

## ABSTRACT

Title of Dissertation: ANTIBODY RESPONSES DURING INFECTION WITH *LEISHMANIA SPP.*

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Classically activated macrophages produce high levels of IL-12 and moderate levels of IL-10. When IgG immune complexes ligate the Fc $\gamma$  receptors on activated macrophages, they shut off IL-12 synthesis and increase IL-10 production. These macrophages are termed 'type II' activated macrophages. The anti-inflammatory responses of type II activated macrophages could be harmful to the host when an inflammatory response is critical to pathogen clearance. The clearance of intracellular pathogens, such as *Leishmania spp.*, relies upon an inflammatory response. Inflammatory cytokines lead to classical macrophage activation, which is critical to the destruction and clearance of phagocytosed parasites. Without a robust inflammatory response, *Leishmania* parasites are free to replicate within macrophages, leading to disease pathology. Based on this knowledge, it was hypothesized that IgG coated *Leishmania* amastigotes would ligate Fc $\gamma$  receptors on host macrophages and induce the production of IL-10. Therefore, the role of IgG in mediating host defense to *Leishmania* was examined. For these studies, J<sub>H</sub> mice, which lack IgG and are on the *Leishmania major* susceptible BALB/c background,

were used. We show that IgG not only fails to provide protection against this intracellular pathogen, but it actually contributes to disease progression. J<sub>H</sub> mice were more resistant to disease than control BALB/c mice, and reconstitution with  $\alpha$ -*L. major* antisera resulted in increased disease, with larger lesions and higher numbers of parasites. Antibody administration correlated with an increase in IL-10 production in lesions, however, this IgG mediated exacerbation of disease could be reversed by simultaneously treating with  $\alpha$ -*L. major* antisera and a monoclonal antibody against the IL-10 receptor. The  $\alpha$ -*L. major* IgG profiles of resistant C57BL/6 mice and susceptible BALB/c mice were examined. BALB/c mice exhibited higher levels of IgG, in both circulating titers and on the surfaces of amastigotes, than C57BL/6 mice. The differences in the  $\alpha$ -*L. major* IgG titers between resistant and susceptible strains provide evidence of the integral role of IgG during leishmaniasis. These studies demonstrate that IgG can cause a novel form of immune enhancement due to its ability to induce IL-10 production from macrophages.

ANTIBODY RESPONSES DURING INFECTION WITH *LEISHMANIA SPP.*

By

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## DEDICATION

This dissertation is dedicated to my Mom, and my grandparents (Mamaw Janice and Papaw Jimmy). Most people have two parents, I was lucky enough to have three. I couldn't have done this without you. I always knew that you all believed in me, even when I didn't. Thank you so much for everything you've given me (and for financially supporting me to the ripe old age of 27....sorry about that!). I love you all very much!

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## LIST OF ABBREVIATIONS

|   |               |
|---|---------------|
| alkaline phosphatase                      | AP            |
| antibody dependent immune enhancement     | ADE           |
| antigen presenting cell                   | APC           |
| axenic amastigote                         | AA            |
| B cell receptor                           | BCR           |
| bone marrow derived macrophage            | BMM $\phi$    |
| CD4 <sup>+</sup> T helper cell type 1     | Th1           |
| CD4 <sup>+</sup> T helper cell type 2     | Th2           |
| cutaneous leishmaniasis                   | CL            |
| cytomegalovirus                           | CMV           |
| cytotoxic T lymphocyte                    | CTL           |
| delayed-type hypersensitivity             | DTH           |
| dendritic cell                            | DC            |
| Dulbecco's Modification of Eagle's Medium | DMEM          |
| Enzyme Linked Immunosorbent Assay         | ELISA         |
| Epstein-Barr virus                        | EBV           |
| Fc receptor                               | FcR           |
| Fc $\gamma$ receptor                      | Fc $\gamma$ R |
| fluorescein isothiocyanate                | FITC          |
| heat inactivated fetal calf serum         | HI-FCS        |
| human IL-10                               | hIL-10        |

|                                       |               |
|---------------------------------------|---------------|
| immunoglobulin                        | Ig            |
| immunoglobulin G                      | IgG           |
| incomplete Freund's adjuvant          | IFA           |
| inducible NO synthase                 | iNOS          |
| interleukin                           | IL            |
| IL-10 receptor                        | IL-10R        |
| interferon $\gamma$                   | IFN- $\gamma$ |
| Langerhans cell                       | LC            |
| lipopolysaccharide                    | LPS           |
| lipoteichoic acid                     | LTA           |
| low molecular weight-hyaluronic acid  | LMW-HA        |
| macrophage                            | M $\phi$      |
| macrophage colony stimulating factor  | m-CSF         |
| major histocompatibility complex      | MHC           |
| multiplicities of infection           | MOIs          |
| natural killer cell                   | NK            |
| optical density                       | OD            |
| ovalbumin                             | OVA           |
| pathogen associated molecular pattern | PAMP          |
| peripheral blood mononuclear cell     | PBMNC         |
| phycoerythrin                         | PE            |
| p-nitrophenyl phosphate               | PNPP          |
| Schneider's complete medium           | SCM           |

|                              |        |
|------------------------------|--------|
| soluble leishmania antigen   | SLA    |
| systemic lupus erythematosus | SLE    |
| T cell receptor              | TCR    |
| T regulatory cell            | Treg   |
| toll-like receptor           | TLR    |
| tumor necrosis factor        | TNF    |
| type II complement receptor  | CR2    |
| viral IL-10                  | vIL-10 |
| visceral leishmaniasis       | VL     |

# CHAPTER 1: INTRODUCTION

## INNATE IMMUNITY

### Overview

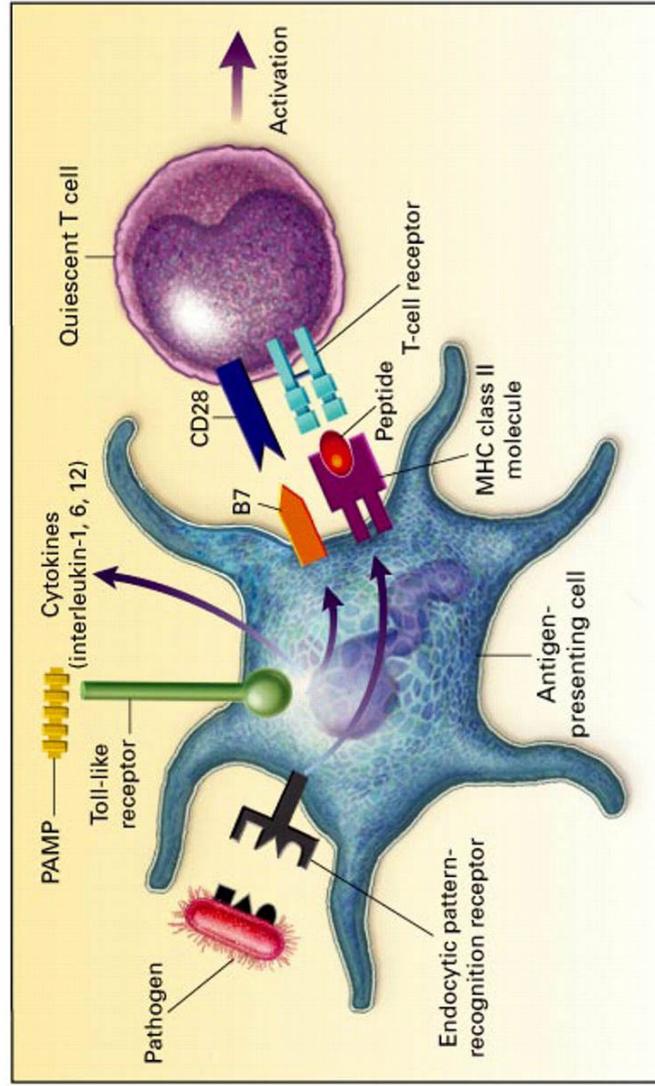
The immune system is divided into innate and acquired components, each with distinct immunological functions. Innate immunity is phylogenetically the oldest mechanism of defense against infection, and is present in all multicellular organisms<sup>1</sup>. Acquired immunity relies upon T and B lymphocytes, and their ability to recognize a large number of diverse antigens, based on their individual expression of rearranged antigen receptors. Following antigen recognition, lymphocytes are activated and proliferate in a process termed clonal expansion. Although this process is necessary for an efficient immune response, the time required for lymphocyte expansion and differentiation to occur allows pathogens to infect and damage the host. This window of susceptibility is covered by innate immunity<sup>2</sup>.

Innate immunity is the first line of defense. It is rapidly activated by microbes, and in some situations, it can eliminate them. The effectors of innate immunity, composed primarily of complement, cytokines, neutrophils, macrophages (M $\phi$ ), and natural killer (NK) cells, are immediately available upon encountering an antigen.

The cells of the innate immune system have inheritable germ line encoded 'pattern recognition receptors', which allow these cells to recognize a number of highly conserved patterns that are associated with microorganisms

exclusively. These patterns, termed pathogen associated molecular patterns (PAMPs)<sup>3</sup>, include lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan, unmethylated CpG nucleotides, and mannans. These molecules are characteristics of microbial pathogens, and are not typically found on mammalian cells. This allows the components of the innate immune response to distinguish between self and non-self. Because these molecules are essential to microbial survival, the microbes cannot simply discard them in an attempt to go unnoticed by the immune system.

These pattern recognition receptors are found on many effector cells of the innate immune system, and their expression is not clonal. Pattern recognition receptors fall into three categories, secreted, endocytic, and signaling. Secreted receptors, such as mannan-binding lectin, opsonize microorganisms and allow for recognition by complement and phagocytes. Endocytic receptors, such as the macrophage mannose and scavenger receptors, are expressed on the surface of phagocytes. These receptors recognize PAMPs and bind the organism for uptake and delivery to the lysosome<sup>2</sup>. Signaling receptors, such as toll-like receptors (TLRs)<sup>4</sup>, recognize PAMPs and activate signal transduction pathways that induce the expression of genes involved in the immune response against the microorganism. TLRs also induce the upregulation of co-stimulatory molecules, which provide the secondary signal necessary for the activation of the cells of the adaptive immune response (Figure 1). Thus, the adaptive response depends on innate immunity, and only occurs after a pathogen has first been detected by the



**Figure 1. Pathogen Detection by the Innate Immune System Activates the Adaptive Immune System.** PAMPs recognized by pattern recognition receptors, such as TLRs and endocytic receptors, generate the signals necessary for the activation of the adaptive immune response. From Medzhitov and Janeway, Jr., 2000.

innate immune system. Through the recognition of microbes and the activation of signal transduction pathways, the innate immune system triggers and controls the major aspects of the adaptive immune response<sup>2</sup>.

### Macrophages

Macrophages have a central role in both the innate and adaptive immune responses, and are important for the elimination of microbes. They can be found at all the sites where a microbe could gain entry to the host, and are resident in the liver, spleen, subepithelial connective tissue, lymphatics, and the interstitial of parenchymal organs. These cells express a variety of surface receptors, such as Fc $\gamma$ , complement, scavenger, TLRs, and mannose, which allow efficient binding and phagocytosis of pathogens. Microbes are ingested into phagosomes, which subsequently fuse with lysosomes to create phagolysosomes. Inside the phagolysosomes, microbes come into contact with proteolytic enzymes, as well as a lowered pH, which contribute to the destruction of the organisms. Certain pathogens avoid killing by preventing phagosomes-lysosome fusion (*T.gondii*), preventing phagolysosome acidification (*Chlamydia spp.*), escaping into the cytosol (*L. monocytogenes*), or thriving in acidified vacuoles (*Leishmania spp.*)<sup>5</sup>.

Opsonization with immunoglobulin G (IgG), in most cases, targets particles to Fc receptors, where they enter phagolysosomes, trigger a respiratory burst, and are destroyed<sup>6</sup>. The Fc receptor (FcR) is composed of a

region that binds the Fc portion of antibody molecules, called the  $\alpha$  chain, associated with one or more signal transduction regions, the  $\beta$  and  $\gamma$  chains. The  $\gamma$  chain is a 7 kD polypeptide that is homologous to the  $\zeta$  chain of the T cell receptor. There are three different receptors for the Fc portion of IgG, termed Fc  $\gamma$  receptors (Fc $\gamma$ Rs). These receptors play a critical role in many immune responses, such as phagocytosis, respiratory burst, cytokine production, and clearance of immune complexes<sup>7</sup>. Fc $\gamma$ RI, also known as CD64, is the high affinity receptor, and can bind monomeric IgG. In the human system, this receptor binds IgG1 and IgG3 tightly, while in the mouse, IgG2a and IgG2b are tightly bound. Fc $\gamma$ RII and Fc $\gamma$ RIII, also known as CD32 and CD16 respectively, are low affinity receptors which bind poorly, if at all, to monomeric IgG, but can bind IgG coated antigens. In July 2005, it was reported that a fourth Fc $\gamma$ R existed, which was termed Fc $\gamma$ RIV<sup>8</sup>. Fc $\gamma$ RIV expression is restricted to myeloid cells, and it binds IgG2a and IgG2b exclusively. This receptor was discovered after it was observed that mice deficient in the  $\alpha$  subunit of the Fc $\gamma$ R, which binds the IgG, of the known activation Fc $\gamma$ Rs (Fc $\gamma$ RI and Fc $\gamma$ RIII), displayed either partial or no reduction in the ability to trigger effector cell responses<sup>9-11</sup>. This suggested that IgG was still binding, and that other  $\gamma$ -chain dependent Fc $\gamma$ R  $\alpha$  subunits were involved in the in vivo activity of IgG2a and IgG2b. These data suggest an important role for Fc $\gamma$ RIV in IgG2a and IgG2b induced inflammatory responses, and shows the powerful influence that these two isotypes have over protective and pathogenic properties<sup>8</sup>.

### Macrophage Activation

Macrophage activation was first described in 1964<sup>12</sup>, showing that the infection of mice with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or *Listeria monocytogenes* enhanced the antimicrobial activities of macrophages. LPS and interferon  $\gamma$  (IFN- $\gamma$ ) are the stimuli responsible for this type of macrophage activation, known as ‘classical’ activation of macrophages. Since the discovery of macrophage activation, it has become clear that there is more than one way to activate a macrophage. The different modes of macrophage activation, lead to the development of different populations of macrophages with distinct biological functions. There currently appear to be at least three populations of activated macrophages, the classical, alternative, and type II activated macrophages.

The classically activated macrophage was discovered first, and is the most well described of the three populations. The classical activation of macrophages is dependent upon two signals, IFN- $\gamma$  and tumor necrosis factor (TNF), or an inducer of TNF. IFN- $\gamma$  primes macrophages for activation<sup>13</sup>. TNF itself can act as the second signal, but generally the second signal is a result of TLR ligation, which induces the production of TNF by the macrophage. Once activated, these cells upregulate surface molecules such as major histocompatibility complex (MHC) class II and B7, which allows them to be effective antigen presenting cells. These cells also secrete a myriad of

inflammatory cytokines including, interleukins (IL) 1, IL-6, IL-12, and TNF, which enhances their ability to kill intracellular organisms. Activated macrophages accomplish the killing of phagocytosed organisms by increasing the production of toxic oxygen species, and activation of inducible NO synthase (iNOS). Thus, the classically activated macrophage is invaluable in the clearance of pathogens. However, this inflammation must be carefully regulated, lest injury to the host occur. When activated macrophages are left unchecked, the excessive inflammation can lead to tissue destruction, autoimmune diseases, and chronic granuloma formation<sup>14</sup>.

The alternatively activated macrophage was described in 1992<sup>15</sup>, when it was noticed that IL-4 induced the expression of the mannose receptor on these macrophages. These macrophages showed signs of activation, but the activation profile was different than that of classically activated macrophages. Thus, macrophages treated with IL-4 were ‘alternatively’ activated. These cells do not produce NO, and are ineffective at killing phagocytosed microbes. Although these cells moderately upregulate MHC II, they aren’t effective in antigen presentation. Rather than the inflammatory cytokines secreted by classically activated macrophages, alternatively activated macrophages produce IL-10 and IL-1 receptor antagonist, and have been described by some as having regulatory functions<sup>16</sup>.

Using the murine model of *Schistosoma mansoni*, it was discovered that when macrophages are treated with Th1 cytokines, the iNOS2 enzyme

produced NO from arginine. However, exposing macrophages to Th2 cytokines induces Arg-1, which metabolizes arginine to yield urea and ornithine<sup>17</sup>. These products give rise to polyamines, which are important in cell proliferation, and proline, which leads to the production of collagen. The production of polyamines and proline, which in turn cause cell proliferation and collagen production, suggests that these cells may also be involved in wound and tissue repair<sup>18</sup>. Therefore, alternatively activated macrophages are important for regulatory and repair functions, in stark contrast to the inflammation induced by classically activated macrophages.

The type II activated macrophage was described in 1997<sup>19</sup>, during an investigation into the termination of IL-12 upon FcγR ligation. It was later revealed that by ligating FcγRs on macrophages, the production of the proinflammatory cytokine IL-12 is terminated, and the production of the anti-inflammatory cytokine, IL-10, is induced<sup>20</sup>. The name 'type II' activated macrophage is indicative of their ability to induce Th2 immune responses<sup>21</sup>. Like the classically activated macrophage, type II activated macrophages also require two signals. The first signal required is the FcγR ligation, which is accomplished by the binding of IgG opsonized antigens. The receptor ligation must be coupled with a second, stimulatory signal, necessary for cytokine production. The cytokine switch occurs in both primed and unprimed macrophages. This cell is biologically distinct from the classically activated as well as the alternatively activated macrophages, and does not induce

arginase. The switch from IL-12 to IL-10 is dramatic, while the secretion of other cytokines, such as TNF, IL-1, and IL-6, is not affected<sup>21;22</sup>.

This profound cytokine reversal from proinflammatory to anti-inflammatory response was found to rescue mice from LPS toxicity. This effect was shown to be directly related to the secretion of IL-10 by utilizing macrophages from IL-10<sup>-/-</sup> mice. IL-10<sup>-/-</sup> macrophages were unable to rescue mice from endotoxemia<sup>21;23</sup>. Thus, the type II activated macrophage has potent anti-inflammatory properties.

The type II activated macrophage was also shown to influence antibody responses in mice<sup>24</sup>. In these studies, mice were injected with either classically, or type II activated macrophages, along with ovalbumin (OVA) antigen. Mice treated with OVA and classically activated macrophages made a modest antibody response to antigen. However, when mice were treated with OVA and type II activated macrophages, IgG levels increased significantly. It was determined that the majority of IgG produced was of the IgG1 isotype, and that type II activated macrophages were able to induce IgG class switching. These studies demonstrated the ability of type II activated macrophages to induce the production of IL-4 from T cells, which then induced B cells to produce IgG1. Thus, type II activated macrophages have powerful anti-inflammatory properties, and are strong inducers of Th2 responses.

The anti-inflammatory responses produced by type II activated macrophages could be potentially harmful in instances where an inflammatory

response is critical to the host survival. Infections with intracellular pathogens are demonstrative of this point. The clearance of intracellular pathogens, such as infections with *Leishmania spp.*, is reliant upon a robust inflammatory response. Inflammatory cytokines, such as IL-12 and IFN- $\gamma$ , lead to the classical form of macrophage activation. This activation is critical to the destruction and clearance of phagocytosed pathogens.

The ligation of Fc $\gamma$ Rs with IgG coated pathogens shuts off the production of IL-12 and induces the production of IL-10 from macrophages. This reversal in cytokine production blocks the inflammatory response and biases toward an anti-inflammatory response. Without a robust inflammatory response, *Leishmania* parasites are free to replicate within host macrophages, leading to disease pathology. Thus, while type II activated macrophages are important players of the immune system, they could also potentially cause harm to the host during infections with intracellular pathogens.

### Cytokines

Cytokines, which are also commonly referred to as interleukins, are proteins produced and secreted by the cells of innate and adaptive immunity in response to microbes and antigens. Though there are many cytokines, which mediate a diverse array of biological effects, they share several important properties. Cytokines can be pleiotropic, redundant, synergistic, and antagonistic. Being pleiotropic allows a single cytokine to influence several cell types, leading to diverse biological effects. Many cytokines have the

same biological effect, demonstrating a redundancy that allows for immune function in cases of a single cytokine gene knockout, or an antagonist against a single cytokine. Cytokines can be synergistic, with two or more cytokines working in concert to produce a greater biological effect, or antagonistic, with one cytokine inhibiting the effects or synthesis of another<sup>1</sup>. Cytokines are important for both innate (IFN- $\gamma$ , TNF, IL-1, IL-10, IL-12) and adaptive (IFN- $\gamma$ , IL-2, IL-4, IL-5) immunity.

IFN- $\gamma$ , IL-12, and IL-10 are important during infection, and function to initiate and regulate the innate immune response to pathogens. During an infection, IL-12 is rapidly produced and is an important proinflammatory cytokine of the early innate response. IL-12 is a heterodimeric protein made up of two disulfide-linked subunits designated p35 and p40, representing their approximate molecular weights<sup>25</sup>. Studies involving animal models demonstrate the central role IL-12 plays in providing protection from various diseases. Infection of IL-12 p40<sup>-/-</sup> mice with *Mycobacterium tuberculosis* leads to decreased resistance to the disease<sup>26</sup>. Decreased resistance, due to a lack of IL-12, has also been shown with *Listeria monocytogenes*<sup>27;28</sup>, *Toxoplasma gondii*<sup>29</sup>, *Cryptococcus neoformans*<sup>30</sup> and *Leishmania major*<sup>31</sup>.

IL-12 plays a key role in stimulating the production of IFN- $\gamma$  from T-cells and NK cells. The production, and subsequent secretion, of IFN- $\gamma$  acts to activate macrophages, providing a powerful feedback mechanism. These activated macrophages are now able to increase IL-12 production, resulting in

a proinflammatory loop<sup>32</sup>. IL-12 has been shown to induce the production IL-10<sup>33</sup>, which can inhibit the production of IL-12<sup>34</sup>, thus providing negative feedback for regulating the proinflammatory response.

IFN- $\gamma$  is a homodimeric protein produced by NK, CD4<sup>+</sup> Th1, and CD8<sup>+</sup> T cells, primarily in response to IL-12. It is responsible for activating macrophages, and it plays a critical role in both innate and adaptive immunity. IFN- $\gamma$  stimulation results in macrophages that display increased pinocytosis and receptor mediated phagocytosis as well as enhanced microbial killing ability. It has been shown to play a role in host defense against non-tuberculosis *Mycobacteria*<sup>35</sup>, *Leishmania*, *Toxoplasma*, *Rickettsia*, and *Chlamydia*<sup>36</sup>.

IFN- $\gamma$ -activated microbicidal ability includes induction of TNF, the NADPH-dependent phagocyte oxidase (NADPH oxidase) system (respiratory burst), NO production, and up-regulation of lysosomal enzymes promoting microbe destruction<sup>37</sup>. In addition, granuloma formation, and the upregulation of costimulatory molecules and MHC class I and II on antigen presenting cells (APCs), are also stimulated by IFN- $\gamma$ <sup>38</sup>. The net effect of these responses is to create a macrophage-rich inflammatory response.

