Varying magnesium concentration elicits changes in inflammatory response in human umbilical vein endothelial cells (HUVECs)

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Abstract

The aims of this study were to determine whether low concentrations of magnesium *in vitro* exacerbated the human umbilical vein endothelial cell (HUVEC) response to inflammatory challenge, and whether expression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) through the toll-like receptor 4 (TLR4) played a role in this process. HUVECs were incubated with different concentrations of Mg (low- 0.1mM, control- 1mM, high- 5mM) for 72 h before being stimulated with bacterial lipopolysaccharide (LPS) for 4 h. The response of cells to LPS was greater in cells cultured in low Mg, relative to control cells and suppressed in high Mg. Expression of NF-κB was increased in low-Mg and decreased with high Mg. Low Mg increased the expression of TLR4 mRNA, but only in the presence of LPS. Antibody blockade of TLR4 but not TLR2 blunted the reponse of cells to LPS in low Mg, such that they were similar to unblocked 1mM Mg cells. Associations of Mg with cardiovascular disease may therefore relate to inflammatory responses mediated through the TLR4/NF-κB pathway.

Introduction

Magnesium (Mg) is fundamental to endothelial cell function [1] and several studies have drawn attention to the interaction of Mg with ATP and its influence on the biochemical functions of nucleic acids, enzymes, phospholipids, and proteins [1-4] . Endothelial dysfunction associated with inflammation is a key stage in atherosclerosis [5]. Mg undernutrition and deficiency, which some studies have associated with cardiovascular disease [6, 7], has been proposed to alter the function of endothelial cells by activating the potent nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [8]. NF-κB activation can be triggered by a number of factors, including ultraviolet (UV) radiation, reactive oxygen species, and bacterial or viral products, such as lipopolysaccharides (LPS) [9].

The NF-κB family comprises many members, including NF-κB1 (p50), NF-κB2 (p52), REL proto-oncogene, NF-κB subunit (RelA, p65), RelB, and RelC. In the cytoplasm, NF-κB family members are bound to members of the NF-κB inhibitor (IkB) family, which includes six proteins [9, 10]. In unstimulated cells, IkB functions as an inhibitor protein that binds to NF-κB to keep it inactive in the cytoplasm and prevent it from binding to DNA [11]. The translocation of NF-κB to the nucleus occurs when IkB is degraded by phosphorylation by the upstream IkB kinase [12] complex [13]. The toll-like receptor (TLR) transmembrane family plays a role in identifying pathogens and eliciting the innate immune response. Toll-like receptor 4 (TLR4) is activated in response to LPS and induces the activation of the NF-κB pathway [14, 15]. When LPS binds to endothelial TLR4, it activates the downstream signal for these receptors via the myeloid differentiation protein 88 (MyD88) toll-domain containing protein, which phosphorylates the interleukin-1 (IL-1) receptor-associated kinase-1 (IRAK1). This, in turn, activates the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [16]. This upstream activation results in a release of NF-κB from the IkB cytoplasmic inhibitor, [17]. In the presence of LPS, TLR4 expression can result in NF-κB activation via the mitogen-

activated protein kinase (MAPK) pathway [18], which in turn activates p38 MAPK and c-Jun N-terminal kinase (JNK) [19].

Whilst NF-κB has been previously identified as being responsive to Mg deficiency, there has not been a full evaluation of magnesium effects on the NF-κB cascade and toll-like receptor signalling. This study aimed to investigate the effect of Mg on the NF-κB pathway in basal conditions and in the presence of LPS.

Methods and Materials

Cell Culture

Primary HUVECs (C2519A; Lonza Basel, Switzerland) were cultured in endothelial cell growth medium (EGM-2, Lonza) with 2% foetal bovine serum (FBS). The HUVEC cultures were incubated in six well plates at seed density (7500/cm^2), at 37°C (5% CO_2), with medium changed every other day until the cells were grown to 80–90% confluence. At 80% confluence, the HUVECs were cultured in human endothelial Mg-free medium (Invitrogen, USA) supplemented with 10% FBS, 1% penicillin 100× 1%, 5% endothelial cell growth supplement (Sigma-Aldrich, UK), and $MgSO_4$ concentrations of 0.1 mM and 5 mM following the method of Ferre *et al*., and Maier *et al*., [1, 20]. The samples were compared with cells cultured with 1 mM $MgSO_4$, which is the physiological circulating concentration of Mg. After 3 days, the cells were treated for 4 h with 0.5 µg/mL LPS (*Salmonella enterica* serotype Enteritidis, Sigma-Aldrich) and the response was compared with untreated cells. HUVECs were plated onto six-well plates, and the cells were treated with 0.1 mM, 1 mM or 5 mM $MgSO_4$ for 3 day. Next, the cells were blocked for 30 minutes using 10µg/ mL neutralising antibodies (Anti-TLR-2 or Anti-TLR-4; InvivoGen, USA) [21], Anti-TLR2 and Anti-TLR4 together; some cells were not blocked and were used as a control. The cells were then stimulated with 0.5 µg/mL LPS for 4

h. Culture media were collected for ELISA, and cells were collected to measure the amount of cellular protein. In a separate experiment, cells were collected for gene expression analysis.

