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2 **Magnesium deficiency heightens lipopolysaccharide-induced inflammation and**
3 **enhances monocyte adhesion in human umbilical vein endothelial cells**

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21 **Abstract**

22 Given a possible anti-inflammatory role of magnesium in endothelial cells, the aim of this
23 study was to investigate the effects of magnesium on human umbilical vein endothelial
24 cell (HUVEC) viability, gene expression, and the pro-inflammatory response caused by a
25 bacterial endotoxin (LPS). HUVECs were cultured at three different concentrations of
26 magnesium sulphate (0.1mM; control-1mM; 5mM) for 72 hours. Exposing the cells to LPS
27 reduced cell viability in culture with low magnesium, but high magnesium protected the
28 HUVECs from LPS-induced cell death. LPS-treated HUVECs cultured in low magnesium
29 showed up-regulation of mRNA expression for pro-inflammatory factors and the
30 expression of cytokine proteins, including IL-2, IL-3, IL-8, IL-15 and MCP-1. This was
31 associated with greater adhesion of monocytes to the cells. In contrast, high magnesium
32 decreased the expression of inflammatory factors and cytokines. The study found that LPS
33 activation of the expression of many pro-inflammatory factors is exacerbated in the
34 presence of low magnesium concentration whilst a high magnesium concentration partly
35 inhibited the inflammatory response to LPS.

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37 **Introduction**

38 Endothelial cells are present along the length of all blood vessels and control the movement
39 of macroparticles and hormones and regulate blood flow [1], cellular adhesion, vascular
40 inflammation, vessel tone and smooth muscle proliferation [2]. Endothelial dysfunction
41 plays a role in many vascular diseases, such as thrombosis and atherosclerosis. In
42 atherosclerosis, plaques develop in the walls of the arteries in response to the dysfunction
43 of endothelial cells, which express cytokines and adhesion molecules that recruit T-cells
44 and drive monocytes and macrophages to the sub-endothelial space [3].

45 Epidemiological studies suggest that poor intakes of magnesium are associated with
46 greater risk of cardiovascular disease [4]. These studies are complemented by animal
47 studies that have demonstrated magnesium supplementation to limit the development of
48 atherosclerotic lesions in LDL receptor and apolipoprotein E knockout mice [5, 6]
49 Magnesium status may impact on cardiovascular health through a number of mechanisms
50 [7]. Previous work suggests that low extracellular magnesium concentrations have a
51 negative impact on endothelial cell proliferation, increase monocyte adhesion, inhibit cell
52 migration and markedly alter endothelial cell gene expression. Low magnesium levels may
53 also contribute to inflammation. These effects may be due to the activation of many types
54 of cytokines, which induce an overexpression of the inflammatory phenotype in endothelial
55 cells [8]. The current study explored the hypothesis that the expression of adhesion
56 molecules and cytokines would be enhanced in human umbilical vein endothelial cells
57 (HUVECs) cultured under conditions of magnesium depletion, and that the enhanced
58 inflammatory response would increase monocyte adhesion, which may represent an early
59 stage in atherosclerosis.

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64 **Methods and Materials**

65 **Cell Culture**

66 Primary HUVECs (C2519A; Lonza Basel, Switzerland) were cultured in endothelial cell
67 growth medium (EGM-2, Lonza) with 2% foetal bovine serum (FBS). Cells were incubated
68 in six well plates at seed density (7500/cm²), at 37°C (5% CO₂), with medium changed
69 every other day until the cells were grown to 80–90% confluence. At 80% confluence,
70 cells were transferred to human endothelial Mg-free medium (Invitrogen, USA),
71 supplemented with 10% FBS, 1% penicillin 100× 1%, 5% endothelial cell growth
72 supplement (Sigma-Aldrich, UK), and magnesium sulphate (MgSO₄) concentrations of 0.1
73 mM (low Mg) and 5 mM (high Mg) following the method of Ferre *et al.*, and Maier *et al.*,
74 [9, 10]. The samples were compared with cells cultured with 1 mM MgSO₄, which is the
75 physiological circulating Mg concentration. After 3 days, the cells were treated for 4 h with
76 0.1 or 0.5 µg/mL lipopolysaccharide (LPS, *Salmonella enterica* serotype Enteritidis, Sigma-
77 Aldrich) and the response was compared with untreated cells. Separate experiments were
78 performed in order to obtain cell lysates and medium for the measurement of each
79 component of the study (cell viability, adhesion molecule and cytokine mRNA expression,
80 adhesion molecule and protein expression, cell adhesion).

