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Immunogenic and antigenic heterogeneity of *Blastocystis* sp. subtype 3 from symptomatic and asymptomatic individuals

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Abstract. *Blastocystis* sp. subtype 3 (ST3) has been implicated previously to be phenotypically heterogeneous between isolates from symptomatic and asymptomatic persons in previous studies. The aim of this study is to identify the immunogenic and antigenic heterogeneity among three different isolates of *Blastocystis* sp. ST3 isolated from asymptomatic and symptomatic individuals. Immunogenic and antigenic profile of ST3 isolates was assessed through splenocyte proliferation, cytokine assessment and antibody measurements. The total IgG antibody responses in mice immunised with symptomatic (0.736±0.033) and asymptomatic (0.426±0.025) of ST3 *Blastocystis* sp. were significantly higher compared to the controls respectively. High secretions of IgG antibody mixture of IgG1/IgG2a antibodies were detected in sera of mice immunised with symptomatic and asymptomatic solubilised *Blastocystis* sp. Antigen (*Blastocystis*-Ag). Predominant IgG1 production was detected higher in mice injected with *Blastocystis* sp. ST 3 from asymptomatic individuals (0.148±0.016) ($P<0.05$) whereas predominant IgG2a production was detected higher in mice injected with the same subtype but from the symptomatic individuals (0.355±0.021). This data was supported by the production of significantly higher Th1 (IFNγ and IL-2) response (393.56±49.21) and (628.89±31.06) respectively in symptomatic isolates compared to Th2 (IL-4 and IL-10) response (93±7.85) and (103.22±11.33) in asymptomatic isolates after *in-vitro* stimulations. Splenocyte stimulation index (SI) was detected higher in symptomatic *Blastocystis-Ag* group. This study is the first to demonstrate antigenic and immunogenic heterogeneity among ST3 symptomatic and asymptomatic *Blastocystis*-Ag with symptomatic isolates being highly immunogenic and antigenic. No significant variations among the three isolates of asymptomatic and symptomatic groups. The study supports the suggestion from previous study that surface variations between symptomatic and asymptomatic isolates as well their distinct phenotypic variation despite being the same subtype may have caused the differences in the distinct immune responses.

INTRODUCTION

Blastocystis sp. is an unusual enteric protozoan parasite found in gastrointestinal tract of humans and many animals (Moosavi *et al.*, 2012). It has a worldwide distribution and is often the most commonly isolated organism in parasitological stool surveys (Tan *et al.*, 2008). *Blastocystis* sp. in symptomatic patients shows clinical symptoms such as abdominal pain, diarrhoea, constipation, fatigue and allergy whereas asymptomatic infections remain symptomless (Boorom *et al.*, 2008).

Studies in the past provide evidences that there were differences between asymptomatic and symptomatic isolates through cancer cell induction analysis (Chan *et al.*, 2012), antigenic-cross-reactivity (Ho *et al.*, 1994; MuEller 1994; Mansour *et al.*, 1995; Clark 1997) and protein profiling (Kukoschke and MuEller 1991; Boreham *et al.*, 1992). Furthermore we have previously established distinctive phenotypic

differences between symptomatic and asymptomatic isolates of ST3 (Ragavan *et al.*, 2014) using a range of biological and biochemical analysis. The structural differences shown between, *Blastocystis* sp. isolated from asymptomatic and symptomatic may have a bearing on the parasite's antigenicity and immunogenicity as previously suggested (Young *et al.*, 2014; Sachse *et al.*, 2000).

However antigenicity and immunogenicity differences between asymptomatic and symptomatic *Blastocystis* sp. ST3 remains still unclear. There has been no study carried out to evaluate the heterogeneity of specific immune responses between the asymptomatic and symptomatic isolates of ST3. In this study, antibody responses in immunized mice as well as, *in vitro* T-lymphocytes antigenicity and immunogenicity were evaluated. This study aims to investigate if there are specific immune responses induced by soluble proteins of *Blastocystis* sp. ST3 asymptomatic and symptomatic isolates in experimental mice to gain better insights into antigenicity and heterogeneity in immune responses.

MATERIALS & METHODS

Source of *Blastocystis* sp.

