STUDIES TO ELUCIDATE HOST IMMUNE MECHANISMS INVOLVED IN THE *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC INFECTION

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STUDIES TO ELUCIDATE HOST IMMUNE MECHANISMS INVOLVED IN THE *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC INFECTION

ABSTRACT

Blastocystis sp. is an enteric protozoan parasite of humans and many animals. *Blastocystis* sp. ST3 proves to be the highest frequency case in most populations around the world and it is further distinguished into symptomatic and asymptomatic isolates based on the clinical symptoms exhibited by infected individuals. Phenotypic and genotypic studies implicate the distinctiveness of this parasite. However, the pathogenesis of this parasite is still in a grey area. Therefore, this study was aimed to analyse the immunopathogenesis of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates. The immunopathogenesis of this parasite was analysed by assessing the (1) characteristic of antigen immune response (antigen specificity and diversity) and (2) the modulations of innate and adaptive immune responses. The antigen specificity was evaluated by immunising Balb/c mice with 20µg/ml solubilised antigen. Results has shown pre-dominant of Th1 (IFNγ and IL-2) cytokine response and IgG2a antibody response induced by symptomatic antigen immunised group and pre-dominant of Th2 (IL-4 and IL-10) cytokine response and IgG1 antibody response induced by asymptomatic antigen immunised group which has shown diverse specific immune response. Antigen diversity analysis was performed by co-culturing sera (10-fold dilution) obtained from mice immunised with *Blastocystis* sp. symptomatic and asymptomatic antigens and the respective *Blastocystis* sp. live cells through complement dependant cell cytotoxicity (CDC) assay. The sera (at 10^1 concentrations) from symptomatic and asymptomatic antigen immunised mice were able to specifically lyse the respective live cells with an average percentage of 82% and 86% respectively. There was almost 50% cross-reactivity

observed between *Blastocystis* sp. ST3 isolates origin from the same group which proving high antigen diversity. However, there was only 17% cross-reactivity observed between the sera and cells of different group (symptomatic and asymptomatic isolates). Further *in vitro* studies were carried out to investigate the immune modulation triggered by *Blastocystis* sp. antigens towards antigen presenting cells (macrophages and monocytes). *Blastocystis* sp. induced apoptosis in macrophages as early as 6 hours of incubation while monocytes suppressed the secretions of pro-inflammatory cytokines through increased expressions of PD-1 during short- and long-term antigen-exposure resembling acute and chronic infection respectively. This observation implicates the immunosuppressive features of *Blastocystis* sp. which could be utilised to evade host innate defence mechanisms. Next, the effect of *Blastocystis* sp. antigen on T cell immune modulation (adaptive immunity) was assessed by introducing symptomatic and asymptomatic parasite antigens to the blood mononuclear cells (PBMCs) *in vitro*. The antigens resulted in elevated levels of T cell co-inhibitory molecules and reduced functional T cell pro-inflammatory cytokines (IL-2 and IFNγ) suggesting that the parasite is able to cause T cell 'exhaustion' or dysfunction by symptomatic and asymptomatic antigens at 83% and 94% respectively. This study underscores the importance of identifying the differences of immune responses and immunomodulation mechanisms induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates in a host. The study for the first time, had shed light on the distinct host immune response induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates implicating that these isolates could portrayed different immunopathogenesis in the host intestine.

Keywords: *Blastocystis* sp., symptomatic, asymptomatic, Subtype 3, Immunopathogenesis.

KAJIAN UNTUK MENJELASKAN MEKANISME IMUN HOST YANG TERLIBAT DALAM JANGKITAN *BLASTOCYSTIS* SP. SUBJENIS 3 SIMPTOMATIK DAN ASIMPTOMATIK

ABSTRAK

Blastocystis sp. adalah sejenis parasit protozoa enterik pada kebanyakan haiwan dan manusia. *Blastocystis* sp. ST3 terbukti mempunyai kes kekerapan paling tinggi berbanding populasi lain di seluruh dunia. Spesis ini dibezakan ke dalam isolat simptomatik dan asimptomatik berdasarkan gejala klinikal yang dipamerkan oleh individu yang telah dijangkiti. Kajian fenotip dan genotipik menunjuk keterasingan parasit ini. Walaubagaimanapun pathogenesis parasit ini tidak diketahui. Oleh itu, pathogenesis imun parasit simptomatik dan asimptomatik isolat telah diterokai dalam kajian ini. Pathogenesis imun parasite dinilai berdasarkan (1) ciri-ciri tindak balas imun antigenik (kekhususannya dan kepelbagaiannya) (2) modulasi imun antigenik. Kehususan antigen dari parasit ini telah dianalisis melalui imunisasi tikus Balb /c dengan 20 μg / ml antigen solubilised telah menunjukkan tindak balas pre-dominan sitokin Th1 (IFNγ dan IL-2) dan antibodi IgG2a yang disebabkan oleh kumpulan imunisasi antigen simptomatik dan pra- dominan tindak balas sitokin Th2 (IL-4 dan IL-10) dan antibodi IgG1 yang disebabkan oleh kumpulan imunisasi antigen asimtomatik yang menunjukkan tindak balas imun spesifik yang berbeza. Analisis lanjut mengenai kepelbagaian antigen parasit ini dilakukan dengan mengkonsultasikan sera (pencairan 10 kali ganda) yang diperoleh dari tikus yang diimunisasi dengan *Blastocystis* sp. antigen simtomatik dan asimtomatik dan *Blastocystis* sp. sel hidup melalui ujian sitotoksisiti sel bergantung kepada pelengkap (CDC). Sera (pada kepekatan 10^1) dari tikus imunisasi simtomatik dan asimtomatik dapat memecahkan sel-sel hidup dengan purata peratusan sebanyak 82% dan 86% masing-masing. Terdapat hampir 50% reaktiviti silang yang diperhatikan di antara *Blastocystis* sp. ST3 mengasingkan asal dari kumpulan yang sama yang membuktikan kepelbagaian

antigen yang tinggi. Selain itu, terdapat hanya 17% tindak balas silang yang diperhatikan di antara sera dan sel-sel kumpulan yang berlainan (isolat simtomatik dan asimtomatik). Kajian *in vitro* secara lanjutan telah dijalankan untuk menyiasat tindak balas imun yang dicetuskan oleh antigen *Blastocystis* sp. terhadap sel penyerahan antigen (makrofaj dan monosit). *Blastocystis* sp. menginduksi apoptosis dalam makrofaj pada tempoh pengeraman seawal 6 jam, manakala monosit menyekat rembesan sitokin keradangan melalui peningkatan ekspresi PD-1 semasa pendedahan jangka pendek dan jangka panjang yang menyerupai jangkitan akut dan kronik. Pemerhatian ini melibatkan ciri-ciri imunosupresif *Blastocystis* sp. yang boleh digunakan untuk mengelakkan mekanisme pertahanan semula jadi perumah. Seterusnya, kesan antigen *Blastocystis* sp. pada modulasi imun sel T (imuniti adaptif) telah dinilai dengan memperkenalkan parasit simptomatik dan asimptomatik antigen ke sel mononuclear darah (PBMCs) secara *in vitro*. Antigen telah menghasilkan molekul dan sitokin (IL-2 and IFNγ) yang menghalang sel T menunjukkan bahawa parasit boleh menyebabkan 'keletihan' atau disfungsi sel T. Buat pertama kalinya, kajian ini memberi gambaran jelas mengenai tindak balas imun perumah yang diakibatkan oleh isolat simptomatik dan asimptomatik *Blastocystis* sp., ST3 yang menunjukkan bahawa isolat-isolat ini boleh menggunakan mekanisme modulasi imun yang berlainan dalam menjelajahi usus perumah.

Kata kunci: *Blastocystis* sp., simptomatik, asimptomatik, subjenis 3, pathogenesis imun.

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LIST OF SYMBOLS AND ABBREVIATIONS

For example:

%	:	Percentage
°C	:	Degree Celsius
α	:	Alpha
β	:	Beta
µL	:	Microliter
µM	:	Micro Molar
ANOVA	:	Analysis of Variance
ATCC	:	American Type Cell Culture
BSA	:	Bovine Serum Albumin
C-1	:	Complement Component-1
CCR-6	:	Chemokine Receptor-6
CD3	:	Cluster of Differentiation 3
CD4	:	Cluster of Differentiation 4
CD8	:	Cluster of Differentiation 8
cDNA	:	Complementary DNA
DC	:	Dendritic Cells
DMSO	:	Dimethylsulphoxide
DNA	:	Deoxyribonucleic Acid
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme Linked Immunosorbent Assay
FBS	:	Fetal Bovine Serum
g	:	Gram
h	:	Hour

HRP	:	Horseradish Peroxide
MAP	:	Mitogen Activated Protein
mg	:	Milligram
MHC	:	Major Histocompatibility Complex
min	:	Minute
ml	:	Millimeter
NaCl	:	Sodium Chloride
NF-κB	:	Nuclear Factor Kappa B
nM	:	Nano Molar
NO	:	Nitric Oxide
OD	:	Optical Density
PBS	:	Phosphate Buffered Saline
pH	:	Power of Hydrogen
rpm	:	Revolutions Per Minute
RPMI	:	Roswell Park Memorial Institute
SD	:	Standard Deviation
sec	:	Second
SEM	:	Scanning Electron Microscopy
SPSS	:	Statistical Package for the Social Sciences
ST	:	Subtype
v/v	:	Volume over Volume
w/v	:	Weight over Volume
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Research Background

1.1.1 Protozoan Parasites and Immune System

Parasitic infections caused by helminth and intestinal protozoan parasites such as *Entamoeba histolytica*, *Giardia intestinalis*, *Cyclospora cayetanenensis*, *Cryptosporidium* spp and *Blastocystis* sp. are among the most prevalent infections in humans across many developing countries (Haque, 2007). Furthermore, intestinal parasites have proven to cause considerable morbidity and mortality by causing major human health problems worldwide. In general, an intestinal parasite causes damage and inflammation in the intestinal epithelial region and at the same time it obtains sustenance from its host (Allen and Sutherland, 2014). However, there are some parasites such as *Entamoeba coli* which can be a commensal where they neither benefit nor harm their colonized host (Lukeš et al., 2015). Parasites have developed various mechanisms to evade or exploit the host's immune response and establish infection in the human intestinal region for long durations (Boorom et al., 2008; Roberts et al., 2014). There are many *in vivo* studies conducted on animals such as rodents, which were used to investigate the host-parasite interactions. Besides, many *in vitro* studies were focused on investigating the immunoregulation of parasites on host immune cells. The *in vitro* analysis was mainly focused on cytokine profile analysis and immune cell modulations that were linked to the various immune evasion pathways inflicted by the parasites (Faubert, 2000; Maizels and McSorley, 2016; Nakada-Tsukui and Nozaki, 2016).

1.1.2 Intestinal Parasites and Antigenic Variations

There are various mechanisms that allow parasite resistance to the host immune response. For instance, studies have suggested that **antigenic variations** were presented by most of human intestinal parasites such as *Giardia lamblia*, *Entamoeba histolytica* and helminth. Antigenic variation was used as one of the strategies by these parasites to evade the host immune system by causing **host immune diversity** (Bhattacharya et al., 1992; Svärd et al., 1998; Yason and Tan, 2018) which may cause host immune system breakdown due to the ability of the parasites to manipulate the host immune system (Ulrich and Schmid-Hempel, 2012). Moreover, studies have proven that antigenically different surface molecules on parasites possibly lead to generations of chronic and recurrent infections due to expansion of host immune diversity in recognizing the antigen (Deitsch et al., 2009). Hence antigenic variations are contributing to the **"pathogenesis"** of a parasitic infection. Therefore, these protozoan intestinal parasites are able to reside in the host for a long period by evading the host immune system recognitions which includes innate and adaptive immune responses as shown in Figure 1.1 below.

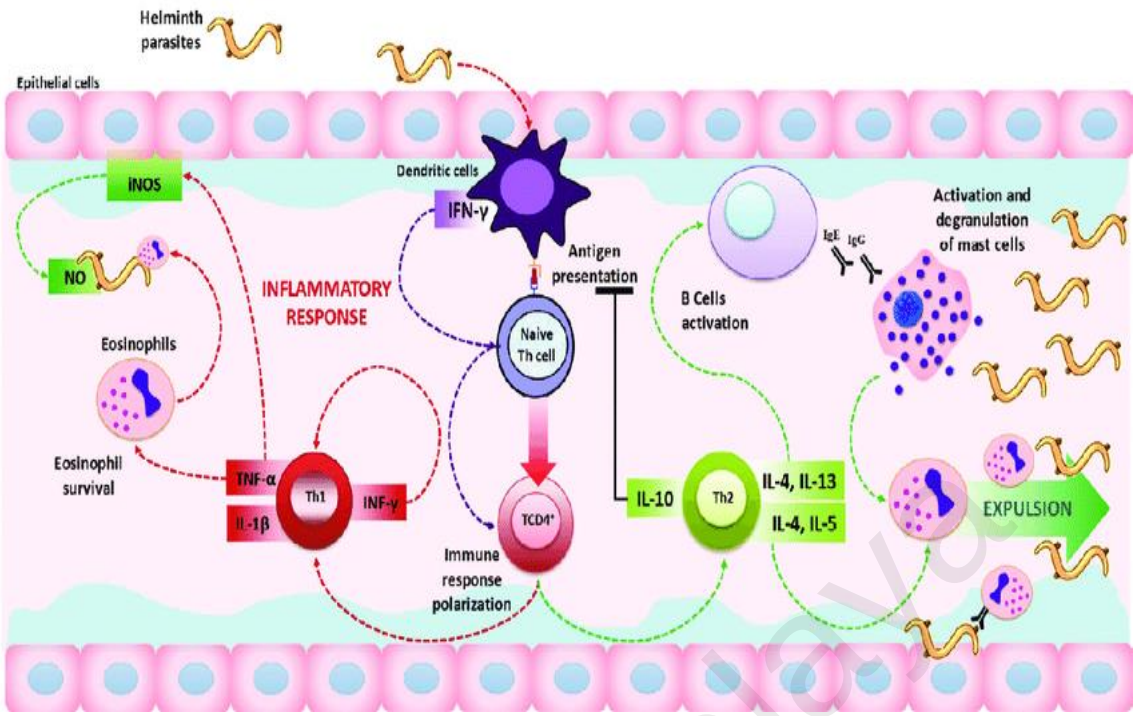


Figure 1.1: The Immune Response Induced by Intestinal Parasite helminth.

(Source: José Luis Muñoz-Carrillo et al., 2018)

1.1.3 *Blastocystis* sp.

Blastocystis sp. is an unusual enteric protozoan parasite in humans and many animals. It has a worldwide distribution and classified as the most commonly isolated organism in parasitological stool surveys. Studies have demonstrated that *Blastocystis* sp. is restricted, with large knowledge gaps especially in our understanding of the parasite's life cycle, transmission mechanisms, incubation period, epidemiology, and treatment options. Since the early 1900s, this parasite has been widely investigated. However, awareness on the biology of this protozoan parasite was only more towards the last decade of the century (Tan, 2008 and Paulos et al., 2018). Number of reports have suggested that *Blastocystis* sp. could be the causative agent of a various gastrointestinal diseases such as diarrhea, colitis, enteritis, irritable bowel disease (IBD) and irritable bowel syndrome (IBS) (Boorom et al., 2008 and Abdul Rani et al., 2016). Humans are prone to *Blastocystis* sp. infections of various subtypes such as subtype 1, 2, 3, 4, 5, 6, 7 and 8. These subtypes can be identified by performing polymerase chain reaction (PCR) based on their genotypes

classifications (Malheiros et al., 2011). However, ST3 seen to be the most frequent subtype in most populations around the world (Figure 1.2).

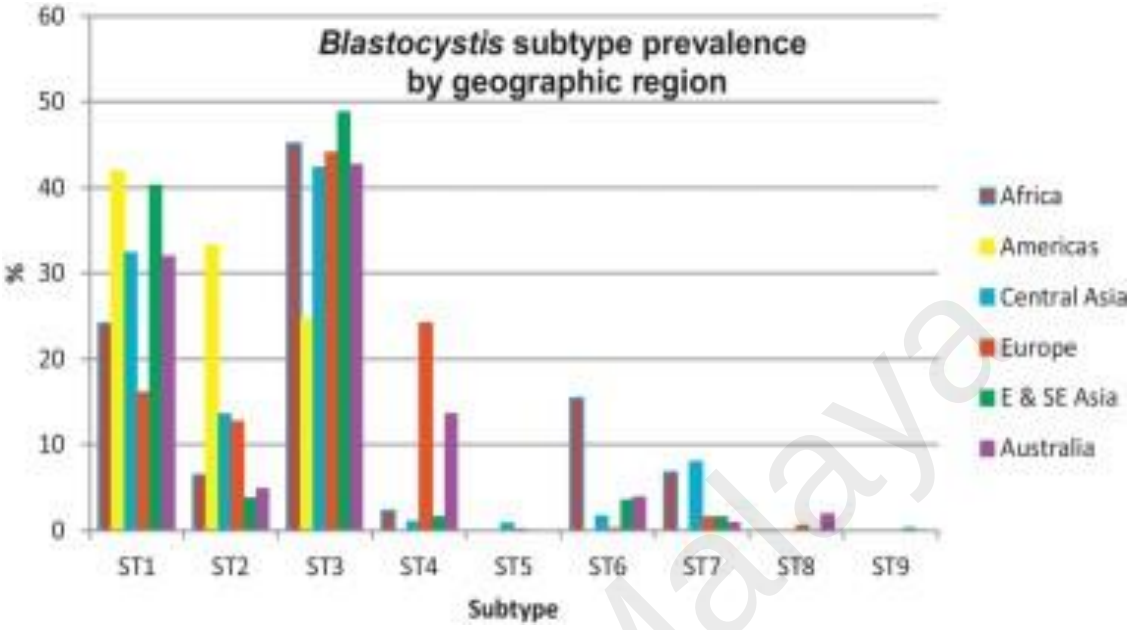


Figure 1.2: Geographical Distributions of *Blastocystis* sp. Infection in Human.

(Source: Alfellani et al., 2013)

1.1.4 *Blastocystis* sp. ST3 Classifications

Blastocystis sp. is highly prevalent among healthy populations and may exist in the gut for years in certain individuals by remaining undiagnosed. This is due to the characteristics of this parasite which can remain silent without causing its usual associated symptoms such as flatulence, bloating, and abdominal discomfort to the infected individuals (Boorom et al., 2008). Therefore, *Blastocystis* sp. infection in humans is clinically distinguished as symptomatic and asymptomatic. However, the probability of symptomatic infection is generally higher compared to asymptomatic infection as reflected in Figure 1.3 below. Studies have suggested that ST3 is the only subtype of human origin because this parasite is predominantly infecting human rather than animals (Tan, 2008 and Noradilah et al., 2017).

Subtype	Symptomatic no. (%)	Asymptomatic no. (%)	Total no
ST 1	43 (89.6 %)	5 (10.4 %)	48
ST 2	5 (71.4 %)	2 (28.6 %)	7
ST 3	33 (62.3 %)	20 (37.7 %)	53
ST 5	20 (60.6 %)	13 (39.4 %)	33
ST 1, 2	1 (100.0 %)	0 (0.0 %)	1
ST 1, 3	13 (92.9 %)	1 (7.1 %)	14
ST 1, 5	4 (100.0 %)	0 (0.0 %)	4
ST 2, 3	1 (50.0 %)	1 (50.0 %)	2
ST 3, 5	3 (75.0 %)	1 (25.0 %)	4
ST 1, 2, 3	3 (75.0 %)	1 (25.0 %)	4
ST 1, 3, 5	4 (100.0 %)	0 (0.0 %)	4

Figure 1.3: *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Distributions.

(Source: Moosavi et al., 2012)

1.1.5 *Blastocystis* sp. ST3 Pathogenicity

The pathogenesis of *Blastocystis* sp. in human hosts has always been a matter of debate because when it is present, it may or may not trigger symptoms in infected individuals. Due to this fact, many studies have been carried out to investigate the possible mechanisms of pathogenesis among symptomatic and asymptomatic *Blastocystis* sp. specific to ST3 isolates. A study carried out by (Tan et al., 2008; Ragavan et al., 2014) speculated symptomatic isolates of ST3 to be highly pathogenic due to their greater size, higher binding affinity and rougher surface structure compared to asymptomatic isolates. Besides, many studies have proven that, there are differences between symptomatic and asymptomatic isolates in terms of protease activity, immune response against cancer cell line induction, caspase activity and apoptosis detection that may describe their pathogenesis (Rajamanikam and Govind, 2013; Balakrishnan and Kumar, 2014; Kumarasamy et al., 2017).

1.2 Justifications of This Study

This study was aimed to investigate the **"immunopathogenesis"** of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates by analysing the immune responses of this parasite as well as describe its pathogenicity. Immunopathogenesis is defined as; "the study of an organism pathogenicity by analyzing the pathogen immune responses". Pathogenicity is defined as "the ability of an organism to cause a disease". The pathogenicity of the *Blastocystis* sp. ST3 has been a matter of debate whereby studies such as surface structure variances (antigenic variances) between symptomatic and asymptomatic isolates were used to describe the pathogenicity of this parasite as stated in 1.1.5 above. However, the pathogenicity of this isolates is still in a grey area as the biological mechanisms in causing pro-longed infection is not known. Furthermore, antigenic variations of a pathogen may contribute to disease progression (pathogenicity) in the host. This scenario will eventually cause host immune system impairment due to

immune diversity caused by the pathogen in the host as mentioned in 1.1.2 above. This describes the correlations between pathogenicity, antigenic variations and immune system as shown in Figure 1.4 below.

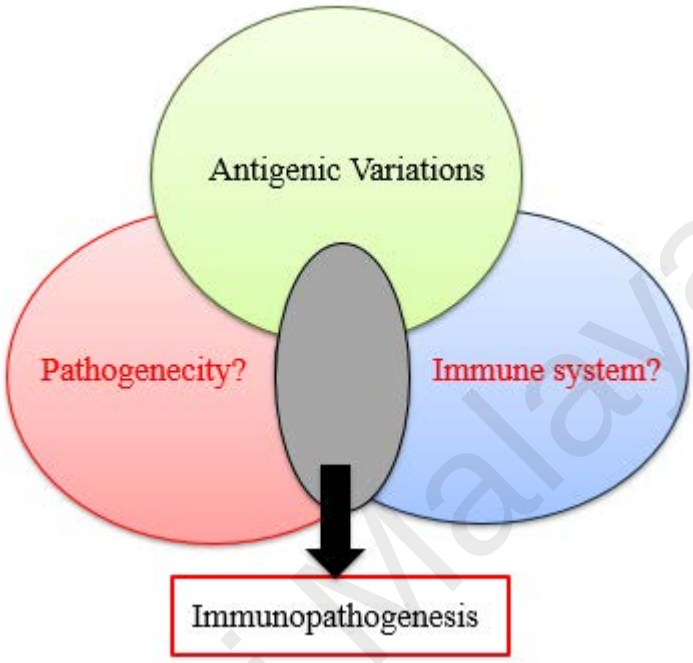


Figure 1.4: The Correlations Between Pathogenicity, Antigen Variation and Immune System of *Blastocystis* sp. ST3.

Therefore, to identify the pathogenicity of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates, it was imperative to investigate the immunopathogenesis of this parasite by analysing the immune system which includes innate and adaptive immune responses involved against this parasite. Hence, the immune response of this parasite was analysed by using two (2) major criteria(s). **The FIRST CRITERIA** was to analyse **"the characteristic of antigen immune response"** against this parasite and the **SECOND CRITERIA** was to analyse **"the modulations of innate and adaptive immune responses"** induced by this parasite. Therefore, to achieve these criteria (s), standardised solubilised antigen concentration of this symptomatic and asymptomatic isolates of this parasite was used to perform the immune response analysis. The *in vivo* host immune response analyses were performed by using mice model, and subsequently *in vitro*

analysis were performed by using primary cell lines derived from animal and human. Following are the study justifications of the specified criteria (s).

1.2.1 Part 1: Characteristic of Antigen Immune Response (Adaptive Immune Response)

In this study the characteristic of antigen immune response against *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates was analysed to enable us to further understand the immunopathogenesis of this parasite. The characterisation of the antigen immune response was evaluated by focusing on the analysis of the host adaptive immune response (s). Generally, there are three important criteria's that are needed to characteristics the adaptive immunity such as **(1)** the ability of the immune system to differentiate between the self and non-self antigens **(2)** the ability of immune cell to act specifically against an antigen and **(3)** the ability of the immune cells to respond faster against an antigen (memory immune cells) upon multiple antigen exposure. Previous studies have demonstrated surface structure and binding affinity differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic individuals which became the basis of pathogenicity differences (Tan et al., 2008; Ragavan et al., 2014). These studies have confirmed that, there are antigenic variations possessed by symptomatic and asymptomatic isolates. Furthermore, antigenic variations are one of the strategies used by many pathogens including intestinal protozoan parasites such as *Giardia lamblia* and *Entamoeba histolytica*. Surface structural variances are known to exploit the recognition of immune cells against an antigen. This allows the pathogen to persist and infect the previously infected (pre-immune) host. Therefore, the antigenic variation between symptomatic and asymptomatic isolates was evaluated by assessing the antigen **specificity** and **diversity**.

1.2.1.1 Antigen Specificity

Antigen specificity refers to the ability of the host immune cells such as B and T lymphocytes cells to recognise the epitope or cell surface molecule of an antigen in a specific manner. The interactions between B and T lymphocytes cells against and antigen can be achieved through the analysis of host immune response after several antigen exposures. In order to correlatively compare the antigen specificity between symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates, the host immune responses against an equal concentration of these isolates are important to be determined. To date, there is only one study available pertaining to infected host immune response against *Blastocystis* sp. ST3 which analysed the IgG and IgA host antibody responses (Mahmoud et al.,2003). In this study the antigen specificity of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates was evaluated by immunising Balb/c mice with specified concentration of the solubilised antigens. The adaptive B and T cell immune responses were evaluated by performing *in vitro* biological sample analysis (sera and spleen obtained from the immunised Balb/c mice).

1.2.1.2 Antigen Diversity

Mirza et al., (2011) proved that, there was antigenic heterogeneity of protein profiles obtained from patients infected with symptomatic *Blastocystis* sp. isolates (not specific to ST3) and it was concluded that, each isolate may display a different pathogenic role. However, the heterogeneity profiles of *Blastocystis* sp. ST3 isolates have yet to be discovered. Therefore, it becomes imperative to investigate the degree of antigen diversity among the symptomatic and asymptomatic isolates of *Blastocystis* sp. ST3 to identify its uniqueness. Hence, to confirm the degree of antigen divergence among symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates, this study was further substantiated by the observation of complement mediated cytotoxicity (CDC) assay. CDC assay is a mechanism which antibodies lyses the targeted cells by activating a cascade of

complement-related reactions. Therefore, the CDC assay was performed by co-culturing the sera (obtained from the immunised mice as mentioned in 1.2.1.1 above) with the live *Blastocystis* sp. ST3 symptomatic and asymptomatic cells. The antigenic diversities were determined based on the degree of cell lysis obtained among these isolates.

1.2.2 Part 2: Innate and Adaptive Immune Modulation

The connections between the host immune system and the invading pathogens start when a pathogenic microorganism infects the human body. This is followed by the activation of innate and adaptive immune mechanisms based on the virulence factors of the invading pathogens. The results from the battle between pathogens and innate immune cells will eventually determine the host recovery or pathogen survival at initial stage of its infection. During a pro-longed infection, many pathogens set up diverse adaptive immune modulation tactics such as **(1)** down-regulation of protective immunity Th1 **(2)** skewing the immune response towards non-protective such as Th2 response **(3)** generating anti-inflammatory immune responses such as IL-10 and **(4)** displaying multiple immune co-inhibitory molecules such as PD-1, CTLA-4 (McNeilly et al.,2014) in the infected host in order to achieve cell invasion and colonization through immune evasions. As a result, exploitation of the host cells occurs by enabling the pathogens to access the targeted areas.

Moreover, further deteriorations of immune system may cause fatality to the host due to the failure in recognizing the antigen. There were many studies demonstrating the innate and adaptive immune evasions inflicted by intestinal protozoan parasites during its pro-longed infection (Faubert, 2000; Maizels and McSorley, 2016; Nakada-Tsukui and Nozaki, 2016). This includes *Blastocystis* sp. which is also reported to establish its infection in the host for a long period (Boorom et al., 2008; Roberts et al., 2014). However, the immune modulations of innate and adaptive immunity inflicted by this

parasite have yet to be discovered. Therefore, the immune modulation analysis of this parasite was initiated by assessing the adaptive and innate immune responses.

1.2.2.1 Innate Immune Modulation: Antigen Presenting Cells

There are close interactions between adaptive and innate immune responses in eradicating pathogens. The innate immune system connects to adaptive immunity through an antigen presenting cells (Rivera et al., 2016). Antigen presenting cells such as macrophages play a major role in influencing the T cell immune response through its cytokine modulations (Muraille et al., 2014). A previous study carried out by (Lim et al., 2014) has shown that macrophages induced with *Blastocystis* sp. whole cell lysate of ST4 and 7 exacerbated various pro-inflammatory cytokines responses. There are instances where the cytokine modulations of macrophages resulted in immune evasion of parasites which caused a progression of the infections (Singh and Agrewala, 2006; Kaiko et al., 2008). Therefore, in this study the immune response of macrophages, mouse derived (RAW 264.7) and human THP-1 derived macrophages were analyzed by inducing the cells with symptomatic and asymptomatic ST3 solubilised antigens. Further analysis on the detrimental effects of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates on innate immune response was observed. Apart from that, monocytes (antigen presenting cells) which is another arm of innate immune response against this parasite was also studied.

Report has shown that, monocytes increased and exhibited toll like receptors upon induction with *Blastocystis* sp. (not specific to ST3) (Lim et al., 2014). Monocytes are one of the first cells after neutrophils and macrophages to be recruited to the infection area during acute infection (Ingersoll et al., 2011). During a chronic or pro-longed pathogenic infection, monocytes initiate the activation of T cells which is an arm of adaptive immune system to eradicate the infection. Researchers had proven that,

monocytes may contribute to the pathogenesis of an infection and are associated with chronic intestinal inflammatory diseases which is associated with *Blastocystis* sp. infection such as IBD, Crohn's and colorectal cancer (Mazlam and Hodgson, 1992; Rugtveit et al., 1994; Shibutani et al., 2017). In order to gain better insights on the innate immune modulations caused by this parasite in the infected host, further analysis was performed to investigate the monocytes response during the short and pro-longed *Blastocystis* sp. infection by inducing *Blastocystis* sp. antigens on THP-1 monocytic cell line and human PBMCs derived monocytes (CD14+). These inductions were incubated for 3 and 6 days *in vitro* to mimic the short- and long-term infection respectively in host.

1.2.2.2 Adaptive Immune Modulation: T cells

Generally, during a chronic or pro-longed pathogenic infection, monocytes initiate the activation of T cells which is an arm of adaptive immune system to eradicate the infection. *Blastocystis* sp. transmits the capability of causing a pro-longed inflammation in the intestine by suppressing the host adaptive immune system (Roberts et al., 2014; Tan, 2008; Boorom et al., 2008). Adaptive immune cell particularly T cells, was suggested to augment pathogenesis of a disease or infection by infiltrating into the targeted tissue and exacerbate damage (Burger, 2000; Burger and Dayer, 2002). Many studies have shown the involvement of T cells during *Blastocystis* sp. infections. Studies have shown that rat induced with *Blastocystis* sp. (not specific to ST3) significantly up-regulated Th1 and Th2 cytokine responses (Roberts et al., 2014; Santos et al., 2009). Furthermore, studies have shown that colorectal cancer cell lines induced with *Blastocystis* sp. solubilised antigens up-regulated Th2 cytokines gene expressions. This result has postulated host immune cells suppression caused by *Blastocystis* sp. due to high secretions of anti-inflammatory cytokines (Chandramathi et al., 2010; Vinoth et al., 2013). Generally, in chronic infections, T cells are exposed to persistent antigen and/or inflammatory signals. This scenario is often associated with the deterioration of T cell function, a state called

"exhaustion". Exhausted T cells lose robust effector functions, express multiple immune co-inhibitory molecules and are defined by an altered transcriptional programme. Therefore, to confirm the T cell immune modulations during pro-longed *Blastocystis* sp. ST3 symptomatic and asymptomatic infection, an *in vitro* PBMC model was set up to study the T cell response induced by solubilized antigen of this parasite.

1.2.3 Objectives

The main objectives of this study include:

1. To study the Balb/c mice immune response against *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens.

    1.1 To elucidate the difference in T and B lymphocyte responses (if any) towards symptomatic and asymptomatic antigens of *Blastocystis* sp. ST3.

    1.2 To elucidate the antigenic differences and cross-reactivity among the symptomatic and asymptomatic antigens of *Blastocystis* sp. ST3.

2. To evaluate macrophage immune response against *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen *in vitro.*

3. To evaluate monocyte immune response against *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen during short- and long-term induction *in vitro*.

4. To evaluate the exppressions of immune co-inhibitory molecules in T cells targeting (T helper (CD4+) and T cytotoxic (CD8+) populations) upon *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen short- and long-term induction *in vitro.*

1.2.4 Study Flow Chart

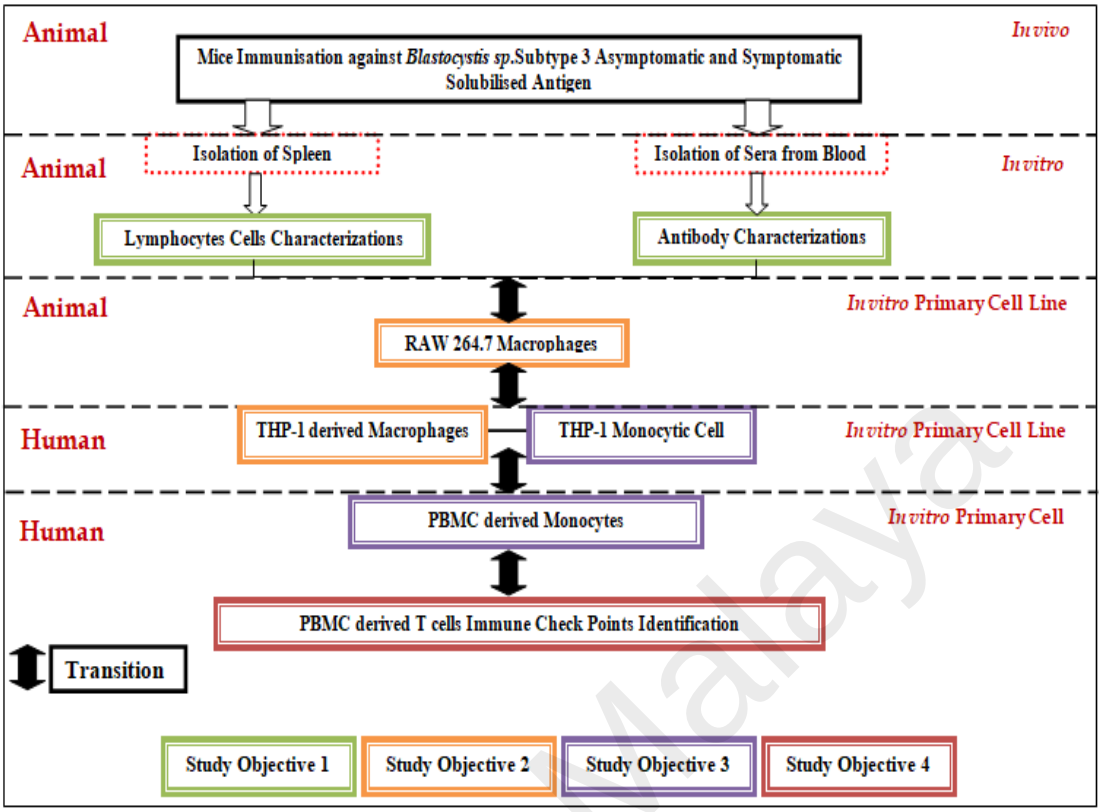


Figure 1.5: Overview of Study Flow Chart.

CHAPTER 2: LITERATURE REVIEW

2.1 Background on *Blastocystis* sp.

2.1.1 Origin

Blastocystis sp. is an anaerobic parasite commonly found in the intestinal tract of humans and various animals (Wawrzyniak et al., 2013). It is classified into stremenophiles. Stramenopiles are defined, based on molecular phylogenies, as a heterogeneous evolutionary assemblage of unicellular and multicellular protist (Wawrzyniak et al., 2013; Denoeud et al., 2011). *Blastocystis* sp. is the most frequently isolated parasite, and it was reported that the prevalence of this parasite ranges from 1.5 to 15% in developed countries, and from 30 to 50% in developing countries (Wu et al., 2014). However, prevalence in underdeveloped countries is found to be the highest. Poor hygiene, consumption of contaminated food or water and contacts with animals inadvertently, facilitate the transmission of this parasite via fecal oral route (Abdulsalam et al., 2013; Duda et al., 2015; Rebolla et al., 2015).

2.1.2 Phenotypic

Interestingly, wide genetic divergence has been observed among numerous isolates from both humans and animals. *Blastocystis* sp. in humans comprises at least 9 ribosomal lineages where 8 of which can be found in other animals as well (Abu-Madi et al., 2015). *Blastocystis* sp. is classified into four major morphologies namely; vacuolar, granular, ameboid and cyst in stools or in vitro cultures as shown in Figure 2.1. However, vacuolar and granular forms are commonly observed in laboratory cultures and stool samples (Tan, 2008; Wawrzyniak et al., 2013). Studies analysed on the phenotypic variations between asymptomatic and symptomatic isolates of *Blastocystis* sp. ST3 concluded that, there are phenotypic differences among asymptomatic and symptomatic isolates (Böhm-Gloning et al., 1997; Ragavan et al., 2014).

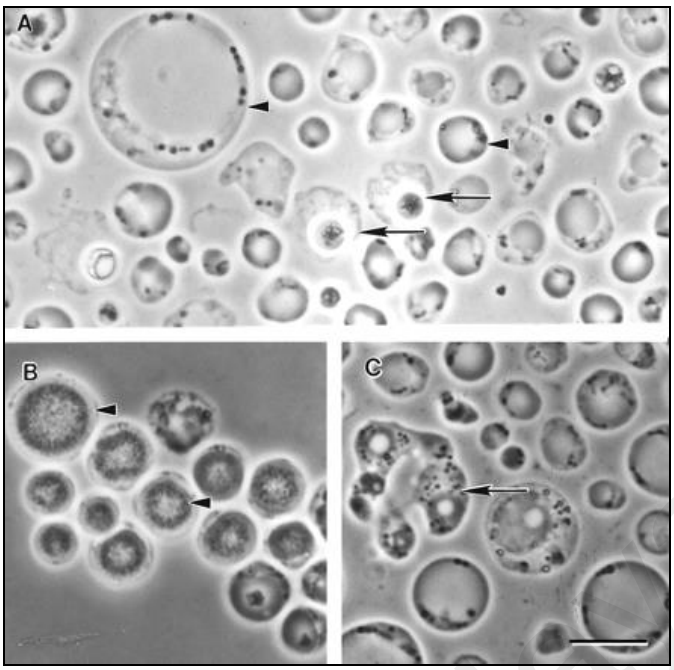


Figure 2.1: Morphological Forms of *Blastocystis* sp. ST4 by Phase-Contrast Microscopy.

(A)Vacuolar and fecal cyst forms *in vitro* axenic culture displaying extensive size variation (arrowheads). Note the refractile appearance and loose outer coat of cysts (arrows). (B) Granular form with distinct granular inclusions within the central vacuole (arrowhead). (C) Amoeboid forms occasionally seen in culture showing pseudopod-like cytoplasmic extension (arrow). Bar,10uM. (Source: Tan, 2008).

2.1.3 Classifications

Based on the study population, *Blastocystis* sp. is classified into nine subtypes (ST); ST1 to ST9 were found in human stool samples with ST1 to ST4 being the most common subtype. However, ST3 is appears as the predominant subtype in the human population study. Even though the phylogenetic studies have proved that ST3 originated from humans, recent publications have demonstrated that ST3 origin is not only limited to human but also from animals (Abu-Madi et al., 2015). On the other hand, another study has concluded that ST3 has been identified as the most dominant genotype has shown an increase of 41.7% to 92.3% among human isolates from four countries, whereas it was

stated that the infection is uncommon among animals, suggesting the route of transmission could be from humans to animals (Yoshikawa et al., 2004). Besides, another study also proved that out of 13 subtypes, *Blastocystis* sp. ST3 shown to be the highest in countries like Thailand, Egypt, Singapore and Turkey with prevalence rates between 41.7-92.3%, 54.55%, 78% and 75.9% respectively (Ragavan et al., 2014).

2.1.4 Pathogenicity

It is proven that pathogenicity is closely associated with the immune system. The pathogenicity of *Blastocystis* sp. is not proven conclusively. Its disease attributes can be divided into symptomatic and asymptomatic carriers (Ragavan et al., 2014). Common disease manifestations related to *Blastocystis* sp. are diarrhea, abdominal pain, vomiting and other gastrointestinal symptoms. In rare occasions, urticaria may also surface (Beyhan et al., 2015). Previous studies had highlighted *Blastocystis* sp. ST3 isolates as highly associated with IBS and acute urticaria. The association of these diseases also has been linked to the serine proteases which cause abdominal aches, muscular contractions and widespread pain. In addition, ST3 is reported to contain high level of serine protease which makes it highly pathogenic. Occurrences of fewer or total absence of symptoms were also detected in asymptomatic *Blastocystis* sp. carriers. For instance, Shlim et al., (1995) has proven that out of 189 populations of Nepal expatriates who 30% were having diarrhea and detected positive for *Blastocystis* sp. However, 36% of the infected individuals showed no symptoms. It was concluded that, that the presence of *Blastocystis* sp. infection in asymptomatic carriers is almost proportionate when compared to symptomatic carriers.

2.1.5 Life Cycle

Thus far, conflicting life cycles have been suggested by previous researchers (Stenzel and Boreham, 1996; Tan, 2004). This is due to the propositions of multiple reproductive processes presented by *Blastocystis* sp. (Singh et al., 1995; Govind et al., 2002) as shown in Figure 2.2. There were many models were proposed which includes schizogony, (Singh et al., 1995), plasmotomy (Tan, 2007), endodyogeny (Zhang et al., 2007), and reproduction involving the formation of sac-like pouches (Govind et al., 2002). Furthermore, it was proven by researchers that *Blastocystis* sp. possess either thick and/or thin-walled cysts. Finally, it was implied to be involved in external transmission in in vivo (Singh et al., 1995). Nevertheless, due to the indecisive modes of reproduction binary fission is accepted as a mode of reproduction for *Blastocystis* sp. Besides that, it was proposed that the life cycle of *Blastocystis* sp. is also associated with animals. Therefore, it was suggested that, humans are vulnerable hosts to various types of zoonotic subtypes. Besides, the most possible route of *Blastocystis* sp. infection is through the ingestion of contaminated food or water contaminated with fecal components contains viable *Blastocystis* sp. cysts which were proven through research analysis (Leelayoova et al., 2008). However, pathogenicity of this parasitic infection may vary in different host due to its genetic diversity.

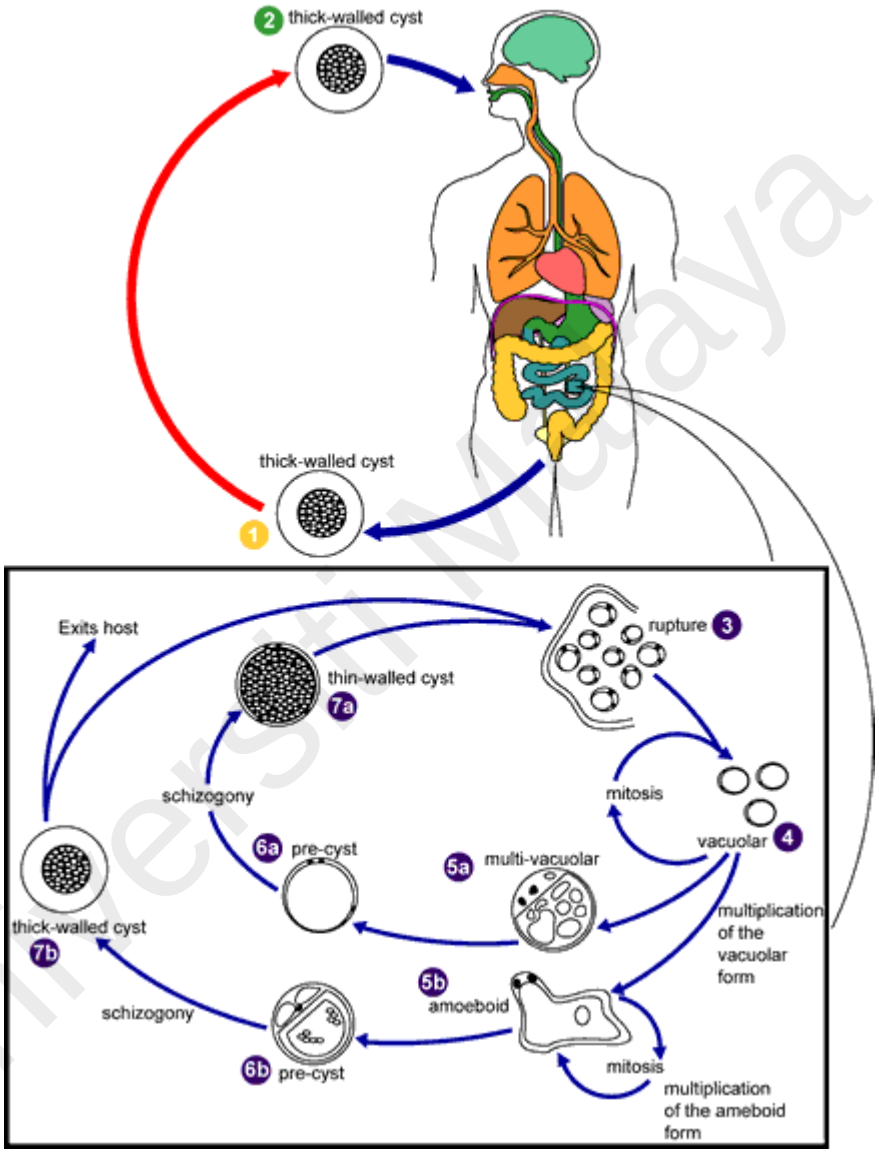


Figure 2.2: Life Cycle of *Blastocystis* sp.

(Source: Centers for Disease Control and Prevention)

2.1.6 Disease Spectrum of *Blastocystis* sp.

Generally, *Blastocystis* sp. infection has been associated with various intestinal and autoimmune diseases such as inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), chronic diarrhea, colorectal cancer and urticaria (Lepczyńska et al., 2017). A study has concluded that solubilised antigen of *Blastocystis* sp. derived from symptomatic isolates was more pathogenic and possess the ability to weaken the cellular immune response compared to the asymptomatic isolates (Chan et al., 2012). Pro-inflammatory and anti-inflammatory cytokines were found to be secreted during the *Blastocystis* sp. infection (Teo et al., 2014; Kumarasamy et al.,2017). Recent studies stated that, antigen isolated from a symptomatic individual was found to be more pathogenic compared to asymptomatic isolates as it caused inflammation when reacted against cancer cells (Kumarasamy et al., 2017). Besides, in the previous reported studies, antigen isolated from a symptomatic individual was found to be more pathogenic compared to asymptomatic isolates, as it caused a more extensive inflammatory reaction as well as more enhanced proliferation of cancer cells (Chan et al., 2012).

2.1.7 *Blastocystis* sp. Excretory or Secretory Products

Proteases are important in order to carry out biological processes such as apoptosis, cell cycle progression and cell migration in normal cells (McKerrow et al., 2006; Puthia et al., 2008). Generally, during parasitic infections, protease may exacerbate the disease pathogenesis by involving parasite-host invasion through the parasite migration. The parasite migrations are characterised as migration of the parasites through tissue barriers, degradation of hemoglobin and other blood proteins, immune evasions and activation of inflammation (Yang et al., 2015). Studies have proven that, proteases played major roles in protozoan parasite pathogenesis where it has been reported to induce pro-inflammatory cytokines secretions in the infected individuals (Puthia et al., 2008; McKerrow et al., 2006; Sajid et al., 2002) such as gastrointestinal inflammatory associated diseases which

indicate the presence of virulence factors and host responses against it (Puthia et al., 2008; Stensvold et al., 2007; Tan, 2004). Furthermore, evidences have shown that the protease activity against *Blastocystis* sp. and its capability in degrading the human secretory immunoglobulin A (IgA) which enable it to have a high virulence and invasion in the infected individuals by changing the immunological responses and disruption of the intestinal barrier function (Sio et al., 2006; Rajamanikam et al., 2013).

A study carried out by (Abdel-Hameed et al., 2011) reported that ST3 *Blastocystis* sp. contained the highest protease activity. Besides, a study to compare the protease activity among symptomatic and asymptomatic isolates has reported that the protease activity is higher in symptomatic isolates. On the other hand, cysteine-type activity of protease was found high in *Blastocystis* sp. infected symptomatic patients (Rajamanikam et al., 2013; Abdel-Hameed et al.,2011). Cysteine protein namely cathepsin B and a legumain, were identified in *Blastocystis* sp. culture which is also found to be immunogenic. These evidences on the protease activity in *Blastocytis* sp. have supported the suggestions of immune response divergence by the isolates.

2.2 Immune System

2.2.1 Innate Immunity

Innate immune system is an immediate non-specific defence against microorganisms and not involving immunological memory as shown in Figure 2.3. The innate immune system is consisting of barriers such as surface mucous layers, chemical influences such as pH and secreted fatty acids which are able to provide effective defence against invasion by many micro-organisms (Mogensen, 2009; Dwivedy et al., 2011). Innate immunity is the early phase of immune response where the immune system utilizes the phylogenitically conserved receptors to recognise and react against varieties of antigens. This results in a quick activation of the immune system cells to release various

inflammatory cytokines (Akira, 2011; Janeway et al., 2001). Cytokines are small molecules secreted by the immune cells to activate the immune cells and induce specific signaling throughout the immune responses (Zhang et al., 2007). Generally, innate immune system is consisting of dendritic cells (DCs), macrophages and natural killer (NK) cells.

On the other hand, the major soluble protein component of the innate immune system is known as the complement system. The complement system consists of a serum protein group that produces biologically active molecules which are capable in lysing the infected cells by attacking and forming pores in membranes of the cells. Apart from cell lysis, it induces inflammatory responses and opsonising targets to enable phagocytosis by granulocytes and macrophages (Nesargikar et al., 2012; Merle et al., 2015). The activation of the complement system is through three major pathways such as (1) the classical pathway through antigen-antibody complexes (2) the alternative complement pathway where the components are activated by the cell wall of a bacteria or yeast and (3) the lectin pathway where the classical pathway will be activated in the absence of the Clq component. The activated complement system in the host will eventually generate the peptides to induce the pathogen phagocytosis. Therefore, complement also acts as an effector system in the host defence against invading pathogens, through its components activation to release the inflammatory mediators whereby promote the tissue injury at sites of inflammation. Besides, complement system has been implicated as a first line defense in the pathogenesis of several parasitic infections (Jokiranta et al., 1995) by providing link between innate and acquired immunity through augmentation of the humoral response to T-cell dependent antigens along with B-cell activation (Janeway et al., 2001).

2.2.2 Adaptive Immunity

Adaptive immunity is more specialised compared to innate immunity as it displays specificity, diversity, memory and discrimination between self and non-self. Its efficiency is evaluated through the recognition of foreign or new material by the lymphoid system. These activated lymphocytes cells differentiate into effector cells that are able to synthesise functional molecules and memory cells that may act spontaneously against the pathogen during the second antigen encounter (Chaplin, 2010). Lymphocytes are central to the adaptive immune response which consist of the two leukocytes subsets namely B lymphocytes (B cells) and T-lymphocytes (T cells) as shown in Figure 2.3. Lymphocytes are able to specifically recognise the individual pathogens intrinsically (inside the host cells) or extrinsically (in the host tissue or circulating blood). The prior contact with the foreign particles leads to the lymphocyte's activation and synthesis of proteins which induce specific reactivity towards the antigen during the subsequent attack by the same foreign particles. Generally, lymphocytes will be circulated in the blood and eventually migrate to sites of entrap antigen which is located in the secondary lymphoid tissue such as the spleen, lymph nodes and Peyers patches (Alberts et al., 2002).

However, during the transition of the innate immune system to the adaptive immune system, the invaded foreign particles will be recognised by T cells which are bound to MHC receptor I where the ingested molecules of the antigen surface molecules will be expressed by antigen presenting cells (APCs). The principle cell which is associated with the translation of information from the innate immunity to the acquired immunity is called antigen presenting cell. Antigen presenting cells known as macrophages, monocytes and dendritic cell, are able to recognise the infection (antigen) and activate the naive T cells. Subsequently, the antigen will be lysed by T cells through cytokine secretions that enable a ligation of pro-apoptotic receptors on the target cell followed by recruitment of inflammatory cells to the targeted area (Zitvogel, 2002; Reis e Sousa, 2004).

On the other hand, the activation of B cell happens through the binding of the antigens to the surface receptors such as B cells to combat extra-cellular pathogens. Normally, binding of B cell receptor to the antigen will form an antigen-receptor complex and this antigen will be internalised and processed through proteolytic cleavage in the endosomes (Bournazos et al., 2016). B cell antigen receptor plays a dual role during the B cell activation. Firstly, antigen-induced clustering of receptors delivers biochemical signals to the B cells to initiate the process of activation. Secondly, the receptor binds protein antigen and internalizes it into endosomal vesicles, which are then processed and presented to helper T cells at the surface with MHC II molecules (Chaplin, 2010). The B cell antigen receptor delivers activating signals to the cells when two or more receptor molecules are brought together, or cross-linked, by the multivalent antigens. Membrane proteins such as Igα and Igβ are linked together by disulphide bonds, covalently linked to membrane Ig and transduce the signals generated by clustering of surface receptors. These two molecules, together with surface antibody form the B lymphocyte antigen receptor complex (Volkmann et al., 2016).

Antigen-specific B cells along with helper T cells engagement enable the antigen recognition. Helper T cells bind to the B cells surface receptor, activate it by secreting specific antibody responses against the antigen. This will eventually lead to the stimulation of B cell clonal expansion, isotype switching, affinity maturation and differentiation into memory B cells. On the other hand, the engagement between helper T cells and B cell also results in T cells differentiations due to high involvements of antigen presentation cells. The antigen presentation helps in T cells differentiations, helper T cell activation and expression of membrane and molecules secretions by the helper T cells. (Alberts B et al., 2002; Janeway CA Jr et al., 2010).

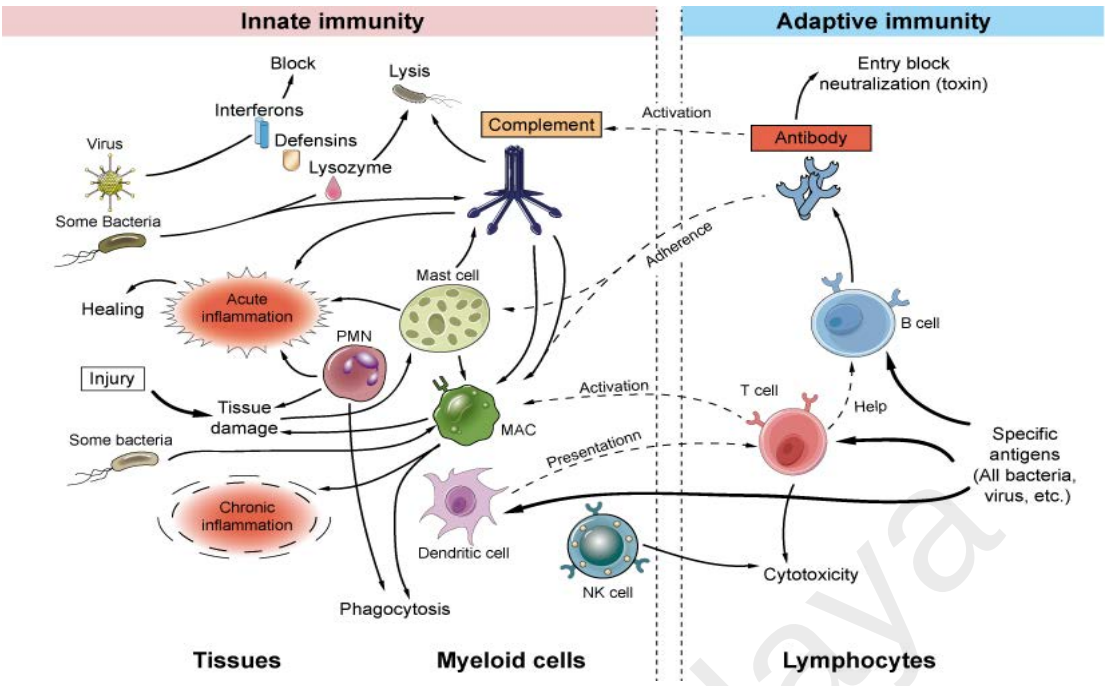


Figure 2.3: Mechanisms of Innate and Adaptive Immune Response.

(Source: https://www.creative-diagnostics.com)

2.2.3 T Helper Cell Dichotomy

T helper (Th) clones are generally differentiated into two distinct populations, namely Thl and Th2 according to the type of cytokines secreted by them as proposed in Figure 2.4. Generally, Thl/Th2 paradigm is subdivided into T cell immune responses into their specialised target defence against pathogens such as Th1 (generally target intracellular pathogens such as viruses and bacteria) and Th2 (generally target the extra-cellular pathogens such as *Blastocystis* sp. and helminths). Eventhough Th1 and Th2 cell populations are derived from Th0 pre-curser, they differentiate the antigens in terms of phenotypic and antigen target differences (Oo et al., 2010; Muraille,1998; Romagnani,1991).

Generally, early stimulations of CD4+ T cells are subdivided into the effector cells, Thl and Th2 cells through manipulation of various factors such as dose of antigen, the source of co-stimulation and the cytokine environment. For instance, Th1 response

secretes IL-2 and IFNγ to mediate broad range of biological effects meant for intracellular pathogens immune response (Magombedze et al., 2014). On the other hand, IFNγ induce the transcription of IgG2a and IgG3 by B cells which interlinked with the activation of the classical complement pathway. This induces phagocytosis of pathogens through the binding of Fc receptor on macrophages. Apart from that, IFNγ also induces the cytocidal and microbicidal activity in macrophages by provoking the production of nitric oxide. These phenomena are known as an antibody-independent immunity, established by the activated macrophages in association with cell mediated immunity to combat against the intracellular pathogens. Besides, macrophages are also able to activate the cell mediated immunity to combat against extracellular parasites such as helmith. However, this phenomenon can be detrimental to the host cells especially during the chronic infections due to association with delayed type II hypersensitivity response (Gallo et al., 2010; Li and Kimberly, 2014; Vogelpoel, 2015).

In contrast, Th2 type response is recognised through the secretions of cytokines such as IL-4, IL-6, IL-10 and IL-13. These Th2 cytokines are able to promote the B cell proliferation to secrete IgGl and IgE (Stone et al., 2010) such as IL-4 cytokine which is able to induce the differentiation of naive T-helper cells into Th2 cell populations and at the same time induce the production of antibody secretions. Besides, studies have proven that IL-4 cytokine plays a major role in the expressions of IgGl (Gallo et al., 2010; Li and Kimberly,2014). Generally, Th2 response triggers the inflammatory reactions during the eradication of extracellular parasites and promotes the mobilisation of the eosinophil. This has been proven in the helminth infections where the IL-13 triggers the promotion of eosinophil to release the toxic cationic compounds. Therefore, the allergic inflammatory eosinophilia reactions through IgE isotype switching was triggered (Shin et al., 2009).

Apart from up-regulations of Th2 cytokine, down-regulations of Th1 cytokine also occurs through the secretion of Th2 cytokine; IL-10 (anti-inflammatory cytokine response). IL-10 cytokine is proven to down-regulate the cellular immune response by inhibiting the Thl-associated cytokines which affect the bacterial and viral eradications during infection. However, Th1 and Th2 cytokines support one another through the counter-regulation of specific cytokines during the specific infection (Kessler et al., 2017). For instance, during the intracellular infection the Th1 cytokine such as IFNγ, is able to down-regulate the Th2 cytokine response. Similarly, Th2 cytokine such as IL-10 is able to inhibit the effects of Th1 cytokine response, IFNγ during the extracellular infection. As a result of the counter-regulation of the opposing response, this phenomenon induces the polarisation of the immune reaction.

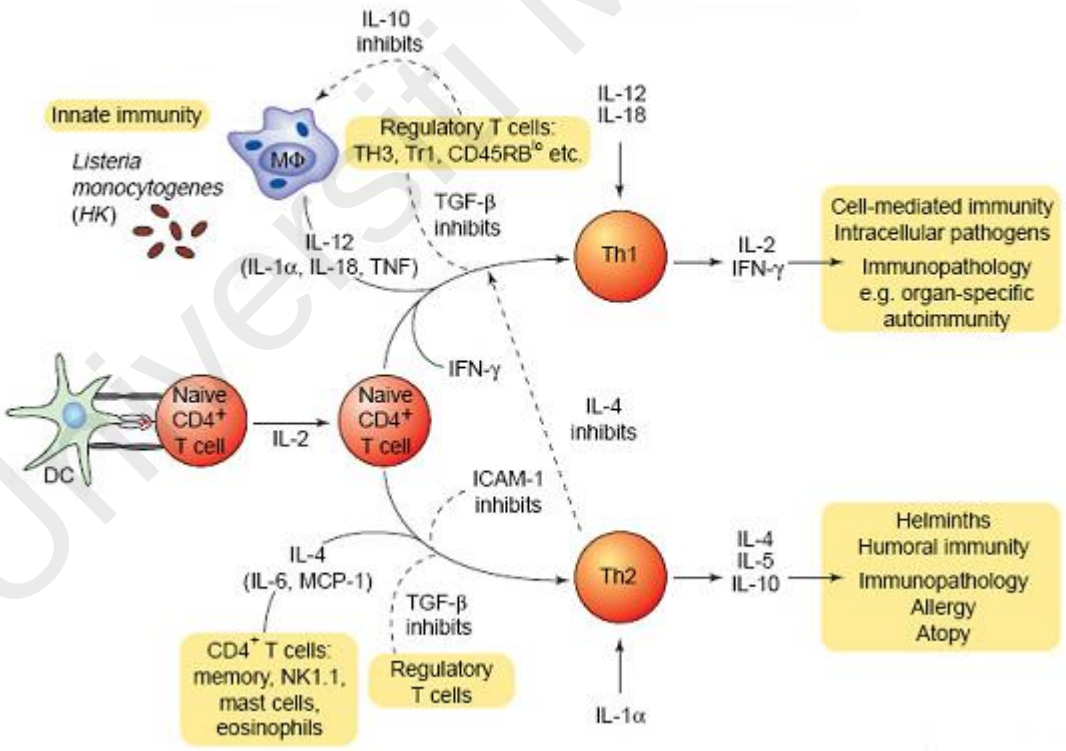


Figure 2.4: Regulation of T Cell Immune Responses.

