

***Lactobacillus pentosus* strain b240 suppresses pneumonia induced by *Streptococcus pneumoniae* in mice**

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ABSTRACT

Aims: Oral administration of probiotics has been known to improve inflammatory responses against infectious diseases. Here, we describe the inhibitory effect of oral intake of heat-killed *Lactobacillus pentosus* strain b240 (b240) on pneumococcal pneumonia in a murine experimental model.

Method and Results: The mice treated with oral b240 for 21 days before *Streptococcus pneumoniae* infection exhibited prolonged survival time and less body weight loss, compared with saline-treated control mice. Mild pneumonia with significantly reduced secretion of inflammatory cytokines/chemokines according to related mitogen-activated protein kinase signalling molecules (phosphorylated c-Jun N-terminal kinase) was found in b240-treated mice, whereas severe pneumonia with hypercytokinemia was evident in control mice.

Prominent reduction in the number of pneumococci and elevated expression of Toll-like receptor 2 and 4 in the lung tissues was concomitantly noted in b240-treated mice.

Conclusions: These findings indicates that b240 has inhibitory effects on pneumococcal pneumonia induced by *S. pneumoniae* infection and improves inflammatory tissue responses, resulting in reduced damages to the respiratory tissues.

Significance and Impact of the Study: These results demonstrate that oral administration of b240 might protect host animals from *S. pneumoniae* infection by augmentation of innate immune response.

Keywords: *Lactobacillus pentosus* strain b240, Probiotics, *Streptococcus pneumoniae*, Cytokines, Toll-like receptors

Role of Authors:

MS managed and coordinated of the experiments.

AK, SY, and KK performed the experiments.

NT, YM, TT, KI, HK, YY, and KY supported the experiments and gave useful comments for the manuscripts.

TT, NK, and SK are the chiefs of the laboratory and this study.

INTRODUCTION

Pneumonia is the fourth leading cause of death in Japan (Anon., 2006). When deaths from pneumonia are analyzed by age, elderly patients (aged, >65 years) account for >90% of total deaths from pneumonia (Anon. 2006; Mandell *et al.* 2007; Seki *et al.* 2008; Anon. 2009). Approximately 25 per 100,000 persons are treated daily for pneumonia in Japan and 75 per 100,000 persons die annually from it (Saito A, et al 2006, Anon 2006, Anon 2009) .

Community-acquired pneumonia (CAP), an acute infection, occurs among individuals participating in ordinary social interactions. The bacterium most frequently isolated from patients with CAP is *Streptococcus pneumoniae*, followed by *Haemophilus influenzae*. The fatality rate associated with *S. pneumoniae* pneumonia is approximately 23%, despite the use of potent antibiotics and aggressive intensive care support (Anon. 2006; Mandell *et al.* 2007). Recently developed therapeutic techniques include effective vaccines, and control of overwhelming inflammatory reactions that are associated with tissue injury have been reported (Fisman DN, et al. 2006, Yanagihara et al. 2007). In fact, growing evidence suggest that the host immune response greatly contributes to the reduction of high mortality rate associated with this life-threatening infection (Seki *et al.* 2004a).

Probiotic lactic acid bacteria have been suggested to have effects, for example, anti-inflammatory properties (Gill et al. 2000; Ménard et al. 2004; Harata et al. 2010; Kawase et al. 2010). Probiotics are defined as live microorganisms that confer a health benefit on the host and are potential measures to regulate infection in the elderly (Fuller 1991; Gill and Guarner 2004). Administration of lactobacillus strain for 3 weeks has been shown to reduce the duration of winter infection, including viral (eg. cold, influenza) and bacterial (eg. pneumonia) infection, in free-living elderly (Turchet *et al.* 2003), and the World Health Organization (WHO) suggested that probiotics are helpful in normalizing nutrition status, especially in children (World Health Organization 2000). However, there is less information on the impact of probiotics on nutrition and immunological status of the elderly showing health-status changes due to infections such as pneumonia.

While most previous studies used viable probiotic bacteria, we used heat-killed *Lactobacillus plantarum* strain b240 (b240) in the present study. We have found that Peyer's patch (PP) cells stimulated with heat-killed b240 produce higher amounts of IgA than those stimulated with 150 other heat-killed lactic acid bacteria, and that IgA production in such PP cells is comparable to that in cells stimulated with viable b240 (Yamahira et al. 2006). In addition, the oral intake of heat-killed b240 for 3 weeks in clinical settings revealed

significant increase of secretory IgA (sIgA) levels in the saliva (Kishi *et al.* 2006). Moreover, oral intake of heat-killed b240 for 12 weeks is known to accelerate salivary IgA secretion even in the elderly (Kotani *et al.* 2010). This ability to efficiently induce sIgA in mucosal tissues, relevant to the adaptive immune response, prompted us to explore the ability of b240 to exert *in vivo* protective effects against various infectious diseases. The effectiveness of oral administration of heat-killed b240 has been confirmed on mouse models of *Salmonella* and influenza infection (Ishikawa *et al.* 2010; Kobayashi *et al.* (in press)). Heat-killed lactic acid bacteria have an extended shelf life, are easier to store and transport, and exhibit less interaction with other components of food products during storage.

In this study, the inhibitory effects of oral heat-killed b240 on pneumococcal pneumonia in mice infected with *S. pneumoniae* were investigated.