RNA Extraction and Measurement of mRNA by qRT-PCR

RNA was extracted from the HUVECs using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Single-stranded cDNA was synthesised using a RevertAid Reverse Transcription Kit (ThermoFisher Scientific, USA) following the manufacturer's instructions. A Thermo Scientific Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to quantify and determine RNA concentrations. RNA integrity was also tested by denaturing gel electrophoresis and running an aliquot of the RNA sample on an agarose gel (1% agarose gel was dissolved in 100 ml 1x TAE buffer) stained with ethidium bromide. Primer Express Software v3.0 (Applied Biosystems, USA) was used to design the primers for quantitative real-time polymerase chain reaction (qRT-PCR). All sequences were taken from the National Centre for Biotechnology Information (NCBI). The design of the primers was confirmed using the Basic Local Alignment Search Tool (BLAST). Quantification of mRNA was performed as previously described. The forward and reverse primer pairs are listed as in Table 1. A LightCycler 480 (Roche) was used to perform Quantitative Real-Time Polymerase Chain Reaction qRT-PCR. The method involved a cycling state of 95°C for 5 min, then 45 cycles for 10 s at 95°C, followed by an annealing for 15 seconds at 60 ºC and elongation step for 15 seconds at 72ºC. Using a SYBR Green Master Mix. All values were normalised to the cyclophilin B housekeeping gene [22]. Using this method, a relative quantification of gene expression can be calculated using a mathematical model known as the comparative threshold ($2^{\Delta\Delta ct}$) [23]. Expression of

cyclophilin B was not significantly influenced by either LPS treatment or variation in Mg concentration.

NFκB Pathway Array

A Human NFκB Pathway Array kit (R&D Systems, Ltd.) was used to quantify the expression of the proteins involved in the NFκB pathway. The array uses captured antibodies spotted on a nitrocellulose membrane. HUVECs were plated onto six-well plates, and the cells were stimulated with 0.5 µg/mL LPS for 4 h. At the end of the stimulation period, the cells were washed with cold PBS, and then 120 µl lysis Buffer RIPA (radio-immunoprecipitation assay) buffer (Sigma Aldrich, UK) was added with 5 µl/ml of protease inhibitor cocktail (Calbiochem, USA). The plate was incubated on ice for 30 minutes. Cells were harvested and the array membrane was blocked according to the manufacturer's instructions (Human NFκB Pathway Array kit; R&D Systems, Ltd.). The spot signal densities were analysed using Quantity One Analysis Software (Bio-Rad).

Enzyme-linked Immunosorbent Assay (ELISA)

The Human CXCL8/IL-8 Quantikine Immunoassay kit and the Human VCAM-1 DuoSet kit (R&D Systems, Ltd.) is a quantitative sandwich enzyme immunoassay microplate-based technique. These kits were used to measure interleukin-8 (IL-8) and vascular cell adhesion molecule-1 (VACM-1) in cell culture supernatants. For both assays, a microplate was precoated with analyte of interest monoclonal antibody, which bonded with a target analyte in the samples. Detection of the bound to the primary antibody was assessed through the addition of a second horseradish peroxidase antibody and the addition of reagent to produce a coloured reactant that can be determined by spectrophotometry.

Statistical Analyses

Each treatment group comprised four samples with a sample being a single well on a culture plate. All data are presented as mean ± standard error of mean [24]; the mRNA expression data were analysed using a two-way analysis of variance (ANOVA; SPSS Statistics 22 software). Data with $p < 0.05$ were regarded as statistically significant. Statistical significance between treatment groups was determined using Dunnett's test as a post-hoc test for the effect of Mg (using 1mM Mg as the control condition) and for the effect of LPS. Protein expression were analysed using a one-way analysis of variance (ANOVA; SPSS Statistics 22 software).

Results

To determine the effect of Mg on the mRNA expression of genes involved in the NF-κB pathway, LPS was used to stimulate the HUVECs in the presence of varying Mg concentrations. As shown in Figure 1A HUVECs expressed TLR4, and there was significant effect of magnesium concentration and LPS on TLR4 mRNA expression (P =0.001 for magnesium concentration × LPS interaction), and a low concentration of magnesium markedly increased the TLR-4 mRNA relative to the physiological concentration of magnesium 1 mM, in the presence of LPS. At the low Mg concentration, the expression of IRAK1, TRAF6 and IkB mRNA expression was not significantly different from the control. Although the impact of LPS on expression of these genes tended to be greater in Mg deficient cells, this was not statistically significant (Figure 1).

