1 **_Prevotella intermedia_ induces severe bacteremic pneumococcal pneumonia**

2 **in mice with up-regulated platelet-activating factor receptor**

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37 **Abstract**

38 *Streptococcus pneumoniae* is the leading cause of respiratory infection.
39 Although oral hygiene has been considered a risk factor for developing
40 pneumonia, the relationship between oral bacteria and pneumococcal
41 infection is unknown. In this study, we examined the synergic effects of
42 *Prevotella intermedia*, a major periodontopathic bacterium, on pneumococcal
43 pneumonia. The synergic effects of the supernatant of *P. intermedia* (Pi Sup)
44 on pneumococcal pneumonia were investigated in mice, and the stimulation
45 of pneumococcal adhesion to human alveolar (A549) cells by Pi Sup was
46 assessed. The effects of Pi Sup on platelet-activating factor receptor (PAFR)
47 transcript levels *in vitro* and *in vivo* were analyzed by quantitative real-time
48 PCR, and the differences between the effects of pneumococcal infection
49 induced by various periodontopathic bacterial species were verified in mice.
50 Mice inoculated with *S. pneumoniae* plus Pi Sup exhibited a significantly
51 lower survival rate, higher bacterial loads in the lungs, spleen, and blood, and
52 higher inflammatory cytokine levels in the bronchoalveolar lavage fluid
53 (macrophage inflammatory protein-2 and tumor necrosis factor-alpha) than
54 those without Pi Sup. In A549 cells, Pi Sup increased pneumococcal adhesion

and PAFR transcript levels. Pi Sup also increased lung PAFR transcript levels in mice. Similar effects were not observed in the supernatants of *Porphyromonas gingivalis* or *Fusobacterium nucleatum*. Thus, *P. intermedia* has the potential to induce severe bacteremic pneumococcal pneumonia with enhanced pneumococcal adhesion to lower airway cells.

73 **Introduction**

74 *Streptococcus pneumoniae* is the leading cause of community-acquired
75 respiratory infections worldwide (1). There are several known risk factors for
76 pneumococcal disease, but limited descriptive data concerning the
77 relationship between oral hygiene and pneumococcal infection.

78 Poor oral hygiene has been suggested to be a risk factor for respiratory disease
79 (2), and several studies indicate that oral care reduces the incidence and
80 mortality of pneumonia in hospitals or nursing homes (3–5). Regarding the
81 relationship between *S. pneumoniae* and oral hygiene, Okuda et al. reported
82 that oral cleansing significantly reduced the detection rates of *S. pneumoniae*
83 in patients that have undergone oral and maxillofacial surgeries (6).

84 Several oral anaerobes, mostly related to periodontitis, are known to interact
85 in a synergistic or antagonistic manner (7,8). To understand the interactions
86 between microorganisms, the enhancement of reciprocal bacterial growth,
87 adhesion/invasion into host cells, and effects on host immunity response have
88 been examined (7–11). Regarding the synergic effects of anaerobes on the
89 pulmonary infection of *Streptococcus* species, Shinzato et al. reported that
90 *Prevotella intermedia* exhibits synergic effects on lower respiratory tract

91 infections of *Streptococcus constellatus* in mice by enhancing reciprocal
92 bacterial growth (9). However, whether oral bacteria exhibit synergic effects
93 on pneumococcal infections remains unclear.
94 Here, we hypothesized that an anaerobe that is ubiquitous in the oral cavity
95 may have synergic effects on pneumococcal respiratory infection. To
96 investigate our hypothesis, we focused on the anaerobe *P. intermedia*.
97 *P. intermedia* is a gram-negative, black-pigmented obligate anaerobic rod,
98 which is often isolated from periodontal lesions associated with various forms
99 of periodontal disease (12,13). In addition, *P. intermedia* has recently been
100 detected in cystic fibrosis airway specimens (14–16). Ulrich et al. reported the
101 pathogenic potential of *P. intermedia* in the respiratory tract and
102 demonstrated that extracellular toxins of *P. intermedia* are cytotoxic for
103 human alveolar type Ⅱ cells and neutrophils (17).
104 In this study, we examined the effects of *P. intermedia* on pneumococcal
105 pneumonia in a murine model. The aims of this study were to determine
106 whether *P. intermedia* exhibits synergic effects on pneumococcal pneumonia
107 and to examine its mechanism of interactions.
108

109 **Materials and Methods**

110 **Bacterial strains and culture conditions.** The *Streptococcus pneumoniae*
111 strain NU83127 (MIC of penicillin G, 0.03 μg/mL; serotype 4), which was
112 clinically isolated at Nagasaki University School of Medicine, was used in the
113 present study. The obligate anaerobes examined are listed in Table 1. All
114 obligate anaerobes were cultured on PV Brucella HK Agar (Kyokuto
115 Pharmaceutical Industrial Co., Tokyo, Japan) for 48−96 h under anaerobic
116 conditions and then scraped and suspended in modified GAM broth (Nissui
117 Pharmaceutical Industrial Co., Tokyo, Japan). To prepare a bacterial
118 suspension, *P. intermedia* was incubated with modified GAM broth in an
119 anaerobic chamber until it reached its late logarithmic growth phase (24 h).
120 Bacteria were then harvested by centrifugation (3000 rpm, 10 min) and
121 resuspended in normal saline.

122 The supernatants of *P. intermedia* and the other anaerobes were obtained as
123 previously reported (18, 19). Briefly, the anaerobes were incubated using
124 modified GAM broth for 48 h in an anaerobic chamber. The supernatants were
125 then collected by centrifugation at 10,000 rpm at 4 °C for 50 min to remove
126 the bacteria and filter-sterilized through a 0.22-μm pore membrane filter

127 (Millipore, Bedford. MA, USA).

128 We conducted all experiments using the PINU499 strain, with the exception
129 of the experiments performed to verify the differences between the effects of
130 periodontopathic bacterial species and strains on pneumococcal infection. We
131 also identified clinical strains at our institution by PCR amplification and 16S
132 rRNA sequence analysis.

133

134 **Mice.** Eight-week-old male BALB/c specific-pathogen-free mice were obtained
135 from SLC Japan Inc., Shizuoka, Japan. All mouse experiments were
136 performed according to the guidelines of the Laboratory Animal Center for
137 Biomedical Research, Nagasaki University School of Medicine. The
138 experimental protocol was approved by the Animal Care Ethics Review
139 Committee at our institution.

140

141 **Intratracheal infection procedure.** The *S. pneumoniae* strain was cultured on
142 blood agar plates (Becton Dickinson Co., Ltd., Japan) for 24 h at 37 °C,
143 scraped and suspended in brain heart infusion broth mixed with horse serum,
144 and cultured with shaking at 37 °C at 250 rpm for 4 h. Bacteria were then

145 harvested by centrifugation (3000 rpm, 10 min). The organism was
146 resuspended in normal saline for a final concentration of approximately 10^8
147 colony forming units (CFU)/mL, as determined by the optical density method.
148 Mice were anaesthetized with pentobarbital, and the trachea was inoculated
149 with 0.05 mL of the bacterial suspension via insertion with a 24-gauge
150 catheter. For mixed-infection experiments with *S. pneumoniae* (Sp) and *P.*
151 *intermedia*, the bacterial suspension of Sp was mixed with the same amount
152 of bacterial suspension of *P. intermedia* or modified GAM broth before
153 inoculating mice. The final bacterial load of Sp was approximately 2×10^6–2
154 $\times 10^7$ CFU/mL (1×10^5–1×10^6 CFU/mouse), and the final bacterial load of *P.*
155 *intermedia* was approximately 2×10^8–2×10^9 CFU/mL (1×10^7–1×10^8
156 CFU/mouse).

