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Dual Ligand Stimulation of RAW 264.7 Cells Uncovers Feedback Mechanisms That Regulate TLR-Mediated Gene Expression$^{1,2,3}$

Xiaocui Zhu,* Mi Sook Chang,* Robert C. Hsueh, † Ron Taussig, ‡ Kelly D. Smith, ‡ Melvin I. Simon,* and Sangdun Choi*§§

To characterize how signaling by TLR ligands can be modulated by non-TLR ligands, murine RAW 264.7 cells were treated with LPS, IFN-$\gamma$, 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-$\gamma$ and LPS cross-regulated the transcriptional response induced by each other: while LPS preferentially enhanced IFN-$\gamma$-induced changes in gene expression at 1 h, IFN-$\gamma$ signaling primarily attenuated LPS-induced changes at 4 h. Our data suggest specific cross-talk mechanisms: 1) LPS enhances the expression of IFN-$\gamma$ response genes by augmenting STAT1 activity and by activating NF-$\kappa$B, which synergizes with IFN-$\gamma$-induced transcriptional factors; 2) IFN-$\gamma$ 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-$\beta$ 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, hypotonic 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-$\beta$ 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 (AICS) 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-$\gamma$, 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-$\gamma$ 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$_\text{s}$-associated

$^{3}$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 GSE1191-18226, 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-$\gamma$-activated sequence; Sox1, 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). PGE2 and ISO can each bind to several receptors coupled to the heterotrimeric G protein whose activation results in cAMP generation. Previous reports showed that IFN-γ-stimulated, while 2MA, PGE2, 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, PGE2, 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), PGE2 (10 μM; Sigma-Aldrich), ISO (50 nM; Sigma-Aldrich), IFN-γ (5 μM; 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). Triplicate experiments were done for each treatment. A total of 100 μM 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 1 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 PP00000184). 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 PGE2, 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 fluorescent signal that was saturated (with Agilent “gsSatuated!” and “gsSaturated?” flags), nonuniform (with Agilent “gsFeatNonUniOL” and “rsFeatNonUniOL” flags), or below background (with Agilent “gsWellAboveBG” and “rsWellAboveBG” flags), its log2(Cy5/Cy3) value was set to NA (not available). The expression change relative to time-matched control (as log2(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 log2(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 PGE1, 1 h, which had four replicates). When examining the profiles of expression changes induced by single and dual ligands, only features with an average log2(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 log2(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, log2(treated/control) of 1 and 2 correspond to net-fold changes of 1 and 3, respectively, while log2(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 replicative 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 log2(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.

\[
\text{ligandEffect} = \frac{\text{power}(x_2)}{\text{power}(x_1)} - 1
\]

if \(x_2 > 0\), \(x_1 > 0\) if \(x_2 < 0\) if \(x_1 < 0\)

where \(x_1\) and \(x_2\) are the expression changes of a feature to ligands 1 and 2, respectively.

2. Calculate the expected additive dual ligand effect, which is the sum of the ligand effect of two single ligands, \(L_1\) and \(L_2\), and the observed effect of the dual ligand, \(L_{1+2}\).

\[
\text{Expected} = \text{ligandEffect}_{L_1} + \text{ligandEffect}_{L_2}
\]

\[
\text{Observed} = \text{ligandEffect}_{L_{1+2}}
\]

3. Calculate the average non-additivity (avg.DIF) and its signal-noise-ratio (SNR) of the dual ligand response

\[
\text{DIF} = \text{Observed} - \text{Expected}
\]

\[
\text{avg.DIF} = \frac{\text{DIF} \times \text{SNR}_{L_1} \times \text{SNR}_{L_2}}{\text{DIF} \times \text{SNR}_{L_{1+2}}}
\]

\[
\text{SNR} = \frac{\text{avg.DIF} \times \text{std.DIF}}{\text{std.DIF} = \text{standard deviation of replicate DIF measurements}}
\]

4. Identify array features with non-additive responses to \(L_{1+2}\).

\[
\text{array features with [avg.DIF] > 1 \times \text{SNR}, [SNR] > 1, and } 50\% \text{ or more expression changes to } L_1, L_2, \text{ or } L_{1+2} \text{ at } p < 0.01
\]

\[
\text{[avg.DIF] and [SNR] are the absolute value of avg.DIF and SNR, respectively}
\]
similarity metrics. The hierarchical clustering program used is implemented in the Multiple Experiment Viewer (www.tigr.org/software/mev.html).

