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Effects of Kumaizasa (*Sasa senanensis*) Leaf Extract on Innate Immune Regulation in HEK293 Cells and Macrophages

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Abstract. *Background/Aim: We investigated the effect of Kumaizasa leaf extract (KLE) on innate immunity using the HEK293 and RAW 264.7 cell lines. Materials and Methods: KLE, lipopolysaccharides (LPS), or KLE with LPS were added to RAW 264.7 cells. The TNF-α and IL-1β mRNA expression was then quantified. The expression of MAPKs, NFκB, TNF-α and IL-1β proteins was also quantified. In addition, KLE was added to HEK293 cells and the IL-8 concentration was measured. Results: In RAW 264.7 cells, KLE increased the levels of TNF-α and IL-1β mRNA. By contrast, when KLE and LPS were added to RAW 264.7 cells, the increase in TNF-α and IL-1β mRNA was ameliorated. Similarly, the expression of JNK and ERK proteins was reduced. The addition of KLE to HEK293 cells induced IL-8 production. Conclusion: Based on these results, a KLE-mediated mechanism may regulate immunity by suppressing the expression of JNK and ERK, which are involved in inflammatory signal transduction.*

The Kumaizasa (*Sasa senanensis*) plant belongs to the Gramineae family and is widely found in northern Japan. Kumaizasa consists primarily of polysaccharides; however, low molecular weight compounds have also been identified, including *p*-coumaric acid, ferulic acid, vanillin, apigenin, luteolin, tricin, syringaresinol, phenylpropanoid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and 3,4-dihydroxybenzaldehyde (1, 2).

Sasa, including Kumaizasa, has been reported to act as a UV protectant (3), antiviral (4), antioxidant (in a rat small intestinal ischemia–reperfusion model) (5), as a suppressor of amyloid-β-mediated neuronal damage (6, 7), and as a suppressor of macrophage-derived nitric oxide (NO) (8) and prostaglandin E_2 (PGE_2) production (9). Other effects include antitumor activity in mouse K562 (human chronic myeloid leukemia) and YAC1 (murine lymphoma) cells, inhibition of histamine release in rat intraperitoneal cells, and improvement of the oral and intestinal environment (10).

Our understanding of the innate immune system, inflammation and biological defense systems has progressed significantly, especially with respect to the discovery of toll-like receptors (TLRs). TLRs are expressed in many types of cells and play important roles in macrophages and dendritic cells of the innate immune system. TLRs recognize foreign substances and stimulate the production of immunomodulatory substances, such as cytokines, through intracellular signaling. These cytokines have direct antibacterial, antiviral and antitumor effects and play various roles, including the regulation of innate and adaptive immune systems (11-13).

The TLR family comprises 11 members. β-Glucan, which is derived from the cell wall of bacteria, is a ligand for TLR2, whereas lipopolysaccharide (LPS), derived from gram-negative bacteria, is a ligand for TLR4. Inflammatory cytokines, including TNF-α, IL-8, IL-1β, are produced following the stimulation of TLR2 and TLR4. TLR signaling activates mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (c-JNK), extracellular signal-regulated kinase (ERK), p38 MAPK pathways and transcription factor nuclear

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Key Words: Kumaizasa, *Sasa senanensis*, HEK293, RAW 264.7, toll-like receptor 2, toll-like receptor 4, tumor necrosis factor-α, interleukin-1β, c-Jun N-terminal kinase, extracellular signal-regulated kinase.

Table I. *List of nutritional information contained in Kumaizasa leaf extract (KLE).*

Nutritional ingredients	Concentration
Protein	17.3 g/100 g
Lipid	<0.1 g/100 g
Ash	19.6 g/100 g
Carbohydrate	82.5 g/100 g
Iron	2.7 mg/100 g
Calcium	56.1 mg/100 g
Potassium	886.6 mg/100 g
Magnesium	776.6 mg/100 g
Glucans (polymerized >7 sugars)	20.1 g/100 g
Lipopolysaccharide (LPS)	40.3 ng/g

Nine nutrients (protein, lipid, ash, carbohydrate, iron, calcium, potassium and magnesium, glucans) were quantified by the Kjeldahl method, Soxhlet extraction method and Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Lipopolysaccharides (LPS) were quantified by immunoassay.

factor κB (NFκB), even with stimulated receptors TLR2 or 4 (14-19).

