Prevotella intermedia induces severe bacteremic pneumococcal pneumonia

in mice with up-regulated platelet-activating factor receptor

Kentaro Nagaoka,a,b,d Katsunori Yanagihara,a,b Yoshitomo Morinaga,a,b Shigeki Nakamura,b Tatsuhiko Harada,b Hiroo Hasegawa,b Koichi Izumikawa,b Yuji Ishimatsu,b Hiroshi Kakeya,b Masaharu Nishimura,d and Shigeru Kohno,a,c

aDepartment of Laboratory Medicine and bSecond Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; cGlobal COE Program, Nagasaki University, Nagasaki, Japan
dFirst Department of Internal Medicine, Hokkaido University Hospital, Hokkaido, Japan

Corresponding author:
Katsunori Yanagihara

Department of Laboratory Medicine, Nagasaki University Graduate School
Funding/support: This research was funded by the Department of Laboratory Medicine and Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. This study was not sponsored by any grants, gifts, or fellowships.

Running title: *Prevotella intermedia* induces severe pneumococcal pneumonia

Keywords: periodontitis, bacteremia, Fusobacterium, *Porphyromonas gingivalis*

Abstract words

226

Text words

3392
**Abstract**

*Streptococcus pneumoniae* is the leading cause of respiratory infection. Although oral hygiene has been considered a risk factor for developing pneumonia, the relationship between oral bacteria and pneumococcal infection is unknown. In this study, we examined the synergic effects of *Prevotella intermedia*, a major periodontopathic bacterium, on pneumococcal pneumonia. The synergic effects of the supernatant of *P. intermedia* (Pi Sup) on pneumococcal pneumonia were investigated in mice, and the stimulation of pneumococcal adhesion to human alveolar (A549) cells by Pi Sup was assessed. The effects of Pi Sup on platelet-activating factor receptor (PAFR) transcript levels *in vitro* and *in vivo* were analyzed by quantitative real-time PCR, and the differences between the effects of pneumococcal infection induced by various periodontopathic bacterial species were verified in mice. Mice inoculated with *S. pneumoniae* plus Pi Sup exhibited a significantly lower survival rate, higher bacterial loads in the lungs, spleen, and blood, and higher inflammatory cytokine levels in the bronchoalveolar lavage fluid (macrophage inflammatory protein-2 and tumor necrosis factor-alpha) than 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.
Introduction

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

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

Several oral anaerobes, mostly related to periodontitis, are known to interact in a synergistic or antagonistic manner (7,8). To understand the interactions between microorganisms, the enhancement of reciprocal bacterial growth, adhesion/invasion into host cells, and effects on host immunity response have been examined (7–11). Regarding the synergic effects of anaerobes on the pulmonary infection of Streptococcus species, Shinzato et al. reported that Prevotella intermedia exhibits synergic effects on lower respiratory tract
infections of *Streptococcus constellatus* in mice by enhancing reciprocal bacterial growth (9). However, whether oral bacteria exhibit synergic effects on pneumococcal infections remains unclear.

Here, we hypothesized that an anaerobe that is ubiquitous in the oral cavity may have synergic effects on pneumococcal respiratory infection. To investigate our hypothesis, we focused on the anaerobe *P. intermedia*.

*P. intermedia* is a gram-negative, black-pigmented obligate anaerobic rod, which is often isolated from periodontal lesions associated with various forms of periodontal disease (12,13). In addition, *P. intermedia* has recently been detected in cystic fibrosis airway specimens (14–16). Ulrich et al. reported the pathogenic potential of *P. intermedia* in the respiratory tract and demonstrated that extracellular toxins of *P. intermedia* are cytotoxic for human alveolar type II cells and neutrophils (17).

