To Study the Alteration in Thymic Functionality in Experimental Visceral Leishmaniasis

Project report submitted

For the award of

M.Sc. Life Sciences (Biochemistry)

By

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Under the Supervision of

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May, 2018

DECLARATION

I declare that the project entitled **"**TO STUDY THE ALTERATION IN THYMIC FUNCTIONALITY IN EXPERIMENTAL VISCERAL LEISHMANIASIS**"** has been prepared by me under the guidance of Dr. Manju Jain, Assistant Professor, Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of project has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Arjun Kumar Mehara has prepared his Project entitled “TO STUDY THE ALTERATION IN THYMIC FUNCTIONALITY IN EXPERIMENTAL VISCERAL LEISHMANIASIS”, for the award of M.Sc. Life Sciences with specialization in Biochemistry degree from Central University of Punjab, under my guidance. He carried out this work at the Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

“To study the alteration in thymic functionality in Experimental Visceral Leishmaniasis”

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Visceral Leishmaniasis (VL) is a neglected tropical disease and is potentially-fatal. Species belonging to *L.donovani* complex are the causative agent of VL in humans. Host immunity against VL critically depends on T cell based cell mediated immune response. VL is associated with lymphopenia such that progression of VL involves depletion of T cells. However, the origin and mechanism of T cell alterations in peripheral blood is not clearly understood. So we have tried to understand the origin of T cell changes in context of thymic functions which is the site of T cell development based on T cell Receptor Excision Circles analysis in experimental murine VL model. The result shows higher copy number of T cell Receptor Excision Circles (TRECs) in peripheral blood and a trend towards increase in developing thymocytes from thymi of infected mice compared to control uninfected group. The results imply that the peripheral T cell repertoires comprise a significant fraction of unexpanded naïve T cells. TREC change in thymocytes is statistically not significant implying no change in thymic output of naïve T cells. Thus our findings show that leishmania parasite can modulate T cell arm of immunity by altering the proliferation capacity of recent thymic naïve T cell emigrants.

Dr. Manju Jain Arjun Kumar Mehara

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Arjun Kumar Mehara

Date

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1.	Antigen Presenting Cells	APC
2.	Cutaneous Leishmaniasis	CL
3.	Cutaneous T cell Lymphoma	CTCL
4.	Deoxyribonucleic Acid	DNA
5.	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
6.	Mucocutaneous Leishmaniasis	MCL
7.	Polymerase Chain Reaction	PCR
8.	Quantitative Real Time PCR	qPCR
9.	Recent Thymic Emigrants	RTE
10.	T cell Receptors	TCR
11.	T-Cell Receptor Excision Circles	TREC
12.	Visceral Leishmaniasis	VL

CHAPTER 1

1. Introduction

Protozoa parasites play a significant role in the progression of several infectious diseases in mammals. Leishmaniasis is one of them that have caused a significant increase in morbidity and mortality of human life all around the world (Torres-Guerrero *et al*.,2017). Leishmaniasis is a major infectious disease just after malaria and is still not eliminated due to the lack of vaccines, effective chemotherapy and development of parasite resistant. It is a disease of poor and mostly prevalent in tropical and sub-tropical regions mostly in developing countries. Environmental factors, poor nutrition, and hygienic conditions contribute the spreading of this vector-borne disease (Oryan & Akbari, 2016). At present 89 countries and territories are affected by several forms of leishmaniasis (Torres-Guerrero *et al*., 2017; WHO, 2016; Oryan & Akbari, 2016).

There are more than 20 species of Leishmania parasites known which lead to different clinical manifestations and progression of disease in humans based on the host immune status and infecting species (Chappuis *et al*., 2007; Nylen & Sacks, 2007). Cutaneous leishmaniasis caused by the *L.tropica* complex result in skin ulcers. *L.braziliensis* complex causes mucocutaneous leishmaniasis such that lesions expand to the mucosal region (WHO, 2016). *Leishmania donovani* complex cause the visceral leishmaniasis also known as Kala-Azar characterized by the long-term low-grade fever, anemia, weight loss, splenomegaly, hepatomegaly etc.(Franceschini *et al*., 2016).

T cells are the backbone of cell-mediated immune response critical in leishmaniasis. They are the major players in deciding the disease outcome which seems to be tightly linked with disease progression in visceral leishmaniasis(Gupta *et al*.,2013). T cell deletion and polarization of CD4+ T cells towards Th1/Th2 helper immune response is seen in the infected humans and animals. The reason for T cell associated changes are not much worked out. Changes in lymphoid compartments of the thymus normal T cell development could be linked to alterations in thymic functionality resulting in peripheral T cell changes (Flavia Nardy *et al.*, 2015).

The alterations of thymic functionality can be tracked by the quantitative T cell Receptor Excision Circles (TRECs), which are the non-replicative, extra chromosomal DNA by-products of T-cell receptor rearrangement processes and are present in recent T cell emigrants from the thymus (Flavia Nardy *et al*., 2015). Thus TRECs analysis is used for evaluating thymic function in patients with T cell associated diseases or individuals receiving extreme chemicals, radiation therapy with suppressed immune system.

This experiment in addition to giving a general overview of peripheral T cell turn over can consolidate the possible alterations in thymic functionality in experimental murine visceral leishmaniasis compared to control group. The results can help in understanding the origin of T cell changes seen in uncured disease.

1.1. Knowledge gap

(a) In Visceral Leishmaniasis, studies are focused mainly on peripheral T cell alterations.

(b) The origin of peripheral T cell alterations are not much worked out.

(c) No single study addressing the role of thymus specific T cell developmental events has been done till date towards understanding peripheral changes.

1.2 Hypothesis

We hypothesize that by evaluating the change in TREC copy number in thymic T cells and peripheral blood cells, possible alterations in turnover of recent thymic emigrants along with proliferative capacity of peripheral naïve T cells can be gauged to understand the origin of peripheral T cell changes in VL.

1.3 Objectives

(a) Quantitative analysis of T Cell Receptor Excision Circles level in thymocytes.

(b) Quantitative analysis of T Cell Receptor Excision Circles levels in peripheral blood.

(c) To correlate thymocytes and blood TREC levels for understanding the origin of peripheral T cell changes in VL.

CHAPTER 2

2. Review of Literature

2.1 Leishmaniasis:

Leishmaniasis is a neglected tropical disease common in developing countries (WHO, 2017). It is a vector-borne disease complex caused by the obligate, intracellular protozoa parasites of the genus Leishmania of family Trypanosomatidae. The infection spread through the bite of an infected female Phlebotomine Sand-fly (of the genus Phlebotomus in Africa, Asia, and Europe and the genus Lutzomyia in the Americas). Most of the people infected by the parasite rarely develop any symptom at all in their life (Bhargava & Singh, 2012).The parasite has a digenetic life cycle with two hosts**;** sandfly and mammal for their proliferation, reproduction, and pathogenicity.

When the infected sandfly bites, it transfers the infectious flagellated promastigotes to and replicate as aflagellated amastigotes after phagocytized by macrophages in a mammalian host, soon the parasites start to multiply in the cells of various tissues lead to disease progression (Figure 1)(Stamperetal.,2011). Till now more than 20 species of *Leishmania* parasites are known and different groups contribute different clinical manifestations based on the infecting species and immune status of the host in humans (Georgiadou *et al*.,2015). According to WHO, leishmaniasis is one of the world's most important protozoa infectious diseases that cause a significant morbidity and mortality just after malaria, and its severity range from self-healing cutaneous leishmaniasis to fatal disseminated visceral leishmaniasis. Leishmaniasis belongs to the category of Neglected Tropical Disease (NTD), prevalent in tropical and subtropical regions as the geographical and environmental factors favor progression of leishmania parasites. Different parasitic species are divided in two main groups: In old World: *L.donovani complex, L.tropica, L. major and L. aethiopica* and in New World: *L.viannia complex, L.mexicana, and L.amazonensis* (WHO, 2017; Mauricio *et al*.,1999).The resolution of infection is dependent on the coordinated interactions between components of cell-mediated immune response, specifically the activation of targeted T-cell populations for appropriate cytokine production and activation of macrophages (Shahi *et al*.,2013).

In a murine model, it's been observed that the development of Th1 response is associated with the control of infection whereas the Th2 response is associated with disease progression. However, in human infection, Th1, and the Th2 dichotomy is not as distinct as in mice and the murine model does not strictly apply to human leishmaniasis (Sharma & Singh, 2009).

