1 **TLR4 agonistic antibody promotes innate immunity against severe pneumonia**
2 **induced by co-infection with influenza virus and *Streptococcus pneumoniae***
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18

19 **Running title**

20 Efficacy of UT12 against secondary bacterial pneumonia

21

22

23 **Abstract**

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

41 secondary pneumococcal pneumonia and makes it an attractive candidate for treating
42 severe infectious diseases.
43
44 **Key words**
45 Secondary bacterial pneumonia, innate immunity, influenza virus, *Streptococcus*
46 *pneumoniae*, macrophage
47

48 **Introduction**

49 Acute respiratory infections account for a large proportion of deaths worldwide
50 [1]. In particular, influenza virus infection is life threatening for elderly individuals and
51 immunocompromised patients. Pneumonia is a serious complication associated with
52 influenza virus infection, and influenza-associated pneumonia can be classified into 2
53 categories: primary viral pneumonia and secondary bacterial pneumonia. While influenza
54 infection can be lethal in and of itself, a substantial number of postinfluenza deaths are
55 due to secondary bacterial pneumonias, most commonly caused by *Streptococcus*
56 *pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* [2-9]. Our previous
57 study demonstrated that cytokine storms caused by an excessive host immune response
58 are often the cause of the synergistic effect of influenza virus and *S. pneumoniae*,
59 resulting in a shorter survival period and more severe lung inflammation in co-infected
60 mice compared to mice infected with either influenza or *S. pneumoniae* alone [10].

61 Toll-like receptor (TLR), a receptor protein found on the surface of animal cells,
62 plays a critical role in the innate immune system. When microbes invade the host, TLR
63 recognizes the pathogen associated molecular patterns (PAMPs), such as
64 lipopolysaccharide (LPS), lipoprotein, flagellin of the flagellum, and double-stranded
65 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**

84 UT12 was a gift from Dr. Fukudome (Saga Medical School, Saga, Japan). Clodronate
85 liposomes were purchased from FormuMax Scientific (Palo Alto, CA, USA). All primary
86 antibodies for western blotting were purchased from Abcam (Cambridge, UK).
87 Secondary antibodies for western blotting were purchased from Santa Cruz
88 Biotechnology (Santa Cruz, CA, USA). Inhibitors of JNK (SP600125), p38 (SB203580),
89 MEK-1 (PD98059), and NF-κB (parthenolide) were obtained from Sigma-Aldrich Japan
90 (Tokyo, Japan).
91
92 **Mice**
93 CBA/JNCrlj mice (6-week-old males) were purchased from Charles River Laboratories
94 Japan (Yokohama, Japan). C3H/HeJ and C3H/HeN mice (6-week-old males) were
95 purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed
96 in accordance with the guidelines of the Laboratory Animal Center for Biomedical
97 Research, Nagasaki University School of Medicine.
98
99 **Virus and bacteria**
100 A mouse-adapted influenza virus A/Puerto Rico 8/34 (H1N1) (PR8; a gift from Dr.
101 Watanabe, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki,

102 Japan) was grown in cultured MDCK cells. After 3 days, the supernatant was collected
103 and stored at -80°C until use. The stored supernatant was thawed and diluted with
104 phosphate-buffered saline (PBS) to the desired concentration just before inoculation. *S.*
105 *pneumoniae* ATCC 49619 clinical isolate with capsular serotype 19F was prepared as
106 previously described [18]. Maintenance and storage of bacteria was performed as
107 reported previously [10]. Bacteria were grown in Mueller-Hinton II broth (Eiken
108 Chemical, Tokyo, Japan) with Strepto Haemo supplement (Eiken Chemical Tokyo,
109 Japan) at 37°C for 6 h or until reaching log phase. The concentration of bacteria in the
110 broth was determined by measuring the absorbance at 660 nm and then plotting the
111 optical density on a standard curve generated by known CFU values. The bacteria culture
112 was then diluted to the desired concentration for co-infection studies.
113
114 **Mouse co-infection studies and UT12 treatment**
115 We performed viral challenge by intranasal inoculation of 5×10^3 plaque-forming units of
116 PR8 in 50 µL PBS into mice anesthetized with pentobarbital. To induce pneumococcal
117 superinfection, we intranasally inoculated 1×10^5 CFU of pneumococcus in 50 µL of PBS
118 into anesthetized mice 2 days after PR8 inoculation. Two hours prior to each inoculation,
119 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

138 Cytospin3 centrifuge (Shandon, Pittsburgh, PA, USA) at 750 × g for 2 min and stained
139 with Diff-Quik staining for differential cell counts.
140
141 **Isolation and culture of peritoneal macrophages**
142 Three days after intraperitoneal injection of 4% sterile thioglycolate medium (2 mL),
143 peritoneal macrophages were isolated by peritoneal lavage with Hank's buffer (without
144 Ca^{2+} and Mg^{2+}) containing 0.1% gelatin. Contaminating erythrocytes, granulocytes, and
145 dead cells were removed by density gradient centrifugation for 45 min at 800 × g in
146 Mono-Poly resolving medium according to the manufacturer's protocol (MP
147 Biomedicals). Purified peritoneal macrophages were washed 3 times and cultured
148 overnight in Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS)
149 with 100 U/mL penicillin and 100 μg/mL streptomycin.
150
151 **Macrophage depletion**
152 Macrophages were depleted using clodronate liposomes as previously described [20].
153 Clodronate liposomes (100 μL/mouse) were administered i.p. 24 h prior to
154 pneumococcus inoculation. Administration of clodronate liposomes led to a more than
155 80% decline in the number of monocyte/macrophages compared with controls, as

156 assessed in cytospin preparations 24 h after administration.