As shown in Figure 1 E a significant increase in NF-κB2 mRNA was observed in the magnesium-deficient cells that were stimulated with LPS (P=0.032, Dunnett test), Moreover, a marked decreased in NF-κB2 mRNA in the high-magnesium LPS-stimulated HUVECs was observed (P=0.048, Dunnett test) relative to the 1 mM physiological concentration. Mg concentration had no significant effect on JUN and C-JUN mRNA (Figure 1 F and G).

In an attempt to gain more detailed understanding of the NF-κB pathway's response to Mg, HUVECs were treated with LPS (0.5 µg/ml) for 4 hours. Protein extraction was performed and concentration of proteins in the pathway was assessed using an NF-κB array kit. The array considered expression of 41 proteins, but for this analysis we focused on the 11 proteins involved in inflammatory processes TLR-2, MYD88, TRAF2, IRAK1 Ikkα, Ikkβ, Ikkγ, IkBα, IkBε, JNK1/2, JUN2. Under LPS stimulation, there were no significant effects of either low or high Mg upon concentration of TLR2, MyD88, TRAF3, IRAK1, JNK1/2 and JUN2 proteins, consistent with the mRNA analyses for JUN, IRAK and TRAF2 (Table 2). Protein expression of the components of the MyD88-independent pathway was not altered by varying Mg concentration (Ikkα, Ikkβ, Ikkγ, IkBα and IkBε; Table 2).

To investigate the role of toll-like receptors in mediating the effects of Mg on the response to LPS challenge, we repeated experiments in the presence of antibodies to block TLR2 and TLR4. The effects of the treatments on mRNA expression of NF-κB, intercellular cell adhesion molecule (ICAM1) and VCAM1 were generally similar (Figure 2). Blocking LPS-stimulated HUVECs with anti-TLR-2, anti-TLR-4 and both anti-TLR-2+TLR-4 antibodies significantly decreased the mRNA expression in cells cultured in 0.1 mM Mg or 1mM Mg, when compared to LPS treatment alone at the same magnesium concentration. As expected, a high magnesium concentration (5 mM) markedly reduced expression of mRNA in LPS-stimulated HUVECs compared to the HUVECs cultured in 1 mM $MgSO_4$ of the same treatment group. No effect of antibody blockade was noted in cells cultured in high magnesium. The effects of antibody blockade were greater with anti-TLR4 than anti-TLR2, suggesting that the former was the major mediator of LPS-stimulated effects on expression of these genes. Similar effects were noted in the analysis of IL-8, IL-6 and monocyte chemoattractant protein-1 (MCP-1) mRNA expression (Figure 2). However, unlike the observations for NF-κB, ICAM1 and VCAM1, anti

TLR-4 blockade of cells cultured in high Mg resulted in further suppression of expression for IL-8 and MCP-1 ($P<0.05$).

To confirm that mRNA expression effects were also present at the protein level, VCAM-1 and IL-8 concentrations were determined by ELISA. The results, as shown in Figure 3 A, demonstrated that magnesium concentration and blocker had a significant interactive effect ($P< 0.001$) on the protein concentration of IL-8. Low magnesium considerably increased secretion of IL-8 by stimulated HUVECs, compared to 1 mM $MgSO_4$ in the same treatment groups. In addition, the blocking HUVECs with anti-TLR-4 antibody decreased IL-8 protein concentration markedly compared to the LPS treated cells, but anti TLR-2 had the opposite effect. Figure 3 B shows that there was a significant effect of magnesium concentration ($P< 0.001$), and the antibody blockade on VCAM-1 expression ($P< 0.001$).

Discussion

Under normal circumstances endothelial cells produce IκB, which inhibits NF-κB subunit activity, but in endothelial dysfunction, NF-κB is activated via the degradation of IκB by proteasomes, generally in response to inflammatory signals [25]. This results in release of activated NF-κB subunits p50 and p65, which then translocate to the nucleus leading to transcription of inflammatory genes [9, 17]. The TLR-4 signalling pathway plays a key role in inducing the NF-κB pathway [26]. As hypothesised, the results of the present study confirmed that HUVECs cultured in a medium with a low concentration of Mg for 72 hours significantly increased NF-κB mRNA expression, as measured by qRT-PCR. In contrast, HUVECs treated with 5mM Mg showed a reduced expression of NF-κB. Other studies have reported that TNF stimulation of HUVECs upregulated the gene expression of NF-κB and IkB [13]. Our findings are consistent with this and with other studies that showed HUVECs cultured in a medium with

a low Mg concentration (0.1 mM) had an enhanced NF-κB activity, which was further increased after treatment with 1 µg/mL LPS [8]. However, we did not observe widespread changes in expression of components of the NF-κB pathway and Mg appeared to have relatively selective effects on NF-κB2.