Blastocystis sp. parasites were obtained from random stool sample collection in a survey carried out at a particular rural area in, Malaysia. Isolates respectively from symptomatic and asymptomatic individuals were continuously cultured in Jones' medium. Individuals with symptoms showed flatulence, abdominal pain, diarrhoea and constipation. This information was obtained using a questionnaire. Samples with *Blastocystis* sp. were selected through direct faecal screening and cyst concentration technique to select the samples with only *Blastocystis* sp. as the sole symptom causative agent. ST3 identification of the isolates was determined through Polymerase Chain Reaction (PCR) technique (Ragavan *et al.*, 2014).

Axenization of *Blastocystis* sp. and isolation of solubilised antigen

Harvested cysts were washed in sterile saline, cultured in Jones' medium supplemented with 10% heat-inactivated horse serum and incubated at 37°C (Suresh and Smith, 2004). After 2 days, parasites from all isolates were assessed using direct microscopy to confirm the presence of the parasite (vacuolar form). The xenic *Blastocystis* sp. cultures were axenised following the method of Tan *et al.*, 2008. The clear layer containing the parasites were harvested and re-suspended in basal Jones' medium (without supplementation). The axenic cells were sonicated and the homogenates were incubated at 4°C overnight. The homogenates were the centrifuged at 13,000×g for 15 min. The supernatant (solubilised *Blastocystis*-Ag), was filter sterilized, and the protein concentration was determined by Bradford assay (Chandramathi *et al.*, 2010).

Animal Selection, Housing and Ethical Clearance

24 female balb/c mice with the age range from 4-6 weeks old were selected. The balb/c mice were purchased from Animal Laboratory, University Putra Malaysia, Serdang, Malaysia and were kept in an IVC cage at Animal Satellite Laboratory, Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia during the course of this study. Prior to the experiment, the mice were acclimatised to the new environment for one week by housing in groups of four in IVC cages. The animal protocols were approved by Institutional Animal Care and Use Committee, University of Malaya (approved ethical number: 2014-04-01/PARA/R/SKG).

Immunization

24 female balb/c mice were randomly divided into groups of three. The mice were immunised intraperitoneally (IP) by using a 27-G needle. The priming dose of 100 µl (40 µg/ml) aliquots of solubilised *Blastocystis*-

Ag of ST3 AS (Isolate 1-3) and S (Isolate 1-3) respectively were emulsified in equal volume of Freund's complete adjuvant (CFA) to 100 µl (ratio of 1:1). The subsequent three injections with incomplete Freund's adjuvant (IFA) of 100 µl emulsion with the booster dose of 100 µl (20 µg/ml) aliquots of *Blastocystis*-Ag of ST3 AS (Isolate 1-3) and S (Isolate 1-3). 200 µl of solubilised *Blastocystsis*-Ag of is ST3 AS (Isolate 1-3) and S (Isolate 1-3) solubilised antigen (20 µg/ml) with PBS was injected as the final boost but without adjuvant, at day 59. Mice which served as negative control were injected with PBS instead of solubilised antigen (Tan *et al.*, 2008).

Mice Spleen Harvesting

Mice were euthanized through cervical dislocation. Prior to cervical dislocation, animal were anesthetised by the injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). Spleens were aseptically removed from the mice 3 days after the last immunisation and were pressed through stainless steel meshes in Hank's balanced salt solution. The harvested spleens were kept in Hanks Balanced Salt solution before proceeding to *in vitro* studies (Yin *et al.*, 2013).

Cytokine Assays

Cytokines were measured as described by Yin *et al.*, 2013. Spleen cells were cultured in triplicates using flat-bottom 24-well microtiter plates. Supernatants from the cultured splenocytes ($1.5x10^6$) were collected after 24, 72 or 96 hours of stimulation with solubilised *Blastocystis*-Ag. of ST3 AS (Isolate 1-3) and S (Isolate 1-3) (10 µg/ml) respectively. Respective supernatant were then assayed for; Interleukin-2 (IL-2) and Interleukin-4 (IL-4) at 24 h; Interleukin-10 (IL-10) at 72 h ; and interferon-gamma (IFN-γ) at 96 h. IL-2, IL-4, IL-10 and IFN-γ using a commercial ELISA Kit (CUSABIO, Biotech, USA) according to the manufacturer's instructions. All the assays were performed in triplicates.