157 In experiments that examined the effects of culture supernatants of *P.*
158 *intermedia* and the other periodontopathic bacteria on pneumococcal
159 pneumonia, a bacterial suspension of Sp was mixed with the same amount of
160 culture supernatant of anaerobes or modified GAM broth before inoculating
161 mice. The final bacterial load of Sp was approximately 5×10^7 CFU/mL ($2.5 \times$
162 10^6 CFU/mouse). The control group was inoculated with an equal volume of

163 broth and normal saline. For the group inoculated with the supernatant of *P.*

164 *intermedia* (Pi Sup) without Sp, equal volumes of Pi Sup and normal saline

165 were used. The pH of modified GAM broth was adjusted to that of the

166 anaerobe's supernatant (pH 5.6 for the Pi Sup and pH 6.8 for the supernatant

167 of *Fusobacterium nucleatum* or *Porphyromonas gingivalis*).

168

169 **Bacteriological and histopathological examinations.** Each group of animals

170 was sacrificed at specific time intervals by cervical dislocation. After

171 exsanguination, the lungs and spleen were dissected and removed under

172 aseptic conditions. Blood was collected by right ventricular puncturing using

173 heparin-coated syringes. For bacteriological analyses, the organs were

174 suspended in normal saline (1 mL) and homogenized with a Polytron

175 homogenizer (AS One Co., Osaka, Japan). Each specimen (blood, lung, and

176 spleen) was quantitatively inoculated onto blood agar plates by serial dilution,

177 followed by incubation at 37 °C for 24 h. The lowest level of detectable

178 CFU/mL was 50 CFU/mL (1.7 log CFU/mL). The lung tissue used for

179 histological examination was fixed in 10% buffered formalin and stained with

180 hematoxylin-eosin.

181

182 **Bronchoalveolar lavage (BAL) and cytokine enzyme-linked immunosorbent**

183 **assays (ELISA).** BAL was performed as previously described (20). The

184 recovered fluid fractions were pooled for each animal, and the total cell counts

185 were calculated using Turk staining. For differential cell counts, cells were

186 centrifuged at 850 rpm for 2 min onto slides that were then stained with Diff–

187 Quick stain. Differential cell counts were performed by counting 100 cells.

188 Various concentrations of macrophage inflammatory protein (MIP)-2 and

189 tumor necrosis factor-alpha (TNF-α) in BAL fluid (BALF) were assayed using

190 mouse cytokine ELISA test kits (R&D Systems, Minneapolis, MN) according

191 to the manufacturer's instructions.

192

193 **Cell culture.** The NCI-A549 (Human type II pneumocyte cell line) was

194 cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum,

195 100 U of penicillin/mL, and 100 μg of streptomycin/mL. The cells were grown

196 at 37 °C with 5% CO_2 in fully humidified air. Cells were exposed to Pi Sup for

197 pneumococcal adhesion studies. For controls, cells were incubated with

198 modified GAM broth, and the pH was adjusted to that of Pi Sup.

199

200 **Pneumococcal adhesion to airway cells exposed to Pi Sup *in vitro*.** The
201 adhesion of pneumococci to airway cells *in vitro* was performed as previously
202 described (21). Briefly, A549 cells were seeded in 24-well plates. Pi Sup was
203 added to cell monolayers, incubated at 37 °C for 4 h, and subsequently
204 removed by washing twice with RPMI medium. Pneumococci was then added
205 and incubated for 2 h. Cell monolayers were washed five times, and cells were
206 removed from the tissue culture plate with trypsin-EDTA and lysed with ice-
207 cold sterile distilled water for 10 min. The lysates were then plated to
208 determine the CFU/mL.

209 The functional relevance of platelet-activating factor receptor (PAFR) was
210 also assessed by co-incubating cells with the competitive PAFR antagonist
211 CV-3988 (Sigma Aldrich). A stock solution of CV-3988 was prepared in ethanol
212 and then diluted in medium to a final concentration of 10 μM. The adhesion
213 data are representative of at least three separate experiments performed on
214 different days.

215

216 **PAFR transcript levels in airway cells exposed to Pi Sup *in vitro*.** Transcript

217 levels of PAFR were assessed in A549 cells using quantitative real-time PCR.

218 The total RNA was extracted from A549 cells cultured in 6-well plates using

219 QuickGene-Mini80 and QuickGene RNA cultured cell kits (FUJIFILM Co.,

220 Tokyo, Japan) according to the manufacturer's instructions. The total RNA (1

221 μg) was reverse transcribed into cDNA using oligo(dT) primers and

222 SuperScript III reverse transcriptase (Invitrogen) and then treated with

223 RNaseH. To quantify the expression of the PAFR gene, PCR primers and

224 Taqman probes were used as previously reported (Hs00265399_S1) (21). To

225 normalize PAFR expression, the housekeeping gene hypoxanthine

226 phosphoribosyltransferase 1 (HPRT1) was also measured using the primer

227 set (Hs01003267_m1) according to the manufacturer's instructions (Life

228 technologies). The data are presented as a ratio of HPRT1.

229

230 **Lung PAFR transcript levels in mice exposed to Pi Sup *in vivo*.** Lung PAFR

231 transcript levels were examined in Pi Sup-inoculated mice and Sp-infected

232 mice with/without Pi Sup. Each group of animals was sacrificed at specific

233 time intervals and a partial lung was preserved in RNA later (Life

234 technologies). The tissue samples were homogenized, and RNA was extracted

235 using the RNeasy Mini kit (QIAGEN) according to the manufacturer's
236 instructions. First strand cDNA synthesis was performed as described above.
237 mRNA transcript levels of PAFR and the housekeeping gene HPRT1 were
238 determined by quantitative real-time PCR using the TaqMan primer and
239 probe sets Mm02621061_m1 and Mm00446968_m1, respectively. Mouse
240 PAFR mRNA transcript levels were normalized to the housekeeping gene
241 HPRT1 (22).

242

243 **Statistical analysis.** All data were expressed as the mean and standard error
244 of the mean (SEM). Differences between groups were evaluated using the
245 Mann-Whitney U test. Survival analysis was performed using the log rank
246 test, and the survival rates were calculated by the Kaplan-Meier method. *P*
247 values less than 0.05 were considered to be statistically significant.

