DNA Microarray Analysis of Signaling Pathway in Macrophages Stimulated by Lignin–Carbohydrate Complex from *Lentinus edodes* Mycelia (LEM) Extract

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Abstract. *In order to investigate the action point of lignin–carbohydrate complex (Fr4) from Lentinus edodes mycelia extract, DNA microarray analysis was performed, using mouse macrophage-like J774.1 cells. Among seven lignin-carbohydrate complex fractions, Fr4 showed the highest stimulatory activity of tumor necrosis factor production by mouse macrophage-like J774.1 cells, as well as its previously reported anti-HIV activity. Fr4 is composed of lignin precursors such as vanillic acid, syringic acid, p-coumaric acid and ferulic acid, with trace amounts of flavonoids and tannins, and negligible amount of lipopolysaccharides (LPS), confirming the authenticity of Fr4 as a lignin. DNA microarray analysis suggested that Fr4 may affect immune response-related gene expression; however, it may not affect the expression of as many genes as LPS does.*

Lentinus edodes mycelia extract (LEM) has shown anti-hepatopathic (1-3), antitumour (4-8), immunopotentiating (9, 10), anti-vascularisation (11). anti-arteriosclerosis (12), skin-protective (13, 14) and undocumented activities such as improvement of hepatic function, menorrhagia, melancholia, nausea and vomiting. Polysaccharides isolated from *L. edodes* such as lentinan (15) and KS-2 (16) have shown immunopotentiating and antitumour activities. On the contrary, studies of anti-HIV (17) and immunopotentiating (18) activity of lignin fractions of LEM have been limited.

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Recently, seven lignin–carbohydrate fractions have been isolated from LEM (19): Three fractions (Fr1-3) were prepared from the water extract by successive ethanol fractionation. Fr4 and 5-7 were prepared by acid and stepwise ethanol precipitation from the NaOH extract, respectively. All fractions showed higher anti-HIV activity than the water extract. Fr4 showed the highest anti-HIV activity and most potently inhibited the NO production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells (RAW264.7, J774.1) (19). These data suggest that the action point of lignin–carbohydate complexes from LEM in macrophages may be different from that of LPS. To test this hypothesis, this study performed a preliminary DNA microarray analysis aiming to investigate how LEM lignin–carbohydrate complex affects mammalian gene expression, using J774.1 cells stimulated with either Fr4 or LPS.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), LPS from *Escherichia coli* (Serotype 0111:B4); dimethyl sulfoxide (DMSO) (Wako Pure Chem Ind, Osaka, Japan).

Preparation of Fr4. Fractionation of lignin–carbohydrate complexes from LEM was described in our previous paper (19). In brief, LEM was first suspended in cold water, and centrifuged at 14.400×*g* for 10 min to collect the supernatant (referred to as water extract). To this water extract, 1-, 2- and 5-fold volumes of ethanol were added successively to precipitate Fr1, 2 and 3. The residue was extracted with 1% NaOH at room temperature. The NaOH extract was acidified to pH 5 with dropwise addition of acetic acid to precipitate Fr4, and the resultant supernatant was mixed successively with 1-, 2- and 5-fold volumes of ethanol to precipitate Fr5, 6 and 7. All these fractions were dialysed against distilled water and lyophilised to dryness.