(Source https://www.mangosteenrd.com)

2.3 Host Immune Response against Parasitic Infections

2.3.1 Immune Response against helminth Infection

Helminth is an intestinal parasite which resides in intestines of the infected individuals. Reports have suggested that, T cell response against this parasite is mediated towards Th2 polarization which is also known to down-regulate the Th1 response. However, the efficiency of Th2 mediated protective immunity to eradicate this parasite remains unclear (McSorley and Maizels, 2012). Soil transmitted helminth which is known as Schistosoma mansoni secretes Th1 response in the early stage. However, the Th2 response is more prevalent after the egg production stage. The immune response against helminth infection and pathological alterations occur when the primary signs of the disease surfaces. For instance, during the Schistosoma mansoni infection, the egg produced by this parasite can be trapped in the sinusoids of the liver which induces Th2 response. This results in the development of granulomatous lesions. In contrast, infection of Schistosoma mansoni in mice model failed to form granulomatous lesions which has led to toxic effects of the egg protein in hepatocytes region that eventually killed the mice (Gravitt et al., 2016; Hotez et al., 2008).

Generally during helminth infection, immunoglobulin IgE and tissue eosinophilia are up-regulated by the Th2 subset cytokines such as IL-5, IL-4 and IL-3. However, this parasite can be killed via *in vitro* condition by IgE regulated mechanisms which involves platelets, mast cells, basophils, eosinophils and macrophages (NEGRÃO-CORRÊA and Deborah, 2001). However, eosinophils are found to be largely involved as the immune defence mechanisms against helminths. This is based on the observation that (1) eosinophils are able to de-granulate and kill helminths *in vitro* (2) eosinophils are found to be accumulated in helminth infected areas (3) eosinophils degranulate in the vicinity of invading helminth. However, *in vivo* evidences to elucidate eosinophils involved in the host protection are still lacking (Klion and Nutman, 2004).

2.3.2 Immune Response against Protozoan Parasites

2.3.2.1 *Entameoba histolytica* infection

Generally, an immune response against a pathogen will create an impact to the infected host such as damage to the host tissue. *Entamoeba histolytica* has been reported to adhere to the colonic mucus and epithelial cells by using Gal/GalNAc-specific lectin-mediated mechanism, which contributes to the binding activity. This is followed by the release of amoeba pores, a pore forming polypeptides. This results in the disruption of the intestinal mucus and epithelial barrier of the colonic region (Begum et al., 2015; Nakada-Tsukui and Nozaki, 2016). On the other hand, intestinal epithelial cells are considered the first line of defence against this pathogen where it is able to detect the infection and retaliate against it by secreting pro-inflammatory cytokines and soluble factors including IL-1, IL-8, monocyte chemoattractant protein-1, granulocyte-macrophage colony-stimulating factor (GMCSF), inducible nitric oxide (NO) synthase and tumor necrosis factor alpha (TNF-α) (Lissner et al., 2015).

Entamoeba histolytica infection causes tissue destruction in amebic colitis and host gut inflammation. The immune mechanism's ability to fight against this parasite is through the release of TNF-α. This cytokine will help to activate the neutrophils and macrophages in order to release the Reactive Oxygen Species (ROS) and Nitric Oxide (NO). However excessive release of TNF-α may cause further damage to the host tissue. Study has proven the correlations of high TNF-α release and the percentage increase of parasite load. Furthermore, the blocking of TNF-α with a monoclonal antibody enhanced the reduction of the host inflammation and damage in amoebic colitis in mice (Moonah et al., 2013). Generally, to counter regulate the over expression of pro-inflammatory cytokine such as TNF-α during the infection, the anti-inflammatory cytokine IL-10 will be secreted to maintain the immune homeostasis. For instance, to study the effects of cytokine homeostatis impairment in inflammatory bowel disease by inhibiting the IL-10

genes in mice models has resulted in amebic colitis formation. Besides, the effects of IgG antibody protective effects on the susceptibility to amoebic infections are also proven to be dependent on the IgG1 and IgG2 isotypes or subclasses which induce Th2 and Th1 cell proliferation respectively during the infection (Leach et al., 1999).

2.3.2.2 *Giardia Iamblia* infection

Giardia lamblia infection causes illness to humans worldwide. This infection is normally found in the upper intestinal region of the infected inviduals (Dormond et al., 2016). During the infection, the host immune system reacts against this parasite by nitric oxide secretion and activation of complement immune system in the epithelial cells regions as a main immune defence mechanisms. Basically, nitric oxide, a water and lipid soluble are able to cross the cell membranes and begin a chemical reaction in host cells whereby the complement system is activated through the presence of pro-enzymatic plasma proteins. Moreover, the humoral immune system is activated through the secretions of specific IgA antibody which is reported to be the main as well as the largest type of protection against this parasite (Pavanelli et al., 2010).

Cell mediated immunity against *Giardia lamblia* depends on the T-lymphocytes population namely CD8+ T lymphocytes cytotoxic cells and CD4+ T lymphocytes helper cells which resides in the lamina propria region of the digestive tracts. Upon *Giardia trophozoite* infection in the host, the parasite initiates a binding to the epithelial lining where the antigen fragment will be presented to the MHC-II pathway. This will be followed by the activation of CD4+ T lymphocytes helper cells and finally pro-inflammatory cytokines; IFNγ and IL-6 cytokines will be secreted along with B lymphocytes cells activation to secrete antibodies (Faubert, 2000). Apart from pro-inflammatory cytokine response, this parasite has been reported to secrete the anti-inflammatory cytokine which is IL-10 cytokine response. This anti-inflammatory

cytokine induces the up-regulations of CCR-6 expressions in dendritic cells (Ringqvist et al., 2008).

2.3.2.3 *Blastocystis* sp. infection

The pathogenesis of *Blastocystis* sp. in human hosts has always been a matter of debate as it is present in both symptomatic and asymptomatic individuals. Due to this fact, several lines of evidence shed light on the possible mechanisms of pathogenesis among asymptomatic and symptomatic *Blastocystis* sp. ST3 isolates. As a clear example, it was implied that, symptomatic ST3 isolates are speculated to be pathogenic due to the fact that symptomatic isolates exhibited a rough surface compared to asymptomatic isolates where it exhibited a smooth surface (Ragavan et al., 2014). Nevertheless, the number of studies pertaining to immunological assessment between asymptomatic and symptomatic isolates of *Blastocystis* sp. ST3 are few.

Several studies have proven that *Blastocystis* sp. is able to provoke immune responses in the infected individuals such as secretory and humoral antibody secretions such as IgG and IgA. However, these immune responses are signifcantly higher in individuals infected with *Blastocystis* sp. symptomatic isolates in comparison with *Blastocystis* sp. asymptomatic isolates infected individuals (Tan, 2008; Hussain et al., 1997; Pasqui et al., 2004). A study conducted to identify the effect of *Blastocystis* sp. isolates on the expression of IFNγ and pro-inflammatory cytokines in the cecal mucosa of rats has showed significant up-regulations of type 1 and pro-inflammatory cytokines (IFNγ, IL-12 and TNF-α) gene transcription (Iguchi et al., 2009). This suggests that *Blastocystis* sp. infection stimulates the specific local host immune responses which includes T cells, monocytes, macrophages and natural killer cells when exposed to live *Blastocystis* sp. cells. However, there is no study that sheds light on the immune response of soluble or attenuated *Blastocystis* sp. The highly immunogenic and antigenic soluble or attenuated

antigen has been known to induce significant amount of immune response upon a few exposures to the immune cells *in vitro* by forming memory B and T-lymphocyte cells. Upon a few exposures of soluble or attenuated antigen, the memory B and T lymphocyte cells have the competency in identifying the specific antigen and responded quicker by eliciting higher specific immune response (Janeway et al., 2001). This approach has yet to take into consideration in *Blastocytis* sp. research as there are several anti-parasitic drugs such as metronidazole which is available to treat this infection. Unfortunately, a recent study conducted has proven that, *Blastocystis* sp. isolates are resistant to metronidazole. Furthermore, *Blastocystis* sp. is also reported to down-regulate the intestinal epithelial inducible nitrix oxide synthase (iNOS) and enhance its survival by escaping host defences. It might also assist other pathogens to evade nitrosative stress and promote the colonization of the gut lumen (Mirza et al., 2011).

2.4 Immune Evasion Strategies Inflicted by Parasites

2.4.1 Immune Evasion by helminth

Helminth, is able to survive in their host immune system for a long period due to its complex structure (MacDonald, 2002). For instance, *Schitosomes*, a soil transmitted helminth is able to evade the host immune defence through the interactions of the surface and internal membrane within the host molecules thus reducing its antigenicity (Mascarini-Serra, 2011). Many pathogens demonstrate antigenic heterogeneity during the stage of its development. This will eventually lead to immune evasions against the invading pathogens due to high antigenic variations. This phenomenon is seen during the infection of helminth in the host where it secrets vast amount of antigenic materials in order to induce the host immune response. Reports has suggested that, helminth causes high immune suppression that affects the specific immune response such as overload of antigen, antigenic differences, the activation of suppressor cells and the generations of lymphocytes-specific suppressor factors (Loukas and Prociv, 2001) For instance,

Schistome is reported to induce non-specific antibodies upon its infection in the host, where it has the potential to halt the protective specific immunity in the infected host (Colley and Secor, 2014). This phenomenon will eventually affect the parasite eradications while prolongs the survival and increase other infection susceptibility in the host. Besides that, study has also proven that the immune evasion strategy imposed by helminth is also co-related to cancer cell proliferations. These phenomena were observed where the monocytes exposed to helminth were able to alter its characteristics by demonstrating an innate immune evasion strategy as similar as cancer cells. This immune evasion strategy is also reported to co-related with the expression of immune inhibitory ligands expressions which is Programme Cell Death Ligand-1 (PDL-1) and Programme Cell Death Ligand-2 (PDL-2) (Narasimhan et al., 2018).

2.4.2 Immune Evasion by Protozoan Parasites

2.4.2.1 *Entameoba histolytica*

Entameoba histolytic carries different strategies to invade the host immune system to sustain its survival. This parasite is capable of inducing a host cell killing by apoptosis mechanism that includes multiple stepwise processes of apoptosis without inducing the inflammatory reactions and toxic release from the dead cells. This will eventually lead to the dead cells clearance in the host system through phagocytosis process (Adam Sateriale and Christopher Huston, 2011). Another immune evasion mechanism imposed by this parasite namely *ameobic trogocytosis,* where it induces cell death by actively biting the live cells (Ralston, 2015). Moreover, *Entameoba histolytica* is able to alter or obliterate the immune system through the induction of neutrophil apoptosis and inhibition of nitric oxide (NO) production in macrophages.

During the late stage of infection, due to the constant inflammatory response, the host immune system will eventually activate the complement mediated system and antibodies

to combat against this parasite (Begum Quach and Chadee, 2015). However, *Entamoeba histolytica* is able to inhibit IgA and IgG serum through the cleavation with cysteine proteases. This will eventually activate the serum complement to inhibit the complement mediated lysis. It was reported that, cysteine protease secreted by *Entamoeba histolytica* trophozoites is able to activate the complement system by cleaving C3 and C5 in order to produce C3b. However, due to the active antibody and cysteine protease cleavation factors, the C3 and C5 were degraded. This method is used by this parasite to diminish the pro-inflammatory response which activates the complements for the lysis (Ortiz-Ortiz et al., 1978). Meanwhile, Gal/GalNac lectins are said to be important in the *Entamoeba histolytica* resistance against complement since they show a similar cross-reactivity with human leukocyte antigen to prevent the C5b9 attack complex (Petri, 2008).

2.4.2.2 Giardia lamblia

Giardia lamblia is able to survive in the host immune response due to its own characteristics which enable them to reproduce in the host immune system. Besides, *Giardia lamblia* is also able to inhibit the nitric oxide production in the human intestinal region. The inhibition or the impairment of the nitric oxide production is reported to be due to its unique characteristics in consuming arginine, a group of a protein which needed in the induction of the nitric oxide production (Eckmann et al., 2000).

Moreover, another strategy to combat the host immunological system is through the presence of specific heterogeneous parasite surface marker. This will allow the parasite to evade the host's immune response and produce chronic and recurrent infections (Carranza et al., 2002). *Giardia trophozoites* is also able to survive in the different environment of the host through its biological adaptation in the host immune system. For instance, this parasite is capable in differentiating into cyst in order to survive outside the

intestinal region of the infected host such as feces and encystations occur when it colonise a new host again (Luján et al., 1995).

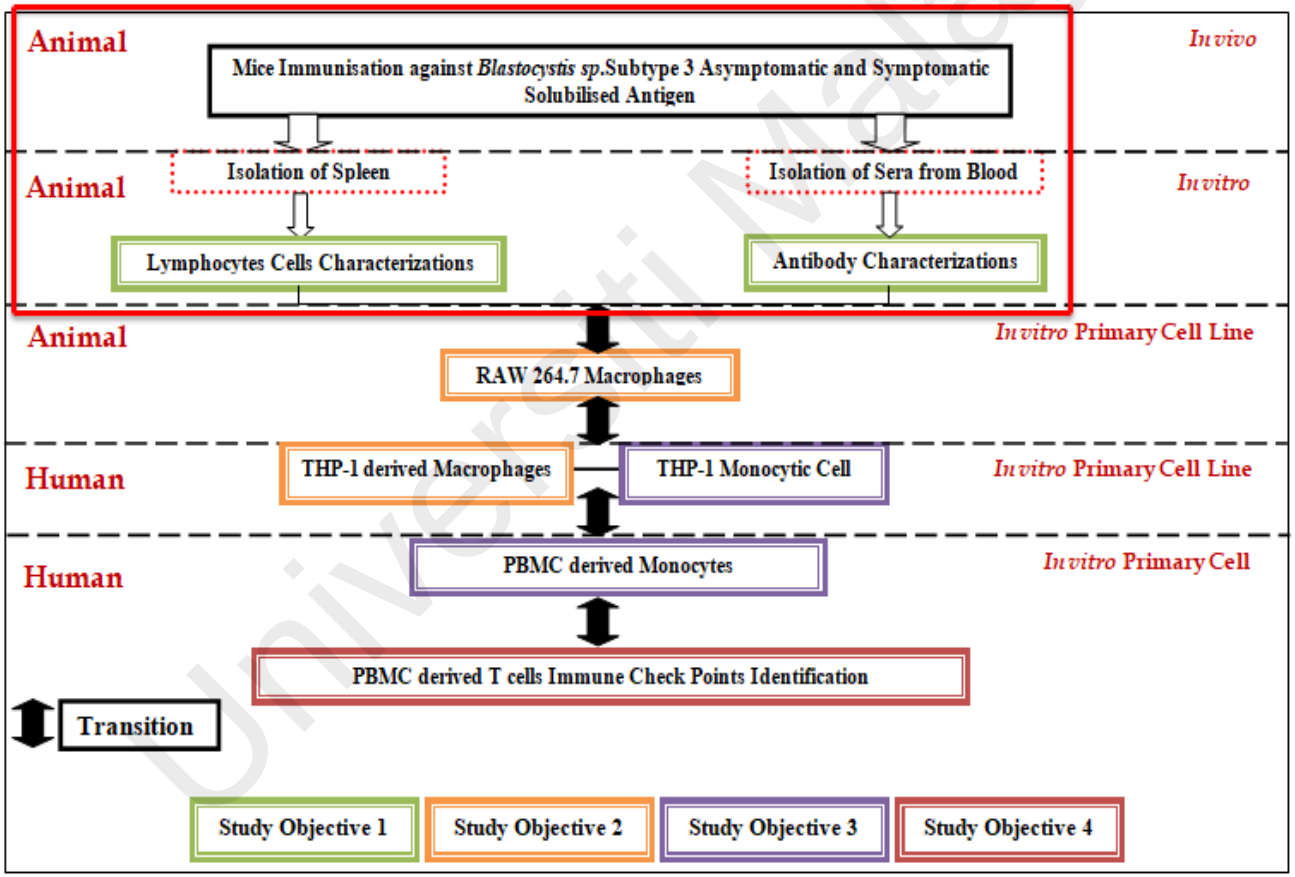
2.4.2.3 *Blastocystis* sp.

Blastocystis sp. is reported to have strain to strain variations which enable it to conclude its pathogenicity. There are findings suggesting that, *Blastocystis* sp. is able to evade the immune system through the inhibition of nitric oxide. Studies also suggest that, intestinal epithelial cells may produce nitric oxide to target the invading pathogens. The nitric oxide production by the intestinal epithelial cells may be a host defence mechanism against *Blastocystis* sp. However, it was reported that, *Blastocystis* sp. is causing a down-regulation of inducible nitric oxide synthase (iNOS) and eventually leads to the evasion of host immune mechanism by inhibiting the nitric oxide production in the host intestinal region (Mirza et al., 2011).

Furthermore, higher arginase activity contained by *Blastocystis* sp. strains is correlated to induce higher level of nitric oxide inhibition. Therefore, *Blastocystis* sp. escape the host immune mechanisms by lowering the amount of nitric oxide concentration in the host gut system. This scenario enables *Blastocystis* sp. to escape from the nitrosative stress while promotes its colonization in the host gut lumen ((Mirza et al., 2011). Besides, stress is also reported as a factor on how the *Blastocystis* sp. evades the host immune system. A study conducted by Chandramathi et al., (2014) reported that, *Blastocystis* sp. is able to down-regulate the immune response of the host infected with *Blastocystis* sp. The study has also implied that, stress in the *Blastocystis* sp. infected host had further influence the down-regulations of the host immunity which eventually suppressed the PBMC and immunoglobulins followed by the occurance of imbalance of oxidant-antioxidant system. Therefore, it was proven that, stress is also plays a part as an additional factor that leads to immune evasion of *Blastocystis* sp. in the host.

Apart from that, proteases are also classified as a part of the *Blastocystis* sp. immune evasion strategy. Cysteine proteases stimulate the intestinal mucosal cells to produce interleukin-8 (IL-8) which can cause fluid loss in the bowel which will eventually leads to the inflammation in the affected intestinal area. Besides, protease is also known to cleave secretory IgA and help in immune evasion and survival of the parasite (Parija et al.,2013). Secretory Immunoglobulin A (IgA), is the main immune defense against *Blastocystis* sp. infection at the mucosal surfaces of the host intestinal tract. However, study has proven that, *Blastocystis* sp. secretory namely cysteine and aspartic proteinases were able to degrade human secretory IgA. This was reported as one of the immune evasion strategies inflicted by this parasite to retain its survival in the host (Puthia et al., 2005).

CHAPTER 3: *IN VIVO* AND *IN VITRO* STUDY TO CHARACTERISE *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC ANTIGEN INDUCED SPECIFIC IMMUNE RESPONSE IN BALB/C MICE.



3.1 Introduction

A pathogen which infects an organism will encounter the host immune responses. However, in order to colonise its host, it should have the capability to defeat or evade the innate and adaptive immune responses. Many intestinal parasites including *Blastocystis* sp. is able to evade the adaptive immune responses mediated by B/T cells. This results in a long-term colonization in the host which is also known as chronic infection. The ability of an antigen to provoke humoral and/or cell-mediated immune responses in the infected host is defined as "immunogenicity" while the ability of an antigen to bind specifically with an antibody and/or cell surface receptor (immunoglobulins or T cell receptor) is defined as "antigenicity" (Ilinskaya et al., 2016). The highly immunogenic and antigenic antigen is capable of inducing a significant level of immune responses upon a few exposures of antigens and form memory lymphocyte cells including B and T cells. The memory lymphocyte cells have the competency in identifying the specific antigen and respond faster by eliciting higher specific immune response following subsequent encounter of the same infection (Ilinskaya and Dobrovolskaia, 2016).

However, antigenic variations are one of the strategies used by many pathogens to evade the host immune system. Antigenic variations such as the surface structural variances are able to alter the immune cells recognition against an antigen. Therefore, this allows the pathogen to persist and infect the previously infected (pre-immune) host. Studies in the past provide evidence that there were differences between symptomatic and asymptomatic isolates of *Blastocystis* sp. 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, established studies have shown distinctive phenotypic differences between ST3 symptomatic and asymptomatic isolates (Ragavan et al., 2014) using a range of biological and biochemical analyses (Figure 3.1). Therefore, it was speculated that, the

structural differences shown between, *Blastocystis* sp. isolated from symptomatic and asymptomatic infected individuals may have a bearing on the parasite's antigenicity and immunogenicity as previously suggested (Young et al., 2014; Sachse et al., 2000).

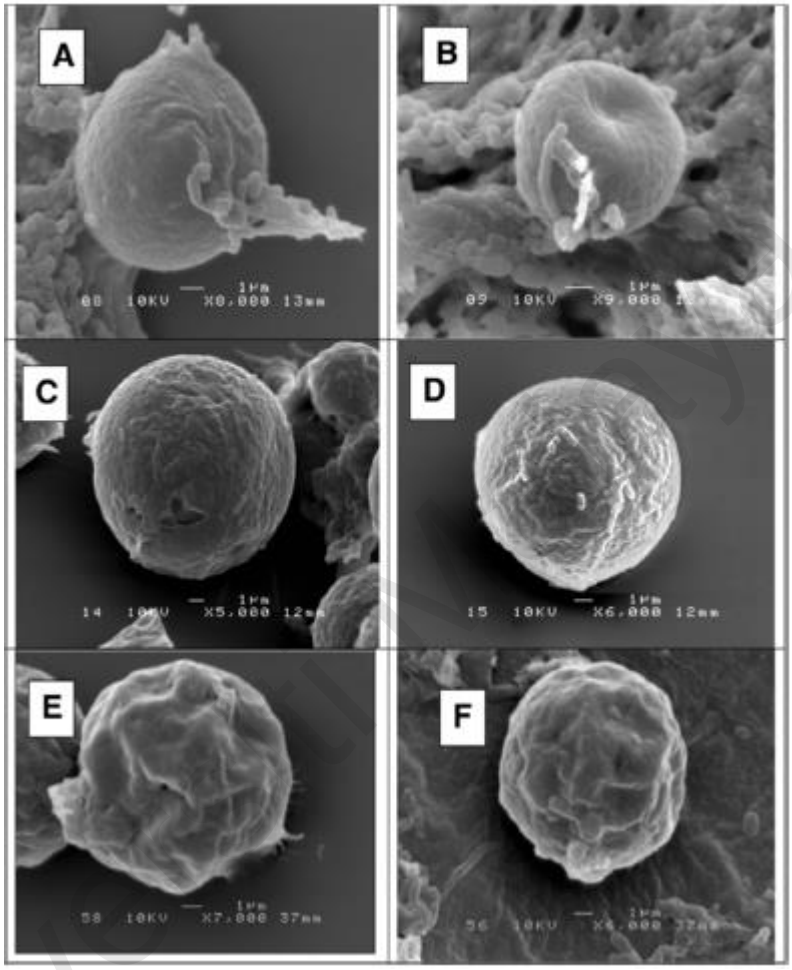


Figure 3.1: Phenotypic Differences of Symptomatic and Asymptomatic *Blastocystis* sp. ST3 Isolate Through Scanning Electron Microscopy.

(A and B): Asymptomatic *Blastocystis* sp. isolate has shown a smooth surface. (C and D): Symptomatic *Blastocystis* sp isolate has shown a slightly rough surface. (E and F): Symptomatic *Blastocystis* sp. isolate from IBS infected patient has shown a coarse and folded surface. (Source: Ragavan et al., 2014).

The production of the **cytokines activates** the immune system so that it could control the survival and infection of a pathogen. Therefore, inappropriate cytokine responses could facilitate the spread of infection and in due course aggravate the level of a disease or infection. Research has proven that, *Blastocystis* sp. (not specific to any subtype) induced stress and suppressed the host immune system by causing imbalance immune response through alterations of oxidant-antioxidant levels (Chandramathi et al., 2014). Besides, pathogenesis of several chronic inflammatory intestinal diseases such as IBD, ulcerative colitis and Crohn's disease were related to imbalance of Th1/Th2 paradigm (Huang and Chen et al., 2016; Silva et al., 2016; Imam et al., 2018). As an example, immune response dysfunction and overstimulation of Th2 response may result in B cells to increase antibody production and cause an allergic response, one of the symptoms associated to *Blastocystis* sp. infection (Pasqui et al., 2004; Deo et al., 2010). Therefore, it is imperative to analyse the balance of Th1/Th2 immune responses induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates.

This study was divided into *in vivo* and *in vitro* analysis to identify the specific immune responses induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens. The *in vivo* analysis was performed by assessing the B cell response targeting IgG and its isotypes IgG1 and IgG2a antibodies obtained from sera of the injected Balb/c mice. At the same time, spleen isolated from the injected Balb/c mice was subjected for *in vitro* analysis to assess the lymphocytes cell proliferation and T cell responses by assessing the Th1 and Th2 cytokine secretions. Besides, **factors influencing the specific immune responses** such as **antigen dose** and the **antigenic and immunogenic relationship** between symptomatic and asymptomatic isolates of *Blastocystis* sp. ST3 were also assessed. Therefore, these analyses were used to assess the **heterogeneity** in immune responses between symptomatic and asymptomatic isolates and

its possible relationship with pathogenicity. The concise details of the analysis are described below.

3.1.1 IgG/IgG1/IgG2a Antibody Responses

IgG dominates the humoral responses in humans and mice which is 75% of the total immunoglobulin concentration in serum. On average, IgG1 isotype appear in a range of 1.2 to 5mg.ml in mice sera (Beutier et al., 2017). IgG1 antibody is primarily secreted by soluble protein antigens and membrane proteins (Vidarsson et al., 2014). Meanwhile, IgG2a isotype appears in a range of 1.3 to 2.9 mg/ml in mice sera. IgG2a isotype interact efficiently with Fc-gamma receptors (FcgammaR), the binding region of IgG (Dekkers et al., 2017). The IgG1 and IgG2a isotype profiles of serum antibodies were used as a marker for Th1 and Th2 *in vitro* analysis in this study.

3.1.2 T Helper Cell (CD4+)

In recent years, there has been a number of emerging research programs looking into the role of CD4+ subsets in promoting chronic inflammations. T helper/CD4+ are the major population of T cells which mediates the host defence mechanisms and regulates the homeostatic responses. However, the imbalances resulted by CD4+ T cells are also reported to perpetuate chronic intestinal inflammation that includes ulcerative colitis (UC), Crohn's disease (CD) and Intestinal Bowel Diseases (IBD) (Imam et al., 2018) which are highly associated with *Blastocystis* sp. infection. In this study CD4+ subsets of Th1 and Th2 cytokines were analysed.

3.1.2.1 Type 1 and 2 Helper- T lymphocyte Cells Response (Th1/Th2)

(a) **Th1 (IL-2 and IFNγ) Cytokine Response**

Th1 type cells mediate pro-inflammatory cytokine responses which are mainly responsible for eradicating the infected cells or intracellular infections (Figure 3.2). IL-2 will be main cytokine which will be produced during primary response of Th cells. IL-2 cytokine levels decline as the Th effector cells (Th1/Th2) differentiations occurs due to the major cytokines of IFNγ and IL-4 secretions by Th cells (Hwang,2005). Even though, IL-2 is characterised under Th1 cytokine, evidence showed that, in some instances, it may work along with downstream signaling molecules by inducing Th2 anti-inflammatory cytokine response (Zhu et al., 2003). Th1 type cells are the main producers of IFNγ which is a strong pro-inflammatory cytokine by T cells during acute and chronic infections. However, aberrant IFNγ cytokine response may lead to severe tissue damage therefore Th2 cytokines are needed to counteract the overstimulation of Th1, pro-inflammatory cytokines (Chen et al., 2017).

(b) **Th2 (IL-4 and IL-10) Cytokine Response**

Th2 type cells stimulate B cells productions and promotion of IgM antibody class switching to IgG1 isotype and IgE (Choi and Reiser, 1998). Th2-type cells secrete IL-4 as the major cytokine where it is responsible for the promotion of allergic response. IL-4 cytokine plays a major influence in differentiations of Th2 dominated immune response especially during gastrointestinal parasite infection (Else et al., 1994). Meanwhile, IL-10 appears to be the key immunoregulator (anti-inflammatory cytokine) during infection, such as protozoan and bacteria and fungi (Figure 3.2). It plays a major role as anti-inflammatory cytokine by inhibiting the Th1 cytokine response during pathogen clearance. However, excess productions of Th2 mediated cytokines will counteract the Th1 mediated microbicidal action (Iyer and Cheng, 2012).

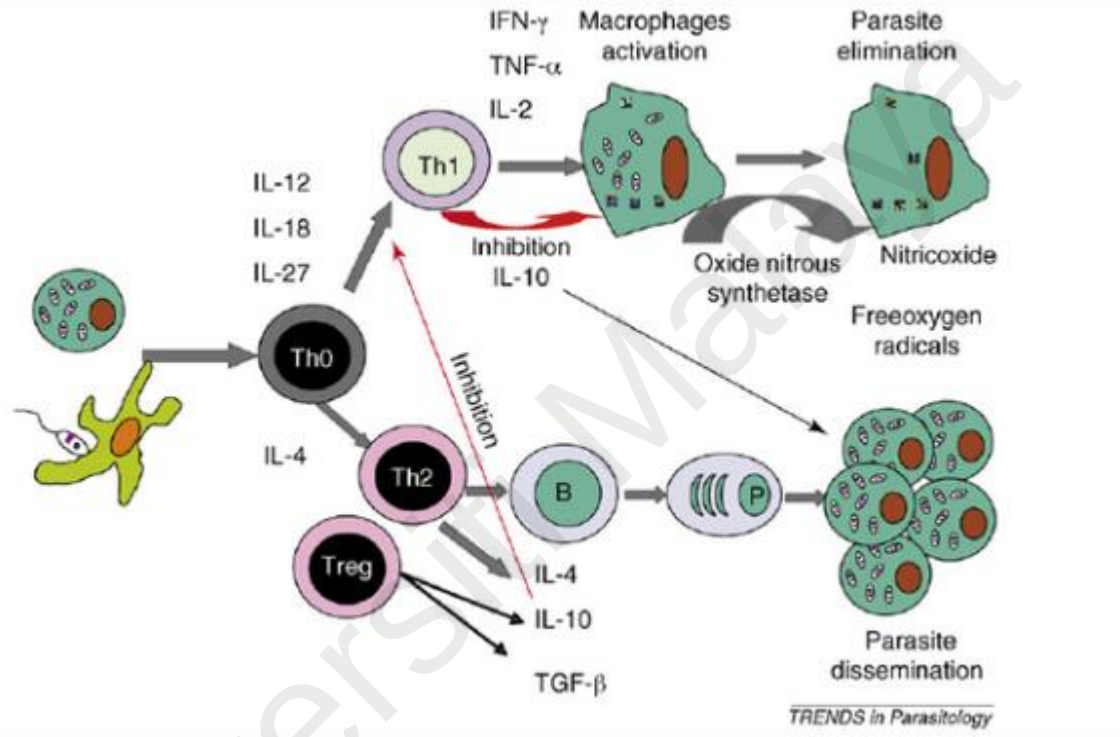


Figure 3.2: Hallmark of Th1 and Th2 Immune Responses.

(Source: Liew et al., 2002).

3.2 Materials and Method

3.2.1 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. *Blastocystis* sp. isolates respectively from symptomatic and asymptomatic individuals were continuously cultured in Jones' basal medium. Individuals with symptoms showed flatulence, abdominal pain, diarrhea and constipation. This information was obtained using a questionnaire. Samples with *Blastocystis* sp. were selected through direct fecal 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).

3.2.2 Axenization of *Blastocystis* sp. and Preparation of Solubilised Antigen

Harvested cells 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). The 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 (2008) (Fig 3.3-A). 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^{o}C overnight. The homogenates were then centrifuged at 13,000×g for 15 min (Fig 3.3-B). The supernatant which was *Blastocystis* sp. solubilised antigen, was filter sterilized, and the protein concentration was determined by Bradford assay (Chandramathi et al., 2010).

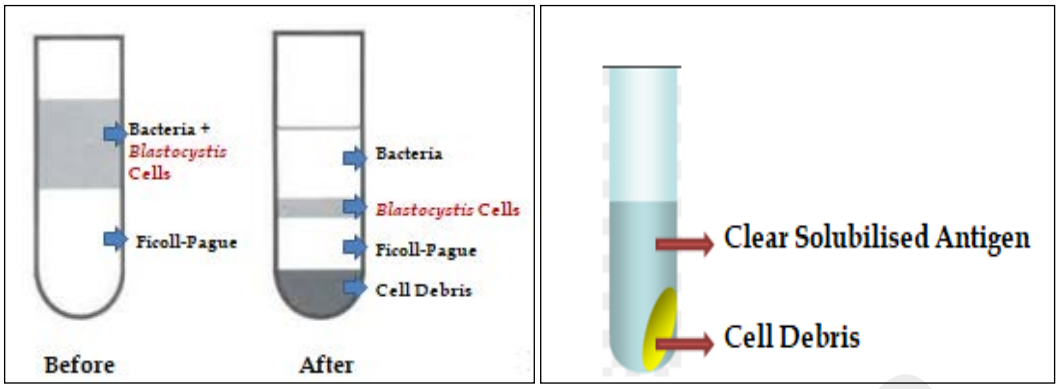


Figure 3.3: The process of *Blastcosystis* sp. ST3 Symptomatic (S1-3) and Asymptomatic (AS1-3) Solubilised Antigen Preparation.

(A) Axenization of xenic *Blastocystis* sp. cells using ficoll-paque method (B) Final product of axenised *Blastocytis* sp. solubilised antigen.

3.2.3 Bradford Assay

Bradford assay used to quantify concentration of solubilised proteins present in the sample. The binding of an acidic dye (Coomassie G-250) to protein in solutions was measured at 595nm wavelength (Bradford, 1976) and performed according to the manufacturer's protocol with the Protein Assay Dye reagent (Bio-Rad, USA). To prepare the protein assay dye reagent, dye stock was diluted at a ratio of 1 ml dye stock to 4 ml of distilled water. This mixture was stored in a dark bottle at 4°C and was stable to use for several weeks. The protein concentrations of the samples were determined using standard (bovine serum albumin, BSA) (Appendix A). The sample or the standard at 10 µl were mixed with 200 µl of diluted Bradford reagent and incubated at room temperature for 5 minutes. The absorbance was measured at 595nm against a reagent blank by using a microplate reader. The quantifications of protein concentrations were done using the obtained BSA standard curve in the range of 0.05-0.5mg/ml.

3.2.4 Animal Selection, Housing and Ethical Clearance

36 female Balb/c mice aged between 4 and 6 weeks were selected. The Balb/c mice were purchased from Animal Laboratory, University Putra Malaysia, Serdang, Malaysia and were kept in an individually ventilated (IVC) cage at Animal Satellite Laboratory, Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia during the course of this study (Appendix G). 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) (Appendix F).

Table 3.1: Distributions of Balb/c mice for intraperitoneal injection with *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens (SA).

Group	No of Isolates /Group	(A) No of Mice for Optimization Injection at (10, 20,30 and 40 µg/ml) SA	(B) No of Mice for Injection at 20µg/ml SA
Asymptomatic	3	2 mice/Isolate	3 mice/Isolate
Symptomatic	3	2 mice/Isolate	3 mice/Isolate
PBS (Control)	-	2 mice/Group	3 mice/Group
TOTAL		**16**	**24**

Table 3.2: Balb/c mice intraperitoneal (IP) injection with *Blastocystis* sp. solubilised antigen.

Immunization Day	Types of Injection	Site	Adjuvant
0	Primary Immunization	IP	CFA
14	First Booster	IP	IFA
28	Second Booster	IP	IFA
42	Third Booster	IP	IFA
56	Final Boost	IP	None
59	Harvesting of spleen and ready for fusion	NA	NA

The *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens were mixed with adjuvant at 1:1 ratio and were injected every two weeks of interval for a period of 56 days.

Figure 3.4: Balb/c Mice Intraperitoneal Injection.

(Source: http://www.procedureswithcare.org.uk/intraperitoneal-injection-in-the-mouse/)

3.2.5 Balb/c mice Immunization

3.2.5.1 Antigen Dose Optimization

16 Female Balb/c mice were immunized intraperitoneally (IP) by using a 27-G needle as shown in Figure 3.4 above. The distribution of Balb/c mice for injection was done as according to Table 3.1. The injections were administered following the schedule in Table 3.2. The priming doses for injection were done by mixing the *Blastocystis*-SA AS (1-3) and S (1-3) with Freund's complete adjuvant (CFA) at the ratio of 1:1. 100 µl of (20 µg/ml) (40 µg/ml) (60 µg/ml) and (80 µg/ml) aliquots of *Blastocystis*-SA were emulsified with 100 µl of Freund's complete adjuvant (CFA). Subsequent booster doses consisted half the concentrations (10 µg/ml) (20 µg/ml) (30 µg/ml) and (40 µg/ml) of priming doses of *Blastocystis*-SA were injected into the Balb/c mice for a period of two weeks intervals by mixing with incomplete adjuvant (IFA) at 1:1 ratio, 100 µl each. Finally, 200 µl of *Blastocystis*-SA at (10 µg/ml) (20 µg/ml) (30 µg/ml) and (40 µg/ml) mixed with PBS were injected as the final boost. Mice served as negative control were injected with PBS instead of solubilised antigen.

3.2.5.2 Balb/c Mice Immunization after Optimization

24 Female Balb/c mice were immunized intraperitoneally (IP) by using a 27-G needle. The selection of Balb/c mice for injection was done as according to Table 3.1. The injections were done following the schedule in Table 3.2. The priming doses for injection were done by mixing the 100 µl of (40 µg/ml) *Blastocystis*-SA AS (1-3) and S (1-3) with 100 µl of Freund's complete adjuvant (CFA) at the ratio of 1:1. Subsequent booster doses consisting of half the concentration (20µg/ml) of priming doses of *Blastocystis*-SA were administered to the Balb/c mice for a period of two weeks intervals by mixing with incomplete adjuvant (IFA) at 1:1 ratio, 100 µl each. Finally, 200 µl of *Blastocystis*-SA at (20 µg/ml) mixed with PBS were injected as the final boost. Mice served as negative control were injected with PBS instead of solubilised antigen.

3.2.6 Balb/c mice Blood Sera Extraction

Blood extractions from the injected mice were done on day 59. The bleeding was done after the mice were aneasthised by the injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). Cardiac puncture was implied to the mice before proceeding to cervical dislocation. The collected blood sample was centrifuged at 210 rpm for 8 minutes to separate the serum. Serum collected were kept in -20ºC freezer until further use.

3.2.7 Balb/c mice Spleen Harvesting

Prior to cervical dislocation, the animals were anesthetised as mentioned in method 3.2.6. Spleens were aseptically removed and were kept in Hanks Balanced Salt solution before proceeding to *in vitro* studies (Yin et al., 2013) (Appendix G).

3.2.8 *In vitro* Th1 and Th2 Cytokine Assays

Th1 and Th2 cytokines analysis were measured as described by Yin et al., 2013. Spleens were removed from immunised Balb/c mice as mentioned in method 3.2.7. The spleens were pressed through stainless steel meshes, pelleted and re-suspended in erythrocyte lysis buffer (Sigma, USA). After centrifugation at 110xg for 10 min at 4ºC, splenocyte pellets were washed three times in PBS and re-suspended in 2 ml of PBS. The splenocytes were suspended in RPMI-1640 complete medium and seeded at $1.5x10^6$ in triplicates using flat-bottom 24-well microtiter plates. 10 µg/ml of *Blastocystis*-SA ST3 asymptomatic (1-3) and symptomatic (1-3) were stimulated in the wells containing splenocytes. Supernatants from the cultured splenocytes were collected at 24, 72 or 96 hours of stimulations. Respective supernatant was then assayed for Th1 and Th2 cells secreted cytokines. IL-2 and IL-4 (supernatant collected at 24 hours); IL-10 (supernatant collected at 72 hours); and IFNγ (supernatant collected at 96 hours). The ELISA tests were performed using a commercial ELISA Kit (CUSABIO, Biotech, USA) according to

the manufacturer's instructions (Appendix B). All the assays were performed in triplicates.

3.2.9 Lymphocytes Proliferation Assay

Lymphocyte proliferation assay was performed based on the protocol described by Yin et al., 2013. The splenocytes preparations before seeding were done as according to the methods mentioned in as 3.2.8. The splenocytes pellet were layered on 2 ml of Ficoll-paque (Sigma, USA), and centrifuged at 450xg for 10 min at RT (Figure 3.5-A). After centrifugation, the lymphocytes layer observed in the tube (Figure 3.5-B). The layer containing lymphocytes were 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 4ºC. The supernatant was then aspirated and discarded. The cell pellet was diluted to 1 ml with RPMI-1640 complete medium. The lymphocytes cells were counted on a hemocytometer using tryphan blue count. The cells were then seeded in triplicates using flat-bottom 96-well plates (Corning) at a density of $5x10^5$ cells per well. After cell seeding, the cells were stimulated with 10 µg/ml, *Blastocystis* sp. ST3 asymptomatic (1-3) and symptomatic (1-3), Concanavalin A (Con A; 10 µg/ml; Sigma, USA; positive control) or medium alone (negative control) and incubated at 37ºC in a 5% CO_2 incubator for 72 hours.

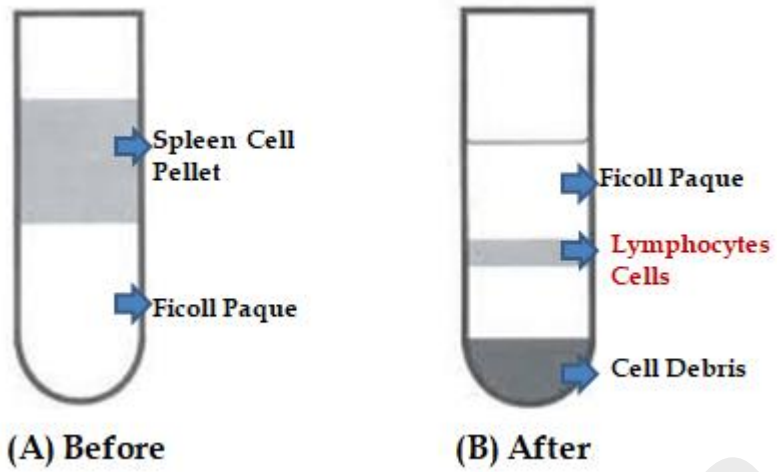


Figure 3.5: Lymphocytes Isolation.

(A) The splenocytes were layered on ficoll paque (B) Lymphocytes layer were observed after centrifugation.

3.2.10 Cell Counting Kit-8 (CCK-8) Analysis

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, following manufacturer protocol. The absorbance was measured in triplicate cultures at 450 nm to quantitatively evaluate cell viability (Appendix C).

3.2.10.1 Assay Principle

CCK-8 is a sensitive colorimetric assay which is use for cell viability determination or cell cyto-toxicity analysis. Dojindo's highly water-soluble tetrazolium salt, WST-8, will be reduced by dehydrogenase activities in cells and yellow-formazan dye, which is soluble in the media. The amount of the formazan dye, generated by dehydrogenases activities in cells, is directly proportional to the number of viable cells. The sensitivity of CCK-8 is higher than the other tetrazolium salts such as MTT.

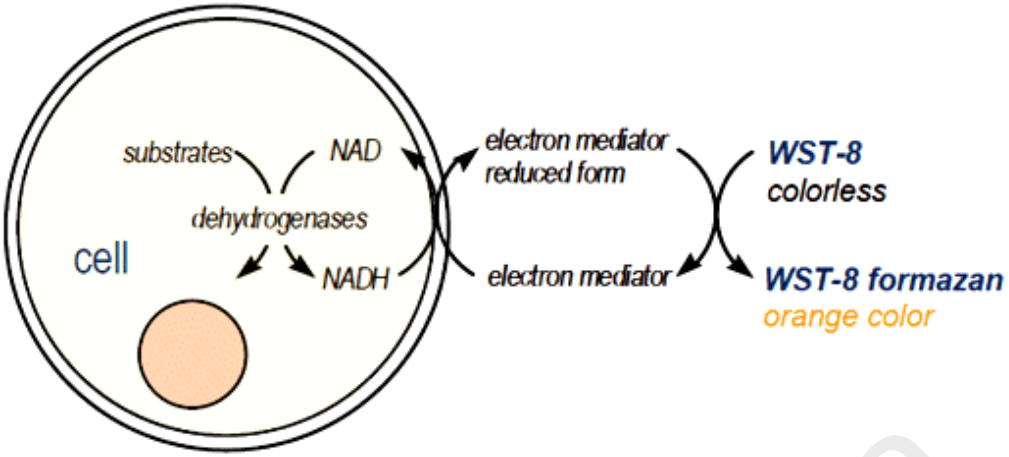


Figure 3.6: Mechanisms of CCK-8 Kit.

(Source: https://www.dojindo.com/store/p/456-Cell-Counting-Kit-8.html)

3.2.10.2 General Method

10 µl of the CCK-8 solution were added to each well of the 96 well plates. The plates were incubated for 1 to 4 hours in the incubator and the absorbance was measured at 450 nm using a microplate reader.

3.2.10.3 Calculations

$$\% \text{ Stimulating Index (SI)}: \frac{\text{Average OD450 of wells containing antigen stimulated cells}}{\text{Average OD450 unstimulated cells}} \times 100$$

3.2.11 *Blastocytsis* sp. Specific Enzyme-linked Immunosorbent Assay (ELISA) IgG Antibody and IgG1/IgG2a Isotype Assessment

The serum samples obtained from mice as mentioned in method 3.2.6 were tested for the presence of specific IgG by performing ELISA test. 96-well plates (Corning) were coated with (100 µl/well); 10 µg/ml *Blastocystis* sp. ST3 asymptomatic (1-3) and symptomatic (1-3) solubilised antigen mixed with PBS, followed by incubation overnight at 4ºC. The negative control wells included diluent only six known negative serum

samples. The plates were then washed with PBS containing 0.05% Tween20 (PBST), blocked for 1 hour at 37ºC in PBS containing 5% FCS, and then washed with PBS. Thereafter, the serum samples with dilution of (1:200) (100 µl/well) were added to each well and incubated for 1 h at 37ºC. After incubation, the wells were washed and 100 µl of goat anti-mouse HRP antibody (Sigma Aldrich; diluted 1:60000 in PBS) were added and incubated for 1 h at 37ºC. Thereafter, the washing step was repeated and followed by addition of 100 µl substrate solution and incubation for 30 min at 37ºC. 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).

3.2.12 Statistical analysis

Data, including antibodies, cytokine and lymphocyte proliferation was analysed by one-way analysis of variance (ANOVA) using SPSS software 22. The graphs were plotted using Graph Pad Prism 8.

3.3 Results

3.3.1 Lymphocytes Cells Proliferation Assay

The splenocyte derived lymphocytes proliferations or stimulation index (SI) upon stimulation with 10 µg/ml soluble antigen of *Blastocystis* sp. ST3 symptomatic and asymptomatic is shown in Figure 3.7. The splenocytes showed highest proliferation when induced with a lectin, Con-A (Concanavalin-A carbohydrate-binding protein from jack beans, Sigma, USA) which targets the proliferation of T cells. The SI of the mice immunised with symptomatic and asymptomatic isolates is significantly higher compared to the mice immunised with PBS (negative control group) which increased 47-fold and 27-fold respectively. Meanwhile, the SI comparison between symptomatic and asymptomatic group showed that symptomatic isolates induced a 2-fold increase of lymphocytes cells compared to symptomatic group with significant difference of $P<0.001$ as reflected in Table 3.3. However, there were no significant differences shown between the isolates of the same group.

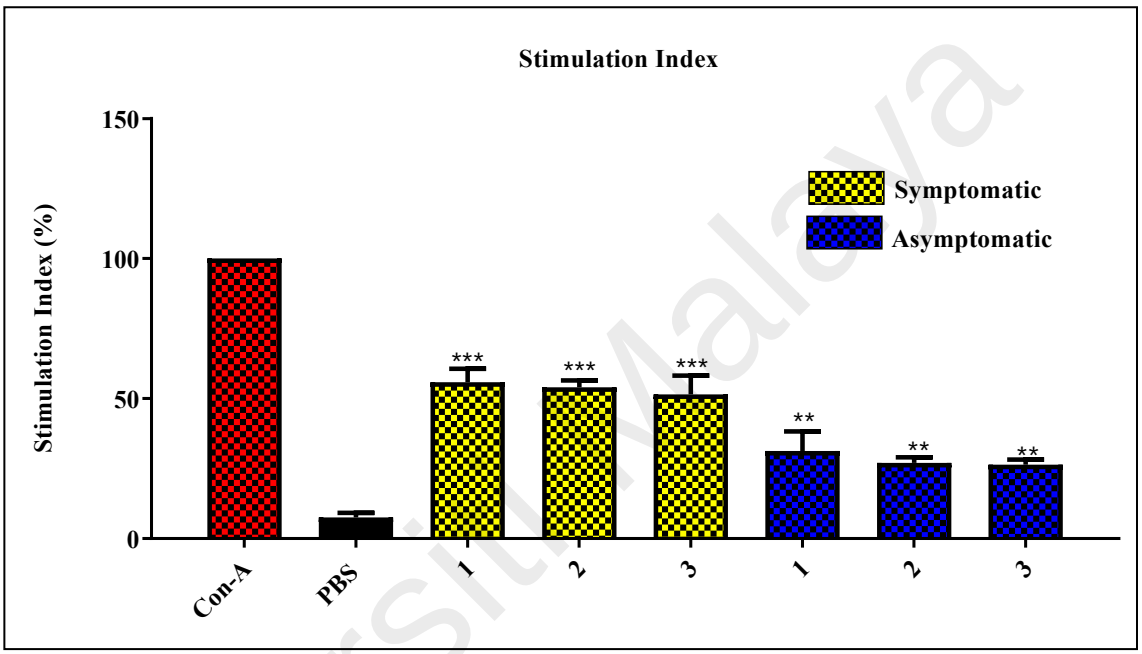


Figure 3.7: Stimulation Index of Splenocytes Derived Lymphocytes Cells Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Concanavalin–A (Con-A) was used as a positive control and spleen derived from PBS injected mice were served as negative control. Values are given in mean ± SD (n=9). **P < 0.01 and ***P < 0.001 was the comparison made against negative control (n=3).

Table 3.3: Lymphocyte cell proliferation upon stimulations with 10 µg/ml *Blastocystis* sp. solubilised antigen.

Group	Cell Proliferation (Number)	SI (%)
Symptomatic	2422564±97985**	5.4±0.46**
Asymptomatic	1274370±229334	3.2±0.96

Data is given as mean ± SD. The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). The differences were significant with, **P<0.001.

3.3.2 Th1 and Th2 Cytokine Assessment

In the present study, solubilised antigen of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates stimulated up-regulations of Th1 and Th2 cytokines in splenocytes. Table 3.4 indicate the summary of Th1 and Th2 response secreted by splenocytes derived T lymphocytes cells.

3.3.2.1 Th1 Cytokines

Th1 cytokines which are known as pro-inflammatory cytokine were assessed. In our study, we measured Th1 cytokines (IFNγ, IL-2) effects on splenocytes upon exposure to 10 μg/ml of solubilised antigen from ST3 *Blastocystis* sp. symptomatic and asymptomatic isolates. Splenocytes exposed to *Blastocystis* sp. symptomatic solubilised antigens significantly increase Th1 cytokines IFNγ (3.2-fold increase) and IL-2 (1.5-fold increase) as compared to asymptomatic solubilised antigens as reflected in Figure 3.8.

3.3.2.2 Th2 Cytokines

Th2 cytokines which are known as anti-inflammatory cytokine were assessed. In our study, we measured Th2 cytokines (IL-4 and IL-10) effects on splenocytes upon exposure to 10 μg/ml of solubilised antigen from *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates. Splenocytes exposed to *Blastocystis* sp. asymptomatic isolates significantly increase Th2 cytokines IL-4 (4.2-fold increase) and IL-10 (4.3-fold increase) as compared to symptomatic isolates as reflected in Figure 3.9.

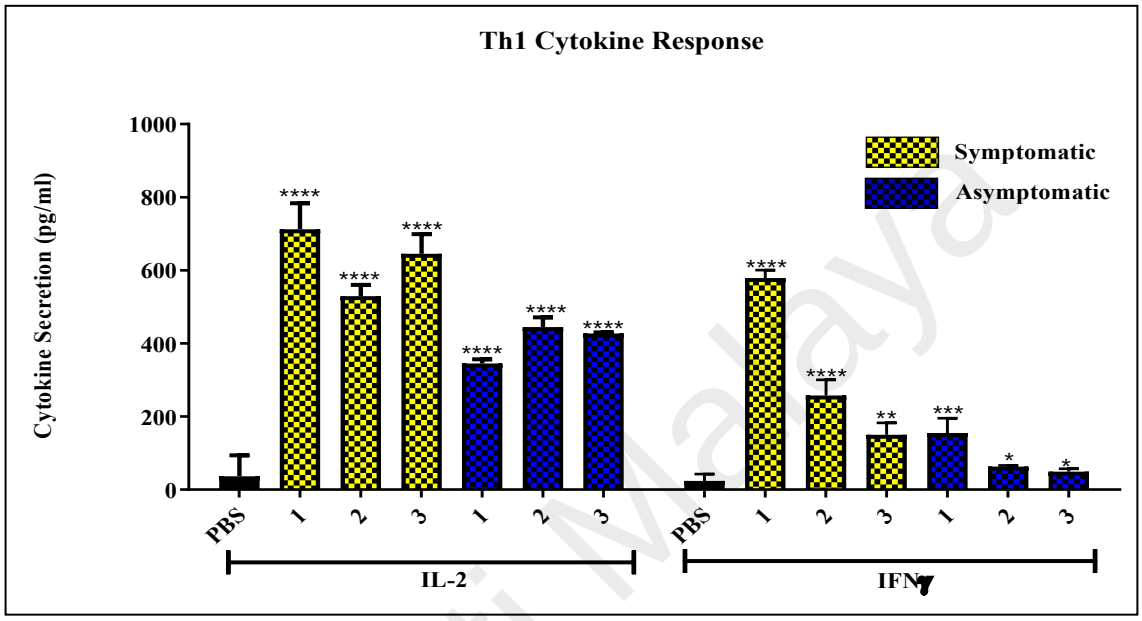


Figure 3.8: Th1 Cytokine Response of Splenocytes Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Values are given in mean ± SD (n=9). * P < 0.05 **P < 0.01 ***P < 0.001 ****P < 0.0001 was the comparison made against stimulation of the spleen derived from PBS injected mice was served as negative control (n=3).

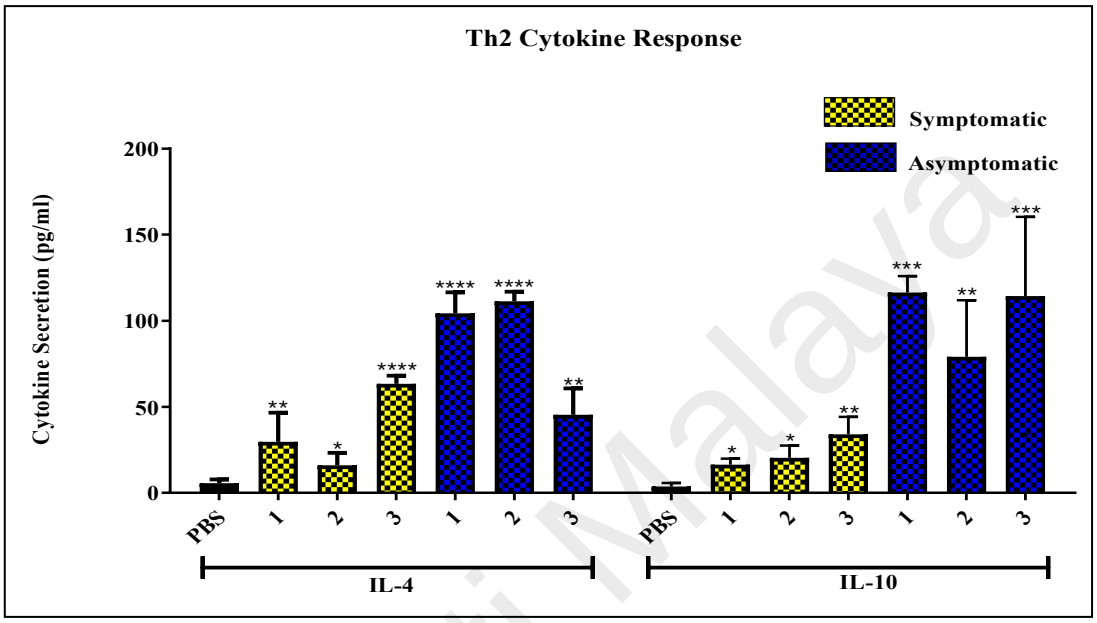


Figure 3.9: Th2 Cytokine Response of Splenocytes Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Values are given in mean ± SD (n=9). * P < 0.05 **P < 0.01 ***P < 0.001 ****P < 0.0001 was the comparison made against stimulation of spleen derived from PBS injected mice was served as negative control (n=3).

Table 3.4: Th1 and Th2 cytokine secretions by splenocytes upon stimulations with 10 µg/ml *Blastocystis* sp. ST3 solubilised antigen.

Isolates	Th1/Th2 Cytokine (pg/ml)	
Symptomatic	**IL2**	628.89±31.06**
Asymptomatic		405.89±48.25
Symptomatic	**IFNγ**	393.56±147.62**
Asymptomatic		122.67±51.62
Symptomatic	**IL4**	21.78±11.05
Asymptomatic		93.00±23.54*
Symptomatic	**IL10**	23.56±33.98
Asymptomatic		103.22±10.29*

Data is given as mean ± SD. The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). The differences (pg/ml) were significant with *P<0.05 and **P<0.01.

3.3.3 Antigen Dose Optimization through Specific IgG Assessment

Antigen dose optimization with different concentrations (10, 20, 30 and 40 µg/ml) of *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens were injected to the mice. The sera obtained from the mice after euthenisation, were subjected for IgG assessment. It was shown that *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen injected mice secreted highest IgG antibodies secretions at concentration of 20 and 40 µg/ml (Figure 3.6). Therefore 20 µg/ml soluble antigens were chosen as the optimal concentration for the mice injection as it required smaller amount of antigen.

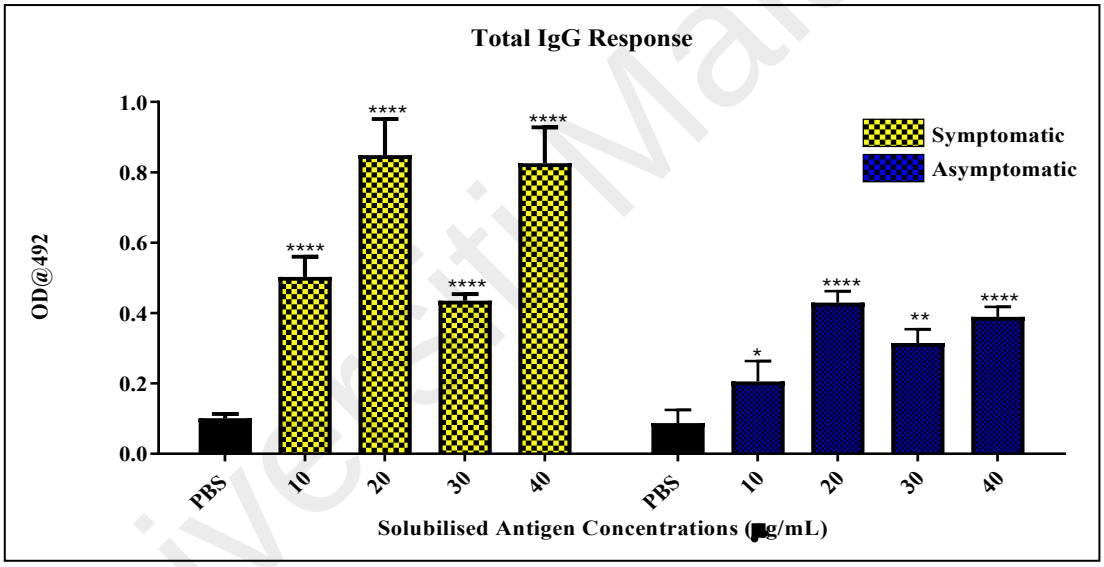


Figure 3.10: Total IgG Response in Sera Obtained from Mice Immunised with 10, 20, 30 and 40 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Values are given in mean ± SD (n=4) with significant difference of IgG secretions with * P < 0.05 **P < 0.01 ***P < 0.001 ****P < 0.0001. The comparison was made against sera obtained from mice injected with PBS injected mice and served as negative control (n=4).

3.3.4 Total Specific IgG/IgG1/IgG2a Antibody Assessment

In this study, total IgG response and its isotypes IgG1 and Ig2a was analysed from sera obtained from mice immunised with 20 µg/ml *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens as shown in Figure 3.11. The total IgG antibody response significantly increased by 9.4-fold in symptomatic group and 6.8-fold in asymptomatic group in comparison to PBS control. Meanwhile, IgG antibodies were secreted higher in symptomatic group at 1.4-fold difference in comparison to asymptomatic group. On the other hand, the IgG1 isotype increased 1.7-fold higher in asymptomatic group in comparison to symptomatic group while IgG2a isotype was significantly higher in symptomatic group with 1.8-fold difference with asymptomatic group. The comparison was carried out between *Blastocystis* sp. ST3 symptomatic and asymptomatic was reflected in Table 3.5.

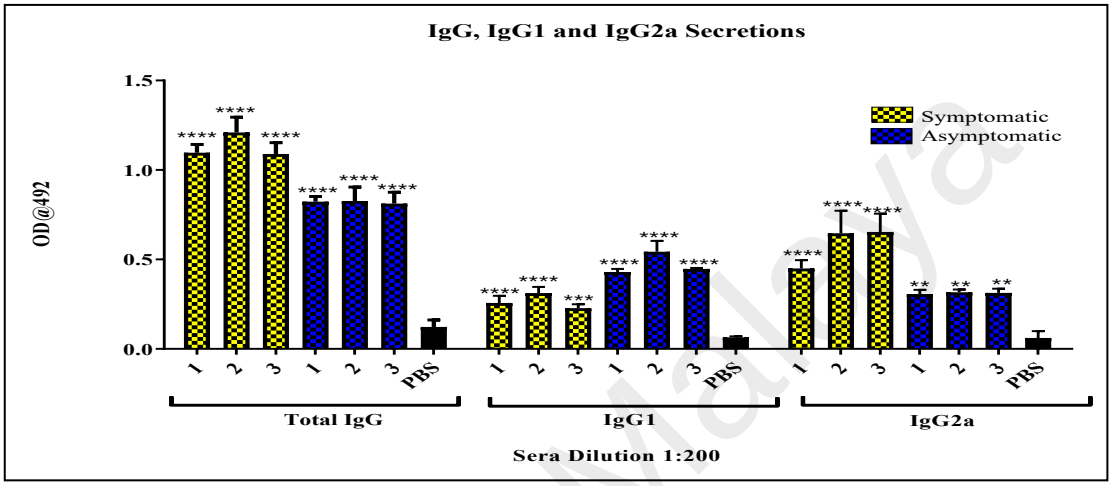


Figure 3.11: Total IgG, IgG1 and IgG2a Antibody Response in Sera Obtained from Mice Immunised with 20 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Values are given in mean ± SD (n=9) with significant difference of IgG, IgG1 and IgG2a secretions with **P < 0.01 ***P < 0.001 ****P < 0.0001. The comparison was made against sera obtained from mice injected with PBS injected mice and served as negative control (n=3).

Table 3.5: IgG, IgG1 and IgG2a antibody secretions in sera of mice immunised with 20 µg/ml *Blastocystis* sp. ST3 solubilised antigens.

Isolates	IgG	IgG1	IgG2a
Symptomatic	1.13±0.82***	0.27±0.05	0.58±0.13**
Asymptomatic	0.82±0.05	0.47±0.06***	0.31±0.02

Data is given as mean ± SD. The comparison was carried out between ST3 Blastocystis sp. symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). The differences (OD420) were significant with **P<0.01 and ***P<0.001.

