Enhancement of *Dectin-2* Gene Expression by Lignin–Carbohydrate Complex from *Lentinus edodes* Mycelia Extract (LEM) in a Mouse Macrophage-like Cell Line

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Abstract. *Background: Lignin fractions of Lentinus edodes mycelia extract (LEM) have shown anti-HIV and immunopotentiating activity. However, the action point of lignin–carbohydrate complex has not been elucidated. In order to elucidate their action point, DNA microarray analysis was performed, using mouse macrophage-like J774.1 cells. Materials and Methods: RNA was isolated with Qiagen RNeasy Plus Mini kit, hydridized with GeneChip MouseGene 1.0 ST arrays, and scanned with Affymetrix GeneChip Command Console software. Results: One of the seven lignin–carbohydrate fractions isolated from LEM (Fr4) enhanced the expression of dectin-2 (4.2-fold) and toll-like receptor (TLR)-2 (2.5-fold) prominently, but only slightly modified the expression of dectin-1 (0.8-fold), complement receptor 3 (0.9-fold), TLR1, 3, 4, 9 and 13 (0.8- to 1.7-fold), spleen tyrosine kinase (Syk)b, zeta-chain (TCR) associated 70 kDa protein kinase (Zap70), Janus tyrosine kinase (Jak)2 (1.0- to 1.2-fold), nuclear factor (Nf)κb1, NFκb2, reticuloendotheliosis viral oncogene homolog (Rel)a, Relb (1.0- to 1.6-fold), Nfκbia, Nfκbib, Nfκbie, Nfκbil2 and Nfκbiz (0.8- to 2.3-fold). On the other hand, lipopolysaccharide did not affect the expression of dectin-2 or TLR-2. Conclusion: These data suggest the significant role of the activation of the dectin-2 signaling pathway in the action of lignin–carbohydrate complex on macrophages.*

Lentinus edodes mycelia extract (LEM) has shown anti-hepatopathic (1-3), antitumour (4-8), immunopotentiating (9, 10), antivascularisation (11), anti-arteriosclerosis (12), skin-protective (13, 14) and undocumented activities, such as improvement of hepatic function, menorrhagia, melancholia, nausea and vomiting. Lentinan (15) and KS-2 (16), polysaccharides isolated from *L. edodes* have shown immunopotentiating and antitumour activities. Lignin fractions of LEM have shown anti-HIV (17) and immunopotentiating (18) activity. However, the action point of these lignin fractions has not been elucidated. We recently isolated seven lignin-carbohydrate fractions from LEM, and among them, fraction 4 (Fr4) showed the highest anti-HIV activity and most potently inhibited NO production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells (RAW264.7, J774.1) (19). Fr4 was found to be 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 LPS, confirming the authenticity of Fr4 as lignin (20). Preliminary DNA microarray analysis suggested that Fr4 may affect immune response-related gene expression, but may not affect the expression of as many genes as LPS did (20). Dectin-1 and dectin-2 have been identified as the receptors for β-glucan and a-mannan, respectively (21, 22). We previously reported that the polysaccharide portion of lignin-carbohydrate complex from pine cone extract of *Pinus parviflora* Sieb et Zucc is comprised of arabinose, mannose, galactose and glucose (23), suggesting that lignin–cabohyrate complex may bind to dectin-1 or dectin-2. In order to elucidate the action point of Fr4, DNA microarray analysis of the effect of Fr4 at different doses and in combination with LPS was performed for J774.1 cells.

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Key Words: Microarray analysis, *dectin-2* gene expression, signaling pathway, macrophage, lignin-carbohydrate complex, LPS, LEM.

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. Fr4 was isolated from LEM by alkaline extraction and acid precipitation, as described previously (20).

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 a humidified 5% CO_2 atmosphere (20). 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 'Control') or with 125 or 250 μg/ml Fr4 (referred to as 'Fr125' or 'Fr250'), or 0.1 μg/ml LPS (referred to as 'LPS') or combination of 250 μg/ml Fr4 and 0.1 μg/ml LPS (referred to as 'Fr250+LPS'). In some experiments, the cells were treated with RLT plus buffer (RNeasy Plus Mini Kit; Qiagen Inc., Tokyo, Japan).

Assay for TNF production. The tumor necrosis factor (TNF) released into the culture medium was determined by ELISA (Quantikine ELISA kit, R&D systems, Minneapolis, MN, USA).

RNA isolation and preparation. Total RNA was isolated from cell samples according to the manufacturer's instructions using a Qiagen RNeasy Plus Mini kit (Qiagen Inc.). The quality of the RNA was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA, USA). Total RNA was used with Ambion's ArrayControl RNA (Ambion, Inc., Austin, TX, USA) spikes, and *in vitro* synthesised polyadenylated *Bacillus subtilis* T3 RNA spikes, and amplified according to the Eberwine procedure (24) using Ambion's MessageAmp™ kit.

Array hybridization. The five prepared RNA samples, namely Fr250, Fr125, LPS (as positive control for Fr250 and Fr125), Fr250+LPS, and Control (as negative control for Fr250 and Fr125), 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 measure the expression of at least 28,000 genes (using at least 7,500,000 probes). After hybridization and washing, the Affymetrix GeneChip Command Console software (AGCC) was used for 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). 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/). Normalized raw data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Extraction of differentially expressed genes. Normalized raw data were analyzed using Microsoft Excel (Microsoft Corporation). First, from 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 (FC) of at least 2, were extracted. FC was calculated as the ratio of the expression value of a gene in one array to that in another array. The total number of these DEG was 1,314 genes in five arrays.

