pept Sci. 2010;16(4):186-9.

18. Kuczer M, Czarniewska E, Rosinski G. Novel biological effects of

alloferon and its selected analogues: structure-activity study. Regul Pept. 2013;183:17-22.

19. Kadej A, Kuczer M, Czarniewska E, Urbanski A, Rosinski G, Kowalik-Jankowska T. High stability and biological activity of the copper(II) complexes of alloferon 1 analogues containing tryptophan. J Inorg Biochem. 2016;163:147-61.

20. Kim H, Im JP, Kim JS, Kang JS, Lee WJ. Alloferon Alleviates Dextran Sulfate Sodium-induced Colitis. Immune Netw. 2015;15(3):135-41.

21. Kim Y, Lee SK, Bae S, Kim H, Park Y, Chu NK, et al. The anti-inflammatory effect of alloferon on UVB-induced skin inflammation through the down-regulation of pro-inflammatory cytokines. Immunol Lett. 2013;149(1-2):110-8.

22. N.V. Konovalova NIK, L. N. Velichko, A. Ia. Novik. Changes in clinical condition of the eye and in parameters

of cell and humoral immunity in iridocyclitis patients

under the influence of anti-viral allokin-alpha. Journal of Ophthalmology (Ukraine). 2016;6.

23. Jeon J, Kim Y, Kim H, Kang JS, Lee WJ. Anti-inflammatory Effect of Alloferon on Ovalbumin-induced Asthma. Immune Netw. 2015;15(6):304-12.

24. Lee N, Bae S, Kim H, Kong JM, Kim HR, Cho BJ, et al. Inhibition of lytic reactivation of Kaposi's sarcoma-associated herpesvirus by alloferon. Antivir Ther. 2011;16(1):17-26.

25. Ryu MJ, Anikin V, Hong SH, Jeon H, Yu YG, Yu MH, et al. Activation of NF-kappaB by alloferon through down-regulation of antioxidant proteins and IkappaBalpha. Mol Cell Biochem. 2008;313(1-2):91-102.

26. Chernysh S, Kozuharova I. Anti-tumor activity of a peptide combining patterns of insect alloferons and mammalian immunoglobulins in naive and tumor antigen vaccinated mice. Int Immunopharmacol. 2013;17(4):1090-3.

27. Lalani S, Gew LT, Poh CL. Antiviral peptides against Enterovirus A71 causing hand, foot and mouth disease. Peptides. 2021;136:170443.

28. Trebbien R, Pedersen SS, Vorborg K, Franck KT, Fischer TK. Development of oseltamivir and zanamivir resistance in influenza A(H1N1)pdm09 virus, Denmark, 2014. Euro Surveill. 2017;22(3).

29. O'Shea LK, Abdulkhalek S, Allison S, Neufeld RJ, Szewczuk MR. Therapeutic targeting of Neu1 sialidase with oseltamivir phosphate (Tamiflu(R)) disables cancer cell survival in human pancreatic cancer with acquired chemoresistance. Onco Targets Ther. 2014;7:117-34.

30. Zhang W, Xu H, Guan S, Wang C, Dong G. Frequency and distribution of H1N1 influenza A viruses with oseltamivir-resistant mutations worldwide before and after the 2009 pandemic. J Med Virol. 2022;94(9):4406-16.

31. Medeiros JJS, Costa TM, Carmo MP, Nascimento DD, Lauro ENC, Oliveira CA, et al. Efficient drug development of oseltamivir capsules based on process control, bioequivalence and PBPK modeling. Drug Development and Industrial Pharmacy. 2022:1-36.

32. Sette DESPH, Costa MJF, Araujo FAC, Alencar EN, Amaral-Machado L. Two phytocompounds from Schinopsis brasiliensis show promising antiviral activity with multiples targets in Influenza A virus. An Acad Bras Cienc. 2021;93(suppl 4):e20210964.