In this study, we examined the effects of *P. intermedia* on pneumococcal pneumonia in a murine model. The aims of this study were to determine whether *P. intermedia* exhibits synergic effects on pneumococcal pneumonia and to examine its mechanism of interactions.
Materials and Methods

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

The supernatants of *P. intermedia* and the other anaerobes were obtained as previously reported (18, 19). Briefly, the anaerobes were incubated using modified GAM broth for 48 h in an anaerobic chamber. The supernatants were then collected by centrifugation at 10,000 rpm at 4 °C for 50 min to remove the bacteria and filter-sterilized through a 0.22-µm pore membrane filter.
We conducted all experiments using the PINU499 strain, with the exception of the experiments performed to verify the differences between the effects of periodontopathic bacterial species and strains on pneumococcal infection. We also identified clinical strains at our institution by PCR amplification and 16S rRNA sequence analysis.

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

**Intratracheal infection procedure.** The *S. pneumoniae* strain was cultured on blood agar plates (Becton Dickinson Co., Ltd., Japan) for 24 h at 37 °C, scraped and suspended in brain heart infusion broth mixed with horse serum, and cultured with shaking at 37 °C at 250 rpm for 4 h. Bacteria were then
harvested by centrifugation (3000 rpm, 10 min). The organism was resuspended in normal saline for a final concentration of approximately $10^8$ colony forming units (CFU)/mL, as determined by the optical density method. Mice were anaesthetized with pentobarbital, and the trachea was inoculated with 0.05 mL of the bacterial suspension via insertion with a 24-gauge catheter. For mixed-infection experiments with *S. pneumoniae* (Sp) and *P. intermedia*, the bacterial suspension of Sp was mixed with the same amount of bacterial suspension of *P. intermedia* or modified GAM broth before inoculating mice. The final bacterial load of Sp was approximately $2 \times 10^6$–$2 \times 10^7$ CFU/mL ($1 \times 10^5$–$1 \times 10^6$ CFU/mouse), and the final bacterial load of *P. intermedia* was approximately $2 \times 10^8$–$2 \times 10^9$ CFU/mL ($1 \times 10^7$–$1 \times 10^8$ CFU/mouse).

In experiments that examined the effects of culture supernatants of *P. intermedia* and the other periodontopathic bacteria on pneumococcal pneumonia, a bacterial suspension of Sp was mixed with the same amount of culture supernatant of anaerobes or modified GAM broth before inoculating mice. The final bacterial load of Sp was approximately $5 \times 10^7$ CFU/mL ($2.5 \times 10^6$ CFU/mouse). The control group was inoculated with an equal volume of
broth and normal saline. For the group inoculated with the supernatant of *P. intermedia* (Pi Sup) without Sp, equal volumes of Pi Sup and normal saline were used. The pH of modified GAM broth was adjusted to that of the anaerobe’s supernatant (pH 5.6 for the Pi Sup and pH 6.8 for the supernatant of *Fusobacterium nucleatum* or *Porphyromonas gingivalis*).

**Bacteriological and histopathological examinations.** Each group of animals was sacrificed at specific time intervals by cervical dislocation. After exsanguination, the lungs and spleen were dissected and removed under aseptic conditions. Blood was collected by right ventricular puncturing using heparin-coated syringes. For bacteriological analyses, the organs were suspended in normal saline (1 mL) and homogenized with a Polytron homogenizer (AS One Co., Osaka, Japan). Each specimen (blood, lung, and spleen) was quantitatively inoculated onto blood agar plates by serial dilution, followed by incubation at 37 °C for 24 h. The lowest level of detectable CFU/mL was 50 CFU/mL (1.7 log CFU/mL). The lung tissue used for histological examination was fixed in 10% buffered formalin and stained with hematoxylin-eosin.
**Bronchoalveolar lavage (BAL) and cytokine enzyme-linked immunosorbent assays (ELISA).** BAL was performed as previously described (20). The recovered fluid fractions were pooled for each animal, and the total cell counts were calculated using Turk staining. For differential cell counts, cells were centrifuged at 850 rpm for 2 min onto slides that were then stained with Diff–Quick stain. Differential cell counts were performed by counting 100 cells. Various concentrations of macrophage inflammatory protein (MIP)-2 and tumor necrosis factor-alpha (TNF-α) in BAL fluid (BALF) were assayed using mouse cytokine ELISA test kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Cell culture.** The NCI-A549 (Human type II pneumocyte cell line) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U of penicillin/mL, and 100 µg of streptomycin/mL. The cells were grown at 37 °C with 5% CO₂ in fully humidified air. Cells were exposed to Pi Sup for pneumococcal adhesion studies. For controls, cells were incubated with modified GAM broth, and the pH was adjusted to that of Pi Sup.
Pneumococcal adhesion to airway cells exposed to Pi Sup in vitro. The adhesion of pneumococci to airway cells in vitro was performed as previously described (21). Briefly, A549 cells were seeded in 24-well plates. Pi Sup was added to cell monolayers, incubated at 37 °C for 4 h, and subsequently removed by washing twice with RPMI medium. Pneumococci was then added and incubated for 2 h. Cell monolayers were washed five times, and cells were removed from the tissue culture plate with trypsin-EDTA and lysed with ice-cold sterile distilled water for 10 min. The lysates were then plated to determine the CFU/mL.

