Detailed research report for International Society for Neurochemistry - the Committee for Aid and Education in Neurochemistry (ISN-CAEN)
(Category 1: Research A. visit by the applicant to another laboratory)

Name of the fellowship recipient: Jayaraman Tharmalingam.

Country: India.

Address: PhD student,

Neurochemistry Laboratory,

Department of Neurological Sciences,

Christian Medical College,

Vellore, Tamil Nadu, India.

Hosts: Prof. Josef Vercruysse & Prof. Pierre Dorny

Department of Virology, Parasitology and Immunology,

Faculty of Veterinary Medicine,

Ghent University, Belgium.

PhD guide: Dr. Anna Oommen,

Head, Neurochemistry Laboratory,

Christian Medical College, Vellore, India.

Duration: 21st March 2011 – 30th April 2011.

Title of the project:

 Characterization of host immunity that determines susceptibility to neurocysticercosis

Project supervisor and guide: Dr. Anna Oommen & Prof. Pierre Dorny.

Outcome of the project:

1. We observed there is an immune response against *Taenia solium* infection specific antigens in a *Taenia* naïve population.

2. A strong pro-inflammatory Th1 response and down regulation of Th2 anti-inflammatory cytokines to *Taenia* antigens characterized the *Taenia* naïve population.

3. The *Taenia* naïve population exhibited the ability to increase their effector memory T cells against *Taenia* on *Taenia* antigen stimulation.

4. Although the *Taenia* naïve population can protect them self against *Taenia solium* infection through their Th1 response, the lack of a Th2 response preventing development of antibody mediated protection indicates the population will develop severe disease if infected with *Taenia solium*.

Methods learnt:

Flow cytometric analysis of T cell markers in peripheral blood mononuclear cells.

Other academic activities during the short term project:

Discussed my PhD research work with faculty in the Department of Parasitology and Immunology, University of Ghent, Belgium

Attended the regular weekly discussions of the PhD students in the Parasitology laboratory.

Visited the laboratories in the Department of Veterinary Medicine, University of Ghent, Belgium.

Benefit earned through the ISN-CAEN funding:

1. The visit to the University of Ghent enabled me to obtain data for one arm of my PhD work: i.e immune response against *Taenia* infection in a Taenia naïve population.

2. Time spent in another laboratory that works on *Taenia* and host parasite immunology has increased my understanding of the field related to my PhD.

3. New scientific contacts in the area of my research.

Acknowledgments:

I would like to extend my gratitude to Prof. Josef Vercruysse for providing laboratory space to carry out my research work and Prof. Pierre Dorny for guiding me and arranging all facilities to fulfill my study at the University of Ghent. I thank Dr. Peter Geldhof for his guidance and supporting my entire study in Ghent. I acknowledge my friends in Ughent, Dr**.** Manuela Rinaldi for helping me in RNA extraction and Real Time PCR, Miss Rika Grit for teaching me FACS analysis, Dr. Bruno Levecke, Dr. Johannes Charlier and Mr.Belgacem Mihi for day to day help in the laboratory.

Picture: Mr. Jayaraman Tharmalingam working in Laboratory of Parasitology, University of Ghent

Description of work:

Taenia solium infections are endemic in many parts of the world since they are sustained by pork consumption, free-range pigs and poor sanitation. Infection with *Taenia* eggs that develops into cysts in the brain, neurocysticercosis (NCC), is the most common parasitic infection of the CNS and cause of recent onset seizures in adults in endemic countries (1). In India NCC underlies a third of all active epilepsy in the population of 2 to 60 years of age (2).

Despite the magnitude and clinical seriousness of NCC, the pathogenesis of the infection remains inadequately detailed. The number of infecting cysts, stage of the cyst and activation of the host inflammatory response, mainly determine the clinical course and severity of NCC (3). Inflammation induced by degenerating cysts underlies seizures seen in almost half of all NCC patients (4). NCC is a silent disease in 40% of patients in whom parasite survival, death, degeneration and calcification occurs without evoking an inflammatory response from the host (5). Host cellular mechanisms that underlie severity of infection and those that define the different stages of cyst development and degeneration are not known. This has contributed to the debates that surround best treatment protocols especially for infections of low cyst number.