The production of inflammatory cytokines is necessary for eliminating foreign bodies. However, when inflammatory cytokines are produced chronically and excessively, they may cause allergies, cancer, and other immune diseases. Therefore, the immune response must maintain a balance (20, 21).

Many plant-derived phytochemicals can regulate the immune system balance. For example, a type of low molecular weight polyphenol suppresses the overproduction of inflammatory cytokines (22). Many of these compounds represent folk medicines that are derived from plants for the prevention and treatment of immune-related diseases, including pollinosis, asthma, atopic dermatitis and malignant tumors (23, 24).

We studied the effect of the Kumaizasa leaves, which have been used as a folk medicine, on the immune system. We used two cell culture systems and investigated the effect of KLE on inflammation-related mRNA and protein expression.

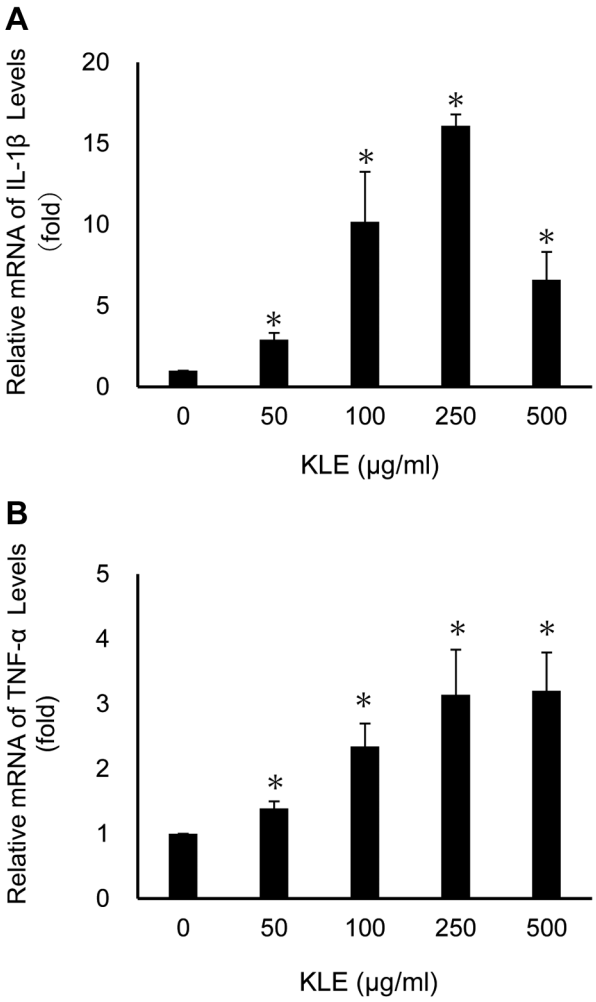

Figure 1. *Effects of Kumaizasa leaf extract (KLE) on pro-inflammatory cytokine levels. RAW 264.7 cells were treated with 50-500 μg/ml of KLE for 24 h. IL-1β (A) and TNF-α (B) mRNA expression levels were measured. Values are mean±SD of three experiments. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test *Significant difference (p<0.05 vs. untreated cells).*

Materials and Methods

Kumaizasa leaf extract. KLE (Lot.Hus_ws01) was obtained from the Hakuju Institute for Health Science Co., Ltd. (Tokyo, Japan). KLE is a hot water extract of Kumaizasa leaves. The nutritional composition of the solid is shown in Table I.

Cell culture. Cells were grown to 80-90% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, L-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2.

Quantification of mRNA levels in KLE- and LPS-treated RAW 264.7 cells. RAW 264.7 cells were purchased from the European Collection of Authenticated Cell Cultures. RAW 264.7 cells were treated with 100 ng/ml LPS alone or with 50-500 μg/ml of KLE. Quantitative RT-PCR analysis was used to quantify mRNA levels. Total RNA from treated cells was extracted with the FastGene™ RNA Basic Kit (Nippon Genetics Co. Ltd., Tokyo, Japan) according to the manufacturer's protocol. mRNAs were reverse-transcribed into cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative RT-PCR was performed with the 7500 Fast Real-Time PCR system (Applied Biosystems). Primers for mouse IL-1β and mouse TNF-α were purchased from Applied Biosystems. mRNA levels were quantified using values of the threshold cycle (Ct) of IL-1β and TNF-α, normalized to that of GAPDH. Relative mRNA levels were compared with control levels. Data are representative of three experiments.

