ABSTRACT

Title of Document:	THE INFLUENCE OF PARASITE-DERIVED CHEMOKINES IN LEISHMANIASIS
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Transgenic chemokine-secreting parasites were generated and used to actively recruit immune cells into *Leishmania* lesions. It was hypothesized that the chemokine induced cell migration would influence the magnitude and character of the immune response and thereby effect the outcome of disease. Two different transgenic chemokine-secreting parasites were engineered. One transgenic parasite secretes murine MCP-1, a CC chemokine primarily responsible for macrophage recruitment. The other transgenic parasite secretes murine IP-10, a CXC chemokine known to attract activated T-cells. Both transgenic parasites transcribed murine chemokine mRNA, translated murine chemokine protein, and infected and replicated inside resting peritoneal macrophages similar to wild-type parasites. However, the two transgenic parasites caused diverse phenotypes in infected mice. The MCP-1 secreting parasites caused little or no detectable lesions, while the IP-10 secreting parasites caused lesions that were significantly larger than the wild-type infected mice. The healing phenotype caused by MCP-1 secreting parasites was further analyzed.

Infection of BALB/c, C57BL/6, or MCP-1 knockout (KO) mice with MCP-1 secreting parasites resulted in minimal lesion development compared to mice infected with wild-type parasites. MCP-1 secreting parasites caused substantial lesions with relatively high numbers of parasites in CCR2 KO mice indicating that the parasites are viable and healthy, and that the lack of lesion development is CCR2- dependent. The enumeration of transgenic MCP-1 parasites in lesions demonstrated a significant reduction in parasite numbers, which coincided with an increase in CCR2-positive macrophage migration on day 7. CCR2-positive macrophages isolated from ears of mice infected with transgenic MCP-1 parasites contained virtually no parasites, whereas infection with wild-type parasites resulted in heavily-infected macrophages in lesions. The lack of parasite survival in mice infected with MCP-1 secreting parasites suggests that parasite-derived MCP-1 is recruiting a population of CCR2-positive macrophages to the lesion that efficiently kill *Leishmania* parasites. In-vitro studies revealed that optimal parasite killing required the recruitment of CCR2-positive macrophages followed by stimulation with a combination of both MCP-1 and IFN-γ. This work suggests that the parasite-derived MCP-1 can recruit a restrictive population of CCR2-positive macrophages into lesions that can be optimally stimulated by MCP-1 and IFN-γ to efficiently kill *Leishmania* parasites.

THE INFLUENCE OF PARASITE-DERIVED CHEMOKINES IN LEISHMANIASIS

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2006

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Dedication

This dissertation is dedicated to my parents, Rachel, and Noah. Not everyone is blessed to be surrounded by such a wonderful supporting cast. Both my parents have instilled in me a great desire to do my best and give 100% at everything that I do in life. I could not imagine having more loving and supportive parents than you. The only person more supportive than my parents during my graduate career was my wife Rachel. She always seemed to understand when I had to work for 18+ hours straight or disappear at night to go work in the lab including the night that she went into labor with Noah (Sorry about that!). She always had much more confidence than me in my abilities and my experimental outcomes. Thank you for everything that you did for me during my 5 years in graduate school. I highly doubt that I could have done it without you! Finally, I would like to dedicate this dissertation to my grandfather, Jesse, who passed away during my third year of graduate school. He always seemed to have the most faith in my abilities while in school and also was an ultimate source of encouragement to be the best at what I do in life. Thank you grandpap for everything!

Acknowledgements

I would like to first thank my graduate advisor David M. Mosser for all of his guidance and support. David has made life in the lab "really interesting" the last five years with his energy and love for science. He always maintained the perfect balance between seriousness and humor, which is a definite necessity in this business. He also always seemed to be looking out for my best interests and future. In addition, I would like to thank the members of my graduate dissertation committee: Dan Stein, Ken Frauwirth, Raymond St. Leger, and Zack Howard. Thank you for all your time, encouragement, and suggestions that helped to strengthen my research project.

I would like to thank all of the members of the "Mosser Lab" both past and present. Each of you has made my time in the lab a very pleasant experience. In particular, I would like to thank Suzanne Miles, Anni Field, and Justin Edwards because they have spent the most time with me in the lab. Suzanne, thank you for helping me to adjust during my first year of graduate school. You always seemed to have a smile on your face and were extremely easy to work with. I would like to thank my fellow "transgenic partner-in-crime" Anni for all of her support over the last 4 years. It was really great to have someone that I could share the high and lows of my research project that actually understood. Justin Edwards, thank you for being the one person that I knew I could count on to joke around each and everyday. I also would like to thank Ron Yahil, Ziyan Yang, and Melba Munoz Roldan for their friendship during the last few years.

I would like to thank Dalit Strauss-Ayali, who really seemed to take me under her wing over the last year and help me with my project. You always seemed to ask me questions that really made me think about my project in different ways. I really feel that

you helped to make me a better scientist. In addition, I would like to thank Xia Zhang and Shanjin Cao for their technical/scientific advice and friendship over the last two years. I really enjoyed my time with the two of you. Finally, I would like to thank Tricia Darrah for all of her suggestions, help (i.e. ear injections), and reagents. Tricia helped me out with my research project in so many ways that I will never be able to repay her. Thank you for everything!

A special thanks to Sam Bish. Sam and I really bounced our frustrations off one another usually every night. Without that time to vent, I don't know if I would have made it through graduate school with much sanity.

Finally, I would like to thank my family, particularly my wife. With all the support that you have given me over the last 5 years, I am proud that you're my wife. Few people are as understanding as you. Thank you for everything!

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LIST OF ABBREVIATIONS

antigen presenting cell	APC
B-cell receptor	BCR
bone marrow derived macrophages	BMDM
bovine serum albumin	BSA
CD4+ T helper cell type 1	Th1
CD4+ T helper cell type 2	Th2
complement receptor	CR
constant region	C
cytoxic T lymphocyte	CTL
dendritic cell	DC
deoxynucleotide triphosphates	dNTPs
deoxyribonucleic acid	DNA
diffuse cutaneous leishmaniasis	DCL
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
forkhead transcription factor	FOXP3
G-protein coupled receptor	GPCR

gene splicing by overlap extension	SOE
green fluorescent protein	GFP
horseradish peroxidase	HRP
immunoglobulin	Ig
induced T regulatory cell	iTreg
inducible NO synthase	iNOS
interleukin	IL
interferon-γ	IFNγ
interferon-γ inducible protein-10	IP-10
knockout	KO
kilodalton	Kda
L. amazonensis MCP-1	La-Tg
L. major IP-10	Lm-TgIP-10
L. major MCP-1	Lm-Tg
langerhans cell	LC
localized cutaneous leishmaniasis	LCL
lipophosphoglycan	LPG
lipopolysaccharide	LPS
lymph node	LN
major histocompatibility complex	MHC
monocyte chemoattractant protein-1	MCP-1
mucocutaneous leishmaniasis	MCL
multiplicities of infection	MOI

natural killer cell	NK
naturally occurring T regulatory cell	nTreg
nitric oxide	NO
nuclear factor kappa B	NFkB
pathogen-associated molecular patterns	PAMP
pattern recognition receptor	PRR
phycoerythrin	PE
polymerase chain reaction	PCR
ribonucleic acid	RNA
ribosomal RNA subunit	SSU
schneider's complete medium	SCM
soluble *Leishmania* antigen	SLA
streptothricin	SAT
T-cell receptor	TCR
T regulatory cell	Treg
Tetramethylrhodamine isothiocyanate	TRITC
toll-like receptor	TLR
tumor necrosis factor	TNF
variable region	V
visceral leishmaniasis	VL
wild-type *L. amazonensis*	La-WT
wild-type *L. major*	Lm-WT
World Health Organization	WHO

CHAPTER 1: INTRODUCTION

Innate Immunity

Overview

The immune system is composed of two branches innate and adaptive immunity. Innate immunity occurred evolutionarily prior to adaptive immunity. Thus, some form of innate immunity exists in all multi-cellular organisms[1].

Both the innate and adaptive components contain different cells, functions, and roles. Cells that make up the innate component of the immune system include neutrophils, macrophages, natural killer (NK) cells, and dendritic cells (DCs). One major difference between innate and adaptive immunity is the receptors and mechanisms that are used to recognize foreign antigens. Innate immune cells have germ-line encoded receptors, meaning that the specificity toward particular microbial products is pre-determined and evolved through natural selection. Innate immune cells are not able to recognize every particular antigen to which the host is exposed, but merely focus on highly conserved antigens from large classes of microorganisms. These highly conserved structures are known as pathogen-associated molecular patterns (PAMPs) and they are recognized by pattern recognition receptors (PRRs) located on the surface of innate immune cells[2]. Examples of PAMPs include bacterial lipopolysaccharide, peptidoglycan, lipotechoic acid, bacterial DNA, and double-stranded RNA. PAMPs share several common features including that they are usually produced by microbial pathogens, but not the host. Second, the PAMPs are usually vital for the microbial pathogen's survival

or pathogenicity. Third, these structures are usually shared by large classes of microbial pathogens[3].

The PRRs that recognize the PAMPs can be divided into three groups based on function: secreted, endocytic, and signaling. Secreted PRRs opsonize foreign pathogens to be recognized by the complement system or taken up by phagocytes. An example includes mannan-binding lectin, which can bind to microbes leading to the activation of the lectin dependent complement pathway. Endocytic pattern recognition receptors are found on the surface of innate immune cells. These receptors bind and take up microbes for them to be broken down inside the phagolysosome and packaged into MHC II receptors for presentation to T-cells. Two examples include the mannose receptor and scavenger receptors. Signaling receptors are PRRs that bind highly conserved microbial patterns to induce cell signaling. The cell signaling leads to the expression of immune-response genes such as inflammatory cytokines and the upregulation of co-stimulatory molecules[3] (Figure 1). An example is Toll-like receptors such as (TLR)4 and TLR2.

Monocytes

Mononuclear phagocytes are a diverse group of cells that express a wide range of phenotypes[4]. Recently, two murine monocyte subsets have been identified. These subsets include inflammatory and resident monocyte subsets. These subsets were identified from experiments involving knock-in mice expressing GFP in place of one allele of the chemokine receptor CX_3CR1[5]. Analysis of subsets that were green fluorescent protein (GFP)-positive compared to those that were GFP-negative revealed that these subsets comprise two major phenotypes that correspond to resident tissue

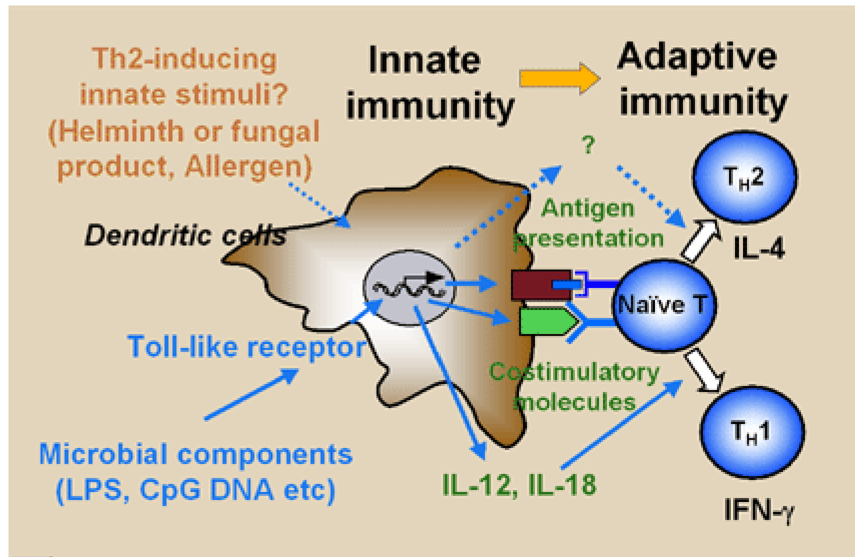


Figure 1. Recognition of microbial product by toll-like receptors activates adaptive immunity. Toll-like receptor ligands such as LPS and CpG DNA activate APCs to produce the pro-inflammatory cytokine IL-12 and upregulate co-stimulatory molecules, which help to activate adaptive immunity. From www.tay.fi/imt/ramet/figure1.html website.

macrophages (CX_3CR1+, CCR2-, Gr-1-) compared to those that migrate into sites of inflammation (CX_3CR1-, CCR2+, Gr-1+). The Gr-1+ monocytes express the chemokine receptor CCR2 and respond to MCP-1.

Recently, CD68+Gr-1+ restrictive macrophages have been identified[6] that are capable of controlling *T. gondi* infection. The CCR2+ Gr-1+ monocytes are believed to become CD68+Gr-1+ restrictive macrophages. During *T. gondi* infection, MCP-1 is produced and is believed to recruit the inflammatory CCR2+Gr-1+ monocytes from the bloodstream and into the site of infection. The inflammatory monocytes become CD68+Gr-1+ restrictive macrophages and control the parasite infection[7].

<u>Macrophages</u>

Macrophages are a heterogenous population of cells with diverse physiologies and distinct immunological functions. One of the first macrophage populations identified is known as the classically activated macrophage. Classically activated macrophages require two signals to become fully activated. They require IFN-γ to prime the macrophages, however, IFN-γ alone will not activate the macrophages[8]. These cells also require a second signal: TNFα or an inducer of TNFα. This usually comes in the form of a TLR agonist, such as LPS, lipotechoic acid, bacterial flagellin, or any microbial product that will ligate a TLR and induce TNFα. Macrophages that receive both signals will produce toxic oxygen radicals (RO) and stimulate the inducible NO synthase (iNOS) gene to produce nitric oxide (NO)[9]. The RO and NO enhance the ability of classically activated macrophages to kill intracellular microorganisms. However, NO production will not occur, nor will the macrophages be classically activated to kill intracellular microorganisms, by merely treating the cells with IFN-γ. In addition, classically

activated macrophages secrete the pro-inflammatory cytokines TNFα, IL-12, IL-1, and IL-6 and also upregulate MHC II molecules and co-stimulatory molecules such as CD86[10].

Two other types of activated macrophages have been identified: alternatively activated and type II-activated macrophages. Alternatively activated macrophages are macrophages that, when treated with IL-4, become activated in a manner different than classically activated macrophages. These cells when activated secrete IL-10 and IL-1R antagonist. In addition, they do not produce NO[10], but instead induce arginase[11], which affects their ability to kill intracellular microorganisms. These cells are poor antigen presenting cells and are believed to play a role in wound repair and angiogenesis. Type II-activated macrophages require two signals like classically activated macrophages. Type II-activated macrophages require a stimulatory signal such as LPS that by itself would lead to high amounts of IL-12 production. In addition to the stimulatory signal, the type II-activated macrophages require Fcγ receptor (FcγR) ligation to suppress IL-12 production and enhance production of IL-10[10]. Immune complexes, which are any antigen-antibody complex, ligate the Fcγ receptor.

Chemotaxis & Chemokines

Chemotaxis is the directed migration of leukocytes toward a chemoattractant gradient. Cells in the immune system use chemotaxis to migrate toward injury or infection. The first step in this directed migration is the exiting of leukocytes from the blood into tissue. This process is called diapedesis. There are several distinct steps taken by a leukocyte to undergo diapedesis and leave the bloodstream toward a chemoattractant gradient located in the tissue. First, the leukocyte loosely adheres to the vascular

endothelium via a family of cell-surface adhesion molecules called selectins. Selectin-mediated adhesion is of low affinity. Therefore, in the presence of the shear force provided by the flow of blood, selectin-mediated adhesion causes the leukocyte to appear to roll on the endothelium. Second, the leukocyte adheres firmly to the endothelium via integrins. Integrins are heterodimeric cell surface receptors, which mediate firm adhesion and allow leukocytes to move out of the blood vessel into tissue through small openings called fenestrations[1] (Figure 2).

The classic chemoattractants include bacterial N-formylated peptides (f-Met-Leu-Phe), complement anaphylotoxins C3a and C5a, and lipid molecules such as leukotriene B_4, and platelet-activating factor. Another category of chemoattractants are the chemotactic cytokines known as chemokines. Chemokines are basic proteins with molecular masses ranging 6 to 14 kilodaltons (kDa) and chemoattractant properties[12]. Most often chemokines are believed to act as monomers[13]. Most chemokines have at least four cysteines with two disulfide bonds. One disulfide bond is located between the first and the third and the other is located between the second and fourth cysteine. These bonds result in an overall structure that involves three β-sheets with short loops in a Greek key formation[12]. Chemokines are divided into four families based on the location of the two cysteines present in N-terminal portion of the chemokine protein sequence. The four families include the CC, CXC, C, and CX_3C chemokines. Both classic chemoattractants and chemokines signal through seven-transmembrane-spanning G-protein coupled receptors (GPCR) (Figure 3). The chemokine receptors include: CXCR1-5, and CCR1-9, CR1, and CX_3CR1[14].

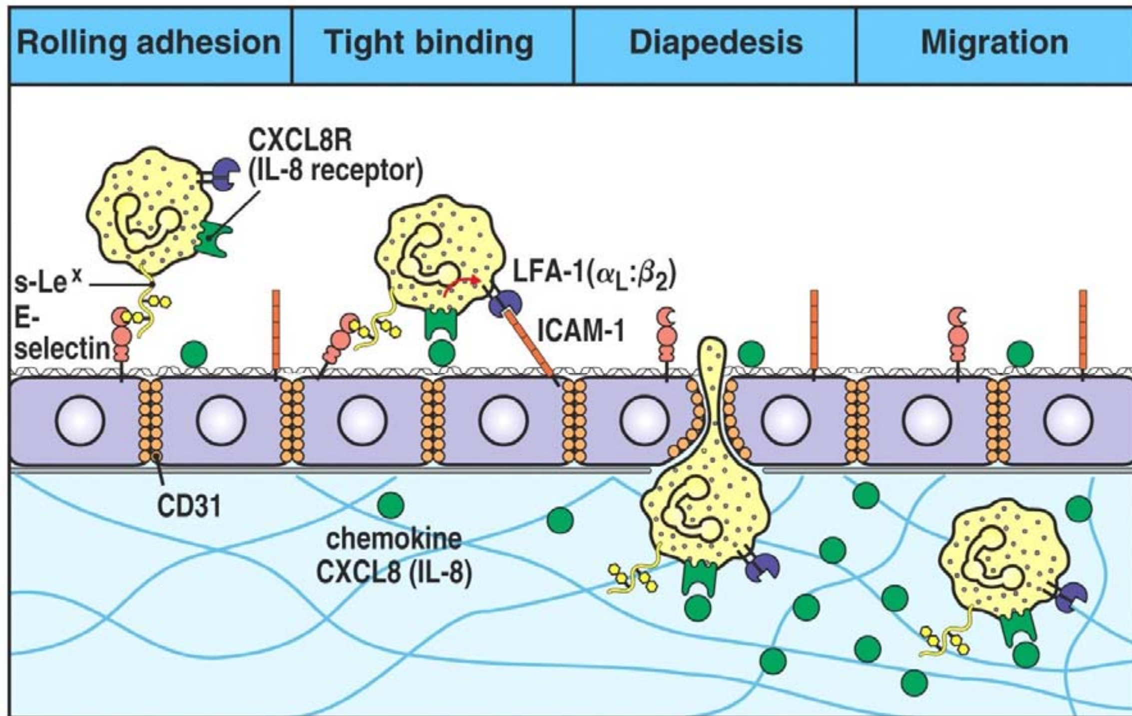


Figure 2. Chemotaxis. Leukocytes go through a series of steps to migrate from the bloodstream toward a chemoattractant located in the tissue. These steps include rolling mediated by selectins, tight binding by integrins, diapedesis, and cell migration toward a chemoattractant. From www.bio.davidson.edu.