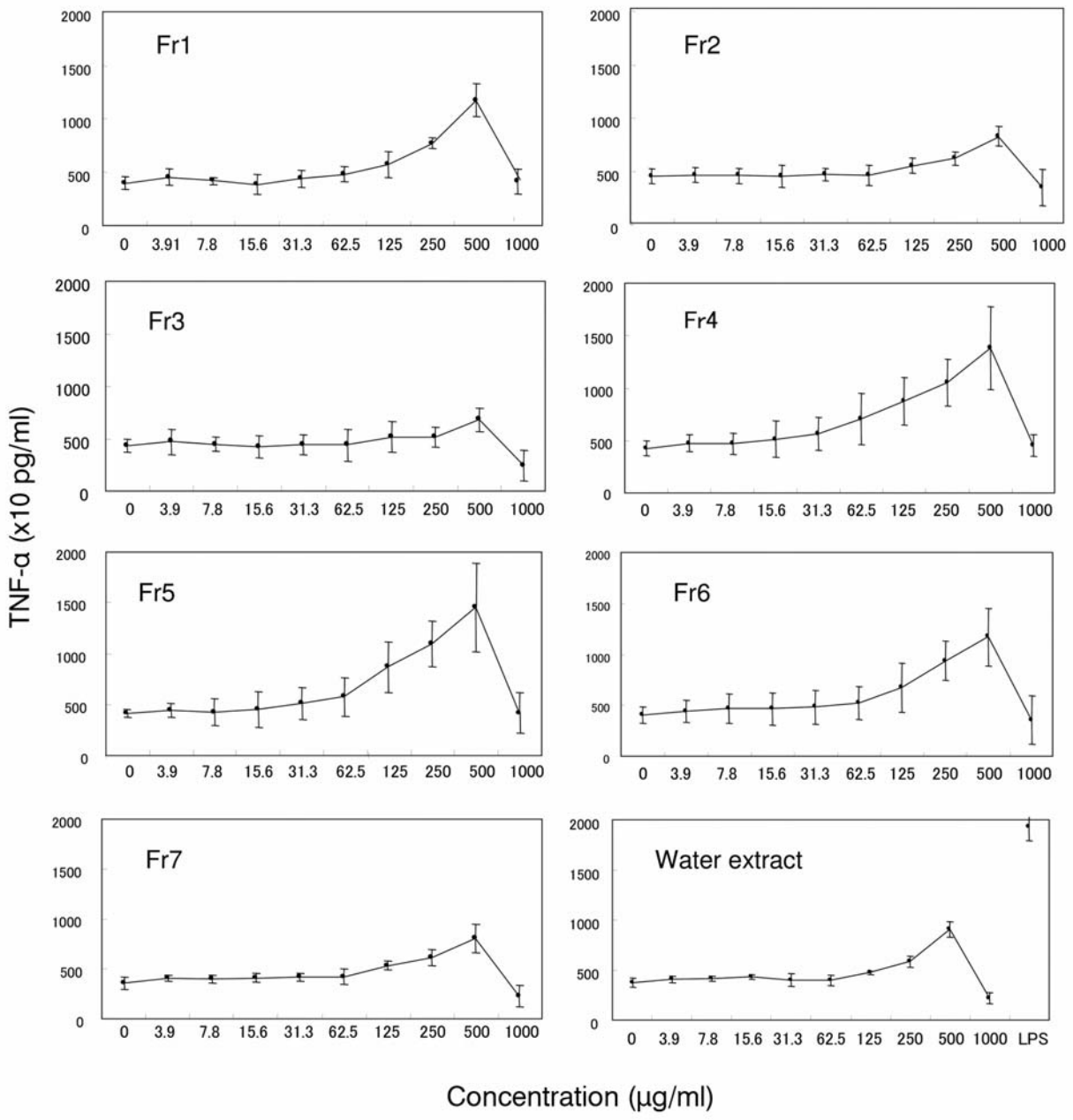

Figure 1. *Stimulation of TNF-α production by lignin–carbohydrate complex fractions of LEM in J774.1 cells. Cells were incubated for 24 hours with the indicated concentrations of each fraction or 0.1 μg/ml LPS. Each value represents the mean±S.D. of three independent experiments.*

Analysis of composition of Fr4. Chemical composition of LEM was analysed by HPLC (Otsuka Pharmaceutical Co, Ltd, Tokyo, Japan). LPS concentration was determined by lipid A-specific colorimetric method (Peptide Door Ltd, Takasaki, Japan).

Cell culture. Mouse macrophage-like J774.1 cells (RIKEN Bioresource Center, Tsukuba, Japan) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under humidified 5% CO_2 atmosphere (20).

Assay for tumor necrosis factor a (TNFa) production. Cells were inoculated at 0.25×10^6/ml (10 ml) in a 8.5 cm-dish and incubated for 36 hours. Near confluent cells were treated for 24 hours without (referred to as 'None'), or with 250 μg/ml Fr4 (referred to as 'Fr4_250'), or 0.1 μg/ml LPS (referred to as 'LPS_0.1'). In some experiments, the cells were treated with RLT plus buffer (RNeasy Plus Mini Kit; Qiagen Inc, Tokyo, Japan). The TNFa released into the culture medium was determined by ELISA (Quantikine ELISA kit; R&D systems, Minneapolis, MN, USA).

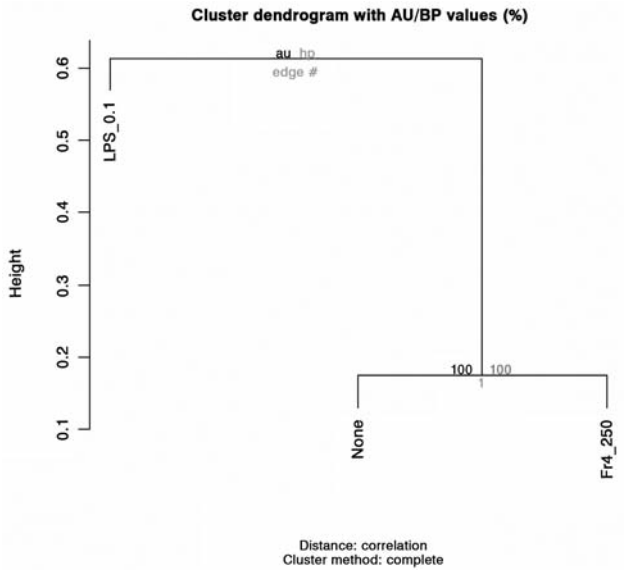

Figure 2. *Hierarchical clustering dendrogram of 4 arrays on the base of gene expression pattern. Approximately unbiased (AU) p-Value computed by multiscale bootstrap resampling; bootstrap probability (BP) value.*