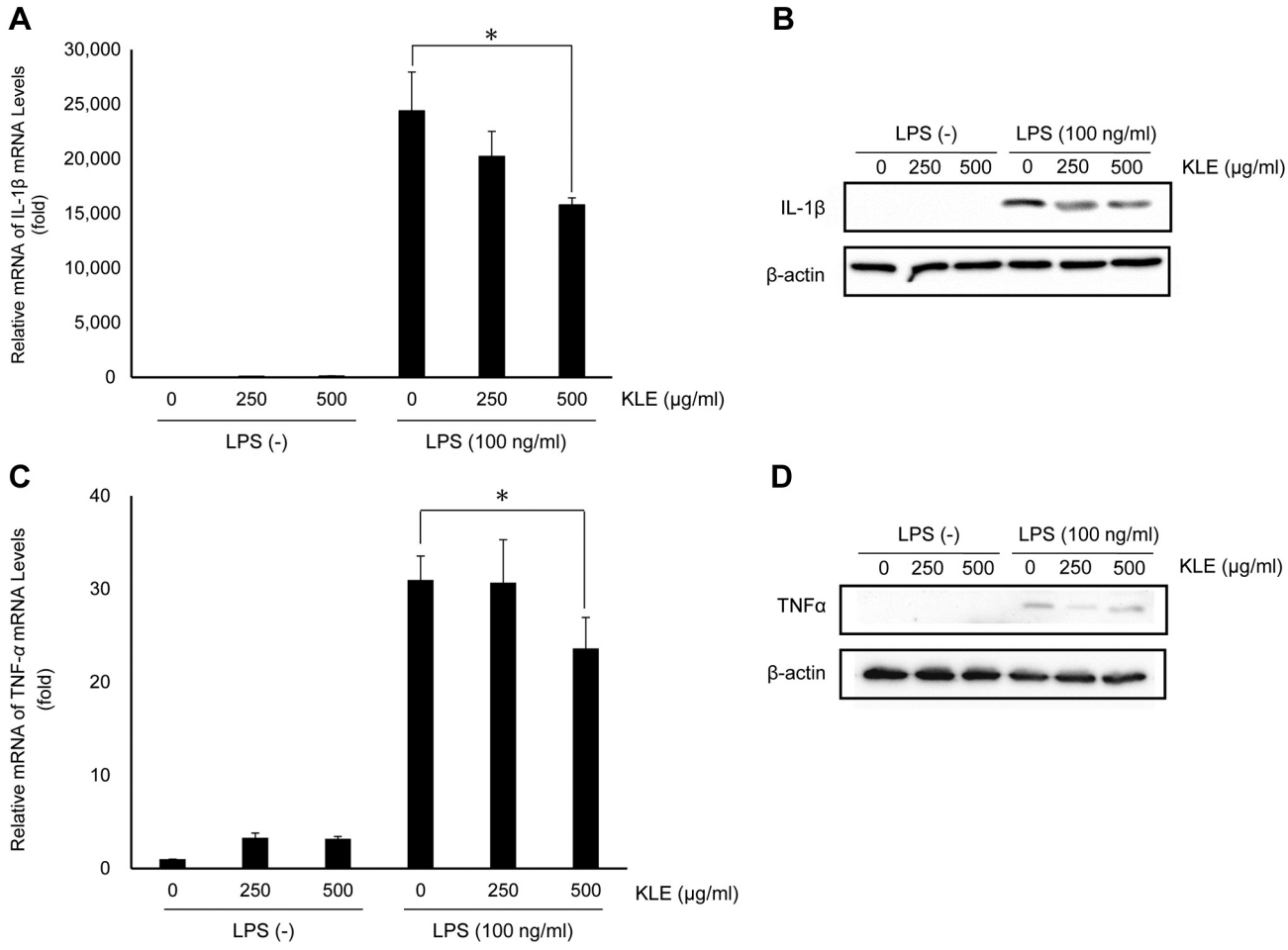

Figure 2. *Effect of Kumaizasa leaf extract (KLE) on lipopolysaccharide (LPS)-induced pro-inflammatory cytokine levels. RAW 264.7 cells treated with or without LPS (100 ng/ml) were incubated with 250 or 500 μg/ml of KLE for 4 h. IL-1β mRNA (A), IL-1β protein (B), TNF-α mRNA (C) and TNF-α protein (D) levels were measured. Values are mean±SD of three experiments. Statistical significance was determined using two-way ANOVA with Tukey's post-hoc test. *Significant difference (p<0.05).*

