Genomics & Informatics Vol. 4(2) 57-64, June 2006

Single and Dual Ligand Effects on Gene Expression Changes in Mouse Macrophage Cells

Sangdun Choi[1]* and Jeong-Sun Seo[2]

[1]Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Korea, [2]Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul 110-799, Korea

Abstract

We identified differentially expressed genes in RAW 264.7 cells in response to single and double ligand treatments (LPS, IFNγ, 2MA, LPS plus IFNγ, and LPS plus 2MA). The majority of the regulated transcripts responded additively to dual ligand treatment. However, a significant fraction responded in a non-additive fashion. Several cytokines showing non-additive transcriptional responses to dual ligand treatment also showed non-additive protein production/secretion responses in separately performed experiments. Many of the genes with non-additive responses to LPS plus 2MA showed enhanced responses and encoded pro-inflammatory proteins. LPS plus IFNγ appeared to induce both non-additive enhancement and non-additive attenuation of gene expression. The affected genes were associated with a variety of biological functions. These experiments reveal both dependent and independent regulatory pathways and point out the specific nature of the regulatory interactions.

Keywords: macrophage, gene expression, signal transduction, toll-like receptor (TLR)

Introduction

Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) of Gram-negative bacteria (Akira *et al.*, 2006). Binding of PAMPs to TLRs triggers a complicated series of events leading to increased expression of proinflammatory genes. To date, 10 human and 13 murine proteins related to the *Drosophila* Toll have been characterized (Kawai and Akira, 2006). For example, TLR2 is essential for the signaling of a variety of ligands, such as peptidoglycan, a main wall component of Gram-positive bacteria, lipopeptides and lipoproteins (Takeuchi *et al.*, 2000). TLR2 cooperates with other TLR family members, TLR1 and TLR6, to discriminate among different microbial components. TLR3 mediates the response to double-stranded viral RNA (Alexopoulou *et al.*, 2001). TLR4 is considered to be the critical component of the LPS receptor complex (Hayashi *et al.*, 2001; Hoshino *et al.*, 1999; Poltorak *et al.*, 1998). TLR5 binds bacterial flagellin from both Gram-positive and Gram-negative bacteria (Hayashi *et al.*, 2001). TLR9 binds unmethylated cytosine-guanosine (CpG) dinucleotide, a sequence typifying bacterial and viral DNA (Hemmi *et al.*, 2000). Depending on the type of cell, TLR activation could lead to a variety of activities, such as antigen-presenting cell activation, dendritic cell maturation and B cell activation.

LPS is the major constitute of the outer membrane of Gram-negative bacteria. In blood, LPS forms a complex with serum protein LBP (LPS-binding protein). LPS binds cell surface receptor CD14 to enhance TLR4-dependent LPS recognition. After TLR4 forms a complex with protein MD-2, the complex is transported to the cell surface (Akira *et al.*, 2001; Takeda *et al.*, 2003; Yamamoto *et al.*, 2003). TLR4 is a membrane protein with an extracellular leucine-rich repeat domain and a cytoplasmic TIR (Toll-like/interleukin-1 receptor) domain. In addition to LPS, TLR4 recognizes several other ligands including Taxol and HSPs (heat shock proteins).

TLR4 activates two downstream pathways, MyD88-dependent pathway and MyD88-independent pathway, each of which is thought to directly activate NFκB. The MyD88-dependent pathway recruits the kinases, interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4, which phosphorylate TNF receptor-associated factor 6 (TRAF6), leading to the activation of the IKK complex. The MyD88-independent pathway is dependent on the TIR domain-containing adaptor inducing interferon-β (Trif) adaptor molecule and Trif-related adaptor molecule (Tram). However, the genes regulated by TLR4 activation are not fully understood.

In an attempt to characterize how TLR4-mediated signal transduction can be activated by LPS, we applied LPS in the murine macrophage-like cell line RAW 264.7 for a series of time points. In addition, to determine whether simultaneous stimulation with two ligands had non-additive effects on gene expression in RAW cells,

*Corresponding author: E-mail sangdunchoi@ajou.ac.kr, Tel +82-31-219-2600, Fax +82-31-219-1615
Accepted 29 May 2006

- RAW cells were treated with:
  - LPS/LBP (100ng/100pM) for 0.5 h, 1 h, 2 h, 4 h
  - IFNγ (5nM) for 1 h, 2 h, 4 h
  - 2MA (500uM) for 1 h, 2 h, 4 h
  - LPS/LBP (100ng/100pM) + IFNγ (5nM) for 1 h, 2 h, 4 h
  - LPS/LBP (100ng/100pM) + 2MA (500uM) for 1 h, 2 h, 4 h
  - Media for 1 h, 2 h, 4 h
  - Triplicate experiments, and triplicate cell samples for each condition
- RNA samples were extracted from treated and time-matched control RAW cells
- Gene expression changes were measured with 16K oligo chips using dye-swap design:
  - Chip #1 : treated-Cy5, control-Cy3, measurement #1 = $\log_2$(Cy5/Cy3)
  - Chip #2 : treated-Cy3, control-Cy5, measurement #2 = $\log_2$(Cy3/Cy5)
  - Expression changes: (measurement #1 + measurement #2) / 2

Fig. 1. Experimental design.

three ligands [LPS, IFNγ and 2MA (2-methyl-thio-ATP)] which activate distinct proximal signaling pathways were used alone or in combination as dual ligands (LPS plus 2MA and LPS plus IFNγ). Gene expression changes at multiple time points relative to the time-matched media control were determined using homemade 16K oligonucleotide microarrays.

