# **Host and Parasite factors that regulate anti-*Leishmania* immunity in mice**

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# Dedication

This work is dedicated to my son and my wife:

My beloved Mohan & Tingting

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# Abstract

The outcome of *leishmaniasis* has been shown to be both host genotype and parasite strain dependent. Understanding the role of host and parasite molecules in disease outcome will provide important information for the development of new drugs, new therapies and new vaccines against this disease.

In this study, we investigated the role of a host molecule, Phosphatidylinositol 3-kinases (PI3Ks) and a parasite molecule, phosphoglycans in primary and secondary immune response against *Leishmania major*. We hypothesized that these host and pathogen factors coordinately influence the quality and magnitude of primary and secondary (memory) immune responses (and immunity) against *Leishmania major*. In the first part of my study, my results show that in the absence of phosphoglycan, antigen-presenting cells (APCs) are able to present parasite antigens to T cells more efficiently and promote a Th1 type of immune responses. However, as phosphoglycan containing molecules are important for parasite survival and virulence, the initial T cell clonal burst is impaired in *lpg2*-infected mice. This in turn leads to significantly impaired antigen-specific recall responses (measured by proliferation, IFN-g production and delayed-type hypersensitivity response) both in vitro and in vivo. Interestingly, despite this impairment, *lpg2*- L. major-infected mice were protected against secondary virulent *L. major* challenge similar to those that healed from WT *L. major* infections. In the second part of my study, I demonstrate that PI3K deficient mice are highly resistant to primary *L. major* infection despite impaired T cells response (proliferation and effector cytokines production). Interestingly, this enhanced resistance was not due to enhanced innate immunity or humoral immunity, but was related to reduced regulatory T cell expansion and differentiation. Surprisingly, results from healed PI3Ks

deficient mice suggest that an excellent primary resistance to *L. major* infection does not automatically translate to secondary protective responses because healed p110δ KI mice failed to efficiently control secondary *L. major* challenge infection. We showed that the impaired memory response was due to defective proliferation of *Leishmania*-specific memory T cells and the inability of central memory T cells to convert to effector memory T cells, which negatively impact on the ability of memory T cells to exit peripheral lymphoid organs and home to the cutaneous site of infection to mediate effector functions.

These findings have significant impacts on our knowledge in understanding of host/pathogen interaction and will shed light on future developments of vaccine, vaccination strategy and new drugs.

# Abbreviations

| | |
|---|---|
| AICD | Activation induced cell death |
| APC | Antigen-presenting cell |
| Blimp-1 | B-lymphocyte-induced maturation protein 1 |
| BMDM | Bone marrow-derived macrophage |
| BMDC | Bone marrow-derived dendritic cell |
| CCR-7 | CC chemokine receptor 7 |
| CD | Cluster of differentiation molecules |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CL | Cutaneous leishmaniasis |
| CNS | Central nervous system |
| CTL | Cytotoxic T cell |
| CXCR | Chemokine, CXC Motif, Receptor |
| DC | Dendritic cell |
| DMEM | Dulbecco’s modified eagle medium |
| EAE | Experimental autoimmune encephalomyelitis |
| EDTA | Ethylenediaminetetraacetic acid |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinases |
| FBS | Fetal bovine serum |
| GM-CSF | Granulocyte-macrophage colony stimulating factor |
| HEV | High endothelial venule |

| | |
|---|---|
| ICOS | Inducible T-cell co-stimulator |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IL | Interlukin |
| iNOS | Inducible nitric oxide synthase |
| *i.p.* | Intraperitoneal |
| IRF | Interferon regulatory factor |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| ITIM | Immunoreceptor tyrosine-based inhibition motif |
| JNK | Jun N-terminal Kinase |
| LACK | Leishmania homologue of receptors for Activated C Kinase |
| LAT | Linker for activation of T cells |
| LPG | Lipophosphoglycan |
| LPS | Lipopolysaccharide |
| MCL | Mucoutaneous leishmaniasis |
| MCP-1 | Monocytic chemotactic protein 1 |
| mDC | Myeloid dendritic cell |
| MAPK | Mitogen-Activated Protein Kinase |
| MIP | Macrophage inflammatory protein |
| MHC | Major histocompatibility complex |
| mTOR | Mammalian target of rapamycin |
| MyD88 | Myeloid differentiation factor 88 |
| NO | Nitric oxide |

| | |
|---|---|
| NTDs | Neglected tropical diseases |
| OVA | Ovalbumin |
| pDC | Plasmacytoid dendritic cell |
| PAMPs | Pathogen-associated molecular patterns |
| PKDL | Post-kala-azar dermal *leishmaniasis* |
| PMNs | Polymorphonuclear neutrophil |
| PRR | Pattern recognition receptors |
| RPMI-1640 | Roswell Park Memorial Institute culture media |
| *s.c.* | Subcutaneous |
| *scid* | Severe combined immunodeficiency |
| SLA | Soluble *leishmanial* antigen |
| STAT | Signal transducer and activator of transcription |
| T-bet | T box transcription factor |
| Tcm | Central memory T cell |
| Tem | Effector memory T cell |
| Tef | Effector T cell |
| TGF | Transforming growth factor |
| Th | T helper cell |
| TLR | Toll-like receptor |
| TNF | Tumor Necrosis Factor |
| Tr1 | Regulatory T cell type 1 |
| Treg | Regulatory T cell |
| VL | Visceral *leishmaniasis* |

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# CHAPTER I Introduction

## 1.1 General Introduction

In history, infectious diseases are the biggest threat for human health. A plague wiped out 25 millions of people in three years. Some experts estimated that the 1918 Spanish flu killed 50 millions of people in Europe, which consisted of one third of total European population at that time. A few decades ago, because of the advances in medical science, scientists thought would soon find an effective way to conquer some of the most terrifying diseases—smallpox, whooping cough, polio—with miracle vaccines. However, infectious diseases are far more complicated than what people used to believe. In fact, the top killer diseases in the developing world are still infectious diseases, they account for one in two deaths in those countries (http://www.who.int/infectious-disease-report/pages/textonly.html). Millions of people are killed every year by infectious diseases. To fight more efficiently with our enemies, it is very crucial for us to have a better understanding of host-pathogen interaction. Infectious diseases are very complicated, and there are complex, dynamic relationships between hosts and pathogens. During the millions of years of evolution, hosts have evolved the many kinds of effective defensive mechanism to against various pathogens. However, pathogens have been successfully evolved many strategies for evading and manipulating host immune system. The obligate intracellular protozoan parasite *Leishmania* is one of these very successful pathogens, which have developed highly successful strategies to manipulate host immune system and avoid killing by the host.

# 1.2 *Leishmania* and *Leishmaniasis*

*Leishmania spp.* is the causative agent of zoonotic disease-*leishmaniasis*, which is prevalent in four continents and considered endemic in 88 countries (WHO *Leishmaniasis* home: http://www.who.int/leishmaniasis/en/). According WHO's estimation, about 2 million new cases occur every year and approximately 50% of these patients are children [1]. More than 12 million people have been presently infected and a population of over 350 millions at risk and around 7000 death annually are due to the disease[2]. Furthermore, *Leishmania* has emerged as an opportunistic pathogen of HIV-infected adults as well as children [3, 4]. There is evidence indicating that co-infection with HIV increases the risk of developing active visceral *leishmaniasis* (VL) by between 100 and 2320 times [4].

The clinical manifestations of this disease can range from mild cutaneous lesion to severe visceral *leishmaniasis* depending on the strain of the parasite and the genetic background of its mammalian host. *L. infantum* and *L. donovani* infect internal organs like liver and spleen, which could cause very high mortality rate (almost 100% for symptomatic VL within two years of infection) in the absence of treatment [5]. However, the most common and extensively studied *leishmaniasis* is the cutaneous form, which is caused by *L. major* in the Old World, or *L. braziliensis* in the New World. The spectrum of disease manifestations and severity reflects the interaction between the genome of the host and that of the parasite, and the pathology is caused by a combination of host factors and parasite molecules. Table 1. is the comprehensive list for different diseases caused by different *Leishmania spp*.

Although *leishmaniasis* has been considered as a major health problem worldwide, it

is largely neglected by drug companies and health agencies due to the fact that it is endemic mainly in low-income developing nations (72 are developing countries among all 88 countries). Because of these reasons, WHO characterizes *Leishmaniasis* and *Trypanosomiasis* as the most challenging of neglected tropical diseases (NTDs) for intensive study[6].

**Table 1. Species of *Leishmania* that cause human disease. *[5]***

| Diseases | *Leishmania* spp. | Main geographical distribution |
|---|---|---|
| CL | *Leishmania major* | Old World (African, Middle East) |
| | *Leishmania tropica* | Old World (African, Middle East) |
| | *Leishmania aethiopica* | Old World (African, Middle East) |
| | *Leishmania venezuelensis* | New World (South American) |
| | *Leishmania mexicana* | New World (South American) |
| | *Leishmania amazonensis* | New World (South American) |
| | *Leishmania braziliensis* | New World (South American) |
| | *Leishmania panamensis* | New World (South American) |
| | *Leishmania peruviana* | New World (South American) |
| | *Leishmania guyanensis* | New World (South American) |
| | *Leishmania donovani* | Mediterranean and Caspian sea region |
| | *Leishmania infantum* | Mediterranean and Caspian sea region |
| MCL | *Leishmania braziliensis* | New World (South American) |
| | *Leishmania panamensis* | New World (South American) |
| | *Leishmania guyanensis* | New World (South American) |
| | *Leishmania amazonensis* | New World (South American) |
| | *Leishmania mexicana* | New World (South American) |
| VL | *Leishmania donovani* | Old World (African, Middle East, India) |
| | *Leishmania chagasi* | Old World (African, Middle East, Mediterranean basin) |
| | *Leishmania infantum* | Old World (African, Middle East, Mediterranean basin) |
| | *Leishmania archibaldi* | Old World (African, Middle East, Mediterranean basin) |
| | *Leishmania tropica* | Old World (Middle East) |
| | *Leishmania amazonensis* | New World (South American continent) |
| PKDL | *Leishmania donovani* | Old World (African continent, Middle East, India) |

# 1.3 Life Cycle of *Leishmania spp.*

All types of *leishmaniasis* are transmitted by female *phlebotomine* sandflies, which infect a range of animal hosts including humans, rodents and dogs. Among 800 known species of the sandflies, about 70 of them can harbor *Leishmania* and 20 species are capable of transmitting the disease to human [7, 8]. During their life cycle, *Leishmania* parasites alternate between two distinct developmental stages: the flagellated, motile 'promastigote' form residing in the midgut of sandfly vectors, and the non-motile 'amstigote' form within macrophages of the infected mammalian host. The life cycle of *Leishmania* is initiated when the infected sandfly ingests blood from the host, the promastigotes will be injected into the host during the bloodmeal taking procedure. The injected promastigotes will be uptaken by phagocytic cells, including macrophages and neutrophils. Since the neutrophils have a very short life span, the macrophages are the final hosts of *Leishmania* parasites [9]. After internalization into the phagolysosome of host macrophages, the promastigotes start to differentiate into small, non-motile amastigote forms, which divide many times by binary fission, eventually rupture the macrophages and infect more surrounding macrophages. If the infected host is bitten by another sandfly, these amastigotes can be uptaken with the bloodmeal. In the sandflies digestive system, the amastigotes will undergo a serial of morphological and developmental changes to differentiate into mature or infectious form of promastigotes (metacyclic) and move to the mouthpart of the sandflies. Once the infected sandfly bites another mammalian host, this life cycle will be complete and the new cycle starts.

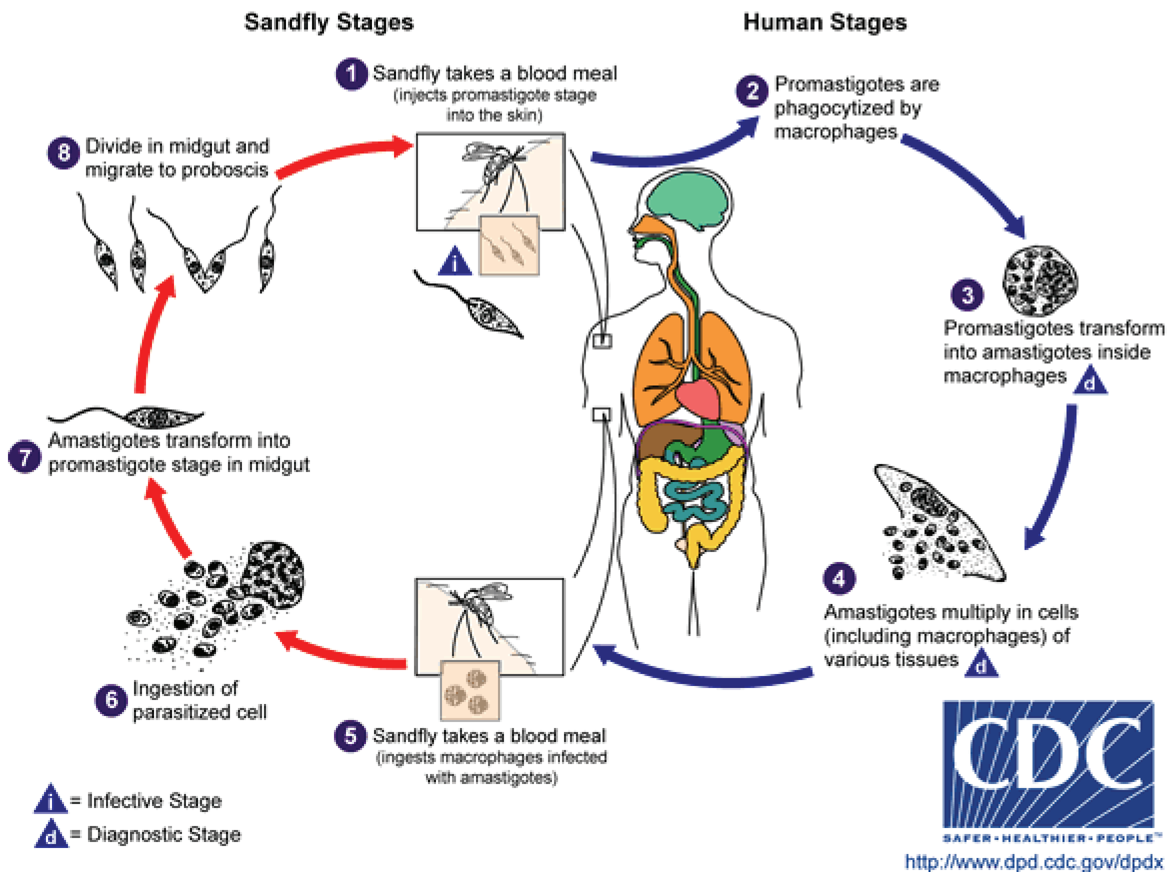



**FIGURE 1.** The *Leishmania* life cycle.

*Leishmania* are transmitted by the bite of infected female sandflies, which regurgitate a small number of infectious metacyclic promastigotes into a pool of blood in the skin. These forms are phagocytosed by blood monocytes or macrophages and targeted to phagolysosomes where they transform into the non-motile amastigotes. Amastigotes are taken up by sandflies with their blood meal into the gut where they transform into the flagellated promastigotes. The promastigotes undergo a maturation program culminating with the infectious metacyclic forms in the mouthparts ready for transmission to the vertebrate hosts.------From http://dpd.cdc.gov/dpdx/HTML/Leishmaniasis.htm

# 1.4 Surface molecules of *Leishmania* parasites and their role in virulence

*Leishmania* are dimorphic parasites exhibiting a life cycle consisting of extracellular promastigotes in the midgut of sandfly vectors and in the phagolysosomal vacuoles of mammalian host macrophages. It is pretty challenging for these organisms to live/survive in a harsh and diverse environment, so the identification and the study of molecules, which are involved in these processes, are the major focus of the research in this area.

The surface of *L. major* parasites is covered by a dense, stage specific glycocalyx coat, which has been shown to play an important role in parasite survival and virulence. In metacyclic promastigotes, the glycocalyx is composed of GPI-anchored glycoproteins, lipophosphoglycan (LPG), glycoinositol phospholipid (GIPL), proteophosphoglycans (PPG, including both membrane bind and secreted forms), and members of a secreted family of heavily glycosylated proteins. In intracellular amastigotes, the glycocalyx coat becomes thinner and the glycoprotein and LPG are highly downregulated, leaving GIPL as the dominant surface glycoconjugates [10]. Since *Leishmania* mutants with various surface molecules deficiency were only generated recently, the role of different surface glycophosphate conjugates in the induction of protective and pathogenic immune responses have not been fully investigated.

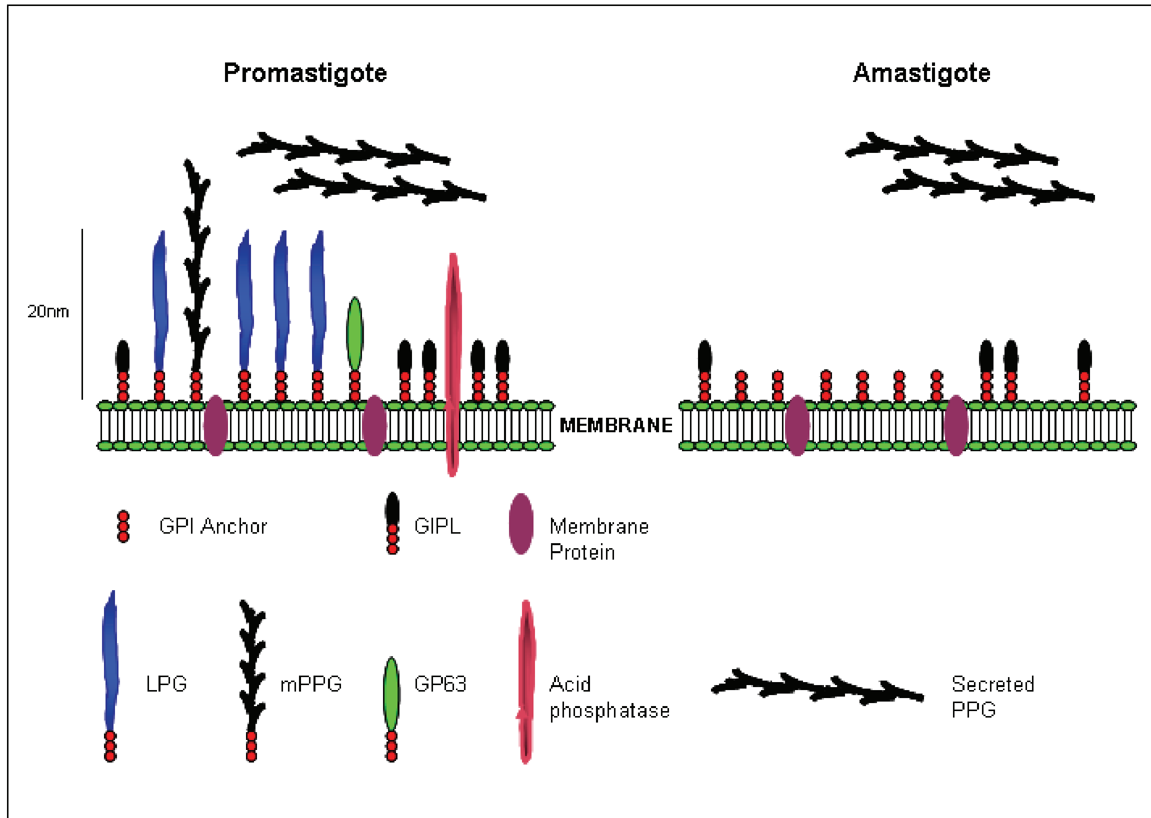



**FIGURE 2.** Schematic representation of the stage-specific expression of different classes of *Leishmania* plasma membrane and secreted components.

### 1.4.1 Lipophosphoglycan (LPG) and other phosphoglycan (PG) containing molecules

LPG is found in all species of *Leishmania* promastigotes that infect humans and is the major out leaf glycoconjugate, which makes it the most well studied surface molecules of *Leishmania*. The structure of LPG consists of a 1-O-alkyl-2-lyso-phosphatidyl (myo) inositol lipid anchor, a heptasaccharide glycan core, repeating Galβ1-4Manα1-PO4 units, and a small oligosaccharide cap [11, 12]. The basic structure elements are conserved in all *leishmania* LPGs, although there are some subtle variations between species or stages. In the

digestive tract of sandfly vector, the LPG glycocalyx may protect promastigotes against the hydrolytic enzymes [13]. Uptake of *Leishmania* promastigotes by macrophages is a receptor mediated phagocytic process through the interaction of complement receptors and LPG or GP63 [14, 15]. There is no question that LPG as a multifunctional virulence factor is essential for parasite survival in the sandfly host and the mammalian host [16]. However, LPG seems to be not very important for the survival of amastigote, since it is absent (e.g. *L. donovani*) or highly downregulated on the surface of *Leishmania* amastigotes [17]. It has been proposed and demonstrated that LPG might be involved in a series of events that are crucial for the control of *Leishmania* infection, such as inhibition of complement lysis, attachment and uptake of promastigotes by macrophages, inhibition of phagolysosome fusion and downregulation of the expression of inducible nitric oxide synthase and synthesis of IL-12 [13, 18, 19]. However, most of the early studies have used purified LPG, which makes it hard to interpret whether LPG would mediate all these effects when on the parasite surface. To solve this problem, a number of null mutants of *L. major* defective in the biosynthesis of LPG and other PG containing molecules were generated by using targeted gene methodology. This approach has allowed us to dissect the exact roles of different PGs in parasite survival and pathogenicity [13].

The synthesis of Galβ1-4Manα1-PO4 repeating units in Golgi lumen requires the mannose 1 phosphate and galactose transporters which are encoded by LPG2 and LPG5A+:LPG5B genes, respectively. By homologous gene replacement, null mutants deficient in Golgi nucleotide sugar transporters for GDP-Man and UDP-Gal were generated [18, 19]. In contrast to LPG2 and LPG5A+:LPG5B genes, the LPG1 gene, encoding a putative galactofuranosyl transferase (GalfT), is responsible for adding Galf to the LPG core

structure. As a result, the LPG1 deficient parasites lack LPG only, while LPG2 deficient mutants lack all PG-containing molecules [15, 20]. It has been shown that LPG1 deficient parasites fail to persist in the midgut of sandfly vectors [13, 21] and are highly attenuated in mammalian macrophages in their initial infectivity [13, 19]. However, some mutants can escape the killing and differentiate into amastigotes. Because amastigotes do not express LPG, these escapees eventually grow and cause progressive diseases in susceptible mice [13, 19]. Unlike LPG1 mutants, *L. major* deficient in LPG2 gene do not cause any lesion in susceptible mice despite persist for a long time after infection [20], suggesting that amastigote-specific glycophosphate conjugates play distinct role from those of promastigotes. Moreover, susceptible BALB/c mice vaccinated with LPG2 mutants are able to resist the virulent challenge without inducing very strong DTH response and IFN-γ production [22]. For some unknown reason, however, in certain rare cases, some LPG2 mutants can regain the virulence to induce disease pathology through a compensatory change [23]. Interestingly, these revertants are still unable to synthesize LPG2 gene dependent PGs, suggesting that other genes, in the absence of LPG2 gene products, are playing important role in *L. major* survival. Fortunately, this revertance has never been observed in my own experiments.

One fact needs to be remembered that not all *Leishmania* species require LPG for their virulence. For example, LPG deficient *L. mexicana* does not display any impairment in establishing infections in macrophage. Moreover, LPG deficient *L. mexicana* parasites are at lest as virulent as their wild type controls in both BALB/c and C57BL/6 mice [24].

## 1.4.2 Major Surface Glycoprotein (GP63)

GP 63 is a 63 kDa zinc-dependent metalloproteinase that is abundantly expressed on the cell surface of all *Leishmania* promastigotes [14], while it is around 10 folds less abundant than LPG on promastigotes [25, 26]. In all *Leishmania* species studied so far, Gp63 proteins are encoded by multiple tandem or closely linked genes (7 in *L. major*, 15 in *L. Mexicana* and 18 in *L. chagasi*) that are highly conserved across the genus [14, 27]. It has been suggested that GP63 plays important role in facilitating promastigote survival in both sandfly midgut and mammalian macrophage phagolysosome compartment [14]. Moreover, GP63 molecule is also capable of preventing promastigotes from complement mediated lysis and enhancing parasite uptake via complement receptors [28]. Recent genetic manipulation of GP63 demonstrated that *L. major* promastigotes with GP63 deficiency were much more sensitive to complement lysis in human serum, and showed less virulence even in highly susceptible mouse strains [29]. In contrast, GP63 mutant amastigotes were as virulent as wild type parasites, suggesting that this molecule is not essential for continued survival in the mammalian host.

## 1.4.3 Glycoinositol phospholipids (GIPLs)

Glycoinositol phospholipids (GIPLs) are the family of small GPI-linked glycolipids containing the Man$\alpha$1,4GlcN$\alpha$1,6 myo-inositol-1-phospholipid structure [30-32], which are the most abundant *Leishmania* promastigotes surface molecule (10 times more than LPG, approximately $10^7$ molecules per cell). Unlike LPG and GP63, GIPLs are actively expressed on the parasite surface by both at both the promastigote and amastigote stages. Although GIPLs are more abundant than LPGs on promastigotes, their much shorter lengths indicate that they are essentially buried under a sea of LPG. Since LPG is highly downregulated in

amastigotes and GIPLs are left to be the dominant molecules on the parasite surface and it has been proposed that GIPLs are crucial for amastigote survival and virulence in mammalian hosts [25]. However, this view was challenged by recent studies using *L. major* mutant lacking the enzyme alkyldihydroxyacetone phosphate synthase (ADS1). This enzyme is critical for the synthesis of ether phospholipids and both LPG and GIPLs are absent on the surface of the *ads1*- null mutant parasites [33]. Interestingly, they found that *ads1*- mutants showed similar phenotypes like *lpg1*- mutants in parasite survival and virulence. In another word, *ads1*-mutants are able to cause pathology in susceptible mice similar to *lpg1*- mutants, and the ability of the mutants to inhibit macrophage activation is still maintained [33]. These results suggest that GIPLs may not play a major role in the parasite virulence during the amastigote stage.

There are also other surface molecules which are not described here (See **Figure 2.**). Nevertheless, the scarcity of these molecules suggests that they may not play a significant role in parasite virulence and thus may play less important role in modulating host immune responses.

# 1.5 Mouse Model in Cutaneous *Leishmaniasis*

Since *leishmaniasis* is a zoonotic disease, rodents are among one of its natural reservoirs and the major focus of epidemiological studies. In fact, the rodent models, especially mice, are the mostly studied animal models of cutaneous *leishmaniasis*. There are a number of advantages to use mouse model to study *leishmaniasis*. Firstly, mice can be infected by the same strains of parasites that infect humans. Secondly, unlike humans, inbred mice are pretty homogenous population in terms of genetic background and many of the environmental variables can be well controlled. Most importantly, their immune system is well studied and a large number of tools have been developed which aid in their genetic analysis and manipulation, including the availability of many inbred strains of mice, specific gene deficient mice and many reagents. In addition, mice also show many similarities to human in their anatomy, physiology, immunology as well as genome organization, which would allow transfer of the knowledge we gain from mice studies to the human diseases. However, unlike homogenous in-bred mouse strains, human are very heterogeneous population, so the results from mouse model should be applied to human very carefully.

Subcutaneous or intradermal inoculation with *L. major* promastigotes is the most commonly used method to decipher the mechanism of murine immune responses to this infection. After infection, most strains of mice would develop self-healing disease with small lesion and strong immunity against reinfection (called “resistant” strains, e.g., C3H, C57BL/6, CBA, A/J, DBA/1), while a few strains of mice would develop uncontrolled disease with the spreading of parasites to other non-draining lymphoid organs (called “susceptible” strains, e.g., BALB/c, SWR/J).

The first direct in vivo evidence that the T Helper1/T Herlper2 balance controls the

outcome of some infectious diseases was obtained from murine cutaneous *leishmaniasis* model. Following subcutaneous injection of virulent parasites, the susceptible BALB/c mice develop progressive diseases in terms of non-resolving lesions and high parasite burdens which may disseminate from site of infection to the viscera leading to systemic disease and death [34]. This susceptibility is due to early, high and sustained production of IL-4 by CD4+ T cells in response to parasite antigen. In contrast to highly susceptible BALB/c mice, the relatively resistant C57BL/6 mice only develop small lesions and can eventually heal from the primary infection. This resistance to *L. major* infection is mediated by IFN-γ produced by CD4+ T cells that activate infected macrophages to kill the intracellular parasites. The balance between IFN-γ and IL-4 defines a key parameter determining the outcome of *L. major* infection.

# 1.6 Immunology of Murine Cutaneous *Leishmaniasis*

The immune system provides protection to the host against infections, which are caused by various pathogens such as viruses, bacteria, fungi, protozoa and helminth. The mammalian immune system is comprised of two distinct but cooperative arms, the innate immunity and the adaptive immunity.

## 1.6.1 The Innate Immune Response in Murine *Leishmaniasis*

We encounter numerous pathogens everyday, but only very a few of them can cause diseases. This is because we have a natural defense system which is also called innate immune system. Innate immune system is the first line of host defense against a variety of pathogens non-specifically; the effective innate responses can clear pathogens before they cause any problems and it can also fundamentally influence and shape the adaptive immune responses.

### 1.6.1.1 Polymorphonuclear neutrophil granulocytes

Once the pathogens break the physical barriers (skin or mucosa etc.) of the host and initiate an inflammatory response (tissue damage etc.), polymorphonuclear neutrophil granulocytes (PMNs) are the first cells to be rapidly recruited to the site of infection via transmigration through the vascular endothelium, followed by a wave of macrophages about 2 days later [35]. PMNs can internalize infectious microorganisms and destroy them by a number of microbicidal mechanisms including both oxygen-independent and oxygen-

dependent killings. Most pathogens are killed by hydrolytic enzymes and bactericidal proteins such as elastase and defensins in an oxygen-independent mechanism or by oxidative burst which generates highly bactericidal reactive oxygen species (ROS) in an oxygen-dependent manner. In most cases, these two mechanisms are very efficient to kill the internalized pathogens [36]. However, there are some evidences suggested that persistent PMNs infiltration can cause immunopathology and exacerbate diseases, as observed in chronic arthritis and gout [37, 38]. Furthermore, some pathogens have evolved certain strategies to resist the killing by PMNs, such as *Helicobacter pylori*, *Francisella tularensis* and *Anaplasma phagocytophilum [39, 40]*. *Leishmania* parasites are also one of these pathogens, which can survive transiently within neutrophils. Laskay and his colleagues have reported that PMNs can phagocytose *Leishmania* promastigotes. Interestingly, in contrast to most readily killed pathogens, *Leishmania* parasite can survive and persist inside PMNs without multiplication [41]. They further demonstrated that the apoptosis of *L. major* infected PMNs was delayed and secreted high levels of the chemokine MIP-1β, which is known to be able to attract macrophages [42]. Once macrophages are recruited to the site of infection, these infected apoptotic PMNs will be phagocytosed by macrophages eventually. These data suggested a previously unknown role of PMNs as “Trojan horses” for the parasite to enter into macrophages “silently” and unrecognized [43].

With the availability of transgenic mice in which neutrophils have been modified to express green fluorescent protein (GFP) and advances in in vivo imaging, Peters et al [43] reinvestigated the recruitment of neutrophils in real time at the site of sand fly bite and their interaction with macrophages. They visualized and confirmed that the majority of cells containing viable parasites early after inoculation of parasites by sand fly were neutrophils.

Furthermore, their data showed that the neutrophil depletion led to reduced parasite burden and disease progression [44]. However, 6-7 days after a sand fly bite, the predominant infected cells became macrophages, instead of acquiring infected neutrophils, macrophages phagocytose parasites released from apoptotic neutrophils [44]. They also examined the numbers of neutrophils and macrophages at the infection sites and found that early after the sandfly bite, the number of macrophages was similar as the number of neutrophils. Despite this similarity in cell numbers, these macrophages have decreased phagocytotic capacity compared to neutrophils [44]. Interestingly, in the absence of neutrophils, macrophages were recruited to the site of infection and were more efficient to phagocytose parasites. Unlike parasite acquired from infected apoptotic neutrophils, the ability of establishing infection for these "naive" parasites in macrophages was significantly compromised [44]. This enhanced killing might be due to an increase of spontaneous release of the proinflamatory cytokines interleukin-1$\alpha$ and 1$\beta$ by ear cells after neutrophil depletion. It seems that the parasites released from apoptotic neutrophils acquired something to "silence" the ear cells or macrophages to escape the killing. However, the exact mechanism needs to be further investigated.

More recently, in the work published by Novais *et al.* [45], they showed that neutrophils play a protective role in *L. braziliensis* infection. They found *in vivo* depletion of neutrophils led to a significant increase in parasite burden in BALB/c mice, while coinoculation with both parasites and live neutrophils resulted in much lower parasite load at the infection site and in the dLNs.

Taken together, current data indicate that neutrophils may play distinct role between the murine models of Old World (*L. major*) and New World (*L. braziliensis*).

### 1.6.1.2 Macrophages

Macrophages are white blood cells that reside within the tissue and are crucial for host immune defense. Macrophages are differentiated from monocytes after emigration from blood vessels in response to different stimuli. Macrophages are well known for their phagocytosis and ability to destroy microorganisms.

Because *Leishmania* parasites are obligatory intracellular pathogens, the intracellular stage in macrophages is indispensible for parasite replication and differentiation. After initial infection, both neutrophils and macrophages are recruited to the infection site and phagocytose the parasites, although neutrophils are more efficient in parasite uptake. Since neutrophils are short-lived phagocytes, macrophages are the final definitive host cells for parasite survival and replication as well as effector cells responsible for the eradication of parasites.

The uptake of *Leishmania* promastigotes is a classical receptor-mediated process, which initiates phagocytosis. A number of parasite and macrophage surface molecules have been implicated to be involved in the interaction between parasites and macrophages. In the case of promastigotes, the complement receptors (CR)1, CR3 (Mac-1), fibronectin receptor and the mannose-fucose receptor on the surface of macrophages play import roles in promastigote binding/attachment [46]. The surface lipophosphoglycan (LPG), GP63 and proteophophoglycans (PPG) on *L. major* promastigotes have been suggested to be also involved [14, 47], as they are the target molecules for various opsonins such as C3b/iC3b

[28], mannan-binding protein [48], and galectins [49, 50]. However, the surface molecules such as LPG might not be essential for attachment, as phagocytosis of LPG mutant parasites is not compromised or even better when compared to their wild type controls [51]. In the case of *Leishmania* amastigote stage, a recent paper demonstrated that host IgG coated parasites can bind Fc receptor for IgG (FcγR) on macrophages for entering into these cells. This engagement and subsequent entry activate downstream signalling pathways which prevent killing and promote parasite growth intracellularly [52].

Within macrophages, *Leishmania* promastigotes undergo a serial of morphological changes and eventually differentiate into non-motile amastigotes. The amastigotes multiply within the phagolysosomes of the macrophages and then rupture host cells to infect more surrounding tissue macrophages. The macrophage, however, is also the major effector cell responsible for the elimination of the parasite. Macrophages can be activated through different signals and develop into functionally distinct subsets, thus the appropriate activation of macrophages is crucial for eliminating this intracellular pathogen, The activation of macrophage is generally divided into two extreme spectrums: classical and alternative activation. Classical activation of macrophages is dependent on the products produced by Th1 cells and NK cells—in particular, IFN-γ, which stimulates macrophages to produce inducible nitric oxide (iNOS, also known as NOS2), an enzyme which catalyzes *L-arginine* to generate nitric oxide (NO) [53]. NO is a toxic molecule which plays a major role in killing intracellular parasites, including *Leishmania*. The importance of iNOS in facilitating parasite control was confirm in an iNOS deficient mouse on a resistant background, which was highly susceptible to *L. major* infection despite mounting a strong Th1 type response [54]. The activation of iNOS is induced by IFN-γ, iNOS mRNA and

protein in macrophages are undetectable in the absence of IFN-γ or microbial products, suggesting an important role of IFN-γ in macrophage activation. Besides IFN-γ, a number of other inflammatory cytokines, such as IL-1, TNF, IFN-α or IFN-β, are also involved in macrophage activation by upregulating iNOS expression [55]. In contrast, alternative activation of macrophages is induced by Th2 cytokines, such as IL-4 and IL-13 [56]. It has been shown that IL-4-induced polyamine biosynthesis favor *L. major* parasite survival in macrophages [57]. Apart from factors controlling classical or alternative activation of macrophages, IL-10 is believed to be the true deactivator of macrophages, which inhibits the respiratory burst and inflammatory cytokine productions, particularly TNF, by macrophages [56].

To evade killing by activated macrophages, the parasites have to manipulate this pathway to favor their survival. Interleukin 12 (IL-12) is required for the production of IFN-γ from activated T cells. *In vitro* infection of macrophages from both susceptible and resistant mice demonstrated that IL-12 production is inhibited after infection, while other proinflammatory cytokines were only slightly affected [58]. Actually, it has been found that infection of macrophages by *Leishmania* can lead to the production of immuno-regulatory cytokines such as IL-10, TGF-β, which are well known for inhibition/deactivation of macrophages functions [59]. The final outcome (or fate) of the parasites within macrophage is determined by the factors which control the activation / deactivation of macrophages, which will be further discussed in the later sections.

## 1.6.1.3 Natural Killer Cell

Natural Killer (NK) cells are a lymphocyte subset with the ability to mediate cytolytic activity and produce large amount of pro-inflammatory cytokines in response to various tumors or infections. NK cells represent approximately 10% of peripheral blood mononuclear cells (PBMC) and 0.4–5% of mononuclear cells in secondary lymphoid organs [60]. NK cells are important innate components, and their contribution to protective immunity against *Leishmania* infection has also been studied extensively both *in vitro* using human donor cells and *in vivo* using mouse models of infection. Several groups have shown that NK cells purified from unexposed human PBMCs are able to proliferate and secrete IFN-γ in response to *Leishmania* antigen [61, 62]. Mouse model studies further demonstrated that NK cells depletion leads to reduced IFN-γ production and significantly higher parasite burden [61, 63], and this exacerbation was observed as early as day 7 post infection, suggesting an important role of NK cells during the early innate response to *Leishmania* infection. NK cells secreted IFN-γ is thought to stimulate macrophage-mediated killing and promote protective Th1 response. In support this, NK induced early IFN-γ-mediated protective response against *L. major* infection in resistant C3H/HeN mice was observed as compared to the diminished NK activity in susceptible BALB/c mice [64]. However, the NK cells from BALB/c mice are not intrinsically unresponsive to parasites, just because they do not receive the correct stimuli. In the presence of appropriate stimuli, NK cells from susceptible mice can be activated normally [65].

NK cells are not only the main producer of IFN-γ during early *L. major* infection, but they are also major cytotoxic lymphocytes. A recent study showed that activated NK can also control the infection by direct lysis of infected macrophages or parasites [65]. However,

NK mediated killing seems not to be essential for the resistance, as the *Leishmania tropica*-infected beige mice only showed slightly decrease in parasite control [60]. The lytic pathway of NK cells in the beige mice is selectively disrupted but the cytokine secretion pathway is not affected [66].

Taken together, current data suggested that the mechanism of NK cell effector function in the control of *leishmaniasis* is mainly cytokine-mediated rather than cytotoxicity-mediated.

### 1.6.1.4 Dendritic Cell

Dendritic cells (DCs) are hemopoietic bone marrow progenitor derived leukocytes that are widely distributed all over the body [67]. Although dendritic cell was first described by *Paul Langerhans* more than one century ago, its role as a central immune coordinator was not firmly established until a few decades [68, 69]. DCs are professional antigen presenting cells (APC) specialized in antigen uptake, process and presentation to T cells. They are considered to be the most potent APC as only DCs are capable of inducing naive T cells activation [68].

Although macrophages are also professional APCs and the main host for harboring *Leishmania* parasites as well as effector cells for parasite killing, *Leishmania*-infected macrophages are incapable of producing IL-12 [69] and unable to stimulate Ag-specific CD4+ T cell response [70]. Indeed, recent data emphasized a central role for DCs in orchestrating immune responses in *leishmaniasis*. Early studies demonstrated that epidermal langerhan cells (MHC $II^{high}$, $CD11c^{+}$, $CD11b^{low}$, $CD8\alpha^{inter}$, $CD205^{high}$, and $Langerin^{+}$) can phagocytose *L. major* and migrate to draining lymph nodes for presentation to antigen-

specific T cells [71]. Surprisingly, however, more recent findings indicated that DCs harboring parasites in dLN are LC marker langerin negative, but rather express dermal DC markers (MHC $II^{high}$, $CD11c^{+}$, $CD11b^{+}$, $CD8\alpha^{-}$, and $CD205^{low}$)[72, 73]. Also, mice genetically modified with MHC class II deficiency in LCs but not dermal DCs control infection similar to wild type animals [71], suggesting that LCs are dispensable for triggering T cell response during *Leishmania* infection. Some studies showed that parasite harbouring macrophages most likely migrate out of the skin and transport the parasites to the draining lymph node [75, 76]. Other studies suggested that blood monocyte derived DC may differentiate within the inflamed skin, then transport parasites to dLN where they present antigen to T cells [72].