157

158 **ELISA**

159 Concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6,

160 keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, and

161 MCP-1 in lung homogenates were assayed using mouse Quantikine ELISA kits (R&D

162 Systems, Minneapolis, MN, USA). Concentrations of total and phosphorylated NF-κB

163 were assayed using PathScan Sandwich ELISA kits (Cell Signaling Technology, Danvers,

164 MA, USA). These cytokine analyses were performed according to the manufacturers'

165 protocols.

166

167 **Western blotting**

168 Protein separation, transfer, blocking, and development of signals were performed as

169 described previously [10]. For detection of intact and phosphorylated (activated) forms of

170 JNK, mitogen-activated protein kinase (MAP)-extracellular signal-regulated kinase

171 (ERK) kinase 1 (MEK-1), and p38, rabbit primary antibodies against each kinase (total

172 JNK: ab7964, p-JNK: ab32447, total MEK-1: ab75608, p-MEK-1: ab5613, total p38:

173 ab7952, p-p38: ab32557, Abcam Inc.) were used. Incubation with primary antibodies was

174 followed by incubation with secondary antibodies conjugated to horseradish peroxidase

175 (sc-2030, Santa Cruz Biotechnology).

176

177 **Statistics**

178 All data were expressed as means ± SDs and analyzed by using StatView software

179 (Abacus Concepts, Cary, NC, USA). Survival curves were estimated by the

180 Kaplan-Meier method, and their homogeneity was evaluated by the log-rank test.

181 Differences between 2 groups were tested for significance using unpaired t-tests.

182 Differences between multiple groups were tested for significance using 2-way analysis of

183 variance (ANOVA). Differences with P-values of less than 0.05 were considered

184 statistically significant.

185

186 **Results**

187 **UT12 administration improved survival in co-infected mice**

188 Mice co-infected with influenza A virus followed 2 days later by pneumococcus had a

189 higher rate of mortality than mice infected with a single pathogen (Fig. 2a), as reported

190 previously [10]. To assess the effects of UT12 in co-infected mice, we compared survival

191 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

210 insult and recruit inflammatory cells that can potentially destroy tissue [21, 22]. The
211 percent survival in co-infected mice was increased by UT12 administration, and we
212 hypothesized that UT12 might protect a host from severe lung injury by preventing
213 cytokine storms through the reduction of host sensitivity against pneumococcal infection.
214 As shown in Fig. 4c, after co-infection, the levels of TNF-α, IL-6, KC, and MIP-2 were
215 significantly suppressed in UT12-treated mice as compared to control mice (TNF-α: $P <$
216 0.001, IL-6: $P < 0.001$, KC: $P < 0.01$, MIP-2: $P < 0.05$).
217 Furthermore, we assayed concentrations of NF-κB, a transcription factor that
218 plays critical roles in inflammation, in the lungs of mice co-infected with the 2 pathogens.
219 Excessive activation of NF-κB can induce a cytokine storm, resulting in septic shock [23].
220 In the current study, the levels of activated NF-κB in the lung homogenates after
221 co-infection were significantly suppressed by UT12 administration ($P < 0.05$, Fig. 5).
222 These data suggested that UT12 might be able to attenuate the expression of cytokines
223 and activation of intracellular signal transduction pathways via TLR signaling.
224 Histopathological analysis of co-infected lungs revealed marked reductions in
225 tissue injury, inflammatory cell accumulation, pulmonary hemorrhage, and edema in
226 UT12-treated mice (Fig. 6). Taken together, our data indicated that UT12 might have a
227 substantial therapeutic effect toward severe pneumococcal pneumonia induced by

228 co-infection with influenza virus through inhibition of inflammatory cell responses and
229 suppression of pro-inflammatory cytokine/chemokine production in the lungs.
230
231 **The anti-inflammatory effects mediated by UT12 were TLR4 specific**
232 Mice with intact (C3H/HeN) and nonfunctional (C3H/HeJ) TLR4 were treated with
233 UT12 prior to individual inoculation. Treatment with UT12 delayed mortality, but did not
234 impact overall survival in co-infected C3h/HeN mice as compared to those treated with
235 vehicle (Fig. 7a). However, percent survival was not significantly different between
236 C3H/HeJ mice treated with UT12 and those treated with vehicle (Fig. 7a). An analysis of
237 TNF-α, IL-6, KC, and MIP-2 concentrations in the lungs of co-infected mice showed that
238 UT12 significantly attenuated pro-inflammatory cytokine production in C3H/HeN mice
239 but not in C3H/HeJ mice (Fig. 7b). These results indicated that the anti-inflammatory
240 effects of UT12 during co-infection with influenza and pneumococcus were primarily
241 TLR4 specific, although the TLR4-dependent inflammatory response was not completely
242 abolished in C3H/HeJ mice.
243
244 **UT12 induced the migration of mononuclear cells into the lower respiratory tract**
245 **by promotion of MCP-1 production from alveolar macrophages**

