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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 is 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 hr 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- κ B and B-cell receptor pathways). The second peak reflected processes associated with cell division (e.g., cell cycle and DNA replication & 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.

Keywords

Rodent; gene regulation; signal transduction; molecular biology

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; Sparwasser *et al.*, 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).

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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- κ B 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 hr - 3 days after CpG administration (Gao *et al.*, 2002; Kato *et al.*, 2003; Klaschik S *et al.*, 2009; Klaschik *et al.*, 2007; 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 S *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 S *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 hr) corresponded to a period when TLR9 ligation rapidly up-regulated genes functionally associated with activation of the immune system, as typified by the NF- κ B 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.

MATERIALS AND METHODS

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 μ g of an equimolar mixture of CpG ODNs 1555 (GCTAGACGTTAGCGT) and 1466 (TCAACGTTGA) or control ODNs 1612 (GCTAGATGTTAGCGT) and 1471 (TCAAGCTTGA).

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 hr, 3 hr, 9 hr, 24 hr, 3 days, 5 days, 7 days, 9 days, and 14 days, diced, and stored at -80°C in RNeasy lysis buffer (Qiagen).

Production of labeled cDNA

Total RNA was extracted from spleens using TRIzol reagent (Invitrogen) as specified by the manufacturer. 20 μ g of total RNA was reverse-transcribed using 3 μ l of 10 $\times$ first strand buffer (Stratagene), 2 μ l (5 μ g) of anchored Oligo(dT) (Invitrogen), 150 U of reverse transcriptase (StrataScript HC RT, Stratagene), 2 μ l 20 $\times$ aminoallyl-dUTP/dNTP mix, and 3 μ l of 0.1 M DTT in a final volume of 30 μ l at 42 $^{\circ}\text{C}$ 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 μ l of cDNA was labeled with Cy5 (sample cDNA) or Cy3 (universal reference cDNA) reactive dyes (Amersham Biosciences) diluted in 5 μ l of DMSO plus 1.7 μ l of 1M NaHCO₃.

for 90 min in the dark. Labeled cDNA was purified using MinElute PCR Purification Kits (Qiagen).

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, denatured 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 Genpax 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.

Analysis of gene expression

Data from 4 independent experiments/time point and 6 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](https://analysis.ingenuity.com/pa/info/help/Ingenuity_Network_Algorithm_Whitepaper_FINAL(2).pdf) ; and (Calvano *et al.*, 2005) for details).

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)-labelled antibodies for 30 min. PE-labelled CD19, CD4, CD8, DX5 and F4/80 antibodies were purchased from BD Pharmingen (San Jose, CA). PE-labelled 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.

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.

Accession codes

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

RESULTS

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

Normal BALB/c mice were injected i.p. with 400 μ g of immunostimulatory CpG ODN. Changes in splenic gene expression were monitored at 9 time points over the subsequent 14 days. Data from at least 4 independently studied animals per time point were combined to identify reproducible changes in gene expression ($R^2 = 0.91 \pm 0.03$). A stringency cutoff of $p < 0.0001$ was used to distinguish genes whose level of expression differed significantly from untreated controls.

A bimodal pattern of gene up-regulation was observed. Gene activation peaked 3 hr after CpG ODN administration and gradually declined over the next 3 days (Fig. 1). This decrease in gene expression was somewhat unexpected, since functional studies previously established that the *in vivo* effects of CpG ODN treatment persist for up to 2 weeks (Ito *S et al.*, 2004; Krieg *et al.*, 1998; Elkins *et al.*, 1999; Verthelyi *et al.*, 2003). This puzzle was resolved when a second peak of gene activation was observed on day 5, which slowly declined towards background by day 14 (Fig. 1).

The nature and function of genes activated by CpG ODN change over time

A. Analysis of the first peak of gene activation—The pattern of gene expression observed during the initial peak of gene activation (at 3 hr) was examined. Ingenuity Pathway Analysis (IPA) was used to categorize the genes up-regulated during this period based on their function and role in signaling pathways. Results showed that up-regulated genes dominated over this period, and fell primarily into three functional groups: “immune response”, “cell signaling” and “cell movement” (Fig. 1; $p < 0.0001$ for all 3 groups). These functional classes are consistent with the known biological effects of TLR9-dependent cell stimulation (Ballas *et al.*, 1996; Broide *et al.*, 1998; Klinman *et al.*, 1996; Roman *et al.*, 1997; Sparwasser *et al.*, 1997; Sparwasser *et al.*, 1998; Bertin *et al.*, 2008; Haining *et al.*, 2008; Stacey *et al.*, 1996; Sun *et al.*, 1998).

