PARASITE INTERACTIONS WITH DENDRITIC CELLS AND MACROPHAGES: IMPLICATIONS FOR CUTANEOUS LEISHMANIASIS CAUSED BY *LEISHMANIA AMAZONENSIS*

APPROVED BY THE SUPERVISORY COMMITTEE

_____
Lynn Soong, M.D., Ph.D.

_____
David M. Mosser, Ph.D.

_____
Vivian L. Braciale, Ph.D.

_____
Barbara L. Doughty, Ph.D.

_____
Rolf König, Ph.D.

_____
Joseph M. Vinetz, M.D.

_____
Dean, Graduate School

PARASITE INTERACTIONS WITH DENDRITIC CELLS AND MACROPHAGES: IMPLICATIONS FOR CUTANEOUS LEISHMANIASIS CAUSED BY *LEISHMANIA AMAZONENSIS*

by

Hai Qi

DISSERTATION

Presented to the Faculty

of

THE UNIVERSITY OF TEXAS
GRADUATE SCHOOL OF BIOMEDICAL SCIENCES AT GALVESTON

in partial fulfillment

of the requirements

for the degree of

Doctor of Philosophy

THE UNIVERSITY OF TEXAS MEDICAL BRANCH
AT GALVESTON

May 2003.

To my wife, the brightest star shinning in my heaven, and to my parents, for giving me this life so full of joy, wonder, and inspiration.

谨献给我的妻子与父母。因为他们，我的生活充满幸福与希望。

ACKNOLEDGEMENTS

I am forever indebted to my mentor, Dr. Lynn Soong. When I first came to her lab, my knowledge and skills were very limited. Rather than being too critical, she showed tremendous patience for me to learn the unknown and gain the skill. I also thank her for giving me the freedom and opportunity to explore on my own—even though that meant an increased risk of failures. Her scientific guidance and moral support have been a source of strength that I can rely on to confront and overcome difficulties, and none of my accomplishments would have been possible without it.

I also want to express my deepest gratitude to Dr. Vivian Braciale for her tremendous contribution to my graduate education. She is one of the few faculty members who are willing to spend hour upon hour in scientific discussion with a “mere” student like myself. I have learned volumes by simply listening to her insightful comments on many of my questions. Her utter enthusiasm for immunology is infectious and has helped to inspire my desire to become an immunologist. I am also very much grateful to Dr. Rolf König for his tireless support of my dissertation research. Whenever needed, Dr. König has always provided expert assistance and shared his ideas. I must especially thank him for providing valuable career advice and consultation.

I thank all the members of my dissertation committee, Dr. Lynn Soong, Dr. Vivian Braciale, Dr. Barbara Doughty, Dr. Rolf König, Dr. David Mosser, and Dr. Joseph Vinetz, for their guidance, advice, and criticism. Their investment of time and effort towards my graduate training is very much appreciated.

I want to thank present and past members of Dr. Soong's laboratory who have provided valuable assistance during my training. They are Dr. Jiaxiang Ji, Mr. Xin Li, Ms. Kimberly Campbell, Ms. Hong Diao, and Ms. Nanchaya Wanasen. Many people from other laboratories at UTMB or other institutions have also been tremendously helpful to my education. In particular, I want to thank Dr. Vsevolod Popov for his expertise on electron microscopy, which has been crucial to one of my research projects. I thank Dr. James Leary for his excellent class on flow cytometry and Mr. Mark Griffin for teaching me the practical operation of a cytometer. Such knowledge and skills have been instrumental to my dissertation study and future research in immunology. I acknowledge Dr. Xuejie Yu, Dr. Tim Denning, Dr. Helene Häeberle, Dr. Roberto Garofalo, Dr. Tonyia Eaves-Pyles, Dr. Xiaoli Shen, and Dr. Wenhong Zhou for stimulating discussions and for collaborative efforts in my studies. I thank Dr. David Sacks and Dr. Yasmine Belkaid at NIAID for providing reagents and sharing thoughts on numerous occasions. I thank Dr.

Bali Pulendran at the Emory University for sharing his expertise on dendritic cell biology and offering constructive criticism on my manuscripts.

I thank Dr. Norbert Herzog and Ms. Harriet Ross for their excellent work in administering the Experimental Pathology program that has been home for me for the last five and half years. Since Dr. Soong's laboratory is primarily hosted in the Department of Microbiology and Immunology, I have also enjoyed all the benefits provided by the Microbiology and Immunology graduate program, thank to ex-director Dr. David Niessel, current director Dr. Thomas Hughes, and the coordinator Ms. Martha Lewis. I must also thank Ms. Theresa Kingsley, Ms. Sonia Gonzalez, Ms. Sarah Daniel, Ms. Sandra Vasquez, Ms. Lyska Morrison, Ms. Mardelle Susman, and Ms. Tresha Goldsmith for their excellent administrative and secretarial work, without which I would have failed on too many things to mention. I thank Dr. Robert Bennett for his editorial assistance during my preparation of this document. I also want to extend my gratitude to the McLaughlin Fellowship Fund for its generous financial support of my dissertation research.

On a more personal level, I want to thank many friends who I have been so fortunate to know during my study at UTMB. When I first came to this country, Dr. Xuejie Yu and Dr. Jingxin Qiu helped me tremendously during the initial settling period and made my adjustment to a completely unfamiliar environment a smooth one. Their continuous caring friendships are why I find

my life here enjoyable. I am very grateful to Ms. Kimberly Campbell, my fellow student and wonderful companion in Dr. Soong's lab, for being an excellent English teacher and sharing fascinating conversations about American culture, religions, and politics with me. I would also like to thank Dr. Charlie Gullo and Dr. Rania Milleron, who have long left UTMB but whose friendship to me will always be memorable. I must thank my dear friend Dr. Helene Häeberle, whose characteristic German will to succeed is what I truly admire and look up to whenever I have to confront a difficult task. I would like to thank Dr. Bali Pulendran for his sheer willingness to befriend a student, despite being an established scientist well known to his field. His warm friendship and enthusiastic support for my career development is one reason that I feel academia is so enjoyable. It has been my privilege and fortune to come to know Dr. Tim Denning. As a fellow student and a brilliant scientist, Tim has shared with me countless inspiring and educational discussions from which I learned volumes. His passion, high standard, and scientific vigor for research represent what I aspire to. As a dear friend, he has always been the first to extend a helping hand whenever I needed. We had a dream to publish *Nature* papers together, but we have not been able to, yet our untiring attempts have made the training so inspirational and forever memorable.

I thank my family, particularly my parents, who have given me this life full of joy, wonder, and inspiration. Mom and Dad, for your unconditional love and support, I will do my best to make you proud.

Finally, I want to thank my beautiful loving wife, Ying Hong. Throughout the years, she has selflessly stood by me and tirelessly supported me for being what I want to be. Ying, because of you, I want to live my life to its fullest potential; because of you, I have the courage to go from failure to failure with a sense of destiny.

PARASITE INTERACTIONS WITH DENDRITIC CELLS AND MACROPHAGES: IMPLICATIONS FOR CUTANEOUS LEISHMANIASIS CAUSED BY *LEISHMANIA AMAZONENSIS*

Publication No. _____

Hai Qi, M.D.

The University of Texas Medical Branch
at Galveston, 2003

Supervising Professor Lynn Soong, M.D., Ph.D.

Protozoan *Leishmania* is an important human pathogen that affects millions people worldwide. Investigation of experimental *Leishmania* infection in mice has been instrumental to our understanding of interactions between the parasite and the host immune system. Previous studies have established the model of Th1-Th2 paradigm: interferon (IFN)-γ-secreting Th1 cells protect the host from developing progressive diseases, while interleukin (IL)-4-producing Th2 cells drive the disease pathogenesis. Focused on *L. amazonensis* infection in mice, this dissertation study is mainly intended to understand the cellular mechanism underlying the generation of parasite-specific Th2 cells and to ascertain the role for IFN-γ in parasite-macrophage (MΦ) interactions. We showed that *L. amazonensis* parasites infected and activated dendritic cells (DCs), a population of phagocytic antigen-presenting cells specialized in activating naïve T cells. We found that DCs from susceptible or resistant mice differentially responded to amastigotes in CD40-dependent cytokine production and that amastigote-infected DCs favor Th2 priming in susceptible but not resistant mice. IFN-γ is believed to be crucial for activating MΦs to kill intracellular parasites such as *L. major*. However, we found that *L. amazonensis* amastigotes but not promastigotes could not only survive but also replicate better in IFN-γ-activated MΦs. The promastigote was evidently killed in IFN-γ-activated MΦs. On the other hand, MΦs activated with IFN-γ and LPS were able to limit intracellular amastigote replication. When tested *in vivo*, endogenous IFN-γ apparently

exerted minimal effects on the course of amastigote infection. It is likely that IFN-γ plays a bidirectional role during *L. amazonensis* infection: when optimally coupled with other factors, it can activate MΦs to control parasite infection; while in the absence of such synergy, it would promote amastigote propagation by itself. Collectively, results presented in this dissertation have pointed to the unique ability of *L. amazonensis* amastigotes to modulate host immune system to the advantage of their own survival.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
Ag	Antigen
B10	C57BL/10
B6	C57BL/6
BM	Bone marrow
CCR	CC chemokine receptor
CR	Complement receptor
DC	Dendritic cell
DLN	Draining lymph node
DTH	Delayed-type hypersensitivity
GPI	Glycosylphosphotidylinositol
IFN	Interferon
IL	Interleukin
La	*Leishmania amazonensis*
LACK	*Leishmania* homolog of receptors for activated C kinase
LC	Langerhans cell
LPG	Lipophosphoglycan
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MΦ	Macrophage
RAG	Recombinase activating gene
SCID	Severe combined immunodeficiency
Th	T helper
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α

1 *LEISHMANIA* AND LEISHMANIASES

From Leishman-Donovan bodies to *Leishmania donovani*

Leishmania parasites are named after Sir William Boog Leishman, a British army surgeon who served in India during his early military career. To the surprise of his comrades in India, Leishman took with him a microscope (Scott, 1939). Among other tropical diseases affecting British soldiers in India, he was interested in studying kala-azar, a prevalent and often fatal tropical fever accompanied by severe anemia, progressive weight loss, and enlarged liver and spleen. As can be expected, the cause of kala-azar was not known at the time. While little historical accounts are available on how Leishman's initial work was conducted, it is probably safe to assume that he was not testing a hypothesis of any sort, unlike the way we are compelled to do today. What is clear though is that his curiosity remained strong despite his apparently fruitless work in India that had hardly yielded any hints on the cause of Kala-azar. In November of 1900, already back to England working as an Assistant Professor of Pathology in the Army Medical School, Leishman did an autopsy on a soldier who died shortly after being invalided home to Britain from Dum-

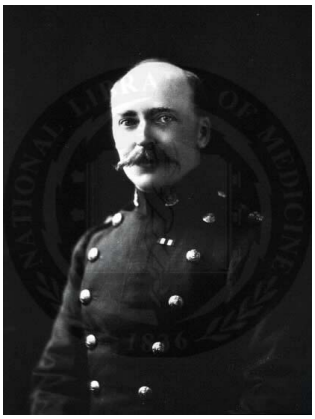

Illustration 1. Sir William B. Leishman (1865-1926). *Photo courtesy of the National Library of Medicine.*

dum of Calcutta (now Kolkata), India. At the time, Dum-dum was a site of British army training camps and arsenal, and many soldiers contracted chronic illness there. Their illness was characterized by irregularly intermittent type of fever, anaemia, progressive muscular atrophy, and enlargement of the spleen (Leishman, 1903). Leishman referred to these cases as Dum-Dum fever. This might be a more revealing name than Kala-azar, considering the fact that Dum-Dum was also the place where British army first produced so-named dumdum bullets, which are so lethal that they have been banned by the Geneva Convention. Leishman did smear preparations of the spleen from that deceased soldier and, under microscope, found small oval bodies of 2 to 3 micron in diameter, which were usually isolated, but occasionally aggregated into "clumps composed of twenty to fifty members" (Leishman, 1903). Perhaps the most interesting observation was that when stained with Romanowsky's method[1], these bodies displayed two separated masses of chromatin, a lager one in circular form and a smaller short rod-like one. While having no immediate explanation for these bodies, Leishman proposed, in a paper published in the *British Medical Journal* on May 30th of 1903, that these bodies were trypanosomes undergoing *post-mortem* degeneration. This view was

[1] In 1900, Leishman developed a simple method for staining malaria and other parasites by modifying the original Romanowsky staining protocol. This is so-called Leishman's stain, which contains Methylene blue and eosin (Scott, H. H. (1939). Leishmaniases. In *A history of tropical medicine* (Baltimore, Williams & Wilkins Co.), pp. 547-570.)

supported by his additional observations of similar “degenerate” bodies in rats that died of experimental trypanosomiasis (Leishman, 1903).

Leishman’s proposed explanation was soon questioned by Captain Charles Donovan, an army physician working for the Government General Hospital in Madras (now Chennai), India. Since April of that year, Donovan had done autopsies on three native Indians “said to have died of chronic malaria” and found unknown bodies from their spleens, which, subjected to the same Romanowsky staining technique, exhibited extreme similarity to what Leishman described. Most importantly, Donovan also found these bodies in the blood and spleen tissues obtained *intra vitam* from a 12-years-old Indian boy, who suffered from “irregular pyrexia” but had no malarial parasites and “nothing resembling trypanosomata in the peripheral blood.” Donovan reported these findings in a short memorandum published in the July 11th issue of the *British Medical Journal* (Donovan, 1903) and suggested that these bodies were not likely to be something merely a result of *post-mortem* degeneration of either trypanosomes or malaria. Considering that the year of 1903 was even before the development of teleprinting machines, what Donovan did feels like a real miracle: he was able to read Leishman’s paper, write the memo, send it back to London, and get it published all in 42 days! (In the year of 2003, it takes almost two weeks to receive my copy of *Science* in Galveston.) To clarify the issue, Donovan sent his samples to the Professor of Tropical

Medicine at the University of Liverpool, Major Ross. Ross invited Leishman to examine these samples together and concluded that Donovan and Leishman were seeing exactly the same organism. He suggested that these Leishman-Donovan bodies did not represent disintegrating trypanosomes but new "parasites of some kind, probably protozoa" and that clinical cases associated with these bodies were most likely to be kala-azar rather than malaria or trypanosomiasis (Ross, 1903). Subsequently, the genus *Leishmania* was created and the parasite that Leishman-Donovan bodies represented was properly named as *Leishmania (L.) donovani*.

According to current taxonomy, *Leishmania* genus belongs to the Subkingdom Protozoa, Phylum Sarcomastigophora, Order Kinetoplastida, and Family Trypanosomatidae (Mendoza-Leon *et al.*, 1996). There are a large number of *Leishmania* species in addition to the founding member *L. Donovani* (Dedet, 2002; Lainson and Shaw, 1987). Approximately 20 of them are known human pathogens. They significantly differ in geological distribution, insect vectors, vertebrate reservoirs, and clinical manifestations of human diseases.

Life cycle of *Leishmania* parasites

One criterion Ross originally used to differentiate Leishman-Donovan bodies from "involution stages of trypanosomes" was the absence of flagella in

the former (Ross, 1903). Since Rogers succeeded in cultivating these organisms in 1904 and noted that they passed into a flagellated stage in culture (Scott, 1939), it was clear that these parasites had at least two developmental stages. This finding led Leishman to suggest the probability of an insect vector, probably because of knowledge on malaria transmission already available at the time. Shortt *et al.* provided hints on the identity of such vectors as they demonstrated that hamsters could be infected by *L. donovani* through the bite of sandfly *Phlebotomus (P.) argentipes* (Shortt *et al.*, 1931). A decade later, the transmission of *L. tropica* and *L. donovani* to man by infected *P. papatasi* and *P. argentipes*, respectively, was demonstrated (Adler and Ber, 1941; Swaminath *et al.*, 1942). These findings, together with those by others (Lewis and Ward, 1987), have led to our current understanding of the *Leishmania* life cycle (*Illustration 2*; also, an animated version can be viewed on the WHO/TDR website). All *Leishmania* parasites exist in two forms: the flagellated promastigote, which parasitize sandflies, and the aflagellated amastigote, which results from transformation of sandfly-delivered promastigotes and live inside macrophages (MΦs) of the vertebrate host. Both promastigote and amastigote replicate asexually by binary fission.

The life cycle of *Leishmania* parasites in sandflies is a complicated multi-stage developmental process (Kamhawi, 2002; Lewis and Ward, 1987), which is not apparent in *Illustration 2* and well beyond the scope of this

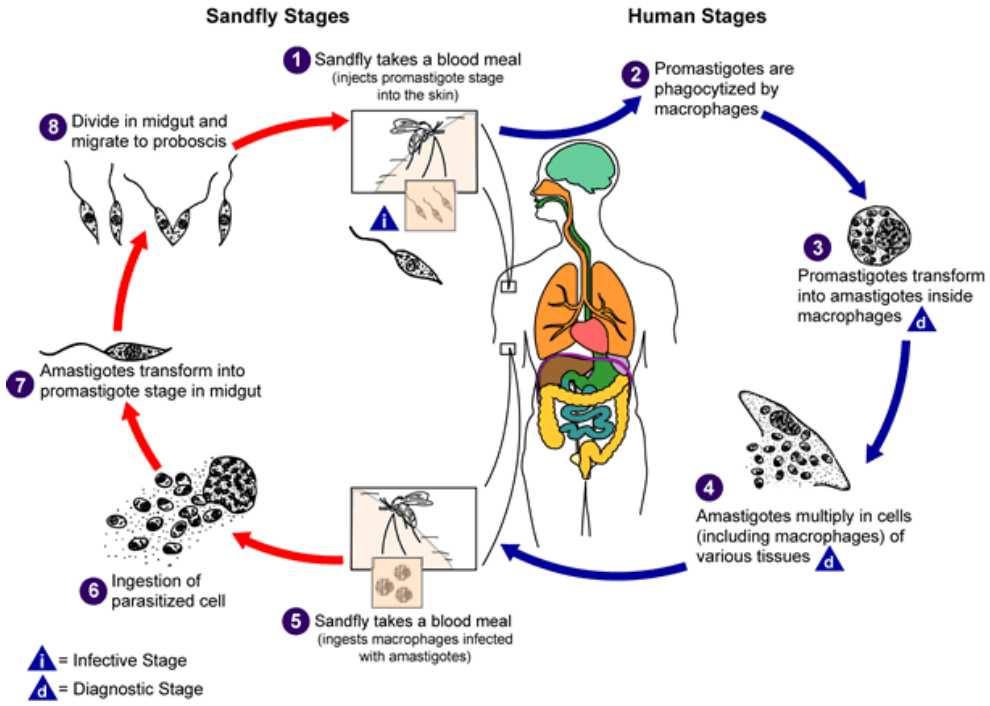

Illustration 2. The life cycle of *Leishmania* parasites. *Leishmania* parasites are transmitted by female phlebotomine sandflies. During a blood meal through biting a man, sandflies inject infective promastigotes ❶, which are phagocytosed by tissue macrophages around the puncture wound ❷. Flagellated promastigotes eventually transform into aflagellated amastigotes inside phagolysosomes of macrophages ❸. All diagnoses are virtually made from this stage on. Amastigotes multiply in infected cells and affect different tissues, depending in part on the parasite species ❹. This originates the clinical manifestations of leishmaniasis. Sandflies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes (❺, ❻). In the sandfly's midgut, amastigotes transform back into promastigotes ❼, which multiply and migrate to the proboscis ❽. *Diagram adapted from the Parasite Image Library, Division of Parasitic Diseases, Centers for Disease Control and Prevention.*

dissertation. However, *Leishmania* metacyclogenesis, a differentiation process by which parasites become pre-adapted to the invasion of vertebrate hosts while still in insect vectors, is certainly of medical significance and of close relevance to my studies. Sacks and Perkins compared promastigotes recovered from sandflies at different time points after a blood meal and found that these parasites became progressively more virulent to mice over time

(Sacks, 1989; Sacks and Perkins, 1984; Sacks and Perkins, 1985). This differentiation phenomenon was further confirmed with cultured promastigotes of various species (Blackwell *et al.*, 1985; Franke *et al.*, 1985; Rizvi *et al.*, 1985; Sacks and Perkins, 1984). When less virulent procyclic *L. major* promastigotes (or procyclics) differentiate to metacyclic promastigotes (or metacyclics), the dominant surface glycoconjugates, lipophosphoglycan (LPG), undergoes major structural modifications. Procyclics express shorter LPG with terminal β-galactose residues that bind to lectin-like molecules on the epithelium of the sandfly gut. During metacyclogenesis, terminal β-galactose residues are capped with α-arabinose and the chain of repeating disaccharide units is extended by two- to three-fold in length (McConville *et al.*, 1992). Due to such changes, parasite binding to the sandfly gut is blocked and metacyclics can be released for transmission (Pimenta *et al.*, 1992). Further, these changes increase parasite resistance to complement-mediated lysis and to intracellular killing by MΦs (Kamhawi, 2002; Turco and Descoteaux, 1992).

Epidemiology of human leishmaniases

Based on ecological studies, leishmaniases are largely zoonotic (Ashford, 1996). Many *Leishmania* species are maintained in reservoir vertebrate hosts such as rodents and canines. Animals can have severe clinical diseases upon infections with some *Leishmania* species but remain

asymptomatic with others. Human infection is often incidental despite being productive and transmissible. For example, *L. major* in the Old World is maintained in an enzootic cycle that involves the great gerbil, *Rhombomys opimus*, as the reservoir and *P. papatasi* as the vector (Ashford and Bettini, 1987). *L. amazonensis* (*La*) in the New World is maintained by the spiny rat *Proechimys guyanensis* and sandfly *Lutzomyia flaviscutellata* (Shaw and Lainson, 1987). In addition, wild and domestic dogs are the main reservoir for *L. infantum* in Europe, Africa and Asia and for *L. chagasi* in Americas (Campino, 2002). However, an important but complicated exception is *L. donovani*. In India, man is the only known vertebrate host for this parasite. Yet, abundant dogs and rats are found to be infected with *L. donovani* in Africa, and they presumably constitute a reservoir in an enzootic cycle (Ashford and Bettini, 1987).

Leishmania infection in human is distributed throughout the world. Based on statistics provided by World Health Organization (WHO Communicable Disease Surveillance and Response: Leishmaniasis), it currently affects at least 12 million people in 88 countries, with 350 million at risk worldwide. Human leishmaniases are presented with a spectrum of clinical manifestations mainly in four types: cutaneous, mucocutaneous, diffuse cutaneous and visceral forms (*Illustration 3*). The number of new cases is estimated to be 1 to 1.5 million for cutaneous and 500,000 for visceral

leishmaniases annually. While cases of all forms can be seen in most

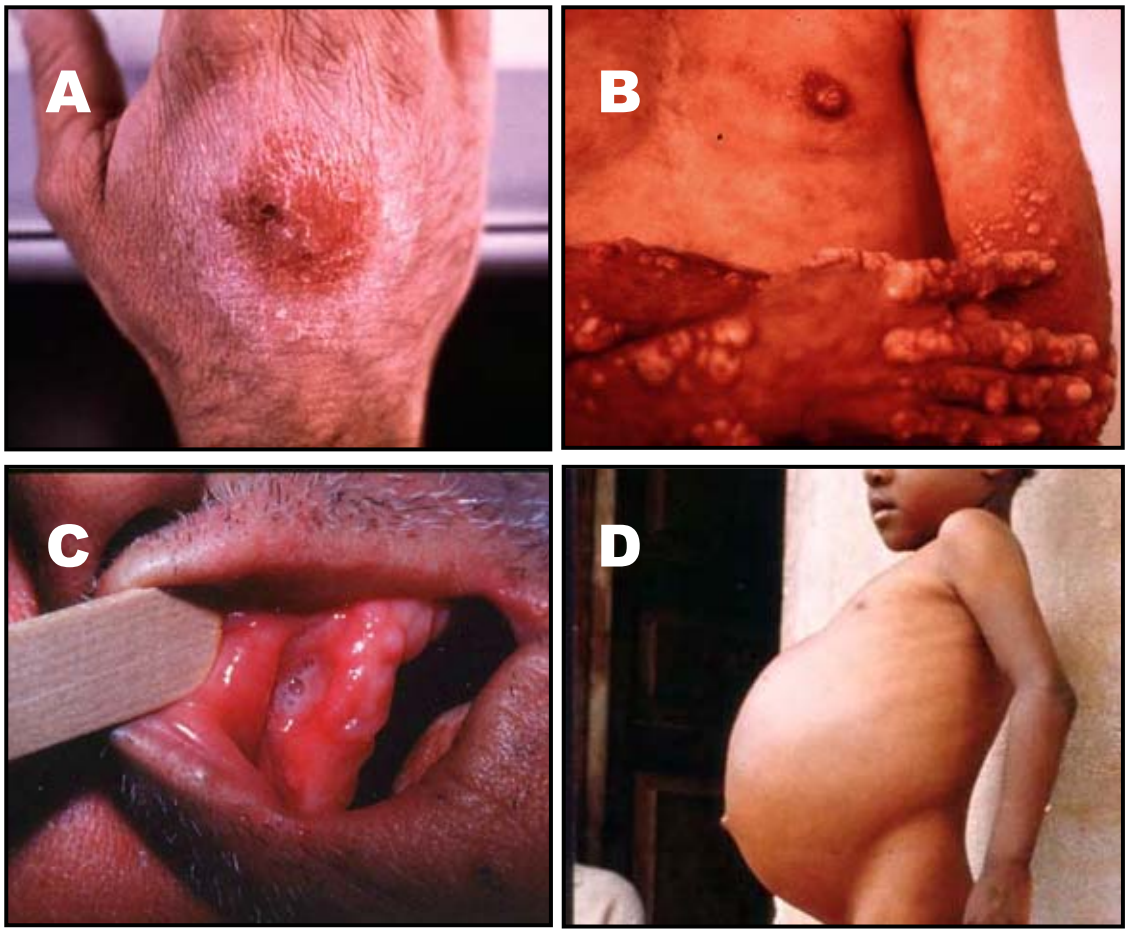

Illustration 3. Various clinical manifestations of leishmaniasis. ***A.*** An initial skin lesion due to cutaneous leishmaniasis (Switzerland, 1990). ***B.*** Numerous lesions on forearms of a patient with diffuse cutaneous leishmaniasis (Switzerland, 1990). ***C.*** Lesions in the oral cavity due to mucocutaneous leishmaniasis in a patient who was also HIV seropositive (Photographed by Dr. D. Carnaciba, Brazil, 1991). ***D.*** Outward signs of visceral leishmaniasis: muscle wasting and markedly distended abdomen due to enlargement of liver and spleen (photographed by Dr. P. Desjeux, Bolivia, 1992). *All photos courtesy of WHO/TDR.*

of the endemic regions, there clearly are regions in which cases of a particular form are concentrated. For example, 90% of all cutaneous and diffuse cutaneous cases occur in Afghanistan, Iran, Saudi Arabia, Syria, Brazil, and Peru; 90% of patients with mucocutaneous leishmaniases are seen in Bolivia,

Brazil, and Peru; 90% of people who contract visceral leishmaniases are in Bangladesh, India, Nepal, Sudan, and Brazil. For a particular parasite species, clinical manifestations are not uniform but can be quite diverse among infected individuals. For example, age and gender differences are readily observable in *L. chagasi* infection in the Latin America and in *L. donovani* infection in India.

Over the last decade, an unexplained sharp increase of leishmaniasis cases was seen in many endemic areas. For example, Afghanistan had 14,200 new cases of cutaneous leishmaniasis but the number went up to 200,000 in 1999. Even more troubling is the fact that an increasing number of leishmaniasis patients are co-infected with HIV and such co-infection greatly precipitates progression of both diseases (Alvar *et al.*, 1997).

The U.S. has apparently seen few if any native cases of human leishmaniasis, despite having vector-competent sandflies and potential reservoir hosts. On the other hand, Americans who travel to endemic regions are clearly at risk. For example, some soldiers returning from the Operation Desert Storm were diagnosed as having visceral leishmaniasis caused by *L. tropica* (Magill *et al.*, 1992; Magill *et al.*, 1993; Magill *et al.*, 1994). Interestingly, approximately 12% of 11,000 hunting dogs tested in 21 states of the U.S. and the Ontario province of Canada were found to be infected by *Leishmania* parasites, although most of them remained asymptomatic. These findings

highlight the possibility of *Leishmania* enzootics in the North America (Enserink, 2000).

Given the enormous variation of biological and clinical properties of leishmaniases, development of general therapeutics remains a daunting challenge, if at all possible. Individualized therapy is clearly desirable but remains to be a distant future, as *Leishmania* infection mostly affects the poorest rural population of developing countries, to which accurate diagnosis is not even readily available. Most of current chemotherapies have been defined empirically rather than developed systematically based on accurate understanding of the disease pathogenesis. Nonetheless, recent development of Miltefosine (Jha *et al.*, 1999; Murray, 2000; Sundar *et al.*, 2002), an abandoned anti-cancer drug turned anti-*Leishmania*, may help India eventually eliminate kala-azar, as this drug produces a cure rate approaching 100% against visceral leishmaniasis caused by *L. donovani* (WHO TDR News no. 68, June 2002).

Given the enzootic feature of many *Leishmania* parasites, vector and reservoir control can be an effective strategy for disease prevention, particularly in domestic or peridomestic situations (Grimaldi and Tesh, 1993). It is worth of noting that in 1950s, Chinese government had a campaign of systematic house-spraying of DDT and wholesale onslaught on dogs, and the incidence of visceral leishmaniasis in China has significantly reduced ever

since. While such draconian measures could only be possible in that era, the result does emphasize the effectiveness of vector and reservoir control.

Vaccination has been systematically pursued as a means to control human leishmaniases for almost 80 years. In *L. major*-endemic regions such as Israel, an ancient practice was to deliberately inoculate the pus from active lesions to non-exposed body areas of people who had not contracted leishmaniasis. The recipient of such crude “immunization” would develop self-healing lesion but life-long immunity against natural infection. Similarly, infected cultured *L. major* promastigotes were first used in Russia in 1937 to induce protection against natural infection (Campos-Neto, 2002). Over the years, a variety of subunit vaccines based on recombinant proteins or DNA plasmids have been tested in experimental animal models or in phase I or II clinical trials but vaccines effective to human leishmaniasis have not been produced (Modabber *et al.*, 1999). Failures of subunit vaccine development are not unique to leishmaniasis. Hepatitis B virus infection is perhaps the only disease for which an effective and widely-used subunit vaccine is available. The most successful vaccines, such as those for small pox, poliomyelitis, and yellow fever, have all been live attenuated ones. Apparently, in order to make those “dead parts” safe and effective as vaccines, we have to learn more from the living. Our vaccine-developing efforts would remain to be shooting in the dark unless we thoroughly understand the pathogenesis of specific pathogens

and host immune mechanisms in general. This dissertation study has been conducted with the hope that it could be one step toward this ultimate goal.

L. amazonensis and human infection

L. amazonensis (*La*) is the particular species under study in this dissertation. It is a New World species identified by Lainson and Shaw in 1972 (Lainson and Shaw, 1972). It is mainly localized to the Amazon Basin and the Bahia state of Brazil (Lainson and Shaw, 1987; Pratt and David, 1981). As mentioned above, this parasite is maintained in an enzootic cycle with the spiny rat *Proechimys guyanensis* as the primary reservoir host and sandfly *Lutzomyia flaviscutellata* as the sole vector (see *Illustration 4*). The infection rate of a wild population of *Proechimys* rats may be as high as 30% (Arias and Naiff, 1981). In these animals, infection is restricted to skin and cutaneous lesions are not pronounced (Shaw and Lainson, 1987). The prevalence of *La* infection in man is relatively low, presumably due to the fact that its vector *L. flaviscutellata* sandflies rarely bite man in large numbers (Lainson and Shaw, 1968; Shaw and Lainson, 1968). Infected people develop cutaneous or diffuse cutaneous leishmaniasis, and the latter is frequently incurable.

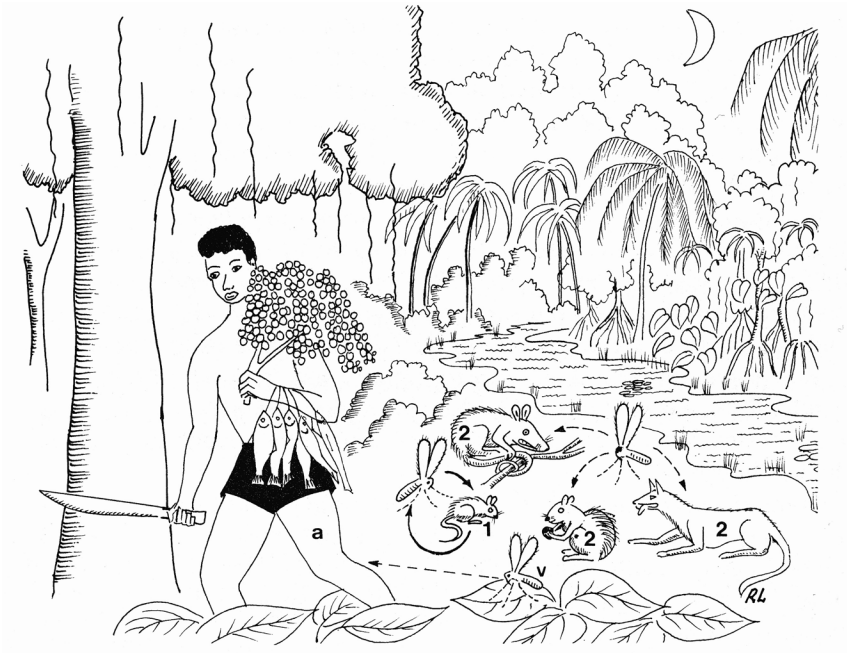

Illustration 4. The enzootic cycle of *Leishmania amazonensis* and its zoonotic transmission to human in the Amazon Basin. The spiny rat, *Proechimys guyanensis* (1) is the primary mammalian host for *Leishmania amazonensis*. Other wild mammals such as other terrestrial rodents and foxes could be secondary host (2). The only known vector (v) *Lutzomyia flaviscutellata* usually feeds at night and is not greatly attracted to man (a). Therefore, human is an accidental host in essence. *Original figure by R. Laison (1983) Trans. R. Soc. Trop. Med. Hyg. 77:569. Reproduced with permission from the Royal Society of Tropical Medicine and Hygiene (see Appendix).*

Pathology and clinical immunology of cutaneous leishmaniases

Cells of the mononuclear phagocytic system are the primary host cell for *Leishmania* parasites. On the other hand, smaller numbers of amastigotes can also be found in cells that are not normally phagocytic or of only a low phagocytic potential. These cells include vascular or lymphatic endothelial cells (Klotz and Lindenberg, 1923) and hepatocytes (Meleney, 1925). Human fibroblasts were also shown to be able to engulf certain species of *Leishmania*

parasites *in vitro* (Chang, 1978). It is worth noting that in 1960s, two groups independently reported that some cells resembling MΦs contained large numbers of amastigotes in the epidermis covering lesions of patients or of experimentally infected animals (Coutinho-Ablath and Coelho, 1965; Lainson and Strangway-Dixon, 1963a; Lainson and Strangway-Dixon, 1963b). Those authors suggested it was epidermal infiltration of infected MΦs, a hypothetical process that has not been demonstrated subsequently. With the benefit of hindsight, I feel what they observed might have been parasite-harboring epidermal dendritic cells (Langerhans cells), which resemble MΦs in many histological aspects but were not defined as a distinct lineage of phagocytes at the time. As to be discussed in the next chapter, the involvement of dendritic/Langerhans cells in harboring *Leishmania* parasites was not demonstrated until 1990s when it was first shown in experimentally infected mice (Moll *et al.*, 1993) and then in skin biopsies of infected men (Gaafar *et al.*, 1995a; Gaafar *et al.*, 1999). Earlier studies have also revealed in skin lesions the existence of extra cellular *Leishmania* amastigotes (Convit, 1958), which were probably in transit from lysed to new host cells.

In addition to the essential feature of amastigote colonization of mononuclear phagocytes system, the development of cutaneous leishmaniasis is also associated with lesional infiltration of other immune cells such as T lymphocytes and plasma cells. Incorporating studies of human

cases in different areas such as Ethiopia, Namibia, Greece, and Brazil (Bryceson, 1969; Grove, 1978; Nicolis *et al.*, 1978), Ridley has proposed a five-group classification for the wide histopathological spectrum of cutaneous leishmaniases, from the anergic form with heavily infected MΦs to the hypersensitive or allergic form with scarce amastigotes and a tuberculoid response (Ridley, 1979; Ridley, 1980; Ridley *et al.*, 1980). The anergic form, Ridley Group I, is characterized by extensive aggregation of heavily infected MΦs, which has no signs of activation and is accompanied by scarce lymphocyte infiltration with no necrosis at the histological level or ulceration at the gross level. This type of lesions is not self-healing, frequently associated with diffuse cutaneous form of the disease, and requires active treatment. On the other extreme of the spectrum, the allergic form or Ridley Group V, exhibits a good tuberculoid response characterized by epitheloid granuloma, large Langhans giant cells, and lymphocyte infiltration to various degrees. Importantly, while parasites usually are not found in histological sections, lesions of this type are also not self-healing and poorly respond to treatments, suggesting the pathology is triggered and perpetuated by the host response that can efficiently limit parasite propagation but not itself. When treated with anti-*Leishmania* drugs, the anergic pathology (Group I) would evolve into a form that is characterized by heavy lymphocyte infiltration, suggesting immune activation, and giant cells of foreign body and Langhans type, indicating active

clearance of the parasite. This is Ridley Group IV pathology that would eventually heal. The other two types of pathology as classified by Ridley, Group II and III, are characterized with focalized granuloma, some primitive giant cells, central necrosis of the granuloma, and significant levels of lymphocyte and plasma cell infiltration. Since these types of pathology tend to spontaneously evolve to the Group IV form, it appears reasonable to view them as ongoing host responses that would lead to the successful resolution of the clinical disease.

In addition to accumulation of infected MΦs and infiltrating lymphocytes, pathological changes also occur to the epidermis and the connective tissue in the dermis during cutaneous leishmaniasis. For example, epidermal hyperplasia is frequently found as pseudo-epitheliomatous downgrowths deep into the dermis. In Ridley group II and III lesions, ulceration frequently occurs and leads to the ejection of free parasites and parasite-infected MΦs from the lesion after destruction of the epidermis. More often in cutaneous leishmaniasis of the New World than that of the Old World, dermal connective tissue can be severely disorganized and destructed, leading to significant influx of fibroblasts followed by bands of fibrosis (Ridley, 1987).

From the discussion above, it appears that the resolution of lesion pathology would require active host anti-*Leishmania* responses (e.g.

lymphocyte infiltration and MΦ activation seen in the Ridley Group IV). On the other hand, strong tuberculoid granuloma inflammation may perpetuate tissue damages even after parasites are largely cleared, as seen in the Ridley Group V. Thus, the host response must strike a balance between effective control of parasites and minimizing tissue damage in order to achieve a clinical cure of the disease.

The precise nature of protective or pathogenic anti-*Leishmania* immune responses in human is not clear. This is partly because only a limited number of methodologies are available for dissecting parasite-host interactions in human. Clinically measurable immunological parameters for a patient of cutaneous leishmaniasis are usually confined to the level of circulating antibodies, presence or absence of delayed type hypersensitivity (DTH), lymphocyte reaction to *Leishmania* antigens, and the phenotype and abundance of various immune cells in blood or in lesional tissues. None of these parameters are indicative of a state of resistance to the parasite or predictive of the disease outcome (Grimaldi and Tesh, 1993). For example, regardless whether their infections would resolve or become chronic, patients of cutaneous leishmaniasis mount strong antibody responses and their lesion biopsies exhibit numerous plasma cells that secret immunoglobulin of various isotypes (Anthony *et al.*, 1980; Behforouz *et al.*, 1976; Menzel and Bienzle, 1978; Moriearty *et al.*, 1982; Roffi *et al.*, 1980). Although it is clear that a self-

healing cutaneous infection is always associated with a strong DTH response (Carvalho *et al.*, 1985; Castes *et al.*, 1984; Mendonca *et al.*, 1986), as elicited by inoculation of leishmanin—a suspension of phenolized promastigotes, a non-healing disease, especially that of the mucocutaneous form, can also exhibit potent DTH responses (Grimaldi and Tesh, 1993).

