CpG- and LPS-activated MAPK signaling in *in vitro* cultured salmon (*Salmo salar*) mononuclear phagocytes

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Abbreviations: MAPK, mitogen-activated protein kinase; PAMPs, pathogen-associated molecular patterns; TLRs, the toll-like receptors; LPS , lipopolysaccharide; JNK, c-Jun NH terminal kinase; ERK, extracellular signal-regulated kinase; ODNs, oligodeoxynucleotides, IL-1β, interleukin-1β; MKK, MAPK kinase; MK2, MAPK-activated protein kinase2; DCs, dendritic cells; NFkB, nuclear factor kappa B; NRF2, nuclear factor (erythroid-derived 2)-like 2; PI3K, phosphatidylinositol 3-kinase; WB, Western blot; M-CSF, macrophage colony stimulating factor; HRP, horseradish peroxidase; eEF2, eukaryotic elongation factor 2; FBS, foetal bovine serum;

Keywords: mitogen-activated protein kinase (MAPK); CpG oligodeoxynucleotides (ODNs); lipopolysaccharide (LPS); Atlantic salmon (*Salmo salar*); mononuclear phagocytes

Abstract

The Mitogen-Activated Protein Kinases (MAPK) are involved in transmitting intracellular signals downstream of diverse cell surface receptors and mediate the response to ligands such as growth factors, hormones and cytokines. In addition, MAPK are critically involved in the innate immune response to pathogen-derived substances, commonly referred to as pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS) and bacterial DNA rich in CpG dinucleotides. Currently, a great deal of knowledge is available about the involvement of MAPK in the innate immune response to PAMPs in mammals; however, little is known about the role of the different MAPK classes in the immune response to PAMPs in lower vertebrates. In the current study, p38 phosphorylation was induced by CpG oligonucleotides (ODNs) and LPS in primary salmon mononuclear phagocytes. Pre-treatment of the cells with a p38 inhibitor (SB203580) blocked the PAMP-induced p38 activity and suppressed the upregulation of most of the CpG- and LPS-induced transcripts highlighting the role of this kinase in the salmon innate immune response to PAMPs. In contrast to p38, the phosphorylation of extracellular signal-regulated kinase (ERK), a MAPK involved primarily in response to mitogens, was high in resting cells and, surprisingly, incubation with both CpG and control ODNs downregulated the phospho-ERK levels independently of p38 activation. The basal phospho-ERK level and the CpG-inducible p38 phosphorylation were greatly influenced by the length of *in vitro* incubation. The basal phospho-ERK level increased gradually throughout a 5-day culture period and was PI3K-dependent as demonstrated by its sensitivity to wortmannin suggesting it is influenced by growth factors. Overall these data indicate that both basal and PAMP-induced activity of MAPKs might be greatly influenced by the differentiation status of salmon mononuclear phagocytes.

1. Introduction

Ligands for innate immune receptors such as the toll-like receptors (TLRs) activate leukocytes by triggering complex intracellular signaling cascades. In mammals, it has been shown that TLR ligands, such as LPS and DNA rich in unmethylated CpG dinucleotides activate all major groups of MAPK in leukocytes, including monocytes, macrophages and dendritic cells (DCs) ([1], [2, 3] [4]). The major groups of MAPK include the extracellular signal regulated kinase (ERK), the c-Jun NH terminal kinase (JNK), and the p38 MAP kinase. Upon activation, MAPK are phosphorylated on the threonine (Thr) and tyrosine (Tyr) residues at their Thr-Xaa-Tyr (TXY) motif found in a highly conserved activation loop near the active site. The ERK signaling is activated mainly by mitogenic stimuli such as growth factors [3] .On the other hand, JNK and p38 pathways are activated by stress stimuli such as UV irradiation and osmotic shock, and by proinflammatory cytokines [5]

The MAPK proteins and functions are well conserved across vertebrates and it has been demonstrated that in lower vertebrates, TLR ligands such as LPS and CpG ODNs, activate the innate immune response through MAPK. More specifically, LPS, CpG ODNs and recombinant trout interleukin-1β (IL-1β)-induced phosphorylation of endogenous p38 in salmon head kidney macrophages in a dose-dependent manner[6]. In addition, in whole salmon head kidney leukocyte cultures, p38 activation was shown to be crucial for LPS induced upregulation of immune genes including IL1-β, TNF, COX2 and, to a lesser extent, CD83 [7]

A major goal of the current study has been to further investigate the involvement of p38 in the response of salmon mononuclear phagocytes to LPS and CpG ODNs. Cultures of primary cells were stimulated with *E. coli* LPS and CpG ODNs alone or in the presence of a chemical inhibitor of p38 (SB203580). This inhibitor has been widely used in mammalian

systems [8]; [9], however, data about its efficacy and specificity in lower vertebrates is scarce. In the current study, SB203580 was able to completely abolish the CpG- and LPS-induced p38 activity as determined by analysis of MAPK-activated protein kinase 2 (MK2, a direct target of p38) phosphorylation. A microarray analysis demonstrated that the upregulation of most of the CpG and LPS-induced genes was p38-dependent, highlighting the importance of the p38 kinase for the innate immune response of salmon leukocytes against pathogens. Additional data indicate that the MAPK activation by PAMPs is affected by a PI3K-dependent mechanism and the differentiation of the cells.

