Dual Ligand Stimulation of RAW 264.7 Cells Uncovers Feedback Mechanisms That Regulate TLR-Mediated Gene Expression[1,2,3]

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To characterize how signaling by TLR ligands can be modulated by non-TLR ligands, murine RAW 264.7 cells were treated with LPS, IFN-γ, 2-methyl-thio-ATP (2MA), PGE_2, and isoproterenol (ISO). Ligands were applied individually and in combination with LPS, for 1, 2, and 4 h, and transcriptional changes were measured using customized oligo arrays. We used nonadditive transcriptional responses to dual ligands (responses that were reproducibly greater or less than the expected additive responses) as a measure of pathway interaction. Our analysis suggests that cross-talk is limited; <24% of the features with significant responses to the single ligands responded nonadditively to a dual ligand pair. PGE_2 and ISO mainly attenuated, while 2MA enhanced, LPS-induced transcriptional changes. IFN-γ and LPS cross-regulated the transcriptional response induced by each other: while LPS preferentially enhanced IFN-γ-induced changes in gene expression at 1 h, IFN-γ signaling primarily attenuated LPS-induced changes at 4 h. Our data suggest specific cross-talk mechanisms: 1) LPS enhances the expression of IFN-γ- response genes by augmenting STAT1 activity and by activating NF-κB, which synergizes with IFN-γ-induced transcriptional factors; 2) IFN-γ attenuates the late LPS transcriptional response by increasing the expression of suppressor of cytokine signaling 1 and cytokine-inducible SH2-containing protein expression; 3) 2MA modulates LPS secondary transcriptional response by increasing *IFN-β* and inhibiting *IL-10* gene expression; 4) PGE_2 and ISO similarly regulate the LPS transcriptional response. They increase IL-10 transcription, resulting in attenuated expression of known IL-10-suppressed genes. ***The Journal of Immunology,* 2006, 177: 4299–4310.**

Signal transduction pathways often intersect at multiple levels, generating feedback and cross-talk and forming complex circuitry. At the level of receptor, for instance, receptor tyrosine kinase activities can be modulated by heterologous signals, such as integrin- or E-cadherin-mediated cell adhesion, hyperosmotic conditions, UV irradiation, and G protein-coupled receptor agonists (1–7). At the level of intracellular signal transduction, signals induced by one ligand can regulate the activity of signaling molecules downstream of another ligand. For example, ERK activated by epidermal growth factor or hepatocyte growth factor can phosphorylate SMAD1 at specific serine residues, resulting in reduced SMAD1 nuclear accumulation and inhibition of TGF-β response (8). Finally, signals from multiple pathways may interact at the level of transcription. Transcriptional regulators activated by different upstream signals can bind to *cis*-elements of the same gene, resulting in cooperative activation or inhibition of gene expression.

In an attempt to characterize how TLR-mediated signal transduction can be modulated by other ligands, we applied a series of ligands singly and in pairs with LPS and looked for nonadditive transcriptional responses resulting from dual ligand treatments. This approach is similar to that used in previous Alliance for Cellular Signaling (AfCS) studies (9) where nonadditive responses were used to determine points of interaction between signal transduction pathways. If the pathways activated by two single ligands regulate the transcription of a gene independent of each other, stimulation with both ligands at the same time would result in an additive response equal to the sum of the responses induced by the two ligands individually. In contrast, if the pathways modulate each other, application of the ligand pair would lead to nonadditive responses, which can either be greater or less than the expected additive response. We used microarrays to identify nonadditive transcriptional changes of RAW 264.7 cells in response to four pairs of ligands, namely, LPS plus IFN-γ, LPS plus 2-methyl-thio-ATP (2MA), LPS plus PGE_2, and LPS plus isoproterenol (ISO)[5]. Of the four ligands paired with LPS, IFN-γ is a cytokine that activates the JAK1/2-STAT1 pathway, while 2MA is an ATP analog that can bind to multiple P2Rs, including the G_q-associated

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[2] A complete listing of the Alliance for Cellular Signaling sponsors can be found at ⟨www.signaling-gateway.org/aboutus/sponsors.html⟩.

[3] The microarray data used in this study were deposited into the Gene Expression Omnibus (⟨www.ncbi.nlm.nih.gov/geo/⟩, Gene Expression Omnibus) under the accession numbers of GSM18191-18226, GSM111939-112028, and GSM112042-112129.

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[5] Abbreviations used in this paper: ISO, isoproterenol; 2MA, 2-methyl-thio-ATP; iNOS, inducible NO synthase; QRT-PCR, quantitative RT-PCR; RGS, regulator of G protein signaling; GAS, IFN-γ-activated sequence; Socs1, suppressor of cytokine signaling 1; Cish, cytokine-inducible SH2-containing protein; Mlp, MARCKS-like protein.

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P2Y1 and ATP-gated ion channel P2X7, which are expressed in RAW 264.7 cells (data not shown). PGE_2 and ISO can each bind to several receptors coupled to the heterotrimeric G_s protein whose activation results in cAMP generation. Previous reports showed that IFN-γ stimulated, while 2MA, PGE_2, and ISO inhibited, LPS responses including TNF-α transcription and secretion, inducible NO synthase (iNOS) gene expression, and NO production (10–21). However, the full extent of cross-talk between gene regulatory pathways activated by LPS and those by IFN-γ, 2MA, PGE_2, or ISO has not been thoroughly investigated in previous studies.

Materials and Methods

Reagents, cell culture, and RNA preparation

LPS (100 ng/ml; Sigma-Aldrich), PGE_2 (10 μM; Sigma-Aldrich), ISO (50 nM; Sigma-Aldrich), IFN-γ (5 nM; R&D Systems), and 2MA (500 μM; Sigma-Aldrich) were applied individually and in combination with LPS to stimulate RAW 264.7 cells for 1, 2, and 4 h, and their RNA was extracted using TriPure (Roche) following AfCS protocol PP0000018600 (supplementary *Materials and Methods*).[6] Triplicate experiments were done for each treatment. A total of 100 pM LPS binding protein (R&D Systems) was combined with LPS before LPS addition.

Oligo array fabrication and annotation

A total of 15,822 oligomers 65- to 75-bp long was printed on 15,840 spots on the custom-made arrays. The oligomers were purchased from Operon and Sigma-Genosys and were inkjet-printed onto glass slides by Agilent Technologies. As of the annotation results generated on January 19, 2005, the entire collection of the oligomers represents ~12,237 genes, each identified by a unique LocusLink ID. Of these, 9,629 are known genes. See supplementary Table I in the supplemental material for the annotation table.

Oligo array analysis

Each array was hybridized with the Cy5-labeled antisense RNA prepared from the RNA of ligand-treated cells and the Cy3-labeled antisense RNA prepared from the RNA of time-matched control cells (AfCS protocol PP000000184). Dye-swap labeling and array hybridization were performed for each pair of cDNA samples. Thus, each treatment condition had three independent biological samples (except for LPS plus PGE_2 1 h, which had two replicate biological samples), with the expression changes in each sample measured with a pair of dye-swap microarrays. The arrays were scanned using Agilent Scanner G2505A (Agilent Technologies) with the scan resolution set to 10 μm and the laser intensity adjusted so that both the maximum red and green (Cy5 and Cy3) fluorescence intensity were around 20,000. 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 Agilent G2566AA Extraction Software version A.6.1.1. The entire raw data sets are available through the RAW cell double ligand screen link at the AfCS Data Center website: ⟨www.signaling-gateway.org/data/⟩.

Data selection

For each array, we removed the control features. For any features with a green or red fluorescence signal that was saturated (with Agilent "glsSaturated" and "rlsSaturated" flags), nonuniform (with Agilent "gIsFeatNonUnifOL" and "rIsFeatNonUnifOL" flags), or below background (with Agilent "gIsWellAboveBG" and "rIsWellAboveBG" flags), its $\log_2$-(Cy5/Cy3) value was set to NA (not available). The expression change relative to time-matched control (as $\log_2$-(treated/control)) of each feature measured from a ligand-treated sample was the average of the two dye-swap measurements, and was set to NA if one or both dye-swap measurements were NA. The expression change of each feature under a ligand treatment condition was the average of the measurements made with replicate biological samples, and was set to NA if there was only one non-NA measurement.