The novel element of this study was the investigation of the role of toll-like receptors in mediating Mg-related effects on inflammatory responses in HUVECs. Cells cultured with a low concentration of Mg and challenged with LPS showed a significant increase in the expression of TLR4. Other studies have shown that endothelial cells expressed TLR4 to activate NF-κB in response to LPS exposure [16, 27]. Many researchers have noted that LPS activates NF-κB in HUVECs via TLR4 and MyD88 [28, 29]. Similarly, TLR4, MyD88, and TRAF have been reported to be upregulated after HUVECs were treated with 100 ng/mL LPS [30]. However, the present study found no difference in the gene expression of IRAK1, TRAF6, IkB, MyD88, Ikk, and IkB proteins in the NF-κB pathway in response to varying Mg concentration. Additionally, incubation at different magnesium concentrations did not significantly affect the expression of c-Jun and Jun.

One of the main limitations of the present study is that measurements focused on the effects of varying magnesium concentration and LPS treatment upon expression of key targets at the mRNA and protein level only. Functional activity of the NF-κB pathway is critically determined by post-translational modification of proteins and translocation within the cell. These modifications at the protein level were not studied and would be a high priority for further studies that can categorically determine the involvement of the pathway in magnesium concentration-dependent modulation of the inflammatory response.

Most current research focuses on the role of TLR4 in eliciting the response of HUVECs to LPS. TLR2 has also been implicated in the LPS pathway, but no study has previously investigated the effect of blocking TLR-2 on NF-κB activation in HUVECs. Our results

confirm the hypothesis that TLR-4 and, to a lesser extent, TLR-2 play a role in regulation of NF-κB activation, as blocking these receptors significantly inhibited the effect of LPS on NF-κB translocation and activation in the HUVECs cultured in a low Mg concentration. This showed that Mg deficiency can influence processes which depend on both receptors. These results are consistent with those reported by [31], which indicated that blocking TLR-4 in HUVECs inhibits the effect of LPS in activating NF-κB. Similarly, blocking TLR-4 in human dermal microvascular endothelial cells (HMEC) stimulated with LPS results in inhibition of NF-κB activity [27].

Interestingly, the high magnesium concentration was shown to inhibit the response to LPS more efficiently than anti-TLR-2 and anti-TLR-4 antibodies. Magnesium appears to have a direct impact the TLR-4 receptors, based on the fact that magnesium does not modify the expression of MyD88 and, subsequently, TRAF6. The anti-inflammatory impact of magnesium in the TLR-LPS pathway may occur via several mechanisms. Firstly magnesium can directly inhibit the LPS induced expression of the TLR-4 receptor (as confirmed in this study) and therefore limit NF-κB activation. Additionally, magnesium acts as a calcium channel blocker and effect on the intracellular calcium concentration [32, 33]. Calcium is a major promoter of inflammation [34, 35] due to its role in the activation of the TLR-4 receptors [36]. Finally high magnesium blocks the NMDA receptors, which regulate the pre-inflammatory response [37, 38]. Such blocking leads to reduced activity of the TLR-4 receptor, and magnesium has an antagonistic effect on the NMDA receptor [39]. Mg^{2+} can suppress NMDA receptors through a voltage dependent manner [38].

Another substantial finding of this study is that a high magnesium concentration significantly inhibits the expression of the adhesion molecules; ICAM-1 and VCAM-1 in LPS-stimulated HUVECs. TLR-2 and TLR-4 play a significant role in ICAM-1 and VCAM-1 release, and the blocking of these receptors also significantly downregulated the expression of ICAM-1 and

VCAM-1 when cells were exposed to a low magnesium concentration. The results of the present study agree with the findings reported by Nakamura et al. [40] who found that incubating stimulated HUVECs with anti-TLR-2 and anti-TLR-4 antibodies significantly inhibited mononuclear cell adhesion to the HUVEC's surface.

One of the main findings of this study was that inhibition of TLR-4 by neutralising antibodies in stimulated HUVECs, cultured in a low magnesium concentration, decreased the LPS induced expression of the inflammatory cytokines IL-8, IL-6 and MCP-1 mRNA. Moreover, this inhibitory effect of the antibodies was enhanced with exposure to a high magnesium concentration. The addition of magnesium significantly inhibited the release of IL-8, IL-6 and MCP-1 in cells following LPS treatment. These results are consistent with the findings reported by Hippenstiel et al. [41], who clarified that activated NF-κB was important in upregulating IL-8 expression and that IL-8 expression was inhibited in LPS-stimulated HUVECs when Rho was blocked. The work of Masood et al. [42] also indicated that, after stimulating HUVECs with the serum of patients with bacterial infections and incubating them with TLR antibodies, blockade with anti-TLR-4 markedly decreased the expression of IL-8 by 40%, whereas blockade with anti-TLR-2 only slightly decreased the expression of IL-8. The present study similarly found that the effects of TLR-4 blockade were greater than seen with TLR-2 blockade. Shimamoto et al. [43] observed that the TLR-4 mutation in mice inhibited the expression of IL-6 and MCP-1 mRNA in the animals' hearts. Furthermore, incubating HUVECs with siRNA to silence TLR-4 for 24 hours, then stimulating them with LPS significantly decreased the expression of MCP-1 [44].