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

PAFR transcript levels in airway cells exposed to Pi Sup in vitro. Transcript
levels of PAFR were assessed in A549 cells using quantitative real-time PCR. The total RNA was extracted from A549 cells cultured in 6-well plates using QuickGene-Mini80 and QuickGene RNA cultured cell kits (FUJIFILM Co., Tokyo, Japan) according to the manufacturer’s instructions. The total RNA (1 µg) was reverse transcribed into cDNA using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen) and then treated with RNaseH. To quantify the expression of the PAFR gene, PCR primers and Taqman probes were used as previously reported (Hs00265399_S1) (21). To normalize PAFR expression, the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) was also measured using the primer set (Hs01003267_m1) according to the manufacturer's instructions (Life technologies). The data are presented as a ratio of HPRT1.

Lung PAFR transcript levels in mice exposed to Pi Sup in vivo. Lung PAFR transcript levels were examined in Pi Sup-inoculated mice and Sp-infected mice with/without Pi Sup. Each group of animals was sacrificed at specific time intervals and a partial lung was preserved in RNA later (Life technologies). The tissue samples were homogenized, and RNA was extracted
using the RNeasy Mini kit (QIAGEN) according to the manufacturer’s instructions. First strand cDNA synthesis was performed as described above. mRNA transcript levels of PAFR and the housekeeping gene HPRT1 were determined by quantitative real-time PCR using the TaqMan primer and probe sets Mm02621061_m1 and Mm00446968_m1, respectively. Mouse PAFR mRNA transcript levels were normalized to the housekeeping gene HPRT1 (22).

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

**Results**

**Mixed infection of S. pneumoniae and P. intermedia.** There were no significant differences observed between the survival rates of mixed-infection experiments of Sp with/without the bacterial suspension of *P. intermedia*
In preliminary experiments, in which BALB/c mice were inoculated with only *P. intermedia* via the trachea, changes in inflammation and the proliferation of *P. intermedia* in the lungs were not observed. Based on these results, the synergic effects of *P. intermedia* on pneumococcal pneumonia were difficult to assess in the mixed-infection experiments because the virulence of only *P. intermedia* was less significant. Therefore, we did not conduct additional experiments using bacterial suspensions of *P. intermedia*.