IFN-γ is required for macrophage activation to kill intracellular *Leishmania* parasites, while optimal IL-12 polarizes T cells towards Th1 subset and subsequent IFN-γ production [69]. It has been reported that infection of DC results in IL-12p70 production and different DC subsets are differentially permissive to infection and this seems to be inversely correlated with their ability of making IL-12p70 [73]. They found that $CD4^{-}CD8\alpha^{+}$ DCs are the lest permissive host cells for *L. major* amastigotes compared to $CD4^{-}$ $CD8\alpha^{-}$ DCs, $CD4^{+}CD8\alpha^{-}$ DCs, but they are the most powerful IL-12p70 producers in response to infection [73]. The mechanism controlling the induction of IL-12 from DCs and the functional differences between the IL-12-producing DCs and the non-producers are still to be determined. It has been speculated that different outcome of *Leishmania* infection between BALB/c and C57BL/6 may be due to their DC functions in promoting the biased helper T cell responses. However, one study done by von Stebut *et al.* [69], using fetal skin-derived DCs, demonstrated that both BALB/c and C57BL/6 DCs can upregulate their

constimulatory molecules and produce cytokines, including IL-12p70, efficiently in response to *L. major* infection. Moll *et al.*[74], on the other hand, investigated the expression of cytokine receptors on the surface of *Langerhans* cells from the ear epidermis. They found that DCs from BALB/c mice could up-regulate IL-4 receptor in response to *L. major* infection and down-regulate IL-12p40 production, suggesting that *Leishmania* parasites have evolved several strategies to inhibit DC functions. In studies *in vivo*, using the metacyclic promastigotes-ear infection model of cutaneous leishmaniasis, Baldwin et al. [75] investigated dendritic cell populations in L. major- infected skin and draining lymph nodes in both BALB/c and C57BL/6 mice. They found that the BALB/c mice have an increased number of plasmacytoid DCs in their lymph nodes. In consistent with this, there was a much higher cell recruitment occurred in the BALB/c skin early after infection compared to C57BL/6. However, it is not clear whether the observed differences in DCs between susceptible and resistant mice are relevant to the pathogenesis of disease. At present, there is still limited information on initial DC responses to other species of *Leishmania* at the site of infection and on the priming of pathogenic T cells.

## 1.6.2 The Adaptive Immune Response in Murine *Leishmaniasis*

Once the innate immune system is unable to effectively contain the pathogens, the adaptive immunity will be initiated to fight the invading microorganisms. Unlike the "non-specific" and evolutionarily older innate immune system, the adaptive immune system is highly specialized/specific and responds only to the microorganism that induces the response. Another hallmark of adaptive immunity is immunological memory. It "remembers" the previously encountered antigens/pathogens and responds much faster on re-exposure to the same antigen/pathogen.

### 1.6.2.1 T cells

T lymphocytes play a central role in immune protection against various infectious diseases. They are heterogeneous populations consisting of different subsets, each with distinct function, such as helping B cell make antibodies, enhancing microbicidal activity by macrophages and recruiting inflammatory cells to the infection sites, as well as making cytokines or chemokines. Because *Leishmania* parasites are obligate intracellular pathogen, cell mediated immunity is required for control of the infection and T cells is indispensable in resistance to *leishmaniasis*. This is well established by the fact that T cell deficient mice are highly susceptible to *Leishmania* infection, and adoptive transfer normal T cells can restore the lost resistance [76, 77].

**CD4+ Helper T lymphocytes in infection control**

In 1986, Mosmann *et al*. first categorized mouse CD4 effector T cells into two

distinct functional subsets, Th1 and Th2 cells, based on their cytokine profiles [78]. Soon after, *Killar et al.* also subdivide CD4 T cell lines in inflammatory and helper CD4 T cells based on the functional criteria [79]. Scott *et al.* demonstrated that in the mouse model of cutaneous *leishmaniasis*, adoptive transfer polarized T cell clones can actually change the outcome of the disease [77]. They found that the Th1 phenotype is ‘protective’ while the Th2 type is ‘non-protective. Around the same time, Heinzel *et al.* also demonstrated that an increased IFN-γ production was associated with the healing of *L. major* infected C57BL/6 mice, while an increased IL-4 production was found in susceptible BALB/c mice after infection [80]. Holaday *et al.* further confirmed this finding by transferring Th1-like line or Th2-like line into SCID mice, which could manipulate the recipient SCID mice to become resistant or susceptible, respectively [81]. These data provided the first *in vivo* evidence that the balance of T helper1/helper2 (Th1/Th2) cytokines determines the disease outcome in mouse model of cutaneous *leishmaniasis*.

The decision of a naïve T cell to differentiate into a variety of effector subsets is governed by the cytokine microenvironment and, to some extent, by the strength of T cell receptor/antigen interaction [82]. In the presence of dominant IL-12 production by DCs as well as IFN-γ produced by NK cells and T cells, naive T cells are driven to differentiate along a Th1 direction. Polarized Th1 cells subsequently produce IFN-γ and TNF and are involved in cell-mediated immunity against intracellular microorganisms. Alternatively, in the presence of Th2 polarizing factors monocytic chemotactic protein 1 (MCP-1) and OX40 ligand (OX40L), the interaction between DCs and T cells directs T cells to differentiate along a Th2 direction where they produce IL-4 and IL-13 and support humoral immune

responses [83]. Other cell types, such as mast cells, eosinophils, basophils and NKT cells, are also able to produce IL-4 to promote Th2 cell differentiation [84, 85].

The parasite host cells, macrophages, are the main target of Th1 or Th2 cytokines. The 'signature' Th1 cytokine IFN-γ induces classical activation of macrophages leading to parasite clearance [86], while the 'signature' Th2 cytokine IL-4 induces alternative activation of macrophages resulting in parasite survival and replication [87].

A number of autoimmune diseases were believed to be Th1 cells mediated diseases. However, studies on the induction experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, revealed that Th1 cells in deed are protective in this disease. These observations eventually lead to the discovery of new helper T cell subset, Th17 [88, 89]. Recent studies suggested that Th17 cells are not only responsible for certain autoimmune diseases, but also critical for some infectious diseases. Th17 cells have been implicated in the control of certain extracellular bacterial and fungal pathogens, as well as intracellular pathogens, such as *Mycobacterium tuberculosis [90]*. There is growing evidence that Th17 cells may also be critical for host defense against leishmaniasis. One recent study using susceptible BALB/c mice demonstrated that Th17 cells play a pathogenic role in disease progression via sustained IL-23 production by infected DCs [91]. However, another study reported that vaccination of C57BL/6 mice with live *L. major* plus CpG DNA provided long-term immunity and this immunity is associated with a protective role of Th17 cells [92]. The live vaccine in the presence of CpG DNA enhances early proinflammatory cytokine production, which promotes both Th1 and Th17 expansion. This discrepancy might be mouse genetic background related but could be also due to strain-related mechanisms. Further studies need to be carried out to define the relative contributions of mouse genetics

and parasite strains in the induction and the development as well as the establishment of Th17 responses in the context of leishmanial infections.

The lineage decision and differentiation of Helper T cell subsets is in involved in a number of fine regulations [93, 94]. In deed, different Th subsets do reciprocally regulate one another. For example, IL-4 produced by Th2 cells prevent Th1 development and Th1 cytokine IFN-γ inhibits Th2 differentiation [95]. Both IFN-γ and IL-4 are also able to inhibit the initial development of Th17 cells [89, 96]. However, fully matured Th17 cells are resistant to the suppression mediated by IFN-γ and IL-4 *in vitro* [89, 96], indicating Th17 subset represents a committed phenotype.

Th1/Th2/Th17 paradigm provides us a useful framework to understand biological phenomena under a variety of disease settings, including leishmaniasis. However, the dogma is highly simplistic, and Th1, Th2 and Th17 immune responses never occur in isolation at the whole animal level. In fact, it has been shown that a Th2 response can be observed in resistant C57BL/6 mice after *L. major* infection and T cells capable of protecting recipient mice have been isolated from susceptible BALB/c mice [97, 98]. The likely outcome of disease is depend on the timing, amounts and quality of cytokine produced as well as the microenvironment during infection other than the mere presence of one cytokine over another.

**Cytotoxic CD8+ T lymphocytes**

CD8+ T cells are essential for the immunological control of intracellular pathogens, particularly viruses, yet their role in other infectious diseases, such as parasitic diseases, is quite controversial. Although anti-*Leishmania* immunity is mainly mediated by CD4+ T

cells, several groups have identified parasite-specific CD8+ T cells from *Leishmania*-infected patients [105, 106]. Since IFN-γ produced by CD4+ T cells is essential for macrophage activation to kill harbored parasites, it is easily to assume that CD8+ T cells should play similar role in controlling *leishmaniasis* as they are also major IFN-γ producers. Surprisingly, a number of studies demonstrated that mice lacking CD8+ T cells or MHC-Class I expression were not impaired in controlling primary infections [99-101]. However, recently, there is clear evidence suggesting that CD8+ T cells are still important either in secondary memory responses or during low dose infections [102-104]. Most early studies on the role of CD8+ T cells used the conventional challenge model involving *s.c.* injection of high doses ($10^5$–$10^7$) of parasites in the footpad, however, in a natural infection only a low number(<1000) is transmitted into the dermis by a sandfly bite [105]. To mime natural infection, Belkaid *et al.* inoculated 100 metacyclic promastigotes into the ear dermis of C57BL/6 mice with CD8+ T cell deficiencies, including CD8-/- and CD8 depleted mice [103]. They found in the absence of CD8+ T cells, the mice failed to control parasite growth. They further demonstrated that co-transfer of CD4+ and CD8+ T cells from naive donor into RAG-/- mice can restore resistance, indicating that CD8+ T cells play an essential role in low dose primary infection. Uzonna *et al.* further investigated the possible mechanism of CD8+ T involved protection during low dose infection [104]. They found low parasite dose induced a transient Th2 response in naive C57BL/6 mice, while in the absence of CD8+ T cells, the Th2 response was maintained. However, either anti-IL4 treatment or IL-12 administration promoted a Th1 response in low dose infected CD8-deficient mice, suggesting that the major role of CD8+ T cells is to modulate CD4+ T cell function.

**Regulatory T lymphocytes**

Regulatory T cells (Treg) are a specialized T cell subset which is able to suppress activation of immune system and therefore is important for maintaining immune cell homeostasis and self-tolerance. The concept of immune suppressor cells is not new, and it was proposed by Gershon *et al.* in the early 1970s [106]. For a quite long period of time, however, the intensive studies of Tregs were prevented by the lack of reliable markers for distinguishing this population from other T cells, and other technical difficulties. It was not until Sakaguchi *et al.* [107] in 1995 reported a T cell subset with markedly increased expression of CD25, immune suppressor T cells (later referred as regulatory T cells) regained the interest from the immunology society. Based on the cell surface markers and cytokine profiles, Tregs can be classified into two groups: naturally occurring Tregs (nTregs) and induced Tregs (iTregs). nTregs represent a distinct thymus-derived T cell lineage and play an important role in maintaining immune homeostasis and self tolerance. Although CD25 originally was used as nTreg marker, Foxp3, an X-linked transcription factor belonging to the fork-head family, was recently identified as a highly specific marker for nTregs [108]. Thus, Foxp3 is currently used as the most reliable and specific marker for nTregs. In contrast to nTregs, inducible regulatory T cells (also referred to as adaptive/acquired regulatory T cells) have been shown to be generated in the periphery from conventional CD4+ T cells under certain antigenic stimulation *in vitro* and *in vivo* and during natural infection *in vivo* [109].

Although initial studies were mainly focused on their suppressive role in T cell-mediated autoimmune diseases [107], regulatory T cells (Treg) have now been suggested to be critical for some infectious diseases [110, 111]. Early studies have further emphasized the

role of Tregs in limiting/controlling excessive tissue damages by immune-mediated pathology during infection [111, 112]. In the case of *Leishmania* infecdtion, Treg cells are found to accumulate at the infection site of *L. major* infected mice and, through a series of adoptive transfer studies, have been shown to suppress the magnitude of both the Th1 response in resistant C57BL/6 mice and the Th2 response in susceptible BALB/c mice. WT C57BL/6 mice received Tregs from a chronic infection site become highly susceptible to subsequent L. major challenge, while removal of Tregs in BALB/c mice results in an significant increase in disease severity[110]. Interestingly, however, Treg cells accumulated at the sites of chronic infection are antigen-specific [113] and Treg depletion also results in the loss of immunity to reinfection, suggesting a critical role of Treg cells in the persistence of concomitant immunity. This immunity to reinfection is dependent on the persistence of parasites in the immune animals, and the survival of Treg cells is maintained through the production of IL-10, which in turn is regulated by the Treg cells [110]. It is clear that IL-10 producing Tregs are essential for long-term parasite persistence [110]. However, IL-10 produced by other cellular sources also play important role in parasite persistence, because CD25+ T cells depletion was less efficient in eliminating persistent parasites than IL-10 receptor blockade [113-115]. In further studies, it has been demonstrated that superinfection at the secondary site can cause disease reactivation at the primary site. They confirmed that depletion of CD25+ cells at the time of challenge prevent the disease reactivation, suggesting that Treg cells are responsible for recrudescence [115].

Recently, we found inoculation of killed parasites to healed C57BL/6 mice resulted in a rapid loss of infection-induced immunity and this phenomenon is associated with rapid expansion of IL-10-producing Tregs in dLNs [121]. Treatment with anti-CD25 or anti-IL-

10R could reverse killed antigen induced immunity loss. Our study suggests that Tregs have a previous overlooked role in maintaining vaccine efficacy and might have important application in vaccine design and vaccination strategies against human cutaneous *leishmaniasis*.

Although the available information on the role of Treg cells in human cutaneous *leishmaniasis* is limited, it is tempting to speculate that they are a key regulatory factor in establishing and maintaining the latent infection seen in humans. A recent study demonstrated that Treg cells with immunosuppressive functions accumulate at sites of patients infected with *L. braziliensis* [116]. It is conceivable that Treg cells are responsible for the recurrence of the disease in human when changes in the host immune system occur, whether due to disease such as HIV/AIDS co-infection, immunosuppressive therapy or just due to ageing.

Taken together, from current published data, it is conceivable that Treg cells may play important roles in the persistence of parasites and disease reactivation in both mice and humans.

### 1.6.2.2 B cells

Early studies did not support a role of B cells or antibodies in resistance against *leishmaniasis* [117] as the parasites hide inside of parasitophorous vacuole of macrophages and hence may not be accessible for antibody-mediated effector mechanisms. The results from irradiated BALB/c mice reconstituted with different cell types further confirmed a role for CD4+ T cells and not B cells or CD8+ T cells [118]. Also, transfer of serum from recovered animals did not confer any protective effect, suggesting that B cells play no role in

resistance to leishmania infection [119].

Since highly susceptible BALB/c mice usually produce very high titers of anti-*leishmania* specific antibodies than resistant mice, it has been proposed that B cells (antibodies) can exacerbate the disease [120]. A recent study investigated the role of IgG immune complexes in *L. major*-infected BALB/c mice. The authors concluded that IgG antibody not only fails to protect mice against this intracellular pathogen, but it contributes to disease progression [52]. They demonstrated that IgG administration leads to IL-10 production, which in turn exacerbate the disease. Moreover, the authors investigated the correlation between disease progression and antibody response in visceral *leishmaniasis* (VL) patients, they found in VL patients antibody levels correlate with, and are predictive of, active disease. Buxbaum *et al.* also demonstrated that IgG1 is pathogenic in murine model of *L. mexicana* infection by inducing IL-10 from macrophages [121]. In their study, they found that although IgG2a/c and IgG1 are equally capable of inducing IL-10 from macrophages in vitro, only IgG1 deficiency results in resistance in vivo. This may be due to IgG1–parasite immune complexes act almost exclusively through FcγRIII. Interestingly, however, another recent paper showed the opposite result in *Leishmania* infected C57BL/6 mice [122]. They demonstrated that uptake of *L. major* parasites via FcγR ligation is associated with DC activation and IL-12 production, which lead to subsequent protective Th1 response.

Taken together, these data suggested that IgG-mediated effects differ significantly, and may depend on the genetic background of the mice or the strain of the parasites. Probably, the dose of the parasite and the Ig isotype might also affect the final IgG-mediated effects, which needs further investigations.

## 1.6.3 The Role of Cytokines in Murine *Leishmaniasis*

Many cytokines and other immunomodulatory molecules have been suggested to play important roles in the response to *Leishmania* infection.

### 1.6.3.1 Interleukin-12/Interferon-γ/Tumor Necrosis Factor

IL-12 is a key cytokine involved in the generation of protective immunity against *leishmaniasis*. Early studies using anti-IL-12 neutralizing antibodies showed that depletion of IL-12 abrogated a Th1 type immune response, which leads to the Th2 cell development [123, 124]. Conversely, *in vivo* administration of recombinant IL-12 enhanced the Th1 type response and protected BALB/c mice from an lethal dose of *L. major* infection [125]. Bioactive IL-12p70 protein is a heterodimer consisting of a p35 and a p40 subunits. Genetic manipulation of either of the two subunits further confirmed that IL-12 is essential for Th1 response and effective control of this disease [126].

IL-12 is mainly produced by antigen presenting cells, and macrophages were proposed to be the main source of IL-12. However, although macrophages phagocytose *Leishmania* efficiently, there is evidence that their ability to produce IL-12 is selectively impaired by ingested parasites [18, 127, 128]. In contrast to macrophages, dendritic cells can uptake *Leishmania* parasites and release IL-12 to promote Th1 immunity both *in vitro* and *in vivo* [69, 129, 130].

Upon infection, IL-12 produced by innate cells as well as IFN-γ produce by both NK and T cells act on dendritic cells or macrophages to induce more IL-12 production, which strongly polarize CD4+ T cells to differentiate into Th1 cells, which are capable of making

Th1 'signature' cytokine---IFN-γ. The important role of IFN-γ is to activate macrophages to enhance microbicidal activity by inducing the production of NO and oxygen radicals required for parasite eradication and the microbicidal activity can be further enhanced by TNF [131]. Moreover, these Th1 cytokines cross-talk to one another, as well as with the Th2-type cytokines, inhibit the Th2 responses. The association of IFN-γ production with resistance has been firmly established even before the Th1/Th2 paradigm [132]. It has been demonstrated that a single injection of monoclonal anti-IFN-γ antibodies to resistant mice 2 days before infection resulted in Th2 cytokine productions and increased susceptibility [133], while administration of recombinant IFN-γ to the susceptible BALB/c mice delayed the disease development and dramatically reduced their lesion sizes [134]. In agreement with these findings, resistant mice with IFN-γ or IFN-γ receptor deficiencies were unable to resolve the lesions after *L. major* infection [135]. Interestingly, IFN-γ deficient mice developed a Th2 type response, whereas the IFN-γ receptor deficient mice are able to maintain a Th1 response [135]. Although IFN-γ was present in IFN-γ receptor deficient mice, it was not able to activate macrophages to generate NO to kill parasites due to the absence of its receptor.

There is no question that IFN-γ and IL-12 play critical roles in the initiation, development and maintenance of a Th1 response. It has been proposed that IL-12 initiate Th1 response by promoting early NK cell activities (including IFN-γ production and cytotoxicity) [136]. However, the exact mechanisms for driving a naive T cell to select a Th1 or Th2 direction are still not clear but are likely to be dictated by the cytokines produced in the early hours after infection [137].

Tumor necrosis factor (TNF) is an inflammatory cytokine which is mainly produced by activated macrophages, but also by a number of other cell types, including T and B lymphocytes. TNF is also known for synergizing with IFN-γ in macrophage activation, which leads to NO-mediated *leishmanicidal* activity [53]. In the mouse model of *leishmaniasis*, the role of TNF has been examined extensively due to its potential effector function. Indeed, Titus *et al.* [138] have reported that treatment of infected mice with recombinant TNF resulted in reduced lesion size and lower parasite burden, while the administration of neutralizing anti-TNF antibodies could result in significantly larger lesion sizes and higher parasite burden of *L. major* infected mice. In agreement with these observations, C57BL/6 mice with disrupted TNF gene developed an unexpected progressive visceral form of leishmaniasis and eventually died within a few weeks [139]. Moreover, a few recent clinical report showed that some patients treated with TNF antagonists developed clinical symptoms of latent *leishmaniasis* [140, 141], suggesting an important role of TNF for controlling this disease. Taken together, current data support a protective role of TNF in *L. major* infection.

### 1.6.3.2 Interleukin-4/Interleukin-13

Interleukin-4 (IL-4) is a pleiotropic cytokine produced mainly by CD4+ T cells which can potently induce naive CD4+ T cells to differentiate into Th2 cells. IL-4 is not only able to bind to IL-4 receptor to promote IL-4 producing Th2 cell differentiation but also strongly inhibit IFN-γ responses [84, 142]. Initial IL-4 burst and similar Vβ4, Vα8 CD4+ T cells number were observed in both resistant and susceptible mice, while resistant mice soon have an outburst of IL-12 and IFN-γ, which result in inhibition of IL-4 [143, 144]. In

contrast to elevated IFN-γ level in resistant mice, sustained high levels of IL-4 has been found in susceptible BALB/c mice and is associated with disease progression [80]. Administration of IL-4 neutralizing antibodies could convert susceptible BALB/c mice into resistant phenotype [147, 148]. To further understand the role of IL-4 in various disease settings, mice with disrupted IL-4 locus were generated. Unexpectedly, IL-4 deficient BALB/c mice remain susceptibility to *L. major* (LV39 substrain) infection [145]. However, IL-4Rα deficient BALB/c mice are highly resistant to *L. major* substrain IR173 compared to IL-4 deficient BALB/c mice, in some cases even more resistant to C57BL/6 mice. These results suggested that another cytokine which signals through the same receptor (IL-4Rα) plays a role in susceptibility to *L. major*. It has been shown that IL-4α is not only used for IL-4 binding, but is also a shared subunit for IL-13 receptor [150]. So the results observed in IL-4Rα deficient mice are not only due to unresponsive to IL-4 but also IL-13.

Clinical studies found that localized cutaneous *leishmaniasis* lesions contain high levels of IL-13 mRNA, and IL-13 not IL-4 is detectable in most of VL patients [146], suggesting a possible regulatory role for IL-13. Although some studies suggest a protective role for IL-13 in chronic phase of *L. major* infection [147, 148], most of the studies still support that IL-13 promotes disease exacerbation as it is a major Th2 cytokine [147, 149-151]. For example in a recent study, transgenic C57BL/6 mice that overexpress IL-13 cannot resolve *L. major* lesions and this susceptibility was IL-4 dependent, demonstrating the additive effect of these cytokines [149, 151]. Surprisingly, murine lymphocytes do not express any IL-13 receptors [152], indicating that the effects of IL-13 on immune responses are mediated through other cell types, most likely APCs. Indeed IL-13 has been found to downregulate such macrophage functions as IL-12 [153], iNOS [154, 155], and TNF [156,

157] production. IL-13 may also mediate some if its effects indirectly. For example, IL-13 can upregulate $PGE_2$ production [158] which can in turn inhibit IL-12Rβ2 expression [159] and is a susceptibility factor in *L. major* infection [160, 161].

## 1.6.3.3 Interleukin 10

IL-10 was originally discovered as a cytokine synthesis inhibitory factor (CSIF) and was thought to be produced by Th2 cells to inhibit cytokine secretion by Th1 cell clones [162]. It was initially classified as a Th2 cytokines. However, it is now clear that IL-10 can inhibit both Th1 and Th2 responses and it can be produced by many cell types including Th2 cells, macrophages, mast cell, eosinophils, myeloid DC, keratinocytes, B cells NK cells and regulatory T cells [163]. IL-10 has broad anti-inflammatory effects by inhibiting the production of TNF, ROI, IL-12 and IL-18 by macrophages and dendritic cells [163]. IL-10-/- mice or IL-10Rβ-/- mice develop spontaneous colitis [164, 165], suggesting an important role for IL-10 in limiting excessive immune response induced by normal microbial flora in the gut. Although the major role of IL-10 is to dampen the host immune response to limit the damage to the host, IL-10 can also enhance certain immune responses, such as IL-10 production by Treg, the stimulation of mast cells, the promotion of CTL differentiation and migration [166].

By using neutralizing IL-10-specific or IL-10 receptor (IL-10R)-specific monoclonal antibodies or IL10-/- mice, a pathogenic role of IL-10 has been identified in a number of intracellular pathogen infections [163]. In the case of *Leishmania* infection, it has been shown that IL10-/- BALB/c mice are resistant to *L. major* infection [59]. It has been demonstrated that the susceptibility of BALB/c mice is IL-10 dependent, because IL-4Ra

deficient BALB/c mice remain highly susceptible to *L. major* infection, despite the absence of IL-4/IL-13 signaling [167]. Healing from primary infection with *L. major* is typically accompanied with parasite persistence even on resistant background mice [168]. However, Belkaid *et al.* have reported that C57BL/6 mice can completely clear the persistent parasites in the absence IL-10 [110, 114], IL-10 produced by Treg is responsible for a persistent chronic infection. Interestingly, IL10-/- mice on C57BL/6 background do not show any enhanced resistance to *L. amazonensis*, despite mounting a stronger Th1-type response [169]. Moreover, another study reported that IL10-/- BALB/c mice infected with *L. Mexicana* and *L. amazonensis* fail to control the disease progression but are less severe than their wild type controls, suggesting the genetic background and parasite species may be associated with a different requirement for IL-10 [170].

Taken together, these data clearly indicated a central role for IL-10 in immunopathology and parasite persistence.

### 1.6.4 Phosphatidylinositol 3-kinase Signaling in Immune System

Phosphatidylinositol 3-kinases (PI3Ks) were first identified and further characterized by Whitman and his colleagues in the middle and late of 80's [171, 172]. PI3Ks belong to a large family of lipid kinases that phosphorylate phosphoinositides at the 3' position of the inositol ring, generating phosphatidylinositol (3)-monophosphate (PIP) phosphatidylinositol (3,4)-biphosphate (PIP2), and phosphatidylinositol (3,4,5)-triphosphate (PIP3) [173, 174]. These lipid products can serve as second messengers to initiate a serial of downstream signaling events, which play important roles in cellular functions such as cell growth, cell differentiation, cell proliferation, cell migration, cell survival and apoptosis.

Based on structural similarities, PI3Ks family can be divided into three different classes—class I, class II and class III. Class II and III are widely expressed in a variety of cell types, and there is little knowledge about the role of classII and classIII of PI3Ks in lymphocytes. Class I PI3Ks can be further divided into two subclasses—class IA and class IB. Since Class IA is mainly expressed in hematopoietic cells, most studies on the role of PI3Ks in immune response have been are focused on class IA PI3Ks. Class IA PI3Ks are heterodimeric lipid kinases composed of one regulatory subunit and one catalytic subunit. There are three genes encoding five subunits—p85α, p55α, p50α, p85β and p55γ. There are also three genes encoding the three catalytic subunits—p110α, p110β, and p110δ. PI3Ks pathway is activated by receptor tyrosine kinases (RTKs) in two different ways. The p85 regulatory subunit contains two SH2 domains, which can serve as binding sites for an activated receptor. Alternatively, PI3Ks can also be recruited to the plasma membrane by

RTKs activated small G protein Ras and undergo a subsequent conformational change [175]. Upon activation, Class IA PI3Ks generate the second messenger PIP3, which will bind to Pleckstrin homology (PH) domain containing molecules, such as protein kinase AKT, to mediate membrane recruitment. The biological function of PI3Ks is antagonized by lipid phosphatases, including the phosphatase and tensin homolog deleted on chromosome ten (PTEN) and the SH2 domain-containing inositol polyphosphatase (SHIP), which can remove the 3-phosphate and 5-phosphate from PIP3, respectively [176].

Published literatures have demonstrated that PI3K signaling regulates the activation and function of T cells, B cells, neutrophils, mast cells, dendritic cells and natural killer cells. In this thesis we will focus on the regulatory roles of PI3Ks in T cell activation and differentiation. To elucidate the functional importance of different isoform of PI3Ks, genetic and pharmacological approaches are widely used. Various knockout mice are established by using genetic approach to target PI3K catalytic or regulatory subunit. Of these, mice with single deletion of p110δ, p110γ or p85α are viable and extensively studied. Advances in PI3K pharmacological inhibitors have now allowed us to selectively inhibit the activity of individual PI3K family member or catalytic subunit in vivo and in vitro, thus pharmacological approach is another powerful tool for functional studies.

In T cells, PI3K is immediately activated upon recognition / ligation of cell surface immune receptors, including T cell receptors (TCRs), co-stimulatory receptors (such as CD28 and inducible T-cell co-stimulator ICOS), cytokine and chemokine receptors. TCR-induced PI3K activation has been suggested to be mainly mediated by TCR-interacting molecule (TRIM) containing a cytoplasmic YXXM domain, a docking site for the SH2 domain of a PI3K regulatory subunit [184]. Co-stimulatory receptors CD28 and ICOS also

contain this YXXM domain, which can further recruit and activate PI3Ks [185]. It is less clear about how cytokine binding leads to PI3K activation, however it has been suggested that cytokine receptor downstream insulin receptor substrate (IRS) or Gab proteins may control PI3K activation via YXXM domain [174].

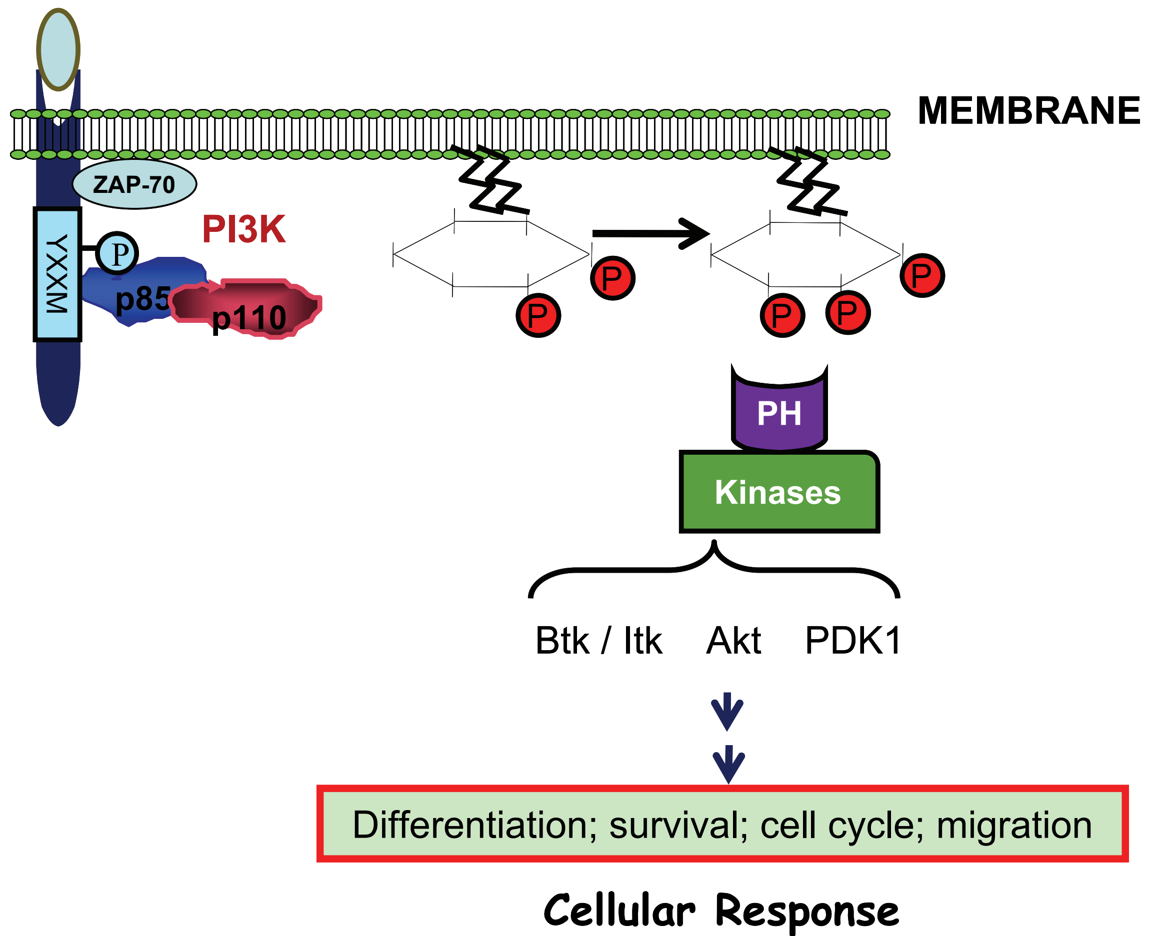



**Fig. 3** A simplified model of the PI3K signalling pathway and its interconnection in lymphocytes.

Gene-targeting regulatory subunit p85α or catalytic subunit p110δ results in different effect on T cell function. No T cell defects are observed in $p85\alpha^{-/-}$ and $p85\alpha/p55\alpha/p50\alpha^{-/-}$ mice [187, 188]; in contrast, p110δ-inactivated (a point mutation in the catalytic domain was introduced to produce a kinase-inactive protein ($p110\delta^{D910A}$) [177]) T cells demonstrate

attenuated PI3K activation and calcium flux upon TCR ligation. And the total number of peripheral mature T cells is greatly reduced in p110δ$^{D910A}$ mice [177]. p110δ$^{D910A}$ mice seems to be a good model to study PI3K roles in T cells since p110δ is responsible for 50% of p85-associated PI3K activity in T cells and appears to be the principal isoform involved in antigen receptor signaling [174, 177]. The Class IB PI3K p110γ subunit, which in contrast to other class IA PI3Ks is regulated by GPCRs (G-protein-coupled receptors), has been targeted by gene deletion and has been shown, in some cases, to contribute to PI3K signaling in concert with p110 delta or other class IA PI3Ks in T-cells [174, 176].

After activation, helper T cells differentiate into different Th subsets. The two best characterized of these are the Th1 and Th2 cells, which produce the characteristic cytokines IFN-γ and IL-4, respectively. Secretion of these cytokines was dramatically reduced in p110δ$^{D910A}$ T-cells [178]. Moreover, the addition of exogenous cytokines failed to rescue the capacity of p110δ$^{D910A}$ cells to differentiate along the Th1 or Th2 lineages. T helper cell differentiation processes require the cells to undergo multiple divisions to successfully remodel the chromatin at their cytokine loci during which the Th1 locus is closed down and the Th2 locus opened, or vice versa [174, 176, 179]. Thus p110δ appears to control a developmental programme that directs T-cells to differentiate to Th1 or Th2 cells. Studies using p110δ-slective pharmacological inhibitor IC87114 also suggest that p110δ signaling regulates Th1 and Th2 differentiation, and blockage of p110δ signaling may be beneficial for the treatment of Th1 / Th2-related diseases [180, 181]. Interestingly, p110δ$^{D910A}$ mice develop inflammation of the lower intestine characteristic of a mild form of colitis [177]. A similar disease is seen in many mice with impaired development of Tregs [182]. In the thymus of p110δ$^{D910A}$ mice, there were increased proportions of Foxp3+ Tregs [183]. It has

been proposed that some autoreactive thymocytes that do not undergo negative selection can instead be diverted to the Treg lineage [183]. Thus the enhanced proportions of thymic Tregs in p110δ$^{D910A}$ mice may be connected to decreased TCR signaling strength causing some cells that normally would be negatively selected to develop into Tregs instead. By contrast, there was a reduction in the proportions of Foxp3+ cells in all peripheral immune organs studied ref. This may reflect impaired emigration from the thymus, decreased peripheral conversion of conventional CD25- T-cells into Foxp3-expressing T-regulatory cells or decreased survival of Tregs in the peripheral lymphoid organs. In co-culture experiments, p110δ$^{D910A}$ Treg showed decreased capacity to suppress the proliferation of CD4+CD25- cells [183]. Interestingly, no IL-10 was detected in the supernatant of p110δ$^{D910A}$ Tregs that had been activated in presence of IL-2.

Collectively, PI3K signaling is required for T cell activation, helper T cell differentiation, regulatory T cell development and T cell migration. Dysregulations of PI3K signaling (enhanced PI3K activity) are often found in a variety of tumors [184-186] and sustained PI3K activation may cause autoimmune diseases. Selective inhibitor of PI3K isoform may be used to for the treatment of immune system-mediated diseases.

## 1.6.5 Immunological memory

Immunological memory is the capacity of immune system to remember a previously seen antigen and respond to it in a much faster and more efficient way on a secondary exposure [187-189]. Immunological memory is one of the hallmarks of adaptive immunity and is the theoretical basis for vaccination practice against various infectious diseases. Upon the first encounter of the antigen/pathogen in the secondary lymphoid organs, a few of antigen-specific cells undergo clonal expansion and differentiate into effector cells which are able to home to peripheral tissues to combat the invading organisms. After the expansion phase has subsided, the effector cells will have a contraction phase via activation induced cell death (AICD) and then a memory T cell pool is generated. Compared to naive antigen specific T cells, these memory T cells are not only higher in the number of the specific precursors, but also have enhanced kinetics in response to the secondary challenge in terms of accelerated proliferation, cytokine secretion and less requirement for co-stimulatory signals. Immunological memory is not restricted to T cells because B cell memory also can be generated during a primary infection. However, since the resistance to cutaneous *leishmaniasis* is mainly mediated by T cells, the focus of my study will be memory T cells.

### 1.6.5.1 Subsets of Memory T Cells

Memory T cells are heterogeneous populations. According to the model proposed by Lanzavecchia and Sallusto, memory T cells can be classified into two distinct subpopulations: central memory T cells (Tcm) and effector memory T cells (Tem), based on their homing properties and effector functions [190]. In their proposed model, protection is mediated by effector memory T cells (Tem), which are able to migrate to inflamed

peripheral tissues and mediate effector functions. The propagation of reactive effector memory T cells (Tem) is ascribed to central memory T cells (Tcm) that home to secondary lymphoid organs, have very limited effector function, but could generate effector T cells and maintain the antigen-specific memory T cells pool.

Similar to naive T cells, memory T cells also continuously circulate around the body through the blood, lymphatics and secondary lymphoid organs. The constant circulation will dramatically increase the chance for memory T cells to re-encounter the same antigenic peptide presented by antigen-presenting cells with the appropriate costimulatory molecules in the secondary lymphoid organs. Entry into the secondary lymph nodes requires well controlled migration of cells and is dependent on chemokine receptors, such as CCR-7, as well as molecules that mediate lymphocyte adhesion, such as CD62L [191]. These molecules are also called homing receptors. The circulating memory T cells enter lymph nodes through highly specialized high endothelial venules (HEVs). The transmigration is initiated by an adhesive interaction between CD62L on T cells and peripheral node addressin (PNAd), a set of sialomucins express on HEVs [192](**Fig. 4**).

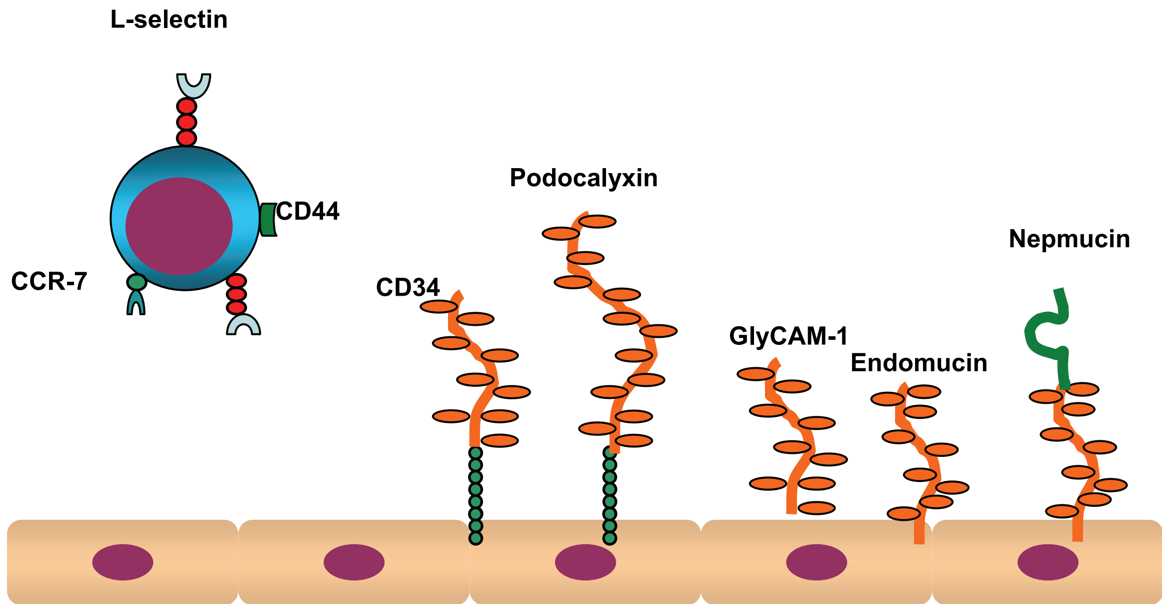



**Fig. 4** The ligands of L-selectin in HEVs of the lymph node. [192]

The interaction leads to the rolling and tethering of memory T cells and eventually migrates into lymph node from blood vessels. In this context, Tcm and Tem express significantly different levels of the lymph node homing receptors CD62L and CCR7. Tcm express high level of CD62L and CCR7 and are preferentially recruited to the lymphoid organs, whereas Tem express low level of CD62L and CCR7 but make a large amount of effector cytokines, such as IFN-γ and are recruited to the infection sites [190]. Together with increased expression of adhesion molecules such as CD44, memory T cells can respond more efficiently to lower amounts of antigen upon the secondary challenge compared to naive T cells [188].

The memory T cell pool consists of both CD4+ and CD8+ T-cells that can rapidly acquire effector functions to kill infected cells and/or secrete inflammatory cytokines that inhibit replication of the pathogen. Effector CD4+ T cells also help B-cell responses and enhance CD8+ T-cell development, through the activation of antigen presenting cells (APCs) or secretion of cytokines, such as interleukin-2 (IL-2), IL-4 and IL-5. In some situations,

protective immunity can be mediated by just one of the branches of the immune system — such as by antibodies or CD8+ T cells — but for optimal control of pathogens, both the humoral and cellular immune responses need to be mobilized.