Identification of differentially expressed features with Linear Models for Microarray Data (LIMMA)

Differentially expressed features in response to ligand stimulation were identified using LIMMA, a software package for the analysis of gene expression microarray data (⟨http://bioinf.wehi.edu.au/limma/⟩) (22, 23). Each dye-swap measurement of the $\log_2$-(treated/control) was considered as an independent replicate in the statistical analysis. Thus, each array feature had six replicates for each treatment (except LPS plus PGE_2 1 h, which had four replicates). When examining the profiles of expression changes induced by single and dual ligands, only features with an average $\log_2$-(treated/control) (in absolute values) ≥ 1, which corresponds to 2-fold expression changes, at $p < 0.01$ were considered differentially expressed. When correlating transcriptional responses to single ligands with nonadditive responses to dual ligands, features with an average $\log_2$ (treated/control) (in absolute values) >0.58, which corresponds to 50% changes in expression, at $p < 0.01$ were considered differentially expressed instead. In LIMMA, the p value is obtained from t-statistics with SE moderated across genes using Empirical Bayesian methods, adjusted for multiple testing with the Benjamini and Hochberg's method to control the false discovery rate.

Identification of nonadditive transcriptional responses to dual ligand treatments

We used net fold changes as a measurement of responses induced by ligands (Fig. 1), so that, for example, $\log_2$-(treated/control) of 1 and 2 correspond to net-fold changes of 1 and 3, respectively, while $\log_2$-(treated/control) of -1 and -2 correspond to net-fold changes of -1 and -3, respectively. Adopting a metric used to measure the costimulatory effect of CD28 signaling on TCR-mediated gene expression (24), we then calculated the nonadditivity of a dual ligand response per feature per time point per replicate sample (DIF) as the difference between the observed response and the expected additive response to the dual ligand treatment, where the latter was the sum of the feature's responses to the two single ligands (Fig. 1). The reproducibility of the nonadditivity of a dual ligand response was evaluated using the signal:noise ratio, which was the ratio of the average of replicate measurements of DIF (avg.DIF) and their SD (Fig. 1). The transcriptional change of a feature to a ligand pair was considered nonadditive if the response had an absolute avg.DIF $>=1$ and an absolute signal:noise ratio $>=1$, and the feature had 50% or more expression changes at $p < 0.01$ to ligand 1, ligand 2, or the ligand pair. A positive or a negative avg.DIF indicates that the response of a gene to the dual ligand treatment was greater than or less than additive, respectively.

Visualization of patterns of gene expression changes and nonadditivity

The average $\log_2$-(treated/control) and the avg.DIF were hierarchically clustered to visualize the patterns of gene expression changes and the nonadditivity, respectively. Only features with significant expression changes and significant nonadditive responses at one or more time points were included, respectively. Clustering was done one-way across the features with experimental conditions aligned in ligand followed by time-course orders. Euclidean correlation coefficient and complete linkage were used as

1. Calculate the ligandEffect, the net fold-change of gene expression, induced by single and dual ligand treatments
- ligandEffect = power(2,x) - 1 if x >= 0; 1 - 1/(power(2,x)) if x < 0
  - x is the average $\log_2$-(treated/control) of a pair of dye-swap measurements.
  - ligandEffect is set to NA if x is NA.

2. Calculate the expected additive dual ligand effect, which is the sum of the ligandEffect of two single ligands, L_1 and L_2, and the observed effect of the dual ligand, $L_{1\&2}$
- Expected = ligandEffect(L_1) + ligandEffect(L_2)
  - Expected is set to NA if the ligandEffect of L_1 or L_2 is NA.
- Observed = ligandEffect($L_{1\&2}$)

3. Calculate the average non-additivity (avg.DIF) and its signal-noise-ratio (SNR) of the dual ligand response
- DIF = Observed - Expected
  - DIF is set to NA if the Observed or the Expected dual ligand effect is NA.
- avg.DIF = (∑DIF / N) * (∑Expected / |∑Expected|)
  - ∑DIF and ∑Expected are the sum of the non-NA replicate measurements of DIF and expected additive dual ligand effect, respectively
  - N is the number of non-NA replicate measurements of DIF for a feature
  - |∑Expected| is the absolute value of ∑Expected
  - avg.DIF is set to blank if the feature has less than two non-NA DIF measurements
- SNR = avg.DIF / stdev.DIF
  - stdev.DIF is the standard deviation of replicate DIF measurements

4. Identify array features with non-additive responses to $L_{1\&2}$
- array features with |avg.DIF| >= 1, |SNR| >= 1, and 50% or more expression changes to L_1, L_2, or $L_{1\&2}$, at p < 0.01
  - |avg.DIF| and |SNR| are the absolute value of avg.DIF and SNR, respectively

FIGURE 1. Mathematical formulas used to identify nonadditive transcriptional changes to dual ligand treatments.

[6] The online version of this article contains supplemental material.

similarity metrics. The hierarchical clustering program used is implemented in the Multiple Experiment Viewer (⟨www.tigr.org/software/tm4/mev.html⟩).

Results

Characterization of gene expression responses induced by LPS, IFN-γ, 2MA, PGE_2, and ISO

To delineate the kinetics of the transcriptional response to LPS in RAW 264.7 cells, the cells were sampled at eight time points in a 48-h period after addition of LPS. We found that ~60% of the differentially expressed features over the 48 h time course in response to LPS showed significant changes (> = 50%) after 4 h of stimulation (supplementary Table II). Thus, we limited our dual ligand study to the first 4-h period.

RAW 264.7 cells were treated with LPS, IFN-γ, 2MA, PGE_2, and ISO applied as single ligands and in pairs with LPS for 1, 2, and 4 h, and the expression changes relative to time-matched control were measured with oligo arrays. See supplementary Table III for the list of array features with 2-fold or more expression changes at $p < 0.01$ in each condition. As shown in Fig. 2*A*, compared with 2MA, PGE_2, and ISO, LPS and IFN-γ individually induced changes in the greatest number of genes especially at 2 and 4 h. The number of changes induced by LPS and IFN-γ increased with time, while the number of changes induced by 2MA, PGE_2, or ISO began to decrease at 4 h, indicating that transcriptional responses to 2MA, PGE_2, and ISO are potentially transient while LPS- and IFN-γ-induced responses are persistent and expansive during the 4-h period.

As shown in the dendrogram of hierarchically clustered expression changes, the most robust changes in transcription were induced by LPS with most affected genes being up-regulated (Fig. 2*B*). Similar trends, albeit with smaller magnitudes, were observed for IFN-γ-responsive genes. Many of the genes that were up-regulated by both LPS and IFN-γ are likely primary and secondary targets of the JAK-STAT pathway. IFN-γ and several LPS-induced cytokines, including IFN-β (supplementary Table III) (25), G-CSF, IL-6, and IL-10 (⟨www.signaling-gateway.org/data/cgi-bin/ligscr.cgi?assay=cytokine&lig=LPS&cellabbr=RW⟩), activate STAT transcriptional activity. LPS and IFN-γ target genes identified in RAW 264.7 cells include a number of previously reported immediate early genes and secondary genes of LPS and IFN-γ responses (supplementary Table III). Examples of immediate early genes include *TNF-α*, *IL-1β*, *Irg1*, *IFN-β*, *Ccl5*, and *Cxcl10 for LPS*, and *Icsbp1*, *Irf1*, and *Tap1* for IFN-γ (25, 26). Examples of secondary genes include *Mx1*, *Ifl1*, *Ifl204*, and *Irf7* for LPS, and *Gbp1* and *Gbp2* for IFN-γ (25–27). Consistent with previous characterization of LPS- and IFN-γ-induced transcriptional responses in macrophages using microarrays, a large number of genes induced by LPS and IFN-γ in RAW 264.7 cells encode cytokines, chemokines, signaling molecules, and transcriptional factors that are involved in innate immunity and inflammation (28–30).

Changes in response to 2MA, PGE_2, or ISO are masked in the dendrogram (Fig. 2*B*) because these responses were significantly weaker than responses to LPS or IFN-γ. Therefore, for features with significant responses to 2MA, PGE_2, or ISO, their changes were hierarchically clustered in the absence of the LPS and IFN-γ data set (Fig. 2*C*). The dendrogram showed that PGE_2 and ISO induced similar patterns of induction and repression; the response to 2MA differs from LPS and IFN-γ, as the majority of the changes in the levels of expression were up-regulation (Fig. 2*C*). Stimulation with 2MA, PGE_2, and ISO result in statistically significant changes in 102, 42, and 65 unique genes, respectively (supplementary Table III). Aside from the induction of gene expression of *IL-1β* and several early response genes such as *Dusp1*, *Dusp2*, and