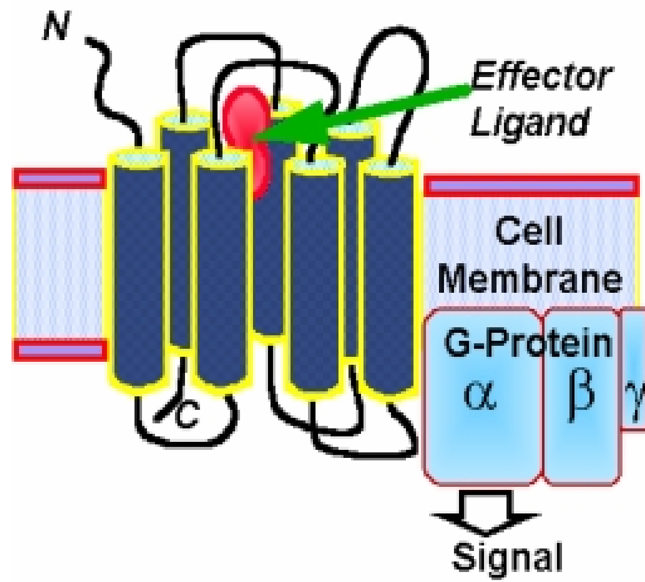


Figure 3. G-protein coupled receptor (GPCR). GPCRs contain seven transmembrane spanning regions with the N-terminal segment facing the outside of the plasma membrane and the C-terminal segment facing the cytoplasmic side. Ligand binding to GPCRs activates a complex of associated G-proteins leading to cell signaling. From www.udel.edu.

Chemokines are major contributors to both innate and adaptive immunity. They are important for innate immunity to attract monocytes, immature DCs, and NK cells to sites of infection as the first line of defense. In addition, chemokines are required in adaptive immunity for lymphocyte development and function by guiding the migration of various types of lymphocytes: immature, mature/naïve, effector, and memory[15]. For example, several chemokines control the migration of lymphocytes within primary lymphoid organs[16]. Finally, chemokines play an important role in bridging innate and adaptive immunity by bringing T-cells, B-cells, and DCs together in the secondary lymphoid organs for activation purposes[17].

IP-10

Interferon-γ inducible protein, also known as IP-10 or CXCL10, is a CXC chemokine known to attract NK cells, CD8+ T-cells, and both CD4+ T-cell subsets[18], but not neutrophils[19]. Structurally, IP-10 exists as three different crystal forms; monomer, dimer, and tetramer.[20] IP-10 binds to and activates the chemokine receptor, CXCR3. IP-10 has long been considered a Th1 chemokine, and it can recruit Th1 cells and bias the immune response toward a Th1 response[21]. This could create a healing phenotype in mice infected with *L. major* IP-10. However, recently there has been major focus on CD4+ CD25+ T-regulatory cells (Treg) that express CXCR3[22]. T-regulatory cells have been shown to suppress the immune response against *Leishmania* through both IL-10 dependent and independent mechanisms, which leads to parasite survival[23]. Therefore, *L. major* IP-10 parasites could possibly recruit T-reg cells and exacerbate the disease.

MCP-1

Monocyte chemotactic protein, also known as CCL2, is a CC chemokine known to attract monocytes, NK cells, both CD4+ T-cell subsets, eosinophils, basophils, and DCs, but not neutrophils[24]. Structurally, MCP-1 can exist in several crystal forms including as a monomer, dimer, and tetramer[25], although it is uncertain at this time which crystal form activates the MCP-1 receptor, CCR2[26]. CCR2 exists in two forms: CCR2A and CCR2B. Both forms have identical 5' untranslated and transmembrane regions, but differ in their carboxyl terminus[27]. Post-translational modification of MCP-1 has been shown to affect its biochemical and biological activities[28;29]. MCP-1 is believed to influence T-helper cell development. In some cases, MCP-1 has been shown by others to stimulate Th2 polarization making MCP-1 KO mice more resistant to intracellular parasite infection involving *Leishmania*[30]. However, CCR2, the receptor for MCP-1, has been shown to stimulate Th1 polarization making CCR2 KO mice susceptible to intracellular parasite infection involving *Leishmania*[31]. There are several explanations for the differences in T-cell polarization. First, CCR2 has high affinity ligands (MCP-1 – MCP-5) in mice and four in humans, and these could possibly exert different biological effects on CCR2. Secondly, there could be a receptor for MCP-1 that has yet to be discovered. MCP-1 binding to this unknown receptor could promote Th2 biasing, whereas binding to CCR2 could drive a Th1 response. Activation of this unknown receptor would override the CCR2 activation when both receptors are present[26]. MCP-1 has received the majority of its attention from its ability to recruit monocytes. For example, MCP-1 expression occurs in a variety of disease states that involve monocyte infiltration such as atherosclerosis and multiple sclerosis[26]. A transgenic mouse was

developed that overexpresses MCP-1. These mice express very high levels of MCP-1 in multiple organs and serum, and showed no evidence of monocyte infiltration. These mice were more susceptible to infection with the intracellular pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis,*[32] and this susceptibility is believed to be a result of the high levels of MCP-1 that desensitized the CCR2 receptor, thus, preventing monocyte infiltration into the sites of infection.

Adaptive Immunity

Overview

Innate immunity can not always completely eliminate certain microorganisms. Therefore, the adaptive component of the immune system has evolved to provide a more versatile line of defense against infectious pathogens. The cells that make up adaptive immunity are known as lymphocytes. There are two types of lymphocytes, B-cells and T-cells, that have completely different functions and eliminate different types of microorganisms.

There are several properties that distinguish adaptive immunity from innate immunity. These properties include specificity, diversity, regulation, memory, and non-reactivity to self. Specificity refers to an immune response to one particular antigen. Lymphocytes possess rearranged receptors that will specifically bind one particular antigen. When the lymphocyte receptor encounters its antigen than the receptor binds the antigen, leading to cell proliferation. This will result in the establishment of a lymphocyte clone with the specificity for a single antigen. Diversity refers to the vast repertoire of receptor possibilities that the lymphocytes can possess. By recombining

their receptors, lymphocytes have the capacity to recognize $10^9 - 10^{11}$ antigen specific epitopes. Regulation refers to the adaptive immune response returning to homeostasis after the host encounters a foreign entity and adaptive immunity eliminates this entity. Memory refers to the established protection that a host develops after adaptive immunity responds and eliminates an infectious pathogen. Non-reactivity to self refers to the fact that lymphocytes are part of adaptive immunity and do not recognize self-antigens so as to prevent attacking the host. Auto-immunity occurs in those cases where lymphocytes recognize self-antigens[33].

The adaptive immune response may be divided into three phases—the recognition of antigen, activation of lymphocyte, and effector phases. Each lymphocyte has a receptor specific for an individual antigen. During the recognition phase, the lymphocyte locates and binds to a specific antigen that it recognizes and thus the lymphocyte undergoes clonal expansion. Clonal expansion results in lymphocyte proliferation so that there is a large lymphocyte clone with receptors of the same specificity. After the lymphocyte recognizes its particular antigen, the activation phase begins. Activation requires two distinct signals. The first signal is the antigen and the second signal is either a microbial product or component of the innate immune response. This is the two-signal hypothesis for lymphocyte activation, which ensures that the adaptive immune response is specific and also necessary. The activation phase involves the synthesis of new proteins, cell proliferation, differentiation into effector cells, or differentiation into memory cells. Finally, the effector phase usually results in the elimination of the antigens[33].

There are two types of adaptive immune responses—humoral immunity and cell-mediated immunity. These immune responses differ by the cells involved and by the different types of microorganisms that each immune response responds. Humoral immunity involves B-lymphocytes and antibody production and is necessary for defending against extracellular toxins and extracellular pathogens such as extracellular bacteria and worms. Cell-mediated immunity involves T-lymphocytes and is required for eliminating intracellular pathogens.

<u>T-cells</u>

T-lymphocytes originate in the bone marrow, but migrate to the thymus for maturation. It is during the maturation period that the T-cell receptor (TCR) on T-cells undergoes gene rearrangement, positive selection, and negative selection. During positive selection, T-lymphocyte precursors with antigen receptors that bind some self-ligand with low affinity are selected to survive[34]. During negative selection, T-lymphocytes whose antigen receptors bind self-antigen with high affinity are selected for cell death[35]. The TCR consists of two polypeptides called TCRα and TCRβ chains linked by a disulfide bond. This receptor allows the T-cells to have a single specificity for one particular antigen. The TCR is able to recognize antigens when they are degraded, processed, and presented in MHC molecules present on the surface of professional antigen presenting cells (APCs)[36]. APCs include dendritic cells (DCs), macrophages, and B-cells. The APCs present antigens to T-cells using MHC receptor molecules. Exogenous antigens taken up by APCs are degraded in the phagolysosome before being packaged into MHC II molecules to be presented to T-cells. Endogenous

antigens are packaged into MHC I molecules in the endoplasmic reticulum (ER) and the MHC I molecules are sent to the cell surface for presentation to T-cells.

There are two types of T-cells that are divided based on surface markers and effector functions. The two T-cell types can also be distinguished based on the cell surface proteins—CD4 or CD8. CD4+ and CD8+ T-cells differ in the class of MHC molecule that they recognize. CD4 binds to the MHC II molecule and CD8 binds to MHC I molecules. CD8+ cytotoxic T-cells (CTLs) are specialized to kill any cell that expresses an MHC I molecule with their peptide loaded inside of it. The main function of the CD4+ T-cells is to recognize their particular peptide in MHC II molecules on the surface of APCs and activate these cells. For example, CD4+ T-cells recognize peptides bound to MHC II molecules on B-cells and then stimulate the B-cells to produce antibody. CD4+ T-cells can also recognize peptides bound to MHC II molecules on macrophages and activate the macrophages to destroy intracellular pathogens present inside the macrophage. CD4+ T-cells can differentiate into two different T-cell subsets, Th1 and Th2 cells depending on the cytokine environment. Th1 cells produce proinflammatory cytokines such as IFN-γ and TNFα, which are required for macrophage activation and intracellular killing in cell-mediated immunity. Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-5, and IL-13 and are required for B-cell activation and antibody production in humoral responses[33].

CD4+ regulatory T-cells (Treg)

The immune system has established a system of checks and balances to be able to both protect against infectious agents and maintain tolerance to autoantigens. Therefore, a network of CD4+ regulatory T-cells (Treg) is present to maintain the balance between

these two very different immunological outcomes. CD4+ Treg cells can be divided into two categories: induced (iTreg) and naturally occurring (nTreg). iTreg cells produce certain signature cytokines as a result of localized environment and these signature cytokines serve as the main suppressive activity. Examples of iTregs include IFN-γ producing Th1 cells, IL-4 producing Th2 cells, IL-10 producing Tr1 cells, and TGF-β secreting Th3 cells. nTregs develop normally through T-cell maturation in the thymus and represent 1-10% of the total CD4+ T-cells. nTregs constitutively express CD25 and Forkhead transcription factor (FOXP3)[37]. These cells play a major role preventing self-reactivity and help to establish tolerance when necessary. Recently, nTregs have been shown to express the chemokine receptor CXCR3, the receptor for the CXC chemokine, IP-10[22].

Cell-mediated Immunity & Macrophage Activation

Many microorganisms live inside host cells where it is impossible for humoral immunity (i.e. antibodies) to reach them. Intracellular microorganisms replicate inside cells, particularly macrophages, because of the protection that it offers from the immune system. These intracellular pathogens can survive inside macrophages by suppressing the innate killing mechanisms. Cell-mediated immunity has evolved to help deal with this problem through T-cell mediated immune responses. T-cells in the presence of the pro-inflammatory cytokine IL-12 become Th1 cells that secrete IFN-γ. IFN-γ along with a second signal, usually a TLR agonist, will activate macrophages infected with intracellular pathogens. Activated macrophages make oxygen radicals and NO to help enhance parasite killing. In addition, natural killer (NK) cells are a source of IFN-γ and can help activate macrophages infected with intracellular microorganisms[33].

Leishmania

Overview

William Leishman and James Donovan each individually discovered parasites of the genus *Leishmania* between 1900-1903[38]. These single-celled intracellular parasites are a member of the order *Kinetoplastida* and family *Trypanosomatidae*. Humans are infected by about 21 of 30 *Leishmani*a species that infect mammals. A few of these species include *L. major*, *L. tropica*, the *L. mexicana* complex (*L. mexicana*, *L. amazonensis*, and *L. venezuelensis*) and the *L. donovani* complex (*L. donovani*, *L. infantum*, and *L. chagasi*)[39;40]. These species can be best distinguished through DNA probe analysis, PCR analysis, or ELISA.

Life cycle

Leishmania are dimorphic organisms that exist in two developmental forms: the promastigote and amastigote. During the promastigote phase the parasite is long, slender, and flagellated. The parasite usually exists in its promastigote form when present inside the sandfly gut. During the amastigote form the parasite is oval-shaped and non-motile. The parasite exists in this form when present inside macrophages. *Leishmania* are transferred via sandfly vectors of the genus *Phlebotomus* and *Lutzomyia*[41;42]. Specifically female sandflies, which require blood for egg maturation, transfer *Leishmania* during blood meals. When an infected sandfly takes a blood meal it transfers *Leishmania* in its promastigote form to the site of the blood meal. The promastigotes are taken up by macrophages, which attempt to destroy the intracellular parasites using the acidic environment of the phagolysosome and various degradative enzymes, such as acid hydrolases and proteases[42]. Despite the harsh conditions of the phagolysosome, the

promastigote differentiates into its amastigote form in the acidic environment leading to parasite survival[43]. Amastigotes replicate, eventually leading to macrophage cell lysis and amastigote dispersal to neighboring cells. The parasite life cycle continues when an uninfected sandfly takes a blood meal and ingests amastigotes, which transform into promastigotes inside the sandfly gut[42] (Figure 4).

Disease manifestation

Leishmaniasis is endemic in 88 countries located throughout 5 continents including North and South America, Asia, Africa, and Europe. The World Health Organization (WHO) believes that 12 million humans are infected by *Leishmania* worldwide, including people with no distinct symptoms[44]. There are three main clinical categories of leishmaniasis: cutaneous, mucocutaneous, and visceral. Cutaneous leishmaniasis exists in two forms, localized cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL). In LCL, skin lesions appear at the site of the sandfly bite. The lesions ulcerate, but eventually heal leaving a scar. *L. major*, *L. amazonensis*, and *L. tropica* are known to cause LCL. In DCL, skin lesions originate the same way that LCL lesions develop; however, the DCL lesion eventually spreads to other areas on the skin and will not heal without treatment. *L. tropica* is most often responsible for DCL lesions, although *L. amazonensis* and *L. mexicana* are found to cause the disease. The incubation period for cutaneous leishmaniasis can range from 1-2 weeks to 1-2 months[45]. Ninety-percent of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria[46].

Mucocutaneous leishmaniasis (MCL) initially resembles the cutaneous form of the disease, except that lesions eventually spread to cartilaginous areas in cavities of the

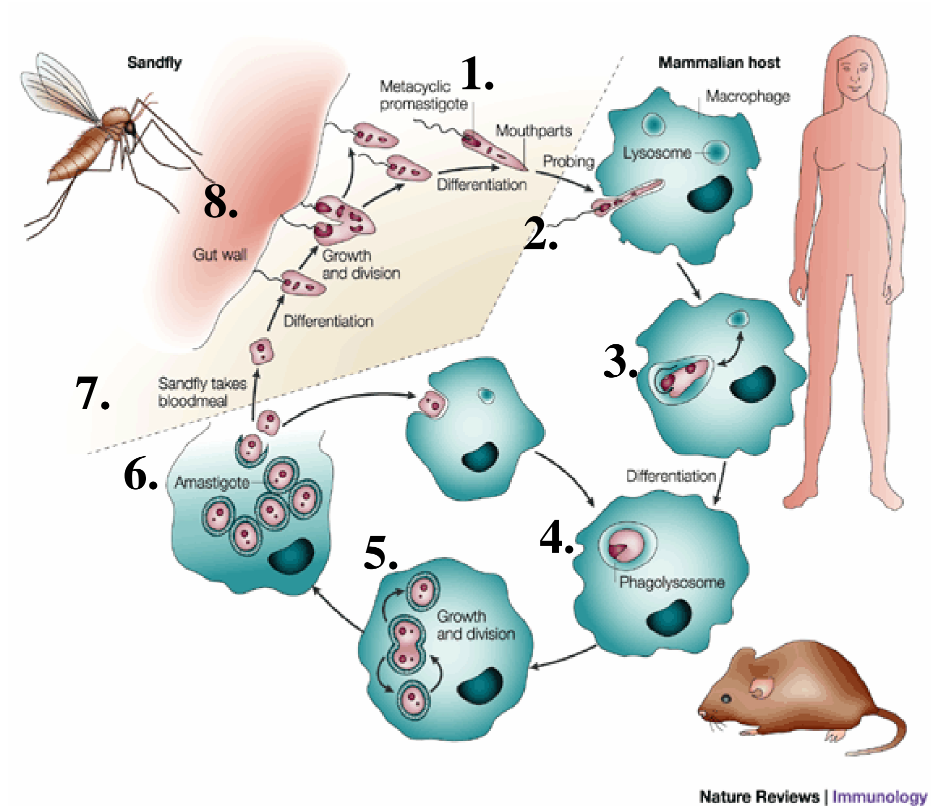


Figure 4. ***Leishmania*** **life cycle.** (1) *Leishmania* infected, female sandfly takes a blood meal and transfers metacyclic promastigotes to mammalian host. (2) Macrophage takes up promastigote. (3) Promastigote is exposed to macrophage phagolysosome. (4) Promastigote transforms to amastigote form permitting survival in phagolysosome. (5) Amastigote replicates. (6) Amastigote replication leads to macrophage lysis. (7) Uninfected sandfly takes a blood meal and ingests amastigotes. (8) In the sandfly gut, each amastigote differentiates into promastigote form preparing for eventual transfer. From Nature reviews[42].

nose, mouth, and throat. Mucocutaneous lesions are destructive and disfiguring and can result in extensive scarring. The mucocutaneous manifestations of MCL may be due to the host immune response to cross-reactive parasite antigens[47]. *L. braziliensis* is known to cause mucocutaneous leishmaniasis[45]. Ninety-percent of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil, and Peru[46].

Visceral leishmaniasis (VL) (also known as kala-azar) is an insidious form of the disease. Many patients with visceral disease fail to realize that they are infected, and the disease can be fatal if it goes untreated. Symptoms of VL include fever, weight loss, splenomegaly, hepatomegaly, and anemia. *L. donovani*, *L. chagasi*, and *L. infantum* are known to cause visceral leishmaniasis. The incubation period of visceral leishmaniasis is 1-3 months[45]. Ninety-percent of visceral leishmaniasis cases occur in Bangladesh, Brazil, India, Nepal, and Sudan[46].