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

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

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

264 pneumococcal infection (P < 0.001, Fig. 9). Improved survival mediated by UT12 was
265 not observed in macrophage-depleted mice (Fig. 9). Taken together, these data indicated
266 that existing macrophages were essential for MCP-1-dependent enhancement of
267 macrophage recruitment and phagocytosis mediated by UT12. Alveolar macrophages
268 appeared to have a crucial role in initial bacterial killing within the lower respiratory tract
269 (LRT), and UT12 augmented host innate immunity against severe pneumococcal
270 pneumonia occurring after influenza infection.
271
272 **UT12 induced MCP-1 production via an NF-κB and JNK-dependent pathway**
273 To investigate the UT12-mediated TLR4 signaling pathways involved in the production
274 of MCP-1, we examined the concentration of activated NF-κB and the expression of
275 MAPK family proteins (JNK, p38, and MEK-1) in the lungs after UT12 administration.
276 Compared with vehicle-treated mice, UT12 pretreatment significantly increased the
277 levels of activated NF-κB in uninfected mice (P < 0.001; Supplementary Fig. 1). In
278 addition, the level of phosphorylated JNK was also clearly increased at 2 h after UT12
279 administration (Fig. 10a), whereas the levels of phosphorylated p38 and MEK-1 were
280 unchanged throughout the experiment (data not shown). To confirm the importance of the
281 NF-kB and JNK-dependent pathway for UT12-mediated MCP-1 production, peritoneal

282 macrophages were pretreated with specific MAPK inhibitors, i.e., SP600125 (a specific
283 inhibitor of JNK), SB203580 (an inhibitor of p38), PD98059 (an inhibitor of MEK-1),
284 and parthenolide (an inhibitor of NF-κB), for 30 min and then cotreated with UT12 for 4
285 h prior to the detection of MCP-1 in the supernatant. Pretreatment with SP600125 or
286 parthenolide inhibited MCP-1 production, indicating that both JNK and NF-kB were
287 involved in the production of MCP-1 in UT12-stimulated macrophages (Fig. 10b). These
288 results suggested that activation of the JNK and NF-kB pathway was required for the
289 promotion of MCP-1 production in UT12-treated macrophages.
290
291 **Discussion**
292 Influenza infection predisposes the host to secondary bacterial infection of the respiratory
293 tract, which is a major cause of death in influenza-related disease, even if appropriate
294 antibiotics are administered. Vaccination is the primary tool to prevent influenza infection,
295 but its effectiveness is not 100%. Annual influenza epidemics result in an estimated 3–5
296 million cases of severe illness and 250,000–500,000 deaths every year.
297 The innate immune system recognizes and rapidly responds to microbial
298 pathogens, providing the first line of host defense. It is becoming clear that induction of
299 innate immunity may be useful for preventing bacterial infection. Indeed, Clement et al.

300 reported that the stimulation of innate immunity by bacterial lysates induces the
301 augmentation of antimicrobial polypeptides in lung lining fluid and protects the lungs
302 against lethal pneumococcal pneumonia [17]. Moreover, providing insight into the
303 specific mechanisms that regulate this response of the innate immune system, Yu et al.
304 reported that intranasal pretreatment of mice with purified P. aeruginosa flagellin induces
305 strong protection against Pseudomonas infection via TLR5 and markedly improves
306 bacterial clearance [26]. Therefore, TLR agonists are being developed as adjuvants for
307 potent new vaccines to prevent or treat infectious diseases [27]. For example,
308 monophosphoryl lipid A (MPLA), which is isolated from bacterial cell walls and
309 detoxified, acts through TLR4 as an immune stimulator. TLR4 mediates LPS
310 responsiveness and recognizes gram-negative bacteria via the LPS moiety on the surface
311 of these microorganisms. Some researchers have reported that MPL treatment promotes
312 neutrophil recruitment to the infectious source and mediates protection against both lethal
313 systemic bacterial infection and nasopharyngeal colonization [28, 29]; however, little is
314 known about whether the induction of innate immunity via TLR4 contributes to the
315 protective immune response against bacterial infection. Therefore, in the current study,
316 we used UT12, a new TLR4 agonistic monoclonal antibody, to address whether the
317 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,

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

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

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

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

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

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

390 agonistic monoclonal antibody UT12 caused resistance to severe pneumonia,
391 characterized by attenuation of systemic pro-inflammatory cytokine production and
392 improved clearance of bacteria by enhanced recruitment of macrophages to sites of
393 infection. Based on a limited case series and accumulated clinical experience, bacterial
394 pneumonia following influenza virus infection appears to be more difficult to treat and
395 has a high fatality rate. The ability of UT12 to improve survival, reduce inflammation,
396 and enhance bacterial clearance makes it an attractive agent for potential application in
397 patients that are at high risk of complications from influenza infection.
398
399
400
401
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405

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531 **Figure legends**
532 **Figure 1.** Schedule of co-infection experiments. Mice were administered i.n. influenza

533 (*PR8* strain, 5×10^3 PFU in 50 µl PBS), followed 2 days later by i.n. *S. pneumoniae* ($1X10^5$

534 CFU in 50 µl PBS). UT12 (1.0µg) was intraperitoneally administered two hours prior to

535 each inoculation. PR8: influenza virus A/Puerto Rico 8/34 (H1/N1) ; Sp: *S.*

536 *pneumoniae*.