IL-10 is a noncovalent homodimer that is acid sensitive<sup>39</sup>. IL-10 is a potent anti-inflammatory cytokine, and acts to limit an excessive inflammatory response, in order to avoid inflammation related pathology. It

affects cytokine production, soluble mediators, and cell surface molecules, having profound effects on the ability of immune effector cells to initiate and sustain an immune response. IL-10 blocks production of chemokines that are involved in inflammation<sup>40</sup>, and in addition enhances expression of their antagonists<sup>41</sup>. IL-10 inhibits the activation of macrophages. Since it is produced by activated macrophages, it is an example of negative feedback in the regulation of immunity. Thus, IL-10 functions in the termination of the inflammatory state, and the return to a resting state, as a microbial infection is cleared.

IL-10 enhances the expression of Fc $\gamma$ Rs, specifically CD16 and CD64, on monocytes<sup>42;43</sup>. Upregulation of the Fc $\gamma$ Rs by IL-10 correlates with enhanced phagocytosis of opsonized particles<sup>44</sup>, but a reduced ability to kill ingested organisms<sup>45</sup>. Ligation of Fc $\gamma$ Rs also induces the production of IL-10, therefore suggesting another regulatory mechanism where IL-10 upregulates Fc $\gamma$ R expression, and their subsequent ligation induces enhanced IL-10 production and a termination of the inflammatory response<sup>20</sup>.

The inhibitory effects of IL-10 on inflammation suggest that it could lead to the inhibition of the inflammatory response during infection *in vivo*<sup>46</sup>, and in fact, IL-10 was shown to rescue BALB/c mice from LPS induced toxic shock<sup>47</sup>. During the administration of endotoxin, IL-10 was shown to be induced in several animals including mice<sup>48</sup>, chimpanzees<sup>49</sup>, as well as in humans<sup>50</sup>. IL-10 production during the administration of endotoxin reduced the levels of IFN- $\gamma$ <sup>51</sup>, and regulated leukocyte-endothelial cell interactions and

microvascular permeability<sup>52</sup>. IL-10 production during septicemia can be correlated to the intensity of the inflammatory response, severity of injury, and clinical outcome<sup>53-55</sup>. These observations suggest that IL-10 plays an important role in the regulation of systemic inflammation.

High levels of IL-10 have been thought to predispose a person to systemic lupus erythematosus (SLE), and precede onset of the disease<sup>56</sup>. An increased rate of mortality in meningococcal disease is also associated with a genetic predisposition of a higher level of IL-10 expression<sup>57</sup>. IL-10 gene homologs have been found in the genomes of several viruses, such as Epstein-Barr (EBV)<sup>58</sup>, human cytomegalovirus (CMV)<sup>59</sup>, and equine herpes virus type 2<sup>60</sup>. Except for CMV, the conservation of the amino acid sequence between viral (vIL-10) and human (hIL-10) IL-10 is 84%. Almost all anti-hIL-10 antibodies cross react with vIL-10<sup>61</sup>. The capture of an IL-10 gene by a virus, which could then be evolved to suit its needs in terms of host interaction, could be extremely beneficial to viral survival, especially considering the deactivating effects of IL-10 on the inflammatory response.

EBV vIL-10 is able to mimic some functions of IL-10, such as macrophage deactivation<sup>62</sup>. The ability of EBV to express vIL-10 indicates that it may be beneficial to the survival of the virus. vIL-10 is expressed during the lytic phase<sup>63</sup>. A target cell of vIL-10 is the macrophage, which is relevant during the lytic phase. vIL-10 is able to inhibit macrophage activation, which suppresses an anti-viral immune response and allows the virus to establish latency<sup>46</sup>.

During infection, the immune system must respond to, and clear, a pathogen while minimizing damage to the host. IL-10 plays a significant role in helping to maintain the delicate balance between protecting the host from pathogens, and also from excessive inflammation. The anti-microbial effects of the inflammatory immune response can cause damage to the host that is often more destructive than the infection. Excessive inflammation can lead to damage ranging from localized destruction of infected cells to widespread tissue necrosis, and chronic granuloma formation<sup>46</sup>. These effects can have life threatening consequences when critical tissues, such as cardiac muscle, are involved. A systemic response to microbes can also lead to sepsis, which can result in death. Thus, ironically, the efficient clearance of an infection could ultimately lead to the demise of the host. IL-10 plays a vital role in the regulation of the inflammatory response to pathogens, and in protecting against inflammation related pathology.

Manipulating IL-10 levels during infections in animal models, demonstrated the crucial role IL-10 plays in the regulation of pathology and protection. Normal levels of IL-10 can impair the immune response to pathogens, and experiments in animal models have shown that by reducing IL-10, resistance to infection is almost always improved<sup>46</sup>. If IL-10 levels are increased during infection, using rIL-10, mice are more susceptible to intracellular pathogens such as *L. monocytogenes*<sup>64</sup>, *Streptococcus pneumoniae*<sup>65</sup>, *Candida albicans*<sup>66</sup>, and *Trypanosoma cruzi*<sup>67</sup>. However, if IL-10 levels are reduced during infection, mice are more resistant to the

intracellular pathogens listed previously<sup>65;68-70</sup>, as well as *T. gondii*<sup>71</sup>, and *L. major*<sup>72</sup>. There is also strong evidence that supports the role of IL-10 in human diseases such as visceral leishmaniasis<sup>73;74</sup>, leprosy<sup>75</sup>, malaria<sup>76</sup>, and tuberculosis<sup>77</sup>.

## **ADAPTIVE IMMUNITY**

### Overview

The adaptive immune response is comprised of lymphocytes and their products, and is more highly evolved than the innate response. When stimulated by a pathogen, the adaptive immune response is able to adapt and mount an appropriate response based on the characteristics of a particular organism. The innate and adaptive immune responses work together to protect the host from infectious organisms. The innate immune response encounters pathogens and initiates a response, which in turn stimulates adaptive immunity. The adaptive immune response then uses the effector molecules of innate immunity to eliminate the infection, frequently by enhancing the microbicidal functions of the innate response. The defining characteristics of the adaptive response are its specificity, and its ability to remember and later respond to repeated exposures of that pathogen more vigorously.

The fundamental properties of the adaptive immune response are specificity, diversity, specialization, self-limitation, memory, and non-

reactivity to self. Specificity ensures that a particular antigen elicits the appropriate, specific response. Lymphocytes express membrane receptor that distinguish between various antigens. When a receptor encounters its specific antigen, it responds and that particular clone proliferates. This process is termed clonal expansion. Diversity refers to the number of antigenic specific lymphocytes, which is termed the lymphocyte repertoire. The immune system can respond to  $10^9$ - $10^{11}$  antigen specific epitopes, allowing an antigen specific response to a large number of pathogens. The distinct way the immune system responds to different organisms is termed specialization. These responses are specialized to the microbe, in order for optimal host defense. Self-limitation is the return to homeostasis. After encountering an organism, the immune response is regulated, and returns to its resting state. Memory develops after exposure to an antigen, and allows for enhanced responses upon subsequent exposures of the same antigen. These secondary immune responses are larger, and occur faster than the primary response. Non-reactivity to self is one of the most important and remarkable features of adaptive immunity. This prevents the injury and attack of host cells from its own lymphocytes, and is also referred to as self-tolerance. Problems with the maintenance of self-tolerance often lead to the development of autoimmune diseases. These hallmarks of adaptive immunity are essential to the function of host defense.

The adaptive response is composed of three phases, recognition, activation, and effector. During the recognition phase, a lymphocyte receptor

encounters its specific antigen, and undergoes clonal expansion. Clonal expansion yields numerous lymphocytes from a single clone, which are capable of reacting with a specific antigen. The activation phase requires two signals, the antigen itself and either microbial products or components of the innate response to the microbe. This two-signal requirement ensures that activation occurs only when needed, and that it is specific to the antigen. The activation phase involves the synthesis of proteins, cellular proliferation, differentiation into effector cells that function to eliminate antigens, or differentiation into memory cells that provide enhanced responses to subsequent exposures. During the effector phase, activated lymphocytes perform the functions needed in order to eliminate organisms. This phase often involves the cooperation of innate and adaptive immunity for effective clearance of the organism.

There are two types of adaptive immune responses, humoral and cell mediated. These two responses are mediated by different immune cells, and eliminate different types of organisms. Cell mediated immunity is mediated by T lymphocytes, and is also known as cellular immunity. This response is generated against intracellular microbes, which survive and proliferate inside host cells. Cell mediated immunity leads to the destruction of the microbes, or the lysis of the infected cells. Humoral immunity is mediated by antibodies in the blood, which are produced by B lymphocytes. Antibodies specifically bind to antigens, neutralizing their infectivity and targeting them for elimination by other effector mechanisms, such as complement and

phagocytosis. This response is generated against extracellular microbes and their toxins.

Immunity against a microbe can be induced by the host's own immune response, or by the transfer of microbe specific antibodies or lymphocytes. Immunity induced by the host's immune response against exposure to a microbe is termed active immunity. This is because the host's immune response played an active role in responding to, and clearing, the microbe. Immunity can also be induced by the transfer of serum (antibodies) or lymphocytes from an immunized individual. This process is known as adoptive transfer, and results in immunity without exposure to the microbe. Since the host's immune response plays no active role in conferring this immunity, it is known as passive immunity. Passive immunity provides rapid resistance, since there is no wait for an active immune response to develop. Both types of immunity are antigen specific and provide resistance to infection, but only active immunity leads to the development of memory cells.

### T cells

T cells originate in the bone marrow and mature in the thymus. T cells exhibit specificity when recognizing antigen due to the polymorphic T cell receptor (TCR). The TCR undergoes gene rearrangement during maturation in the thymus. This process results in a TCR specific for a single antigen, and commits the TCR to this specificity for the remainder of the life span. T cells recognize their specific antigen only when presented as a processed peptide.

These peptides are loaded onto the MHC expressed on antigen presenting cells (APCs)<sup>78</sup>. Since recognition of antigen is dependent upon MHC recognition, T cells must be selected for their ability to engage MHC during the maturation process. T cells are first selected for their ability to recognize self MHC in a process known as positive selection<sup>79</sup>. T cells then undergo negative selection, which involves the apoptosis of T cells which react to self antigens<sup>80</sup>. This step is important for self tolerance and ensuring that the adaptive immune response is not initiated against the host.

T cells are further divided into individual subsets which direct distinct responses. These cells differ in their mutually exclusive expression of either CD4 or CD8. The subsets are referred to as CD4<sup>+</sup> helper T cells, and CD8<sup>+</sup> cytotoxic T cells. CD4 and CD8 determine which MHC complex can interact with the T cell. CD4<sup>+</sup> T cells are restricted to interaction with the MHC class II, while CD8<sup>+</sup> T cells are restricted to MHC class I. CD8<sup>+</sup> T cells mature into cytotoxic T lymphocytes (CTLs) which function in the lysis of pathogen infected cells. CTLs have cytolytic proteins that allow for the destruction of targeted cells. CD4<sup>+</sup> T cells are further divided into distinct subsets based on their function and cytokine production. These cells initiate, maintain, and regulate the humoral and cell mediated immune responses<sup>81</sup>.

There are two distinct subsets of CD4<sup>+</sup> helper cells (Th1 and Th2), and a subset of T regulatory (Treg) cells, and which are defined based on their cytokine production<sup>82</sup>. The T helper cell subsets enhance, or 'help', in the innate, cellular, and humoral responses to antigens, and are defined by the

cytokines they produce. Th1 cells are defined by their production of IL-2 and IFN- $\gamma$ , while Th2 cells produce IL-4 and IL-5. Th1 responses are characterized as being inflammatory, and help in the clearance of intracellular pathogens. Conversely, Th2 responses are characterized as anti-inflammatory, and function in the control and clearance of extracellular pathogens and helminths. T regs are characterized by the production of IL-10 and TGF $\beta$ , and function to control the immune response<sup>83</sup>.

Activation and clonal expansion of T cells occurs in response to two signals. The first signal is the recognition of antigen by the TCR. This must occur simultaneously with the second signal, the recognition of co-stimulatory molecules on the surface of the APC<sup>84</sup>. The maturation process of T cells involves the development of memory cells. Memory cells are a sub population of clonally expanded, long lasting, T cells that revert to a quiescent state<sup>85</sup>. Maximum efficiency in responding to pathogens is achieved by preferentially recirculating naïve cells through the secondary lymph organs, while memory cells remain in the peripheral tissues and blood<sup>86</sup>.

### B cells

B lymphocytes are an important part of adaptive immunity, and are capable of producing and secreting millions of antigen specific antibodies. Antibodies have a common core structure of two identical covalently linked heavy chains and two identical light chains, each of which is linked to one of the heavy chains. Each antibody recognizes a different antigen, and their

diversity is due to the complex mechanism of V(D)J recombination of the immunoglobulin genes<sup>87</sup>, as well as somatic hypermutation, and class switching. B cells recognize antigen via the B cell receptor (BCR), which is composed of two immunoglobulin (Ig) heavy chains, two Ig light chains, and two heterodimers of Ig $\alpha$  and Ig $\beta$ <sup>88</sup>.

B cells originate and mature in the bone marrow, and are the only cells capable of differentiating into plasma cells, which produce antibodies. An important characteristic that sets them apart from T lymphocytes is their ability to recognize soluble antigen. Immature B cells develop into mature B cells, which express IgM and IgD on their surface. This is a tightly controlled process, with approximately 75% of developing cells becoming apoptotic during pre-B cell development<sup>89</sup>. Apoptosis is induced if heavy chain gene rearrangement is incomplete or inappropriate.

During maturation, the immunoglobulin genes undergo rearrangement, allowing for production of a unique receptor on each cell. This gene rearrangement allows an individual to produce approximately  $10^{11}$  distinct antibody specificities<sup>88</sup>. Apoptosis may also be induced at this point if the surface Ig recognizes and responds to self-antigens. This induction of apoptosis is known as negative selection. Antigen binding is required for activation, and B cells can become activated on their own in response to non-protein antigens (T-independent), or with the help of T cells in response to protein antigens (T-dependent). In addition to antigen binding, the activation

of B cells also requires a second signal which is fulfilled by the binding of complement protein C3d to the type II complement receptor (CR2)<sup>1</sup>.

During activation, B cells become enlarged, and undergo clonal expansion. Co-receptors are important during activation, and provide regulation of B cell signaling. CD40 is essential for T-dependent antibody responses, while CD22 and the FcγRIIB inhibit B cell activation. Following proliferation, some B cells will undergo differentiation into plasma and memory cells. Plasma cells produce antibodies, and exhibit a large endoplasmic reticulum and Golgi apparatus, which are necessary in the production of antibody. Since plasma cells produce only secreted antibody, Ig mRNAs increase, while surface Ig expression decreases.

Two major genetic changes occur during differentiation, somatic mutations and class switching. Somatic mutations change the antigen binding properties, allowing antigens to bind more tightly to B cells. Class switching results in a change in the heavy chain constant region within the immunoglobulin, and affects the way B cells and antibody are recognized by effector cells<sup>89</sup>.

The main function of B cells is to produce and secrete antibodies. Antibodies protect in several ways, including, neutralizing toxins, preventing the invasion of viruses and bacteria into cells, and coating antigens to enhance phagocytosis, as well initiate the complement cascade. There are 5 classes of Ig (IgM, IgD, IgA, IgG, and IgE).

IgM and IgD are found on the surface of B cells. IgD is not secreted, and is only found as an antigen receptor on naïve B cells. IgM is expressed on the surface of B cells, as well as secreted, and is the first class of Ig to be expressed after antigen binding. It is secreted as a pentamer, and subsequently is particularly good at activating the complement cascade. IgA functions in mucosal immunity, and is secreted as a monomer, dimer, and trimer. IgE is secreted as a monomer, and functions in immediate hypersensitivity reactions and the eosinophil mediated killing of helminths. IgG is secreted as a monomer, and functions in opsonization, feedback inhibition of B cells, complement activation, and antibody-dependent cytotoxicity<sup>1</sup>. The class switching of antibody molecules allows for changes in the antibody, while still retaining its specificity.

Changes in the isotypes of antibodies during a humoral response allow a single clone of B cells to produce antibodies with identical specificity for antigen, but different functions. These changes in isotypes are referred to as isotype switching, and influence how the antibodies work to eradicate antigen. The cytokine environment heavily influences class switching. Cytokines play an important role in antibody production, with different cytokines having distinct, but frequently overlapping roles. These cytokines not only influence isotype switching, but also have significant impact on the production of various subclasses of Ig isotypes, such as IgG (Table 1). In humans, the IgG isotype can be further divided into subclasses known as IgG1, IgG2, IgG3,

and IgG4. The IgG isotype of mice can also be subdivided into IgG1, IgG2a, IgG2b, and IgG3.

| <b>CHARACTERISTICS OF IgG</b>             |  |
|---|--|
| Subclasses:                               | IgG1, IgG2, IgG3, IgG4 (human)<br>IgG1, IgG2a, IgG2b, IgG3 (mouse)   |
| Concentration in Sera                     | IgG1 > IgG2 > IgG3 > IgG4  |
| Complement Activation                     | IgG3 > IgG1 > IgG2 >> IgG4 (human)<br>IgG2a, IgG2b (mouse)   |
| FcγR Binding                              | High affinity receptor -can bind monomeric IgG<br>FcγRI: IgG1, IgG3 (weakly binds IgG4)<br><br>Low affinity receptors – cannot bind monomeric IgG<br>FcγRII: IgG1, IgG2, IgG3<br>FcγRIII: IgG1, IgG3 |
| Cytokine Influence over Isotype Switching | IgE: IL-4<br>IgA: IL-5, TGF-β<br>IgM: IL-2, IL-4, IL-5<br>IgG: IL-2, IL-4, IL-6, IFN-γ   |
| Th Response                               | Th1: IgG1, IgG3 (human)<br>IgG2a (mouse)<br><br>Th2: IgG4 (human)<br>IgG1 (mouse)  |

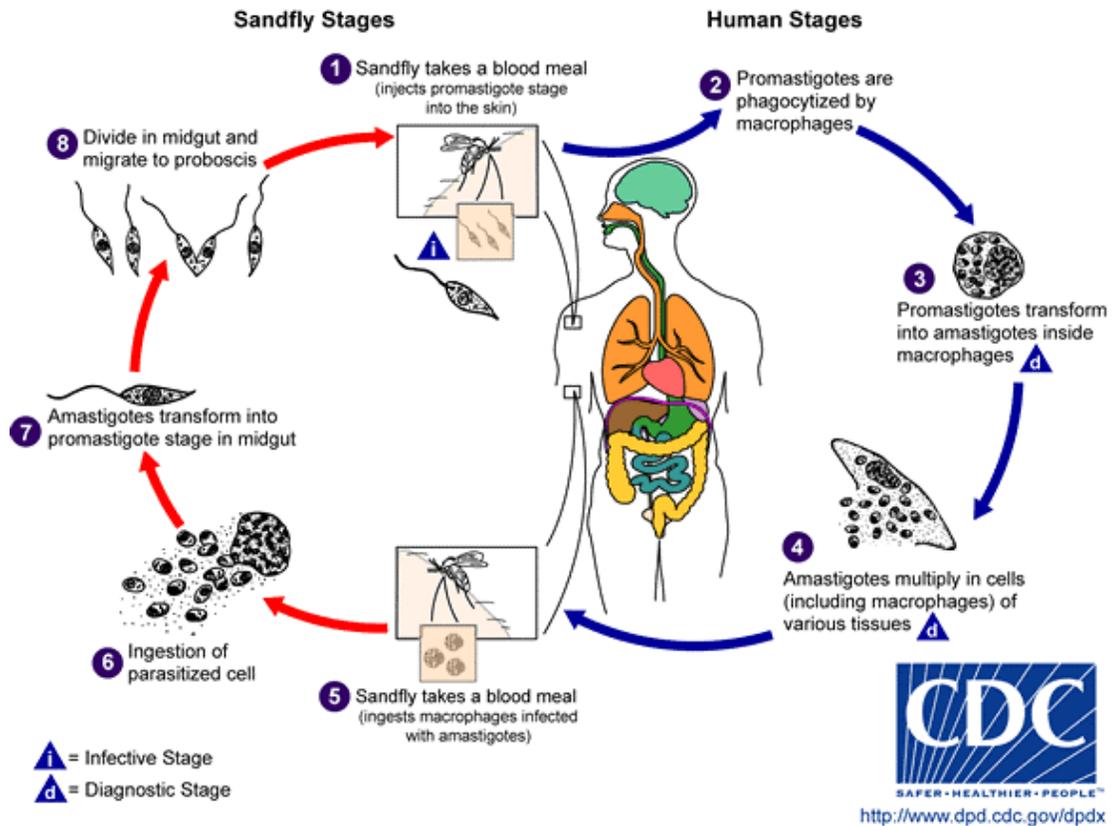
**Table 1. Characteristics of the IgG Isotype.** Compilation of the various characteristics and functions of the IgG subclasses.

## LEISHMANIA

Parasites of the genus *Leishmania* are named after W. B. Leishman. In 1901 he gave one of the first accounts of the parasites and their association with the lesions that are the hallmark symptom of the disease<sup>90</sup>. *Leishmania* are single celled, intracellular, protozoan parasites. They belong to the order kinetoplastida and the family trypanosomatidae. There are various species of the parasite, all belonging to the genus *Leishmania*. Humans are affected by 21 of approximately 30 species that infect mammals, causing a broad spectrum of disease<sup>91</sup>.

### Life cycle

*Leishmania spp.* reside and replicate primarily in host tissue macrophages. There are two developmental stages of the *Leishmania* life cycle (Figure 2), the flagellated promastigote and the nonmotile intracellular amastigote. The promastigote resides in the gut of a phlebotomine sand fly. A small pool of blood is formed when a female fly takes a blood meal. Infectious promastigotes (metacyclics) are regurgitated into this pool of blood, where they enter the human host<sup>92</sup>. Phagocytic cells recruited to the site of infection rapidly internalize the promastigotes. Once inside the cell, the parasite will find itself in the phagolysosome, where various enzymes reside, waiting to kill and digest any invading organisms. It is in this hostile environment that the promastigote transforms into the



**Figure 2. The *Leishmania* Life Cycle.** (1)The sandflies inject the infective stage, promastigotes, during blood meals. (2)Promastigotes that reach the puncture wound are phagocytized by macrophages (3)and transform into amastigotes. (4)Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species. (5-6)This originates the clinical manifestations of leishmaniasis. Sandflies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes. (7)In the sandfly's midgut, the parasites differentiate into promastigotes, (8) which multiply and migrate to the proboscis. From the Centers for Disease Control, [www.cdc.gov](http://www.cdc.gov)

nonmotile amastigote. The parasites not only survive but replicate within the acidic phagolysosome. Replication continues until the host cell is lysed, releasing parasites, which in turn infect neighboring cells.

### Disease manifestation

Approximately 350 million people live in the 88 countries where leishmaniasis can be found. Most of these countries are in the tropics and subtropics, however, leishmaniasis is found from the rain forests in Central and South America to the deserts in West Asia<sup>91</sup>. In humans, *Leishmania* spp. cause a wide spectrum of diseases ranging from confined cutaneous lesions to a progressive and sometimes fatal visceral disease. The degree of disease depends upon the species of the parasite and immune competency of the host. The various disease manifestations can be classified into three major categories: cutaneous (CL), mucocutaneous, and visceral leishmaniasis (VL).