Methods

Experimental design

RAW 264.7 cells were treated with LPS (Sigma- Aldrich), IFNγ (R & D Systems), 2MA (Sigma-Aldrich), LPS plus IFN γ, and LPS plus 2MA for up to 4 h. Cells were collected at multiple time points and RNAs were extracted. The same experiment was repeated three times. RNA samples collected at each replicate experiment were processed for microarray hybridization as described in Zhu *et al.* (Zhu *et al.*, 2004). The expression changes in each treated RNA sample were measured with two oligonucleotide chips using the dye-swap design. Fig. 1 contains further details of the experimental design.

Data processing

The arrays were scanned using Agilent Scanner G2505A (Agilent Technologies). The image files were extracted with background-subtraction (the Local background subtraction method) and dye-normalization (the Rank consistent filter and the LOWESS algorithm) using the Agilent G2566AA Extraction Software Version A.6.1.1. Log_2(Cy5/Cy3) readouts from each array were first filtered by several criteria to mainly remove unreliable array spots. For features that were control, saturated (with "gIsSaturated" and "rIsSaturated" flags), non-uniform (with "gIsFeatNonUnifOL" and "rIsFeatNonUnifOL" flags), or below background (with "gIsWellAboveBG" and "rIsWellAboveBG" flags), their $\log_2$(Cy5/Cy3) value was set to blank. Additional filters were applied to eliminate inconsistent measurements. The average $\log_2$(treated/media) of the pair of dye-swap measurements of a feature was set to blank if one of the measurements was blank. Further, the average $\log_2$(treated/media) of the triplicate measurements of a feature was set to blank if more than one of the measurements were blank.

Identify differentially expressed genes in response to ligand treatment

We limited our analysis to genes whose expression significantly changed in response to at least one of the treatments. We have identified differentially expressed genes using B statistics generated by the Limma package (http://bioinf.wehi.edu.au/limma/) (Smyth, 2004 and 2005). Briefly, the B statistics is the log odds of differential expression [$\log_{10}$ (p1/p2), where p1 is the probability that the two populations are different and p2 is the probability that the two populations are the same]. The B statistics has been adjusted for multiple tests in Limma package. The greater B statistics

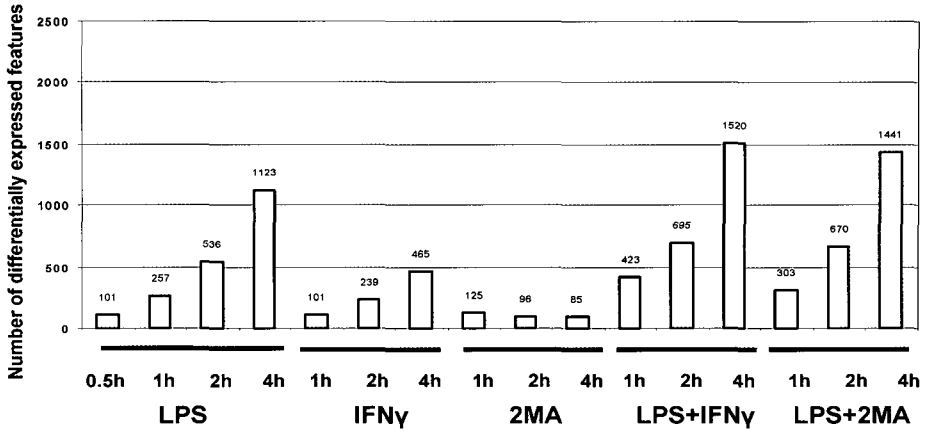

Fig. 2. The number of differentially expressed features (B ≥2 & fold-change ≥ 1.7).

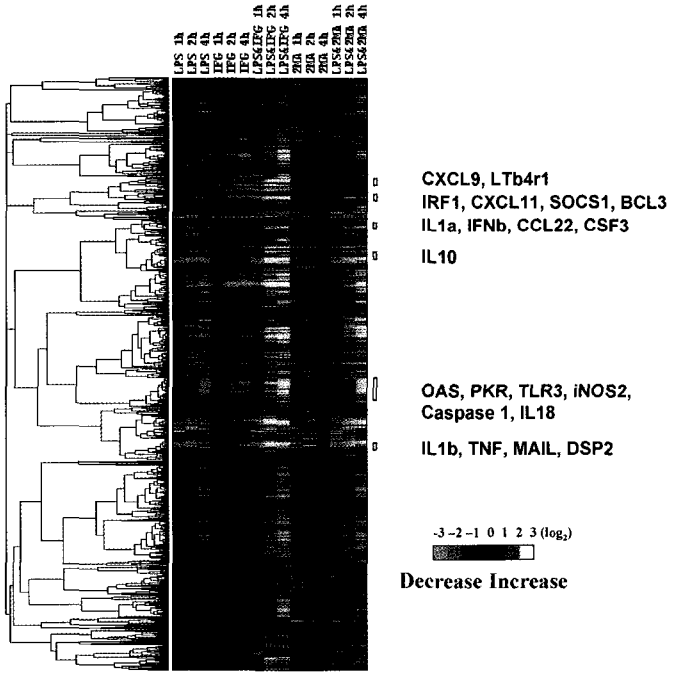

Fig. 3. The dendrogram of hierarchically clustered significant expression changes in response to single or dual ligands.

a gene has, the greater chance a gene of differential expression. In general, B increases with $\log_2$(treated/ control). In the current analysis, features with B ≥ 2 and with an average of $\log_2$(treated/control) ≥ 0.75 (fold-change of 1.7) are considered differentially expressed.

