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$CD4^+$ T CELLS IN THE PATHOGENESIS OF VISCERAL LEISHMANIASIS

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$CD4^+$ T CELLS IN THE PATHOGENESIS OF VISCERAL LEISHMANIASIS

by

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Dedication

-To my parents, Robert Medina & Lorraine Gonzalez, for their love and support

-To my grandparents, Tomas & Gloria Castellanos, for their love and life's lessons

-To my fiancé, Anthony Colorado, for his infinite love, support and memorable laughs

-To my siblings, Robert Medina, Jr. & Zoey Gonzalez,

my cousins, Samantha Deleon & Gabriella Belmontes, and all my friends,

for always entertaining me and keeping a smile on my face even from miles away

&

-To my great grandparents, Audon & Linda Jaime, who I lost along this journey.

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Visceral leishmaniasis, caused by infection with the intracellular protozoan *Leishmania donovani,* is a chronic disease that affects the spleen, liver and bone marrow. Patients present with high parasite burdens in visceral organs, splenomegaly, cachexia, pancytopenia and will progress to death if not treated. Control of *Leishmania* infection is dependent on Th1 (IFNγ producing) $CD4^+$ T cells, which activate macrophages to produce nitric oxide and kill the intracellular parasites. Despite there being an expansion of $CD4^+$ T cells and an increase in pro-inflammatory molecules in the spleen, humans with active visceral leishmaniasis cannot control infection. We have established an experimental model of chronic progressive visceral leishmaniasis in hamsters, which mimics clinical features seen in humans, to determine the mechanisms of disease. The studies presented here focus on the role splenic $CD4^+$ T cells play during chronic visceral leishmaniasis. A global view of the hamster splenic transcriptome during chronic infection provided insights into potential genes involved in disease pathogenesis. The characterization of the responding $CD4^+$ T cells in chronic progressive visceral leishmaniasis in hamsters is described here as a mixed Th1 and Th2 response marked by increased mRNA of associated transcription factors, cytokines and chemokines. The capacity for the $CD4^+$ T cells from the chronically infected hamsters to activate macrophages and induce parasite killing was present but marginally effective. Increased markers of T cell exhaustion in total spleen tissue led to the exploration of this as a potential contributor to suboptimal T cell effector function. We discovered that the splenic $CD4^+$ T cell and macrophage populations expressed inhibitory receptors and ligands, respectively. Blocking PD-L2 led to a significant decrease in parasite burden revealing a pathogenic role for the PD-1 pathway in chronic visceral leishmaniasis. Inhibitory molecules are possible targets for immunotherapeutics or combinatory treatment regimens for chronic visceral leishmaniasis. Further studies into the role of PD-1 expression in $CD4^+$ T cells during disease will provide better understanding of the mechanisms involved in immunopathogenesis of chronic visceral leishmaniasis.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

VL	Visceral leishmaniasis
Th(#)	T helper type (#) cell
Treg	T regulatory helper cell
Ld	*Leishmania donovani*
iNOS	Inducible nitric oxide synthase
Arg1	Arginase I
BMMO	Bone marrow derived macrophages
RT_2-PCR	Real-time reverse transcribed polymerase chain reaction
IFN	Interferon
IL	Interleukin
APC	Antigen presenting cell
MΦ	Macrophage
DC	Dendritic cell
NK	Natural killer cell
FDR	False discovery rate
FC	Fold change
GAS	Interferon gamma activated sequence

CHAPTER 1: GENERAL INTRODUCTION

Leishmaniasis is caused by intracellular protozoan parasites of the genus *Leishmania*. There are more than 20 known species of *Leishmania* parasites that cause human disease. *Leishmania* species are distinguished by the different clinical disease aspects they cause and their geographical origin (Table 1.1) [1-3]. *Leishmania* parasites are responsible for many human diseases ranging from cutaneous, mucosal and visceral leishmaniasis.

Table 1. 1 Leishmaniasis: Diseases and causative parasite species

Clinical form of disease	*Species*[A]	*Geographical distribution*
Cutaneous (CL)	*L. (L.) chagasi/infantum*	Central America, South America, Mexico, North Africa
	L. (L.) mexicana	Central America, Mexico
	L. (L.) amazonensis	Central and South America
	L. (L.) major	China, Indian subcontinent, Middle East, North Africa
	L. (L.) tropica	Indian subcontinent, Mediterranean basin, Middle East, West Asia
	L. (L.) aethiopica	Ethiopia, Kenya, Yemen
	L. (V.) braziliensis	Central and South America
	L. (V.) panamensis	Central America
	L. (V.) guyanensis	Central and South America
	L. (V.) peruviana	South America
		Peru
Diffuse cutaneous (DCL)	*L. (L.) amazonensis*	Brazil
	L. (L.) mexicana	Central America, Mexico
Mucosal (ML)	*L. (V.) braziliensis*	Central and South America
	L. (V.) panamensis	Central and South America
	L. (V.) guyanensis	South America

Table 1.1 continued

Visceral (kala-azar) (VL)	*L. (L.) donovani* *L. (L.) infantum/chagasi*	Africa, India, East Asia Mediterranean basin, Southwestern Asia, Middle East
Post kala-azar diffuse cutaneous (PKDL)	*L. (L.) donovani*	Africa, China, Indian subcontinent

[A] *(L.)* subgenus *Leishmania; (V.)* subgenus *Viannia*

GLOBAL SIGNIFICANCE OF LEISHMANIASIS

The incidence of leishmaniasis is increasing and spreading. This is a result of international travel, the AIDS epidemic, poor vector control, poor field diagnostics, inadequate treatment regimens and lack of a vaccine. The available treatments currently used have several problems including cost, availability, toxicity, long hospital stays, severe side effects, and development of resistance in *Leishmania* parasite strains. Moreover, the immune responses to each clinical form of leishmaniasis differ, making it difficult for vaccine development.

LEISHMANIA PARASITES AND TRANSMISSION CYCLE

Leishmania are protozoan parasites from the family Trypanomasomatidae and order Kinetoplastida. They are dimorphic parasites that exist in two morphological forms in their life cycle, the amastigote and the promastigote form (Figure 1.1) [4]. Leishmaniasis is a vector borne disease with the distinct replicative stages specific for the insect and mammalian hosts (Figure 1.2).

Leishmania metacyclic promastigotes are transmitted to a mammalian host through the bite of a female *Phlebotomus spp.* sand fly [5]. The promastigote form is

found extracellulary in the midgut of the insect vector. It is characterized by the flagellum located on the anterior point of the promastigote, which pulls the body forward in movement [3, 6]. Promastigotes are larger than the amastigote form, ~15-25 μm in length, with a longer elliptical shape. There are two types of promastigotes, the procyclic and metacyclic forms. These are distinguished by their morphology, infectivity, and surface lipophosphoglycan (LPG). LPG is a glycolipid that forms a coating around the entire body and flagellum of the parasite. The basic structure of LPG, the lipid anchor and glycan core, is highly conserved in all *Leishmania* species [7, 8]. The LPG also has repeating carbohydrate regions that vary between *Leishmania* species and aid in their infectivity. *Leishmania* promastigotes undergo metacyclogenesis in the midgut of the sandfly from the less infective procyclic form to the infectious metacyclic stage [9, 10]. Procyclic promastigotes have shorter LPG chains that prevent them from detaching from the wall of the midgut and being transmitted when the vector feeds [11]. The longer LPG of metacyclic promastigotes enables their release from the midgut wall [9]. They then can move from the midgut into the foregut allowing them to be transmitted when the vector bites and takes a bloodmeal [6, 11, 12]. The metacyclic promastigotes are more infective because the longer and larger repeat LPG residues aid in their survival in the mammalian host [6]. When the parasites are transmitted into a mammalian host, they are phagocytosed by dendritic cells and macrophages. LPG inhibits the fusion between the phagosome and endosome thus limiting the phagocyte innate defense mechanisms to destroy intracellular parasites [13]. This allows the survival of the promastigotes and the transformation into their replicating amastigote form.

Temperature and pH differences in the mammalian host trigger differentiation into the amastigote form. The amastigote form does not have a flagellum, is non-motile and replicates intracellularly in myeloid cells in the mammalian host [3]. This form is smaller and round in size (~2-10 μm). Amastigotes replicate within host cells, eventually leading to their lysis and infection of more phagocytes. When a new sand fly takes a

blood meal from an infected host, it takes up intracellular amastigotes. The cycle starts again, transforming the parasite into the promastigote form that can be transmitted with the next bite. In cases of progressive visceral disease, parasite replication is not controlled leading to high parasite burden in the bone marrow and secondary lymphoid organs.

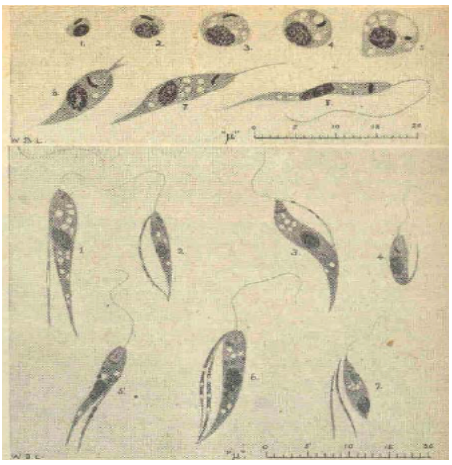


Figure 1.1 Drawings of the *Leishmania* parasite. William B. Leishman was the first to discover *Leishmania* parasites and cultivate them in 1905. His drawings show the transformation of the amastigote into the promastigote form over days of culture. The top figure depicts the amastigote growing its flagellum. The bottom picture shows the many developmental stages of fully transformed flagellated promastigotes at which time, Leishman believed the parasite to replicate by binary fission giving rise to two to four flagellated promastigotes. *Permission granted by Journal of Royal Army Medical Corps.*

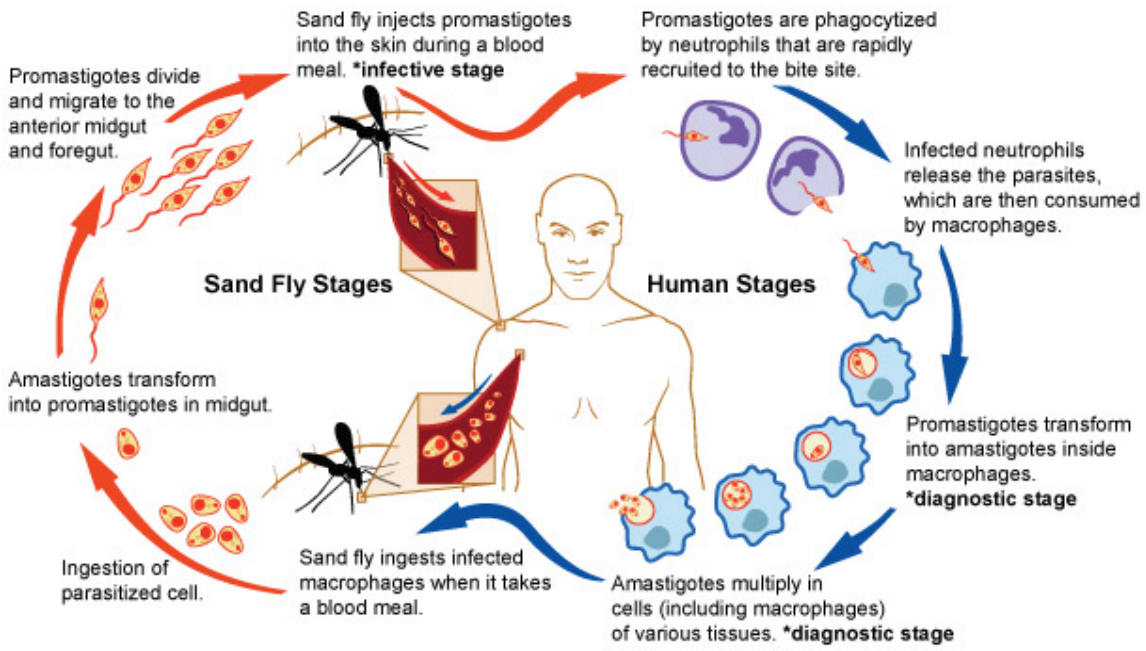


Figure 1.2 Life cycle of *Leishmania* parasites. *Diagram courtesy of National Institute of Allergy and Infectious Disease (NIAID).*

CLINICAL AND PATHOLOGICAL FEATURES OF LEISHMANIASIS

The outcome of infection is dependent on the species of the *Leishmania* parasite, host genetics and the immune response elicited in the host [14]. According to the World Health Organization, there are an estimated 1.3 million new cases of leishmaniasis that occur each year. More than 350 million people are at risk for infection in ~90 countries. The three main forms are cutaneous, mucosal and visceral leishmaniasis.

The most common form of leishmaniasis is the cutaneous disease, which the Center for Disease Control and Prevention reports to account for 0.7-1.2 million cases worldwide. The clinical features of cutaneous leishmaniasis include a range of lesions on the surface of the skin. The lesion usually starts as a small, reddened papule. It remains localized to the site of the insect bite. Over time, the lesion increases in size, becoming nodular and eventually ulcerating [3, 15]. Typical chronic lesions have raised borders made of dry, rough skin (Figure 1.3A). Some lesions produce an exudate appearing wet

and others are dry and crusty [3]. The progression of the lesion depends on the infecting species of the *Leishmania* parasite and on the host's immune response. Ulcerative lesions have a risk of contracting secondary bacterial or fungal infections. Cutaneous leishmaniasis lesions are usually found on exposed areas of the affected individuals such as the face, neck, ears and arms where the sand fly can bite [15]. They can persist for several months or even years. The cutaneous lesions may spontaneously heal, leaving a scar with discoloration in skin [3].

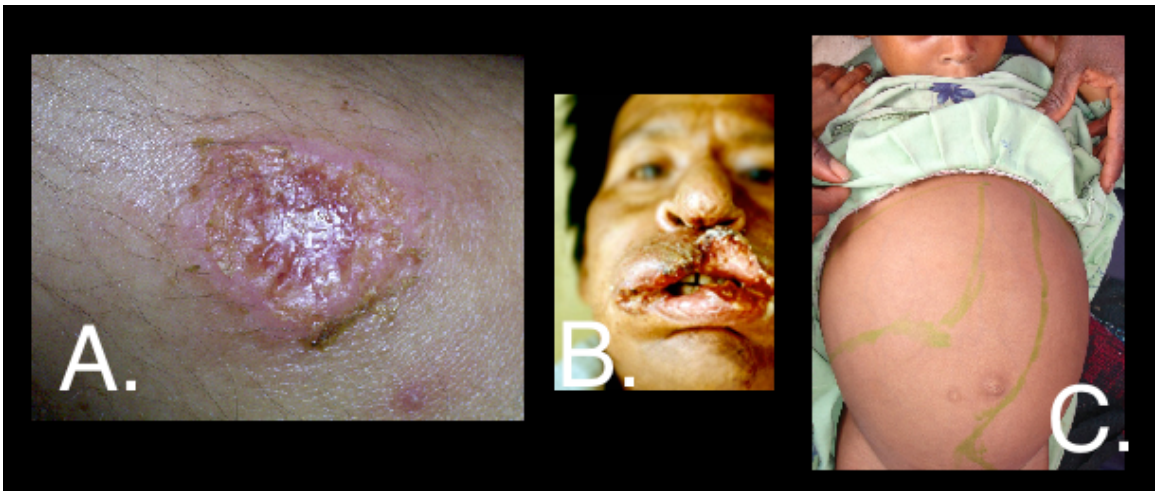


Figure 1.3 Clinical forms of leishmaniasis disease. (A) Cutaneous leishmaniasis ulcerative lesion (B) Patient suffering from deforming lesions of mucocutaneous leishmaniasis (C) Enlarged spleen and liver causing distended abdomen in visceral leishmaniasis patient. *Images courtesy of The World Health Organization*.

A sequela of cutaneous leishmaniasis is mucosal leishmaniasis. The pathogenesis of this disease is unclear. It can affect the host simultaneously with cutaneous infection or occur years after the initial lesion has cleared. An estimated <5% of individuals that suffer cutaneous leishmaniasis caused by *L. braziliensis* develop mucosal disease [16]. The development of mucosal leishmaniasis is believed to be influenced by differences in host immunity, genetics, number of lesions from the initial infection, and inadequate treatment of the initial cutaneous leishmaniasis [17]. The majority of mucosal leishmaniasis cases are found in Brazil, Peru and Bolivia. Initial symptoms of mucosal

leishmaniasis are inflammation of the nasal, oral and pharyngeal cavities, but patients do not usually seek medical attention until there is significant lesion development [18]. Symptoms worsen and lesions become destructive and ulcerating (Figure 1.3B). This is primarily the result of intense host inflammatory response. Despite having low parasite burden in the nasal mucosa lesions, patients with mucosal disease have increased inflammatory cytokine production compared to lesions from cutaneous leishmaniasis patients [19]. Patients that develop this slow and progressive mucosal disease are at risk of secondary respiratory infections because the mucosal tissues become exposed due to massive tissue destruction.

Visceral leishmaniasis is a systemic infection that primarily affects the spleen, liver and bone marrow of the host. It is the most serious form of the disease. It accounts for 500,000 cases annually and is usually fatal if not treated [3, 20]. Even with treatment, the mortality rate can be as high as 20% [20, 21]. Most cases of visceral leishmaniasis are found in India, Bangladesh, Ethiopia, Sudan and Brazil. Only 10% of infected individuals develop clinically evident disease. Clinical symptoms include a long-term low-grade fever, loss of appetite, weight loss, enlarged liver and spleen, and death if not treated (Figure 1.3C). Patients with progressive disease experience pancytopenia and loss of antigen-induced lymphoproliferative and cytokine responses in peripheral blood mononuclear cell cultures [22, 23]. Also during active disease, patients have increased accumulation of monocytic cells in the spleen and a mixed pro- and anti-inflammatory immune response in serum and spleen [24-26]. The other 90% of infected individuals experience asymptomatic infection without progression of disease. The outcome of infection is dependent on host immune response, genetics and environmental factors such as malnutrition [27, 28]. However, asymptomatic individuals may contribute to the transmission of this disease in endemic areas.

IMMUNITY IN LEISHMANIASIS AND THE TH1/TH2 PARADIGM

The study of the interaction between host and pathogen is necessary to develop therapeutics and vaccines to cure and prevent the disease. When a pathogen invades a host it induces an immune response involving specific immune cells [29]. Host immunity to leishmaniasis involves communication between the innate and adaptive immune systems. When the vector bites and transmits the parasite to the host, innate cells (neutrophils, macrophages, dendritic cells) are first responders to the invading foreign pathogen. The APCs that engulf the parasites prime T cells to mount an adaptive immune response.

Naïve $CD4^+$ T cells become activated when they encounter foreign antigen expressed on the surface of APCs in the context of the MHC class II antigen. Depending on the antigen and the cytokine milieu created by APCs, $CD4^+$ T cells differentiate into one of several T helper subsets (Figure 1.4). They are then directed towards maturation and proliferation through a series of signaling pathways [29-31]. The first discovered phenotypes of $CD4^+$ T helper cells are T helper type 1 (Th1) and T helper type 2 (Th2) cells [32]. These were first characterized using experimental models of cutaneous leishmaniasis in resistant and susceptible mice.

Th1 $CD4^+$ T cells differentiate in response to the type of antigen being presented by APCs and the nature of the inflammatory environment. They further enhance and direct the immune response. They are induced by the type 1 cytokine, IL-12, which is produced by dendritic cells [33, 34]. Th1 $CD4^+$ T cells have increased responses to IL-12 due to their higher expression of the IL-12 receptor. IL-12 induces the STAT4 pathway to activate the master transcriptional regulator of Th1 cells, Tbet (gene symbol Tbx21) [33]. Activation of Tbet leads to production of IFNγ, TNFα and IL-2 [29, 35]. The production of IFNγ acts as a positive feedback loop to enhance Th1 differentiation through the STAT1 pathway and upregulation of IL-12 receptor. Th1 $CD4^+$ T cells play important

roles in clearance of intracellular pathogens by IFNγ-induced macrophage microbicidal activity. These macrophages are considered classically activated, or M1 macrophages.

Th2 $CD4^+$ T cells play a role in humoral immunity by triggering B cell production of antibodies [29]. This subset of $CD4^+$ T cells plays an important role in the clearance of extracellular pathogens, such as helminths, but also drive the pathogenesis of hyper-inflammatory allergy and asthma [36, 37]. Th2 $CD4^+$ T cells have high expression of the IL-4 receptor, allowing induction by the type 2 cytokine IL-4 [38]. IL-4 signals the naïve $CD4^+$ T cells to induce the STAT6 pathway which leads to expression of the master regulatory transcription factor of Th2 cells, GATA3 [39]. Polarized Th2 $CD4^+$ T cells produce cytokines IL-4, IL-5 and IL-13 that stimulate macrophage activation. Unlike macrophages activated by Th1 CD4+ T cells, macrophages activated by Th2 cytokines take on a type 2 phenotype that dampens inflammation induced by tissue damage and promotes wound healing. These macrophages are considered alternatively activated, or M2, macrophages.

Regulatory T cells play an important role in maintaining immune homeostasis through self-tolerance and abrogating autoimmunity by their immunosuppressive activity [40]. They can be found as either natural Tregs (nTreg) originating in the thymus or inducible Tregs (iTreg) that are generated in the periphery [41]. Although all activated $CD4^+$ T cells express the surface marker CD25, Treg cells express higher levels [42]. Their differentiation is triggered by the cytokines TGFβ and IL-2, which induce the transcription factor, Foxp3 [40]. Treg cells are characterized by the production of the immunosuppressive cytokines IL-10, TGFβ and IL-35.

One of the first experimental *in vivo* studies examining the differences in Th1 and Th2 cellular responses used the murine model of cutaneous *L. major* infection [43]. It compared the resistant C57BL/6 mouse strain and the susceptible Balb/c mouse strain. These mouse strains showed distinctly polarized immune responses in concordance with their different disease outcomes with *L major* infection. The Balb/c mouse elicits a Th2

response leading to the production of type 2 cytokines like IL-4 and IL-5. This leads to the development of a progressive skin lesion from which parasites ultimately disseminate. In contrast, the C57BL/6 strain has a strong Th1 response with the production of IL-12 and IFNγ, resulting in small, self-healing lesions. This study initiated the Th1/Th2 paradigm that identified that a Th1 response was protective and a Th2 response led to exacerbation of leishmaniasis.

The differentiation of each $CD4^+$ T cell population is regulated by other T cell subsets. For instance, cytokine production by Th1 cells inhibits IL-4 production and the development of Th2 cells, and vice versa [44, 45]. Also, Treg cells suppress Th1 and Th2 cell differentiation [46]. Despite this well-established phenomenon, there are recent findings that do not fit a clear Th1 or Th2 cell differentiation pathway and population. For instance, a novel $CD4^+$ T cell subset was identified as a Th1/Th2 hybrid expressing both Tbet and GATA3 transcription factors [47]. There are also the finding of dual production of IFNγ and IL-10 by $Tbet^+CD4^+$ T cells [48-50]. The concepts today of T cell populations are more convoluted than previously thought. Ongoing studies to identify and understand the role of the many different subsets of $CD4^+$ T cells are vital.

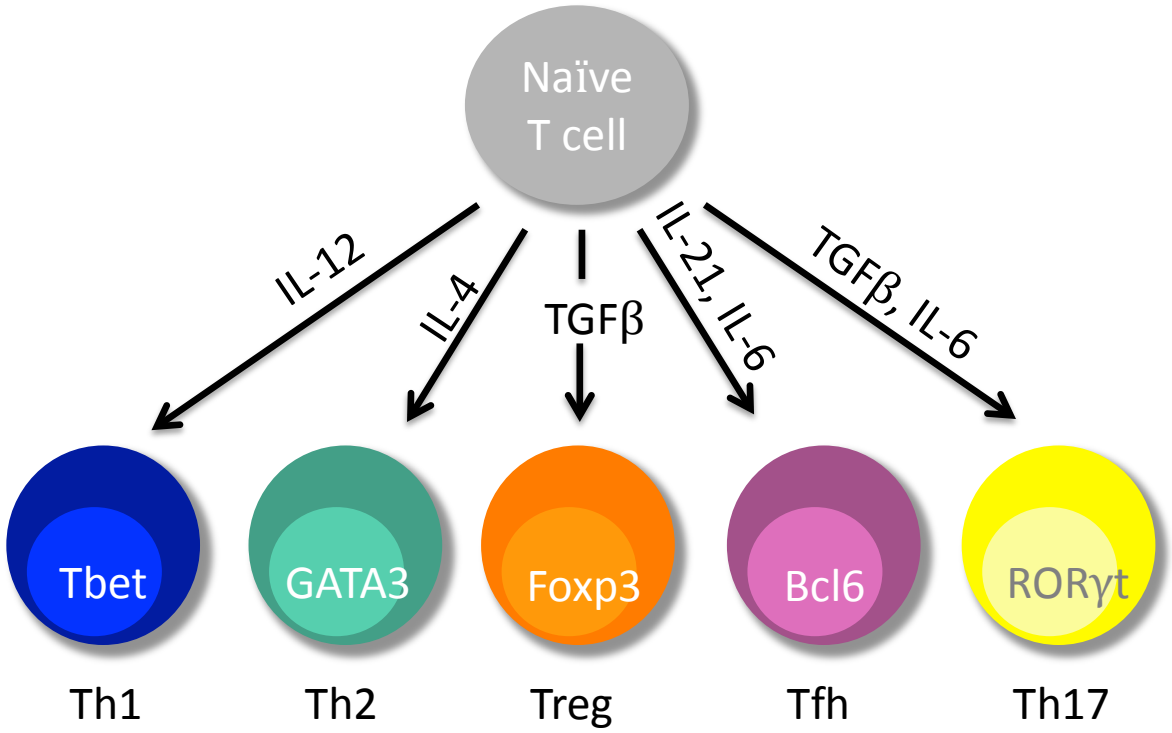


Figure 1.4 CD4$^+$ T cell subset populations

ANIMAL MODELS OF LEISHMANIASIS

Leishmaniasis is a major worldwide health problem and is one of the most neglected tropical diseases. Vector control, along with early therapeutic approaches, is important for control of the disease. But prevalence is increasing and a successful vaccine is needed. Animal models to study the disease pathogenesis and preventative treatment effects on immunity are key components to achieving this goal.

The mouse model is the most common animal used in scientific research. The Th1/Th2 paradigm was established in the cutaneous leishmaniasis mouse model and revealed the regulation of disease outcome by different T helper responses. Genetically different mouse strains mount different immune responses and exhibit different disease outcomes to *L. major* infection [43, 51]. As previously mentioned, the Balb/C mouse

strain is susceptible to *L. major* infection due to a Th2 dominant immune response with high levels of IL-4 production. In contrast, the C57BL/6 strain mounts a strong Th1 response with early production of IFNγ allowing control of *L. major* replication and dissemination [52]. The mouse model is used to study different forms of leishmaniasis including visceral disease.

However, the immune response to visceral leishmaniasis in the mouse model differs from cutaneous leishmaniasis. Both Balb/c and C57BL/6 mice are considered susceptible to infection with *L. donovani* and *L. infantum*, the causative species for visceral leishmaniasis [53]. The outcome of infection in these mice largely mimics asymptomatic or subclinical infection in humans. After infection in the mouse, there is rapid replication of the parasite in the liver. After ~8 weeks, production of IL-2 and IFNγ leads to granuloma formation and clearance of parasites from the liver [54, 55]. However, parasite replication persists in the spleen leading to splenomegaly and destruction of tissue architecture [55, 56]. Eventually parasite replication is controlled and the host does not experience the fatal outcome of progressive disease as seen in active visceral leishmaniasis in humans. This organ specific immunity leads to protection from reinfection for the mice [54]. Although the pathological features presented by the mouse differ from what is seen in humans, it has led to outstanding discoveries and advances in understanding immunopathogenesis in asymptomatic visceral leishmaniasis.

A model to study chronic and progressive visceral leishmaniasis was found in the golden Syrian hamster (*Mesocricetus auratus*) [57, 58]. Hamsters are susceptible to systemic infection with *L. donovani* and progress to chronic disease. They experience weight loss, splenomegaly, progressive parasite replication and ultimately death [59]. The clinical signs in the hamster are similar to chronic human visceral leishmaniasis. As in humans, hamsters have mixed cytokine responses in the sera and spleen, including the type 1 cytokines IL-2, IL-12, IFNγ, TNFα and the type 2 cytokines IL-4, IL-10, IL-13, IL-21, respectively (Table 1.2) [25, 59, 60]. Indeed, the hamster model is helpful in

understanding the complex pathogenicity of chronic and progressive visceral leishmaniasis. However, limitations to using this model are the lack of available reagents.

Canine species are natural reservoirs of visceral leishmaniasis. In endemic regions, dogs have a high prevalence of infection with *L. infantum* [61]. This animal model is helpful to study the disease in a natural infection and transmission patterns of visceral leishmaniasis within endemic environments.

Non-human primates are ideal animal models that are most similar to human anatomy, physiology and immunology. However, they are costly and difficult to work with. In the study of leishmaniasis, they are more commonly used as a final experimental animal model to evaluate the effects of potential therapeutics and vaccine candidates.

