Premedication with clarithromycin is effective against secondary bacterial pneumonia during influenza virus infection in a pulmonary emphysema mouse model

Tatsuhiko Harada, Yuji Ishimatsu, Atsuko Hara, Towako Morita, Shota Nakashima, Tomoyuki Kakugawa, Noriho Sakamoto, Kosuke Kosai, Koichi Izumikawa, Katsunori Yanagihara, Hiroshi Mukae, and Shigeru Kohno

Department of infectious diseases, Unit of Molecular Microbiology and Immunology (T.H., K.I.), Department of Cardiopulmonary Rehabilitation Sciences (Y.I.), Department of Respiratory Medicine (N.S., H.M.) and Department of Laboratory Medicine (K.K, K.Y.), Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan (T.H., A.H., T.M., S.N., T. K., N.S., H.M., S.K)

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Running title: Effect of CAM on SBP during influenza in emphysema mice

Address for correspondence: Dr. Yuji Ishimatsu, Department of Cardiopulmonary

Rehabilitation Sciences, Nagasaki University Graduate School of Biomedical Sciences,

1-7-1 Sakamoto, Nagasaki 852-8520, Japan. E-mail: yuji-i@nagasaki-u.ac.jp

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Abbreviations

ATCC: American Type Culture Collection; **BALF:** bronchoalveolar lavage fluid**; CAM:**

clarithromycin; **COPD:** chronic obstructive pulmonary disease; **CFU:** colony forming

unit; **ELISA**: enzyme-linked immunosorbent assay; **Flu:** influenza virus**; HE**:

hematoxylin-eosin; **IFN-α:** interferon-alpha; **IFN-β:** interferon-beta; **IFN-γ:**

interferon-gamma; **IL**: interleukin; **MIC:** minimum inhibitory concentration; **MIP-2**:

macrophage inflammatory protein-2. **PBS**: phosphate-buffered saline; **SBP:** secondary

bacterial pneumonia; **TCID$_{50}$:** 50% tissue culture infective dose.

Abstract

Secondary bacterial pneumonia (SBP) during influenza increases the severity of and

mortality associated with chronic obstructive pulmonary disease (COPD). Macrolide

antibiotics, including clarithromycin (CAM), are potential treatments for a variety of

chronic respiratory diseases owing to their pharmacological activities, in addition to their

antimicrobial action. We examined the efficacy of CAM for the treatment of SBP after

influenza infection in COPD. Specifically, we evaluated the effect of CAM in

elastase-induced emphysema mice that were inoculated with influenza virus (strain

A/PR8/34) and subsequently infected with macrolide-resistant *Streptococcus pneumoniae*.

CAM was administered to the emphysema mice 4 days prior to influenza virus

inoculation. Premedication with CAM improved pathological responses and bacterial

load 2 days after *S. pneumoniae* inoculation. Survival rates were higher in emphysema

mice than control mice. While CAM premedication did not affect viral titers or exert

antibacterial activity against *S. pneumoniae* in the lungs, it enhanced host defense and

reduced inflammation, as evidenced by the significant reductions in total cell and

neutrophil counts and interferon (IFN)-γ levels in bronchoalveolar lavage fluid (BALF)

and lung homogenates. These results suggest that CAM protects against SBP during

influenza in elastase-induced emphysema mice by reducing IFN-γ production, thus

enhancing immunity to SBP, and by decreasing neutrophil infiltration into the lung to

prevent injury. Accordingly, CAM may be an effective strategy to prevent secondary

bacterial pneumonia in COPD patients in areas in which vaccines are inaccessible or

limited.

Introduction

Chronic obstructive pulmonary disease (COPD) is the third-leading cause of death

globally (World Health Organization, 2012) and is of particular concern in middle- and

low-income countries (Mathers and Loncar, 2006). Secondary infections by viral or

bacterial pathogens often trigger acute exacerbations of COPD, resulting in morbidity

and mortality (Bautista et al., 2010). The influenza virus is associated with 5–26% of

cases of COPD exacerbation (Rohde et al., 2003; Kurai et al., 2013; Dai et al., 2015) and

predisposes the host to secondary bacterial pneumonia (SBP). *S. pneumoniae* is

associated with 10–25% of all cases of acute exacerbation of COPD (Sethi et al., 2002;

Sapey and Stockley, 2006). Co-infection with influenza virus and *S. pneumoniae*

contributes substantially to the increased morbidity and mortality associated with

seasonal and pandemic influenza (Murata et al., 2007; Morens et al., 2008; van der Sluijs

et al., 2010; Cilloniz et al., 2012). In fatal cases of influenza virus infection, the time to

death from the onset of illness is significantly shorter in patients with bacterial

co-infections (Brundage and Shanks, 2008).

