

Chemokine CCL9 (MIP- 1 γ), a gene identified for its Up-regulation by CpG-ODN and analysis of its signaling pathways in mouse macrophage and microglial cells

C. Ravindran*[†] Yung-Chih[†] and Shu-Mei Liang[†]

[†]Institute of Bio Agricultural Sciences, Academia Sinica, Taipei, Taiwan

Abstract.

CpG-ODN are bacterial DNA sequences that induce inflammatory signals via TLR9. CpG-ODN stimulation is currently under investigation in patients with allergic diseases like inflammation. However, the mechanisms involved are not fully understood. Here we demonstrate for the first time that the CpG-ODN stimulates the chemokine CCL9. The gene CCL9, is responsible for activation of osteoclasts through its receptor CCR1, suggesting an important role in bone resorption. The results showed the signaling pathways of CpG-ODN induced CCL9 expression goes through different pathways in Bv2 and Raw 264.7 cells. The expression of CCL9 induced by Amyloid beta in microglial (Bv2) cells was also observed and discussed, which may further contribute in understanding the mechanism that link in clearing the amyloid plaques in Alzheimer disease.

Key words: Chemokine; Bacterial; macrophages; Neuroimmunology; Molecular biology

*Address for correspondence: Dr. C. Ravindran, National institute of oceanography, DonaPaula, Goa, India, 403 004, Email-cravindran@nio.org

Phone no.: - +91-(0)832-2450442 Fax no. : - +91-(0)832-2450602

Introduction

The mammalian Toll-like receptors (TLRs) are expressed on macrophages and dendritic cells, which are primarily involved in innate immunity. Pathogen recognition by TLRs provokes rapid activation of innate immunity by inducing production of proinflammatory cytokines and up-regulation of co-stimulatory molecules. The activated innate immunity subsequently leads to effective adaptive immunity [28, 34, 4, 21]. The vertebrate immune system has evolved innate immune defense pattern recognition receptors (PRRs) that detect unmethylated CpG motifs within bacterial DNA requires Toll-like receptor (TLR) 9, which triggers alterations in cellular redox balance and the induction of cell signaling pathways including the mitogen activated protein kinases (MAPKs) and NF κ B [35, 22]. It had been showed that TLR9 responds very specifically to DNA sequences that distinguish bacteria from mammals. The key discovery was that the TLR9^{-/-} mice did not respond to a specific bacterial DNA sequence [12].

Bacterial DNA consists of high frequency of unmethylated CpG motifs than vertebrate genomic DNAs due to combination of CpG suppression and CpG methylation [7, 26, 33]. These CpG motifs flanked by two 5' purines and two 3' pyrimidines activate host defense mechanisms leading to innate and acquired immune responses [20, 17]. A molecular understanding of cellular recognition of CpG-DNA is only now beginning to emerge [19, 36, 1]. Synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODN), like bacterial DNA with the CpG moiety (CpG-DNA), induce potent Th1-like immune responses that are protective against several infectious agents and immune disorders in animal models [16]. Bacterial DNA stimulates the innate immune system cells such as macrophages/monocytes and dendritic cells to produce various inflammatory cytokines including IL-1, IL-6, IL-8, IL-12, TNF- α , and IFN- γ [17, 2]. Thus, CpG-ODN serves as an adjuvant and immunomodulator in vaccines against a wide variety of targets, including infectious agents, cancer antigens and allergens [32]. Analysis on BALB/c mice to CpG-ODN indicated that normal macrophages from the spleen and lymph nodes are triggered by CpG-ODN to increase MIP-1 α , MIP-1 β , MIP-1 γ , RANTES, JE/ MCP-1, and IP-10 mRNA expression [33]. This was confirmed by studies of RAW 264.7 murine macrophages. Further, they confirmed that the increase in chemokine mRNA levels occurred very rapidly and did not require other cell types, indicating that CpG-ODN directly stimulate macrophages to produce these chemokines [33].

The mechanism of bacterial pathogens induce inflammatory responses in CNS (Central nervous system) is unclear due to the blood-brain barrier (BBB). Recent evidences showed the role of TLR9 in

inflammation suggest that in the brain, TLR9 may function as a receptor for CNS-infiltrating bacterial DNA and may thus initiate inflammatory responses in the CNS [33]. The recent studies on intracisternally administered bacterial DNA containing CpG motifs and intracranial injection of CpG-ODN in microglial cells suggests that TLR9 is expressed in the region of the brain parenchyma that mediates the CpG-ODN induced inflammatory signal. These results were further supported by a study that used cultured purified astrocytes and microglia, in which the production of several proinflammatory cytokines and chemokines, including IL-1h, IL-6, IL-12, TNF- α , MIP-1 α , and MIP-1 β , was rapidly up-regulated after being stimulated with CpG-ODN [33]. Thus, in addition to increased cytokine and Ig production, the immune response elicited by CpG DNA is characterized the production of chemokines that may help recruit immunologically relevant cells to sites of ODN administration. The precise mechanism of action of CpG-DNA and CpG-ODN and the TLR9-specific signaling have not been clearly studied.

In the present study, the chemokine CCL9 is identified for the first time for its induction by CpG-ODN in both mouse microglial (Bv2) and macrophage (RAW 264.7) cells and the signaling pathways of CpG-ODN induced CCL9 expression in both the cells were compared and analyzed. The expression of CCL9 induced by Amyloid beta in microglial cells was also observed and discussed for its possible role in clearing the amyloid plaques in Alzheimer disease.