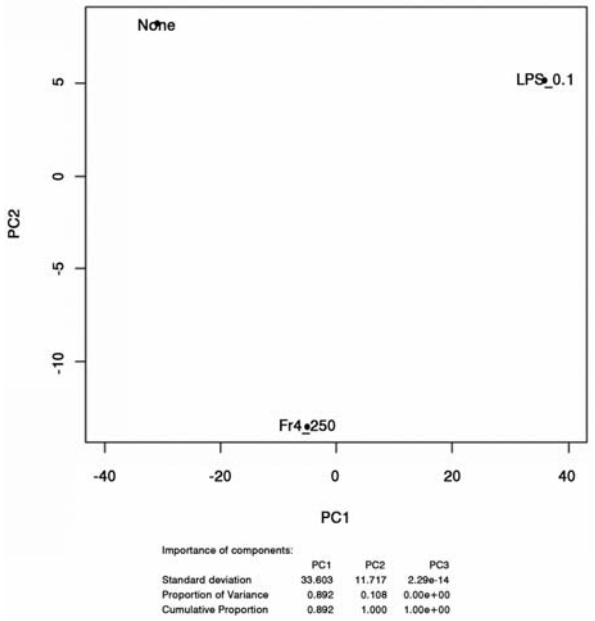

Figure 3. *Arrays plotted with respect to the first and second principal components of principal component analysis.*

RNA isolation and preparation. Total RNA was isolated from cell samples according to the manufacturer's instructions using the Qiagen RNeasy Plus Mini kit (Qiagen Inc, Tokyo, Japan). The quality of the RNA was checked with Agilent 2100 Bioanalyzer. Total RNA was supplied with Ambion's ArrayControl RNA spikes and *in vitro* synthesised polyadenylated *Bacillus subtilis* T3 RNA spikes, and amplified according to the Eberwine procedure (21) using Ambion's MessageAmp™ kit.

Array hybridization. The three prepared RNA samples, namely 'Fr4_250', 'None' as negative control of Fr4_250 and 'LPS_0.1' as positive control of Fr4_250, were submitted to GLab Pathology Center Co, Ltd (Hokkaido, Japan), and the labeling, hybridization, washing, and scanning were performed there. For hybridization, GeneChip MouseGene 1.0 ST arrays (Affymetrix Inc, Tokyo, Japan) were used, which could measure the expression of at least 28,000 genes (using at least 7,500,000 probes). After hybridization, the Affymetrix GeneChip Command Console software (AGCC) was used for washing and scanning. All these procedures were conducted according to the manufacturer's instructions. The microarray data were normalized by robust multi-array analysis (RMA) using GeneSpring GX 10.0.2 (Agilent Technologies, Inc, Santa Clara, CA, USA). Statistical analysis was conducted using the R 2.9.2 statistical software (available from http://www.r-project.org/) and Bioconductor (available from http://www.bioconductor.org/).

Extraction of differential expressed genes. Normalized raw data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). First, on each array, 1,441 probes which had the lowest 5% of expression intensity and low-trust data were removed from 28,815 probes for quality control. Next, from the remaining 27,374 probes, differentially expressed genes (DEG), identified as genes which showed a fold change of at least 2, were extracted. Fold change (FC) was calculated as the ratio of the expression value of a gene in one array to that in another array.

DEG values were: (i) 'None' relative to 'Fr4_250': 97 genes, (ii) 'None' relative to 'LPS_0.1': 545 genes, (iii) 'Fr4_250' relative to 'None': 178 genes, (iv) 'Fr4_250' relative to 'LPS_0.1': 192 genes, (v) 'LPS_0.1' relative to 'None': 534 genes and (vi) 'LPS_0.1' relative to 'Fr4_250': 326 genes. The number of these DEG was 1,314 genes.

Hierarchical clustering (HCL). The three arrays, namely 'None', 'Fr4_250', and 'LPS_0.1', were classified on the basis of the expression profiles of the 1,314 DEG among arrays using a hierarchical clustering method. The hierarchical clustering was performed using the R 2.9.2 statistical software together with the pvclust package (available from http://cran.r-project.org/web/packages/pvclust/index.html) (22), after a log2 transformation of expression raw data were performed. The parameter values 'correlation' and 'complete' were used as method.dist and method.hclust, respectively.

