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Global changes in gene expression and synergistic interactions induced by TLR9 and TLR3

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Abstract

The innate immune system is triggered when pathogen associated molecular patterns (PAMPs) expressed by infectious microorganisms interact with toll-like receptors (TLR) present on immune cells. Individual TLRs signal through distinct molecular pathways. For example, TLR9 interacts with unmethylated CpG motifs expressed by bacterial DNA and triggers via a MyD88 dependent pathway whereas TLR3 recognizes viral RNA through a MyD88-independent pathway. Bioinformatic analysis of microarray data was used to identify the regulatory patterns underlying changes in gene expression induced when RAW 264.7 macrophages were stimulated via TLR9 by CpG oligonucleotides (ODN) and/or via TLR3 by poly (I:C). While the genes activated by each ligand mediated similar functions, poly (I:C) elicited a larger and more diverse change in gene expression. Co-stimulation with both ligands accelerated gene expression and synergistically activated genes primarily associated with immune function. This is the first work to compare global changes in gene regulation triggered by distinct TLR pathways and clarify their impact on gene expression.

Keywords

gene regulation; CpG oligodeoxynucleotides; poly (I:C); TLR; synergy; molecular biology

1. Introduction

The innate immune system is triggered by the recognition of pathogen-associated molecular patterns expressed by a variety of infectious microorganisms. Members of the toll-like receptor family contribute to the recognition of these PAMPs. For example, TLR9 is stimulated by the unmethylated CpG motifs present at high frequency in bacterial DNA (an effect mimicked by synthetic ODN expressing identical CpG motifs) (Hemmi *et al.*, 2000; Klinman *et al.*, 1996; Krieg *et al.*, 1995) while TLR3 recognizes double-stranded viral RNA (an effect mimicked by poly (I:C)) (Alexopoulou *et al.*, 2001; Kulka *et al.*, 2004; Matsumoto *et al.*, 2002; Yamamoto *et al.*, 2003). Microarrays provide a powerful tool for analyzing changes in gene expression induced when the innate immune system is activated. Previous work from this laboratory examined the network of gene interactions induced when CpG ODN were used to stimulate murine spleen cells. Results showed that a small group of regulatory genes mediated global

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changes in gene expression over time (Klaschik *et al.*, 2007). The current work extends those findings by comparing the effects of poly (I:C) vs CpG ODN on gene regulation in a murine macrophage cell line and delineating the synergy resulting from the co-administration of both ligands.

In addition to responding to distinct ligands, individual TLRs use different adaptor proteins. For example, TLR9 signals via MyD88 whereas TLR3 signals via TRIF (Boonstra *et al.*, 2006; Hoebe *et al.*, 2003; Honda *et al.*, 2005; Latz *et al.*, 2004). Individual pathogens frequently express multiple PAMPs and thus can trigger the innate immune system via multiple discrete TLR pathways, potentially culminating in a synergistically enhanced immune response (Gautier *et al.*, 2005; Napolitani *et al.*, 2005; Trinchieri and Sher, 2007; Zhu *et al.*, 2008). Consistent with such a possibility, previous studies showed that the expression of IL6, IL12B, TNF and NOS2 were synergistically enhanced by co-stimulation with CpG DNA plus poly (I:C) (He *et al.*, 2007; Whitmore *et al.*, 2004; Zheng *et al.*, 2008). However, neither the full impact on gene up-regulation nor basis of this synergy is known (Whitmore *et al.*, 2007). The current work examines genome-wide changes in gene expression induced by treating RAW264.7 macrophages with CpG ODN and/or poly(I:C). Results provide novel insights into the activation patterns and regulatory gene networks induced with both ligands and identifies that subset of genes whose expression is synergistically enhanced.

2. Material and methods

2.1 Reagents and cells

Unmethylated CpG-containing phosphorothioate oligonucleotides 1555 (5'-GCTAGACGTTAGCGT-3') and 1466 (5'-TCAACGTTTGA-3') were synthesized at the CBER core facility (CBER/FDA, Bethesda, MD) and used as an equimolar mixture (Takeshita and Klinman, 2000). Polyinosine-polycytidylic acid (poly I:C) was purchased from InvivoGen (San Diego, CA). The mouse RAW 264.7 macrophage-like cell line was obtained from ATCC (catalogue number TIB-71, Manassas, VA).

2.2 ELISA assay and Intracellular staining

Bone marrow derived dendritic cells (BMDC) were produced by culturing BALB/c BM in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 ng/ml GM-CSF for 6-7 days at 37° in a 5% CO₂ in air incubator. BM-derived macrophages were produced by culturing BALB/c BM in DMEM supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate and 50 µg/ml M-CSF (R&D, Minneapolis, MN) for 5 days.

Cultured DC and macrophages were washed and stimulated *in vitro* for 20 h with CpG ODN and/or poly(I:C). Following surface staining of CD11c and MHC class II cells were fixed and permeabilized with Cytotfix/Cytoperm (BD Biosciences, San Jose, CA) and then incubated with antibodies against IL-12p70/40 (BD Biosciences, San Jose, CA). Sample data were acquired on a FACSCalibur or LSR II (BD, Sunnyvale, CA) and analyzed with FlowJo software (TreeStar Inc, Ashland, OR). IL12, IL-6, and IL-10 protein levels were quantified by ELISA as described previously (Verthelyi *et al.*, 2001).