Table 1. 2 Different immune responses in models of visceral leishmaniasis

	Human		***Hamster***	***Mouse***	
	Spleen	*Sera*	*Spleen*	*Spleen*	*Sera*
IL-4	+++	+++	++	-	-
IL-13	+++	+++	++	+	+
IL-10	+++	+++	+++	+++	+++
Arginase	+	++	+++	-	-
IFNγ	+++	+++	+++	+++	+++
TNFα	+++	+++	+++	+++	?
IL-12	+++	+++	+++	+++	?
NO	+/-	?	+	+++	+++

NECESSITY TO STUDY THE IMMUNOPATHOGENESIS OF LEISHMANIASIS

Much work has contributed to elucidating the immune response of leishmaniasis, but there are still many challenges to control the disease. The limited chemotherapeutic options available for patients remain inadequate, suggesting that adjunctive immunotherapies could have a therapeutic role. Each infecting *Leishmania* species elicits a different immune response that is speculated to be influenced by host genetics. The animal models to study host-pathogen interactions for the different leishmaniases have been a remarkable tool, but the differences in the disease manifestations pose a paradox. Investigations to fully understand the development of chronic and progressive visceral leishmaniasis are ongoing. The mechanisms underlying the pathogenesis and the failure of the host to clear the infection are not completely understood. A thorough understanding of the immunopathogenesis of progressive visceral leishmaniasis can aid in the development of new therapeutics and vaccines.

OBJECTIVES OF THE DISSERTATION

The main objective of this dissertation is to study the host immune response in the experimental hamster model of progressive visceral leishmaniasis. This model was chosen because we want to understand the immunological determinants that permit, or lead to, progressive fatal disease. The second chapter discusses the use of RNA sequencing and a *de novo* assembled hamster transcriptome to identify differentially expressed T cell-related genes at the site of chronic infection (spleen). This part of my study provides some broad insights into T cell function in VL. The third chapter characterizes the $CD4^+$ T cell population responding during chronic disease within the complex inflammatory environment of the chronically infected spleen. Lastly in chapter 4, the interaction of the responding $CD4^+$ T cells with infected macrophages is evaluated.

Together, the experimental data in this dissertation will add to the understanding of the ineffective T cell response during chronic and progressive visceral leishmaniasis. It is hoped that this information can contribute to improved therapeutics and potential vaccine candidates for this disease.

CHAPTER 2: TRANSCRIPTIONAL PROFILING OF THE EXPERIMENTAL CHRONIC VISCERAL LEISHMANIASIS HAMSTER MODEL

Introduction

We used the Syrian golden hamster to study the immunopathogenesis of visceral leishmaniasis. Hamsters are susceptible to infection with *L. donovani* and progress to chronic disease and death. The clinical signs in infected hamsters are similar to chronic human visceral leishmaniasis. The systemic infection leads to uncontrolled parasite replication in the spleen and liver in both the hamster model and in human patients. Both hamsters and humans with visceral leishmaniasis have a fatal outcome in the absence of treatment. The cytokine response in hamsters with progressive visceral leishmaniasis has been studied previously [59, 60, 62, 63]. It mimics what is found in humans with active disease [25, 64, 65]. The complexity of the cytokine response has raised questions about the mechanistic importance of the elevated markers of Th1 and Th2 $CD4^+$ T cells.

RNAseq was used to identify differentially expressed genes in 28-day chronically infected hamster spleens compared to uninfected controls. RNAseq is a high-throughput sequencing approach that provides efficient genome-wide analyses for improved gene identification during disease in a specific tissue. The advantage of using RNAseq for the hamster model is that it allows the assembly of transcripts without prior knowledge of sequences (since a fully sequenced and annotated hamster genome is not available). This technology also has a large dynamic range of expression levels that common microarray techniques do not detect because the gene expression is too low or too high. It is a rapid and efficient technique that produces large data sets that can be used for large-scale genetic analysis. Briefly, RNA is reverse transcribed to cDNA that is then sequenced resulting in small sequences, or reads, that are used to align to reference transcripts or

assemble a transcriptome *de novo.* The result is a map of the entire transcriptome during a disease [66]. By mapping the entire transcriptome, we will gain a global outlook on the molecular constituents in the spleen during visceral leishmaniasis that will aid in understanding the development of disease. For the scope of this dissertation, transcripts related to $CD4^+$ T cells were selected for analysis. We found transcripts for several markers of $CD4^+$ T cell activation and inhibition of effector function to be significantly upregulated in 28-day infected hamsters compared to uninfected controls.

Materials and Methods

PARASITES

Leishmania donovani (MHOM/SD/001S-2D) promatigotes were cultured in M199 media supplemented with 0.1mM adenine (in 50mM HEPES), 5g/mL hemin (in 50% triethanolamine), 20% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and grown at 26°C with no CO_2. Parasites were passaged every 5-7 days. For infections, metacyclic promastigotes were isolated from early passage 7-day cultures by peanut agglutination as previously described. *Leishmania donovani* promastigote infectivity was maintained by regular *in vivo* passages through Syrian golden hamsters.

HAMSTERS

Age matched female outbred Syrian golden hamsters (*Mesocricetus auratus*) were obtained from Harlan Laboratories. They were kept in conditions following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The research protocol was approved by the Institutional

Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas where all animal experiments were conducted.

IN VIVO INFECTIONS

Hamsters were used when 6-8 weeks old. They were infected by intracardiac injection using 10^6 metacyclic *L. donovani* promastigotes in 50μL Dubelcco's Modified Eagle's Medium (DMEM) or Phosphate Buffered Saline (PBS). The total spleen of uninfected (n=4) and 28 day infected (n=4) hamsters were collected for RNA isolation using the RNAqueous Micro Total RNA isolation kit and protocol (Ambion).

TRANSCRIPTIONAL PROFILING BY RNA SEQUENCING

Next generation sequencing was carried out as described in a recent submitted manuscript and depicted in Figure 2.1. In short, the total RNA was used to construct libraries for deep sequencing using the Illumina TruSeq RNA Sample Preparation Kit. Agilent Bioanalyzer confirmed the quality of the library and Truseq SBS kit v3 was used to sequence paired-end 50 base reads on an Illumina HiSeq 1000. *De novo* assembly of the hamster genome required removal of reads that aligned to the *Leishmania donovani* BPK282A1 genome. *De novo* assembly of a complete hamster transcriptome was performed using Trinity and BRANCH software in collaboration with Texas Advanced Computing Center (TACC) at the University of Texas at Austin. At a false discovery rate (FDR) cutoff of <0.01 and fold change (FC) cutoffs being ≥2 or ≤-2, a total of 4,360 transcripts were found to be differentially expressed. Gene identification was achieved by BLAST alignment to the *Rattus norvegicus* and *Mus musculus* reference genomes as explained in a newly submitted manuscript. To explore the biological context of the differentially expressed genes during chronic *L. donovani* infection in the experimental hamster model, the upregulated (≥2 FC) or downregulated (≤-2 FC) genes were uploaded into WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) online software using the

Mus musculus reference genome and matched gene symbols [67]. WikiPathways Enrichment Analysis was run using default settings and a significance level cutoff of 0.01. Entrez Gene IDs were translated back to Gene symbols using UniProt resource database [68].

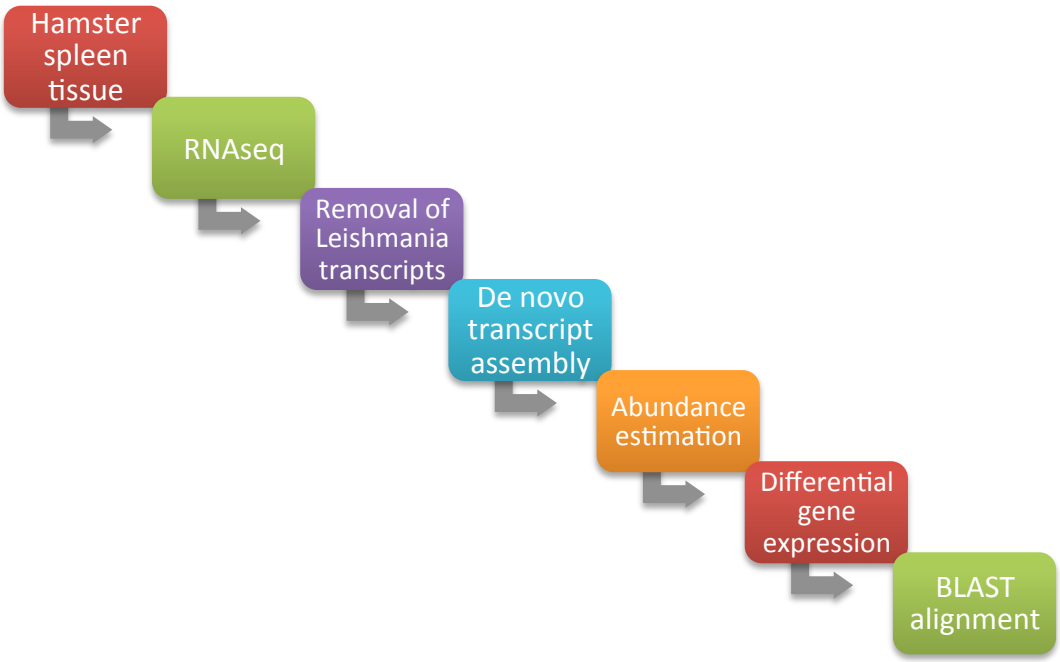


Figure 2. 1 Flow chart of methods for RNA sequencing

Results

PROGRESSIVE INCREASE OF PARASITE BURDEN IN EXPERIMENTAL VISCERAL LEISHMANIASIS

There have been several studies characterizing the hamster as an experimental model for chronic visceral leishmaniasis [59, 69-71]. The hamster is highly susceptible to developing chronic disease when inoculated with *L. donovani* [58]. It models the clinicopathological features seen in humans, including progressive cachexia, relentless

visceral parasite replication, splenomegaly, pancytopenia and death [58, 59]. The increase in organ size is first seen at about two weeks of infection with the spleen doubling in size. Parasite replication is significantly higher by this time and progressively increases until death. By the time hamsters have succumbed to the fatal disease, the spleen is ~7 times the size of an uninfected animal. The muscle wasting and weight loss is evident in hamsters after approximately 2 months of infection with 18% body mass lost [59, 69]. For the purpose of this dissertation project, hamsters were used at 28 days of chronic infection when the spleen size and parasite burden are drastically increased [60, 62]. The progressive increase in splenic parasite burden is shown in Figure 2.2.

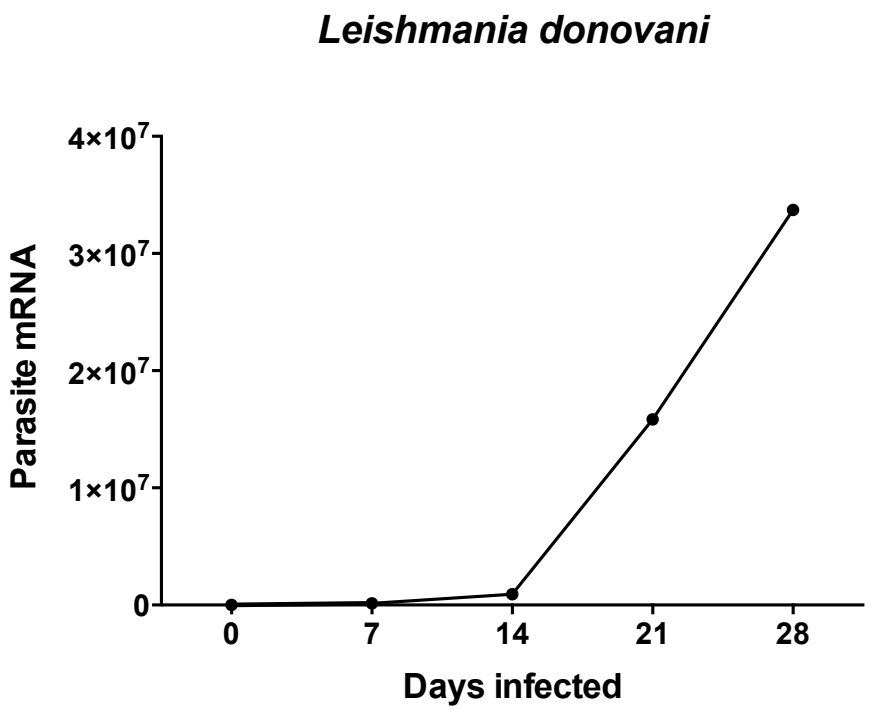


Figure 2. 2 Splenic parasite burden over course of chronic infection. Hamsters were infected by intracardiac injection with 1 million metacyclic wild type *Leishmania donovani* promastigotes. Total spleen tissue was collected and immediately processed to detect *L. donovani* 18s mRNA for each time point (0=uninfected, 7, 14, 21, 28 days infected).

ENRICHMENT OF PATHWAYS THAT INFLUENCE T CELL ACTIVATION AND FUNCTION DURING CHRONIC VISCERAL LEISHMANIASIS

Differentially expressed genes were identified by calculation of the False Discovery Rate (FDR) to control for false positives among multiple comparisons. With a FDR of ≤0.01, we discovered a total of 4,360 genes differentially expressed during chronic visceral leishmaniasis (2,340 upregulated and 2,020 downregulated). Molecular pathways that were upregulated in the spleen during chronic disease were identified. Genes from the differentially expressed gene data set that met the FC≥2 and FDR≤0.01 cutoffs were uploaded into WebGestalt and WikiPathways Enrichment Analysis was performed. The pathways that were enriched with $p \leq 0.01$ and involved in $CD4^+$ T cell activation and effector function were selected with the list of genes involved in the signaling pathway. The FC for each gene in the selected pathways was pulled from the RNAseq data set. The gene signaling events involved in the enriched pathways during chronic disease compared to control are described in detail below.

The IFNγ signaling pathway is the most enriched pathway involved in T cell activation in chronically infected hamster spleen tissue (*adj p*=2.5E-9) (Table 2.1). When IFNγ (FC=52.2) binds its IFNγ receptor it activates the pathway. Upon initiation of the IFNγ pathway, a signaling cascade involves activation and autophosphorylation of Janus Kinase 1 (JAK1), which phosphorylates JAK2. This leads to the recruitment and binding of STAT1 (FC=3.4) to the IFNγ receptor. The STATs also become phosphorylated for release and formation of STAT1 homodimer, which is translocated into the nucleus. The STAT1 homodimer binds the IFNγ activated sequence (GAS) promoter element to begin transcription of interferon-stimulated genes (ISG) [72]. Among these include upregulated genes in the hamster transcriptome pathway analysis: CXCL9 (FC=29.4), CXCL10 (FC=12.9), IRF2 (FC=6.6), ISG15 (FC=3.8), IRF1 (FC=3.2), IFIT2 (FC=2.8),

ICAM1 (FC=2.7), and IRF4 (FC=2). IFNγ also drives the differentiation of Th1 $CD4^+$ T cells and classically activated macrophages (M1).

The second most enriched signaling pathway involved in T cell activation is the T cell receptor pathway (*adj p*=7.67E-7). T cell receptor engagement with antigen presenting MHC molecules induces T cell activation. The first molecules activated in the T cell receptor pathway are Src (FC=10.1) family kinase members Lck (FC=2.2) and Fyn (FC=10.1). Lck is expressed in all naïve T cells to maintain homeostasis and prevent activation of T cells to self-antigen. If foreign-antigen is detected, Lck and Fyn bind the cytoplasmic tail of the T cell receptor complex to initiate the signaling cascade. Lck induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) that signals recruitment of Zap70 (FC=2.6) to also bind the T cell receptor complex, which then gets phosphorylated by Lck. Zap70 induces phosphorylation of linker for activation of T cells (LAT) that recruits several molecules to form the LAT signalosome, which initiates signaling cascades leading to T cell gene expression through assisting transcription factor translocation into the nucleus [73, 74].

The IL-2 signaling pathway is another enriched signaling pathway during chronic hamster visceral leishmaniasis (*adj p*=9.6E-6). IL-2 is known to induce T cell differentiation and maintain T cell homeostasis. IL-2 binds to its receptor complex made of IL-2Rα/CD25, IL-2Rβ/CD122 (FC=2) and the common cytokine receptor gamma chain/CD132 to induce pathway signaling [75]. Signal transduction begins with JAK1 and JAK3 autophosphorylation, which initiates the STAT5, phosphoinositide kinase 3 (PI3K/Akt) and/or mitogen-activated protein kinase (MAPK) pathways to induce transcription of genes including IL-2Rα and more IL-2 to maintain cytokine levels [76].

The IL-4 signaling pathway (*adj p*=2E-4) plays a role in the differentiation of Th2 $CD4^+$ T cells. IL-4 is produced by T cells and APCs and binds its receptor complex expressed on T cells, B cells and APCs. There are two types of IL-4 receptor complexes, type 1 made of the IL-4Rα chain and common γ chain and type 2 made of IL-4Rα chain

and IL-13Rα1 chain, which either IL-4 or IL-13 can bind to. Signaling through the IL-4 pathway leads to JAK 1 activation and phosphorylation of STAT6. The dimerization and translocation of STAT6 to the nucleus leads to transcription of type 2 cytokines, IL-4 and IL-13 [77].

These data show evidence of T cell activation and signaling of Th1 and Th2 pathways in the spleen during chronic visceral leishmaniasis.

Table 2. 1 Pathway analysis

Pathway	Gene Symbol	Name	FC
Type II interferon signaling (IFNγ)	Ifng	Interferon gamma	52.2
Adjusted p value = 2.5E-9	Cxcl9	C-X-C motif chemokine 9	29.4
	Cxcl10	C-X-C motif chemokine 10	12.9
	Gbp2b	Guanylate binding protein 1	11.2
	Irf2	Interferon regulatory factor 2	6.6
	Socs1	Suppressor of cytokine signaling 1	5.3
	Socs3	Suppressor of cytokine signaling 3	4.4
	Cybb	Cytochrome b-245 heavy chain	4.1
	Isg15	Ubiquitin like protein ISG15	3.8
	Stat1	Signal transducer and activator of transcription 1	3.4
	Irf1	Interferon regulatory factor 1	3.2
	Ifit2	Interferon induced protein with tetracopeptide repeats 2	2.8
	Tap1	Antigen peptide transporter 1	2.7
	Icam1	Intercellular adhesion molecule 1	2.7
	Stat2	Signal transducer and activator of transcription 2	2.1
	Irf4	Interferon regulatory factor 4	2
T cell receptor signaling	Tuba4a	Tubulin alpha-4A chain	601.1
Adjusted p value = 7.67E-7	Fyn	Proto-oncogene c-Fyn	10.1
	Src	Neuronal proto-oncogene tyrosine-protein kinase Src	10.1
	Sos2	Son of sevenless homolog 2	7.2
	Sh2d2a	SH2 domain containing protein 2A	6.5
	Skap1	Src kinase-associated phosphoprotein 1	4
	Abi1	Abelson interactor 1	4
	Cd8a	T cell surface glycoprotein CD8 alpha	3.8
	Cebpb	CCAAT/enhancer-binding protein beta	3.7
	Stat1	Signal transducer and activator of transcription 1	3.4
	Ptk2b	Protein-tyrosine kinase 2-beta	3
	Tubb5	Tubulin beta-5 chain	2.8
	Cd2	T cell surface antigen CD2	2.7
	Ptprc	Protein tyrosine phosphatase receptor type C	2.7
	Zap70	Tyrosine-protein kinase zeta chain associated protein-70	2.6
	Stat5a	Signal transducer and activator of transcription 5A	2.2
	Stat5b	Signal transducer and activator of transcription 5B	2.2
	Lck	Proto-oncogene tyrosine-protein kinase LCK	2.2

Pathway	Gene Symbol	Name	FC
IL-2 signaling *Adjusted p value = 9.6E-6*	Plcb1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1	347.1
	Fyn	Tyrosine protein kinase Fyn	10.1
	Socs1	Suppressor of cytokine signaling 1	5.3
	Socs3	Suppressor of cytokine signaling 3	4.4
	Ets2	E26 avian leukemia oncogene 2,3' domain	3.7
	Stat1	Signal transducer and activator of transcription 1	3.4
	Ptk2b	Protein tyrosine kinase 2 beta	3
	Icam1	Intercellular adhesion molecule 1	2.7
	Stat5a	Signal transducer and activator of transcription 5 alpha	2.2
	Stat5b	Signal transducer and activator of transcription 5 beta	2.2
	Lck	Proto-oncogene tyrosine-protein kinase LCK	2.2
	Hsp90aa1	Heat shock protein 90 alpha class A member 1	2.1
	Il2rb	Interleukin 2 receptor subunit beta	2
IL-4 signaling *Adjusted p value = 2.0E-4*	Fyn	Tyrosine protein kinase Fyn	10.1
	Src	Neuronal proto-oncogene tyrosine-protein kinase Src	10.1
	Socs1	Suppressor of cytokine signaling 1	5.3
	Ncf1	Neutrophil cytosol factor 1	4.4
	Socs3	Suppressor of cytokine signaling 3	4.4
	Stat1	Signal transducer and activator of transcription 1	3.4
	Il13ra1	Interleukin 13 receptor subunit alpha 1	3.2
	Bcl2l1	B cell lymphoma 2 like protein	3.1
	Stat5a	Signal transducer and activator of transcription 5 alpha	2.2
	Lck	Proto-oncogene tyrosine-protein kinase LCK	2.2

DIFFERENTIALLY EXPRESSED GENES RELATED TO T CELL ACTIVATION AND FUNCTION DURING CHRONIC VISCERAL DISEASE

In addition to the unbiased approach to identify pathway enrichment shown above, genes known to be involved in $CD4^+$ T cell activation or effector function were manually curated (Table 2.2). Only transcripts with BLAST scores >400 were selected, and for transcripts with multiple entries the one with the highest BLAST score was used. The upregulated genes showed a mix of $CD4^+$ T cell activation/effector and exhaustion

markers. The simultaneous increase in activation and exhaustion markers is an interesting phenomenon that suggests dysfunctional T cell effector function. The cell populations expressing these markers will be investigated in later chapters.

Table 2. 2 Genes related to T cell activation and function

Gene Symbol	Protein Name	Blast Score	FC
IFNG	Interferon gamma	706	52.2
LAG3	Lymphocyte activation gene 3	1844	27.3
PDCD1LG2	Programmed cell death 1 ligand 2	1328	26.9
CCR5	C-C chemokine receptor 5	1737	19.8
TNFRSF4	OX40, Tumor necrosis factor receptor superfamily member 4	1317	9.7
CTLA4	Cytotoxic T-lymphocyte associated protein 4	1968	7.5
TNF	Tumor necrosis factor alpha	1076	6.8
IL12RB2	Interleukin 12 receptor subunit beta 2	2852	4.8
CXCR3	C-X-C chemokine receptor 3	1954	3.6
PDCD1	Programmed cell death 1	1682	3.3
TNFRSF9	CD137, Tumor necrosis factor receptor superfamily member 9	567	3.1
TBX21	Tbet, T-box transcription factor 21	2953	3.0
PTPRC	CD45 antigen, protein tyrosine phosphatase receptor type c	4538	2.7
TNFRSF14	CD270, Tumor necrosis factor receptor superfamily member 14	812	2.6
CD274	Programmed cell death 1 ligand 1	1633	2.6
CD44	CD44 antigen	2913	2.4
IL2RB	Interleukin 2 receptor subunit beta	1921	2.0
CD4	Cluster of differentiation 4	1344	1.6
IL-10	Interleukin 10	1397	-1.27

NETWORK CONNECTION BETWEEN DIFFERENTIALLY EXPRESSED GENES DURING CHRONIC VISCERAL LEISHMANIASIS

The genes identified in the signaling pathways that are upregulated during chronic disease overlapped with the other enriched signaling pathways (Table 2.1). The genes shared between the IFNγ, IL-2 and IL-4 signaling pathways included SOCS1 (FC=5.3), SOCS3 (FC=4.4) and STAT1 (FC=3.4). The suppressor of cytokine signaling (SOCS) proteins play a role in inhibiting cytokine signaling pathways. SOCS1 has been found to inhibit Janus Kinase (JAK) signaling in the IFNγ and TLR4 pathways. SOCS3 inhibits the IL-6 and IL-10 cytokine pathways through its kinase inhibitory region [78]. Together, SOCS1 and SOCS3 have been found to reduce the response of macrophages to IL-6 and IFNγ stimulation [79]. STAT1 is a transcription factor involved in the JAK-STAT pathway induced by IFNγ, which is also inhibited by the SOCS proteins [78]. The IFNγ signaling pathway is the most enriched and the gene IFNγ has the highest FC (52.2) of the upregulated cytokines (Table 2.1). This could suggest the production of the SOCS proteins is a regulatory response to dampen the massive inflammation in the spleen during chronic disease. The enriched TCR, IL-2 and IL-4 signaling pathways share the upregulated genes Fyn (FC=10.1), Lck (FC=2.2), STAT1 (FC=3.4) and STAT5a (FC=2.2). Fyn and Lck are members of the Src family kinases, which aid in the early events of TCR signal transduction for T cell development, activation and homeostasis [73]. IL-2 is a cytokine involved in T cell differentiation, proliferation and apoptosis. Once a naïve T cell encounters antigen, it activates the production of IL-2 and its receptor for a positive feedback loop [80]. IL-4 is a type 2 cytokine involved in $CD4^+$ Th2 differentiation that was recently discovered to require Fyn for its production [81]. The gene sharing between the enriched signaling pathways suggests pathway crosstalk during chronic visceral leishmaniasis in hamsters.

Discussion

In this chapter, we gained a global view of tissue-specific gene activity during chronic visceral leishmaniasis. This set a platform for understanding the biological relevance and function of upregulated genes during disease at the site of infection. We chose to conduct our studies in the hamster as it closely models the chronic and progressive disease seen in visceral leishmaniasis patients. We confirmed the relentless increase in parasite burden over the course of the first 28 days in *Leishmania donovani* infected hamsters (Figure 2.2). Previous studies in this model have shown the parasite burden continuously increases until the hamster's death [59]. A closer look into the splenic immune response to chronic infection using a model that mimics human chronic disease will help answer the question of why the host cannot control parasite replication and development of chronic disease. We present the gene set enrichment analysis from a perspective of $CD4^+$ T cell activation and function.

The role of $CD4^+$ T cells in chronic visceral leishmaniasis has been widely studied in the experimental animal models and in human patients. We show pathways involved in T cell receptor (p=7.67E-7) and signaling pathways for cytokines IFNγ (p=2.5E-9), IL-2 (p=9.6E-6) and IL-4 (p=2.0E-4) to be enriched in chronically infected hamster spleen tissues (Table 2.1). IFNγ is involved in the differentiation of Th1 $CD4^+$ T cells, which also produce IL-2. The increase of IFNγ in active visceral leishmaniasis patients is well characterized [82, 83]. The activity of IFNγ in the spleen was evident by the large number of IFN-responsive genes that were upregulated. IL-4 is a type 2 cytokine that induces differentiation of Th2 $CD4^+$ T cells that produce more IL-4. The Th2 response seen in patients was thought to be responsible for disease pathology, but this hypothesis has since been revised [24, 25]. The pathway analysis data correlates with reports of mixed Th1 and Th2 immune responses in active visceral leishmaniasis. The dysregulation of multiple genes in connected pathways is an important consideration

when understanding a complex disease like visceral leishmaniasis and for identifying targets for development of host-directed therapeutics.

Focusing on $CD4^+$ T cells, a manually curated gene set was generated highlighting genes considered to be relevant to active visceral leishmaniasis according to literature review and our current avenues of research (Table 2.2). IFNγ was the most highly upregulated cytokine in the spleen during chronic visceral leishmaniasis in the hamster (FC=52.2). This is surprising given that IFNγ mediates macrophage-induced killing of *Leishmania* parasites [84-86]. While the requirement for IFNγ in parasite control is well established, these data corroborate other reports that indicate it is not sufficient. The gene TBX21, or Tbet, (FC=3.0) is the master transcription factor for Th1 $CD4^+$ T cells, which are a significant source of IFNγ. Th1 CD4+ T cells are characterized by chemokine receptors CCR5 (FC=19.8) and CXCR3 (FC=3.6), which aid in cell recruitment. PTPRC, or CD45, is expressed on all lymphocytes and plays a crucial role in T cell activation. It is a phosphatase that regulates the activation of Lck (FC=2.2) and Fyn (FC=10.1) in the TCR signaling pathway [87]. CD44 is a surface marker expressed on activated T cells and promotes the differentiation of memory Th1 $CD4^+$ T cells [88]. These upregulated genes favor a protective T cell response during experimental chronic visceral leishmaniasis.

Despite the increased expression of genes involved in T cell activation, several upregulated genes were suggestive of T cell exhaustion (Figure 2.3). CTLA-4 (FC=7.5) and PDCD1, or PD-1 (FC=3.3), are common receptors known to negatively regulate the activation of T cells. The expression of these inhibitory receptors on $CD4^+$ and $CD8^+$ T cells in models of visceral leishmaniasis has been demonstrated [89-91]. Both known ligands of PD-1, PD-L1 (FC=2.6) and PD-L2 (FC=26.9), were significantly increased as well. The common conception of T cell exhaustion is if expression of an inhibitory receptor is found, it marks T cell exhaustion. But a recent study suggests the co-expression of inhibitory receptors and markers of T cell activation or differentiation is

rather linked to T cell activation [92]. The expression and role of inhibitory molecules have yet to be defined in the hamster model of chronic *L. donovani* infection. The effector function of $CD4^+$ T cells, despite increased genes of T cell exhaustion, will be investigated in later chapters.

The transcriptome of hamsters with chronic visceral leishmaniasis provides insight into the molecular functions and pathways involved in the disease pathogenesis. It is a basis for identifying key genes that could be potential targets for development of new immunotherapeutics. The next chapter uses these targets to characterize immune cells involved in cell-mediated immunity.