Principal component analysis (PCA). The three arrays were classified on the basis of the expression profiles of the 1,314 DEG using principal component analysis (PCA). PCA was performed using the R 2.9.2 statistical software together with the prcomp package (available from http://rss.acs.unt.edu/Rdoc/ library/stats/html/prcomp.html).

Gene annotation using the DAVID annotation tool. In order to identify gene expression trends in each array, gene annotation enrichment analysis (or ORA: over-representation analysis) was performed for each DEG set using the DAVID2008 knowledgebase (available from http://david.abcc.ncifcrf.gov/)

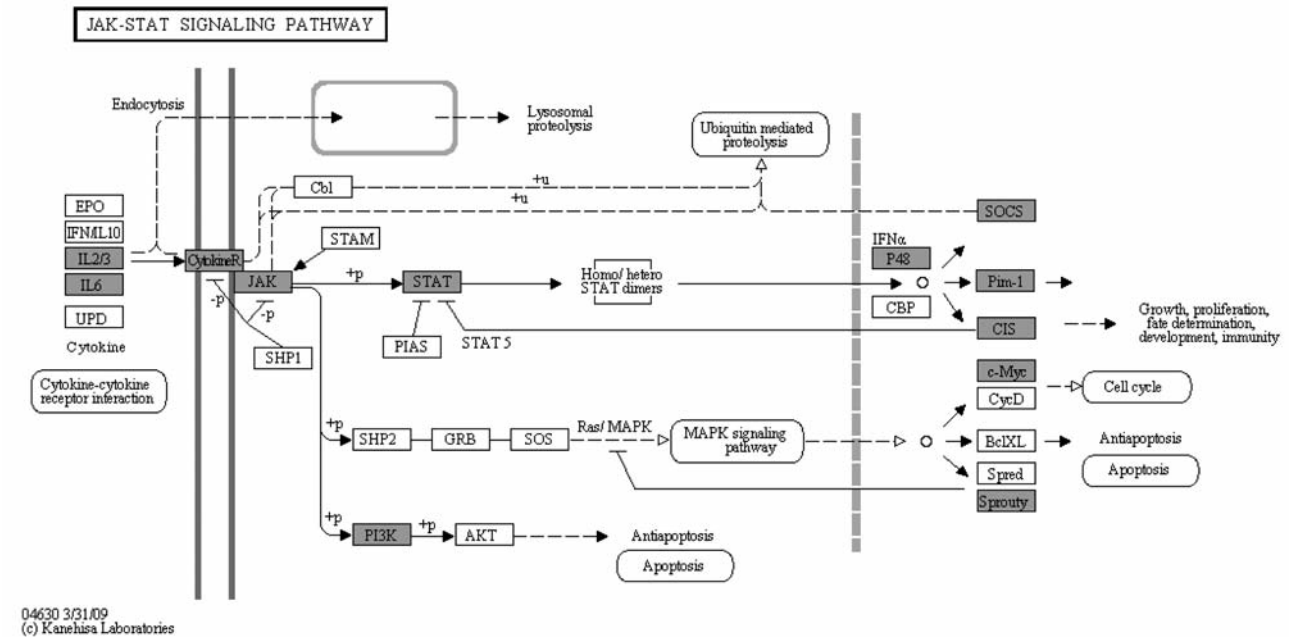

Figure 4. *JAK-STAT signaling pathway of KEGG pathway (mmu4630). Gray-colored rectangles indicate differentially expressed genes of 'LPS_0.1' as compared to 'None' (FC≥2).*

(23). Each DEG set was annotated by the enriched (over-represented) GeneOntology categories (available from http://www.geneontology.org/) (24) and KEGG pathways (available from http://www.genome.jp/kegg/pathway.html) (25), with a threshold of FDR (false discovery rate) <0.05.

Gene annotation using the GeneTrail annotation tool. Furthermore, gene set enrichment analysis (GSEA) (26) was used to identify gene expression trends of each array. Here, the input data set were sorted by FC values in ascending order of genes, instead of the set of DEG which showed a FC of at least 2. GSEA has the advantage that it does not require any predefined threshold, which may not have substantial cause, for DEG. The advanced gene set enrichment analysis tool GeneTrail (available from http://genetrail.bioinf.uni-sb.de/) (27) was used to perform functional annotation by GeneOntology categories and KEGG pathways.