Results and Discussion

The number of differentially expressed features

As shown in Fig. 2, single ligand treatment with LPS and IFNγ for up to 4 h induced increasing numbers of gene expression changes, with approximately twice as many significant changes in LPS responses. 2MA induced a similar number of changes as IFNγ at 1 h and the number of differentially expressed features appeared to decrease with time. Greater numbers of differentially expressed genes were found in response to LPS plus IFNγ or LPS plus 2MA than those found in single ligand responses, and this may be attributable to both additive and non-additive effects of the two single ligands.

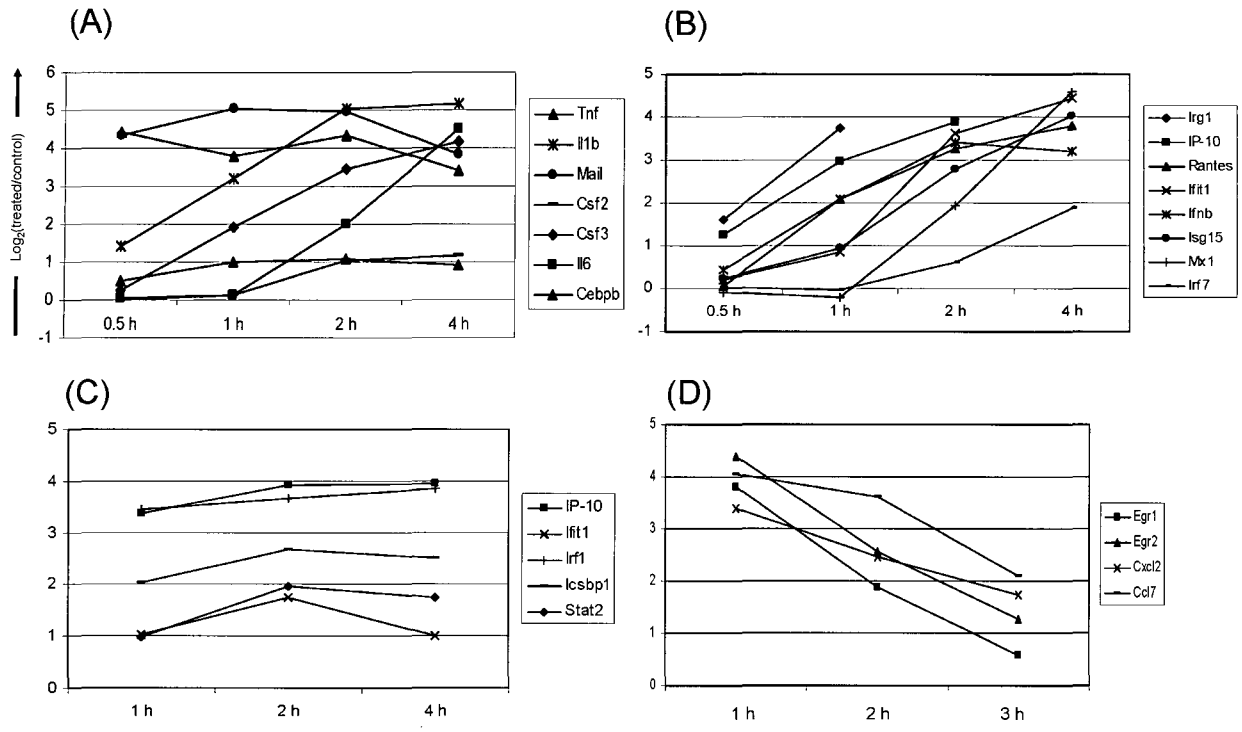

Fig. 4. Genes regulated by ligands. (A) LPS-induced MyD88-NFκB pathway targets and secondary response genes. (B) LPS-induced MyD88 independent, IRF3 pathway targets and secondary response genes. (C) IFNγ-induced immediate early genes. (D) 2MA-induced immediate early genes.

Calculate the ligand effect, the net fold-change of gene expression:

ligandEffect = power(2, x) - 1 if x >=0;

= 1 - 1/(power(2,x)) if x <0;

where x is the avg. $\log_2$-(treated/control)

Calculate the combined effect of single ligand treatments, combinedSgl:

combinedSgl = ligandEffect(ligand 1) + ligandEffect(ligand 2)

Calculate the difference of dual ligand effect and combined single ligand effect:

Fchange Diff = ligandEffect(dual) - combinedSgl

Fig. 5. Calculation of the difference between the dual ligand-induced expression changes and the sum of expression changes induced by single ligands.