The expression of genes within these functional groups changed dramatically over time. Representative examples are provided by a detailed analyses of the components of the “NF κ B pathway” (triggered as part of the innate immune response (Li and Verma, 2002; Krakauer, 2008) and the “B-cell receptor pathway” (B cells express TLR9 and are early targets of CpG-induced activation of the adaptive immune system (Klinman *et al.*, 1998)). CpG administration

led to the rapid up-regulation of a majority of the genes in the “NFkB pathway” (Fig. 2, group A). Many of these were functionally associated with the induction of innate immunity (e.g. IL1A, IL1B, NFKB1, REL, RELB, TLR9, MyD88, TNF). mRNA levels for most of these genes (85%) fell back to or below baseline by day 3 (Fig. 2, group A). Indeed, $\approx 40\%$ of the genes in the “NFkB pathway” were significantly down-regulated by days 5–7 (expression levels significantly below baseline, $p < 0.001$, group C and part of group A), an effect that slowly resolved by day 14 (Fig. 2).

Many of the genes in the “B-cell receptor pathway” were also rapidly up-regulated following CpG ODN administration ($p < 6 \times 10^{-3}$; Fig. 3). This was particularly true of genes linked to the initiation of innate and adaptive immune responses (e.g. NFKB1, REL, RELB, FOS, IKBKE, MAP3K2, MAP2K1, NFATC2, BCAP, BFL-1, RAS), a pattern similar to that observed in the “NFkB pathway” (Fig. 3, group A). Such activation was typically short lived, with 93% of the up-regulated genes returning to baseline by 24 hr (Fig. 3, group A). In contrast, genes in groups “B” and “C” were up- or down-regulated late (during the second peak of gene activation). The expression of these genes generally did not vary from baseline at early time points. However, several genes (particularly those associated with the p38 signaling cascade, e.g. MAPK14, MAPK2K6, CREB3, CREB1) were significantly down-regulated over the first day ($p < 0.001$), and expression of $\approx 50\%$ of all genes in the B-cell receptor pathway were reduced to significantly below baseline by day 5 ($p < 0.001$, group C). This suppression persisted through day 7 ($p < .000006$) and resolved by day 14, replicating the pattern observed with NFkB. The behavior of genes in the “cell signaling” and “cell movement” categories followed a similar pattern (data not shown). These findings are consistent with a process by which TLR9 ligation initially up-regulates and then suppresses the expression of genes functionally associated with the generation of innate and adaptive immune responses.

B. Analysis of the second peak of gene expression—As noted above, a second peak of gene activation was observed 5 days after CpG ODN administration. This effect involved nearly 1,100 genes and occurred when most genes in the early peak had been down-regulated (see above). The second peak of gene activation was not due to experimental or statistical artifact, as it was observed in multiple animals in different experiments and was confirmed by the pattern of gene activation detected on days 5, 7 and 9.

Of note, functional groups associated with the genes up-regulated at 3 hours were significantly down-regulated by 5 days (Fig. 1). The genes stimulated on day 5 were distinct from those triggered during the initial peak of CpG induced gene expression ($< 3.8\%$ overlap between these two groups, $p < 0.00001$). The function of these genes also differed, with those in the second peak being primarily associated with the functional pathways “cell cycle regulation” and “DNA damage response” ($p < 3 \times 10^{-9}$, Fig. 4 and $p < 10^{-12}$, Fig. 5, respectively). These pathways both promote and support the fidelity of cell division, consistent with the B cell proliferation and splenomegaly observed 4–7 days after CpG administration (Sparwasser et al., 1999).

Similar patterns of gene expression characterized both the “cell cycle regulation” and “DNA damage response” pathways. As seen in Fig 4 and Fig 5, $< 10\%$ of genes in these pathways were up-regulated immediately after CpG ODN administration (Group A). Rather, activation of genes in these pathways typically began on days 3–5, remained high through day 7, and then fell progressively towards baseline through day 14 (Group B in Fig 4, Fig 5).

C. Changes in spleen cell composition following CpG ODN administration—CpG ODN treatment induces changes in the number and frequency of cell subpopulations in the spleen {Sparwasser et al., 1999}. We therefore monitored that absolute and relative number of cell types over time. As seen in Fig 6, splenomegaly developed by day 5, consistent with

the observed induction of genes associated with the cell cycle and DNA replication pathways (Fig 4, Fig 5). B cell frequency changed by less than 10% over this period, and thus could not account for the 2 – 10 fold changes in “B cell pathway” associated mRNA levels (Fig. 3). Indeed, the cellular composition of the spleen remained fairly constant from 1 – 14 days post CpG activation, indicating that changes in cell activation and function rather than frequency must account for the reproducible patterns of gene expression observed in this study (Fig. 6).