MATERIALS AND METHODS

Bacteria Preparation and Infection

Penicillin-sensitive *S. pneumoniae* ATCC 49619 strain was used. Bacteria were incubated in horse blood agar for 20 h at 37°C, scraped and suspended in Mueller-Hinton II Broth (Eiken Chemicals, Tokyo, Japan), mixed with Strepto Haemo supplement (Eiken Chemicals, Tokyo, Japan), and cultured for 6 h at 37°C at 250 rpm (BR-22FP; TAITEC Co., Ltd, Saitama, Japan). Bacteria were harvested by centrifugation for 10 min at $3000 \times g$. The harvested bacteria were suspended in saline at a concentration of 1×10^6 colony-forming units (CFU)/ml, as determined by the optical density method.

The mice were challenged with or without pneumococci on the day following the end of b240 treatment. *S. pneumoniae* infection was induced by intranasal inoculation of 0.05 ml of bacterial suspension (5×10^4 CFU/mouse) into mice under anesthesia (pentobarbital sodium, 70 mg kg⁻¹ intraperitoneally (ip)).

Laboratory Animals

Five-week-old male CBA/J specific-pathogen-free mice (body weight, 16–20 g)

were purchased from Charles River, Japan. The details of the CBA/J mouse model of pneumococcal pneumonia has been previously described (Yanagihara *et al.* 2007). All mouse experiments were performed in accordance with the guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Lactobacillus pentosus strain b240 and treatment regimes

Lactobacillus pentosus strain b240 (ONRIC b0240: b240) was isolated from fermented tea leaves by Okada *et al.* (Okada *et al.* 1986). Strain b240 was initially identified as *L. plantarum*. Recently, Bringel *et al.* proposed re-classification of the *L. plantarum* group on the basis of the *recA* gene sequence (Bringel *et al.* 2005). On the basis of their proposal, we re-classified b240 as *L. pentosus*.

Strain b240 was grown in MRS broth (Beckton Dickinson, Franklin Lakes, NJ, USA) at 33°C for 24 h. The culture was washed twice with saline and suspended in deionized water, followed by autoclaving at 121°C for 15 min. The heat-killed bacterial suspension was freeze-dried for storage. It was resuspended in saline before use.

Mice received orally by gavage b240 at a rate of 500 mg kg^{-1} once in a day (total: $12.5 \text{ mg kg}^{-1} \text{ day}^{-1}$) for 21 days before *S. pneumoniae* infection. Saline was administered orally as negative control.

For survival study, mice were observed and evaluated for 14 days. For sample collection, mice were killed under anesthesia 2 days after infection, and the lungs were dissected under aseptic condition and suspended in saline. Lung tissue fixation was performed as described previously (Seki *et al.* 2004a). For tissue sectioning, lungs were excised and immersed in 10% phosphate buffered formalin. Paraffin embedding and tissue staining with hematoxylin and eosin (H&E) were performed using the standard methods.

Bacteriological Examination

Mice were killed under anesthesia 2 days after *S. pneumoniae* inoculation. For bacteriological examination, lungs were dissected under aseptic conditions and suspended in saline (1 ml). They were homogenized using a Shake Master NEO (Bio Medical Science, Tokyo, Japan). The lung homogenates were quantitatively inoculated onto blood agar plates by serial dilution, followed by incubation for 20 h. The lowest detectable bacterial level was 50 CFU ml^{-1} .

Enzyme-linked immunosorbent assay

Concentrations of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), keratinocyte-derived cytokine (KC), and macrophage inflammatory protein (MIP)-2 in aqueous lung extracts were assayed by enzyme-linked immunosorbent assay using mouse Quantikine Kits (R&D Systems, Inc., Minneapolis, MN, USA). The lung homogenates were centrifuged for 60 min at $2,000 \times g$ at 4°C , and the supernatants were collected as aqueous lung extracts and used in the assay.

Western blotting

Proteins in the lung homogenates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS; pH 7.2–7.4) containing 0.05% Tris-buffered saline with Tween 20 (TBS-T) and incubated with goat antibody (Ab) directed against toll-like receptor 2 (TLR-2) (Santa Cruz, CA, USA; diluted 1:500), TLR-4 (Santa Cruz, CA, USA; diluted 1:8000), Total c-Jun N-terminal kinase (abcom, USA; diluted 1:8000), phosphorylated c-Jun N-terminal kinase (abcom, USA; diluted 1:8000) or rabbit Ab directed

against β -actin (Sigma-Aldrich; diluted 1: 8,000) for overnight at 4°C. The membranes were then washed with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (Santa Cruz, CA, USA; diluted 1:2,000) or HRP-conjugated anti-rabbit IgG (Santa Cruz, CA, USA; diluted 1:8,000) for 1 h at room temperature. After 2 additional washes, the signals were developed using an enhanced chemiluminescence (ECL) plus western blot detection kit (Amersham, Arlington Heights, IL).

Statistical Analysis

All data were expressed as means \pm SD and compared between the saline and b240 administration groups. Survival curves after *S. pneumoniae* infection were estimated by the Kaplan–Meier method, and their homogeneity was evaluated by the log-rank test. The body weight of mice infected with *S. pneumoniae* was analysed by repeated measures analysis of variance (ANOVA). Differences between groups were tested for significance using unpaired *t*-test. Values with $P < 0.05$ were considered statistically significant and analysed using the SAS software R 9.1 (SAS Institute Japan, Tokyo, Japan).