Lymphocytes Proliferation Assay

Lymphocyte proliferation assay was performed based on the protocol described by Yin *et al.*, 2013. Single-cell preparations of spleen cells were pelleted and re-suspended in erythrocyte lysis buffer (Sigma). After centrifugation at 110xg for 10 min at 4°C, cell pellets were washed three times in PBS and re-suspended in 2 ml of PBS. The cells were layered on 2 ml of Ficoll-paque (Sigma), and tubes were then centrifuged at 450xg for 10 min at RT. The lymphocyte layer was carefully transferred into a fresh 5 ml polypropylene centrifuge tube. The cell pellets were washed twice in PBS and centrifuged at 110xg for 10 min at 4°C. The supernatant was then aspirated and discarded. The remaining cell pellet was diluted to 1 ml with RPMI-1640 complete medium. The lymphocytes cells were counted on a haemocytometer. The cells were then plated in triplicates using flat-bottom 96-well plates (Corning) at a density of $5x10^5$ cells per well. The cells were the stimulated with 10 µg/ml, solubilised *Blastocystis*-Ag ST3 AS (Isolate 1-3) and S (Isolate 1-3), Concanavalin A (Con A; 10 µg/ml; Sigma; positive control) or medium alone (negative control) and incubated at 37°C in a 5% CO_2 incubator for 72 hours. After 72 hours of incubation, the plates were pulsed with 10 µl of Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories; Kumamoto, Japan) per well for 4 hours. The absorbance was measured in the triplicate cultures at 450 nm to quantitatively evaluate cell viability.

Specific IgG/IgG1/IgG2a ELISA

The serum samples were tested for the presence of specific IgG/IgG1/IgG2a by ELISA. 96-well plates (Corning) were coated with 10 µg/ml solubilised *Blastocystis*-Ag ST3 AS (Isolate 1-3) and S (Isolate 1-3) (100 µl/well) respectively in PBS overnight at 4°C. The plates were then washed with PBS containing 0.05% Tween20 (PBST), blocked for 1 hour at 37°C in PBS containing 5% FCS, and then washed with PBS. Thereafter, the serum samples (1:200) were incubated in different wells (100 µl/well) for 1 h at 37°C. After washing, the wells were incubated with 100 µl of goat anti-mouse HRP antibody (Sigma Aldrich; diluted 1:60000 in PBS) for serum specific IgG/IgG1/IgG2a for 1 h at 37°C. The plates were washed extensively

and incubated with 100 µl of substrate solution for 30 min at 37°C. The optical density was measured at 492 nm (OD492) with a microplate reader (VICTOR) followed by 50 µl 2 N H_2SO_4 to stop the enzyme reaction. All the samples were run in triplicate (Yin *et al.*, 2013).

Statistical analysis

Data, including antibodies, cytokine and lymphocyte proliferation were analysed by one-way analysis of variance (ANOVA). The graphs were plotted using Graph Pad Prism 6. The cytokine analysis, cell proliferation assay were analysed using SPSS software. Levels of significance of the differences between groups were determined by the Post-Hoc Test. Two-sided P values<0.05 were considered to indicate statistical significance. Tests of normality for the data within each group were analysed by the Q-Q plot.

RESULTS

Cell mediated response

Cell mediated immunity was evaluated by measuring cytokine levels (IFN-γ, IL-2, IL-4 and IL-10) in the supernatants of asymptomatic and symptomatic *Blastocystis*-Ag stimulated spleen cell cultures. Significantly higher IFNγ (393.56±49.21 and IL-2 (628.89±31.06) response were observed in the spleen cell cultures from the mice immunised with symptomatic group compared to the mice immunised with PBS (P<0.05) (Fig. 1a-b).