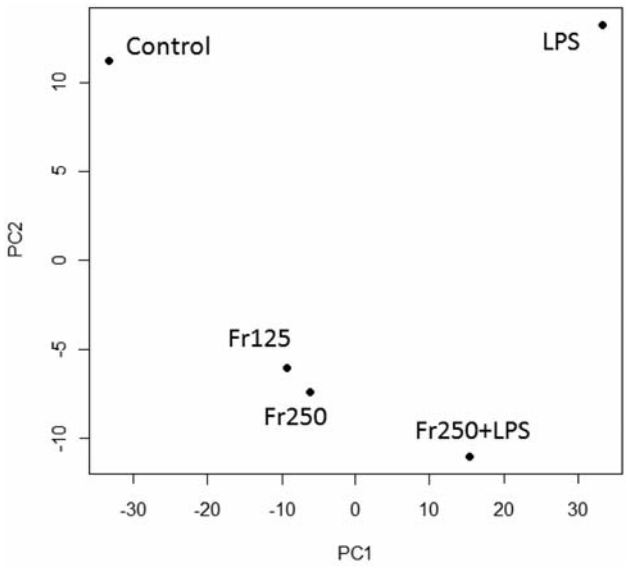


Figure 1. *Arrays plotted with respect to the first and second principal components of principal component analysis*.

Principal component analysis (PCA). The five arrays were classified on the basis of the expression profiles of the 1,314 DEGs using principal component analysis (PCA). PCA was performed using the R 2.11.1 statistical software together with the prcomp package (available from http://rss.acs.unt.edu/Rdoc/library/stats/html/prcomp.html).

Hierarchical clustering (HCL). The five arrays, namely Fr125, Fr250, LPS, Fr250+LPS, and Control, were classified on the basis of the expression profiles of the 1,314 DEG among arrays using a hierarchical clustering (HCL) method. First, a $\log_2$ transformation of raw expression data was performed, and then the data were z-score transformed so that each gene has a mean 0 and a variance of 1 using R 2.11.1 software together with the genefilter package (available from http://www.bioconductor.org/packages/2.6/bioc/html/genefilter.html) (25).

Next, HCL was performed for the gene clustering and the array clustering using open source clustering software Cluster 3.0 (available from http://bonsai.hgc.jp/~mdehoon/software/cluster/) (26). 'Correlation [uncentered]' and 'Complete linkage' were used as 'Similarity Metric' and 'Clustering method', respectively. The results of the HCL were visualized using Java TreeView (available from http://jtreeview.sourceforge.net/) (27).

Results and Discussion

TNF production. Fr4 dose-dependently stimulated the production of tumour necrosis factor-α (TNF-α) by mouse-macrophage-like J774.1 cells. The TNF-α concentration in the culture medium was elevated from 132 pg/ml (Control) to 706 pg/ml (Fr125), 881 pg/ml (Fr250), 1,741 pg/ml (LS) and 1,880 pg/ml (LPS+Fr250). The RNA was prepared from these cells for the subsequent DNA microarray analysis.