The relevance of this in vitro study to human health needs to be considered in the context of magnesium intake, particularly as a number of studies have identified relationships between intake and cardiovascular disease risk [6, 7, 45, 46]. Magnesium nutrition is often overlooked, but low intakes are commonplace in Western populations. The data presented in this paper

demonstrate that the inflammatory response of HUVECs to bacterial endotoxin is exacerbated by low Mg concentrations in vitro, but suppressed with high Mg. Toll-like receptors and the NF-κB pathway may be central to these effects.

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FIGURE LEGENDS

Figure 1. Effect of different concentrations of magnesium on NF-κB pathway gene expression. Data is shown as Mean±SEM for n=4. * indicates $P<0.05$, compared to 1 mM $MgSO_4$ in the same treatment (Dunnett's test), + indicates $P<0.05$, ++ indicates $P<0.01$ compared to LPS at the same magnesium concentration (t-test).
A: Expression of TLR-4 mRNA. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS ($P=0.001$). There was a significant effect of magnesium concentration ($P=0.019$) and LPS ($P=0.039$).
B: Expression of IRAK1 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS
C: Expression of TRAF6 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS.
D: Expression of IKB mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS. There was a significant effect of LPS ($P= 0.002$).
E: Expression of NF-KB 2 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS. There was a significant effect of magnesium concentration ($P<0.001$) and LPS ($P< 0.001$).
F: Expression of JUN mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS. There was a significant effect of LPS ($P= 0.04$).
G: Expression of C-JUN mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS. There was a significant effect of LPS ($P= 0.043$).

Figure 2. Effect of different concentrations of magnesium on HUVEC gene expression when treated or not treated with LPS and TLR antibodies. Data are shown as mean ±SEM for n=4. * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$ compared to 1mM $MgSO_4$ in same treatment (Dunnett t). + indicates $P<0.05$, ++ indicates $P<0.01$, +++ indicates $P<0.001$ compared to LPS at same magnesium concentration (Dunnett t).
A: Expression of NF-κB mRNA. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P< 0.001$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).
B: Expression of ICAM-1 mRNA. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P< 0.001$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).

C: Expression of VCAM-1 mRNA. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P< 0.001$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).
D: Expression of IL-8 mRNA. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P< 0.001$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).
E: Expression of IL-6 mRNA. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P< 0.001$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).
F: Expression of MCP-1 mRNA. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P= 0.005$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).

Figure 3. Effect of different concentrations of magnesium on HUVECs protein concentration treated or not with LPS and TLR antibodies. Data are shown as mean ±SEM for n=4. * indicates $P<0.05$ compared to 1mM $MgSO_4$ in same treatment (Dunnett t). + indicates $P<0.05$, ++ indicates $P<0.01$ compared to LPS at same magnesium concentration (Dunnett t).
A: Protein concentration of IL-8. Two-way ANOVA showed a significant interaction of magnesium × blocker ($P< 0.001$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).
B: Protein concentration of VCAM-1. Two-way ANOVA showed no significant interaction of magnesium × blocker. There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).