Table 3.6: Overall immune response induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens.

Isolate	Cytokine Response (pg/ml)					Antibody (OD420nm)		Cells Stimulation Index (SI) %
	Th1		Th2		Th1:Th2	IgG		
	IL-2	IFNγ	IL-4	IL-10		IgG1	IgG2a	
S	629	394	22	24	22:1	0.27	0.58	5.4
AS	406	123	93	103	3:1	0.47	0.31	3.2

Data mean ± SD shows the overall immune response of Th1/Th21 cytokine response, IgG/ IgG1/IgG2a antibody secretions, and lymphocyte cells proliferations in mice induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens.

3.4 Discussion

The associations between antigenic variations, colonization and survival of a pathogen in the host were proven in studies which include intestinal protozoa parasites such as *Giardia lamblia* and *Entamoeba histolytica* (Svard et al., 1998 and Nakada-Tsukui et al., 2016). However, this association remains unexplored in *Blastocystis* sp. Therefore, this study investigates the fact whether the antigenic differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates could demonstrate distinct immune response profiles which possibly be implicated as an immune evasion strategy by this parasite. In order to assess the immune response profiling, comparison of antigenicity and immunogenicity between symptomatic and asymptomatic *Blastocystis* sp. isolates were made through mice immunization with standardised concentration of solubilised antigen (20 µg/ml) by using three different isolates. A pathogen which carries antigenic variations extensively involves immune response targeting; B cell (antibodies) and T cell response covering helper T cell (CD4+) and cytotoxic T cell (CD8+) recognition. This is because; B and T cells are exquisitely antigen-specific, especially CD4+ cells which play an important role in recognising extracellular and intracellular pathogens (Kitchen et al., 2004; Moretto et al., 2004).

The efficiency of the immunogenicity of an antigen can be evaluated by assessing the proliferation rate of pre-sensitised lymphocytes cells against specific antigens (Chandramathi et al., 2014). Therefore, in this study the *in vitro* effect of solubilised *Blastocystis* sp. antigen of symptomatic and asymptomatic isolates and its effect on previously sensitised lymphocytes (derived from splenocytes of immunised mice) proliferation were investigated by using the stimulation index (SI) calculations. Concanavalin-A (Con-A) was used as a positive control since it activates lymphocytes by binding to the various glycoproteins and also used in previous *Blastocystis* sp. study (Ragavan et al., 2014). Therefore, the comparison revealed that lymphocytes cells

established a significant proliferation *in vitro* and showed a higher establishment of immune responses against antigen re-stimulation. This observation implies a general perception that the symptomatic and asymptomatic pre-infected host immune cells are able to respond against the infection through establishment of lymphocyte memory cells. Generally, the lymphocytes cells obtained from the pre-infected host were proven to be antigenic and immunogenic where they possess the capability in establishing a quick and specific response. However, the comparison made between symptomatic and asymptomatic solubilised antigen injected groups revealed that significantly different stimulation index was observed. The symptomatic isolates exhibited higher stimulation index in comparison to asymptomatic isolates. This difference has shown the distinctiveness of these isolates in carrying a diverse degree of immunopathogenicity which could possibly hamper the immune system of the infected host despite being able to respond against the re-infection. Therefore, to further analyse this data, the results obtained from T cell cytokine response was assessed.

Generally, the subdivisions of helper T cells namely Th1 and Th2 type cells are able to produce cytokines that subdivided into pro-inflammatory and anti-inflammatory (Berger et al., 2000). In this study, the cytokine profile of T cells against stimulated *Blastocystis* sp. soluble antigens were evaluated through the assessment of Th1 and Th2 cytokine responses. The assessment was done as early as 24 hours of *Blastocystis* sp. solubilised antigen stimulation where Th1 mediated cytokine (IL-2) and Th2 mediated cytokine (IL-4) was assessed. In general, IL-2 cytokine secreted by activated T cells and is a key regulator for Th1 mediated immune response whereby IL-4 cytokine helps to induce the proliferations of Th2 type cells. In this study, IL-2 cytokine was secreted highly by symptomatic solubilised antigen induced group. However, IL-2 cytokine was also secreted by asymptomatic solubilised antigen induced group despite highly secreted IL-4 cytokine which is a Th2 cells inducer. It was reported that, IL-2 cytokine function is

not limited to Th1 cells proliferation inducer, but it may also help to induce the proliferations of Th2 type cells to secrete IL-4 cytokine (Zhu et al., 2003). This observation was proven in this study where significantly high secretions of IL-4 cytokines were induced by splenocytes stimulated with asymptomatic solubilised antigen in the presence of IL-2 cytokine secretions at 24 hours. This observation was supported by the comparison with lymphocyte cells proliferations in asymptomatic solubilised antigen induced group which was significantly lower in comparison to symptomatic solubilised antigen stimulated group. This was due to the significantly higher secretions of IL-4 cytokine which is being an anti-inflammatory cytokine possibly enhanced by IL-2 cytokine secretions. This could be due to IL-4 cytokine which may have caused the down-regulations of Th1 cytokine response by secreting higher anti-inflammatory cytokine response during long-term stimulations. Therefore, the IL-2 and IL-4 cytokine analysis in this study can be interpreted as; different T cell response may induce in host infected with symptomatic and asymptomatic *Blastocystis* sp. ST3 antigens. This is due to the fact that, these isolates were able to skew the Th cells towards Th1 and Th2 as early as 24 hours.

Further observations on Th1/Th2 cytokine responses were performed by assessing pro-inflammatory cytokine response, IFNγ and anti-inflammatory cytokine response, IL-10 which were analysed after 72 and 96 hours of stimulations respectively. The IL-10 cytokine significantly increased in splenocytes culture stimulated with asymptomatic solubilised antigen. In contrast, high secretions of IFNγ cytokine was observed in splenocytes culture stimulated with symptomatic solubilised antigen. Therefore, in this study, IL-10 and IFNγ cytokine analysis supported the evidence above that, symptomatic and asymptomatic infection in host may trigger the stimulations of T cells to diverse the host immune response towards Th1 and Th2 cytokine at early infection (acute) and extend it during a longer infection (chronic). Moreover, the imbalance of Th1/Th2 profiles upon stimulation with *Blastocystis* sp. symptomatic and asymptomatic isolates which were

shown in Table 3.6 implicated that, the symptomatic *Blastocystis* sp. infection induce higher proliferations of Th1 cells to secrete higher pro-inflammatory cytokine. On the other hand, asymptomatic infected individuals may exhibit lower secretions of pro-inflammatory cytokine due to enhanced anti-inflammatory response by Th2 proliferated cells. Both the isolates exhibited an unbalanced secretion of Th1/Th2 cytokine responses during re-stimulation. Therefore, this study may also implicate that, during pro-longed symptomatic isolate infection in host, higher intestinal damage may occur in the host intestinal region due to high pro-inflammatory cytokine establishment. In contrast, asymptomatic isolate may cause lesser intestinal damage in the host due to high anti-inflammatory cytokine establishment.

In addition to the above evidences of immunogenicity and antigenicity triggered by symptomatic and asymptomatic solubilised antigen, the assessment of humoral response was included. In this study, a high elevation of IgG response was observed in sera of Balb/c mice, when injected with *Blastocystis* sp. symptomatic and asymptomatic soluble antigens. The IgG antibodies were mainly assessed due to the fact that, IgG is the most versatile immunoglobulin to act against invading pathogens. Besides, IgG involves in phagocytosis and in activation of complement system to eradicate pathogen infection which makes up the largest percentage of 80% from the circulating immunoglobulin (Chandramathi et al., 2014). Research has also proven that, IgG humoral response is highly elicited when the antibodies produced by the host are able to recognize and bind to epitopes on the surface of antigens with high specificity (Wang et al., 2011). Therefore, these characteristics of B cells have instigated to the assessment of IgG response and its possible associations with antigenic heterogeneity among mice injected with ST3 symptomatic and asymptomatic solubilised antigen. The results in this study has shown significantly high level of humoral IgG antibodies were secreted with a fold increase of 9.4 and 6.8 in symptomatic and asymptomatic solubilised antigen injected mice in

comparison to PBS control group. Further assessment of IgG isotypes, IgG1 and IgG2a antibodies were analysed because these isotypes are used as an indicators Th1 and Th2 cytokine responses in previous study (Mounford et al., 1994). It was found that sera of mice immunised with symptomatic and asymptomatic solubilised antigen contained a mixture of IgG1/IgG2a isotypes. It was observed that, asymptomatic antigen injected group has shown predominance of IgG1 antibody secretions whereas symptomatic antigen injected group induced predominance of IgG2a antibody secretions which are correlated with the results of Th1 and Th2 response induced by symptomatic and asymptomatic antigens respectively.

In this study the immunogenicity and antigenicity assessment were assessed through the correlations of lymphocytes expansion, Th1 and Th2 cytokine responses along with IgG antibody secretions modulated by *Blastocystis* sp. symptomatic and asymptomatic isolates. It was observed that, Th1 and Th2 cells proliferation induced by *Blastocystis* sp. symptomatic and asymptomatic isolates were capable of supporting antigen-specific B cell clonal expansion and IgG antibody secretions. This is because, despite being cellular mediated immune response, Th1 type cells were able to trigger 1.4-fold higher of total IgG response in symptomatic antigen injected group compared to asymptomatic antigen injected group which dominated a Th2 type (antibody mediated response). This was possibly due to the expressions of CCR7 on Th1 cells which may help in B cell clonal expansions in *in vivo* conditions as postulated by previous researchers upon ovalbumin antigen stimulation in mice (Smith et al., 2000; Weinstein et al., 2012; Rao, 2018). Generally, CCR7 receptor is found in various lymphoid tissues and this receptor is able to activate B and T lymphocytes. It has been shown to regulate T cell activation especially during chronic inflammation by associating with CXCR5 signalling on B cells to induce the B cell access to T helper cells (McGovern et al., 2013). Moreover, studies have also proven that transition from antibody mediated response to cell mediated

response occurred in protozoan parasite infections such as helminth, *Toxoplasma Gondi*, *Plasmodium* and *Trypnasomes*. These parasites mediated B cell activation where it up-regulates CCR7 ligand and to facilitate the migration toward T cells zone which will subsequently induced T cell proliferation (McGovern et al., 2013). Therefore, the T cell proliferation induced by *Blastocystis* sp. in this study may also activated through CCR7 ligand activation. However further investigation is needed to confirm this associations.

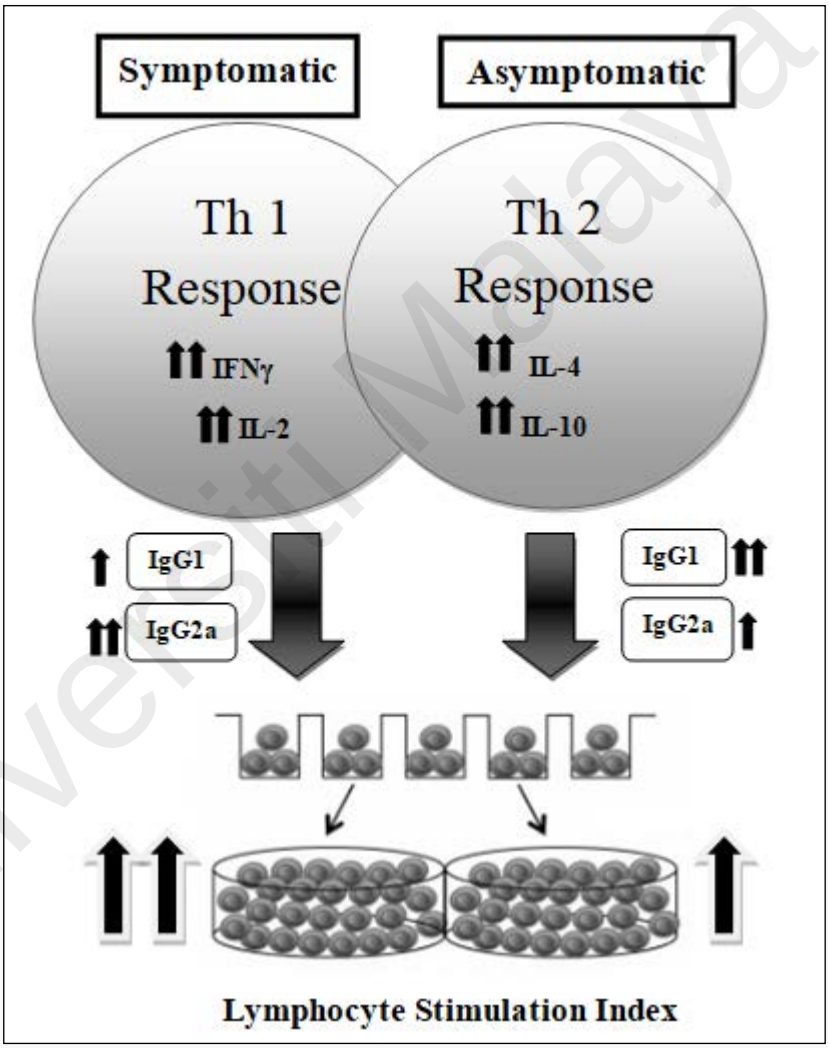
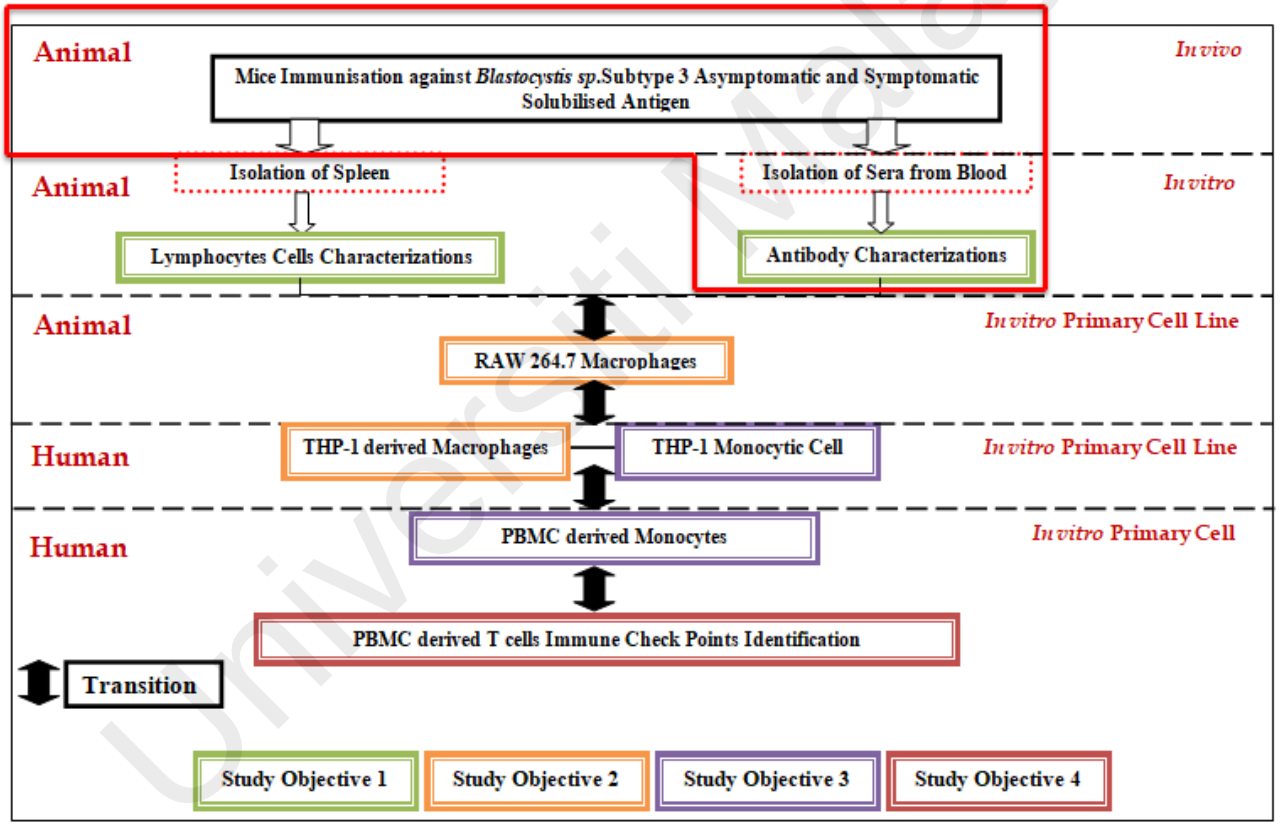


Figure 3.12: Summary of Adaptive Immune Response Induced by *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Antigens.

3.5 Conclusion

There has been thus far no information on whether antigenicity and immunogenicity differences portrayed by symptomatic and asymptomatic *Blastocystis* sp. antigens can influence immune modulations in the infected host. Therefore, this *in vivo* and *in vitro* analysis basically provides a better understanding of how host immunity would possibly react against *Blastocystis* sp. symptomatic and asymptomatic antigens of ST3 origin. It is possible to hypothesise that surface variations between asymptomatic and symptomatic antigens may have contributed to the distinctive immune responses as shown in Figure 3.12 above. In this study for the first time it was demonstrated that, symptomatic antigens have proven to be highly antigenic and immunogenic compared to asymptomatic antigens due to higher Th1 immune response establishment and thus can be concluded as being more pathogenic. This has also shown the antigenic differences demonstrated by symptomatic and asymptomatic isolates. Therefore, it becomes imperative to investigate the degree of **antigen diversity** among the *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates to further identify its uniqueness. Hence, to confirm the degree of antigen divergence among symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates, this study was substantiated by the observation of complement mediated cytotoxicity (CDC) assay which were discussed in the following Chapter 4.

CHAPTER 4: *IN VITRO* ASSESSMENT OF ANTIGEN SPECIFICITY AND CROSS-REACTIVITY AMONG *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC ISOLATES.



4.1 Introduction

The **antigenic specificity** of *Blastocystis* sp. ST3 isolates was demonstrated through the characterization of the adaptive immune response by performing antigenicity and immunogenicity assessments. It was observed that, the symptomatic and asymptomatic isolates of this parasite demonstrated distinct adaptive immune responses which may indicate their unique pathogenic role. However, there was difference of immune response observed among the isolates of the same group (symptomatic and symptomatic isolates/asymptomatic and asymptomatic isolates). A study conducted by Mirza et al., (2011) had proven that, there were antigenic heterogeneity of protein profiles obtained from patients infected with symptomatic *Blastocystis* sp. isolates. It was concluded that, each of the isolates may display a different pathogenic role. Nevertheless, they were no antigen diversity assessment performed among the isolates of symptomatic and asymptomatic *Blastocystis* sp. ST3 to identify the degree of the **antigenic uniqueness** among them.

Generally, antigen specificity defines the ability of an immune system to differentiate between various antigens whereas antigen cross-reactivity evaluates the degree where various antigens were recognised as similar by the immune system. The molecular determinants play a major role in dictating the specificity and **cross-reactivity** across all antigens by segregating the populations based on its **unique variations** (Frank, 2002). The cross-reactivity of antibodies between symptomatic and asymptomatic *Blastocystis* sp. ST3 has not been characterised so far. It was reported that, parasite surfaces share molecules which immensely overlap with antibody binding sites also known as epitopes (Frank, 2002). Previous studies have also reported antibody cross-reactivity among *Blastocystis* sp. isolates of human and animal origins (Tan et al., 2001; Tan et al., 1997). Therefore, it becomes imperative to investigate the degree of specificity and cross-reactivity between symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates to

confirm the differences of adaptive immune response demonstrated by these isolates. Therefore, in this study the antigen diversity of these parasite was determined through the analysis of complement mediated cytotoxicity (CDC) assay. Past studies have proven CDC assay as an efficient method in differentiating and identifying cross-reactivity mediated by using polyclonal antibodies (Konishi et al., 2007; Kitai et al., 2010. In this study, the antigenic heterogeneity of this parasite was further substantiated by performing CDC assay. CDC assay, is a mechanism where antibodies lyse the targeted cells by activating a cascade of complement-related reactions. The **antigenic diversities** were determined based on the **degree of cell lysis** obtained among these isolates. Further descriptions pertaining to CDC assay are as described below.

4.1.1 Cell Mediated Cell Lysis (CDC) Assay

The CDC mechanism usually involves IgG antibodies and its subtypes such as IgG1 and IgG2. The antigen specificity of this parasite covering ST3 symptomatic and asymptomatic isolates in inducing antibodies was validated in last chapter through the analysis of specific IgG, IgG1 and IgG2a responses obtained from mice sera. In order to obtain the sera, mice were injected with *Blastocystis* sp. solubilised antigens for the duration of 56 days by two weeks interval. Research has proven that, IgG antibodies are highly dominant in comparison to IgM antibodies especially during secondary immune response against an antigen (Goldammer et al., 2002) which is reflected in Figure 4.1 below.

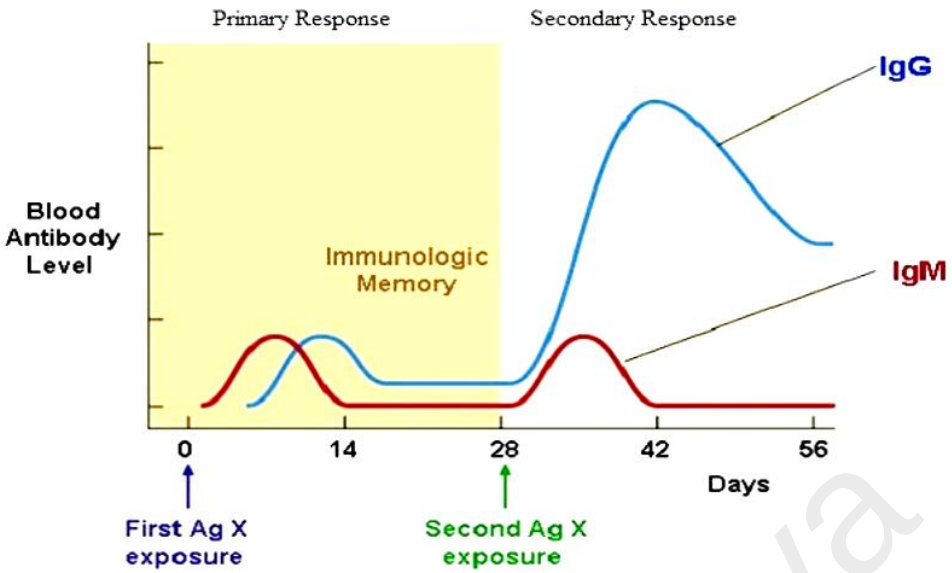


Figure 4.1: IgG and IgM Antibody Responses During Primary and Secondary Ag Exposure.

(Source: http://microbiologynotes.com)

Therefore, in this chapter IgG antibody OD readings were used to analyse the cell lysis mechanism. Besides it was reported that, specific non-covalent interactions between binding segments of IgG antibodies and antigen binding resulted in the formation antibody hexamers. These hexamers will be recruited to activate C1, the first component of complement, thus the complement cascade will be triggered and complement activation as well as killing of target cells will occur. Besides, CDC assay is reliable in measuring low levels of specific antibodies.

4.1.2 IgG Antibody

Immunoglobulin G (IgG) is dominating the humoral responses in humans and mice which is 75% of the total immunoglobulin concentration in sera being IgG. The immunoglobulin molecule characterised as a membrane bound on B lymphocyte cells and in a secreted form that is produced by activated B lymphocyte cells which is known as the plasma cells. IgG is a later development that owes its value to the ability of its Fc portion to bind to the receptors on phagocytic cells. It also gains access to the extra-

vascular spaces and (via the placenta) to the fetus. In most species, IgG has become further diversified into subclasses. While IgG antibody has been the dominant class of immunoglobulin utilized in research and therapeutic applications, attention is turning to other classes such as IgM antibodies which have their own distinct features and corresponding strengths (Vidarsson et al., 2014).

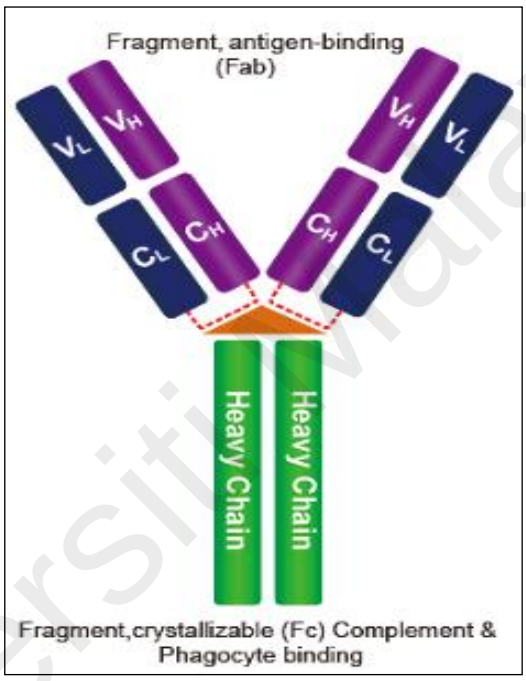


Figure 4.2: IgG Antibody Structure.

(Source: https://www.genscript.com)

4.1.3 IgG Antibody Cross Reactivity with Various Antigens

Antibody-antigen interactions typically take place between the paratope (antigen binding site) at where the antigen binds, and the epitope (antigen molecule) being the site on the antigen. During a pathogenic infection, in *in vivo* B cells will produce immunoglobulins against the intact antigens (in a form of soluble proteins), and thus specifically identify surface epitopes. The ability of the immunoglobulins to identify the antigen epitopes enable the B cell to discriminate its antibody response against an antigen. Moreover, it may permit the same antibody to bind to divergent antigens which may share similar epitopes. This phenomenon is referred as cross-reactivity (Sela-Culang et al., 2013; Frank, 2004).

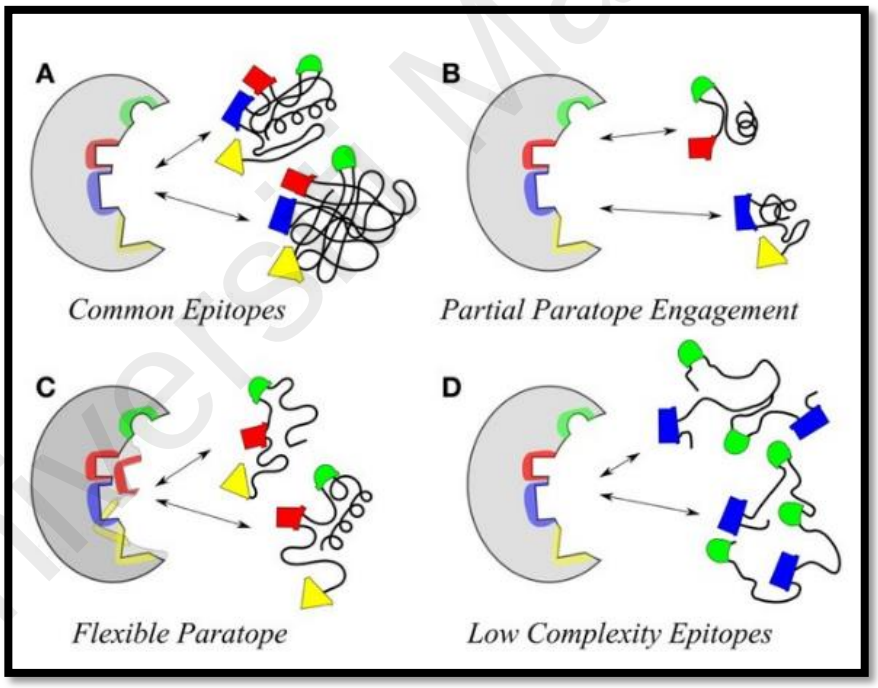


Figure 4.3: The Epitopes Sharing Among The Antigen-Antibody

(Source: Kieber-Emmons et al., 2014)

The figure above (4.3) shows the cross-reactivity between antigens and an antibody. (A) Different antigen molecules (epitopes) may share the same structure to bind to the same antibody (B) The same antibody may accommodate various antigens (epitope) in different part of its binding region (paratope). (C) The flexibility of the binding region may allow interaction with various epitopes. (D) Different flexible antigen molecules with repetitive low complexity structure containing common structure have a high probability of fitting in the same paratope. These are aspects of cross-reactivity binding, may occur in any of the combination such as (A and D) or (A and C) (Kieber-Emmons et al., 2014).

4.1.4 Complement Activated Cell Lysis

The complement system consists of approximately 30 proteins which are widely found in the blood plasma or cell surfaces and these proteins are responsible for detecting and killing the invading pathogens. The main element that induces the pathogens killing is the C3 convertase, an enzyme that resides on the surface of the pathogens that marks them for destructions by immune cells. This C3 convertase enzyme will amplify signals and induce various effector functions by the immune cells to eliminate the pathogens (Swe et al., 2017). During the phase of infection, the formation of active C3 convertases for pathogen eliminations is initiated through three different pathways.

Step 1: Complements will be formed and activated spontaneously on almost every cell surface. Due to this fact, the cells will start expressing regulatory proteins on their surfaces which eventually lead to the C3 convertases inactivation.

Step 2: Complement provides its own Pattern Recognition Receptors (PRRs) through Mannan Binding Lection (MBL) -Pathway which allows the binding of MBL to mannose or N-acetyl-glucosamine on bacterial surfaces and eventually activates serine proteases that form an active C3 convertase.

Step 3: Complement is activated thorough the classical pathway mediated by a main component of the complement which is C1-complex. C1 consists of a pattern recognition molecule C1q and branching into two serine proteases which are C1r and C1s. The binding of C1q to the targets like MBL and antigen-antibody, will activate the serine proteases (C1r and C1s) and leads to the activation of C3 convertase. The activation of C3 convertase activates the antibody mediated effector functions and therefore it mediates a bridge between innate and adaptive immunity (Janeway et al., 2001).

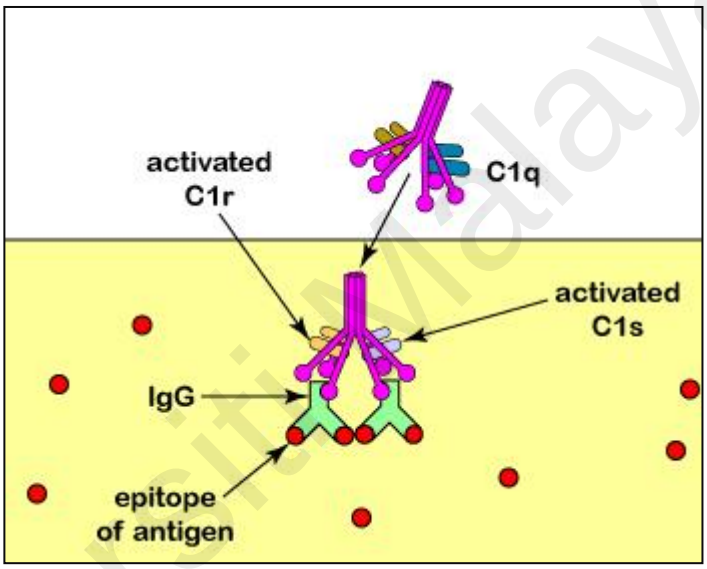


Figure 4.4: Complement Binding with IgG Antibody

(Source: https://bio.libretexts.org)

In this study the classical pathway of complement mediated cell lysis was initiated. The classical pathway can be activated by several factors such as IgG antibodies. IgG involvements are dominant during secondary immune response. The IgG subclasses such as IgG1 and IgG2 are said to be highly involved in the C1q activation and membrane cell lysis (Nesargikar et al., 2012; Noris and Remuzzi, 2013).

4.2 Materials and Methods

4.2.1 Source of *Blastocystis* sp.

As mentioned in 3.2.1.

4.2.2 Axenization of *Blastocystis* sp. Isolates

As mentioned in 3.2.2.

4.2.3 Animal Selection, Housing and Ethical Clearance

As mentioned in 3.2.4.

4.2.4 Immunization

As mentioned in 3.2.5.

4.2.5 Balb/c mice Blood Sera Extraction

As mentioned in 3.2.6. The sera were heat inactivated at 56ºC for 20 minutes.

4.2.6 Sera and *Blastocystis* sp. ST3 Cells Cytotoxicity Analysis

For the CDC assay, symptomatic (S1-3) and asymptomatic (AS1-3) *Blastocystis* sp. ST3 cell suspension containing 5×10^4 cells in 50 µl of sera-free Jones Medium (10-fold serial diluted) was mixed with an equal volume of test sera diluted in Jones Medium incubated on ice for 30 min (Appendix E). 11µL of human complement (Sigma, USA) was added to make a final concentration of 10% and was incubated at 37°C for 4 hours. After 4 hours the cells were subjected for CCK-8 analysis to quantitate the viable cells following protocol by Konishi et al., 2007.

4.2.7 Cell Counting Kit-8 (CCK-8) Analysis

As mentioned in 3.2.10.

4.2.8 Calculations

The percentage of specific cell lysis was calculated according to the manufacturer's instructions by using the following formula:

$$\% \text{ Viable Cells: } \frac{\text{Average OD450 of wells containing antigen stimulated cells}}{\text{Average OD450 unstimulated cells}} \times 100$$

4.2.9 Cell Cytotoxicity and Cross-reactivity Study Design

The cell lysis was observed between the cells and sera of ST3 *Blastocystis* sp. following the experimental matrix design in Table 4.0. The following terms were used to describe the cell cytotoxicity reactions.

1. **Same isolates** described for the reaction between eg: (S1 cells with S1 Sera) and (AS1 cells with AS1 sera)
2. **Same group** described for the reaction between eg: (S1 cells with S2 and S3 sera) and (AS1 cells with AS2 and AS3 sera)
3. **Different group** described for the reaction between eg: (S1 cells with AS1, AS2 and AS3 sera).

Table 4.1: The matrix experimental design of cell cytotoxicity and cross-reactivity analysis between *Blastocystis* sp. ST3 symptomatic and asymptomatic cells and sera obtained from immunised mice

Cell / Sera	AS1	AS2	AS3	S 1	S 2	S 3
AS 1	√	√	√	√	√	√
AS 2	√	√	√	√	√	√
AS 3	√	√	√	√	√	√
S 1	√	√	√	√	√	√
S 2	√	√	√	√	√	√
S 3	√	√	√	√	√	√

4.2.10 Statistical analysis

Data for cell lysis and was analysed by one-way analysis of variance (ANOVA) using SPSS software 22. The graphs were plotted using Graph Pad Prism 8.

4.3 Results

4.3.1 Cell Lysis at 1:10 Sera Dilution

4.3.1.1 Cell Lysis of Same Isolates

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) isolates were co-cultured with sera obtained from mice injected with symptomatic (S1-3) and asymptomatic (AS1-3) isolates diluted at 1:10. The percentage cell lysis is reflected in Table 4.2. It was observed that *Blastocystis* sp. symptomatic (S1-3) and asymptomatic (AS1-3) isolates induced significantly higher cell lysis when they were co-cultured with the sera of the same isolates. The average percentage cell lysis for symptomatic group was 82% whereby those of the asymptomatic group was 86%. Therefore, the average of 83% was used as benchmark for specific cell lysis for the subsequent comparisons.

Table 4.2: *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Isolates Specific Cell Lysis

Symptomatic Isolates	**S1**	**S2**	**S3**	**Average A &B** 84%
% Lysis	96.0±0.1	79.4±0.9	71.9±0.1	
Asymptomatic Isolates	**AS1**	**AS2**	**AS3**	
% Lysis	84.0±0.3	87.2±1.9	85.7±0.6	

Data is given as mean ± SD (n=2). The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). There was no significant difference observed.

4.3.1.2 Cell Lysis of Same Group (Symptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) cells were co-cultured with sera obtained from mice injected with symptomatic (S1-3) isolates diluted at 1:10. The percentage lysis among the isolates is shown in Table 4.3. It was observed that *Blastocystis* sp. symptomatic (S1-3) cells induced average cell lysis of 44% when they were co-cultured with the sera of the same group (S1-3).

Table 4.3: Cross-reactivity among *Blastocystis* sp. ST3 symptomatic group

***Blastocystis* sp. Cells**	**Sera (1:10)**	**Lysis (%)**
S1	S2	49.0±2.3
	S3	29.3±1.4
S2	S1	37±2.1
	S3	55.5±1.5
S3	S1	39.3±1.2
	S2	53.1±0.2

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.1.3 Cell Lysis of Same Group (Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) isolates diluted at 1:10. The percentage cell lysis among the isolates is shown in Table 4.4. It was observed that *Blastocystis* sp. asymptomatic (AS1-3) cells induced average cell lysis of 48% when they were co-cultured with the sera of the same group (AS1-3).

Table 4.4: Cross-reactivity among *Blastocystis* sp. ST3 asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:10)**	**Lysis (%)**
AS1	AS2	49.7±0.7
	AS3	59.5±0.6
AS2	AS1	48.6±0.54
	AS3	37.4±0.1
AS3	AS1	41.3±1
	AS2	48.7±0.1

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.1.4 Cell Lysis of Different Group (Symptomatic with Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) and symptomatic (S1-3) isolates diluted at 1:10. The percentage lysis among the isolates is shown in Table 4.5. It was observed that *Blastocystis* sp. asymptomatic and symptomatic groups induced average cell lysis of 17% cross-reactivity upon co-culture.

Table 4.5: Cross-reactivity between *Blastocystis* sp. ST3 symptomatic and asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:10)**	**Lysis (%)**	***Blastocystis* sp. Cells**	**Sera (1:10)**	**Lysis (%)**
S1	AS1	16.6±0.2	AS1	S1	25.4±0.6
	AS2	20.2±0.4		S2	13.5±0.6
	AS3	13.4±0.4		S3	11.5±0.2
S2	AS1	5.7±0.3	AS2	S1	18.4±0.3
	AS2	16.8±0.04		S2	21.9±1.1
	AS3	4.2±0.1		S3	29.0±0.2
S3	AS1	9.9±0.04	AS3	S1	19.7±0.1
	AS2	11.4±0.6		S2	22.7±1.3
	AS3	13.7±0.3		S3	36.7±0.2

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

			% Cell Lysis							
			Serum (1:10)						PBS Control	IgG (OD492)
			Symptomatic			Asymptomatic				
			1	2	3	1	2	3		
Blastocystis sp. ST3 Live Cells	Symptomatic	1	92	37	39	25	18	20	0.68	2.63
		2	49	79	53	14	22	23	0.16	1.83
		3	29	55	72	12	29	37	0.99	2.37
	Asymptomatic	1	17	6	10	84	49	41	0.37	1.48
		2	20	17	11	49	87	49	1.76	1.13
		3	13	4	14	60	37	86	0.44	1.10

Low <1%
Medium <84%
High >84%

Figure 4.5: Summary Percentage of *Blastocystis* sp. ST3 Cell Lysis at 1:10 sera Dilution

4.3.2 Cell Lysis at 1:100 Sera Dilution

4.3.2.1 Cell Lysis of Same Isolates

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) isolates were co-cultured with sera obtained from mice injected with symptomatic (S1-3) and asymptomatic (AS1-3) isolates diluted at 1:100. The percentage cell lysis is reflected in Table 4.6. It was observed that *Blastocystis* sp. symptomatic (S1-3) and asymptomatic (AS1-3) isolates induced significantly higher cell lysis when they were co-cultured with the sera of the same isolates. The average percentage cell lysis for symptomatic group was 42% whereas for the asymptomatic group was 41%.

Table 4.6: *Blastocystis* sp. ST3 symptomatic and asymptomatic Isolates Specific Cell Lysis

Symptomatic Isolates	**S1**	**S2**	**S3**	**Average A &B =42%**
% Lysis	39.6±0.6	46.3±1.2	40.1±0.4	
Asymptomatic Isolates	**AS1**	**AS2**	**AS3**	
% Lysis	33.4±1.0	30.2±1.4	61.4±0.7	

Data is given as mean ± SD (n=2). The comparison was carried out between *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). There was no significant difference observed.

4.3.2.2 Cell Lysis of Same Group (Symptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) cells were co-cultured with sera obtained from mice injected with symptomatic (S1-3) isolates diluted at 1:100. The percentage cell lysis is reflected in Table 4.7. It was observed that *Blastocystis* sp. symptomatic (S1-3) cells induced average cell lysis of 24% when they were co-cultured with the sera of the same group (S1-3).

Table 4.7: Cross-reactivity among *Blastocystis* sp. ST3 Symptomatic group

***Blastocystis* sp. Cells**	**Sera (1:100)**	**Average Lysis (%)**
S1	S2	29.1±1.4***
	S3	21.4±1.6
S2	S1	20.8±2.1
	S3	23.6±1.1
S3	S1	22.7±0.6
	S2	24.7±0.2

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.2.3 Cell Lysis of Same Group (Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) isolates diluted at 1:100. The percentage cell lysis is reflected in Table 4.8. It was observed that *Blastocystis* sp. asymptomatic (AS1-3) cells induced average cell lysis of 25% when they were co-cultured with the sera of the same group (AS1-3).

Table 4.8: Cross-reactivity among *Blastocystis* sp. ST3 Asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:100)**	**Average Lysis (%)**
AS1	AS2	26.9±2.4
	AS3	31.5±0.7***
AS2	AS1	22.5±0.1
	AS3	23.3±0.5
AS3	AS1	19.1±0.3
	AS2	26.5±0.5****

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.2.4 Cell Lysis of Different Group (Symptomatic with Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) and symptomatic (S1-3) isolates diluted at 1:100. The percentage cell lysis is reflected in Table 4.9. It was observed that *Blastocystis* sp. asymptomatic and symptomatic groups induced average cell lysis of 9% cross-reactivity upon co-culture.

Table 4.9: Cross-reactivity between *Blastocystis* sp. ST3 symptomatic and asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:100)**	**Lysis (%)**	***Blastocystis* sp. Cells**	**Sera (1:100)**	**Lysis (%)**
S1	AS1	8.9±0.4	AS1	S1	16.7±0.3
	AS2	13.1±1.1		S2	12.6±0.1
	AS3	12.4±0.5		S3	3.8±0.7
S2	AS1	12.7±0.03	AS2	S1	10.7±0.03
	AS2	6.2±0.1		S2	4.3±1.2
	AS3	4.1±0.1		S3	6.4±0.7
S3	AS1	20.5±0.6	AS3	S1	2.4±0.7
	AS2	8.7±0.4		S2	5.2±0.2
	AS3	6.5±0.01		S3	-2±3.2
Average Lysis (%) (A)		**10**	**Average Lysis (%) (B)**		**7**

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

			% Cell Lysis							
			Serum (1:100)						PBS Control	IgG (OD492)
			Symptomatic			Asymptomatic				
			1	2	3	1	2	3		
Blastocystis sp. ST3 Live Cells	Symptomatic	1	40	21	23	17	11	2	0	1.357
		2	30	46	25	13	4	5	0	1.246
		3	21	24	40	4	6	-2	0	1.208
	Asymptomatic	1	9	13	21	33	23	19	0	1.029
		2	13	6	9	27	30	27	0	1.030
		3	12	4	7	32	23	61	0	0.941

Low	<1%
Medium	<84%
High	>84%

Figure 4.6: Summary Percentage of *Blastocystis* sp. ST3 Cell lysis at 1:100 Sera Dilution.

4.3.3 Cell Lysis at 1:1000 Sera Dilution

4.3.3.1 Cell Lysis of Same Isolates

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) isolates were co-cultured with sera obtained from mice injected with symptomatic (S1-3) and asymptomatic (AS1-3) isolates diluted at 1:1000. The percentage cell lysis is reflected in Table 4.10. It was observed that *Blastocystis* sp. symptomatic (S1-3) and asymptomatic (AS1-3) isolates induced significantly higher cell lysis when they were co-cultured with the sera of the same isolates. The percentage cell lysis for symptomatic group was 16% whereby asymptomatic group was 26%.

Table 4.10: *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Isolates Specific Cell Lysis

Symptomatic Isolates	**S1**	**S2**	**S3**	**Average A &B =21%**
% Lysis	14.4±1.8	13.6±1.6	21.1±2.3	
Asymptomatic Isolates	**AS1**	**AS2**	**AS3**	
% Lysis	22.8±2.3	12.9±2.1	41.4±0.2	

Data is given as mean ± SD (n=2). The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). There was no significant difference observed.

4.3.3.2 Cell Lysis of Same Group (Symptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) cells were co-cultured with sera obtained from mice injected with symptomatic (S1-3) isolates diluted at 1:1000. The percentage cell lysis is reflected in Table 4.11. It was observed that *Blastocystis* sp. symptomatic (S1-3) cells induced average cell lysis of 11% when they were co-cultured with the sera of the same group (S1-3).

Table 4.11: Cross-reactivity among *Blastocystis* sp. ST3 Symptomatic group

***Blastocystis* sp. Cells**	**Sera (1:1000)**	**Average Lysis (%)**
S1	S2	10.3±1.4
	S3	24.4±1.2****
S2	S1	3.1±0.5
	S3	7.5±1.6
S3	S1	8.1±0.9
	S2	10.3±0.2

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.3.3 Cell Lysis of Same Group (Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) isolates diluted at 1:1000. The percentage cell lysis is reflected in Table 4.12. It was observed that *Blastocystis* sp. asymptomatic (AS1-3) cells induced average cell lysis of 11% when they were co-cultured with the sera of the same group (AS1-3).

Table 4.12: Cross-reactivity among *Blastocystis* sp. ST3 Asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:1000)**	**Average Lysis (%)**
AS1	AS2	12.6±1.3
	AS3	10.1±0.8
AS2	AS1	12.4±0.1
	AS3	13.3±1.0
AS3	AS1	6.9±0.1
	AS2	12.9±0.1

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.3.4 Cell Lysis of Different Group (Symptomatic with Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) and symptomatic (S1-3) isolates diluted at 1:1000. The percentage cell lysis is reflected in Table 4.13. It was observed that *Blastocystis* sp. asymptomatic and symptomatic groups induced average cell lysis of 3.8% cross-reactivity upon co-culture.

Table 4.13: Cross-reactivity between *Blastocystis* sp. ST3 symptomatic and asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:1000)**	**Lysis (%)**	***Blastocystis* sp. Cells**	**Sera (1:1000)**	**Lysis (%)**
S1	AS1	1.8±0.1	AS1	S1	5.7±0.03
	AS2	0.59±0.01		S2	4.8±0.9
	AS3	7.3±0.2		S3	0.13±0.035
S2	AS1	5.9±0.2	AS2	S1	0.64±0.1
	AS2	6.1±0.2		S2	-0.42±0.23
	AS3	-0.42±0.1		S3	0.61±0.05
S3	AS1	11.6±0.2	AS3	S1	0.68±0.12
	AS2	17.3±0.04		S2	0.85±0.2
	AS3	5.6±0.3		S3	-2.0±3.0
Average Lysis (%) (A)		**6.2**	**Average Lysis (%) (B)**		**1.3**

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

			% Cell Lysis						IgG (OD492)
			Serum (1:1000)						
			Symptomatic			Asymptomatic			
			1	2	3	1	2	3	
Blastocystis sp. ST3 Live Cells	Symptomatic	1	14	3	8	6	0.6	0.7	0.643
		2	10	14	11	5	-0.4	0.9	0.568
		3	24	8	21	0.1	0.6	-2	0.538
	Asymptomatic	1	2	6	12	23	12	7	0.279
		2	0.6	6	17	13	13	13	0.624
		3	7.3	-0.4	5.6	10	13	41	0.424

Low <1%
Medium <84%
High >84%

Figure 4.7: Summary Percentage of *Blastocystis* sp. ST3 Cell Lysis at 1:1000 Sera Dilution

4.3.4 Cell Lysis at 1:10000 Sera Dilution

4.3.4.1 Cell Lysis of Same Isolates

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) isolates were co-cultured with sera obtained from mice injected with symptomatic (S1-3) and asymptomatic (AS1-3) isolates diluted at 1:10000. The percentage cell lysis is reflected in Table 4.14. It was observed that *Blastocystis* sp. symptomatic (S1-3) and asymptomatic (AS1-3) isolates induced significantly highest cell lysis when they were co-cultured with the sera of the same isolates. The percentage cell lysis for symptomatic group was 12% whereas for the asymptomatic group was 10%.

Table 4.14: *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Isolates Specific Cell Lysis

Symptomatic Isolates	**S1**	**S2**	**S3**	**Average A &B =11%**
% Lysis	5.9±1.1	12.1±2.9	17.3±1.4	
Asymptomatic Isolates	**AS1**	**AS2**	**AS3**	
% Lysis	7.6±0.6	8.5±0.7	14.5±1.6	

Data is given as mean ± SD (n=2). The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). There was no significant difference observed.

4.3.4.2 Cell Lysis of Same Group (Symptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) cells were co-cultured with sera obtained from mice injected with symptomatic (S1-3) isolates diluted at 1:10000. The percentage cell lysis is reflected in Table 4.15. It was observed that *Blastocystis* sp. symptomatic (S1-3) cells induced average cell lysis of 4% when they were co-cultured with the sera of the same group (S1-3).

Table 4.15: Cross-reactivity among *Blastocystis* sp. ST3 Symptomatic group

***Blastocystis* sp. Cells**	**Sera (1:10000)**	**Average Lysis (%)**
S1	S2	8.8±1.1
	S3	4.7±1.8
S2	S1	3.8±0.7
	S3	0.51±0.1
S3	S1	-0.63±1.2
	S2	3.5±0.5

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.4.3 Cell Lysis of Same Group (Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) isolates diluted at 1:10000. The percentage cell lysis is reflected in Table 4.16. It was observed that *Blastocystis* sp. asymptomatic (AS1-3) cells induced average cell lysis of 0.9% when they were co-cultured with the sera of the same group (AS1-3).

Table 4.16: Cross-reactivity among *Blastocystis* sp. ST3 Asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:10000)**	**Average Lysis (%)**
AS1	AS2	3.8±0.3
	AS3	2.9±0.1
AS2	AS1	0.11±0.3
	AS3	0.004±0.14
AS3	AS1	-0.55±0.32
	AS2	-1.41±0.05

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

4.3.4.4 Cell Lysis of Different Group (Symptomatic with Asymptomatic Isolates)

The *Blastocystis* sp. ST3 cells symptomatic (S1-3) and asymptomatic (AS1-3) cells were co-cultured with sera obtained from mice injected with asymptomatic (AS1-3) and symptomatic (S1-3) isolates diluted at 1:10000. The percentage cell lysis is reflected in Table 4.17. It was observed that *Blastocystis* sp. asymptomatic and symptomatic groups induced average cell lysis of -1% cross-reactivity upon co-culture.

Table 4.17: Cross-reactivity between *Blastocystis* sp. ST3 symptomatic and asymptomatic group

***Blastocystis* sp. Cells**	**Sera (1:10000)**	**Lysis (%)**	***Blastocystis* sp. Cells**	**Sera (1:10000)**	**Lysis (%)**
S1	AS1	1.54±0.07	AS1	S1	9.7±0.35
	AS2	2.45±0.06		S2	6.1±1.9
	AS3	-1.26±0.21		S3	0.043±0.054
S2	AS1	5.24±1.0	AS2	S1	-0.28±0.04
	AS2	4.1±0.36		S2	-1.56±0.47
	AS3	4.2±0.24		S3	-0.69±0.004
S3	AS1	9.8±0.2	AS3	S1	-1.54±0.61
	AS2	8.7±0.46		S2	-7.67±0.28
	AS3	8.1±0.17		S3	-12.29±2.1
Average Lysis (%) (A)		**5**	**Average Lysis (%) (B)**		**-7**

Data is given as mean ± SD (n=2) using One-Way ANOVA (SPSS Version 22).

			% Cell Lysis						IgG (OD492)
			Serum (1:10000)						
			Symptomatic			Asymptomatic			
			1	2	3	1	2	3	
Blastocystis sp. ST3 Live Cells	Symptomatic	1	6	4	-0.6	10	-0.2	-1.5	0.242
		2	9	12	4	6	-1.5	-7.6	0.242
		3	5	0.51	17	0.04	-0.6	-12	0.143
	Asymptomatic	1	1.5	5.2	10	8	0.1	-0.5	0.216
		2	2.5	4	9	4	9	-1.4	0.216
		3	-1.2	4	8	3	0	14	0.116

Low <1%
Medium <84%
High >84%

Figure 4.8: Summary Percentage of *Blastocystis* sp. ST3 Cell Lysis at 1:10000 Sera Dilution

			% Cell Lysis																								
			Serum (1:10)						Serum (1:100)						Serum (1:1000)						Serum (1:10000)						PBS
			Symptomatic			Asymptomatic			Symptomatic			Asymptomatic			Symptomatic			Asymptomatic			Symptomatic			Asymptomatic			Control
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	(1:10)
Blastocystis sp. ST3 Live Cells	Symptomatic	1	92	37	39	25	18	20	40	21	23	17	11	2	14	3	8	6	0.6	0.7	6	4	-0.6	10	-0.2	-1.5	0.68
		2	49	79	53	14	22	23	30	46	25	13	4	5	10	14	11	5	-0.4	0.9	9	12	4	6	-1.5	-7.6	0.16
		3	29	55	72	12	29	37	21	24	40	4	6	-2	24	8	21	0.1	0.6	-2	5	0.51	17	0.04	-0.6	-12	0.99
	Asymptomatic	1	17	6	10	84	49	41	9	13	21	33	23	19	2	6	12	23	12	7	1.5	5.2	10	8	0.1	-0.5	0.37
		2	20	17	11	49	87	49	13	6	9	27	30	27	0.6	6	17	13	13	13	2.5	4	9	4	9	-1.4	1.76
		3	13	4	14	60	37	86	12	4	7	32	23	61	7.3	-0.4	5.6	10	13	41	-1.2	4	8	3	0	14	0.44

Low <1%
Medium <84%
High >84%

Figure 4.9: Summary Percentage of *Blastocystis* sp. ST3 Cell Lysis from 1:10 to 1:10000 Sera Dilutions

4.4 Discussion

The sera of the mice injected with symptomatic and asymptomatic *Blastocystis* sp. antigen produced polyclonal antibodies. In order to identify the degree of polyclonal antibodies specificity and efficacy, the antibodies and cells were interacted at dose dependent manner from 10-fold dilutions (10^1, 10^2,10^3 and 10^4). We have proven in our previous Chapter 3, that there was a presence of dominant IgG antibodies in the sera sample of the injected mice. Therefore, various dilutions of sera containing polyclonal antibodies were used in this study to observe the cell lysis from highest to lowest concentrations. In theory, the specific antibodies which were present in the mice sera, will initiate the binding of the respective antigens expressed on the surface of the *Blastocystis* sp. cells *in vitro* with antibodies. This will eventually form an antigen-antibody complex which may induce the complement activation and lyse the *Blastocystis* sp. cells. CCK-8 cell cytotoxicity kit was used to measure the NADH content of the live *Blastocystis* sp. cells which denotes the metabolic activity of the live cells. In this study the positive and negative cut off points were set based on the specific sera and cell lysis of the respective isolates.

In this study, the positive cut off value was set based on the average value of specific percentage cell lysis by symptomatic and asymptomatic group which was 84%. In contrast the negative cut off point were set based on the specific cell lysis between cells and sera obtained from PBS injected mice. It was shown that PBS immunised mice did not exert specific cell lysis in this assay even at the lowest dilution which is at (1:10). Therefore, negative (PBS control cell lysis) and specific *Blastocystis* sp. cell lysis at more than (>84%) and lower than (<1%) were used as a high and low cut off point for all the cell lyses.

The specificity of an antibody is defined by its ability to recognize one specific antigen. As a general example, an antibody that recognizes the *Blastocystis* sp. antigen will not be able to recognize *Entameoba histolytica* parasite, on the contrary, an antibody that recognizes *Entameoba histolytica* will not able to recognize the *Blastocystis* sp. (Tan et al., 2001). This phenomenon is defined as antibody specificity where each and every B lymphocyte cell is able to produce antibody of one kind. In this study, the specificity of the *Blastocystis* sp. from the symptomatic and asymptomatic groups was evaluated and it was shown to induce high specific cell lysis. The polyclonal antibodies produced against each isolate of *Blastocystis* sp. ST3 symptomatic and asymptomatic group were able to recognise the cell surface membrane of its specific cells.

The specific cell lysis at 1:10 sera concentration was observed in both symptomatic and asymptomatic group with an average percentage value of 82% and 86% respectively with no significant difference between the two isolates. These results were compared with the IgG antibody OD reading obtained at 1:10 dilution. However, the IgG OD readings have shown a contrast results which has shown higher IgG content in symptomatic isolates where average readings of 2.3 and 1.2 OD were observed in sera obtained from mice injected with symptomatic and asymptomatic isolates respectively with a significant difference of $P<0.01$. This scenario could be due to the higher influence of IgG1/IgG2a antibody isotype contents. Studies suggested that, IgG1 isotype performs a higher classical complement mediated lysis effector function in comparison to IgG2 isotype (Beenhouwer et al., 2007; Wang et al., 2017). In the previous Chapter 3 it was proven that, asymptomatic isolates were Th2 dominant with higher IgG1 isotype and symptomatic were Th1 dominant with higher IgG2a isotype secretions. Therefore, the differences between cell lysis and sera IgG content could be due to higher IgG1 isotype in comparison to sera obtained from mice injected with symptomatic isolates.

Further investigation on the specific lysis at (1:10) sera concentration demonstrated that, S1 isolate induced the highest cell lysis at 92% compared to other isolates of symptomatic and asymptomatic group. The comparison of S1 isolate with IgG antibody OD reading has shown that this isolate exhibited the highest IgG. Besides, the previous Chapter 3 had also reported S1 as the highest lymphocyte cells proliferator which enormously increased the Th1 response compared to the other isolates. Therefore, the specific cell lysis of S1 (symptomatic group) had confirmed that the isolate showed higher pathogenicity in comparison to asymptomatic isolates which have bearing on higher immune cells binding affinity which were also proven by previous researcher through the phenotypic analysis (Tan et al., 2008; Ragavan et al.,2014). It was implicated by the researchers that, symptomatic isolates depicted higher cell binding with Con-A in comparison to asymptomatic isolates. In this study it was observed that, the specific lysis results showed gradual decrease in cells co–cultured with different concentrations (10^1 to 10^4) 10-fold diluted sera obtained from mice injected with symptomatic and asymptomatic isolates. However, the lowest sera concentration (10^4) of symptomatic and asymptomatic origin exhibited more than 1% specific cell lysis which is higher than the negative cut off point.

The diversity of the antibodies raised against symptomatic and asymptomatic isolates of *Blastocystis* sp. ST3 were further evaluated through the cross-reactivity assay in this study. The antibodies were cross reacted (1) among symptomatic isolates (2) among asymptomatic isolates (3) between symptomatic and asymptomatic isolates. The antibodies which were cross reacted among symptomatic isolates at sera dilution of 1:10 showed an average of 44% cell lysis. On the other hand, the cell lysis among asymptomatic isolates showed an average of 48% cell lysis. This showed that symptomatic and asymptomatic isolates shared almost half the epitopes among its own group which showed a medium cell lysis at lesser than 84%. In contrast, the cross-

reactivity between symptomatic and asymptomatic group showed average cell lysis of 17%. The cell lysis between symptomatic and asymptomatic group were exerted higher than the negative cut off value more than 1% but significantly lower than positive cut off value which is higher than 84%. Therefore, a minimal cross-reactivity between symptomatic and asymptomatic isolates was demonstrated. This is aligned with the findings in Chapter 3 which have shown the antigen diversity between *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates by the characterization of adaptive immune response. Therefore, CDC cross reactivity analysis has further proven the distinctiveness of the adaptive immune response between these isolates.

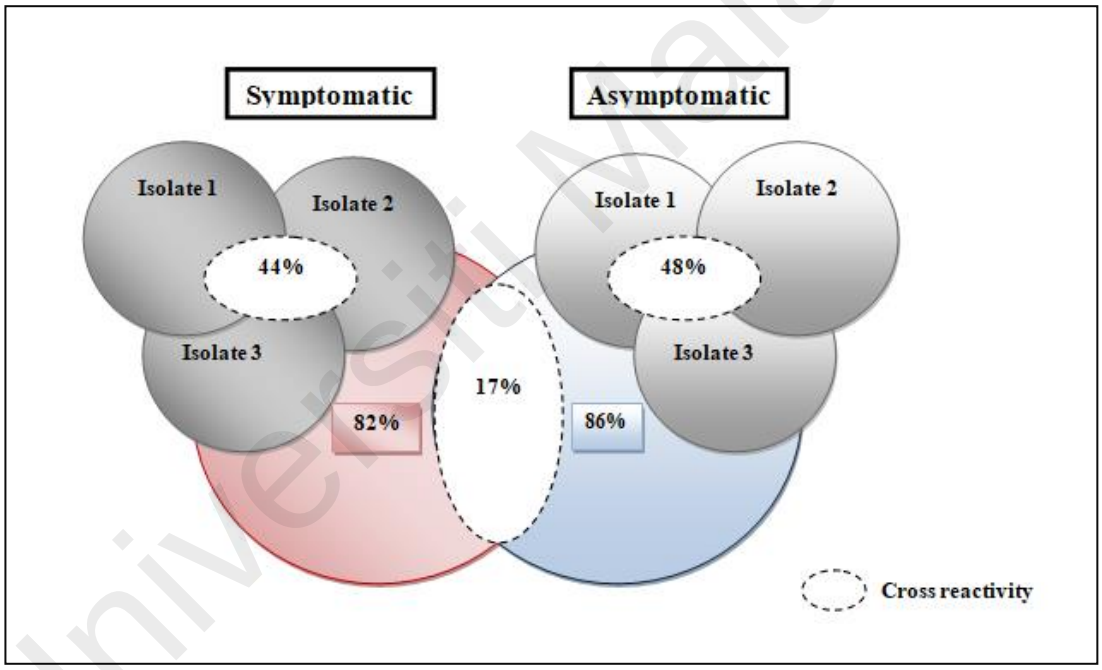
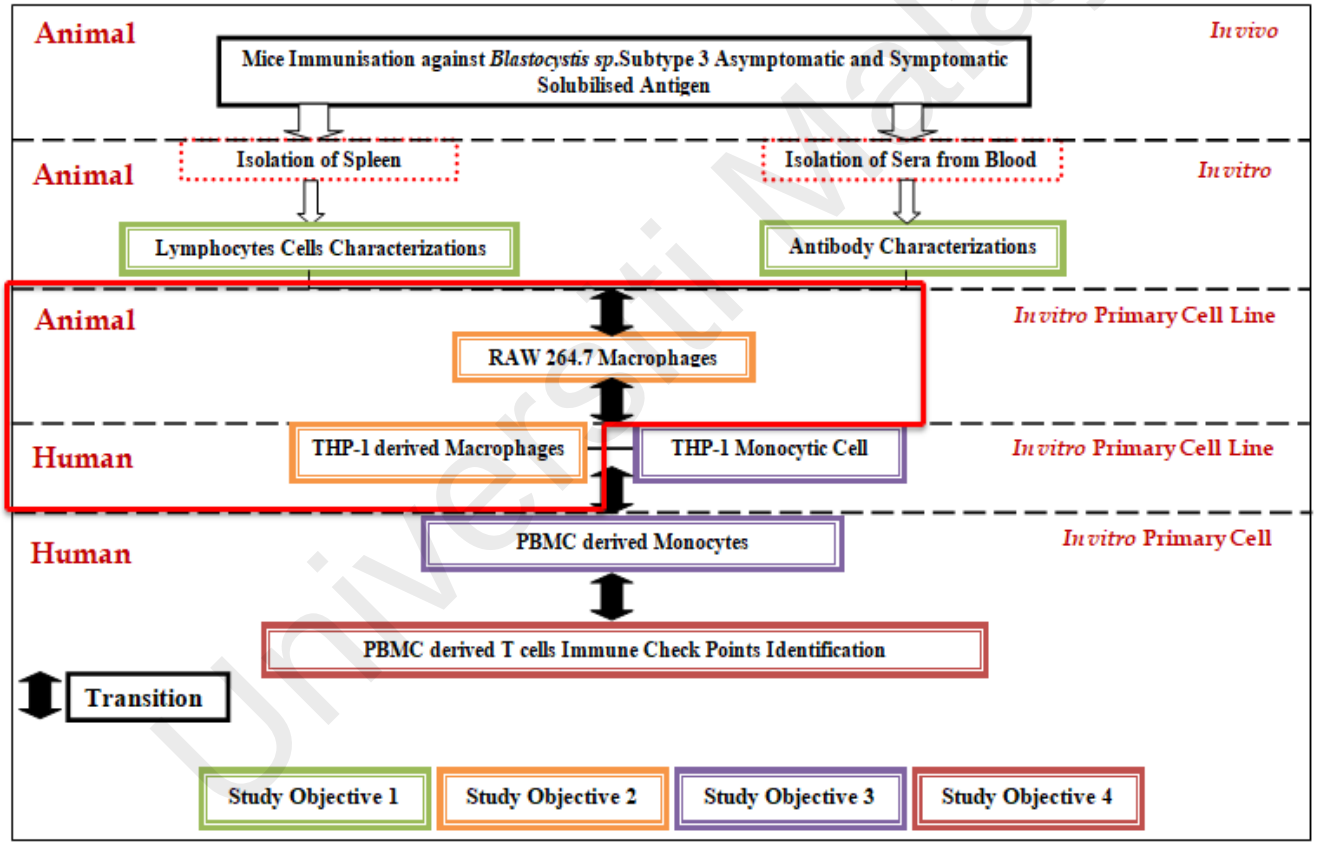


Figure 4.10: Summary Percentage of Cell-Cytotoxicity and Cross-Reactivity Induced by *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Cells.