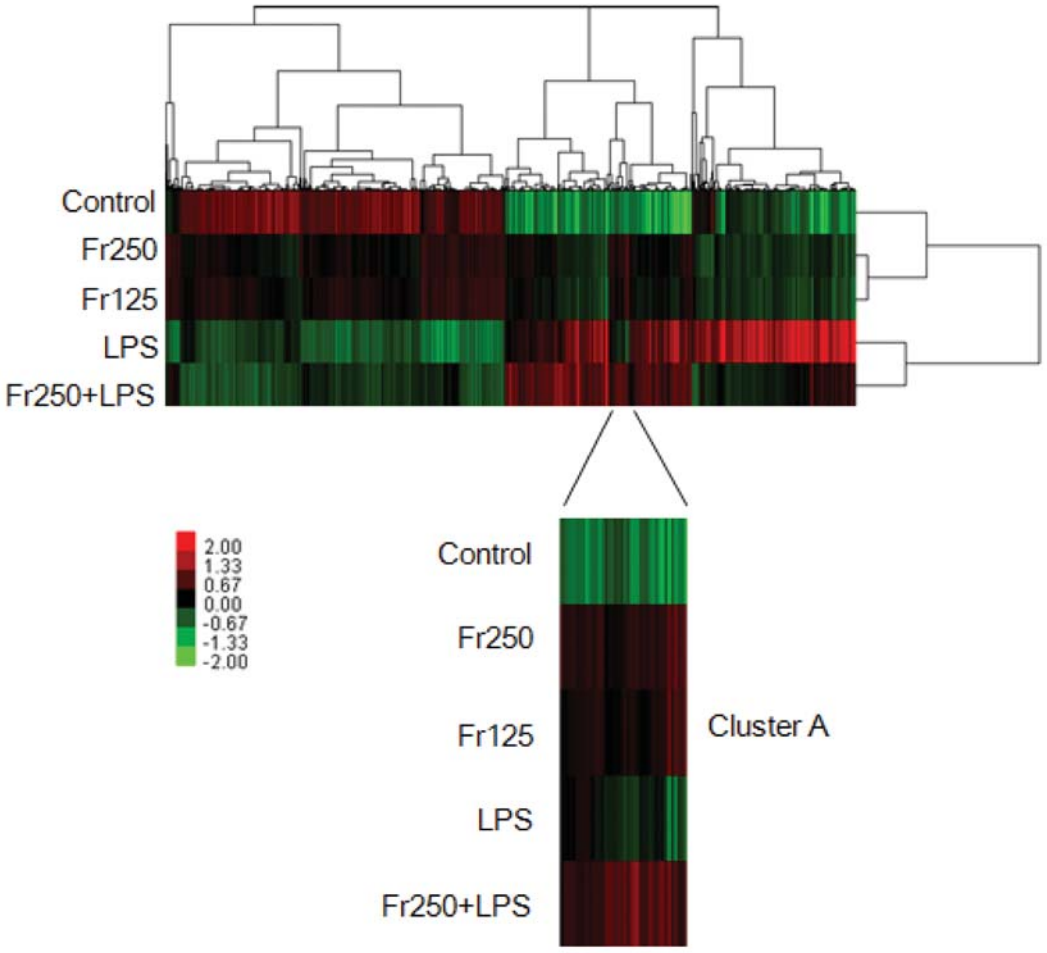


Figure 2. *Hierarchical clustering dendrogram of 5 arrays. The upper dendrogram displays the result of 1,314 gene clustering, and that below displays the result of 5 arrays, with Control, Fr250, Fr125, LPS, and Fr250+LPS treatments. In the heat map, red represents overexpressed genes, and green represents underexpressed genes. Cluster A indicates a cluster consisting of 40 genes, including Clec4n, and Clec4d of the Dectin-2 family, and Srxn1, and Txnrd1 for antioxidant genes, which were overexpressed with Fr250, Fr125, and Fr250+LPS treatment compared with Control and LPS treatment.*

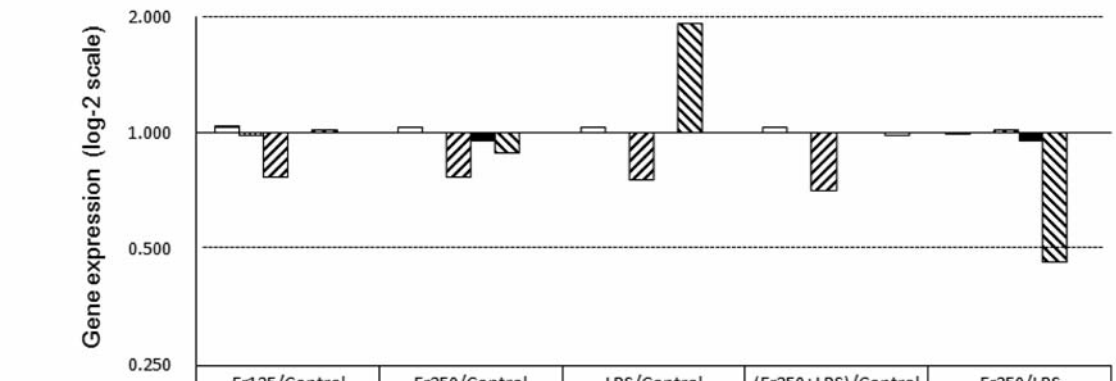
Classification of each treatment using PCA. Figure 1 shows a visualization of the five treatments in the space of the first and second principal components of PCA. The horizontal axis indicates the first component and the vertical axis indicates the second component. The cumulative proportion from first to second components was 96.3%. Similarity between the gene expression pattern under treatment with Fr125 and Fr250 was recognized.

Classification of each treatment using HCL method. Figure 2 shows the results of hierarchical clustering for 1,314 genes and 5 treatments. The dendrogram on the right indicates the gene expression patterns with Fr125 and Fr250 were the most similar, and those of LPS and Fr250+LPS were also similar, and then that of Control was more similar to Fr125 and Fr250 than LPS and Fr250+LPS.

Gene expression pattern of Dectin-1 and Dection-2 family. The expression patterns of Dectin-1 and Dectin-2 family genes (28) which are strongly suggested as receptors of polysaccharides of the cell walls in the fungus (Figures 3 and 4). For example, 'Fr125/Control' on the x-axis denotes the expression of each gene under Fr125 treatment in comparison to that with Control. The dotted lines represent FC in gene expression rate of above 2.00 and below 0.50 (Figures 3-7), changes which were considered significant.

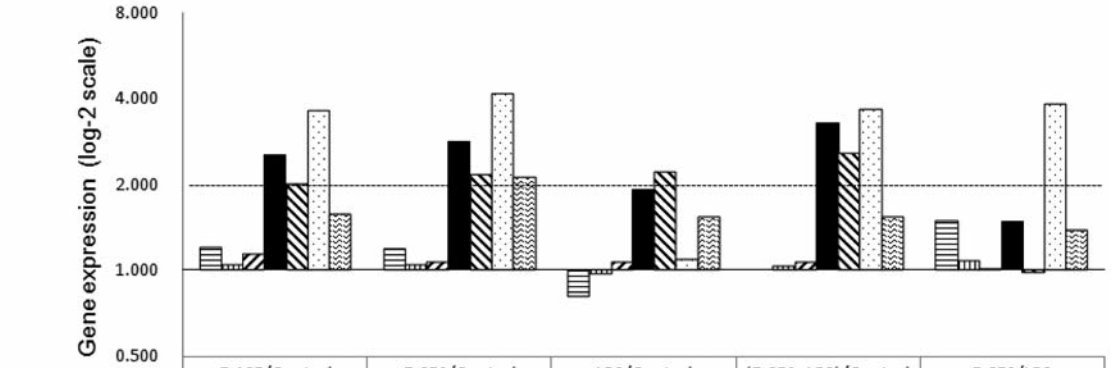
For Dectin-1 family genes, it was confirmed that there were no differences between the expression of *Clec1a, Clec1b, Clec7a (Dectin-1), Clec9a, Clec12a* and *Clec12b* genes with each treatment and that of Control.

