

1 ***Acanthamoeba* activates macrophages predominantly through TLR4 and MyD88-**  
2 **dependent mechanisms to induce IL-12 and IL-6**

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12 **Running Title:** *Acanthamoeba* TLR4 and MyD88 dependent macrophage activation

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21 **Abstract**

22 *Acanthamoeba castellanii* is a free-living ubiquitous amoeba, with a worldwide distribution,  
23 that can occasionally infect humans, causing particularly severe infections in immune  
24 compromised individuals. Dissecting the immunology of *Acanthamoeba* infections has been  
25 considered problematic due to the very low incidence of disease despite the high exposure rates.  
26 Whilst macrophages are acknowledged as playing a significant role in *Acanthamoeba* infections  
27 little is known about how this facultative parasite influences macrophage activity. Therefore, in  
28 this study we investigate the effects of *Acanthamoeba* on the activation of resting macrophages.  
29 Consequently, murine bone marrow derived macrophages were co-cultured with trophozoites  
30 of either the laboratory Neff strain, or a clinical isolate of *A. castellanii*. *In vitro* real-time  
31 imaging demonstrated that trophozoites of both strains often established evanescent contact with  
32 macrophages. Both *Acanthamoeba* strains induced a pro-inflammatory macrophage phenotype  
33 characterized by significant production of IL-12 and IL-6. However, macrophages co-cultured  
34 with the clinical isolate of *Acanthamoeba* produced significantly less IL-12 and IL-6 in  
35 comparison to the Neff strain. The utilization of macrophages derived from MyD88, TRIF,  
36 TLR2, TLR4, TLR2/4 deficient mice indicated that *Acanthamoeba*-induced pro-inflammatory  
37 cytokine production was through MyD88-dependent, TRIF independent, TLR4-induced events.  
38 This study shows for the first time the involvement of TLRs, expressed on macrophages in the  
39 recognition and response to *Acanthamoeba* trophozoites.

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## 43 **Introduction**

44 *Acanthamoeba castellanii* is a ubiquitous free-living amoeba that has been isolated from both  
45 outdoor and indoor environments. It exists as active feeding, dividing trophozoites and the  
46 dormant environmentally resistant, cyst. Despite its ability to proliferate and survive as a free  
47 living organism, *Acanthamoeba* is also a facultative parasite of humans, most frequently causing  
48 a painful, potentially blinding infection of the eye, called *Acanthamoeba* keratitis (AK), in  
49 immune-competent individuals. *Acanthamoeba* is also an opportunistic parasite and in immune-  
50 compromised individuals it is responsible for a often fatal infection of the brain, named  
51 granulomatous amoebic encephalitis (GAE) (1). The ability of the vast majority of immune-  
52 competent humans to resist infection coupled with the susceptibility of the immune-  
53 compromised demonstrates the importance of the immune system in resistance to infection.  
54 However, and surprisingly, there is little available data regarding the immune response to  
55 *Acanthamoeba* although approximately 50-100% of people are known to be seropositive (2).

56 *Acanthamoeba* preferentially infects immune privileged sites such as the brain and the eye  
57 which are characterized by a limited regenerative capability (3). It has been demonstrated that  
58 both the innate and the adaptive immune responses are involved during *Acanthamoeba* infection  
59 (2, 4). Amongst those elements of the innate immune response that have been implicated in  
60 protective immunity are the phagocytic cells, primarily neutrophils and macrophages both of  
61 which are capable of killing *Acanthamoeba* (5). However, macrophages have been demonstrated  
62 to persist at the site of infection (6, 7) and therefore they may be involved, not only in initiating  
63 and maintaining an effective immune response, but also may have a role in tissue repair (8). To  
64 date the majority of studies have examined the interaction between corneal epithelial cells (9–  
65 11) and *Acanthamoeba* with comparatively few examining the interaction of these organisms

66 with macrophages. Macrophages can be either long-lived cells patrolling the host's tissues  
67 (resident macrophages), or they can originate from recruited blood-derived monocytes at the  
68 site of infection-(elicited macrophages) (12). Resident macrophages, strategically distributed  
69 within all tissues, are responsible for the first recognition of pathogens, through Pattern  
70 Recognition Receptors (PRRs) including Toll like receptors (TLRs). The interaction of these  
71 PRRs with pathogen associated molecular patterns (PAMPs) is important in initiating effector  
72 mechanisms for the eradication of pathogens and also directing the developing adaptive immune  
73 response and initiating tissue repair (13). To date the role of TLRs expressed on macrophages  
74 in recognizing and responding to *Acanthamoeba* has not been addressed. To address this in this  
75 study, bone marrow derived (BMD) macrophages were co-incubated with either a classical  
76 laboratory strain of *A. castellanii*, named Neff or a clinical isolate of *A. castellanii*, isolated from  
77 a case of bilateral keratitis. Both strains utilised were of the T4 genotype that has been associated  
78 with the majority of human infections. The kinetics of pro-inflammatory cytokine released by  
79 macrophages upon exposure to trophozoites of *A. castellanii* has been quantified, and the TLRs  
80 and key signalling molecules responsible for these immunological events identified.

81

## 82 **Materials and Methods**

### 83 ***Acanthamoeba castellanii* cultures**

84 Trophozoites of *A. castellanii* Neff strain, a classical laboratory strain, isolated from soil over  
85 60 years ago and kindly donated by the late Prof. Keith Vickerman (Glasgow, United Kingdom)  
86 and a *A. castellanii* genotype T4, isolated in 1992 from a patient affected by bilateral keratitis  
87 in Ancora, Italy (clinical isolate) (14–17) were used in this study. Both strains were grown in a  
88 medium consisting of 0.9% w/v D-(+)-maltose monohydrate 95% (AlfaAesar, Heysham UK)

89 and 2% w/v mycological peptone (Oxoid, Basingstoke, UK) and supplemented with 125µg/ml  
90 of Penicillin/Streptomycin (Sigma Chemical Co, Poole, UK) and the Neff strain was also treated  
91 with 125µg/ml Amphotericin B (Sigma Chemical Co, Poole, UK). Trophozoites were cultured  
92 in 75 cm<sup>2</sup> tissue culture flasks (Corning, NY, USA) and incubated at room temperature. They  
93 were used when confluent and harvested by mechanical detachment.