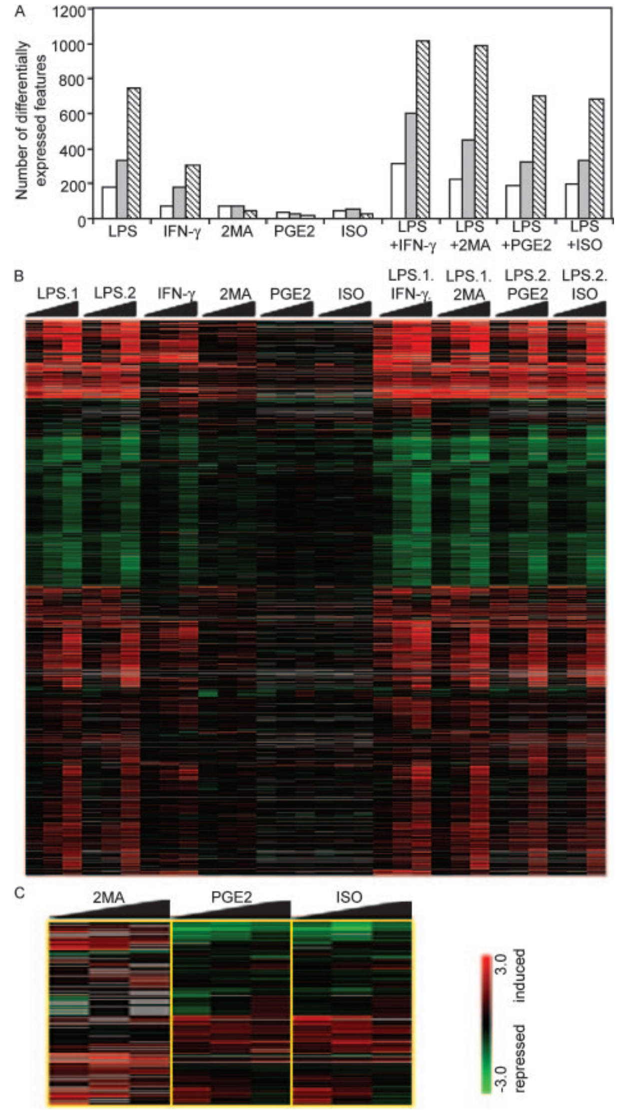


FIGURE 2. Gene expression changes induced by LPS, IFN-γ, 2MA, PGE_2, and ISO individually and in combination with LPS. *A*, The number of differentially expressed features at 1 h (□), 2 h (▨), and 4 h (▧). *B*, The dendrogram of hierarchically clustered transcriptional changes of differentially expressed features. Two different LPS data sets, one generated in dual ligand experiments with LPS, IFN-γ, and 2MA, and the other in dual ligand experiments with LPS, PGE_2, and ISO, were shown separately. *C*, The dendrogram of hierarchically clustered transcriptional changes of differentially expressed features regulated by 2MA, PGE_2, and ISO only. ▲, Each represents a time course of 1, 2, and 4 h.

Ier3 by all three ligands, we noticed that 2MA by itself increased the gene expression of *TNF-α*, as reported previously (31), and a number of chemokines including Ccl2, Ccl4, Ccl7, Cxcl2, Cxcl10, and Cxcl11. PGE_2 and ISO did not significantly induce the expression of these genes. Instead, they inhibited the expression of Ccl4. The three ligands differentially regulated regulator of G protein signaling (*RGS*) genes as well; 2MA increased *RGS16* expression, while PGE_2 and ISO inhibited *RGS1* and *RGS2* expression (supplementary Table III).

Low resolution of the dendrogram obscured nonadditive responses to combinations of ligand, and initially the patterns of changes induced by the ligands combined with LPS appear similar to the patterns of changes induced by LPS alone (Fig. 2*B*). To identify features that responded nonadditively to combinations of ligands with LPS, additional quantitative measures were applied systematically (see *Materials and Methods*).

An overview of nonadditive responses to dual ligands

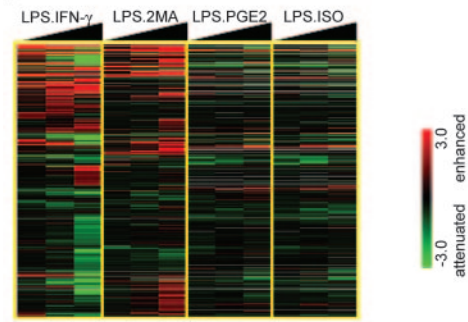
A total of 619 features, representing 503 unique genes, showed above-threshold nonadditive responses in at least one of the dual ligand conditions (supplementary Table V). Although LPS plus IFN-γ and LPS plus 2MA elicited distinct profiles of nonadditive expression responses, as shown in the dendrogram of hierarchically clustered, nonadditivity, LPS plus PGE_2 and LPS plus ISO induced similar patterns of nonadditive responses (Fig. 3*A*). Furthermore, the majority of genes with nonadditive responses after 1 h of LPS plus IFN-γ stimulation had enhanced expression (greater than additive). However, the number of attenuated (less than additive) response increased with time and by 4 h, the majority of nonadditive responses to LPS plus IFN-γ were attenuated. The increased number of attenuated responses is not caused by the saturation of microarray signals or pathways activated by both LPS and IFN-γ (addressed below). In contrast, LPS plus 2MA induced mostly enhanced responses throughout the time course, while LPS plus PGE_2 and LPS plus ISO induced mostly attenuated responses. The observation that LPS plus PGE_2 and LPS plus ISO induced similar nonadditive responses and that PGE_2 and ISO alone induced similar gene expression profiles, together with the fact that both ligands can activate G_s and induce cAMP, suggest that pathways downstream of cAMP regulate most of the transcriptional changes induced by PGE_2 and ISO and are involved in cross-talk with the gene regulatory pathways activated by LPS.

To examine how the transcriptional response to one ligand was affected by signals activated by another ligand, we grouped features with nonadditive responses to a ligand pair into three categories: those that had significant responses to both single ligands, to either ligand 1 or 2, and to neither (supplementary Table IV-IX, Fig. 3*B*). We considered transcriptional changes >50% at $p < 0.01$ as significant. This helps to take into account the changes that were moderate yet statistically significant. We specifically focused on the nonadditive responses of features that responded significantly to only one ligand of a ligand pair when applied individually, which directly indicate how the response induced by this ligand is modulated by the presence of another ligand.

We found that many LPS-induced transcriptional changes were affected nonadditively by simultaneous addition of 2MA, PGE_2, or ISO, while few 2MA-, PGE_2-, or ISO-specific features were influenced by LPS signaling. In contrast, numerous unique transcriptional targets regulated by either LPS or IFN-γ responded nonadditively to LPS plus IFN-γ (Fig. 3*B*). With each ligand pair, there were distinct trends in the nonadditive interactions. In response to LPS plus 2MA, 77–83% of the nonadditive responses of unique LPS transcriptional targets were enhancement by 2MA at 1, 2, and 4 h; while in response to LPS plus PGE_2 and LPS plus ISO, 61–71% and 65–75% of the nonadditive responses of LPS transcriptional targets were attenuation by PGE_2 and ISO, respectively (Fig. 3*B*). In response to LPS plus IFN-γ, there was a distinct time-dependent trend in the interactions. At 1 h, around 80% of the nonadditive responses of unique IFN-γ transcriptional targets were LPS-dependent enhancement, while >70% of the nonadditive responses of the unique transcriptional targets of LPS at 4 h were IFN-γ-dependent attenuation.

Finally, by measuring nonadditive transcriptional changes using microarrays, we were able to ask the following question: of the expression changes induced by two single ligands individually, what percentage is affected by the presence of another ligand? The percentage provides a measure of the extent of gene regulatory pathway cross-talk for the chosen ligands. We found that 18–24%

A



B

	LPS.IFN-γ			LPS.2MA			LPS.PGE2			LPS.ISO		
	1 h	2 h	4 h	1 h	2 h	4 h	1 h	2 h	4 h	1 h	2 h	4 h
TOTAL	126	191	353	46	78	160	33	30	59	34	65	71
LESS	29	79	238	10	26	33	21	23	36	24	52	52
Changed.in.BOTH	2	22	150	4	17	11	4	4	1	8	16	7
Changed.in.LPS	17	41	74	5	9	20	14	17	34	13	31	40
Changed.in.LIG2	7	12	6	1	0	1	3	2	1	3	5	3
Changed.in.NEITHER	3	4	8	0	0	1	0	0	0	0	0	2
GREATER	97	112	115	36	52	127	12	7	23	10	13	19
Changed.in.BOTH	15	34	49	17	12	18	3	0	0	3	1	1
Changed.in.LPS	24	33	27	17	35	98	9	7	21	7	10	16
Changed.in.LIG2	34	27	13	0	0	1	0	0	0	0	0	0
Changed.in.NEITHER	24	18	26	2	5	10	0	0	2	0	2	2

