DISSERTATION

EARLY CELLULAR IMMUNE RESPONSE TO *LEISHMANIA MAJOR* AND MODIFYING EFFECT OF SAND FLY SALIVARY GLAND PROTEINS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KATHLEEN A. ROGERS ENTITLED EARLY IMMUNE RESPONSE TO *LEISHMANIA MAJOR* AND MODIFYING EFFECTS OF SAND FLY SALIVARY GLAND PROTEINS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

EARLY CELLULAR IMMUNE RESPONSES TO *LEISHMANIA MAJOR* AND MODIFYING EFFECT OF SAND FLY SALIVARY GLAND PROTEINS

The protozoan parasite *Leishmania major* is transmitted by the bite of an infected phlebotomine sand fly. The resulting disease, leishmaniasis, manifests itself as ulcerated skin lesions that can take months to resolve. This model has been used to characterize the T helper type-1 (Th1)/ T helper type-2 (Th2) paradigm for cellular immune responses. Th1/Th2 responses are distinguished by the cytokines secreted and selective activation of the cells, resulting in the development of a cellular or humoral immune response respectively. In leishmaniasis, a Th1 response, primarily characterized by the production of IFN-γ, will result in an inflammatory response capable of controlling most intracellular infections. On the other hand, a Th2 response, characterized by the production of IL-4, IL-5 and IL-10, drives primarily a humoral response, the hallmark being the production of high titers of antibodies. In *L. major* infections, a Th2 response results in disease exacerbation.

Most experiments studying the human immune response to the parasite involve subjects already infected with the disease. As cytokine production can begin within hours to days of a *Leishmania* infection and could in turn establish the cytokine microenvironment leading to a Th1 or Th2 response, we wanted to examine the human

response during the acute infection phase. Peripheral blood mononuclear cells (PBMC) from *Leishmania*-naïve donors were isolated and exposed to *L. major*. The interactions of the cells and the parasite were evaluated during the first week of infection, during primary exposure, as well as upon restimulation with *L. major* after a week of primary stimulation. This system was characterized by phenotypic analysis (e.g. CD4 expression) as well as by cytokine secretion (e.g. IFN-γ). We found that both CD4+ and CD8+ T cells responded to *L. major* and that Type 1 cytokines were primarily produced.

The influence of exogenous cytokines or neutralizing antibodies on the production of Th1 and Th2 cytokines at 3 and 7 days was also examined. We found that that the cytokine most influenced by either Th1-like cytokines (e.g., IL-12) or Th2-like cytokines (e.g., TGF-β) was IFN-γ, suggesting that in humans, this cytokine has dominant role in immune modulation during the infection.

When sand flies inject *L. major* into the vertebrate host, they also inject immunomodulatory salivary proteins. Using the in vitro system developed, the modifying effects of sand fly salivary gland lysate, as well as Maxadilan, a potent vasodilatory peptide isolated from sand fly saliva, were determined on PBMC and monocyte/macrophage cultures. Both Maxadilan and saliva decreased IFN-γ production of PBMC and increased IL-6 production in monocyte/macrophages. Maxadilan appears to interact with macrophages through the neuropeptide receptor, pituitary adenylate cyclase activating peptide (PACAP).

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DEDICATION

To Arlin.

The best and most understanding husband a girl could ever dream to marry.

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INTRODUCTION

Leishmaniasis is a vector borne parasitic disease transmitted by the bite of an infected phlebotomine sand fly. This disease is quite prevalent in both tropical and sub-tropical areas of the world. Outbreaks of leishmaniasis have occurred in over eighty-eight countries and have affected twelve million people world-wide (WHO, 2000). Currently, one tenth of the world's population is at risk to contract an infection and over 2 million new cases are diagnosed each year. There are at least 22 species of *Leishmania* that can cause disease (Marsella and Ruiz de Gopegui, 1998) and over 30 species of sand flies are capable of transmitting the parasite (Killick-Kendrick, 1999). With the trend of global climate change, there is concern that the distribution of vector-borne pathogens may expand in both time and space, exposing host populations to longer transmission seasons and immunologically naïve populations to new diseases (Patz and Reisen, 2001). Giving merit to this theory, leishmaniasis has been increasing in incidence, and endemic regions have been spreading over the past ten years (WHO, 2000). Another disturbing trend has been the increase in co-infections of visceral leishmaniasis and AIDS, particularly in southern Europe (Pintado et al., 2001). In the United States, leishmaniasis is on the rise in dog populations. In 1999-2000, 11,000 foxhounds were tested for leishmaniasis after outbreaks in 21 states, and 12% of the dogs were found to have antibodies to the parasite (Enserink, 2000). This discovery is a particular concern as leishmaniasis is a zoonotic disease and in many areas of the world, dogs are the primary reservoir host (Marsella and Ruiz de Gopegui, 1998). It is conceivable that