Characterization of gene expression changes in response to single ligand treatment with LPS, IFN γ, or 2MA

Before we proceeded to determine the non-additive effect on gene expression of dual ligand treatment, we characterized gene expression changes in single ligand responses. This will allow us (a) to validate previously reported expression changes induced by the single ligands, and (b) to identify potential novel target genes of

specific signaling pathways if the genes co-cluster with well-characterized known targets. Information of the latter kind can be useful in identifying pathway interaction mechanisms if some of the novel targets are affected non-additively by dual ligand treatments.

We used hierarchical clustering to characterize patterns of gene expression changes. Clustering was done across the features with experimental conditions aligned according to the order of time points. Euclidean correlation coefficient and complete linkage were used as similarity metrics. The hierarchical clustering program used is implemented in the Multiple Experiment Viewer (http://www.tigr.org/software/tm4/mev.html). Single ligand treatment with LPS, IFNγ, and 2MA all induced strong expression changes as early as 1 h (Fig. 3). As shown in Fig. 3, additional expression changes were induced at 2 h and 4 h in LPS and IFNγ responses, and many 2MA-induced changes were transient and subsided with time.

A number of the known LPS- or IFNγ- regulated genes showed expected expression changes in our experiment. As shown in Fig. 4A, known direct target genes of the MyD88-NFκB pathway activated by LPS through the TLR4, such as TNFα, IL1β and Mail, were strongly induced at 0.5 h, while Csf2, Csf3, IL6 and Cebpb, the known transcription targets of Mail, were induced later at 1 h or 2 h (Kawai, 2001; Yamamoto *et al.*, 2004). Similarly, known primary target genes of another pathway activated by LPS, the Myd88-independent IRF3 pathway, such as Irg1, IP-10, Rantes, Ifit1, Ifnb and Isg15, were induced at 0.5 h or 1 h, while Mx1 and Irf7, genes induced by secreted Ifnb, were up regulated later by LPS at 2 h and 4 h, respectively (Fig. 4B) (Doyle *et al.*, 2002; Hoshino *et al.*, 2002). As shown in Fig. 4C, several reported IFNγ -induced immediate early genes regulated by the STAT1 pathway, such as Irf1, Icsbp1, Stat2, IP-10 and Ifit1, also increased their expression significantly at 1 h [reviewed in (Boehm *et al.*, 1997)]. We also found several known MAPK-induced genes, such as Egr1, Egr2, and Dusp2, in clusters of early induced genes by 2MA (Fig. 4D).

Identify features whose expression was non-additively affected by dual ligand treatments

We computed the net expression fold-change in response to each single ligand and to dual ligand treatment at each time point for each gene using the average of the triplicate $\log_2$-ratio measurements after filtering as follows: (1) Sets that do not have at least two data points are not used (e.g., blank, blank, 2). (2) If two of the triplicates show different signs and have a standard deviation above 0.5, the data is not used (e.g., -2, 2, 0.5). (3) If one data set shows more than a 2 fold difference ($\log_2$ ratio of 1) and the other points do not, that data is not used, unless the standard deviation is less than 0.5. (e.g., 3, 0.2, 0.2) (4) The remaining data points are averaged and used in the analysis. The difference between the fold-change induced by dual ligand and the sum of fold-changes induced by single ligands was used as a measure of the non-additive effect (Fig. 5).

We then added the fold-change differences at 1 h, 2 h, and 4 h together, and used the resulting sum to rank genes in descending order. In the current analysis, we generally considered genes that ranked at the top (the sum being positive) as candidates whose expression was enhanced non-additively by the dual ligands, and