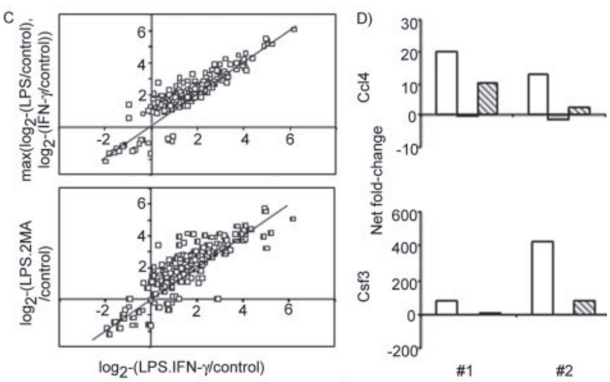


FIGURE 3. Nonadditive responses to dual ligands. *A*, The dendrogram of hierarchically clustered average nonadditivity (avg.DIF) of dual ligand responses to LPS plus IFN-γ, LPS plus 2MA, LPS plus PGE_2, and LPS plus ISO. A total of 619 features with nonadditive responses in at least one dual ligand condition were included. *B*, The number of nonadditive features with significant responses to both single ligands, to LPS only, to the second ligand only (LIG2), or to neither. TOTAL, all features with nonadditive responses; LESS/GREATER, features with less than/greater than additive responses; Changed.in.BOTH, Changed.in.LPS, Changed.in.LIG2, and Changed.in.NEITHER correspond to the number of features that showed above threshold nonadditive responses to a ligand pair, and had significant responses to both single ligands, LPS only, LIG2 only, and neither ligand, respectively. *C*, The less-than-additive transcriptional changes induced by LPS plus IFN-γ at 4 h were not mainly a result of signal saturation. *Top graph*, The average log_2-(treated/control) in response to LPS plus IFN-γ on the *x*-axis were graphed against the average log_2-(treated/control) in response to LPS alone or IFN-γ alone (the greater of the two was graphed) on *y*-axis. Approximately 80% of the points were above the $y = x$ line. *Bottom graph*, The average log_2-(treated/control) in response to LPS plus IFN-γ on *x*-axis were graphed against the average log_2-(treated/control) in response to LPS plus 2MA on *y*-axis. Approximately 80% of the points were above the $y = x$ lone. *D*, *Ccl4* and *Csf3* showed less-than-additive responses to LPS plus IFN-γ at 4 h as measured by QRT-PCR in two independent biological samples nos. 1 and 2. Net-fold changes in gene expression induced by ligands were graphed on the *y*-axis with different ligand treatments graphed on the *x*-axis. □, ▨, and ▧, The net fold-change induced by LPS, IFN-γ, and LPS plus IFN-γ, respectively. For features with increased gene expression, the net fold-change = expression ratio treated/control − 1; for features with decreased gene expression, the net fold-change = 1 − expression ratio control/treated. See supplementary *Materials and Methods* for the detail of QRT-PCR procedure and expression ratio calculation method.

Table I. *The percentage of features with nonadditive responses to dual ligand treatments*[a]

	LPS IFN-γ (%)			LPS 2MA (%)			LPS PGE_2 (%)			LPS ISO (%)		
	1 h	2 h	4 h	1 h	2 h	4 h	1 h	2 h	4 h	1 h	2 h	4 h
All	24	19	18	8	9	9	7	4	3	8	7	4
Changed in both	44	35	36	30	25	22	23	ND	ND	21	26	14
Changed in LPS	11	11	9	6	6	7	6	3	3	6	5	3
Changed in LIG2	39	22	9	ND	ND	ND	ND	ND	ND	ND	9	ND

[a] All, the percentage of all features with significant responses to either LPS or LIG2 (the ligand paired with LPS in dual ligand treatment) only, or to both LPS alone and LIG2 alone; both, the percentage of features with significant responses to both single ligands; LPS, the percentage of features with significant responses to LPS only; LIG2, the percentage of features with significant responses to only LIG2. If a category had less than five features with nonadditive responses, the percentage was not calculated (ND), as the percentage calculated with small numbers may not be accurate.

of all features differentially expressed in single ligand responses to LPS and IFN-γ showed nonadditive responses to LPS plus IFN-γ, compared with 8–9% for LPS and 2MA, 3–7% for LPS and PGE_2, and 4–8% for LPS and ISO (Table I), indicating that only a small fraction of transcriptional targets of single ligands were affected by pathway cross-talk in our experiments. Higher percentages of transcriptional targets shared by two single ligands showed nonadditive responses than unique targets of each single ligand. For example, in response to LPS plus IFN-γ, 35–44% of shared target genes showed nonadditive responses compared with 9–11% of LPS targets and 9–39% of IFN-γ targets (Table I). This suggests that ligand-induced gene regulatory pathways are more likely to interact when the ligands share transcriptional targets.

Attenuated response to LPS plus IFN-γ at 4 h is not primarily due to the saturation of microarray signals or pathways activated by both LPS and IFN-γ

The number of attenuated responses to LPS plus IFN-γ increased with time. By 4 h, 230 features had significant responses to LPS or IFN-γ alone and showed attenuated responses to LPS plus IFN-γ, which account for approximately two-thirds of all the features with nonadditive responses to LPS plus IFN-γ at 4 h. One concern is that most of these attenuated responses might be an artifact of signal saturation. Because both LPS and IFN-γ alone induced robust expression changes of numerous genes at 4 h (Fig. 2*B*), the responses of these genes to LPS plus IFN-γ may well exceed the detection limit of the microarray, or the signals induced by the dual ligand may saturate the capacity of signaling pathways downstream of LPS and IFN-γ stimulation. If expression changes induced by LPS plus IFN-γ saturated the microarray detection limit or the capacity of signaling pathways, then we expect that the expression level would be greater than that induced by LPS or IFN-γ alone. We found that for ~80% of the features with less-than-additive responses to LPS plus IFN-γ at 4 h, LPS plus IFN-γ-induced smaller expression changes than LPS or IFN-γ alone at 4 h (Fig. 3*C*). Furthermore, compared with changes induced by another dual ligand pair, LPS plus 2MA, 80% of the features had smaller changes to LPS plus IFN-γ at 4 h (Fig. 3*C*). For two of the features, we confirmed using quantitative RT-PCR (QRT-PCR) that their response to LPS plus IFN-γ was greatly attenuated compared with their response to LPS alone (Fig. 3*D*). Together, these observations suggest that the less-than-additive transcriptional changes mainly result from the attenuation of either the LPS- or IFN-γ-induced response, and not through saturation of the detection platform or the signaling pathways themselves.

Nonadditive responses to ligand pairs: mechanisms of cross-talk

To search for clues to mechanisms of cross-talk between gene regulatory pathways, we examined nonadditive responses of unique transcriptional targets of single ligands. We looked for coordinated enhancement or attenuation of the expression of known transcriptional targets of specific signal transduction pathways, which would reflect corresponding changes in the activity of upstream signals. We also looked for indications of feedback and autocrine loops, where significantly increased or reduced transcriptional responses of cytokines or signal transduction modulators in response to dual ligands compared with responses induced by single ligands alone were followed by later nonadditive responses of their downstream transcriptional targets.

LPS plus IFN-γ: enhanced expression of IFN-γ-primary and secondary response genes at 1 h suggests the enhancement of STAT1 activity and cooperative transcriptional activation STAT1 and NF-κB

A total of 41 features significantly changed their transcription in response to IFN-γ while having no response to LPS at 1 h, and their IFN-γ-induced changes were modulated in the presence of LPS. Although only 7 of the 41 features showed attenuated response to LPS plus IFN-γ (Figs. 3*B* and 4*B*), for 34 of the 41 features (83%), their response to IFN-γ was enhanced by LPS (Figs. 3*B* and 4*A*). These features included *Icsbp1*, *CXCL9*, and *TAP1*, documented IFN-γ primary response genes induced through binding of STAT1 dimers to the IFN-γ-activated sequence (GAS) element, and *Gbp1* and *Gbp2*, known IFN-γ secondary response genes whose expression is dependent on IFN-γ primary response genes such as *IRF1* (Fig. 4*A*) (26, 27). The synergistic induction of *Icsbp1* and *TAP1* by LPS plus IFN-γ was demonstrated previously in murine peritoneal macrophages and human THP-1 cell line, respectively (32, 33). LPS also enhanced the expression of additional IFN-induced genes, including *Ifi202b*, *Ifi203*, *Ifi204*, *Ifi47*, *Ligp2*, *Gbp4*, and *Tgtp*.