Innate and acquired host immunity that govern parasitic infections function through T cell mediated pro and anti-inflammatory cytokines, memory and effector T cells and protective antibodies. Studies by Chavarría *et al*, (2003) show that NCC patients from regions endemic for *T solium* exhibit suppressed cellular immunity (6). They also found that asymptomatic NCC patients exhibited suppressed Th2 anti-inflammatory responses while symptomatic patients manifested suppressed mixed Th1/Th2 responses and elevated IgG, IgE, IgM. Ostrosky-Zeichner *et al* in 1996 (7) found an enhanced immune response was associated with increased severity of NCC, characterized by increased CSF cells, high levels of IgG antibodies and an anti-inflammatory Th2 profile of high levels of IL-10, IL-5, IL-6. Multi-cyst infections are considered to stimulate a stronger anti-inflammatory response (IL-5, IL-6) compared to single cyst infections. However in Indian NCC patients Thussu *et al* (1997) found the T cell response did not differ between patients with multiple or single cyst infections (8).

High numbers of activated T cells, especially CD8^{+} cells effector cells, have been noted during inflammatory phases of NCC compared to inactive phases of the infection (4). *In vitro* lymphoproliferation studies of cells from the inflammatory active phase (determined by imaging) of NCC

show a predominant Th1 response while those the inflammatory inactive phase of NCC exhibit a mixed Th1/Th2 response (4).

Broadly these studies indicate an anti-inflammatory cytokine response is associated with infection that is enhanced with severity of infection in humans, and the inflammatory phase of NCC is associated with a pro-inflammatory response. The role of central memory and effector cells that protect against infection remains largely unstudied in the pathogenesis of NCC.

Studies in mice indicate that susceptibility to *Taenia* infection is accompanied by an anti-inflammatory Th2 response that permits the infection to establish while a strong pro-inflammatory Th1 response is associated with elimination or reduced *Taenia* infections (9). Animal studies also indicate a role for protective antibodies in preventing disease and vaccines that stimulate production of protective antibodies have been developed that reduce and prevent cysticercosis in pigs (10).

We would like to understand the role of host immunity in the pathogenesis of NCC, specifically of resistance and susceptibility to infection, and thus severity of infection. This would require the study of three populations. 1) A population not exposed to *Taenia* (naïve), 2) A population living in an endemic region but not infected, 3) Neurocysticercosis patients from the endemic region.

The *Taenia* naive population because of no prior exposure to *Taenia solium* would be characterized by:

a) A Th1 pro-inflammatory response.
b) Normal naive T cells that expand on stimulation with *Taenia* antigens.
c) Normal central memory and effector cell populations that expand on stimulation with *Taenia* antigens.
d) Low central memory effector cell populations that expand on stimulation with *Taenia* antigens.

In this report we present our study on host immunity of a healthy *Taenia* naive population in response to *Taenia* antigen exposure.

Aim

To determine the immune response of a healthy *Taenia* naïve population against *Taenia solium* antigens.

Objective

Determine the immune response that develops in the *Taenia* naïve population against *Taenia solium* antigens, by measuring pro/anti-inflammatory cytokines and T cell populations (naïve, central and effector memory cells), in peripheral blood mononuclear cells cultured with *Taenia solium* antigens for 8, 24, 72 hours.

Cytokines determined from mRNA levels by real time PCR and T cell populations by FACS.

SUBJECTS

Twelve healthy normal Belgians, who had no prior exposure to *T. solium* or who had not travelled to an endemic country in the previous two years, provided 10ml blood with consent.