Table 1. Gene expression enhanced by the non-additive effects of LPS and IFNγ

		LPS				IFNgamma			Dual				Fchange Diff			
Symbol	Gene Name	LPS.5	LPS1	LPS2	LPS4	IFNg1	IFNg2	IFNg4	L&I1	L&I2	L&I4	# /Cell	L&I1	L&I2	L&I4	F(LI)
Cxcl9	Small inducible cytokine B subfamily (Cys-X-Cys), member 9	0.164	-0.18	0.15	0.5	2.48	4	4.9	5.17	5.58	5.77	12	31	32	24	86.5
F3	Coagulation factor III (F3)	0.64	1.59	2.16	3.61	0.51	1.99	3.49	4.45	5.61	5.12	19	18	41	12	72.2
F3	*Coagulation factor III*	0.409	1.25	1.77	3.2	0.5	1.65	3.08	3.84	5.37	4.9	19	12	36	13	60.5
Homer1	Homer, neuronal immediate early gene, 1	0.22	0.45	1.44	1.43	0.11	0.75	1.35	2.27	4.57	4.01	37	3.4	20	12	35.6
Gbp1	Guanylate nucleotide binding protein 1	0.038	0.23	2.84	5.99	3.18	6.12	ND	5.48	ND	ND	18	35	ND	ND	35.5
Tnfrsf5	Tumor necrosis factor receptor superfamily, member 5	0.155	1.6	3.11	4.6	0.62	2.02	3.51	3.46	4.82	5.44	14	7.4	17	8.9	32.8
Gbp2	Guanylate nucleotide binding protein 2	0.154	0.28	2.58	5.1	2.69	5.18	ND	4.74	5.72	ND	12	20	11	ND	31.4
Ccl12	Small inducible cytokine A12 (Scya12)	-0.15	0.23	0.52	3.06	0.77	1.13	1.59	3.29	3.57	4.27	6	7.9	9.3	8.9	26.1
Cxcl11	Small inducible cytokine subfamily B (Cys-X-Cys), member 11	0.189	0.4	1.75	4.26	ND	3.38	4.77	3.06	5.54	5.11	21	ND	34	-11	22.9
Il6	Interleukin 6	0.034	0.17	2.01	4.5	-0.16	0.26	0.45	0.33	3.03	5.27	14	0.2	4	16	19.8
Gbp3	Guanylate nucleotide binding protein 3	0.201	0.39	2.65	5.48	2.18	3.9	5.14	3.92	5.18	6.14	23	10	16	-8.2	18.1
Sgk	Serum/glucocorticoid regulated kinase	0.219	0.09	0.13	ND	-0.1	-0.36	-0.94	3.17	3.37	ND	85	8	9.4	ND	17.4
Cd83	*CD83 antigen (Cd83)*	2.272	3.42	3.98	2.7	1.6	2.26	2.11	4.25	4.58	3.8	80	6.4	4.3	4.1	14.8
CCl 12	Small inducible cytokine A12	0.109	-0.11	0.7	2.69	0.62	1.19	1.51	2.48	3.06	3.71	7	4.1	5.5	4.8	14.3
Icsbp1	Interferon concensus sequence binding protein	0.03	-0.18	0.27	1.01	2.04	2.69	2.54	2.91	3.71	3.26	133	3.5	6.4	2.8	12.7
Ptgs2	Prostaglandin-endoperoxide synthase 2	0.744	1.67	2.86	3.64	-0.23	0.39	2.25	2.35	3.43	4.56	7	2.1	3.2	7.3	12.6
Procr	Protein C receptor, endothelial	0.002	0.22	0.5	1.79	0	0.03	1.04	0.15	1.61	3.82	57	-0.1	1.6	9.6	11.2
Marcks	Myristoylated alanine rich protein kinase C substrate	0.035	ND	3.46	4.38	0.13	ND	0.31	2.1	4.22	5.01	6	ND	ND	11	11.1
Cish	Mus musculus cytokine inducible SH2-containing protein (Cish), mRNA	0.068	0.77	2.11	2.54	ND	1.16	1.47	2.64	3.81	3.16	15	ND	8.5	1.4	9.87
Nola1	Nucleolar protein family A, member 1 (H/ACA small nucleolar RNPs)	0.07	0.1	-0	-0.1	0.64	0.71	0.88	2.52	2.12	1.81	11	4.1	2.7	1.7	8.55
Crem	CAMP responsive element modulator	0.006	0.08	0.04	0.57	0.83	1.33	1.43	2.18	2.65	2.33	9	2.7	3.7	1.8	8.27
Jak2	Janus kinase 2	-0.04	ND	2.09	2.89	0.32	0.83	1.6	ND	2.99	3.78	203	ND	2.9	4.3	7.17
Socs1	Cytokine inducible SH2-containing protein 1	0.151	0.35	1.21	2	3.18	3.38	3.04	3.15	3.68	4.04	59	-0.4	1.1	5.2	5.92

* The copy number of each transcript per cell was calculated by the method described in Park *et al.* (Park *et al.*, 2004).

Table 2. Gene expression attenuated by the non-additive effects of LPS and IFNγ

		LPS				2MA			Dual				Fchange Diff			
Symbol	Gene Name	LPS.5	LPS1	LPS2	LPS4	IFNg1	IFNg2	IFNg4	L&I1	L&I2	L&I4	# /Cell	L&I1	L&I2	L&I4	F(LI)
Pdcd1	Programmed cell death 1	-0.14	ND	2.26	2.7	0.13	0.33	0.28	0.3	1.25	0.33	60	ND	-2.7	-5.5	-8.1
Rgs16	Regulator of G-protein signaling 16	0.328	0.75	2.57	2.7	-0.64	0.24	1.24	-0.1	1.67	1.42	20	-0.4	-3	-5.2	-8.5
	RIKEN cDNA 9130002C22 gene	0.044	0.03	ND	3.63	ND	2.54	3.78	1.6	2.72	4.01	64	ND	ND	-9	-9
	Sjogren syndrome antigen A1	-0.07	-0.36	1.74	2.8	1.88	2.18	1.31	1.28	1.77	1.92	31	-1	-3.4	-4.6	-9.1
Irf1	Interferon regulatory factor 1	1.16	0.71	2.38	2.71	3.45	3.67	3.86	3.87	3.81	3.45	29	3	-2.9	-9.2	-9.1
Ifit2	Interferon-induced protein with tetratricopeptide repeats 2 (Ifit2)	-0.11	-0	2.41	5.39	ND	2.06	1.98	0.92	3.21	5.12	15	ND	0.8	-10	-9.3
Il4i1	Interleukin-four induced gene 1	-0.03	1.05	3.31	2.85	-0.04	0.11	0.1	ND	1.92	1.61	13	ND	-6.2	-4.2	-10
Ifit3	Interferon-induced protein with tetratricopeptide repeats 3 (Ifit3)	3.026	2.57	3.27	3.87	0.18	0.62	0.38	2.83	2.73	2.76	6	1.1	-3.5	-8.2	-11
	Interferon-inducible GTPase	0.026	-0.29	0.32	3.35	2.96	4.93	5.69	3.1	4.83	5.68	11	1	-2.2	-9.6	-11
	RIKEN cDNA 5830443L24 gene	-0.04	-0.13	ND	3.64	1.14	3.48	5.14	1.78	3.86	5.06	9	1.3	ND	-13	-12
Cirbp	Interferon-induced protein with tetratricopeptide repeats 1	0.192	0.85	3.63	4.46	1.18	1.75	1.11	2.12	3.39	3.77	28	1.3	-4.2	-9.5	-12
Igtp	Interferon gamma induced GTPase	0.033	-0.07	1.65	3.41	2.63	3.85	4.14	2.67	3.83	4.05	81	0.2	-2.3	-11	-13
Rgs-r	Retinally abundant regulator of G-protein signaling mRGS-r	0.51	0.85	2.93	3.35	ND	0.46	1.62	-0.3	1.91	1.84	21	ND	-4.3	-8.7	-13
	Interferon-g induced GTPase	0.031	-0.28	2.14	3.52	3.09	4.19	3.92	3.59	4.12	3.71	156	3.7	-4.3	-12	-13
Csf3	Colony stimulating factor 3 (granulocyte)	0.244	1.93	3.45	4.18	-0.24	0.17	0.03	1.11	2.54	3.22	12	-1.5	-5.2	-8.8	-16
Lynx1	Ly6/neurotoxin 1 (Lynx1)	3.227	3.57	4.21	4.62	0.34	2.08	2.84	3.74	4.17	4.11	25	1.3	-3.8	-13	-16