94

### 95 **Culture of bone marrow derived macrophages**

96 Bone marrow derived (BMD) macrophages, obtained from the femurs of either 7-weeks-old  
97 male BALB/c or C57BL/6 Toll like receptor 4 (TLR4<sup>-/-</sup>), Toll like receptor 2 (TLR2<sup>-/-</sup>), Toll  
98 like receptor 2/4 (TLR2/4<sup>-/-</sup>), myeloid differentiation primary response gene 88 (MyD88<sup>-/-</sup>) or  
99 TIR-domain-containing adapter-inducing interferon β (TRIF<sup>-/-</sup>) deficient mice and their wild-  
100 type counterparts, were used to perform the experiments. Briefly, bone marrow stem cells were  
101 flushed from the femur with 5 ml of Dulbecco's medium (DMEM) (Life technologies, Paisley,  
102 UK) supplemented with 20% v/v of heat inactivated foetal calf serum (HI-FCS) (Biosera,  
103 Sussex, UK), 30% v/v of L-cell conditioned medium, 2 mM L-glutamine (Sigma Chemical Co,  
104 Poole, UK), 125 U/ml Penicillin and 125 µg/ml Streptomycin and grown for 10 days, at 37°C,  
105 5% CO<sub>2</sub> atmosphere. L-cell conditioned medium was obtained by harvesting the metabolized  
106 medium from cultured murine fibroblastic cell line L-929. This conditioned medium provides a  
107 source of macrophage colony stimulating factor (M-CSF) necessary for the growth and  
108 differentiation of bone marrow stem cells into mature macrophages (18).

109

110

111 **Co-incubation of BMD-macrophages with *Acanthamoeba castellanii* trophozoites.**

112 At day 10, macrophages were harvested with RPMI-1640 medium (Lonza Biowhittaker,  
113 Virviers, Belgium), and centrifuged at x 300 g 5 minutes (min). Pellets were re-suspended in  
114 RPMI supplemented with 10% v/v HI-FCS, 2 mM L-glutamine, 125µg/ml 100 U/ml Penicillin  
115 and 100 µg/ml Streptomycin (complete RPMI, cRPMI) and centrifuged for 5 min at x 300 g.  
116 Supernatants were discarded and macrophages were then re-suspended in cRPMI and counted  
117 using the Neubauer Chamber (Superior Marienfeld, Germany). Macrophage suspensions at  
118 required density were prepared, seeded in the appropriate vessel, and incubated over night at  
119 37°C, 5% CO<sub>2</sub>, to allow macrophages to adhere.

120 The day after, trophozoites were harvested by mechanical detachment. Trophozoite suspensions  
121 were centrifuged at x 360 g for 10 min and subsequently, washed once in sterile Phosphate  
122 Buffered Saline without Ca<sup>2++</sup> or Mg<sup>2++</sup> or Phenol Red (PBS) (Lonza, Walkersville, USA) (x  
123 360 g 5 min) and once in cRPMI (x 360 g 5 min). Trophozoites were then suspended in cRPMI  
124 and counted using the Neubauer Chamber. Trophozoite suspensions were used for the co-  
125 incubation experiments at specified ratios. Macrophages were co-incubated with trophozoite  
126 suspension in cRPMI of either the Neff strain (Neff) or the clinical isolate (clinical).

127

128 **Real time microscopy of a co-culture of murine BMD macrophages and trophozoites of *A.***  
129 ***castellanii* at 37°C**

130 Murine macrophages were harvested and suspended to 7 x 10<sup>5</sup> cells/ml in cRPMI. 30 µl of cell  
131 suspension was added into 3 channels of the µ-slide VI0.4 (ibidi GmbH, Martinsried, Germany).  
132 After replacing the lid to cover the reservoirs, the µ-slide was incubated at 37°C 5%CO<sub>2</sub> for 1

133 h and 30 min to allow macrophages to adhere. Afterwards, 60  $\mu$ l of free cell cRPMI was added  
134 to each well, and then the  $\mu$ -slides were incubated at 37°C 5%CO<sub>2</sub> over night. After incubation  
135 cRPMI was removed from the wells, and 30  $\mu$ l of either Neff or clinical trophozoites ( $7 \times 10^5$   
136 trophozoites/ml suspensions in cRPMI) were added. In the control channel (macrophages alone)  
137 30  $\mu$ l of cRPMI were applied; in the co-culture channels, 60  $\mu$ l of cRPMI were then added to all  
138 wells. Prepared  $\mu$ -slides were then observed, using an inverted epifluorescence microscope  
139 (Nikon, Eclipse TE300), provided with a 37°C chamber that allowed maintenance of the  
140 samples at the appropriate temperature while images were acquired at X10, X20 or X40  
141 magnification. After manually focusing, images were acquired every 30 sec for a period of 1 h  
142 using the software MetaMorph (Molecular devices, Sunnyvale, CA, USA). Thereafter, images  
143 were processed using the programme Volocity (Perkin Elmer, Massachusetts, USA).

144

#### 145 **Stimulation of BMD macrophages with specific agonists**

146 Lipopolysaccharide (LPS) from *Salmonella enterica* serotype *abortus equi* (Sigma Chemical  
147 Co, Poole, UK), the synthetic tripalmitoylated lipopeptide (PAM3CSK4) (Invivo Gen, San  
148 Diego, CA, USA), and the synthetic double stranded RNA polyinosinic-polycytidylic acid  
149 (POLY I:C) were used to stimulate macrophages via TLR4, TLR2 and TLR3, respectively as  
150 positive controls. The final concentration used in wells, was 200 ng/ml for LPS, 320 ng/ml for  
151 PAM3CSK4 and 10  $\mu$ g/ml for POLY I:C.

#### 152 **Quantitative analysis of murine cytokines production**

153 At specific time points after co-incubation, plates were centrifuged for 1 min at 480 x g,  
154 supernatants were collected and Enzyme Linked-Immuno-Sorbent Assay (ELISA) was

155 performed, using paired purified rat anti-mouse primary antibody and secondary biotin rat anti-  
156 mouse antibody (BD Bioscience), to determine the concentration of IL-12 p40/p70 and IL-6  
157 released by murine macrophages. The optical density (OD) of each well was determined at 405  
158 nm using SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA,  
159 USA). SOFTMAX PRO software (Molecular Devices, Sunnyvale, CA, USA) was used to  
160 obtain the values of OD and to calculate cytokine concentration in relation to the standard curve  
161 (BD Bioscience). Cytokine concentration was expressed in ng/ml.

162

### 163 **Statistical analyses**

164 Experiments were performed in triplicate and repeated twice. Data are shown as the mean  $\pm$   
165 standard errors of means (SEM) of 3 replicates. Statistical analyses were performed using  
166 GraphPad Prism 5 program. Data, that were normally distributed, were analysed using the  
167 parametric statistical tests one way analysis of variance (ANOVA) and student's *t* test  
168 accordingly with the nature of experiments and of the hypothesis to be investigated. One way  
169 ANOVA was used to analyse statistical significance within several conditions (more than two)  
170 and post-hoc tests, either Tukey's or Dunnett's, were applied respectively to set all pairwise  
171 comparisons or to compare each condition mean with the control. Student's *t* test was applied  
172 to evaluate significant differences between two conditions. Differences were considered  
173 significant with a value of  $P < 0.05$ .