Cutaneous leishmaniasis begins as small lesions on the skin where an infected sand fly has bitten the host. This form of the disease is often caused by *L. tropica*, *L. major*, *L. amazonensis*, and *L. amazonensis*. The incubation period may range from 1-2 weeks, up to 1-2 months. Lesions may persist for up to a year without treatment, but most will eventually heal<sup>93</sup>. This is the most prevalent form of the disease with the number of new cases estimated to be about 1.5 million per year<sup>91</sup>. Ninety percent of these cases occur in Afghanistan, Brazil, Peru, Iran, Saudi Arabia, and Syria<sup>90</sup>. As recently as June 28, 2002, the World Health Organization appealed for \$1.2 million to help

tackle an epidemic that was ongoing in Afghanistan with an estimated 200,000 cases in Kabul alone<sup>90</sup>.

Mucocutaneous leishmaniasis is thought to be the result of metastasis from a primary cutaneous lesion to mucosal membranes, since most patients have a history of infection with the cutaneous form. *L. braziliensis* is often responsible for this manifestation. These lesions progress slowly, but result in damage to the mucosal areas in the throat and nose. Three countries, Bolivia, Brazil, and Peru, are responsible for 90% of these cases<sup>90</sup>.

Visceral leishmaniasis, also known as kala-azar, results in parasitization and subsequent enlargement of the liver and spleen. *L. infantum*, *L. chagsi*, and *L. donovani* are responsible for this form, which is an important cause of death in India, Brazil, Bangladesh, and Africa. The incubation period is generally long, 1-3 months, with disease onset accompanied by sweating, fever, and weakness. This is the most serious of the disease forms and if left untreated, death may occur<sup>93</sup>. There are approximately 500,000 new cases of VL per year<sup>91</sup>.

### Diagnosis and treatment

Diagnosing leishmaniasis is a difficult task. Laboratory tests can yield negative results even when the person is infected with the organism. The most important and definitive test is the identification of parasites by microscopic observation or growth in culture. Biopsies of the lesion provide samples for examination and testing. Samples are obtained by punch biopsies

and needle aspirations of the active lesion. Giemsa staining slides of the biopsied tissue is still the technique most commonly used to detect the parasite. Needle aspirations are used to recover parasites for growth in culture. A needle is placed under the skin, and fluid is aspirated out of the lesion. This fluid is then placed into culture medium and observed for parasite growth. Blood tests to detect antibody against the parasite can be helpful for cases involving the visceral form, but these test are frequently not sensitive enough to be of use in diagnosing the cutaneous form<sup>91</sup>.

Cutaneous lesions will generally heal without treatment, however they often leave disfiguring scars. Other forms of the disease are very difficult to treat and often require prolonged treatment with pentavalent antimony drugs (Pentostam) or amphotericin B.

#### Macrophages, Leishmania, and the evasion of intracellular killing

The uptake of *Leishmania* by macrophages is a receptor-mediated process<sup>94</sup>. Due to the obligate intracellular nature of the pathogen, it is important that they utilize various receptors to ensure their phagocytosis. Complement<sup>95;96</sup>, fibronectin<sup>97</sup>, mannose-fucose<sup>98</sup>, and Fcγ receptors<sup>72;99</sup> have all been shown to be involved in the phagocytosis of these organisms. Once the promastigotes have entered the macrophage, they must contend with the harsh environment of the phagolysosome. The ability of *Leishmania spp.* to not only survive, but replicate in the macrophage phagolysosome is one of the most intriguing aspects of *Leishmania* virulence.

*Leishmania* parasites enter cells by a quiescent mechanism that fails to activate innate immune responses, and fails to induce macrophage cytokine expression. Infected macrophages fail to induce TNF- $\alpha$ , IL-12, NO and fail to activate NF- $\kappa$ B. NF- $\kappa$ B activation is critical for inducing co-stimulatory molecules on APCs, and for the induction of several genes which encode important proinflammatory cytokines<sup>100</sup>. Amastigotes<sup>101</sup>, as well as promastigotes<sup>102</sup>, have previously been observed to not only fail to induce IL-12 production, but to actively downregulate it. This proinflammatory cytokine is responsible for the production of IFN- $\gamma$ , which is the hallmark of a Th1 immune response. The downregulation of IL-12 leads to a failure in macrophage activation, and ultimately a failure in the intracellular killing of *Leishmania*.

### Immune response and deviation

The disease spectrum described above is also largely observed in murine systems, allowing the mouse to be a reasonable *in vivo* model. The availability of various strains of inbred mice has allowed extensive characterization of the immune response to *Leishmania spp.*<sup>103-105</sup>. These studies have found that the type of immune response mounted against the parasite determines the outcome of the disease<sup>106</sup>. The mouse model is especially useful in the study of cutaneous leishmaniasis where disease progression can be tracked by lesion size. The murine model of cutaneous leishmaniasis caused by *L. major* has served as a paradigmatic model in which

to study cellular immunity to an intracellular pathogen. This model has done much to help us understand the events leading to T cell biasing and immune deviation.

The outcome of *L. major* infections in mice has been linked to the type of immune response, Th1 or Th2, which is mounted in response to the parasite. CD4<sup>+</sup> T lymphocytes from resistant C57BL/6 mice produce high levels of IFN- $\gamma$  and other Th1 cytokines, such as IL-12, while T cells from susceptible BALB/c mice produce high levels of IL-4 and IL-10<sup>107</sup>. The predictability and dramatic differences of this model of disease has led it to become the premier model for studying the Th1/Th2 paradigm in vivo.

In response to *L. major*, C57BL/6 mice mount a Th1 response, characterized by high levels of IFN- $\gamma$  and IL-12 cytokine production. This appropriate immune response leads to activation of macrophages, which promotes healing, parasite clearance, and resolution of infection. BALB/c mice, conversely, mount an inappropriate Th2 response, characterized by IL-4<sup>106;108</sup>. This blocks IFN- $\gamma$  production and the subsequent macrophage activation. Without macrophage activation, the parasites continue to replicate increasing lesion size, and the mice ultimately succumb to the disease. Additionally, differences in parasite dissemination have been found in resistant and susceptible strains. Parasite dissemination occurs much more rapidly in BALB/c mice, while parasites are contained at the site of infection and in draining lymph nodes in resistant C57BL/6 mice<sup>109</sup>. This early

dissemination in BALB/c mice results in IL-4 producing CD4<sup>+</sup> T cells in the liver and spleen as early as 2 weeks after infection<sup>110</sup>.

Resistance to disease can be conferred upon *L. major* susceptible BALB/c mice by treatment with  $\alpha$ -IL-4 antibody<sup>111;112</sup>, or by knocking out the IL-4 gene<sup>113;114</sup>. These data established the idea that IL-4 was the driving force behind the polarization of the Th2 response in response to *L. major*, and the suppression of Th1 cell development. The suppression of a Th1 response inhibits the production of IFN- $\gamma$ , which is needed to activate infected macrophages for the clearance of parasites.

It has been shown that a population of CD4<sup>+</sup> T cells that recognize the *Leishmania* antigen LACK, a homologue of receptors for activated C kinase, was responsible for the early production of IL-4<sup>115;116</sup>. However, it was determined that this early induction of IL-4 by LACK responsive CD4<sup>+</sup> T cells was not sufficient to induce susceptibility, because both BALB/c and C57BL/6 mice were found to have similar numbers of these cells and thus, a production of IL-4 upon infection with *L. major*<sup>106;117</sup>. Additionally, treatment of resistant mice with IL-4 or  $\alpha$ -IL-12 antibodies at the time of parasite infection, leads to a strong Th2 response, but this response is temporary and unable to reverse the normal resistance throughout the duration of the infection<sup>111;118</sup>. Thus, these data indicate that the redirection of the initial Th2 response is most likely responsible for conferring resistance in *L. major* infected mice.

The absence of Th1 related cytokines, receptors, transcription factors, and co-stimulatory molecules can lead to the susceptibility of resistant mice to *L. major*. Resistant mice which are deficient in IL-12<sup>119</sup>, IFN- $\gamma$ <sup>120</sup>, T-bet<sup>121</sup>, or CD40-CD40L<sup>122;123</sup> interactions, are rendered susceptible to infection with *L. major*, and mount a Th2 immune response, rather than the protective Th1 response. Additionally, mice which are deficient in either iNOS<sup>124</sup> or TNF<sup>125</sup> are unable to control *L. major* infections, and develop disease. Interestingly, resistant mice deficient in IFN- $\gamma$  receptor<sup>126</sup> or STAT4<sup>127</sup> resulted in an increased susceptibility to *L. major*, but are still able to suppress a Th2 response. In the absence of IL-12, other Th1 promoting factors such as IL-18 and a class-1 cytokine receptor, WSX1, can inhibit a Th2 response and influence resistance in the early stages of *L. major* infection<sup>31;128;129</sup>. These factors contribute to the redirection of the early Th2 response in resistant mice; however the presence of IL-12 and IL-12 receptor signaling is needed to maintain a curative Th1 response<sup>130</sup>.

The necessity of IL-12 in maintaining resistance was established through the use of IL-12 deficient mice. These normally resistant mice are susceptible to infection with *L. major*, and while intermittently treating with IL-12 slows lesion development, they eventually produce progressive lesions. Additionally, Th1 cells from healed mice were unable to provide IL-12 deficient mice with immunity to infection<sup>31</sup>. The lesions of healed C57BL/6 mice, which have achieved clinical, but not sterile cure, reactivate when treated with  $\alpha$ -IL-12 antibodies<sup>131</sup>. Thus the continued presence of IL-12 is

necessary to prevent the differentiation of uncommitted cells into Th2 promoting cells. The conditions which induce the early onset of an initial Th2 response are able to reappear upon the depletion of IL-12<sup>132</sup>.

Though macrophages are the main target of *Leishmania* parasites, other innate immune cells are important during infection with *L. major*, especially as source of IL-12. While macrophages are effective at ingesting *Leishmania* parasites, their ability to effectively produce IL-12 is impaired<sup>102</sup>. Dendritic cells (DCs) and epidermal Langerhans cells (LCs) ingest *L. major* parasites and produce IL-12, thus promoting the development of the Th1 response<sup>133;134</sup>. Natural killer cells have also been implicated in the promotion of a Th1 response during *L. major* infections, because of their ability to produce the IFN- $\gamma$  which is needed to optimize the production of IL-12, upregulate expression of the IL-12 receptor on T cells, and activate macrophages. It should be noted however, that while NK cells help promote a Th1 response, mice deficient in NK cells are still capable of mounting a protective Th1 response, and controlling *L. major* infections<sup>135;136</sup>.

There is little doubt that the susceptibility to *L. major* of BALB/c mice is the result of a sustained Th2 response. However, there is evidence that IL-4 alone may not be sufficient for susceptibility. Mice deficient in IL-13 have shown that this cytokine is important in mediating susceptibility to *L. major*, and the deletion of both IL-4 and IL-13 has an additive effect on increasing disease<sup>137</sup>. Additionally, IL-10 is another cytokine capable of suppressing Th1 responses, as well as macrophage activation, and CD4<sup>+</sup> T cells from *L.*

*major* infected BALB/c mice have been shown to express high levels of both IL-4 and IL-10 mRNA<sup>117</sup>. Accordingly, the role of IL-10 in leishmaniasis has been demonstrated for both murine<sup>72;138;139</sup>, and human disease<sup>74</sup>.

IL-10 has been identified to be an important mediator of susceptibility in both cutaneous and visceral leishmaniasis. This cytokine has long been shown to be elevated in humans suffering with visceral leishmaniasis<sup>74</sup>. In a recent study, Murray and colleagues demonstrated that macrophage-derived IL-10 can increase susceptibility to *L. major*<sup>140</sup>. In addition to macrophages<sup>72;140</sup>, T regulatory cells<sup>141</sup> have also been implicated in the production of IL-10 during *L. major* infections. It is probable that both play a role, however, recent evidence supports the role of IL-10 producing Tregs in the persistence of parasites after clinical cure, as mice deficient in IL-10 were able to achieve sterile cure<sup>142</sup>. Mice which obtained a sterile cure from *L. major* infection, whether achieved by IL-10 knockout or treatment with an antibody blocking the IL-10 receptor<sup>143</sup>, were not immune to subsequent reinfections, indicating that the maintenance of memory T cells is dependant upon parasite persistence. Tregs were determined to be the source of IL-10 in resistant C57BL/6 mice, preventing sterile cure and thus persevering immunity. Thus, macrophages which are the main target of *Leishmania* parasites may be the dominant source of IL-10 during active infection.

While the classically activated macrophage is necessary for parasite clearance, the type II macrophage may be responsible for the production of IL-10, and thus Th2 responses, during *L. major* infections. It has been

previously shown that macrophages, activated in the presence of immune complexes, shut off production of IL-12<sup>19</sup> and produce high levels of IL-10<sup>20</sup>. These previous observations suggest that immune complexes could adversely influence the development of cell mediated immunity to *Leishmania spp.*, by inducing this alteration in cytokine production<sup>21</sup>. In fact, previous studies have shown that the levels of immune complexes are high in patients with visceral leishmaniasis<sup>144;145</sup>. Immune complexes during *Leishmania* infections would result from the opsonization of parasites with IgG. Interestingly, in patients with visceral leishmaniasis, high IgG titers correlate with, and are indicative of disease.

This would also seem to agree with *L. major* infections in BALB/c mice, which develop high antibody titers, with parasites frequently metastasizing to the bone marrow, liver, and spleen<sup>146</sup>. High antibody titers have also been observed in canine leishmaniasis<sup>147</sup>. Additionally, the delayed-type hypersensitivity (DTH) response in BALB/c mice is smaller and more transient than in resistant mice<sup>148</sup>. Interestingly, 20 years ago it was demonstrated that the suppression of DTH responses in BALB/c mice required the presence of B cells<sup>149</sup>. Therefore, it is possible that antibody could play a role in disease by contributing to the formation of immune complexes, which in turn induce the production of IL-10 from host macrophages.

Increased levels of IgG, and its subclasses, are present during infection with *Leishmania spp.*<sup>147;150-152</sup>. Though the presence of high anti-*Leishmania*

antibody titers and *Leishmania* immune complexes, as well as a possible role for B cells during *Leishmania* infection have been well documented over the last 20 years, no one has offered definitive evidence as to what role B cells and/or immunoglobulin plays in mediating the host responses to *Leishmania* infections. As early as 1982, a role was suggested for humoral factors in immunosuppression during visceral leishmaniasis<sup>153</sup>. Pearson and colleagues repeatedly reported on the presence of immune complexes and their possible potential to suppress the immune response during visceral leishmaniasis throughout the 1980s<sup>145;154-156</sup>. These studies indicated that circulating immune complexes were prevalent in patients suffering from visceral leishmaniasis, and should be investigated further to determine the role of humoral factors in visceral disease and the immunosuppression of the immune response during these infections. Additionally, a role for polyclonal B cell activation was suggested in the formation of immune complexes during visceral leishmaniasis by Galvao-Castron *et al.*, in 1984. These studies noted the close association between a marked increase in IgG and IgM levels and the presence of immune complexes.

Murine infections involving B cell depletion, B cell knockouts, and IgG deficient mice, seem to support a role for the humoral response in mediating the host response to leishmaniasis. Numerous theories have been suggested, but to date, no specific mechanisms involving the host's humoral response to *Leishmania spp.* have been proven. In the years leading up to a 1999 report involving the polarization of T cell responses in the absence of B

cells, by Brown and Reiner, almost every report published on the subject of B cells and *Leishmania* infection offered a new theory, but little direct evidence, about their involvement during disease.

Over 20 years ago, studies were performed in BALB/c mice which had been depleted of B cells. These mice lacked any detectable anti-*Leishmania* antibodies during the course of infection, and were capable of controlling lesions. It was determined that these BALB/c mice displayed a sustained DTH response to leishmanial antigen, leading to controlled infection. It was concluded that B cells and antibodies were required for the suppression of the DTH response, and subsequent susceptibility in BALB/c mice<sup>149</sup>. An additional report by Titus and colleagues echoed the similar theme of the role of B cells in the development of DTH responses. In contrast to the previous report by Sacks, *et al.*, these studies showed that  $\mu$ MT mice, which lack B cells, on the resistant C57BL/6 background, were unable to maintain a sustained DTH response following secondary infections, and produced lower amounts of IFN- $\gamma$  than control mice. The authors conclude that B cells have a major role in the development of DTH responses<sup>157</sup>. These 2 papers offer conflicting theories about the role of B cells during *Leishmania* infections. In fact, over the past 20 years, there have been several papers reporting on the involvement of B cells and/or immunoglobulin during infection with *Leishmania spp.*, none of which seem to agree upon the method or mechanisms by which B cells and immunoglobulin exert their influence.

In 1994, Hoerauf, *et al.*, conducted studies in Xid BALB/c mice during the course of *L. major* infection. These mice showed slower lesion development, 10-30 fold lower parasite burdens, and significant decreases in  $\alpha$ -*L. major* antibody levels<sup>158</sup>. The authors conclude that B cells contribute to the susceptibility in *L. major* infections by skewing toward a Th2 immune response, but offered no mechanism of action. The authors followed up this report in 1995 with similar studies involving Xid BALB/c mice treated with IL-7. These studies show that IL-7 results in a total increase in B cells, and a 100 fold higher parasite load. The authors conclude that IL-7 enhances lymphopoiesis, increasing the number of B cells, and resulting in aggravation of *L. major* disease<sup>159</sup>. A similar report by Gessner, *et al.*, in the same year also observed the course of leishmaniasis in IL-7 treated mice. The authors treated *L. major* infected BALB/c mice with IL-7, and observed an increase in IL-10, IL-4, and lesion size, as well as accelerated death, and a 40 fold increase in parasite burden<sup>160</sup>. They concluded that disease enhancement is due to the augmentation of B cell lymphopoiesis. Additional reports the following year implicated an increase in B cell number with susceptibility to *L. major*<sup>161;162</sup>, but again failed to provide a direct mechanism of action.

Reports by Peters, *et al.*, in 1995, Kima, *et al.*, in 2000, and Colmenares *et al.*, in 2002 investigated infections with *Leishmania spp.* in J<sub>H</sub> mice, which are B cell deficient, on the susceptible BALB/c background. These studies illustrated a decrease in disease severity. Upon treatment with *Leishmania* specific IgG, lesion size increased, suggesting that IgG played a

role in increased susceptibility to disease<sup>97;163</sup>. These three reports echo each other in their conclusions that IgG is important for parasite uptake during infection, suggesting that the opsonization of parasites with IgG allows uptake through Fc receptors on macrophages, which is the key to susceptibility. A 1997 report involving *L. major* infected BALB/c mice correlated IgG serum antibodies with the formation of skin lesions, suggesting that they played a role in disease manifestation<sup>164</sup>.

In 1999, a report published by Brown and Reiner suggested that neither B cells nor antibody play any role influencing the T cells response during infections with *L. major*. These studies examined  $\mu$ MT mice on both the resistant C57BL and the susceptible BALB/c backgrounds. In these studies, there were no differences in the lesion size or development of  $\mu$ MT mice, as compared to their appropriate background controls<sup>165</sup>. C57BL mice, both control and  $\mu$ MT, were able to control infection and mount an appropriate Th1 response. BALB/c mice, control as well as  $\mu$ MT, developed progressive lesions and mounted inappropriate Th2 responses upon infection with *L. major*. Thus, the authors conclude that neither B cells, nor antibody mediated effector functions are required for the development of polarized T helper cell responses during *L. major* infections. The following year another paper, published by Smelt, *et al.*, suggested that while B cell deficient mice were highly resistant to infection with *L. donovani*, it was not related to the presence, or absence, of IgG. These studies suggested, rather, that IgG protected mice from the neutrophil mediated pathology that occurs during

infection<sup>166</sup>. These papers reversed the idea of a humoral requirement during *Leishmania* infection, despite almost 20 years of reports indicating a potential involvement for B cells, IgG, and/or immune complexes during both murine and human leishmaniasis. In the 5 years following these reports, there has been little subsequent investigation into the involvement of humoral factors during leishmaniasis.

### Summary

Previous work in our lab has shown that IgG coated immune complexes are capable of ligating the Fc $\gamma$ R on macrophages. This leads to the abrogation of IL-12, and an increase in the production of IL-10. We hypothesize that IgG coated amastigotes ligate macrophage Fc $\gamma$ Rs, increasing IL-10. Increased production of IL-10 blocks macrophage activation. Without macrophage activation, *Leishmania* amastigotes are free to replicate unchecked within macrophage phagolysosomes, allowing for the continued progression of the disease. These studies examine this hypothesis and the correlation of antibody with the progression of leishmaniasis.

This work makes the unexpected observations that IgG accesses *Leishmania* within M $\phi$ s and that IgG opsonization can lead to disease exacerbation. This points to an unexpected role for IgG in inducing susceptibility to intracellular infections.

## CHAPTER 2: MATERIALS AND METHODS

### Parasites

The Friedlin strain of *L. major* (WHO MHOM/IL/80/Friedlin)<sup>167</sup> was isolated from an Israeli patient with cutaneous leishmaniasis, and was provided to us by David Sacks at the National Institutes of Health, Bethesda, MD. The Josefa isolate of *L. amazonensis*<sup>168</sup> was isolated from a Brazilian patient with cutaneous leishmaniasis and was provided to us by Janet Keithly at the Wadsworth Center, Albany, NY. *L. chagasi* was isolated from a Brazilian patient with visceral leishmaniasis, and provided to us by Selma Jeronimo at the Universidade Federal Rio Grande do Norte, Natal, Brazil.

Promastigotes were cultured in Schneider's complete medium (SCM), which consisted of Schneider's insect medium (Sigma-Aldrich, St. Louis, MO), 20% heat-inactivated (30 min at 56°C) fetal calf serum (HI-FCS, Hyclone Laboratories, Logan, UT), 2mM glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin G (Mediatech, Herndon, VA). Lesion-derived *L. major* amastigotes were isolated from infected BALB/c, or C57BL/6 mice as described previously<sup>168</sup>. Axenic *L. chagasi* and *L. amazonensis* amastigotes were grown as described previously<sup>169</sup>. Briefly, promastigotes were resuspended in pH adjusted SCM, and incubated at 33°C, to allow transformation to the amastigote stage. For opsonization of amastigotes with IgG, axenically grown amastigotes were incubated for 20 min at 4°C in immune serum. Soluble Leishmania Antigen (SLA) was prepared by freezing

late log phase cultures (4-6 days) in liquid nitrogen and immediately thawing in a 56°C water bath. This procedure was performed a total of seven times. The culture was then observed by microscopy to confirm the deterioration of the parasites. The parasites were then centrifuged at 2500 rpm for 10 min, and the supernatant containing SLA was collected. A Coomassie Blue assay was used to determine the concentration of SLA.

### Mouse Strains and Infections

These studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. BALB/c and C57BL/6 were purchased from the National Cancer Institute Charles River Laboratories, or from Taconic (Germantown, NY). J<sub>H</sub> mice<sup>170</sup> and FcR  $\gamma$ -chain knockout<sup>171</sup> mice ( $\gamma^{-/-}$ ) were purchased from Taconic.

For infection, mice were injected in the hind footpad with either  $2 \times 10^5$  or  $2 \times 10^6$  amastigotes. Lesion size was determined by using a caliper to measure the thickness of the infected footpad and subtracting the thickness of the contralateral uninfected footpad as described previously<sup>72</sup>. Parasite burdens were determined by a serial dilution of single cell suspensions made from excised lesions as described previously<sup>72</sup>.