2. Materials and methods

2.1. Fish and reagents

Non-vaccinated healthy Atlantic salmon, *Salmo salar* L., strain Aquagen standard (Aquagen, Kyrksæterøra, Norway), 500–1000 g, was obtained from Tromsø Aquaculture Research Station (Tromsø, Norway). The fish were kept at 10°C in tanks supplied with running filtered sea water and were fed on commercial dry food. All experiments were performed according to the guidelines from the national committee for animal experimentation (Forsøksdyrutvalget, Norway).

The phosphorothioate-modified ODNs were purchased from Thermo Scientific. The sequences are as follows, where phosphorothioate modifications are marked with * : CpG-B: T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T; CpG-A: G*G*G*G*G*A*C*G*A*T*C*G*T*C*G*G*G*G*G*G. In the control, non-stimulatory ODN (GpC) the places of the cytosines and guanines in the CpG-B sequence are switched. LPS from *E. coli* 0111:B4 was obtained from Sigma Aldrich (cat.# L2630). The p38 inhibitor (SB203580) was purchased from Alexis Biochemicals. Wortmannin was purchased from Invivogen. Rabbit antibodies against phosphorylated p38 (p-p38), p-MKK3/6, p-MK2, p-ERK, p-Akt and eukaryotic elongation factor 2 (eEF2) were obtained from Cell Signaling Technology. The secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG-HRP was purchased from Santa Cruz Biotechnology

2.2. Isolation and stimulation of primary mononuclear phagocytes from Atlantic salmon

Head kidney (HK) leukocytes were isolated as described previously [10]. The organs were passed through a 100-mm pore size cell strainer (Falcon) in L-15 medium containing penicillin (60 mg/ml), streptomycin (100 mg/ml), 2% foetal bovine serum (FBS) and heparin (20U/ml). The resulting suspension was placed on a 25/54% discontinuous Percoll gradient and centrifuged at 400g for 40 min at 4°C. The cells at the interface were collected and washed twice in L-15 medium. The cells were seeded in 24-well plates at a density of $7x10^6$

cells per well. After 1 hour of incubation in L-15, supplemented with 0.1% FBS at 14°C, the cells were washed by vigorous pipetting with fresh L15 medium and the adherent mononuclear phagocytes were further incubated in L-15 supplemented with 5% FBS. In the different experiments the cells were stimulated immediately after washing or after 1, 3 and 5 days as indicated in the Results section and in the figure legends. For the Western blot analysis, the cells were lysed with 50 µl of NuPAGE LDS sample buffer, sonicated and heated for 10 min at 70°C. For RT-PCR microarray analyses, the cells were lysed with TRIzol Reagent (Invitrogen).

2.3. Western blot analysis

Cell lysates were separated by SDS⁄PAGE (4–12% precast NuPAGE; Invitrogen), followed by transfer to a 0.45 µm pore size polyvinylidene difluoride membrane (Millipore). The blots were pre-blocked for 1 hour (Tris-buffered saline, 5% BSA, 0.1% Tween-20) and incubated for 24 hours with 1:1000 dilution of the primary antibodies followed by 1 hour incubation with 1:10000 dilution of the secondary antibody in blocking solution. Detection was performed with Super-Signal West Pico substrate (Pierce Biotechnology). The membranes were stripped for 10 min in 0.2M NaOH followed by washing, blocking and antibody incubation. The size of the proteins was estimated using the MagicMark Western protein standard (Invitrogen).

2.4. Real-time PCR

The RNA isolation and the cDNA synthesis were performed with RNeasy Mini Kit (Qiagen) and TaqMan Reverse Transcription Reagents kit (Applied Biosystems) as previously described ([11]). The Real-time PCR reactions were assembled using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences, the reaction conditions and

the data analysis have previously been described [12]. Statistical analysis was carried out using the Student t test.

2.5. Microarray analysis

The cDNA microarray containing 1800 unique clones, the reagents and the procedures have been previously described [12].

3. Results

3.1. Time course of the MK2, p38, ERK and MKK3/6 phosphorylation in CpG and LPS-stimulated mononuclear phagocytes; the p38 inhibitor effectively inhibits the CpG- and LPS-induced MK2 phosphorylation

In order to analyze the efficacy of the p38 inhibitor, the cells were stimulated alone or in the presence of the inhibitor for 0.5, 2 and 4 hours prior to assessment of the phosphorylation of MK2 which is a target of p38 (Fig. 1). The CpG-B and the LPS-induced MK2 phosphorylation were completely suppressed in the presence of the p38 inhibitor at all of the tested time points. These data also showed that the LPS stimulation induced a faster response and there was a good correlation between the levels of phospho-p38 (p-p38) and p-MK2 in cells stimulated in the absence of the p38 inhibitor.