Quantification of protein levels in RAW 264.7 cells treated with KLE and LPS. TNF-α, IL-1β, phospho-NFκB (p-NFκB), NFκB, phospho-p38 (p-p38), p38, phospho-JNK (p-JNK), JNK, phospho-ERK (p-ERK) and ERK protein levels were analyzed by western blotting. Cells were treated with 100 ng/ml LPS alone or with 50-500 μg/ml *Sasa senanensis*, washed with Dulbecco's phosphate buffered saline and lysed in lysis buffer [50 mM HEPES (pH 7.4), 5 mM EDTA, 120 mM NaCl, 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate)]. The lysate was centrifuged at 10,000 × *g* for 15 min and 20-50 μg of protein from the supernatant was resolved using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were blotted onto a polyvinylidene difluoride membrane. The membrane was incubated with the following primary antibodies: anti-mouse IL-1β (#12426), anti-rabbit TNF-α (#11948), anti-rabbit phospho-NFκB (#3033), anti-rabbit NFκB (#6956), anti-rabbit phospho-p38 (#9211), anti-rabbit p-38 (#9212), anti-rabbit phospho-JNK (#4668), anti-rabbit JNK (#9252), anti-rabbit phospho-ERK (#4377) and anti-rabbit ERK (#4695) (Cell signaling Technology, Danvers, MA, USA) and anti-mouse β-actin polyclonal antibody (A5441) (Sigma-Aldrich Co., St. Louis, MO, USA). The membrane was then incubated with horseradish-peroxidase-conjugated secondary antibodies. Chemiluminescence was detected with Immobilon (Merck KGaA, Darmstadt, Germany).

Quantification of IL-8 production in HEK293 cells with KLE. Two types of cell lines were used. One had the mouse mTLR4/MD2/CD14 gene inserted into human embryonic kidney (HEK)293 cells and the other had the vector inserted into HEK293 cells (null) as a control [purchased from Invitrogen (Waltham, MA, USA)].

KLE was used as a test sample, and LPS (purified from *Pantoea agglomerans*) was used as a positive control. KLE (0.4-400 μg/ml, solid content) was added to mouse HEK293 mTLR4/MD2/CD14

