

REPORT

Effect of macrophage alone or primed with cytokines on *Balamuthia mandrillaris* interactions with human brain microvascular endothelial cells *in vitro*

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Abstract: *Balamuthia mandrillaris* is well known to cause fatal *Balamuthia* amoebic encephalitis (BAE). Amoebic transmission into the central nervous system (CNS), haematogenous spread is thought to be the prime step, followed by blood–brain barrier (BBB) dissemination. Macrophages are considered to be the foremost line of defense and present in excessive numbers during amoebic infections. The aim of the present investigation was to evaluate the effects of macrophages alone or primed with cytokines on the biological characteristics of *Balamuthia in vitro*. Using human brain microvascular endothelial cells (HBMEC), which constitutes the BBB, we have shown that *Balamuthia* demonstrated >90% binding and >70% cytotoxicity to host cells. However, macrophages further increased amoebic binding and *Balamuthia*-mediated cell cytotoxicity. Furthermore macrophages exhibited no amoebicidal effect against *Balamuthia*. Zymography assay demonstrated that macrophages exhibited no inhibitory effect on proteolytic activity of *Balamuthia*. Overall we have shown for the first time macrophages has no inhibitory effects on the biological properties of *Balamuthia in vitro*. This also strengthened the concept that how and why *Balamuthia* can cause infections in both immuno-competent and immuno-compromised individuals.

Keywords: *Balamuthia mandrillaris*, macrophages, cytokines, human brain microvascular endothelial cells, *Balamuthia* amoebic encephalitis.

INTRODUCTION

Balamuthia mandrillaris is a protozoan pathogen and member of free living amoeba family. It may causes life threaten human infection which involves CNS (Anzil *et al.*, 1991; Visvesvara *et al.*, 1993). BAE can be distinguished by fever, headache, stiff neck, characteristic skin lesions, vomiting, nausea, acute confused state, with cerebral haemorrhagic necrotizing lesions, cranial nerve palsies, seizures and finally death (Schuster and Visvesvara, 2004; Matin *et al.*, 2008). Predisposing factors of BAE are not known but recent studies have shown clearly that unlike *Acanthamoeba*, *Balamuthia* can cause fatal infections in comparatively immuno-competent individuals. BAE can be developed in patients with no history of diabetes mellitus, syphilis, malignancies, or fungal and mycobacterial infections and are negative for HIV-1 and HIV-2. In addition the route of entry into CNS is believed to be through olfactory neuroepithelium (Kiederlan and Laube, 2004) or lower respiratory tract, followed by haematogenous spread. On the other hand skin lesions can provide the amoeba direct

entry into bloodstream, by evading the lower respiratory tract. However in haematogenous spread, *Balamuthia mandrillaris* may enters into the CNS most probably through BBB (Martinez *et al.*, 2001; Schuster and Visvesvara, 2004). Skin and lungs infections can last for months, but in case of CNS involvement always results in death within days. Recently it has been proven that *Balamuthia* exhibited multifactorial properties which turn out to be HBMEC damage, which are important component of BBB (Jayasekera *et al.*, 2005; Matin *et al.*, 2007a). Macrophages are believed to be the initial defense line during many infections; however, the effect of macrophages on biological properties of *Balamuthia* is not known and thus the objective of the present investigation.

MATERIALS AND METHODS

Cytokines

Recombinant human tumor necrosis factor- α (TNF- α), Interferon- γ (INF- γ), transforming growth factor- β 1 (TGF- β 1) and Interleukin-6 (IL-6) were purchased from Roche (Germany). Cytokines were used to determine whether in combination with macrophage exhibited

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cytolytic activity for *B. mandrillaris* (Fischer-Stenger *et al.*, 1990; Fischer-Stenger, K. and Marciano-Cabral 1992).

Culture of mouse macrophage (RAW 264.7) cells

Mouse leukaemic monocyte macrophage cell line (RAW 264.7) was kindly provided by Dr. Sanjeeb Bukhta, Department of Biology, Birkbeck College, University of London. Briefly, RAW were routinely grown in 10% heat-inactivated fetal bovine serum, 2mM glutamine, 1mM streptomycin (100µg/mL), penicillin (100U/mL) and RPMI-1640 (Invitrogen, Paisley, UK). RAW were cultured in T-75 tissue culture flasks and incubated for 24 h in the presence of 5 % CO₂ at 37°C.

