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

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

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Abstract

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

Introduction

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

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

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

Materials and Methods

Acanthamoeba castellanii cultures

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

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

Culture of bone marrow derived macrophages

Bone marrow derived (BMD) macrophages, obtained from the femurs of either 7-weeks-old male BALB/c or C57BL/6 Toll like receptor 4 (TLR4-/-), Toll like receptor 2 (TLR2-/-), Toll like receptor 2/4 (TLR2/4-/-), myeloid differentiation primary response gene 88 (MyD88 -/-) or TIR-domain-containing adapter-inducing interferon β (TRIF-/-) deficient mice and their wild-type counterparts, were used to perform the experiments. Briefly, bone marrow stem cells were flushed from the femur with 5 ml of Dulbecco's medium (DMEM) (Life technologies, Paisley, UK) supplemented with 20% v/v of heat inactivated foetal calf serum (HI-FCS) (Biosera, Sussex, UK), 30% v/v of L-cell conditioned medium, 2 mM L-glutamine (Sigma Chemical Co, Poole, UK), 125 U/ml Penicillin and 125 µg/ml Streptomycin and grown for 10 days, at 37°C, 5% CO2 atmosphere. L-cell conditioned medium was obtained by harvesting the metabolized medium from cultured murine fibroblastic cell line L-929. This conditioned medium provides a source of macrophage colony stimulating factor (M-CSF) necessary for the growth and differentiation of bone marrow stem cells into mature macrophages (18).

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

At day 10, macrophages were harvested with RPMI-1640 medium (Lonza Biowhittaker, Virviers, Belgium), and centrifuged at x 300 *g* 5 minutes (min). Pellets were re-suspended in RPMI supplemented with 10% v/v HI-FCS, 2 mM L-glutamine, 125µg/ml 100 U/ml Penicillin and 100 µg/ml Streptomycin (complete RPMI, cRPMI) and centrifuged for 5 min at x 300 *g*. Supernatants were discarded and macrophages were then re-suspended in cRPMI and counted using the Neubauer Chamber (Superior Marienfeld, Germany). Macrophage suspensions at required density were prepared, seeded in the appropriate vessel, and incubated over night at 37°C, 5% CO_2, to allow macrophages to adhere.

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

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

Murine macrophages were harvested and suspended to 7 x 10^5 cells/ml in cRPMI. 30 µl of cell suspension was added into 3 channels of the µ-slide VI0.4 (ibidi GmbH, Martinsried, Germany). After replacing the lid to cover the reservoirs, the µ-slide was incubated at 37°C 5%CO2 for 1

h and 30 min to allow macrophages to adhere. Afterwards, 60 µl of free cell cRPMI was added to each well, and then the µ-slides were incubated at 37°C 5%CO_2 over night. After incubation cRPMI was removed from the wells, and 30 µl of either Neff or clinical trophozoites (7 x 10^5 trophozoites/ml suspensions in cRPMI) were added. In the control channel (macrophages alone) 30 µl of cRPMI were applied; in the co-culture channels, 60 µl of cRPMI were then added to all wells. Prepared µ-slides were then observed, using an inverted epifluorescence microscope (Nikon, Eclipse TE300), provided with a 37°C chamber that allowed maintenance of the samples at the appropriate temperature while images were acquired at X10, X20 or X40 magnification. After manually focusing, images were acquired every 30 sec for a period of 1 h using the software MetaMorph (Molecular devices, Sunnyvale, CA, USA). Thereafter, images were processed using the programme Volocity (Perkin Elmer, Massachusetts, USA).

Stimulation of BMD macrophages with specific agonists

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

Quantitative analysis of murine cytokines production

At specific time points after co-incubation, plates were centrifuged for 1 min at 480 x g, 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 ±
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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Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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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-α, 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,

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

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

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

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.