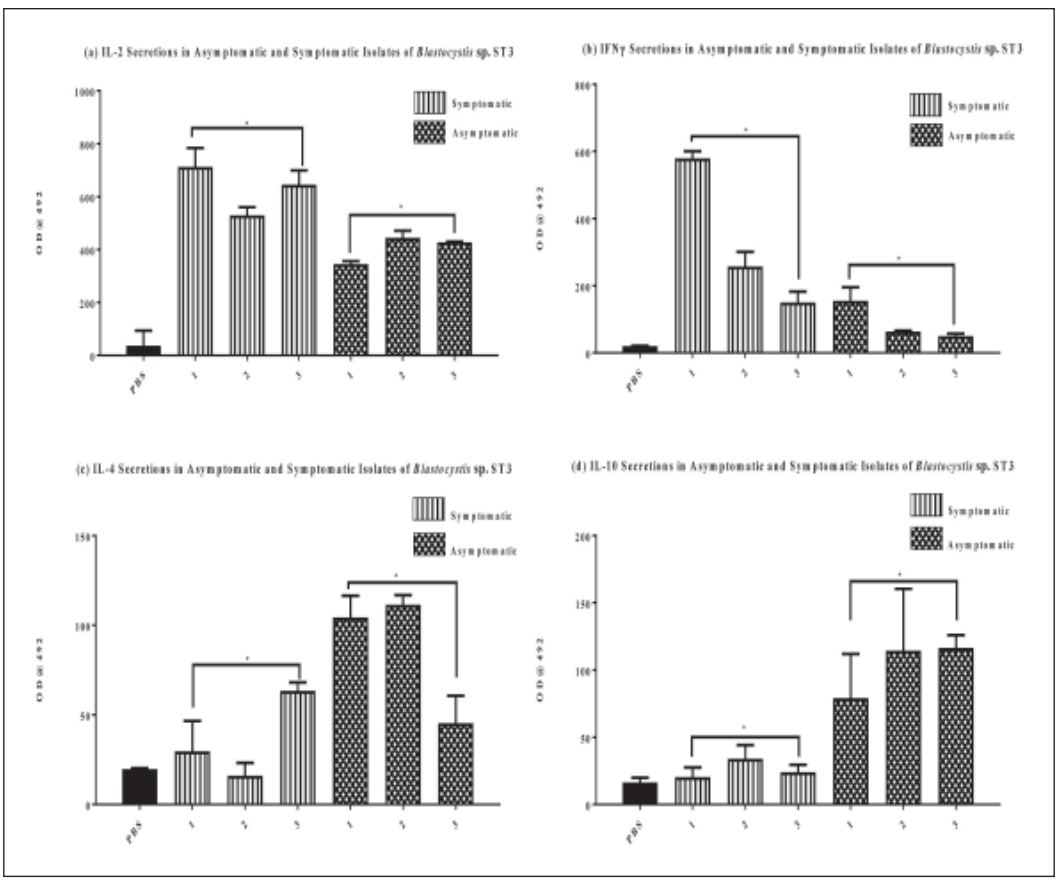

Figure 1. (a)-(b) *In vitro* Th-1 and (c)-(d) Th-2 cytokine secretions of isolated splenocyte of mice immunised mice stimulated with symptomatic and asymptomatic *Blastocystis* sp.-Ag (10µg/ml. Results are mean ± SD values (each group n = 9) for three different isolates; *, P < 0.05 compared to the PBS group (n=6).

Likewise, asymptomatic group secreted IFNγ (122.67±17.21 and IL-2 (405.89±16.09) and respectively when compared to PBS group (P<0.05). Similarly the secretions of IL-4 and IL-10 in the splenocyte supernatants from immunised mice were observed. The IL-10 levels in the asymptomatic group were higher than symptomatic group with (103.22±11.33) and (23.56±3.43) respectively compared to PBS group (P<0.05) (Fig. 1b). The levels of IL-4 showed a slight but significant increase in the splenocyte from mice immunised with asymptomatic (93±7.85) group compared to symptomatic (21.78±3.69) and PBS control group (P<0.05) (Fig. 1b). Th1 response (IFNγ and IL-2) are significantly higher in symptomatic group and Th2 response (IL-4 and IL-10) were significantly higher in asymptomatic group with no significant difference of cytokine secretions observed among the three isolates of asymptomatic and symptomatic group (P>0.05).