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177



178 **Results**

179 ***In vitro* real time imaging of *A. castellanii* infection shows cell-cell interactions between**  
180 **murine BMD macrophages and trophozoites of either the Neff strain or clinical isolate.**

181 Macrophages and Neff strain trophozoites were found to engage in mutual interactions a few  
182 minutes after infection. Trophozoites showed characteristic pseudopodia and amoeboid  
183 locomotion. Macrophages were healthy, actively moving and their lamellipodia and filopodia  
184 were visible. However, macrophages were generally less motile than the trophozoites (S1-a).  
185 Neff trophozoites were observed to establish contact with macrophages and to roll on the  
186 macrophage's surface. Although macrophages briefly attached to trophozoites with either  
187 lamellipodia or filopodia they were unable to maintain this contact and to phagocytose them  
188 (S1-b). Indeed, no phagocytic invagination was observed at any time during observation of up  
189 to 1 hour (S1-a and S1-b). In contrast, real time interaction between macrophages and  
190 trophozoites of the clinical isolate showed some differences in comparison with macrophage  
191 infection with the Neff strain. In this instance, trophozoites of the clinical isolate did not show  
192 rolling behaviour over macrophage's surface (S2-a and S2-b); nevertheless, macrophages were  
193 able to interact with trophozoites through lamellipodia and filopodia (S2-a and S2-b) and  
194 sometimes this resulted in trophozoite disruption (S2-a), but we also observed trophozoite  
195 division occurred during the observation period (S2-b).

196

197 ***A. castellanii* Neff induces IL-12 and IL-6 production in murine BMD macrophages in a**  
198 **time and trophozoite density dependent manner**

199 In order to evaluate if the production of pro-inflammatory cytokines depends on trophozoite  
200 density or on duration of infection, murine macrophages were stimulated with Neff trophozoites  
201 at three different ratios of trophozoite:macrophage (ratio 1:1, 1:2, 1:10) and the production of  
202 IL-12 and IL-6 was assessed at 4, 6, 8, 10, and 24 h after infection.

203

204 IL-12 production increased progressively throughout the course of the study in all trophozoite  
205 infected experimental groups, reaching the highest concentrations at 24 h post-infection  
206 compared to unstimulated control cultures. At 24 h the trophozoite/macrophage co-culture ratios  
207 of 1:1 and 1:2 had induced similar levels of IL-12 and significantly higher than the other  
208 experimental groups (22.08 ng/ml  $P < 0.0005$  and 20.92 ng/ml  $P < 0.0005$ ). LPS stimulated  
209 macrophages produced IL-12 in a time dependent manner, peaking at 24 h post-stimulation. No  
210 IL-12 was detectable in control macrophage cultures at any time (Fig 1-A).

211

212 IL-6 production by murine macrophages co-cultured with trophozoites increased throughout the  
213 24 h time course of study and levels recorded were significantly greater from the  
214 trophozoite/macrophage co-culture ratios of 1:1 and 1:2 than control macrophage cultures from  
215 8 h. The trophozoite/macrophage co-culture ratios of 1:1 and 1:2 were equally effective at  
216 inducing IL-6 production and significantly more than a trophozoite/macrophage co-culture ratio  
217 of 1:10 (Fig 1-B). Interestingly the dynamics and profile of LPS-induced macrophage IL-6  
218 production are quite dissimilar from that induced by trophozoite co-culture. LPS induced a  
219 significantly more rapid IL-6 production than trophozoite/macrophage co-culture ratios of 1:1  
220 and 1:2 that peaked at 8 h post-stimulation. No IL-6 was detectable in non-infected macrophage  
221 cultures at any time.

222 **A clinical isolate of *A. castellanii*, belonging to genotype T4, stimulates lower cytokine**  
223 **production than Neff, the classical laboratory strain T4 genotype.**

224 Differences in the levels of cytokines induced by macrophages co-cultured with either the Neff  
225 or the clinical strains were investigated. Based on the effectiveness of cytokine induction in the  
226 studies above, murine BMD macrophages were infected at the ratios of  
227 trophozoites:macrophages 1:1 and 1:2. Samples were collected at 8 h and 24 h after co-  
228 incubation, an early and late time point respectively. IL-12 production by macrophages was  
229 greater following incubation with Neff strain trophozoites than the clinical isolate at both 8 h  
230 and 24 h ( $p < 0.0005$ ) (Fig 2-A). These results were consistent at both trophozoite/macrophage  
231 infection levels. IL-6 production by macrophages was significantly induced by Neff strain  
232 trophozoites compared with non-infected macrophage cultures at both 8 and 24 h ( $p < 0.0005$ )  
233 post-infection. In addition, macrophages incubated with Neff strain trophozoites produced  
234 significantly more IL-6 than macrophages incubated with clinical strain trophozoites at both 8h  
235 and 24h ( $p < 0.0005$ ) post-infection. Clinical strain trophozoites failed to induce IL-6 production  
236 significantly above that produced by unstimulated control macrophages at 8 h post-infection  
237 (Fig 2-B). In addition, Neff strain trophozoites induced IL-6 at a later time point than the LPS  
238 control.

239

240 ***Acanthamoeba*-induced IL-12 and IL-6 production is MyD88-dependent and partially**  
241 **TRIF-dependent**

242 Studies were performed using gene-deficient mice to identify key signalling pathways involved  
243 in the induction of cytokines by *Acanthamoeba*. BMD macrophages were obtained from  
244 C57BL/6 wild-type mice, or C57BL/6 mice deficient in either MyD88 or TRIF genes and co-

245 incubated with Neff strain or the clinical isolate trophozoites at ratios of 1:1 and 1:2. As positive  
246 controls macrophages were stimulated with LPS, natural TLR4 ligand, or with the synthetic  
247 double stranded RNA POLY I:C, a selective TLR3 agonist. Production of IL-12 and IL-6 was  
248 then measured at 24 h post co-culture.

249 Significant production of IL-12 and IL-6 by WT macrophages was induced by trophozoites of  
250 either Neff strain or clinical isolate at both co-culture ratios studied (Fig 3-A1 and 3-B1).  
251 However, MyD88<sup>-/-</sup> macrophages co-incubated with trophozoites of either Neff strain or clinical  
252 isolate failed to produce detectable levels of either IL-12 or IL-6 (Fig 3-A2 and 3-B2). By  
253 comparison IL-12 production was significantly inhibited though not ablated in TRIF<sup>-/-</sup>  
254 macrophages co-incubated with trophozoites of either the Neff strain or clinical isolate  
255 compared to WT macrophages (Fig 4–A1 and A2). In addition, IL-6 production by the Neff  
256 strain significantly decreased in TRIF<sup>-/-</sup> macrophages (p=0.0014) (Fig 4-B1 and B2).