33. Ison MG, Hayden FG, Hay AJ, Gubareva LV, Govorkova EA, Takashita E, et al. Influenza polymerase inhibitor resistance: Assessment of the current state of the art - A report of the isirv Antiviral group. Antiviral Res. 2021;194:105158.

34. Du W, de Vries E, van Kuppeveld FJM, Matrosovich M, de Haan CAM. Second sialic acid-binding site of influenza A virus neuraminidase: binding receptors for efficient release. FEBS J. 2021;288(19):5598-612.

35. Wagner R, Matrosovich M, Klenk HD. Functional balance between

haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol. 2002;12(3):159-66.

36. Leser GP, Lamb RA. Influenza virus assembly and budding in raft-derived microdomains: a quantitative analysis of the surface distribution of HA, NA and M2 proteins. Virology. 2005;342(2):215-27.

37. Qin HJ, Li S, Zhu YB, Bao YB, Tang Q, Liu WB, et al. Oseltamivir modified bovine serum albumin inhibits neuraminidase activity and accumulates virion particles to disturb influenza virus replication. Carbohydr Res. 2022;520:108631.

38. Shim SJ, Chan M, Owens L, Jaffe A, Prentice B, Homaira N. Rate of use and effectiveness of oseltamivir in the treatment of influenza illness in high-risk populations: A systematic review and meta-analysis. Health Sci Rep. 2021;4(1):e241.

39. Holmes EC, Hurt AC, Dobbie Z, Clinch B, Oxford JS, Piedra PA. Understanding the Impact of Resistance to Influenza Antivirals. Clin Microbiol Rev. 2021;34(2).

40. Li Y, Lin Z, Zhao M, Xu T, Wang C, Hua L, et al. Silver Nanoparticle Based Codelivery of Oseltamivir to Inhibit the Activity of the H1N1 Influenza Virus through ROS-Mediated Signaling Pathways. ACS Appl Mater Interfaces. 2016;8(37):24385-93.

41. Mahal A, Duan M, Zinad DS, Mohapatra RK, Obaidullah AJ, Wei X, et al. Recent progress in chemical approaches for the development of novel neuraminidase inhibitors. RSC Adv. 2021;11(3):1804-40.

42. Rather IA, Kamli MR, Sabir JSM, Paray BA. Potential Antiviral Activity of Lactiplantibacillus plantarum KAU007 against Influenza Virus H1N1. Vaccines (Basel). 2022;10(3).

43. Vahid F, Rahmani D. Can an anti-inflammatory diet be effective in preventing or treating viral respiratory diseases? A systematic narrative review. Clin Nutr ESPEN. 2021;43:9-15.

44. Yin H, Jiang N, Shi W, Chi X, Liu S, Chen JL, et al. Development and Effects of Influenza Antiviral Drugs. Molecules. 2021;26(4).

45. Colunga Biancatelli RML, Berrill M, Marik PE. The antiviral properties of vitamin C. Expert Rev Anti Infect Ther. 2020;18(2):99-101.

46. Miranda-Massari JR, Toro AP, Loh D, Rodriguez JR, Borges RM, Marcial-Vega V, et al. The Effects of Vitamin C on the Multiple Pathophysiological Stages of COVID-19. Life (Basel). 2021;11(12).

47. Kim Y, Kim H, Bae S, Choi J, Lim SY, Lee N, et al. Vitamin C Is an Essential Factor on the Anti-viral Immune Responses through the Production of Interferon-alpha/beta at the Initial Stage of Influenza A Virus (H3N2) Infection. Immune Netw. 2013;13(2):70-4.

48. Kim H, Jang M, Kim Y, Choi J, Jeon J, Kim J, et al. Red ginseng and vitamin C increase immune cell activity and decrease lung inflammation induced by influenza A virus/H1N1 infection. J Pharm Pharmacol. 2016;68(3):406-20.

49. Vollbracht C, Kraft K. Feasibility of Vitamin C in the Treatment of Post Viral Fatigue with Focus on Long COVID, Based on a Systematic Review of IV Vitamin C on Fatigue. Nutrients. 2021;13(4).