Materials and Methods

Reagents

Tlr9 ligands, CpG ODN 1668 (5'-TCC ATG ACG TTC CTG ATG CT-3'), CpG-ODN 2006 (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3'), IS ODN 6, D19 (5'-GGT GC A TCG ATG CAG GGG GG-3'), and TLR ligands PAM (Tlr2), Poly I: C (Tlr3), LPS (Tlr4), Flagellin (Tlr5), R848 (Tlr7), obtained from MDBio, Inc. (Taipei, Taiwan). The inhibitors, PI3K, ERK, p38, IKK and Chloroquine are purchased from Sigma (USA). A β 1-40, A β 1-42, A β 25-35 are purchased from Sigma (USA). Phosphorothioate-modified CpG-B ODN and GpC-ODN were synthesized by MDBio (Taipei, Taiwan). The mouse specific ODN sequences used are as follows, CpG-B ODN, 5'-TCC ATG ACG TTC CTG ATG CT -3' and GpC-ODN, 5'-TCC ATG AGC TTC CTG ATG CT-3'.

Cell culture

Cell lines were obtained from the American Type Culture Collection (Rockville, MD). Mouse RAW264.7 macrophage and BV2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 200 mmol/L *L*-glutamine, and 50 µM β-mercaptoethanol in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 days for all experiments. RAW 264.7 cells, which have been shown to express TLR9 and respond to CpG-DNA stimulation [33, 2], were cultured in RPMI1640. Cells were preincubated with or without inhibitors for 1 h before CpG-B ODN treatment unless specified otherwise. The duration of CpG-B ODN treatment varied depending on the requirement of the experiments.

RNA isolation and Quantitative RT-PCR analysis

Total RNA extraction from Raw and BV2 cells induced by several Tlr and Tlr9 ligands and kinase inhibitors were made according to the manufacturer protocols (Viogene RNA extraction kit, Taiwan). The RNA preparations were treated with ribonuclease-free deoxyribonuclease (Dnase) I (invitrogen) to remove residual genomic DNA. Total RNA were subjected to quantitative RT-PCR for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and oligo (dT)₁₅ primer for 1 h at 42°C or according to manufacturer's (invitrogen) instructions. Thereafter, equal amounts of cDNA products were amplified for CCL9 expressions. The primers used in this study are CCL9 (forward 5'-AAC AGA GAC AAA AGA AGT CCA GAG-3', reverse 5'-CTT GCT GAT AAA AGA TGA TGC CC-3' (NM_011338)), CCR1 (forward 5'-AAG AGC CTG AAG CAG TGG AAG-3', reverse 5'-GCA GCC ATT TTG CCA GTG-3' (NM_009912)); TLR9 (forward 5'-AGG CTG TCA ATG GCT CTC AGT T-3', reverse, 5'-TGA ACG ATT TCC AGT GGT ACA AGT-3); β-actin (forward, 5'-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3'; reverse, 5'-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3') as control using specific set of primers, with the required cycle numbers of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1min. The amplified PCR products were fractionated on at 1% agarose gel in 1X tris-acetate-EDTA (TAE) buffer for 60 min at 80V and 25°C and visualized by staining with 0.6 µg/mL of ethidium bromide. The intensities of the bands were measured with aid of IMAGE GUAGE V. 12 analysis software, and the ratios to β-actin was determined.

Stable transfections

The DNA plasmids used for transfections encoded dominant negative (dn) version of MyD88. For transfections, the cells were seeded at a density of 5×10^6 cells/25-cm² plate and left for 24 h. The cells were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Two days after transfection, the antibiotic G418 (1 mg/ml) was used for clonal selection. The G418-resistant clones were expanded and then screened.

Results

Effect of CpG ODN on TLR9 expression in Raw 264.7, BV2 and Neuron cells.

The time course study was performed in the CpG-ODN induced expression levels of Tlr9 in RAW 264.7, BV2 and neuron cells at different time intervals (Fig. 1a, 1b). Tlr9 expression level was found to increase constantly from 0 to 24th hour in Raw 264.7 cells (Fig. 1a) and also the expression levels was found high with other Tlr's tested (data not shown). The CpG-ODN stimulation levels in BV2 and neuron cells were significantly found high at 8th hour. Since preliminary experiments in our lab showed that 1.5 μ M CpG-ODN caused more contrasting results between normal and CpG-ODN treated cells, 1.5 μ M CpG-ODN was used throughout the experiments. To distinguish CpG-ODN treated samples from the control, total cellular RNA of normal and CpG-ODN treated cells was isolated.

Effect of CpG ODN on CCL9 expression at different time intervals in Raw and BV2 cells.