RESULTS

Long-term survival rates and nutrition status

In order to assess the effects of b240, long-term survival rate was analyzed in *S. pneumoniae*-infected mice that were treated with b240 or saline (Fig. 1). In the controls (saline-treated mice), the mice started to die around day 5 after infection, around 50% of mice died by day 9, and all mice died by day 14, which was the end of the observation period. In contrast, all mice treated with b240 survived the first 9 days after infection, and 30% of mice survived even on day 14 (saline vs. b240 treated; $P = 0.0664$).

Although the difference of survival mice number was not significant between saline or b240-treatment, we next investigated the nutrition status assessed by body weight loss (Fig. 2). The mice treated with b240 significantly maintained the starting body weight until day 5 after infection. The average body weights of the mice in the b240 group remained higher than those of the mice in the control group throughout the observation period. Notably, the weight of the mice in the b240 group were statistically higher than those of the mice in the control group between day 2 and day 5 after *S. pneumoniae* infection (saline vs. b240 treated; $P < 0.05$, from Day 2 to Day 5).

Inhibitory effects of bacterial growth and reduction of pneumonia severity

The number of *S. pneumoniae* in lung tissues showed a 10–100-fold decrease in b240-treated mice on day 2 after infection significantly ($P = 0.032$), compared with that in the saline-treated mice (Fig. 3). However, there were no statistically significant differences in bacterial numbers in the lungs between the 2 groups after day 4 of infection (data not shown).

Pathological examination of the lungs of control mice showed severe pneumonia (Fig. 4A and E), whereas those of b240-treated mice exhibited only mild to moderate pneumonia 2 days after infection (Fig. 4B and F). These differences are confirmed in terms of pathological features such as airspaces filled with inflammatory cells (arrow) and epithelial hyperplasia (arrowhead) (Fig. 4A and E), whereas b240-treated mice showed only very mild bronchopneumonia 2 days after *S. pneumoniae* infection (Fig. 4B and F). Similar pathological differences were also observed on day 4, although the differences became relatively smaller compared with those observed on day 2 (data not shown).

In mice treated with either b240 or saline, but without pneumococcus infection, we did not find inflammatory changes (b240 only; Fig. 4C and G, saline only: Fig. 4D and H, respectively).

Reduced induction of inflammatory cytokines in the lungs

Diverse cytokines are known to play a pivotal role within the airways during the course of infectious diseases, and the deleterious activation of inflammatory cells, including neutrophils, macrophages, and T cells, is associated with tissue injury (Seki *et al.* 2004a; Yanagihara *et al.* 2007; Seki *et al.* 2010). In line with this notion, the amounts of inflammatory cytokines in the lung tissues were investigated. TNF- α is expressed early in animal models of pulmonary infection caused by *S. pneumoniae*. It is a main trigger in the production of IL-6 as one of the most important mediators of inflammation (Kosai *et al.* 2008). KC, as vELR⁺ CXC chemokines, including growth-related genes (GRO- α ; CXCL1) and MIP-2(CXCL2/3; GRO- β/γ) were identified in the lung preparations obtained from mice with pneumonia (Strieter *et al.* 2002). Accordingly, the levels of TNF- α , IL-6, MIP-2, and KC were analyzed in the lung tissue homogenates on day 2 after *S. pneumoniae* infection. Intrapulmonary levels of TNF- α , IL-6, MIP-2 cytokines or chemokines were elevated significantly in the lungs by *S. pneumoniae* infection, indicating the presence of aggravated pulmonary inflammation induced by the infection (Fig. 5A-C). The KC level tended to decrease by the oral uptake of b240 (Fig. 5D), but the difference from that of the control

group was not statistically significant at Day 2. In the mice without pneumococcal infection, no production of cytokines and chemokines were observed either saline-or b240 treated.

The suppression of all of them was markedly evident on day 2 after the infection and became marginal thereafter at Day 4 and 7 (data not shown).

Mitogen-activated protein (MAP) kinase signalling in b240 treated lungs

We next performed kinetics of another immune-related molecules, such as MAP kinase to investigate more detailed mechanisms of this probiotic effect. We found activation of p38 in *S. pneumoniae* infected mice, compared with non-infected mice. However, neither saline or b240 treatment decreased its activation,

In contrast, activation of JNK was suppressed significantly in the lungs of b240 treated mice although *S. pneumoniae* infection. According to cytokines/chemokines expressions and pathological changes (Fig. 6).

Expression of Toll-like receptors

Furthermore, we analysed the expression level of TLR2, known as key pattern recognition-type receptor for *S. pneumoniae* on mucus membrane (Seki *et al.* 2004a; Akira

2006; Miyauchi *et al.* 2008). Innate immune systems have been suggested to play a critical protective role against pathogens on the mucus membrane in the airways (Gill *et al.* 2000; Gill and Guarner 2004; Racedo *et al.* 2006; Villena *et al.* 2008). Significant upregulation of TLR2 was observed 2 days after the infection in whole lung tissues of mice treated with oral b240 (Fig. 6). In addition, expression level of TLR4 was increased significantly in the lungs of mice treated with b240. These expression were not observed in the lungs of non-infected mice either saline or b240 treated.

DISCUSSION

Oral intake of heat-killed *Lactobacillus pentosus* strain b240 did not show the prolongation of survival time, but led to significant reduction of body weight loss, as well as reduced bronchitis, in the present pneumococcal pneumonia model. There was a significant reduction in the secretion level of inflammatory cytokines in b240-treated mice in accordance with the alleviated pathological severity of pneumonia. We tried more 3 days administration of b240 after infection, but could not continue more prolonged oral administration because the mice became weakened after *S. pneumoniae* infection. In addition, survival and weight loss rates in above regimen were same as the regimen stopped after infection (data not shown).