537

538 **Figure 2.** Administration of UT12 prior to pathogen exposure improves survival in

539 co-infected mice. Influenza virus was inoculated 2 days before *S. pneumoniae* exposure

540 (Day 0). Percent survival of mice singly infected with influenza virus A/Puerto Rico 8/34

541 (H1/N1) (PR8) or *S. pneumoniae* (Sp), and co-infection of influenza virus and

542 *S.pneumoniae* with or without UT12 was examined. Kaplan-Meier curve with survival

543 rates of mice (PR8: n=10, Sp: n=8, PR8+Sp: n=10, PR8+Sp+UT12: n=10). Statistical

544 significance was determined using the log-rank test. $^{**}P < 0.01$.

545

546 **Figure 3.** Body weight change was monitored. The body weight of UT12-treated mice

547 was higher at day1, 3 and 4 significantly than saline-treated control mice. UT12-treated

548 group (solid line; n = 10), control group (dotted line; n = 10). PR8: influenza virus

549 A/Puerto Rico 8/34 (H1/N1); Sp: *S. pneumoniae*; Values represent means ± SD, $^{*}P < 0.05$,

550 $^{**}P < 0.01$.

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

569 **Figure 7.** UT12-mediated protection against pneumococcal infection is TLR4 specific. a)
570 Percent survival in co-infected C3H/HeN and C3H/HeJ mice with or without UT12
571 treatment. b) Concentration of proinflammatory cytokines and chemokines in the lung at
572 day 2 after *S. pneumoniae* co-infection. C3H/HeJ mice treated with UT12 (n = 4),
573 C3H/HeJ mice treated with saline (n = 4), C3H/HeN mice treated with UT12 (n = 5) and
574 C3H/HeN mice treated with saline (n = 4), respectively from left to right. Sp: *S.*
575 *pneumoniae*; Kaplan-Meier curve with survival rates of mice (C3H/HeN with UT12
576 treatment group: n=15, The other groups: n=10). Statistical significance was determined
577 using the log-rank test. Values represent means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, n.s.; not
578 significant.
579
580
581 **Figure 8.** The resident macrophages are required for UT12-mediated promotion of
582 recruitment of macrophages via MCP-1 dependent. a) Mononuclear cell count in BALF 4
583 h after saline or UT12 administration was examined by cytospin. b) Time course of the
584 level of MCP-1 concentration in the lung after UT12 administration. Values at each time
585 point after UT12 administration were compared with untreated mice. c) Concentration of
586 MCP-1 in the lung 4 h after UT12 administration with or without UT12 and clodronate

587 liposomes (C.L.) treatment. Values represent means ± SD, *P < 0.05, **P < 0.01, ***P <
588 0.001.
589
590 **Figure 9.** Percent survival of mice in pneumococcal pneumonia model with or without
591 clodronate liposome (C.L.) administration. Sp: *S. pneumoniae;* Kaplan-Meier curve with
592 survival rates of mice [(solid line: Saline+Sp) :n=10, (dashed line: C.L.+Sp) : n =10,
593 (dotted line: C.L.+UT12+Sp) : n=6]. Statistical significance was determined using the
594 log-rank test. The survival of Sp infected mice without both C.L. and UT12 was longer
595 than that of mice with C.L. pretreatment. ***P < 0.001.
596
597 **Figure 10.** UT12-mediated MCP-1 production is required for the phosphorylation of both
598 JNK and NF-kB. a) Activation of c-Jun N-terminal kinase (JNK) in the lung after UT12
599 administration. b) The levels of MCP-1 production 4 h after UT12 stimulation from the
600 peritoneal macrophage pretreated inhibitors of JNK, p38, MEK-1, and NF-κB. Values
601 represent means ± SD, **P < 0.01.
602
603

