TLR4 agonistic antibody promotes innate immunity against severe pneumonia induced by co-infection with influenza virus and *Streptococcus pneumoniae*

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Running title

Efficacy of UT12 against secondary bacterial pneumonia

Abstract

Co-infection with bacteria is a major cause of mortality during influenza epidemics. Recently, toll-like receptor (TLR) agonists have been shown to have immunomodulatory functions. In the present study, we investigated the effectiveness and mechanisms of the new TLR4 agonistic monoclonal antibody UT12 against secondary pneumococcal pneumonia induced by co-infection with influenza virus in a mouse model. Mice were intranasally inoculated with *Streptococcus pneumoniae* 2 days after influenza virus inoculation. UT12 was intraperitoneally administered 2 h before each inoculation. Survival rate and body weight loss were significantly improved by UT12 administration. Additionally, the production of inflammatory mediators was significantly suppressed by administration of UT12. In a histopathological study, pneumonia in UT12-treated mice was very mild compared to that in control mice. UT12 increased antimicrobial defense through acceleration of macrophage recruitment into the lower respiratory tract induced by c-Jun N-terminal kinase (JNK) and nuclear factor-kappaB (NF-κB) pathway-dependent monocyte chemoattractant protein (MCP)-1 production. Collectively, these findings indicated that UT12 promoted pulmonary innate immunity and may reduce the severity of severe pneumonia induced by co-infection with influenza virus and *S. pneumoniae*. This immunomodulatory effect of UT12 improves the prognosis of

secondary pneumococcal pneumonia and makes it an attractive candidate for treating severe infectious diseases.

Key words

Secondary bacterial pneumonia, innate immunity, influenza virus, *Streptococcus pneumoniae*, macrophage

Introduction

Acute respiratory infections account for a large proportion of deaths worldwide [1]. In particular, influenza virus infection is life threatening for elderly individuals and immunocompromised patients. Pneumonia is a serious complication associated with influenza virus infection, and influenza-associated pneumonia can be classified into 2 categories: primary viral pneumonia and secondary bacterial pneumonia. While influenza infection can be lethal in and of itself, a substantial number of postinfluenza deaths are due to secondary bacterial pneumonias, most commonly caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* [2-9]. Our previous study demonstrated that cytokine storms caused by an excessive host immune response are often the cause of the synergistic effect of influenza virus and *S. pneumoniae*, resulting in a shorter survival period and more severe lung inflammation in co-infected mice compared to mice infected with either influenza or *S. pneumoniae* alone [10].

Toll-like receptor (TLR), a receptor protein found on the surface of animal cells, plays a critical role in the innate immune system. When microbes invade the host, TLR recognizes the pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoprotein, flagellin of the flagellum, and double-stranded viral RNA. PAMPs are broadly shared by pathogens but distinguishable from host

66 molecules, and detection of PAMPs by TLR proteins activates immune cell responses.
67 Moreover, some TLR agonists were recently found to have anti-infective, antitumor, and
68 anti-allergic effects based on their functions as immune activators [11-14].

69 UT12 is an antibody generated against BaF3 cells overexpressing mouse TLR4.
70 UT12 acts as an agonist of the TLR4/MD-2 complex and induces a stimulatory signal
71 similar to the original ligand LPS [15]. UT12 can induce the production of NF-κB and
72 inflammatory cytokines involved in the innate immune system from peritoneal exudate
73 cells in vitro [15]. Previous studies have demonstrated that prophylactic treatment with
74 TLR ligands enhances host immunity against influenza virus infection or pneumococcal
75 infection alone [16, 17]. However, no report has verified the effectiveness of the TLR
76 agonist for an influenza virus/bacteria co-infection, which is more lethal than when either
77 pathogen is delivered alone.

78 Therefore, in the present study, we sought to elucidate the mechanistic basis of
79 the effects of UT12 treatment against severe pneumococcal pneumonia following
80 influenza virus infection in mice.

81

82 **Materials and methods**

83 **Reagents**

UT12 was a gift from Dr. Fukudome (Saga Medical School, Saga, Japan). Clodronate liposomes were purchased from FormuMax Scientific (Palo Alto, CA, USA). All primary antibodies for western blotting were purchased from Abcam (Cambridge, UK). Secondary antibodies for western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Inhibitors of JNK (SP600125), p38 (SB203580), MEK-1 (PD98059), and NF-κB (parthenolide) were obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Mice

CBA/JNCrlj mice (6-week-old males) were purchased from Charles River Laboratories Japan (Yokohama, Japan). C3H/HeJ and C3H/HeN mice (6-week-old males) were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed in accordance with the guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Virus and bacteria

A mouse-adapted influenza virus A/Puerto Rico 8/34 (H1N1) (PR8; a gift from Dr. Watanabe, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki,

Japan) was grown in cultured MDCK cells. After 3 days, the supernatant was collected and stored at -80°C until use. The stored supernatant was thawed and diluted with phosphate-buffered saline (PBS) to the desired concentration just before inoculation. *S. pneumoniae* ATCC 49619 clinical isolate with capsular serotype 19F was prepared as previously described [18]. Maintenance and storage of bacteria was performed as reported previously [10]. Bacteria were grown in Mueller-Hinton II broth (Eiken Chemical, Tokyo, Japan) with Strepto Haemo supplement (Eiken Chemical Tokyo, Japan) at 37°C for 6 h or until reaching log phase. The concentration of bacteria in the broth was determined by measuring the absorbance at 660 nm and then plotting the optical density on a standard curve generated by known CFU values. The bacteria culture was then diluted to the desired concentration for co-infection studies.

Mouse co-infection studies and UT12 treatment

We performed viral challenge by intranasal inoculation of 5×10^3 plaque-forming units of PR8 in 50 μL PBS into mice anesthetized with pentobarbital. To induce pneumococcal superinfection, we intranasally inoculated 1×10^5 CFU of pneumococcus in 50 μL of PBS into anesthetized mice 2 days after PR8 inoculation. Two hours prior to each inoculation, 1.0 μg of UT12 was intraperitoneally (i.p.) administered. A scheme of the study protocol

120 is shown in Fig. 1. Samples of lungs and bronchoalveolar lavage fluid (BALF) were
121 collected 2 days after pneumococcal inoculation.
122
123 **Whole-lung preparations for CFU determination and histopathology**
124 Whole lungs were removed under aseptic conditions and homogenized in 1.0 mL PBS
125 using a Shake Master NEO (Bio Medical Science, Tokyo, Japan). *S. pneumoniae* was
126 quantified by placing serial dilutions of the lung homogenates onto blood agar plates and
127 incubating them at 37°C in a 5% CO_2 atmosphere. The remaining homogenates were
128 centrifuged at 10000 × g for 30 min, and the supernatants were used for enzyme-linked
129 immunosorbent assay (ELISA). Lung tissue sections were paraffin-embedded and stained
130 with hematoxylin and eosin (HE) using standard procedures [10, 19].
131
132 **Bronchoalveolar lavage and BALF cell analysis**
133 Bronchoalveolar lavage was performed to assess inflammatory cell accumulation in the
134 air space. The chest was opened to expose the lungs after the mice were anesthetized, and
135 a disposable sterile feeding tube (Toray Medical Co., Chiba, Japan) was inserted into the
136 trachea. Bronchoalveolar lavage was performed using 1.0 mL PBS, and the recovered
137 fluid was pooled for each animal. The BALF was then centrifuged onto a slide using a

Cytospin3 centrifuge (Shandon, Pittsburgh, PA, USA) at 750 × g for 2 min and stained with Diff-Quik staining for differential cell counts.

