

**Myocardial depressant effects of Interleukin 6 in meningococcal sepsis are regulated by p38MAPK.**

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**At a glance commentary:** Myocardial dysfunction is a major feature in the pathophysiology of septic shock. IL-6 has been identified as a key mediator of a negative inotropic state in meningococcal sepsis. In this study we demonstrate that the p38MAPK pathway is a central regulator in the negative inotropic activity of interleukin 6 and is highly dysregulated in meningococcal septic shock. Modulators of the p38MAPK pathway could have therapeutic potential for patients with cardiac dysfunction in meningococcal and other forms of septic shock.

**Abstract:***Objectives:*

Myocardial failure, leading to inotrope-unresponsive shock, is the predominant cause of death in meningococcal and other forms of septic shock. Pro-inflammatory cytokines released in septic shock are known to have myocardial depressant effects. We previously showed that Interleukin 6 (IL-6) is a major myocardial depressant factor in children with meningococcal septicaemia. In the current study, we aimed to investigate the mechanisms by which IL-6 induces myocardial failure in meningococcal sepsis, and to identify potential novel therapeutic targets.

*Design:* Laboratory based study

*Setting:* University hospital and laboratories

*Patients:* Children with a clinical diagnosis of meningococcal septic shock

*Methods:*

We studied IL-6-induced signaling events, both *in vitro* using isolated rat ventricular cardiac myocytes as a model of myocardial contractility, and in whole blood from children with meningococcal sepsis.

*Measurements and Main results:*

We demonstrated involvement of JAK2, PI3K, Akt and p38MAPK in IL-6-induced negative inotropy in isolated cardiac myocytes. Inhibition of p38MAPK not only reversed IL-6-induced myocardial depression in both rat and human myocytes, but restored inotrope responsiveness. Cardiomyocytes transduced with dominant-negative p38MAPK showed no IL-6-induced myocardial depression. To investigate p38MAPK *in vivo*, we profiled global RNA expression patterns in peripheral blood of children with meningococcal septicaemia. Transcripts for genes mapping to the p38MAPK pathway showed significantly altered levels of abundance, with a high proportion of genes of this pathway affected.

*Conclusions:* Our findings demonstrate an integral role of the p38MAPK pathway in IL-6-mediated cardiac contractile dysfunction and inotrope insensitivity. Dysregulation of the p38MAPK pathway in meningococcal septicemia suggests that this pathway may be an important target for novel therapies to

reverse myocardial dysfunction in patients with meningococcal septic shock who are not responsive to inotropic support.

(273 words)

**Introduction:**

Sepsis and septic shock remain major causes of morbidity and mortality worldwide, with reported mortality rates of 10-50% [1-4]. An important feature in the pathophysiology of septic shock is the development of progressive hemodynamic and cardiovascular instability and depressed myocardial contractility [5, 6].

Over the past three decades, intense efforts to develop immunomodulatory treatments for septic shock have targeted early mediators in the inflammatory cascade triggered by bacterial infection. Despite success in animal models, therapeutic trials of inhibitors of up-stream factors such as endotoxin [7], cytokines [8, 9] and key mediators of coagulation and inflammation [10, 11] have been largely disappointing. It is increasingly clear that by the time septic shock is recognised, hours or days after the initial onset of infection, downstream inflammatory pathways dominate as causal factors in the ongoing pathophysiology and may present more logical clinical targets for immune modulating therapies.

Meningococcal septicaemia is among the most fulminant causes of septic shock, and is a major infectious cause of death in children in many developed countries. Since meningococcal sepsis generally occurs in otherwise healthy children and adults, it provides a unique model to study the mechanisms of septic shock. We previously showed that Interleukin 6 (IL-6) is a major myocardial depressant factor in meningococcal shock [12].

IL-6 induces a wide range of cellular and physiological responses [13]. Upon binding to its receptor, IL-6 causes homodimerization of the membrane-bound receptor gp130 which in turn leads to activation of associated protein tyrosine kinases including the Janus kinase (JAK) family [14], tyrosine phosphorylation of gp130 [15], and activation of multiple signal transducing factors such as signal transducer and activator of transcription (STAT) [16]. Following phosphorylation, STATs migrate to the nucleus where they upregulate transcription of a number of intracellular signaling cascades. The p38 mitogen-activated protein kinase (MAPK) [17] and phosphatidylinositol 3-kinase (PI3K) [18] pathways are known to be activated in cardiac

myocytes following inflammatory activation. The functional significance of these intracellular signaling pathways in the context of IL-6 mediated negative inotropy in sepsis is not well defined. We set out to investigate the role of key downstream signaling mediators in the myocardial depression induced by IL-6.

## **Methods:**

We undertook a series of *in vitro* and *in vivo* studies to explore the pathway of IL-6 induced myocardial depression in meningococcal sepsis (Figure 1).

### *Patients and controls*

Children were enrolled in the study at the time of admission to the Intensive Care Unit with suspected meningococcal sepsis. Following informed parental consent, and with approval of the hospital Local Research Ethics Committee, venous blood was collected on admission to the PICU. Diagnosis was confirmed by isolation of the organism from blood, CSF, or by PCR amplification of meningococcal DNA from peripheral blood, as described previously [19]. Venous blood samples from healthy adult volunteers were obtained following informed consent.

### *Biological and chemical reagents*

Antibodies to p38MAPK, phospho-p38MAPK, Akt, phospho-Akt, STAT3 and phospho-STAT3 were obtained from Cell Signaling Technology (Danvers, MA). IL-6 was obtained from Biomyx Technology (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (Gillingham, UK).

### *Isolation of Adult rat and human ventricular myocytes*

All studies complied with the United Kingdom Home Office Regulation Governing the Care and Use of Laboratory Animals. Ventricular myocytes were isolated from adult male Sprague–Dawley rats (250-350 grams) by collagenase digestion, using the standard enzymatic technique as previously described [12].

Human tissue from the left or right ventricle was obtained from explanted hearts in adult patients undergoing cardiac transplantation following informed consent. Procedures for collecting human heart tissue conformed to the Ethics Committee requirements of the Royal Brompton and Harefield Hospital, UK. Tissue was immediately placed in cardioplegia solution at 4°C and ventricular myocytes isolated using a standard enzymatic technique [12].

For adenoviral transfection, cells were infected with adenovirus at a multiplicity of infection of 500 for the dominant negative p38MAPK (Ad. p38DN) construct, which had the phosphorylation motif (T-G-Y) mutated to (A-G-F) (kindly provided by Drs Menick, Charleston and Wang, University of California, San Diego). Cells were incubated at 37°C for 48 hours before use in myocyte contraction assays.