Figure 4 shows the expression of Dectin-2 family genes under Fr125, Fr250, LPS, and Fr250+LPS treatment in comparison to Control. The expressions of *Clec4d, Clec4e,* and *Clec4n* (*Dectin-*



	Fr125/Control	Fr250/Control	LPS/Control	(Fr250+LPS)/Control	Fr250/LPS
Clec1a	1.042	1.028	1.031	1.031	0.996
Clec1b	0.980	1.000	1.000	1.000	1.000
Clec7a (Dectin-1)	0.766	0.766	0.752	0.705	1.018
Clec9a	1.000	0.954	1.000	1.000	0.954
Clec12a	1.000	0.883	1.914	1.000	0.461
Clec12b	1.000	1.000	1.000	0.980	1.000

Figure 3. *Comparison of expression profiles regarding the Dectin-1 gene family.*

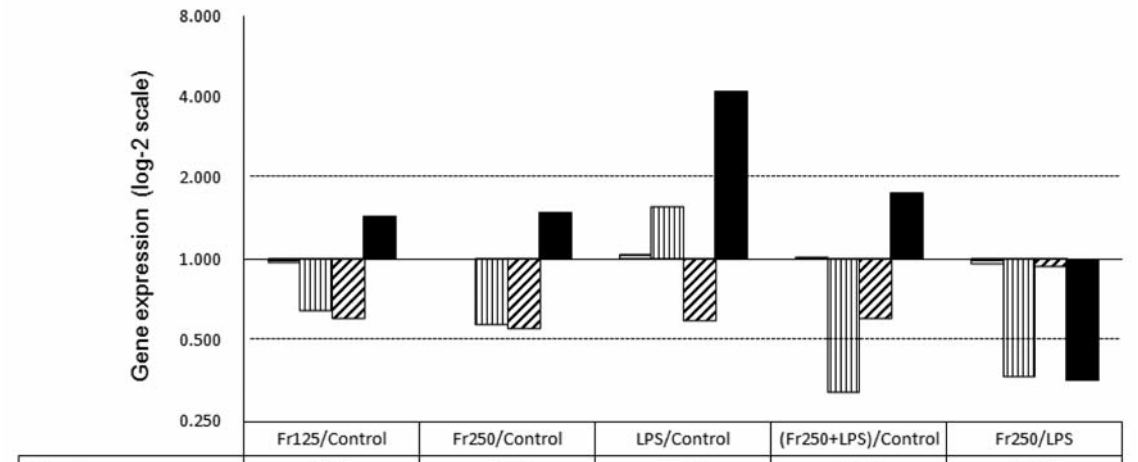


	Fr125/Control	Fr250/Control	LPS/Control	(Fr250+LPS)/Control	Fr250/LPS
Clec4a2	1.191	1.187	0.802	1.000	1.480
Clec4b1	1.040	1.035	0.968	1.031	1.069
Clec4b2	1.139	1.061	1.059	1.061	1.002
Clec4d	2.523	2.815	1.910	3.292	1.474
Clec4e	2.005	2.168	2.216	2.566	0.979
Clec4n (Dectin-2)	3.619	4.156	1.082	3.646	3.842
Clec4a4	1.563	2.111	1.529	1.529	1.380

Figure 4. *Comparison of expression profiles regarding the Dectin-2 gene family.*

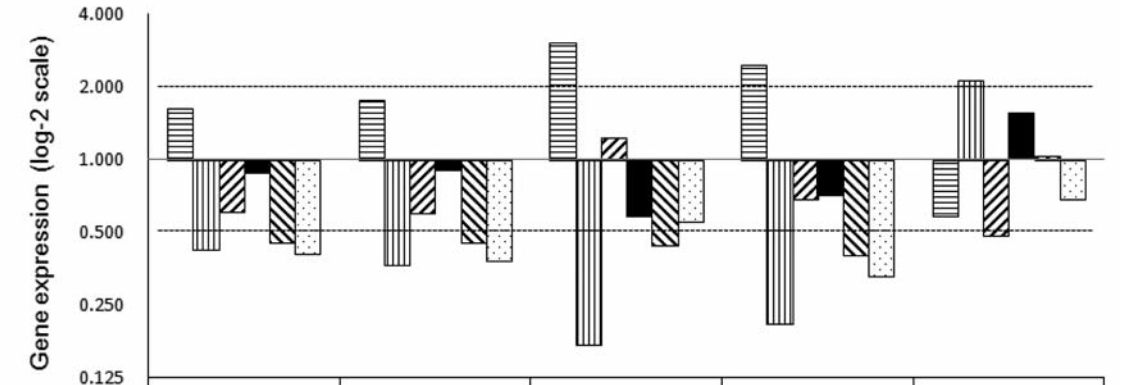
2) genes with both Fr125 and Fr250 were more than twice as high as those of Control. Furthermore, the expression of *Clec4n* (*Dectin-2*) in both of those treatments increased by more than threefold as compared with Control. On the other hand, LPS treatment did not change the expression of *Clec4n* (*Dectin-2*). This indicates that *Clec4n* (*Dectin-2*) was specifically overexpressed under treatment with Fr125 and Fr250.