The enhanced IFN-γ transcriptional response at 1 h by LPS can result from an increase in STAT1 transcriptional activity. STAT1 transcriptional activity is regulated by phosphorylation of both the tyrosine 701 and serine 727 residues (34–40). Although LPS did not have a significant effect on IFN-γ-induced STAT1 tyrosine 701 phosphorylation in our AfCS data (⟨www.signaling-gateway.org/data/cgi-bin/llr.cgi?assay=western&lig1=IFG&lig2=LPS&protein=ST1A⟩; ⟨www.signaling-gateway.org/data/cgi-bin/llr.cgi?assay=western&lig1=IFG&lig2=LPS&protein=ST1B⟩), a previous study showed that LPS increased IFN-γ-induced STAT1 serine phosphorylation and GAS-driven luciferase transcription in mouse macrophage cells (41). For two primary IFN-γ response genes, *Icsbp1* and *Tap1*, which showed enhanced gene expression response to LPS plus IFN-γ both in this study (Fig. 4, *A* and *C*) as well in published reports (32, 33), promoter deletion analysis indicated that the STAT1 dimer binding site, GAS, was obligatory for the synergistic effect of LPS and IFN-γ while the NF-κB binding site was not required (32, 33). This observation is consistent with the possibility that LPS increases the expression of

A

FeatureNum	LLID	SYMBOL	LPS	IFN-γ	LPS.IFN-γ	avg.DIF
14709	66526	2210012G02Rik	-0.30	0.66	1.41	1.32
2521	74481	4933430F08Rik	-0.11	1.75	2.81	3.89
11555	76074	5830443L24Rik	-0.13	1.13	1.78	1.77
17418	57370	B4galt3	0.02	1.26	1.92	1.40
13862	20293	Ccl12	0.21	0.77	3.19	7.26
8030	12916	Crem	0.09	0.82	2.18	2.70
12552	17329	Cxcl9	0.03	2.48	5.17	32.18
75	14468	Gbp1	0.23	3.03	5.47	34.97
8212	14469	Gbp2	0.27	2.69	4.65	18.67
18514	55932	Gbp4	0.12	1.23	2.55	3.52
17465	14546	Gdap10	-0.13	1.40	1.85	1.14
18468	80885	Gpr109b	-0.24	1.35	2.09	1.96
14186	15900	Icsbp1	-0.24	2.04	2.90	3.47
12729	26388	Ifi202b	0.24	1.60	2.36	1.96
14550	26388	Ifi202b	0.17	1.55	2.35	2.07
9058	15950	Ifi203	-0.07	0.81	1.42	1.01
12878	15951	Ifi204	0.42	1.47	2.08	1.14
12833	15953	Ifi47	-0.09	2.20	2.56	1.31
15211	54396	Iigp2	-0.28	3.09	3.59	3.74
18555	16174	Il18rap	0.27	1.16	2.13	1.42
16649	16995	Ltb4r1	0.23	2.09	2.65	2.04
9767	381308	Mnda	0.00	0.99	1.89	1.71
18146	432411	Parp14	0.28	1.76	2.24	1.12
17735	60533	Pdcd1lg1	-0.09	1.97	3.07	4.57
11089	19730	Ralgds	0.24	0.97	1.83	1.40
18288	74748	Slamf8	0.05	1.69	2.71	3.30
3210	20848	Stat3	0.32	0.86	1.71	1.18
8943	20848	Stat3	0.22	0.85	1.68	1.24
5402	21354	Tap1	-0.17	0.96	1.61	1.25
9604	21822	Tgtp	0.02	1.82	2.57	2.29
10743	21822	Tgtp	0.03	3.92	4.84	13.21
560	21937	Tnfrsf1a	0.09	0.97	1.63	1.17
12618	21937	Tnfrsf1a	0.14	1.20	1.82	1.12
15424	81630	Zfp297	0.77	0.79	1.83	1.11

B

FeatureNum	LLID	SYMBOL	LPS	IFN-γ	LPS.IFN-γ	avg.DIF
7232	11542	Adora3	-0.24	1.40	0.31	-1.09
12257	11542	Adora3	-0.13	1.35	0.15	-1.33
15860	14281	Fos	0.08	-0.93	0.80	-1.67
9034	23882	Gadd45g	0.54	1.10	0.64	-1.10
7841	15901	Idb1	0.22	1.57	0.77	-1.48
16170	50930	Tnfsf14	0.10	1.45	0.60	-1.28
16933	50930	Tnfsf14	0.10	1.43	0.65	-1.21

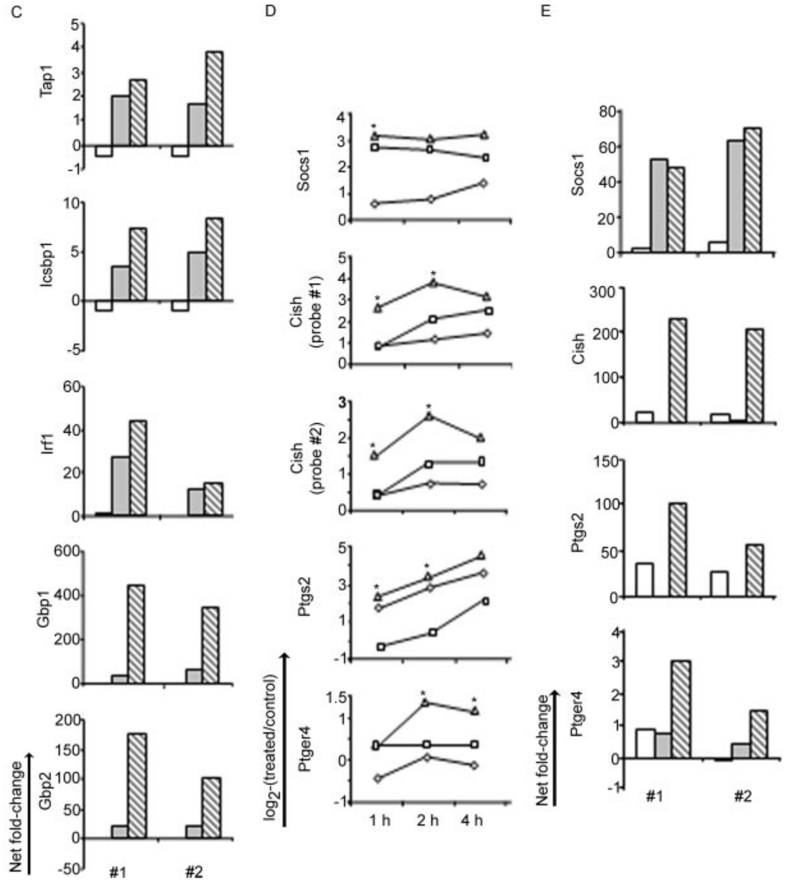


FIGURE 4. Nonadditive responses to LPS plus IFN-γ. *A*, IFN-γ-induced gene expression changes that were enhanced by LPS at 1 h. *B*, IFN-γ-induced gene expression changes that were attenuated by LPS at 1 h. Columns FeatureNum, LLID, and SYMBOL list the featureNumber, LocusLink ID, and gene symbol of array features, respectively. Columns LPS, IFN-γ, and LPS.IFN-γ list the average $\log_2$-(treated/control) in response to LPS, IFN-γ, and LPS plus IFN-γ at 1 h, respectively; avg.DIF lists the average difference of observed and expected dual ligand response in fold-changes. *C*, LPS enhanced the expression of IFN-γ primary response genes, *Tap1*, *Icsbp1*, and *Irf1*, and secondary response genes, *Gbp1* and *Gbp2*, at 1 h as measured by QRT-PCR in two independent biological samples 1 and 2. Figure legends are similar as those in Fig. 3*D*. *D*, *Socs1*, *Cish*, *Ptgs2*, and *Ptger4* were induced by LPS plus IFN-γ at higher levels than by LPS alone as detected by microarray. *X*-axis represents the time points; *y*-axis scales the average $\log_2$-(treated/control) in response to LPS (◇), IFN-γ (□), and LPS plus IFN-γ (△) at 1, 2, and 4 h. Reproducible nonadditive expression changes induced by LPS plus IFN-γ were marked by ∗. *E*, Socs1, Cish, Ptgs2, and Ptger4 showed significantly higher levels of induction by LPS plus IFN-γ than by LPS alone at 2 h, as measured by QRT-PCR in two independent biological samples nos. 1 and 2. Figure legends are similar as those in Fig. 3*D*.

IFN-γ-induced primary response genes by directly augmenting the transcriptional activity of STAT1. Several reports suggest that kinases including p38 MAPK, IL-1R-associated kinase, and PI3K/AKT are involved in STAT1 serine phosphorylation (42–44). Because these kinases also participate in signal transduction downstream of LPS stimulation, it is possible that they act as cross-talk points between LPS-activated pathways and the pathway mediating IFN-γ-induced STAT1 activation.

