ABSTRACT

Title of Document: THE DEVELOPMENT AND CHARACTERIZATION OF TRANSGENIC *LEISHMANIA MAJOR* EXPRESSING MURINE CD40L.

Ann Elizabeth Field, Ph.D., 2007

Directed By: David Mosser, Professor,
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Leishmanization is the inoculation of live *Leishmania* into the host to vaccinate against subsequent infections. This approach has been largely discontinued due to safety concerns. We have previously shown that combining CD40L with *Leishmania* antigen preferentially induces a type 1 immune response and provides some protection to vaccinated mice. In the present study, we developed transgenic *L. major* which express and secrete the extracellular portion of CD40L (*L. major* CD40LE). We hypothesized that these organisms would be less virulent but more immunogenic than wild-type organisms, and therefore be more effective at leishmanization. Transgenic parasites expressing CD40L mRNA and protein were developed. These parasites had similar growth characteristics to wild-type organisms. Susceptible BALB/c mice infected with these parasites developed significantly smaller lesions containing fewer parasites than animals infected with wild-type organisms. Infection of C57BL/6 CD40L$^{-/-}$ mice with transgenic *L. major* resulted in significantly smaller lesions than infection with wild-type *L. major*, indicating *in vivo* biologi-

cal activity of the transgenic protein. Infection of resistant C57BL/6 mice with low doses of transgenic parasites induced a significant amount of protection against subsequent high dose infection with wild-type organisms. These results demonstrate that transgenic organisms expressing CD40L are less virulent than wild-type organisms while retaining full immunogenicity. The implications of this study are that parasites expressing immune-modulatory molecules may be improved alternatives to traditional leishmanization.

THE DEVELOPMENT AND CHARACTERIZATION OF TRANSGENIC *LEISHMANIA MAJOR* EXPRESSING MURINE CD40L.

by

Ann Elizabeth Field

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Professor David Mosser, Chair
Professor Catherine Carr, Dean's Representative
Professor Daniel Stein
Assistant Professor Kenneth Frauwirth
Associate Professor Louisa Wu

Dedication

This dissertation is dedicated to my family, especially to my late grandfather. He got me started in science by getting me a microscope. Then, when I called to say I'd joined the Mosser lab and would be working on Leishmania, his first question was, "Is that a trypanosome?"

Acknowledgements

First, I must thank my advisor, David Mosser, for his support and guidance during these last few years. He was always able to help me find a new direction when I felt like I was hitting a wall. He was great at knowing what I needed to hear to get motivated and back in the lab. I learned a lot from him, not just about science, but about how to run a good lab. I'd also like to thank the other members of my dissertation committee, Ken Frauwirth, Dan Stein, Louisa Wu, and Catherine Carr. Thanks for all your time, suggestions, and help to focus and further my project.

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List of Abbreviations

antigen presenting cell	APC
B-cell receptor	BCR
bone marrow derived dendritic cells	BMDC
bone marrow derived macrophages	BMMϕ
B cell stimulating factor-1 (now IL-4)	BSF-1
CD4+ T helper cell type 1	Th1
CD4+ T helper cell type 2	Th2
CD40 Ligand	CD40L
CD40 Ligand Extracellular Domain	CD40LE
caspase activating recruitment domain	CARD
chinese hamster ovary cells	CHO
complement receptor	CR
cytoxic T lymphocyte	CTL
dendritic cell	DC
deoxynucleotide triphosphates	dNTPs
deoxyribonucleic acid	DNA
diffuse cutaneous leishmaniasis	DCL
diversity region	D
double negative	DN
dulbecco's modified eagle's medium	DMEM
endoplasmic reticulum	ER
enzyme-linked immunosorbent assay	ELISA
Fcγ receptor	FcγR
fetal bovine serum	FBS
fluorescein isothiocyanate	FITC
gene splicing by overlap extension	SOE
granulocyte monocyte colony stimulating factor	GM-CSF
Hanks Balanced Salt Solution	HBSS
heat inactivated FBS	HI-FBS
high efficiency particulate air (filter)	HEPA
horseradish peroxidase	HRP
hypoxanthine phosphoribosyltransferase	HPRT
immunoglobulin	Ig
inducible NO synthase	iNOS
interleukin	IL
interferon-γ	IFNγ
janus kinase domain	Jak
joining region	J
knockout	KO

kilodalton	kDa
L929 cell conditioned medium	LCCM
localized cutaneous leishmaniasis	LCL
lipophosphoglycan	LPG
lipopolysaccharide	LPS
lymph node	LN
major histocompatibility complex	MHC
membrane attack complex	MAC
mucocutaneous leishmaniasis	MCL
muramyl dipeptide	MDP
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
natural killer cell	NK
naturally occurring Treg	nTreg
nitric oxide	NO
nuclear factor kappa B	NF-κB
nucleotide binding site and leucine rich repeat	NBS-LRR
pathogen-associated molecular patterns	PAMP
pattern recognition receptor	PRR
phosphate buffered saline	PBS
polymerase chain reaction	PCR
Leishmania vector pIR1SAT	pSAT
pyrin domain	PYD
Reactive oxygen intermediate	ROI
regulatory T cell	Treg
ribonucleic acid	RNA
ribosomal RNA subunit	SSU
soluble *Leishmania* antigen	SLA
streptothricin acetyl transferase	SAT
T-cell receptor	TCR
toll-like receptor	TLR
transforming growth factor	TGF
trinitrobenzene sulfonic acid induced-colitis	TNBS-colitis
tumor necrosis factor	TNF
tumor necrosis factor receptor	TNF-R
TNF receptor associated family	TRAF
visceral leishmaniasis	VL

CHAPTER 1: INTRODUCTION

Innate Immunity

Overview

The immune system is divided into two components, innate and adaptive immunity. Innate immunity is the body's first line of defense against invasion by pathogens. It was evolutionally developed prior to adaptive immunity, and virtually every multi-cellular organism has some form of innate immunity [1–5]. Innate immunity utilizes a variety of mechanisms to protect the host. First, contact with possible pathogens is minimized through barriers such as skin and mucous membranes. Mucus traps potential pathogens and cilia expel mucus from the body, preventing attachment of potential pathogens. Body fluids contain anti-microbial proteins including defensins and complement that help to destroy microbes [1].

The innate immune system also contains cells that patrol the body for infection, including macrophages and dendritic cells. These cells can ingest material from their surroundings which initiates several different pathways. They can directly destroy microbes, or present antigen to alert the adaptive immune system. First, however, these cells must be able to distinguish self from non-self. The innate im-

mune system uses pattern recognition receptors (PRRs) that identify certain highly conserved structures found on numerous pathogens. These highly conserved structures are referred to as pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharide (LPS), peptidoglycan, CpG DNA, double stranded RNA, and many others. These PAMPs are not produced by the host, so their presence signifies an infection. Often, these microbial products are essential for pathogen survival, which means loss or mutation of these structures is unlikely. Thus, recognition of essential structures reduces the chance of the pathogen simply changing the structure to evade innate immunity [4]. There are several types of PRRs, including Toll-like receptors (TLRs), Nods/NALPs, mannose receptors, and scavenger receptors [5]. These receptors have different functions. Mannose receptors and scavenger receptors improve phagocytosis, allowing microbes to be taken up and destroyed. Toll-like receptors and Nods/NALPs, however, provide signaling to alert the cell that the material being ingested is foreign.

Toll-like receptors are membrane associated receptors present on phagocytes and other cells that lead to activation of the innate immune system. Toll was first discovered as a gene having a role in dorsal-ventral polarity in *Drosophila melanogaster* by Nusslein-Volhard in 1985 [6]. Its role in immunity was later discovered by Lemaitre in 1996 [7]. Lemaitre showed that a mutation in Toll led to an inability to fight off fungal infection. A mutation in imd was shown to be responsible for immunity to bacterial infection [7]. Eleven different TLRs have been identified to date in humans [8]. These are defined by a common TIR domain in the

cytoplasmic region [9]. TLRs are capable of recognizing a wide variety of pathogen patterns. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS), while TLR9 recognizes bacterial DNA containing unmethylated CpG motifs, and TLR5 recognizes bacterial flagellin [10]. Other TLRs recognize peptidoglycan (TLR2), fungal cell wall (TLR6), dsRNA (TLR3), and ssRNA (TLR7) [10–12]. In addition, TLRs can combine to increase specificity. TLR2 can dimerize with TLR6 or TLR1 to increase specificity and induce signaling [10]. Ligation of toll-like receptors leads to signaling through adaptor molecules that induces inflammatory cytokines, interferon inducible genes, and maturation of dendritic cells [10, 13]. Different TLRs use different combinations of adaptor molecules. For example, TLR4 uses both MyD88 dependent signaling and a MyD88 independent mechanism to induce inflammatory cytokines, while TLR2 only uses MyD88 dependent signaling to induce these cytokines [13]. The different pathways induced through different TLRs illustrate how different TLRs could induce specific responses to a pathogen.

Nods/NALPs belong to a family of intracellular proteins containing neucleotide binding domains and leucine rich repeats (NBS-LRR)[14]. These molecules are differentiated by N-terminal effector motifs. Nods have caspase-activating and recruitment domains (CARD), while NALPs have an N-terminal pyrin domain (PYD) [14]. Nod1 has been shown to bind procaspase-9 and RICK, and induce apoptosis and NF-κB [15]. It has been established that Nod1 recognizes gram-negative bacterial peptidoglycan components and is important in the innate response to these organisms [16, 17]. Nod 2 has been shown to recognize muramyl dipeptide (MDP),

the minimal active peptidoglycan motif present in both gram-positive and gram-negative bacteria [18].

In addition to these receptors, others recognize self-generated molecules that enhance phagocytosis, like complement receptors. Complement proteins deposit on pathogen surfaces, allowing for recognition by complement receptors and improved phagocytosis or direct pathogen lysis. When the cells of the innate immune system encounter a pathogen, they do their best to destroy it through nitric oxide (NO), reactive oxygen (ROI), acidic environments, and proteases. If innate immunity fails to eliminate the intruder, the innate immune cells fulfill one critical function: to alert the adaptive immune reponse [1].

Macrophages

Macrophages play an important role in innate immunity and provide antigen interaction and co-stimulation to adaptive immune cells. These cells are efficient at phagocytosis, and their primary function is to destroy ingested material in their highly acidic phagolysosomes. Upon phagocytosis, macrophages also produce a variety of other microbicidal products including nitric oxide, superoxide, and hydrogen peroxide [19]. They are present in the periphery and in virtually all endothelial surfaces, and thus have access to entering pathogens. In addition to their direct destruction of pathogens, they serve another important function. Macrophages are a part of an elite group of cells known as the professional antigen presenting cells (APCs), which are known for their ability to present antigen to CD4+ T cells

through their expression of the major histocompatibility complex (MHC) class II. Dendritic cells and B cells make up the other members of the professional APCs. These cells serve the critical function of activating adaptive immunity, thus linking innate and adaptive immunity.

To efficiently activate T cells, macrophages must express MHC class II with antigen and a second signal or "danger" signal to alert T cells that the antigen is from a foreign body (Figure 1)[19]. Examples of danger signals (also referred to as co-stimulation) include CD40, CD80, and CD86. Resting macrophages express few MHC class II molecules and co-stimulatory molecules. Macrophages must undergo an activation process due to their encounter with a pathogen to express the danger signal that will induce T cell activation. In addition to these cell surface markers, macrophage activation can lead to cytokine release that assists in T cell activation or activation of neighboring macrophages [19].

Macrophage activation is a complex process with multiple stimuli resulting in different activation patterns and subsequent influence over the character of the adaptive immune response. To date three major classifications of macrophage activation have been identified that that can be reliably reproduced *in vitro*. The first type of macrophage activation to be discovered is now known as classical activation. This is characterized by activation through IFN-γ and TNF-α or an inducer of TNF-α such as signaling through Toll-like receptors. This stimulation induces NF-κB translocation, and secretion of IL-12 and TNF-α, and many other cytokines as well as upregulation of co-stimulatory molecules CD40, CD86, and CD80. Classically acti-

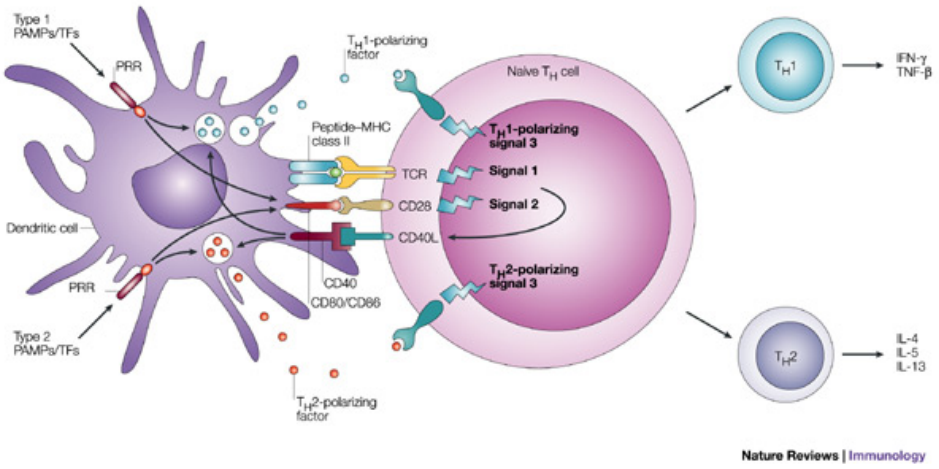

Figure 1: Activation of adaptive immunity through antigen presentation.

Adapted from Kapsenberg [20]. Macrophages and DCs identify pathogens through PRRs. Recognition of PAMPs leads to improved antigen presentation to T cells and up-regulation of co-stimulatory molecules like CD28. Upon interacting with a naïve T cell specific for the pathogen antigen, APCs initiate T cell activation through co-stimulation and secreted factors that instruct the T cell on the type of response to make. T cells then differentiate into effector cells of the Th1 or Th2 variety.

vated macrophages also produce nitric oxide and reactive oxygen species [21]. The second type of activation was termed "alternative" activation, and is accomplished through exposure of macrophages to the Th2 cytokines IL-4 or IL-13. These cells can be characterized by high arginase expression, expression of FIZZ-1 and YM-1 [22], and the expression of many components of the extracellular matrix [21, 23]. Alternatively activated macrophages do not produce NO, unlike their classically activated counterparts [23]. Thus they are impaired in their ability to kill pathogens. Although they do express some MHC class II, they have been shown to be poor antigen presenting cells unable to induce high levels of T cell proliferation [23]. It is thought that these cells play a role in dampening of the acute inflammatory response and assisting with wound-healing [21].

The third type of macrophage activation is termed "type II" activation [24]. In this type of activation, TLR signals are coupled with FcγR ligation. Type II activation results in down-regulation of IL-12, and up-regulation of IL-10 [25]. These macrophages have been shown to induce a Th2, or humoral adaptive immune response [26]. When type II macrophages were used as antigen presenting cells, T cells were stimulated to secrete IL-4, a hallmark cytokine of the humoral response. In contrast, when classically activated macrophages were used as APCs, T cells expressed IFN-γ [26]. Immunization of mice with type II macrophages led to a humoral response characterized by high levels of IgG1, indicating that these macrophges are sufficient to activate humoral immunity and induce class switching [24]. These macrophages play an important role in amplifying the humoral

response *in vivo*, and have been shown in some cases, for example in leishmaniasis, to exacerbate disease by skewing towards an inappropriate response [27].

Dendritic Cells

Dendritic cells (DCs) are the most potent of all the professional APCs, and are particularly adept at priming naïve T cells [2, 3]. They are derived from precursors in the bone marrow, and migrate to the periphery where they take up antigen. When they are activated by a pathogen they return to lymph nodes where they prime T cells to respond to the infection.

DCs are derived from a number of different bone marrow precursors. They leave the bone marrow and travel the blood and lymphatics as immature dendritic cells. These immature DCs migrate to tissue, where they are efficient phagocytes, sampling the surrounding area for infection. They mature when activated, up-regulating co-stimulatory molecules (CD40, CD80, CD86) and stably expressing MHC class II complexes on the surface. They lose their phagocytic capabilities, but become professional antigen presenting cells [2, 28]. They also alter their chemokine receptor expression profile, down-regulating receptors that would keep them in the periphery and up-regulating CCR7 to initiate migration to the lymph nodes [29].

All DCs follow this general maturation progression, however all DCs are not identical. Different DC subsets exist, and currently there is much debate as to the importance of these different subsets. Defining the subsets and their function is a complex study. The cells are relatively rare *in vivo*, which makes them difficult to

isolate and study. *Ex vivo* studies are complicated by the tendency of DCs to spontaneously mature during handling, drastically altering their phenotype [30]. Several categories of DCs have been identified to date. These include pre-DCs (a subset of which are plasmacytoid DCs), conventional DCs (further split into migratory and lymphoid-resident), and inflammatory DCs (i.e. Tip DCs that can occur during disease states) [31]. Plasmacytoid DCs are known for their ability to produce type 1 interferons and induce antiviral immunity. Conventional migratory DCs are those that patrol the periphery for foreign antigen and mature to report to the lymph nodes. Lymphoid-tissue-resident DCs sample lymphoid organs for foreign material and can be further subdivided into many subsets, including splenic and thymic DCs. Inflammatory DCs are not normally present in the steady state and are induced following antigen encounter. For example, Tip DCs are inflammatory DCs designated by their ability to produce TNF-α and iNOS in response to infection [31]. It is still unclear whether the different subsets are actually different populations or represent different maturation states of the same population [32].

There is also debate over whether the different subsets respond to specific kinds of pathogens, or if DCs of different subsets are flexible enough to respond to many different pathogens. Originally, it was thought that different subsets were preprogrammed to respond to either Th1 or Th2 stimuli [20]. This was based on the different TLR profiles of human DC subsets. Initial evidence indicated that CD8α+ DCs primarily induce a Th1 response, while CD8α- DCs primarily induce a Th2 response [30]. However, it seems that DCs should be able to discriminate among

various pathogens to be most effective. Recent literature seems to suggest that DCs are flexible, and may respond to a variety of pathogens, regardless of subset [20, 30, 33, 34]. For example, de Jong *et al*. found that immature DCs receiving antigen from *Shistosoma mansoni* matured as a DC2 phenotype, capable of inducing a stable Th2 response from T cells. Immature DCs receiving antigen from the intracellular bacterium *Bordetella pertussis* matured in a DC1 phenotype, generating a Th1 response from T cells [33]. In addition, previous work in our lab has shown that the stimulation of immature bone-marrow derived DCs (BMDCs) with the TLR ligands LPS and CpG DNA resulted in a Th1 response from T cells with the production of IFN-γ from these cells [35]. When immature BMDCs were stimulated with immune complexes in addition to LPS and CpG DNA, T cell responses were skewed towards a Th2 response, with IL-4 detected instead of IFN-γ. Immunization of mice to ovalbumin through transfer of BMDCs activated with immune complexes induced high IgG1 antibody titers indicative of an *in vivo* Th2 response. When TLR ligands were used without immune complexes as stimulation for cell transfer, little antibody response was detected [35]. These data would support the hypothesis that DCs are able to respond to the maturation stimuli received to induce an appropriate adaptive immune response. The literature surrounding this topic is complex and the underlying theories are still being challenged. Much work still needs to be done to determine the role of the different DC subsets in regulating and activating the immune system, however, the literature is starting to indicate that the stimulus a DC receives is the most important determinant of fate and thus can

usually override the innate preference of a DC subset for supporting a Th1 or a Th2 response.

Adaptive Immunity

Overview

When the innate immune system is unable to rid the host of an invading organism, adaptive immunity is activated. Adaptive immunity can be differentiated from innate immunity based upon the cells involved, the receptors used, and the specificity of the response. As previously mentioned, innate immune cells recognize patterns or classes of molecules. These receptors are encoded in the germline, and all cells that express a receptor have the same specificity [1, 4, 5]. The cells of the adaptive immune system are far more specific in their response. The adaptive response is composed of two major cell types: B and T cells. Each naïve cell has a unique receptor generated through random gene segment recombination at the loci encoding the BCR or TCR to allow for exquisite specificity in pathogen antigen recognition. This allows for incredible diversity in a large repertoire of cells with the potential to respond to virtually all pathogens. The receptors on B cells recognize particulate antigen, and these cells are responsible for producing antibody. T cells recognize antigen in the context of MHC expressed on the surface of an APC or other cell. There are two major classes of T cell, CD4+ T cells and CD8+ T

cells. CD4+ T cells recognize antigen in the context of MHC class II present on APCs. CD8+ T cells, or cytotoxic T cells (CTLs) recognize antigen in MHC class I molecules present on all cells in the body.

There are three major subsets of mature B and T cells: naïve, effector, and memory cells. Naïve cells are mature cells that have yet to encounter antigen. Effector cells are cells that have recently encountered antigen and have been induced to proliferate and act upon other cells to eradicate the pathogen. The differentiation from naïve to effector cell is the primary immune response. During this reponse, memory cells are generated that can provide protection against subsequent challenge with the same pathogen.