Effective pulmonary host defense against respiratory pathogens is believed to be mediated via innate and adaptive immune responses such as the stimulation of phagocytosis by alveolar macrophages and recruited neutrophils, IgA secretion, and upregulation of pattern recognition receptors, including TLR2 and 4 (Akira 2006). If pneumococci overcome these host defenses and gain entry into the bloodstream, systemic protection is afforded by anti-capsular antibodies (Lee *et al.* 2005). Such defenses are also produced by a spatiotemporally regulated inflammatory response coordinated by diverse cytokines (Seki *et*

al. 2004a; Akira 2006; Yanagihara *et al.* 2007).

In our infection model, b240 induced faster clearance of the pathogen, which was reflected by the reduced number of pneumococci in the lung and reduced body weight loss in the treated group compared to that in the control group. Administration of b240 was able to induce a rapid and early increase in polymorphonuclear leukocyte (PMN) cells (data not shown). Although PMNs are a key component of the host defense response against invading pathogens, they have also been implicated as mediators of tissue injury (Seki *et al.* 2004a, 2004b; Yanagihara *et al.* 2007; Seki *et al.* 2010). The regulation of inflammatory responses by anti-inflammatory cytokines prevents damage to the host. *Lactobacillus* has been suggested to participate in tissue protection against the deleterious effect of an ongoing inflammatory processes (Blum *et al.* 1999). Intrapulmonary levels of TNF- α , IL-6, and MIP-2 were elevated in the lung tissue by *S. pneumoniae*, indicating the presence of aggravated pulmonary inflammation. The reduced induction of these cytokines by oral b240 administration clearly indicates the beneficial effect of b240 to ameliorate the tissue injury caused by local inflammation. The histopathological examination of lung tissues showed that both the inflammatory response and tissue damage were significantly reduced in mice treated with b240. These results suggest that b240 treatment beneficially regulates the balance

between Th1 and Th2 cytokines, biased to a protective inflammatory response against infection. TNF- α could also inhibit the onset of bacteremia by stimulating antibody production and neutrophil recruitment and by promoting the antimicrobial activity of phagocytic cells (Takashima *et al.* 1997). The suppression was markedly evident on day 2 after the infection and became marginal thereafter.

In order to evaluate the relationships between cytokines and the pathological changes, the related molecules, not only TLRs but also MAPK were investigated in terms of activation in b240-treated lungs. The production of cytokines and chemokines in response to components of microbiological agents is stimulated by TLRs and mediated by MAPK signaling (Xu *et al.* 2008). Activation of MAPK-related molecules, such as JNK, was observed. These data suggest that MAPK signalling cytokine secretion pathways may be involved in the lung damage noted in pneumococcal infected mice and b240 could suppress the inflammation via this pathway. However, we could find suppression of JNK, but not p38 in this study. Kenzel S. *et al.* reported P38, but not JNK, is critical for phagocytosis of GBS by macrophages. RAW 264.7 cells (Kenzel *et al.* 2006). Further investigation about these discrepancy will be needed.

The mucosal immune system is able to respond to invading pathogens in the

respiratory tract by producing pathogen-specific sIgA Ab (Lee *et al.* 2005; Racedo *et al.* 2006, Fukuyama *et al.* 2006., Tamura *et al.* 1990). Mice treated with *L. casei* for 2 days showed higher levels of anti-pneumococcal serum IgG and bronchoalveolar lavage (BAL) IgA than those in the control group (Racedo *et al.* 2006). In this study, we investigated the production of total IgA Ab in the nasal lavage fluid (NLF) that may cause non-specific reactions to streptococcal colonization, however, those in NLF of b240 group on day 2 was not significantly higher than that of the control group (data not shown). In this context, *S. pneumoniae*-specific sIgA level should be measured for precisely understanding the protective effect of b240 through pathogen-specific sIgA Ab in a future investigation.

TLRs play a key role in the innate immune recognition of multiple bacterial pathogens, including *S. pneumoniae* (Seki *et al.* 2004a; Akira 2006; Miyauchi *et al.* 2008). Among them, TLR2 is usually involved in the recognition of gram-positive bacteria, and its upregulation was induced by the administration of *L. paracasei* F19 (Akira 2006; Cammarota *et al.* 2009). We observed significant expression of TLR2 protein in the b240-treated mice, indicating the augmentation of innate immune response by b240. In addition, TLR4 was also upregulated in b240-treated mice. These results suggested b240 could induce novel innate immune reactions and might be beneficial for protection against bacteria invasion. The

detailed mechanism underlying the upregulated expression of TLR2 and 4 by b240 needs to be urgently with further.

Collectively, we found not only TLR2, but also TLR4 were significantly induced in the mice lungs treated by b240 oral-intake. Furthermore, we examined kinetics of another immune-related molecules, such as MAP kinases and found significant down-regulated of these molecules' activation according to decrease of inflammatory cytokines/chemokines in b240-treated mice. These interesting results believed to explain anti-inflammatory and host defense mechanisms of b240-treated pneumococcal pneumonia by TLRs activation.