2.3 Stimulation of cells for microarray analysis

RAW 264.7 cells were cultured for 5 days in DMEM supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 1mM sodium pyruvate at 37°C in a 5% CO₂ in air incubator. Cells were washed, transferred into new flasks, and rested for 16 h before being stimulated with 3.2 µg/ml CpG ODN and/or 32 µg/ml poly(I:C) for 4 - 12 h. These ligand concentrations were identified in preliminary dose-ranging experiments as representing the

minimal concentration of each ligand capable of inducing optimal RAW cell stimulation (data not shown). All experiments were independently repeated three times, and data from all experiments combined for analysis.

2.4 Microarray studies

Total RNA was extracted from resting and stimulated RAW 264.7 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) as specified by the manufacturer. The RNA was quantified using an ND-1000 (NanoDrop Technologies, Wilmington, DE) and qualified for the absence of degradation by electrophoresis through a 1% agarose denaturing gel. Twenty micrograms of total RNA was reverse-transcribed using 3 ul 10× first strand buffer (Stratagene, La Jolla, CA), 2 ul (1 ug) oligo(dT)₁₂₋₁₈ (Invitron, Carlsbad, CA), 3 ul (150 U) of AffinityScript Reverse Transcriptase, 2 ul 20× aminoallyl-dUTP/dNTP mix, and 3 ul 0.1 M DTT in a final volume of 30 ul at 42° C for 1 h as previously described (Klaschik *et al.*, 2007). In all studies, a reference murine RNA (Stratagene, La Jolla, CA) sample was processed in parallel. Both cDNA's were purified using a MinElute PCR Purification Kit (Qiagen Sciences, Valencia, CA) and labeled with Cy5 (sample cDNA) or Cy3 (universal reference cDNA) as described (Klaschik *et al.*, 2007). The probes were mixed, diluted in 5 ul DMSO plus 1.7 ul of 1 M NaHCO₃ and hybridized to 36K mouse 60-mer oligonucleotide array slides (NCI Mouse Array Set Mm-MEEBO-v1.3) at 42°C for 18 h in a MAUI hybridization system (BioMicro Systems, Salt Lake City, UT) followed by washing, centrifugation and air drying.

2.5 Analysis of gene expression

Arrays were scanned using a GenePix 4000B Scanner (Axon Instruments, Union City, CA), and analyzed using the GenePix Pro 6.0 Software Tool (Axon Instruments/Molecular Devices, Union City, CA) using GAL files provided by the manufacturer at <http://nciarray.nci.nih.gov>. Data were uploaded to CIT/BIMAS-NCI/CCR Microarray Database (mAdb; <http://nciarray.nci.nih.gov>) and formatted via export function to BRB Array Tools. Gene expression analysis was performed using BRB Array Tools Version 3.6.0 developed by the NCI Biometric Research Branch (Bethesda, MD). Expression ratios were log base 2 transformed, lowess normalization was used to adjust for differences in labeling intensities and analyses restricted to genes present in >50% of the arrays. Based on this filtering, 30,703 features could be analyzed.

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc) was used to identify the regulatory interactions and functional characteristics of all significantly up-regulated genes. IPA maps each gene using a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Gene networks were generated algorithmically based on their connectivity in terms of expression, activation, transcription, and/or inhibition. A 'network' in IPA is defined as a graphical representation of the molecular relationships between genes. Genes are represented as nodes, and the biological relationship between nodes is shown by a connecting line. All connections are supported by published data stored in the Ingenuity Pathways Knowledge Base or PubMed. IPA ranks all genes based on their connectivity, using a generalization of the concept of node degree, which measures the number of single genes to which a gene is connected. (see

<https://analysis.ingenuity.com/pa/info/help/>

Ingenuity_Network_Algorithm_Whitepaper_FINAL(2).pdf; and (Calvano *et al.*, 2005) for details). Genes identified by IPA as influencing the expression of >10% of the genes up-regulated at any given time point were defined as "regulators".

Microarray data were deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) and are accessible through GEO series number GSE15416.

2.6 Quantitative PCR

Total RNA was extracted and first-strand cDNA synthesized as described above for microarray studies. Quantitative-PCR (Q-PCR) analysis was performed using TaqMan probes (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions in 384-well microtiter plates (Applied Biosystems, Foster City, CA) in a final volume of 10 μ l. Specific primers for Q-PCR of GAPDH and IL33 genes were purchased from Applied Biosystems (Foster City, CA). Thermocycling conditions using an Applied Biosystems ABI-7900 SDS were as follows; 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. mRNA levels were quantified by TaqMan PCR. Relative mRNA levels were then determined using the comparative cycle threshold method. This approach calculates the amount of target mRNA by normalization to an endogenous reference gene (GAPDH). Microarray studies showed that GAPDH mRNA levels did not differ significantly between stimulated vs unstimulated cells (data not shown). mRNA levels in stimulated cells were then compared to those of non-stimulated cells.

2.7 Statistical Analysis

The gene expression profile of treatment and control groups was compared using a random variance t-test. The random-variance t-test permits sharing information among genes about within-class variation without assuming that all genes have the same variance (Wright and Simon, 2003). Genes were considered significant if their p value was <0.000001 . Differences between groups were measured using Student's t test.

