Activation by CpG Oligodeoxynucleotides Protects Bone Marrow-Derived Dendritic Cells from Apoptosis: A Transcriptomic and Bioinformatic Study

Min Yang[*,1], Nitya Krishnan[1], Stephen Coade[1], Roger S. Buxton[1], Mike Hubank[2], Douglas B. Young[1] and Ricardo E. Tascon[1]

[1]*Division of Mycobacterial Research, MRC National Institute for Medical Research, London, NW7 1AA, UK*

[2]*Institute of Child Health, University College London, London, WC1N 1EH, UK*

Abstract: Despite the essential role of dendritic cells in the priming of immune responses, the cellular and molecular mechanisms involved in regulating apoptosis and survival of dendritic cells are still poorly documented. Experimental data has suggested that activation of dendritic cells through either T cells or by engagement of pathogen-associated molecular patterns recognition suppresses dendritic cell apoptosis and promotes survival. In this study we investigated the mechanisms involved in regulating bone marrow derived dendritic cells (BMDCs) survival and apoptosis after cytidine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN) treatment. We found that addition CpG-ODN to BMDC cultures protected cells from spontaneous apoptosis; in addition, CpG-ODN also protected BMDCs from camptothecin-induced apoptosis. To identify transcription factors controlling CpG-ODN-mediated BMDCs survival we employed DNA microarrays, gene clustering and transcription element listening system (TELiS), a sequence-based bioinformatic tool that identifies transcription factor binding motifs that are over-represented among the promoters of up- or down-regulated genes. Our analysis revealed that several transcription factors may play key roles in regulating CpG-ODN-induced BMDCs survival. Interestingly, the CCAAT/enhancer binding protein alpha (C/EBPα) was significantly over-represented among the promoters of the up-regulated genes; however its expression levels in nuclear extracts was significantly reduced following CpG-ODN treatment, suggesting that CpG-ODN-mediated survival of BMDCs is associated with decreased activation of C/EBPα. In conclusion, our study suggested that in addition to NF-κB and AP-1, other transcription factors, such as C/EBPα, also contribute to the regulation of CpG-ODN induced BMDC survival.

INTRODUCTION

Dendritic cells (DCs) are short lived, efficient professional antigen presenting cells (APCs), which bridge innate and adaptive immune responses [1]. Immature DCs detect microbial infections through their pattern recognition receptors [2]. Toll-Like Receptors (TLRs) represent a major group of these receptors that detect multiple pathogen associated molecular patterns (PAMPs), for example, TLR2 recognises bacterial lipoproteins and lipoteichoic acid, TLR4 detects LPS and major glycolipidic component of Gram-negative bacteria, whilst TLR9 recognizes unmethylated cytidine-phosphate-guanosine DNA (CpG-DNA) of bacteria and viruses [3]. Activation of DCs to efficient APCs for T cell priming can be initiated by engagement of TLRs. During activation DCs increase their ability to present and process antigens, up-regulate MHC class II and costimulatory molecules and produce several inflammatory cytokines [4]. Experimental data also suggests that DC activation induced by engagement of PAMPs recognition promotes DC survival, for example, LPS and CpG oligodeoxynucleotide (CpG-ODN) have been demonstrated to inhibit cellular apoptosis [5-7]; in addition it has been shown that promotion of DC survival can promote specific immune responses [8, 9]. Thus, activation and regulation of DC survival are critical for mounting efficient innate immune responses to pathogens as well as for the development of effective T cell-mediated adaptive immune responses.

Previously we demonstrated that activation of DCs by CpG-ODN promotes priming and activation of CD8 T cells [10]. Despite the increasing knowledge in the mechanisms regulating priming of T cells by activated DCs [8-11], the cellular and molecular mechanisms involved in regulating activation and survival of DCs in response to CpG-ODN are still poorly documented, therefore, uncovering the molecular mechanisms regulating activation and survival of DCs would enhance our understanding of the intracellular pathways involved in promoting T cell priming. In addition, this information ultimately will be useful in developing DC-based immunotherapies and vaccines for diseases such as cancer and tuberculosis.

It has been reported that CpG-ODN induced inhibition of spontaneous DC apoptosis by up-regulating cellular inhibitor of apoptosis proteins (cIAPS) [7]. Sester and co-workers [12] demonstrated that CpG-ODN activated survival of murine macrophages through TLR9 and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. It is well characterised that CpG-ODN-induced DC maturation is regulated principally

*Address correspondence to this author at the Division of Mycobacterial Research, MRC National Institute for Medical Research, London, NW7 1AA, UK; Tel: 44 20 88162677; Fax: 44 20 88162564;
E-mail: myang@nimr.mrc.ac.uk

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by the MyD88/IL-1-receptor-associated kinase (IRAK)/ TNF-receptor-associated factor (TRAF) signalling pathway [13]; however, signalling dependent on the DNA-dependent protein kinase (DNA-PK) has also been reported [14]. Although the proximal events regulating signalling in DCs by CpG-ODN have been extensively studied, the global transcriptional responses involved in controlling activation and survival are less well characterised. In this study we demonstrate that treatment of bone marrow derived DC (BMDCs) with CpG-ODN results in the activation of BMDCs as well as prevention of apoptosis. We also characterise these responses using Affymetrix genome-wide DNA microarrays (mouse 430-2) combined with Transcription element listening system (TELiS) [15] to identify changes in gene expression and involvement of transcription factors that were over represented among the promoters of up-regulated genes. We have validated the expression levels of several important genes regulating cellular apoptosis and inflammatory response by real time PCR (RT-PCR) and examined the role of the CCAAT/enhancer binding protein alpha (C/EBPα) in controlling BMDC survival in response to CpG-ODN.

MATERIALS AND METHODOLOGY

Materials

Camptothecin (CPT) was purchased from Sigma (Dorset, UK). Endotoxin-free phosphorothioate-stabilized CpG-ODN (GCATGACGTTGAGCT) and its control GpG-ODN (GCATGAGGTTGAGCT) were from Eurogentec (Seraing, Belgium). Anti-phospho-C/EBPα was bought from Cell Signaling Technology (Beverly, MA). LY294002 was purchased from Superarray Bioscience (Frederick, MD). Cell permeable NF-κB inhibitor peptide, SN 50, and its control peptide, SN50M, were obtained from Calbiochem (Nottingham, UK).

Cell Culture and Treatment

BMDCs were extracted from C57BL/6 mice and cultured for 6 days in the presence of recombinant GM-CSF (R&D system, Minneapolis, MN). Cells were then purified using CD11c (N418) microbeads (Militenyi Biotech, Germany) and 92%-95% purity was achieved. On day 7, the BMDCs were stimulated with CpG-ODN or GpG-OND for 24 hrs. After 24 hrs conditioned medium was collected for cytokine measurement and the cells were processed for further experiments such as flow cytometry analysis and RNA isolation.