leishmaniasis could become a disease of concern in the United States, such as the West Nile Virus has in the past few years. As this disease is so wide-spread, it is important to recognize and treat cases efficiently. Thus, it is necessary to study how the parasite interacts with the immune system to enable the development of efficacious drug treatments and vaccines.

Morphology, physiology and life cycle

There are two main life-stages of the parasite, the amastigote, which lives within the mammalian host and the promastigote, which is found in the vector. All species that cause leishmaniasis are obligate intracellular parasites in a mammalian host. The sand flies that transmit the parasite in the Old World are of the genus *Phlebotomus* and those that transmit *Leishmania sp.* in the New World are of the genus *Lutzomyia.* All species of *Leishmania* share a common life-cycle (reviewed in Dedet et al., 1999; Solbach and Laskay, 2000).

To gain entry into the mammalian host, the parasite utilizes the feeding strategy of the sand fly. The mouthparts of sand flies are too short to cannulate vessels in the skin, and their stylets are wider than the capillary loops they can reach in the dermis (Titus et al., 1994). In order to feed, the sand fly lacerates dermal capillaries and then feeds on pools of blood which form. In the process, parasites and sand fly saliva are regurgitated into the bite wound. At this point, the parasite is in its promastigote form, however, once *Leishmania* is engulfed by phagocytic cells, it will transform into an amastigote.

Leishmania do not actively force their way into cells, rather they are engulfed by a number of cell types. The parasite is most commonly found in macrophages, neutrophils and dendritic cells (Rittig and Bogdan, 2000). When exposed to the parasite, macrophages undergo coiling phagocytosis, which involves wrapping multiple layers of pseudopods around

the parasite (Rittig et al., 1998). Shortly after phagocytosis, the parasite is located within phagosomes. Normally, the phagosomes then fuse with endocytic compartments and phagolysosomes are formed. However, in the case of *Leishmania* infections, this process is altered. Desjardins and Descoteaux (1997) demonstrated that lipophosphoglycan (LPG), which is found on the surface of promastigotes parasites could inhibit phagosome-endosome fusion temporarily, protecting the parasite and allowing for the conversion to an amastigote. Inside the parasitophorous vacuole (PV) the promastigotes will transform into amastigotes, triggered by the elevated temperature in a mammal and a decreased pH in the PV (Zilberstein and Shapira, 1994). The transformation is also accompanied by the down-regulation of parasite LPG, which allows for the fusion of endocytic organelles within the macrophage (Scianimanica et al., 1999). At this stage, the parasite has a round or oval body around 2-6 μm in diameter, does not have an external flagellum and hence, is non-motile (Dedet et al., 1999). Amastigotes are capable of surviving the acidic environment of the PV. There are at least two strategies used by the parasite to withstand the harsh environment of the PV. The first adaptation is the presence of stage-specific proton pumps involved in the capture of metabolites, and the second adaptation is stage-specific metabolite transporters, whose activity is optimum at acidic pH (Zilberstein and Shapira, 1994). *Leishmania* also possess a large concentration of glycosphingolipids in the plasma membrane which have been suggested to protect the parasite (Antoine et al., 1998). As the parasite's life cycle continues, the amastigotes divide and are released via the exocytic machinery of the host cells (Rittig et al., 2000).