Human brain microvascular endothelial cells cultures

Primary human brain cells were cultured as described previously (Matin *et al.*, 2006a: 2013). Briefly, cells were cultured in 20% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (100U/ml), vitamins, 1mM pyruvate, non-essential amino acids, streptomycin (100 µg/ml) and RPMI-1640 (Invitrogen, Paisley, UK). For assays, HBMEC (5 x 10⁵ cells/ml/well) were grown in 24-well plates and cultured at 37°C with 5% CO₂ for 24h. This cell density forms confluent monolayer which was used for further different assays afterwards.

Cultures of *Balamuthia mandrillaris*

Balamuthia mandrillaris was previously isolated from the brain of a mandrill baboon (ATCC 50209). *Balamuthia* were regularly cultured on human brain cells as a food source. Briefly, *Balamuthia* were incubated (10⁶ parasites in 10ml of RPMI-1640) with host cells grown in T-75 tissue culture flasks. *Balamuthia* consumed brain cells within 48h and gained approximately 5-8 x10⁶ parasites/10ml (>99% in trophozoite forms), which were subsequently used for further assays.

Adhesion assays

To establish whether macrophages affects *Balamuthia* binding to host cells, adhesion assays were performed as described before (Matin *et al.*, 2007a). Briefly HBMEC were cultured until confluent in 24-well plates. Next, *Balamuthia* (2 x 10⁵ amoebae/well) was incubated with endothelial cells in 24-wells plates for 60 min with 5% CO₂ at 37°C. In some experiments macrophages were pre-incubated with various cytokines {INF-γ, TGF-β, TNF-α and IL-6 (1 and 10ng/ml) and LPS (10µg/ml)} for 1h followed by incubation with co-culture of *Balamuthia* and HBMEC. The unbound amoebae were calculated by haemocytometer and the numbers of bound amoebae were calculated.

Cytotoxicity assays

For *Balamuthia*-mediated endothelial cells cytotoxicity, assays were performed as reported previously (Matin *et al.*, 2007b). Briefly, *Balamuthia* were incubated with

endothelial cells in 24-well plates as described above. Plates were kept for 24h at 37°C in the presence of 5% CO₂. After this incubation, cell free supernatants [conditioned media (CM)] were obtained after centrifugation and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release (according to manufacture instructions; Roche, UK). Briefly, CM of co-cultures of amoebae and endothelial cells were obtained and % LDH was measured. In some experiments macrophages were pre-incubated with various cytokines for 1h followed by incubation with co-culture of *Balamuthia* and HBMEC.

Amoebicidal assays

To evaluate the effect of macrophages on *Balamuthia* viability amoebicidal assays were performed as described previously (Matin *et al.*, 2007b). Briefly macrophages were grown as described above. Next *Balamuthia* trophozoites were incubated with macrophages in equal numbers (2 x 10⁵/ml/well) in 24-well plates for 24h with 5 % CO₂ at 37°C. After this incubation *Balamuthia* were numbered using haemocytometer and the total numbers of amoebae were calculated by using the following formulae.

$$\frac{\text{No. of haemocytometer counted amoebae}}{\text{Total number of amoebae}} = \text{Amoebae increase/decrease}$$

The plates were further incubated up to a week (170h) and number of amoeba was counted at various time intervals (48, 72, 96, 122, 146 and 170h). *Balamuthia* incubated alone without macrophages were considered as control. In some experiments macrophages were pre-treated with different cytokines and were incubated co-cultures (amoeba and macrophages) together up to a week.