3. Results

3.1 Effect on gene expression of treating of RAW 264.7 macrophages with CpG ODN versus poly (I:C)

RAW 264.7 macrophages were stimulated with CpG ODN and/or poly (I:C). mRNA was isolated from these cells after 4 - 12 h of culture, converted in Cy5-labeled cDNA, and hybridized to murine microarrays in the presence of a Cy3-labeled cDNA reference standard (facilitating inter-sample comparison). Reproducible patterns of gene up-regulation were observed in similarly treated cells from three independent experiments ($R^2 = 0.941 \pm 0.02$).

Data from all experiments were combined, and genes whose level of expression increased significantly ($p < 10^{-6}$) identified. RAW 264.7 cells stimulated with CpG ODN activated 190 genes at 4 h and 105 genes at 12 h (Table I). Poly (I:C) treatment led to the stimulation of 574 genes at 4 h and 1,051 at 12 h. Thus, the number of genes triggered by poly (I:C) significantly exceeded the number stimulated by CpG ODN at both time points ($p < .01$, Table I).

Additional analysis focused on those genes most strongly stimulated at each time point by each treatment. Whereas 97% of the genes most strongly activated by CpG ODN were also stimulated by poly (I:C), only 66% of the genes most strongly activated by poly (I:C) were triggered by CpG ODN (Table II). The magnitude by which the most strongly up-regulated genes was activated also differed significantly between groups, with poly (I:C) triggering a 37.2 ± 3.1 fold increase in mRNA level vs only 15.4 ± 2.8 fold in the CpG ODN treated group ($p < .001$). These findings are consistent with the observation that poly (I:C) induced broader gene up-regulation than CpG ODN.

3.2 Comparison of the regulatory networks triggered by CpG ODN versus poly (I:C)

Ingenuity Pathway Analysis (IPA) was used to identify the regulatory pathways underlying the patterned changes in gene expression induced by CpG ODN and/or poly (I:C) stimulation. Results show that TNF was the dominant regulator of gene expression at both 4 and 12 h in RAW 264.7 cultures stimulated with CpG ODN (Table III). IFN γ and IL1 β were also important

regulators of CpG-driven macrophage activation, as together with TNF they impacted the expression of $\approx 95\%$ of all genes at both time points (Table III). Several other genes, such as IL1A and CSF2, had detectable but limited effects on the CpG-induced response of these cells (Table III).

The pattern of gene regulation observed following poly (I:C) stimulation of RAW 264.7 cells differed from that induced by CpG ODN. Poly (I:C) significantly increased mRNA levels for many type I interferons, an effect that was absent from CpG ODN stimulated cultures. TNF and IFNG were the strongest regulators of poly (I:C) induced gene activation (Table IV), together accounting for 84% of the up-regulated network. Regulators other than IFNG and TNF uniquely accounted for the stimulation of $\approx 10\%$ of the poly (I:C) induced network (data not shown). Of interest, nearly half of the genes strongly stimulated by poly (I:C) but not CpG ODN were controlled by IFN β 1, suggesting that this gene has a greater impact on the breadth and magnitude of the poly (I:C) response than other minor regulators (Table II and data not shown). The fold rise in mRNA level of genes stimulated by multiple regulators significantly exceeded that of genes triggered by a single regulator ($p = .033$), suggesting that these regulatory networks interacted synergistically. TNF continued to dominate poly (I:C) driven gene regulation at 12 h whereas the role of IFNG declined (impacting only 41% of genes at 12 h vs 58% at 4 h, Table IV).

IPA was then used to examine the function of the genes up-regulated by CpG ODN and poly (I:C). Despite differences in the regulatory patterns noted above, both ligands activated genes in similar functional categories, specifically immune response, immune system development and cell death (Table V).

3.3 Co-stimulation of TLR9 and TLR3 synergistically increases cytokine production

Bone marrow derived macrophages and dendritic cells (BMDC) express both TLR9 and TLR3, and thus can be stimulated by both CpG ODN and poly (I:C). Preliminary experiments were conducted to confirm previous reports showing that co-stimulation by these two ligands synergistically enhanced immune activation. These cells were cultured *in vitro* with CpG ODN and/or poly (I:C) for 20 h and analyzed for the production of IL-12, IL-6, and/or IL-10. As seen in Fig 1, the production of all three cytokines was synergistically enhanced ($p < 0.01$).

3.4 Effect of CpG ODN plus poly (I:C) on gene activation

RAW cells stimulated simultaneously with CpG ODN plus poly (I:C) up-regulated significantly more genes than cells treated with either ligand alone at 4 h ($p < 0.001$, Table I). The gene networks activated by the ligand combination included elements of the regulatory networks triggered by each ligand independently, and mirrored the extent of that activation (Table III). Of interest, IL-6 was identified as contributing to the regulation of gene expression triggered by the combination of CpG ODN plus poly (I:C) at 4 h, yet this regulator was not induced by either CpG ODN or poly (I:C) alone at that time point. IL-6 was up-regulated by poly (I:C) at 12 h, suggesting that the synergy observed when RAW cells were co-stimulated via TLR3 plus TLR9 reflects an acceleration of the gene expression profile induced by each ligand independently.

