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Short- and long-term changes in gene expression mediated by the activation of TLR9

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ABSTRACT

CpG DNA binds to Toll-like receptor 9 to stimulate a strong innate immune response. The magnitude, duration and scope of CpG-induced changes in gene expression are incompletely understood despite extensive studies of TLR9 mediated signal transduction pathways. In particular, the prolonged effects of CpG DNA on gene activation have not been investigated despite evidence that a single dose of CpG DNA alters immune reactivity for several weeks. This study used gene expression analysis to monitor changes in mRNA levels for 14 days, and identified the genes, pathways and functional groups triggered *in vivo* following CpG DNA administration. Two discrete peaks of gene activation (at 3 h and 5 days) were observed after CpG injection. Both the behavior and function of genes activated during the second peak differed from those triggered shortly after CpG administration. Initial gene up-regulation corresponded to a period when TLR9 ligation stimulated genes functionally associated with the generation of innate and adaptive immune responses (e.g. the NF-κB and B-cell receptor pathways). The second peak reflected processes associated with cell division (e.g. cell cycle and DNA replication and repair). The complex bimodal pattern of gene expression elicited by CpG DNA administration provides novel insights into the long-term effects of TLR9 engagement on genes associated with immunity and cell proliferation.

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1. Introduction

Bacterial DNA expresses "CpG motifs" that interact with TLR9 to stimulate an innate immune response characterized by the production of a variety of Th1 and pro-inflammatory cytokines, and the maturation and proliferation of immune cells, including B lymphocytes and dendritic cells (Sun et al., 1998; Ballas et al., 1996; Broide et al., 1998; Klinman et al., 1996; Roman et al., 1997; Sparwasser et al., 1997, 1998; Stacey et al., 1996). Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA (Klinman et al., 1996; Krieg et al., 1995; Yamamoto et al., 1992).

The signaling pathway triggered when CpG interacts with TLR9 proceeds through the recruitment of myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase (IRAK), and tumor necrosis factor receptor-associated factor 6 (TRAF6), and subsequently involves the activation of several mitogen-activated kinases (MAPK) and transcription factors (such as NF-κB and AP-1) (Akira et al., 2001) culminating in the transcription of pro-inflammatory chemokines and cytokines. Previous works identified the diverse changes in gene expression elicited from 1 h to 3 days after CpG administration (Gao et al., 2002; Kato et al., 2003; Klaschik et al., 2007, 2009; Schmitz et al., 2004). *In vitro* analysis of the long-term effects of CpG stimulation on normal cells were hampered by the changes in gene expression induced by cell culture alone (unassociated with CpG stimulation, Kato et al., 2003; Klaschik et al., 2007). *In vivo* studies indicated that CpG treatment triggers a rapid rise in gene activation, with mRNA levels largely returning to background after 2–3 days (Klaschik et al., 2009). The current work extends the duration of *in vivo* analysis through 14 days, as functional data indicates that CpG treatment can impact immune activation for several weeks (Ito et al., 2004; Krieg et al., 1998; Verthelyi et al., 2003).

There were important differences in the type and behavior of genes activated in the early vs. late peak of gene activation. The initial peak (at 3 h) corresponded to a period when TLR9 ligation rapidly up-regulated genes functionally associated with activation of the immune system, as typified by the NF-κB and B-cell receptor pathways. The second distinct peak (at 5 days) reflected the activation of genes primarily associated with cell division. Identifying these regulatory patterns improves our understanding of TLR-mediated host defense and may aid in the development of interventions designed to optimize the ensuing response.

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2. Materials and methods

2.1. Oligodeoxynucleotides

Endotoxin-free phosphorothioate ODN were synthesized at the CBER core facility (FDA, Bethesda, MD) as previously described (Takeshita et al., 2000). Mice were injected intraperitoneal (i.p.) with 400 μg of an equimolar mixture of CpG ODNs 1555 (GCTAGACGTTAGCGT) and 1466 (TCAACGTTGA) or control ODNs 1612 (GCTAGATGTTAGCGT) and 1471 (TCAAGCTTGA).

2.2. Mice and cell culture conditions

Two-month-old female BALB/c mice were housed in the NCI specific pathogen-free animal facility. All experiments were conducted under Animal Care and Use Committee approved protocols. Spleens were surgically removed from mice under sterile conditions after 0, 3, 9, 24 h, 3, 5, 7, 9, and 14 days, diced, and stored at −80 °C in RNAlater (Qiagen).