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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 $F4/80^{+}$

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

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

Figure Legends

Fig 1. Release of IL-12 (A) and IL-6 (B) at 4, 6, 8, 10, 24 h after co-incubation with *A. castellanii* Neff trophozoites. 1 x 10^6 murine macrophages, obtained from BALB/c mice, were challenged with either $1x10^6$ (Ratio 1:1) or $5x10^5$ (Ratio 1:2) or $1x10^5$ (Ratio 1:10) trophozoites of *A. castellanii* Neff strain. LPS was used as a positive control at a concentration of 200 ng/ml, whereas uninfected macrophages (Control) were considered the negative control. Experiments were repeated three times. Results represent the mean ± standard error of n=6. Two way ANOVA could not be applied, since the interaction between the stimuli and time was statistically significant and the statistical analysis for the time and stimuli effects are therefore difficult to interpret. For this reason, one way ANOVA was applied for each time points. Tukey's multiple comparison test was performed to evaluate differences within the conditions means at each time point. In the graphs, significances within the different conditions are indicated as follow: for values of $p<0.005$ **; $p<0.0005$ ***. Note that trophozoite/macrophage infection higher ratios induce higher cytokine production and IL-6 and IL-12 production peaks at 24 hrs.

Fig 2. Release of IL-12 (A) and IL-6 (B) at 8 and 24 h after co-incubation with *A. castellanii* trophozoites. $1x10^6$ murine macrophages, obtained from BALB/c mice, were challenged with either $1x10^6$ or $5x10^5$ trophozoites of either the Neff strain (respectively Neff ratio 1:1 and Neff ratio 1:2) or clinical isolate (respectively Clinical ratio 1:1 and Clinical ratio 1:2). LPS, at a concentration of 200 ng/ml, (LPS) was used as a positive control, whereas uninfected macrophages (Control) were considered the negative control. The experiment was repeated twice. Results represent the mean ± standard error of n=3. One way ANOVA was applied for

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

Fig 3. Release of IL-12 (A) and IL-6 (B): comparison between C57BL/6 WT (A1 and B1) and C57BL/6 MyD88$^{-/-}$ (A2 and B2) macrophages at 24 h after co-incubation with *Acanthamoeba* trophozoites. $1x10^6$ murine macrophages, obtained from either C57BL/6 WT or C57BL/6 MyD88$^{-/-}$ mice, were challenged with either $1x10^6$ or $5x10^5$ trophozoites of either *A. castellanii* Neff strain (respectively Neff 1:1 and Neff 1:2) or clinical isolate (respectively clinical 1:1 and 1:2). LPS and POLY I:C at respectively 200 ng/ml (LPS) and 10 μg/ml (POLY I:C) were used to stimulate macrophages as positive controls. Uninfected macrophages (Control) were considered the negative control. The experiment was performed twice. Results represent the mean ± standard error of n=3. Student's *t*-test was applied to evaluate differences between C57BL76 WT and the C57BL/6 MyD88$^{-/-}$ macrophage cytokine production. Values below the detectable levels are indicated in the graphs as ND (not detected). IL-12 and IL-6 production in trophozoites/MyD88$^{-/-}$ macrophages co-incubation is completely ablated in comparison to trophozoites/WT macrophages co-incubation, suggesting that the production of these pro-inflammatory cytokines by macrophages, in response to *Acanthamoeba*, is MyD88-dependent.

Fig 4. Release of IL-12 (A) and IL-6 (B): comparison between C57BL/6 WT (A1 and B1) and C57BL/6 TRIF$^{-/-}$ (A2 and B2) macrophages at 24 h after co-incubation with *Acanthamoeba* trophozoites. 1x10^6 murine macrophages, obtained from either C57BL/6 WT or C57BL/6 TRIF$^{-/-}$ mice, were challenged with either 1x10^6 or 5x10^5 trophozoites of either *A. castellanii* Neff strain (respectively Neff 1:1 and Neff 1:2) or clinical isolate (respectively clinical 1:1 and clinical 1:2). LPS and POLY I:C at respectively 200 ng/ml (LPS) and 10 µg/ml (POLY I:C) were used to stimulate macrophages as positive controls. Uninfected macrophages (Control) were considered the negative control. The experiment was performed twice. Results represent the mean ± standard error of n=3. Student's *t*-test was applied to evaluate differences between C57BL76 WT and the C57BL/6 TRIF$^{-/-}$ macrophages. Values below the detectable levels are indicated in the graphs as ND (not detected). IL-12 and IL-6 production in trophozoites/TRIF$^{-/-}$ macrophages co-incubation is significantly diminished in comparison with trophozoites/WT macrophages co-incubation condition suggesting that the production of these pro-inflammatory cytokines by macrophages in response to *Acanthamoeba*, might be in part TRIF-dependent, although this does not appear to be the main signalling pathway involved during *Acanthamoeba* stimulation.