It has been previously shown that salmon MKK6 is involved in the activation of p38 [13] .In the current study, the LPS stimulation induced the phosphorylation of MKK6b/c and the putative MKK4 at all time points. The CpG stimulation upregulated detectably p-MKK6b/c only at the latest time point (4 hours). The band corresponding to salmon p-MKK6a, which migrates slightly faster than p-MKK6b/c [13], was observed in all of the samples and it was not detectably influenced by any of the treatments. Interestingly, the p38 inhibitor induced the MKK6b/c phosphorylation at all of the time points suggesting that p38 may exert a negative feedback on the MKK6b/c activity.

Unlike the p38 whose activity is mostly controlled by stress and proinflammatory stimuli [5], the activation of the ERK is controlled primarily by mitogens which signal through receptors involved in cell growth, proliferation and differentiation including G-protein coupled receptors, receptor tyrosine kinases, Integrins, and Ion Channels [14, 15]. Surprisingly, the CpG stimulation down-regulated the levels of p-ERK at all sampling time points indicating that this process is not transient but it is both fast and relatively long-lasting

(Fig. 1). Like MKK6b/c, the p38 inhibitor upregulated p-ERK in CpG-treated samples at 30 min and 2 hours of stimulation, while at the latest time-point (4 hours) positive effect was not clearly visible.

3.2. Microarray analysis – influence of the p38 inhibitor SB203580 on the CpG and LPS-modulated gene expression in salmon mononuclear phagocytes.

The microarray data shown in Fig. 1A are derived from a larger experiment and it should be pointed out that the values for the gene expression in cells stimulated with CpG and LPS in the absence of the p38 inhibitor have been previously published [12]. In the current study they have been used solely as control values for the effect of SB203580.

The chosen experimental conditions, including the concentrations of the stimulants (2 µM CpG ODNs and 50 µg/ml of LPS) and the p38 inhibitor (10µM) and the preincubation period with the inhibitor (1 hour) were based on a previous study [6] .

The microarray results in the current study demonstrate that the upregulation of most of the CpG and LPS-inducible transcripts was affected by SB203580 which highlighted the importance of the p38 signaling for the activation of the cells by these immunostimulants. Most of the genes that were weakly upregulated by the PAMP stimulation were completely suppressed by the inhibitor. Of note, DUSP5 which is involved in MAPK dephosphorylation [16] was highly upregulated by the PAMPs and it was completely suppressed in the presence of the p38 inhibitor indicating its induction by CpGs and LPS might be a part of a negative feedback mechanism. On the other hand, other highly-inducible genes involved in regulation of NF-kappaB activation, including two NF-kappaB inhibitor alpha isoforms, and also p62 and TRAF2 were partially inhibited suggesting involvement of p38-independent signaling pathways in their induction by PAMPs

The microarray analyses were performed with pooled RNA from individually stimulated samples from 3 fish. SYBR Green Real time PCR (RT-QPCR) was further used to validate microarray results using the individual RNA samples. The results shown in Fig. 2B confirmed that the p38 inhibitor consistently suppressed the CpG- and LPS-induced upregulation of the surface marker gene CD40, the macrophage inflammatory protein CCL4 and MMP9. In addition, IL-1β, an important proinflammatory cytokine was also suppressed by the p38 inhibitor.

3.3. CpG-induced p-ERK downregulation and p-p38 upregulation - dose response

Salmon mononuclear phagocytes were stimulated with increasing CpG ODN concentrations for 2 hours prior to assessment of the p-ERK and p-p38 levels. The western blot results shown in Fig. 3 indicate that the p-ERK level is slightly downregulated even in cells stimulated with the lowest concentration of CpG ODNs (0.5 μM) and the intensity of the p-ERK band further declines with increased CpG ODN concentrations. The blot was also reprobed with the p-p38 antibody to confirm the cell activation. The p-p38 band was induced by CpG ODNs at 2 μM and higher concentrations.