Adaptive immunity systematically and specifically rids the body of pathogens. However, adaptive immunity must initially be activated by innate immunity through antigen presentation and co-stimulation typically provided by macrophages and DCs. Naïve B and T cells that receive antigen and co-stimulation are signaled to undergo clonal expansion. This is a process that allows generation of an expanded population of cells with specificity for the pathogen antigen. During clonal expansion, cells develop into effector cells or memory cells. Once activated, the adaptive immune system develops one of two general responses: a cell-mediated (Th1) or humoral (Th2) response . Th1 responses are generally mounted in response to intracellular pathogens, while Th2 responses are generated to extracellular pathogens. The most important component of adaptive immunity is memory. This function is unique to adaptive immunity. The mechanism of memory cell generation is poorly

understood, but these cells provide a rapid, ready response upon re-infection with a pathogen, and can prevent illness. This is why once an adaptive immune response has been generated to a pathogen, an individual is usually immune to a second exposure [19].

T cells

T cells originate in the bone marrow, and very early in development lymphoid progenitors travel to the thymus, where they complete development into T cells. Early in development, cells are "double negative," (DN) meaning that they do not yet express CD4 or CD8. T cells that undergo successful rearrangement of the TCRβ chain become "double positive" cells, characterized by their expression of both CD4 and CD8 and enter the late phase of T cell development. Double positive cells must undergo rigorous selection before maturing into single positive naïve cells that are permitted to leave the thymus. Double positive cells continue to rearrange their TCR-α chain until a functional receptor is formed that recognizes self-MHC expressed by thymic epithelial cells. 90% of cells are unable to produce a functional TCR capable of recognizing self-MHC and die by neglect. Approximately 5% of cells will generate a TCR that reacts strongly to self-MHC. These cells are deleted through apoptosis in a process called negative selection [36]. The mechanism of negative selection is not fully understood, but it has been proposed that the strength of signal through the TCR determines the fate of the cell, whether it is survival or death. Cells with low affinity for self-MHC are permitted to mature

into single positive cells (positive selection), while those with high affinity for self-MHC are deleted [37]. The successfully selected single positive cells are permitted to leave the thymus and circulate to the periphery and secondary lymphoid organs where they may encounter antigen.

CD8+ cells are also referred to as cytoxic T lymphocytes, or CTLs. They recognize antigen presented in the context of MHC class I molecules present on all cells in the body. CTL activation is especially important in the case of viral infection, where virus proteins can be expressed in the context of MHC class I on different cell types. CTLs must receive two signals for activation, antigen stimulation and co-stimulation, for example IL-2 [38]. Once a CTL becomes activated, it targets cells expressing MHC class I containing specific antigen and directs killing of target cells through the release of perforin from preformed cytolytic granules or Fas-Fas ligand interactions. These cells also produce IFN-γ that can promote macrophage activation and Th1 responses, but these levels are typically lower than those of CD4+ Th1 cells [39].

CD4+ T helper cells were originally thought to be a single population of cells. However, many subsets of CD4+ cells with different functions have been identified to date. Figure 2 illustrates several major subsets with cytokines demonstrated to be relevant in their development and the signature cytokine they produce. Tada *et al.* in 1978 first provided evidence that two distinct subsets of T helper cells existed, separable by passage through a nylon wool column [40]. They termed those cells found to be nonadherent to nylon "Th1", and those cells found to be adher-

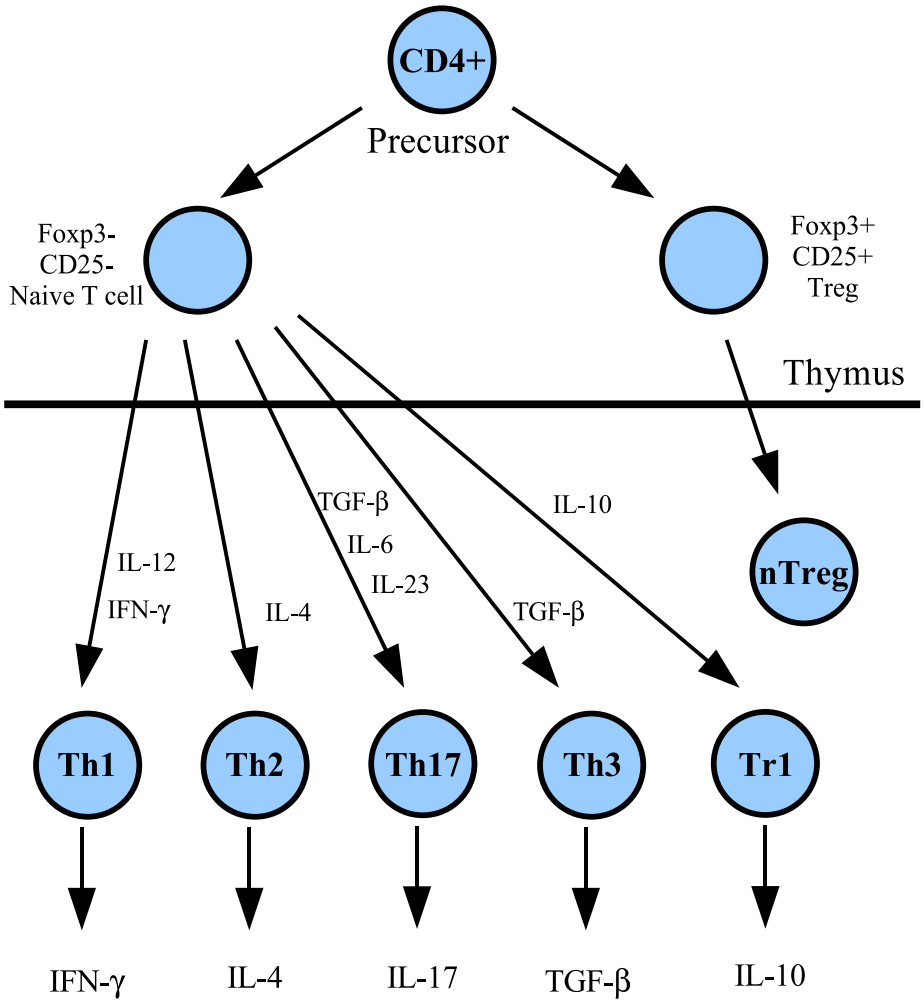

Figure 2: Overview of CD4+ T cell subsets.

Naïve CD4+ T cells differientate into many types of effector cells upon encountering antigen. Th1 and Th2 cells drive cell-mediated and humoral immunity respectively. IL-12 and IFNγ have been shown to give rise to Th1 cells, and IL-4 can induce differentiation of Th2 cells. Th1 cells primarily produce IFN-γ, which helps to activate macrophages. Th2 cells produce IL-4, among other cytokines that drive antibody production and class switching. Th17 cells have been shown to be involved in autoimmunity and some cancers, and arise from TGF-β and IL-6 signals. IL-23 can act on activated or memory Th17 cells. Th17 cells produce IL-17, which can act on macrophages to induce Il-1β and TNF-α. Th3 cells arise in the presence of TGF-β and produce TGF-β. Tr1 cells arise in the presence of high IL-10, and produce high levels of IL-10. nTregs are generated during negative selection in the thymus.

ent to nylon "Th2". Mosmann *et al.* in 1986 followed Tada's nomenclature and further defined these two subsets by differential expression of lymphokine proteins [41]. They found that Th1 cells expressed IL-2, IFN-γ, and IL-3, while Th2 cells were found to produce B cell stimulating factor 1 (BSF-1, later known as IL-4) and enhanced IgG1 and IgE production. Th1 cells were shown to be responsible for delayed type hypersensitivity (DTH) reactions, while Th2 cells were unable to induce DTH responses [42]. Further characterization of these two subsets showed that Th1 cells produce IFN-γ, IL-2, and lymphotoxin, while Th2 cells produce IL-4 and IL-5 [41]. Th1 cells are important in intracellular infections, such as leishmaniasis [43], while Th2 cells play a role in extracellular infections. Exactly how naïve T cells are instructed to become Th1 or Th2 cells is still not fully understood, but several cytokines and methods of stimulation have been shown to preferentially induce one response over the other. It has been shown that IFN-γ [44] or IL-12 [45, 46] can induce Th1 cell development, while IL-4 can promote Th2 cell development. In addition, the stimulus received by antigen presenting cells can play a role in naïve T cell instruction. LPS induces IL-12 from macrophages, promoting Th1 cells, while coupling LPS with FcγR stimulation causes macrophages to produce IL-10 and promote Th2 cell development [25].

In addition to Th1 and Th2 cells, CD4+ suppressor T cells or regulatory T cells (Treg) have been described. Treg cells have been shown to be composed of many subsets. Three of the populations receiving the most attention are "natural" Tregs, Tr1 cells, and Th3 cells. Tregs were first described in a series of elegant experi-

ments that demonstrated the ability of a population of CD4+CD25+ cells to control autoimmunity [47]. When CD25+ cells were depleted, mice developed severe autoimmune disease. Co-transfer of CD25+ cells was able to prevent autoimmunity, demonstrating that this population was responsible for maintaining self-tolerance.

Naturally occurring Tregs (nTreg) are defined by their expression of CD25 and Foxp3. Fontenot *et al.* demonstrated that Foxp3 expression was specific to CD4+CD25+ T cells [48]. Mice deficient in Foxp3 developed lethal autoimmune disease that could be treated early on with CD25+ cell transfer and prevent lethality. Ectopic expression of Foxp3 was also shown to confer suppressor function onto CD4+CD25- cells, indicating the important role of this transcription factor in development of a suppressor population. Natural Tregs develop in the thymus during negative selection, and act in a cell-contact dependent, cytokine independent manner [49, 50].

Tr1 cells are induced from naïve CD4+ cells by high IL-10 levels [51]. These cells require stimulation through the TCR in addition to IL-10, and then are able to induce bystander suppression through IL-10 and TGF-β secretion [52]. The primary role of these cells is thought to be in gut tolerance. IL-10 knockout mice have been shown to be prone to inflammatory bowel disease that is prevented through adoptive transfer of IL-10 competent CD45RBlow cells [53].

Th3 cells were first discovered in studies of oral tolerance [54]. These cells require TCR stimulation and TGF-β for development [55]. They secrete TGF-β, IL-4, and IL-10, and promote IgA production. They are generated in gut associated

lymphoid tissue in the presence of high levels of TGF-β primarily after ingestion of foreign antigen. They also express CTLA-4 on their surface, and stimulation through CTLA-4 results in TGF-β secretion. Upon stimulation with TGF-β, Th3 cells upregulate CD25 and Foxp3, however these cells differ from natural Tregs through the TGF-β mechanism of suppression rather than the natural Treg cell-contact dependent method [56].

Recently, a new subset of CD4+ T cells was discovered that are characterized by their production of IL-17 [57]. Thus, these cells have been termed Th17 cells. The story of this subset begins with the discovery of IL-23, a cytokine that shares the p40 subunit with IL-12, but instead of the p35 subunit, has a p19 subunit [58]. This cytokine was found to be expressed by activated macrophages and DCs and to act upon T cells. The common p40 subunit with IL-12 threw into question the use of p40 knockout systems to study the role of IL-12 in disease. Indeed, study of experimental autoimmune encephalomyelitis (EAE) revealed that IL-23, not IL-12 (as previously thought) was responsible for disease progression [59]. IL-23 was shown to drive an IL-17 producing T cell population that was demonstrated to have encephalogenic properties when adoptively transferred [57]. In addition, mice deficient in the p19 subunit of IL-23 were found to lack the population of T cells expressing IL-17 [60].

Th17 cells has recently been shown to be generated through TGF-β and IL-6 stimulation of naïve T cells, and it has been established that the effects of IL-23 are primarily on memory or activated T cells [61]. Some Tregs are known to be

generated through exposure to TGF-β, and it was demonstrated that the addition of IL-6 was sufficient to exclude Treg development and drive Th17 developent. IL-17 has been shown to induce IL-1β and TNF-α from human macrophages among other cytokines [62], and these cells have been implicated in a number of autoimmune disorders some of which include multiple sclerosis [63], lupus [64], and encephalomyelitis [59] through elevation of IL-17 levels [63, 64]. IL-23 has also been demonstrated to be elevated in tumors [59] and to provide limited resistance to toxoplasmosis in the absence of IL-12 [65].

Humoral Immunity

Many pathogens that cause infections replicate in the extracellular spaces of the body. These extracellular spaces are protected by humoral immunity. In this form of adaptive immunity, naïve mature B cells are activated to produce antibody. These antibodies contribute to the destruction of invading pathogens in a variety of ways (Figure 3). Antibodies can neutralize pathogens by binding molecules required for invasion of tissues and toxin action. They also target pathogens for uptake by phagocytic cells either directly by Fc receptors, or indirectly by activating the classical pathway of complement (Figure 3). Complement activation opsonizes microbes for phagocytosis and can also induce direct killing through membrane attack complex (MAC) pore formation [19].

Antibodies from different subtypes have different effector functions in humoral immunity [19]. IgM exists as both a membrane bound form and a secreted form.

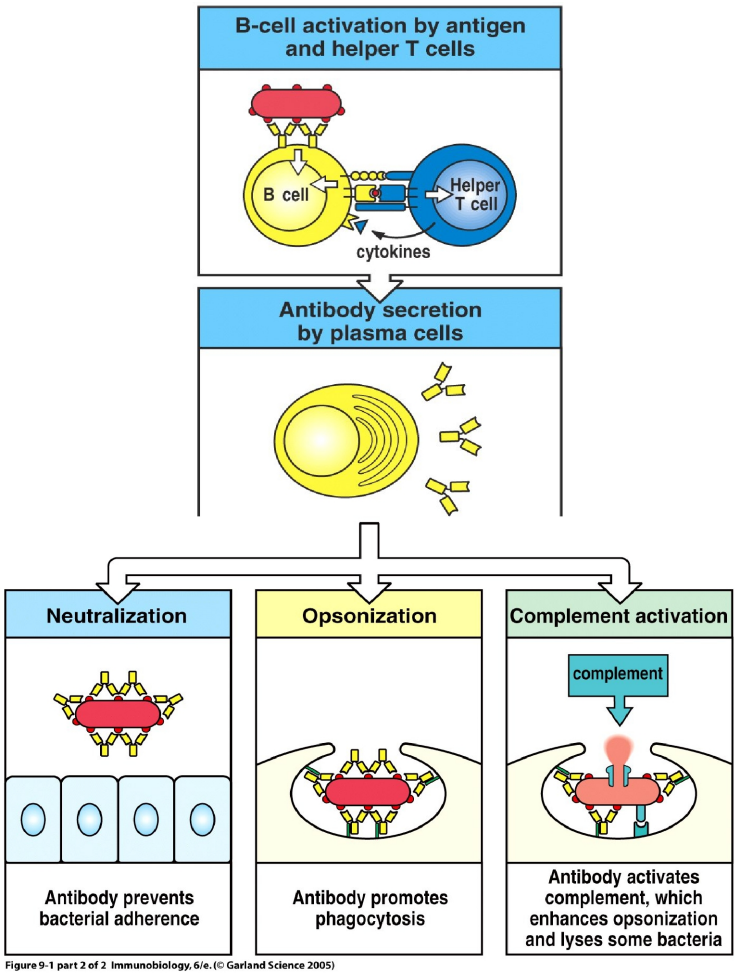

Figure 3: B cell effector functions.

Adapted from Janeway [19]. When activated, some B cells differentiate in to effector B cells, or plasma cells. Plasma cells secrete antibody that mediate three major mechanisms of pathogen eradication: neutralization, opsonization, and complement activation. Neutralization prevents pathogens from invading, opsonization targets pathogens for efficient phagocytosis, and complement activation leads to direct pathogen killing and improved phagocytosis.

The secreted form of IgM forms pentamers and is primarily involved in complement activation. IgA molecules form monomers or dimers, and are found at mucosal surfaces where their major function is to neutralize pathogens and prevent pathogen attachment to mucosal surfaces. IgG antibody is usually of higher affinity than IgM antibodies and has many functions. IgG can neutralize toxins, viruses, and bacteria, opsonize them for phagocytosis and activate the complement system. In mice, IgG1 antibodies are associated with type 2 responses, while IgG2a antibodies are associated with type 1 responses. IgE is primarily associated with mast cells, and is involved in atopic reactions and mast cell signaling. Thus, the antibodies produced during the humoral response use a wide variety of methods to eradicate invading organisms.

Cell-Mediated Immunity

Intracellular pathogens like *Leishmania* hide inside host cells, and evade humoral immunity. They can only be eradicated through macrophage activation, or direct cytotoxic effects provided by cell-mediated immunity. There are two major T cell effectors involved in cell-mediated immunity, CD4+ Th1 cells, and CD8+ CTLs. Th1 cells secrete IFN-γ, which activates the microbicidal properties of macrophages. CTLs induce direct killing of infected cells through interactions with MHC class I and also provide a source of IFN-γ.

Naïve CD4+ T cells must encounter antigen in the context of an MHC class II molecule only present on dendritic cells, macrophages, or B cells. When a T cell

receives antigen plus co-stimulation, it is activated to become an effector T cell. Exactly how T cells are instructed is not fully understood, but the cytokines found in the microenvironment have been shown to play a major role in this process. IL-12 is a cytokine that can be produced by macrophages or dendritic cells in response to certain PRR, like TLRs. IL-12 can help to instruct T cells to differentiate into Th1 cells, and thus is one of the driving cytokines of the Th1 response [45]. Th1 cells produce IFN-γ, which is important for macrophage activation. As mentioned previously, macrophages require two signals to become activated in the classical manner. One signal is IFN-γ, and the other can be provided in a variety of ways and sensitizes the macrophage to respond to IFN-γ. Th1 effector cells can provide both signals. IFN-γ is the primary cytokine produced by these cells, and CD40L interacts with CD40 on the macrophage to deliver the second signal. Macrophage activation transforms these cells into potent antimicrobial effector cells, inducing NO, IL-12, TNF-α, MHC class II expression, CD40, and B7 expression. They become better antigen presenters, and amplify the immune response through cytokine production and antigen presentation [19].

Th1 cells are the coordinators of the cell-mediated immune response. In addition to inducing macrophage activation, they also recruit phagocytic cells to the site of infection through the secretion of chemokines, such as CCL2. Th1 cells help to activate the other effectors of the cell-mediated response, CD8+ T cells. These cytotoxic cells recognize antigen in the context of MHC class I expressed on every cell in the body. Their activation is tightly regulated, and they require more co-

stimulation than CD4+ T cells to become activated. Naïve CD8+ cells can receive co-stimulation either through high levels of B7 expression on APCs like dendritic cells, or through indirect T cell help primarily through the induction of high levels of co-stimulatory molecules on the APC mediated by CD40-CD40L interactions [66–68]. Once activated, CTLs target cells displaying the pathogen antigen in their MHC class I on the cell surface. CTLs then release lytic granules containing perforin and granzymes, inducing apoptosis in the target cell. This response is especially important in the case of an infection, such as a virus, within the cytoplasm of non-antigen presenting cells that do not bear MHC class II, and thus do not respond to CD4+ T cell activation [19].

CD40-CD40L

The murine CD40L cDNA predicts a polypeptide of 260 amino acids (aa), with a 22aa cytoplasmic domain, a 24aa transmembrane domain, and a 214aa extracellular domain [69]. It is a type II membrane protein that presents an extracellular carboxy terminus. The human gene is organized into 5 exons [70]. The first exon encodes the intracellular, transmembrane, and a small portion of the extracellular domain, and the remaining exons encode for the rest of the extracellular domain. The murine gene also contains 5 exons [69].

CD40L is expressed primarily on activated T cells, but mRNA has been detected in CD8+ and γ:δ T cells, as well as NK cells. Initial studies suggested a molecular mass of 39kDa, but full length CD40L has been shown to be 32-33 kDa. The amino

acid backbone predicts a protein of 29kDa, which suggests post-translational modifications. An N-linked glycosylation site has been identified in human, murine, and cattle CD40L. Human and mouse CD40L share 75% identity over their extracellular domains. CD40L can be expressed as a soluble molecule in addition to the cell surface form [71, 72]. Two shorter versions of 31 and 18kDa have been identified. These shorter soluble forms retain their ability to form trimers, bind CD40, and induce biological signals. This indicates that CD40L may also act in part as a cytokine [69].

CD40L interacts with cell-surface CD40 expressed on macrophages, DCs, and B cells, as well as other cells. Cross-linking of CD40 with CD40L leads to many signaling events. CD40 itself has no kinase domain, but several second messenger systems are activated through CD40. There is evidence for recruitment of a variety of TRAFs (TRAF-2, 3, 5, and 6), and Jak3 has also been shown to associate with CD40 [73–76]. This signaling leads to a number of downstream events, including NF-κB, NF-AT and AP1 activation [69, 74].