4.5 Conclusion

In conclusion this study has demonstrated that, *Blastocystis* sp. ST3 symptomatic and asymptomatic cells were able to induce complement mediated antibody cell lysis as depicted in Figure 4.10 above. It was demonstrated in this study that, sera obtained from mice injected with 20 µg/ml symptomatic and asymptomatic solubilised antigens were able to exert specific lysis which showed high specific cell and antibody binding which supporting the evidence of high antigen specificity. This study has also proven that, there are almost 50% cross-reactivity was observed between *Blastocystis* sp. ST3 isolates origin from the same group which proving high antigen diversity. Besides, there was only 17% cross-reactivity observed between the sera and cells of different group (symptomatic and asymptomatic isolates). Therefore, this study clearly implicates that, large level of epitopes dissimilarities between *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates may allow the parasite to set up diverse innate and adaptive immune modulations tactics (observation of Chapter 3) in order to maintain its survival in the host. The following Chapter 5 describes the investigations that were performed to explore on how these symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates modulate the host innate immune system by using animal and human primary cell lines.

CHAPTER 5: *IN VITRO* STUDIES TO EVALUATE MACROPHAGE IMMUNE RESPONSE AGAINST *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC ANTIGENS



5.1 Introduction

It was proven through the results obtained from Chapter 3 and 4 that, *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates demonstrated antigenic variations. Studies have suggested that antigenic variations were presented by most of human intestinal parasites such as *Giardia lamblia*, *Entamoeba histolytica* and helminth. These parasites evaded the host immune system by diversifying the modulations of innate and adaptive immunity (Bhattacharya et al., 1992; Svärd et al., 1998; Yason and Tan, 2018). Generally, adaptive and innate immunity interact closely to eradicate pathogens. Furthermore, adaptive immune response will be initiated when the innate immune system fails to eliminate an infection or antigen through the activation of the antigen-presenting cells (Janeway et al., 2001; Rivera et al., 2016). Antigen presenting cells such as macrophages play a major role in influencing the Th1 and Th2 immune responses through its cytokine modulations (Muraille et al., 2014). In the previous Chapters 3, we have demonstrated the adaptive immune modulation mediated by *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. Adaptive immune modulation is usually evaluated by the T cell cytokine response that includes **(1)** down-regulation of protective immunity Th1 **(2)** skewing the immune response towards non-protective such as Th2 response **(3)** generating anti-inflammatory immune responses such as IL-10 as similarly demonstrated in helminth by McNeilly et al., (2014). Generally, T cell response is largely depending on antigen presenting cells such as macrophage where the interactions are influenced by cytokine release by the macrophage upon encountering an antigen. This current chapter focuses on macrophage cell proliferation and cytokine modulation upon exposure with *Blastocystis* sp. ST3 antigen that may reflect the innate immunity of the infected host. Past studies have shown that, cytokine modulations caused by macrophages resulted in immune evasions of parasites and resulted in progression of infections (Singh and Agrewala, 2006; Kaiko et al., 2008).

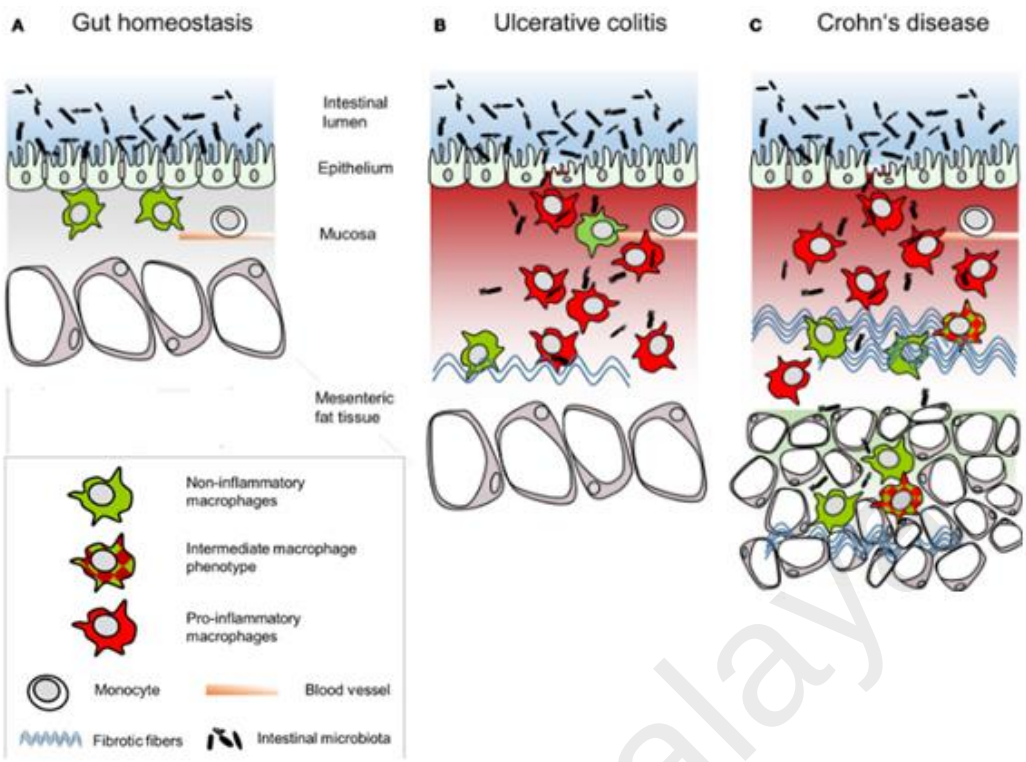


Figure 5.1: Summary of Intestinal Macrophage Distribution During Gut Homeostasis and Inflammatory Disease Conditions

(Source: Kühl et al., 2015)

Generally, the intestinal resident macrophages are responsible in maintaining mucosal balance and protective immunity in the gut region during intestinal barrier damage. Lim et al., (2014) suggested that, macrophages are to be the first cells to encounter invading pathogens which cross the intestinal epithelium to eradicate the infection. Macrophages are suggested to be abundantly found in the intestinal targeting the region of lamina propria (the mucosa region) as shown in Figure 5.1. Therefore, since *Blastocystis* sp. has been reported to cause damage in the intestinal barrier integrity, the probability of this parasite in interacting with the macrophages in the intestinal region will be enormous. The intestinal macrophages also play an essential role in exacerbating intestinal inflammations and pathogenesis of intestinal inflammatory diseases (Figure 5.1) such as IBD and IBS (Mahida,2000; Distrutti et al., 2016) which are highly associated with *Blastocystis* sp. infection (Boorom et al., 2008). Previous studies have proven that,

Blastocystis sp. subtype 4 and 7 induced various pro-inflammatory cytokines through mitogen-activated protein kinase (MAPK) activation and toll like receptor (TLR) expressions which possibly contribute to the pathogenesis of intestinal inflammation during its infection (Teo et al., 2014; Lim et al., 2014).

In this Chapter, we aimed to demonstrate the innate immune modulation induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates on macrophages. The *in vitro* analysis was performed on macrophage cell line derived from Balb/c mice (RAW 264.7) due to the previous results Chapter 3 which analysed the T cell cytokine response on Balb/c mice model. Following this, in order to compare the macrophage immune response on human model, the analysis was transitioned to human cell line (THP-1 monocyte derived macrophages). Generally, these cell lines are widely used as an *in vitro* model to analyse host immune modulations and also were used against *in vitro* parasitic infection analysis such as *Leshmania* (Donovan et al., 2009; Rai et al., 2017). Following are the descriptions of the explored areas in this chapter.

5.1.1 Cytokines

Macrophages secrete the following cytokines which modulates the intestinal tissues with the following cytokines:

Interleukin-6 (IL-6) is a cytokine which generally classified as pro-inflammatory cytokine. Nevertheless, for the past years, studies had demonstrated that in some instances, macrophages may attenuate IL-6 by promoting anti-inflammatory effects (Covarrubias and Horng, 2014; Allison Reiss et al., 2017)

Tumor necrosis factor-α (TNF-α) acts as a major-regulator of pro-inflammatory cytokines which engages in a number of critical cell activities such as cell proliferation, survival, differentiation, and apoptosis (Parameswaran and Patial, 2010). Macrophages are the major producers of TNF-α and respond highly to the secretions. Imbalances of TNF-α production have been widely associated with the pathogenesis of intestinal inflammatory diseases, including Crohn's disease and inflammatory bowel disease (IBD) (Sanchez-Munoz et al., 2008).

Interleukin-10 (IL-10) acts as an anti-inflammatory cytokine by limits inflammatory responses by macrophages during its activation. It is also known as a key inhibitor of many aspects of the pro-inflammatory response activated by macrophages such as IL-6, TNFα and generation of nitric oxide and up-regulation of surface antigen expression such as MHC class II, CD80 and CD86 (Williams et al., 2004).

Interleukin-12p70 (IL-12p70) is known as the active heterodimer of IL-12, consisting two subunits; IL-p35 and IL-p40. It is produced by antigen presenting cells; dendritic cells, monocytes, macrophages, and B-cells in response to pathogens. The pro-inflammatory IL-12p70 subunits, IL-12p40 acts as both stimulatory and inhibitory of IL-23 cytokine secretion; Th17 cells differentiations. Therefore, IL-12 controls the inflammatory induced by antigen presenting cells such as macrophages (Hamza et al., 2010)

5.1.2 Apoptosis or Programmed Cell Death

Cell death is divided into two different processes namely apoptosis and necrosis. Apoptosis causes changes and loss of nucleus and mitochondrial functions to maintain cell integrity. Meanwhile, necrotic cell death occurs when cells are injured and cause cell swelling or lysis (Fink and Cookson, 2005). Apoptosis benefits the host by regulating tissue homeostasis when the host is uninfected. However, apoptosis may cause harmful effects to the host during parasitical infection according to the specific host-parasite conditions. It was reported that parasite evolutions are capable of developing mechanisms to induce or evade host cell apoptosis for survival (Bienvenu et al., 2010). The role of *Blastocystis* sp. in inducing apoptosis to the macrophage cells is unknown and was explored in this chapter.

5.2 Materials and Methods

5.2.1 Source of *Blastocystis* sp.

As mentioned in method 3.2.1

5.2.2 Axenization of *Blastocystis* sp. and Isolation of Solubilized Antigen

As mentioned in method 3.2.2

5.2.3 RAW 264.7 Balb/c Mice Macrophage Cell Line Culture and Inductions with *Blastocystis* sp. solubilised antigen

In this study, the RAW 264.7 Balb/c Mice Macrophage Cell Line (ATCC) was used to investigate host and *Blastocystis* sp. interactions. Cells were maintained in T-75 flasks in a humidified incubator at 37°C and 5% CO_2. The RAW 264.7 cells were cultured in RPMI 1640 media (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, USA) and 10% heat-inactivated fetal bovine serum (Gibco). Culture viability was evaluated every 3 to 4 days using the trypan blue assay and only cultures with >95% viability was used for the experiment.

5.2.4 RAW 264.7 Balb/c Mice Macrophage Inductions with *Blastocystis* sp. solubilised antigen

Briefly, 0.3×10^5 cells per well were seeded in sterile 96-well plates in the presence of ST3 asymptomatic (1-3) and symptomatic (1-3) (0.01-10 µg/ml) respectively in RPMI complete medium. The plates were then incubated for the duration of 6, 24 and 48 hours. The experimental plate was terminated at 6, 24 and 48 hours by centrifugation at 300xg. The supernatant was aspirated and stored in -80°C till further use. The cell pellet was subjected to cell proliferation assay using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, USA) (Following method 5.2.6).

5.2.5 Human THP-1 Monocytic Cell Line Culture

In this study, the THP-1 human monocytic cell line (ATCC) was used to investigate host–*Blastocystis* sp. interactions. THP-1 cells were maintained in T-75 flasks in a humidified incubator at 37°C and 5% CO_2. The THP-1 cells were cultured in RPMI 1640 (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, USA) and 10% heat-inactivated fetal bovine serum (Gibco). Culture viability was evaluated every 3 to 4 days using the trypan blue assay and only cultures with >95% viability was used for the experiment.

5.2.6 Human THP-1 Monocytic Cell Line Differentiations to Macrophage

Briefly, 2×10^6 cells were stimulated with 200 nM phorbol-12-myristate-13-acetate (PMA) for 24 hours. After 24 hours of incubation, the non-attached cells were removed by aspiration whereas the adherent cells were washed with RPMI media three times and the cells were scraped using cell scraper and were centrifuged at 800xg for 5 minutes and the cells were ready to use for the experiment.

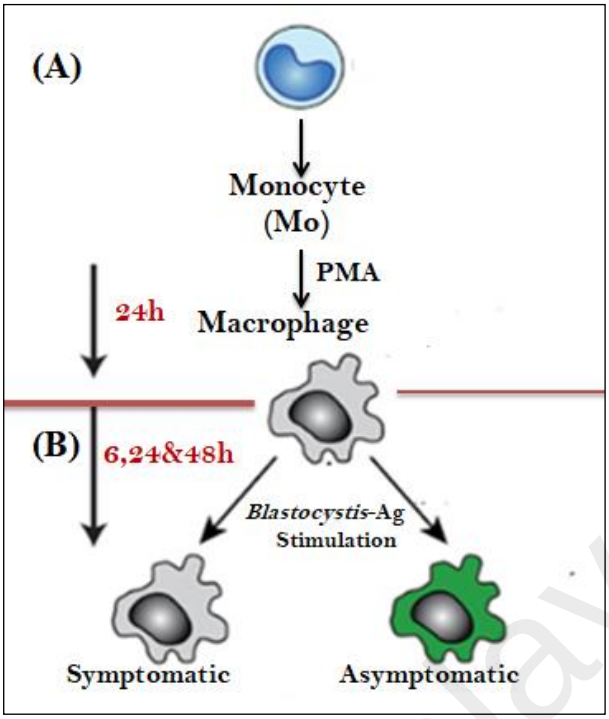


Figure 5.2: THP-1 Monocytic Cell Line Differentiations to Macrophages and Inductions with Symptomatic and Asymptomatic *Blastocystis* sp. ST3 Solubilised Antigens

(A) THP-1 monocytic cell line were differentiated to macrophage by stimulation with PMA for 24 hours (B) THP-1 derived macrophages inductions with *Blastocystis* sp. solubilised antigen and incubations at 6, 24 and 48 hours.

5.2.7 Human THP-1 Macrophage Induction with *Blastocystis* sp. Solubilised Antigen

Briefly, 3×10^4 cells per well were seeded in sterile 96-well plates in the presence of ST3 asymptomatic (1-3) and symptomatic (1-3) (0.01-10 µg/ml) respectively in RPMI complete medium. The plates were then incubated for the duration of 6, 24 and 48 hours. The experimental plate was terminated at 6, 24 and 48 hours by centrifugation at 300xg. The supernatant was aspirated and stored in -80°C till further use. The cell pellet was subjected for cell proliferation assay using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, USA) (Following method 5.2.6).

5.2.8 CellTiter-Glo® Luminescent Cell Viability Assay

The pellet of stimulated macrophages was subjected to cell quantifications. The cell quantifications of the cell pellets after 6, 24 and 48 hours were performed by using CellTiter-Glo® (Promega, USA) luminescent cell viability assay kit.

5.2.8.1 Assay Principle

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in the culture. The CellTiter-Glo® Assay generates a "glow-type" luminescent signal, produced by the luciferase reaction, which has a half-life of longer than five hours (Fig 5.2). This extended half-life eliminates the need for reagent injectors and provides flexibility for continuous or batch-mode processing of multiple plates. The unique homogeneous format reduces pipetting errors that may be introduced during the multiple steps required by other ATP measurement methods.

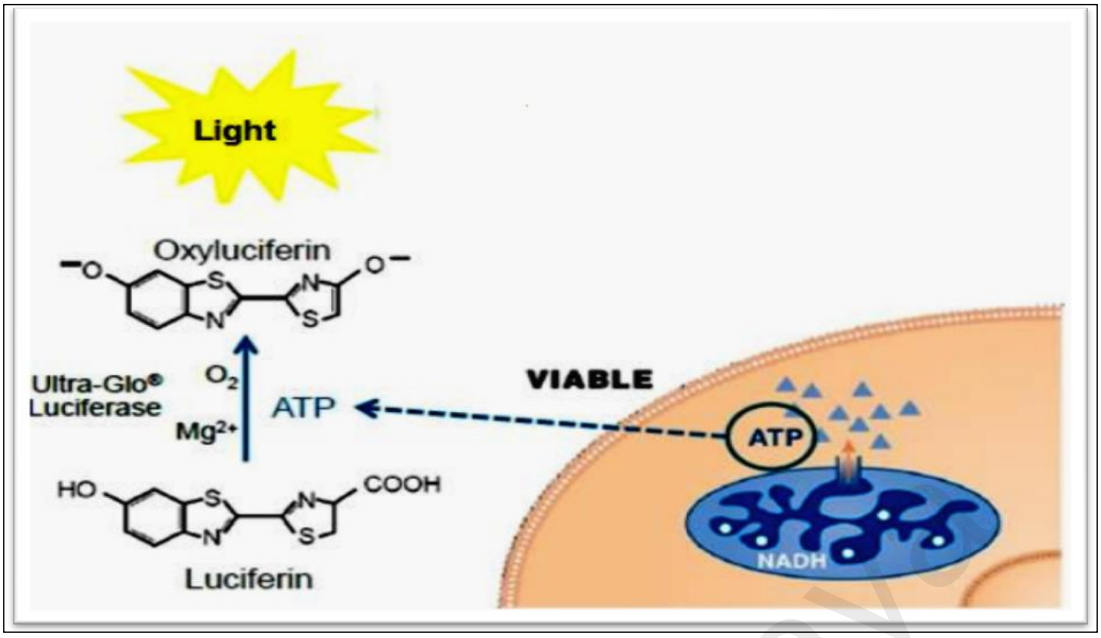


Figure 5.3: The Diagram Showing the Mechanisms of CellTiter-Glo® Luminescent Cell Viability Assay

(Source: Promega Data Sheet)

5.2.8.2 General Method

Cell viability assay was performed by quantifying the ATP generated by metabolically active cells. This was done using a CellTiter-Glo luminescent cell viability assay according to the manufacturer's instructions. Briefly, the cultured cells in sterile 96-well plates were terminated by centrifugation at 300xg for 5 minutes. The supernatant was aspirated and stored in -80ºC till further use. The pellet containing cells was added with 100uL of PBS. The plates were then added with 100 µl of CellTiter-Glo reagent. The content was mixed using orbital shaker for 5 minutes to induce cell lysis. After 10 minutes incubation at room temperature, the 100 µL of the contents were transferred to the 96 opaque-walled well plates. The luminescence was recorded using microplate reader (VICTOR, USA) with an integration time of 1 second per well. The luminescence signals for the treated cells were normalized by the luminescence signal obtained from non-treated cells.

5.2.9 Nitric Oxide Detection with Griess Reaction Assay

5.2.9.1 Assay Principle

The Griess reaction is used to analyze through nitrate catalytic reduction to nitrite. In this assay, the sulfanilic acid is converted to diazonium salt through the reaction with nitrite in acid solution. The diazonium salt is then combined with N-(1-naphthyl) ethylenediamine, forming an azo dye.

5.2.9.2 General Method

Griess Reagent was prepared by mixing equal volumes of N-(1-naphthyl) ethylenediamine (Component A) and sulfanilic acid (Component B). Using 96 well microplate, the following were mixed in each well: 20 µL of Griess Reagent 150 µL of the nitrite-containing sample collected after 6, 24 and 48 hours and 130 µL of deionized water (limiting to volume of 300 µL per well). The mixture was incubated for 30 minutes at room temperature. All the assays were performed in triplicates and the absorbance at 548 nm (A548) was compared to a $NaNO_2$ standard curve.

5.2.10 ELISA Test

Supernatants obtained from the macrophages culture were collected after 48 hours of stimulation with ST3 *Blastocystis* sp. asymptomatic (1-3) and symptomatic (1-3) (10 µg/ml) respectively. Respective supernatant was then assayed for; Interleukin-6 (IL-6) and Interleukin-10 (IL-10); Interleukin-12p70 (IL-12p70) and tumor necrosis factor alpha (TNF-α) using a commercial ELISA Kit (R&D Systems, USA) according to the manufacturer's instructions (Appendix B). All the assays were performed in triplicates.

5.2.11 Flow Cytometry

Macrophages pellets obtained from the 48 hours incubation assays (induced and un-induced), were comprehensively characterized by multicolour flow cytometry for the expression of the surface marker PD-1 molecule. Briefly, the cells were centrifuged at 300×g for 10 minutes and the pellet was re-suspended with 9.8 µL cold phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA and kept at 2−8 °C. The cell suspension was added with 0.2 µL of the human PD-1 antibody (CD279-PE-PD1, Miltenyi Biotech, USA, Cat No: 130-117-384) and mouse antibody (CD279-PE-PD-1, Miltenyi Biotech, USA, Cat No: 130-102-299). After antibody addition, the cell suspension was thoroughly mixed using a micropipette and was incubated for 10 minutes in dark in the refrigerator (2−8 °C). The cell suspension was washed by adding 1 mL of cold PBS buffer and centrifuged at 300×g for 10 minutes. The cell pellet was re-suspended with 300 µL of cold PBS buffer and were analysed for PD-1 surface molecule detection using multicolour flow cytometry machine (BD, USA).

5.2.12 Annexin-V Apoptosis Detection

5.2.12.1 Assay Principle

Annexin V-FITC kit detects annexin V fluorescent by binding to apoptotic cells. The cells which underwent apoptosis will be quantitated by using flow cytometry. Annexin V conjugated with fluorescein isothiocyante (FITC) will be use to label phosphatidylserine sites on the cell membrane surface. The necrotic cells are able to be detected through labelling of propidium iodide (PI) to the cellular DNA. Thus, this kit is able to detect (1) early apoptotic cells (annexin V positive, PI negative) (2) necrotic cells (annexin V positive, PI positive), and (3) viable cells (annexin V negative, PI negative).

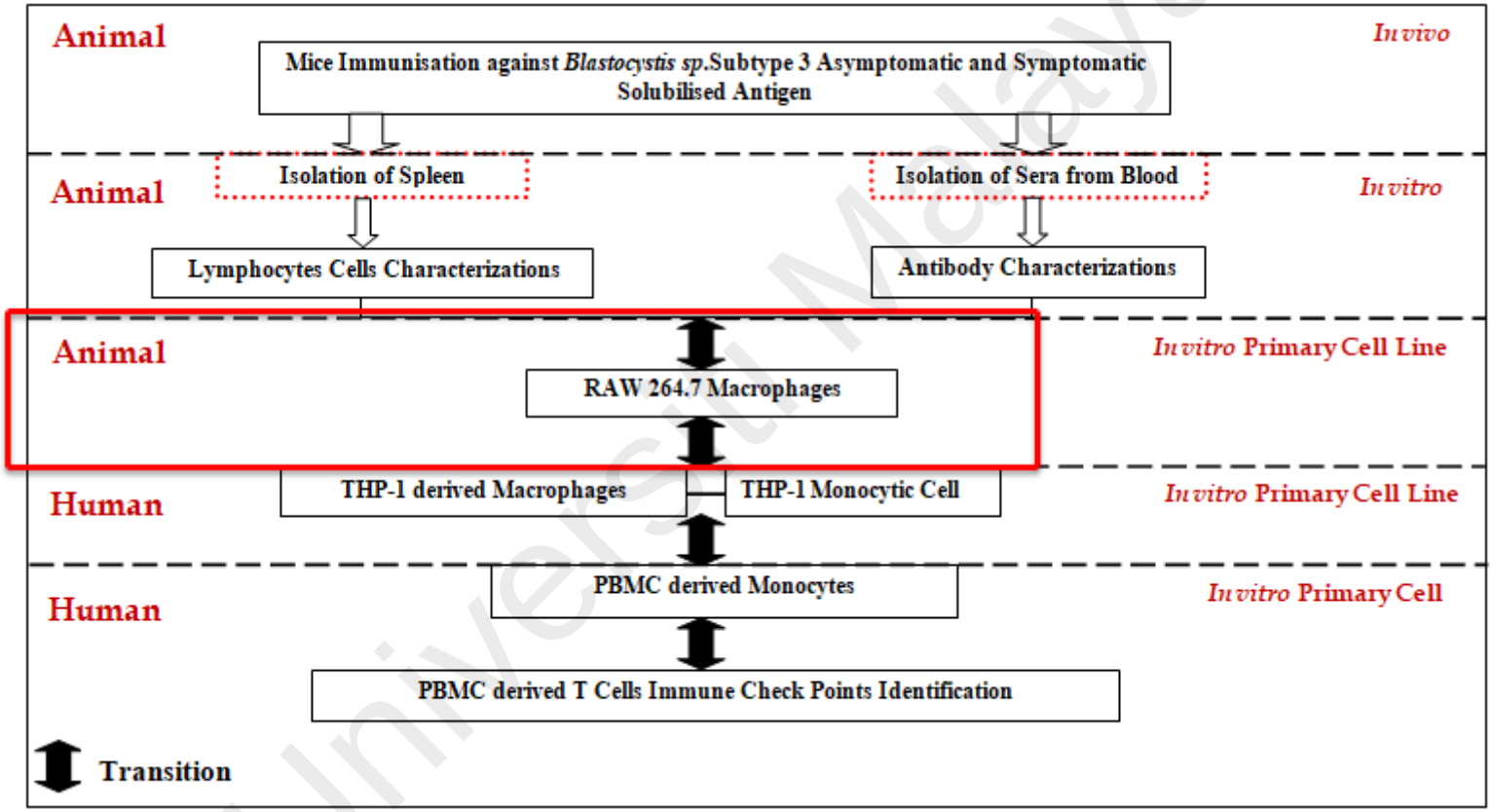
5.2.12.2 General method

The macrophages were stimulated with *Blastocystis* sp. ST3 asymptomatic (1-3) and symptomatic (1-3) (10 µg/ml) and were incubated for 48 hours in CO_2 incubator at 37°C and 5% CO_2. Meanwhile, macrophages were induced with DMSO with a final concentration of 10% (v/v) and were used as a positive control was incubated for 24 hours in CO_2 incubator at 37°C and 5% CO_2. The macrophages were then subjected for Annexin V staining to measure apoptosis (Appendix D).

5.2.13 Statistical analysis

Data was analysed by one-way analysis of variance (ANOVA) using SPSS software 22. The graphs were plotted using Graph Pad Prism 8.

PART 1: ANIMAL CELL DERIVED MACROPHAGES



5.3 Results

5.3.1 Cell Viability Assessment of RAW264.7 Macrophage Stimulated with Symptomatic and Asymptomatic *Blastocystis* sp. ST3 Solubilised Antigen

5.3.1.1 Solubilised Antigen Concentrations Optimization

Solubilised antigen dose optimization with different concentrations (0.01, 0.1, 1 and 10 µg/ml) of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens inductions in RAW264.7 macrophages were observed. It was shown that 10 µg/ml of *Blastocystis* sp. symptomatic and asymptomatic antigens induced macrophages, induced the highest cell inhibitions with inhibition percentage of 35% and 36% respectively as shown in Figure 5.4. Therefore 10 µg/ml soluble antigens were chosen as the optimal concentration to expedite the investigation on cell inhibition. The cell viability differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic antigen inductions are shown in Table 5.1.

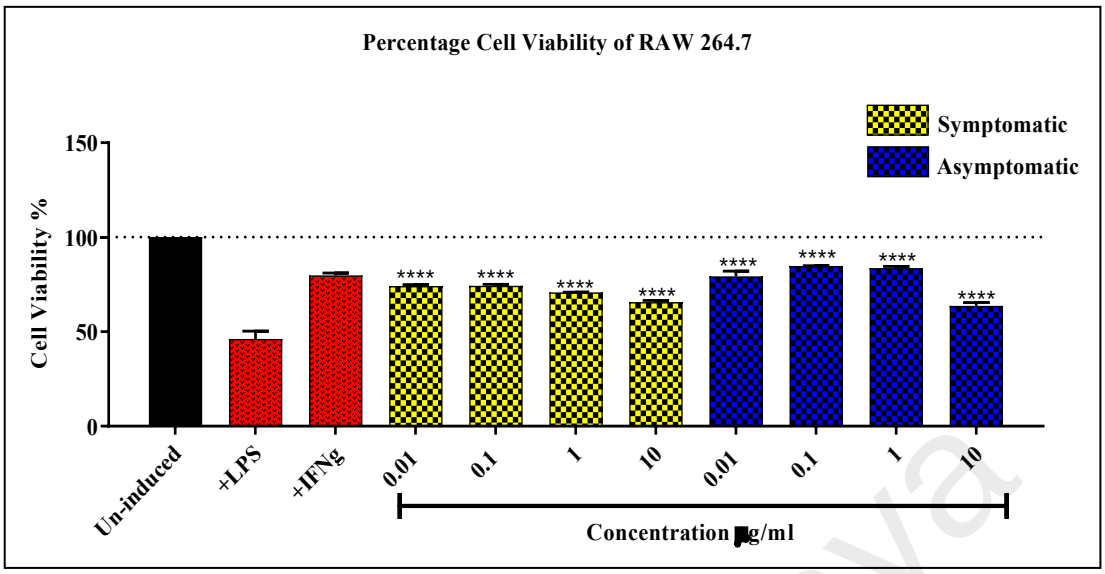


Figure 5.4: Cell Viability Assessment of RAW264.7 Macrophages Stimulated with 0.001, 0.01, 1 and 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced RAW264.7 group served as negative control (n=6).

Table 5.1: RAW 264.7 cell viability upon inductions with different concentrations of *Blastocystis* sp. solubilised antigens

Group	Solubilised Antigen (µg/ml)			
	0.01	0.1	1.0	10.0
Symptomatic	74.18±0.76	74.31±0.71	70.77±0.22	65.68±0.92
Asymptomatic	79.36±2.74*	84.79±0.14****	83.69±0.82****	63.68±1.72

Data is given as mean ± SD (n=9). The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). The differences were significant with, *P<0.05 and ****P<0.0001.

5.3.1.2 Induction at 6, 24 and 48 hours

The RAW 264.7 cells stimulation with 10 µg/ml of solubilised antigen (optimal concentration) of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates was carried out at 6, 24 and 48 hours. It was observed that *Blastocystis* sp. symptomatic and asymptomatic antigens induced significantly high cell inhibitions at 48 hours of soluble antigen induction with percentage of 45.6% and 40.3% respectively as shown in Figure 5.5. Therefore 48 hours of soluble antigens induction were chosen for the subsequent observations. The cell viability differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic inductions at different timing are shown in Table 5.2.

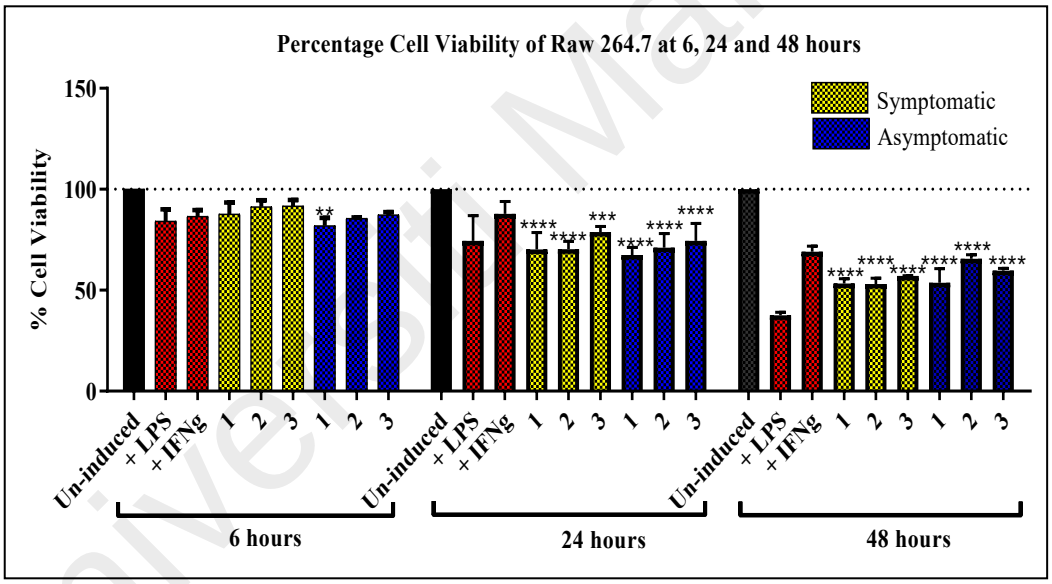


Figure 5.5: Cell viability Assessment of RAW264.7 Macrophages Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic And Asymptomatic Solubilised Antigens at 6, 14 and 48 hours

Values are given in mean ± SD (n=9). **P < 0.01 ***P < 0.001 ****P < 0.0001 was the comparison made against un-induced RAW264.7 group served as negative control (n=6).

Table 5.2: RAW 264.7 cell viability upon inductions with 10 µg/ml of *Blastocystis* sp. ST3 solubilised antigen incubation at different timing

Group	Incubation Time (hours)	
	6 vs 24	24 vs 48
Symptomatic	90.22±3.87***	73.10±3.87***
Asymptomatic	84.92±3.87**	70.97±3.87*

Data is given as mean±SE difference between (6 with 24 hours) and (24 and 48 hours) of RAW 264.7 stimulated with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). The differences were significant with, *P<0.05, **P<0.01 and ***P<0.001.

5.3.1.3 Induction at 48 hours

The RAW 264.7 cells inductions with 10 µg/ml of ST3 *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens were carried out at 48 hours. It was observed that *Blastocystis* sp. symptomatic and asymptomatic antigens induced cell inhibitions at 31% and 35% respectively as shown in Figure 5.6. The cell viability differences between ST3 *Blastocystis* sp. symptomatic and asymptomatic inductions are shown in Table 5.3.

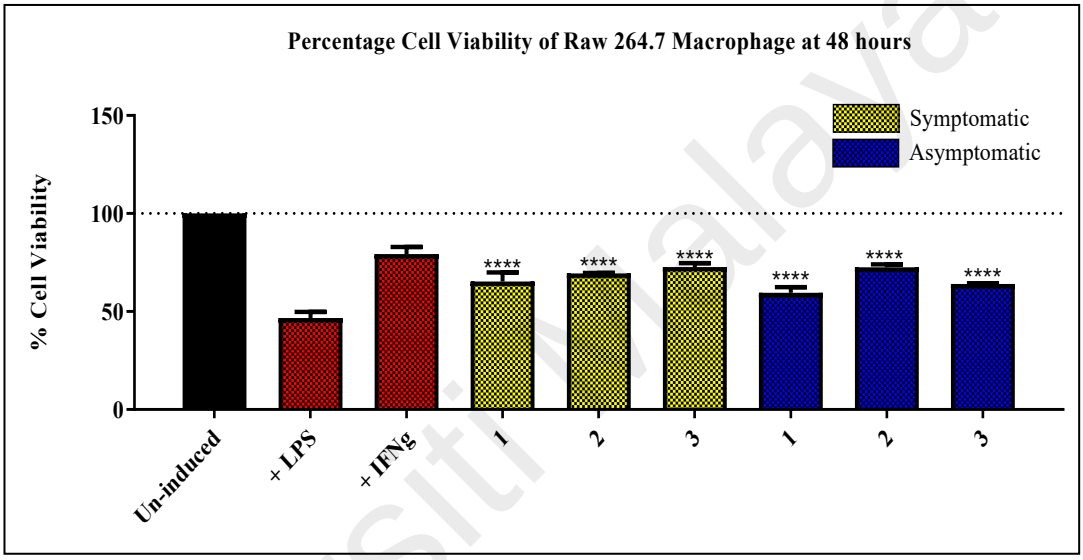


Figure 5.6: Cell viability Assessment of RAW264.7 Macrophages Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic And Asymptomatic Solubilised Antigens at 48 hours

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced RAW264.7 group served as negative control (n=6).

Table 5.3: RAW 264.7 cell viability upon inductions with 10µg/ml of *Blastocystis* sp. solubilised antigen incubation at 48 hours

Group	Cell Viability (%)
Symptomatic	69.14±3.72
Asymptomatic	65.37±6.61

Data is given as mean±SD are difference between RAW 264.7 stimulated with ST3 *Blastocystis* sp. symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22) (n=6). The differences were not significant.

5.3.2 Nitric Oxide Release Assessment

The RAW 264.7 cells inductions with 10 µg/ml of *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens were carried out at 48 hours. It was observed that *Blastocystis* sp. symptomatic and asymptomatic solubilised antigen induced macrophages, secreted significant nitric oxide release compared to un-induced group as shown in Figure 5.7. The cell nitric oxide release was observed at 17 µg/ml and 15 µg/ml in symptomatic and asymptomatic antigens induced group respectively. The nitric oxide level differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic inductions are shown in Table 5.4.

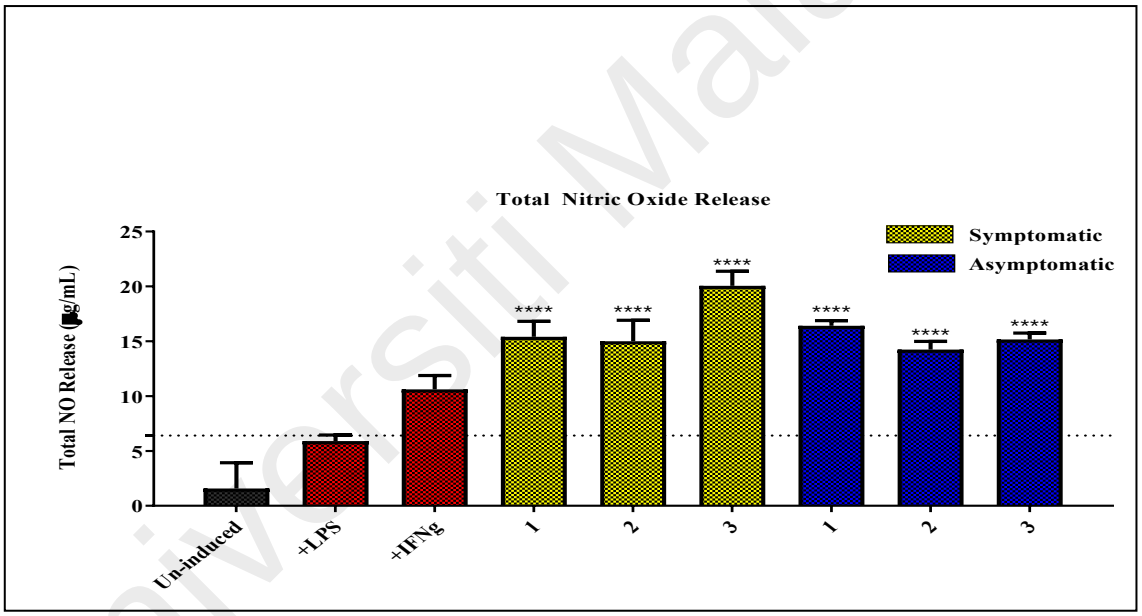


Figure 5.7: Assessment of Nitric Oxide Release (µg/ml) by RAW264.7 Macrophages Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens at 48 hours.

Values are given in mean ± SD (n=6). ****P < 0.0001 was the comparison made against un-induced RAW264.7 group served as negative control (n=6).

Table 5.4: RAW 264.7 nitric oxide release upon inductions with 10 µg/ml of *Blastocystis* sp. ST3 solubilised antigen incubation at 48 hours

Group	Nitric Oxide Release (µg/ml)
Symptomatic	16.82±2.8
Asymptomatic	15.27±1.1

Data is given as mean±SD difference between RAW 264.7 stimulated with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22) (n=6). The differences were not significant.

5.3.3 ELISA Test Results

5.3.3.1 Cytokine Assessment

In the present study, solubilised antigen of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens induced up-regulations of IL-6 and TNF-α cytokines secretion in RAW 264.7 macrophages as shown in Figure 5.8 and 5.9. The cell cytokine release differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic inductions are shown in Table 5.5.

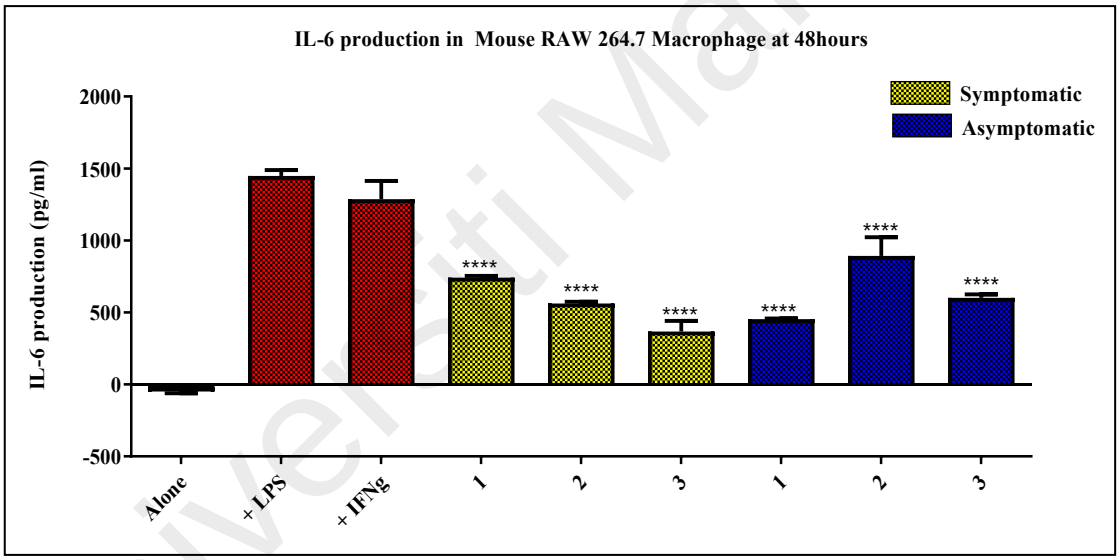


Figure 5.8: IL-6 Cytokine Response Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). ****P < 0.0001 was the comparison made against un-induced RAW264.7 group served as negative control (n=3).

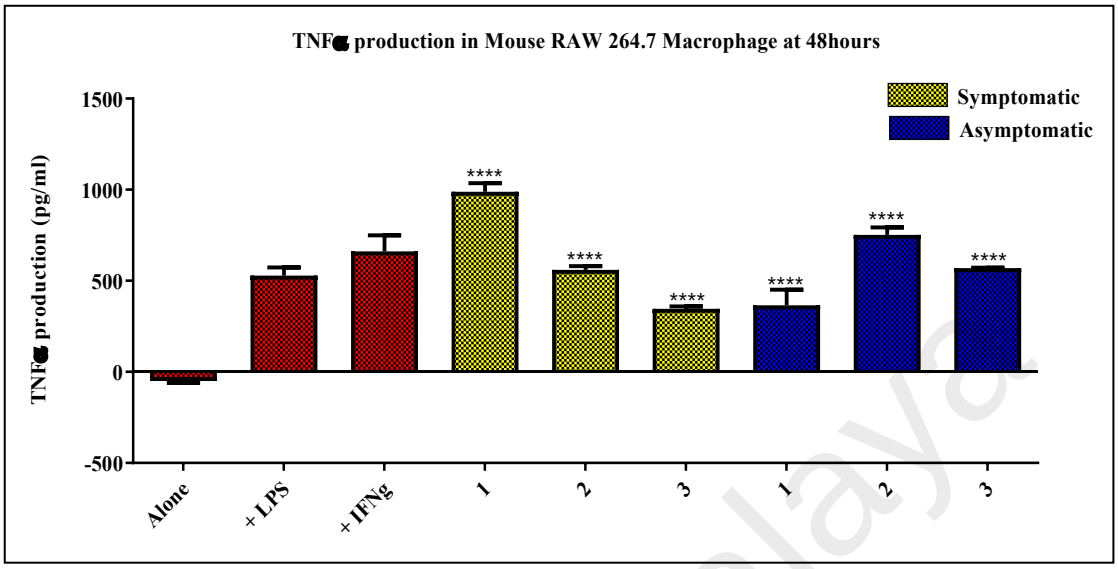


Figure 5.9: TNF-α Cytokine Response Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). ****P < 0.0001 was the comparison made against un-induced RAW264.7 group served as negative control (n=3).

Table 5.5: IL-6 and TNF-α cytokine secretion in RAW 264.7 cells upon inductions with 10µg/ml *Blastocystis* sp. solubilised antigen

Cytokine	Isolates	Secretions (pg/ml)
IL-6	Symptomatic	577.4±186.8
	Asymptomatic	649.3±223.2
TNF-α	Symptomatic	631.3±327.3
	Asymptomatic	562.3±193.6

Data is given as mean ± SD. The comparison was carried out between cytokines released by THP-1 cells stimulated with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). No significant differences (pg/ml) were observed.

5.3.4 Cell Culture Images

Cell proliferation was noticed in RAW264.7 macrophage upon induction with 10 µg/ml *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. Cell death was observed in cells induced with *Blastocystis* sp. antigens (as indicated by the arrows in Figure 5.10). However, no cell apoptosis observed in un-induced RAW264.7 macrophages.

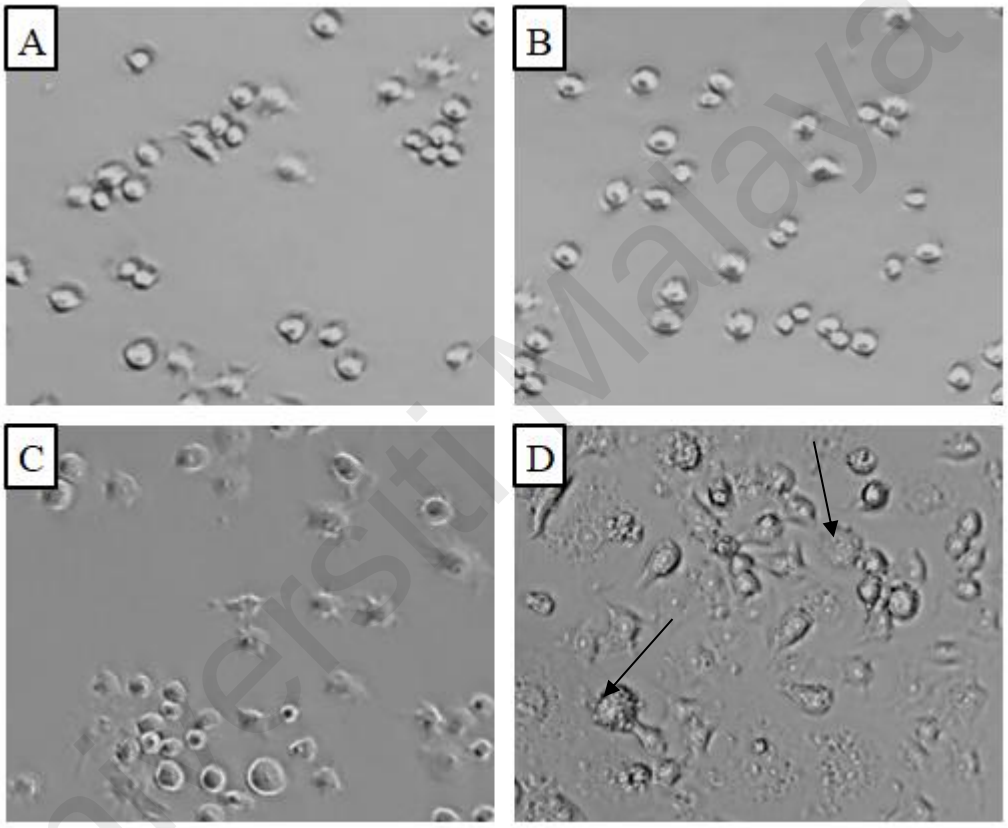


Figure 5.10: Phenotypic Differences of Un-Induced and *Blastocystis* sp. ST3 Induced RAW 264.7 Cells Through Phase Contrast Microscopy at 40X after 48 hours of Incubation

(A): Un-induced cells (B) LPS induced cells (C) IFNγ induced cells (D) symptomatic/asymptomatic solubilised antigens induced cells triggered cell death. Images were captured using inverted microscope with magnification at 40X.

5.3.5 Annexin V-Apoptosis Detection

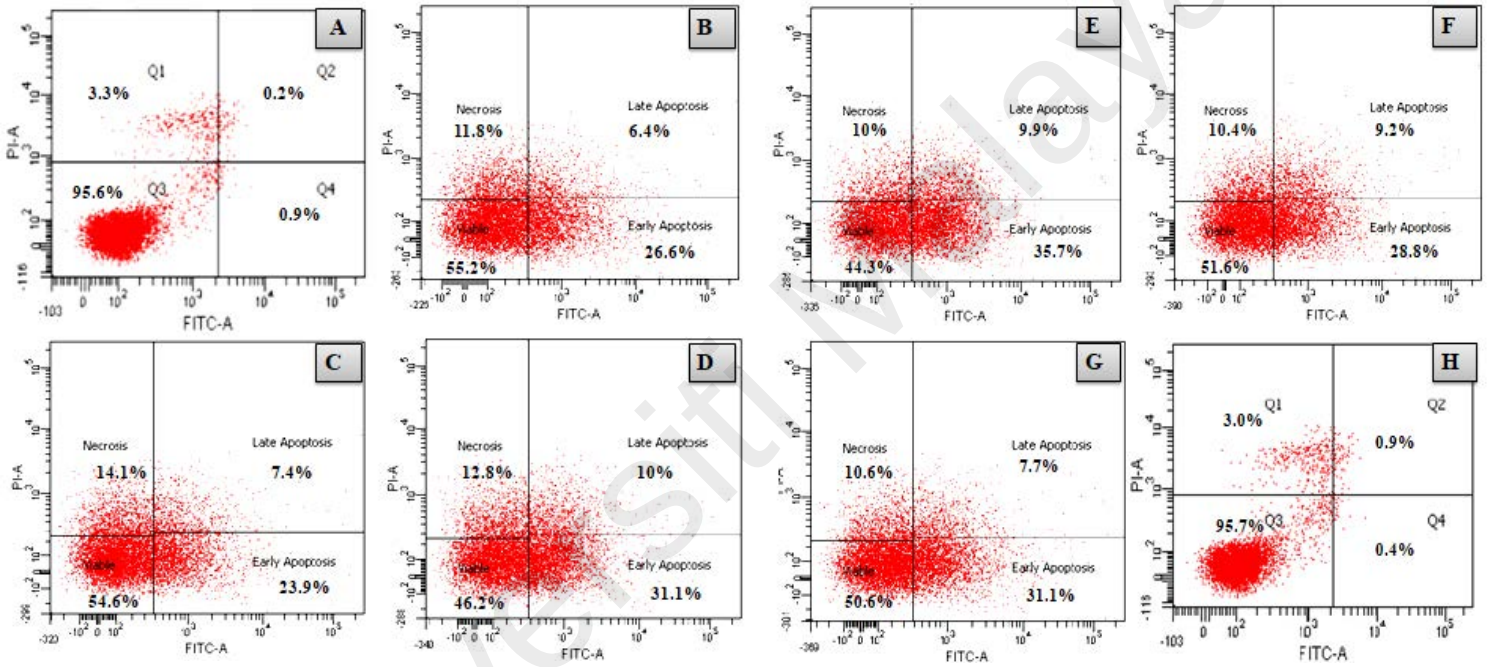


Figure 5.11: Apoptosis Detection in RAW 264.7 Macrophages

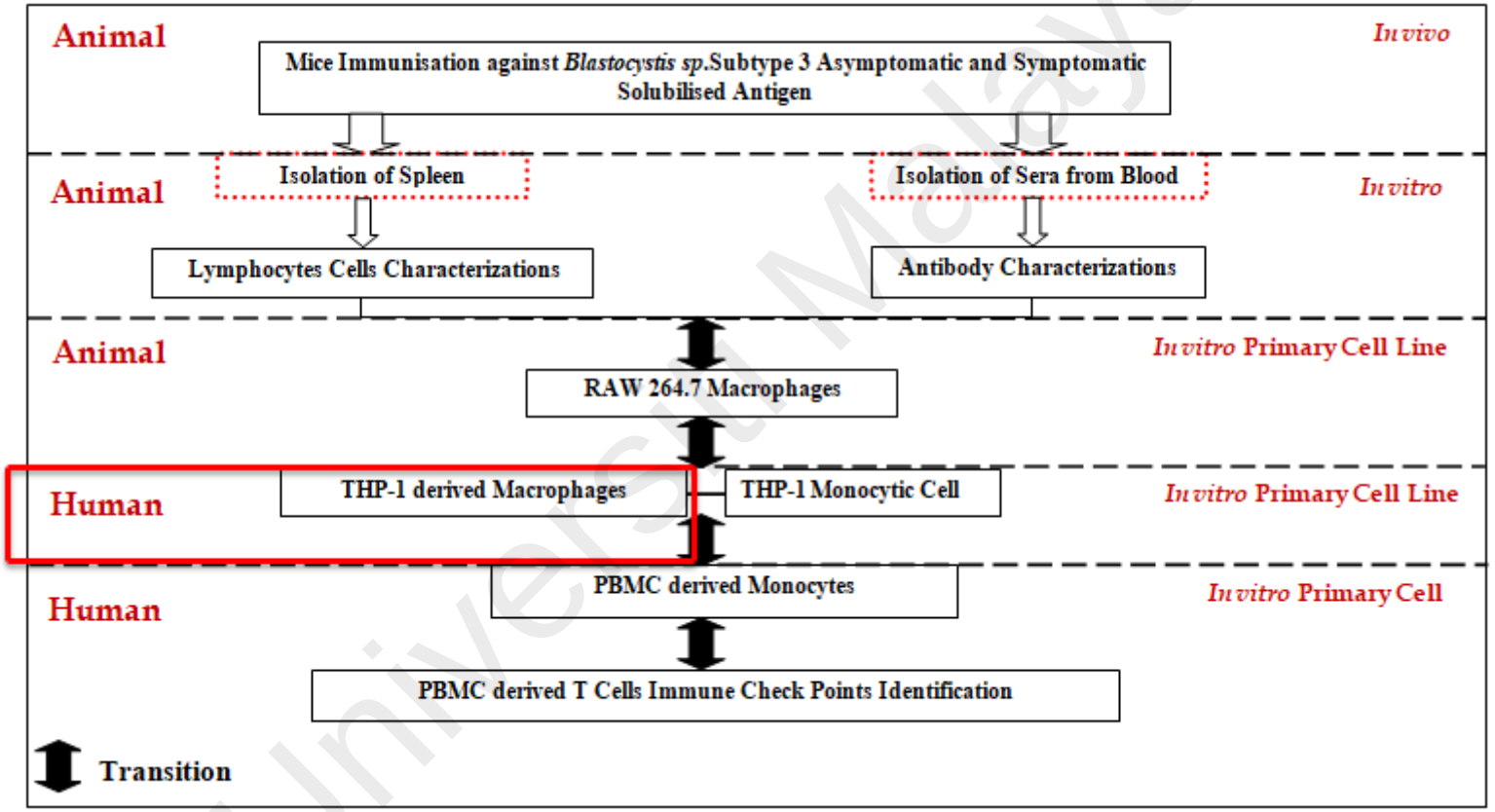
Induced with (A-C) S1-3 (D-F) AS1-3 (G) LPS-induced (H) un-induced. The quadrants specifying the phases of apoptosis were labelled as (Clockwise from top Necrosis; Late Apoptosis; Early Apoptosis and Viable).

Table 5.6: Summary of RAW 264.7 cells stimulation results with 10 µg/ml *Blastocystis* sp. solubilised antigen

Group	Cell Suppressions (%)	Cytokine (pg/ml)		NO Release (µg/ml)	Apoptosis Detection				
		IL-6	TNF-α		Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)	PD-1 Marker (%)
S1	38	741.6	988.5	15	55	27	6	12	13.5
S2	35	562.6	559.7	15	55	24	7	14	14.4
S3	31	368.0	345.8	20	46	31	10	13	12.5
Average	**35**	**577.4**	**631.3**	**17**	**52**	**27**	**8**	**13**	**13.4**
AS1	42	453.9	365.0	16	44	36	10	5	15.0
AS2	37	892.5	752.2	15	52	29	9	10	13.9
AS3	29	601.4	569.8	14	51	31	8	11	13.7
Average	**36**	**649.3**	**562.3**	**15**	**49**	**32**	**9**	**9**	**14.2**
Un-Induced	**0**	**0**	**0**	**1.6**	**96**	**0.4**	**0.9**	**3.0**	**0.3**

Data is given as mean. The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic antigen inductions.

PART 2: HUMAN CELL DERIVED MACROPHAGES



5.3.6 Cell Viability Assessment of THP-1 Derived Macrophages Stimulated with *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigen

5.3.6.1 Solubilised Antigen Concentrations Optimization

Solubilised antigen dose optimization with different concentrations (0.01, 0.1, 1 and 10 µg/ml) of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens inductions in THP-1 derived macrophages were observed. The analysis has shown that 10 µg/ml of *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens induced highest macrophage inhibitions with percentage of 40% and 31% respectively (Figure 5.12). Therefore 10 µg/ml soluble antigens were chosen as the optimal concentration to expedite the investigation of cell inhibition. The cell viability differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen inductions at different concentrations are shown in Table 5.7.

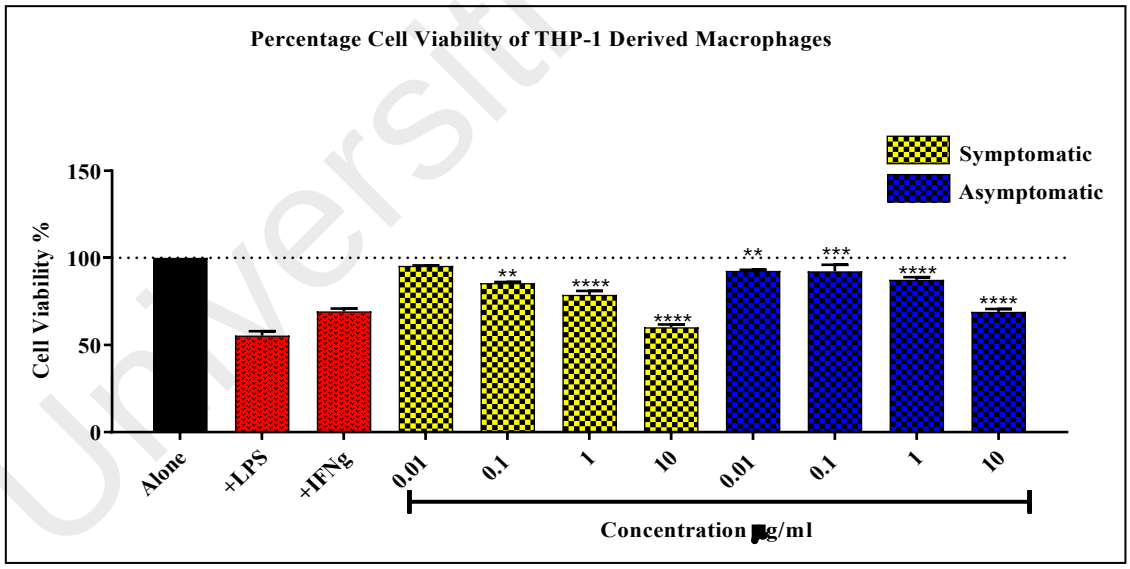


Figure 5.12: Cell Viability Assessment of THP-1 Derived Macrophages Stimulated with 0.001, 0.01, 1 and 10 µg/ml Solubilised Antigen of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Isolates

Values are given in mean ± SD (n=9). *P<0.05, **P<0.01, ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced THP-1 derived macrophages group served as negative control (n=6).

Table 5.7: THP-1 derived macrophages cell viability upon inductions with different concentrations of *Blastocystis* sp. solubilised antigen

Group	Solubilised Antigen (µg/ml)			
	0.01	**0.1**	**1.0**	**10.0**
Symptomatic	95.39±0.27	85.55±0.62	78.83±2.22	60.23±1.53
Asymptomatic	92.55±0.66	92.35±3.71***	87.39±1.53****	69±1.70****

Data is given as mean ± SD. The comparison was carried out between THP-1 derived macrophages induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). The differences were significant with, ***P<0.01 and ****P<0.0001.

5.3.6.2 Induction at 6, 24 and 48 hours

The THP-1 derived macrophages cells induced with 10 µg/ml of solubilised antigen (optimal concentration) of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens were carried out at 6, 24 and 48 hours. It was observed that *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens incubated for 48 hours induced highest macrophage inhibitions with percentage of 37.8% and 36.6% respectively as shown in Figure 5.13. Therefore 48 hours of soluble antigens induction were chosen for the subsequent observations. The cell viability differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen inductions at different timing are shown in Table 5.8.