Our lack of sufficient knowledge regarding to the nature of protective or pathogenic anti-*Leishmania* immune responses in human has made it imperative to study the parasite-host interactions in animal models. Indeed, our current understanding of the disease pathogenesis mainly comes from studies of experimental *Leishmania* infection in mice, which, as to be discussed in the next chapter, have produced the mechanistic model that can explain the complex parasite-host interactions and provide the theoretical foundation for development of anti-*Leishmania* vaccines (Grimaldi and Tesh, 1993; Mauel and Behin, 1987; Sacks and Noben-Trauth, 2002). Toward the end of next chapter, I will also summarize some of more recent clinical immunological studies of human leishmaniasis, which are essentially descriptive but very meaningful in the light of mechanistic studies in mice.

2 MURINE LEISHMANIASES AND PARASITE-HOST INTERACTIONS

Leishmania infection and Th1-Th2 dichotomy

Our knowledge of *Leishmania* pathogenesis has mainly come from studies of experimental infections in animals. The pursuit of suitable animal models had begun soon after the identification of *Leishmania* as a human pathogen. The earliest documented experimental *Leishmania* infection was that by Nicolle and Sicre, who in 1908 successfully produced clinical diseases in monkeys and dogs through inoculation of *Leishmania* parasites (Nicolle and Sicre, 1908a; Nicolle and Sicre, 1908b). *Leishmania* infection in mice was first developed by Laveran and Pettit in 1909 (Laveran and Pettit, 1909). Currently, *L. major* infection in mice is the most well-studied animal model for cutaneous leishmaniases.

Kellina was the first to describe differential susceptibilities of various inbred mice to *L. major* infection (Kellina, 1973). Subsequently, the exceptional susceptibility seen in BALB/c mice was found to correlate with the lack of parasite-specific DTH (Nasseri and Modabber, 1979). A study by Howard *et al.* offered the first evidence that such susceptibility was due to impairment of otherwise protective cell-mediated immune responses by “suppressor T cells” (Howard *et al.*, 1980). Since the discovery of type-1 and type-2 T helper cells

(Th1 and Th2) in murine T cell clones (Mosmann *et al.*, 1986), a large number of studies have indicated a crucial role of Th1/Th2 balance in determining the outcome of *L. major* infection in mice. Locksley *et al*. was the first to show that following footpad infection with *L. major*, the draining lymph node of non-healing BALB/c mice expressed a large amount of Interleukin(IL)-4, the signature Th2 cytokine, whereas resistant C57BL/6 (B6) mice expressed little IL-4 but much higher levels of Interferon(IFN)-γ (Locksley *et al.*, 1987). This was later confirmed by Morris *et al.* who examined *L. major* infection in 13 inbred strains of mice with various degrees of susceptibility and found the level of IL-4 production a very good indicator for the disease severity (Morris *et al.*, 1993). Subsequently, a series of Th cell transfer studies, involving immunodeficient or sublethally irradiated naïve mice as recipients, demonstrated that *Leishmania*-specific $CD4^+$ cells that conferred resistance to *L. major* infection produced Th1 cytokines, while those that exacerbated the disease produced a Th2-like pattern (Heinzel *et al.*, 1989; Holaday *et al.*, 1991; Moll *et al.*, 1988; Scott *et al.*, 1990; Scott *et al.*, 1988). Further, negation of biological effects of IL-4, either through the administration of an anti-IL-4 monoclonal antibody (mAb) (Chatelain *et al.*, 1992; Sadick *et al.*, 1990) or by genetic deletion of the *IL-4* gene (Kopf *et al.*, 1996), significantly ameliorate *L. major* infection in BALB/c mice. In parallel, B6 mice deficient in IFN-γ or its receptor were found to succumb to *L. major* infection, unlike their wild-type

counterparts (Swihart *et al.*, 1995; Wang *et al.*, 1994). Importantly, Belkaid *et al*. further confirmed the Th1-Th2 dichotomy in a natural model of *L. major* infection, in which 1000 metacyclics were given intradermally to mice (Belkaid *et al.*, 1998b). Since such an inoculum is very similar to natural transmission by sandflies (10-1000 metacyclics (Warburg and Schlein, 1986)), this study largely ruled out the possibility that Th1-Th2 dichotomy was an artifact due to the large-dose inoculum usually used (10^5 to 10^7 stationary-phase promastigotes). Taken together, these results have conclusively established that IL-4-dominated Th2 responses underlie the murine susceptibility to *L. major* infection, while the IFN-γ-dominated Th1 responses are responsible for the healing phenotype of B6 mice.

While the Th1-Th2 dichotomy in association with murine resistance to leishmaniasis has been largely, if not exclusively, built upon studies of *L. major* infection, this model may not be extrapolated to other *Leishmania* species in its entirety. For example, while most non-BALB strains are able to heal following *L. major* infection, they all fail to control *La* infection (Barral *et al.*, 1983; Colmenares *et al.*, 2002). While the resistance to *L. major* infection in B6 mice strictly requires $CD4^+$ T cells (Chakkalath *et al.*, 1995; Erb *et al.*, 1996; Holaday *et al.*, 1991; Mitchell, 1983), the disease in *La*-infected B6 mice is rather dependent on a functional $CD4^+$ T cell compartment. Soong and colleagues have shown that lesion development and tissue parasite load are

markedly reduced in nude mice and mice deficient in MHC class II molecules or Recominase Activating Gene (RAG)-2, and that reconstitution of $RAG2^{-/-}$ mice with wild-type naive $CD4^+$ T cells results in disease development (Soong *et al.*, 1997). While a deficiency in B cells does not change the course of *L. major* infection in either resistant B6 or susceptible BALB/c mice (Brown and Reiner, 1999), the disease caused by *La* infection requires the circulating antibody (Kima *et al.*, 2000). Collectively, these studies reveal some distinctive aspects of *La*-host interactions not seen in *L. major* infection and suggest a key role played by $CD4^+$ T cells during the pathogenesis of *La* infection.

Murine infection by *La* parasites in mice also shares some common features with that by *L. major*. For example, parasite-specific Th2 cells clearly promote parasite propagation and disease development following *La* infection: the disease in *La*-infected BALB/c mice can be fully controlled with anti-IL-4 treatment (Afonso and Scott, 1993), while its development can be significantly accelerated by the adoptive transfer of a *La*-specific Th2 cell line prior to infection (Ji *et al.*, 2002). On the other hand, Th2-dependent mechanisms could not fully account for the disease pathogenesis during *La* infection in C57BL mice. For example, the disease of *La*-infected C57BL/10 mice can be ameliorated but not fully cured by neutralizing anti-IL-4 treatment (Afonso and Scott, 1993). In correlation, B6 mice succumb to *La* infection without producing a high level of IL-4 (Soong *et al.*, 1996). Together, these results have

confirmed the pathogenic role for Th2 cells during *La* infection but also revealed the possibility that both Th2-dependent and Th2-independent mechanisms may operate in *La*-infected mice, particularly those on the C57BL background.

Th subset development during *Leishmania* infection

Given the fact that different effector subsets of $CD4^+$ T cells (e.g. Th1 and Th2 cells) are crucially involved in host interactions with *Leishmania* parasites, it is important to understand how Th1 and Th2 subsets are differentially generated during *Leishmania* infection.

The current concept is that the same naïve $CD4^+$ T cell is directed to assume distinct effector phenotypes by different cytokine environment that naïve T cells experience at the time of antigenic activation (Seder and Paul, 1994). Among cytokines that influence Th1 and Th2 differentiation, IL-12 and IL-4 are believed to exert the greatest impact (O'Garra, 1998). Shortly after its isolation and initial designation as the NK cell stimulatory factor (Kobayashi *et al.*, 1989), IL-12 was shown to strongly induce IFN-γ production from both NK and T cells (Chan *et al.*, 1991). With an *in vitro* priming system with purified $CD4^+$ T cells being stimulated with anti-CD3 monoclonal antibody (mAb), Seder *et al.* showed that IL-12 directly acted on T cells to promote their differentiation to IFN-γ-producing Th1 cells (Seder *et al.*, 1993). Further, Hsieh

et al. demonstrated that heat-killed *Listeria* bacteria could stimulate MΦs to promote Th1 differentiation by inducing their IL-12 production (Hsieh *et al.*, 1993). Similarly, the role of IL-4 in promoting Th2 differentiation was initially defined in T cell culture systems. It was found that when IL-4 was given to naïve T cells that were being activated with either polyclonal stimuli or specific antigens (Ags), the frequency of primed IL-4-producing Th2 cells was dramatically increased (Le Gros *et al.*, 1990; Swain *et al.*, 1990). These results on Th differentiation *in vitro* are the foundation on which studies of Th1 and Th2 polarization during *Leishmania* infection are interpreted.

In studies of interventional strategies aimed at reversing the course of *L. major* infection in BALB/c mice, it was found that all successful regimens must be given around the time of parasite infection. This included treatment with anti-CD4 (Sadick *et al.*, 1987; Titus *et al.*, 1985), anti-IL-2 (Heinzel *et al.*, 1993a), or anti-IL-4 mAbs (Sadick *et al.*, 1990) and administration of CTLA-4-Ig to block CD28-B7 interactions (Corry *et al.*, 1994). The common effect of these treatments was the reduction of early IL-4 expression in infected BALB/c mice. Based on these results and the fact that IL-4 is established as a potent Th2-driving cytokine as summarized above, a view has emerged that the early burst of IL-4 is responsible for driving subsequent Th2 polarization and disease progression in these mice (Reiner and Locksley, 1995). This view has been further supported by findings related to a dominant *L. major* Ag,

Leishmania homolog of receptors for activated C kinase (LACK). A series of work by Scott *et al.* led to the identification of a T cell clone that is capable of conferring resistance when adoptively transferred to BALB/c mice prior to *L. major* infection (Scott *et al.*, 1990; Scott *et al.*, 1988; Scott *et al.*, 1987a; Scott *et al.*, 1987b). Defining the Ag recognized by this T cell clone as LACK, Mougneau *et al.* showed that immunization with this single Ag with IL-12 as adjuvant was able to protect BALB/c mice against *L. major* infection (Mougneau *et al.*, 1995). The endogenous LACK-reactive T cells in BALB/c mice were found to be a population of $CD4^+$ T cells that express Vβ4Vα8 T cell receptors and constitute the main source for the IL-4 production shortly after *L. major* infection (Launois *et al.*, 1997). When this population of T cells is absent, either as a result of Vβ4-targeted superantigen-driven deletion or LACK-specific tolerance induced by its transgenic expression in the thymus, BALB/c mice are rendered able to mount a Th1 response and control the disease following *L. major* infection (Himmelrich *et al.*, 2000; Julia *et al.*, 1996). Together, these data strongly indicate that an early burst of IL-4 production by a small population of T cells that recognize a single Ag drives BALB/c mice onto a path of irreversible Th2 polarization and disease progression. However, these studies do not fully address the mechanism by which *L. major*-specific Th2 cells are generated in BALB/c mice. By all possible criteria, those LACK-reactive and IL-4-producing T cells are Th2 cells. Thus, an important unknown

is, by what mechanisms are these LACK-reactive T cells driven to produce IL-4 in the first place? Are they pre-programmed to do so once activated by Ags or is there an external trigger? Accumulating evidence indicate that an important external push could be responsible for the generation of Th2 effectors, such as those LACK-specific T cells activated during *L. major* infection. Detailed background information on this point is provided in the section *Leishmania-dendritic cell interactions*, beginning on page 34.

While aforementioned studies have established that the early IL-4 production from LACK-reactive T cells determines the susceptibility of BALB/c mice, they have not addressed the mechanism of Th1-mediated resistance in B6 mice. Interestingly, during the first week of infection, $CD4^+$ T cells from both B6 and BALB/c mice similarly produce IL-4 (Belkaid *et al.*, 2000a; Heinzel *et al.*, 1995; Morris *et al.*, 1992; Reiner *et al.*, 1994; Scott *et al.*, 1996). Further, LACK-reactive $CD4^+$ T cells were found to effect the early IL-4 production at a similar level in B6 and BALB/c mice (Julia and Glaichenhaus, 1999; Stetson *et al.*, 2002). The true distinction in the pattern of Th cytokines actually emerges one to two weeks after infection, when IL-4 is down-regulated in B6 but not in BALB/c mice (Sacks and Noben-Trauth, 2002). Convincing evidence suggest that in *L. major*-infected B6 mice, IL-12 is responsible for the timely down-regulation of IL-4 and subsequent Th1 polarization, which leads to the control of parasite growth and the resolution of cutaneous lesions. First, anti-IL-12

treatment ablates the down-regulation of IL-4 and upregulation of IFN-γ seen in these mice (Heinzel *et al.*, 1995). Second, B6 mice deficient in either IL-12 or its downstream signaling mediator, the signal transducer and activator of transcription 4, are highly susceptible to *L. major* infection (Mattner *et al.*, 1996; Stamm *et al.*, 1999). Third, endogenous IL-12 is still required in B6 mice that have healed their lesions, as anti-IL-12 treatment reactivates growth of latent parasites and induces new pathology (Stobie *et al.*, 2000). Further, Th1 cells can confer protection RAG-deficient B6 mice against *L. major* infection, only if the recipient mice are not deficient in IL-12 (Park *et al.*, 2000). Finally, administration of recombinant IL-12 to BALB/c mice during the first week of infection diminishes Th2 but greatly enhances Th1 responses, leading to the resolution of the infection (Heinzel *et al.*, 1993b; Sypek *et al.*, 1993). Collectively, these results demonstrate that IL-12 is the key to the down-regulation of an early IL-4 production and to the induction and maintenance of a polarized Th1 response following *L. major* infection in B6 mice. Perhaps more important is the fact that when endogenous IL-12 is neutralized even after B6 mice heal their cutaneous lesions, polarized Th1 responses would still wane and latent parasites would reactivate (Park and Scott, 2001; Stobie *et al.*, 2000). Intriguingly, perhaps the parasite may somehow be able to effect a Th2-favoring environment so that whenever the counterbalancing IL-12 is weakened or absent, deviation to Th2 responses ensues. This proposition also

provides a viable explanation for the fact that LACK-specific Th2 cells are activated early after parasite infection in both resistant B6 and susceptible BALB/c mice: they both fail to produce IL-12 initially and a parasite-orchestrated mechanism favors Th2 development. When B6 mice later become competent to produce IL-12, this potential Th2 polarization is halted and redirected. However, what cells are producing the initial IL-12 that is so crucial to the healing phenotype of B6 mice? Why is IL-12 production delayed but not immediate following infection? To these questions, *Leishmania*-dendritic cell (DC) interactions may hold the answers.

Dendritic cells and the induction of anti-microbial immunity

DCs were first identified by Steinman and Cohn in pursuit of the accessory cell crucial for T cell-dependent antibody production in culture (Steinman and Cohn, 1973; Steinman *et al.*, 1974). Their critical role in activating T cells were not clear until when Inaba *et al.* demonstrated that DCs could activate naïve T cells both *in vitro* and *in vivo* (Inaba *et al.*, 1990; Inaba and Steinman, 1985). Since then, a large number of studies have collectively established that DCs are the professional antigen presenting cell (APC) that is uniquely competent to present Ags to T lymphocytes and initiate a primary immune response *in vivo* (Banchereau and Steinman, 1998). This unique competence is determined by at least two distinctive features of DCs, namely,

the migration from non-lymphoid peripheral tissues to the immediate draining lymph node (DLN) and the maturation *en route*.

Langerhans cells (LCs) are probably the best described DCs located in the periphery. Specifically, they are derived from hematopoietic cells in the bone marrow (BM) but reside in skin (Katz *et al.*, 1979). Based on morphological criteria, LCs were identified in the afferent lymph and subsequently in the T cell area of the DLN following contact sensitization (Hoefsmit *et al.*, 1982; Lens *et al.*, 1983; Silberberg-Sinakin *et al.*, 1976). Further, within a day after the skin is painted with FITC, FITC-labeled DCs can be found in the DLN (Cumberbatch and Kimber, 1990; Kripke *et al.*, 1990; Macatonia *et al.*, 1987). These results revealed the migratory property of DCs following immune challenge. The maturation process was first suggested in studies of isolated LCs. Upon culture *in vitro*, epidermal LCs were found to become much more stimulatory to T cells (Inaba *et al.*, 1986; Schuler and Steinman, 1985; Teunissen *et al.*, 1990). Subsequent studies have established that DCs located in the periphery are immature. These cells efficiently capture soluble and particulate Ags (Inaba *et al.*, 1993; Reis e Sousa *et al.*, 1993; Sallusto *et al.*, 1995; Svensson *et al.*, 1997), maintain large amounts of major histocompatibility complex (MHC) molecules intracellularly, and are not potent T cell stimulators (Cella *et al.*, 1997; Pierre *et al.*, 1997). During the migration, DCs gradually gain the ability to present Ags in the

immunogenic form to T cells (Romani *et al.*, 1989; Streilein and Grammer, 1989). This involves transporting peptide-MHC complexes to the cell surface (Chow *et al.*, 2002; Turley *et al.*, 2000) and upregulating co-stimulatory molecules such as B7 and CD40 (Inaba *et al.*, 1994; Larsen *et al.*, 1992). Arriving at the DLN, mature DCs express high levels of peptide-MHC complexes coupled with co-stimulatory molecules and become the potent stimulator for naïve T cells. In essence, by transforming periphery-deposited Ags that are not recognizable to T cells into the Ag derivative optimal for T cell activation, DCs constitute an efficient conduit for relaying antigenic information from the periphery to the organized lymphoid tissues.

Initial studies on DC biology were largely confined to interactions of immune cells themselves but had little connection to microbial infection. Studying *L. major* infection in mice, Moll and colleagues are probably the first to provide direct evidence for the involvement of DCs in anti-microbial immunity (Blank *et al.*, 1993). In 1994, bacterial LPS was found to induce the maturation of human DCs (Sallusto and Lanzavecchia, 1994), suggesting for the first time that DC maturation, an important check-point in the induction of T cell responses, could be a response to microbial stimuli. In 1997, Medzhitov *et al.* showed that a human homolog to *Drosophila* Toll protein could signal to induce upregulation of B7 co-stimulatory molecules (Medzhitov *et al.*, 1997), which was already recognized as a hallmark of DC maturation at the time.

Finally, Poltorak *et al.* conclusively mapped the genetic defect that is responsible for the LPS hyporesponsiveness in C3H/HeJ and C57BL/10ScCr mice to the gene coding for Toll-like receptor 4 (TLR4) (Poltorak *et al.*, 1998). This finding was further confirmed in TLR4 knock-out mice (Hoshino *et al.*, 1999). Together, these results have pointed to an intriguing connection between innate recognition of microbial products and the induction of T cell immunity by DCs. A total of 10 TLRs have so far been identified, and they recognize an array of conserved microbial molecular structures (Janeway and Medzhitov, 2002). Importantly, DCs are found to express the widest TLR repertoire, further emphasizing the significance of pathogen recognition by DCs in the initiation of anti-microbial T cell immunity (Kaisho and Akira, 2001). While a majority of known TLR stimuli are of a bacterial origin, glycosylphosphotidylinositol anchors of *Trapanosoma cruzi*, a *Leishmania*-related parasite in the Trypanosomatidae family, activates TLR2 and represents the first protozoan TLR ligand (Campos *et al.*, 2001).

Dendritic cells and Th differentiation

DCs are not only crucial to the initiation of T cell responses but also important in determining the effector phenotype of activated T cells. Seminal work by Macatonia *et al.* showed for the first time in 1995 that DCs could induce Th1 polarization from naïve $CD4^+$ T cells by virtue of IL-12 secretion

(Macatonia *et al.*, 1995). Subsequently, it has become increasingly clear that DCs has tremendous plasticity in the ability to polarize Th responses (Kalinski *et al.*, 1999). For example, rat DCs isolated from the respiratory tract preferentially induced Th2-like responses (Stumbles *et al.*, 1998). Murine DCs from the Peyer's patch but not the spleen favored Th2 polarization, at least in part due to their preferential production of IL-10 (Iwasaki and Kelsall, 1999). These two studies suggest DCs may respond to tissue-specific factors to adjust their ability to polarize Th responses. A series of studies by Kapsenberg's group clearly showed that factors such as IFN-γ and prostaglandin E_2, when given to human DCs during their maturation, greatly influence their capacity to produce IL-12 and their ability to prime for distinct Th effectors (Hilkens *et al.*, 1997; Kalinski *et al.*, 1997; Kalinski *et al.*, 1998; Vieira *et al.*, 2000). Perhaps more importantly, it is increasingly clear that pathogens or pathogen-derived products can condition DCs to favor distinct Th effector phenotypes. For example, murine DCs stimulated with a filarial nematode-secreted protein or soluble egg antigens from *Schistosoma mansoni* induced Th2 responses, while LPS or gram-positive bacterium *Propionebacterium acnes* activated DCs to drive Th1 polarization (MacDonald *et al.*, 2001; Whelan *et al.*, 2000). In response to yeast and hyphae of *Candida albicans*, interestingly, DCs were found to produce IL-12 and IL-4 and prime Th1 and Th2 responses, respectively (d'Ostiani *et al.*, 2000). More recently,

the filamentous hemagglutinin from *Bordetella pertussis* bacteria was found to inhibit IL-12 but enhance IL-10 production by DCs and to condition these cells to prime for pathogen-specific regulatory T cells (McGuirk *et al.*, 2002). Taken together, these results suggest that DCs direct Th subset development by incorporating information of the tissue environment that they reside in and of the invading pathogen that they encounter (Kalinski *et al.*, 1999). Therefore, it is an intriguing possibility that the outcome of *Leishmania*-DC interactions would influence the type of parasite-specific Th responses.

As already mentioned above, TLR-mediated innate pathogen recognition by DCs is critical to the induction of T cell immunity. Since different pathogens may require different types of T cell responses, TLRs may constitute the sensor by which DCs differentiate pathogens and mount various types of T cell responses. Studies presented in Chapter 5 were designed to begin to test this possibility.

Leishmania-dendritic cell interactions and Th1-Th2 dichotomy

Initial evidence of DC involvement in the *Leishmania* infection came from studies of LC distribution and turnover in the skin lesion of human patients (Caceres-Dittmar *et al.*, 1992; Kaplan *et al.*, 1987). Notably, the phenotype of LCs (e.g. expression of MHC and ICAM-1 molecules) was found to be different between lesions of cutaneous and diffuse cutaneous

leishmaniasis, suggesting a correlation between LC functions and the disease severity (Caceres-Dittmar *et al.*, 1992). With *L. major* infection in mice as a model, Moll and colleagues revealed that LCs could be infected by amastigotes *in vitro* and *in vivo* (Blank *et al.*, 1993). They further showed that *Leishmania*-carrying LCs migrated to the DLN and that activation of *Leishmania*-specific T cells was exclusively mediated by DLN DCs following parasite challenge in the skin (Moll *et al.*, 1993). More recently, they also demonstrated that in B6 mice that had resolved cutaneous lesions, DLN DCs persistently harbored *L. major* parasites and were able to stimulate a vigorous T cell response (Moll *et al.*, 1995). Together, these studies established a critical role for DCs in initiating and maintaining T cell responses during *Leishmania* infection.

Given this evidence of *Leishmania*-DC interactions and the fact that DCs direct Th differentiation, it is possible that parasite-infected DCs play a key role to the differential Th polarization during *Leishmania* infection. To explore this possibility, von Stebut *et al.* studied a fetal skin-derived LC-like cell line exposed to *L. major* parasites and found that amastigotes induced DC maturation and release of IL-12 p40 (von Stebut *et al.*, 1998). Similarly, Konecny *et al.* found that *L. major* promastigotes could stimulate IL-12 p40 production from splenic DCs (Konecny *et al.*, 1999). Interestingly, however, production of bioactive IL-12 p70 by *L. major*-exposed human DCs appears to

strictly depend on co-engagement of CD40 molecules (Marovich *et al.*, 2000). These studies strongly suggest that DCs are probably the source of IL-12 that is indispensable for the control of *Leishmania* infection (see discussion in *Th subset development during* Leishmania *infection*). However, it remains unknown whether *Leishmania* parasites could modulate DC functions so as to induce Th2 responses in a susceptible host. Studies presented in the Chapter 3 specifically address this possibility.

Leishmania-macrophage interactions and Th1-Th2 dichotomy

MΦs are the primary host cells in which *Leishmania* parasites reside and propagate. Numerous groups have independently demonstrated the importance of complement in mediating the entry to MΦs by promastigotes of Old World species such as *L. major* and *L. donovani*. In the presence of serum, these parasites activate complement cascade and as a result, surface-fixed C3b and/or C3bi bind to MΦ CR1 and/or CR3, leading to the phagocytic entry of the parasite (Da Silva *et al.*, 1989; Mosser and Edelson, 1984; Mosser and Edelson, 1985; Mosser *et al.*, 1992; Puentes *et al.*, 1988; Wozencraft *et al.*, 1986). The reason that promastigotes could activate complement without being lysed is probably due to active shedding of the membrane attack complex (Puentes *et al.*, 1990). Complement fixation not only enhances promastigote entry but also promotes its intracellular survival (Da Silva *et al.*,

1989; Mosser and Edelson, 1987). Interestingly, however, promastigotes of New World species such as *L. mexicana* and *L. amazonensis* do not require complement to enter MΦs, although they do activate the cascade (Russell and Talamas-Rohana, 1989). Instead, *L. mexicana* appear to use a surface glycoprotein to engage CR3 directly (Russell and Wright, 1988), while *L. amazonensis* may utilize fibronectin receptor (Rizvi *et al.*, 1988; Wyler *et al.*, 1985). This disparity between New World and Old World species may have arisen from the fact that they differ in the structure of lipophosphoglycan (LPG) (Ilg *et al.*, 1992), which is the most abundant surface macromolecule on promastigotes and believed to mediate complement fixation by *L. major* metacyclics (Puentes *et al.*, 1988). This point is further underscored by the recent finding that LPG is a virulence factor of *L. major* but not of *L. mexicana* (Ilg, 2000; Spath *et al.*, 2000)

In contrast to promastigotes, much less is known about MΦ receptors for amastigotes. While amastigotes can attach to all adherent cells through binding to cellular proteoglycans (Love *et al.*, 1993), they primarily infect phagocytes. In the case of *L. amazonensis*, the amastigote entry does not involve CR1, CR3 or the mannose receptor (Mosser and Rosenthal, 1993), while *L. major* amastigotes were found to be able to use CR3 to enter MΦs (Guy and Belosevic, 1993). Perhaps more important is the fact that virtually all amastigotes in tissues are coated with host Abs, and thus Fc receptors are

likely to be a dominant way of entry for them (Guy and Belosevic, 1993; Pearson and Roberts, 1990; Peters *et al.*, 1995). Attesting to the importance of this pathway, a recent study by Kima *et al.* showed that infection by *L. amazonensis* and *L. pifano* was significantly ameliorated in the absence of either circulating Abs or the common γ chain of Fc receptors (Kima *et al.*, 2000).

Once inside MΦs, promastigotes or amastigotes could be killed by reactive oxygen species during an respiratory burst (Murray, 1982; Murray and Cartelli, 1983; Pearson *et al.*, 1983; Zarley *et al.*, 1991). However, the major route of entry by promastigotes, CR1- or CR3-mediated phagocytosis, does not trigger an oxygen burst (Wright and Silverstein, 1983). Further, amastigotes do not elicit a significant respiratory burst (Channon *et al.*, 1984; Haidaris and Bonventre, 1982; Pearson *et al.*, 1983). Thus, under physiological conditions, MΦs that are not somehow activated are unlikely to be able to clear intracellular *Leishmania* parasites. Murray *et al.* was the first to report that lymphokine activation was required for inducing optimal leishmanicidal activities in MΦs and identified IFN-γ as a crucial effector cytokine in this process (Murray and Cartelli, 1983). These authors also noted that IFN-γ-activated MΦs could kill *Leishmania* parasites independent of reactive oxygen intermediates. Similarly, Scott *et al.* found that when activated by lymphokines, a MΦ cell line that was incapable of generating oxygen burst

could still kill *Leishmania* (Scott *et al.*, 1985). This lymphokine-induced leishmanicidal activity was later found to be mediated by L-arginine-dependent nitric oxide (NO) production (Green *et al.*, 1990b; Liew *et al.*, 1990c; Mauel *et al.*, 1991). Further, It was found that IFN-γ-induced leishmanicidal effects could be blocked by neutralizing autocrine tumor necrosis factor-α (TNF-α) (Green *et al.*, 1990a), which by itself activated MΦs to produce NO and kill *Leishmania* parasites (Liew *et al.*, 1990b; Theodos *et al.*, 1991a). The indispensability of the NO-mediated killing mechanism has been demonstrated by the finding that resistance to *L. major* infection in B6 mice is abrogated if they are deficient in inducible NO synthase (iNOS) (Wei *et al.*, 1995) or treated with iNOS inhibitors (Liew *et al.*, 1990c). In terms of the relative contribution of reactive oxygen species and NO to MΦ leishmanicidal activities, Murray *et al.* showed that while both pathways operated *in vivo* during the first two weeks of *L. donovani* infection, the production of NO but not reactive oxygen species was ultimately required for the disease resolution (Murray and Nathan, 1999). Collectively, these studies have uncovered the fundamental pathway that is responsible for killing *Leishmania* parasites by host MΦs. At the level of parasite-MΦ interactions, these results also provide a firm explanation for why Th1 cytokines IFN-γ and TNF-α are crucial to the control of *Leishmania* infection.

On the issue of how Th2 cytokines such as IL-4 and IL-10 could exacerbate the disease, numerous studies have shown that these two cytokines inhibit IFN-γ- and/or TNF-α-induced iNOS activity and NO production in MΦs, and thereby prevent the parasite killing (Bogdan *et al.*, 1994; Cunha *et al.*, 1992; Gazzinelli *et al.*, 1992; Lehn *et al.*, 1989; Vieth *et al.*, 1994; Vouldoukis *et al.*, 1997; Wu *et al.*, 1993). More recently, Corraliza and colleagues have revealed that IL-4 and IL-10 could also directly enhance the intracellular replication of *L. major* through arginase induction (Iniesta *et al.*, 2001; Iniesta *et al.*, 2002). As depicted in the *Illustration 5*, arginase competes with iNOS for the same substrate, L-arginine. It converts arginine into L-ornithine, the precursor for polyamine biosynthesis (Mills, 2001). Since polyamines are essential to the replication of eukaryotic cells, including protozoa such as *Leishmania* (Pegg, 1988; Yarlett and Bacchi, 1994), increased polyamine production in the host cell would favor parasite growth. Given the interconnectivity between the iNOS-NO and the arginase-polyamine pathways, it is difficult to clearly separate inhibition of killing from promotion of parasite growth by Th2 cytokines. What is clear though is that at the level of parasite-MΦ interactions, a cellular mechanism has been defined that explains very well why a Th1 response is protective against *L. major* infection while a Th2 one is pathogenic.

However, conclusions made with one *Leishmania* model may not always be generalizable to other parasite species. This is particularly important when *La* parasites are concerned, since very limited knowledge is

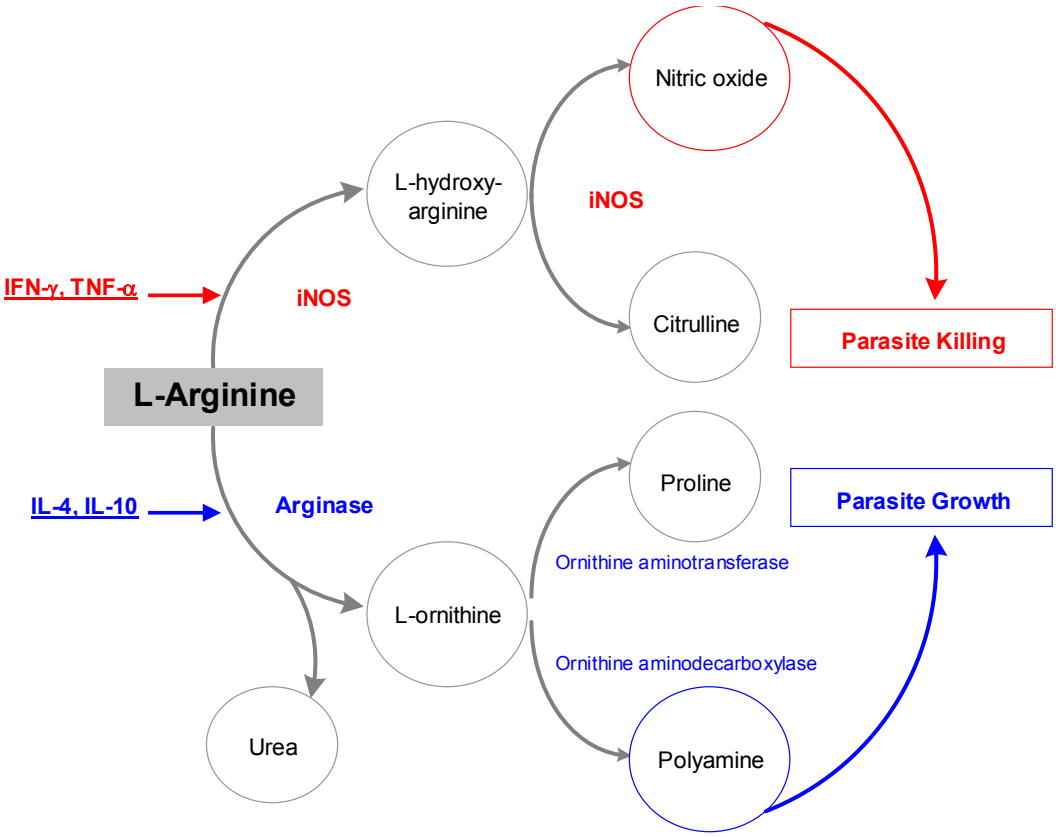

Illustration 5. L-Arginine metabolism in MΦs and the regulation of parasite growth. Inducible nitric oxide synthase, which is upgregulated by Th1 cytokines IFN-γ and TNF-α, catalyses the generation of nitric oxide and thereby promotes the killing of *Leishmania* parasites. Arginase, on the other hand, is induced by Th2 cytokines IL-4 and IL-10, converts L-arginine to L-ornithine. L-ornithine is the precursor for praline and polyamines, which are required for collagen production and cell proliferation, respectively, and thus very important in wound healing process. However, replication of *Leishmania* parasites requires polyamines as well, and they can utilize MΦ-produced polyamines to promote their own growth.

available about their fate in MΦs that are activated with Th1 or Th2 cytokines. Notably, it was shown that unlike *L. major*, *La* amastigotes (maria strain) resisted killing by MΦs that were activated with “lymphokine” (culture

supernatants of Ag-stimulated splenocytes from immune hosts) (Scott *et al.*, 1983). On the other hand, Soong *et al.* showed that *La* promastigotes could be killed by IFN-γ-activated MΦs (Soong *et al.*, 1997). Studies presented in Chapter 4 were designed to further ascertain the role of IFN-γ in activating murine MΦs to kill *La* parasites.

The natural model of *Leishmania* infection in mice

In an overwhelming majority of studies on murine leishmaniasis, the infection has been established through footpad injection of a large number of *Leishmania* promastigotes, usually in the order of one to ten million organisms. This experimental protocol does not reflect the biology of natural *Leishmania* transmission in two important aspects. First, during a natural infection, sandflies would only deliver 100-1,000 parasites (Warburg and Schlein, 1986). Second, sandflies inject parasites together with their saliva, which contains antihemostatic and immunomodulatory contents and thereby may influence the course of parasite infection (Ribeiro, 1987; Titus, 1998; Warburg and Schlein, 1986). Therefore, the Th1-Th2 paradigm built upon the needle infection protocol would have to be verified in settings closer to the natural infection.

The Titus group pioneered studies of potential effects of sandfly saliva on *Leishmania* infection. They showed that *L. major* infection was severely

exacerbated when promastigotes were co-injected with homogenized salivary glands from *Lutzomyia longipalpis* (Theodos *et al.*, 1991b; Titus and Ribeiro, 1988). Further, such disease-exacerbating effects were found to correlate with the ability of salivary gland lysates to inhibit IFN-γ-induced NO production from MΦs (Hall and Titus, 1995). In an intradermal model of *L. major* infection in mice, Belkaid and colleagues demonstrated that co-injection of 1,000 metacyclic promastigotes and salivary gland sonicate not only led to a more severe disease in susceptible BALB/c mice but also abrogated the self-healing process associated with *L. major* infection in resistant B6 mice (Belkaid *et al.*, 1998b). Importantly, these authors revealed that frequencies of IL-4- or IL-5-producing cells at the infection site were significantly elevated in infected mice given the sonicate, that these mice, either BALB/c or B6, exhibited a heightened Th2 response throughout the course of infection, and that IL-4-deficient mice were not sensitive to the disease-exacerbating effect of the salivary gland sonicate. These observations strongly suggest that the disease exacerbation was mediated by Th2-enhancing activities in the sandfly saliva. Overall, these studies imply that during natural *Leishmania* infection in man, the vector may modulate host immune responses to create an environment that favors the parasite survival. Importantly, involving vector biology as an experimental parameter, these studies have validated the Th1-Th2 paradigm during *L. major* infection in a model one step closer to the natural infection.

One caveat of these studies, however, is the use of whole salivary gland lysates. Such lysates contain homogenized tissue/cellular materials of the gland, in addition to the salivary contents a sandfly would normally inject into a host during a blood meal. More recent evidence indicates that the saliva actually delivered by sandflies does not appear to contain the Th2-biasing activity observed in the whole gland lysates. For example, in both man and mouse, repeated sand fly bites lead to the development of strong saliva-specific DTH responses, which are indicative of a pro-inflammatory Th1 condition (Belkaid *et al.*, 2000b). Indeed, such saliva-specific DTH responses are associated with increased local IFN-γ and IL-12 production and are rapidly induced in mice that have previously been bitten by uninfected flies (Kamhawi *et al.*, 2000). When these mice are infected with parasites delivered by sandflies, the rapid saliva-specific DTH response at the site of infection can drastically inhibit the parasite growth (Kamhawi *et al.*, 2000). These results tend to question the relevance of disease-exacerbating effects by whole salivary gland lysates, and a formal proof or disproof requires experimental parasite infection in the presence of purified salivary contents. Nonetheless, results obtained in this essentially natural, sandfly-transmitted model of *L. major* infection lend further support to the validity of Th1-Th2 paradigm: a Th1-dominant inflammatory environment facilitates parasite killing, while a Th2 response favors parasite survival.

Clinical immunology in light of the Th1-Th2 dichotomy in mice

As discussed in the previous chapter, systematic investigation of anti-*Leishmania* immune responses in human is at present still difficult. Nonetheless, accumulating results from descriptive human studies do support the relevance of Th1 or Th2 responses to the disease resolution or pathogenesis (Kemp *et al.*, 1994b). For example, severe forms of leishmaniasis are usually associated with higher levels of Th2 cytokine production. Pirmez *et al.* examined lesion biopsies from ten cutaneous and eleven mucocutaneous cases due to *L. braziliensis* infection and found that the level of IL-4 was several orders of magnitude greater in latter form, while IFN-γ mRNA levels were comparable in both forms of the disease (Pirmez *et al.*, 1993). Similarly, Gaafar *et al.* found much higher levels of IL-4 were produced by peripheral blood $CD4^+$ T cells isolated from patients whose *L. major* infection was severe than those from patients who only suffered from mild diseases (Gaafar *et al.*, 1995b). Furthermore, people who had subclinical *Leishmania* infection or who were recovering from clinical diseases displayed Th1-like parasite-specific responses (Ajdary *et al.*, 2000; Bourreau *et al.*, 2003; Gaafar *et al.*, 1999; Kemp *et al.*, 1998; Kemp *et al.*, 1994a). Collectively, these data support the Th1-Th2 paradigm established in experimental studies of murine leishmaniasis.