CD40 and CD40L belong to the tumor necrosis factor receptor family (TNF-R) and the TNF family respectively. CD40 was first described on B cells, and CD40L is primarily expressed on activated CD4+ T cells. The interaction between CD40-CD40L was first described in humoral immunity. This was shown by the finding that patients suffering from hyper-IgM syndrome were characterized by mutations in their CD40L gene [69, 77, 78]. These patients displayed impaired class-switching and a susceptibility to bacterial infections. The role of

CD40L in humoral immunity was examined further in knockout mice deficient in CD40L [79, 80]. These mice showed impaired class-switching to T cell dependent antigens, similar to the effect of CD40L deficiency in humans. In addition, germinal center formation was impaired in these mice. Treatment with an anti-CD40L antibody also prevented germinal center formation and demonstrated a lack of B cell memory from adoptive transfer [81]. Studies in CD40 deficient mice obtained similar results [82], indicating that CD40-CD40L interactions are crucial for T cell dependent antibody responses and B cell memory.

Many studies have also shown the importance of CD40-CD40L in cell-mediated immunity [83–90]. CD40L is a potent inducer of IL-12 from macrophages and DCs, indicating that CD40-CD40L interactions may be important in priming cell-mediated immunity [83, 90, 91]. Incubation of T cell depleted splenocytes with a Th1 clone resulted in IL-12p40 mRNA and protein that was abrogated with an anti-CD40L antibody [83]. CD40L expressed on CHO cells was also shown to induce IL-12p40 from DCs and macrophages [91]. The role of CD40L in cell-mediated immunity is supported by the finding that hyper-IgM patients have been shown to be more susceptible to opportunistic infections requiring a cell-mediated response [92]. HIGM patients were impaired in their ability to respond to the intracellular pathogen *Toxoplasma gondii* through lack of specific T cell proliferation, IL-12 or IFN-γ [88]. CD40L was shown to induce IL-12 from macrophages and DCs in response to *T. gondii* in healthy humans, leading to IFN-γ production and T cell proliferation. Addition of soluble CD40L trimer was able to reverse the impairment

in IFN-γ production from an HIGM individual and was able to reconstitute IL-12 induction in PBMCs from an infected patient. CD40L has also been shown to be important in Th1-mediated autoimmune disease [87]. Blocking CD40L-CD40 interactions through anti-CD40L antibody can prevent Th1-mediated TNBS-induced colitis when administered during the induction phase of colitis. Administration of anti-CD40L antibody prevented IL-12 induction, Th1 T cell priming, and subsequent IFN-γ production responsible for inflammation associated with disease. Treatment with anti-CD40L antibody following disease establishment was ineffective, demonstrating that CD40L may play an important role in the initiation of T cell priming. A correlation between CD40-CD40L expression and disease state in leprosy has also been observed [89]. CD40 and CD40L were readily detected in lesions of patients with tuberculoid leprosy, but not in those with lepromatous disease. Tuberculoid leprosy is characterized by a Th1 response, while lepromatous leprosy is characterized by a Th2 response. These data provide evidence for a significant role of CD40-CD40L interaction in cell-mediated immunity.

CD40 ligation can induce differential outcomes, depending on the cell type expressing CD40. B cells are stimulated to proliferate, differentiate, and produce Ig. As mentioned, CD40L stimulation is critical for germinal center formation and class switching in response to T cell dependent antigens [79, 80]. CD40L stimulation of B cells also leads to release of IL-6, IL-10, TNF-α, and expression of adhesion molecules and co-stimulatory markers. In macrophages and DCs, CD40 activation results in enhanced survival, secretion of cytokines including IL-1, IL-6,

IL-8, IL-10, IL-12, TNF-α, and NO synthesis [69, 93, 94]. CD40 ligation also induces maturation of macropahges and dendritic cells, up-regulating expression of ICAM-1, LFA-3, CD80, and CD86. Disruption of CD40-CD40L interactions leads to reduced T cell proliferation and cytokine release [69, 93].

Leishmania

Overview and Life Cycle

Leishmania spp. were discovered by William Leishman and James Donovan working independently in 1901-1903 [95]. *Leishmania* belong to the order *Kinetoplastida* and the family *Trypanosomatidae*. *Leishmania* are single celled protozoa, and are the etiological agents of the disease leishmaniasis. Leishmaniasis is endemic in nearly 100 countries worldwide, with almost all cases occurring in tropical and sub-tropical areas. Humans are infected by 21 of the approximately 30 species of *Leishmania* that infect mammals. *Leishmania* species are divided into Old World and New World species by geographic location of endemic species [95]. The parasite is carried by the female phlebotamine sandfly of the genus *Phlebotamus* in the Old World or *Lutzomyia* in the New World.

Leishmania spp. have a two stage life cycle, consisting of the elongated, flagellated promastigote, and the oval, non-motile amastigote (Figure 4). The promastigote is found in the mid-gut of the sandfly vector, where it matures into the

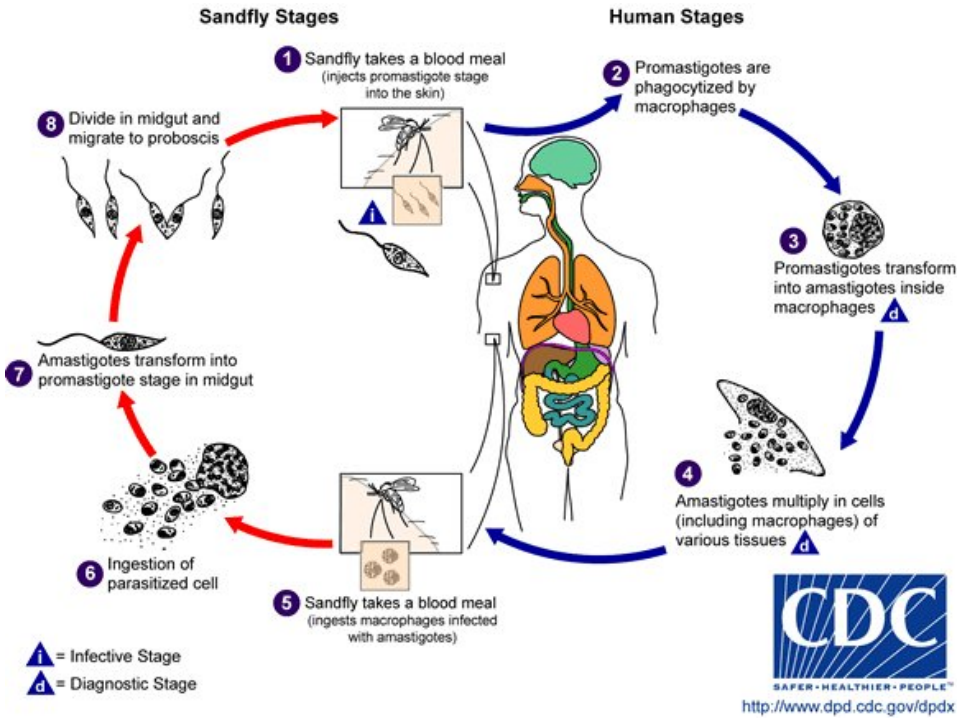

Figure 4: *Leishmania* Life Cycle.

Adapted from CDC (http://www.dpd.cdc.gov/dpdx). *Leishmania* are intracellular parasites with 2 life stages, the promastigote, which resides in the gut of the sandfly, and the amastigote, an obligate intracellular usually found in host tissue macrophages. **1)** A sandfly takes a blood meal injecting promastigotes. **2-4)** Promastigotes are taken up by host tissue macrophages, where they transform into amastigotes, replicate, and rupture the cell to infect others. **5-6)** The sandfly takes a blood meal from an infected individual, ingesting macrophages containing amastigotes. **7-8)** Amastigotes transform back into promastigotes in the midgut of the sandfly and replicate.

infective metacyclic form. When the sandfly takes a blood meal, promastigotes are injected into the skin of the host. Promastigotes are rapidly taken up by host tissue macrophages where they transform into amastigotes within the phagolysosome. Amastigotes replicate within phagolysosomes and eventually rupture host cells, allowing them to infect neighboring cells. The cycle is completed when a sandfly takes a blood meal from an infected individual, ingesting macrophages containing amastigotes. These amastigotes transform back into promastigotes in the gut of the sandfly, and prepare to infect another host [95, 96].

Disease Manifestations

Leishmania spp. can cause three major clinical disease manifestations: cutaneous, mucocutaneous, and visceral leishmaniasis. The type of disease contracted depends primarily upon the species of *Leishmania* involved. Cutaneous leishmaniasis generally presents as a localized skin lesion (LCL) around the area of the bite that self-heals over the course of weeks to months. Occasionally, diffuse cutaneous leishmaniasis (DCL) can develop, where multiple non-healing skin lesions appear as parasites disseminate from the original site of infection. *Leishmania major*, *Leishmania tropica*, and *Leishmania amazonensis* are some species that primarily cause the cutaneous form of the disease [96]. Mucocutaneous leishmaniasis is caused by dissemination of parasites to the mucous membranes, resulting in large lesions in the nose, mouth, and throat, and cause severe disfigurement and scarring. Mucocutaneous leishmaniasis can be caused by *L. braziliensis*. The most

perilous form of the disease is visceral leishmaniasis, with parasite dissemination to the visceral organs. Symptoms of visceral leishmaniasis include splenomegaly, hepatomegaly, fever, diarrhea, and weight loss, and this disease is often fatal if not treated. The *Kala-azar*, or black fever, refers to visceral leishmaniasis [95]. Species known to cause viscerial leishmaniasis include *L. chagasi*, *L. donovani*, and *L. infantum* [96].

Diagnosis and Treatment

Infection with *Leishmania* spp. is diagnosed most accurately by biopsy of the lesion and visual detection of the parasite by microscopic observation or culture of parasites from the lesion. Other methods of diagnosis include PCR, ELISA, serology, and DTH responses. These methods are improving, but visual detection of the parasite is still the best way to diagnose this disease [95, 96].

Treatments are costly and difficult to administer. The most common treatments are intravenous injections of pentavalent antimonials like sodium stibogluconate [95]. Treatment periods typically last three to four weeks, and side effects are common. Other treatments include amphotericin B. A new oral drug, Miltefosine has shown promise in treating some forms of the disease [97, 98].

No safe, effective vaccines exist for this disease. The only way to vaccinate against leishmaniasis is to inoculate live organisms into the host. Historically, it was shown that once an individual had contracted leishmaniasis, they were resistant to re-infection. Children were exposed to the bite of a sandfly or were inoculated with

material from a lesion in the buttocks to induce immunity and prevent disfiguring lesions on the face. Later, inoculation of adults with live, virulent organisms was used to induce protective immunity. This process is called leishmanization. In the 1980s a large-scale vaccination program was instituted in approximately 1.3 million soldiers and civilians during the Iran-Iraq war [99]. Leishmanization led to lesions approximately 5-10mm in diameter that self-healed in 4-6 months. However, 2-3% of vaccinated individuals developed large, non-healing lesions that required treatment. Vaccination with live parasites induces powerful immunity, as shown by a recent study of volunteers in Iran [100]. Twenty-three human volunteers were inoculated with $5x10^5$ live *L. major*. 19 volunteers were "takes," developing measurable lesions that healed (two required treatment) within 6-13 months. Following lesion resolution, 11 participants volunteered to be challenged with *L. major* to test their immunity level. In participants who were "takes," 100% protection from reinfection was observed. Conversely, the administration of killed parasites with BCG as an adjuvant had no effect on the incidence of natural infection [101]. However, the incidence of lesions requiring treatment and the potential for secondary infection and other side effects from leishmanization has been deemed unacceptable in most of the world. Thus, traditional leishmanization has been largely discontinued due to safety concerns [99]. Vaccine development is a priority to reduce infection levels and improve quality of life for at-risk individuals in endemic countries.

Leishmania Genetics

Leishmania spp. are eukaryotes with unusual genetic characteristics. The genome contains approximately 35.5Mb of DNA, and this is organized into 34-36 chromosomes. The absolute number of chromosomes can change with species [102]. *Leishmania* chromosomes are generally diploid, but these parasites have a tendency to become aneuploid or haploid for some chromosomes. The diploid state of the parasite would seem to suggest a form of sexual reproduction, however, the parasite replicates by asexual binary fission [102]. *Leishmania* spp. can also maintain small linear or circular chromosomes, called mini-chromosomes. It is thought that these arise from gene amplification under stress conditions, and may be stably maintained or unstable, depending on the stress encountered. In addition to their nuclear chromosomal DNA, *Leishmania* contain a kinetoplast, which is a mitochondrial-like structure with many circular pieces of DNA [103]. The circular DNA is divided into the maxi-circles and the mini-circles. Maxi-circles are analogous to the mitochondrial DNA of higher eukaryotes. Mini-circles are thought to serve a role in RNA editing as guide RNAs for addition or deletion of uracils.

Gene arrangement in *Leishmania* also has some differences from higher eukaryotes. Introns and cis-splicing, two characteristics of higher eukaryotes have not been demonstrated in *Leishmania* [102]. The ribosomal RNA genes are organized similar to those of higher eukaryotes, with multiple head to tail repeats with coding regions separated by non-transcribed spacers. Non ribosomal genes are found as either single copy, paired loci, or tandem repeats. Highly expressed proteins

are present in multiple copies in the genome, and are usually organized as tandem repeats.

Trypanosomatids have clear homologs of the three major RNA polymerases. Polymerase I promoters have been identified in rRNA loci and upstream of surface proteins in *T. brucei*. Few RNA polymerase II promoters exist in these organisms as of yet, although RNA polymerase II transcription has been demonstrated in multiple leishmanial genes by susceptibility to α-amanitin [102]. It is not understood how transcription of genes by RNA polymerase II is initiated in these organisms. Small RNA genes and tRNA genes are transcribed by RNA polymerase III, as determined by polymerase inhibitors α-amanitin and tagetitoxin [104, 105].

Leishmania also have an unusual way of processing mRNA. In higher eukaryotes, genes are transcribed individually, then processed to remove introns and add the 5' cap and poly-A tail. *Leishmania* generate polycistronic RNA, similar to prokaryotes. However, they have a unique method of processing RNA post-transcription. In eukaryotes, a 5' cap is added during transcription. An RNA triphophatase clips the phosphate off the end of the growing RNA, leaving a diphosphate. A guanylyl transferase attaches GMP from GTP to the 5' end. Finally, two methyl transferases methylate the capping guanosine and the penultimate nucleotide [106]. *Leishmania* have a trans-splicing event following transcription that attaches a 39bp spliced-leader sequence (also called the mini-exon) to the 5' intergenic region at a splice acceptor site. Trans-splicing is followed by polyadenylation at the 3' end. These two events are spatially and temporally coupled, with polyadenylation occurring a fixed

distance upstream of the mini-exon splice site [107, 108]. The trans-splicing reaction of the downstream gene precedes the polyadenylation of the upstream gene.

Higher eukaryotes typically regulate gene expression through transcriptional regulation. In *Leishmania*, however, most gene regulation occurs post-transcriptionally, either through RNA stability, or protein stability. It is proposed that similar to other organisms, regions of the 3'UTR may target mRNAs for deadenylation and subsequent decapping and degradation. Several studies have identified regions of the 3'UTR that are responsible for mRNA stability, but the exact machinery and mechanism of this regulation is as yet unknown [109].

Immune evasion

For a pathogen to be successful, it must evolve ways to evade destruction by the immune system. As mentioned, innate immunity is the first line of defense against microbes, and by definition a pathogen must be able to circumvent innate immunity. *Leishmania* have evolved several mechasisms of immune evasion. Immediately upon being introduced into the host, *Leishmania* must battle complement present in the blood. This organism has developed a mechanism that not only prevents complement lysis, but subverts this host defense mechanism to improve targeting to the host cell of choice, the macrophage. *Leishmania* metacyclic promastigotes are resistant to complement lysis. Two surface molecules, lipophosphoglycan (LPG), and gp63 are thought to be responsible for this resistance [110–112]. Both are efficient binders of the C3 molecule of complement. The LPG on metacyclic promastigotes

is nearly twice as long as that on log phase promastigotes, and prevents insertion of the membrane attack complex (MAC) into the parasite membrane [112]. The metalloprotease gp63 has been shown to transform cells into efficient activators of complement [110]. Proteolytic activity was not required for complement activation, but was required to prevent subsequent complement lysis, indicating that gp63 may play a role in converting active C3b to the inactive iC3b. This has the added effect of targeting these organisms to the CR3 receptor on macrophages, allowing for their efficient phagocytosis [110, 111].

One of the most important adaptations to evade the immune response is the development of an intracellular lifestyle. This allows the parasite to hide from detection within host cells. The parasite lacks the ability to directly invade cells, and relies on receptor mediated phagocytosis to infect macrophages. As mentioned, they are efficiently opsonized by complement, and utilize the complement receptors CR1 and CR3 for phagocytosis. CR3 is the primary complement receptor for parasite uptake, as C3b is rapidly converted to iC3b by gp63 [110, 111]. In addition, parasites can be ingested through mannose-fucose receptors, scavenger receptors, and Fc receptors. Once inside the macrophage, they face a new set of challenges. As described previously, the main function of the macrophage is to ingest and destroy pathogens. *Leishmania* have evolved to subvert this cell and thrive in the highly acidic phagolysosome. It has been shown the promastigote LPG may delay phagolysosome fusion through steric hindrance of membrane fusion, allowing time for the organism to transform into the amastigote stage [111, 113]. This stage is highly

resistant to the acidic environment found in the phagolysosome, and amastigotes have been found to be more metabolically active at lower pH.

Usually, ingestion of an organism by the macrophage leads to activation of the cell through PRRs, destruction of the organism, and presentation of antigen to activate adaptive immunity. *Leishmania* enter macrophages quiescently, without activating NO and ROI [111]. They fail to activate NF-κB, and fail to induce up-regulation of co-stimulatory molecules. Some studies have shown that *Leishmania* are able to modulate MHC class II expression or antigen loading to interfere with antigen presentation. In addition, they actively inhibit IL-12 production, and render infected macrophages refractory to stimulation with IFN-γ [111, 114]. The mechanisms involved in this subversion of events in the macrophage are still unclear, but many scenarios have been proposed. It has been shown that cross-linking of Fc, complement, or scavenger receptors specifically inhibits IL-12 production through calcium influx, and *Leishmania* are known to enter macrophages through these paths [115]. Ligation of CR3 is also advantageous, because it does not activate NO and ROI. It has also been suggested that *Leishmania* inhibit PKC signaling, thus inhibiting ROI [114]. In addition to inhibition of activating cytokines, infection leads to the up-regulation of detrimental anti-inflammatory cytokines, like IL-10. It is well known that *Leishmania* amastigotes are coated in host antibody, and studies have shown that ligation of FcγR leads to a down-regulation of IL-12 and a subsequent up-regulation of IL-10 [25, 27]. Finally, *Leishmania* have developed ways to

inhibit apoptosis signaling pathways, prolonging the life of the host cell and their own survival [116].

In summary, *Leishmania* have adapted many ways of evading innate immunity, from entering host cells to actively inhibiting host cell activation. Knowledge of the mechanisms of immune evasion can suggest targets for drug and vaccine development.

Murine Models of Leishmaniasis

The spectrum of human disease is also largely observed in murine systems, allowing the mouse to be a reasonable *in vivo* model. The availability of various strains of inbred mice and knockout animals has provided extensive characterization of the immune response to *Leishmania* spp. Susceptible and resistant strains have contributed to our knowledge of how this disease progresses and the important immune cells and cytokines involved in disease resolution. Early on, it was noted that many strains of mice were resistant to leishmanial infection, developing small healing lesions. Some strains, however, were observed to be susceptible to disease, eventually succumbing to large necrotic lesions [117]. The differences in disease outcomes were determined to be related to differences in the T cell subsets activated and the cytokines induced during infection [118, 119]. Resistant strains develop a Th1 immune response to leishmanial infection, characterized by IFN-γ production. Conversely, susceptible strains were found to develop a Th2 response to infection, characterized by IL-4 and antibody production [43, 119]. This led to the murine

model of *L. major* being used for many studies of the molecules pertaining to Th1 or Th2 responses.

Studies with resistant mice identified IFN-γ as the dominant cytokine in a healing response. Normally resistant mice deficient in IFN-γ [120] or the IFN-γ receptor [121, 122] were shown to be more susceptible to leishmanial infection and treating with anti-IFN-γ antibody also increased susceptibility to disease [123]. IL-12 has been shown to be a major driving cytokine of the Th1 response, and this has been demonstrated in *Leishmania* infection. Mice on a resistant background deficient in IL-12 [124] or the IL-12Rβ2 [125] were found to be more susceptible to *Leishmania* infection. In addition, treatment with recombinant IL-12 was shown to improve disease outcome in susceptible BALB/c animals [126].