257

258 ***Acanthamoeba*-induced macrophage IL-12 and IL-6 production is TLR4-dependent but**  
259 **TLR2-independent**

260 TLR2 and TLR4 were considered potential targets for trophozoite induced macrophage cytokine  
261 induction since they are localized on the cell surface and associated with the MyD88-dependent  
262 signalling pathway. Therefore, macrophages derived from C57BL/6 mice deficient in TLR2 or  
263 TLR4 or TLR2/TLR4 were utilized to determine their specific roles in this process. Neff strain  
264 and clinical strain trophozoites were used in co-incubation with isolated macrophages at a ratio  
265 of 1:1. As positive controls, macrophages were stimulated with either LPS or PAM3CKS4 or  
266 RNA POLY I:C. Production of IL-12 and IL-6 was then measured at 24 h after infection.

267 C57/BL6 WT macrophages produced IL-12 and IL-6 when induced by trophozoites of either  
268 Neff or clinical strains (Fig 5-A1 and B1). Production of these cytokines was not diminished in  
269 TLR2<sup>-/-</sup> macrophages (Fig 5-A2 and B2) whereas, IL-12 production by TLR4<sup>-/-</sup> macrophages  
270 was significantly lower in comparison to WT macrophages (Neff p=0.0009; clinical p=0.0002)  
271 (Fig 5-A3), and IL-6 was completely ablated (Fig 5-B3). The simultaneous absence of both TLR2  
272 and TLR4 on macrophages was characterized by significantly lower IL-12 production when co-  
273 incubated with either Neff strain (p=0.0009) or clinical isolate trophozoites (p=0.0002) (Fig 5-  
274 A4) and no IL-6 was produced, compared to WT macrophages (Fig 5-B4).

275

## 276 **Discussion**

277 *A. castellanii* is a ubiquitous free-living micro-organism, so much so that individuals are  
278 constantly exposed to these amoebae in their everyday life. Although the opportunities of  
279 becoming infected with this protist are high, few cases of facultative or opportunistic parasitism  
280 and disease have been reported. Patients with an immune deficiency are particularly susceptible  
281 to infection with these organisms and they usually present the most severe and deadly amoebic  
282 disease, GAE. On the other hand, AK can also occur in immune competent individuals  
283 predominantly, but not exclusively, in contact lens wearers (19). While, AK is generally  
284 attributed to either bad hygiene or to corneal trauma caused by the lens, a recent study  
285 demonstrating that prolonged use of contact lenses may impair innate immunity at the ocular  
286 surface provides an additional mechanism that potentially contributes to increased incidence  
287 of AK in contact lens wearers (20, 21). Overall therefore, it is the general consensus that the  
288 immune system is critical in determining whether infection occurs following an encounter with  
289 *Acanthamoeba* in humans.

290

291 Our studies have shown, for the first time, the profile and kinetics of *Acanthamoeba*-induced  
292 IL-12 and IL-6 production in murine BMD-macrophages during co-culture with trophozoites.  
293 These cytokines, not only play important roles in the activation of the innate immune cell  
294 functions, but they are also involved in the activation and proliferation of the adaptive immune  
295 cells (22, 23). IL-12, in particular, plays a pivotal role in controlling microbial infections (24)  
296 as it stimulates IFN- $\gamma$  production by natural killer (NK) cells and promotes type-1 immune  
297 responses and classical macrophage activation (25). IL-6 is known to play both “early” as well  
298 as a “late” roles at the site of infection/inflammation (22). Thus in the absence of IL-6 mice are  
299 highly susceptible to infection as seen with a variety of organisms including, *Listeria*  
300 *monocytogenes* (26, 27), *Candida albicans* (28) and *Toxoplasma gondii* (29). However, IL-6 is  
301 also necessary to down-regulate ongoing inflammatory responses by inhibiting the production  
302 of TNF- $\alpha$  and IL-1 by macrophages (30–33).

303

304 *Acanthamoeba* can grow axenically in laboratory conditions. However, this can lead to loss of  
305 virulence factors, encystment capability and reduced susceptibility to drugs (34). Therefore, we  
306 compared the ability of *A. castellanii* Neff strain, a classical laboratory strain, and *A. castellanii*  
307 isolated from a case of bilateral keratitis to induce macrophage cytokine production. This  
308 clinical strain of *Acanthamoeba* was chosen as it is known to be capable of infecting immune  
309 competent hosts. Interestingly, while both clinical and Neff strains induced macrophage IL-12  
310 and IL-6 production, with similar kinetics, macrophages incubated with the clinical isolate  
311 trophozoites produced significantly less cytokines than macrophages incubated with the Neff  
312 strain under similar conditions. While it could be argued that these results are predictable, as

313 limiting the production of pro-inflammatory cytokines would enhance the virulence of a  
314 pathogen, it would require a more comprehensive study comparing a number of clinical and  
315 laboratory isolates of the T4 genotype to establish such a relationship. Lower cytokine  
316 production by macrophages incubated with the clinical strain could be due to this strain inducing  
317 comparatively more damage to macrophages than the Neff strain, although this has not been  
318 investigated in this present study. Certainly, real time imaging experiments indicated differences  
319 in macrophage responses to the pathogenic and non-pathogenic *Acanthamoeba* strains. In  
320 particular, the results indicate that macrophages respond to trophozoites of the clinical isolate  
321 in a contact-dependent manner not observed with the laboratory strain, with some cytolysis  
322 observed and phagocytosis attempted.

323

324 All this considered, our data demonstrate that *Acanthamoeba* trophozoites induce production of  
325 both IL-12 and IL-6 by murine macrophages. The higher production was observed at 24 h post  
326 co-incubation, in accordance with their role in modulating immune responses, at the highest co-  
327 incubation ratios. In contrast, a recent study from our laboratories has demonstrated that  
328 *Acanthamoeba* trophozoites of the clinical, fail to induce pro-inflammatory cytokines, such as  
329 TNF- $\alpha$ , IL-6 and IL-12, by human monocyte-derived macrophages. This discrepancy could be  
330 explained by differences between murine and human cells, or experimental techniques  
331 optimised for each cell type. This would include differences in numbers of trophozoites used  
332 in the co-incubation models, as well as the time of observation which were each optimised to  
333 allow co-culture with minimal cell death, but with sufficient interaction to induce measurable  
334 levels of cytokines (35). In addition, we have previously demonstrated that conditioned media  
335 derived from *Acanthamoeba* cultures can mimic some of the observed effects on human cells,

336 but find conditioned media to be less effective in murine cells. These differences could be  
337 species dependent or due differences in culture conditions including media components such as  
338 the presence of absence of heat inactivated bovine calf serum.