Owing to the significant increase in morbidity and mortality associated with

influenza and SBP infection in COPD patients, the Global Initiative for Chronic

Obstructive Lung Disease (GOLD) recommends the annual administration of the

influenza vaccine and the pneumococcal polysaccharide vaccine to high-risk COPD

patients (Global Initiative for Chronic Obstructive Lung Disease, 2015). However, these

vaccines are often not readily accessible in low-income countries and in low-resource

settings. Accordingly, alternative preventive and prophylactic measures are necessary.

Long-term treatment with macrolide antibiotics decreases the risk of acute

exacerbation of COPD according to some studies (Albert et al., 2011; Yamaya et al.,

2012; Donath et al., 2013; Spagnolo et al., 2013). A variety of pharmacological activities,

including direct antibacterial, anti-viral (e.g., against influenza virus and rhinovirus),

anti-inflammatory, and immunomodulatory activity, as well as inhibitory effects on

mucus secretion, bacterial virulence, and biofilm formation (Kanoh and Rubin, 2010;

Zarogoulidis et al., 2012; Spagnolo et al., 2013) are thought to contribute to this

protective effect, but the precise mechanisms of action and the effects of long-term

macrolide therapy on influenza virus and *S. pneumoniae* co-infection in COPD patients

have not been clarified.

In the present study, we investigated the effects and mechanism of action of

clarithromycin (CAM) in a mouse model of COPD with secondary infection by influenza

virus and subsequent co-infection by CAM-resistant *S. pneumoniae*.

Materials and Methods

Microorganisms

Influenza virus (strain A/PR8/34: H1N1 type) was purchased from the American Type

Culture Collection (ATCC, Manassas, VA, USA). The virus was cultured in Madin-Darby

canine kidney cells for 3 days. Cells were then centrifuged at $2000 \times g$ for 15 min and the

supernatant was stored at -80°C as a primary virus stock. A mouse-adapted influenza

virus was prepared. Briefly, primary virus stock solutions were inoculated intra-nasally

to anesthetized C57BL/6J mouse. After 3 days, lung homogenates were collected and

centrifuged at $2000 \times g$ for 15 min. The supernatant was termed the first-generation

mouse-adapted influenza virus; a third-generation mouse-adapted influenza virus was

prepared by repeating the above methods and storing the stock solutions at -80°C. Viral

stocks were thawed and diluted with phosphate-buffered saline (PBS) to the desired

concentration prior to use.

The macrolide-susceptible *Streptococcus pneumoniae* strain ATCC49619 and a

clinical isolate of macrolide-resistant *S. pneumoniae* strain NU4471 were used.

ATCC49619 (minimum inhibitory concentration (MIC) of clarithromycin <0.125 µg/mL;

serotype 19F) was purchased from ATCC. NU4471 (MIC of clarithromycin >256 µg/mL;

serotype 19) was obtained from the Nagasaki University School of Medicine (Nagasaki,

Japan). The presence of both *erm* B and *mef* E/A resistance genes in NU4471 was

confirmed by polymerase chain reaction as previously reported (Fukuda et al., 2006).

Bacteria were stored at -80°C until use.

Viral titer and 50% tissue culture infective dose

The virus titer in Madin-Darby canine kidney cells was determined by measuring the

50% tissue culture infective dose ($TCID_{50}$) following the methods described by Reed and

Muench (Reed, 1938). Briefly, when cells reached 80% confluence in a 96-well plate,

they were infected with 50 µL of ten-fold serial virus dilutions (four replicates). Minimal

essential medium supplemented with 0.5% bovine serum albumin and 2.5 µg/mL trypsin

was used for the virus dilutions. After 2 h, the solutions were removed and the cells were

incubated at 37°C. Seventy-two hours later, the cytopathic effect was monitored using an

inverted microscope and $TCID_{50}$ was determined.