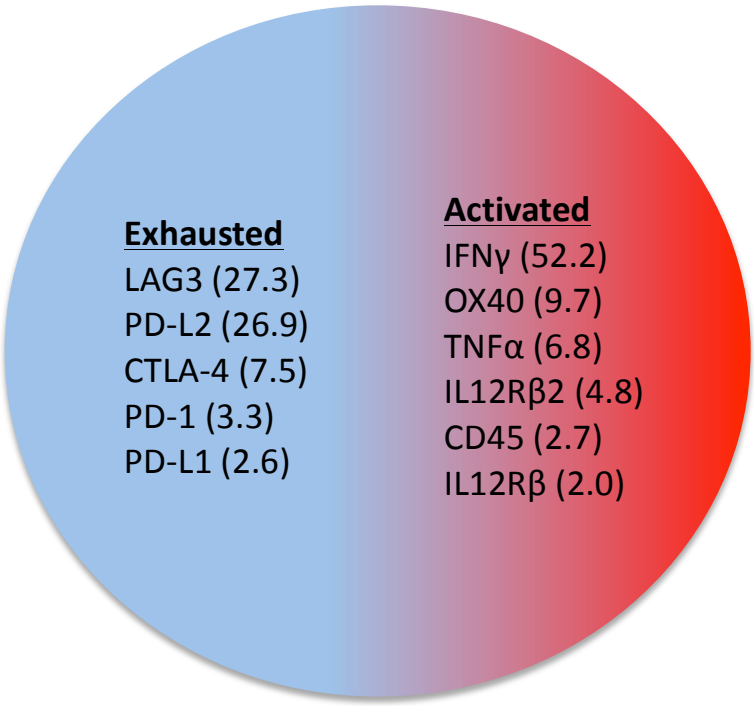


Figure 2. 3 Markers of T cell exhaustion and activation simultaneously increased. Gene symbols with corresponding fold change in parentheses taken from Table 2.2.

CHAPTER 3: PROFILE OF $CD4^+$ T CELLS THAT ACCUMULATE IN THE SPLEEN DURING CHRONIC VISCERAL LEISHMANIASIS

Introduction

VISCERAL LEISHMANIASIS

The most serious form of leishmaniasis is the visceral form. It differs from the other leishmaniases in that it is a systemic infection that affects the spleen, liver and bone marrow. Visceral leishmaniasis is most prevalent in South Asia (India, Bangladesh, and Nepal), Africa (Sudan, Ethiopia and Kenya), and South America (Brazil). The main causative agents of visceral leishmaniasis are *Leishmania donovani* and *L. infantum/chagasi*. It is transmitted by female phlebotomine sandflies. A majority of infected individuals have a subclinical infection with no sign of disease. In contrast, about 10% of infected patients experience uncontrolled visceral parasite replication resulting in chronic progressive disease. Patients suffering from active visceral leishmaniasis have fatal outcomes if effective treatment is not given [93].

In humans, chronic visceral leishmaniasis symptoms include a long term, low-grade fever, cachexia, pancytopenia and splenomegaly. They have increased type 1 cytokines in serum such as IFNγ, IL-1, IL-6, IL-12 and TNFα [25, 64]. Patients also have increased type 2 cytokines like IL-4, IL-5, IL-10 and IL-13 in the serum [24]. It has been established in experimental models of visceral leishmaniasis that control of parasite replication requires an early and strong Th1 response with production of IL-12 and IFNγ [94, 95]. But recent studies in human visceral leishmaniasis have shown this strong Th1 response at the site of infection in active visceral leishmaniasis patients [25]. It is crucial to identify the cellular source of protective and non-protective cytokines for understanding the pathogenesis of this disease and for vaccine development.

ROLE OF $CD4^+$ T CELLS IN VISCERAL LEISHMANIASIS

Visceral leishmaniasis is a very complex disease whose immunopathogenesis is not completely understood. As described earlier, the cell mediated immune response is critical in controlling the infection. $CD4^+$ T cells play a central role in adaptive immune response and outcome of Leishmania infection. Rather than the clear Th1/Th2 dichotomy seen in murine models of cutaneous leishmaniasis, there is a mixed response in chronic human and hamster visceral leishmaniasis. Our understanding of immunity against this chronic disease is further complicated by the discovery of distinct $CD4^+$ T cell subsets that can be protective or pathogenic.

The Th1 $CD4^+$ T cell population is characterized by the production of IFNγ and mediation of an inflammatory macrophage-activating immune response. Other markers of Th1 cells include chemokine receptors such as CCR5 and CXCR3, which aid in the migration of the Th1 cells to sites of inflammation [96]. In the susceptible mouse model of visceral leishmaniasis, there is organ specific immunity. Chronic infection occurs in the spleen and bone marrow while an acute self-resolving infection occurs in the liver. At about 2-4 weeks, there are increased IFNγ levels in the liver leading to granuloma formation and control of parasite replication [55]. Chronic infection develops slowly in the spleen and bone marrow. There is increased parasite replication, splenomegaly and production of the immunosuppressive cytokine IL-10 [97]. After ~8 weeks of infection, visceral parasite replication stabilizes as IFNγ and IL-2 production is increased [54].

On the contrary, in human and hamster, despite having high levels of IFNγ production at the site of infection, there is disease progression [59, 98]. Active visceral leishmaniasis patients present with decreased $CD4^+$ T cell numbers in the blood, but increased numbers in the spleen accompanied by increased IFNγ [25, 65]. This suggests involvement of Th1 $CD4^+$ T cells as a source of inflammation. Along with this ineffective Th1 cellular response, there is also evidence of immunosuppression from

regulatory T cell populations. There have been several studies, both in experimental models and humans, showing increase of IL-10 in the spleen during chronic visceral leishmaniasis [25, 59, 99, 100]. But the source of IL-10 in human visceral leishmaniasis has been controversial. One study found that natural Treg $Foxp3^+$ cells are not a major source of IL-10 in the spleen and not the immunosuppressive population. Instead a $CD4^+Foxp3^-$ cell population was the major immunosuppressive source of IL-10 [25, 101]. However, contradicting findings showed an increase in $Foxp3^+$ T cells in the bone marrow of visceral leishmaniasis patients and these cells were the major source of IL-10 production [102].

Apart from Th1 and Th2 $CD4^+$ T cells subsets, recent findings suggest a protective role for Th17 cells. Th17 cells play a role in the inflammatory immune response as well. The transcription factor, RORγ, is the master regulator of the differentiation of this subset. It is induced by the cytokines TGFβ and IL-6 or IL-21 [103]. Th17 $CD4^+$ T cells are differentiated by their cytokine profile (IL-17A, IL-17F, IL-17AF, IL-21, IL-22) [104]. It was recently discovered that *L. donovani* induces IL-17 and IL-22 cytokine production in healthy patient PBMCs. Th17 cells were the primary source of these cytokines. The levels of IL-17 and IL-22 showed strong association with protection in patients that were resistant to developing disease compared to those that progressed to chronic visceral leishmaniasis [105]. The authors concluded Th17 cells and their products aid in the Th1 response, but are dampened due to high production of immunosuppressive cytokines.

T CELL-ATTRACTING CHEMOKINES

It is crucial that the proper host immune response be elicited to determine the outcome of disease for leishmaniasis. Chemokines are important in the early stages of infection as they recruit immune cells to the initial site of parasite deposition to initiate

host defense [106]. Therefore, it is important that the first responders produce the proper chemokine ligands to recruit the appropriate immune cells.

Chemokines are small inducible cytokine proteins that are chemoattractants for different immune cells [106-108]. They are grouped into different families based on the number of amino acids between the first two cysteine residues; C, CC, CXC and CX3C (Figure 3.1) [107, 109]. Receptors for the chemokine ligands are expressed on multiple immune cells including activated and non-activated antigen presenting cells and T cells. Chemokines and chemokine receptors play a large role in the immune cell network maintaining a homeostatic environment during infection, inflammation and autoimmunity [108]. Their roles consist of immune cell differentiation, inflammatory response, and leukocyte trafficking to secondary lymphoid organs [107]. Several immune cells express chemokines, and specific chemokines play different roles as shown in Table 3.1 [109-111]. Dysregulation of cytokine and chemokine networks have been implicated in many autoimmune diseases and infections. Pathogens have evolved to utilize chemokine systems in the host for their own survival [107, 108, 112].

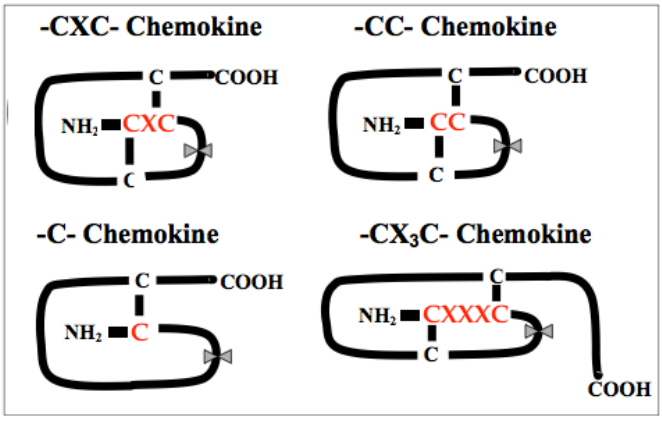


Figure 3. 1 Chemokine structure. Chemokines have a similar structure characterized by three β-sheets and at least one α-helix at the C terminus. The different families of chemokines are grouped based on the cysteine residue spacing originating from the N terminus.

In the case of leishmaniasis, it has been shown *L. major* can induce chemokines that recruit cells that the parasite can hijack for persistent intracellular survival and replication [113, 114]. There is a lack of proper immune mechanisms in *L. donovani* chronically infected patients that keeps them from clearing the parasite. Chemokines and chemokine receptors that are involved in T cell recruitment may be the key to understanding the ineffective immune responses in the spleen during chronic visceral leishmaniasis.

Table 3. 1 Chemokine ligands, receptors and function

Ligand	*Receptor*	*Cells recruited*
CCL1	**CCR8**	Mo, DC, Th2, Treg
CCL2/8	**CCR2**	Mo, NK, DC, T and B cells
CCL3	**CCR1, CCR5**	Mo, MΦ, DC, Th1, NK
CCL4	**CCR5**	Mo, MΦ, Th1, NK
CCL5	**CCR1, CCR3, CCR5**	Mo, MΦ, Th1, Th2, NK, Eo, Ba
CCL7	**CCR1, CCR2, CCR3**	Mo, MΦ, DC, NK, Eo, Ba, Th2
CCL11/24/26	**CCR3**	Eo, Ba, Th2
CCL13	**CCR2, CCR3**	Mo, DC, Eo, Ba, Th2, NK, B cells
CCL14/23	**CCR1**	Mo, MΦ, DC, NK
CCL15/16	**CCR1, CCR3**	Mo, MΦ, DC, NK, Eo, Ba, Th2
CCL17/22	**CCR4**	DC, Th2, NK
CCL18	**Unknown**	DC, Naïve T cells
CCL19/21	**CCR7**	DC, MΦ, T cells
CCL20	**CCR6**	DC, T and B cells
CCL25	**CCR9, CCR11**	T cells
CCL27/28	**CCR10**	T cells
CXCL1/2/3/5/7	**CXCR2**	Neutrophils
CXCL4/9/10/11	**CXCR3**	Th1, NK
CXCL6/8	**CXCR1**	Neutrophils
CXCL12	**CXCR4**	Unknown
CXCL13	**CXCR5**	B cells
CXCL14	**Unknown**	Mo
CXCL16	**CXCR6**	T cells
CX3CL1	**CX3CR1**	Mo, DC, NK, Th1
XCL1/XCL2	**XCR1**	T cells, NK

SPLENIC ARCHITECTURE IN VISCERAL LEISHMANIASIS

The spleen is one of the primary sites of infection during chronic visceral leishmaniasis. It is a secondary lymphoid organ that is important in immunity against blood borne pathogens [115]. It filters the blood of any foreign antigen and removes old red blood cells [116]. The spleen consists of fibrous and connective tissue made of two main compartments known as the red pulp and white pulp. The red pulp mostly contains macrophages and other antigen presenting cells that are involved in the immune response against blood borne pathogens.

Leishmania parasites primarily localize in the red pulp during infection where they are phagocytosed by macrophages [56]. The white pulp of the spleen contains naïve T cells in the periarteriolar lymphoid sheath (PALS) compartment and B cells localized in the B cell zone [115, 116]. During murine subclinical visceral leishmaniasis infection, the T cells are predominantly located in the white pulp until 4 weeks after infection when they migrate into the red pulp where infected macrophages reside [56]. Chronic infection in the mouse spleen leads to destruction of the splenic architecture [56, 97].

This destruction of splenic architecture is also seen in the hamster experimental model of chronic visceral leishmaniasis [117]. But it is unclear about the hamster $CD4^+$ T cell compartmentalization during chronic infection with *L. donovani*. Cytokine and chemokine responses are most commonly measured in the peripheral blood of infected hosts, which does not give a clear understanding of the immune responses in the infected tissue. As demonstrated in the previous chapter, there are increased transcripts of markers for Th1 and Th2 cells in the spleens of chronically infected hamsters. This suggests that $CD4^+$ T cell recruitment is occurring, and evaluation of the chemokine profile of the chronically infected spleen tissue could provide insights into the mechanisms behind this.

Materials and Methods

HAMSTERS AND *IN VIVO* INFECTIONS

Age-matched female outbred Syrian golden hamsters (*Mesocricetus auratus*) were obtained from Harlan Laboratories. Hamsters were used when 6-8 weeks old. They were infected by intracardiac injection using 10^6 metacyclic *L. donovani* (MHOM/SD/001S-2D) promastigotes in 50μL Dubelcco's Modified Eagle's Medium (DMEM) or Phosphate Buffered Saline (PBS). The total spleen of uninfected and 7-, 14-, 21- and/or 28-day infected hamsters were collected for RNA isolation (Qiagen) or isolation of $CD4^+$ T cells.

ISOLATION OF SPLENIC $CD4^+$ T CELLS

Hamsters were infected for 28 days, after which the total spleen was collected in ice cold RPMI 1640 medium supplemented with Glutamax™, 10% heat inactivated FBS, 0.5 mM EDTA (Gibco) and 0.6 μg DNase (Sigma). Spleens were digested for 10 minutes at 37°C by injecting with collagenase D (Roche) at 2 mg/ml in buffer containing (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Hepes pH 7.4). The tissue was further minced and strained through a 100μM cell strainer to obtain a single cell suspension that was plated in large tissue culture flasks to separate adherent cells. Splenocytes were cultured for 30 minutes at 37° C in 5% CO_2 in 10% DMEM culture medium. The non-adherent cell population was collected. More culture medium was added to the culture flasks and cells were cultured for 30 more minutes and the non-adherent cell population was collected once more. The cells were washed once in 10% RPMI and resuspended in 1x red blood cell lysis buffer (0.2mM NH_4Cl, 0.01M $NaHCO_3$, 0.1mM EDTA, pH 7.4) for 10 minutes and washed again with 10% RPMI. Cells were labeled with anti-mouse $CD4^+$ magnetic particles (clone GK1.5) and resuspended in ice-

cold separation buffer (1x PBS, 0.5% bovine serum albumin, 2mM EDTA, pH 7.2). Cells were placed on a BD magnet and separated following manufacture protocol collecting positive selections (BD™ iMag Cell Separation System, BD Biosciences). $CD4^+$ cell populations were resuspended in 10% RPMI and counted for FACs staining and RNA isolation.

FLOW CYTOMETRY

Single cell suspensions of $CD4^+$ splenocytes obtained from 28 day infected and uninfected control hamsters described above were adjusted to a concentration of 500,000-10^6 cells per 100μL of blocking buffer containing 2% normal mouse serum and 2% normal rat serum in PBS. Cells were blocked for 30 minutes at room temperature, stained with surface antibodies, APC-Cy™7 conjugated rat anti-mouse $CD4^+$ or recommended isotype control (BD Biosciences) and FITC conjugated mouse anti-rat TCRα/β or recommended isotype control (AbD Serotec) for 30 minutes in the dark at 4°C followed by extensive washing in PBS with 2% FBS and 0.1% sodium azide. For intracellular staining, cells were fixed/permeabilized using Foxp3/transcription factor staining buffer set and stained with PE-Cy5 conjugated anti-mouse/rat Foxp3 or manufacture recommended isotype control, and eFluor® 660 conjugated anti-human/mouse Tbet or recommended isotype control (eBioscience). All flow cytometric analyses were performed on a Stratedigm SE520EX6 flow cytometer using software CellCapTure v3.1.0. Data were analyzed using FlowJo v10.0.7 (Treestar).

PRIMER DESIGN

Primer sequences were designed for the Syrian Golden hamster using Genscript Primer Design Tool and Ensembl genome database to map exons and introns according to the mouse genome. For some genes, another primer design tool was used, National Center for Biotechnology Information (NCBI) Primer-BLAST which provided a wider

selection of parameters. Target genes for which primers were designed are detailed in Table 3.2. Each primer set was designed to span an intron on the hamster target gene and confirmed by analysis of dissociation curves for true amplification with no primer dimers.

Table 3. 2 Hamster primer sequences designed for real time RT-PCR

Target gene	*Primer sequences*
Tbet	*For:* 5' – ACA AGG GGG CTT CCA ACA AT – 3' *Rev:* 5' – CAG CTG AGT GAT CTC GGC AT – 3'
GATA3	*For:* 5' – GAA GGC AGG GAG TGT GTG AA – 3' *Rev:* 5' – GTC TGA CAG TTC GCA CAG GA – 3'
Foxp3	*For:* 5' – AGG TCT TCG AGG AGC CAG AA – 3' *Rev:* 5' – GCC TTG CCC TTC TCA TCC A – 3'
CCR5	*For:* 5' – TGT GAC ATC CGT TCC CCC T – 3' *Rev:* 5' – GGC AGG GTG CTG ACA TAC TA – 3'
CXCR3	*For:* 5' – CAA GTG CCA AAG CAG AGA AGC – 3' *Rev:* 5' – CAA AGT CCG AGG CAT CTA GCA – 3'
CCR4	*For:* 5' – GCT TGG TCA CGT GGT CAG TG – 3' *Rev:* 5' – GTG GTT GCG CTC CGT GTA G – 3'
CXCL9	*For*: 5' – TGG GTA TCA TCC TCC TGG AC – 3' *Rev*: 5' – AAT GAG GAC CTG GAG CAA AC – 3'
CXCL10	*For:* 5' – TGG AAA TTA TTC CTG CAA GTC A – 3' *Rev*: 5' – GTG ATC GGC TTC TCT CTG GT – 3'
CXCL11	*For:* 5' – TGG CTG TGA TCA GTT GTG CT – 3' *Rev:* 5' – GTG CTT TCA GGG TAA CAA TCA CT – 3'
CCL4	*For:* 5' – TCT CTC TCC TCC TGT TCG TGG – 3' *Rev:* 5' – TTT GCT TGC CTT TTC TGG TCA – 3'
CCL5	*For:* 5' – CTA CGC TCC TTC ATC TGC CTC – 3' *Rev:* 5' – CCT TCG GGT GAC AAA AAC GAC – 3'
CCL17	*For:* 5' – GTG CTG CCT GGA GAT CTT CA – 3' *Rev:* 5' – TGG CAT CCC TGG GAC ACT – 3'
CCL22	*For:* 5' – CGT GGC TCT CAT CCT TCT TGC – 3' *Rev:* 5' – CAG ATG CTG TCT TCC ACG TTG G – 3'
IL-4	*For:* 5' – CCT GCT CTG CCT TCT AGC AT – 3' *Rev:* 5' – GCC CTG CAG ATG AGG TCT TT – 3'
IFNγ	*For*: 5' – AAT ATC TTG ACG AAC TGG CAA A – 3' *Rev*: 5' – CCT TCA AGG CTT CAA AGA GTT T – 3'
IL-10	*For:* 5' – TAA GGG TTA CTT GGG TTG CC – 3' *Rev:* 5' – TTC ACC TGT TCC ACA GCC TTG – 3'
PD-1	*For:* 5' – CTG AAA AGG GTT AAG CCA GC – 3' *Rev:* 5' – GCC TCC AGG ATT CTC TCT GTT – 3'

PD-L1	*For:* 5' – TGA TCA TCC CAG ACC CGC TC – 3' *Rev:* 5' – CTC CTC GAA CTG CGT ATC GT – 3'
PD-L2	*For:* 5' – CAG TAC CGC TGT CTG GTC AT – 3' *Rev:* 5' – CTT CAG GGG TCC TGA TGT GG – 3'
CTLA-4	*For:* 5' – GTT ATT GAA CCA GAA CCA TGC CC – 3' *Rev:* 5' – ACC CCT GTT GTA AGG GGA CT – 3'

DETERMINATION OF GENE EXPRESSION BY REAL-TIME RT-PCR

RNA was isolated using Qiagen RNeasy Mini Kit for concentrations larger than 1ug or Ambion RNAqueous®-Micro Total RNA Isolation Kit for concentrations less than 1ug RNA. Total RNA was DNase treated with Life Technologies Turbo DNA-Free™ kit and reverse transcribed into cDNA according to manufacture protocols (High-Capacity cDNA Reverse Transcription Kit, Life Technologies). Hamster gene expression for primers designed for specific gene targets outlined in Table 3.2 was determined in total spleen tissue, baby hamster kidney cells (BHK) and $CD4^+$ splenocytes by SYBR green PCR on ViiA™ 7 Real-Time PCR System. Data was analyzed using comparative Ct method relative to uninfected BHK controls or uninfected hamster controls and using the 18S ribosomal RNA gene as the normalizer.

STATISTICAL ANALYSIS

Comparison between two groups was performed using Student's T test or Mann-Whitney test (non-parametric). Comparison between more than 2 groups was performed using one-way ANOVA (parametric) or Kruskall-Wallis (non-parametric) depending on the normalcy of the data. A correction for multiple comparisons was used when appropriate. P-values < 0.05 were considered significant. All analyses were conducted using GraphPad Prism version 5.04 for Windows or Mac, GraphPad Software, San Diego, California, USA.

Results

$CD4^+$ T CELLS INCREASE IN SPLEEN DUE TO CHRONIC *LEISHMANIA DONOVANI* INFECTION

Many studies of human patients with active visceral leishmaniasis have analyzed cytokine responses in serum and peripheral blood mononuclear cells (PBMCs). As previously stated, it has been found that patients have increased type 1 and type 2 cytokines in serum [64]. In PBMCs from cured visceral leishmaniasis patients, the major cellular source of IFNγ, IL-10 and IL-4 was found to be $CD3^+CD4^+$ T cells [118]. To examine the $CD4^+$ T cell response in the spleen during experimental chronic visceral leishmaniasis, we compared uninfected with 28 day infected hamsters. Total spleen cells were analyzed using FACs by gating on the $CD4^+$ lymphocyte population (Figure 3.2A). The percentage of $CD4^+$ T cells in the spleen increases during chronic stages of the disease ($p=0.0357$) (Figure 3.2B) although the total number was not significant (Figure 3.2C).

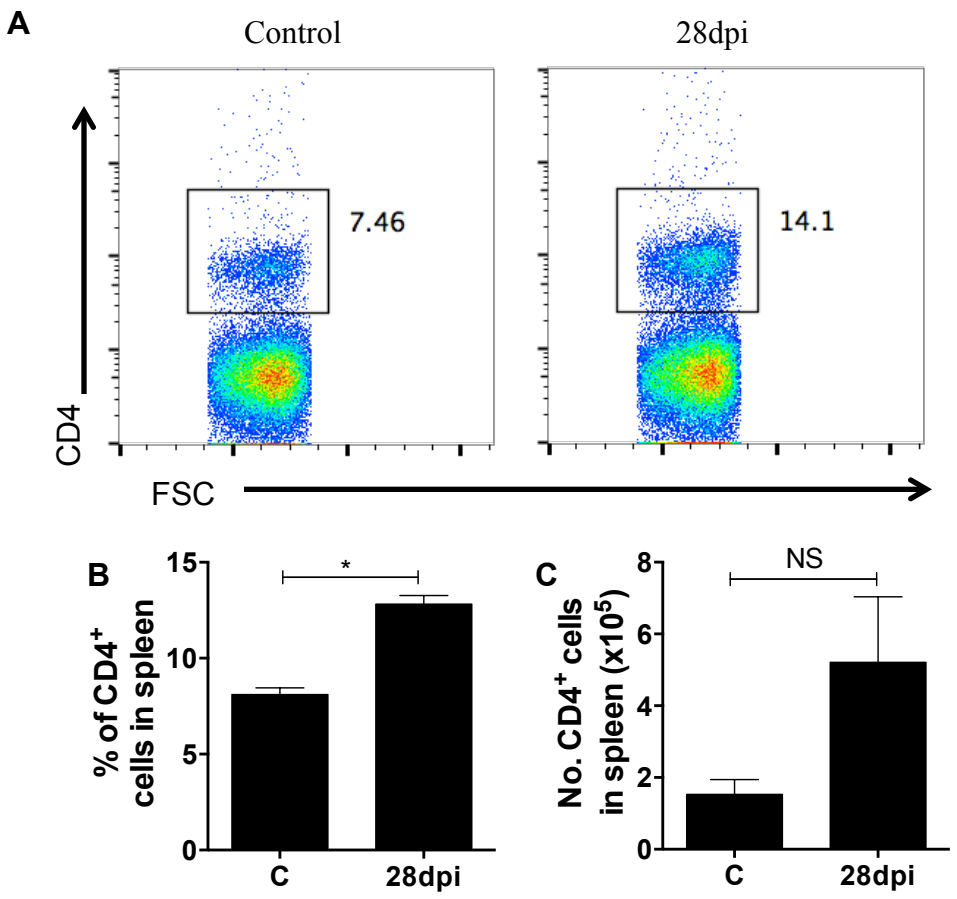


Figure 3. 2 Increase of $CD4^+$ cells in spleen due to chronic infection. Uninfected and 28 day infected total spleen cells were labeled with PE conjugated CD4 antibody. Total lymphocytes were gated on based on FSC and SSC. (A) Frequency of $CD4^+$ lymphocytes from total spleen cells. (B) Percentage and (C) Total number of $CD4^+$ cells in spleen (n=4-6 hamsters). *p<0.05.

INCREASED MARKERS OF TH1 AND TH2 CELLS IN SPLEENS OF CHRONICALLY INFECTED HAMSTERS

During chronic visceral leishmaniasis, patients have high IFNγ in the plasma, spleen and bone marrow [25, 64]. Along with this strong Th1 response, patients that progress to chronic disease also have increased IL-4, IL-5, IL-10 and IL-13 in the spleen and serum [24, 25]. This suggests there is a mixed population of $CD4^+$ T cells accumulating in the spleen during chronic infection. To explore this possibility we

determined mRNA expression of Th1, Th2 and Treg markers in the spleens of *L. donovani* infected hamsters over the course of disease.

We found mRNA expression of the Th1 transcription factor, Tbet, and the inflammatory cytokine commonly produced by Th1 $CD4^+$ T cells, IFNγ, to be increased throughout the chronic stages of infection (Figure 3.3). This finding is counterintuitive in that the parasite burden is increasing in parallel over the course of infection (Figure 2.2), and IFNγ should be a protective, macrophage activating cytokine. However, a similar increase in markers for Th2 cells, which are considered to be disease-promoting, was seen in infected hamster spleen tissue. The master regulator transcription factor for Th2 $CD4^+$ T cells, GATA3, and the commonly associated Th2 cytokine, IL-4, were significantly increased throughout the course of disease (Figure 3.3). In addition to the mixed Th1 and Th2 markers being upregulated, mRNA of the transcription factor that regulates the differentiation of Treg $CD4^+$ T cells, Foxp3, was transiently increased at 14 days post-infection (Figure 3.3). This could suggest a regulatory $CD4^+$ T cell response early in infection before Th1 and Th2 $CD4^+$ T cells significantly increase. The regulatory cytokine produced by Treg, Th2 and some Th1 cells, IL-10, shows significant increase in the spleen up to 28 days of infection (Figure 3.3).

Overall, these data suggest there is a mixed population of $CD4^+$ T cell subsets responding throughout the course of infection. Alternatively, these cytokines can be produced by cells other than $CD4^+$ T cells.