81 For the cell viability assay, cell suspensions were mixed with 10 µl of 0.4% Trypan blue
82 solution (Sigma Aldrich, UK) and pipetted onto a haemocytometer. The cells were
83 examined under the microscope, and the number of viable and nonviable cells was
84 counted.

85 **Cell Adhesion Assay**

86 The Vybrant™ Cell Adhesion Assay kit (V13181, Molecular Probes, ThermoFisher Scientific)
87 was used to evaluate the adherence of monocytes to HUVECs. The procedure used
88 fluorescent dye to label the human monocyte cells, which were then added to culture
89 HUVECs. To assess monocyte adhesion, human monocytic THP-1 cells were labelled with
90 5 µM of calcein for 30 minutes. The calcein-labelled cells were co-cultured with the

91 HUVECs, and then incubated for 60 minutes. Relative fluorescence was used as an index
92 of adhesion

93 **RNA Extraction and Synthesis of Single-stranded cDNA**

94 RNA was extracted from the HUVECs using a High Pure RNA Isolation Kit (Roche,
95 Mannheim, Germany) according to the manufacturer's protocol. Single-stranded cDNA was
96 synthesised using a RevertAid Reverse Transcription Kit (ThermoFisher Scientific, USA)
97 following the manufacturer's instructions. A Thermo Scientific Nano Drop ND-1000
98 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to quantify and
99 determine RNA concentrations. RNA integrity was also tested by denaturing gel
100 electrophoresis and running an aliquot of the RNA sample on an agarose gel (1% agarose
101 gel was dissolved in 100 ml 1x TAE buffer) stained with ethidium bromide (EtBr). Primer
102 Express Software v3.0 (Applied Biosystems, USA) was used to design the primers for real-
103 time polymerase chain reaction (RT-PCR). All sequences were taken from the National
104 Centre for Biotechnology Information (NCBI). The design of the primers was confirmed
105 using the Basic Local Alignment Search Tool (BLAST). Quantification of mRNA was
106 performed as previously described. The forward and reverse primer pairs are listed as in
107 Table 1. A LightCycler 480 (Roche) was used to perform Real-Time Polymerase Chain
108 Reaction RT-PCR. The method involved a cycling state of 95°C for 5 min, then 45 cycles
109 for 10 s at 95°C, followed by an annealing for 15 seconds at 60 °C and elongation step for
110 15 seconds at 72°C. Using a SYBR Green Master Mix. All values were normalised to the
111 cyclophilin B housekeeping gene [11]. Expression of cyclophilin was not significantly
112 influenced by either LPS treatment or variation in Mg concentration.

113 **Western Blot Analysis**

114 The cells were lysed in 120 µl RIPA (radio-immunoprecipitation assay) buffer (Sigma
115 Aldrich, UK) was added with 5 µl/ml of protease inhibitor cocktail (Calbiochem, USA). The
116 samples were denatured at 90°C for 5 minutes. Then, 30 µg of protein was loaded into
117 each well in 4-10% mini-protean TGX precast polyacrylamide gradient gel. Next, the
118 samples were transferred to a nitrocellulose membrane, and then the membrane was

119 blocked. The intercellular cell adhesion molecule-1 (ICAM-1) proteins were detected by
120 using a primary antibody anti-ICAM (Abcam), followed by a secondary antibody to detect
121 the primary antibody. The membranes were then impregnated with enhanced
122 chemiluminescence (ECL) detection (Prime Western Blotting Detection Reagent Kit, GE
123 Healthcare) and visualised by X-ray film exposure.

124 **Human Cytokine Array**

125 The protein concentration of several cytokines in lysed cells was measured using a Human
126 Cytokine Antibody Array Membrane Kit (Abcam), HUVECs were plated onto six-well plates,
127 and the cells were stimulated with 0.5 µg/ml LPS for 4h. At the end of the stimulation
128 time, the cells were lysed in RIPA buffer with protease inhibitor cocktails. The plate was
129 incubated on ice for 30 minutes. Cells were harvested and the array membrane was
130 blocked according to the manufacturer's instructions. The spot signal densities were
131 analysed using Quantity One Analysis Software (Bio-Rad).