Increased expression of IFN-γ primary and secondary response genes can also be due to synergistic interaction between NF-κB activated by LPS or LPS-induced TNF-α and IFN-γ-activated STAT1. Several genes induced by IFN-γ including *Irf1*, *Cxcl10*, and *Icam-1* were found to respond synergistically to IFN-γ and TNF-α, and such responses required binding sites for both STAT1 and NF-κB in their promoters (45–47). In addition, the enhanced expression of IFN-γ secondary response genes can result from an earlier increase of the expression of transcription factors encoded by IFN-γ primary genes. *Irf1* is an IFN-γ primary response gene which encodes a transcription factor regulating the expression of IFN-γ secondary response genes including *Gbp1* and *Gbp2* (26, 27). In the current study, the expression of *Irf1* was induced to a greater extent by LPS plus IFN-γ than IFN-γ alone at 1 h (supplementary Tables III, V, and VI). It is conceivable that increased *Irf1* expression at an earlier time point contributes to the observed synergistic expression of its downstream targets such as *Gbp1* and *Gbp2* at 1 h. Using TOUCAN (〈http://homes.esat.kuleuven.be/~saerts/software/toucan.php〉), we analyzed the promoter region (within 1 kb 5′ of the first exon) of IFN-γ transcriptional targets whose expression was enhanced by LPS at 1 h, and identified the IRF1 site as the most overrepresented site. This is consistent with the possibility that the IRF1 protein plays an important role in the LPS-enhanced expression of IFN-γ response genes.

It is worth noting that of the features that were rapidly induced by IFN-γ and showed no response to LPS at 1 h in Fig. 4*A*, the majority (~90%) had significant response (with 50% or more expression changes at $p < 0.01$) to LPS at later time points (2 or 4 h). These features are likely to be induced by autocrine production of IFN-β in response to LPS. The delayed induction of these genes by LPS can be explained by the requirement for IFN-β induction by LPS, which functions in an autocrine loop, to activate the STAT1 pathway, whereas IFN-γ immediately activates the STAT1 pathway. This is supported by the AfCS phosphoprotein data that shows IFN-γ induces early (<5 min) and strong STAT1 phosphorylation (also STAT3 and STAT5), while LPS only begins to induce STAT1 phosphorylation after 30 min (⟨www.signaling-gateway.org/data/cgi-bin/dligscr.cgi?assay=western&lig1=IFG&lig2=LPS&cellabbr=RW⟩).

LPS plus IFN-γ: the attenuation of LPS-induced late transcription involves feedback/autocrine loops

Although similar numbers of LPS-induced expression changes were enhanced and attenuated by IFN-γ at 1 and 2 h, there was a bias toward IFN-γ suppression of LPS effects at 4 h, where 74 of 101 LPS transcriptional targets showed attenuated responses at 4 h in the presence of IFN-γ (Fig. 3*B*). Many of these features significantly changed expression in response to LPS alone while showing no response to IFN-γ, and their expression changes induced by LPS plus IFN-γ were smaller than those induced by LPS alone (Fig. 3, *C* and *D*). The delayed inhibition of LPS-mediated gene expression suggests the involvement of secondary effects, such as feedback and/or autocrine inhibition loops. One potential feedback loop involves suppressor of cytokine signaling 1 (Socs1) and cytokine-inducible SH2-containing protein (Cish). *Socs1* and *Cish*, genes encoding inducible inhibitors of the JAK-STAT pathway (48–53), had no or moderate response to LPS alone, but were strongly induced by LPS plus IFN-γ at 1 and 2 h (Fig. 4, *D* and *E*). It was recently proposed that Socs1 and Cish protein also regulate the LPS response by inhibiting LPS-induced NF-κB transcriptional activation and the secondary response of IFN-β (54–57). In agreement with their inhibitory effect on IFN-β response, $Socs1^{-/-}$ macrophages showed increased levels of STAT1 phosphorylation in response to IFN-β and LPS, and the overexpression of Socs1 inhibited LPS-induced STAT1 phosphorylation (55–57). Similarly, the overexpression of Cish in RAW 264.7 cells repressed STAT1 phosphorylation and reduced the expression and production of CXCL10 in response to LPS (56). Conflicting results were reported regarding the effect of Socs1 on LPS-induced NF-κB activation. Although some studies showed that the overexpression of Socs1 inhibited NF-κB activation and the production of TNF-α, which is transcriptionally regulated by NF-κB (54, 55), others failed to detect any such effects (56, 57). The inhibitory effect of Socs1 and Cish on LPS-induced STAT1 phosphorylation suggests that Socs1 and Cish attenuate STAT1-regulated transcriptional responses to LPS plus IFN-γ. It is likely that the increased and more rapid induction of Socs1 and Cish contributes to the inhibition of the late transcriptional changes in response to LPS plus IFN-γ.

PGE_2 induced in response to LPS plus IFN-γ can also contribute to the attenuation of transcriptional response at 4 h. Exogenously added PGE_2 inhibits LPS-induced gene expression and production of cytokines, including TNF-α (18, 58–60). PGE_2 is also induced after 2–4 h of LPS stimulation in macrophages (61–66), and endogenous PGE_2 can reach a level that is effective in inhibiting TNF-α production (21, 67). However, a recent report suggested that in RAW 264.7 cells, PGs induced by LPS is incapable of inhibiting TNF-α production, as treatment with COX inhibitors did not alter *TNF-α* gene expression and production (68). The authors argued that this was in part due to the low level of PGE_2 produced during the first 2 h of LPS stimulation as a result of the slow induction of *Ptgs2*, the gene encoding COX-2, the inducible PG synthase critical for PGE_2 production in response to inflammatory stimuli (62, 65, 66). In our experiment, LPS plus IFN-γ increased *Ptgs2* gene expression at a faster rate compared with LPS alone (Fig. 4, *D* and *E*). In addition, the expression of *Ptger4*, the gene encoding EP4, which is the main constitutively expressed PGE_2 receptor coupled to the G_s protein and mediates the early inhibition of cytokine production/release by PGE_2 (21, 58), was increased by >2-fold at 2 and 4 h in response to LPS plus IFN-γ (Fig. 4, *D* and *E*). It is conceivable that the increased expression of Ptgs2 and Ptger4 together results in increased PGE_2 production and signaling and the subsequent inhibition of LPS response at 4 h via the PGE_2/EP4 autocrine loop.

LPS plus IFN-γ: synergistic induction of genes that have no response to LPS or IFN-γ alone suggests cooperative interaction between transcriptional factors activated by LPS and IFN-γ

A number of features that had no responses to LPS or IFN-γ alone showed nonadditive responses, mostly enhanced induction, to LPS plus IFN-γ. Of these, 22 genes including *Sgk*, *Cebpd*, *Homer1*, *Pmaip1* (*Noxa*), *IL-15*, and *CCL12* at 1 h, 17 genes including *Sgk*, *Mad*, *Nos2* (*iNOS*), *Pmaip1* (*Noxa*), *Ptger4*, and *Mad* at 2 h, and 26 genes including *Idb2* and *Ptger4* at 4 h, were synergistically induced (supplementary Table VI). It was shown that LPS and IFN-γ synergistically induce iNOS expression in murine macrophages (69, 70) and such an effect requires an iNOS promoter that contains binding sites for NF-κB, STAT1, and IRF1 (71–73). It was further reported that NF-κB and IRF1 interacted with each other while binding to their respective binding sites in the iNOS promoter in response to IFN-γ and TNF-α, a cytokine rapidly induced by LPS in macrophage cells (74). Thus, one mechanism mediating the synergistic gene induction in response to LPS plus IFN-γ may involve cooperative transcriptional activation by STAT1 or IRF1 activated by IFN-γ and NF-κB activated by LPS or LPS-induced TNF-α (74, 75).

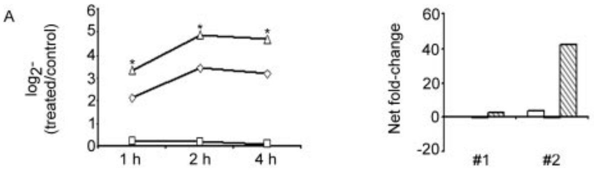
Many of the genes such as *iNOS*, *IL-15*, and *Ccl12* induced by LPS plus IFN-γ at 1 or 2 h did respond to LPS alone and/or IFN-γ alone at later time points (supplementary Tables IV and VI). The more rapid gene induction of some of the genes may be because IFN-γ functions as an equivalent to the IFN-β autocrine loop, but the lag time required for IFN-β production in LPS response has been eliminated by the addition of IFN-γ as a second ligand.

LPS plus IFN-γ: summary

Overall, we identified patterns of nonadditive responses suggesting multiple modes of cross-talk between gene regulatory pathways mediated by LPS and those by IFN-γ. Three of the modes are consistent with a proposed model by which LPS signaling modulates IFN-γ response (75): 1) LPS enhances IFN-γ-activated signals, possibly STAT1 activity, during early response to LPS plus IFN-γ; 2) cooperative transcriptional activation by NF-κB induced by LPS or LPS-induced TNF-α and STAT1 or IRF1 induced by IFN-γ; 3) LPS synergizes with IFN-γ in inducing IFN-γ primary response genes encoding transcriptional factors that mediate the expression of IFN-γ secondary response genes. We also found a delayed attenuation of a large number of LPS-regulated expression changes at 4 h. We propose that such attenuation is at least in part due to the feedback inhibition by Socs1 and Cish, and the autocrine loop mediated by PGE_2.