There were no significant differences between the expression of *Clec4n* (*Dectin-2*) with Fr125 and Fr250. In terms of other Dectin-1 and Dectin-2 family genes, and thus the tendency of similarity between Fr125 and Fr250 was recognized (Figures 3, 4). Furthermore, there were no significant differences between the expressions of these genes among Fr250, LPS, Fr250+LPS treatment, and thus



	Fr125/Control	Fr250/Control	LPS/Control	(Fr250+LPS)/Control	Fr250/LPS
Fcer1g	0.974	1.000	1.043	1.005	0.959
Fcgr1 (CD64)	0.645	0.567	1.558	0.320	0.364
Fcgr3 (CD32A homology)	0.599	0.553	0.589	0.599	0.937
Fcgr4 (CD16)	1.449	1.496	4.204	1.758	0.356

Figure 5. *Comparison of expression profiles regarding the Fc receptor gene family.*

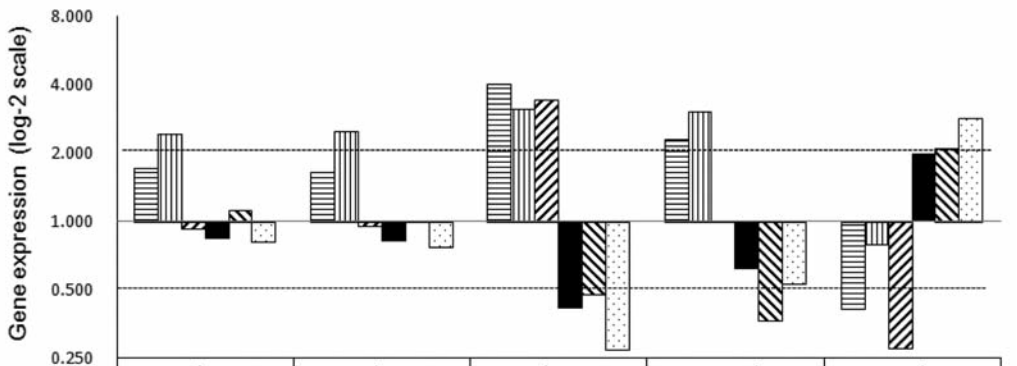


	Fr125/Control	Fr250/Control	LPS/Control	(Fr250+LPS)/Control	Fr250/LPS
Itga5	1.637	1.762	3.027	2.475	0.582
Itga6	0.421	0.367	0.172	0.210	2.135
Itgal	0.608	0.598	1.228	0.683	0.487
Itgam (CR3)	0.883	0.901	0.579	0.705	1.555
Itgav	0.452	0.452	0.439	0.401	1.030
Itgb5	0.407	0.380	0.555	0.327	0.685

Figure 6. *Comparison of expression profiles regarding the Integrin gene family.*

the synergy effect of the combination of Fr250 and LPS on the enhancement of gene expression were not observed (Figures 3, 4).

Expression pattern regarding the Fc receptor family genes. Activated *Clec4n* by its ligands would control the transcription of cytokines through Fcer1g which is a member of the Fc receptor family, with immune receptor tyrosine-based activation motif (ITAM) domain. Following the phosphorylation of the ITAM of Fc receptor after lectin receptor (Dectin-2) activation, spleen tyrosine kinase (Syk)b (which is a member of non-receptor tyrosine kinase family that has two SH2 domains) is recruited to activate caspase recruitment domain family, member 9 (Card9)-dependent



	Fr125/Control	Fr250/Control	LPS/Control	(Fr250+LPS)/Control	Fr250/LPS
Tlr1	1.718	1.653	4.027	2.313	0.411
Tlr2	2.425	2.497	3.132	3.056	0.797
Tlr3	0.931	0.953	3.431	1.005	0.278
Tlr4	0.840	0.823	0.416	0.623	1.979
Tlr9	1.120	1.011	0.479	0.367	2.110
Tlr13	0.816	0.773	0.272	0.529	2.845

Figure 7. *Comparison of expression profiles regarding the Tlr gene family.*

signaling pathway leading to the activation of NFKb (29). It is also known that Fcer1g co-expresses and co-localizes with Clec4n. Figure 5 shows the expression of Fc receptor family genes, including *Fcer1g*. There was no difference in the expression of Fcer1g between Fr125, Fr250, and LPS treatments. On the other hand, we observed a tendency for an increase of *Fcgr4* expression with LPS treatment.

Expression pattern of integrin family genes. Itgam (CR3), which is a member of the integrin family and expressed in macrophages, is considered as a receptor for β-glucan. Figure 6 shows the expression pattern of integrin family genes *Itga5, Itga6, Itgal, Itgam, Itgav*, and *Itgb5*. There was no difference in the expression of *Itgam* among treatments. It was confirmed that the expression of *Itga5* significantly increased only with LPS treatment. On the other hand, the expression of *Itga6, Itgav*, and *Itgb5* decreased with LPS, Fr125, and Fr250 treatments.