Co-stimulation with CpG ODN plus poly (I:C) also resulted in a significant increase in the magnitude of gene activation. Restricting the analysis to those genes up-regulated by the combination of CpG plus poly (I:C) at 4 h, synergistic stimulation increased mRNA level by 5.9-fold on average versus only 4.2- and 2.7-fold for the same set of genes when stimulated by poly (I:C) or CpG ODN alone, respectively ($p < 0.001$). Having found in Table III that mRNA levels increased significantly when genes stimulated by multiple regulators ($p = 0.003$), this outcome may reflect the contribution of IL-6 to the other regulators activated by each ligand.

At 12 h, there were fewer genes up-regulated in cultures stimulated with the combination of CpG ODN plus poly (I:C) than with poly (I:C) alone. This is consistent with the decrease in gene activation mediated by CpG ODN alone at the same time point.

3.5 Identification of genes synergistically up-regulated by CpG ODN plus poly (I:C)

Evidence that the combination of CpG ODN plus poly (I:C) synergistically enhanced the secretion of certain cytokines led us to examine whether this ligand combination had broader synergistic effects on mRNA expression. To insure that inter-experimental variability in mRNA levels was not misinterpreted as evidence of synergy, only those genes whose level of expression when treated with CpG ODN plus poly (I:C) exceeded the mean + 3 SD of the sum induced by each ligand individually were included in this analysis. By this criterion, 60 genes were identified as being synergistically up-regulated by CpG ODN plus poly (I:C). IPA analysis of these genes showed that 80% fell into the “immune response” category and 22 encoded proteins of immunological interest (cytokines or chemokines, Table VI).

At 4 h, the synergistically up-regulated genes included those encoding IL-1A, CSF3, PTGS2, several type I IFN's and the chemokine CCL17. At 12 h, the synergistically up-regulated group included the interleukin genes IL33, IL12A, IL12B, IL10, IL19, and IL1F6, the adipokine genes LCN2 and SAA3, the neuropeptide-encoding genes NPY and NTS, and the chemokine genes CCL7 and S100A8. IL6 and TSLP were the only 2 cytokines/chemokines synergistically up-regulated at both 4 and 12 h. Of interest, neither CpG ODN nor poly (I:C) alone effected IL33, IL19, IL12A, S100A8, DUSP1, TIMP1, SCN9A, CCL17, HAMP, MARCKS, PFKFB3, CSN1S1, VAV2 or NPY expression, yet all of these genes were significantly up-regulated by this ligand combination.

RT-PCR was performed to confirm that the increases in cytokine mRNA levels identified by microarray were valid. As seen in Fig 2, the concentration of IL-33 mRNA increased 60 fold after 12 h of stimulation with CpG ODN plus poly (I:C), consistent with the 46 fold increase predicted by the microarray data (Fig 2 vs Table VI). This is the first evidence of a synergistic increase in IL-33 after dual TLR stimulation.

4. Discussion

Microarrays facilitate the evaluation of global changes in gene expression induced by immune stimuli (Huang *et al.*, 2006; Jenner and Young, 2005; Klaschik *et al.*, 2008; Klaschik *et al.*, 2007). By combining microarray with IPA analysis, this work examined the effect of stimulating the RAW 264.7 macrophage cell line via TLR9 and/or TLR3. Results indicate that the pattern of signaling triggered via these distinct TLR receptors are broadly similar but include reproducible differences. In general, poly (I:C) stimulation via TLR3 activated a larger and broader regulatory network than CpG stimulation of TLR9. Gene activation was accelerated when poly (I:C) was co-administered with CpG ODN, and triggered the synergistic up-regulation of many immune-related genes including a subset that was not activated by either ligand alone.

Simultaneous exposure to TLR9 and TLR3 ligands is likely to occur under physiologic conditions. For example, an infected host would recognize the CpG motifs expressed by adenovirus, herpesvirus and other DNA viruses via TLR9 while the double-stranded stem-loop RNA structures produced during the replication of the same viruses would be recognized via TLR3 (Jenner and Young, 2005; Sorensen *et al.*, 2008; Tabeta *et al.*, 2004; Wells *et al.*, 2008).

RAW 264.7 cells were selected for this study because they respond to stimulation via both TLR3 and TLR9 and can be expanded to yield a large and uniform macrophage population.

The genes triggered by key regulators following CpG-driven activation of RAW cells in the current study represented a consistent subset of those detected in an earlier study of CpG-stimulated whole spleen cells (Klaschik *et al.*, 2007). In both cases TNF, IFNG, and IL1B contributed to global changes in gene expression. These observations suggest that i) data generated from the study of RAW cells appears to be reliable and ii) while macrophages comprise only a small fraction of the mixed cell population present in whole spleen (Johansson *et al.*, 2006) the regulatory elements that dominate CpG mediated signaling are conserved among cell types. However, several “minor regulators” were induced by CpG ODN treatment of whole spleen that were not present after the stimulation of RAW cells, including NFKB1, IL6, IL12B, MYC, and STAT1. As mouse spleen contains multiple cell types capable of responding to CpG stimulation, these differences may reflect cell-specific patterns of gene activation, the impact of cell-cell interactions on global mRNA expression, or underlying differences in the behavior of RAW cells vs resting macrophages. Ongoing studies of a B cell line are consistent with the conclusion that individual phenotypes contribute to the overall pattern of CpG-induced gene regulation.