Zymography assays

To determine whether macrophages had any effect on *Balamuthia* proteases, zymography was performed with few modifications in already reported protocol (Matin *et al.*, 2006b). Briefly, HBMEC were cultured for overnight with 5 % CO₂ at 37°C. *Balamuthia* was pre-incubated with macrophages up to 1 h and co-cultures were incubated together with HBMEC for 24h. After this incubation culture was centrifuged at 13,000 rpm for 5 min and cell free medium (supernatant) was obtained and used as a CM. CM were mixed (1:1) with sample buffer and electrophoresed on SDS-polyacrylamide gel electrophoresis containing gelatin. After electrophoresis, gels were left in 2.5% Triton X-100 (w/v) solution for 1h to remove SDS. Next, gels were further incubated in a developing buffer at 37°C for overnight followed by rinsing and staining with Coomassie brilliant blue. Areas of gelatin digestion by protease activity were visualized as non-staining regions in the gel. In some experiments macrophages were activated with cytokines and further used to investigate their effect on proteolytic activities of *Balamuthia*.

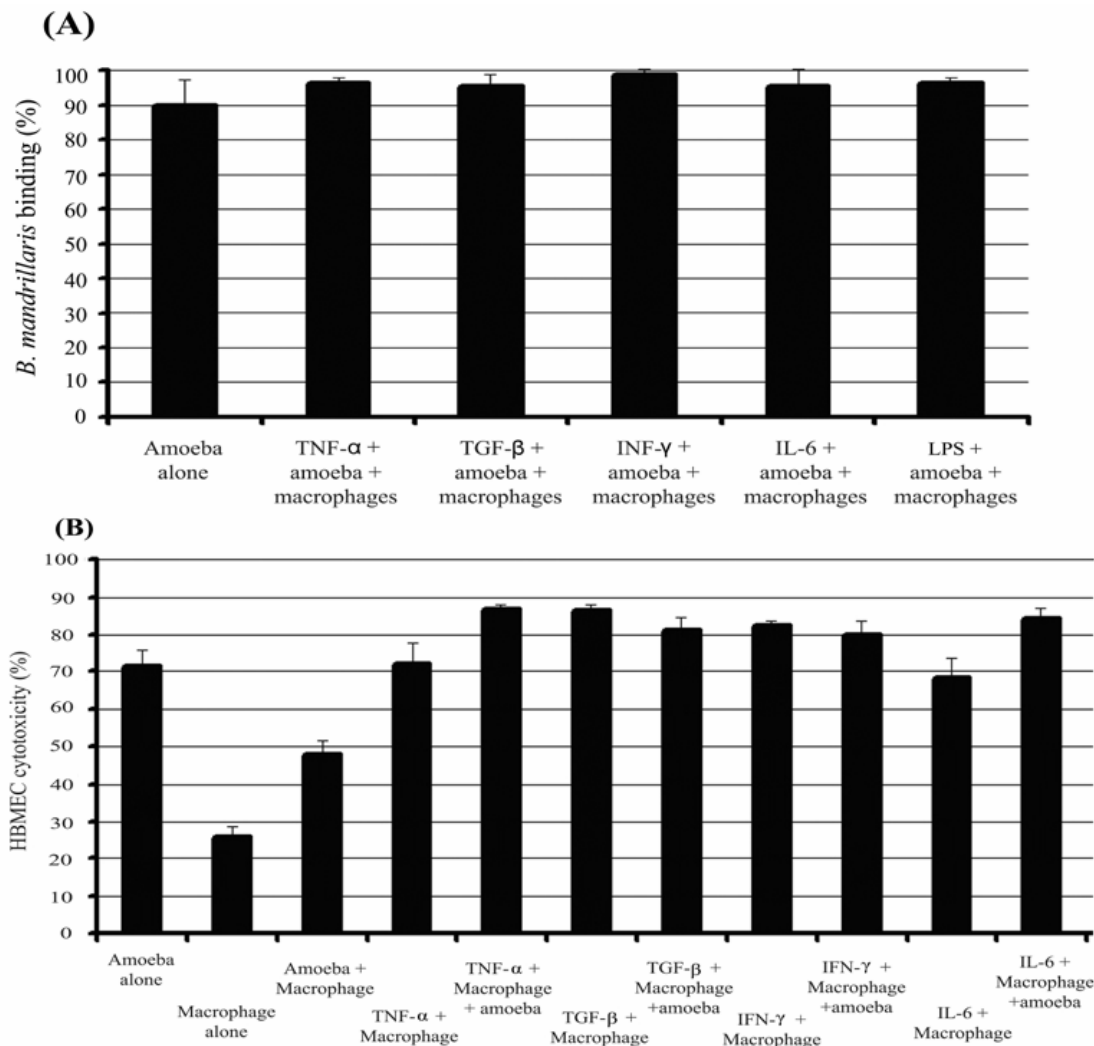


Fig. 1: Macrophages alone or primed with cytokines exhibited an increase in *Balamuthia* binding and cytotoxicity to HBMEC. (A) To determine the impact of macrophages on *Balamuthia* binding to HBMEC adhesion assay were performed as described in Materials and Methods. Our results revealed *Balamuthia* binding to HBMEC was further increased in the presence of macrophages or primed with various cytokines. *Balamuthia* incubated with HBMEC (without macrophages and cytokines) is considered