Studies of the susceptible BALB/c mouse showed that these mice developed a Th2 response to *Leishmania* that resulted in the production of high levels of IL-4 [43, 118, 119]. IL-4 was shown to be largely responsible for the detrimental effects of this response, because removing IL-4 increased resistance. IL-4 gene knockout BALB/c mice were more resistant to infection with the MHOM/IL/81/FEBN1 substrain of *L. major* [121]. Somewhat surprisingly, in these mice IFN-γ mRNA in draining lymph nodes was found to be unchanged from that of wild-type animals, as was mRNA expression of several other cytokine genes tested, indicating that in these animals, removal of IL-4 is sufficient to improve disease outcome [121]. Other studies showed that treating mice before or early in infection with anti-IL-4 antibodies improved disease outcome [118, 127]. This effect was shown to be in-

dependent of IFN-γ, because although treatment led to a slight increase in IFN-γ mRNA in draining lymph nodes, addition of an anti-IFN-γ antibody had no effect on anti-IL-4 mediated reduction in disease [127]. The addition of neutralizing anti-IL-4 antibodies was also shown to enhance vaccine efficacy in BALB/c mice [128]. Studies of mRNA levels of IL-4 in draining lymph nodes from resistant and susceptible strains of mice have shown that IL-4 expression correlates with disease severity, with more susceptible strains expressing higher levels of this cytokine [129]. IFN-γ levels were less informative, as the range of mRNA expression was smaller, and had overlap among resistant and susceptible strains. These studies would seem to indicate that in resistant mice, IFN-γ is important for resistance, but in susceptible BALB/c mice, resistance is dependent on interference with, or suppression of, the induction of the initial Th2 response.

It has been shown that IL-4 is not the only cytokine responsible for susceptibility in leishmaniasis. Noben-Trauth reported in 1996 that mice deficient in IL-4 were not more resistant to the LV39 substrain of *Leishmania major* [130]. This work called into question the role of IL-4 in susceptibility to Leishmaniasis. It was later reported that the role of IL-4 in susceptibility differed according to the substrain of *Leishmania major* used for infections [131]. IL-4 deficient mice infected with the LV39 substrain were similarly susceptible when compared to wild-type controls. However, IL-4 deficient mice infected with the IR173 substrain were more resistant than wild-type mice. A role for IL-13 was shown in enhanced resistance to infection seen in IL-4Rα knockouts when compared to IL-4 gene knockouts or wild-

type controls [131]. This study also found a lack of increased IFN-γ production even in the presence of a more resistant phenotype and the lack of Th2 cytokines. They later proposed that the differences in susceptibility seen among the different strains were due to the intrinsic differences in susceptibility to killing by IFN-γ activated macrophages [132]. The IR173 and Friedlin V1 substrains were found to be more susceptible to macrophage killing induced by IFN-γ than the LV39 strain, and Noben-Trauth *et al.* proposed that in this case, resistance is achieved by the absence of deactivating signals without the requirement for IFN-γ upregulation.

IL-10 has also been shown to play an important role in disease progression. Mice deficient in IL-10 were more resistant to leishmanial infection, and treating mice with anti-IL-10R also improved disease outcome [27, 133]. Noben-Trauth *et al*. showed in 2003 that IL-10 might be the additional factor controlling disease susceptibility [132]. They found that when IL-10 was removed in addition to IL-4Rα, BALB/c mice became resistant to the substrain LV39 that they were still susceptible to with IL-4Rα or IL-10 deficiency alone.

The powerful mouse model has also been used to examine potential vaccine candidates. As previously mentioned, there is no safe vaccine for leishmaniais, and with increasing drug resistance there is a focus on development of an improved vaccine candidate. Due to the safety concerns of leishmanization, many vaccination approaches have been examined. Studies with killed parasites, subunit vaccines, and DNA vaccines have had varying degrees of success [134]. Some suggest that parasite antigen persistence is required for continued immunity to *Leishmania* in-

fection, because when sterile cure is achieved mice lose immunity to re-infection [135, 136]. This may account for the lack of long term protection with killed or subunit vaccines and the improved ability of DNA vaccines to provide protection [134]. Thus many labs have devoted efforts to developing attenuated organisms for use as vaccine candidates. Attenuation with γ-radiation led to protection of resistant CBA mice [137]. In addition, attenuation of *L. major* and *L. mexicana* under gentamycin pressure induced significant protection against challenge with wild-type organisms [138]. However, these methods are impractical for the clinic because of the potential for revertants. Genetically defined knockout parasites have also been developed to generate attenuated vaccine candidates, and have less potential for reversion to virulent forms because of irreversible genome changes [134]. *L. major dhfr-ts-* parasites are double knockout auxotrophs that require thymidine for survival in macrophages. These parasites were incapable of causing disease in BALB/c mice, but persisted for up to 2 months [139]. When challenged with wild-type organisms, BALB/c mice that had been vaccinated were significantly protected. In addition, this strain was shown to provide partial protection against *L. amazonensis* in both BALB/c and C57BL/6 mice, with mice developing lesions 40-75% smaller in BALB/c mice, and 57% smaller in C57BL/6 mice [139]. *L. mexicana* lacking cysteine proteinase genes are attenuated in BALB/c mice and were able to provide some protection against challenge [140]. *L. major* lacking lpg2 (Golgi guanosine diphosphate-mannose transporter) failed to induce lesions, but persisted at low lev-

els [141]. These parasites were shown to induce protection in BALB/c animals [142], but protection in C57BL/6 mice required CpG DNA as an adjuvant [143].

One potential problem of attenuation is loss of immunogenicity to generate a protective response. Streit *et al*. showed in *L. chagasi* that attenuated versions of the parasite with *dhfr-ts* knockouts were unable to provide protection against challenge, while infection with virulent parasites provided protective immunity [144]. In addition, the protective immunity observed in mice with *dhfr-ts* mutants was not transferable to the rhesus monkey model. While the attenuated strain persisted without pathogenicity, it was unable to provide protection against challenge [145]. Thus it is likely that adjuvants of some variety will be required for attenuated parasite vaccines to transfer to the clinic.

CD40L and Leishmaniais

The co-stimulatory molecule CD40L is well characterized, and has been demonstrated in multiple studies to play important roles in disease progression in leishmaniais. Mice lacking CD40 or CD40L have been shown to be more susceptible to *Leishmania* infection [84, 146–149]. Treatment with exogenous soluble CD40L could restore resistance in $CD40L^{-/-}$ C57BL/6 mice [147]. Furthermore, administration of anti-CD40 antibodies was shown to induce IL-12 and protect mice from *Leishmania* challenge [150]. Anti-CD40 antibodies were also shown to induce macrophage killing of parasites and act synergistically with antimony therapy to improve disease outcome [148, 149].

Previously, we have shown that the addition of CD40L to leishmanial antigens improved disease outcome and provided protection against challenge with virulent organisms [151]. In addition, CD40L trimer DNA has been successfully used to vaccinate mice against *Leishmania* infection [152]. Thus, we hypothesized that transgenic organisms expressing murine CD40L would be less virulent and more immunogenic than wild-type organisms, and would be an improved vaccine alternative to traditional leishmanization.

CHAPTER 2: MATERIALS AND METHODS

Mice

Six to eight week old BALB/c or C57BL/6 female mice were purchased from Charles River Laboratories (Wilmington, MA) and Taconic Inc. (Rockville, MD) respectively. SCID mice on a BALB/c background, $CD40^{-/-}$, and $CD40L^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Labs (Bar Harbor, ME). All mice were maintained in HEPA-filtered caging units (Thoren Caging Systems, Hazelton, PA) at the University of Maryland (College Park, MD). Mice were used for experiments at 6-10 weeks of age. All protocols were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC). See Table 1 for a list of mouse strains used in this study.

Parasite culture

Promastigotes of the *Leishmania major* Friedlin strain, clone V1 (MHOM/IL/80/Friedlin) were used throughout. Parasites were maintained in BALB/c mice and cultured *in vitro*. Promastigotes were cultured in 50:50 media, which is composed

Table 1: Mouse strains used in study.

Strain	**Rationale**	**Phenotype of *L. major***	**Expected phenotype of *L. major* CD40LE**
BALB/c	Susceptible mouse model	Progressive Lesions	Attenuated
C57BL/6	Resistant mouse model	Small, healing lesions	Attenuated
SCID (BALB/c)	Lack B and T cells, thus no adaptive immunity	Progressive Lesions	Progressive lesions
$CD40L^{-/-}$ (C57BL/6)	Deficient in CD40L. Used to determine if parasite derived CD40L responsible for attenuated phenotype observed in wild-type mice.	Progressive Lesions	Attenuated
$CD40^{-/-}$ (C57BL/6)	Lack CD40, the receptor for CD40L. Used to confirm CD40L responsible for attenuated phenotype observed in wild-type mice.	Progressive Lesions	Progressive Lesions

of 50% M199 media (CellGro) and 50% Schneider's *Drosophila* Medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS), 100U of penicillin G per mL, 100μg of streptomycin per mL, and 2mM glutamine. 100μg/mL of nourseothricin (Werner BioAgents, Germany) was added to cultures of transgenic parasites to select for resistant parasites.

Infections, Immunizations, and Parasite Quantitation

For mouse infections, parasites were washed in Hanks balanced salt solution (HBSS) (CellGro, Lawrence, Kansas), centrifuged at 1000 x *g* for 10 minutes, and resuspended in HBSS for subcutaneous injection into the footpad in a volume of approximately 20μL. For standard BALB/c infections $1x10^5$ organisms were injected into the right hind footpad. $5x10^5$ parasites were used for the more resistant C57BL/6 mice. For immunizations with live parasites, mice were infected with $5x10^4$ wild-type, empty vector, or CD40LE *L. major* in the right hind footpad. Three to five weeks later they were challenged with wild-type *L. major* in the contralateral footpad ($1x10^5$ for BALB/c, $5x10^5$ for C57Bl/6). When mice were infected in the ear dermis, $1x10^4$ parasites were injected in a 10μL volume. Disease progression was monitored twice weekly using a digital caliper to measure footpad thickness. The lesion size for standard infections was calculated as the difference between the thickness of the infected and uninfected footpad as previously described [27]. For immunizations where both footpads were infected, lesion

size was reported as the thickness of the infected footpad only. Ear lesions were measured as the diameter of the lesion. Parasite burdens were determined by serial dilution of single cell suspensions made from excised footpads, lymph nodes, or spleens as previously described [153]. Briefly, organs were harvested into HBSS and were then forced through a cell strainer. Cells were washed through the strainer with 10mLs of HBSS and centrifuged at 500 x *g* to remove cell debris. Supernatants were transferred to a new tube and centrifuged at 1000 x *g* to harvest amastigotes. Cell pellets were resuspended in 1mL of HBSS. For footpad burdens, samples were diluted 1000 fold with HBSS before dilution plating in a 1:5 serial dilution scheme. Parasite burden was determined as the final well containing live parasites after 5-7 days incubation at 25°C. For *in vitro* infections of macrophages, parasites were washed and counted as for mouse infections and added to wells in a small volume of HBSS (20-50μL).

Gene splicing by overlapping extension (SOE) PCR

The 126bp gp63 signal sequence from *L. amazonensis* was amplified from the plasmid U11 using the forward primer 5'TAACCCGGGATGTCCGTCGACAGCAG3' (F1) and the reverse primer 5'AAATTGCCTTCTCATGGCGTGTGCCCACGC3' (R1) from IDT DNA Technologies, Inc. (Coralville, IA). The CD40L extracellular domain (aa#87-260) was amplified from the plasmid pORF-mCD40L from Invivogen (San Diego, CA) using the forward primer 5'TGGGCACACGCCAT-

GAGAAGGCAATTT3' (F2) and the reverse primer 5'TTAGCTAGCGAAGACT-GCCAGCATCAGC3' (R2) also from IDT. The hybrid gp63CD40LE product was amplified using splice overlap extension PCR as previously described [154]. The gp63CD40LE product was then ligated into the TA cloning vector pCRII (Invitrogen, San Diego, CA) and grown in DH10B competent *E. coli* (Invitrogen). Colonies were grown on LB plates containing ampicillin and X-Gal, 10 white colonies were chosen, and plasmids were extracted using the mini-prep kit (Qiagen). Purified plasmid was digested with XmaI and NheI (New England BioLabs [NEB], Beverly, MA) to detect clones containing the insert. Clones positive for the insert by enzymatic digest were then sent for sequencing (Core Sequencing Facility, University of Maryland, College Park, MD). One clone with the correct sequence was excised from the TA vector using the restriction enzymes XmaI and NheI and ligated into the *Leishmania* vector pIR1SAT (pSAT) (a kind gift from Stephen Beverley, Washington University, St. Louis, MO) that had been digested with XmaI and XbaI (NEB). This plasmid contains 5' and 3' portions of the 18srRNA gene of *L. major* to allow for insertion into the genome, as well as intergenic regions necessary for mini-exon splicing and polyadenylation. This allowed for stable transgenic constructs to be generated through homologous recombination. The plasmid also contains a streptothricin acetyl transferase (SAT) gene for selection with antibiotics from the streptothricin family, such as nourseothricin. Insertion into the pSAT vector was confirmed using XmaI and BglIII (NEB) (another downstream site in

pSAT). pSAT CD40LE was linearized using the restriction enzyme SwaI (NEB) and gel purified using a kit from Qiagen.

Electroporation

Leishmania major were transformed with 10μg of linearized plasmid using electroporation as previously described [154]. Briefly, 1x10^8 parasites were resuspended in 400μL of electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM Na2PO4) and mixed with plasmid. Following electroporation, cells were plated on blood agar plates containing 100μg/mL of nourseothricin. Single colonies were selected and grown in 50:50 broth culture containing 100μg/mL nourseothricin. Clones were labeled no. 13 for the clone of pSAT-CD40LE used for the transfection and sequentially numbered from colonies chosen at random from blood agar plates, resulting in the nomenclature *L. major* CD40LE 13.2 (no. 13 for pSAT clone,no. 2 for colony from blood agar plate).

RT-PCR

RNA was isolated using the Trizol method according to the manufacturer's protocol (Invitrogen) as previously described [154]. RNA was then converted to cDNA using random hexamer primers (Invitrogen). Then gp63 was amplified using the

forward primer 5'ATCCTCACCGACGAGAAGCGCGAC3' (F3) and the reverse primer 5'ACGGAGGCGACGTACAACACGAAG3' (R3) from IDT. CD40L was amplified using the same primers used to generate the original PCR product (F2, R2).

Western Blot

$1x10^8$ parasites were harvested from late log/stationary phase cultures, washed once with HBSS and resuspended in 1mL of ice-cold lysis buffer as previously described [155]. Lysates were cleared by centrifugation (13,000 rpm, 10 min, 4°C). Equal amounts were then loaded on 18% SDS-polyacrylamide pre-cast gels (BioRad). After separation, proteins were transferred onto a polyvinylidene difluoride membrane for 1h at 100V. Membranes were then blocked in 5% nonfat milk in TBS-T for 1h at room temperature, washed briefly and incubated with the primary antibody (1:200) (polyclonal goat anti-murine CD40L, R&D Systems cat#AF1163) overnight at 4°C. Membranes were washed in TBS-T and incubated with secondary antibody (anti-goat HRP diluted 1:5000) for 1h at room temperature. Membranes were then developed using ECL chemiluminescent substrate (Amersham Biosciences) according to the manufacturer's instructions. For non-denaturing gels, samples were resuspended in sample buffer without reductance and immediately loaded onto gels without SDS for native PAGE.

ELISA

ELISAs were done using standard protocols as previously described [35] with the following antibody pair from R&D Systems: anti-CD40L (cat#AF1163) and biotinylated anti-CD40L (cat#BAF1163). Cytokine production was determined using the following antibody pairs from BD Pharmigen (San Jose, CA): IL-12p40, C15.6 and C17.8; TNFα, G281-2626 and MP6-XT3.

MTT assay

MTT assays were performed as previously described [154]. 1x10^6 *L. major*, *L. major* pSAT or *L. major* CD40LE 13.2 were inoculated into 5mL of 50:50 media. 100μL of culture was harvested at each time point and loaded onto a 96 well plate. 20μL of MTT (stock 5mg/mL) (Sigma, St. Louis, MO) was added to wells for 2h at room temperature, followed by 100μL of DMSO to lyse cells for 30min at room temperature in the dark. Plate was read on an ELISA plate reader at 550nm.

Macrophages

Bone marrow derived macrophages (BMMϕ) were generated as previously described [155]. Briefly, bone marrow was flushed from BALB/c femurs and tibias and the cells were plated in Dulbecco's Modification Eagle's Medium supplemented

with F12 (DMEM-F12) (DIFCO) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (Hyclone, Logan, UT), 100U/mL of penicillin (Fisher Scientific), 100μg/mL of streptomycin (Fisher Scientific), 2mM glutamine (Fisher Scientific), and 20% L-cell conditioned medium (LCCM). Cells were fed with 10mL of this media on day 2, and adherent cells were used on day 7-14.

L929 Cells

Control L929 cells and L929 cells stably transfected with CD40L were provided by Brian Kelsall (National Institutes of Health). Cells were cultured in RPMI suplemented with 10% HI-FBS, 100U/mL penicillin, 100μg/mL of streptomycin, and 2mM glutamine. 300μg/mL of genetecin was added to transfected cells to maintain CD40L expression.

Macrophage Stimulation

For some experiments, macrophages were primed with 100U/mL of IFN-γ overnight. Macrophages were washed three times with PBS prior to stimulation to remove residual IFN-γ. LPS was used at a concentration of 10ng/mL. L929 and L929 CD40L cells were added at a 1:1 ratio to BMMϕ. When soluble *Leishmania* antigen was used, it was added at a concentration of 50μg/mL. For certain experiments, par-

asites were opsonized prior to infection by incubation in 5% C5d serum in HBSS for 15 minutes at 37°C. Parasites were washed with HBSS before macrophage infection.

Immunofluorescent staining of amastigotes

Bone marrow derived macrophages from BALB/c mice were plated $1x10^5$ cells per coverslip and infected with a 20:1 ratio of *L. major*, *L. major* pSAT, or *L. major* CD40LE for 2 hours. Monolayers were thoroughly washed and fixed with methanol for 15 minutes at 4°C. Coverslips were blocked with 5% FBS in PBS for 15 minutes and stained with polyclonal murine anti-*L. major* serum diluted 1:250 in blocking buffer for one hour at room temperature. The cells were washed and a FITC conjugated goat anti-murine secondary antibody (1:100) was added for one hour. The cells were counterstained with propidium iodide. Coverslips were mounted with Mowiol and viewed using a fluorescent microscope (Zeiss).

Immunofluorescent staining of NF-κB

BMMϕ were plated with $1x10^5$ cells per coverslip and infected with a 20:1 ratio of *L. major* or *L. major* CD40LE for 45 minutes at 37°C. Cells were washed with warm PBS and fixed in 4% paraformaldehyde for 15 minutes. Cells were

then washed and incubated in blocking buffer (1% normal goat serum and 0.1% saponin) for 15 minutes at room temperature. Cells were incubated with primary mouse monoclonal anti-p65 (clone F6) for 1 hour. Cells were washed and stained with FITC conjugated goat anti-mouse antibody diluted 1:100 in blocking buffer for 1 hour. During the last 10-15 minutes of this incubation, the cytoplasm was counterstained with 0.008% final concentratin of Evan's Blue. Coverslips were then mounted on slides with mowiol and viewed under a fluorescent microscope.

Nitric Oxide Production

BMMϕ were plated with $2x10^5$ cells per well in 24 well tissue culture dishes and stimulated under various conditions. Supernatants were collected after 24 hours of incubation at 37°C, and nitric oxide concentrations were determined using the Griess assay. Standards were created by 1:10 serial dilution of 100μM $NaNO_2$. Standards and samples were loaded onto a 96 well plate and Griess Reagent was added. Griess reagent consists of 2% phosphoric acid, 1g sulfanilamide, and 0.1g naphthylethylene diamine hydrochloride with H_2O to a final volume of 100mL. Plates were read approximately 5 minutes later on an ELISA plate reader at 550nm.

Flow Cytometry for CD40

BMMϕ were plated with $2x10^6$ cells per well in 6 well tissue culture dishes. Cells were infected with a 20:1 ratio of parasites to macrophages overnight. Cells were then harvested into flow tubes using Cell Stripper. Cells were fixed with 4% paraformaldehyde for 15 minutes, washed, and were then stained for surface expression of CD40 with a PE conjugated anti-CD40 antibody for 45 minutes in the dark at 4°C. Cells were then run on a FACSCalibur machine and analyzed using CellQuest software.

