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ORIGINAL ARTICLE

Immunomodulatory effects of specific bacterial components of *Lactobacillus plantarum* KFCC11389P on the murine macrophage cell line RAW 264·7

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Abstract

Aims: The objective of this study was to investigate the ability of specific bacterial components of *Lactobacillus plantarum* KFCC11389P to induce anti-inflammatory mediators in cell cultures of the murine macrophage cell line, RAW 264·7.

Methods and Results: The RAW 264·7 cells were stimulated with viable bacterial cells (VC), heat-killed (HK) cells, cell walls (CW) or ultrafiltrates of metabolic products (UF). An increase in the levels of tumour necrosis factor (TNF)-α was observed in VC, HK and CW, but this effect was much lower in UF. VC stimulated higher levels of interleukin (IL)-6 releases as well as nitric oxide production than HK. In contrast, UF and its separated molecule, fraction 4, were much strong IL-10 inducers. Fraction 4 (8·1 kDa), especially, inhibited the production of pro-inflammatory cytokines, IL-6 (89% decrease) and TNF-α (55% decrease), in lipopolysaccharide (LPS)-stimulated murine macrophages.

Conclusions: The results of this study indicate that metabolic products of *Lact. plantarum* KFCC11389P could influence the immune-modulating activity via IL-10, and pretreatment with this specific molecule could inhibit LPS-induced release of IL-6 and TNF-α.

Significance and Impact of the Study: Our findings suggest that the specific molecules of *Lact. plantarum* KFCC11389P may be useful for the treatment of acute inflammatory responses such as Crohn's disease or ulcerative colitis.

Introduction

Lactic acid bacteria are Gram-positive and widespread in nature, fresh and fermented vegetables, meats, fish, dairy products, as well as colonizing the gastrointestinal tract of animals. Certain members of the *Lactobacillus* genus are believed to have beneficial effects on the health of the host when they are ingested. Possible health effects include: modulating the immune system; increasing the antibacterial, anticancer and antimutagenic activities; and preventing cancer recurrence (Gill *et al.* 2000; Nikoskelainen *et al.* 2003; Haza *et al.* 2004; Nomoto 2005; Suzuki *et al.* 2008).

Macrophages are tissue-based phagocytes derived from monocytes and they play the central role in initiating the first defence line of host immunity. Macrophages can be activated by microbial components, such as endotoxin, lipopolysaccharides (LPS) and lipoteichoic acids (LTA). Activated macrophages phagocytize micro-organisms, secrete pro-inflammatory cytokines and nitric oxide (NO) and present antigens to helper T cells (Schereiber *et al.* 1985; Celada and Nathan 1994). Macrophages may regulate immunity via enhanced production of several cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α. These cytokines contribute to defence mechanisms of the host immunity in response to external

invasion, but they may induce immuno-pathological disorders when secreted in excess. TNF-α elicits several physiological effects, such as septic shock, inflammation and cytotoxicity, and IL-6 is believed to be an endogenous mediator of LPS-induced fever (Sarih *et al.* 1993; Fukuo *et al.* 1995; Laskin and Pendino 1995; Brown *et al.* 2007).

Several lactic acid bacterial strains were reported to exert *in vitro* stimulatory properties on innate immune cells including macrophages and natural killer cells, and to induce the production of both pro-inflammatory and anti-inflammatory cytokines. Some components of lactic acid bacterial cell walls (CW), such as peptidoglycans and LTA, have been shown to be involved in cytokine induction. Most studies have demonstrated that viable bacterial cells (VC), heat-killed (HK) cells or ultraviolet (UV)-killed cells of *Lactobacilli* and *Bifidobacterium* species, as well as their cell components, are capable of stimulating the production of NO and cytokines such as IL-6 and TNF-α in macrophage cell lines (Park *et al.* 1999; He *et al.* 2002; Morita *et al.* 2002; Matsuguchi *et al.* 2003; Kim *et al.* 2007). However, some investigators also indicated that the effects of inducing cytokines in VC are higher than those of HK cells (Miettinen *et al.* 1996; Cross *et al.* 2004), and hence some controversy still remains.

Studies for anti-inflammatory effects of lactic acid bacteria have also been reported. Earlier study of *Lactobacillus acidophilus* showed IL-10 secretion from J774·1, murine macrophage cell line, with the treatment of heat-inactivated forms of lactobacilli (Morita *et al.* 2002). Bifidobacteria, especially those from healthy infants, stimulated macrophage-derived IL-10 production (He *et al.* 2002). However, anti-inflammatory cytokine induction by innate immune cells was solely focused on the HK forms of bacterial cells, and these strains also induced the secretion of much pro-inflammatory IL-6 and TNF-α. The overexpression of the pro-inflammatory cytokines may impair the organ-specific auto-immunity balance. In contrast, IL-10 blocks the induced synthesis of TNF-α, IL-1β and IL-6 by human monocytes (Waal-Malefyt *et al.* 1991) and mouse peritoneal macrophages (Fiorentino *et al.* 1991; Mosmann 1994), and stimulates B cells (Hessle *et al.* 1999, 2000). Presumably, IL-10 may be useful in the treatment of endotoxic shock and could play an important role in acute inflammatory responses. In addition, the balance between the pro-inflammatory and anti-inflammatory cytokines must be important for the host immunity (Park *et al.* 1999; Morita *et al.* 2002).