Emphysema mouse model with influenza and secondary bacterial pneumonia

The mouse model is described in Figure 1A. All experimental protocols and procedures

were carried out in accordance with the Guide for the Care and Use of Laboratory

Animals as adopted and promulgated by the U.S. National Institutes of Health and

performed in compliance with Nagasaki University guidelines for animal experiments.

Specific-pathogen-free male C57BL/6J mice (8 to 9 weeks old, Charles River

Laboratories, Japan, Inc., Yokohama, Japan) were anesthetized by intraperitoneal

injection of 1.25% (w/v) pentobarbital. Pulmonary emphysema was induced by injecting

3 units of porcine pancreatic elastase (Wako Pure Chemical Industries Ltd., Osaka,

Japan) dissolved in 50 µL of sterile PBS into the trachea (Day -21). Pulmonary

emphysema was verified by microscopic examination 14 days later in a preliminary

evaluation. Twenty-one days after the porcine pancreatic elastase injection (Day 0), mice

were inoculated intra-nasally with 1×10^4 TCID$_{50}$/mouse influenza virus. Four days later

(Day 4), a sub-group of mice were also inoculated with 1×10^6 colony forming units

(CFU) of *S. pneumoniae* NU4471 to induce secondary bacterial pneumonia. Eight to ten

mice were used in each experimental group.

Clarithromycin medication and survival analysis

CAM was obtained from Taisho Toyama Pharmaceutical (Tokyo, Japan). CAM powder

was dissolved in 5% gum arabic solution and diluted to a concentration of 25 mg/mL.

This solution was stored at 4°C for no more than 7 days. CAM was administered orally

once daily (200 mg/kg/day) either from Day -4 (premedication group) or from Day 1

(post-exposure medication group) until Day 14. In the premedication and post-exposure

medication groups, sets of mice were administered 5% gum arabic solution (200 µL)

orally once daily as the vehicle control group. A survival analysis was performed using

the log-rank test, and the Kaplan–Meier method was used to estimate short-term (14 day)

survival for comparisons between the CAM premedication and post-exposure medication

groups and the respective vehicle controls.

Pathological and bacteriological examination of lung tissues

On Day 6 (i.e., 6 days after inoculation with influenza virus and 2 days after co-infection

with *S. pneumoniae* NU4471), a subset of mice from the CAM premedication group were

sacrificed and lung tissue was taken for pathological (n = 3 mice) and bacteriological (n

= 7 mice) examinations. For the pathological examination, the tissue sample was fixed in

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10% buffered formalin and stained with hematoxylin-eosin (HE). For the bacteriological

examination, the number of bacteria in the lungs was determined as described previously

(Otsu et al., 2003). Briefly, the lungs were dissected under aseptic conditions and

suspended in 1 mL of sterile saline at Day 6. Organs were homogenized, quantitatively

inoculated onto blood agar plates by serial dilution, and incubated at 37°C for 24 h.

Colony numbers were counted and the number of bacteria was calculated as CFU/mL.

Local antibacterial activity and viral titers in lung tissue

On Day 4 (i.e., 4 days after inoculation with influenza virus and immediately prior to

co-infection with *S. pneumoniae* NU4471), a subset of mice from the CAM

premedication group were sacrificed. Local antibacterial activity in the lung was

determined using the paper disc agar diffusion method. Petri dishes containing blood

agar (TSA II 5% Sheep Blood Agar M, Japan Becton, Dickinson and Company, Tokyo,

Japan) were prepared and each plate was inoculated with cultures of

macrolide-susceptible *S. pneumoniae* (ATCC49619) or macrolide-resistant *S.*

pneumoniae (NU4471) adjusted to McFarland No. 0.5 by swabbing on the whole agar

surface with cotton wool. Lungs were homogenized and centrifuged at 3000 rpm for 10

min, and the supernatant was evenly spread on both sides of filter paper discs (6 mm

diameter, Paper disc for antibiotic assay; Toyo Roshi Kaisha, Ltd. Tokyo, Japan). The

discs were aseptically placed on the plates. CAM discs (15 µg; Eiken Chemical Co., Ltd.

Tokyo, Japan) and garenoxacin discs (5 µg; Eiken Chemical Co., Ltd.) were used as

positive controls. The Petri dishes were incubated at 37.0°C for 24 h. At the end of this

period, inhibition zones that formed on the media were recorded.