Isolation and culture of peritoneal macrophages

Three days after intraperitoneal injection of 4% sterile thioglycolate medium (2 mL), peritoneal macrophages were isolated by peritoneal lavage with Hank's buffer (without Ca^{2+} and Mg^{2+}) containing 0.1% gelatin. Contaminating erythrocytes, granulocytes, and dead cells were removed by density gradient centrifugation for 45 min at 800 × g in Mono-Poly resolving medium according to the manufacturer's protocol (MP Biomedicals). Purified peritoneal macrophages were washed 3 times and cultured overnight in Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS) with 100 U/mL penicillin and 100 μg/mL streptomycin.

Macrophage depletion

Macrophages were depleted using clodronate liposomes as previously described [20]. Clodronate liposomes (100 μL/mouse) were administered i.p. 24 h prior to pneumococcus inoculation. Administration of clodronate liposomes led to a more than 80% decline in the number of monocyte/macrophages compared with controls, as

assessed in cytospin preparations 24 h after administration.

ELISA

Concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, and MCP-1 in lung homogenates were assayed using mouse Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA). Concentrations of total and phosphorylated NF-κB were assayed using PathScan Sandwich ELISA kits (Cell Signaling Technology, Danvers, MA, USA). These cytokine analyses were performed according to the manufacturers' protocols.

Western blotting

Protein separation, transfer, blocking, and development of signals were performed as described previously [10]. For detection of intact and phosphorylated (activated) forms of JNK, mitogen-activated protein kinase (MAP)-extracellular signal-regulated kinase (ERK) kinase 1 (MEK-1), and p38, rabbit primary antibodies against each kinase (total JNK: ab7964, p-JNK: ab32447, total MEK-1: ab75608, p-MEK-1: ab5613, total p38: ab7952, p-p38: ab32557, Abcam Inc.) were used. Incubation with primary antibodies was

followed by incubation with secondary antibodies conjugated to horseradish peroxidase (sc-2030, Santa Cruz Biotechnology).

Statistics

All data were expressed as means ± SDs and analyzed by using StatView software (Abacus Concepts, Cary, NC, USA). Survival curves were estimated by the Kaplan-Meier method, and their homogeneity was evaluated by the log-rank test. Differences between 2 groups were tested for significance using unpaired t-tests. Differences between multiple groups were tested for significance using 2-way analysis of variance (ANOVA). Differences with P-values of less than 0.05 were considered statistically significant.

Results

UT12 administration improved survival in co-infected mice

Mice co-infected with influenza A virus followed 2 days later by pneumococcus had a higher rate of mortality than mice infected with a single pathogen (Fig. 2a), as reported previously [10]. To assess the effects of UT12 in co-infected mice, we compared survival and body weight changes between UT12-treated mice and untreated mice. Treatment

192 with UT12 increased the survival rate from 0% (control mice) to 60% at the end of the
193 observation period ($P < 0.0001$, Fig. 2b). Control mice lost an average of 15% of their
194 body weight at day 4 after co-infection; in contrast, although body weight loss was
195 observed later, UT12-treated mice maintained their body weight at least 4 days after
196 secondary pneumococcal challenge (Fig. 3). These data indicated that UT12 decreased
197 the mortality and body weight loss induced by co-infection with influenza virus and
198 *S.pneumoniae*.
199
200 **Bacterial burden and inflammation were reduced in the lungs of co-infected mice**
201 **following administration of UT12**
202 There was a significant difference in the bacterial burdens of co-infected mice with and
203 without UT12 treatment ($P < 0.05$, Fig. 4a). While we attempted to examine viral titers
204 after UT12 administration in mice infected influenza alone and in mice co-infected with
205 influenza and *S. pneumoniae*, no significant differences were observed (data not shown).
206 Total cell counts in the BALF were significantly lower in UT12-treated mice than in
207 control mice ($P < 0.05$). In addition, neutrophil counts were also decreased by UT12
208 treatment, although the difference was not significant ($P = 0.10$, Fig. 4b).
209 Robust innate pro-inflammatory cytokine expression can cause direct tissue

insult and recruit inflammatory cells that can potentially destroy tissue [21, 22]. The percent survival in co-infected mice was increased by UT12 administration, and we hypothesized that UT12 might protect a host from severe lung injury by preventing cytokine storms through the reduction of host sensitivity against pneumococcal infection. As shown in Fig. 4c, after co-infection, the levels of TNF-α, IL-6, KC, and MIP-2 were significantly suppressed in UT12-treated mice as compared to control mice (TNF-α: $P < 0.001$, IL-6: $P < 0.001$, KC: $P < 0.01$, MIP-2: $P < 0.05$).

Furthermore, we assayed concentrations of NF-κB, a transcription factor that plays critical roles in inflammation, in the lungs of mice co-infected with the 2 pathogens. Excessive activation of NF-κB can induce a cytokine storm, resulting in septic shock [23]. In the current study, the levels of activated NF-κB in the lung homogenates after co-infection were significantly suppressed by UT12 administration ($P < 0.05$, Fig. 5). These data suggested that UT12 might be able to attenuate the expression of cytokines and activation of intracellular signal transduction pathways via TLR signaling.

Histopathological analysis of co-infected lungs revealed marked reductions in tissue injury, inflammatory cell accumulation, pulmonary hemorrhage, and edema in UT12-treated mice (Fig. 6). Taken together, our data indicated that UT12 might have a substantial therapeutic effect toward severe pneumococcal pneumonia induced by

co-infection with influenza virus through inhibition of inflammatory cell responses and suppression of pro-inflammatory cytokine/chemokine production in the lungs.

The anti-inflammatory effects mediated by UT12 were TLR4 specific

Mice with intact (C3H/HeN) and nonfunctional (C3H/HeJ) TLR4 were treated with UT12 prior to individual inoculation. Treatment with UT12 delayed mortality, but did not impact overall survival in co-infected C3h/HeN mice as compared to those treated with vehicle (Fig. 7a). However, percent survival was not significantly different between C3H/HeJ mice treated with UT12 and those treated with vehicle (Fig. 7a). An analysis of TNF-α, IL-6, KC, and MIP-2 concentrations in the lungs of co-infected mice showed that UT12 significantly attenuated pro-inflammatory cytokine production in C3H/HeN mice but not in C3H/HeJ mice (Fig. 7b). These results indicated that the anti-inflammatory effects of UT12 during co-infection with influenza and pneumococcus were primarily TLR4 specific, although the TLR4-dependent inflammatory response was not completely abolished in C3H/HeJ mice.