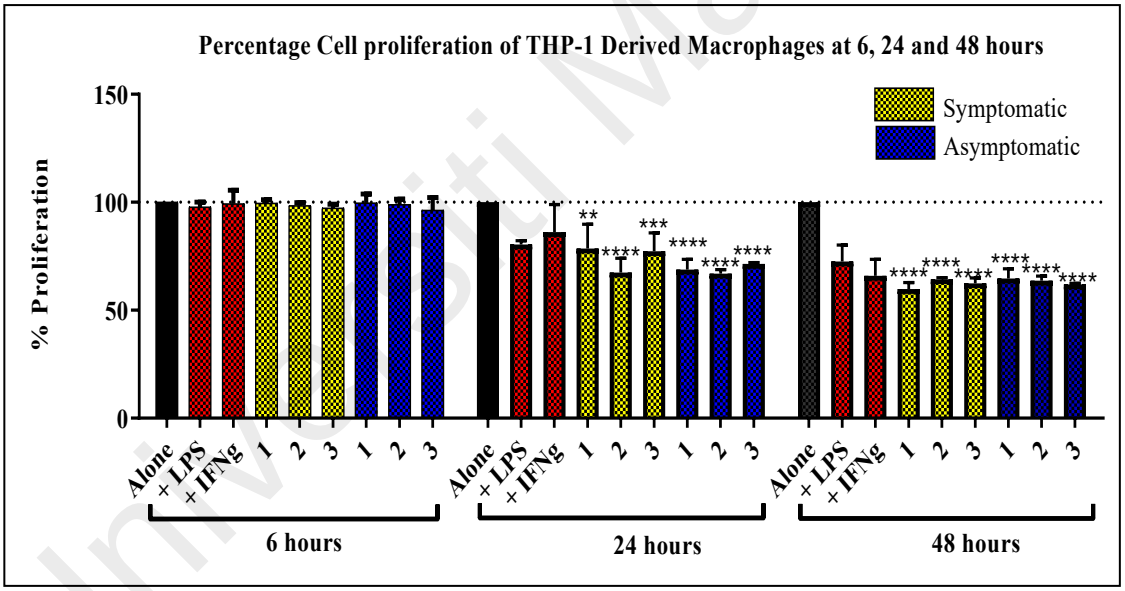


Figure 5.13: Cell viability Assessment of THP-1 Derived Macrophages Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens at 6, 14 and 48 hours

Values are given in mean ± SD (n=6). **P < 0.01 ***P < 0.001 ****P < 0.0001 was the comparison made against un-induced THP-1 derived macrophages group served as negative control (n=6).

Table 5.8: THP-1 derived macrophages cell viability upon inductions with 10 µg/ml of *Blastocystis* sp. solubilised antigen incubation at different timing

Group	Incubation Time (hours)	
	6 and 24	24 and 48
Symptomatic	23.98±4.01****	12.21±4.01*
Asymptomatic	29.25±4.01****	5.62±4.01*

Data is given as mean±SE difference between (6 and 24 hours) and (24 and 48 hours) of THP-1 derived macrophages induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). The differences were significant with, *P<0.05, and ****P<0.0001.

5.3.6.3 Induction at 48 hours

The THP-1 derived macrophages cells induction with 10 µg/ml of *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens was carried out at 48 hours. It was observed that *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens induced highest macrophages inhibitions at 40.7% and 56.2% respectively. The cell viability differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic inductions at 48 hours are shown in Table 5.9.

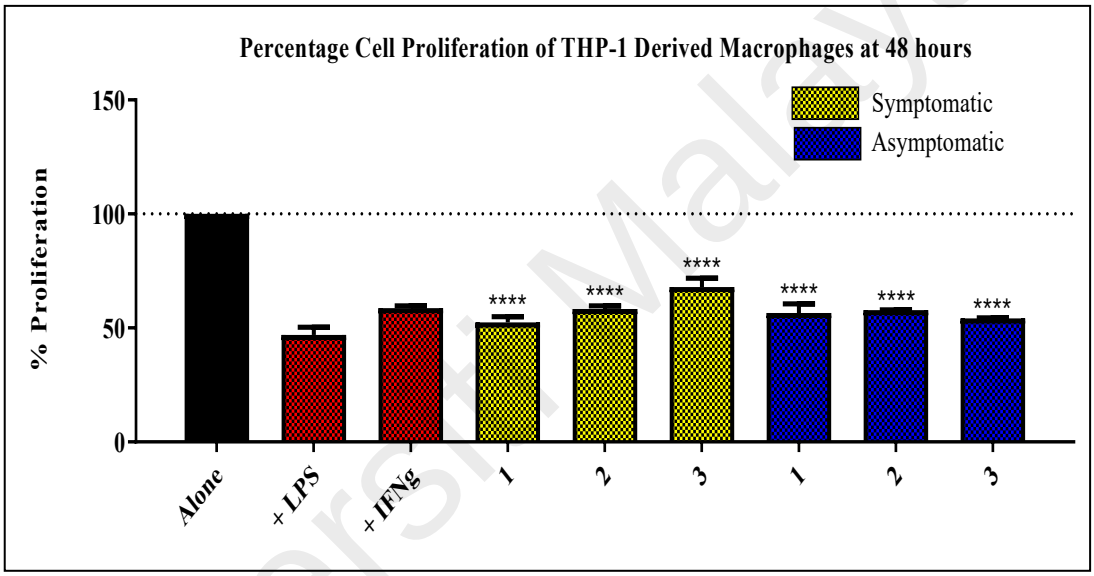


Figure 5.14: Cell viability Assessment of THP-1 Derived Macrophages Induced with 10 µg/ml *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens at 48 hours

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced THP-1 derived macrophages group served as negative control (n=6).

Table 5.9: THP-1 derived macrophages cell viability upon inductions with 10µg/ml of *Blastocystis* sp. solubilised antigen incubation at 48 hours

Group	Cell Viability (%)
Symptomatic	59.54±7.83
Asymptomatic	56.15±1.85

Data is given as mean±SD difference between THP-1 derived macrophages induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22) (n=6). The differences were not significant.

5.3.7 Nitric Oxide Release Assessment

The THP-1 derived macrophages cells induction with 10 µg/ml of ST3 *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens was carried out at 48 hours. It was observed that *Blastocystis* sp. asymptomatic and symptomatic antigens induced high nitric oxide release compared to un-induced group as shown in Figure 5.15. The cell nitric oxide release was observed at 25.6 µg/ml in both symptomatic and asymptomatic induced group. The nitric oxide differences between ST3 *Blastocystis* sp. symptomatic and asymptomatic inductions at different timing are shown in Table 5.10.

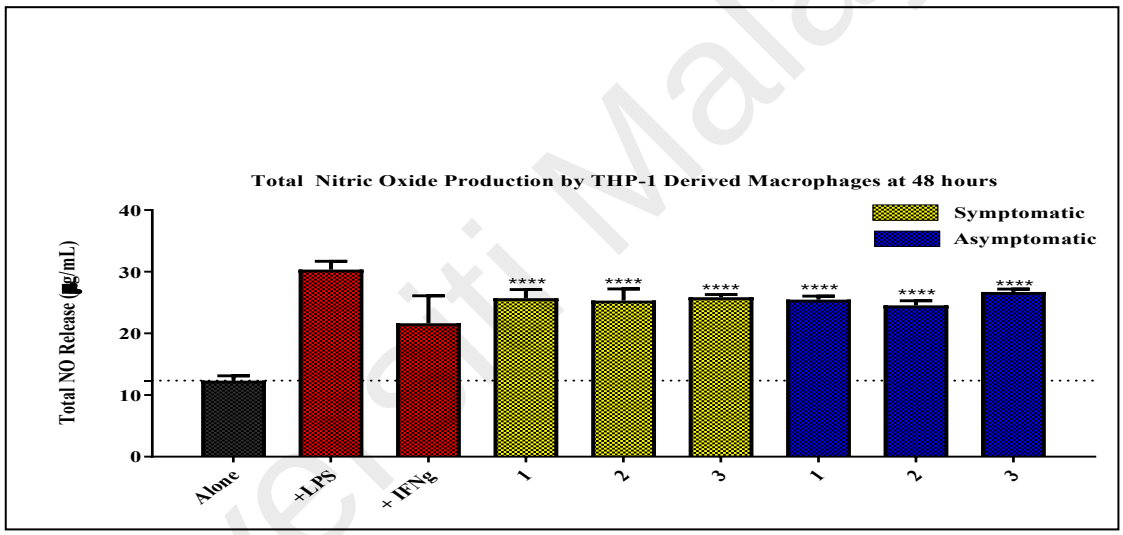


Figure 5.15: Assessment of Nitric Oxide Release (µg/ml) by THP-1 Derived Macrophages Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens at 48 hours.

Values are given in mean ± SD (n=6). ****P < 0.0001 was the comparison made against un-induced THP-1 derived macrophages group served as negative control (n=6).

Table 5.10: THP-1 derived macrophages nitric oxide release upon inductions with 10 µg/ml of *Blastocystis* sp. solubilised antigen incubation at 48 hours

Group	Nitric Oxide Release (µg/ml)
Symptomatic	25.64±0.29
Asymptomatic	25.58±1.1

Data is given as mean±SD difference between THP-1 derived macrophages induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22) (n=6). The differences were not significant.

5.3.8 ELISA Test Results

5.3.8.1 Cytokine Assessment

In the present study, solubilised antigen of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens induced up-regulations of IL-6 and TNF-α cytokines secretion in THP-1 derived macrophages. The differences of IL-6 and TNF-α cytokine release between ST3 *Blastocystis* sp. symptomatic and asymptomatic inductions are shown in Table 5.11.

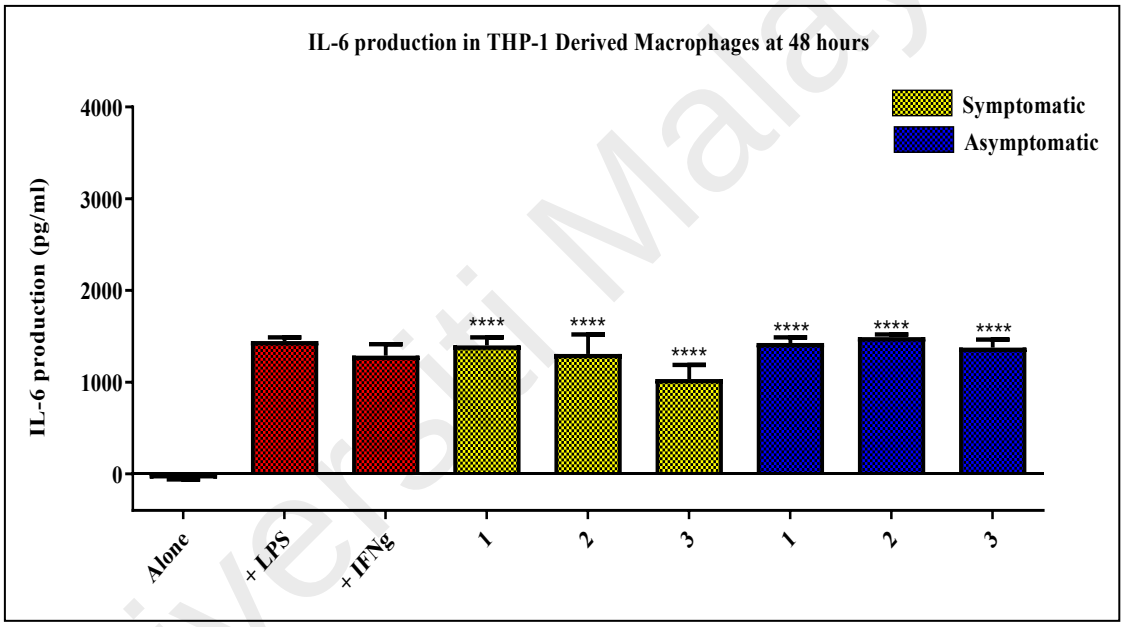


Figure 5.16: IL-6 Cytokine Response by THP-1 Derived Macrophages Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Values are given in mean ± SD (n=3). ****P < 0.0001 was the comparison made against un-induced THP-1 derived macrophages group served as negative control (n=3).

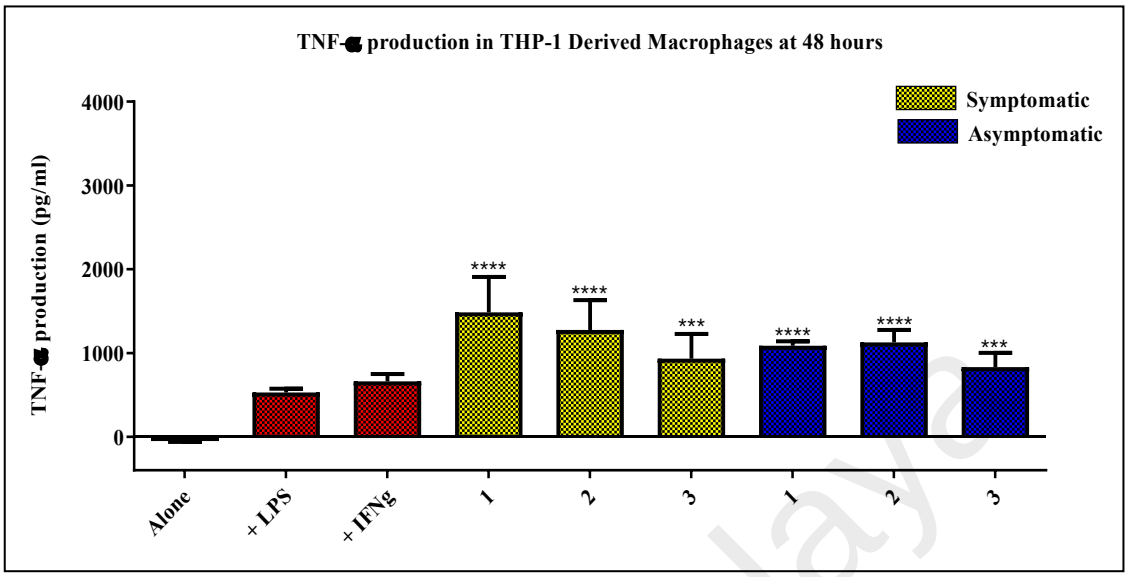


Figure 5.17: TNF-α Cytokine Response by THP-1 Derived Macrophages Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced THP-1 derived macrophages group served as negative control (n=3).

Table 5.11: IL-6 and TNF-α Cytokine Secretion by THP-1 Derived Macrophages Upon Inductions with 10 µg/ml *Blastocystis* sp. Solubilised Antigens

Cytokine	Isolates	Secretions (pg/ml)
IL-6	Symptomatic	1246.01±190.54
	Asymptomatic	1431.08±58.30
TNF-α	Symptomatic	1230.57±278.32
	Asymptomatic	1014.34±160.45

Data is given as mean ± SD. The comparison was carried out between cytokines released by THP-1 induced macrophages stimulated with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). No significant differences (pg/ml) were observed.

5.3.9 Cell Culture Images

Cell proliferation was noticed in THP-1 derived macrophage upon induction with 10 µg/ml *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. Cell shrinkage and blebbing was observed in cells induced with *Blastocystis* sp. antigens (as indicated by the arrows in Figure 5.18). However, no cell death observed in un-induced THP-1 macrophages.

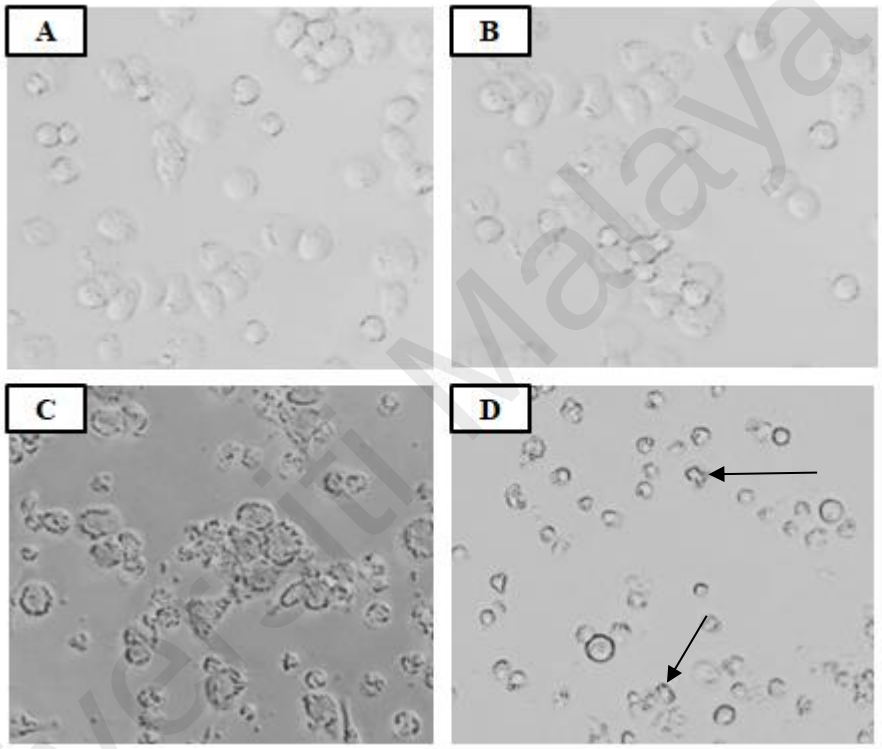


Figure 5.18: Phenotypic Differences of Un-Induced and *Blastocystis* sp. ST3 Antigens Induced THP-1 Macrophages Through Phase Contrast Microscopy at 40X after 48 hours of Incubation

(A): Un-induced cells (B) LPS induced cells (C) IFNγ induced cells (D) Symptomatic/asymptomatic solubilised antigens induced cells triggered cell shrinkage. Images were captured using inverted microscope with magnification at 40X.

5.3.10 Annexin V Results-Apoptosis Detection

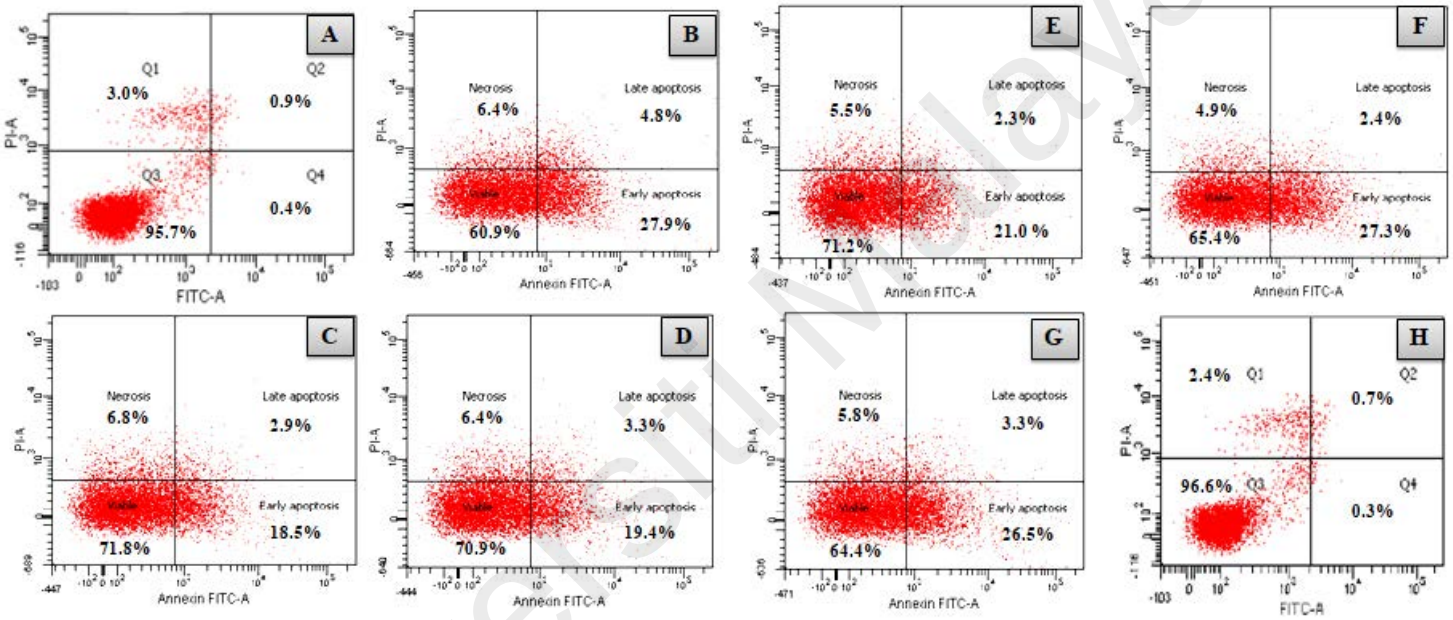


Figure 5.19: Apoptosis Detection in THP-1 Macrophages

Induced with (A-C) S1-3 (D-F) AS1-3 (G) LPS-induced (H) un-induced. The quadrants specifying the phases of apoptosis were labelled as (Clockwise from top Necrosis; Late Apoptosis; Early Apoptosis and Viable).

Table 5.12: Summary of THP-1 derived macrophages induction results with 10 µg/ml *Blastocystis* sp. solubilised antigens.

Group	Cell Suppressions (%)	Cytokine (pg/ml)		NO Release (µg/ml)	Apoptosis Detection				
		IL-6	TNF-α		Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)	PD-1 Marker (%)
S1	48	1399	1484	26	61	28	5	6	26.6
S2	42	1307	1275	25	72	19	3	7	26.1
S3	32	1033	933	26	71	19	3	6	27.2
Average	**41**	**1246**	**1231**	**26**	**68**	**22**	**3.7**	**6.3**	**26.1**
AS1	46	1376	831	25	64	27	3	6	25.6
AS2	43	1426	1086	25	71	21	2	6	27.0
AS3	42	1735	1126	27	65	27	2	5	23.9
Average	**44**	**1512**	**1014**	**26**	**67**	**25**	**2.3**	**5.7**	**25.5**
Un-Induced	**0**	**0**	**0**	**12**	**96.6**	**0.3**	**0.7**	**2.4**	**0.4**

Data is given as mean. The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic (S) and asymptomatic (AS) antigen inductions.

5.4 Discussion

Previous researchers have shown that *Blastocystis* sp. was able to influence macrophage functions by activating MAP kinases, up-regulating NFkβ along with exacerbation of pro-inflammatory cytokine responses (Lim et al., 2014; Teo et al., 2014). However, *Blastocystis* sp. isolates which were used for the analysis was devoid of ST3. Therefore, the role of macrophages in regulating immune response against ST3 *Blastocystis* sp. and its failure to combat against this luminal parasite seems important to be identified. To date there has been no focused investigation to study its association with macrophages. Further insights into the interactions between *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens provided more in-depth characterization of its pathogenicity. The present study has explored the fact that *Blastocystis* sp. ST3 derived antigens influenced its pathogenic potentials towards macrophages by mediating cell death which possibly displayed as a part of its immune evasion strategy in the infected individuals. It was demonstrated in this study that, *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens were capable of inducing cell inhibition of macrophages. The cell inhibition induced by macrophages was first detected with induction of various concentrations of *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens. The increasing concentrations of solubilised antigens have showed to induce cell inhibitions in macrophages in both symptomatic and asymptomatic isolates which may reflect the actual scenario in infected host whereby high number of *Blastocystis* sp. cells infection may lead to higher suppression of macrophages. The macrophage cells inhibition was further analysed at different time points (6, 24 and 48 hours). The introduction of *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens to macrophages was capable of inducing cell inhibitions as early as 6 hours of inductions. Furthermore, the cell inhibitions increased as the stimulation of time

increases from 6, 24 to 48 hours of incubations in both RAW264.7 and THP-1 derived macrophages. However, 48 hours incubation depicted the highest macrophage inhibition.

The induced and un-induced macrophages were further analysed for phenotypic changes. The macrophage induced with symptomatic and asymptomatic solubilised antigens has demonstrated cell shrinkage and blebbing which may indicate macrophage cell death. This was further validated through Annexin V assay to quantify the viable and non-viable cells. The results have shown that, macrophages induced with symptomatic and asymptomatic solubilised antigens have induced cells apoptosis and necrosis at 48 hours. Therefore, this analysis was further substantiated by analysing inflammatory cytokines response. Even though macrophages are constitutively resistant against apoptosis, factors such as pathogenic infection may trigger macrophage apoptosis which enable pathogen survival in host by evading the innate immune system. Furthermore, macrophage apoptosis may prevent the host immune mechanism to act against the invading pathogens by delaying the inflammation resolution.

The cytokine analysis in this study revealed that, the inductions of symptomatic and asymptomatic *Blastocytis* sp. ST3 solubilised antigens in macrophages (RAW264.7 and THP-1) cell lines *in vitro*, were able to stimulate the secretions of inflammatory cytokines (TNF-α and IL-6). Generally, mucosal intestinal inflammation and inflammatory disorders are caused by immune cells such as macrophages and their associated cytokines such as TNF-α that have appeared in various protozoan parasites such as *E. histolytica*, *T. cruzi*, *T. vaginalis* and *Leishmania*. (Lim et al., 2014). Furthermore, researchers have proven that TNF-α cytokine secretion possibly enhance the apoptosis induction in macrophages (Liu et al., 2004). Studies have also reported that, macrophages are the main cells which secrete high IL-6 cytokine in the intestinal regions of many chronic inflammatory intestinal diseases (Palmer et al., 2009). Therefore, this

study has shown that IL-6 and TNF-α cytokines may have exacerbated the process of macrophage apoptosis.

Previous experiments in Chapter 3 and 4 have highlighted the distinctiveness between symptomatic and asymptomatic isolates with disparity within their same origin through adaptive immune response characterization. However, the non-specific immune response against *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens in this study has shown non-significant immune response against macrophages. These were proven through cell viability test, apoptosis detection analysis and nitric oxide analysis. It was found that, *Blastocystis* sp. symptomatic and asymptomatic antigens mediated contrary apoptosis induction by possessing different cytokine modulations as shown in (Table 5.6 and 5.12). The macrophage apoptosis induced by symptomatic solubilised antigen caused exacerbation of pro-inflammatory cytokines (IL-6 and TNF-α). However, macrophage apoptosis induced by asymptomatic solubilised antigens resulted in decrease of pro-inflammatory cytokines secretions. This scenario is similar to the adaptive T cell response in Chapter 3 which has shown a pro-longed exacerbation of pro-inflammatory cytokines by symptomatic isolates whereas increase of anti-inflammatory cytokines by asymptomatic solubilised antigens. Since IL-6 portray a dual role as either pro-inflammatory or anti-inflammatory cytokine response, in this study it was clearly demonstrated that, IL-6 act as a pro-inflammatory cytokine for macrophages induced with symptomatic antigens whereas as an anti-inflammatory cytokine for macrophages induced with asymptomatic antigens.

As a result, this experiment has clearly demonstrated that higher pro-inflammatory cytokines are elevated by macrophages induced with symptomatic solubilised antigens. Meanwhile, the pro-inflammatory cytokines mediated by asymptomatic solubilised antigens was impaired through high elevations of IL-6 cytokine which triggered an anti-inflammatory effect instead. Therefore, it can be postulated in real scenario that, symptomatic isolates infected host may experience early symptoms due to triggering of high inflammation which may lead to intestinal damage in the host. However, asymptomatic isolates infected host potentially showed no early symptoms due to the attribute of asymptomatic isolates in being a "silent killer" by mediating high anti-inflammatory cytokine response and evade the macrophage innate immune mechanism.

In this study, further analysis on the nitric oxide (NO) results have shown high elevations in macrophages induced with *Blastocystis* sp. symptomatic and asymptomatic antigens. Generally, NO is described as an intracellular messenger that plays a major role is immune system. Innate immune cells such as macrophages utilise the pattern recognition receptors to recognise the molecular patterns of associated pathogens to release various effector molecules such as NO. Besides, studies have reported that, nitric oxide is highly triggered during the presence of inflammatory cytokines during infection (Tripathi et al., 2007). It was also reported that high release of NO may cause suppressions and damage to the immune cells including macrophages (Hortelano et al.,2002; Weigert et al., 2008). Therefore, in this study it can be also speculated that, the release of NO during the antigen inductions may have contributed to the macrophage apoptosis.

The macrophage apoptosis was further validated through analysis of PD-1 molecule (pro-immune inhibitory molecule). The immunophenotyping results obtained from this study has shown high expression of PD-1 by macrophages induced with symptomatic and asymptomatic antigens. Studies have reported that, PD-1 molecule was elevated during chronic inflammation on a variety of immune cells, including macrophages (Huang et al., 2009; Bally P et al., 2015; Roy et al., 2017). As such it was proven in this study that high inflammation induced by symptomatic and asymptomatic *Blastocystis* sp. antigens contributed to the acceleration of PD-1 molecule expression in macrophages which may directly correlated to the inflammatory cytokine exacerbation. Research has proven that, the up-regulations of PD-1 molecule during parasitical infection are highly linked to macrophage apoptosis and possibly be a mechanism of immune evasion inflicted by parasites (Roy et al., 2017). However, this study is the first to investigate on PD-1 expression and macrophage apoptosis associated with *Blastocystis* sp. This data may also suggest that PD-1 expression may not only be a dysfunctional marker for macrophages, but also be a potential therapeutic target for designing measures to prevent innate immune modulation by *Blastocystis* sp. ST3. Therefore, this may result in preventing the detrimental effects of apoptosis and necrosis in macrophages caused by *Blastocystis* sp. ST3.

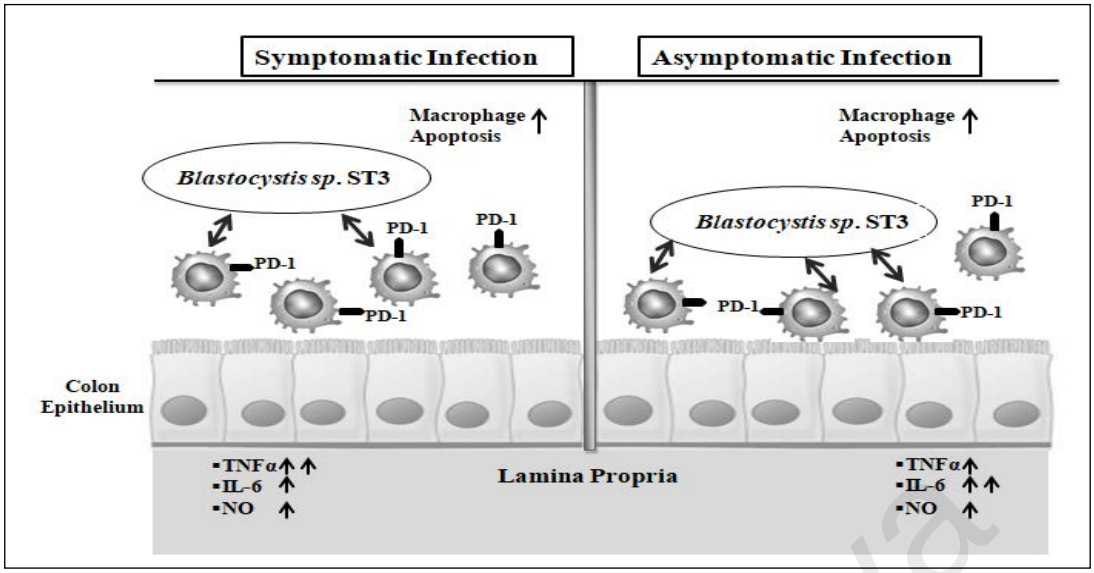


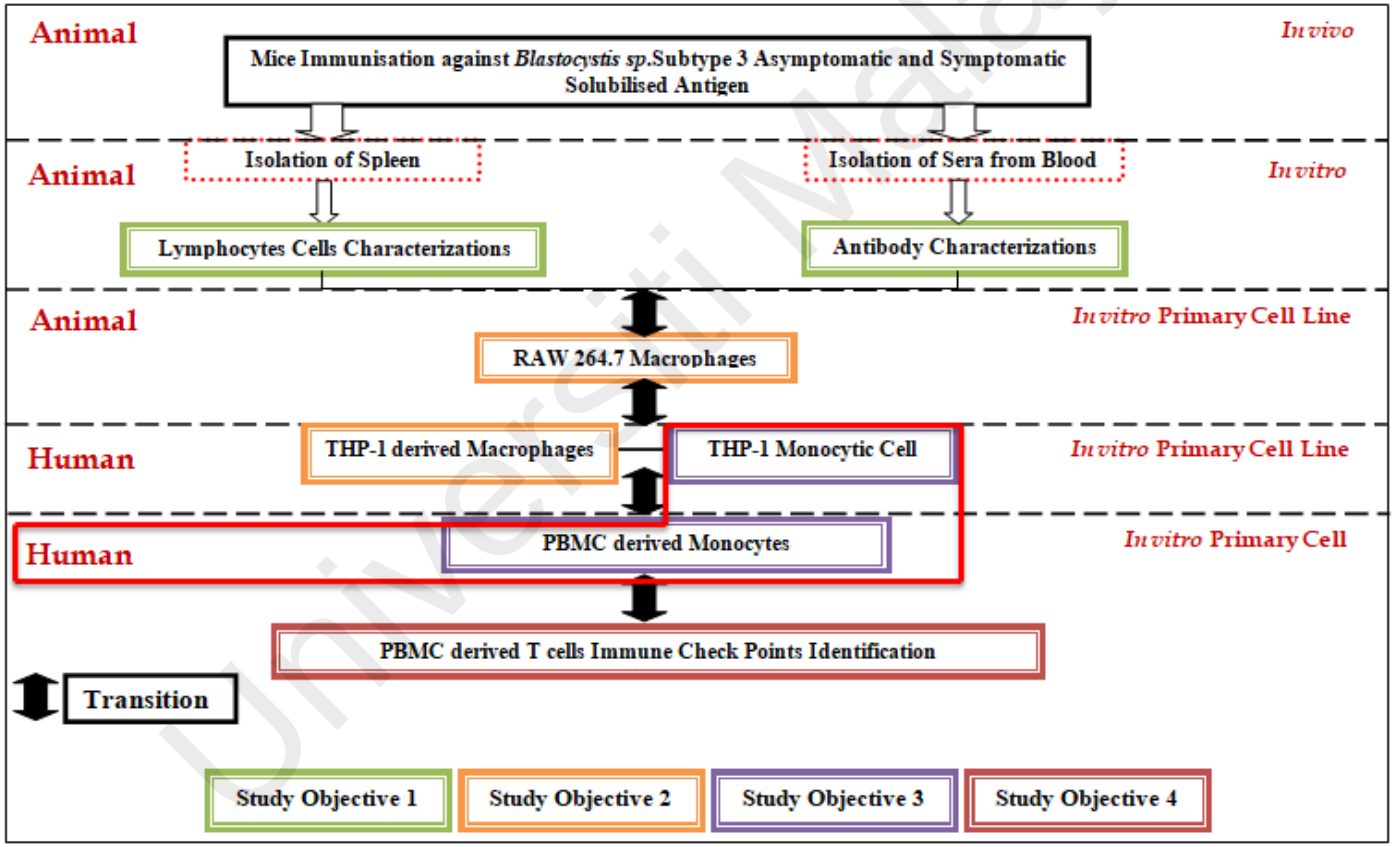
Figure 5.20: Summary of Macrophages Immune Response Upon Induction with *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Isolates

5.5 Conclusion

There has been no study thus far which elucidated the effects of *Blastocystis* sp. in causing dysfunctional effects on macrophages. Therefore, it has been proven for the first time that, *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates causes cell senescence during its association with macrophages by releasing high inflammatory cytokines secretions and increased PD-1 molecule expression as shown in Figure 5.20. The increased cell suppressions observed in mouse and human macrophages cell lines suggesting that, *Blastocystis* sp. ST3 may prevent macrophages from combating this parasite invasion at the earliest infectious phase. These propositions became more evident with the apoptosis and necrosis exhibition by the macrophages incubated with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. It was also proven that there were similar macrophage immune responses induced by this parasite on macrophages of animal and human origin. In a nutshell, for the first time the immune evasions strategies of symptomatic and asymptomatic isolates were demonstrated in this study and it showed different innate immune modulations imposed by antigens of these

isolates. Therefore, further analysis on the detrimental effects of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates in causing innate immune modulation were investigated on a different antigen presenting cell (monocytes) which were covered in the following Chapter 6.

CHAPTER 6: *IN VITRO* STUDY TO EVALUATE MONOCYTES IMMUNE RESPONSE AGAINST *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC ANTIGENS DURING SHORT- AND LONG-TERM INDUCTIONS.



6.1 Introduction

In the previous Chapter 5 antigen presenting cell immune modulation against *Blastocystis* sp. ST3 was studied. It was shown that the macrophages function was impaired upon introduction with solubilised antigens of this parasite. In this chapter, further investigations on *Blastocystis* sp. innate immune modulation was performed by using monocytes model. Generally, during acute infection, monocytes from the blood stream are one of the immune cells after neutrophils to be recruited to the infection area (Ingersoll et al., 2011). During a pro-longed or chronic infection, monocytes will initiate the activation of T cells (the arm of adaptive immune system) to eradicate the infection by differentiating into macrophages and/or dendritic cells (Arango Duque et al., 2014). (Figure 6.1). Researchers have proven that, monocytes contribute to the increase of infection or disease pathogenesis. The correlations between monocytes and disease pathogenesis were proven in various chronic intestinal inflammatory diseases such as IBD, Crohn's and colorectal cancer (Mazlam MZ and Hodgson, 1992; Rugtveit et al., 1994; Shibutani et al., 2017). These chronic intestinal inflammatory diseases are also highly linked with *Blastocystis* sp. infection.

Thus far there has been no study to investigate the associations between monocytes and ST3 *Blastocysis* sp. symptomatic and asymptomatic isolates especially in identifying the role of monocytes in exacerbating the pathogenicity of this parasite. The correlations between monocytes and *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates were studied to gain better insights on the innate immune modulations by this parasite and subsequently identify its immunopathogenesis during short and pro-longed infection. Thus, in this study, *in vitro* analysis was performed by inducing *Blastocystis* sp. antigens on monocytes cells (THP-1 monocytic cell line) and (human PBMCs derived monocytes for 3 and 6 days. Experimental design of this study is described below:

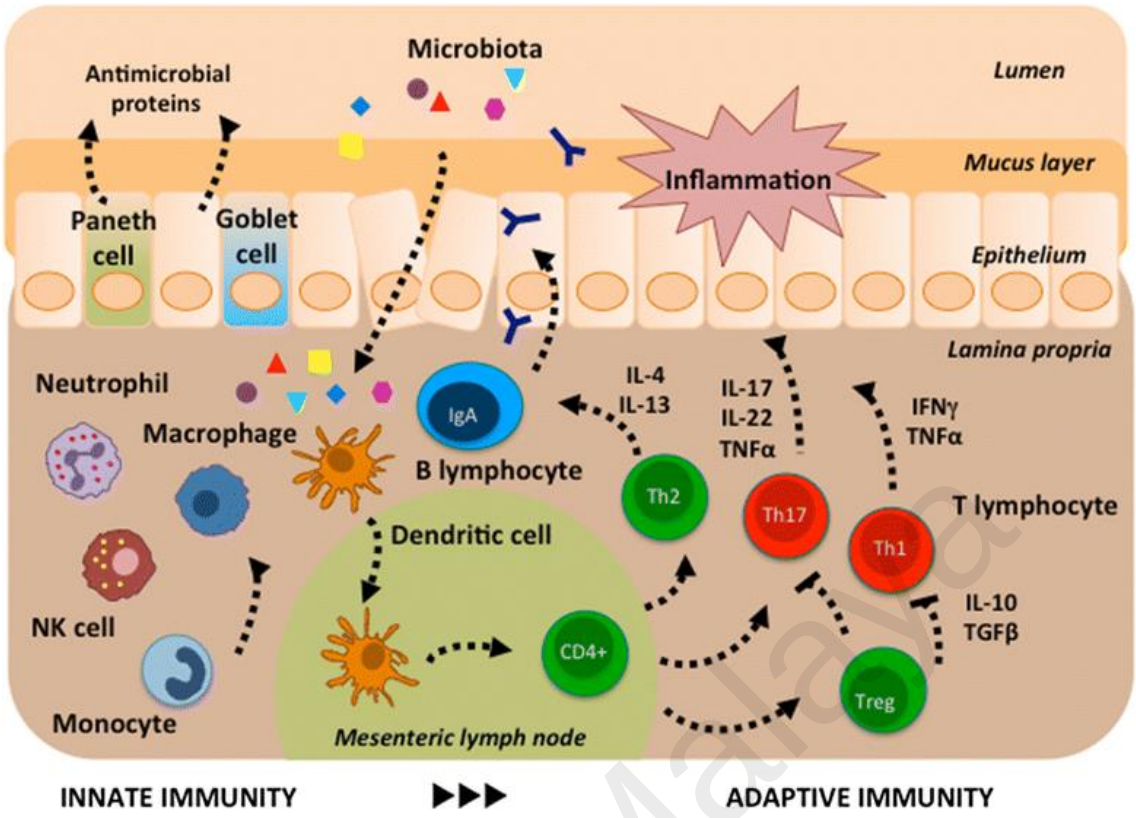


Figure 6.1: Mechanisms of Monocytes in Regulating Immune Response During an IBD infection

(Source: Leal et al., 2015)

6.1.1 Study Design Description

In this study human monocytic cell line THP-1 was used as it is a common model to estimate monocyte immune modulations (Chanput et al., 2014; Bosshart et al., 2016) and subsequently the analysis was transitioned to human PBMCs derived monocytes (CD14+) to compare the results with human primary cells. On the other hand, 3- and 6-days antigen inductions were used to resemble short- and long-term infections. The induction of antigen for short- and long-term infections for 3 and 6 days was impersonated from previous studies where different timeframe of immune cells inductions created impact on the cellular growth and survival during short-term and long-term phases of infection (De Mattia et al., 1999; Anguille et al., 2009; Liu et al., 2016; Keilhack and Chang, 2017; Forsythe et al., 2004). Concanavalin-A (Con-A) a lectin from jack-bean (Canavalia ensiformis), was used as a ConA was chosen as a negative or weak stimulus of the innate immunity, since it was reported to modulate low immune responses against THP-1 monocytic cell line (Chanput et al., 2010).

6.2 Materials and Methods

6.2.1 Source of *Blastocystis* sp.

As mentioned in 3.2.1

6.2.2 Axenization of *Blastocystis* sp. and Isolation of Solubilised Antigen

As mentioned in 3.2.2

6.2.3 Human THP-1 Monocytic Cell Line Culture

In this study, the THP-1 human monocytic cell line (ATCC) was used to investigate host-*Blastocystis* sp. interactions. THP-1 cells were maintained in T-75 flasks in a humidified incubator at 37°C and 5% CO_2. The THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (PAN-Biotech) and 10% heat-inactivated fetal bovine serum (Gibco, USA). Culture viability was evaluated every 3 to 4 days using the trypan blue assay and only cultures with >95% viability was used for the experiment.

6.2.4 THP-1 cells Inductions with *Blastocystis* sp. Solubilised Antigen

Briefly, 3×10^4 cells per well was suspended in RPMI complete medium and seeded in sterile 96-well plates. The seeded cells were induced with *Blastocystis* sp. ST3 solubilised Ag (0.01-10 µg/ml) from symptomatic (S) isolate and asymptomatic (AS) isolates. Three isolates were used for symptomatic (S1-3) and asymptomatic (AS1-3) groups respectively. The un-induced THP-1 cells were served as negative control. The plates were then incubated for 72 hours. After 72 hours the short-term induction was terminated by centrifugation at 300xg. While for the long-term induction, the THP-1 cells were subjected to continued culture by media replacement and re-induction of ST3 AS (1-3) and S (1-3) (0.01-10 µg/ml) *Blastocystis* sp. solubilised Ag; incubated for 72 hours. Meanwhile the un-induced THP-1 cells were subjected for media change only. After 72 hours, the long-term induction was terminated by centrifugation at 300xg.

6.2.5 Human Peripheral Blood Mononuclear Cells (hPBMCs) Isolation

Fresh human peripheral blood samples were collected in 10 ml of heparin tubes. The PBMCs were then isolated from the blood sample by using Histopaque®-1077 (Sigma-Aldrich, USA). 15ml of peripheral blood was gently layered onto 12 ml of Histopaque in a 50 ml conical centrifuge tube (BD, USA) and was centrifuged at 400 x g for 30 minutes at room temperature. After centrifugation, the layers containing PBMCs was transferred into a sterile 50ml conical centrifuge tube. The isolated PBMCs were then washed with PBS three times and centrifuged at 250 x g for 10 minutes. After the final washing step, the resulting cell pellet was re-suspended in 2 ml of RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin-streptomycin and cultured in a T75 cell culture flask prior to the monocytes (CD14+) cell isolation.

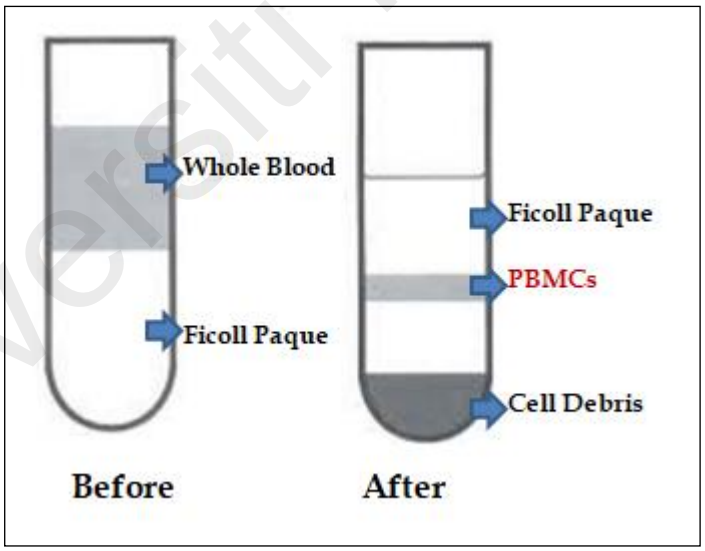
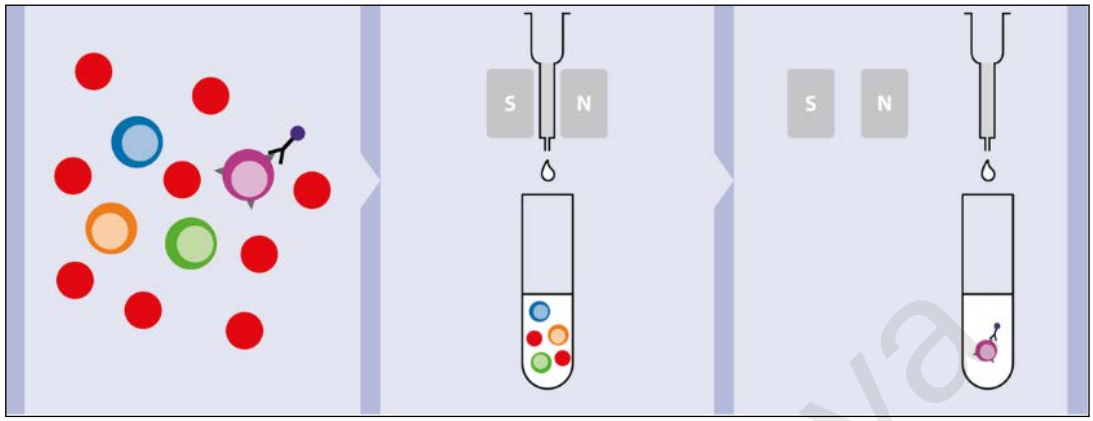


Figure 6.2: PBMCs Isolation from Peripheral Whole Blood

6.2.6 Human Monocytes Cell (CD14+) Isolation from PBMCs



(A) Step 1 **(B) Step 2** **(C) Step 3**

Figure 6.3: Monocytes Isolation from PBMCs using MACS Cell Separation Kit

(Adapted and modified from: Miltenyi Biotec Product Sheet)

6.2.6.1 Step 1: Magnetic Labelling

The magnetic labelling was performed as illustrated in Figure 6.3 (A). The cells were re-suspended in buffer (0.5% BSA 2 mM EDTA in PBS) at a concentration of 10^7 cells per 90 µl. 10 µl CD14 micro beads were added into 10^7 of cells. The suspension was incubated for 15 min at 4°C. After incubation, 10 ml of the buffer were added to the cells and centrifuged for 10 min at 300xg. The supernatant was removed and the cells pellet was re-suspended in 500 µl buffer.

6.2.6.2 Step 2: Magnetic Separation

LS column as shown in Figure 6.3 (B) was placed in the magnetic separation unit and washed with 3 ml of buffer. A collecting 50ml Centrifuge tube was placed under the LS Column before adding the cell suspension into the column. The non CD14+ cell flow-through was collected in the collecting tube. The column was further washed 3 times with 3 ml buffer and the flow-through was collected in the same collecting tube. The flow through contained the non CD14+ cells whereas the CD14+ cells were retained in the column.

6.2.6.3 Step 3: Elution of the Labelled Cells

Elution of the CD14+ labelled cells was performed as illustrated in Figure 6.3 (C). The column was removed out of the separation and was placed on 50 ml centrifuge tube. Thereafter, 5 ml buffer was added to the column followed by plunging the column with a plunger to elute CD14+ cells. The cells were collected in the 50 ml centrifuge tube followed by centrifugation for 10 minutes at 300 g. After centrifugation, the supernatant was removed and the pellet containing CD14+ cells was re-suspended and cultured in X-vivo 10 media at a concentration of $5x10^5$ cells per ml. The cells were counted using tryphan blue exclusion dye. An aliquot of the cells was subjected for FACS analysis to confirm its marker.

6.2.6.4 Human Primary Monocytes Inductions with *Blastocystis* sp. Solubilised Antigen

Briefly, 3×10^4 cells per well was suspended in RPMI complete medium and seeded in sterile 96-well plates. The seeded cells were induced with *Blastocystis* sp. ST3 solubilised Ag (0.01-10 µg/ml) from symptomatic (S) isolate and asymptomatic (AS) isolates. Three isolates were used for symptomatic (S1-3) and asymptomatic (AS1-3) groups respectively. The un-induced monocytes cells were served as negative control.

The plates were then incubated for 72 hours. After 72 hours the short-term induction was terminated by centrifugation at 300xg. While for the long-term induction, the monocytes cells were subjected to continued culture by media replacement and re-induction of ST3 AS (1-3) and S (1-3) (0.01-10 µg/ml) *Blastocystis* sp. solubilised Ag; incubated for 72 hours. Meanwhile the un-induced monocytes cells were subjected for media change only. After 72 hours, the long-term induction was terminated by centrifugation at 300xg.

6.2.7 Cell Viability Assay

Following assay termination in method 6.2.4 and 6.2.7, the cell pellet (induced and un-induced) were subjected to cell viability test using Cell Titter Glow Assay. The protocol is as mentioned in 5.2.8.2.

6.2.8 ELISA Test

Following assay termination in method 6.2.4 and 6.2.7, the respective supernatant was then assayed for; Interleukin-6 (IL-6) and Interleukin-10 (IL-10); Interleukin-12p70 (IL-12p70) and tumor necrosis factor alpha (TNF-α) using a commercial Human ELISA Kit (R&D Systems, USA) according to the manufacturer's instructions (Appendix B). All the assays were performed in triplicates.

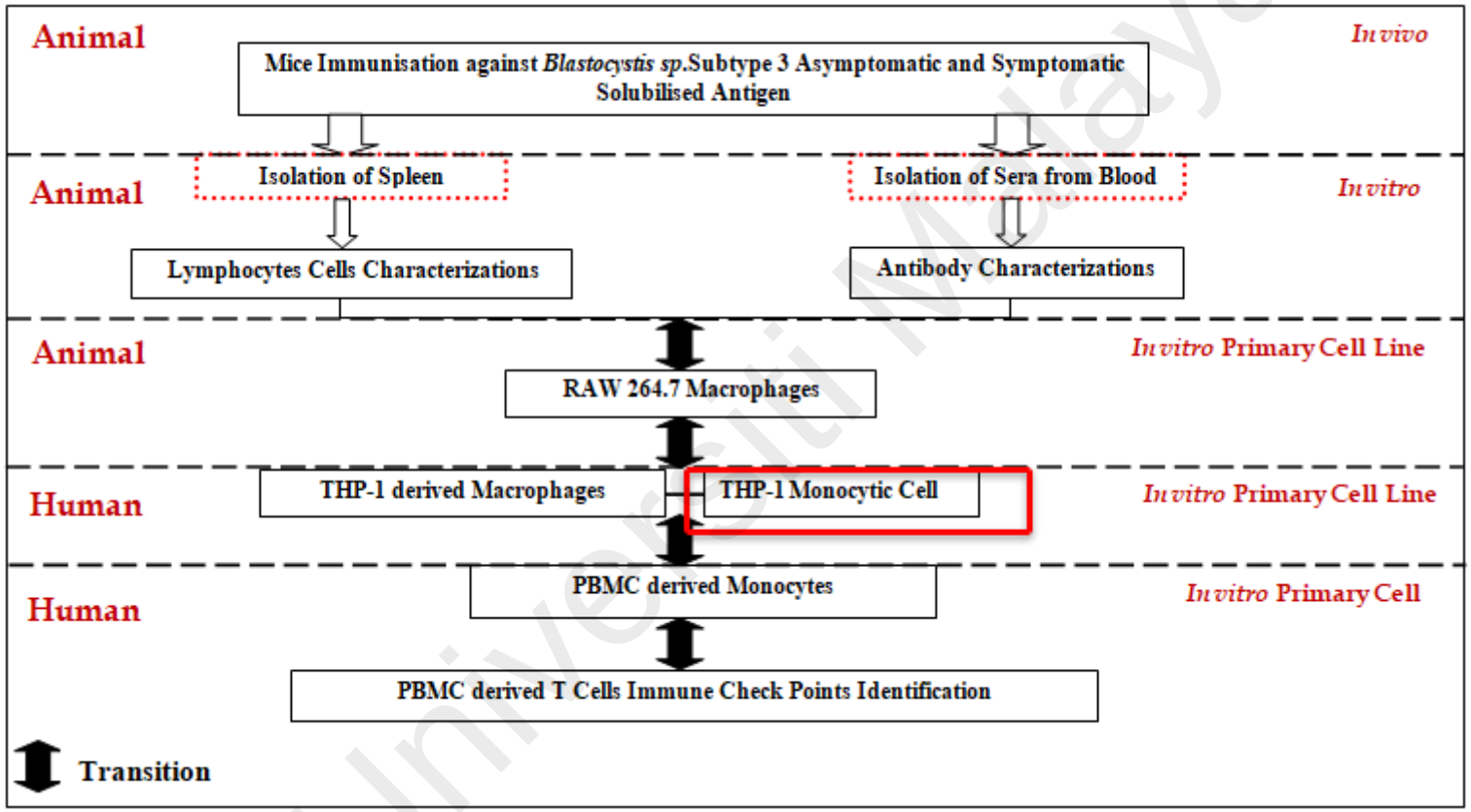
6.2.9 Flow Cytometry

Following assay termination in method 6.2.4 and 6.2.7, monocytes pellets obtained from the assays (induced and un-induced), were comprehensively characterized by multicolour flow cytometry for the expression of the surface marker PD-1 molecule, CD14+ and CD86 following the protocol mentioned in method 5.2.11.

6.2.10 Statistical analysis

Data was analysed by one-way analysis of variance (ANOVA) using SPSS software 22. The graphs were plotted using Graph Pad Prism 8.

PART 1-HUMAN CELL LINE DERIVED MONOCYTES



6.3 Results

6.3.1 Cell Viability Assessment of THP-1 Monocytes Stimulated with *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigen

6.3.1.1 Solubilised Antigen Concentrations Optimization at 3- and 6-days Induction

Dose optimization with different concentrations (0.01, 0.1, 1 and 10 µg/ml) of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigen response in THP-1 monocytes were assessed (Figure 6.4). *Blastocystis* sp. symptomatic and asymptomatic isolates induced the highest cell proliferations at 10 µg/ml soluble antigen with 3.4-fold and 1.6-fold increase respectively from 3 to 6 days induction as reflected in Table 6.1. Therefore 10 µg/ml solubilised antigen was chosen as the optimal concentration for subsequent experiments. Asymptomatic and Symptomatic antigen at this concentration induced highest percentage of cell proliferation which is 123% and 76% respectively at 6-day incubation assay. The cell proliferation differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic antigen induction were shown in Table 6.1.

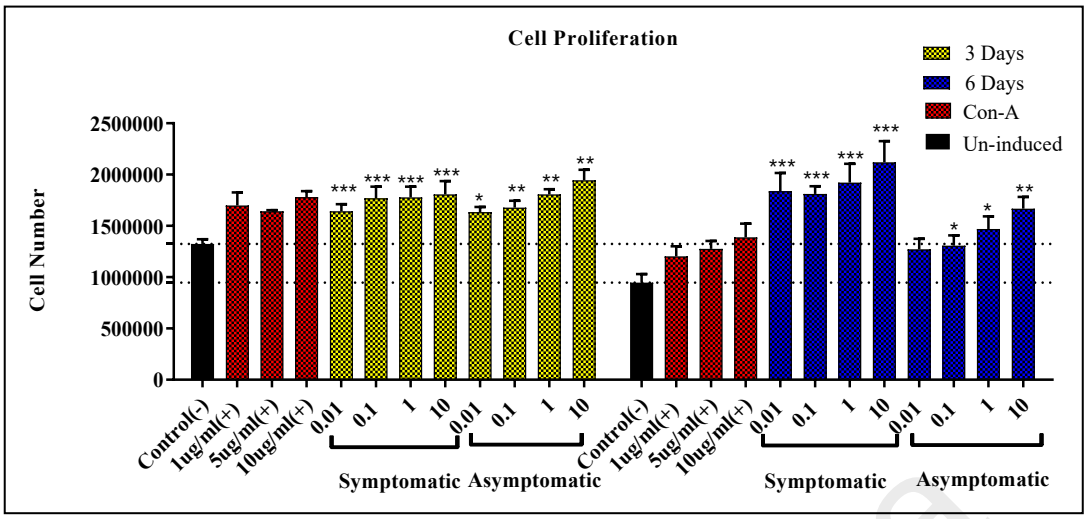


Figure 6.4: Cell Viability Assessment of THP-1 Cells Stimulated with 0.001, 0.01, 1 and 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). *P<0.05, **P<0.01, ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced THP-1 cells served as negative control (n=6).

Table 6.1: Summary percentage of THP-1 cell proliferation at 3- and 6-days inductions

	THP-1 Cell Proliferation							
Isolates	**Symptomatic**				**Asymptomatic**			
Blastocystis-Ag (µg/ml)	**0.01**	**0.1**	**1**	**10**	**0.01**	**0.1**	**1**	**10**
3 Days Incubation (%)	24.1	33.7	34.2	36.5	23.5	26.7	36.6	46.8
6 Days Incubation (%)	94.2****	91.4****	103****	123.8****	34.3	55.3	32.4	76.0
% Increase	**70.1**	**57.7**	**68.8**	**87.3**	**10.8**	**28.6**	**-4.2**	**29.2**

Data is given as mean ± SD (n=9). The comparison was carried out between 3- and 6-days proliferation of THP-1 cells induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). The differences were significant with, ****P<0.0001.

6.3.2 ELISA Test Results

6.3.2.1 Cytokine Assessment

In the present study, solubilised antigen of ST3 *Blastocystis* sp. symptomatic and asymptomatic antigens induced up-regulations of pro-inflammatory cytokines (IL-6, IL-12p70 and TNF-α) and anti-inflammatory cytokine IL-10 secretions in THP-1 cells. The level of cytokine secretions induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens in THP-1 monocytes were shown in Figure 6.5 to 6.8. The pro- and anti-inflammatory cytokine level differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic induction were shown in Table 6.2.

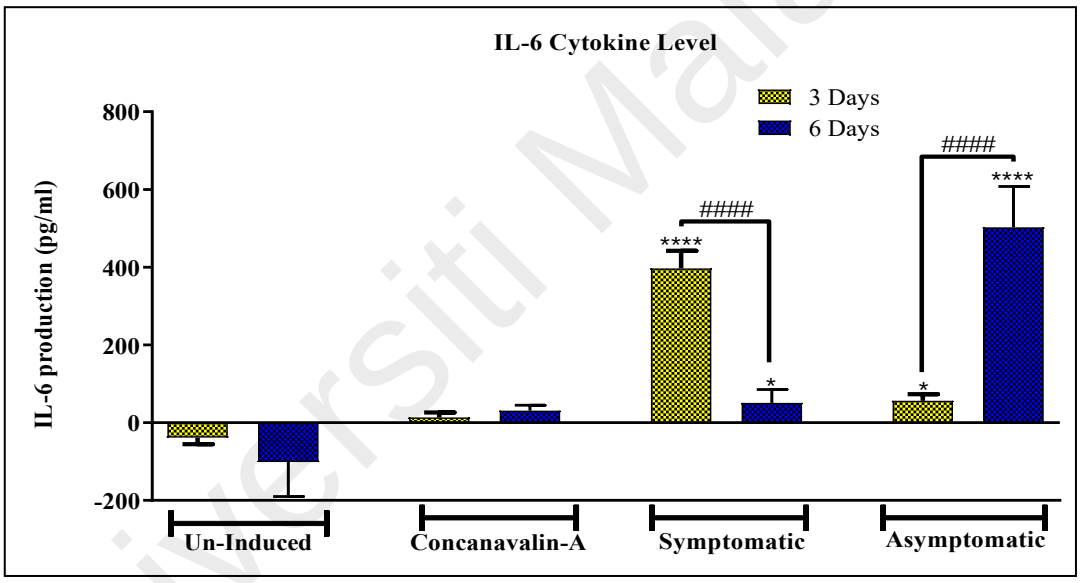


Figure 6.5: IL-6 Cytokine Level by THP-1 Cells Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). *P<0.05 and ****P < 0.0001 was the comparison made against un-induced THP-1 cells served as negative control (n=3). ####P<0.0001 was the comparison made between 3 and 6 days of induction.

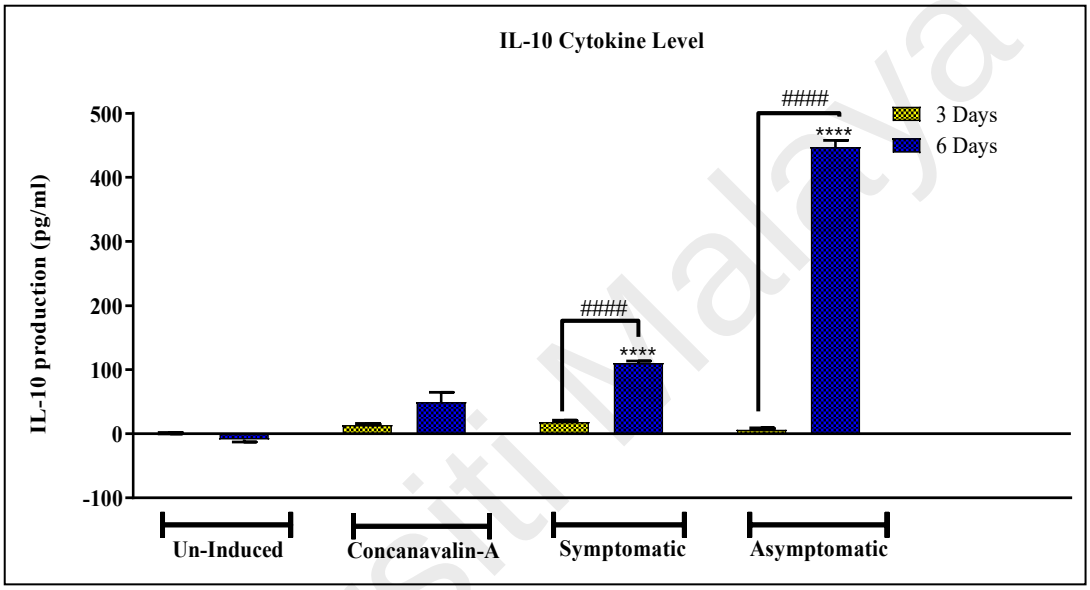


Figure 6.6: IL-10 Cytokine Level by THP-1 Cells Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced THP-1 cells served as negative control (n=3). ####P<0.0001 was the comparison made between 3 and 6 days of induction.