Table 3. Gene expression enhanced by the non-additive effects of LPS and 2MA

		LPS				2MA			Dual				Fchange Diff			
Symbol	Gene Name	LPS.5	LPS1	LPS2	LPS4	2MA1	2MA2	2MA4	L&2M1	L&2M2	L&2M4	# /Cell	L&2M1	L&2M2	L&2M4	F(L2)
Il1a	Interleukin 1 alpha	0.5	1.4	3.2	3.6	0.1	0.1	0.1	3.2	4.7	5.1	9.3	6.5	16.1	22.2	44.8
Il1a	Interleukin 1 alpha	0.4	1.2	3.2	3.6	0.1	0.2	0.1	3.4	4.5	5.0	12.7	8.4	13.6	20.8	42.8
Ifnb	Interferon beta, fibroblast	0.4	2.1	3.4	3.2	0.2	0.1	0.2	3.4	4.9	4.8	8.5	6.1	18.2	17.8	42.1
Cxcl11	Small inducible cytokine subfamily B (Cys-X-Cys), member 11	0.2	0.4	1.8	4.3	0.3	0.0	0.5	0.9	2.8	5.5	20.8	0.4	3.6	25.4	29.4
Marcks	Myristoylated alanine rich protein kinase C substrate	0.0	ND	3.5	4.4	-0.1	0.2	0.3	0.7	3.8	5.5	5.5	ND	3.1	23.1	26.2
Il6	Interleukin 6	0.0	0.2	2.0	4.5	-0.1	0.0	0.0	0.4	2.5	5.5	14.2	0.2	1.7	22.6	24.4
Gbp3	Guanylate nucleotide binding protein 3	0.2	0.4	2.6	5.5	0.0	0.1	0.4	ND	2.9	6.1	22.7	ND	1.0	23.1	24.2
Dusp1	Protein tyrosine phosphatase, non-receptor type 16 (Ptpn16)	4.1	4.2	3.2	2.2	1.6	ND	0.4	5.2	4.4	3.1	33.6	17.9	ND	3.7	21.6
Il1b	Interleukin 1 beta	1.4	3.3	5.0	5.2	0.9	ND	1.0	4.3	5.3	5.6	39.2	9.2	ND	12.3	21.4
	T-cell death associated gene	2.8	4.3	3.5	2.5	1.9	2.1	1.4	5.1	4.2	3.6	17.2	11.0	4.0	4.6	19.6
Cd83	CD83 antigen (Cd83), mRNA	2.3	3.4	4.0	2.7	1.5	1.3	0.6	4.1	4.5	3.9	80.1	4.8	5.8	7.9	18.4
Gbp2	Guanylate nucleotide binding protein 2	0.2	0.3	2.6	5.1	0.1	0.1	0.6	0.4	2.9	5.6	11.8	0.0	1.6	14.4	16.1
Ccl22	Small inducible cytokine subfamily A, member 22 (Scya22)	0.1	ND	2.3	2.6	-0.1	0.0	0.3	1.4	3.5	3.8	5.1	ND	6.4	7.8	14.3
Marcks	Myristoylated alanine rich protein kinase C substrate (Macs)	-0.1	0.4	2.4	3.3	0.0	-0.2	-0.1	0.2	2.9	4.4	4.3	-0.2	2.4	11.4	13.6
Gdnf	Plasminogen activator inhibitor, type I (Planh1), mRNA	1.9	3.1	3.3	2.5	2.4	2.1	0.8	4.1	4.3	3.1	60.7	4.3	6.3	2.5	13.1
Egr2	Early growth response 2	4.0	3.5	1.7	ND	4.4	2.6	1.3	5.5	2.8	ND	8.5	14.2	-1.2	ND	13.0
Il4i1	Interleukin-four induced gene 1	0.0	1.1	3.3	2.9	0.4	0.9	1.4	1.4	3.9	4.1	13.0	0.3	4.2	8.4	12.8
Serpine1	Plasminogen activator inhibitor, type I	2.3	3.5	3.4	2.6	2.3	2.0	ND	4.3	4.4	3.3	182.9	4.7	7.7	ND	12.5
Ccl22	Small inducible cytokine subfamily A, member 22	-0.1	ND	1.8	2.2	0.0	0.1	-0.2	ND	3.0	3.5	3.5	ND	4.5	6.8	11.3
Dusp1	Protein tyrosine phosphatase, non-receptor type 16	3.5	3.7	3.1	2.1	1.4	ND	0.5	4.5	4.0	2.9	39.1	7.7	ND	3.0	10.7