UT12 induced the migration of mononuclear cells into the lower respiratory tract by promotion of MCP-1 production from alveolar macrophages

Macrophages are responsible for the majority of cell-mediated bacterial clearance after infection and are key participants in the acute inflammatory response. Therefore, we next assessed the effects of UT12 treatment on the recruitment of macrophages to the primary site of infection. BALF was obtained from mice 4 h after i.p. treatment with UT12. Interestingly, the number of mononuclear cells in the BALF of UT12-treated mice was significantly increased compared to that in vehicle-treated mice ($P < 0.05$, Fig. 8a).

MCP-1 is a chemokine that recruits mononuclear cells to the infectious source. The production of MCP-1 in the lungs was markedly increased after UT12 administration; MCP-1 levels peaked at 4 h and remained high at 48 h after UT12 administration (Fig. 8b). Because much of the MCP-1 in the lung is produced by alveolar macrophages [24], we examined whether resident macrophages were involved in the UT12-mediated production of MCP-1 by performing macrophage depletion experiments with clodronate liposomes [20, 25]. As shown in Fig. 8c, macrophage depletion suppressed the production of MCP-1 in response to UT12 stimulation.

To confirm the importance of macrophages for protection against pneumococcal infection, we compared survival after *S. pneumoniae* infection between macrophage-depleted mice and mice with intact macrophages. All macrophage-depleted mice died within 6 days of infection; however, all intact mice survived at least 6 days after

pneumococcal infection ($P < 0.001$, Fig. 9). Improved survival mediated by UT12 was not observed in macrophage-depleted mice (Fig. 9). Taken together, these data indicated that existing macrophages were essential for MCP-1-dependent enhancement of macrophage recruitment and phagocytosis mediated by UT12. Alveolar macrophages appeared to have a crucial role in initial bacterial killing within the lower respiratory tract (LRT), and UT12 augmented host innate immunity against severe pneumococcal pneumonia occurring after influenza infection.

UT12 induced MCP-1 production via an NF-κB and JNK-dependent pathway

To investigate the UT12-mediated TLR4 signaling pathways involved in the production of MCP-1, we examined the concentration of activated NF-κB and the expression of MAPK family proteins (JNK, p38, and MEK-1) in the lungs after UT12 administration. Compared with vehicle-treated mice, UT12 pretreatment significantly increased the levels of activated NF-κB in uninfected mice ($P < 0.001$; Supplementary Fig. 1). In addition, the level of phosphorylated JNK was also clearly increased at 2 h after UT12 administration (Fig. 10a), whereas the levels of phosphorylated p38 and MEK-1 were unchanged throughout the experiment (data not shown). To confirm the importance of the NF-kB and JNK-dependent pathway for UT12-mediated MCP-1 production, peritoneal

macrophages were pretreated with specific MAPK inhibitors, i.e., SP600125 (a specific inhibitor of JNK), SB203580 (an inhibitor of p38), PD98059 (an inhibitor of MEK-1), and parthenolide (an inhibitor of NF-κB), for 30 min and then cotreated with UT12 for 4 h prior to the detection of MCP-1 in the supernatant. Pretreatment with SP600125 or parthenolide inhibited MCP-1 production, indicating that both JNK and NF-kB were involved in the production of MCP-1 in UT12-stimulated macrophages (Fig. 10b). These results suggested that activation of the JNK and NF-kB pathway was required for the promotion of MCP-1 production in UT12-treated macrophages.

Discussion

Influenza infection predisposes the host to secondary bacterial infection of the respiratory tract, which is a major cause of death in influenza-related disease, even if appropriate antibiotics are administered. Vaccination is the primary tool to prevent influenza infection, but its effectiveness is not 100%. Annual influenza epidemics result in an estimated 3–5 million cases of severe illness and 250,000–500,000 deaths every year.

The innate immune system recognizes and rapidly responds to microbial pathogens, providing the first line of host defense. It is becoming clear that induction of innate immunity may be useful for preventing bacterial infection. Indeed, Clement et al.

reported that the stimulation of innate immunity by bacterial lysates induces the augmentation of antimicrobial polypeptides in lung lining fluid and protects the lungs against lethal pneumococcal pneumonia [17]. Moreover, providing insight into the specific mechanisms that regulate this response of the innate immune system, Yu et al. reported that intranasal pretreatment of mice with purified P. aeruginosa flagellin induces strong protection against Pseudomonas infection via TLR5 and markedly improves bacterial clearance [26]. Therefore, TLR agonists are being developed as adjuvants for potent new vaccines to prevent or treat infectious diseases [27]. For example, monophosphoryl lipid A (MPLA), which is isolated from bacterial cell walls and detoxified, acts through TLR4 as an immune stimulator. TLR4 mediates LPS responsiveness and recognizes gram-negative bacteria via the LPS moiety on the surface of these microorganisms. Some researchers have reported that MPL treatment promotes neutrophil recruitment to the infectious source and mediates protection against both lethal systemic bacterial infection and nasopharyngeal colonization [28, 29]; however, little is known about whether the induction of innate immunity via TLR4 contributes to the protective immune response against bacterial infection. Therefore, in the current study, we used UT12, a new TLR4 agonistic monoclonal antibody, to address whether the promotion of innate host resistance through TLR4 mediates protection against secondary

318 pneumococcal pneumonia following influenza virus infection. We found that UT12
319 pretreatment significantly improved survival, attenuated the levels of pro-inflammatory
320 cytokine production, and enhanced the clearance of bacteria in our co-infection model of
321 influenza and pneumococcus. In a separate set of experiments of co-infection, we tested
322 the effects of a single dose of UT12 prior to influenza virus or *S. pneumoniae* exposure;
323 improvement of survival observed in co-infected mice with UT12 administration prior to
324 both influenza virus and *S. pneumoniae* inoculation was lost, indicating that each
325 prophylactic inoculation of UT12 may be relevant to its protective effects against severe
326 lung injury induced by co-infection of influenza virus and *S. pneumoniae*. Thus, our data
327 suggested that stimulation of the innate immune system protected against co-infection in
328 this system.

329 Our previous study showed that in animals with prior influenza infection, a
330 bacterial burden was detected as early as 48 h after secondary infection with S.
331 *pneumoniae*, and extreme production of inflammatory cytokines and chemokines was
332 induced (cytokine storm), resulting in severe host tissue injury [10]. In the present study,
333 the viable *S. pneumoniae* count in the lungs of UT12-treated mice was significantly
334 reduced compared with control mice 2 days after pneumococcal inoculation. In addition,
335 cytokine storms induced by co-infection were suppressed in UT12-treated mice,

suggesting that UT12 inhibited the growth of *S. pneumoniae* and attenuated the excessive host immune response. Thus, UT12-mediated reduction of host sensitivity against secondary pneumococcal exposure may be able to inhibit the development of cytokine storms after influenza virus infection. These results are similar to those of a previous study that investigated the effects of the TLR4 agonist MPLA against postburn wound infection by *P. aeruginosa* [30].