#### *Myocyte Contraction Measurements*

Myocyte contraction experiments were performed as previously described [12]. In brief, cells in Kerbs–Henseleit (KH) solution equilibrated to pH 7.4 with 95% O<sub>2</sub>-5% CO<sub>2</sub> were placed in a perspex chamber on the stage of an inverted microscope. Myocytes were left for 10 minutes to allow time for adherence to the cover slip. After this time, cells were perfused with KH solution supplemented with calcium (1-2 mM, rat; 2-6 mM, human). Experiments were carried out at 37°C with field stimulation at 0.5 Hz in rat myocytes, while human myocytes were paced at 0.2 Hz, 32°C. Individual cells were digitally tracked and the contraction amplitude measured continually using video edge detection as previously described [12] (Ion Optix, MA, USA). Myocyte contraction was expressed as the percentage shortening of cell length upon field stimulation.

#### *Analysis of the IL-6 signaling pathway using inhibitors of key intracellular mediators.*

To obtain a model that closely resembled the clinical situation of septic shock, where patients are supported with inotropic medication, we employed catecholamine stimulation. Isoprenaline (10 nM) was

added to perfusion buffer, and a baseline measurement taken when contraction amplitude reached a stable plateau..

For evaluation of PI3K, JAK2 and p38MAPK, cells were perfused with the specific inhibitors (10 $\mu$ M LY94002, 20 $\mu$ M AG490, 3 $\mu$ M SB203580 respectively) for 15 minutes before exposure to IL-6.

To evaluate the role of G<sub>i</sub> signaling, isolated rat ventricular myocytes were incubated with 1.5  $\mu$ g/ml pertussis toxin (an inhibitor of this pathway) for 24 hours and then used in contraction assays. For individual myocytes, the efficiency of G<sub>i</sub> inactivation was confirmed by carbachol challenge in the presence of isoprenaline.

#### *Western Blotting*

Western blotting was performed to identify and quantify STAT3, Akt and p38MAPK protein levels. We also used this method to examine p38MAPK activation in dominant negative myocytes. Cultured myocytes were centrifuged (14,000 g, 10 minutes, 4°C), snap frozen in liquid nitrogen and stored at -80°C. Cells were subsequently resuspended in Phospho-Safe extraction buffer (Novagen) and protein concentrations determined (Bio-Rad). Using a 10% gel, 40  $\mu$ g of protein was separated by SDS-PAGE and transferred to PVDF membrane. Blots were incubated with antibodies to the target mediator overnight at 4°C. Secondary antibodies were either conjugated with horseradish peroxidase (Amersham pharmaceuticals) or fluorescence (QDot) to detect protein bands. Protein levels were quantified using densitometry (Gene Genius Gel Imaging, Syngene).

#### *Gene expression*

Whole blood (2.5 ml) from patients and controls was collected into RNA stabilisation fluid (PaxGene Blood RNA tubes, Beckton Dickinson). Patient samples were obtained on admission to the ICU within 24 hours of clinical presentation. Total RNA was extracted using PreAnalytix RNA extraction kit (Qiagen, Crawley, UK).



After linear amplification (MessageAmp II, Applied Biosystems), and reverse transcription to cDNA, sample cDNA was labelled with Cy5-dUTP, and combined with Cy3-dUTP-labelled reference cDNA (Stratagene) [20]. Samples were then washed, concentrated, and hybridised to custom-printed cDNA microarrays containing 37632 elements representing approximately 18000 unique human genes [21]. The hybridized slides were scanned using a GenePix 4000A microarray scanner (Axon Instruments), with every spot on the array given a relative fluorescence ratio for sample and reference RNA. Areas of the array with blemishes or poor quality were excluded from analysis using GenePix Pro 6.0 (Axon Instruments).

For the rat microarray experiment, isolated cardiac ventricular myocytes were cultured in M199 medium with addition of 5 mM creatine, 5 mM taurine, 2 mM carnitine, 0.1 M insulin, 100 mM ascorbate and penicillin/streptomycin. Cells were incubated with recombinant human IL-6 (20ng/ml) or in medium alone for 6 hours at 37°C. Myocyte RNA was extracted using the RNEasy extraction kit (Qiagen, Crawley, UK). RNA was amplified and labelled using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems), and hybridised to the RatRef-12 Expression BeadChip (Illumina). Arrays were scanned using a BeadArray Reader (Illumina) and probe IDs associated with intensity data using BeadStudio v3.1.3.0

The datasets discussed in this publication have been deposited in the Stanford Microarray Database and Array Express under accession number E-MTAB-344.

### *Statistics*

All quantitative data are expressed as median  $\pm$  IQR. Continuous data were analysed using the Wilcoxon signed rank test to compare control and experimental conditions. A value of  $p < 0.05$  was considered statistically significant.

Raw expression data from 37 individuals (23 healthy controls, 14 patients) were downloaded from Stanford Microarray Database. Data were filtered to include array elements that were present on more than 80% of the arrays. Within-slide normalization was performed through within-print-tip-group intensity dependent

location normalization using the loess function, to correct for systematic variation that is introduced by the overall spot intensity, dye and spatial effects. Scale normalization was applied to equalize the range of  $\log_2$  ratios between slides. Differential expression analysis followed a “Two-Groups: Common Reference” experimental design and was performed using Limma [22]. Linear models were fitted and moderated  $t$ -statistics were calculated for each gene. Adjustment for multiple hypothesis testing was performed via the Benjamini and Hochberg method that controls the false discovery rate [23]. The significance level was set to  $\alpha=0.05$ . Enrichment of the p38MAPK pathway, was assessed by the hypergeometric distribution (Fischer’s exact). The analysis was conducted with the use of Bioconductor software packages in R.

In order to investigate the role of p38MAPK in the clinical pathophysiology of meningococcal sepsis, we used whole genome RNA expression profiling to study the regulation of activators and downstream effectors of p38MAPK. **We identified genes widely accepted as being part of the p38MAPK signaling pathway using** KEGG,[24] Panther version 6.1,[25] Ingenuity Pathways Analysis 6 and GeneAssist™ Pathway Atlas (www.ambion.com)), as well as published literature and websites,[24, 26-30] ([www.cellsignal.com](http://www.cellsignal.com)).