**Pneumococcal infection with *P. intermedia* supernatant caused severe bacteremic pneumonia.** Figure 1A illustrates the survival rates of Sp-infected mice with/without Pi Sup. In the controls (broth- or Pi Sup-inoculated mice), no deaths were observed during the 10-day observation period. In contrast, 90% of Sp-infected mice without Pi Sup died 3 days after inoculation, and all Sp-infected mice with Pi Sup died within 3 days. The survival rates of Sp-infected mice with Pi Sup were significantly shorter than those of Sp-infected mice without Pi Sup (p < 0.01). The change in the number of viable Sp in the lungs, blood, and spleen over time following infection is shown in Figure 1B—
D. The mean bacterial count in each organ/blood of Sp-infected mice with Pi Sup began to increase 24 h after inoculation (p < 0.005, Sp with Pi Sup vs Sp without Pi Sup), with the exception of the spleen, in which the increase was observed starting as early as 6 h after inoculation (p < 0.05). Because these results indicate that Pi Sup induces early exacerbation of Sp-infection in mice within 6–48 h, we examined the pathological changes in the lungs 24 h after inoculation (Fig. 2). Pathological examination of the lungs of Sp-infected mice with Pi Sup showed severe bronchopneumonia with massive hemorrhaging (Fig. 2d). Pi Sup-inoculated mice also exhibited mild hemorrhaging (Fig. 2b), whereas the lungs of Sp-infected mice without Pi Sup only exhibited mild pneumonia 24 h after inoculation (Fig. 2c). Broth-inoculated (control) mice did not exhibit any inflammatory changes in the lungs.

In order to examine peak inflammatory changes in the lungs of Sp-infected mice with Pi Sup, we performed BAL 36 h after inoculation. The total cell and neutrophil counts (Table 2) were significantly higher in Sp-infected mice with Pi Sup and Pi Sup-inoculated mice than those of Sp-infected mice without Pi Sup. To further examine the differences, inflammatory cytokine levels in BALF were analyzed. TNF-α and MIP-2 concentrations were significantly
higher in Sp-infected mice with Pi Sup than those of the other group (Fig. 3). TNF-α levels also increased slightly in Pi Sup-inoculated groups and were still significantly higher than those of Sp-infected mice without Pi Sup. To confirm the inflammatory effects of Pi Sup, we also performed BAL 12 h and 24 h after Pi Sup-inoculation. BALF of Pi Sup-inoculated mice demonstrated that the total cell and neutrophil counts increased 12 h after inoculation, and the concentrations of MIP-2 and TNF-α also increased after inoculation. However, the peak concentrations of TNF-α and MIP-2 in Pi Sup-inoculated mice were 183.0 ± 30.3 ng/mL (12 h) and 58.4 ± 39.4 ng/mL (24 h), respectively (data not shown), which were lower than those of Sp-infected mice with Pi Sup.

Culture supernatant of P. intermedia stimulated PAFR in vitro and in vivo. To further understand the effects of Pi Sup on pneumococcal pneumonia, we hypothesized that Pi Sup possesses a stimulatory effect on pneumococcal adhesion to lower airway cells, contributing to rapid bacterial proliferation and invasion. Regarding pneumococcal adhesion, there is increasing evidence that PAFR is a major epithelial receptor used by S. pneumoniae to invade
airway epithelium cells (23). Up-regulation of PAFR transcripts *in vivo* has been described in several animal models as a result of interleukin 1 stimulation (24), influenza infection (25), and exposure to cigarette smoke (21). However, the relationship between periodontopathic bacteria and PAFR transcript levels has not been described previously. Thus, we sought to examine the effects of Pi Sup on pneumococcal adhesion and PAFR expression. Pi Sup increased pneumococcal adhesion to A549 cells (*p* < 0.05 vs control; Fig. 4A). CV-3988 decreased pneumococcal adhesion stimulated by Pi Sup (*p* < 0.05, Pi Sup + antagonist vs Pi Sup + Ethanol; Fig. 4B), and PAFR mRNA levels increased in Pi Sup-stimulated cells (*p* < 0.005 vs control; Fig. 4C).