50. Wei Li NM, Melinda A. Beck. Vitamin C Deficiency Increases the Lung Pathology of Influenza Virus–Infected Gulo−/− Mice. The Journal of Nutrition. 2006;136(10):2611-6.

51. May CN, Bellomo R, Lankadeva YR. Therapeutic potential of megadose vitamin C to reverse organ dysfunction in sepsis and COVID-19. Br J Pharmacol. 2021;178(19):3864-8.

52. Kim H, Bae S, Yu Y, Kim Y, Kim HR, Hwang YI, et al. The analysis of vitamin C concentration in organs of gulo(-/-) mice upon vitamin C withdrawal. Immune Netw. 2012;12(1):18-26.

53. Harrison FE, Meredith ME, Dawes SM, Saskowski JL, May JM. Low ascorbic acid and increased oxidative stress in gulo(-/-) mice during development. Brain Res. 2010;1349:143-52.

54. Kaur N, Singh R, Dar Z, Bijarnia RK, Dhingra N, Kaur T. Genetic

comparison among various coronavirus strains for the identification of potential vaccine targets of SARS-CoV2. Infect Genet Evol. 2021;89:104490.

55. Alluwaimi AM, Alshubaith IH, Al-Ali AM, Abohelaika S. The Coronaviruses of Animals and Birds: Their Zoonosis, Vaccines, and Models for SARS-CoV and SARS-CoV2. Front Vet Sci. 2020;7:582287.

56. Daubeuf F, Frossard N. Performing Bronchoalveolar Lavage in the Mouse. Curr Protoc Mouse Biol. 2012;2(2):167-75.

57. Swierczynska M, Mirowska-Guzel DM, Pindelska E. Antiviral Drugs in Influenza. Int J Environ Res Public Health. 2022;19(5).

58. Ishiguro N, Koseki N, Kaiho M, Ariga T, Kikuta H, Oba K, et al. Clinical effectiveness of four neuraminidase inhibitors (oseltamivir, zanamivir, laninamivir, and peramivir) for children with influenza A and B in the 2014-2015 to 2016-2017 influenza seasons in Japan. J Infect Chemother. 2018;24(6):449-57.

59. Tappenden P, Jackson R, Cooper K, Rees A, Simpson E, Read R, et al. Amantadine, oseltamivir and zanamivir for the prophylaxis of influenza (including a review of existing guidance no. 67): a systematic review and economic evaluation. Health Technol Assess. 2009;13(11):iii, ix-xii, 1-246.

60. Jackson RJ, Cooper KL, Tappenden P, Rees A, Simpson EL, Read RC, et al. Oseltamivir, zanamivir and amantadine in the prevention of

influenza: a systematic review. J Infect. 2011;62(1):14-25.

61. Antipov EA, Pokryshevskaya EB. The effects of adverse drug reactions on patients' satisfaction: Evidence from publicly available data on Tamiflu (oseltamivir). Int J Med Inform. 2019;125:30-6.

62. Butler CC, van der Velden AW, Bongard E, Saville BR, Holmes J, Coenen S, et al. Oseltamivir plus usual care versus usual care for influenza-like illness in primary care: an open-label, pragmatic, randomised controlled trial. Lancet. 2020;395(10217):42-52.

63. Brown SK, Tseng YY, Aziz A, Baz M, Barr IG. Characterization of influenza B viruses with reduced susceptibility to influenza neuraminidase inhibitors. Antiviral Res. 2022;200:105280.

64. Caceres CJ, Seibert B, Cargnin Faccin F, Cardenas-Garcia S, Rajao DS, Perez DR. Influenza antivirals and animal models. FEBS Open Bio. 2022;12(6):1142-65.

65. Liu X, Luo W, Zhang B, Lee YG, Shahriar I, Srinivasarao M, et al. Design of Neuraminidase-Targeted Imaging and Therapeutic Agents for the Diagnosis and Treatment of Influenza Virus Infections. Bioconjug Chem. 2021;32(8):1548-53.