DO11.10 T cell assay

BMMϕ were plated $2x10^5$ cells per well in a 48 well plate. Macrophages were infected with wild-type or transgenic *L. major* and concurrently given ovalbumin (150μg/mL). T cells were isolated from the spleens of DO11.10 mice using the murine SpinSep T cell enrichment kit from Stem Cell Technologies. The procedure was performed according to the manufacturer's instructions. Briefly, spleens were passed through cell strainers with PBS containing 2% FBS (recommended medium). Cells were pelleted at 200 x *g* for 10 minutes and resuspended in 2mL recommended medium. 20μL of antibody cocktail was added, and cells were mixed and incubated for 15 minutes at 4°C. Cells were washed and resuspended in 2mL of recommended medium. 500μL of dense particles were added for 20 minutes on

ice. Then cell suspensions were diluted with 6mL of recommended medium and layered over density medium. T cells were at the interface of the density medium after centrifugation at 1200 x *g* for 10 minutes with the brake off. Harvested cells were washed with PBS before use. T cells were resuspended in 500μL of warm PBS. Stock CFSE (5μM) was diluted 1:100 in PBS and added 1:1 to resuspended cells. Cells were mixed and incubated for 7 minutes at room temperature in the dark. Staining was stopped with the addition of an equal volume of FBS. Cells were then centrifuged and counted for use in T cell proliferation assay. T cells were added to macrophages approximately 3 hours post macrophage stimulation, and cells were incubated for 96 hours before CFSE dye dilution was measured by flow cytometry. Positive proliferation was determined by multiple peaks shifting left due to dye dilution following cell division.

Real Time PCR

Real Time PCR was performed on draining lymph nodes from ear infections. Lymph nodes were placed in Trizol immediately following removal. Tissue was disrupted with small pestles (Quiagen) in 1.5mL microfuge tubes. RNA was quantified by spectrophotometry, and cDNA was generated using the ThermoScript kit from Invitrogen according to the manufacturer's instructions. Real Time PCR was performed for cytokines IL-10, IL-4, and IFN-γ using the following primer sets from IDT DNA:

IL-10 forward 5'AAGGACCAGCTGGACAACAT 3';

IL-10 reverse 5' TCTCACCCAGGGAATTCAAA 3';

IL-4 forward 5'TCAACCCCCACGTAGTTGTC 3';

IL-4 reverse 5'ACGTTTGGCACATCCATCTC 3';

IFN-γ forward 5' GCGTCATTGAATCACACCTC 3';

IFN-γ reverse 5' TGAGCTCATTGAATGCTTGG 3'.

HPRT was used as a housekeeping gene for normalization with the primers: forward 5'AAGCTTGCTGGTGAAAAGGA 3'; reverse 5' TTGCGCTCATCTTAGGCTTT 3'. Dissociation curves were performed for every run, and data was only analyzed if the curve showed a single peak. Samples from all primer sets were also run on a gel initially to ensure singe band products.

CHAPTER 3: RESULTS

Plasmid construction and transformation of *Leishmania major*

Transgenic *L. major* parasites expressing the extracellular domain of CD40L were developed using Splicing Overlap Extension (SOE) PCR [154, 156, 157]. This technique attached the gp63 signal sequence from *L. major* to the extracellular domain of CD40L (CD40LE) (Figures 5 and 6). It has previously been shown that the extracellular domain of CD40L can be expressed and secreted in a biologically active trimer by *E. coli*, and we used the same amino acids (#87-260) from the 18kDa protein described in that study for our molecule [72]. The PCR products were generated as described in Materials and Methods. First, the gp63 signal sequence from *L. major* was generated, as was the CD40L extracellular domain (Figure 6A). These two products were joined in the SOE PCR reaction (Figure 6B). The hybrid SOE PCR product was placed in the TA vector for stabilization. Clones with identified inserts were then sent for sequencing. A clone with a perfect sequence (number 14) was digested and gel purified for ligation into the leishmanial vector pIR1SAT (pSAT).

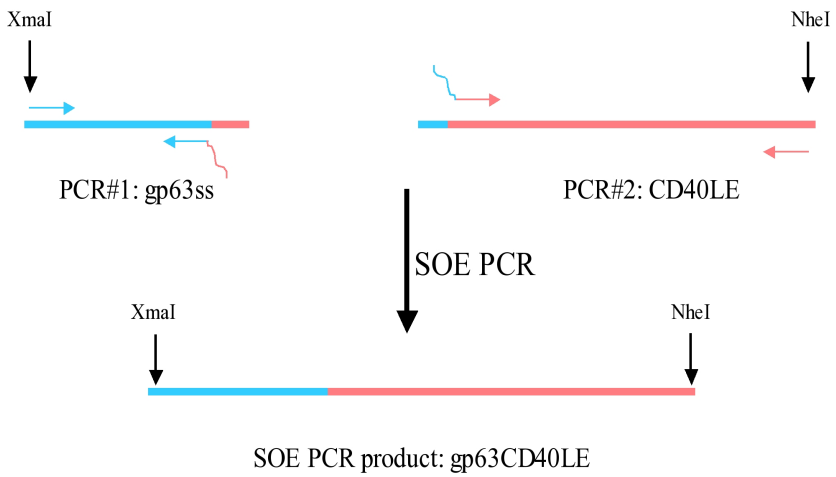

Figure 5: SOE PCR Reactions.

The CD40LE construct was generated using SOE PCR. First, the gp63 signal sequence was amplified. Then the CD40L extracellular domain was separately amplified. These first reactions used primers that contained overhangs complementary to the other sequence. The two products were then put into the SOE PCR reaction, which allowed the generation of the hybrid gp63CD40LE product.

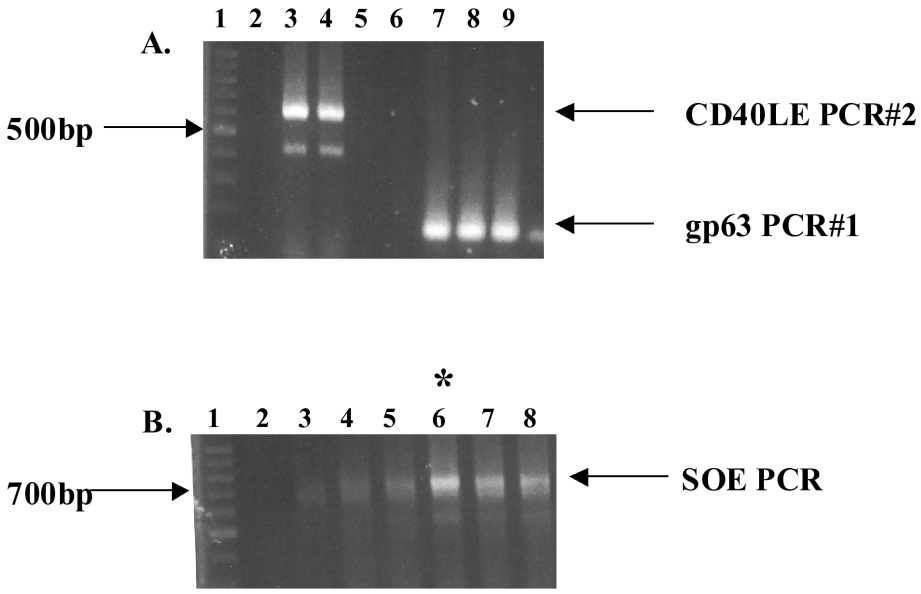

Figure 6: SOE PCR Reactions. Gel Photos.

A) The signal sequence from gp63 was amplified in the first PCR reaction (PCR#1, Lanes 7-9). Its size is 126bp. The 590bp extracellular domain from CD40L (CD40LE) was amplified in the second PCR reaction (PCR#2, Lanes 3-4). **B)** The SOE PCR reaction was done using a temperature gradient and generated a 716bp hybrid containing the gp63 signal sequence and the CD40L. The product was gel purified from Lane 6 (also marked with an asterisk) for ligation into the TA vector.

This vector has several important features that allow for gene expression in *Leishmania* (Figure 7). First, it contains the 5' and 3' ends of the 18srRNA (SSU) gene of *L. major* to allow for stable integration of the plasmid into the genome. This can be exposed by digestion of the plasmid with SwaI and gel purification of the larger product, resulting in linear plasmid for transformation and insertion (Figure 8). The plasmid also contains intergenic regions required for mini-exon attachment and polyadenylation for mRNA processing. Finally, it contains both an ampicillin resistance gene for plasmid selection in bacterial cells, and a streptothricin acetyl transferase (SAT) gene for resistance to the streptothricin family of antibiotics for selection of parasites containing the plasmid. Following ligation into pIR1SAT, DH10B cells were transformed, and ampicillin resistant colonies selected. Plasmid was isolated from these colonies and assayed for CD40LE insertion by digestion with XmaI and BglIII. Again, clones that were determined to have the insert by digestion were sent for sequencing. Clone 13 was chosen for transformation of *L. major*. The plasmid was then linearized with the restriction enzyme SwaI and gel purified to remove the ampicillin resistance gene. *Leishmania major* were electroporated with the linearized plasmid and plated onto blood agar plates containing nourseothricin. Colonies were chosen and sequentially numbered from the electroporation reaction resulting in the nomenclature of 13.2; 13 for the pSAT clone used, and 2 for the colony chosen from electroporation.

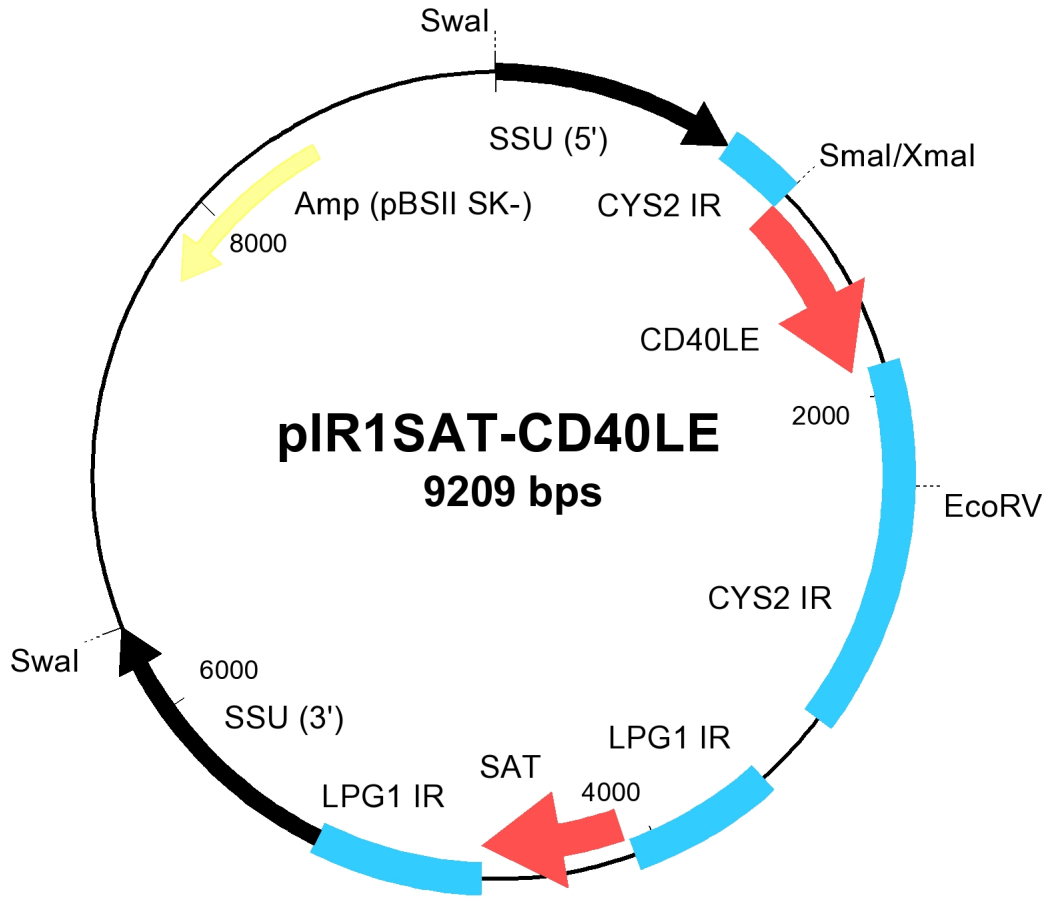

Figure 7: pIR1SAT Leishmania vector.

The vector contains the 5' and 3' ends of the *L. major* 18srRNA (SSU) gene for stable insertion into the *Leishmania* genome and intergenic regions (CYSIR) required for mini-exon attachment and polyadenylation. It contains a multiple cloning site (at the restriction enzymes SmaI/XmaI) where the CD40LE SOE PCR product was inserted. The plasmid also contains a streptothricin acetyl transferase gene to provide resistance to this family of antibiotics.

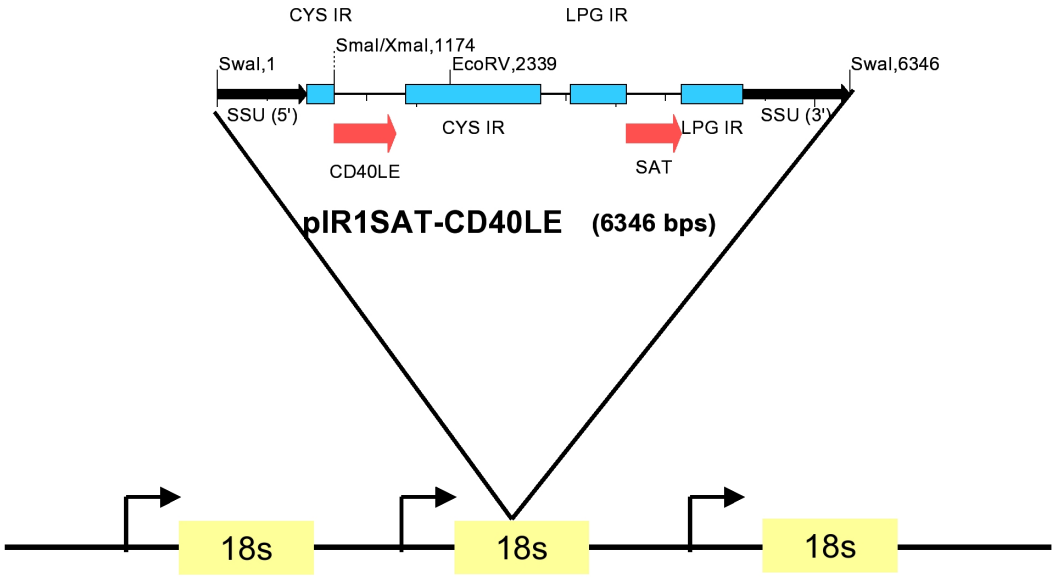

Figure 8: Insertion of pIR1SAT into the genome.

pIR1SAT CD40LE was linearized using the restriction enzyme SwaI. After linearization and gel purification of the plasmid, *Leishmania major* were transformed and the plasmid was able to integrate into the 18srRNA gene for stable expression.

Transgenic parasites express CD40L

Antibiotic resistant clones were assayed for expression of CD40L using a variety of methods. First, we examined mRNA expression in promastigotes by RT-PCR. CD40L mRNA was present in three clones of CD40LE *L. major*, designated 13.1, 13.2, and 13.4 (Figure 9A, lanes 2-4). No CD40L mRNA was detected in wild-type organisms (Figure 9A, lane 1). A leishmanial surface protein, gp63, was used as a loading control. It was detected in both wild-type and transgenic *Leishmania major* promastigotes (Figure 9A lower panel). To confirm the mRNA data, a western blot was performed on whole cell lysates (Figure 9B). L929 cells stably transfected with full-length CD40L were used as a positive control (Figure 9B lane 1). Wild-type *L. major* organisms were used as a negative control (Figure 9B lane 2). All three clones were also shown to express CD40L protein by western blot (Figure 9B, Lane 3-5). CD40L from transgenic *L. major* CD40LE was smaller than CD40L detected in L929 cells because L929 cells expressed full length CD40L. *L. major* CD40LE lack the transmembrane domain, allowing for the secretion of the protein. Secretion of CD40L was measured by ELISA (Figure 10). CD40L protein was detected in the supernatants of cultures of transgenic parasites in a dose dependent manner (Figure 10), but not in the wild-type cultures (data not shown), indicating that CD40L was secreted from the parasite.

After determining that promastigotes expressed CD40L, we examined expression by the amastigote form of the organism. BMMϕ were infected at a 20:1 ratio of

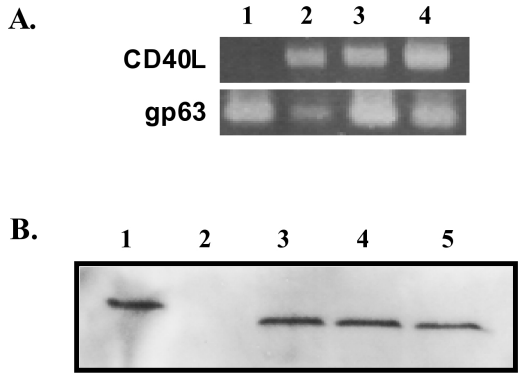

Figure 9: *L. major* CD40LE promastigotes express CD40L mRNA and protein.

A) Promastigote mRNA was isolated from wild-type *L. major* (Lane 1) and three clones of *L. major* CD40LE (Lanes 2-4) and assayed for CD40L (upper panel) and gp63 (lower panel) by RT-PCR. gp63 was used as a loading control. CD40L was detected in transgenic parasites, but not in wild-type organisms. **B)** CD40L protein was detected by western blotting of whole cell lysates from control L929 cells stably transfected with CD40L (Lane 1) and *L. major* CD40LE (Lanes 3-5). CD40L was not detected in wild-type *L. major* (Lane 2).

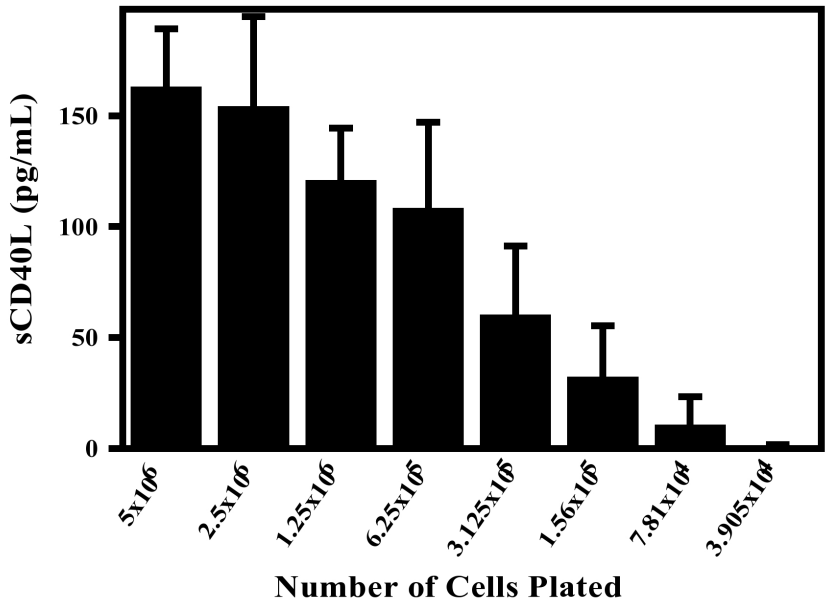

Figure 10: Secretion of CD40L by transgenic parasites.

Promastigotes were plated in 1:2 serial dilutions for 24hr. Secretion of CD40L into the supernatant was measured by ELISA.

parasites to macrophages with either wild-type or CD40LE organisms. Two hours after infection, monolayers were washed thoroughly to remove unbound parasites and visually checked to ensure the removal of promastigotes. At indicated times following infection, RNA was harvested and assayed for CD40L. CD40L was detected from monolayers infected with transgenic parasites, but not from uninfected BMMϕ or from BMMϕ infected with wild-type *L. major* (Figure 11, top panel). The presence of parasites was confirmed by the detection of gp63 mRNA (Figure 11 middle panel). HPRT was used as an indicator of equal loading of macrophage cDNA (Figure 11, lower panel). *L. major* CD40LE promastigotes were used as a positive control (Figure 11, far left lane). To examine protein expression by amastigotes, BMMϕ were infected with transgenic parasites and supernatants were harvested at the indicated time points (Figure 12) to measure soluble CD40L levels. CD40L was detected in supernatants of Mϕ infected with transgenic parasites, confirming amastigote protein expression. Taken together, these data demonstrate that the transgenic *Leishmania* organisms we generated are able to express and secrete CD40L. Clone 13.2 was chosen for the remainder of the study.