132 **Enzyme-linked Immunosorbent Assay (ELISA)**

133 The Human vascular cell adhesion molecule-1 (VCAM-1) DuoSet kit (R&D Systems, Ltd.)
134 is a quantitative sandwich enzyme immunoassay microplate-based technique. This kit was
135 used to measure VCAM-1 in cell culture supernatants.

136 **Statistical Analyses**

137 Each treatment group comprised four samples with a sample being a single well on a
138 separate culture plate. All data are presented as mean ± standard error of mean [12]. The
139 mRNA expression data were analysed using a two-way analysis of variance (ANOVA; SPSS
140 Statistics 22 software). Data with $P < 0.05$ were regarded as statistically significant.
141 Statistical significance between treatment groups was determined using Dunnett's test
142 (1mM magnesium group as reference) as a post-hoc test for the effect of Mg where
143 univariate effects were indicated by the ANOVA. No post-hoc testing was performed where
144 interactions of Mg concentration x LPS treatment were observed. This compared low- and
145 high-Mg concentrations to the control, when variances were homogeneous among the

146 tested groups. Protein expression data were analysed using a one-way analysis of variance
147 (ANOVA; SPSS Statistics 22 software) with Dunnett's test as a post-hoc test (1mM
148 magnesium group as reference).

149 **Results**

150 The viability of HUVECs in culture was impacted by the concentration of MgSO₄ in the
151 medium. Overall, low magnesium conditions resulted in lower cell viability while a
152 significantly greater proportion of viable cells were observed when HUVECs were cultured
153 in high magnesium concentrations, compared to the control 1 mM (Figure 1). After HUVECs
154 were stimulated with 0.1 µg/ml LPS or 0.5 µg/ml LPS for 24 hours, there was a significant
155 loss of viability in both a Mg concentration-dependent and LPS concentration-dependent
156 manner ($P < 0.001$). The effect of 48 hours LPS-stimulation on viability varied with Mg
157 concentration (interaction of LPS and Mg concentration, $P = 0.018$). Viability of cells grown
158 in 5 mM MgSO₄ after treatment with 0.5 µg/ml LPS for 24 and 48 hours was improved
159 relative to the control. Conversely, LPS treatment of HUVECs cultured in 0.1 mM MgSO₄
160 resulted in markedly lower cell viability (Figures 1 a and b). Cell viability was also assessed
161 using the MTT assay method [13] which showed the same trends as identified using trypan
162 blue staining (data not shown).

163 The effects of varying Mg concentration and inflammatory challenge on the expression of
164 adhesion molecules by HUVECs were evaluated at both the mRNA and protein level. The
165 expression of ICAM-1 and VCAM-1 mRNA was influenced by a significant interaction of Mg
166 concentration and presence of LPS ($P = 0.001$, $P < 0.001$ respectively). In the absence of
167 LPS, low Mg increased expression of ICAM-1, but not VCAM-1, whilst high Mg had the
168 opposite effect (Figure 2 a and b). Following the treatment of cells with LPS to induce an
169 inflammatory response, both ICAM-1 and VCAM-1 mRNA was significantly increased in the
170 presence of low Mg, compared to the physiological concentration 1mM. In contrast, the
171 higher Mg concentration significantly reduced the mRNA expression for both ICAM-1 and
172 VCAM-1, relative to 1 mM controls (Figure 2 a and b). Western blot analysis showed a
173 significant increase in ICAM-1 protein ($P = 0.048$) in response to low magnesium

174 concentrations (Figure 2 c) in the absence of LPS, but no impact of high magnesium
175 concentration. Concentrations of VCAM-1 in the culture medium significantly increased in
176 magnesium deficient cells that were stimulated with LPS ($P=0.032$) (Figure 2 d).