*3.4. The intensity of ERK phosphorylation in cultured mononuclear phagocytes correlates with PI3K activity and the spontaneous morphological differentiation of mononuclear phagocytes***.**

In the current study, preliminary experiments indicated that ERK and p38 phosphorylation might be influenced significantly by the length of *in vitro* incubation preceding the activation of the cells with PAMPs. The results presented in Fig.4A demonstrate that the p-ERK levels in non-stimulated cells increased gradually between day 1 and day 5 of *in vitro* incubation and correlated with the phosphatidylinositol 3-kinase (PI3K)

activity as indicated by the increased levels of p-Akt, which is a downstream target of PI3K. Induction of ERK phosphorylation by CpGs and inverted (GpC), control ODNs could inconsistently be detected on day 1 when p-ERK levels were relatively low. In contrast, after 3-5 days of incubation, stimulation with both ODN types exerted a negative effect on the p-ERK levels. The ERK phosphorylation in resting cells was highly sensitive to wortmannin indicating it was PI3K-dependent. In contrast, the p38 phosphorylation was not detectably affected by the PI3K inhibitor. The CpG-induced p38 phosphorylation was affected by the length of *in vitro* incubation and it was substantially lower on day 3 and barely detectable on day 5.

It has been previously demonstrated that trout mononuclear phagocytes differentiate spontaneously *in vitro* and over the course of several days develop a mature, macrophage-like phenotype [12, 17]. In the current study the morphology of the cells was examined under a microscope at each time point prior to taking the samples for Western blot (WB) (Fig. 3B). Similar to trout, salmon mononuclear phagocytes were also able to differentiate spontaneously as shown by development of a macrophage-like morphology within 5 days of *in vitro* culture.

4. Discussion

The pyridinyl imidazole SB203580 has been described as a specific p38 inhibitor and has been widely used in studies aimed to characterize the function of this kinase [8].The data presented in the current study show that it effectively inhibits the PAMP-induced p38 activity in primary salmon mononuclear phagocytes and highlight the role of p38 in the response of these cells to CpG ODNs and LPS. SB203580 acts on p38 by competitive binding within the ATP pocket but does not inhibit phosphorylation of p38 MAPK by upstream kinases [18]. Therefore, the efficacy of the inhibitor was confirmed by its potential to completely suppress the CpG- and LPS-induced phosphorylation of MK2, a p38 substrate [8]. The data correlates well with an earlier study, showing that the same inhibitor totally prevented phosphorylation of MK2 in sodium arsenite stressed CHSE-cells, in which p-p38 was upregulated [13]

In mammals MKK3, MKK6 and, in some cases, MKK4 are activators of p38 [19], however, in fish the activation of p38 by upstream kinases is poorly understood. A study has shown that teleosts lack MKK3 and salmon MKK6 homologs are involved in stress-induced phosphorylation of p38 [13]. Nevertheless, the results in this study also suggested that salmon p38 may be activated by additional kinases depending on the type of the stimulus. In the current study, the intensity of the p-MKK6 and the putative p-MKK4 bands did not unambiguously correlate with the level of p38 phosphorylation. MKK6a was constitutively phosphorylated whereas MKK6b/c (very likely MKK6c, since a previous study has shown that salmon macrophages do not express MKK6b mRNA [13]) and the putative MKK4 were weakly and inconsistently phosphorylated following LPS and CpG stimulation. These results indicate that other kinase/s may be involved in the activation of p38 by PAMPs in salmon mononuclear phagocytes.

In contrast with its inhibitory effect on p38 activity, SB203580 upregulated pMKK6b/c and had a positive effect on p-ERK levels in CpG-treated cells. It has for a long time been

known that p38 activates a negative feedback loop which in addition to p38 also affects ERK and JNK activity [20]. Upregulation of various phosphatases such as dual specificity phosphatases (DUSP) is an important mechanism through which MAPK activity is controlled [21], [22]. In the current study the CpG- and LPS-induced upregulation of DUSP5, a molecule which in mammals specifically dephosphorylates ERK, appeared to be completely p38-dependent as indicated by the microarray analysis and may have been involved in negative regulation of ERK activity by p38. On the other hand, it should also be considered that SB203580 has potential to stimulate Raf1 [23]. This kinase is involved in ERK activation and a model has shown that it may also be involved in MKK6 phosphorylation [24]. Therefore the relatively fast SB203580-induced phosphorylation of salmon MKK6b/c and ERK may also be due to a direct positive effect of the inhibitor on Raf-1 or other upstream signalling components.

Upregulation of ERK phosphorylation by SB203580 was detected only in CpG-stimulated cells in which the constitutive p-ERK levels were, surprisingly, downregulated. This downregulation was not dependent on PAMP-induced p38 activity since LPS upregulated faster p38 phosphorylation as compared to CpGs but it did not suppress the p-ERK levels at any of the assayed time points. In studies with mammalian antigen-presenting cells it has been shown that PAMPs, such as CpGs and LPS induce phosphorylation of both p38 and ERK [25] [26]. However, another study has demonstrated that, following *in vitro* differentiation for several days, stimulation of murine dendritic cells with CpG ODNs, but not LPS, suppressed ERK activity [27]. It has been shown that *in vitro* cultured trout HK-derived adherent monocytes differentiate spontaneously and following incubation for several days develop a more mature, macrophage-like phenotype. This is manifested by changes in their morphology and enhanced potential to respond to LPS stimulation [17, 28, 29]. In the current study, the non-stimulated salmon primary cells displayed similar morphological changes