Cytokine Measurements

Sandwich ELISAs were used to measure the level of cytokines produced by cultured cells; these cytokines were IL-1β, IL-6, IL-10, IL-12p40, TNFα (*e*-Bioscience, Germany), IL-1α and IP-10 (R&D system). The sensitivity ranges of the ELISAs were: IL-1β (8pg/ml), IL-6 (4pg/ml), IL-10 (15pg/ml), IL-12p40 (8pg/ml), TNFα (8pg/ml), IL-1α (10pg/ml) and IP-10 (30pg/ml).

Apoptosis Detection

BMDCs were harvested from the culture after appropriate treatment and washed twice in PBS. After blocking Fc receptors using anti-mouse CD16/CD32 (BD Bioscience Pharmingen, Oxford, UK) for 15 min at room temperature, the cells were stained with PI and annexin V (Annexin V-FITC Apoptosis Detection Kit 1, BD Biosciences) for 45 min in dark at 4°C. Acquisition was performed on a FACScan (Becton Dickinson, Mountain View, CA). Data were analysed using WINMDI 2.6.

Differential Expression of Gene Profile by DNA Microarray Analysis

After BMDCs were treated with CpG-ODN for 24 hrs, total RNA was extracted using Trizol (Invitrogen, Paisley, UK). The quality of the RNA was checked using a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). Microarray expression experiments (n=3) were performed using total RNA from three different sets of BMDC preparations. Briefly, 5μg of total RNA was used for one-cycle target labelling and hybridisation to the Affymetrix mouse genome-MOE430 2.0 array according to Affymetrix's standard protocol (http://www.affymetrix.com). Labelled GeneChips were scanned and data files scaled to 100 prior to analysis with GeneSpring 7.3 software (Agilent Technologies). Genes were excluded if the signal strength did not significantly exceed background values and if expression did not reach a threshold value for reliable detection (based on the relaxed Affymetrix MAS 5.0 probability of detection ($p \leq 0.1$) [16] in each of the three separate studies. Expression was median normalised per array and per gene, and genes were excluded if the level of expression did not vary by more than 1.4 fold between CpG-ODN treated compared with untreated control BMDCs. The remaining genes were subjected to non-parametric Welch tests and were reported with their respective fold changes and *p*-values.

The functional enrichment analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database that organises the genes (gene products) into pathway reaction maps [17] and can be used to illustrate causal relationships between genes (gene products).

To predict which transcription factors control differential expression of a set of genes driven by CpG-ODN stimulation, the data of differentially expressed genes was then analysed using the TELiS database [15] which contains information on the prevalence of transcription factor binding motifs (TFBMs) in the promoters of mouse genes (24,384 genes). The results are organised as a ranked list of transcription factors which may control the gene expression caused by CpG-ODN.

RT-PCR Analysis

The same RNA samples used in the microarray study were analysed by RT-PCR. Equal amounts of RNA were used for cDNA synthesis using Superscript RT (Invitrogen) and RT-PCR products were detected using QuantiTect SYBR green PCR kit (Qiagen, Crawley, UK) and ABI prism 7000 (Applied Biosystems, Warrington, UK). Commercially available primers (Superarray Biosciences, USA) were used for the real-time PCR reactions. Controls for the real-time PCR reactions were performed using reverse transcriptase negative samples to exclude any DNA contamination. The test genes and normalizing gene were assayed along with a set of standard samples (genomic DNA). The expression value of individual genes was normalised to the 18sRNA house keeping gene and the transcript level of each gene was

expressed as an induction ratio of the sample with CpG-ODN treated relative to the untreated control.

Detecting DNA-Protein Interactions

Nuclear extracts were prepared from the BMDCs by using BD™ Transfactor Extraction Kit (BD Bioscience). Protein levels in nuclear extracts of BMDCs were measured using the Coomassie Protein Assay Regent Kit (Pierce, Rockford, IL). Protein levels of C/EBPα and NF-κB in nuclear extracts were determined using BD Transfactor Kit (ELISA).

Statistical Analysis

Data are expressed as mean ± SD, unless otherwise stated. Comparisons between untreated control, CpG-ODN or GpG-ODN treated cultures were performed using Student's *t*-test for paired data. Values for $p<0.05$ were considered significant.

RESULTS

CpG-ODN Activates BMDCs and Protects Them from Apoptosis

Addition of 10 μM CpG-ODN to BMDCs for 24 hrs resulted in elevated levels of IL-1α, IL-1β, IL-6, IL-10, IL-12p40 and IP-10 in culture medium (Fig. **1**) indicating BMDC activation. Consistent with our previously reported data [18], costimulatory molecules CD80 (B7-1) and CD86 (B7-2) were also found to be up-regulated in response to CpG-ODN by flow cytometer (data not shown).

We then examined the effect of CpG-ODN on BMDC survival. BMDCs were cultured with CpG-ODN for 24 hrs, and cell apoptosis was examined using flow cytometry with annexin V and PI (Fig. **2A**). The survival rate of BMDCs was significantly enhanced from 77.49% to 87.05% ($p<0.003$, Fig. **2B**). This effect was not seen with GpG-ODN.

In order to examine whether CpG-ODN can prevent drug-induced apoptosis of BMDCs, camptothecin (CPT), an anticancer drug that induces cellular apoptosis by inhibiting the activity of DNA topoisomerase-I [19] was added during the last 4 hrs of CpG-ODN or GpG-ODN treatment. As demonstrated in Fig. (**2**), treatment of BMDCs with CPT resulted in profound apoptosis within BMDCs, the survival of BMDCs decreasing from 77.497% to 14.59% ($p<0.0001$). However, CpG-ODN treatment protected BMDCs undergoing CPT-induced apoptosis, and cell survival returned to 50.84% from 14.59% ($p<0.0001$). The effect of CpG-ODN mediated protection of apoptosis was CpG motif specific since GpG-ODN showed no protection towards CPT-induced BMDCs apoptosis. Thus, our data showed that CpG-ODN protected BMDCs from both spontaneous and drug-induced apoptosis.

Transcriptomic Analysis of CpG-ODN Activated BMDCs

To understand the molecular mechanisms regulating BMDCs activation and survival in response to CpG-ODN treatment, we employed Affymetrix DNA microarray analysis to obtain profiles of differentially expressed genes from BMDCs after treatment with 10 μM CpG-ODN for 24 hrs. Our microarray analysis revealed that 2039 out of 39000 genes were differently expressed after CpG-ODN treatment when a minimum 1.4 fold change in their expression levels was applied.