In this study, we compared the effects of VC, their CW, HK bacterial cells as well as metabolic products of *Lactobacillus plantarum* KFCC11389P on induction of mediators for pro-inflammatory and anti-inflammatory responses at the cellular level. The main focus of this study was to contrast the cytokine response patterns observed following the treatment of metabolic products in LPS-stimulated macrophages by concentrating on the relative levels of induction of known pro-inflammatory and anti-inflammatory cytokines. Finally, we characterized the partially purified active molecule separated from the metabolic products of *Lact. plantarum* KFCC11389P using gel filtration chromatography and SDS-PAGE.

Materials and methods

Preparation of various bacterial components of *Lactobacillus plantarum* KFCC11389P

Lactobacillus plantarum KFCC11389P was grown in modified de Mann Rogosa Sharpe (MRS) medium (peptone : glucose : yeast extract : sodium citrate : dipotassium phosphate = 1: 2 : 0·5 : 0·5 : 0·5; Vision Biochem Co., Sungnam, Korea) at 37°C for 18 h in 300-l Pilot Fermentation System and harvested by centrifugation (Tubular Bowl Type Centrifuge, Kansai, Japan). Half of the VC were lyophilized (Ushin Engineering Co., Yangju, Korea), and the other half were used for CW preparations following rupture by five successive passages in a cell homogenizer (Avestin, Ottawa, Canada) at 30 000 psi. The lysate was heated at 75°C for 30 min in order to inactivate autolytic enzymes, and the unbroken cells and cell debris were harvested by centrifugation (Tubular Bowl Type Centrifuge). Then, the walls were recovered from the supernatants by centrifugation at 20 000 ***g*** for 30 min at 4°C (Vision Biochem Co). Finally, the pellets of CW were resuspended in phosphate buffer saline (PBS; pH 7·0; Gibco BRL) and frozen until use (Vinderola *et al.* 2004). For HK cell preparations, the VC were heated for 30 min at 90°C (Park *et al.* 1999; Suzuki *et al.* 2008). The culture supernatants were filtered by membrane filtration fitted with dual filtering system (Sartorius, Germany). To dialyse the metabolic products, they were subject to ultrafiltration by Centriprep centrifugal filter devices with Ultracel YM-10 membrane (Millipore, USA) at 3000 ***g*** for 30 min. Retained and filtered fractions were adjusted to the initial concentration (Ménard *et al.* 2004). For the treatment of RAW 264·7 cells, each component was diluted with Dulbecco's modified Eagle's medium (DMEM; Hyclone, UT, USA) to the desired concentration.

Murine macrophage cells maintenance

The RAW 264·7 murine macrophage cell line was maintained in DMEM, supplemented with 10% (v/v) foetal bovine serum and gentamycin (20 μg ml^{-1}; Sigma) at 37°C in a 5% CO_2 humidified incubator. For experiments involving VC, RAW 264·7 cells were cultured in medium

containing gentamycin (50 μg ml^{-1}) as described previously (Cross *et al.* 2004).

Cell viability

The cytotoxicity of the various bacterial components on RAW 264·7 cells was assayed by MTT (3-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) according to the method previously described (Mosmann 1983; Todorov *et al.* 2005). The RAW 264·7 cells at a density of $5{\cdot}0 \times 10^5$ cells ml^{-1} were treated with indicated concentrations for 48 h. Then, MTT (0·5 μg ml^{-1}) were added (10%, v/v) to the cell culture and the cells were continued to be incubated for 4 h. Then, 0·04 N HCl/isopropanol was added to solubilize the formazan. Optical density (OD) at 540 nm was determined with a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as the OD of formazan formed in the cells, and was calculated as follows: cell proliferation (%) = (OD of treatment group/OD of control group) × 100.

NO determination

The same procedures were followed for NO production as previously described. After 48 h, the culture supernatants were collected and analysed for NO production via the Griess reaction (Park *et al.* 1999; Kim *et al.* 2007). Nitrite, which is produced from NO in the presence of H_2O and O_2, accumulates in the culture medium and reflects the amount of NO production. The concentration of nitrite was determined by mixing 100 μl of the culture supernatant with equal volumes of Griess reagent [1% sulfanilamide in 50% H_3PO_4 (w/v), 0·1% *N*-1-napthylethylenediamine dihydrochloride in 1 : 1; Ding *et al.* 1988]. The colour development was measured by the absorbance at 540 nm. The positive control, LPS (2 μg ml^{-1}, from *Escherichia coli*; Sigma), was used and then the NO production was measured by a 96-well plate reader (Molecular Devices) at 540 nm.

Induction of cytokine release

The cells were stimulated as described before, except that the culture time was changed to 24 h (Park *et al.* 1999; Kim *et al.* 2007). The levels of cytokines produced were compared against cytokine levels observed in RAW 264·7 cells in DMEM alone as a negative control and cells cultured with LPS (2 μg ml^{-1}) as a positive control. Modified MRS medium (1%) was also used as another control group. For the experiments of the effect of pretreatment of fraction 4 on LPS-induced release of IL-6, TNF-α and IL-10, the cells were serum-starved for 4 h before treatment. The pretreated cells were then further cultured with LPS (2 μg ml^{-1}) for 24 h. Overall culture supernatants were collected and stored at −20°C until cytokine analysis (Waseem *et al.* 2008). The induction of various cytokines was assayed by commercial ELISA kit (BD OptEIA™ Set; BD Biosciences, CA, USA) for TNF-α, IL-1β, IL-6 and IL-10 according to the manufacturer's recommendations, and absorbance was measured at 490 nm using the 96-well plate reader (Molecular Devices) (Morita *et al.* 2002; Kim *et al.* 2007).