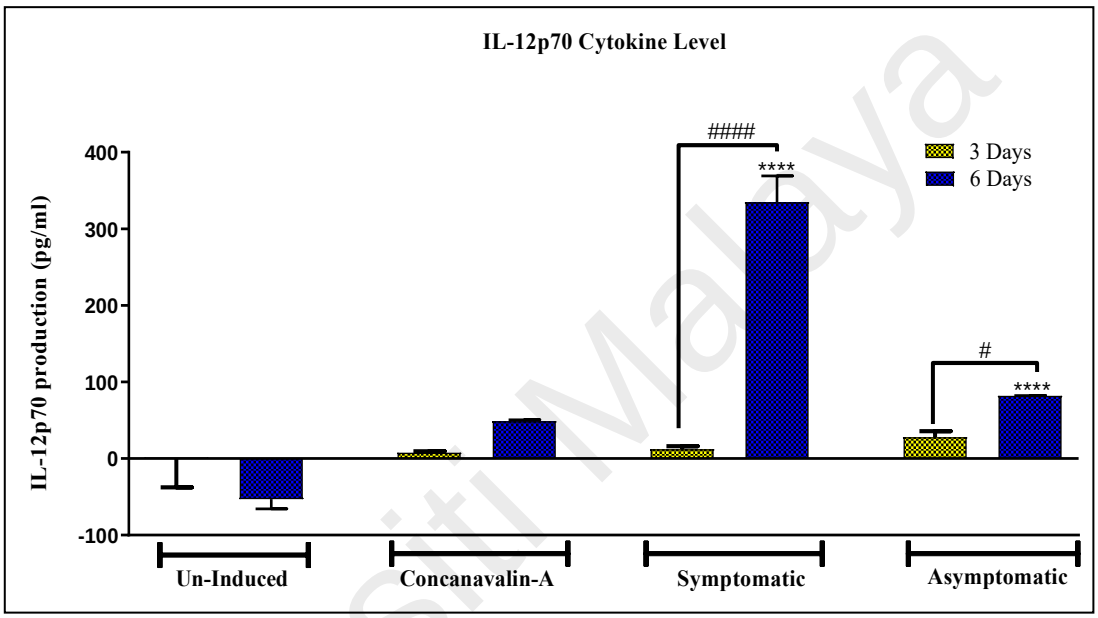


Figure 6.7: IL-12p70 Cytokine Level by THP-1 Cells Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced THP-1 cells served as negative control (n=3). #P<0.05 and ####P<0.0001 was the comparison made between 3 and 6 days of induction.

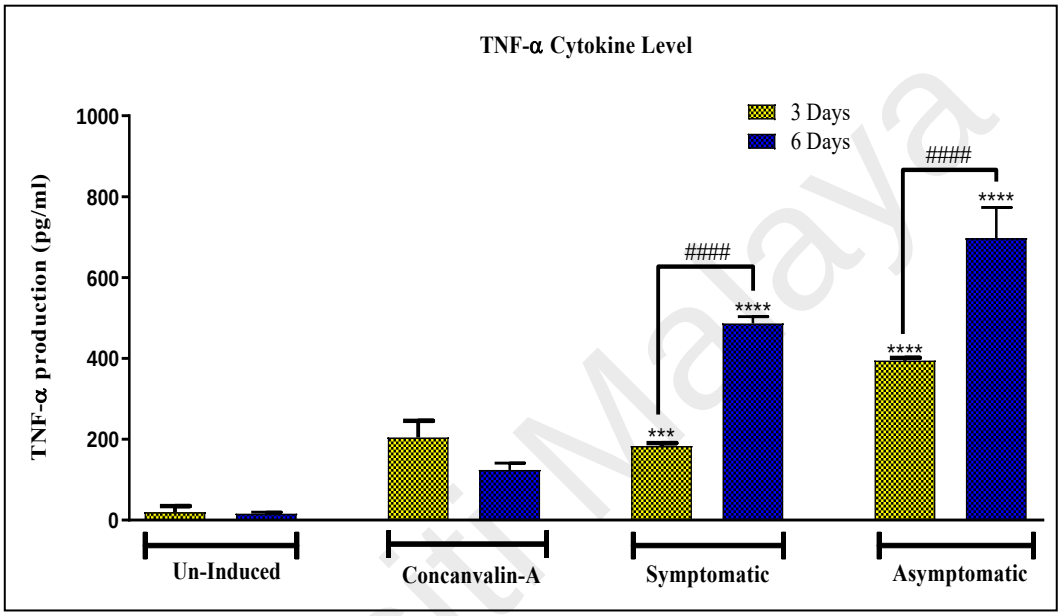


Figure 6.8: TNF-α Cytokine Level by THP-1 Cells Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced THP-1 cells served as negative control (n=3). ####P<0.0001 was the comparison made between 3 and 6 days of induction.

Table 6.2: IL-6, IL-10, IL-12p70 and TNF-α cytokine secretions induced by THP-1 cells upon inductions with 10 µg/ml *Blastocystis* sp. solubilised antigens

Cytokine	Isolates	Secretions (pg/ml)	
		3 Days	6 Days
IL-6	Symptomatic	396.97±45.22****	51.13±34.11
	Asymptomatic	56.23±17.25	502.30±105.45****
IL-10	Symptomatic	18.37±1.72***	110.47±2.96
	Asymptomatic	6.40±1.82	447.17±10.58****
IL-12p70	Symptomatic	12.03±3.95	334.77±34.23****
	Asymptomatic	28.00±7.67*	81.95±0.35
TNF-α	Symptomatic	183.13±7.41	486.57±17.05
	Asymptomatic	394.80±6.29****	697.13±75.82****

Data is given as mean ± SD. The comparison was carried out between cytokine secretion induced by THP-1 cells induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens using One-Way ANOVA (SPSS Version 22). The differences were significant with, *P<0.05, ***P<0.001 and ****P<0.0001.

6.3.3 Cell Culture Images

Cell proliferation was noticed in THP-1 cells upon induction with 10µg/ml *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. Clustering of cells were seen in day 3 antigen inductions and gradually increased in day 6 antigen inductions (as indicated by the arrows in Figure 6.9). However, no cell clustering was noticed in un-induced THP-1 cells.

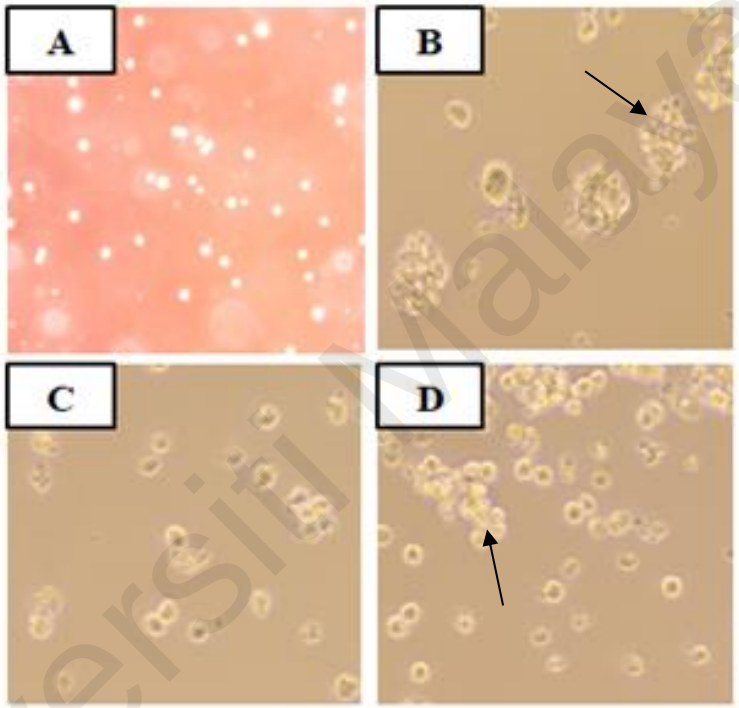


Figure 6.9: THP-1 Cell Culture Images

(A) Day 3 culture of un-induced cells (B) Day 3 culture of *Blastocystis* sp. antigen induced cells (C) Day 6 culture of un-induced cells (D) Day 6 culture of *Blastocystis* sp. antigen induced cells. Images were captured using inverted microscope with magnification at 40X.

6.3.4 Immuno-Phenotyping Analysis

THP-1 cells induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens (3 and 6 days induction) were subjected for cell surface receptor/marker (CD14+) and immune co-inhibitory PD-1 molecule analysis using flow cytometry analysis. The results for these expressions are as shown in Table 6.3. The CD14+ expressions remain unchanged from 3 to 6 days of inductions despite high cell proliferation was observed in cells induced with symptomatic and asymptomatic antigens. However, the cells induced with symptomatic and asymptomatic antigens have shown significant increase of PD-1 molecule from 3 to 6 days of induction at (17% to 29%) and (16% to 28%) respectively. Besides, the comparison between un-induced and antigen induced cells for 3 days revealed that, PD-1 molecule increased by 7.0-fold and 6.7-fold in cells introduced with symptomatic and asymptomatic antigens. On the other hand, the comparison between un-induced and antigen induced cells (symptomatic and asymptomatic antigens) for 6 days has shown to increase by 5.6-fold and 5.9- fold respectively.

Table 6.3: Cell surface marker CD14+ and PD-1 molecule expressions on THP-1 cells upon induction with 10 µg/ml *Blastocystis* sp. solubilised antigens

Group	3 Days Induction		6 Days Induction	
	CD14+	PD-1	CD14+	PD-1
Symptomatic	33.9±0.27	17.03±0.83	34.6±2.19	28.6±1.35****
Asymptomatic	29.8±1.5	16.23±2.06	35.47±2.73*	28.03±1.86****
Un-induced	35.33±1.53	2.43±0.058	36.0±1.0	5.07±0.12

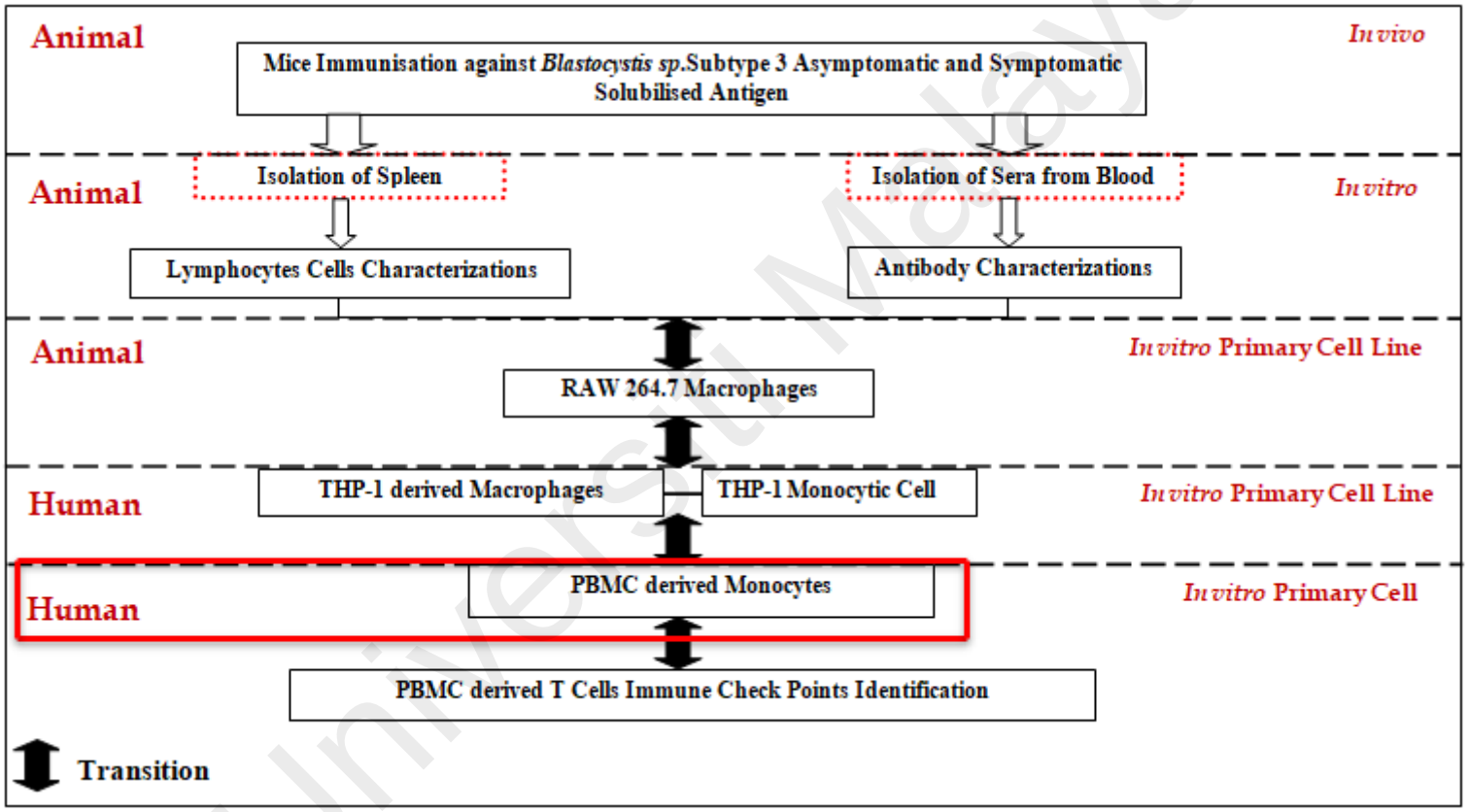
Data is given as mean ± SD. The comparison was carried out with between cells stimulated with *Blastocystis* sp. ST3 symptomatic, asymptomatic and un-induced group using One-Way ANOVA (SPSS Version 22). The comparison was carried out between 3 and 6 days of inductions. The differences were significant with, *P<0.05 and ****P<0.0001.

Table 6.4: Overall Immune Response of THP-1 cell Induced with 10 µg/ml *Blastocystis* sp. solubilised antigen at 3 and 6 days

Group	Induction	Cell Proliferation (%)	PD-1 Molecule Expression (%)	Cytokine Response (pg/ml)		Ratio A: B
				Pro-Inflammatory (IL-6+IL-12p70+ TNF-α) (A)	Anti-Inflammatory (IL-10) (B)	
Symptomatic	3 Days	37	17	592	18	33:1
	6 Days	124	29	872	110	8:1
Asymptomatic	3 Days	47	16	479	6	80:1
	6 Days	76	28	1265	447	3:1

Data is given as mean ± SD of cells induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens at 3 and 6 days.

PART 2: HUMAN PRIMARY CELL DERIVED MONOCYTES



6.3.5 Cell Viability Assessment of Human PBMCs Derived Primary Monocytes Induced with *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

6.3.5.1 Primary Monocytes Proliferation at 3 and 6 days Induced with 10 µg/ml Solubilised Antigen

The primary monocytes induction with 10 µg/ml of *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens were carried out for 3- and 6-days durations in three different healthy donors. The results are as in shown Figure 6.10. It was observed that *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens induced the highest cell proliferations from 3 to 6 days in healthy Donor 2 with percentage of (112.07% to 154%) with the increase by 1.4-fold in the symptomatic isolates induced group and (116.06% to 125.51%) with the increase by 1.1-fold in the asymptomatic isolates induced group. The cell proliferation differences between monocytes induced with ST3 *Blastocystis* sp. symptomatic and asymptomatic inductions were shown in Table 6.5.

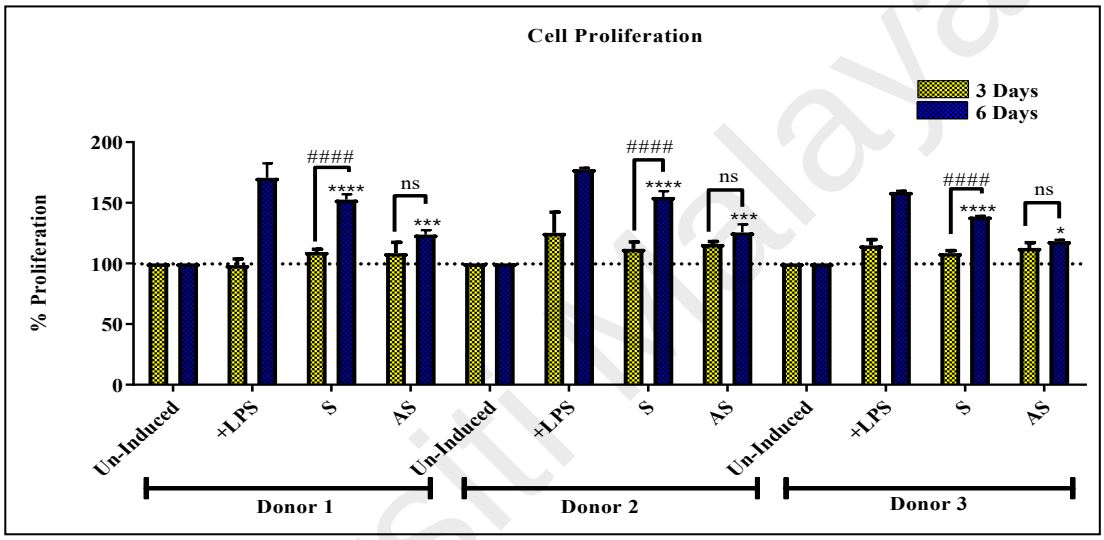


Figure 6.10: Percentage Cell Proliferation of Primary Monocytes Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens for 3 and 6 days in Donor 1, 2 and 3

Values are given in mean ± SD (n=3). *P<0.05, ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced monocytes served as negative control (n=3). ####P<0.0001 was the comparison made between 3 and 6 days of induction.

Table 6.5: Primary monocytes proliferation upon inductions with 10 µg/ml of *Blastocystis* sp. solubilised antigen incubation for 3 and 6 days in healthy (Donor 1, 2 and 3)

Group	Donor 1		Donor 2		Donor 3	
	3 Days	6 Days	3 Days	6 Days	3 Days	6 Days
Symptomatic	109.49±2.29	152.58±4.48***	112.07±5.7	154.87±4.84**	108.54±1.99	138.57±0.64****
Asymptomatic	108.50±8.88	123.79±3.72	116.06±2.04	125.51±6.67	112.77±4.30	118.33±1.17

Data is given as mean ± SD. The comparison was carried out between *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen inductions. The differences were significant with, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.6 ELISA Test Results

6.3.6.1 Cytokine Assessment

In the present study, solubilised antigen of *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens induced higher response of pro-inflammatory cytokines (IL-6, IL-12p70 and TNF-α) and anti-inflammatory cytokine IL-10 secretions in primary monocytes derived from healthy Donor 1, 2 and 3 as shown in Figure 6.11 to 6.14. However, the un-induced cells have not shown any cytokine secretions. The pro- and anti-inflammatory cytokine level differences between *Blastocystis* sp. ST3 symptomatic and asymptomatic inductions were shown in Table 6.6.

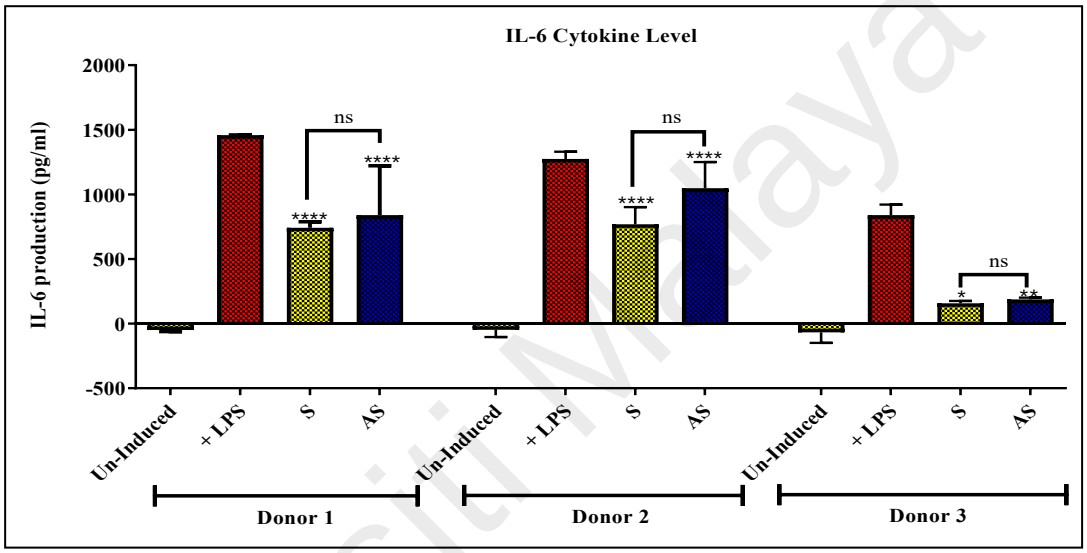


Figure 6.11: IL-6 Cytokine Level by Primary Monocytes Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). *P<0.05, **P<0.01 and ****P < 0.0001 was the comparison made against un-induced monocytes served as negative control (n=3). The comparison between symptomatic and asymptomatic induction showed no significant (ns) difference.

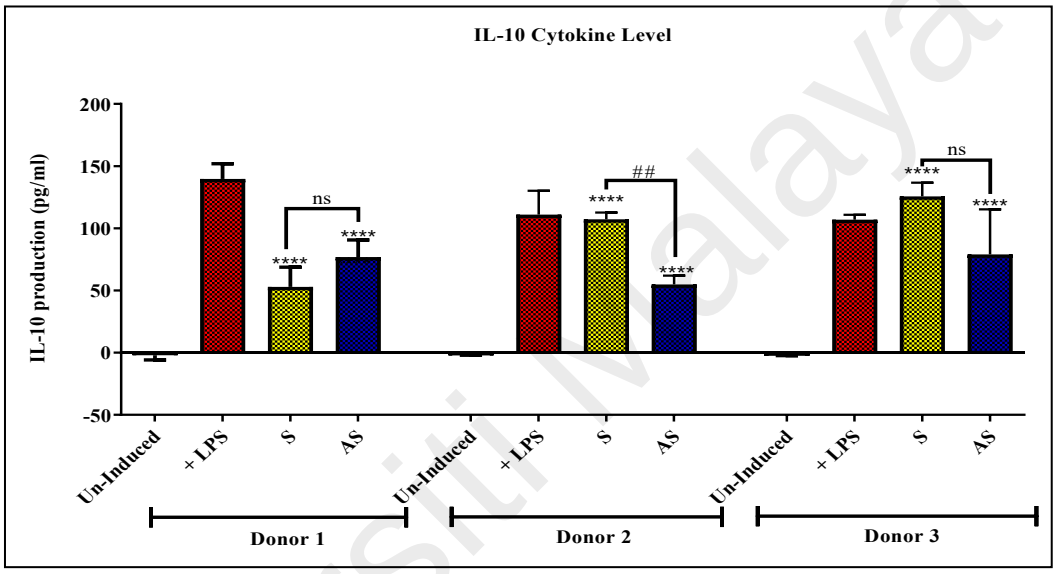


Figure 6.12: IL-10 Cytokine Level by Primary Monocytes Induced with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). ****P < 0.0001 was the comparison made against un-induced monocytes served as negative control (n=3). ##P<0.01 was the comparison made between symptomatic and asymptomatic induction.

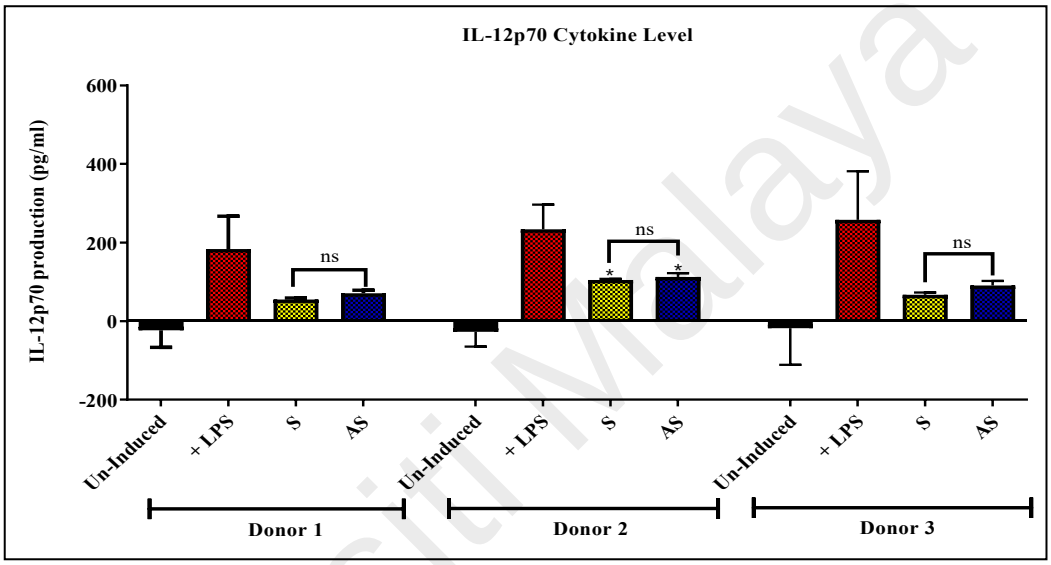


Figure 6.13: IL-12p70 Cytokine Level by Primary Monocytes Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). *P < 0.05 was the comparison made against un-induced monocytes served as negative control (n=3). The comparison between symptomatic and asymptomatic induction showed no significant (ns) difference.

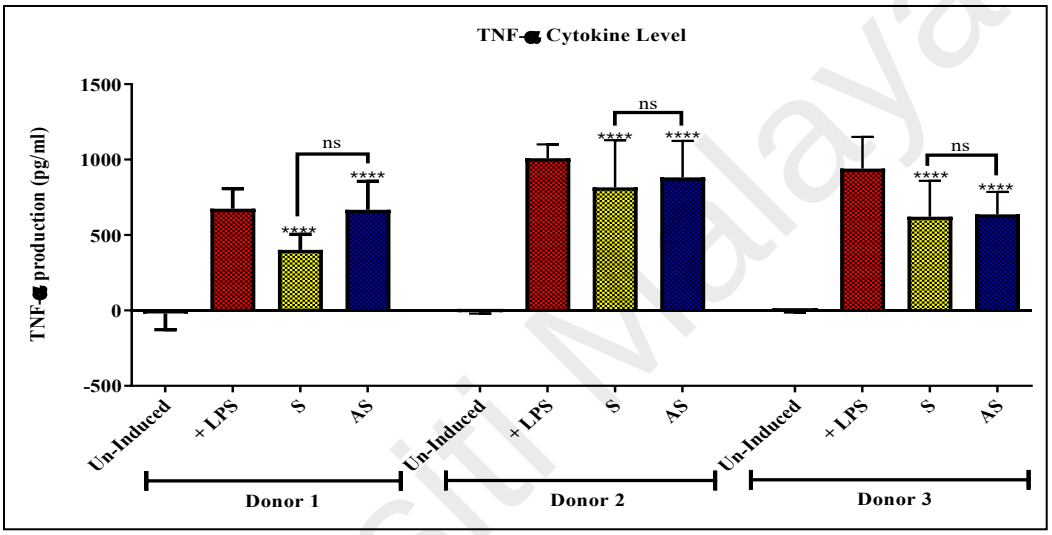


Figure 6.14: TNF-α Cytokine Level by Primary Monocytes Stimulated with 10 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=3). ****P < 0.0001 was the comparison made against un-induced monocytes served as negative control (n=3). The comparison between symptomatic and asymptomatic induction showed no significant (ns) difference.

Table 6.6: IL-6, IL-10, IL-12p70 and TNF-α cytokine secretion in primary monocytes upon Inductions with 10 µg/ml *Blastocystis* sp. solubilised antigens

Cytokine	Isolates	Cytokine Secretion (pg/ml)		
		Donor 1	Donor 2	Donor 3
IL-6	Symptomatic	742±45.91	769.33±131.85	157.92±17.65
	Asymptomatic	839.33±382.48	1047.88±202.01	187.39±13.80
IL-10	Symptomatic	52.87±15.91	90.60±29.11	104.06±38.26
	Asymptomatic	76.96±13.68	54.78±7.20	79.06±36.17
IL-12p70	Symptomatic	54.88±3.93	106.81±1.79	66.9±5.87
	Asymptomatic	70.80±7.67	112.22±9.81	90.56±11.39
TNF-α	Symptomatic	401.98±101.67	815.52±313.24	621.33±237.72
	Asymptomatic	666.94±188.71	881.74±241.97	637.07±148.14

Data is given as mean ± SD among *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigen inductions was tabulated using One-Way ANOVA (SPSS Version 22).

6.3.7 Cell Culture Images

Cell proliferation was noticed in primary monocytes upon induction with 10 µg/ml *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. High cell density was observed in day 3 antigen inductions and gradually increased in day 6 antigen inductions as shown in Figure 6.15 below. However, low cell density was observed in un-induced primary monocytes.

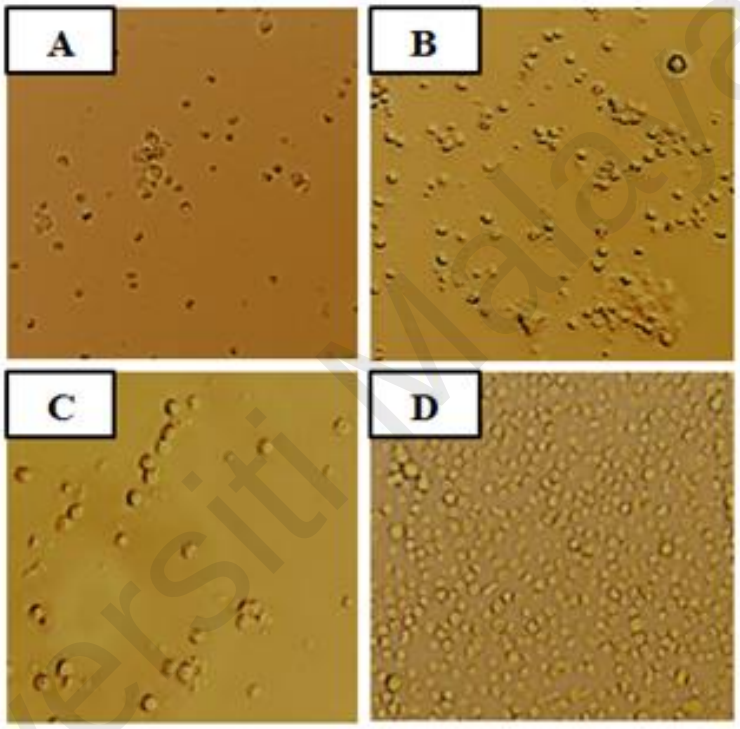


Figure 6.15: Primary Monocytes Cell Culture Images

(A) Day 3 of un-induced cells (B) Day 3 of *Blastocystis* sp. antigen induced cells (C) Day 6 of un-induced cells (D) Day 6 of *Blastocystis* sp. antigen induced cells. Images were captured using inverted microscope with magnification at 40X.

6.3.8 Immuno-Phenotyping Analysis

Primary monocytes induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens (3 and 6 days induction) were subjected for cell surface receptor/marker immuno- phenotyping analysis. Cell surface marker CD14+, immune T cell co-stimulatory marker (CD86) and immune co-inhibitory marker (PD-1) molecules were analysed. The results for these expressions are shown in Table 6.7. The CD14+ cell surface marker decreased significantly from 3 to 6 days of induction in cells stimulated with symptomatic antigens (70% to 45%) and asymptomatic antigens (69% to 46%) antigens. Meanwhile, CD86 immune co-stimulatory marker increased significantly from 3 to 6 days by 2.1-fold in cells stimulated with symptomatic and asymptomatic antigens. Analysis of PD-1 molecule has shown significant increase from 3 to 6 days inductions in cells induced with symptomatic (4.7% to 28%) and asymptomatic (6.5% to 30%) antigens. Besides, the comparison between un-induced and antigen induced cells for 3 days revealed that, PD-1 molecule increased by 4.7-fold and 6.5-fold in cells introduced with symptomatic and asymptomatic antigens. On the other hand, the comparison between un-induced and antigen induced cells (symptomatic and asymptomatic antigens) for 6 days has shown to increase by 28-fold and 30-fold respectively.

Table 6.7: CD14+, CD86 and PD-1 cell surface marker on monocytes upon inductions with 10 µg/ml *Blastocystis* sp. solubilised antigen

Group	3 Days			6 Days		
	CD14 +	CD86	PD-1	CD14+	CD86	PD-1
Symptomatic	70.3±5.45	26.43±1.53	4.7±0.78	45.30±0.46****	54.33±2.15****	28.17±2.70****
Asymptomatic	68.6±2.81	26.17±0.59	6.47±2.60	46.77±1.88****	55.13±0.76****	30.23±1.19****
Negative	73.80±1.51	13.3±1.53	0.23±0.06	76.23±0.90	16.0±1.0	0.35±0.05

Data is given as mean ± SD. The comparison was carried out between 3- and 6-days inductions in *Blastocystis* sp. ST3 symptomatic and asymptomatic antigen inductions. The differences were significant with, ****P<0.0001.

Table 6.8: Overall Immune Response of Human PBMC derived Monocytes

Group	Cell Proliferation Increase (%)		Cytokine Response (pg/ml)		Ratio (A: B)
	3 Days	6 Days	Pro-Inflammatory (IL-6+IL12p70+TNFα) (A)	Anti-Inflammatory (IL-10) (B)	
Symptomatic			**6 days**	**6 days**	
Donor 1	10	53	1066	53	20:1
Donor 2	17	55	1692	91	19:1
Donor 3	9	34	846	104	8:1
Average	**12**	**47**	**3040**	**179**	**17:1**
Asymptomatic			**6 days**	**6 days**	
Donor 1	13	24	1577	80	19:1
Donor 2	16	26	2041	55	37:1
Donor 3	11	18	915	79	12:1
Average	**13**	**23**	**4533**	**214**	**21:1**

Data is given as mean ± SD of cells induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens at 3 and 6 days.

6.4 Discussion

6.4.1 Cell Proliferation

Generally, during pathogenic infections, monocytes will be recruited to the inflamed tissue where it will be actively proliferating to eradicate the infection. Similarly, in this study it was observed that, THP-1 monocytic cell line and primary monocytes highly proliferated upon induction with *Blastocystis* sp. ST3 antigens during a short- and long-term inductions. It was observed that, induction of monocytes with various concentrations of *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens (0.01 to 10 µg/ml) resulted in various cell proliferation patterns. However, in this dose response study, the induction of 10 µg/ml of solubilised antigen showed the highest proliferation rate in monocytes cell induced with asymptomatic and symptomatic antigens at both time points (3 days and 6 days). This observation may implicate the immunopathogenesis between *Blastocystis* sp. ST3 antigens and monocytes during short-term and long-term infections. Therefore 10 µg/ml of solubilised antigens of symptomatic and asymptomatic concentration was used to compare the monocytes cell modulations.

Further observations on cell proliferation at 10 µg/ml of solubilised antigens have shown higher cell proliferation by cells induced with symptomatic antigens at 3 days and 6 days. On average, THP-1 and primary monocytes (CD14+) increase by 3.4 and 1.4-fold respectively in symptomatic antigen induced cells and 1.6 and 1.1-fold cell increase in asymptomatic antigen induced cells from 3 to 6 days. Thus, in this study higher increase of monocytes were induced by symptomatic antigens which may indicate its higher pathogenicity. The increase number of monocytes suggesting the increase in inflammatory response caused by *Blastocystis* sp. ST3 infection which can lead to cytokine storm at the affected site. Therefore, further investigation was done to observe the pro- inflammatory cytokine secretions by the monocytes.

6.4.2 Pro-Inflammatory Cytokine Responses

It was shown that during a prolonged antigen induction, symptomatic antigens induced significantly higher monocytes proliferation compared to asymptomatic antigen. Likewise mentioned above, higher cell proliferation induces higher pro-inflammatory cytokine unless there is a secretion of anti-inflammatory cytokine which may suppress the cell proliferation by inhibiting the release of pro-inflammatory cytokine secretions. However, monocytes induced with asymptomatic antigen have shown non-corresponding results of cell proliferation and pro-inflammatory cytokine release where, lower cell proliferation has induced higher pro-inflammatory cytokine release. It was shown that, during a short-term (3 days) antigen induction, THP-1 cells introduced with asymptomatic antigens showed significantly higher pro-inflammatory cytokine secretions (IL-6, TNF-α and IL-12p70) compared to cells introduced with symptomatic antigen. However, during a pro-longed antigen induction (6 days) the pro-inflammatory cytokine release was surpassed in cells introduced with asymptomatic antigen as reflected in Table 6.2. Similar results were seen in primary monocytes (CD14+) during a long-term antigen induction as reflected in Table 6.6. This could be potentially influenced by IL-6 cytokine release due to the study observations that, IL-6 is being the major contributor of pro-inflammatory cytokine after TNF-α in both THP-1 and primary monocytes. Generally, IL-6 plays a dual role as pro- or anti-inflammatory response as mentioned in previous Chapter 5. Therefore, to confirm its effect as either pro- or anti-inflammatory, the IL-6 cytokine level was compared with the percentage monocytes proliferation. Therefore, suggesting that, IL-6 may act as an anti-inflammatory cytokine in monocytes induced with asymptomatic antigens. However, monocytes induced with symptomatic antigen have shown corresponding results of cell proliferations and pro-inflammatory cytokine release which suggesting IL-6 acting as a pro-inflammatory cytokine.

Further investigations on other pro-inflammatory cytokines such as TNF-α and IL-12p70 have shown high exacerbation of these cytokines in monocytes upon pro-longed induction of symptomatic and asymptomatic antigens. This finding is concurring with previous studies which reported that TNF-α cytokine was highly secreted in *Blastocystis* sp. infected individuals which may cause chronic intestinal inflammation (Jimenez-Gonzalez et al., 2012; Lim et al., 2014; Teo et al., 2014). Further investigations on IL12-p70 cytokine analysis have shown similar results where this cytokine was significantly increased during the pro-longed *Blastocystis* sp. antigen induction. Studies have proven that IL-12p70 cytokine are able to up-regulate TNF-α cytokine secretions during pathogenic infections (Shin et al., 1999; Louis et al., 2010). Therefore, it can be suggested that, the increase of TNF-α cytokine in monocytes induced with symptomatic and asymptomatic antigens is enhanced by IL-12p70 cytokine which may increase the pathogenesis of *Blastocystis* sp. ST3 during pro-longed infection.

6.4.3 Anti-Inflammatory Cytokine Response

The overall observation of cell proliferation and total pro-inflammatory cytokine release by monocytes induced by *Blastocystis* sp. antigens has demonstrated high immunopathogenesis induced by this parasite during a pro-longed infection. However, studies have suggested that, immense regulations of pro-inflammatory cytokine may induce monocytes impairment which may eventually initiate the induction of anti-inflammatory cytokine secretions (Shi et al., 2011; Pan et al., 2015). Therefore, further assessment of anti-inflammatory cytokine (IL-10) was performed. It was demonstrated that, the pro-longed symptomatic and asymptomatic antigen exposures for 6 days increased the IL-10 cytokine secretions. Nevertheless, significantly higher IL-10 cytokine was increased in monocytes induced with asymptomatic antigen compared to symptomatic antigen. A study conducted by Kovacs (2010) reported that, IL-6 cytokine is capable in enhancing the IL-10 cytokine secretion. Therefore, higher IL-6 cytokine

secretions observed in monocytes induced with asymptomatic antigen may possibly enhanced due to the increase of IL-10 during the pro-longed antigenic induction. In general, this study proved that, asymptomatic antigen is capable of inducing higher anti-inflammatory cytokine release in monocytes compared to symptomatic antigen.

In overall our observations suggest that there is an imbalance between the levels of pro- and anti-inflammatory cytokines in both 3 and 6 days of monocytes inductions with *Blastocystis* sp. antigen. Study has suggested that, imbalance between pro- and anti-inflammatory cytokines especially higher level of pro-inflammatory cytokines response by monocytes during a pathogenic infection may allow the pathogens to evade the host immune system which can lead to a disease development (Kurokawa et al., 2007). Besides, different pattern of pro- and anti-inflammatory cytokine responses also was observed in monocytes induced with symptomatic and asymptomatic antigen (reflected in Table 6.4 and 6.8) from 3 to 6 days of induction. There was a decrease of 27-fold and 4-fold were observed in THP-1 antigen induction as reflected in Table 6.4. However, the comparison in primary monocytes was not shown since no cytokine analysis performed due to low cell proliferation (not significant with un-induced cells) observed during short-term antigen induction.

Therefore, the increase of anti-inflammatory cytokine response in this study suggesting monocytes impairment during a pro-longed *Blastocystis* sp. ST3 infection where this cytokine possibly underpin the disease symptoms especially the asymptomatic phase. It was reported by Maizels (2016) that, asymptomatic carriers of helminth parasite have shown increased level of IL-10 cytokine response and decreased pro-inflammatory cytokine response which contributed to reduced disease symptoms and additionally increased the susceptibility of co-infection in the infected host. Another study, conducted by Tuero (2016) revealed that, a high increase of anti-inflammatory cytokine, (IL-10)

response induced by *Taenia solium cysts* leads to dissemination of the parasite due to failure of the immune cells in regulating inflammation in the infected host. Therefore, further investigation was performed to analyse the monocytes functionality by analysing the PD-1 molecule expression.

6.4.4 Programme Cell Death-1 (PD-1) Molecule Analysis

Generally, a PD-1 molecule (immune co-inhibitory molecule) is expressed mainly on the surface of activated T/ B/NK cells. However, it is also found on antigen presenting cells such as monocytes and dendritic cells (Zasada et al., 2017). A PD-1 molecule is also known to down-regulate the immune system by inhibiting the pro-inflammatory cytokine activity. The PD-1 expression analysis in this study has shown significant increase from 3 to 6 days of inductions in monocytes induced with symptomatic and asymptomatic antigens. However, no significant differences were noticed between cells induced with symptomatic and asymptomatic antigen. Past studies have revealed that, antigen presenting cells such as monocytes and macrophages elevated high level of PD-1 molecules expressions during chronic inflammatory disease conditions (Zasada et al., 2017 and Gordon et al., 2017). Furthermore, a study conducted by Pan (2015) has shown similar observations as this study, where prolonged stimulation of THP-1 cells with LPS has triggered significantly high elevations of PD-1 molecule expressions along with secretions of high-level anti-inflammatory cytokines release and inhibition of pro-inflammatory cytokine response which may indicate the THP-1 cell dysfunction.

Therefore, the present study proves that, PD-1 expression does play a part in monocytes impairment caused by *Blastocystis* sp. ST3 isolates especially during a longer antigen induction. Gorentla et al., (2012) have reported that, a negative signal transmitted from the activated PD-1 molecules leads to decreased activity of the adaptive immune system especially during a long-term infection which could be possibly due to the

impairment of connecting signaling pathways between antigen presenting cells and T cell receptor (TCR). Generally, naive T cells will be activated upon TCR engagement with foreign peptides presented by dendritic cells and other antigen-presenting cells in response to a pathogenic infection (Gorentla et al., 2012). Nevertheless, limited studies have navigated on the expressions of PD-1 molecule on antigen presenting cells especially covering a parasitic infection. Therefore, for the first time it was demonstrated that, *Blastocystis* sp. ST3 antigen is capable in causing monocytes dysfunction which were proven through high PD-1 (immune co-inhibitory molecules) expressions during its long-term infection. It can also be speculated that, monocytes connections with T cells will further impaired especially during pro-longed *Blastocystis* sp. infection.

6.4.5 T cell Co-Stimulatory Molecule (CD86) Analysis

Therefore, further analysis was performed by assessing the T cell co-stimulatory molecule (CD86 molecule) on monocytes cells during short- and long-term induction with *Blastocystis* sp. ST3 antigens. Generally, CD86 molecule is known as a co-stimulatory molecule which mediates T cells activation. Usually, this co-stimulatory molecule is located on the surface of antigen presenting cells (APCs) such as monocytes and dendritic cells. Upon activation of the APCs by antigens, CD86 molecules will be up-regulated on its cell surfaces to activate the adaptive arm targeting T cell populations (CD4+ and CD8+). The latest findings suggested that, CD86 molecule on monocytes cell surfaces was able to influence the immune suppression effect in asymptomatic patients infected with *Trypanosoma cruzi* parasite by elevating immune inhibitory receptors on T cells (Pinto et al., 2018). In this study, primary monocytes showed high expressions of CD86 (2.1-fold increase) from short to long-term inductions of monocytes introduced with symptomatic and asymptomatic antigens. These results were compared with the monocytes cell proliferation. It was observed that, during pro-longed *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens induction, monocytes increased by (1.4-fold)

and (1.6-fold) respectively. Therefore, it can be suggested that, *Blastocystis* sp. antigens may trigger high activations of T cells even though the phagocytic functions of monocytes were impaired.

6.4.6 T Cell Surface Marker (CD14+) Analysis

The monocytes dysfunction was further confirmed by the expressions of CD14+ cell surface marker. Previous studies had stated that, primary monocytes will up-regulate its CD14+ expressions during chronic antigenic stimulations which also indicate the cell activation (Nockher and Scherberich, 1995). In this study, the CD14+ expression was significantly reduced from short to long-term inductions which may prove its loss of property or dysfunction due to associations with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens.

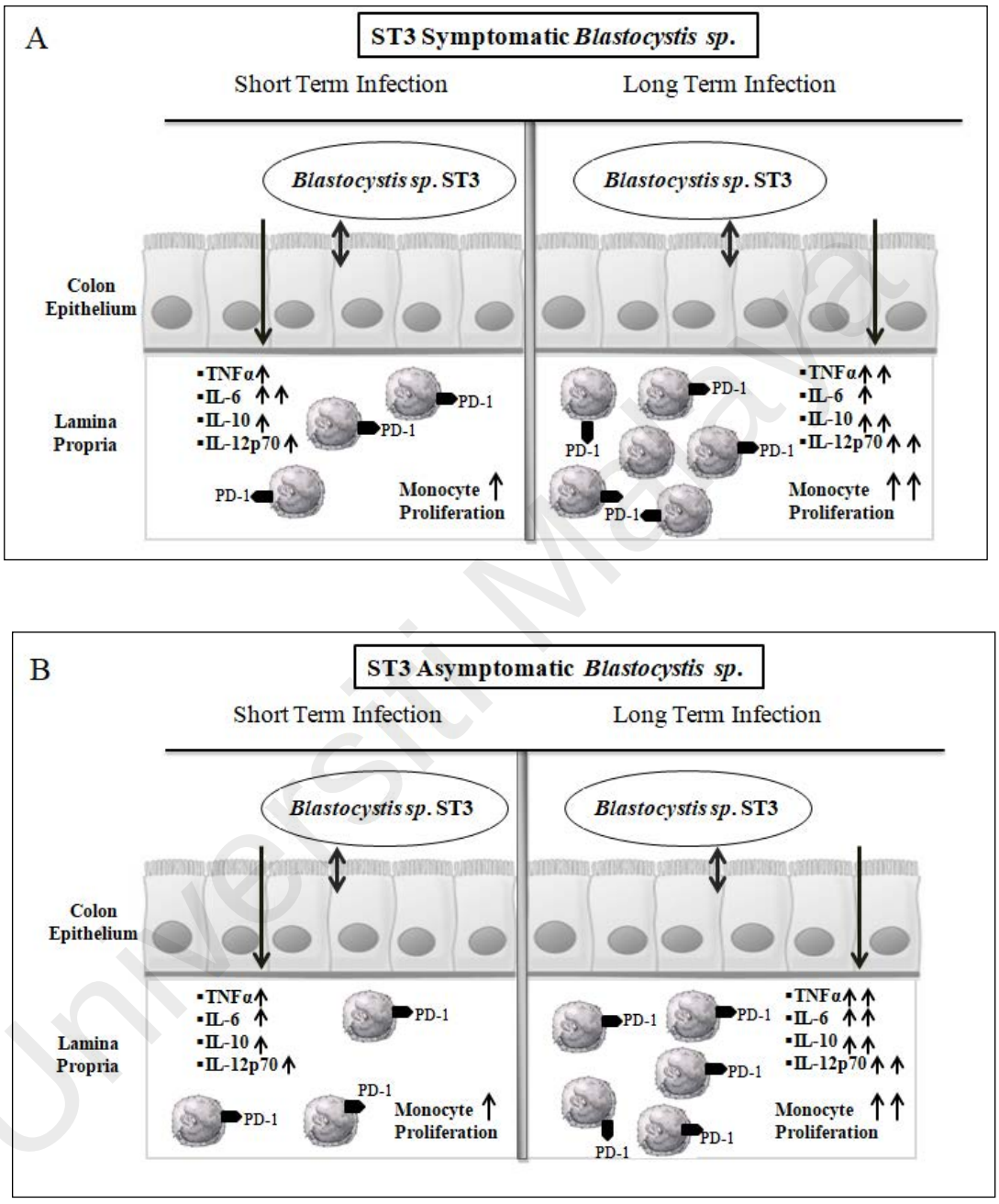


Figure 6.16: Illustrations of Monocytes Immune Response Upon Stimulation with *Blastocystis* sp. ST3 In Human Intestinal Region

(A) symptomatic and (B) asymptomatic isolates during a short- and long-term infection.

6.5 Conclusion

Therefore, the present study has revealed the immunopathogenesis between *Blastocystis* sp. ST3 (symptomatic and asymptomatic isolates) and monocytes. The suggested immune modulations of this parasite on monocytes during a short- and long-term infection in human intestinal region were illustrated in Figure 6.16 (A) and (B).

During a short-term *Blastocystis* sp. ST3 infection:

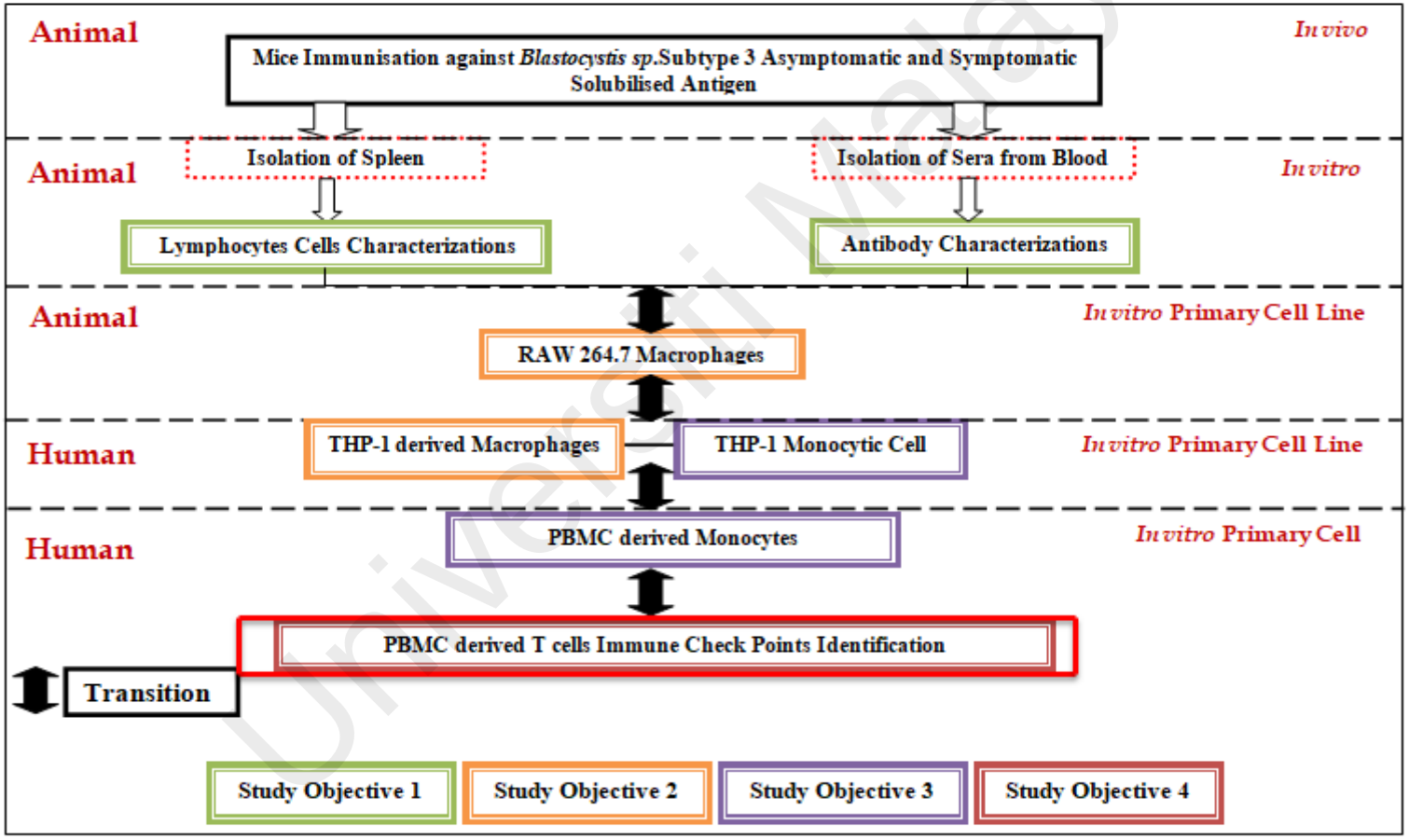
1. Symptomatic and asymptomatic antigens have stimulated monocytes response by inducing high cell proliferation and immense pro-inflammatory cytokine secretions which may possibly cause intestinal damage to the infected host.

During a long-term *Blastocystis* sp. ST3 infection:

1. Monocytes proliferated higher against symptomatic and asymptomatic antigen. However, the exacerbation of anti-inflammatory cytokines and increased level of PD-1 molecule expressions on monocytes cell surface confirmed the monocytes impairment.
2. Elevation of anti-inflammatory cytokine level and PD-1 molecule expression were more evident in the asymptomatic isolates induced monocytes. This may underpin the disease symptoms in the infected host suggesting it to be a "silent killer" and highly detrimental compared to symptomatic isolates.
3. Despite having impaired monocytes, high level of T cell co-stimulatory molecule CD86+ indicates possibility of high T cell activation. However, the involvements of impaired monocytes in causing high T cell activation needs further investigation. These aspects were investigated in the following Chapter 7.

In a nutshell, for the first time it was proven that *Blastocystis* sp. ST3 antigens are able to impair monocytes function which include the phagocytic activity (a key function of innate immunity) and possibly impair the T cell functions (key player of adaptive immunity). Therefore, the following Chapter 7 uncover the T cell immune modulation caused by *Blastocystis* sp. ST3 isolates.

CHAPTER 7: *IN VITRO* STUDY TO EVALUATE T CELL IMMUNE CHECK POINTS EXPRESSIONS INDUCED BY *BLASTOCYSTIS* SP. SUBTYPE 3 SYMPTOMATIC AND ASYMPTOMATIC ANTIGENS DURING SHORT- AND LONG-TERM INDUCTIONS



7.1 Introduction

In previous Chapter 6, it was highlighted that *Blastocystis* sp. ST3 antigens were able to induce high monocytes proliferations and increased expressions of T cell co-stimulatory molecule (CD86) during pro-longed *Blastocystis* sp. antigen induction. Generally, antigen presenting cells such as monocytes play a major role in T cell activation during a prolonged infection. However, past evidences have proven that T cells were capable in enhancing the pathogenesis of inflammatory diseases by infiltrating into the targeted tissue and exacerbate damage (Burger, 2000; Burger and Dayer, 2002; Chen et al., 2017). There are many instances that have shown the intestinal protozoan parasite in causing down-regulation of T cells function (Zander et al., 2013). Therefore, the observations of T cell co-stimulatory molecules on monocytes upon exposure of *Blastocystis* sp. ST3 implicate the involvement of T cells as described in Figure 7.1 (A). Studies have revealed that during pro-longed pathogenic infections, immune down-regulations have occurred through high expressions of T cell immune inhibitory molecules (Zasada et al., 2017; Pan et al., 2017) as shown in Figure 7.1 (B).

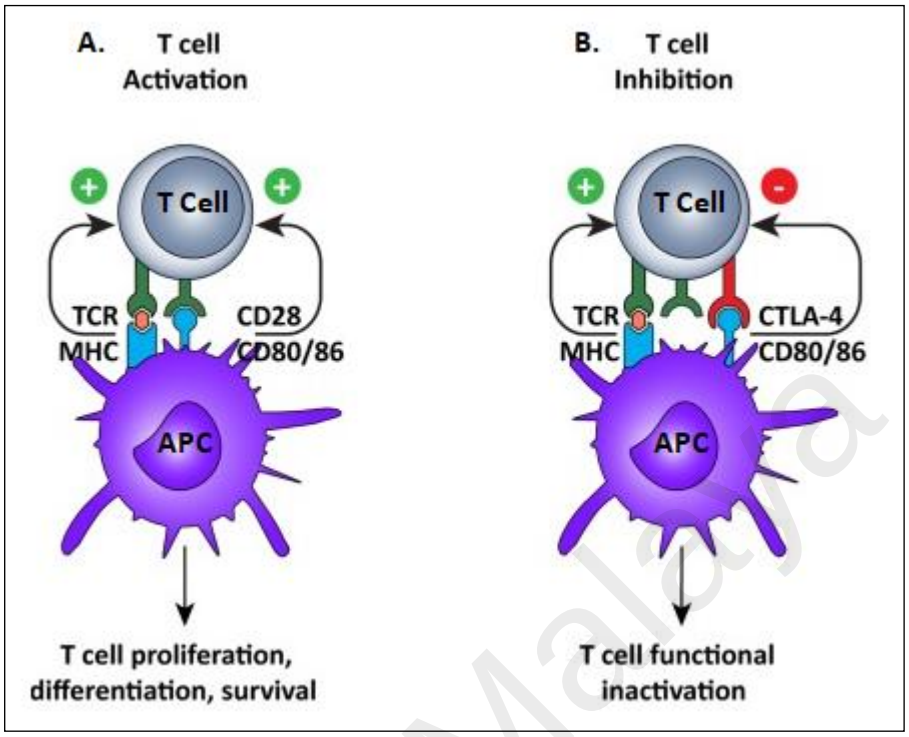


Figure 7.1: Mechanisms of T cell Activation and Inactivation by Antigen Presenting Cells (APC)

(A) T cell activation by co-stimulatory molecule (CD28) and CD80/86 (B) T cell Inhibition by co-inhibitory molecule (CTLA-4) and CD80/86. (Source: Vasaturo et al., 2013)

It was also proven in previous Chapter 3 that, there were active involvements of T cells after multiple exposures of *Blastocystis* sp. ST3 antigens to the mice where the memory T cells established higher immune cell response. However, there were imbalance of Th1 and Th2 cytokines were observed suggesting impairment of adaptive T cell response caused by symptomatic and asymptomatic isolates. Therefore, the T cell immunopathogenesis of *Blastocystis* sp. ST3 symptomatic and asymptomatic infection was analysed by using human PBMC model. Further descriptions on T cell analysis are explained below.

7.1.1 T cell (CD4+ and CD8+) Response During a Pathogenic Infection

It was also reported in many studies that persistence of antigen stimulations or prolonged infections, will eventually impair the function of antigen-specific T cell populations particularly CD4+ and CD8+ subsets which function as an effector cells of adaptive immunity (Mueller and Ahmed, 2009). Therefore, in this study, the investigations of T cells immune modulations by *Blastocystis* sp. antigens was assessed targeting helper (CD4+) and cytotoxic (CD8+) T cell populations. Generally, CD4+ and CD8+ T cells are extensively involved during long-term infections. Upon encountering antigens during an infection, CD4+ cells will secrete its cytokines such as IL-2 to activate the CD8+ cells as shown in Figure 7.2 below. Eventually, the activated CD8+ cell will secrete IFNγ cytokines to eradicate the infections. Extracellular parasite such as *Blastocystis* sp. may involve T cells response if the respective antigen particles escapes phagosome and lives in the blood and lymph of infected host. Therefore, in this study we have demonstrated the immune mechanisms of *Blastocystis* sp. ST3 antigen as described in 7.1.2 below.

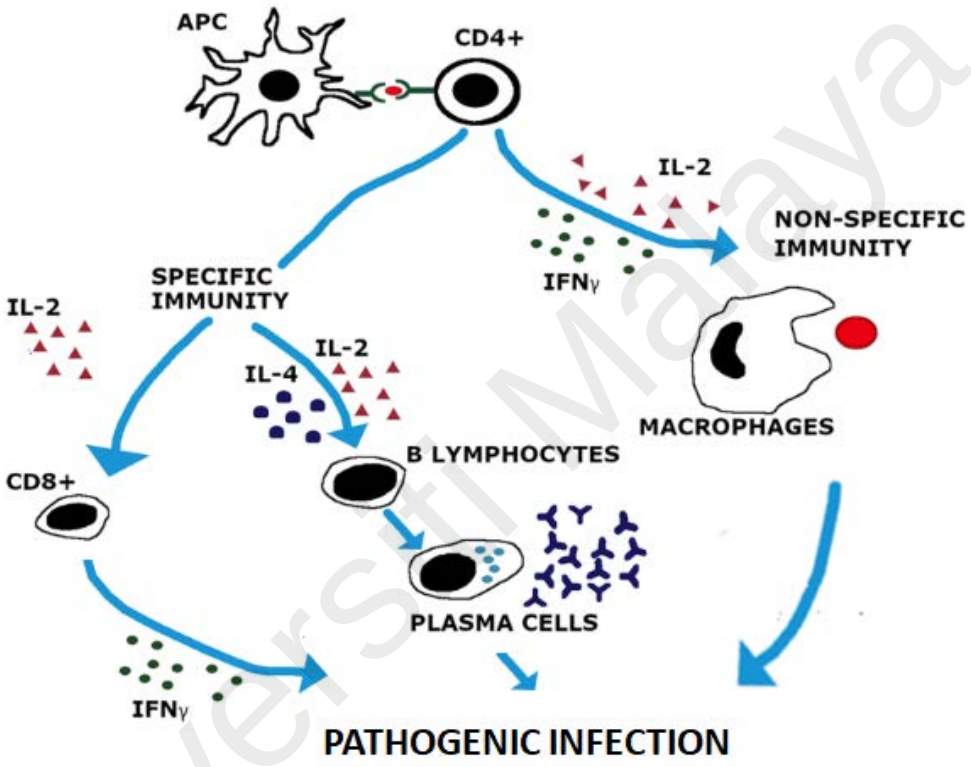


Figure 7.2: The Mechanisms of CD4+ and CD8+ Cells Activations During a Pathogenic Infection

(Adapted and modified from: Ashfaq et al., 2019)

7.1.2 Intestinal Parasite Infection and Immune System

Generally, T cell interactions between T cells and intestinal parasite such as *Giardia lamblia* and *Trypanosoma* and *Giardia intestinalis* happens through invasion of the intestinal mucosal layer in the host as shown in Figure 7.3 below.