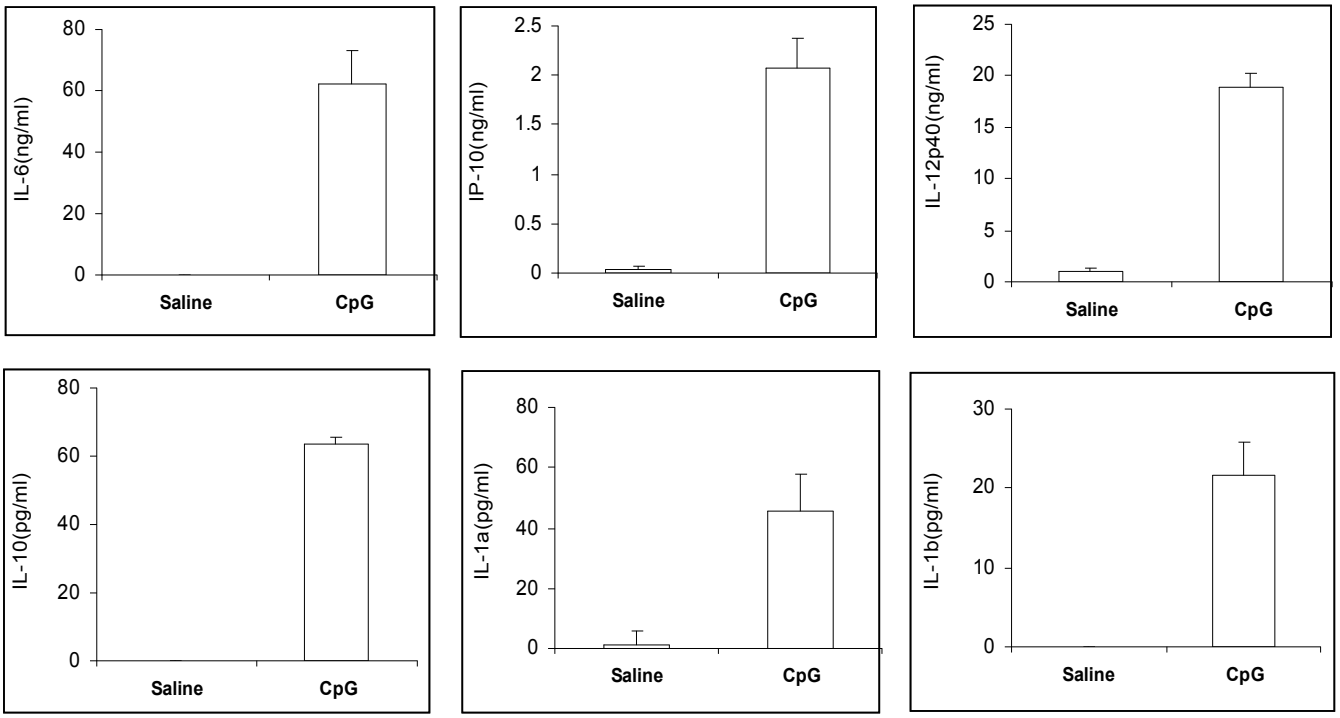
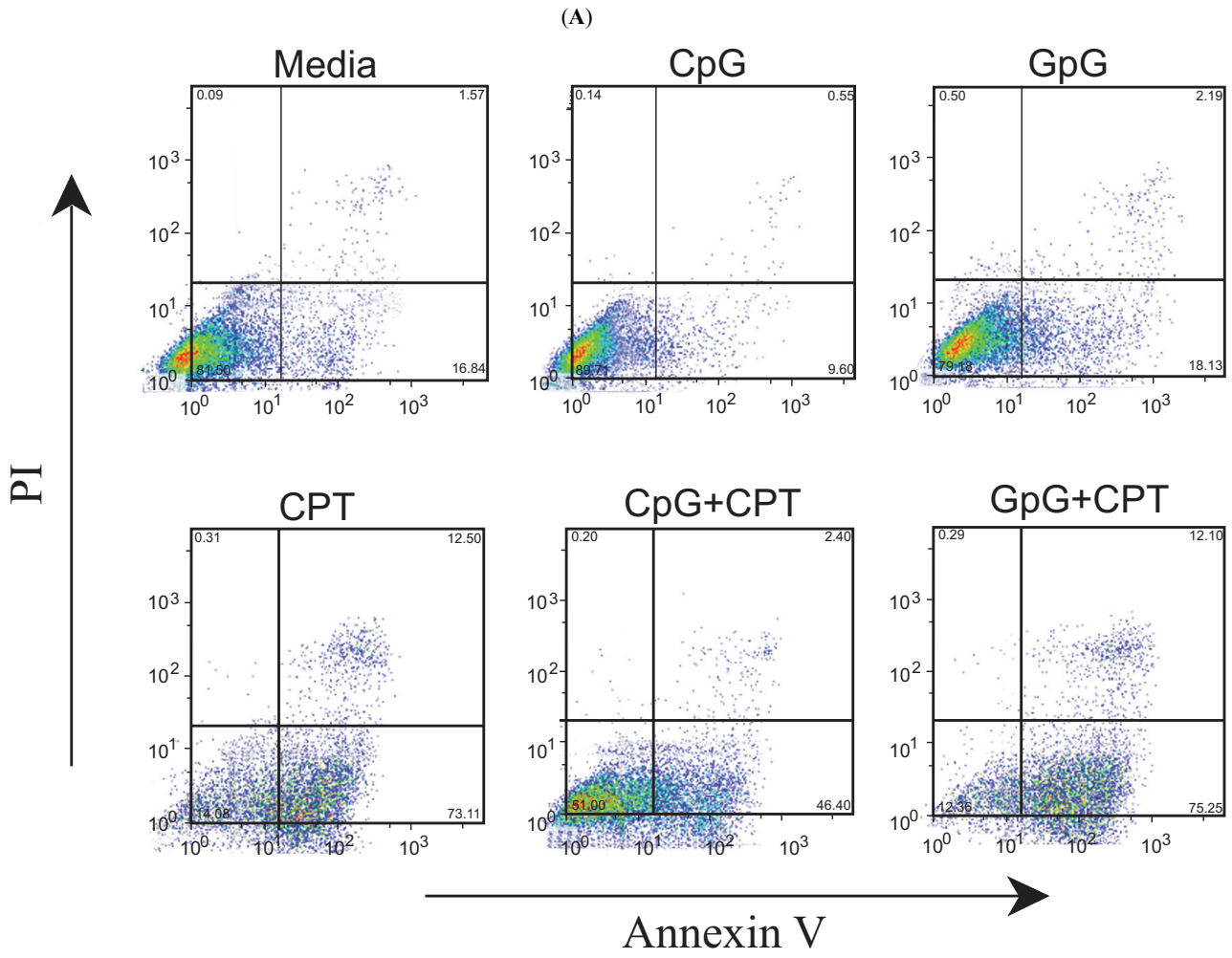


Fig. (1). The effect of CpG-ODN on the concentration of IL-1α, IL-1β, IL-6, IL-10, IL-12p40 and IP-10 present in culture medium of BMDCs. BMDCs ($5x10^5$ cells/ml) were activated by CpG-ODN (10 μM) for 24 hrs, cytokines released in the culture medium were measured by ELISA. Data are representative of three experiments.