In addition, we focused on probiotics *L. pentosus* strain b240 in this study because we previously found this strain showed much higher induction of IgA in vitro experiments (Yamahira S. et al. 2006). In brief, we extracted peyer's patch cells from small intestine of mice, and soluble IgA in supernatant of the cell culture was measured by ELISA. *L. pentosus* strain b240 was the most effective strain for IgA production among many different bacterial strains. These results were also believed to explain the mechanisms how the Lactobacilli decrease the severity of pneumonia in vivo. We could not investigate another strain unfortunately in this study, but further examination to detect the potential of other probiotic strain in vivo will be needed.

In conclusion, the results obtained in this study may lead to new insights concerning the use of b240 as an oral adjuvant or as an oral vaccine vector for a wide range of infectious lung diseases. Further detailed studies are necessary to elucidate the molecular mechanisms underlying the effects of b240 in pneumonia in animal models and humans.

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FIGURE LEGENDS

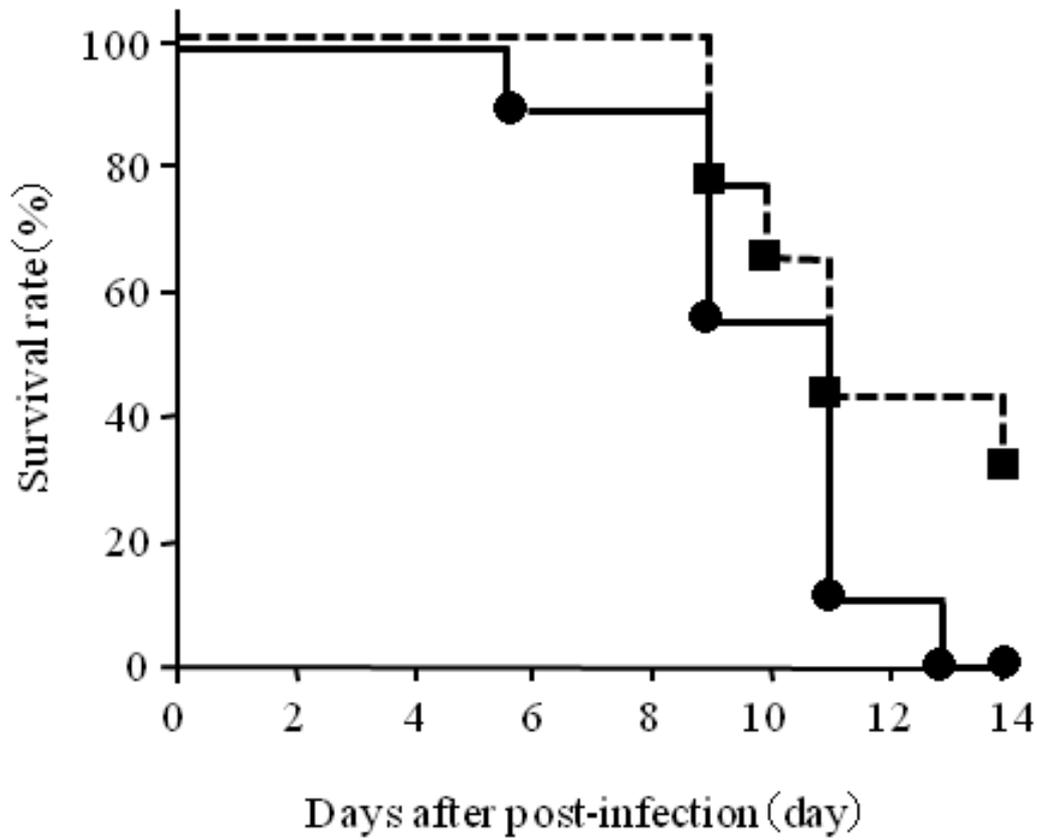


Fig. 1 Survival rates of mice treated with saline or b240 infected with *S. pneumoniae*. Mice were treated with saline or b240 from 21 days before *S. pneumoniae* infection. Each group comprised 9 mice (● and normal line: saline-treated mice, and ■ and dotted line: b240-treated mice).