Poly (I:C) treatment resulted in the up-regulation of nearly 3-fold more genes at 4 h and 10-fold more at 12 h than did CpG ODN. Several notable differences in the pattern of gene activation induced by these distinct TLR ligands were identified. The CpG-mediated response was primarily regulated by TNF, IFNG and IL1B. IL1B played a lesser role in the regulation of the poly (I:C) dependent response while a number of other inducers (including IFNB1, IFNA2, IL15, IL18, and IL3) played more important roles. The poly (I:C) response was more complex and persistent than that elicited by CpG ODN. At 12 h, the number of genes up-regulated by CpG ODN fell by nearly half whereas the number of poly (I:C) stimulated genes doubled over the same period. The temporal pattern of gene regulation elicited by CpG treatment of RAW cells was entirely consistent with that observed in studies of whole spleen cells *in vivo* (Klaschik *et al.*, 2008). Analysis of those genes most highly up-regulated by each treatment (Table II) also showed that components of the poly (I:C) induced response were not activated by CpG ODN. This effect could be largely attributed to the impact of IFNB1 on the breadth and magnitude of the poly (I:C) response. Despite these differences, both CpG ODN and poly (I:C) triggered genes within the same broad functional groups: immune response, immune system development and cell death (Table V), consistent with both contributing to the induction of an innate immune response.

Co-administering poly (I:C) with CpG ODN led to the activation of more genes than either ligand alone at 4 h. The regulatory networks triggered when RAW cells were treated with CpG ODN plus poly (I:C) primarily reflected the combined effect of each ligand individually. Thus, IFNB1, IL18, IFNA2 and IL3 were stimulated by poly (I:C) but not CpG ODN, CSF2 was stimulated by CpG ODN but not poly (I:C), yet all were stimulated by combination treatment. IL6 was unique in being activated by combination treatment at 4 h but neither ligand alone until 12 h. In addition, the magnitude of activation (fold increase in mRNA level) of those genes stimulated by each ligand separately was significantly lower than the same genes stimulated by the combination of CpG ODN plus poly (I:C). These findings suggest that exposure to both ligands magnified and accelerated the response elicited by each individual ligand.

Previous studies show that certain PAMP combinations synergistically enhance cytokine secretion ((Napolitani *et al.*, 2005; Zhu *et al.*, 2008) and Fig 1). New insight into the extent of this synergy was gained by monitoring global changes in gene expression by microarray. We found that the expression level of 60 genes was significantly elevated following treatment with CpG ODN plus poly (I:C) vs either ligand alone. Studies of protein production by ELISA and mRNA levels by Q-PCR confirmed the synergistic activation of members of this group (including IL12a, IL12b, IL6, IL10 and IL-33, Figs 1, 2 and Table VI (Napolitani *et al.*,

2005; Zhu *et al.*, 2008). IPA analysis showed that 87% of the synergistically activated genes were immune related. Indeed, over one-third of all synergistically up-regulated genes, including the most highly up-regulated, were cytokines or chemokines (Table VI). These findings support the hypothesis that stimulation via multiple PAMPs synergistically broadens and increases the magnitude of the host's protective immune response.

The mechanism(s) underlying the synergistic up-regulation of immune genes by PAMP combinations is of interest. Napolitani *et al.* concluded that TLR synergy occurred primarily at late time points and involved only a small number of genes (Napolitani *et al.*, 2005). They hypothesized that synergy resulted from a prolongation of signaling due to dual TLR engagement. Our results suggest that synergy is instead characterized by the early and persistent activation of one or more regulatory pathways, resulting in a prolonged signaling cascade that increases, magnifies, and diversifies gene expression. Our work establishes that the pattern of gene regulation triggered by the interaction of CpG ODN with TLR9 and poly (I:C) with TLR3 are distinct despite sharing several features in common. The level of gene activation triggered by poly (I:C) exceeds that induced by CpG ODN. The resulting expansion in gene activation reflects both the acceleration and prolongation of gene up-regulation, processes that are initiated shortly after ligand administration.

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Abbreviations

ODN	oligodeoxynucleotide
BM	bone marrow
TLR	toll-like receptor
PAMP	pathogen associated molecular pattern
DC	dendritic cell
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony stimulating factor
M-CSF	macrophage colony stimulating factor

Figure 1.

Cytokine production by cells treated with TLR ligands.

Cells were cultured *in vitro* for 20 h with 3.2 ug/ml of CpG ODN and/or 32 ug/ml of poly (I:C).

A,B) The percent of BMDCs containing IL-12 was determined by intracytoplasmic staining and confirmed by monitoring secreted IL-12. C,D) The amount of IL-6 (C) or IL-10 (D) present in culture supernatants from BM derived macrophages was examined.

*, p < .01.

Figure 2.

Synergistic increase in IL-33 mRNA following CpG +poly(I:C) stimulation.

RAW264.7 cells were stimulated with CpG plus poly (I:C) for 12 h as described in Fig 1 and IL-33 mRNA level determined by RT-PCR.

Table I

Number of genes significantly up-regulated following TLR stimulation.

TLR Ligand	Timepoint	
	4 h	12 h
CpG	190 ^{**}	105 ^{**}
Poly (I:C)	574	1051
CpG+ Poly (I:C)	773 ^{**}	943 ^{**}

RAW 264.1 cells were stimulated with 3.2 ug/ml of CpG ODN and/or 32 ug/ml of poly (I:C) in 3 independent experiments. The number of genes significantly up-regulated after 4 and 12 h is shown ($p < 0.000001$).