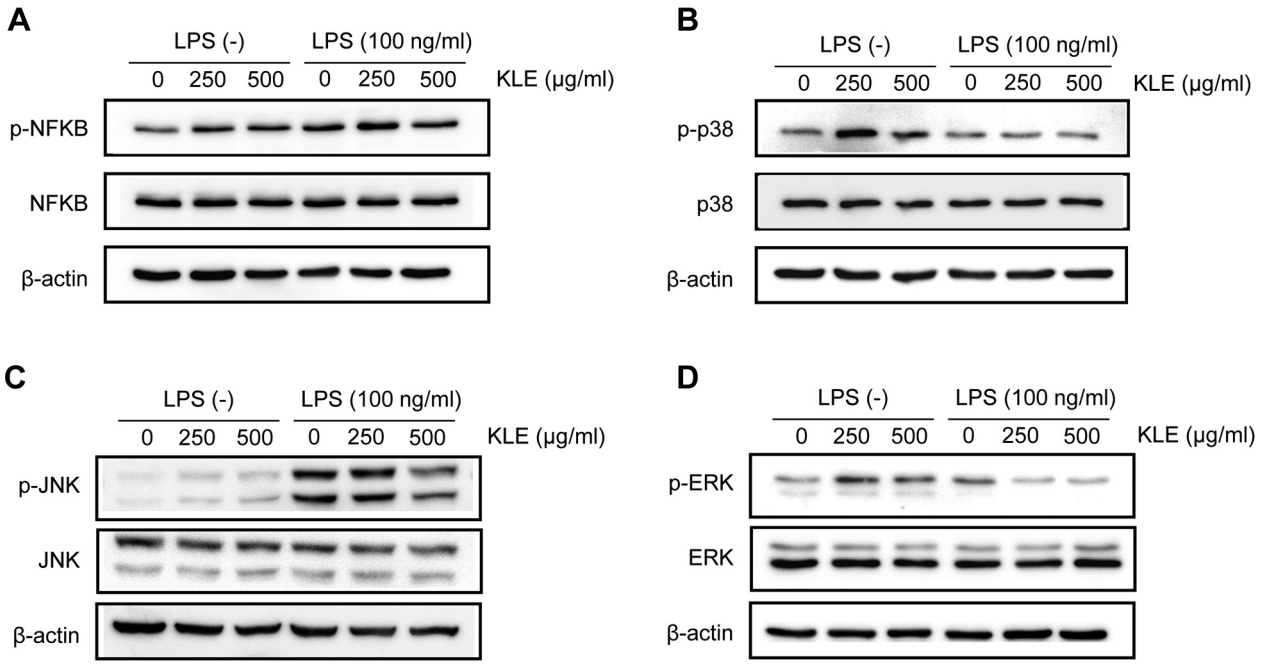

Figure 3. *Effect of Kumaizasa leaf extract (KLE) on lipopolysaccharide (LPS)-induced transcription factor nuclear factor κB (NFκB) and mitogen-activated protein kinase (MAPK) activation. RAW 264.7 cells treated with or without LPS (100 ng/ml) were incubated with 250 or 500 μg/ml KLE for 4 h. NFκB (A), p38 (B), c-Jun N-terminal kinase (JNK) (C), and extracellular signal-regulated kinase (ERK) (D) protein levels were measured.*

and null cells (4×10^5 cells/ml) and cocultured for 24 h at 37˚C. The culture supernatant was then collected. Quantification of IL-8 was performed from the obtained supernatant using an ELISA kit (BioLegend Co., Ltd., CA, USA).

Statistical analysis. Results are expressed as mean±standard deviation (SD). Statistical analysis was performed using Excel 2015 v.1.15 (Microsoft Japan Co., Ltd. Tokyo, Japan). Statistical significance was determined using the Dunnett's multiple comparison test, one-way analysis of variance (ANOVA) and two-way ANOVA with Tukey's *post-hoc* test. $*p<0.05$ and $**p<0.01$ were considered statistically significant.

Results

Effect of KLE on pro-inflammatory cytokines in RAW 264.7 cells. Treatment of RAW 264.7 cells with KLE (50-500 μg/ml) for 24 h significantly increased the levels of IL-1β and TNFα mRNA (Figure 1). RAW 264.7 cells were treated with 100 ng/ml LPS alone or with 250 or 500 μg/ml KLE. Levels of IL-1β and TNFα mRNA and protein increased in LPS-stimulated RAW 264.7 cells. KLE significantly suppressed the mRNA expression levels of IL-1β and TNFα (Figure 2).

Effect of KLE on LPS-induced NFκB and MAPK activation. Effect of KLE on activated NFκB and MAPKs was examined in RAW 264.7 cells by LPS addition. As shown in Figure 3C and D, KLE resulted in a decrease of phosphorylated JNK and ERK by LPS. By contrast, there was no effect of KLE on LPS-activated p-NFκB and p-p38 (Figure 3).

Effects of KLE on IL-8 production in HEK293 cells. HEK293 and null cells were treated with KLE (0.4-400 μg/ml) for 24 h (Figure 4). IL-8 production in the medium was significantly increased after KLE (400 μg/ml) treatment. IL-8 production was also enhanced by treatment with LPS, which is a positive control (Figure 5).

To understand the results of these *in vitro* experiments, the relationship between KLE and immunity is summarized in Figure 6. KLE stimulated either TLR2 or TLR4 in RAW 264.7 cells to promote the production of IL-1β and TNF-α. However, when LPS and KLE are combined in RAW cells, KLE inhibits the expression of JNK and ERK protein levels and suppresses the production of IL-1β and TNF-α. KLE stimulates TLR4 and enhances IL-8 production in HEK cells.