Effects of anti-IL-10 monoclonal antibodies (mAb) on pro-inflammatory cytokine release

In a separate experiment, RAW 264·7 cells were incubated with different concentrations of anti-rat IL-10 mAb (R&D Systems Inc., Minneapolis, MN, USA) to elucidate that the fraction 4 from UF could influence the immune-modulating activity via IL-10 and the role of IL-10 in regulating pro-inflammatory cytokine release. Briefly, RAW 264·7 cells were incubated with fraction 4 (0·1%) supplemented with different concentrations of anti-IL-10 mAb for 6 h, and subsequently stimulated with LPS for 24 h. The cytokine levels were then measured by ELISA (Spittler *et al.* 1999; Yokoyama *et al.* 2004).

Preparation and partial purification of ultrafiltrates of metabolic products by gel filtration chromatography

The ultrafiltrate samples after 10-kDa cut-off ultrafiltration and dialysis were fractionated by gel filtration chromatography (TSK-Gel Super SW 2000; Tosoh Bioscience, Montgomeryville, PA, USA). The gel filtration column was equilibrated with an equilibrium buffer composed of 100 mmol l^{-1} of phosphate and 100 mmol l^{-1} of sodium chloride at pH 7·4. The sample was manually fractionated by six peaks, and the molecular weight of the fractionated samples was estimated by gel filtration calibration kit from GE Healthcare Biosciences (Little Chalfont, Buckinghamshire, UK). The absorbance was monitored at 280 nm and used for assay of pro-inflammatory and anti-inflammatory cytokines. The modified MRS medium after 10-kDa cut-off ultrafiltration and dialysis was also applied on gel filtration chromatography as a control. SDS-PAGE was performed on 20% polyacrylamide slab gel as described by Laemmli (1970). Proteins were stained with 0·25% Coomassie Brilliant Blue R-250 (ICN Biomedicals, Aurora, OH, USA). Molecular weight marker was purchased from Bio-Rad (USA).

Statistical analysis

All statistical analyses were performed using SPSS software (ver. 13.0; SPSS Inc., Chicago, IL), and mean values were

expressed with SD of three independent experiments. Data were evaluated with one-way ANOVA and compared using Scheffé and Duncan's multiple tests at $P \leq 0{\cdot}05$ between groups.

Results

Morphology of RAW 264·7 and cell proliferation

When RAW 264·7 cells were cultured with LPS, normal proliferation was suppressed, and change in the circular morphology was observed. When cells were stimulated with VC, HK or CW, morphological alterations were clearly observed in association with macrophage activation, whereas treatment with UF and a negative control showed circular morphology and normal proliferation (Fig. 1). Figure 2 shows the inhibitory effects of VC, HK and CW treatments on the viability of RAW cells in a dose-dependent manner through MTT assay. In addition, there were no differences between negative control, modified MRS medium and UF treatment on cell viability.

NO production of *Lactobacillus plantarum* KFCC11389P

The RAW 264·7 cells in DMEM only generated little amounts of NO in the culture supernatant, whereas the cells cultured with LPS (2 μg ml^{-1}), a macrophage activator, generated 12·4 ± 1·9 μmol l^{-1}. When cells were cultured with VC or CW, NO production increased to the same level as LPS. NO production induced by HK cells at the same density was found to be lower than the production induced by VC. NO production under UF treatment appeared to be less profound in the macrophages (Table 1).

Cytokine induction of *Lactobacillus plantarum* KFCC11389P and partial purification of active molecules

The concentration for cytokine inductions was determined to be $5{\cdot}0 \times 10^6$ CFU ml^{-1} for VC and HK, 1% for CW and 0·1% for UF. Treatments at higher concentrations resulted in cell death and perturbed inflammatory cytokines (Hessle *et al.* 1999; Suzuki *et al.* 2008). IL-1β levels in the RAW 264·7 cells cultured with LPS (2 μg ml^{-1}) were evaluated at 20·3 ± 3·0 pg ml^{-1}, and little IL-1β was detected in the negative control RAW 264·7 cells. The IL-1β induction by VC and CW was equal to that of LPS stimulation. The most noticeable effects of VC, HK and CW were in the secretion of TNF-α. These treatments generated at least three times as much TNF-α as UF, and these elevated levels were equal to that of LPS (3129 ± 169 pg ml^{-1}). TNF-α production was equally induced by HK and CW treatments, which was also comparable with VC. However, IL-6 was elevated only under VC treatment (117·8 ± 7·2 pg ml^{-1}), which was at least twice as the production by HK, CW and UF treatments. Interestingly, the crude preparations of the metabolic products, UF, induced higher levels of IL-10 (57·3 ± 13·2 pg ml^{-1}) when compared with other treatments, and reached levels that were equal to those generated in the LPS treatment (43·2 ± 3·1 pg ml^{-1}) as a positive stimulant. Another control group, modified MRS

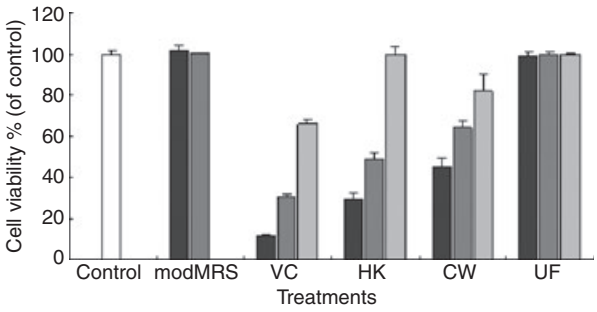

Figure 2 Cell cytotoxicity of various bacterial components on RAW 264·7 cells. Proliferation of macrophage cells was determined by Mosmann's method. Results are representative of three independent experiments and presented as the means ± SD. Black, 1% or $5{\cdot}0 \times 10^7$ CFU ml^{-1}; dark grey, 0·1% or $5{\cdot}0 \times 10^6$ CFU ml^{-1}; grey, 0·01% or $5{\cdot}0 \times 10^5$ CFU ml^{-1}.