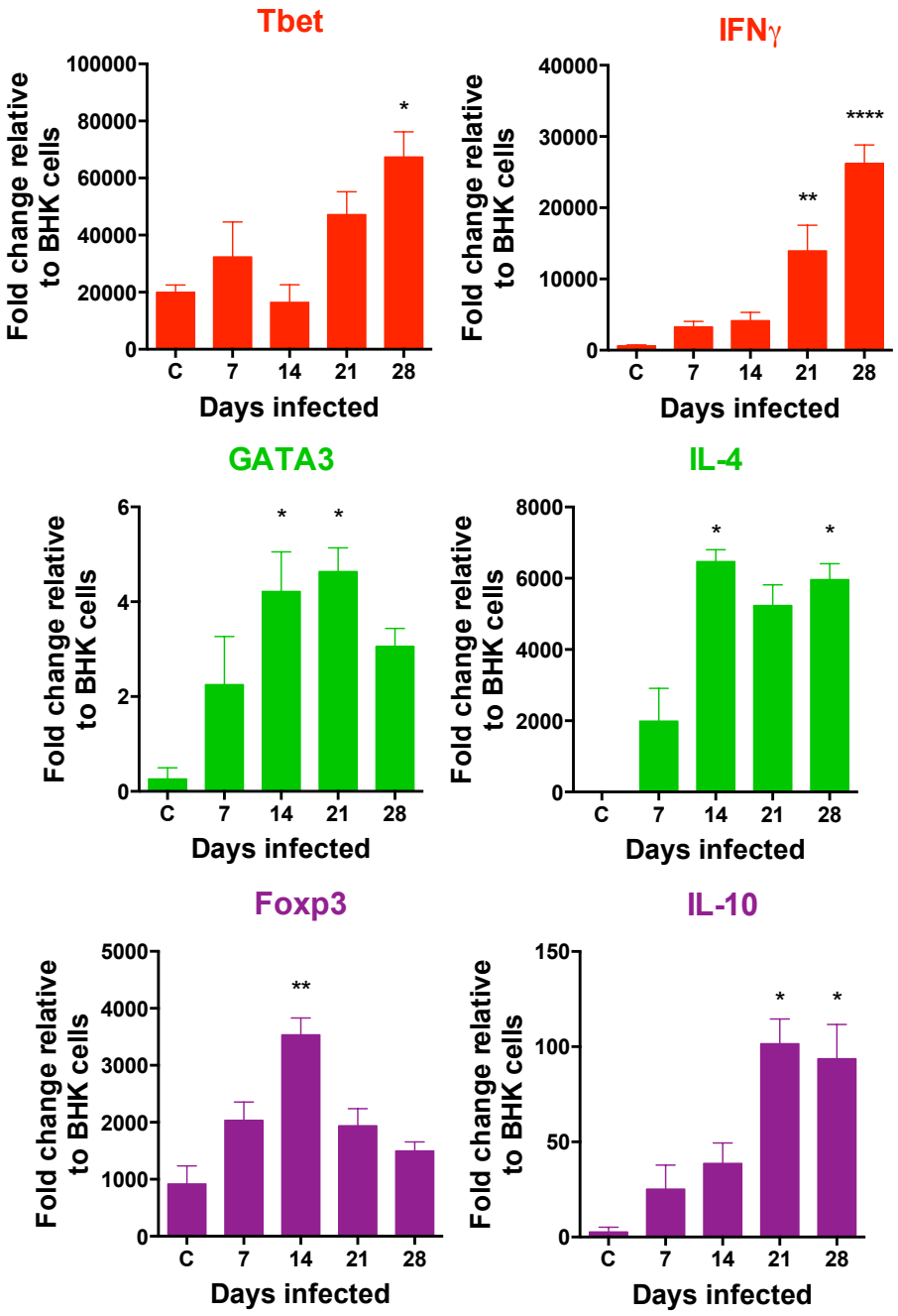


Figure 3. 3 Markers of CD4$^+$ T cell subpopulations. Hamsters were infected by intracardiac injection with 1 million *L. donovani* metacyclic promastigotes and total spleen was collected at 7, 14, 21 and 28 days post-infection. RNA was isolated from total spleen tissue and processed for detecting expression of target genes by real time RT-PCR compared to uninfected animals (C). Fold change was calculated relative to basal gene expression in uninfected baby hamster kidney (BHK) cell line. (n=3-6) Figures are representative of at least 3 independent experiments. *p<0.05, **p<0.01, ****p<0.0001. ■Th1-associated genes ■Th2-associated genes ■Treg-associated genes.

MIXED CHEMOKINE RESPONSE IN SPLEEN OF CHRONICALLY INFECTED HAMSTERS

Chemokines are a diverse group of chemoattractants important for recruitment of immune cells and determination of the type of immune response that will be initiated [112]. There are many immune cells that accumulate in the spleen during chronic progressive visceral leishmaniasis. The differential expression of chemokines has a major role in determining the nature of the inflammatory infiltrate. Chemokines have a critical role in homing, priming and directing differentiation of T cells [106, 107, 119] (Figure 3.4).

We investigated the mRNA expression of Th1 (type 1), Th2 (type 2) and Treg (regulatory type) $CD4^+$ T cell-attracting chemokine ligands and their receptors in the spleens of chronically infected hamsters. We found mRNAs of the type 1 chemokine ligands and their receptors (CCL4, CCL5, CCR5, CXCL9, CXCL10, CXCL11 and CXCR3) increased at 21-28 days post-infection compared to uninfected controls (Figure 3.5). Expression of the type 2 chemokine ligands, CCL17 and CCL22 increased as early as 7 days post-infection and stayed significantly upregulated up to 21 days of infection (Figure 3.5). The type 2 chemokine ligands were upregulated, but somewhat surprisingly their known receptor, CCR4, was not (Figure 3.5). It could be that the recruitment of Th2 and Treg $CD4^+$ T cells is dependent on another chemokine receptor known to be expressed on Th2 and Treg cells, such as CCR8. Together, this data suggests a mixed chemokine response, which could translate to a mixed recruitment of $CD4^+$ T cell subsets.

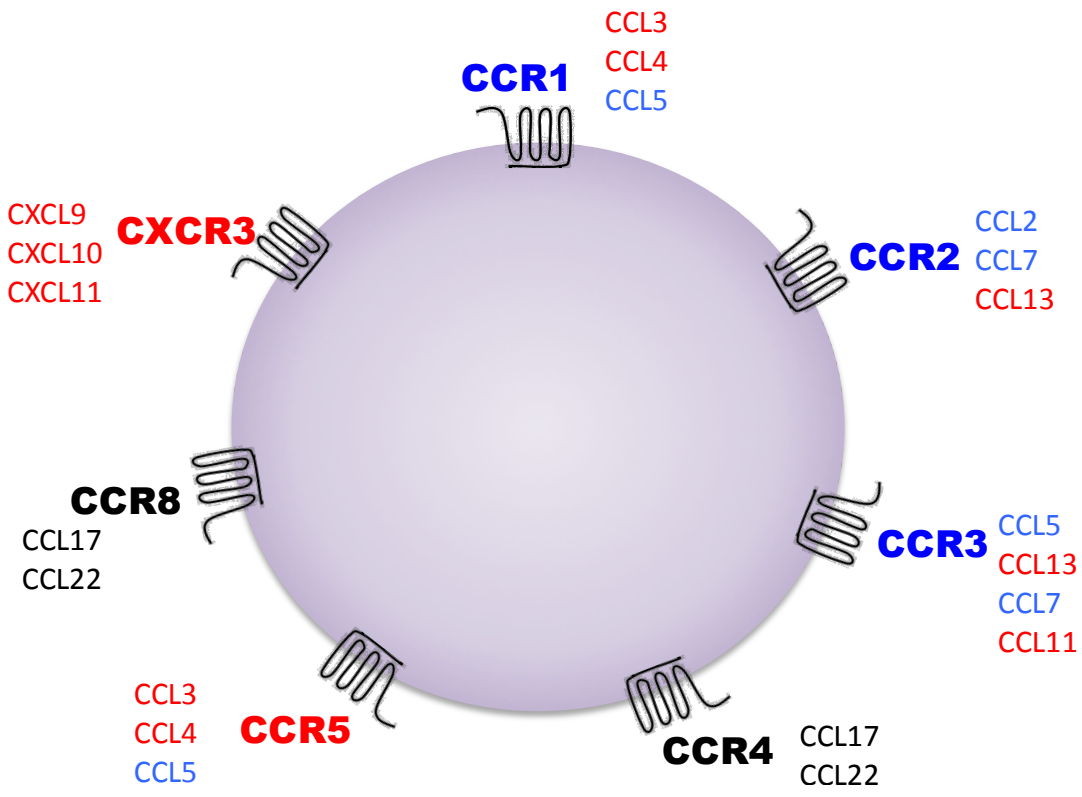
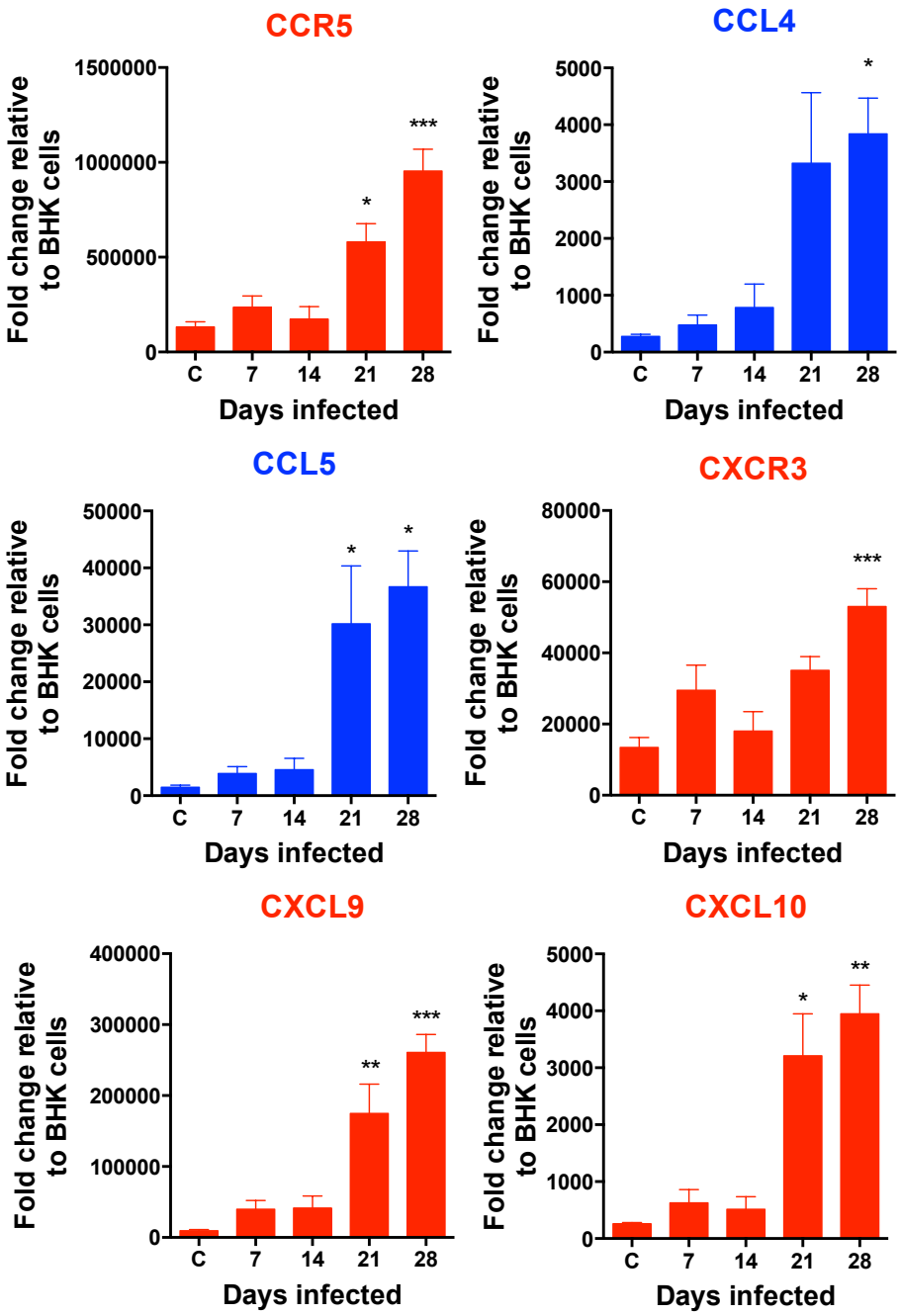


Figure 3. 4 Chemokine receptors and ligands in the recruitment of T cells. Chemokine receptors expressed on $CD4^+$ T cell subsets are represented by the larger text. The ligands considered to be involved in T cell recruitment are listed next to their respective receptor in smaller text. ■Th1-associated genes ■Th2/Treg-associated genes ■Th1/Th2-associated genes.


CCR5
Fold change relative to BHK cells
1500000
1000000
500000
0
C
7
14
21
28
Days infected
*
***
CCL4
5000
4000
3000
2000
1000
0
*
CCL5
50000
40000
30000
20000
10000
0
*
*
CXCR3
80000
60000
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20000
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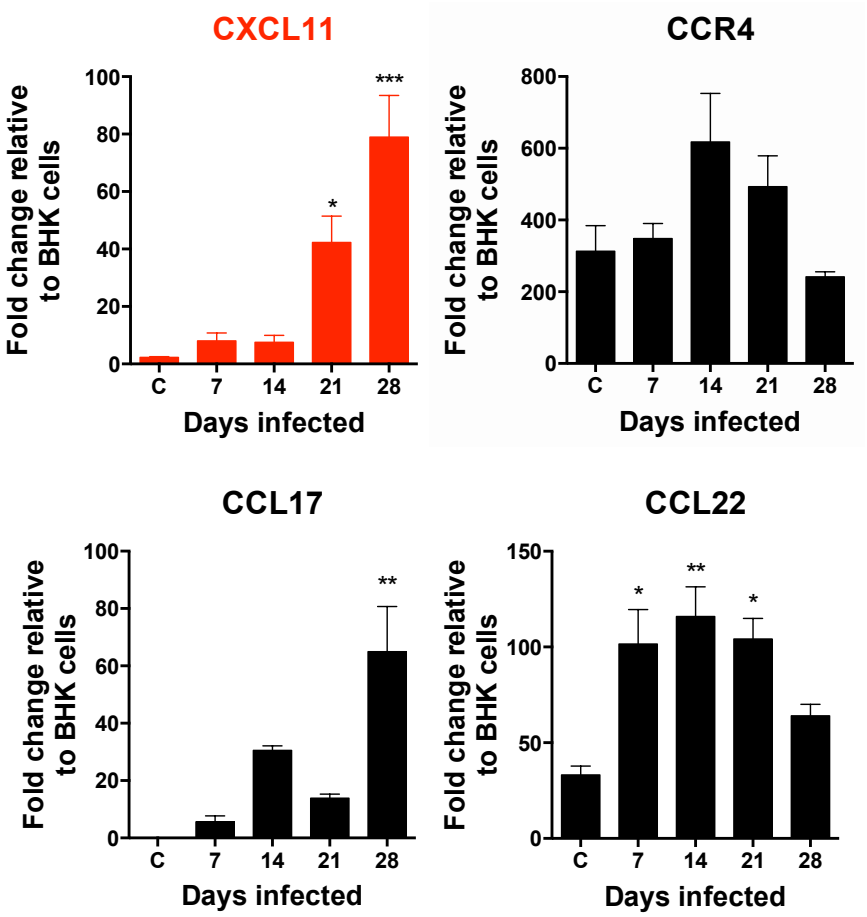


Figure 3. 5 Chemokine receptor and ligand mRNA expression in hamster spleen tissue over a course of chronic *L. donovani* infection. Total spleen tissue was obtained from uninfected hamsters or hamsters infected with 1 million *L. donovani* metacylcic promastigotes for 7, 14, 21 and 28 days. mRNA expression of type 1, type 2 and regulatory type chemokine ligands and their corresponding receptors was determined by real time RT-PCR. Results are expressed as a relative fold increase between experimental samples and uninfected BHK cells to which the value of 1 was arbitrarily assigned. Shown is the mean and SEM of a single experiment representative of 2 independent experiments from 3-6 hamsters per time point. *p<0.05; **p<0.01, ***p<0.001. ■Th1-associated genes ■Th2/Treg-associated genes ■Th1/Th2-associated genes.

SPLENIC MACROPHAGES ARE A CELLULAR SOURCE OF TH1 AND TH2 T CELL-ATTRACTING CHEMOKINES

In order to determine the cellular source of the chemokine ligands, we next examined isolated splenic macrophages from chronically infected hamster spleen tissue. We have previously shown this cell population is highly permissive to infection with *L. donovani* [60, 120]. Macrophage cell counts are markedly increased during chronic disease, which led us to explore this cell population [26]. In a recently submitted manuscript, RNAseq data revealed that spleen cells isolated by adherence were a pure macrophage population based on enriched transcripts of markers for the macrophage lineage. We determined the mRNA expression of T cell attracting chemokine ligands in splenic macrophages during chronic *L. donovani* infection of hamsters. The chemokine ligands that bind the Th1 chemokine receptor CCR5 (CCL4 and CCL5) were significantly upregulated in splenic macrophages during chronic stages of the disease compared to uninfected animals (Figure 3.6). Similar increases were found for chemokine ligands CXCL9, CXCL10, CXCL11 that bind the Th1 chemokine receptor CXCR3 (Figure 3.6). Interestingly Th2 attracting chemokines, CCL17 and CCL22, showed a trend of increased mRNA expression, although it was not significant (Figure 3.6). As previously shown (Figure 3.5), the receptor for CCL17 and CCL22, CCR4, was not increased during the chronic stage of disease.

The increase in chemokine ligands in this isolated cell population is consistent with the data from total spleen tissue (Figure 3.5). This data suggests the splenic macrophage cell population is a significant source of chemokine ligand production during chronic visceral leishmaniasis.

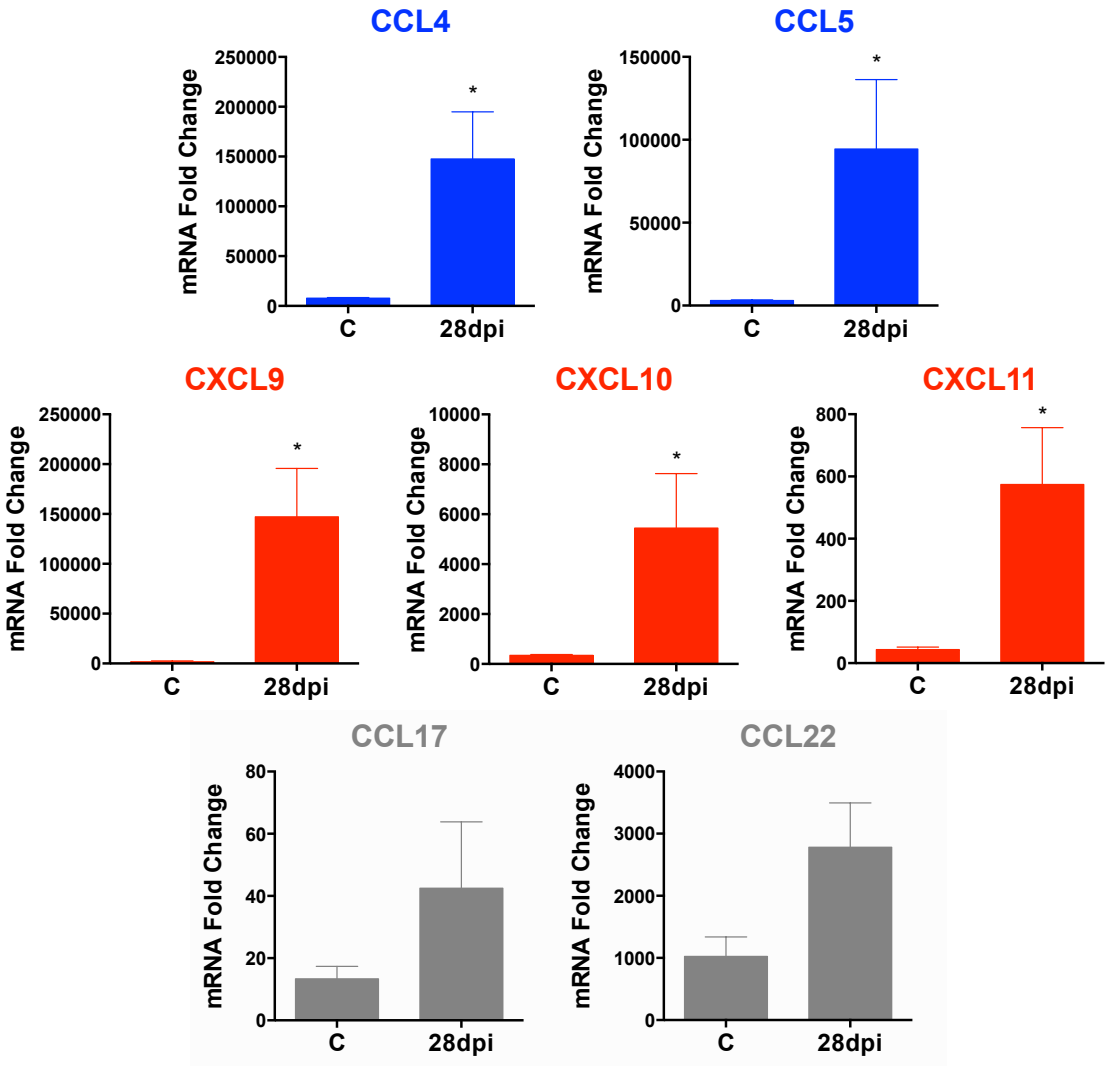


Figure 3. 6 Chemokine ligand mRNA expression in splenic macrophages from chronically infected hamster spleen tissues. Splenic macrophages were isolated from uninfected hamsters or 28 day infected hamsters. mRNA expression of type 1, type 2 and regulatory type chemokine ligands was determined by real time RT-PCR. Results are expressed as a relative fold increase compared to uninfected BHK cells to which the value of 1 was arbitrarily assigned. Shown is the mean and SEM of a single experiment representative of 2 independent experiments from 4 hamsters per time point. Student's t-test was performed to compare control uninfected (C) and chronically 28 day infected (28dpi) samples. *$p<0.05$. ■Th1-associated genes ■Th1/Th2-associated genes ■Th2/Treg-associated chemokines.

$CD4^+$ T CELLS FROM CHRONICALLY INFECTED HAMSTERS EXPRESS MARKERS OF TH1 AND TH2 T CELLS

Progressive human visceral leishmaniasis is associated with increased $CD4^+$ T cells in the spleen and low $CD4^+/CD8^+$ T cell ratio in peripheral blood [25, 65]. It has also been reported that there is no accumulation of natural Treg $CD4^+$ T cells in the spleen of human visceral leishmaniasis patients [25, 101]. As mentioned before, the regulatory cytokine IL-10 plays a pathogenic role in visceral leishmaniasis. It was found that a $CD4^+CD25^-Foxp3^-$ T cell population is the major source of IL-10 [25, 121]. Despite these findings, and the evidence of mixed cytokine responses, there have been no further studies on identifying the specific phenotype of the responding $CD4^+$ T helper cells in the spleen during chronic human infection.

To determine the phenotype of $CD4^+$ T cells that accumulated in the spleen during chronic disease, we investigated transcription factor mRNA expression in isolated $CD4^+$ T cells. $CD4^+$ splenocytes were separated using magnetic particles conjugated with anti-CD4 antibody which yielded a >90% pure $CD3^+CD4^+$ population that was used for real time RT-PCR analysis (Figure 3.7). There was an evident increase in mRNA (Figure 3.8) and protein (Figure 3.9) expression of the $CD4^+$ T cell transcription factor, Tbet. The mRNA for chemokine receptors associated with Th1 cells, CCR5 and CXCR3, was significantly increased (Figure 3.8). The type 1 cytokine IFNγ that is commonly produced by Th1 T cells also had increased mRNA in the isolated splenic $CD4^+$ T cell population (Figure 3.8). The Th2 transcription factor, GATA3, and Th2 cytokine, IL-4, was also significantly increased (Figure 3.8). Consistent with what was found in total spleen (Figure 3.3), the transcription factor that regulates the differentiation of Treg $CD4^+$ T cells, Foxp3, showed no increase in mRNA (Figure 3.8) or protein (Figure 3.9). The chemokine receptor commonly expressed on Th2 and Treg cells, CCR4, was not increased either (Figure 3.8). IL-10 and IL-21, which are thought to have a disease-

promoting effect in VL, were significantly upregulated in splenic $CD4^+$ T cells at 28 days of chronic *L. donovani* infection (Figure 3.8).

All together, these data support the hypothesis that both Th1 and Th2 $CD4^+$ T cells develop during chronic visceral leishmaniasis, and that $CD4^+$ T cells are a major source of the mixed cytokine response.

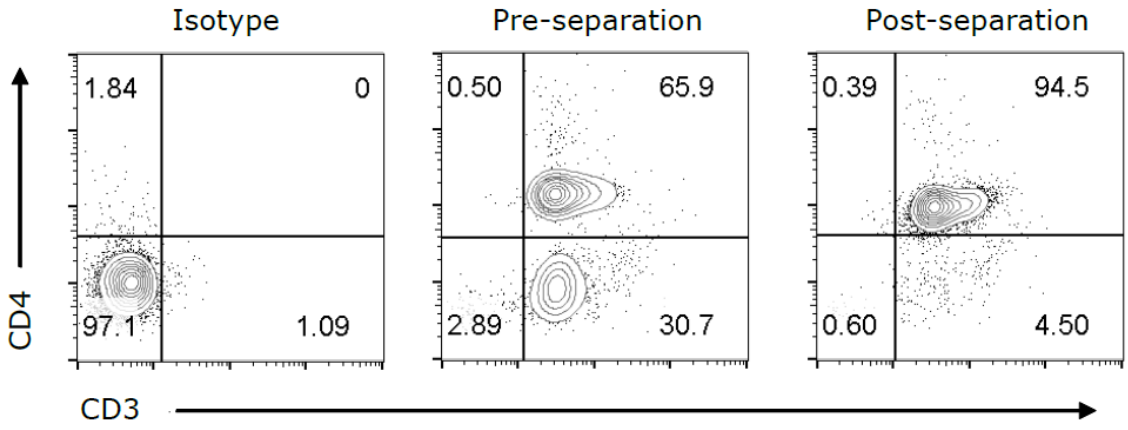
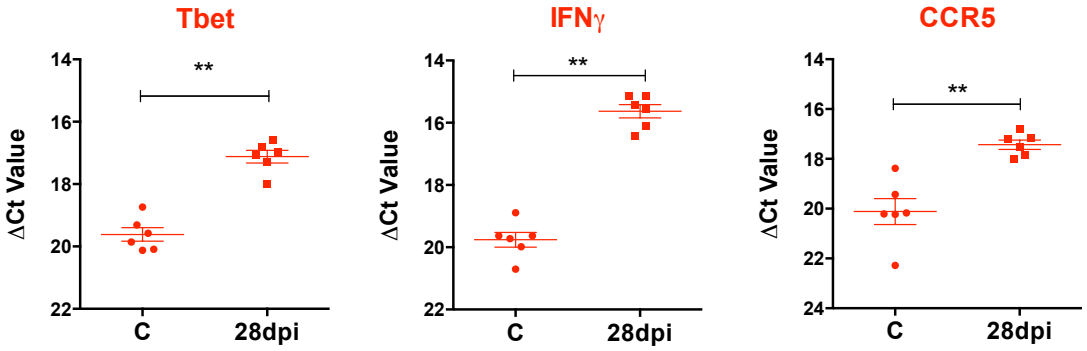


Figure 3. 7 Purity of isolated $CD4^+$ T cells from hamster spleen tissue. Total spleen tissue from uninfected or 28 day infected hamsters was processed for the positive selection of $CD4^+$ T cells by MACs column separation. Isolated cells were labeled with TCRα/β, CD4 and corresponding isotype flow cytometry antibodies to determine purity. Cells were gated on the lymphocyte population based on SSC and FSC. Percentage of the different cell populations is shown in each quadrant. Isotype-matched negative control antibody is the left plot, cell percentages before magnetic bead separation is the middle plot and cell percentages after magnetic bead separation is the plot on the right. This was observed in 6 hamsters per experimental group from at least 2 independent experiments.



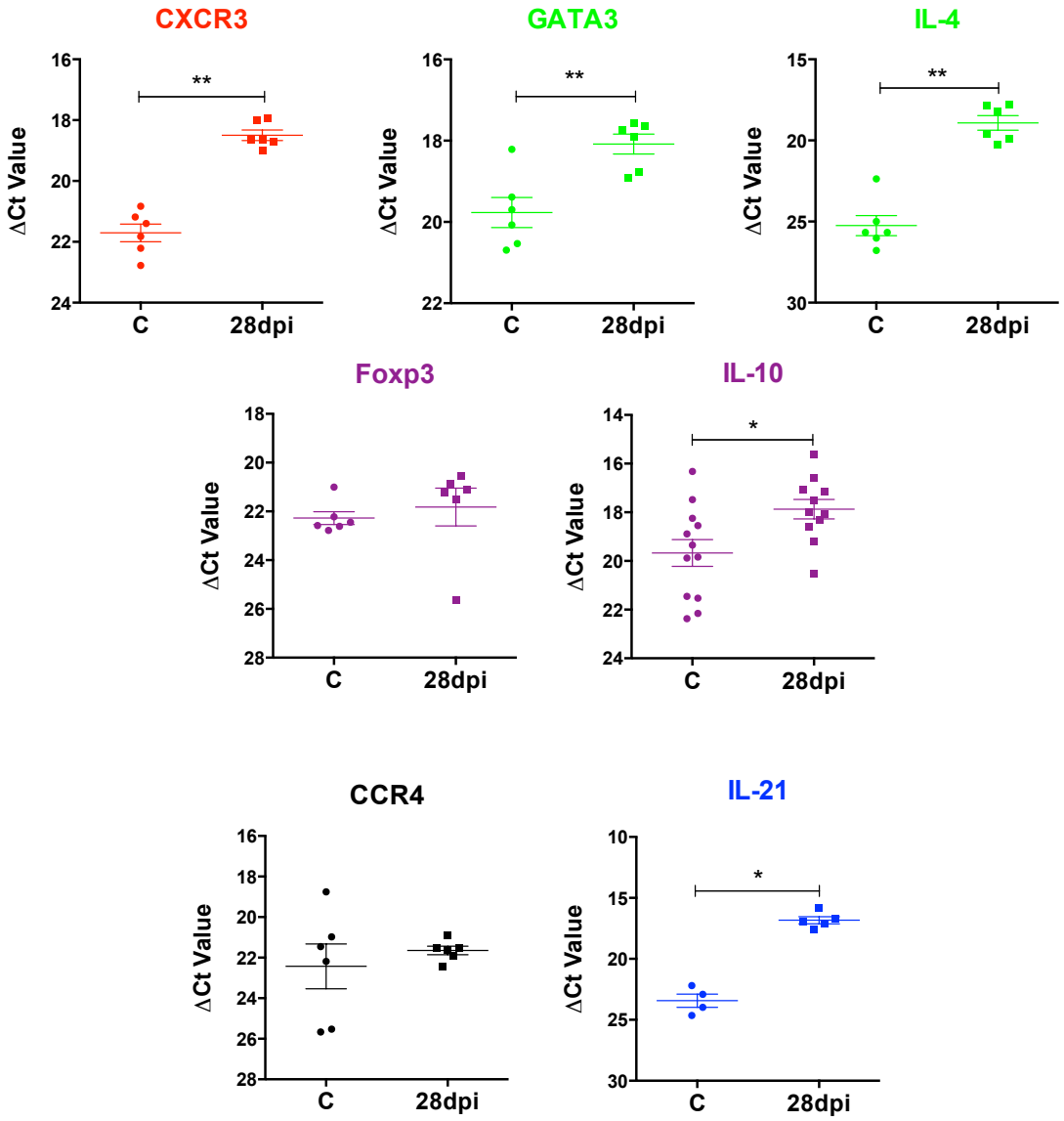


Figure 3. 8 Markers of different T helper cell subsets in chronically infected hamster splenic CD4$^+$ T cells. RNA was isolated from the pure splenic CD4$^+$ T cell population and mRNA expression of Th1, Th2 and Treg cells was determined by real time RT-PCR. Results are expressed as delta Ct values comparing uninfected (C) with 28 days post-infection (28dpi) samples. Shown is the mean and SEM of a single experiment representative of 2 independent experiments from 6 hamsters per experimental group. Student's t-test was performed to compare control and 28dpi samples. *p<0.05, **p<0.01. ■Th1-associated genes ■Th2-associated genes ■Treg-associated genes ■Th2/Treg-associated genes ■Th1/Th2-associated genes.