For immunizations against ovalbumin (OVA), C57BL/6 mice were immunized with 25  $\mu$ g of OVA in 250  $\mu$ L of Incomplete Freund's Adjuvant (IFA; Difco Laboratories, Livonia, MI), by i.p. injection. At 2 and 4 wks after the initial injection, the mice received a "booster" dose of 25  $\mu$ g of OVA in

250  $\mu$ L of IFA. After receiving the final boost, mice were bled to confirm the presence of antibodies, and subsequently infected in the right hind footpad.

### Primary Cells

Murine bone marrow derived macrophages (BMM $\phi$ ) were established as previously described<sup>19</sup>. Briefly, bone marrow was flushed from the femurs of mice using 5 mL cold PBS, using a 23 gauge needle, and collected by centrifugation. Bone marrow cells were suspended in D-10, which consisted of Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech) supplemented with 10% HI-FCS, 2mM glutamine, 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin G. Cells were then plated in petri dishes, in D-10 supplemented with 20% L-929 conditioned media, as a source of macrophage colony stimulating factor (m-CSF). Cells were incubated at 37°C with 5% CO<sub>2</sub>. For infection of monolayers, macrophages were cultured overnight in 24-well plates at a density of  $2 \times 10^5$  cells/well, or on glass coverslips at  $1 \times 10^5$ . When required, cells were stimulated with 150  $\mu$ g/mL low molecular weight-hyaluronic acid (LMW-HA, Worthington, Lakewood, NJ) before the addition of a 10:1 ratio of *L. major* amastigotes.

Human monocytes were isolated from the blood of normal donors. These studies were approved by the IRB of the University of Maryland. Blood was collected in heparinized tubes (BD Biosciences, San Jose, CA), and then transferred to 50 mL tubes, in 23 mL aliquots. Total volume was brought to 36 mL using PBS. 14 mL of Ficoll-Paque (Amersham

Biosciences, Piscataway, NJ) was underlaid by slowly dispensing at the bottom of the tube. Tubes were centrifuged for 20 min at room temperature, 2500 rpm, and with the brake set to zero. After the spin, the opaque middle layer of PBMNCs, containing the monocytes, was transferred to another 50 mL tube. Cells were diluted 1:1 with PBS and centrifuged for 10 min at 4°C and 2500 rpm. The pellet was resuspended in 5 mL PBS, transferred to a 15 mL tube, and centrifuged at 1500 rpm and 4°C for 10 min. The PBMNCs were then counted and either resuspended in a volume appropriate to the specific experiment, or plated at  $1 \times 10^6$  cells/mL in R-20, which consisted of RPMI (Mediatech), 20% HI-FCS, 2mM glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin G, supplemented with 100 µL HEPES per 5 mL of media. For infections, monocytes were given a 10:1 ratio of *L. chagasi* amastigotes. In some instances, monocytes were stimulated with 10 ng/mL lipopolysaccharide (LPS).

### Antibodies

For opsonization and reconstitution experiments, antibodies to *L. major* and *L. amazonensis* were obtained by bleeding uninfected or infected mice. The blood was allowed to clot, and then centrifuged to recover sera. Purified IgG against *L. major* was obtained by passing serum from infected BALB/c, or C57BL/6 mice through a protein G column on a GradiFrac System (Amersham Biosciences).  $\alpha$ -IL-10R monoclonal antibody<sup>172</sup> was provided by K. Moore (DNAX Research Institute, Palo Alto, CA). IgG and

$\alpha$ -IL-10R treatments were administered by i.p. injections at designated times during the experiment. Serum against *L. chagasi* was obtained from patients with parasitological confirmed cases of visceral leishmaniasis. Use of human sera was reviewed and approved by the Universidad Federal do Rio Grande do Norte Ethical Committee (CEPUFRN19/01), by the Brazilian National Ethical Committee of Research (CONEP 4572), and the IRB of the University of Maryland.

### Staining

To visualize intracellular amastigotes, infected BMM $\phi$ , which were adhered to glass coverslips, were fixed in methanol at 4°C for 15 min. Monolayers were washed with PBS. Non-specific antibody binding was prevented by incubation in blocking buffer (PBS + 10% HI-FCS) for 30 min at 4°C. Monolayers were stained for 45 min at 4°C with antisera from *Leishmania* infected BALB/c mice (1:200). After washing with PBS, a 1:500 dilution of fluorescein isothiocyanate (FITC) conjugated goat  $\alpha$ -mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) was added to monolayers for 45 min at 4°C. Macrophages were counterstained for 15 min with 0.008% Evans Blue (Sigma-Aldrich), or for 1 min with 1% Propidium Iodide.

Intracellular opsonization of axenic *L. amazonensis* amastigotes was determined by infecting monolayers of BMM $\phi$  adhered to glass coverslips, with a 10:1 ratio axenically grown amastigotes. After 45 min, monolayers

were washed with PBS to remove any unbound amastigotes. Infected monolayers were incubated for 1 hour to allow complete internalization of bound amastigotes. Antisera (5%), from infected BALB/c mice, was added to the monolayers for 2 hours, after which time the monolayers were washed to remove extracellular IgG. Monolayers were fixed in methanol at 4°C for 15 min, and then washed with PBS. Non-specific antibody binding was prevented by incubation in blocking buffer (PBS supplemented with 10% HI-FCS) for 30 min at 4°C. The presence of surface IgG on the internalized amastigotes was visualized by staining for 45 min at 4°C with a 1:500 dilution of FITC conjugated goat  $\alpha$ -mouse IgG, specific for total as well as isotype specific IgG (Southern Biotechnology, Birmingham, AL). Macrophages were counterstained for 15 min with 0.008% Evans Blue, or for 1 min with 1% Propidium Iodide.

Intracellular opsonization of axenic *L. chagsi* amastigotes was determined by infecting monolayers of primary human M $\phi$ , isolated from blood donors then adhered to glass coverslips, with a 10:1 ratio axenically grown amastigotes. After 45 min, monolayers were washed with PBS to remove any unbound amastigotes. Infected monolayers were incubated for 1 hour to allow complete internalization of bound amastigotes. Antisera (5%), from patients with parasitological confirmed cases of visceral leishmaniasis, was added to the monolayers for 2 hours, after which time the monolayers were washed to remove extracellular IgG. Monolayers were fixed in methanol at 4°C for 15 min, and then washed with PBS. Non-specific

antibody binding was prevented by incubation in blocking buffer for 30 min at 4°C. The presence of surface IgG on the internalized amastigotes was visualized by staining for 45 min at 4°C with a 1:500 dilution of FITC conjugated mouse  $\alpha$ -human IgG, specific for total as well as isotype specific IgG (Sigma-Aldrich). Macrophages were counterstained for 15 min with 0.008% Evans Blue, or for 1 min with 1% Propidium Iodide.

Coverslips with adhered, immunostained macrophages were mounted on slides using mowiol (Calbiochem, San Diego, CA). Slides were allowed to dry at room temperature for at least 30 min, while being protected from light, before visualizing at either 400x or 630x (oil immersion) magnification using a Zeiss Axioplan 2 fluorescent imaging research microscope and Zeiss KS300 imaging software.

### Real Time PCR

J<sub>H</sub> mice were infected with  $2 \times 10^5$  *L. major* amastigotes in the right hind footpad. 21 days after infection, mice were given a single i.p. injection of 600  $\mu$ l  $\alpha$ -*L. major*-IgG. Total RNA was extracted from the footpad lesions 48 hours after treatment with  $\alpha$ -*L. major* IgG, using the RNAqueous-4PCR kit obtained from Ambion (Austin, TX). IL-10 mRNA levels were detected by real-time PCR. Real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System and SYBR green core PCR reagents (Applied Biosystems, Foster City, CA). The murine IL-10 primer sequences used for this analysis were as follows: sense, 5'-CCACAAAGCAGCCTTGCA- 3' and

antisense, 5' AGTAAGAGCAGGCAGCATAGCA- 3'. The hypoxanthine phosphoribosyltransferase primers used were as follows: sense, 5'-GTTGGATACAGGCCAGACTTTGTTG-3' and antisense, 5'-AGGGTAGGCTGGCCTATAGGCT-3'.

#### Enzyme Linked Immunosorbent Assay (ELISA)

Cytokine production from stimulated macrophages was measured by ELISA. Macrophages were seeded in 24-well plates at a density of  $2 \times 10^5$  cells/well and incubated overnight at 37°C. Macrophages were stimulated with 150 µg/mL LMW-HA, and axenic, or axenic-opsonized, or lesion derived amastigotes at a ratio of 10:1. Cell supernatants were harvested 24 hours after infection. Levels of murine secreted IL-12p40 and IL-10 protein concentrations in cell supernatants were determined by sandwich ELISA according to protocols provided by BD PharMingen. Capture and detection (biotinylated) antibody pairs (IL-12p40, C15.6 and C17.8; IL-10, JES5-2A5 and JES5-16E3) and recombinant cytokine protein standards were purchased from BD PharMingen. Streptavidin alkaline phosphatase (AP) and p-nitrophenyl phosphate (PNPP) substrate were purchased from Southern Biotechnology and were used according to the manufacture's instructions. Samples were placed in 96-well flat bottom plates, and measured at 405nm.

The levels of IL-10 in active footpad lesions were also measured by ELISA. J<sub>H</sub> mice were infected with  $2 \times 10^5$  *L. major* amastigotes in the right hind footpad. 21 days after infection, mice were given a single i.p. injection

of 600  $\mu$ l  $\alpha$ -*L. major*-IgG. After the administration of  $\alpha$ -*L. major*-IgG, IL-10 protein levels in the infected feet were measured by ELISA. Lesions were homogenized in lysis buffer (15 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>) as described previously<sup>173</sup>. Samples were incubated on ice for 30 min and centrifuged at 850 g for 30 min. Supernatants were then harvested and analyzed by ELISA as described above.

Cytokine production by lymph node T cells was determined by ELISA. Mice were infected with  $2 \times 10^5$  *L. major* amastigotes in the presence or absence of 600  $\mu$ l  $\alpha$ -*L. major*-IgG. At day 21 after infection, lymph nodes were removed, and unfractionated lymph node cells were stimulated with 50  $\mu$ g/mL SLA. Supernatants were harvested 72 hours later, and IFN- $\gamma$  and IL-4 protein concentrations were measured by ELISA as described above. Capture and detection antibody pairs (IFN- $\gamma$ , R4-6A2 and XMG1.2; IL-4, 11B11 and BVD6-24G2) and recombinant cytokine protein standards were purchased from BD PharMingen.

After infection with *L. major*, antigen specific IgG isotypes in circulating sera were determined by ELISA. 96-well plates were coated overnight with 200 ng/well of SLA in coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 34 mM NaHCO<sub>3</sub>, pH = 9.6). The following day, non-specific binding was blocked by incubating wells in blocking buffer for 1 hour at room temperature. Dilutions of the sera (1:1000 through 1:500,000) were placed in the wells, and incubated at room temperature for 2 hours. After washing, 100  $\mu$ L of AP conjugated antibodies for total as well as isotype specific IgG (Southern Biotechnology)

was added to wells at a 1:2000 dilution, and incubated for 1 hour at room temperature. PNPP was added to wells after washing, and plates were incubated in the dark until being read at 405 nm. The antibody titer was determined as the final dilution of sera which had an optical density (OD) greater than 0.100.

### Flow Cytometry Analysis

IL-10 production by human monocytes infected with *L. chagasi* amastigotes was measured by flow cytometry. Freshly isolated human monocytes were infected with a 10:1 ratio of serum opsonized *L. chagasi* amastigotes in the presence of 10 ng/mL LPS. After 15 min, monolayers were treated with Golgi Stop (BD Biosciences) for an additional 2 hours. Surface staining to identify monocytes was performed first, by staining with a 1:100 dilution of a FITC conjugated mouse  $\alpha$ -human CD14 antibody (M5E2, BD Biosciences) for 30 min at 4°C. Monocytes were washed in PBS, and then fixed and permeablized using the CytoFix/CytoPerm solution (BD Biosciences) for 20 min at 4°C. Intracellular IL-10 was detected using a 1:100 dilution of a phycoerythrin (PE) conjugated rat  $\alpha$ -human IL-10 monoclonal antibody (JES3-19F1, BD Biosciences) for 30 min at 4°C.

IgG on the surface of lesion derived amastigotes was measured by flow cytometry. Surface IgG from lesion derived *L. major* amastigotes was carried out immediately after amastigotes were isolated from the footpad lesions of BALB/c or C57BL/6 mice. Amastigotes were incubated in a 1:500

dilution of FITC conjugated goat  $\alpha$ -mouse isotype specific IgG for 45 min at 4°C. Amastigotes were washed with PBS, and fixed in fresh 4% paraformaldehyde.

Intracellular opsonization of axenic *L. amazonensis* amastigotes was determined by flow cytometry. Monolayers of BMM $\phi$ , seeded at a density of  $1 \times 10^6$  BMM $\phi$ /well in 6-well plates, were infected with a 10:1 ratio axenically grown amastigotes. After 45 min, monolayers were washed with PBS to remove any unbound amastigotes. Infected monolayers were incubated for 1 hour to allow complete internalization of bound amastigotes. Antisera (5%), from infected BALB/c mice, was added to the monolayers for 2 hours, after which time the monolayers were washed to remove extracellular IgG. Macrophages were taken up by incubating in Cell Stripper (Mediatech) at 37°C for 10 min, washed in PBS, and lysed using a series of syringes, as described in the amastigote isolation procedure. Isolated amastigotes were fixed in fresh 4% paraformaldehyde at 4°C for 15 min. The presence of surface IgG was visualized by staining for 45 min at 4°C with a 1:500 dilution of FITC conjugated goat  $\alpha$ -mouse IgG, specific for total as well as isotype specific IgG (Southern Biotech, Birmingham, AL).

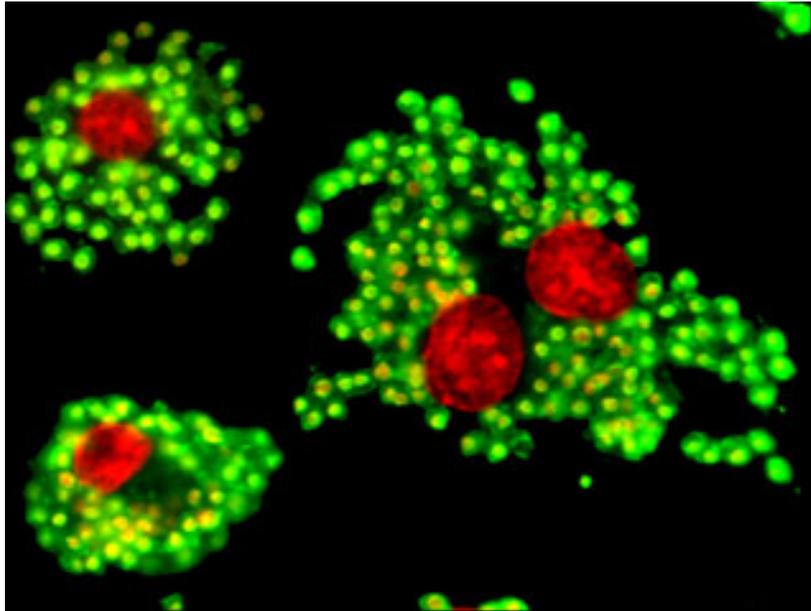
Following fixation, cells were washed in PBS and analyzed by flow cytometry using a Becton Dickinson FACSCalibur benchtop flow cytometer and CellQuest software.

## CHAPTER 3: A ROLE FOR IgG IMMUNE COMPLEXES DURING INFECTION WITH *LEISHMANIA*

### *Opsonization and Association with Macrophages*

*Leishmania* amastigotes are known to have host IgG on their surfaces<sup>174;175</sup>. It was thought that this provided opsonization of the organisms, facilitating amastigote entry into macrophages. Although previous publications have demonstrated the presence of IgG on amastigote surfaces<sup>72</sup> (Figure 3), no experiments have been performed to determine how the amastigotes and IgG come into contact. It was assumed that as the parasites replicate, bursting host macrophages, the amastigotes were released into the extracellular milieu, and there they encountered host IgG. We hypothesized that IgG can access amastigotes intracellularly.

The neonatal receptor is instrumental in the regulation of IgG serum levels, salvaging IgG from degradation within macrophage phagolysosomes. IgG binds to the receptor in the acidic phagolysosome environment. Upon receptor recycling to the cell surface, the neutral pH causes the IgG to be released from the receptor, thus maintaining IgG levels<sup>176-178</sup>. It is possible that amastigotes and IgG could come into contact during this process, and result in amastigotes which had been opsonized intracellularly. An experiment was performed to determine whether IgG could bind to axenic *L.*

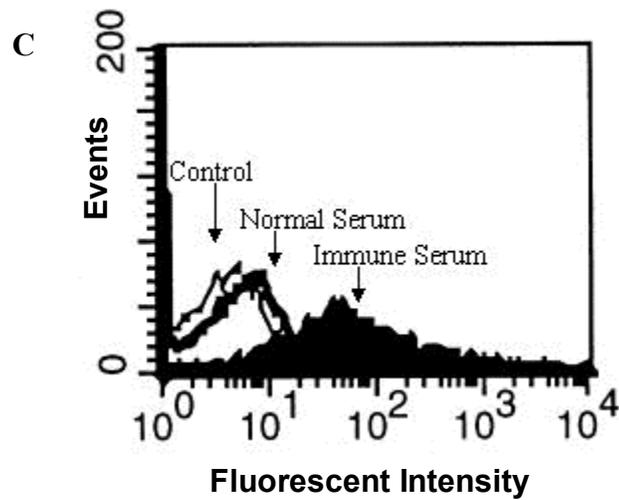
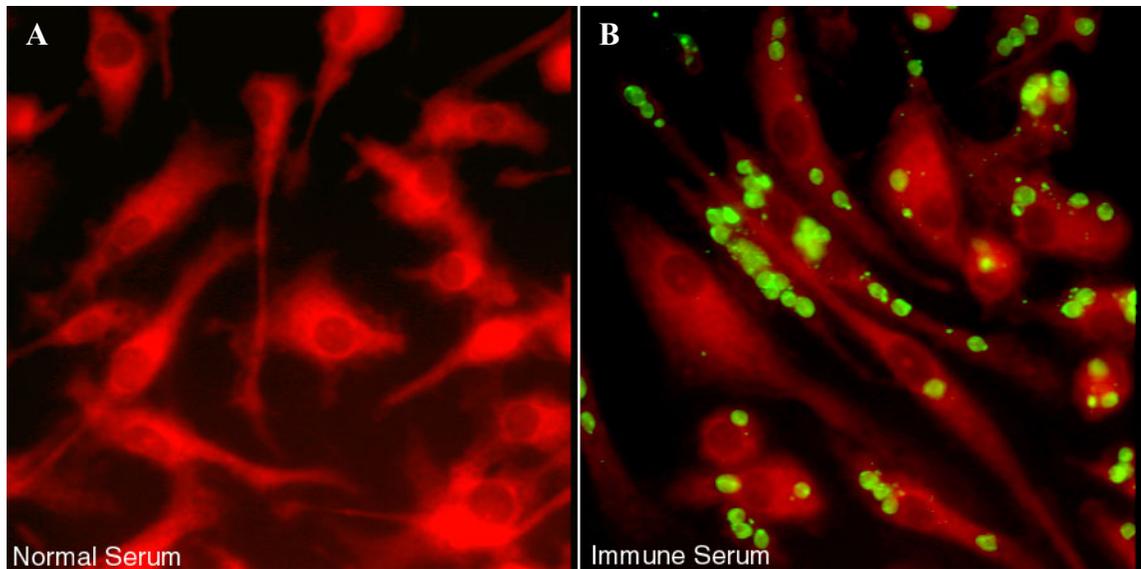


**Figure 3. Lesion Derived Amastigotes Have Host IgG on Their Surfaces.** Amastigotes isolated from active BALB/c lesions were added to macrophage monolayers. After a 45 min incubation, unbound parasites were washed away, and the amastigote containing macrophages were fixed. A FITC conjugated goat  $\alpha$ -mouse IgG antibody (1:500) allowed the visualization of host (murine) IgG on the surface of amastigotes (green). Macrophage nuclei (red) were counterstained with propidium iodide (1:1000).

*amazonensis* amastigotes after their uptake into macrophages, and opsonize them intracellularly. Axenic amastigotes were grown in cell free culture, and thus have no IgG on their surface.

Monolayers of macrophages were infected 10:1 with axenic *L. amazonensis* amastigotes. After an initial incubation, unbound amastigotes were washed away, and 5% sera from either normal, naïve BALB/c mice, or chronically infected mice, was added to the media and allowed to incubate for 2 hours. After staining with a FITC-conjugated anti-IgG antibody, it was evident by both fluorescent microscopy (Figure 4 A,B) and flow cytometry (Figure 4 C) that immune serum was able to access the amastigotes, which were presumably within the macrophage, and bind to its surface. These data suggest that antigen specific IgG may be able to enter macrophages and opsonize amastigotes intracellularly.

To determine whether host IgG was an opsonin that was necessary for amastigotes to gain entry into host cells, we infected BALB/c macrophage monolayers with IgG-free axenic, axenic amastigotes opsonized with IgG, and with lesion derived amastigotes. Axenic opsonized amastigotes were made by incubating axenic amastigotes in 5% sera to allow them to become opsonized with IgG. We also used lesion derived amastigotes, which were isolated from active lesions in mice, and are thus coated with murine IgG<sup>72</sup>. Over a 120 min time course, the *L. amazonensis* axenic, axenic-opsonized, and lesion



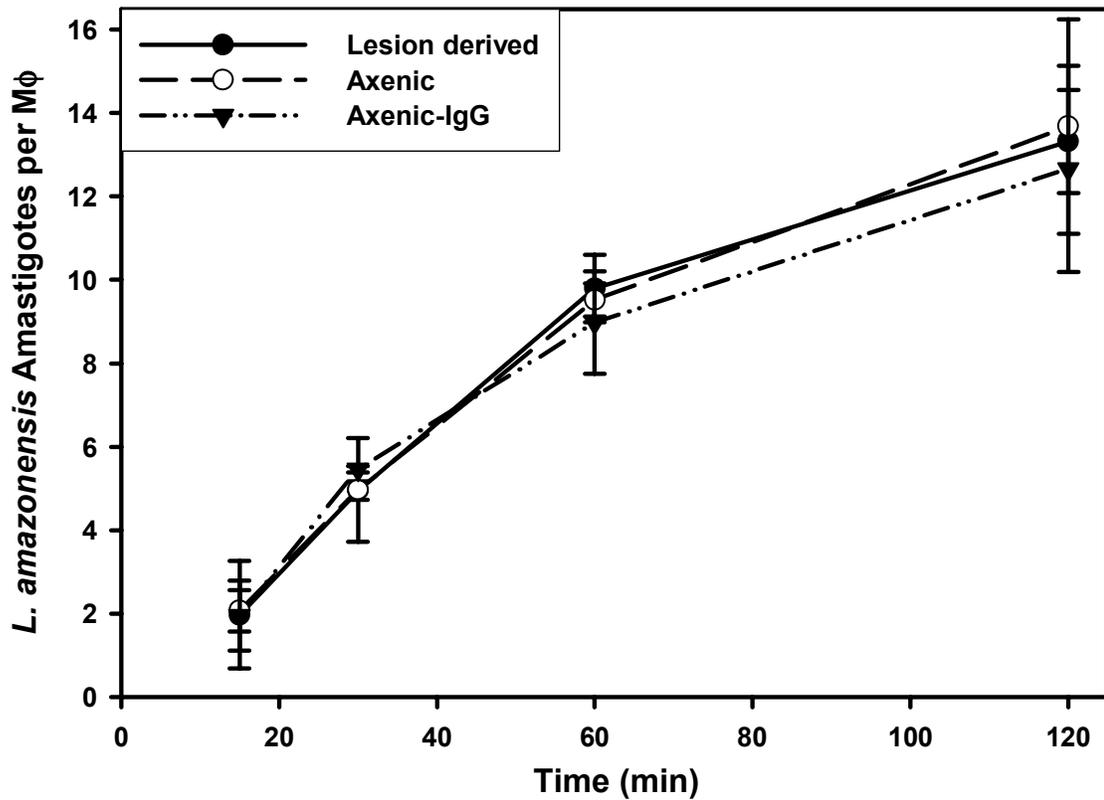
**Figure 4. The Intracellular Opsonization of *L. amazonensis* Amastigotes.** Macrophages were infected with axenic *L. amazonensis* amastigotes, and then incubated in either normal or immune sera. After the incubation with sera, monolayers were washed, and amastigotes were stained for the presence of surface IgG (green). Macrophages were counterstained with Evans Blue (red). Immune sera (B), but not normal sera (A) was able to opsonize amastigotes. Flow cytometry on the amastigotes (C) was performed to quantify surface staining. These findings suggest that only immune sera was able to opsonize amastigotes intracellularly.

derived amastigotes displayed a similar ability to associate with macrophages (Figure 5). Thus, the presence of IgG does not increase the ability of amastigotes to associate with macrophages.