#### 1.6.5.2 Factors Regulating Subsets Generation/Maintenance/Loss of Memory T Cells

Following the optimal antigenic or pathogenic stimulation, the primary antigenic T cells response shows distinct kinetics and can be divided into three separate phases: expansion phase, contraction phase and memory phase [193]. Following initial vigorous expansion, the newly generated effector T cells are recruited to the infection site to exert their effector functions by making cytokines or killing infected cells directly. After the clearance of the invading pathogens, the majority (>95%) of antigen-specific effector T cells undergo apoptosis. This phenomenon, termed activation-induced cell death (AICD), plays essential roles and serves as major mechanism in regulating antigen-specific T cell pools and maintaining T cell homeostasis. However, some antigen specific T cells escape the fate of cell death and undergo further differentiation to become memory cells. The memory T cell generated during this process can persist for many years[193]. Although considerable effort has been made in this area, the exact mechanism for the generation of memory T cells is still unclear. However, one thing that is pretty clear is that the conversion to memory cells is not a random event but rather a highly regulated process. It has been shown that the expression of IL-7 receptor alpha chain on activated T cells is correlated with cell survival, suggesting that IL-7 signaling might be responsible for memory cell generation [203, 204]. The strength of the signal may also influence the generation of memory T cells. Williams *et al.* found that

defective memory differentiation is independent of precursor frequency, but correlated with a lower TCR avidity [194]. This finding might be one explanation that why PI3K deficient mice have defective memory T cell generation, since TCR signaling is significantly impaired in these mice [206].

### 1.6.5.3 Immunological Memory in Cutaneous *Leishmaniasis*

Most of our knowledge for immunological memory is from bacteria and virus studies, while we have very limited knowledge in immunologic memory following parasitic infections. A most significant difference of immune memory between the majority of parasitic infections and most bacterial or viral infections is long-term antigen persistence. For example, an important feature of *leishmaniasis* is the persistence of parasites after resolution from the primary infection. It is still unclear why the host can not eliminate the parasites completely. Recent studies suggested that regulatory T cells may contribute to the parasite persistence, depletion of regulatory T cells leading to the complete elimination of the parasites [110]. Interestingly, several studies demonstrated that the animals, which completely cleared the parasites, also lost their resistance to the secondary challenge [195], indicating that persistent parasites may be required for maintaining anti-*Leishmania* immunity [110].

Although plenty of studies have been done on mechanisms of immunity to cutaneous *leishmaniasis*, there is still no clinically approved vaccine available for preventing this disease. This is in part related to our poor understanding of mechanisms of immunologic memory in infected animals. Understanding the factors that regulate the generation and maintenance of immunologic memory in cutaneous *leishmaniasis* is critical for the

development of effective vaccines and vaccination strategies against this disease. It has been argued whether real immunologic memory (and memory cells) develops in infected animals due to the fact that leishmaniasis is a persistent infection and the host can never clear the parasites. However, a recent paper provided compelling evidence for the generation of real memory T cells during *L. major* infection [196]. To examine the dynamics of CD4+ T cells, Zaph *et al.* first showed that central memory T cells from *L. major*–infected mice could mediate protection after adoptive transfer into naive mice. In this study, central memory T cells were purified from lymph nodes and spleens of healed mice still harboring small numbers of parasites. Moreover, such cells were also purified from lymph nodes of mice immunized with a live, attenuated parasite strain in which infection is completely cleared. Hence, central memory T cells could be maintained in the absence of parasites in vivo and are capable of mediating protection upon adoptive transfer into naïve mice. An understanding of how immunologic memory is generated, maintained and lost is essential for the development of effective vaccines and vaccination strategies against infectious agents and their diseases.

# 1.7 General Project Rationale

Although there are extensive publications on the murine model of experimental leishmaniasis has been published, the complex nature of the interactions between leishmania parasites and its murine hosts still remain unresolved. The outer surface membrane of *L. major* parasites is covered by a dense glycoconjugates, which are the first parasite components that are encounter by the host immune system. Thus, these molecules, particularly phosphoglycan-containing molecules, must play a crucial role in modulating host immune responses in order to support parasite survival. Therefore, it is important to dissect the exact role of different glycoconjugates during infection. Most of the roles proposed for these parasite surface components have been deduced by using purified molecules, which could be misleading because these purified molecules may not be in their natural conformation, may not be highly pure and could have been contaminated by LPS. The use of null mutant parasites lacking different surface glycoconjugates will be a better way to unequivocally study the role of these molecules in parasite virulence and their impact on the host immune responses. In our study, we used *lpg1*- and *lpg2*- mutants to dissect different glycoconjugates in early and memory immune responses.

The outcome of *Leishmania major* infection is determined by the parasite species and host genetic background. Studies have identified several host factors that regulate the outcome of infection with L. major. Yet the role played by several important host immune regulating factors in the pathogenesis of cutaneous leishmaniasis remain unknown. Among these host factors, the class IA phosphoinositide 3 kinases (PI3Ks) play a unique role in cell differentiation/proliferation, cell migration, cell survival, etc. PI3Ks are p85/p110

heterodimeric lipid kinases and are ubiquitously expressed in mammalian cells. In contrast to other isoforms, p110δ isoform is mainly expressed by leukocytes. By using mice with an inactivating knock-in mutation in the p110δ isoform (p110$\delta^{D910A}$) of PI3Ks, we are able to investigate the specific role of PI3Ks in immune system. In our study, we comprehensively evaluated the primary and secondary anti-*Leishmania* immune responses in p110$\delta^{D910A}$ mice.

# 1.8 Global Hypothesis

Parasite and host factors modulate primary and secondary immunity in experimental murine cutaneous leishmaniasis.

# 1.9 Overarching Goal & Specific Aims

The overarching goal is to determine how host and parasite factors contribute to the development, maintenance and loss of anti-Leishmania immunity.

**Specific Aims:**

1) Determine the role of phosphoglycan-containing molecules in modulating dendritic cell functions in terms of antigen presentation and cytokine production in primary *L. major* infection.

2) Investigate the influence of phosphoglycan-containing molecules in shaping adaptive immunity in primary *L. major* infection.

3) Determine the role of phosphoglycan-containing molecules in secondary anti-*Leishmania* (memory) responses.

4) Investigate the role of p110δ isoform of PI3Ks in primary *L. major* infection.

5) Determine the role of p110δ isoform of PI3Ks in regulating memory (secondary) anti-*Leishmania* immunity.

6) What in Quality of memory (secondary) anti-*Leishmania* immunity in healed p110δ$^{D910A}$ mice following secondary challenge

# CHAPTER II MATERIALS AND METHODS

## 2.1 Mice

C57BL/6 (B6) mice (wild type, WT), and CB-17/lcr-Prkdcscid/Crl (BALB/c SCID) were purchased from Charles River, St Constante PQ, Canada. Female BALB/c mice (WT) 6- to 8-wk-old were purchased from GMC, Central Animal Care Services (CACS), University of Manitoba, or from The Jackson Laboratory. B6.PL-Thy1a/CyJ (Thy1.1) mice were purchased from Jackson Lab, Bar Harbor, Maine. C57BL/6 mice that expressed an inactive form of p110δ isoform of PI3K (termed p110δ$^{D910A}$) were generated by introducing a point mutation into the P110 δ gene as previously described [177]. BALB/c p110δ$^{D910A}$ mice were generated by backcrossing B6 p110δ$^{D910A}$ mice unto BALB/c background (> 12 generations). BALB/c OVA TCR transgenic mice (DO11.10) mice were kindly provided by Dr. Xi Yang, Department of Medical Microbiology, University of Manitoba. All mice were maintained at the University of Manitoba Animal Care facility under specific pathogen-free conditions and used according to guidelines stipulated by the Canadian Council for Animal Care.

## 2.2 Parasites

*lpg1-, lpg2-, lpg2-*+LPG2 gene (Addbacks, *lpg2*AB) and lpg5A-/lpg5B- mutants were derivatives of the wild-type (WT) *L. major* strain LV39 clone 5 (Rho/SU/59/P), and were made by homologous gene targeting as described previously [19, 197-199]. Parasites

were cultured at 26$^o$C in M199 medium (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FBS (Cansera, Mississauga, ON, Canada), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies, Burlington, ON Canada). For the *lpg1-, lpg2-* and lpg5A-/lpg5B- lines, selective drugs were added to the culture media as appropriate for the selective markers used in their construction (puromycin, 15 µg/ml; G418, 15 µg/ml; hygromycin B, 20 µg/ml; blasticidin S, 15 µg/ml).

*Leishmania major* Fredlin (MHOM/80/Fredlin) strain parasites were grown in Grace's insect culture medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine, 100U/ml penicillin, and 100 g/ml streptomycin.

## 2.3 Infection protocol and parasite quantification.

For infection of mice, stationary phase promastigotes (7 days post-passage) were washed three times in cold PBS, resuspended in PBS at $10^8$/ml and 50 µl containing 5 x $10^6$ (C57BL/6) or 2 × $10^6$ (BALB/c) parasites was injected into the right or left hind footpad. Lesion sizes were monitored weekly by measuring footpad swellings with calipers. In some experiments, mice were infected by inoculating $10^6$ stationary phase parasites suspended in 10 µl cold PBS in the ear (intradermal). The lesion progression was also monitored weekly by measuring the diameter of ear induration with vernier calipers.

Parasite burden in the infected footpads was determined by limiting dilution assay[200]. In brief, mice were sacrificed and feet were cut off just above the ankle. Removed feet were placed in 2 ml of PBS with 2% Pen/Strep on ice for further use. First, foot was placed in 70% EtOH for 5 minutes. Then foot was transferred into chlorhexiderm

disinfectant for 5 minutes. Foot was washed in 70% EtOH then left in PBS with 2% Pen/Strep on ice. To obtain lesion material, the toes and ankle were removed with razor blade. Then an incision along the top of the foot was made to peel back the skin with the forceps. The peeled skin need to be removed with forceps. Place lesion/foot in tissue grinder with 1-2 ml of PBS with 2% Pen/Strep and grind. Transfer grinded tissue to 15 ml conical tube (B-D falcon 15ml/High clarity polypropylene conical tube, Ref 352096). Wash grinder with PBS with 2 ml 2% Pen/Strep, transfer to the same tube. Fill the tube to 15 ml with PBS with 2% Pen/Strep and spin at 500 rpm (50 g) for 5 minutes. Transfer the supernatant to another tube and spin at 3000 rpm for 15 minutes. The pellet was resuspended in 2 ml of complete Grace's or Schneider media. Seed the parasites in 96 well flat bottomed plates. Add 180ul of complete Grace's or Schneider media to each well. Add 20 ul of responded pellet into first well and log dilute. The parasite growth was visually assessed 5-7 days later under microscopes.

The parasite burden in the ear was quantified as described previously [214] with some modifications. In brief, the dorsal and ventral layers of the ear were separated with forceps and incubated for 30 min at 37°C in complete Grace's medium containing 1 mg/ml collagenase/dispase (Sigma-Aldrich). Thereafter, the ears were minced and homogenized in 2 ml tissue grinder and the homogenate was serially diluted (1:2) in 96-well flat bottom plates and incubated at 26°C. The number of viable parasites in each tissue was calculated from the highest dilution at which parasites were observed after 7 days.

## 2.4 Dendritic cell isolation from infected mice

After 3 days of infection, mice were sacrificed and CD11c+ DCs were isolated from the draining popliteal lymph nodes of mice infected with CFSE-labeled WT, lpg2- or lpg2AB *L. major* as described previously [75] with some modifications. Briefly, pooled popliteal lymph nodes (dLN) from 6-8 mice were cut into small pieces and digested at 37 $^{o}$C with 1 mg/ml collagenase D/Dispase (Roche Scientific, Laval, PQ, Canada) and 50 µg/ml DNase I (Sigma Aldrich, Mississauga, ON Canada) in 1 ml RPMI (Invitrogen) medium containing 2% fetal bovine serum (Cansera). Frequent mixing for 20 min was sufficient to disperse the cells, after which 600 µl of 0.1 M EDTA, pH 7.2, was added and stirred continuously for another 5 min to break up DC-T-cell conjugates. Undigested fibrous materials were removed by passing through a 70 µm cell strainer (Falcon, BD bioscience, Canada). The digested lymph node cell suspensions were counted and labeled with anti-CD11c antibodies coated microbeads and passed through autoMACS separator (Miltenyi Biotec, Auburn, CA) according to manufacturer's suggested protocols. The purity of the isolated DCs was between 90-95 %.

## 2.5 Bone marrow derived dendritic cells (BMDCs) and bone marrow derived macrophages (BMDM) generation, in vitro infection and stimulation

Bone marrow cells were isolated from the femur and tibia of 6-8 weeks old C57BL/6 or BALB/c mice. Briefly, after depletion of erythrocytes with ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA, pH 7.2-7.4), the cells were seeded in 100x15 mm Petri dishes (BD falcon, Ref 351029 ) at 2 x $10^5$/ml and differentiated in the presence of recombinant murine GM-CSF (20 ng/ml, Peprotech, Indianapolis, IN) at 37°C in $CO_2$

incubator. The culture media were changed twice on day 3 and on day 6, and on day 7, the non-adherent cells (dendritic cells) were collected for in vitro experiments. Immature DCs were assessed for the expression of CD11c, CD40, CD80, CD86 and MHC class II by flow cytometry. The purity of the cells was between 85-92% (CD11c+ cells).

The bone marrow cells were seeded in 100x15 mm Petri dishes (BD falcon, Ref 351029 ) at 5 x $10^5$/ml and differentiated into macrophages using complete RPMI-1640 medium supplemented with 30% L929 cell culture supernatant as previously described [201].

For infection, BMDCs or BMDMs were incubated with unlabeled or CFSE-labeled parasites for 5 hours at a BMDC or BMDM to parasite ratio of 1:10. The free parasites were washed away (3 times washing with complete media) and infected cells were cultured at 37 $^{o}$C for 24 hours. In some experiments, infected BMDCs or BMDMs were further stimulated with different concentrations of agonistic stimuli, including anti-CD40 mAb, IFN-γ and LPS for 24 hours. The ability of the cells to kill parasites was determined by counting Giemsa stained cytospin preparations under light microscope at 100x (oil) objective.

## 2.6 5- (6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling protocol

CFSE labeling protocol has been described previously [202]. Briefly, single cell suspensions from spleen or dLNs were counted with hemocytometer, then spin at 300 g for 5 minutes. The pellets were resuspended at 8 x$10^6$/ml in warm (prewarmed in 37 $^{o}$C waterbath) PBS. The CFSE stock solution (5 mM in DMSO) is diluted 1:2000 in warm PBS, then 1:1 mix with cell suspension. The final concentration of CFSE was 1.25 μM. The cell and CFSE

mixtures were kept in the dark with continuous rocking. After 5 min, staining was quenched with 4-5 ml heat-inactivated FBS and the cells were washed, counted, resuspended in complete medium and used for in vitro cultures.

For labeling parasites with CFSE, the stationary parasites were washed 3 times in warm PBS, resuspended at 40 x $10^6$/ml in PBS and mixed with equal volume of 1.6 μM CFSE for 5 min at room temperature.

## 2.7 Dendritic cells and T cells co-culture experiments

T cells were purified from the spleens of DO11.10 mice by positive selection using CD90.2 coated microbeads and autoMACS separator system according to manufacturer's suggested protocols (Miltenyi) and labeled with CFSE. The infected BMDCs were pulsed with full-length OVA protein (100 μg/ml) overnight, then co-cultured with CFSE-labeled DO11.10 cells at different T: DC ratio (ranging from 5:1, to 1000:1) for 4 days and cell proliferation and intracellular IFN-γ production were assessed by flow cytometry. In some experiments, infected DCs were co-cultured with LACK-specific CD4+ T cell hybridoma expressing I-Ad [203] (LMR-7.5, kindly provided by Dr Nicholas Glaichenhaus, (Institut National de la Sante et de la Recherche Medicale, Valbonne, France). After 3 days, proliferation and cytokine production were assessed.

## 2.8 In vitro recall response and intracellular cytokine staining

At various times after infection, dLNs were harvested and made into single cell suspensions. The cells were washed, resuspended at 4 x $10^6$ cells/ml in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), plated at 1 ml/well in 24-well tissue culture plates (Falcon) and stimulated with soluble *leishmanial* antigen [204] (SLA, 50 μg/ml). After 72 hr, the culture supernatants were collected and stored at –20 $^{o}$C until assayed for cytokines by ELISA. Some cells were used for intracellular cytokine staining as previously described [196]. Briefly, cells were stimulated with 20 ng/ml PMA, 1μM Ionomycin, and 10 μg/ml Brefeldin A (all from Sigma), for 4-6 hours before surface staining. Surface stained cells were washed and fixed with 2% paraformaldehyde for 10 min, then permeabilized with 0.1% saponin (Sigma) in staining buffer for another 10 min. The cell pellets were stained with specific fluorochrome-conjugated mAbs against IFN-γ (XMG1.2), IL-4 (11B11), IL-10 and TNF (Biolegend, San Diego, CA). Samples were acquired on either a FACSCalibur or a FACSCanto II flow cytometer (BD Bioscience, Mississauga, ON, Canada) and analyzed with FlowJo software (TreeStar).

## 2.9 Cytokine ELISA

Three days after culture, the supernatant fluids were collected and assayed for cytokines (IFN-γ, TNF, IL-4 IL-10 and IL-12) by sandwich ELISA using the following Ab pairs from BioLegend as follows: IL-12p40, C15.6 and C17.8; IL-10, JES5-16E3 and JES5-2A5; IFN-γ, R4-6A2 and XMG1.2; IL-4, 11B11 and BVD6-24G2. The sensitivities of the ELISA are as follows: IL-4, 15 pg/ml; IL-10, 15 pg/ml; IL-12, 64 pg/ml, and IFN-γ, 30 pg/ml).

# 2.10 Real time PCR assay

Three days post-infection, total RNA was extracted from draining lymph node cells using RNA Stat-60 (Tel-Test, Friendswood, TX), according to manufacturer's specifications and reverse transcribed into cDNA using the RT-transcription kit (Gibco-BRL, Gaithersburg, MD). Real-time PCR reactions were performed with the Smart Cycler II system (Cepheid, Sunnyvale, CA). PCR cycling conditions included 2 min at 50°C, 10 min at 95°C then 40 cycles of 5 sec at 95°C and 1 min at 60°C. The quantitative PCR was performed according to the manufacturer's protocol using the pre-developed TaqMan assay reagents for murine IL-4, IL-10, IL-12p40 and IFN-γ genes and the SYBR Green Master mix (SuperArray, Frederick, MD). Differences in starting amount of RNA or cDNA were corrected by normalizing with β-actin RNA (housekeeping gene). Data were analyzed with the Smart Cycler software v2.0 (Cepheid). The results are expressed as the relative increase in gene expression in infected animals over the uninfected ones.

***Real Time PCR for Blimp-1 expression***

Splenic T cells from WT and p110$\delta^{D910A}$ mice were purified using T cell enrichment kit according to manufacturer's suggested protocols (StemCell Technologies, Seattle, WA). Purified T cells were stimulated *in vitro* for 3 days with plate-bound anti-CD3 mAb (BD Biosciences) in the presence of soluble anti-CD28 and rIL-2 (50 U/ml, BD Bioscience). Total cellular RNA was extracted from stimulated T cells using Trizol reagent (Invitrogen) and cDNA was synthesized from 1 μg of RNA using SuperScript II Reverse Transcriptase (Invitrogen). RT-PCR was performed using LightCycler System (Roche Diagnostics, Laval,

PQ, Canada). The primers for Blimp-1 were selected according to previously published report [205].

## 2.11 In vitro proliferation assays.

To measure proliferation, cells labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) as previously described [206]. The cells were resuspended at $10^6$/ml, plated onto 96-well round bottom plates and stimulated with SLA or anti-CD3 and anti-CD28. In some experiments, CFSE labeled cells were co-culture with infected BMDCs at different ratios. After 5 days, proliferation was analyzed by flow cytometry.

## 2.12 Serum antibody ELISA and treatment with anti-*Leishmania* immune serum.

Purified IgG, IgG1 and IgG2a standards are purchased from BD Bioscience, and the starting concentration for IgM and IgG1 standards is 20 ng/ml. Serial dilution of serum samples begins at $1:10^6$ for WT, at $1:10^5$ for p110$\delta^{D910A}$ mice to detect total IgG.

| | **Capture antibodies** | **Detection antibodies** |
|---|---|---|
| Total IgG | 1 μg/ml | 1 μg/ml |

For determination of *Leishmania*-specific IgG1 and IgG2a, ELISA plates were coated with $10^7$ freeze/thaw killed parasites in 100ul volume of PBS overnight. Serum samples were added to the wells by serial dilution from $1:10^5$ (WT mice) or $1:10^4$

(p110δ$^{D910A/D910A}$ mice). Same biotinylated mAbs for measuring total Igs are used for detection.

At sacrifice, immune serum was obtained from infected mice and used to determine the levels of anti-*Leishmania*-specific antibody titers by ELISA as previously explained [207]. At 2 and 3 weeks post-infection, WT or p110δ$^{D910A}$ mice were injected i.p. with pooled serum (500 μl per mouse per injection) from naïve or 6 week old *L. major* infected mice.

## 2.13 Purification of T cell subsets and adoptive transfer experiments.

Thy1.2+, CD4+CD25+ and CD4+CD25- T cells were purified from pooled spleen and lymph node cells from naïve or infected mice by using autoMACS column and antibodies according to manufacturer's suggested protocols (Miltenyi Biotec, Auburn, CA). Recipient mice were subsequently infected with *L. major* the next day. In some experiments, SCID BALB/c mice were reconstituted with 10 million Thy1.2+ cells from WT or p110-/- BALB/c mice and infected the next day with *L. major*.

Healed WT and p110δ$^{D910A}$ mice (on Thy 1.2 background) were sacrificed after 10-14 wk and single cell suspensions from the draining (popliteal) lymph nodes and spleens were made. T (Thy1.2+) cells were enriched by depleting non-T cell fractions using autoMACS column and antibodies according to manufacturer's suggested protocols (Miltenyi Biotec, Auburn, CA). Enriched T cells (> 98% pure) were labeled with CFSE dye and 10 million cells were adoptively transferred into naïve congenic (Thy1.1) mice by tail

vein injection. In some experiments, CFSE-labeled cells were further separated into CD62Lhi and CD62Llo fractions by autoMACS (> 98% purity). Twenty-four hr after cell transfer, the recipient mice were challenged with 2 x$10^6$ *L. major* parasites. At 5 and 14 days post-challenge, mice were sacrificed to examine cell proliferation, homing to lymph nodes and site of infection, CD62L expression, IL-2 and IFN-γ production by gating on Thy1.2+ (donor) cells.

## 2.14 Isolation of cells from infected footpads

To determine the numbers (percentage) and subsets of cells recruited into the challenged footpads, mice were sacrificed and the footpads were removed with scissors and then dipped into 70% ethanol for 5 minutes. After peeling off the skin, the footpads were homogenized gently with tissue grinders. The crude homogenized tissues were resuspended in 7 ml of cold PBS and were carefully layered on top of 5 ml Ficoll, then centrifuged according to manufacturer's suggested protocols for cell separation from tissue homogenates. The interface of Ficoll and PBS was collected carefully and transferred into 50 ml centrifuge tube. After washing with complete DMEM, the cells were resuspended in 5 ml complete medium, counted by using hemocytometer, stained directly for expression of various cell surface molecules and analyzed by flow cytometer.

## 2.15 Flow cytometry analysis for intracellular cytokines

The donor cells were analyzed for intracellular cytokine staining as previously described [196]. Briefly, cells were directly stimulated ex vivo with 50 ng/ml PMA, 500

ng/ml Ionomycin, and 10 μg/ml Brefeldin A (all from Sigma-Aldrich, Oakville, ON, Canada) for 4 hr before surface staining. Fixed and surface-stained cells were permeabilized with 0.1% saponin (Sigma-Aldrich) in staining buffer and then stained with specific fluorochrome-conjugated mAbs (Biolegend) against IFN-γ and IL-2. Samples were acquired on a FACSCanto II (BD Biosciences, Mississauga, ON, Canada) and analyzed using Flowjo software (Treestar Inc.).

## 2.16 Statistical analysis

Results are shown as the mean ± SEM. A two-tailed Student's t-test was used to compare means of lesion sizes, parasites burden, and cytokine production from different groups of mice. Significance was considered if $p \leq 0.05$.

# CHAPTER III Regulating anti-*Leishmania* Primary Immunity by LPG2 Gene Products

## Specific Introduction

*Leishmania* species are the causative agents of the zoonotic disease *leishmaniasis,* which is prevalent in six continents and considered endemic in 88 countries. According to WHO, more than 12 million people are currently suffering from *leishmaniasis* and a population of over 350 millions are at risk of contracting the disease [208, 209]. Human cutaneous *leishmaniasis* is caused by members of the *L. major* subspecies and is the most common form of the disease. Various forms of pentavalent antimonial components are used for treatment of human *leishmaniasis*, but treatment failures and drug resistances are common [210, 211]. Therefore, there is a critical need for new drugs and drug targets, as well as vaccines.

The outer membrane of *Leishmania major* is covered by a dense glycocalyx consisting predominantly of lipophosphoglycan (LPG) and other phosphoglycan (PG)-containing molecules including proteophosphoglycans (PPG), and a heterogeneous group of glycoinositolphospholipids (GIPLs) [15, 212]. These molecules are thought to be important virulence factors and play key roles in entry, survival and proliferation of parasites inside host cells [213-216]. *In vitro* and *in vivo* studies using purified parasite molecules have identified LPG as a multifunctional virulence factor required for the establishment of

infections [15, 19]. The roles ascribed to LPG by *in vitro* testing include inhibition of complement lysis, inhibition of phagolysosome fusion, modulation of macrophage signal transduction pathways and down-regulation of the expression of inducible nitric oxide synthase and synthesis of IL-12 [15, 18, 213, 214, 217, 218]. However, because of close structural similarities of the glycoconjugate molecules [15, 212], there is a strong potential for cross activity of shared domains between LPG and other molecules. Moreover, these studies explore LPG in an artificial context, rather than in the natural membranous context during parasite infection [15].

To overcome these limitations, we and others have used targeted gene inactivation to generate mutants which specifically lack the expression of one or more glycoconjugates [19, 20, 33, 219, 220]. This allows dissection of the role of these molecules in virulence in the context of the parasite *in situ*, and their influence on the host immune response as well. For example, *L. major* mutants generated by deleting the *LPG1* gene encoding the putative galactofuranosyl transferase involved in the biosynthesis of the LPG core glycan specifically lack LPG [221]. *lpg1*$^{-}$ *L. major* shows deficiencies in the initial steps of the infectious cycle including complement resistance, phagolysosomal fusion, and macrophage survival [19, 222]. However, those parasites that go on to survive can form amastigotes that are still virulent *in vivo* [19], consistent with the observation that amastigotes do not express LPG. In contrast, *lpg2*$^{-}$ parasites, generated by deleting the *LPG2* gene encoding the transporter required for GDP mannose uptake into the Golgi lumen, lack both LPG and other PG-containing molecules [223], and other potential LPG2-dependent metabolites [220]. These parasites are highly attenuated *in vivo,* showing deficiencies in the early steps of promastigote survival, and fail to induce any overt pathology in infected mice [20].

Based on the analysis of known glycoconjugates, the virulence defect of *lpg2⁻ L. major* has been attributed to its clear PG deficiency by most researchers. However, our recent studies of another PG-deficient *L. major*, obtained through inactivation of the UDP-Gal transporters encoded by the *LPG5A* and *LPG5B* genes (*lpg5A⁻/lpg5B⁻*), question this assumption. Unexpectedly, the *lpg5A⁻/lpg5B⁻* mutant resembled the *lpg1⁻* rather than the *lpg2⁻* parasites, by displaying a virulence defect when tested as promastigotes, but showing normal virulence as amastigotes [199, 220]. This suggests that a loss of *LPG2*-dependent glycoconjugates, other than PGs, may underlie the virulence defect of *lpg2⁻ L. major*. These data likewise raise the possibility that PG-deficiency alone may not be responsible for alterations in the immune response seen in *lpg2⁻* infections [22].

We previously showed that vaccination with *lpg2⁻ L. major* protects mice against virulent WT challenge [22]. In that report, we speculated the protected effect of *lpg2⁻* parasites could be related to their differential interaction with cells of the immune system (including dendritic cells) early after infection. Here we have investigated the early host innate and adaptive immune responses to *lpg2⁻* parasites and compared them to those induced by wild type (WT), *lpg2⁻*+LPG2 gene (add-backs) and *lpg1⁻* parasites. Our results show that the outcome of *lpg2⁻* interaction with dendritic cells (DCs) and the host early immune response against these mutants are qualitatively and quantitatively different from those of WT *L. major*. Specifically, we show that although *lpg2⁻ L. major* infection does not up-regulate the expression of costimulatory molecules on DCs, it significantly enhanced their production of IL-12, thereby altering the host early immune response such that ratio IL-4 and IFN-γ production by the responding T cells is altered. Thus, in response to infection by

*lpg2*$^-$ *L. major*, the antigen-presenting abilities of DCs are skewed towards a more effective Th1 response.

# Results

## WT and *lpg2⁻ L. major* induce similar pattern of expression of costimulatory molecules but differential IL-12 production in BMDCs

We previously showed that similar to WT, *lpg2⁻ L. major* can invade macrophages but are progressively destroyed beyond 24 hrs [20] . Dendritic cells (DCs), which are among the first cells infected by *Leishmania* [224, 225], are the most efficient antigen presenting cells and hence are essential for inducing an efficient anti-*Leishmania* adaptive immune response [226]. To investigate the possible effect of *LPG2*-dependent molecules on DC function, we generated bone marrow-derived dendritic cells (BMDCs) and determined their expression of costimulatory molecules after infection with WT and *lpg2⁻ L. major*. In our initial experiments, at all times tested, there was no difference in the expression (percent expression and mean fluorescence intensities [MFI]), of MHC class II, CD40, CD80 and CD86 molecules by BMDCs infected with WT or *lpg2⁻*. However, because the infectivity of the DCs in our system was low (~47-54% for WT, *lpg2*AB and *lpg2⁻*, **Fig. 5A**), we considered the possibility that moderate changes in expression of these molecules by infected cells may be masked by normal or enhanced expressions in uninfected cells. Therefore, we infected the cells with CFSE-labeled parasites, and analyzed the expression of costimulatory molecules on only infected ($CFSE^{high}$) DCs. The percentage of WT, *lpg2*AB

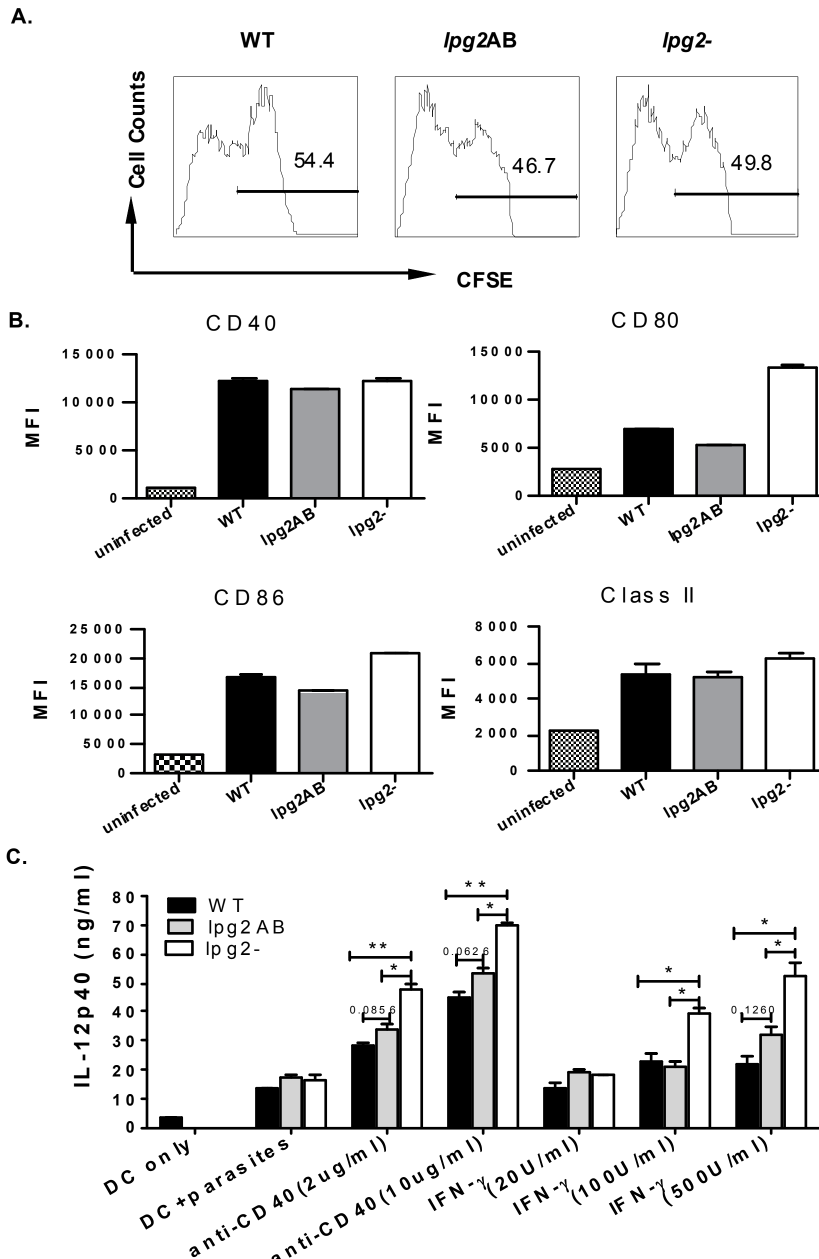



**FIGURE 5.** *L. major* glycoconjugate molecules do not regulate the expression of costimulatory molecules but influences IL-12 production by DCs.

Bone marrow-derived dendritic cells (BMDCs) were infected with CFSE-labeled wild type (WT), *lpg2*-+LPG2 gene (Add-back, *lpg2*AB) and *lpg2* deficient (*lpg2*-) *L. major* stationary phase promastigotes (BMDC-parasite ratio = 1:10) and the rate of infection was determined after 24 hours by gating on infected cells (CD11c+/CFSE+ cells) **(A)**. The expression of MHC class II, CD40, CD80 and CD86 on infected (CFSE+) on total (CD11c+) DCs was determined by flow cytometry. Data are presented as percent mean fluorescence intensity (MFI) of gated infected cells **(B)**. Some uninfected and infected BMDCs were stimulated with varying concentrations of anti-CD40 and IFN-γ for 24 hours and the culture supernatant fluids were collected and assayed for IL-12p40 by ELISA **(C)**. Data presented are representative of 3 independent experiments with similar results. * $p < 0.05$, ** $p<0.01$.

or *lpg2*$^-$-infected (CFSE$^{high}$) DCs was comparable (**Fig. 5A**), suggesting that the loss of cell surface glycoconjugates did not affect uptake of *lpg2*$^-$ parasites by DCs. By focusing only on infected DCs, there was still no difference in expression (mean fluorescence intensity, MFI) of these molecules by WT and *lpg2*$^-$ *L. major*-infected BMDCs (**Fig. 5B**). These data suggest that expression of *LPG2*-dependent glycoconjugates does not greatly affects the expression of costimulatory molecules on DCs.

IL-12 produced by DCs also plays a critical role in the development of $CD4^+$ Th1 cells and resistance to *L. major* infection in mice [226-229]. Thus, we measured the production of IL-12p40 by BMDCs after 24 hours of infection. *lpg2*$^-$ *L. major*-infected BMDCs produced significantly ($p < 0.05$ to 0.01) more IL-12p40 than those infected with WT parasites (**Fig. 5C**) following stimulation with several IL-12-inducing agonists, including anti-CD40 mAb, IFN-γ and LPS (**Fig. 5C**, and Supplementary Fig. A). Cells infected with AB parasites showed a pattern of cytokine production similar to those infected with WT parasites (**Fig. 5C**). Interestingly, we were unable to detect significant levels of IL-12p70 subunit by ELISA despite the high levels of IL-12p40 subunit as has been previously reported [230, 231]. Taken together, these results indicate that although infection with *lpg2*$^-$ *L. major* did not lead to significant alteration in the expression of co-stimulatory molecules on DCs, they are capable of positively enhancing their secretion of IL-12p40, a critical Th1-inducing cytokine.

## Freshly isolated (ex vivo) DCs from WT and *lpg2*⁻-infected mice express similar levels of costimulatory molecules

Since we were unable to demonstrate differential effects of WT and *lpg2*⁻ parasites on the expression of costimulatory molecules on infected DCs *in vitro*, we asked whether similar effects occurred *in vivo*. We infected mice in the footpad with CFSE-labeled WT, *lpg2AB* and *lpg2*⁻ *L. major* and after 3 days purified DCs from pooled popliteal lymph nodes draining the infection site (dLNs) and analyzed their number, subsets and expression of costimulatory molecules. Labeling with CFSE allowed us to determine the percentages of infected DCs and also to focus our assessment of costimulatory molecule expression on only these infected cells. As expected, infection with both parasite lines led to significant increase in the number of cells recovered from the dLN (**Fig. 6A**). However, there was no significant difference in the total numbers of cells (**Fig. 6A**), $CD11c^{+}$ (**Fig. 6B**) or $CFSE^{high}$ (i.e. infected, **Fig. 6C**) DCs recovered from the dLNs of mice infected with either parasite line, suggesting that the deficiency of PGs does not impair the early inflammatory response, migration and/or expansion of cells (including DCs) into the dLNs. Consistent with the *in vitro* findings, we found no difference in the expression of costimulatory molecules (MHC II, CD40, CD80 and CD86) by directly *ex vivo* total or infected ($CFSE^{high}$) DCs isolated from mice infected with WT, *lpg2AB* or *lpg2*⁻ parasites (**Fig. 6D**). Because the relative expression of CD8α on DCs may affect their ability to present *Leishmania* antigens and activate *Leishmania*-specific T cells [224, 232], we determined whether infection with *lpg2*⁻ affected the expression of this subpopulation of DCs. There was no difference in percentage or mean fluorescence intensity (**Fig. 6E**) of

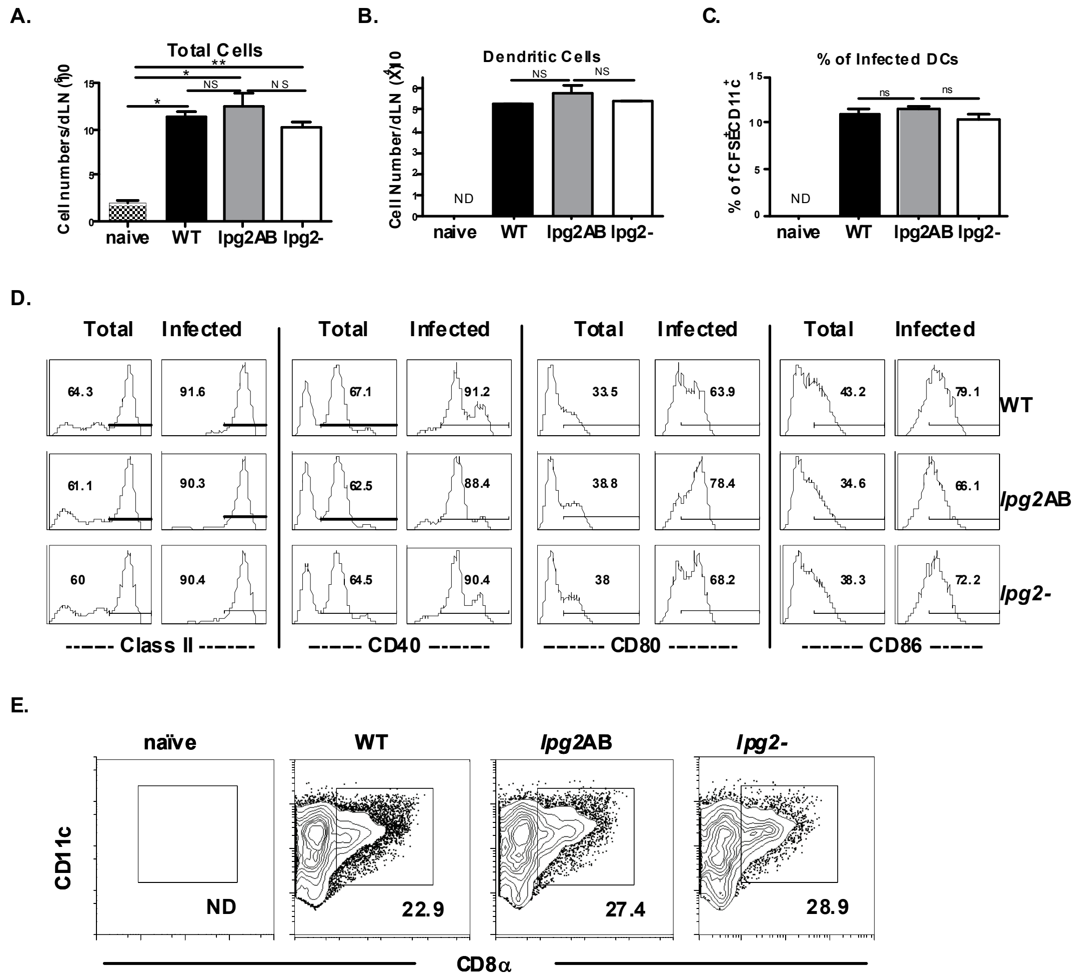



**FIGURE 6.** The glycoconjugate molecules of *L. major* do not modulate DC activation in vivo.
BALB/c mice infected in the footpad with CFSE-labeled WT, Add-back or *lpg2- L. major* were sacrificed at 3 days and the dLNs were collected, digested with collagenase and the total number of cells was enumerated by direct cell count using a hemocytometer **(A)**. DCs were isolated from pooled dLN cells by positive selection using autoMACS column, and stained routinely with fluorochrome-labeled anti-CD11c mAb and other various cell surface markers (CD40, CD80, CD86 and MHC II). The stained cells were analyzed by flow cytometry to enumerate the total number of DCs i.e. CD11c+ cells **(B)**, or percentage of infected cells (i.e. $CFSE^{high}$CD11c+ cells **(C)**. The expression of MHC class II, CD40, CD80 and CD86 **(D)** and CD8$\alpha$ molecules **(E)** on both infected ($CFSE^{high}$) and total DCs were analyzed after gating on CD11c+ cells. Data presented are representative of 3 (A-C) and 2 (D) independent experiments with similar results. ND = not done. * $p < 0.05$, ** $p < 0.01$.