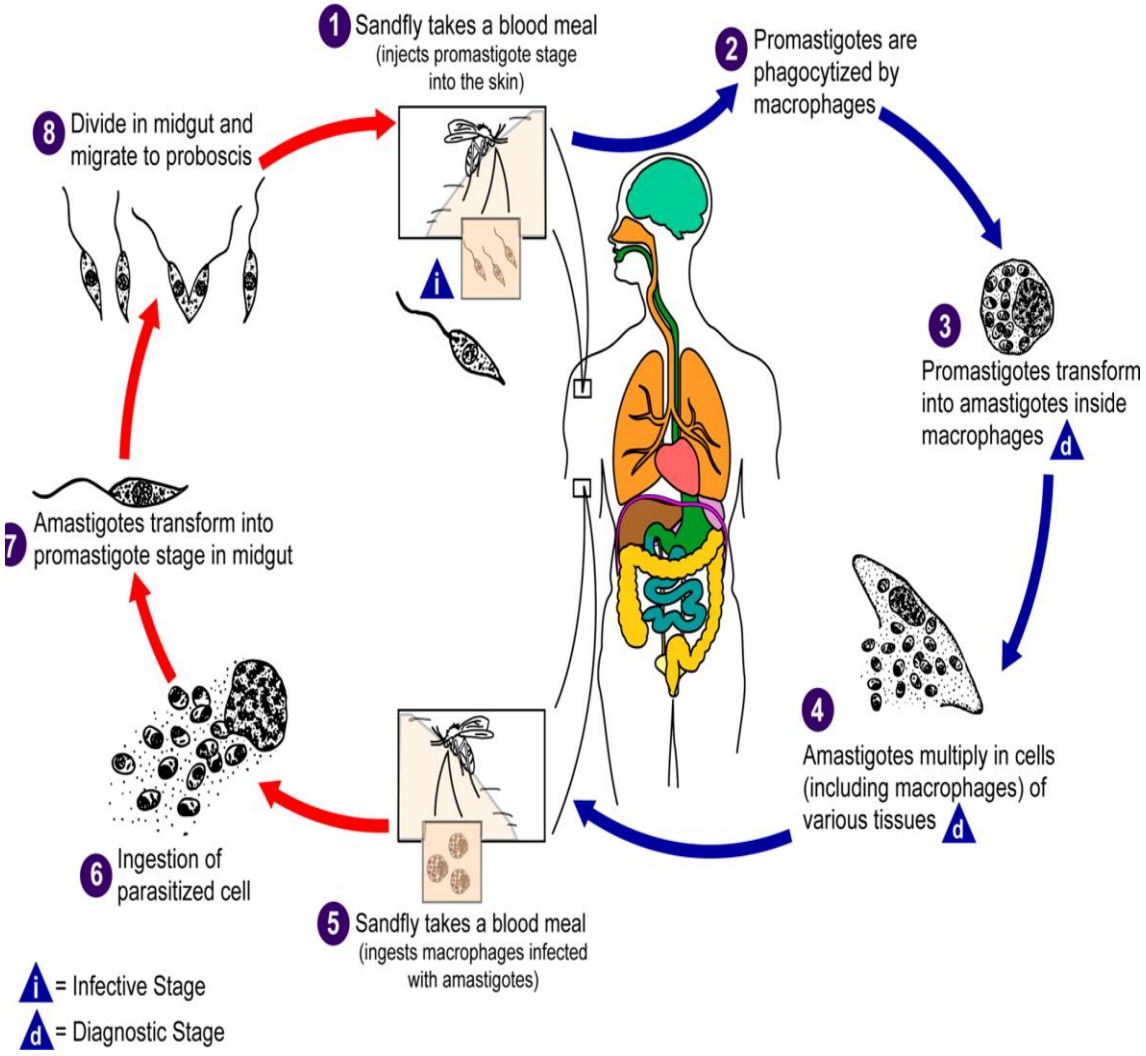


Figure 1: Life cycle of Leishmania parasites.

Source: Centre for Disease Control and Prevention,CDC

2.2 Epidemiology:

Leishmaniasis is endemic in 89 countries and approx. ~12 million people are infected (Table 1). The annual incidence is of around 2 million and is rapidly rising with secondary co-infections like Human Immunodeficiency Virus (HIV) (Torres-Guerrero *et al*., 2017; Roberts, 2005).

Parameter	**Statistics or Information**
Geographical location	Worldwide tropical and subtropical regions
Population at risk in 2016	~350 million
Number of people affected	~12 million
Number of deaths per year	~20,000 – 70,000
Number of new cases each year	~1.5 to 2 million
Global disease burden (DALYs)	~1.7 million
Multidrug-resistance	Resistance to antimonials only
Visceral Leishmaniasis (VL) .	~200 000 to 400 000 new cases of VL occur worldwide each year. Over 90% of new cases occur in seven countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, Kenya and Sudan
Cutaneous Leishmaniasis (CL)	~One-third of CL cases occur in the Americas, the Mediterranean basin, and the Middle East and Central Asia. An estimated 0.7 million to 1.3 million new cases occur worldwide annually
Mucocutaneous Leishmaniasis	Reported in Bolivia, Brazil and Peru.
Major risk factors	Socioeconomic conditions, Malnutrition, Environmental changes

Table1: Fact file: Worldwide leishmaniasis statistics for 2017 (Adapted from Torres-Guerrero *et al*., 2017; Leishmaniasis: a review; WHO factsheets, 2017).

2.3 Leishmaniasis is a complex disease with three main clinical manifestations:

(a) Cutaneous Leishmaniasis (CL): It is the most common and prevalent clinical form of the disease, usually characterized by ulcers on the exposed parts of the body, such as the face, arms, and legs. If not treated properly it may progress to advance form. The Leishmania species involved in cutaneous manifestations *are L.tropica, L.major, L.mexicana, and L.aethiopica* (Scorza *et al*., 2017).

(b) Mucocutaneous Leishmaniasis (MCL): MCL is usually the advanced form of CL, the lesions spread to the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. MCL is caused by 4 different species of Leishmania protozoa; *L. amazonensis, L. (Viannia) braziliensis, L. (V.) guyanensis,* and *L. (V.) panamensis*. MCL is characterized by skin and mucosal involvement with the formation of lesions that spread locally and severely mutilate the face, accompanied by fever, fatigue, and general illness (Scorza *et al*., 2017; da Silva *et al*., 2014).

(c) Visceral Leishmaniasis (Kala-Azar): is the most severe form of leishmaniasis caused by: *L. donovani.* The disease progress to all organs but particularly in the reticulo-endothelial system; aspirates of lymph node, bone marrow, and spleen. VL is characterized by fever, weakness, loss of weight, anemia, swelling of the liver and spleen, weight loss, fever and fatigue, lymphadenopathy , low pancytopenia etc. (Harizanov *et al*., 2013; WHO, 2017). People, who generally belong to economically weaker sections in the endemic regions, have a high risk of infection. But infected individuals who are immune compromised have severe signs and symptoms. Under such conditions visceral leishmaniasis can be fatal (Hirve *et al*., 2017). The VL treated patients and asymptomatic infected individuals may suffer post infection complications in the form of Post-Kala-Azar Dermal Leishmaniasis (PKDL); mainly distinguished by a nodular rash, macular, and maculopapular. During VL, peripheral blood mononuclear cells (PBMC) does not produce efficient interferon gamma but soon after treatment of VL, PBMC start reacting to Leishmania antigens and IFN gamma production, which coincides with the appearance of PKDL lesions and there is skin inflammation as a reaction to persisting parasites in the skin (Zijlstra *et al*., 2017).

2.4 Immunology of Visceral leishmaniasis: T cells are the major player in controlling the disease:

Immunity is predominantly mediated by T cells or T lymphocytes both in human and experimental leishmaniasis (Sacks and Noben-Trauth, 2002). T cells are the major player in controlling and eliminating the intracellular pathogens and act as effectors and regulators in cell-mediated immune responses. Initial studies, using nude BALB/c and T cell-depleted murine models have shown the importance of T cells for protection against *L. donovani* infection (Murray *et al*., 1987; Skov & Twohy, 1974).