Results and Discussion

Polyphenol analysis and LPS contamination in Fr4. Polyphenol analysis demonstrated that lignin carbohydrate complex from LEM (Fr4) is composed of lignin precursors such as vanillic acid (25.9 μg/g), syringic acid (25.7 μg/g), *p*-coumaric acid (157.7 μg/g) and ferulic acid (13.4 μg/g), with trace levels (<0.1 μg/g) of flavonoids (fisetin, daizein, genistein, quercetin, kaempferol, apigenin and chrysin), tannins and related compounds (gallic acid, gallo catechin, catechin, epicatechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, catechin gallate, ellagic acid, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-gallate), chlorogenic acid and edugenol, and negligible amount (0.0395 μg/g) of LPS (data not shown). This confirmed the authenticity of Fr4 as a lignin.

TNF-α production. Among seven lignin–carbohydrate complex fractions, Fr4 most potently stimulated the production of TNF-α by mouse macrophage-like J774.1 cells (Figure 1), in addition to its highest anti-HIV activity and inhibition of LPS-stimulated NO production (19). For DNA microarray analysis, near confluent J774.1 cells were treated without ('None'), or with 250 μg/ml Fr4 ('Fr4_250') or 0.1 μg/ml LPS ('LPS_0.1'). The TNF-α concentration in the culture medium was elevated from 1317 pg/ml ('None') to 8809 ('Fr4_250') and 17414 pg/ml ('LPS_0') (data not shown). The RNA was prepared from these cells for the following DNA microarray analysis.

Classification of genes and arrays using HCL. Figure 2 shows the HCL dendrogram of the 3 arrays on the basis of gene expression patterns. Similarity of gene expression patterns among arrays is indicated by the vertical distance. As a result, the gene expression of 'Fr4_250' and 'None' was found to be similar, while, that of 'LPS_0.1' and 'Fr4_250' was found to be less similar.

Classification of arrays using PCA. Figure 3 shows a visualization of the 3 arrays in the space of the first and second principal components of PCA. The horizontal axis indicates the first component and the vertical axis indicates

Table I. *GeneOntology analysis of differentially expressed genes DEG in 'None', 'Fr4_250' and 'LPS_0.1' using the DAVID knowledge base (over-representation analysis).*

		GeneOntology	
	No. of DEG	Biological process	Molecular function
'Fr4_250' in comparison to 'None'	178	Immune response Immune system process Inflammatory response Iesponse to external stimulus Response to wounding Chemotaxis Taxis Response to stress Response to stimulus Response to chemical stimulus	Cytokine activity GTPase activity Receptor binding Guany1 ribonucleotide binding Guany1 nucleotide binding GTP binding
'None' in comparison to 'Fr4_250'	97		
'LPS_0.1' in comparison to 'None'	534	Immune response Immune system process Inflammatory response Apoptosis Programmed cell death Cell death Response to wounding Death Response to external stimulus Defense response	Cytokine activity Chemokine activity Chemokine receptor binding G-protein-coupled receptor binding Protein binding Receptor binding GTPase activity
'None' in comparison to 'LPS_0.1'	545	DNA replication DNA metabolic process Biological regulation Regulation of biological process Cell cycle Regulation of cellular process Positive regulation of biological process Cell activation Immune system process DNA-dependent DNA replication	Protein binding DNA binding
'LPS_0.1' in comparison to 'Fr_205'	326	Immune response Immune system process Inflammatory response Defense response Response to stimulus Response to wounding Response to other organisms Response to virus Cytokine metabolic process Response to external stimulus	Cytokine activity GTPase activity Receptor binding Nucleoside triphosphatase activity Pyrophosphatase activity Hydrolase activity, acting on acid Anhydrides, in phosphorus-containing
'Fr4_250' in comparison to 'LPS_.01'	192		

The row 'Fr_250 in comparison to None' represents annotation results for differentially expressed genes of 'Fr_250' to 'None' (FC≥2). Top 10 categories which showed the threshold of FDR<0.05 and at least 10 genes contained within each category were used for the functional annotation. Blank columns indicate that there are no statistically significant categories. The results of the cellular component category in Gene Ontology were omitted.