## Results

### *Patients*

There were 14 children (aged 2 months to 16 years of age) included in the study, 8 children were female, 6 were male. Median age was 51 months (Interquartile range 21-157 months). There were 2 deaths (14.3%). All patients had clinical evidence of meningococcal septicaemia with petechiae or purpura. Some had additional signs or symptoms of meningitis.

*IL-6 reduces both basal cardiac myocyte contractility and inotrope responsiveness.*

Addition of 20 ng /ml IL-6 (the median serum level of IL-6 seen in our patients **in a previous study** [12]) to the perfusion buffer not only acutely depressed basal myocyte contraction as previously shown, but significantly diminished inotrope sensitivity. After establishing baseline myocyte contractility for 15 minutes, addition of isoprenaline (30nM) to the perfusion buffer increased myocyte contraction amplitude

by 52% : from 6.1% (IQR 3.9 - 8.5) to 9.3% (IQR 6.7 - 11.7), n=45. After 15 minutes, IL-6 was added to the perfusion buffer, significantly decreasing myocyte contraction by 19.9% to 7.45% (IQR 6.0-9.6), n=45 (Figure 2a). This inotrope insensitivity persisted for at least 15 minutes after IL-6 was removed from the perfusion buffer.

We previously showed meningococcal serum had both acute and latent myocardial depressant effects [31]. Similarly, IL-6 significantly reduced contraction amplitude of cardiac myocytes following incubation (48 hours), with a median (IQR) percentage change in contraction amplitude of -24.2% (-4.9 to -56.3) compared to control cells, n=14, p=0.03.

#### *JAK2 and PI3K/Akt are involved in IL-6 induced negative inotropy*

Activation of JAK/STAT signaling has previously been associated with gp130-related stimulation by IL-6 in adult ventricular cardiac myocytes [16]. Addition of AG490 (a specific inhibitor of Jak 2) to the perfusion buffer reduced the negative inotropic effect of IL-6 on isoprenaline-stimulated myocytes: Median (IQR) change in contraction amplitude of -9.8% (-22.5 to -2) (control) to -0.85% (-18.5 to 10.2), n=9 (JAK2-inhibited), although the change was not statistically significant (Figure 2b). Further, STAT3 phosphorylation was not significantly increased. Median (IQR) ratio of phosphorylated to total STAT3 in control cells was 0.381 (0.17-0.60) and in IL-6 exposed cells was 0.39 (0.22-0.93), n=10 p=0.06. This suggests that pathways other than JAK/STAT may be involved.

Several groups have implicated PI3K in the negative regulation of cardiac contractility [18, 32]. In agreement with these previous findings, we found that the PI3 Kinase inhibitor LY294008 ablated the myocardial depressant effect of IL-6. Control cells had a median (IQR) change of -17.8% (-46.6 to -8.2), n=6 in contraction amplitude, whilst pre-treatment of myocytes with LY294008, resulted in no significant change (Median, IQR: 0.1; -6.8 to 5.8) in contraction amplitude following exposure to IL-6 (n=5), (Figure 2b). In addition, in myocytes exposed to IL-6, phosphorylation of Akt, a downstream signaling protein in the PI3K pathway was increased by 1.5 fold over control cells (Figure 2c).

*p38MAPK regulates inotropic insensitivity in IL-6 exposed myocytes.*

We used the p38MAPK inhibitor, SB203580, to investigate the role of p38MAPK in IL-6-induced negative inotropy. Pretreatment with SB203580 not only inhibited IL-6-induced myocardial depression, but restored isoprenaline responsiveness in both rat and human myocytes (Fig. 1d). In rat myocytes, a reduction in contractility of control cells (median, IQR -15%, -27.7 to -5.7) compared to isoprenaline alone was converted to a (median, IQR 5.6%, -3 to 56.5) increase in contractility in response to IL-6 in SB203580-exposed cells (again compared to contractility with isoprenaline alone) (n=8). In human myocytes, IL-6 reduced myocyte contractility (median, IQR -27.4, -41.8 to 0.8), but SB203580 pre-treatment led to a median increase in contraction amplitude of 107.1 (IQR 93.5 to 116.1) following IL-6-exposure (n=7). There was no effect of any of the inhibitors on basal myocyte contraction amplitude or on the response to isoprenaline (data not shown). This is to our knowledge the first study to demonstrate in human cardiac myocytes that inhibition of p38MAPK reverses IL-6-mediated contractile depression. Indeed in the absence of p38MAPK activity, IL-6 had a paradoxical effect and increased myocyte contractility.

To confirm the key role of the p38MAPK pathway in the negative inotropy of IL-6, rat myocytes were transfected with an adenovirus encoding a dominant negative p38MAPK (p38DN), to reduce p38MAPK activity. Western blotting confirmed that transduced cells showed greatly reduced p38MAPK phosphorylation, although a small amount of residual p38MAPK phosphorylation was retained (data not shown). P38DN myocytes were also resistant to the negative inotropy of IL-6 (Fig. 2d). In control cells, IL-6 exposure reduced contraction amplitude (median, IQR -13.1, -29 to -2.9). In p38DN myocytes, IL-6 induced no significant change in contraction amplitude (median;IQR 5.35% -9.7 to 10.7, n=10), confirming the importance of p38MAPK in the myocardial depressant activity of IL-6.

Finally, we investigated changes in gene expression of the p38MAPK pathway in cardiac myocytes exposed to IL-6. Adult rat ventricular myocytes were cultured with recombinant human interleukin 6 for a period of 6 hours. 740 genes were significantly differentially expressed (fold change of treated cells compared to

control cells of  $p < 0.05$ ) (supplementary table 1). Of these, 8 genes belonged to the p38-MAPK pathway (out of 115 MAPK-associated genes represented on the array). The hypergeometric probability of this occurring by chance was  $p = 0.004$ . Table 1 lists these genes, their log fold changes and p-values. Taken together, our data establish an important functional role for p38MAPK signaling in the negative inotropic activity of IL-6. It is interesting to note that in the absence of p38MAPK activity, the net effect of IL-6 on rat myocytes was positive inotropy.