Chemicals

Histopaque 1077, PHA-P, Chloroform RNase free, Isopropanol were from Sigma Chemical Co, USA. Phosphate Buffered Saline, RPMI-1640, Fetal Bovine Serum, Trizol, RNase free water were from Invitrogen, USA. iScript cDNA synthesis kit from Bio-Rad, USA, Fast SYBR Green master mix from Applied Biosystems, USA and primers from Sigma Genosys, India. Fluorescent-tagged mouse IgG antibodies to human T cell surface markers CD4+ CD62L, CCR7, CD45RA and mouse IgG1 were from BD Biosciences, USA.

Lentil lectin specific *Taenia solium* glycoproteins were purified as given by Prabhakaran et al (11).

METHODS

PBMC isolation and culture

PBMCs from 10ml heparinised blood were isolated over Histopaque 1077 as per manufacturer's instructions, washed with RPMI 1640 media and re-suspended in RPMI media containing 10% FCS to 2 million cells / ml .

Two million PBMCs /well, stimulated with 10 µg/ml lentil lectin specific *T. solium* antigens, were set up in 24 well plates and cultured under standard conditions for 8, 24 and 72 hours. Cells stimulated with PHA (10 µg/ml) served as stimulation controls.

Culture supernatants were stored at -20°C for further tests and cells studied for INF-γ and IL-4 gene expression and T cell markers.

RNA extraction and cDNA conversion

RNA was extracted from cells with Trizol as per standard protocol and 200ng converted to cDNA with the BioRad iScript cDNA synthesis kit.

Real time PCR amplification of interferon-γ, IL-4, β-actin genes

Amplification was carried out in a 20µl reaction mixture of 10µl Fast SYBR Green master mix from Applied Biosystems, 1µl primer, 2µl cDNA (diluted 10X in RNase free water), 7µl RNase free water following the steps : AmpliTaq® Fast DNA Polymerase activation for 20 sec at 95°C, denaturation for 3 sec at 95°C, annealing and extension for 30 sec at 60°C. Denaturation, annealing and extension were carried through 40 cycles to complete the reactions.

Primer sequences for interferon-γ (INF-γ), IL-4 and β-actin genes were from the Real Time primer Database Bank.

Genes amplified and primers used

Gene	Description	Primers
β-actin	House keeping gene	Forward: 5'-TCCCTGGAGAAGAGCTACG-3' Reverse: 5'-TAGTTTCGTGGATGCCACA-3'
γ-interferon	Pro-inflammatory Th1 cytokine	Forward: 5'-TCGGTAACTGACTTGAATGTCCA-3' Reverse: 5'-TCCTTTTTCGCTTCCCTGTTTT-3'
IL-4	Anti-inflammatory Th2 cytokine	Forward: 5'-CGAGTTGACCGTAACAGACAT-3' Reverse: 5'-CGTCTTTAGCCTTTCCAAGAAG-3'

Flow cytometry determination of naive, central memory and effector T cells.

T cells of 5 subjects were analyzed by FACS. Half a million cells from stimulated and unstimulated 24 hour cultures were plated in V bottomed microplates, washed twice with PBS/1% FCS and incubated on

ice for 20 minutes in the dark with 50µl appropriately diluted fluorescent-tagged antibodies to T cell markers : CD4+, CD62L, CCR7, CD45RA. The reaction was stopped by dilution of cells with 250µl PBS, cells washed with PBS, transferred to FACS tubes and gated and counted for live/dead cells (with Sytox stain) and different T cell populations in a BD FACSAria flow cytometer.

T cell markers: CD4+ - marker for T helper cells: CD62L – marker for naïve cells directed toward lymphoid organs; CD45RA – marker for central memory T cells; CCR7 – marker for effector memory T cells.

Statistical analysis

INF-γ and IL-4 gene expression at different times of culture were analyzed for differences by repeated measure ANOVA with post hoc Bonferroni multiple comparisons.

Th1 and Th2 responses were analyzed for difference by the Mann Whitney U test.