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

stimulate macrophages as positive controls. Uninfected macrophages (Control) were considered the negative control. The experiment was performed twice. Results represent the mean ± standard error of n=3. Student's *t*-test was applied to evaluate differences between C57BL76 WT and the C57BL/6 TLR2-/-, TLR4-/- and TLR2/4-/- conditions. Values below the detectable levels are indicated in the graphs as ND (not detected). IL-12 and IL-6 production in trophozoites/TLR2-/- macrophages co-incubation was not significantly diminished in comparison with trophozoites/WT macrophages co-incubation condition, suggesting that the production of these pro-inflammatory cytokines by macrophages in response to *Acanthamoeba* is not TLR2-dependent. On the other hand, in the absence of TLR4, trophozoites-induced IL-12 production by macrophages was significantly decreased, whereas IL-6 production was completely ablated. The same pattern was observed when both TLR2 and TLR4 were not expressed. Therefore, TLR4 appeared to be the main TLR involved in the recognition and response to *A. castellanii*.

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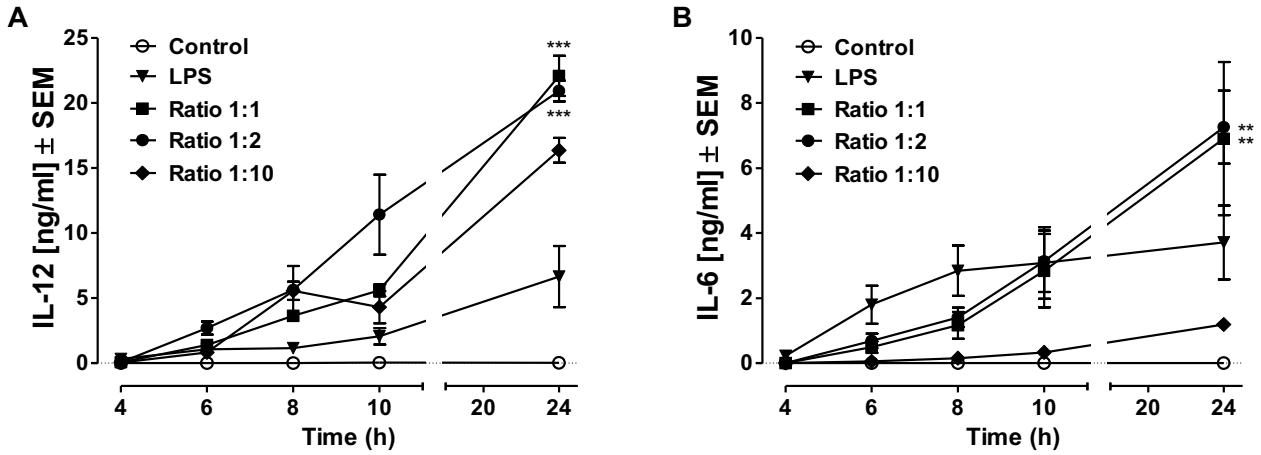
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Figure 1