After the last immunization, spleen cells were prepared to assess the systemic proliferative responses to asymptomatic and symptomatic group isolates. The splenocyte stimulation index (SI) from the mice immunised with symptomatic group were higher compared to the asymptomatic group. Both were shown to be higher compared to the PBS group (P<0.05) (Fig. 2a) with the mean ± SD of (4.63±0.48) for symptomatic group and (2.40±0.48) asymptomatic group respectively. However there are no significant differences between the SIs of the isolates among the groups (P>0.05). In addition, splenocyte from all the experi-

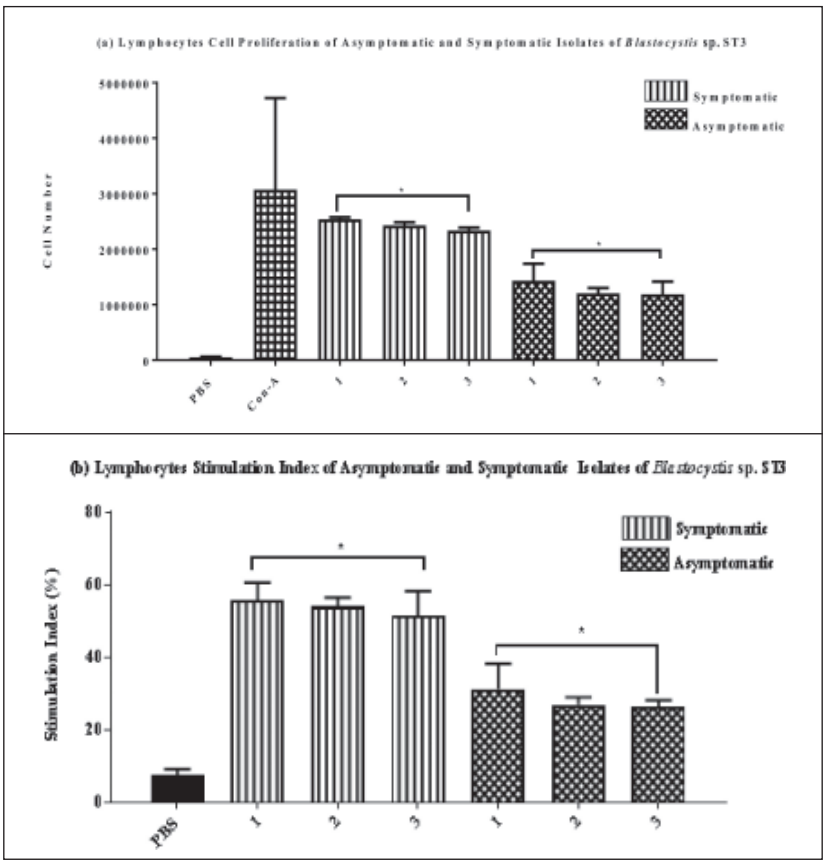

Figure 2. (a) Lymphocyte Proliferation (b) Stimulation Index of lymphocyte cell proliferation upon exposure to symptomatic (S) and asymptomatic (AS) *Blastocystis* sp.-Ag with Con-A (5µg/ml) was used as a positive control. Results are mean ± SD values (each group n = 9) for three different isolates; *, P < 0.05 compared to the PBS group (n=6).