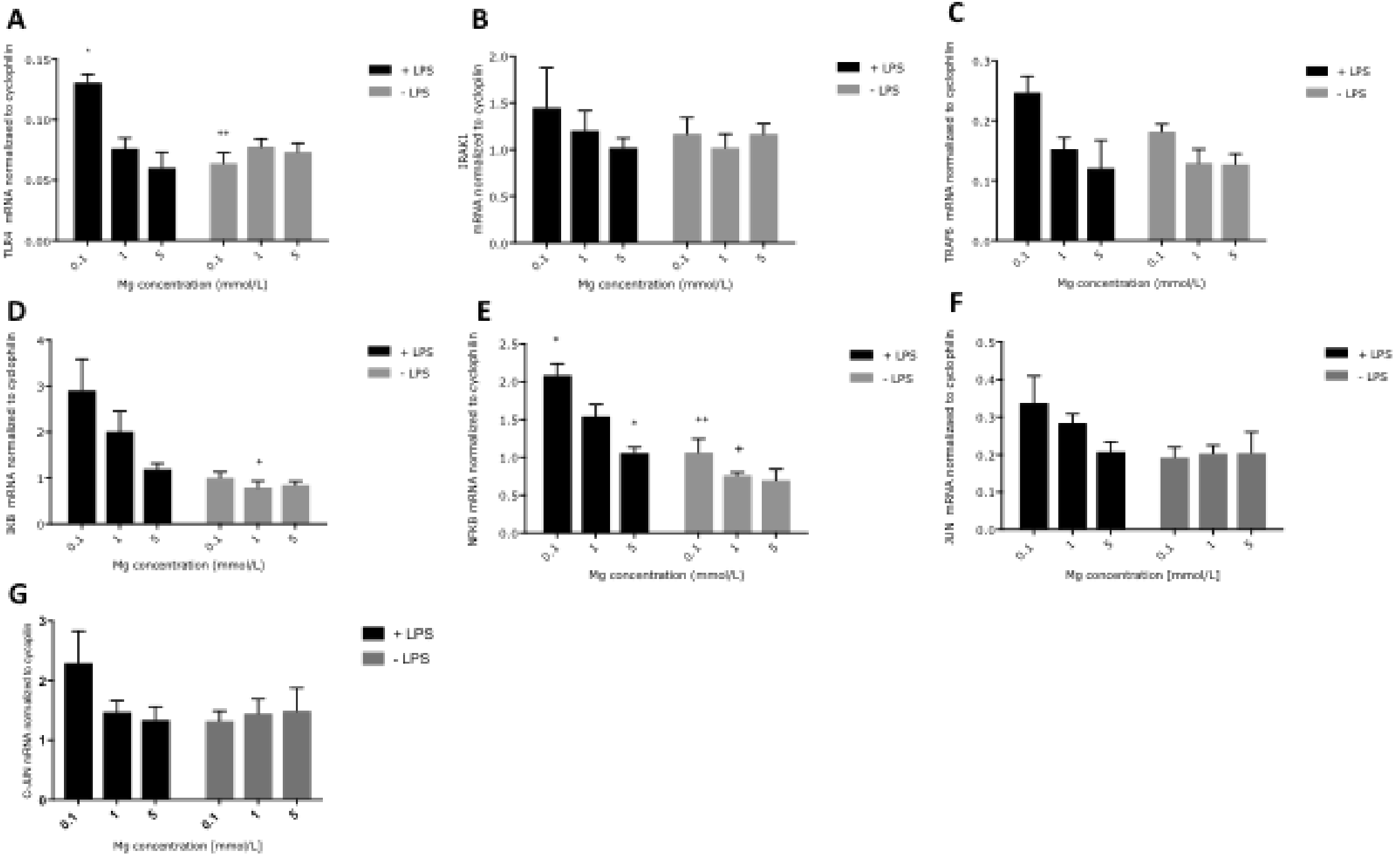
456 Table 1. Primer sequences used for qRT-PCR