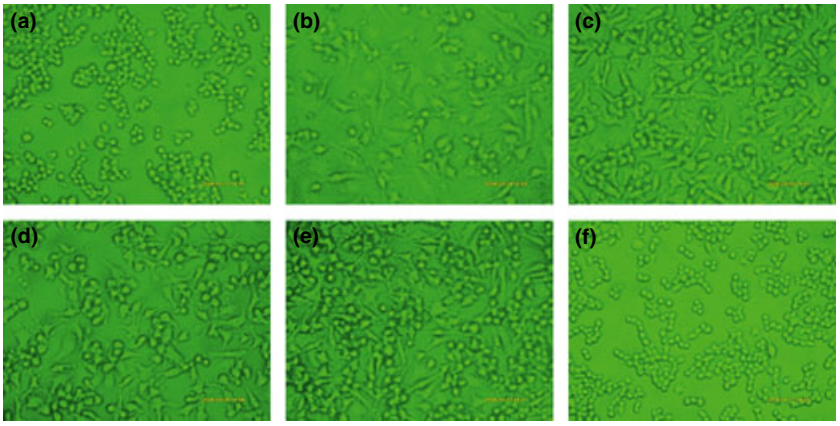

Figure 1 Morphology of RAW 264·7 cells cultured with bacterial components for 48 h. (a) Control; (b) lipopolysaccharide (2 μg ml^{-1}); (c) viable cells ($5{\cdot}0 \times 10^6$ CFU ml^{-1}); (d) heat-killed cells ($5{\cdot}0 \times 10^6$ CFU ml^{-1}); (e) cell walls (1%); (f) ultrafiltration (0·1%).

Table 1 Nitric oxide (NO) production by RAW 264·7 cells with various bacterial components

	Control	Mod. MRS*	LPS†	VC	HK	CW	UF
	1·5 ± 0·8	1·5 ± 0·1	12·4 ± 1·9				
5×10^7				UD‡	UD		
5×10^6				14·1 ± 1·6	4·0 ± 0·6		
5×10^5				3·6 ± 0·5	1·6 ± 0·8		
1%						10·1 ± 1·0	1·2 ± 0·9
0·1%						2·7 ± 0·3	2·2 ± 0·4
0·01%						1·9 ± 0·3	1·9 ± 0·5

The RAW 264·7 cells at a density of $5{\cdot}0 \times 10^5$ cells ml^{-1} were stimulated with indicated concentrations of viable cells (VC), heat-killed (HK) cells, cell walls (CW) or ultrafiltrates of metabolic products (UF) for 48 h; the culture supernatants were collected and analysed for NO production. Results are expressed as μmol l^{-1}and presented as means ± SD.

*Modified de Mann Rogosa Sharpe (MRS) medium was also used as another control group.

†Lipopolysaccharide or LPS, 2 μg ml^{-1}.

‡UD, undetectable levels owing to cell cytotoxicity.

Table 2 Effects of various bacterial components of *Lactobacillus plantarum* KFCC11389P on pro-inflammatory and anti-inflammatory cytokine production in RAW 264·7 cells

	Control	Mod. MRS	LPS	VC	HK	CW	UF
IL-1β	9·3 ± 1·5^a	13·5 ± 5·9$^{a, b}$	20·0 ± 3·0^b	19·7 ± 2·1^b	14·0 ± 3·5$^{a, b}$	18·0 ± 3·2^b	11·6 ± 2·5^a
IL-6	11·7 ± 5·7^a	30·1 ± 6·9^a	207·4 ± 6·0^c	117·8 ± 7·2^b	54·0 ± 5·0^a	59·7 ± 4·0^a	43·0 ± 5·0^a
TNF-α	221 ± 45^a	1572 ± 65^b	3129 ± 169^c	3595 ± 126^c	3442 ± 162^c	2830 ± 549^c	837 ± 50^b
IL-10	7·6 ± 0·1^a	16·2 ± 8·1^a	43·2 ± 3·1^b	5·7 ± 0·6^a	5·7 ± 0·6^a	15·0 ± 1·4^a	57·3 ± 13·2^b

The RAW 264·7 cells at a density of $5{\cdot}0 \times 10^5$ cells ml^{-1} were stimulated with viable cells (VC) or heat-killed (HK) cells ($5{\cdot}0 \times 10^6$ CFU ml^{-1}), cell walls (CW; 1%) or ultrafiltrates of metabolic products (UF; 0·1%) for 24 h. The culture supernatants were collected and analysed for cytokine induction. Modified de Mann Rogosa Sharpe (MRS) medium (1%) was also used as another control group. Data are expressed in pg ml^{-1} and presented as means ± SD. Means with different superscripts are significantly different within the level ($P \leq 0{\cdot}05$).