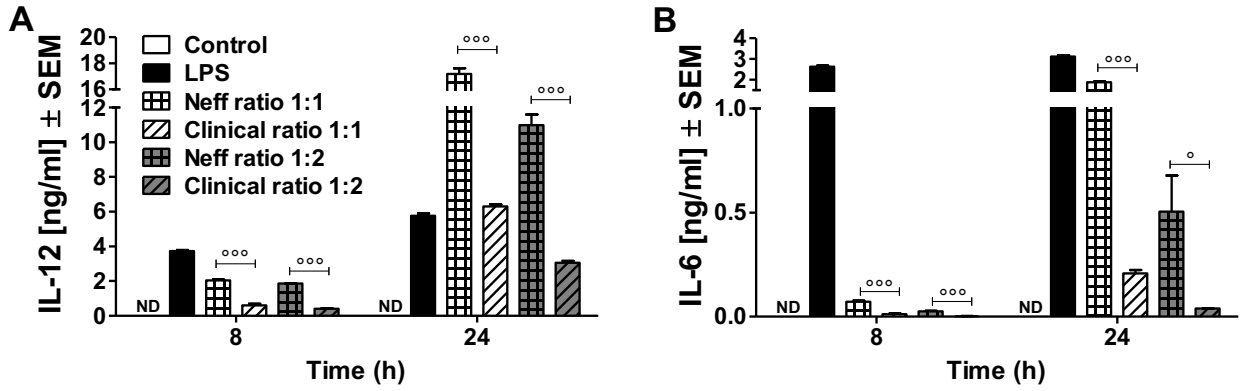
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Figure 2

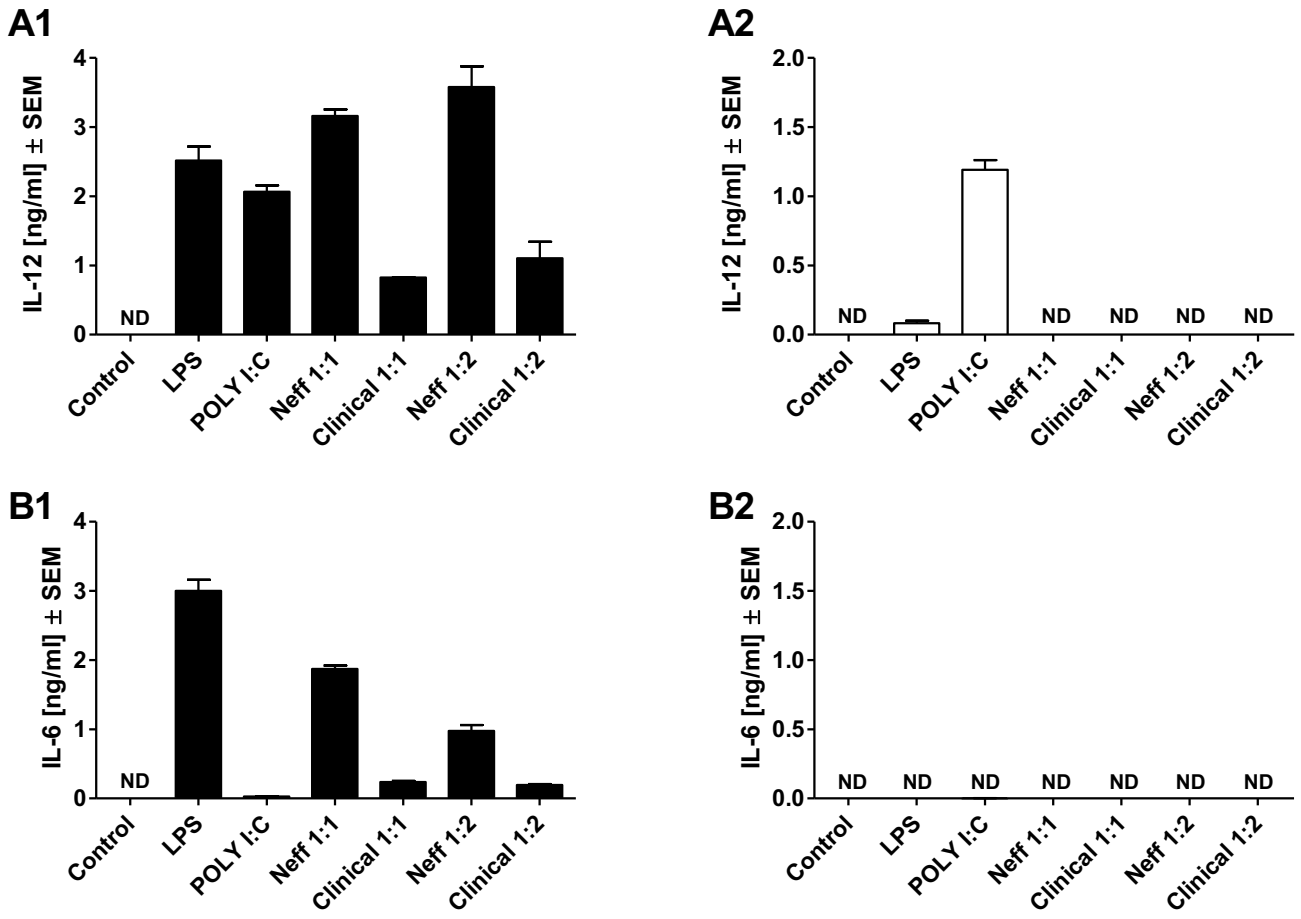
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Figure 3