66. Sergey Chernysh SIK, G. Bekker, V. A. Pleskach, N. A. Filatova, V. B. Anikin, V. G. Platonov and Philippe Bulet. Antiviral and antitumor

peptides from insects. PNAS. 2002;99:12628–32.

67. Duan XY, Sun Y, Zhao ZF, Shi YQ, Ma XY, Tao L, et al. Baicalin attenuates LPS-induced alveolar type II epithelial cell A549 injury by attenuation of the FSTL1 signaling pathway via increasing miR-200b-3p expression. Innate Immun. 2021;27(4):294-312.

68. Li YY, Wang XJ, Su YL, Wang Q, Huang SW, Pan ZF, et al. Baicalein ameliorates ulcerative colitis by improving intestinal epithelial barrier via AhR/IL-22 pathway in ILC3s. Acta Pharmacol Sin. 2021.

69. Park BK, Kim D, Park S, Maharjan S, Kim J, Choi JK, et al. Differential Signaling and Virus Production in Calu-3 Cells and Vero Cells upon SARS-CoV-2 Infection. Biomol Ther (Seoul). 2021;29(3):273-81.

70. Pasman T, Baptista D, van Riet S, Truckenmuller RK, Hiemstra PS, Rottier RJ, et al. Development of an In Vitro Airway Epithelial-Endothelial Cell Culture Model on a Flexible Porous Poly(Trimethylene Carbonate) Membrane Based on Calu-3 Airway Epithelial Cells and Lung Microvascular Endothelial Cells. Membranes (Basel). 2021;11(3).

71. Howard FHN, Kwan A, Winder N, Mughal A, Collado-Rojas C, Muthana M. Understanding Immune Responses to Viruses-Do Underlying Th1/Th2 Cell Biases Predict Outcome? Viruses. 2022;14(7).

72. Wohlleben G, Muller J, Tatsch U, Hambrecht C, Herz U, Renz H, et

al. Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. J Immunol. 2003;170(9):4601-11.

73. Dienz O, Rud JG, Eaton SM, Lanthier PA, Burg E, Drew A, et al. Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung. Mucosal Immunol. 2012;5(3):258-66.

74. Lin SW, Shen CF, Liu CC, Cheng CM. A Paper-Based IL-6 Test Strip Coupled With a Spectrum-Based Optical Reader for Differentiating Influenza Severity in Children. Front Bioeng Biotechnol. 2021;9:752681.

75. Morris G, Bortolasci CC, Puri BK, Marx W, O'Neil A, Athan E, et al. The cytokine storms of COVID-19, H1N1 influenza, CRS and MAS compared. Can one sized treatment fit all? Cytokine. 2021;144:155593.

76. Bhavsar I, Miller CS, Al-Sabbagh M. Macrophage inflammatory protein-1 alpha (MIP-1 alpha)/CCL3: as a biomarker. General methods in biomarker research and their applications. 2015:223.

77. Patricia Menten AS, Chris Dillen, Anja Wuyts, Sofie Struyf, Paul Proost,, Alberto Mantovani JMWaJVD. Role of the autocrine chemokines MIP-1α and MIP-1β in the metastatic behavior of murine T cell lymphoma. Journal of Leukocyte Biology. October 2002;Volume 72.

78. Arabpour M, Saghazadeh A, Rezaei N. Anti-inflammatory and M2 macrophage polarization-promoting effect of mesenchymal stem cell-derived exosomes. Int Immunopharmacol. 2021;97:107823.

79. Patricia Menten AW, Jo Van Damme. Macrophage inflammatory protein-1. Cytokine & Growth Factor Reviews. December 2002;Volume 13, Issue 6.

80. Tyno YY, Jarigina EI, Ustinova VA, Vidrashko MT, Morosova GV, Bakaeva EV. Antiviral Activity of Allokin-Alpha against Herpesvirus in Birds. Russian Agricultural Sciences. 2018;44(1):97-100.