LPS, lipopolysaccharide at 2 μg ml^{-1}; IL, interleukin; TNF, tumour necrosis factor.

medium, induced little amounts of NO, IL-1β, IL-6 and IL-10 with moderate induction of TNF-α (Table 2).

The ultrafiltrated UF was fractionated by gel filtration chromatography, and each fraction was collected for further studies except for fractions 5 and 6 (Fig. 3a). The last two fractions were not capable of being retrieved in abundant amounts for analysis. As shown in Fig. 3b, the modified MRS medium after 10-kDa cut-off dialysis showed a different profile compared with UF. This suggests that the fractions obtained from UF do not arise from the composition of the modified MRS medium. Later, SDS-PAGE analysis revealed that an active molecule is estimated to be 8·1 kDa (Fig. 4).

As shown in Fig. 5, all treatments induced little TNF-α release to a level that was equal to the negative control, but fraction 1 induced slightly higher IL-6 release (64·4 ± 5·5 pg ml^{-1}) than the negative control (12·1 ± 2·1 pg ml^{-1}). IL-10 induction in all treatments tended to be higher than the negative control. Fractions 2, 3 and 4 separated from UF led to a strong increase in the capacity to induce IL-10 induction, 39·4 ± 2·4, 51·4 ± 8·9 and 31·9 ± 2·2 pg ml^{-1}, respectively. Figure 6 shows the effects of LPS-induced release of pro-inflammatory and anti-inflammatory cytokines by pretreatment of fractions 1–4 by RAW 264·7 cells. IL-6 induction was strongly inhibited by pretreatment of these separated fractions when compared with cells treated with LPS alone. TNF-α induction was elevated in LPS-stimulated cells when compared with cells treated with fractions alone, but still significantly inhibited by pretreatment of fractions when compared with cells treated with LPS alone. IL-10 production in pretreatment with fractions 1–3 was not enhanced in LPS-stimulated cells. In contrast, fraction 4 (38·8 ± 6·8 pg ml^{-1}) treatment had levels that were nearly equivalent to treatment with LPS alone (43·2 ± 3·1 pg ml^{-1}), and still statistically different from the negative control. Therefore, fraction 4 seems to be important in inducing anti-inflammatory activities.

To determine whether fraction 4 on LPS-induced cytokine production could influence the immune-modulating activity via IL-10, RAW 264·7 cells were pretreated with fraction 4 supplemented with different concentrations of anti-IL-10 mAb, and the levels of IL-6, TNF-α and IL-10 in the supernatant were measured (Fig. 7). Treatment

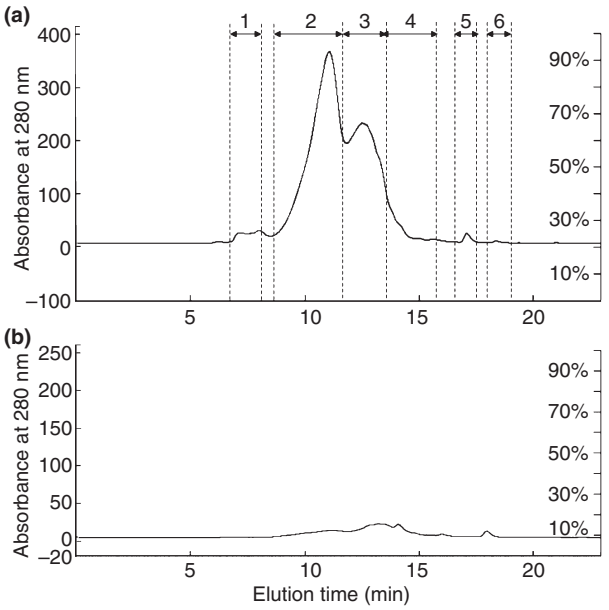

Figure 3 Elution pattern of ultrafiltrate by gel filtration chromatography. (a) The ultrafiltrated sample was fractionated by each peak. (b) The elution pattern of control ultrafiltrate preparation is shown, following 10-kDa cut-off dialysis of modified de Mann Rogosa Sharpe medium applied on gel filtration chromatography as a control.

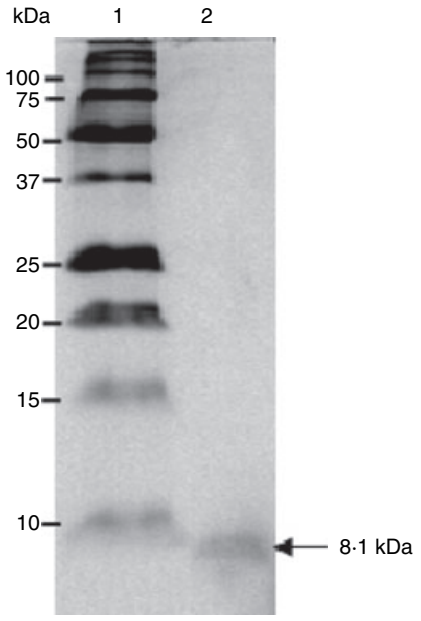

Figure 4 Determination of molecular weight of fraction 4 obtained from ultrafiltration (UF) by SDS-PAGE. The fraction 4 obtained from UF was electrophoresed and stained with Coomassie Brilliant Blue R-250. Lanes 1 and 2 indicate the standard proteins and fraction 4 obtained from gel filtration, respectively.

with fraction 4 supplemented with an anti-IL-10 mAb has been shown to increase the production of IL-6 and TNF-α by abolishing the regulatory role of IL-10. However, neutralization of anti-inflammatory cytokines with anti-IL-10 mAb did not produce any change in the induction of IL-10.