177 Having established that varying Mg concentration could alter the expression of adhesion
178 molecules by HUVECs, we evaluated the possibility that the expression of cytokines in
179 response to inflammatory challenge was also modulated by extracellular magnesium
180 concentration (Table 2). Magnesium had a significant effect on interleukin 8 (IL-8) and
181 monocyte chemotactic protein 1 (MCP-1) mRNA expression in a dose- and LPS-dependent
182 ($P=0.004$, $P<0.001$ respectively for magnesium concentration \times presence of LPS
183 interaction). In the presence of LPS, cells cultured in low Mg had significantly greater
184 mRNA concentrations for IL-8 and MCP-1 relative to cells cultured in control medium (Table
185 2). In contrast, a marked decline in IL-8 and MCP-1 mRNA was seen at the high Mg
186 concentration (5 mM) with LPS treatment (Table 2). Mg and LPS also had a significant
187 effect on expression of interleukin 6 (IL-6) mRNA ($P=0.002$, $P<0.001$ respectively). A
188 marked decline in IL-6 mRNA ($P=0.043$) was seen at the highest magnesium concentration
189 5 mM with LPS treatment compared to control (Table 2). No significant change in the
190 expression of interleukin-1 (IL-1B) and tumour necrosis factor- beta (TNF- β) was found
191 between the magnesium treatment groups and within the same magnesium concentration
192 groups compared to LPS-treated group (Table 2).

193 Given that measurements of the mRNA expression of selected cytokines showed variation
194 in the inflammatory response with both low and high Mg, additional evidence was sought
195 to establish whether different concentrations of magnesium could modulate the protein
196 concentration of a broad spectrum of inflammatory cytokines. Cytokine proteins were
197 measured using the human cytokine antibody array membrane kit. The array was designed
198 to detect 42 human cytokine proteins; for this study we concentrated on the 18 cytokine
199 proteins actually expressed in HUVECs. The assay confirmed that Mg insufficiency notably
200 enhanced the expression of many cytokines from HUVECs following challenge with LPS,
201 including interleukin-2 (IL-2; $P=0.029$), interleukin-3 (IL-3; $P=0.023$), IL-8 ($P=0.038$),

202 MCP-1 ($P=0.029$), growth-related oncogene (GRO; $P=0.003$) and $GRO\alpha$ ($P=0.044$)
203 compared to the 1mM Mg concentration (Table 3). Conversely, the results showed that
204 the high Mg concentration (5 mM) inhibited the protein concentration of IL-2 ($P=0.05$) and
205 IL-6 ($P=0.01$) (Table 3). Consistent with the mRNA expression data magnesium did not
206 affect IL-1 and TNF protein expression. Additionally, no effect of Mg was observed on the
207 cellular concentrations of interleukin-4 (IL4), interleukin-5 (IL5), interleukin-7 (IL7),
208 interleukin-10 (IL10), interleukin-12 (IL12), interleukin-13 (IL13) and interleukin-15
209 (IL15).

210 Having demonstrated that low Mg exacerbates the HUVEC response to LPS challenge and
211 that high Mg alleviates this, a further experiment was conducted to demonstrate the
212 functional impact of these effects. Monocyte adhesion was measured through co-
213 incubation of labelled THP-1 cells with HUVECs grown in varying Mg conditions and subject
214 to inflammatory challenge. Adhesion was markedly increased by LPS treatment only in the
215 cells cultured in 5 mM $MgSO_4$ (interaction of LPS and Mg concentration $P=0.044$). In the
216 presence and absence of LPS, the adhesion of THP-1 cells was significantly higher when
217 HUVECs were cultured in low Mg, compared to the control group. In contrast, treating
218 HUVECs with 5mM Mg significantly inhibited the adhesion of THP-1 cells to HUVECs (Figure
219 5).

220 **Discussion**

221 The aim of the present study was to investigate whether exposure to Mg- deficient or high
222 Mg concentrations would modulate the response of HUVECs to an inflammatory challenge.
223 The key findings were that low Mg concentrations resulted in an increase in expression of
224 adhesion molecules and inflammatory cytokines, even in the absence of inflammatory
225 stimulus and that this enhanced binding of monocytes to endothelial cells. Providing high
226 concentrations of Mg dampened the response to inflammatory stimulus.

227 A number of studies have reported decreased proliferation of HUVECs after culturing in
228 low Mg concentrations [9, 10, 14]. The findings of the present study suggest that low Mg
229 is associated with greater cell death, particularly following an inflammatory challenge. In

230 Mg-deficient cells reduced cell viability was seen at both concentrations of LPS that were
231 used and this can be taken as an indicator of LPS-induced cell death, though further work
232 would be needed to establish whether this was necrosis or apoptosis. In contrast, the high
233 Mg concentration protected the HUVECs from LPS-induced cell death, when stimulated
234 with 0.5 µg/ml LPS for 24 hours. These data are consistent with other studies which have
235 reported that higher Mg concentrations promote HUVEC proliferation [15, 16].