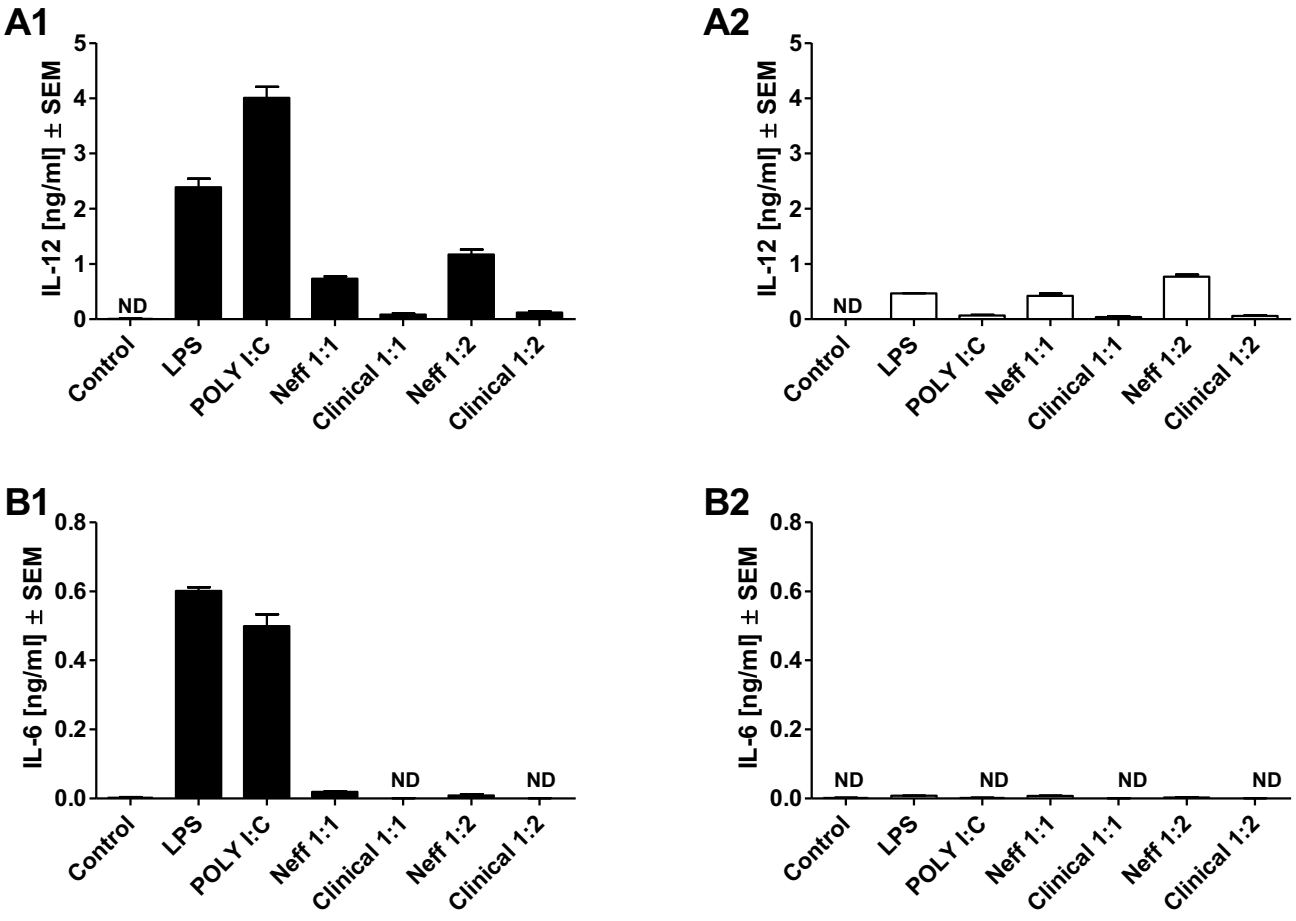
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Figure 4