Figure 1

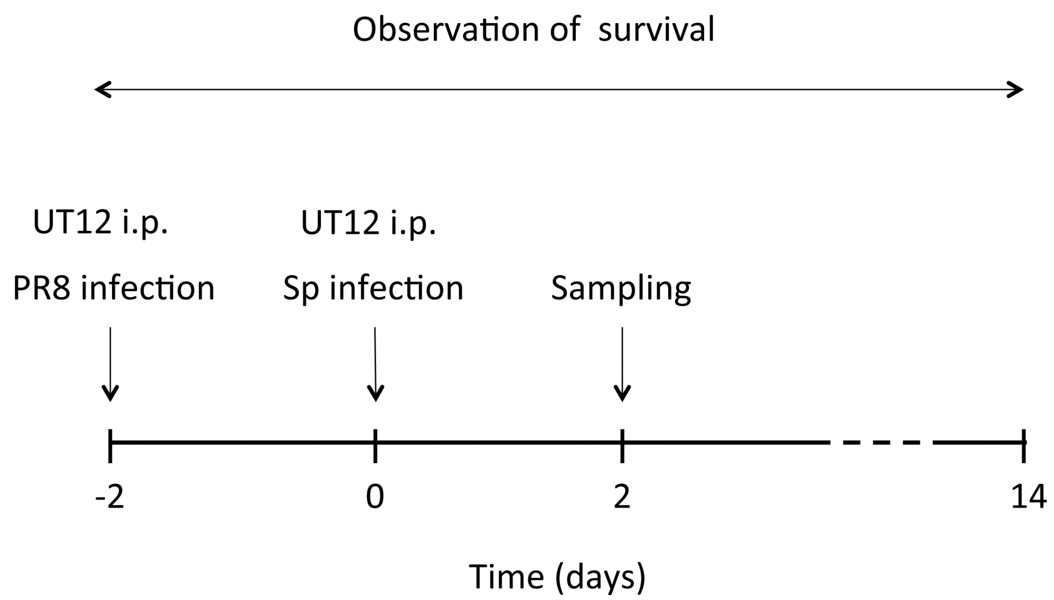
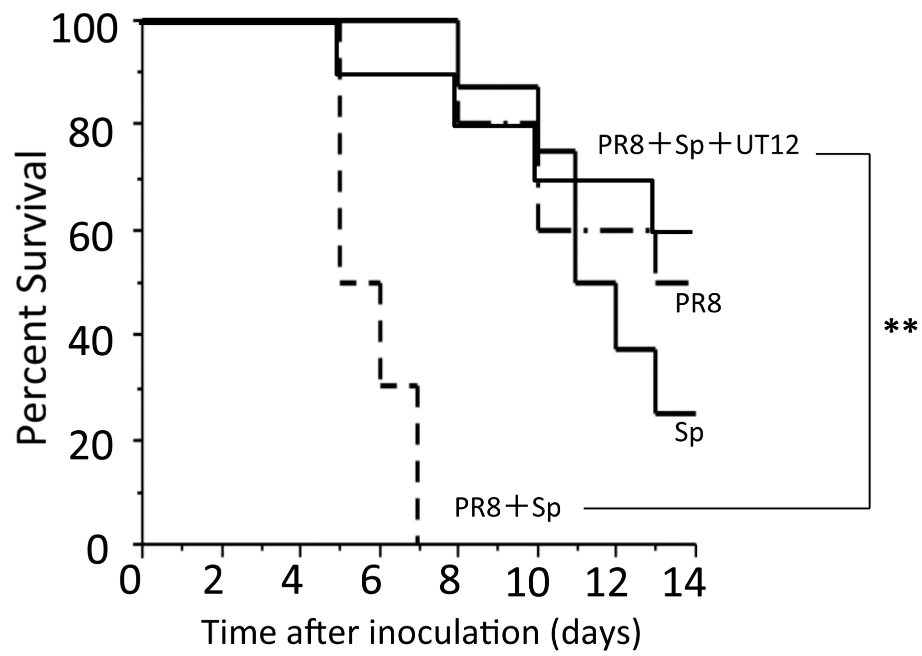


Figure 2



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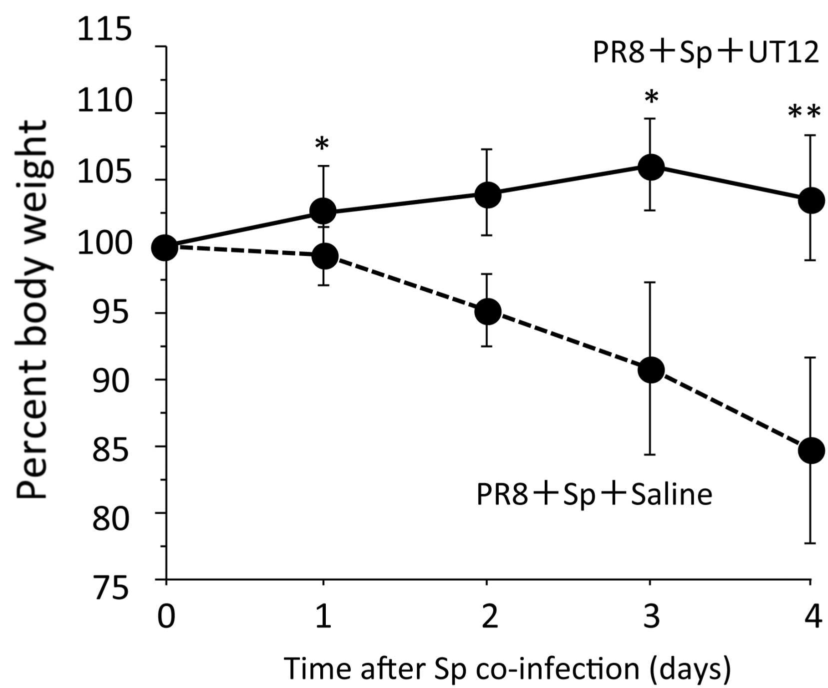