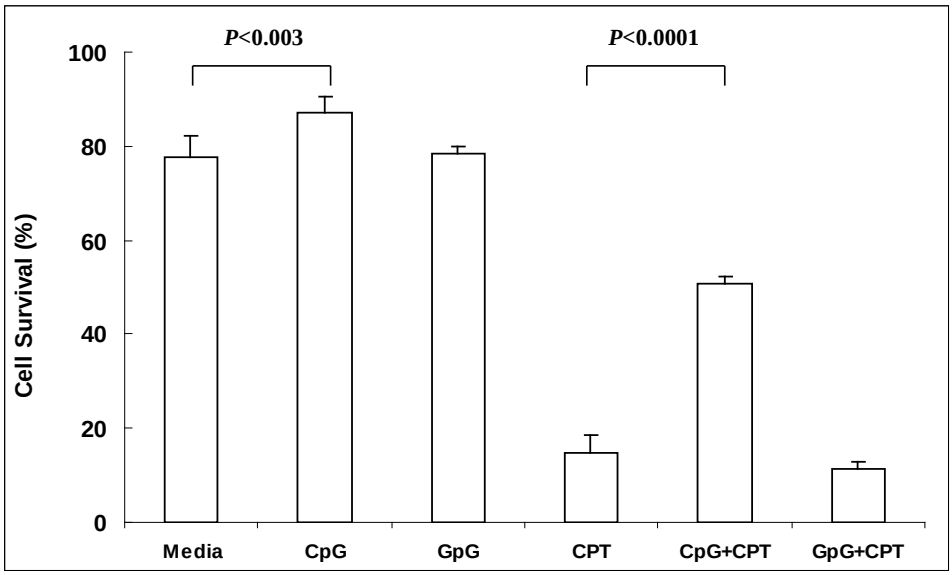


Fig. (2). CpG-ODN protects BMDCs from apoptosis. BMDCs ($2x10^5$ cells/ml) were treated with either CpG-ODN (10μM) or GpG ODN (10μM) for 20 hrs, and then CPT (2μg/ml) was added for a further 4 hrs. Cell apoptosis was examined using flow cytometry after cells were stained with anti-annexin V and propidium iodide (PI). One representative experiment of 3 is shown (**A**). Percentage cell survival was determined as negative for both markers and results represent the mean of 3 separate experiments ± SD (**B**).

Table 1. Microarray Results were Analysed Using Genespring and Welch Test. Genes with 1.4 Fold Change and Overlapping with KEGG Pathways are Listed (A). The *p*-Value in the Gene List Indicates the Likehood of Random Overlap. Apoptosis-Related Genes with Expression Levels Changed > 2 Fold in CD11C Purified BMDCs After CpG-ODN Stimulation (B), and Cytokine-Cytokine Receptor Interaction Related Genes (C)

(A) The Number of Genes which were Significantly Overlapped with KEGG Pathways

Genes Overlapping with Kegg Pathways	No. of Common Genes with Each Pathway	GeneList *vs* Pathway Random Overlap *p*-Value
Jak-STAT signaling pathway	43	2.33E-11
Cytokine-cytokine receptor interaction	**52**	**2.57E-11**
Cell cycle	38	2.63E-09
T cell receptor signaling pathway	33	1.47E-07
Toll-like receptor signaling pathway	**27**	**3.77E-07**
Focal adhesion	45	5.38E-07
Cell adhesion molecules (CAMs)	35	9.94E-07
Type I diabetes mellitus	17	1.81E-06
Purine metabolism	32	2.25E-06
B cell receptor signaling pathway	24	2.47E-06
ECM-receptor interaction	22	4.59E-05
Antigen processing and presentation	18	8.53E-05
Complement and coagulation cascades	16	9.44E-05
Hematopoietic cell lineage	17	0.00034
Methionine metabolism	8	0.000362
Pyrimidine metabolism	17	0.00178
Glycosphingolipid metabolism	10	0.00179
MAPK signaling pathway	48	0.00194
Riboflavin metabolism	6	0.00445
Adipocytokine signaling pathway	15	0.00461
Reductive carboxylate cycle (CO2 fixation)	5	0.00468
Nicotinate and nicotinamide metabolism	9	0.00507
Arachidonic acid metabolism	11	0.00644
Nitrogen metabolism	6	0.00768
Cysteine metabolism	5	0.00774
Regulation of actin cytoskeleton	33	0.0165
Glycan structures - degradation	6	0.0205
Pantothenate and CoA biosynthesis	5	0.0221
Sulfur metabolism	3	0.0231
Apoptosis	**16**	**0.0265**
Leukocyte transendothelial migration	18	0.0325
DNA polymerase	6	0.0344
N-Glycan degradation	4	0.0359
TGF-beta signaling pathway	14	0.042
Pyruvate metabolism	8	0.0949

(Table 1) contd…..

(B) Gene Associated with Cytokine-Cytokine Receptor Interaction

Common Name	Fold Change	Gene Bank	Description
IL-1β	86.62	BC011437	interleukin 1 beta
CXCL1	58.42	NM_008176	chemokine (C-X-C motif) ligand 1
IL-6	41.39	NM_031168	interleukin 6
CXCL2	37.68	NM_009140	chemokine (C-X-C motif) ligand 2
IL-1α	34	BC003727	interleukin 1 alpha
CXCL5	30.8	NM_009141	chemokine (C-X-C motif) ligand 5
Inhibin βA	16.56	NM_008380	inhibin beta-A
CXCL10	12.05	NM_021274	chemokine (C-X-C motif) ligand 10
TNFRSF5	10.35	AI385482	tumor necrosis factor receptor superfamily, member 5
IL-12p40	7.829	AF128214	interleukin 12b
TNFSF9	6.906	NM_009404	tumor necrosis factor (ligand) superfamily, member 9
TNFSF7	6.134	NM_011617	tumor necrosis factor (ligand) superfamily, member 7
Lta	5.342	NM_010735	lymphotoxin A
IL-2R	4.799	AF054581	interleukin 2 receptor, alpha chain
TNFSF4	3.564	NM_009452	tumor necrosis factor (ligand) superfamily, member 4
CXCL9	3.327	NM_008599	chemokine (C-X-C motif) ligand 9
VEGF-C	3.246	BB089170	vascular endothelial growth factor C
LIF	3.192	AF065917	Leukemia inhibitory factor
IL-17Rb	2.779	NM_019583	Interleukin 17 receptor B
TNFSF11b	2.779	AB013898	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
IL-18	2.479	NM_008360	Interleukin 18
Acvr2a	2.262	BB818297	activin receptor IIA
IL-15R	2.248	NM_008358	interleukin 15 receptor, alpha chain
Acvr1	2.097	NM_007394	activin A receptor, type 1
CXCL16	2.083	BC019961	Chemokine (C-X-C motif) ligand 16
IL-15	2.046	NM_008357	interleukin 15
PDGFα	0.484	BB371842	platelet derived growth factor, alpha
IL-1R2	0.463	NM_010555	interleukin 1 receptor, type II
IL-6st	0.445	BI102913	interleukin 6 signal transducer
VEGF-B	0.433	U48800	vascular endothelial growth factor B
TGF-βRI	0.416	BM248342	transforming growth factor, beta receptor I
IL-6Rα	0.384	X53802	interleukin 6 receptor, alpha
TGF-βRII	0.327	BG793483	transforming growth factor, beta receptor II
IL-11Rα1	0.309	BC004619	interleukin 11 receptor, alpha chain 1
TNFSF13	0.298	NM_023517	tumor necrosis factor (ligand) superfamily, member 13
KL	0.286	BB815530	kit ligand
CCR2	0.172	BB148128	chemokine (C-C) receptor 2
CXCL14	0.161	AF252873	Chemokine (C-X-C motif) ligand 14