CD8α-positive DCs isolated from dLNs of *lpg2*⁻ and WT *L. major*-infected mice. Together with the *in vitro* data, these results show that infection with *lpg2*⁻ *L. major* does not significantly alter the expression of costimulatory molecules on DCs relative to that seen with WT *L. major*.

## DCs from *lpg2*⁻ *L. major*-infected mice are more efficient antigen-presenting cells than those from WT *L. major*-infected mice

IL-12 is a critical cytokine that skews the development of naïve CD4+ T cells into Th1 cells. To determine if the higher IL-12 production by *lpg2*⁻-infected DCs affect their ability to activate naïve T cells, we pulsed freshly isolated DCs from 3 day *lpg2*⁻ and WT *L. major*-infected mice with whole-length OVA protein overnight, and then co-cultured them *in vitro* with CFSE-labeled OVA-specific (DO11.10) T cells for 4 days. As shown in **Fig. 7A and B**, DCs from mice infected with *lpg2*⁻ *L. major* stimulated OVA-specific T cells to produce higher amounts of IL-2 and IFN-γ than those from WT-infected controls. Interestingly and consistent with lack of differences in the expression of costimulatory molecules (**Figs. 5B and 6D**), there was no significant difference in antigen-specific proliferation of OVA-specific T cells following presentation with OVA by DCs from mice infected with either parasite (**Fig. 7A**).

To determine the relevance of the enhanced IFN-γ production by DO11.10 T cells following OVA presentation by freshly isolated DCs from *lpg2*⁻-infected mice, we compared the ability of freshly isolated DCs from WT or *lpg2*⁻-infected mice to activate *Leishmania* specific T cell hybridoma, LMR7.5, *in vitro* [233]. LMR7.5 cells

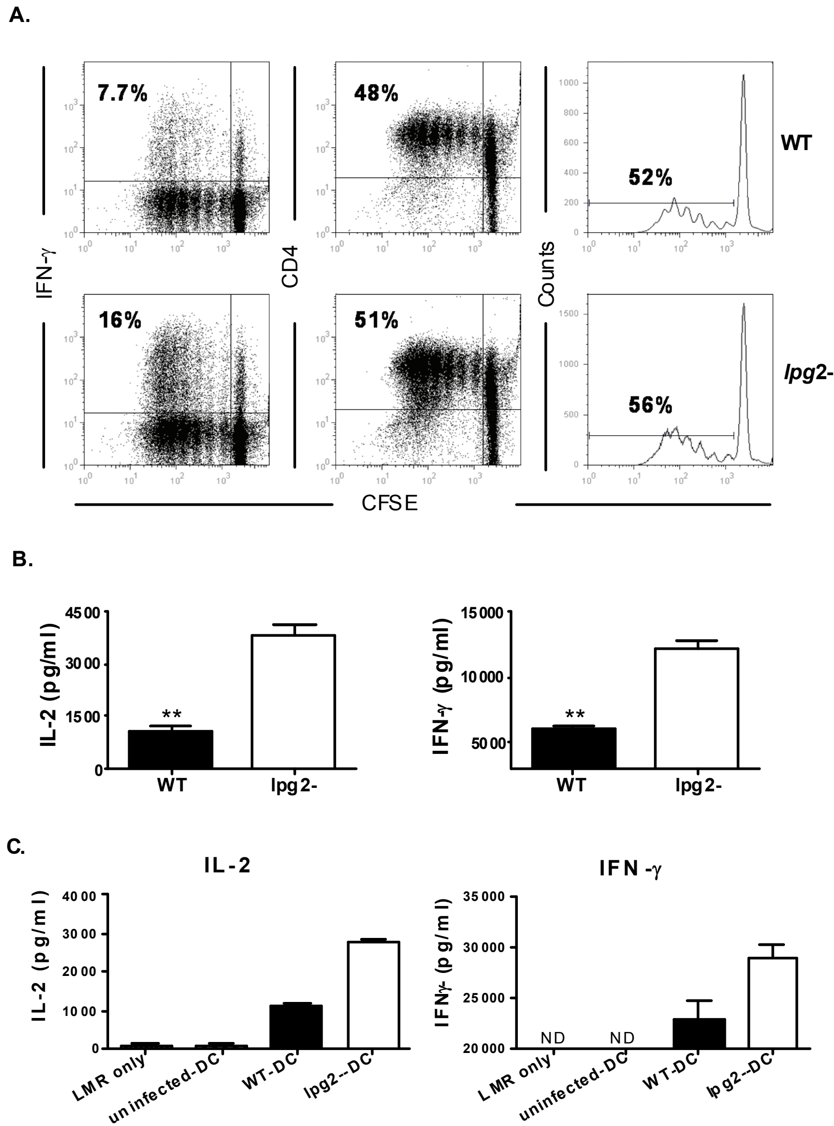



**FIGURE 7.** More efficient induction of IL-2 and IFN-γ responses by DCs from *lpg2- L. major*-infected mice.

DCs isolated from BALB/c mice infected for 3 days with WT or *lpg2- L. major* were pulsed overnight with full-length OVA protein (100 mg/ml) and then co-cultured with purified CFSE-labeled CD4+ T cells from DO11.10 transgenic mice. After 4 days, proliferation and IFN-γ production was determined by flow cytometry **(A)**. The culture supernatant fluids were collected and assayed for IL-2 and IFN-? by ELISA **(B)**. Some purified DCs were co-cultured with LACK-specific T cell hybridoma (LMR 7.5) for 72 hours and the culture supernatant fluids were assayed for IL-2 and IFN-γ by ELISA **(C)**. Data presented are representative of 3 (A and B) and 2 (C) independent experiments (n = 4-5 mice/group, A and B) with similar results. * $p < 0.05$, ** $p < 0.01$.

co-cultured with DCs from *lpg2*$^-$-infected mice produced more IL-2 and IFN-γ than those stimulated with cells from WT-infected mice (**Fig. 7C**). Taken together, these results indicate that the absence of glycoconjugate molecules influences the expression of costimulatory molecules and in the process modulates the antigen-presenting functions of dendritic cells.

## *L. major* glycoconjugates regulate the host early immune response.

We previously showed that despite parasite persistence, the effector T cell response in mice infected with *lpg2*$^-$ parasites for 4 weeks is weak [22]. This suggests that the early immune response in *lpg2*$^-$-infected mice may be defective. Here we examined the early immune response induced by *lpg2*$^-$, *lpg2*AB and WT parasites in the first few days post-infection. The number of cells in the dLN of mice infected with *lpg2*$^-$ increased at 24 hours, reaching peak at 3 days post-infection, and were comparable to those seen in mice infected with WT. However, from day 7 post-infection, the cell numbers began to decline in *lpg2*$^-$-infected mice, while the number of cells in dLNs from WT-infected mice was sustained (**Fig. 8A**). Interestingly and consistent with similar infectivity seen in DCs (**Fig. 6C**), similar numbers of WT and *lpg2*$^-$ parasites were recovered at the site of infection on day 3 and 7 post-infection (**Fig. 8B**), suggesting that *lpg2*$^-$ parasites were not rapidly destroyed by the innate immune system.

Next, we determined the early antigen-specific cytokine production by dLNs following stimulation with soluble *Leishmania* antigen (SLA). Although the antigen-specific proliferation of dLN cells from *lpg2*$^-$ and WT *L. major*-infected mice was comparable at 3 days post-infection (**Fig. 8C**), similar to that seen by *ex vivo* DC stimulation of DO11.10 T

cells (**Fig. 7A**), cytokine production was dramatically different. Intracellular cytokine staining showed that the numbers of IFN-γ -producing cells were comparable, whereas the numbers of IL-4- (and to a lesser degree IL-10) producing cells in the dLNs from mice infected with *lpg2*⁻ were lower than those from WT-infected mice (**Fig. 8D**). Interestingly, most of the IL-4 producing cells also co-produced IL-10 (Supplementary Fig. B), an important disease promoting cytokine in experimental cutaneous *leishmaniasis*. The significant reduction in IL-4 production skewed the IFN-γ/IL-4 ratio ($12.5 \pm 4$ vs. $36.6 \pm 9.3$, $p < 0.01$, for WT and *lpg2*⁻ parasites, respectively). As expected and consistent with our previous report [22], the pattern of cytokine production by dLN cells from mice infected with *lpg2*⁻ parasites complemented with the *LPG2* gene (*lpg2*⁻/+*LPG2, lpg2AB*) was similar to those infected with WT parasites (**Fig. 8D**), indicating that decreased IL-4 response by *lpg2*⁻ parasites is related to deficiency of PGs and not a result of the genetic manipulation processes. This pattern of intracellular cytokine response (normal IFN-γ and low IL-4 in *lpg2*⁻-infected mice) was also confirmed by ELISA (**Figs. 8F**). Furthermore, cells from mice infected with *lpg2*⁻ parasites produced more IL-12p40 than those from WT *L. major*-infected mice (**Fig. 8E**). By day 14 post-infection, the production of IFN-γ and IL-4 was dramatically reduced in *lpg2*⁻-infected mice (**Figs. 8G**). This sharp decline in cytokine production parallels the decline in parasite numbers in the footpads of *lpg2*⁻-infected mice, suggesting that parasite number is directly correlated with the magnitude of immune response in infected mice.

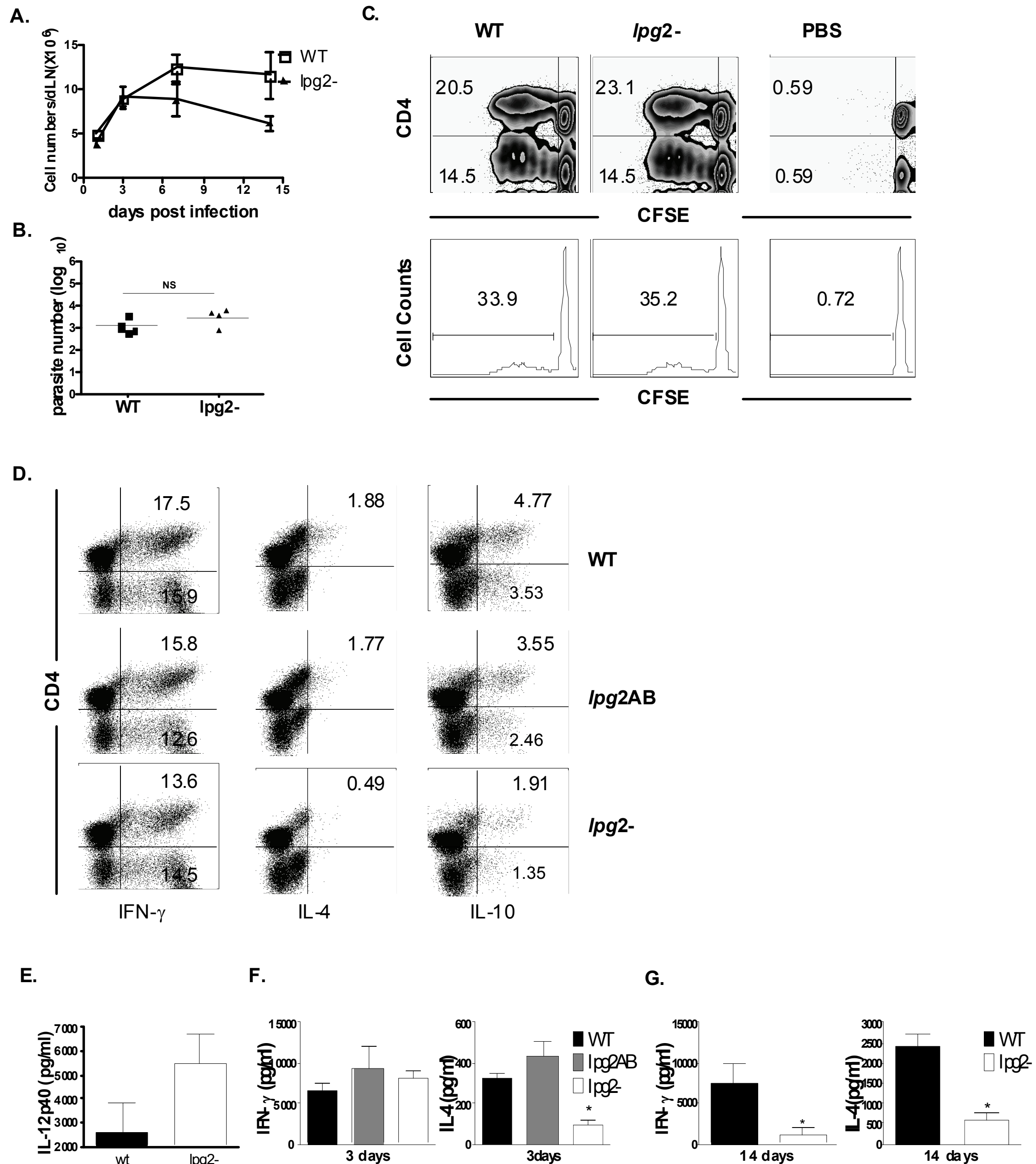



**FIGURE 8.** Modulation of the host early immune response by *L. major* glycoconjugate molecules. BALB/c mice infected in the footpad with WT, *lpg2*AB and *lpg2- L. major* were sacrificed at the indicated days and the number of cells in the dLN was enumerated by direct cell count **(A)**. Parasite burden in the infected footpad was determined by limiting dilution **(B)**. Some cells were labeled with CFSE dye and stimulated in vitro with SLA (50 mg/ml) for 3 days and proliferation was assessed by flow cytometry **(C)**. After 3 days of culture, some cells were stained for intracellular production/expression of IFN-γ, IL-4 and IL-10 and analyzed by flow cytometry **(D)**. The cell culture supernatants were collected and assayed for cytokines (IL-12p40, IL-4 and IFN-γ) by ELISA **(E-G)**. Data presented are representative of 3-5 independent experiments (n = 3-5 mice/group) with similar results. * $p < 0.05$.

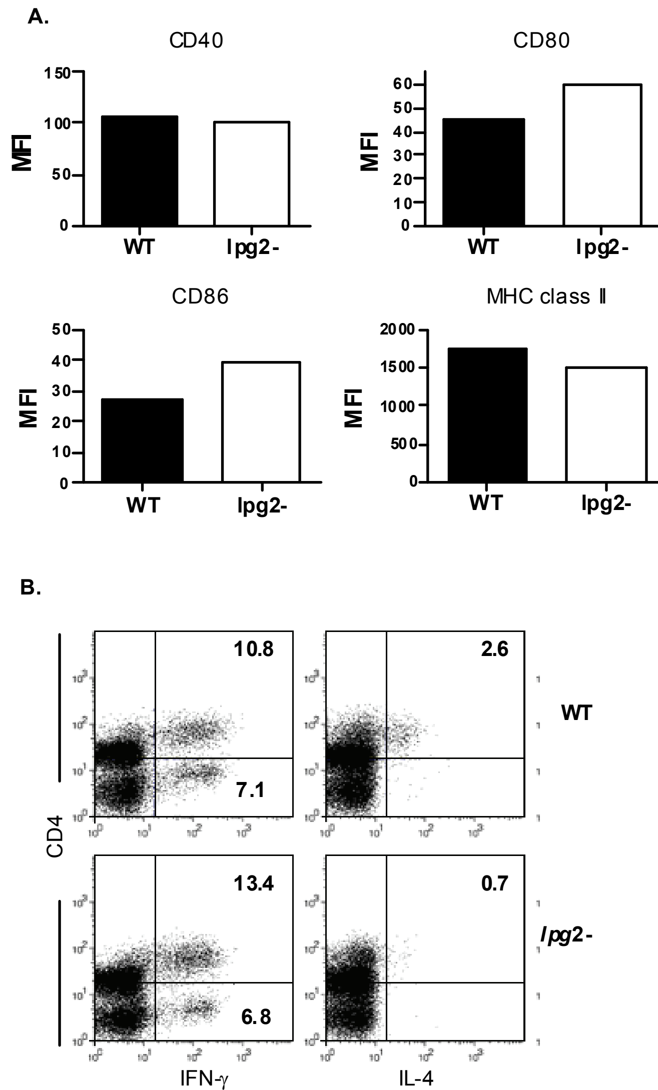



**FIGURE 9.** The immunomodulatory effect of *L. major* glycoconjugate molecules is independent of site of infection.

BALB/c mice infected in the ear (intradermal) with 5 million WT or *lpg2-* L. major were sacrificed after 3 days and CD11c+ DCs were purified from pooled draining lymph node (dLN) cells, stained for surface expression of MHC class II, CD40, CD80 and CD86 molecules and analyzed by flow cytometry **(A)**. Some unfractionated dLN cells were stimulated with SLA for 3 days and the percentage of IFN-γ- and IL-4-producing cells was determined by intracellular cytokine staining **(B)**. Data presented are representative of 2 independent experiments (n = 4-5 mice/group) with similar results.

As the route of infection with *L. major* influences the nature of the immune response [103, 234], we investigated whether modulation of DC function and early host immune response by *lpg2*$^-$ *L. major* is a general phenomenon that is independent of route of infection. Therefore, we assessed the early immune response in mice infected intradermally in the ear with *lpg2*$^-$ and WT *L. major*. As shown in **Fig. 9A and B**, the pattern of DC activation and early cytokine response was similar to that observed after subcutaneous (footpad) infection, such that cells from *lpg2*$^-$-infected mice expressed equivalent (MHC II, CD40 and CD86) or slightly higher (CD80) levels of costimulatory molecules and produced less IL-4 than those from WT-infected mice. Taken together, these results show that infection with *lpg2*$^-$ parasites alters the host early immune response towards a more robust Th1 phenotype.

## Deficiency of PGs may contribute to the differences in the early immune response in mice infected with *lpg2*$^-$ *L. major*

As summarized in the introduction, *lpg2*$^-$ parasites lack all PGs including those attached to LPG and proteophosphoglycans (PPGs), which exist in both secreted and membrane bound forms through out the parasite infectious cycle. However, recent data showed that PG deficiency is not responsible for the amastigote virulence defects of the *lpg2*$^-$ mutant [220], likewise raising the possibility that PG-deficiency alone may not be responsible for alterations in the early immune response seen in *lpg2*$^-$ infections. To further probe the nature of the molecule(s) responsible for the LPG2-dependent effects on DCs *in vitro* and *in vivo* (seen above), we made use of additional *L. major* mutants deficient in

various PGs through inactivation of other LPG genes, and differing in virulence from WT or *lpg2*$^-$ parasites.

*lpg1*$^-$ deficient parasites specifically lack LPG but maintain WT levels of other PGs such as PPG [19]. *In vivo*, *lpg1*$^-$ infection show defects in the initial phases of macrophage establishment, but parasites that survive go on to form fully virulent amastigotes able to cause progressive lesions in susceptible BALB/c mice [19]. As shown in **Fig. 10A**, there was no significant alteration in the expression of costimulatory molecules on DCs from mice infected for 3 days with WT, *lpg1*$^-$ or *lpg2*$^-$ *L. major*. In contrast, whereas the early cytokine responses induced by *lpg1*$^-$ parasites were similar to those of WT parasites (no decrease in IL-4 and IL-10 responses), the frequency of IL-4- and IL-10-producing cells from mice infected with *lpg2*$^-$ parasites were reduced (**Fig. 10B)**, consistent with previous results (see **Figs. 8D and 9B**).

To confirm whether the decreased IL-4 response following infection with *lpg2*$^-$ *L. major* was due to of lack of phosphoglycans (PG) or related to PG-associated loss of virulence, we examined IL-4 production in infections by the *lpg5A*$^-$*/lpg5B*$^-$ *L. major* mutant (LPG5 KO), which lacks all PGs due to inactivation of the parasite's Golgi UDP-Gal transporters [199], yet show virulent characteristics similar to *lpg1*$^-$ *L. major* [199, 220]. As shown in **Figs. 10C and D**, both IL-4 message and protein were suppressed 2-3 fold in mice infected with *lpg2*- and LPG5 KO *L. major*, suggesting that the absence of PG molecules and not lack of virulence was primarily responsible for the decrease in IL-4 response in mice infected with *lpg2*$^-$ *L. major*.

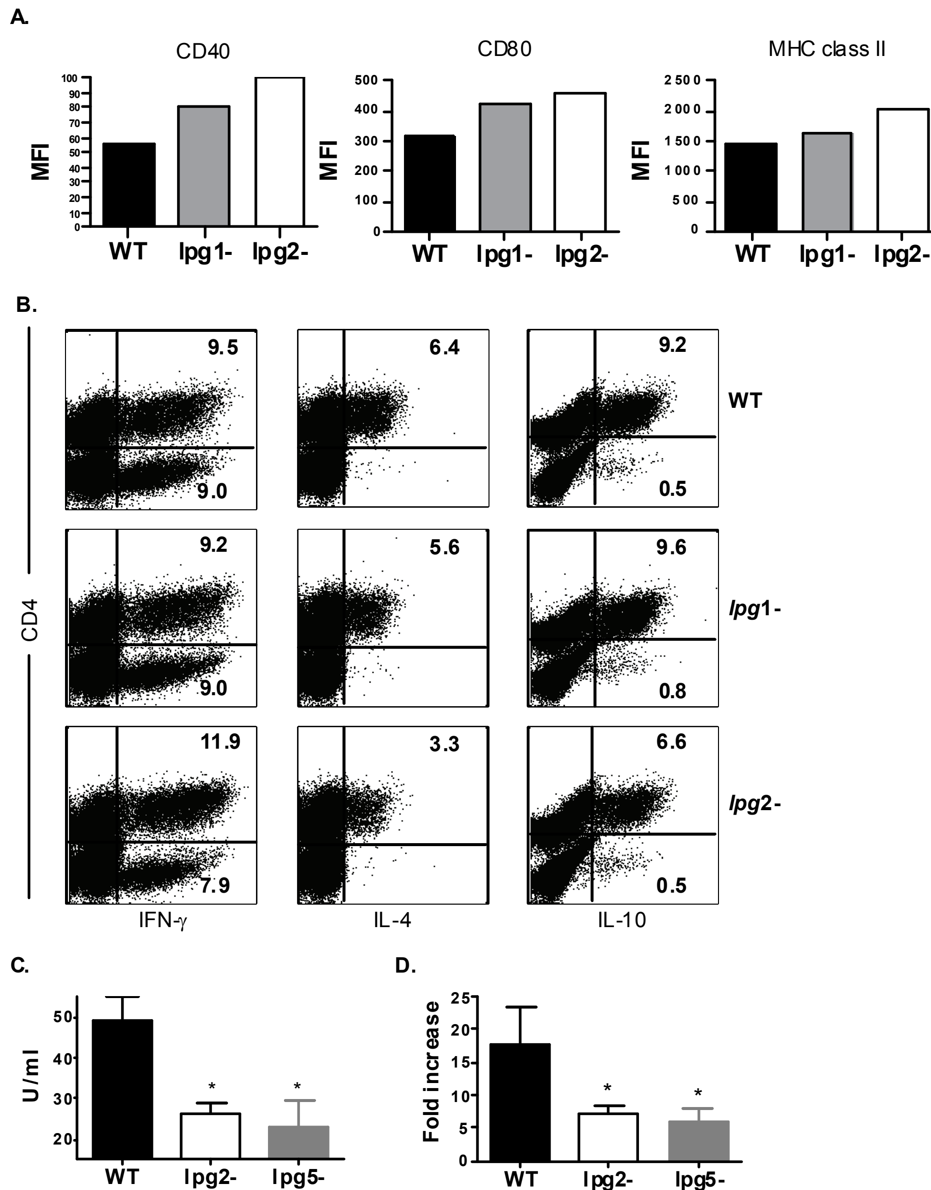



**FIGURE 10.** Differences in the early immune response in mice infected with *lpg2- L. major* is unrelated to absence of lipophosphoglygans (LPG).

BALB/c mice were infected in the footpad with 5 million WT, *lpg1-* and *lpg2- L. major* stationary phase promastigotes. After 3 days, mice were sacrificed and CD11c+ cells (DCs) were isolated from pooled dLN cells, stained for surface expression of MHC class II, CD40 and CD80 molecules and analyzed by flow cytometer **(A)**. Some unfractionated dLN cells were also stimulated with SLA for 3 days and the percentage of IFN-g-, IL-4- and IL-10-producing cells was determined by intracellular cytokine staining **(B)**. In some experiments, dLN cells from mice infected with WT, *lpg2-* and *lpg5A-/ lpg5B- L. major* for 3 days were cultured for 72 hr and the culture supernatant fluid was assayed for IL-4 by ELSA **(C)**. Total cellular RNA was isolated from some dLN cells and quantified by real time PCR **(D)**. Data presented are representative of 2 independent experiments (n = 3-5 mice/group) with similar results. * $p < 0.05$.

# Discussion

The primary aim of this study was to determine whether differences in the early immune response to WT and *lpg2*⁻ *L. major* could account for the protection observed in mice vaccinated with *lpg2*⁻ parasites [235]. We investigated the early immune response and DC functions following infection with WT and *lpg2*⁻ *L. major*. First we focused on the expression of costimulatory molecules and antigen presentation by DCs. We found that WT and *lpg2*⁻ *L. major* induced comparable expression of costimulatory molecules on infected DCs *in vitro* and *in vivo*. After 3 days, infection with both WT and *lpg2*⁻ parasites did not lead to appreciable changes in the expression of MHC class II and other costimulatory molecules, including CD40, CD80 and CD86 on total DCs isolated from the dLN of infected mice. However, these molecules were upregulated equally on the minor population (~ 10%) of infected DCs, suggesting that *LPG2*-dependent PG-containing molecules do not alter the expression of costimulatory molecule expression on DCs. Interestingly, infection with the specifically LPG-deficient *lpg1*⁻ mutant also failed to induce any significant changes in the expression of costimulatory molecules on DCs (**Fig. 6A**). Quantitative studies show that in promastigotes, LPG constitutes well over 90% of the parasite PGs in the *L. major* line studied here [19]. The present study suggests that neither LPG nor PG-containing molecules play any significant role in altering the expression of costimulatory molecules on DCs.

Functional antigen presentation studies showed that *lpg2*⁻-infected DCs exhibited an increased ability to present a test antigen (OVA) relative to those infected with WT parasites as manifested by increased production of IL-2 and IFN-γ by OVA-specific T cells. Since infection with *lpg2*⁻ *L. major* did not alter the expression of costimulatory molecules on

infected DCs, the results suggest that the increased Th1 response following *lpg2*$^{-}$ *L. major* infection may not be related to a more efficient T cell activation resulting from more costimulatory molecules interaction with their ligands, which leads to stronger signals on T cells. However, it is conceivable that altered T cell response could also occur from altered antigen presentation that could have arisen through other *LPG2*-dependent effects such as transient delay in the formation of fusogenic phagolysosomes [18, 218, 236]. However, because full length OVA were used in our studies and should be mostly taken up by endocytosis, it is most likely that altered phagolysosomal fusion in *lpg2*$^{-}$-infected cells may not have significantly affected the rate of antigen processing in this system. Therefore, the exact mechanism through which DCs from *lpg2*$^{-}$-infected mice enhanced IFN-γ response without affecting proliferation remains unknown.

Previous reports showed that the LACK antigen is mostly released, processed and presented to T cells by macrophages only following internalization of parasites with reduced infectivity that are subsequently killed [233, 237]. This raises the possibility that the increased presentation of LACK observed by DCs from *lpg2*$^{-}$ infected mice could reflect a superior killing of the mutant parasites. However, we were able to recover only about 1% of the infecting dose (~ close to 5 x $10^{6}$, **Fig. 8B**) after 3 days post-infection with WT and *lpg2*$^{-}$ parasite indicating that greater than 95% of the infecting parasites are killed within few hours or days of infection. This high and similar rate of early destruction of WT and *lpg2*$^{-}$ parasites suggests that release of LACK and subsequent priming of LACK-specific T cells in mice infected with WT and *lpg2*$^{-}$ parasites are similar. Therefore, it is unlikely that the increased presentation of LACK and induction of higher IFN-γ and IL-2 production by *lpg2*$^{-}$ -infected DCs over WT-infected cells is related to faster killing of *lpg2*$^{-}$ parasites after

infection. Another important consideration is that differences between WT and *lpg2⁻* parasites could reflect differences in infectivity since unselected stationary phase promastigotes used here may contain different numbers of infective metacyclic promastigotes. However, given that our studies and analyses concentrated on the early phase of infection (3 days post-infection) when there was no difference in both the percentage of infected DCs and parasite burden in WT and *lpg2⁻* infected mice (see **Figs 6C** and **8B**), we believe the use of unselected parasites in these studies have not significantly affected the results and their interpretation.

A previous report showed that LPG of *L. major* negatively modulates the expression of costimulatory molecules on *Langerhans* cells (a subtype of DCs) and inhibits their migratory activities [238]. However, since this report was based on studies using purified LPG, its biological relevance is difficult to assess. In contrast, a recent report suggests a critical role for *L. mexicana* LPG in the upregulation of costimulatory molecules (CD40, MHC class II and CD80) on DCs [239]. Interestingly, the complementary add backs were unable to restore the WT phenotype on infected cells. We found that as WT *L. major*, both *lpg1*- and *lpg2⁻ L. major* infection of DCs altered the expression of costimulatory molecules to the same extent over basal levels seen in uninfected cells. These differences in observation may be related to the use of different *Leishmania* species (*L. mexicana* vs. *L. major*). For instance, the ability of *Leishmania* to induce IL-12 production by DCs has been shown to vary depending on the parasite species [240-242]. Furthermore, while *lpg2⁻ L. major* parasites are highly attenuated [197, 222], *lpg2⁻ L. mexicana* retain amastigote virulence [219]. It is conceivable that different species of *Leishmania* may use different mechanisms to subvert the host immune response. Interestingly, infection of DCs with *lpg2⁻ L. major* significantly enhanced their

production of IL-12p40 in response to various DC stimulating agonists (**Fig. 5C**). Thus, while the glycoconjugate molecules of *L. major* do not influence DC expression of costimulatory molecules, they may act to prevent their effective antigen-presentation capacity (in an as yet undetermined way) thereby affecting their effector Th1 cell-inducing abilities.

In addition to antigen processing and presentation, DCs also influence the nature of immune response by producing cytokines including IL-12. While *in vitro* infection of DCs with WT *L. major* resulted in minimal production of IL-12p40 over basal levels, infection with *lpg2*$^{-}$ *L. major* resulted in more than 180% increase in IL-12p40 production (**Fig. 5C**). This differences in IL-12 production between WT and *lpg2*$^{-}$ *L. major*-infected DCs persisted following stimulation with various stimuli, including anti-CD40, IFN-γ and LPS (**Fig. 5C and** Supplementary Fig. A). Similar effects were seen *in vivo,* where IL-12p40 production by cells from the dLNs of mice infected with *lpg2*$^{-}$ *L. major* was higher than those from infected with WT parasites (**Fig. 8E**). Despite the high production of IL-12p40 subunit, we were unable to detect measurable amounts of the bioactive IL-12p70 as has been reported by others [230, 231]. Since p40 subunit is common to both IL-12 and IL-23, we could not rule out the possibility that some of the molecules detected in this study are components of IL-23. Consistent with the DC findings, cells from the dLN of mice infected for 3 days with *lpg2*$^{-}$ parasites produced lesser amounts of macrophage-deactivating cytokines (IL-4/IL-10) than those from WT-infected mice **(Figs. 8D, 9B and 10B and C**). Interestingly, infection with LPG-deficient *lpg1*$^{-}$ parasites did not lead to reduction in IL-4 production by dLN cells (**Fig. 10B**), whereas infection with PG-deficient *lpg5A*$^{-}$/*lpg5B*$^{-}$ parasites (which lack PGs similar to *lpg2*$^{-}$) also led to reduction of the early IL-4 response in the dLN (**Fig. 10C and D**).

Surprisingly, the reduced IL-4 response in mice infected with *lpg2-* parasites was not associated with a concomitant increase in IFN-γ production, a cytokine that has been shown to suppress the production of IL-4 by T cells [243]. Wang *et al.* [244] showed that IL-12 could directly downregulate IL-4 in the absence of IFN-γ. Thus, it is possible that higher IL-12 production by cells from *lpg2*⁻-infected mice directly suppress IL-4 response, in an IFN-γ-independent manner. These results suggest that the PGs play important role in regulating early IL-4 and IL-12p40 responses following infection with *L. major*. It should be emphasized that these data do not completely rule out a role for LPG in this process; however, if active, its role is fully redundant with that of other PGs unaffected in the *lpg1*⁻ mutant. Furthermore, we do not know whether the reduced IL-4 levels in cultures of cells from *lpg2*⁻ *L. major*-infected mice was related to suppression or inhibition of IL-4 production in the absence of PG-containing molecules.

The contribution of PGs and other *LPG2*-dependent molecules in modulating the host immune response, particularly in the induction of protective IFN-γ and pathogenic IL-4 responses have not been investigated. The early immune response to *L. major* in mice is dominated by a unique population of Vβ4 Vα8 CD4+ T cells recognizing the immunodominant epitope of LACK protein [245-248]. These MHC class II-restricted cells rapidly expand in the dLN, accumulate IL-4 transcripts within hours after infection and secrete high levels of this cytokine *in vivo* and *in vitro* [246, 247]. The reduction in IL-4 production in mice infected with *lpg2*⁻ and *lpg5A*⁻/ *lpg5B*⁻ parasites closely mimics the failure to produce early IL-4 burst in mice made tolerant to LACK by transgenic expression [248]. Thus, it is tempting to suggest that the PG-containing molecules of *L. major* influence the host immune response to LACK protein. Recently, LACK TCR transgenic (ABLE) mice

on a BALB/c background [249] were developed and used to study T cell response to LACK protein [250]. These mice will be valuable for studying the influence of PG-containing molecules on the induction of Vβ4 Vα8 CD4+ T cells and early IL-4 burst during *L. major* infection.

Overall, our results show that PGs and/or *LPG2*-dependent glycoconjugate molecules of *L. major* are responsible for the reduction in the production of IL-12p40 by infected DCs, and the consequent induction of increased Th1 cell response. Thus, in addition to their role in subverting macrophage function, the glycoconjugates of *Leishmania major* influence the initial pathways that lead to effective T cell activation, thereby ensuring optimal survival of the parasites in infected host. We speculate that reduction of IL-4 (and to some extent IL-10) response in mice infected with *lpg2*⁻ *L. major* could lead to a more efficient IFN-γ-mediated activation of macrophages and dendritic cells leading to rapid early parasite control. The protection induced by *lpg2*⁻ parasites is striking despite the absence of a large IFN-γ response [235]. Our results suggest that one reason underlying this phenomenon is the possibility of a heightened early immune response to the *lpg2*⁻ parasites due to the absence of PGs and LPG, in conjunction with the inability of this parasite to induce lesion pathology.

# CHAPTER IV Regulation of Secondary anti-*Leishmania* (memory) Immunity by LPG2 Gene Products

## Specific Introduction

*Leishmaniasis* is a serious global health problem that affects millions of people world wide, especially in developing countries. A number of mammals, such as wild rodent, canids and marsupials, can be the natural reservoirs for this disease. Although there is no direct evidence of human infection in North America, a recent serosurvey of foxhounds and other canids suggested that *leishmania* infection appears to be widespread in canids and the probability of human exposure will be likely increased in the near future [251]. Moreover, due to the difficulties in control of the wild reservoirs population, the development of human vaccines becomes the only and most effective way to prevent this disease. As we have shown in the previous chapter that LPG2 gene products can modulate dendritic cells functions and shape subsequent adaptive immune responses, the next logical question is whether and how LPG2 gene products also influence secondary anti-*leishmania* immunity in vaccinated mice. Since *lpg2-* mutant parasites persist without causing any disease even in the highly susceptible BALB/c mice, this attribute makes it a promising live-attenuated anti-*Leishmania* vaccine candidate. In fact, Uzonna *et al.* [23] demonstrated that *lpg2-* mutants vaccinated BALB/c mice vaccinated with *lpg2-* mutants were protected from virulent *L. major* challenge, and this protection was not associated with a strong Th1 response. However, the exact mechanism of *lpg2*-mediated protection is still largely unknown.

Following recovery of primary natural or experimental infection with wild-type (WT) *L. major*, a state of immunity develops that is able to rapidly protect healed mice animals

(both humans and mice) against an otherwise pathogenic secondary challenge [125]. This so called infection-induced immunity is very durable (sometimes lasting the entire life of infected mice) and is the strongest indication that vaccination against cutaneous *leishmaniasis* is feasible. Studies indicated that this infection-induced immunity is mediated by IFN-γ-producing T cells with primary characteristics of central and effector memory-like cells [206, 210]. This is the underlying principle behind leishmanization, which is still practiced in some countries today [252]. The significant morbidity and secondary infections-related chronic lesions seen in leishmanized individuals have hampered leishmanization as an acceptable vaccination strategy. For *lpg2-* parasites to become a contending vaccine candidate, it must, in addition to not causing any overt disease, be able to induce secondary (memory) immune response comparable to those of WT parasites. Here, we compared the quality and quantity of anti-*Leishmania* memory immunity induced by WT and *lpg2*-parasites. We show that despite significant differences in quantity, the anti-*Leishmania* immunity induced by WT and *lpg2-* parasites are qualitatively similar. These findings further support the consideration of *lpg2-* parasites as live attenuated vaccine candidate against cutaneous *leishmaniasis*.

# Results

## *lpg2⁻ L. major* parasites induce memory T cell population in susceptible mice

BALB/c mice are highly susceptible to *L. major* and develop progressive lesion after parasite inoculation in the footpad. As previously reported [18, 23], we found that *lpg2⁻ L. major* persists in the footpad of these mice without causing any lesion for greater than 16 weeks post-infection (**Fig. 11A**). Interestingly, spleen and draining lymph node cells from 16 wk-these infected mice produce very minimal IFN-γ following short-term (3 days) *in vitro* restimulation with soluble *Leishmania* antigen ([23], left panel of **Fig. 11B**).

Because mice infected with *lpg2⁻ L. major* are protected against virulent *L. major* challenge despite minimal IFN-γ response [22], we wondered whether there is any *Leishmania*-specific memory T cells existed that are responsible for mediating the observed resistance. Therefore, we restimulated cells labeled with CFSE isolated from the draining lymph nodes (dLNs) and spleens of *lpg2-* vaccinated BALB/c mice *in vitro* with soluble *Leishmania* antigen (SLA) for 5 days. As shown in the right panel of **Fig. 11B**, CD4+ T cells from *lpg2*-infected mice proliferated and produced IFN-γ in response to *leishmania* antigen restimulation *in vitro*, indicating that parasites-specific memory T cells are maintained in the presence of low level of parasites. These memory T cells might be responsible for previously demonstrated resistance in *lpg2-* vaccinated BALB/c mice [23].

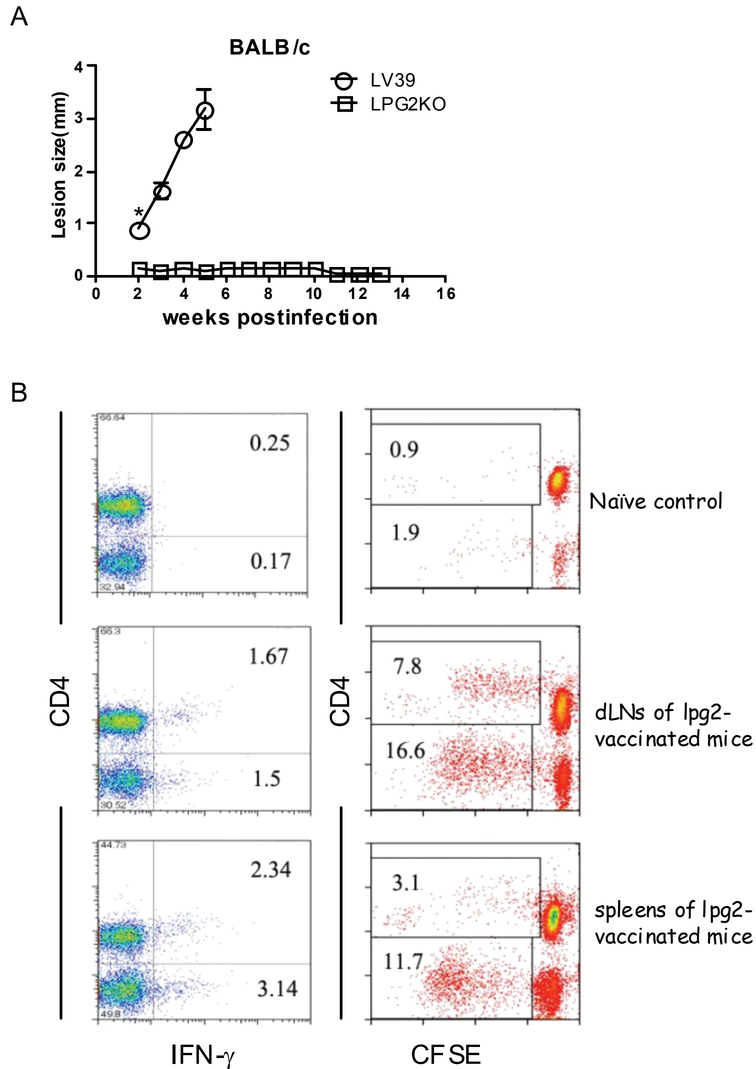



**FIGURE 11.** *lpg2- L. major* parasites induce memory T cell population in susceptible mice .

BALB/c mice were infected in the footpad with 5 million 7 day's stationary WT and *lpg2- L. major* stationary phase promastigotes. Lesion size was monitored weekly by measuring with a Vernier caliper the footpad thickness relative to that of the uninjected foot **(A)**. >16 wks post infection, draining lymph node cells and splenocytes from *lpg2-* infected BALB/c mice were labeled with CFSE and restimulated *in vitro* with soluble leishmania antigen for 5 days. The intracellular IFN-γ and proliferation were determined by flow cytometry **(B)**.