The influenza viral titer in the lung was determined using the supernatant of the

lung homogenates as viral solutions as described previously (50% Tissue Culture

Infective Dose; $TCID_{50}$/mL).

Bronchoalveolar lavage cell counts and cytokine analysis

On Day 4 (i.e., 4 days after inoculation with influenza virus and immediately prior to

co-infection with *S. pneumoniae* NU4471), a subset of mice from the CAM

premedication group and vehicle control group were sacrificed for a bronchoalveolar

lavage fluid (BALF) analysis. Additional control mice that were inoculated with PBS

instead of influenza virus on Day 0 were also investigated. The effect of CAM

premedication on inflammation in the lung was examined.

The anesthetized mice were sacrificed by incision of the axillary artery and vein.

The lung blood was perfused by injection of 3 mL of sterile saline into the right ventricle.

BAL was performed by washing the lungs and airways with 1 mL of sterile saline three

times per mouse. The number of live cells in the BALF was determined using a

LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems, Inc., Annandale, VA,

USA). BALF was centrifuged at 1100 rpm for 2 min using the Cytospin III (Thermo

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Fisher Scientific K.K., Kanagawa, Japan) and differential cell counts were analyzed

using Diff Quick staining. The BALF supernatants were analyzed for cytokines and

chemokines associated with the immune response to viral infections (interleukin-4 (IL-4),

interleukin-10 (IL-10), interleukin-12 (IL-12), macrophage inflammatory protein-2

(MIP-2), interferon-alpha (IFN-α), IFN-beta (IFN-β), and IFN-gamma (IFN-γ)) by

enzyme-linked immunosorbent assay (ELISA; R&D Systems, Inc., Minneapolis, MN,

USA) according to the manufacturer's instructions.

Cytokine and chemokine analysis in lung homogenates and serum

Based on the ELISA results from the BALF analysis, the levels of IL-10 and IFN-γ were

examined in lung homogenates and serum on Day 4 using ELISA.

Statistical analysis

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Data are presented as means ± standard error (SE). A survival analysis was performed

using the log-rank test, and survival rates were calculated using the Kaplan-Meier

method. Comparisons between two groups were performed using Mann–Whitney U tests.

Comparisons among four groups were performed using the Tukey–Kramer method. A

P-value of <0.05 was considered statistically significant.

Results

Premedication with CAM improved survival rates

Post-exposure treatment with CAM did not improve the survival rate when compared with

that of the vehicle control. However, premedication with CAM resulted in a significantly

higher survival rate than that of the vehicle control ($P < 0.05$, Figure 2A, B).

CAM premedication suppressed lung inflammation and bacterial counts

Figure 3 shows representative images of HE-stained lung sections from the premedication

group at Day 6. Vehicle control mice exhibited bronchopneumonia, characterized by

abundant neutrophils, particularly in peri-bronchiolar lesions as well as in airways, alveolar

spaces, and the interstitium (Figure 3A, B). In contrast, the premedication group showed

significantly less neutrophilic inflammation than that of the control group (Figure 3C, D)

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based on a quantitative analysis of histological images (control group: 102 ± 6

neutrophils/mm^2, premedication group: 65 ± 4 neutrophils/ mm^2; P < 0.0001). The bacterial

count was significantly lower in the lungs of the premedication group than those of the

control group (P < 0.05, Figure 4).

CAM did not exhibit antibacterial activity against NU4471 in the lungs.

The discs with lung homogenates (Figure 5A, lower left) and those with 15 µg of CAM

(Figure 5A, lower right) did not form inhibition zones on the macrolide-resistant NU4471

plate, but both discs formed inhibition zones on the macrolide-susceptible ATCC49619

plate (Figure 5B).

CAM did not affect viral titers at the time of inoculation with NU4471

There was no significant difference between the premedication and control groups in the

viral titers of the lung homogenates on Day 4 (Figure 6).

CAM reduced the number of neutrophils in BALF

The total number of cells and neutrophils in BALF from the CAM-/Flu+ group was

significantly higher than that observed in the BALF from the CAM-/Flu- group. Although

no significant differences in cell number were observed after CAM medication, there was a

significant reduction in the number of neutrophils compared to the control group (Figure 7).

No significant differences between groups were observed for other cell subsets

(lymphocytes, basophils, and eosinophils; data not shown).