Results

Characterization of gene expression responses induced by LPS, IFN-γ, 2MA, PGE₂, 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, PGE₂, 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. 2A, compared with 2MA, PGE₂, 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, PGE₂, or ISO began to decrease at 4 h, indicating that transcriptional responses to 2MA, PGE₂, 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. 2B). 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=RAW), 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, Irf1, IFN-β, Cxcl5, and Cxcl10 for LPS, and Icsbp1, Irf1, and Tap1 for IFN-γ (25, 26). Examples of secondary genes include Mx1, Ifi1, Ifi204, and Ifr7 for LPS, and Ghp1 and Ghp2 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, PGE₂, or ISO are masked in the dendrogram (Fig. 2B) because these responses were significantly weaker than responses to LPS or IFN-γ. Therefore, for features with significant responses to 2MA, PGE₂, or ISO, their changes were hierarchically clustered in the absence of the LPS and IFN-γ data set (Fig. 2C). The dendrogram showed that PGE₂ 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. 2C). Stimulation with 2MA, PGE₂, 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 leri3 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, Cxc2, Cxc10, and Cxc11. PGE₂ 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 PGE₂ 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. 2B). 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 PGE2 and LPS plus ISO induced similar patterns of nonadditive responses (Fig. 3A). 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 PGE2 and LPS plus ISO induced mostly attenuated responses. The observation that LPS plus PGE2 and LPS plus ISO induced similar nonadditive responses and that PGE2 and ISO alone induced similar gene expression profiles, together with the fact that both ligands can activate Gαs and induce cAMP, suggest that pathways downstream of cAMP regulate most of the transcriptional responses of the unique transcriptional targets of LPS at 4 h were LPS-dependent enhancement, while 61–71% of the nonadditive responses of unique IFN-γ responses to LPS plus IFN-γ (Fig. 3B). 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 PGE2 and LPS plus ISO, 61–71% and 65–75% of the nonadditive responses of LPS transcriptional targets were enhancement by PGE2, and ISO, respectively (Fig. 3B). 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% 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.
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 PGE₂, 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. 2B), 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. 3C). 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. 3C). 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. 3D). 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. 3B and 4B), for 34 of the 41 features (83%), their response to IFN-γ was enhanced by LPS (Figs. 3B and 4A). 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. 4A) (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-γ-induced genes, including Ifi202b, Ifi203, Ifi204, Ifi47, Lig2p, 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 AICS data (www.signaling-gateway.org/data/cgi-bin/lr.cgi?assay=western&lig1=IFG&lig2=LPS&protein=ST1A); (www.signaling-gateway.org/data/cgi-bin/lr.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

<table>
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<td>22</td>
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Table I. The percentage of features with nonadditive responses to dual ligand treatments

*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 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 only to 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.
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/~saets/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. 4A, 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 ACS phosphoprotein data that shows IFN-γ induces early (<5 min) and strong STAT1 phosphorylation (also STAT3 and STAT5), while LPS only begins to

![Image](http://www.jimmunol.org/Downloaded_from http://www.jimmunol.org)
induce STAT1 phosphorylation after 30 min (www.signaling-gateway.org/data/cgi-bin/dligscr.cgi?assay=western&lig1=FGF&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. 3B). 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 suppression of Socs1 and Cish contributes to the inhibition of the late transcriptional changes in response to LPS plus IFN-γ.