Diagnosis & Treatment

Leishmania infection should be suspected of anyone that lives or has visited a *Leishmania* endemic area and develops a suspicious-looking lesion or experiences visceral-like symptoms, which include anemia, splenomegaly, hepatomegaly, fever, and weight loss. Still it is difficult to diagnose leishmaniasis because the cutaneous lesions often resemble leprosy or fungal infections, while disease symptoms of visceral leishmaniasis can be similar to typhoid, malaria, syphilis, and tuberculosis[44].

The best way to identify a cutaneous infection is to take a biopsy from the edge of the lesion and analyze it microscopically for the parasite. In addition, PCR-based methods that amplify mitochondrial (kinetoplast) DNA common to all *Leishmania* species and serodiagnosis can be used to properly diagnose leishmaniasis.

There are several current therapies used to treat leishmaniasis, but no vaccine. The most commonly used treatment for VL is a 20-28 day intravenous regimen of pentavalent antimonials. Unfortunately, the treatment is not without side-effects including nausea, pain, anorexia, myalgia, arthralgia, headache, and malaise. Two other drugs that are more expensive include amphotericin B, a 4-8 week intravenous regimen and pentamidine, a 35-63 day intravenous regimen. A new therapy that was recently introduced in June 2002 is miltefosin, a 28-day regimen that is the most affordable of all the *Leishmania* therapies[46;48].

Leishmania molecular genetics

The *Leishmania* nuclear genome is approximately 35.5 Mb and organized into 34-36 chromosomes depending on the species. The genome contains 58-60% GC content and 30% is composed of repeat elements. None of the *Leishmania* protein-encoding genes contain introns. Most, if not all, of these genes are initially transcribed into large polycistronic precursor RNAs of 60 kb (kilobase) or more and are cleaved into monocistronic mRNAs by two intergenic RNA cleavage reactions. The two reactions include trans-splicing of a 39-nt spliced leader sequence (also called a mini-exon) that attaches to the 5' ends of all monocistronic mRNAs and 3' cleavage/polyadenylation at the 3' ends of mRNAs. Trans-splicing and polyadenylation are coupled reactions that allow the trans-splicing reaction on a downstream gene to be coupled with the polyadenylation reaction of the closest upstream gene[49;50].

Regulation of gene expression in *Leishmania* is primarily posttranscriptional, unlike most prokaryotic and eukaryotic organisms, which primarily regulate genes at the

transcriptional level. *Leishmania* gene expression is controlled by trans-RNA splicing, polyadenylation, mRNA half-lives, protein synthesis, and protein stabilities[49;50].

Leishmania lack promoters for RNA polymerase II, the enzyme that transcribes most protein-encoding genes. Transcription in these organisms is, however, strand specific and is performed by RNA polymerases with α-amanitin-sensitivities similar to mammalian RNA polymerases I, II, III. Promoters for α-amanitin-resistant RNA polymerase I activity have been readily detected, as expected, in front of ribosomal RNA genes. A typical α-amanitin sensitive RNA polymerase II activity has been shown to transcribe most protein-encoding genes[49;50].

Leishmania evasion of host response

Leishmania have been shown to primarily infect macrophages[51;52] and DCs[53]. In order to survive long-term, *Leishmania* must evade cellular microbicidal mechanisms and gain entry into an intracellular environment provided primarily by host macrophages.

Leishmania uses two major surface molecules for entry into cells. Lipophosphoglycan (LPG), the major cell surface glycoconjugate on *Leishmania* promastigotes, plays an active role in protecting parasites from the harsh phagolysosome environment[54] and is a ligand for several macrophage receptors including the mannose receptor, and the β-glucan receptor[52]. The major surface protein on *Leishmania* promastigotes, gp63, is a 63 kDa glycoprotein required for cellular entry. The gp63 proteolytic activity prevents complement mediated lysis via membrane attack complex[55]. Promastigotes become opsonized with complement proteins and enter macrophages via complement receptors (CR)1 and CR3[56]. In addition, gp63 may be able to bind directly to Mac-1[57] and fibronectin receptors[58]. *Leishmania* does not enter DCs as efficiently as

macrophages. It has been reported that the number of parasites phagocytized by macrophages is higher possibly due to fact that the macrophages have higher levels of CR3 on their surface than the splenic DCs. This evidence suggests that macrophages may be the preferential target cell for *Leishmania*[59].

Macrophages and DCs serve as APCs for naïve T-cells and thus regulate cell-mediated immunity. However, *Leishmania*-infected macrophages generally lack the ability to induce primary stimulation of specific naïve T-cells. This lack of stimulation may be due to an active suppression of APC functions by the parasite. Parasite suppression of MHC II synthesis[60], parasite inhibition of antigen processing, and MHC II loading with immunogenic peptides[61;62] along with deficient expression of the co-stimulatory molecule CD80 have been reported[63]. In addition, *Leishmania* fails to induce innate immune responses in infected macrophages. Parasite infection fails to activate NFκB (Mosser Lab, unpublished observations), which is critical for inducing proinflammatory cytokines such as IL-12 and TNFα and for inducing co-stimulatory molecules on APCs[64]. *Leishmania* infected macrophages not only fail to induce IL-12, the main inducer of IFN-γ, Th1 differentiation, and *Leishmania* resistance[65], but actively downregulate IL-12 transcription[66]. Only *Leishmania*-infected macrophages that are activated by TNFα and IFN-γ to create nitric oxide (NO) can kill intracellular parasites.

Despite the inhibitory effect that *Leishmania* has on macrophage IL-12 production, in vitro studies have shown that *Leishmania*-infected DCs are still capable of producing IL-12[53;67] suggesting that macrophages are exploited to a higher degree than DCs. This may explain the relatively high levels of IFN-γ production observed in human cutaneous leishmaniasis. The DC mechanism for processing the parasite antigen and

loading it into the DC MHC II molecules is unknown. It is also unknown whether the parasite is capable of disrupting DC activation, although our lab has developed preliminary evidence that is consistent with *Leishmania*-infected DCs failing to induce co-stimulatory molecules on the surface of infected DCs (Mosser Lab, unpublished observations).

IP-10 in leishmaniasis

IP-10 has been shown to be important in the killing of intracellular parasites by virtue of its ability to recruit Th1 effector cells, CD8-positvie T-cells and NK cells[68]. All these cells produce IFN-γ that is required for macrophage activation. Despite IP-10's role in Th1 protection, it has been shown that with high concentrations of exogenous recombinant IP-10 *Leishmania* lesions can actually become larger[69]. Our work confirms this by showing that IP-10 secreting transgenic parasites cause larger lesions. This unexpected observation can be explained by the work of Bonecchi who showed that increasing concentrations of IP-10 led to diminished Th1 migration[70]. In addition, others have shown that patients with visceral leishmaniasis (VL) have high levels of IP-10 in their plasma[71], again suggesting that IP-10 may play a role in exacerbating the disease.

nTreg's role in leishmaniasis

Recently, it was discovered that CD4+ CD25+ T-regulatory cells can express CXCR3, the receptor for IP-10. Others have shown that the survival of *Leishmania* parasites in the skin of resistant mice infected with wild-type *L. major* parasites is controlled by a population of CD4+ CD25+ FOXP3+ regulatory T-cells. These natural regulatory T-cells downregulate parasite-specific immunity, thus preventing CD4+

CD25- T-cell mediated elimination of parasites from infected skin. This is done in both a IL-10 dependent and IL-10 independent manner[23]. Therefore, the parasites are able to persist at small base-line numbers and maintain a sterile cure[72]. It is possible that high levels of IP-10 can play a role in recruiting the natural T-regulatory cells to the *Leishmania* lesion in order to help maintain disease.

MCP-1 in leishmaniasis

MCP-1 is a CC chemokine known to attract monocytes, DCs, NK cells, and memory T-lymphocytes[14;26]. Both human monocytes[73] and bone marrow derived macrophages (BMDM)[74] have been shown to produce MCP-1 one hour after *L. major* infection, but the MCP-1 returns to uninduced levels by 4 hours in the BMDM and 12 hours in human monocytes. In human leishmaniasis, there is evidence of elevated MCP-1 levels in localized self-healing cutaneous lesions (LCL). In contrast, there is no detectible MCP-1 in diffuse, non-healing cutaneous lesions[75-77]. CCR2, the receptor that binds MCP-1[14;27], has been shown to be vital for host defense to a number of pathogens[7;78-84]. Likewise, several groups have suggested that MCP-1/CCR2 may play a variety of roles in host defense against *Leishmania*[82]. It has been reported that high doses of MCP-1 can directly activate anti-*Leishmania* macrophage killing mechanisms either directly[85], by inducing reactive oxygen intermediates[86], or via nitric oxide production[87-89]. In addition, MCP-1 has been reported to activate NK cells[69], which could indirectly activate macrophages via IFN-γ production. Mice normally resistant to *L. major* infection become susceptible when lacking the CCR2 receptor[82]. Paradoxically, MCP-1 KO mice have been shown to develop resistance to *Leishmania* implicating a possible role for MCP-1 in Th2 biasing[30]. There are several possible explanations for the

conflicting phenotypes between *Leishmania* infected MCP-1 versus CCR2 KO mice. These explanations were previously mentioned in the section on MCP-1.

As mentioned above, previous studies by others have demonstrated the presence of MCP-1 during self-healing *Leishmania* infections, but they did not establish the cause-and-effect relationship between MCP-1 production, CCR2-positive cellular recruitment, and disease resolution. To recruit CCR2-positive cells into lesions, we developed transgenic *Leishmania* that secrete the murine CC chemokine MCP-1. Thus, the transgenic parasites would secrete MCP-1 and thereby take an active role in manipulating immune cell migration into lesions. We hypothesized that the MCP-1-induced cell migration could influence the magnitude and character of the immune response[90] and thereby affect the outcome of disease.

CHAPTER 2: MATERIALS AND METHODS

Animal Studies

These studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. BALB/c and C57BL/6 mice were purchased from Taconic (Rockville, MD). CCR2 KO mice on a C57BL/6 background, RAG KO mice on a C57BL/6 background, and C5 deficient mice were purchased from Jackson Laboratories (Bar Harbor, Maine). MCP-1 KO mice[91] on a BALB/c background were obtained from Dr. Barrett Rollins (Harvard Medical School, Boston, MA).

Cell/parasite culture

D10 media, which consists of Dulbecco's modified eagle's medium (DMEM) (Fisher Scientific, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/ml penicillin (Fisher Scientific), 100 µg/ml streptomycin (Fisher Scientific), and 2mM glutamine (Fisher Scientific), was used for culturing peritoneal and bone-marrow derived macrophages. Both wild-type and transgenic parasites were grown in 50:50 media [50% Schneider's insect medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine and 50% M199 media (Invitrogen, Rockville, MD)]. Transgenic parasites were grown in the presence of 100 µg/ml nourseothricin (SAT) (WERNER BioAgents, Germany). *Leishmania amazonensis* axenic amastigotes were cultured as previously described[92]. Blood agar plates[93] were prepared as previously described containing SAT. A concentration of 100 µg/ml SAT was used to select for

transgenic *L. major* parasites, while 50 µg/ml SAT was used to select for transgenic *L. amazonensis* parasites.

<u>Parasites, infection, and parasite quantitation</u>

Lesion-derived wild-type and transgenic *L. major* (WHO MHOM/IL/80/Friedlin) and *L. amazonensis* (WHOM/BR/75/Josefa) were isolated from infected mice as previously described[56]. Mice were injected in the right hind footpad with $1x10^5$ or $5x10^6$ wild-type or transgenic stationary *L. major* promastigotes depending on the experiment. Lesion size was determined using a caliper to measure the thickness of the infected footpad and subtracting the thickness of the contralateral uninfected footpad as described previously[94]. For ear infections, mice were injected in the right and left ears with $5x10^4$ wild-type or transgenic *L. major* parasites. Ear lesion progression was monitored by measuring the diameter of the lesion using an Absolute Digimatic Caliper (Mitutoyo, Ontario, Canada) as previously described[72]. For protection experiments, C57BL/6 mice were infected with $5x10^4$ wild-type *L. major* or transgenic *L. major* MCP-1 parasites in the right footpad. After 5 weeks, these mice along with non-infected (unprotected) mice were infected in both ears with $1x10^5$ wild-type *L. major* parasites and monitored for lesion development over the next 5 weeks. For all the in-vivo experiments, error bars represent the standard error of the mean of three separate experiments done with a minimum of five mice per group.

Parasite burdens were determined by serial dilution of single cell suspensions made from excised footpads, ears, lymph nodes, or spleens as previously described[94].

Gene Splicing by Overlapping Extension (SOE) PCR

Two different PCR products were created in separate PCR reactions and used to generate a hybrid SOE PCR product[95;96] that begins with a 5'-*SmaI* restriction site followed by the *L. major* gp63 signal sequence and the entire murine MCP-1/JE gene and ends with a 3'-*SmaI* site.

The first PCR reaction generated a 148 basepair (bp) PCR product that contained a 5'-*SmaI* restriction site followed by the *L. major* gp63 signal sequence and a short sequence corresponding to the murine MCP-1/JE gene. This fragment was created using the following primers: sense 5'-TATCCCGGGATGTCCGTCGACAGCAG-3' and antisense 5'-GCATGACAGGGACCTGAGCGGCGTGTGCCCACGC-3'. These primers were used along with a plasmid template that contained the entire *L. major* gp63 gene (accession #: Y00647). After the gp63 PCR #1 product was generated, it was gel purified using a gel extraction kit (Qiagen, Valencia, CA). The second PCR reaction generated a 450 bp PCR product that contains the murine MCP-1/JE gene, followed by a 3'-*SmaI* restriction site, using the following primers: sense 5'-GCTCAGGTCCCTGTCATGCTTCTGGGC-3' and antisense 5'-TACCCGGGGTTCACTGTCACACTGGTCACTCCTAC-3'. These primers were used along with the template pORF-mMCP-1 (Invivogen, San Diego, CA) to create MCP-1 PCR #2.

The hybrid PCR product MCP-1SOE was created using gp63 PCR #1 and MCP-1 PCR #2 as templates. A series of short melting (95 °C) and extension (72 °C) steps were performed prior to running the full PCR cycle. The two templates were added alone prior to the addition of Taq polymerase, primers, and deoxynucleotide triphosphates (dNTPs)

in order to generate a few hybrid templates before starting the true PCR cycling. The PCR product was purified using a PCR purification kit (Qiagen) and ligated into the TA cloning vector, pCRII (Invitrogen).

The hybrid PCR product was excised from the TA cloning vector with *SmaI*, gel purified, and ligated into the multiple cloning site of the *Leishmania* expression plasmid, pIR1SAT, which was generously provided by Dr. Steven Beverley (Washington University, St. Louis, MO). The ligated expression plasmid, pIR1SAT-MCP-1/JE was transformed into Max Efficiency DH10B competent cells (Invitrogen) by Heat-shock method. The pIR1SAT-MCP1/JE plasmid was isolated from DH10B cells using a plasmid maxi kit (Qiagen), digested with *SwaI*, and transfected into *Leishmania* parasites to permit integration into the parasite genome. The same approach was taken to create pIR1SAT-IP-10 and integrate it into the *Leishmania* genome.

<u>*Transfection of Leishmania*</u>

L. major or *L. amazonensis* parasites ($1x10^8$ parasites) were resuspended in 400 µl of electroporation buffer [21 mM HEPES (pH 7.5), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, and 6 mM glucose]. This suspension was mixed with 5 µg of linearized pIR1SAT-MCP-1/JE, added to a 0.4 cm Gene Pulser cuvette (BIORAD, Hercules, CA), and electroporated (0.5 kV, 0.5 µFd) using BIORAD's Gene Pulser II. The cuvette was put on ice for 10 minutes. Electroporated parasites were added to blood agar plates containing SAT.

RNA isolation

RNA was isolated from $1x10^6$ wild-type or transgenic *L. major* or *L. amazonensis* parasites during either promastigote or axenic development using Trizol RNA prep (Invitrogen). The RNA was converted to cDNA using the manufacturer's protocol. Murine MCP-1/JE was amplified from the cDNA samples using the following primers: sense 5'-GCTCAGGTCCCTGTCATGCTTCTGGG-3' and antisense 5'-GTTCACTGTCACACTGGTCACTCCTAC. gp63 was amplified using the following primers: sense 5'-ATCCTCACCGACGAGAAGCGCGAC-3' and antisense 5'-ACGGAGGCGACGTACAACACGAAG-3'.

Transgenic MCP-1 detection

Costar high-binding ELISA plates (Fisher Scientific) were coated with monoclonal goat anti-mouse MCP-1/JE antibody (capture antibody) from the DuoSet MCP-1/JE ELISA kit (R&D systems, Minneapolis, MN). Wild-type and transgenic parasites ($5x10^6$) were added to the ELISA plate wells for 24 hours. The following day, the parasites were washed away and the MCP-1 ELISA was completed according to the manufacturer's protocol using biotinylated anti-mouse MCP-1/JE detection antibody, streptavidin-horseradish peroxidase (HRP), and HRP substrate.

MTT assay

Parasite viability and metabolism was measured using an MTT assay as previously described[97]. Briefly, wild-type and transgenic parasites ($1x10^6$) were grown in 50:50 media. During the first seven days, 100 µl of the parasite culture was removed and added to a 96-well plate in triplicate. 20 µl of MTT [3-(4,5-dimethylthiazolyl-2)-2,5

diphenyltetrazolium bromide] (Sigma-Aldrich)(5mg/mL) was added to the 100 μl parasite culture for 2 hours at room temperature followed by 100 μl of DMSO (Sigma-Aldrich) for an additional 1 hour. The absorbance was then measured at 550 nm.

Leishmania survival assay and staining

Peritoneal macrophages were isolated from mouse peritoneal cavities using cold PBS as previously described[56]. Macrophages ($1x10^5$) were added to coverslips in 100 μl bubbles for 2 hours to allow macrophage attachment. D10 media was added and the cells were rested for 2 hours. Prior to infection, parasites were opsonized with 10% serum from C5 deficient mice for 20 minutes at room temperature. The peritoneal macrophages were then infected at a multiplicity of infection (MOI) of 10:1 using opsonized wild-type or transgenic parasites for 2 hours. Cells were washed and fixed with methanol for 10 minutes immediately after infection or after further 72 hours of incubation. The experiment was also performed using lesion-derived amastigotes from either wild-type or transgenic infected CCR2 KO mice. The amastigotes were used at an MOI of 10:1 and added for 1 hour before washing. Cells were fixed immediately after 1 hour of infection or after further 72 hours of incubation. For immunofluoresence visualization, glass coverslips were blocked with PBS containing 5% FBS. Mouse anti-*Leishmania* serum (1:250) was used to stain *Leishmania* amastigotes for 1 hr at 4 °C. The coverslips were then washed twice and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H+L) (1:100) was added for 30 minutes. After the coverslips were washed again, they were treated with a 1:1000 dilution of 1 mg/ml propidium iodide for 2 minutes. Coverslips were then mounted to slides using MOWIOL (EMD Biosciences, San Diego, CA).