248

249 **Results**

250 **Mixed infection of *S. pneumoniae* and *P. intermedia*.** There were no
251 significant differences observed between the survival rates of mixed-infection
252 experiments of Sp with/without the bacterial suspension of *P. intermedia*

253 (data not shown). In preliminary experiments, in which BALB/c mice were
254 inoculated with only *P. intermedia* via the trachea, changes in inflammation
255 and the proliferation of *P. intermedia* in the lungs were not observed. Based
256 on these results, the synergic effects of *P. intermedia* on pneumococcal
257 pneumonia were difficult to assess in the mixed-infection experiments
258 because the virulence of only *P. intermedia* was less significant. Therefore, we
259 did not conduct additional experiments using bacterial suspensions of *P.*
260 *intermedia*.
261
262 **Pneumococcal infection with *P. intermedia* supernatant caused severe**
263 **bacteremic pneumonia.** Figure 1A illustrates the survival rates of Sp-infected
264 mice with/without Pi Sup. In the controls (broth- or Pi Sup-inoculated mice),
265 no deaths were observed during the 10-day observation period. In contrast,
266 90% of Sp-infected mice without Pi Sup died 3 days after inoculation, and all
267 Sp-infected mice with Pi Sup died within 3 days. The survival rates of Sp-
268 infected mice with Pi Sup were significantly shorter than those of Sp-infected
269 mice without Pi Sup ($p < 0.01$). The change in the number of viable Sp in the
270 lungs, blood, and spleen over time following infection is shown in Figure 1B–

271 D. The mean bacterial count in each organ/blood of Sp-infected mice with Pi
272 Sup began to increase 24 h after inoculation ($p < 0.005$, Sp with Pi Sup vs Sp
273 without Pi Sup), with the exception of the spleen, in which the increase was
274 observed starting as early as 6 h after inoculation ($p < 0.05$). Because these
275 results indicate that Pi Sup induces early exacerbation of Sp-infection in mice
276 within 6–48 h, we examined the pathological changes in the lungs 24 h after
277 inoculation (Fig. 2). Pathological examination of the lungs of Sp-infected mice
278 with Pi Sup showed severe bronchopneumonia with massive hemorrhaging
279 (Fig. 2d). Pi Sup-inoculated mice also exhibited mild hemorrhaging (Fig. 2b),
280 whereas the lungs of Sp-infected mice without Pi Sup only exhibited mild
281 pneumonia 24 h after inoculation (Fig. 2c). Broth-inoculated (control) mice
282 did not exhibit any inflammatory changes in the lungs.
283 In order to examine peak inflammatory changes in the lungs of Sp-infected
284 mice with Pi Sup, we performed BAL 36 h after inoculation. The total cell and
285 neutrophil counts (Table 2) were significantly higher in Sp-infected mice with
286 Pi Sup and Pi Sup-inoculated mice than those of Sp-infected mice without Pi
287 Sup. To further examine the differences, inflammatory cytokine levels in
288 BALF were analyzed. TNF-α and MIP-2 concentrations were significantly

289 higher in Sp-infected mice with Pi Sup than those of the other group (Fig. 3).

290 TNF-α levels also increased slightly in Pi Sup-inoculated groups and were

291 still significantly higher than those of Sp-infected mice without Pi Sup. To

292 confirm the inflammatory effects of Pi Sup, we also performed BAL 12 h and

293 24 h after Pi Sup-inoculation. BALF of Pi Sup-inoculated mice demonstrated

294 that the total cell and neutrophil counts increased 12 h after inoculation, and

295 the concentrations of MIP-2 and TNF-α also increased after inoculation.

296 However, the peak concentrations of TNF-α and MIP-2 in Pi Sup-inoculated

297 mice were 183.0 ± 30.3 ng/mL (12 h) and 58.4 ± 39.4 ng/mL (24 h),

298 respectively (data not shown), which were lower than those of Sp-infected

299 mice with Pi Sup.

300

301 **Culture supernatant of *P. intermedia* stimulated PAFR *in vitro* and *in vivo.***

302 To further understand the effects of Pi Sup on pneumococcal pneumonia, we

303 hypothesized that Pi Sup possesses a stimulatory effect on pneumococcal

304 adhesion to lower airway cells, contributing to rapid bacterial proliferation

305 and invasion. Regarding pneumococcal adhesion, there is increasing evidence

306 that PAFR is a major epithelial receptor used by *S. pneumoniae* to invade

307 airway epithelium cells (23). Up-regulation of PAFR transcripts *in vivo* has
308 been described in several animal models as a result of interleukin 1
309 stimulation (24), influenza infection (25), and exposure to cigarette smoke
310 (21). However, the relationship between periodontopathic bacteria and PAFR
311 transcript levels has not been described previously. Thus, we sought to
312 examine the effects of Pi Sup on pneumococcal adhesion and PAFR expression.
313 Pi Sup increased pneumococcal adhesion to A549 cells ($p < 0.05$ vs control;
314 Fig. 4A). CV-3988 decreased pneumococcal adhesion stimulated by Pi Sup (p
315 < 0.05, Pi Sup + antagonist vs Pi Sup + Ethanol; Fig. 4B), and PAFR mRNA
316 levels increased in Pi Sup-stimulated cells ($p < 0.005$ vs control; Fig. 4C).
317 In mice, Pi Sup increased lung PAFR transcript levels 6–24 h after inoculation
318 (Fig. 5A). To examine the differences between the PAFR transcript levels of
319 Sp-infected mice with/without Pi Sup, we collected the lungs of mice 24 h after
320 inoculation. The highest increase in PAFR transcript levels was observed in
321 the lungs of Sp-infected mice with Pi Sup ($p < 0.005$ vs Sp without Pi Sup; p
322 < 0.05 vs Pi Sup). The Pi Sup-inoculated group exhibited higher PAFR
323 transcript levels than Sp-infected mice without Pi Sup ($p < 0.005$).

324

325 ***In vivo* effects of culture supernatant of periodontal bacteria on pneumococcal**

326 **pneumonia.** To estimate the effects of periodontopathic bacteria on

327 pneumococcal infection, we examined the survival rates of Sp-infected mice

328 inoculated with the supernatants of *Prevotella intermedia* (Fig. 6A),

329 *Fusobacterium nucleatum* (Fn; Fig. 6B), and *Porphyromonas gingivalis* (Pg;

330 Fig. 6C). Each group was composed of three different strains, including a

331 reference strain. The survival rates of Sp-infected mice with the supernatant

332 of PINU499 were significantly lower than that of Sp-infected mice without Pi

333 Sup ($P < 0.01$). The survival rates of Sp-infected mice with Pg Sup were

334 significantly higher than those of Sp-infected mice without Pg Sup ($P < 0.05$),

335 whereas there was no significant difference between the survival rates of Sp-

336 infected mice with/without Fn Sup.

337

338 **Discussion**

339 The present study is the first to demonstrate that the products of *P.*

340 *intermedia* induce severe bacteremic pneumococcal pneumonia as well as the

341 enhancement of pneumococcal adhesion to lower airway cells. Several lines of

342 evidence support this notion.

343 First, Sp-infection with Pi Sup exhibited significant lower survival rates with
344 earlier increases in Sp bacterial load in the lungs, spleen, and blood compared
345 to those of Sp-infected mice without Pi Sup. Significant increases in
346 inflammatory cytokines were observed in the early phases of Sp-infected mice
347 with Pi Sup, indicating the severity of bacteremia compared to that of Sp-
348 infected mice without Pi Sup. Although belated bacteremia was observed in
349 Sp-infected mice without Pi Sup, a high bacterial load in the lungs was only
350 observed in Sp-infected mice with Pi Sup. These data suggest that Pi Sup
351 enhances Sp invasion into blood circulation as well as Sp adhesion and
352 proliferation in the lungs.

353 Second, Pi Sup enhanced pneumococcal adhesion to lower airway cells *in vitro*.
354 We also observed the up-regulation of PAFR expression in airway cells upon
355 Pi Sup stimulation and attenuation of pneumococcal adhesion by CV-3988,
356 suggesting that Pi Sup enhances pneumococcal adhesion via PAFR up-
357 regulation.