Table 4. Gene expression attenuated by the non-additive effects of LPS and 2MA

		LPS				2MA			Dual				Fchange Diff			
Symbol	Gene Name	LPS.5	LPS1	LPS2	LPS4	2MA1	2MA2	2MA4	L&2M1	L&2M2	L&2M4	# /Cell	L&2M1	L&2M2	L&2M4	F(L2)
Il10	Interleukin 10	0.313	1.76	2.2	2.62	0.33	0.32	0.462	1.331	1.597	2.298	16	-1.131	-1.834	-1.611	-4.6
Bcl6b	BcL6-associated zinc finger protein	-0.19	2.19	3.47	2.12	0.71	2.15	1.796	1.5674	3.368	2.864	8	-2.249	-4.191	0.4502	-6
Il10	Interleukin 10 (Il10)	0.262	1.97	2.38	2.82	0.44	0.36	0.525	1.55	1.781	2.289	8	-1.356	-2.045	-2.605	-6
Csf3	Colony stimulating factor 3 (granulocyte)	0.244	1.93	3.45	4.18	0.11	0.02	-0.09	1.8947	2.962	3.879	12	-0.184	-3.154	-3.345	-6.7
Saa3	Serum amyloid A 3	0.944	1.3	2.97	4.68	-0.08	-0.1	0.188	1.6691	2.508	4.144	41	0.776	-2.102	-8.135	-9.5

genes that ranked at the bottom (the sum being negative) as candidates whose expression was attenuated non-additively by the dual ligand treatments. Tables 1-4 showed gene expression changes (as average $\log_2$-ratios) of the single time point and the sum fold-change differences of these top/bottom ranked genes in responses to LPS + IFNγ or LPS + 2MA. Table 1 listed genes whose expression was enhanced non-additively by LPS + IFNγ. Non-additively enhanced genes including IL6 and IL10 were also measured in cytokine secretion assays. Secreted IL6 in response to LPS + IFNγ was clearly non-additively enhanced at 4 h and a slight non-additive effect was seen in IL10 production at 4 h (data not shown). Other interesting genes including Sgk, Nola1, Crabp2, and Fst did not show significant changes at most time points in response to either LPS or IFNγ, but showed strong changes when LPS and IFNγ were applied together. Whether these changes are reflected at the protein level, what mechanisms underlie the changes, and the nature of the biological significance of these changes remain to be seen. Similar results are found in Tables 2-4.