In mice, Pi Sup increased lung PAFR transcript levels 6–24 h after inoculation (Fig. 5A). To examine the differences between the PAFR transcript levels of Sp-infected mice with/without Pi Sup, we collected the lungs of mice 24 h after inoculation. The highest increase in PAFR transcript levels was observed in the lungs of Sp-infected mice with Pi Sup (*p* < 0.005 vs Sp without Pi Sup; *p* < 0.05 vs Pi Sup). The Pi Sup-inoculated group exhibited higher PAFR transcript levels than Sp-infected mice without Pi Sup (*p* < 0.005).
In vivo effects of culture supernatant of periodontal bacteria on pneumococcal pneumonia. To estimate the effects of periodontopathic bacteria on pneumococcal infection, we examined the survival rates of Sp-infected mice inoculated with the supernatants of Prevotella intermedia (Fig. 6A), Fusobacterium nucleatum (Fn; Fig. 6B), and Porphyromonas gingivalis (Pg; Fig. 6C). Each group was composed of three different strains, including a reference strain. The survival rates of Sp-infected mice with the supernatant of PINU499 were significantly lower than that of Sp-infected mice without Pi Sup (P < 0.01). The survival rates of Sp-infected mice with Pg Sup were significantly higher than those of Sp-infected mice without Pg Sup (P < 0.05), whereas there was no significant difference between the survival rates of Sp-infected mice with/without Fn Sup.

Discussion

The present study is the first to demonstrate that the products of P. intermedia induce severe bacteremic pneumococcal pneumonia as well as the enhancement of pneumococcal adhesion to lower airway cells. Several lines of evidence support this notion.
First, Sp-infection with Pi Sup exhibited significant lower survival rates with earlier increases in Sp bacterial load in the lungs, spleen, and blood compared to those of Sp-infected mice without Pi Sup. Significant increases in inflammatory cytokines were observed in the early phases of Sp-infected mice with Pi Sup, indicating the severity of bacteremia compared to that of Sp-infected mice without Pi Sup. Although belated bacteremia was observed in Sp-infected mice without Pi Sup, a high bacterial load in the lungs was only observed in Sp-infected mice with Pi Sup. These data suggest that Pi Sup enhances Sp invasion into blood circulation as well as Sp adhesion and proliferation in the lungs.

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

Third, we also observed PAFR up-regulation by Pi Sup in vivo. Higher levels of PAFR up-regulation were observed in Sp-infected mice with Pi Sup compared to those of Pi Sup-inoculated mice, suggesting that Pi Sup may
possess synergic effects on PAFR up-regulation with pneumococcal infection. PAFR is a major epithelial receptor that binds to phosphorylcholine in the bacterial cell wall. Thus, the effects of Pi Sup on PAFR expression could be synergic not only for *S. pneumoniae* infection but for other bacteria containing phosphorylcholine, including *Pseudomonas aeruginosa* (26) and *Acinetobacter baumanii* (27).

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

The main goal of our study was to determine the extent by which PAFR expression affects the susceptibility of *S. pneumoniae* in mice administered Pi Sup, and the data obtained were inconclusive. We treated Sp-infected mice with Pi Sup with CV3988 (PAFR antagonist) but could not determine any significant improvement in survival or attenuation of pneumococcal bacterial load in the lungs or blood (data not shown). However, Pi Sup-induced PAFR up-regulation in our murine model was consistent up to at least 24 h after
inoculation. As were able to administer CV3988 only once at the initiation of inoculation, we could not thoroughly determine that treatment failure by CV3988 was due to insufficient drug administration. To investigate the role of PAFR expression induced by Pi Sup in Sp-infected mice, additional experiments that focus on specific *P. intermedia* products and use PAFR knock-out mice will be necessary.

In this study, we also examined the effects of other periodontopathic bacteria on our murine model. *P. gingivalis* is a major pathogen of chronic periodontitis (28), and *F. nucleatum* is a pathogen frequently detected in the lesions of gingivitis, chronic periodontitis, and lower respiratory tract specimens (29, 30). One possible mechanism that could increase the presence of periodontopathic bacteria in the pathogenesis of respiratory infection is saliva aspiration, which contains periodontal disease-associated enzymes, cytokines, or other biologically active molecules (31, 32). Considering the frequency of saliva aspiration, Marik et al. reported that approximately half of all healthy adults aspirate small amounts of oropharyngeal secretions while sleeping (33). On the basis of these reports, periodontopathic bacteria may have pathogenic
effects on the respiratory tract via saliva aspiration. The results of our study indicate that the presence of *P. intermedia* in the oral cavity or lower respiratory tract may be a risk factor for severe pneumococcal pneumonia. In addition, our study suggests that differences in the pathogenicity of pneumococcal pneumonia may exist among periodontopathic bacterial species. Based on our data, there is a possibility that the constituents of periodontopathic species could play an important role in how periodontitis affects pneumococcal pneumonia.