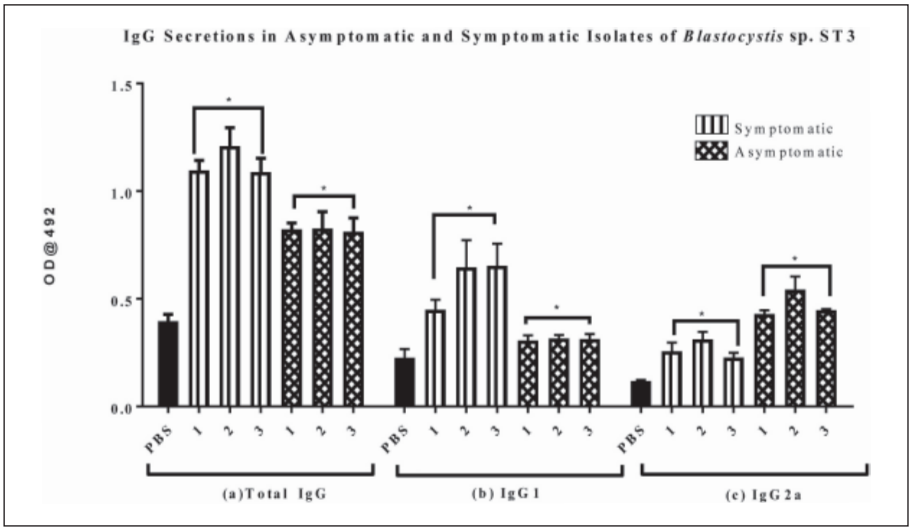

Figure 3. (a)-(c) Specific IgG/IgG1/Ig2a antibody responses from sera samples collected from the immunized mice. Sera were collected two weeks after the last immunization; comparison was made with control mice (PBS). Titers were the average of triplicate readings. Results are mean ± SD values (each group n = 9) for three different isolates; *, $P < 0.05$ compared to the PBS group (n=6).

mental and control groups proliferated to comparable levels in response to ConA. The splenocyte proliferation showed a 2-fold increase in the symptomatic *Blastocystis*-Ag groups compared to asymptomatic group. However when compared to PBS control group, a 47-fold increase in symptomatic group and and a 27 fold increase in asymptomatic were detected (Fig. 2b). These results demonstrated that intraperitoneal injection of symptomatic and asymptomatic solubilised *Blastocystis*-Ag triggered a systemic cell mediated immune response.

Humoral response

The total IgG antibody responses of the mice immunised with symptomatic and asymptomatic of ST3 *Blastocystis* sp. group were significantly higher compared to the mice injected with PBS ($P<0.05$) (Fig. 3a) were (0.736±0.033) and (0.426±0.025) respectively. Significant difference ($P<0.05$) of IgG antibody response between symptomatic and asymptomatic groups was (0.311±0.032) with symptomatic group showed the highest antibody secretions. A mixed of IgG1/IgG2a response with greater IgG2a response were detected among the group (Fig. 3a). Predominant IgG1 production was detected higher in mice injected with asymptomatic group (0.148±0.016) ($P<0.05$) (Fig. 3b) whereas predominant IgG2a production was detected higher in mice injected with symptomatic group (0.355± 0.021) compared to PBS control group. However, there was no significant difference of IgG/IgG1/IgG2a antibodies observed among the three isolates of asymptomatic and symptomatic group ($P>0.05$).

DISCUSSION

To date this is the first study to report on the *in vivo* and *in vitro* antigenicity and immunogenicity heterogeneity of ST3 soluble *Blastocystis*-Ag immunization against three different isolates derived from asymptomatic and symptomatic individuals. Previous study has demonstrated phenotypic differences between asymptomatic and symptomatic individuals (Ragavan *et al.*, 2014). It was reported that, ST3 is the only genotype of human origin shown to be geographically heterogeneous (Chan *et al.*, 2012). A study conducted by, Lanuza *et al.*, 1999 has clearly implicated that soluble antigen of *Blastocytis* sp. symptomatic isolates has

proven to be pathogenically and antigenically heterogenic with existence of demes.

It is important to know the characteristics of a host protective immune response(s), antigen characteristics, and the delivery required before inducing responses to the host. Several studies have shown that, intraperitoneal administration of *Blastocystis* sp. has often been reported to have effect on the immune response (Zhou *et al.*, 2010 and Kumarasamy *et al.*, 2017). In this study, intraperitoneal injection with previously optimised antigen concentrations (data not shown) has been used before injecting to the host. In this study, balb/c mice immunised with 20 µg/ml of solubilised antigen has acquired humoral and cell mediated response.