Several recent reports indicate that CpG ODN can stimulate the production of chemotactic factors in vivo [33]. To define the source and extent of this chemokine production, Takeshita and his group [33] cultured lymphocyte, NK and macrophage cell lines in vitro with 0.1- 3 μ M CpG-ODN. In the present study we performed the chemokine CCL9 expression levels in Raw 264.7 and BV2 cells treated with 1.5 μ M CpG-ODN. The time course study showed the CpG ODN induced expression levels in BV2 cells high at 8th hour and low during 24th hour (Fig. 2a). The similar observation was found in RAW macrophage cells, that is high expression level at the 6th hour and gradual decrease in 24th hour (Fig. 2a). As there was considerable increase in the expression levels during the 8th hour with CpG ODN, the other different Tlr9 ligands also were treated with macrophage and microglial cells at 8 hours for their stimulation levels. The results showed that the Tlr9 ligand, CpG-ODN treated cells showed maximum expression levels than the other Tlr ligands (Fig. 2b). The Tlr ligands like R848, PAM, Poly I: C also showed some CCL9 expression level in BV2 cells, but lower than the Tlr 9 ligand (Fig. 2b). The

Tlr and Tlr9 ligands treated RAW macrophage cells showed higher amount of CCL9 expression levels than BV2 cells (Fig 2b).

Effect of CpG ODN on CCL9 receptor CCR1 expression in Raw/Bv2 cells at different time intervals

CCR1, the high affinity receptor for CCL9 was also analysed for the CpG-ODN induced expression levels in RAW and BV2 cells at different time intervals (Fig. 3). The CCR1 expression induced by CpG-ODN was found high at 8th hour and decreased at 24th hour in BV2 cells (Fig. 3). The similar observation was found in RAW macrophage cells, that is high expression level at the 6th hour and gradual decrease in 24th hour (Fig. 3). The time course study of CCR1 expression levels correlates with the time course experiment of CpG-ODN stimulation of CCL9 by having elevated levels at the 8th hour.

The Tlr ligands such as R848, PAM, Poly I: C, D-19, Flagellin, and Tlr 9 ligand CpG-ODN were treated with the Raw and Bv2 cells. The maximum level of expression of CCR1 was found in CpG-ODN treated macrophage and microglial cells. The other Tlr ligands PAM, R848, LPS also showed some distinct stimulated expression levels. This was the similar result with that of the CCL9 expression studies (Fig.3).

Effect of treatment of Bv2 / Raw 264.7 cells with inhibitors and dnMyD88 for CCL9 pathway analysis

We also analyzed the signaling pathways for CCL9 expression using chloroquine, an inhibitor of lysosomal acidification known to abolish signaling of the intracellularly located TLRs [29]. The treatment of chloroquine (CQ) in Raw and BV2 cells showed that the coincubation of CpG-ODN with chloroquine was completely blocked and no activation was observed in CCL9 expression (Fig4a, b). Thus, the DnMyD88 (Fig. 4c) and chloroquine (Fig4a, b) treated cells confirms that the CCL9 expression adopts MyD88 Pathway.

Hacker [10], showed CpG DNA also activates stress kinases such as p38 MAPK and PI3K. Stress kinase activation is essential for CpG-DNA-induced cytokine release of TNF- α and IL-12 [10]. These kinases and NF- κ B are also involved in TLR4 and TLR2 signal transduction pathways [31, 9]. Recently, it was found that inhibition of PI3K with wortmannin prevents activation of not only NF- κ B, but also p38 MAPK and ERK in TLR2-stimulated neutrophils, and suggested that the PI3K signaling

cascade occupies a central role in TLR2 induced activation of neutrophils [30]. Although the interaction between CpG DNA and TLR9 has been shown to be dependent on wortmannin-sensitive members of the PI3K family [13], it is unclear whether the PI3K signaling cascade also plays a central role in the cells activated via the TLR9 pathway. Kuo [22], the senior researcher from our lab, clearly demonstrated that class I and class III PI3Ks play distinct roles in TLR signaling pathways and selective activation or inhibition of these PI3Ks might be useful for certain immunological or therapeutic applications. Thus the macrophage and microglial cells were treated with different inhibitors for signaling pathways of CCL9 expression and found that the CCL9 expression is inhibited by P38, ERK and IKK inhibitors in BV2 cells and only P38 in Raw 264.7 cells (Fig. 4d).

Discussion:

We initiated this study by characterizing the expression of inflammatory chemokine CCL9 mRNA induced by CpG-ODN in primary mouse macrophages and microglial cells. Earlier studies have shown that the CpG-ODN activate macrophages and microglia in vitro, which leads to the production of various cytokines and chemokines [33]. Chemokines are a superfamily of many small (8-16 kDa) cytokines with structural similarity and chemotactic activity. According to the numbers and positions of conserved cysteine residues, chemokines are divided into four subfamilies: CXC, CC, C, and CX3C [27]. Chemokines are secreted by leukocytes and various types of stromal cells, and they play a pivotal role in lymphocyte migration and recirculation, modulation of the immune response, and leukocyte recruitment to inflammation sites [25]. They serve as chemotactic agents to induce the migration and adhesion of leukocytes. The chemokines like MIP-1 α , MIP-1 β , MIP-1 γ , RANTES, JE/ MCP-1, and IP-10 had been analyzed for its CpG ODN induction [33] but so far no studies had been made on chemokine, CCL9 for its induction by CpG-ODN. Thus, we attempted for the first time and elucidated CCL9 for CpG-ODN induction.