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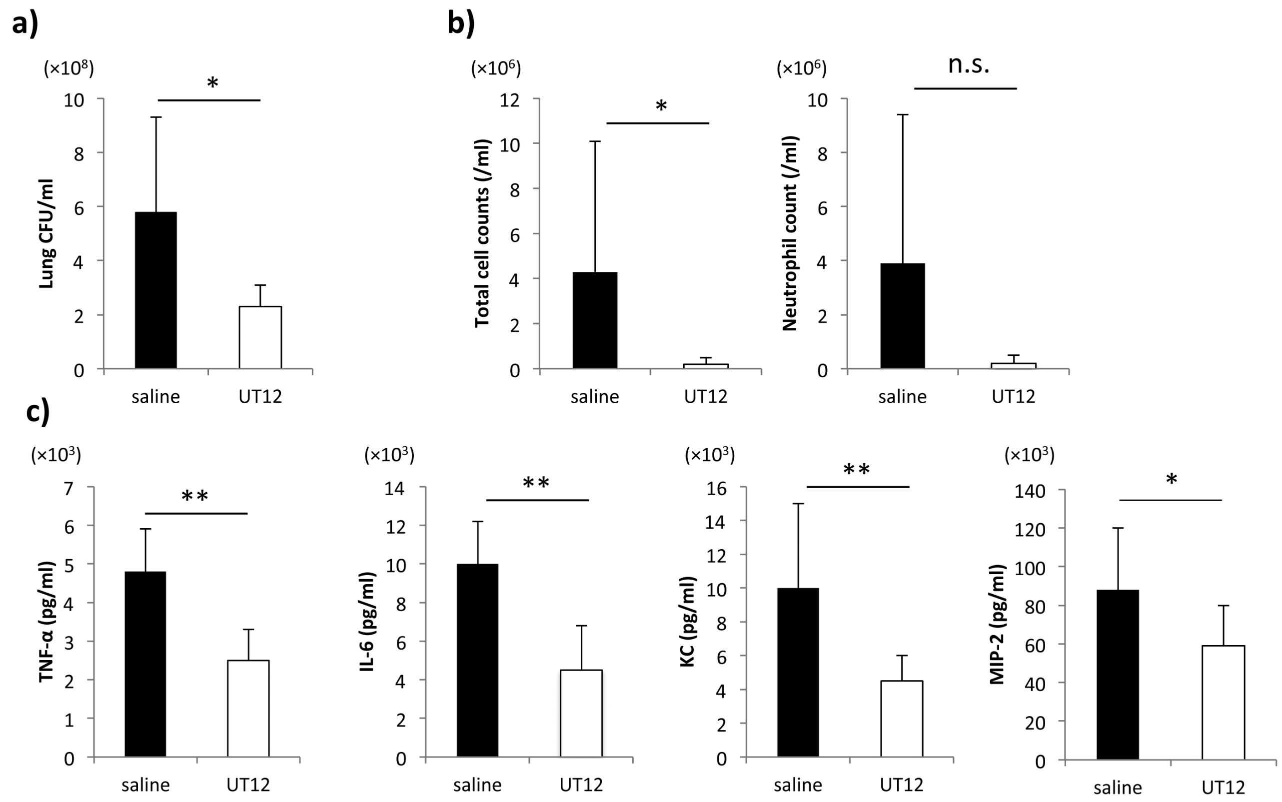

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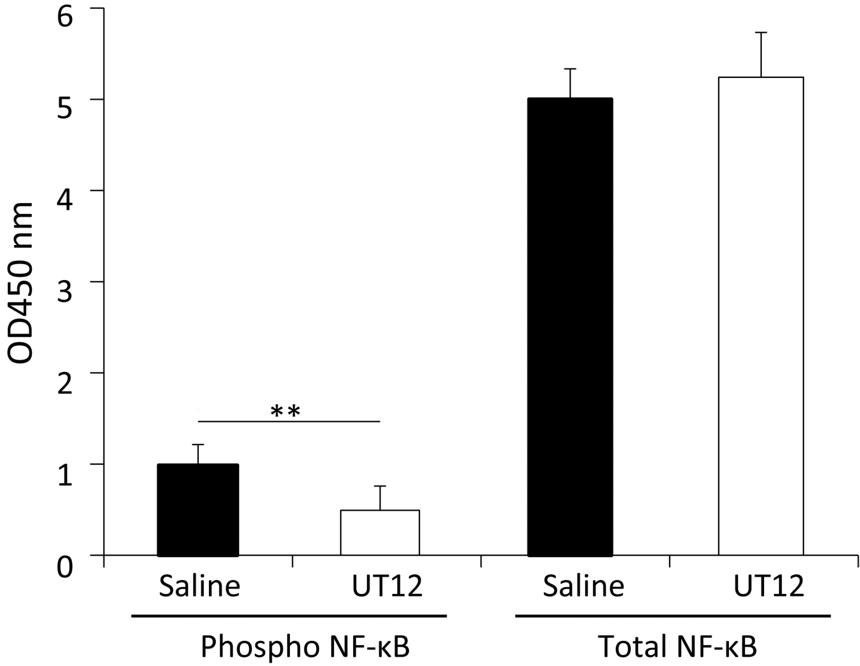