when cultured *in vitro* for a period of 3 to 5 days (Fig. 3 B). It is very likely that the observed *in vitro* differentiation of salmonid mononuclear phagocytes depends on the action of autocrine growth factors, such as the macrophage colony stimulating factor (M-CSF) which is necessary for the development and the differentiation of mononuclear phagocytes to mature macrophages [30]. The action of M-CSF is mediated by ERK [31] and PI3K/Akt-mediated signaling cascades [32]. The gradually increasing levels of p-ERK and p-Akt (a downstream mediator of PI3K signalling) over the course of *in vitro* incubation correlated with the morphological differentiation of the salmon mononuclear phagocytes and could be explained by the activity of such growth factors. It has also been shown that, in mouse osteoclasts the M-CSF-induced phosphorylation of ERK is dependent on upstream PI3K activation [33]. Therefore, it is likely that the high sensitivity of ERK phosphorylation to wortmannin, a specific PI3K inhibitor [34], and the relatively good correlation between the levels of phosphorylated ERK and Akt might reflect a similar interplay between these two kinases in salmon mononuclear phagocytes as well.

In the current study, suppression of ERK phosphorylation by CpG and non-stimulatory GpC ODNs was observed only in cells incubated *in vitro* for at least 3 days. The p-ERK levels in ODN-treated cells were not down-regulated below these detected in control cells on day 1 indicating the negative effect might be due to suppression of growth factor receptor/s signalling. Interestingly, stimulation with bacterial DNA was shown to quickly downregulate the M-CSF receptor in murine macrophages [35]. However, the same effect was not induced by non-stimulatory calf thymus DNA and therefore, the observation that the p-ERK downregulation in salmon mononuclear phagocytes was also observed following treatment of with control ODNs would be difficult to explain with this phenomenon and it, most likely, depends on a different mechanism/s.

Another unexpected observation was that the CpG-induced p38 phosphorylation appeared to be gradually supressed throughout the course of *in vitro* incubation. It is possible that this might be due to changes in the kinetics of p38 activation – either a transient peak or a delayed phosphorylation. However, another explanation is the potential autocrine action of M-CSF, or analogous growth factors, since in primary murine macrophages the p38 activation by CpGs, but not LPS, was suppressed by pretreatment with M-CSF which at the same time upregulated ERK phosphorylation [36]. In addition, it has been shown that the PI3K signalling suppresses TLR-induced p38 phosphorylation in innate immune cells including DCs [37] and macrophages [38]. Enhanced activity of PI3K in macrophages results in increased DUSP1 (a p38 specific phosphatase) expression, IL-10 production and, as a result, diminished p38 activation and lowered production of proinflammatory cytokines in response to pathogens [39].

In summary, the data presented in the current study indicate that p38 signalling is crucial for activation of salmon mononuclear phagocytes by CpG ODNs and LPS. In contrast to p38, ERK is constitutively phosphorylated *in vitro* through a mechanism that is, most likely, PI3K-dependent. Explaining the downregulation of p-ERK by both CpG and control ODNs may be an interesting direction for future research.

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Figure captions

Figure 1. Time course of the MK2, p38, ERK and MKK3/6 phosphorylation in CpG and LPS-stimulated mononuclear phagocytes; the p38 inhibitor effectively inhibits the CpG- and LPS-induced MK2 phosphorylation at all of the tested time points. The cells were incubated *in vitro* for 3 days prior to stimulation with CpG and LPS alone or in presence of the p38 inhibitor prior to sample harvesting and WB analysis with the indicated Abs. Reprobing with the eEF2 Ab was used as a loading control Representative data from experiments with cells isolated from two individuals are shown

Figure 2. The p38 ihibitor SB203580 interferes with the upregulation of most of the CpG- and LPS-induced transcripts in salmon mononuclear phagocytes. A, Microarray analysis of cells stimulated with CpG ODNs and LPS for 4 hours. The values show the fold upregulation as compared to non-stimulated controls. The color intensity correlates with the level of upregulation. The microarray was probed with pooled RNA from individually stimulated samples from 3 individuals. B, The negative effect of p38 inhibition of immune genes was confirmed with SYBR Green Real Time PCR. The samples from the 3 individuals used for the microarray were analyzed separately. The values are mean fold upregulation as compared to non-stimulated cells. Statistical analysis was carried out using the Student t test (*$P < 0.05$).