Table II. *GeneOntology analysis of 'None', 'Fr4_250' and 'LPS_0.1' by GSEA using GeneTrail (GSEA). For example, the row 'Fr_250' in comparison to 'None' represents the results when a set of sorted FC values of 'Fr_250' to 'None' were used as the input data set of GeneTrail.*

	GeneOntology	
	Biological process	Molecular function
'Fr4_250' in comparison 'None'	Chemotaxis Taxis Locomotory behavior Multi-organism process Response to other organisms Response to biotic stimulus Response to chemical stimulus Behavior Response to external stimulus Response to virus	Cytokine activity Cytokine receptor binding Growth factor receptor binding Chemokine activity Chemokine receptor binding Receptor binding G-protein-coupled receptor binding
'None' in comparison to 'Fr4_250'	DNA replication Organelle organization Cell cycle DNA metabolic process Regulation of cellular process Cellular component organization Biological regulation Cellular process Negative regulation of transcription, DNA-dependent Negative regulation of RNA metabolic process	Enzyme activator activity Phosphatase activity Small GTPase regulator activity DNA binding GTPase activator activity Phosphoric ester hydrolase activity
'LPS_0.1' in comparison to 'None'	Defence response Response to other organism Immune response Response to biotic stimulus Multi-organism process Response to virus Chemotaxis Taxis Behavior Locomotory behavior	Cytokine activity Chemokine receptor binding Cytokine activity Chemokine receptor binding Receptor binding Growth factor receptor binding G-protein-coupled receptor binding GTPase activity Regulation of DNA binding Regulation of binding
'None' in comparison to 'LPS_0.1'	DNA replication DNA metabolic process Organelle organization Cellular component organization Cell cycle DNA repair Cellular process Regulation of cellular process Cell cycle phase M phase	Enzyme activator activity Enzyme regulator activity GTPase regulator activity
'LPS_0.1' in comparison to 'Fr_205'	Defence response Response to virus Multi-organism process Response to other organism Response to biotic stimulus Inflammatory response Response to external stimulus Response to wounding Immune receptors Negative regulation of molecular function	Cytokine activity Chemokine receptor binding GTPase activity G-protein-coupled receptor binding Cytokine activity Chemokine receptor binding Receptor binding Growth factor receptor binding

Top 10 categories which showed the threshold of FDR<0.05 and at least 10 genes contained within each category were used for the functional annotation. Blank columns indicate that there are no statistically-significant categories. The results of the cellular component category in GeneOntology were omitted.

Table II. *continued*

Table II. *continued*

	DNA replication	Enzyme regulator activity
	DNA metabolic process	GTPase regulator activity
	Nervous system development	Nucleoside triphosphatase regulator activity
'Fr4_250' in comparison to 'LPS_.01'	DNA repair	Enzyme activator activity
	Cellular component organization	GTPase activator activity
	Cellular process	Cytokine receptor activity
	Cell cycle	Small GTPase regulator activity
	Central nervous system development	Actin binding
	Biological process	Cytokine binding
	Brain development	Hydrolase activity, acting on ester bonds

Table III. *KEGG pathway analysis of differentially expressed genes (DEG) in 'None', 'Fr4_250' and 'LPS_0.1' using the DAVID knowledge base (over representation analysis).*

	No. of DEG	KEGG Pathway
'FR4_250' in comparison to 'None'	178	Cytokine-cytokine receptor interaction Toll-like receptor signaling pathway
'None' in comparison to 'Fr4_250'	97	
'LPS_0.1' in comparison to 'None'	534	Toll-like receptor signaling pathway Cytokine-cytokine receptor interaction Jak-STAT signaling pathway
'None' in comparison to 'LPS_0.1'	545	Cell cycle
'LPS_0.1' in comparison to 'FR_250'	326	Cytokine-cytokine receptor interaction Toll-like receptor signaling pathway
'LPS_0.1' in comparison to 'Fr4_250'	192	

The row 'Fr_250 in comparison to None' represents annotation results for differentially expressed genes of 'Fr_250' to 'None' (FC≥2). Top 10 categories which showed the threshold of FDR<0.05 and at least 10 genes contained within each category were used for the functional annotation. Blank columns indicate that there are no statistically significant categories.

Table IV. *KEGG pathway analysis of 'None', 'Fr4_250' and 'LPS_0.1' by GSEA using GeneTrail (GSEA). For example, the row 'Fr_250 in comparison to None' represents the results when a set of sorted FC values of 'Fr_250' to 'None' were used as the input data set of GeneTrail.*

	KEGG Pathway
'FR4_250' in comparison to 'None'	
'None' in comparison to 'Fr4_250'	
'LPS_0.1' in comparison to 'None'	Toll-like receptor signaling pathway Cytokine–cytokine receptor interaction Chemokine signaling pathway RIG-I-like receptor signaling pathway Graft-*versus*-host disease Type I diabetes mellitus Allograft rejection Cell adhesion molecules (CAMs)
'None' in comparison to 'LPS_0.1'	Cell cycle DNA replication Axon guidance Wnt signaling pathway Chronic myeloid leukemia Pancreatic cancer
'LPS_0.1' in comparison to 'FR_250'	Toll-like receptor signaling pathway RIG-I-like receptor signaling pathway CAMs Graft-*versus*-host disease Type I diabetes mellitus Allograft rejection Cytokine–cytokine receptor interaction
'LPS_0.1' in comparison to 'Fr4_250'	DNA replication Cell cycle Axon guidance Prostate cancer Melanoma

Top 10 categories which showed a threshold of FDR<0.05 and at least 10 genes contained within each category were used for the functional annotation. Blank columns indicate that there are no statistically significant categories.

the second component. The cumulative proportion from first to second components was 100.0%. In the diagram, there are not so apparent similarities between the gene expression pattern of 'None', 'Fr4_250' and 'LPS_0.1'.