PGE2 induced in response to LPS plus IFN-γ can also contribute to the attenuation of transcriptional response at 4 h. Exogenously added PGE2 inhibits LPS-induced gene expression and production of cytokines, including TNF-α (18, 58–60). PGE2 is also induced after 2–4 h of LPS stimulation in macrophages (61–66), and endogenous PGE2 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 PGE2 produced during the first 2 h of LPS stimulation as a result of the slow induction of Ptgss2, the gene encoding COX-2, the inducible PG synthase critical for PGE2 production in response to inflammatory stimuli (62, 65, 66). In our experiment, LPS plus IFN-γ increased Ptgss2 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 PGE2 receptor coupled to the G protein and mediates the early inhibition of cytokine production/release by PGE2 (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 PGE2 production and signaling and the subsequent inhibition of LPS response at 4 h via the PGE2/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 Id2 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 PGE2.

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. 3B). 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. 5A),
greater-than-additive induction in response to LPS plus 2MA at 4 h, including Bcl2a1d, C3, C3d2, IL-1α, IL-1β, IL-6, IL-18, Pim1, and Socs1 (80) (Fig. 4D). 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. 3B). 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. NF-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/lrl.cgi?assay=cytokine&ligl=1&ISO&lig2=LPS&cytokine=IL-10). Correlating with the increase of IL-10 production, genes whose LPS-induced expression was reported to be 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, Gadd45a, Ire2, Myd116, Dusp1, and Dusp2 at 1 or 2 h, and a number of cytokine genes such as Ccl4, Ccl9, Csf2, and LTBβ, at 1, 2, or 4 h (supplementary Tables IV, V, and IX).

LPS plus PGE2 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 PGE2, 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, PGE2, 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) PGE2 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 Ptg2 and Ptgdr, 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. 3B). 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. 3B). 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/lignscr.cgi?assay=c
calium&lig=2MA&cellabbr=RAW) 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-β 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 endothotoxic shock, which can be helpful for the development of better treatment strategies.

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Disclosures
The authors have no financial conflict of interest.

References


Supplemental Material and Methods

Quantitative real-time PCR (QRT_PCR)

1 \( \mu \)g total RNA from two of the triplicate RNA samples used for microarray experiments was treated with DNase I (Invitrogen) to remove contaminating genomic DNA. First-strand cDNA was synthesized with SuperScript II (Invitorgen) and random hexamer priming (Invitrogen). Quantitative real-time PCR was performed by using i-Cycler (Bio-Rad) and SYBR green detection. Reactions were performed in 25 \( \mu \)l in triplicate wells in 96-well plates with the following ingredients: 5’ and 3’ primers (200 nM each), iQ\textsuperscript{TM} SYBR Green Supermix (Bio-Rad), and cDNA corresponding to 40 ng of total RNA. Mouse \( \beta \)-actin was amplified in separate reactions in the same plate to be used as an internal control for variances in the amount of cDNA in PCR reactions. See Supplementary Table X for primer sequences and amplicon lengths. PCR cycle setup was as the following: 95 Cº for 1 min, 95 Cº for 3 min, 40x (95 Cº for 30 sec, 60 Cº for 10 sec, 72 Cº for 10 sec). The Pfaffl’s equation (1) was used to calculate the expression ratio (R) of ligand-treated versus control B cells. Briefly,

\[
R = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target (control-treated)}}}}{(E_{\beta\text{-actin}})^{\Delta \text{CP}_{\beta\text{-actin (control-treated)}}}}
\]

Where \( E = 10^{(-1/\text{slope})} \), with the slope being the slope of the standard curve of the target genes or \( \beta \)-actin; \( \Delta \text{CP}_{\text{target (control-treated)}} \) is the average cross point cycle number of the control minus that of the treated sample for the target gene; \( \Delta \text{CP}_{\beta\text{-actin (control-treated)}} \) is the average cross point cycle number of control minus the treated sample for \( \beta \)-actin.
Ligand treatment and RNA preparation

1. Suspend RAW 264.7 cells to $3.3 \times 10^5$ cells/ml in RAWGM1 (AfCS solution protocol PS00000510) and distribute 3 ml into individual wells of 6-well tissue culture plates as needed.