^{**} significantly different from the number of genes up-regulated by poly (I:C) alone at the same time point, $p < 0.001$.

Table II

Highly up-regulated genes

Gene Symbol	CpG ODN		Poly (I:C)	
	4 h	12 h	4 h	12 h
Ccl2	-----	7.70	5.41 [^]	12.10 [^]
Ccl3	4.35 [^]	5.61	7.58 [^]	-----
Ccl4	16.44	12.09	12.29 [^]	12.96 [^]
Cd69	7.04	3.45 [^]	10.31 [^]	11.98 [^]
Cpm	-----	4.04 [^]	31.16	53.81
Csf2	27.07	11.61	-----	14.86 [^]
Csf3	7.60	12.12	-----	5.06 [^]
Cxcl2	33.82	22.57	9.12 [^]	38.46
Edn1	12.08	-----	4.66 [^]	35.66
Fas	8.17	-----	6.96 [^]	13.50 [^]
Gadd45b	9.70	5.29 [^]	3.71 [^]	8.60 [^]
Gch1	4.82 [^]	5.85	4.42 [^]	15.28 [^]
Glr3	6.13 [^]	5.70	3.89 [^]	16.28 [^]
Gp49a	5.92 [^]	7.74	5.26 [^]	16.48 [^]
Ier3	6.57	6.61	2.34 [^]	11.77 [^]
Ifi202b	6.38 [^]	9.40	47.73	51.14
Ifi204	-----	6.28	52.48	75.86
Ifit2	3.53 [^]	-----	47.51	66.26
Ifit3	6.44 [^]	5.59 [^]	50.99	52.02
Il1a	61.73	-----	-----	86.52
Il1b	76.25	42.52	10.74 [^]	76.61
Irg1	9.12	6.34	9.33 [^]	6.75 [^]
Lilrb4	5.73 [^]	6.67	5.45 [^]	13.41 [^]
Lipg	14.32	4.78 [^]	4.38 [^]	21.11 [^]
Mx1	4.02 [^]	-----	14.52	14.95 [^]
Nfkbiz	13.61	5.20 [^]	2.41 [^]	5.98 [^]
Oasl2	-----	2.58 [^]	13.71	12.77 [^]
Phlda1	7.15	-----	3.26 [^]	10.40 [^]
Ptgs2	28.46	25.86	7.08 [^]	109.98
Rpph1	4.87 [^]	7.05	-----	3.73 [^]
Socs3	14.15	15.43	-----	5.33 [^]
Spry1	9.59	5.25 [^]	-----	14.53 [^]
Tnc	8.53	5.38 [^]	4.74 [^]	10.42 [^]
Tnf	6.65	5.75	4.09 [^]	4.60 [^]
Usp18	5.37 [^]	-----	13.61	11.03 [^]
BC013672	-----	-----	17.51	17.56 [^]
Bear3	-----	-----	-----	21.29
Csrp3	-----	-----	9.40 [^]	22.31
Cxcl10	-----	-----	23.31	17.76 [^]

Gene Symbol	CpG ODN		Poly (I:C)	
	4 h	12 h	4 h	12 h
Cxcl11	-----	-----	22.61	28.62
Gbp1	-----	-----	27.09	9.29^
Gbp2	-----	-----	22.48	15.48^
Gbp3	-----	-----	16.18	13.30^
Ifi205	-----	-----	11.37^	29.52
Ifih1	-----	-----	15.68	15.55^
Ifnb1	-----	-----	19.06	50.55
Iigp1	-----	-----	46.05	26.99
Oasl1	-----	-----	14.71	15.92^
Plk2	-----	-----	3.54^	55.74
Rgs1	-----	-----	4.05^	21.15
Rsad2	-----	-----	18.51	28.67
Tyki	-----	-----	16.34	15.54^
Zfp644	-----	-----	13.38^	24.66
Expi	3.88^	8.29	-----	-----

RAW 264.1 cells were stimulated with 3.2 ug/ml of CpG ODN and/or 32 ug/ml of poly (I:C) in 3 independent experiments. The 20 most activated genes at each time point/treatment are listed. Data represent the fold increase in expression over baseline. Genes whose level of expression did not increase significantly are shown by (---), while those whose level of expression increased significantly but were not among the top 20 genes at that timepoint/treatment are shown by (^).

Table III

Regulators of gene expression following TLR stimulation.

Stimulation:	CpG ODN	Poly (I:C)	CpG + Poly (I:C)
Regulators at 4 hr:	TNF (67%) IFNG (51%) IL1B (51%) IL1A (22%) CSF2 (14%)	IFNG (58%) TNF (56%) IL1B (37%) IL15 (17%) IFNB1 (16%) IFNA2 (14%) IL18 (10%) IL3 (9%)	TNF (57%) IFNG (46%) IL1B (33%) IL6 (17%) IL1A (16%) CSF2 (11%) IL3 (11%) IFNB1 (11%) IL18 (10%) IFNA2 (8%)
Regulators at 12 hr:	TNF (67%) IFNG (58%) IL1B (51%) CSF2 (19%)	TNF (53%) IFNG (41%) IL1B (34%) IL1A (13%) IL15 (12%) CSF2 (11%) IL18 (10%) IL6 (10%) IFNB1 (9%)	TNF (56%) IFNG (41%) IL1B (35%) IL6 (16%) IL1A (15%) IFNB1 (10%)

Regulators of the expressed gene networks (and % of genes regulated) were identified by IPA based on the microarray data shown in Table I.