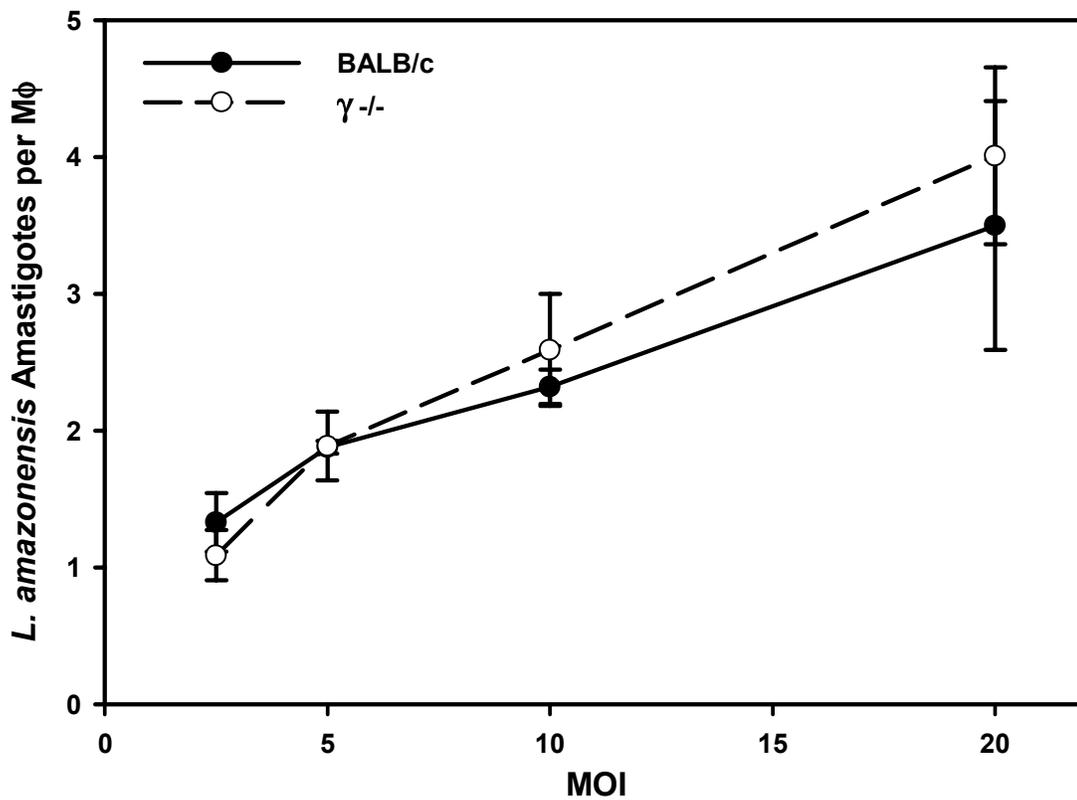
A similar study was performed using macrophages from  $\gamma^{-/-}$  mice. These mice are deficient in the FcR  $\gamma$  chain. The association of the Fc binding chain ( $\alpha$ ) and the signaling chain ( $\gamma$ ) is necessary for the expression of FcRs. Thus, knocking out the  $\gamma$  chain results in the reduced activity of these receptors. The association of amastigotes with  $\gamma^{-/-}$  macrophages was compared to macrophages from BALB/c mice. Lesion derived amastigotes, which are coated in IgG, associated similarly to both BALB/c macrophages and macrophages that are deficient in the FcR  $\gamma$  chain (Figure 6). Various multiplicities of infection (MOIs) were used, and parasite association with both types of macrophages was similar, regardless of parasite number. Together, these figures show that neither IgG on the surface of amastigotes, nor the Fc $\gamma$ R on the surface of macrophages affects amastigote association with macrophages.

#### *Mice Lacking IgG Are More Resistant to Leishmania Infection*

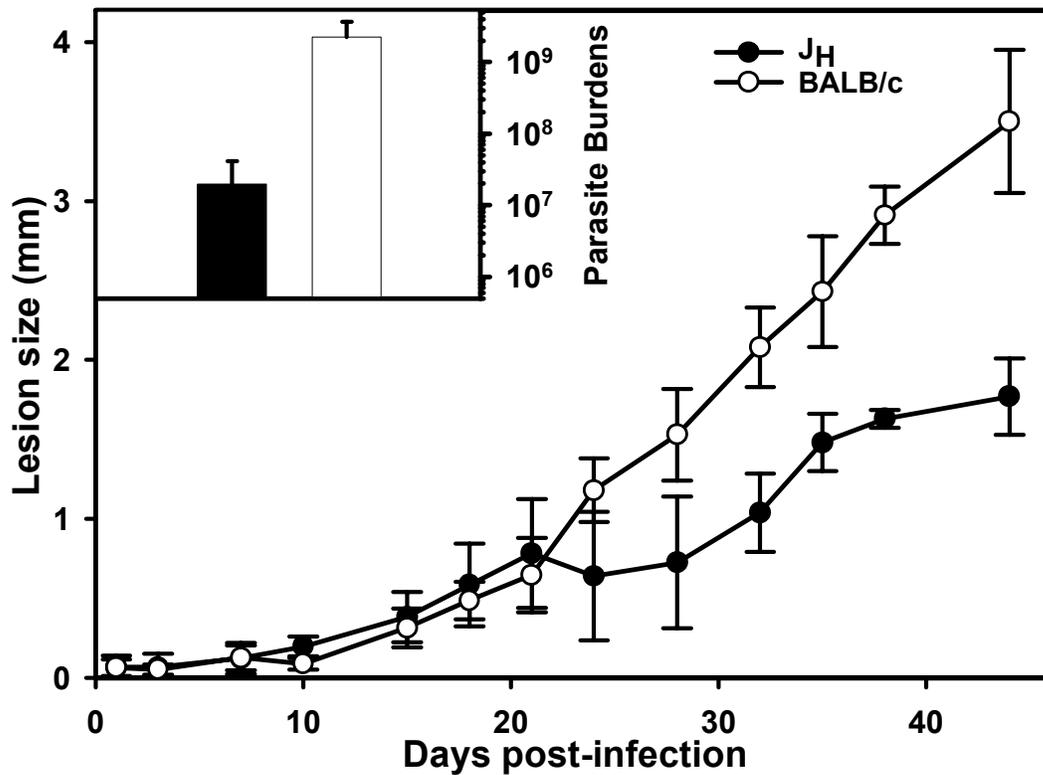
To determine a role for IgG antibodies in the host immune response to *Leishmania spp.*, we infected normal BALB/c mice with *L. amazonensis* (Figure 7) parasites and compared the course of infection to that which occurred in the J<sub>H</sub> strain of mice on the BALB/c background. J<sub>H</sub> mice have a



**Figure 5. The Role of IgG in Amastigote Association.** Lesion derived (closed circles), Axenic (open circles), and axenic-IgG (closed triangles) *L. amazonensis* amastigotes (10:1) were added to Mφ monolayers for 15, 30, 60, and 120 minutes. Monolayers were then washed, fixed and stained to allow visualization of bound amastigotes. Error bars represent the standard error of the mean of 3 separate experiments.



**Figure 6. Fc $\gamma$ R<sub>s</sub> in Amastigote Association.** Association of lesion derived *L. amazonensis* amastigotes with macrophages from  $\gamma^{-/-}$  (open circles) and BALB/c (closed circles) mice. Parasites were added to monolayers at MOIs of 2.5:1, 5:1, 10:1, and 20:1, for 45 min. Monolayers were then washed, fixed and stained to allow visualization of bound amastigotes. Error bars represent the standard error of the mean of 3 separate experiments.

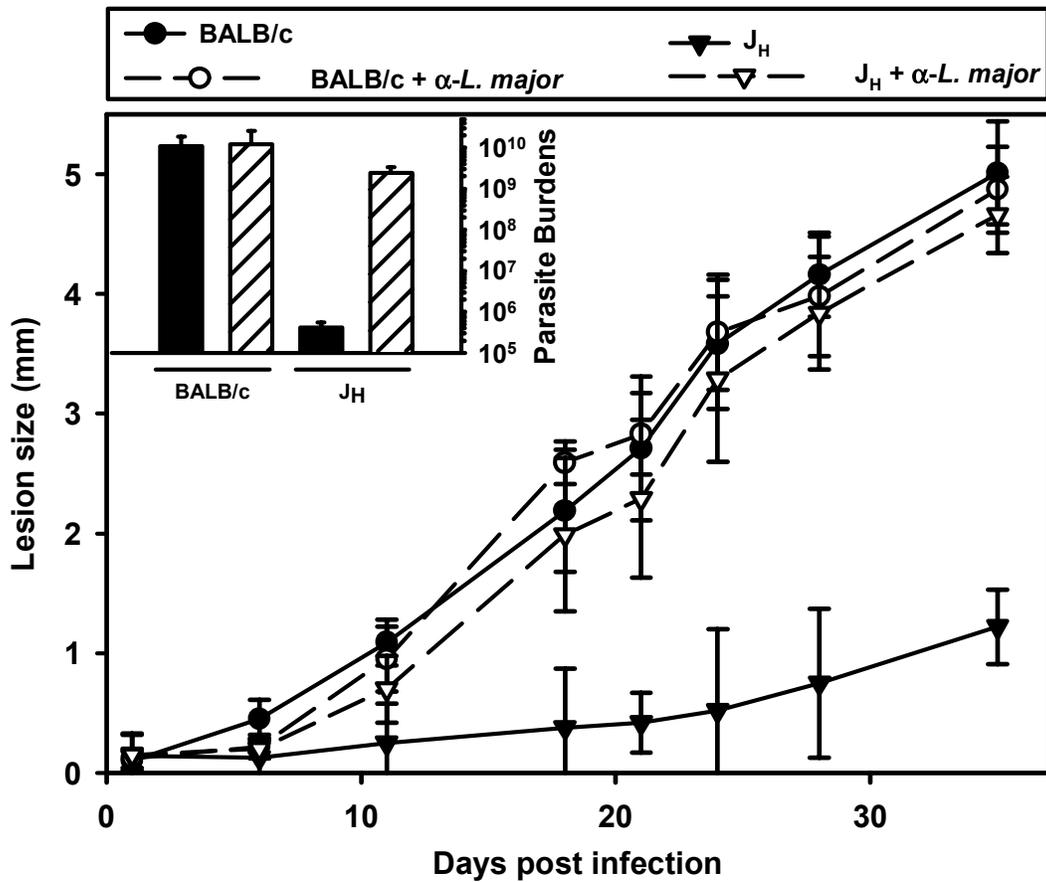


**Figure 7.** *L. amazonensis* progression in J<sub>H</sub> vs. BALB/c mice. Footpad swelling in J<sub>H</sub> (closed circles, n=4) and BALB/c mice (open circles, n=4) was compared following infection in the right hind footpad with  $2 \times 10^6$  amastigotes. Groups begin to differ after day 21, with J<sub>H</sub> developing significantly smaller lesions than their wild type counterparts. Parasite burdens (inset) mirror the findings of the footpad measurements. Error bars represent the standard deviation of the mean of 4 determinants.

targeted deletion of the immunoglobulin heavy chain J locus and, therefore, make no antibody. Normal BALB/c mice are susceptible to *Leishmania* infections<sup>179</sup> and developed large lesions (Fig.7 open circles). As expected, J<sub>H</sub> mice develop smaller lesions (Fig.7 closed circles) with fewer parasites within the lesion (Fig.7 closed bar, inset). These data suggest that the absence of antibody in J<sub>H</sub> mice leads to increased resistance to *L. amazonensis*.

#### Addition of IgG Exacerbates Disease in J<sub>H</sub> Mice

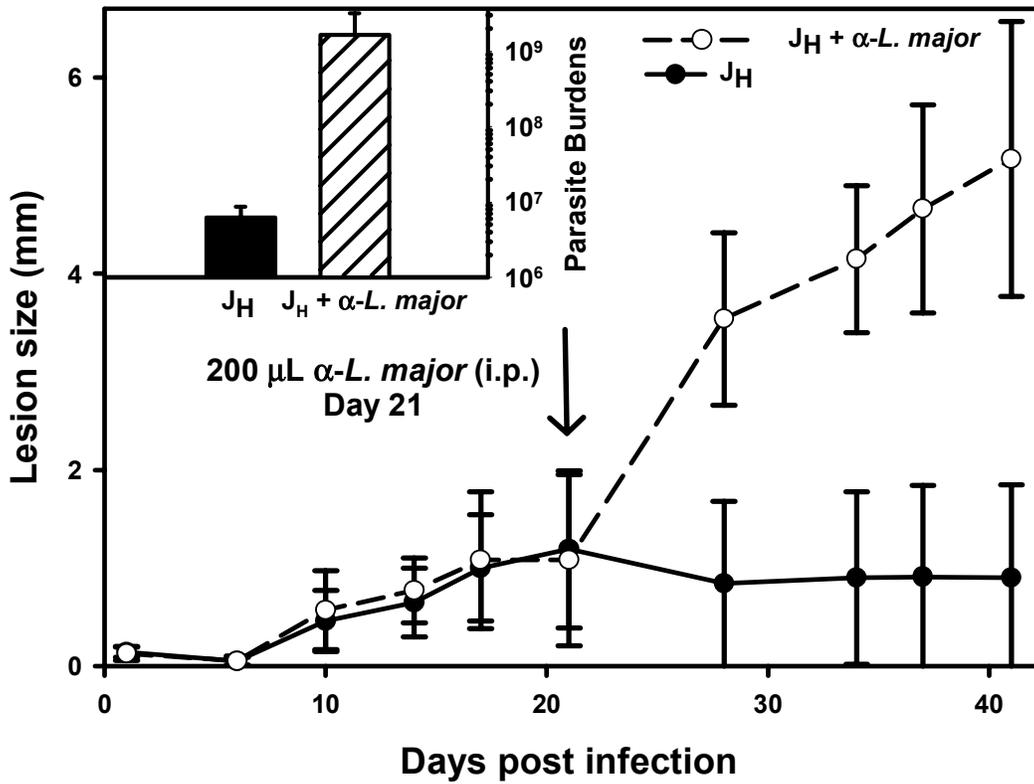
A similar experiment was performed using J<sub>H</sub> mice infected with *L. major*. J<sub>H</sub> mice were relatively resistant to disease, developing modest lesions (Figure 8, filled triangles), which contained several orders of magnitude fewer parasites (Fig.8, inset) than the wild-type BALB/c mice. The passive administration of polyclonal  $\alpha$ - *L. major* antiserum to wild-type BALB/c mice had essentially no effect on lesion progression (Fig. 8, open circles). These mice developed lesions with the same size and kinetics as mice that received no antisera. Furthermore, the two groups of BALB/c mice had similar numbers of parasites within their lesions at the conclusion of the observation period 35 days post infection (Fig.8, inset). In 3 separate experiments, using a minimum of 4 mice per experimental group, the mean lesion size of infected J<sub>H</sub> mice was 1.22 +/- 0.31 mm at 35 days post-infection, whereas infected



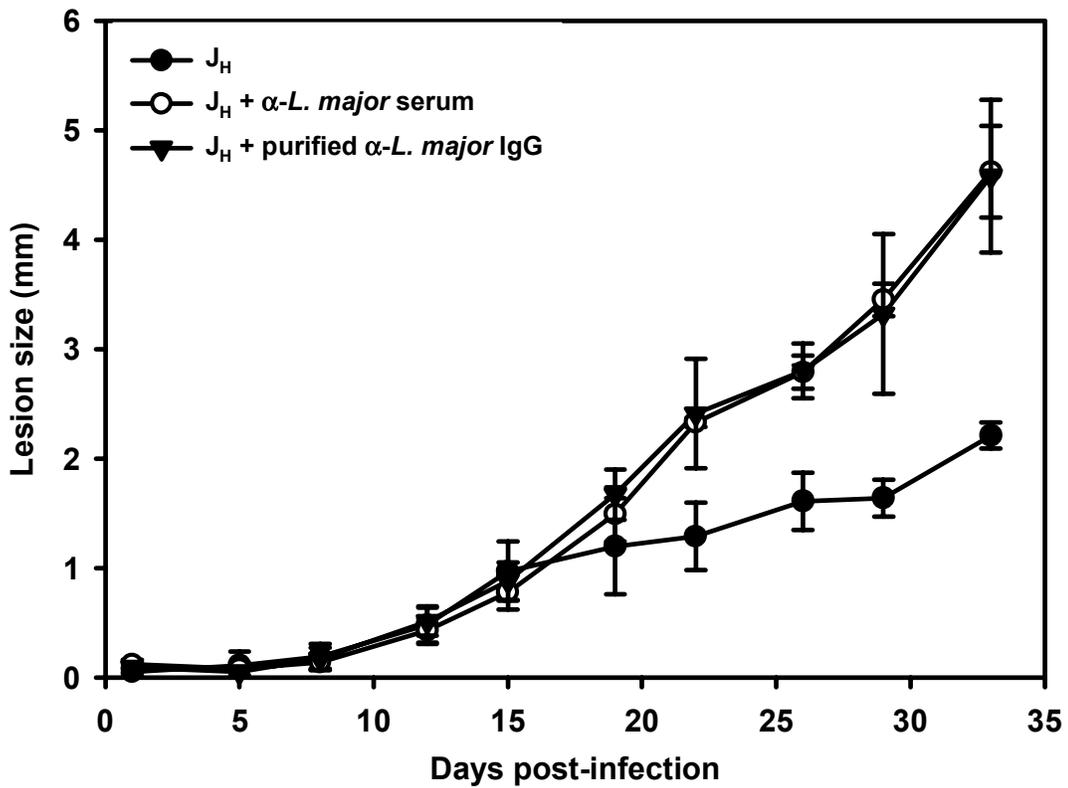
**Figure 8. Serum Reconstitution of J<sub>H</sub> Mice.** Lesion size of J<sub>H</sub> mice (filled triangles) and BALB/c mice (filled circles) was compared to J<sub>H</sub> mice (open triangles) and BALB/c mice (open circles) given 200  $\mu$ l antisera to *L. major* on days 1, 7 and 14. Parasite burdens (inset) in BALB/c and J<sub>H</sub> mice that were infected with (striped bars) or without (solid bars)  $\alpha$ -*L. major* antisera were determined on Day 35, by limiting dilution assays. Error bars represent the standard error of the mean of 3 separate experiments, with a minimum of 4 mice per group.

BALB/c mice developed lesions that were 5.01 +/- 0.43 mm. The passive administration of immune serum to J<sub>H</sub> mice reversed this resistant phenotype and actually exacerbated disease, restoring lesion size to that observed in wild-type BALB/c mice (Fig.8, open triangles). The number of parasites in these lesions was comparable to that observed in wild-type BALB/c mice (Fig.8, inset).

A similar study was performed on J<sub>H</sub> mice, except that  $\alpha$ -*L. major* antiserum was administered to the mice after the infection had progressed for 3 weeks. This was done to determine the speed with which antibody to *L. major* could exacerbate infections (Figure 9). In these studies, two groups of J<sub>H</sub> mice were infected with *L. major* for 3 weeks. One of the groups was then given a single 200  $\mu$ L i.p. injection of  $\alpha$ -*L. major* antiserum. Within 1 week of antiserum administration, footpad lesions in this group were already significantly larger than the controls. Three weeks after the injection of antiserum, the lesions had progressed to a point where their size (Fig. 9, open circles), and the number of parasites within them (Fig. 9, inset) was comparable to susceptible BALB/c mice. Similar studies were performed using IgG that was affinity purified from the immune serum used in Figure 9. A single i.p. injection of 600  $\mu$ g of purified  $\alpha$ -*L. major* IgG into mice at 3 weeks post-infection caused a similar increase in lesion formation with kinetics that were similar to unfractionated immune serum (Figure 10).



**Figure 9. Serum Reconstitution of J<sub>H</sub> Mice 3 Weeks Post-Infection.** Lesion size of J<sub>H</sub> mice (filled circles) were compared to those of J<sub>H</sub> mice given 200 μl α-L. major antiserum on day 21 post-infection (open circles). Parasite burdens (inset) were determined at 42 days post-infection by limiting dilution assays, as described. Error bars represent the standard deviation of the mean of 4 determinations. This experiment is representative of three.

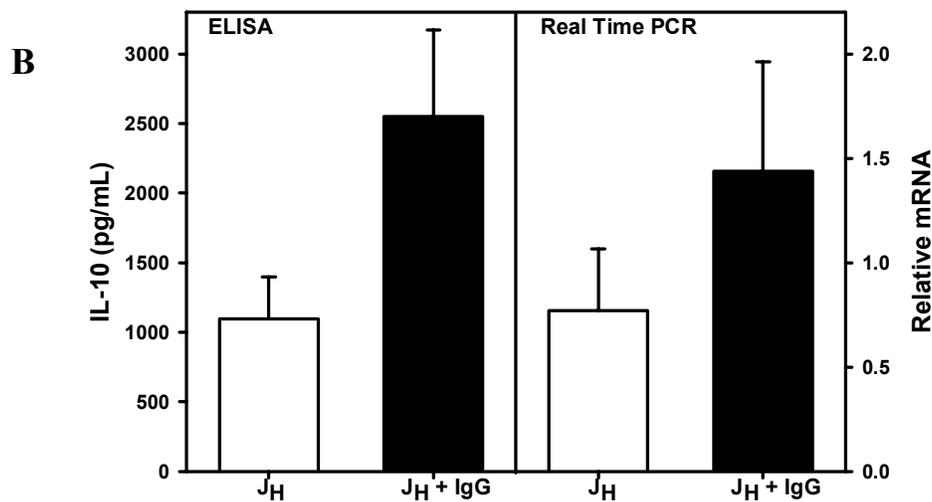
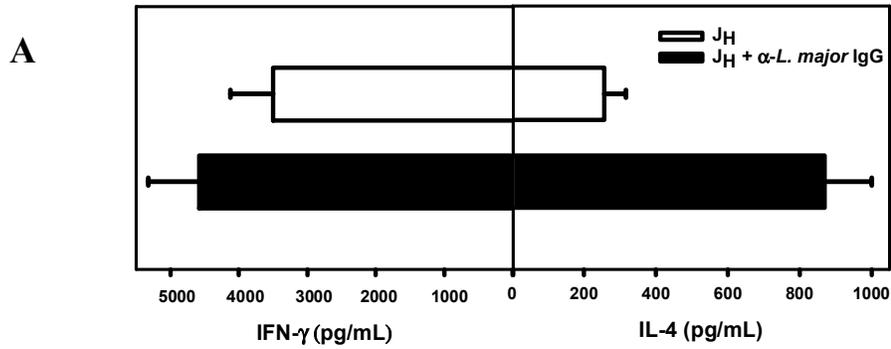


**Figure 10. Reconstitution of J<sub>H</sub> mice 3 Weeks After Infection with Either *α-L. major* Antiserum or Purified *α-L. major* IgG.** Lesion size of J<sub>H</sub> mice (closed circles) was compared to those of J<sub>H</sub> mice given 200  $\mu$ l *α-L. major* antiserum (open circles) or 600  $\mu$ l *α-L. major* IgG (closed triangles) on day 21 after infection. Error bars represent the standard deviation of the mean of 4 determinations.