236 Several studies have reported that stimulating endothelial cells with TNF results in the
237 upregulation of VCAM-1 and ICAM-1 expression [17-20]. Similarly, activating HUVECs with
238 IL-4 results in an increased VCAM-1 expression that is dependent on activated NF-κB
239 binding to the transcription site on the VCAM gene [21]. Increased expression of the
240 adhesion molecules by endothelial cells is one of the steps that initiate atherosclerosis and
241 Cybulsky, *et al.*, [22] found that among mice expressing a low level of VCAM-1, there was
242 a significantly reduced occurrence of early atherosclerosis lesions in the aorta compared
243 to the control group. Humans with high levels of ICAM-1, and VCAM-1 are at greater risk
244 of coronary heart disease [23]. The findings of the present study confirmed that HUVECs
245 grown in 0.1mM magnesium significantly increased ICAM-1 mRNA and protein
246 concentrations and showed sharp and significant increases in ICAM-1 and VCAM-1
247 expression on exposure to LPS. Rochelsen *et al.*, reported that when HUVECs were cultured
248 in 10mM MgSO₄ for just 30 minutes before adding LPS, there was suppressive effect on
249 ICAM-1 protein expression (40% less than the control). Adding 10mM MgSO₄ concurrently
250 with the LPS lowered the ICAM-1 protein expression to 25% [24]. The current study has
251 shown that culturing HUVECs in high concentrations of Mg (5 mM) similarly decreased
252 gene expression for both ICAM-1 and VCAM-1. We found that the high expression of
253 adhesion molecules in the 0.1 mM Mg condition was associated with greatly increased
254 monocyte adhesion to HUVECs. However, only cells incubated in 5mM Mg showed a
255 significant increase in adhesion with LPS treatment. This is unexplained as it did not relate
256 to either protein or mRNA expression of ICAM-1 and VCAM-1.

257 Culturing HUVECs in a low Mg concentration and then subjecting the cells to an
258 inflammatory challenge increased the gene and protein expression of IL-8 and MCP-1
259 relative to the physiological concentration of magnesium. Low Mg concentration also
260 significantly increased the protein expression of GRO, GRO α , IL-2 and IL-3. These findings
261 are consistent with the idea that Mg deficiency increases the inflammatory response to
262 LPS and with the report of Ferre *et al.*, who showed that low Mg concentrations (0.1 mM)
263 increased the expression of IL-8 protein in HUVECs, but did not affect the expression of
264 TNF- α [25]. To the best of our knowledge there are no other studies examining the effect
265 of low Mg on the protein expression of IL-6, MCP-1, GRO, GRO α , IL-2 and IL-3. Whilst
266 Tam *et al.*, reported that treatment of pregnant rats with supplemental Mg suppressed the
267 inflammatory response to LPS *in vivo* [26], there are no other studies which have shown
268 the blunting of inflammatory cytokine production by cultured endothelial cells. Our study
269 therefore provides a new insight into the potential role for magnesium in limiting the
270 inflammatory cascade.

271 Low Mg status has been shown to be related to inflammation *in vivo* [15, 27, 28]. The
272 Harvard Nurses Study found a negative relationship between Mg intake and the level of
273 inflammatory response in plasma [29]. Similarly, the Nurse's Health Study showed that
274 women who had a high Mg intake had lower plasma inflammatory markers, such as C-
275 reactive protein (CRP) and E-selectin [30]. Furthermore, Sugimoto, *et al.*, [31] showed
276 that the inflammatory response to LPS was decreased in human umbilical cord blood
277 following treatment with MgSO₄. Similarly, rat placentas collected 4 hours after injection
278 of LPS with MgSO₄ showed reduced expression of IL-6, TNF-1 and MCP-1 [32]. Malpuech-
279 Brugère, *et al.*, [33] found that rats fed a Mg-deficient diet for four days had higher IL-6
280 in their plasma compared to controls. The present study is therefore consistent with a
281 broad body of literature including an *in vitro* study in which microvascular endothelial cells
282 cultured in low magnesium (0.1 mM) increased the production of VCAM-1, IL-1 and IL-6
283 in response to LPS challenge [15]. The mechanisms for the anti-inflammatory effect of

284 Mg are not clear [31, 33-35], but we have recently demonstrated involvement of the toll-
285 like receptor 4 in mediating the magnesium-LPS interaction [36].