*p38MAPK is highly dysregulated in children with meningococcal sepsis.*

As shown in Figure 2, a large proportion of genes of the p38MAPK pathway were significantly differentially expressed in children with meningococcal septicaemia compared to healthy controls. Hierarchical clustering of p38 MAPK genes and subjects showed that patients and controls showed significant differentiation in the levels of expression of p38MAPK pathway genes (Figure 4a). Of the 150 MAPK genes on the array, 115 were differentially expressed. In order to examine those genes of this pathway which best discriminated patients from controls we displayed the data on a volcano plot as demonstrated in Figure 4b. p38MAPK $\alpha$  (MAPK14), the isoform most commonly associated with negative inotropy had a high log odds (17.54) and significance (adjusted p value =  $7.88 \times 10^{-10}$ ) in patients compared to healthy controls. In addition, a large number of genes upstream and downstream of p38MAPK showed significant differential expression in children with severe meningococcal infection on admission to intensive care, relative to expressed levels of these genes in healthy controls (Figures 4a and 4b and Supplementary Figure 1; Supplementary table 2). The probability that this group of p38MAPK genes was differentially expressed by chance was  $2.12 \times 10^{-11}$  (calculated using the hypergeometric distribution), which strongly supports the hypothesis that the differential regulation of the p38MAPK pathway is a key feature of meningococcal sepsis.

## **Discussion**

Interleukin 6 is an established mediator of organ dysfunction in septic shock. We and others have highlighted the role of IL-6 in the pathogenesis of organ dysfunction in children with septicemia [12, 33]. In a murine cecal ligation and puncture model of septic shock, levels of IL-6 at 6 hours following onset of sepsis were predictive of outcome [34]. Whilst the effect of IL-6 on cardiac myocytes has largely been assumed to be due to transsignaling from binding of IL-6 to soluble receptor in serum and subsequent activation via cell surface gp130 receptor, our data in both rat and human cardiac myocytes show a direct negative inotropic effect of IL-6 in the absence of the soluble receptor [12], suggesting that classical signaling via a cell surface IL-6 receptor also occurs.

Our study has demonstrated that the myocardial depressant effects of IL-6 appear to be dependent on signaling through the p38MAPK pathway, and that inactivation of p38MAPK abolishes this activity, and restores sensitivity to the inotropic agent isoprenaline.

p38MAPK regulates a broad range of cellular responses, including inflammation, proliferation, apoptosis, cellular defense and differentiation [35, 36]. Activation of p38MAPK has been demonstrated in myocardial, lung and brain tissue in sepsis [37-40], suggesting that p38MAPK has widespread effects in the pathophysiology of multi-organ dysfunction in septic shock. Inhibition of p38MAPK has been shown to attenuate LPS-induced acute lung injury through downregulation of the NF $\kappa$ B pathway [41].

In animal models, the p38MAPK pathway has been shown to contribute to the cardiodepressant activity of TNF $\alpha$  [17] and endotoxin [42]. During myocardial ischemia and reperfusion, p38MAPK activation induces myocyte apoptosis, increases expression of adhesion molecules, and reduces cardiac myofilament calcium sensitivity [43]. Moreover, inhibition of p38MAPK prevents myocardial TNF $\alpha$  expression and improves cardiac function and survival in endotoxemic rats [42].

Our RNA expression studies in children with meningococcal sepsis has revealed elevated abundance of transcripts for genes that encode upstream activating factors for p38 $\alpha$  MAPK (MAP3K7, MAP3K5, MAP2K4 and MAP2K6); the IL6 receptor; JAK2 and Akt, as well as downstream mediators, including transcription factors (such as Nuclear Factor KappaB and Activating Transcription Factor), MAPK activated protein

kinases and Inflammatory cytokines such as IL-1 $\beta$ . These data confirm that the p38 MAPK pathway is highly activated in acute meningococcal sepsis in humans. **Data from the Genomics of Pediatric SIRS/Septic Shock Investigators confirms in a larger group of children with septic shock that the IL-6 and p38MAPK pathways are significantly dysregulated on admission to PICU [44].**

We have demonstrated that the p38MAPK pathway is dysregulated in cardiac myocytes following exposure to IL-6, and that the negative inotropy of IL-6 is reversed by blockade of the p38MAPK pathway. Our data suggest that the p38MAPK pathway is of major importance in the development of inotrope-unresponsiveness **both acutely and after a prolonged incubation time.** The dramatic effect of inhibitors of P38MAPK not only in abolishing the myocardial depressant effect of IL6 on cardiac contractility *in vitro*, but in restoring inotrope sensitivity, suggests that inhibition of p38MAPK may be of benefit in treatment of meningococcal shock, as the condition is characteristically associated with reduced inotrope responses, and the requirement for escalating doses of catecholamines. **Other inflammatory mediators known to have myocardial depressant effects such as the complement component C5a and the pro-inflammatory cytokine TNF $\alpha$  have also been shown to activate the p38MAPK pathway [45, 46].**

**An important limitation of our study is the unavailability of human septic myocardial tissue. Checchia et al examined the effects of age on transcriptional responses to sepsis in the myocardium of young (6 weeks) and old (20 months) septic mice. Genes of the IL-6 and p38MAPK pathway were among 53 significantly differentially expressed genes comparing young and old sepsis and sham groups of animals [47].**

As IL-6 is released in response to all bacterial infections, our findings may be relevant to the treatment of myocardial depression in other forms of septic shock. **In children with septic shock of varying etiology (both Gram positive and Gram negative), Cvijanovich identified the p38MAPK pathway being significantly upregulated [48].** Furthermore, our findings may also be relevant to other forms of systemic inflammatory activation such as burns [49], ischemia-reperfusion [50] and cardiopulmonary bypass [51, 52], where p38MAPK activation and its relation to multi-organ dysfunction is becoming increasingly clear. Inhibitors of the p38MAPK pathway are being investigated in animal and human studies of sepsis and other pro-

inflammatory disease states [52-56]. Modulation of up- or downstream regulators of this pathway could also be of interest therapeutically.

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## Figure Legends:

### Figure 1: Experimental approach to studying the pathway of IL-6 induced myocardial depression

We utilised both an *in vitro* model of cardiac function to explore the mediators activated by IL-6 (1a) and blood samples from children with meningococcal sepsis to examine changes in the regulation of the p38MAPK pathway (1b).