Expression pattern of Tlr family genes. Figure 7 shows the expression pattern of toll-like receptor (Tlr) family genes *Tlr1, Tlr2, Tlr3, Tlr4, Tlr9,* and *Tlr13*. The expression of *Tlr1, Tlr2*, and *Tlr3* was increased by LPS treatment, but, that of *Tlr4* (a known receptor of LPS), *Tlr9* and *Tlr13* were reduced by LPS treatment. On the other hand, the expression of *Tlr2* was increased more by Fr125, and Fr250 treatments, as well as by LPS treatment, than by Control. In the case of the expression of *Tlr1, Tlr3, Tlr4, Tlr9* and *Tlr13*, there was no difference between Control and Fr125, or Fr250 treatments.

Expression pattern of genes for inflammatory cytokines. The expression patterns of genes that are involved in the mammalian immune system, such as those in toll-like receptor signaling pathway, are listed in Table I. The expression of *Il1a, Il1b, Il6, Osm, Csf2,* and *Il12b* in LPS treatment was increased more than threefold that in Control. In particular, *Il6* gene expression was increased by more than two hundredfold that in Control. The expressions of *Il1a, Il1b, Il6, Osm*, and *Csf2* with Fr250 treatment were increased by more than twofold that of Control, as were the expressions of *Il1a, Il1b*, and *Il6* with Fr125.

The expressions of *Il1a, Il1b, Il6, Csf2*, and *Il12b* in Fr125 and Fr250 were reduced to less than one seventh level of that with LPS treatment. The findings indicate that Fr125 and Fr250 enhanced the expressions of genes for inflammatory cytokines, such as *Il1a, Il1b, Il6, Osm, Csf2*, and *Il12b*, but the effects of Fr125 and Fr250 were lower than those of LPS. The expression of *Osm* with Fr125, Fr250, and LPS increased by more than twofold compared to Control, but the ratio expression of *Osm* with Fr250, compared to LPS was 0.8, and no difference between gene expression induced by Fr250 and LPS was confirmed. The expressions of genes for other inflammatory cytokines, such as *Csf1, Csf3, Il18* and *Il1f6*, which are not listed in Table I, with LPS, Fr125, and Fr250 treatments were increased much more than that of Control. In the case of *Ifng*, *Il12, Il17a, Il17b, Il17c, Lif* and *Tnf*, there was no difference among their gene expressions induced with LPS, Fr125 and Fr250.

Table I. *Comparison of relative gene expressions under each treatment. For example, Fr125/Control lists the expression rate of each gene under Fr125 treatment in comparison with that of Control. Bold figures represent expression rates which are ≥2.00 or ≤0.50 i.e. significantly changed. Underlined figures represent gene expression rates which are ≤0.50.*

	Gene	Fr125/ Control	Fr250/ Control	LPS/ Control	Fr250+LPS/ Control	Fr250/ LPS
Inflammatory cytokines	*Il1a*	**2.45**	**3.49**	**105.15**	**66.02**	**0.03**
	Il1b	**3.28**	**5.39**	**43.65**	**35.65**	**0.12**
	Il6	**10.20**	**15.53**	**266.17**	**221.40**	**0.06**
	Osm	**2.70**	**3.06**	**3.85**	**6.74**	0.80
	Csf2	1.92	**2.36**	**28.25**	**21.76**	**0.08**
	Il12b	1.41	1.47	**32.33**	**6.27**	**0.05**
Anti-inflammatory cytokines	*Il4*	0.88	0.94	0.94	0.94	1.00
	Il10	1.09	1.02	1.40	0.91	0.73
	Il1rn	**5.10**	**5.65**	**14.38**	**12.28**	**0.39**
	Tgfb1	1.05	1.06	0.78	1.00	1.36
	Tgfb2	0.90	1.01	1.04	1.00	0.97
	Tgfb3	**0.30**	**0.25**	**0.35**	**0.21**	0.71
Non-receptor tyrosine kinase	*Sykb*	1.06	1.15	1.73	1.86	0.67
	Zap70	0.99	0.99	0.97	0.99	1.02
	Jak2	0.95	1.14	**4.28**	**2.69**	**0.27**
	Fes	0.73	0.71	**0.47**	**0.47**	1.52
	Hck	**2.53**	**2.81**	**5.91**	**3.68**	**0.47**
Card-Bcl10- Malt1	*Card11*	0.85	0.85	0.82	0.82	1.03
	Sgsh	**0.49**	**0.42**	**0.21**	**0.22**	**2.03**
	Card6	0.61	0.56	0.65	0.48	0.86
	Card9	0.91	0.87	0.91	0.83	0.95
	Bcl10	1.11	1.11	1.14	1.31	0.97
	Malt1	1.35	1.33	**2.52**	**3.71**	0.53
Ikbs	*Nfkbia*	1.78	1.86	**3.77**	**3.37**	**0.49**
	Nfkbib	1.13	1.16	1.69	1.61	0.69
	Nfkbid	1.30	1.53	1.14	1.42	1.34
	Nfkbie	0.90	1.07	1.60	1.23	0.67
	Nfkbil2	0.81	0.78	**0.36**	**0.42**	**2.17**
	Nfkbiz	**2.62**	**2.43**	**6.51**	**5.22**	0.37
Rel/Nfkb family	*Nfkb1*	1.32	1.44	**2.06**	1.86	0.70
	Nfkb2	1.55	1.64	**2.98**	**2.75**	0.55
	Rela	1.02	1.02	1.02	1.09	1.00
	Relb	1.63	1.62	1.84	**2.12**	0.88
	Rel	1.26	1.25	**2.05**	1.72	0.61

Expression pattern of genes for anti-inflammatory cytokines. The expression of *Il1rn*, an anti-inflammatory cytokine, with LPS, Fr125 and Fr250 treatments was increased by more than fivefold that in Control, and that of *Il1rn* with Fr250 was reduced to 0.39-fold that with LPS treatment. Meanwhile, the expression of *Tgfb3* in Fr125, Fr250, and LPS treatment was reduced to less than 0.35 times that of Control, and there were no differences in *Tgfb3* gene expression among Fr125, Fr250 and LPS treatments. In the case of the gene expressions of other anti-inflammatory cytokines, such as *Il4, Il10, Tgfb1, Tgfb2, and Il11* and *Il13*, which are not listed in Table I, there were no differences among Fr125, Fr250, LPS, LPS+Fr250, and Control.