358 Third, we also observed PAFR up-regulation by Pi Sup *in vivo*. Higher levels
359 of PAFR up-regulation were observed in Sp-infected mice with Pi Sup
360 compared to those of Pi Sup-inoculated mice, suggesting that Pi Sup may

361 possess synergic effects on PAFR up-regulation with pneumococcal infection.
362 PAFR is a major epithelial receptor that binds to phosphorylcholine in the
363 bacterial cell wall. Thus, the effects of Pi Sup on PAFR expression could be
364 synergic not only for *S. pneumoniae* infection but for other bacteria containing
365 phosphorylcholine, including *Pseudomonas aeruginosa* (26) and
366 *Acinetobacter baumanii* (27).

367 For the reason that *P. intermedia* itself does not exhibit significant
368 inflammatory or synergic effects on pneumococcal pneumonia in mice, we
369 consider the instability of *P. intermedia* in lungs. Because of the aerobic
370 environment in the lungs, *P. intermedia* may not be stable in the lungs,
371 preventing proliferation and the secretion of virulent products.

372 The main goal of our study was to determine the extent by which PAFR
373 expression affects the susceptibility of *S. pneumoniae* in mice administered
374 Pi Sup, and the data obtained were inconclusive. We treated Sp-infected mice
375 with Pi Sup with CV3988 (PAFR antagonist) but could not determine any
376 significant improvement in survival or attenuation of pneumococcal bacterial
377 load in the lungs or blood (data not shown). However, Pi Sup-induced PAFR
378 up-regulation in our murine model was consistent up to at least 24 h after

379 inoculation. As were able to administer CV3988 only once at the initiation of
380 inoculation, we could not thoroughly determine that treatment failure by
381 CV3988 was due to insufficient drug administration. To investigate the role
382 of PAFR expression induced by Pi Sup in Sp-infected mice, additional
383 experiments that focus on specific *P. intermedia* products and use PAFR
384 knock-out mice will be necessary,.

385 In this study, we also examined the effects of other periodontopathic bacteria
386 on our murine model. *P. gingivalis* is a major pathogen of chronic periodontitis
387 (28), and *F. nucleatum* is a pathogen frequently detected in the lesions of
388 gingivitis, chronic periodontitis, and lower respiratory tract specimens (29,
389 30).

390 One possible mechanism that could increase the presence of periodontopathic
391 bacteria in the pathogenesis of respiratory infection is saliva aspiration,
392 which contains periodontal disease-associated enzymes, cytokines, or other
393 biologically active molecules (31, 32). Considering the frequency of saliva
394 aspiration, Marik et al. reported that approximately half of all healthy adults
395 aspirate small amounts of oropharyngeal secretions while sleeping (33). On
396 the basis of these reports, periodontopathic bacteria may have pathogenic

397 effects on the respiratory tract via saliva aspiration. The results of our study
398 indicate that the presence of *P. intermedia* in the oral cavity or lower
399 respiratory tract may be a risk factor for severe pneumococcal pneumonia. In
400 addition, our study suggests that differences in the pathogenicity of
401 pneumococcal pneumonia may exist among periodontopathic bacterial species.
402 Based on our data, there is a possibility that the constituents of
403 periodontopathic species could play an important role in how periodontitis
404 affects pneumococcal pneumonia.
405 Our results provide novel evidence that *P. intermedia* may contribute to the
406 pathophysiology of pneumococcal pneumonia. Additional studies are required
407 to elucidate a more detailed mechanism of interactions between *P. intermedia*
408 and *S. pneumoniae*.
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424

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426

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545
546 **Figure Legends**
547 Fig. 1
548 (A) Survival rates of mice infected by *Streptococcus pneumonia* (Sp)
549 with/without supernatant of *Prevotella intermedia* (Pi Sup). Inocula for all
550 groups contained an equal amount of modified GAM broth and normal saline.
551 Each group was composed of 6–12 mice (○, broth-inoculated mice; ×, Pi Sup-
552 inoculated mice; □, Sp-infected mice without Pi Sup; and ▽, Sp-infected
553 mice with Pi Sup). The survival rates of both Sp-infected groups were
554 significantly lower compared to those of broth- or Pi Sup-inoculated groups (*,
555 $p < 0.05$). The survival rates of Sp-infected mice with Pi Sup were also
556 significantly lower than those of Sp-infected mice without Pi Sup (†, $p < 0.01$).
557 Similar results were obtained in two independent experiments.
558 (B–D) Bacterial load in the lungs (B), blood (C), and spleen (D) of Sp-infected

559 mice with/without Pi Sup were compared at different times (6 h, 24 h, and 48

560 h) after inoculation. Each point represents the value for a mouse (●, Sp-

561 infected mice without Pi Sup; and □, Sp-infected mice with Pi Sup). The

562 mean bacterial count in each organ/blood of Sp-infected mice with Pi Sup

563 increased 24 h after inoculation (**, $p < 0.005$, Sp with Pi Sup vs Sp without

564 Pi Sup), with the exception of the spleen showing an increase as early as 6 h

565 after inoculation (*, $p < 0.05$, Sp with Pi Sup vs Sp without Pi Sup). The bars

566 represent mean bacterial counts. The broken horizontal line represents the

567 detection limit (1.7 log cfu/mL or organs). The data represent two independent

568 experiments.

569

570 Fig. 2

571 Pathological analysis of the lungs of *Streptococcus pneumoniae* (Sp)-infected

572 mice with/without supernatant of *Prevotella intermedia* (Pi Sup). Lungs were

573 collected 24 h after inoculation. (A–D) Hematoxylin-eosin-stained tissue

574 sections at magnifications of ×400. (A) Broth-inoculated (control) mice, (B) Pi

575 Sup-inoculated mice, (C) Sp-infected mice with broth, and D) Sp-infected mice

576 with Pi Sup.

577

578 Fig. 3

579 Changes in the levels of inflammatory cytokines (36 h after inoculation),
580 tumor necrosis factor-alpha (TNF-α, A), and macrophage inflammatory
581 protein-2 (MIP-2, B), in bronchoalveolar lavage fluid in *Streptococcus*
582 *pneumoniae* (Sp)-infected mice with/without supernatant of *Prevotella*
583 *intermedia* (Pi Sup) (n = 8, respectively) and Pi Sup-inoculated mice (n = 7).
584 All groups contained an equal amount of modified GAM broth and normal
585 saline. TNF-αand MIP-2 levels were significantly higher in Sp-infected mice
586 with Pi Sup than in other groups. TNF-α levels also slightly increased in the
587 Pi Sup-inoculated group. The data are expressed as means (SEM).
588 Statistically significant differences are indicated as follows: **, $p < 0.001$.

589

590 Fig. 4

591 Pneumococcal adhesion to airway cells (A549 cells) exposed to the
592 supernatant of *Prevotella intermedia* (Pi Sup) *in vitro*. (A) Incubation with 5–
593 10 fold diluted Pi Sup increased *Streptococcus pneumoniae* colony-forming
594 units (CFU), indicating increased adhesion (*, $p < 0.05$ vs modified GAM

595 broth control). The data are representative of three separate experiments.

596 (B) Co-infection with a platelet-activating factor receptor (PAFR) blocker (10

597 μM, CV-3988) reduced Pi Sup-stimulated adhesion (*, $p < 0.05$ vs without

598 PAFR blocker). The data are representative of three separate experiments.

599 (C) Pi Sup increased PAFR transcript levels (*, $p < 0.01$ vs the broth control).

600 The data are representative of two experiments with six replicates. All data

601 represent the mean and SEM.