Our results provide novel evidence that *P. intermedia* may contribute to the pathophysiology of pneumococcal pneumonia. Additional studies are required to elucidate a more detailed mechanism of interactions between *P. intermedia* and *S. pneumoniae*.

**Acknowledgements** The authors thank Mariko Naito and Koji Nakayama in the Division of Microbiology and Oral Infection, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Japan. We also thank Kaori Tanaka and Kunitomo Watanabe in the Division of Anaerobe Research, Life Science Research Center,
Gifu University, Japan for providing bacterial strains. We are grateful to Takeshi Yamanaka and Kazuyoshi Yamane from the Department of Bacteriology, Osaka Dental University, Japan for their technical advice regarding *Prevotella intermedia*, and also thank Atsushi Yokoyama for providing technical support.

**Funding** This research was funded by the Department of Laboratory Medicine and Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

**Competing interests** None.

**References**


**Figure Legends**

Fig. 1

(A) Survival rates of mice infected by *Streptococcus pneumonia* (Sp) with/without supernatant of *Prevotella intermedia* (Pi Sup). Inocula for all groups contained an equal amount of modified GAM broth and normal saline. Each group was composed of 6–12 mice (〇, broth-inoculated mice; ×, Pi Sup-inoculated mice; □, Sp-infected mice without Pi Sup; and ▽, Sp-infected mice with Pi Sup). The survival rates of both Sp-infected groups were significantly lower compared to those of broth- or Pi Sup-inoculated groups (*, p < 0.05). The survival rates of Sp-infected mice with Pi Sup were also significantly lower than those of Sp-infected mice without Pi Sup (†, p < 0.01). Similar results were obtained in two independent experiments.

(B–D) Bacterial load in the lungs (B), blood (C), and spleen (D) of Sp-infected
mice with/without Pi Sup were compared at different times (6 h, 24 h, and 48 h) after inoculation. Each point represents the value for a mouse (●, Sp-infected mice without Pi Sup; and □, Sp-infected mice with Pi Sup). The mean bacterial count in each organ/blood of Sp-infected mice with Pi Sup increased 24 h after inoculation (**, p < 0.005, Sp with Pi Sup vs Sp without Pi Sup), with the exception of the spleen showing an increase as early as 6 h after inoculation (*, p < 0.05, Sp with Pi Sup vs Sp without Pi Sup). The bars represent mean bacterial counts. The broken horizontal line represents the detection limit (1.7 log cfu/mL or organs). The data represent two independent experiments.

Fig. 2

Pathological analysis of the lungs of Streptococcus pneumoniae (Sp)-infected mice with/without supernatant of Prevotella intermedia (Pi Sup). Lungs were collected 24 h after inoculation. (A–D) Hematoxylin-eosin-stained tissue sections at magnifications of ×400. (A) Broth-inoculated (control) mice, (B) Pi Sup-inoculated mice, (C) Sp-infected mice with broth, and (D) Sp-infected mice with Pi Sup.
Fig. 3

Changes in the levels of inflammatory cytokines (36 h after inoculation), tumor necrosis factor-alpha (TNF-α, A), and macrophage inflammatory protein-2 (MIP-2, B), in bronchoalveolar lavage fluid in *Streptococcus pneumoniae* (Sp)-infected mice with/without supernatant of *Prevotella intermedia* (Pi Sup) (n = 8, respectively) and Pi Sup-inoculated mice (n = 7).