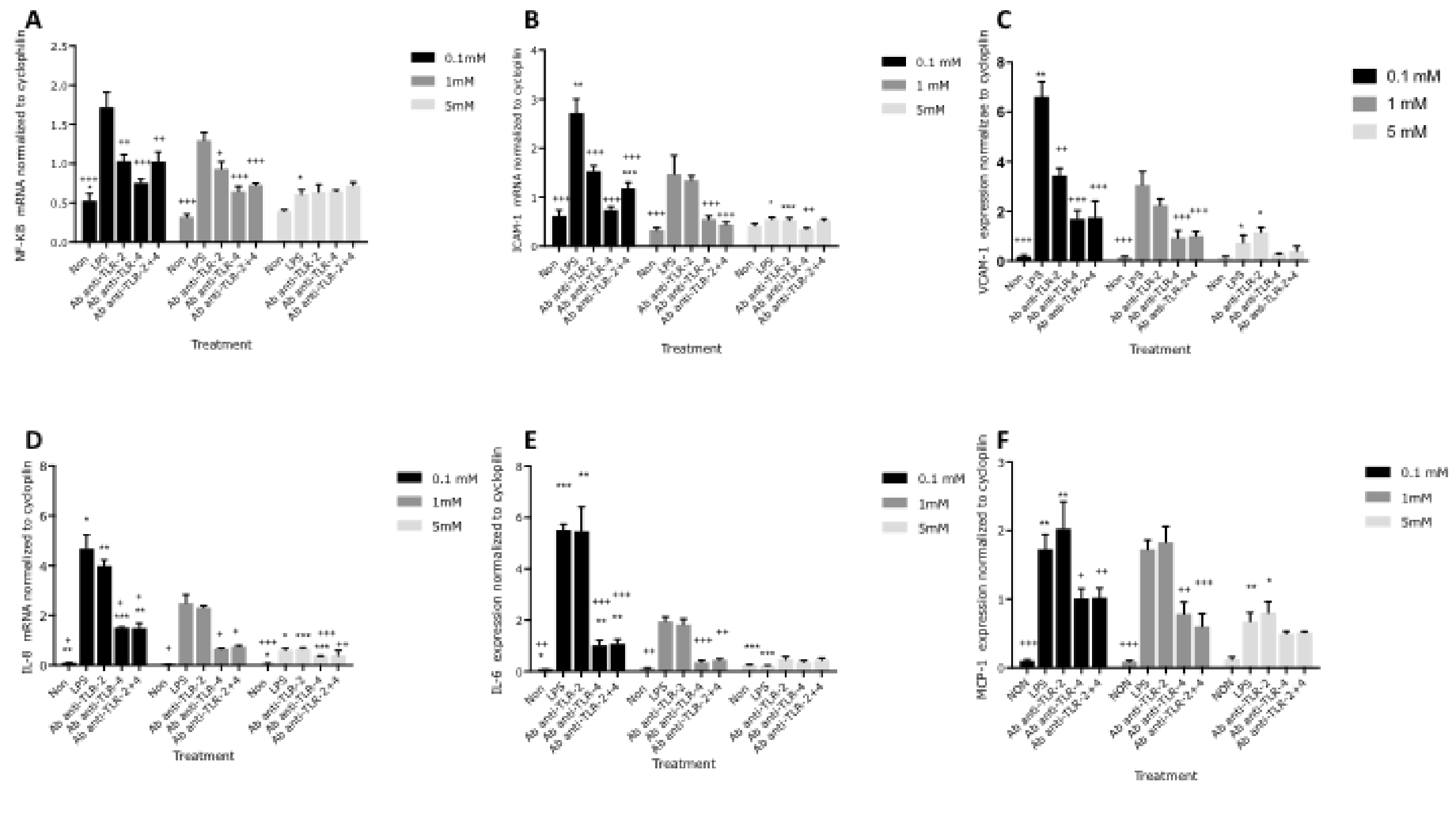
Symbol	Gene name	Foreword primer	Reverse primer
TLR4	Homo sapiens Toll-like receptor 4	GGGTCCAACACTTGTTCAGTTAATAA	TGCATATCTAGTGCACCATGGAA
IRAK	Interleukin-1 receptor-associated kinase 1	CGCCCCTTTCCGTTTTG	GATCTTGAGCTCCTCCGAGAAG
TRAF6	TNF receptor-associated factor 6	TCAGTACTTTTGGTTGCCATGAA	AGCCTGGGCCAACATTCTC
IKB	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	GCTGAAGAAGGAGCGGCTACT	TCGTACTCCTCGTCTTTCATGGA
NFkB	Nuclear translocation of nuclear factor kappaB	GGGATCTGCGCCGTTTCT	TCCCCATCATGTTCTTCTTAGTCA
Jun	Jun N-terminal kinase	ATGACTGCAAAGATGGAAACGA	GCTCTCGGACGGGAGGAA
C-JUN	c-Jun N-terminal kinase kinase 2	ATTGTGAAGGCGCTGTACTACCT	CGTCCAGCAGGATGTTGGA
PPI B	Cyclophilin B	GGAGATGGCACAGGAGGAAA	CGTAGTGCTTCAGTTTGAAGTTC TCA
IL-8	Interleukin 8	ACCGGAAGGAACCATCTCACT	ATCAGGAAGGCTGCCAAGAG
ICAM-1	Intercellular adhesion molecule	TCCCCCCGGTATGAGATTG	GCCTGCAGTGCCCATTATG
VCAM	Vascular cell adhesion molecule	GCAAGGTTCCTAGCGTGTAC	GGCTCAAGCATGTCATATTCAC
IL-6	Interleukin 6	CCGGGAACGAAAGAGAAGCT	GCGCTTGTGGAGAAGGAGTT
MCP-1	Homo sapiens chemokine (C-C motif) ligand 2 (CCL2)	CGCCTCCAGCATGAAAGTCT	GGAATGAAGGTGGCTGCTATG