Additional studies have revealed that the precise MAPK signaling pathways involved in this process differ depending on the cell types. For example, TLR4 activates ERK in macrophages, whereas it has no effect on ERK activity in microglial cells [37]. In addition, TLR9 induces ERK activation in macrophages, while it fails to induce the ERK activity in dendritic cells [11]. One outcome of these TLR signaling pathways is the transcriptional induction of a series of proinflammatory genes. Interestingly, the signaling pathways needed to induce each target gene are also unique. For example, in dendritic cells, TLR9-mediated TNF- α production requires ERK activation, whereas this signaling pathway

inhibits IL-12 production [11]. The studies on the signal transduction pathways mediated by TLRs have demonstrated that TLRs induce inflammatory signals through the activation of NF- κ B and MAPKs. By and large, the results of these reports suggest that some of the signaling pathways activated by TLRs are cell type-specific. Therefore, the present study was analyzed for the signal transduction pathways of CCL9 stimulation that are mediated by TLR9 in microglial and Raw 264.7 cells.

We found that CpG ODN induce CCL9 gene expression in both the cells but with high CpG ODN stimulation at the time 8th hour in BV2 cells and at the 6th hour in RAW 264.7 and gradual decrease in 24th hour. The Tlr and Tlr9 ligands treated RAW macrophage cells showed higher amount of CCL9 expression levels than BV2 cells. In addition, we found that CCR1 the receptor of CCL9 which also showed robust stimulation by CpG ODN with both the cells but more activation microglial cells than RAW 264.7. The macrophage and microglial cells were treated with different inhibitors for signaling pathways of CCL9 expression and found that the CCL9 expression is inhibited by p38, ERK and IKK inhibitors in BV2 cells and only p38 in Raw 264.7 cells. It was previously reported that CpG ODN activates ERK in macrophages resulting in downregulation of IL-12 expression [11]. This is in contrast to what occurs in dendritic cells, in which CpG ODN fail to induce ERK activation and induce strong IL-12 expression [11]. Inhibitor p13 does not show any inhibition in both the cells, but it was observed that the induction level of CCL9 was more in RAW 264.7 cells than the microglial cells. Further the treatment of chloroquine (CQ) showed that CCL9 adopts the MyD88 pathway. Thus characterization of the TLR9-activated signaling molecules in macrophages and microglial cells by an in vitro assay showed that the expression of CCL9 adopts different pathways in the above cells tested.

In central nervous system (CNS), there is limited understanding as to the cellular sources of chemokine production, or even which cells have chemokine receptors. Immunohistochemical analysis of involved brain tissues from patients with Alzheimer's disease (AD) revealed high level expression of CXCR2 receptor in the neuritic portion of plaques, surrounding deposits of amyloid [14]. This immunoreactivity was detected in regions of the diseased brain which under normal circumstances showed very low level expression. These findings were later confirmed by another group also showing hippocampal neuronal expression of CXCR2 in AD plaques co-localizing β -amyloid (β A) polypeptides [38]. Recently [14], a marked increase in CCR1 expression in neurons of the entorhinal cortex and somatic staining of hippocampal neurons of the CA1 and CA3 region was found. However, they also not observed any expression of CCR1 in cortical or hippocampal neurons of normal age matched brain tissue. CCR1 staining was also present in dystrophic processes around senile plaques. Confocal

microscopy studies showed these processes to be neurofilament positive and CD68 negative. These findings suggest that CCR1 is up-regulated in affected neurons in individuals with Alzheimers disease, perhaps in response to the disease process. Previous results of the chemokine MIP-1 α or CCL3, a chemokine expression, highly sensitive to A β (1-42) stimulation [8] and the CCR1 up-regulation in Alzheimer affected neurons, the major receptor of CCL9 initiated our study to extend the analysis of CCL9 with Alzheimer disease. We found CCL9 can be expressed by A β in microglial cells (Fig. 5). A β stimulated CCL9 expression extended our knowledge that CCL9 may contribute further understanding of mechanism that link in clearing the amyloid plaques in Alzheimer disease. We also confirmed CCR1, the major receptor of CCL9 is expressed in both BV2 and raw cells by CpG-ODN. Thus the hypothesis is made that the Tlr9 recognition of CpG-ODN enhances both, CCL9 expression and the FPR2 (Formyl peptide receptor) uptake of A β (Fig. 6). Amyloid beta is formed by the sequential cleavage of APP (amyloid precursor protein) by β -secretase and amyloid beta enters the cell via FPR2 and form plaques. The A β uptake via FPR2 also stimulates the CCL9 secretion (Fig. 6). The secreted CCL9 enters via CCR1 possibly by an autocrine pathway and attracts the microglial cells into the plaque area for the clearance of A β Plaques.

In conclusion, the results of this study demonstrate that CpG-ODN stimulate CCL9 expression in macrophages and microglial cells via the activation of MyD88, further the signaling differs in other kinases for the CpG-ODN induced chemokine CCL9 gene expression in Raw 264.7 and microglial cells. The novel findings of A β stimulated CCL9 expression may contribute further understanding the mechanism that link in clearing the amyloid plaques in Alzheimer disease.

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Figure Legend

Figure 1a and 1b: Induction of TLR9 by CpG-ODN. Raw 264.7 (1a) and Bv2 cells (1b)

were stimulated with 1.5 μ M CpG-ODN for the indicated times. RT-PCR was then performed to analyze gene expression levels. β -actin was used as an internal control. The experiment was repeated three times with similar results.