602

603 Fig. 5

604 (A) Pulmonary platelet-activating factor receptor (PAFR) transcript levels in

605 mice inoculated with the supernatant of *Prevotella intermedia* (Pi Sup) were

606 examined over time. PAFR expression significantly increased 6 h after

607 inoculation with Pi Sup for up to 24 h compared to that of control mice (†, p

608 < 0.05 vs control). (B) PAFR transcript levels in the lungs of *Streptococcus*

609 *pneumoniae* (Sp)-infected mice with/without Pi Sup. Statistically significant

610 differences are indicated as follows: *, $p < 0.05$; and **, $p < 0.001$.

611 All groups were inoculated with an equal amount of modified GAM broth
612 and normal saline. Each group was composed of 6 mice. The data represent
613 the mean and SEM.
614
615 Fig. 6
616 Survival rates of mice infected by *Streptococcus pneumonia* (Sp) with
617 supernatant (Sup) of *Prevotella intermedia* (Pi, A), *Fusobacterium*
618 *nucleatum* (Fn, B), and *Porphyromonas gingivalis* (Pg, C). All groups
619 contained an equal amount of modified GAM broth and normal saline. The
620 survival rates of Sp-infected mice with PINU499 Sup were significantly
621 lower than those of Sp-infected mice without Pi Sup (†, $p < 0.01$). The
622 survival rates of Sp-infected mice with Pg Sup were significantly higher
623 than those of Sp-infected mice without Pg Sup (*, $p < 0.05$), whereas there
624 was no significant difference between Sp-infected mice with/without Fn Sup.
625
626
627
628

629 Table 1. Strains used in this study.

Microorganism	Strain	Source
Prevotella intermedia	PINU499	A
	PINU046	A
	ATCC 25611	B
Fusobacterium nucleatum	FNU191	A
	GAI 03017	C
	ATCC 10953	B
Porphyromonas gingivalis	W83	B
	TBC60	B
	ATCC 33277	B

630

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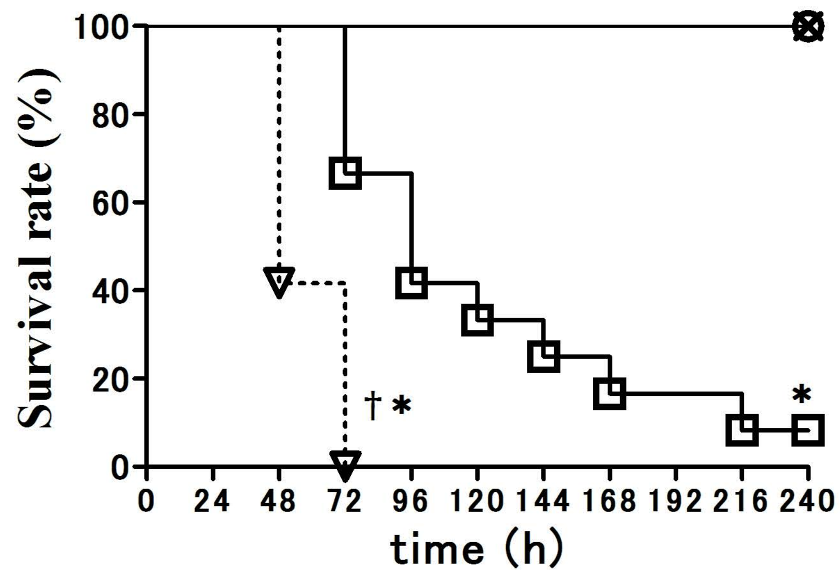
653 Table 2. Inflammatory cells in the bronchoalveolar lavage fluid of mice
654 infected with *Streptococcus pneumoniae* with/without supernatant of
655 *Prevotella intermedia* 36 h after inoculation.

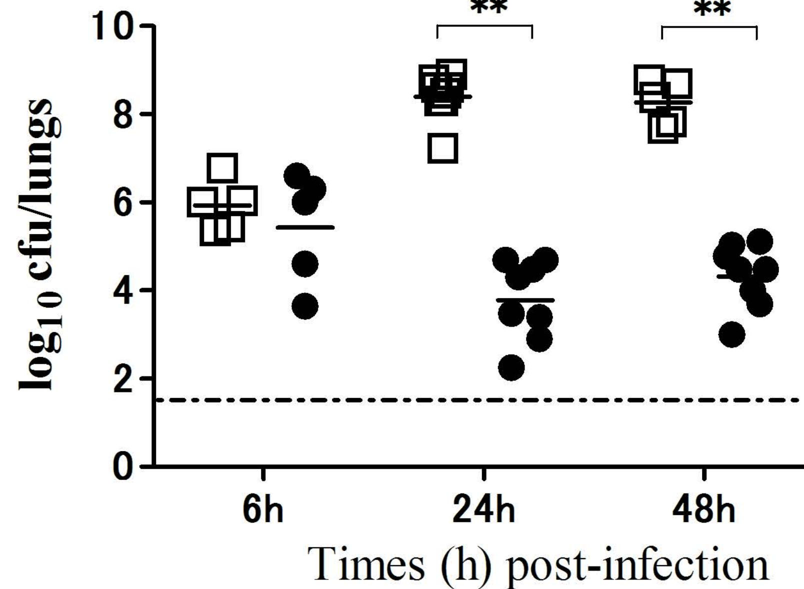
	Control	Sp	Pi Sup	Sp + Pi Sup
Cell density				
10^4 cells・mL^{-1}				
Total cells	7.2 ± 3.0	15.1 ± 4.2*,†	45.1 ± 2.0*,#	63.3 ± 16.9*,#
Neutrophils	0.82 ± 0.86	6.8 ± 3.0*,†	38.2 ± 19.8*,#	58.6 ± 16.0*,#
Macrophages	6.0 ± 3.0	8.0 ± 4.5	6.3 ± 3.0	4.2 ± 3.9
Lymphocytes	0.33 ± 0.29	0.35 ± 0.29	0.49 ± 0.56	0.45 ± 0.55

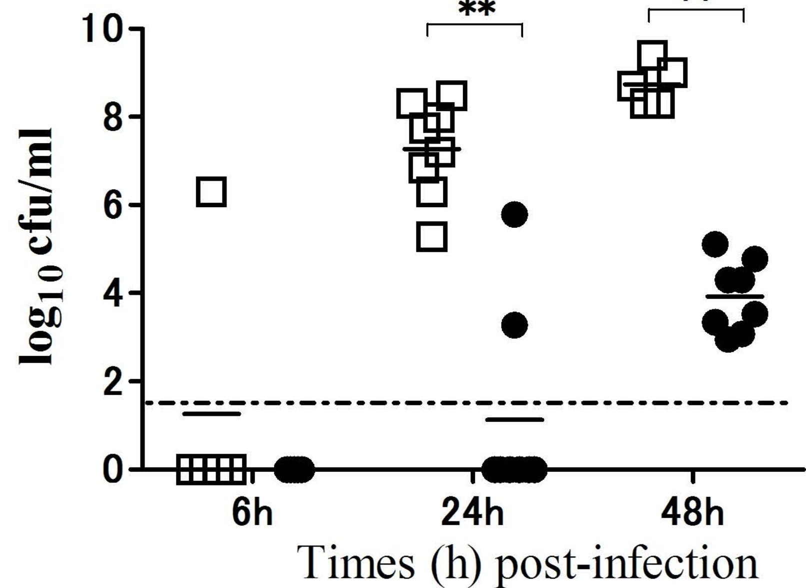
656

657 Data are presented as mean ± SEM (n = 6–9). *, $p < 0.05$ versus control group
658 mice; #, $p < 0.05$ versus *S. pneumoniae*-infected mice; †, $p < 0.05$ versus Pv
659 Sup-inoculated mice and Sp + Pv Sup-inoculated mice.
660 Sp, *Streptococcus pneumoniae*; and Pi Sup, Supernatant of *Prevotella*
661 *intermedia.*