Discussion

Fungal β-glucan is a ligand for TLR2, and LPS derived from gram-negative bacteria is a ligand for TLR4. Such stimulation induces the production of inflammatory and antitumor cytokines, including TNF-α and IL-1β. Fungal-derived

lentinan is composed primarily of β-glucan and is used as an immunostimulant and anticancer agent (25, 26). LPS derived from gram-negative bacteria (*Pantoea*) is also used as an immunomodulator in health foods and cosmetics (27, 28).

LPS and β-glucan are often used as target materials for immunological studies using macrophages. The mechanism of TNF-α and NO production by TLR stimulation of LPS or β-glucan has been studied in detail (14). For example, it is important to study the effect of using LPS or β-glucan alone and the inhibitory or synergistic effect of using other materials in combination (29, 30). In other words, by comparing test samples with substances such as LPS or beta-glucan, it is possible to estimate the effect of the test samples on the immune system (31, 32). The combination of LPS and KLE can confirm whether KLE enhances or inhibits the effects of LPS on the production of inflammatory cytokines.

Naturally occurring compounds may be ligands for TLR2 and TLR4. For example, palmitic acid activates cytokine production through TLR4 and its downstream signal transduction pathway (33). Curcumin from turmeric and xanthohumol from the hop plant (*Humulus lupulus*) suppress TLR4 signaling (34-36). In addition, plant- and seaweed-derived polysaccharides activate signal transduction and cytokine production downstream of TLR2 (26, 28). The citrus flavonoid, naringenin, has been reported to suppress TLR2 signaling (37, 38). Based on these studies, low molecular-weight polyphenols, such as plant-derived flavonoids, may act by suppressing TLR2 and TLR4, whereas plant-derived polysaccharides activate TLR4.

In HEK293 mTLR4/MD2/CD14 cells, stimulation with a TLR4 ligand promotes IL-8 production. The chemokine IL-8 functions as a neutrophil chemotactic factor. Thus, when the production of IL-8 is induced, a concomitant increase in chemotaxis of neutrophils occurs at the infected site (39). Neutrophils are the primary target of IL-8; however, but they also induce chemotaxis of other granulocytes. When these cells reach the affected area, foreign bodies, such as bacteria and cancer cells, are eliminated through phagocytosis. RAW 264.7 is a macrophage-like cell that expresses both TLR2 and 4. Moreover, RAW 264.7 produces TNF-α and IL-1β through the stimulation of TLR2 and 4.

Table I shows KLE contains polysaccharides and LPS, according to the nutritional analysis conducted by the Hakuju Institute for Health Science Co., Ltd and Japan Food Research Laboratories foundation (Tokyo, Japan). Based on metabolome analysis using liquid chromatography-mass spectrometry (LC/MS) and the community energy management system (CE/MS), 466 water-soluble and 61 fat-soluble components have been identified. Palmitic acid and 29 polyphenols, including 7-hydroxycoumarin, apigenin-8-C-glucoside, isoorientin, isofraxidin, liquiritigenin, eriodictyol, eleutheroside B, eleutheroside E, capillarisin, chrysoeriol, chlorogenic acid, genistein, quercetin-3-rutinoside, quercetin, kaempferol,

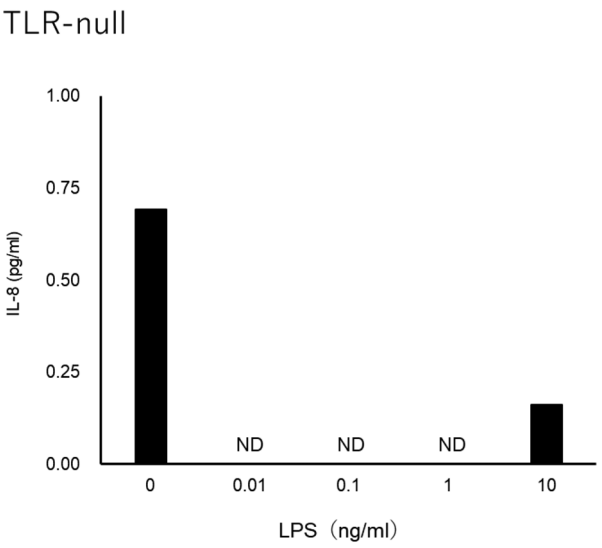

Figure 4. *Effect of Kumaizasa leaf extract (KLE) on IL-8 levels in null cells. Null cells treated with 0.1-10 ng/ml lipopolysaccharide (LPS) for 24 h. IL-8 levels were measured in the medium. Values are mean±SD of three experiments. ND: Not detectable.*