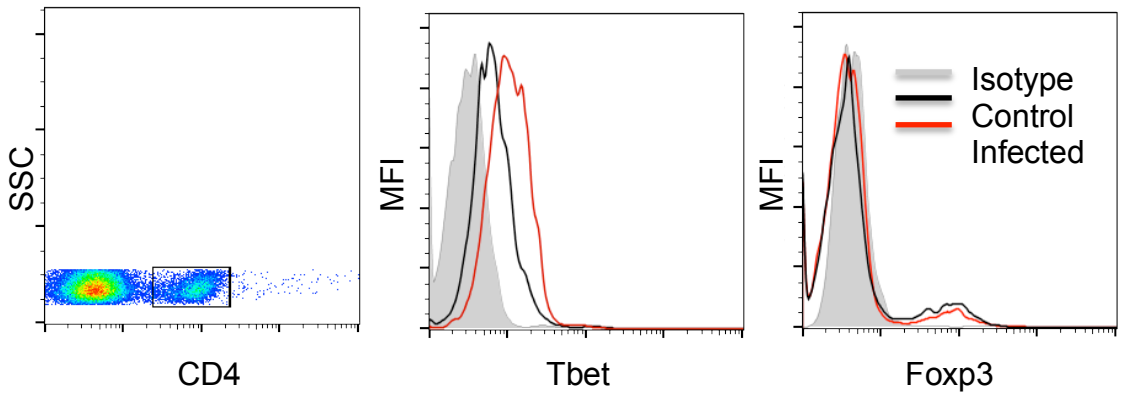


Figure 3. 9 Tbet and Foxp3 expression in total hamster spleen tissue. Expression of Tbet and Foxp3 was verified in CD4 splenocytes. Chronically infected hamster spleens were collected and processed for flow cytometry antibody labeling with CD4, Tbet and Foxp3. Cells were gated on the lymphocyte population based on SSC and FSC and then CD4 to determine the mean fluorescent intensity (MFI) of Tbet and Foxp3. Data is representative of at least 2 independent experiments.

Discussion

In this chapter, we set out to characterize the responding splenic $CD4^+$ T cells during chronic visceral leishmaniasis in hamsters. Understanding the distinct roles of the different subsets of $CD4^+$ T helper cells has been challenging. It has been established that T cells are critical in controlling early infection [122-125]. The percentage of $CD4^+$ T cells in the plasma of active visceral leishmaniasis patients is significantly lower than healthy or cured patients [65]. Among these circulating T cells, there is reduced effector and memory function with decreased IFNγ production and decreased responses to antigen [65, 126, 127]. However, other studies looking at the spleen, the site of chronic infection, found increased accumulation of $CD4^+$ T cells and *Leishmania* specific IFNγ producing $CD4^+$ T cells [25, 128]. In this study, we also show an increase of $CD4^+$ T cells in the spleen of 28 day chronically infected hamsters compared to the uninfected (Figure 3.2). This could mean that $CD4^+$ T cells are proliferating within the spleen.

Another hypothesis is that $CD4^+$ T cells are being recruited from sources outside the spleen, like blood or thymus. In support of this, we found increased chemokine receptor (CCR5, CXCR3) and chemokine ligand (CCL4, CCL5, CXCL9/10/11, CCL17, CCL22) mRNA in the spleen over the course of infection (Figure 3.5). A closer look into the source of the chemokine ligands showed splenic macrophages to play an important role (Figure 3.6). A recent study shows pathogenic $CD4^+$NKT cells migrate from peripheral blood to the infection site [129]. This would support our hypothesis that recruitment of $CD4^+$ T cells responding during chronic infection in the spleen are the cause for pathology and disease progression.

We found that the phenotype of $CD4^+$ T cells in the spleen during chronic *L. donovani* infection is a mixed Th1 and Th2. There were increased markers of Th1 (Tbet, IFNγ, CCR5, CXCR3) in the total spleen tissue as well as the isolated $CD4^+$ T cell population (Figures 3.3, 3.8 and 3.9). Some, but not all markers of Th2 (GATA3, IL-4)

were also significantly increased with chronic visceral leishmaniasis in the total spleen and splenic $CD4^+$ T cells (Figures 3.3 and 3.8). This mixed response is contrary to initial studies of peripheral blood T cells, which proposed that development of chronic visceral leishmaniasis was due to strong Th2 responses and a lack of Th1 responses [130, 131]. In agreement to what we describe here in the hamster model, several studies have reported there is in fact a mixed Th1 and Th2 cytokine response during chronic infection in humans [64, 82, 83, 132]. But despite strong Th1 responses, patients show no increased NO and still progress to the fatal disease.

Among the mixed Th1 and Th2 immune responses at the site of infection, there is also an increase in immunosuppressive cytokines like IL-10 and IL-21 (Figures 3.3 and 3.8). There have been several studies finding increased IL-10 in patients and experimental models of visceral leishmaniasis [25, 64, 65, 133, 134]. Also, increased IL-21 is described in human patients [121]. Moreover, the pathogenic role of IL-10 and the effects IL-21 signaling has on inducing IL-10 has been well characterized [25, 100, 121, 135]. The most common IL-10 producing T cell subsets are Treg cells, which can be classified as $CD4^+CD25^+Foxp3^+$ natural Treg (nTreg) or $CD4^+CD25^-Foxp3^-$ adaptive/inducible Treg (Tr1/iTreg) [136]. Here we show a transient increase in the Treg transcription factor, Foxp3, in the total spleen that was not evident in splenic $CD4^+$ T cells by 28 days of infection (Figures 3.3, 3.8 and 3.9). Interestingly, the source of IL-10 is ambiguous since both monocyte and lymphocyte populations are known producers. One study showed that IL-10 was mainly produced by $CD4^+CD25^+Foxp3^+$ nTreg cells that accumulated in the bone marrow of active visceral leishmaniasis patients [102]. Soon after, another study discovered $CD4^+CD25^+Foxp3^+$ nTreg cells did not accumulate in spleen and the major producers of IL-10 were in fact $CD4^+CD25^-Foxp3^-$ Tr1 cells [25, 101]. This could suggest nTreg are playing a role early in *L. donovani* infection in the hamster spleen, but the source of IL-10 during the chronic stage is a $CD4^+Foxp3^-$ cell population (Figure 3.8 and 3.9).

Collectively, our data confirm what is seen in human chronic visceral leishmaniasis. This further strengthens the applicability of the hamster model for progressive disease. The $CD4^+$ T cell population showed enhanced mRNA of Th1 and Th2 markers, and they were significant producers of the mixed array of cytokines. Two significant questions remain. First, it is unclear if these $CD4^+$ T cells are recruited to the spleen or if they are proliferating at the site of infection. The $CD4^+$ T cells are expressing chemokine receptors and the splenic macrophages are expressing the corresponding chemokine ligands, suggesting that there is recruitment, but more conclusive studies are required. Second, the mixed phenotype of the CD4 T cells leaves open the question of whether they are protective or pathologic. Characterization of the immune cell population in the spleen during chronic disease is an initial effort to determine the immunological mechanisms responsible for the progression of chronic visceral leishmaniasis.

CHAPTER 4: CD4$^+$ T CELLS RESPONDING TO CHRONIC VISCERAL LEISHMANIASIS INHIBIT PARASITE REPLICATION IN HOST CELLS

Introduction

The T cell responses in human and experimental models of visceral leishmaniasis are diverse, and can be categorized as protective or pathologic. For example, CD4$^+$ T cells may play a role in immunosuppression by the production of the regulatory cytokine, IL-10, which inhibits phagocyte effector function in killing intracellular parasites [137, 138]. Other subsets of CD4$^+$ T cells, like Treg, can aid in disease progression [25, 139]. In the murine experimental model of visceral leishmaniasis, which is a model of a controlled infection, T cells producing IFNγ play an effective role in activating immune cells to control parasite replication [140]. It is unclear why some infected individuals develop progressive chronic disease and others do not, but a closer look into the effector function of CD4$^+$ T cells may yield important insights.

CD4$^+$ T CELL ACTIVATION AND EXHAUSTION

The CD4$^+$ T cell response to infection is a critical determinant of host immunity. CD4$^+$ T cells produce cytokines, like IFNγ and IL-4, which induce the activation of APCs to promote cell-mediated immunity. Some produce chemokines, which direct the recruitment of naïve T cells and APCs to the site of infection. But it has been discovered that T cells can lose their functionality, especially in chronic infections.

CD4$^+$ T cell activation occurs when naïve T cells encounter a foreign antigen via a unique surface T cell receptor (TCR) that recognizes the antigen being presented by the MHCII molecules on APCs. The costimulatory receptor, CD28, also expressed on all naïve CD4$^+$ T cells, binds CD80/86 on APCs and further enhances T cell activation. Once

TCR/MHCII and CD28/CD80/86 engagement has taken place, a cascade of signaling pathways in both cells takes place [141]. The responses from both cell types include proliferation, differentiation into the appropriate phenotype, production of cytokines and chemokines, and promotion of adaptive immunity.

T cell activation can be restrained by the engagement of inhibitory receptors and lead to T cell exhaustion. T cell exhaustion is a damaging result of persistent antigen stimulation that was first described in the chronic lymphocytic choriomeningitis virus infection [142]. Cytotoxic lymphocyte antigen 4 (CTLA-4) is expressed on T cells and has a higher affinity to bind to CD80/86 than CD28 [143]. If T cells are induced to express this inhibitory receptor, it can lead to a signaling pathway towards T cell exhaustion rather than activation. Exhaustion can also be mediated by synergism of CTLA-4 with other inhibitory receptors such as programmed death cell 1 (PD-1) [144]. PD-1 binds programmed death cell 1 ligands 1 and 2 (PDL-1 and PDL-2) that are expressed on APCs. T cell exhaustion is characterized by the loss of T cell effector function, such as decreased cytokine production, decreased proliferation and increased apoptosis [145]. As mentioned, these events are a result of chronic antigen stimulation during chronic infections. It is a regulatory mechanism to downregulate inflammatory responses and protect against tissue damage [146]. But T cell exhaustion can have detrimental effects on host defense by reducing the immune response against the pathogen.

Visceral leishmaniasis in its active form of disease is persistent and chronic. Many studies have demonstrated decreased T cell function. The T cell response in patients and dogs (a naturally infected reservoir host) with active disease were shown to be impaired upon *ex vivo* stimulation with *Leishmania* antigen [22, 147]. Recent work in chronic visceral leishmaniasis has found increased expression of exhaustion markers in the mouse and dog models, and also in human patients with active disease [50, 89, 90]. In the mouse model, blockade of the CTLA-4 ligand, CD86, led to increased T cell

responses and reduction of parasite burden in the liver [148]. In dogs, the increase in inhibitory receptor, PD-1, correlated with decreased function of phagocytic cells and blocking of PD-1 improved $CD4^+$ T cell IFNγ-induced production of NO and parasite killing [89]. In humans with active disease, increased CTLA-4 and PD-1 were found in $CD8^+$ T cells from whole blood and splenic aspirates. But, unlike the other models, blocking these inhibitory molecules did not enhance cytokine production (IFNγ) or decrease parasite burden [90].

T cell exhaustion during chronic visceral leishmaniasis could be a limiting factor in host defense. It poses limitations for the development of optimal chemotherapeutic treatments and vaccines. A better understanding of the responding $CD4^+$ T cells and their exhaustion status in experimental chronic visceral leishmaniasis is necessary. Preliminary experiments to address this will be described in this chapter.

MACROPHAGE ACTIVATION: M1/M2 PHENOTYPES

Macrophages are an important APC involved in innate and adaptive immune responses. They respond to infection and inflammation recognized by first responder neutrophils. Macrophages' primary roles in immunity consist of phagocytosing pathogens, killing of intracellular pathogens, production of cytokines, presenting antigen to $CD4^+$ T cells for the aid in difficult pathogen removal and producing chemokines for the recruitment of other immune cells to the site of infection [85]. Macrophages also have many roles in immune homeostasis. For the scope of this dissertation, only their role in host immunity will be described.

Like $CD4^+$ T cells, the activation of macrophages is an important event in host defense. Macrophages respond to external stimuli like cytokines or pathogen-associated molecular patterns (PAMPs). Innate immune cells produce cytokines like IFNγ and TNFα in response to stress signals or infections. This activates the classical inflammatory phenotype of macrophages (M1). The M1 phenotype can also be induced

by bacterial lipopolysaccharide (LPS) through the toll like receptor 4 (TLR4) pathway. As a result of polarization, M1 macrophages produce more inflammatory cytokines (IFNγ, IL-12, IL-6, IL-23) and chemokines (CXCL9, CXCL10, CXCL11) to activate and attract more immune cells. M1 macrophages also generate intracellular reactive oxygen and nitrogen species like nitric oxide, which aids in killing intracellular pathogens [85]. These effector functions of M1 macrophages require activation of intracellular signaling pathways. The macrophage priming with IFNγ engages the IFNγ receptor. This signals for a cascade of events. In short, the receptor subunits activate receptor-associated Janus Kinases (JAK1 and JAK2) to be phosphorylated, which in turn phosphorylate the IFNγ receptor subunits. This leads to binding, dimerization and phosphorylation of the transcription factor STAT1, which then translocates into the nucleus of the cell to regulate transcription of IFNy responsive genes like interferon regulatory factor 1 (IRF1) [72]. Together with IFNγ cell priming, LPS activation of the TLR4 pathway promotes production of antimicrobial reactive oxygen and nitrogen species. TLR4 activation leads to the recruitment of several adapter molecules and kinases that ultimately results in activation of nuclear factor κB (NF-κB) that together with IRF-1, binds the IFNβ and iNOS promoters in the nucleus. This leads to production of type I interferons and iNOS [149]. The role of iNOS and its pathway in anti-leishmanial activity will be discussed in the next section.

M1 macrophages in an inflammatory response have an antimicrobial effect but lead to tissue injury. In response to injury, cytokines IL-4 and IL-13 signal for an alternative phenotype of macrophage polarization (M2) that aids in tissue remodeling and angiogenesis and promote a Th2 immune environment [85]. IL-4 engagement of the IL-4 receptor leads to JAK1 and JAK3 activation to recruit and phosphorylate the transcription factor STAT6. STAT6 then dimerizes and translocates to the nucleus that together with C/EBPβ, a product of the IL-6 pathway, binds the arginase I promoter. Production of

arginase leads to more type 2 cytokines and synthesis of polyamines that aid in collagen repair and wound healing [150, 151].

THE ROLES OF ARGINASE AND NITRIC OXIDE IN *LEISHMANIA* REPLICATION

Leishmania parasites have sophisticated subversion mechanisms when infecting a host to avoid immune cell activation and allow for intracellular replication [152]. Their surface glycoproteins, like LPG, protect the parasites from activating complement and from degradation [153]. Once inside the host cells, LPG aids in delaying endosome-lysosome fusion. Quick transformation into its more resilient amastigote form protects it from the harsh conditions of the cell phagolysosomes.

LPG and other surface lipids directly inhibit intracellular macrophage production of reactive oxygen species. In addition to the surface molecules, *Leishmania* parasites secrete molecules that degrade nitrite derivatives and reactive oxygen intermediates (ROI) that are key anti-leishmanial molecules [152]. The amino acid, arginine, is a common substrate for the enzymes arginase (Arg) and inducible nitric oxide synthase (iNOS). The pathway through which arginine is metabolized determines the fate of host-parasite interaction. The metabolism of arginine by iNOS will form nitric oxide (NO) and L-citrulline. The production of NO by M1 macrophages results in intracellular parasite killing by nitrosylation of cysteine residues on critical proteins involved in physiological processes such as DNA transcription and replication [154]. If arginine is metabolized by Arg, it results in production of urea and L-ornithine, which leads to the synthesis of polyamines [155]. Host polyamines produced by M2 macrophages are scavenged by *Leishmania* to promote parasite growth [156].

The activation and phenotype of macrophages studied in both human disease and the hamster model of progressive visceral leishmaniasis has become an important

consideration in the pathogenesis of disease. We will show $CD4^+$ T cells interact with macrophages to have a limited effect on parasite control.

Materials and methods

HAMSTERS AND INFECTION MODEL

For co-culture experiments, an inbred Chester Beatty hamster colony was maintained in the animal resource center at the University of Texas Medical Branch. Inbred hamster litters were weaned at 3 weeks old and then used at 4-6 weeks of age. Experiments were set up using cells from sex-matched hamsters.

ISOLATION OF HAMSTER BONE MARROW-DERIVED MACROPHAGES

Femurs from uninfected hamsters were collected and bone marrow cells were flushed using GlutaMax™ (Gibco) RPMI 1640 culture medium supplemented with 10% heat inactivated FBS, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin and 20 ng/mL recombinant human macrophage-colony stimulating factor (M-CSF) (eBioscience). Cells were adjusted to 8 x 10^6/mL and cultured for 3 days at 37°C in 5% CO_2 after which the culture medium was replenished and cells were allowed to differentiate for 3 more days. On the last day of culture, cells were washed 3 times with PBS and detached with Trypsin/EDTA (Gibco). The cells were plated overnight in starvation RPMI 1640 culture medium with 2% FBS to avoid serum activation. Cells were stimulated with either recombinant hamster IL-4 (2.5% v/v) or IFNγ (10% v/v) supernatants [157]. Infected and stimulated cells were cultured for 24 hours before collection for analysis.

MACROPHAGE ACTIVATION AND PRIMING

Bone marrow-derived macrophages were plated and allowed to adhere overnight in 2% serum medium. The next day, media was replenished and cells were primed for activation with IFNγ (10% v/v in supernatants from CHO cells expressing recombinant hamster IFNγ) [157] for 1-2 hours and then stimulated with 20 ng/uL LPS before infections. For *in vitro* infections, stationary phase *L. donovani* promastigotes from 6-7 day old cultures were used at a parasite to cell ratio of 5:1 in 2% complete RPMI 1640 medium. For co-culture experiments, a *Leishmania donovani* strain transfected with an episomal vector containing the Luciferase reporter gene was used [158]. Parasites were allowed to be phagocytosed for 4 hours and then the monolayer was carefully washed with pre-warmed medium to remove extracellular parasites. Media was replenished and cells were incubated for 48 hours at 37°C in 5% CO_2.

T CELL – MACROPHAGE CO-CULTURE AND TRANSWELL ASSAYS

$CD4^+$ T cells were isolated from control or chronically infected inbred hamster spleens as described in the previous chapter. Co-cultures and transwell assays were set up at a T cell to macrophage 5:1 ratio. For co-cultures, control or chronically stimulated $CD4^+$ T cells were added to wells with uninfected or infected macrophages. For transwell assays, the macrophages were cultured in the bottom chamber, and purified $CD4^+$ T cells were cultured in a maximum of 100uL culture medium in top transwell inserts with 0.4 uM pore polycarbonate membrane (Corning). Cells were cultured for 1 and 48 hours at 37°C in 5% CO_2. At each time point, culture medium was aspirated and cells were lysed for RNA isolation as described earlier. To determine the parasite burden, the macrophages were lysed using Promega luciferase assay kit to determine parasite luciferase activity on the FluorStar Model 403 [60, 158].

STATISTICAL ANALYSIS

Comparison between two groups was performed using Student's *t* test or Mann-Whitney test (non-parametric). For experiments using 2 independent variables, comparisons were done using two-way ANOVA. Bonferroni's multiple comparisons test was used. P-values < 0.05 were considered significant. All analyses were conducted using GraphPad Prism version 5.04 for Windows or Mac, GraphPad Software, San Diego, California, USA.

Results

IFNγ PRIMED MACROPHAGES ARE EFFECTIVE IN KILLING INTRACELLULAR PARASITES *IN VITRO*

Macrophages stimulated with IFNγ differentiate into the M1 phenotype leading to production of reactive oxygen and nitrogen species [86, 124, 159]. Inducible nitric oxide synthase (iNOS) is essential for intracellular parasite killing, but the relentlessly progressive disease in human VL suggests that the macrophage response to the high IFNγ levels is ineffective [160, 161]. This has led to the notion that macrophages infected with *Leishmania* parasites are rendered unresponsive to the strong Th1 host immune response.

To investigate this, we first tested the effector function of hamster macrophages in an *in vitro* activation system. Hamster bone marrow-derived macrophages were first primed with IFNγ for an hour before LPS stimulation and *L. donovani* infection. The macrophage response showed a drastic increase in the uptake of parasites compared to unstimulated control macrophages after 1 hour (Figure 4.1A). This indicates that IFNγ induces macrophage phagocytic function *in vitro.* After 48 hours, the IFNγ- and LPS-stimulated macrophages showed significantly reduced parasite burden (Figure 4.1A) and increased expression of iNOS (Figure 4.1B). This data suggests the increase in iNOS expression results in increased intracellular *Leishmania* parasite killing. These results are

evidence that in a controlled *in vitro* system, hamster macrophages have the capacity to respond to IFNγ and increase iNOS to reduce intracellular parasite burden. While iNOS as a mechanism to control *Leishmania* infection is well established, we did not use inhibitors to prove causality in these experiments.

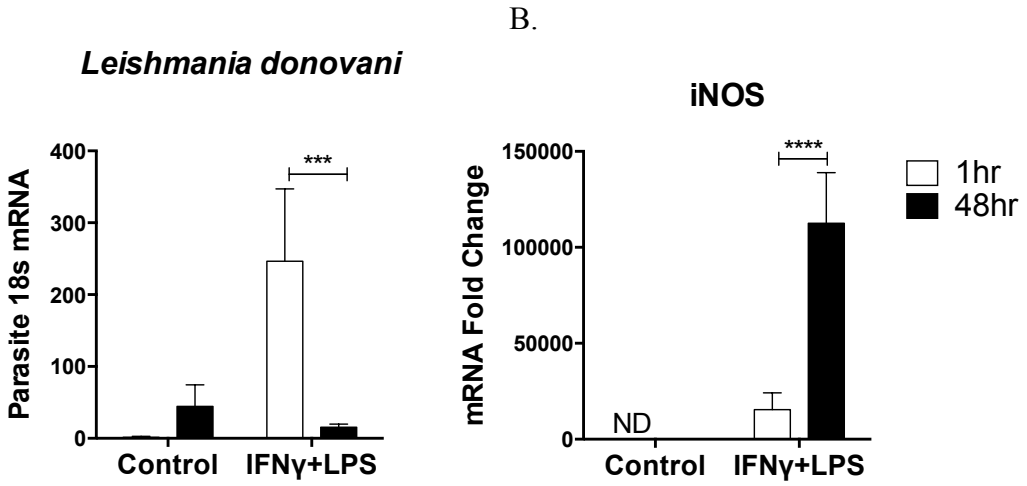


Figure 4. 1 IFNγ primes macrophages to increase iNOS and decrease parasite burden. Hamster bone marrow-derived macrophages were primed with IFNγ before being triggered with LPS and infected with *L. donovani.* Extracellular parasites were removed after 1 hour and RNA was isolated to obtain initial parasite burden □. The other infected cells were incubated for 48 hours more before RNA isolation n. Gene expression of *L. donovani* 18s (A) and hamster iNOS (B) was determined by real time RT-PCR. Results are expressed as a relative fold change in comparison to the initial time point of each treatment group. Data shown is representative of at least 2 independent experiments with 6 replicates per group. ND=Not Detected. ***p<0.001, ****p<0.0001

SPLENIC CD4$^+$ T CELLS FROM CHRONICALLY INFECTED HAMSTERS RESTRAIN INTRACELLULAR PARASITE REPLICATION *IN VITRO*

Understanding the clear role of CD4$^+$ T cells in immunity against leishmaniasis has been a longstanding question. It has been demonstrated that CD4$^+$ T cell clones from an asymptomatically infected individual induce anti-leishmanial responses *in vitro* [162]. Studies have also shown increased IFNγ responses in whole blood but not PBMCs when stimulated with *Leishmania* antigen [22, 163]. So we next evaluated splenic CD4$^+$ T cell

function in activating macrophages to kill intracellular *L. donovani* parasites. We compared $CD4^+$ T cells from uninfected hamsters to $CD4^+$ T cells from 28 day chronically infected hamsters. To avoid confusion from any parasite DNA contamination in the purified $CD4^+$ T cell preparation, we used the luciferase transfected *L. donovani* strain for the *in vitro* macrophage infections and measured parasite burden by luciferase expression rather than by PCR. After phagocytosis of parasites, $CD4^+$ T cells were co-cultured with infected macrophages. After 48 hours, there was less luciferase activity in macrophages co-cultured with $CD4^+$ T cells from chronically infected hamster spleens. The percent of parasite burden showed a modest but statistically significant decrease in the infected macrophages co-cultured with the *Leishmania* exposed $CD4^+$ T cells compared to those from uninfected hamsters (Figure 4.2). This correlated well with a significant increase in iNOS expression (Figure 4.3A). Surprisingly, there was also an increase in Arg1 expression, which could be owed to the intracellular parasites that remained (Figure 4.3B). These data indicate that $CD4^+$ T cells, when removed from the environment of the chronically infected spleen during visceral leishmaniasis, have effector function that can control intracellular parasite replication (approximately a 25% reduction) by inducing iNOS expression in infected macrophages.

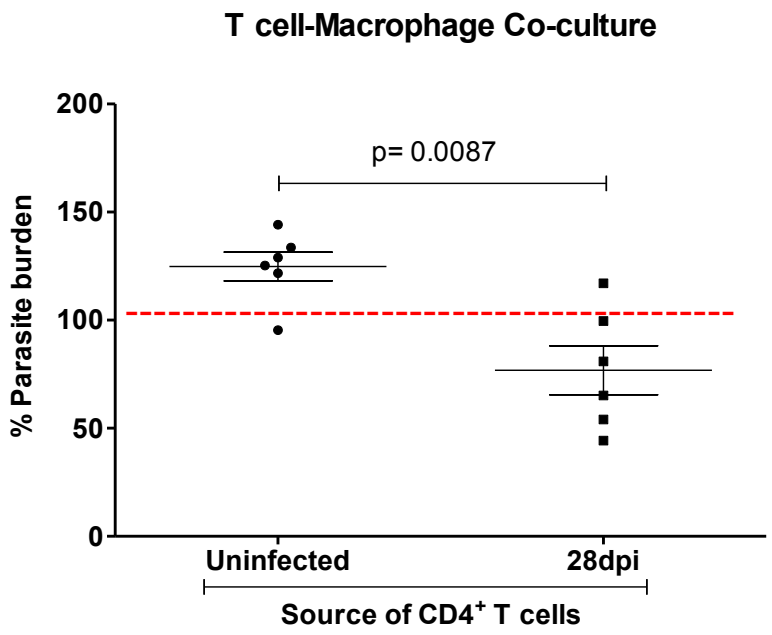


Figure 4. 2 Splenic CD4$^+$ T cells from chronically infected hamsters reduces intracellular parasite burden in macrophages. Bone marrow-derived macrophages from uninfected hamsters were infected *in vitro* with *L. donovani* luciferase transfected promastigotes (LUC). CD4$^+$ T cells were isolated from uninfected or 28 day infected hamster spleen tissue. Infected macrophages were co-cultured with either CD4$^+$ T cell population at a 5:1 T cell to macrophage ratio for 48 hours. The monolayer was washed before cell lysis to measure luciferase activity and determine parasite burden. Relative fold change was calculated using relative luminescent unit values. The fold change was then used to calculate percentage of parasite increase or decrease from the initial parasite burden (100%) for each group of co-cultured CD4$^+$ T cells. Each group had 6 replicates and data shown is representative of 2 independent experiments.