## T cells from *lpg2*⁻-infected C57BL/6 mice proliferate and produce IFN-γ in response to parasite antigens *in vitro*

BALB/c mice do not naturally heal WT infections, which makes it difficult to compare memory responses following *lpg2*- and WT *L. major* infections in this strain. Fortunately, similar to BALB/c mice, *lpg2*⁻ *L. major* parasites also persist indefinitely in the resistant C57BL/6 mice and *lpg2*⁻ vaccinated mice are also protected against virulent WT challenge. As shown in **Fig. 12A**, *lpg2*-infected C57BL/6 mice did not develop any lesion for up to 20 weeks, while mice infected with WT parasites only developed small and self-healing lesion (**Fig. 12A**). After 16-20 weeks following primary infection, both WT and *lpg2*- infected C57BL/6 mice contain comparable number of parasites (~1000) in their footpads as determined by limiting dilution assay (**Fig. 12B**). As IFN-γ production by *lpg2*-infected BALB/c mice was very low, we wondered whether our inability to detect significant IFN-γ production was related to suboptimal antigen presentation following SLA restimulation. Therefore, we restimulated cells from the draining lymph nodes and spleens of *lpg2*- vaccinated C57BL/6 mice *in vitro* with *L. major*-infected bone marrow-derived dendritic cells (BMDCs) as antigen-presenting cells. Similar to our observation in BALB/c mice, T cells from *lpg2*- vaccinated C57BL/6 mice recognized leishmania antigen presented by BMDCs as evidenced by strong proliferative and IFN-γ production (**Fig. 12C and , 12D**). Surprisingly, both cell proliferation and IFN-γ production by T cells from *lpg2*-infected mice were significantly lower than those from WT *L. major*-infected mice (**Fig. 12C and , 12D**), suggesting that *lpg2*- parasites are less effective in at inducing memory T cells than WT parasites.

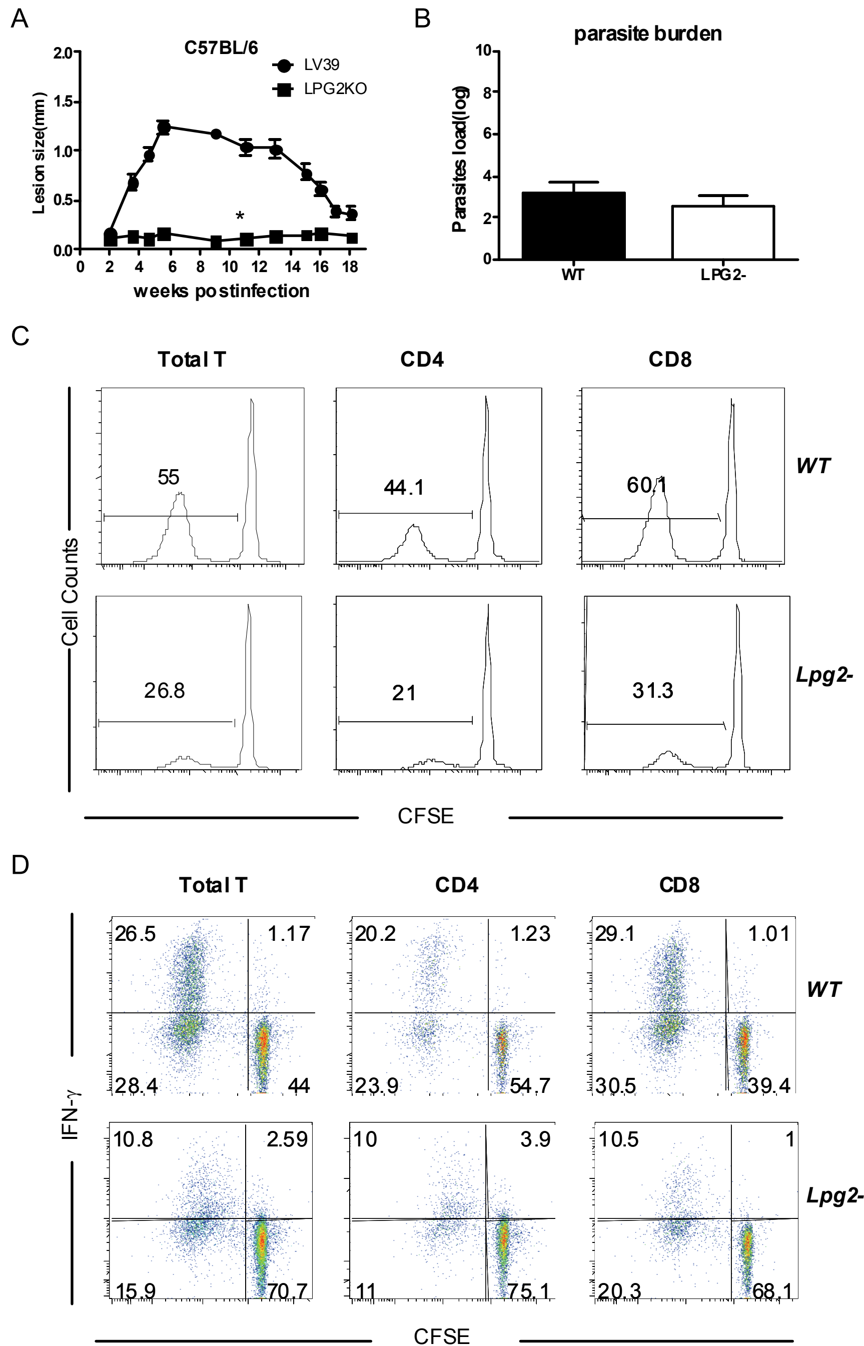



**FIGURE 12.** T cells from the spleens of WT and *lpg2-* infected C57BL/6 mice proliferate and make IFN-γ in response to *L. major*–infected DCs.

Lesion progression in WT and *lpg2- L. major* infected C57BL/6 mice **(A)**. >16-20 weeks after infection WT and *lpg2-* infected mice were sacrificed and parasite burdens were determined by limiting dilution assay **(B)**. $2\times10^5$ CFSE-labeled T cells from infected mice were cultured for 5 d in 200 µl culture medium per well of a 96-well U-bottom plate with $2\times10^3$ BMDCs *L. major*–infected BMDCs. Cells were analyzed by flow cytometry. The percentage of cells that divided is indicated **(C)**. The percentage of IFN-γ producing cells is indicated **(D)**. Results are representative of 2 independent experiments.

## Proliferation and IFN-γ production by T cells from *lpg2- L. major*-infected mice were impaired *in vivo*.

We previously showed that *Leishmania* phosphoglycans molecules influence primary adaptive immune responses to *L. major* by modulating DC functions [261]. To further determine whether the impairment of secondary recall (proliferative and IFN-γ responses) observed in cells from *lpg2- L. major* infected mice is strictly related to T cells and in a more physiologic environment, we adoptively transferred CFSE labeled *Thy1.2* (CD90.2+) cells purified from healed WT and *lpg2-* infected mice or naive mice into congenic *Thy1.1* (CD90.1+) naive recipients. Twenty-four hours post-transfer, the recipient mice were challenged WT *L. major* into the contra-lateral footpads. Challenged mice were sacrificed on days 5, day 14 and 21 following post-challenge and cells from the draining lymph nodes and spleens were assessed for cell proliferation and effector cytokine (IFN-γ) production by flow cytometry by gating on donor's (CD90.2+) T cells (**Fig. 13A**). As early as 5 days post-challenge, the donor cells from *lpg2*-infected mice were less proliferative (**Fig. 13B**) and produced significantly lesser IFN-γ in response to virulent *L. major* challenge than those from WT-infected mice (**Fig. 13C**). Moreover, this recall defect in responding to virulent challenge can be observed up to day 21, suggesting that *lpg2-* parasites do not induce memory T cells as efficient as WT parasites *in vivo*.

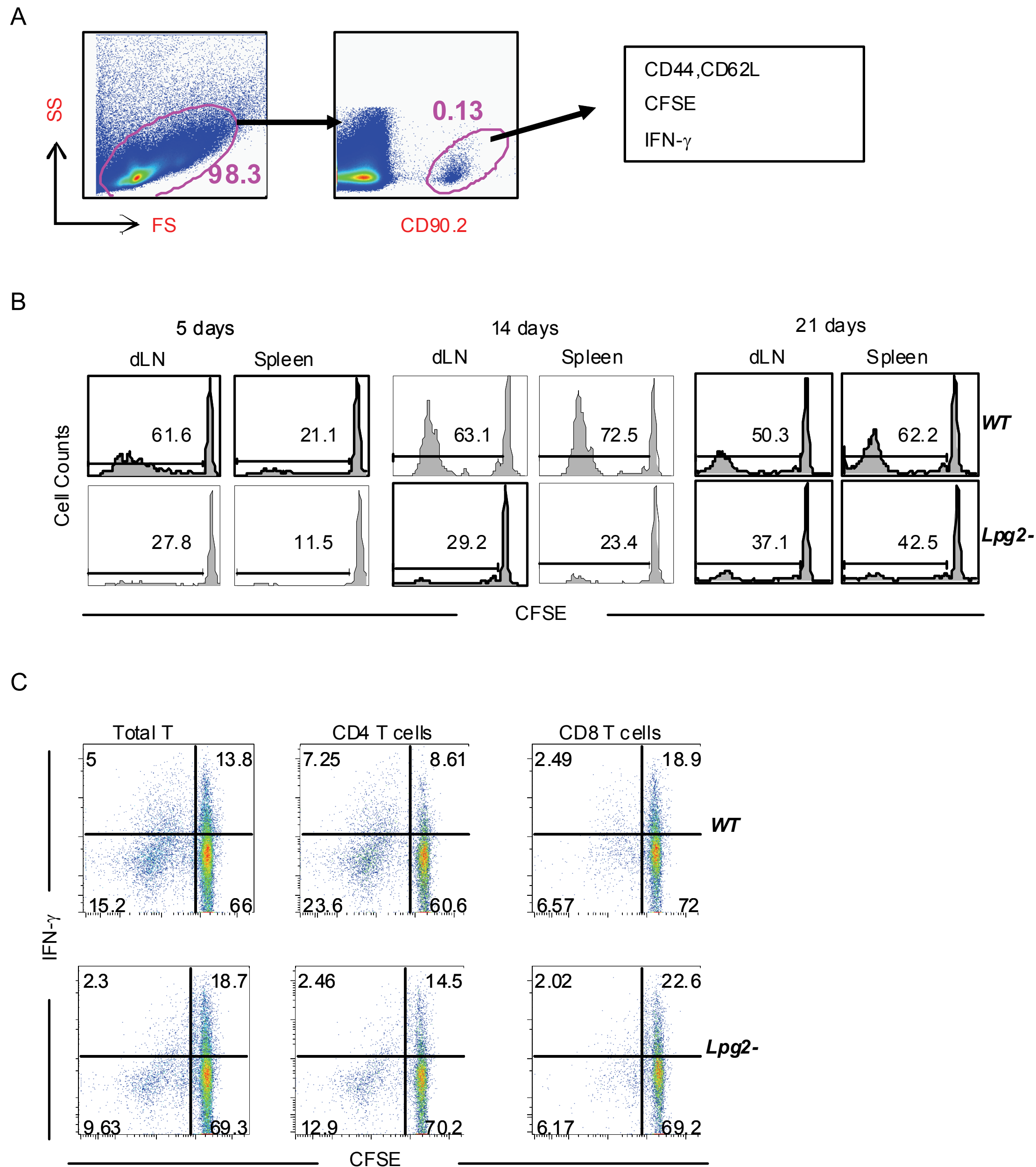



**FIGURE 13.** *L. major lpg2-* parasites induce less number of effector T cells and IFN-γ production than their wide type controls.

Naïve Thy1.1 mice received (by i.v. injection) CFSE-labeled highly purified (> 98% pure) CD90.2+ T cells (purified by autoMACS) from healed WT or *lpg2-* (Thy1.2) mice. Recipient mice were challenged with $2\times10^6$ WT *L. major* the next day and were sacrificed at indicated times. Splenocytes or dLNs cells were stained with anti-CD3, anti-CD90.2, anti-CD4, anti-CD8, anti-IFN-γ antibodies conjugated with different fluorochromes and analyzed by flow cytometry. All analyses were gated on CD90.2+ donor population **(A)**. Cell proliferations were determined **(B)**. Also, the production of IFN-γ by *Leishmania*-reactive (proliferating) cells was determined by gating on donor cells (Thy1.2) **(C)**.

## Quantitative differences in memory T cells in WT and *lpg2- L. major*-infected mice.

To directly determine whether there are quantitative differences in the memory numbers of memory T cells generation in mice infected with WT and *lpg2-* parasites, we collected spleens and draining popliteal lymph nodes (dLNs) from 20 weeks WT and *lpg2-*-infected mice and assessed for the expression of CD62L on CD44+ T cells (CD3+) by flow cytometry directly *ex vivo*. CD44 is a surface protein required for lymphocyte extravasation to inflammatory sites and its upregulation is a marker of previous T cell activation and hence is expressed by all memory T cells [253]. CD62L is a lymph node homing receptor for lymphocytes, which allows them to enter the high endothelial venules and is downregulated upon activation. and usually expressed by central memory-like T cells [263]. (see introductory for more information) By using these markers, we are able to discriminate central memory-like T cells ($CD44^{hi}CD62L^{hi}$, Tcm) and effector memory-like T cells ($CD44^{hi}CD62L^{lo}$, Tem) [206, 210]. Consistent with *in vitro* co-culture results, our direct *ex vivo* results show that the percentages of memory-like T cells (both Tcm and Tem populations) in these lymphoid tissues of *lpg2*-infected mice were significantly lower than those from WT-infected mice (**Fig. 14A**).

Next, we used the highly sensitive adoptive transfer studies to determine whether there were differences in CD62L expression on proliferating (*Leishmania*-experienced) donor cells *in vivo*. At day 14 post challenge, donor T cells from both groups have started to downregulate their CD62L expression (**Fig. 14B**), although T cells from WT-infected mice were more proliferative (as previously shown above) and downregulated their CD62L more efficiently than those from *lpg2*-infected mice. Thus, despite lower proliferative response,

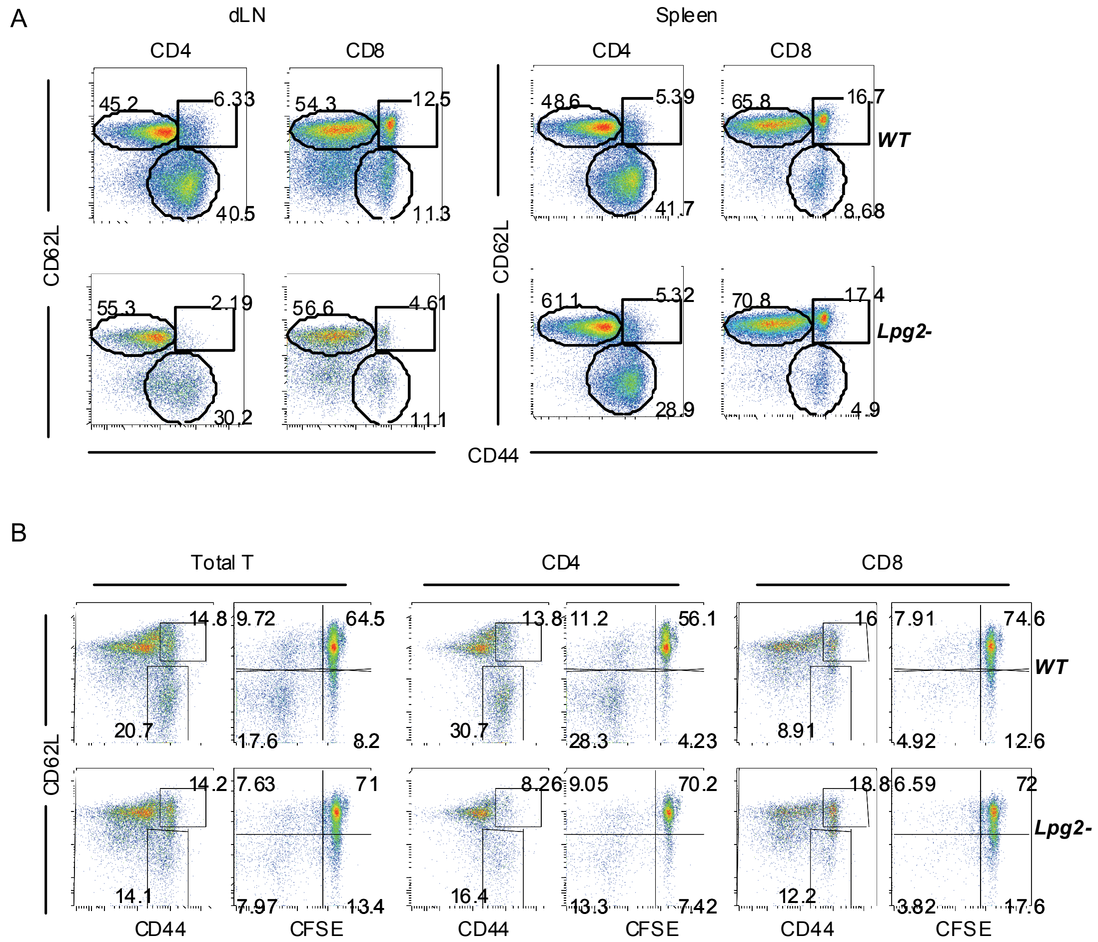



**FIGURE 14.** Quantitative differences in memory T cells in WT and *lpg2- L. major*-infected mice.

Cells from spleens and popliteal draining lymph nodes (dLNs) from 20 weeks wide type and *lpg2-*-infected C57BL/6 mice were stained with anti-CD3, anti-CD4, anti-CD62L and anti-CD44 antibodies conjugated with different fluorochromes and analyzed by flow cytometry. Expression of CD44 and CD62L on T cell subsets **(A)**. Thy1.1 mice received CFSE-labeled cells were sacrificed on day 14 post-challenge. Splenocytes or dLNs cells were stained with anti-CD3, anti-CD90.2, anti-CD4, anti-CD62L and anti-CD44 antibodies conjugated with different fluorochromes and CD90.2+ cells were analyzed by flow cytometry. The expression of CD44 and CD62L on donor T cell subsets **(B)**. Data presented are representative of 2 independent experiments (n = 3-5 mice per group) with similar results.

*Leishmania*-reactive cells from *lpg2*-infected mice could downregulate their CD62L expression, suggesting that they could efficiently home to the site of infection to mediate effector function.

## *lpg2⁻ L. major* mediated protection in vaccinated C57BL/6 mice is not associated with a strong DTH response

It has been shown that *lpg2- L. major* vaccinated BALB/c mice were protected from virulent challenge. However, whether this mutant parasite is able to mediate protection in C57BL/6 mice directly in the absence of adjuvant or by adoptive transfer is still unknown. Therefore, we challenged WT and *lpg2-* infected C57BL/6 mice (> 16 weeks) with virulent WT *L. major* parasites and 24 or 72 hrs later, we measured delayed-type hypersensitivity (DTH) response. We found that WT *L. major*-infected mice exhibited very strong DTH response in terms of footpad swelling following challenge (**Fig. 15A**). In contrast and consistent with our previous report in BALB/c mice [23], *lpg2*-infected mice did not exhibit any significant footpad swellings and its DTH response was similar to naïve mice (**Fig. 15A**), consistent with the observation of fewer numbers of memory-like cells in *lpg2*-infected mice. Interestingly, when challenged mice were sacrificed at 3 weeks post-challenge to determine parasite burden, we found that *lpg2-* infected mice were protected as well as WT-infected mice (**Fig. 15B**). Furthermore, adoptive transfer of highly purified T cells from both WT and *lpg2*-infected mice conferred protection to naïve mice against virulent *L. major* challenge (**Fig. 15C**). Taken together, these results indicate that despite quantitative differences, cells from *lpg2*-infected mice are qualitatively as efficient as those from WT-*L. major*-infected mice in mediating secondary immunity, an observation that has very important vaccination implications. Thus, despite the lack of a strong IFN-γ production, cells from *lpg2-* infected

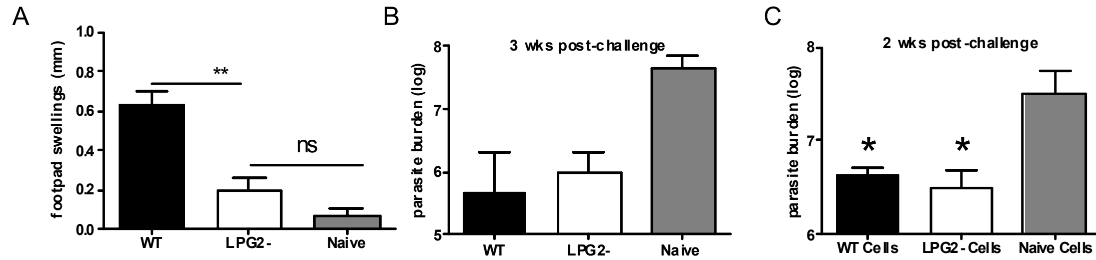



**FIGURE 15.** *lpg2- L. major* mediated protection in vaccinated C57BL/6 mice is not associated with a strong DTH response.

20 weeks post infection, both WT and *lpg2*- infected mice were challenge with 5 millions of WT parasites. Footpad swelling of DTH response at 24hr **(A)** and parasite burden (3 wk post challenge) **(B)** in the footpads of challenged mice. Parasite burden of mice received T cells from WT infected mice or lpg2 infected mice. **(C)**. Data presented are representative of 2 independent experiments (n = 2-4 mice per group) with similar results.

mice were able to confer considerable protection to naive recipients at levels that were comparable to those from WT *L. major*-infected mice (**Fig. 15C**).

## Protection in *lpg2*-infected mice is strongly dependent on IFN-γ but is independent of CD8+ T cells

It has been reported previously that cells from *lpg2*- vaccinated BALB/c mice produced very low levels of IFN-γ in response to *Leishmania* antigen stimulation [23]. Our results also demonstrated that cells from *lpg2*- vaccinated C57BL/6 mice were less effective in making IFN-γ when co-cultured with infected BMDCs *in vitro* and following *in vivo* restimulation in adoptive transfer studies (**Fig. 12D, Fig. 13C**). *lpg2*- parasites are sensitive to lysis by human complement and therefore are highly attenuated in macrophages after infection [18]. In addition, *lpg2*- parasites persist at levels of about 100 to 1000 in infected mouse [18], which is similar to the doses number of parasites commonly used in low dose infections. Moreover, we have reported that primary infection with *lpg2- L. major* induced a strong CD8+ T cell response [260]. Since CD8+ T cells are important in secondary memory anti-*Leishmania* immunity responses and following during low dose infections [110-112], we speculated that secondary resistance in *lpg2*–infected mice might be dependent on CD8+ T cells. To determine the role of CD8+ T cells in *lpg2*-induced secondary immunity, we treated WT and *lpg2*-infected mice with anti-CD8+ mAb (TIB 210 1 mg/mouse weekly) and challenged them with virulent *L. major*. As shown in **Fig. 16A and 16B**, depletion of CD8+ T cells had no effect on *lpg2*-induced protection, suggesting that CD8+ T cells are dispensable for this immunity.

Because IFN-γ recall response by cells from *lpg2*-infected mice is significantly impaired *in vitro* and *in vivo*, we investigated whether protection following *lpg2*-infection is

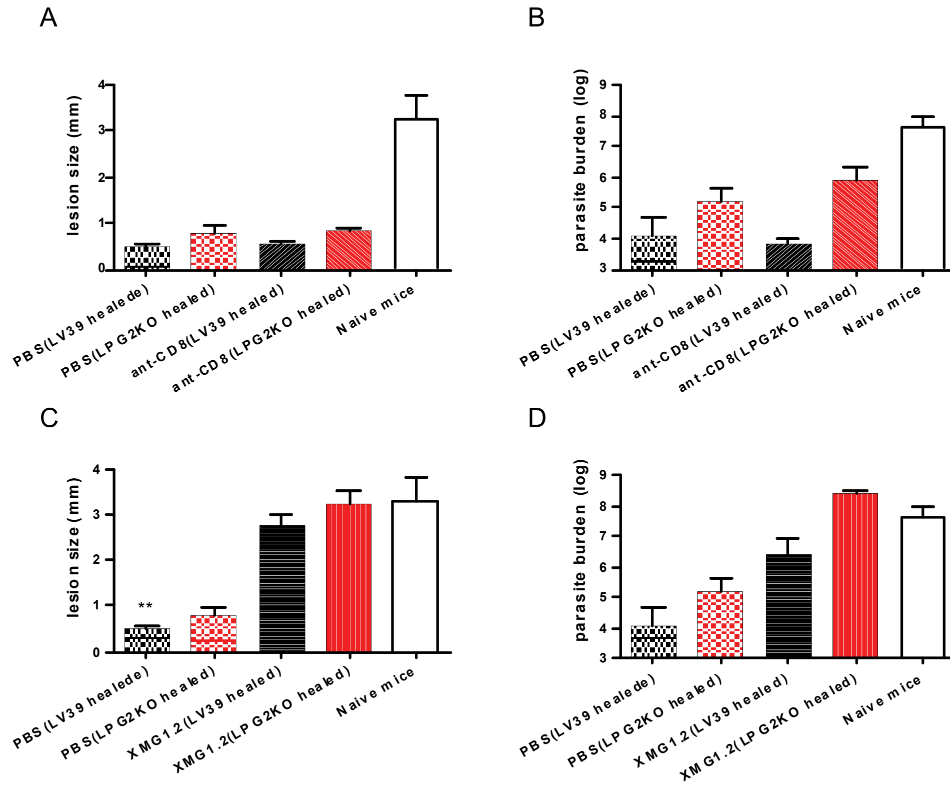



**FIGURE 16.** Protection in *lpg2*-infected mice is strongly dependent on IFN-γ but is independent of CD8+ T cells.

C57BL/6 mice were infected in the right footpad with 5 million 7 day's stationary WT and *lpg2- L. major* stationary phase promastigotes. >16 weeks post-vaccination, mice were treated with anti-IFN-γ or anti-CD8 monoclonal antibodies. 24 hrs later, mice were challenged with 5 millions WT virulent *L. major* in the left footpads. 3 weeks post-challenge, lesion sizes **(A, C)** and parasite burdens **(B, D)** in lesions were also assessed. Data are representative of two or more experiments.

dependent on this cytokine, IFN-γ. The data shown in **Fig. 16C and 16D** shows that IFN-γ is stringently required for *lpg2*-induced protection as neutralization of this cytokine by anti-IFN-γ mAb completely abrogated the protection. To further assess the ability of T cells from WT and lpg2- infected mice to provide protection in naive recipient, we adoptively transferred immuned T cells (CD90+) from WT and lpg2- infected C57BL/6 mice into naive mice. Interestingly, despite the lack of a strong IFN-γ production, cells from *lpg2*- infected mice were able to confer considerable protection to the naive recipients at levels that were comparable to cells from WT infected mice (**Fig. 15C**). These results are consistent with our previous observation [205], suggesting that the quality of anti-*Leishmania* secondary immunity is regulated not only by the quantity of IFN-γ but also by other immunoregulatory networks that may be operation during infection.

# Discussion

In this study, we investigated the possible mechanism of *lpg2*- mediated protection in murine cutaneous *leishmaniasis*. First we demonstrated that *lpg2*- vaccinated mice contained cells that are rapidly recalled by challenge with *Leishmania* antigen *in vitro* or *in vivo*. These *Leishmania*-reactive (memory) T cells proliferate and produce IFN-γ in response to *Leishmania* antigen stimulation. Nevertheless, both *in vitro* and *in vivo* data suggested that *lpg2*- parasites were less effective than WT parasites at inducing and/or maintaining *Leishmania*-specific memory T cells. Although memory T cells generated by *lpg2*- parasites did not proliferate as well and make much lower IFN-γ compared to WT parasites, they were capable of mediating protection against virulent challenge similar to WT parasites. Interestingly, *in vivo* depletion and neutralization studies with mAbs demonstrated that *lpg2*- *L. major*-mediated resistance was strongly dependent on IFN-γ, but independent of CD8+ T cells.

It is widely believed that long-term immunological protection depends not only on the quantity but also the quality of the memory T cells that are generated. The quantity of memory T cells formed is determined primarily by the burst size (clonal expansion) during the initial infection or immunization [254]. In contrast, the quality of memory T cells are mostly measured by their effector cytokine responses, which are directly related to their ability to perform effector functions [255]. The production of IFN-γ by T cells is one of the most widely used parameter for assessing vaccine efficiency (and hence immunologic memory) because this cytokine has been shown to play important roles in controlling a number of intracellular bacteria and parasites [256-258], including *Leishmania*. However, our *in vitro* and *in vivo* data demonstrated that cells from *lpg2*-infected mice only produce

very little IFN-γ, yet these mice were strongly protected against virulent *L. major* challenge. These observations suggest that other factors may also contribute the *lpg2-* mediated resistance. Apart from IFN-γ, tumor necrosis factor (TNF) has also been shown to play important role in protective immunity against *leishmaniasis* [138, 259]. Indeed, our preliminary data show that cells from *lpg2-* vaccinated mice were able to make TNF as efficiently as those from WT controls (Supplementary Fig. C). It is conceivable that this unimpaired production of TNF in *lpg2*$^{-}$-infected may partly compensate the defective IFN-γ production found in *lpg2-* infected mice.

Besides effector cytokines, the homing properties of the memory T cells are also strongly correlated with the quality of immunological memory. It is crucial for antigen-specific memory T cells to migrate to the infection sites to mediate effector functions. The paradigm of central memory T cells (Tcm) and effector memory T cells (Tem) provides us a conceptual advance in understanding the importance of T cell trafficking and their effector functions [190]. According to this paradigm, Tcm cells express high level of CD62L and CCR7 with little effector functions, while Tem cells are able to downregulate CD62L and migrate to the peripheral site to combat with the pathogen. Our previous data demonstrated that the impaired transition from Tcm to Tem was responsible for increased susceptibility to *L. major* re-infection in PI3K deficient mice [260]. In contrast to those from infected PI3K deficient mice, memory T cells from lpg2- infected mice showed no defect in the transition from Tcm to Tem (**Fig. 14B**), suggesting that these cells could effectively migrate to the site of infection to mediate effector functions (activation of infected macrophages for parasite killing). The efficient cell migration might also partially compensate for the defective IFN-γ production and contribute to the superb resistance conferred by *lpg2-* parasites.

Vaccination is the most effective and economic way to prevent infectious diseases. Unfortunately, there is currently no clinical approved vaccine for human cutaneous *leishmaniasis* [261]. Interestingly, recovery from natural or experimental infection leads to a long-lasting protective immunity against re-infection, an observation that formed the basis for the century long *leishmanization* practice in many parts of the world [262]. *Leishmanization* involves inoculation of live parasites or tissue extracts from infected lesion into hidden area of non-immune individuals, with the intention of preventing a more serious and disfiguring lesion from developing following natural infection. The success of *leishmanization* practice suggests that it is possible to vaccinate against cutaneous *leishmaniasis*. The significant morbidity and secondary infections-related chronic lesions seen in *leishmanized* individuals have hampered *leishmanization* as an acceptable vaccination strategy. To overcome these problems, a number of live attenuated mutant parasites have been generated [20, 221, 263, 264]. In contrast to other vaccines, *lpg2-* parasites have several advantages as a potential vaccine candidate. First, *lpg2-* parasites are not pathogenic and do not cause any disease even in highly immunocompromised *scid* mice [22]. Second, similar to WT mice, *lpg2-* parasites persist almost indefinitely in vaccinated mice [20], which eliminates the need for repetitive inoculations. Lastly, *lpg2-* parasites protect vaccinated host against virulent challenge without inducing “nasty” DTH response, which makes it perfect for human use. We referred to DTH as being “nasty” because very few individuals would want to have a huge vaccination-induced swelling (DTH response) on their faces following a bite from an infected Sandfly.

Overall, our results show that *lpg2- L. major* parasites induce protective immunity in both BALB/c and C57BL/6 mice against virulent challenge, despite the absence of delayed

type hypersensitivity (DTH) response and significantly reduced proliferative and IFN-γ recall responses. Interestingly, this protection is still dependent on IFN-γ production by CD4+ T cells. However, our findings in this study again support our position that a stronger IFN-γ response does not necessarily translate to better protection [265], contrary to the current thinking. We hypothesize that other factors, including polyfunctionality of the effector and memory T cells and immunoregulatory networks act together to determine the outcome of primary and secondary anti-*Leishmania* immunity in mice. Whatever the mechanisms of protection are, our findings lend support for the consideration of *lpg2*- parasites as live-attenuated vaccine or *leishmanization* candidates against cutaneous leishmaniasis, particularly in parts of the world where the latter is still being practiced with WT parasites. This would at least reduce the morbidity and lesion chronicity associated with WT *leishmanization* since *lpg2*- parasites do not cause any disease. We are currently examining the pathogenesis of *lpg2*- parasites in non-human primates and will also determine whether infection with these mutant parasites could confer protection against virulent challenge (as in mice).

# CHAPTER V PI3K Signaling in primary anti-*Leishmania* immune responses

## Specific Introduction

*Leishmaniasis*, one of the six major tropical diseases identified by the WHO for intense further study, is a chronic protozoan disease that is endemic in 88 countries and affects more than 12 million people. A key event in the life cycle of this protozoan is infection of macrophages and dendritic cells, which need to become activated in order to kill these intracellular parasites. The activation of infected macrophages is regulated by the availability of interferon-γ (IFN-γ) produced by activated T cells. Thus, a strong T cell response is believed to be necessary for resistance to *L. major* in humans.

*L. major*-infected mice mimic the human cutaneous disease, with healing and non-healing disease in specific mouse strains, dependent on the type of $CD4^+$ helper T (Th) cell subset that is generated [266, 267]. Traditionally (and as in humans), healing has been associated with the development of a strong Th1 response, leading to the production of IFN-γ which activates macrophages, ultimately resulting in killing of parasite in these cells [266, 268]. Non-healing disease has been associated with the development of Th2 cells that produce interleukin-4 (IL-4), a cytokine which deactivates macrophages, making them unable to kill the intracellular parasites [266, 268]. IL-10 is another cytokine that has been shown to regulate disease outcome. IL-10-deficient mice are highly resistant to *L. major* [59], and transgenic overexpression of IL-10 renders resistant mice susceptible [269]. The cellular source of IL-10 has been shown to include natural $CD4^+CD25^+$ regulatory T cells [110, 270],

conventional $CD4^+$ T cells [271] and in some cases, macrophages [272]. Accordingly, C57BL/6 mice, in which Th1 immune responses predominate, clear *Leishmania* effectively whereas BALB/c mice, which preferentially mount Th2 immune responses, are susceptible and fail to control *Leishmania* infections.

The class IA phosphoinositide 3-kinases (PI3Ks) are a family of p85/p110 heterodimeric lipid kinases that control multiple cellular processes including cell differentiation, growth, proliferation, migration, metabolism and survival [174]. There is accumulating evidence for an important role of PI3Ks in the immune response [174, 273, 274]. Mammals have 3 catalytic subunits of class IA PI3Ks (p110α, p110β and p110δ) [275] with the p110δ isoform being highly enriched in leukocytes [276]. *L. major* promastigotes can activate PI3K/AKT signaling in infected host macrophages and confer resistance to apoptosis, thereby giving ample time for parasites to complete their replication cycle [277]. In contrast, macrophage-specific inhibition of PTEN, a phosphatase that negatively regulates PI3K pathway [278, 279], abrogates efficient killing of parasites by infected macrophages [280]. Thus the PI3K pathway has been shown to either promote or impede parasite growth in macrophages in different experimental model systems. PI3Ks have also been shown to negatively regulate TLR signaling in DCs. Thus, $p85\alpha^{-/-}$ mice show enhanced production of IL-12 leading to increased Th1 responses and resistance to *L. major* infection [281]. However, $p85\alpha^{-/-}$ mice show no T cell defects and hence the role for T cell-intrinsic PI3K activity was not addressed in these studies.

By contrast, inactivation of p110δ protein by germline knock-in of an inactivating mutation (herein referred to as $p110\delta^{D910A}$) results in impaired T cell proliferation and cytokine (IL-2, IL-4 and IFN-γ) production in response to antigen stimulation in the

presence of LPS [177, 178]. However, in response to challenge by antigen adsorbed to alum, IFN-γ responses were either unaffected or slightly enhanced, probably due to impaired IL-10 production [180]. Indeed, p110δ$^{D910A}$ mice have fewer peripheral $CD4^{+}CD25^{+}Foxp3^{+}$ T cells which secrete less IL-10 and which fail to suppress colitis [177, 273]. Given that p110δ inhibition can variably block both inflammatory cytokine signaling and T cell regulation, it was difficult to predict how p110δ$^{D910A}$ mice might respond to challenge with a parasite. To address this, we investigated the outcome of infection of p110δ$^{D910A}$ mice with the intracellular pathogen, *L. major*. Our results reveal that even in the context of diminished Th1 responses, p110δ-deficiency restricts the growth of *L. major in vivo* by preventing expansion, tissue homing and effector activities of natural regulatory T cells.

# Results

## Mice with inactive p110δ PI3K are highly resistant to *L. major* infection

p110δ$^{D910A}$ mice have impaired T cell responses (proliferation and cytokine production) following polyclonal and/or model antigen stimulation [178]. Surprisingly, *L. major*-infected p110δ$^{D910A}$ mice developed smaller cutaneous lesions that resolved faster than the usually resistant C57BL/6 (WT) mice (**Fig. 17A**). The accelerated lesion resolution was accompanied by faster parasite control at infection site (**Fig. 17B**) and its draining lymph nodes (dLNs). By two weeks post-infection, p110δ$^{D910A}$ mice harbored fewer parasites than WT mice and this reduction was maintained for several weeks (**Fig. 17B**, wk 5 post-infection is shown). At 10 weeks post-infection, p110δ$^{D910A}$ and WT mice completely resolved their footpad lesion and harbor similar low but detectable numbers of parasites at the infection site consistent with the characteristic persistence of parasites in healed mice [168, 282].

The enhanced resistance in p110δ$^{D910A}$ mice prompted us to assess their T cell responses following *Leishmania* infection. Infected p110δ$^{D910A}$ mice had fewer leukocytes than WT mice in the infected footpads, dLNs, and spleens **(Fig. 17C**). Interestingly, while there was no different in the percentage of CD11b+ cells in the footpad of infected WT and p110δ$^{D910A}$ mice (**Fig. 17D**, left panel), there was a significant difference in the quality (subset) of T cell infiltration into this tissue (**Fig. 17D**, right panel). The

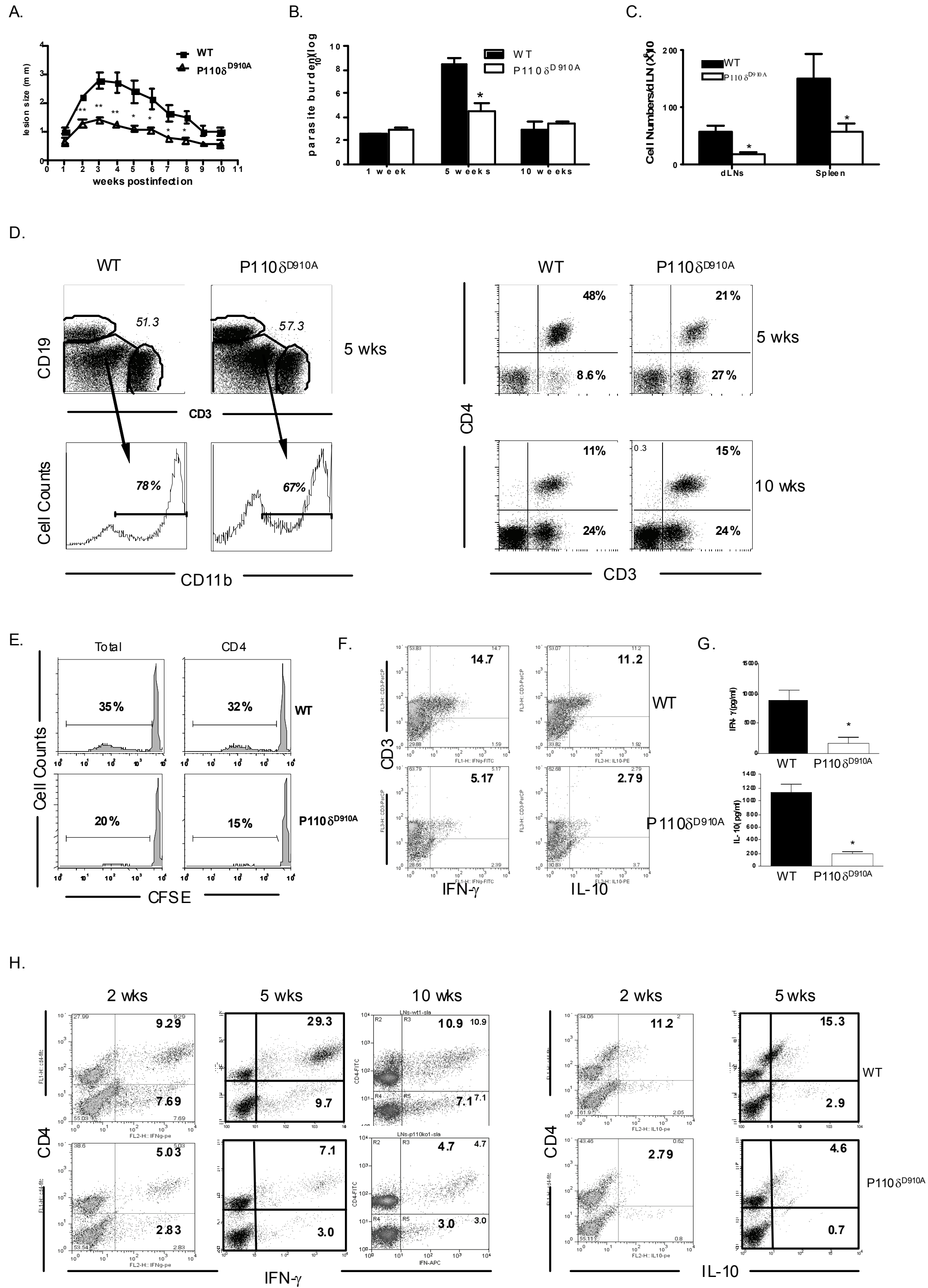

A.
B.
C.
D.
E.
F.
G.
H.
WT
P110δD910A
lesion size (mm)
weeks postinfection
parasite burden (log10)
1 week
5 weeks
10 weeks
Cell Numbers/dLN (X10)
dLNs
Spleen
CD19
CD3
5 wks
10 wks
Cell Counts
CD11b
CD4
CFSE
Total
IFN-γ
IL-10
2 wks

**FIGURE 17.** p110δ$^{D910A}$ mice are hyper-resistant to *L. major* despite having impaired T cell expansion and cytokine responses.