We further examined changes in the immune cell population in the LRT to determine which cells were responsible for UT12-induced protection since neutrophils, macrophages, and dendritic cells are important cellular mediators of innate immune defense in severe pneumococcal pneumonia induced by co-infection with influenza virus. In particular, inflammatory macrophages respond rapidly to microbial stimuli by secreting cytokines and antimicrobial factors. In addition, they express the CCR2 chemokine receptor and traffic to sites of microbial infection in response to MCP-1 (also known as chemokine [C-C motif] ligand 2 [CCL2]) secretion. In murine models, monocyte recruitment mediated by the CCL2-CCR2 axis is essential for defense against several bacterial, protozoan, and fungal pathogens. Moreover, in pneumococcal studies, alveolar macrophages have also been shown to be essential for the initial clearance of pneumococci within the respiratory tract. Winter et al. demonstrated that

MCP-1-dependent macrophage recruitment contributes to lung protective immunity against pneumococcal infection [31, 32]. In the current study, the survival of pneumococcus-infected mice was similarly reduced by depletion of macrophages, and survival after pneumococcal pneumonia was not restored by UT12 administration in macrophage-depleted mice. Likewise, we showed that the accumulation of macrophages in the LRT and production of MCP-1 in the lungs were induced after UT12 administration. The disappearance of the benefit from UT12 mediated macrophage recruitment and MCP-1 production were observed in macrophage-depleted mice, indicating that resident macrophages may be responsible for producing MCP-1 after stimulation with UT12.

Our results also demonstrated that UT12 administration increased the phosphorylation of JNK and NF-κB. Moreover, JNK and NF-κB inhibitors significantly reduced MCP-1 production in macrophages, and UT12 exerted protective effects in C3H/HeN mice, but not in C3H/HeJ mice, which have low responsiveness to TLR4 agonists. In addition, the UT12-mediated reduction of excessive inflammatory cytokine production induced by co-infection also disappeared in C3H/HeJ mice. These results indicated that sufficient innate immune activation against secondary pneumococcal infection via a TLR4-specific signaling pathway was induced by UT12 prophylactic treatment.

There are some limitations in this study. First, the importance of endotoxin tolerance induced by UT12 for the suppression of the cytokine storm was not demonstrated. The clearance of some pathogens is promoted during the LPS-tolerant state, despite attenuated cytokine production [35]. The pneumococcal pore-forming toxin, pneumolysin, is also recognized by TLR4 [36, 37]. Additional studies are required to determine the effects of UT12-mediated tolerance against the inflammation induced by *S. pneumoniae* and pneumolysin in particular. Second, we did not investigate the interaction between UT12 and other types of immune cells. For instance, we cannot exclude CD4-positive T cells or dendritic cells as sources of MCP-1 production, and we did not examine the phagocytic function of neutrophils. Therefore, additional cell deletion studies may be necessary to confirm which cells were the most important for UT12-induced MCP-1 production. However, our data demonstrated that macrophages play a crucial role in the activation of innate immunity against pneumococcal pneumonia induced by co-infection with influenza virus. Finally, we did not examine the signaling crosstalk between TLRs, which regulates the host inflammatory reaction to bacterial infection [33, 34]. Thus, in future experiments, we will investigate the role of other TLRs in UT12-induced signaling pathways.

In conclusion, the present study demonstrated that treatment with the TLR4

agonistic monoclonal antibody UT12 caused resistance to severe pneumonia, characterized by attenuation of systemic pro-inflammatory cytokine production and improved clearance of bacteria by enhanced recruitment of macrophages to sites of infection. Based on a limited case series and accumulated clinical experience, bacterial pneumonia following influenza virus infection appears to be more difficult to treat and has a high fatality rate. The ability of UT12 to improve survival, reduce inflammation, and enhance bacterial clearance makes it an attractive agent for potential application in patients that are at high risk of complications from influenza infection.

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Figure legends

Figure 1. Schedule of co-infection experiments. Mice were administered i.n. influenza

(*PR8* strain, 5×10^3 PFU in 50 μl PBS), followed 2 days later by i.n. *S. pneumoniae* ($1X10^5$ CFU in 50 μl PBS). UT12 (1.0μg) was intraperitoneally administered two hours prior to each inoculation. PR8: influenza virus A/Puerto Rico 8/34 (H1/N1) ; Sp: *S. pneumoniae*.

Figure 2. Administration of UT12 prior to pathogen exposure improves survival in co-infected mice. Influenza virus was inoculated 2 days before *S. pneumoniae* exposure (Day 0). Percent survival of mice singly infected with influenza virus A/Puerto Rico 8/34 (H1/N1) (PR8) or *S. pneumoniae* (Sp), and co-infection of influenza virus and *S.pneumoniae* with or without UT12 was examined. Kaplan-Meier curve with survival rates of mice (PR8: n=10, Sp: n=8, PR8+Sp: n=10, PR8+Sp+UT12: n=10). Statistical significance was determined using the log-rank test. **P < 0.01.

Figure 3. Body weight change was monitored. The body weight of UT12-treated mice was higher at day1, 3 and 4 significantly than saline-treated control mice. UT12-treated group (solid line; n = 10), control group (dotted line; n = 10). PR8: influenza virus A/Puerto Rico 8/34 (H1/N1); Sp: *S. pneumoniae*; Values represent means ± SD, *P < 0.05, **P < 0.01.

551

552 **Figure 4.** Prophylactic UT12 administration inhibits bacterial burden and excessive
553 proinflammatory cytokine production induced by co-infection in the lung. a) The
554 numbers of viable *S. pneumoniae* after co-infection. b) Total cell and neutrophil count in
555 BALF. c) Concentration of proinflammatory cytokines/chemokines. Each examination
556 was performed 2 days after *S. pneumoniae* infection. Each group contained 7 mice.
557 Values represent means ± SD, *P < 0.05, **P < 0.01, n.s.; not significant.

558

559 **Figure 5.** Phosphorylated and total NF-κB concentration in the lung homogenates. Each
560 examination was performed 2 days after *S. pneumoniae* infection. Each group contained 7
561 mice. Values represent means ± SD, **P < 0.01.

562

563 **Figure 6.** UT12 administration protects host from acute lung injury induced by
564 co-infection. Histopathological analysis of the lungs. Lungs were collected 2 days after *S.*
565 *pneumoniae* co-inoculation. Photographs of whole lungs and haematoxylin and
566 eosin-stained tissue sections at magnifications of ×40 and ×200. PR8: influenza virus
567 A/Puerto Rico 8/34 (H1/N1); Sp: *S. pneumoniae.*

568

Figure 7. UT12-mediated protection against pneumococcal infection is TLR4 specific. a) Percent survival in co-infected C3H/HeN and C3H/HeJ mice with or without UT12 treatment. b) Concentration of proinflammatory cytokines and chemokines in the lung at day 2 after *S. pneumoniae* co-infection. C3H/HeJ mice treated with UT12 (n = 4), C3H/HeJ mice treated with saline (n = 4), C3H/HeN mice treated with UT12 (n = 5) and C3H/HeN mice treated with saline (n = 4), respectively from left to right. Sp: *S. pneumoniae*; Kaplan-Meier curve with survival rates of mice (C3H/HeN with UT12 treatment group: n=15, The other groups: n=10). Statistical significance was determined using the log-rank test. Values represent means ± SD, *P < 0.05, **P < 0.01, n.s.; not significant.