339

340 The role of innate immune receptors such as TLRs has been widely described during protist  
341 infections. TLR2 and TLR4 are the main TLRs involved in the recognition of parasitic protists  
342 such as *Leishmania* spp., *Trypanosoma cruzi*, *T. gondii*, *Plasmodium falciparum* and  
343 *Entamoeba histolytica*. Studies have demonstrated that *Acanthamoeba* trophozoites can activate  
344 TLR4 expressed on corneal epithelial cells, inducing pro-inflammatory cytokines and  
345 chemokines at the ocular surface (10, 36). The activation of TLRs at the ocular surface by  
346 *Acanthamoeba* and the release of cytokines and chemokines, can be the triggering event for the  
347 recruitment of innate immune cells such as macrophages and neutrophils (37).

348

349 The results described herein demonstrate that recognition of *Acanthamoeba* trophozoites is  
350 predominately through TLR4 that induces MyD88 dependent, with a small contribution of TRIF  
351 dependent signalling culminating in cytokine production. This is in accordance with the unique  
352 ability of TLR4 to induce both MyD88-dependent and TRIF-dependent signalling pathways  
353 (38). Our study demonstrates TLR4 as the main receptor involved in the recognition and  
354 response to *Acanthamoeba* trophozoites, in macrophages and this is in agreement with studies  
355 utilising corneal epithelial cells (10, 36).

356



357 The results obtained suggest that *Acanthamoeba* might present on its surface, molecules that are  
358 recognized by TLR4, thus inducing an innate immunological response. GPI-anchors are highly  
359 expressed in several parasitic protists (39) and are highly immunogenic, inducing a response by  
360 cells of both the myeloid and lymphoid lineages (40). These structures are recognized by TLRs,  
361 mainly TLR2, TLR4 and are therefore considered as protist PAMPs (41). According to an early  
362 study, the plasma membrane of *A. castellanii* is composed for 31% of its mass by  
363 lipophosphoglycans (LPG) (42). This data has been confirmed and further characterised by a  
364 more recent study using Gas Chromatography-Mass Spectrometry techniques, where the  
365 chemical nature of *Acanthamoebic* LPG was identified and recognized as  
366 glycoinositolphosphosphingolipids (GIPSL) (43). Although it has yet to be confirmed, these  
367 structural moieties are strong candidates to be the *Acanthamoeba*-associated molecular patterns  
368 that stimulate the TLRs expressed on macrophages.

369

370 The studies described herein used murine bone marrow derived macrophages as these can be  
371 generated consistently in high numbers from both wild-type and gene-deficient mice. The results  
372 are intuitively relevant to systemic infections and most tissues where macrophages are known  
373 to be resident. Although macrophages have are known to be present within the eye, they are  
374 predominantly found adjacent to the pigment epithelium of the iris, the ciliary body and in the  
375 retina. It was previously thought that antigen presenting cells such as macrophages and DCs  
376 are generally not present in the cornea which is the site of AK. However, a series of recent  
377 studies have demonstrated the presence of resident macrophages, albeit in low numbers within  
378 both murine and human cornea and reviewed (1). Furthermore, these macrophages have been  
379 found to be important in a murine model of *Pseudomonas aeruginosa* infection. Thus F4/80<sup>+</sup>

380 cells present in the corneal stroma expressing TLR4, TLR5 and TLR2 where found to respond  
381 to *Pseudomonas* by releasing of cytokines in a TLR4/TIRAP/MyD88 and TLR4/TRIF- Nf-kB  
382 translocation dependent manner (2). Thus the studies described herein could also have relevance  
383 to AK.

384

385 In conclusion, we have demonstrated that *Acanthamoeba* interact and activate macrophages,  
386 inducing the production of IL-12 and IL-6 in a time and density dependent manner. Furthermore,  
387 the clinical isolate examined in this study induces lower cytokine production by macrophages,  
388 in comparison with the classical laboratory strain Neff. Both strains induce IL-12 and IL-6  
389 production by macrophage through a predominantly TLR-4/MyD88 dependent mechanism. The  
390 study of the immunological mechanisms involved in these rare, but insidious infectious diseases,  
391 is essential to develop appropriate therapeutic strategies. Indeed the modulation of receptor  
392 activity and of signalling pathways at the site of infection, might not only help control infection  
393 but also mitigate over exuberant immune/inflammatory responses.

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401 **Figure Legends**

402 **Fig 1. Release of IL-12 (A) and IL-6 (B) at 4, 6, 8, 10, 24 h after co-incubation with *A.***  
403 ***castellanii* Neff trophozoites.**  $1 \times 10^6$  murine macrophages, obtained from BALB/c mice, were  
404 challenged with either  $1 \times 10^6$  (Ratio 1:1) or  $5 \times 10^5$  (Ratio 1:2) or  $1 \times 10^5$  (Ratio 1:10) trophozoites  
405 of *A. castellanii* Neff strain. LPS was used as a positive control at a concentration of 200 ng/ml,  
406 whereas uninfected macrophages (Control) were considered the negative control. Experiments  
407 were repeated three times. Results represent the mean  $\pm$  standard error of  $n=6$ . Two way  
408 ANOVA could not be applied, since the interaction between the stimuli and time was  
409 statistically significant and the statistical analysis for the time and stimuli effects are therefore  
410 difficult to interpret. For this reason, one way ANOVA was applied for each time points.  
411 Tukey's multiple comparison test was performed to evaluate differences within the conditions  
412 means at each time point. In the graphs, significances within the different conditions are  
413 indicated as follow: for values of  $p < 0.005$  \*\*;  $p < 0.0005$  \*\*\*. Note that trophozoite/macrophage  
414 infection higher ratios induce higher cytokine production and IL-6 and IL-12 production peaks  
415 at 24 hrs.

416

417 **Fig 2. Release of IL-12 (A) and IL-6 (B) at 8 and 24 h after co-incubation with *A. castellanii***  
418 **trophozoites.**  $1 \times 10^6$  murine macrophages, obtained from BALB/c mice, were challenged with  
419 either  $1 \times 10^6$  or  $5 \times 10^5$  trophozoites of either the Neff strain (respectively Neff ratio 1:1 and Neff  
420 ratio 1:2) or clinical isolate (respectively Clinical ratio 1:1 and Clinical ratio 1:2). LPS, at a  
421 concentration of 200 ng/ml, (LPS) was used as a positive control, whereas uninfected  
422 macrophages (Control) were considered the negative control. The experiment was repeated  
423 twice. Results represent the mean  $\pm$  standard error of  $n=3$ . One way ANOVA was applied for

424 each time point and Tukey's multiple comparison test was performed to evaluate differences  
425 within the conditions means at each time point. In the graphs, significant differences between  
426 Neff and clinical strains are indicated as follow: for values of  $p < 0.05$  °;  $p < 0.0005$  °°. Values  
427 below the detectable levels are indicated in the graphs as ND (not detected). Note that  
428 *Acanthamoeba* Neff strain induces higher levels of macrophage pro-inflammatory cytokines  
429 than *Acanthamoeba* clinical isolates. This event is observed both at the early time points (8 hrs  
430 post co-incubation) and later time points (24 hrs post co-incubation).