saponarin, datiscetin, naringenin-7-*O*-neohesperidoside, baicalein, formononetin-7-*O*-β-D-glucoside, formononetin, hesperidin, poncirin, malvidin, myricetin 3-*O*-rhamnoside, luteolin-7-*O*-glucoside, luteolin, resveratrol and epigallocatechin gallate have been qualitatively confirmed in KLE (36). These data suggest that β-glucan, LPS, and a type of lipid may stimulate the immune system, and components of KLE may exert an inhibitory effect on TLR2 and TLR4. In other words, palmitic acid and LPS are involved in the TLR4-stimulating effect of KLE. In this report, we showed that both palmitic acid and LPS are components of KLE. Therefore, it is possible that these two components represent TLR4 ligands in KLE. Similarly, glucans in KLE may function as ligands for TLR2. These components of KLE promote the production of IL-8, TNF-α and IL-1β by stimulating TLR2 and 4.

NFκB is a major regulator of immune and inflammatory responses and is highly involved in the pathophysiology of cancer (18, 19). It has been reported that the MAPK (ERK, JNK, and p38 MAPK) pathway plays important roles in controlling inflammation through the production of inflammatory mediators. TLR signaling activates MAPKs and NFκB through stimulated TLR2 or 4, and these pathways promote the production of IL-1β, TNFα and IL-8 (37).

When LPS and KLE were co-incubated with macrophages expressing both TLR2 and TLR4, a significant suppression of TNF-α and IL-1β production and a significant decrease in p-JNK and p-ERK protein expression was observed. This is probably because some components in KLE suppress the action of TLR2 and 4, JNK and ERK.

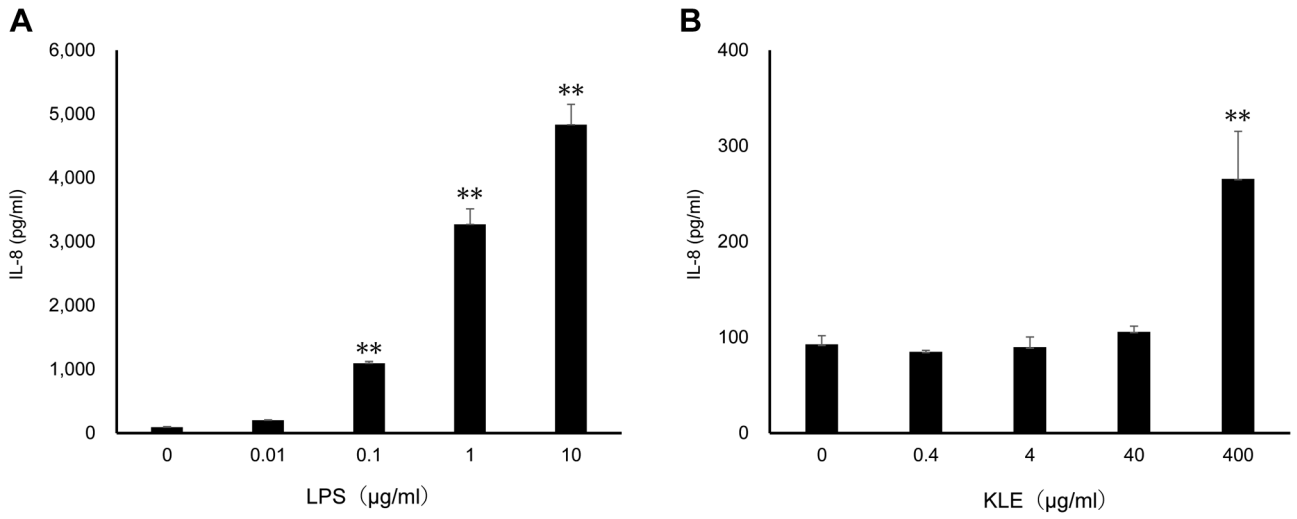

Figure 5. *Effect of Kumaizasa leaf extract (KLE) on IL-8 levels in HEK293 cells. HEK236 cells were treated with 0.1-10 ng/ml lipopolysaccharide (LPS) (a) or 0.4-400 μg/ml KLE (b) for 24 h. IL-8 levels were measured in the medium. Values are mean±SD of three experiments. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparison test. *Significant difference (p<0.05 vs. untreated cells).*