Table IV

Multiple Regulators increase gene expression in poly (I:C) stimulated cultures.

TLR Ligand	# of regulators	Mean fold increase	p value
CpG ODN	1	6.7 ± 2.8 (26)	0.840
	>1	7.3 ± 1.3 (43)	
poly(I:C)	1	4.5 ± 0.5 (46)	0.033
	>1	7.0 ± 0.9 (72)	
CpG ODN +poly (I:C)	1	4.8 ± 0.4 (67)	0.003
	>1	9.5 ± 1.1 (113)	

The effect of single vs multiple regulators on the magnitude of gene expression is shown. Data reflect the mean fold increase in mRNA levels ± SEM (N shown in parentheses) at 4 h for all significantly activated genes in each treatment group.

Table V

Top Functional Categories of Genes Up-regulated by CpG ODN and poly (I:C)

Functional Category	CpG ODN p-value	Poly (I:C) p-value
Cell Death	$< 4.7^{-06}$	$< 1.1^{-05}$
Cellular Growth and Proliferation	$< 5.0^{-06}$	$< 8.7^{-06}$
Immune Response	$< 4.9^{-06}$	$< 1.3^{-05}$
Cell-to-Cell Signaling and Interaction	$< 5.0^{-06}$	$< 1.2^{-05}$
Hematological System Development and Function	$< 4.9^{-06}$	$< 1.3^{-05}$
Immune and Lymphatic System Development and Function	$< 4.6^{-06}$	$< 1.2^{-05}$
Gene Expression	$< 4.0^{-6}$	$< 1.1^{-05}$
Cellular Development	$< 4.0^{-6}$	$< 1.0^{-05}$
Tissue Morphology	$< 4.6^{-06}$	$< 3.5^{-06}$
Tissue Development	$< 4.2^{-06}$	$< 9.8^{-06}$

IPA was used to assess the functional categories associated with CpG ODN or poly (I:C) up-regulated genes.

Table VI

Synergistically up-regulated genes.

I. 4 h time point					Transcript Level (Fold over Non-Stimulated control)			
Accession No	Gene Symbol	Gene Description	CpG	Poly IC	CpG + Poly IC	Synergistic increase		
NM_031168	IL6	Interleukin 6	4.1	2.2	100.3	15.9		
NM_010554	IL1A	Interleukin 1, alpha	7.1	1.5	48.9	5.7		
NM_021367	TSLP	Thymic stromal lymphopoietin	2.3	1.4	20.1	4.2		
NM_020521	V1RB5	Vomerolasal V1r-type receptor V1rb5	2.6	1.9	12.7	2.8		
NM_013642	DUSP1	Dual specificity phosphatase 1	0.7	1.2	5.1	2.7		
NM_008335	IFNA6	Interferon alpha 6	0.9	4.6	14.7	2.6		
NM_011198	PTGS2	Prostaglandin-endoperoxide synthase 2	25.1	6.2	80.9	2.6		
NM_011593	TIMP1	Tissue inhibitor of metalloproteinase 1	1.6	1.3	7.0	2.4		
NM_010720	LIPG	Lipase, endothelial	20.5	6.3	63.5	2.4		
NM_010510	IFNB1	Interferon beta 1, fibroblast	1.6	17.8	46.1	2.4		
NM_010104	EDN1	Endothelin 1	20.5	7.9	64.1	2.3		
XM_196271	SCN9A	Sodium channel, voltage-gated, type IX, alpha	1.1	1.7	6.2	2.3		
NM_009971	CSF3	Colony stimulating factor 3 (granulocyte)	8.2	0.8	19.4	2.2		
NM_178909	WDR92	WD repeat domain 92	3.0	2.0	10.2	2.0		
NM_011607	TNC	Tenascin	5.6	3.2	17.4	2.0		
NM_008655	GADD45B	Growth arrest and DNA-damage-inducible 45 beta	10.2	3.9	27.0	1.9		
NM_010505	BFSP2	Beaded filament structural protein 2	2.9	1.3	8.0	1.9		
NM_206867	IFNA6T	Predicted gene interferon alpha 13	1.0	3.2	8.0	1.9		
NM_011332	CCL17	Chemokine (C-C motif) ligand 17	1.0	1.1	3.9	1.8		
NM_010505	INFIA5	Interferon alpha 5	1.0	2.9	6.9	1.8		

II. 12 h time point

II. 12 h time point					Transcript Level (Fold over Non-Stimulated control)			
Accession No	Gene Symbol	Gene Description	CpG	Poly IC	CpG + Poly IC	Synergistic increase		

I. 4 h time point

Transcript Level (Fold over Non-Stimulated control)