DISCUSSION

Synthetic ODN containing unmethylated CpG motifs mimic the immunostimulatory activity of bacterial DNA. Through their ability to support the development of innate and adaptive immune responses these agents are finding use in the treatment of cancer, allergy and infectious diseases (Ashkar *et al.*, 2003; Carpentier *et al.*, 2003; Dong *et al.*, 2003; Halperin *et al.*, 2003; Heckelsmiller *et al.*, 2002; Hegele *et al.*, 2005; Horner and Raz, 2002; Jahrsdorfer and Weiner, 2003; Jahrsdorfer *et al.*, 2005; Krieg *et al.*, 1998; Krieg, 2007; Leonard *et al.*, 2007; Walker *et al.*, 1999; Weighardt *et al.*, 2000; Zimmermann *et al.*, 1998). While the signalling pathway activated when CpG ODN trigger via TLR9 is well described (Aderem and Ulevitch, 2000; Bauer *et al.*, 2001; Hacker *et al.*, 2000; Hemmi *et al.*, 2000; Takeshita *et al.*, 2001; Yamamoto *et al.*, 2002), the breath, time course and regulation of CpG-induced changes in gene expression *in vivo* are incompletely understood. This work is the first to examine the effects of TLR9 stimulation on gene activation over the multi-week period of CpG-induced changes in immune function (Elkins *et al.*, 1999; Ito S *et al.*, 2004; Krieg *et al.*, 1998; Verthelyi *et al.*, 2003). Microarrays were employed to monitor changes in mRNA levels over time, and the results analyzed by IPA to identify the genes, pathways and functional groups triggered *in vivo* by CpG ODN administration.

Several groups previously examined how CpG stimulation affected gene expression (Gao *et al.*, 2002; Kato *et al.*, 2003; Schmitz *et al.*, 2004). Studies of a murine macrophage cell line showed that several NF- κ B-dependent genes were up-regulated following CpG treatment (e.g., CCL3, IL1RN, PTGS2, and TNF) (Gao *et al.*, 2002; Schmitz *et al.*, 2004). Another group analyzed gene expression by human peripheral blood cells stimulated *in vitro* for 24 h with CpG ODN (Kato *et al.*, 2003). Consistent with current results, they identified a cluster of pro-inflammatory genes whose early up-regulation was presumably NF- κ B dependent.

Unfortunately, the long term impact of CpG ODN on gene expression could not be studied *in vitro*, as artifacts induced by cell culture non-specifically altered gene expression within 24 hr. Short-term *in vivo* studies showed that CpG DNA stimulated a rapid rise in gene activation that returned nearly to baseline by day 3 (Klaschik S *et al.*, 2009). Those findings suggested that CpG ODN induced only short term changes in gene expression - a conclusion inconsistent with functional data showing that TLR9 stimulation impacted the innate and adaptive immune system for several weeks (Ito S *et al.*, 2004; Krieg *et al.*, 1998; Verthelyi *et al.*, 2003) (Elkins *et al.*, 1999). Current findings resolve this dilemma by documenting that a complex bimodal pattern of gene up- and down- regulation is elicited *in vivo* following CpG ODN administration.

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 hr) corresponded to a period when TLR9 ligation up-regulated genes functionally associated with the generation of innate and adaptive immune responses (e.g. the NF- κ B and B-cell receptor pathways; Fig. 2 and Fig 3, respectively). Previous studies showed that the genes triggered during this early phase were stimulated primarily by TNF and IFNG, and were then selectively down-regulated via a process involving active suppression (Klaschik S *et al.*, 2009; Klaschik *et al.*, 2007). Of interest, current results show that the expression of virtually every gene rapidly up-regulated in the B cell and NF- κ B pathways returned to or below baseline by day 5 (Fig 1–Fig 3). This pattern is consistent with the hypothesis that TLR ligation not only triggers genes that contribute to the induction

of immune responses but also “suppressor” genes whose function is to down-regulate the induced response and thus avoid excessive/persistent immune stimulation (Klaschik S *et al.*, 2009; Liew FY *et al.*, 2005).

The current study found that >1,000 genes were significantly up-regulated and nearly 800 down-regulated 5 days after CpG ODN administration. This unexpected finding could not be attributed to experimental or statistical artifact, since it was observed repeatedly in multiple animals/group, in different experiments, and at multiple time points from days 5 through 9. There was <3% overlap between the genes triggered during the first and second peaks of gene expression. Of note, many of the genes activated during this first peak were down-regulated (expression levels significantly below baseline) by day 5 (Fig. 1–3). This finding extends previous work showing that the expression of genes up-regulated during the early peak was actively suppressed at later time points (Klaschik et al, 2009). We hypothesize that this down-regulation (which includes critical genes such as IL-1) serves to prevent excessive/prolonged immune stimulation. The second peak reflected processes associated with cell division (e.g., “cell cycle” and “DNA replication & repair”), consistent with the proliferation of splenocytes observed at that time (Fig. 6).