LPS plus 2MA: 2MA enhanced secondary transcriptional responses to LPS by increasing IFN-β and inhibiting IL-10 *gene expression*

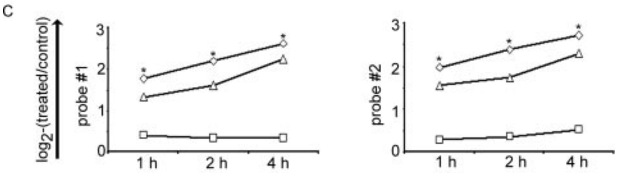
LPS plus 2MA primarily induced greater-than-additive responses. The bias toward greater-than-additive responses is especially pronounced in features uniquely regulated by LPS but not 2MA. Over 75% of LPS-regulated features showed greater-than-additive responses at 1, 2, and 4 h (Fig. 3*B*). At 1 h, the expression of 17 LPS-regulated features was nonadditively enhanced by 2MA, including cytokine-encoding genes such as *IL-1a*, *IL-1b*, *Cxcl11*, *Ccl5*, and *IFN-β* (supplementary Tables IV, V, and VII). Correlating with the enhanced gene expression of *IFN-β* at 1 h (Fig. 5*A*), A



B

FeatureNum	LLID	SYMBOL	LPS	2MA	LPS.2MA	avg.DIF
8212	14469	Gbp2	2.58	0.09	2.94	2.10
15677	16362	Irf1	2.29	0.09	2.84	2.13
6573	17857	Mx1	1.95	0.16	2.48	1.81
15328	17858	Mx2	2.49	0.27	2.81	1.12

C



D

FeatureNum	LLID	SYMBOL	LPS	2MA	LPS.2MA	avg.DIF
2127	12047	Bcl2a1d	2.93	0.46	3.13	1.26
6549	12266	C3	3.02	0.40	3.61	2.91
15307	12444	Ccnd2	1.07	0.36	3.14	7.31
7848	16173	Il18	2.85	0.44	3.37	2.72
15422	16173	Il18	2.45	0.26	2.94	1.86
2818	16175	Il1α	3.42	0.11	4.98	22.73
16708	16175	Il1α	3.62	0.07	5.11	27.03
1321	16176	Il1β	4.75	0.69	5.26	10.59
7872	16193	Il6	4.50	-0.03	5.50	23.16
3634	18712	Pim1	2.69	0.26	3.20	2.65
42	12703	Socs1	1.97	0.15	2.54	1.75
9168	12703	Socs1	1.40	0.07	2.03	1.41

FIGURE 5. The enhancement of IFN-β and inhibition of *IL-10* gene expression in response to LPS by 2MA was followed by the corresponding changes in their target genes later. *A*, Enhanced *IFN-β* gene expression in response to LPS by 2MA as early as 1 h as detected by microarray (*left graph*) and QRT-PCR (*right graph*). *Left graph*, *x*-axis represents the time points; *y*-axis scales the average $\log_2$-(treated/control) in response to LPS (◇), 2MA (□), and LPS plus 2MA (△) at 1, 2, and 4 h. Reproducible nonadditive expression changes induced by LPS plus IFN-γ were marked by *. *Right graph*, Figure legends are similar as those in Fig. 3*D*. *B*, Enhanced expression of IFN-induced genes in response to LPS by 2MA at 2 h. Columns FeatureNum, LLID, and SYMBOL list the featureNumber, LocusLink ID, and gene symbol of array features, respectively. Columns LPS, 2MA, LPS.2MA list the average $\log_2$-(treated/control) in response to LPS, 2MA, and LPS plus 2MA at 2 h, respectively. avg.DIF lists the average difference of observed and expected dual ligand response in fold changes. *C*, LPS-induced *IL-10* expression was attenuated by 2MA at 2 h. The symbols are similar as those used in the *left graph* of *A*. *D*, Enhanced expression of genes known to be repressed by IL-10 in response to LPS by 2MA at 4 h. For information listed by different columns, see *B*.

several IFN-β response genes, including *Irf1*, *Mx1*, *Mx2*, and *Gbp2* (25, 76) showed nonadditively increased expression in the presence of 2MA at 2 h (Fig. 5*B* and supplementary Table VIIB), suggesting that the 2MA-enhanced gene expression at 2 h is at least in part attributed to the augmentation of IFN-β-mediated autocrine loop (77).

LPS-induced *IL-10* expression was attenuated in the presence of 2MA at 1, 2, and 4 h at the transcript level (Fig. 5*C*), and at 3 and 4 h at the protein production/secretion level (⟨www.signaling-gateway.org/data/cgi-bin/llr.cgi?assay=cytokine&lig1=2MA&lig2=LPS&cytokine=IL-10⟩). IL-10 can suppress the production of proinflammatory cytokines such as IL-1α, TNF-α, IL-12p40, and IL-6 produced by macrophages, and is thought to mediate its immunosuppressive effects mainly at the level of transcription (78, 79). It was shown that the expression of 20–25% of genes induced by LPS in macrophages at 45 min and 3 h was inhibited by IL-10 (80). Notably, these include a number of genes that showed greater-than-additive induction in response to LPS plus 2MA at 4 h, including *Bcl2a1d*, *C3*, *Ccnd2*, *IL-1α*, *IL-1β*, *IL-6*, *IL-18*, *Pim1*, and *Socs1* (80) (Fig. 4*D*). We suggest that in the presence of 2MA, the decreased production of IL-10 in response to LPS results in a relative increase in transcription of genes inhibited by IL-10.

LPS plus ISO: the attenuated expression of genes known to be repressed by IL-10 may result from the earlier enhancement of LPS-induced IL-10 expression by ISO

Of the LPS target features with nonadditive responses to LPS plus ISO, >65% of them had attenuated response to LPS plus ISO at 1, 2, and 4 h (Fig. 3*B*). It is proposed that cAMP induced by ISO mediates the attenuation of LPS-induced gene expression by inhibiting the short-term as well as long-term activity of NF-κB (81). In short-term within 1 h of LPS stimulation, cAMP-activated protein kinase A phosphorylates CREB, which then competes with p65 for shared activation partners, CREB binding protein and p300 (82–84). In long-term via unknown mechanisms, cAMP can stabilize IκB and increase LPS-induced IL-10, which leads to decreased IκB kinase activity and NF-κB DNA binding activity (85–88). Because both the MyD88-dependent and the MyD88-independent pathways involve NF-κB activity for transcription, we examined the nonadditive responses of known primary transcriptional targets of the pathways. *TNF-α* and *IL-1α* of the MyD88-dependent pathway and *Cxcl10* and MARCKS-like protein (*Mlp*) of the MyD88-independent pathway (25, 89) responded nonadditively to LPS plus ISO. Consistent with published reports (20), the expression of *TNF-α* was attenuated at all three time points. However, the expression of *IL-1α* was enhanced at 1 and 2 h. In contrast, the expression of *Cxcl10* and *Mlp* was attenuated, with *Cxcl10* expression reduced at 1 and 2 h and *Mlp* expression reduced at 2 and 4 h. Thus, a few primary transcriptional targets of LPS displayed nonadditive responses to LPS plus ISO in our study. ISO enhanced LPS-induced *IL-10* gene expression at 1 and 2 h (supplementary Tables IV, V, and IX) and protein production/secretion at 3 h and 4 h (⟨www.signaling-gateway.org/data/cgi-bin/llr.cgi?assay=cytokine&lig1=ISO&lig2=LPS&cytokine=IL-10⟩). Correlating with the increase of IL-10 production, genes whose LPS-induced expression was reportedly inhibited by IL-10, including *Ccl2*, *TNF-α*, *Pim1*, and *Bcl2a1d* (80), showed attenuated expression to LPS plus ISO at 2 or 4 h (supplementary Table IV, V, and IX). Other genes whose LPS-induced expression was attenuated by ISO include a number of early response genes such as *Egr1*, *Egr2*, *Gadd45α*, *ler2*, *Myd116*, *Dusp1*, and *Dusp2* at 1 or 2 h, and a number of cytokine genes such as *Ccl4*, *Ccl9*, *Csf2*, and LTβ, at 1, 2, or 4 h (supplementary Tables IV, V, and IX).