Transfer of adoptive T cells resistant to leishmania antigen, conferred protection against *L. donovani* infection (Rezai *et al*.,1980). Both CD4+ and CD8+ T cells are seems to be necessary in protection against infection as shown by using euthymic mice (with cell depletion experiments) and nude BALB/c mice in reconstitution experiments with monoclonal anti-CD4 or anti-CD8 antibodies (Stern *et al*.,1988). T cells or T lymphocytes produce different cytokines in response to infection as in euthymic *L. donovani*-infected BALB/c mice (Figure 2). Initially they are able to control infection with granuloma formation and IFN- g and IL-2 production but with time the IL 4 and IL 10 cytokines levels increases and shifting to Th2 immune response (Nylen & Sacks, 2007).

2.5 Immune response in murine model

Although the experimental *L. donovani* infected murine model is not good for the study of immune responses but it gives some insights in understanding and progression of the disease. Based on extensive studies in mice, the development of Th1-type immunity in host is prerequisite for protection against leishmanial infection and control of the parasites(Figure 2) (Nylen & Sacks, 2007). In TH1-cell development following infection with leishmania parasite antigens trigger the antigen-presenting cells, through toll-like receptors (TLRs), and secrete cytokines (IL-12), promoting the differentiation of naive T cells into IFNγ secreting TH1 cells. The inability of antigen to activate dendritic cells to produce IL-12 lead to differentiation of naive T-cell into IL-4-secreting TH2 cells (Ezra N *et al*.2010).

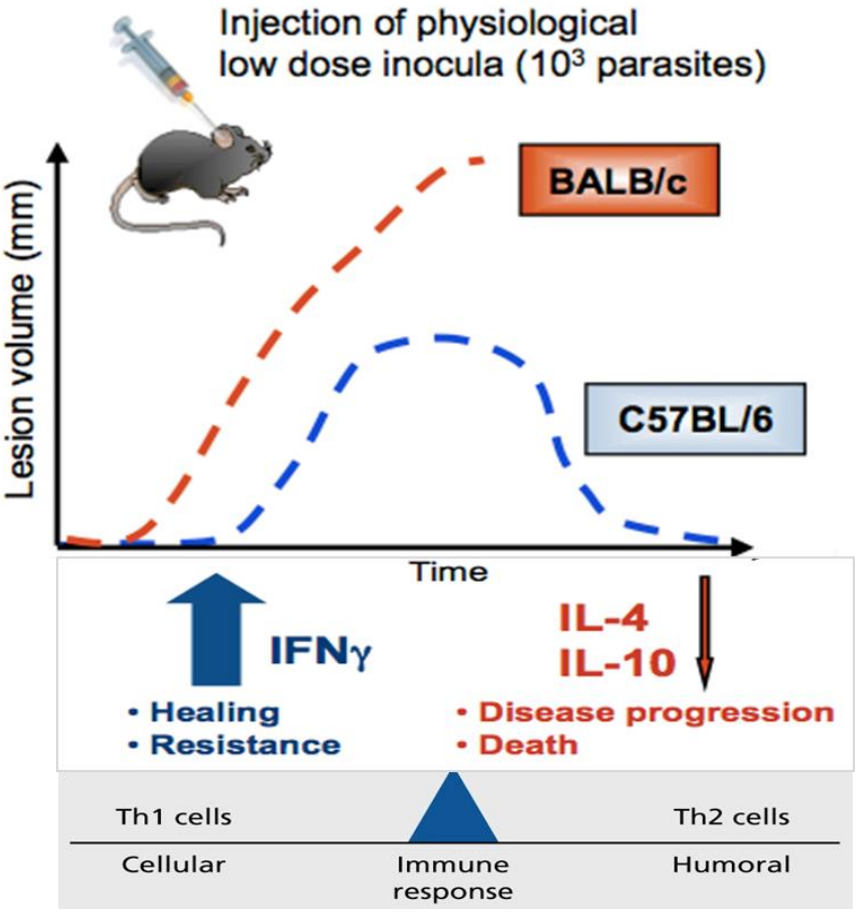


Figure 2: Th1 (Resistant) to Th2 (Suseptible) response in Experimental Visceral leishmaniasis in a BALB/c murine model.

Source: Centre for Disease control and Prevention,CDC

Although, most susceptible mouse strains (BALB/c) develop resistance against parasite growth at a later stage (H-2 loci; Blackwell, 1983). The leishmania parasites multiply rapidly in the liver following intravenous infection for the first weeks then its growth is controlled by granuloma formation and cell-mediated immune responses.

The parasites were eventually cleared from the liver and become resistant to reinfection over a period of 2-3 months (Murray *et al*., 1987).Similar to observations made in humans the immune response in the murine spleen is characterized by a mixed regulatory and inflammatory response. Both IL-10 and tumor necrosis factor (TNF) α production is elevated in the spleen. TNFα, which is critical for the development of protective immunity in the liver cause destruction of the marginal zone macrophages and stromal cells (Carrión*et*al, 2006; Stanley and Engwerda, 2007; Murray*et*al, 2000).

2.6 Immune response in human VL

In Human majority of individuals infected with VL by *L. donovani* never develops any disease, and remain asymptomatic suggesting that some genetic factors may be involved in resistance and susceptibility (Kumar & Nylen, 2012). The coordination between the environment, the parasite, and the host genetics factors determine whether a person develops fatal VL or remain asymptomatic, however the inducing factors to be developing VL is at large still unclear (Nylen & Sacks, 2007). Examining the Peripheral blood mononuclear cells (PBMCs) from infected individuals with subclinical or asymptomatic infection (i.e. leishmanin skin-test-positive individuals, with no history of disease) respond to stimulation with leishmanial antigen and produce IL-2, IFNγ, and IL-12 (Kumar & Nylen, 2012).

The peripheral blood mononuclear cells from active VL patient's shows inability to proliferate or produce IFNγ by T cells in response to Leishmania antigens (Sacks *et al*., 1987; White *et al*., 1992) also have a negative skin test in most patients with active VL disease (Kumar & Nylen, 2012). However, cured individuals after completion of therapy are resistant to reinfection which seems to be no inherent defect in antigen-induced Th1 responsiveness (White *et al*., 1992).

2.7 T Cell Development

To maintain the peripheral T cell repertoire and T cell diversity the generation of T cells through the thymic dependent pathway is essential and necessary for potential immune responses to a vast number of antigens. T cells are derived from hematopoietic stem cells found in the bone marrow and mature in thymus. Thymus is a multi-lobed central lymphoid organ composed of cortical and medullary regions surrounded by a capsule (Figure 3). A specialized microenvironment is provided by the thymus for maturation and selection for the majority of developing progenitors and identified based on the expression of different types of specific cell surface markers.

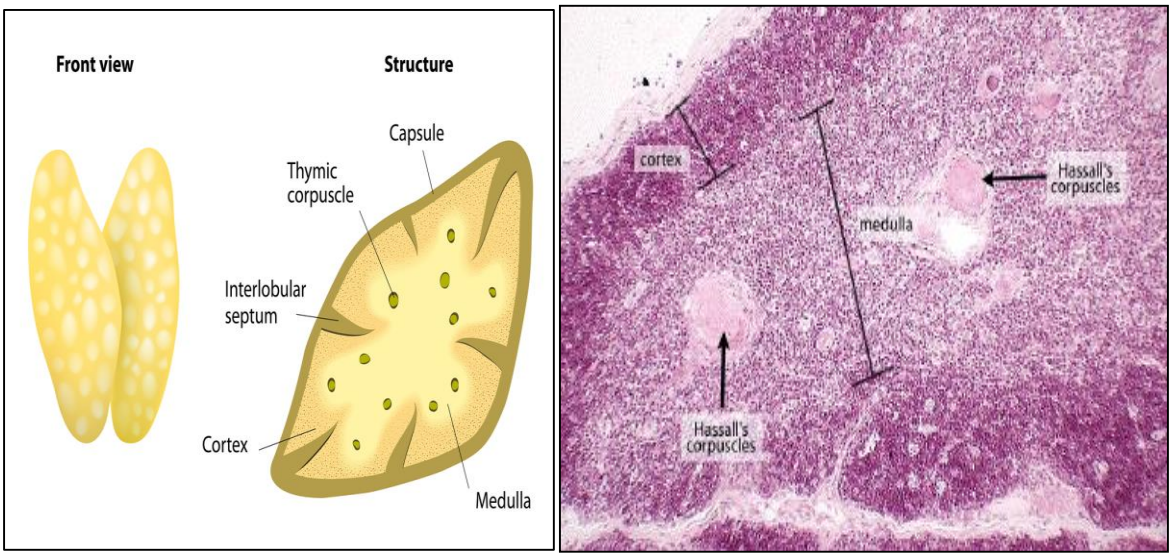


Figure 3: a) Structure of Thymus (b) Cross section of thymic tissue showing details of internal architecture.