(Table 1) contd…..

(C) Genes Associated with Apoptosis

Common Name	Fold Change	Gene Bank	Description
IL-1β	86.62	BC011437	interleukin 1 beta
IL-1α	34	BC003727	interleukin 1 alpha
Cflar	3.55	NM_009805	CASP8 and FADD-like apoptosis regulator (RIKEN cDNA A430105C05 gene)
Chuk	2.896	AU045682	conserved helix-loop-helix ubiquitous kinase
Akt3	2.738	AF124142	thymoma viral proto-oncogene 3
PI3K	2.398	BE647269	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
Ppp3cc	2.199	NM_008915	protein phosphatase 3, catalytic subunit, gamma isoform
1500003O03Rik	2.108	NM_019769	RIKEN cDNA 1500003O03 gene
NFκBIα	2.057	NM_010907	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha

Functional enrichment analysis based on KEGG pathways has been used to illustrate causal relationships between genes. Table **1A** lists the number of genes (from the 2039 differentially expressed) which significantly overlapped with KEGG pathways. As expected, cytokine and cytokine receptor interaction, TLRs signalling and apoptosis pathways were amongst those pathways with large overlaps of genes. For example, 52 genes expressed significantly in CpG-ODN treated BMDCs overlapped with the cytokine and cytokine receptor interaction pathway (Table **1B**), whilst 18 genes were involved with the apoptosis pathway (Table **1C**). The up-regulated expression of cytokines and cytokine receptor and costimulatory molecules in response to CpG-ODN treatment (Table **1B**) were in a good agreement with our ELISA data (Fig. **1**). Changes in the expression levels of selected genes which were involved in different functional pathways, such as IL-1α, IL-1β, IL-6, IL-12p40, IP-10, Akt, PI3K, conserved helix-loop-helix ubiquitous kinase (Chuk) and CASP8 and FADD-like apoptosis regulator (Cflar), were further validated using RT-PCR. RT-PCR data were in good agreement with the microarray data (Fig. **3**).

Expression-Based Monitoring of Transcription Factor Activity

To predict which transcription factors control differentially expressed genes driven by CpG-ODN stimulation, we employed the analysis of the TELiS database to report the potential involvement of transcription factors. PromoterStats of Web-link database retrieves the appropriate population prevalence matrix and generates a sample prevalence matrix containing transcription factor binding motifs (TFBMs) fre-

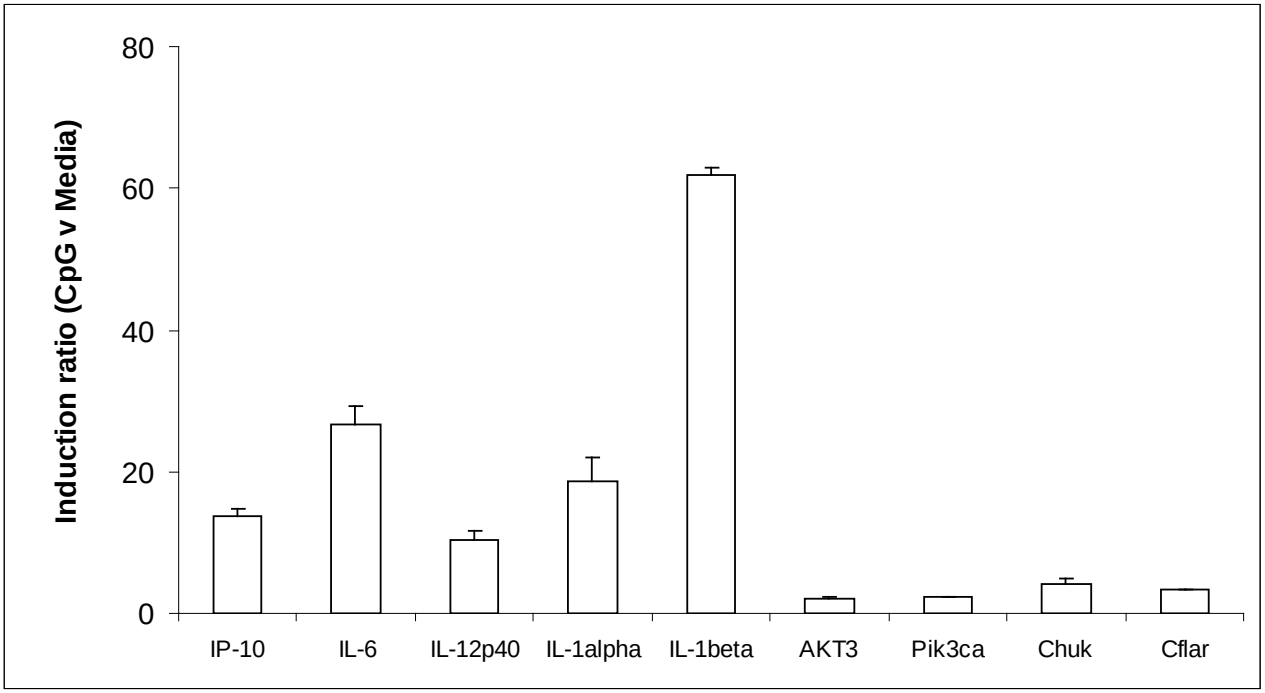


Fig. (3). Selected differentially expressed genes were evaluated by RT-PCR. After BMDCs were stimulated with CpG-ODN for 24 hrs, mRNA was isolated and the expression of mRNA for IP-10, IL-6, IL-12p40, IL-1α, IL-1β, AKT, PI3K, Chuk and Cflar was analysed by RT-PCR. Data are representative of three experiments.

quencies for the imported differentially expressed genes [15]. A list of predicted transcription factors were listed according to their *p*-values. The data in Table **2A** suggested that both NF-κB and AP-1 were involved in regulating gene expression in BMDCs activated after CpG-ODN treatment. NF-κB is the most well-documented transcription factor involved in regulating DCs activation by CpG-ODN, and our TELiS data is in a good agreement with results previously reported [4,13]. Interestingly, in addition to NF-κB and AP-1, the TELiS analysis revealed several other transcription factors that may be involved in CpG-ODN-mediated BMDCs activation and survival (Table **2B**). These include C/EBPα which is involved in controlling cell proliferation and differentiation [20].