Intracellular staining of parasite-derived MCP-1

Bone-marrow derived macrophages (BMDM) were prepared from MCP-1 KO mice as previously described[98]. Macrophages were plated, infected for 48 hours with C5d serum opsonized wild-type or transgenic MCP-1 parasites, and fixed as described above. Coverslips were blocked with 6% bovine serum albumin (BSA) for 1 hour prior to staining. Parasite-derived MCP-1 was stained using 100 ng/mL goat anti-mouse MCP-1 antibody (R&D systems) for 45 minutes at 4 ºC followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) (1:100) for 30 minutes at 4 ºC. The parasites were then stained as described above using mouse anti-*Leishmania* serum for 45 minutes along with FITC-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch) (1:100) for 30 minutes at 4 ºC. The macrophage nucleus was stained with Hoechst stain (1:4000) for 2 minutes. Coverslips were mounted as described above.

Thymidine incorporation assay

Lymph node cells were isolated from C57BL/6 mice that were infected with wild-type or transgenic *L. major* parasites in the footpad. After 1, 2, and 3 weeks post-infection, cells ($5x10^5$) from each group were added to a 96-well round bottom plate and stimulated with 25 µg soluble *Leishmania* antigen (SLA) for 72 hours. After 72 hours, 1 µCi Thymidine (^3H) (MP Biomedicals Inc., Irvine, CA) was added to each well and mixed with the stimulated and non-stimulated lymphocytes for 6 hours. Proliferation was measured using a cell harvester 96 (Tomtec, Hamden, CT) and 1450 Microbeta Trilux liquid scintillation and luminescence counter (EG&G Wallac, Finland). SLA was prepared as previously described[99].

Cytokine measurement

IFN-γ and IL-4 were measured from supernatants of SLA stimulated lymphocytes by sandwich ELISA using capture (clone R4-6A2) and detection (clone XMG1.2) anti-IFN-γ antibodies and capture (clone 11B11) and detection (clone BVD6-24G2) anti-IL-4 antibodies (BD Pharmingen, San Diego, CA).

Isolation of cells from infected mouse ears

Ears infected with wild-type or transgenic *L. major* MCP-1 parasites were excised, soaked in 70% ethanol, and air-dried for 5-10 minutes. The ears were then split into ventral and dorsal portions and placed in liberase (5mg/mL) (Roche, Indianapolis, IN) diluted 1:100 in PBS for 2 hours at 37 ºC as previously described[72]. The ears were put into a 50 μM medicon homogenizer (BD biosciences) with 1 ml PBS and homogenized in BD's Medimachine (BD biosciences) for 1 minute. The liquefied, homogenized ears were then passed through a 50 μM syringe filcon filter (BD biosciences) and centrifuged at 300g for 10 minutes. The cells isolated from the ears were then resuspended in PBS containing 5% FBS and 5mM EDTA and labeled with antibodies for flow cytometry.

Flow Cytometry

Cells isolated from ears were labeled with the following antibodies: PE conjugated anti-mouse F4/80 (clone BM8, eBioscience, San Diego, CA), PerCp-Cy5.5 conjugated anti-mouse CD11b (clone M1/70), and FITC conjugated anti-mouse Gr-1 (clone RB6-8C5)(BD Pharmingen). MC-21 (rat anti-mouse CCR2)[100] was provided by Dr. Matthias Mack (University of Munich, Germany) and used along with the secondary

antibody FITC conjugated goat anti-rat Ig (BD biosciences) to identify CCR2-positive cells. Cells were sorted using BD FACSAria (BD biosciences) at Johns Hopkins Bloomberg School of Public Health.

Staining sorted, infected CCR2-positive macrophages

Sorted CCR2-positive macrophages were cytospun using Cytospin 4 (Thermo Shandon, Pittsburgh, PA) at 600 rpm for 6 minutes. Cells were fixed with methanol and then stained in 1:20 diluted Giemsa stain (Sigma-Aldrich) for 20-30 minutes. The number of parasites per 100 CCR2-positive macrophages was counted with a maximum of 10 parasites per macrophage as a limit for heavily infected macrophages.

MCP-1 co-activation in-vitro studies

Mice were injected intraperitoneally (i.p.) with 1 ng of recombinant MCP-1 (Peprotech Inc., Rocky Hill, NJ) or with PBS. Three days later peritoneal macrophages were isolated from both groups and plated as described above. Macrophages were treated with 100 U IFN-γ, 1 ng MCP-1, or both IFN-γ and MCP-1 for 10 hours. The cells were then washed twice with warm PBS and fresh D10 was added. Infection was done with an MOI 10:1 of wild-type *L. major* parasites for 2 hours. Staining was performed as described above. Intracellular survival was analyzed at 0 and 72 hours post-infection.

CHAPTER 3: THE GENERATION OF CHEMOKINE-SECRETING *LEISHMANIA* PARASITES

SOE-PCR and Plasmid Construction

Previously, our lab made the observation that *Leishmania* parasites infect macrophages via a quiesent mechanism and do not induce the proinflammatory cytokines IL-12 and TNF-α due to a failed NFκB translocation (data not shown). Based on these earlier studies we became interested in genetically engineering chemokine-secreting parasites that would recruit immune cells to the site of infection. These parasites were engineered by first using a technique called Gene Splicing by Overlap Extension (SOE-PCR). In SOE-PCR, two different PCR products were created in separate PCR reactions and used together as templates to generate a hybrid SOE PCR product (Figure 5). This approach has been shown to avoid the need for repeated digestions and ligations during regular cloning[95;96]. The first hybrid SOE-PCR product to be constructed was MCP-1SOE, a 579 base pair (bp) PCR product that began with a 5'-*SmaI* restriction site followed by the *Leishmania* gp63 signal sequence and the entire murine MCP-1 gene and ended with a 3'-*SmaI* restriction site. The gp63 signal sequence portion of MCP-1SOE was required for protein transport and protein secretion, while the murine MCP-1 gene sequence encoded the chemokine MCP-1.

The second hybrid SOE-PCR product constructed was IP-10SOE, a 426 bp PCR product that was constructed in the same manner as described above except that the gp63 signal sequence was attached to the entire murine IP-10 gene.

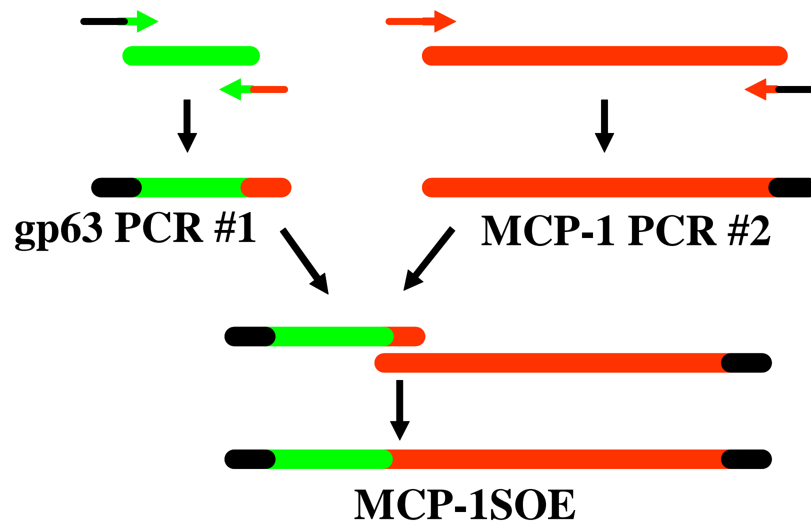


Figure 5. Gene Splicing by Overlap Extension. gp63 PCR#1 and MCP-1 PCR#2 were created in separate PCR reactions. Both PCR products were used together in a separate PCR reaction to create the hybrid PCR product MCP-1SOE.

After MCP-1SOE and IP-10SOE were generated via PCR they were ligated into a TA vector. MCP-1SOE and IP-10SOE were then cut from the TA vector by digestion and ligated into the *Leishmania* expression plasmid pIR1SAT, creating pIR1SAT-MCP-1 (Figure 6) and pIR1SAT-IP-10 (plasmid not shown). The *Leishmania* expression plasmid pIR1SAT contains several key components. First, it contains a multiple cloning site to ligate in the hybrid PCR products. Second, it contains a streptothricin (SAT) resistance marker for selection purposes. Finally, the plasmid contains the 5' and 3' portion of the *L. major* 18S ribosomal RNA subunit on opposite ends of the plasmid. Therefore, we were able to digest both pIR1SAT-MCP1 and pIR1SAT-IP10 with the restriction enzyme *SwaI* and linearize the plasmids so that the 5' and 3' portions of the 18S rRNA genes were on opposite sides of the linearized plasmid for easy integration into the *Leishmania* genome (Figure 7).

Transgenic *Leishmania* parasites secreting MCP-1

Transgenic *L. major* MCP-1 (Lm-Tg) and *L. amazonensis*-MCP-1 (La-Tg) transcribed MCP-1 mRNA during the promastigote stage of development (Figure 8). Wild-type (WT) non-transfected parasites did not transcribe MCP-1 mRNA as expected. Axenically grown amastigotes of transgenic *L. amazonensis* MCP-1 also transcribed murine MCP-1 (Figure 8). The level of MCP-1 produced by these parasites during the extracellular promastigote stage of development was measured by ELISA. Transgenic *L. major* MCP-1 and *L. amazonensis* MCP-1 secreted 161 $\pm$ 14.6 and 199 $\pm$ 51 pg/ml respectively (Figure 9). Wild-type parasites produced no detectible MCP-1, as expected. The two clones represented in Figure 9 secreted the highest level of MCP-1 of the parasites tested. In order to verify that the transgenic parasites did not lose their ability to

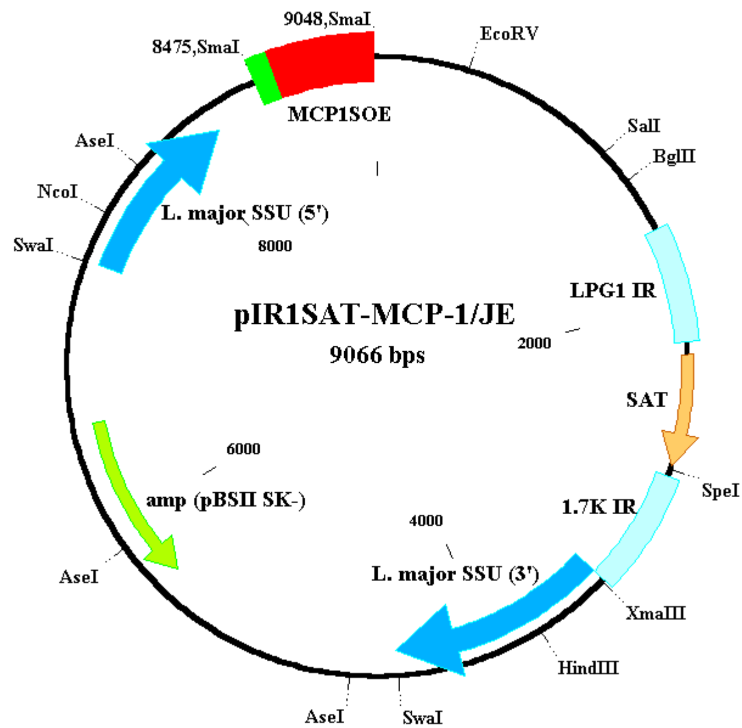


Figure 6. pIR1SAT-MCP-1. The plasmid contains a multiple cloning site for insertion of the MCP-1SOE hybrid product. The multiple cloning site is flanked by genes involved in transplicing and polyadenylation. The plasmid contains a streptothricin (SAT) resistance marker. The plasmid also contains the 5' and 3' portions of the *L. major* 18S ribosomal RNA subunit (SSU) for integration into the *Leishmania* genome.

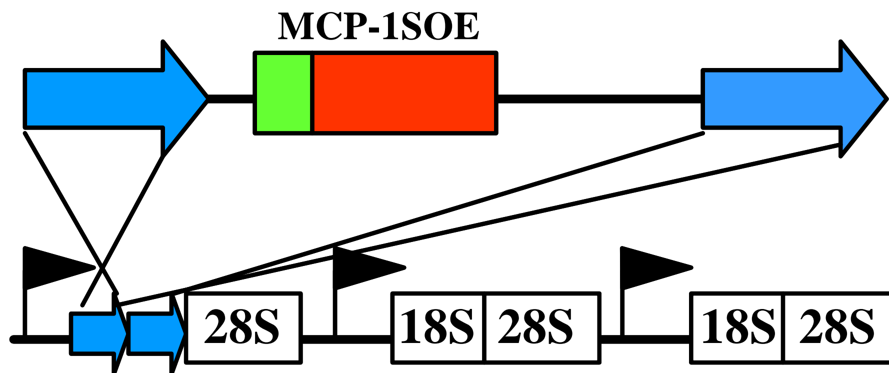


Figure 7. Integration of linearized pIR1SAT-MCP-1 into the *Leishmania* genome. pIR1SAT-MCP-1 was linearized using the restriction enzyme *Swa*I. After linearization, the 5' and 3' portions of the 18 S ribosomal RNA subunit were on opposite sides of the linearized plasmid. *Leishmania* parasites were transfected with the linearized plasmid to allow for integration into the genome.

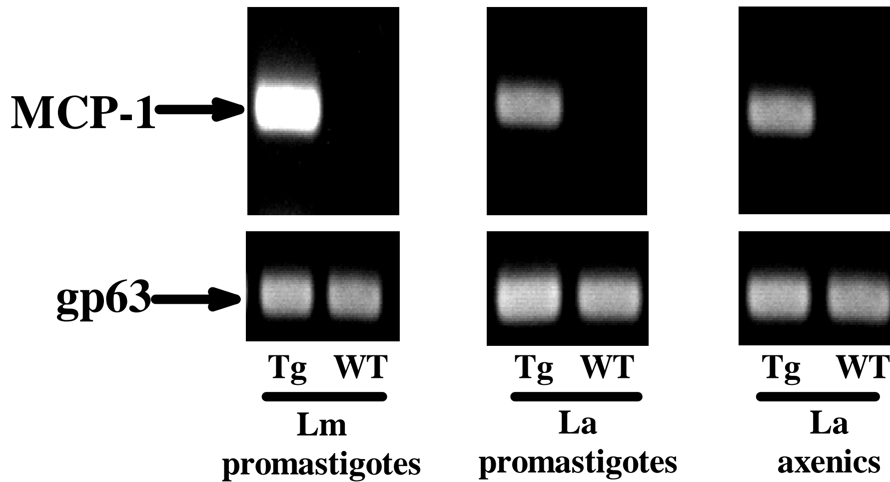


Figure 8. Transgenic parasites transcribe MCP-1 mRNA. Total RNA was isolated from wild-type and transgenic MCP-1 parasites during promastigote development. Murine MCP-1/JE was amplified from the cDNA of transgenic *L. major* MCP-1 (Lm-Tg) and *L. amazonensis* MCP-1 (La-Tg) parasites, but not from the cDNA of wild-type *L. major* (Lm-WT) or *L. amazonensis* (La-WT). Axenically grown *L. amazonensis* MCP-1 and wild-type *L. amazonensis* amastigotes were also analyzed. MCP-1/JE was amplified from transgenic amastigotes, but not from the wild-type. The gene gp63 was amplified from the cDNA as a loading control.

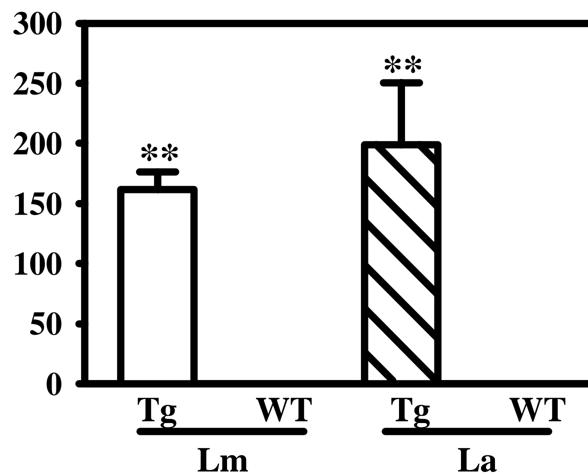


Figure 9. Transgenic MCP-1 parasites secrete MCP-1. A total of 5x10^6 Lm-Tg, Lm-WT, La-Tg, or La-WT parasites were added to an ELISA plate coated with monoclonal goat anti-mouse MCP-1 antibody. After 24 hours, levels of MCP-1 production were determined. (**, P<0.01)

secrete MCP-1 in-vivo, BALB/c mice were infected with transgenic MCP-1 parasites and four weeks later the parasites were isolated from footpads and screened for MCP-1 production. Despite the absence of antibiotic selective pressure in the mouse, the transgenic MCP-1 parasites isolated from infected footpads continued to secrete high levels of MCP-1 after isolation (Figure 10). This expression in the absence of selection is consistent with the stable integration of the linearized constructs into the *Leishmania* genome.

The presence of parasite-derived MCP-1 was examined in *L. major* MCP-1 infected BMDM from MCP-1 KO mice. Parasite-derived MCP-1 was detected in the cytoplasm of Lm-Tg infected BMDM (Figure 11A), but not in wild-type infected macrophages (Figure 11B) confirming that the transgenic MCP-1 parasites are capable of secreting MCP-1 during their intracellular stage of development. MCP-1 was not detected in the supernatants of Lm-Tg infected BMDM (MCP-1 KO) by ELISA during various time points up to 96 hours post-infection (Figure 12). However, considerable amounts of MCP-1 (318 $\pm$ 3 pg/ml) were detected in the supernatants after the Lm-Tg infected macrophages were lysed at 96 hours post-infection. Macrophages infected with wild-type parasites did not release MCP-1 after lysing as expected (Figure 12). These results suggest that the parasite-derived MCP-1 does not leak out of the infected cells and must be released at the same time the amastigotes are released when they burst free from infected cells.