### IL-10 Production During Leishmania Infection

Cytokine production from lymph node T cells was analyzed in vitro, following the infection of J<sub>H</sub> mice with *L. major* in the presence or absence of antibody, in vivo. The administration of  $\alpha$ -*L. major* IgG resulted in a significant increase in the production of IL-4 by lymph node T cells which were restimulated in vitro (Figure 11 A), but little to no decrease in the production of IFN- $\gamma$  levels by these cells. Thus, the presence of antibody to *L. major* correlates with an increase in IL-4 production by parasite-specific T cells.

The alteration in the production of these T cell cytokines did not appear to be substantial enough to account for the large differences in lesion formation observed in these mice. Consequently we examined a possible role for IL-10. Our lab<sup>72</sup> and others<sup>138;139</sup> have previously shown that mice lacking IL-10 were relatively resistant to leishmaniasis. We examined the production of IL-10 in the lesions of mice infected with *Leishmania*. IL-10 mRNA and protein were measured in infected feet 2 and 4 days, respectively, after the administration of  $\alpha$ -*L. major* IgG. J<sub>H</sub> mice given a single dose of purified  $\alpha$ -*L. major* IgG i.p. produced approximately twice as much IL-10 protein and mRNA in their lesions, relative to parallel groups of mice infected at the same time, which did not receive IgG (Figure 11 B).



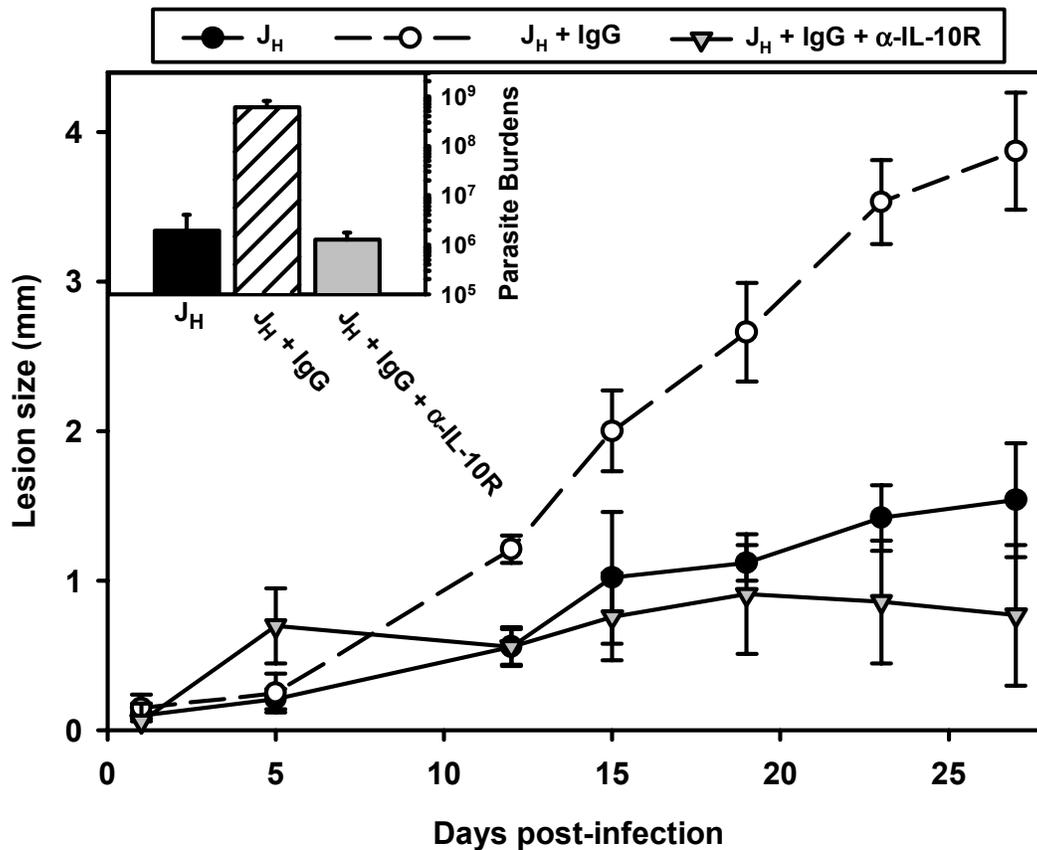
**Figure 11 A. Cytokine Production in Mice Administered  $\alpha$ -*L. major* Antiserum.** Cytokine production by lymph node T cells from infected  $J_H$  mice (open bar) was compared to  $J_H$  mice administered 200  $\mu$ l  $\alpha$ -*L. major* antiserum on days 1, 7, and 14 post-infection (solid bar). Lymph nodes were removed on day 21 and stimulated with SLA. Supernatants were harvested 72 hr later and assayed for IFN- $\gamma$  and IL-4 by ELISA.

**Figure 11 B. IL-10 Production in Lesions of  $J_H$  Mice.** IL-10 protein (left) and mRNA (right) were determined in two groups of  $J_H$  mice infected with  $2 \times 10^5$  *L. major* amastigotes. On day 21 of infection, one group was administered 600  $\mu$ g purified  $\alpha$ -*L. major* IgG i.p. IL-10 protein levels in the lesions of three infected mice were measured by ELISA, 4 days following the administration of  $\alpha$ -*L. major* IgG (left axis). RNA was isolated from footpad lesions on day 2 post-IgG administration, and real time PCR was performed to determine relative IL-10 mRNA (right axis). Levels represent the average from three infected mice, and mRNA was normalized to HPRT levels in a single infected foot.

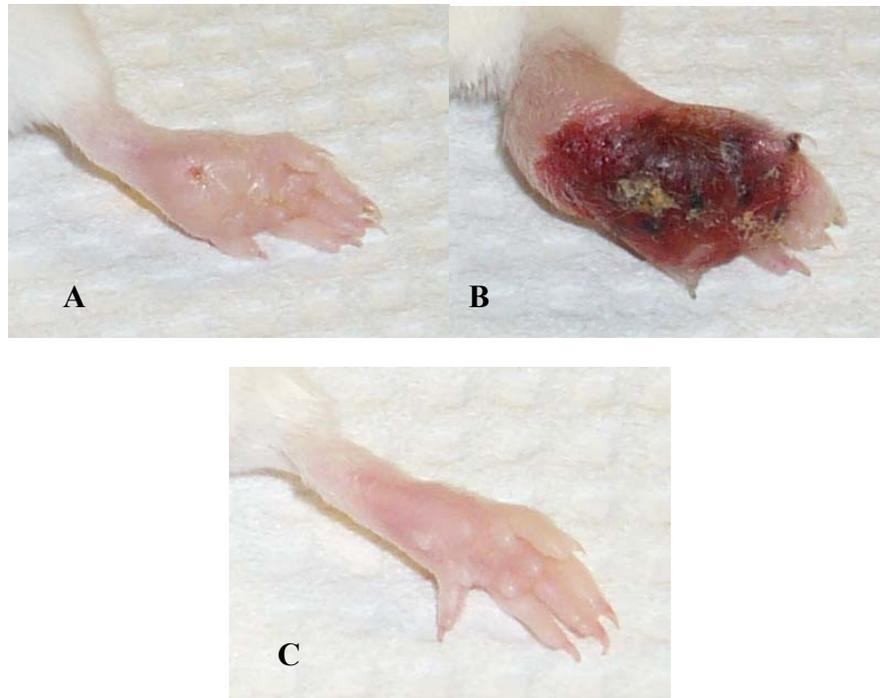
To confirm a role for IL-10 in lesion progression, J<sub>H</sub> mice were infected with *L. major* and administered purified  $\alpha$ -*L. major* IgG along with a monoclonal antibody to the IL-10 receptor. Infected J<sub>H</sub> mice developed modest lesions (Figure 12, filled circles; Figure 13 A), whereas those administered  $\alpha$ -*L. major* IgG developed large lesions (Fig.12, open circles; Figure 13 B) with high numbers of parasites (Fig.12, inset). The administration of a monoclonal antibody to the IL-10 receptor completely prevented the exacerbation of disease caused by  $\alpha$ -*L. major* IgG (Fig. 12, grey triangles; Figure 13 C). In fact, the footpad lesions in mice treated with  $\alpha$ -*L. major* IgG plus  $\alpha$ -IL-10R, essentially resembled an uninfected footpad.

To further show the relationship between macrophage, IgG, and IL-10, axenic amastigotes were opsonized and examined for their ability to induce the production of IL-10 from macrophages. Axenic *L. amazonensis* amastigotes were opsonized with sera from normal and infected BALB/c or J<sub>H</sub> mice, and added to monolayers of macrophages. J<sub>H</sub> mice are unable to produce IgG, and therefore will not be able to ligate Fc $\gamma$ R<sub>s</sub> on macrophages. This experiment tests the hypothesis of IL-10 induction being the result of IgG immune complexes ligating the Fc $\gamma$ R<sub>s</sub> on macrophages. Without the ligation of Fc $\gamma$ R<sub>s</sub>, the cytokine production should not be altered, and the production of IL-10 should not be induced.

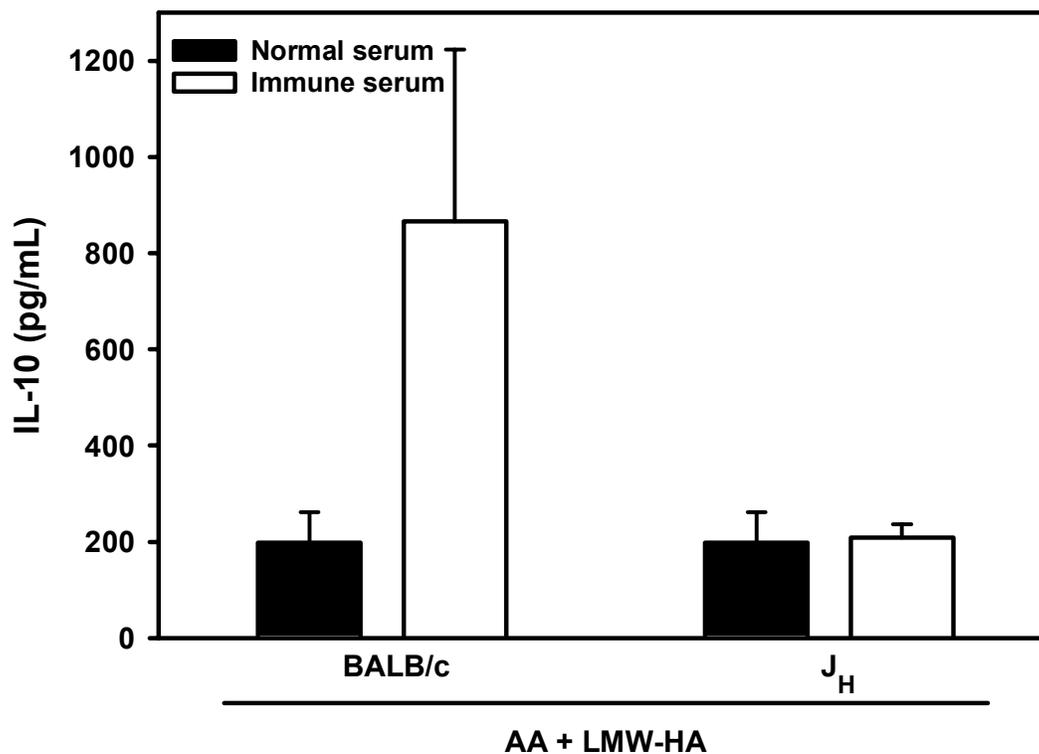
Amastigotes opsonized with normal sera from either strain of mouse were unable to induce the production of IL-10 (Figure 14, closed bars). When axenic amastigotes were opsonized with sera from BALB/c mice, they were



**Figure 12. IgG Reconstitution of J<sub>H</sub> Mice and the Effect of α-IL-10R mAb.** Three parallel groups of J<sub>H</sub> mice were infected with  $2 \times 10^5$  *L. major* amastigotes. One group (open circles) was administered 600 μg purified α-*L. major* IgG i.p. on days 1, 8, and 15. Another group (grey triangles) was given the same dose of α-*L. major* IgG, along with α-IL-10R on days 0 [1mg], 7 [200 μg], and 14 [200 μg]. The third group (filled circles) received no treatment. Lesion size was measured at semi-weekly intervals. Parasite burdens (inset) were determined by limiting dilution. Error bars represent the standard deviation of the mean of 4 determinations. This experiment is representative of 3.



**Figure 13. IgG Reconstitution of  $J_H$  Mice and the Effect of  $\alpha$ -IL-10R mAb.** Pictures of footpads from Fig. 12. Footpads of  $J_H$  mice were infected with  $2 \times 10^5$  *L. major* amastigotes. One group (B) was administered 600  $\mu$ g purified  $\alpha$ -*L. major* IgG i.p. on days 1, 8, and 15. Another group (C) was given the same dose of  $\alpha$ -*L. major* IgG, along with  $\alpha$ -IL-10R on days 0 [1mg], 7 [200  $\mu$ g], and 14 [200  $\mu$ g]. A third group (A) received no treatment after infection.



**Figure 14. In vitro Cytokine Production.** Axenic *L. amazonensis* amastigotes (AA) were opsonized with sera from either normal (closed bars) or immune (open bars) BALB/c or J<sub>H</sub> mice. Amastigotes were added to macrophages in the presence of inflammatory low molecular weight hyaluronic acid (LMW-HA) at a ratio of 10:1. Supernatants were harvested 24 hrs later and IL-10 production measured by ELISA. This experiment is representative of 3.

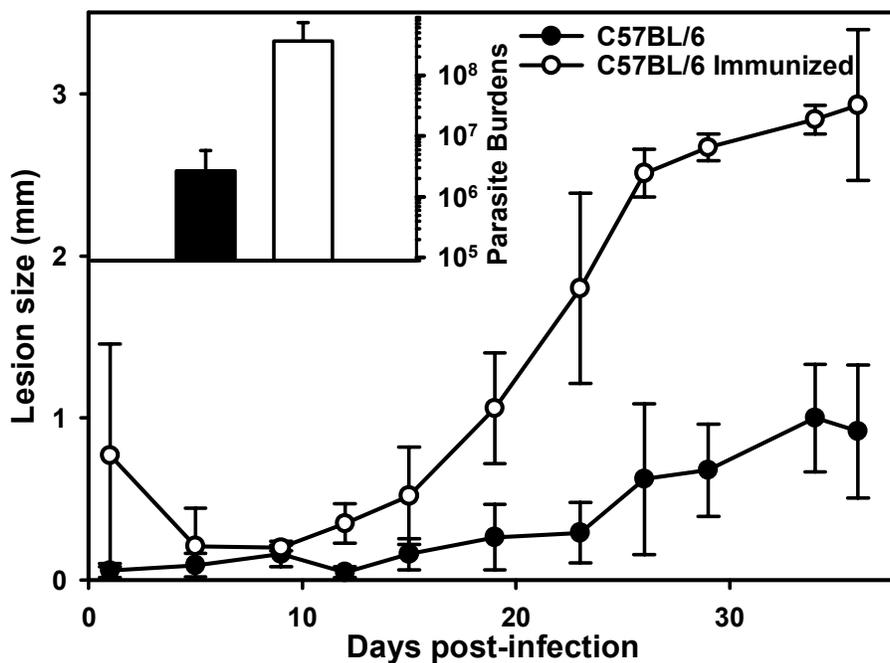
able to induce macrophages to produce a robust IL-10 response (Fig.14, open bar). Immune sera from J<sub>H</sub> mice (Fig.14, open bar) was unable to induce the production of IL-10. The failure of these amastigotes to induce IL-10, demonstrates the necessity for IgG containing immune complexes in this process.

#### Immunization of C57BL/6 Mice Leads to Increased Disease

To determine whether irrelevant immune complexes, which are unrelated to the ongoing *L. major* infection, could have an effect on lesion progression, we immunized the genetically resistant C57BL/6 mice with ovalbumin (OVA) in incomplete Freund's adjuvant (IFA). Mice were boosted 2 weeks later, and 4 weeks after the initial immunization. These mice developed high titers of IgG antibody to OVA. They were then infected with *L. major* suspended in a solution of PBS containing 50 µg/mL OVA. Immunized mice developed larger lesions (Figure 15, open circles) with more parasites (Fig. 15, inset), compared to control mice receiving IFA alone and the same *L. major* infection (Fig. 15, closed circles).

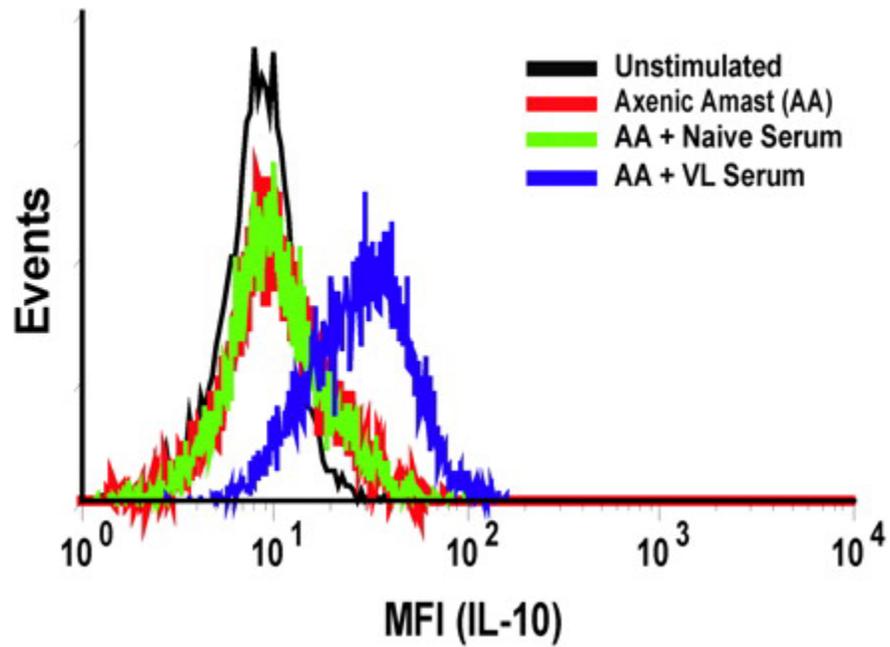
#### IgG antibodies in human visceral leishmaniasis

In vitro infection studies were performed to measure IL-10 production from human monocytes infected with *L. chagasi* amastigotes. For these studies IgG-free axenically grown *L. chagasi* amastigotes were used to infect normal human monocytes. Axenic amastigotes induced little to no detectible



**Figure 15. The Immunization of C57BL/6 Mice with OVA.** Lesion development in OVA-immunized mice (open circles) was compared to control mice given IFA alone (filled circles). Immunized mice were given 25 $\mu$ g OVA in 500 $\mu$ L of IFA, and boosted 2 weeks later. Both groups of mice were infected with *L. major* resuspended in PBS containing 50  $\mu$ g/mL OVA. Parasite burdens (inset) were determined on Day 34. Error bars represent the standard deviation of the mean of 4 determinants. This experiment is representative of 3.

IL-10 production from human monocytes (Figure 16, red line). However, incubating these organisms in serum from a patient with visceral leishmaniasis induced the production of high levels of IL-10 from monocytes (Fig. 16, blue line). Parallel groups of axenic amastigotes incubated in normal serum from uninfected volunteers failed to induce IL-10 production from monocytes (Fig. 16, green line). Thus, serum from infected patients opsonizes amastigotes to induce the production of IL-10 from infected monocytes.



**Figure 16. Flow Cytometry to Detect Intracellular IL-10.** Human monocytes were infected with a 10:1 ratio of axenic amastigotes of *L. chagasi*. Prior to infection some amastigotes were incubated at 4C for 15 minutes in 5% serum from either an uninfected volunteer (naïve serum) or from a patient with visceral leishmaniasis (VL serum). Monocytes were incubated in Golgi-stop for 2 hrs, fixed, and permeabilized and stained for intracellular IL-10 expression. Cells were gated on CD14 expression. This experiment is representative of 3.

## Discussion

In the present studies, we examine the host response during the development of leishmaniasis, using murine and human models. *Leishmania* amastigotes have been shown to have host IgG on their surfaces<sup>174;175</sup>, and it has been accepted for some time that IgG played a role in leishmaniasis. It was assumed that the role IgG fulfilled was as an opsonin, allowing the parasite more efficient entry in to the macrophage<sup>97</sup>. We examined how IgG and amastigotes came into contact with each other, and whether amastigote opsonization was essential for entry into neighboring cells.

IgG can enter macrophages, where it is saved from degradation by the neonatal receptor, also known as the IgG protective receptor<sup>176-178</sup>. A study involving the intracellular bacterium, *Listeria monocytogenes*, found that once inside the macrophage, antibody molecules could traffic within the endosomal compartments of the cell and encounter *Listeria* containing vacuoles<sup>180</sup>. Since *Leishmania spp.* also reside inside macrophages, we examined the possibility of intracellular amastigote opsonization as they reside within the cell. We determined that immune sera from chronically infected BALB/c mice, but not normal sera from naïve mice, could enter macrophages, and coat amastigotes intracellularly. This suggests that IgG must be specific to the amastigote for opsonization to occur, and this opsonization can occur inside, as well as outside, host macrophages. We show that *L. amazonensis* amastigotes gain entry into host macrophages regardless of opsonization. Axenic amastigotes

were able to invade wild type (Fig. 5) macrophages with kinetics similar to IgG opsonized, and lesion derived amastigotes. Also, lesion derived amastigotes were able to bind with similar kinetics to both wildtype and  $\text{Fc}\gamma\text{R}^{-/-}$  macrophages (Fig. 6). This ruled out the necessity of both IgG opsonization, and the binding to the  $\text{Fc}\gamma\text{R}$ , for the entry of amastigotes into cells. Thus, IgG must be serving some other role in the pathogenesis of leishmaniasis.