as a control. (B) To investigate the role of macrophages on *Balamuthia*-mediated HBMEC death, cytotoxicity assays were performed. Our results revealed *Balamuthia*-mediated HBMEC cytotoxicity was increased in the presence of macrophages or primed with various cytokines.

RESULTS

Macrophages exhibited an increase in Balamuthia binding and cytotoxicity to HBMEC

To investigate the impact of macrophages alone or primed with various cytokines in *Balamuthia* binding to HBMEC adhesion assay were performed. Our results revealed normally *Balamuthia* shows more than 90% binding to HBMEC which was further increased in the presence of macrophages and or primed with various cytokines TNF- α , TGF- β , IL-6 and INF- γ (1, 10ng/ml final concentrations) and LPS (10 μ g/ml) (fig. 1A). *Balamuthia* alone with HBMEC (without macrophages and cytokines) is considered as a control. To determine the role of macrophages alone or primed with cytokines on

Balamuthia-mediated HBMEC death, cytotoxicity assays were performed. Our results revealed that *Balamuthia* caused sever HBMEC cytotoxicity i.e. >70% within 24h which was further increased in the presence of macrophages alone or primed with various cytokines (fig. 1B). It is worthy to note that macrophages alone (without *Balamuthia* and cytokines) also cause ~30% HBMEC cytotoxicity which support the previous finding to some extent (Diez-Roux and Lang 1997).

Macrophages could not induce amoebicidal effects against Balamuthia

To determine the effects of macrophages on *Balamuthia* biological properties amoebicidal assays were performed as described above in amoebicidal assays in the method