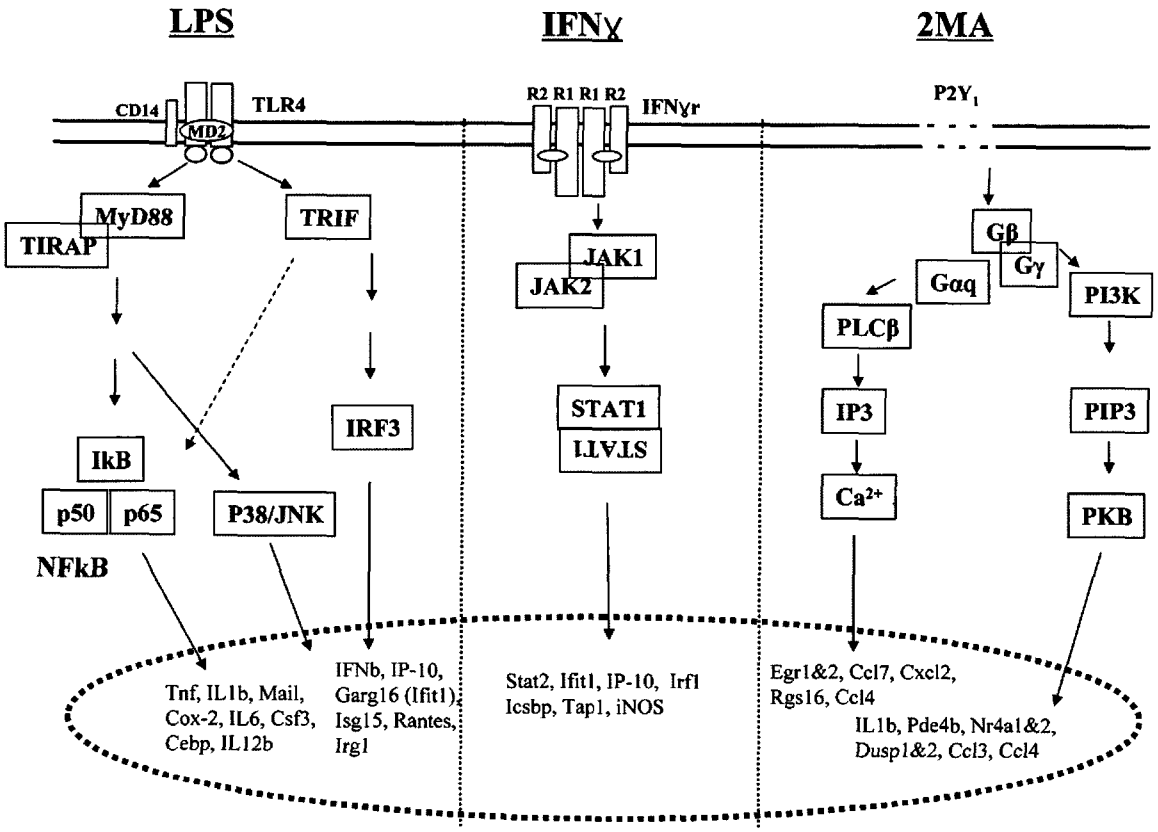

Fig. 6. Identification of the expression changes indicative of the activation of specific signaling pathways.

Socs1- and Cish-mediated feedback inhibition and PGE2 (prostaglandin E2) negative feedback seem to attenuate the transcriptional responses to LPS plus IFNγ (Table 1). The increased number of less-than-additive transcriptional changes induced by LPS plus IFNγ at 4 h (Table 2) was preceded by a significant induction of Socs1 and Cish (Table 1), genes encoding putative inhibitors of LPS, and Ptgs2 and Ptger4, genes encoding COX-2 and EP4, an enzyme and a receptor for the synthesis and binding of PGE2, respectively.

In response to LPS plus 2MA, the enhancement of LPS-induced expression of IFNβ at 1 h and 2 h (Table 3) was followed by the increased expression of multiple interferon induced genes, such as Gbp2, Irf1, Mx1, and Mx2, and the attenuation of LPS-induced expression of IL10 at 1 h and 2 h (Table 4) correlated with the increased expression of IL10 repressed genes later at 4 h including IL1α, IL1β, IL6, IL18, Pim1, and Socs1. It can be hypothesized that 2MA-induced Ca^{2+} mobilization activates NFAT, and NFAT synergizes with IRF3 activated by LPS in the transcriptional induction of IFNβ. Consistent with this possibility, the 5' upstream region of the mouse and human IFNβ genes have a NFAT site, an IRF site overlapped with the NFAT site, and a NFκB site. The IRF site and the NFκB site mediate IFNβ transcriptional activation by IRF3 and NFκB in response to LPS (Doyle *et al.*, 2002).

In summary, analysis of gene expression response to single ligands allowed us to identify expression changes indicative of the activation of specific signaling pathways (Fig. 6). While genes showing such changes include known target genes of the pathway, novel target genes were also found. Computational analysis of transcriptional regulatory pathways activated by various ligands, such as TLR ligands, with palpable waves of transcription will not only reveal the independent pathway of each ligand but also give further ideas of cross signaling networks within or with other signal transduction pathways by further exploring the data.

Acknowledgements

This work was supported by 2006 Ajou University Internal Research Grant.

References

Akira, S., Takeda, K., and Kaisho,T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2, 675-680.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124, 783-801.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413, 732-738.

Boehm, U., Klamp, T., Groot, M., and Howard, J. C. (1997). Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* 15, 749-795.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R., and Cheng, G. (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17, 251-263.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099-1103.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740-745.

Hoshino, K., Kaisho, T., Iwabe, T., Takeuchi, O., and Akira, S. (2002). Differential involvement of IFN-[beta] in Toll-like receptor-stimulated dendritic cell activation. *Int. Immunol.* 14, 1225-1231.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide evidence for TLR4 as the Lps gene product. *J. Immunol.* 162, 3749-3752.

Kawai,T. (2001). Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* 167, 5887-5894.

Kawai, T. and Akira, S. (2006). TLR signaling. *Cell Death Differ.* 13, 816-825.

Park , P.J., Cao, Y.A., Lee, S.Y., Kim, J.W., Chang, M.S., Hart, R., and Choi, S. (2004). Current issues for DNA microarrays: platform comparison, double linear amplification, and universal RNA reference. *J. Biotechnol.* 112, 225-245.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085-2088.

Smyth, G.K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3, 3.

Smyth, G.K. (2005). Limma: linear models for microarray data. In *Bioinformatics and Computational Biology Solutions Using R and Bioconductor,* V.C.R. Gentleman, S. Dudoit, R. Irizarry, W. Huber, ed. (New York: Springer Press).

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. *Annu. Rev. Immunol.* 21, 335-376.

Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Muhlradt, P.F., and Akira, S. (2000). Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* 164, 554-557.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4, 1144-1150.

Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K., and Akira, S. (2004). Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* 430, 218-222.

Zhu, X., Hart, R., Chang, M.S., Kim, J.W., Lee, S.Y., Cao, Y. A., Mock, D., Ke, E., Saunders, B., Alexander, A., Grossoehme, J., Lin, K.M., Yan, Z., Hsueh, R., Lee, J., Scheuermann, R.H., Fruman, D.A., Seaman, W., Subramaniam, S., Sternweis, P., Simon, M. I., and Choi, S. (2004). Analysis of the major patterns of B cell gene expression changes in response to short-term stimulation with 33 single ligands. *J. Immunol.* 173, 7141-7149.