Interestingly, Peters, *et al.*, demonstrated in 1995 that B cell deficient mice injected with specific IgG, showed surface bound IgG upon isolation of lesion amastigotes. However, amastigotes isolated from B cell deficient mice injected with naïve sera were IgG negative<sup>97</sup>. The authors suggested that specific antibodies injected into the tail vein had access to lesion formation in the footpad.

The investigation into B cells, IgG, and leishmaniasis has been well documented<sup>97;149;157;158;161;163;165;166;181;182</sup>. Most of these papers agree that B cells and IgG play a role in leishmaniasis, but there is no agreement as to what extent, and how they influence the course of disease. While several groups acknowledge a direct role for IgG in leishmaniasis<sup>97;163;166;182</sup>, no one has shown a definitive mechanism by which B cells and IgG exert their influence.

We reexamined the role of IgG in host defense to *Leishmania spp.* using  $J_H$  mice. The  $J_H$  strain of BALB/c mice lacks IgG because they have a targeted deletion in the immunoglobulin heavy chain (J) locus.  $J_H$  mice were more resistant to infections with *L. major* than were normal BALB/c mice.

When these mice were reconstituted with sera from chronically infected BALB/c mice, they exhibited larger lesions, with higher numbers of parasites residing in those lesions. These data show that the presence of antibody to *L. major* did not protect BALB/c mice, and in the case of the IgG deficient J<sub>H</sub> mice, the presence of IgG actually exacerbated disease. This provides evidence that intracellular organisms can exploit IgG, normally considered to be a protective component of our immune system, and use it to their benefit, thus causing harm to the host.

In patients with visceral leishmaniasis, high IgG titers correlate with disease. IL-10 production in both cutaneous and visceral leishmaniasis has been previously reported<sup>72;138;139</sup>. A role for IL-10 has been demonstrated for both murine<sup>72;138;139</sup>, and human disease<sup>74</sup>. We examined the production of IL-10 during murine *L. major* infections, and showed the effect of IL-10 receptor (IL-10R) blockade on disease progression. We showed that by blocking IL-10, using a monoclonal antibody against the IL-10 receptor, we were able to reverse the IgG mediated increase in disease susceptibility. This provides direct evidence for the requirement of IgG and IL-10 in the susceptibility of mice to *L. major*. Providing further evidence for the role of IL-10, we showed that IL-10 protein and mRNA levels were higher in the lesions of antibody treated mice.

The cells responsible for the production of IL-10 during infection with *Leishmania spp.* have not been definitively identified. Previously it was determined that regulatory T cells play a role in sustaining murine cutaneous

leishmaniasis<sup>183</sup>. Tregs can produce IL-10, and these cells may be a source of IL-10 during disease progression. We showed that the IL-10 production during *L. major* infections had a detrimental effect on the host by exacerbating the infection. Previous studies<sup>20;23</sup> have shown that macrophages produce IL-10 upon the ligation of their FcγRs by immune complexes. We examined whether this phenomenon could apply to *L. major* infections, with *L. major* – α-*L. major* IgG serving as the immune complex responsible for FcγR ligation. Further showing that the production of IL-10 is from macrophages, we isolated peripheral blood mononuclear cells (PBMNCs) from the blood of normal human donors, and infected them with opsonized *L. chagasi*. *L. chagasi* amastigotes opsonized with sera from infected patients were able to induce the production of IL-10 from monocytes. These data show that infected macrophages can also produce IL-10 in response to FcγR ligation by *L. major* – α-*L. major* IgG, and *L. chagasi* – α-*L. chagasi* immune complexes. We conclude that IgG can cause a novel form of immune enhancement, due to its ability to induce IL-10 production from macrophages.

The IL-10 produced by macrophages has both a direct and an indirect effect on the progression of leishmaniasis. IL-10 is a potent inhibitor of macrophage activation, providing a direct way in which immune complexes can prevent the elimination of parasites, even in the presence of an ongoing immune response. Immune complexes also have an indirect effect by influencing APC cytokine production, to bias T cell responses (Fig. 11 A). Our data show that irrelevant immune complexes (Fig. 15), as well as

amastigote-IgG immune complexes, were sufficient to increase disease. This illustrates that this phenomenon is not parasite specific, but rather due to the induction of IL-10 by type II activated macrophages. In lesions, macrophages and dendritic cells<sup>19;134</sup> are in close association with T cells. This means that they are in a prime position to influence T cell cytokine production, and both macrophages<sup>19</sup> and dendritic cells<sup>184</sup> shut off their production of IL-12 in response to immune complexes. In fact, when T cells from infected lymph nodes were restimulated in vitro, we showed that these cells produced high levels of the Th2 cytokine, IL-4.

Interestingly, 20 years ago it was demonstrated that the susceptibility of BALB/c mice required the presence of B cells<sup>149</sup>. Also a more recent study has demonstrated that IgG could increase susceptibility to *L. amazonensis* infections in mice<sup>163</sup>, but did not identify the mechanism by which this increased susceptibility occurred. We show a role for IgG immune complexes in leishmaniasis, and conclude that IgG not only fails to provide protection against infection with *Leishmania spp.*, but it can actually contribute to the disease progression. We demonstrate that the mechanism of this exacerbation is due to the induction of the anti-inflammatory cytokine, IL-10, rather than the production of the inflammatory and macrophage activating cytokine, IL-12.

Previous studies have identified immune complexes to be present in patients with visceral leishmaniasis<sup>144;145</sup>. High antibody titers have been observed in canine, as well as in human, visceral leishmaniasis<sup>147;151;185</sup>. The

observations made in the murine model of leishmaniasis were confirmed in in vitro studies involving human cells and the causative agent in human visceral leishmaniasis, *L. chagasi*. Infection of human monocytes with axenic *L. chagasi* amastigotes opsonized with antisera from infected patients, resulted in the production of IL-10. This data confirmed our hypothesis of IgG immune complexes inducing the production of IL-10 by ligating Fc $\gamma$ Rs on macrophages. This data also provides a mechanism by which these two factors contribute to disease progression.

In many ways the infection of BALB/c mice with *L. major* may be a better model for human visceral leishmaniasis than for cutaneous leishmaniasis. BALB/c mice are susceptible to infection with *L. major*, and develop high antibody titers. During infection, the parasites frequently metastasize to the bone marrow, liver, and spleen<sup>146</sup>, and similar to human visceral leishmaniasis, BALB/c mice will typically succumb to infection. Thus, it is possible that some of the observations made in the BALB/c model of infection with *L. major* can be extended to human visceral leishmaniasis.

These data suggest a novel form of antibody-dependent immune enhancement, where the immune complexes influence cytokine production, and prevent the eradication of intracellular pathogens. These observations may also provide a general mechanism to help explain antibody dependent immune enhancement (ADE). The phenomenon of ADE has been observed in Dengue hemorrhagic fever<sup>186</sup>, where the presence of antibody causes a marked increase in viremia and viral pathogenesis. IL-10 gene homologs

have also been found in the genomes of several viruses. Considering the inhibitory effects of IL-10 on the inflammatory response, the capture of an IL-10 gene by a virus could be extremely beneficial to viral survival. An increased rate of mortality in meningococcal disease is also associated with a genetic predisposition of a higher level of IL-10 expression<sup>57</sup>. Intracellular bacteria have been shown to use IL-10 as a mechanism to establish disease, as studies have associated the production of IL-10 from macrophages with the progression of lepromatous leprosy<sup>187</sup>. The present studies would suggest that antibody-mediated immune enhancement, resulting in IL-10 production from macrophages, should be considered as a potential risk for some vaccination protocols.

## CHAPTER 4: IgG DIFFERENCES BETWEEN SUSCEPTIBLE AND RESISTANT MICE

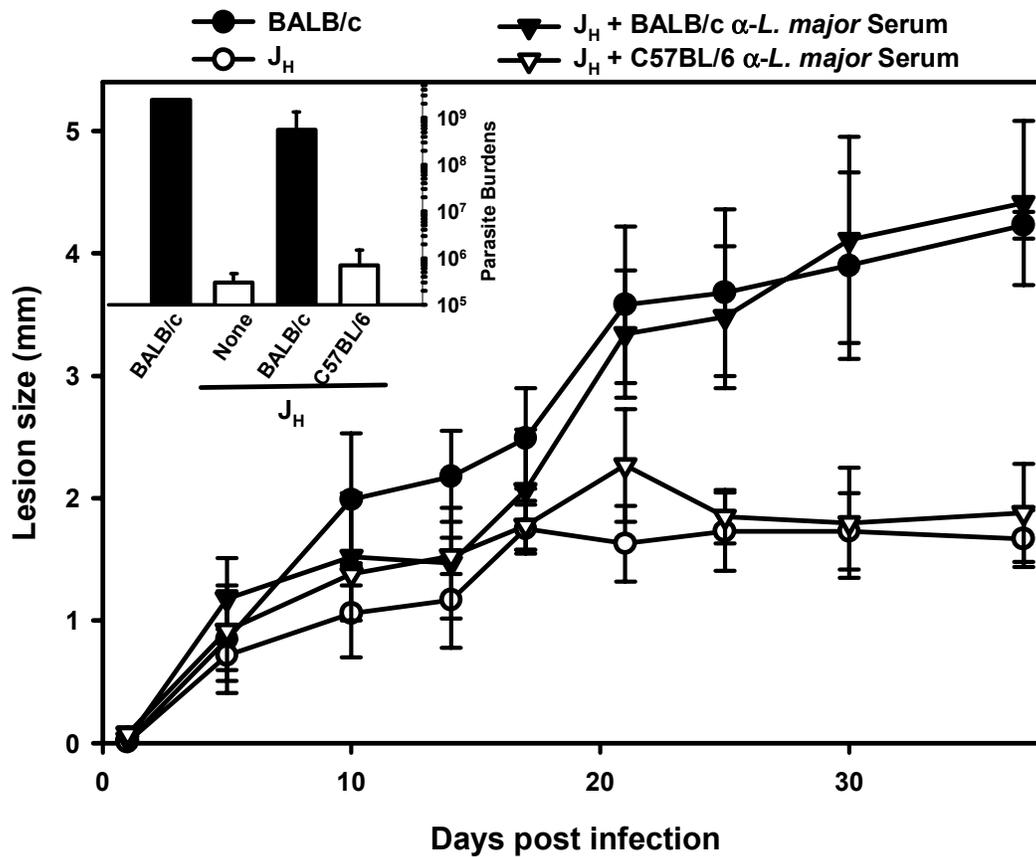
### Addition of BALB/c Antisera, but not C57BL/6, Influences the Progression of Disease

*Leishmania major* infections have been extensively studied in mice, and are an excellent model for the Th1/Th2 paradigm. It has been shown that the type of immune response mounted results in either resolution or susceptibility to disease<sup>103-106;108</sup>. *L. major* infections in C57BL/6 mice result in a Th1 response, leading to the resolution of the infection. The same infection in BALB/c mice results in a Th2 response, leading to disease susceptibility. BALB/c mice are known to be exceptionally susceptible, regardless of infective dosage, displaying high antibody titers, parasite metastasis<sup>146;188</sup>, and eventually, loss of infected extremities<sup>188</sup>.

We demonstrate that J<sub>H</sub> mice, which lack IgG, are more resistant to infection with *Leishmania spp.* than the control BALB/c mice (Figs. 7, 8). However, reconstitution with  $\alpha$ -*L. major* sera from BALB/c mice rendered J<sub>H</sub> mice more susceptible to infection with *L. major* (Fig. 8). The goal of these studies was to determine whether *L. major* antiserum from C57BL/6 mice was similar to, and could exert the same effects as, BALB/c antisera. An experiment was performed to determine the effects of the reconstitution of J<sub>H</sub> mice with  $\alpha$ -*L. major* antisera from C57BL/6 mice. The administration of C57BL/6 derived  $\alpha$ -*L. major* antisera had no effect on the progression of disease in J<sub>H</sub> mice (Figure 17, open triangles). These mice developed lesions

similar to J<sub>H</sub> mice which received no antisera. These two groups also had similar numbers of parasites within their lesions at the conclusion of the experiment on day 35 (Fig. 17, inset). Both BALB/c (Fig. 17, closed circles) and J<sub>H</sub> mice administered BALB/c derived antisera (Fig.17, closed triangles) are susceptible to infection with *L. major*, and develop large lesions with high numbers of parasites within their lesions (Fig. 17, inset). These data suggest that while the passive administration of immune serum from BALB/c mice can exacerbate disease, the antiserum obtained from C57BL/6 mice is unable to influence the progression of *L. major* infections in J<sub>H</sub> mice.

We previously showed a role for IgG in the progression of *L. major* infections<sup>189</sup>. The presence of IgG was linked to the formation of immune complexes, which ligate FcγRs on macrophages and induce the production of IL-10 from these macrophages. These data, along with the differences discovered between the pathogenicity of BALB/c antisera in J<sub>H</sub> mice, with respect to the inability of C57BL/6 antisera to affect disease, pose interesting questions regarding how these IgG differences relate to disease progression. These data suggest that IgG could influence leishmaniasis in several ways. The presence of immune complexes influences cytokine production by macrophages, resulting in a Th2 environment, which leads to the production of other Th2 cytokines, including those that stimulate a robust IgG response and isotype switching. The resulting increase of total IgG, or the switching to an IgG subclass that promotes disease progression, could mean the difference between resistance and susceptibility. Another question raised by these data,

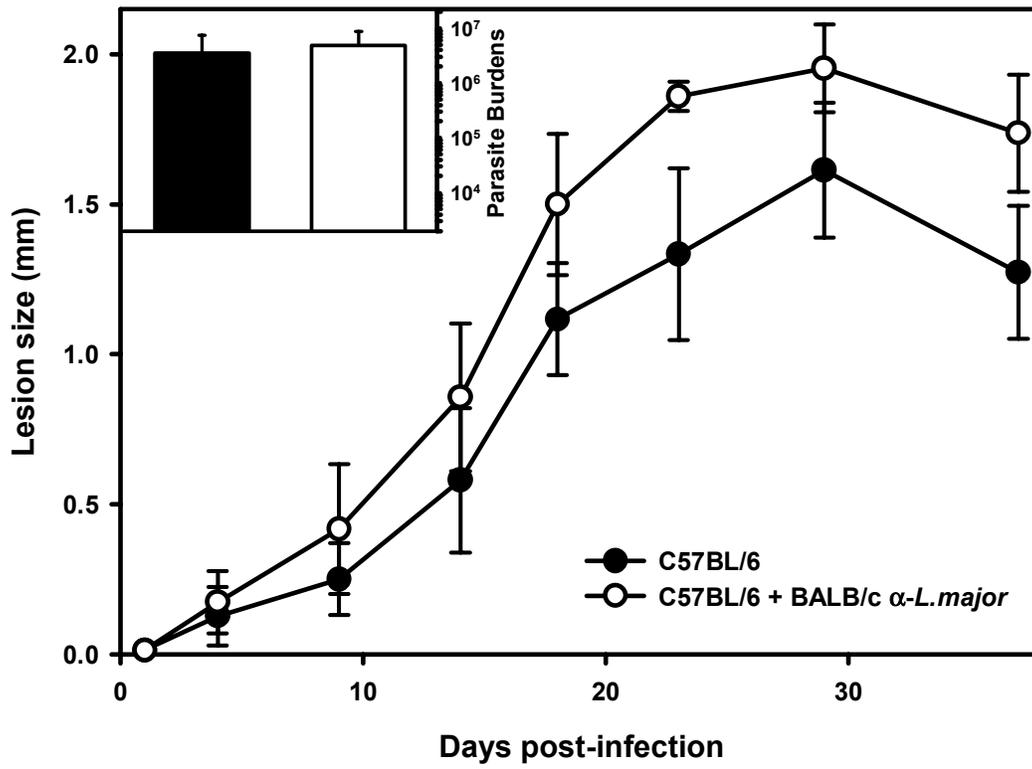


**Figure 17. Reconstitution of J<sub>H</sub> Mice with C57BL/6 Sera.** Lesion size of BALB/c mice (closed circles) and J<sub>H</sub> mice (open circles) was compared to J<sub>H</sub> mice given 200 μl antisera to *L. major* from C57BL/6 mice (open triangles) and J<sub>H</sub> mice given 200 μl antisera to *L. major* from BALB/c mice (closed triangles) on days 1, 7 and 14. Parasite burdens (inset) were determined by limiting dilution. Error bars represent the standard deviation of the mean of 5 determinations. This experiment is representative of 3.

is whether the presence of IgG alone is sufficient for increased disease, or whether the presence of IgG must be coupled with an ongoing Th2 response to cause disease exacerbation.

*Disease in C57BL/6 Mice is Not Affected by BALB/c Serum*

We examined whether the addition of BALB/c  $\alpha$ -*L. major* antisera could affect the progression of leishmaniasis in the normally resistant C57BL/6 strain of mice. Two groups of C57BL/6 mice were infected with identical amounts of parasites, with or without the addition of BALB/c immune sera. The passive administration of polyclonal  $\alpha$ -*L. major* antiserum to C57BL/6 mice had essentially no effect on lesion progression (Figure 18, open circles). These mice developed lesions with a similar size and kinetics as the control mice that received no antisera. Furthermore, the two groups of mice had similar numbers of parasites within their lesions at the conclusion of the observation period (Fig. 18, inset). Thus, unlike the J<sub>H</sub> mouse, the addition of BALB/c antisera to C57BL/6 mice was not capable of influencing disease outcome. This could be due to the inherent differences in the immune responses mounted by these mice. Since C57BL/6 mice mount a robust Th1 response and are capable of controlling infections with *L. major*, the addition of antibody may not be enough to overcome this genetic tendency toward an inflammatory response. These data suggest that IgG is required for disease



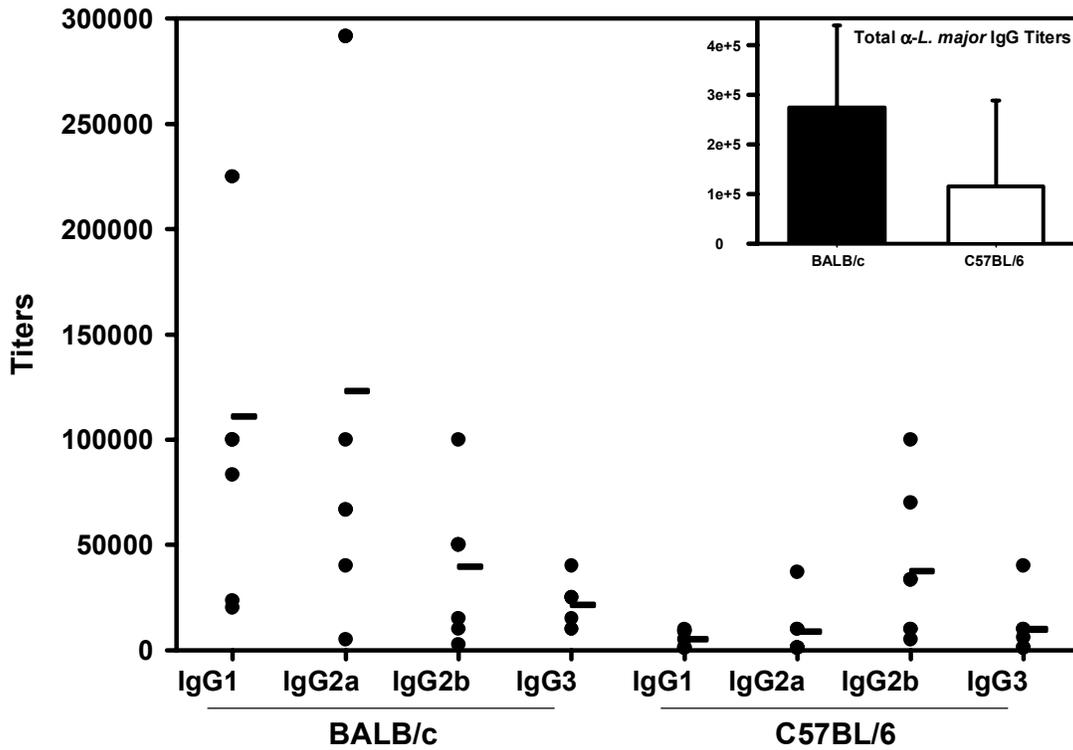
**Figure 18. Reconstitution of C57BL/6 Mice with BALB/c Sera.** Lesion size of C57BL/6 mice (closed circles) was compared to C57BL/6 mice given 200  $\mu$ l antisera to *L. major* from BALB/c mice (open circles) on days 1, 7 and 14. Parasite burdens (inset) were determined by serial dilution. Error bars represent the standard error of the mean of 3 separate experiments with a minimum of 5 mice per group.

susceptibility, as shown in the J<sub>H</sub> model, but it may not be sufficient to overcome the inherent resistance of C57BL/6 mice.

### IgG Titers in Resistant and Susceptible Strains

The administration of BALB/c derived, but not C57BL/6 derived,  $\alpha$ -*L. major* antisera influenced the course of *L. major* infections in J<sub>H</sub> mice. This suggests a fundamental difference in the antisera of these animals, which leads to the difference in disease severity. Consequently we examined a possible difference in the IgG titers of these animals.