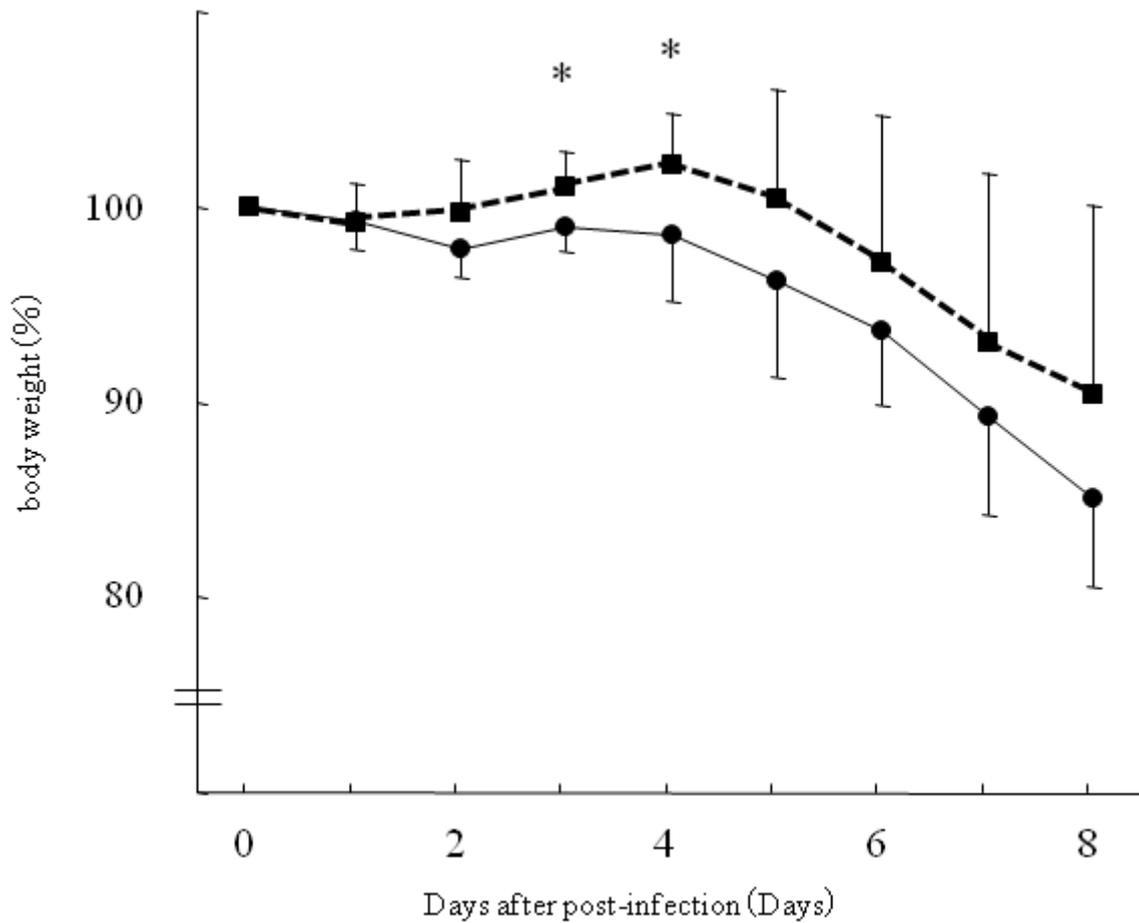


Fig. 2 The body weight of *S. pneumoniae*-infected mice treated with saline or b240. Mice were treated with saline or b240 from 21 days before *S. pneumoniae* infection. Each group comprised 9 mice (● and normal line: saline-treated mice, and ■ and dotted line: b240-treated mice). Body weight loss was significantly reduced in b240-treated mice compared with that in saline-treated mice, from day 2 to day 5 after infection ($P = 0.0133$, 0.0256 , respectively). Statistically significant differences are indicated as follows: * $P < 0.05$ compared to saline and b240 groups at day 2, 3, and 4.

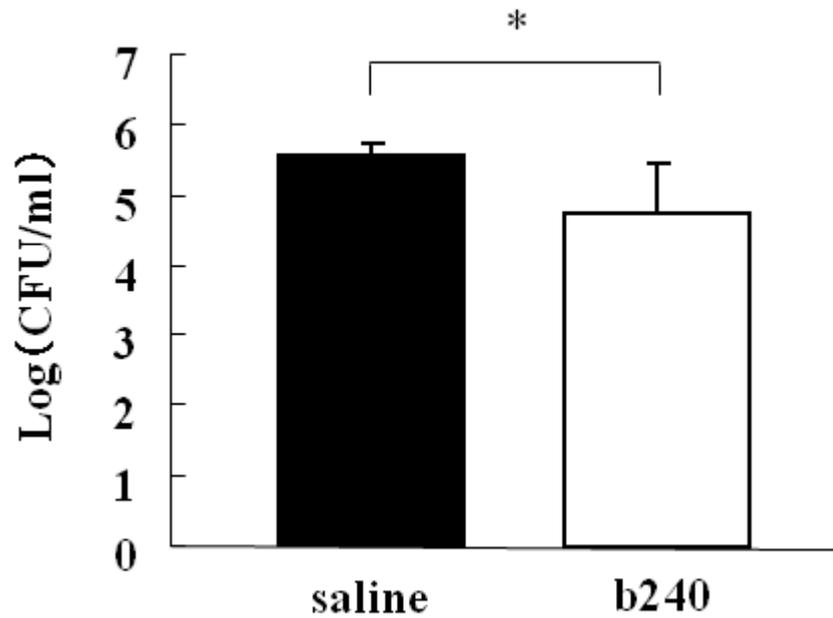


Fig. 3 Bacterial numbers in the lungs of *S. pneumoniae*-infected mice treated with saline or b240. Bacterial numbers were significantly reduced in b240-treated mice compared with that in the saline-treated mice at day 2. Each group comprised 6 mice. * indicates a statistically significant difference ($P = 0.032$) compared to saline and b240 groups.