286 In summary, these data show that low Mg concentrations enhance the inflammatory
287 response to LPS, eliciting a specific profile of cytokine expression. Supplementing cells
288 with Mg appears to suppress the inflammatory response and protect the endothelium from
289 both damage. The findings add weight to the argument that Mg is an important
290 determinant of vascular endothelial cell function. The capacity for Mg to modulate
291 inflammatory responses in the vasculature may, at least partly, explain why Mg deficiency
292 is associated with increased risk of atherosclerotic cardiovascular disease.

293

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296 The authors have no conflict of interest to declare.

297

298 **Figure legends**

299 **Figure 1: Dependence of HUVECs viability on MgSO₄ concentration as determined by the**
300 **trypan blue exclusion assay.** Data are shown as mean \pm SEM for n=4. A: Viable cells stimulation
301 for 24 hours. Two-way ANOVA showed a significant interaction of magnesium concentration \times LPS
302 concentration ($P < 0.001$). There was significant effect of magnesium concentration ($P < 0.001$) and
303 LPS ($P < 0.001$). B: Viable cells Stimulation for 48 hours. Two-way ANOVA showed a significant
304 interaction of magnesium concentration \times LPS concentration ($P < 0.018$). There was significant effect
305 of magnesium concentration ($P < 0.002$) and LPS ($P < 0.001$).

306

307 **Figure 2: Effect of different concentrations of magnesium on adhesion molecule**
308 **expression.** Data are shown as mean \pm SEM for n=4. **A: Expression of ICAM-1 mRNA.** Two-way
309 ANOVA showed significant interaction of magnesium concentration \times presence of LPS ($P = 0.001$).
310 There was a significant effect of magnesium concentration ($P < 0.001$), and presence of LPS ($P <$

311 0.001). **B: Expression of VCAM-1 mRNA.** Two-way ANOVA showed significant interaction of
312 magnesium concentration \times presence of LPS ($P < 0.001$). There was a significant effect of
313 magnesium concentration ($P < 0.001$), and presence of LPS ($P < 0.001$). **C: Expression of ICAM-1**
314 **protein.** One way ANOVA showed significant effect of magnesium concentration ($P=0.038$), with a
315 marked increase in ICAM-1 protein concentration in low magnesium concentration ($P=0.048$,
316 Dunnett t). Data are shown as mean \pm SEM for n=3. * indicates $P < 0.05$, compared to 1mM MgSO₄
317 in the same treatment (Dunnett t). **D, Expression of VCAM-1 protein.** Data are shown as
318 mean \pm SEM for n=4. Two-way ANOVA showed no significant interaction of magnesium concentration
319 \times presence of LPS. There was a significant effect of magnesium concentration ($P = 0.001$), and
320 presence of LPS ($P = 0.021$).

321

322 **Figure 3:** THP-1 adherence to HUVECs. Two-way ANOVA showed significant interaction of
323 magnesium concentration \times presence of LPS ($P = 0.044$). There was a significant effect of
324 magnesium concentration ($P < 0.001$), and presence of LPS ($P = 0.047$).

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Table 1 Primer sequences used for RT-PCR

Symbol	Gene name	Forward primer	Reverse primer
IL-8	Interleukin 8	ACCGGAAGGAACCATCTCACT	ATCAGGAAGGCTGCCAAGAG
TNF-Beta	Tumor necrosis factor Beta Lymphotoxin Alpha	TGTTGGCCTCACACCTTCAG	TGCTGTGGGCAAGATGCAT
IL-1B	Interleukin 1, beta	TCTGCCCAGTTCCTCAACT	TGGTCCCTCCCAGGAAGAC
ICAM-1	Intercellular adhesion molecule	TCCCCCGGTATGAGATTG	GCCTGCAGTGCCATTATG
VCAM	Vascular cell adhesion molecule	GCAAGGTTCTAGCGTGTAC	GGCTCAAGCATGTCATATTCAC
IL-6	Interleukin 6	CCGGGAACGAAAGAGAAGCT	GCGCTTGTGGAGAAGGAGTT
MCP-1	Homo sapiens chemokine (C-C motif) ligand 2 (CCL2)	CGCCTCCAGCATGAAAGTCT	GGAATGAAGGTGGCTGCTATG
PPI	Cyclophilin B	GGAGATGGCACACAGGAGGAAA	CGTAGTGCTTCAGTTTGAAGTTCTCA