Figure 2a and 2b: Induction of Ccl9 by CpG-ODN and TLR ligands. Raw 264.7 and

Bv2 cells were stimulated with 1.5 μ M CpG-ODN (2a) and TLR ligands (2b) for the indicated times. GpC-ODN (1.5 μ M) was used as the negative control in 2b. RT-PCR was then performed to analyze gene expression levels. β -actin was used as an internal control. Data represent mean \pm SEM. ($n = 3$). Con = Control

Figure 3a and 3b: Induction of Ccr1 by CpG-ODN and TLR ligands. Raw 264.7 and

Bv2 cells were stimulated with 1.5 μ M CpG-ODN (3a) and TLR ligands (3b) for the indicated times. GpC-ODN (1.5 μ M) was used as the negative control in 3b. RT-PCR was then performed to analyze gene expression levels. β -actin was used as an internal control. Data represent mean \pm SEM. ($n = 3$). Con = Control.

Figure 4a and 4b: Ccl9 expression in Raw 264.7 and Bv2 cells treated with CpG ODN

and Chloroquine (CQ). Raw 264.7 and Bv2 cells were stimulated with different doses of CpG-ODN and Chloroquine (CQ) as indicated. Untreated cells were used as the control. RT-PCR was then performed to analyze gene expression levels. β -actin was used as an internal control. The experiment was repeated three times with similar results. Data represent mean \pm SEM. ($n = 3$).

Figure 4c: Expression of Ccl9 using Dn-MyD88 in Raw 264.7 cells were stimulated with

1.5 μ M CpG-ODN. GpC-ODN (1.5 μ M) was used as the negative control. RT-PCR was then performed to analyze gene expression levels. β -actin was used as an internal control. Data represent mean \pm SEM. ($n = 3$). C = Control.

Figure 4d: Raw 264.7 and Bv2 cells was treated with inhibitors (P13, P38, ERK, IKK)

for Ccl9 signaling pathway analysis. RT-PCR was then performed to analyze gene expression levels. β -actin was used as an internal control. Data represent mean \pm SEM. ($n = 3$). Con = Control.

Figure 5: The expression of Ccl9 induced by amyloid beta ($A\beta$) in Bv2 cells. RT-PCR

was then performed to analyze gene expression levels. β -actin was used as an internal control. The experiment was repeated three times with similar results. Data represent mean \pm SEM. ($n = 3$). Con = Control.

Figure 6: A pictorial hypothesis on possible role of Ccl9 induction by amyloid beta, $A\beta$ and CpG-ODN in Alzhemeir disease