Table 2. TELiS Results of TFBMs Over-Represented in the Promoters of the Differentially Expressed Genes in Response to CpG-ODN. TELiS Results Confirmed that Both NF-κB and AP-1 are Involved in Controlling Gene Expression in BMDCs Activation and Survival After CpG-ODN Treatment (A). TELiS Results for Novel Transcription Factors Involved in Regulating BMDCs Activation and Survival are Listed in (B)

(A)

TFBM Matrix	Sample Mean	Population Mean	*p* Value
NFκB-01	3.4%	1.07%	$3.29x10^{-6}$
NFκB-C	1.39%	0.26%	$6.34x10^{-5}$
NFκB-Q6	1.23%	0.24%	0.0002
NFκB65-01	3.09%	1.06%	$2.90x10^{-5}$
CRel-01	5.40%	3.24%	0.0027
AP-1-C	7.41%	4.90%	0.0035

(B)

TFBM Matrix	Sample Mean	Population Mean	*p* Value
ISRE-01	1.08%	0.12%	$2.0x10^{-5}$
MycMax-02	0.15%	1.32%	0.0018
Oct-C	0.77%	0.26%	0.0282
DeltaEF1-01	13.58%	16.61%	0.0181
Max-01	0.77%	2.00 %	0.0103
C/EBPα-01	3.24%	2.01%	0.0242
GATA3-01	9.57%	11.96%	0.0300

Anti-apoptotic Effect Mediated by CpG-ODN is Not Fully Reversed by an Inhibitor of NF-κB

As NF-κB may be involved in these responses, we examined whether an inhibitor of NF-κB, SN50, could reverse the protective effect of CpG-ODN on CPT-induced BMDCs apoptosis. Again CpG-ODN, but not GpG-ODN, significantly improved BMDC survival at 24 hrs from 14.59% to 50.84% ($p<0.0001$); in the presence of 18 μM SN50, this effect was reduced but not fully abolished with 34.95% of cells surviving ($p<0.0003$). The control SN50M did not reduce the effect of CpG-ODN with 48.45% survival (Fig. **4**). These results suggest that NF-κB activation is partially involved in mediating the effect of CpG-ODN on BMDCs survival but that transcription factors, such as C/EBPα may also be involved in the process.

Activation of NF-κB and C/EBPα are Modulated in Response to CpG-ODN Treatment and Regulate BMDCs Survival

To examine whether the activation of NFκB and C/EBPα were modulated in BMDCs in response to CpG-ODN treatment, nuclear proteins were extracted from cells incubated with 10 μM CpG-ODN for 24 hrs. ELISAs for NFκB and C/EBPα were used to compare the levels of activated nuclear C/EBPα and NFκB in CpG-ODN-activated BMDCs; the level of activated NFκB was significantly increased after CpG-ODN treatment ($p<0.001$) (Fig. **5**). However, in the same experiment CpG-ODN treated BMDCs expressed significantly lower levels of nuclear C/EBPα compared with control cells ($p=0.012$) (Fig. **5**). This data suggested that the role of CpG-ODN on nuclear translocation of NFκB and C/EBPα activation was different, i.e. CpG-ODN enhanced the accumulation of NFκB, but at the same time reduced the level of C/EBPα in the nucleus. This phenomenon reflects the functional differences between the two transcription factors; thus NFκB contributes to the development, activation, and survival of many cell types including DCs, whereas C/EBPα is a transcription factor with growth-inhibitory activity [20] and displays its inhibitory function through interactions with cell cycle proteins, such as cyclin-dependent kinase 4 (CDK4) [21].

It has been demonstrated that C/EBPα suppression of growth inhibitory activity is controlled by the protein phospatase 2A (PP2A) and the activation of PI3K/Akt [22]. Due to the lack of a C/EBPα inhibitor, we examined whether blocking PI3K using the specific inhibitor LY294002 would modulate CpG-ODN-mediated BMDC survival in the presence and absence of CPT. Fig. (**6A**) illustrates that in the presence of CpG-ODN the percentage survival of CPT-treated BMDCs was increased from 12.82% to 47.89% ($P<0.0003$). However, in the presence of LY294002, CpG-ODN-induced survival was reduced to 41.13% ($P<0.0007$). The similar pattern was also seen in the experiments in the absence of CPT, but the effect of PI3K inhibitor was much less obvious than one with CPT. Our data suggests that blocking PI3K could reverse CpG-ODN-mediated cell survival *via* C/EBPα. The production of IL-1α, IL-1β, IL-6, IL-10, IL-12p40 and IP-10 were elevated after CpG-ODN stimulation (Fig. **1**). We also found that LY294002 treatment resulted in reduction of IL-6, IL-12p40 and TNFα in the medium compared with their CpG-ODN controls and the reduction of IL-6 was statistically significant (Fig. **6B**). These results demonstrate that nuclear C/EBPα activity is regulated by CpG-ODN and suggests that dephosphorylation of this transcription factor is also involved in regulating CpG-ODN-induced BMDC survival.