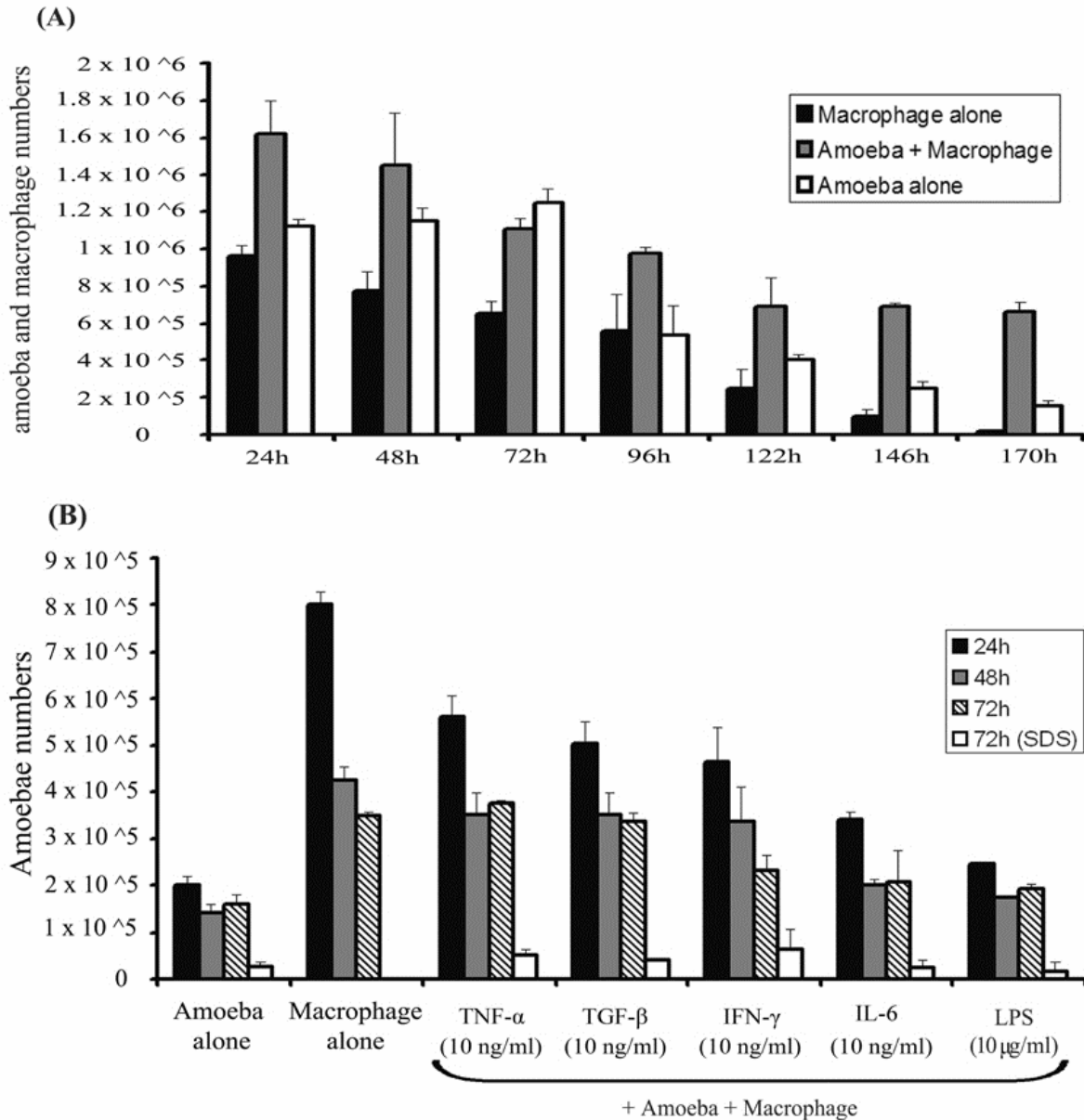


Fig. 2: Macrophages could not induce amoebicidal effects against *Balamuthia*. (A) To determine the amoebicidal effects of macrophages against *Balamuthia*, assay was performed as described in “Materials and Methods”. Amoeba and macrophages were incubated together for various time intervals. Amoeba alone is considered as a standard. (B) To determine the number of amoeba and macrophages, wells were treated with SDS and counted the amoeba in each well. Results are representative of three independent experiments performed in triplicate. Bars represent standard error.

section. Our findings revealed that macrophages exhibited no activity against *Balamuthia*. Amoeba exhibited optimal numbers in the presence of macrophages within 24h (fig. 2A). To determine the amoebicidal effects, assays were performed in the presence of macrophages. The co-cultures (amoeba and macrophages) were incubated together for various time intervals (24, 48, 72, 96, 122, 146 and 170h). Our results showed *Balamuthia* increased in number in the presence of macrophages and reached to highest number after 24h incubation and became static after 122h (fig. 2B). In some experiments

macrophages were pre-treated with different cytokines and were incubated with amoeba together up to 72h. Our results showed macrophages alone or primed with various cytokines do enhance the amoeba numbers till 24h and then start declining gradually up to 72h (fig. 2B). Off interest it is important to note that SDS treatment showed the presence of amoeba cyst after 72h (fig. 2B). Overall our findings suggest neither macrophages alone nor primed with cytokines have any inhibitory effects on *Balamuthia in vitro*.

Macrophages have no inhibitory effects on proteolytic ability of *Balamuthia*

To demonstrate the proteolytic ability of amoebae zymography was performed. Our results revealed CM exhibited gelatin degradation activities (protease bands) below 82 kDa which is not affected by macrophages incubated with *Balamuthia* in the presence of HBMEC (fig. 3A). Interestingly, when macrophages were primed with various cytokines and further incubated together with *Balamuthia* in the presence of HBMEC, we observed another protease band above 82 kDa which became prominent (fig. 3B). Overall, these results suggest that macrophages alone or primed with cytokines have no major inhibitory effect on proteolytic ability of *Balamuthia*.