When a sand fly takes a blood meal, *Leishmania*-infected macrophages release amastigotes into the sand fly gut. These amastigotes then transform into promastigotes (Sacks

and Perkins, 1985). In contrast to the non-motile amastigotes, the promastigote has a long, slender body approximately 15-30 μm by 2-3 μm and has a long anterior flagellum (Dedet et al., 1999). The promastigote resides within the midgut of the sand fly (Dedet et al., 1999). In order to protect itself from the digestive enzymes in the sand fly gut, procyclic promastigotes express LPG and the metalloprotease gp63 (Davies et al., 1990; Pimenta et al., 1991). LPG also assists the parasite in attaching to the wall of the midgut and thus preventing passage from the gut with the digested bloodmeal (Sacks et al, 1994; Sacks et al., 2000). During this phase, procyclics divide rapidly. After 4-7 days, the parasites stop dividing and differentiate into metacyclic promastigotes which have altered LPG that prevents them from binding in the midgut (Sacks et al., 1995). At this point, *Leishmania* migrate to the foregut and the esophagus, where they are suspended in the sand fly's saliva (Schlein et al., 1992). The cycle continues the next time the sand fly takes another bloodmeal.

Clinical manifestations of Leishmaniasis, treatment and vaccination

The clinical manifestations of leishmaniasis depend not only upon the species of parasite infecting the host, but the general health and genetic constitution of the infected individual. Interestingly, evidence suggests that in many cases the infection is subclinical and self-limiting and detectable only by the development of *Leishmania*-specific antibodies or T cells responses to parasite antigens (Badaro et al., 1986; Kurtzhals et al., 1992; Kemp et al., 1993). There are three main forms of the disease: cutaneous, mucocutaneous and visceral leishmaniasis. Cutaneous leishmaniasis (CL) usually lasts for less than one year (Salman et al., 1999). The hallmark of CL is a lesion that occurs at the site of the insect bite. The incubation period can be anywhere from one week to three months (Salman et al., 1999). The

lesion enlarges and a seropurulent discharge develops that dries and forms a crust. The characteristic lesion forms an ulcerated crater (Salman et al., 1999). It can take several weeks before the lesion begins to heal and a scar develops. This disease is generally caused by *L. major, L. tropica* and *L. aethiopica* in the Old World and *L. braziliensis* and *L. mexicana* in the New World. Oftentimes, lesions resolve without treatment. If treatment for chronic lesions is needed, however, a number of therapies have been approached including thermotherapy, cryotherapy, electrotherapy, ultrasound and intralesional antimony treatment (Moskowitz and Kurban, 1999). Unfortunately, none of these therapies have been proven to be ideal (Herwaldt, 1999).

Mucocutaneous leishmaniasis (ML) is caused by *L. braziliensis* and *L. panamensis*. Generally, ML manifests within several years after the healing of the original CL skin lesion (Herwaldt, 1999). The characteristics of this kind of infection include the formation of disfiguring lesions in the nasal mucosa and pharynx. Respiratory blockage, anemia and secondary infections can be serious complicating problems (Etges and Muller, 1999; Martin et al., 1998). Approximately 3% of *L. braziliensis* patients develop ML (Barrel-Netto et al., 1998). ML is thought to be caused by either a hyper-cell mediated immune response to the parasite or by a genetic predisposition, such as carrying certain alleles for the genes encoding tumor necrosis factor α and β (Barrel-Netto et al., 1998; Cabrera et al., 1995; Blackwell, 1999).

The third form of the disease is visceral leishmaniasis (VL). VL causes the greatest morbidity and mortality of all the manifestations of the disease. VL is characterized by long-term fever, hepatosplenomegaly, anemia, leukopenia, weight loss and can be fatal if not treated (Rees and Kager, 1987). The parasites that cause VL in the Old World are *L. donovani*

and *L. infantum*. In the New World, *L. chagasi* infection can result in VL (Martin et al., 1998). A complication of VL can be post-kala-azar dermal leishmaniasis (PKDL), which occurs several months or years after successful treatment of VL (Kharazmi et al., 1999). Approximately 20-50% of VL patients in India and Sudan develop PKDL. The condition is characterized by inflammation and a low number of parasites in the skin (Salman et al., 1999). There are few drugs available to treat either ML or VL. The two most common drugs used to control the disease are sodium stibogluconate, a pentavalent antimony compound (Pentostam, Glaxo Wellcome) and meglumine antimonite (Glucantime, Rhone-Poulenc) (Herwaldt, 1999; Davidson, 1998). Recently, doctors in India and other areas with high endemnicity have reported that *Leishmania* is becoming resistant to pentavalent antimonies (Mattock, 1999). Up to 40% of infected patients are resistant to current treatments (Mattock, 1999). These drugs are also rather difficult to administer, requiring intravenous or intramuscular injections daily for a month (Herwaldt, 1999). The side effects can be severe, including fatigue, body aches, electrocadiographic abnormalities, pancreatitis and diabetes (Davidson, 1998; Herwaldt, 1999; Mattock, 1999).