2.3. Production of labeled cDNA

Total RNA was extracted from spleens using TRIzol reagent (Invitrogen) as specified by the manufacturer. 20 μg of total RNA was reverse-transcribed using 3 μl of 10× first strand buffer (Stratagene), 2 μl (5 μg) of anchored Oligo(dT) (Invitrogen), 150 U of reverse transcriptase (StrataScript HC RT, Stratagene), 2 μl 20× aminoallyl-dUTP/dNTP mix, and 3 μl of 0.1 M DTT in a final volume of 30 μl at 42 °C for 90 min. A reference mouse RNA sample (Stratagene) was processed in parallel. Both cDNA were purified using a MinElute PCR Purification Kit (Qiagen).

10 μl of cDNA was labeled with Cy5 (sample cDNA) or Cy3 (universal reference cDNA) reactive dyes (Amersham Biosciences) diluted in 5 μl of DMSO plus 1.7 μl of 1 M $NaHCO_3$ for 90 min in the dark. Labeled cDNA was purified using MinElute PCR Purification Kits (Qiagen).

2.4. Oligonucleotide microarray hybridization

Murine oligonucleotide microarrays were produced by the NCI microarray facility (Gaithersburg, MD). Cy3 labeled reference and Cy5 labeled sample cDNAs (10 μl each) were combined, denaturated by heating for 2 min at 98 °C and mixed with 36 μl of hybridization solution at 42 °C (Ambion). Microarrays were overlaid with this solution and hybridized for 18 h at 42 °C using an actively mixing MAUI hybridization system (BioMicro Systems). Post-hybridization, the arrays were washed in 1× SSC/0.05% SDS and 0.1× SSC, centrifuged to remove remaining liquid with unbound cDNA, and dried. Arrays were scanned and intensity values generated using an Axon scanner and Genpix software 5.1 (Axon Instruments). Data were up-loaded to the mAdb database (Microarray Database, a collaboration of CIT/BIMAS and NCI/CCR at the NIH; http://nciarray.nci.nih.gov/) and formatted via the export function for use with BRB ArrayTools.

2.5. Analysis of gene expression

Data from four independent experiments/time point and six untreated controls were used for all statistical analyses. Reproducibility was established by comparing gene expression profiles among similarly treated mice from independent experiments in mAdb (referenced above). Expression analyses were performed using BRB ArrayTools (Biometric Research Branch, NCI). Data were background corrected, flagged values were removed, spots in which both signals were <100 were filtered out, ratios were log base 2 transformed and lowess intensity dependent normalization was used to adjust for differences in labeling intensities of the Cy3 and Cy5 dyes (Yang et al., 2002). Analysis was restricted to genes present on >70% of the arrays after filtering. In toto, 30,601 features were reproducibly tracked in all microarrays. The gene expression profile of all treatment groups was compared to that of the control groups.

Data were evaluated using Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc.). IPA maps each gene within a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Genes up-regulated during the time course of the study were categorized based on their function and role in signaling pathways. 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 and/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).

2.6. Flow cytometry

BALB/c mice were injected i.p. with 400 μg of CpG ODN. The total number of splenocytes and the relative frequency of individual cell types was determined on days 0, 1, 3, 5, and 14 by FACS ($N = 4$/group). Spleen cells were washed with PBS, fixed in 4% paraformaldehyde for 5 min at RT, and stained with phycoerythrin (PE)-labeled antibodies for 30 min. PE-labeled CD19, CD4, CD8, DX5 and F4/80 antibodies were purchased from BD Pharmingen (San Jose, CA). PE-labeled PDCA-1 antibody was purchased from Miltenyi Biotec (Auburn, CA). Stained cells were washed, re-suspended in PBS/0.1% BSA plus azide, and analysed by FACSort (BD Biosciences, San Jose, CA). In each experiment 30,000 events were analyzed per sample.

2.7. Statistical analysis

Genes that were differentially expressed in the treatment groups were identified using a random-variance *t*-test. The random-variance *t*-test is an improvement over the standard separate *t*-test as it 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 statistically significant if their p value was less than 0.0001. Differences between functional groups in terms of number of genes regulated were established by Chi-square analysis and Fisher exact test.

2.8. Accession codes

Microarray data were deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE16465.

3. Results

3.1. CpG ODN treatment triggers a bimodal pattern of gene expression in vivo

Normal BALB/c mice were injected i.p. with 400 μg of immunostimulatory CpG ODN. Changes in splenic gene expression were monitored at 9 time points over the subsequent 14 days. Data