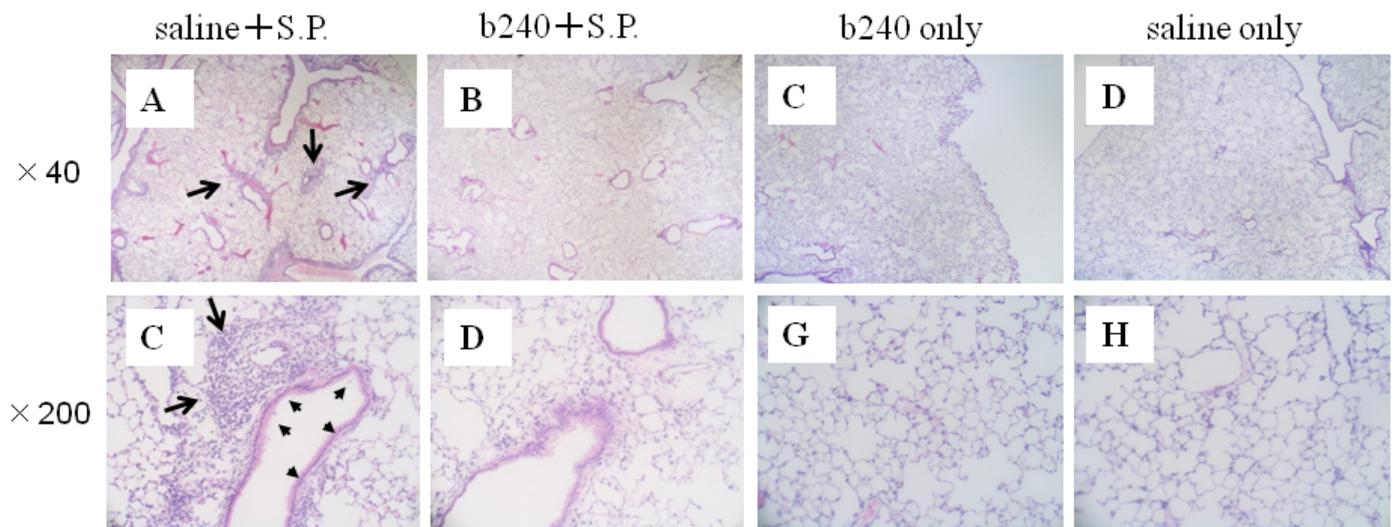


Fig. 4 Pathological analysis of the lungs of *S. pneumoniae*-infected or non-infected mice treated with saline or b240. Lungs were collected 2 days after with or without *S. pneumoniae* infection and representative photos are shown. A–H) eosin-stained tissue sections at magnifications of A) -D): ×40, and E) - H): ×200. A) and E): saline-treated (control) mice with pneumococcal infection, B) and F): b240-treated mice with pneumococcal infection, C) and G): b240-treated without pneumococcal infection, and D) and H): saline-treated without pneumococcal infection, respectively.

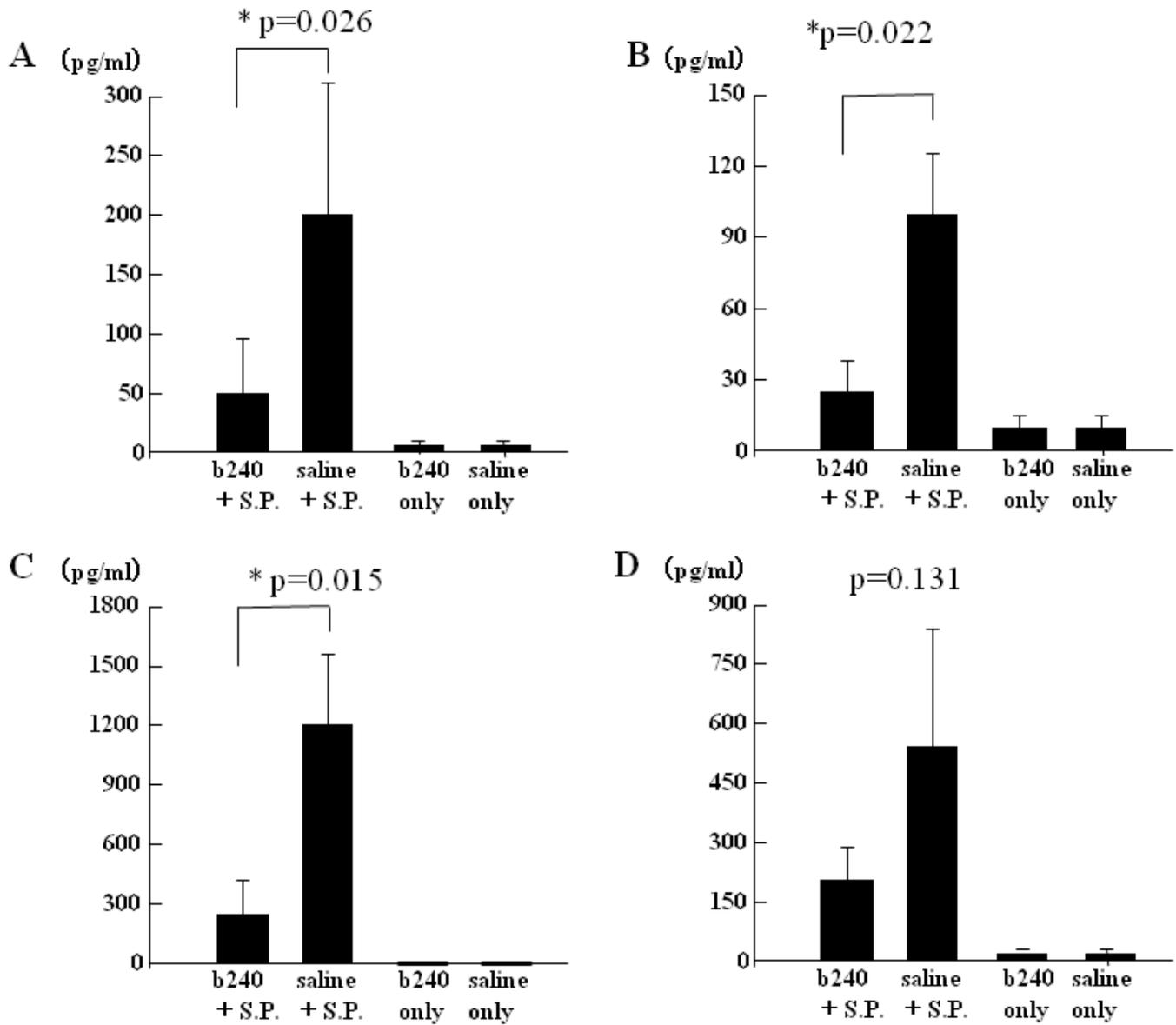


Fig. 5 Concentrations of inflammatory cytokines on day 2 in the lungs of mice treated with saline or b240. These mice were infected or non-infected with *S. pneumoniae*. (A) TNF- α , (B) IL-6, (C) MIP-2, and KC (D), respectively. Each group comprised 4–7 mice. (day 2 and 4, n = 4; day 7, n = 7). * indicates statistically significant difference ($P < 0.05$) compared to all other groups.