Figure 1a

Raw cells

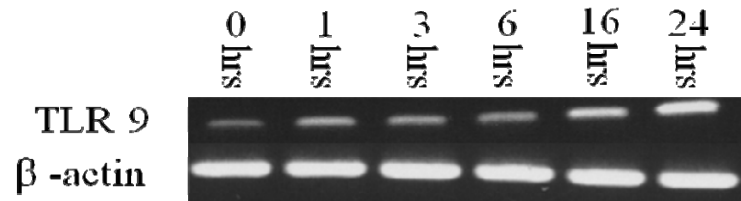


Figure 1b

Bv2 cells

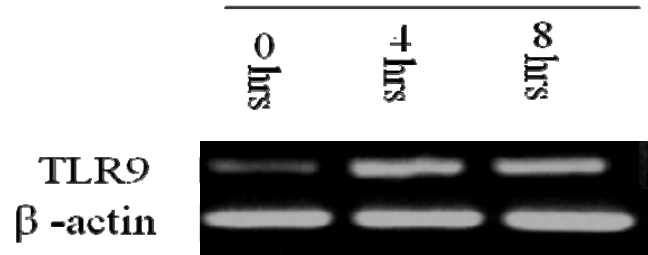


Figure 2a

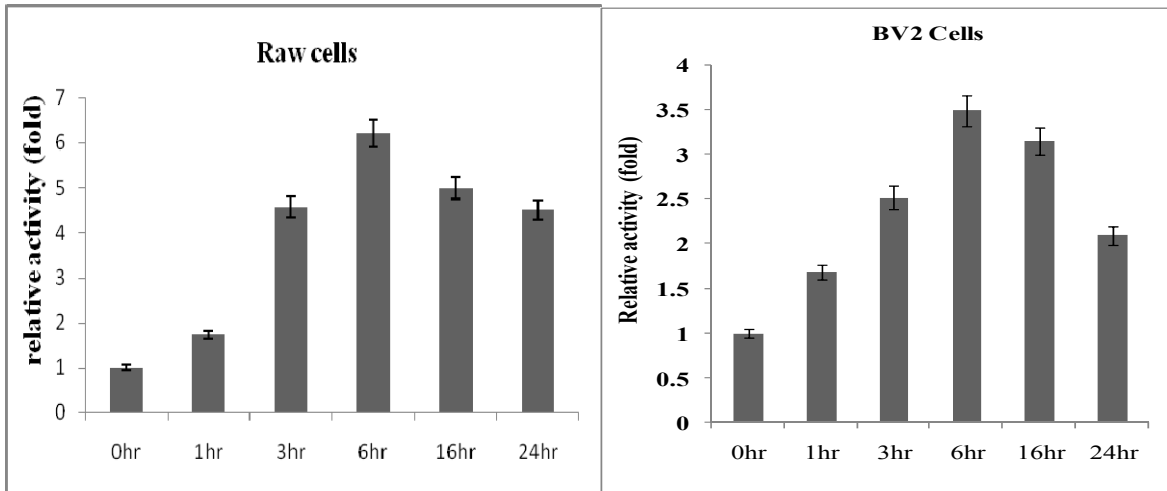


Figure 2b