Expression of CD40L by transgenic parasites is stable

To determine if the CD40L produced by the transgenic parasites was stably expressed, BALB/c mice were infected with *L. major* CD40LE 13.2. Six weeks later, parasites were harvested and assayed for sCD40L production by ELISA (Figure

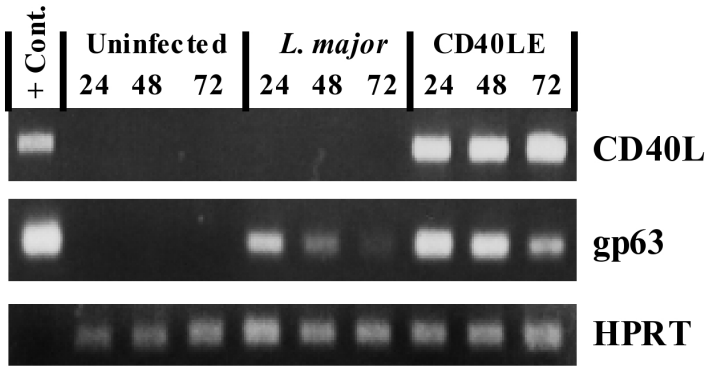

Figure 11: Amastigote mRNA expression.

BMMϕ were infected for 24, 48, or 72 hours with wild-type or CD40L organisms, or left uninfected. At each time point, RNA was isolated and RT-PCR was performed for CD40L (top panel), gp63 (middle panel), and HPRT (bottom panel). Transgenic promastigotes were used as a positive control.

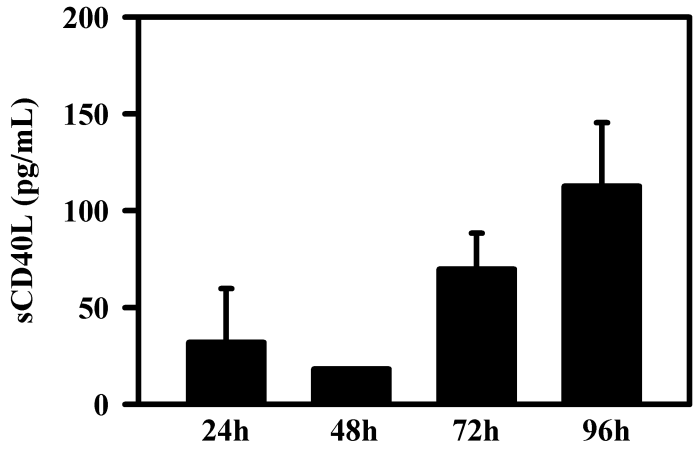

Figure 12: CD40L release from macrophages.

Macrophages were plated $2x10^5$ cells/well in a 48 well dish and infected with a 30:1 ratio of CD40L parasites. Two hours later, unbound parasites were washed away and 300μL on media was added. At indicated timepoints, supernatants were harvested and CD40L was detected by ELISA.

13). Promastigotes in culture are shown as a positive control (hatched bars). Parasites isolated from the footpads of mice (filled bars) expressed comparable amounts of sCD40L to parasites that had been maintained under antibiotic selection, indicating that the expression of CD40L by the transgenic parasite is stable. This would suggest that the plasmid successfully integrated into the *L. major* genome.

sCD40L produced by transgenic *Leishmania* forms trimers

Previous studies have shown that sCD40L can form trimers and be biologically active [72]. To examine the ability of our transgenic parasite to produce trimers, a non-denaturing gel was run, and a western blot was performed. L929 cells stably transfected with CD40L were used as a positive control. CD40L from L929 cells as well as parasite derived CD40L formed trimers, which appeared at around 60kDa on the non-denaturing gel (Figure 14A), compared to ≈20kDa on a standard SDS-PAGE gel (Figure 14B). This indicates that the sCD40L produced by our parasites is capable of forming trimers.

Transgenic parasites are as healthy as wild-type

We next determined whether the insertion in the 18srRNA gene had any effect on the viability and growth of the parasite. To assay the growth rate of the organ-

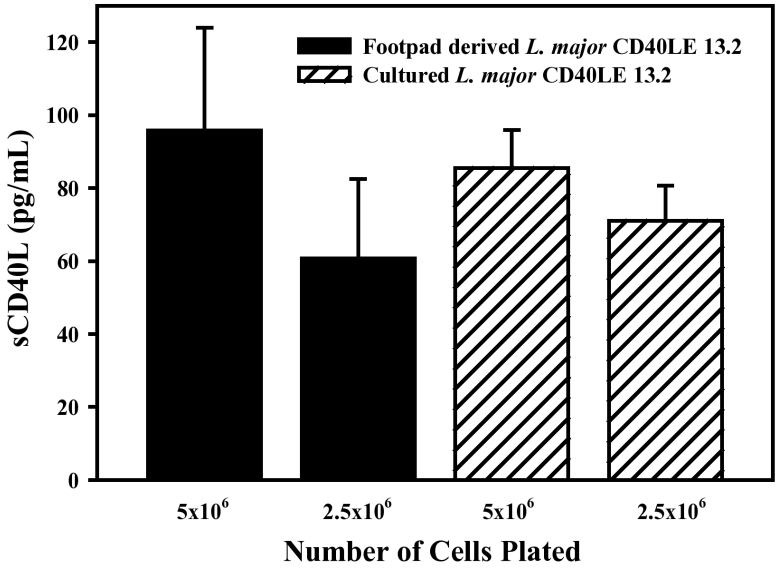

Figure 13: CD40L secretion from footpad derived parasites.

Parasites were isolated from the footpads of mice infected with *L. major* CD40LE for six weeks (filled bars) and allowed to return to promastigotes without addition of antibiotic selection. Promastigotes were then plated in two dilutions in 50:50 media for 24hr. Supernatants were harvested and assayed for CD40L by ELISA. Promastigotes that had been cultured in antibiotic selection and were not footpad derived were used as a positive control (hatched bars).

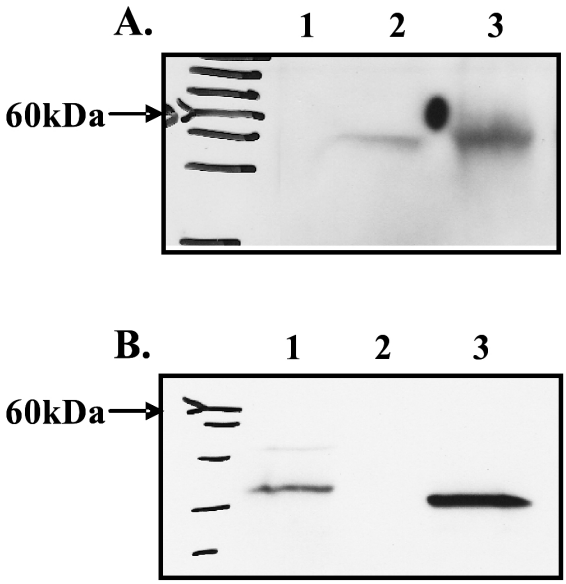

Figure 14: Parasite derived CD40L forms trimers.

A) Whole cell lysates from *L. major* (Lane 1), L929 cells transfected with CD40L (Lane 2), and *L. major* CD40LE (Lane 3) were run on a non-denaturing gel. No CD40L is detected in wild-type parasites, while CD40L trimers are detected in L929 and CD40LE cells at ≈50-60kDa. **B)** Whole cell lysates from L929 CD40L cells (Lane 1), *L. major* (Lane 2), and *L. major* CD40LE (Lane 3) were run on an SDS-PAGE gel. Monomer CD40L was detected ≈25 kDa in L929 and CD40LE cells, but not in wild-type *L. major*.

isms, an MTT assay was performed. This assay measures the metabolic rate of the organism by the conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. All three organisms have the same growth curve, indicating that the insertion has no effect on replication and metabolism rate of the parasite (Figure 15). Next, we infected bone marrow derived macrophages with the transgenic organism to ensure that the parasites were still able to invade host cells and turn into amastigotes. Unstimulated BMMϕ were plated on coverslips and infected for two hours with either *L. major* (Figure 16A), *L. major* pSAT (Figure 16B), or *L. major* CD40LE 13.2 (Figure 16C). Cells were fixed with methanol and stained with murine polyclonal anti- *L. major* serum. There was no difference between the numbers of wild-type or transgenic organisms at two hours post-infection (Table 2), indicating that there is no defect in the ability of transgenic organisms to invade host cells and transform into amastigotes.

To confirm the viability of the transgenic organisms, we infected SCID mice on a BALB/c background with $1x10^5$ wild-type or transgenic promastigotes in the right hind footpad. Disease progression was monitored using calipers to measure footpad thickness. Lesion size was determined as the thickness of the infected foot minus the thickness of the uninfected foot. Transgenic CD40LE organisms generated lesions that were comparable to those of wild-type organisms (Figure 17). Lesions contained similar parasite numbers as determined by limiting dilution assay (Figure 17, inset). These data indicate that the insertion of the transgene does

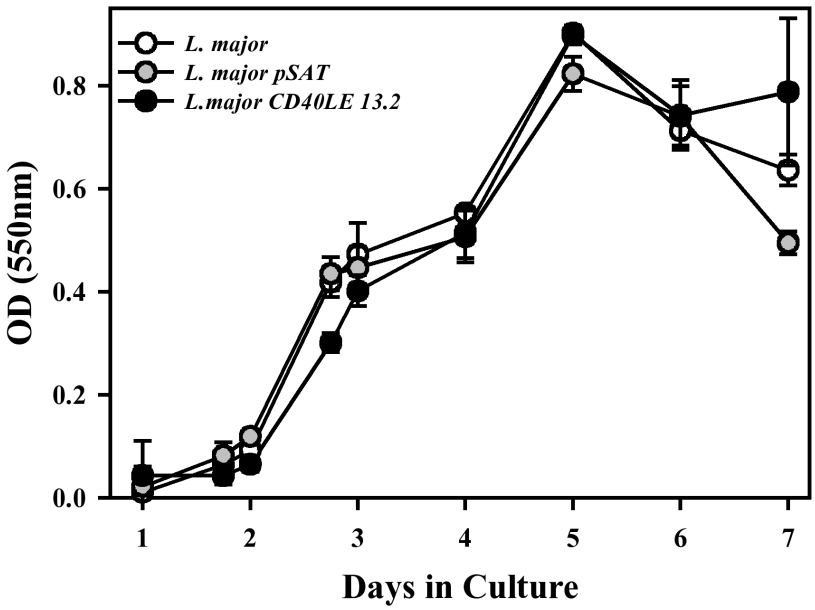

Figure 15: Parasite growth curves.

$1x10^6$ wild-type, empty vector, or CD40LE parasites were inoculated into 5mL of 50:50 media. 100μL of culture was harvested at each indicated time point and metabolic activity was measured using an MTT assay. Growth curves of wild-type (open circles), pSAT (gray circes), and CD40LE organisms (closed circles) were compared (Mean$\pm$SD). There were no differences observed among the growth curves.

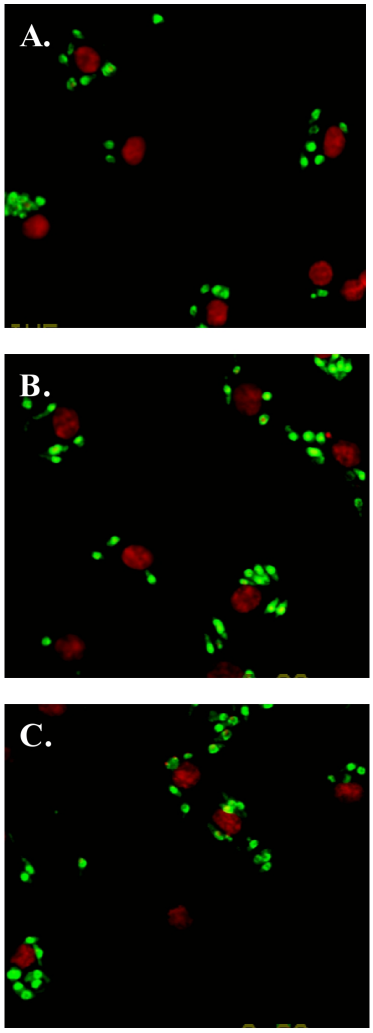

Figure 16: Infection of BMMϕ with *L. major*, *L. major* pSAT, and *L. major* CD40LE.

BMMϕ were infected for 2 hr with wild-type (A), empty vector (B), or CD40LE (C) promastigotes and stained with anti-*L. major* serum to visualize parasites. Propidium iodide was used to counterstain the nucleus. Results are representative of at least three experiments. No differences among samples were observed.

Table 2: Two hour infection of BMMϕ with wild-type or transgenic parasites.

	Number of macrophages	**Infected macrophages**	**Total number of parasites**	**Total number of macrophages infected with >3 parasites**
L. major	100	90[a]±7.18[b]	327±81.67	58±15.57
L. major CD40LE 13.2	100	87±7.09	327±58.99	58±13.03

[a]Mean from 5 independent experiments.
[b]Standard Deviation.

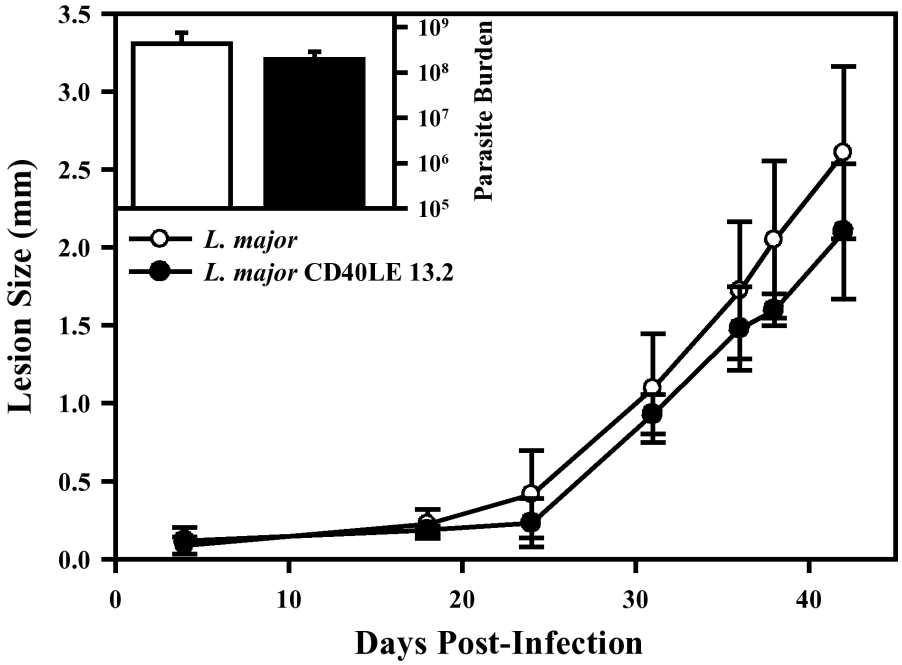

Figure 17: CD40LE parasites cause lesions in SCID mice.

SCID mice on a BALB/c background were infected with $1x10^5$ parasites in the right hind footpad. Lesion size was monitored, and parasite burdens were determined at the completion of the experiment (inset). Wild-type (open circles/bars) and CD40LE organisms (filled circles/bars) induced comparable lesions with similar numbers of parasites within them.

not affect parasite viability, nor does it affect their ability to infect and replicate in immuno-compromised hosts.

Transgenic *Leishmania major* CD40LE delay lesion development in susceptible BALB/c mice.

To examine whether or not *L. major* CD40LE had an effect on disease outcome, BALB/c mice were infected with $1x10^5$ wild-type, pSAT, or CD40LE 13.2 *L. major* parasites in the hind footpad. Lesion size was monitored over the course of the next 6 weeks. BALB/c mice are susceptible to *L. major* infections, and therefore mice infected with wild-type organisms developed large lesions (Figures 18A and 19A). Littermate mice infected with *L. major* CD40LE 13.2 developed significantly smaller lesions (Figures 18A and 19A) with fewer parasites within the lesions (Figure 18B). Dissemination to the lymph nodes was also reduced (Figures 18C and 19B). Mice infected with CD40L parasites exhibited reduced splenomegaly (Figure 19C) and parasites were virtually undetectable in the spleen of animals receiving CD40LE expressing organisms (Figure 18D). Mice infected with empty vector organisms developed lesions comparable to those of mice infected with wild-type organisms. These data indicate that *L. major* CD40LE 13.2 parasites are less virulent than wild-type organisms in the susceptible BALB/c host.

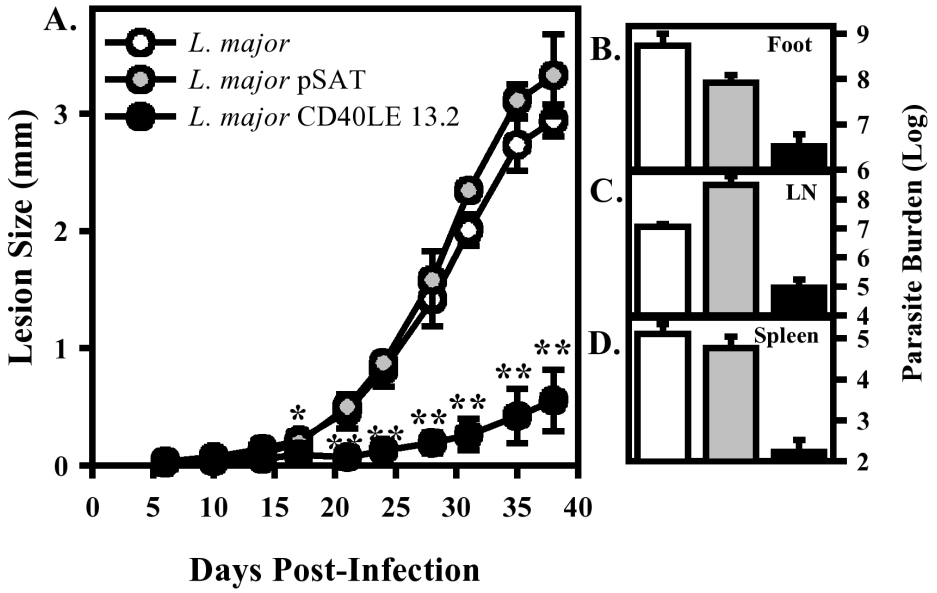

Figure 18: CD40LE parasites exhibit delayed pathology in BALB/c mice.

BALB/c mice were infected in the hind footpad with $1x10^5$ *L. major* (open circles/bars), *L. major* pSAT (gray circles/bars), or *L. major* CD40LE 13.2 (black circles/bars). **A)** Lesion development was measured with calipers twice weekly. **B-D)** Parasite burdens were determined in the footpad (B), popliteal lymph node (C), and spleen (D) at the end of the infection. Results are compiled from 3 independent experiments, with a minimum of 5 mice per goup, and are expressed as the mean $\pm$ SEM. Asterisks indicate significance (*$p<0.05$, **$p<0.01$).

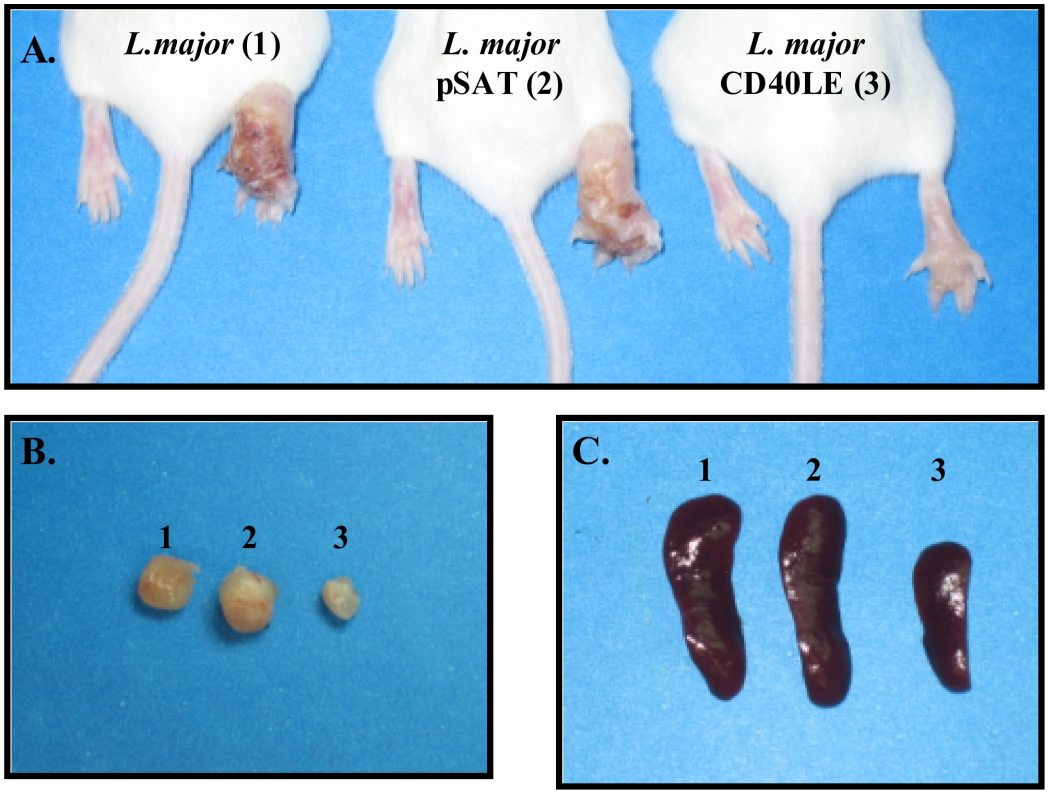

Figure 19: Disease outcome in BALB/c mice.