### Figure 2: Pathway analysis of IL-6 mediated cardiac dysfunction

JAK2, PI3K, Akt and p38MAPK modulate the negative inotropic response of cardiac myocytes to IL-6. *a. Acute effect of IL-6 on cardiac myocyte contractility.* Measurements were taken in the presence of perfusion buffer alone (baseline) and then following addition of 30nM Isoprenaline to the buffer (isoprenaline stimulation), addition of 20ng/ml IL-6 to the perfusion buffer (addition of IL-6), and removal of IL-6 from the buffer (IL-6 washout). *b. Role of JAK2 and PI3K in IL-6 induced myocyte contractile depression.* The difference in contractility of cardiac myocytes in the presence of isoprenaline and IL-6 was measured with and without a JAK2 or PI3K inhibitor. IL-6 depressed contractility, and this depression was reversed by inhibition of JAK2 using AG490, or by inhibition of PI3K using LY294008. *c. IL-6 increases phosphorylation of Akt in rat cardiac myocytes.* Western blots demonstrate increased phosphorylation of Akt (pAkt) in rat cardiac myocytes incubated with IL-6 compared to control cells. *d. Role of p38MAPK in IL-6 induced myocyte contractile depression.* Inhibition of p38MAPK using SB203580 not only reversed the negative inotropic effect of IL-6 in both rat (i) and human (ii) cardiac myocytes, but a positive inotropic activity was seen in response to IL-6 in these cells. In addition, in p38 Dominant Negative myocytes, the effect of IL-6 was significantly diminished.

### Figure 3: Genes of the p38MAPK pathway are differentially regulated in meningococcal sepsis

Schematic diagram showing the genes that encode key members of the p38MAPK signaling pathway, showing upstream triggers and downstream effector molecules of p38MAPK (MAPK14). Genes with significantly elevated transcript levels in patients are shown in red, genes with significantly decreased transcript levels are shown in green, while non-differentially expressed genes are shown in black. Genes that were dysregulated in cardiac myocytes following exposure to IL-6 are marked with an asterisk.

**Figure 4: The p38MAPK pathway is significantly dysregulated in blood from septic patients compared to healthy controls. a. Heatmap of gene expression patterns in whole blood from meningococcal patients compared to control patients.** Patterns of transcript abundance of genes involved in the p38MAPK pathway in whole-blood samples from 14 children with meningococcal sepsis compared to 23 healthy adults. Genes and subjects were organized using hierarchical clustering; each row represents a single gene, and each column a single subject (sample). Black indicates the median level of transcript abundance, red indicates higher transcript abundance than the median, green, lower transcript abundance, and white, missing data. The genes in the heatmap are listed in supplementary table 1. **b. Log-odds ratios of differentially expressed genes of the p38MAPK pathway in meningococcal patients compared to healthy children.** The volcano plot arranges genes along dimensions of biological and statistical significance. The horizontal axis is the average log fold difference between meningococcal patients and the healthy controls for genes of the p38MAPK pathway. The vertical axis is the log-odds ratio that the gene is differentially expressed, and indicates the statistical evidence, or reliability of the difference.

**Table 1. Differentially expressed genes of the p38MAPK pathway in cardiac myocytes exposed to IL-6.**

Changes in transcript levels of differentially expressed genes associated with the p38MAPK pathway in rat cardiac myocytes exposed to interleukin 6. “logFC” is the  $\log_2$  fold difference between IL-6 treated and untreated cardiac myocytes. “adj.P.Val” is the associated p-value before and after adjustment for multiple testing. Genes are ordered by the magnitude of the adjusted p value.

**Supplementary Figure 1. Boxplots showing significantly regulated genes of the p38MAPK pathway. a.**

**Upregulated genes of the p38MAPK pathway.** Genes that encode members of the p38MAPK pathway that are significantly over-expressed in meningococcal patients as compared to healthy children. **b.**

**Downregulated genes of the p38MAPK pathway.** Genes that encode members of the p38MAPK pathway that are significantly under-expressed in meningococcal patients as compared to healthy children.

**Supplementary Table 1.** List of all differentially expressed genes in rat cardiac myocytes exposed to IL-6.

“logFC” is the  $\log_2$  fold difference between cases and controls. “*t*” is the moderated *t*-statistic[22].

“adj.P.Val” is the associated p-value before and after adjustment for multiple testing. “*B*-statistic” is the log-odds that the gene is differentially expressed[22]. Genes are ordered by magnitude of the adjusted p value, and are identified by official gene symbol (Gene ID, column A) and by the Illumina Probe number (column B).

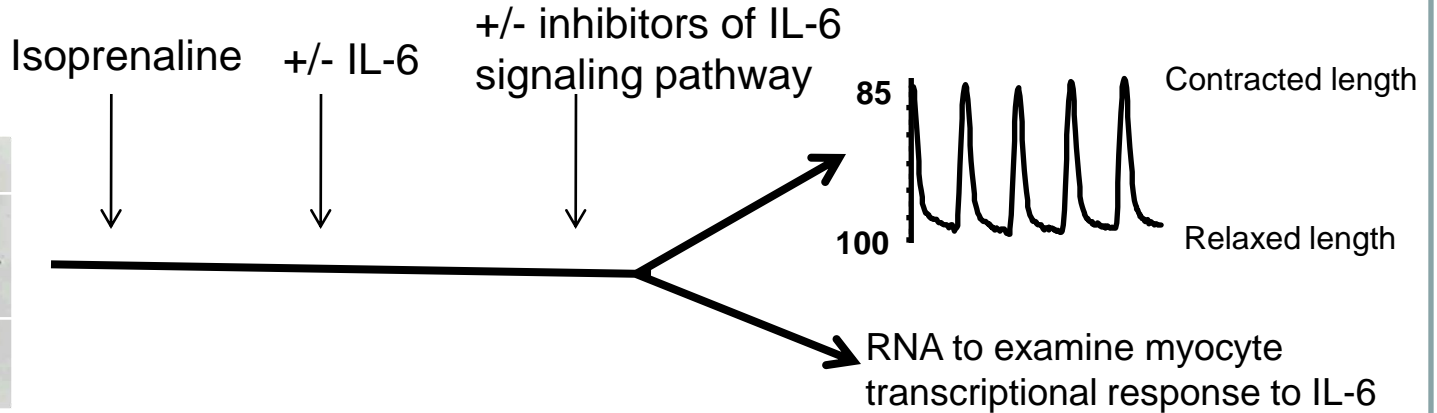
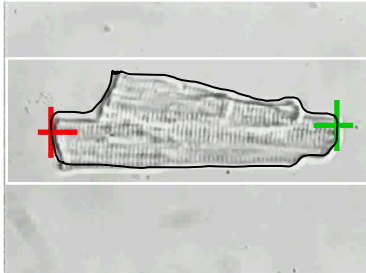
**Supplementary Table 2. Summary statistics of changes in expression of p38MAPK pathway genes.**

List of differences in transcript levels for genes associated with the p38MAPK pathway and differentially expressed in children with and without meningococcal sepsis. “logFC” is the  $\log_2$  fold change between cases and controls. “*t*” is the moderated *t*-statistic[22]. “adj.P.Val” is the associated p-value before and after adjustment for multiple testing. “*B*-statistic” is the log-odds that the gene is differentially expressed[22]. Genes are ordered by log fold change.