Discussion

In this article, we demonstrated the ability of various bacterial components of *Lact. plantarum* KFCC11389P to induce the production of different pro-inflammatory and anti-inflammatory responses in murine macrophage cell line, RAW 264·7 cells. In the induction of TNF-α in RAW 264·7 cells, HK cells of this strain did not show any differences when compared with VC. However, the ability of this strain to induce IL-1β and IL-6 was reduced after subjecting the VC to heat treatment. These results suggest that the stimulus required for TNF-α induction is heat stable and presumably different from those required for IL-1β and IL-6 induction. These results contradict a recent study where the ability of *Lactococcus* strains to induce TNF-α and IL-6 was demonstrated. Production of TNF-α was induced in the *Lactococcus* strains, and this effect was lost after heat treatment (Suzuki *et al.* 2008).

When RAW 264·7 cells were stimulated with VC, HK or CW, majority of the cells suppressed the proliferation and induced the morphological alterations such as vacuolization and extrusion of cellular processes in association with macrophage activation. The activation also resulted in the release of TNF-α and NO (Park *et al.* 1999; Péna and Versalovic 2003; Kim *et al.* 2007). However, in our study, cells that induced NO production appeared to be similar to cells that induced IL-1β release, because higher NO production was detected only in VC- and CW-treated cells. NO production is assumed to be related to induction of IL-1β because of the previous suggestions that prolonged incubation with IL-1 stimulated the release of large amounts of NO from cultured vascular smooth muscle cells and NO-mediated IL-1β induction in rat ovary (Ellman *et al.* 1993; Fukuo *et al.* 1995).

It has been reported that viable probiotic lactic acid bacteria are more potent inducers of cytokine production than the HK bacteria. Viable forms of *Lactobacillus casei* Shirota stimulated higher levels of TNF-α than HK bacteria in the murine macrophage cell line, J774·1 (Cross *et al.* 2004). Production of TNF-α and IL-6 in human peripheral blood mononuclear cells that were stimulated with live bacteria of *Lactobacillus rhamnosus* and *Lact. acidophilus* was induced in amounts even greater than those obtained with LPS (Miettinen *et al.* 1996; Gill and Rutherfurd 2001). This observation has been confirmed in this study, because VC of *Lact. plantarum* KFCC11389P were capable of inducing RAW 264·7 cells to secrete higher levels of IL-1β and IL-6 as well as NO production than those observed in the HK cells of this

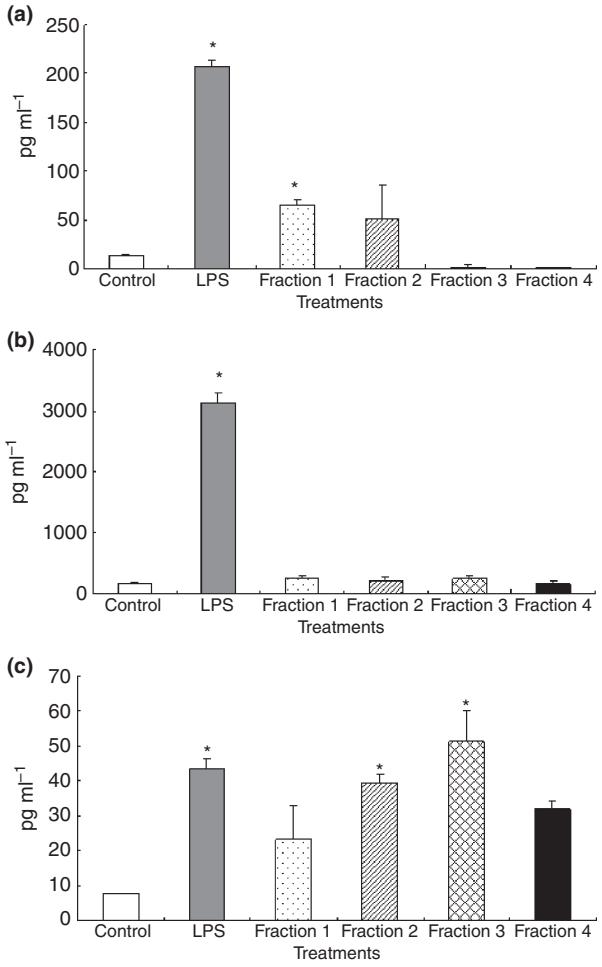

Figure 5 Effects of specific molecules separated by ultrafiltration on pro-inflammatory and anti-inflammatory cytokine production by RAW 264·7 cells. The secretion of interleukin (IL)-6 (a), tumour necrosis factor-α (b) and IL-10 (c) in the culture supernatant after 24 h of incubation with the separated molecules was measured by ELISA. The bars represent cytokine production in the negative control (cells with no treatment), positive control (lipopolysaccharides, 2 μg ml^{-1}) and fractions 1–4 (0·1%), respectively. Data are presented as the means ± SD and expressed in pg ml^{-1} units, and the asterisk indicates the treatment group that produces statistically significant ($P \leq 0{\cdot}05$) higher cytokine induction than the control group.

strain. However, there are some conflicting results have been reported in other studies that suggest VC are not necessarily required for pro-inflammatory cytokine induction (Hessle *et al.* 1999; Park *et al.* 1999; Morita *et al.* 2002).