Expansions of T cell populations on *Taenia* antigen stimulation were analyzed between 24 hour stimulated and unstimulated cultures by the Kruskal-Wallis test (FACS data).

p<0.05 was considered significant. Statistical analysis was with Graphpad Prism version 5.04 software.

RESULTS

PBMC INF-γ (Th1) and IL-4 (Th2) cytokine gene expression of a healthy *Taenia* naive population in response to *Taenia* antigen stimulation.

The changes in INF-γ and IL-4 gene expression in PBMCs stimulated with *Taenia* antigens over 72 hours of all 12 subjects are given in Table 1.

INF-γ gene expression was significantly upregulated and IL-4 gene expression significantly down regulated in PBMCs of the *Taenia* naive population stimulated with infection specific *T solium* antigens over 8 to 72 hours (p<0.05) (Table 1 and Fig 1).

INF-γ expression was significantly higher in cells stimulated with antigens for 72 hours compared to 8 hours (p<0.05) (Table 1).

IL-4 expression was significantly suppressed in cells stimulated with antigens for 24 and 72 hours compared to 8 hours of stimulation (p<0.05) (Table 1).

INF-γ expression was significantly higher than IL-4 expression in the cells at all times upto 72 hours of antigen stimulation (p<0.05) (Table 1 and Fig 1).

<u>T helper, central memory and effector T cells of a *Taenia* naive population and in response to *Taenia* antigen exposure</u>

T helper, central memory and effector T cell populations and their expansion in response to *Taenia* antigens of 5 *Taenia* naïve subjects are given in Table 2. A representative T cell FACS analysis of one subject is shown in Figure 2.

PBMCs of the *Taenia* naive population comprised of 18% CD4 T cells, 16.6% naive T cells and equal populations of central memory and effector T cells (`10%). The population exhibited low central memory effector T cell populations (6.5%). All cell populations expanded on stimulation with *Taenia* antigens for 24 hours with greatest expansion of effector cells (3.68%) and naive T cells directed to lymphoid organs (Table 2).

INFERENCE

A strong pro-inflammatory Th1 and suppressed anti-inflammatory Th2 response was elicited from PBMCs stimulated with *Taenia* antigens in the *Taenia* naive population. This is in support of protective responses to *T solium* infection in this population. However the strong anti-inflammatory suppression on long term culture (72 hours) implies susceptibility of the *Taenia* naive population to infection on chronic exposure. This is because constant down regulation of the anti-inflammatory response does not support development of antibody-mediated immunity and absence of protective antibodies is associated with establishment of severe infection.

Normal T helper, memory and effector cell populations noted in the *Taenia* naive population reflect an immune system that can mount a response against infection. Expansion of T cells in response to *Taenia* antigen stimulation for 24 hours indicates their ability to respond to the antigens. Since the greatest expansion was noted with effector cells, it is presumed the *Taenia* naive population would clear an initial *T. solium* infection. The increase of CD62L T cells on *Taenia* antigen stimulation reflect the characteristic to home to secondary lymphoid organs where the cells proliferate and differentiate, again indicating the ability of the *Taenia* naive population to respond to *Taenia* antigens.

This study provides baseline host immune data of a *Taenia* naive population to *Taenia* antigen stimulation for a larger study on host immunity contributing to the pathogenesis of NCC.

Table 1: Change in γ-interferon and IL-4 gene expression of *Taenia* naïve PBMCs stimulated with *Taenia* antigens.