Figure 8. The resident macrophages are required for UT12-mediated promotion of recruitment of macrophages via MCP-1 dependent. a) Mononuclear cell count in BALF 4 h after saline or UT12 administration was examined by cytospin. b) Time course of the level of MCP-1 concentration in the lung after UT12 administration. Values at each time point after UT12 administration were compared with untreated mice. c) Concentration of MCP-1 in the lung 4 h after UT12 administration with or without UT12 and clodronate

liposomes (C.L.) treatment. Values represent means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 9. Percent survival of mice in pneumococcal pneumonia model with or without clodronate liposome (C.L.) administration. Sp: *S. pneumoniae;* Kaplan-Meier curve with survival rates of mice [(solid line: Saline+Sp) :n=10, (dashed line: C.L.+Sp) : n =10, (dotted line: C.L.+UT12+Sp) : n=6]. Statistical significance was determined using the log-rank test. The survival of Sp infected mice without both C.L. and UT12 was longer than that of mice with C.L. pretreatment. ***P < 0.001.

Figure 10. UT12-mediated MCP-1 production is required for the phosphorylation of both JNK and NF-kB. a) Activation of c-Jun N-terminal kinase (JNK) in the lung after UT12 administration. b) The levels of MCP-1 production 4 h after UT12 stimulation from the peritoneal macrophage pretreated inhibitors of JNK, p38, MEK-1, and NF-κB. Values represent means ± SD, **P < 0.01.

Figure 1

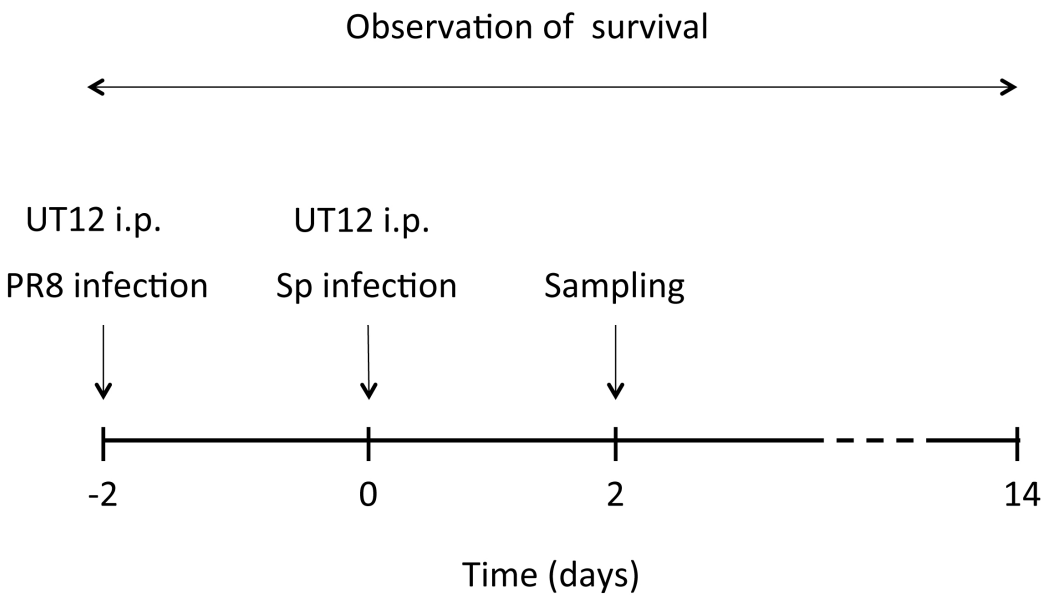

Observation of survival
UT12 i.p.
PR8 infection
UT12 i.p.
Sp infection
Sampling
-2
0
2
14
Time (days)

Figure 2

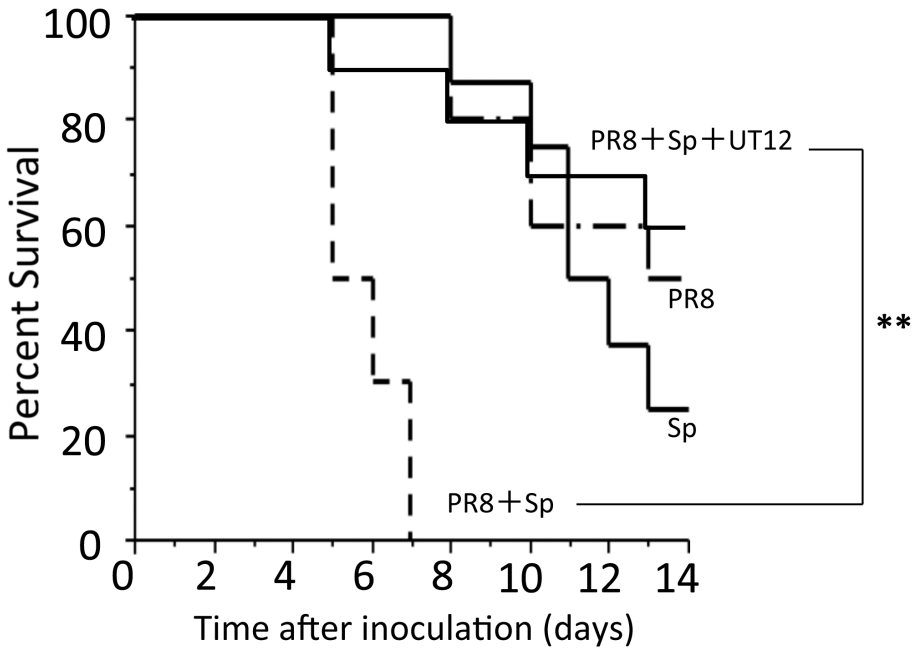

100
80
60
40
20
0
Percent Survival
0
2
4
6
8
10
12
14
Time after inoculation (days)
PR8＋Sp＋UT12
PR8
Sp
PR8＋Sp
**

Figure 3

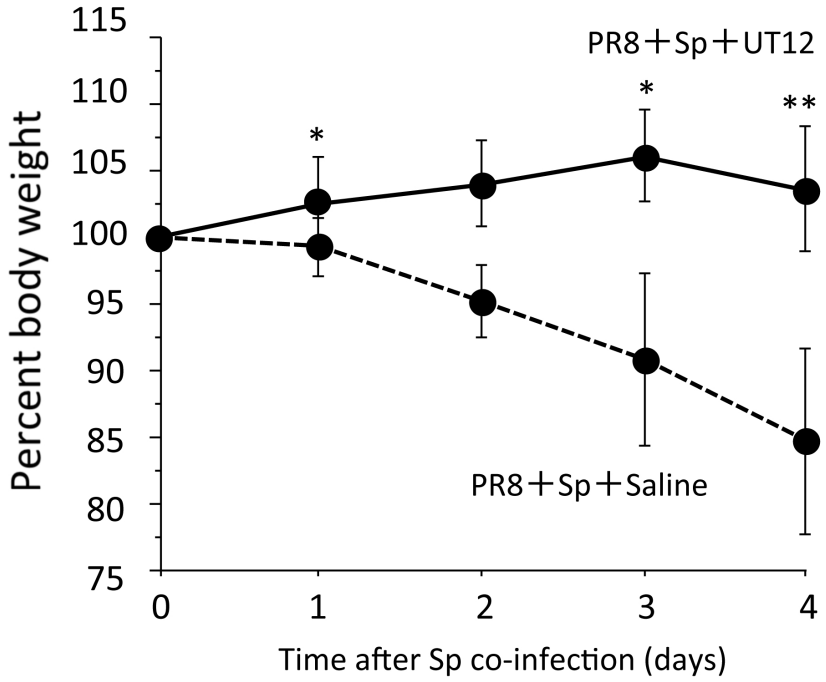

115
110
105
100
95
90
85
80
75
Percent body weight
PR8＋Sp＋UT12
PR8＋Sp＋Saline
*
*
**
0
1
2
3
4
Time after Sp co-infection (days)

