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CpG-mediated changes in gene expression in murine spleen cells identified by microarray analysis

Sven Klaschik, Ihsan Gursel, Dennis M. Klinman *

Section of Retroviral Research, Center for Biologics Evaluation and Research, Food and Drug Administration, Building 29A, Room 3D10, Bethesda, MD 20892, USA

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Abstract

Unmethylated CpG motifs interact with Toll-like receptor 9 (TLR9), triggering an innate immune response characterized by the production of cytokines, chemokines and immunoglobulins. Microarray analysis of cDNA from murine spleen cells stimulated with CpG oligodeoxynucleotides (ODN) identified reproducible changes in gene expression over time. Eight genes are significantly up-regulated 2 h post CpG ODN stimulation, most of which contribute to the induction of innate or adaptive immune responses. Network analysis indicates that *TNF* and *NFKB1* are key regulators of gene expression at this early time point. At 4 h, *IL1B* in addition to *TNF* and *NFKB1* play dominant roles in the up-regulation of immune gene expression, whereas by 8 h this function is mediated by *TNF*, *IFNG*, and *MYC*. Genes responsible for down-regulating CpG-induced responses were also identified, dampening what would otherwise be a continuous positive feedback loop. This work provides novel insights into the regulatory process embedded in the gene expression profile induced by CpG ODN, identifies novel genes associated with CpG-induced immune stimulation, and clarifies the breadth of the immune response elicited via TLR9.
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1. Introduction

Bacterial DNA expresses "CpG motifs" that interact with toll-like receptor 9 to stimulate an innate immune response characterized by the production of a variety of Th1 and pro-inflammatory cytokines, and the functional maturation of immune cells, including B lymphocytes and dendritic cells (Klinman et al., 1996; Ballas et al., 1996; Broide et al., 1998; Sparwasser et al., 1997, 1998; Sun et al., 1998; Stacey et al., 1996; Roman et al., 1997). Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA.

The signaling pathway triggered by the interaction of CpG DNA 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.

Several groups have examined the effect of CpG stimulation on gene expression using microarrays (Gao et al., 2002; Kato et al., 2003; Kuo et al., 2005; Schmitz et al., 2004). Schmitz et al. (2004) and Gao et al. (2002) evaluated the response of a murine macrophage cell line to CpG and lipopolysaccharide (LPS) stimulation and found that LPS induced greater gene activation. Kato et al. (2003) analyzed gene expression in a single CpG-stimulated human peripheral blood mononuclear cell (PBMC) sample. That group detected an "early" and "late" gene response cluster, and concluded that CpG effects were mediated via an interferon (IFN)-α/β receptor paracrine pathway.

CpG ODN have broad and well documented effects on multiple types of immunologically relevant cells. These include the ability to directly trigger B cells and dendritic cells to produce factors that subsequently activate additional cell types, including natural killer cells and T cells (Klinman et al., 1996; Ballas et al., 1996; Broide et al., 1998; Sparwasser et al., 1997, 1998; Sun et al., 1998; Stacey et al., 1996; Roman et al., 1997). To gain a clearer understanding of the breadth of these CpG-induced

* Corresponding author. Tel.: +1 301 827 1707; fax: +1 301 496 1810.
E-mail address: dennis.klinman@fda.hhs.gov (D.M. Klinman).

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interactions, changes in gene expression were monitored using whole spleen cell preparations. Spleen cells were chosen on the basis of earlier studies showing that the response of this population reflected the breadth of immunity induced by CpG ODN in vivo (Takeshita et al., 2000; Klinman et al., 1996; Ishii et al., 2002; Zelenay et al., 2003; Datta et al., 2003; Ito et al., 2005). In contrast, studies examining CpG-mediated activation of cloned cell lines are not designed to detect interactions between different cell types (Gao et al., 2002; Kuo et al., 2005; Schmitz et al., 2004).

The current study analyzed the expression of 11,300 genes at 2, 4, and 8 h after CpG stimulation of murine spleen cells. Results provide insight into the nature, breadth and magnitude of the response elicited by CpG DNA, and identify genes that play a central role in the up- and down-regulation of CpG-mediated cellular activation.