Humoral immune response such as IgG response is highly elicited when the antibodies produced by the host is able to recognize and bind to epitopes on the surface of antigens with high specificity (Wang *et al.*, 2011). In this study, the antigenic heterogeneity of the asymptomatic and symptomatic ST3 isolates were evaluated through IgG, IgG1 and IgG2a antibody responses with no significant difference among the isolates but significant difference among the groups. Results showed that, high level of humoral antibodies and a mixed of IgG1/IgG2a response were secreted in the sera of the immunised mice. Asymptomatic group of *Blastocystis* sp. isolates showed predominance of IgG1 productions whereas symptomatic group of *Blastocystis* sp. isolates showed predominance of IgG2a secretions. IgG1 and IgG2a isotypes are the indicated as the markers for Th1 and Th2 lymphocytes (Mounford *et al.*, 1994). This result has been associated with the secretions of pro-inflammatory and anti-inflammatory cytokine production. It was evidenced that, *in vivo* conditions of both Th1 and Th2 cells are capable of supporting antigen-specific B cell clonal expansion and antibody production (Smith *et al.*, 2000). Thus, they had balanced expression of IgG1 and IgG2a, despite having a dominant Th1 and Th2 lymphocyte population in the respective groups like shown in Diagram 1. Therefore, this study provided evidence that several inoculations of antigens from symptomatic and asymptomatic isolates in mice can stimulate the switch to Th1 and Th2 respectively.

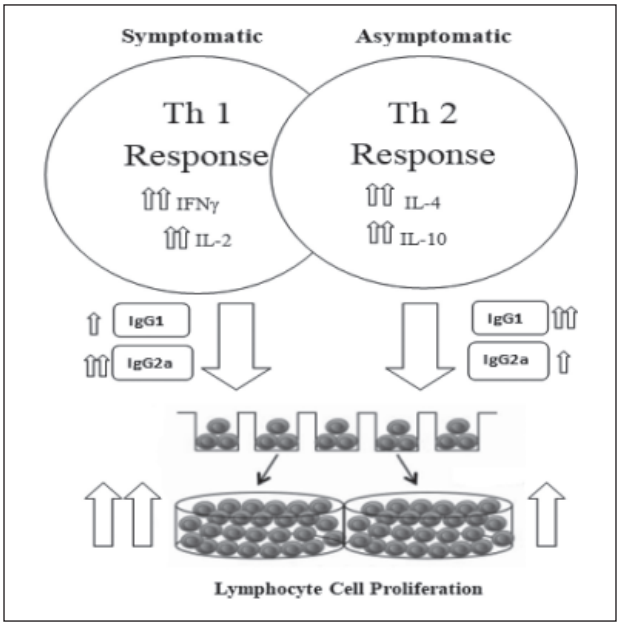

Diagram 1. Schematic Diagram of Overall Immune Response induced by Symptomatic and Asymptomatic Groups.

Interestingly, the kinetics of T lymphocyte cell clonal expansion was almost seen to be similar when inoculated with antigens from both symptomatic and asymptomatic isolates. However, Th2 cells expanded significantly lesser than Th1 cells in symptomatic group. Despite the differences, both groups were able to trigger a similar degree of B cell clonal expansion and IgG production. This might imply that Th2 cells are more proficient at supporting B cell responses on a per cell basis, as suggested by earlier studies (Noelle *et al.*, 1991 and Stevens *et al.*, 1998). Although this might require further detailed analysis, this study clearly indicates that Th1 cells which was highly triggered when inoculated with antigens from symptomatic isolates were able to support B cell clonal expansion and antibody production. Alternatively, it is possible to suggest that Th1 and Th2 cells could have achieved optimal B cells to help in different ways i.e. Th1 cells helping less well on an individual cell basis but proliferating to provide more cells, whereas Th2 cells provide more help per cell but expand less, this in turn creates a net effect amounting to the same. In addition to that, studies had also proved that transition from B cell response to adaptive immunity happens in other parasites infection such as *Helminth*, *Toxoplasma*, *Plasmodium* and *Trypanosomes*. These parasites mediate B cell activation where it up-regulate CCR7 ligand to facilitate the migration toward T lymphocytes cells proliferation (McGovern *et al.*, 2013).

On the other hand, the immunogenic variations between Th1 and Th2 cells in responding to asymptomatic and symptomatic groups may happen in the arrays of receptors on their outer surface that they use to respond to cytokines and other messenger substances (Kid *et al.*, 2003). This was supported by previous evidences that, epitope variations and cross reactivity of *Blastocystis* sp. isolates which exhibited the same morphology but with a different pathogenic prospective (Kukoschke and MuEller, 1991; Lanuza *et al.*, 1999; Mirza *et al.*, 2011). The immunogenicity heterogeneity evaluation through the *in vitro* stimulated splenocyte cytokine analysis implicated that, soluble antigen of symptomatic isolates predominantly increased production of Th1 (IFNγ and IL-2) mediated cytokine response whilst asymptomatic isolates elicited higher Th2 (IL-4) and Treg (IL-10) cytokine response. Symptomatic isolates has triggered inflammatory reactions in animal models with higher pro-inflammatory cytokine secretions.