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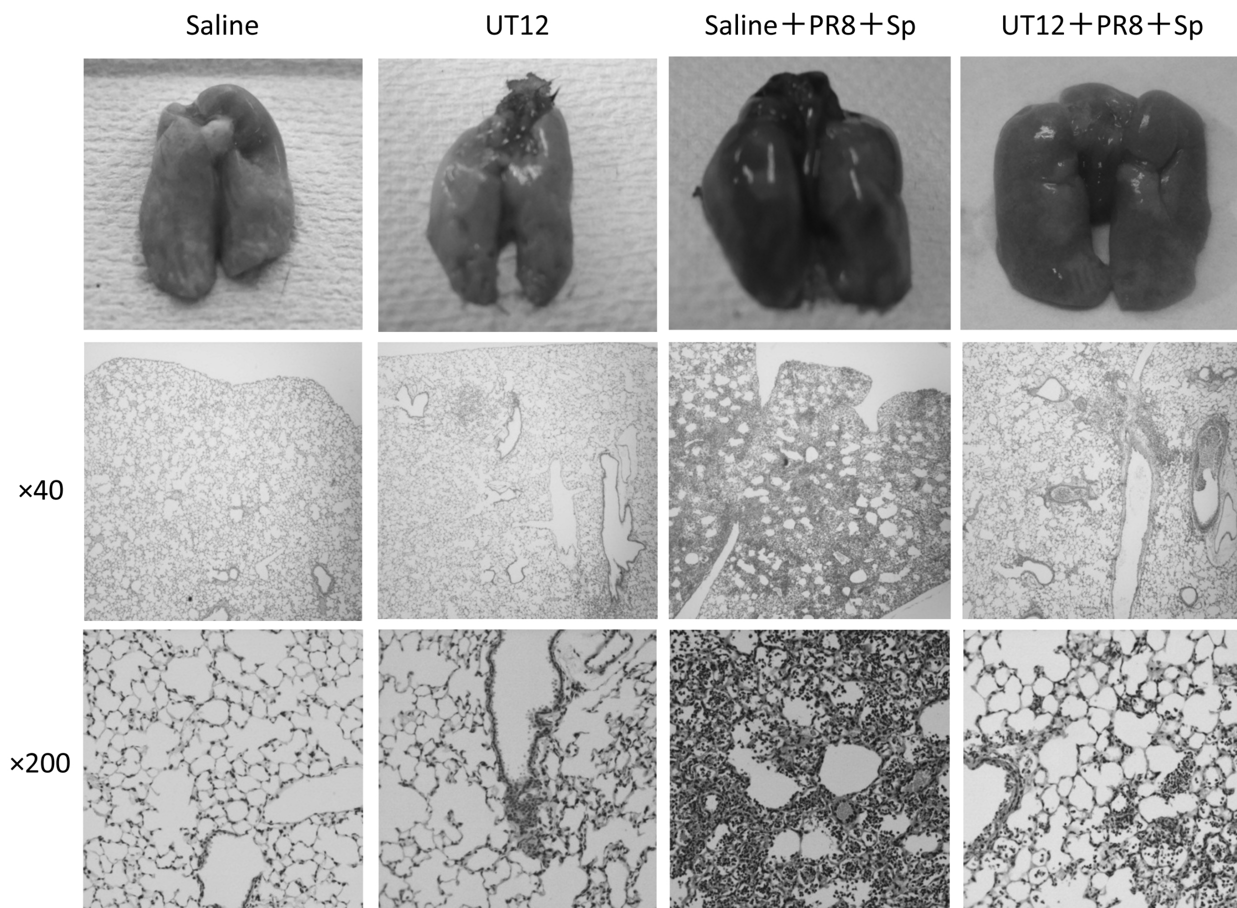
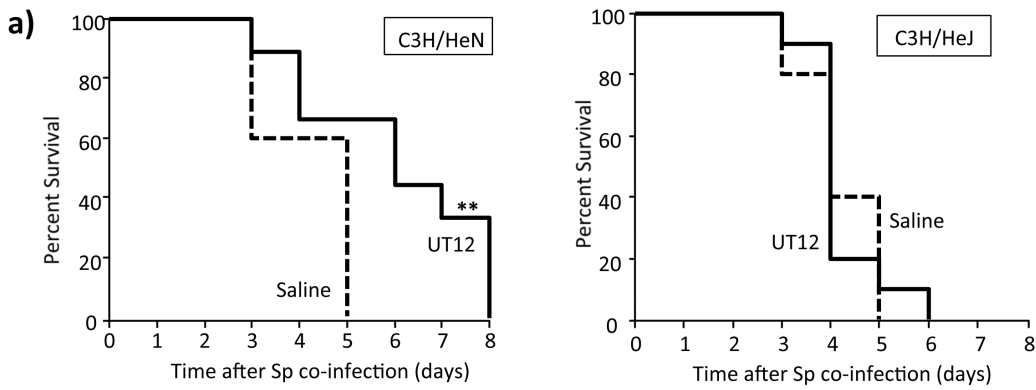


Figure 7

a)