Source: Anatomyinner.com

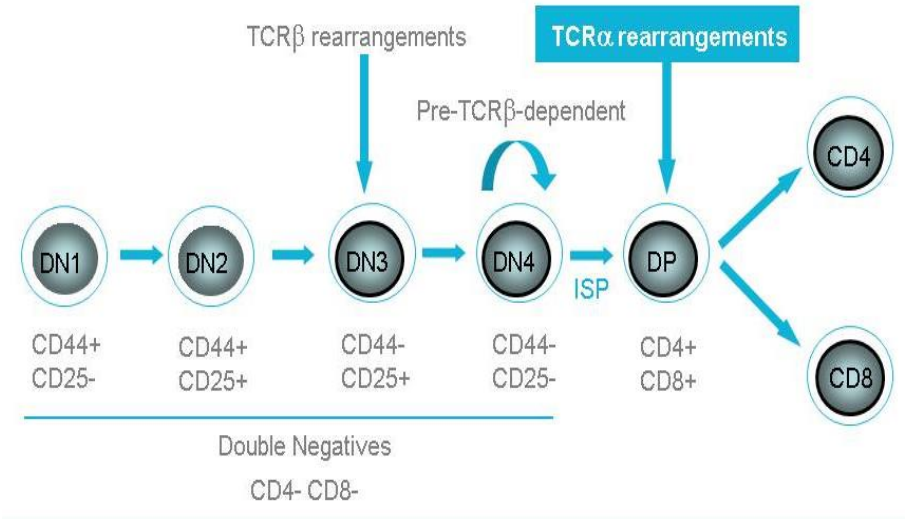
Double negative (DN) cells which are the earliest developing thymocytes lack the expression of the co-receptors CD4 and CD8 and further sub-divided by the expression of CD44 (an adhesion molecule) and CD25 (Interleukin-2 receptor α chain).

Beta-selection starts when cells express CD25 (DN3) and lacks the expression of CD44, selects cells which have successfully rearranged their TCR-β chain locus (Shah & Zuniga-Pflucker, 2014). A pre TCR is formed when the β chain pairs with pre-Tα, and forms a complex with CD3 molecules. This complex leads to the survival, proliferation, arrest in further β chain loci rearrangement, and further differentiation by up-regulation and expression of CD4 and CD8, these cells are termed double positive (DP) cells(Figure 4a). Cells that do not undergo beta-selection die by apoptosis (T-cell development in the thymus, Divya K. Shah).

Double Positive cells further rearrange their TCR-α chain loci, to produce a αβ-TCR. These cells enter in thymic cortex, go through the positive selection and select those T cells which are capable of self-interacting with MHC molecules which express either MHC Class I or II receptors. Thymocytes that interact with MHC-I or MHC-II appropriately and send surviving signal if not will die (Klein *et al*., 2014). Now they enter in the medulla, where negative selection starts, selects those cells which appropriately interact with self-antigen MHC1 or MHC 2 molecules. Thymocytes which interact too strongly with self-antigens undergo apoptosis. Following selection, down-regulation of either co-receptor produces either naïve CD4 or CD8 single positive cells that exit the thymus and circulate the periphery (Figure 4b) (Klein *et al*., 2014).

Naïve T lymphocytes are activated upon antigen recognition by their specific T-cell receptors (TCR) formed by developmentally regulated TCR gene rearrangements and intra-thymic selection processes. Failure of this system involved in many diseases (Wang & Reinherz, 2012). With age or in diseased conditions the thymic involution starts which causes decrease in lymphatic thymic mass. Some data suggest that the adult thymus may remain active even at old ages supplying functional T cells to the periphery.

(a)



(b)

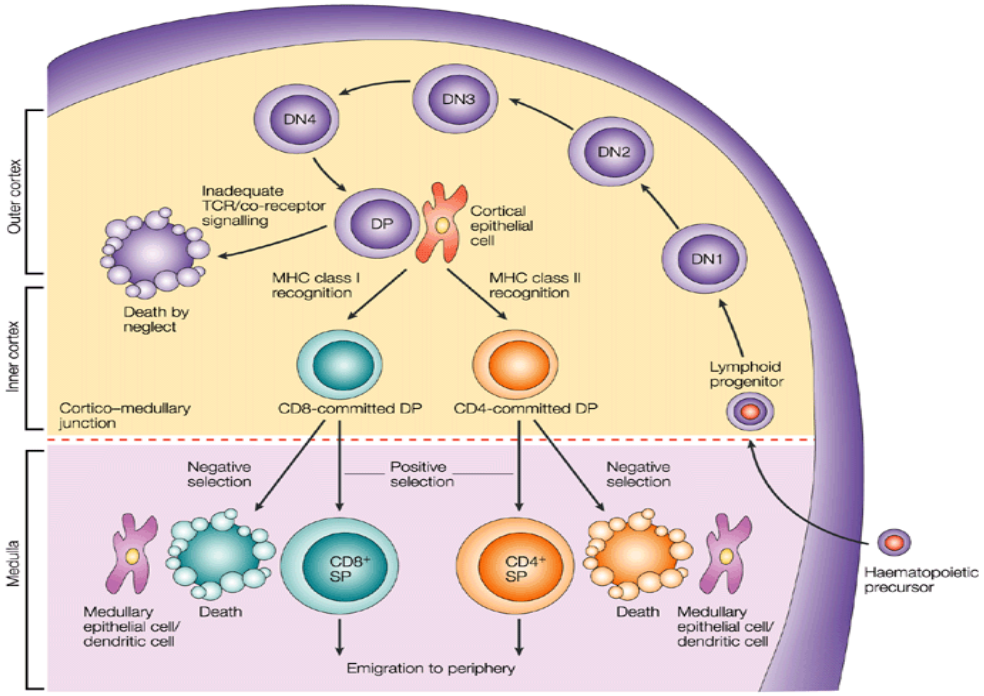


Figure 4: (a) Developmental stages of thymocytes in thymus with stage specific markers (b) Sequential stages in development of thymocytes in thymic microenvironments.

Source: Divya K. Shah; Hsieh, *et al.* 2012

The CD4+ and CD8+ T cells progress through several stages during their long lifespan (Weinreich & Hogquist, 2008). Both mature CD4 and CD8 thymocytes first emigrate from the thymus into the periphery as recent thymic emigrants (RTE).After interaction with antigens they further mature, continually circulate through the blood and lymphoid tissues. Measuring the number of RTE produced per day by the thymus allows for assessment of the thymic contribution (Weinreich & Hogquist, 2008).

2.8 T cell receptor gene rearrangement

About 95% cells in the thymus give rise to alpha and beta T cells, approximately 5% bear the gamma and delta T cell receptor. The t-cell receptor of α and β chains like immunoglobulin chain, consist of a variable (V) amino-terminal region and a constant (C) region. The organization of the gene segments is broadly homologous to that of the immunoglobulin gene segments. The TCRα locus contains V and J gene segments (Vα and Jα) and TCRβ locus, contains D gene segments in addition to Vβ and Jβ gene segment.

During the development of T cell, discrete segments of TCRα- and β-chain genes are joined by somatic recombination. For the chain, to create a functional V-region exon a (Vα) gene segment rearranges to a (Jα) gene segment. T cell receptor α-chain protein is yielded by mRNA produced during transcription and splicing of the VJα exon to Cα. The variable domain is encoded in three gene segments in β chain i.e., Vβ, Dβ, and Jβ.