Figure 4

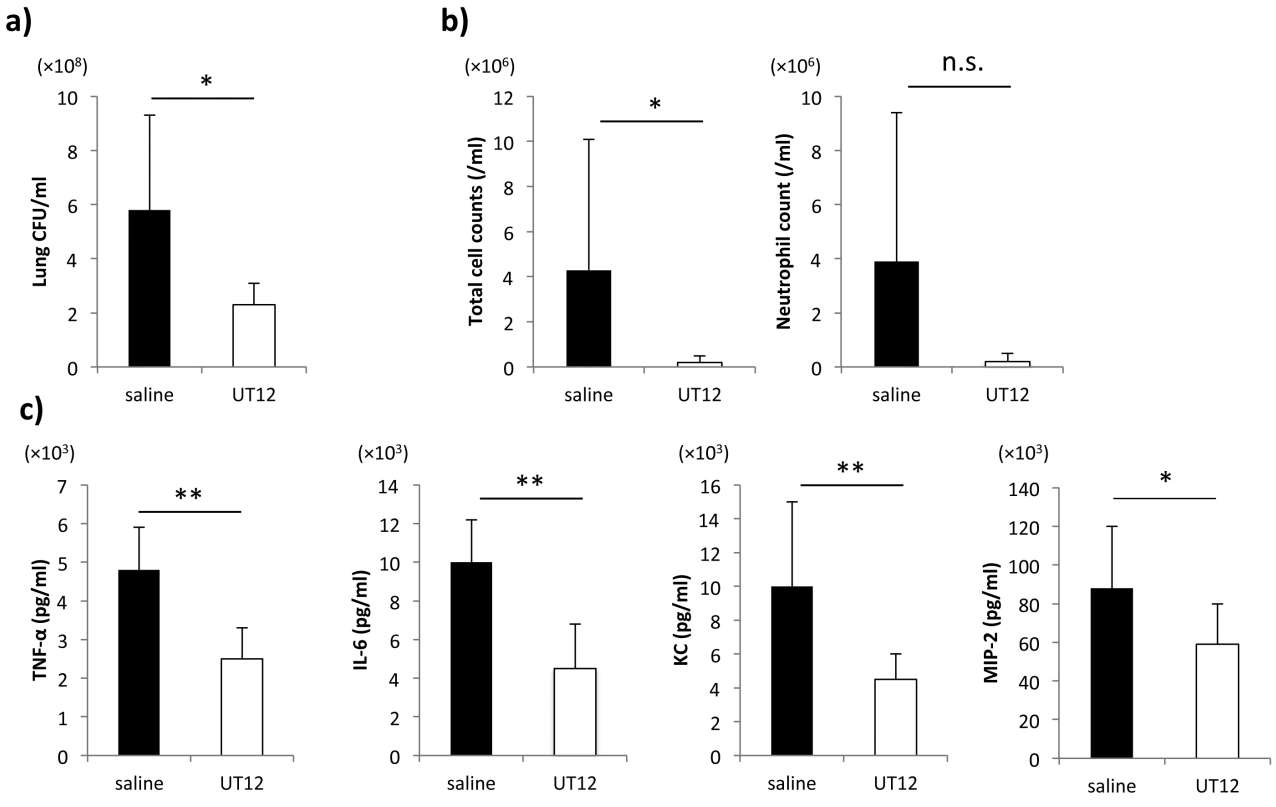

a)
b)
c)
(×10⁸)
Lung CFU/ml
(×10⁶)
Total cell counts (/ml)
Neutrophil count (/ml)
n.s.
(×10³)
TNF-α (pg/ml)
IL-6 (pg/ml)
KC (pg/ml)
MIP-2 (pg/ml)
saline
UT12
*
**