Figure 3. CpG ODN-induced p-ERK downregulation and p-p38 upregulation - dose response. The cells were incubated *in vitro* for 3 days prior to stimulation with different concentrations of CpG ODNs for 2 hours and WB analysis with p-ERK and p-p38 antibodies. Reprobing with the eEF2 Ab was used as a loading control. Representative data from experiments with cells isolated from two individuals are shown

Figure 4. The intensity of ERK phosphorylation in *in vitro* cultured mononuclear phagocytes correlates with PI3K activity and the spontaneous morphological differentiation of mononuclear phagocytes. The cells on day 1 were stimulated immediately after removal of non-adherent cells (1 hour after seeding the whole HK population). The stimulations were performed with 2 μM concentration of CpG ODNs and inverted, control GpC ODNs for 2 hours prior to isolation of proteins and WB analysis with the indicated Abs (panel A) or microscopy imaging (panel B). In order to block PI3K activity, on day 5, the cells were pretreated for 1 hour with 2 μM Wortmannin prior to stimulation as indicated. Representative data from experiments with cells isolated from two individuals are shown.

Figure 1

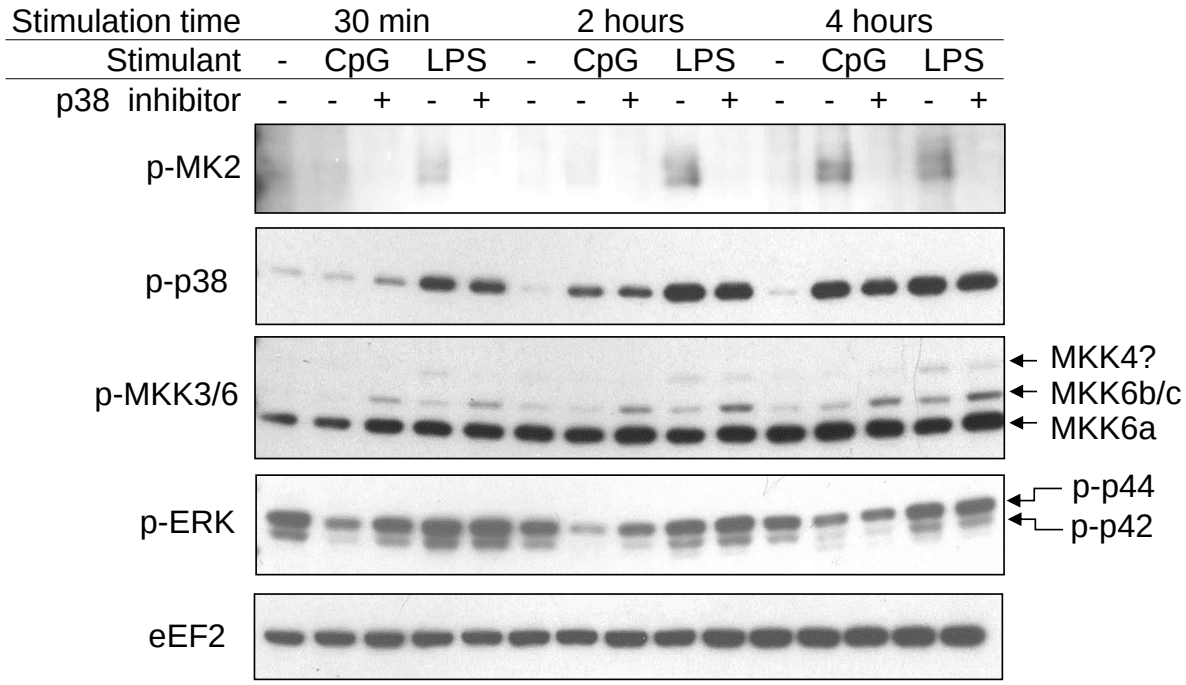

Stimulation time
30 min
2 hours
4 hours
Stimulant - CpG LPS - CpG LPS - CpG LPS
p38 inhibitor - - + - + - - + - + - - + - +
p-MK2
p-p38
p-MKK3/6
MKK4?
MKK6b/c
MKK6a
p-ERK
p-p44
p-p42
eEF2