BALB/c mice were infected with $1x10^5$ *L. major* (1), *L. major* pSAT (2), or *L. major* CD40LE (3). Six weeks later, mice were sacrificed, and photos were taken of footpad lesions (A), popliteal draining lymph nodes (B), and spleens (C). Mice infected with CD40LE parasites had much smaller lesions, lymph nodes (although lymph nodes were enlarged compared to uninfected), and spleens than those mice infected with either wild-type or pSAT organisms.

Transgenic parasites fail to directly activate macrophages *in vitro* (data not shown).

We examined biological roles for CD40L *in vitro* utilizing a variety of techniques. First, bone marrow derived macrophages were infected for 45 minutes with either wild-type or transgenic parasites and NF-κB translocation was observed through immunofluorescence. Macrophages stimulated for 45 minutes with LPS were used as a positive control. Neither wild-type or transgenic parasites showed evidence of NF-κB translocation into the nucleus, although translocation was readily observed in macrophages treated with LPS. These data suggest that transgenic parasites, like wild-type parasites fail to activate NF-κB translocation.

We examined the ability of macrophages to produce IL-12, TNF-α, and nitric oxide because CD40L is known to be a potent inducer of IL-12 and play a role in TNF-α and NO activation. Macrophages were infected for 24 hours with wild-type or transgenic parasites and supernatants were harvested and assayed for IL-12, TNF-α, and NO production. Macrophages stimulated with LPS or L929 cells expressing CD40L induced IL-12, although only LPS induced measurable levels of TNF-α and NO. Macrophages infected with either wild-type or transgenic parasites failed to induce measurable levels of IL-12, TNF-α, or NO. When wild-type *L. major* were added to L929 cells expressing CD40L and used to stimulate macrophages, IL-12 induction was abrogated. These data taken together suggest that the ability of

the parasite to actively suppress IL-12 production overrides the CD40L stimulation provided by the transgenic parasite in this *in vitro* experiment.

We also measured the level of CD40 present on the surface of infected macrophages to detect any up-regulation of this co-stimulatory molecule in response to infection with transgenic parasites. Similar to wild-type parasites, transgenic parasites fail to induce CD40 up-regulation on infected macrophages. Stimulation with LPS was used as a positive control, and 91% up-regulation of CD40 was observed on these macrophages compared to 1% on infected macrophages.

The transgenic parasites appear not to directly activate macrophages, but are clearly playing a role *in vivo*. To determine if interaction with T cells was required, we examined the ability of macrophages infected with transgenic parasites to affect T cell proliferation. Macrophages were infected with either wild-type or transgenic parasites and concurrently given ovalbumin. Approximately three hours later, CFSE labeled DO11.10 T cells were added for 96 hours and T cell proliferation was measured by flow cytometry. Macrophages infected with transgenic parasites induced robust T cell proliferation, while macrophages infected with wild-type parasites induced no proliferation above background. These data suggest that parasite derived CD40L may act to improve antigen presentation, or that T cells may be required for the effects of parasite derived CD40L to be observed *in vitro*.

Transgenic parasites induce lower levels of the Th2 cytokine IL-4 in susceptible hosts.

To examine cytokine induction from draining lymph nodes *ex vivo*, we used the ear model of infection, and isolated mRNA from lymph nodes. Disease progression was monitored using calipers, and lesion size was determined as the diameter of the ear lesion. As in the footpad model, wild-type L major caused large, non-healing lesions (Figure 20, open circles), whereas transgenic organisms induced significantly smaller lesions (Figure 20, filled circles). At week four post-infection, draining lymph nodes were harvested directly into trizol to assay *in vivo* cytokine mRNA levels (Figure 21A-C). Cytokine mRNA levels were determined using real-time PCR and normalized to HPRT. Data are expressed as relative fold changes when compared to uninfected lymph nodes, which were normalized to 1. The transgenic parasites induced significantly less IL-4 than wild-type parasites ($p<0.05$) (Figure 21A). In three separate experiments, IL-4 levels were significantly lower in the nodes of transgenic infected mice relative to wild-type infected mice. IL-10 transcripts in the two groups were also compared. In two of three experiments, IL-10 levels were also reduced in the transgenic infected mice ($p<0.05$), but when the three experiments were compiled and compared to wild-type, the differences were not significant (Figure 21B). Wild-type and transgenic parasites induced comparable levels of IFN-γ (Figure 21C).

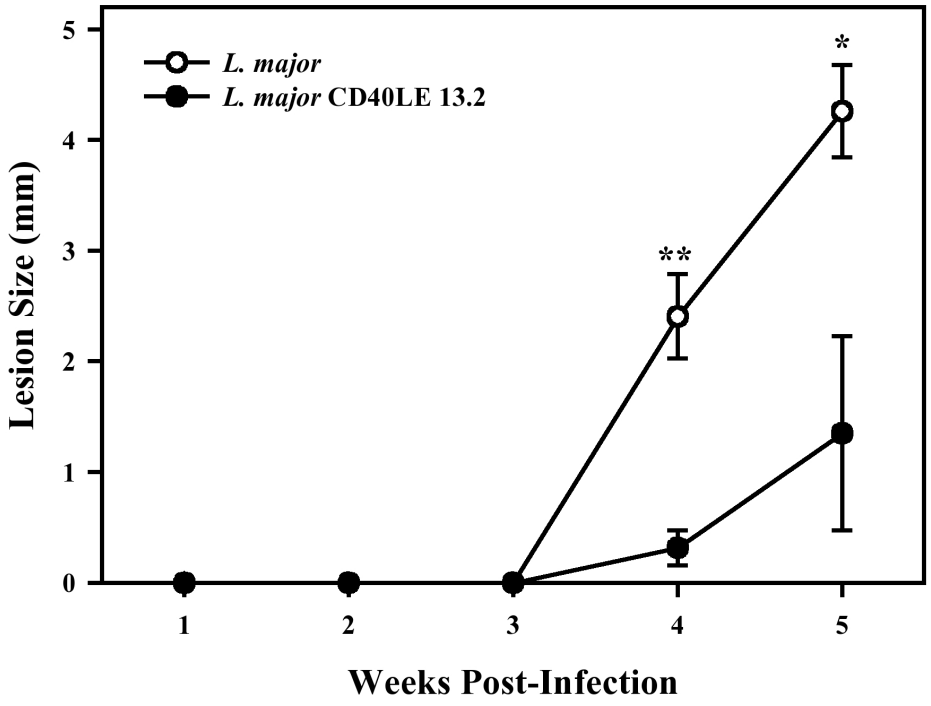

Figure 20: BALB/c mice infected in the ear with wild-type or transgenic parasites.

BALB/c mice were infected in the ear dermis with $1x10^4$ promastigotes. Lesion progression was followed by measuring lesion diameter with calipers. *L. major* (open circles) induce large, non-healing lesions. CD40LE organisms (filled circles) induce smaller lesions in this low dose model. Asterisks indicate significance (*$p<0.05$, **$p<0.01$). Data are compiled from three independent experiments and are expressed as the mean $\pm$ SEM.

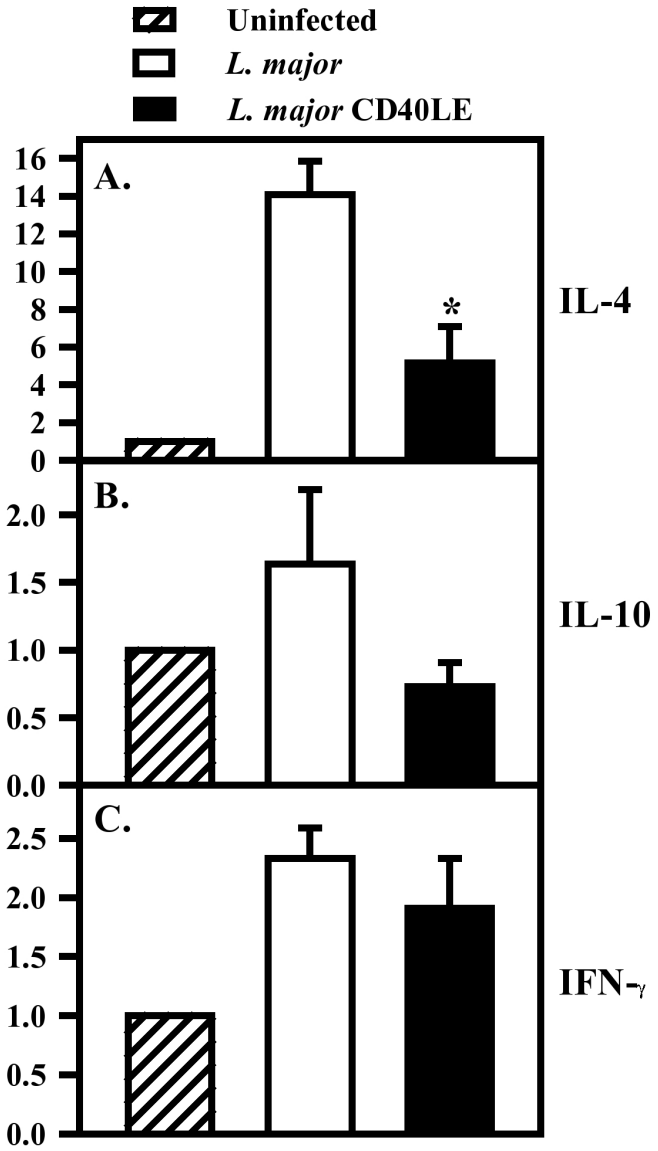

Figure 21: *L. major* CD40LE induce lower levels of IL-4 in BALB/c lymph nodes.

BALB/c mice were infected in the ear dermis with $1x10^4$ promastigotes. **A)** IL-4, **B)** IL-10, and **C)** IFN-γ levels were assayed by real-time PCR at week four post-infection. Asterisk indicates significantly less IL-4 ($p<0.05$) induced in response to transgenic relative to wild-type infection. Data were normalized to HPRT and are expressed as fold change relative to uninfected controls. Data are compiled from three independent experiments and are expressed as the mean ±SEM.

Parasite derived CD40L is biologically active

To determine if the parasite derived CD40L was active, we infected $CD40L^{-/-}$ mice on a C57BL/6 background with $1x10^5$ wild-type or transgenic organisms. Lesion development was monitored over the course of the next six weeks. C57BL/6 mice lacking CD40L were relatively susceptible to *L. major* infection, and developed progressive lesions (Figure 22, open circles). Mice infected with transgenic organisms developed significantly smaller lesions ($p<0.01$), indicating that the CD40L produced by the parasite was biologically active *in vivo*.

We also examined disease progression in $CD40^{-/-}$ mice on a C57BL/6 background to confirm that the attenuated phenotype was due to parasite derived CD40L. We would expect that parasites expressing CD40L would have no phenotype in mice lacking CD40, and that is what we observed. $CD40^{-/-}$ mice infected with transgenic parasites developed lesions that were similar in size to those caused by wild-type parasites (Figure 23). These lesions contained comparable numbers of parasites as determined by limiting dilution assay (Figure 23, inset). Taken together, these data suggest that parasite derived CD40L is biologically active and responsible for the attenuated phenotype observed.

As a control for these experiments, wild-type C57BL/6 mice were infected with $5x10^5$ wild-type or transgenic parasites (Figure 24). Mice infected with transgenic parasites developed significantly smaller lesions than those infected with wild-type

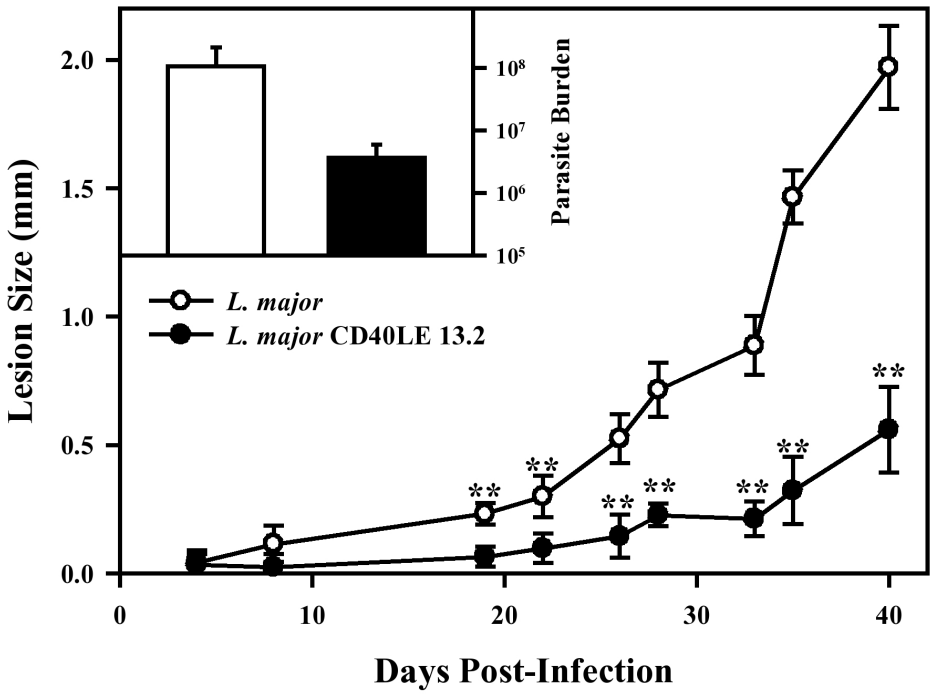

Figure 22: *L. major* CD40LE cause smaller lesions in CD40L$^{-/-}$ mice.

CD40L$^{-/-}$ mice on a C57BL/6 background were infected with 1x10^5 wild-type (open circles/bars) or transgenic (filled circles/bars) parasites. Mice infected with wild-type parasites developed large lesions, while those infected with transgenic parasites developed smaller lesions with fewer parasites. Asterisks indicate significance (**$p<0.01$).

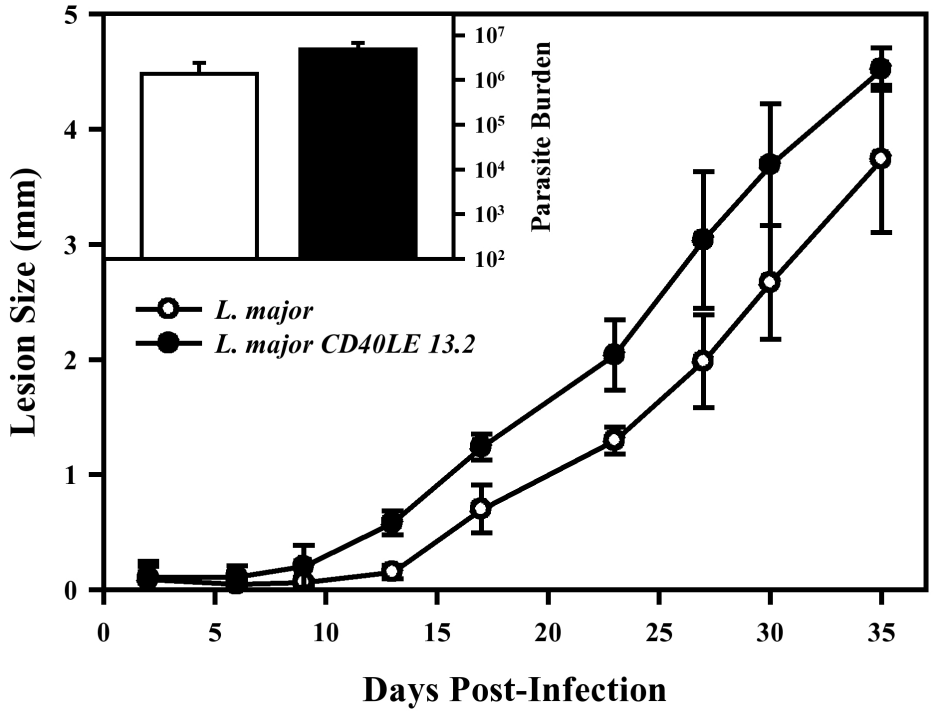

Figure 23: Transgenic parasites cause lesions in C57BL/6 CD40KO mice.

$CD40^{-/-}$ mice on a C57BL/6 background were infected with $5x10^5$ wild-type (open circles) or transgenic parasites (filled circles). Disease progression was monitored and parasite burdens were determined at the end of the experiment (inset). Mice infected with transgenic parasites developed lesions comparable to those in mice infected with wild-type parasites, with comparable numbers of parasites within them.

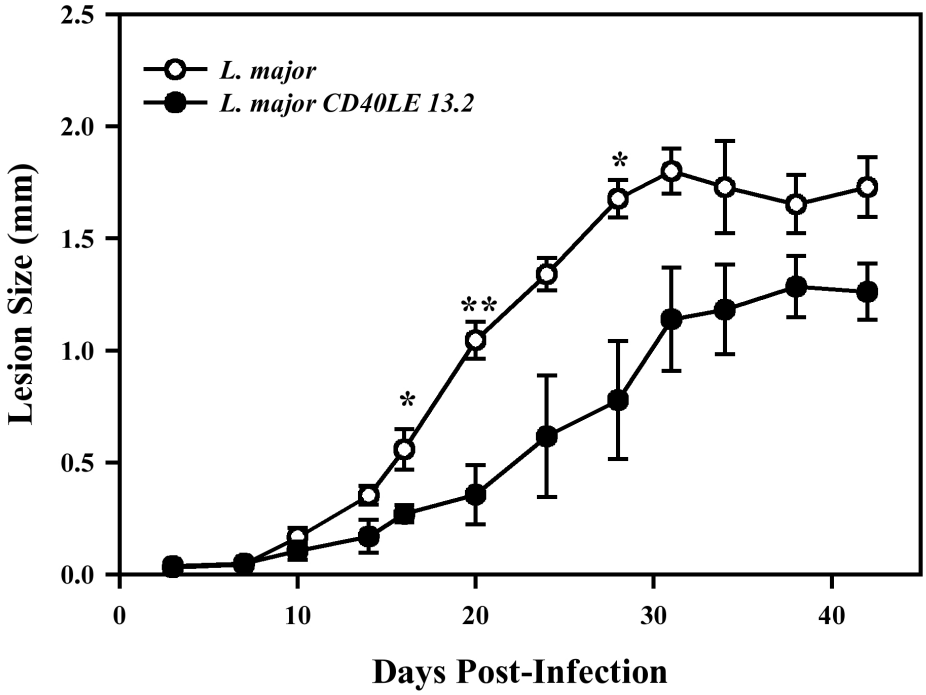

Figure 24: *L. major* CD40LE reduce disease in C57BL/6 mice.

C57BL/6 mice were infected with $5x10^5$ wild-type or transgenic parasites in the right hind footpad. Disease progression was monitored. Wild-type infected mice (open circles) developed small lesions that plateaued around 4-5 weeks post-infection. Infection with transgenic parasites (filled circles) resulted in smaller lesions that also plateaued at 4-5 weeks post infection. Asterisks indicate significance (*$p<0.05$, **$p<0.01$). Data are compiled from three independent experiments and are expressed as the mean $\pm$ SEM.

organisms (Figure 24). However, this phenotype was less dramatic that that observed in the CD40L$^{-/-}$ and BALB/c mice.

Live *L. major* CD40LE organisms establish protection against challenge with wild-type organisms

We examined the ability of the transgenic organisms to protect in a vaccination model of leishmaniasis. For these studies, the resistant C57BL/6 strain of mice was used. Several groups have demonstrated that these mice resolve infection with wild-type parasites and develop immunity to reinfection [117, 158]. C57BL/6 mice were infected with a low dose ($5x10^4$) of either wild-type *L. major*, *L. major* pSAT, or *L. major* CD40LE 13.2 in the right hind footpad. Four to five weeks following initial infection, mice were re-challenged with $5x10^5$ wild-type *L. major* parasites in the contralateral footpad and disease progression was monitored as above. As previously reported, mice that had cleared an infection with wild-type parasites were resistant to re-infection (Figure 25)[159]. Mice that were previously infected with transgenic parasites were similarly protected from re-infection. Mice that were not previously infected developed larger lesions with higher parasite numbers within them (Figure 25 inset). These data suggest that the attenuated transgenic organisms retain the immunogenicity of their wild-type counterparts.