Expression pattern of genes for non-receptor tyrosine kinases. We compared the expression pattern of genes for several non-receptor tyrosine kinases, such as *Sykb, Zap70, Jak2, Fes* and *Hck*. Sykb contains two SH2 domains and binds to the ITAM domain of FcRgamma, such as Fce1g (28, 30), and Zap70 contains two SH2 domains and binds to the ITAM domain of TCR (CD3) (31). There was no difference in the expression of *Sykb* among Fr125, Fr250, LPS, LPS+Fr250, and Control. The expression of *Fes* with LPS treatment was reduced to less than 50% that of Control. The gene expression of *Hck,* which contains an SH2 domain, an SH3 domain, and a membrane-associated domain, with Fr125, Fr 250, LPS, Fr250+LPS treatments were more than doubled as compared with Control. The gene expression for *Jak2*, which does not contain SH2 domains but binds to Il6 receptor and activates a transcription factor Stat, in LPS treatment was increased by more than fourfold that of Control.

Expression pattern of Card, Bcl10 and Malt1. We investigated the expression patterns of *Card11, Sgsh(Card14), Card6, Card9, Bcl10,* and *Malt1*, which are downstream of *Sykb* (32). The expressions of these genes were not clearly changed by Fr125, Fr250 or LPS (Table I).

Expression pattern of Ikb family genes. The expression of Iκb family genes, which are known as negative regulators for transcription factor Nfkb, were investigated. LPS stimulated the expression of Iκb family genes, except for *Nfκbil2*. Fr125 and Fr250 showed similar effects, but to a much lesser extent (Table I).

Expression pattern of Rel/Nfκb family genes. It is known that the Rel/Nfκb family, such as Nfκb1, Nfκb2, Rela, Relb and Rel, regulates the expression of cytokines, and chemokines. LPS stimulated the expression of *Nfκb1, Nfκb2* and *Rel*, whereas Fr125 and Fr250 were much less active (Table I).

Conclusion

The present DNA microarray study demonstrated for the first time that lignin–carbohydrate complex from LEM preferentially induced the expression of *Dectin-2* mRNA. This data suggests the involvement of Dectin-2 signaling pathway in the defense mechanism of lignin–carbohydrate complex. Dectin-2 is a specific receptor for α-mannan, and plays significant role in the regulation of protection of the body from *Candida* infection *via* induction of differentiation of IL-17-producing T-cells (29). It is expected that the activation of Dectin-2 may be significantly affected by the compositional changes in the lignin–carbohydrate complex. Further structure–activity study is underway in our laboratory.

References

1 Watanabe A, Kobayashi M, Hayashi S, Kodama D, Isoda K, Kondoh M, Kawase M, Tamesada M and Yagi K: Protection against D-galactosamine-induced acute liver injury by oral administration of extracts from *Lentinus edodes* mycelia. Biol Pharm Bull *29*: 1651-1654, 2006.

2 Akamatsu S, Watanabe A, Tamesada M, Nakamura R, Hayashi S, Kodama D, Kawase M and Yagi K: Hepatoprotective effect of extracts from *Lentinus edodes* mycelia on dimethylnitrosamine-induced liver injury. Biol Pharm Bull *27*: 1957-1960, 2004.