Kinectics and progression of cutaneous lesion **(A)** and parasite burden **(B)** in the footpads of WT C57BL/6 and p110δ$^{D910A}$ mice (n = 8), infected with $5\times10^6$ stationary phase promastigotes of *L. major*. **(C)** Total number of cells in the lymph nodes draining the infection sites (dLNs) and spleens of WT and p110δ$^{D910A}$ C57BL/6 mice at 5 weeks post-infection. **(D)** Flow cytometry of cells from *L. major*-infected footpads of WT and p110δ$^{D910A}$ mice at 5 and 10 weeks post-infection. Cells were gated as shown and non-T and B cells were further analyzed for CD11b expression. In some experiments, cells were also analyzed by for CD3 and CD4 expression at 5 and 10 wk after infection (right panel). **(E)** Antigen-specific proliferation of cells from the dLNs of WT and p110δ$^{D910A}$ mice infected with *L. major*. Cells were labeled with CFSE dye, stimulated with soluble *Leishmania* antigen (SLA) for 5 days and analyzed by flow cytometry. **(F)** Flow cytometry of dLN cells from WT and p110δ$^{D910A}$ mice infected with *L. major*. Cells were directly stained *ex vivo* for IFN-g and IL-10. **(G)** ELISA values for IFN-γ and IL-10 in culture supernatant fluids of dLN cells from infected WT and p110δ$^{D910A}$ mice restimulated in vitro with SLA for 3 days. **(H)** Flow cytometry of dLN cells from infected WT and p110δ$^{D910A}$ mice. Draining lymph node cells (obtained at different times after infection) were stimulated with SLA for 3 days, pulsed with PMA, ionomycin and brefeldin A (BFA) during the last 5 h and stained for intracellular IFN-γ and IL-10. Numbers in the box indicate % cytokine-positive cells. Results are representative of 3 independent experiments (n = 5-8 mice per group) with similar results. Error bars, +/- SEM; *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$; ns, no significant difference.

majority of T cells in the infected footpad of WT mice at 5 weeks post-infection were $CD4^+$ (85%) while only about 40% of cells recovered from footpads of infected p110δ$^{D910A}$ mice were $CD4^+$, with the majority being $CD8^+$ cells (**Fig. 17D**, right panel). However, following lesion resolution (10 weeks), the percentage of $CD4^+$ T cells in the footpad became similar (34% *vs.* 39% in WT and p110δ$^{D910A}$ mice, respectively). These data suggest that $CD4^+$ cells from infected p110δ$^{D910A}$ mice may be defective in clonal expansion in the dLNs or that their recruitment to inflamed sites is impaired. Consistent with the observed lower number of cells in the dLNs and tissues, cells isolated from infected p110δ$^{D910A}$ mice were significantly impaired in recall proliferative response to soluble leishmanial antigen *in vitro* (SLA; **Fig. 17E**) and polyclonal (anti-$CD3^+$ anti-CD28 mAb) stimulation. Interestingly, cells from p110δ$^{D910A}$ mice were significantly impaired in their ability to make cytokines, particularly IFN-γ, IL-10 and TNF (**Fig. 17F** and **G**). This impaired response was observed throughout the course of the infection (**Fig. 17H**). Thus, despite showing blunted IFN-γ response, p110δ$^{D910A}$ mice exhibit enhanced resistance to *Leishmania major*.

## The enhanced resistance of p110δ $^{D910A}$ mice to *L. major* is independent of genetic background and route of infection

Next, we investigated if the enhanced resistance in the absence of p110δ signaling is mouse-strain specific. BALB/c mice are highly susceptible to *L. major* infection because they develop strong *Leishmania*-specific T cell proliferation associated with high IL-4 and IL-10 production [287]. In contrast to WT BALB/c mice, which developed progressive and non-

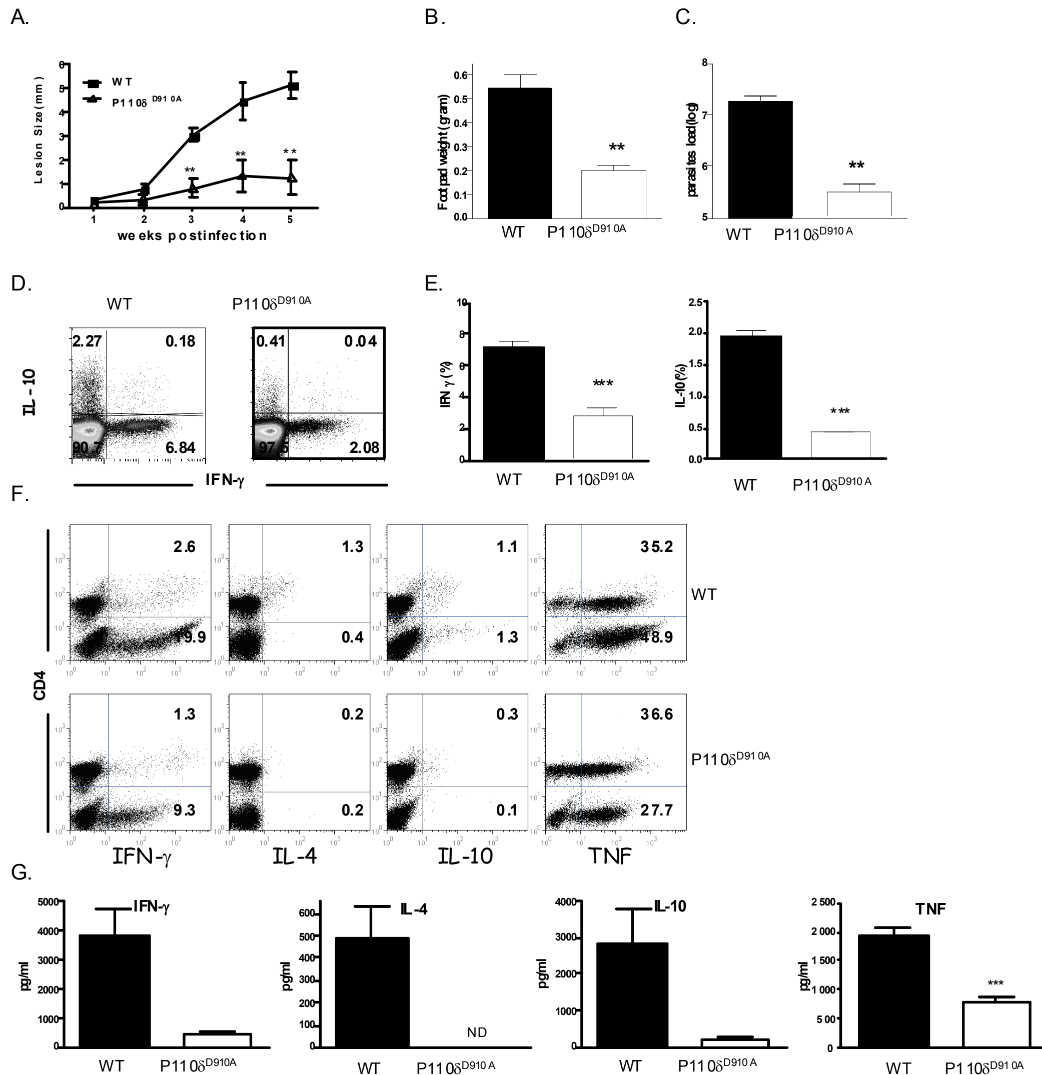



**FIGURE 18.** p110δ$^{D910A}$ mice BALB/c mice are highly resistant to *L. major* despite mounting impaired T cell responses.

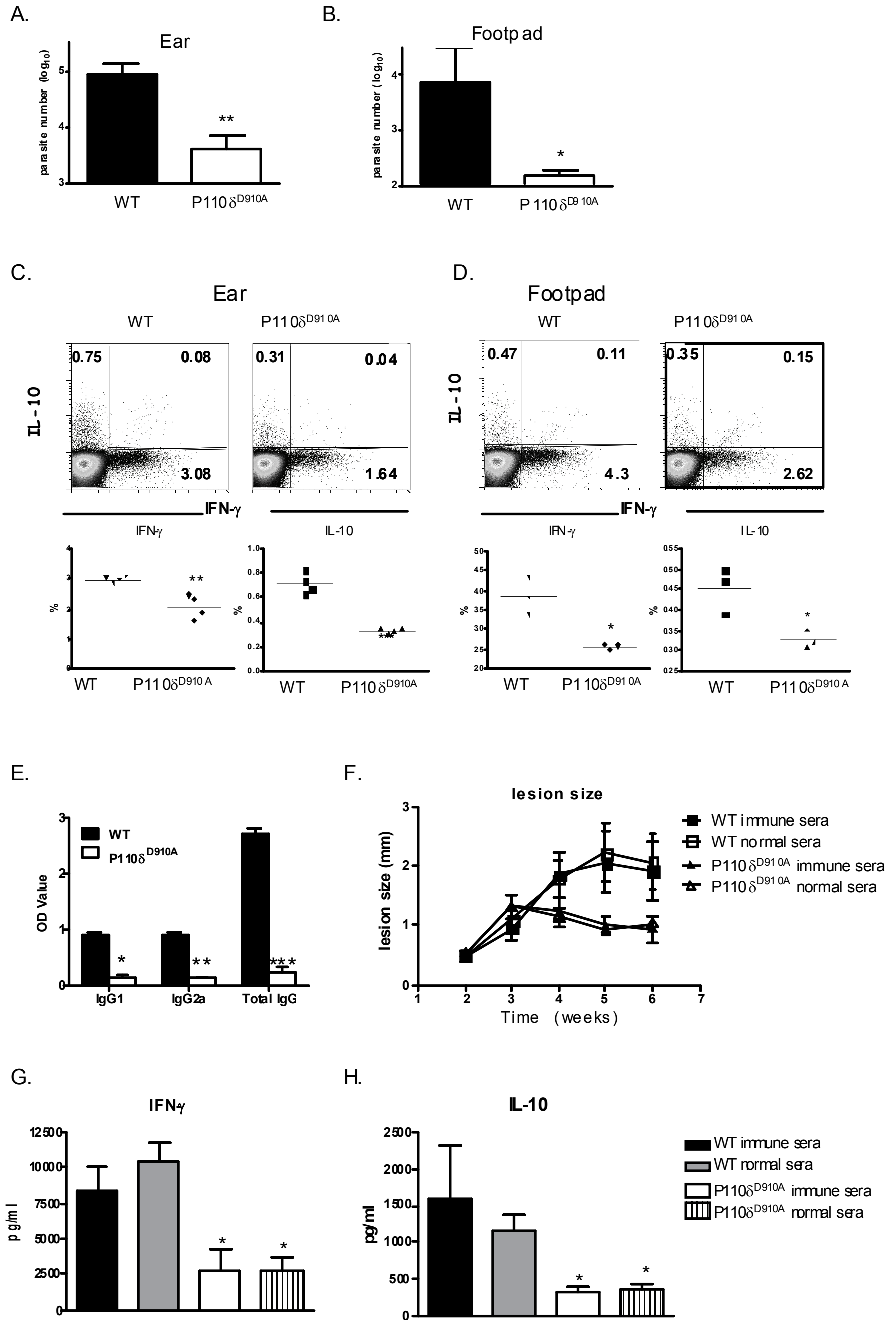
(A) Development and progression of cutaneous lesion in the footpads of WT and p110δ$^{D910A}$ mice BALB/c mice (n = 7) infected with $2 \times 10^6$ stationary phase promastigotes of *L. major*. The experiment was terminated only after 5 weeks for ethical reasons because the footpads of WT mice were ulcerated. (B) Weight of footpads of infected (5 weeks post-infection) WT and p110δ$^{D910A}$ mice. (C) Parasite burden in the footpads of infected WT and p110δ$^{D910A}$ mice (BALB/c) mice, determined by limiting dilution analysis at 5 weeks post-infection. (D-F) Flow cytometry of dLN cells from infected WT and p110δ$^{D910A}$ mice (5 weeks post-infection). Cells were directly stained *ex vivo* IFN-γ and IL-10 (D and E or restimulated in vitro with SLA for 3 days, pulsed with PMA, ionomycin and BFA for 5 hr and stained intracellularly for IFN-γ, IL-4, IL-10 and TNF. Numbers in the box indicate % cytokine-positive cells. (G) ELISA values of IFN-γ, IL-4, IL-10 and TNF in culture supernatant fluids of cells from the dLNs of infected WT and p110δ$^{D910A}$ mice (BALB/c) mice restimulated in vitro with SLA for 3 days. Results are representative of 3 independent experiments (n = 4-7 mice per group). Error bars, +/- SEM; **, $p < 0.01$; ***, $p < 0.001$.

healing ulcerative lesions, *L. major*-infected p110δ$^{D910A}$ BALB/c mice (>12$^{th}$ generation backcross) developed minimal lesions (and in some cases no lesion at all) upon infection with *L. major* (**Fig. 18A-C**, note that this experiment was terminated after 5 weeks when the footpads of WT mice began to ulcerate). Similar to infected p110δ$^{D910A}$ C57BL/6 mice, T cells from the dLNs and spleens of infected p110δ$^{D910A}$ BALB/c mice proliferated less and fewer cells produced cytokines (IL-4, IL-10, IFN-γ and TNF) after stimulation with SLA than those from WT mice (**Fig. 18D-G**). We conclude that the loss of p110δ activity is sufficient to convert the normally susceptible BALB/c mice to become resistant to *Leishmania* infection and the enhanced resistance to *L. major* following inactivation of p110δ is independent of genetic background.

Because route of *L. major* infection influences the nature of the immune response [283, 284], we investigated whether the concomitant suppression of immune response and enhanced resistance of p110δ$^{D910A}$ mice is dependent on the route of infection. As shown in **Fig. 19A-D**, the pattern of resistance and immune response in p110δ$^{D910A}$ mice following intradermal (ear) infection was similar to those seen following subcutaneous (footpad) infection, suggesting that the enhanced resistance of p110δ$^{D910A}$ mice to *L. major* is independent of route of infection.

## Impaired antibody response is not responsible for the enhanced resistance of p110δ$^{D910A}$ to *L. major*

p110δ$^{D910A}$ mice have reduced numbers of peripheral B cells as well as impaired B cell signaling leading to a reduction in serum antibody levels and total numbers of circulating



A.
Ear
parasite number ($log_{10}$)
WT
P110δ$^{D910A}$
B.
Footpad
C.
Ear
D.
Footpad
IL-10
IFN-γ
E.
OD Value
IgG1
IgG2a
Total IgG
F.
lesion size
lesion size (mm)
Time (weeks)
WT immune sera
WT normal sera
P110δ$^{D910A}$ immune sera
P110δ$^{D910A}$ normal sera
G.
IFNγ
pg/ml
H.
IL-10

**FIGURE 19.** Enhanced resistance of p110δ$^{D910A}$ mice is independent of route of infection and not related to impaired antibody response.

Parasite burden in the ear **(A)** and footpad **(B)** of WT and p110δ$^{D910A}$ mice infected with *L. major* for 5 weeks. (**C** and **D**) Frequency of IFN-γ- and IL-10-producing cells in the lymph nodes draining the ear **(C)** and footpad **(D)** of WT and p110δ$^{D910A}$ mice. Cells were directly stained ex vivo and analyzed for cytokine secretion by flow cytometry. Upper and lower panels: representative and collective dot plots, respectively. **(E-H)** Enhanced resistance of p110δ$^{D910A}$ mice cannot be reversed by administering parasite-specific immune serum. **(E)** ELISA values of total and *Leishmania*-specific immunoglobulin isotypes in the sera of WT and p110δ$^{D910A}$ mice (n = 6) infected with *L. major* for 5 weeks. (F) Kinetics and lesion sizes of WT and p110δ$^{D910A}$ mice infected with *L. major* and given 500 ml (per mouse; n = 4 normal or anti-*Leishmania* hyperimmune serum at 0, 1, 2 and 4 weeks post-infection. **(G, H)** IFN-γ and IL-10 concentration in supernatant fluids of dLN cells from infected WT and p110δ$^{D910A}$ mice given normal or hyperimmune serum; cells were restimulated *in vitro* with SLA for 3 days. Results are representative of 2-3 independent experiments (n = 3-6 mice per group). Error bars, +/- SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

plasma cells [177, 285, 286]. Serum levels of anti-*Leishmania* IgG antibodies became detectable at 2 wk post-infection infection, peaked at 5 weeks and plateaued thereafter in both WT and p110$\delta^{D910A}$ mice. Consistent with the reported impaired B cell signaling, the total IgG as well as parasite-specific IgG1 and IgG2a levels in the sera of infected p110$\delta^{D910A}$ mice were significantly lower than in WT controls (**Fig. 19E**).

High levels of anti-*Leishmania* antibodies may enhance disease by facilitating uptake of amastigotes by macrophages via the FcγRII [287, 288], although this concept has recently been challenged [122]. Nonetheless, to determine if the low antibody response (see **Fig. 19E**) contributed to the enhanced resistance of p110$\delta^{D910A}$ mice to *L. major*, we injected them with normal serum (from uninfected) or serum obtained from 6-wk *L. major*-infected WT mice (a time when anti-*Leishmania* IgG levels were highest in infected WT mice) weekly from -1 to +3 wk post-infection and compared their lesion development after infection. p110$\delta^{D910A}$ mice given either normal or infected sera were still highly resistant to *L. major* despite mounting impaired cytokine responses (Fig. 3*F-H*), suggesting that impaired B cell responses in p110$\delta^{D910A}$ mice are not responsible for their enhanced resistance to *L. major.*

## Macrophages and dendritic cells from p110$\delta^{D910A}$ mice produce more IL-12 but are not superior in controlling parasite replication *in vitro*

Next, we determined if the enhanced resistance of p110$\delta^{D910A}$ mice was related to hyperactivity of their macrophages and/or dendritic cells. Bone marrow derived macrophages (BMDMs) and dendritic cells (BMDCs) from p110$\delta^{D910A}$ mice produced comparable or higher levels of IL-12 in response to IFN-γ (**Fig. 20A**) or LPS (**Fig. 20B** and

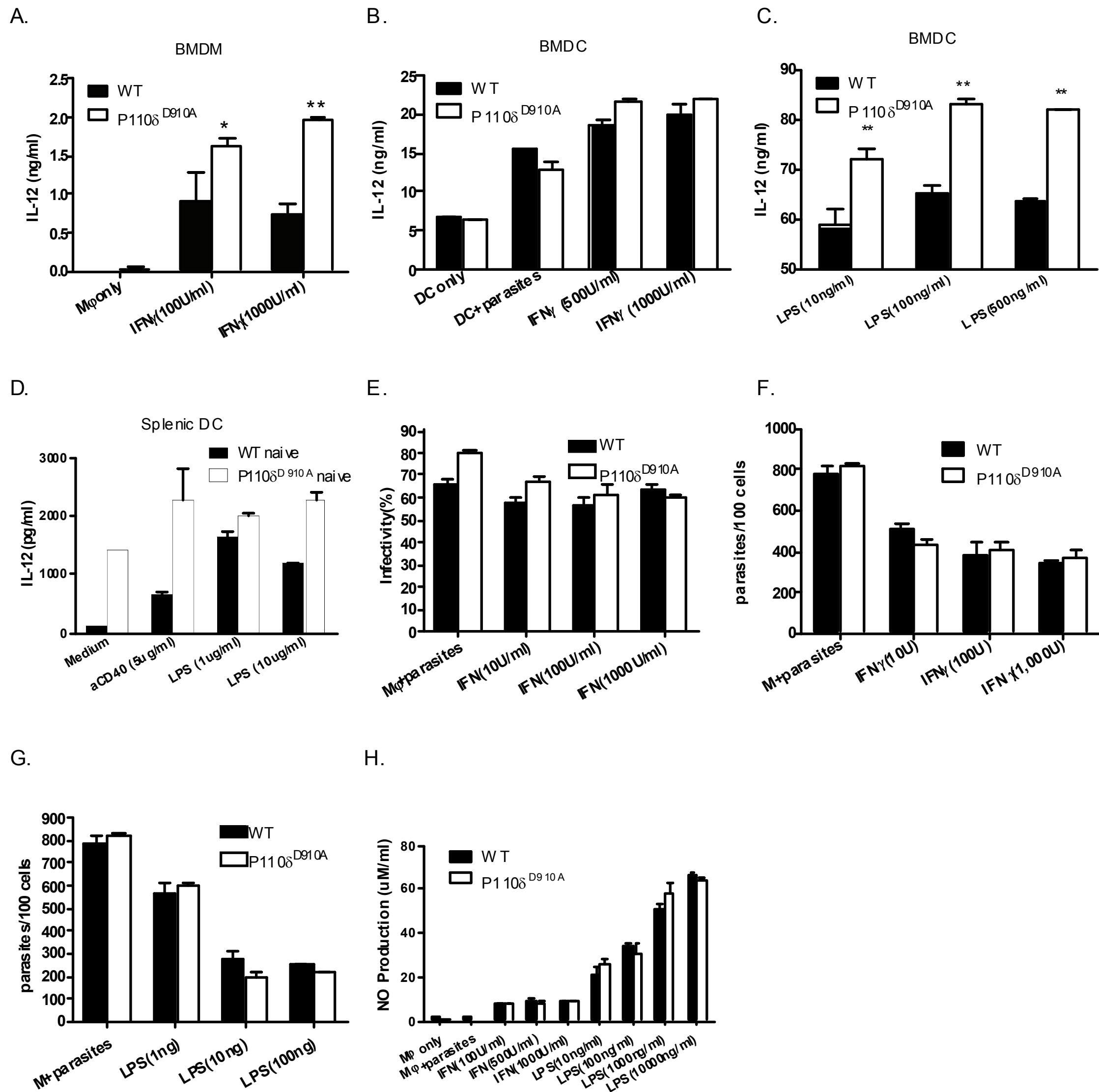



**FIGURE 20.** Enhanced resistance of p110δ$^{D910A}$ mice is not due superior parasiticidal ability of their macrophages and dendritic cells in vitro.

**(A-D)** ELISA values of IL-12 produced by BMDM **(A)**, BMDC **(B, C)** and splenic DCs **(D)** from WT and p110δ$^{D910A}$ mice after stimulation *in vitro* for 24 h with IFN-γ **(A, B)**, LPS **(C, D)** and anti-CD40 mAb **(D)**. **(E)** Percent infectivity of BMDM from WT and p110δ$^{D910A}$ mice infected *in vitro* for 72 hr with *L. major*; cytospin preparations were stained with Giemsa and counted using a light microscope. **(F, G)** Number of parasites per 100 cells (BMDM) at 72 h post infection in unstimulated and IFN-γ or LPS-stimulated cells from WT and p110δ$^{D910A}$ mice. **(H)** *L. major*-infected BMDM from WT and p110δ$^{D910A}$ mice produce comparable amounts of nitric oxide following stimulation with IFN-γ or LPS. Results are representative of 3 independent experiments. Error bars, +/- SEM; *, $p < 0.05$; **, $p < 0.01$.

**C**). Similarly, *ex vivo* splenic DCs from p110$\delta^{D910A}$ mice also produced more IL-12p40 spontaneously or upon LPS and anti-CD40 mAb stimulation than those from WT mice (**Fig. 20D**). However, both WT and p110$\delta^{D910A}$ BMDM and DMDC were equally permissive to *L. major* infection, allowing parasite proliferation *in vitro* at comparable levels (**Fig. 20E-G**), and there was no significant difference in the ability of p110$\delta^{D910A}$ and WT cells to kill *L. major* following stimulation with IFN-γ (**Fig. 20F**) or LPS (**Fig. 20G**). Consistent with this, uninfected and *L. major*-infected macrophages from WT and p110$\delta^{D910A}$ mice produced similar levels of nitric oxide and reactive oxygen radicals following IFN-γ and LPS stimulation (Fig. 20H). Taken together, these results show that the enhanced resistance of p110$\delta^{D910A}$ mice to *L. major* infection is not due to enhanced macrophage responsiveness. The reduced T cell IFN-γ response (**Figs 17** and **18**) also argues against a significant role for the enhanced secretion of IL-12 by the DCs and macrophages in this model system.

## IFN-γ and T cells mediate enhanced resistance of p110$\delta^{D910A}$ mice to *L. major*

Resistance to *L. major* is primarily dependent on IFN-γ produced by $CD4^+$ and $CD8^+$ T cells. Given that IFN-γ production by cells from *L. major*-infected p110$\delta^{D910A}$ mice is significantly impaired, we considered the possibility that this cytokine may be dispensable for resistance in absence of PI3K signaling. Therefore, we treated infected p110$\delta^{D910A}$ mice with neutralizing anti-IFN-γ antibody and assessed the outcome of infection over time. As shown in **Fig. 21A** and **B**, treatment with anti-IFN-γ mAb significantly increased lesion size and parasite burden in p110$\delta^{D910A}$ mice, suggesting that as in WT mice, IFN-γ is critically

important for resistance of p110δ$^{D910A}$ mice to *L. major*.

Many leukocyte types, including B cells and antigen-presenting cells (macrophages and DCs), express the p110δ isoform of PI3K. Because we found that the enhanced resistance of p110δ$^{D910A}$ mice to *L. major* was unrelated to differences in B cell and macrophage functions, we determined if defects in T cells are primarily responsible. We used a system where p110δ signaling is intact in leukocytes other than T cells by adoptively transferring purified CD3$^{+}$ T cells (> 99% pure) from WT and p110δ$^{D910A}$ mice into severe combined immunodeficiency (*scid*) mice that were then infected with *L. major*. As previously reported (Powrie, Correa-Oliveira et al. 1994), *scid* recipients of WT T cells became susceptible to *L. major* as evidenced by the development of large ulcerative lesions and uncontrolled parasite proliferation (**Fig. 21C** and **D**). In contrast, *scid* recipients of T cells from p110δ$^{D910A}$ mice were highly resistant, which associated with lower production of IFN-γ and IL-10 by cells from the spleen and dLNs (**Fig. 21E** and data not show). Interestingly, the expansion of CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ cells in SCID mice that received cells from p110δ$^{D910A}$ mice was severely impaired (**Fig. 21F**). Taken together, these results indicate that the defect in p110δ$^{D910A}$ mice that result in enhanced resistance to *L. major* is T cell intrinsic. They further suggest that signaling via the p110δ isoform of PI3K in T cells may be important for expansion of Treg cells in *L. major*-infected mice.

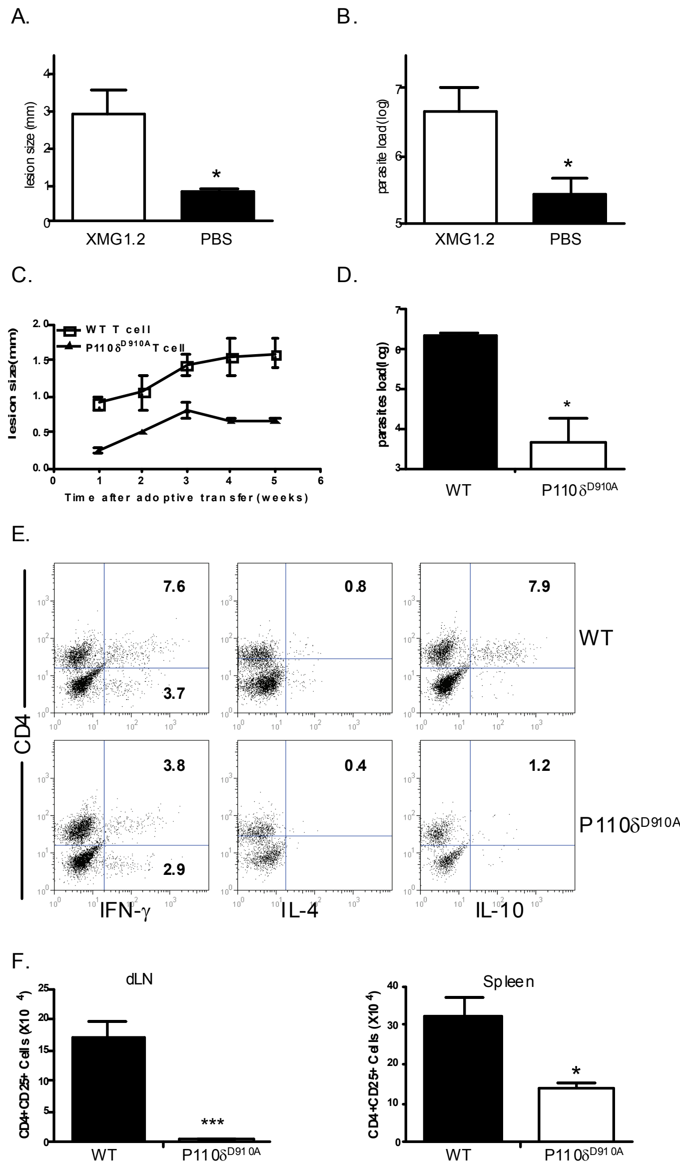



**FIGURE 21.** IFN-γ and T cells are required for enhanced resistance of p110δ$^{D910A}$ mice to *L. major*. p110δ$^{D910A}$ mice were treated with anti-IFN-γ mAb (XMG 1.2, 1 mg/mouse i.p.) or PBS one day before and once weekly after infection with *L. major*. At 6 weeks post-infection, lesion size and parasite burden were determined **(A, B)**. **(C)** Kinetics of footpad swelling in *L. major*-infected SCID mice reconstituted with highly purified (> 98%) CD3+ cells ($10^7$/mouse) from WT or p110δ$^{D910A}$ mice. CD3+ cells were purified from spleens of WT and p110δ$^{D910A}$ mice by magnetic beads using autoMACS technology, injected intravenously into recipient SCID mice that were subsequently challenged with 5 million *L. major* the next day. **(D)** Parasite burden (at 5 weeks post-infection) in the infected footpads of reconstituted SCID mice. **(E)** Impaired cytokine (IFN-γ, IL-10 and IL-4) response in SCID recipients of T cells from p110δ$^{D910A}$ mice. **(F)** Absolute numbers of CD4+CD25+ T cells in the dLNs and spleens of SCID mice reconstituted with T cells from WT or p110δ$^{D910A}$ mice and infected with *L. major*. Results are representative of 2 independent experiments (n = 4-5 mice per group). Error bars, +/- SEM; *, $p < 0.05$; ***, $p < 0.001$.

## Reduced numbers of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells in spleens, dLNs and infection sites of *L. major*-infected $p110\delta^{D910A}$ mice

$p110\delta^{D910A}$ mice have impaired expansion of regulatory T cells (Tregs) [177, 273]. Tregs have been shown to promote parasite persistence and disease chronicity following *L. major* infection [110, 270, 289]. Because we found greatly reduced numbers of CD4+CD25+Foxp3+ cells in *scid* mice reconstituted with cells from $p110\delta^{D910A}$ mice, we determined whether the enhanced resistance of $p110\delta^{D910A}$ mice to *L. major* is related to impaired induction and/or expansion of Tregs. As shown in **Fig. 22A-D**, the percentages of $CD4^{+}CD25^{+}$ T cells in the spleens, lymph nodes and infected footpads of $p110\delta^{D910A}$ mice were about 3-4-fold lower than in WT mice. Most of the $CD4^{+}CD25^{+}$ T cells in infected mice also expressed the transcription factor Foxp3 (**Fig. 22A** and **B**), a key Treg signature gene [290, 291]. Most of the IL-10-producing cells in infected mice were $CD3^{+}$ and the majority of these IL-10 producers were from the $CD4^{+}CD25^{+}$ population (**Fig. 22C**). Interestingly, the expression of Blimp-1, a T cell lineage-specific transcription factor that plays a role in function of Tregs [292, 293], was completely absent in activated T cells from $p110\delta^{D910A}$ mice (**Fig. 22E**). These results suggest that impaired expansion and/or function of Tregs may be responsible for the enhanced resistance of $p110\delta^{D910A}$ mice to *L. major*.

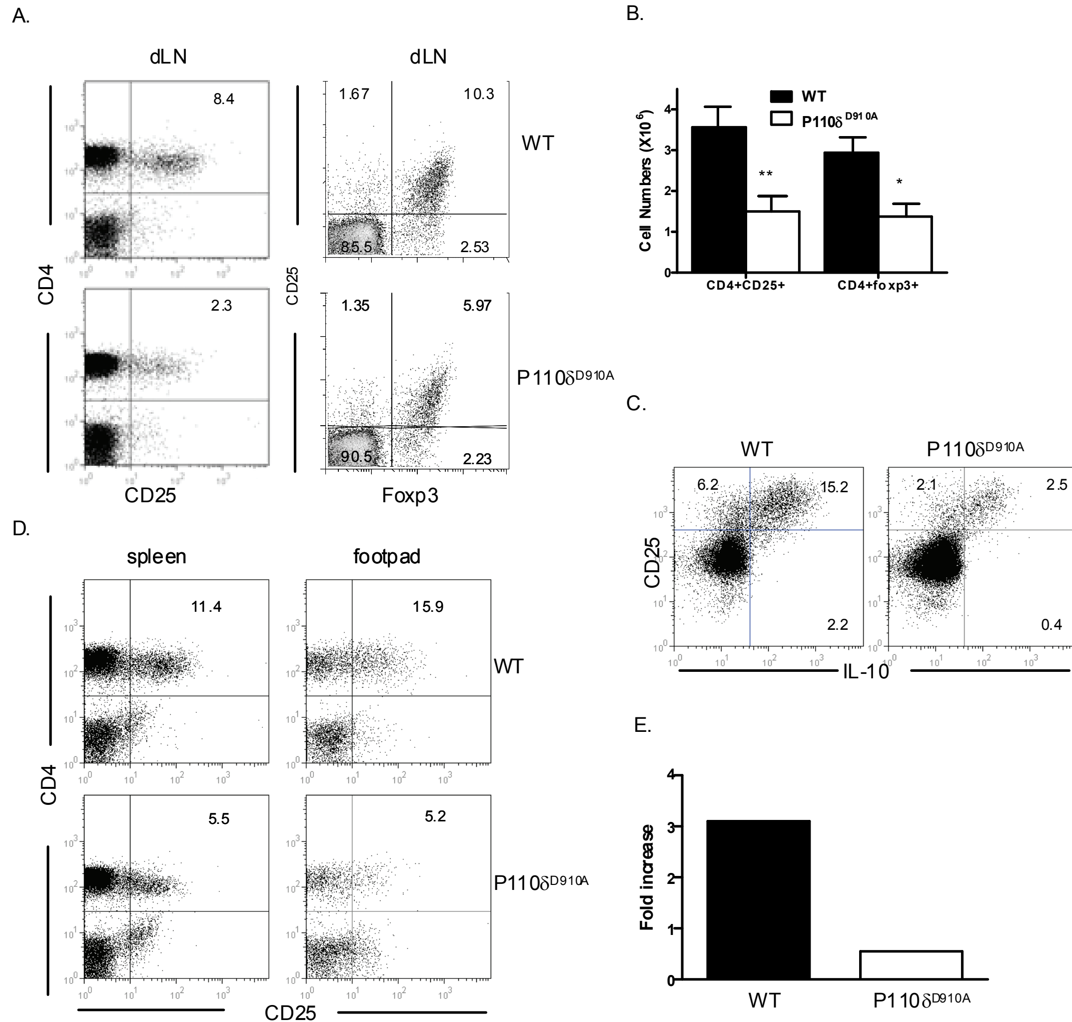



**FIGURE 22.** The p110δ isoform of PI3K regulates expansion of CD4+CD25+Foxp3+ T cells (Tregs). **(A)** Flow cytometry showing the percentage of CD4+CD25+ (left panel) and CD25+Foxp3+ (right panel) T cells in the dLNs of WT and p110δ$^{D910A}$ mice infected with *L. major* (5 weeks post-infection). **(B)** Absolute numbers of CD4+CD25+ and CD4+Foxp3+ cells in the draining lymph nodes of infected WT and p110δ$^{D910A}$ mice. **(C)** Most of IL-10-secreting CD4+ T cells in infected co-express CD25. Flow cytometry showing the percentage of CD25 and IL-10 expression by CD4+ T cells from infected WT and p110δ$^{D910A}$ mice. Numbers in the box indicate % double positive (CD25+IL-10+) cells. **(D)** Reduced number of CD4+CD25+ cells in the spleens and impaired homing at infection site in p110δ$^{D910A}$ mice. Flow cytometry showing the percentage of CD4+CD25+ in spleens and infection sites (footpad) of *L. major*-infected (5 weeks post-infection) WT and p110δ$^{D910A}$ mice. Numbers in the box indicate % double positive (CD4+CD25+) cells. **(E)** Deficiency of p110d impairs expression of Blimp-1 by T cells. WT and p110d$^{D910A}$ T cells were stimulated *in vitro* with anti-CD3/anti-CD28 and rIL-2 for 3 days and the expression of Blimp-1 mRNA was assessed by real-time PCR. Results are representative of 3 (independent experiments (n = 6-8 mice per group). Error bars, +/- SEM; *, $p < 0.05$ **, $p < 0.01$.

## Adoptive transfer of WT $CD4^{+}CD25^{+}$ T cells into $p110\delta^{D910A}$ mice abolishes their enhanced resistance to *L. major*

Next, we investigated whether the paucity of $p110\delta^{D910A}$ mice Tregs could account for their enhanced resistance to *L. major*. We injected 2 million $CD4^{+}CD25^{+}$ cells (> 98% pure) into naïve $p110\delta^{D910A}$ mice and infected them with *L. major* the next day. $p110\delta^{D910A}$ mice that received naïve or infected WT $CD4^{+}CD25^{+}$ cells lost their enhanced resistance and became susceptible to *L. major* (**Fig. 23A** and **B).** Interestingly, adoptive transfer of $CD4^{+}CD25^{-}$ cells from infected WT mice into $p110\delta^{D910A}$ mice also abolished their enhanced resistance *L. major* and this was associated with increased numbers of $CD4^{+}CD25^{+}$ cells in these mice (**Fig. 23C** and **D)**.

Next, we investigated whether impaired function of CD25+ T cells in $p110\delta^{D910A}$ mice was due to impaired expansion, intrinsic suppressive defects or both. Adoptive transfer of CD25+ cells from infected (but not naïve) $p110\delta^{D910A}$ mice into naïve $p110\delta^{D910A}$ mice also abolished their enhanced resistance to *L. major* (**Fig. 23E** and **F**), suggesting that the few CD25+ cells generated in infected $p110\delta^{D910A}$ mice are nonetheless functionally suppressive *in vivo*. In contrast to WT cells, CD4+CD25- cells from infected $p110\delta^{D910A}$ mice were unable to transfer susceptibility to naive $p110\delta^{D910A}$ mice (**Fig. 23G** and **H**).

Taken together, these results suggest that impaired expansion and recruitment of CD4+CD25+ cells (i.e. inducible Tregs) in $p110\delta^{D910A}$ mice could largely account for their enhanced resistance to *L. major*.