431

432 **Fig 3. Release of IL-12 (A) and IL-6 (B): comparison between C57BL/6 WT (A1 and B1)**  
433 **and C57BL/6 MyD88<sup>-/-</sup> (A2 and B2) macrophages at 24 h after co-incubation with**  
434 ***Acanthamoeba* trophozoites.**  $1 \times 10^6$  murine macrophages, obtained from either C57BL/6 WT  
435 or C57BL/6 MyD88<sup>-/-</sup> mice, were challenged with either  $1 \times 10^6$  or  $5 \times 10^5$  trophozoites of either  
436 *A. castellanii* Neff strain (respectively Neff 1:1 and Neff 1:2) or clinical isolate (respectively  
437 clinical 1:1 and 1:2). LPS and POLY I:C at respectively 200 ng/ml (LPS) and 10 µg/ml (POLY  
438 I:C) were used to stimulate macrophages as positive controls. Uninfected macrophages  
439 (Control) were considered the negative control. The experiment was performed twice. Results  
440 represent the mean ± standard error of n=3. Student's *t*-test was applied to evaluate differences  
441 between C57BL/6 WT and the C57BL/6 MyD88<sup>-/-</sup> macrophage cytokine production. Values  
442 below the detectable levels are indicated in the graphs as ND (not detected). IL-12 and IL-6  
443 production in trophozoites/MyD88<sup>-/-</sup> macrophages co-incubation is completely ablated in  
444 comparison to trophozoites/WT macrophages co-incubation, suggesting that the production of  
445 these pro-inflammatory cytokines by macrophages, in response to *Acanthamoeba*, is MyD88-  
446 dependent.

447 **Fig 4. Release of IL-12 (A) and IL-6 (B): comparison between C57BL/6 WT (A1 and B1)**  
448 **and C57BL/6 TRIF<sup>-/-</sup> (A2 and B2) macrophages at 24 h after co-incubation with**  
449 ***Acanthamoeba* trophozoites.** 1x10<sup>6</sup> murine macrophages, obtained from either C57BL/6 WT  
450 or C57BL/6 TRIF<sup>-/-</sup> mice, were challenged with either 1x10<sup>6</sup> or 5x10<sup>5</sup> trophozoites of either *A.*  
451 *castellanii* Neff strain (respectively Neff 1:1 and Neff 1:2) or clinical isolate (respectively  
452 clinical 1:1 and clinical 1:2). LPS and POLY I:C at respectively 200 ng/ml (LPS) and 10 µg/ml  
453 (POLY I:C) were used to stimulate macrophages as positive controls. Uninfected macrophages  
454 (Control) were considered the negative control. The experiment was performed twice. Results  
455 represent the mean ± standard error of n=3. Student's *t*-test was applied to evaluate differences  
456 between C57BL/6 WT and the C57BL/6 TRIF<sup>-/-</sup> macrophages. Values below the detectable  
457 levels are indicated in the graphs as ND (not detected). IL-12 and IL-6 production in  
458 trophozoites/TRIF<sup>-/-</sup> macrophages co-incubation is significantly diminished in comparison with  
459 trophozoites/WT macrophages co-incubation condition suggesting that the production of these  
460 pro-inflammatory cytokines by macrophages in response to *Acanthamoeba*, might be in part  
461 TRIF-dependent, although this does not appear to be the main signalling pathway involved  
462 during *Acanthamoeba* stimulation.

463

464 **Fig 5. Release of IL-12 (A) and IL-6 (B): comparison between C57BL/6 WT (A1 and B1)**  
465 **and C57BL/6 TLR2<sup>-/-</sup> (A2 and B2), TLR4<sup>-/-</sup> (A3 and B3), TLR2/4<sup>-/-</sup> (A4 and B4)**  
466 **macrophages 24 h after co-incubation with *Acanthamoeba* trophozoites.** 1x10<sup>6</sup> murine  
467 macrophages, obtained from C57BL/6 WT and KO mice, were challenged with 1x10<sup>6</sup>  
468 trophozoites of either *A. castellanii* Neff strain (Neff) or clinical isolate (Clinical). LPS,  
469 PAM3CSK4 and POLY I:C at respectively 200 ng/ml, 320 ng/ml and 10 µg/ml were used to

470 stimulate macrophages as positive controls. Uninfected macrophages (Control) were considered  
471 the negative control. The experiment was performed twice. Results represent the mean  $\pm$   
472 standard error of n=3. Student's *t*-test was applied to evaluate differences between C57BL/6  
473 WT and the C57BL/6 TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup> and TLR2/4<sup>-/-</sup> conditions. Values below the detectable  
474 levels are indicated in the graphs as ND (not detected). IL-12 and IL-6 production in  
475 trophozoites/TLR2<sup>-/-</sup> macrophages co-incubation was not significantly diminished in  
476 comparison with trophozoites/WT macrophages co-incubation condition, suggesting that the  
477 production of these pro-inflammatory cytokines by macrophages in response to *Acanthamoeba*  
478 is not TLR2-dependent. On the other hand, in the absence of TLR4, trophozoites-induced IL-12  
479 production by macrophages was significantly decreased, whereas IL-6 production was  
480 completely ablated. The same pattern was observed when both TLR2 and TLR4 were not  
481 expressed. Therefore, TLR4 appeared to be the main TLR involved in the recognition and  
482 response to *A. castellanii*.