Figure 5

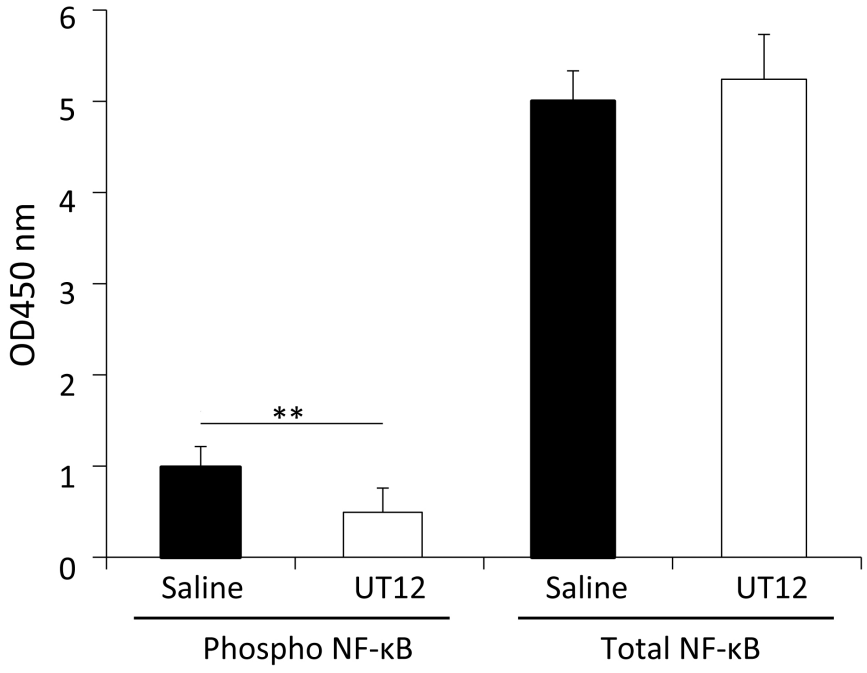

6
5
4
3
2
1
0
OD450 nm
**
Saline
UT12
Saline
UT12
Phospho NF-κB
Total NF-κB

Figure 6

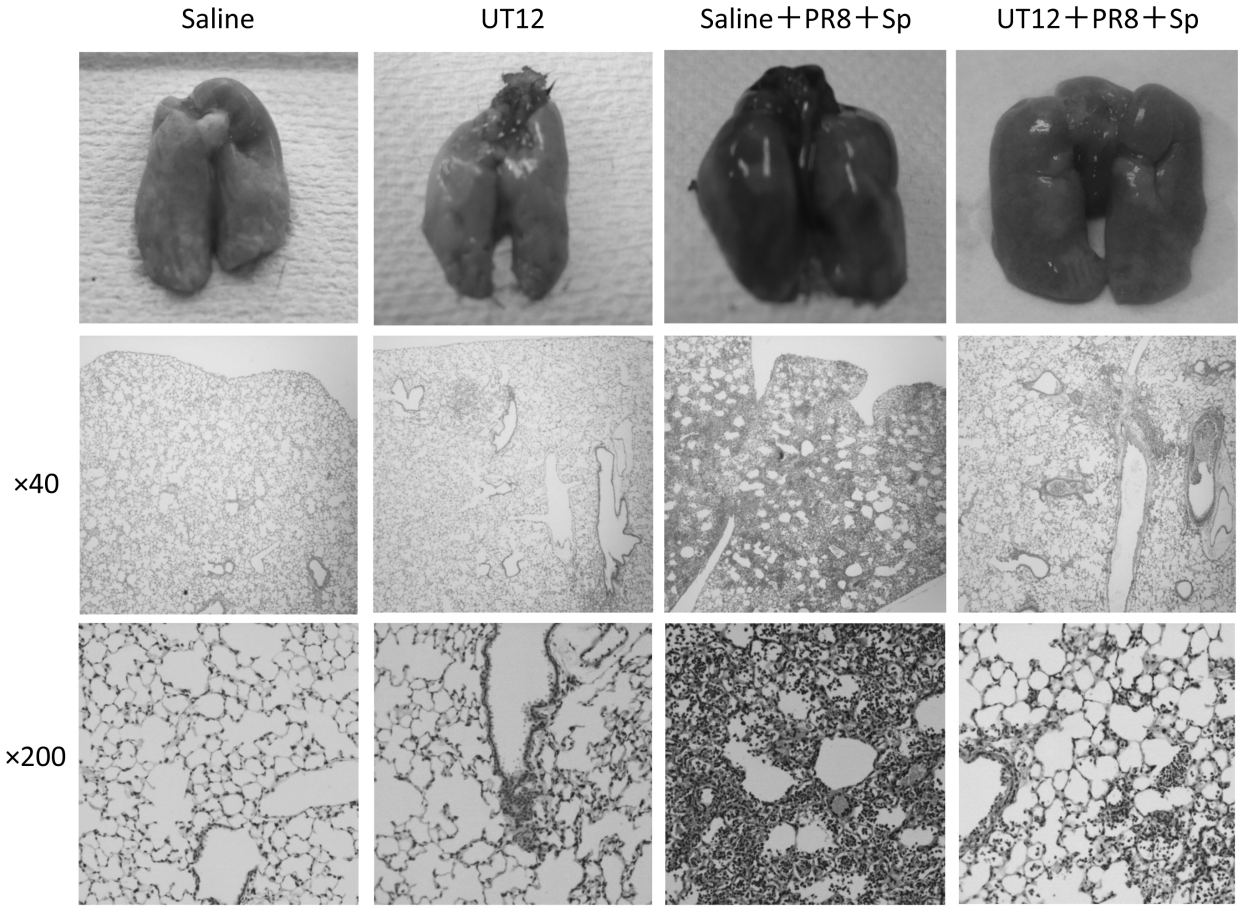


Figure 7

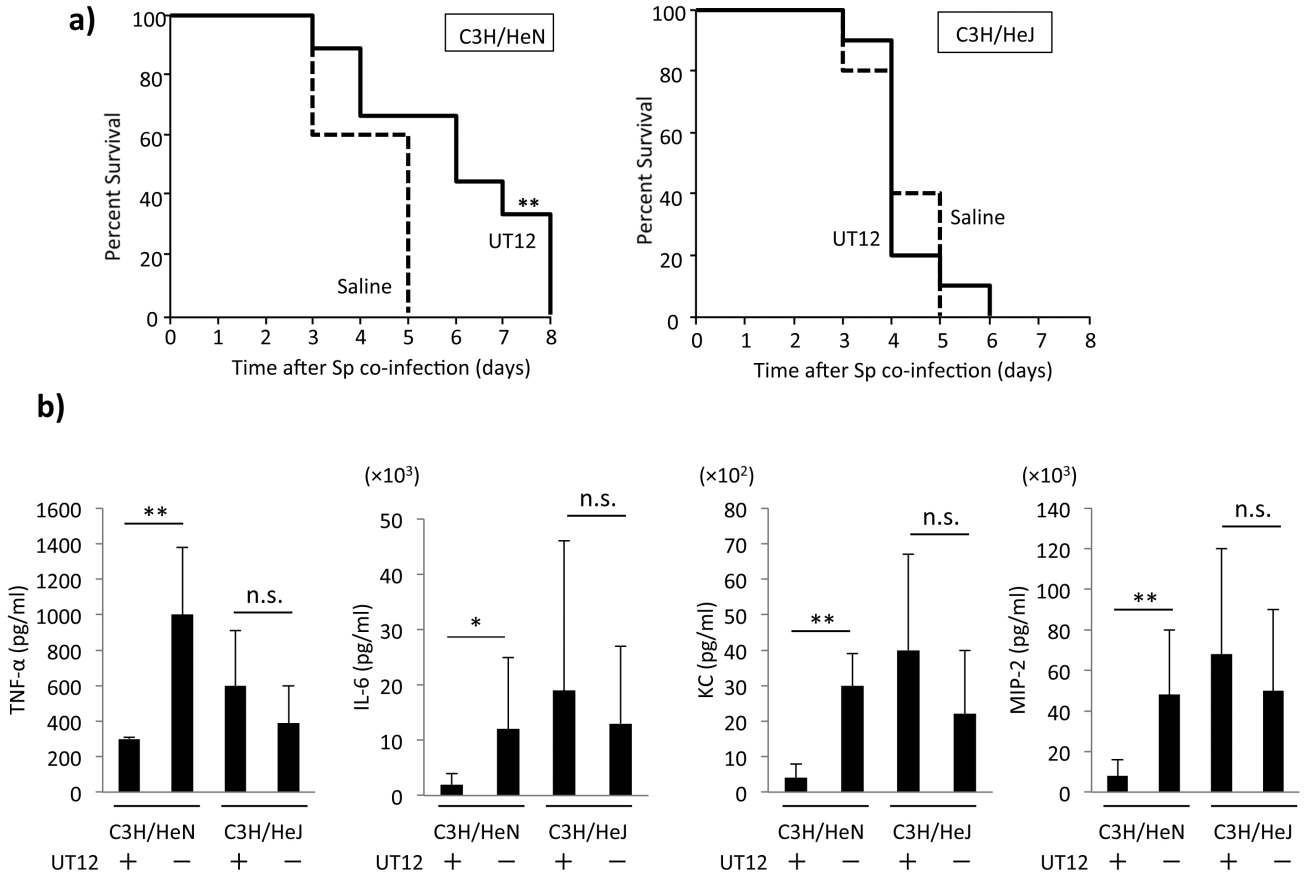

a)
C3H/HeN
Percent Survival
Time after Sp co-infection (days)
**
UT12
Saline
C3H/HeJ
UT12
Saline
b)
TNF-α (pg/ml)
IL-6 (pg/ml)
KC (pg/ml)
MIP-2 (pg/ml)
(×10³)
(×10²)
(×10³)
**
*
n.s.
C3H/HeN
C3H/HeJ
UT12