Fig. 1







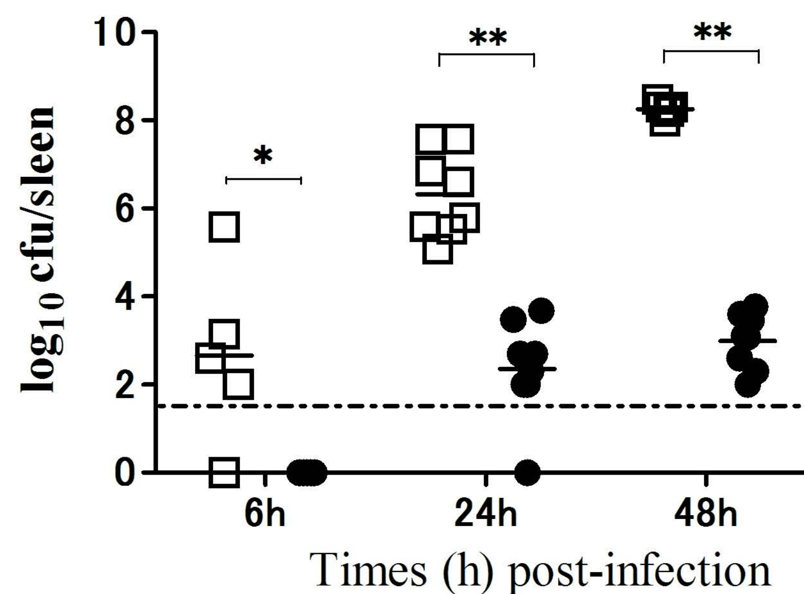
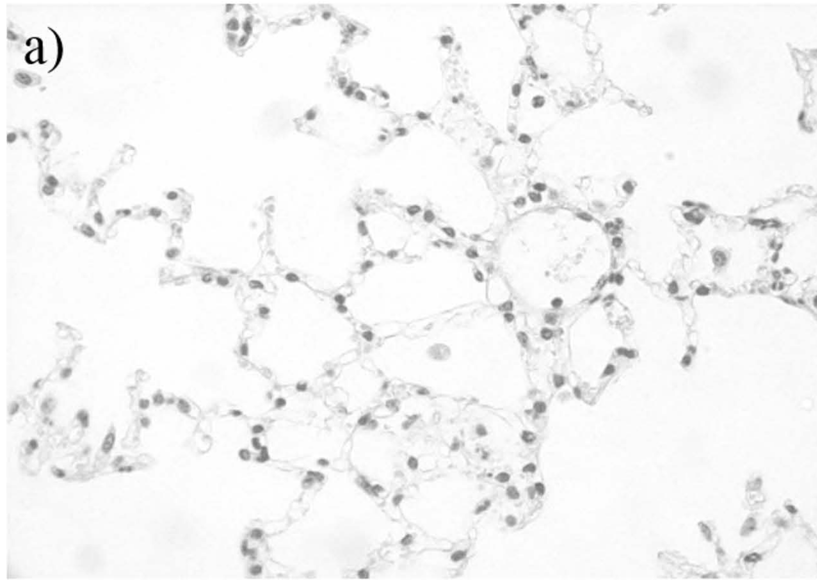
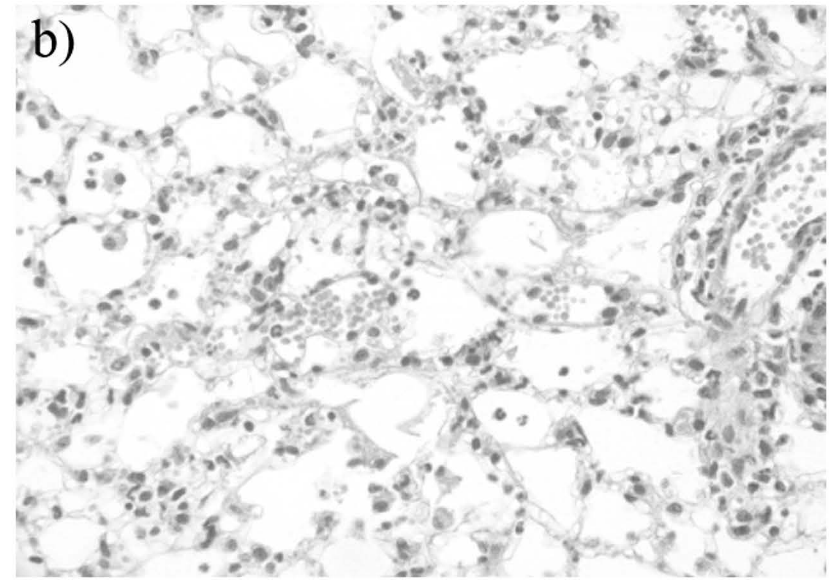
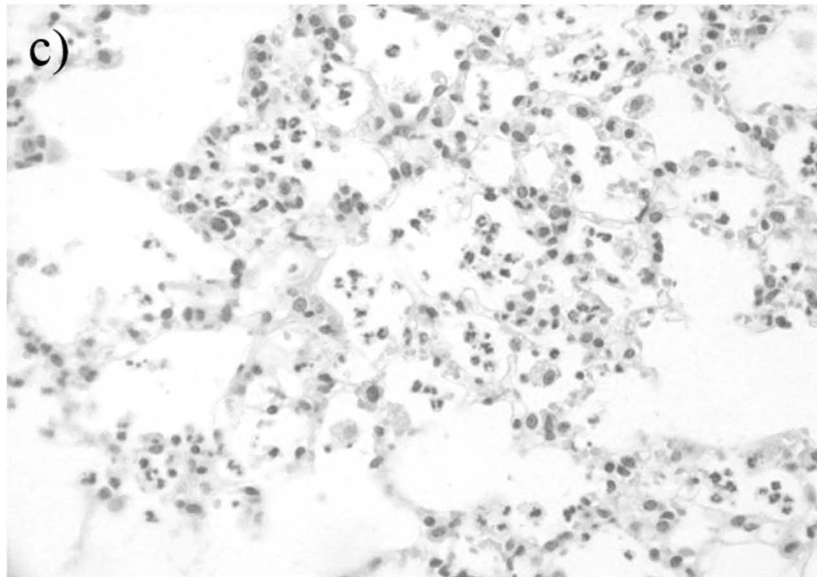