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431 Table 2 Effect of different concentrations of magnesium on cytokine mRNA expression

Genes		0.1 mM MgSO ₄	1mM MgSO ₄	5mM MgSO ₄	<i>P</i> value Mg	<i>P</i> value LPS	<i>P</i> value Mg× LPS interaction
IL-8	- LPS	0.48 ± 0.068	0.33 ± 0.04	0.31 ± 0.11	0.001	0.004	< 0.001
	+ LPS	2.6 ± 0.44	1.4 ± 0.27	0.63 ± 0.06			
IL-6	- LPS	1.2 ± 0.08	1.15 ± 0.2	0.97 ± 0.14	0.002	0.053	< 0.001
	+ LPS	2.6 ± 0.37	2.2 ± 0.07	1.5 ± 0.09			
MCP-1	- LPS	0.32 ± 0.015	0.21 ± 0.029	0.18 ± 0.001	< 0.001	< 0.001	< 0.001
	+ LPS	2.5 ± 0.32	1.4 ± 0.23	0.053 ± 0.04			
IL-1β	- LPS	0.15 ± 0.027	0.11 ± 0.026	0.12 ± 0.028	0.31	0.46	0.19
	+ LPS	0.17 ± 0.04	0.12 ± 0.03	0.21 ± 0.05			
TNF-β	- LPS	0.12 ± 0.02	0.097 ± 0.04	0.21 ± 0.01	0.66	0.87	0.94
	+ LPS	0.18 ± 0.07	0.11 ± 0.02	0.13 ± 0.06			

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433 Data are shown as mean ± SEM for n=4 observations.

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458 Table 3 Effect of different concentrations of magnesium on cytokine protein concentration

Genes	0.1 mM MgSO ₄	1mM MgSO ₄	5mM MgSO ₄	<i>P</i> value 0.1mM vs 1mM	<i>P</i> value 5mM vs 1mM
IL-1 α	0.067 \pm 0.034	0.03 \pm 0.009	0.052 \pm 0.022	0.24	0.55
IL-1 β	0.17 \pm 0.02	0.12 \pm 0.055	0.11 \pm 0.05	0.65	0.99
IL-2	0.08 \pm 0.003	0.049 \pm 0.007	0.022 \pm 0.007	0.029	0.05
IL-3	0.27 \pm 0.02	0.16 \pm 0.03	0.13 \pm 0.019	0.023	0.5
IL-4	0.063 \pm 0.025	0.042 \pm 0.012	0.037 \pm 0.013	0.5	0.65
IL-5	0.097 \pm 0.016	0.07 \pm 0.01	0.029 \pm 0.007	0.26	0.096
IL-6	0.042 \pm 0.001	0.036 \pm 0.006	0.011 \pm 0.002	0.49	0.01
IL-7	0.138 \pm 0.04	0.066 \pm 0.016	0.033 \pm 0.01	0.38	0.21
IL-8	0.6 \pm 0.12	0.28 \pm 0.05	0.15 \pm 0.003	0.038	0.42
IL-10	0.12 \pm 0.02	0.094 \pm 0.02	0.089 \pm 0.05	0.81	0.99
IL-12	0.3 \pm 0.019	0.23 \pm 0.07	0.15 \pm 0.04	0.56	0.42
IL-13	0.074 \pm 0.018	0.044 \pm 0.013	0.038 \pm 0.018	0.95	0.24
IL-15	0.29 \pm 0.05	0.14 \pm 0.007	0.2 \pm 0.03	0.046	0.44
MCP-1	0.71 \pm 0.036	0.47 \pm 0.03	0.31 \pm 0.07	0.029	0.12
GRO	0.63 \pm 0.03	0.23 \pm 0.036	0.18 \pm 0.075	0.003	0.79
GRO- α	0.3 \pm 0.007	0.14 \pm 0.049	0.11 \pm 0.045	0.044	0.78
TNF- α	0.061 \pm 0.035	0.052 \pm 0.017	0.07 \pm 0.027	0.92	0.59
TNF- β	0.13 \pm 0.019	0.13 \pm 0.03	0.11 \pm 0.037	0.99	0.94

459 Data are shown as mean \pm SEM for n=3 observations.

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