<u>Transgenic parasites secrete IP-10</u>

We also engineered an IP-10 secreting parasite. Transgenic *L. major* IP-10 (Lm-TgIP-10) parasites were able to transcribe IP-10 mRNA during their promastigote stage

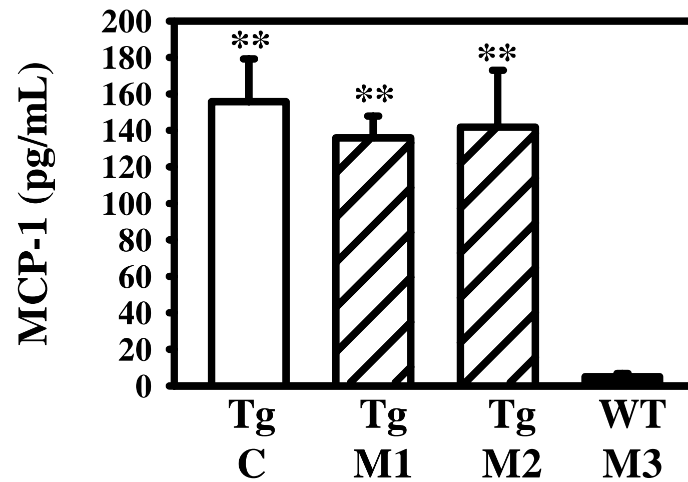


Figure 10. MCP-1 production from footpad-derived parasites. MCP-1 production by transgenic *L. major* MCP-1 parasites grown in culture (Tg-C, open bars) was determined by ELISA. MCP-1 production by transgenic parasites isolated from two different mice infected 4 weeks prior (Tg-M1 and Tg-M2, striped bars) or wild-type *L. major* parasites (WT-M3, solid bars). (**, P<0.01)

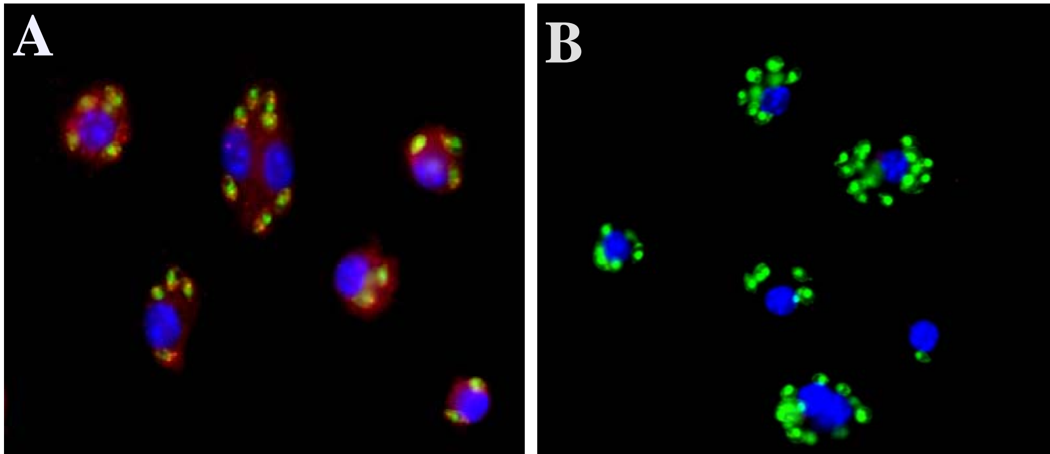


Figure 11. Parasite-derived MCP-1 expressed during intracellular growth.
Bone-marrow derived macrophages (BMDM) from MCP-1 KO mice were infected with (A) Lm-Tg or (B) Lm-WT parasites at an MOI for 10:1. After 48 hours, the infected cells were washed, fixed with methanol, and stained with separate polyclonal antibodies against *Leishmania* (stained green) and murine MCP-1 (stained red). Nuclei were counter-stained with Hoechst (blue stain).

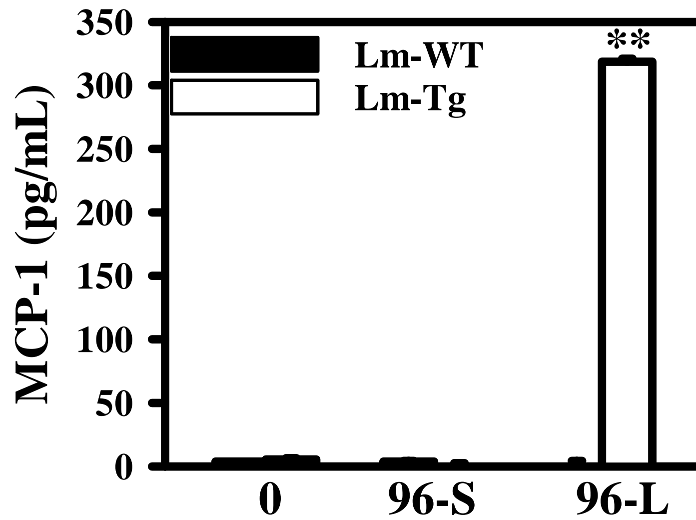


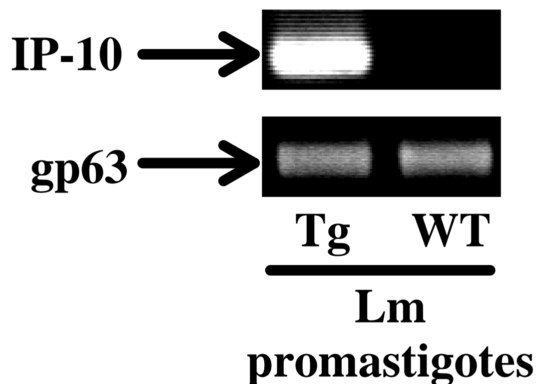
Figure 12. Intracellular accumulation of parasite-derived MCP-1. MCP-1 KO BMDM were infected at an MOI of 10:1 using Lm-WT or Lm-Tg parasites. Supernatants were collected at 0 and 96 hours (96-S) post-infection. In addition, cells infected for 96 hours were lysed (96-L) using 0.01% Triton X + 5mM $MgCl_2$ in H_20. An MCP-1 ELISA was used to detect the level of MCP-1. (**, $P<0.01$)

of development (Figure 13A). The wild-type *L. major* parasites were unable to transcribe IP-10 mRNA. In addition, transgenic *L. major* IP-10 were capable of secreting low levels of IP-10 (35 pg), while the wild-type parasites secreted no IP-10 protein (0 pg) (Figure 13B).

Transgenic parasites are as healthy as wild-type parasites

The metabolic activity of the transgenic parasites was evaluated and compared to wild-type parasites by measuring their ability to reduce MTT to formazan. Both transgenic *L. major* MCP-1 and *L. major* IP-10 were able to reduce MTT to formazan. The rate and extent of MTT reduction was not different between wild-type and transgenic parasites (Figure 14). To determine whether the transgenic parasites were equally as infective as wild-type parasites, we infected resting resident peritoneal macrophages with both transgenic parasites and compared their intracellular survival to wild-type parasites 72 hours later. Equal numbers of infected macrophages were found at 0 hours (data not shown). At 72 hours, there was an equivalent number of wild-type *L. major* (Figure 15 A), *L. major* MCP-1 (Figure 15B), and *L. major* IP-10 (Figure 15C) parasites present inside macrophages (Table 1). Similar results were obtained with the amastigote form. The numbers of wild-type and transgenic MCP-1 amastigotes present inside infected macrophages were similar at 1 hour post-infection (data not shown), and these numbers remained similar at 72 hours post-infection (Figure 16 and Table 2).

A



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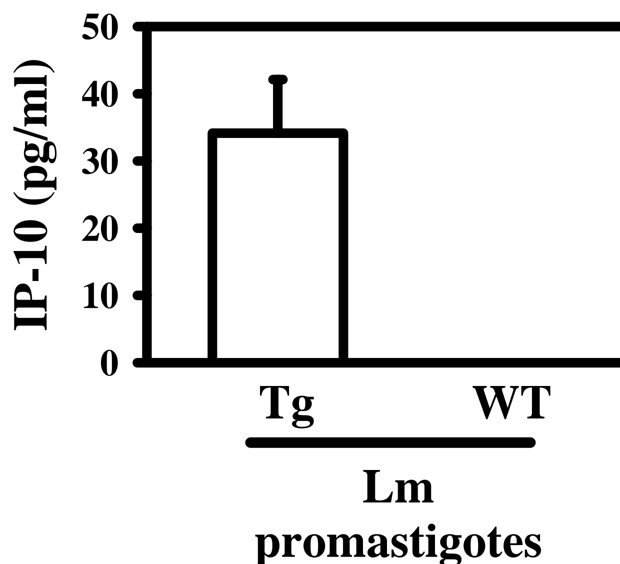

Figure 13. Transgenic IP-10 secreting parasites transcribe IP-10 mRNA and secrete IP-10 protein. (A) Total RNA was isolated from wild-type and transgenic IP-10 parasites during promastigote development. Murine IP-10 was amplified from the cDNA of transgenic *L. major* IP-10 (Lm-TgIP-10) parasites, but not from the cDNA of wild-type *L. major* (Lm-WT). The gene gp63 was amplified from the cDNA as a loading control. (B) A total of $5x10^6$ Lm-TgIP-10, or Lm-WT parasites was added to an ELISA plate coated with monoclonal goat anti-mouse IP-10 antibody. After 24 hours, levels of IP-10 production were determined.

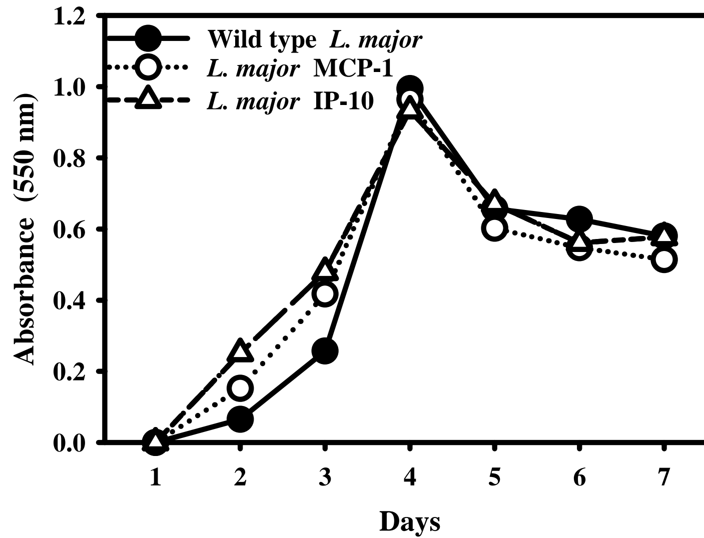

Figure 14. Transgenic parasites are as healthy as wild-type. Wild-type *L. major* (closed circles), *L. major* MCP-1 (open circles), and *L. major* IP-10 (open triangles) ($1x10^6$) were added to separate flasks. On days 0-7, 100 µl aliquots were taken from each parasite culture, mixed with 20 µl MTT (5 mg/ml), and added to a 96-well round bottom plate for 2 hours at room temperature. After the incubation period, 100 µl DMSO was added to each well and the plate was incubated for 1 hour at room temperature. The absorbance was measured at 550 nm.

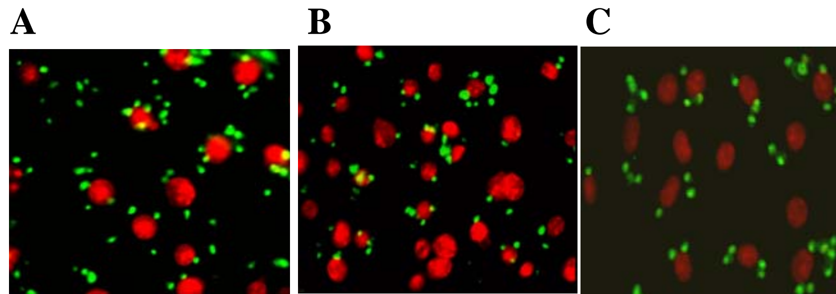

Figure 15. Transgenic parasites efficiently infect monolayers of resident macrophages. Peritoneal macrophages ($1x10^5$) were infected at an MOI of 10:1 for 2 hours with (A) wild-type *L. major*, (B) *L. major* MCP-1, or (C) *L. major* IP-10 promastigotes. After a 72 hour incubation, monolayers were washed, fixed, and stained with a polyclonal antibody to *Leishmania* (stained green). Nuclei were counter-stained with propidium iodide (stained red).

TABLE 1. **Transgenic parasites efficiently infect monolayers of resident macrophages**

Parasite[a]	Number of macrophages	Infected macrophages	Total number of parasites	Macrophages infected with 4 $\geq$ parasites
Wild type *L. major*	100	83 ± 4	255 ± 11	33 ± 4
L. major MCP-1	100	80 ± 5	258 ± 20	34 ± 4
L. major IP-10	100	87 ± 3	271 ± 19	37 ± 7

[a] Parasites in promastigote stage of development were used for infection

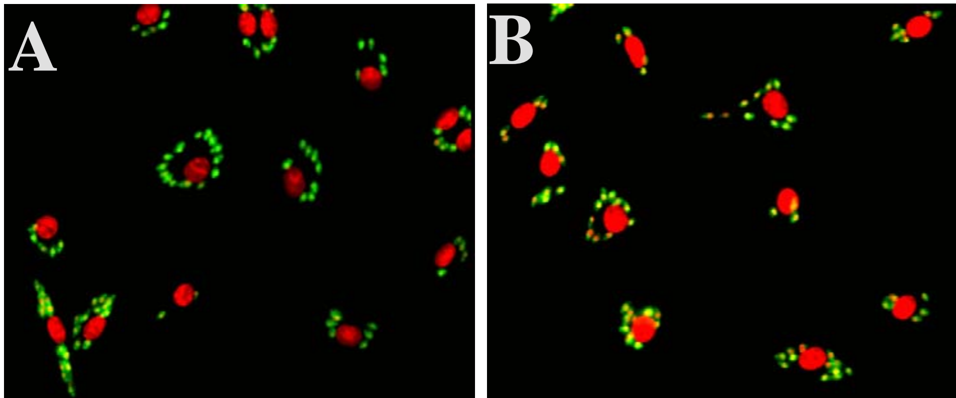

Figure 16. Transgenic MCP-1 amastigotes are equally as infective as wild-type amastigotes. Peritoneal macrophages ($1x10^5$) were infected using lesion-derived amastigotes from either (A) wild-type *L. major* or (B) transgenic *L. major* MCP-1 infected CCR2 KO mice. The amastigotes were used at an MOI of 10:1 and added for 1 hour before washing. Cells were fixed and stained after 72 hours of incubation as described previously.

TABLE 2. **Transgenic MCP-1 amastigotes are equally as infective as wild-type amastigotes**

Parasite[a]	Number of macrophages	Infected macrophages	Total number of parasites	Macrophages infected with 4 $\geq$ parasites
Wild type *L. major*	100	87 ± 11	284 ± 21	38 ± 6
L. major MCP-1	100	84 ± 7	274 ± 29	32 ± 3

[a] Parasites in amastigote stage of development were used for infection

Discussion

We developed two transgenic *Leishmania* parasites that secrete either the murine CC chemokine MCP-1 or CXC chemokine IP-10. Both transgenic parasites thereby could take an active role in manipulating immune cell migration into lesions. Other groups have engineered transgenic parasites in the past[101-104], but never has a parasite been constructed that was shown to recruit innate immune cells to the site of infection. We hypothesized that the chemokine induced cell migration created by either transgenic parasite could influence the magnitude and character of the immune response, and thereby affect the outcome of disease.

By all criteria available to us, the transgenic parasites that we developed appear to be as healthy as wild-type parasites. They grow in culture with the same kinetics and to the same density (data not shown). They reduce MTT to formazan and they invade and persist in resting peritoneal macrophages similar to wild-type parasites.

The transgenic MCP-1 parasites are capable of transcribing MCP-1 mRNA and secreting MCP-1 protein. The MCP-1 protein was secreted during both the extracellular and intracellular stages of development. No MCP-1 was detected in the supernatant of MCP-1 KO macrophages following infection with transgenic MCP-1 parasites. However, when the cells that had been infected with transgenic MCP-1 parasites for 96 hours were lysed, then parasite-derived MCP-1 was released from the infected cells. We believe that MCP-1 is secreted by the transgenic MCP-1 parasites into the macrophage cytoplasm and that the MCP-1 accumulates within infected cells. When the cells burst due to overwhelming parasite replication then the parasite-derived MCP-1 spills out of the infected cell.

Transgenic IP-10 secreting parasites were also engineered to recruit immune cells to the site of infection. We hypothesized that the immune cell recruitment in response to the IP-10 secreting parasites would be distinct from the MCP-1 secreting parasites and that this recruitment could lead to a different phenotype in mice infected with transgenic IP-10 parasites. These IP-10 secreting parasites transcribe IP-10 mRNA and secrete IP-10 protein.

CHAPTER 4: TRANSGENIC MCP-1 PARASITES CAUSE A HEALING PHENOTYPE IN MICE; TRANSGENIC IP-10 PARASITES CAUSE A NON-HEALING PHENOTYPE

Lack of lesion development in mice infected with *L. major* MCP-1

MCP-1 secreting parasites were engineered and demonstrated to secrete MCP-1 protein during both stages of development. We were interested in determining whether the MCP-1 producing parasites would have any effect when injected into mice. We infected BALB/c mice in the hind footpad with $1x10^5$ transgenic *L. major*- MCP-1 or wild-type *L. major* parasites and monitored lesion development over 35 days. The transgenic *L. major* MCP-1 caused little to no detectable lesions, with the mean peak swelling of only 0.14 $\pm$ 0.03 mm (Figure 17). In contrast, wild-type *L. major* infected mice developed progressively larger lesions, which reached an average diameter of 4.95 $\pm$ 0.15 mm. When parasites were isolated from the foot, lymph node, and spleen after 35 days, there were significantly fewer parasites in the foot of the transgenic *L. major* MCP-1 infected mice (274 $\pm$ 38 parasites) compared to the wild-type infected mice ($2.17x10^8$ $\pm$ $6x10^7$ parasites) (Figure 17, inset). There were also fewer parasites in the lymph nodes (54 $\pm$ 23 compared to $1.22x10^5$ $\pm$ $3.7x10^4$), and no transgenic parasites were detected in the spleen. In initial infectivity experiments, *L. major* that were transfected with the empty plasmid (*L. major* pIR1SAT) were also used. They caused lesions that were comparable in size to wild-type infected mice and had comparable numbers of parasites (data not shown).

A similar infection was performed in mice lacking the MCP-1/JE gene[91]. Similar to what was observed in BALB/c mice, the MCP-1 KO mice developed essentially no

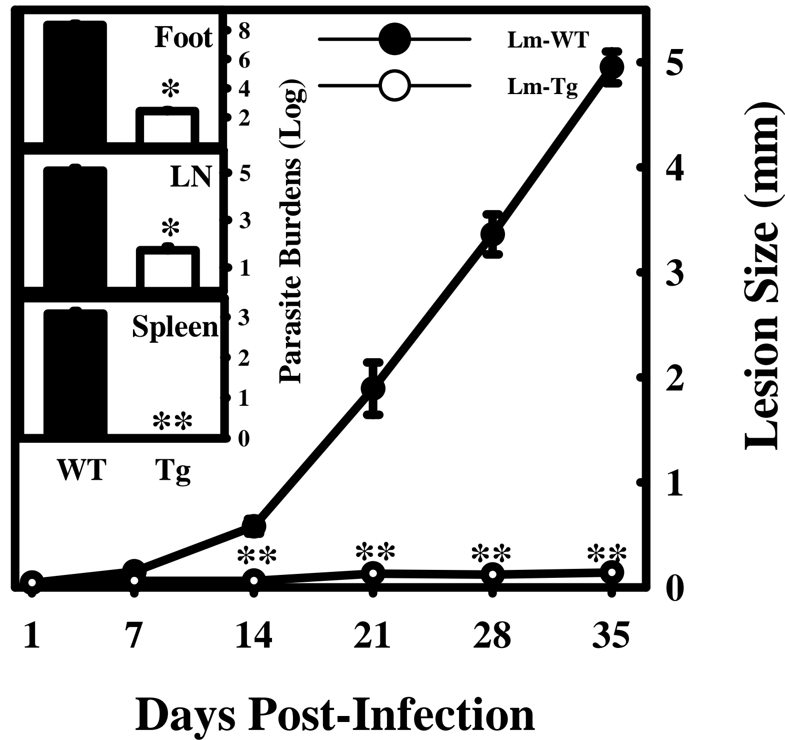

Figure 17. Lack of lesion development in Lm-Tg infected BALB/c mice. Lesion size of BALB/c mice infected with $1x10^5$ Lm-WT parasites in the footpad (closed circles) were compared with those of BALB/c mice infected with Lm-Tg (open circles). Lesions were measured at weekly intervals. Parasite burdens (inset) were determined for Lm-WT (solid bars) and Lm-Tg (open bars) on day 35 post-infection by limiting dilution assays. Parasite burdens in footpads, lymph nodes (LN), and spleens were determined. Error bars represent the standard error of the mean of three separate experiments done with a minimum of five mice per group. (*, $P<0.05$, **, $P<0.01$)

lesions (0.12 $\pm$ 0.02 mm) by day 35 following infection with *L. major*-MCP-1 (Figure 18). They also had minimal to no parasites in their feet (37 $\pm$ 13), lymph nodes (50 $\pm$ 15), or spleens (0$\pm$ 0) (Figure 18, inset). Infection of the MCP-1 KO mice with wild-type *L. major* resulted in substantial lesion development (4.47 $\pm$ 0.43 mm) and higher numbers of parasites in the foot ($4.43 \times 10^7 \pm 1.45 \times 10^7$), lymph node ($2.66 \times 10^6 \pm 8.8 \times 10^5$), and spleen (4300 $\pm$ 1914). These results suggest that parasite-derived MCP-1 was sufficient to induce the healing phenotype that was observed following infection with transgenic *L. major* MCP-1.