At the moment, there is no vaccine available to protect people in endemic areas. The development of effective vaccines has been hindered by a significant amount of antigenic diversity between different parasite species and the fact that *Leishmania* has morphologically and chemically different life stages which alternate between the sand fly and the mammalian host (Handman, 2001). Historically, various studies have been undertaken where groups of people were infected with less destructive forms of *Leishmania* (i.e. *L. major*) and then challenged with either the same parasite or a more lethal form (i.e. *L. donovani*) (reviewed in Melby, 1991). Many human vaccination studies have used live parasites (reviewed in Jaffe,

1999), but the chance that a person may get a serious infection is too great of a risk to continue the use of this sort of vaccine. Overall, most of the vaccination experiments were not definitive, although occasionally there appeared to be limited protection. For instance, Armijos et al. (1998) performed a phase III vaccination study in children in endemic areas using killed parasite with BCG as an adjuvant and found the efficacy of the vaccine to be 73%. Further studies are needed to determine the long-term protection of this type of vaccine. It is necessary to determine more efficacious vaccine candidates that can be inexpensively produced and distributed to the mostly third world countries where leishmaniasis is a serious and widespread problem.

The mouse model of leishmaniasis

The Th1/Th2 paradigm

The investigation of leishmaniasis in mice involves far more than just studying simple host-parasite interactions. By intricately dissecting the murine immune response to *Leishmania*, investigators have been able to study the basis of immune function. The murine model of leishmaniasis has developed into a prime tool to observe the interactions between T helper cells, cytotoxic T cells, antigen presenting cells, cytokines, chemokines and hormones in vivo and in vitro. The primary paradigm which has been defined by many discoveries studying murine leishmaniasis is Type 1/Type 2 immunity, also called T helper cell type one (Th1) and type two (Th2) immunity. The hypothesis of Type 1/Type 2 immunity is that the level of antibody elicited in an immune response is inversely proportional to the level of cell mediated immunity (Spelberg and Edwards, 2001). Mosmann et al. (1986) first described the existence of subpopulations of CD4+ T

helper cells from mice. These subpopulations arose from a common naïve CD4+ T cell precursor (Rocken et al., 1992). These populations were classified based on their production of cytokines and termed T helper type 1 and T helper type 2 lymphocytes. Cytokines are soluble mediators produced by many different kinds of cells involved in the immune response (Launois et al., 1998). In an overall classification scheme, Th1 cells are defined on the basis of production of interleukin (IL)-2 and IFN-γ, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 (Seder and Paul, 1994; Romagnani, 1997). The cytokine milieu surrounding the cells influences the development of an uncommitted Th cell into either a Th1 or Th2 cell (Nakamura et al., 1997). Once a T cell commits to either being Th1 or Th2, it will usually become refractory to cytokines that will lead to the opposite development (Launois et al., 1997). Generally, Type 1 immunity allows for cure of intracellular infections via cell mediated immunity, such as in leishmaniasis and tuberculosis, while Type 2 immunity is best suited for eradicating extracellular infections, such as helminth infections, and certain bacterial and viral infections. Type 2 responses are characterized by antibody production.

Th1 cells are responsible for directing an inflammatory response to pathogens and other stimuli. The cytokine that is key to this response is IFN-γ (Bradley et al., 1996). IFN-γ stimulates the activation of macrophages (Murray et al., 1983; Cradin and Mauel, 1991; Wyler et al., 1989), the oxidative burst (Johnson and Kitagawa, 1985), and the subsequent killing of intracellular microbes (Dellacasagrande et al., 1999; Ritter and Moll, 2000). IFN-γ, produced by Th1 cells, appears to be able to inhibit the proliferation of Th2 CD4+ T cells (Pernis et al., 1995). Also, a Th1 response results in delayed type hypersensitivity (DTH) (Reed et al., 1986; Wolday et al., 1999). Type 1 immunity

causes the classic symptoms of inflammation: rubor (redness), dolar (pain), tumor (swelling) and calor (warmth) (Spelberg and Edwards, 2001).