In a study of *Lact. casei* Shirota, VC also stimulated higher levels of TGF-β, which is another anti-inflammatory cytokine, than HK bacteria in J774·1 cells. However, viable *Lact. casei* Shirota did not induce IL-10 production (Cross *et al.* 2004). In another study of cytokine production in J774·1 cells, HK *Lact. acidophilus* (10^8 CFU ml^{-1}) significantly induced the production of IL-10 (Morita *et al.* 2002). However, the anti-inflammatory effects of neither the VC forms nor the HK forms could be detected in our study, which suggest that the bacterial cells from *Lact. plantarum* did not influence anti-inflammatory cytokines, thus could not exert the immune-modulating activities.

Little is known about the bacterial metabolic products of lactobacilli for cytokine production. UF of *Lact. plantarum* KFCC11389P basically induced neither pro-inflammatory cytokines nor NO production. We also examined the effect of UF and its separated molecule, fractions 1–4, in LPS-induced release of IL-6, TNF-α and IL-10 by the murine macrophages. The inhibition of TNF-α in LPS-stimulated macrophages has been crucially considered in acute inflammation as TNF-α elicits septic shock, cachexia, cytotoxicity as well as inflammation (Aggarwal and Natarajan 1996; Kim *et al.* 2007). Pretreatment of fraction 4 led to a strong inhibition of IL-6 and TNF-α induction as well as maintaining IL-10 release in LPS-induced macrophages at a level that was equal to cells treated with fraction 4 without LPS stimulation. In contrast, intracellular metabolites of this strain showed similar levels of IL-6 and TNF-α when compared with the same concentration (0·1%) of UF. However, intracellular metabolites induced little amounts of IL-10. Pretreatment of intracellular metabolites also led to a strong augmentation of TNF-α induction in LPS-induced macrophages at a level that was equal to cells treated with LPS stimulation alone (data not shown).

Several previous studies indicated that some peptides or other molecules inhibit the production of pro-inflammatory cytokines IL-1β and TNF-α in LPS-stimulated murine macrophages (Park *et al.* 1999; Waseem *et al.* 2008). Other observations showed that soluble factors or cytoplasmic bacterial components, such as CpG motifs containing genomic DNA, play a major role in IL-10 production. However, the molecule for IL-10 release is not always explained by bacterial immuno-stimulatory DNA, as synthetic CpG and CpG DNA have also been shown to activate macrophage cells to produce several pro-inflammatory cytokines such as TNF-α (Hartmann *et al.* 2000; Utaisincharoen *et al.* 2002). Bifidobacteria from healthy infants stimulated macrophage-derived IL-10 production, but the same species in allergic infants induced little or no IL-10 production from the macrophages (He *et al.* 2002). From these results, they hypothesized that compounds other than LPS in bifidobacteria probably contribute to the ability of these bacteria to stimulate an anti-inflammatory response, and that lactobacilli are capable of downregulating pro-inflammatory cytokines such as diminishing TNF-α or NO production (He *et al.* 2002; Péna and Versalovic 2003). Generally, Gram-positive

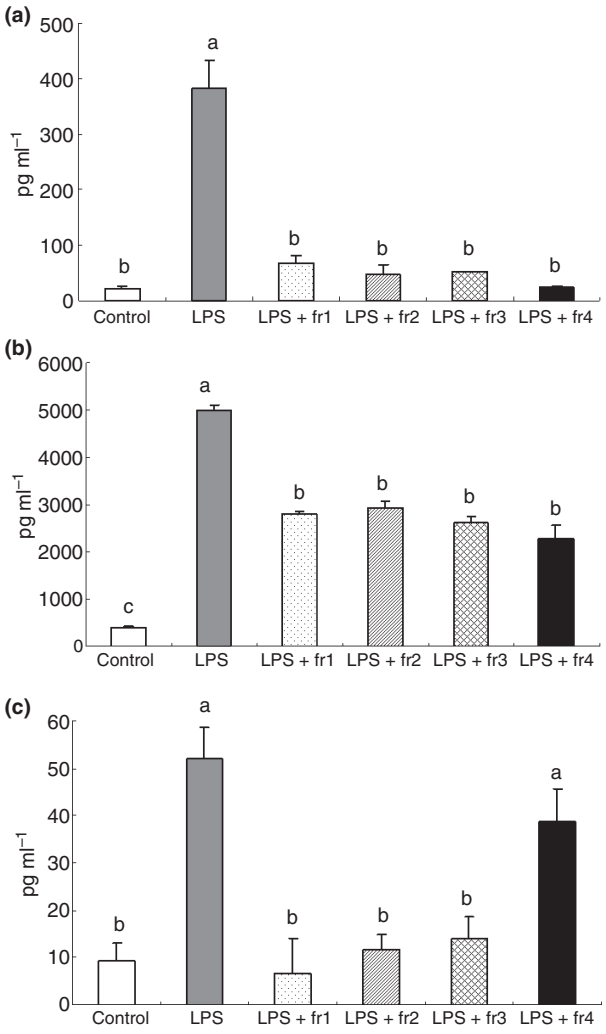

Figure 6 Lipopolysaccharide (LPS)-induced releases of pro-inflammatory and anti-inflammatory cytokines by pretreatment with specific molecules separated by ultrafiltration by RAW 264·7 cells. The secretion of interleukin (IL)-6 (a), tumour necrosis factor-α (b) and IL-10 (c) in the culture supernatant after 24 h of incubation with fractions 1–4, for 4 h before LPS treatment (2 μg ml^{-1}) was measured by ELISA. Data are presented as the mean ± SD and expressed in pg ml^{-1} units. $^{a–c}$ indicate that means with different superscripts are significantly different within the level ($P \leq 0{\cdot}05$).