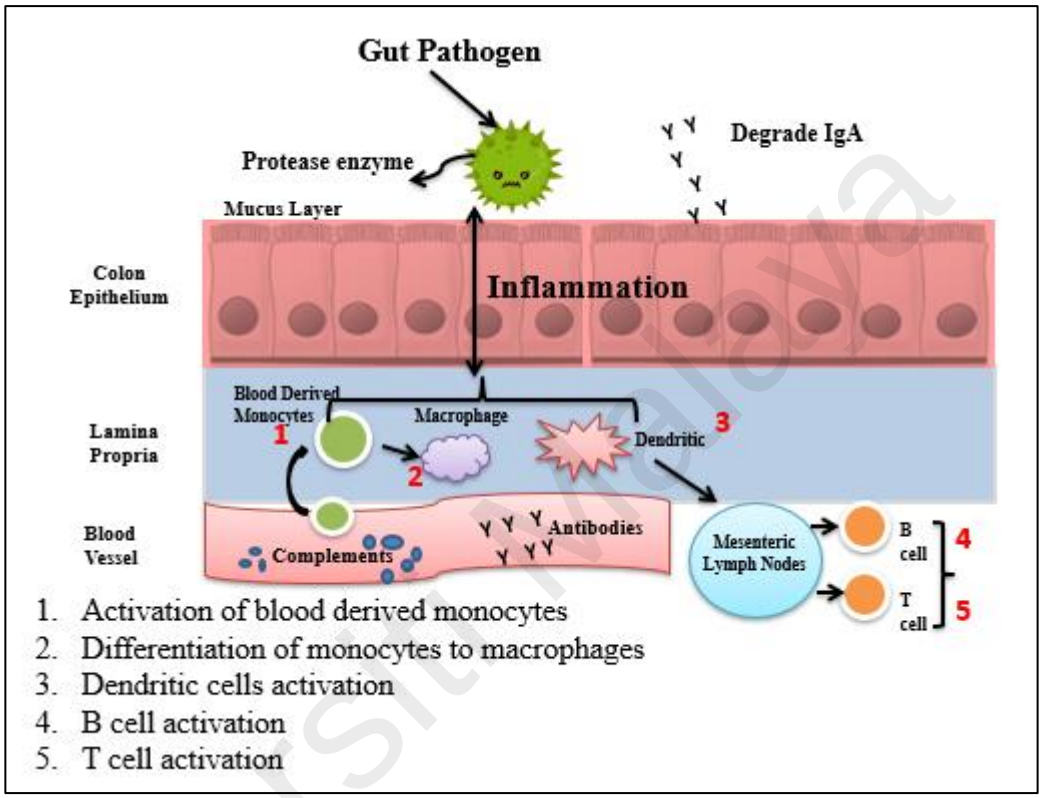


Figure 7.3: Intestinal Parasite Infection and Immune Mechanism

As a result, the invaded pathogen will cause damage to the intestinal region and release its antigens in the lamina propria region. This will activate the antigen presenting cells such as monocytes, macrophages or dendritic cells to activate the CD4+ helper cells and CD8+ cytotoxic T cells to clear the invaded pathogens as described in Figure 7.2 above. During an acute infection, antigen-specific cytotoxic T cells differentiate into effector cells to clear the infected cells and remain in the host as long-lived memory cells. During a chronic infection the memory T cells establish a quick secondary immune response against the pathogen through proliferation of cytotoxic T cell CD8+ (Kalia et al., 2000-2003). As a result, the antigen-specific T cells populations persist throughout the infection

and become dysfunction. This state of T cell dysfunction with progressive loss of its functions is referred to as T-cell exhaustion (Figure 7.4).

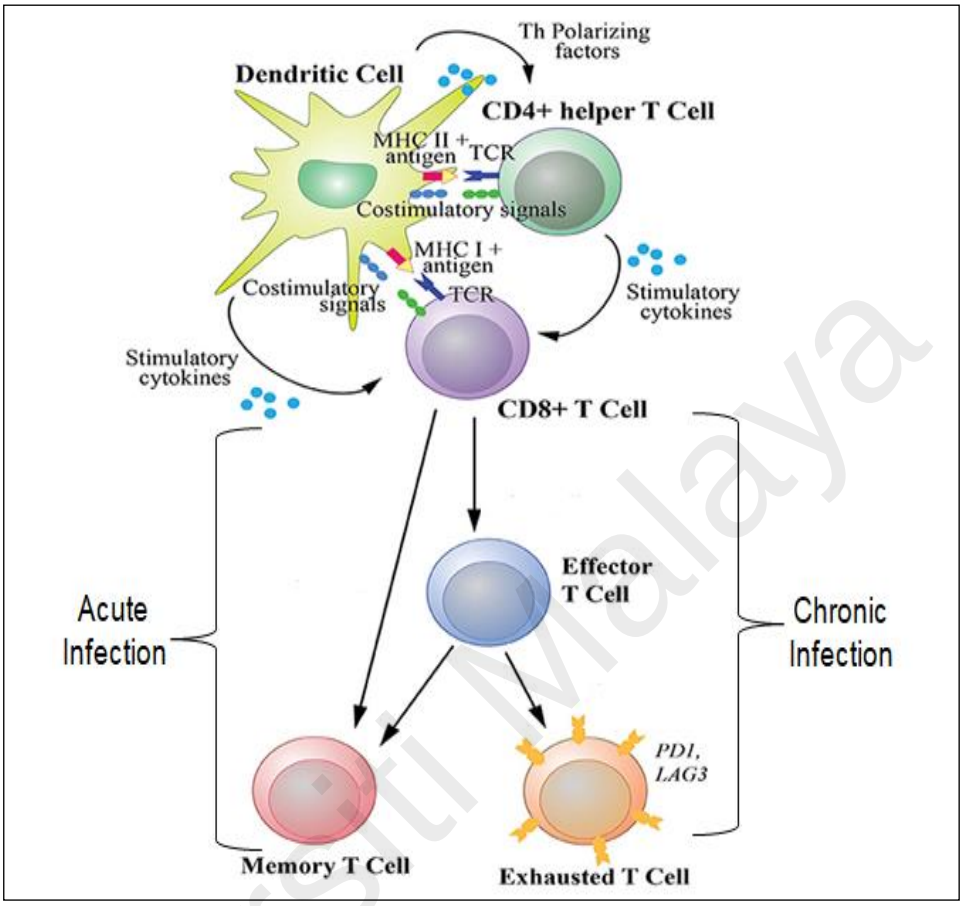


Figure 7.4: Mechanisms of T cells (CD4+ and CD8+ T) During Acute and Chronic Infection

(Adapted and modified from: Kartikasari et al., 2019)

7.1.3 T Cell Dysfunction and Immune Check Points Regulations

Generally, T cells response is regulated by the expressions of immune check point which includes co-stimulatory and co-inhibitory receptors according to the state of immune response against an infection (acute/chronic). Generally during acute or chronic infection, the T cell signaling cascade is activated or "switched ON" through the binding of T cell receptor (TCR) to MHC I or II complex as shown in Figure 7.5 below. It induces the transcriptions factors that are required for gene expression regulations (Laugel et al., 2011; Lin and Yan, 2018). The T cell activation can be further accelerated with the binding of co-stimulatory molecules such as CD28 and CD80/86 to the antigen presenting cells which lead to T cell proliferations, cytokines secretions and survival during the battle against an infection. However, during acute infections, the immune response will be down regulated or "switched OFF", after the antigen battling ended and will be stored as memory T cell. In contrast, during a chronic infection co-inhibitory molecule such as PD-1 will be expressed on the surface of T cells.

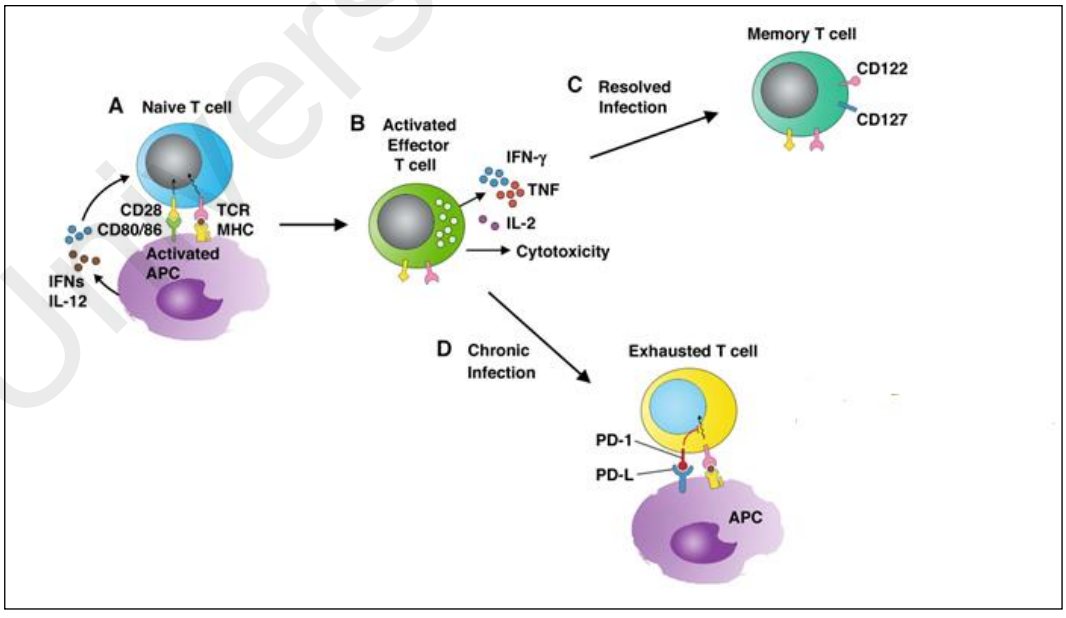


Figure 7.5: Mechanisms of T cell Exhaustion During a Chronic or Pro-Longed Infection

(Source: Freeman et al., 2006)

Generally, the down-regulations of the T cell activation is initiated by the co-inhibitory receptors on T cells such as BTLA-4, KLRG1 and 2B4 cells (Beyersdorf et al., 2015). These co-inhibitory receptors are able to inhibit the T cell receptor (TCR) signaling. This will eventually prevent the damage of the surrounding tissues. Therefore, co-stimulatory and co-inhibitory receptors are vital in retaining balanced immune regulations in order to prevent damage to the surrounding tissue. Imbalances of these receptors have been reported during a chronic pathogenic infections and antigen persistence which dampens the immune system (Attanasio and Wherry, 2016). This is due to multiple types of co-inhibitory molecules expressions on exhausted T cells such as PD-1, CTLA-4 and TIGIT during a prolonged infection. Subsequently the eradication of the pathogenic infection will be impaired due to the binding of T cell co-inhibitory molecules instead of co-stimulatory molecules to the antigen presenting cell surfaces as shown in Figure 7.6 below.

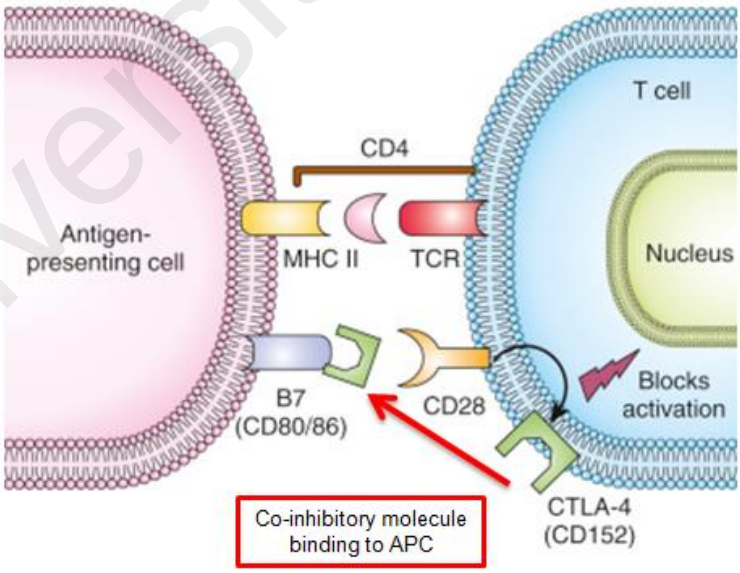


Figure 7.6: Binding of T Cell Co-Stimulatory Molecule (CTLA-4) to Antigen Presenting Cell

(Adapted and modified from: Peter Taylor, 2017)

T cell exhaustion has been widely documented during chronic intestinal inflammatory diseases such as IBD, IBS (Mavrangelos et al., 2018) and colorectal cancer (Cantero-Cid et al., 2018) where all these diseases are highly linked with *Blastocystis* sp. infection (Poirier et al., 2012; Kumarasamy et al., 2017). Besides, T cell exhaustion was also reported during chronic parasitical infections such as *Schistosoma*, *Leishmania*, *Trypanosoma*, *Toxoplasma* and *Plasmodium* (Zander et al., 2013). T cell exhaustion will eventually lead to its functional inept such as reduced cell proliferation and pro-inflammatory cytokine secretions during pro-longed antigen stimulation as shown in Figure 7.7 below.

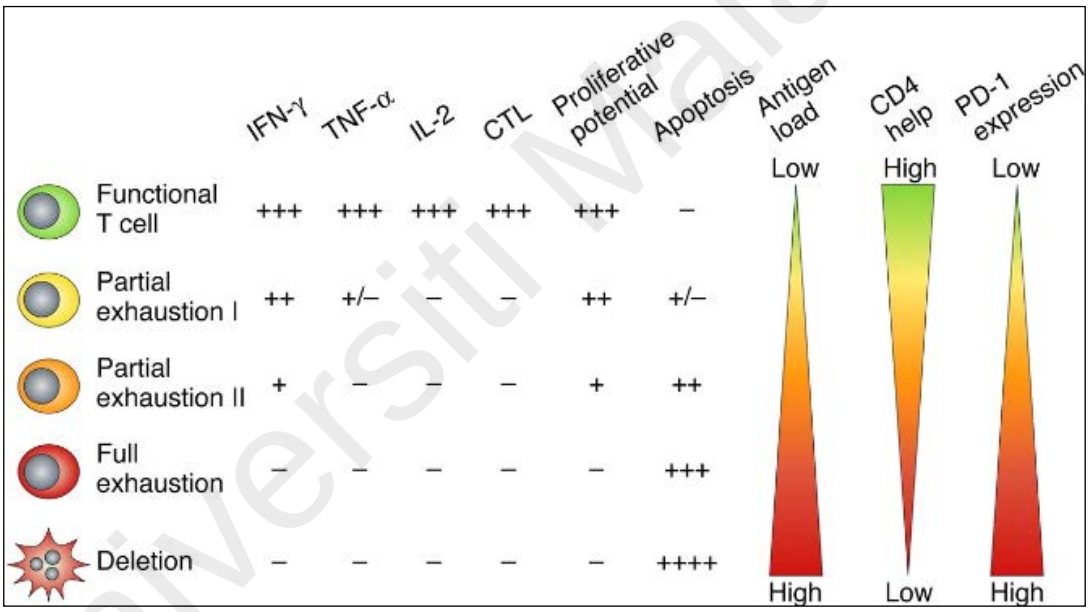


Figure 7.7: Parameters of Functional T Cell (CD4+) During an Antigenic Stimulation

(Source: Freeman et al., 2006)

Therefore, in this chapter further analysis was carried out to investigate the T cell immunomodulations during pro-longed *Blastocystis* sp. ST3 infection by assessing immune check point (CTLA-4, PD1 and TIGIT). Following is the detailed description of the immune check point analysis.

7.1.4 Programme Cell Death-1 (PD-1)

The programmed cell death-1 (PD-1, CD279) receptor is classified as immunoglobulin family which holds immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibition motif (ITIM) on its expressing immune cells such as myeloid and lymphoid lineage cells. The expression of PD-1 is up-regulated upon activation of these cells. This receptor will bind to its ligands which are PD-L1 (B7H1, CD274) and PD-L2 (B7DC, CD273), members of the immune-regulatory B7 family as shown in Figure 7.8. Therefore, the binding of PD-1 of the activated T cells to the ligand PD-L1/PD-L2 will eventually inhibit its proliferation and reduced cytokine release in T cell populations (Riley, 2009).

7.1.5 Cytotoxic T Lymphocyte Antigen-4 (CTLA-4)

Cytotoxic T-lymphocyte antigen 4 (CTLA-4), is classified as a member of the immunoglobulin superfamily. It is reported to exhibit similar homology with CD28 and completely bound to the CD80 or CD86 proteins which are located on the antigen presenting cells as shown in Figure 7.8. CTLA-4 expression has been known to down-regulate T cell function by decreasing its proliferation (Rowshanravan et al., 2017).

7.1.6 T-cell immunoreceptor with Ig and ITIM domains (TIGIT)

TIGIT is classified as a co-inhibitory receptor expressed on lymphoid cells, T-cell and natural killer (NK) cells. Binding of TIGIT to its ligand triggers a negative signal in T cells and NK cells, preventing their activation. TIGIT expression in effector and regulatory T cells is highly up regulated in the inflammatory microenvironment. Both NK and T cells compete to bind to its ligand CD155 (Poliovirus receptor (PVR)) and exhibit the inhibitory function during infection as shown in Figure 7.8. TIGIT competes with CD226, a T cell co-stimulatory molecule, to bind with CD155 on APCs in order to initiate anti-viral, anti-tumor and anti-parasitic responses (Sher et al., 2003).

7.1.7 DNAM-1 receptor (CD226)

DNAM-1 (DNAX accessory molecule 1) is an activating receptor which is also known as CD226, a glycoprotein expressed on the surface of NK cells, monocytes and a subset of B/T lymphocytes cells. The molecule CD226 will bind to either CD155 or CD112 identified as ligands for DNAM-1 on NK or T cells which augment the cell cyto-toxicity activity by mediating cytokine production (Xiong et al., 2015; Lenac Rovis et al., 2016) as shown in Figure 7.8.

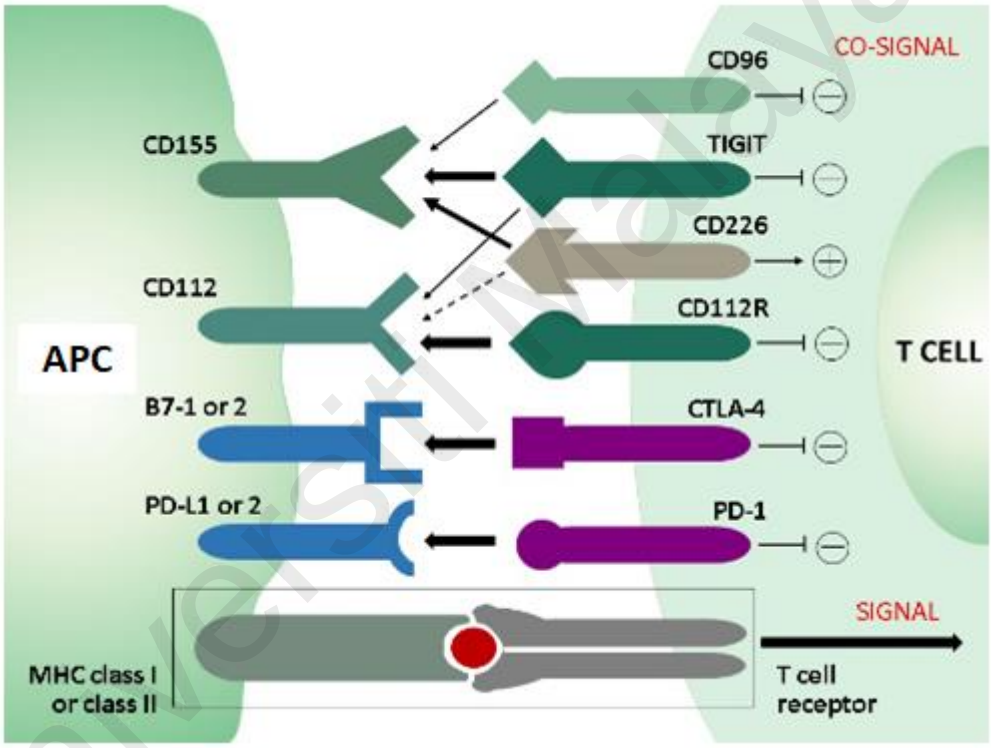


Figure 7.8: Immune Check Points Expressions by Antigen Presenting Cell and T Cell

(Source: https://www.dianova.com)

7.2 Materials and Methods

7.2.1 Source of *Blastocystis* sp.

As mentioned in 3.2.1

7.2.2 Axenization of *Blastocystis* sp. and Isolation of Solubilized Antigen

As mentioned in 3.2.2

7.2.3 Human Peripheral Blood Mononuclear Cells Isolations

As mentioned in 5.2.6

7.2.4 Induction of hPBMC Cells with *Blastocystis* sp. Solubilised Antigen for 3 and 6 days

Briefly, 3×10^4 cells per well was seeded in sterile 96-well plates in the presence of ST3 asymptomatic (1-3) and symptomatic (1-3) (0.01-10 µg/ml) respectively in RPMI complete medium. The plates were then incubated for the duration of 3 and 6 days. The experimental plate was terminated at 6, 24 and 48 hours by centrifugation at 300xg. The supernatant was aspirated and stored in -80ºC till further use. The cell pellet was subjected to cell proliferation assay using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, USA) (Following method 5.2.6).

7.2.5 Cell Viability Assay

Following assay termination in method 7.2.4 the cell pellet (induced and un-induced) were subjected to cell viability test using Cell Titter Glow Assay. The protocol is as mentioned in 5.2.8.2.

7.2.6 ELISA Test

Following assay termination in method 7.2.4 the respective supernatant was then assayed for; pro-inflammatory cytokines (IL-2, IL-6, IFNγ, IL-12p70 and TNF-α) and anti-inflammatory cytokine IL-10 analysis using a commercial Human ELISA Kit (R&D Systems, USA) according to the manufacturer's instructions (Appendix B). All the assays were performed in triplicates.

7.2.7 Flow Cytometry Analysis

7.2.7.1 Anti-human Antibodies

Following assay termination in method 7.2.4 PBMCs pellets obtained from the assays (induced and un-induced), were comprehensively characterized by multicolour flow cytometry for the expression of the cell surface marker. The following human fluorescent conjugated antibodies were used:

1. CD4-PE: (Miltenyi Biotech, USA, Cat No: 130-113-254)
2. CD8-PE: (Miltenyi Biotech, USA, Cat No:130-110-678)
3. CD152-FITC-CTLA-4: (Miltenyi Biotech, USA, Cat No:130-116-809)
4. Inside Stain Kit (Miltenyi Biotech, USA, Cat No: 130-090-477)
5. CD279-PE-PD1: (Miltenyi Biotech, USA, Cat No: 130-117-384)
6. TIGIT-PerCp: (E-Bioscience, USA, Cat No: E14967-107)
7. CD3-PE-CY7: Becton Dickson, USA, (Cat No: 5019706)
8. CD14-APC-H7: (Becton Dickson, USA, Cat No:5082736)
9. CD226- PE (Becton Dickson, USA, Cat No:4038706).

7.2.7.2 Flow Cytometry Protocol

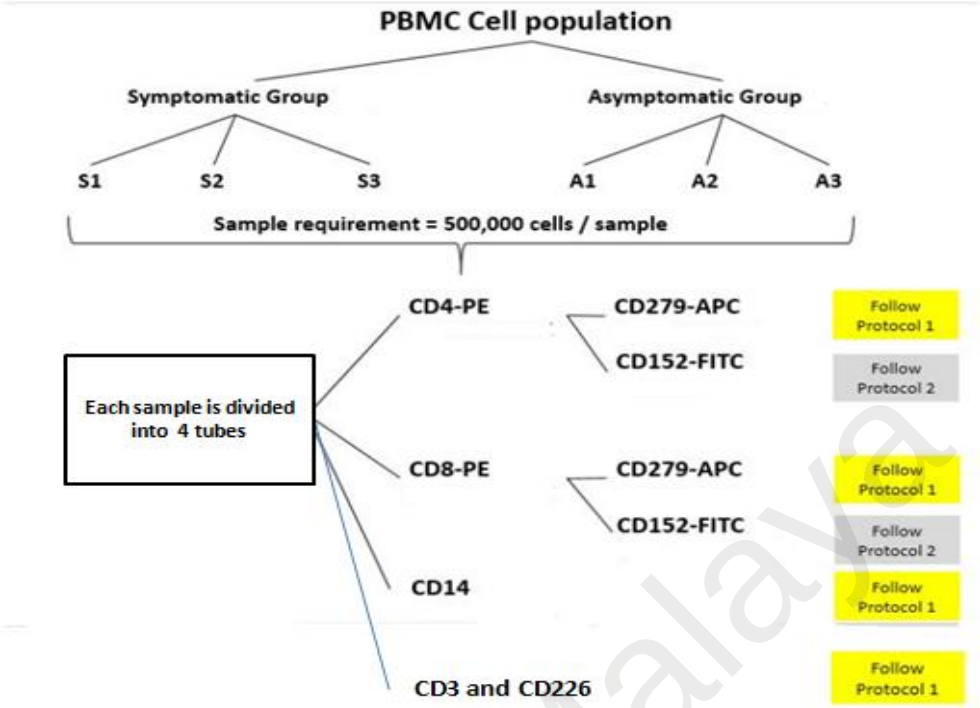


Figure 7.9: Study Plan for Flow Cytometry Analysis

7.2.7.3 Protocol 1

Briefly, the cells were centrifuged at 300×g for 10 minutes and the pellet was re-suspended with 9.8µL cold phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA and kept at 2−8 °C. The cell suspension was added with 0.2 µL of the human antibody. After antibody addition, the cell suspension was thoroughly mixed using a micropipette and was incubated for 10 minutes in dark in the refrigerator (2−8 °C). The cell suspension was washed by adding 1 mL of cold PBS buffer and centrifuged at 300×g for 10 minutes. The cell pellet was re-suspended with 300 µL of cold PBS buffer and were analysed for respective surface molecule detection using multicolour flow cytometry machine.

7.2.7.4 Protocol 2

Briefly, the cells were centrifuged at 300×g for 10 minutes and the pellet was re-suspended with 9.8µL cold phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA and kept at 2−8 °C. The cell suspension was added with 0.2 µL of the human antibody. After antibody addition, the cell suspension was thoroughly mixed using a micropipette and was incubated for 10 minutes in dark in the refrigerator (2−8 °C). The cell suspension was washed by adding 1mL of cold PBS buffer and centrifuged at 300×g for 10 minutes. Following centrifuge, the cell pellet was re-suspended at 10^5 cells in 25 µL of buffer and was added with 25 µL of inside stain kit. The mixture was mixed well and incubated for 20 minutes in the dark at room temperature followed by centrifugation at 300×g for 5 minutes. After centrifugation, the supernatant was aspirated carefully and the cell pellets were washed by adding 1 mL of buffer and followed by centrifugation at 300×g for 5 minutes.

After centrifugation, the supernatant was aspirated carefully and the cell pellet was washed by adding 0.5 mL of inside stain kit and was centrifuged at 300×g for 5 minutes. After centrifugation, the supernatant was carefully aspirated and the cell pellet was re-suspended in 9.8 µL of inside stain kit again and 0.2 µL of antibody was added. The mixture was incubated for 10 minutes in the dark at room temperature followed by the addition of 0.5 mL of inside stain kit. The mixture was centrifuged at 300×g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended with 300 µL of cold PBS buffer and were analysed for respective surface molecule detection using multicolour flow cytometry machine.

7.2.8 Statistical analysis

Data was analysed by one-way analysis of variance (ANOVA) using SPSS software 22. The graphs were plotted using Graph Pad Prism 8.

7.3 Results

7.3.1 Cell Viability Assessment of PBMCs Stimulated with *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigen

7.3.1.1 Solubilised Antigen Concentrations Optimization at 3- and 6-days Induction

Solubilised antigen dose optimization with different concentrations (0.01, 0.1, 1 and 10 µg/ml) of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates stimulations in PBMCs were observed. The analysis has shown that 1 µg/ml of *Blastocystis* sp. symptomatic and asymptomatic solubilised antigens induced highest PBMCs proliferation with percentage of 22% and 33% respectively as shown in Figure 7.10. Therefore 1 µg/ml soluble antigens were chosen as the optimal concentration for further investigation of cell inhibition. The comparisons between PBMCs proliferations induced with different concentrations of symptomatic and asymptomatic solubilised antigen for 3 and 6 days are as reflected in Table 7.1.

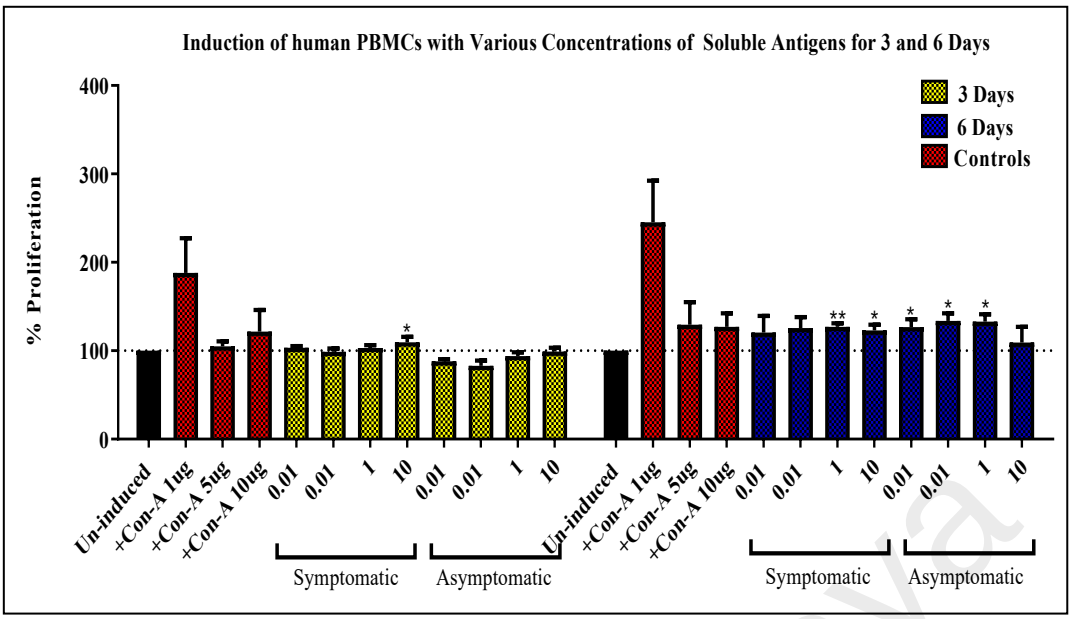


Figure 7.10: Cell Viability Assessment of PBMCs Induced with 0.001, 0.01, 1 and 10 µg/ml Solubilised Antigen of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Isolates

Values are given in mean ± SD (n=18) obtained from three healthy donors. *P<0.05 and **P<0.01 was the comparison made against un-induced PBMCs served as negative control (n=6).

7.3.1.2 Percentage Proliferation Increase from 3 to Day 6 Induction

Table 7.1: Summary Percentage Proliferations of PBMCs induced for 3 and 6 days

	PBMCs Proliferation (%)							
Isolates	**Symptomatic**				**Asymptomatic**			
Blastocystis-antigen (µg/ml)	**0.01**	**0.1**	**1**	**10**	**0.01**	**0.1**	**1**	**10**
3 Days Incubation	3.4	2.7	4.8	9.6	2.4	-	-	-
6 Days Incubation	20.7	25.7	27.1*	22.9	33.7*	33.8*	32.9*	9.2
Fold Difference	**17.3**	**23.0**	**22.3**	**13.3**	**31.3**	**33.8**	**32.9**	**9.2**

Data is given as mean ± SD (n=18) obtained from three different healthy donors. The comparison was carried out between 3 and 6 days of proliferation in *Blastocystis* sp. ST3 symptomatic and asymptomatic group using One-Way ANOVA (SPSS Version 22). The differences were significant with, *P<0.05.

7.3.1.3 PBMCs Induced with 1µg/ml Solubilised Antigen for 3 and 6 days

The PBMCs obtained from three different healthy donors were induced with 1 µg/ml of *Blastocystis* sp. ST3 symptomatic and asymptomatic solubilised antigens for 3 and 6 days. It was observed that *Blastocystis* sp. antigens stimulated the cell proliferations from 3 to 6 days with the percentage increase of (5.75% to 24.79%) and (0% to 35.21%) in symptomatic and asymptomatic antigens inductions respectively as shown in Figure 7.11 below. The comparisons between PBMCs proliferations induction at 1µg/ml symptomatic and asymptomatic solubilised antigen for 3 and 6 days are as reflected in Table 7.2.

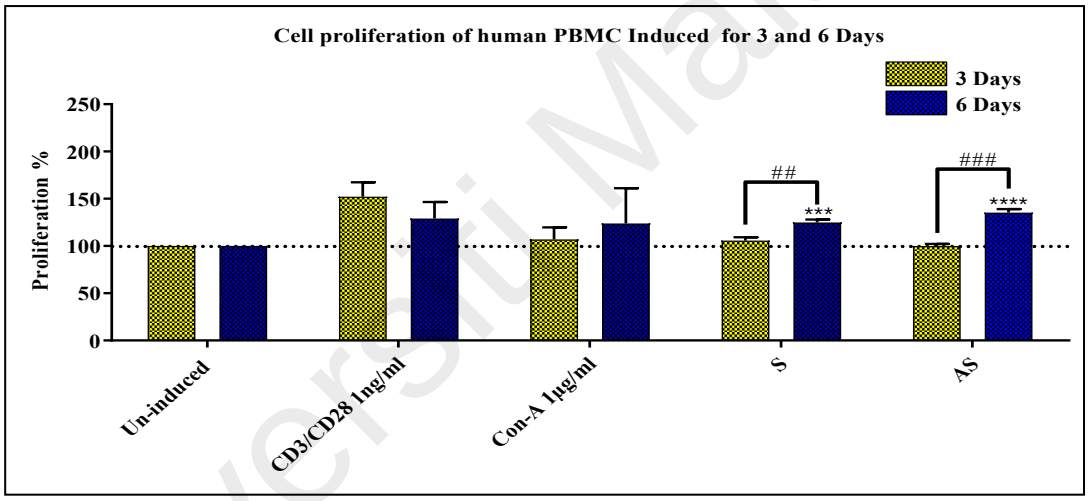


Figure 7.11: Cell Proliferations of PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigen for 3 and 6 days

Values are given in mean ± SD (n=18) obtained from three different healthy donors. **P<0.01, ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=6). ##P<0.01 and ###P<0.001 was the comparison made between 3 days and 6 days of induction.

Table 7.2: PBMCs proliferation upon inductions with 1 µg/ml of *Blastocystis* sp. solubilised antigen for 3 and 6 days

Group	Cell Viability (%)	
	3 Days	6 Days
Symptomatic	5.75±3.58	24.79±3.32
Asymptomatic	-0.11±2.35	35.22±3.90

Data is given as mean±SD. The comparison was carried out with between *Blastocystis* sp. ST3 symptomatic and asymptomatic using One-Way ANOVA (SPSS Version 22) (n=18). No significant differences were observed.

7.3.2 ELISA Test Results

7.3.2.1 Cytokine Assessment

In the present study, solubilised antigen of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates induced the up-regulations of pro-inflammatory cytokines (IL-2, IL-6, IFNγ, IL-12p70 and TNF-α) and anti-inflammatory cytokine IL-10 secretions in PBMCs as shown in Figure 7.12 to 7.17. The comparisons between cytokine secretions by PBMCs induced at 1 µg/ml symptomatic and asymptomatic solubilised antigen for 3 and 6 days are as reflected in Table 7.3.

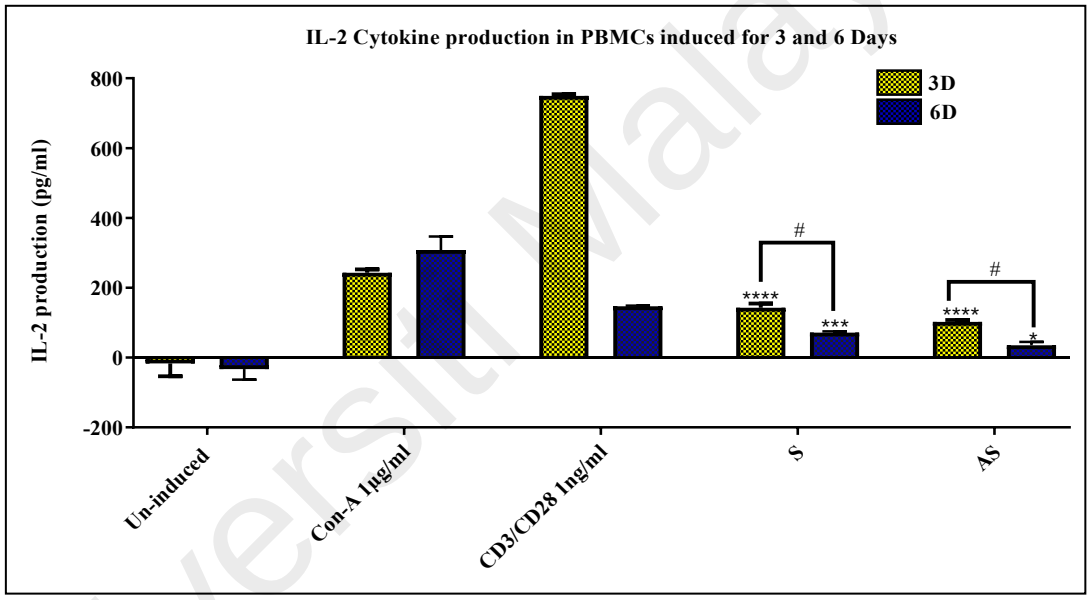


Figure 7.12: IL-2 Cytokine Response by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens for 3 and 6 days.

Values are given in mean ± SD (n=18). *P<0.05, ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=9). #P<0.05 was the comparison made between 3 and 6 days of induction.

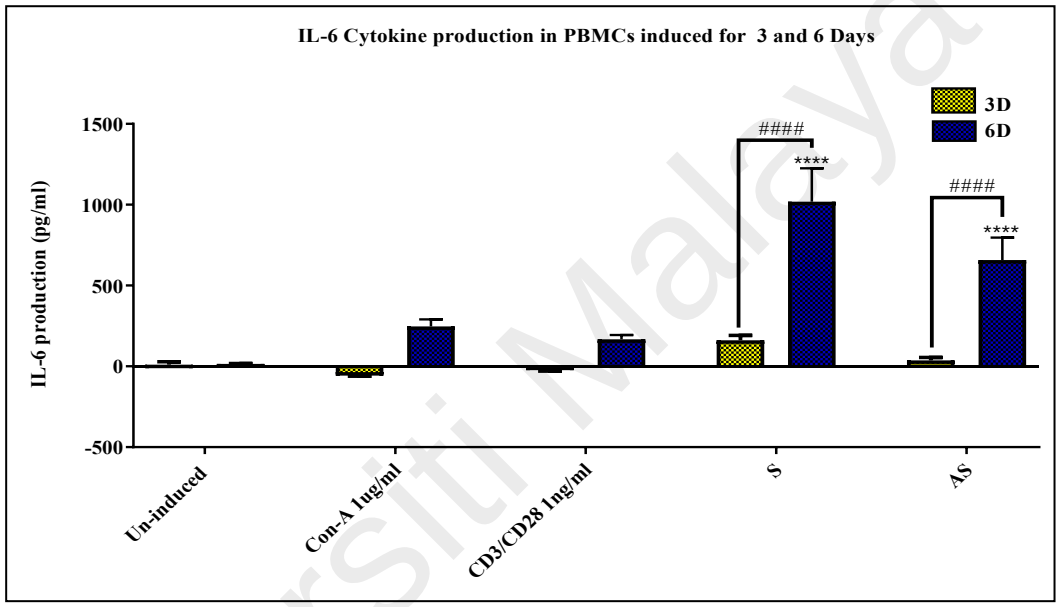


Figure 7.13: IL-6 Cytokine Response by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=18). ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=9). ###P<0.001 was the comparison made between 3 and 6 days of induction.

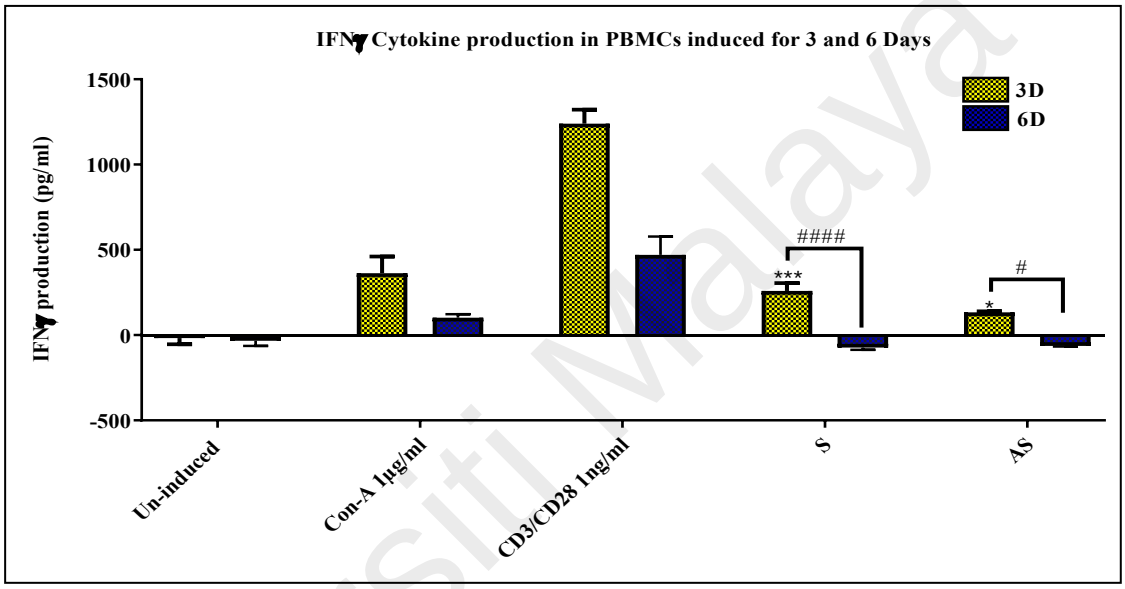


Figure 7.14: IFNγ Cytokine Response by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=18). *P<0.05 ***P < 0.001 was the comparison made against un-induced PBMCs served as negative control (n=9). #P<0.05 and ###P<0.001 was the comparison made between 3 and 6 days of induction.

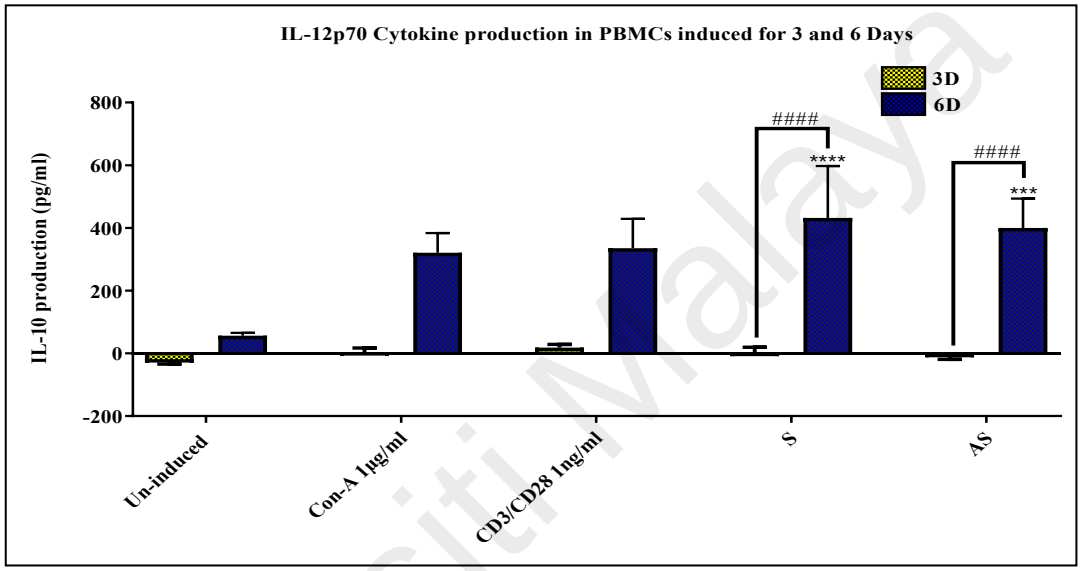


Figure 7.15: IL-12p70 Cytokine Response by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=18). ***P<0.01 and ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=9). #P<0.05 and ####P<0.001 was the comparison made between 3 and 6 days of induction.

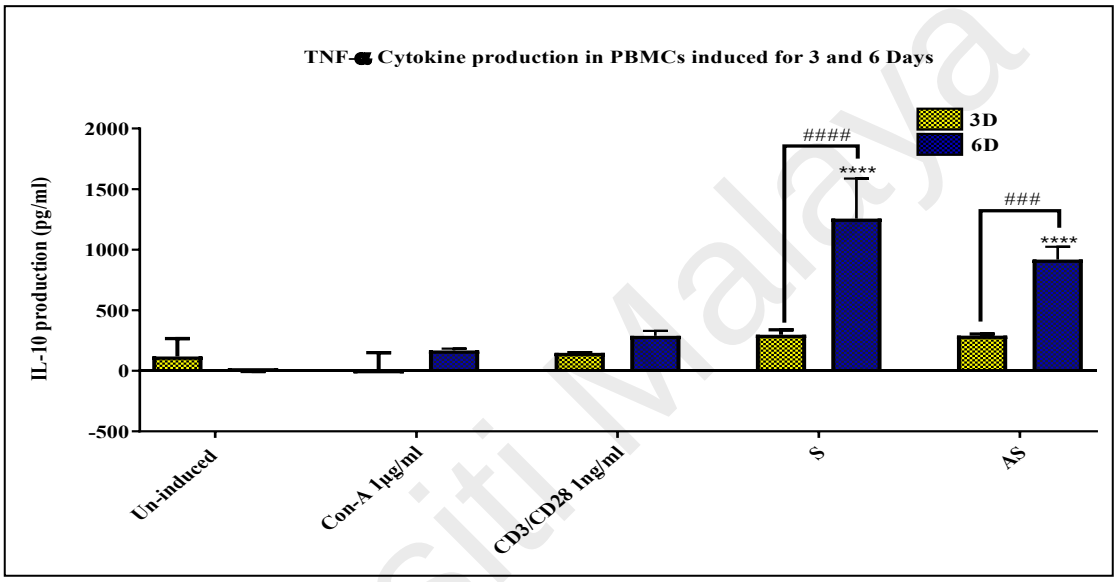


Figure 7.16: TNF-α Cytokine Response by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=18). ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=9). ###P<0.001 and ####P<0.0001 was the comparison made between 3 and 6 days of induction.

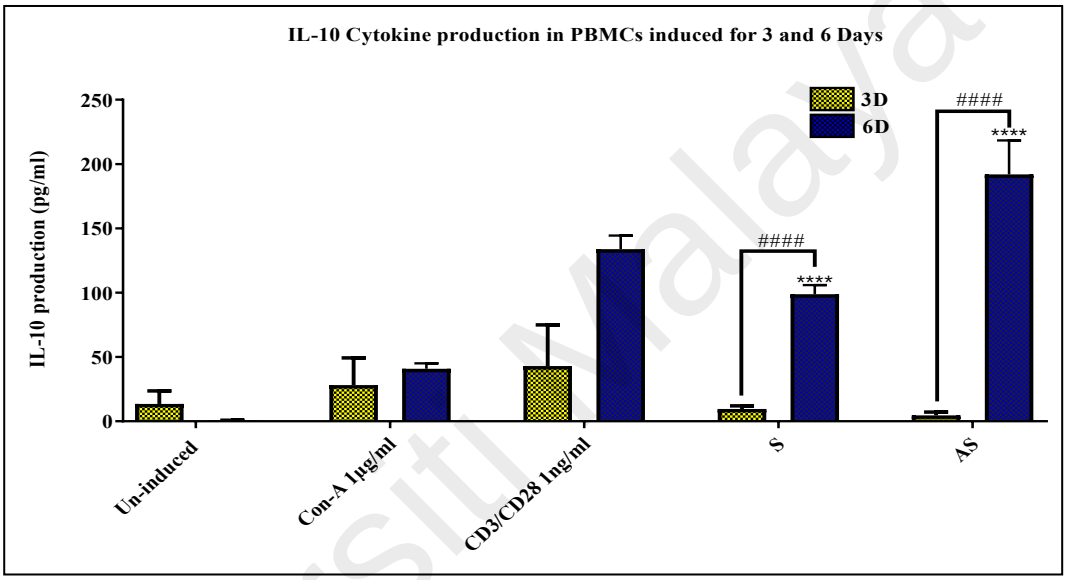


Figure 7.17: IL-10 Cytokine Response by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=18). ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=9). ####P<0.0001 was the comparison made between 3 and 6 days of induction.

Table 7.3: Summary of cell proliferation and cytokine response of PBMCs induced with symptomatic and asymptomatic of *Blastocystis* sp. ST3 (1 µg/ml) of soluble antigens

Group	Cell Proliferation Increase (%)		Cytokine Response (pg/ml)				Ratio of Pro: Anti-Inflammatory Cytokines	
			Pro-Inflammatory (IL-2+IL-6+IL-12p70+TNF-α+IFNγ)		Anti-Inflammatory (IL-10)			
	3 Days	6 Days	3 Days	6 Days	3 Days	6 Days	3 Days	6 Days
Symptomatic								
Donor 1	9	32	1300	2977	5	74	260:1	40:1
Donor 2	6	23	2454	2806	17	115	144:1	24:1
Donor 3	11	24	1465	2821	7	107	209:1	26:1
Average	**9**	**26**	**1740**	**2868**	**10**	**99**	**174:1**	**29:1**
Asymptomatic								
Donor 1	5	40	877	2423	3	171	292:1	14:1
Donor 2	2	34	1115	1329	7	205	159:1	6:1
Donor 3	0	32	1325	2337	2	200	662:1	12:1
Average	**2**	**35**	**1106**	**2030**	**4**	**192**	**277:1**	**11:1**

Data is given as mean of PBMCs stimulations in *Blastocystis* sp. ST3 symptomatic and asymptomatic group at 3- and 6-days induction obtained from three different healthy donors.

7.3.3 Immune Check Points Analysis on PBMCs Induced with *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

7.3.3.1 PD-1 Molecule Analysis

PBMCs was analysed for PD-1 molecule through flow cytometry analysis. The PD-1 molecule expressions on CD4+ and CD8+ T cell populations significantly increased from 3 to 6 days of inductions in PBMCs induced with symptomatic and asymptomatic antigens as shown in Figure 7.18. It was observed that PBMCs induced with symptomatic antigen increased PD-1 molecules on CD4+ and CD8 by (4.5 and 2.1–fold) respectively. On the other hand, PBMCs induced with asymptomatic antigens increased PD-1 molecules on CD4+ and CD8+ by (3 and 1.3-fold) respectively.

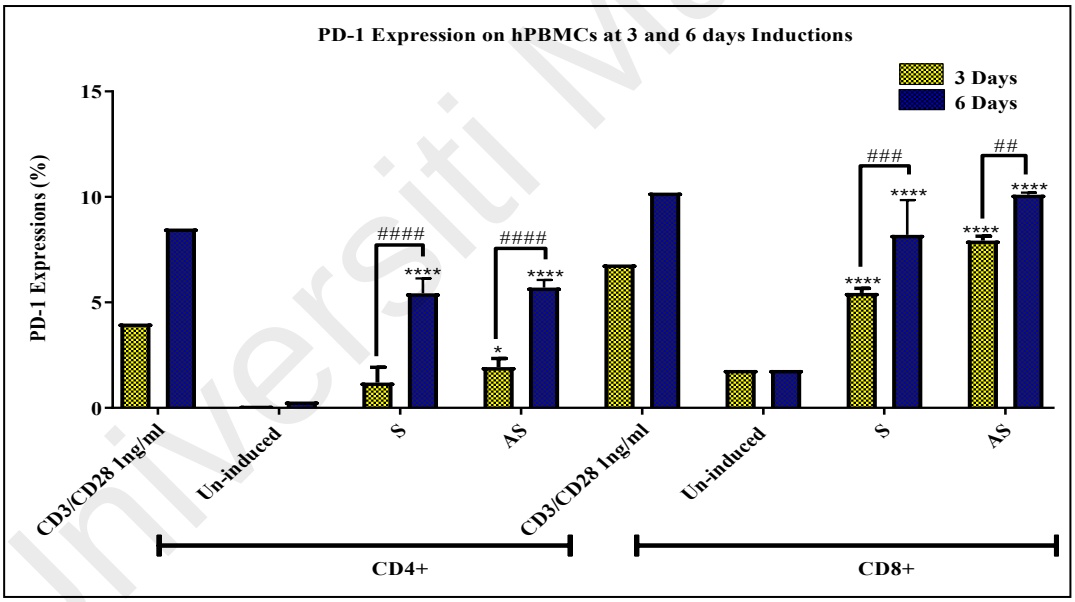


Figure 7.18: PD-1 Expression by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens.

Values are given in mean ± SD (n=9). *P<0.05 and ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=3). ##P<0.01, ###P<0.001 and ####P<0.0001 was the comparison made between 3 and 6 days of induction.

7.3.3.2 CTLA-4 Molecule Analysis

PBMCs was analysed for CTLA-4 molecule through flow cytometry analysis. The CTLA-4 molecule expressions on CD4+ and CD8+ T cell populations significantly increased from 3 to 6 days of inductions in PBMCs induced with symptomatic and asymptomatic antigens as shown in Figure 7.19. It was observed that PBMCs induced with symptomatic antigen increased CTLA-4 molecules on CD4+ and CD8 by (1.3-fold) respectively. On the other hand, PBMCs induced with asymptomatic antigens increased CTLA-4 molecules on CD4+ and CD8+ by (1 and 1.3-fold) respectively.

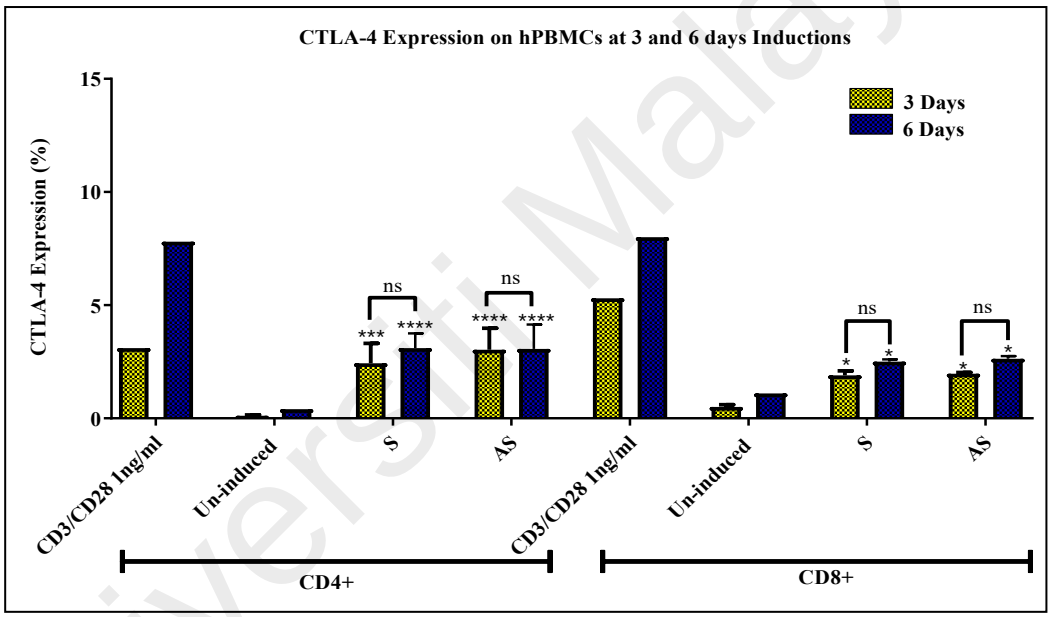


Figure 7.19: CTLA-4 Expression by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). *P<0.05, ***P<0.001 and ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=3). There was no significant difference observed between 3 and 6 days of induction.

7.3.3.3 TIGIT Molecule Analysis

PBMCs was analysed for TIGIT molecule through flow cytometry analysis. The TIGIT molecule expressions on CD4+ and CD8+ T cell populations significantly increased from 3 to 6 days of inductions in PBMCs induced with symptomatic and asymptomatic antigens as shown in Figure 7.20. It was observed that PBMCs induced with symptomatic antigen increased TIGIT molecules on CD4+ and CD8+ by (3.7 and 3.3-fold) respectively. On the other hand, PBMCs induced with asymptomatic antigens increased TIGIT molecules on CD4+ and CD8+ by (2.8 and 2.9-fold) respectively.

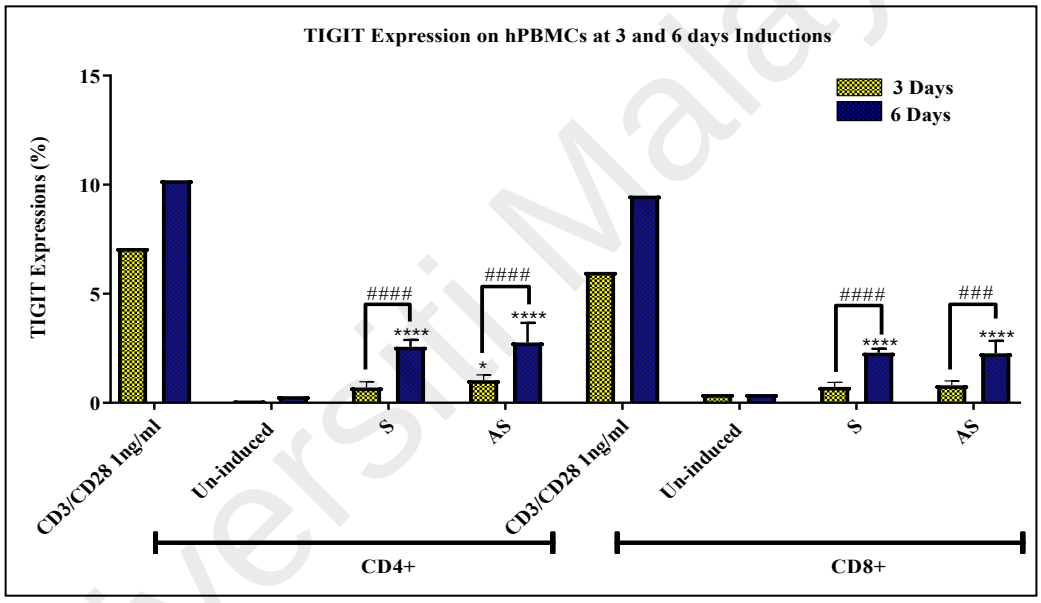


Figure 7.20: TIGIT Expression by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). *P<0.05 and ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=3). ###P<0.001 and ####P<0.0001 was the comparison made between 3 and 6 days of induction.

7.3.3.4 Total T Cell Surface Marker (CD3) Analysis

PBMCs was analysed for total T cells populations by targeting its cell surface marker (CD3+) through flow cytometry analysis. The CD3+ expressing T cells significantly increased from 3 to 6 days of inductions in PBMCs induced with symptomatic and asymptomatic antigens as shown in Figure 7.21. It was observed that PBMCs induced with symptomatic and asymptomatic antigen increased CD3+ marker by 1.2-fold respectively.

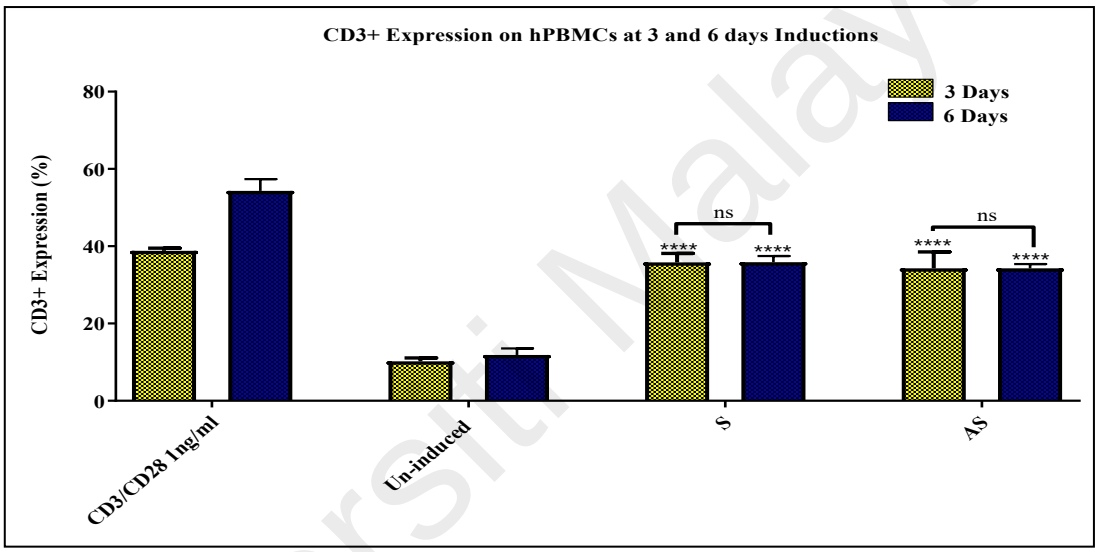


Figure 7.21: CD3+ Expression by PBMCs Induced with 1 µg/ml of *Blastocystis* sp. ST3 Symptomatic And Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=3). No significance difference was observed between 3 and 6 days of induction.

7.3.3.5 Total Antigen Presenting Cells Surface Marker (CD14+) Analysis

PBMCs was analysed for total antigen presenting cells populations (monocytes and macrophages) by targeting its cell surface marker (CD14+) through flow cytometry analysis. The CD14+ expressing antigen presenting cells significantly increased from 3 to 6 days of inductions in PBMCs induced with symptomatic and asymptomatic antigens as shown in Figure 7.22. It was observed that PBMCs induced with symptomatic and asymptomatic antigen increased CD14+ marker by 5.5 and 7.4-fold respectively.

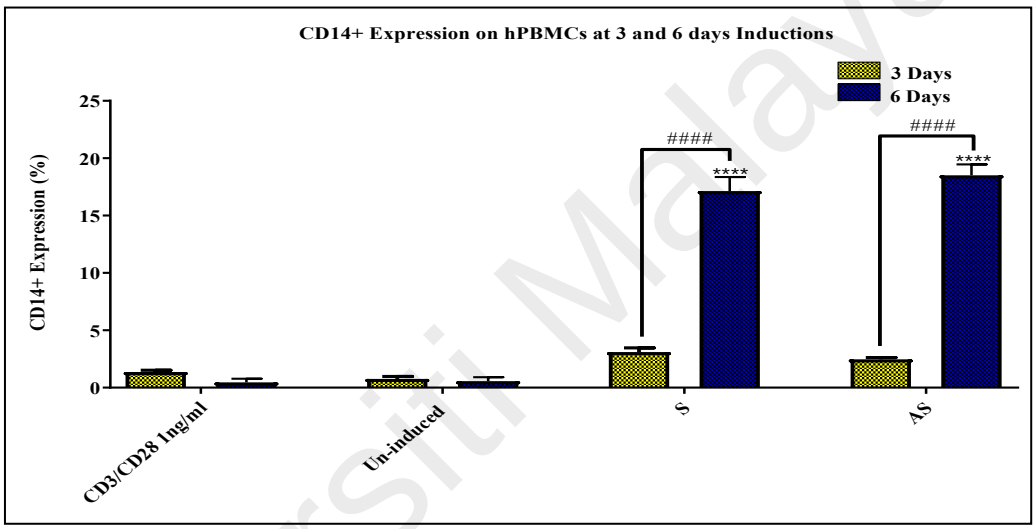


Figure 7.22: CD14+ Expression by PBMCs Induced with 1 µg/ml *Blastocystis* sp. ST3 Symptomatic and Asymptomatic Solubilised Antigens

Values are given in mean ± SD (n=9). ****P < 0.0001 was the comparison made against un-induced PBMCs served as negative control (n=3). ####P<0.0001 was the comparison made between 3 and 6 days of induction.

Table 7.4: PBMC Induction for 3 days

Group	Inhibitory and Stimulatory Receptors	Targeting T cells Population (%)			Total (%)	Group	Total Cell Populations (%)			
		Blastocystis sp. Antigen Induced					*Blastocystis* sp. Antigen Induced			
		CD4+	CD8+	Non CD4+/CD8+			CD3+	CD4+	CD8+	CD14+
S	PD-1	1.2	5.5	7.6	14.2	S	30.2	12.1	12.7	3.1
AS		1.9	7.9	14 .1	23.9	AS	28.7	10.9	14.7	2.5
S	CTLA-4	2.4	1.9	4.5	8.8	Un-Induced				
AS		3.0	2.0	6.0	11.0		CD3+	CD4+	CD8+	CD14+
S	TIGIT	0.7	0.7	3.5	4.9		10.5	1.8	3.0	0.8
AS		1.0	0.8	4.4	6.2					
S	CD226			14.5	14.5					
AS				15.7	15.7					

Data is given as mean value (n=3) obtained through immuno-phenotyping analysis from one healthy donor. The PBMCs were stimulated with *Blastocystis* sp. ST3 symptomatic and asymptomatic group for 3 days of duration.