Figure 2

A

CpG	CpG+inh	LPS	LPS+inh	Probe
12.45	2.13	4.83	1.41	CCL4-like chemokine
6.84	1.42	2.04	1.25	Dual specificity protein phosphatase 5 (DUSP5)
6.13	2.4	3.2	1.51	P62 (sequestosome-1)
5.14	4.8	3.78	2.82	NF-kappaB inhibitor alpha
4.98	2.4	3.59	2.21	Tumor necrosis factor receptor associated factor 2
4.21	-1.27	1.59	1.16	Secretory granule proteoglycan core protein
3.98	2.98	3.72	2.34	NF-kappaB inhibitor alpha-like
3.94	1.16	3.87	-1.15	Aminolevulinate, delta-, synthetase 1
3.86	1.44	2.48	1.62	Tumor necrosis factor receptor superfamily member 5 (CD40)
3.7	1.66	1.51	1.57	Transcription factor jun-B
3.65	2	2.1	2.25	Growth arrest and DNA-damage-inducible protein alpha
3.44	1.81	1.1	-1.12	High affinity immunoglobulin gamma Fc receptor I
3.19	1.86	1.78	1.45	4F2 cell-surface antigen heavy chain
3.07	1.1	3.62	1.56	Tumor necrosis factor receptor superfamily member 9 (CD137)
3	2.14	1.73	1.74	Interferon regulatory factor 1
2.56	2.11	1.54	1.41	C type lectin receptor B
2.53	1	3.57	1	Aminolevulinate, delta-, synthetase 1
2.36	1.14	1.86	1.21	Matrix metalloproteinase-9 (MMP9)
2.28	1.61	1.33	1.48	C-Jun protein
2.24	1.27	2.9	1.39	High-mobility group box 1
2.23	1.91	1.16	1.04	High affinity immunoglobulin epsilon receptor alpha
2.13	1.07	1.09	-1.21	Sulfotransferase 3
2.02	1	1.78	-1.05	T-cell activation Rho GTPase-activating protein
2	1.8	1.75	1.75	Cation transport regulator-like protein 1
1.92	-1.21	-1.2	1.26	JAK2-like
1.86	1.21	1.75	1.1	Tissue inhibitor of metalloproteinase 2
1.83	-1.34	1.13	1.17	40S ribosomal protein S29
1.82	-1.32	1.29	-1.03	Dynein light chain 2, cytoplasmic
1.77	ND	2.35	1.09	TNF decoy receptor (DCR3)
1.74	1.32	1.34	1.27	Nuclear factor NF-kappa-B
1.7	1.06	1.94	1.63	Chemokine 5a receptor-like
1.69	1.35	1.77	1.38	NACHT-, LRR- and PYD-containing protein 2
1.68	1.46	1.43	1.36	CASP8 and FADD-like apoptosis regulator precursor
1.68	1.09	2.11	1.25	Matrix metalloproteinase-13
1.64	-1.14	1.18	1.77	Tolloid-like 2 protein (nephrosin)
1.64	1.14	1.32	1.25	Nuclear factor kappa-B, subunit 1
1.57	1.5	1.16	1.48	60S ribosomal protein L32-2
1.57	1.33	2.22	1.37	p53-like transcription factor
1.54	1.31	1.73	1.51	Cytochrome B-245 heavy chain-1
1.54	1.42	1.18	1.49	60S ribosomal protein L7
1.54	1.75	1.61	1.28	60S ribosomal protein L39
1.52	1.48	1.5	1.49	Tapasin-1
1.52	1.16	2.13	1.37	CCAAT/enhancer binding protein beta
1.52	1.22	1.13	1.31	Lymphocyte pore forming protein
1.5	-1.47	1.39	1.29	Chemokine receptor-like 1
1.5	1.59	1.03	1.02	WD-repeat containing protein Ciao 1