A functional VDJβ V-region exon is generated by rearrangement of these gene segments further transcribed and spliced to join to Cβ; resulting mRNA is translated to yield the T-cell receptor β chain as shown in (figure 5). The αβ T-cell receptor heterodimer is formed by α and β chains (Janeway CA Jr., *et al*. 2001)**.**

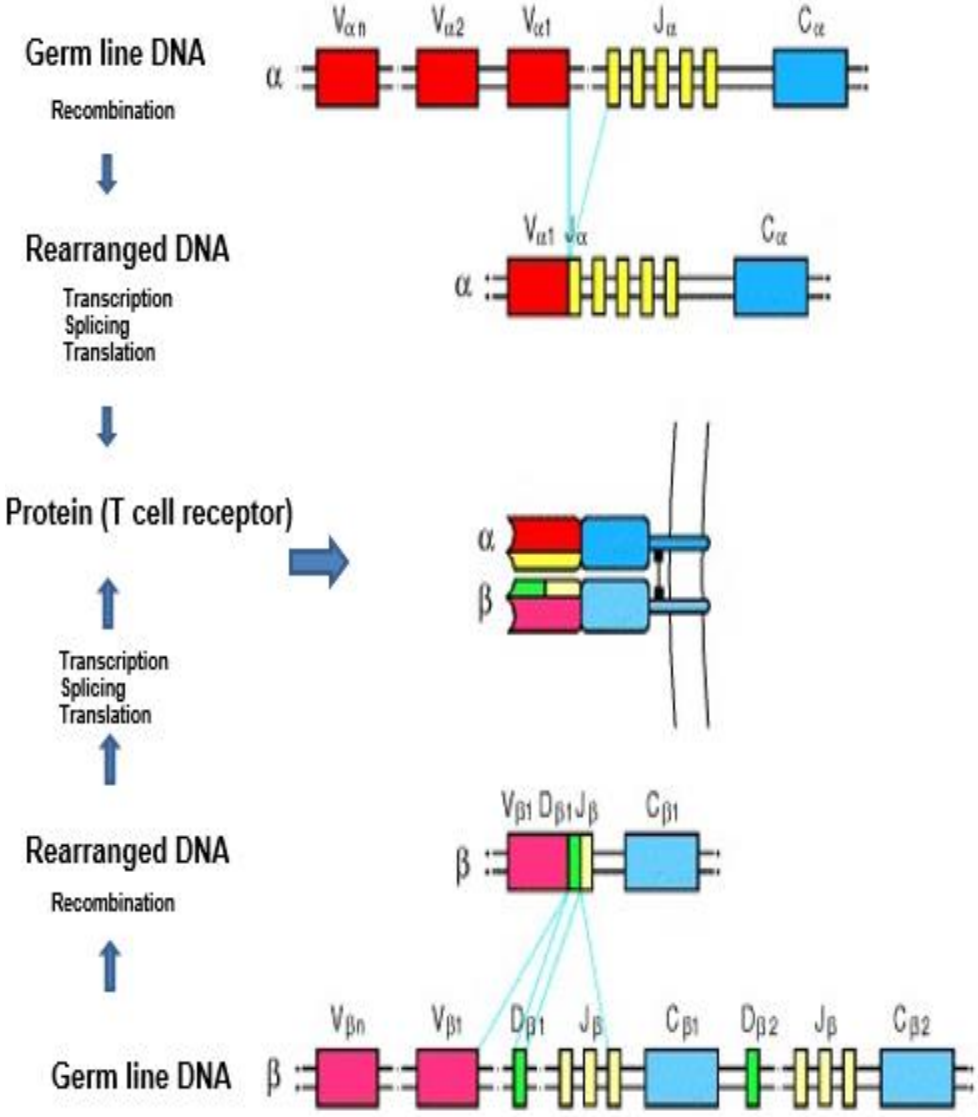


Figure 5: Formation of T-cell receptors (α- and β-chain gene rearrangement and expression).

(Source: Janeway CA Jr *et al*., 2001)

2.9 TREC (T cell Receptor Excision Circles) formation

T cells are generated in two ways in thymus: T lymphopoiesis and post thymic expansion of T cells. In the former process newly created T cells contain TRECs, and the latter process does not. During the event of rearrangement process (both for beta and alpha chain) the intervening DNA sequences are deleted and circulised into episomal DNA molecules, called sj-TREC (Figure 6)(signal joint T cell Receptor Excision Circles) and cj-TREC (Coding joint T cell Receptor Excision Circles).

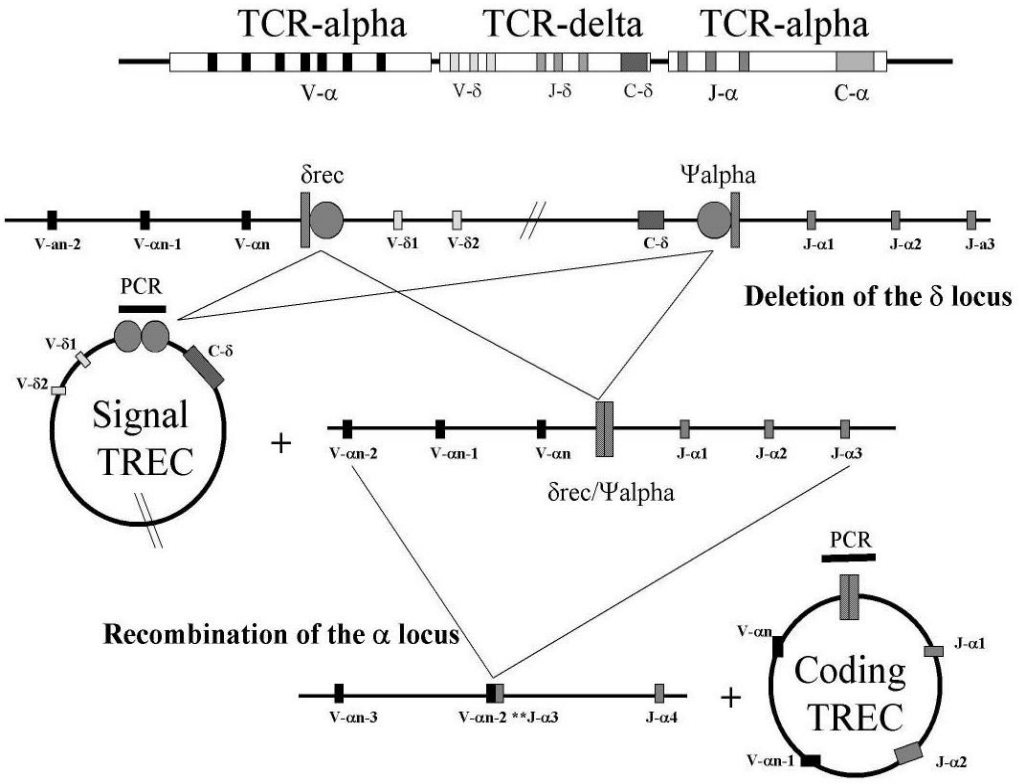


Figure 6: Molecular events in formation of T cell Receptor Excision Circles (TRECs) during T cell development.

Source: (Ponchel *et al*., 2003)

The presence of TRECs in peripheral blood could be used as a biomarker to read out the thymic functionality as its concentrations changes in diseased conditions, like visceral leishmaniasis (Ye & Kirschner, 2002b). As we know that the newly generated T cells are exported to the periphery by thymus and no markers are available right now for these recent thymic emigrants (RTE), which makes pretty impossible to measure human thymic output (Ye & Kirschner, 2002b).

T cell Receptor Excision Circles (TREC) seems to be a promising assay technique for evaluating thymic output and function in disease conditions (Ye & Kirschner, 2002a). TREC assay is very sensitive to origin of quantitative changes specially in vivo, therefore a number of factors such as T cell degradation, T cell death or thymic output can influence the result (Figure 7).

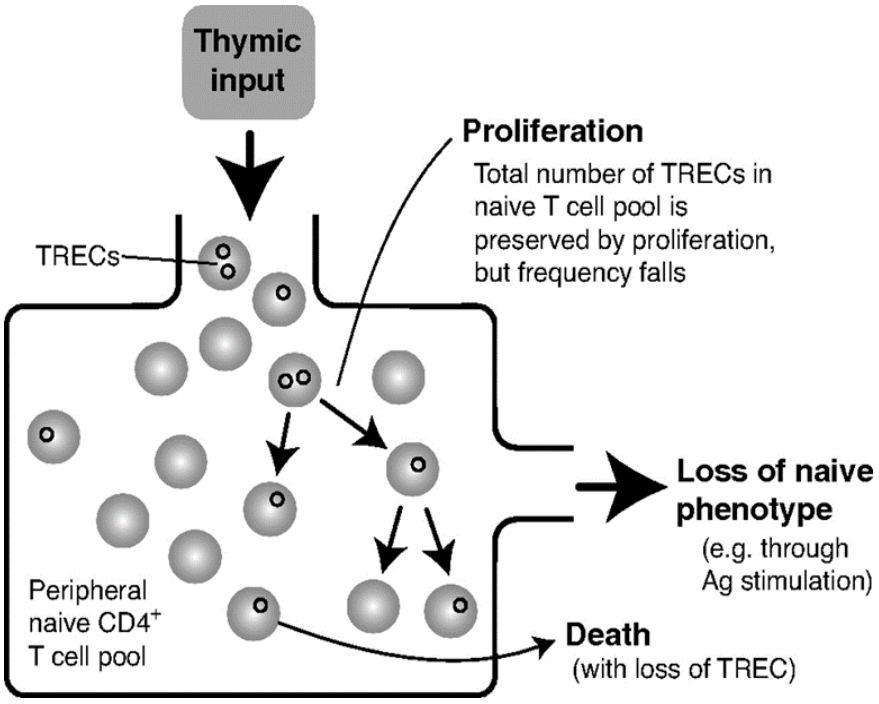


Figure 7: Possible origin of quantitative changes in TRECs: Thymic output, T cell division, T cell death, TREC degradation.