2. Incubate at 37 °C in air with 5% CO$_2$ overnight.

3. Next day aspirate the medium, and add 2.7mls of warm equilibrated RAWGM1.

4. Place at 37 °C in air with 5% CO$_2$ for 1 hour.

5. Prepare ligands as needed.

6. Add 300ul of 10X ligands to the appropriate well, and swirl to mix. Incubate at 37 °C in air with 5% CO$_2$ for desired duration

7. Aspirate the medium.

8. Add 3ml of ice-cold TriPure (Roche) to each of the wells and pipet up and down with 5-ml sterile glass pipettes to lyse the cells.

9. Transfer the lysates with 5-ml sterile glass pipettes to the corresponding 13-ml barcoded polypropylene tube.

10. Vortex the tubes.

11. Place the samples in a –80 °C freezer and store at –80 °C until ready for isolation of RNA. Otherwise, proceed immediately to step 12.

12. Remove the samples from –80 °C freezer and thaw at room temperature.

13. Add 0.6 ml of chloroform (20% of TriPure volume) immediately after the samples are thawed.

14. Cap the tubes tightly and shake for 30 sec.
15. Incubate the mixtures at room temperature for 5 min.

16. Loosen the cap and centrifuge the samples at 4 °C for 15 min at 12,000 x g (e.g., 9,500 rpm in Beckman JA-17 rotor).

17. Transfer the colorless upper aqueous phase (about 1.8 ml) of each sample to a new 13-ml barcoded polypropylene tube.

18. Repeat the extraction by adding 2 ml of chloroform to the transferred, separated aqueous phase of each sample.

19. Cap the sample tubes tightly and shake for 30 sec.

20. Loosen the cap and centrifuge the samples at 4 °C for 15 min at 12,000 x g.

21. Transfer the colorless upper aqueous phase to a new 13-ml barcoded polypropylene tube.

22. Add 1.5 ml of isopropanol to each of the final, isolated aqueous phases (step 23), cap tightly, and mix by inverting.

23. Incubate at room temperature for 5 min.

24. Centrifuge the samples at 4 °C for 10 min at 12,000 x g.

25. Remove most of the supernatant gently, but leave about 0.5 ml containing the pellet of RNA.

26. Vortex briefly to disperse the pellet and transfer to a new barcoded Eppendorf tube.

27. Centrifuge for 5 min at 4 °C in a microfuge (Microfuge®18 Beckman Coulter) at 18,000 x g.

28. Remove the supernatant carefully without disturbing (losing) the pellet of RNA.

29. Add 1 ml of room temperature RNase-free 70% ethanol.
30. Vortex briefly to loosen the pellet and centrifuge at 18,000 x g in the microfuge for 5 min at 4 °C.

31. Remove the supernatant carefully and air-dry the sample for 30 min.

32. Add 10 µl of nuclease-free water to each sample.

33. Incubate the sample on ice for 20 min, vortexing at 5 min intervals.

34. Determine the concentration of total RNA by diluting 1 µl of the sample in 79 µl of Tris-EDTA buffer and reading the absorbance at 260 nm in a spectrophotometer.

35. Determine the integrity of total RNA by electrophoresis of 1 µg of total RNA on a 1.0% agarose gel and staining with ethidium bromide for 18S and 28S ribosomal RNA.

36. Place samples in a storage box and store at –80 °C prior to shipment until microarray analysis.
Supplemental Table Legends

Table I. The annotation table of the customized Agilent Oligo Arrays.