Objectives of this dissertation study

From preceding discussion, it is clear that murine infection by *La* parasites shares common features with that by *L. major* but also exhibits unique aspects of parasite-host interactions. Perhaps the most important common feature is the immunopathogenesis mediated by parasite-specific Th2 cells (see discussion on page 23). At the cellular level, this can be largely explained by the fact that Th2 cytokines such as IL-4 and IL-10 inhibit leishmanicidal activities of MΦs and directly promote intracellular parasite growth (see discussion starting on page 36). Therefore, one objective of this dissertation is to understand how parasite-specific Th2 cells are generated. Given the prominent role that DCs play in regulating T cell differentiation, parasite-DC interactions have been specifically examined to determine whether *La* parasites could modulate DC functions in favor of priming of Th2 cells. Results pertinent to this aim are presented in Chapter 3. An interesting distinction of murine *La* infection is the apparent operation of Th2-independent pathogenic mechanisms, in addition to the Th2-dependent one (see discussion on page 23). Given the fact that Th cells are absolutely required for the disease development following *La* infection, it is tempting to speculate perhaps Th1 cells are involved in the pathogenesis of *La* infection. This is a provocative thought. Yet the role of Th1 cytokine IFN-γ during *La* infection is not entirely clear. Therefore, the second objective of this dissertation is to

begin to fill this gap. Specifically, the fate of *La* parasites, both promastigotes and amastigotes, in IFN-γ-activated MΦs is examined, and the role of endogenous IFN-γ during *La* amastigote infection is also evaluated. Results pertinent to this aim are presented in Chapter 4. In the process of these studies, our understanding of innate immunity has been drastically advanced by a series of discoveries related to TLR biology. TLRs are crucially involved in innate recognition of various microbes by DCs and MΦs. Importantly, they also appear to exert a great influence on the nature of adaptive immune responses to pathogens. Given the possibility that protozoan parasites such as *T. cruzi* (see discussion on page 32), a close "relative" to *Leishmania*, are recognized by TLR2, it is of interest to study the outcome of TLR2 activation in DCs or MΦs. This is the third objective of this dissertation. Specifically, a series of known microbial TLR2 and TLR4 agonists are compared for their effects on DC cytokine production and on DC ability to differentially prime Th cells. Results pertinent to this aim are presented in Chapter 5. Collectively, these studies are intended to improve our understanding of *La* parasite-host interactions.

3 *LEISHMANIA AMAZONENSIS*-DENDRITIC CELL INTERACTIONS *IN VITRO* AND THE PRIMING OF PARASITE-SPECIFIC $CD4^+$ T CELLS *IN VIVO*[2]

Introduction

Leishmania parasites are intracellular protozoa that are transmitted to humans and other vertebrates by sandflies in the form of flagellated promastigotes (Sacks and Perkins, 1984). After entering vertebrate hosts, the promastigote transforms into aflagellated amastigotes, which primarily propagate inside tissue macrophages (MΦs) (Alexander and Russell, 1992). Depending upon parasite species and host immune responses, *Leishmania* infection can cause various forms of diseases with different clinical manifestations (Tapia *et al.*, 1996b). Cutaneous leishmaniasis is the most common form that can be caused by *L. major* in the Old World and *L. mexicana* or *L. amazonensis* (*La*) in the New World (Tapia *et al.*, 1996b).

In experimental cutaneous leishmaniasis caused by *L. major* infection, the susceptibility of BALB/c mice is attributable to selective expansion of Th2 cells, whereas resistance seen in most other mouse strains, such as C57BL/6 (B6) mice, is associated with predominant Th1 responses (Reiner and Locksley, 1995). Although it is not entirely clear how these functionally distinct

[2] Published in *Journal of Immunology 167:4534-4542, 2001*, with minor modifications

$CD4^+$ T subsets are primed during *L. major* infection, studies with B6 mice deficient in $CD4^+$ or $CD8^+$ T cells indicate an essential role for MHC class II-restricted $CD4^+$ T cells in controlling *L. major* infection. Disruption of the MHC class II gene renders otherwise resistant B6 mice susceptible (Chakkalath *et al.*, 1995; Erb *et al.*, 1996). In addition, nude and SCID mice cannot control *L. major* infection unless they are reconstituted with $CD4^+$ T cells (Holaday *et al.*, 1991; Mitchell, 1983).

Different from that of *L. major*, murine *La* infection leads to progressive diseases in a majority of inbred strains of mice. Further, lesion development and tissue parasite load are remarkably reduced in immunodeficient MHC class $II^{-/-}$, $RAG2^{-/-}$, and nude mice on a B6 background (Soong *et al.*, 1997), as well as in BALB/c SCID mice (unpublished results). Strikingly, reconstitution of $RAG2^{-/-}$ mice with wild-type $CD4^+$ T cells from syngeneic naive mice results in disease development (Soong *et al.*, 1997). Therefore, parasite propagation and lesion pathology following *La* infection require $CD4^+$ T cells that are primed to a pathogenic phenotype. While the nature of pathogenic T cells in *La*-infected B6 mice is still unclear, accumulating evidence indicates that in *La*-infected BALB/c mice, IL-4-producing Th2 cells promote the lesion development and parasite growth. For example, administration of a neutralizing anti-IL-4 mAb ameliorates *La* infection in BALB/c mice (Afonso and Scott, 1993), and adoptive transfer of *La*-specific Th2 cell lines exacerbate

the disease (Ji *et al.*, 2002). Yet, it is not understood how these pathogenic Th2 cells are generated. One possibility is that functional modulation of APCs by the parasite may establish a priming environment that aberrantly favors generation of pathogenic Th cells.

MΦs are not only the primary host cell for *Leishmania* parasites, but also an important population of APCs. Thus, infected MΦs could constitute the aberrant priming environment. Indeed, ample evidence has suggested that *Leishmania* infection impairs the ability of MΦs to produce IL-12 (Belkaid *et al.*, 1998a; Carrera *et al.*, 1996; Weinheber *et al.*, 1998). As a result, parasitized-MΦs probably cannot stimulate robust Th1 and thus inherently favor Th2 responses (McDowell and Sacks, 1999). However, *Leishmania* infection also significantly impairs the antigen-presenting function of MΦs (Fruth *et al.*, 1993; Kima *et al.*, 1996; Prina *et al.*, 1993), making parasitized-MΦs an unlikely candidate to prime naïve T cells.

It is believed that naïve $CD4^+$ T cells are primed mainly in secondary lymphoid organs by dendritic cells (DCs), a unique population of professional APCs (Banchereau and Steinman, 1998; Steinman, 1991). Immature DCs are located in most non-lymphoid tissues and can efficiently uptake soluble and particulate antigens (Inaba *et al.*, 1993; Sallusto *et al.*, 1995). Upon exposure to microbial pathogens and their components, DCs migrate to lymphoid organs

where they eventually mature into potent APCs, which express high levels of MHC products and co-stimulatory molecules and prime naïve T cells (Steinman *et al.*, 1997). Depending upon maturation environment and/or lineage origin, DCs can express distinct cytokine profiles to regulate Th cell differentiation and thereby to ensure the induction of protective immunity (Kalinski *et al.*, 1999; Pulendran *et al.*, 2001a). However, certain pathogens such as HIV (Cameron *et al.*, 1992), measles virus (Grosjean *et al.*, 1997), *Plasmodium falciparum* (Urban *et al.*, 1999), and *Trypanosoma cruzi* (Van Overtvelt *et al.*, 1999) have been found to impair DC functions and the induction of effective host immune responses. Similarly, *La* parasites may have evolved mechanisms to modulate functions of DCs in a susceptible host to promote the generation of pathogenic T cells. To explore this possibility, we investigated parasite-DC interactions *in vitro* and directly examined effector phenotypes of T cells primed *in vivo* by parasite-exposed DCs.

Materials and Methods

Mice. Female wild-type, SCID and IL-$4^{-/-}$ BALB/c (H-2^{d}) mice and C3H/HeJ (H-2^{k}) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions and used for experiments at 6-10 weeks of age with protocols approved by the

Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasite culture and lysates preparation. L. amazonensis (MHOM/BR/77/LTB0016) parasites were maintained by regular passage through BALB/c mice. Promastigotes were cultured at 23°C in 20% FBS-supplemented Schneider's *Drosophila* medium (Life Technologies, Rockville, MD). Stationary promastigote cultures of less than five *in vitro* passages were used for animal infection and purification of metacyclics by negative selection with the 3A1 mAb (a kind gift by Dr. David Sacks, NIAID), according to N. Courret *et al*. (Courret *et al*., 1999). Lesion-derived amastigotes were cultured at 32°C in acidified (pH 5.0) complete Schneider's medium for 48 to 72 h and were used for infection of DCs. To prepare amastigote lysates, parasites (2×10^8/ml in PBS) were subjected to 6 freeze-thaw cycles and 15-min sonication in an ice bath, and then stored at -70°C in aliquot. To label metacyclics or amastigotes with the fluorescent tracking dye, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Inc., Eugene, OR), parasites were suspended at 5×10^7/ml in PBS containing 1.25 μM CFSE and incubated at room temperature for 5 min. Labeled parasites were washed four times with PBS and culture medium prior to

infection of DCs. Amastigotes or metacyclics (8×10^7/ml in PBS) were incubated in a 60°C water bath for 15 min to prepare heat-killed parasites.

Evaluation of parasite infection of mice. Age- and sex-matched mice (5-6 per group) were infected s.c. in the right hind foot with 2×10^6 stationary *La* promastigotes. Lesion size was monitored over time with a digital caliper (Control Company, Friendswood, TX). Tissue parasite burdens were measured via a limiting dilution assay as previously described (Soong *et al.*, 1997). To examine parasite-specific Th cytokine production in infected mice, draining lymph node (DLNs) cells (10^6/well in 200 μl medium) from individual infected mice were restimulated with amastigote lysates (equivalent to 10^6 parasites), and supernatants were harvested at 48-72 h to determine levels of IL-4, IL-10, and IFN-γ by ELISA.

Generation of DCs from the bone marrow. The protocol for generating bone marrow-derived DCs (BM-DCs) was originally described by Inaba *et al.* (Inaba *et al.*, 1992) and modified by Lutz *et al.* (Lutz *et al.*, 1999). Briefly, a single marrow cell suspension was prepared from the femurs and adjusted to 2×10^6 per 10 ml complete IMDM medium (Iscove's modified DMEM containing 10% FBS, 1mM sodium pyruvate, 50 μM 2-ME, 50 μg/ml gentamycin, and 100 U/ml penicillin). DC culture medium was supplemented with 20 ng/ml rGM-CSF (PharMingen, San Diego, CA) or with 2% culture supernatants of J558L

cells that were stably transfected with the murine *gm-csf* gene (the transfected cell line was a kind gift from Dr. Charles Janeway, Yale University.) At day 3, 10 ml of fresh GM-CSF-containing medium was added, and 10 ml of the culture medium was replaced with fresh GM-CSF-containing medium at day 6. Usually, 8-day cultures contained >70% $CD11c^+$ cells as judged by FACS analysis and were used for all experiments. Media used for DC culture were routinely monitored for potential LPS contamination, and the LPS levels were <0.06 EU/ml as measured by the Limulus Amebocyte Lysate pyrogen test kit (BioWhittaker, Inc., Walkersville, MD).

Infection of BM-DCs with Leishmania parasites. BM-DCs were adjusted to 2.5×10^6/well in 6-well plates or 10^6/ml in 24-well plates and incubated with parasites at a 4:1 parasite-to-DC ratio at 34°C for 12 h, and then at 37°C for an additional period of time as required in specific experiments. For the sham-exposed control, a parasite suspension was centrifuged to pellet parasites, and the resulting supernatant was added to DC cultures.

Electron microscopy (EM). Following 24 or 48 h of co-culture with parasites, BM-DCs were harvested and fixed in Ito's fixative (1.25% formaldehyde, 2.5% gultaraldehyde, 0.03% $CaCl_2$ and 0.03% trinitrophenol in 0.05 M cacodylate buffer, pH 7.3) at room temperature for 1 h and then overnight at 4°C. After washing, samples were post-fixed in 1% osmium

tetraoxide for 1 h and *en bloc* stained with 1% uranyl acetate in 0.1 M maleate buffer. After dehydration in a graded series of ethanol, samples were embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Sorvall MT-6000 ultramicrotome (RMC, Tucson, AZ), stained with uranyl acetate and lead citrate, and examined in a Philips 201 transmission electron microscope (Philips Electron Optics, Eindhoven, Netherlands) at 60 kV. For each sample, approximately 100 cells were examined.

Cytokine assays for amastigote-exposed DCs. BM-DCs were co-cultured with live or heat-killed amastigotes in 24-well plates as described above. Ammonium sulfate-purified anti-CD40 mAb was then added at 1:10 dilution (clone FGK45 (Rolink *et al.*, 1996a), a kind gift from Dr. Antonius Rolink, Basel Institute for Immunology, Switzerland). This dilution was chosen based upon pilot experiments measuring DC cytokine production. Supernatants were harvested at 24 h and saved at -70°C until ELISA measurement. For detection of intracellular cytokines, parasite-exposed DCs were incubated with anti-CD40 mAb for 12 h before GolgiStop™ (BD PharMingen) was added for additional 6 h. Cells were harvested, fixed, and stained as described below.

Flow cytometric analysis of surface and intracellular antigens. For blocking non-specific Ab binding, normal mouse IgG (Caltag, Burlingame, CA), hamster IgG, rat IgG (Pierce, Rockford, IL) and culture supernatants of 2.4G2 hybridoma (a kind gift from Dr. Rolf König, UTMB) were used. The following specific mAbs were purchased from BD PharMingen: FITC-conjugated anti-I-A^d/E^d (2G9, also reactive to I-E^k but not I-A^k); PE-conjugated anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-IL-4 (BVD4-1D11), anti-IL-10 (JES5-16E3), and anti-IL-12 p40/p70 (C15.6); and biotinylated anti-CD11c (clone HL3). Isotype control Abs included FITC-conjugated rat IgG2a, PE-conjugated rat IgG1, IgG2a, and IgG2b, PE-conjugated hamster IgG, and biotinylated hamster IgG. TriColor-labeled streptavidin, the secondary reagent for biotinylated antibody, was purchased from Caltag. All staining steps were done on ice. After washing, cells were incubated for a total of 30 min with 2.4G2 hybridoma supernatants (200 μl/10^6 cells) to block Fc receptors and then with a cocktail containing 5 μg/ml each of hamster IgG, rat IgG and mouse IgG. Thereafter, cells were stained in a final volume of 200 μl for 20 min in the presence of specific mAbs against surface antigens of interest (1 μg/10^6 cells). For detecting intracellular cytokines, cells were first stained for surface antigens, fixed/permeablized with Cytofix/Cytoperm Kit (BD PharMingen), and then incubated for 20 min with mAbs specific to cytokines in the presence of 5 μg/ml rat and mouse IgGs. Cells were washed and analyzed

on a FACScan (Becton Dickinson, Franklin Lakes, NJ). For characterization of DCs, at least 10,000 $CD11c^+$ events were collected. Data were analyzed with FlowJo software (TreeStar Inc., San Carlos, CA).

Priming T cells in vivo by DC transfer. For *in vivo* T cell priming by DCs, a protocol previously established by Inaba *et al.* (Inaba *et al.*, 1990) was used with some modifications. Briefly, 3×10^5 parasite-exposed DCs in 10 μl PBS were injected *s.c.* into the right hind foot (4-5 mice per group). Popliteal draining lymph nodes (DLNs) were harvested at day 8. To evaluate primary T cell cytokine responses induced by DC transfer, 10^6 DLN cells of individual mice were cultured in 200 μl medium containing amastigote lysates (equivalent to 10^6 parasites). In some cases, DLN cells from the same experimental group were pooled to purify $CD4^+$ T cells with Dynabeads Mouse CD4 in combination with DETACHaBEAD Mouse CD4 (Dynal Inc., Lake Success, NY) according to the manufacturer's protocol. Purified $CD4^+$ T cells (~95% pure) were seeded in 96-well plates at 10^5/well in 200 μl medium and incubated with titrated doses of amastigote lysates together with 10^6 syngeneic splenocytes (pretreated with 50 μg/ml mitomycin C; Sigma, St. Louis, MO). Culture supernatants were harvested at 48 h to measure IL-4 or at 72 h to measure IL-10 and IFN-γ. All media used for *ex vivo* assays of DLN and $CD4^+$ T cells from DC-transferred mice were IMDM supplemented with 3% mouse serum (Gemini Bio-Products, Woodland, CA). Cytokine contents in

supernatants were determined by ELISA, in which paired mAbs specific to IL-4, IL-10, and IFN-γ and their corresponding protein standards (BD PharMingen) were used following manufacturer's suggestion. Detection limits were: 16 pg/ml for IL-4, 31 pg/ml for IL-10, and 16 pg/ml for IFN-γ.

Statistical analysis. For comparison of mean values of different experimental groups, the two-tailed *t* test was used and *p* values were calculated in SigmaPlot software (SPSS Inc., Chicago, IL). A difference in mean values was deemed significant when $p<0.05$ or very significant when $p<0.01$.

Results

Differential susceptibilities of BALB/c and HeJ mice to *L. amazonensis* infection. BALB/c mice are known to be susceptible to *La* infection. As shown in Fig. 1A, these mice developed progressive lesions when challenged with 2×10^6 promastigotes. However, similarly infected HeJ mice showed no measurable lesions during the observation period. Analysis of the tissue parasite burden at 10 wk post-infection revealed that the number of parasites per foot was approximately 1,700-fold lower in HeJ mice than in BALB/c mice (Fig. 1B), indicating that HeJ mice are resistant to the infection. To determine parasite-specific cytokine production in these mice, DLN cells were restimulated *in vitro* with amastigote lysates. At 10 wk post-infection,

significantly higher levels of IL-4 and IL-10 were detected in BALB/c mice than in HeJ mice (p<0.01), while IFN-γ production was similar in both strains of

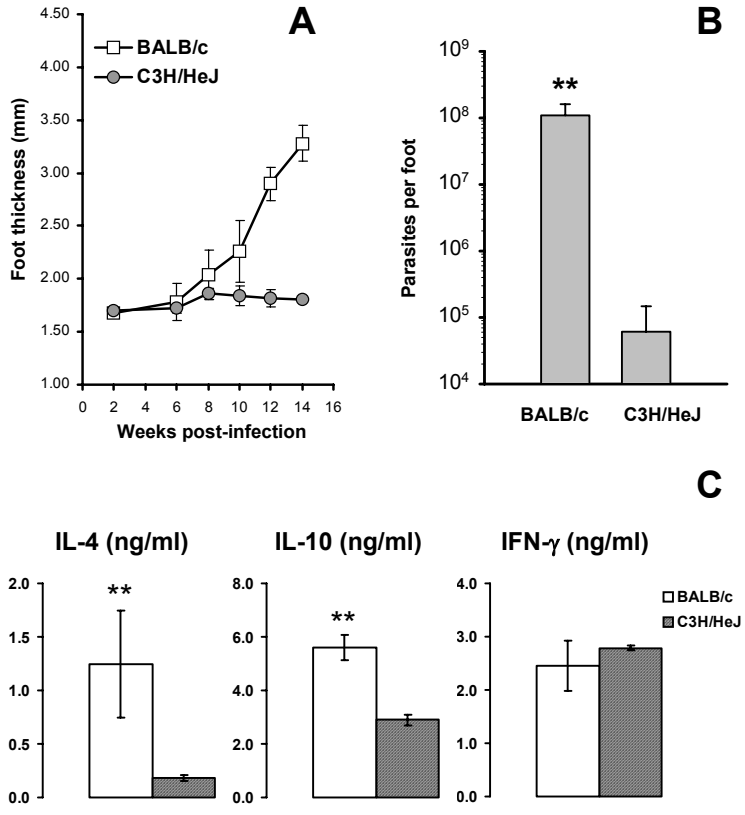

Figure 1. Differential susceptibilities of BALB/c and HeJ mice to *L. amazonensis* infection. (A) Lesion development in BALB/c and HeJ mice following infection with $2x10^6$ promastigotes were monitored by measuring the thickness of the infected foot. Each data point represents the mean ± SD for the respective experimental group. Results represent three independent experiments. (B) Parasite burdens of infected BALB/c and HeJ mice were examined in a limiting dilution assay at 10 weeks post-infection. The mean ± SD of parasite number per foot is shown for the two strains of mice (** p<0.01, n=5). (C) At 10 weeks post-infection, 10^6 DLN cells from individual BALB/c or HeJ mice were collected and cultured in the presence of amastigote lysates (equivalent to 10^6 parasites). Culture supernatants were harvested at 48-72 h to measure concentrations of IL-4, IFN-γ and IL-10. For each cytokine, the mean concentration ± SD is shown for the two strains of mice (** p<0.01, n=5). For (B) and (C), results are representative of two independent experiments.

mice (Fig. 1C). Therefore, the susceptibility of BALB/c mice to *La* infection is linked to an enhanced production of IL-4 and IL-10, while the resistance of HeJ mice is associated with markedly reduced Th2 responses. These data are consistent with previous findings that Th2 cells promote disease progression in *La*-infected BALB/c mice (Afonso and Scott, 1993; Ji *et al.*, 2002).

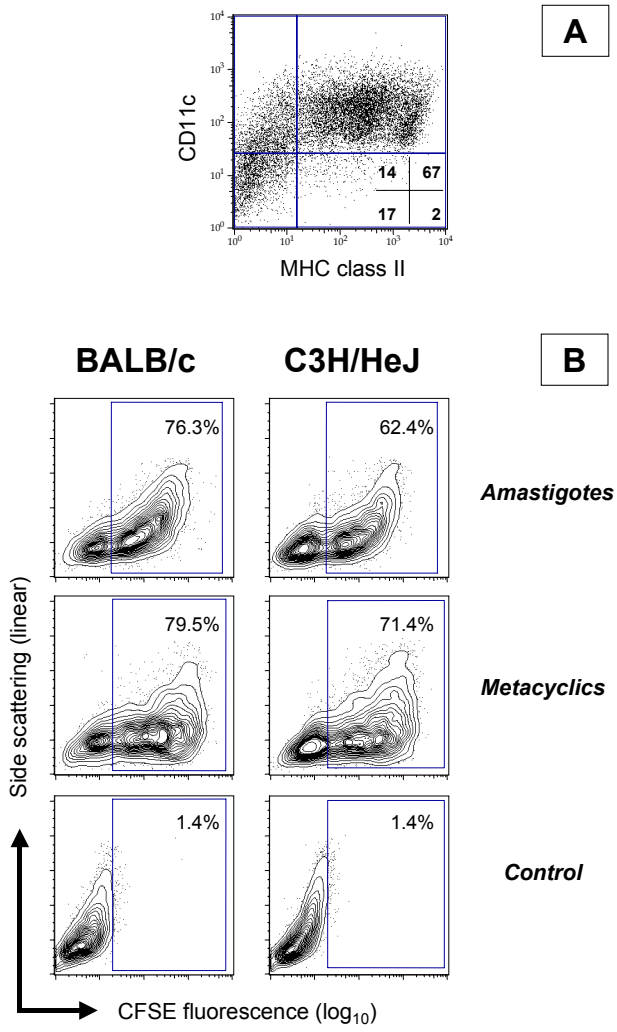

Figure 2. DCs from both susceptible and resistant backgrounds can engulf *L. amazonensis* parasites. DCs of BALB/c and HeJ mice were co-cultured for 24 h with CFSE-labeled amastigotes or purified metacyclic promastigotes at 4:1 parasite-to-DC ratio (see *Materials and Methods* for parasite-labeling procedure). Cells were stained for CD11c before being subjected to FACS analysis. Contour plots for $CD11c^+$ cells are shown. The Y-axis represents the side scattering intensity, while the X-axis represents green fluorescence intensity due to CFSE-labeled parasites. The boundary was set according to the autofluorescence level of DCs exposed to unlabeled parasites. Similar results were obtained from more than three independent experiments.

Efficient uptake of *L. amazonensis* parasites by DCs of susceptible and resistant mice. To explore the possibility that *La* parasites may modulate DC functions to favor the priming of pathogenic Th2 cells in susceptible BALB/c mice, we compared parasite-induced responses in DCs of BALB/c and HeJ backgrounds. Since immature DCs are phagocytic (Inaba *et al.*, 1993) and capable of engulfing *L. major* parasites (Marovich *et al.*, 2000; von Stebut *et al.*, 1998), we first examined whether DCs of susceptible and resistant backgrounds differ in parasite uptake. Immature

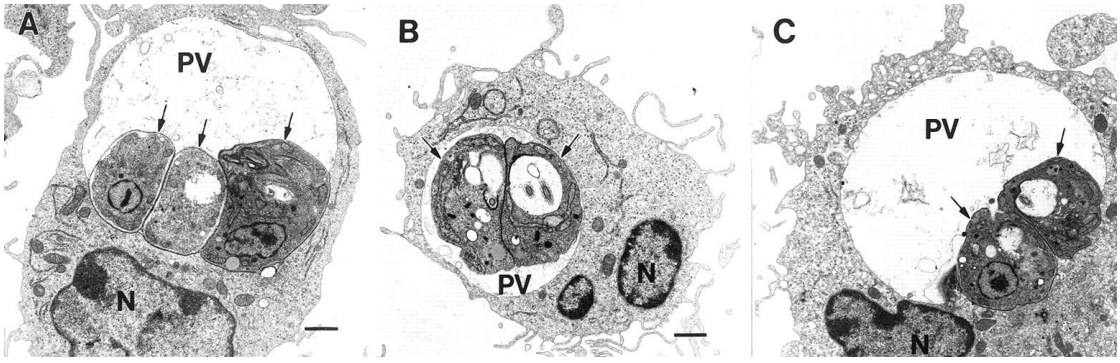

Figure 3. Transmission EM of parasite-carrying DCs. *L. amazonensis* amastigotes were co-cultured with DCs of BALB/c (A, C) or HeJ (B) background at a 4:1 parasite-to-DC ratio. At 24 h, structurally intact amastigotes (marked with arrows) were found inside a parasitophorous vacuole of a BALB/c DC (A) or HeJ DC (B). The structural integrity of the parasites was maintained for at least 48 h (C). Results represent more than 10 samples prepared from DCs of different bone marrow donors. Approximately 100 cells were examined for each sample. The original magnification for these pictures was ×5,600. *PV: parasitophorous vacuole; N: DC nuclei; Bar = 1 μm.*

BM-DCs from BALB/c or HeJ mice were co-cultured with CFSE-labeled *La* amastigotes or metacyclics. Flow cytometric analyses revealed that the parasite-associated CFSE fluorescence co-localized with a majority of $CD11c^+$ DCs (62-80%) of both BALB/c and HeJ backgrounds. The increased side scattering intensity in $CFSE^+$ DCs indicated the presence of intracellular parasites (Fig. 2). The percentages of fluorescent DCs were reduced by merely 5% when heat-killed amastigotes or metacyclics were used (not shown), consistent with the passive nature of parasite entry to phagocytic host cells (Love *et al.*, 1998). To further examine the parasite inside DCs, we conducted EM studies. After exposure to amastigotes for 24 h, DCs of BALB/c and HeJ mice typically contained several ultrastructurally intact parasites within one or two parasitophorous vacuole(s) (Figs. 3A and 3B). The structural integrity of intracellular parasites was maintained after 48 h of co-culture (Fig.

3C; HeJ DCs, not shown). Similar studies were carried out with promastigote bulk culture or purified metacyclics. While intact amastigote-like parasites were found inside vacuoles of metacyclic-exposed DCs, most parasites were destroyed by 24 h in bulk culture-exposed DCs of both strains of mice (to be reported elsewhere). Taken together, these data demonstrate that both BALB/c and HeJ DCs can efficiently uptake *La* parasites and that amastigotes and metacyclics are capable of surviving inside DCs.

L. amazonensis parasites activate both BALB/c and HeJ DCs. Upon exposure to microbes or their products, DCs usually undergo an activation/maturation process characterized by upregulation of MHC products and co-stimulatory molecules (Banchereau and Steinman, 1998). Thus, we examined whether differential activation of DCs following exposure to *La* parasites could contribute to the development of distinct Th responses observed in BALB/c and HeJ mice (Fig. 4). We found that exposure to amastigotes or metacyclic promastigotes resulted in upregulation of MHC class II, CD40, CD80 and CD86 in DCs of both genetic backgrounds (Fig. 4). Upregulation of these molecules was not seen in sham-exposed DCs and could not be blocked by LPS-neutralizing polymixin B (not shown). Given that DCs might be exposed to dead parasites *in vivo* and that they can engulf heat-killed parasites *in vitro* (unpublished data), we also examined whether heat-killed parasites could activate DCs. Heat-killed amastigotes activated

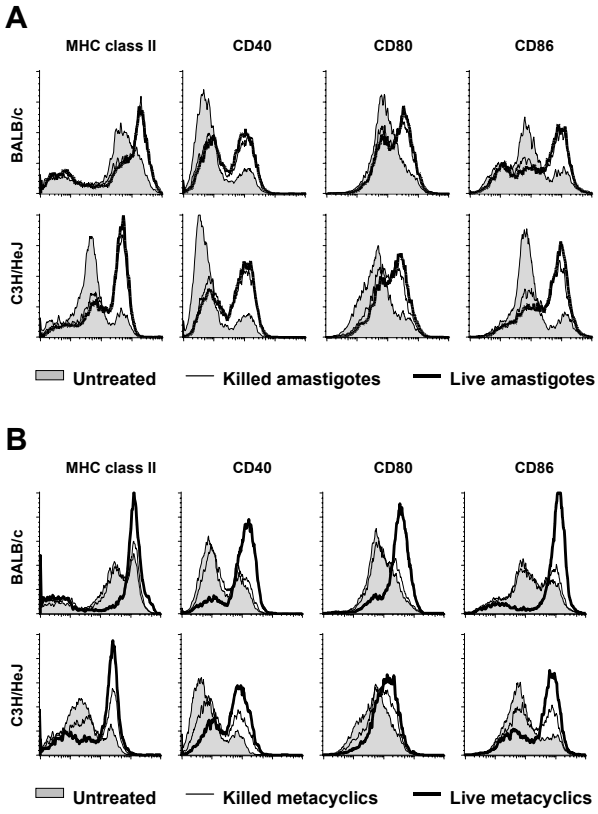

Figure 4. *L. amazonensis* parasites upregulate expression of MHC class II and co-stimulatory molecules on DCs. BALB/c and HeJ DCs were co-cultured with live or heat-killed amastigotes (A) or metacyclic promastigotes (B) for 24 h prior to FACS analysis for surface expression of MHC class II and co-stimulatory molecules (CD40, CD80 and CD86). Histograms depict expression profiles of the indicated molecules on $CD11c^+$ cells. Blocking steps described in *Materials and Methods* essentially brought non-specific staining of DCs by isotype control Abs to the level of autofluorescence, which was always adjusted to the first decade on the 4-decade log scale. For clarity, staining profiles of isotype controls were not included in the histogram overlay. The difference in MHC class II-staining intensity between BALB/c and HeJ DCs was due to the fact that FITC-conjugated 2G9 mAb reacts to the $I\text{-}A^d$, $I\text{-}E^d$, and $I\text{-}E^k$, but not to the $I\text{-}A^k$, molecules. Results represent three independent experiments on promastigote-exposed DCs and more than five on amastigote-exposed DCs.

DCs of both mouse strains in patterns similar to those of their live counterparts (Fig. 4A). However, heat inactivation significantly attenuated the ability of metacyclics to activate DCs (Fig. 4B). Regardless of developmental stages of the parasite, DCs from both susceptible and resistant backgrounds are comparable in surface expression of MHC class II and co-stimulatory molecules after parasite exposure. Thus, it is unlikely that the susceptibility to *La* infection in BALB/c mice is due to gross defects in parasite-induced DC activation.

Differential effects of *L. amazonensis* amastigotes on cytokine production by BALB/c or HeJ DCs. Activated DCs can produce a variety of cytokines (e.g., IL-12, IL-10, and IL-4) that polarize Th subset development (de Saint-Vis *et al.*, 1998; Kanangat *et al.*, 1995). Therefore, it is possible that differential production of DC cytokines following parasite exposure could contribute to distinct Th2 responses in BALB/c and HeJ mice. To test this possibility, DCs were exposed to parasites for 12 h and then treated with an agonistic anti-CD40 mAb (Rolink *et al.*, 1996b) for another 18 h. This was to mimic the engagement of CD40 molecules on DCs by T cell-derived CD40 ligands, which can greatly augment DC cytokine production (Cella *et al.*, 1996; Koch *et al.*, 1996).

As revealed in flow cytometric analyses, only a small fraction (1~4%) of BALB/c or HeJ DCs produce IL-12 following amastigote exposure without anti-CD40 treatment (not shown). When further treated with the anti-CD40 mAb, amastigote-exposed HeJ DCs consistently exhibited a two-fold increase in the frequency of IL-12 producers, as compared to the control (Fig. 5). However, pre-exposure of BALB/c DCs to live or heat-killed amastigotes did not lead to increased CD40-dependent IL-12 production (Fig. 5). Since IL-4 or IL-10 production by DCs was not detectable by intracellular staining (not shown), we sought to measure these two cytokines by ELISA. As shown in Fig. 6, CD40 engagement was sufficient to induce IL-10 secretion in both BALB/c and HeJ

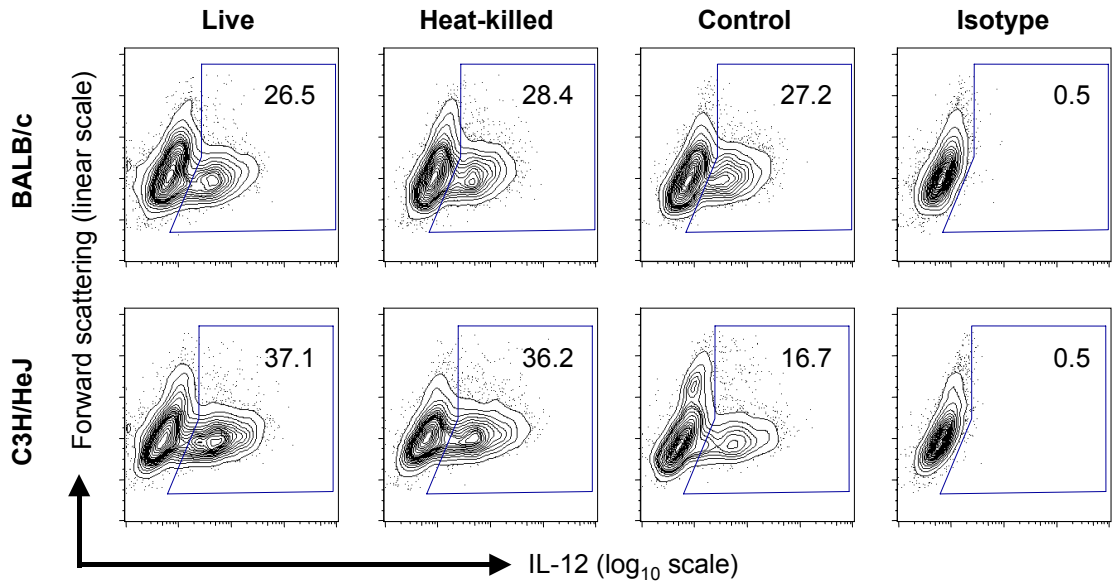

Figure 5. IL-12 production by amastigote-exposed DCs. BALB/c and HeJ DCs were co-cultured with live or heat-killed *La* amastigotes or left untreated for 12 h, and were further incubated with an anti-CD40 mAb for 18 h. Monensin was present during the last 6 h. Cells were stained for surface CD11c and then for intracellular IL-12. The IL-12 staining pattern of $CD11c^+$ cells is shown. The gate enclosing $IL\text{-}12^+$ population was determined according to staining with an isotype control Ab. Since autofluorescent levels varied among DCs of different forward scattering intensities, we set up the $IL\text{-}12^+$ gate in bivariate contour plots instead of histograms. Results were representative of three independent experiments. *Control: DCs not exposed to parasites; Heat-killed: DCs exposed to heat-killed amastigotes; Live: DCs exposed to live amastigotes.*

DCs, and this was not affected by pre-exposure to the parasite. IL-4 production was detected in BALB/c DCs that were exposed to amastigotes and then treated with the anti-CD40 mAb. However, IL-4 was not detectable in HeJ DCs under the same conditions (Fig. 6). Additionally, exposure to amastigotes for 24 h led to a two-fold increase of IL-4 mRNA levels in BALB/c but not HeJ DCs, as measured by RT-PCR (not shown). Collectively, these data show that *La* amastigotes fail to enhance CD40-dependent IL-12 production, but rather potentiate IL-4 production in BALB/c DCs.

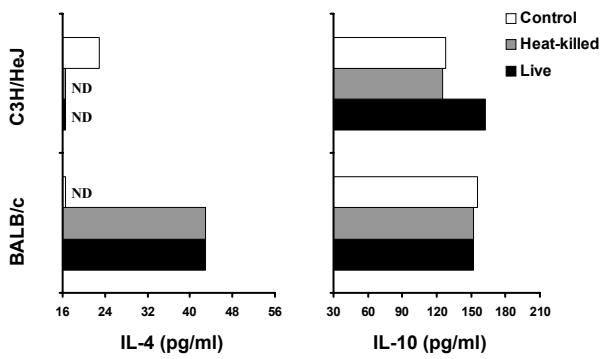

Figure 6. IL-4 and IL-10 production by amastigote-exposed DCs. BALB/c and HeJ DCs were co-cultured with live or heat-killed *La* amastigotes or left untreated for 12 h, and were further incubated with an anti-CD40 mAb for 24 h. Culture supernatants were harvested to determine cytokine concentrations by ELISA. Results for one out of three independent experiments were shown. *Control: DCs not exposed to parasites; Heat-killed: DCs exposed to heat-killed amastigotes; Live: DCs exposed to live amastigotes; ND: not detectable.*

Amastigote-carrying BALB/c or HeJ DCs prime distinct effector Th cells *in vivo.* IL-12 and IL-4 can skew Th responses toward a type-1 or type-2 phenotype, respectively. To test whether amastigote-exposed BALB/c or HeJ DCs prime distinct Th responses *in vivo*, a DC transfer protocol was used (Inaba *et al.*, 1993; Inaba *et al.*, 1990). Pilot studies revealed that at 7 to 9 days after s.c. transfer with 3×10^5 amastigote-carrying DCs (62-80% carrying the parasite, Fig. 2), DLNs reached a peak size of $1.5\text{-}3\times10^7$ cells/node and displayed a strong T cell proliferative response *in vitro* (not shown). Subsequently, DLN cells from individual mice were collected at 8 days post-transfer to examine cytokine production by Th cells *in vitro*. Similar studies were conducted with DCs derived from IL-$4^{-/-}$ BALB/c mice to directly evaluate the significance of DC-derived IL-4 in priming parasite-specific Th cells.