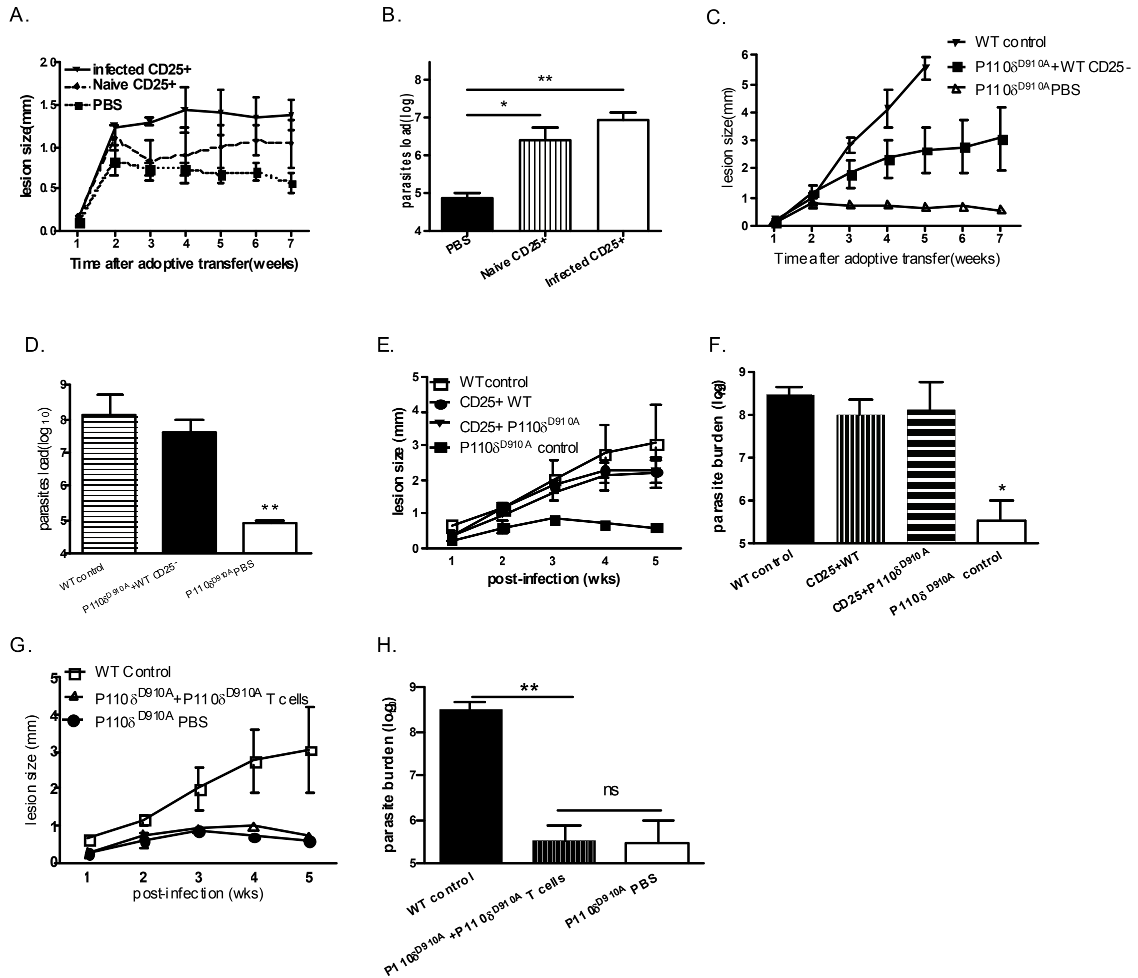



**FIGURE 23.** Adoptive transfer of CD4+CD25+ T cells from WT mice into naïve p110δ$^{D910A}$ mice abolished their enhanced resistance to *Leishmania major*.

Kinetics of footpad swelling **(A)** and parasite burden **(B)** in *L. major*-infected p110δ$^{D910A}$ mice given PBS or enriched CD4+CD25+ cells (2×10$^{6}$/mouse) from naïve or *L. major*-infected (5 weeks post-infection) mice. p110δ$^{D910A}$ mice that received CD4+CD25+ cells were infected after one day with 5 million parasites and the progression of cutaneous lesion size was measured weekly. Mice were sacrificed at 7 wk post-infection to determine parasite burden. **(C, D)** Lesion size and parasite burden in p110δ$^{D910A}$ mice that received CD4+CD25- cells from WT mice and infected with *L. major* as above. **(E, F)** CD4+CD25+ cells from p110δ$^{D910A}$ mice abolish hyper-resistance of naïve p110δ$^{D910A}$ mice to L major. CD4+CD25+ cells (2×10$^{6}$/mouse) from 5 wk *L. major*-infected WT or p110δ$^{D910A}$ mice were transferred intravenously into naïve p110δ$^{D910A}$ mice that are then challenged with *L. major*. **(G, H)** CD4+CD25- cells from infected p110δ$^{D910A}$ mice do not transfer susceptibility to naïve p110δ$^{D910A}$ mice. Lesion size and parasite burden in p110δ$^{D910A}$ mice that received CD4+CD25- cells from infected p110δ$^{D910A}$ mice. Results are representative of 2 independent experiments (n = 3-5 mice per group). Error bars, +/- SEM; *, $p < 0.05$; **, $p < 0.01$.

# Discussion

The immune response against *Leishmania major* has served as the single most important paradigm to explore the Th1-Th2 dichotomy. Th1 prone mice are protected whereas Th2 prone mice are susceptible. Our expectation was therefore that p110δ$^{D910A}$ mice, which show attenuated Th1 responses, would be susceptible to *Leishmania*. The opposite is true - they are protected against infection even in the normally susceptible BALB/c background. Our work therefore challenges the current paradigm and instead focuses the attention towards regulatory mechanisms that control inflammation. Moreover, this is the first time the inhibition of a kinase has been shown to offer protection against *leishmaniasis* (and indeed any parasitic infection). Other than going against the dogma that the quantity and quality of Th1/Th2 cell responses regulate the outcome of infection with *L. major* and perhaps other intracellular infections, our studies also highlight the importance of p110δ isoform of PI3K signaling in the regulation of T cell-mediated immunity. These findings have important and direct implications for immunomodulation and immunotherapy *in vivo*.

A robust IFN-γ response is generally believed to be critical for resistance to *L. major* [294, 295] by activating macrophages to produce nitric oxide, an effector molecule for killing intracellular parasites, and inhibition of IL-4 and IL-10 production by Th2 cells. However, more recent studies suggest that events distinct from the quantity and quality of Th1/Th2 cell responses might play a more dominant role in regulating the outcome of infection with *L. major* and perhaps other intracellular infections. We previously showed that although BALB/c mice infected with non-pathogenic phosphoglycan-deficient (termed *lpg2-*) *L. major* do not produce any significant IFN-γ recall responses, these mice were strongly protected against virulent *L. major* challenge [235]. Similarly C57/BL/6 mice

infected with *L. major* clone SD (MHOM/SN/74/SD) developed chronic non-healing lesions despite mounting a very strong *Leishmania*-specific IFN-γ responses and resolution of cutaneous lesions occurred only after blockade of IL-10 or depletion of $CD4^+CD25^+$ Tregs [296]. Together with previous studies, our data on $p110\delta^{D910A}$ mice suggest that a strong T cell and IFN-γ response may be required for resistance to *L. major* only in situations where a substantial number of Tregs are induced. In the absence of optimal Treg activation (as in $p110\delta^{D910A}$ mice), low levels of IFN-γ can more efficiently activate macrophages leading to more effective intracellular parasite killing *in vivo*. Indeed, we found that neutralization of IFN-γ by injecting monoclonal anti-IFN-γ antibody into *L. major*-infected $p110\delta^{D910A}$ mice results in progressive disease associated with uncontrolled parasite proliferation. This indicates that the low levels of IFN-γ produced the T cells in infected $p110\delta^{D910A}$ mice is nonetheless required for effective parasite control and enhanced resistance to *L. major* infection. The regulation of excessive IFN-γ production in the absence increased numbers of Tregs makes physiologic sense because this cytokine has been associated with many deleterious side effects and death in many diseases including *toxoplasmosis* [297], *trypanosomiasis* [298-300] and many viral infections [301, 302].

There is accumulating evidence suggesting that $CD4^+CD25^+$ Tregs play important roles in resistance to many pathogens [270, 289, 303]. IL-10-producing natural Tregs accumulate at the primary site of *L. major* infection in both humans and mice [110, 116, 270], mediate disease chronicity [110, 270, 289], and their depletion leads to parasite clearance [110, 115]. We found lower numbers of $CD4^+CD25^+Foxp3^+$ and antigen-specific $IL\text{-}10^+$ $CD4^+CD25^+$ T cells in the peripheral lymphoid organs and at infection sites of $p110\delta^{D910A}$ mice indicating that defects in homing, expansion and/or function of Treg cells may contribute to the

enhanced resistance of p110δ$^{D910A}$ mice to in *L. major*. However, because p110δ$^{D910A}$ mice have impaired peripheral T cell expansion *in vivo* [178] (see Figs. 2), it is possible that defective expansion of non T regs ($CD4^+CD25^-$) may also contribute to the enhanced resistance of p110δ$^{D910A}$ mice to *L. major*. Indeed, recent reports indicate that $CD4^+CD25^-$ $Foxp3^-$ IL-10-producing cells play important roles in regulating the outcome of parasitic infections including *L. major* [271] and *Toxoplasma gondii* [304]. Interestingly, while $CD4^+CD25^+$ cells from infected WT and p110δ$^{D910A}$ mice were able to transfer susceptibility to naïve p110δ$^{D910A}$ mice, only $CD4^+CD25^-$ cell from infected WT were able to transfer susceptibility to p110δ$^{D910A}$ mice and this was associated with increased numbers of $CD25^+$ cells in the lymph nodes and site of infection. These observations support the conclusion that $CD4^+$ T cells from p110δ$^{D910A}$ mice are intrinsically defective in differentiating into inducible Tregs following infection with *L. major*.

Several cell types including macrophages, neutrophils and dendritic cells express p110δ isoform of PI3K and thus could potentially contribute to the observed phenotype of p110δ$^{D910A}$ mice to *L. major* infection. Using adoptive transfer studies, we critically confirmed that defects in T cells (specifically impaired expansion of Tregs) are responsible for the enhanced resistance of p110δ$^{D910A}$ mice to *L. major*. The enhanced resistance of p110δ$^{D910A}$ mice could be reproduced in *scid* mice by adoptive transfer of highly purified $CD3^+$ cells from p110δ$^{D910A}$ mice. In contrast, transfer of $CD4^+CD25^+$ T cells from naïve (uninfected) and infected WT mice into p110δ$^{D910A}$ mice abolished their enhanced resistance leading to susceptibility to *L. major*. Although macrophages and DCs from p110δ$^{D910A}$ mice produce more IL-12 when stimulated with LPS *in vitro*, they did not produce more nitric oxide (the major effector molecule for killing intracellular *Leishmania* parasites) or reactive

oxygen species than cells from WT mice. Similarly, macrophages and DCs from p110δ$^{D910A}$ mice were equally permissive to infection and were not better at killing *L. major* than WT cells following stimulation with IFN-γ of LPS *in vitro*. Cumulatively, these observations implicate intrinsic T cell defects in p110δ$^{D910A}$ mice as the major contributor of their enhanced resistance to *L. major*.

Previously, we showed that p110δ$^{D910A}$ mice have higher numbers of CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ T cells in their thymus than WT mice [273]. By contrast, the numbers of both CD4$^{+}$CD25$^{+}$ and CD4$^{+}$CD25$^{-}$ T cells in the draining lymph nodes and spleens of infected p110δ$^{D910A}$ mice is lower than in those of infected WT mice, indicating that p110δ signaling play important role in peripheral expansion of effector and regulatory T cells. Interestingly, we found that while CD25$^{+}$ cells from naïve and infected WT mice could abrogate enhanced resistance of p110δ$^{D910A}$ mice to *L. major*, only those from infected p110δ$^{D910A}$ mice were capable of abrogating enhanced resistance of p110δ$^{D910A}$ mice to *L. major.* Thus, our data suggest a role for p110δ in peripheral induction and expansion of inducible Treg cells. In line with this, we have found that the expression of Blimp-1 is completely absent in activated T cells from p110δ$^{D910A}$ mice. T cell lineage-specific Blimp-1-deficient mice develop T cell hyper-proliferative disorders including mild colitis (similar to p110δ$^{D910A}$ mice) due to impaired expansion and function of Tregs [292, 293]. Hence, signaling via p110δ may control expansion of Tregs by regulating Blimp expression on T cells. Alternatively, signaling via p110δ may regulate other transcription factors such as Foxo3a and Foxj1, which have been shown to inhibit T cell activation and their absence results in T cell hyper-proliferation and multi-organ inflammation [305, 306]. In line with this, p110δ$^{D910A}$ mice also show a concomitant profound and generalized defective Th cell

clonal expansion and differentiation, which may help to protect them from severe autoimmune manifestations that would otherwise occur.

The finding that p110δ$^{D910A}$ mice have impaired expansion of T cells *in vivo* has important therapeutic implications for regulation and/or treatment of diseases caused by excessive or chronic activation of Th1 and Th2 cells. Indeed, pharmacologic blockade of p110δ in mice ameliorates symptoms of allergic airway inflammation and smooth muscle hyper-responsiveness [307], and selective inhibition of PI3K is proposed to yield beneficial effects in thrombosis [308] and other T cell-mediated autoimmune diseases [309, 310]. However, it is conceivable that the dampening effects on inflammation seen in *L. major*-infected p110δ$^{D910A}$ mice may be specific to this infection model. It is possible that in other inflammatory conditions, inhibition of p110δ signaling may have opposite effects from those reported here, possibly by altering recruitment and retention of inflammatory cells to inflammatory sites. Current treatment of human cutaneous *leishmaniasis* involves the use of highly toxic pentavalent antimonial compounds but treatment failures and drug resistance are common [311]. Given the dramatic hyper-resistance seen in p110δ$^{D910A}$ mice infected with *L. major*, we speculate that pharmacological inhibitors of p110δ may have beneficial effects in the treatment of human cutaneous *leishmaniasis*, despite these currently being developed with anti-inflammation as therapeutic indication [310]. Indeed, we have preliminary evidence showing that specific blockade of p110δ signaling with a pharmacologic inhibitor ameliorates disease outcome in *L. major*-infected mice.

In summary, we have demonstrated the critical importance of the p110δ isoform of PI3K in controlling the outcome of *L. major* infection by regulating the expansion of Tregs. Mice with inactive p110δ have fewer $CD4^+CD25^+Foxp3^+$ T cells and are highly resistant to

*L. major* despite having an impaired *in vivo* development of both Th1 and Th2 cells. We propose that an impaired regulatory T cell activity in p110δ$^{D910A}$ mice leads to effective and unopposed macrophage function *in vivo* resulting in more efficient parasite control and enhanced resistance to *L. major*.

# CHAPTER VI PI3K Signaling in secondary anti-*Leishmania* immune responses

## Specific Introduction

Although the type of immune response required for anti-*Leishmania* immunity is well known to involve CD4$^+$ Th1 cells that produce IFN-γ [266, 267, 312], and the pathways that lead to Th1 development are well defined, there is still no effective vaccine against human leishmaniasis. This is in part due to our poor understanding of the factors that regulate anti-*Leishmania* (in particular) and anti-protozoan (in general) memory immunity. Understanding the factors that regulate memory T cell induction and maintenance is crucial for effective vaccine designs and vaccination strategies against *leishmaniasis*.

Studies suggest that the memory T cell pool contains at least two distinct subsets [313-316]. One subset, central memory T cells (Tcm), express high levels of the adhesion molecule CD62L (L-selectin), which is responsible for their retention in peripheral lymphoid organs [313, 317]. It is these cells that are able to renew the effector T cell pool when needed. The other subset, effector memory T cells (Tem), produces effector cytokines (e.g. IL-4 or IFN-γ) upon antigenic challenge and migrates to tissues to mediate effector functions. Thus, the memory T cell pools are heterogeneous, containing cells with different migratory and effector capacities [189, 314, 318, 319].

We had previously described the existence of *Leishmania*-specific memory cells with characteristics of Tcm and Tem subsets [196]. However, the relative contribution of these memory T cell subsets in secondary anti-*Leishmania* immunity is not well understood,

although it has been speculated that Tem cells mediate rapid secondary resistance while Tcm cells act to replenish the Tem pool [320, 321]. Furthermore, the host factors that regulate the generation and maintenance of these subsets of anti-*Leishmania* memory cells are not known.

Phosphatidylinositol-3 kinases (PI3Ks) are heterodimeric lipid kinases that consist of regulatory (p85) and catalytic (p110) subunits. PI3Ks regulate multiple downstream signaling cascades by producing phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3), which can provide the binding sites for the pleckstrin homology (PH) domain containing molecules, including the serine-threonine kinase AKT [174]. Mammalian cells express three catalytic subunits of class IA PI3K namely p110α, p110β and p110δ. The p110δ isoform is mainly expressed by immune cells and is an important signaling protein for lymphocytes. Although there are several studies on the role of PI3Ks in immune responses [177, 281, 322, 323], few have focused on their role in immunologic memory. Recently, there is accumulating data suggesting that PI3K may also play an important role in the maintenance and recruitment of memory T cells [324, 325].

We previously showed that the expression of inactive form of p110δ protein by targeted knock-in mutation (referred to here as $p110\delta^{D910A}$) results in hyper-resistance to primary *L. major* infection in both the genetically resistant and susceptible strains of mice, despite having impaired IFN-γ response [326]. In the present study, we investigated the nature of secondary (memory) immunity in $p110\delta^{D910A}$ mice that have completely resolved their primary infection. We show that despite mounting superb resistance to primary *Leishmania major* infection, healed $p110\delta^{D910A}$ mice exhibited impaired secondary anti-*Leishmania* responses, manifested as poor DTH response, impaired IFN-γ recall response and absence of faster and efficient parasite control at the secondary challenge site. We further show that

*Leishmania*-reactive memory T cells from p110$\delta^{D910A}$ mice were unable to downregulate CD62L expression upon secondary *L. major* challenge and failed to home to the site of infection, suggesting that the impaired secondary anti-*Leishmania* response of p110$\delta^{D910A}$ mice was due to the inability of their Tcm to convert into Tem.

# Results

## Healed p110δ$^{D910A}$ mice contain non-functional *Leishmania*-specific memory T cells in their peripheral lymphoid organs.

Following primary infection with *L. major*, p110δ$^{D910A}$ mice developed significantly smaller lesion and harbored 100-1000-fold less parasites at the primary infection site than WT control mice at the peak of lesion development (**Fig. 24A** and **B**). However, by 10 weeks post-infection, both infected WT and p110δ$^{D910A}$ mice completely resolved their footpad lesion and harbor similar low but consistently detectable parasite load at the primary infection site (**Fig. 24A** and **B**)).

Given that p110δ$^{D910A}$ mice exhibited enhanced primary anti-*Leishmania* resistance, we hypothesized they would also mount superior secondary (memory) anti-*Leishmania* response. Therefore, we challenged healed WT and p110δ$^{D910A}$ mice with *L. major* in the contra-lateral footpad and assessed delayed-type hypersensitivity (DTH) response after 3 days and sacrificed challenged mice after 3 weeks to determine parasite burden. Surprisingly, healed p110δ$^{D910A}$ mice exhibited significantly ($p < 0.05$) weaker DTH response than their WT counterpart mice and their parasite burden was not significantly different from those of naïve (primary infection) p110δ$^{D910A}$ mice at 3 wk post-challenge (**Fig. 24C** and **D**). In contrast, healed WT mice mounted robust DTH response and significantly ($p < 0.01$) contain fewer parasites than naïve WT mice (**Fig. 24C** and **D**). In particular, the footpads of healed p110δ$^{D910A}$ mice that were challenged with virulent *L. major* contained significantly (p <

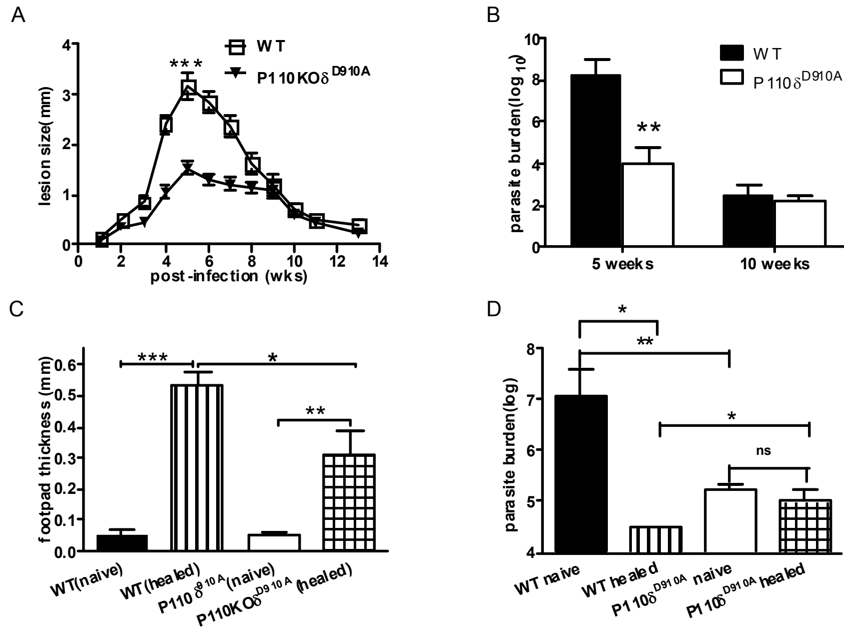



**FIGURE 24.** Impaired secondary anti-*Leishmania* immunity in healed p110δ$^{D910A}$ mice.

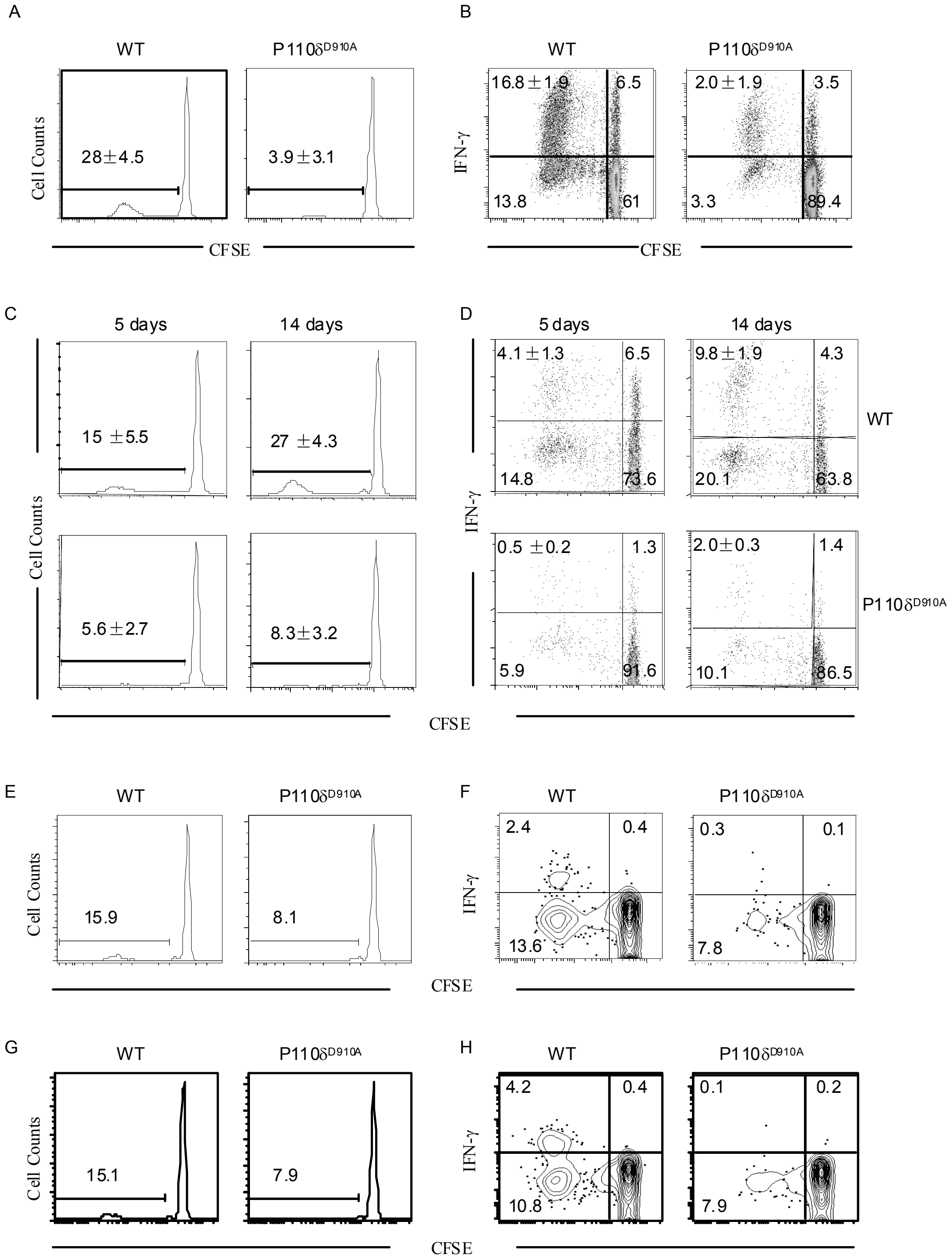
Kinetics of footpad swelling **(A)** and parasite burden **(B)** in wild type (WT) and p110δ knock-in (p110δ$^{D910A}$) mice following primary infection with *L. major*. Lesion size was monitored weekly and parasite burden was determined at 5 and 10 wk after infection. At 14 wk after primary infection, healed mice were challenged with 2 million *L. major* in the contra-lateral footpad and delayed-type hypersensitivity (DTH) was measured after 72 hr **(C)**. Three weeks after challenge, mice were sacrificed to determine parasite burden at the secondary challenge site **(D)**. Data presented are representative of 4 independent experiments (n = 4-6 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

0.05) more parasites than their WT counterpart mice (**Fig. 24D**). Collectively, these results suggest that either there are fewer numbers of *Leishmania*-specific memory cells in healed p110δ$^{D910A}$ mice or these cells are impaired or dysfunctional in their effector activities.

## *Leishmania*-reactive T cells from healed p110δ$^{D910A}$ mice are defective in proliferation and cytokine production *in vitro* and *in vivo*.

Because healed p110δ$^{D910A}$ mice are unable to mount faster protective immunity against secondary virulent *L. major* challenge, we hypothesized that effector functions of their T cells may be dysfunctional or impaired. Therefore, we assessed and compared effector cell functions (proliferative and cytokine responses) of cells from healed WT and p110δ$^{D910A}$ mice in response to *Leishmania* antigen *in vitro*. Antigen-specific proliferation (**Fig. 25A**) and IFN-γ production (**Fig. 25B**) by cells from healed p110δ$^{D910A}$ mice were significantly ($p < 0.01$) lower than those from WT mice. Furthermore, the median percentages of IFN-γ-producing proliferating cells from p110δ$^{D910A}$ mice were also significantly lower than those from healed WT mice (38% vs. 55% for p110δ$^{D910A}$ and WT mice, respectively, $p < 0.05$).

Next, we investigated whether the *in vitro* defects in effector activities of cells from healed p110δ$^{D910A}$ mice are also reproducible *in vivo*. Although we previously showed that the enhanced resistance of p110δ$^{D910A}$ mice to primary *L. major* infection is T cell intrinsic [326], we still could not completely exclude the impact of functional inactivity of p110δ protein in other immune cells on secondary anti-*Leishmania* immunity. To exclude this possibility, we adoptively transferred CFSE-labeled highly purified $CD3^+$ cells from healed WT and p110δ$^{D910A}$ mice (on a Thy 1.2 background) into naïve Thy1.1 (congenic) mice



A
WT
P110δ D910A
Cell Counts
28±4.5
3.9±3.1
CFSE
B
WT
P110δ D910A
IFN-γ
16.8±1.9
6.5
13.8
61
2.0±1.9
3.5
3.3
89.4
CFSE
C
5 days
14 days
15 ±5.5
27 ±4.3
5.6±2.7
8.3±3.2
Cell Counts
D
5 days
14 days
4.1±1.3
6.5
14.8
73.6
9.8±1.9
4.3
20.1
63.8
WT
0.5 ±0.2
1.3
5.9
91.6
2.0±0.3
1.4
10.1
86.5
P110δ D910A
IFN-γ
CFSE
E
WT
P110δ D910A
Cell Counts
15.9
8.1
CFSE
F
WT
P110δ D910A
IFN-γ
2.4
0.4
13.6
0.3
0.1
7.8
G
WT
P110δ D910A
Cell Counts
15.1
7.9
CFSE
H
WT
P110δ D910A
IFN-γ
4.2
0.4
10.8
0.1
0.2
7.9

**FIGURE 25.** Impaired *in vitro* and *in vivo* antigen-specific proliferative and IFN-γ responses by T cells from healed p110δ$^{D910A}$ mice.

Twelve wk after infection, cells from the lymph nodes draining the primary infection sites (dLN) of WT and p110δ$^{D910A}$ mice were stimulated *in vitro* with soluble *Leishmania* antigen (SLA) for 72 hr, pulsed with BFA, PMA and Ionomycin for 5 hr, stained intracellularly with fluorochrome-labeled anti-CD4 and anti-IFN-γ mAb and analyzed by flow cytometry for proliferation **(A)** and IFN-γ production **(B)**. Similar results were also obtained with spleen cells. In some experiments, CD3+ cells were purified (> 95% purity) from the dLNs and spleens of healed WT and p110δ$^{D910A}$ mice (on Thy1.2 background) by autoMACS technology, labeled with CFSE dye and adoptively transferred into naïve congenic (Thy1.1) mice. Recipient mice were challenged with *L. major* the next day and at 5 and 14 days post-challenge, mice were sacrificed and the percentages of proliferating **(C)** and IFN-γ-secreting **(D)** donor CD4+ T cells were determined directly *ex vivo* by flow cytometry. In some experiments, Thy1.2+ donor T cells from lymph nodes draining the challenge site (**E** and **F**) and spleens (**G** and **H**) were further gated on CD4 expression and analyzed for proliferation (**E** and **G**) and IFN-γ production (**F** and **H**). Data presented are representative of 3 independent experiments (n = 4-6 mice per group) with similar results.

and then challenged the recipient mice with virulent *L. major* 24 hr later. This polyclonal transfer of cells does not cause homeostatic proliferation of donor cells and has previously been used to study T cell memory in experimental cutaneous *leishmaniasis* [196]. At days 5 and 14 post-challenge, mice were sacrificed and proliferation and cytokine (IFN-γ) production by donor cells in the lymph nodes draining the challenge sites (dLN) were assessed directly *ex vivo*. As shown in **Fig. 25C** and **D**, proliferation (CFSE dilution) and IFN-γ production by total $CD3^+$ T cells from $p110\delta^{D910A}$ mice were significantly (3-5-fold) lower than those from WT mice at all times tested, suggesting that as *in vitro* (**Fig. 25A** and **B**), effector functions of memory cells from healed $p110\delta^{D910A}$ mice are also impaired *in vivo.* The defects in cell proliferation and IFN-γ production were also observed within the donor $CD4^+$ T cell populations in both the draining lymph nodes (**Fig. 25E** and **F**) and spleens (**Fig. 25G** and **H**) of challenged mice.

## *Leishmania*-reactive T cells from healed $p110\delta^{D910A}$ mice exhibit central memory-like phenotype.

The expression of CD62L defines functionally two distinct populations of memory T cells; effector memory (Tem) and central memory (Tem) cells [190]. We previously showed that Tem cells mediate faster and more effective anti-*Leishmania* immunity than Tcm cells following secondary virulent *L. major* challenge [196]. To determine whether there are differences in numbers and effector functions of these two memory T cell populations in healed WT and $p110\delta^{D910A}$ mice, we again used the congenic adoptive transfer experiment as above and examined the expression of CD62L on proliferating donor cells (from WT and $p110\delta^{D910A}$ mice) at different times after secondary *L. major* challenge. Our results show that

the majority of proliferating (*Leishmania*-experienced/reactive) cells from healed p110δ$^{D910A}$ mice were CD62L$^{hi}$ (Tcm) at Day 5 and this phenotype was maintained even at Day 14 post-challenge (**Fig. 26A**). In contrast, while the percentage of cells expressing CD62L$^{hi}$ and CD62L$^{lo}$ were almost equal in proliferating donor cells from WT mice on day 5 post-challenge, the majority (~ 2 fold) of these cells downregulated their CD62L expression and became CD62L$^{lo}$ (Tem) by day 14 post-challenge (**Fig. 26A**). Similar results were also obtained within the proliferating donor CD4$^{+}$ T cell population (**Fig. 26B**). Furthermore, intracellular cytokine staining showed that whereas the percentage of IL-2 single positive cells were similar, the percentage of IL-2/IFN-γ double positive or IFN-γ single positive cells (characteristic of Tem population [327]) were highly reduced in p110δ$^{D910A}$ donor T cells (**Fig. 26C**). These results suggest that either the majority of memory T cell subset in healed p110δ$^{D910A}$ mice bear characteristics of central memory T cells (Tcm), or that Tcm cells from p110δ$^{D910A}$ mice are defective in converting to T effector cells (i.e. downregulating CD62L expression), a crucial step that is important for homing of effector cells to tissues and for mediating rapid anti-*Leishmania* immunity.

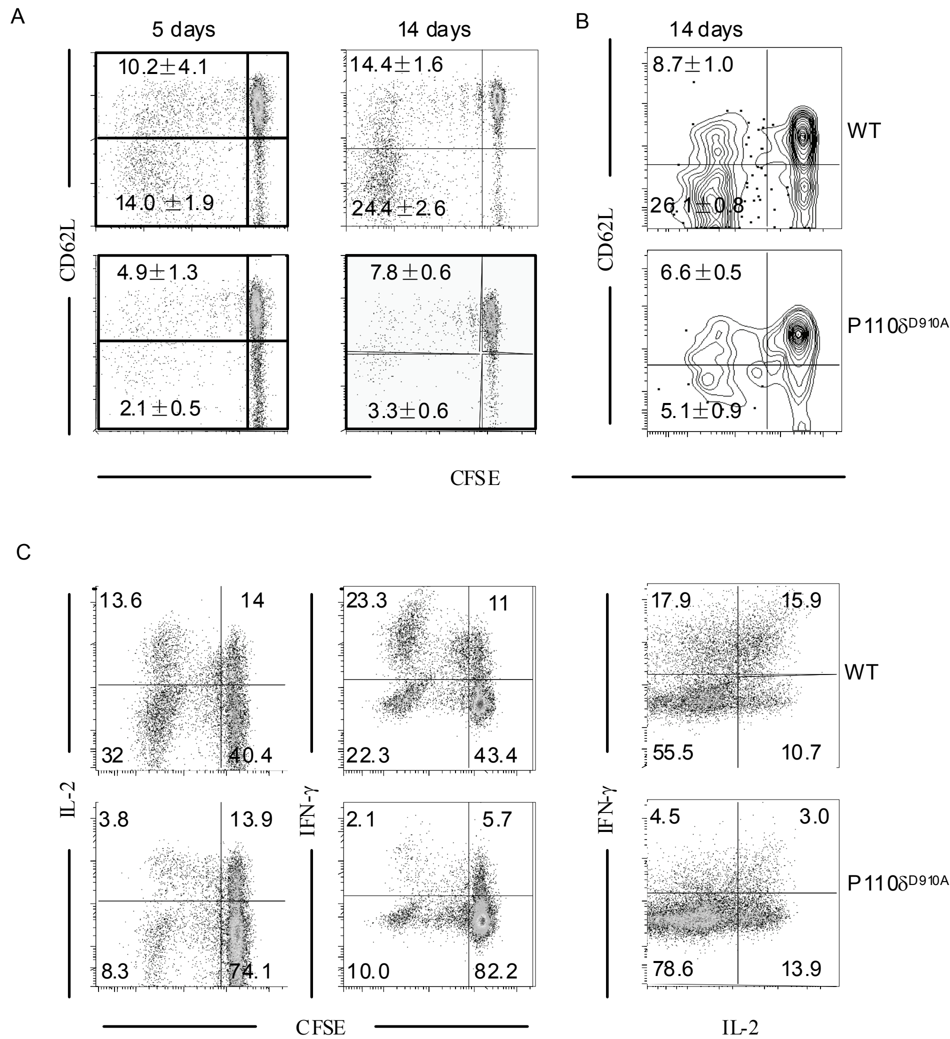
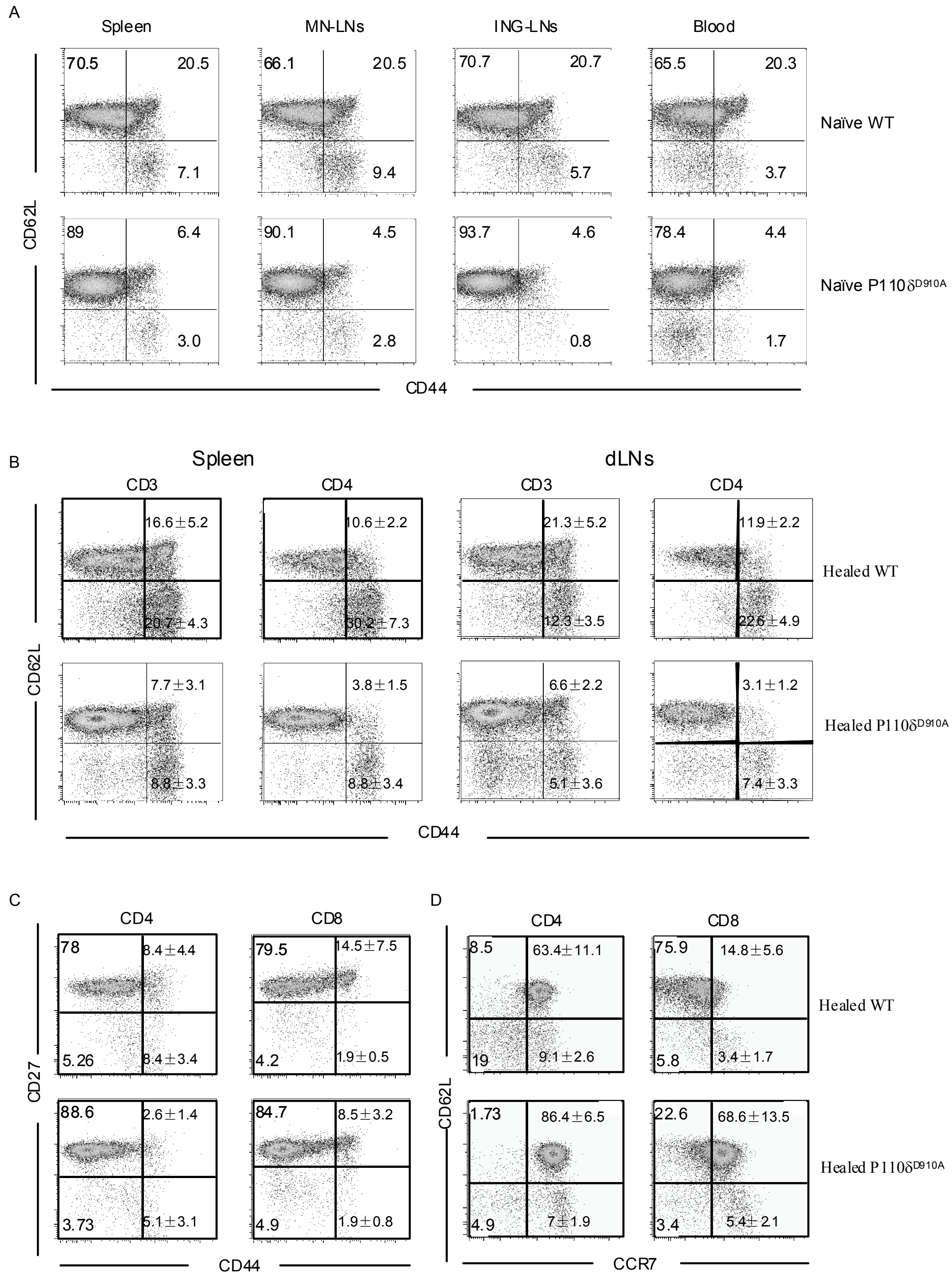



**FIGURE 26.** *Leishmania*-reactive T cells from healed p110δ$^{D910A}$ mice are phenotypically and functionally distinct from those from healed WT mice.

Naïve Thy1.1 mice adoptively received (by i.v) CFSE-labeled highly purified CD3+ T cells isolated from pooled spleen and dLN cells from healed Thy1.2 WT or p110δ$^{D910A}$ mice and then challenged with 2 million *L. major* the next day. At indicated times, mice were sacrificed and the expression of CD62L on *Leishmania*-reactive (proliferating) donor CD3+ **(A)** and CD4+ **(B)** T cells in the lymph nodes draining the challenge site was analyzed by flow cytometry directly *ex vivo*. Also, the production of cytokines (IFN-γ and IL-2, **C**) by *Leishmania*-reactive (proliferating) cells was determined by gating on donor cells (Thy1.2) cells. Data presented are representative of 3 independent experiments (n = 4-5 mice per group) with similar results.

## Naïve and healed p110δ$^{D910A}$ mice contain fewer activated and memory cells in their blood and peripheral lymphoid organs

Next, we investigated whether defective secondary anti-*Leishmania* immunity in healed p110δ$^{D910A}$ mice was also related to decreased peripheral numbers of effector and memory cells by determining the relative expression of CD44 and CD62L on WT and p110δ$^{D910A}$ T cells. CD44 is a surface protein required for lymphocyte extravasation to inflammatory sites and its upregulation is a marker of previous T cell activation and hence is expressed by all memory T cells [253]. CD62L is a lymph node homing receptor for lymphocytes, which allows them to enter the high endothelial venules and is downregulated upon activation of T cells [328]. Our results show that CD44 expression on total (CD3$^+$) and CD4$^+$ T cells in blood, spleens and peripheral lymph nodes of healed and naïve p110δ$^{D910A}$ mice was 2-4-fold ($p < 0.05$-$0.01$) lower than those from WT mice (**Fig. 27A** and **B**). Similarly, the percentage of CD44$^{hi}$CD62L$^{hi}$ cells (central memory cells) in these tissues in p110δ$^{D910A}$ mice was lower than those of WT mice (**Fig. 27A** and **B**). In contrast, the percentages of CD44$^{lo}$CD62L$^{hi}$ (naïve cells) T cells from p110δ$^{D910A}$ mice were higher than those of WT mice. Similar results were also obtained in the blood, mesenteric and inguinal lymph nodes of mice that have healed their primary *L. major* infection (**Fig. 27B**). In addition, healed p110δ$^{D910A}$ mice also have significantly lower number of cells that co-express CD44 and CD27, a T cell costimulatory molecule required for generation and maintenance of memory T cells [329] (**Fig. 27C**). Furthermore, p110δ$^{D910A}$ mice have fewer



A
Spleen
MN-LNs
ING-LNs
Blood
Naïve WT
Naïve P110δ D910A
CD62L
CD44
B
Spleen
dLNs
CD3
CD4
Healed WT
Healed P110δ D910A
C
D
CD27
CCR7
CD8

**FIGURE 27.** p110δ$^{D910A}$ mice have lower numbers of CD44$^{hi}$CD62L$^{lo}$ and CD44$^{hi}$CD27$^{hi}$ cells in their peripheral tissues.