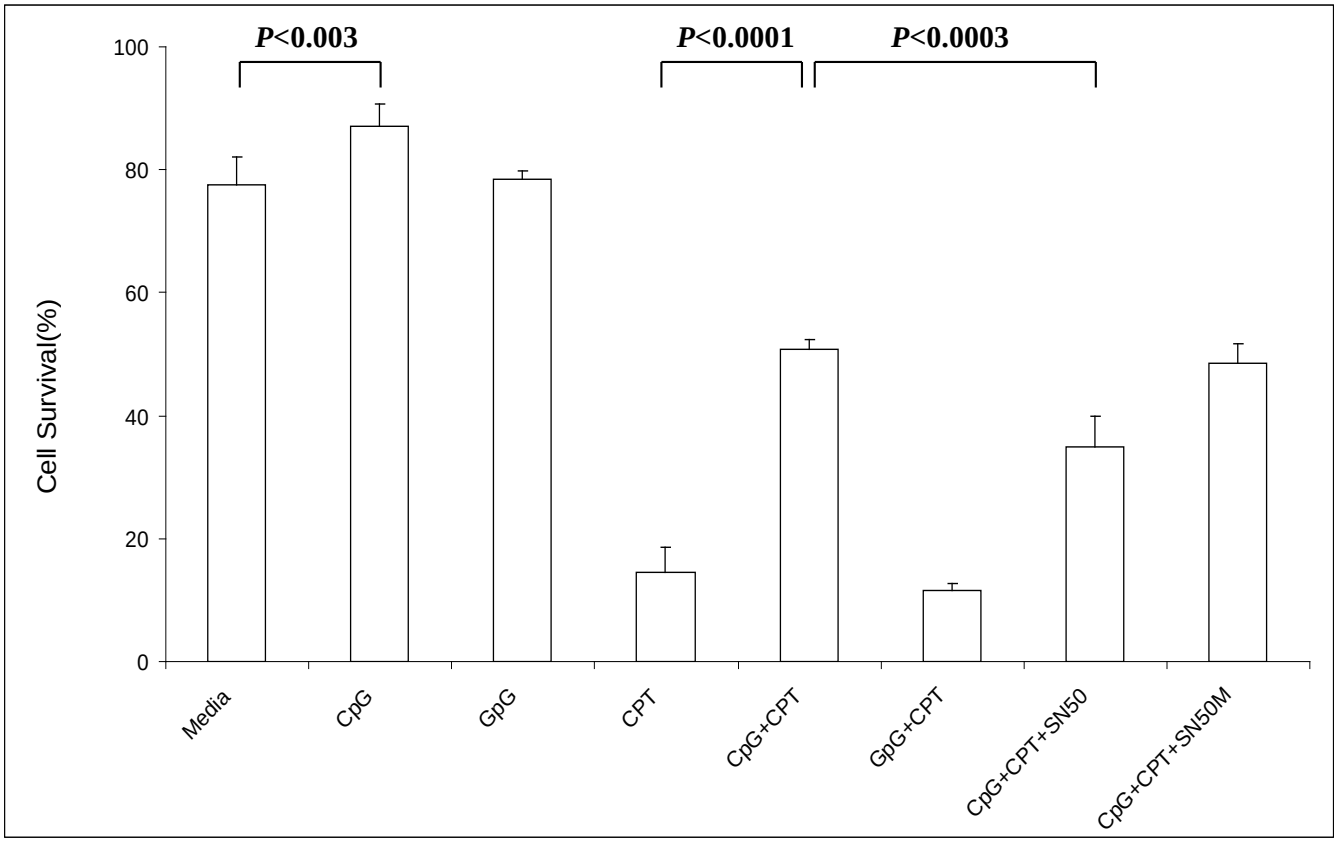


Fig. (4). CpG-ODN-induced survival of BMDCs is partially blocked by the NF-κB inhibitor SN50. BMDCs ($2x10^5$ cells/ml) were treated with 10 μM CpG-ODN in the presence or absence of the NFκB inhibitor SN50 (18 μM) for 20 hrs and then 2 μg/ml CPT was added for a further 4 hrs. Cell apoptosis was examined using flow cytometry after cells were stained with anti-annexin V and PI. Percentage cell survival was determined as negative for both markers and results represent the mean of 3 separate experiments ± SD.

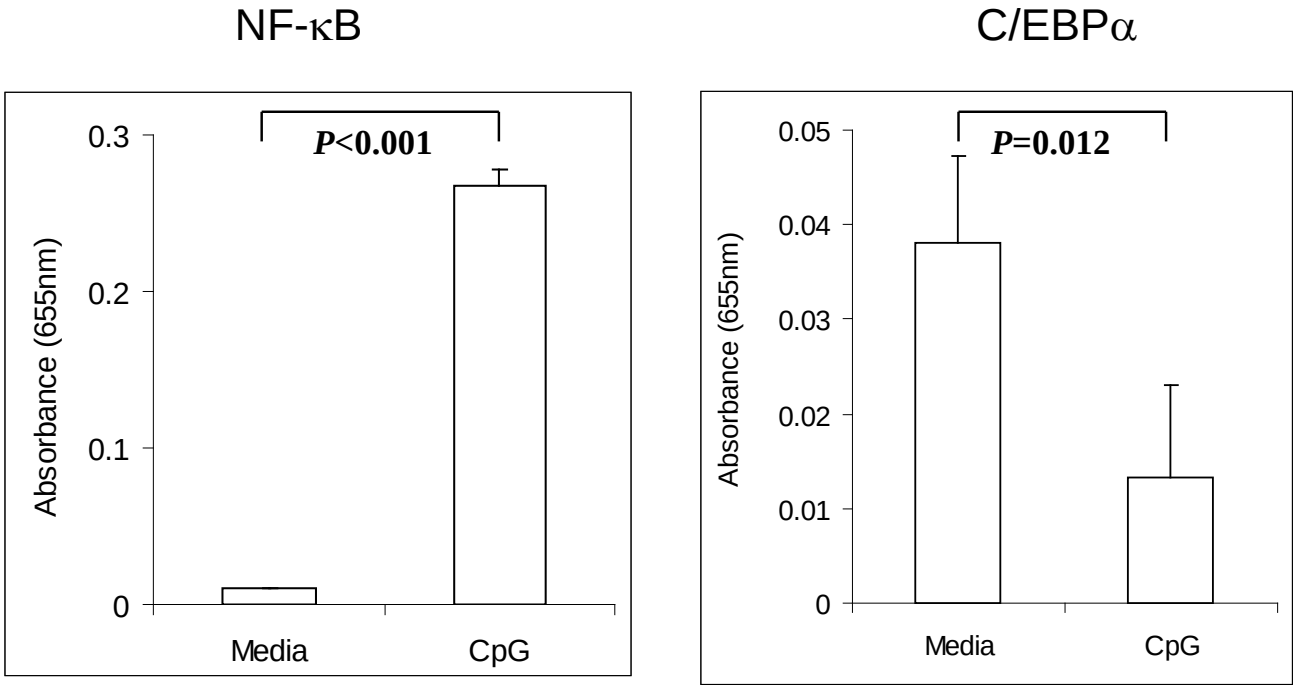
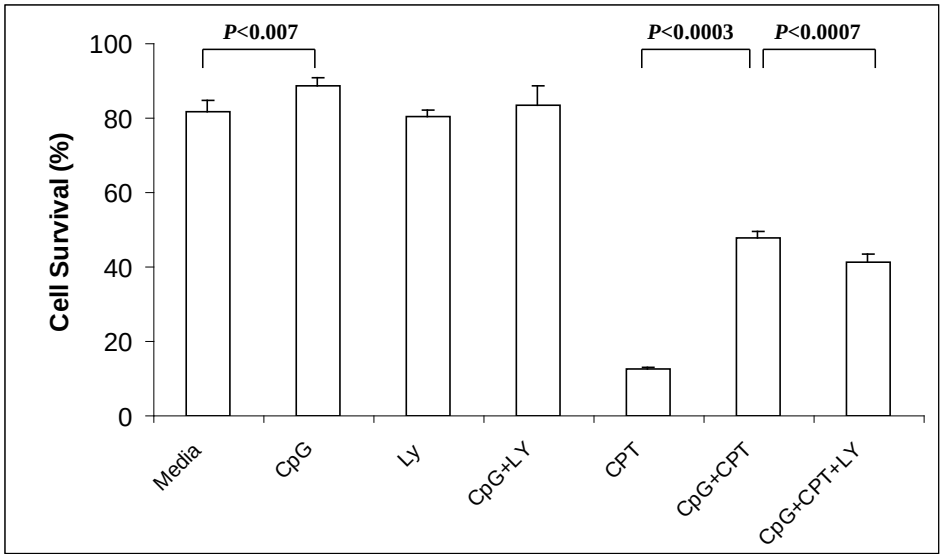