Fig. 2







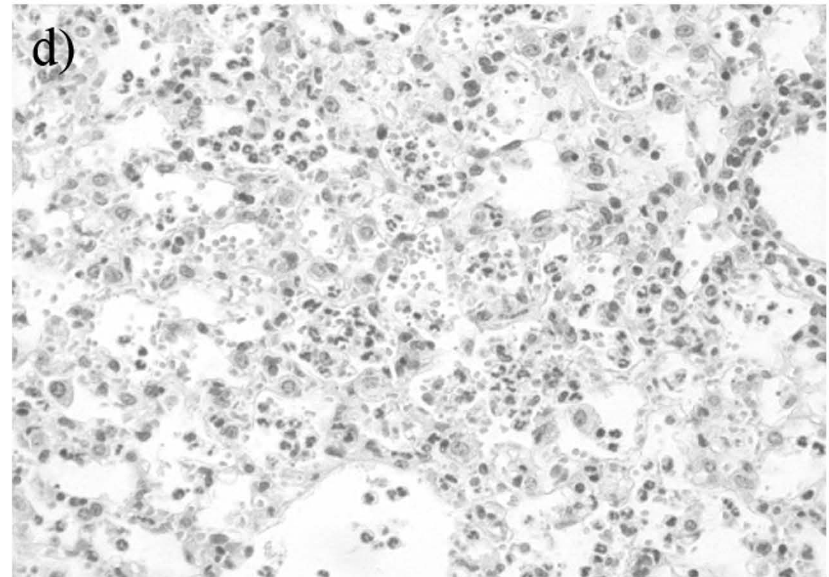
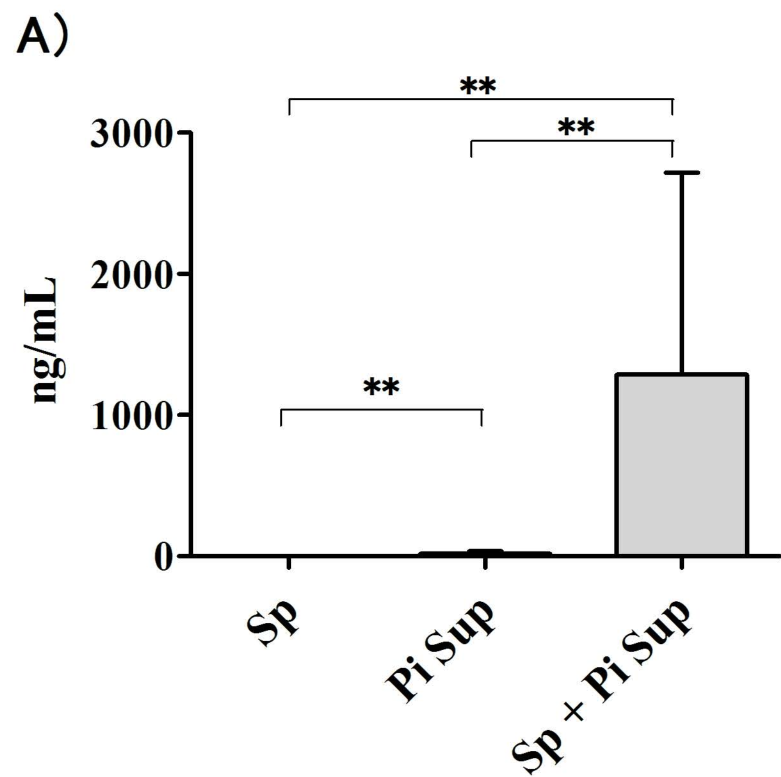


Fig. 3



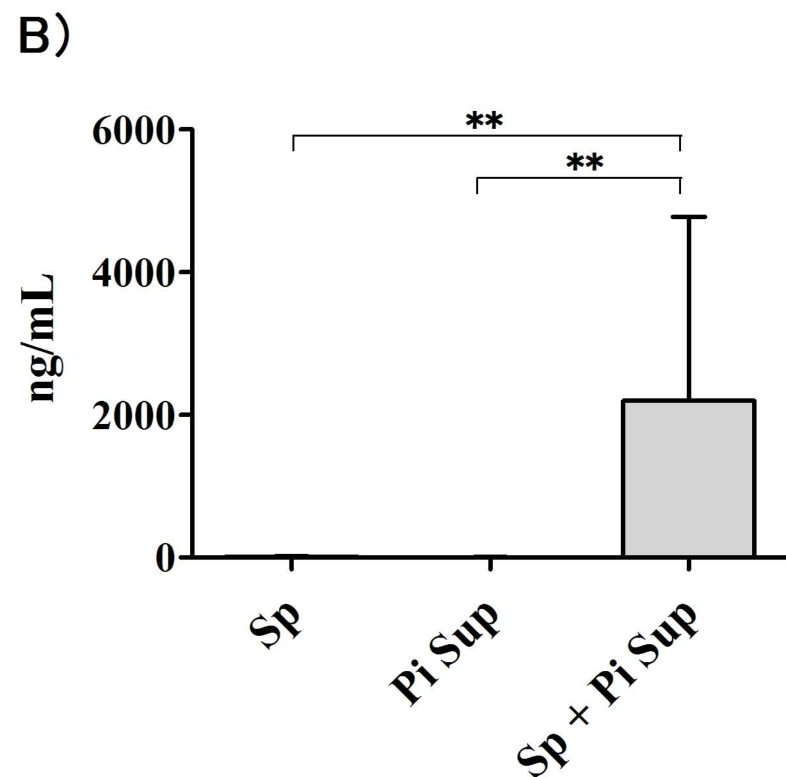


Fig. 4

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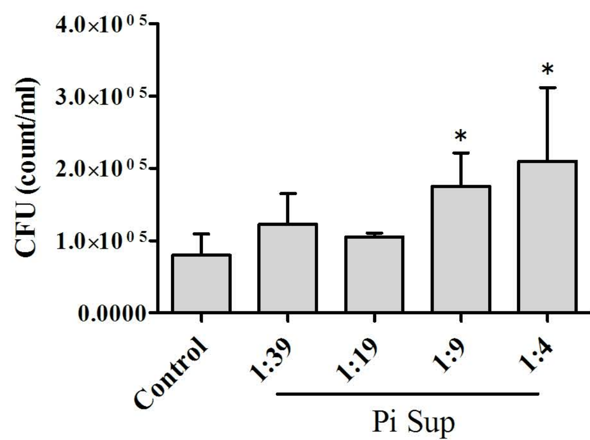

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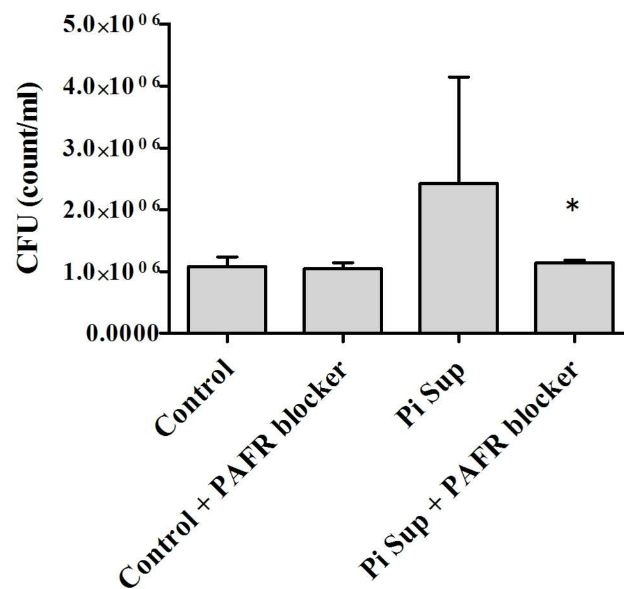