The expression pattern of most genes fell into one of three patterns. Those in group A were rapidly up-regulated and then fell to or below baseline by day 5. Those in group B were up-regulated late (during the second peak gene activation) and typically remained at baseline at earlier time points. Those in group C were down-regulated during the second peak and were typically at or below baseline at earlier time points. There were a few genes whose pattern of expression followed a less consistent pattern (less than 3% of all regulated genes), fluctuating between increased and decreased expression over time (e.g. SHP-2). Such complexity was also observed in the expression levels of several gene families rather than individual genes (e.g. the PIK3- gene family). Further study is required to determine whether such fluctuations are of physiologic relevance (reflecting genes whose mRNA levels show unusual variability) or experimental artifact.

In this context, several lines of evidence support the quality of the microarray data generated in this study. A minimum of 4 animals/group were analyzed independently to generate each data set and identify reproducible changes in gene expression ($R^2 = 0.91 \pm 0.03$). A stringency cutoff of $p < 0.0001$ was used to distinguish genes whose level of expression differed significantly from untreated controls ($N = 6$). Furthermore, consistent results were observed when the current findings were compared to those generated in earlier studies {Tross et al., 2009, Klaschik et al. 2007, Klaschik et al. 2009}. Of note, previous studies in our lab confirmed that differences in mRNA levels detected by microarray could also be detected by RT-PCR and ELISA (Tross et al., 2009).

FACS analyses were performed to examine the possibility that changing patterns of gene expression reflected alterations in the frequency or distribution of various cell types within the spleen. While the absolute number of splenocytes increased significantly by day 5 ($p < 0.002$), there was little difference in the relative frequency of specific cell subpopulations over time (Fig. 6). This is important, since microarray studies monitor relative rather than absolute changes in mRNA expression. For example, despite CpG ODN treatment inducing an absolute increase in B cell number, the relative frequency of such cells changed by less than 10%. This change could not account for the 2 – 10 fold increase in mRNA levels for B cell associated genes observed in this study. Similarly, the frequency of other cell types generally varied by 10 – 25%, far below the magnitude of change in relevant mRNA levels. The changes in gene expression described in this work may reflect either the direct stimulation of TLR9 expressing cells or secondary events mediated by factors (such as cytokines/chemokines) produced by other cells over the duration of the study. It is not currently possible to differentiate between

direct vs. indirect effects occurring over prolonged periods *in vivo* or to isolate and independently study each cell type of interest. However current results are of relevance, as they document the changes in gene expression that occur under physiologic conditions *in vivo*.

Based on current findings, we propose a model in which CpG-TLR9 ligation initially induces a pro-inflammatory response characterized by the activation of regulatory genes (e.g. TNF, IFNG) and pathways (e.g. NF- κ B and B cell receptor) functionally associated with stimulation of the innate and adaptive immune systems. This initial burst of activity is specifically down-regulated over the ensuing 3 days, but is replaced by a new and distinct population of genes and pathways (e.g. “cell cycle”, “DNA damage response”) that ensure the persistence of CpG-induced activation (Fig. 4, Fig 5). This secondary peak of gene expression is consistent with functional data showing that CpG treatment can alter immune cell number and function for several weeks (Elkins *et al.*, 1999; Ito S *et al.*, 2004; Krieg *et al.*, 1998; Sparwasser *et al.*, 1999; Verthelyi *et al.*, 2003; Zheng *et al.*, 2008). Further definition of these regulatory patterns should improve our understanding of TLR-mediated host defense and facilitate the development of interventions designed to optimize the nature and duration of the ensuing response.

Acknowledgments

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Fig. 1. Time course of gene regulation induced by CpG ODN *in vivo*

BALB/c mice were injected i.p. with 400 ug CpG ODN. Gene expression in the spleen was monitored over time by microarray. At least 4 biological replicates were analyzed independently at each time point in at least 2 independent experiments. Differentially regulated genes were identified by comparison to untreated mice (n=6) using a stringency cut-off of $p < 0.0001$. Ingenuity Pathway Analysis (IPA) was used to identify those genes differentially regulated during this period based on their function and role in signaling pathways.

B-cell receptor pathway

Fig. 3. Regulation of genes in the “B-cell receptor” pathway
See legend to Fig 2 for details.

Cell cycle regulation

Fig. 4. Regulation of genes in the “Cell cycle regulation” pathway
See legend to Fig 2 for details.

Fig. 5. Regulation of genes in the “DNA damage response” pathway
See legend to Fig 2 for details.

Fig. 6. Number and frequency of cell types in the spleen after CpG ODN administration
BALB/c mice were injected i.p. with 400 ug CpG ODN. The 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). Note that spleen cell number increased significantly by day 5 ($p < 0.002$; 45% increase).