Source: (Bains *et al*., 2009)

During TCR gene rearrangement process the excised DNA fragments from alpha and beta chains are circulised episomally as T cell receptor excision circles. The signal-joint (sj-TREC) and coding-joint TREC are the two species which are the products of the excision of TCR delta locus from TCR alpha chain during the first TCR alpha gene rearrangement. Both are produced sequentially by all alpha and beta T cells (Petrie *et al*., 1993).

In most of them, these TREC are generated without prior delta rearrangement and are hence identical and can be detected in 70% of alpha beta T cells. Maximum two sj-TREC and two cj-TREC can present within one alpha beta T cell if the corresponding rearrangement event occurs in both alleles (Ye & Kirschner, 2002b).

The sequences remaining after recombination provides the target for a PCR detection of TREC. Each mature T-cell exiting the thymus and released into the circulation contains episomal TRECs. As TRECs cannot replicate they are diluted in peripheral blood compared to thymocytes where TREC concentrations are higher. That is why naive T cells have higher TREC concentrations than memory T cells (Zhang *et al*. 1999; Sodora et.al 2000; McFarland *et al.,* 2000). TREC is exported from the thymus to the periphery within Recent Thymic Emigrants. Thus, TREC levels in the periphery reflect RTE numbers.

So in visceral leishmaniasis the recent thymic function can be evaluated by measuring the proportion of peripheral T-cells containing TRECs and understanding the TH1 to the TH2 response in peripheral blood mononuclear cells (Frederique Ponchel *et al*. 2003).

CHAPTER 3

3. Materials and Methods

Sample collection

Peripheral blood and thymocytes were collected from infected and healthy experimental BALB/c murine model by senior.

Time point

(a) **Experimental blood** and thymocytes of murine samples were of 4 weeks and 8weeks after acclimatization period infected with L.donovani.

(b) **Control blood** and thymocytes samples were of 4 weeks and 8weeks from healthy BALB/c mice samples.

Chemical reagents required for DNA isolation, Simple PCR and Quantitative Real Time PCR

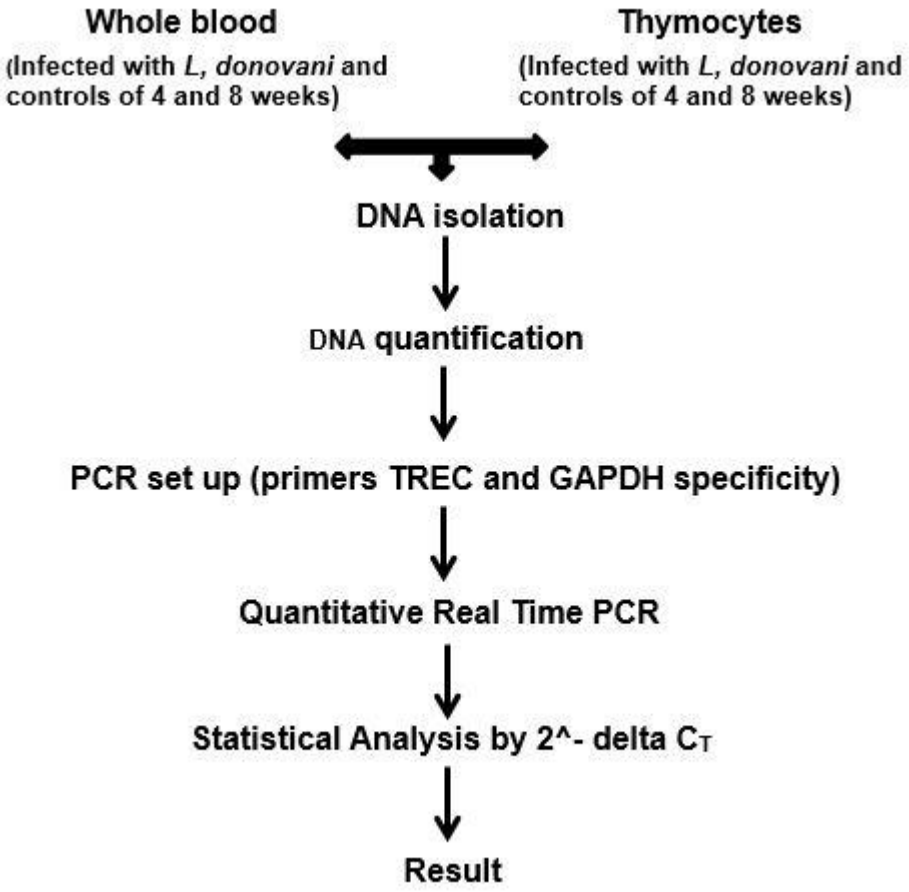
For DNA isolation: Lysis buffers, Proteinase K, Phenol-chloroform isoamyl alcohol(25:24:1), Chloroform isoamyl alcohol(24:1), Ice, cold 95-100% ethanol,70% Ethanol ,Ultrapure (DNA- & DNase-free) water,TE buffer, Extraction buffer

For PCR and qPCR: A set of specific appropriate primers (Forward and Reverse primers for GAPDH and TREC),SYBR Green Dye, PCR Master Mix, Taq DNA polymerase and buffer, Template DNA, DNase/RNase-free molecular-biology grade water, 2% agarose for Gel electrophoresis and set up, TBE buffer etc.

Plastic wares: Hand gloves, Simple PCR and qPCR tubes and caps, tips, a PCR tube rack, an ethanol-resistant marker, Eppendorf tubes.

Instruments: A thermal cycler, PCR. qPCR machine, Table top centrifuge with swinging 96-well plate holders, Micro-centrifuge, 96-well PCR plates. A set of micropipettes that dispense between 1 - 10 µl (P10), 0.2 - 20 µl ,20 - 200 µl (P200) and 100 - 1000 µl (P1000).Nano Drop UV spectrophotometer, Gel visualization instrument, ice bucket etc.

Methodology



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3.1 DNA isolation from blood and thymocytes:

The peripheral blood and thymocytes for the murine model were provided by the senior and obtained from the healthy (for control) and infected BALB/c mice (of visceral leishmania of four and eight weeks after acclimatization period).

For DNA isolation, 100 µl whole blood and 100 micrograms of thymocytes were taken in a 2ml Eppendorf tubes with 900 µl NET lysis buffer solution and 10 µl proteinase K and incubated overnight at 37 °C in water bath. Phenol, chloroform and isoamyl alcohol (25:24:1) were added in equal amounts to the sample (1ml sample+1ml PCI) in Eppendorf tubes and centrifuged at 16000G for 20 minutes at RT (25 °C). The aqueous phase was taken out and transferred in new tubes.

Again Chloroform and isoamyl alcohol (24:1) solution were added in equal amounts in the aqueous phase and centrifuged at 16000G for 12 minutes at RT, repeating the same procedure again for 10 minutes. The aqueous phase was taken out and absolute ethanol (98%) and sodium acetate (1/10) were added in new tubes and left for an incubation period of 1 hour at 20 °C. Then centrifuged it for 25 minutes at 16000 G (RT) then water is removed and 70% ethanol is added (500) to each sample, shake at centrifuged again at 16000G for 15 minutes (RT).

Now water is removed, left it for dry for 15 minutes then 45 micro litres of nuclease free water is added in each tube and left it to dissolve at 4°C.

3.2 DNA Quantification

DNA absorbs light at a wavelength of 260 nm. For pure DNA, a ratio of 260/280 is widely considered ~1.8.Therefore the average concentrations of DNA and their purity were quantified using nano drop UV spectrophotometer. The DNA samples are used for this project is close to ~1.8

3.3 PCR set up

A PCR is set up to amplify the isolated DNA from thymocytes and whole blood using GAPDH and TREC specific primers (Apeksha Ashok Gulvady, 2011**)** also the reaction is standardized at different annealing temperatures to minimize the non-specific bands. To know the specificity of primers a gel electrophoresis is run .It is found that the primers are working efficiently at 62 °C annealing temperature. A picture of gel is shown in Figure 8.