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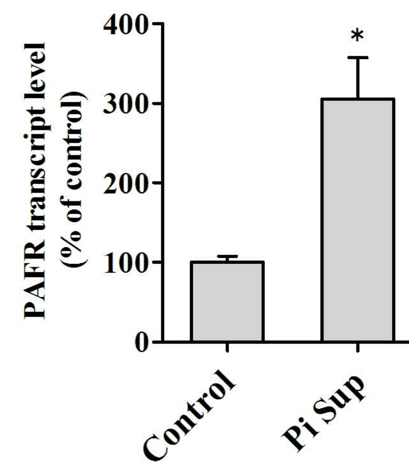
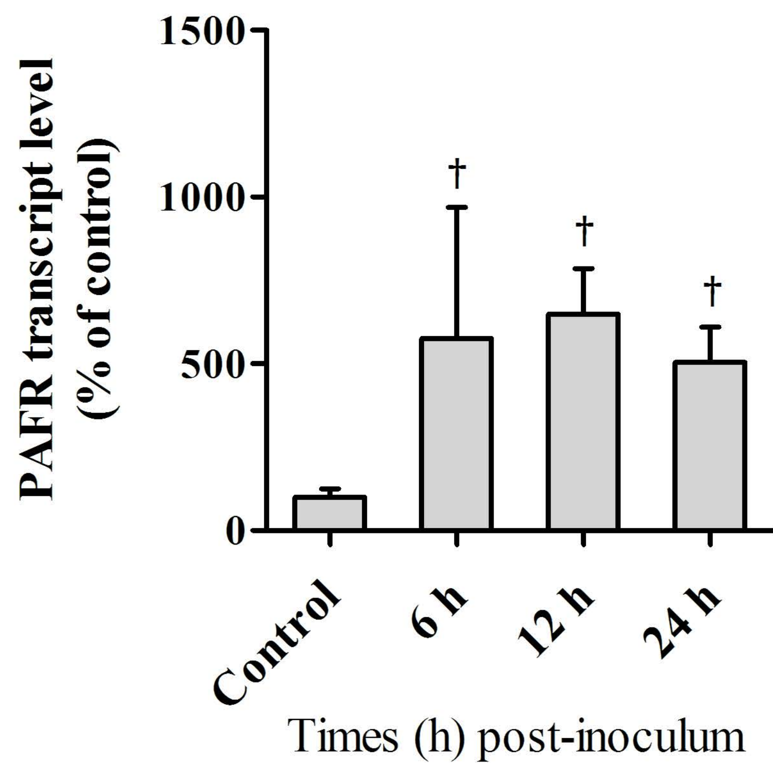


Fig. 5



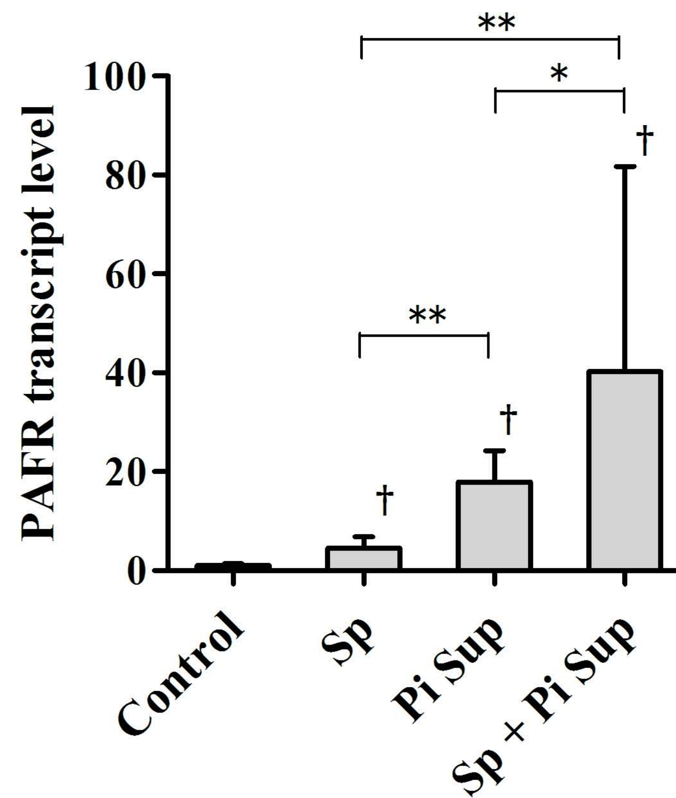
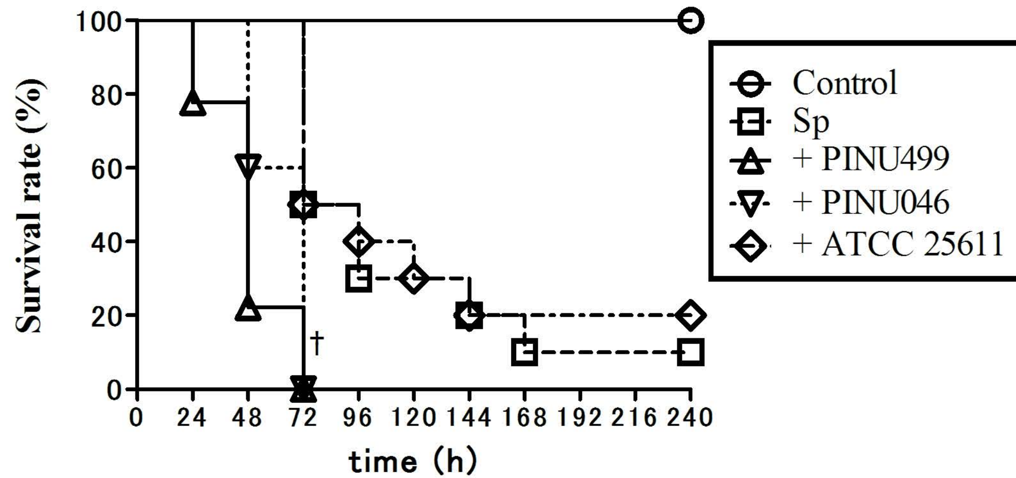


Fig. 6

A)



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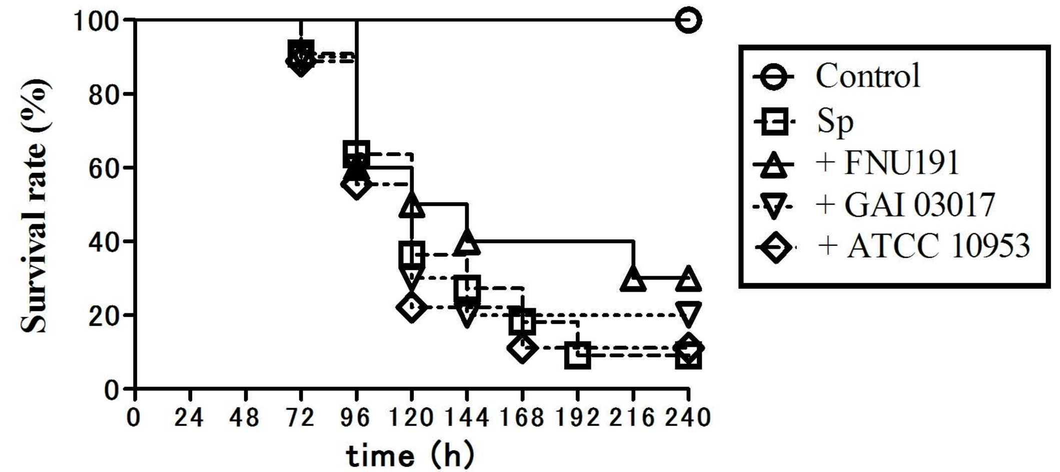

C)