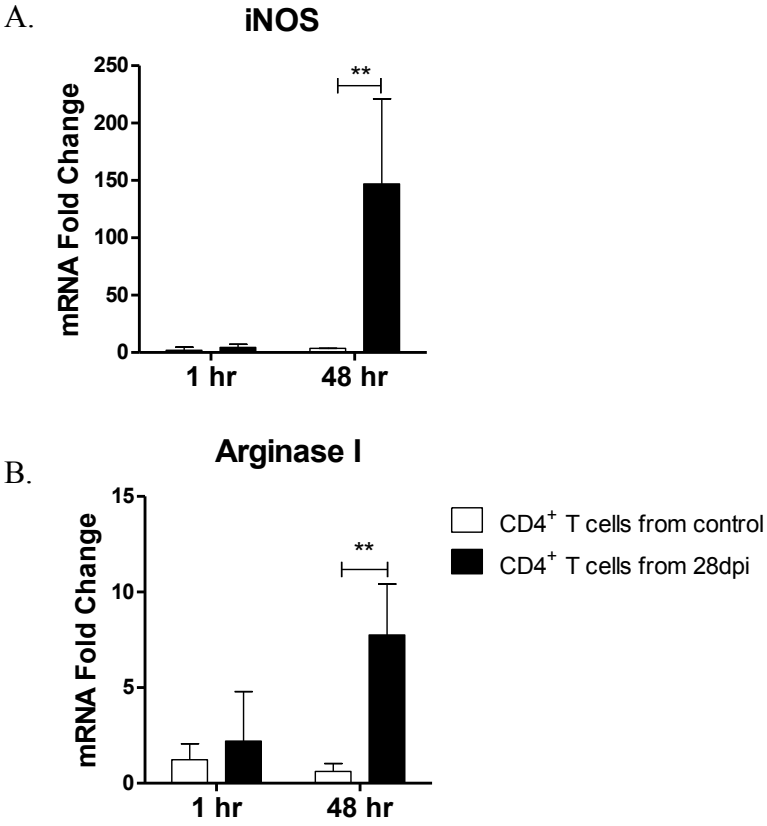


Figure 4. 3 Splenic CD4$^+$ T cells from chronically infected hamsters increase iNOS and Arg1 expression. Infected macrophages were co-cultured with either CD4$^+$ T cell population at a 5:1 T cell to macrophage ratio for 48 hours. (A) iNOS and (B) Arg1 mRNA expression in macrophages co-cultured with CD4$^+$ T cells from uninfected (control) or 28 day infected (28dpi) hamsters was determined by real time RT-PCR at baseline time point 1hr and 48 hours later. Each group had 6 replicates and data shown is representative of 2 independent experiments. $^{**}p<0.01$

INCREASED mRNA EXPRESSION OF T CELL EXHAUSTION MARKERS IN SPLEENS OF CHRONICALLY INFECTED HAMSTERS

Cellular immune function is ineffective in abating the relentless disease progression in visceral leishmaniasis, but removal of CD4$^+$ T cells from the splenic environment and co-culture with *in vitro* infected macrophages uncovered T cell effector activity. We therefore examined other mechanisms that might impair T cell function in the infected spleen. Our transcriptional profiling data identified the upregulation of a number of receptors/ligands that function to inhibit T cell responses and are associated

with T cell exhaustion (Table 2.2). To further analyze the effector phenotype of $CD4^+$ T cells during chronic visceral leishmaniasis, we therefore investigated markers of T cell exhaustion in more detail. First, looking at total spleen tissue over the course of disease, we found that the inhibitory receptors PD-1 and CTLA-4, were significantly increased in later stages of the disease (Figure 4.4). The ligands for PD-1 also showed upregulation. PDL-1 showed a trend of progressive upregulation, but it was not significant. The other PD-1 ligand, PDL-2, increased at 21 days of infection and was significant by day 28 (Figure 4.4). The cell populations known to express these inhibitory molecules within the spleen were evaluated next. Splenic macrophages were isolated from uninfected or 28-day infected hamsters by adherence. Macrophages are among the APCs that express inhibitory ligands to bind T cells. The PD-1 ligand, PDL-1, was increased in chronically infected splenic macrophages although it was not significant (p=0.3429), while the ligand PDL-2 showed a trend in increase (p=0.0571) (Figure 4.5). The inhibitory receptors, PD-1 and CTLA-4, that were shown to be upregulated in total spleen tissue with chronic disease, were both significantly increased in splenic $CD4^+$ T cells from 28 day chronically infected animals (Figure 4.6). Collectively, this data suggests inhibitory signaling between infected macrophages and $CD4^+$ T cells may occur in the spleen during chronic stages of visceral leishmaniasis.

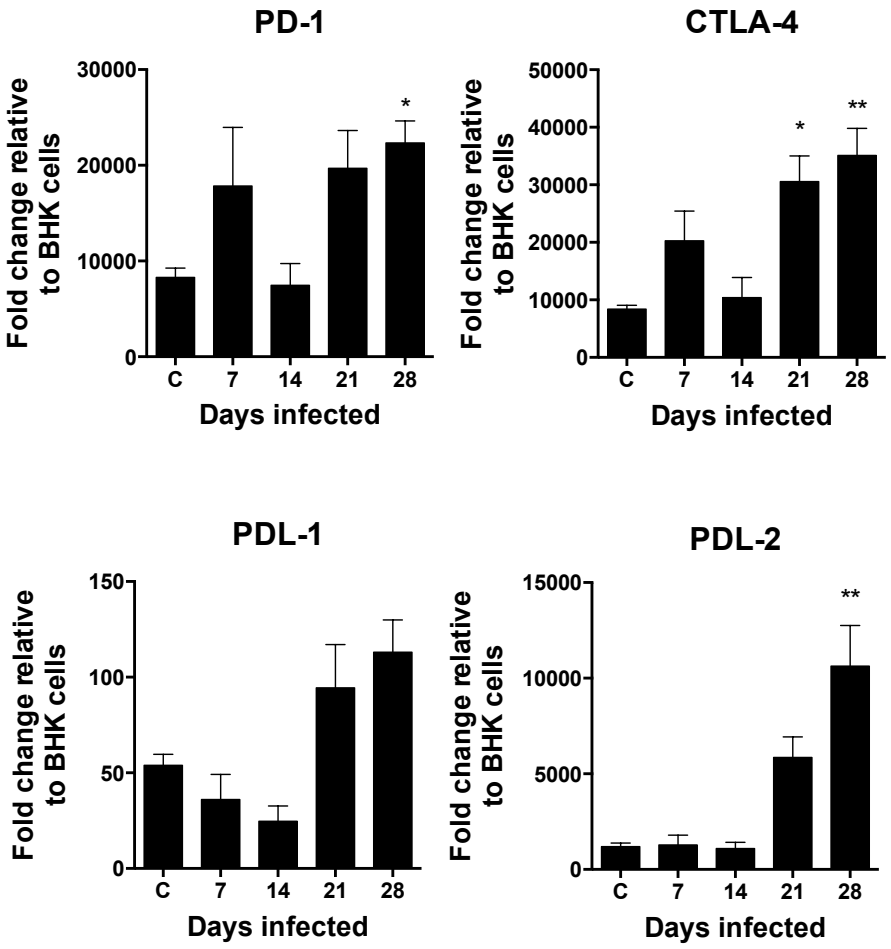


Figure 4. 4 Inhibitory markers increased in spleen over course of disease. Total spleen tissue was obtained from uninfected hamsters or hamsters infected with 1 million *L. donovani* metacylcic promastigotes for 7, 14, 21 and 28 days. mRNA expression was determined by real time RT-PCR. Results are expressed as a relative fold increase between experimental samples and uninfected BHK cells to which the value of 1 was arbitrarily assigned. Shown is the mean and SEM of a single experiment representative of 2 independent experiments from 3-6 hamsters per time point. *$p<0.05$; **$p<0.01$.

Splenic macrophages

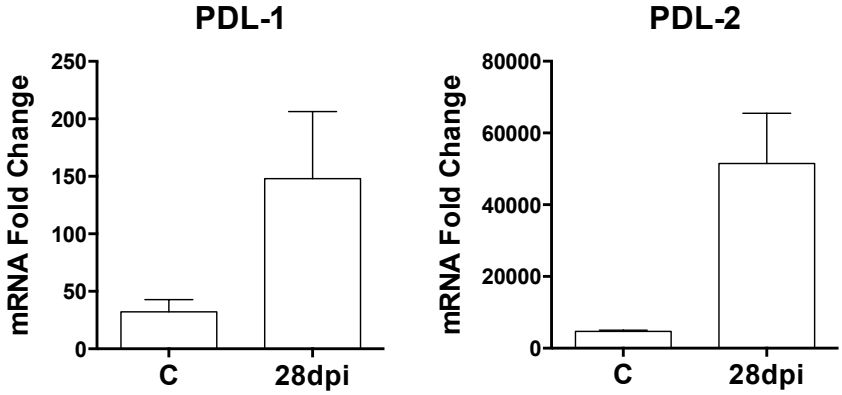


Figure 4. 5 Increased mRNA expression of inhibitory ligands in splenic macrophages during chronic visceral leishmaniasis. Splenic macrophages were isolated from uninfected hamsters or 28 day infected hamsters. mRNA expression was determined by real time RT-PCR. Results are expressed as a relative fold increase compared to uninfected BHK cells to which the value of 1 was arbitrarily assigned. Shown is the mean and SEM of a single experiment representative of 2 independent experiments from 4 hamsters per time point. Student's t-test was performed to compare control uninfected (C) and chronically 28 day infected (28dpi) samples.

$CD4^+$ T cells

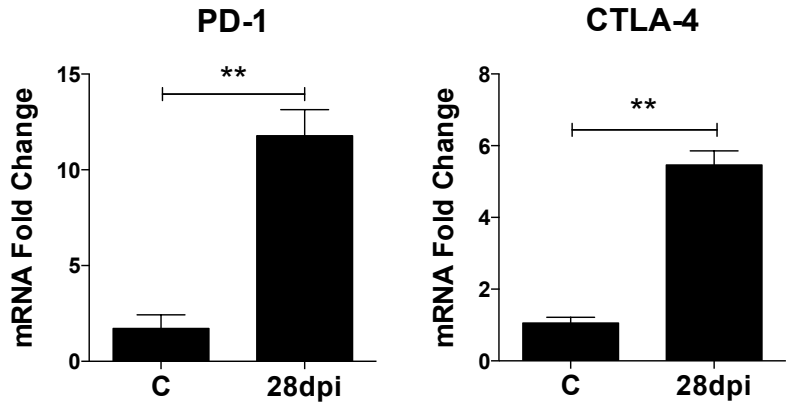


Figure 4. 6 Chronically stimulated $CD4^+$ T cells express markers of exhaustion in hamster spleen. Splenic $CD4^+$ T cells were isolated from uninfected or 28 day infected hamsters. mRNA gene expression was determined by real time RT-PCR to detect inhibitory markers. Results are expressed as a relative fold increase compared to uninfected BHK cells to which the value of 1 was arbitrarily assigned. Shown is the mean and SEM of a single experiment representative of 2 independent experiments from 4 hamsters per time point. Student's t-test was performed to compare control uninfected (C) and chronically 28 day infected (28dpi) samples. $^{**}p<0.01$

BLOCKADE OF PD-1/PDL-2 INTERACTION LEADS TO ENHANCED PARASITE CONTROL IN *EX VIVO* SPLEEN CELL CULTURE

The evidence we show of increased inhibitory markers in the spleen of hamsters during chronic disease agrees with other studies in other models of visceral leishmaniasis. In the mouse model, PDL-1 was increased in DCs. *In vivo* blockade enhanced *Leishmania* specific $CD8^+$ T cell proliferation and reduced parasite burden in the spleen [91]. Canine progressive visceral leishmaniasis exhibits $CD4^+$ and $CD8^+$ T cell exhaustion. Blocking PD-L1 reversed T cell exhaustion, enhanced $CD8^+$ T cell proliferation and increased superoxide production in monocytes [89]. To investigate the role of the increased inhibitory molecules on hamster macrophages and $CD4^+$ T cells, we initiated a pilot experiment to block the PD-1 ligand, PDL-2 in *ex vivo* spleen cultures (containing both macrophages and T cells). We found the parasite burden was significantly reduced after 48 hours incubation with α-PD-L2 antibody compared to isotype control (Figure 4.7). These findings need to be confirmed, but suggest activation of the PD-1 signaling pathway in the spleen plays a pathological role in hamster progressive visceral leishmaniasis and that this can be reversed.

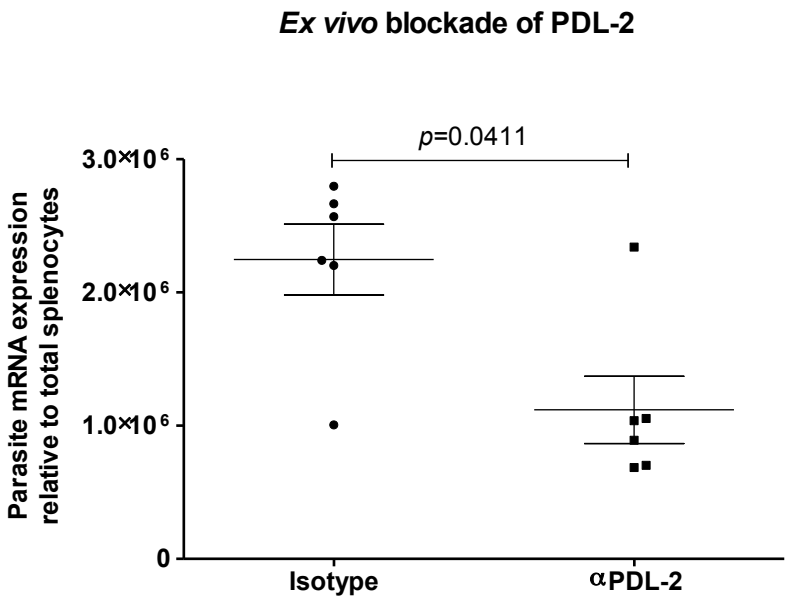


Figure 4. 7 *Ex vivo* blockade of PDL-2 reduced parasite burden. Total spleen cells from 28 day chronically infected hamsters were cultured with αPDL-2 antibody or isotype control for 48 hours. Parasite burden was determined by detecting *L. donovani* 18s mRNA by real time RT-PCR. Results are expressed as a relative fold increase compared to uninfected total splenocytes to which the value of 1 was arbitrarily assigned. Shown is the mean and SEM of a single preliminary experiment with 6 replicates. Student's t-test was performed to compare isotype control and αPDL-2 treatments.

CLOSE PROXIMITY BETWEEN T CELLS AND MACROPHAGES IS REQUIRED FOR ANTI-LEISHMANIAL EFFECTOR CD4$^+$ T CELL FUNCTION

As noted, CD4$^+$ T cells from chronically infected hamster spleens maintain an effector phenotype to limit parasite replication that is evident when removed from the splenic environment. But the hamster progresses to fatal chronic disease from *L. donovani* infection. Another possibility for an ineffective T cell response in visceral leishmaniasis is that the CD4$^+$ effector T cells are not localizing to the site of infected macrophages in the red pulp of the spleen. To test the hypothesis that close proximity between T cells and macrophages is required, we repeated the same co-culture protocol but separated the CD4$^+$ T cells from infected macrophages in a transwell system. As

hypothesized, we did not see a difference in parasite burden (Figure 4.8) nor did we see an increase in iNOS (Figure 4.9A) or Arg1 (Figure 4.9B) expression. This preliminary data suggests that cell-to-cell contact, or at least close proximity of T cell to macrophage, may be required for anti-leishmanial activity. It also raises the possibility that effector $CD4^+$ T cells may not be reaching infected macrophages in the spleen of hamsters during chronic visceral leishmaniasis.

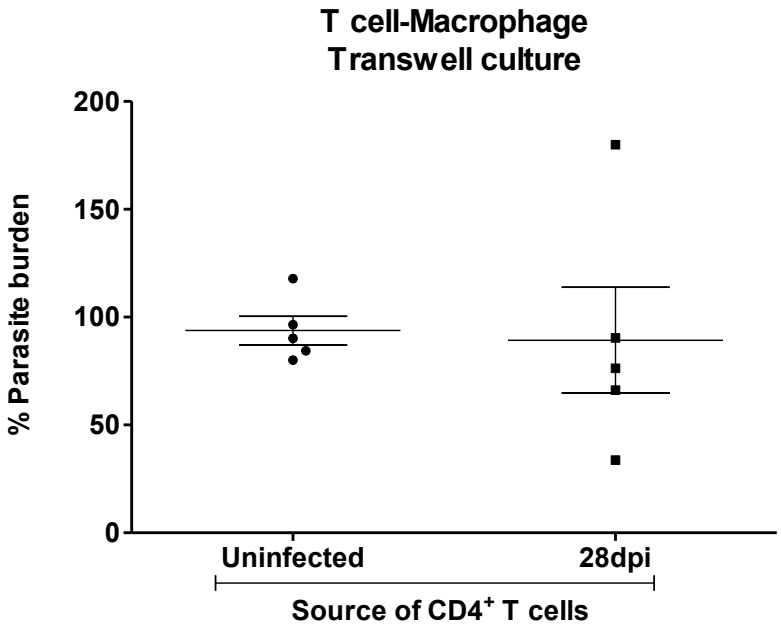


Figure 4. 8 $CD4^+$ T cell and macrophage interaction is necessary to induce parasite killing. *L. donovani* LUC infected macrophages were cultured in the bottom wells of a 24 well plate. Isolated $CD4^+$ T cells from uninfected or 28 day infected hamster spleens were added to a 0.4μm membrane transwell on top and cultured for 48 hours. The monolayer was washed before cell lysis to measure luciferase activity and determine parasite burden. Relative fold change was calculated using relative luminescent unit values to determine percentage using each group of $CD4^+$ T cells initial parasite burden time point as 100%. Each group had 6 replicates and data shown is representative of 2 independent experiments.

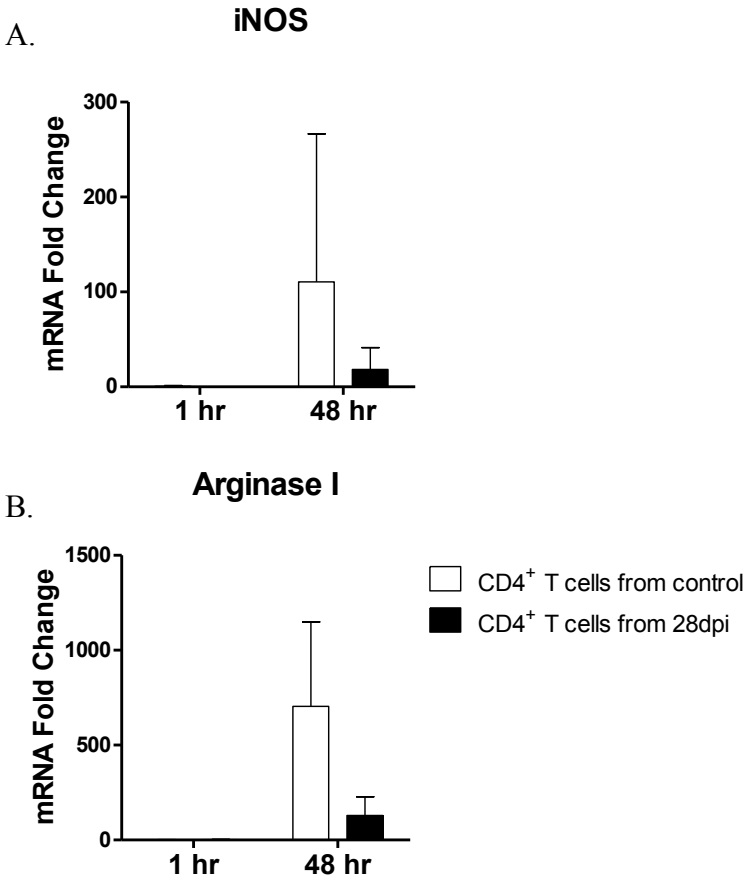


Figure 4. 9 Close proximity of CD4$^+$ T cells and macrophages is necessary to induce parasite killing. Infected macrophages were cultured in the bottom well with either CD4$^+$ T cell population in the top transwell at a 5:1 T cell to macrophage ratio for 48 hours. (A) iNOS and (B) Arg1 mRNA expression in macrophages was determined by real time RT-PCR at baseline time point 1hr and 48 hours later. Each group had 6 replicates and data shown is representative of 2 independent experiments. $^{**}p<0.01$

CD4$^+$ T CELLS FROM CHRONICALLY INFECTED HAMSTERS DO NOT SHOW ENHANCED MIGRATION TO SPLEEN DURING CHRONIC VISCERAL LEISHMANIASIS

We next wanted to determine the migration capabilities of chronically stimulated CD4$^+$ T cells from 28 day infected hamsters compared to naïve CD4$^+$ T cells from uninfected animals. To achieve this, we isolated CD4$^+$ T cells as described earlier from uninfected and 28-day infected hamster spleens and labeled them with different

fluorescent markers. Both cells types were transferred together at a 1:1 ratio to 28-day infected hamsters. Spleens were collected after 6 hours to allow for cell migration. The accumulated labeled cells were enumerated by flow cytometry analysis. Interestingly, we found the chronically stimulated $CD4^+$ T cells migrated no more than naïve $CD4^+$ T cells (Figure 4.10). This finding suggests that, despite having increased chemokine receptor expression (Figure 3.8), chronically stimulated $CD4^+$ T cells do not migrate any more efficiently to the site of chronic *L. donovani* infection than do unstimulated $CD4^+$ T cells. It is possible that analysis of different time points after transfer might identify a difference in accumulation.

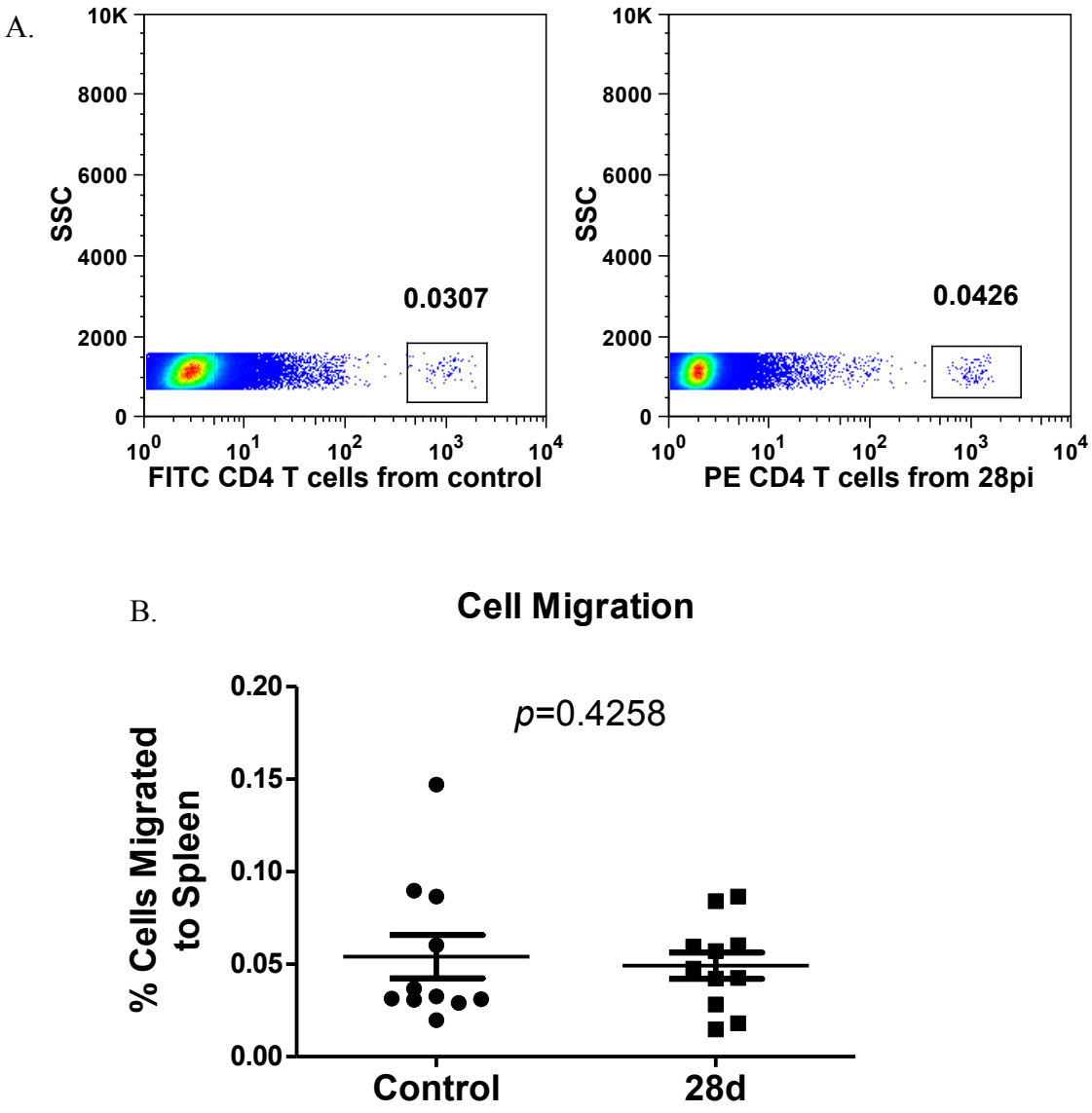


Figure 4. 10 Chronically stimulated CD4$^+$ T cells show comparable migration in chronic visceral leishmaniasis. Splenic CD4$^+$ T cells from uninfected (Control) or chronically infected (28d) hamsters were isolated and labeled separately with PKH cell tracker dyes. Once labeled, both cell populations were transferred to 28 day infected hamsters and migration to the spleen was analyzed 6 hours later by flow cytometry. (A) Representative graph for percentage of labeled cells captured from spleen. (B) Quantification of the proportion of transferred cells that migrated to the infected spleen after 6 hours. Data shown is compiled from 2 independent experiments for a total of 12 replicates per group.

Discussion

In the previous chapter we characterized the splenic environment during chronic *L. donovani* infection in the hamster model. We found mixed Th1 and Th2 immune responses, which is in agreement with what is found in the literature. This complexity of disease begs for a better understanding of the contributing factors in progression to fatal visceral leishmaniasis. This chapter focused on the main cell population targeted by the parasites, macrophages, and their interaction with $CD4^+$ T cells in the spleen during chronic stages of the disease.

We show the hamster is unable to control the persistent parasite replication in the spleen (Figure 2.2). As previously described, *Leishmania* parasites have adapted sophisticated mechanisms to evade host immune responses. It has been reported that *L. donovani* infection of macrophages inactivates key transcription factors involved in the type 1 inflammatory signaling cascade [152, 164, 165]. Thus, we first examined the effector function of *L. donovani* infected macrophages. *In vitro* cultured macrophages were primed with IFNγ and then triggered with LPS at the time of *L. donovani* infection. Determination of parasite burden demonstrated that the stimulated infected cells were in fact capable of producing NO and inducing intracellular killing of the parasite (Figure 4.1). This led us to believe the responding $CD4^+$ T cells are pathogenic and may render the macrophages unresponsive to IFNγ and unable to take on the M1 anti-leishmanial phenotype.

Previous studies in experimental models of leishmaniasis show contrasting roles for $CD4^+$ T cells in the development of chronic infection. In a model of cutaneous *L. amazonensis* infection, the lack of $CD4^+$ T cell function renders the host resistant to lesion development and able to maintain low parasite burden. This indicates that $CD4^+$ T cells are associated with pathogenesis [166]. But in a cutaneous *L. major* model of infection, $CD4^+$ T cells are necessary to control infection and inhibit progression to lesion

development [167]. Thus, the regulation of host immune responses to control infection seems to be *Leishmania* species dependent. Therefore, we next determined the role of $CD4^+$ T cells in chronic *L. donovani* infection in the hamster model. We set up a co-culture assay using the same technique of infecting macrophages *in vitro* but without IFNγ priming. Splenic $CD4^+$ T cells from uninfected animals were compared to $CD4^+$ T cells from chronically infected animals each co-cultured with infected macrophages. To our surprise, the chronically stimulated (activated) $CD4^+$ T cells reduced the intracellular parasite burden while increasing iNOS expression compared to the naïve $CD4^+$ T cells from uninfected animals (Figure 4.2 and 4.3A).

Chronic infection is associated with high levels of inflammatory cytokines. Prolonged inflammation leads to tissue damage and calls for the type 2 inflammatory immune response. A constant maintenance of immune homeostasis is critical for a host to recover from an inflammatory insult, but often this restoration of balance fails. Just as chemokines maintain immune balance by recruiting appropriate cell types, negative costimulatory receptors also play a role in immune homeostasis by negatively regulating T cell activation and proliferation [145, 168]. Since we showed that $CD4^+$ T cells from chronically infected hamsters couldn't efficiently migrate to the site of infection, we considered cellular exhaustion as a possible underlying cause. In total spleen tissue over a course of infection, we found inhibitory receptors, CTLA-4 and PD-1, are significantly increased in the chronic stage of disease (Figure 4.4). The engagement of these receptors with their ligands is required to induce their negative regulatory effects. The ligands for PD-1, PD-L1 and PD-L2, are expressed on activated APCs and even some lymphocytes [169]. Looking at ligands for PD-1, we found PD-L1 showed a trend of progressive increase although it was not significant. The second ligand of PD-1, PD-L2, did not show significant increase until 28 days post infection (Figure 4.4). Different studies in mouse, dog and human have showed increase of exhaustion markers on T cells, which would limit their role in protective immunity [89-91]. We looked at exhaustion markers

in both the $CD4^+$ T cell population and splenic macrophage compartment during chronic stages of disease. At 28 days of chronic infection in the hamster, the splenic macrophages show increased mRNA of the ligands PD-L1 and PD-L2 although it was not significant (Figure 4.5). Looking specifically at the $CD4^+$ T cell population, we found CTLA-4 and PD-1 were significantly upregulated at 28 days infection (Figure 4.6). This data suggests $CD4^+$ T cell exhaustion during chronic visceral leishmaniasis in the hamster. However, our previous data showed splenic $CD4^+$ T cells to have increased mRNA of cytokines and chemokine receptors (Figure 3.8), which appears to contradict this notion. T cell exhaustion has been thoroughly characterized and a recent study found that throughout a course of chronic disease, patients displayed a gradual decrease in T cell function, including cytokine production. Patients in later stages of chronic Chagas disease showed monofunctional $CD8^+$ T cells (single cytokine producers) compared to patients in early stages with polyfunctional $CD8^+$ T cells (multiple cytokine producers) [170]. This could explain the co-expression of inhibitory receptors, (T cell exhaustion) and cytokines (T cell effector function).