As shown in Fig. 7A, when re-stimulated *in vitro* with amastigote lysates, DLN cells from DC-transferred BALB/c mice produced significantly

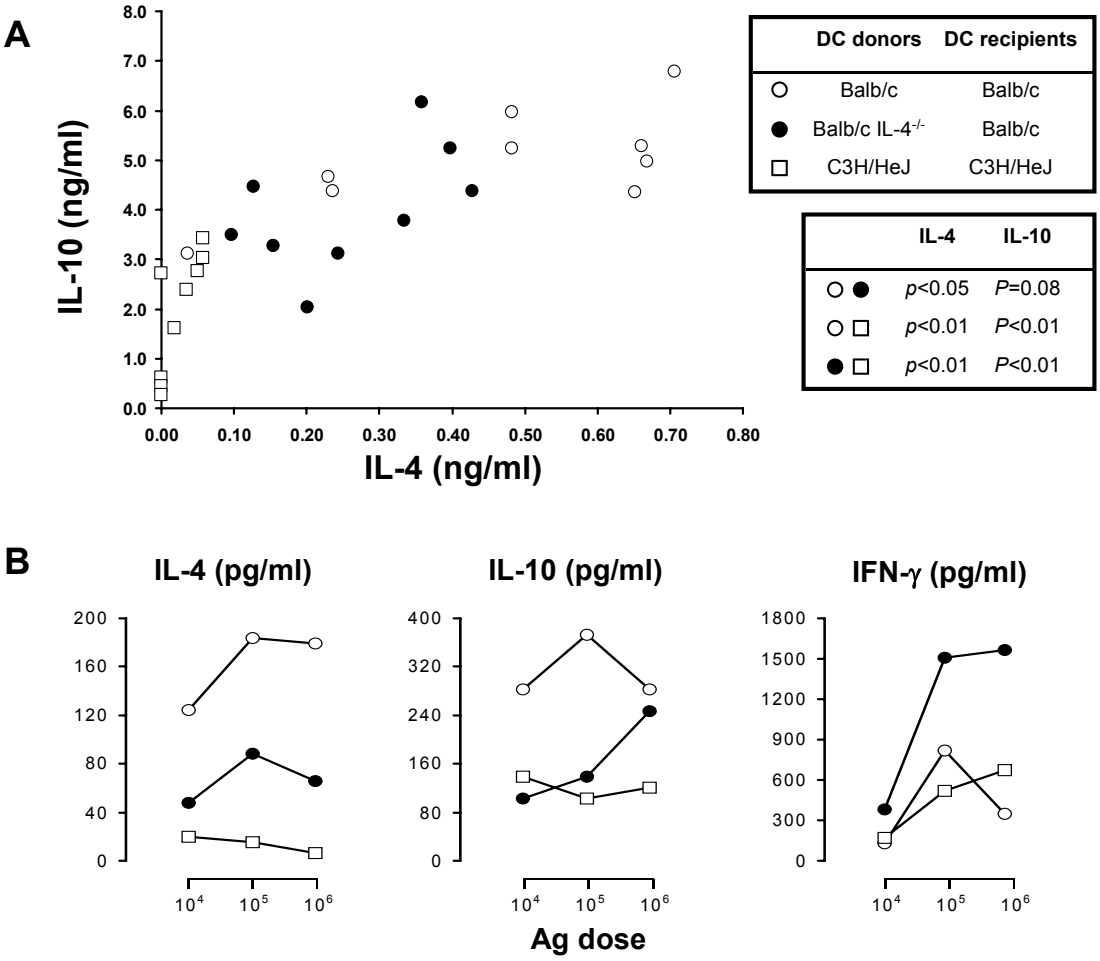

Figure 7. Distinct T cell cytokine profiles induced *in vivo* by amastigote-carrying DCs of resistant or susceptible background. DCs that had been co-cultured with live amastigotes for 24 h were s.c. transferred (3×10^5 DC/mouse) to the right hind foot of syngeneic wild-type mice. The cytokine profiles of DLN cells were examined in three groups of mice: BALB/c mice receiving wild-type BALB/c DCs (open circles), BALB/c mice receiving IL-$4^{-/-}$ BALB/c DCs (closed circles), and HeJ mice receiving HeJ DCs (open squares). (A) 10^6 DLN cells from individual mice were restimulated with amastigote lysates (equivalent to 10^6 parasites). The levels of IFN-γ (not plotted), IL-4 and IL-10 in the supernatants were determined by ELISA. Data from two independent experiments were pooled, and each data point represents individual recipient. Two-tailed *t* tests were conducted for comparison between groups, and *p* values are given in the inset. (B) DLN cells of DC recipients (4-5 per group) were pooled for isolation of $CD4^+$ T cells using magnetic beads. $CD4^+$ T cells (10^5, ~95% pure) were restimulated with 10^6 mitomycin-treated syngeneic splenocytes in the presence of indicated doses of amastigote lysates (in parasite equivalent). IL-4, IL-10 and IFN-γ in the supernatants were measured by ELISA. In the absence of amastigote lysates and/or feeder splenocytes, cytokine concentrations in the supernatants were minimal (<16 pg/ml for IL-4, <50 pg/ml for IL-10, and <100 pg/ml for IFN-γ). Similar results were obtained from two independent experiments.

higher levels of IL-4 and IL-10 than did the HeJ counterpart (p<0.01 for both cytokines, n=9). When the IL-$4^{-/-}$ DCs were used in the transfer, the level of parasite-specific IL-4 production by BALB/c DLN cells was significantly reduced (p<0.05, n=9), but that of IL-10 was not (p=0.08, n=9). Levels of IFN-γ production by DLN cells did not significantly differ among the three groups (ranging from 2.7$\pm$1.1 ng/ml to 3.1$\pm$1.3 ng/ml, p>0.5, n=9). Further, $CD4^+$ T cells were purified from recipient DLNs and re-stimulated with amastigote lysates in the presence of syngeneic mitomycin-treated splenocytes. Culture supernatants were harvested to examine the cytokine profile of Th cells. As shown in Fig. 7B, $CD4^+$ T cells from BALB/c mice given wild-type DCs produced higher levels of IL-4 and IL-10 than did those transferred with IL-$4^{-/-}$ DCs or those of the HeJ counterpart. In addition, $CD4^+$ T cells from BALB/c mice transferred with IL-$4^{-/-}$ DCs produced a higher level of IFN-γ than did cells from the other two groups. These data demonstrate that amastigote-carrying BALB/c DCs are significantly more potent in priming Th2 responses than their HeJ counterparts, and that DC-derived IL-4 is partially responsible for enhanced IL-4 and IL-10 production in Th cells.

Discussion

Previous studies have indicated that IL-4-producing Th2 cells mediate the pathogenesis following *La* infection in BALB/c mice (Afonso and Scott,

1993). In correlation, we have found that the magnitude of parasite-specific Th2 responses is a major distinction between susceptible BALB/c and resistant HeJ mice following the infection. Specifically, DLN cells from *La*-infected BALB/c mice produce moderate levels of both Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines, while resistant HeJ mice exhibit significantly reduced Th2 responses without developing stronger Th1 responses (Fig. 1C). It is known that an otherwise susceptible host can be rendered resistant to *La* infection by virtue of strong Th1 induction through vaccination (Soong *et al.*, 1995) or direct transfer of Th1 cells (Ji, J., Sun, J., and L. Soong, submitted[3]). In light of these findings, data presented in Fig. 1C suggest that the magnitude of Th2 responses could determine the outcome of *La* infection when the level of Th1 responses is only moderate.

It is noticeable that amastigote-carrying BALB/c DCs prime $CD4^+$ T cells to produce much higher levels of IL-4 and IL-10 than their HeJ counterparts (Fig. 7). This observation correlates with our data describing cytokine profiles of DLN cells from infected mice (Fig. 1C), implying that the effector phenotype of $CD4^+$ T cells activated during *La* infection may be determined by amastigote-exposed DCs. Accumulating evidence has indicated the existence of direct interactions between *Leishmania* amastigotes

[3] Jiaxing Ji, Jiaren Sun, and Lynn Soong. Deficient expression of inflammatory cytokines and chemokines at early stages of infection contributes to disease progression in *Leishmania amazonensis*-infected mice.

and DCs *in vivo*. In genetically resistant B6 mice, viable *L. major* amastigotes have been found to persist in DCs, and these DCs could stimulate a vigorous T cell recall response (Moll *et al.*, 1995). Dr. Sacks and colleagues have shown that in a natural *L. major* infection model, the DC compartment is mobilized to initiate T cell responses only after tissue parasitization by amastigotes reaches a threshold level (Belkaid *et al.*, 2000a; Lira *et al.*, 2000). Further, we have detected parasite-carrying DCs in DLNs within 3 days of s.c. inoculation with CFSE-labeled *La* amastigotes (unpublished data). Therefore, future studies are warranted to determine how amastigote-DC interactions *in vivo* can influence T cell effector phenotypes and shape the outcome of *Leishmania* infection.

La parasites could induce different responses in BALB/c and HeJ DCs, and thus potentially account for the different abilities of these DCs to prime Th2 cells (Fig. 7). First, the ability to engulf *La* parasites could be different between the two DCs. However, the observation that BALB/c and HeJ DCs similarly take up not only *La* amastigotes but also metacyclics (Fig. 2) clearly argues against this possibility. Further, EM studies reveal no major differences between BALB/c and HeJ DCs in terms of quantity, location, ultrastructure, and apparent fate of intracellular parasites (Fig. 3 and unpublished data). Thus, no gross difference in parasite engulfment by DCs of these two backgrounds could explain their distinct abilities to prime Th2 cells.

Secondly, enhanced Th2 priming by amastigote-carrying BALB/c DCs could result from defects in parasite-induced DC activation, as the strength and nature of co-stimulation provided by DCs to T cells could skew the balance of Th1/Th2 subset development (Constant and Bottomly, 1997). In particular, DCs that are not optimally activated and consequently provide insufficient co-stimulation can preferentially prime IL-10-producing regulatory T cells that suppress bystander Th1 development, and thereby favor Th2 responses (Dhodapkar *et al.*, 2001; Jonuleit *et al.*, 2000). While some protozoan parasites suppress DC activation (Urban *et al.*, 1999; Van Overtvelt *et al.*, 1999), data reported herein (Fig. 4) and results from others (Marovich *et al.*, 2000; von Stebut *et al.*, 1998) demonstrate that *Leishmania* parasites can activate DCs. Regardless of whether parasites are metacyclics or amastigotes, live or dead, they upregulate surface expression of MHC class II and co-stimulatory molecules in BALB/c and HeJ DCs in a similar fashion. These *in vitro* studies indicate no major difference at the level of parasite-induced DC activation in these two strains of mice. Bennett *et al.* have recently showed that *L. mexicana* parasites that carry the gene for a green fluorescent protein do not activate DCs of the CBA background (Bennett *et al.*, 2001). The difference between this report and our study (Fig. 4) could be due to the *Leishmania* species used, potential distinctions between freshly prepared wild-type parasites versus a genetically-engineered parasite clone, and/or

differences in responsiveness to the parasite among DCs of different genetic backgrounds.

Although the quality and strength of co-stimulatory signals delivered by DCs to $CD4^+$ T cells can affect development of distinct Th subsets, the cytokine environment during T cell priming can be a dominant factor shaping the cytokine profile of activated T cells (Seder and Paul, 1994). Thus, another possibility to explain the differential Th2 priming by amastigote-carrying BALB/c or HeJ DCs is that *La* amastigotes may condition these DCs to produce different cytokines. IL-12 is a potent Th1-driving factor indispensable in the initiation of protective immunity against *L. major* infection (Mattner *et al.*, 1996; Sypek *et al.*, 1993). Given that IL-12 production in MΦs is selectively inhibited by various *Leishmania* species (Belkaid *et al.*, 2000a; Carrera *et al.*, 1996; Weinheber *et al.*, 1998), it is generally believed that DCs are the major source of IL-12 during *L. major* infection (Marovich *et al.*, 2000; von Stebut *et al.*, 1998). In this study, we have shown that CD40 engagement enhances IL-12 production in amastigote-exposed HeJ but not BALB/c DCs (Fig. 5). This is consistent with our finding that amastigote-carrying BALB/c DCs prime stronger Th2 responses, as compared to the HeJ counterparts (Fig. 7). Since *La* amastigotes do upregulate CD40 expression in BALB/c DCs (Fig. 4), current efforts are directed to investigate whether amastigotes could render a portion of those $CD40^+$ DCs incapable of producing IL-12 in response to CD40

engagement. Of note, in spite of producing higher levels of IL-12, amastigote-exposed HeJ DCs do not induce stronger Th1 responses than their BALB/c counterparts. This might relate to the finding that IL-12R expression in $CD4^+$ T cells is down-regulated during *La* infection (Jones *et al.*, 2000).

DCs may induce Th2 development by producing IL-10 and IL-4 (d'Ostiani *et al.*, 2000; Iwasaki and Kelsall, 1999). Of particular interest, d'Ostiani *et al.* have shown that hyphae of *Candida albicans* directly stimulate murine DCs to produce a high level of IL-4 (d'Ostiani *et al.*, 2000). Our data indicate that amastigote exposure potentiates IL-4 production from BALB/c but not HeJ DCs (Fig. 6). However, exposure to *La* amastigotes alone is not sufficient, subsequent CD40 engagement is also needed for IL-4 secretion from BALB/c DCs. These observations suggest that direct DC-T cell interactions may enhance IL-4 production from amastigote-carrying BALB/c DCs. We are currently investigating whether factors other than CD40 are also involved in signaling these DCs to produce IL-4. Although the level of IL-4 produced by amastigote-exposed BALB/c DCs is low under our *in vitro* experimental conditions, this DC-derived IL-4 contributes to the enhanced Th2 response *in vivo*. The transfer study reveals that $CD4^+$ LN T cells produce significantly lower levels of IL-4 but higher levels of IFN-γ in IL-$4^{-/-}$ DC-transferred mice than in mice given wild-type amastigote-carrying DCs (Fig. 7). Importantly, IL-$4^{-/-}$ and wild-type DCs show no major differences in amastigote

uptake, surface marker expression (CD11c, MHC class II, CD40, CD80, and CD86), and IL-12 production after exposure to amastigotes (not shown). Therefore, production of IL-4 by amastigote-carrying DCs could be a significant Th2-biasing mechanism during *La* infection of BALB/c mice.

A lack of the IL-4 gene in BALB/c DCs does not totally abrogate priming of IL-4-producing $CD4^+$ T cells *in vivo* (Fig. 7). This could be because some amastigotes escape from carrier DCs to induce host responses independent of transferred DCs, or because amastigotes potentiate DCs to produce other Th2-driving factors in addition to IL-4. The observation that IL-$4^{-/-}$ and wild-type DCs indeed induce distinct Th cytokines *in vivo* (Fig. 7) suggests that donor DCs rather than cells of the recipient initiate T cell responses. Therefore, potential production of other Th2-skewing cytokines/chemokines by amastigote-exposed DCs warrants further investigation.

Inappropriate T cell responses could exacerbate diseases caused by *Leishmania* parasites and other pathogens. Given the crucial role of DCs in T cell-mediated immune responses, it is tempting to speculate that a pathogen may modulate the DC compartment, and thereby the host response to the pathogen. Our studies of murine leishmaniasis have suggested that *Leishmania* parasites can modulate DCs to skew $CD4^+$ T cell responses toward a phenotype that could lead to pathogenesis and uncontrolled parasite

growth. This study would also improve our general understanding of the complex interactions among intracellular pathogens, DCs, and T cells.

4 ENHANCED REPLICATION OF *LEISHMANIA AMAZONENSIS* AMASTIGOTES IN INTERFERON-γ-ACTIVATED MURINE MACROPHAGES: IMPLICATIONS FOR THE PATHOGENESIS OF CUTANEOUS LEISHMANIASIS[1]

Introduction

Leishmania parasites are dimorphic protozoa. They are transmitted to humans or other mammals by sandfly vectors in the form of flagellated promastigotes, but propagate inside tissue macrophages (MΦs) in the form of aflagellated amastigotes (Alexander and Russell, 1992; Sacks and Perkins, 1984). *Leishmania* infection exhibit a spectrum of clinical manifestations, from relatively benign cutaneous pathology to life-threatening visceral diseases, depending on the infective parasite species and host immune responses (Tapia *et al.*, 1996a).

Studies of experimental *Leishmania* infection in mice have been instrumental to our understanding of the disease pathogenesis. In the model of murine infection by *L. major*, susceptibility or resistance is due to the development of IL-4-dominated Th2 or IFN-γ-dominated Th1 responses in the infected host, respectively (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). At the cellular level, in accordance, IFN-γ activates microbicidal mechanisms of MΦs to kill intracellular *L. major* parasites (Green *et al.*, 1990a;

[1] Submitted to *Journal of Immunology*

Green *et al.*, 1990b; Kane and Mosser, 2001), while cytokines such as IL-4, IL-10, and TGF-β not only inhibit IFN-γ-mediated parasite killing (Kane and Mosser, 2001; Vieth *et al.*, 1994; Vouldoukis *et al.*, 1997) but also directly promote parasite growth inside MΦs (Iniesta *et al.*, 2001; Iniesta *et al.*, 2002). Although such Th1/Th2 dichotomy is well established in the model of *L. major* infection, it may not adequately explain the pathogenesis of murine infection by other *Leishmania* species. For example, infection by parasites of the New World species *L. amazonensis* (*La*) exhibits many unique aspects (Colmenares *et al.*, 2002). While most inbred mouse strains are susceptible to *La* infection, this susceptibility is not associated with polarized Th2 responses (Afonso and Scott, 1993; Soong *et al.*, 1997). C3H/HeJ mice are found to be relatively resistant to *La* infection, yet their cytokine profile during infection is not highly Th1-polarized (Qi *et al.*, 2001a). Further, the propagation of *La* parasites *in vivo* is significantly reduced when either $CD4^+$ T cell function or the B cell-mediated antibody response is abrogated (Kima *et al.*, 2000; Soong *et al.*, 1997). In contrast, mice deficient in $CD4^+$ T cells succumb to *L. major* infection (Chakkalath and Titus, 1994; Erb *et al.*, 1996; Holaday *et al.*, 1991; Mitchell, 1983). These immunological studies indicate that important differences exist between *L. major* and *La* parasites in the biology of their interactions with the host. This point is further strengthened by the recent finding that lipophosphoglycan is an essential virulent factor for *L. major* but

not at all for *L. mexicana* (Ilg, 2000; Spath *et al.*, 2000). Thus, conclusions drawn from studies of one *Leishmania* species may not always be extended to other ones. It is therefore necessary, in the context of *La* infection, to revisit some fundamental aspects of *Leishmania*-host interactions that are mainly built upon *L. major* infection. Given the fact that MΦs are the primary host cells for all *Leishmania* parasites, in this study we sought to ascertain the role of Th1 cytokine IFN-γ in the dynamic interactions between *La* parasites and host MΦs. Our efforts led to the surprising observation that IFN-γ may promote the replication of *La* amastigotes.

Materials and Methods

Mice. Wild-type and IFN-γ-deficient BALB/c and B6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). They were maintained under specific pathogen-free conditions and used at 6-10 weeks of age. All protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Reagents. Recombinant murine IFN-γ and the neutralizing mAb against mouse TGF-β (clone 1D11) were purchased from R&D systems (Minneapolis, MN), while recombinant IL-10, TNF-α, and neutralizing mAb against IL-10 (clone JES5-16E3) were purchased from BD PharMingen (San Diego, CA).

LPS from *Salmonella typhimurium* and FITC-conjugated goat anti-mouse IgG (Fab specific) were purchased from Sigma (St. Louis, MO). The HRP-conjugated goat anti-mouse IgG (H+L) was purchased from Bio-Rad Laboratories (Hercules, CA). The *La*-specific antisera for staining intracellular parasites were harvested from BALB/c mice that had been infected for 4 months.

Parasites. L. amazonensis (MHOM/BR/77/LTB0016) parasites were maintained by regular passage through BALB/c mice. To culture parasites, 20% FBS-supplemented Schneider's *Drosophila* media (Life Technologies, Rockville, MD) were used, with pH 7 for promastigotes and pH 5 for amastigotes. Promastigotes were cultured at 23°C. Metacyclics of *L. amazonensis* were purified through negative selection with the 3A1 mAb (a gift by Dr. David Sacks, NIAID) as previously described (Courret *et al.*, 1999). Tissue-derived amastigotes were harvested from foot tissues of infected BALB/c mice and cultured at 33°C for 24 to 48 h before use. To prepare MΦ-derived amastigotes, MΦs that were infected with tissue-derived amastigotes for 96 h were lysed to release intracellular parasites (see below). These released amastigotes were rested at 33°C overnight in complete Schneider's *Drosophila* media (pH 5) prior to use. Unlike amastigotes immediately harvested from lesion tissues, MΦ-derived amastigotes had no FACS-detectable surface opsonization of antibodies (our unpublished data).

La infection in mice and the evaluation of disease course. Mice (5 to 8 per group) were subcutaneously inoculated at the right hind foot with 2×10^6 stationary-phase promastigotes or 10^5 tissue-derived amastigotes. The lesion size was measured with a digital caliper (Control Company, Friendswood, TX). At indicated time points, mice were sacrificed to determine the parasite burden by a limiting dilution assay as previously described (Qi *et al.*, 2001a). For certain experiments as indicated, mice were i.v. transferred through the tail vein with 10^7 S1A Th1 cells in 150 μl PBS one day before the infection. Mice receiving PBS were used as the control. The generation and characterization of the Th1 line S1A was reported elsewhere (Ji, J., Sun, J., and L. Soong, submitted).

Macrophage culture. Cells were cultured in IMDM media supplemented with 10% FBS, 1 mM sodium pyruvate, 50 μM 2-ME, 50 μg/ml gentamycin, and 100 U/ml penicillin. Bone marrow-derived MΦs (BM-MΦs) were generated as described previously (Soong *et al.*, 1996). Briefly, marrow cells were seeded in a Petri dish at 2×10^6 per 10 ml media supplemented with 10% L929 culture supernatants. After 5 days, non-adherent cells were discarded, and adherent cells were maintained for additional 2 to 4 days before being detached from the Petri dish with cold PBS containing 2 mM EDTA. These BM-MΦs were washed twice with warm media and then cultured on 4-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at 1.5×10^5

cells/well for enumeration of intracellular parasites with fluorescence microscopy. For other assays, cells were cultured in 24-well tissue culture plates at 3×10^5 cells/well. Peritoneal MΦs were obtained from the peritoneal lavage of mice that were intraperitoneally injected with 1 ml 3% thioglycolate broth 7 days ago.

MΦ stimulation and parasite infection. BM-MΦs or peritoneal MΦs that had been rested for at least 12 h in tissue culture plates or on chamber slides were washed once with warm media. They were then given different cytokines, LPS, or their various combinations 4 h before infection with *Leishmania* parasites. Alternatively, MΦs were first infected at the indicated parasite-to-cell ratio and then given cytokine stimulation 4 h later. Unless indicated otherwise, parasite-exposed MΦs were kept at 33°C, a temperature consistent with that of *Leishmania*-induced cutaneous lesions in mice (Scott, 1985). In some experiments as noted in figure legends, promastigotes were incubated with 2% antisera or freshly prepared mouse sera for 20 min at 33°C before used for infection. To synchronize amastigote binding to MΦs, the culture plate was spun at 100 ×*g* for 5 min immediately after parasites were added. MΦ culture was processed to evaluate intracellular parasite burdens at various time points for up to 96 h postinfection, beyond which the spontaneous

lysis of MΦs with huge parasitophorous vacuoles made it difficult to accurately measure parasite loads (our unpublished observation).

Enumeration of parasites in MΦs with fluorescent microscopy. Fluorescent labeling of intracellular parasites was done according to a previously described method (Kane and Mosser, 2001). Briefly, the infected MΦ monolayer was fixed on the chamber slide with methanol at 4°C for 20 min and then washed twice with PBS. It was subsequently stained with antisera (1:200 in PBS, for 20 min at 4°C), washed 3× with PBS, and then stained with FITC-conjugated goat anti-mouse IgG(H+L) (1:200 in PBS, at 4°C for 20 min). Cells were counter-stained with DAPI and then examined under a coverslip with a Zeiss AxioPlan II fluorescent microscope (Thornwood, NY). To enumerate intracellular parasites, each well of the chamber slide was approximately divided into three areas along the long axis, and a random field from each of the three areas was pictured in both FITC and DAPI channels with Plan-Neofluar 40×/0.75 lens. The software-merged image was then evaluated, and data representing the three random fields were pooled. Typically, 500 to 700 cells were counted for each chamber well.

Evaluation of the parasite burden in MΦs after cell lysis by SDS. The exposure to a low concentration of SDS (0.01%, w/v) has been used to lyse infected MΦs in order to release intracellular parasites (Pham and Mauel,

1987). Accordingly, at different time points following infection, MΦs in 24-well tissue culture plates (3 wells per condition) were washed with PBS and then exposed to 200 μl of 0.01% SDS in PBS at 37°C. The process of cell lysis was monitored under an inverted microscope. The lysis was typically completed within 10 to 15 min, with virtually all intracellular parasites released. This parasite suspension was immediately supplemented with 0.8 ml complete culture media and thoroughly re-suspended by repeated pipetting. The number of parasites per well was counted with a hemocytometer.

Data analysis. Two-tailed *t* tests were used to evaluate statistical significance of the difference between experimental groups. All graphs were generated with SigmaPlot software (SPSS Inc., Chicago, IL).

Results

Polarized Th1 cells fail to control *La* amastigote infection. Murine infection by *La* parasites does not exhibit Th1/Th2 dichotomy in association with resistance/susceptibility. Nonetheless, a highly polarized Th1 response is thought to be sufficient for controlling *La* infection in susceptible hosts. This is not only because Th1 responses play a protective role against many protozoan infections including that caused by *L. major* (Sacks and Noben-Trauth, 2002), but also because vaccine protection against *La* infection is associated with much enhanced Th1 responses (Soong *et al.*, 1995). We have

recently generated a *La*-specific Th1 cell line, S1A, from splenocytes of infected B6 mice through repeated *in vitro* stimulation with amastigote lysates in the presence of IFN-γ and a neutralizing anti-IL-4 mAb. Intracellular staining and FACS analysis indicates that more than 90% of S1A cells produce IFN-γ and TNF-α upon antigenic or polyclonal stimulation with no detectable IL-4 or IL-10 production (Ji *et al.*, submitted[4]). Upon *i.v.* transfer into naïve B6 mice that were challenged with *La* promastigotes one week later, S1A helped control the lesion development and reduced the parasite burden by more than 3 orders of magnitude (Fig. 8A). Curiously, however, when amastigotes were used for infection, the S1A transfer failed to reduce the parasite burden and slightly accelerated the lesion development (Fig. 8B). Importantly, S1A-transferred mice that were later infected with amastigotes did exhibit a strong Th1 cytokine pattern: when 10^6 lymph node cells were stimulated with amastigote lysates in 200 μl complete media for 72 h, the IFN-γ level was 220±45 ng/ml, accompanied by 0.12±0.04 ng/ml IL-4 (n=5). Thus, S1A cells failed to control the amastigote infection in spite of orchestrating a highly Th1-polarized response. Thus, a polarized Th1 response, albeit being able to confer protection against promastigote infection, may not suffice to limit

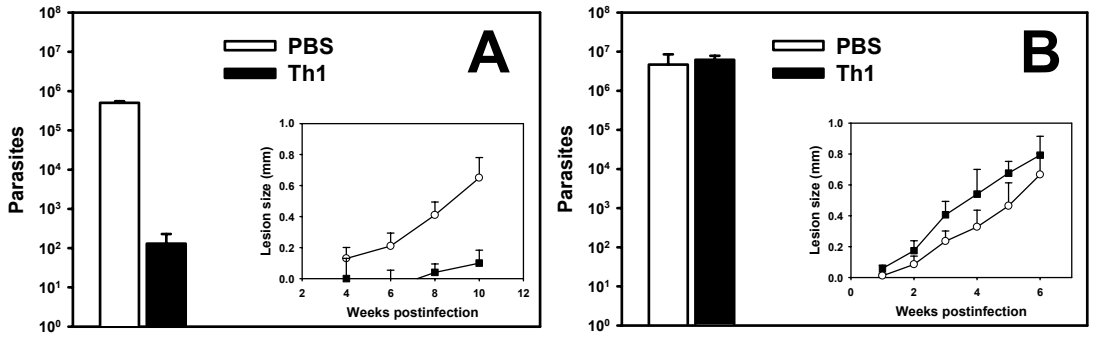

Figure 8. *La* infection in mice following adoptive transfer of Th1 cells. Groups of 5 to 8 B6 mice were i.v. transferred with 10^7 S1A Th1 cells or with PBS alone one day prior to infection with (A) 2×10^6 stationary-phase promastigotes or (B) 10^5 tissue-derived amastigotes. Parasite burdens were assayed at 10 wk post promastigote infection (A) or 6 wk post amastigote infection (B). Inset figures show the lesion size at indicated time points. Data represent 3 independent experiments.

amastigote propagation in tissues. One explanation for this dramatic distinction in the effectiveness of Th1 transfer against promastigote- or amastigote-established infection is that the two forms of parasites may have quite different fates in immune-activated MΦs. Since MΦs are able to kill *La* promastigotes following IFN-γ activation *in vitro* (Soong *et al.*, 1997), we sought to test the fate of amastigotes in IFN-γ-activated MΦs.

Enhanced amastigote replication in IFN-γ-treated murine MΦs. In agreement with the previous report (Soong *et al.*, 1997), MΦs treated with 20 ng/ml IFN-γ harbored fewer parasites than did untreated counterparts 48 h after promastigote infection (Fig. 9A). Strikingly, when MΦs were infected with *La* amastigotes, the same IFN-γ treatment significantly increased the number of parasites per cell, from 3.18 parasites per control MΦ to 5.08 parasites per

IFN-γ-treated MΦ (p<0.01, Fig. 9A). This increase of the average number of parasites per MΦ can be appreciated from the micrographs in Fig. 9B and 9C. It thus appeared that IFN-γ promoted the amastigote replication in MΦs.

A

		Control	IFN-γ-treated
Ama.	Exp. 1	$\frac{2205}{643} \approx 3.43$	$\frac{2618}{500} \approx 5.24$
	Exp. 2	$\frac{1456}{586} \approx 2.48$	$\frac{3250}{638} \approx 5.09$
	Exp. 3	$\frac{1755}{590} \approx 2.97$	$\frac{2367}{557} \approx 4.25$
	Exp. 4	$\frac{2469}{621} \approx 3.98$	$\frac{3098}{545} \approx 5.68$
	Exp. 5	$\frac{1816}{601} \approx 3.02$	$\frac{3159}{617} \approx 5.12$
	Mean±SD	3.18±0.56	5.08±0.52[a]
Pro.	Exp. 2	$\frac{1073}{621} \approx 1.73$	$\frac{443}{579} \approx 0.77$
	Exp. 5	$\frac{1389}{678} \approx 2.05$	$\frac{631}{701} \approx 0.90$

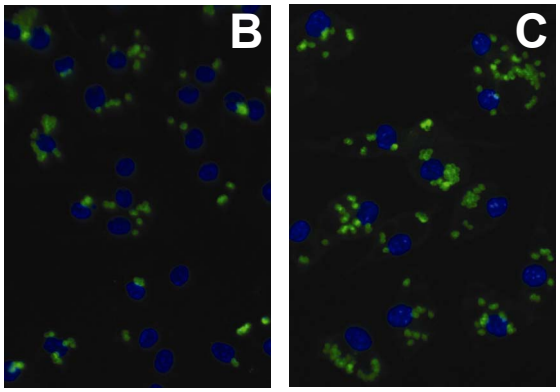

Figure 9. Microscopic evaluation of *La* infection in MΦs. BM-MΦs from BALB/c mice (Exp. 1 and 2) or B6 mice (Exp. 3, 4, and 5) were seeded at 1.5×10^5 cells/chamber on chamber slides. Cells were left untreated or treated with 20 ng/ml IFN-γ for 4 h prior to infection with 3×10^5 amastigotes or 7.5×10^5 stationary-phase promastigotes. After 48-h incubation, cells were processed for immuno-staining of the parasite. (A) The summary of the average parasites/cell, which was calculated by the total number of parasites divided by the total number of MΦs examined. Representative images of amastigote-infected control (B) and IFN-γ-treated MΦs (C) are shown.

To further address this possibility, we directly determined the total parasite burden in infected MΦ culture by counting the number of parasites released after lysing MΦs with 0.01% SDS in PBS (Pham and Mauel, 1987). The kinetics of amastigote replication were examined in MΦs that were untreated or treated with 20 ng/ml IFN-γ for 4 h prior to infection. As shown in

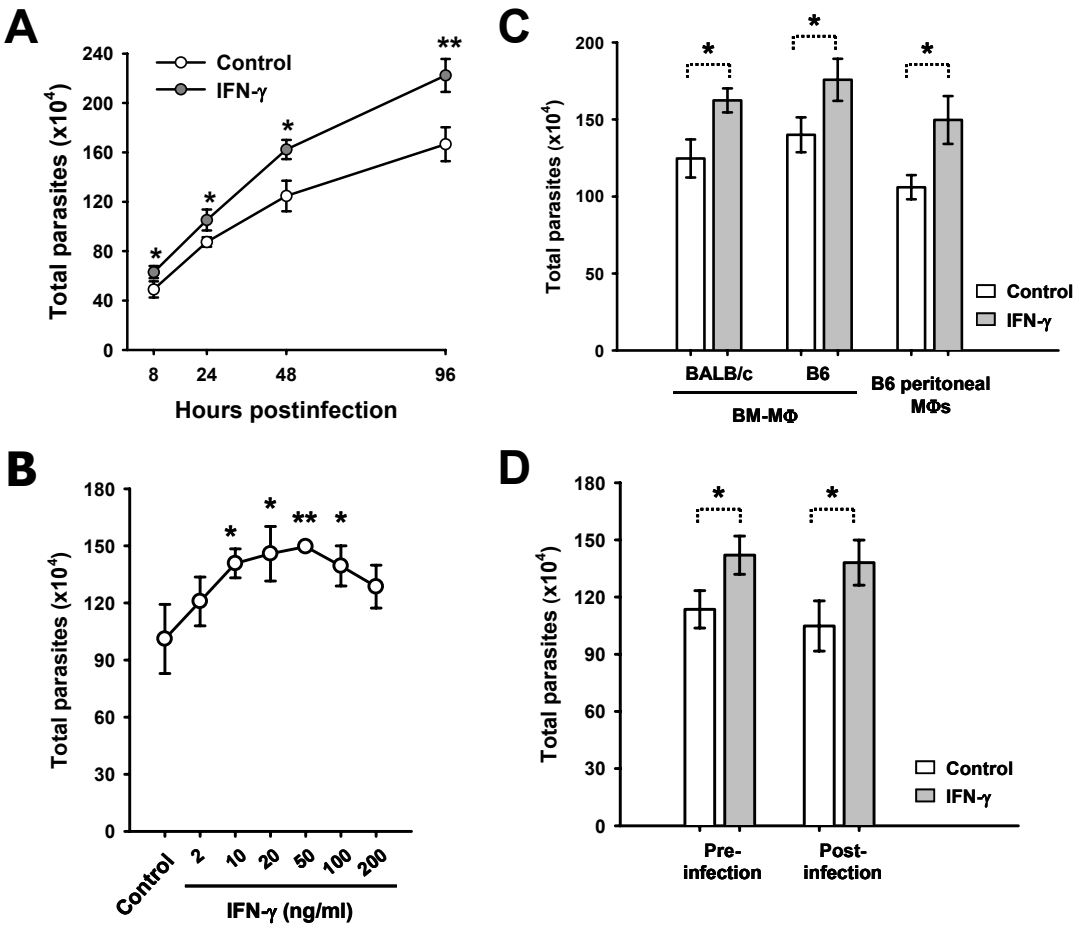

Figure 10. Enhanced amastigote replication in IFN-γ-treated MΦs. (A) B6 BM-MΦs (3×10^5 cells/well) were left untreated or treated with 20 ng/ml IFN-γ for 4 h prior to infection with 4.5×10^5 amastigotes. The number of parasites in each well was determined at indicated time points. One of three experiments with similar results is shown. (B) Different concentrations of IFN-γ were tested with a similar procedure as in (*A*). The number of parasites in each well of MΦ culture was determined at 48 h postinfection. Data represent three independent experiments. (C) MΦs from indicated strains of mice and sources were treated with 20 ng/ml IFN-γ for 4 h and then infected with amastigotes. The number of parasites was determined at 48 h postinfection. One of two experiments with similar results is shown. (D) B6 BM-MΦs were treated with 20 ng/ml IFN-γ 4 h before or 4 h after amastigote infection. The parasite binding to the MΦ monolayer was synchronized as described in *Materials and Methods*. Data represent three independent experiments. Each data point in *A-D* is presented as the mean ± SD for a given culture in triplicate (* $p<0.05$, ** $p<0.01$).

Fig. 10A, significantly more amastigotes were recovered from IFN-γ-treated MΦs than from control cells at 8, 24, 48, and 96 h postinfection. Next, MΦs

were treated with different doses of IFN-γ, and the parasite load was tested 48 h after infection. As shown in Fig. 10B, the amastigote burden was significantly augmented in MΦs treated with 10-100 ng/ml IFN-γ. MΦs treated with 2 ng/ml or 200 ng/ml IFN-γ harbored more amastigotes, although the increase was not always statistically significant. To exclude the possibility that the increase of intracellular amastigote burdens by IFN-γ treatment is peculiar to a certain type of MΦs, different MΦ preparations were examined. As shown in Fig. 10C, the IFN-γ treatment augmented the total number of amastigotes regardless of the genetic background (B6 vs. BALB/c) or the source of MΦs (BM-MΦs vs. peritoneal MΦs). Taken together, these data clearly demonstrate that IFN-γ treatment augments the amastigote load in murine MΦs.

This unexpected phenomenon may be because MΦs are actually more permissive to intracellular amastigote replication after IFN-γ treatment. Alternatively, it could result from an increase of amastigote uptake in IFN-γ-treated MΦs. This possibility is particularly relevant in that a fraction of amastigotes harvested from lesion tissues remain opsonized by host IgG even after 24 to 48 h in culture ((Hodgkinson and Soong, 1997) and our unpublished data), and that IFN-γ could enhance Fc receptor(FcR)-mediated phagocytosis by MΦs (Warren and Vogel, 1985). In addition, IFN-γ might have direct effects on *La* amastigotes in shortening their doubling time. For

example, IL-2 and IFN-γ were reported to promote *in vitro* growth of *La* promastigotes and *Trypanosoma brucei*, respectively (Bakhiet *et al.*, 1996; Mazingue *et al.*, 1989). To exclude these possibilities, we treated MΦs with IFN-γ after they had internalized amastigotes. Phagocytosis of *La* amastigotes by MΦs is known to be a rapid and efficient process, taking approximately 30 minutes to complete from the time of parasite binding (Love *et al.*, 1998). In our hands, when the binding of amastigotes to the MΦ monolayer was synchronized by gentle centrifugation, virtually no amastigotes could be seen outside of MΦs by 1 h. At this time point, the number of parasites recovered from lysed MΦs was essentially equal to the number in the initial inoculum (not shown), indicating complete and synchronized uptake of amastigotes. Under this condition of synchronized parasite uptake, when MΦs were stimulated with IFN-γ 4 h after the onset of amastigote infection, they still harbored significantly more amastigotes than untreated counterparts (Fig. 10D). Therefore, the increased parasite burden was not because enhanced parasite uptake following IFN-γ treatment or IFN-γ acting directly on the parasite. In a total of more than 20 experiments, the amastigote load at 48 h postinfection was on average enhanced by 34±13% in 20 ng/ml IFN-γ-treated MΦs, regardless whether the IFN-γ treatment was 4 h prior to or after the onset of infection. Together, these data strongly suggest that IFN-γ stimulation of MΦs promotes the replication of intracellular amastigotes.

Enhanced replication in IFN-γ-treated MΦs is a unique property of amastigotes not shared by metacyclic promastigotes. Data presented in Fig. 10, as well as in a previous report (Soong *et al.*, 1997), clearly indicated that promastigotes were being killed in IFN-γ-activated MΦs. However, these experiments involved the use of stationary-phase promastigotes, which typically contain only 10% of highly-infective metacyclic promastigotes. Metacyclics directly give rise to amastigotes in a natural infection, and are more resistant to innate killing by hosts (Sacks and Perkins, 1984; Sacks and Perkins, 1985). This prompted us to further examine whether enhanced growth in IFN-γ-activated MΦs is a property shared by amastigotes and metacyclics. As shown in Fig. 11A, significantly fewer parasites were recovered from metacyclic-infected IFN-γ-treated MΦs. This was also true when metacyclics were pre-incubated with freshly harvested normal sera or anti-sera, despite the fact that serum opsonization may assist certain *Leishmania* species in attaching to and surviving in MΦs (Blackwell *et al.*, 1985; Mosser and Edelson, 1987). Clearly, the ability to grow better in IFN-γ-activated MΦs is a unique feature of *La* amastigotes.