In contrast to Th1 cells, Th2 cells stimulate the production of high titers of antibodies. Type 2 immunity can also be characterized by eosinophilic and basophilic tissue infiltration and mast cell degranulation (Spelberg and Edwards, 2001). The primary Th2 related cytokines are IL-4, IL-10 and IL-13, which can activate B cell proliferation, antibody production and class switching, particularly from IgG to IgE (Lundgren et al., 1989; Punnonen et al., 1993; Punnonen and de Vries, 1994; Lai and Mosmann, 1999). IL-4 is also essential in mice for the development of Th2 cells (Seder et al., 1992; Hsieh et al., 1993). Another important Th2 cytokine is IL-5, which can activate and stimulate eosinophil and basophil chemotaxis (Warringa et al., 1992; Simon et al., 1999). The Th2 response results in antibody mediated control of infections.

Not only are there Th1 and Th2 cells, but a Th0 subpopulation, which is an intermediate phase of Th cell. Th0 cells secrete both Th1 and Th2 cytokines. For instance, some Th0 cells produce IFN-γ and IL-5 (Palmer and van Seventer, 1997; Russo et al., 1998). It is unclear what role Th0 cells play in infection (Russo et al., 1998).

Murine leishmaniasis

The study of inbred strains of mice infected with *Leishmania* has allowed for the exploration of the immunoregulation that occurs during infection with an intracellular parasite. Because there are both *Leishmania*-susceptible and resistant strains of mice and these strains have a clear-cut dichotomy of response, the *Leishmania* model has become the workhorse of defining Type 1 and 2 immunity in intracellular infections. The basic

finding using this mouse model is that the control of *L. major* infection is dependant on a vigorous Th1 response that ensures production of IFN-γ, which will then activate macrophages and will allow for parasite replication to be controlled (Reiner and Locksley; 1995).

The genetic constitution of the mouse strain plays an important role in determining whether IL-4 (Type 2 cytokine) or IL-12 (Type 1 cytokine) is the dominant cytokine when both are present during the initiation of the immune response (Hondowicz and Scott, 1999). Strains of mice that can cure the infection efficiently include CBA, C3H/He, 129Sv/Ev and B10.D2 (Handman et al., 1979; Launois et al., 1997; Soares et al., 1997; Hondowicz and Scott, 1999). These mice develop a small lesion at the site of *L. major* inoculation that heals spontaneously, and the mice become resistant to infection (Launois et al., 1998).

At the other end of the spectrum are mice on the BALB background. BALB mice develop progressive lesions that do not heal and this strain is susceptible to reinfection (Behin et al., 1979). These mice develop a Th2 response, characterized by very high IL-4 production and thus, they are unable to control the infection efficiently (Howard et al., 1980; Howard et al., 1982; Reiner et al., 1994). BALB/c mice rapidly produce a burst of IL-4 from CD4+ T cells after injection with *Leishmania* (Launois et al., 1995). The production of IL-4 down regulates the expression of the IL-12-Receptor (R)-β2 subunit of CD4+ T cells within 48 hours of exposure to the parasite and thus, the T cells become refractory to the Th1-differentiating signals of IL-12 (Himmelrich et al., 1998). Several studies have indicated that the susceptibility of BALB/c mice to *L. major* infection is due to the recognition of a parasite protein called LACK, by a population of Vβ4Vα8 T cells

(Mougneau et al., 1995; Julia et al., 1996; Launois et al., 1997). LACK is a 24 kDa protein that shares homology with intracellular receptors for protein kinase C (RACK) and was thus termed *Leishmania* homologue of receptors for activated C kinase (LACK)(Mougneau et al., 1995).

It is possible to change the cytokine profile and thus the outcome of the disease by intervention early in *Leishmania* infection. Some of these treatments include the administration of anti-CD4 (Titus et al. 1985), anti -IL-4 (Sadick et al., 1990; Launois et al., 1997), or IL-12 (Heinzel et al., 1993; Launois et al., 1997), all of which allow BALB/c mice to control *L. major* infection. Conversely, the infection can be exacerbated in resistant mouse strains when either IFN-γ or IL-12 are neutralized or deficient (Sadick et al., 1990; Sypek et al., 1993; Wang et al., 1994; Heinzel et al., 1995). Treating resistant mice with IL-4 augments infection in these animals (Himmelrich et al., 2000), driving them toward Type 2 immunity. Some of these experimental approaches could potentially be used clinically to enable the control of infections in humans.