LPS plus PGE_2 induced similar profiles of nonadditive responses as LPS plus ISO. Similar genes were affected in a similar direction. We assumed that the main mechanisms of cross-talk of these two pairs of ligands are similar. Therefore, we only discussed the nonadditive responses induced by LPS plus ISO, as for the response of some genes, the pair induced greater magnitudes of attenuation.

Discussion

Our study identified nonadditive transcriptional changes to LPS plus IFN-γ, LPS plus 2MA, LPS plus PGE_2 and LPS plus ISO, and used them as readouts to investigate the extent and mechanisms of cross-talk between gene regulatory pathways activated by LPS and those by IFN-γ, 2MA, PGE_2, and ISO. Our major findings are: 1) IFN-γ preferentially attenuated late LPS transcriptional response at 4 h; 2) 2MA mainly enhanced LPS-induced gene expression changes; 3) PGE_2 and ISO function through common pathways to preferentially inhibit LPS transcriptional response. Some of the

PGE_2 and ISO effect may be indirect, and may be due to the increased expression of IL-10.

We suggest that Socs1- and Cish- mediated feedback inhibition and PGE_2-EP4 negative feedback loops attenuate the transcriptional responses to LPS plus IFN-γ at 4 h. The increased number of less-than-additive transcriptional changes induced by LPS plus IFN-γ at 4 h was preceded by a significant induction of gene expression of *Socs1* and *Cish*, genes encoding putative inhibitors of LPS-induced IFN-β autocrine response, and *Ptgs2* and *Ptger4*, genes encoding COX-2 and EP4, an enzyme and a receptor for the synthesis and binding of PGE_2, respectively (Fig. 4, *D* and *E*). However, it is worth pointing out that the inhibition mediated by endogenous PGE_2 is likely to be limited in RAW 264.7 cells. We found that 10 μM exogenous PGE_2 added with LPS induced less-than-additive responses of 21 to 36 features in our experiment (Fig. 3*B*). This number was considerably smaller than the number (223) of features with less-than-additive responses to LPS plus IFN-γ at 4 h (Fig. 3*B*). This suggests that even if the endogenous PGE_2 reaches the level of 10 μM, it is likely to have a limited contribution to the attenuation of gene expression responses to LPS plus IFN-γ at 4 h. Considering the report that only ~7 nM PGE_2 was induced by LPS alone at 4 h in RAW 264.7 cells (68), we expect that PGE_2 induced by LPS plus IFN-γ during a 4-h period would be lower than 10 μM, even though *COX-2* gene induction by the dual ligand was 2- to 3-fold higher than that by LPS alone (Fig. 4, *D* and *E*).

Time course correlation also suggests that autocrine loops play a role in mediating the effect of 2MA on the LPS response. In response to LPS plus 2MA, the enhancement of LPS-induced expression of *IFN-β* at 1 h was followed by the increased expression of multiple IFN-induced genes at 2 h, and the attenuation of LPS-induced expression of *IL-10* at 1 and 2 h correlated with the increased expression of IL-10-repressed genes later at 4 h (Fig. 5). The effect of 2MA on LPS-induced *IFN-β* and *IL-10* gene expression in macrophages has not been reported. Although it is unclear to us how 2MA-activated signals attenuated LPS-induced *IL-10* gene expression, we hypothesize that 2MA-induced Ca^{2+} mobilization (⟨www.signaling-gateway.org/data/cgi-bin/ligscr.cgi?assay=calcium&lig=2MA&cellabbr=RW⟩) activates NFAT, and NFAT synergizes with IRF3 activated by LPS in the transcriptional induction of *IFN-β*. Consistent with this possibility, we found a conserved noncoding sequence in the 5′ noncoding region of the mouse and human *IFN-β* genes, and both the human and mouse segments have a NFAT site, an IRF site overlapped with the NFAT site, and a NF-κB site. While the IRF site and the NF-κB site mediate IFN-β transcriptional activation by IRF3 and NF-κB in response to LPS (25), the NFAT site can recruit NFAT activated by 2MA. NFAT then cooperates with IRF3 bound to the overlapping IRF3 site, leading to the enhancement of *IFN-β* expression. In fact, NFAT has been reported to interact and synergize with IRF4 and IRF8 in *IL-4* and *IL-12 p40* transcriptional activation, respectively (90–92). Experiments combining treatment with inhibitors of NFAT activation and IFN-β promoter analysis should help to elucidate the role of NFAT in mediating the enhancement of LPS-induced *IFN-β* by 2MA.

Several substances elevating intracellular cAMP levels including PGE_2 and ISO have been reported to enhance LPS-induced *IL-10* gene expression (86, 87, 93–101). It is possible that intracellular cAMP activates protein kinase A, which in turn phosphorylates CREB, and the phosphorylated CREB contributes to the enhancement of LPS-induced *IL-10* transcription. Two CREB sites have been identified in the 5′ noncoding sequence of human *IL-10* that to which CREB-1 and ATF-1 can bind, and mutations of the two sites reduced the level of cAMP-stimulated transactivation in reporter gene assays by 20–50% (86, 102). We found that one of the sites, CRE4, located ~400 bp 5′ of the first human *IL-10* exon, was also present in a similar location in the mouse *IL-10* gene within a 300-bp segment highly conserved between human and mouse. Furthermore, a recent report suggested that LPS signaling induces CREB transcriptional activity by inhibiting glycogen synthase kinase 3 via the PI3K/AKT pathway, and activated CREB promotes LPS-induced *IL-10* transcription (103).

There are differences between the effects of IFN-γ and 2MA on LPS-induced expression changes identified in this study and those previously reported. Although we found that IFN-γ attenuated the expression of many genes in response to LPS including *IFN-β1* at 2 h, *Csf3*, *Ccl4*, *TLR1*, and *TLR2* at 2 h (supplementary Tables IV-VI), multiple reports showed that IFN-γ synergized with LPS in *TNF-α* gene expression and production and *iNOS* gene expression in human and murine primary macrophages and RAW264.7 cells (10–13, 69, 70), and that blocking IFN-γ signaling protected mice from endotoxin shock (104–107). Although our data showed synergistic induction of *iNOS* gene expression by LPS plus IFN-γ, we did not detect any enhancing effects of IFN-γ on LPS-induced *TNF-α* expression. The discrepancy between the reported and observed effects on LPS-induced *TNF-α* by IFN-γ could be due to differences between the ligand dosages, type of cells, and other culture conditions used in our work and the conditions used in other studies.

Although we found that 2MA mainly enhanced LPS-induced gene expression, several reports showed that 2MA inhibited various LPS-induced responses. In RAW264.7 cells, 2MA inhibited GTPase activity induced by LPS plus ATP/ADP (14, 16, 108) and decreased nitrite production induced by LPS (16); in primary resident exudate cells from CD-1 mice, 2MA also attenuated NO generation and iNOS protein/mRNA expression induced by LPS plus IFN-γ (16); in vivo, 2MA reduced serum TNF-α and IL-1α levels in LPS-treated BALB/c mice (14) and iNOS protein expression in peritoneal macrophages from LPS-treated CD-1 mice (16); finally, at the whole animal level, 2MA protected C57BL/6 mice from lethal challenges of LPS (14, 15). The differences in experimental conditions, such as LPS and 2MA dosages and endpoints of cellular responses, may contribute to the inconsistency between the reported effects of 2MA and those observed in the present study.

It is worth noting that although in many instances, RAW264.7 cells show similar signaling and gene expression responses to stimulation as primary macrophages (65, 109–113), quantitative and temporal differences in some LPS responses have been reported recently between RAW cells and primary macrophages (68). It was found that compared with murine resident peritoneal macrophages, RAW cells produce higher levels of TNF-α and the peak TNF-α levels sustain for a longer period of time in response to LPS (68). Such a difference is thought, in part, to be due to the lack of feedback inhibition of TNF-α production by endogenous PGs (68), as RAW cells produce lower total amount of PGs in response to LPS than resident peritoneal macrophages, and they do not express the receptor (DP1) specific for the primary PGs they produce, PGD2 (68). Therefore, we caution to keep in mind potential differences between RAW 264.7 cells and macrophages when extrapolating our results to primary macrophages.

In all, our report characterized nonadditive responses to LPS plus IFN-γ, LPS plus 2MA, LPS plus PGE_2, and LPS plus ISO, and provided evidence suggesting that specific mechanisms, including feedback/autocrine loops, by which IFN-γ, 2MA, PGE_2, and ISO can modulate LPS-induced gene expression. It would be important to identify the variables that regulate the effect of these ligands on LPS response by characterizing the transcriptional responses induced by dual ligands in primary macrophages under different conditions, such as at different dosages and in different orders of ligand treatments. Such studies would provide insight into the mechanisms regulating LPS-induced inflammation and endotoxic shock, which can be helpful for the development of better treatment strategies.

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