Fold change
>10
>4
>2
>1.5

B

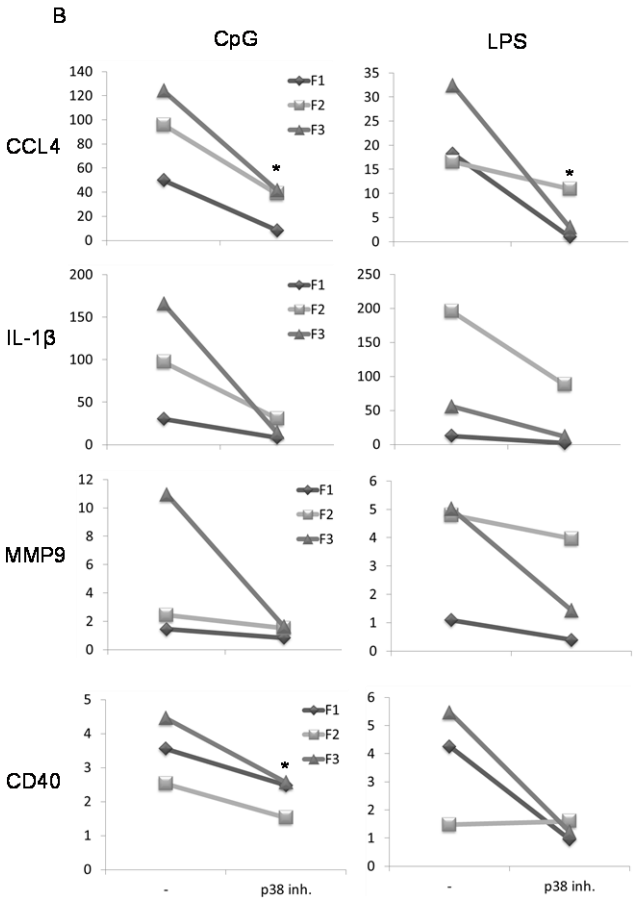


Figure 3

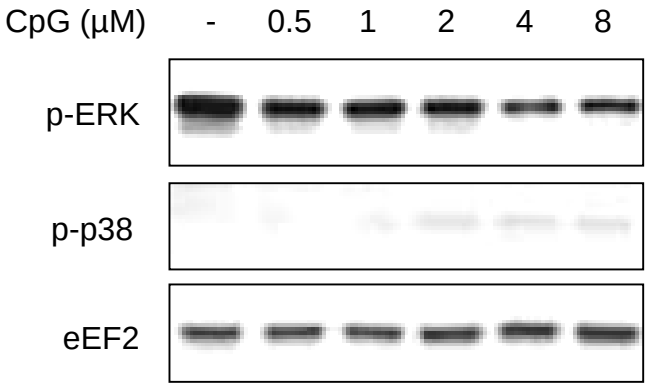
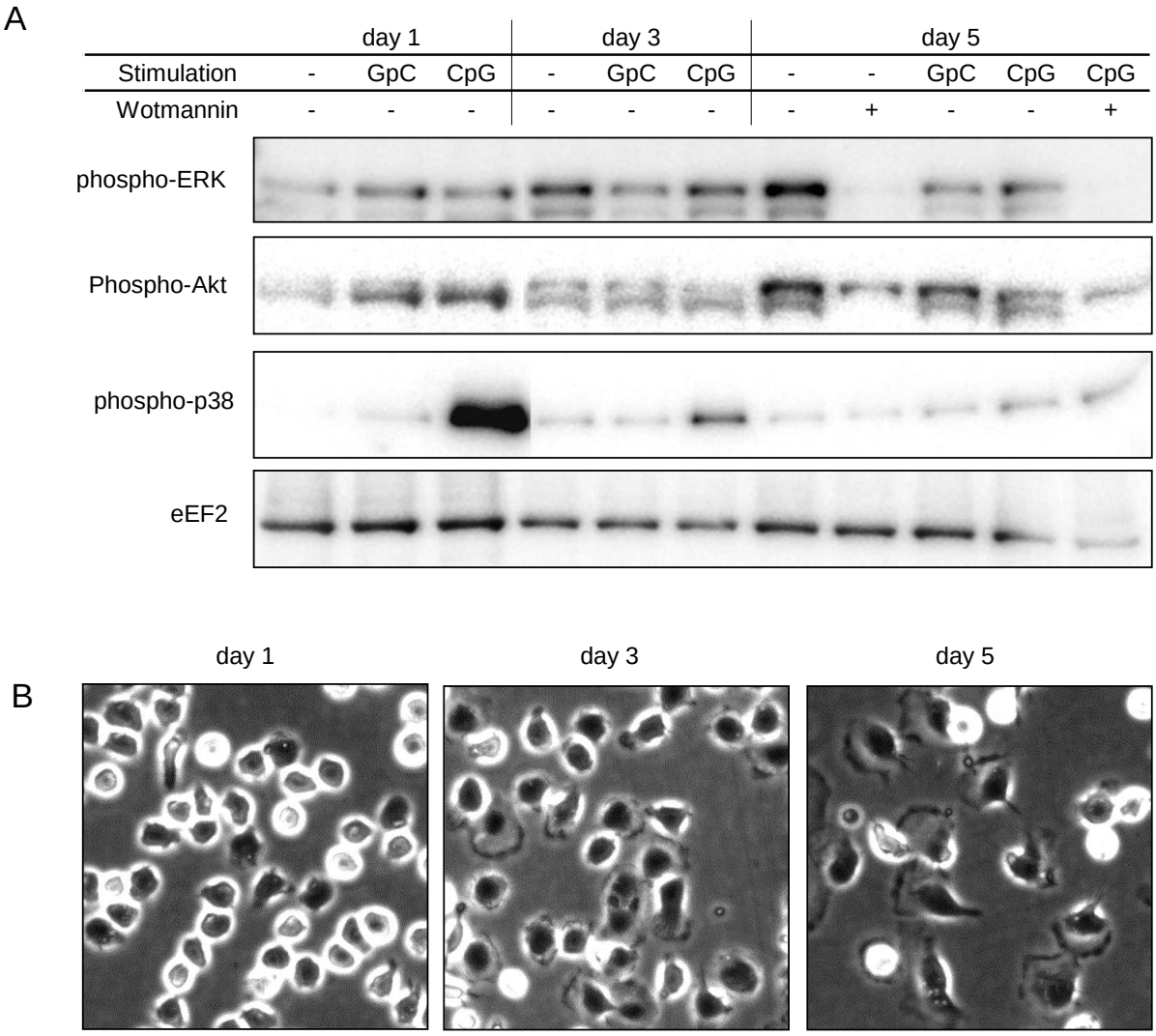


Figure 4


A
day 1
day 3
day 5
Stimulation - GpC CpG - GpC CpG - - GpC CpG CpG
Wotmannin - - - - - - - + - - +
phospho-ERK
Phospho-Akt
phospho-p38
eEF2
B
day 1
day 3
day 5