Fig. (5). In responding to CpG-ODN treatment, the nuclear level of C/EBPα in BMDCs was significantly decreased, while the nuclear level of NFκB was significantly increased. After BMDCs ($2x10^5$ cells/ml) were treated with 10 μM CpG-ODN, nuclear proteins were extracted. ELISAs for NFκB and C/EBPα were used to compare the levels of activated nuclear C/EBPα and NFκB in CpG-ODN-activated BMDCs. Results represent the mean of 3 separate experiments ± SD.

(**A**)



(**B**)

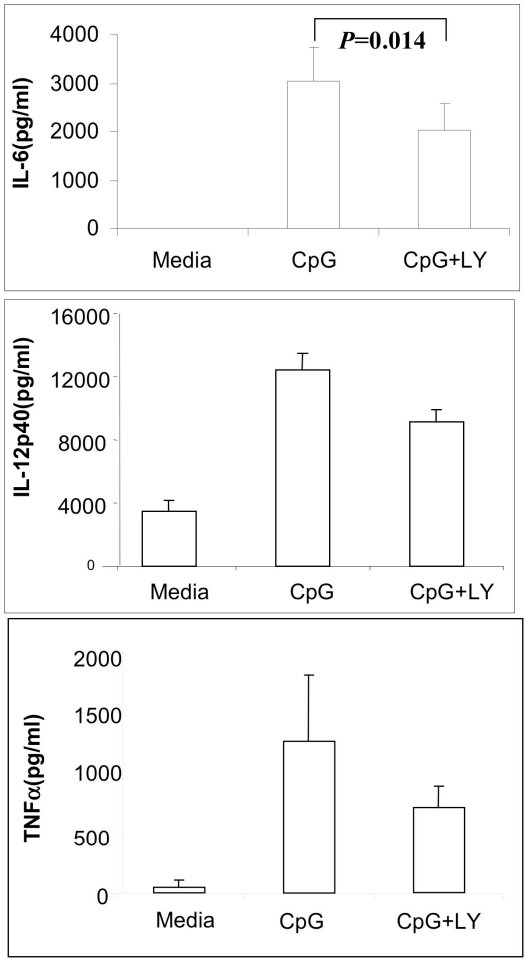


Fig. (6). Effect of PI3K inhibitor on BMDC survival and cytokine production. BMDCs ($2x10^5$ cells/ml) were treated with LY294002 (1.4 μM) for 1 hr and cells were further treated with CpG-ODN (10 μM) for 24 hrs. Cell apoptosis was examined using flow cytometry after cells were stained with anti-annexin V and PI. Percentage cell survival was determined as negative for both markers (**A**). The concentration (pg/ml) of IL-6, IL-12p40 and TNFα present in the culture medium was determined (**B**). The results represent the mean of 3 separate experiments ± SD.

DISCUSSION

CpG-ODN activates DCs and promotes adaptive immune responses [4] and this activation is critical for mediating the adjuvant effect of CpG-DNA in promoting *in vivo* productive Th1 and cytotoxic T lymphocyte (CTL) responses. TLR9 is a necessary component of the CpG-ODN receptor [23]. Unlike other TLRs, TLR9 is mainly expressed at the endosome with an extracellular leucine-rich repeat and a cytoplasmic TLR/IL-1R (TIR) domain [24]. CpG-DNA uptake activates the TIR signalling pathway *via* MyD88 and TRAF6, leading principally to activation of transcription factors NFκB and AP-1 and regulating inflammatory responses. Several studies have reported that CpG-ODN promoted survival of macrophages [12, 25], DCs [7], neutrophils [26] and B cells [27]. However the transcriptional responses and the associated regulatory mechanisms involved in regulating DC survival are largely unclear. In this study we report that activation of BMDCs by CpG-ODN resulted in inhibition of cellular apoptosis. We used DNA microarray and TELiS database analysis to predict activated transcription factors that control BMDC activation and survival in response to CpG-ODN treatment. We found that, in addition to NFκB activation, other transcription factors such as C/EBPα are also involved in BMDC activation and survival by CpG-ODN treatment.

Both NFκB and AP-1 are well-documented transcription factors identified in CpG-ODN-induced DCs activation reported previously [4, 13]. Our microarray and TELiS analysis correctly predicated the involvement of these two key transcription factors in BMDC activation and survival. Thus the combination of microarray and TELiS data analysis appears to be a useful tool to investigate the involvement of transcription factors in important biological functions. Most importantly, the expression-based monitoring of transcription factor activity analysis also predicted the involvement of several novel transcription factors in response to CpG-ODN-mediated BMDCs activation and survival. These included IFNγ inducible transcription factor (IRF-1), a transcription factor that binds to the promoters of IFN-stimulated response element ISRE [28], Max [29], C/EBPα [25] and others (see Table **2**).

C/EBPα is a potent regulator of cell-cycle arrest by blocking cell-cycle progression at the G1-S boundary through interaction with a number of factors, such as p21 [30], CDK2 and CDK4 [21], retinoblastoma tumor suppressor (pRB) [31] and PI3K/Akt/PP2A [22]. Based on these biological functions of C/EBPα and our bioinformatics analysis, it is possible that C/EBPα activity is modulated in response to CpG-ODN. We therefore examined C/EBPα protein expression levels in BMDCs in response to activation by CpG-ODN and found that nuclear levels of C/EBPα were significantly reduced in response to CpG-ODN treatment, the opposite effect to the increased level of NFκB found in the nucleus after activation.

As both PP2A and PI3K are likely to be up-stream signalling components of C/EBPα [22], we also examined the involvement of PI3K in controlling C/EBPα dephosphorylation by using the PI3K inhibitor LY294002 in BMDCs. We found that LY294002 treatment induced significant suppression of BMDC survival mediated by CpG-ODN (Fig. **5**). This result suggested that PI3K partially controlled C/EBPα activity in CpG-ODN-mediated BMDC survival and activation since blocking PI3K with LY294002 also suppressed the release of cytokines by BMDCs.

CONCLUSION

The treatment of BMDCs with CpG-ODN causes their activation and prevention of apoptosis, and activates several transcription factors such as NFκB and C/EBPα, which may play some critical roles in regulating cell survival and fate.

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