A number of other cytokines also play integral roles in modulating *Leishmania* infection. Both IL-12 and TNF-α influence Type 1 immunity. IL-12 is produced by activated APC such as macrophages and dendritic cells and acts to induce IFN-γ production in T cells and Natural Killer (NK) cells (Reiner and Locksley, 1995; Kobayashi et al., 1989; Scharton and Scott, 1993; Laskay et al., 1993). Likewise, IL-12 plays a critical role in promoting the differentiation of Th1 cells from naïve T cells (Chan et al., 1991). Not only important for the initiation of Type-1 immunity, IL-12 is also necessary to maintain this response in later stages of the infection (Sypek et al., 1993;

Park et al., 2000). Finally, IL-12 is capable of inhibiting IL-4 production, thus inhibiting a Th2 response (Wang et al., 1994; Launois et al., 1998).

Similarly, TNF-α is also a proinflammatory cytokine and is secreted by macrophages. TNF-α participates in the induction of NO and macrophage activation leading to parasite elimination (Solbach and Laskay, 2000). Likewise, TNF-α appears to be required to activate IL-12 production (Taylor and Murray, 1997). The ability of mice to resist infection with *L. major* correlates directly with the ability of their lymph node cells to produce TNF in response to *in vitro* parasite challenge (Titus et al., 1989). Along the same line, vaccinated BALB/c mice produced high levels of TNF and were protected from a parasite challenge, suggesting that this cytokine is important in resistance to *L. major* (Boom et al., 1990). In studies using TNF knock out mice infected with either *L. donovani* or *L. major*, infections were fatal, demonstrating that TNF is essential in the control of the parasite (Murray et al., 2000; Wilhelm et al, 2001).

Much like Type 1 immunity, several other cytokines secreted by non-T cells appear to play a role in Type-2 immunity. Although IL-4 appears to have the most influential and incontrovertible role in the differentiation of a naïve CD4+ T cell to a Th2 cell, transforming growth factor-beta (TGF-β), IL-10 and IL-6 can influence the Type 2 response. TGF-β and IL-10 inhibit Type 1 cytokines and contribute to the low IFN-γ response seen in BALB/c mice (Scharton and Scott; 1993). TGF-β is produced by macrophages and dendritic cells. When normally resistant C3H/HeJ mice were exposed to a recombinant adenovirus expressing TGF-β, these mice could not control an infection of *L. chagasi* (Wilson et al., 1998). In this case, TGF-β appeared to down-regulate IFN-γ production. Similarly, IL-10 inhibits IFN-γ production and has macrophage deactivating

properties (Vieth et al., 1994). Recent work indicates that high production of IL-10 early in *L. major* infection and the resulting inhibition of IFN-γ may assist in driving a Th2 response (Chatelain et al., 1999). Murine macrophages treated with IL-10 are less capable of killing *L. major* parasites (Lohoff et al., 1998; Kane and Mosser, 2001), while IL-10 KO mice on a susceptible background are able to better control *L. major* infection (Kane and Mosser, 2001) since Th1 cytokines were not suppressed. Another Type 2 cytokine is IL-6. This cytokine is produced by macrophages and lymphocytes and plays a role in the regulation of acute inflammation (Durum and Oppenheim, 1993). IL-6 can modulate a Type 1 or Type 2 immune response. The production of IL-6 can suppress the production of TNF-α, as well as IFN-γ, which in turn could decrease a Type 1 response (Durum and Oppenheim, 1993; Kopf et al., 1994; Rincon et al., 1997). In certain intracellular infections, such as with *Listeria monocytogenes*, IL-6 knock-out (KO) mice have reduced inflammatory responses (Durum and Oppenheim, 1993; Kopf et al., 1994). Interestingly, IL-6 can inhibit Th1 differentiation by interfering with IFN-γ receptor gene expression during T cell activation (Diehl et al., 2000). IL-6 can also act directly upon Th cells to drive a Th2 response. IL-6 can stimulate IL-4 production, leading to the polarization of naive CD4+ T cells into Th2 cells (Rincon et al., 1997). In spite of these studies, experiments with *Leishmania* and IL-6 KO mice have not yet elucidated the role IL-6 plays in susceptibility to infection (Moskowitz et al., 1997; Titus et al., 2001).