| | TH1 RESPONSE | | | | TH2 RESPONSE | | |
| | INF-γ gene Fold change from 0hr on stimulation with *Taenia* antigens for | | | | IL-4 gene Fold change from 0hr on stimulation with *Taenia* antigens for | | |
Subject	8hr*	24hr	72hr*		8hr*#	24hr*	72hr#
1	1.02	0.74	3.65		1.97	0.34	0.78
2	0.68	0.44	0.92		0.3	0.11	0.11
3	2.09	6.58	9.12		0.24	0.42	0.10
4	4.05	4.0	8.51		1.54	1.16	0.95
5	9.31	6.06	23.26		1.97	1.8	0.72
6	2.42	4.65	4.78		0.23	0.14	0.02
7	4.05	1.74	0.95		2.02	0.52	0.01
8	1.12	1.54	4.46		0.96	0.60	0.44
9	1.77	3.09	8.16		1.85	0.52	0.26
10	1.93	8.87	29.85		0.95	0.59	0.36
11	1.76	2.53	11.04		0.83	0.21	0.12
12	1.95	9.71	4.14		0.77	1.05	1.24
MEAN ± SD	2.67±2.33	4.16±3.08	9.07±8.86		1.13±0.70	0.62±0.49	0.42±0.40
	*P< 0.05				#P< 0.05		

Fig 1: INF-γ (Th1) and IL-4 (Th2) response of *Taenia* naïve PBMCs stimulated with *Taenia* antigens.
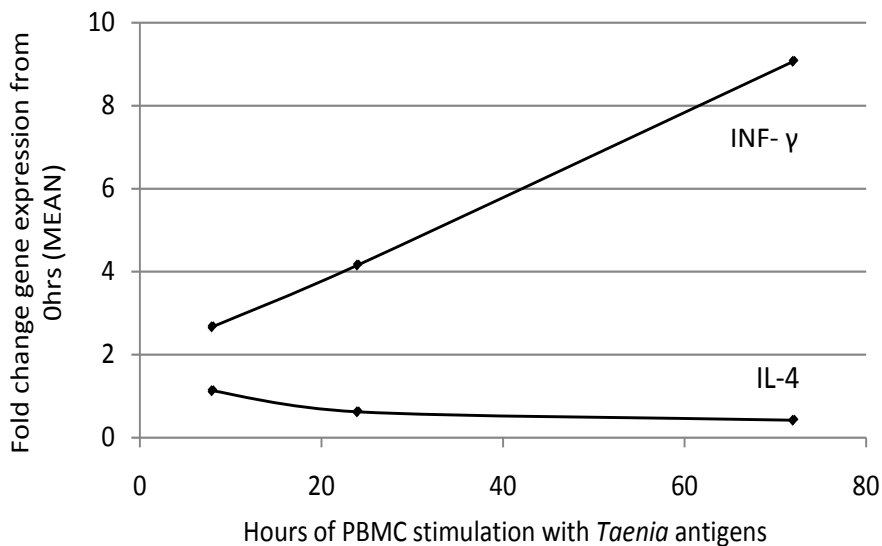
Table 2: T cell populations of the *Taenia* naïve population and their response to *Taenia* antigens.

T cell (N=5)	Unstimulated cell population (Mean)	Cell population on 24 hour stimulation (Mean)	Expansion of cell population on 24hr stimulation (Mean)
CD4	18.16%	20.08%	1.92%
CD4/CD62L	16.62%	19.76%	3.14%
CD45RA	10.88%	12.34%	1.46%
CD4/CCR7	9.38%	12.27%	3.68%
CD45RA/CCR7	6.5%	8.44%	1.94%