3.4 SYBR green-based TREC Analysis by Real-Time PCR: The sj TREC DNA from blood and thymocytes were detected by qPCR, based on SYBR-Green fluorescence. The reaction was set up for TREC with 10 µl reactions, containing 5.0 µl of SYBR Green dye, 0.3µM each of sj-TREC forward 5'-CCAAGCTGACGGCAGGTTT-3' and sj-TREC reverse 5'-AGCATGGCAAGCAG-CACC-3' primers and 5.6 µl mixture of purified DNA(ng) and nuclease-free water.

Similarly, for GAPDH samples with 10 µl reactions, containing 5.0 µl of SYBR Green dye with 0.3µM each of forward 5'-GTGAGGCCGGTGCTGAGTAT-3' and reverse 5'-TCATGAGCCCTTCCACAATG-3' primers and 5.6 µl mixture of purified DNA(ng) and nuclease-free water were taken.

Samples were briefly short spun. The thermo-cycling conditions were set up as 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 30 seconds and annealing/extension at 60°C for 60 seconds followed by 95 °C for 60 seconds. Samples were transferred to real-time PCR

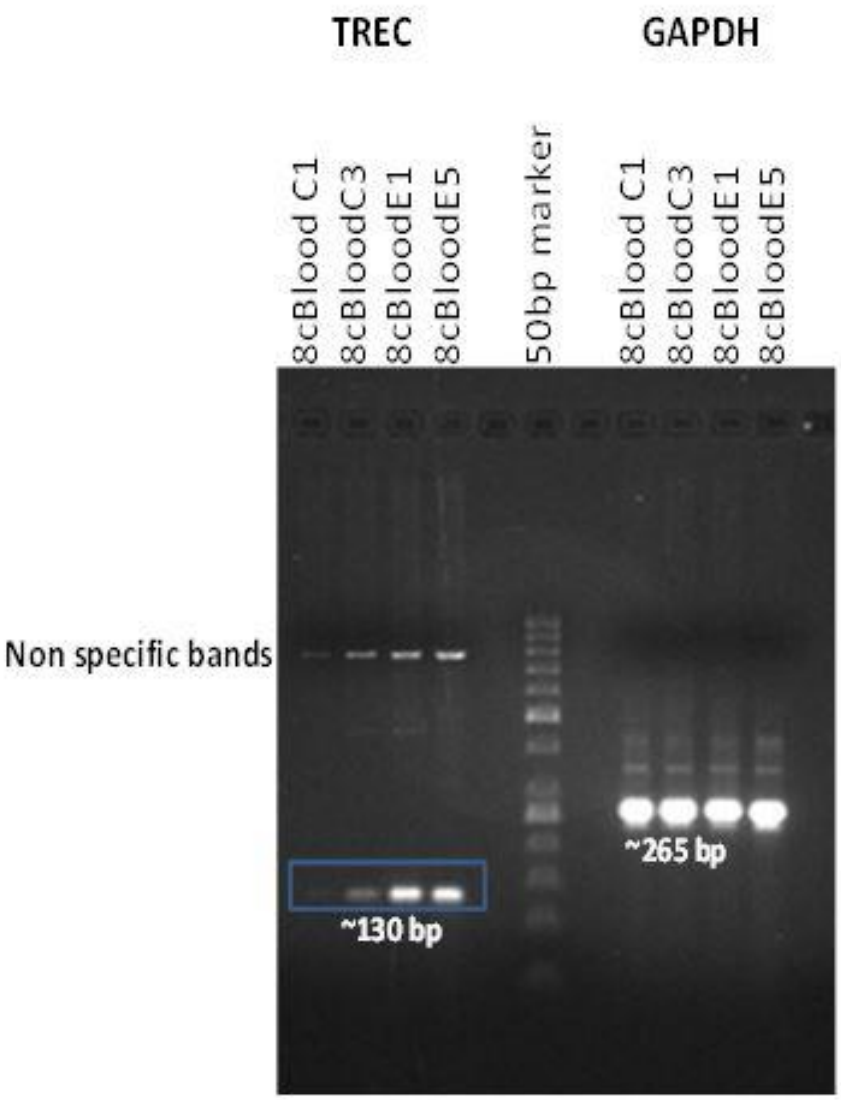


Figure 8: Agarose Gel showing TREC and GAPDH specific PCR amplification products in Control and Experimental samples (TREC: expected size- ~130bp, GAPDH: expected size ~ 265bp)

3.5 Statistical analysis

Delta Ct method was used to calculate gene expression. Cycle threshold (Ct) values were obtained after Real time-PCR. The $^{\Delta Ct}$ value for control was obtained by subtracting the Ct value of control of GAPDH from the control of TREC, similarly, $_{\Delta Ct}$ value for experimental was obtained by subtracting experimental GAPDH Ct value from TREC experimental Ct value, separately for each sample.

Then $2^{-\Delta Ct}$ values were calculated separately for each sample (control and experimental) and an average of $2^{-\Delta Ct}$ values for controls and experimental samples were recorded. Finally, fold change for control is obtained by dividing $2^{-\Delta Ct}$ mean values for control by itself.

Then fold change for experimental samples were obtained by dividing the $2^{-\Delta Ct}$ mean values of experimental from $2^{- -\Delta Ct}$ mean values of control. Now if the values of fold change were (>1) then it will indicate up-regulation in the expression and if the values of fold change were (<1), then the values were divided by 1 and the resulted value will indicate down-regulation in the gene expression.

Statistical analysis was performed using the analytical tool Graph Pad Prism 7.04 software. Unpaired t test was used to analyze the data by non-parametric Mann-Whitney Test and results were expressed as mean ± SD.

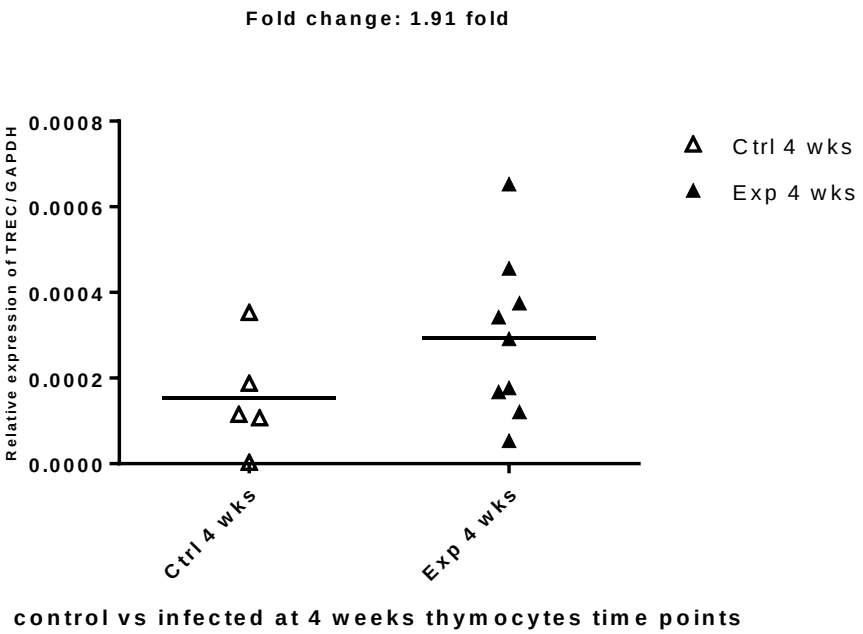
CHAPTER 4

4. Result

Up regulation of TREC in thymocyte samples prepared from thymi of control versus parasite infected mice: The relative quantification of TREC normalized against GAPDH in DNA preparation from 4 weeks and 8 weeks thymocytes samples, showed an up-regulation of TREC in infected mice compared to control group. There is fold change of 1.91(4weeks) and 1.38 (8weeks) respectively however the p value =0.18 (4 weeks) and (>0.99) (8weeks) (figure 9) were not significant**.**

Up regulation of TREC in whole blood sample from control versus parasite infected mice: Relative quantification of TREC normalized against GAPDH in DNA preparation from 4 weeks and 8 weeks blood samples from control and *L. donovani* infected mice, showed an up-regulation of TREC in infected mice compared to control as shown in (Figure 10). The fold change increase at 4 weeks was 7.32 and p value (<0.05) 0.008 showing statistically significant however at 8 weeks the increased fold change was 3.003 and p value (<0.005) 0.10 which was not significant.

a.



b.