To evaluate the infectivity of our transgenic parasites in a more resistant strain of mouse, we proceeded with infections in C57BL/6 mice. These mice were infected in the hind footpad with 5×10^6 transgenic *L. major*-MCP-1 or wild-type *L. major* parasites and lesion development was monitored over the course of 31 days. The transgenic parasites caused essentially no lesions (0.12 $\pm$ 0.01 mm), while the mice infected with Lm-WT developed lesions that reached maximum size on day 21 (2.41 $\pm$ 0.04 mm) (Figure 19A). Consistent with the healing phenotype of these mice, lesions had diminished to 1.87 $\pm$ 0.02 mm on day 31 post-infection. Mice infected with transgenic parasites contained fewer parasites in the foot (108 $\pm$ 23), lymph node (74 $\pm$ 13), and spleen (0$\pm$ 0) compared to mice infected with Lm-WT; foot ($4.85 \times 10^6 \pm 5.5 \times 10^5$), lymph node ($1.9 \times 10^4 \pm 6.54 \times 10^2$), and spleen (52 $\pm$ 7) (Figure 19B). Thus, mice that are relatively resistant to infection with wild-type *L. major* parasites show significantly higher resistance to infection with the transgenic *L. major* MCP-1 parasites.

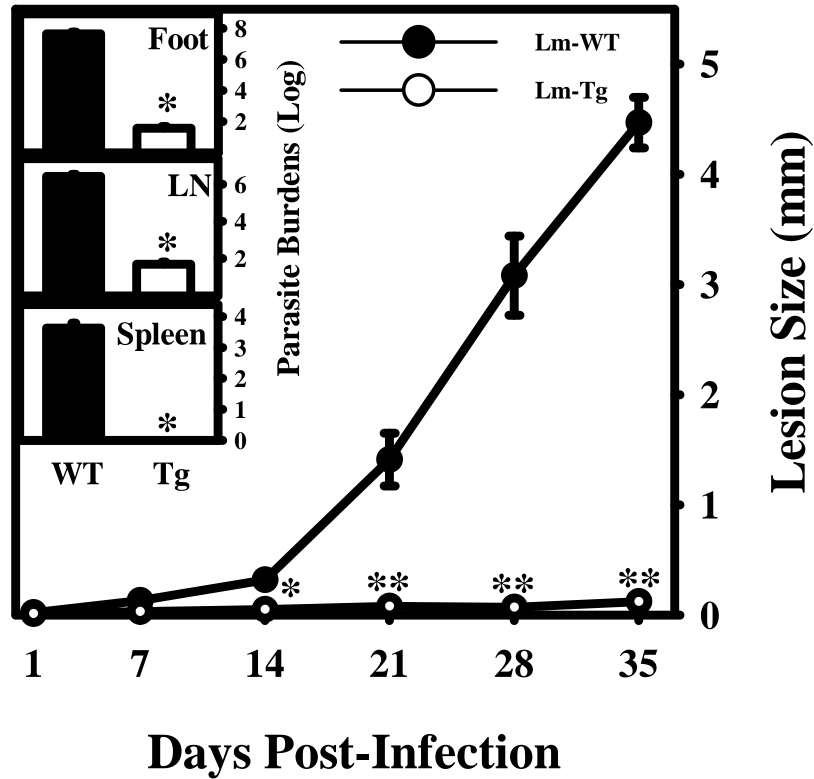

Figure 18. Lack of lesion development in MCP-1 KO mice infected with Lm-Tg. MCP-1 KO mice on a BALB/c background were infected with 1×10^5 Lm-WT (closed circles) or Lm-Tg (open circles). Lesions were measured in weekly intervals. Parasite burdens (inset) were determined on day 35 post-infection for Lm-WT (solid bars) and Lm-Tg (open bars). Error bars represent the standard error of the mean of three separate experiments done with a minimum of five mice per group. (*, $P<0.05$, **, $P<0.01$)

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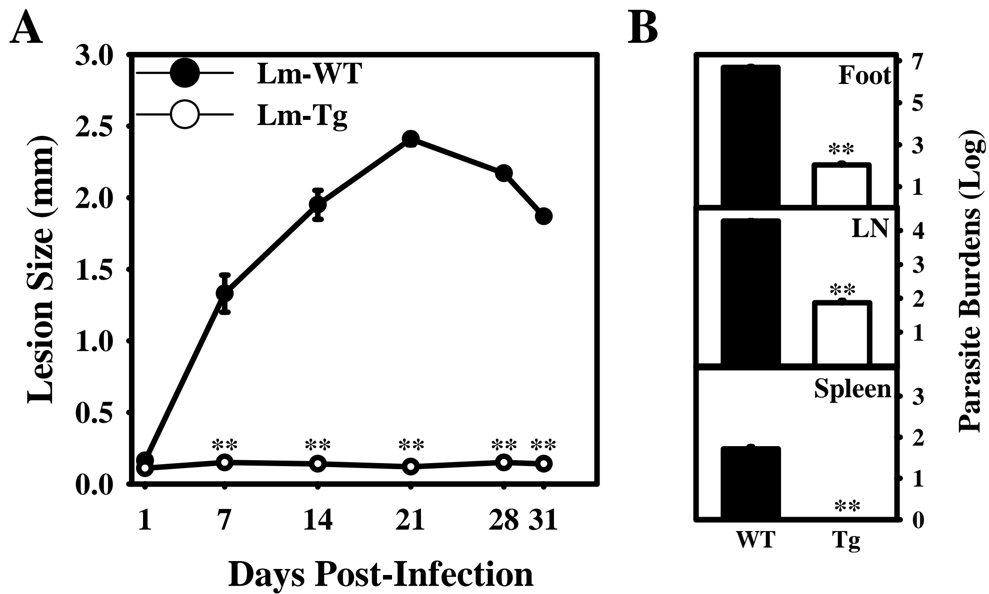


Figure 19. Lack of lesion development in C57BL/6 mice infected with Lm-Tg. (A) C57BL/6 mice were infected with $5x10^6$ Lm-WT (closed circles) or Lm-Tg (open circles) and lesion development was measured at weekly intervals. Parasite burdens were quantitated on day 31 post-infection for C57BL/6 mice infected with Lm-WT (solid bars) and Lm-Tg (open bars). Error bars represent the standard error of the mean of three separate experiments done with a minimum of five mice per group. (**, P<0.01)

Transgenic *L. major* IP-10 are hypervirulent

Transgenic IP-10 secreting parasites were used to infect BALB/c and C57BL/6 mice in separate in-vivo experiments. BALB/c mice were infected in the footpad with $1x10^5$ *L. major* IP-10 parasites and compared to wild-type *L. major* infected BALB/c mice. The transgenic *L. major* IP-10 caused larger lesions, with the mean peak swelling of 4.97 $\pm$ 0.21 mm on day 35 post-infection (Figure 20A) compared to mice infected with wild-type *L. major*, which had a mean peak swelling of 3.25 $\pm$ 0.19 mm. Parasites were isolated from the foot on day 35 post-infection. There were significantly more parasites in the foot of those mice infected with the IP-10 secreting parasites compared to mice infected with the wild-type parasite ($3.51x10^8 \pm 5.5x10^7$ compared to $1.1x10^7 \pm 1.5x10^7$) (Figure 20B)

C57BL/6 mice were also infected with $5x10^6$ *L. major* IP-10 parasites and compared to mice infected with wild-type parasites. Wild-type parasites caused lesions of 1.83 $\pm$ 0.13 mm on day 31 post-infection. Transgenic *L. major* IP-10 parasites caused large lesions with the mean peak swelling of 5.07 $\pm$ 0.38 mm (Figure 21A). These infected mice were monitored biweekly and lesion development was monitored for 31 days. At the end of 31 days parasite burdens were prepared from mice infected with transgenic IP-10 parasites and compared to wild-type infected mice. The C57BL/6 mice infected with transgenic IP-10 parasites contained significantly higher numbers of parasites in the infected foot ($5.39x10^8 \pm 3.24x10^8$), lymph node ($1.1x10^6 \pm 5.6x10^5$), and spleen (6250 $\pm$ 551) compared to mice infected with wild-type parasites; foot ($5.93x10^6 \pm 4.8x10^6$), lymph node ($2x10^4 \pm 1.1x10^4$), and spleen (50 $\pm$ 9) (Figure 21B).

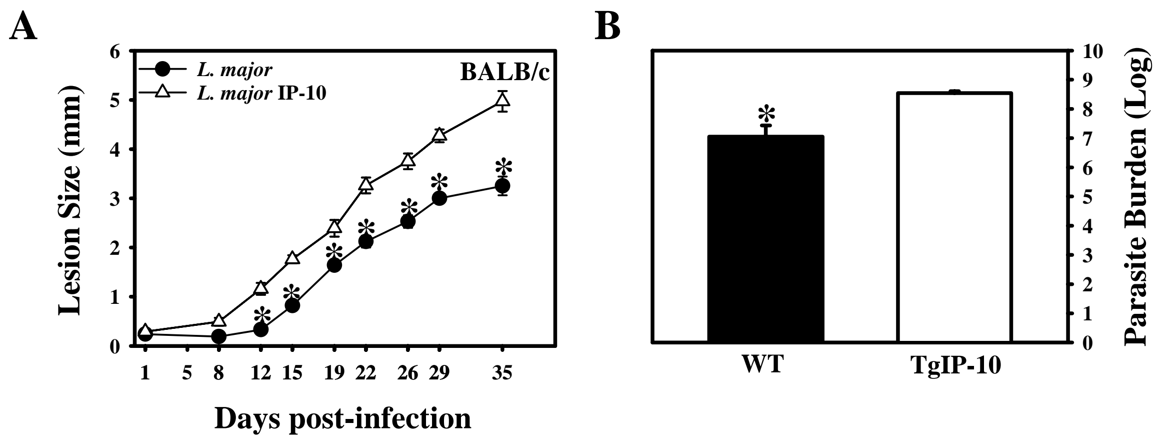
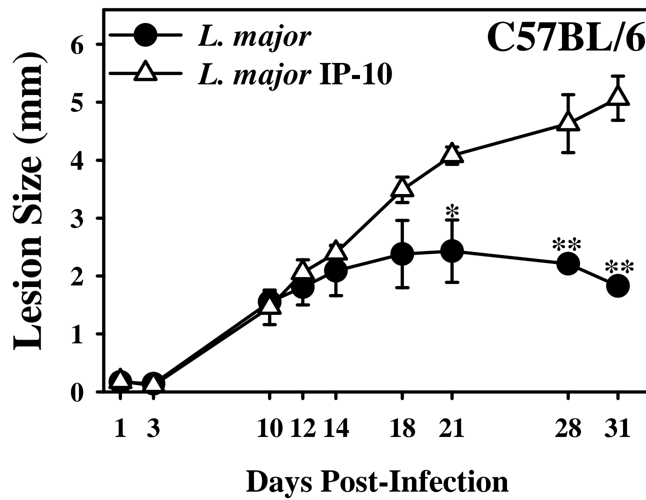


Figure 20. *L. major* IP-10 are hypervirulent. (A) BALB/c mice were infected with $1x10^5$ Lm-WT (closed circles) or Lm-TgIP-10 (open triangles) and lesion development was measured at weekly intervals. (B) Parasite burdens were quantitated on day 35 post-infection from BALB/c mice infected with Lm-WT (solid bars) and Lm-TgIP-10 (open bars). ($P<0.05$)

A



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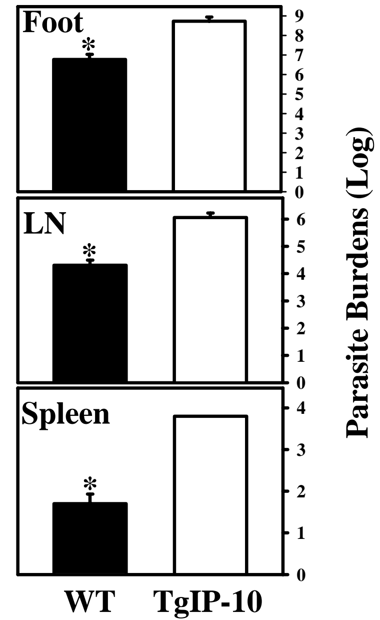


Figure 21. *L. major* IP-10 causes significantly larger lesions in resistant C57BL/6 mice. (A) C57BL/6 mice were infected with $5x10^6$ Lm-WT (closed circles) or Lm-TgIP-10 (open triangles) and lesion development was measured at weekly intervals. Parasite burdens were quantitated on day 31 post-infection for C57BL/6 mice infected with Lm-WT (solid bars) and Lm-TgIP-10 (open bars).

Discussion

The transgenic MCP-1 secreting parasites caused minimal disease in several strains of mice. BALB/c mice, which are normally susceptible to *L. major* infection, did not develop lesions when infected in the footpad, nor did these parasites efficiently disseminate to other organs, as did wild-type parasites. Transgenic MCP-1 parasites were also attenuated in MCP-1 deficient mice suggesting that parasite-derived MCP-1 was responsible for the attenuated phenotype. Others have demonstrated that wild-type *Leishmania* parasites can induce MCP-1 production 1 hour after infection and that the MCP-1 levels return to uninduced a few hours later[73;74]. Therefore, reproducing the healing phenotype in MCP-1 KO mice infected with Lm-Tg was important to prove that the parasite-derived MCP-1 was the cause of the healing phenotype and not host MCP-1. Transgenic parasites also failed to cause lesions in C57BL/6 mice that are resistant to *L. major* infection, even with high doses of transgenic *L. major* MCP-1. Normally C57BL/6 mice infected with wild-type parasites develop lesions and eventually resolve the lesions. However, C57BL/6 mice infected with Lm-Tg were even more resistant and failed to develop lesions. We have tried to reproduce the healing phenotype in the footpad by using high amounts of recombinant MCP-1 prior to *Leishmania* infection, but were not successful (data not shown). This may indicate that the constitutive expression of MCP-1 by the transgenic parasites is important for the continued recruitment of immune cells into lesions.

The transgenic IP-10 secreting parasites caused large lesion development in both BALB/c and C57BL/6 mice. Much to our surprise, the C57BL/6 mice infected with transgenic IP-10 parasites developed large lesions that did not appear to resolve like the

wild-type infected C57BL/6 footpads. The disease exacerbation that occurs in C57BL/6 infected with Lm-TgIP-10 is worse than the exacerbation in BALB/c mice infected with Lm-TgIP-10. BALB/c mice infected with wild-type *L. major* develop a Th2 response that results in high levels of IL-4 production and a lack of intracellular parasite killing. Ultimately, this results in the mouse's death. C57BL/6 mice infected with wild-type *L. major* develop a Th1 response that results in high levels of IFN-γ production and the activation of macrophage intracellular parasite killing mechanisms, which controls the disease and leads to disease resolution. The hypervirulent Lm-TgIP-10 causes an additive effect in BALB/c mice and further impairs the disease. However, the hypervirulent Lm-TgIP-10 has a more profound effect in C57BL/6 mice by causing very large lesions that do not appear to resolve. Instead, these lesions continue to increase over time and the mice appear unable to control the infection despite developing a Th1 response. We hypothesized that a population of cells was migrating into the footpad and exacerbating the disease. Recently, it was discovered that nTregs express CXCR3, the receptor for IP-10, on their surface. nTregs have been shown to produce high levels of IL-10 during leishmaniasis. It is possible that the transgenic IP-10 secreting parasites are recruiting nTregs, which are producing IL-10 and exacerbating the disease.

CHAPTER 5: CHARACTERIZATION OF HEALING PHENOTYPE CAUSED BY TRANSGENIC MCP-1 PARASITE

L. major MCP-1 cause lesions in CCR2 KO mice

Previously it was demonstrated that transgenic MCP-1 parasites cause a healing phenotype in three different strains of mice including C57BL/6 mice. We hypothesized that the parasite-derived MCP-1 was responsible for the healing phenotype in each of these strains of infected mice. CCR2 KO mice lack the receptor for MCP-1. Therefore, we were interested in repeating the in-vivo experiment with CCR2 KO mice. If the parasite-derived MCP-1 was responsible for the healing phenotype than the phenotype should not be reproduced when CCR2 KO mice were infected with transgenic MCP-1 secreting parasites because the MCP-1 would not have any CCR2 receptors to bind and signal. Lesion progression was measured over a 51-day period. Infection of CCR2 KO mice with either transgenic *L. major* MCP-1 (open squares) or wild-type parasites (closed squares) resulted in similar lesion progression (Figure 22A). On day 51, wild-type parasites caused lesions of 1.95 ± 0.18 mm, whereas transgenic parasites caused lesions of 1.75 ± 0.11 mm. The number of parasites on day 51 in the Lm-Tg infected CCR2 KO foot ($4.16 \times 10^5 \pm 1 \times 10^5$), lymph nodes ($3475 \pm 664$), and spleen ($210 \pm 84$) were comparable to the number of parasites in CCR2 KO mice infected with wild-type *L. major* parasites; foot ($6.2 \times 10^5 \pm 2 \times 10^5$), lymph nodes ($4250 \pm 342$), and spleen ($425 \pm 53$) (Figure 22B). Parallel infection of C57BL/6 mice with transgenic parasites (open circles) caused essentially no lesions (0.15 ± 0.01 mm) as previously described, with fewer

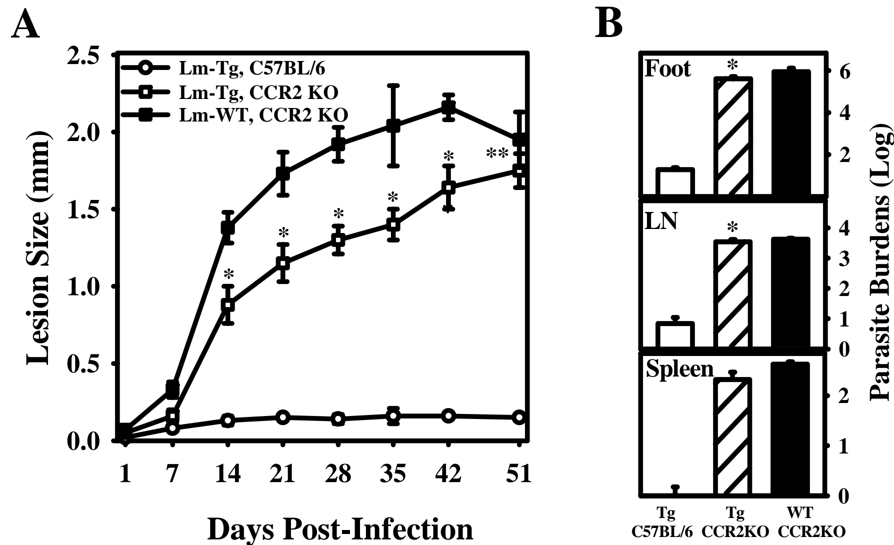


Figure 22. Lesion development in CCR2 KO mice. CCR2 KO mice were similarly infected with Lm-WT (closed squares) or Lm-Tg (open squares) and compared to infections of Lm-Tg in C57BL/6 (open circles). Parasite numbers were determined on day 51 for the CCR2 KO mice infected with Lm-WT (solid bars) and Lm-Tg (striped bars). Error bars represent the standard error of the mean of two separate experiments done with a minimum of five mice per group. (*, $P<0.05$) (**, $P<0.01$)

parasites in the foot, lymph node, and spleen. These observations suggest that the presence of CCR2, the receptor for MCP-1, on immune cells is required for the healing phenotype that we observe.