Effects of CAM on cytokines and chemokines

The levels of IFN-α, IFN-β, INF-γ, and IL-10 were significantly elevated in BALF at Day 4

of influenza infection compared with the levels observed in mice without influenza (Figure

8). There were no significant differences in the levels of IL-4, IL-12, and MIP-2 (data not

shown). CAM medication significantly reduced IFN-γ levels in BALF, while there were no

significant changes in IFN-α, IFN-β, and IL-10 levels after CAM medication (Figure 8).

Further investigation of INF-γ levels in the serum and lung revealed that INF-γ levels were

significantly higher in the lung and serum during influenza infection than in the controls

(Figure 9). CAM medication significantly reduced IFN-γ levels in lung homogenates, but

did not significant influence its serum levels (Figure 9).

Discussion

Based on our results, premedication with CAM initiated 4 days before influenza virus

inoculation in COPD-model mice, but not post-exposure medication, improved the survival

of mice against subsequent co-infection with the influenza virus and *S. pneumoniae*. Mice

with improved survival also had a less severe inflammatory response in lung tissues than

those of mice in the control group. While viral titers were not affected by CAM

premedication, viable bacterial counts decreased in lung tissues, even though *S.*

pneumoniae NU4471 is macrolide-resistant. CAM accumulates to higher concentrations in

the bronchial epithelial fluid than in the serum (McCarty, 2000; Kikuchi et al., 2008);

accordingly, we examined the local antimicrobial activity of CAM in lung tissues (Figure

5) and confirmed that the lung homogenates did not possess antibacterial activity against *S.*

pneumoniae NU4471. Further investigation revealed that CAM premedication significantly

reduced the number of total inflammatory cells, including neutrophils, in BALF and

significantly attenuated IFN-γ levels in BALF and lung homogenates. These results suggest

that the improvement in survival rates associated with CAM premedication could be

attributed to an anti-inflammatory effect exerted prior to the inoculation of mice with *S.*

pneumoniae.

Taken together, CAM premedication reduced the susceptibility of COPD mice

infected with influenza virus to subsequent *S. pneumoniae* infection by decreasing the

overall inflammatory response and ultimately improved the survival of COPD model mice

infected with both pathogens. Our results obtained using COPD model mice are similar to

those of previous reports demonstrating that macrolides offer clinical benefits in reducing

acute exacerbations of COPD (Albert et al., 2011; Ni et al., 2015). Erythromycin or

azithromycin therapy for 6–12 months could effectively reduce the frequency of

exacerbations and improve the quality of life in patients with COPD (Albert et al., 2011; Ni

et al., 2015). Unlike previous studies reporting that macrolides exert direct antiviral effects

on the influenza virus (Miyamoto et al., 2008; Yamaya et al., 2010), CAM premedication

did not influence viral titers in our study. Rather, CAM premedication appeared to act via a

chemokine-mediated mechanism by disrupting the cycle of infection-inflammation and

strengthening lung defenses, as reported previously (Spagnolo et al., 2013).

Interferons play critical and complex roles in host defense against viral and

bacterial infections. For example, IFN-α and IFN-β (Type-1 IFNs) prevent the progression

of local lung infection with *S. pneumoniae* (Trinchieri, 2010; LeMessurier et al., 2013) and

IFN-γ (Type-2 IFN, type-1 T-helper cell cytokine) combats the early stages of viral

infection (Muller et al., 1994; Schroder et al., 2004). However, high levels of IFN-α, IFN-β,

and IFN-γ-induced by influenza virus infection enhance susceptibility to secondary

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pneumococcal infection (Sun and Metzger, 2008; Nakamura et al., 2011; Li et al., 2012;

Lee et al., 2015) via various mechanisms, including IFN-γ-mediated inhibition of initial

bacterial clearance from the lung by alveolar macrophages (Sun and Metzger, 2008). The

trends toward reduced IFN-α and IFN-β and the significantly lower levels of IFN-γ

observed after CAM premedication in our study and in previous studies (i.e., reduced

IFN-γ levels were detected after macrolide therapy in mice with influenza virus-induced

lung injury (Sato et al., 1998)) might have enhanced bacterial clearance from the lung and

reduced the susceptibility of influenza-infected COPD mice to secondary pneumococcal

pneumonia.

Influenza-infected COPD mice showed a more advanced pathology, characterized

by significantly more neutrophils in the lung tissue and BALF compared with control mice.