Figure 1:

a. Examining IL-6 induced contractile depression using isolated ventricular myocytes in an *in vitro* model of cardiac function

Continuous edge detection monitoring of myocyte length



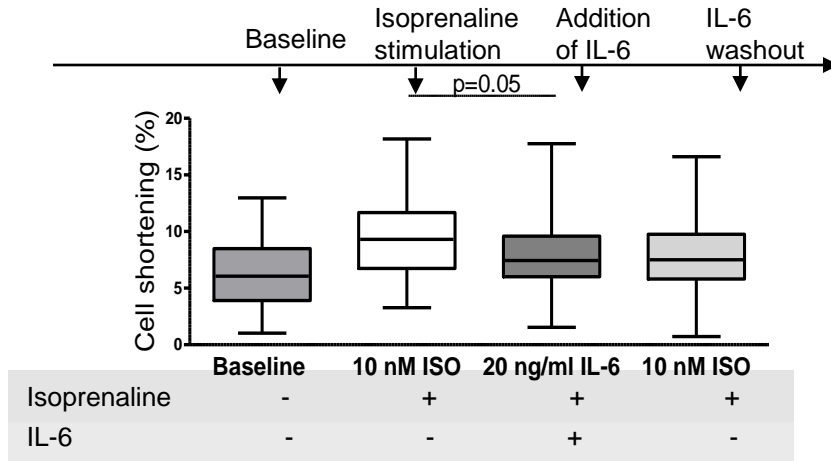
b. Examining the p38MAPK pathway involvement in transcriptional responses to sepsis in children with meningococcal disease



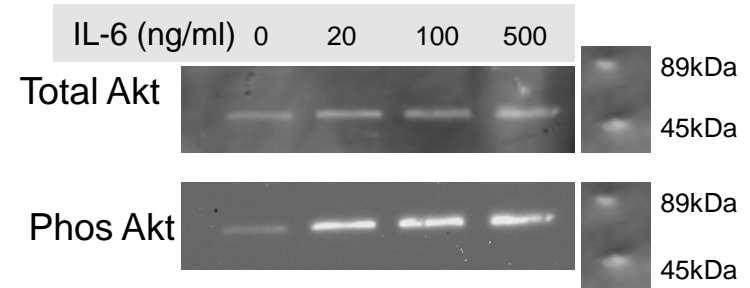


# Figure 2

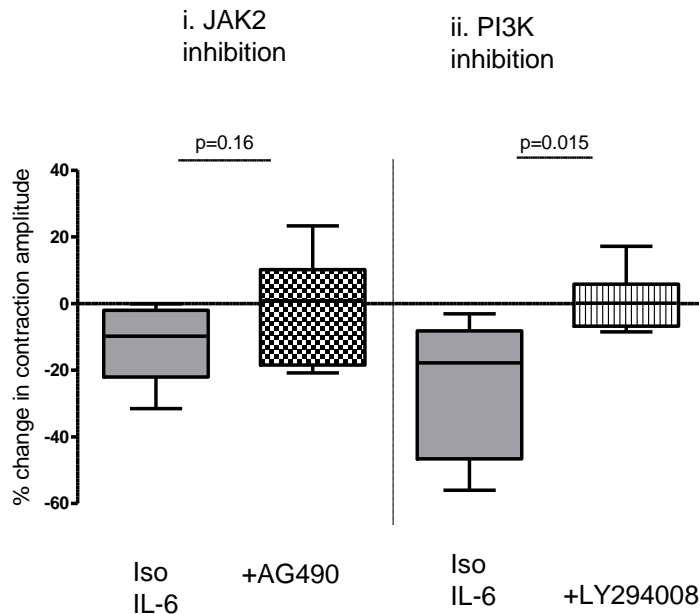
## 2a: Acute effect of IL-6 on cardiac myocyte contraction.



## 2c: IL-6 increases phosphorylation of Akt in rat cardiac myocytes.



## 2b: Role of JAK2 and PI3K in IL-6 induced myocyte contractile depression



## 2d: Role of p38MAPK in IL-6 induced myocyte contractile depression

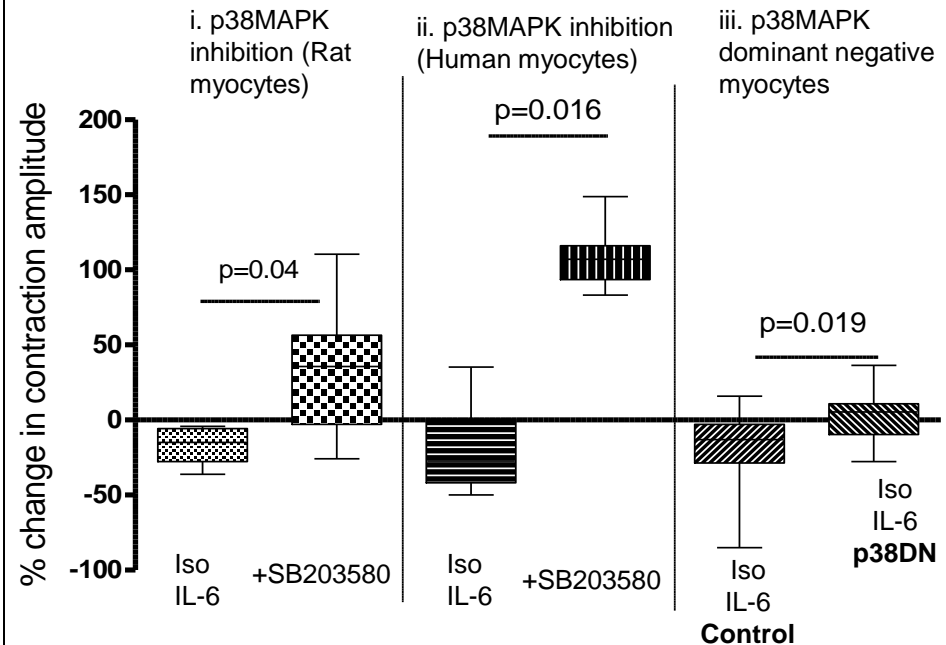


Table 1: list of differentially expressed genes of the p38MAPK pathway in cardiac myocytes exposed to IL-6