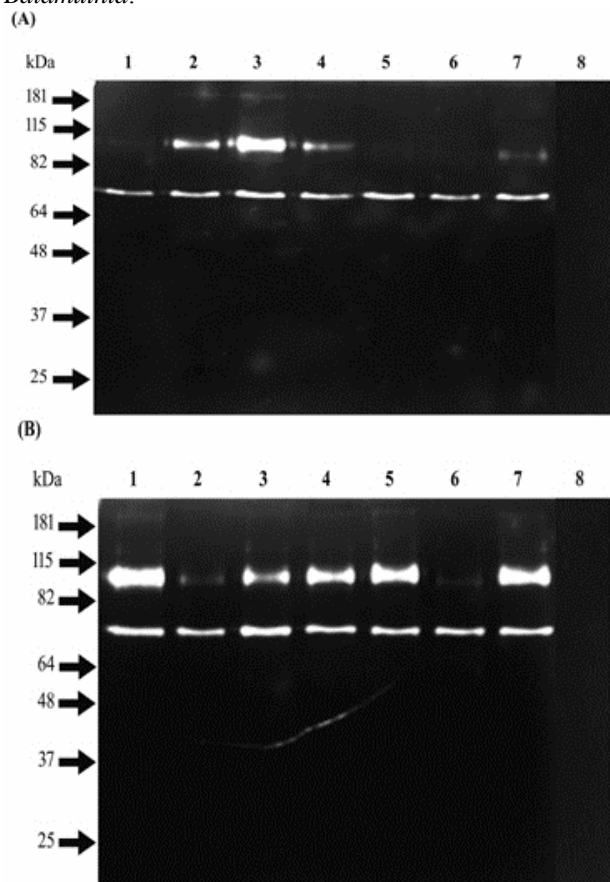


Fig. 3: Cytokines exhibited no effect on proteolytic ability of *Balamuthia*. To determine the proteolytic ability (secretions) of amoebic conditioned media, in the presence of HBMEC, zymography assays were performed as described in "Materials and Methods". (A) Lane 1: *Balamuthia* without cytokines (control); Lane 2: *Balamuthia* + TNF- α ; Lane 3: *Balamuthia* + TGF- β ; Lane 4: *Balamuthia* + LPS; Lane 5: *Balamuthia* + IFN- γ ; and Lane 6: *Balamuthia* + IL-6; Lane 7: HBMEC without *Balamuthia* and cytokines and Lane 8: *Balamuthia* alone (without HBMEC + cytokines) (B) Lane 1: *Balamuthia* without cytokines and macrophages (control); Lane 2: *Balamuthia* + TNF- α + macrophages; Lane 3: *Balamuthia* + TGF- β + macrophages; Lane 4: *Balamuthia* + LPS + macrophages; Lane 5: *Balamuthia* + IFN- γ + macrophages;

Lane 6: *Balamuthia* + IL-6 + macrophages; Lane 7: HBMEC without *Balamuthia*, cytokines and macrophages and Lane 8: *Balamuthia* alone (without HBMEC + cytokines + macrophages). We observed macrophages alone or primed with cytokines don't have any inhibitory effect on proteolytic activity of *Balamuthia*. Results are representative of three independent experiments.

DISCUSSION

In the present investigation, mouse macrophage cell line alone or primed with various cytokines were used to investigate their interaction with *Balamuthia*. Macrophages are believed to be the primary line of defense in many infections and are found to be present in huge numbers during amoebic infection. For example, macrophage depletion affected the incidence, severity, and chronicity of keratitis. The profound exacerbation of *Acanthamoeba* keratitis in hamsters strongly suggests that macrophages play a significant role in corneal infection of *Acanthamoeba*, probably by acting as a primary line of defense and eliminating substantial numbers of *Acanthamoeba* trophozoites (van Klink *et al.*, 1996). *Balamuthia* is a free-living amoeba and being a close relative of *Acanthamoeba* (belong to the same family, i.e., Acanthamoebidae), it is quite possible the same mechanism prevails in BAE also.