2. Material and methods

2.1. Oligodeoxynucleotides

Endotoxin-free phosphorothioate ODN were synthesized at the CBER core facility as previously described (Takeshita et al., 2000). Cells were stimulated with an equimolar mixture of CpG ODN 1555 (GCTAGACGTTAGCGT) and 1466 (TCAACGTTGA) or control ODN 1612 (GCTAGATGTTAGCGT) and 1471 (TCAAGCTTGA).

2.2. Mice and cell culture conditions

Two-month-old female BALB/c mice (NCI, Frederick, MD) were housed in CBER's specific pathogen-free facility. All experiments were conducted under Animal Care and Use Committee approved protocols. Spleens were surgically removed from mice under sterile conditions and dissociated in warm complete medium by gently pressing with a sterile syringe plunger. Single cell suspension were prepared in warm RPMI 1640 supplemented with 10% FBS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM Hepes buffer, 0.11 mg/ml sodium pyruvate, and 0.5 mM β-mercaptoethanol. Five milliliters of cells (10^7 cells/ml) were cultured at 37 °C in a 5% CO_2 incubator ±1 μM ODN for 2–8 h.

2.3. Production of labeled cDNA

Total RNA was extracted from spleen cell cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) as specified by the manufacturer. Twenty micrograms of total RNA was reverse-transcribed using 3 μl 10× first strand buffer (Stratagene, La Jolla, CA), 2 μl (1 μg) $Oligo(dT)_{12–18}$ (Invitrogen, Carlsbad, CA), 3 μl (150 U) reverse transcriptase (StrataScript HC RT, Stratagene, La Jolla, CA), 2 μl 20× aminoallyl-dUTP/dNTP mix, and 3 μl 0.1 M DTT in a final volume of 30 μl at 42 °C for 1 h. A reference mouse RNA sample (Stratagene, La Jolla, CA) was processed in parallel. Both cDNA's were purified using a MinElute PCR Purification Kit (Qiagen, Valencia, CA). Ten microliters of cDNA was labeled with Cy5 (sample cDNA) or Cy3 (universal reference cDNA) reactive dyes (Amersham Biosciences, UK) 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, Valencia, CA).

2.4. Oligonucleotide microarray hybridization

Murine oligonucleotide microarrays were produced by the CBER microarray facility (Bethesda, MD). Cy-labeled reference and sample cDNAs (7.5 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, Austin, TX). A low volume active mixing chamber was applied to the microarray via an adhesive gasket (MAUI mixer FL, BioMicro Systems, Salt Lake City, Utah), the hybridization solution containing the sample was added to the array, and was placed for 18 h at 42 °C in the base unit of the actively mixing MAUI hybridization system (BioMicro Systems, Salt Lake City, Utah).

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, Union City, CA). Data were up-loaded to the mAdb database (Microarray Database, a collaboration of CIT/BIMAS and NCI/CCR at the NIH) and formatted via the export function for use with BRB ArrayTools.

2.5. Analysis of gene expression

Data from three to four independent experiments were used for all statistical analyses. The signal:noise ratio of the resultant dataset was calculated with mAdb (reference see above). Reproducibility was established by comparing gene expression profiles among similarly treated cultures from independent experiments. Expression analyses were performed using BRB ArrayTools (Biometric Research Branch, NCI). Data were background corrected, flagged values were removed, spots with both signals less than 190 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). Only genes present on >70% of the arrays after filtering were included in the subsequent analysis.

The effect of CpG ODN stimulation was compared to the gene expression profile of two combined control groups. Genes that were differentially expressed among the two classes were identified using a random-variance *t*-test. The test is based on comparing the differences in mean log-ratios between the predefined classes relative to the variation expected in the mean differences. It is computed for each gene using the normalized log-ratios from the independent experiments. 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 0.001 (2 and 4 h time point) or 0.0001 (8 h time point).

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