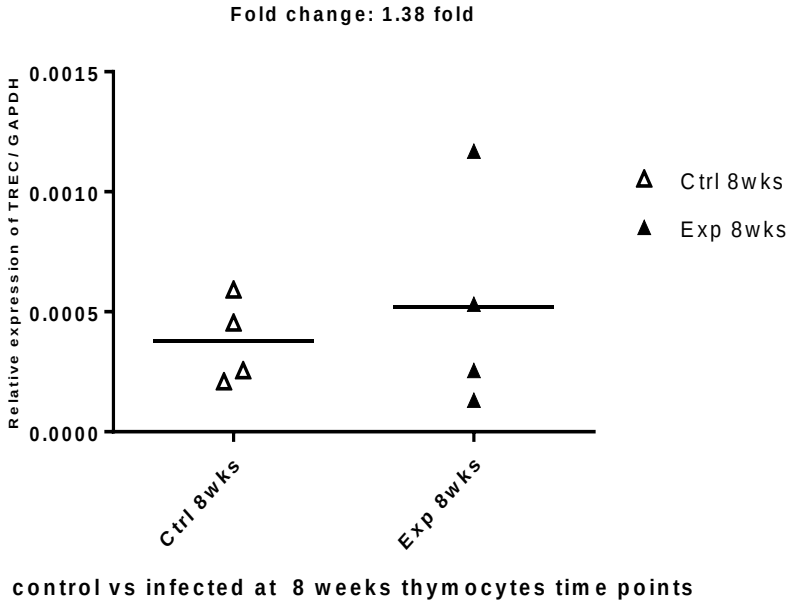
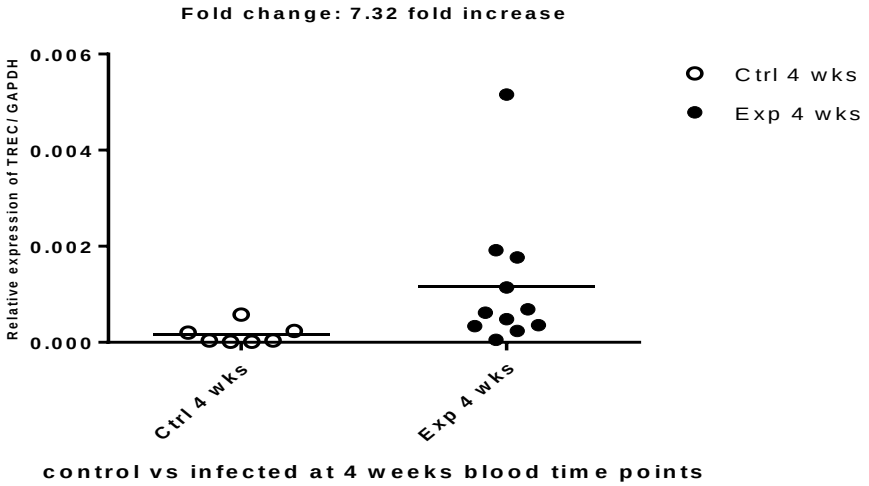


Figure 9. Scatter plot of overall delta CT values of (a) 4weeks thymocytes (b) 8 weeks thymocytes (Experimental versus Control).The up regulation in thymocytes with p value(<0.05) 0.18 in 4 weeks and in 8 weeks p value(<0.05) 0.99 were not significant.

a.



b.

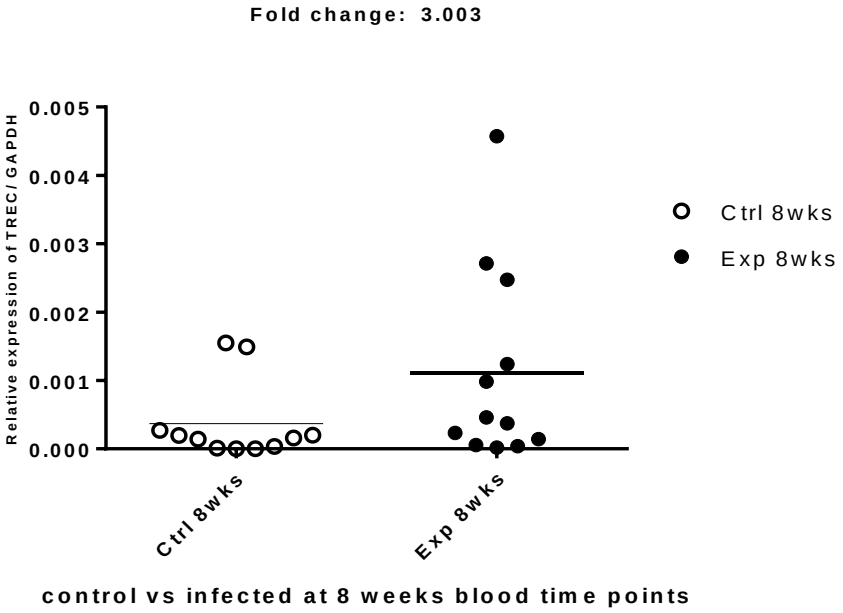


Figure 9: Scatter plot of overall delta CT values of (a) 4 weeks blood (b) 8 weeks blood (Experimental versus Control). The up-regulation in peripheral 4 weeks blood with p value (<0.05) 0.008 statistically significant however not significant in 8 weeks blood p value (<0.05) 0.10.

CHAPTER 5

5. Discussion

Thymus is the source of newly generated naïve T cells which are exported to the peripheral blood to form the T cell repertoire. Naive T cells are those cells which have not diluted their TREC copies (T-cell receptor excision circles) by antigen-driven cell division and referred as Recent Thymic Emigrants (RTE). Peripheral T-cell homeostasis is maintained by a balance of thymic output and peripheral T-cell expansion. In diseased conditions the number of T cells might decrease based on the severity of infection (Ye & Kirschner, 2002b).

A sustained T cell response is required to control the intracellular protozoan parasites as in Leishmaniasis there is high parasite load for many weeks following infection (Sacks & Noben-Trauth, 2002). It has been observed in acute viral and bacterial infection that Ag presentation is down regulated to control immune responses following infection (Gray *et al*., 2006). TREC levels in Cutaneous T cell Lymphoma (CTCL) patients in comparison to normal donors shows reduced T cells and statistically significant differences between normal controls and patients at all stages of CTCL (Yamanaka *et al*., 2005).In human VL peripheral blood it has been observed that the capacity to produce IFN gamma by T cells (CD8+) are severely reduced and impaired due to the up-regulation of CD94, CD158a and CD158b (Miralles *et al*., 1994; Gautam *et al*., 2014).

In our work with thymocyte samples(4 weeks and 8 weeks), the increase level is not significant, p value = 0.18 and 0.99 respectively implying that there is no change in thymic output of RTEs and that thymus functionality is not altered in terms of T cell output.

In 4 weeks whole blood sample, significant increase in TRECs level reflect that there is a decreased proliferation of Recent Thymic Emigrants in peripheral T cell pool, confirmed by p value = 0.008.However, at 8 weeks blood, there is increase in TRECs level but compared to 4 weeks it is low, which reflect that there is a increase in proliferation of Recent Thymic Emigrants in peripheral T cell pool, however it is not-

significant confirmed by p value = 0.10. In one experiment it is demonstrated that the activation of naive T cells diminishes following *L. major* infection (Gray *et al*., 2006).
It is possible that the activation of naïve T cells by Leishmania antigens and proliferation capacity might have reduced with time points following *L.donovani* infection.

CHAPTER 6

6. Conclusion

In both 4 weeks and 8 weeks thymocytes samples, no change in thymic output of RTEs and that thymus functionality is not altered in terms of T cell output.
In our 4 weeks data from blood samples we can say that Leishmania parasites have the capacity to modulate T cell-mediated immune response by regulating the expression of Leishmanial antigens result in decrease in proliferation capacity of naïve T cells. However, in 8 weeks there is proliferation in RTEs, but was not significant.
These alterations of T cells in host system may lead to polarization of Th1 to Th2 response.

Future work

TREC assay is very sensitive assay as the experiments were conducted in vivo, a number of factors such as T cell degradation, T cell death or thymic output can influence the result. So, to understand the alterations in thymic functionality in Visceral Leishmaniasis more experiments need to be carried out.

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