It has been observed that macrophages are important effector cells in *Acanthamoeba* infections (van Klink *et al.*, 1996). It has been reported previously that *Acanthamoeba culbertsoni* exhibited the ability to resist macrophage amebicidal activity due to its high pathogenicity. Furthermore, this specie also destroys macrophage monolayers more rapidly than other less pathogenic species like *Acanthamoeba castellanii*. Our findings reports *Balamuthia* destroy macrophages alone or primed with various cytokines which suggest the virulence potential of the amoeba. Furthermore in some experiments cytokines are also used in combination to activate macrophages before incubation with *Balamuthia*, but could not observe any difference in results (data not shown). That might be one of the reason *Balamuthia* can cause infection in both immunocompetent and immunocompromised individuals.

BAE infection is difficult to treat and immunotherapeutic intervention could be a substitute and potential option of treatment. Macrophage cell line RAW264.7 was utilized because RAW macrophages produce high levels of cytokines and nitric oxide, substances with tumoricidal, protozoacidal and microbicidal activities (Lachman, and Metzgar. 1980; Ichinose *et al.*, 1988). Thus, here we examined the possibility whether macrophages primed with various cytokines could destroy *Balamuthia* by the production of cytolytic factors. In response lack of amebicidal activity was observed when *Balamuthia* were co-cultured with macrophage which may be due to the

lack of production of the appropriate amoebicidal factors and/or the production of insufficient quantities of cytolytic factors. This also indicates that cytokines used in the present *in vitro* trial did not play any role in killing *Balamuthia*. Our current investigation confirmed that combination of various cytokines did not exhibit amoebicidal effects against *Balamuthia*. In contrast previous studies have shown *Naegleria fowleri* were injured when cultured with activated RAW264.7 cells or with BCG-activated peritoneal macrophages (Cleary and Marciano-Cabral 1986; Fischer-Stenger and Marciano-Cabral 1992). Similarly in support the hypothesis of the first line of defense by macrophages demonstrating that macrophages exhibit a strong chemotactic response to *Acanthamoeba* and can completely kill trophozoites *in vitro* (Stewart *et al.*, 1992).

We currently investigated whether macrophage alone or primed with various cytokines have any amoebicidal effect on *Balamuthia*. Our results demonstrated clearly strong virulence ability of the amoeba suggesting neither macrophage alone nor in combination with various cytokines (INF- γ , TGF- β , TNF- α and IL-6) have inhibitory effect against *Balamuthia* biological properties. It has been shown previously TNF- α and IL-1 was cytolytic when used in combination against *Naegleria* (Fischer-Stenger and Marciano-Cabral 1992). In contrast in the present investigation cytokines were also used in combination but could not show any cytotoxic effects against *Balamuthia* (data not shown). Furthermore *Naegleria fowleri* hardly ever form cysts in macrophage cultures and *Naegleria* are not eventually ingested by activated macrophages (Fischer-Stenger and Marciano-Cabral 1992). But interestingly *Balamuthia* form cysts when co-cultured with macrophages or primed with various cytokines (fig. 2B), suggesting the cyst formation ultimately protects *Balamuthia* from cytolytic factors of macrophage.

The higher pathogenicity of *Balamuthia* may be elucidated by the ability and destruction of macrophages alone or primed with various cytokines and high rate of amoebic growth. We have shown previously (Matin *et al.*, 2014) that cytokines have no effect on virulence properties of *Balamuthia* which enforce our interests to investigate further the role of macrophages and/or primed with various cytokines to study *Balamuthia* virulence response.

CONCLUSION

There is no such report available in literature so far demonstrating the interactions of macrophages with *Balamuthia*. Therefore, here for the first time we are reporting macrophages have no inhibitory effects on the pathogenic properties of *Balamuthia in vitro*. It is speculated that various factors are involved for the

increase of pathogenicity of *Balamuthia*, which still need to be further addressed. In fact the capability of *Balamuthia* to oppose amoebicidal activity of the macrophages and/or primed with various cytokines may be a vital contributing factor to the virulence of *Balamuthia*.

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