Cells from spleens, mesenteric (MN-Ls) and inguinal (ING-Ls) lymph nodes and peripheral blood from naïve **(A)** and 12 wk-infected **(B)** WT and p110δ$^{D910A}$ mice were stained with anti-CD3, anti-CD4, anti-CD62L and anti-CD44 antibodies conjugated with different fluorochromes and analyzed by flow cytometry. In some experiments, the co-expression of CD27 and CD44 **(C)** or CD62L and CCR7 **(D)** CD4+ and CD8+ T cells was also determined. All dot plots of cells from naïve mice were gated on CD4+ T cells while those from healed mice were gated on CD3+ and CD4+ or CD4+ and CD8+cells. Data presented are representative of 2 independent experiments (n = 5-6 mice per group) with similar results.

$CD62L^{lo}CCR7^{lo}$ $CD4^{+}$ and $CD8^{+}$ T cells (**Fig. 27D**), a population that are commonly described as memory cells [190]. Collectively, these results show that $p110\delta^{D910A}$ mice have fewer numbers of memory cells and suggest that this reduction in cell number could contribute to the failure of healed $p110\delta^{D910A}$ mice to mount effective secondary anti-*Leishmania* immunity.

## The conversion from Tcm to Tem is defective in $p110\delta^{D910A}$ mice

The experiment reported in **Fig. 26** utilized unfractionated (total) T cells from healed mice, containing both $CD62L^{hi}$ and $CD62L^{lo}$ cells. Therefore, the increased numbers of $CD62L^{lo}$ effector cells in recipients of healed WT cells at day14 could simply reflect enhanced proliferation of WT $CD62L^{lo}$ T cells (see **Fig. 26**), and not necessarily due to their conversion (transition) from $CD62L^{hi}$ (Tcm) to $CD62L^{lo}$ (Tem) cells. To exclude this possibility, we transferred CFSE-labeled highly enriched (> 99% pure) $CD62L^{hi}$ or $CD62L^{lo}$ T cells from healed WT or $p110\delta^{D910A}$ mice (Thy1.2) into naïve Thy1.1 mice and then challenged the recipient mice with *L. major*. By day 5 post-challenge, donor T cells from $p110\delta^{D910A}$ mice proliferated less than those from WT mice (**Fig. 28A**), consistent with previous findings (see Fig. 2). Furthermore, most of the proliferating donor T cells remained $CD62L^{hi}$ such that 75% and 76% of proliferating cells in both recipients of WT and $p110\delta^{D910A}$ cells respectively, were $CD62L^{hi}$ (**Fig. 28B**). Thereafter, the majority of proliferating cells from healed WT mice downregulated their CD62L expression such that by day 14 post-challenge, 37% and 63% of the cells were $CD62L^{hi}$ and $CD62L^{lo}$, respectively (**Fig. 28B**). In contrast, most of the proliferating cells from healed $p110\delta^{D910A}$ mice were unable to downregulate their CD62L expression such that 71% of the cells remained

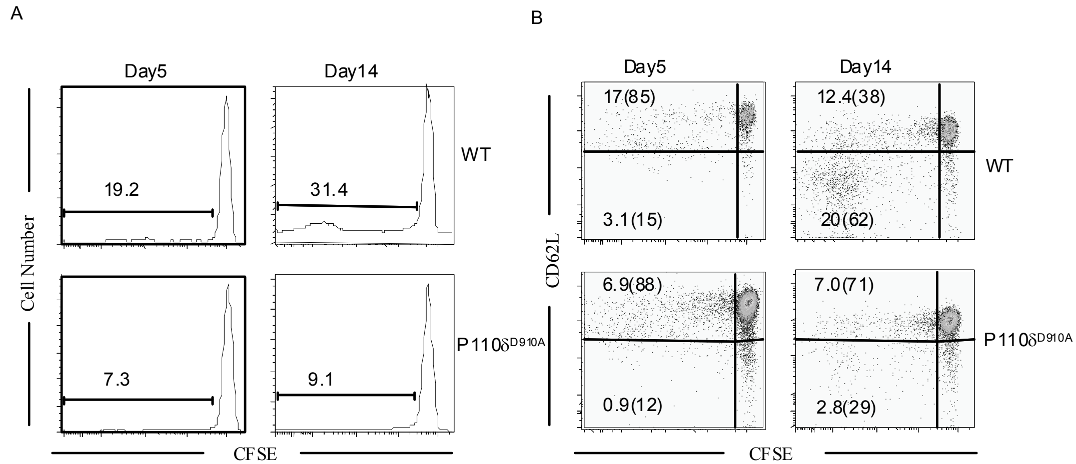



**FIGURE 28.** The transition from CD62L$^{hi}$ to CD62L$^{lo}$ cells following antigenic restimulation is impaired in the absence of p110δ signaling.

Naïve Thy1.1 mice received (by i.v. injection) CFSE-labeled highly purified (> 98% pure) CD62hi CD3+ T cells (purified by autoMACS) from healed WT or p110δ$^{D910A}$ (Thy1.2) mice. Recipient mice were challenged with $2\times10^6$ *L. major* the next day and at indicated times, mice were sacrificed and proliferation **(A)** and CD62L expression on proliferating donor cells **(B)** were determined directly *ex vivo*. All dot plots and histograms were gated on donor cells based on Thy1.2 (CD90.2) expression. Number in brackets (**Fig. B**) represents the percentage of proliferating cells that are CD62Lhi/lo at each time point. Data presented are representative of 2 independent experiments (n = 4-5 mice per group) with similar results.

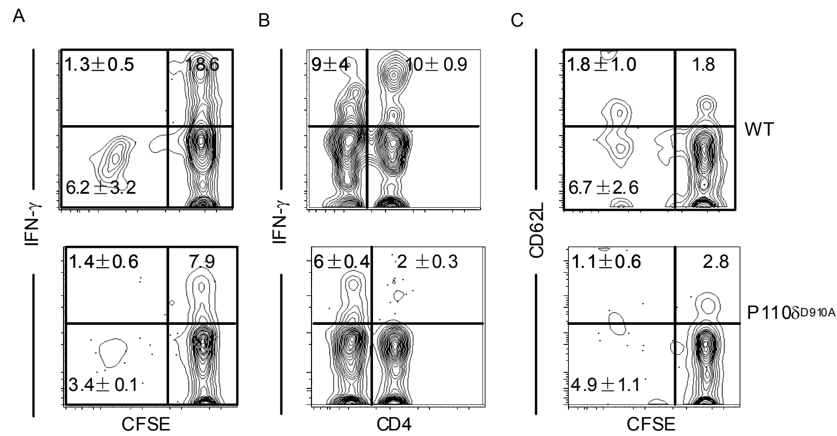



**FIGURE 29.** CD62L$^{lo}$ cells from healed p110δ$^{D910A}$ mice are defective in proliferation and IFN-? production following secondary *L. major* challenge.

Naïve Thy1.1 mice received (by i.v. injection) CFSE-labeled highly purified (> 98% pure) CD62lo CD3+T cells (purified by autoMACS) from healed WT or p110δ$^{D910A}$ (Thy1.2) mice. Recipient mice were challenged with 2×10$^6$ *L. major* the next day and after 5 days, mice were sacrificed and proliferation **(A)**, IFN-? expression **(B)** and CD62L **(C)** expression on proliferating donor cells were determined directly *ex vivo*. All dot plots and histograms were gated on donor cells based on Thy1.2 (CD90.2) expression. Data presented are representative of 2 independent experiments with similar results.

$CD62L^{hi}$ while only 29% were $CD62L^{lo}$ (**Fig. 28B**). Interestingly, proliferation and IFN-γ production by donor $CD62L^{lo}$ cells from healed $p110\delta^{D910A}$ mice were also impaired relative to $CD62L^{lo}$ WT cells (**Fig. 29A** and **29B**). However, unlike the result obtained following transfer of $CD62L^{hi}$ cells, most of the proliferating donor $CD62L^{lo}$ cells from WT and $p110\delta^{D910A}$ mice remained $CD62L^{lo}$ to a similar degree (**Fig. 29C**). Taken together, these results show that *Leishmania*-specific Tcm-like cells from healed $p110\delta^{D910A}$ mice are unable to convert to effector cells following secondary encounter with their specific antigen.

## Memory T cells from $p110\delta^{D910A}$ mice are preferentially recruited to lymphoid tissues but fail to home to cutaneous site of infection.

Due of their differential expression of CD62L, the homing properties of Tcm and Tem cells are different. Tcm cells are preferentially recruited to lymphoid organs whereas Tcm home to tissues were they mediate effector functions [190]. Because we found *Leishmania*–reactive T cells from healed $p110\delta^{D910A}$ mice express higher levels of CD62L molecule and do not downregulate this molecule following secondary *L. ma*jor challenge, we hypothesized that these cells will home preferentially to lymphoid organs. Therefore, we examined the donor T cells percentages in draining (dLN) and non-draining (non-dLN) lymph nodes and footpads of recipient mice following *L. major* challenge. By 14 days post-transfer, we found that the percentage of donor T cells from healed $p110\delta^{D910A}$ mice were either similar or slightly higher than those from WT mice in all peripheral lymphoid organs (spleens and lymph nodes), especially in non-draining lymph nodes (**Fig. 30A**). In contrast, donor T cells from healed $p110\delta^{D910A}$ mice were almost absent

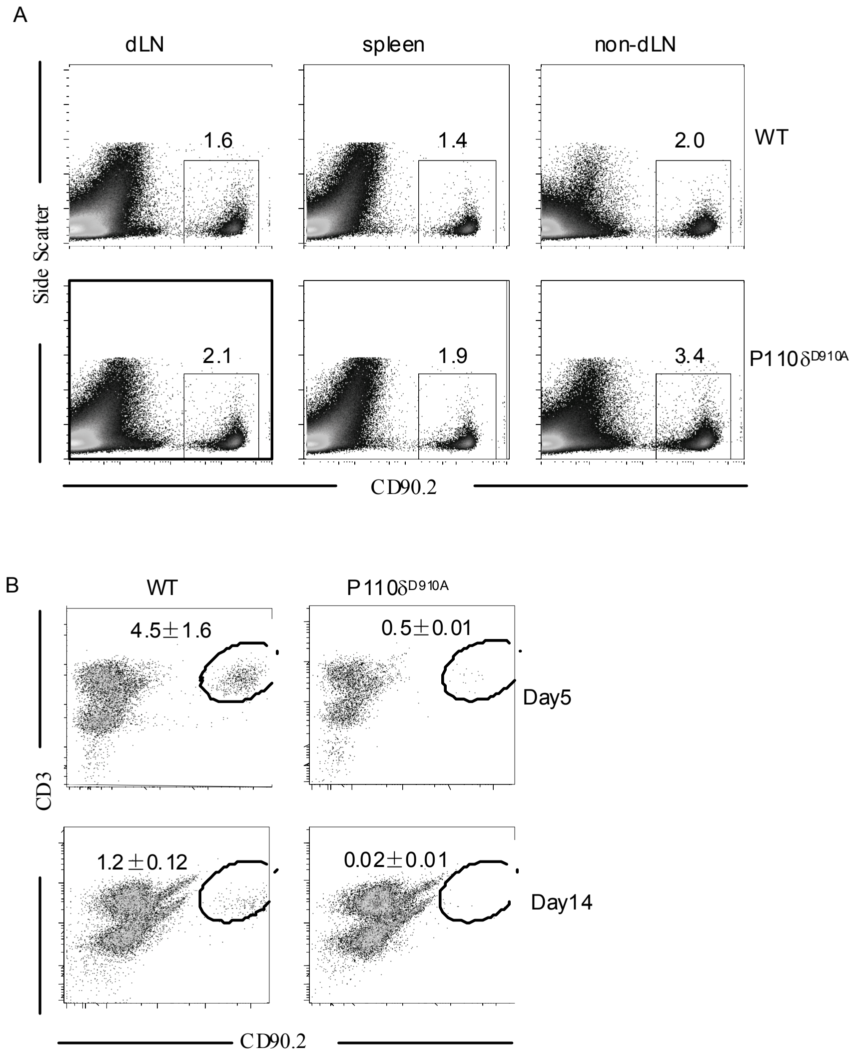



**FIGURE 30.** *Leishmania*-reactive cells from healed mice home preferentially to lymphoid tissues. Naïve Thy1.1 mice were given (by i.v transfer) CFSE-labeled highly purified CD3+ T cells (isolated by autoMACS) from pooled spleen and dLN cells of healed WT or p110$\delta^{D910A}$ (Thy1.2) mice. The recipient mice were challenged with 2 million *L. major* the next day and at indicated times, mice were sacrificed and the percentage of donor cells in the spleens, draining (dLN) and non draining (non-dLN) lymph nodes **(A)** and the footpads **(B)** was determined by gating on donor (Thy1.2) cells. Data presented are representative of 2 independent experiments (n = 4 mice per group) with similar results

in peripheral tissues (infected footpads) of recipient Thy1.1 mice (**Fig. 30B**), suggesting that memory cells from p110δ$^{D910A}$ mice are unable to exit the lymph nodes and home to the site of infection due likely to inability to downregulate CD62L and CCR7 expression (**Fig. 28B**). Collectively, these results show that the impaired secondary anti-*Leishmania* immunity in healed p110δ$^{D910A}$ mice is related to failure of their Tcm cells to proliferate, downregulate CD62L expression, home to the tissues (site of secondary infection) and mediate IFN-γ-dependent effector function.

# Discussion

Despite accelerated control of parasites and resolution of primary cutaneous lesion [265], the ability of p110δ$^{D910A}$ mice to control secondary *L. major* challenge is impaired compared to their WT counterpart mice. A detailed phenotypic analysis revealed that healed p110δ$^{D910A}$ mice have lower numbers of CD44$^{hi}$CD27$^{hi}$ T cells compared to their WT counterpart mice, suggesting the inability of healed p110δ$^{D910A}$ mice to rapidly resolve their secondary *L. major* challenge may be due to impaired generation of memory T cells. By adoptively transferring CFSE-labeled T cells from healed WT and p110δ$^{D910A}$ (Thy1.2) mice into naïve congenic (Thy1.1) mice, we were able to show that *Leishmania*-specific T cells from healed p110δ$^{D910A}$ mice were also grossly defective in terms of cell proliferation and effector cytokine (IFN-γ) production upon *L. major* challenge. Interestingly, the majority of memory T cells in healed p110δ$^{D910A}$ mice were CD62L$^{hi}$ and exhibit characteristics reminiscent of central memory T cells. Upon secondary *L. major* challenge, these cells proliferated (albeit at lower rate than those from WT mice), produced comparable amounts of IL-2 but were defective in IFN-γ production and did not downregulate their CD62L expression. Furthermore, these CD62L$^{hi}$ central memory-like T cells were unable to home to the footpads (site of infection) where parasite clearance is conducted, an effect that is consistent with their inability to downregulate CD62L expression in response to *Leishmania* antigen.

The impaired secondary *L. major* response in p110δ$^{D910A}$ mice was surprising given the fact that these mice exhibited enhanced resistance to primary *L. major* infection. We had expected similar enhanced (or at least comparable) secondary immunity following challenge

with *L. major*. Instead, healed p110δ$^{D910A}$ mice displayed lower DTH response, impaired proliferation and effector cytokine response and showed no enhanced parasite control over naïve p110δ$^{D910A}$ mice. Further analysis showed that both naïve and healed p110δ$^{D910A}$ mice have lower numbers of CD44$^{hi}$CD62L$^{lo}$ cells (effectors or effector-like memory cells) in their blood and lymphoid tissues. We demonstrated that the reduced number of CD44$^{hi}$CD62L$^{lo}$ cells was related to failure of p110δ$^{D910A}$ mice to downregulate CD62L expression, perhaps as a consequence of impaired proteolysis of CD62L ectodomain or suppression of gene transcription in the absence of p110δ signaling [330].

The concept of central (Tcm) and effector (Tem) memory T cells provides a useful framework to understand T-cell dynamics and the relative importance of secondary lymphoid organs, peripheral T cell homing and tissue localization in mediating effective protective immunity. Whereas Tem cells are able to home to peripheral tissues and mediate immediate effector functions (such as cytokine production and cytolysis of infected cells), Tcm cells home preferentially to lymphoid organs where they proliferate extensively, downregulate their CD62L expression and exit the lymphoid organs to repopulate the Tem pool [317]. We previously showed that both Tem and Tcm cells are generated following recovery from primary *L. major* infection in WT mice. While both Tcm and Tem cells can confer resistance to secondary *L. major* challenge, only Tem cells are capable of mediating rapid and more effective resistance [196]. Our present data corroborate these findings and further show that signaling via the p110δ isoform of PI3K critically regulates the generation and/or maintenance of effector and/or effector memory T cells *in vivo* following *L. major* infection by acting to downregulate CD62L expression on activated T cells.

Although the concept of Tem and Tcm cells is widely accepted, there is still controversy about the origin and lineage commitment of these cells following antigen exposure. While some studies show the origin of Tem and Tcm cells are distinct, others suggest they are from a common memory precursor and are interconvertible [314, 331]. We found that following transfer of highly enriched CD62L$^{hi}$ cells from healed WT into naïve congenic mice, the majority of these donor cells proliferated extensively following *L. major* challenge, downregulate their CD62L expression and produced high levels of IFN-γ. In contrast, cells from p110δ$^{D910A}$ mice proliferated poorly and failed to downregulate CD62L expression. This failure to generate substantial numbers of CD62L$^{lo}$ cells was associated with impaired IFN-γ production and inability to mount secondary immunity. Interestingly, CD62L$^{lo}$ cells retained their phenotype upon transfer and did not result in significant generation of CD62L$^{hi}$ cells (Tcm) following *L. ma*jor challenge (data not shown). Furthermore, we found that the proliferation and IFN-γ production by donor CD62L$^{lo}$ cells from healed mice were also impaired following secondary *L. major* challenge. Collectively, these results support the idea that effector memory T cells (CD62L$^{lo}$) are derived from central memory T cells (CD62L$^{hi}$) upon antigen restimulation. They further show that the defect in p110δ$^{D910A}$ mice is not only intrinsic to their Tcm cells and that both effector and effector memory T cells are also affected.

The impaired memory response in healed p110δ$^{D910A}$ mice was surprising given their enhanced primary resistance to *L. major* [265]. We speculate that the impaired generation of Tregs in p110δ$^{D910A}$ mice could lead to rapid parasite clearance during primary infection despite a concomitant defect in clonal T cell expansion and generation of effector T cells. This impaired generation of effector cells results in significantly lower numbers of memory

T cells in p110δ$^{D910A}$ mice. This effect when combined with defective proliferation, IFN-γ production and failure to downregulate CD62L expression by memory cells additively contribute to impaired resistance in healed p110δ$^{D910A}$ mice following secondary *L. major* challenge (see **Fig. 31**).

It has been suggested that the memory T cell lineage commitment as well as the quality of memory T cells may be influenced by initial T cell frequency and magnitude of primary T cell clonal expansion [332]. We propose that the quality and quantity of memory T cell pools generated following primary *L. major* infection is directly related to the magnitude and quality of initial T (Th1) cell responses (clonal expansion and cytokine response). Thus, the magnitude and quality of Th1 response in primary *L. major* infection are crucial determinants of optimal memory response upon secondary challenge. Following primary *L. major* infection of p110δ$^{D910A}$ mice, the magnitude and quality of primary anti-*Leishmania* response (T cell proliferation and effector cytokine production) is highly blunted [265]. This grossly impaired primary response impacts negatively on the generation of memory T cells resulting in impaired secondary anti-*Leishmania* immunity. While low proliferation and effector cytokine responses may be adequate for controlling primary infection particularly in the absence of regulatory T cells, a low response may be inadequate during secondary infection. Such differences could reflect the relative importance of regulatory T cells during primary and secondary *L. major* infections.

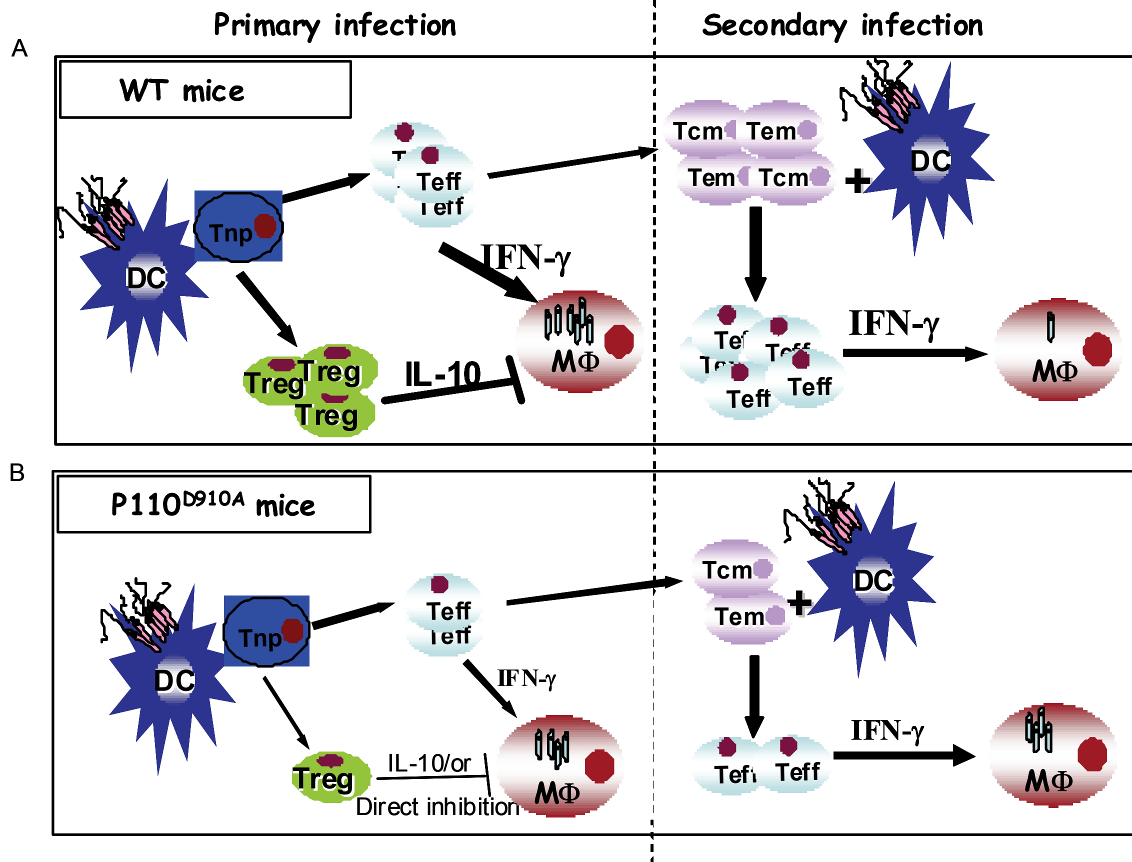



**FIGURE 31.** A simplified model to explain impaired secondary anti-*Leishmania* immunity in p110δ$^{D910A}$ mice. **(A)** Following infection of the resistant WT mice with *L. major*, dendritic cells (DC process and present *Leishmania*-derived antigens to *Leishmania*-specific naïve T (Tnp) cells. These cells undergo clonal expansion leading to the generation of IFN-γ-producing effector T (Teff) cells that activate infected macrophages (MΦ) for parasite clearance. Some Tnp cells also develop into IL-10-producing regulatory T (Treg) cells by a p110δ-influenced pathway, which limits rapid parasite clearance. Some Teff cells develop into central- (Tcm) and effector- (Tem) like memory cells. Following secondary *L. major* challenge, the memory cells proliferate rapidly and generate more IFN-γ-producing effector cells, which downregulate their CD62L expression and emigrate to cutaneous site of infection and activate macrophages for parasite clearance. **(B)** In the absence of p110δ signaling, the impaired generation of Tregs leads to rapid parasite clearance during primary infection despite a concomitant defect in clonal T cell expansion and generation of Teff cells (see ref Moulton et al. 2006, J. Immunol.). The impaired generation of Teff cells results in significantly impaired generation of Tcm and Tem cells. Furthermore, Teff cells generated from memory cells upon secondary antigenic challenge are impaired in their proliferation and IFN-γ production, do not downregulate their CD62L expression and thus are unable to exit the lymphoid organs and emigrate to site of infection to mediate rapid parasite clearance. These combined defects lead to impaired resistance in healed p110δ$^{D910A}$ mice following secondary *L. major* challenge.

In summary, we have demonstrated that the catalytic subunit p110δ of PI3K plays an important role in secondary anti-*Leishmania* immunity by regulating differentiation, expansion, effector (IFN-γ) cytokine production and interconversion of subsets of memory T cells. Furthermore, in the absence of p110δ signaling, the downregulation of L-selectin (CD62L) by proliferating *Leishmania*-reactive cells is impaired leading to defective secondary anti-*Leishmania* immunity. These collective and mutually non-exclusive defects lead to impaired resistance in healed $p110\delta^{D910A}$ mice following secondary *L. major* challenge. Collectively, these observations identify signaling via the PI3K pathway as important events in memory T cells differentiation, generation, effector function and recruitment to peripheral tissue sites, suggesting that manipulating bio-activities of PI3K pathways could provide means of enhancing generation of desired memory T cell subset and/or response during vaccination.

# CHAPTER VII General Discussion, Significance & Future Direction

In the past six years, my research has primarily focused on the complex nature of the interaction between a pathogen and its host, specifically *L. major* and inbred mouse strains. Although it is generally accepted that the outcome of experimental murine *Leishmaniasis* is dependent on both parasite *spp.* and the genetic background of the host, a number of factors involved in regulating parasite/host interaction still remain unknown. Understanding the role of host and parasite molecules in disease outcome could provide important information for the development of new drugs, new therapies and new vaccines against this disease. My overarching goal was to get a better understanding of how these genetic factors of host/parasite influence resistance and susceptibility to murine cutaneous *leishmaniasis* and to explore the possibility of using this information for effective vaccine design and vaccination strategies against the disease. To achieve this goal, *lpg2-* parasites and p110$\delta^{D910A}$ mice were used in my studies to dissect the role of phosphoglycans and PI3K, respectively in disease pathogenesis. Thus, my whole thesis can be roughly divided into two major parts. The first part focused on the role of LPG2 gene products in modulating innate and primary adaptive immunity to *L. major* infection. Studies were also designed to investigate the potential of using lpg2- deficient parasites as live-attenuated vaccine candidates by understanding the quality of memory responses induced by *lpg2-* parasites. In the second part, I investigated the possible role of host PI3K in primary and secondary anti-*Leishmania* response by using mice with inactive knock-in mutation in the catalytic subunit p110δ of PI3K.

In the first part of my study, I investigated the early host innate and adaptive immune responses to *lpg2⁻* parasites and compared them with those induced by wild type (WT), *lpg2⁻* +LPG2 gene (add-backs) and *lpg1⁻* parasites. Both our *in vitro* and *in vivo* results demonstrated that *lpg2⁻* infection resulted in qualitative and quantitative alternation of dendritic cells (DCs) function. Although *lpg2⁻ L. major* infection does not up-regulate the expression of costimulatory molecules on DCs, it significantly enhanced their production of IL-12, thereby altering the host early immune response such that ratio of IL-4 to IFN-γ production by the responding T cells is altered. The antigen presentation capacity of lpg2-infected DCs was also enhanced. Thus, in response to infection by *lpg2⁻ L. major*, DCs tend to promote a Th1 type response, while inhibiting Th2 type response. Previous reports show that *lpg2- L. major* can persist in mice for a long period without causing overt lesion [18], suggesting *lpg2-* could be a promising vaccine candidate against cutaneous *leishmaniasis*. Indeed, our data showed that *lpg2-* vaccination could generate and maintain a considerable amount of memory T cells up to 20 wks in both susceptible and resistant mice. However, the frequencies of *Leishmania*-reactive T cells (as measured by proliferation and IFN-γ production) were much lower in *lpg2-* infected mice than WT *L. major*-infected mice. This defective memory T cell response might be related to smaller burst size during primary infection because *lpg2-* parasites induced significantly lesser early cell recruitment in the local draining lymph nodes than WT parasites. Surprisingly, despite significant differences in quantity, the secondary anti-*Leishmania* immunity induced by WT and *lpg2-* parasites are qualitatively comparable. We further demonstrated that lpg2-induced resistance was IFN-γ dependent but CD8+ T cells independent. The next obvious question was why the quality of lpg2-induced secondary immunity was comparable to those induced by WT parasites despite

the inability of lpg2- parasites to induce strong IFN-γ response akin to WT parasites. Because TNF is also crucial for macrophage activation and NO-mediated killing of *Leishmania* parasites [333], we speculated that the production of TNF by *lpg2*-infected mice could compensate the defect in IFN-γ response during 2$^{nd}$ challenge. In agreement with this, our preliminary data showed that the production of TNF by cells from lpg2-infected mice was comparable to those from WT controls (Supplementary Fig. C). Taken together, our findings strongly support the consideration of *lpg2-* parasites as live attenuated vaccine candidate against cutaneous *leishmaniasis.* We propose that the mechanism of *lpg2-* parasites-mediated protection is different from that induced by WT parasites.

In the second part of my study, we investigated the crucial role of the p110δ isoform of PI3K in primary and secondary (memory) immune responses against *L. major* infection. We demonstrated that with targeted inactive knock-in mutation in the p110δ gene, one of the catalytic subunits of PI3K, (referred to as p110δ$^{D910A}$), were highly resistant to *L. major* infection, despite having impaired *in vivo* development of both Th1 and Th2 cells. Further experiments confirmed that this hyper-resistance is not due to differences in number and function of macrophages, DCs or B cells, as manipulations of these cells could not change the observed resistant phenotype of p110δ$^{D910A}$ mice. The results of transferring T cells from p110δ$^{D910A}$ mice into SCID mice indicated that this hyper-resistance of p110δ$^{D910A}$ mice to *L. major* is associated with T cells. Phenotypic analysis of T cell subsets revealed that the expansion of CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ T cells (nTregs) is highly impaired in p110δ$^{D910A}$ mice. Indeed, adoptive transfer of regulatory T cells from WT mice into naive p110δ$^{D910A}$ mice could abolish their enhanced resistance and restore their susceptibility to *L. major*.  Hence, we propose that an impaired regulatory T cell activity in p110δ$^{D910A}$ mice leads to effective

and unopposed macrophage function *in vivo* resulting in more efficient parasite control and enhanced resistance to *L. major*. These results strongly suggest that manipulation PI3K pathway might be a novel treatment for human leishmaniasis. In fact, we have preliminary evidence showing that using a specific p110δ inhibitor reduces the disease severity in *L. major*-infected mice (data not shown).

Since p110$\delta^{D910A}$ mice were highly resistant to primary *L. major* infection, one could speculate that these mice might also be resistant to secondary *L. major* challenge. Surprisingly, healed p110$\delta^{D910A}$ mice were as susceptible as naïve mice to secondary virulent challenge, suggesting that memory T cell development is defective in the absence of p110δ signaling. We further demonstrated that p110$\delta^{D910A}$ mice were not only defective in memory T cell generation but also in the conversion of central memory to effector memory T cells. In the absence of p110δ signaling, the downregulation of L-selectin (CD62L) by proliferating *Leishmania*-reactive cells is impaired leading to defective interconversion from Tcm to Tem, which is responsible for impaired secondary anti-*Leishmania* immunity. Taken together, these observations revealed that PI3K signaling pathway plays distinct roles in primary and secondary immune response against leishmaniasis, and provide unique perspectives for developing new treatment and vaccination strategy against this disease.

In summary, the significance of my study shows as follow: 1) We provided compelling evidence that LPG2 gene products direct T cell response by modulating DC functions. 2) We provided the first evidence that *lpg2-* parasites can protect C57BL/6 mice, which more closely resembles the situation in the human disease. We also proposed a distinct mechanism of the protection mediated by *lpg2-* vaccination. 3) Experimental infection of p110$\delta^{D910A}$ mice suggests that specific blockade of p110δ signaling with a pharmacologic inhibitor

might be a promising treatment for *L. major*-infection. 4) During vaccination, manipulating bio-activities of PI3K pathways might be a way to enhance generation of preferred memory T cell subset and/or response.

## Future Directions:

Although our studies reveal very important aspects of host-parasite interactions that regulate resistance to cutaneous *leishmaniasis*, several questions still remain unanswered. Further experiments need to be performed to elucidate these questions in the future studies.

We have demonstrated that *lpg2-* - mediated protection is not associated with a strong IFN-γ response, but this protection was lost by *in vivo* administration of anti-IFN-γ neutralizing antibodies. These results strongly suggest that other cytokines might play an important role in *lpg2-* parasites mediated protection. Tumor necrosis factor might be a possible player in this protection. Hence, we are currently using anti-TNF receptor blocking antibodies (Enbrel®) to treat *lpg2-* parasites vaccinated mice and assess their ability to control secondary *L. major* challenge. Nevertheless, we cannot exclude the possible role of Th2 cytokines, such as IL-4, IL-10 and IL-13, in these *lpg2-* parasites vaccinated mice. Because our results showed the early inhibition of IL-4 production in *lpg2-* parasites infected BALB/c mice, it is possible that pathogenic cytokines (or factors) have also been inhibited during $2^{nd}$ challenge. This might be another explanation why *lpg2-* parasites vaccinated mice are protected from virulent challenge without inducing a strong IFN-γ response. Since we have shown that *lpg2-* parasites vaccination can protect murine host from virulent challenge, the next question is whether this protection can be observed in other mammals, even human. Future experiments might be to test whether vaccination with *lpg2-* parasites could protect larger mammals, such as non-human primates, against virulent *L. major* challenge. This

would provide direct evidence of the efficacy of this live-attenuated parasite as a potential vaccine candidate for human cutaneous *leishmaniasis*.

Regulatory T cells development in the thymus of p110δ$^{D910A}$ mice is not defective, while they show impaired numbers and functions in periphery [197]. Whether the defect we observed in periphery is Treg intrinsic or is a consequence of interaction with other cell types is not known. Since p110δ deficiency is not restricted to T cells, we can not exclude the role of other cell types in the peripheral Treg development. T cell specific knock out might be a desirable way to exclude the influence of other cells and for more precise investigation of the role of p110δ in infectious disease. It will be also interesting to investigate the role of PI3Ks in other infectious diseases. In experimental *Trypanosoma congolense* infection, we found in contrast to hyper-resistance in *L. major* infection, p110δ$^{D910A}$ mice were more susceptible than WT controls, suggesting PI3K signaling pathway may play a distinct role in extracellular pathogen infections (unpublished observations). To make things even more complicated, our collaborators recently found that p110δ$^{D910A}$ mice were more susceptible to another intracellular pathogen- *Chlamydia pneumoniae* (unpublished observations). p110δ$^{D910A}$ mice showed functional defects in multiple cell populations, and a number of cell surface molecules are also affected by PI3K signaling, such as L-selectin (CD62L), CCR-7 and CD44. For example, L-selectin is not only indispensible for T cells but also for NK cell migration to lymph nodes [334]. It has been demonstrated that the inability to downregulation of L-selectin and CCR-7 in p110δ$^{D910A}$ mice is due to impaired preteolysis in the absence of PI3K-mTOR signaling [330]. However, there was no molecular mechanism presented for other molecules in these mutant mice, eg CD44. CD44 is an adhesion molecule that is expressed by most mammalian

cells and mediates cell-cell interaction, cell adhesion and migration via its only known *in vivo* ligand, the hyaluronic acid (HA) [335]. In the results presented in this thesis, we showed that p110δ$^{D910A}$ mice could not upregulate the expression of CD44 on their cell surfaces. A recent paper reported that CD44 is essential for the memory Th1 cells survival by limiting Fas-mediated death in Th1 cells through its engagement with PI3K signaling pathway [336]. This result further supports the idea that enhancing desired memory responses can be achieved by manipulating PI3Ks signaling pathway.

Taken together, data presented in this thesis show important aspects of the interaction between host and pathogen and these results provide important evidence to understand the molecular and cellular mechanisms underlying the interaction. The ultimate goal for my study was to provide theoretical basis for future vaccine development and vaccination strategy against infectious diseases. My current studies in *lpg2*- parasites and p110δ$^{D910A}$ mice greatly improved our knowledge in understanding of host/pathogen interaction and will shed light on future vaccine development.

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# Appendix

## **1.** List of My Publications

1. **Liu D**, Uzonna JE. The p110delta isoform of phosphoinositide 3 kinase controls the quality of secondary anti-Leishmania immunity by regulating expansion and effector function of memory T cell subsets. ***J Immunol.*** 2010 Mar 15;184(6):3098-105.

2. Okwor I, **Liu D**, Beverley SM, Uzonna JE. Inoculation of killed Leishmania major into immune mice rapidly disrupts immunity to a secondary challenge via IL-10-mediated process. ***Proc Natl Acad Sci U S A***. 2009 Aug 18;106(33):13951-6.

3. **Liu D**, Zhang T, Marshall AJ, Okkenhaug K, Vanhaesebroeck B, Uzonna JE. The p110delta isoform of phosphatidylinositol 3-kinase controls susceptibility to Leishmania major by regulating expansion and tissue homing of regulatory T cells. ***J Immunol***. 2009 Aug 1;183(3):1921-33.

4. **Liu D**, Kebaier C, Pakpour N, Capul AA, Beverley SM, Scott P, Uzonna JE. Leishmania major phosphoglycans influence the host early immune response by modulating dendritic cell functions. ***Infect Immun***. 2009 Aug;77(8):3272-83.

5. Okwor I, **Liu D**, Uzonna J. Qualitative differences in the early immune response to live and killed Leishmania major: Implications for vaccination strategies against Leishmaniasis. ***Vaccine***. 2009 Apr 28;27(19):2554-62.

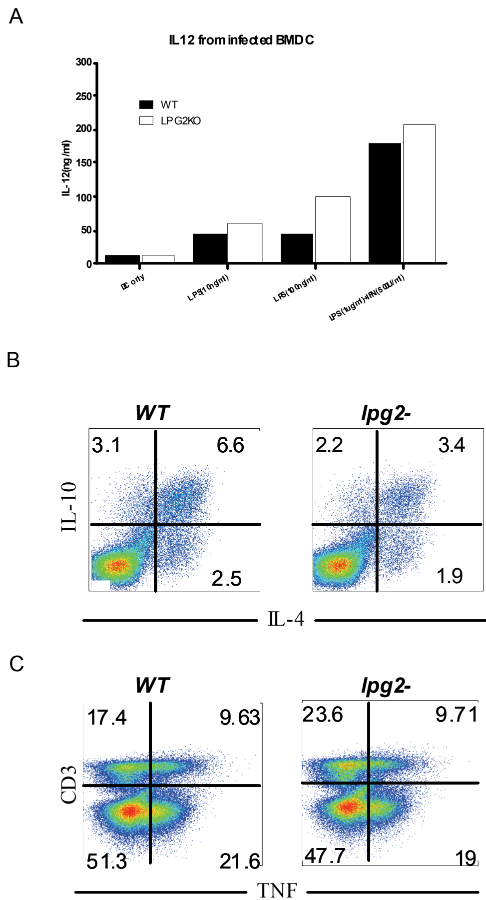
6. Xu G, **Liu D**, Okwor I, Wang Y, Korner H, Kung SK, Fu YX, Uzonna JE. LIGHT Is critical for IL-12 production by dendritic cells, optimal CD4+ Th1 cell response, and resistance to Leishmania major. ***J Immunol***. 2007 Nov 15;179(10):6901-9.

7. Xu G, **Liu D**, Fan Y, Yang X, Korner H, Fu YX, Uzonna JE. Lymphotoxin alpha beta 2 (membrane lymphotoxin) is critically important for resistance to Leishmania major infection in mice. ***J Immunol***. 2007 Oct 15;179(8):5358-66.

**Paper in Preparation**

Enhanced Secondary anti-*Leishmania* (memory) Immunity by LPG2 Gene Products without a strong IFN-γ response. *Liu D, Mou Z, Okwor I, Uzonna JE.* 2011 ***Ready for submission***

# **2.** Supplementary Figures




**SUPPLEMENTARY FIGURES**

**A.** *L. major* glycoconjugate molecules influence IL-12 production by BMDCs. Some uninfected and infected BMDCs were stimulated with varying concentrations of LPS for 24 hours and the culture supernatant fluids were collected and assayed for IL-12p40 by ELISA. Data presented are representative of 3 independent experiments with similar results. * $p < 0.05$, ** $p<0.01$.

**B.** Differences in the early immune response in mice infected with *lpg2- L. major*. BALB/c mice were infected in the footpad with 5 million WT and *lpg2- L. major* stationary phase promastigotes. After 3 days, mice were sacrificed, some unfractionated dLN cells were stimulated with SLA for 3 days and the percentage of IL-4- and IL-10-producing cells was determined by intracellular cytokine staining.

**C.** >16 wks post infection, draining lymph node cells and splenocytes from *lpg2-* infected BALB/c mice were restimulated *in vitro* with PMA, Ionomycin in the presence of Brefeldin A for 6 hrs. The intracellular TNF was determined by flow cytometry.