GeneOntology annotation using the DAVID and GeneTrail tools. The results of the GeneOntology analysis by ORA using DAVID, and by GSEA using Genetrail are shown in Tables I

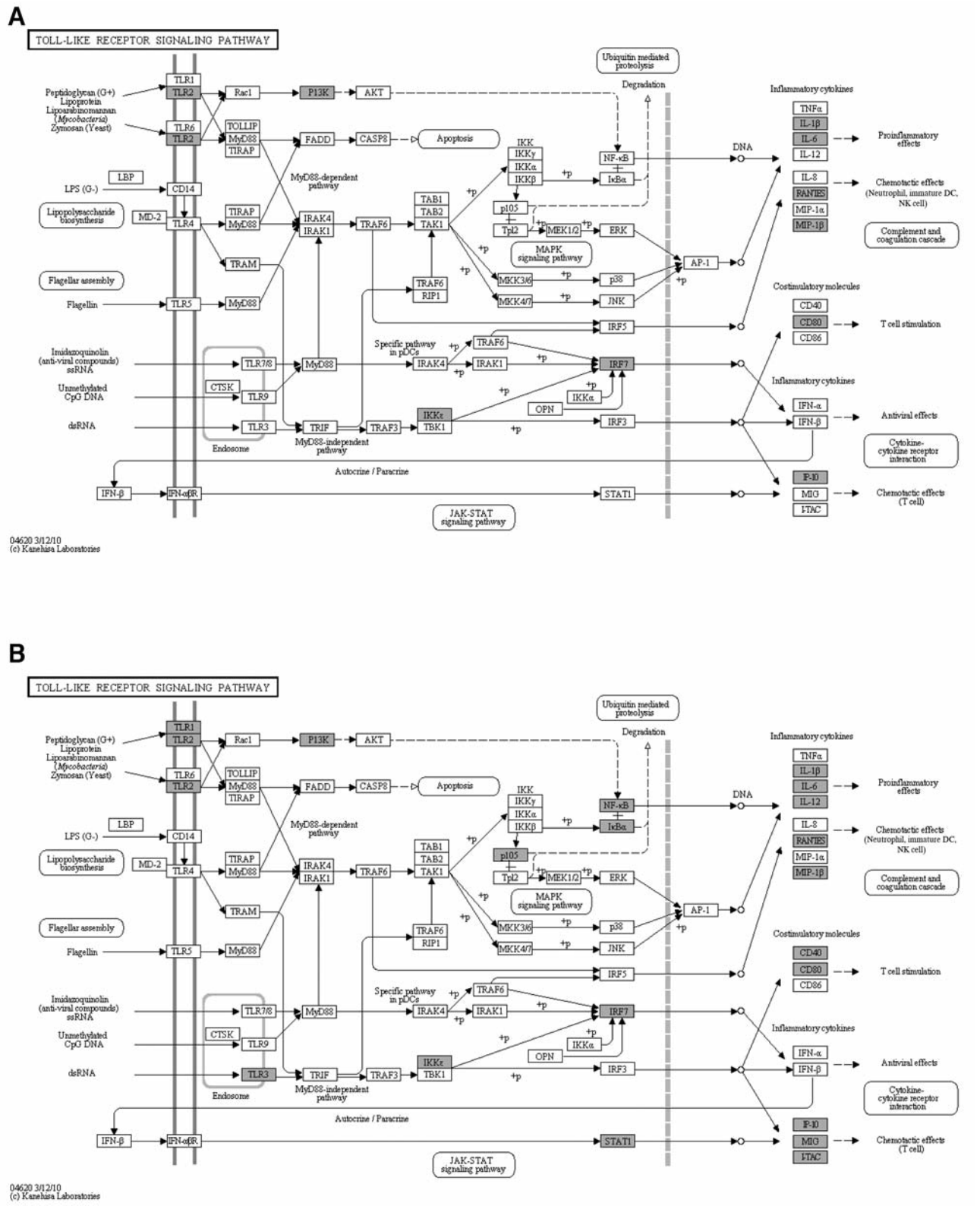

Figure 5. *Toll-like receptor signaling pathway of KEGG pathway (mmu04620). In diagram A, gray-colored rectangles indicate differentially expressed genes of 'Fr4_250' compared to 'None' (FC>=2). In diagram B, gray-colored rectangles indicate differentially expressed genes of 'LPS_0.1' compared to 'None' (FC≥2).*