Several studies have blocked inhibitory receptors to restore T cell effector function. It has been demonstrated in dogs with active visceral leishmaniasis that blocking increased inhibitory molecules can restore macrophage-mediated killing of parasites. The $CD4^+$ and $CD8^+$ T cell populations regained capacity to proliferate and $CD4^+$ T cell IFNγ production was restored [89]. We show evidence of increased inhibitory receptors during chronic visceral leishmaniasis and hypothesized that blockade of the PD-1 pathway will induce intracellular killing of parasites. In a preliminary experiment using a hamster *ex vivo* model, the blockade of PD-L2 in total splenocyte cultures from 28 day infected animals showed a significant decrease in parasite burden (Figure 4.7). Thus, restoration of macrophage and T cell effector function by blocking the inhibitory PD-1 pathway led to increased parasite killing.

Another possible explanation for $CD4^+$ T cell failure *in vivo* could be T cells and macrophages are not in close proximity for appropriate cellular signaling. We showed in Figure 4.2 co-culture of $CD4^+$ T cells from chronically infected spleen tissue reduced parasite burden in macrophages and increased iNOS expression (Figure 4.3A). We wanted to assess whether this phenomenon was due to cytokine signaling from the activated $CD4^+$ T cells or if cell-to-cell contact induces macrophage effector function. This required a transwell assay, which allowed the $CD4^+$ T cells and infected cells to share the same cytokine environment but to not make contact. Interestingly, we did not see the same effect of intracellular parasite killing (Figure 4.8). Nor did we see increased iNOS expression suggesting $CD4^+$ T cell-macrophage engagement, or at least close proximity, is required for NO-induced parasite killing in macrophages (Figure 4.9A). This would require sufficient recruitment of effector $CD4^+$ T cells to the site of infection *in vivo*.

The establishment of immunity to leishmaniasis requires an efficient cell-mediated immune response. The recruitment of appropriate effector immune cells depends on chemokines. The role of chemokines expressed in lesional tissue in human visceral leishmaniasis is not completely understood but experimental models have provided some insight. As previously described, the experimental mouse model of *L. donovani* infection displays organ specific immunity. Studies investigating chemokine responses in the liver, where the infection is controlled, show increases of the type 1 chemokine CXCL10, which aids in recruitment of $CD4^+$ and $CD8^+$ T cells to promote granuloma formation [171]. But in human patients, it was reported that increased CXCL9 and CXCL10 in the plasma correlated with active visceral leishmaniasis. Once patients underwent treatment, chemokine levels significantly decreased, which they concluded that CXCL9 and CXCL10 play a pathogenic role in active disease [127]. Arguably, this decrease in chemokine ligand expression after treatment could be due to removal of the inflammatory stimulus once the infection is under control. Alternatively,

it is possible that these chemokines are adding to the massive inflammatory response that is damaging to the host. The balance of an inflammatory immune environment is important in host immunity against chronic infections. We showed an array of type 1 and type 2 chemokines upregulated in the spleen during chronic stages of visceral leishmaniasis (Figures 3.5). Furthermore, we showed infected splenic macrophages are a key source of these chemokines (Figure 3.6). To determine if this increased chemokine profile in the spleen favored the recruitment of activated $CD4^+$ T cells, an adoptive transfer of naïve and activated $CD4^+$ T cells was performed. We looked at the migration of the two groups of $CD4^+$ T cells to the site of infection during chronic visceral leishmaniasis. The activated $CD4^+$ T cells from infected hamsters migrated at similar proportions to naïve $CD4^+$ T cells (Figure 4.10). This suggests that $CD4^+$ T cells from chronically infected hamsters may not effectively reach the site of infection, and perhaps is the reason persistent parasite replication cannot be controlled.

The model systems used in these experiments allowed close examination of immune effector responses of key immune cells during chronic visceral leishmaniasis. In summary, this chapter first revealed the capacity of hamster *L. donovani* infected macrophages to kill the intracellular parasites when activated by IFNγ and a TLR4 ligand. This led to increased mRNA expression of NO by the infected macrophages which correlated with decreased parasite mRNA. Furthermore, macrophages infected *in vitro* and co-cultured with chronically stimulated $CD4^+$ T cells were also able to reduce intracellular parasite burden, which required close proximity of the T cells and macrophages. This led us to believe effector $CD4^+$ T cells may not be interacting with infected APCs to induce activation and control of parasite replication in chronic disease. Adoptive transfer of naïve and activated effector $CD4^+$T cells showed no difference in migration to the infection site. This suggests that $CD4^+$ T cells may not be protective *in vivo* because they do not effectively reach the site of infection.

The expression of exhaustion markers in the spleen during chronic visceral leishmaniasis led to further speculation of $CD4^+$ T cell effector function *in vivo.* Inhibitory molecules were not only expressed on the $CD4^+$ T cell population, but also on splenic macrophages. Blocking the PD-1 inhibitory pathway in an *ex vivo* culture of splenocytes from chronically infected hamsters revealed a significant decrease in parasite burden. Although initial experiments showed co-culture of chronically stimulated $CD4^+$ T cells with infected macrophages enhanced intracellular parasite killing, these experiments were performed with bone marrow derived macrophages. A more accurate representation of *in vivo* immune responses would have best been displayed in chronically infected splenic macrophages, as the phenotype could differ from *in vitro* infected macrophages. However, the long-term culture of splenic macrophages poses a challenge, as they are very fragile and do not survive well. The findings in this chapter suggest the insufficient immune responses seen in chronic human visceral leishmaniasis are in part due to $CD4^+$ T cell exhaustion. Despite showing increased markers of activation and effector functions in the $CD4^+$ T cell population, blocking an important molecule in the inhibitory pathway enhances effective immune responses to control parasite replication *ex vivo.* Confirmatory experiments are required and *in vivo* blocking experiments would strengthen this conclusion.

Defining the role of $CD4^+$ T cells during chronic visceral leishmaniasis in the hamster model is an important tool in understanding the pathogenesis of the disease in humans. For the first time, it has been demonstrated in the hamster that $CD4^+$ T cells and macrophages have the capacity to induce appropriate effector function in response to *L. donovani* infection *in vitro*, but there are multiple factors *in vivo* influencing the failure in cell-mediated immunity. The hamster is a great model that closely mimics human disease and the data presented here strengthen the applicability of this animal model.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Visceral leishmaniasis can progress to a chronic and fatal disease in humans. The causative species are *Leishmania donovani* and *L. infantum/chagasi,* which are transmitted by the sand fly vector. Visceral leishmaniasis accounts for 500,000 new cases reported annually. The majority of infected individuals develop a subclinical, or asymptomatic, infection that is controlled and they do not develop disease. Other individuals experience persistent parasite replication in the spleen, liver and bone marrow leading to splenomegaly, pancytopenia, cachexia and ultimately death [58]. It is not completely understood why some individuals progress to disease while others do not. Differences in host genetics, environmental factors and infecting parasite species are associated with susceptibility to disease. Differences in host immune responses also play a role in the outcome of disease, but it is not well defined. The purpose of this dissertation was to gain a better understanding of the role $CD4^+$ T cells play in the immunopathogenesis of chronic *L. donovani* infection using the hamster model, which displays similar clinical features as humans with active visceral leishmaniasis.

$CD4^+$ T CELL RESPONSE DURING CHRONIC VISCERAL LEISHMANIASIS

The Th1/Th2 paradigm defined the resistant/susceptible immunity against intracellular pathogens in experimental models of cutaneous leishmaniasis. Th1 cells are defined by their production of the cytokines IFNγ, TNF-α and IL-2, which are linked to resistance against development of disease. In contrast, Th2 cells induce a counter immune response with cytokines IL-4, IL-5 and IL-13, which dampen inflammation leading to increased susceptibility [32, 51, 172]. However, recent studies have found this dichotomy of immune responses is not as clear-cut in human and other non-murine models of leishmaniasis. Active visceral leishmaniasis patients are found to have

increased type 1 and type 2 cytokines (IFNγ, IL-1, IL-12, IL-6, TNFα, IL-4, IL-13 and IL-10) in plasma, spleen and bone marrow [22, 25, 64, 82, 173]. In chapter 3 we report similar increases of type 1 and type 2 cytokines (IFNγ, IL-4, and IL-10) in the spleen of chronically infected hamsters. This suggests involvement of both Th1 and Th2 $CD4^+$ subsets.

It is evident that $CD4^+$ T cells play an important role in immunity against progressive visceral leishmaniasis. The T cell subsets based on cytokine profiles have been widely studied in human visceral leishmaniasis. Immune responses have been demonstrated in both asymptomatic patients and those with the active form of disease. PBMCs from patients with asymptomatic infection respond to *Leishmania* soluble antigen by producing IFNγ and proliferation. On the other hand, PBMCs from patients with active visceral leishmaniasis do not show such a response [22]. But, a more recent study has demonstrated that whole blood cell cultures from patients with active disease do in fact respond to *Leishmania* soluble antigen stimulation with increased IFNγ production, and the main source of IFNγ was revealed as $CD4^+$ T cells [128].

Some studies found fewer $CD4^+$ T cells in the peripheral blood of patients with active disease [65, 129]. Another study revealed that the highest percentage of cells in the infected spleen were lymphocytes [25]. Our findings confirm an increase in accumulation of $CD4^+$ T cells in the spleen during chronic infection compared to healthy controls. A closer look into the hamster splenic $CD4^+$ T cell population revealed there to be mixed Th1 and Th2 cells marked by the increase of transcription factors Tbet and GATA3, respectively. This increase was correlated with increased expression of IFNγ and IL-4. Surprisingly, we did not find increase in $CD4^+Foxp3^+$ cells despite an increase in the regulatory cytokine IL-10, suggesting the source is a $CD4^+Foxp3^-$ subset. Studies in human visceral leishmaniasis also demonstrate the source of splenic IL-10 is from a $CD4^+Foxp3^-$ cell population, which interestingly is also a primary source of IFNγ [25]. These double producing $CD4^+$ T cells have been found in several other models of chronic

disease including *Toxoplasma gondii* and *Mycobacterium tuberculosis* [48, 174]. The distinction of $CD4^+$ T cells subsets based on cytokine profiles has become more complex with the availability of multicolor flow cytometry and new T helper cell markers. It is likely there are double- or multiple-cytokine producing splenic $CD4^+$ T cells in chronic visceral leishmaniasis in the hamster.

CHRONIC DISEASE AND EFFECTOR FUNCTION OF HOST IMMUNE CELLS

It is well established that chronic infectious diseases involve the exhaustion of T cells resulting in the diminished capacity of effector function. T cell exhaustion was first described in a chronic viral infection of lymphocytic choriomeningitis (LCMV). The establishment of chronic LCMV infection was in part due to clonal deletion of $CD8^+$ T cells, which was preceded by T cell exhaustion marked by loss of effector function and proliferation. They concluded exhaustion was a result of long-term antigen stimulation of T cells [175]. In many chronic infections, pathogens have evolved to utilize this host mechanism for their benefit and establish chronicity [145]. The role of the receptors PD-1 and CTLA-4 have been defined as negative regulators of T cell function during chronic infections. They are primarily expressed on $CD8^+$ and $CD4^+$ T cells, but can also be found on B cells [176]. Their expression has been shown to increase soon after T cell activation is initiated. With chronic stimulation of T cells, the regulatory functions of CTLA-4 and PD-1 control overly aggressive T cell responses that are damaging to the host. These inhibitory receptors lead to the gradual loss of activation and expansion of T cells, decreased cytokine production and in some cases clonal deletion of T cell populations [145]. As described in chapter 2, the hamster model displays expression of both $CD4^+$ T cell activation and exhaustion markers.

The increase in exhaustion markers has been demonstrated in different models of visceral leishmaniasis including mouse, dog and humans [89-91]. We described in chapters 2 and 4 there is increased mRNA expression of PD-1, CTLA-4, PD-L1 and PD-

L2 in chronically infected hamster spleen tissue. More specifically, we identified in the $CD4^+$ T cell population significant expression of the inhibitory receptors CTLA-4 and PD-1. Despite showing increased exhaustion markers, we showed $CD4^+$ T cells from 28 day infected spleen co-cultured with *in vitro* infected macrophages inhibited intracellular parasite growth. This suggests the $CD4^+$ T cells responding at the site of chronic infection have some level of effector function. The migration of immune effector cells to the site of infection is necessary to initiate the proper immune responses. However, our adoption transfer experiments did not show enhanced migration in the splenic $CD4^+$ T cells from chronically infected hamsters.

Our data add to the growing evidence of a strong type-1 (IFNγ) immune response during active visceral leishmaniasis. But the question remains, why are macrophages in this Th1 environment not able to control persistent *L. donovani* infection? As noted, several studies have focused on exhaustion markers and the effects on T cell effector function. However, few studies have investigated the effects of the inhibitory receptors from the APC point of view. It has been hypothesized that *Leishmania* infection drives subversive mechanisms that render macrophages unresponsive to cytokine stimulation [25, 165, 177, 178]. This allows the parasites to keep proliferating in cells uninterrupted. In chapter 4, we showed that total spleen tissue and splenic macrophages had increased expression of the inhibitory ligands PD-L1 and PD-L2. It is possible that the interaction between PD-1 and PD-L1/2 has a bi-directional effect. Recently it was shown that engagement of the inhibitory receptors on macrophages and dendritic cells delivers a suppressive signal from the T cell to the myeloid cell [179-181]. Our T cell-macrophage co-cultures used *in vitro* differentiated bone marrow macrophages, which displayed enhanced capabilities of reducing intracellular parasite burden after 48 hours. It is possible if these experiments were conducted using splenic macrophages from chronically infected spleen tissue, the results would be different. It would have been beneficial to detect inhibitory ligand expression in *in vitro* infected macrophages

compared to splenic macrophages. The immune phenotype of bone marrow derived macrophages differentiated and infected *in vitro* may not be the best representation of macrophages chronically stimulated over a disease course of 28 days.

T cell exhaustion is exhibited by the gradual decline in effector functions [145]. We described in chapters 2 and 4 the upregulation of T cell exhaustion markers. This led us to explore the role T cell exhaustion in disease pathogenesis. $CD8^+$ T cell exhaustion has been described in human patients with active visceral leishmaniasis. Blockade of CTLA-4 and PD-1 was performed in isolated $CD8^+$ T cells from PBMCs but showed no difference in response to *Leishmania* soluble antigen [90]. Chronic visceral leishmaniasis in dogs also led to increased $CD4^+$ T cell exhaustion markers. By blocking PD-L1 *in vitro* $CD4^+$ T cell proliferation and production of IFNγ was rescued [91]. Similarly, we were able to demonstrate a reduction in parasite burden by blocking PD-L2 in total hamster splenocytes. This indicates a pathogenic role for the PD-1 pathway in chronic visceral leishmaniasis. Whether this effect was mediated by a recovery of T cell responsiveness or macrophage effector function (or both) is unknown. Further investigation into the role of inhibitory receptors/ligands on the interaction between $CD4^+$ T cells and macrophages is warranted.

CONCLUDING REMARKS

The work described in this dissertation provides novel insights of $CD4^+$ T cell effector function during chronic visceral leishmaniasis in an experimental model that closely mimics human disease. Our findings indicate the involvement of $CD4^+$ T cells and induction of immunity against *L. donovani* infection is a complex process. *In vivo* experiments would be optimal in understanding complete mechanisms in the immune response, but work in the hamster model is limited due to the lack of available reagents. The primary conclusions of the work presented in this dissertation are that 1) $CD4^+$ T cells in the spleen during chronic progressive visceral leishmaniasis have a mixed Th1

and Th2 phenotype; 2) Despite the ineffective cellular immune response during progressive disease, removal of the $CD4^+$ T cells from the splenic environment enables them to promote inhibition of intracellular parasite growth in normal *in vitro* macrophages infected; 3) Splenic $CD4^+$ T cells and macrophages express an array of chemokines and chemokine receptors that should contribute to the accumulation of these cells, but their co-localization in the spleen remains uncertain; 4) Splenic $CD4^+$ T cells and macrophages express a number of inhibitory receptors and ligands that may contribute to impaired T cell and macrophage effector function. Blockade of the interaction between PD-1 and PD-L2 led to reduced parasite burden in total spleen cell cultures. For continuation of the work presented in this dissertation and to further advance the leishmaniasis field, I recommend the following future experiments to compliment the findings reported here: 1) Evaluate the localization of $CD4^+$ T cells in reference to infected macrophages in the spleen by immunohistochemistry. 2) Fully characterize the macrophage effector phenotype induced by the $CD4^+$ T cells by measuring NO and Arg1 expression in the co-culture assay. 3) Determine if the mixed Th1 and Th2 markers are indicative of two different T cell populations or if $CD4^+$ T cells have a double-positive phenotype by RNA-Flow (RNA amplification coupled with in situ fluorescence hybridization and flow cytometry) methods. 4) Develop *in vivo* methods to block PD-1 in hamsters to better characterize the chronic immune responses in the spleen. 5) Evaluate the role of other inhibitory receptors-ligands in the pathogenesis of visceral leishmaniasis.

Appendix

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References

1. Awasthi A, Mathur RK, Saha B. Immune response to Leishmania infection. Indian J Med Res. 2004;119(6):238-58. PubMed PMID: 15243162.
2. Grimaldi G, Tesh RB, McMahon-Pratt D. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. The American journal of tropical medicine and hygiene. 1989;41(6).
3. Pearson RD, Sousa AdQ. Clinical spectrum of Leishmaniasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1996;22(1).
4. Leishman WB. On the possibility of the occurence of trypanosomiasis in India. On the possibility of the occurence of trypanosomiasis in India. 1903;1(2213). doi: 10.1038/068123b0.
5. Lewis DJ. The Biology of Phlebotomidae in Relation to Leishmaniasis. Annual Review of Entomology. 1974. doi: 10.1146/annurev.en.19.010174.002051.
6. Sacks DL, da Silva RP. The generation of infective stage Leishmania major promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. Journal of immunology (Baltimore, Md : 1950). 1987;139(9).
7. Turco SJ, Descoteaux A. The lipophosphoglycan of Leishmania parasites. Annu Rev Microbiol. 1992;46:65-94. doi: 10.1146/annurev.mi.46.100192.000433. PubMed PMID: 1444269.
8. Descoteaux A, Turco SJ. Glycoconjugates in Leishmania infectivity. Biochim Biophys Acta. 1999;1455(2-3):341-52. PubMed PMID: 10571023.
9. Da Silva RP, Sacks DL. Metacyclogenesis is a major determinant of Leishmania promastigote virulence and attenuation. Infection and immunity. 1987;55(11).
10. Sacks DL. Metacyclogenesis in Leishmania promastigotes. Exp Parasitol. 1989;69(1):100-3. PubMed PMID: 2659372.
11. King DL, Chang Y-D, Turco SJ. Cell surface lipophosphoglycan of Leishmania donovani. Molecular and biochemical parasitology. 1987;24(1). doi: 10.1016/0166-6851(87)90114-9.
12. Sacks DL, Pimenta PFP, McConville MJ, Schneider P, Turco SJ. Stage-specific binding of Leishmania donovani to the sand fly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. The Journal of experimental medicine. 1995;181(2).
13. Desjardins M, Descoteaux A. Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. J Exp Med. 1997;185(12):2061-8. PubMed PMID: 9182677; PubMed Central PMCID: PMC2196352.
14. Colmenares M, Kar S, Goldsmith-Pestana K, McMahon-Pratt D. Mechanisms of pathogenesis: differences amongst Leishmania species. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2002;96:S3-S7. doi: 10.1016/s0035-9203(02)90044-1.
15. Dowlati Y. Cutaneous leishmaniasis: clinical aspect. Cutaneous leishmaniasis: clinical aspect. 1996.

16. Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. Infect Immun. 2002;70(12):6734-40. PubMed PMID: 12438348; PubMed Central PMCID: PMC132996.
17. Camuset G, Remy V, Hansmann Y, Christmann D, Gomes de Albuquerque C, Sena Casseb GA. [Mucocutaneous leishmaniasis in Brazilian Amazonia]. Med Mal Infect. 2007;37(6):343-6. doi: 10.1016/j.medmal.2007.03.012. PubMed PMID: 17512150.
18. Strazzulla A, Cocuzza S, Pinzone MR, Postorino MC, Cosentino S, Serra A, et al. Mucosal leishmaniasis: an underestimated presentation of a neglected disease. Biomed Res Int. 2013;2013:805108. doi: 10.1155/2013/805108. PubMed PMID: 23853773; PubMed Central PMCID: PMC3703408.
19. Faria DR, Gollob KJ, Barbosa J, Jr., Schriefer A, Machado PR, Lessa H, et al. Decreased in situ expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis. Infect Immun. 2005;73(12):7853-9. doi: 10.1128/IAI.73.12.7853-7859.2005. PubMed PMID: 16299275; PubMed Central PMCID: PMC1307048.
20. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7(5):e35671. doi: 10.1371/journal.pone.0035671. PubMed PMID: 22693548; PubMed Central PMCID: PMCPMC3365071.
21. Rai K, Cuypers B, Bhattarai NR, Uranw S, Berg M, Ostyn B, et al. Relapse after treatment with miltefosine for visceral leishmaniasis is associated with increased infectivity of the infecting Leishmania donovani strain. MBio. 2013;4(5):e00611-13. doi: 10.1128/mBio.00611-13. PubMed PMID: 24105765; PubMed Central PMCID: PMC3791894.
22. Sacks DL, Lal SL, Shrivastava SN, Blackwell J, Neva FA. An analysis of T cell responsiveness in Indian kala-azar. Journal of immunology (Baltimore, Md : 1950). 1987;138(3).
23. Carvalho EM, Bacellar O, Barral A, Badaro R, Johnson WD, Jr. Antigen-specific immunosuppression in visceral leishmaniasis is cell mediated. J Clin Invest. 1989;83(3):860-4. doi: 10.1172/JCI113969. PubMed PMID: 2522103; PubMed Central PMCID: PMC303759.
24. Sundar S, Reed SG, Sharma S, Mehrotra A, Murray HW. Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis. Am J Trop Med Hyg. 1997;56(5):522-5. PubMed PMID: 9180602.
25. Nylén S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med. 2007;204(4):805-17. doi: jem.20061141 [pii]
10.1084/jem.20061141. PubMed PMID: 17389235; PubMed Central PMCID: PMCPMC2118563.
26. Osorio Y, Travi BL, Renslo AR, Peniche AG, Melby PC. Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system. PLoS Negl Trop Dis. 2011;5(2):e962. doi: 10.1371/journal.pntd.0000962. PubMed PMID: 21358812; PubMed Central PMCID: PMCPMC3039689.

27. Cerf BJ, Jones TC, Badaro R, Sampaio D, Teixeira R, Johnson WD, Jr. Malnutrition as a risk factor for severe visceral leishmaniasis. J Infect Dis. 1987;156(6):1030-3. PubMed PMID: 3680989.
28. Anstead GM, Chandrasekar B, Zhao W, Yang J, Perez LE, Melby PC. Malnutrition Alters the Innate Immune Response and Increases Early Visceralization following Leishmania donovani Infection. Infection and Immunity. 2001;69(8). doi: 10.1128/IAI.69.8.4709-4718.2001.
29. Bucy RP, Karr L, Huang GQ, Li J, Carter D, Honjo K, et al. Single cell analysis of cytokine gene coexpression during CD4+ T-cell phenotype development. Proc Natl Acad Sci U S A. 1995;92(16):7565-9. PubMed PMID: 7638231; PubMed Central PMCID: PMC41380.
30. Becattini S, Latorre D, Mele F, Foglierini M, De Gregorio C, Cassotta A, et al. T cell immunity. Functional heterogeneity of human memory CD4+ T cell clones primed by pathogens or vaccines. Science (New York, NY). 2015;347(6220):400-6. doi: 10.1126/science.1260668.
31. Hedrick SM, Engel I, McElligott DL, Fink PJ, Hsu ML, Hansburg D, et al. Selection of amino acid sequences in the beta chain of the T cell antigen receptor. Science. 1988;239(4847):1541-4. PubMed PMID: 2832942.
32. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of immunology (Baltimore, Md : 1950). 1986;136(7).
33. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-69. PubMed PMID: 10761931.
34. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol. 2010;28:445-89. doi: 10.1146/annurev-immunol-030409-101212. PubMed PMID: 20192806; PubMed Central PMCID: PMC3502616.
35. Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science. 2002;295(5553):338-42. doi: 10.1126/science.1065543. PubMed PMID: 11786644.
36. Koyasu S, Moro K, Tanabe M, Takeuchi T. Natural helper cells: a new player in the innate immune response against helminth infection. Adv Immunol. 2010;108:21-44. doi: 10.1016/B978-0-12-380995-7.00002-1. PubMed PMID: 21056728.
37. Harris N, Gause WC. To B or not to B: B cells and the Th2-type immune response to helminths. Trends Immunol. 2011;32(2):80-8. doi: 10.1016/j.it.2010.11.005. PubMed PMID: 21159556; PubMed Central PMCID: PMC3076625.
38. Kubo M, Yamashita M, Abe R, Tada T, Okumura K, Ransom JT, et al. CD28 costimulation accelerates IL-4 receptor sensitivity and IL-4-mediated Th2 differentiation. J Immunol. 1999;163(5):2432-42. PubMed PMID: 10452978.
39. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity. 1998;9(5):745-55. PubMed PMID: 9846495.
40. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4(4):330-6. doi: 10.1038/ni904. PubMed PMID: 12612578.

41. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol. 2001;2(4):301-6. doi: 10.1038/86302. PubMed PMID: 11276200.
42. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155(3):1151-64. PubMed PMID: 7636184.
43. Locksley RM, Heinzel FP, Sadick MD, Holaday BJ, Gardner KD, Jr. Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets. Ann Inst Pasteur Immunol. 1987;138(5):744-9. PubMed PMID: 2964247.
44. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med. 1997;185(5):817-24. PubMed PMID: 9120387; PubMed Central PMCID: PMC2196166.
45. Naoe Y, Setoguchi R, Akiyama K, Muroi S, Kuroda M, Hatam F, et al. Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. J Exp Med. 2007;204(8):1749-55. doi: 10.1084/jem.20062456. PubMed PMID: 17646405; PubMed Central PMCID: PMC2118685.
46. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol. 2007;8(2):191-7. doi: 10.1038/ni1428. PubMed PMID: 17136045.
47. Peine M, Rausch S, Helmstetter C, Fröhlich A, Hegazy AN, Kühl AA, et al. Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation. PLoS Biol. 2013;11(8):e1001633. doi: 10.1371/journal.pbio.1001633. PubMed PMID: 23976880; PubMed Central PMCID: PMCPMC3747991.
48. Gerosa F, Nisii C, Righetti S, Micciolo R, Marchesini M, Cazzadori A, et al. CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. Clin Immunol. 1999;92(3):224-34. doi: 10.1006/clim.1999.4752. PubMed PMID: 10479527.
49. Jagannathan P, Eccles-James I, Bowen K, Nankya F, Auma A, Wamala S, et al. IFNgamma/IL-10 co-producing cells dominate the CD4 response to malaria in highly exposed children. PLoS Pathog. 2014;10(1):e1003864. doi: 10.1371/journal.ppat.1003864. PubMed PMID: 24415936; PubMed Central PMCID: PMC3887092.
50. Owens BM, Beattie L, Moore JW, Brown N, Mann JL, Dalton JE, et al. IL-10-producing Th1 cells and disease progression are regulated by distinct CD11c(+) cell populations during visceral leishmaniasis. PLoS Pathog. 2012;8(7):e1002827. doi: 10.1371/journal.ppat.1002827. PubMed PMID: 22911108; PubMed Central PMCID: PMC3406093.
51. Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. The Journal of Experimental Medicine. 1989;169(1). doi: 10.1084/jem.169.1.59.
52. Kopf M, Brombacher F, Kohler G, Kienzle G, Widmann KH, Lefrang K, et al. IL-4-deficient Balb/c mice resist infection with Leishmania major. J Exp Med.