Excessive neutrophil infiltration into the lungs has been reported during influenza virus

infection and disrupts the alveolar-capillary barrier, leading to acute lung injury and death

(Seki et al., 2010; Galani and Andreakos, 2015; Herold et al., 2015). Conversely, reductions

in neutrophils or neutrophil function increase the severity of influenza virus infection (Tate

et al., 2009). CAM has been reported to enhance the non-phlogistic clearance of apoptotic

neutrophils by alveolar macrophages (Yamaryo et al., 2003). Indeed, CAM premedication

in our study reduced neutrophil numbers in the lung tissue and BALF of influenza-infected

COPD mice and might have attenuated the potential for inflammation-induced lung

damage.

While vaccination strategies are most efficient with respect to protection against

influenza and pneumococcal infection in COPD patients, the global supply of influenza

vaccines is inadequate during influenza pandemics and is particularly concerning in low-

and middle-income countries (Partridge and Kieny, 2013), where the incidence of COPD

and the demand for influenza vaccinations are expected to increase. Our results suggest that

macrolide premedication may be an effective strategy in COPD patients at a high risk of

infection with influenza virus and *S. pneumoniae* to prevent severe or even fatal secondary

bacterial pneumonia in these high-risk and low-resource settings. Additional studies are

required to identify the ideal patient group and the optimal timing and duration of

macrolide premedication to avoid injudicious administration over extended periods (Ni et

al., 2015).

In conclusion, the administration of CAM initiated 4 days prior to influenza virus

infection in COPD mice reduced IFN-γ production and enhanced the immune response

against secondary bacterial pneumonia to reduce bacterial counts and prolong survival rates.

Future studies should evaluate the use of macrolide premedication in COPD patients at a

high risk of infection with influenza virus and *S. pneumoniae* to prevent severe secondary

bacterial pneumonia.

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Authorship Contributions

Participated in research design: Harada, Ishimatsu, Hara, and Morita.

Conducted experiments: Harada, Hara, Morita, Nakashima, Kakugawa, Sakamoto, and

Kosai.

Contributed new reagents or analytic tools: Kosai, Izumikawa, Yanagihara, and Kohno.

Performed data analysis: Harada, Nakashima, and Ishimatsu.

Wrote or contributed to the writing of the manuscript: Harada, Ishimatsu, Kakugawa,

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Sakamoto, and Mukae.

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Footnotes

Figure Legends

Figure 1. Experimental schedule of treatment. Mice were treated with clarithromycin

(CAM; 200 mg/kg/day) or vehicle once daily. The post-exposure medication group was

treated from Day 1, and the premedication group was treated from Day -4.

Figure 2. Survival curve of secondary bacterial pneumonia (SBP) mice treated with or

without CAM. (A) Survival curve of the post-exposure medication group. Open triangle:

mice treated with vehicle from Day 0 (control) (n=6); Solid triangle: mice treated with

CAM from Day 0(n=10). (B) Survival curve of the premedication group. Open circle: mice

treated with vehicle from Day-4 (control) (n=9); Solid circle: mice treated with CAM from

Day -4 (n=9); N.S: no significant difference versus control; *: significant difference ($P <$

0.05) versus control.

Figure 3. Representative HE-stained tissue sections of the vehicle control group (A, B) and

CAM premedication group (C, D) showing pathological inflammatory changes and

bacterial proliferation on Day 6. Magnification: 40× (A, C) and 200× (B, D).

Figure 4. Bacterial counts in lung homogenates. n = 7 mice/group. Data are presented as

means ± SE, n= 7 mice. *: significant difference ($P < 0.05$) versus control.

Figure 5. Local antibacterial activity of CAM in the lung. Agar plates inoculated with (A)

NU4471 (macrolide-resistant *S. pneumoniae*) and (B) ATCC49619 (macrolide-susceptible

S, pneumoniae). Four paper discs were arranged as follows: upper left, lung homogenates

of vehicle-treated mice; lower left, lung homogenates of CAM-treated mice; upper right,

garenoxacin; and lower right, CAM.

Figure 6. Influenza viral titers of lung homogenates at Day 4. Data are presented as means

± SE, n = 4 mice. N.S: no significant difference between two groups. *: significant

difference ($P < 0.05$) versus the vehicle control group.