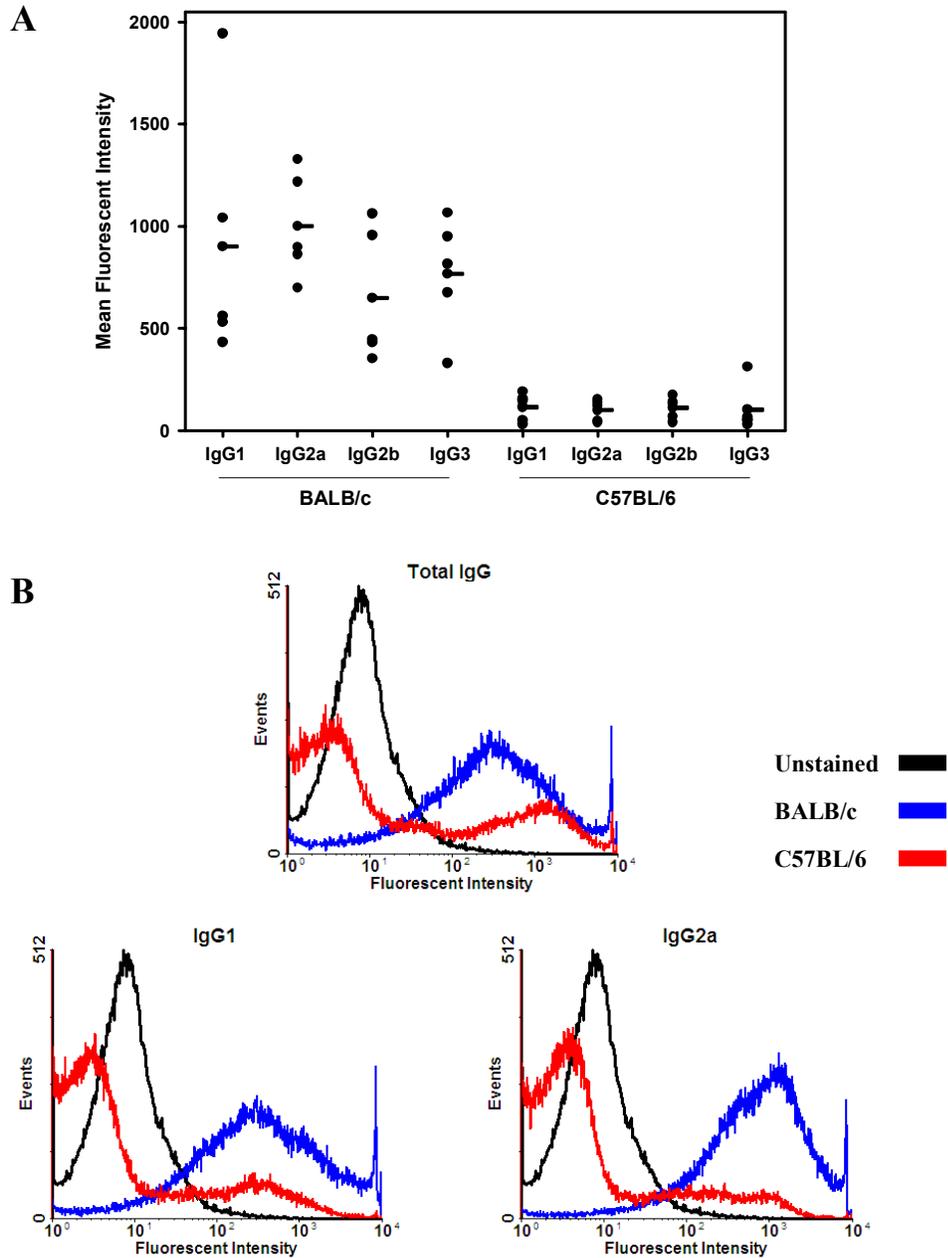
Infected C57BL/6 and BALB/c mice were bled, and their serum isolated. A serial dilution of the sera was plated in 96 well plates, and *L. major* specific serum IgG levels, for total as well as isotype subclasses (IgG1, IgG2a, IgG2b, IgG3), were determined by ELISA. IgG titers were consistently higher in *L. major* susceptible BALB/c mice, than in resistant C57BL/6 mice (Figure 19). In 7 separate experiments, the total *L. major* specific IgG titers were  $2.75 \times 10^5 \pm 1.65 \times 10^5$  for BALB/c, and  $1.15 \times 10^5 \pm 1.74 \times 10^5$  for C57BL/6 mice. While all BALB/c IgG titers were higher than those of C57BL/6 mice, the titers of subclasses IgG1 and IgG2a were found to be statistically significant using the Student's t test. In 7 separate experiments, IgG1 and IgG2a titers were  $1.11 \times 10^5 \pm 8.46 \times 10^4$  and  $1.23 \times 10^5 \pm 1.19 \times 10^5$  respectively for BALB/c and  $5143 \pm 4180$  and  $8714 \pm 1.32 \times 10^4$  respectively for C57BL/6 mice. These data demonstrate that the circulating  $\alpha$ -*L. major* titers are 22 fold



**Figure 19. The Circulating  $\alpha$ -L. major IgG Titers of Infected C57BL/6 vs BALB/c Mice.** Sera were isolated from infected BALB/c or C57BL/6 mice. Titers of specific IgG subclasses were determined using ELISA. The mean of 5 separate determinations is represented by a solid bar. The circulating titers of total  $\alpha$ -L. major IgG (IgG1, IgG2a, IgG2b, IgG3) for BALB/c mice (closed bar) and C57BL/6 mice (open bar) are shown in the inset graph.

higher for IgG1 and 14 fold higher for IgG2a in susceptible BALB/c mice, relative to resistant C57BL/6 mice.

Amastigotes are known to be coated in host IgG, and consequently we examined whether surface IgG on lesion derived amastigotes mirrored the relative titers of circulating serum IgG. *L. major* amastigotes were isolated from the footpads of BALB/c and C57BL/6 mice, and stained for the presence of surface IgG. FITC conjugated antibodies were utilized to allow for the quantitation of total, as well as the specific isotype subclasses. Flow cytometric analysis provided a direct comparison between and within the strains. Amastigotes isolated from BALB/c footpad lesions had consistently higher levels of IgG on their surfaces (Figure 20). In 5 separate experiments, the mean fluorescence intensities of amastigote surface IgG from BALB/c mice were determined to be statistically significant in all four subclasses using the Student's t test. Total IgG, IgG1 and IgG2a titers were  $842 \pm 223$ ,  $901 \pm 628$  and  $1000 \pm 262$  respectively for BALB/c, and  $437 \pm 183$ ,  $114 \pm 70$  and  $99 \pm 52$  respectively for C57BL/6 mice.



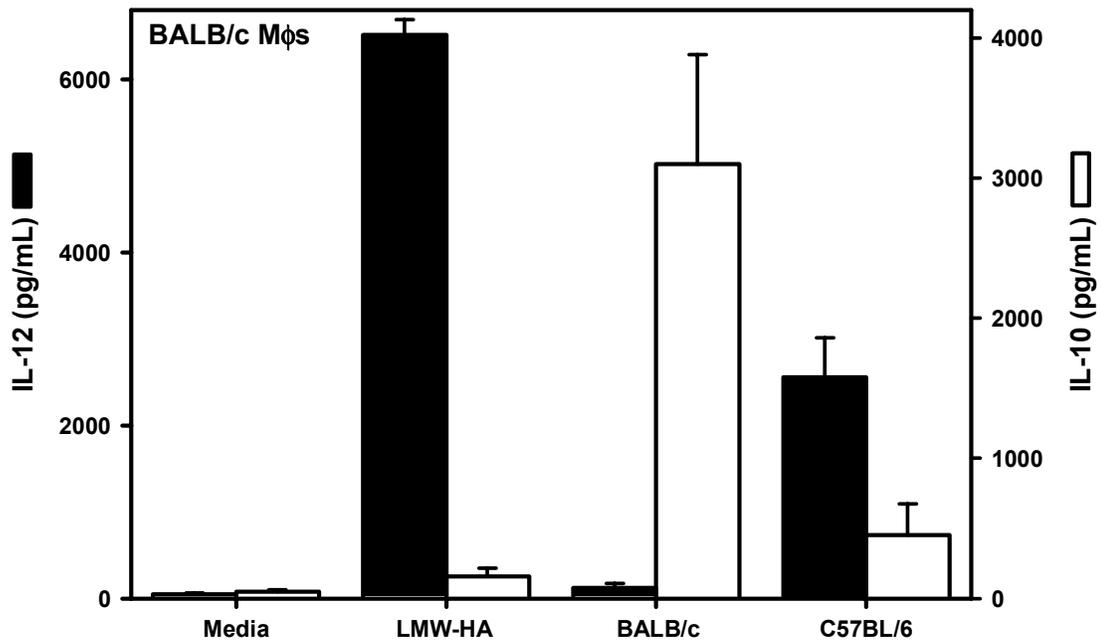
**Figure 20. Composition of IgG Subclasses on the Surface of Lesion Derived *L. major* Amastigotes.** Amastigotes were isolated from the footpads of BALB/c and C57BL/6 mice. These amastigotes were stained for the presence of IgG on their surfaces. Total IgG and its various subclasses were quantitated by flow cytometry. (A) The mean fluorescent intensity of BALB/c and C57BL/6 subclasses are plotted. The mean of 5 determinants is plotted as a solid bar. (B) Flow profiles of BALB/c (blue line) and C57BL/6 (red line) are compared to the unstained control (black line). These profiles are representative of 5 separate experiments.

### Amastigote Induced Macrophage Cytokine Profile Differs Between Strains

Lesion derived amastigotes, which are coated in host IgG, coupled with an inflammatory stimulus such as LMW-HA, induce the production of IL-10 from macrophages. This is an IgG dependent effect because axenically grown amastigotes, which are grown in cell free culture and thus lack surface IgG, are unable to induce macrophages to produce IL-10<sup>72</sup>. The original observations showing IL-10 production by amastigotes were determined using lesion derived amastigotes isolated from BALB/c mice. It had never been tested using amastigotes isolated from the resistant C57BL/6 strain. Consequently we examined the ability of C57BL/6 lesion derived amastigotes to induce the production of IL-10 from macrophages.

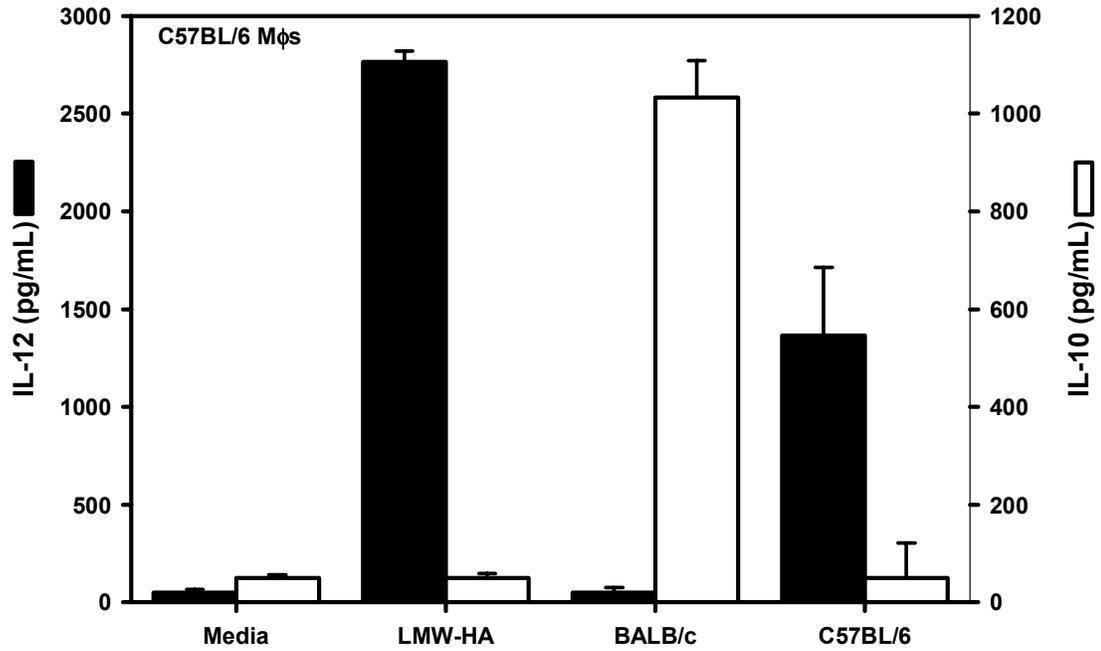
When lesion derived *L. major* amastigotes isolated from BALB/c footpads were added to BMM $\phi$  from BALB/c mice, macrophages shut off the production of IL-12, and secreted high levels of IL-10. Amastigotes derived from the lesions of C57BL/6 mice, however, were unable to completely abrogate the production of IL-12, and induced only modest levels of IL-10 (Figure 21). To ensure that this response was a direct result of the differences in the lesion derived amastigotes, and not an artifact of the origin of the BMM $\phi$ , we repeated the experiment using BMM $\phi$  isolated from C57BL/6 mice.

Similar to the previous experiment, *L. major* amastigotes isolated from BALB/c mice induced the C57BL/6 derived macrophages to shut off



**Figure 21. In vitro Cytokine Production of BALB/c vs C57BL/6 Derived Footpad Amastigotes.** Lesion-derived *L. major* amastigotes from either BALB/c or C57BL/6 mice were added to BALB/c bone marrow derived macrophages (10:1) in the presence of inflammatory low molecular weight hyaluronic acid (LMW-HA). Supernatants were harvested 24 hrs later and cytokines IL-12 (closed bars) and IL-10 (open bars) were measured by ELISA. Error bar represent 3 determinations. This experiment is representative of 4.

production of IL-12 and secrete high levels of IL-10. Similar to the previous experiment, amastigotes isolated from the lesions of C57BL/6 mice failed to shut off production of IL-12 and induce IL-10 production (Figure 22). These data suggest that amastigotes isolated from BALB/c lesions, but not those isolated from C57BL/6 lesions, are capable of altering the cytokine profile of macrophages, regardless of macrophage origin. This observation, along with our preceding experiments, demonstrates the differences in IgG titers and amastigote surface IgG between susceptible BALB/c and resistant C57BL/6 mice.



**Figure 22. In vitro Cytokine Production of BALB/c vs C57BL/6 Derived Footpad Amastigotes.** Lesion-derived *L. major* amastigotes from either BALB/c or C57BL/6 mice were added to C57BL/6 bone marrow derived macrophages (10:1) in the presence of inflammatory low molecular weight hyaluronic acid (LMW-HA). Supernatants were harvested 24 hrs later and cytokines IL-12 (closed bars) and IL-10 (open bars) were measured by ELISA. Error bar represent 3 determinations. This experiment is representative of 4.

## Discussion

Murine *Leishmania spp.* infections have been extensively studied. In response to *L. major*, C57BL/6 mice mount a Th1 response, leading to activation of macrophages and resolution of infection. BALB/c mice mount an inappropriate Th2 response, blocking macrophage activation, leading to uncontrolled parasite replication and disease susceptibility. These responses against *L. major* have been exploited, and yielded valuable data, which has furthered the knowledge of the Th1/Th2 paradigm.

Resistance to *L. major* in murine infections is dependent upon a Th1 response. These responses are characterized by the production of IFN- $\gamma$ . In response to antigen, classically activated macrophages produce high levels of IL-12. This IL-12 stimulates the production of IFN- $\gamma$  from CD4<sup>+</sup> Th1 cells, and CD8<sup>+</sup> T cells. The increase in the production of IFN- $\gamma$  allows macrophages to become properly activated, and thus induces the destruction of phagocytosed microbes. IFN- $\gamma$  also stimulates the differentiation of naïve T cells into the Th1 subset. IFN- $\gamma$  not only stimulates a Th1 environment, but blocks the production of Th2 cytokines, and prevents the proliferation of naïve T cells into the Th2 subset. B cells induce class switching to IgG2a in response to IFN- $\gamma$ , and inhibit switching to the Th2 subclass, IgG1.

Susceptibility to *L. major* is associated with a Th2 response. IL-4 is the hallmark cytokine of this response, and induces naïve CD4<sup>+</sup> T cells to develop into Th2 cells. IL-4 promotes the production of IgG1 from B cells, and class switching to IgE, while inhibiting the production of the IgG2a and

IgG3 subclasses of IgG. The abrogation of IL-4 during *L. major* infections, either by IL-4 knockout<sup>113;114</sup> or a monoclonal antibody to IL-4<sup>111;112</sup>, has demonstrated its importance in disease susceptibility. Another Th2 related cytokine, IL-10, has also been shown to be important in the susceptibility to *L. major* murine infections<sup>72;139;189</sup>. IL-10 inhibits the activation of macrophages, and blocks the production of IL-12. This deactivation of macrophages leaves them unable to kill the ingested *L. major* parasites, which are now free to replicate unchecked, leading to increased susceptibility.

We previously showed a role for IL-10 and IgG in the disease progression of *L. major* infections<sup>189</sup>. The presence of IgG, in otherwise resistant mice, lead to the formation of immune complexes that influenced disease by inducing the production of IL-10 from macrophages. As discussed, IL-10 blocks macrophage activation and the production of IL-12. IL-12 is needed for enhanced IFN- $\gamma$  production, and a biasing toward the protective Th1 response. Thus IgG immune complexes, and the subsequent production of IL-10, could be the key in determining the path of the murine immune response. The goal of these experiments was to examine possible differences in the IgG between resistant and susceptible mice.

Previous work in the *Leishmania* field has examined the role that B cells and IgG play in disease progression. The role of B cells, and the subsequent lack of IgG, has been met with mixed reviews. The examination of a role for B cells, and/or IgG, in leishmaniasis has been well documented in the literature<sup>97;149;157;158;161;163;165;166;181;182;189</sup>. While most agree that there is

indeed a role for B cells and/or IgG, the mechanisms of action proposed, vary greatly. Brown and Reiner are the lone group to determine that B cells play no roll in *L. major* infections<sup>165</sup>. Using  $\mu$ MT mice from both the BALB/c and C57BL backgrounds, they determined that infections with *L. major* remained unchanged in the absence of B cells. However, as early as 1984, Sacks, *et al.* demonstrated that B cells were required for the susceptibility of BALB/c mice to *L. tropica* and *L. mexicana* infections<sup>149</sup>.

While several groups acknowledge a role for IgG in leishmaniasis<sup>97;163;166;182</sup>, we are the only group to offer a definitive mechanism and establish this role in immune complex formation and subsequent induction of macrophage IL-10<sup>189</sup>. In light of our previous observations showing the role of IgG in disease progression, we examined the IgG titers in resistant mice and compared them to the IgG titers of susceptible mice. In these experiments,  $\alpha$ -*L. major* antisera from BALB/c mice, but not from C57BL/6 mice, was able to influence the progression of *L. major* infections in J<sub>H</sub> mice. These data were interesting because they reiterated the effect of BALB/c antisera on J<sub>H</sub> mice. Despite this however, antisera from C57BL/6 mice could not induce susceptibility to disease. This gives further evidence of a distinct difference in the antisera, most likely in IgG titers, of resistant and susceptible strains of mice. In accordance with this theory, we determined that *L. major* infected BALB/c mice had consistently higher titers of circulating IgG, as well as higher levels of IgG on the surface of their lesion

derived amastigotes, than C57BL/6 mice also infected with *L. major* in the same manner.

IgG contributes to the progression of *L. major* infections by forming immune complexes. These complexes ligate the FcγRs on macrophages, altering their cytokine production. These macrophages shut off the production of the inflammatory cytokine IL-12, and begin to produce large amounts of the anti-inflammatory cytokine IL-10. This change in cytokine production leads to the development of a Th2 environment, and prevents the activation of macrophages, which is crucial to the eradication of the parasite. *L. major* amastigotes derived from BALB/c lesions were able to shut off production of the inflammatory cytokine IL-12 from macrophages and induce them to produce high levels of the anti-inflammatory cytokine IL-10. Interestingly, amastigotes isolated from the lesions of C57BL/6 mice were unable to induce this response.

Without macrophage activation, *L. major* amastigotes are able to replicate unchecked inside the macrophage, and develop into rapidly progressing lesions which eventually lead to metastasis and loss of infected extremities in mice. Thus, IgG exacerbates disease by inducing the production of IL-10 from macrophages. Therefore, high levels of IgG on the surface of BALB/c derived amastigotes could be a possible explanation of the production of IL-10 from macrophages stimulated with these parasites, and the failure of low level IgG C57BL/6 amastigotes to induce this similar response.

It is possible that high antibody titers are needed for the ligation of Fc $\gamma$ Rs, leading to the production of IL-10 from macrophages. Also, this alteration of cytokine secretion, allows for the deactivation of macrophages and ultimately, the continued survival of the *Leishmania* parasites residing within them. The pronounced differences between the titers of the specific subclasses of IgG, IgG1 and IgG2a, between the resistant and susceptible strains suggests that IL-10 induction could be the result of a specific subclass, rather than total amounts of IgG. A new Fc $\gamma$ R, Fc $\gamma$ RIV, was recently discovered by Ravetch and colleagues<sup>8</sup>, which interacts exclusively with IgG2a and IgG2b. The interaction of IgG1 with Fc $\gamma$ RIII, along with the affinity of Fc $\gamma$ RIV for IgG2a and IgG2b, may account for their protective and pathogenic activities. Future exploration of this Fc $\gamma$ R-IgG interaction may provide additional insight into any role this phenomenon may play in the resistance or susceptibility of mice to *L. major* infections.

We have thus far been unable to determine whether it is the quantity of IgG produced, or a specific isotype, which is responsible for our observations. Thus, we are currently working on purifying each IgG subclass from the  $\alpha$ -*L. major* antisera of both C57BL/6 and BALB/c mice. In doing this, it will make it possible to determine whether it is simply an abundance of IgG that causes disease susceptibility in BALB/c mice, or whether the increased production of a specific IgG subclass is responsible. Currently, we see the most dramatic increase in the titers of IgG1 and IgG2a of BALB/c mice, when compared to C57BL/6 mice. This is also an interesting observation due to the opposing

responses normally associated with these subclasses. IgG1 is a predominantly Th2 subclass, induced by IL-4 driven class switching of B cells. In contrast, IgG2a is a predominantly Th1 subclasses, induced by IFN- $\gamma$ . Thus, it is surprising to see the titers of both subclasses increased in the robust Th2 response associated with BALB/c disease.

The increase in opposing IgG subclasses reiterates that more may be involved than the simple upregulation of IgG subclasses. Therefore, we cannot rule out the possibility of a specific Fc $\gamma$ R being involved. Fc $\gamma$ RI and Fc $\gamma$ RIII are known to induce the production of IL-10 upon ligation with immune complexes<sup>20;23;24</sup>, and Fc $\gamma$ RIII is known to associate predominantly with IgG1. Very little is known about Fc $\gamma$ RIV, as it was only recently discovered<sup>8</sup>, however it interacts with IgG2a and IgG2b exclusively. The distinct and opposing IgG subclass specificities may play a role in shaping the immune responses mounted by resistant and susceptible strains of mice. These different Fc $\gamma$ Rs, along with their IgG subclass preferences, must be examined further to determine what role, if any they play in the IgG mediated exacerbation of *L. major* infections.

Additionally, human as well as murine monocytes studies will be undertaken to determine the role, and specificities, of IgG and Fc $\gamma$ Rs involved in disease. It is currently known that human visceral disease results in a marked increase of IgG titers<sup>145;147;185;189</sup>, and the production of IL-10<sup>74;138;189</sup>. In fact these two determinants are actually predictive of disease. Due to the similarities between human visceral and BALB/c *L. major* infections (high

IgG responses, increased IL-10 production), we proposed that the BALB/c model of *L. major* infection is comparative to human visceral disease<sup>189</sup>.

Control monocytes from healthy donors, or naïve BALB/c mice, as well as monocytes from patients with confirmed visceral disease, or *L. major* infected BALB/c mice, will be analyzed based on their expression of FcγRIII. We would expect to see an upregulation of FcγRIII in infected samples, thus correlating with the published reports of increased IL-10, and susceptibility to disease. Additionally, the same experiment repeated in monocytes isolated from humans infected with cutaneous forms, such as *L. major*, could be used in comparison with C57BL/6 *L. major* infections. The low IgG titers, as well as the ability of C57BL/6 mice to control and cure *L. major* infections would make it an ideal model for the comparison of human cutaneous disease. These data will help provide a distinction between healthy and infected monocytes and provide further insight into the populations of monocytes that are present during ongoing disease.

The ability of BALB/c immune serum, which has higher titers of *L. major* specific IgG, but not C57BL/6 serum with low titers of *L. major* specific IgG, to influence the progression of leishmaniasis in J<sub>H</sub> mice suggests the involvement of IgG in susceptibility. Although the presence of *L. major* specific IgG exacerbated disease in J<sub>H</sub> mice, which are on the susceptible BALB/c background, it was unable to influence disease in *L. major* resistant C57BL/6 mice. These data suggest that while IgG induces the production of IL-10 and furthers the Th2 response necessary for susceptibility to

leishmaniasis, it cannot overcome the innate resistance and robust Th1 response mounted by the C57BL/6 strain.

This suggests that both the presence of IgG is required, but not substantial enough to increase disease alone. Additionally, the resistance of BALB/c IL-4<sup>-/-</sup> mice<sup>113</sup>, furthers the idea of the necessity of both a sustained Th2 response, and the presence of IgG. This observation, along with our data, would suggest that the development of a Th2 response, along with the presence of IgG, is imperative to the development of lesions and the exacerbation of disease. Additionally, we would like to identify the IgG subclass, if there is one, responsible for inducing the production of IL-10, because it could lead to a therapeutic antibody to increase IL-10 levels during autoimmunity.

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