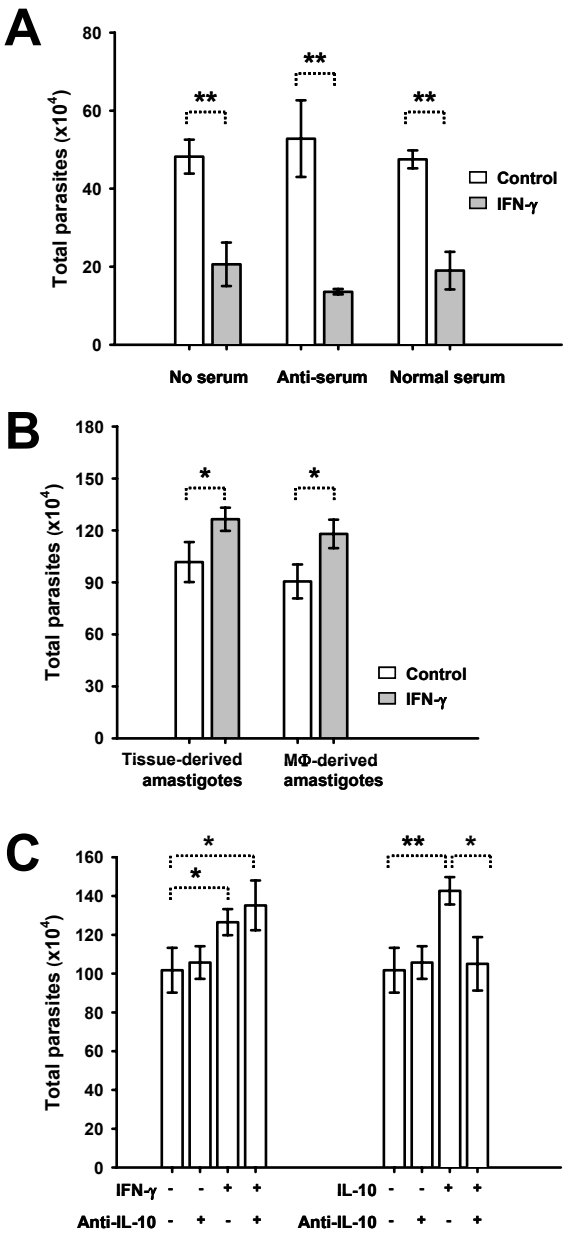

Figure 11. Enhanced replication in IFN-γ-treated MΦs is a unique property of amastigotes and is not mediated by IL-10. MΦs (3×10^5/well) were infected with 1.5×10^6 metacyclics or 4.5×10^5 amastigotes in 24-well plates. All parasite burdens were determined at 48 h postinfection. (A) B6 BM-MΦs were either untreated or treated with 20 ng/ml IFN-γ and then infected with metacyclic promastigotes that had been pre-incubated without serum, with 5% *La*-specific anti-serum, or with normal mouse serum. (B) B6 BM-MΦs were left untreated or treated with 20 ng/ml IFN-γ and then infected with tissue-derived or MΦ-derived amastigotes. (C) Before infection with amastigotes, B6 BM-MΦs were pre-treated with 20 ng/ml IFN-γ in the presence or absence of 10 μg/ml anti-IL-10 mAb. As controls, cells were treated with 20 ng/ml IL-10 with or without 10 μg/ml anti-IL-10 mAb. All data are presented as the mean ± SD of 3 wells for each condition (* $p<0.05$, ** $p<0.01$).

Enhanced replication in IFN-γ-treated MΦs is not mediated by IL-10. To our knowledge, no biological activities that are previously ascribed to IFN-γ are able to directly account for the observed replication-promoting effect on amastigotes inside MΦs. However, IFN-γ might promote amastigote growth in

MΦs through induction of another cytokine(s) that favors parasite replication. In this context, IL-10 is particularly relevant. IL-10 can deactivate nitric oxide(NO)-mediated leishmanicidal effects of MΦs (Kane and Mosser, 2001; Vieth *et al.*, 1994; Vouldoukis *et al.*, 1997). It can also directly promote the growth of *L. major* parasites in MΦs by inducing arginase (Iniesta *et al.*, 2001; Iniesta *et al.*, 2002), a key enzyme in the synthetic pathway of polyamines that are required for intracellular growth of *Leishmania* parasites (Yarlett and Bacchi, 1994). Interestingly, opsonized amastigotes can induce FcR-dependent IL-10 production by MΦs, provided that these MΦs also receive concomitant inflammatory stimuli such as bacterial LPS and hyaluronic acid (Kane and Mosser, 2001). Thus, if IFN-γ constitutes a co-stimulus to induce IL-10 production from infected MΦs, it may explain why IFN-γ can enhance the replication of *La* amastigotes. However, this does not appear to be the case. As shown in Fig. 11B, the growth-enhancing effect of IFN-γ was observed with both tissue-derived amastigotes and MΦ-derived amastigotes, which were bound by or free of host IgG, respectively (see *Materials and Methods* for details). In addition, no IL-10 was detectable by ELISA in amastigote-infected MΦs regardless whether they were treated with IFN-γ or not (not shown). More importantly, while exogenous IFN-γ and IL-10 promoted amastigote replication to a similar extent, the addition of a neutralizing anti-IL-10 mAb totally negated the effect of IL-10 but not that of IFN-γ (Fig. 11C). Similarly, a neutralizing mAb

against TGF-β did not affect the growth-promoting effect of IFN-γ (not shown). Since arginase I upregulation is responsible for the enhanced growth of *L. major* parasites seen in MΦs treated with IL-4, IL-10, or TGF-β (Iniesta *et al.*, 2001; Iniesta *et al.*, 2002), we also examined the level of arginase I expression by western blotting in IFN-γ-treated and *La* amastigote-infected MΦs. We found no major changes of arginase expression by IFN-γ treatment, amastigote infection, or their combination (not shown here, see Fig. 19). Taken together, these results indicate that IFN-γ-enhanced amastigote replication is not likely to be mediated by the induction of IL-10 or other arginase I-enhancing cytokines.

IFN-γ together with LPS limits intracellular replication of *La* amastigotes. Having demonstrated that IFN-γ promotes intracellular replication of *La* amastigotes, we next assessed its effects when combined with other factors such as TNF-α. The reason that *L. major* amastigotes are efficiently killed in IFN-γ-activated MΦs is that the parasite synergizes with IFN-γ in inducing TNF-α from MΦs (Green *et al.*, 1990a; Liew *et al.*, 1990a), and that IFN-γ in combination with TNF-α optimally activates the iNOS-mediated NO production, an essential leishmanicidal mechanism operating *in vitro* and *in vivo* (Green *et al.*, 1990a; Green *et al.*, 1990b; Liew *et al.*, 1990c; Murray and Nathan, 1999; Wei *et al.*, 1995). Since neither *La* amastigotes nor

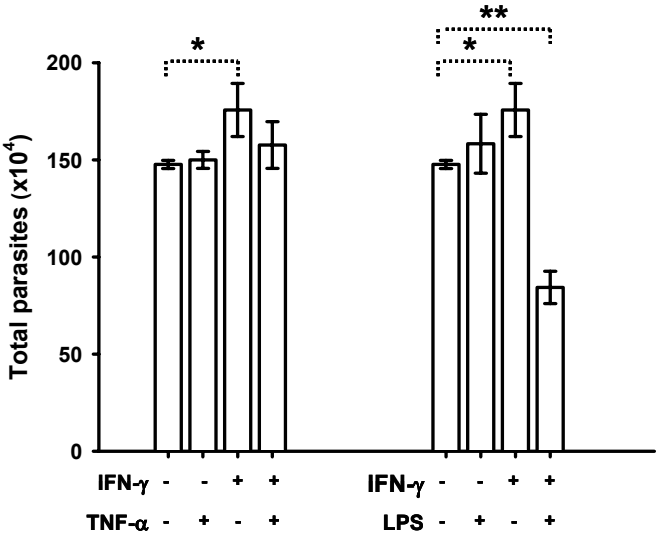

Figure 12. Effects of IFN-γ coupled with TNF-α or LPS on amastigote replication in MΦs. B6 BM-MΦs (3×10^5 cells/well) were left untreated or treated with 20 ng/ml IFN-γ, 20 ng/ml TNF-α, or 10 ng/ml LPS, individually or in combinations as indicated. Infection with 4.5×10^5 amastigotes was carried out 4 h later. The number of parasites in each well of MΦ culture was determined at 48 h postinfection. Results represent two independent experiments. Each data point is mean ± SD of 3 wells for each condition (* $p<0.05$, ** $p<0.01$).

promastigotes induce TNF-α in IFN-γ-treated MΦs (not shown), we sought to determine whether IFN-γ together with exogenous TNF-α can induce MΦ killing of amastigotes. We also tested the combination of IFN-γ and LPS, which is probably the strongest iNOS/NO inducer for murine MΦs (MacMicking *et al.*, 1997). As shown in Fig. 12, TNF-α by itself or in combination with IFN-γ had no discernable effects on amastigote replication in MΦs. IFN-γ coupled with LPS, however, rendered MΦs significantly more resistant to intracellular proliferation of amastigotes, while LPS by itself failed to do so. These data clearly indicate that when combined with other factors such as LPS, IFN-γ is able to stimulate MΦs to limit the amastigote replication.

Temperature dependence of amastigote replication and effects of IFN-γ and LPS treatment. In all of above experiments, MΦ infection with *La*

amastigotes and subsequent incubation was carried out at 33°C. This temperature is consistent with that of *Leishmania*-induced lesions in mice (Scott, 1985). However, it can be argued that microbicidal functions of MΦs are impaired at such a lowered temperature. Thus, it is possible that the requirement for both IFN-γ and LPS to induce a leishmanicidal state in MΦs is peculiar at 33°C, while IFN-γ alone might be sufficient at 37°C. Therefore, we further tested whether at 37°C IFN-γ alone or in combination with LPS can promote killing of *La* amastigotes in MΦs. A sharp loss of intracellular amastigotes was observed at 37°C in infected MΦs without any treatment (Fig. 13A, dashed line). This was particularly evident after 24 h. Such parasite loss was most likely due to spontaneous dying of amastigotes rather than active killing by untreated MΦs, because lesion-derived amastigotes quickly ceased to replicate and spontaneously die when cultured at 37°C ((Hodgkinson and Soong, 1997) and our unpublished data). This spontaneous loss of parasites at 37°C was in sharp contrast to the pronounced parasite propagation at 33°C (compare dashed lines in Fig. 13A and 13B). At 33°C, amastigote replication was clearly reduced in MΦs treated with IFN-γ plus LPS as compared to untreated ones (compare dashed line and filled circle in Fig. 13B). Interestingly, co-treatment with IFN-γ and LPS did not enhance the

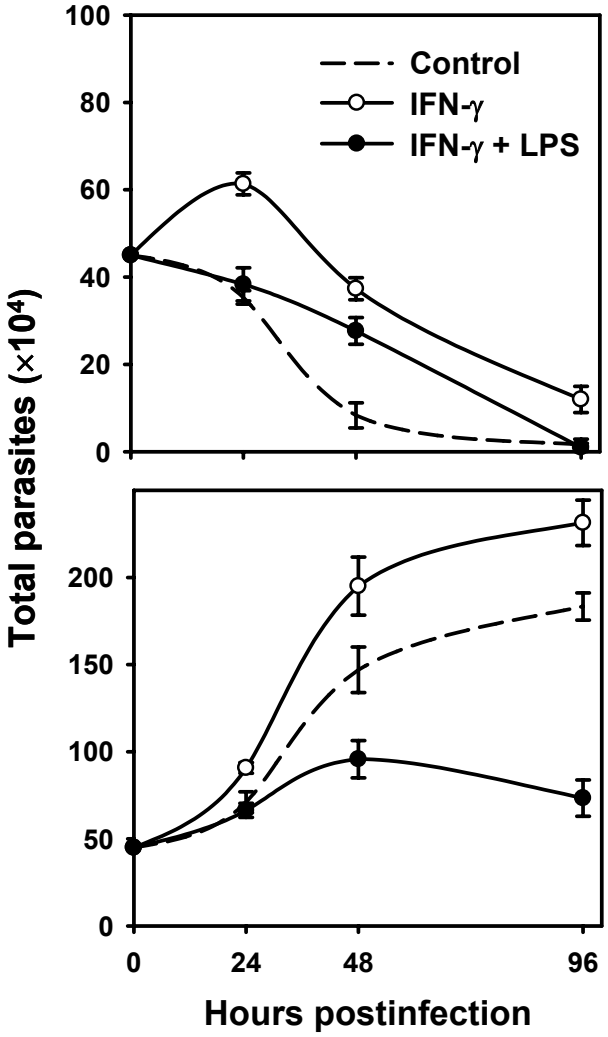

Figure 13. The temperature dependence of amastigote replication in MΦs. B6 BM-MΦs (3×10^5 cells/well) were left untreated, treated with 20 ng/ml IFN-γ alone, or with 20 ng/ml IFN-γ plus 10 ng/ml LPS for 4 h and then infected with 4.5×10^5 amastigotes. Infected MΦ culture was either kept at 37 (A) or 33°C (B) throughout the experiments. At indicated time points, the amastigote burden was determined. Data are presented as mean ± SD of 3 wells. One of two independent experiments is shown.

spontaneous loss of amastigotes at 37°C (Fig. 13A). Importantly, the treatment of MΦs with IFN-γ alone also failed to accelerate the spontaneous loss of intracellular amastigotes at 37°C (compare open circle and dashed line in Fig. 13A). Rather, it induced significantly amastigote replication during the first 24 h after the onset of infection. Thereafter, the parasite load in IFN-γ-treated MΦs was not lower than that in the control at any time points (Fig.

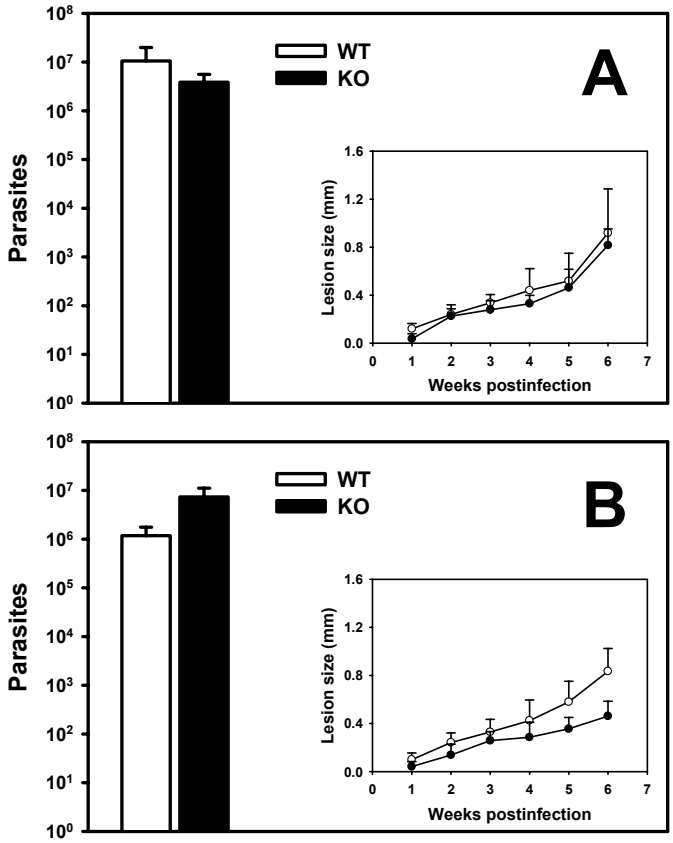

Figure 14. The role of endogenous IFN-γ during *La* amastigote infection in mice. Groups of 5 IFN-$\gamma^{-/-}$ (KO) or their wild-type (WT) control mice of either BALB/c (A) or B6 (B) background were infected with 10^5 *La* amastigotes. Lesion development was monitored weekly, and the parasite burden was assayed at 6 wk postinfection. Shown are data from one of two experiments with similar results.

13A). Taken together, these data demonstrate that IFN-γ-enhanced amastigote replication in MΦs is not peculiar to a lowered temperature. Rather, IFN-γ promotes amastigote growth even at a temperature that does not favor the parasite survival.

The role of endogenous IFN-γ during *La* amastigote infection *in vivo*. Results from *in vitro* studies summarized above suggest that the effect of IFN-γ on amastigote infection is not unidirectional. By itself, IFN-γ renders MΦs more permissible to amastigote replication; in combination with other agents such as LPS or LPS-inducible factors, it can stimulate MΦs to control amastigote growth. Conceivably, the role of IFN-γ during *La* infection *in vivo* may not be as clear-cut as a protective or exacerbating one. To evaluate the

effect of endogenous IFN-γ, wild-type and IFN-γ-deficient mice on a B6 or BALB/c background were infected with *La* amastigotes and the disease course was examined. As shown in Fig. 14A, wild-type and IFN-γ-deficient BALB/c mice harbored a comparable load of parasites (10.6×10^6 vs. 3.9×10^6, p=0.15) and exhibited no difference in lesion development. On the B6 background, IFN-γ-deficient mice exhibited smaller lesions and harbored slightly more parasites than did their wild-type counterparts (7.3×10^6 versus 1.2×10^6, p=0.05). Overall, however, the absence of endogenous IFN-γ did not significantly alter the course of amastigote infection in either BALB/c or B6 mice. The reason that endogenous IFN-γ apparently had a minimal effect could be that wild-type mice simply did not mount a significant IFN-γ response. However, this was clearly not the case when we assayed the cytokine recall response *in vitro* by LN cells from *La* amastigote-infected mice. When stimulated with amastigote lysates for 72 h, 10^6 LN cells of BLAB/c and B6 mice (n=5) produced 61±9 ng/ml and 220±45 ng/ml IFN-γ, accompanied by 1.5±1.1 ng/ml and 0.11±0.05 ng/ml IL-4, respectively. These results are consistent with the duality that IFN-γ exhibits in its effect on amastigote infection *in vitro*: depending on the presence of other factors, it could promote killing or growth of amastigotes in MΦs. These two opposing outcomes are possibly canceled out by each other *in vivo*.

Discussion

Two decades ago, Scott *et al.* showed that despite being able to kill *L. major* amastigotes and *Toxoplasma gondii* parasites, lymphokine-activated MΦs were unable to control intracellular replication by *La* amastigotes (Scott *et al.*, 1983). Yet, no significant enhancement of amastigote replication was observed. This is probably because those authors used complete culture supernatants produced by splenocytes from *La*-sensitized mice as the lymphokine. With recombinant cytokines, we extended their work by showing that amastigote replication is enhanced in IFN-γ-treated MΦs, and thus further unveiled the unique ability of *La* parasites to resist and to take advantage of host defense mechanisms. On the other hand, our data clearly indicate that *La* amastigotes can be killed by MΦs if stimulated properly, e.g. by the combination of IFN-γ and LPS but neither of them alone (Figs. 12 and 13). Because iNOS-deficient MΦs failed to control amastigote replication even when stimulated with IFN-γ and LPS (our unpublished data), this killing is probably NO-mediated, similar to what have been observed for *L. major* parasites (Green *et al.*, 1990a; Green *et al.*, 1990b; Kane and Mosser, 2001). Thus, IFN-γ may exert a pathogenic or a protective effect, depending on other factors. The operation of such duality of IFN-γ *in vivo* is suggested by the observation that a strong IFN-γ response was mounted in *La* amastigote-infected mice, but in its absence, no major changes in the disease course

occurred (Fig. 14). Since LPS is not likely to be present in *La*-infected mice without compounding bacterial infection, it is imperative to identify the endogenous factor(s) that can shut down the replication-promoting effect of IFN-γ and turn on the killing mechanism against amastigotes. Since MΦs stimulated with IFN-γ and TNF-α still failed to control amastigote replication (Fig. 12), TNF-α alone is not sufficient to constitute such a factor. It is worth of mentioning that macrophage inflammatory protein(MIP)-1α and -1β, both of which are strongly induced by LPS, can activate murine MΦs to kill *L. major* and *L. donovani* amastigotes (Bhattacharyya *et al.*, 2002; Titus *et al.*, 1994). Future studies are required to address whether these factors are involved. Nonetheless, this hypothetical duality of IFN-γ *in vivo* would well explain the observed failure of Th1 transfer to control amastigote infection (Fig. 8): the transfer may simply provide a source of excessive IFN-γ without correcting a deficiency in optimal synergy between IFN-γ and the other factor(s) essential to turning on the killing mechanism. It is also consistent with the fact that prolonged administration of IL-12 to *La*-infected mice hardly ameliorates the disease (Jones *et al.*, 2000).

Also revealed in this study is the contrast between *La* promastigotes and amastigotes in terms of their different fates in IFN-γ-activated MΦs. The finding that promastigotes are being killed while amastigotes could grow better

correlates well with the observation that transferred Th1 cells controlled infection with promastigotes but not amastigotes (Fig. 8). During a natural infection, however, all surviving promastigotes in the mammalian host must eventually transform into amastigotes. Thus, there seems to be a significant period, from the time of promastigote entry to MΦs to the time of complete amastigote transformation, during which the parasite is highly vulnerable to Th1-induced microbicidal attacks by host MΦs. Indeed, this period of transformation was estimated to be as long as 5 days (Courret *et al.*, 2001). Conceivably, this 5-day period would be the window of opportunity for vaccine-generated memory Th1 cells to be recalled into the action, activating MΦs to eliminate the parasite. (as seen in successfully immunized mice). Beyond that, a polarized Th1 response would not be beneficial or even have an exacerbating impact. Based on these analyses, it appears that the immune memory induced by an effective anti-*La* vaccine would have to be fast-reacting and able to mount a Th1 response before the complete promastigote-amastigote transformation. Furthermore, Th1 enhancement should not be the sole basis for immunotherapies aimed at resolving *La* infection at a later stage. When amastigotes have established tissue residence in the host, for example, local administration of recombinant IFN-γ to the lesion may be harmful rather than beneficial to the host.

Previous studies have provided conclusive evidence that IFN-γ plays a clear-cut protective role in controlling *L. major* infection in mice (Swihart *et al.*, 1995; Szabo *et al.*, 2002; Wang *et al.*, 1994). Accordingly, the guiding principle for vaccine development and immunotherapeutics against *Leishmania* infection is to enhance Th1 responses. Results in this report reveal a more complicated role of IFN-γ during *La* infection and suggest that this particular infection requires a modified strategy for the development of vaccines and immunotherapies.

5 DIFFERENTIAL INDUCTION OF IL-10 AND IL-12 IN DENDRITIC CELLS BY MICROBIAL TLR ACTIVATORS AND THE SKEWING OF T CELL CYTOKINE PROFILES[1]

Introduction

To detect microbial infection, the immune system utilizes pattern-recognition receptors such as Toll-like receptors (TLRs) to recognize invariant molecular structures of related microbes (Hoffmann *et al.*, 1999; Medzhitov, 2001). TLR activation results in rapid induction of innate defense programs and ultimately the initiation of adaptive immunity (Aderem and Ulevitch, 2000). Dendritic cells (DCs) are critically involved in this process. Upon stimulation by microbial TLR ligands, DCs undergo a maturation process characterized by upregulation of MHC and co-stimulatory molecules and by homing to the secondary lymphoid organ. When matured, DCs are potent antigen presenting cells able to prime naïve T cells and direct T cell differentiation (Banchereau and Steinman, 1998). Therefore, TLRs expressed by DCs constitute a critical link between pathogen recognition and the induction of T cell immunity (Akira *et al.*, 2001; Medzhitov and Janeway, 1998).

The efficient control of microbial infections not only requires immune activation upon pathogen invasion but also demands the generation of appropriate types of immune responses tailored to a particular group of

[1] In press in *Infection and Immunity*, 2003.

pathogens. For example, certain infections require Th1 responses while others may be best countered by Th2 immunity (Abbas *et al.*, 1996; Romagnani, 1994). Accumulating evidence indicate that DCs can shape the Th1/Th2 balance according to the outcome of their microbial interactions (Moser and Murphy, 2000; Reis e Sousa *et al.*, 1999). DCs achieve this at least in part through differential production of IL-10 and IL-12 (Lanzavecchia and Sallusto, 2001), as IL-10 is implicated in priming Th2 responses (Iwasaki and Kelsall, 1999; Stumbles *et al.*, 1998), while IL-12 potently induces IFN-γ-producing Th1 cells (Trinchieri, 1995). However, it is not clear how the stimulation of various TLRs by microbes is connected to this process. Specifically, do microbial TLR activators differentially stimulate IL-10 and IL-12 production from DCs? A recent study showed that *Escherichia coli* LPS and *Porphyromonas gingivalis* LPS, being TLR4 and TLR2 agonists respectively, induced distinct profiles of inflammatory genes in murine macrophage (Hirschfeld *et al.*, 2001) and differentially stimulated IL-12 production by murine DCs (Pulendran *et al.*, 2001b). Further, human monocyte-derived DCs were found to express different cytokines/chemokines following activation by TLR4 or TLR2 stimuli (Re and Strominger, 2001). Thus, triggering different TLRs by various microbial stimuli may drive DCs to assume distinct phenotypes and functions. To further investigate this issue, we examined murine DC interactions with three microbial TLR stimuli: a TLR4 agonist, Gram-negative bacterial LPS

(Hoshino *et al.*, 1999; Poltorak *et al.*, 1998), and two TLR2 agonists, Gram-positive bacterial peptidoglycan (PGN) and yeast Zymosan (Takeuchi *et al.*, 1999; Underhill *et al.*, 1999). Specifically, we examined IL-10 and IL-12 production by DCs in response to these stimuli and tested their abilities to polarize T cell responses.

Materials and Methods

Mice. Female BALB/c (H-2^d) mice and C57BL/6 (H-2^b) mice (6-week old) were purchased from Jackson Laboratory (Bar Harbor, ME), and maintained under specific pathogen-free conditions. All experimental protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Reagents and antibodies (Abs). LPS (*Salmonella typhimurium*) and peptidoglycan (*Staphylococcus aureus*) as well as monensin, PMA, ionomycin, and saponin were purchased from Sigma (St. Louis, MO). Zymosan A from *Saccharomyces cerevisiae* was purchased from Molecular Probes (Eugene, OR). For intracellular staining of T cell cytokines, PE-conjugated anti-IL-4 (BVD4-1D11), anti-IL-10 (JES5-16E3), anti-IFN-γ (XMG1.2), and their respective isotype-matched control Abs were purchased from BD PharMingen (San Diego, CA). Tri-Color™-conjugated anti-CD4 (TC-CD4) was from Caltag (Burlingame, CA).

Dendritic cell culture. Bone marrow-derived DCs (BM-DCs) were generated as previously described (Lutz *et al.*, 1999; Qi *et al.*, 2001b) with certain modifications. Briefly, bone marrow cells were cultured in Petri dish (Fisher Scientific, Houston, TX) at 2×10^6 per 10 ml 10% FBS-supplemented IMDM. Culture supernatants of J558L cells that had been transfected with the murine *gm-csf* gene were used as the source of GM-CSF (the transfected cell line was a kind gift from Dr. Charles Janeway, Yale University.). Non-adherent cells were harvested at day 7 and further cultured in the 6-well plate overnight. Resultant non-adherent cells were typically >80% $CD11c^+$ cells as judged by FACS analysis. Sometimes, $CD11c^+$ cells were directly purified from day-7 culture to >95% purity with Microbeads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA).

Microbial stimulation and anti-CD40 treatment of dendritic cells. DCs were stimulated with different concentrations of LPS, PGN, or Zymosan in 96-well plates at 1.25×10^5 in 200 μl or in 24-well plates at 6.25×10^5 in 0.5 ml. The stimulation culture was not supplemented with any cytokines including GM-CSF. For some experiments, an agonistic anti-CD40 Ab (FGK45, (Rolink *et al.*, 1996b)) was added together with microbial stimuli and supernatants were harvested 24 hours (hrs) later. For other experiments, DCs were stimulated with microbial stimuli for 12 hrs, washed twice, and then cultured in the presence of anti-CD40 Ab for additional 24 hrs before the supernatants were

harvested. The anti-CD40 Ab was used in the form of FGK45 hybridoma supernatants at 1:10 dilution, an optimal titration as determined previously (Qi *et al.*, 2001b). For T cell priming assay, DCs were harvested at 12 hrs, washed twice with complete media, and then co-cultured with T cells. Aliquots of these DCs were used to isolate total RNA with TRI Reagent (Sigma) for measuring mRNA levels of IL-10 and IL-12 p40 (see below).

Isolation of $CD4^+CD45RB^{high}$ T cells and T-DC co-culture. For each isolation, 3 to 5 female C57BL/6 mice were used to minimize individual variation. Total $CD4^+$ T cells were purified from pooled spleens and lymph nodes with Dynabeads Mouse CD4 and DETACHaBEAD (Dynal Inc., Lake Success, NY) to >99% purity as assayed by FACS. The $CD45RB^{high}$ fraction was then purified from the total $CD4^+$ T cell preparation as previously described with certain modifications (Boursalian and Bottomly, 1999). Briefly, cells were consecutively labeled with biotin-CD45RB mAb (BD PharMingen) and streptavidin-conjugated Microbeads and then passed through a positive-selection column on a magnetic separator (Miltenyi Biotec). The unbound $CD45RB^{low}$ fraction was discarded. The bound fraction was eluted and re-applied to a new column and the column-retained fraction was harvested as $CD45RB^{high}$ fraction. $CD4^+CD45RB^{high}$ T cells (2×10^5/well), together with 2×10^4 DCs that were stimulated for 12 hrs, were seeded into 96-well plates in 200 μl IMDM supplemented with 1 ng/ml recombinant IL-2 (BD PharMingen). The T-

DC co-culture was maintained in 96-well plates for 4 days, then transferred to 24-well plates with supplementation of 0.5 ml plain medium, and further cultured for additional 6 days. Primed T cells were then assayed for the cytokine profile by intracellular staining following restimulation with 20 ng/ml PMA, 500 ng/ml ionomycin, and 2 μM monensin for 5 hrs.

Cytokine assays. To measure levels of IL-10, IL-12p70, and TNF-α in DC cultures, ELISA was performed with OptEIA Kits (BD PharMingen). The multi-probe template set mCK2b from RiboQuant RPA system (BD PharMingen) was used to measure mRNA levels of IL-10 and IL-12 p40 in a RNase protection assay according to the manufacturer's instruction. To enumerate IL-4-, IL-10-, and IFN-γ-producers in T cells primed by DCs, cells were first stained for CD4 and then fixed, permeablized, and stained with fluorochrome-conjugated mAbs specific for cytokines. Stained cells were analyzed on a FACScan flowcytometer (BD Biosciences, Franklin Lakes, NJ). Data were analyzed with the FlowJo software (TreeStar, San Carlos, CA).

Results and Discussion

To examine potential differences of LPS, PGN, and Zymosan in inducing DC cytokine production, BALB/c DCs were stimulated for 24 hrs and supernatant IL-10 and IL-12 concentrations were measured. As shown in Fig. 15, when

tested over a 10,000-fold dose range, LPS induced low IL-10 but high-level IL-12 p70 production. In contrast, DCs exposed to PGN produced low-level IL-12 but high-level IL-10. Another profile was observed in Zymosan-exposed DCs: high levels of both IL-10 and IL-12. This observation suggests that LPS, PGN, and Zymosan have inherently distinct abilities to induce DC IL-10 and IL-12 production. Alternatively, this phenomenon might simply reflect different sensitivities of DCs to these microbial stimuli, as the molar concentrations of actual TLR-engaging ligands in these stimuli are not known. However, even at the highest concentration tested, LPS did not induce IL-10 to a level comparable to that induced by PGN or Zymosan, while PGN failed to induce IL-12 to a level comparable to LPS or Zymosan. This in fact argues for the first possibility. To further differentiate the two, we tested TNF-α induction in DCs exposed to LPS, PGN, and Zymosan. Because all microbial TLR activators trigger the NF-κB activation leading to TNF-α production (Medzhitov, 2001), different levels of TNF-α induction would reflect different DC sensitivities to these stimuli. As shown in the lower panel of Fig. 15, while LPS was the most efficient TNF-α inducer at lower concentrations, PGN and Zymosan were able to stimulate similar levels of TNF-α at higher concentrations. Thus, at concentrations where comparable levels of TNF-α were stimulated, LPS, PGN, and Zymosan induced distinct profiles of IL-10 and IL-12. This was further confirmed with sorted DCs (>95% $CD11c^+$) to exclude potential effects

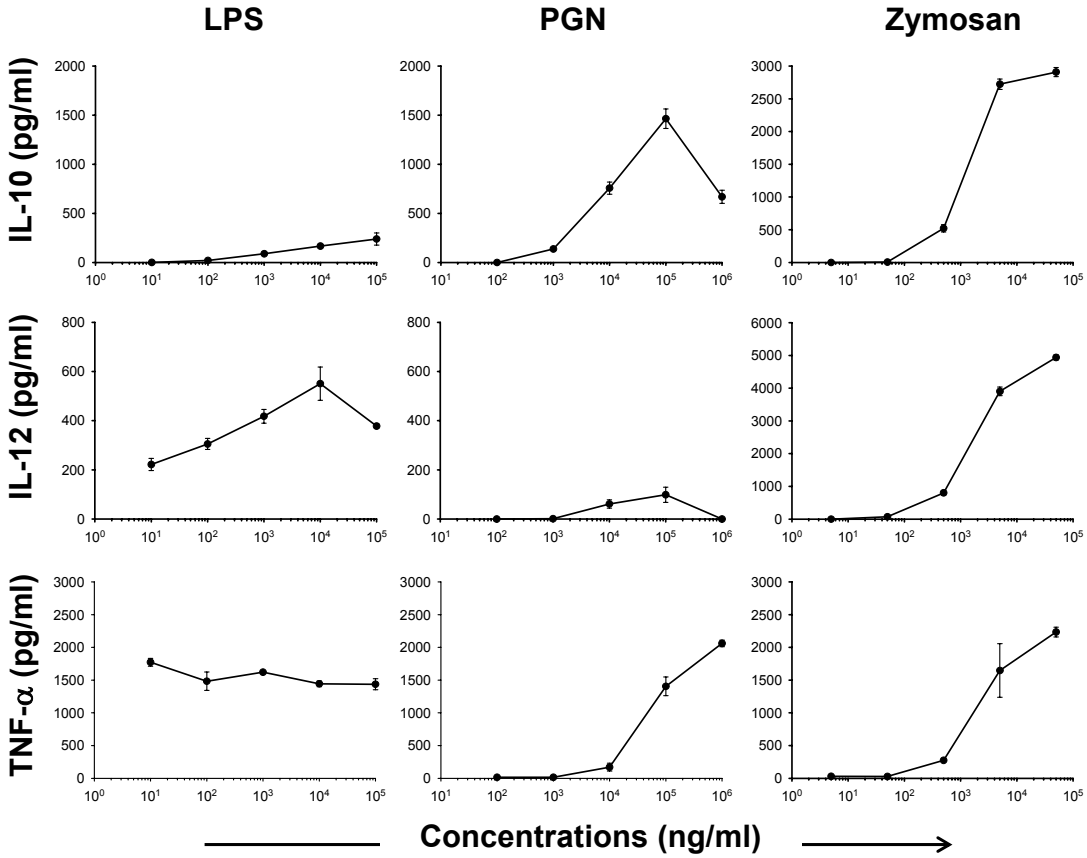

Figure 15. Cytokine production by LPS-, PGN-, and Zymosan-stimulated DCs. Bone marrow-derived BALB/c DCs were stimulated with indicated concentrations of LPS, PGN, or Zymosan, and 24-hr supernatants were harvested to measure IL-10, IL-12p70, and TNF-α by ELISA. None of these cytokines was measurable (<15 pg/ml) in untreated DCs (not shown). Representative results of five independent experiments for IL-10 and IL-12 and two for TNF-α are shown. Data are presented as mean ± SD.

of contaminating non-DC cells in the BM-DC preparation (data not shown). Finally, RNase protection assay was used to test levels of IL-10 and IL-12 p40 mRNA in DCs stimulated with 1 μg/ml LPS, 10 μg/ml PGN, or 5 μg/ml Zymosan. At these respective concentrations, LPS, PGN, and Zymosan induced a similar level of TNF-α (~1500 pg/ml, Fig. 15). As shown in Fig. 16, Zymosan-exposed DCs expressed the highest level of IL-10 mRNA, followed

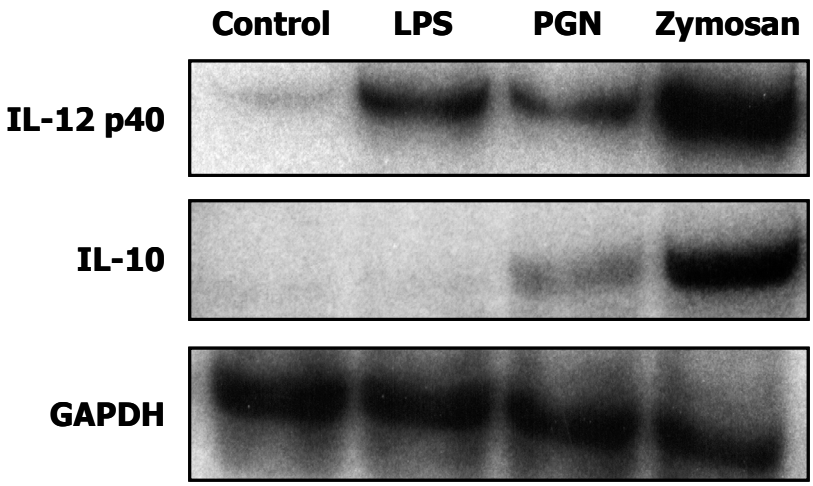

Figure 16. IL-10 and IL-12 p40 mRNA levels in LPS-, PGN-, and Zymosan-stimulated DCs. DCs were stimulated for 12 hrs with 1 μg/ml LPS, 10 μg/ml PGN, or 5 μg/ml Zymosan, and then total RNA was isolated and subjected to RNase protection assay with RiboQuant multi-probe template set mCK2b. The bands corresponding to IL-12p40, IL-10, and GAPDH are shown. Data represent two independent experiments.

by PGN-exposed DCs. IL-10 level was undetectable in LPS-exposed DCs by this assay. For IL-12 p40, Zymosan-exposed DCs remained to be the highest producer, followed by LPS- and PGN-exposed DCs. Of note, all of above experiments were also done with C57BL/6 DCs with similar results obtained (data not shown). Thus, the three microbial TLR activators are inherently different in their abilities to induce IL-10 and IL-12 production from murine DCs.

Upon interactions with T cells, microbe-exposed DCs may be further triggered to produce cytokines that are dependent on T cell-derived signals. Conceivably, such cytokine production would significantly contribute to the cytokine milieu controlling the outcome of T cell differentiation (O'Garra, 1998).