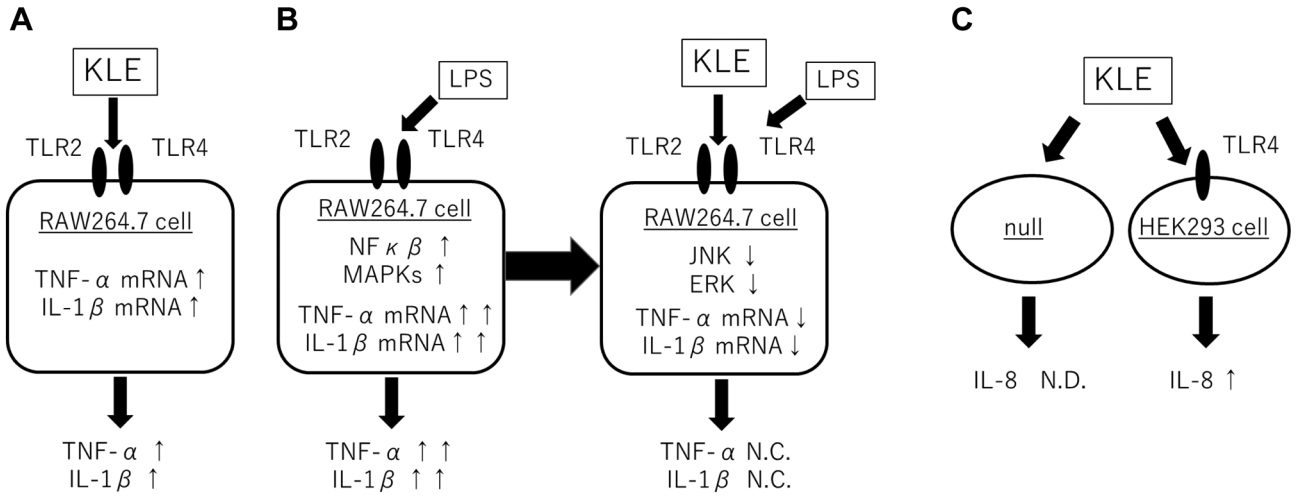

Figure 6. *Schematic diagram of the effect of Kumaizasa leaf extracts (KLE) on HEK293 and RAW 264.7 cells. KLE promotes the production of IL-1β and TNF-α on RAW 264.7 cells (a). Inhibition of c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) protein levels by KLE in RAW cells (b). TLR4-stimulated enhancement of IL-8 production by KLE in HEK cells (c). N.D.: Not detectable; N.C.: no change. The combination of LPS and KLE can confirm whether KLE enhances or inhibits the effects of LPS on the production of inflammatory cytokines.*

Thus, we conclude that KLE induces inflammatory cytokines through TLR2 or 4 stimulation and suppresses the LPS-induced production of inflammatory cytokines. This suggests that KLE regulates the JNK- and ERK-associated immune-response pathways. TLR-mediated immunomodulation is very important in cancer and inflammatory disease therapies. Notably, chronic inflammatory conditions induce carcinogenesis and cancer cachexia, and modulating immunity through naturally-occurring compounds, such as KLE, is desirable (38-40). However, regulatory effects of KLE on TLR2 and TLR4 and specific components involved remain unclear. These issues remain to be resolved in future in studies.

Conflicts of Interest

This study was funded by Hakuju Institute for Health Science Co., Ltd., Tokyo. Shinji Harakawa, Takaki Nedachi and Kaoru Haketa are employees of Hakuju Corporation. Hakuju Corporation has provided test samples for this research.

Authors' Contributions

K Sato, K Komatsu and K Wakame designed the research protocol. T Nedachi and K Haketa provided test samples. K Sato, R Tatsunami and H Inagawa are study investigators and analyzed the physical data for evaluation of adverse events. S Harakawa performed the statistical analysis. K Sato, K Wakame and A Nakata wrote the manuscript. K Komatsu, S Harakawa and T Nedachi reviewed and edited the manuscript. All Authors read and approved the final version of the manuscript.

Acknowledgements

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