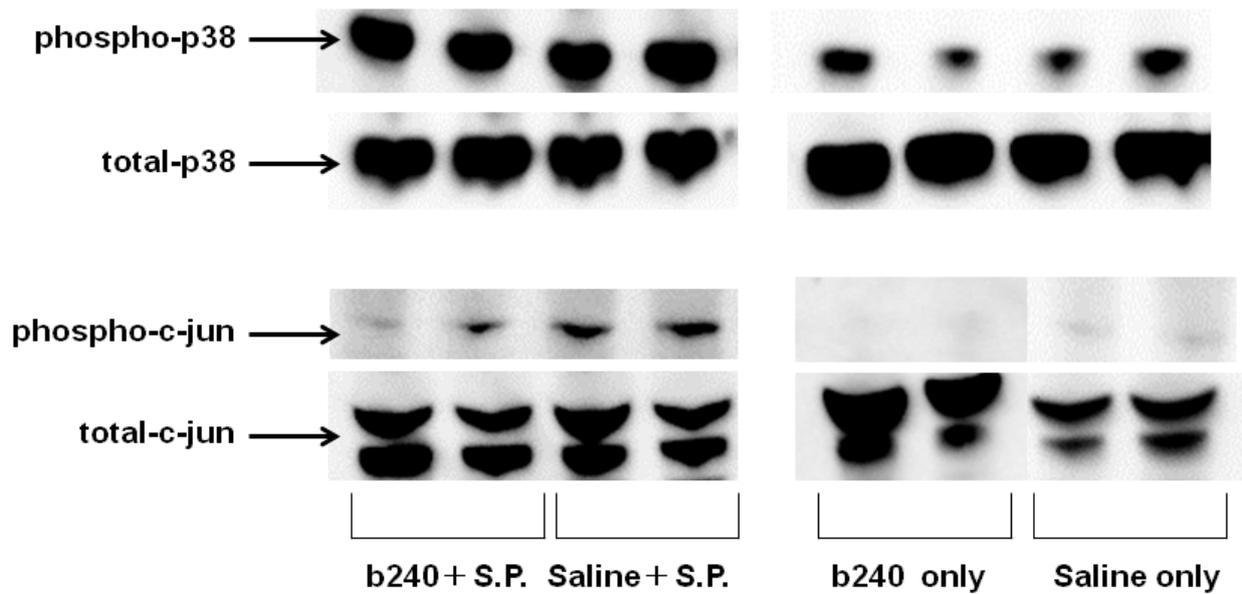


Fig. 6 Activation of p38 and c-Jun N-terminal kinase (JNK), signalling factors in the Toll-like receptor-mediated cytokine production pathway, in the whole lungs of S. pneumoniae-infected or non-infected mice treated with saline or b240 on day 2. Levels of activation were analysed by Western blotting, using antibodies directed against each phosphorylated (activated) form and intact form. The experiment was repeated three times; representative results are shown.

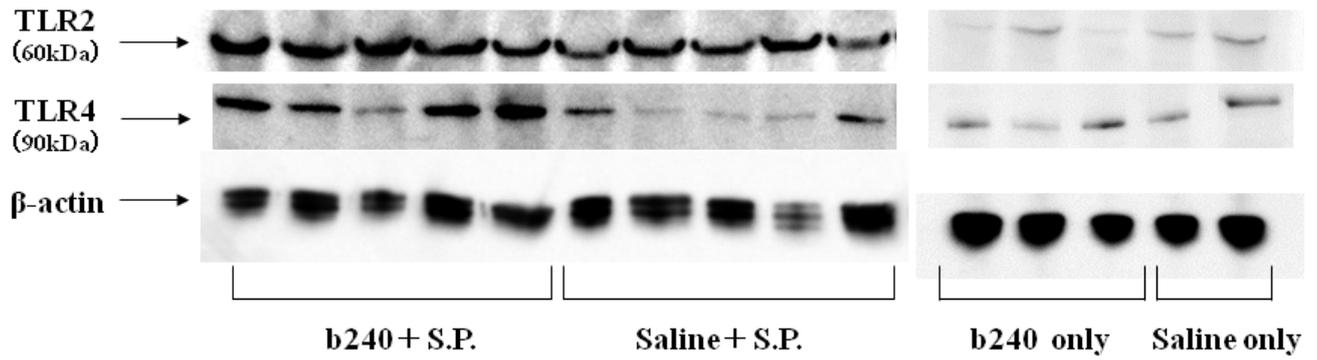


Fig. 7 Increased expression of TLR2 and TLR4 in the whole lungs of *S. pneumoniae*-infected or non-infected mice treated with saline or b240. Lungs were collected 2 days after *S. pneumoniae* infection, and representative photos are shown.