Table 7.5: PBMC Induction for 6 days

Group	Inhibitory and Stimulatory Receptors	Targeting T cells Population (%)			Total (%)	Group	Total Cell Populations (%)			
		Blastocystis sp. Antigen Induced					*Blastocystis* sp. Antigen Induced			
		CD4+	CD8+	Non CD4+/CD8+			CD3+	CD4+	CD8+	CD14+
S	PD-1	5.4	8.2	10.7	24.3	S	35.8	14.5	17.0	17.1
AS		5.7	10.1	11.2	27.0	AS	34.2	14.7	22.0	18.5
S	CTLA-4	3.1	2.5	3.2	8.8	Un-Induced				
AS		3.1	2.6	3.9	9.6		CD3+	CD4+	CD8+	CD14+
S	TIGIT	2.6	2.3	4.4	9.3		11.8	2.7	3.1	0.6
AS		2.8	2.3	4.3	9.4					
S	CD226			19.0	19.0					
AS				20.4	20.4					

Data is given as mean value (n=3) obtained through immuno-phenotyping analysis from one healthy donor. The PBMCs were stimulated with *Blastocystis* sp. ST3 symptomatic and asymptomatic group for 6 days of duration.

7.3.4 Evaluations of Impaired T cell CD4+ and CD8+ populations

As discussed in 7.1 above, the parameters of T cells dysfunctional or exhaustion upon pro-longed antigenic stimulation are evaluated based on (i) reduced T cell proliferation (ii) reduced T cell pro-inflammatory cytokines (iii) increased anti-inflammatory cytokine and (iv) increased multiple T cell co-inhibitory molecules expressions. Following are the observations of T cells (CD4+ and CD8+) populations upon pro-longed (6 days) *Blastocystis* sp. ST3 symptomatic and asymptomatic antigenic induction on PBMCs;

Cell Proliferation:

1. Total T cell (CD3+) increased (not significant) compared to 3 days induction as shown in Figure 7.11.
2. T cell populations CD4+ and CD8+ cells increased (not significant) from 3 to 6 days of symptomatic and asymptomatic antigen induction as shown in Table 7.4 and 7.5.

Cytokines Responses:

1. T cell pro-inflammatory cytokines (IFNγ and IL-2) levels were significantly reduced from 3 to 6 days of symptomatic and asymptomatic antigenic induction as shown in Figure 7.12 and 7.14.
2. Anti-inflammatory cytokine response significantly increased from 3 to 6 days of symptomatic and asymptomatic antigen induction as shown in Figure 7.17.

Co-Inhibitory Molecules Expressions:

1. Multiple co-inhibitory molecules (PD-1, TIGIT and CTLA-4) on T cell populations CD4+ and CD8+ was observed. There were increased of these co-inhibitory molecules from 3 to 6 days of symptomatic and asymptomatic antigen induction as shown in Figure 7.18 to 7.20.

Therefore, it was evident that, T cell exhaustion was observed in PBMCs induced with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. The percentages of T cell exhaustion targeting CD4+ and CD8+ populations are as reflected in Figure 7.23 and 7.24. Percentage values were calculated based on the results obtained from Table 7.5. Calculations for percentage (%) exhausted CD4+ and CD8+ cells are as mentioned in 7.3.4.1 below:

7.3.4.1 Calculations

A. Total Percentage of CD4+/ CD8+ cell populations

$$\frac{\%\ \text{of CD4+/ CD8+}}{\%\ \text{of (CD3+)}} \times 100$$

B. Total percentage of co-inhibitory molecules on CD4+/ CD8+ cell populations

$$\frac{\%\ \text{of co-inhibitory molecules}}{\%\ \text{of CD4+/CD8+ from (A)}} \times 100$$

C. Total percentage of exhausted CD4+/ CD8+ cell populations

$$\frac{(B)}{(A)} \times 100$$

7.3.4.2 Graphs

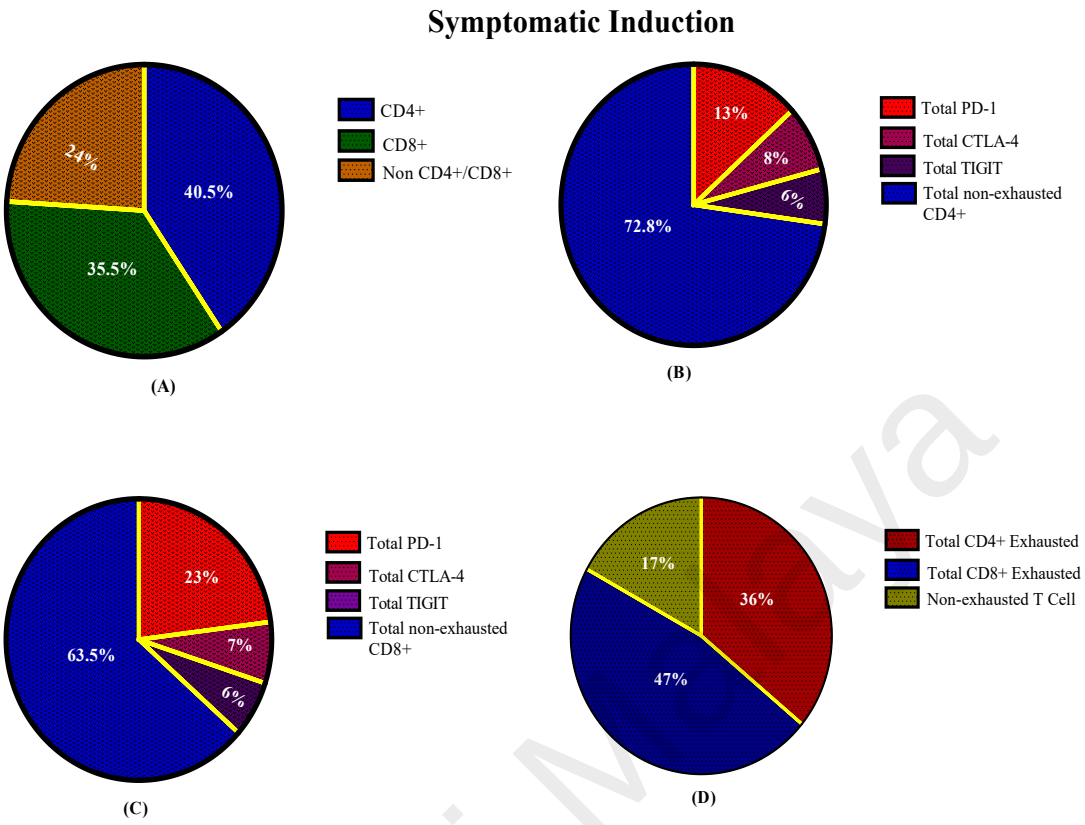


Figure 7.23: Percentage of CD4+ and CD8+ Exhaustion Induced by Symptomatic Antigens at Day 6 Induction

(A) Total Percentage of CD4+ and CD8+ cell populations.

(B) Total percentage of co-inhibitory molecules on CD4+ cell populations.

(C) Total percentage of co-inhibitory molecules on CD8+ cell populations.

(D) Total percentage of exhausted CD4+ and CD8+ populations.

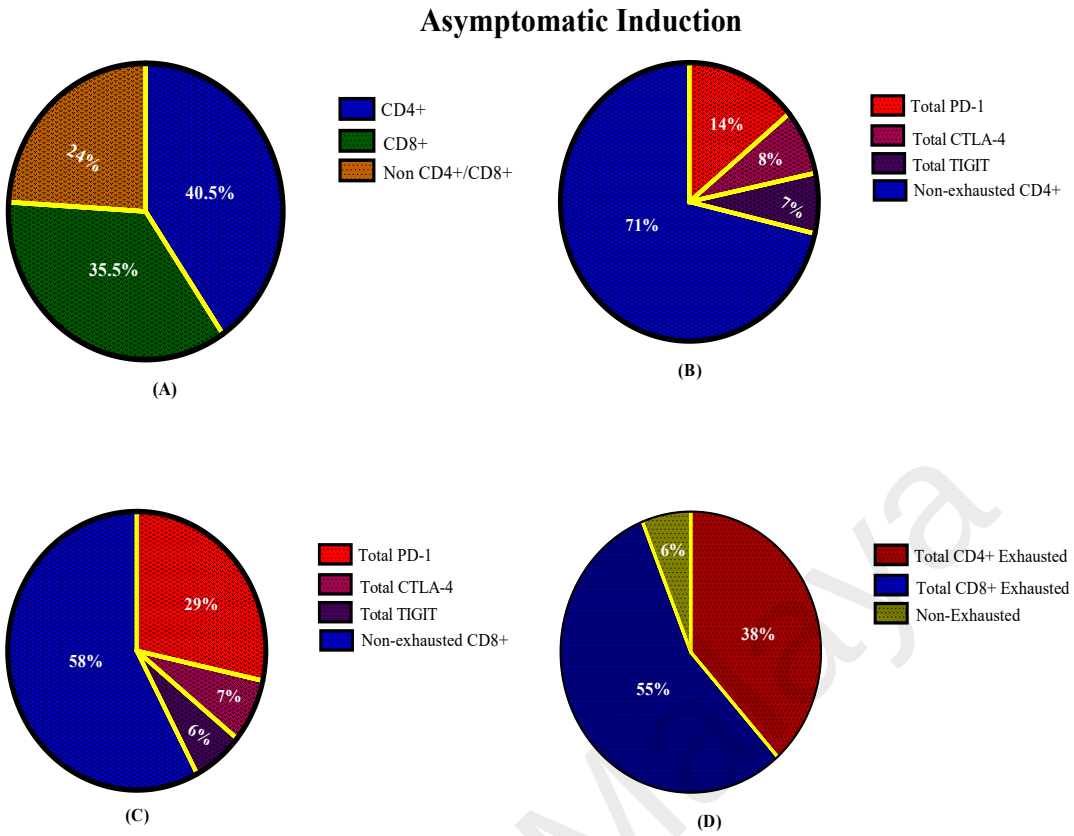


Figure 7.24: Percentage of CD4+ and CD8+ Exhaustion Induced by Asymptomatic Antigens at Day 6 Induction

(A) Total Percentage of CD4+ and CD8+ cell populations.

(B) Total percentage of co-inhibitory molecules on CD4+ cell populations.

(C) Total percentage of co-inhibitory molecules on CD8+ cell populations.

(D) Total percentage of exhausted CD4+ and CD8+ populations.

7.3.5 Cell Culture Images

Cell proliferation was noticed in PBMCs upon induction with 1 µg/ml *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens. High cell density was observed in day 3 antigen inductions and gradually increased in day 6 antigen inductions (as indicated by the arrows in (Figure 7.25). However, low cell density was observed in un-induced PBMCs.

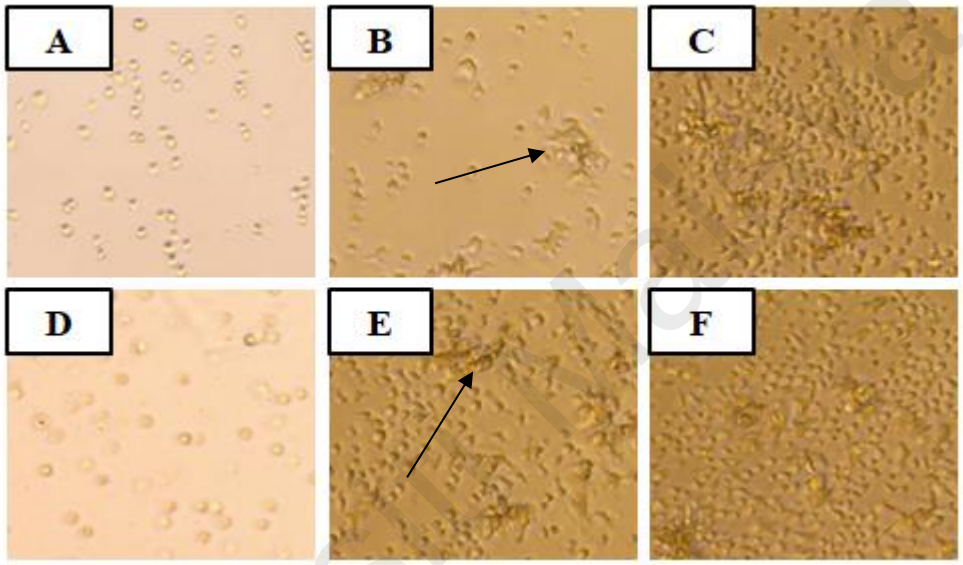


Figure 7.25: PBMCs Cell Culture Images

(A) Day 3 of un-induced cells (B) Day 3 of *Blastocystis* sp. antigen induced cells (C) Day 3 of CD3/CD28 antibody (positive control) induced cells (D) Day 6 of un-induced cells (E) Day 6 of *Blastocystis* sp. antigen induced cells (F) Day 6 of CD3/CD28 antibody induced cells. Images were captured using inverted microscope with magnification at 40X.

7.4 Discussion

In the previous Chapter 6 it was observed that, *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens induced high expressions of PD-1 (immune co-inhibitory molecules) and CD86 (T cell co-stimulatory molecule) on monocytes from 3 to 6 days of inductions. Generally, co-stimulatory molecules expressions either CD80 or CD86 on antigen presenting cells such as monocytes and dendritic cells are highly involved in effective T cells activation (Vasilevko et al., 2002). Previous *in vitro* and *in vivo* studies by researchers demonstrated that, both CD80 and CD86 molecules played an equal role in T cell activation. Furthermore, the authors have concluded that CD86 could be more important and more specific to the activation of T cell responses, while CD80 could be more important for maintaining general pathogenic immune responses which is not specific to T cell response (Subauste et al., 1998; Vasu et al., 2003; Lim TS et al., 2012). Therefore, the immunomodulations by *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens on T cells were investigated by stimulating PBMCs for 3 and 6 days.

7.4.1 Cell Proliferation Analysis

The short-term (3 days) and long-term (6 days) of PBMC inductions with symptomatic and asymptomatic antigens showed increased cell proliferation with significant difference of $P<0.01$ and $P<0.001$ respectively. PBMCs contain lymphocytes populations which includes T cells and monocytes. Therefore, the antigen presenting cells cell surface marker CD14+ was analysed. The immuno-phenotyping results have shown increased populations of CD14+ cell surface marker in PBMCs induced with symptomatic and asymptomatic antigen by 5.5 and 7.4-fold respectively which suggesting increase of monocytes. Generally, CD14+ marker is found on either monocytes or macrophages. The increase of CD14+ on monocytes cell surface was demonstrated in Chapter 6 where it was demonstrated that *Blastocystis* sp. symptomatic and asymptomatic antigens

significantly increased monocytes proliferation from short to long-term induction. The CD14+ cell surface marker also was found on macrophages as shown in Chapter 5. However, upon macrophages induction with *Blastocystis* sp. antigens it was shown to undergo senescence. Therefore, it was confirmed that there was proliferation of monocytes in PBMCs upon induction with symptomatic and asymptomatic *Blastocystis* sp. antigens. In order to confirm the activation of T cell by monocytes during the *Blastocystis* sp. antigenic stimulation, CD3+ cell surface marker was analysed. It was observed that there was an increase of CD3+ T cell surface marker in PBMCs during a short-term induction with symptomatic and asymptomatic antigen with significance of $p<0.0001$ in comparison to un-induced cells. There was an increase of its T cells subsets of CD4+ and CD8+ cells. The cell surface marker (CD3+) indicated the total populations of T cells in PBMCs whereby CD4+ and CD8+ indicating the increase of helper and cytotoxic T cells upon pro-longed antigen inductions. It was observed that, there was a concurrent increase of monocytes and T cells during the antigenic stimulations. Therefore, suggesting the increase of T cells proliferations were activated by monocytes upon *Blastocystis* sp. antigen induction.

7.4.2 Pro-Inflammatory Cytokine Responses

This was further confirmed with the assessment of pro-inflammatory cytokine secretions such as IL-2, IFNγ, IL-6, IL-12p70 and TNF-α. Past chapters of our study, have concluded symptomatic antigens to be more pathogenic compared to asymptomatic antigens due to their ability to secrete high pro-inflammatory cytokines and higher immune cell proliferations (T cells, macrophages and monocytes). Therefore, the pro-inflammatory cytokines levels of IL-2 and IFNγ secreted by PBMCs induced with *Blastocystis* sp. ST3 antigens were assessed. Generally, IL-2 and IFNγ are secreted mainly by T cells subset of CD4+ and CD8+ respectively. It was observed that there was an increase of these cytokines in short-term inductions of PBMCs induced with

symptomatic and asymptomatic antigens. T cell proliferation cytokine (IL-2) significantly increased at $p<0.0001$ in PBMCs induced with symptomatic and asymptomatic antigens in comparison to un-induced cells. The IFNγ cytokine level increase at $p<0.001$ and $p<0.05$ in cells induced with symptomatic and asymptomatic antigen respectively. However, the secretion of these cytokines was significantly dropped during the long-term *Blastocystis* sp. antigen induction. This results also was compared with positive control induction targeting T cell proliferation; CD3/CD28 antibody control where the cytokine level of IL-2 and IFN-γ was impaired during a prolonged cell stimulation as shown in Figure 7.12 and 7.14. Therefore, these evidences have clearly shown that there was an inhibition of T cells activity to combat against the pro-longed antigen exposure.

Further analysis of pro-inflammatory cytokine responses IL-6, IL-12p70 and TNF-α has shown increased level of these cytokine in PBMCs induced with symptomatic and asymptomatic antigens. Even though, TNF-α is usually secreted mainly by activated CD8+ cells, it was demonstrated that there was an increase of this cytokine during a pro-longed *Blastocystis* sp. ST3 antigen stimulations. It was shown in previous Chapter 6 that, there was a significant elevation of TNF-α during pro-longed monocytes stimulations suggesting the cytokine was highly secreted by monocytes and not by T cells. This result was further supported by the evidence of pro-inflammatory cytokine response IL-6 and IL-12p70 (antigen presenting cells secreting cytokine) where high elevation of this cytokine was observed in monocytes and macrophages induced with *Blastocystis* sp. antigen in Chapter 5 and 6. Furthermore, the positive control (CD3/CD28 antibody) induction (Figure 7.16) has demonstrated non-significant increase of TNF-α cytokine level despite high cell proliferation observed. Therefore, it was proven that the TNF-α was secreted by monocytes induced with *Blastocystis* sp. ST3 antigens during the pro-longed induction supporting the evidence of T cell impairment.

7.4.3 Anti-Inflammatory Cytokine Response

Further confirmation on T cell dysfunction was evaluated through the evaluations of IL-10 cytokine response. It was observed that, there was an increase of IL-10 cytokine level in PBMCs during pro-longed symptomatic and asymptomatic antigen inductions with significance of $P<0.0001$. Generally, anti-inflammatory cytokine response is induced by T regulatory cells. In this study, it was observed that there was an average of 24% of non CD4+/CD8+ T cells suggesting the population of regulatory T cells (T reg) present in the PBMCs inductions. It was reported that, T reg causes up-regulations of IL-10 and reduce the activity of CD4+ and CD8+ T lymphocyte cells (Walker et al., 2013; Zhao et al., 2017). Furthermore, the ratio of pro-inflammatory to anti-inflammatory cytokines was tremendously reduced from 3 to 6 days of symptomatic and asymptomatic antigen inductions by 16 and 25-fold respectively as shown in Table 7.3. The reduction of total pro-inflammatory cytokine response in PBMCs induced with symptomatic and asymptomatic antigens once again confirmed the immunomodulation on immune cells caused by *Blastocystis* sp. ST3 antigens which has been proven in our previous findings in Chapter 3, 5 and 6. Therefore, it was proven that the T cells activity was impaired due to higher level of IL-10 cytokine secretions during long-term antigenic exposure. Due to high evidences of T cell impairment which has been proven through its reduced cytotoxicity against the pro-longed antigenic stimulation, further evaluations were done to analyse the presence of immune check point (T cell co-inhibitory molecules) targeting CD4+ and CD8+ cells.

7.4.4 Immune Check Point Analysis

The elevations of multiple co-inhibitory molecules also indicate the T cell dysfunction (Yi et al., 2010; Sperk et al., 2018). In this study, three types of co-inhibitory molecules were assessed namely (PD-1, CTLA-4 and TIGIT). It was observed that, there was an increase of these molecules during pro-longed symptomatic and asymptomatic antigens inductions. There was a significant up-regulations of PD-1 and TIGIT expressions on CD4+ and CD8+ cells induced with symptomatic and asymptomatic antigens as shown in Figure 7.18 and 7.20 respectively. The level of CTLA-4 molecule was increased on cells (CD4+ and CD8+) induced with symptomatic and asymptomatic antigens from 3 to 6 days of induction. However, there was no statistical significance observed. Besides, significant increase of PD-1 and CTLA-4 molecules was observed in short-term inductions despite high secretions of T cell functional cytokine (IL-2 and IFNγ). Generally, PD-1 is transiently expressed on activated T cell. The PD-1 expressions on T cells especially during a pro-longed antigenic exposure will results in T cell impairment upon binding with its ligand PDL-1 which generally expressed on antigen presenting cells as shown in Figure 7.8 above.

It was observed that, besides T cells, NK cells were also impaired by the pro-longed *Blastocystis* sp. inductions. This was due to high elevations of TIGIT expressions in long-term PBMCs induction of symptomatic and asymptomatic antigens targeted non CD4+/CD8+ cells. Generally, TIGIT is widely expressed on NK cells besides T cells (Catakovic et al., 2017 and Yin et al., 2018). Furthermore, high increase of CD226 molecule observed in PBMCs induced with symptomatic and asymptomatic antigens from 3 to 6 days of inductions as shown in Table 7.4 and 7.5. The CD226 molecule is usually expressed on NK cells besides monocytes and T cells as described in the introduction part 7.1.7 Therefore, the TIGIT molecule on non CD4+/CD8+ cells

suggesting to be expressed by NK cell populations. However, needs further investigation by targeting its specific cell surface marker.

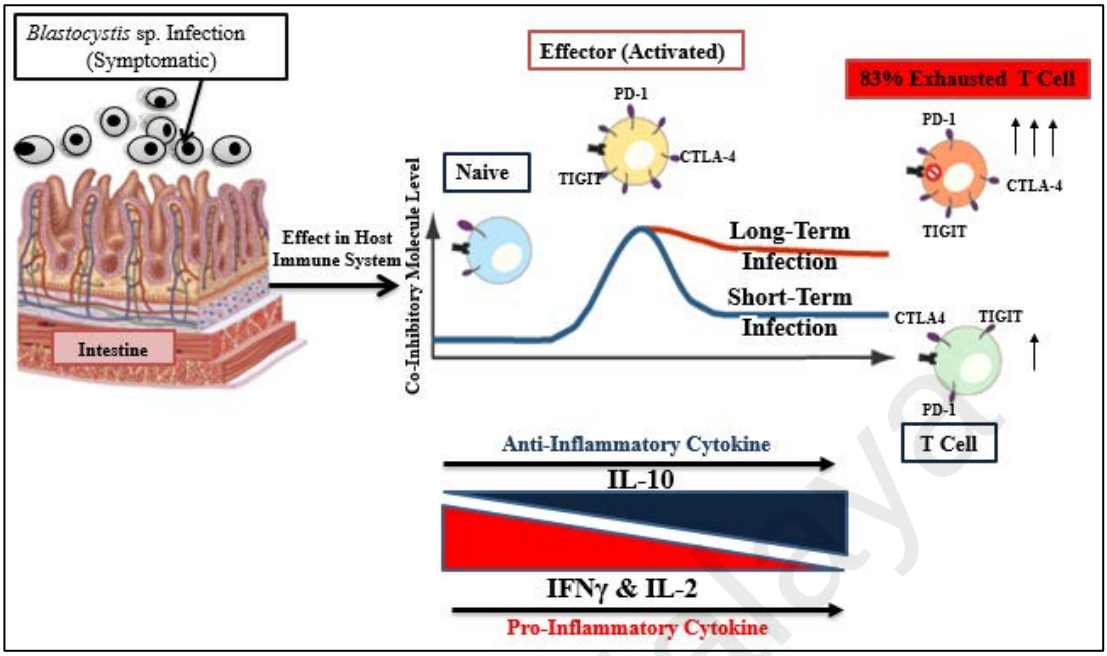
7.4.5 T cell Exhaustion Analysis

Generally, the characteristics of impaired or exhausted T cells were assessed based on several factors such as prolong antigen exposure, increased expressions of multiple inhibitory receptors, loss of effector cells cytokine (IL-2 and IFNγ) secretions along with T cell proliferation assessment (Catakovic et al., 2017; Grywalska et al., 2018; Martinez and Moon, 2019). It was evident through this study that *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates have caused T cell exhaustion during pro-longed antigenic stimulations. Generally, T cell exhaustion happens gradually along with antigen persistence or chronic infection and particularly targeting CD4+ and CD8+ T cells populations as demonstrated in this study. The T cell exhaustion which involves CD4+ and CD8+ populations has been widely demonstrated in chronic parasitic infections such as *Leishmania* spp., *Trypanosoma cruzi*, *Plasmodium* spp., and *Toxoplasma gondii* (Rodrigues et al., 2014). However, these parasites are classified under intracellular parasites where they infect the cells directly unlike *Blastocystis* sp. which may damage the intestinal layers or tissue through its endocytosis secretions.

Generally, *Blastocystis* sp. has been associated with many debilitating chronic intestinal and auto-immune diseases such as colorectal cancer, IBD, IBS which causes extreme inflammation in the intestinal region of the infected host (Cekin et al., 2012; Vinoth et al., 2013; Ragavan et al., 2015). Past study has shown that colorectal cancer cell proliferations were found to be highly triggered by *Blastocystis* sp. ST3 (Vinoth et al., 2013). The establishment of chronic inflammation in cancer such colorectal cancer has been proven to be associated with the expressions of immune check point receptors such as PD-1, TIGIT and CTLA-4 (Kim and Kim, 2018; Shi et al., 2018; Seidel et al.,

2018). These immune check points are highly expressed by colorectal cancer cells which causes immune cell inhibition. This is one of the strategies used by cancer cells to dominate and survive in the host. Therefore, these propositions become more evident through this experiment where *Blastocystis* sp. is proven to be part of the triggering factor in expressing immune co-inhibitory molecules such as PD-1, TIGIT and CTLA-4 in T cells.

Scenario 1-ST3 *Blastocystis* sp. Symptomatic Infection



Scenario 2-ST3 *Blastocystis* sp. Asymptomatic Infection

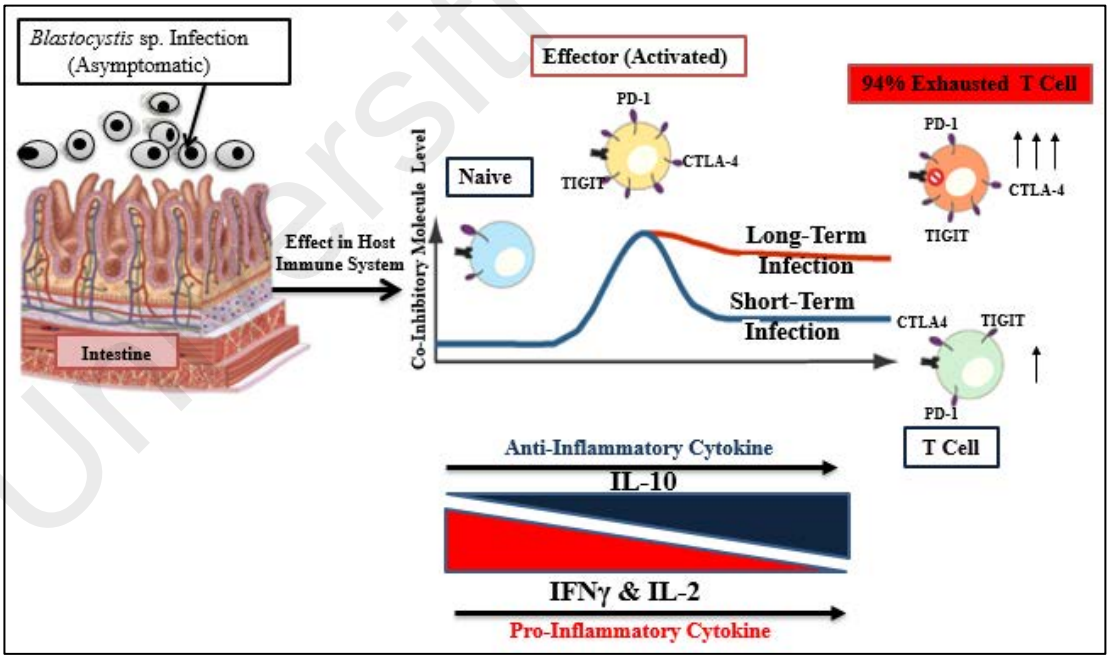


Figure 7.26: Illustrations of T cell Exhaustion During Short and Long-Term *Blastocystis* sp. ST3 Infection

Scenario 1: During symptomatic long-term infection 83% of T cell exhaustion occurred

Scenario 2: During asymptomatic long-term infection 94% of T cell exhaustion occurred

7.5 Conclusion

Thus far, there is no information on whether *Blastocystis* sp. ST3 causes T cell dysfunction during its pro-longed infection in host. This study will be the first to open the Pandora box to elucidate the engagement between T cells and *Blastocystis* sp. ST3 by proving that antigens of symptomatic and asymptomatic isolates of this parasite are able to cause high T cell exhaustion at 83% and 94% respectively as shown in Figure 7.26 above. In a nutshell, this study has achieved a further milestone to prove, ST3 *Blastocystis* sp. symptomatic and asymptomatic isolates has the ability to impair T cell function. This result has created a strong implication on its ability to cause further deterioration of its associated diseases besides proving its pathogenicity and may help to develop novel immunotherapies to reverse the state of exhaustion caused by *Blastocystis* sp. ST3 infection. However, further investigations are needed.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSION

The prevalence of *Blastocystis* sp. ST3 infection remains high worldwide, particularly in developing countries such as Malaysia. The characteristics of this parasite which can remain silent without causing its usual associated symptoms to the infected individuals have raised concerns on public health problems. Furthermore, the pathogenicity of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates is still at a state of limbo. Therefore, the current study was aimed to garner further evidence attest to their virulence. This dissertation was continued based on the previous findings which speculated that pathogenicity of *Blastocystis* sp. ST3 was due to the surface structure differences between the symptomatic and asymptomatic isolates. Generally, cell surface phenotype or antigenic variation is one of the strategies used by various types of intestinal protozoan parasites to evade the host immune system which can result in long term colonisation. This is due to fact that antigenic variations, such as the surface structural variances are able to exploit the immune cells recognition against an antigen and allows the pathogen to persist and infect the previously infected (pre-immune) host.

To date, studies to compare the differences between the host immune responses induced by *Blastocystis* sp. ST3 symptomatic solubilised antigen and asymptomatic solubilised antigen are still scanty. Therefore, the present research was aimed to evaluate the immunopathogenesis of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates by assessing the adaptive immune response characterisation and immune cell modulation induced by this parasite. Therefore, the adaptive immune response characterization was performed by the analysis of host immune response as discussed in Chapter 3. A determined concentration of symptomatic and asymptomatic solublilised antigens was used to evaluate the antigenicity and immunogenicity of these isolates by assessing the IgG antibody and T helper cell responses.

As discussed previously in Chapter 3, the term antigenic is defined as the ability of the immune cells such as T/B lymphocyte cells in responding against an antigen. Whereby, immunogenic is termed as the ability of T/B lymphocyte cells (particularly memory T/B cells) in responding against the previously sensitised antigens. Therefore, the assessment of antigenicity and immunogenicity of the symptomatic and asymptomatic antigens was performed by obtaining the spleen and sera obtained from the immunised Balb/c mice. Generally, spleen contains an abundance of mainly T and B lymphocyte cells. The characterizations of T cells targeting T helper 1 (IFNγ and IL-2) and T helper 2 (IL-4 and IL-10) cytokine analysis has shown a divergent host immune response against symptomatic and asymptomatic antigens. This was due to the skewing of symptomatic isolates towards Th1 response and asymptomatic towards Th2 response. This result was supported by the assessment of IgG1 and IgG2a antibody responses which has been widely used as an identification marker for Th2 and Th1 response respectively.

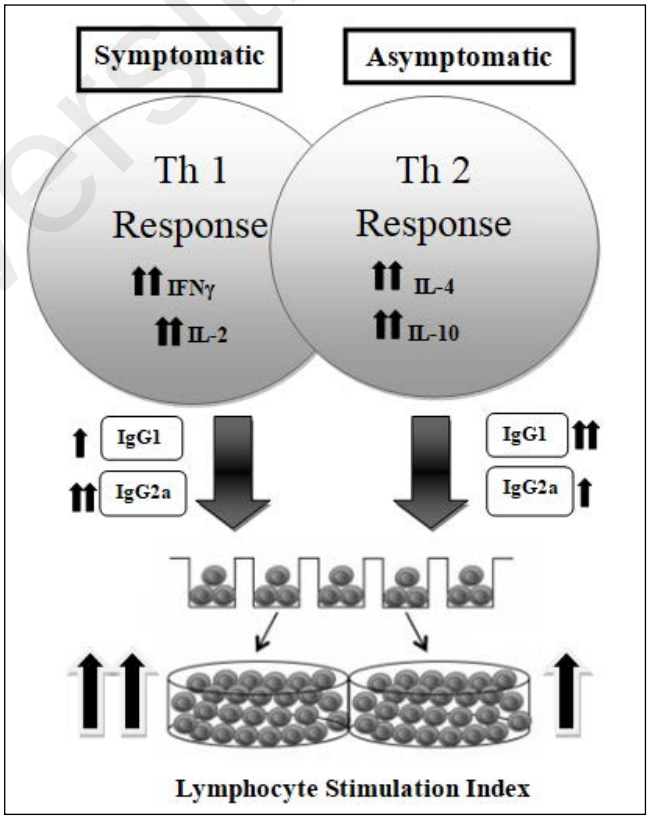


Figure 8.1: Summary Results from Chapter 3

In summary, the results described in Chapter 3 have proven again that, it is possible to hypothesise that surface variations between asymptomatic and symptomatic antigens may have contributed to its distinctive adaptive immune responses as shown in Figure 8.1. Furthermore, symptomatic antigens have been proven to be highly antigenic and immunogenic compared to asymptomatic antigens due to higher Th1 immune response establishment. Therefore, for the first time it was demonstrated that, symptomatic isolates exhibited higher immunopathogenicity compared to asymptomatic antigen. However, to confirm the degree of antigen divergence among symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates, this study was substantiated by the observation of complement mediated cytotoxicity (CDC) assay which were discussed in the following Chapter 4.

Specificity defines the ability of an immune system to differentiate between various antigens whereas cross-reactivity evaluates the degree of various antigens recognition as similar by the immune system. The surface molecule plays a major role in dictating the specificity and cross-reactivity across all antigens by segregating the populations based on its unique variations. The identification of the degree of polyclonal antibodies specificity and efficacy were evaluated by co-culturing sera of mice containing antibodies with respective live *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates. In contrast, sera and *Blastocystis* sp. symptomatic and asymptomatic live cells were co-cultured to measure the percentage of cross-reactivity these isolates. The sera and cells were interacted at dose dependent manner following 10-fold sera dilutions (10^1, 10^2,10^3 and 10^4). The specificity of 82% cell lysis by the symptomatic group and 86% cell lysis by asymptomatic group was demonstrated at 1:10 sera dilution. Besides, cross-reactivity among symptomatic and asymptomatic group at 1:10 sera dilution resulted in 17% of cell lysis. This study has also proven that, there are almost 50% cross-reactivity was observed between *Blastocystis* sp. isolates origin from the same group (symptomatic and

asymptomatic) isolates which showed high epitopes (antigen site) similarities at 1:10 sera dilution as shown in Figure 8.2.

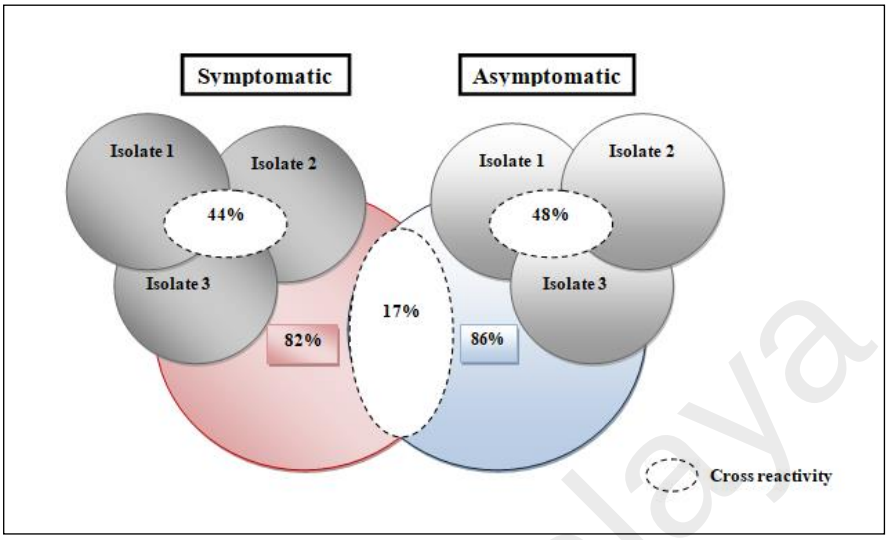


Figure 8.2: Summary Results from Chapter 4

In a nutshell it was demonstrated that, symptomatic and asymptomatic isolates mediated high specific lysis with almost half cross-creativity among its group. This study confirmed that, there are high epitopes disimilarities between *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates which may allow this parasite to set up diverse innate and adaptive immune modulations tactics in order to maintain its survival in the host. Therefore, further investigations were performed in the following Chapter 5 to explore on how these symptomatic and asymptomatic *Blastocystis* sp. ST3 isolates modulate the host innate immune system.

The connections between the host immune system and the invading pathogens start when a pathogenic microorganism infects the human body. This is followed by the activation of innate and adaptive immune response based on the virulence factors of the invading pathogens. The results from the battle between a pathogen and innate immune cells will determine the host recovery or pathogen survival at initial stage of its infection. Generally, during a pro-longed infection, many pathogens set up diverse innate immunity

evasion tactics in the host to achieve cell invasion and colonization. Further deteriorations of host immune system may cause fatality due to the failure in recognising the antigen. Therefore, further study was conducted to determine the correlation between *Blastocystis* sp. ST3 isolates and innate host immune response. Therefore, in Chapter 5, macrophages (the arm of innate immunity) response against *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates was investigated.

. In this study it was observed that, macrophages undergone apoptosis upon introduction with *Blastocystis* sp. ST3 symptomatic and asymptomatic antigens as early as 6 hours and deteriorates at 48 hours. Macrophages are the first cells to encounter invading pathogens such as *Blastocystis* sp. which cross the intestinal epithelium by damaging the intestinal barrier integrity. In the intestinal epithelium such as lamina propria region, the resident macrophages will be recruited to the inflamed area through the cytokine modulations where *Blastocystis* sp. should be present. In this study, it was observed that macrophages exacerbated high pro-inflammatory cytokine (IL-6 and TNFα) upon induction with symptomatic and asymptomatic antigens. However, there was no detection of anti-inflammatory, IL-10 cytokine response induced by these isolates which may further limit its activity. Besides, high elevations of nitric oxide secretions also present in the macrophages induced with symptomatic and asymptomatic antigens. Further analysis of macrophages phenotypic in the culture upon antigen induction has shown cell shrinkage and blebbing which has demonstrated the formation of apoptotic cells after *Blastocystis* sp. antigen induction. This was more evident through Annexin V assay which has shown high apoptosis induction in cells induced with *Blastocystis* sp. ST3 antigen and supported by the results of PD-1 expression (immune co-inhibitory molecules) which confirmed macrophage senescence as shown in Figure 8.3.

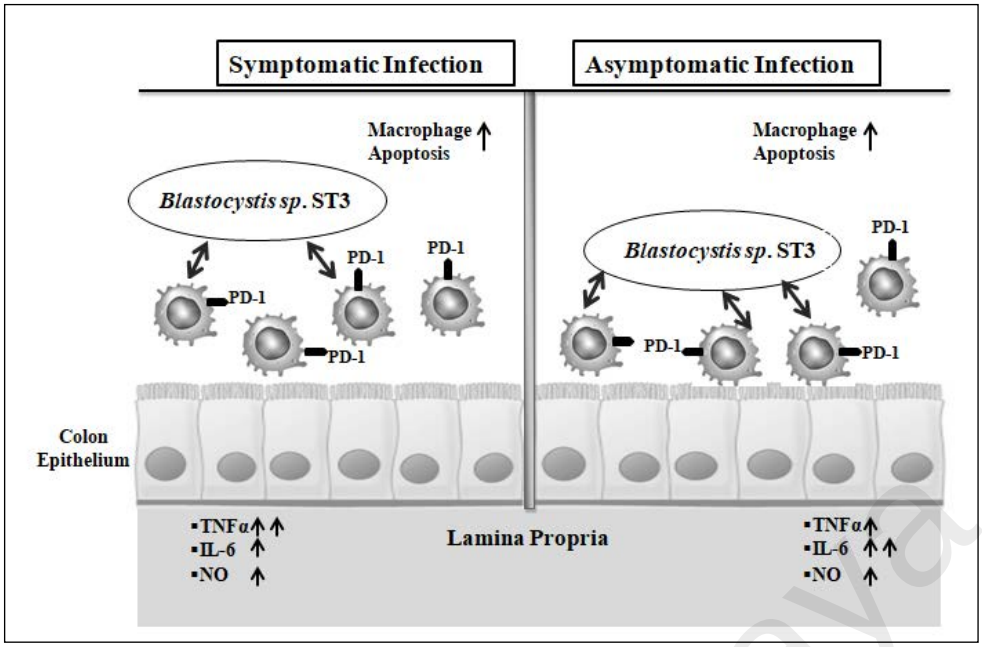


Figure 8.3: Summary Results from Chapter 5

However, it was again proven that symptomatic and asymptomatic isolates demonstrated different cytokine modulations in causing harmful effects to the macrophages. Higher exacerbation of pro-inflammatory cytokines was observed in macrophages induced with symptomatic antigens whereas asymptomatic antigens established as a "silent killer" by expressing higher anti-inflammatory cytokine secretion as the infection time progresses. Thus far, there has been no study which elucidated the effects of *Blastocystis* sp. ST3 in causing dysfunctional effects on macrophages. Therefore, this analysis has enlightened us with the fact that, *Blastocystis* sp. ST3 parasite could cause detrimental effects on macrophages at the earliest stage of its infection suggesting evasion of innate immune system in the host. Therefore, further analysis on the detrimental effects of *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates on innate immune response was observed by analysing monocytes immune response against this parasite which was investigated in Chapter 6.

The immune response of monocytes against *Blastocystis* sp. ST3 antigens were indicated as short- and long-term infection which may represent the acute and chronic infection (proposed by previous researcher through *in vitro* immune cells analysis). Therefore an *in vitro* monocytes model was formed and the cells were introduced with *Blastocystis* sp. ST3 antigens. This was followed by incubation for 3 and 6 days which represent a short- and long-term infection respectively. The short- and long-term induction of monocytes with *Blastocystis* sp. antigens has resulted in increase of cell proliferations compared to un-induced cells. However, pro-longed antigen exposure demonstrated the highest cell proliferation with an increase of 3.4 and 1.6-fold in monocytes induced with symptomatic and asymptomatic antigens respectively. Further evaluations on the cytokine release revealed that, pro-longed exacerbation of pro-inflammatory cytokines (IL-6, IL-12p70 and TNF-α) was observed in monocytes induced with symptomatic and asymptomatic antigens. However, asymptomatic isolates remained as "silent killer" through demonstration of higher anti-inflammatory cytokine (IL-10) release. This study has again proven the immunopathogenicity distinctiveness between symptomatic and asymptomatic on innate immune cells. In this analysis, symptomatic isolates were again confirmed as highly pathogenic due to higher exacerbation of pro-inflammatory cytokines in monocytes compared to asymptomatic antigens. However, there was an imbalance of pro- and anti-inflammatory cytokines and reduced pro-inflammatory cytokine response in monocytes during pro-longed symptomatic and asymptomatic antigen inductions which may implicate its impairment. The monocytes impairment became more evident through the analysis of immune co-inhibitory PD-1 molecule where high elevation of this molecule was observed during pro-longed symptomatic and asymptomatic antigens as shown in Figure 8.4 below.

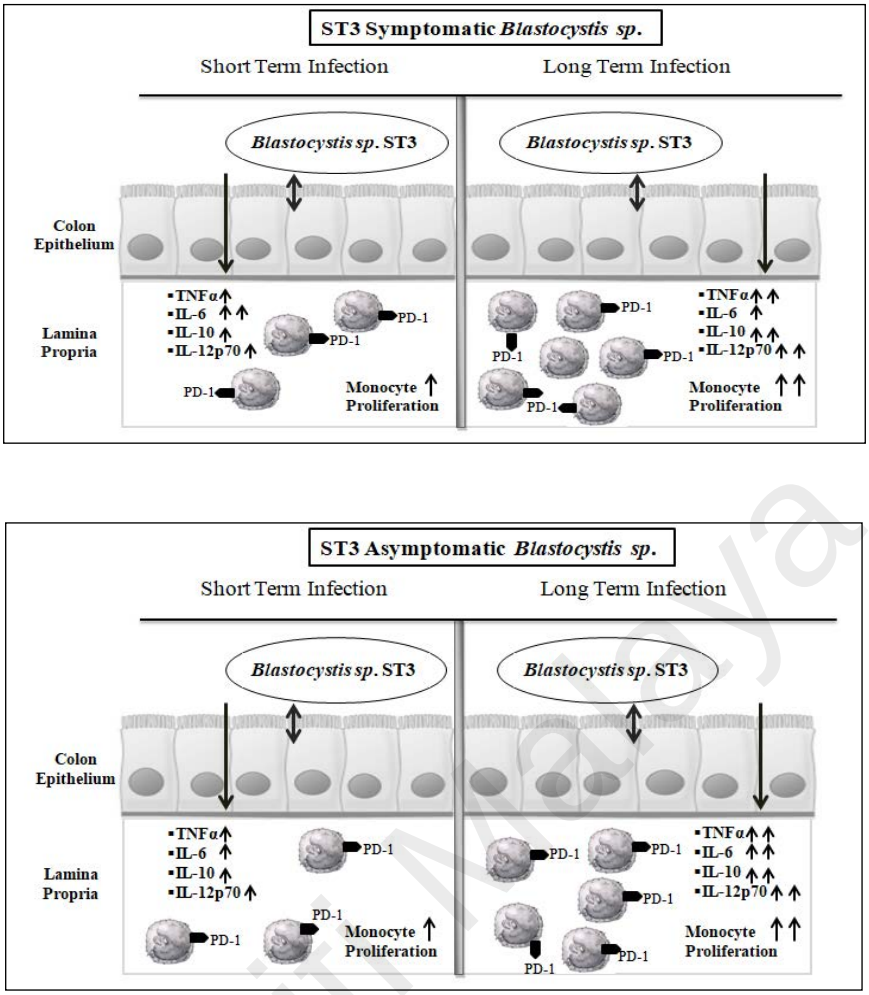
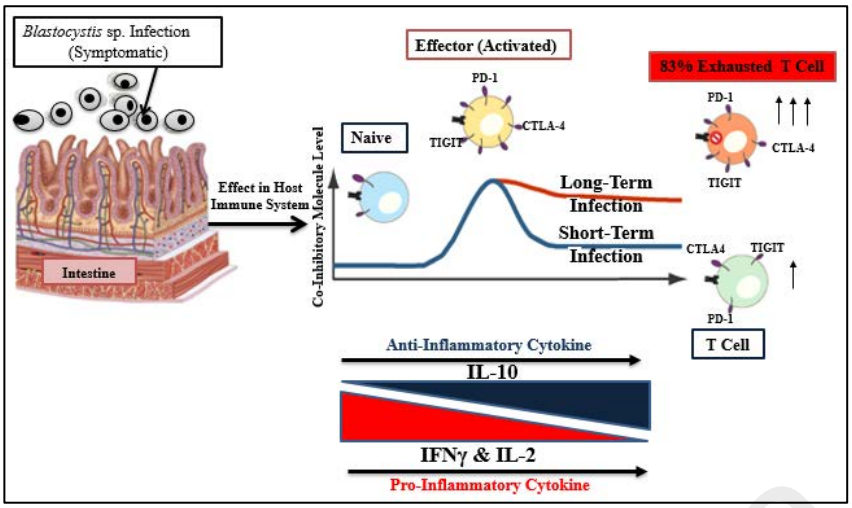


Figure 8.4: Summary Results from Chapter 6

The monocytes modulations were further assessed on their ability in connecting the adaptive arm which is T cell activation during *Blastocystis* sp. ST3 long term infection. The elevations of T cell co-stimulatory molecule (CD86) on monocytes confirmed the engagement of T cells during pro-longed *Blastocystis* sp. ST3 infection. In a nutshell this study has proven for the first time that *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates are able to impair the host innate immune mechanism and may modulate the T cell function (a key player of adaptive immune response). The following Chapter 7 uncovered the T cell immunomodulation caused by *Blastocystis* sp. ST3 antigens during its pro-longed infection.

The results in Chapter 7 highlighted the effects of *Blastocystis* sp. ST3 in causing T cell immune modulations (CD4+ and CD8+) during *Blastocystis* sp. ST3 infection on PBMC *in vitro* model. The results have revealed that, there was an increase of PBMC proliferation which includes T cell (CD3+) and monocytes (CD14+) populations increase from 3 to 6 days of induction with symptomatic and asymptomatic antigens. Further assessment of pro-inflammatory cytokine responses demonstrated high elevations of antigen presenting cells (monocytes/macrophages) induced pro-inflammatory cytokine responses (IL-6, IL-12p70 and TNF-α) during pro-longed antigen stimulation. However, reduced T cell pro-inflammatory cytokines (IL-2 and IFNγ) responses was observed during the pro-longed antigen exposure. There was an imbalance of pro- and anti-inflammatory cytokines observed with exacerbation of anti-inflammatory cytokine response in PBMCs during pro-longed symptomatic and asymptomatic antigen inductions. The reduction of pro-inflammatory cytokines particularly T cell induced cytokines (IL-2 and IFNγ) upon pro-longed antigen exposure implicated the T cell impairment. There was a significant increase of multiple T cell co-inhibitory molecules such as (PD-1, CTLA-4 and TIGIT) were observed in T cells (CD4+ and CD8+) induced with symptomatic and asymptomatic antigens which also suggesting T cell impairment. Generally, T cell impairment or also known as "exhaustion" will be assessed based on several factors such cell proliferation, loss of cytokine secretions (IL-2 and IFNγ) and increase of multiple co-inhibitory molecule expressions. Therefore, the results obtained from this study are correlating with the general term for T cell exhaustion which subsequently revealed the T cell impairment caused by *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates at 83% and 94% respectively as shown in Figure 8.5 below.



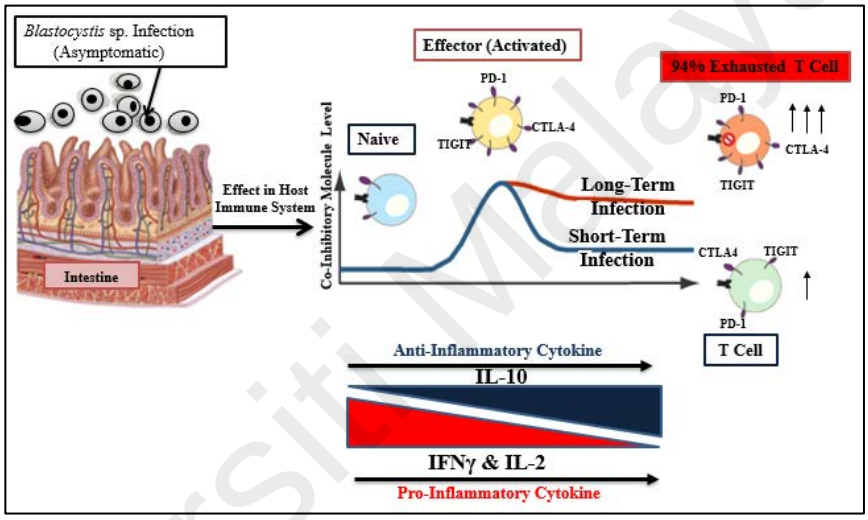


Figure 8.5: Summary Results from Chapter 7

Thus, this study was concluded with the findings of, symptomatic isolates exhibited higher pathogenicity (due to higher pro-inflammatory cytokines response) compared to asymptomatic isolates which remained as "silent killer" (due to higher anti-inflammatory response). In a nutshell, *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates have demonstrated the ability to impair T cell function. This result has created a strong implication on its ability to cause further deterioration on its associated bowel inflammatory diseases besides proving its pathogenicity and may help to develop novel immunotherapies to reverse the state of exhaustion caused by *Blastocystis* sp. ST3 infection which requires further investigations.

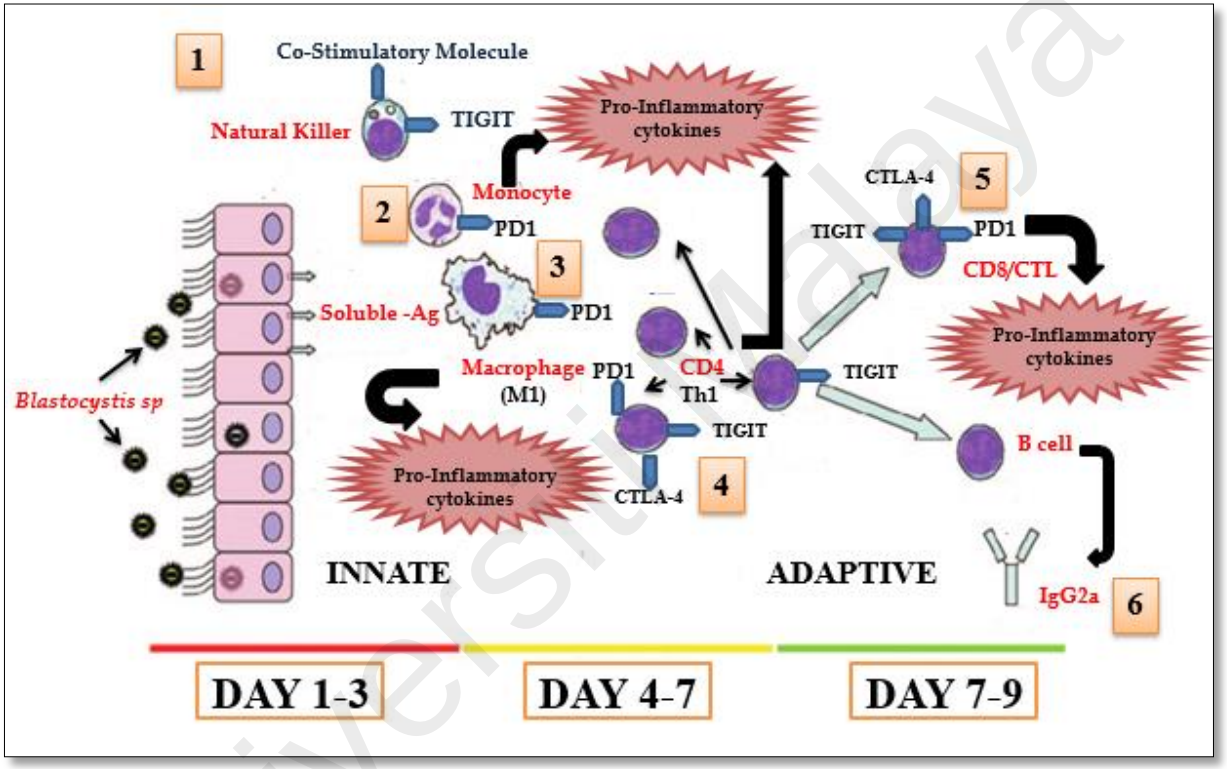


Figure 8.6: Immunopathogenesis Induced by *Blastocystis* sp. ST3 Symptomatic Isolates

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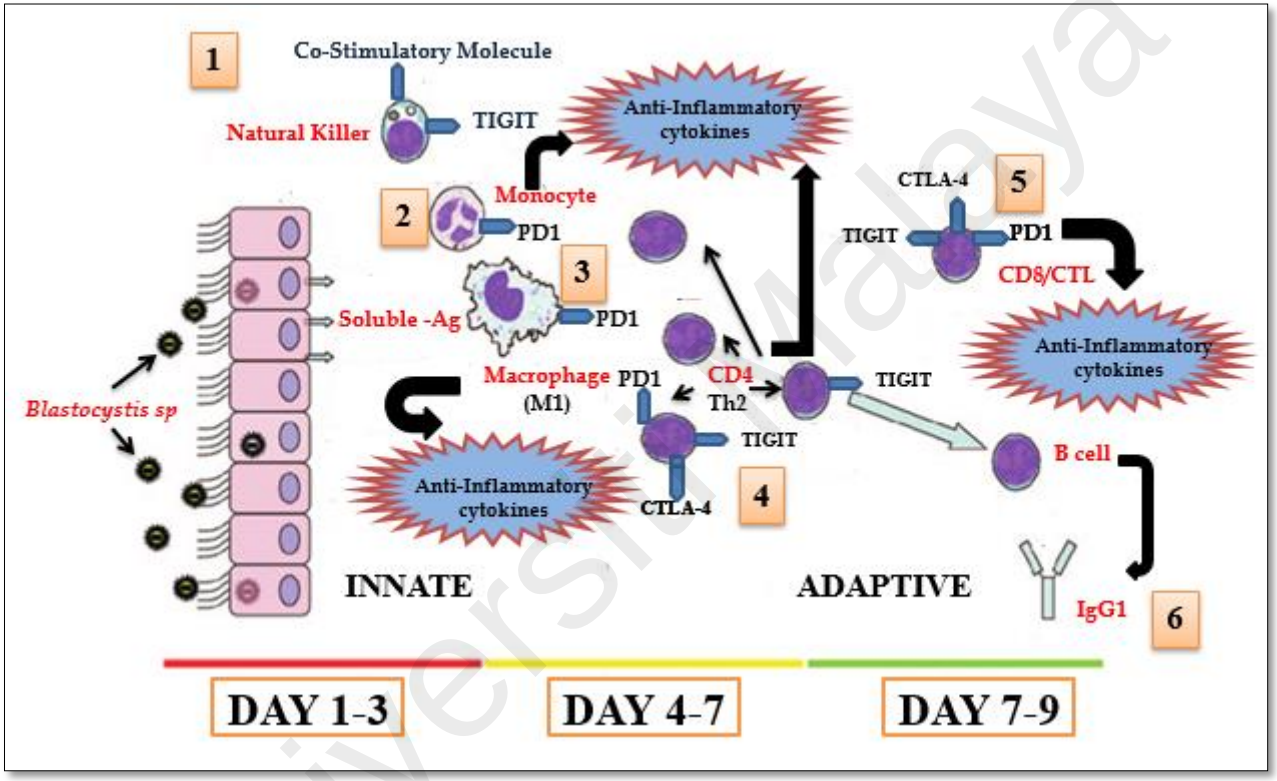

Figure 8.7: Immunopathogenesis Induced by *Blastocystis* sp. ST3 Asymptomatic Isolates

In conclusion, the present dissertation elucidated the host innate and adaptive immune mechanisms involved in *Blastocystis* sp. ST3 symptomatic and asymptomatic infections. It was demonstrated that, during a prolonged *Blastocystis* sp. ST3 infection, symptomatic isolates triggered higher pro-inflammatory cytokine secretions whereby asymptomatic isolates triggered higher anti-inflammatory cytokine response and shall recognise as a **"silent killer"** which can be more detrimental to the infected host. The summary of immunopathogenesis induced by *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates of this study are described in Figure 8.6 and 8.7 above. In a nutshell, it was successfully proven for the first time that these isolates are **divergent** and demonstrated a different pathogenic role in causing detrimental effects to the host innate and adaptive immune cells.

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LIST OF PUBLICATIONS AND SEMINARS

PUBLICATIONS

1. **Sheela Devi, S**., and Suresh, K., Immunogenic and Antigenic Heterogeneity of *Blastocystis* sp. Subytpe from Symptomatic and Asymptomatic Individuals. Tropical Biomedicine Journal, March, Vol. 36 (1) 2019. (**ISI-Indexed**)
2. **Sheela Devi, S**., Suresh, K., and Chandramathi, S. Can *Blastocystis* sp. ST3 evade the innate immune system? Evidence for Impairing Monocytes Function. Scientific Reports Journal. 2019. (**Submitted to ISI-Indexed**).

SEMINARS

1. **Sheela Devi, S**., and Suresh, K. (2017). Systemic Immune Responses Elevated in Mice Immunised against Symptomatic and Asymptomatic Isolates of Subtype 3 *Blastocystis* sp. International Conference on Clinical & Health Sciences, Faculty of Medicine, University Technology Mara (UiTM), 1st and 2nd November 2017, Kuala Lumpur, Malaysia. (Oral Presentation).
2. **Sheela Devi, S**., Suresh, K., and Chandramathi, S. (2019). Monocytes Response During Acute and Chronic Phases of ST3 *Blastocystis* Sp. Subtype 3 Infection. *MSPTM 2019 Annual Scientific Conference*, 13-14 March 2019, Kuala Lumpur, Malaysia. (Oral Presentation).
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1. **Sheela Devi, S**., Suresh, K., and Chandramathi, S. *Blastocystis* sp. Induced Macrophage Apoptosis. (2019).
2. **Sheela Devi, S**., Suresh, K., and Chandramathi S. Asymptomatic *Blastocystis* sp. Exacerbated High T Cell Exhaustion. (2019).
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FUTURE STUDIES

1. In this study Balb/c mice was used to study the host immune responses against *Blastocystis* sp. ST3 symptomatic and asymptomatic isolates. However, in future, FACS analysis targeting spleen derived lymphocytes cell characterization can be performed to identify the specific populations of T and B lymphocytes cells derived from host infected with *Blastocystis* sp. ST3 solubilised antigen.
2. In this study axenic *Blastocystis* sp. ST3 isolates was used. However, in future solubilised antigen protein characterization can be performed by using western blot or proteomics study to further identify the types of proteins present in the solubilised antigens.
3. In this study, PD-1 immune co-inhibitory marker was analysed on the antigen presenting cells (monocytes and macrophages). However, in future, PDL-1 and PDL-2 molecules can be assessed to further identify *Blastocystis* sp. ST3 immune modulation mechanisms.
4. Assessment of dendritic cell (another type of antigen presenting cells) modulation upon induction with *Blastocystis* sp. ST3 solubilised antigen.
5. Assessment of other T cell co-inhibitory molecules derived from patients infected with *Blastocystis* sp. ST3 and analysis of mait (mucosal associated invariant T cell) from the intestinal mucosa region.
6. IgG antibodies can be isolated from the infected mice/rat model and tag it with fluorescent conjugated anti-IgG antibodies. Similarly, the co-inhibitory molecules such as anti-PD1 molecules or anti-Th1 or Th2 cytokines also can be conjugated with fluorescent. These fluorescent conjugates can be injected to the infected mice/rat model to tract the *in vivo* molecular imaging of its intestinal region.