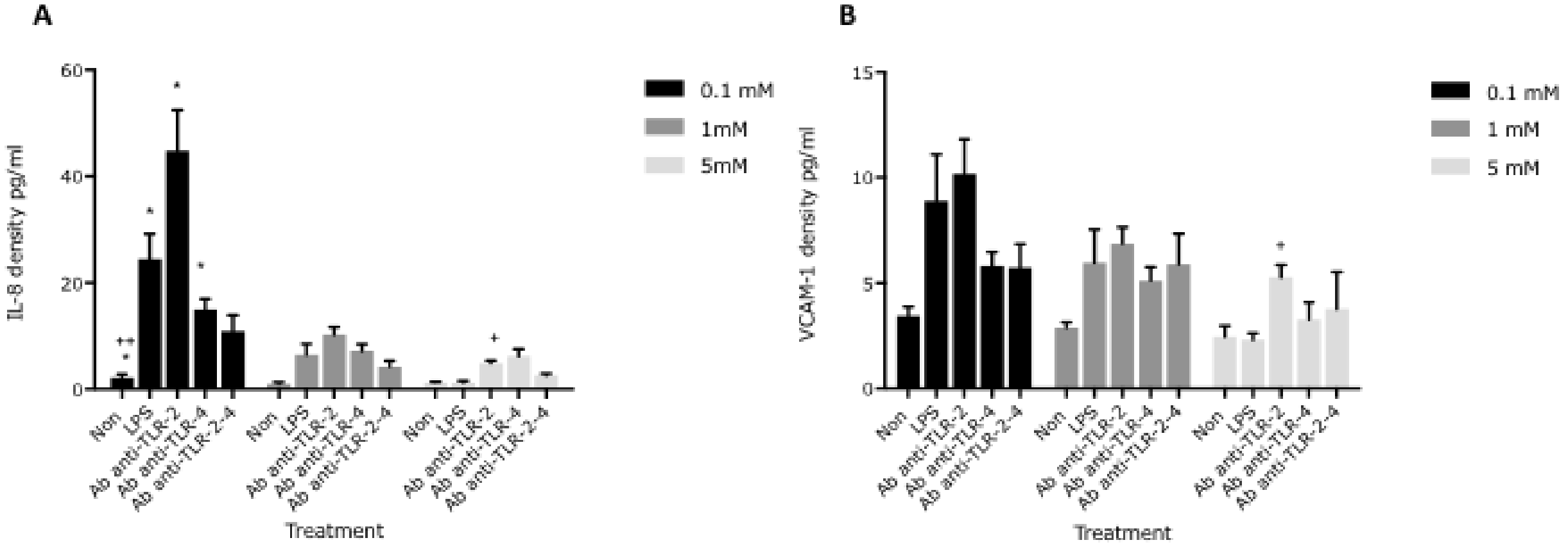
Table 2. Effect of different concentrations of Mg on NF-κB pathway protein concentration.

Genes	0.1 mM $MgSO_4$	1mM $MgSO_4$	5mM $MgSO_4$	*P* value 0.1mM vs 1mM	*P* value 5mM vs 1mM
TLR2	0.21 ± 0.12	0.09 ± 0.016	0.07± 0.011	0.62	0.67
MYD88	0.58± 0.29	0.3 ± 0.089	0.27 ± 0.06	0.46	0.99
TRAF2	0.67 ± 0.26	0.49 ± 0.12	0.35 ± 0.062	0.69	0.79
IRAK1	1.22 ± 0.31	1.05 ± 0.24	0.94 ± 0.17	0.85	0.94
JNK1/2	0.89 ± 0.21	0.71 ± 0.19	0.6 ± 0.09	0.7	0.87
JNK2	0.44 ± 0.25	0.22 ± 0.05	0.02 ± 0.036	0.48	0.99
IKKα	0.64 ± 0.29	0.4 ± 0.11	0.41 ± 0.062	0.74	0.99
IKKβ	0.9 ± 0.36	0.58 ± 0.14	0.65 ± 0.095	0.54	0.96
IKKγ	0.78 ± 0.27	1.13 ± 0.25	0.84± 0.2	0.7	0.95
IKBα	1.4 ± 0.3	1.16 ± 0.31	1.03 ± 0.15	0.71	0.96
IKBε	1.62 ± 0.24	1.54 ± 0.34	1.01 ± 0.2	0.91	0.32

Data is shown as Mean± SEM for n=4.


A
B
C
D
E
F
G
+ LPS
- LPS
Mg concentration (mmol/L)
Mg concentration [mmol/L]
0.1
1
5


A
NF-KB mRNA normalized to cyclophilin
0.1mM
1mM
5mM
Non
LPS
Ab anti-TLR-2
Ab anti-TLR-4
Ab anti-TLR-2+4
Treatment
B
ICAM-1 mRNA normalized to cyclophilin
0.1 mM
1 mM
5mM
Treatment
C
VCAM-1 expression normalized to cyclophilin
0.1 mM
1 mM
5 mM
Treatment
D
IL-8 mRNA normalized to cyclophilin
0.1 mM
1mM
5mM
Treatment
E
IL-6 expression normalized to cyclophilin
0.1 mM
1mM
5mM
Treatment
F
MCP-1 expression normalized to cyclophilin
0.1 mM
1mM
5mM
Treatment


A
IL-8 density pg/ml
60
40
20
0
0.1 mM
1mM
5mM
Non
LPS
Ab anti-TLR-2
Ab anti-TLR-4
Ab anti-TLR-2-4
Treatment
B
VCAM-1 density pg/ml
15
10
5
0
0.1 mM
1 mM
5 mM
Non
LPS
Ab anti-TLR-2
Ab anti-TLR-4
Ab anti-TLR-2-4
Treatment