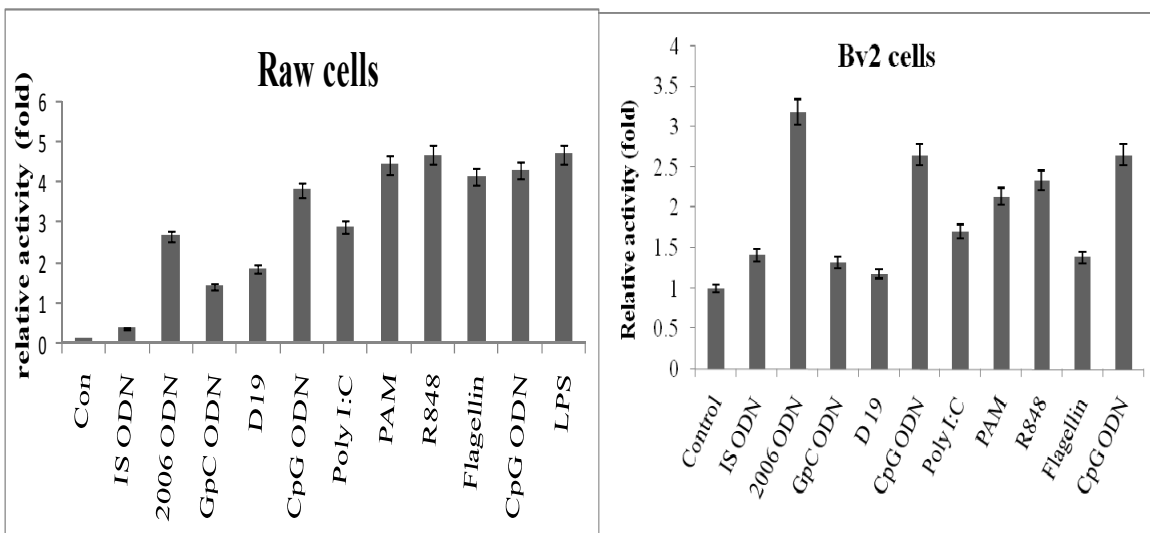


Figure 3a

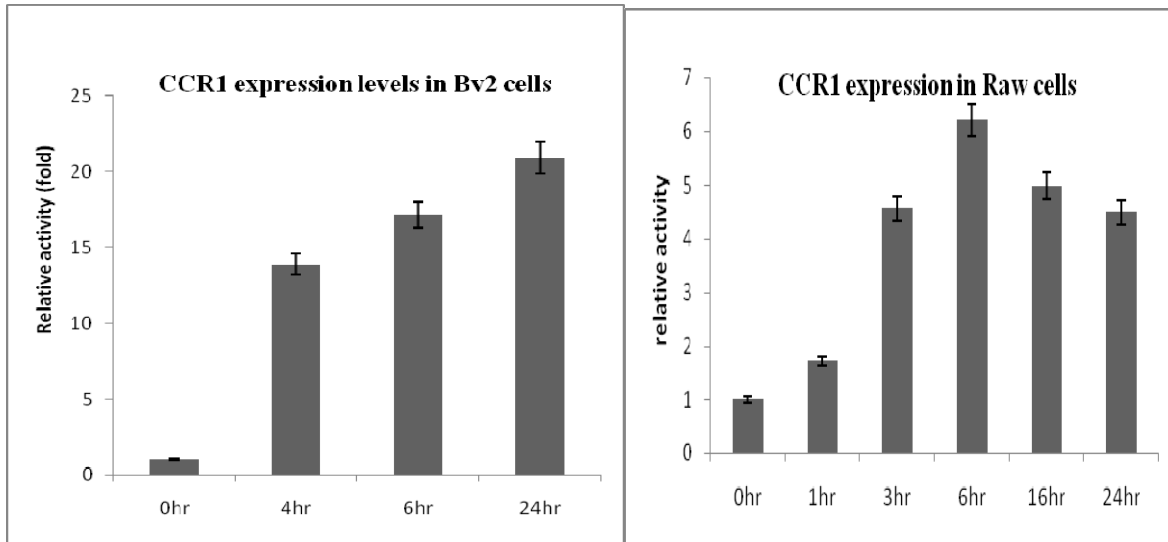


Figure 3b

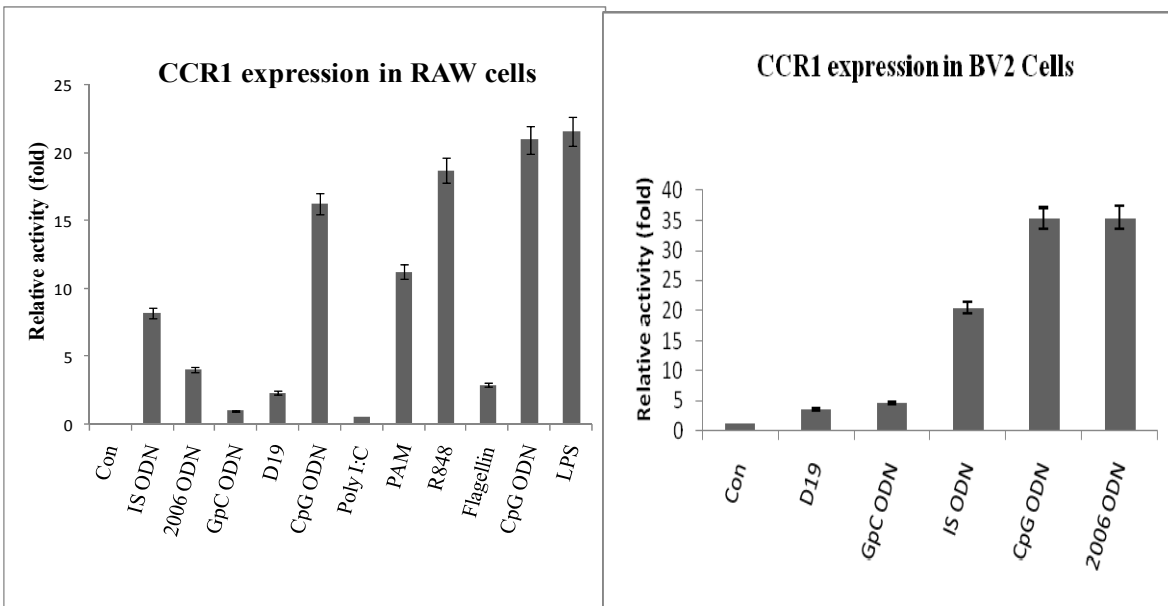


Figure 4a

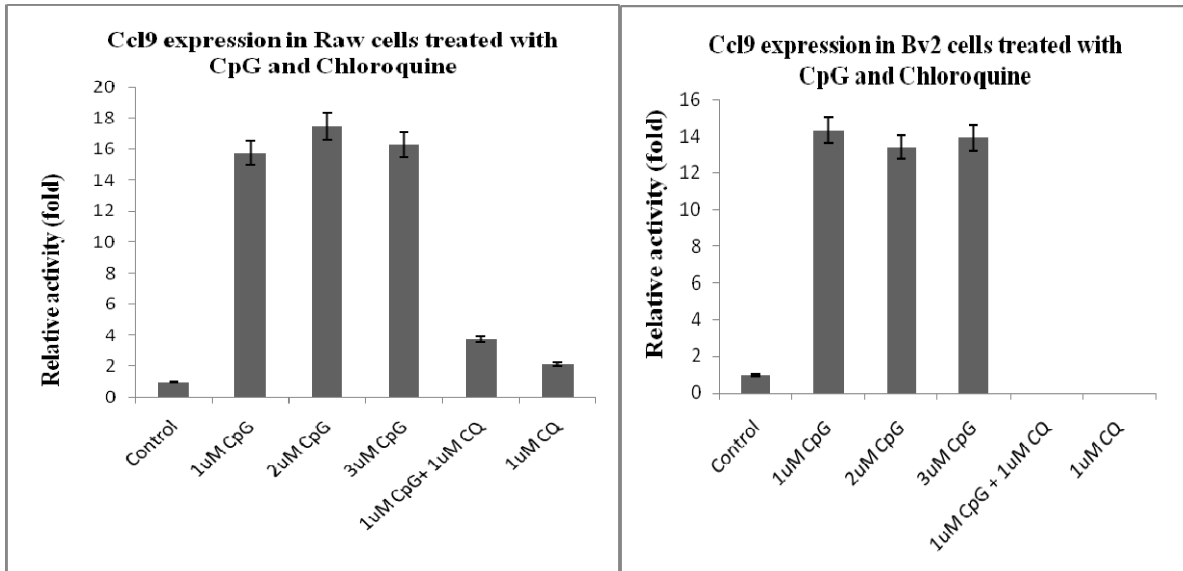


Figure 4b