Not only do cytokines play a role in the outcome of the infection, but parasite dose can also be important. BALB/c mice can develop Type 1 immunity to *L. major* infection when exposed to low doses of parasite (Bretscher et al., 1992; Doherty et al., 1996). Similarly, resistant mice, such as those of the CBA strain can be made susceptible

to infection with high doses of *L. major* (Menon and Bretscher. 1998). The dose of the parasite influences the level of cell mediated or antibody mediated responses and appears to correlate with clinical results found in diseases characterized by DTH. such as leprosy and human leishmaniasis. As suggested by Menon and Bretscher (1998), parasite dose could play an important role in future vaccine strategies by using low levels of attenuated *L. major* to drive a Th1 response.

Although most murine research occurs in vivo. Shankar and Titus (1993) developed an in vitro model to study the early immune response to *L. major*. This model allows for precise control over various experimental conditions, such as antigen dose. cytokines (Soares et al., 1997) and antigen presenting cells (Shankar and Titus, 1997). These conditions can influence the development of Th1 or Th2 cells from naïve precursors. Shankar and Titus (1993) designed a system whereby *Leishmania*-specific T cells were generated from the lymphoid tissue of naïve mice. These cells were found to act in the same fashion as those isolated from infected mice. in terms of MHC-class II expression and cytokine production (Shankar and Titus, 1993; Soares et al., 1997). The T cells derived in this system were found to be *L. major*-specific (Shankar and Titus, 1993). The in vitro system was also used to determine the interactions of antigen presenting cell types with *Leishmania*-specific T cells (Shankar and Titus, 1997). In addition, in vitro models can be used to determine the role of cytokines in specific responses. For instance, the neutralization of IL-10 increased parasite load in splenic macrophages in a naïve cell priming system (Gomes et al., 2000a). Soares et al. (1997) used the model to explore which cytokines could influence Th1 or Th2 responses early after exposure to the parasite and also determined that the in vitro system results compared to those found in models

using animals. This latter finding indicates that results discovered in this type of in vitro system can be viewed with confidence as an indication of the immune responses which occur in vivo. In vitro models allow for the dissection of particular responses to the parasite in a highly controlled environment.

Human leishmaniasis

Leishmaniasis is not only a tool for studying the immune system using inbred strains of mice, but it is also a disease that affects people. Since humans are outbred and are exposed to uncountable numbers of different kinds of antigens in their life-time, some of which may cross react with leishmanial antigens (Akuffo et al., 1992), it is not surprising that the Th1/Th2 dichotomy present in murine infections is not as clear cut in patients or when studying human cells. Similar to the mouse model, however, is the integral role cytokines play in disease development. In humans, as in mice, the local cytokine environment is essential in guiding the outcome of the disease (Kemp et al., 1993; Kemp et al., 1993b; Maasho et al., 1998). Genetic defects in cytokine production can result in the inability to control the disease. For instance, Weinstock et al (1997) reported a case of diffuse cutaneous and mucocutaneous leishmaniasis wherein the patient was unable to resolve the infection with standard treatments. It was determined that the patient was unable to produce IFN-γ and finally healed after intensive IFN-γ therapy.

There is an increasing awareness of *Leishmania* infections, particularly in regions of the world where AIDS is surging and more of the population is immunocompromised, because incidences of visceral leishmaniasis are approaching record levels (Wolday, 1999). The primary reason for this resurgence is thought to be due to latent *Leishmania* infections

becoming virulent as CD4+ T cells are destroyed in the body and thus cytokine levels associated with these activated cells are greatly decreased (Wolday, 1999). The parasites can then recrudesce as AIDS takes its toll on the immune system. Leishmaniasis epidemics are also occurring throughout war torn areas of the world, such as Afghanistan and Sudan (WHO, 2000).