Accession No	Gene Symbol	Gene Description	CpG	Poly IC	CpG + Poly IC	Synergistic increase
NM_133775	IL33	Interleukin 33	1.3	2.7	188.8	46.5
NM_013650	S100A8	S100 calcium binding protein A8	2.4	0.4	55.9	19.5
NM_031168	IL6	Interleukin 6	11.1	23.6	570.7	16.4
NM_008351	IL12A	Interleukin 12a (IL-12 subunit p35)	1.6	1.8	39.3	11.6
NM_008491	LCN2	Lipocalin 2	10.7	2.6	131.0	9.9
NM_013642	DUSP1	Dual specificity phosphatase 1	1.1	2.5	23.7	6.5
NM_008871	SERPINE1	Serine peptidase inhibitor, clade E	3.5	17.7	131.1	6.2
NM_011315	SAA3	Serum amyloid A 3	1.8	1.9	22.8	6.1
NM_032541	HAMP	Hepcidin antimicrobial peptide (Hepcidine)	1.1	1.0	11.7	5.4
NM_023456	NPY	Neuropeptide Y	1.2	1.3	12.6	5.1
NM_008538	MARCKS	Myristoylated alanine rich PKC substrate	1.5	1.1	13.2	5.1
NM_011593	TIMP1	Tissue inhibitor of metalloproteinase 1	1.2	1.9	15.1	5.0
XM_283649	IL19	Interleukin 19	1.1	1.2	10.9	4.7
NM_010548	IL10	Interleukin 10	1.7	0.9	10.2	3.9
NM_013654	CCL7	Macrophage/monocyte chemotactic protein-3	1.6	2.4	14.8	3.7
NM_019450	IL1F6	Interleukin 1 family, member 6	3.9	2.6	21.0	3.2
NM_010444	NR4A1	Nuclear receptor subfamily 4	1.1	2.2	10.4	3.2
XM_133915	1190003J15RIK	5-hydroxyisourate hydrolase	16.4	2.6	57.4	3.0
NM_011607	TNC	Tenascin C	3.8	6.3	30.5	3.0
NM_173869	STFA2L1	Stefin A2 like 1	8.6	1.7	25.8	2.5
NM_008964	PTGER2	Prostaglandin E receptor 2 (subtype EP2)	1.6	2.6	10.3	2.5
NM_013454	ABCA1	ATP-binding cassette, sub-family A (ABC1)	1.8	1.7	8.8	2.5
NM_007969	EXPI	Extracellular proteinase inhibitor	7.6	2.2	24.0	2.5
NM_025288	STFA3	Stefin A3	9.8	2.2	29.2	2.4
NM_133232	PFKFB3	6-phosphofructo-2-kinase	1.0	0.9	4.2	2.3
NM_002569	FURIN	Furin (paired basic amino acid cleaving enzyme)	1.9	1.6	7.7	2.2

I. 4 h time point

Accession No	Gene Symbol	Gene Description	Transcript Level (Fold over Non-Stimulated control)			
			CpG	Poly IC	CpG + Poly IC	Synergistic increase
NM_011171	PROCR	Protein C receptor, endothelial	2.4	7.1	20.3	2.2
NM_172847	ADAMTS4	A disintegrin-like and metalloproteinase	1.1	1.6	5.9	2.2
NM_009632	RPPH1	Ribonuclease P RNA component H1	6.9	3.7	22.8	2.2
NM_172050	CD300E	CD300e antigen	3.0	1.6	9.9	2.2
NM_009705	ARG2	Arginase type II	2.4	3.7	13.2	2.2
NM_007784	CSN1S1	Casein alpha s1	1.5	1.1	5.6	2.1
NM_025549	ARRDC4	Arrestin domain containing 4	2.2	1.2	7.2	2.1
NM_011610	TNFRSF1B	TNF receptor superfamily	3.8	3.3	14.8	2.1
NM_014143	CD274	CD274 antigen	1.8	4.1	12.3	2.1
NM_207673	OLFR100	Olfactory receptor 100	5.1	3.5	17.7	2.1
NM_030612	NFKBIZ	NF kappa light polypeptide gene enhancer	5.5	6.4	24.3	2.1
NM_010442	HMOX1	Heme oxygenase (decycling) 1	1.9	2.9	9.8	2.0
NM_021367	TSLP	Thymic stromal lymphopoietin	1.5	3.2	9.6	2.0
NM_025681	LIX1	Limb expression 1 homolog (chicken)	4.6	1.2	11.5	2.0
NM_024435	NTS	Neurotensin	0.9	5.2	12.0	2.0
NM_172891	STYK1	Serine/threonine/tyrosine kinase I	1.5	0.9	4.8	2.0
NM_008352	IL12B	Interleukin 12b (IL-12 subunit p40)	1.1	1.8	5.5	1.9
NM_008655	GADD45B	Growth arrest and DNA-damage-inducible 45 beta	5.0	8.	24.7	1.9
NM_010720	LIPG	Lipase, endothelial	8.0	35.0	80.5	1.9
NM_145953	CTH	Cystathionase (cystathionine gamma-lyase)	4.4	4.1	15.8	1.9
NM_009500	VAV2	Vav 2 oncogene	1.0	1.0	3.4	1.9

RAW264.7 cells were stimulated for 4 or 12 h with 3.2 ug/ml of CpG ODN and/or 32 ug/ml of poly (I:C). Those genes whose mRNA level when stimulated with both ligands exceeded the sum + 3 SD of the level induced by each ligand independently were considered to have been synergistically up-regulated. Results reflect the mean of three independent experiments/time point. Lines in bold denote cytokines/chemokines.