Fig 2 : FACS of T cells from a *Taenia* naïve subject
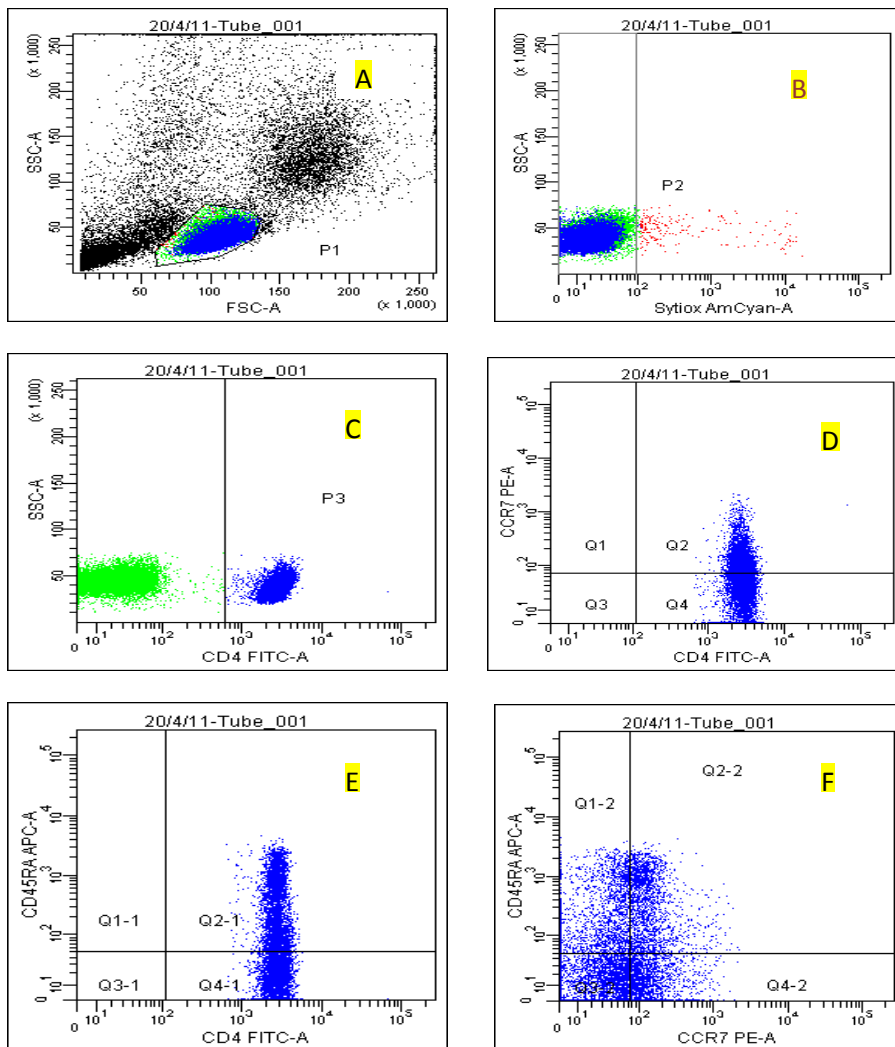
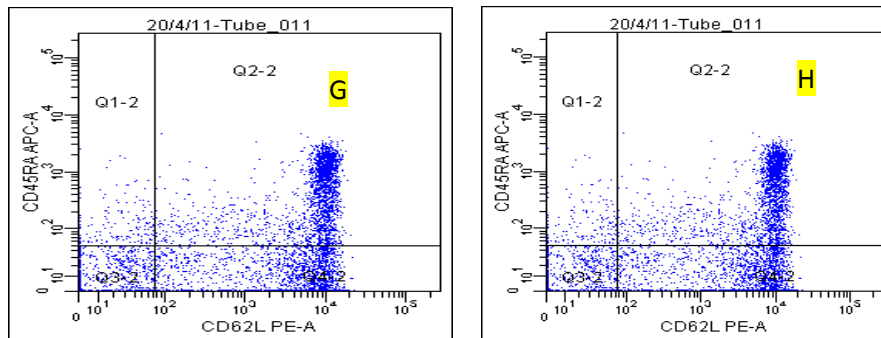**Fig 2 Legend**

A) Forward and side scatter of PBMC cells
B) Live and dead cells marked by Sytox dye (Live cells do not stain)
C) CD4 gating of entire lymphocyte population (P3)
D) CCR positive cells in CD4 cell population (Q2)
E) CD45RA positive cells in CD4 population (Q2-1)
F) CD45RA and CCR double positive cells (Q2-2)
G) CD62L positive in CD4 populations (Q2)
H) CD45RA and CD62L double positive cells(Q2-2)

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