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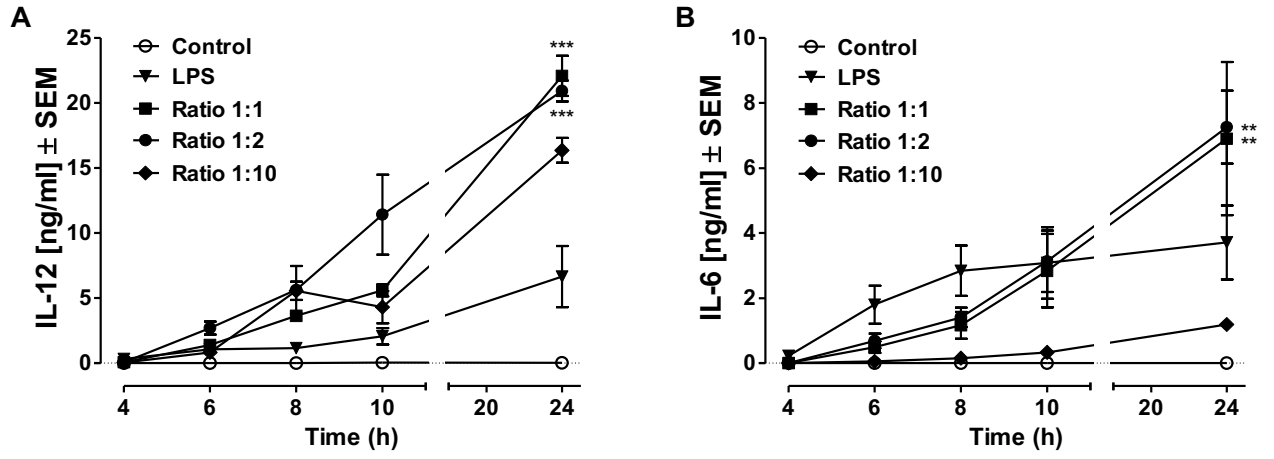