3 Terada H, Ohara T, Yamaguchi Y, Ueda T and Asano K: Protective effects of the *Lentinus edodes* mycelia on carbon

tetrachloride-induced, liver injury. J New Rem Clin *50*: 655-664, 2001 (in Japanese).
4 Shen J, Tanida M, Fujisaki Y, Horii Y, Hashimoto K and Nagai K: Effect of the culture extract of *Lentinus edodes* mycelia on splenic sympathetic activity and cancer cell proliferation. Auton Neurosci *145*: 50-54, 2009.
5 Yamasaki H, Kawanishi T, Matsui Y, Tamesada M and Asano K: Experimental study on the antitumor effect of *Lentinus edodes* mycelia using murine pulmonary metastasis mode (B16F10). Biotherapy *17*: 467-472, 2003 (in Japanese).
6 Itoh M, Sano J, Sugiyama Y, Kunieda K and Saji S: Anti-tumor effect of *Lentinus edodes* mycelia using a rat mode with high lung metastasis (GKS-HL) established in our department. Biotherapy *16*: 605-611, 2002 (in Japanese).
7 Hibino Y, Konishi Y, Koike J, Tabata T, Ohashi Y and Sugano N: Productions of interferon-gamma and nitrite are induced in mouse splenic cells by a heteroglycan-protein fraction from culture medium of *Lentinus edodes* mycelia. Immunopharmacology *28*: 77-85, 1994.
8 Sugano N, Hibino Y, Choji Y and Maeda H: Anticarcinogenic actions of water-soluble and alcohol-insoluble fractions from culture medium of *Lentinus edodes* mycelia. Cancer Lett *17*: 109-114, 1982.
9 Morinaga H, Tazawa K, Tagoh H, Muraguchi A and Fujimaki M: An *in vivo* study of hepatic and splenic interleukin-1 beta mRNA expression following oral PSK or LEM administration. Jpn J Cancer Res *85*: 1298-1303, 1994.
10 Matsui Y, Kawanishi T, Yamasaki H, Uchida K, Yukawa H, Tamesada M and Asano K: Inhibitory effect of *Lentinus edodes* mycelia on angiogenesis. Biotherapy *18*: 543-547, 2004 (in Japanese).
11 Yamada T, Oinuma T, Niihashi M, Mitsumata M, Fujioka T, Hasegawa K, Nagaoka H and Itakura H: Effects of *Lentinus edodes* mycelia on dietary-induced atherosclerotic involvement in rabbit aorta. J Atheroscler Thromb *9*: 149-156, 2002.
12 Nagai K: Mental balance for beauty, Autonomic mechanism for woman who wants to know still more. Proceedings of the 7th Annual Meeting of the Society of Anti-Aging Medicine for Women, Tokyo, 2008 (in Japanese).
13 Hashimoto K and Sugiyama N: Inhibitory effect of *Lentinus edodes* mycelia on melanin production. Proceedings of the 129th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, p. 165, 2009 (in Japanese).
14 Mushroom Biology and Mushroom Products. Chang S-T, Buswell JA and Chiu S-W (eds.). Proceedings of the First International Conference on Mushroom Biology and Mushroom Products 23-26 August 1993, The Chinese University of Hong Kong, Hong Kong, The Chinese University Press. 1993.
15 Keneko Y and Chihara G: Potentiation of host resistance against microbial infections by lentinan and its related polysaccharides. *In*: Microbial Infections, Role of Biological Response Modifiers, pp. 201-215. Friedman H, Klein TW and Yamaguchi H (eds.). New York, Plenum, 1992.
16 Fujii T, Maeda H, Suzuki F and Ishida N: Isolation and characterization of a new antitumor polysaccharide, KS-2, extracted from culture mycelia of *Lentinus edodes*. J Antibiot *31*: 1079-1090, 1978.
17 Suzuki H, Iiyama K, Yoshida O, Yamazaki S, Yamamoto N and Toda S: Structural characterization of the immunoreactive and antiviral water-solubilized lignin in an extract of the culture medium of *Lentinum edodes* mycelia (LEM). Agric Biol Chem *54*: 479-487, 1990.
18 Yamamoto Y, Shirono H, Kono K and Ohashi Y: Immuno-potentiating activity of the water-insoluble lignin rich fraction prepared from LEM – the extract of the solid culture medium of *Lentinus edodes* mycelia. Biosci Biotechnol Biochem *61*: 1909-1912, 1997.
19 Kawano M, Sakagami H, Satoh K, Shioda S, Kanamoto T, Terakubo S, Nakashima H and Makino T: Lignin-like activity of *Lentinus edodes* mycelia extract (LEM). In Vivo *24*: 543-552, 2010.
20 Kawano M, Thet MM, Makino T, Kushida T and Sakagami H: DNA microarray analysis of signaling pathway in macrophages stimulated by lignin–carbohydrate complexes from *Lentinus edodes* mycelia extract (LEM). Anticancer Res *30*: 2567-2576, 2010.
21 Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, Haynes K, Steele C, Botto M, Gordon S and Brown GD: Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat Immunol *8*: 31-38, 2007.
22 Holmig ST, Ariizumi K and Cruz PD: Recognition of non-self-polysaccharides by C-type lectin receptors dectin-1 and dectin-2. Glycobiology *19*: 568-575, 2009.
23 Sakagami H, Ikeda M, Unten S, Takeda K, Murayama J, Hamada A, Kimura K, Komatsu N and Konno K: Antitumor activity of polysaccharide fractions from pine cone extract of *Pinus parviflora* Sieb. et Zucc. Anticancer Res *7*: 1153-1160, 1987.
24 Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD and Eberwine JH: Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proc Natl Acad Sci USA *87*: 1663-1667, 1990.
25 Dudoit S, Gentleman RC and Quackenbush J: Open source software for the analysis of microarray data. Biotechniques *34*: S45-S51, 2003.
26 de Hoon MJ, Imoto S, Nolan J and Miyano S: Open source clustering software. Bioinformatics *20*: 1453-1454, 2004.
27 Saldanha AJ: Java Treeview – extensible visualization of microarray data. Bioinformatics *20*: 3246-3248, 2004.
28 Graham LM and Brown GD: The Dectin-2 family of C-type lectins in immunity and homeostasis. Cytokine *48*: 148-155, 2009
29 Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S and Iwakura Y: Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida* albicans. Immunity *32*: 681-691, 2010.
30 Fütterer K, Wong J, Grucza RA, Chan AC and Waksman G: Structural basis for syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. J Mol Biol *281*: 523-537, 1998.
31 Bu JY, Shaw AS and Chan AC: Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. Proc Natl Acad Sci USA *92*: 5106-5110, 1995.
32 Reid DM, Gow NA and Brown GD: Pattern recognition: recent insights from Dectin-1. Curr Opin Immunol *21*: 30-37, 2009.

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