81. Yang S, Wang L, Pan X, Liang Y, Zhang Y, Li J, et al. 5-Methoxyflavone-induced AMPKalpha activation inhibits NF-kappaB and P38 MAPK signaling to attenuate influenza A virus-mediated inflammation and lung injury in vitro and in vivo. Cell Mol Biol Lett. 2022;27(1):82.

82. Börgeling Y, Schmolke M, Viemann D, Nordhoff C, Roth J, Ludwig S. Inhibition of p38 mitogen-activated protein kinase impairs influenza virus-induced primary and secondary host gene responses and protects mice from lethal H5N1 infection. Journal of Biological Chemistry. 2014;289(1):13-27.

83. Choi M-S, Heo J, Yi C-M, Ban J, Lee N-J, Lee N-R, et al. A novel p38 mitogen activated protein kinase (MAPK) specific inhibitor suppresses respiratory syncytial virus and influenza A virus replication by inhibiting

virus-induced p38 MAPK activation. Biochemical and biophysical research communications. 2016;477(3):311-6.

84. Dai J-P, Wang Q-W, Su Y, Gu L-M, Deng H-X, Chen X-X, et al. Oxymatrine inhibits influenza A virus replication and inflammation via TLR4, p38 MAPK and NF-κB pathways. International journal of molecular sciences. 2018;19(4):965.

85. Nacken W, Wixler V, Ehrhardt C, Ludwig S. Influenza A virus NS1 protein-induced JNK activation and apoptosis are not functionally linked. Cellular Microbiology. 2017;19(7):e12721.

86. Li J, Xu Y, Lin Z, Guan L, Chen S, Zhou L. Isorhamnetin inhibits amplification of influenza A H1N1 virus inflammation mediated by interferon via the RIG-I/JNK pathway. Ann Transl Med. 2021;9(16):1327.

87. Zhang J, Ruan T, Sheng T, Wang J, Sun J, Wang J, et al. Role of c-Jun terminal kinase (JNK) activation in influenza A virus-induced autophagy and replication. Virology. 2019;526:1-12.

88. Su J, Chen D, Zheng R, Liu X, Zhao M, Zhu B, et al. Duvira Antarctic polysaccharide inhibited H1N1 influenza virus-induced apoptosis through ROS mediated ERK and STAT-3 signaling pathway. Mol Biol Rep. 2022.

89. Stephan Pleschka TW, Christina Ehrhardt, Gerd Hobom, Oliver

Planz, Ulf R. Rapp and Stephan Ludwig. Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. NATURE CELL BIOLOGY. MARCH 2001;3.

90. Kim H, Kim Y, Bae S, Lim SH, Jang M, Choi J, et al. Vitamin C Deficiency Causes Severe Defects in the Development of the Neonatal Cerebellum and in the Motor Behaviors of Gulo(-/-) Mice. Antioxid Redox Signal. 2015;23(16):1270-83.

91. Hwang JH, Piao H, Jang JY, Lee SK, Han D, Lee GM, et al. Suppressive effects of vitamin C-treated induced-regulatory T cells on heart allograft rejection under vitamin C-deficient or -sufficient conditions. PLoS One. 2021;16(2):e0246967.

92. Shi L, Rath M, Niedzwiecki A. Dietary Vitamin C and Age-Induced Lipid and Hormonal Metabolic Changes in a Humanized Mouse Model Not Synthesizing Vitamin C and Producing Lipoprotein(a) [Gulo (-/-); Lp(a)+]. J Nutr Metab. 2021;2021:5591697.