It was reported that proteins are more immunogenic due to its complexity and heterogeneity which will eventually influence the antigen presentation to the T-helper (Th) cells (Jonathan *et al.*, 2015). T lymphocytes expressing CD4 are known as helper T cells, and these are regarded as being the most prolific cytokine producers. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines (Berger *et al.*, 2000). Th1 immune response is mediated by strong antigenic stimulation while Th2 immune response is mediated by weak antigenic stimulation (Magombedze *et al.*, 2014). Therefore, these results demonstrated stronger antigenic and immunogenic mediation of T lymphocytes cells in symptomatic isolates.

Besides, efficiency of the immunogenicity of an antigen can be evaluated by assessing the proliferation rate of lymphocytes cells upon the exposure to parasite-specific antigens (Chandramathi *et al.*, 2014). Therefore, in this study the *in vitro* effect of solubilised *Blastocystis*-Ag on the proliferation level of lymphocytes isolated from the splenocyte of all the immunised groups were investigated. The stimulation index was calculated as the ratio of the average OD_{450} value of the wells containing antigen stimulated cells to the average value of wells containing cells with medium. Concanavalin-A (Con-A) was used as positive control to look at the effect of *Blastocystis*-Ag towards the growth of lymphocytes which consist of both B cells and T cells based on the previous studies which supported a good binding affinity of *Blastocystis* sp. ST3 towards Con-A (Ragavan *et al.*, 2014). Therefore, the

Table 1. Summary Results

Humoral Antibody Response@ OD42	Symptomatic vs. Asymptomatic
IgG	0.31±0.032*
IgG1	-0.21±0.26*
IgG2a	0.27±0.044*
Cytokine Response (pg/ml)	
Interferon gamma (IFNγ)	270±52.1*
Interleukin-2	223±34.98*
Interleukin-4	-71±8.66*
Interleukin-10	-79±11.8*
Lymphocyte Proliferation Assay	
Stimulation Index	2.23±0.34*

Table 1. Results are mean ± SD values (each group n = 9) for three different isolates; *, P < 0.05. Symptomatic immunised group compared to asymptomatic immunised group.

comparison revealed that lymphocytes isolated from spleen of immunised mice established a higher level of lymphocyte proliferation *in vitro* and showed a better and faster establishment of immune responses against the infection. This observation implies a general perception that both asymptomatic and symptomatic groups induced cellular mediated response. However, the immunogenicity seems higher in symptomatic group. This data was supported by the T helper cell mediated immune response evidences in this study which implicated symptomatic group as the most prolific cytokine producer like summarised in Table 1.

CONCLUSION

In conclusion, the present study has clearly revealed three strong implications regarding ST3 symptomatic and asymptomatic solubilised *Blastocystis*-Ag immunization and *in vitro* assessment. 1) Symptomatic isolates exhibited higher Th1 cytokines which helps in B cell clonal expansion to secrete higher IgG antibodies 2) Both symptomatic and asymptomatic isolates demonstrated a balanced IgG1 and IgG2a immune equilibrium which helps to stimulate the switch to Th1 and Th2 cells clonal expansion 3) Symptomatic isolates induced higher lymphocytes cells proliferation in previously sensitised lymphocytes.

It is possible to hypothesize that surface variations between asymptomatic and symptomatic isolates may have contributed to the distinctive immune response. Symptomatic isolates have been shown to be highly antigenic and immunogenic compared to asymptomatic isolates due to stronger immune response establishment. However, *Blastocystis* sp. isolates from different individuals which exhibiting the same morphology but with no significant differences of immune response potential does exist. This study does contribute that ST 3 from symptomatic and asymptotic individuals elicit distinct immunogenic response and further establish that we should not be too quick to ascribe subtypes to pathogenicity.

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