Lack of adaptive immunity in mice infected with transgenic parasites

We were interested in determining whether the healing phenotype caused by transgenic MCP-1 parasites was a result of adaptive or innate immunity. Therefore, we infected RAG KO mice with the transgenic MCP-1 parasites to see if the healing phenotype was lost in RAG KO mice or whether the healing phenotype would be reproduced. If the healing phenotype was lost in RAG KO mice, this would suggest that the phenotype is due to adaptive immunity. If the healing phenotype was reproduced in RAG KO mice than it would suggest that only innate immunity is required. RAG KO mice were infected with either $5x10^6$ transgenic MCP-1 parasites or wild-type parasites. RAG KO mice infected with transgenic MCP-1 parasites developed no lesions with the mean peak swelling of 0.11 ± 0.01 mm compared to RAG KO mice infected with wild-type parasites that developed large lesions with a mean peak swelling of 4.36 ± 0.37 mm (Figure 23A). On day 35 post-infection, there were significantly higher numbers of parasites isolated from the foot ($7.03x10^7 \pm 1.1x10^7$), lymph node ($4.87x10^6 \pm 1.4x10^6$), and spleen (2188 ± 442) from the RAG KO mice infected with Lm-WT compared to those isolated from mice infected with Lm-Tg; foot (88 ± 18), lymph node (75 ± 35), and spleen (0 ± 0) (Figure 23B).

We were interested in determining whether the avirulent transgenic MCP-1 parasites could stimulate T-cell responses. C57BL/6 mice were used in these studies because previous reports had shown that they were an excellent model to study

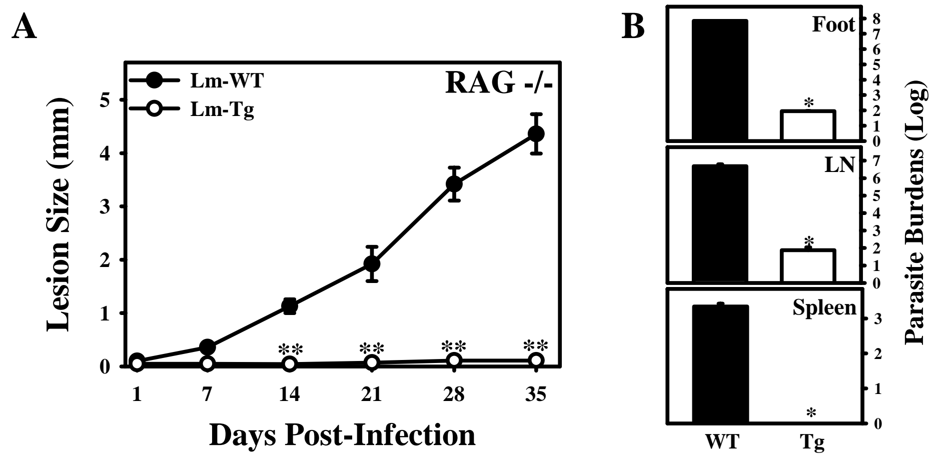


Figure 23. Lack of lesion development in RAG KO mice.
(A) RAG -/- mice on a C57BL/6 background were infected with $5x10^6$ Lm-WT (closed circles) or Lm-Tg (open circles) and lesion development was measured at weekly intervals. (B) Parasite burdens were quantitated on day 35 post-infection for RAG -/- mice infected with Lm-WT (solid bars) and Lm-Tg (open bars).

immunological memory and protection against *L. major*[105]. Lymphocytes isolated from the draining lymph nodes of mice infected with transgenic *L. major* MCP-1 on weeks 1, 2, and 3 post-infection proliferated poorly in response to SLA stimulation (Figure 24, open bars), whereas mice infected with wild-type parasites exhibited strong proliferative responses (Figure 24, solid bars). These SLA-stimulated lymphocytes from transgenic *L. major* MCP-1 infected mice secreted virtually no IFN-γ (Figure 25A, open bars) or IL-4 (Figure 25B, open bars), whereas lymphocytes isolated from wild-type infected mice secreted significantly higher levels of IFN-γ (Figure 25A, solid bars) and IL-4 (Figure 25B, solid bars). There was essentially no spontaneous lymphocyte proliferation or cytokine production from either group in the absence of SLA stimulation (data not shown). These data suggest that the adaptive immune response plays a minimal role in the clearance of transgenic MCP-1 parasites.

This lack of adaptive immunity was confirmed in a vaccination study, in which C57BL/6 mice were infected in the right footpad with $5x10^4$ transgenic *L. major* MCP-1 or wild-type parasites. After 5 weeks, when the lesions were resolving, these mice were challenged in the ear with $1x10^5$ wild-type *L. major* parasites, as previously described.[105] As a control, an unprotected group was also challenged in the ear. The ears were monitored over the course of the next 5 weeks for lesion development. The unprotected group of mice developed progressive lesions as expected (Figure 26A, solid triangles), with substantial numbers of parasites in the ears (Figure 26B, striped bars) and lymph nodes (Figure 26C, striped bars). Mice vaccinated with wild-type parasites were more resistant, developing healing lesions (Figure 26A, solid circles) with few detectable parasites in the ears (Figure 26B, solid bars) and lymph nodes (Figure 26C, solid bars).

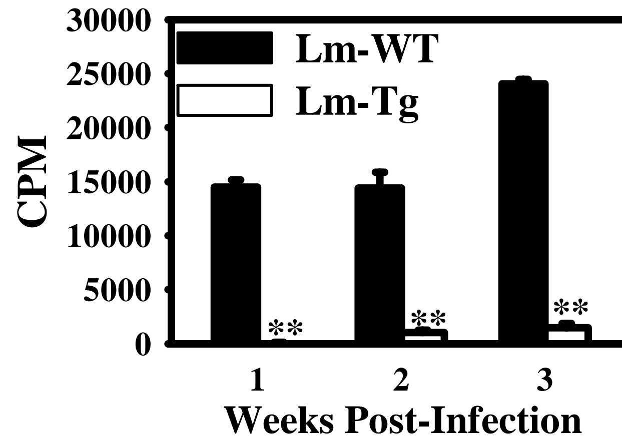
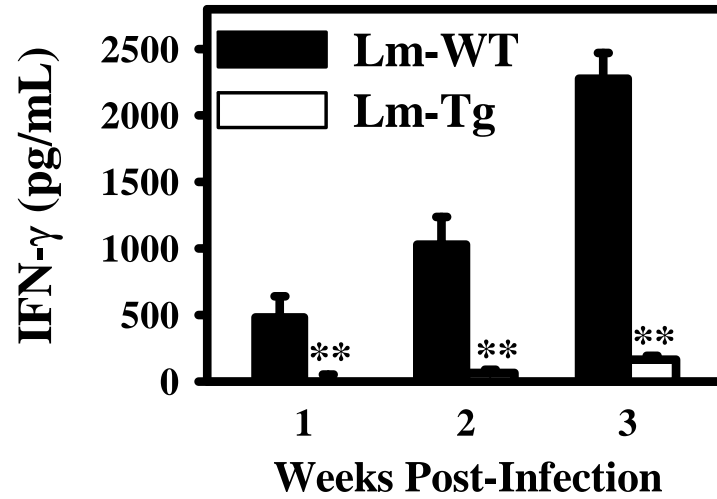


Figure 24. Lack of T-cell proliferation in mice infected with Lm-Tg. C57BL/6 mice were infected with $5x10^4$ Lm-WT or Lm-Tg parasites in the footpad. Popliteal lymph nodes were isolated on weeks 1, 2, and 3 post-infection. The proliferation of $5x10^5$ lymph node cells from mice infected with Lm-WT (solid bars) or Lm-Tg (open bars) was measured by ^{3}H-thymidine incorporation following stimulation with SLA. (**, P<0.01)

A



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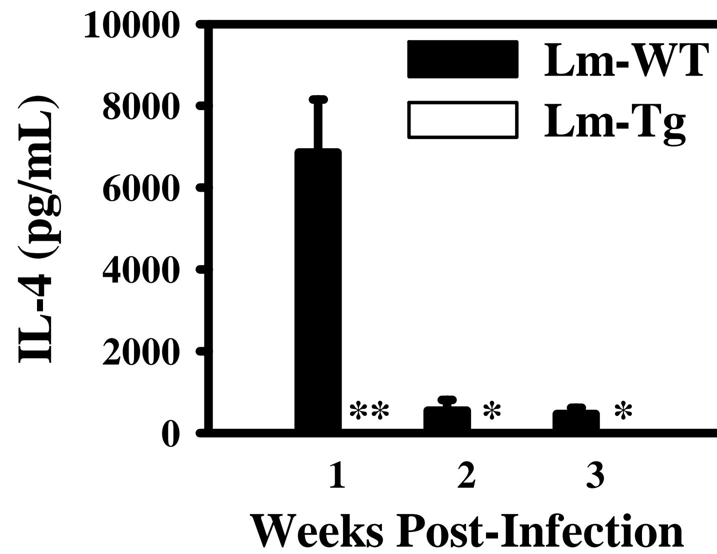

Figure 25. Lack of cytokine production in mice infected with Lm-Tg. (A) IFN-γ or (B) IL-4 was measured by ELISA following stimulation of lymph node T-cells from mice infected with Lm-WT (solid bars) or Lm-Tg (open bars). (*P<0.05, **, P<0.01)

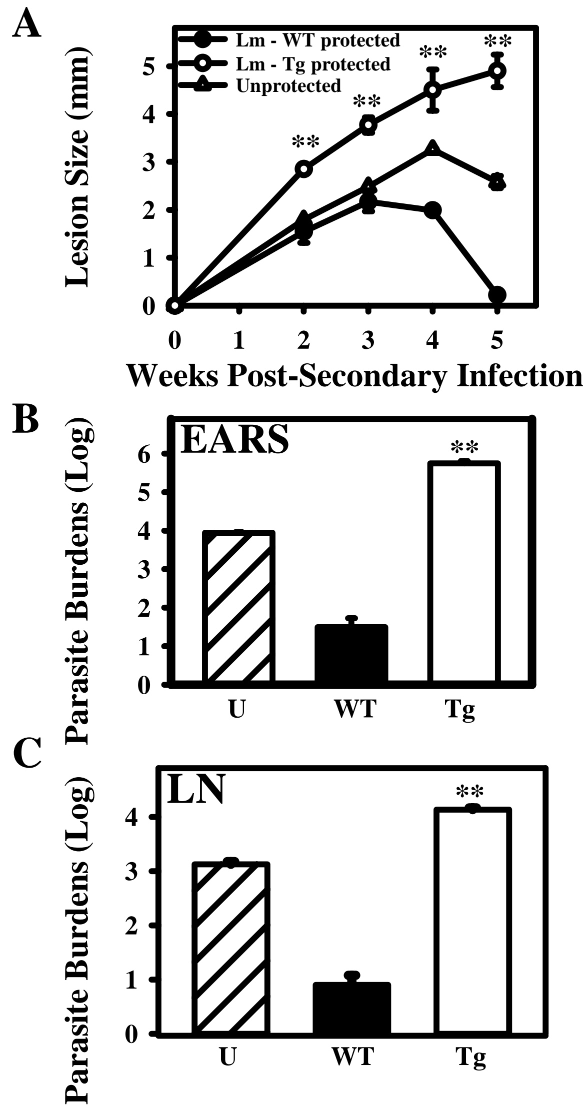

Figure 26. Transgenic MCP-1 parasites are unable to protect mice against secondary wild-type *Leishmania* infection. (A) C57BL/6 mice were vaccinated with 5×10^4 viable Lm-WT or Lm-Tg parasites in the right footpad. Footpad lesions were monitored for 5 weeks. After 5 weeks, when lesions had resolved, 1×10^5 wild-type *L. major* parasites were injected into the ears of non-vaccinated (closed triangles), Lm-WT protected (closed circles), and Lm-Tg protected (open circles) C57BL/6 mice. Ears were monitored over the next 5 weeks for lesion development. (B-C) Parasite burdens in the ears (B) and lymph node (C) were measured at 5 weeks. Data are expressed as the mean + SEM of three experiments with a minimum of five mice per group. (**, P<0.01)

Mice vaccinated with transgenic *L. major* MCP-1, however, developed large lesions (Figure 26A, open circles) with high numbers of parasites in the ears (Figure 26B, open bars) and lymph nodes (Figure 26C,open bars). By week 5, these lesions had begun to spread over the entire length of the ear and were necrotic (data not shown). Thus, prior infection of mice with avirulent transgenic *L. major* MCP-1 parasites did not provide any protection against subsequent challenge with wild-type parasites and may even have inhibited the immune response to the ear challenge.

<u>The control of transgenic MCP-1 parasite infection coincides with increased migration of CCR2-positive macrophages</u>

We quantitated the migration of CCR2-positive (CD11b-positive, F4/80-positive, Gr-1-negative) macrophages into the ears of mice infected with transgenic *L. major* MCP-1 parasites during early stages of the infection. BALB/c mice were infected in the ears with either transgenic *L. major* MCP-1 or wild-type parasites. On various days post-infection, cells were isolated from ears to identify cells migrating into the lesions. At day 7, there was a significant increase in the mean number of CCR2-positive macrophages (41,633 $\pm$ 5994 [4.8% of total ear cells]) in the ears of mice infected with transgenic MCP-1 parasites (Figure 27A) compared to those infected with wild-type parasites (27,831 $\pm$ 5799 [3.02% of total ear cells]) (Figure 27B). This influx was transient and by day 14 the number of macrophages had begun to recede to levels similar to wild-type parasites. There was no difference in the amount of cell migration between the two groups on day 21 post-infection (data not shown). In addition, the number of neutrophils was the same in both groups during early time-points (data not shown).

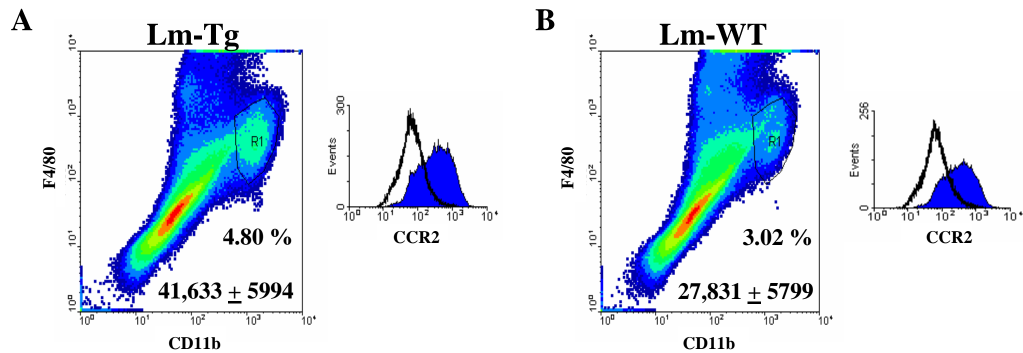

Figure 27. CCR2-positive macrophage migration into lesions. BALB/c mice were infected with $5x10^4$ (A) transgenic *L. major* MCP-1 or (B) wild-type *L. major* parasites. Cells were isolated from infected ears on day 7 post-infection and labeled with antibodies to identify CD11b-positive, F4/80-positive, Gr-1-negative (data not shown) CCR2-positive macrophages by flow cytometry. Percentages and cell numbers are based on the mean percentage of three separate experiments using the R1 gate. Data are expressed as the mean + SEM of three experiments.

To determine the kinetics of disease resolution, we infected BALB/c mice in the ears with either wild-type or transgenic MCP-1 parasites. The number of parasites in infected ears was quantitated at various times post-infection. Significant differences in parasite loads between mice infected with either wild-type parasites or Lm-Tg began to appear as early as day 7 post-infection (Figure 28). The number of parasites in the transgenic *L. major* MCP-1 infected ears decreased from 1292 $\pm$ 84 on day 7 to only 467 $\pm$ 9 on day 14. In contrast, the number of wild-type parasites increased from $1.2 \times 10^4 \pm 2 \times 10^3$ on day 7 to $2.94 \times 10^5 \pm 1.6 \times 10^4$ parasites on day 14 (Figure 28). At this time, parasites began to disseminate into the lymph nodes of wild-type infected mice, but there was no dissemination into the lymph nodes in the Lm-Tg infected mice (data not shown).

To evaluate the level of macrophage infectivity in wild-type *L. major* and transgenic *L. major* MCP-1 infected BALB/c ears, total cells were isolated on days 7, 14, and 21 post-infection. The cells were sorted to isolate a population of CD11b-positive, F4/80-positive, Gr-1-negative, CCR2-positive macrophages. CCR2-positive macrophages isolated from Lm-Tg infected ears contained very few parasites and the low level of parasites remained relatively constant over the entire observation period (Figure 29A). By day 21, CCR2-positive macrophages from Lm-Tg infected ears contained few or no intact parasites (Figure 29B). However, macrophages isolated from wild-type *L. major* infected ears contained high numbers of parasites per 100 macrophages and this number progressively increased over time (Figure 29A). By day 21, the CCR2-positive macrophages from wild-type infected ears were heavily infected with high numbers of parasites (Figure 29C).