Figure 7. Anti-inflammatory effect of CAM on BALF based on the number of (A) total

cells, and (B) neutrophils in each group. Data are presented as means ± SE; n = 5–9 mice.

N.S: no significant difference between two groups; *: significant difference ($P < 0.05$)

between two groups.

Figure 8. Anti-inflammatory effect of CAM on cytokine levels in BALF: (A) IFN-α, (B)

IFN-β, (C) IFN-γ, and (D) IL-10. Data are presented as means ± SE, n = 5–9 mice. N.S: no

significant difference between two groups. *: significant difference ($P < 0.05$) between two

groups.

Figure 9. Anti-inflammatory effect of CAM on IFN-α levels in (A) lung homogenates and

(B) serum samples. Data are presented as means ± SE, n = 5–9 mice. N.S: no significant

difference. *: significant difference ($P < 0.05$).
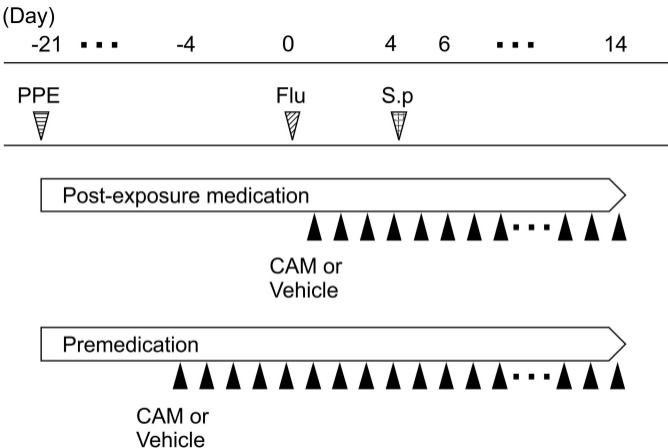
Figure 1
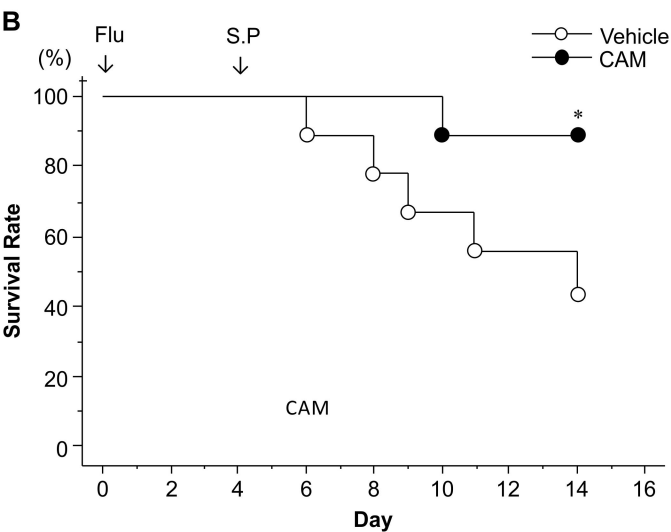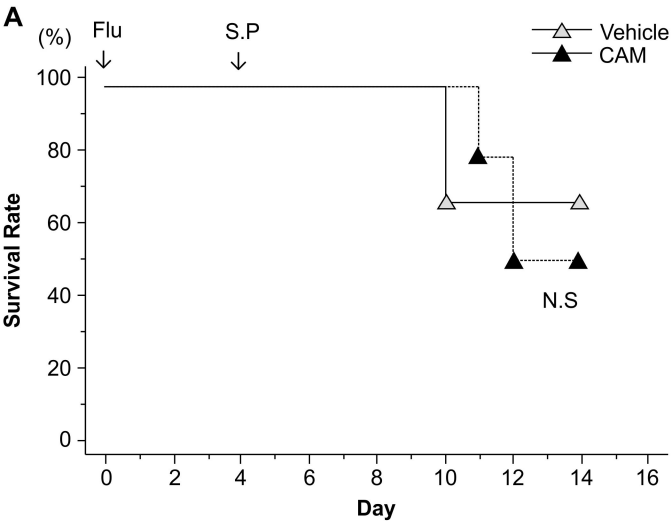
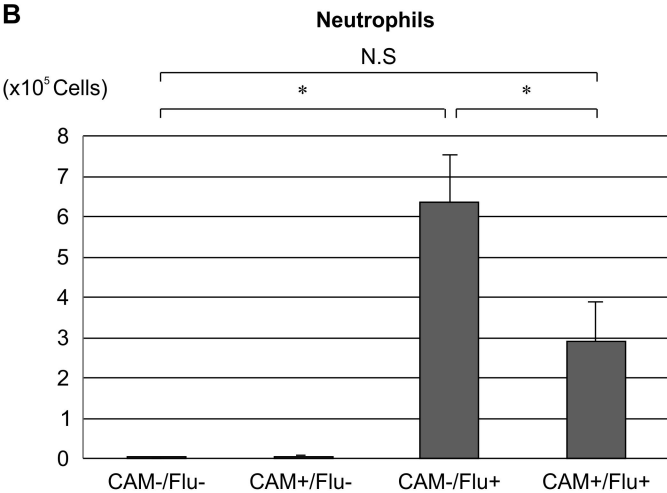**Figure 2**
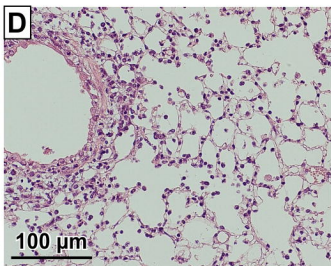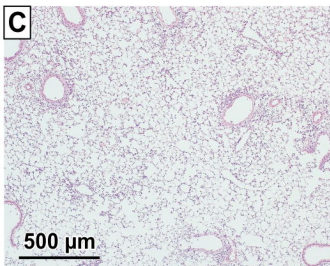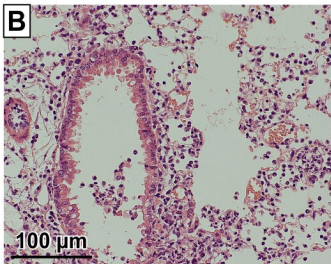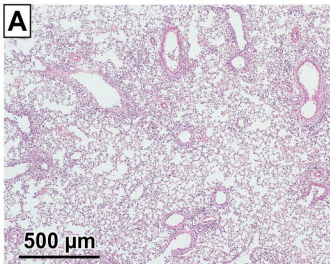
Figure 3