국 문 초 록

인플루엔자 바이러스 (H1N1) 감염에 대한 알로페론과 자나미비르의 효과적인 항바이러스 효능에 관한 연구

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해부 및 세포 생물학 전공 이 다 해

인플루엔자 바이러스는 크고 작은 항원 변이를 일으켜서 기존 항바이러스제에 대한 내성을 가진다. 또한, 기존의 항바이러스제들은 크고 작은 부작용도 가지고 있기 때문에 인플루엔자 바이러스에 의한 감염을 효과적으로 조절하기 위한 치료제의 개발과 치료법의 확립이 필요하다. 본 연구에서는 기존 항바이러스제와 다르게 흡입을 통해 체내에 투여되며 높은 안정성을 보이지만 항바이러스 효능이 상대적으로 낮은 자나미비르와, 면역조절물질로 개발되어 인플루엔자 바이러스 감염에 효과적인 항바이러스 효능이 있는 것으로 알려진 알로페론을 이용하여 두 물질의 병용 투여에 대한 인플루엔자 바이러스 감염 조절 효과와 감염에 따른 폐에서의 염증 반응의 조절 효과에 대하여 연구하였다.

알로페론과 자나미비르의 병용 처리에 의해 인플루엔자 바이러스로 감염된 개 신장세포주와 사람 폐 상피세포주에서

바이러스 증식이 효과적으로 억제됨을 확인하였다. 또한, 두 약물의 병용 처리는 바이러스 감염에 의해 활성화된 p38MAPK와 c-*Jun*의 활성을 억제하고 폐에서의 염증을 유발하는 대표적 사이토카인인 IL-6의 생성과 폐 조직으로의 염증세포 이동을 촉진하는 대식세포 염증성 단백질, MIP-1α의 생성을 억제함을 관찰하였다.

알로페론과 자나미비르의 병용에 따른 항바이러스 효능을 생체 내에서 확인하기 위하여 C57BL/6 생쥐를 이용한 동물실험을 진행한 결과, 바이러스 감염에 의해 감소된 생쥐의 체중과 생존율이 현저하게 개선됨을 확인하였다. 이와 함께, 폐조직에서의 염증 증상 또한 개선되었으며, 이는 세포실험에서의 결과와 동일하게 인터루킨-6와 MIP-1α의 증가 억제를 통하여 매개됨이 확인되었다. 특히 폐포액 내에서의 호중구와 대식세포의 감소는 폐조직에서의 염증 증상 개선과 밀접한 연관이 있을 것으로 생각된다. 폐조직을 이용한 신호전달 기전에 관한 연구에서도 바이러스 감염에 의한 p38MAPK와 c-*Jun*에서의 활성이 두 약물의 병용 처리에 의해 효과적으로 억제됨을 확인하였다. 이와 더불어 p42/44MAPK의 활성 또한 효과적으로 억제됨을 확인하였다. 비타민 C는 항바이러스 효능, 특히 인플루엔자 바이러스 감염에 대한 항바이러스 효능이 있다고 알려져 있으며, 일반 생쥐들의 경우 생체 내에서 높은 수준의 비타민 C 합성능력이 있다고 알려져 있다. 따라서 C57BL/6에 두 약물의 병용 투여하였을 때 확인된 항바이러스 효능에 있어서 비타민 C의 관여 유무를 확인하기 위하여 비타민 C의 생합성에 필수적 효소인 L-gulono-γ-lactone oxidase가 제거된 *Gulo* (-/-) 생쥐를 이용한 연구를 수행하였다. 그 결과 C57BL/6 생쥐에서와 동일한 결과를 얻을 수 있었다.

이상의 결과들을 종합하여 볼 때, 알로페론과 자나미비르의 병용 투여는 인플루엔자 바이러스 감염증 치료에 있어서 자나미비르와 효능을 효과적으로 증대시킬 수 있을 것으로 생각되며, 알로페론은 향후 인플루엔자를 비롯한 다른 호흡기 감염 바이러스와 치료 물질의 효능 증가에 있어서도 효과적인 보조제로 사용될 수 있을 것으로 생각된다.

주 요 어: 알로페론, 자나미비르, 인플루엔자 바이러스, 바이러스 복제, 폐염증

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