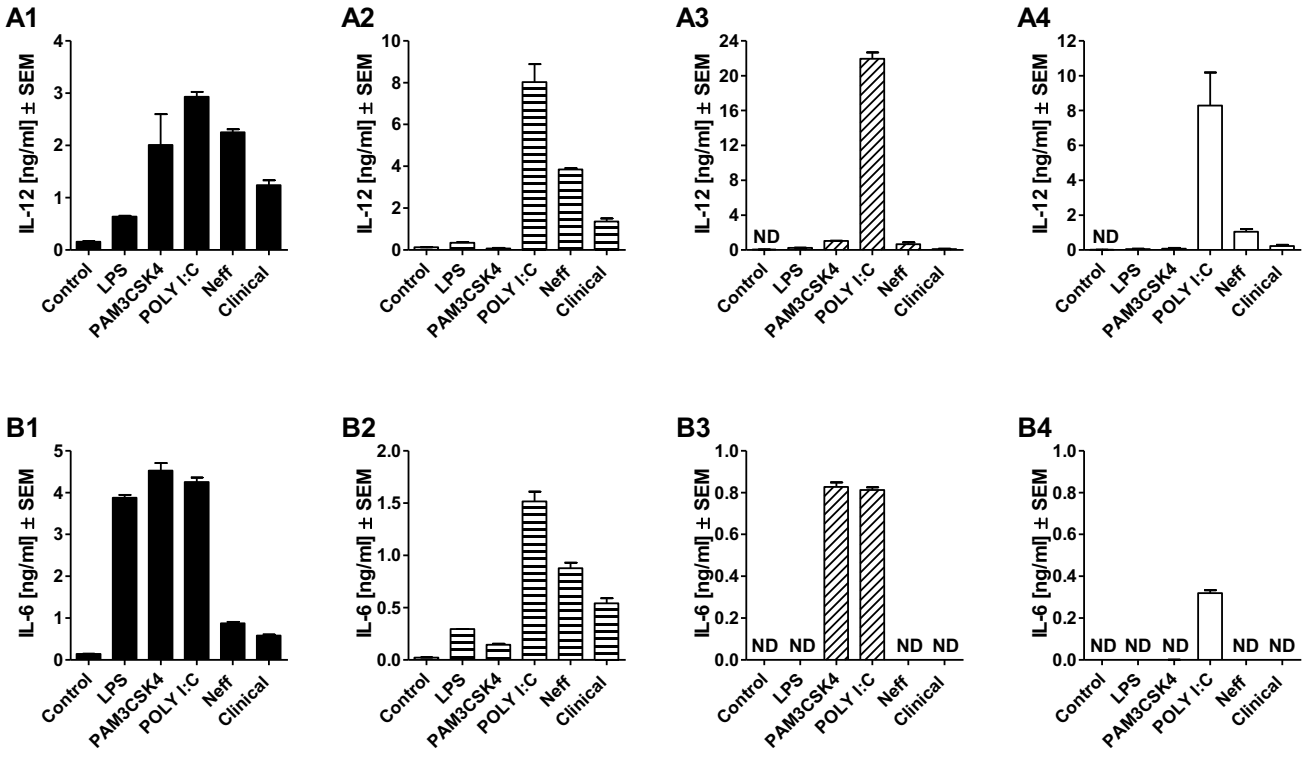
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Figure 5