Figure 1

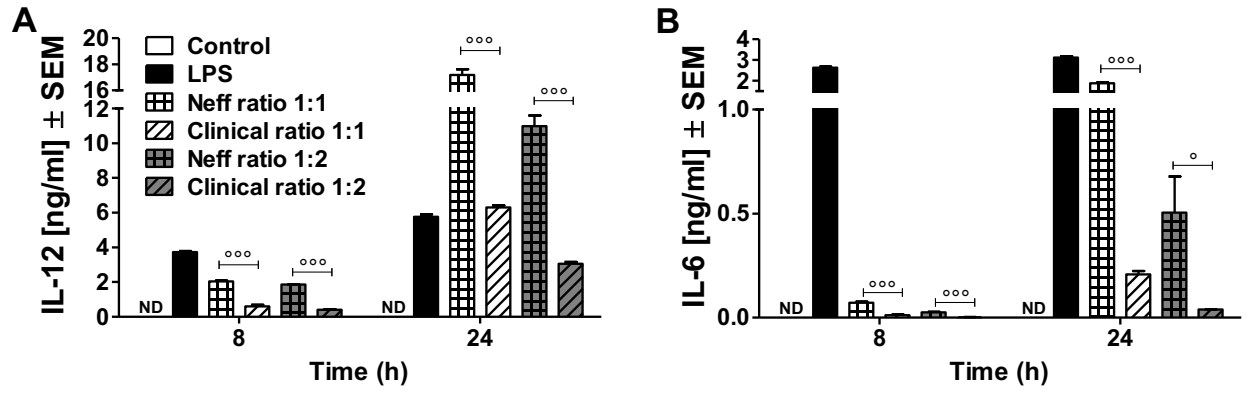


Figure 2

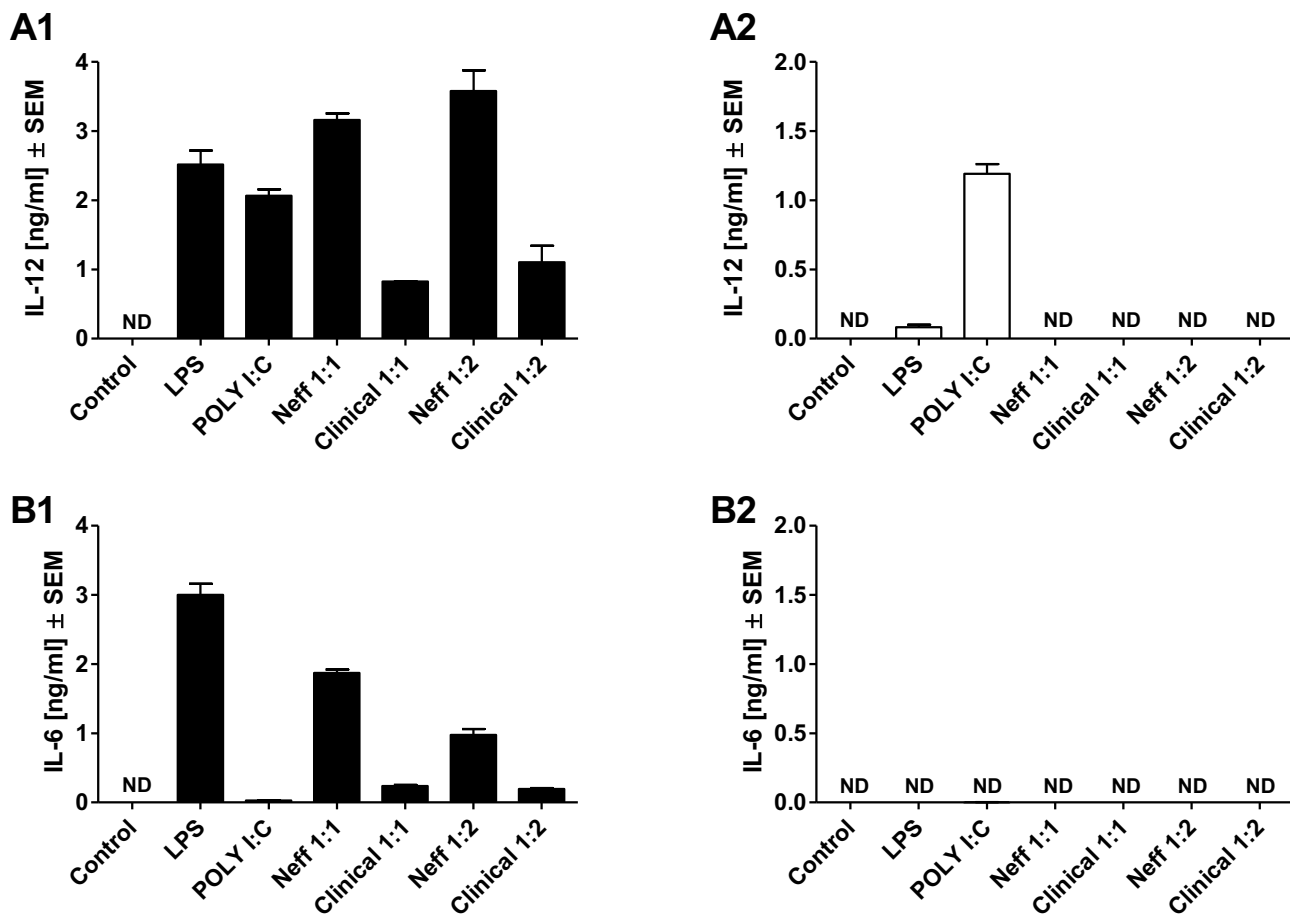
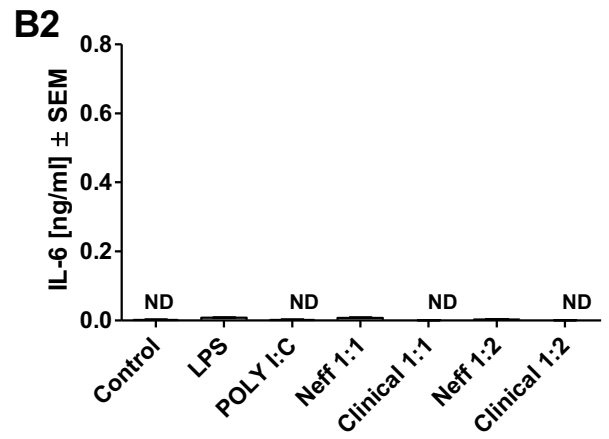
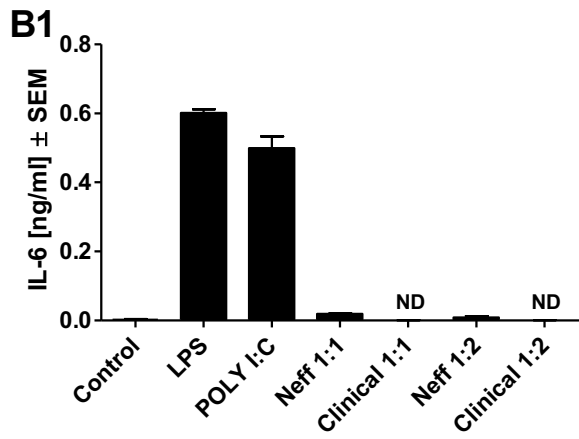
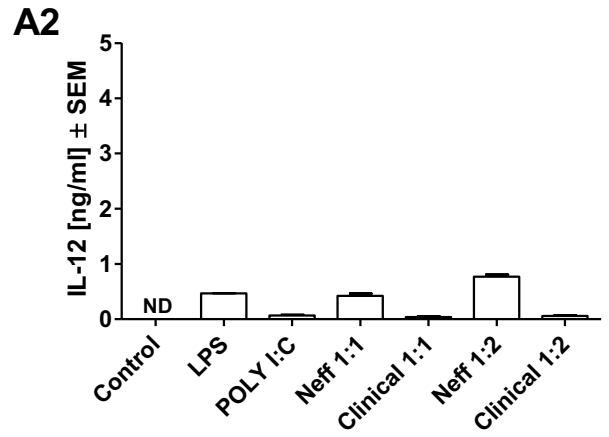
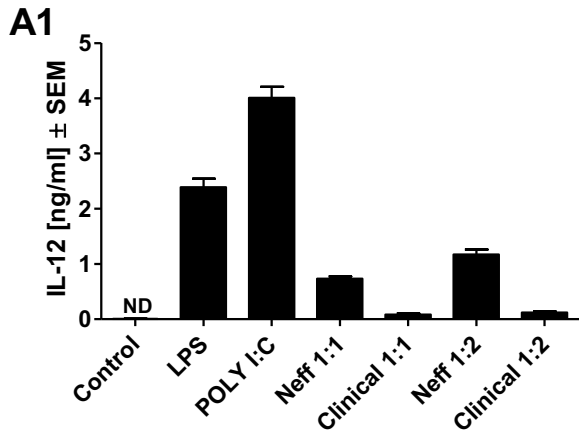
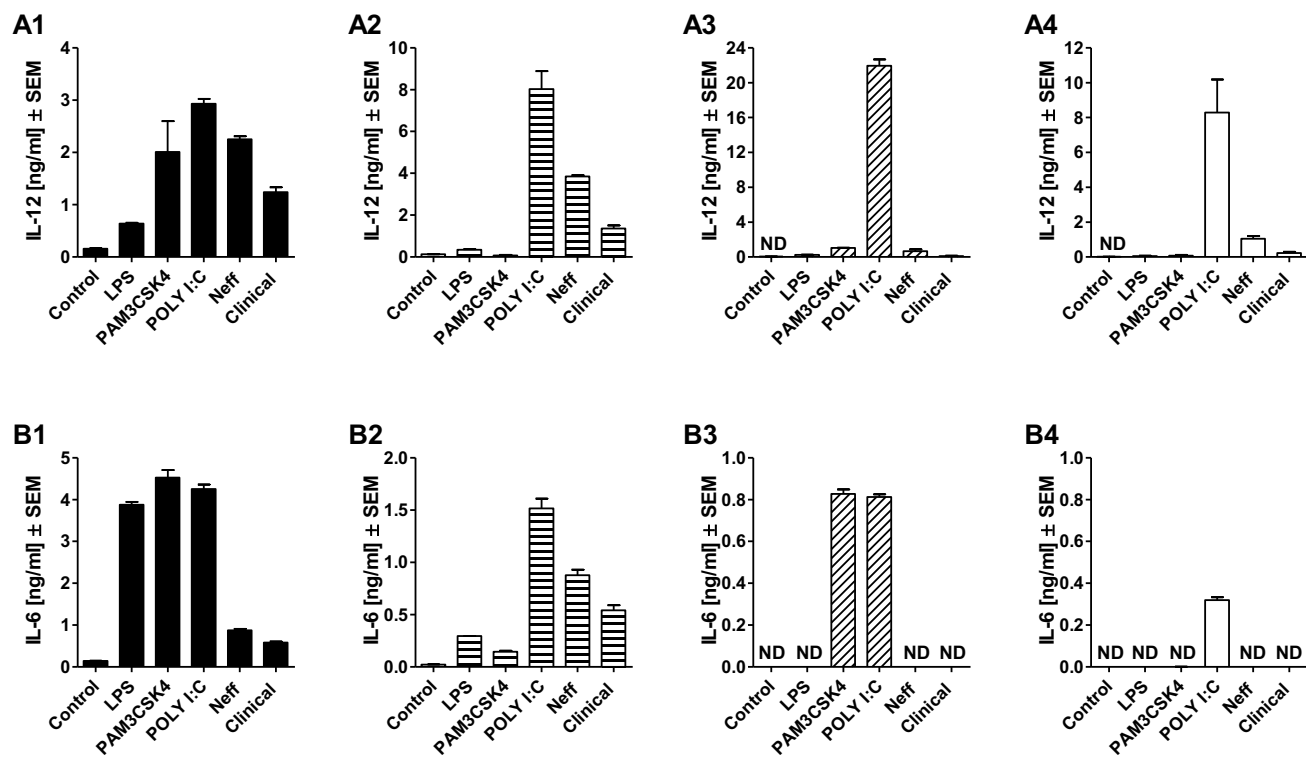


Figure 3



**Figure 4**





**Figure 5**