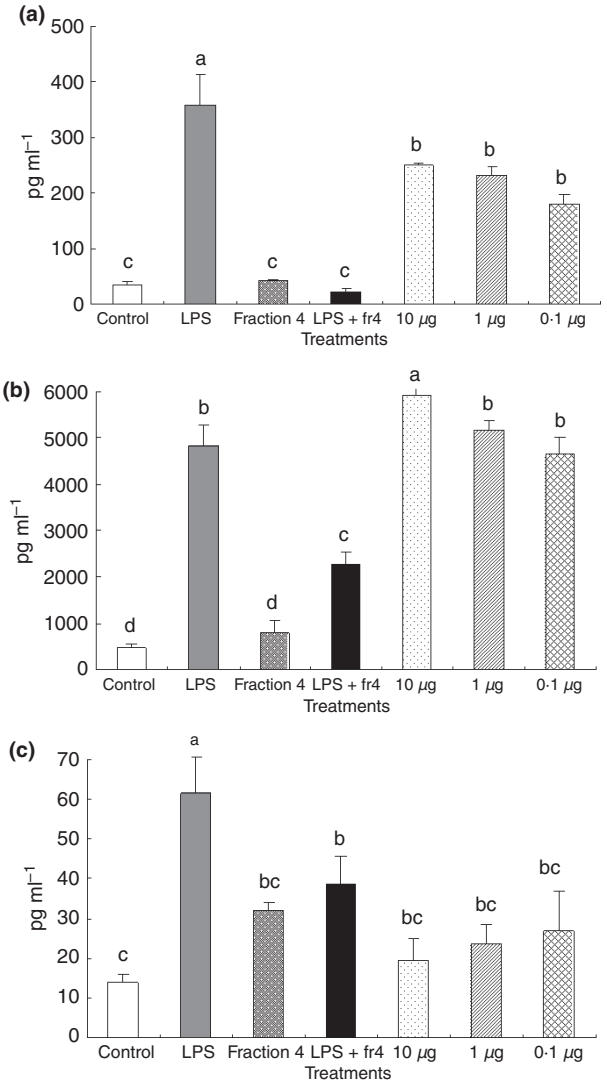

Figure 7 Influence of anti- interleukin (IL)-10 antibodies on lipopolysaccharide (LPS)-induced pro-inflammatory and anti-inflammatory cytokine production. The secretion of IL-6 (a), tumour necrosis factor -α (b) and IL-10 (c) in the culture supernatant was measured by ELISA. Data are presented as mean ± SD and expressed in pg ml^{-1} units. The bars represent control (cells with no treatment), LPS (2 μg ml^{-1}), fraction 4 (0·1%), LPS + fraction 4, 4-h pretreatment of fraction 4 (0·1%) before LPS stimulation; 10, 1 and 0·1 μg, 6-h incubation with fraction 4 (0·1%) supplemented with different concentrations of anti-IL-10 monoclonal antibodies for 24 h before LPS stimulation (2 μg ml^{-1}). $^{a–d}$ indicate that means with different superscripts are significantly different within the level ($P \leq 0{\cdot}05$).

bacteria, such as lactic acid bacteria, seem to stimulate inflammatory cytokines, and Gram-negative bacteria preferentially induce IL-10 production. However, HK *Lact. acidophilus* or UV-killed *Lact. rhamnosus*, which lack LPS, still gave rise to some IL-10 production, suggesting that the other bacterial components may trigger production of this cytokine (Hessle *et al.* 1999; Morita *et al.* 2002). However, more studies are needed in order to obtain more conclusive evidence for this hypothesis.

Our study demonstrated that metabolic product itself or its separated molecule from lactic acid bacteria has inhibitory effects on the production of IL-6 and TNF-α in LPS-stimulated RAW 264·7 macrophages. The LPS-induced production of IL-6 and TNF-α by pretreatment with fraction 4 was significantly increased by inhibiting the activity of IL-10 by using neutralizing anti-IL-10 mAb (Spittler *et al.* 1999; Yokoyama *et al.* 2004). However, the

production of IL-10 was sustained or slightly diminished, so anti-inflammatory effects of IL-10 are not likely to be related with anti-IL-10 mAb but rather with some other molecules such as fraction 4. These results partially support that feedback downregulatory effects of IL-6 and TNF-α are not solely related to IL-10 release on macrophage cells (Yokoyama *et al.* 2004).

In conclusion, we found that fraction 4, presumably 8·1 kDa, separated from the metabolic products of *Lact. plantarum* KFCC11389P was strongly anti-inflammatory against LPS-induced responses in RAW 264·7 cells. From these results, it appears that this specific molecule may be useful for the treatment of acute inflammatory responses such as Crohn's disease and ulcerative colitis. However, the mechanism of effects of this molecule on pro-inflammatory cytokine production in LPS-stimulated murine macrophages was not investigated in this study, and small animal models will also be necessary to facilitate further studies of anti-inflammation.

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