All groups contained an equal amount of modified GAM broth and normal saline. TNF-α and MIP-2 levels were significantly higher in Sp-infected mice with Pi Sup than in other groups. TNF-α levels also slightly increased in the Pi Sup-inoculated group. The data are expressed as means (SEM). Statistically significant differences are indicated as follows: **, p < 0.001.

Fig. 4

Pneumococcal adhesion to airway cells (A549 cells) exposed to the supernatant of *Prevotella intermedia* (Pi Sup) *in vitro*. (A) Incubation with 5–10 fold diluted Pi Sup increased *Streptococcus pneumoniae* colony-forming units (CFU), indicating increased adhesion (*, p < 0.05 vs modified GAM
broth control). The data are representative of three separate experiments.

(B) Co-infection with a platelet-activating factor receptor (PAFR) blocker (10 µM, CV-3988) reduced Pi Sup-stimulated adhesion (*, p < 0.05 vs without PAFR blocker). The data are representative of three separate experiments.

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

The data are representative of two experiments with six replicates. All data represent the mean and SEM.

Fig. 5

(A) Pulmonary platelet-activating factor receptor (PAFR) transcript levels in mice inoculated with the supernatant of Prevotella intermedia (Pi Sup) were examined over time. PAFR expression significantly increased 6 h after inoculation with Pi Sup for up to 24 h compared to that of control mice (†, p < 0.05 vs control). (B) PAFR transcript levels in the lungs of Streptococcus pneumoniae (Sp)-infected mice with/without Pi Sup. Statistically significant differences are indicated as follows: *, p < 0.05; and **, p < 0.001.
All groups were inoculated with an equal amount of modified GAM broth and normal saline. Each group was composed of 6 mice. The data represent the mean and SEM.

Fig. 6

Survival rates of mice infected by Streptococcus pneumonia (Sp) with supernatant (Sup) of Prevotella intermedia (Pi, A), Fusobacterium nucleatum (Fn, B), and Porphyromonas gingivalis (Pg, C). All groups contained an equal amount of modified GAM broth and normal saline. The survival rates of Sp-infected mice with PINU499 Sup were significantly lower than those of Sp-infected mice without Pi Sup (†, p < 0.01). The survival rates of Sp-infected mice with Pg Sup were significantly higher than those of Sp-infected mice without Pg Sup (*, p < 0.05), whereas there was no significant difference between Sp-infected mice with/without Fn Sup.
Table 1. Strains used in this study.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Preventella intermedia</em></td>
<td>PINU499</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>PINU046</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>ATCC 25611</td>
<td>B</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>FNU191</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>GAI 03017</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>ATCC 10953</td>
<td>B</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>W83</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>TBC60</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>ATCC 33277</td>
<td>B</td>
</tr>
</tbody>
</table>

A, Department of Laboratory Medicine, Nagasaki University Hospital, Nagasaki, Japan; B, Division of Microbiology and Oral Infection, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; and C, Division of Anaerobe
Research, Life Science Research Center, Gifu University, Gifu City, Japan.
Table 2. Inflammatory cells in the bronchoalveolar lavage fluid of mice infected with *Streptococcus pneumoniae* with/without supernatant of *Prevotella intermedia* 36 h after inoculation.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sp</th>
<th>Pi Sup</th>
<th>Sp + Pi Sup</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁴ cells · mL⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells</td>
<td>7.2 ± 3.0</td>
<td>15.1 ± 4.2* †</td>
<td>45.1 ± 2.0* †#</td>
<td>63.3 ± 16.9* †#</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.82 ± 0.86</td>
<td>6.8 ± 3.0* †</td>
<td>38.2 ± 19.8* †#</td>
<td>58.6 ± 16.0* †#</td>
</tr>
<tr>
<td>Macrophages</td>
<td>6.0 ± 3.0</td>
<td>8.0 ± 4.5</td>
<td>6.3 ± 3.0</td>
<td>4.2 ± 3.9</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.33 ± 0.29</td>
<td>0.35 ± 0.29</td>
<td>0.49 ± 0.56</td>
<td>0.45 ± 0.55</td>
</tr>
</tbody>
</table>

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