1996;184(3):1127-36. PubMed PMID: 9064329; PubMed Central PMCID: PMC2192785.
53. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. Front Immunol. 2012;3:251. doi: 10.3389/fimmu.2012.00251. PubMed PMID: 22912637; PubMed Central PMCID: PMCPMC3418610.
54. Murray HW, Stern JJ, Welte K, Rubin BY, Carriero SM, Nathan CF. Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin 2 and interferon-gamma. J Immunol. 1987;138(7):2290-7. PubMed PMID: 3104456.
55. Bankoti R, Stager S. Differential Regulation of the Immune Response in the Spleen and Liver of Mice Infected with Leishmania donovani. J Trop Med. 2012;2012:639304. doi: 10.1155/2012/639304. PubMed PMID: 21811511; PubMed Central PMCID: PMC3143424.
56. Melby PC, Tabares A, Restrepo BI, Cardona AE, McGuff HS, Teale JM. Leishmania donovani: evolution and architecture of the splenic cellular immune response related to control of infection. Exp Parasitol. 2001;99(1):17-25. doi: 10.1006/expr.2001.4640. PubMed PMID: 11708830.
57. Adler S. Origin of the golden hamster Cricetus auratus as a laboratory animal. Nature. 1948;162(4111):256. PubMed PMID: 18934878.
58. Gifawesen C, Farrell JP. Comparison of T-cell responses in self-limiting versus progressive visceral Leishmania donovani infections in golden hamsters. Infection and immunity. 1989;57(10).
59. Melby PC, Chandrasekar B, Zhao W, Coe JE. The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. Journal of immunology (Baltimore, Md : 1950). 2001;166(3).
60. Osorio EY, Weiguo Z, Claudia E, Omar S, Leo H, Craig VB, et al. Progressive Visceral Leishmaniasis Is Driven by Dominant Parasite-induced STAT6 Activation and STAT6-dependent Host Arginase 1 Expression. PLoS Pathogens. 2012;8(1). doi: 10.1371/journal.ppat.1002417.
61. Alvar J, Canavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. Adv Parasitol. 2004;57:1-88. doi: 10.1016/S0065-308X(04)57001-X. PubMed PMID: 15504537.
62. Espitia CM, Saldarriaga OA, Travi BL, Osorio EY, Hernandez A, Band M, et al. Transcriptional profiling of the spleen in progressive visceral leishmaniasis reveals mixed expression of type 1 and type 2 cytokine-responsive genes. BMC Immunol. 2014;15:38. doi: 10.1186/s12865-014-0038-z. PubMed PMID: 25424735; PubMed Central PMCID: PMC4253007.
63. Reema G, Pramod KK, Mukesh S, Anil KJ, Rajendra KB, Anuradha D. Treatment of Leishmania donovani-infected hamsters with miltefosine: analysis of cytokine mRNA expression by real-time PCR, lymphoproliferation, nitrite production and antibody responses. Journal of Antimicrobial Chemotherapy. 2012;67(2):440-3. doi: 10.1093/jac/dkr485.
64. Ansari NA, Saluja S, Salotra P. Elevated levels of interferon-gamma, interleukin-10, and interleukin-6 during active disease in Indian kala azar. Clin Immunol. 2006;119(3):339-45. doi: S1521-6616(06)00047-7 [pii]

10.1016/j.clim.2006.01.017. PubMed PMID: 16540374.
65. Hailu A, van Baarle D, Knol GJ, Berhe N, Miedema F, Kager PA. T cell subset and cytokine profiles in human visceral leishmaniasis during active and asymptomatic or sub-clinical infection with Leishmania donovani. Clin Immunol. 2005;117(2):182-91. doi: 10.1016/j.clim.2005.06.015. PubMed PMID: 16125466.
66. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10(1):57-63. doi: 10.1038/nrg2484. PubMed PMID: 19015660; PubMed Central PMCID: PMC2949280.
67. Zhang B, Kirov S, Snoddy J. WebGestalt: an integrated system for exploring gene sets in various biological contexts. Nucleic Acids Res. 2005;33(Web Server issue):W741-8. doi: 10.1093/nar/gki475. PubMed PMID: 15980575; PubMed Central PMCID: PMC1160236.
68. UniProt C. UniProt: a hub for protein information. Nucleic Acids Res. 2015;43(Database issue):D204-12. doi: 10.1093/nar/gku989. PubMed PMID: 25348405; PubMed Central PMCID: PMC4384041.
69. Melby PC, Tryon VV, Chandrasekar B, Freeman GL. Cloning of Syrian hamster (Mesocricetus auratus) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. Infection and immunity. 1998;66(5).
70. Aslan H, Dey R, Meneses C, Castrovinci P, Jeronimo SM, Oliva G, et al. A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. J Infect Dis. 2013;207(8):1328-38. doi: 10.1093/infdis/jis932. PubMed PMID: 23288926; PubMed Central PMCID: PMC3603531.
71. Hommel M, Jaffe CL, Travi B, Milon G. Experimental models for leishmaniasis and for testing anti-leishmanial vaccines. Ann Trop Med Parasitol. 1995;89 Suppl 1:55-73. PubMed PMID: 8745928.
72. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75(2):163-89. doi: 10.1189/jlb.0603252. PubMed PMID: 14525967.
73. Palacios EH, Weiss A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene. 2004;23(48):7990-8000. doi: 10.1038/sj.onc.1208074. PubMed PMID: 15489916.
74. Brownlie RJ, Zamoyska R. T cell receptor signalling networks: branched, diversified and bounded. Nat Rev Immunol. 2013;13(4):257-69. doi: 10.1038/nri3403. PubMed PMID: 23524462.
75. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol. 2012;12(3):180-90. doi: 10.1038/nri3156. PubMed PMID: 22343569.
76. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. Immunity. 2010;33(2):153-65. doi: 10.1016/j.immuni.2010.08.004. PubMed PMID: 20732639; PubMed Central PMCID: PMC2946796.
77. Bhattacharjee A, Shukla M, Yakubenko VP, Mulya A, Kundu S, Cathcart MK. IL-4 and IL-13 employ discrete signaling pathways for target gene expression in alternatively activated monocytes/macrophages. Free Radic Biol Med. 2013;54:1-16. doi: 10.1016/j.freeradbiomed.2012.10.553. PubMed PMID: 23124025; PubMed Central PMCID: PMC3534796.

78. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol. 2007;7(6):454-65. doi: 10.1038/nri2093. PubMed PMID: 17525754.
79. Wormald S, Zhang JG, Krebs DL, Mielke LA, Silver J, Alexander WS, et al. The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling. J Biol Chem. 2006;281(16):11135-43. doi: 10.1074/jbc.M509595200. PubMed PMID: 16473883.
80. Osinalde N, Moss H, Arrizabalaga O, Omaetxebarria MJ, Blagoev B, Zubiaga AM, et al. Interleukin-2 signaling pathway analysis by quantitative phosphoproteomics. J Proteomics. 2011;75(1):177-91. doi: 10.1016/j.jprot.2011.06.007. PubMed PMID: 21722762.
81. Frossi B, Rivera J, Hirsch E, Pucillo C. Selective activation of Fyn/PI3K and p38 MAPK regulates IL-4 production in BMMC under nontoxic stress condition. J Immunol. 2007;178(4):2549-55. PubMed PMID: 17277164.
82. Karp CL, El-Safi SH, Wynn TA, Satti MMH, Kordofani AM, Hashim FA, et al. In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma. Journal of Clinical Investigation. 1993. doi: 10.1172/JCI116372.
83. Kenney RT, Sacks DL, Gam AA, Murray HW, Sundar S. Splenic cytokine responses in Indian kala-azar before and after treatment. J Infect Dis. 1998;177(3):815-8. PubMed PMID: 9498473.
84. Green SJ, Nacy CA, Meltzer MS. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens. J Leukoc Biol. 1991;50(1):93-103. PubMed PMID: 2056250.
85. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008;8(12):958-69. doi: 10.1038/nri2448. PubMed PMID: 19029990; PubMed Central PMCID: PMC2724991.
86. Murray HW, Nathan CF. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani. J Exp Med. 1999;189(4):741-6. PubMed PMID: 9989990; PubMed Central PMCID: PMCPMC2192937.
87. Altin JG, Sloan EK. The role of CD45 and CD45-associated molecules in T cell activation. Immunol Cell Biol. 1997;75(5):430-45. doi: 10.1038/icb.1997.68. PubMed PMID: 9429890.
88. Baaten BJ, Li CR, Deiro MF, Lin MM, Linton PJ, Bradley LM. CD44 regulates survival and memory development in Th1 cells. Immunity. 2010;32(1):104-15. doi: 10.1016/j.immuni.2009.10.011. PubMed PMID: 20079666; PubMed Central PMCID: PMC2858628.
89. Esch KJ, Juelsgaard R, Martinez PA, Jones DE, Petersen CA. Programmed death 1-mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. J Immunol. 2013;191(11):5542-50. doi: 10.4049/jimmunol.1301810. PubMed PMID: 24154626; PubMed Central PMCID: PMCPMC3896087.
90. Gautam S, Kumar R, Singh N, Singh AK, Rai M, Sacks D, et al. CD8 T cell exhaustion in human visceral leishmaniasis. J Infect Dis. 2014;209(2):290-9. doi: 10.1093/infdis/jit401. PubMed PMID: 23922369; PubMed Central PMCID: PMC3873784.

91. Joshi T, Rodriguez S, Perovic V, Cockburn IA, Stager S. B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against Leishmania donovani infections. PLoS Pathog. 2009;5(5):e1000431. doi: 10.1371/journal.ppat.1000431. PubMed PMID: 19436710; PubMed Central PMCID: PMC2674929.
92. Legat A, Speiser DE, Pircher H, Zehn D, Fuertes Marraco SA. Inhibitory Receptor Expression Depends More Dominantly on Differentiation and Activation than "Exhaustion" of Human CD8 T Cells. Front Immunol. 2013;4:455. doi: 10.3389/fimmu.2013.00455. PubMed PMID: 24391639; PubMed Central PMCID: PMC3867683.
93. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol. 2007;5(11):873-82. doi: nrmicro1748 [pii] 10.1038/nrmicro1748. PubMed PMID: 17938629.
94. Lehmann J, Enssle KH, Lehmann I, Emmendorfer A, Lohmann-Matthes ML. The capacity to produce IFN-gamma rather than the presence of interleukin-4 determines the resistance and the degree of susceptibility to Leishmania donovani infection in mice. J Interferon Cytokine Res. 2000;20(1):63-77. doi: 10.1089/107999000312748. PubMed PMID: 10670653.
95. Murray HW. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. J Infect Dis. 1997;175(6):1477-9. PubMed PMID: 9180189.
96. Mackay CR. Chemokine receptors and T cell chemotaxis. J Exp Med. 1996;184(3):799-802. PubMed PMID: 9064339; PubMed Central PMCID: PMC2192795.
97. Smelt SC, Engwerda CR, McCrossen M, Kaye PM. Destruction of follicular dendritic cells during chronic visceral leishmaniasis. J Immunol. 1997;158(8):3813-21. PubMed PMID: 9103448.
98. Nylén S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol. 2007;28(9):378-84. doi: S1471-4906(07)00183-4 [pii] 10.1016/j.it.2007.07.004. PubMed PMID: 17689290.
99. Gautam S, Kumar R, Maurya R, Nylén S, Ansari N, Rai M, et al. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. 2011.
100. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, et al. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. 2002.
101. Maurya R, Kumar R, Prajapati VK, Manandhar KD, Sacks D, Sundar S, et al. Human visceral leishmaniasis is not associated with expansion or accumulation of Foxp3+ CD4 cells in blood or spleen. Parasite Immunol. 2010;32(7):479-83. doi: 10.1111/j.1365-3024.2010.01219.x. PubMed PMID: 20591118; PubMed Central PMCID: PMCPMC2898731.
102. Rai AK, Thakur CP, Singh A, Seth T, Srivastava SK, Singh P, et al. Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis.

PLoS One. 2012;7(2):e31551. doi: 10.1371/journal.pone.0031551. PubMed PMID: 22347492; PubMed Central PMCID: PMC3275558.
103. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485-517. doi: 10.1146/annurev.immunol.021908.132710. PubMed PMID: 19132915.
104. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity. 2008;28(4):454-67. doi: 10.1016/j.immuni.2008.03.004. PubMed PMID: 18400188; PubMed Central PMCID: PMC3424508.
105. Pitta MG, Romano A, Cabantous S, Henri S, Hammad A, Kouriba B, et al. IL-17 and IL-22 are associated with protection against human kala azar caused by Leishmania donovani. J Clin Invest. 2009;119(8):2379-87. doi: 10.1172/JCI38813. PubMed PMID: 19620772; PubMed Central PMCID: PMC2719936.
106. Oghumu S, Lezama-Dávila CM, Isaac-Márquez AP, Satoskar AR. Role of chemokines in regulation of immunity against leishmaniasis. Exp Parasitol. 2010;126(3):389-96. doi: 10.1016/j.exppara.2010.02.010. PubMed PMID: 20206625.
107. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol. 2002;283(1):R7-28. doi: 10.1152/ajpregu.00738.2001. PubMed PMID: 12069927.
108. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. Annu Rev Immunol. 2004;22(1):891-928. doi: 10.1146/annurev.immunol.22.012703.104543. PubMed PMID: 15032599.
109. Townson DH, Liptak AR. Chemokines in the corpus luteum: implications of leukocyte chemotaxis. Reprod Biol Endocrinol. 2003;1:94. doi: 10.1186/1477-7827-1-94. PubMed PMID: 14613530; PubMed Central PMCID: PMC293429.
110. Bonecchi R, Galliera E, Borroni EM. Chemokines and chemokine receptors: an overview. Frontiers in …. 2008.
111. Proudfoot AE. Chemokine receptors: multifaceted therapeutic targets. Nat Rev Immunol. 2002;2(2):106-15. doi: 10.1038/nri722. PubMed PMID: 11910892.
112. Teixeira MJ, Teixeira CR, Andrade BB, Barral-Netto M, Barral A. Chemokines in host-parasite interactions in leishmaniasis. Trends Parasitol. 2006;22(1):32-40. doi: 10.1016/j.pt.2005.11.010. PubMed PMID: 16310413.
113. Campanelli AP, Brodskyn CI, Boaventura V, Silva C, Roselino AM, Costa J, et al. Chemokines and chemokine receptors coordinate the inflammatory immune response in human cutaneous leishmaniasis. Hum Immunol. 2010;71(12):1220-7. doi: 10.1016/j.humimm.2010.09.002. PubMed PMID: 20854864.
114. Katzman SD, Fowell DJ. Pathogen-imposed skewing of mouse chemokine and cytokine expression at the infected tissue site. J Clin Invest. 2008;118(2):801-11. doi: 10.1172/JCI33174. PubMed PMID: 18188454; PubMed Central PMCID: PMC2176190.
115. Cesta MF. Normal structure, function, and histology of the spleen. Toxicol Pathol. 2006;34(5):455-65. doi: 10.1080/01926230600867743. PubMed PMID: 17067939.
116. Mebius RE, Kraal G. Structure and function of the spleen. Nat Rev Immunol. 2005;5(8):606-16. doi: 10.1038/nri1669. PubMed PMID: 16056254.
117. Nieto A, Dominguez-Bernal G, Orden JA, De La Fuente R, Madrid-Elena N, Carrion J. Mechanisms of resistance and susceptibility to experimental visceral leishmaniosis: BALB/c mouse versus Syrian hamster model. Vet Res. 2011;42:39. doi:

10.1186/1297-9716-42-39. PubMed PMID: 21345200; PubMed Central PMCID: PMC3052183.
118. Kemp K, Kemp M, Kharazmi A, Ismail A, Kurtzhals JL, Hviid L, et al. Leishmania▪specific T cells expressing interferon▪gamma (IFN▪γ) and IL▪10 upon activation are expanded in individuals cured of visceral leishmaniasis. Clinical & Experimental Immunology. 1999. doi: 10.1046/j.1365-2249.1999.00918.x.
119. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014;32:659-702. doi: 10.1146/annurev-immunol-032713-120145. PubMed PMID: 24655300.
120. Osorio EY, Bruno LT, Alda MdC, Omar AS, Audrie AM, Peter CM. Growth Factor and Th2 Cytokine Signaling Pathways Converge at STAT6 to Promote Arginase Expression in Progressive Experimental Visceral Leishmaniasis. PLoS Pathogens. 2014;10(6). doi: 10.1371/journal.ppat.1004165.
121. Ansari NA, Kumar R, Gautam S, Nylén S, Singh OP, Sundar S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. J Immunol. 2011;186(7):3977-85. doi: 10.4049/jimmunol.1003588. PubMed PMID: 21357266; PubMed Central PMCID: PMCPMC3076633.
122. Skov CB, Twohy DW. Cellular immunity to Leishmania donovani. I. The effect of T cell depletion on resistance to L. donovani in mice. J Immunol. 1974;113(6):2004-11. PubMed PMID: 4214869.
123. Rezai HR, Farrell J, Soulsby EL. Immunological responses of L. donovani infection in mice and significance of T cell in resistance to experimental leishmaniasis. Clinical and experimental immunology. 1980;40(3):508-14.
124. Murray HW, Rubin BY, Rothermel CD. Killing of intracellular Leishmania donovani by lymphokine-stimulated human mononuclear phagocytes. Evidence that interferon-gamma is the activating lymphokine. Journal of Clinical Investigation. 1983;72(4). doi: 10.1172/JCI111107.
125. Nickol AD, Bonventre PF. Immunosuppression associated with visceral leishmaniasis of hamsters. Parasite Immunol. 1985;7(4):439-49. PubMed PMID: 4034238.
126. Carvalho EM, Bacellar OA, Reed S, Barral A, Rocha H. Visceral leishmaniasis: a disease associated with inability of lymphocytes to activate macrophages to kill leishmania. Braz J Med Biol Res. 1988;21(1):85-92. PubMed PMID: 3179584.
127. Hailu A, van der Poll T, Berhe N, Kager PA. Elevated plasma levels of interferon (IFN)-gamma, IFN-gamma inducing cytokines, and IFN-gamma inducible CXC chemokines in visceral leishmaniasis. Am J Trop Med Hyg. 2004;71(5):561-7. PubMed PMID: 15569785.
128. Kumar R, Singh N, Gautam S, Singh OP, Gidwani K, Rai M, et al. Leishmania specific CD4 T cells release IFNgamma that limits parasite replication in patients with visceral leishmaniasis. PLoS Negl Trop Dis. 2014;8(10):e3198. doi: 10.1371/journal.pntd.0003198. PubMed PMID: 25275531; PubMed Central PMCID: PMC4183461.
129. Kumari S, Jamal F, Shivam P, Thakur A, Kumar M, Bimal S, et al. Leishmania donovani skews the CD56(+) Natural Killer T cell response during human visceral leishmaniasis. Cytokine. 2015;73(1):53-60. doi: 10.1016/j.cyto.2015.01.011. PubMed PMID: 25697139.

130. Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL, Reed SG. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. J Immunol. 1994;152(12):5949-56. PubMed PMID: 8207220.
131. Zwingenberger K, Harms G, Pedrosa C, Omena S, Sandkamp B, Neifer S. Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon-gamma production. Clin Immunol Immunopathol. 1990;57(2):242-9. PubMed PMID: 2145107.
132. Costa AS, Costa GC, de Aquino DM, de Mendonça VR, Barral A, Barral-Netto M, et al. Cytokines and visceral leishmaniasis: a comparison of plasma cytokine profiles between the clinical forms of visceral leishmaniasis. Mem Inst Oswaldo Cruz. 2012;107(6):735-9. PubMed PMID: 22990961.
133. Miralles GD, Stoeckle MY, McDermott DF, Finkelman FD, Murray HW. Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. Infection and Immunity. 1994;62(3).
134. Melby PC, Yang Y-Z, Cheng J, Zhao W. Regional differences in the cellular immune response to experimental cutaneous or visceral infection with Leishmania donovani. Infection and immunity. 1998;66(1).
135. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP. IL-10 mediates susceptibility to Leishmania donovani infection. Eur J Immunol. 2001;31(10):2848-56. doi: 10.1002/1521-4141(2001010)31:10<2848::AID-IMMU2848>3.0.CO;2-T. PubMed PMID: 11592059.
136. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol. 2003;3(3):253-7. doi: 10.1038/nri1032. PubMed PMID: 12658273.
137. Vouldoukis I, Becherel PA, Riveros-Moreno V, Arock M, da Silva O, Debre P, et al. Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation. Eur J Immunol. 1997;27(4):860-5. doi: 10.1002/eji.1830270409. PubMed PMID: 9130636.
138. Wu J, Cunha FQ, Liew FY, Weiser WY. IL-10 inhibits the synthesis of migration inhibitory factor and migration inhibitory factor-mediated macrophage activation. J Immunol. 1993;151(8):4325-32. PubMed PMID: 7691945.
139. Anderson CF, Oukka M, Kuchroo VJ, Sacks D. CD4+ CD25− Foxp3− Th1 cells are the source of IL-10–mediated immune suppression in chronic cutaneous leishmaniasis. CD4+ CD25− Foxp3− Th1 cells are the source of IL-10–mediated immune suppression in chronic cutaneous leishmaniasis. 2007.
140. Murray HW, Hariprashad J, Aguero B, Arakawa T, Yeganegi H. Antimicrobial response of a T cell-deficient host to cytokine therapy: effect of interferon-gamma in experimental visceral leishmaniasis in nude mice. J Infect Dis. 1995;171(5):1309-16. PubMed PMID: 7751708.
141. Broere F, Apasov S, Sitkovsky M, Eden W. T cell subsets and T cell-mediated immunity. T cell subsets and T cell-mediated immunity. 2011. doi: 10.1007/978-3-0346-0136-8_2.
142. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature. 1993;362(6422):758-61. doi: 10.1038/362758a0. PubMed PMID: 8469287.

143. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med. 1995;182(2):459-65. PubMed PMID: 7543139; PubMed Central PMCID: PMC2192127.
144. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol. 2005;25(21):9543-53. doi: 10.1128/MCB.25.21.9543-9553.2005. PubMed PMID: 16227604; PubMed Central PMCID: PMC1265804.
145. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. Immunology. 2010;129(4):474-81. doi: 10.1111/j.1365-2567.2010.03255.x. PubMed PMID: 20201977; PubMed Central PMCID: PMC2842494.
146. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3(5):541-7. PubMed PMID: 7584144.
147. Boggiatto PM, Ramer-Tait AE, Metz K, Kramer EE, Gibson-Corley K, Mullin K, et al. Immunologic indicators of clinical progression during canine Leishmania infantum infection. Clin Vaccine Immunol. 2010;17(2):267-73. doi: 10.1128/CVI.00456-09. PubMed PMID: 20032217; PubMed Central PMCID: PMC2815526.
148. Murphy ML, Engwerda CR, Gorak PMA, Kaye PM. B7-2 blockade enhances T cell responses to Leishmania donovani. B7-2 blockade enhances T cell responses to Leishmania donovani. 1997.
149. Ohmori Y, Hamilton TA. Requirement for STAT1 in LPS-induced gene expression in macrophages. J Leukoc Biol. 2001;69(4):598-604. PubMed PMID: 11310846.
150. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM, Jr. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. Gene. 2005;353(1):98-106. doi: 10.1016/j.gene.2005.04.004. PubMed PMID: 15922518.
151. Pauleau AL, Rutschman R, Lang R, Pernis A, Watowich SS, Murray PJ. Enhancer-mediated control of macrophage-specific arginase I expression. J Immunol. 2004;172(12):7565-73. PubMed PMID: 15187136.
152. Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. 2005.
153. Mosser DM, Edelson PJ. The third component of complement (C3) is responsible for the intracellular survival of Leishmania major. Nature. 1987;327(6120):329-31. doi: 10.1038/327329b0. PubMed PMID: 3035377.
154. Pavanelli WR, Gutierrez FR, Silva JJ, Costa IC, Menezes MC, Oliveira FJ, et al. The effects of nitric oxide on the immune response during giardiasis. Braz J Infect Dis. 2010;14(6):606-12. PubMed PMID: 21340302.
155. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005;5(8):641-54. doi: 10.1038/nri1668. PubMed PMID: 16056256.
156. Iniesta V, Gomez-Nieto LC, Corraliza I. The inhibition of arginase by N(omega)-hydroxy-l-arginine controls the growth of Leishmania inside macrophages. J Exp Med.

2001;193(6):777-84. PubMed PMID: 11257143; PubMed Central PMCID: PMC2193414.
157. Perez LE, Chandrasekar B, Saldarriaga OA, Zhao W, Arteaga LT, Travi BL, et al. Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen. J Immunol. 2006;176(9):5519-28. PubMed PMID: 16622021.
158. Roy G, Dumas C, Sereno D, Wu Y, Singh AK, Tremblay MJ, et al. Episomal and stable expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol Biochem Parasitol. 2000;110(2):195-206. PubMed PMID: 11071276.
159. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol. 2013;229(2):176-85. doi: 10.1002/path.4133. PubMed PMID: 23096265.
160. Green SJ, Meltzer MS, Hibbs JB, Jr., Nacy CA. Activated macrophages destroy intracellular Leishmania major amastigotes by an L-arginine-dependent killing mechanism. J Immunol. 1990;144(1):278-83. PubMed PMID: 2104889.
161. Ray M, Gam AA, Boykins RA, Kenney RT. Inhibition of interferon-gamma signaling by Leishmania donovani. J Infect Dis. 2000;181(3):1121-8. doi: 10.1086/315330. PubMed PMID: 10720539.
162. Melby PC, Valencia-Pacheco G, Andrade-Narvaez F. Induction of macrophage killing of Leishmania donovani by human CD4+ T cell clones. Arch Med Res. 1996;27(4):473-9. PubMed PMID: 8987180.
163. Gidwani K, Jones S, Kumar R, Boelaert M, Sundar S. Interferon-gamma release assay (modified QuantiFERON) as a potential marker of infection for Leishmania donovani, a proof of concept study. PLoS Negl Trop Dis. 2011;5(4):e1042. doi: 10.1371/journal.pntd.0001042. PubMed PMID: 21526219; PubMed Central PMCID: PMC3079582.
164. Forget G, Gregory DJ, Olivier M. Proteasome-mediated degradation of STAT1alpha following infection of macrophages with Leishmania donovani. J Biol Chem. 2005;280(34):30542-9. doi: 10.1074/jbc.M414126200. PubMed PMID: 15983048.
165. Contreras I, Gomez MA, Nguyen O, Shio MT, McMaster RW, Olivier M. Leishmania-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63. PLoS Pathog. 2010;6(10):e1001148. doi: 10.1371/journal.ppat.1001148. PubMed PMID: 20976196; PubMed Central PMCID: PMC2954837.
166. Soong L, Chang C-H, Sun J, Longley JBJ, Ruddle NH, Flavell RA, et al. Role of CD4+ T cells in pathogenesis associated with Leishmania amazonensis infection. Journal of immunology (Baltimore, Md : 1950). 1997;158(11).
167. Chakkalath HR, Theodos CM, Markowitz JS, Grusby MJ, Glimcher LH, Titus RG. Class II major histocompatibility complex-deficient mice initially control an infection with Leishmania major but succumb to the disease. J Infect Dis. 1995;171(5):1302-8. PubMed PMID: 7751707.
168. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. The many roles of chemokines and chemokine receptors in inflammation. 2006.

169. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett. 2004;574(1-3):37-41. doi: 10.1016/j.febslet.2004.07.083. PubMed PMID: 15358536.
170. Lasso P, Mateus J, Pavia P, Rosas F, Roa N, Thomas MC, et al. Inhibitory Receptor Expression on CD8+ T Cells Is Linked to Functional Responses against Trypanosoma cruzi Antigens in Chronic Chagasic Patients. J Immunol. 2015;195(8):3748-58. doi: 10.4049/jimmunol.1500459. PubMed PMID: 26385520.
171. Cotterell SE, Engwerda CR, Kaye PM. Leishmania donovani infection initiates T cell-independent chemokine responses, which are subsequently amplified in a T cell-dependent manner. Eur J Immunol. 1999;29(1):203-14. doi: 10.1002/(SICI)1521-4141(199901)29:01<203::AID-IMMU203>3.0.CO;2-B. PubMed PMID: 9933102.
172. Locksley RM, Heinzel FP, Holaday BJ, Mutha SS, Reiner SL, Sadick MD. Induction of Th1 and Th2 CD4+ subsets during murine Leishmania major infection. Research in immunology. 1991;142(1):28-32.
173. Asrat H, Tom van der P, Nega B, Piet AK. Elevated plasma levels of interferon (IFN)-gamma, IFN-gamma inducing cytokines, and IFN-gamma inducible CXC chemokines in visceral leishmaniasis. The American journal of tropical medicine and hygiene. 2004;71(5):561-7.
174. Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, et al. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. J Exp Med. 2007;204(2):273-83. doi: 10.1084/jem.20062175. PubMed PMID: 17283209; PubMed Central PMCID: PMC2118735.
175. Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, Elliott T, et al. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. J Exp Med. 1998;187(9):1383-93. PubMed PMID: 9565631; PubMed Central PMCID: PMC2212278.
176. McCoy KD, Le Gros G. The role of CTLA-4 in the regulation of T cell immune responses. Immunol Cell Biol. 1999;77(1):1-10. doi: 10.1046/j.1440-1711.1999.00795.x. PubMed PMID: 10101680.
177. Marr AK, MacIsaac JL, Jiang R, Airo AM, Kobor MS, McMaster WR. Leishmania donovani infection causes distinct epigenetic DNA methylation changes in host macrophages. PLoS pathogens. 2014;10(10). doi: 10.1371/journal.ppat.1004419.
178. Ghalib HW, Whittle JA, Kubin M, Hashim FA, el-Hassan AM, Grabstein KH, et al. IL-12 enhances Th1-type responses in human Leishmania donovani infections. J Immunol. 1995;154(9):4623-9. PubMed PMID: 7722314.
179. Cho HY, Choi EK, Lee SW, Jung KO, Seo SK, Choi IW, et al. Programmed death-1 receptor negatively regulates LPS-mediated IL-12 production and differentiation of murine macrophage RAW264.7 cells. Immunol Lett. 2009;127(1):39-47. doi: 10.1016/j.imlet.2009.08.011. PubMed PMID: 19723542.
180. Ma CJ, Ni L, Zhang Y, Zhang CL, Wu XY, Atia AN, et al. PD-1 negatively regulates interleukin-12 expression by limiting STAT-1 phosphorylation in monocytes/macrophages during chronic hepatitis C virus infection. Immunology.

2011;132(3):421-31. doi: 10.1111/j.1365-2567.2010.03382.x. PubMed PMID: 21091911; PubMed Central PMCID: PMC3044908.
181. Lei GS, Zhang C, Lee CH. Myeloid-derived suppressor cells impair alveolar macrophages through PD-1 receptor ligation during Pneumocystis pneumonia. Infect Immun. 2015;83(2):572-82. doi: 10.1128/IAI.02686-14. PubMed PMID: 25404033; PubMed Central PMCID: PMC4294265.