Figure 8

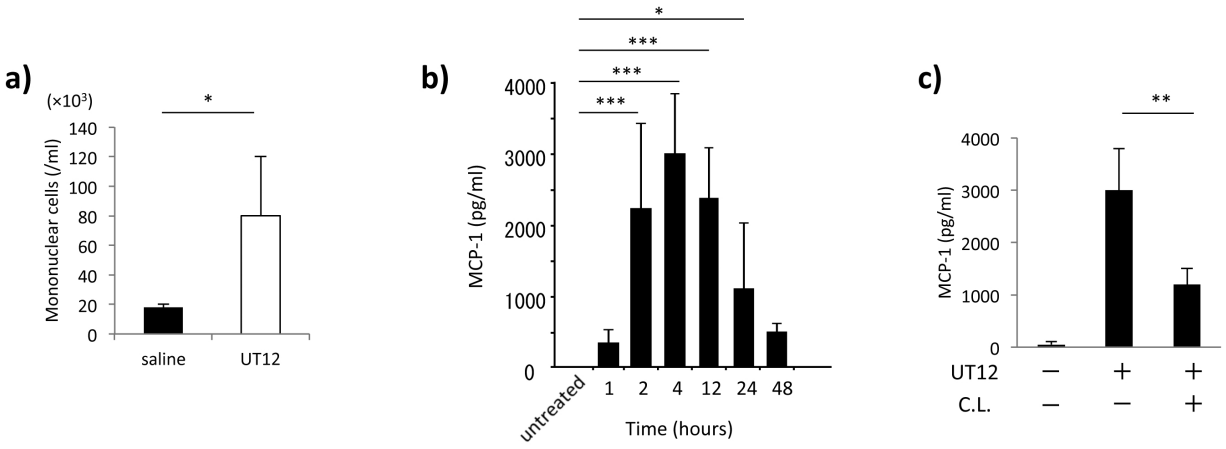

a)
(×10³)
Mononuclear cells (/ml)
140
120
100
80
60
40
20
0
*
saline
UT12
b)
MCP-1 (pg/ml)
4000
3000
2000
1000
0
*
***
***
***
untreated
1
2
4
12
24
48
Time (hours)
c)
MCP-1 (pg/ml)
4000
3000
2000
1000
0
**
UT12
C.L.
–
+
+
–
–
+

Figure 9

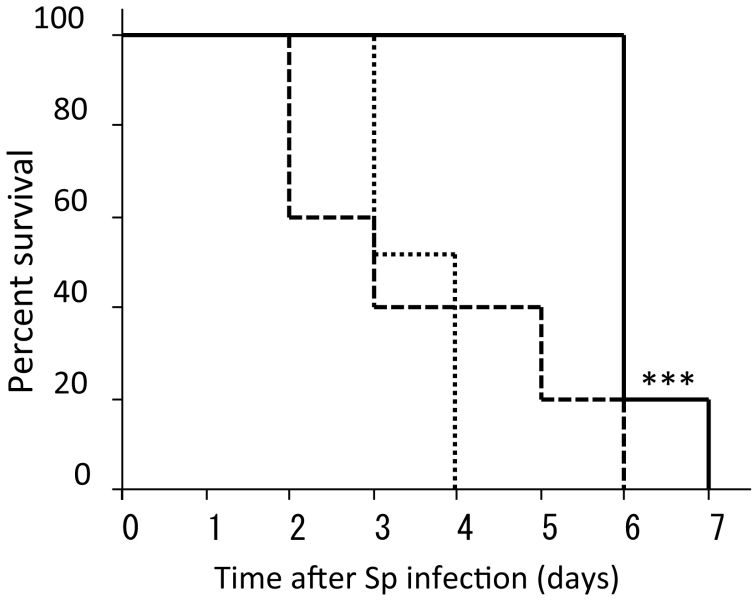
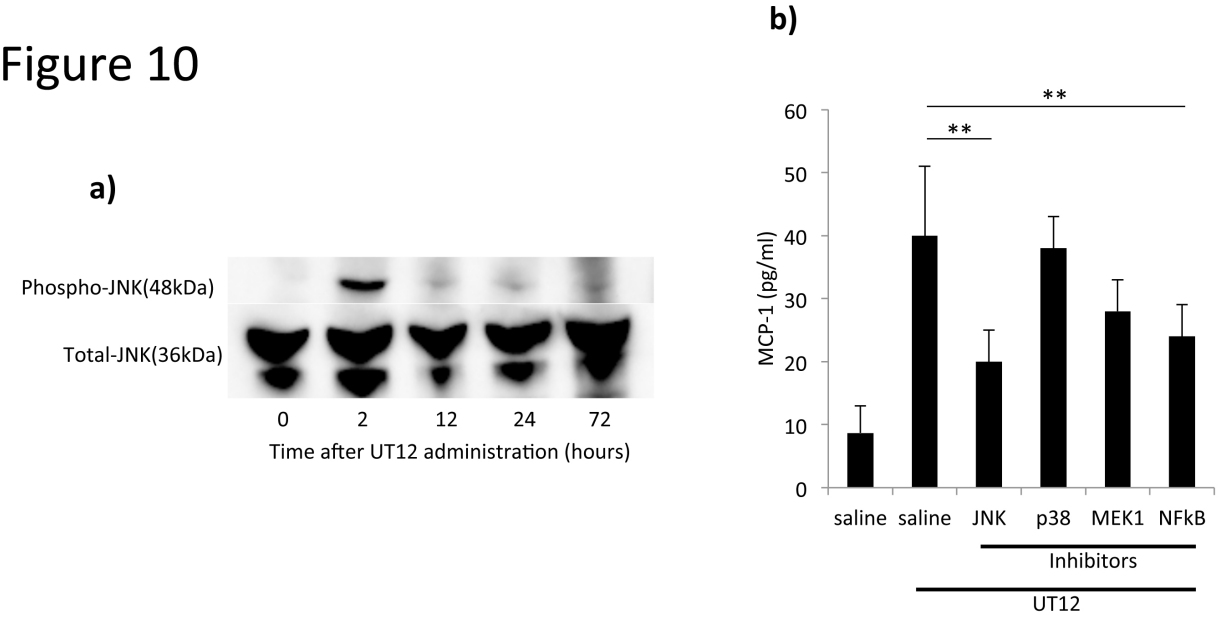

100
80
60
40
20
0
Percent survival
***
0
1
2
3
4
5
6
7
Time after Sp infection (days)

Figure 10


a)
Phospho-JNK(48kDa)
Total-JNK(36kDa)
0
2
12
24
72
Time after UT12 administration (hours)
b)
MCP-1 (pg/ml)
60
50
40
30
20
10
0
**
**
saline
saline
JNK
p38
MEK1
NFkB
Inhibitors
UT12