and II, respectively. The overexpressed genes of 'Fr4_250' in comparison to 'None' were significantly annotated as genes related to the following biological processes, immune response, inflammatory response, response to external stimulus, locomotory behaviour, response to other organisms *etc*. The overexpressed genes of 'LPS_0.1' in comparison to 'None' were significantly annotated as genes of the following biological processes and molecular functions, immune response, response to wounding (injury damage), cytokine activity, defense response, response to other organisms *etc*. (Tables I and II). The enriched categories, as compared with untreated control, annotated in both 'Fr4_250' and 'LPS_0.1' were found to be similar. These findings suggest that extracts of 'Fr4_250' and 'LPS_0.1' may have similar bioactivity with respect to immune response-related gene expression.

In contrast, the overexpressed genes of 'LPS_0.1' in comparison to 'Fr4_250' were significantly annotated as genes related to immune response, inflammatory response, defense response, response to virus, response to other organisms, cytokine activity and other processes related to immune response (Tables I and II). Moreover, the number of DEG of 'LPS_0.1' in comparison to 'None' was three times as many as that of 'Fr4_250' in comparison with 'None' (Table I). Therefore, it is suggested that 'LPS_0.1' may affect immune response-related gene expression more strongly than 'Fr4_250'.

Furthermore, it was confirmed that the overexpressed genes of 'LPS_0.1' in comparison to 'None' were significantly annotated as genes related to biological processes, such as apoptosis, programmed cell death *etc*., whereas no such difference was found between 'Fr4_250' and None' (Table I).

KEGG pathway annotation using DAVID, and GeneTrail tools. The results of the KEGG pathway analysis by ORA using DAVID and by GSEA using GeneTrail are shown in Tables III and IV, respectively. It is largely known that LPS induces the production of cytokines by the toll-like receptor signaling pathway through TLR4 (28). In the present study, the overexpressed genes of 'LPS_0.1' in comparison to 'None' were significantly annotated as genes related to the following pathways, cytokine–cytokine receptor interaction, toll-like receptor signaling pathway and chemokine signaling pathway (Tables III and IV). Furthermore, the overexpressed genes of 'LPS_0.1' in comparison to 'None' were significantly annotated as genes of the JAK-STAT signaling pathway (Table III). It is known that LPS activates JAK2, part of the JAK-STAT signaling pathway (29). The present study demonstrated that in the JAK–STAT signaling pathway of 'LPS_0.1' in comparison to that of 'None', 19 genes, namely *Jak2, Csf2, Il15, Csf3, Il12b, Il6, Il23r, Osm, Ghr Stat1, Stat2, Pik3r5, Irf9, Socs1, Socs3, Pim1, Cish, Myc* and *Spry4,* were over-expressed (FC≥2) (Figure 4).

In contrast, when comparing 'Fr4_250' with 'None', only the cytokine-cytokine receptor interaction and the Toll-like receptor signaling pathway were significantly annotated (Table III). Likewise, the overexpressed genes of 'LPS_0.1' in comparison to 'Fr4_250' were significantly annotated as genes related to the following pathways, toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway and cell adhesion molecules. These results suggest that 'Fr4_250' may not influence innate immune response-related gene expression as strongly as 'LPS_0.1'.

DEG (FC≥2) in toll-like receptor signaling pathway were compared between 'Fr4_250' and 'LPS_0.1' relative to 'None'. The number of DEG of 'Fr4_250' and 'LPS_0.1' was 10 (*Tlr4, Pik3r5, Ikbke, Irf7, Il1b, Il6, Ccl5, Ccl4, Cd80* and *Cxcl10*) and 19 (*Tlr1, Tlr3, Tlr4, Pik3r5, Nfkb1, Nfkbia, Ikbke, Irf7, Stat1, Il1b, Il6, Il12b, Ccl5, Ccl4, Cd40, Cd80, Cxcl10, Cxcl9,* and *Cxcl11*), respectively. Consequently, it was found that the DEG set of 'Fr4_250' was a subset of that of 'LPS_0.1' (Figure 5). These results suggest that 'Fr4_250' would affect innate immune response-related gene expression; however, it may not affect the expression of as many genes as LPS does.

In conclusion, the present study demonstrates that relatively higher concentrations of Fr4 induced a pattern of gene expression similar to that of LPS, however, and the number of genes induced by Fr4 was smaller than that induced by LPS. It remains to be investigated whether lower concentrations of Fr4 induce a pattern of gene expression similar to that induced by LPS.

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