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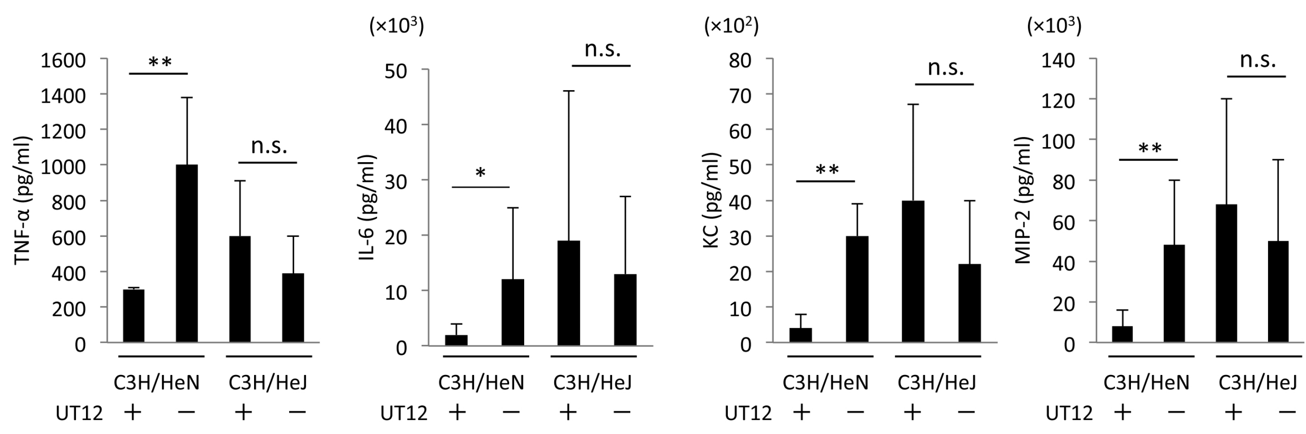

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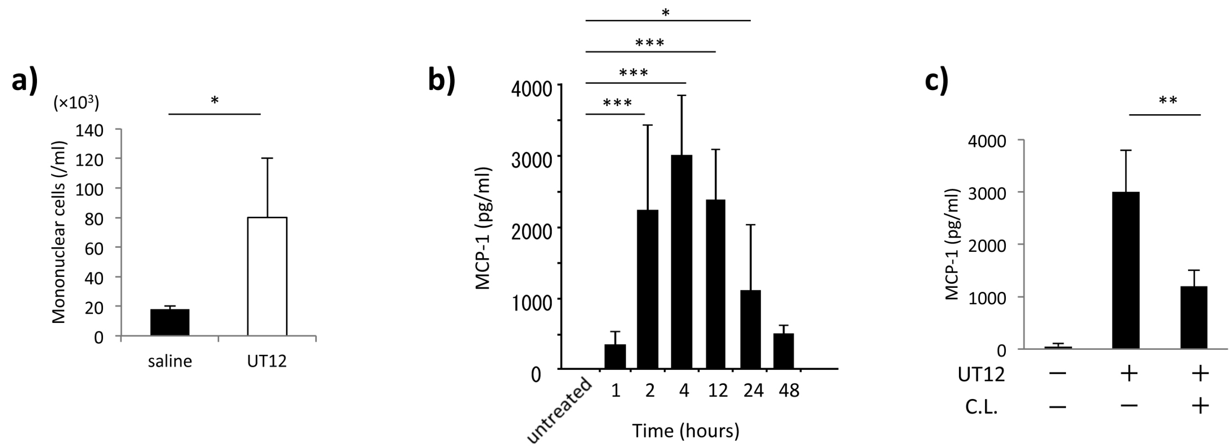

Figure 9

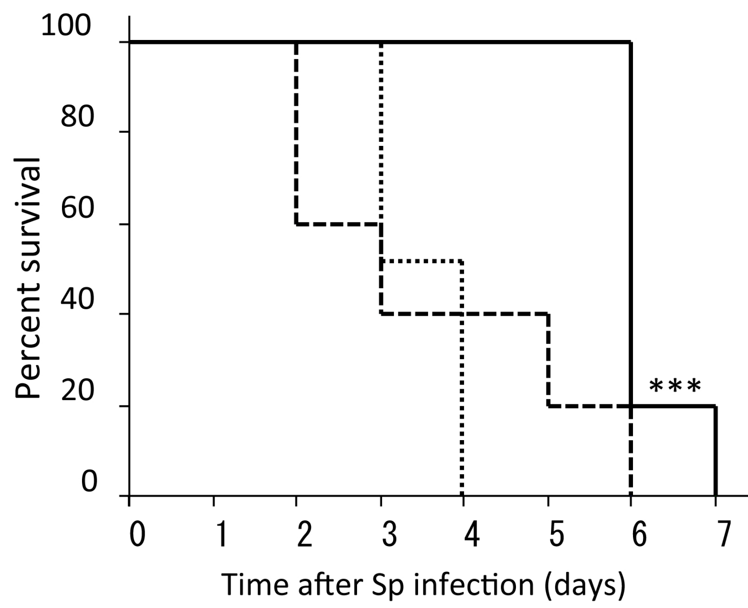

Figure 10