Gene symbol	Gene name	log Fold change	adj.P.Val
JUND	Jun D proto-oncogene	-0.366	0.001
ZFP36	zinc finger protein 36	-0.381	0.009
AKT1	v-akt murine thymoma viral oncogene homolog 1	-0.440	0.013
FAS	Fas (TNF receptor superfamily, member 6)	0.148	0.015
MAPK8	Mitogen-activated protein kinase 8	0.130	0.037
SOS2	Son of sevenless homolog 2	0.104	0.043
ECSIT	Evolutionarily conserved signaling intermediate in Toll pathway	-0.177	0.044
DUSP12	Dual specificity phosphatase 12	-0.163	0.046

Figure 3: Genes of the p38MAPK pathway are differentially regulated in meningococcal sepsis

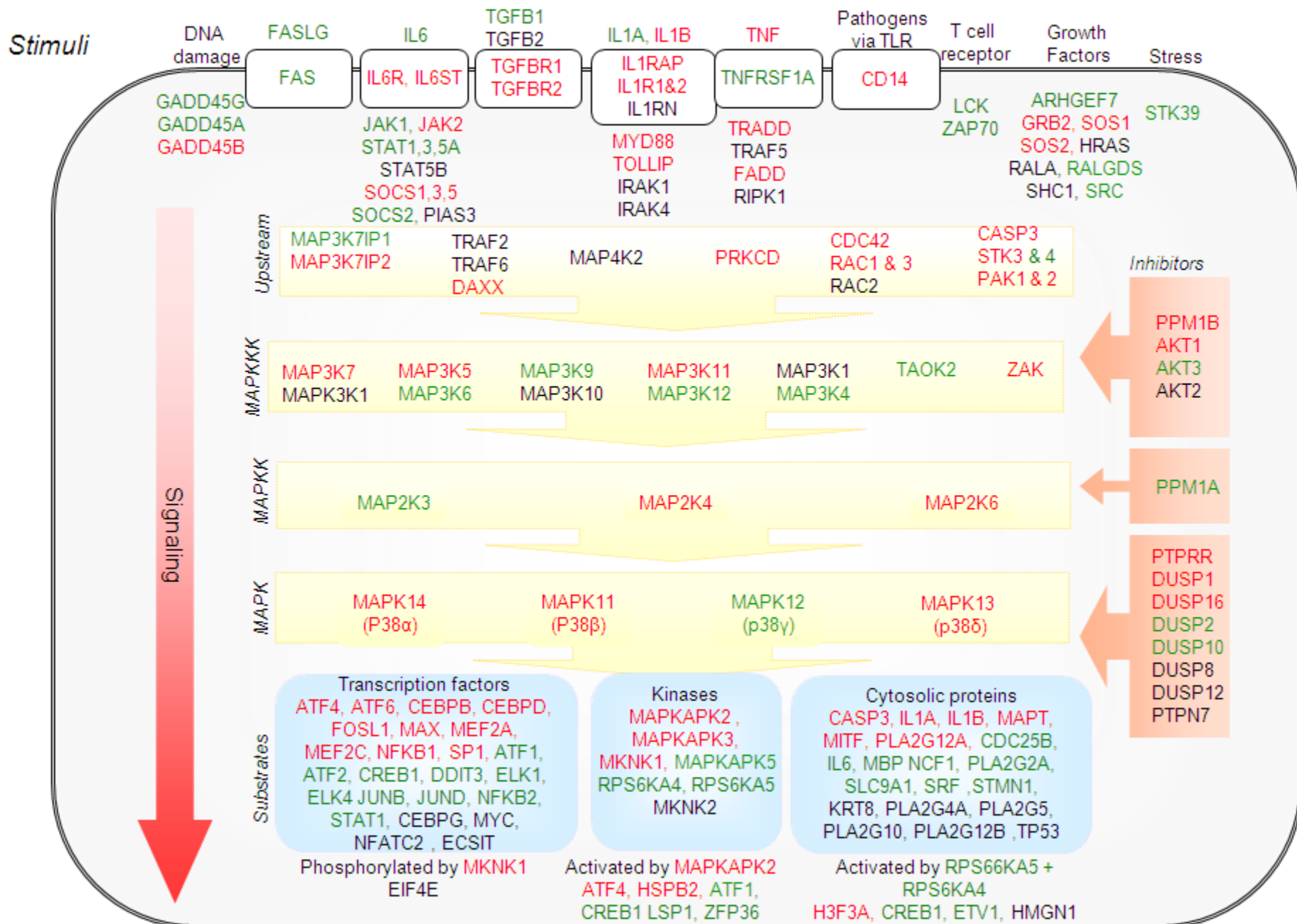
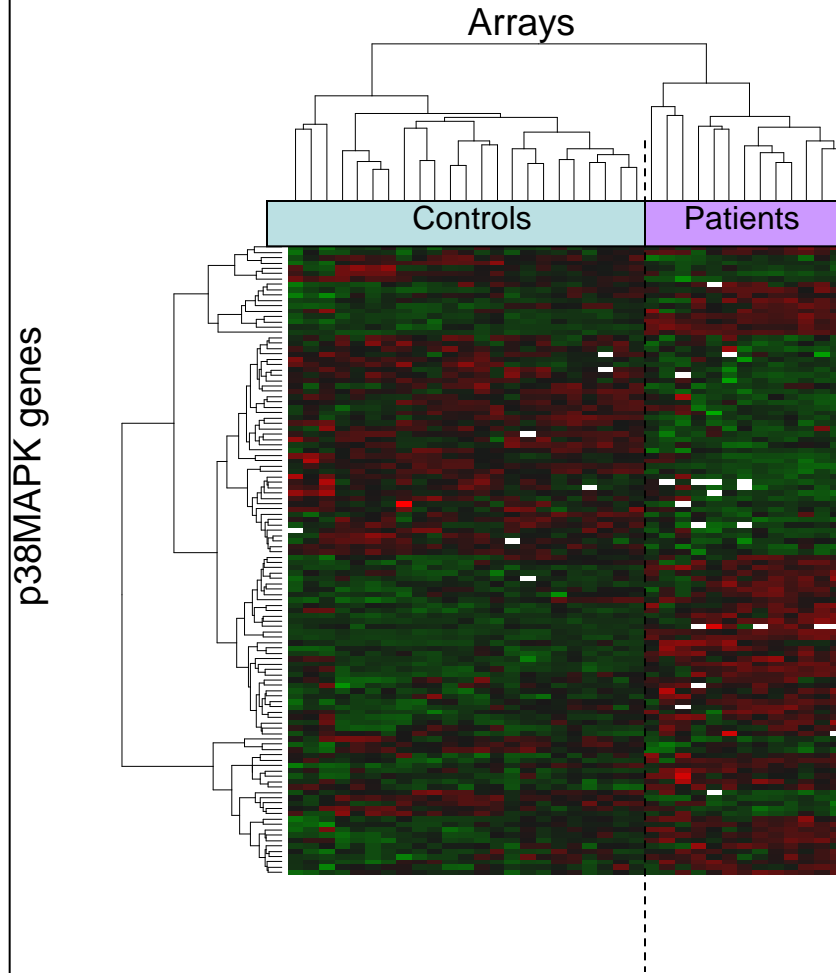
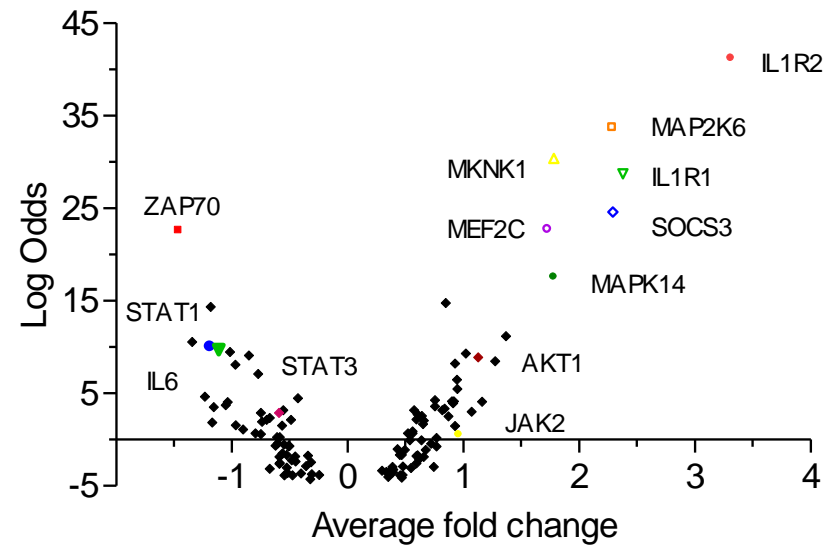