Previous studies have shown that IL-12 production by DCs can be augmented upon ligation of their CD40 receptors by T cell-derived CD40 ligands, which can be mimicked by using agonistic anti-CD40 Abs (Cella *et al.*, 1996; Koch *et al.*, 1996; Schulz *et al.*, 2000). Therefore, given the result that LPS, PGN, and Zymosan trigger distinct programs of innate cytokine production (Figs. 1 and 2), we further tested the impact of CD40 ligation on IL-10 and IL-12 production by DCs exposed to these stimuli. An agonistic anti-CD40 Ab (clone FGK45, (Rolink *et al.*, 1996b)) was used to engage CD40 receptors on DCs. DCs that were stimulated with LPS, PGN, or Zymosan were treated with the anti-CD40 Ab either immediately (Fig. 17A and B) or after being washed following 12-hr microbial stimulation (Fig. 17C and D). The 12-hr time point was chosen because the initial wave of microbe-induced cytokine production was largely completed by this time (our unpublished data), similar to what previously described for human monocyte-derived DCs (Langenkamp *et al.*, 2000). As shown in Fig. 17A, LPS- or Zymosan-induced IL-12 p70 production was significantly reduced when DCs were simultaneously activated with anti-CD40. In sharp contrast, while PGN by itself did not induce a high level of IL-12, simultaneous triggering of CD40 led to approximately 20-fold increase in IL-12 p70 production. When DCs were incubated with these stimuli for 12 hrs, washed, and then cultured for additional 24 hrs in the presence of anti-CD40 Ab, only PGN-stimulated DCs

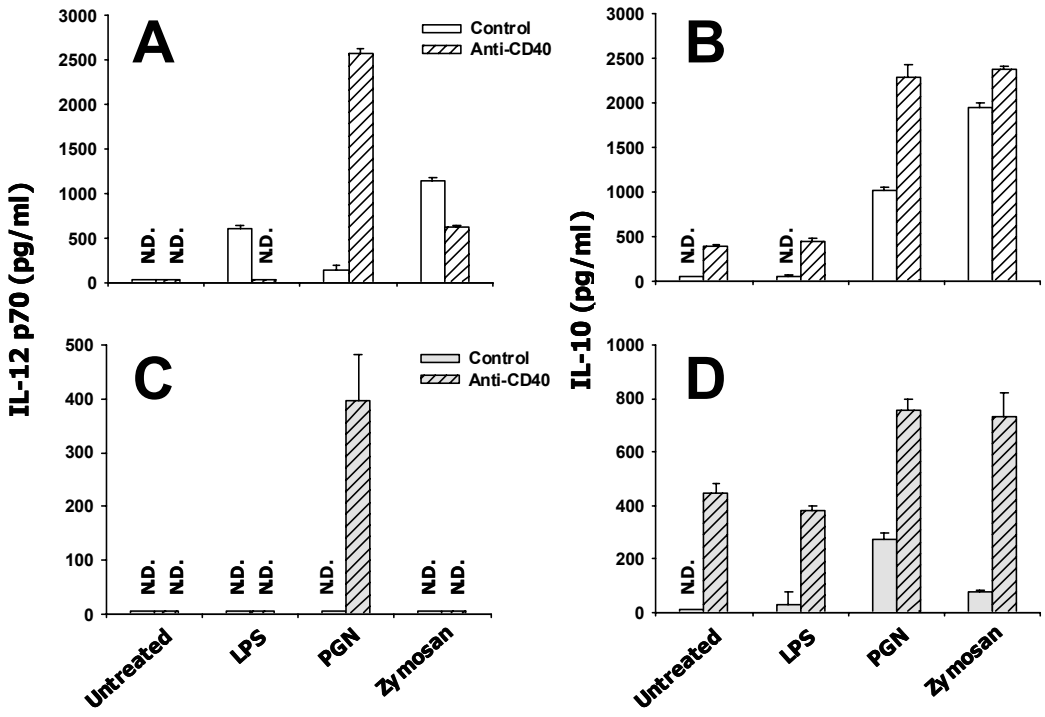

Figure 17. IL-10 and IL-12 p70 production in microbe-exposed DCs in response to CD40 ligation. (A, B) DCs were stimulated for 24 hrs with 1 μg/ml LPS, 10 μg/ml PGN, or 5 μg/ml Zymosan together with an anti-CD40 Ab (hatched) or control Rat IgG (open). (C, D) DCs were stimulated with microbial stimuli for 12 hrs, washed, and then further cultured for 24 hrs in the presence of the anti-CD40 Ab (hatched) or control Ab (open). At the end of these culture periods, supernatants were harvested to measure IL-12 p70 (A and C) and IL-10 (B and D). N.D., not detectable.

produced a significant level of IL-12 p70 (Fig. 17C). Clearly, PGN is distinguished from LPS and Zymosan by its capability of conditioning DCs to produce a high level of IL-12 p70 in response to CD40 engagement. On the other hand, applied either immediately or 12 hrs after microbial stimulation, the anti-CD40 Ab treatment augmented IL-10 production by DCs regardless of the microbial stimulus used (Fig. 17B and D). Of note, similar to isolated murine myeloid DCs (Iwasaki and Kelsall, 2001) but contrary to human monocyte-derived DCs (Cella *et al.*, 1996), murine BM-DCs produce IL-10 but not IL-12

p70 in response to CD40 engagement in the absence of microbial co-stimulation. Taken together, these results suggest that microbial TLR activators such as LPS, PGN, and Zymosan differentially modulate the potential of DCs to produce IL-12 in response to a T cell-derived signal.

To test whether LPS-, PGN-, and Zymosan-conditioned DCs would prime for distinct Th phenotypes *in vitro*, we used a mixed leukocyte reaction, in which BALB/c DCs (H-2^d) were used to activate C57BL/6 $CD4^+$ T cells (H-2^b). To minimize the influence of antigen-experienced memory cells, purified naïve T cells (as defined by $CD45RB^{high}$, see reference (Boursalian and Bottomly, 1999)) were used. In the absence of microbial stimulation, DCs primed T cells to exhibit a mixed cytokine profile as assayed by intracellular staining 10 days after the onset of co-culture. As shown in Fig. 18A, approximately 23% cells produced IFN-γ, while IL-4 and IL-10 producers were 17% and 7%, respectively. A negligible fraction of cells simultaneously produced IFN-γ and IL-4 or IFN-γ and IL-10. As shown in Figure 4B, LPS exposure rendered DCs to prime much less IL-4, while IFN-γ producers were induced to a comparable or slightly higher level. PGN stimulation significantly diminished DC potentials to prime T cells producing IL-4 and IL-10, while evidently enhanced their potency to prime IFN-γ producers. Interestingly, Zymosan-exposed DCs

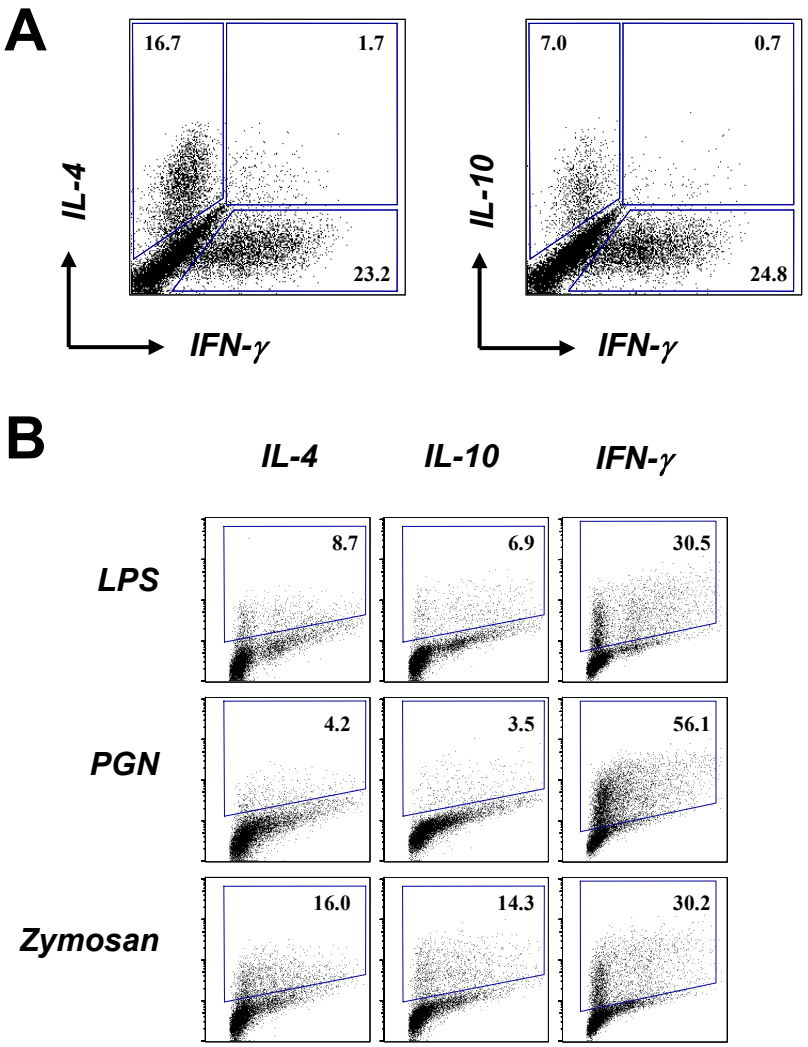

Figure 18. *In vitro* priming of distinct Th effector phenotypes by LPS-, PGN-, and Zymosan-stimulated DCs. BALB/c DCs were left untreated (A) or stimulated with 1 μg/ml LPS, 10 μg/ml PGN, or 5 μg/ml Zymosan for 12 hrs (B), and then co-cultured with 2×10^5 C57BL/6 $CD4^+CD45RB^{high}$ T cells (DC:T ratio=1:10). T cells were harvested 10 days later to enumerate cytokine-producing cells following brief restimulation. (A) T cells primed by untreated DCs were double-stained for IFN-γ and IL-4 or IFN-γ and IL-10. (B) T cells primed by microbe-exposed DCs were stained for individual cytokines separately. Gates were drawn based on isolate controls for individual specific Abs. Y-axis is the fluorescent intensity in $\log_{10}$ scale, while X-axis is the linear-scale forward scatter. Data represent five independent experiments.

induced as much IL-4 producers and even more IL-10 producers as compared to untreated counterparts. When tested at stimulating concentrations that were

10-fold higher or 10-fold lower than those presented in Fig. 18, the three stimuli still exhibited similar differences in their relative potencies to polarize T cell responses (not shown). Importantly, PGN-stimulated DCs was the strongest Th1 inducer, consistent with the fact that PGN potently potentiated CD40-dependent IL-12 p70 production from DCs (Fig. 17). Taken together, these data suggest that LPS, PGN, and Zymosan differentially condition DCs to prime Th effector phenotypes, and that distinct microbial TLR agonists can be a cue that DCs sense in directing Th effector development.

Results presented in this study have provided new information to our understanding of DC activation by microbial TLR ligands. Previously, using LPS as TLR4 and PGN as TLR2 ligand, Re and Strominger showed that human monocyte-derived DCs preferentially expressed IL-12 or IL-10 when stimulated through TLR4 or TLR2, respectively (Re and Strominger, 2001). Our study reported herein has confirmed their finding with murine DCs. Importantly, we tested the stimulants over a wide 10,000-fold dose range and with TNF-α as an internal control, and we still found LPS was a much stronger IL-12 inducer but weaker IL-10 stimulator than PGN (Fig. 15). This result strongly suggests that signaling programs induced by TLR4 and TLR2 are qualitatively rather than quantitatively different in DCs. This point is further supported by the interesting finding that simultaneous anti-CD40 treatment suppressed IL-12 production by DCs stimulated with LPS but enhanced IL-12

production by PGN-stimulated DCs (Fig. 17A). Interestingly, a recent study showed that as a immune-evading strategy, *Yersinia pestis* induced IL-10 through a TLR2-dependent pathway (Sing *et al.*, 2002). This is very much in agreement with our observation that two TLR2 ligands, PGN and Zymosan, directly induced high levels of IL-10 production from DCs (Figs. 15 and 16). The difference between TLR2 and TLR4 signaling is probably not limited to DCs, as Hirschfeld *et al.* studied macrophage responses and found that TLR4 agonists were more potent in inducing pro-inflammatory cytokines/chemokines than TLR2 ligands (Hirschfeld *et al.*, 2001). Collectively, these studies suggest that DCs may be induced to assume distinctive functions through activation of TLR4 or TLR2 and that TLR4 activation favors a stronger pro-inflammatory state than TLR2 activation. On the other hand, as TLR2 receptor has a large number of known ligands (Medzhitov, 2001), all TLR2 ligands are not necessarily equal. For example, PGN and Zymosan, both being able to engage TLR2 and possibly TLR6 (Ozinsky *et al.*, 2000), dramatically differed in the ability to induce DC production of IL-12 (Fig. 15). We speculate that additional TLRs or other receptors are involved in DC activation by Zymosan.

While activation through different TLRs leads to distinct cytokine production in DCs (Figs 15 and 16, (Re and Strominger, 2001)), it was not clear whether and how this would be linked to differential T cell priming. Upon activation by microbe-exposed DCs, antigen-specific T cells rapidly upregulate

CD40 ligands, which engage CD40 receptors on DCs to further modulate their cytokine production (van Kooten and Banchereau, 2000). Consistent with this notion, we found that the ability to produce IL-12 by LPS-, PGN-, and Zymosan-stimulated DCs were significantly modulated by CD40 engagement (Fig. 17). Interestingly, LPS, PGN, and Zymosan not only induced distinct cytokines in DCs directly, but also differentially conditioned CD40-dependent IL-12 production by DCs. More importantly, the direct IL-12 induction by these TLR activators is not necessarily correlated with the conditioned CD40-dependent IL-12 production by DCs. For example, while failing to directly induce a high level of IL-12 (Figs. 15 and 16), PGN strongly potentiated DCs to produce this cytokine following CD40 ligation (Fig. 17C). While LPS and Zymosan directly induced IL-12 from DCs (Figs. 16 and 17), they did not significantly potentiate CD40-dependent IL-12 production. Conceivably, the CD40-dependent cytokine production by microbe-exposed DCs would exert a greater impact on the outcome of T cell differentiation than the innate cytokine response that microbes directly induce in DCs. Indeed, when tested for the ability to skew Th responses, PGN-stimulated DCs, which responded to CD40 engagement by producing a high level of IL-12 while LPS- and Zymosan-stimulated DCs did not, were most potent in priming Th1 effectors (Fig. 18). Perhaps, the potential to orchestrate disparate innate and subsequent CD40-induced cytokine production offers DCs with certain flexibilities to sequentially

and differentially regulate the innate defense program and the adaptive T cell response. On the other hand, for both of the innate and adaptive phases, differential signals channeled through various TLRs are likely to be the crucial cue.

6 SUMMARY AND DISCUSSION

A majority of experimental data collected in this dissertation study have been presented in the form of independent manuscripts through the three preceding chapters. Specific conclusions pertinent to particular chapters have been summarized therein. In this final chapter, I would like to discuss those conclusions in a broader context of *Leishmania* infection. I will try to point out potential caveats of my experiments and elaborate on new directions that might be taken as a result of this dissertation. At certain points, additional data that are not included in previous chapters are incorporated here to aid the discussion.

The role of IFN-γ in *L. amazonensis* infection

As already discussed in the Chapter 2, it has been firmly established that IFN-γ-producing Th1 cells play a protective role in the host defense against murine leishmaniasis caused by *L. major*. IFN-γ-activated, NO-mediated pathogen destruction is also crucial to the control of other microorganisms such as *Toxoplasma* and *Trypanosoma* parasites (Stafford *et al.*, 2002). Indeed, the Th1 response is thought to be instrumental to the cell-mediated immunity against most, if not all, intracellular microbial infections. In the case of *La* infection in mice, however, this notion is probably more applicable to the promastigote than to the amastigote. As shown in Chapter 4,

La amastigotes can grow better in IFN-γ-activated MΦs, while the promastigote is expectedly being killed.

This contrast between amastigotes and promastigotes has two important implications for development of vaccines and immunotherapies. First, it would suggest that at an early stage of infection, when most parasites are still in the form of promastigotes, high levels of IFN-γ could activate MΦs to destroy a majority of parasites, leading to the control of infection before a clinical disease (see *Illustration 6*). This proposition is very much in line with

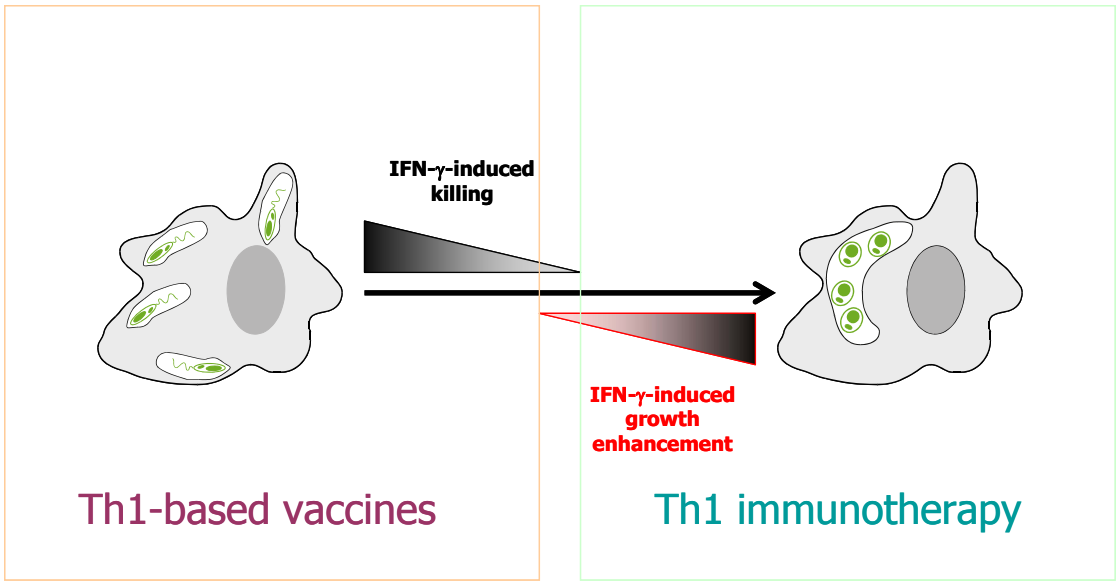

Illustration 6. Implications of the distinct fates of *L. amazonensis* promastigotes and amastigotes in IFN-γ-activated MΦs. During the transformation of promastigotes to amastigotes, which takes approximately 5 days *in vitro*, the parasite gradually loses its sensitivity to IFN-γ-induced MΦ killing, but probably gains the ability to enhance replication in IFN-γ-activated cells. Thus, the window of opportunity for a Th1-based vaccine to be effective is relatively short and probably only confined to first few days following infection (yellow box). Beyond this window of time, immunotherapeutic strategies, if solely aimed at enhancing the level of IFN-γ at the infection site or lesions, may be not only ineffective but also harmful.

the accepted concept of Th1-mediated immunity against intracellular infection. Indeed, anti-*La* vaccines that are aimed to enhance Th1 responses clearly protect mice against infection with promastigotes (K. Campbell, J. Ji, and L. Soong, manuscript in preparation). Similarly, adaptive transfer of parasite-specific Th1 cells is effective in controlling promastigote infection as well (e.g. Fig. 8 on page 85). The second proposition to be made is that if a significant number of promastigotes survive the initial host defense and transform into amastigotes, IFN-γ alone would not only fail to induce parasite killing but may actually enhance amastigote growth (see *Illustration 6*). Consistent with this notion, continuous IL-12 treatment of *La*-infected mice failed to change the disease course, while two-week IL-12 treatment was sufficient to help BALB/c mice cure *L. major* infection (Jones *et al.*, 2000). In light of these two propositions, it is very interesting to learn that after successfully entering MΦs, *La* promastigotes needed almost 5 days to fully differentiate into amastigotes (Courret *et al.*, 2001). Conceivably, this 5-day period would be the window of opportunity for memory Th1 cells to be recalled into the action, activating MΦs to eliminate the parasite (as seen in successfully immunized mice). After that, Th1-based immunotherapeutics may not be effective to *La* infection or even have an exacerbating impact.

As already discussed in Chapter 2, *La* infection in mice, particularly in those of C57BL background, both Th2-dependent and Th2-independent

pathogenic mechanisms operate (see page 23). While the Th2-driven disease development in mice is almost universal to all *Leishmania* parasites, the Th2-independent mechanism appears to be unique to *La* infection yet is unclear in nature. The finding that IFN-γ potentially plays a bidirectional role during *La* infection offers an intriguing explanation: without optimal synergy with other factors, Th1 cytokine IFN-γ may enhance amastigote propagation *in vivo* and thereby constitute a Th2-independent pathogenic mechanism.

At this stage, it is not clear mechanistically how *La* amastigotes could achieve enhanced growth in IFN-γ-activated MΦs. However, at least two mutually non-exclusive possibilities can be speculated. First, the activation of a particular signaling component downstream of the IFN-γ receptor may be sensed by *La* amastigotes as a signal to accelerate their own replication. This accelerated amastigote replication may be sufficiently fast to overcompensate the loss of parasites due to NO-mediated killing, especially when a microbicidal level of NO production requires *de novo* iNOS transcription, translation, and posttranslational modification (Geller and Billiar, 1998). Given the fact that NO serves as a signal-transducing molecule in higher enkaryotic cells (Bredt and Snyder, 1994), it is interesting to consider that NO itself may be sensed by *Leishmania* parasites. For example, NO-mediated signaling pathways apparently operate in *Trypanosoma cruzi* and *Plasmodium falciparum* (Ghigo *et al.*, 1995; Paveto *et al.*, 1995). The second possibility

may be that *La* amastigotes can influence the balance of arginine metabolism in MΦs to their own favor. As already discussed in Chapter 2 and depicted in *Illustration 5* (page 40), L-arginine is metabolized in MΦs through two pathways, iNOS pathway and arginase pathway. The latter one leads to synthesis of polyamines, which are required for *Leishmania* replication and therefore, presumably favorable to the growth of *La* amastigotes. While IFN-γ activates the iNOS pathway, it may also enhance arginine transport into the MΦs (Bogle *et al.*, 1992; Caivano, 1998; MacLeod, 1996). Thus, if *La* amastigotes in IFN-γ-activated MΦs could somehow skew the balance between arginase and iNOS pathways toward the former one, increased polyamine synthesis and thus enhanced parasite replication may ensue. To test this possibility, I measured iNOS and arginase expression in amastigote-infected MΦs. Amastigote infection did not cause significant changes of the iNOS or arginase levels in MΦs (Fig. 19). Thus, this hypothetical process may not involve an increase in arginase or a reduction in iNOS expression. Notably, the expression of arginase I is apparently constitutive in untreated MΦs (Fig. 19), consistent with the fact that in terms of molar consumption, L-arginine is predominantly metabolized through the arginase pathway in murine

MΦs (Granger *et al.*, 1990; Mills *et al.*, 1992; Shearer *et al.*, 1997).

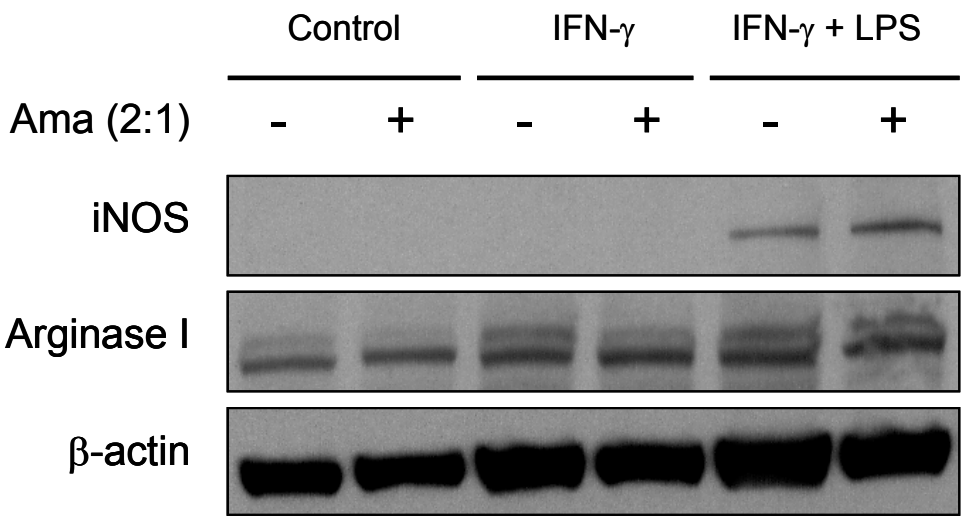

Figure 19. Arginase and iNOS expression in MΦs. BM-MΦs of B6 mice were left in plain medium or treated with 20 ng/ml IFN-γ alone or together with 10 ng/ml LPS for 4 hrs. Then, they were either infected with amastigotes or left untreated. Twenty-four hours later, total proteins were extracted from each sample and subjected to SDS-PAGE followed by Western blotting. The blot was developed with the enhanced chemi-luminescence method. Each lane was loaded with proteins from 2×10^5 MΦs. One of two independent experiments with similar results is shown.

It is important to emphasize that results presented in Chapter 4 do not in any way imply that *La* amastigotes are resistant to NO-mediated killing. I have not tested parasite viability in amastigote culture that is directly subjected to NO. However, MΦs stimulated with IFN-γ and LPS greatly enhanced iNOS expression (Fig. 19) and limited the parasite growth (Fig. 12 and Fig. 13, page 94 and page 95). Further, MΦs derived from iNOS-deficient mice failed to control amastigote replication even if they were stimulated with IFN-γ and LPS (unpublished observation). These data strongly indicate a killing effect of NO

on *La* amastigotes. Clearly, NO-mediated killing induction by IFN-γ and LPS can outweigh IFN-γ-enhanced amastigote replication. In this regard, it remains to be further addressed why IFN-γ and LPS is effective, while IFN-γ plus TNF-α is not (Fig. 12). This is particularly important given the fact that IFN-γ and LPS are synergistic in inducting TNF-α and that IFN-γ together with TNF-α can efficiently kill *L. major* amastigotes (Green *et al.*, 1990a; Liew *et al.*, 1990a). It is possible that IFN-γ and LPS would induce TNF-α to a level much higher than what was tested in Fig 16 (i.e. 20 ng/ml; page 111). Alternatively, additional factors induced by IFN-γ and LPS could be involved. It is interesting to note that macrophage inflammatory protein(MIP)-1α and -1β, both of which are strongly induced by LPS, can activate murine MΦs to kill *L. major* and *L. donovani* amastigotes (Bhattacharyya *et al.*, 2002; Titus *et al.*, 1994). Future studies are required to address these issues.

Endogenous IFN-γ apparently has minimal effect on the course of infection in mice challenged with *La* amastigotes (Fig. 14, page 97). As already discussed in Chapter 4, one explanation is that the IFN-γ-mediated enhancement of amastigote replication is canceled by NO-mediated killing effects induced by IFN-γ together with other factors, which may potentially include LPS-inducible MIP-1, as just mentioned above. However, this somewhat simple-minded hypothesis has an underlying assumption in that

there should be an optimal molar ratio between IFN-γ and the unknown factor for the NO induction. Without actual identification of the unknown factor, it is very difficult to test this assumption *in vivo*. On the other hand, it could be tested *in vitro* by combining various concentrations of IFN-γ with a fixed concentration of LPS. It is important to point out that this hypothesis is consistent with the previous finding that *La* amastigote replication was neither inhibited nor enhanced in MΦs activated by "lymphokine", which was culture supernatants of splenocytes from immune mice stimulated with recall Ags and presumably contained IFN-γ and a host of other cytokines (Scott *et al.*, 1983).

An important caveat of the Fig. 14 experiment (page 97) is that the observation period might has been too short to reveal any difference between wild-type and IFN-γ-deficient mice. However, up to the time of the writing of this dissertation, amastigote infection in BALB/c wild-type and IFN-γ-deficient mice have been monitored for 20 weeks and no significant difference in disease progression was observed. A long-term follow-up experiment for B6 mice is also needed to further confirm these findings.

Overall, these studies have revealed a novel and apparently unique aspect of *La* parasites. They emphasize the importance of individualities of different *Leishmania* species and caution unwarranted generalization of the *L. major* model. As already discussed, it is probably necessary to dissect iNOS

and arginase pathways in *La*-infected MΦs, as the explanation for IFN-γ-enhanced amastigote replication may well lie behind the biochemical balance of arginine metabolism. It is imperative to define factors that are involved in the control of amastigote replication by IFN-γ/LPS-activated MΦs. Such knowledge is likely to be the key to successful immunotherapies against *La* infection.

DCs and the activation of *Leishmania*-specific Th effectors

While the role IFN-γ and Th1 cells in *La* infection is more complicated than previously appreciated, IL-4- and IL-10-producing Th2 cells generally promote the pathogenesis of most *Leishmania* species including *La*. As already discussed in Chapter 2, in the case of *L. major* infection in BALB/c mice, the LACK-specific T cells become committed to the Th2 phenotype during an early stage of the infection. In the case of *La* infection, although LACK-reactive T cells may not be evidently involved (J. Ji and L. Soong, unpublished observation), Th2 cells do rapidly arise following parasite challenge. However, it has not been fully understood how parasite-specific Th2 cells are generated. Through a comparison of BM-DCs from resistant C3H/HeJ and susceptible BALB/c mice, the study presented in Chapter 3 suggests a potential link between DC response to *La* amastigotes and the priming of Th2 cells. Specifically, amastigotes may condition DCs to produce

IL-4, which in turn drive the development of pathogenic Th2 responses in BALB/c mice (Fig. 6 and 7, page 66 and 67).

As briefly mentioned in the Chapter 2, the first and currently the only known protozoan TLR ligand is the GPI anchor from *Trapanosoma cruzi*, which activates TLR2 (Campos *et al.*, 2001). The GPI anchor is a highly conserved molecular structure also shared by *Leishmania* parasites (Ferguson, 1997). For example, many surface proteins and lipids of *Leishmania* promastigotes are anchored to the membrane by the GPI, while amastigotes express high levels of free GPIs (Ilgoutz and McConville, 2001). It is possible that *Leishmania* parasites can also activate TLR2. Interestingly, TLR2 is also the only TLR known to be involved in recognition of fungi, which, similar to *Leishmania*, are also lower eukaryotic organisms. The finding that *La* amastigotes could condition DCs to produce IL-4 appeared to be somewhat connected to the result that hyphae of *Candida albicans* triggered DCs to produce IL-4 (d'Ostiani *et al.*, 2000). I thought TLR2 might be the link underlying both observations. This thought led to the study in Chapter 5, in which two TLR2 ligands and a TLR4 stimulus were compared for their abilities to condition BM-DCs to prime distinct Th effector phenotypes. While those results did not indicate any link between TLR2 activation and DC IL-4 production, they did indicate an interesting contrast between TLR4 and TLR2. As already discussed in Chapter 5, there is a growing body of evidence to

indicate that as compared to TLR4 activation, TLR2 stimulation would lead to a much less pro-inflammatory state of MΦs and DCs, as judged from their profiles of cytokine/chemokine production (Hirschfeld *et al.*, 2001; Re and Strominger, 2001). Of particular interest, TLR2 stimulation somehow favors IL-10 induction from DCs. This has been consistently observed in murine BM-DCs (Fig. 15 and 16, page 110 and 111), murine splenic DCs (Dr. Pulendran, personal communication), and human monocyte-derived DCs (Re and Strominger, 2001). Conceptually, it appears beneficial to *Leishmania* parasites if TLR2 is triggered and IL-10 is induced from DCs. However, it is also entirely possible that *Leishmania* parasites do not stimulate any of the TLRs but induce DC activation through some completely different pathways.

While molecular mechanisms of DC activation by *La* amastigotes are not known, the finding that Th2 priming is favored by amastigote-carrying BALB/c DCs is still important in that it offers an intriguing explanation for how Th2 cells are generated in BALB/c mice following *Leishmania* infection. However, additional observations suggest this is oversimplified.

As an extension to studies presented in Chapter 3, I have done experiments in which DCs that carried amastigotes were transferred to naïve mice at one foot seven days prior to amastigote challenge at the other. As shown in Fig. 20, while transfer of amastigote-carrying DCs did exacerbate the

foot swelling in BALB/c mice (left panel), it did not significantly increase the parasite burden (right panel). These data suggest that the potential Th2 induction by amastigote-infected DCs (Fig. 7, page 67) is not sufficient to

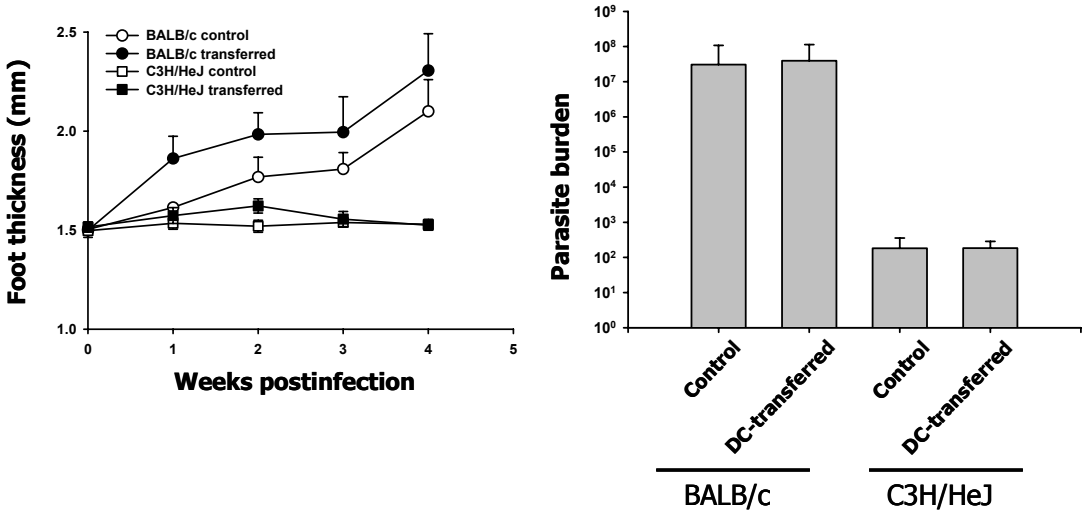

Figure 20. *La* infection following transfer of amastigote-carrying BM-DCs. BM-DCs that had been co-cultured with live *La* amastigotes for 24 h were s.c. transferred (3×10^5 DC/mouse) to the right hind foot of syngeneic wild-type mice. One week later, mice were challenged at the left hind foot with 10^5 tissue-derived amastigotes. The foot thickness at the challenge side was monitored weekly (upper panel), while the parasite burden was measured in a limiting dilution assay 4 weeks after the challenge. Data represent two independent experiments.

significantly alter the disease course following amastigote challenge. However, one compounding factor to this interpretation is the fact that mice receiving amastigote-infected DCs also developed significant lesions at the foot of DC transfer. Thus, the parasite burden at the challenge side alone might not be an appropriate readout for the effect of DC transfer. Alternatively, it is possible that the inoculum of 10^5 amastigotes had infected a sufficient number of endogenous DCs to induce Th2 cells so that the potential exacerbating effect

of transferred exogenous DCs was obscured. As shown in Table 1, when *La* amastigotes were used to infect BALB/c mice, a significant amount of IL-4 (1.49±1.14 ng/ml) and a high level of IL-10 (12.7±2.3 ng/ml) were indeed produced, indicating the generation of Th2 cells. Unexpectedly, however, the amastigote infection also induced a very high level of IFN-γ (61±9 ng/ml). This IFN-γ production was even more striking in B6 mice, reaching 280 ng/ml or so (Table 1). Since phenotypically and functionally

Table 1. Cytokine profiles of amastigote-infected BALB/c and B6 mice [a]

Unit in *ng/ml*	BALB/c			B6		
	IFN-γ	**IL-4**	**IL-10**	**IFN-γ**	**IL-4**	**IL-10**
#1	51.3	0.83	12.1	367.2	0.12	1.3
#2	62.8	0.83	12.2	328.6	0.06	2.3
#3	56.6	2.29	13.6	255.4	0.14	2.8
#4	75.7	0.43	9.6	271.3	0.07	1.6
#5	60.6	3.10	15.9	182.5	0.18	3.1
Mean±SD	**61.4±9.1**	**1.49±1.14**	**12.7±2.3**	**281.0±71.0**	**0.11±0.05**	**2.23±0.8**

[a:] BALB/c and B6 mice were infected with 10^5 *La* amastigotes at the right hind foot. Six weeks later, popliteal DLN cells from individual mice were stimulated with amastigote lysates (10^6 parasite equivalent) in 200 μl for 72 hours. For each DLN, the culture was set up in triplicate (10^6 cells per well in the 96-well plate). Concentrations of IFN-γ, IL-4, and IL-10 in culture supernatants were then measured by ELISA. The numbers (1-5) denote 5 individual mice, for which the mean of triplicate wells is shown. Data represent two separate experiments for B6 one for BALB/c mice.

distinct DC populations clearly exist *in vivo* (Shortman and Liu, 2002), these data would suggest that some endogenous DCs are activated by amastigotes

to prime Th2 cells *in vivo*, while other endogenous DCs are activated to prime IFN-γ-producing Th1 cells. While direct evidence is lacking for the existence and identity of these Th1-driving DCs during amastigote infection, previous studies on DC subsets in general and on *L. major*-DC interactions in particular have provided important hints.

Regarding to the differential regulation of Th effector development, DCs isolated from spleen or LN of a mouse can be distinguished into at least two populations, based on their expression of the CD8α homodimer (Shortman and Liu, 2002). Importantly, $CD8\alpha^-$ and $CD8\alpha^+$ DCs have been found to prime for Ag-specific Th2 and Th1 responses, respectively (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999). Similarly, distinct human DC populations that favor Th1 or Th2 development have also been described (Rissoan *et al.*, 1999). Potentially, $CD8\alpha^-$ DCs, as represented by BM-DCs *in vitro*, could be activated by *La* amastigotes to drive Th2 differentiation, while $CD8\alpha^+$ DCs are the one priming for Th1 cells. However, several lines of evidence question a potential role for $CD8\alpha^+$ DCs in mounting Th responses during microbial infections such as leishmaniasis. First, to my knowledge, $CD8\alpha^+$ DCs have not been demonstrated as being able to phagocytose microbes *in vitro* or *in vivo*. Instead, they appeared to preferentially capture dead cells and induced T cell tolerance by deletion (Iyoda *et al.*, 2002; Kurts *et al.*, 1997; Liu *et al.*, 2002; Suss and Shortman, 1996). Further, it has been suggested that such deletion

of T cells may be proceeded by cell proliferation and high levels of IFN-γ production and require DC-derived IL-12 (Liu *et al.*, 2002; Smith and Fazekas de St Groth, 1999). Second, CD8α^+ DCs were shown as unable to uptake exogenous Ags *in situ*; when pulsed with antigenic peptide in culture and then transferred to naïve mice through footpad injection, they did not home to LNs, although T cell responses were induced in those mice (Smith and Fazekas de St Groth, 1999). Third, when a T cell response was induced *in vivo* following immunization with Ags, CD8α^- but not CD8α^+ DCs were the one that homed from periphery to LNs and established physical contact with T cells (Ingulli *et al.*, 2002). Taken together, these results suggest CD8α^+ DCs may not be relevant to the generation of IFN-γ-producing Th1 cells during microbial infection.

Moll and colleagues have argued that since cutaneous leishmaniasis is a skin disease, LCs are the DC population most relevant to the initiation of anti-*Leishmania* T cell responses. They have demonstrated that LCs can carry *L. major* parasites to LNs and activate Ag-specific T cells (Blank *et al.*, 1993; Moll *et al.*, 1993). Subsequently, Udey and colleagues did a series of studies of *L. major*-DC interactions with fetal skin-derived LC-like cell lines. Most notably, these authors found that LCs from BALB/c mice produced IL-12 but no IL-4 when infected with *L. major* amastigotes. Further, when transferred into naïve BALB/c mice, these amastigote-carrying LCs could protect the

susceptible host against subsequent promastigote challenge (von Stebut *et al.*, 1998; von Stebut *et al.*, 2000). These results suggest that LCs are activated by *L. major* amastigotes to prime Th1 cells. Further, Sato *et al.* showed that CC chemokine receptor 2 (CCR2) was required for LC migration to the DLN after *L. major* infection and that CCR2-deficient B6 mice mounted a Th2 response and developed clinical diseases (Sato *et al.*, 2000). Thus, LCs are indispensable for the priming of protective Th1 cells during *L. major* infection. Possibly, LCs are also the DC population that primes those Th1 cells following *La* amastigote infection *in vivo*. Indeed, there is evidence for the propensity of LCs to favor Th1 priming in general. For example, LCs constitutively express IL-12 p40 (Kang *et al.*, 1996). *L. major* promastigote lysates, while do not seem to induce LC maturation at all (von Stebut *et al.*, 1998; von Stebut *et al.*, 2000), could induce a polarized Th1 response that protects BALB/c mice against the challenge of virulent parasites (Flohe *et al.*, 1998). In a model of contact sensitization, LCs were found to become CD8α-positive after reaching the DLN and capable of producing a high level of IL-12 (Merad *et al.*, 2000).