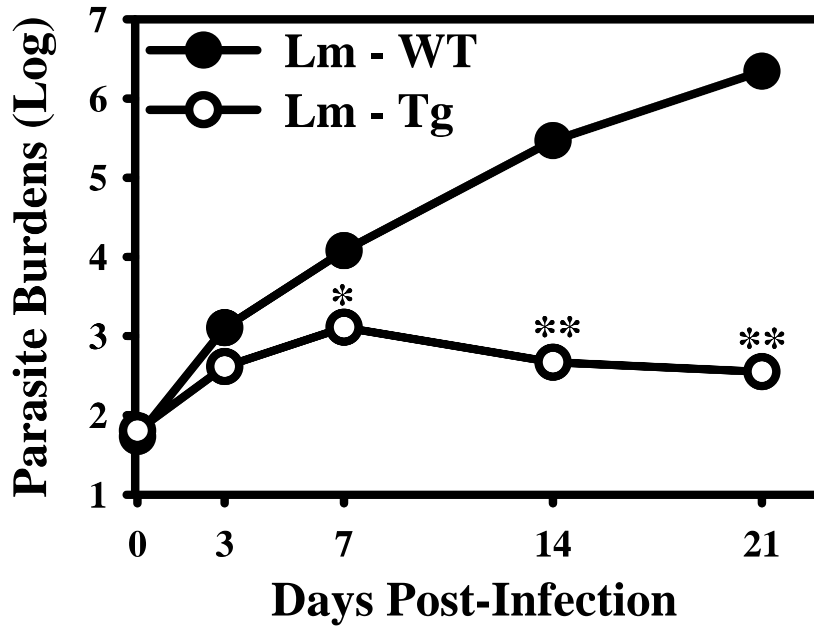

Figure 28. Reduced parasite loads in Lm-Tg infected BALB/c mice by day 7 post-infection. BALB/c mice were infected with $5x10^4$ Lm-WT (closed circles) or Lm-Tg parasites (open circles) in the ear. Parasite burdens in infected ears were enumerated at 2 hours and at days 3, 7, 14, and 21 post-infection. The experiment was repeated three times using a minimum of 5 mice per group. (*, P<0.05, **, P<0.01)

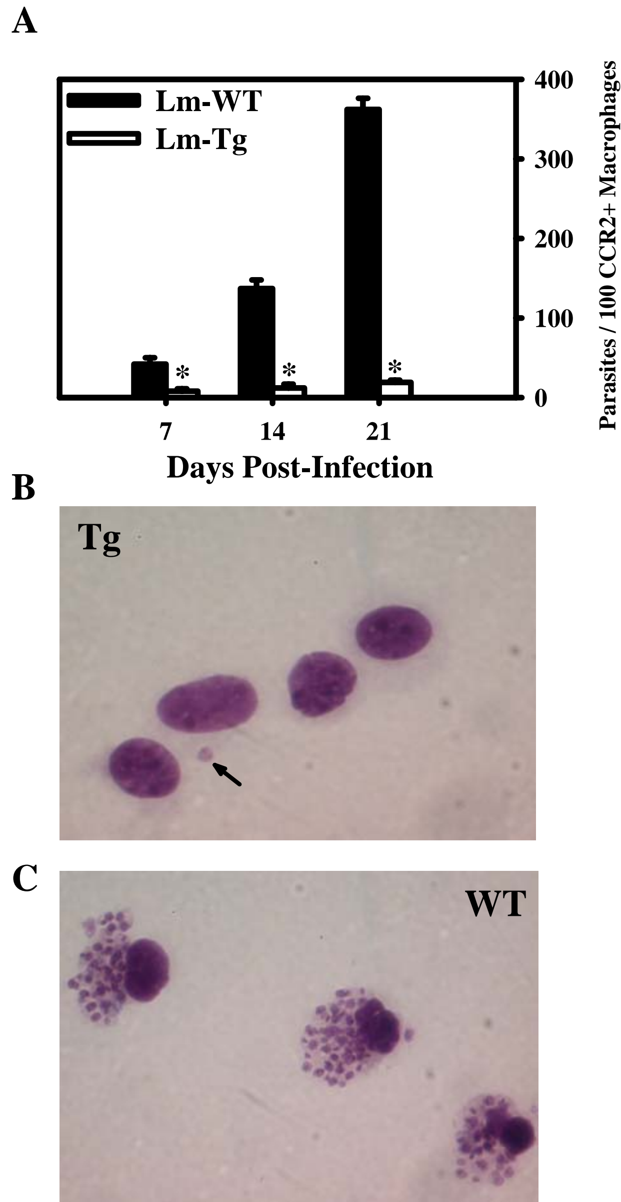

Figure 29. Low infectivity of transgenic infected CCR2-positive macrophages. (A) Total cells were isolated from infected ears on day 7, 14, and 21 post-infection, and CD11b-positive, F4/80-positive, Gr-1-negative, CCR2-positive macrophages were sorted. The number of parasites per 100 CCR2-positive macrophages isolated from Lm-WT (solid bars) or Lm-Tg (open bars) infected mice was compared. (B-C) Sorted CCR2-positive macrophages were cytospun and Giemsa stained to examine macrophage infectivity. Macrophages from day 21 ears infected with (B) Lm-Tg or (C) Lm-WT were compared. (*, $P<0.05$)

The kinetics of disease resolution and the levels of CCR2-positive macrophage infectivity were analyzed in wild-type and Lm-Tg infected C57BL/6 ears as well as CCR2 KO ears. Similar to the observations in BALB/c mice, there were significantly fewer Lm-Tg parasites in the ears of C57BL/6 mice by as early as day 7 (Figure 30A). On day 21, the CCR2-positive macrophages from wild-type infected C57BL/6 ears were heavily infected with parasites (Figure 30B), whereas those from Lm-Tg infected mice were not (Figure 30C).

In contrast to the C57BL/6 mice, infection of CCR2 KO mice with either wild-type or transgenic MCP-1 parasites resulted in the increased parasite accumulation in the ears over the 21 day observation period (Figure 31A). At this time, macrophages from the infected CCR2 KO mice contained high levels of wild-type (Figure 31B) or transgenic MCP-1 (Figure 31C) parasites in them. These data further confirms that a population of CCR2-positive macrophages is migrating toward the parasite-derived MCP-1 and plays a role in eradicating the parasites.

Recruited CCR2-positive macrophages and MCP-1 co-activation

To determine how recruited CCR2-positive macrophages were capable of killing intracellular parasites, mice were injected i.p. with recombinant MCP-1 to recruit restrictive CCR2-positive macrophages to the peritoneum. These cells were isolated and stimulated in-vitro under various conditions. Intracellular killing of wild-type parasites was enhanced when MCP-1 recruited macrophages were activated with both MCP-1 and IFN-γ in-vitro (Table 3). Optimal parasite killing appeared to require both recruitment of CCR2-positive macrophages and stimulation of these cells since the removal of either diminished the killing (Table 3). Normally macrophages become classically activated by

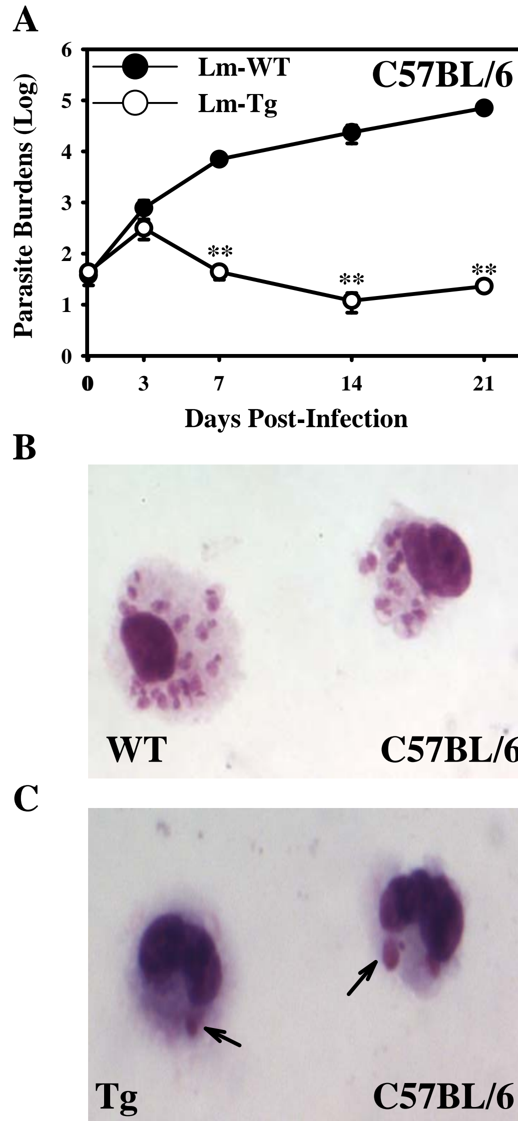

Figure 30. Reduced parasite loads in Lm-Tg infected C57BL/6 mice by day 7 post-infection. (A) Parasite numbers in the ears of C57BL/6 mice were determined at weekly intervals following infection with $5x10^4$ Lm-WT (closed circles) or Lm-Tg parasites (open circles). (B-C) Intracellular parasites within sorted CCR2-positive macrophages isolated from the ears of C57BL/6 mice, 21 days after infection with Lm-WT (B) or Lm-Tg (C) parasites. (**, P<0.01)

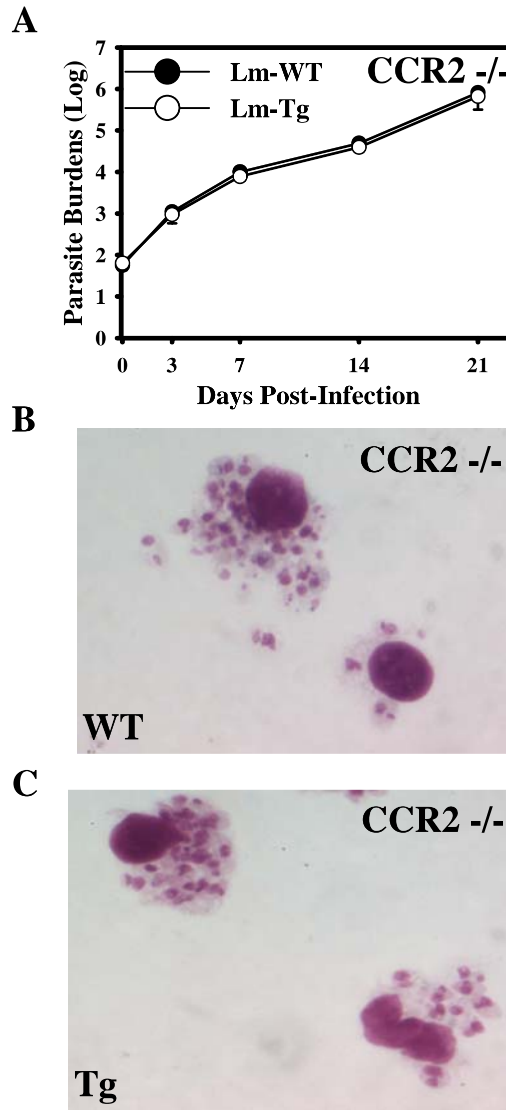

Figure 31. Parasite levels in the infected ears of CCR2 KO mice. Parasite numbers in the ears of CCR2 KO mice were determined at various time points following infection with $5x10^4$ Lm-WT (closed circles) or Lm-Tg (open circles) parasites. (B-C) Intracellular parasites within macrophages isolated from the ears of CCR2 KO mice, 21 days after infection with Lm-WT (B) or Lm-Tg (C) parasites.

TABLE 3. Killing of wild-type *Leishmania* by MCP-1 recruited macrophages

	Treatment	Number of macrophages	Infected[c] macrophages	Total number of parasites	Macrophages infected with 4 ≥ parasites
Resident peritoneal macrophages	No stimulation	100	85[a] ± 3[b]	248 ± 10	38 ± 4
	IFN-γ (100 U)	100	84 ± 4	238 ± 22	35 ± 4
	IFN-γ (100 U) + MCP-1 (1 ng)	100	84 ± 3	249 ± 6	32 ± 2
MCP-1 recruited macrophages	No stimulation	100	81 ± 3	232 ± 18	34 ± 3
	IFN-γ (100 U)	100	84 ± 3	235 ± 16	33 ± 5
	MCP-1 (1 ng)	100	87 ± 2	219 ± 8	30 ± 3
	IFN-γ (100 U) + MCP-1 (1 ng)	100	72 ± 4 *	159 ± 15 **	19 ± 2 **

[a] Mean number counted in three different coverslips
[b] Standard deviation
[c] Macrophages were infected with wild-type *L. major* parasites
* Significantly different when compared to either non-stimulated, IFN-γ or MCP-1 stimulated samples ($p < 0.05$)
** Significantly different when compared to either non-stimulated, IFN-γ or MCP-1 stimulated samples ($p < 0.01$)

stimulating them with two signals: a TLR agonist and IFN-γ. Classically activated macrophages make pro-inflammatory cytokines and NO, which allow them to kill intracellular pathogens such as *Leishmania*. CCR2-positive macrophages are similar in the sense that they also require two signals. After migrating to the site of infection, these CCR2-positive macrophages appear to require both MCP-1 and IFN-γ to enhance parasite killing. This suggests that transgenic parasites expressing MCP-1 both recruit and stimulate a restrictive population of CCR2-positive macrophages to efficiently kill *Leishmania* parasites.

Discussion

Mice lacking the CCR2 receptor on the C57BL/6 background developed relatively normal lesions when infected with Lm-Tg parasites. This suggests that signaling through the CCR2 receptor was required for the healing phenotype. In all of these strains, the phenotype caused by transgenic MCP-1 parasites was very dramatic. While the wild-type parasites caused large lesions with many parasites, the transgenic *L. major* MCP-1 parasites failed to cause measurable lesions and the number of parasites in the lesions and visceral organs was dramatically reduced.

Several experiments were performed to discover whether the CCR2-positive cells that caused the healing phenotype were part of the innate or adaptive immune response that develops in mice infected with transgenic MCP-1 parasites. We first observed that mice lacking a functional adaptive immune response (RAG KO mice) when infected with Lm-Tg developed the healing phenotype. The reproducible healing phenotype in RAG KO mice showed that lymphocytes were not required for the healing phenotype observed in mice previously infected with Lm-Tg. This also suggested that the immune cells responsible for the healing phenotype were part of innate immunity. Next, we observed that lymph node-derived lymphocytes obtained from mice infected with transgenic MCP-1 parasites were not able to proliferate or secrete IFN-γ or IL-4 in response to soluble *Leishmania* antigen, in contrast to lymphocytes from wild-type infected mice. The lack of T-cell proliferation and cytokine production suggested that the transgenic MCP-1 parasites were cleared by an innate immune response before an adaptive immune response was generated. The role of innate immunity in the healing phenotype caused by Lm-Tg was verified by a follow-up vaccination experiment. Mice that were infected in

the footpad with transgenic MCP-1 parasites were not protected 5 weeks later when re-infected in the ear with wild-type parasites. However, mice previously infected with wild-type parasites in the footpad were completely protected when similarly challenged. These results are consistent with the idea that mice infected with transgenic MCP-1 parasites did not develop a detectable adaptive immune response. We therefore decided to focus on the innate immune response in mice infected with transgenic MCP-1 parasites. In an attempt to identify the early events that could be responsible for these lesion differences, we focused on the cells migrating into infected ears during the first week. On day 7 post-infection, there was a 50% increase in the number of CCR2-positive macrophages migrating into the transgenic MCP-1 infected ears relative to the wild-type infected ears. The increased CCR2-positive macrophage migration correlated with significant differences in parasite levels between wild-type and Lm-Tg infected ear lesions in both BALB/c and C57BL/6 mice. These differences in parasite numbers were detected as early as 7 days post-infection and they continued to increase over time. The CCR2-positive macrophages that were sorted on days 7, 14, and 21 post-infection from Lm-Tg infected BALB/c and C57BL/6 ears contained few if any parasites, whereas CCR2-positive macrophages from wild-type infected ears were heavily infected. In contrast, there were no differences in parasite levels in macrophages from infected CCR2 KO mice. These data suggests that transgenic *L. major* MCP-1 may recruit a population of CCR2-positive macrophages that prevent intracellular parasite growth and survival.

One potential scenario that could account for the lack of lesion formation in Lm-Tg infected mice is that macrophages may become activated by MCP-1, leading to

parasite killing. This would be consistent with previous in vitro observations of others[85-89].

A second possibility is that a specific CCR2-positive macrophage subset may be recruited into the lesion and this subset may be particularly adept at killing intracellular organisms. Recently, a CCR2-positive monocyte population has been identified that migrates into inflammatory sites and is believed to play a role in pathogen clearance[5;106]. In addition, a CCR2-positive Gr-1-positive macrophage population was shown to control toxoplasmosis[6;7]. This population, like our restrictive CCR2-positive macrophage population, expresses CCR2 and controls parasite dissemination. However, the population was also reported to express Gr-1, a marker that we did not detect on our CCR2-positive macrophages.

Our in-vitro MCP-1 activation studies using MCP-1 recruited macrophages (Table 3) suggests that both scenarios may be true. We suggest that the transgenic MCP-1 secreting parasites recruit restrictive CCR2-positive macrophages to the site of infection. The MCP-1 produced by the transgenic parasites also helps to activate the CCR2-positive macrophages making them particularly adept at killing *Leishmania* parasites.

Others have shown that CCR2 KO mice on a C57BL/6 background infected with *L. major* developed larger ear lesions with higher numbers of parasites compared to infected CCR2 +/+ C57BL/6 mice[82]. This increased disease severity in infected CCR2 KO mice was attributed to a lack of Langerhans cell (LCs) migration from the ear to the lymph node, thereby affecting antigen presentation to T-cells. Others have questioned the role of LCs in antigen presentation during leishmaniasis[107]. Although we

acknowledge that the lack of DC migration (whether LCs or dermal DCs) contributes to increased lesion progression in wild-type *L. major* infected CCR2 KO, we would speculate that the increased disease severity could also be attributed to a lack of recruitment of the CCR2-positive macrophages into the infected ear late in the infection.

Previously, it has been shown that *L. major* induces MCP-1 production in lymph nodes of C3H mice resistant to *L. major*, but does not induce MCP-1 in BALB/c mice susceptible to *L. major*[108]. In addition, MCP-1 and CCR2 mRNA were detected in the footpads of resistant C57BL/6 mice infected with *L. major* between 2-4 weeks post-infection[109]. Based on our findings with the MCP-1 transgenic parasites, it is possible that the MCP-1 produced in resistant mouse strains is the main chemoattractant recruiting a restrictive CCR2-positive macrophage subset to the localized site of infection. These macrophages may assist in resolving the lesions when co-activated with MCP-1, while the lack of MCP-1 production may prevent any such CCR2-positive macrophage recruitment from ever occurring in susceptible BALB/c mice. A similar idea can be used to explain the events that lead to self-healing cutaneous leishmaniasis in humans, as other groups have already discovered the presence of MCP-1 in self-healing cutaneous lesions and the absence of MCP-1 in the non-healing diffuse cutaneous leishmaniasis[75-77]. The MCP-1 present in self-healing lesions may recruit and co-activate restrictive CCR2-positive macrophages to the site of infection to kill the parasites and resolve the lesions.

Future studies will attempt to identify subpopulations of CCR2-positive macrophages and biochemically define the macrophage subset recruited by the parasite-derived MCP-1 that is important for early parasite control during leishmaniasis.

Overall, we feel that the transgenic MCP-1 secreting parasites are infecting macrophages and producing MCP-1 intracellularly. The parasite-derived MCP-1 accumulates within the infected macrophages until the cell bursts due to parasite replication, releasing transgenic MCP-1 amastigotes and also parasite-derived MCP-1. The parasite-derived MCP-1 recruits restrictive CCR2-positive macrophages to the site of infection and these macrophages are optimally activated with MCP-1 and IFN-γ making the restrictive cells particularly adept at killing *Leishmania* parasites.

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