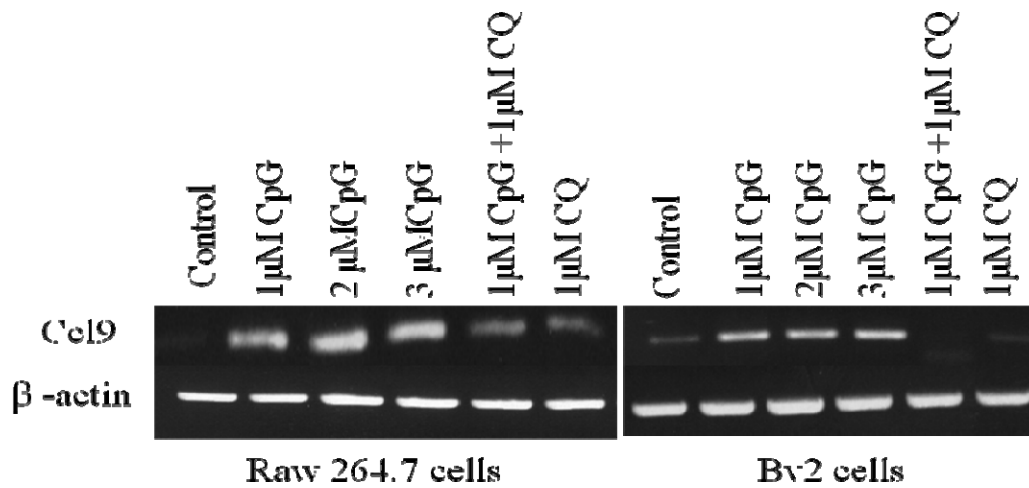


Figure 4c

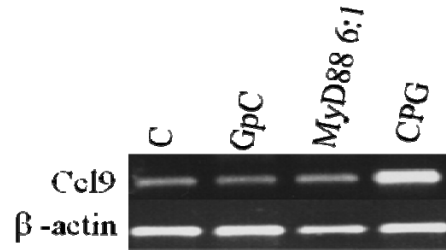


Figure 4d

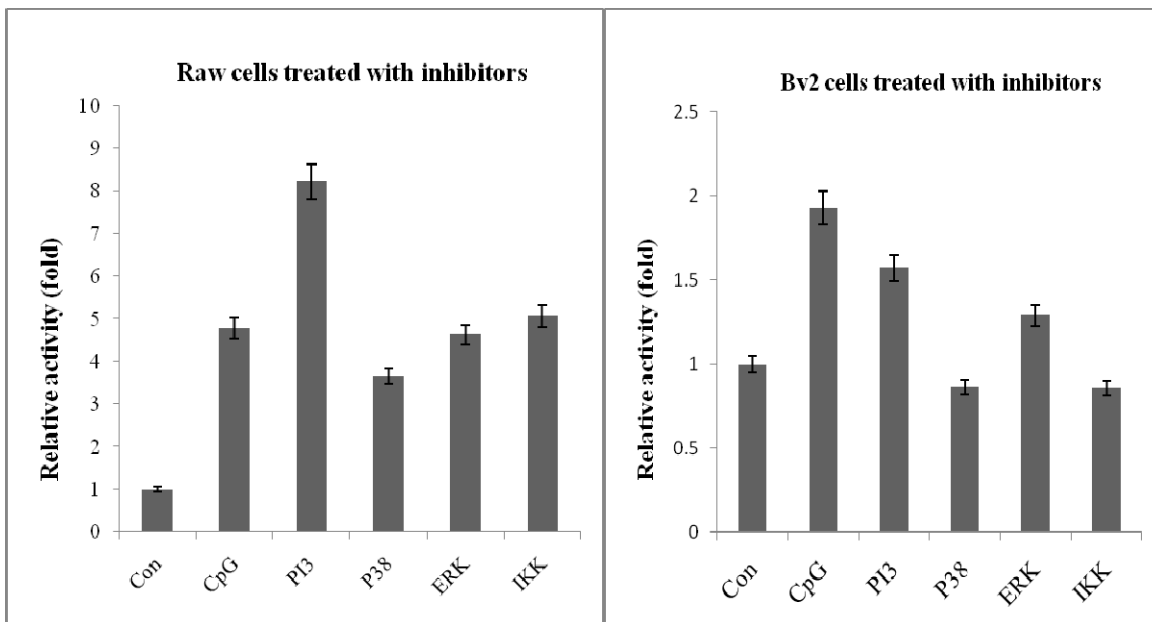


Figure 5

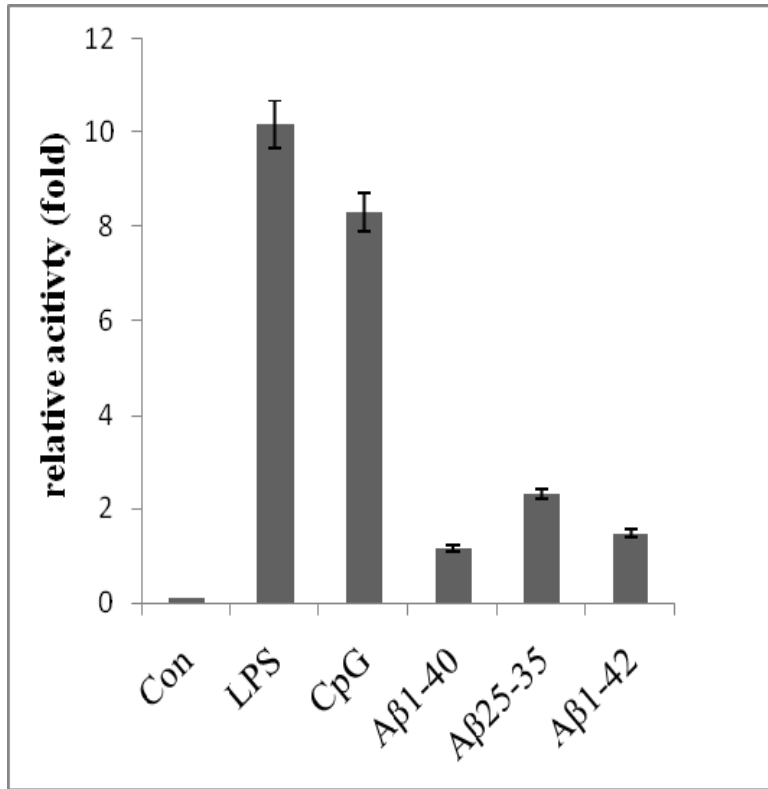


Figure 6