On the other hand, LCs would not be the only DC population involved in mounting anti-*Leishmania* T cell responses. It is important to note that *Leishmania* infection occurs in the skin but it is not limited to the epidermis, in which resting LCs are located. When promastigotes are delivered by a sandfly

bite or by a needle, dermal/interstitial DCs would also be involved. This also appears to be the DC population that massively immigrated into DLNs following subcutaneous immunization with Ags plus adjuvant (Merad *et al.*, 2000). Phenotypically, dermal/interstitial DCs are $CD4^-CD8^-CD11c^+CD11b^+$, very similar to BM-DCs (Shortman and Liu, 2002).

Based on these considerations, it may be proposed that amastigotes infect both epidermal LC and dermal DC populations *in vivo*, and amastigote-infected LCs and dermal DCs preferentially prime Th1 and Th2 cells, respectively. This theory would help explain the apparent contradiction that BM-DCs carrying *La* amastigotes favor Th2 differentiation (Chapter 3), while in addition to Th2 cytokines such as IL-4 and IL-10, a high level of IFN-γ is also produced in amastigote-infected mice (Table 1). This theory may also potentially explain how the Th2 response is generated in *L. major*-infected BALB/c mice. The reason that von Stebut *et al.* did not find Th2-priming DCs is perhaps because they did not look at DCs other than LCs (von Stebut *et al.*, 1998; von Stebut *et al.*, 2000).

It is worth of mentioning that promastigote-DC interactions may be important as well to the Th differentiation during *Leishmania* infection. After all, it is promastigotes that establish a natural infection. However, our knowledge on promastigote-DC interactions is very limited. Interestingly, what we do

know is that promastigotes do not enter or activate LCs (von Stebut *et al.*, 1998; von Stebut *et al.*, 2000). On the other hand, metacyclic promastigotes efficiently enter and activate BM-DCs and some splenic DCs (Chapter 3 and (Konecny *et al.*, 1999)). It is perhaps reasonable to assume that in the skin, dermal DCs are the main DC population to interact with promastigotes. It may also be necessary to investigate potential differences between procyclic and metacyclic promastigotes in terms of influence on DC functions. A sandfly would only deliver 10 to 1000 metacyclics to a mammalian host. However, the model system in which most of *Leishmania*-related immunological phenomena are described involves infecting mice with 0.5 to 10 million stationary-phase promastigotes. A typical stationary-phase culture only contains approximately 10% metacyclics and 90% procyclics. It appears likely that this flood of non-infective procyclics could have certain impacts on DC functions and disease pathogenesis in the given experimental model.

It is interesting to point out that when infected with *La* promastigotes, BALB/c or B6 mice can only produce much lower levels of IFN-γ as compared to their counterparts directly infected with amastigotes. For example, at 10-week post promastigote infection, a time point when all parasites are amastigotes, the IFN-γ level seen in BALB/c mice was 2-3 ng/ml (Fig. 1, page 59), while at 6 weeks after amastigote infection, the IFN-γ level was approximately 60 ng/ml (Table 1, page 132). This comparison might not be

entirely justified, as the two experiments were done two years apart. However, the restimulation assay used in these experiments was standardized in my hand and the parasite lysates used were from the same batch of preparation. Further, the striking magnitude of difference was specific to IFN-γ (20- to 30-fold) but not IL-4 or IL-10. Thus, it appears that promastigotes somehow prevent the subsequent generation of IFN-γ-producing cells, which are otherwise expected to arise when amastigotes dominate. Possibly, promastigote-DC interactions lead to the activation of *La*-specific naïve T cells, and effector phenotypes of these T cells are already relatively fixed when amastigote-DC interactions finally dominate. These issues will have to be systematically addressed in order to gain a further improved understanding of Th development in *Leishmania* infection.

Resistance to *L. amazonensis* infection

While the consensus has been that most inbred mice are susceptible to *La* infection, the study in Chapter 4 indicates that C3H/HeJ mice are quite resistant to promastigote challenge (Fig. 1, page 59). Data presented in Fig. 20 (page 128) further indicate that C3H/HeJ mice are also resistant to direct infection with amastigotes. The mechanism underlying this resistance is not clear. On the other hand, it is worth of noting that in one experiment, a group of 5 C3H/HeJ mice were infected with 2×10^6 promastigotes and then

monitored for 23 weeks. These mice developed lesions (palpable nodules) approximately 20 to 21 weeks post infection. It is not clear whether this represents a prolonged process of natural disease development or is a result of impaired immune functions in aging mice. It would be very informative to test whether or not *La* amastigotes could grow better in C3H/HeJ MΦs stimulated with IFN-γ.

Concluding remarks

This dissertation study has provided intriguing evidence suggesting that *L. amazonensis* parasites can exploit host immune system to the advantage of their own survival. First, the parasite may modulate functions of DCs, their cytokine production in particular, to induce the generation of pathogenic Th cells. Second, the parasite may hijack the IFN-γ-activated microbicidal program in MΦs to enhance its intracellular growth. Finally, if TLRs are involved in the recognition of *Leishmania* parasites by the mammalian immune system, the use of TLR2 might be advantageous to the parasite rather than the host, as TLR2 appears to be exceptional at inducing cytokines such as IL-10 that enhance *Leishmania* replication in MΦs. These studies have improved our understanding of the pathogenesis of leishmaniasis and the *Leishmania*-host interactions in general.

It goes without saying that we have a long way to go towards the ultimate goal of eliminating *Leishmania* or any other pathogens. It has taken millions of years of evolution for pathogens and our immune system to “learn” each other, fight each other, and, at the end as of today, to reach certain compromises. However, newly added into this evolutionary competition is our brains, and one day, we would not have to take those old compromises any more. To this cause, I pledge all my effort, and I begin with this dissertation.

REFERENCE

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787-793.

Aderem, A., and Ulevitch, R. J. (2000). Toll-like receptors in the induction of the innate immune response. Nature *406*, 782-787.

Adler, S., and Ber, M. (1941). The transmission of *Leishmania tropica* by the bite of *Phlebotomus papatasii*. Indian J. Med. Res. *29*, 803-809.

Afonso, L. C., and Scott, P. (1993). Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. Infect. Immun. *61*, 2952-2959.

Ajdary, S., Alimohammadian, M. H., Eslami, M. B., Kemp, K., and Kharazmi, A. (2000). Comparison of the immune profile of nonhealing cutaneous leishmaniasis patients with those with active lesions and those who have recovered from infection. Infect. Immun. *68*, 1760-1764.

Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. *2*, 675-680.

Alexander, J., and Russell, D. G. (1992). The interaction of *Leishmania* species with macrophages. Adv. Parasitol. *31*, 175-254.

Alvar, J., Canavate, C., Gutierrez-Solar, B., Jimenez, M., Laguna, F., Lopez-Velez, R., Molina, R., and Moreno, J. (1997). *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clinical Microbiolgy Review *10*, 298-319.

Anthony, R. L., Christensen, H. A., and Johnson, C. M. (1980). Micro enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of New World leishmaniasis. Am. J. Trop. Med. Hyg. *29*, 190-194.

Arias, J. R., and Naiff, R. D. (1981). The principal reservoir host of cutaneous leishmaniasis in the urban areas of Manaus, Central Amazon of Brazil. Mem. Inst. Oswaldo Cruz *76*, 279-286.

Ashford, R. W. (1996). Leishmaniasis reservoirs and their significance in control. Clinical Dermatology *14*, 523-532.

Ashford, R. W., and Bettini, S. (1987). Ecology and epidemiology: Old World. In The leishmaniases in biology and medicine, W. Peters, and R. Killick-Kendrick, eds. (London, Academic Press), pp. 291-363.

Bakhiet, M., Olsson, T., Mhlanga, J., Buscher, P., Lycke, N., van der Meide, P. H., and Kristensson, K. (1996). Human and rodent interferon-γ as a growth factor for *Trypanosoma brucei*. Eur. J. Immunol. *26*, 1359-1364.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barral, A., Petersen, E. A., Sacks, D. L., and Neva, F. A. (1983). Late metastatic leishmaniasis in the mouse. A model for mucocutaneous disease. Am. J. Trop. Med. Hyg. *32*, 277-285.

Behforouz, N., Rezai, H. R., and Gettner, S. (1976). Application of immunofluorescence to detection of antibody in *Leishmania* infections. Ann. Trop. Med. Parasitol. *70*, 293-301.

Belkaid, Y., Butcher, B., and Sacks, D. L. (1998a). Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in *Leishmania*-infected cells. Eur. J. Immunol. *28*, 1389-1400.

Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., Ribeiro, J., and Sacks, D. L. (1998b). Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J. Exp. Med. *188*, 1941-1953.

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., and Sacks, D. (2000a). A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J. Immunol. *165*, 969-977.

Belkaid, Y., Valenzuela, J. G., Kamhawi, S., Rowton, E., Sacks, D. L., and Ribeiro, J. M. (2000b). Delayed-type hypersensitivity to *Phlebotomus papatasi* sand fly bite: An adaptive response induced by the fly? Proc Natl Acad Sci U S A *97*, 6704-6709.

Bennett, C. L., Misslitz, A., Colledge, L., Aebischer, T., and Blackburn, C. C. (2001). Silent infection of bone marrow-derived dendritic cells by *Leishmania mexicana* amastigotes. Eur. J. Immunol. *31*, 876-883.

Bhattacharyya, S., Ghosh, S., Dasgupta, B., Mazumder, D., Roy, S., and Majumdar, S. (2002). Chemokine-induced leishmanicidal activity in murine macrophages via the generation of nitric oxide. J. Infect. Dis. *185*, 1704-1708.

Blackwell, J. M., Ezekowitz, R. A., Roberts, M. B., Channon, J. Y., Sim, R. B., and Gordon, S. (1985). Macrophage complement and lectin-like receptors bind *Leishmania* in the absence of serum. J. Exp. Med. *162*, 324-331.

Blank, C., Fuchs, H., Rappersberger, K., Rollinghoff, M., and Moll, H. (1993). Parasitism of epidermal Langerhans cells in experimental cutaneous leishmaniasis with Leishmania major. J. Infect. Dis. *167*, 418-425.

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q. W., and Nathan, C. (1994). Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. J. Leukoc. Biol. *55*, 227-233.

Bogle, R. G., Baydoun, A. R., Pearson, J. D., Moncada, S., and Mann, G. E. (1992). L-arginine transport is increased in macrophages generating nitric oxide. Biochem. J. *284 (Pt 1)*, 15-18.

Bourreau, E., Gardon, J., Pradinaud, R., Pascalis, H., Prevot-Linguet, G., Kariminia, A., and Pascal, L. (2003). Th2 responses predominate during the early phases of infection in patients with localized cutaneous leishmaniasis and precede the development of Th1 responses. Infect. Immun. *71*, 2244-2246.

Boursalian, T. E., and Bottomly, K. (1999). Stability of naive and memory phenotypes on resting CD4 T cells in vivo. J. Immunol. *162*, 9-16.

Bredt, D. S., and Snyder, S. H. (1994). Nitric oxide: a physiologic messenger molecule. Annu. Rev. Biochem. *63*, 175-195.

Brown, D. R., and Reiner, S. L. (1999). Polarized helper-T-cell responses against *Leishmania major* in the absence of B cells. Infect. Immun. *67*, 266-270.

Bryceson, A. D. (1969). Diffuse cutaneous leishmaniasis in Ethiopia. I. The clinical and histological features of the disease. Trans. R. Soc. Trop. Med. Hyg. *63*, 708-737.

Caceres-Dittmar, G., Sanchez, M. A., Oriol, O., Kraal, G., and Tapia, F. J. (1992). Epidermal compromise in American cutaneous leishmaniasis. J. Invest. Dermatol. *99*, 95S-98S.

Caivano, M. (1998). Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. FEBS Lett. *429*, 249-253.

Cameron, P. U., Freudenthal, P. S., Barker, J. M., Gezelter, S., Inaba, K., and Steinman, R. M. (1992). Dendritic cells exposed to human immunodeficiency

virus type-1 transmit a vigorous cytopathic infection to $CD4^+$ T cells. Science *257*, 383-387.

Campino, L. M. (2002). Canine reservoirs and leishmaniasis: epidemiology and disease. In *Leishmania*, J. P. Farrell, ed. (Kluwer Academic Publishers), pp. 45-57.

Campos, M. A., Almeida, I. C., Takeuchi, O., Akira, S., Valente, E. P., Procopio, D. O., Travassos, L. R., Smith, J. A., Golenbock, D. T., and Gazzinelli, R. T. (2001). Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J. Immunol. *167*, 416-423.

Campos-Neto, A. (2002). Anti-*Leishmania* vaccine. In *Leishmania*, J. P. Farrell, ed. (Kluwer Academic Publishers), pp. 167-190.

Carrera, L., Gazzinelli, R. T., Badolato, R., Hieny, S., Muller, W., Kuhn, R., and Sacks, D. L. (1996). *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J. Exp. Med. *183*, 515-526.

Carvalho, E. M., Johnson, W. D., Barreto, E., Marsden, P. D., Costa, J. L., Reed, S., and Rocha, H. (1985). Cell mediated immunity in American cutaneous and mucosal leishmaniasis. J. Immunol. *135*, 4144-4148.

Castes, M., Agnelli, A., and Rondon, A. J. (1984). Mechanisms associated with immunoregulation in human American cutaneous leishmaniasis. Clin. Exp. Immunol. *57*, 279-286.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature *388*, 782-787.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J. Exp. Med. *184*, 747-752.

Chakkalath, H. R., Theodos, C. M., Markowitz, J. S., Grusby, M. J., Glimcher, L. H., and Titus, R. G. (1995). Class II major histocompatibility complex-deficient mice initially control an infection with *Leishmania major* but succumb to the disease. J. Infect. Dis. *171*, 1302-1308.

Chakkalath, H. R., and Titus, R. G. (1994). *Leishmania major*-parasitized macrophages augment Th2-type T cell activation. J. Immunol. *153*, 4378-4387.

Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Young, H. A., Wolf, S. F., Young, D., Clark, S. C., and Trinchieri, G. (1991). Induction of interferon-γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med. *173*, 869-879.

Chang, K. P. (1978). *Leishmania* infection of human skin fibroblasts in vitro: absence of phagolysosomal fusion after induced phagocytosis of promastigotes, and their intracellular transformation. Am. J. Trop. Med. Hyg. *27*, 1084-1096.

Channon, J. Y., Roberts, M. B., and Blackwell, J. M. (1984). A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages. Immunology *53*, 345-355.

Chatelain, R., Varkila, K., and Coffman, R. L. (1992). IL-4 induces a Th2 response in *Leishmania major*-infected mice. J. Immunol. *148*, 1182-1187.

Chow, A., Toomre, D., Garrett, W., and Mellman, I. (2002). Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. Nature *418*, 988-994.

Colmenares, M., Kar, S., Goldsmith-Pestana, K., and McMahon-Pratt, D. (2002). Mechanisms of pathogenesis: differences amongst *Leishmania* species. Trans. R. Soc. Trop. Med. Hyg. *96 Suppl 1*, S3-7.

Constant, S. L., and Bottomly, K. (1997). Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. Annu. Rev. Immunol. *15*, 297-322.

Convit, J. (1958). Leishmaniasis tegumentaria difusa. Nueva entidad clinico patologica y parasitaria. Revista de Sanidad y Asistencia Social *23*, 1-28.

Corry, D. B., Reiner, S. L., Linsley, P. S., and Locksley, R. M. (1994). Differential effects of blockade of CD28-B7 on the development of Th1 or Th2 effector cells in experimental leishmaniasis. J. Immunol. *153*, 4142-4148.

Courret, N., Frehel, C., Prina, E., Lang, T., and Antoine, J. C. (2001). Kinetics of the intracellular differentiation of *Leishmania amazonensis* and internalization of host MHC molecules by the intermediate parasite stages. Parasitology *122*, 263-279.

Courret, N., Prina, E., Mougneau, E., Saraiva, E. M., Sacks, D. L., Glaichenhaus, N., and Antoine, J. C. (1999). Presentation of the *Leishmania* antigen LACK by infected macrophages is dependent upon the virulence of the phagocytosed parasites. Eur. J. Immunol. *29*, 762-773.

Coutinho-Ablath, E., and Coelho, M. d. V. (1965). Experimental cutaneous leishmaniasis. II. The pathology of leishmaniasis by *Leishmania mexicana*. Rev. Inst. Med. Trop. Sao Paulo *7*, 145-155.

Cumberbatch, M., and Kimber, I. (1990). Phenotypic characteristics of antigen-bearing cells in the draining lymph nodes of contact sensitized mice. Immunology *71*, 404-410.

Cunha, F. Q., Moncada, S., and Liew, F. Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. Biochem. Biophys. Res. Commun. *182*, 1155-1159.

Da Silva, R. P., Hall, B. F., Joiner, K. A., and Sacks, D. L. (1989). CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. J. Immunol. *143*, 617-622.

de Saint-Vis, B., Fugier-Vivier, I., Massacrier, C., Gaillard, C., Vanbervliet, B., Ait-Yahia, S., Banchereau, J., Liu, Y. J., Lebecque, S., and Caux, C. (1998). The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. J. Immunol. *160*, 1666-1676.

Dedet, J.-P. (2002). Current status of epidemiology of leishamaniases. In *Leishmania*, J. P. Farrell, ed. (Kluwer Academic Publishers), pp. 1-10.

Dhodapkar, M. V., Steinman, R. M., Krasovsky, J., Munz, C., and Bhardwaj, N. (2001). Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J. Exp. Med. *193*, 233-238.

Donovan, C. (1903). Memoranda: on the possibility of the occurence of trypanosomiasis in India. Br. Med. J. *2*, 79-79.

d'Ostiani, C. F., Del Sero, G., Bacci, A., Montagnoli, C., Spreca, A., Mencacci, A., Ricciardi-Castagnoli, P., and Romani, L. (2000). Dendritic cells discriminate between yeasts and hyphae of the fungus *Candida albicans*: Implications for initiation of T helper cell immunity *in vitro* and *in vivo*. J. Exp. Med. *191*, 1661-1673.

Enserink, M. (2000). Has leishmaniasis become endemic in the U.S.? Science *290*, 1881-1883.

Erb, K., Blank, C., Ritter, U., Bluethmann, H., and Moll, H. (1996). *Leishmania major* infection in major histocompatibility complex class II-deficient mice: $CD8^+$ T cells do not mediate a protective immune response. Immunobiology *195*, 243-260.

Ferguson, M. A. (1997). The surface glycoconjugates of trypanosomatid parasites. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *352*, 1295-1302.

Flohe, S. B., Bauer, C., Flohe, S., and Moll, H. (1998). Antigen-pulsed epidermal Langerhans cells protect susceptible mice from infection with the intracellular parasite *Leishmania major*. Eur. J. Immunol. *28*, 3800-3811.

Franke, E. D., McGreevy, P. B., Katz, S. P., and Sacks, D. L. (1985). Growth cycle-dependent generation of complement-resistant *Leishmania* promastigotes. J. Immunol. *134*, 2713-2718.

Fruth, U., Solioz, N., and Louis, J. A. (1993). *Leishmania major* interferes with antigen presentation by infected macrophages. J. Immunol. *150*, 1857-1864.

Gaafar, A., el Kadaro, A. Y., Theander, T. G., Permin, H., Ismail, A., Kharazmi, A., and el Hassan, A. M. (1995a). The pathology of cutaneous leishmaniasis due to *Leishmania major* in Sudan. Am. J. Trop. Med. Hyg. *52*, 438-442.

Gaafar, A., Kharazmi, A., Ismail, A., Kemp, M., Hey, A., Christensen, C. B., Dafalla, M., el Kadaro, A. Y., el Hassan, A. M., and Theander, T. G. (1995b). Dichotomy of the T cell response to *Leishmania* antigens in patients suffering from cutaneous leishmaniasis; absence or scarcity of Th1 activity is associated with severe infections. Clin. Exp. Immunol. *100*, 239-245.

Gaafar, A., Veress, B., Permin, H., Kharazmi, A., Theander, T. G., and el Hassan, A. M. (1999). Characterization of the local and systemic immune responses in patients with cutaneous leishmaniasis due to *Leishmania major*. Clin. Immunol. *91*, 314-320.

Gazzinelli, R. T., Oswald, I. P., James, S. L., and Sher, A. (1992). IL-10 inhibits parasite killing and nitrogen oxide production by IFN-γ-activated macrophages. J. Immunol. *148*, 1792-1796.

Geller, D. A., and Billiar, T. R. (1998). Molecular biology of nitric oxide synthases. Cancer Metastasis Rev. *17*, 7-23.

Ghigo, D., Todde, R., Ginsburg, H., Costamagna, C., Gautret, P., Bussolino, F., Ulliers, D., Giribaldi, G., Deharo, E., Gabrielli, G., and et al. (1995). Erythrocyte stages of *Plasmodium falciparum* exhibit a high nitric oxide synthase (NOS) activity and release an NOS-inducing soluble factor. J. Exp. Med. *182*, 677-688.

Granger, D. L., Hibbs, J. B., Jr., Perfect, J. R., and Durack, D. T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. J. Clin. Invest. *85*, 264-273.

Green, S. J., Crawford, R. M., Hockmeyer, J. T., Meltzer, M. S., and Nacy, C. A. (1990a). *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-γ-stimulated macrophages by induction of tumor necrosis factor-α. J. Immunol. *145*, 4290-4297.

Green, S. J., Meltzer, M. S., Hibbs, J. B., Jr., and Nacy, C. A. (1990b). Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J. Immunol. *144*, 278-283.

Grimaldi, G., Jr., and Tesh, R. B. (1993). Leishmaniases of the New World: current concepts and implications for future research. Clin. Microbiol. Rev. *6*, 230-250.

Grosjean, I., Caux, C., Bella, C., Berger, I., Wild, F., Banchereau, J., and Kaiserlian, D. (1997). Measles virus infects human dendritic cells and blocks their allostimulatory properties for $CD4^+$ T cells. J. Exp. Med. *186*, 801-812.

Grove, S. S. (1978). The clinical and histological features of South West African cutaneous leishmaniasis. S. Afr. Med. J. *53*, 712-715.

Guy, R. A., and Belosevic, M. (1993). Comparison of receptors required for entry of *Leishmania majo*r amastigotes into macrophages. Infect. Immun. *61*, 1553-1558.

Haidaris, C. G., and Bonventre, P. F. (1982). A role for oxygen-dependent mechanisms in killing of *Leishmania donovani* tissue forms by activated macrophages. J. Immunol. *129*, 850-855.

Hall, L. R., and Titus, R. G. (1995). Sand fly vector saliva selectively modulates macrophage functions that inhibit killing of *Leishmania major* and nitric oxide production. J. Immunol. *155*, 3501-3506.

Heinzel, F. P., Rerko, R. M., Ahmed, F., and Pearlman, E. (1995). Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. J. Immunol. *155*, 730-739.

Heinzel, F. P., Rerko, R. M., Hatam, F., and Locksley, R. M. (1993a). IL-2 is necessary for the progression of leishmaniasis in susceptible murine hosts. J. Immunol. *150*, 3924-3931.

Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L., and Locksley, R. M. (1989). Reciprocal expression of interferon-γ or interleukin-4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J. Exp. Med. *169*, 59-72.

Heinzel, F. P., Schoenhaut, D. S., Rerko, R. M., Rosser, L. E., and Gately, M. K. (1993b). Recombinant interleukin 12 cures mice infected with *Leishmania major*. J. Exp. Med. *177*, 1505-1509.

Hilkens, C. M., Kalinski, P., de Boer, M., and Kapsenberg, M. L. (1997). Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood *90*, 1920-1926.

Himmelrich, H., Launois, P., Maillard, I., Biedermann, T., Tacchini-Cottier, F., Locksley, R. M., Rocken, M., and Louis, J. A. (2000). In BALB/c mice, IL-4 production during the initial phase of infection with *Leishmania major* is necessary and sufficient to instruct Th2 cell development resulting in progressive disease. J. Immunol. *164*, 4819-4825.

Hirschfeld, M., Weis, J. J., Toshchakov, V., Salkowski, C. A., Cody, M. J., Ward, D. C., Qureshi, N., Michalek, S. M., and Vogel, S. N. (2001). Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect. Immun. *69*, 1477-1482.

Hodgkinson, V. H., and Soong, L. (1997). *In vitro* maintenance of *Leishmania* amastigotes directly from lesions: advantages and limitations. J. Parasitol. *83*, 953-956.

Hoefsmit, E. C., Duijvestijn, A. M., and Kamperdijk, E. W. (1982). Relation between Langerhans cells, veiled cells, and interdigitating cells. Immunobiology *161*, 255-265.

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313-1318.

Holaday, B. J., Sadick, M. D., Wang, Z. E., Reiner, S. L., Heinzel, F. P., Parslow, T. G., and Locksley, R. M. (1991). Reconstitution of *Leishmania* immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. J. Immunol. *147*, 1653-1658.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. J. Immunol. *162*, 3749-3752.

Howard, J. G., Hale, C., and Liew, F. Y. (1980). Immunological regulation of experimental cutaneous leishmaniasis. III. Nature and significance of specific suppression of cell-mediated immunity in mice highly susceptible to *Leishmania tropica*. Journal of Experiemntal Medicine *152*, 594-607.

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., and Murphy, K. M. (1993). Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science *260*, 547-549.

Ilg, T. (2000). Lipophosphoglycan is not required for infection of macrophages or mice by *Leishmania mexicana*. EMBO J. *19*, 1953-1962.

Ilg, T., Etges, R., Overath, P., McConville, M. J., Thomas-Oates, J., Thomas, J., Homans, S. W., and Ferguson, M. A. (1992). Structure of *Leishmania mexicana* lipophosphoglycan. J. Biol. Chem. *267*, 6834-6840.

Ilgoutz, S. C., and McConville, M. J. (2001). Function and assembly of the *Leishmania* surface coat. Int. J. Parasitol. *31*, 899-908.

Inaba, K., Inaba, M., Naito, M., and Steinman, R. M. (1993). Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens *in vivo*. J. Exp. Med. *178*, 479-488.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. *176*, 1693-1702.

Inaba, K., Metlay, J. P., Crowley, M. T., and Steinman, R. M. (1990). Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific, MHC-restricted T cells *in situ*. J. Exp. Med. *172*, 631-640.

Inaba, K., Schuler, G., Witmer, M. D., Valinksy, J., Atassi, B., and Steinman, R. M. (1986). Immunologic properties of purified epidermal Langerhans cells. Distinct requirements for stimulation of unprimed and sensitized T lymphocytes. J. Exp. Med. *164*, 605-613.

Inaba, K., and Steinman, R. M. (1985). Protein-specific helper T-lymphocyte formation initiated by dendritic cells. Science *229*, 475-479.

Inaba, K., Witmer-Pack, M., Inaba, M., Hathcock, K. S., Sakuta, H., Azuma, M., Yagita, H., Okumura, K., Linsley, P. S., Ikehara, S., and et al. (1994). The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation *in vitro*. J. Exp. Med. *180*, 1849-1860.

Ingulli, E., Ulman, D. R., Lucido, M. M., and Jenkins, M. K. (2002). *In situ* analysis reveals physical interactions between $CD11b^+$ dendritic cells and

antigen-specific CD4 T cells after subcutaneous injection of antigen. J. Immunol. *169*, 2247-2252.

Iniesta, V., Gomez-Nieto, L. C., and Corraliza, I. (2001). The inhibition of arginase by N^{ω}-hydroxy-l-arginine controls the growth of *Leishmania* inside macrophages. J. Exp. Med. *193*, 777-784.

Iniesta, V., Gomez-Nieto, L. C., Molano, I., Mohedano, A., Carcelen, J., Miron, C., Alonso, C., and Corraliza, I. (2002). Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular *Leishmania* parasites. Parasite Immunol. *24*, 113-118.

Iwasaki, A., and Kelsall, B. L. (1999). Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J. Exp. Med. *190*, 229-239.

Iwasaki, A., and Kelsall, B. L. (2001). Unique functions of $CD11b^{+}$, $CD8\alpha^{+}$, and double-negative Peyer's patch dendritic cells. J. Immunol. *166*, 4884-4890.

Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R. M., and Inaba, K. (2002). The $CD8^{+}$ dendritic cell subset selectively endocytoses dying cells in culture and *in vivo*. J. Exp. Med. *195*, 1289-1302.

Janeway, C. A., and Medzhitov, R. (2002). Innate immune recognition. Annu. Rev. Immunol. *20*, 197-216.

Jha, T. K., Sundar, S., Thakur, C. P., Bachmann, P., Karbwang, J., Fischer, C., Voss, A., and Berman, J. (1999). Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N. Engl. J. Med. *341*, 1795-1800.

Ji, J., Sun, J., Qi, H., and Soong, L. (2002). Analysis of T helper cell responses during infection with *Leishmania amazonensis*. Am. J. Trop. Med. Hyg. *66*, 338-345.

Jones, D. E., Buxbaum, L. U., and Scott, P. (2000). IL-4-independent inhibition of IL-12 responsiveness during *Leishmania amazonensis* infection. J. Immunol. *165*, 364-372.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., and Enk, A. H. (2000). Induction of interleukin 10-producing, nonproliferating $CD4^{+}$ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J. Exp. Med. *192*, 1213-1222.

Julia, V., and Glaichenhaus, N. (1999). $CD4^{+}$ T cells which react to the *Leishmania major* LACK antigen rapidly secrete interleukin-4 and are

detrimental to the host in resistant B10.D2 mice. Infect. Immun. *67*, 3641-3644.

Julia, V., Rassoulzadegan, M., and Glaichenhaus, N. (1996). Resistance to *Leishmania major* induced by tolerance to a single antigen. Science *274*, 421-423.

Kaisho, T., and Akira, S. (2001). Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. Trends Immunol *22*, 78-83.

Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G., and Kapsenberg, M. L. (1997). IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J. Immunol. *159*, 28-35.

Kalinski, P., Hilkens, C. M., Wierenga, E. A., and Kapsenberg, M. L. (1999). T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol. Today *20*, 561-567.

Kalinski, P., Schuitemaker, J. H., Hilkens, C. M., and Kapsenberg, M. L. (1998). Prostaglandin E2 induces the final maturation of IL-12-deficient $CD1a^{+}CD83^{+}$ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J. Immunol. *161*, 2804-2809.

Kamhawi, S. (2002). The journey of Leishmania parasites within the digestive tract of phlebotomine sand flies. In *Leishmania*, J. P. Farrell, ed. (Kluwer Academic Publishers), pp. 59-73.

Kamhawi, S., Belkaid, Y., Modi, G., Rowton, E., and Sacks, D. (2000). Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. Science *290*, 1351-1354.

Kanangat, S., Nair, S., Babu, J. S., and Rouse, B. T. (1995). Expression of cytokine mRNA in murine splenic dendritic cells and better induction of T cell-derived cytokines by dendritic cells than by macrophages during *in vitro* costimulation assay using specific antigens. J. Leukoc. Biol. *57*, 310-316.

Kane, M. M., and Mosser, D. M. (2001). The role of IL-10 in promoting disease progression in leishmaniasis. J. Immunol. *166*, 1141-1147.

Kang, K., Kubin, M., Cooper, K. D., Lessin, S. R., Trinchieri, G., and Rook, A. H. (1996). IL-12 synthesis by human Langerhans cells. J. Immunol. *156*, 1402-1407.

Kaplan, G., Nusrat, A., Witmer, M. D., Nath, I., and Cohn, Z. A. (1987). Distribution and turnover of Langerhans cells during delayed immune responses in human skin. J. Exp. Med. *165*, 763-776.

Katz, S. I., Tamaki, K., and Sachs, D. H. (1979). Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature *282*, 324-326.

Kellina, O. I. (1973). [Differences in the sensitivity of inbred mice of different lines to Leishmania tropica major]. Med. Parazitol. (Mosk) *42*, 279-285.

Kemp, M., Handman, E., Kemp, K., Ismail, A., Mustafa, M. D., Kordofani, A. Y., Bendtzen, K., Kharazmi, A., and Theander, T. G. (1998). The *Leishmania* promastigote surface antigen-2 (PSA-2) is specifically recognised by Th1 cells in humans with naturally acquired immunity to *L. major*. FEMS Immunol. Med. Microbiol. *20*, 209-218.

Kemp, M., Hey, A. S., Kurtzhals, J. A., Christensen, C. B., Gaafar, A., Mustafa, M. D., Kordofani, A. A., Ismail, A., Kharazmi, A., and Theander, T. G. (1994a). Dichotomy of the human T cell response to *Leishmania* antigens. I. Th1-like response to *Leishmania* major promastigote antigens in individuals recovered from cutaneous leishmaniasis. Clin. Exp. Immunol. *96*, 410-415.

Kemp, M., Kurtzhals, J. A., Kharazmi, A., and Theander, T. G. (1994b). Dichotomy in the human $CD4^+$ T-cell response to *Leishmania* parasites. APMIS *102*, 81-88.

Kima, P. E., Constant, S. L., Hannum, L., Colmenares, M., Lee, K. S., Haberman, A. M., Shlomchik, M. J., and McMahon-Pratt, D. (2000). Internalization of *Leishmania mexicana* complex amastigotes via the Fc receptor is required to sustain infection in murine cutaneous leishmaniasis. J. Exp. Med. *191*, 1063-1068.

Kima, P. E., Soong, L., Chicharro, C., Ruddle, N. H., and McMahon-Pratt, D. (1996). *Leishmania*-infected macrophages sequester endogenously synthesized parasite antigens from presentation to $CD4^+$ T cells. Eur. J. Immunol. *26*, 3163-3169.

Klotz, O., and Lindenberg, H. (1923). The pathology of leishmaniasis of the nose. American Journal of Tropical Medicine *3*, 117-141.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. *170*, 827-845.

Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kampgen, E., Romani, N., and Schuler, G. (1996). High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J. Exp. Med. *184*, 741-746.

Konecny, P., Stagg, A. J., Jebbari, H., English, N., Davidson, R. N., and Knight, S. C. (1999). Murine dendritic cells internalize *Leishmania major* promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation *in vitro*. Eur. J. Immunol. *29*, 1803-1811.

Kopf, M., Brombacher, F., Hodgkin, P. D., Ramsay, A. J., Milbourne, E. A., Dai, W. J., Ovington, K. S., Behm, C. A., Kohler, G., Young, I. G., and Matthaei, K. I. (1996). IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. Immunity *4*, 15-24.

Kripke, M. L., Munn, C. G., Jeevan, A., Tang, J. M., and Bucana, C. (1990). Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. J. Immunol. *145*, 2833-2838.

Kurts, C., Kosaka, H., Carbone, F. R., Miller, J. F., and Heath, W. R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive $CD8^+$ T cells. J. Exp. Med. *186*, 239-245.

Lainson, R., and Shaw, J. J. (1968). Leishmaniasis in Brazil: I. Observations on enzootic rodent leishmaniasis--incrimination of *Lutzomyia flaviscutellata* (Mangabeira) as the vector in the Lower Amazonian Basin. Trans. R. Soc. Trop. Med. Hyg. *62*, 385-395.

Lainson, R., and Shaw, J. J. (1972). Leishmaniasis of the New World: taxonomic problems. Br. Med. Bull. *28*, 44-48.

Lainson, R., and Shaw, J. J. (1987). Evolution, classification and geographical distribution. In The leishmaniases in biology and medicine, W. Peters, and R. Killick-Kendrick, eds. (London, Academic Press), pp. 1-120.

Lainson, R., and Strangway-Dixon, J. (1963a). The epidemiology of dermal leishmaniasis in British Honduras. I. The human disease. Trans. R. Soc. Trop. Med. Hyg. *57*, 242-265.

Lainson, R., and Strangway-Dixon, J. (1963b). The epidemiology of dermal leishmaniasis in British Honduras. II. Reservoir hosts of *Leishmania mexicana* among the forest rodents. Trans. R. Soc. Trop. Med. Hyg. *58*, 136-153.

Langenkamp, A., Messi, M., Lanzavecchia, A., and Sallusto, F. (2000). Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat. Immunol. *1*, 311-316.

Lanzavecchia, A., and Sallusto, F. (2001). The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. Curr. Opin. Immunol. *13*, 291-298.

Larsen, C. P., Ritchie, S. C., Pearson, T. C., Linsley, P. S., and Lowry, R. P. (1992). Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. J. Exp. Med. *176*, 1215-1220.

Launois, P., Maillard, I., Pingel, S., Swihart, K. G., Xenarios, I., Acha-Orbea, H., Diggelmann, H., Locksley, R. M., MacDonald, H. R., and Louis, J. A. (1997). IL-4 rapidly produced by Vβ4Vα8 CD4$^+$ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. Immunity *6*, 541-549.

Laveran, A., and Pettit, A. (1909). Infection légère du cobaye par la *Leishmania donovani*. C. R. Seances Soc. Biol. Fil. *66*, 911-913.

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D., and Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for *in vitro* generation of IL-4-producing cells. Journal of Experimetnal Medicine *172*, 921-929.

Lehn, M., Weiser, W. Y., Engelhorn, S., Gillis, S., and Remold, H. G. (1989). IL-4 inhibits H_2O_2 production and antileishmanial capacity of human cultured monocytes mediated by IFN-γ. J. Immunol. *143*, 3020-3024.

Leishman, W. B. (1903). On the possibility of the occurence of trypanosomiasis in India. Br. Med. J. *1*, 1252-1254.

Lens, J. W., Drexhage, H. A., Benson, W., and Balfour, B. M. (1983). A study of cells present in lymph draining from a contact allergic reaction in pigs sensitized to DNFB. Immunology *49*, 415-422.

Lewis, D. J., and Ward, R. D. (1987). Transmission and vectors. In The leishmaniases in biology and medicine, W. Peters, and R. Killick-Kendrick, eds. (London, Academic Press), pp. 235-290.

Liew, F. Y., Li, Y., and Millott, S. (1990a). Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. *145*, 4306-4310.

Liew, F. Y., Li, Y., and Millott, S. (1990b). Tumour necrosis factor (TNF-α) in leishmaniasis. II. TNF-α-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology *71*, 556-559.

Liew, F. Y., Millott, S., Parkinson, C., Palmer, R. M., and Moncada, S. (1990c). Macrophage killing of *Leishmania* parasite *in vivo* is mediated by nitric oxide from L-arginine. J. Immunol. *144*, 4794-4797.

Lira, R., Doherty, M., Modi, G., and Sacks, D. (2000). Evolution of lesion formation, parasitic load, immune response, and reservoir potential in C57BL/6 mice following high- and low-dose challenge with *Leishmania major*. Infect. Immun. *68*, 5176-5182.

Liu, K., Iyoda, T., Saternus, M., Kimura, Y., Inaba, K., and Steinman, R. M. (2002). Immune tolerance after delivery of dying cells to dendritic cells *in situ*. J. Exp. Med. *196*, 1091-1097.

Locksley, R. M., Heinzel, F. P., Sadick, M. D., Holaday, B. J., and Gardner, K. D., Jr. (1987). Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets. Ann. Inst. Pasteur. Immunol. *138*, 744-749.

Love, D. C., Esko, J. D., and Mosser, D. M. (1993). A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular proteoglycans. J. Cell Biol. *123*, 759-766.

Love, D. C., Mentink Kane, M., and Mosser, D. M. (1998). *Leishmania amazonensis*: the phagocytosis of amastigotes by macrophages. Exp. Parasitol. *88*, 161-171.

Lutz, M. B., Kukutsch, N., Ogilvie, A. L. J., Rossner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods *223*, 77-92.

Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. S., Culpepper, J. A., Wysocka, M., Trinchieri, G., Murphy, K. M., and O'Garra, A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive $CD4^+$ T cells. J. Immunol. *154*, 5071-5079.

Macatonia, S. E., Knight, S. C., Edwards, A. J., Griffiths, S., and Fryer, P. (1987). Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. J. Exp. Med. *166*, 1654-1667.

MacDonald, A. S., Straw, A. D., Bauman, B., and Pearce, E. J. (2001). $CD8^-$ dendritic cell activation status plays an integral role in influencing Th2 response development. J. Immunol. *167*, 1982-1988.

MacLeod, C. L. (1996). Regulation of cationic amino acid transporter (CAT) gene expression. Biochem. Soc. Trans. *24*, 846-852.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. Annu. Rev. Immunol. *15*, 323-350.

Magill, A. J., Gasser, R. A., Jr. , Oster, C. N., Grogl, M., and Sun, W. (1992). Viscerotropic leishmaniasis in persons returning from Operation Desert Storm. Morbidity and Mortality Weekly Report. Surveillance Summaries *41*, 131-134.

Magill, A. J., Grogl, M., Gasser, R. A., Jr., Sun, W., and Oster, C. N. (1993). Visceral infection caused by *Leishmania tropica* in veterans of Operation Desert Storm. N. Engl. J. Med. *328*, 1383-1387.

Magill, A. J., Grogl, M., Johnson, S. C., and Gasser, R. A., Jr. (1994). Visceral infection due to *Leishmania tropica* in a veteran of Operation Desert Storm who presented 2 years after leaving Saudi Arabia. Clin. Infect. Dis. *19*, 805-806.

Maldonado-Lopez, R., De Smedt, T., Michel, P., Godfroid, J., Pajak, B., Heirman, C., Thielemans, K., Leo, O., Urbain, J., and Moser, M. (1999). $CD8\alpha^+$ and $CD8\alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells *in vivo*. J. Exp. Med. *189*, 587-592.

Marovich, M. A., McDowell, M. A., Thomas, E. K., and Nutman, T. B. (2000). IL-12p70 production by *Leishmania major*-harboring human dendritic cells is a CD40/CD40 ligand-dependent process. J. Immunol. *164*, 5858-5865.

Mattner, F., Magram, J., Ferrante, J., Launois, P., Di Padova, K., Behin, R., Gately, M. K., Louis, J. A., and Alber, G. (1996). Genetically resistant mice lacking interleukin-12 are susceptible to infection with *Leishmania major* and mount a polarized Th2 cell response. Eur. J. Immunol. *26*, 1553-1559.

Mauel, J., and Behin, R. (1987). Immunity: clinical and experimental. In The leishmaniases in biology and medicine, W. Peters, and R. Killick-Kendrick, eds. (London, Academic Press), pp. 731-791.

Mauel, J., Ransijn, A., and Buchmuller-Rouiller, Y. (1991). Killing of *Leishmania* parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen derivatives. J. Leukoc. Biol. *49*, 73-82.

Mazingue, C., Cottrez-Detoeuf, F., Louis, J., Kweider, M., Auriault, C., and Capron, A. (1989). *In vitro* and *in vivo* effects of interleukin 2 on the protozoan parasite *Leishmania*. Eur. J. Immunol. *19*, 487-491.

McConville, M. J., Turco, S. J., Ferguson, M. A., and Sacks, D. L. (1992). Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. EMBO J. *11*, 3593-3600.

McDowell, M. A., and Sacks, D. L. (1999). Inhibition of host cell signal transduction by *Leishmania*: observations relevant to the selective impairment of IL-12 responses. Curr. Opin. Microbiol. *2*, 438-443.

McGuirk, P., McCann, C., and Mills, K. H. (2002). Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J. Exp. Med. *195*, 221-231.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. Nat. Rev. Immunol. *1*, 135-145.

Medzhitov, R., and Janeway, C. A., Jr. (1998). Innate immune recognition and control of adaptive immune responses. Semin. Immunol. *10*, 351-353.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997). A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Meleney, H. E. (1925). The histopathology of kala-azar in the hamster, monkey and man. Am. J. Pathol. *1*, 147-168.

Mendonca, S. C., Coutinho, S. G., Amendoeira, R. R., Marzochi, M. C., and Pirmez, C. (1986). Human american cutaneous leishmaniasis (*Leishmania b. braziliensis*) in Brazil: lymphoproliferative responses and influence of therapy. Clin. Exp. Immunol. *64*, 269-276.

Mendoza-Leon, A., Shaw, J. J., and Tapia, F. J. (1996). A guide for the cutaneous leishmaniasis connoisseur. In Molecular and immune mechanisms in the pathogenesis of cutaneous leishmaniasis, F. J. Tapia, M. Caceres-dittmar, and M. A. Sanchez, eds. (Austin, R.G. Landes Company), pp. 1-23.

Menzel, S., and Bienzle, U. (1978). Antibody responses in patients with cutaneous leishmaniasis of the Old World. Tropenmedizin und Parasitolgie *29*, 194-197.

Merad, M., Fong, L., Bogenberger, J., and Engleman, E. G. (2000). Differentiation of myeloid dendritic cells into CD8α-positive dendritic cells *in vivo*. Blood *96*, 1865-1872.

Mills, C. D. (2001). Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. Crit. Rev. Immunol. *21*, 399-425.

Mills, C. D., Shearer, J., Evans, R., and Caldwell, M. D. (1992). Macrophage arginine metabolism and the inhibition or stimulation of cancer. J. Immunol. *149*, 2709-2714.

Mitchell, G. F. (1983). Murine cutaneous leishmaniasis: resistance in reconstituted nude mice and several F1 hybrids infected with *Leishmania tropica major*. J. Immunogenet. *10*, 395-412.

Modabber, F., Chance, M. L., Evans, D. A., Zijstra, E. E., el Hassan, A. M., Kager, P. A., Ashford, R. W., Hepburn, N. C., Omer, A. H. S., and Dedet, J.-P. (1999). The leishmaniases. In Protozoal diseases, H. M. Gilles, ed. (London, Edward Arnold), pp. 413-529.

Moll, H., Flohe, S., and Rollinghoff, M. (1995). Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. Eur. J. Immunol. *25*, 693-699.

Moll, H., Fuchs, H., Blank, C., and Rollinghoff, M. (1993). Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells. Eur. J. Immunol. *23*, 1595-1601.

Moll, H., Scollay, R., and Mitchell, G. F. (1988). Resistance to cutaneous leishmaniasis in nude mice injected with L3T4$^+$ T cells but not with Ly-2$^+$ T cells. Immunol. Cell Biol. *66 (Pt 1)*, 57-63.

Moriearty, P. L., Grimaldi, G., Jr., Galvao-Castro, B., de Oliveira Neto, M. P., and Marzochi, M. C. (1982). Intralesional plasma cells and serological responses in human cutaneous leishmaniasis. Clin. Exp. Immunol. *47*, 59-64.

Morris, L., Troutt, A. B., Handman, E., and Kelso, A. (1992). Changes in the precursor frequencies of IL-4 and IFN-γ secreting CD4$^+$ cells correlate with resolution of lesions in murine cutaneous leishmaniasis. J. Immunol. *149*, 2715-2721.

Morris, L., Troutt, A. B., McLeod, K. S., Kelso, A., Handman, E., and Aebischer, T. (1993). Interleukin-4 but not gamma interferon production correlates with the severity of murine cutaneous leishmaniasis. Infect. Immun. *61*, 3459-3465.

Moser, M., and Murphy, K. M. (2000). Dendritic cell regulation of TH1-TH2 development. Nat. Immunol. *1*, 199-205.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. *136*, 2348-2357.

Mosser, D. M., and Edelson, P. J. (1984). Activation of the alternative complement pathway by *Leishmania* promastigotes: parasite lysis and attachment to macrophages. J. Immunol. *132*, 1501-1505.

Mosser, D. M., and Edelson, P. J. (1985). The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J. Immunol. *135*, 2785-2789.

Mosser, D. M., and Edelson, P. J. (1987). The third component of complement (C3) is responsible for the intracellular survival of *Leishmania major*. Nature *327*, 329-331.

Mosser, D. M., and Rosenthal, L. A. (1993). *Leishmania*-macrophage interactions: multiple receptors, multiple ligands and diverse cellular responses. Semin. Cell Biol. *4*, 315-322.

Mosser, D. M., Springer, T. A., and Diamond, M. S. (1992). *Leishmania* promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). J. Cell Biol. *116*, 511-520.

Mougneau, E., Altare, F., Wakil, A. E., Zheng, S., Coppola, T., Wang, Z. E., Waldmann, R., Locksley, R. M., and Glaichenhaus, N. (1995). Expression cloning of a protective *Leishmania* antigen. Science *268*, 563-566.

Murray, H. W. (1982). Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. J. Immunol. *129*, 351-357.

Murray, H. W. (2000). Treatment of visceral leishmaniasis (kala-azar): a decade of progress and future approaches. Int. J. Infect. Dis. *4*, 158-177.

Murray, H. W., and Cartelli, D. M. (1983). Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity. J. Clin. Invest. *72*, 32-44.

Murray, H. W., and Nathan, C. F. (1999). Macrophage microbicidal mechanisms *in vivo*: reactive nitrogen versus oxygen intermediates in the

killing of intracellular visceral *Leishmania donovani*. J. Exp. Med. *189*, 741-746.

Nasseri, M., and Modabber, F. Z. (1979). Generalized infection and lack of delayed hypersensitivity in BALB/c mice infected with *Leishmania tropica major*. Infect. Immun. *26*, 611-614.

Nicolis, G. D., Tosca, A. D., Stratigos, J. D., and Capetanakis, J. A. (1978). A clinical and histological study of cutaneous leishmaniasis. Acta Derm. Venereol. *58*, 521-525.

Nicolle, C., and Sicre, A. (1908a). Nouvelles acquisitions sur le kala-azar chez le chien. Orgine canine probable de cette affection. Comptes Rendus des Seances de l Academie des Sciences *146*, 498-499.

Nicolle, C., and Sicre, A. (1908b). Rreproduction expérimentale du bouton d'Orient chez le singe (*Macacus sinicus*). Comptes Rendus des Seances de la Societe de Biologie *64*, 1096-1098.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity *8*, 275-283.

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. Natl. Acad. Sci. USA *97*, 13766-13771.

Park, A. Y., Hondowicz, B. D., and Scott, P. (2000). IL-12 is required to maintain a Th1 response during *Leishmania major* infection. J. Immunol. *165*, 896-902.

Park, A. Y., and Scott, P. (2001). IL-12: keeping cell-mediated immunity alive. Scand. J. Immunol. *53*, 529-532.

Paveto, C., Pereira, C., Espinosa, J., Montagna, A. E., Farber, M., Esteva, M., Flawia, M. M., and Torres, H. N. (1995). The nitric oxide transduction pathway in *Trypanosoma cruzi*. J. Biol. Chem. *270*, 16576-16579.

Pearson, R. D., Harcus, J. L., Roberts, D., and Donowitz, G. R. (1983). Differential survival of *Leishmania donovani* amastigotes in human monocytes. J. Immunol. *131*, 1994-1999.

Pearson, R. D., and Roberts, D. (1990). Host immunoglobulin on spleen-derived *Leishmania donovani* amastigotes. Am. J. Trop. Med. Hyg. *43*, 263-265.

Pegg, A. E. (1988). Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Res. *48*, 759-774.

Peters, C., Aebischer, T., Stierhof, Y. D., Fuchs, M., and Overath, P. (1995). The role of macrophage receptors in adhesion and uptake of *Leishmania mexicana* amastigotes. J. Cell Sci. *108 (Pt 12)*, 3715-3724.

Pham, T. V., and Mauel, J. (1987). Studies on intracellular killing of *Leishmania major* and lysis of host macrophages by immune lymphoid cells *in vitro*. Parasite Immunol. *9*, 721-736.

Pierre, P., Turley, S. J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R. M., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787-792.

Pimenta, P. F., Turco, S. J., McConville, M. J., Lawyer, P. G., Perkins, P. V., and Sacks, D. L. (1992). Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science *256*, 1812-1815.

Pirmez, C., Yamamura, M., Uyemura, K., Paes-Oliveira, M., Conceicao-Silva, F., and Modlin, R. L. (1993). Cytokine patterns in the pathogenesis of human leishmaniasis. J. Clin. Invest. *91*, 1390-1395.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C.*, et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. Science *282*, 2085-2088.

Pratt, D. M., and David, J. R. (1981). Monoclonal antibodies that distinguish between New World species of *Leishmania*. Nature *291*, 581-583.

Prina, E., Jouanne, C., de Souza Lao, S., Szabo, A., Guillet, J. G., and Antoine, J. C. (1993). Antigen presentation capacity of murine macrophages infected with *Leishmania amazonensis* amastigotes. J. Immunol. *151*, 2050-2061.

Puentes, S. M., Da Silva, R. P., Sacks, D. L., Hammer, C. H., and Joiner, K. A. (1990). Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. J. Immunol. *145*, 4311-4316.

Puentes, S. M., Sacks, D. L., da Silva, R. P., and Joiner, K. A. (1988). Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. J. Exp. Med. *167*, 887-902.

Pulendran, B., Banchereau, J., Maraskovsky, E., and Maliszewski, C. (2001a). Modulating the immune response with dendritic cells and their growth factors. Trends Immunol *22*, 41-47.

Pulendran, B., Kumar, P., Cutler, C. W., Mohamadzadeh, M., Van Dyke, T., and Banchereau, J. (2001b). Lipopolysaccharides from distinct pathogens induce different classes of immune responses *in vivo*. J. Immunol. *167*, 5067-5076.

Pulendran, B., Smith, J. L., Caspary, G., Brasel, K., Pettit, D., Maraskovsky, E., and Maliszewski, C. R. (1999). Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. Proc. Natl. Acad. Sci. U. S. A. *96*, 1036-1041.

Qi, H., Popov, V., and Soong, L. (2001a). *Leishmania amazonensis*-dendritic cell interactions *in vitro* and the priming of parasite-specific $CD4^+$ T cells *in vivo*. J. Immunol. *167*, 4534-4542.

Qi, H., Popov, V., and Soong, L. (2001b). *Leishmania amazonensis*-dendritic cell interactions *in vitro* and the priming of parasite-specific $CD4^+$ T cells *in vivo*. J. Immunol. *167*, 4534-4542.

Re, F., and Strominger, J. (2001). Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J. Biol. Chem. *276*, 37692-37699.

Reiner, S. L., and Locksley, R. M. (1995). The regulation of immunity to *Leishmania major*. Annu. Rev. Immunol. *13*, 151-177.

Reiner, S. L., Zheng, S., Wang, Z. E., Stowring, L., and Locksley, R. M. (1994). *Leishmania* promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from $CD4^+$ T cells during initiation of infection. J. Exp. Med. *179*, 447-456.

Reis e Sousa, C., Sher, A., and Kaye, P. (1999). The role of dendritic cells in the induction and regulation of immunity to microbial infection. Curr. Opin. Immunol. *11*, 392-399.

Reis e Sousa, C., Stahl, P. D., and Austyn, J. M. (1993). Phagocytosis of antigens by Langerhans cells in vitro. J. Exp. Med. *178*, 509-519.

Ribeiro, J. M. (1987). Role of saliva in blood-feeding by arthropods. Annu. Rev. Entomol. *32*, 463-478.

Ridley, D. S. (1979). The pathogenesis of cutaneous leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. *73*, 150-160.

Ridley, D. S. (1980). A histological classification of cutaneous leishmaniasis and its geographical expression. Trans. R. Soc. Trop. Med. Hyg. *74*, 515-521.

Ridley, D. S. (1987). Pathology. In The leishmaniases in biology and medicine, W. Peters, and R. Killick-Kendrick, eds. (London, Academic Press), pp. 665-701.

Ridley, D. S., Marsden, P. D., Cuba, C. C., and Barreto, A. C. (1980). A histological classification of mucocutaneous leishmaniasis in Brazil and its clinical evaluation. Trans. R. Soc. Trop. Med. Hyg. *74*, 508-514.

Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R., and Liu, Y. J. (1999). Reciprocal control of T helper cell and dendritic cell differentiation. Science *283*, 1183-1186.

Rizvi, F. S., Afchain, D., Sherlock, I., Sadigursky, M., Capron, A., and Santoro, F. (1985). Infectivity of *Leishmania* promastigotes is associated with surface antigenic expression. Immunol. Lett. *11*, 317-323.

Rizvi, F. S., Ouaissi, M. A., Marty, B., Santoro, F., and Capron, A. (1988). The major surface protein of *Leishmania* promastigotes is a fibronectin-like molecule. Eur. J. Immunol. *18*, 473-476.

Roffi, J., Dedet, J. P., Desjeux, P., and Garre, M. T. (1980). Detection of circulating antibodies in cutaneous leishmaniasis by enzyme-linked immunosorbent assay (ELISA). Am. J. Trop. Med. Hyg. *29*, 183-189.

Rolink, A., Melchers, F., and Andersson, J. (1996a). The SCID but not the RAG-2 gene product is required for S mu-S epsilon heavy chain class switching. Immunity *5*, 319-330.

Rolink, A., Melchers, F., and Andersson, J. (1996b). The SCID but not the RAG-2 gene product is required for Sμ-Sε heavy chain class switching. Immunity *5*, 319-330.

Romagnani, S. (1994). Lymphokine production by human T cells in disease states. Annu. Rev. Immunol. *12*, 227-257.

Romani, N., Koide, S., Crowley, M., Witmer-Pack, M., Livingstone, A. M., Fathman, C. G., Inaba, K., and Steinman, R. M. (1989). Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. J. Exp. Med. *169*, 1169-1178.

Ross, R. (1903). Note on the bodies recently described by Leishman and Donovan. Br. Med. J. *2*, 1261-11262.

Russell, D. G., and Talamas-Rohana, P. (1989). *Leishmania* and the macrophage: a marriage of inconvenience. Immunol Today *10*, 328-333.

Russell, D. G., and Wright, S. D. (1988). Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp-containing region of the major surface glycoprotein, gp63, of *Leishmania* promastigotes. J. Exp. Med. *168*, 279-292.

Sacks, D., and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat. Rev. Immunol. *2*, 845-858.

Sacks, D. L. (1989). Metacyclogenesis in *Leishmania* promastigotes. Exp. Parasitol. *69*, 100-103.

Sacks, D. L., and Perkins, P. V. (1984). Identification of an infective stage of *Leishmania* promastigotes. Science *223*, 1417-1419.

Sacks, D. L., and Perkins, P. V. (1985). Development of infective stage *Leishmania* promastigotes within phlebotomine sand flies. Am. J. Trop. Med. Hyg. *34*, 456-459.

Sadick, M. D., Heinzel, F. P., Holaday, B. J., Pu, R. T., Dawkins, R. S., and Locksley, R. M. (1990). Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon-γ-independent mechanism. J. Exp. Med. *171*, 115-127.

Sadick, M. D., Heinzel, F. P., Shigekane, V. M., Fisher, W. L., and Locksley, R. M. (1987). Cellular and humoral immunity to *Leishmania major* in genetically susceptible mice after *in vivo* depletion of $L3T4^+$ T cells. J. Immunol. *139*, 1303-1309.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med. *182*, 389-400.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor-α. J. Exp. Med. *179*, 1109-1118.

Sato, N., Ahuja, S. K., Quinones, M., Kostecki, V., Reddick, R. L., Melby, P. C., Kuziel, W. A., and Ahuja, S. S. (2000). CC chemokine receptor (CCR)2 is required for Langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells. Absence of CCR2 shifts the *Leishmania major*-resistant phenotype to a susceptible state dominated by Th2 cytokines, B cell

outgrowth, and sustained neutrophilic inflammation. J. Exp. Med. *192*, 205-218.

Schuler, G., and Steinman, R. M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. J. Exp. Med. *161*, 526-546.

Schulz, O., Edwards, A. D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., and Reis e Sousa, C. (2000). CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. Immunity *13*, 453-462.

Scott, H. H. (1939). Leishmaniases. In A history of tropical medicine (Baltimore, Williams & Wilkins Co.), pp. 547-570.

Scott, P. (1985). Impaired macrophage leishmanicidal activity at cutaneous temperature. Parasite Immunol. *7*, 277-288.

Scott, P., Caspar, P., and Sher, A. (1990). Protection against *Leishmania major* in BALB/c mice by adoptive transfer of a T cell clone recognizing a low molecular weight antigen released by promastigotes. J. Immunol. *144*, 1075-1079.

Scott, P., Eaton, A., Gause, W. C., di Zhou, X., and Hondowicz, B. (1996). Early IL-4 production does not predict susceptibility to *Leishmania major*. Exp. Parasitol. *84*, 178-187.

Scott, P., James, S., and Sher, A. (1985). The respiratory burst is not required for killing of intracellular and extracellular parasites by a lymphokine-activated macrophage cell line. Eur. J. Immunol. *15*, 553-558.

Scott, P., Natovitz, P., Coffman, R. L., Pearce, E., and Sher, A. (1988). Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J. Exp. Med. *168*, 1675-1684.

Scott, P., Pearce, E., Natovitz, P., and Sher, A. (1987a). Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. J. Immunol. *139*, 221-227.

Scott, P., Pearce, E., Natovitz, P., and Sher, A. (1987b). Vaccination against cutaneous leishmaniasis in a murine model. II. Immunologic properties of protective and nonprotective subfractions of soluble promastigote extract. J. Immunol. *139*, 3118-3125.

Scott, P., Sacks, D., and Sher, A. (1983). Resistance to macrophage-mediated killing as a factor influencing the pathogenesis of chronic cutaneous leishmaniasis. J. Immunol. *131*, 966-971.

Seder, R. A., Gazzinelli, R., Sher, A., and Paul, W. E. (1993). Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon-γ production and diminishes interleukin 4 inhibition of such priming. Proc. Natl. Acad. Sci. U. S. A. *90*, 10188-10192.

Seder, R. A., and Paul, W. E. (1994). Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. Annu. Rev. Immunol. *12*, 635-673.

Shaw, J. J., and Lainson, R. (1968). Leishmaniasis in Brazil: II. Observations on enzootic rodent leishmaniasis in the Lower Amazon Region--the feeding habits of the vector, *Lutzomyia flaviscutellata* in reference to man, rodents and other animals. Trans. R. Soc. Trop. Med. Hyg. *62*, 396-405.

Shaw, J. J., and Lainson, R. (1987). Ecology and epidemiology: New World. In The leishmaniases in biology and medicine, W. Peters, and R. Killick-Kendrick, eds. (London, Academic Press), pp. 291-363.

Shearer, J. D., Richards, J. R., Mills, C. D., and Caldwell, M. D. (1997). Differential regulation of macrophage arginine metabolism: a proposed role in wound healing. Am. J. Physiol. *272*, E181-190.

Shortman, K., and Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. *2*, 151-161.

Shortt, H. E., Smith, R. O. A., Swaminath, C. S., and Krishnan, K. V. (1931). Transmission of Indian kala-azar by the bite of *Phlebotomus argentipes*. Indian J. Med. Res. *18*, 1373-1375.

Silberberg-Sinakin, I., Thorbecke, G. J., Baer, R. L., Rosenthal, S. A., and Berezowsky, V. (1976). Antigen-bearing langerhans cells in skin, dermal lymphatics and in lymph nodes. Cell. Immunol. *25*, 137-151.

Sing, A., Rost, D., Tvardovskaia, N., Roggenkamp, A., Wiedemann, A., Kirschning, C. J., Aepfelbacher, M., and Heesemann, J. (2002). Yersinia V-antigen exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J. Exp. Med. *196*, 1017-1024.

Smith, A. L., and Fazekas de St Groth, B. (1999). Antigen-pulsed $CD8\alpha^+$ dendritic cells generate an immune response after subcutaneous injection without homing to the draining lymph node. J. Exp. Med. *189*, 593-598.

Soong, L., Chang, C. H., Sun, J., Longley, B. J., Jr., Ruddle, N. H., Flavell, R. A., and McMahon-Pratt, D. (1997). Role of $CD4^+$ T cells in pathogenesis associated with *Leishmania amazonensis* infection. J. Immunol. *158*, 5374-5383.

Soong, L., Duboise, S. M., Kima, P., and McMahon-Pratt, D. (1995). *Leishmania pifanoi* amastigote antigens protect mice against cutaneous leishmaniasis. Infect. Immun. *63*, 3559-3566.

Soong, L., Xu, J. C., Grewal, I. S., Kima, P., Sun, J., Longley, B. J., Jr., Ruddle, N. H., McMahon-Pratt, D., and Flavell, R. A. (1996). Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. Immunity *4*, 263-273.

Spath, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., and Beverley, S. M. (2000). Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc. Natl. Acad. Sci. USA *97*, 9258-9263.

Stafford, J. L., Neumann, N. F., and Belosevic, M. (2002). Macrophage-mediated innate host defense against protozoan parasites. Crit. Rev. Microbiol. *28*, 187-248.

Stamm, L. M., Satoskar, A. A., Ghosh, S. K., David, J. R., and Satoskar, A. R. (1999). STAT-4 mediated IL-12 signaling pathway is critical for the development of protective immunity in cutaneous leishmaniasis. Eur. J. Immunol. *29*, 2524-2529.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. *9*, 271-296.

Steinman, R. M., and Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J. Exp. Med. *137*, 1142-1162.

Steinman, R. M., Lustig, D. S., and Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. J. Exp. Med. *139*, 1431-1445.

Steinman, R. M., Pack, M., and Inaba, K. (1997). Dendritic cells in the T-cell areas of lymphoid organs. Immunol. Rev. *156*, 25-37.

Stetson, D. B., Mohrs, M., Mallet-Designe, V., Teyton, L., and Locksley, R. M. (2002). Rapid expansion and IL-4 expression by *Leishmania*-specific naive helper T cells *in vivo*. Immunity *17*, 191-200.

Stobie, L., Gurunathan, S., Prussin, C., Sacks, D. L., Glaichenhaus, N., Wu, C. Y., and Seder, R. A. (2000). The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc. Natl. Acad. Sci. U. S. A. *97*, 8427-8432.

Streilein, J. W., and Grammer, S. F. (1989). *In vitro* evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. J. Immunol. *143*, 3925-3933.

Stumbles, P. A., Thomas, J. A., Pimm, C. L., Lee, P. T., Venaille, T. J., Proksch, S., and Holt, P. G. (1998). Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. J. Exp. Med. *188*, 2019-2031.

Sundar, S., Jha, T. K., Thakur, C. P., Engel, J., Sindermann, H., Fischer, C., Junge, K., Bryceson, A., and Berman, J. (2002). Oral miltefosine for Indian visceral leishmaniasis. N. Engl. J. Med. *347*, 1739-1746.

Suss, G., and Shortman, K. (1996). A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. J. Exp. Med. *183*, 1789-1796.

Svensson, M., Stockinger, B., and Wick, M. J. (1997). Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. J. Immunol. *158*, 4229-4236.

Swain, S. L., Weinberg, A. D., English, M., and Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. J. Immunol. *145*, 3796-3806.

Swaminath, C. S., Shortt, H. E., and Anderson, L. A. P. (1942). Transmission of Indian kala-azar to man by the bites of *Phlebotomus argentipes*. Indian J. Med. Res. *30*, 473-477.

Swihart, K., Fruth, U., Messmer, N., Hug, K., Behin, R., Huang, S., Del Giudice, G., Aguet, M., and Louis, J. A. (1995). Mice from a genetically resistant background lacking the interferon-γ receptor are susceptible to infection with *Leishmania major* but mount a polarized T helper cell 1-type $CD4^+$ T cell response. J. Exp. Med. *181*, 961-971.

Sypek, J. P., Chung, C. L., Mayor, S. E., Subramanyam, J. M., Goldman, S. J., Sieburth, D. S., Wolf, S. F., and Schaub, R. G. (1993). Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J. Exp. Med. *177*, 1797-1802.

Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P., and Glimcher, L. H. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. Science *295*, 338-342.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443-451.

Tapia, F. J., Caceres-dittmar, M., and Sanchez, M. A. (1996a). Molecular and immune mechanisms in the pathogenesis of cutaneous leishmaniasis (Austin, R.G. Landes Company).

Tapia, F. J., Caceres-dittmar, M., and Sanchez, M. A. (1996b). Molecular and immune mechanisms in the pathogenesis of cutaneous leishmaniasis (Austin, R.G. Landes Company).

Teunissen, M. B., Wormmeester, J., Krieg, S. R., Peters, P. J., Vogels, I. M., Kapsenberg, M. L., and Bos, J. D. (1990). Human epidermal Langerhans cells undergo profound morphologic and phenotypical changes during *in vitro* culture. J. Invest. Dermatol. *94*, 166-173.

Theodos, C. M., Povinelli, L., Molina, R., Sherry, B., and Titus, R. G. (1991a). Role of tumor necrosis factor in macrophage leishmanicidal activity *in vitro* and resistance to cutaneous leishmaniasis *in vivo*. Infect. Immun. *59*, 2839-2842.

Theodos, C. M., Ribeiro, J. M., and Titus, R. G. (1991b). Analysis of enhancing effect of sand fly saliva on *Leishmania* infection in mice. Infect. Immun. *59*, 1592-1598.

Titus, R. G. (1998). Salivary gland lysate from the sand fly *Lutzomyia longipalpis* suppresses the immune response of mice to sheep red blood cells *in vivo* and concanavalin A *in vitro*. Exp. Parasitol. *89*, 133-136.

Titus, R. G., Ceredig, R., Cerottini, J. C., and Louis, J. A. (1985). Therapeutic effect of anti-L3T4 monoclonal antibody GK1.5 on cutaneous leishmaniasis in genetically-susceptible BALB/c mice. J. Immunol. *135*, 2108-2114.

Titus, R. G., and Ribeiro, J. M. (1988). Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. Science *239*, 1306-1308.

Titus, R. G., Theodos, C. M., Shankar, A. H., and Hall, L. R. (1994). Interactions between *Leishmania major* and macrophages. Immunol. Ser. *60*, 437-459.

Trinchieri, G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. *13*, 251-276.

Turco, S. J., and Descoteaux, A. (1992). The lipophosphoglycan of *Leishmania* parasites. Annu. Rev. Microbiol. *46*, 65-94.

Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M., and Mellman, I. (2000). Transport of peptide-MHC class II complexes in developing dendritic cells. Science *288*, 522-527.

Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M., and Aderem, A. (1999). The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature *401*, 811-815.

Urban, B. C., Ferguson, D. J., Pain, A., Willcox, N., Plebanski, M., Austyn, J. M., and Roberts, D. J. (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. Nature *400*, 73-77.

van Kooten, C., and Banchereau, J. (2000). CD40-CD40 ligand. J. Leukoc. Biol. *67*, 2-17.

Van Overtvelt, L., Vanderheyde, N., Verhasselt, V., Ismaili, J., De Vos, L., Goldman, M., Willems, F., and Vray, B. (1999). *Trypanosoma cruzi* infects human dendritic cells and prevents their maturation: inhibition of cytokines, HLA-DR, and costimulatory molecules. Infect. Immun. *67*, 4033-4040.

Vieira, P. L., de Jong, E. C., Wierenga, E. A., Kapsenberg, M. L., and Kalinski, P. (2000). Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J. Immunol. *164*, 4507-4512.

Vieth, M., Will, A., Schroppel, K., Rollinghoff, M., and Gessner, A. (1994). Interleukin-10 inhibits antimicrobial activity against *Leishmania major* in murine macrophages. Scand. J. Immunol. *40*, 403-409.

von Stebut, E., Belkaid, Y., Jakob, T., Sacks, D. L., and Udey, M. C. (1998). Uptake of *Leishmania major* amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-*Leishmania* immunity. J. Exp. Med. *188*, 1547-1552.

von Stebut, E., Belkaid, Y., Nguyen, B. V., Cushing, M., Sacks, D. L., and Udey, M. C. (2000). *Leishmania major*-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis. Eur. J. Immunol. *30*, 3498-3506.

Vouldoukis, I., Becherel, P. A., Riveros-Moreno, V., Arock, M., da Silva, O., Debre, P., Mazier, D., and Mossalayi, M. D. (1997). Interleukin-10 and interleukin-4 inhibit intracellular killing of *Leishmania infantum* and *Leishmania major* by human macrophages by decreasing nitric oxide generation. Eur. J. Immunol. *27*, 860-865.

Wang, Z. E., Reiner, S. L., Zheng, S., Dalton, D. K., and Locksley, R. M. (1994). $CD4^+$ effector cells default to the Th2 pathway in interferon-γ-deficient mice infected with *Leishmania major*. J. Exp. Med. *179*, 1367-1371.

Warburg, A., and Schlein, Y. (1986). The effect of post-bloodmeal nutrition of Phlebotomus papatasi on the transmission of *Leishmania major*. Am. J. Trop. Med. Hyg. *35*, 926-930.

Warren, M. K., and Vogel, S. N. (1985). Bone marrow-derived macrophages: development and regulation of differentiation markers by colony-stimulating factor and interferons. J. Immunol. *134*, 982-989.

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. Nature *375*, 408-411.

Weinheber, N., Wolfram, M., Harbecke, D., and Aebischer, T. (1998). Phagocytosis of *Leishmania mexicana* amastigotes by macrophages leads to a sustained suppression of IL-12 production. Eur. J. Immunol. *28*, 2467-2477.

Whelan, M., Harnett, M. M., Houston, K. M., Patel, V., Harnett, W., and Rigley, K. P. (2000). A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. J. Immunol. *164*, 6453-6460.

Wozencraft, A. O., Sayers, G., and Blackwell, J. M. (1986). Macrophage type 3 complement receptors mediate serum-independent binding of *Leishmania donovani*. Detection of macrophage-derived complement on the parasite surface by immunoelectron microscopy. J. Exp. Med. *164*, 1332-1337.

Wright, S. D., and Silverstein, S. C. (1983). Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. J. Exp. Med. *158*, 2016-2023.

Wu, J., Cunha, F. Q., Liew, F. Y., and Weiser, W. Y. (1993). IL-10 inhibits the synthesis of migration inhibitory factor and migration inhibitory factor-mediated macrophage activation. J. Immunol. *151*, 4325-4332.

Wyler, D. J., Sypek, J. P., and McDonald, J. A. (1985). *In vitro* parasite-monocyte interactions in human leishmaniasis: possible role of fibronectin in parasite attachment. Infect. Immun. *49*, 305-311.

Yarlett, N., and Bacchi, C. J. (1994). Parasite polyamine metabolism: targets for chemotherapy. Biochem. Soc. Trans. *22*, 875-879.

Zarley, J. H., Britigan, B. E., and Wilson, M. E. (1991). Hydrogen peroxide-mediated toxicity for *Leishmania donovani chagasi* promastigotes. Role of hydroxyl radical and protection by heat shock. J. Clin. Invest. *88*, 1511-1521.

APPENDIX

The reproduction permission for *Illustration 4* By the Royal Society of Tropical Medicine and Hygiene

Qi, Hai

From: Caroline Scott [trans@rstmh.org]
Sent: Monday, March 31, 2003 7:06 AM
To: 'Qi, Hai'
Subject: RE: Request for reproduction permission

Dear Hai Qi

I am happy to grant permission for you to include the Figure detailed below in your PhD dissertation provided that full acknowledgement of the original source (including all bibliographical details) is given.

Best wishes with your research.

Caroline Scott

From: Qi, Hai[SMTP:haqi@utmb.edu]
Sent: Thursday, March 27, 2003 5:14 PM
To: 'trans@rstmh.org'
Subject: Request for reproduction permission

<<File: ATT00006.htm>>
Dear Sir or Madam,

I am writing to inquire about the possibility of using a figure from a review article published in the respected Transactions of the Royal Society of Tropical Medicine and Hygiene. The article is "The American
Leishmaniases: some observations on their ecology and epidemiology" by R. Laison, (1983) 77:569-596. The figure of request is Figure 13 in this article. This figure provides a wonderful overview of the ecology of leishmaniasis caused by Leishmania amazonensis, the parasite I have been studying in my Ph.D. dissertation research. For this reason, I would like to request for the permission from the Society to reproduce this figure in the introductory section of my final dissertation. Should it be necessary, I will be more than happy to write a formal letter of request and mail it to your attention. Your prompt response on this matter is very much appreciated.

Sincerely,

Hai Qi, M.D., Ph.D. candidate
Department of Pathology
University of Texas Medical Branch

Keiller 1.104
301 University Blvd.
Galveston, TX 77555-0609
TEL: 409-7728163
FAX: 409-7476869

VITA

Hai Qi, the son of Drs. Guoming Qi and Jing Wang, was born in 1973 in Beijing, China. For his entire childhood, Hai lived with his grandmother in Beijing, while his parents had been working in a distant province. In 1991, he went to Beijing Medical University, presently known as Peking University, Health Science Center, to study medicine. During his tenure as a medical student, Hai gradually lost his interests in clinical medicine. In 1997, he was accepted by the Graduate Program in Experimental Pathology, University of Texas Medical Branch (UTMB).

During his graduate education at UTMB, Hai Qi has received several awards. In 2000, he was honored with the Robert L. Harrison Award for Pathologic/Clinic Research (UTMB), the Award for Excellence in Research, UTMB 5th Life Science Symposium, and the Young Investigator Award from American Society of Tropical Medicine and Hygiene. He also received McLaughlin Travel Award at the 2nd Biennial Cell Biology Poster Session and Forum in 2001, and Arthur V. Simmang Academic Scholarship from the Graduate School in 2002.

Hai Qi has accepted a post-doctoral position in the laboratory of Dr. Ronald N. Germain at the National Institute of Allergy and Infectious Diseases. He will study antigen presenting functions of dendritic cells during the activation of T and B lymphocytes.

Hai Qi married Ying Hong, currently a graduate student at the University of Houston, on April 9, 1997 in Beijing, China.

Education

B.M. (M.D.), August 1996, Beijing Medical University, Beijing, China

Publications

Articles:

1. **Hai Qi**, Vsevolod Popov, and Lynn Soong. *Leishmania amazonensis*-dendritic cell interactions *in vitro* and the priming of parasite-specific $CD4^+$ T cells *in vivo*. *Journal of Immunology. 167:4534-4542, 2001.*
2. **Hai Qi**, Timothy L. Denning, and Lynn Soong. Differential induction of IL-10 and IL-12 in dendritic cells by microbial TLR activators and the skewing of T cell cytokine profiles. (*In press*) *Infection and Immunity. 71:000-000, 2003.*
3. **Hai Qi**, Jiaxiang Ji, and Lynn Soong. Enhanced replication of *Leishmania amazonensis* amastigotes in interferon-γ-activated murine macrophages: implications for the pathogenesis of cutaneous leishmaniasis. (*Submitted*)
4. Jing Wang and **Hai Qi**. [Indoor air quality and diseases]. *Chinese Journal of Epidemiology. 17:370-372, 1996.*
5. S. Wesley Long, Xiao-feng Zhang, **Hai Qi**, Steven Standaert, David H. Walker, and Xue-jie Yu. Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. *Infection and Immunity. 70:1824-1831, 2002.*
6. Jiaxiang Ji, Jiaren Sun, **Hai Qi**, and Lynn Soong. Analysis of T helper cell responses during infection with *Leishmania amazonensis*. *The American Journal of Tropical Medicine and Hygiene*. *66:338-345, 2002.*
7. Timothy L. Denning, **Hai Qi**, Rolf Konig, and Peter B. Ernst. Disruption of CD4-Class II MHC interactions generates $CD4^+$ T cells resembling regulatory (Tr1) Th cells that inhibit inflammatory responses *in vitro* and colitis *in vivo*. (*Submitted*)

Books and Chapters:

1. Guoming Qi, **Hai Qi**, Jie Shen, Xinhua Sun, Gang Fu, and Huiquan Mai. [When AIDS comes]. Popular Science Press, Beijing. 9-37, 1996.
2. Jing Wang, **Hai Qi**, Yan Huang and Hai Huang. [How to use the Internet]. Popular Science Press, Beijing, China. 1998.