Number of bacteria

(log cfu/ml)
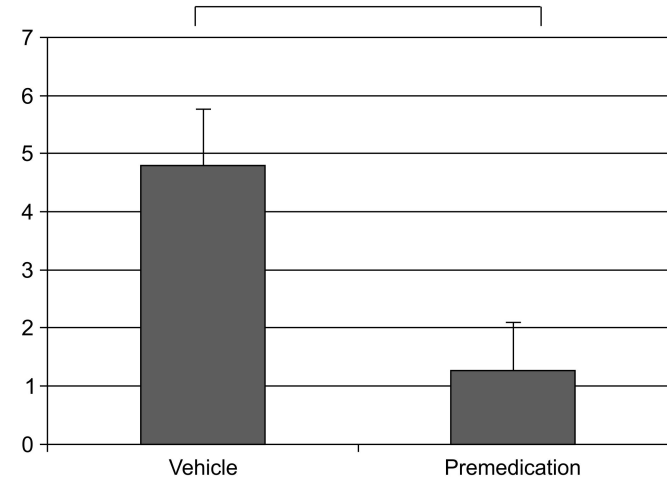
Figure 4

A NU4471   **B** ATCC49619

1cm   1cm

Figure 5

Influenza viral titer

Figure 6

A

Total cells

N.S

*

N.S

(x10^5 Cells)

CAM-/Flu-   CAM+/Flu-   CAM-/Flu+   CAM+/Flu+

B

Neutrophils

N.S

*

*

(x10^5 Cells)

CAM-/Flu-   CAM+/Flu-   CAM-/Flu+   CAM+/Flu+

Figure 7

A s-BALF IFN-α

B s-BALF IFN-β

C s-BALF IFN-γ

D s-BALF IL-10

Figure 8

A

s-Lung IFN-γ

N.S

*

*

(pg/g-lung)

70
60
50
40
30
20
10
0

CAM-/Flu-   CAM+/Flu-   CAM-/Flu+   CAM+/Flu+

B

Serum IFN-γ

N.S

*

N.S

(pg/ml)

200
180
160
140
120
100
80
60
40
20
0

CAM-/Flu-   CAM+/Flu-   CAM-/Flu+   CAM+/Flu+

Figure 9