Figure 4

4a: Heatmap of gene expression patterns in meningococcal patients compared to controls

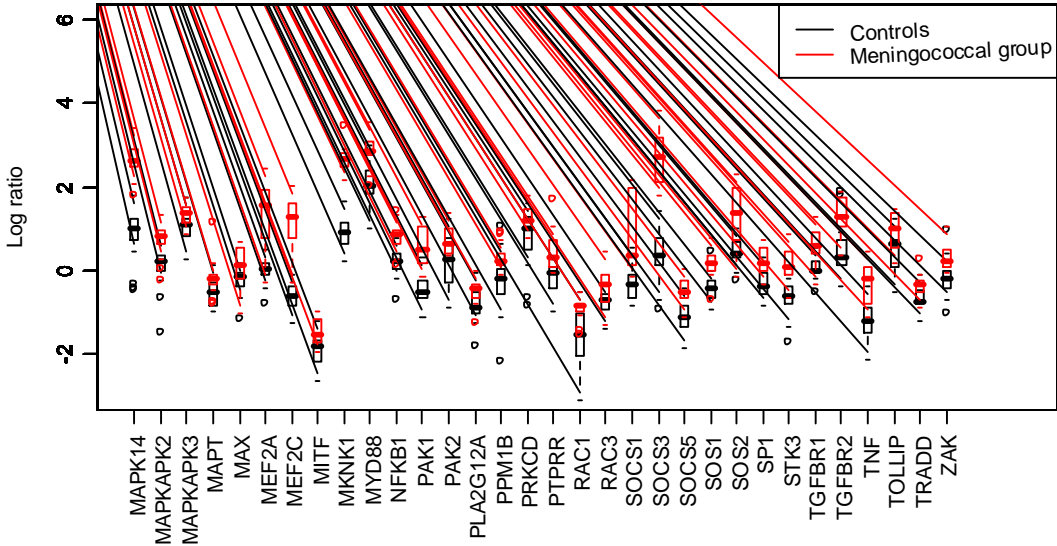
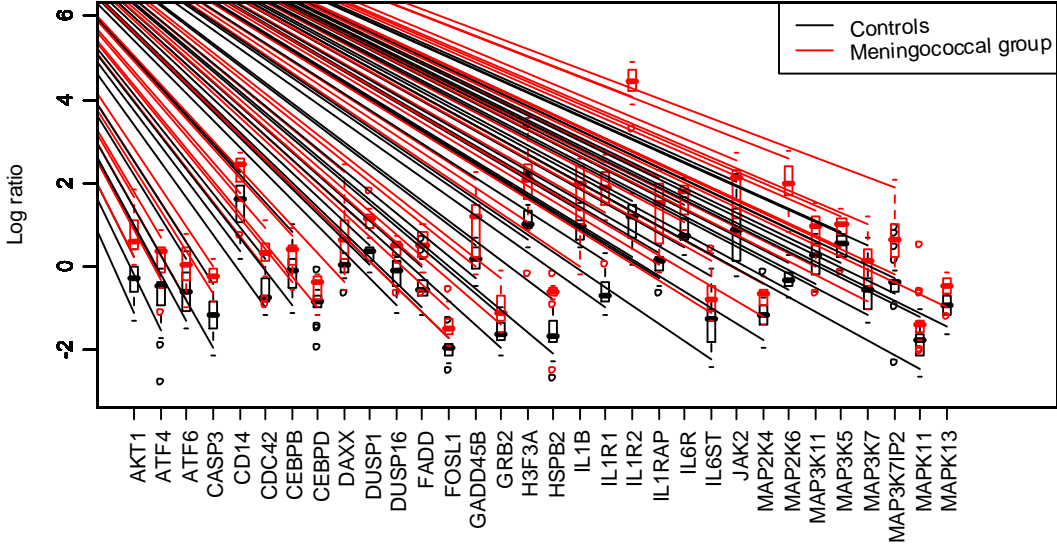


4b: Log-odds ratios of differentially expressed genes of the p38Mapk pathway in meningococcal patients compared to healthy controls:



Supplementary Figure 1a: *Boxplot showing significantly upregulated genes of the p38MAPK pathway.*

Up regulated p38MAPK genes



Supplementary figure 1b: *Boxplot showing significantly downregulated genes of the p38MAPK pathway.*

Down regulated p38MAPK genes