Next we wanted to ascertain if the transgenic parasite would be able to provide protection to the susceptible BALB/c mouse. Vaccination with viable wild-type

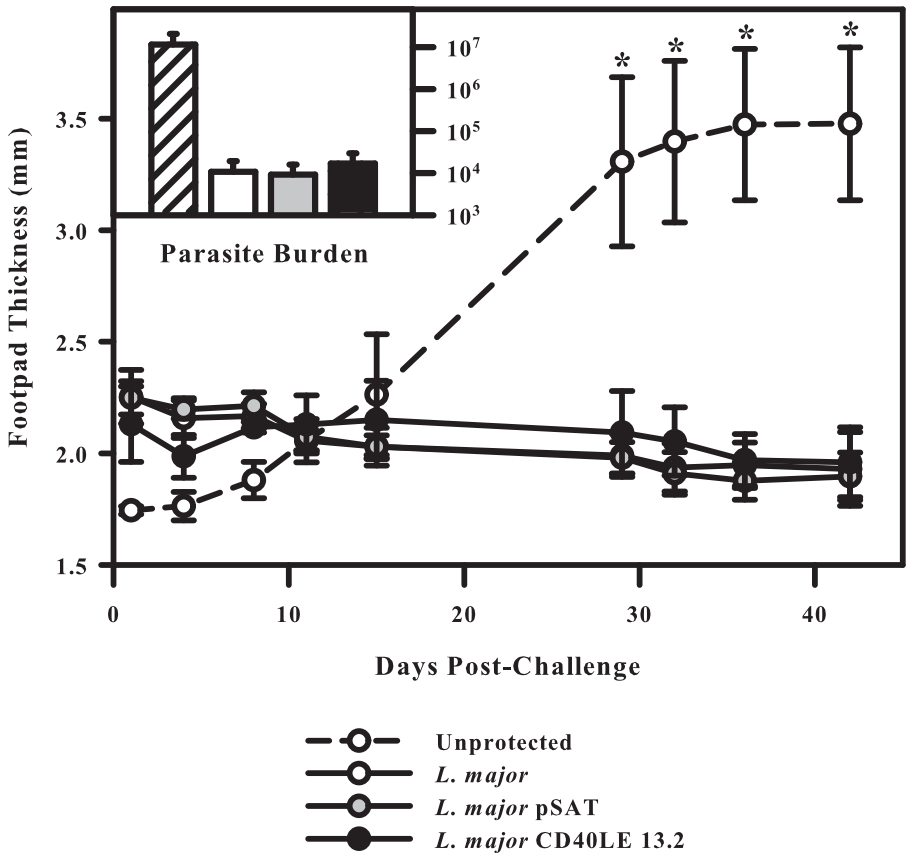

Figure 25: *L. major* CD40LE 13.2 induce protection against wild-type challenge in C57BL/6 mice.

C57BL/6 mice were infected with $5x10^4$ *L. major* (open circles/bars), *L. major* pSAT (gray circles/bars), or *L. major* CD40LE 13.2 organisms (black circles/bars) in the right hind footpad. Lesions were allowed to heal, and then mice were challenged in the contralateral footpad with $5x10^5$ wild-type parasites. Lesion development was monitored and burdens determined as previously described. A group of unprotected mice was used as a control for lesion development (hatched lines/bars). Asterisks indicate significantly larger ($p<0.05$) lesions in unprotected mice when compared to any protected group. Results are compiled from three independent experiments and are expressed as the mean $\pm$ SEM.

organisms is not possible in this model, because BALB/c mice develop large, non-healing lesions and eventually succumb to infection. BALB/c mice were infected with a low dose ($5x10^4$) of CD40LE 13.2 organisms in the right hind footpad. Four to five weeks later, mice were re-challenged with $1x10^5$ wild-type *L. major* promastigotes in the contralateral footpad, and lesions were monitored. Vaccination of susceptible BALB/c mice with *L. major* CD40LE 13.2 resulted in a significant, albeit modest, reduction of lesion size in challenged animals (Figure 26, filled circles). These lesions contained approximately one log fewer parasites than unvaccinated animals (Figure 26 inset), indicating that vaccination with the transgenic organism was unable to completely overcome the Th2 bias in these animals and induce full protection.

Summary

In summary, we have generated transgenic *Leishmania major* parasites that express CD40L mRNA and protein at both life stages. By all criteria, transgenic parasites appear to be as healthy as wild-type organisms. Parasites induce significantly smaller lesions in the susceptible BALB/c mouse model, and induce less IL-4 in this model of infection. Infection of CD40L deficient mice and CD40 deficient mice suggest that attenuated phenotypes are due to parasite derived CD40L. In addition, transgenic parasites are able to provide full protection in the resistant

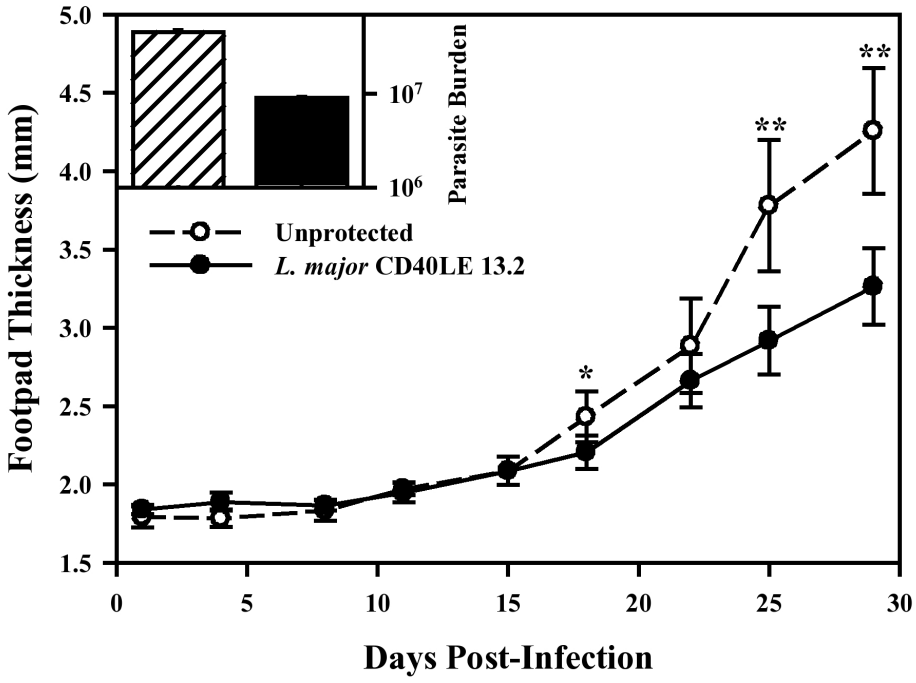

Figure 26: Protection with transgenic parasites in BALB/c mice.

BALB/c mice were injected with $5x10^4$ transgenic parasites (black circles/bars), and four to five weeks later were challenged with $1x10^5$ wild-type parasites. Unprotected mice were used as a control for lesion development (hatched lines/bars). Inset shows parasite burden. Asterisks indicate significance (*$p<0.05$, **$p<0.01$). Results are representative of three independent experiments.

C57BL/6 mouse model, and partial protection in the susceptible BALB/c mouse model.

CHAPTER 4: DISCUSSION

Leishmaniasis is a disease that is endemic in nations with populations that cannot easily afford the costly drugs used to treat it. An effective vaccine is needed to prevent infection and improve the quality of life for at-risk people. As mentioned previously, leishmanization can provide powerful immunity against infection [100], but is not routinely practiced due to the incidence of lesions requiring treatment and the potential for secondary infections. Many vaccine strategies have been examined to improve upon traditional leishmanization, including using killed parasites with adjuvants [101] and generating attenuated strains [137–139, 141, 142]. Attenuated strains have shown some success in animal models, but thus far none have been successfully transferred to the clinic. One major challenge with attenuation was that some strains lost immunogenicity during attenuation [144, 145]. Our approach was to develop a transgenic parasite that would express a co-stimulatory molecule. We hypothesized that the transgenic organisms would induce less pathology in susceptible mice and would provide protection against re-challenge with wild-type organisms. Our goal was to develop an attenuated organism that would retain immunogenicity and thus be an improved alternative to traditional leishmanization.

We chose to use the co-stimulatory molecule CD40L for this study for a number of reasons. CD40L is known to be important for T cell activation and antigen presentation through binding its receptor, CD40. It has been shown to play an important role in cell mediated immunity, and has been demonstrated in a number of studies to be relevant in leishmanial infection [84, 147, 148]. Mice lacking either CD40 or CD40L have been shown to be more susceptible to infection with *Leishmania* spp. [84, 146–148]. Previously, we have shown that combining CD40L with leishmanial antigens can improve disease outcome in susceptible animal models [151]. Also, vaccination with CD40 ligand trimer DNA was shown to provide protection in the BALB/c host [152]. Thus in this study we have developed transgenic *L. major* organisms expressing the extracellular domain of CD40L.

Our parasites were shown to express CD40L mRNA and protein, and secrete CD40L into the media. Parasite derived CD40L was demonstrated to form trimers, required for biological activity. CD40LE expression was detected by both western blot (data not shown) and ELISA (Figure 13) in parasites isolated from footpads of mice infected for six weeks, indicating a stable level of expression. Using a variety of methods, we examined whether this insertion had any effect on the fitness of the organism. By all criteria, the transgenic organisms appear to be as healthy as wild-type organisms. The growth curves for these organisms were virtually identical, and parasites were able to infect macrophages and convert into the amastigote form within equally well. In addition, infection of SCID mice with transgenic parasites resulted in lesions comparable in size to wild-type infected mice with similar num-

bers of parasites within them (Figure 17). From these data, we conclude that the insertion of transgenic DNA into the parasite genome has no discernable effect on the growth and infectivity of the parasite.

The question of how the CD40L secreted by the parasite is able to access CD40 positive cells remains unanswered. We provide evidence that some CD40L is released from monolayers of infected cells (Figure 12), but we have not formally determined whether it was secreted from these cells or released upon parasite destruction of the monolayer. Previous work in our lab demonstrated that MCP-1 secreted by transgenic parasites was unable to escape the macrophage until the cell was lysed [154]. Our observations that some CD40L was released from infected macrophages is consistent with the work of Dumas *et al.* who developed *L. major* secreting GM-CSF and reported 25% escape of GM-CSF from the macrophage [160]. Further studies must be done to determine the extent and mechanism of CD40L release from infected macrophages.

One major concern when using a genetically modified organism is the potential for revertants. Our data from parasites retrieved from infected footpads would suggest stable integration of CD40L into the genome, and the constant expression level over time would suggest little loss of expression. However, we cannot guarantee that no revertants exist. We would suggest for future development of this organism combining CD40L expression with defined gene knockout organisms, such as the lpg2 mutant. This would take advantage of the attenuated lpg2 parasite, and add the co-stimulation of the CD40L molecule. Combination of the different genetic

manipulations may lower the incidence of revertants to virulent forms due to irreversible genome changes and increase efficacy of the vaccine through improved immunogenicity.

We examined the ability of transgenic parasites to influence disease outcome in the susceptible BALB/c mouse. We found that *L. major* CD40LE caused substantially delayed disease in this model of infection. These animals developed significantly smaller lesions than those infected with either wild-type or empty vector organisms. It is important to note that mice infected with empty vector organisms developed lesions comparable to those of mice infected with wild-type organisms. This is another indication that the insertion of pSAT into the genome has no effect on the fitness of the organism. Thus, we conclude that transgenic organisms expressing CD40L are less virulent that wild-type organisms in a susceptible BALB/c mouse model.

We wanted to determine a possible explanation for the improved disease progression in BALB/c mice. We examined possible biological roles for parasite derived CD40L using a variety of *in vitro* methods (data not shown). Since CD40L is known to be an inducer of IL-12 from macrophages [43], and plays a role in the induction of TNF-α and NO, the ability of transgenic parasites to stimulate IL-12, TNF-α, and NO production from macrophages was tested. While macrophages stimulated with L929 cells expressing CD40L produced IL-12, infection with transgenic parasites failed to induce macrophage IL-12 production. In addition, macrophages infected with wild-type or transgenic parasites failed to activate TNF-α or NO pro-

duction. It is well known that wild-type parasites actively suppress IL-12 production [111, 114], and we demonstrated that when wild-type parasites were added to CD40L expressing L929 cells, IL-12 induction was abrogated. These data suggest that the supression of IL-12 by *Leishmania* can override the induction signal from CD40L and the suppression likely occurs downstream of CD40. CD40L induced IL-12 is mediated by the transcription factor NF-κB, thus the ability of the parasite to induce NF-κB translocation was examined. Transgenic parasites, similar to wild-type parasites, fail to activate NF-κB translocation. The ability of transgenic parasites to induce the up-regulation of CD40, the receptor for CD40L on the macrophage surface was also examined. Infection with wild-type parasites does not induce CD40 up-regulation. Macrophages infected with transgenic parasites also failed to up-regulate CD40. Although the BALB/c *in vivo* results suggest that parasite derived CD40L plays a role in decreased parasite virulence, no indcations of direct macrophage activation were observed in these *in vitro* experiments. There are several possible explanations for these results. First, transgenic parasites may not have secreted enough CD40L during *in vitro* incubation times for macrophage activation to be detected. Parasites are typically washed prior to infection, which effectively removed any CD40L present in the culture. L929 cells express membrane bound CD40L, and this expression would not be affected by washing of the cells, thus CD40L would be present immediately in this method of stimulation. In contrast, transgenic parasites would have to produce and secrete CD40L before activation effects could be observed, and the 45 minute to 24 hour *in vitro* incubation

times may not have been long enough for parasite derived CD40L to be produced in sufficient quantities to measure macrophage activation. *In vivo*, however, parasites would have time to express and secrete CD40L and induce local macrophage activation over time. This could explain why parasites are able to show an attenuated phenotype *in vivo* while direct activation of macrophages is not observed *in vitro*. Another possibility is that the assays used to detect macrophage activation were not sensitive enough to detect subtle changes induced by transgenic parasites. Real-time PCR for cytokine mRNA from infected macrophages may have been a more sensitive measure than measurement of protein in supernatant by ELISA. In addition, the unknown mechanism of CD40L could have played a role in the inability of *in vitro* assays to detect macrophage activation. If CD40L must be released through lysis of infected macrophages, longer incubation times would be required to allow parasites to replicate enough to induce macrophage lysis. Alternatively, infected macrophages could be lysed and used as a stimulation for other macrophages to determine if CD40L from infected macrophages would induce activation.

It is also possible that transgenic parasites did not directly activate macrophages. It is unlikely that parasite derived CD40L had no effect on macrophages, because such dramatic phenotypes in BALB/c mice are observed, but other cells may be required to assist CD40L macrophage activation. This hypothesis is supported by our studies in SCID mice. If parasite derived CD40L alone could fully activate macrophages to kill parasites, we would have expected to see a phenotype in these mice. SCID mice lack B and T cells, and we observe no difference in phenotype for

transgenic parasite when compared to the wild-type parasite in this model, which would suggest that adaptive immunity is required for parasite derived CD40L to show an effect. In addition, *in vitro* studies using macrophages as antigen presenting cells to DO11.10 T cells showed that macrophages infected with transgenic parasites were able to induce robust T cell proliferation of naïve T cells, while macrophages infected with wild-type organisms were unable to induce T cell proliferation. These data would suggest that parasite derived CD40L may have an effect on improved antigen presentation, or that T cell help is also required for CD40L to be most effective.

Because the phenotype in BALB/c mice was so dramatic, and the *in vitro* data suggest that macrophage interaction with T cells may be required to observe the effects of parasite derived CD40L, we examined *in vivo* cytokine mRNA levels in draining lymph nodes from lesions. We hypothesized that looking directly at infected lymph nodes would provide insight into the observed phenotype that was lacking with the *in vitro* experiments. We found that the wild-type organisms were better inducers of the anti-inflammatory cytokine IL-4 in BALB/c mice. Transgenic organisms induced a significantly lower level of this cytokine. This appeared to be specific to IL-4 because IFN-γ levels were not significantly altered. We also observed a trend toward reduced IL-10 induction in transgenic infected animals. In two of three independent experiments, transgenic infected animals induced significantly lower levels of IL-10, but when the three experiments were compiled, this difference was no longer significant. It is well known that in BALB/c mice, IL-4

and IL-10 are induced following infection with *L. major* [118, 161]. In the resistant C57BL/6 strain of mice, however, IFN-γ is the dominant cytokine that is produced in response to this infection [43, 159, 161]. IL-4 is thought to be a major contributor in the generation of a detrimental Th2 response in BALB/c animals, and treatment with anti-IL-4 antibody early in infection can confer a resistant phenotype on these genetically susceptible animals [118, 162, 163]. In addition, BALB/c mice deficient in the IL-4 gene are resistant to *L. major* infection [121]. Notably, in these models a converse increase in IFN-γ was not observed, which would suggest that for BALB/c animals, interfering with the Th2 response is sufficient to improve disease outcome. The decreased lesion induction by the transgenic parasites correlates with the decrease in IL-4 production that occurs. We conclude that these data taken in the context of the literature provide a plausible explanation for the attenuated phenotype observed in this model of infection.

We also examined disease progression in the resistant C57BL/6 strain of mice. Mice infected with transgenic parasites developed slightly smaller lesions than wild-type infected animals, indicating that these parasites can have a modest effect on disease outcome in this resistant strain, however this phenotype is less dramatic than that observed in the susceptible mouse model. If the mechanism of action of the transgenic parasite is to reduce Th2 cytokines, this is not unexpected, as C57BL/6 mice preferentially mount a Th1 response to Leishmania, and express lower levels of IL-4 than susceptible BALB/c animals.

Many groups have shown that disruption of CD40-CD40L interactions results in susceptibility in the otherwise resistant C57BL/6 mouse [84, 146–148]. To confirm that parasite derived CD40L was active, we infected CD40L$^{-/-}$ and CD40$^{-/-}$ mice on a C57BL/6 background with wild-type or transgenic parasites. In CD40L$^{-/-}$ mice, transgenic parasites caused reduced disease. Transgenic infected mice developed significantly smaller lesions with fewer parasites than mice infected with wild-type organisms. From these data, we conclude that the CD40L produced by the parasite was biologically active, and provided complementation for the CD40L deficiency in these animals. In CD40 deficient mice, the opposite occurred. Transgenic parasites induced progressive disease with lesions at least as large as those observed in mice infected with wild-type parasites. These data suggest that parasite derived CD40L is responsible for the attenuation seen in wild-type C57BL/6 mice and that it signals through host CD40.

We also examined the ability of the transgenic organism to vaccinate C57BL/6 mice. Animals that were vaccinated with wild-type or transgenic organisms were equally protected against re-challenge, indicating that these organisms, although reduced in virulence, retain immunogenicity required to induce an adaptive immune response. Previous studies in our laboratory demonstrated that combining L929 cells expressing CD40L with parasite antigens was able to provide significant protection in the susceptible BALB/c mouse [151]. Also, CD40L trimer DNA has been used successfully to vaccinate BALB/c mice against *L. major* [152]. Therefore, we hypothesized that the CD40LE organism would be able to provide protection in the

BALB/c model. We found that immunization with transgenic *L. major* CD40LE was able to induce partial protection in susceptible BALB/c mice, with vaccinated animals developing slightly, but significantly smaller lesions. This suggests that CD40L expressing organisms were unable to overcome the overwhelming Th2 bias in these animals to provide complete protection against wild-type challenge. The differences seen in our study when compared to those mentioned above may be explained by differences in study design. In our previous study, transfected cells expressing CD40L were mixed with soluble *Leishmania* antigen (SLA) and mice were vaccinated three times prior to challenge with wild-type organisms. The transgenic parasites in the present study make relatively small amounts of CD40L compared to these previous doses. If the expression level of CD40L were increased, we would expect improved protection in BALB/c mice.

Others have attempted to generate transgenic "suicide" *Leishmania* organisms with varying degrees of success. Tobin *et al.* generated *L. major* expressing IFN-γ [164]. These parasites were able to delay lesion development in nude mice, but had no effect on disease outcome in BALB/c animals [164]. Dumas *et al*. developed GM-CSF secreting organisms with more success [160]. These parasites were able to delay lesion development in BALB/c animals and there was evidence that GM-CSF could be combined with traditional treatments to improve disease outcome [160]. However, this group did not examine the protective capabilities of the GM-CSF secreting organism. The *L. major* organisms we have developed expand upon these previous studies generating transgenic *Leishmania* spp. to manipulate disease

outcome. To our knowledge, this is the first report of the development of a transgenic *Leishmania major* to produce a murine co-stimulatory molecule. Our data correlate well with data obtained by Chamekh *et al*. that showed that *Trypanosoma cruzi* transfected with CD40L are able to reduce disease and induce protection in an animal model [165]. Our data confirm the ability of CD40L to act as an adjuvant to generate an attenuated organism that can induce a protective response in the host. Thus this method of generating less virulent organisms using host immune-stimulatory molecules can have merit in a variety of infections.

In summary, previous observations that CD40L could improve disease outcome and provide protection in leishmaniasis led us to develop transgenic organisms secreting the extracellular domain of this protein. We observed that these transgenic organisms are less virulent than wild-type organisms, while retaining immunogenicity to induce a protective response. We conclude that transgenic parasites expressing immune-stimulatory molecules may provide a better alternative to traditional leishmanization resulting in improved vaccines.

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