Table II. The list of features with 2-fold or more expression changes at p<0.01 in response to LPS treatment for 1 h, 2 h, 4 h, 8 h, 16 h and 48 h. A) Differential calls, with 0, 1 and -1 corresponding to no significant change, up- and down- regulation, respectively. B) Expression changes as average log₂-(treated/control)

Table III. The list of features with 2-fold or more expression changes at p<0.01 in response to LPS, IFN-γ, 2MA, PGE2, and ISO individually or as ligand pairs with LPS. A) Differential calls, with 0, 1 and -1 corresponding to no significant change, up- and down- regulation, respectively. B) Expression changes as average log₂-(treated/control) at 1 h, 2 h, 4 h.

Table IV. The list of features with 50% or more expression changes at p<0.01 in response to LPS, IFN-γ, 2MA, PGE2, and ISO individually or as ligand pairs with LPS. A) Differential calls, with 0, 1 and -1 corresponding to no significant change, up- and down-regulation, respectively. B) Expression changes as average log₂-(treated/control) at 1 h, 2 h, and 4 h.

Table V. The list of features with above-threshold non-additive responses. Features with significant non-additive responses to at least one dual ligand at one time points were included. A) Calls for significant non-additivity, with 0, 1 and -1 corresponding to
additive, greater-than- and less-than-additive, respectively. B) The non-additivity of dual ligand responses of each feature as the avg. DIF calculated according to Figure 1 in the paper.

Table VI. The comparison of single ligand responses and non-additive responses of features with above-threshold non-additive-responses to LPS plus IFN-γ at A) 1 h, B) 2 h; C) 4 h. Column FeatureNum, ProbeName, NCBI GENEID, SYMBOL, , and NAME correspond to the Feature number, probe name, locus link ID, gene symbol, and gene name of the array features, respectively; In columns NOADDITIVE&DE.in.BOTH, LESS&DE.in.BOTH, GREATER&DE.in.BOTH, 1 identifies the array features that had 50% or more expression changes at p < 0.01 to LPS alone and IFN-γ alone, and showed non-additive responses, less-than-additive responses, and greater-than-additive responses to LPS plus IFN-γ, respectively; In columns NOADDITIVE&DE.in.LPS, LESS&DE.in.LPS, and GREATER&DE.in.LPS, 1 identifies the array features that showed non-additive responses, less-than-additive responses, and greater-than-additive responses to LPS plus IFN-γ, respectively, and had 50% or more expression changes at p < 0.01 to LPS alone; In columns NOADDITIVE&DE.in.LIG2, LESS&DE.in.LIG2, GREATER&DE.in.LIG2, 1 identifies the array features that showed non-additive responses, less-than-additive responses, and greater-than-additive responses to LPS plus IFN-γ, respectively, and had 50% or more expression changes at p < 0.01 to IFN-γ alone; In columns NOADDITIVE&DE.in.NEITHER, LESS&DE.in.NEITHER, GREATER&DE.in.NEITHER, 1 identifies the array features that showed non-additive responses, less-than-additive responses, and greater-than-additive responses to LPS plus
IFN-γ, respectively, and had 50% or more expression changes at p < 0.01 to neither LPS alone nor IFN-γ alone.

Table VII. The comparison of single ligand responses and non-additive responses of features with above-threshold non-additive-responses to LPS plus 2MA at A) 1 h, B) 2 h; C) 4 h. Information listed in different columns are similar to those in Table VI.

Table VIII. The comparison of single ligand responses and non-additive responses of features with above-threshold non-additive-responses to LPS plus PGE2 at A) 1 h, B) 2 h; C) 4 h. Information listed in different columns are similar to those in Table VI.

Table IX. The comparison of single ligand responses and non-additive responses of features with above-threshold non-additive-responses to LPS plus ISO at A) 1 h, B) 2 h; C) 4 h. Information listed in different columns are similar to those in Table VI.

Table X. The primers and amplicon length of genes whose transcriptional response to single and dual ligand treatments were determined using QRT-PCR.

Reference