In the human leishmaniasis field, studies of patients most often involve investigating the lesions and the cells present or measuring parameters of isolated peripheral blood cells. In order to obtain a picture of how a patient responds to the parasite, the cellular composition and cytokines present in the local environment are evaluated using biopsies of the lesions. In the lesions, the most predominant cells are CD4+ and CD8+ T cells with lower numbers of macrophages, NK cells, granulocytes and few B cells in both Old and New World infections (Pirmez et al., 1990; Esterre et al., 1992; Lima et al., 1994; Palma and Saravia, 1997; Gaafar et al., 1999). There are few distinct patterns of cytokine expression, however, in the lesions. When active cutaneous leishmaniasis lesions are analyzed, IL-10 and IL-12 are expressed and IL-10 does not appear to down-regulate IL-12 or IFN-γ expression (Melby et al., 1996; Bourreau et al., 2001). IL-12 is measured at the greatest level in chronic lesions (Melby et al., 1996), suggesting that dis-regulation of the inflammatory response may be responsible for the severe pathology sometimes seen in *Leishmania* infections. Other cytokines found in lesion sites include IFN-γ and IL-6 (Melby et al., 1994).

Despite the fact that the majority of the studies evaluating cutaneous lesions do not indicate a clear Th1/ Th2 pattern, several studies comparing chronic and acute lesions or CL and ML do suggest there is a divergence of a Type 1 or Type 2 response. For instance, TNF-α, IL-10 and TGF-β are observed in lesions greater than four months old, compared to lesions

less than two months old (Melby et al., 1994). A comparison of patients with cutaneous leishmaniasis and mucocutaneous leishmaniasis showed that individuals with the cutaneous form of the disease expressed IL-2, IFN-γ and TGF-β mRNA in their lesions while those with the mucocutaneous form had IL-4 mRNA in their lesions (Pirmez et al., 1993). Similarly, IL-13, a Th2 cytokine, also plays a role in chronic lesions. In active lesions, IL-13 mRNA was preferentially expressed compared to IL-4 and was able to down regulate IL-12Rβ2 chain expression, thus allowing a Th2 response to be maintained (Bourreau et al., 2001). A recent study has indicated that cytokine production in lesions allows just a snap shot of information and does not necessarily reflect the entire immune response (Gaafar et al., 1999). These investigators found that despite the fact that a discernable Th1/Th2 cytokine pattern could not be determined via in situ staining for cytokines, peripheral blood mononuclear cells (PBMC) proliferated in response to leishmanial antigens and IFN-γ production increased as patients healed.

In a study comparing patients with subclinical *L. panamensis* infections and those with recurring disease, macrophages isolated from individuals with recurrent disease were more permissive to entry of parasites than the macrophages from people with subclinical infections (Bosque et al., 2000), suggesting that the macrophage response of different individuals may lead to disease susceptibility. Also, T cell proliferation of the patients with recurring disease or severe cutaneous *L. major* infections was reduced compared to control when the cells were restimulated with parasite antigen (Bosque et al., 2000; Gaafar et al., 1995; Ajdary et al., 2000). The variability in an individual's responses to the parasite as measured by lesion composition and in situ cytokine production, as well as the limitations of measuring immune parameters in a system which represents more established infections has led many

investigators to move to analyze cellular and humoral responses of infected and non-infected individuals.

Similar to the murine model, the production of cytokines from peripheral blood can be correlated with a disease state. For instance, subjects with mild infections generally produced a Th1-like cytokine pattern, with high production of IFN-γ and low IL-4 production (reviewed in Kemp, 1997). In order to regulate Type 1 immunity in *Leishnania* infections, IL-12 appears to play an important role (Ghalib et al., 1995). PBMC from patients with active infections did not produce IL-12p40 or IFN-γ when stimulated with the parasite, whereas the PBMC from those who had been treated and cured produced IL-12p40 and IFN-γ (Ghalib et al., 1995). The production of IFN-γ early after exposure is also necessary for controlling *Leishmania* infections (Carvalho et al., 1992). During early *L. braziliensis* infections (<60 days), there was lower IFN-γ production in infected individuals' PBMC, compared to persons with longer durations of illness (Novis Rocha et al., 1999), suggesting the parasite gains a foothold in the host by the suppression of Type 1 cytokines.

The production of Type 2 cytokines also contribute to the exacerbation of human leishmaniasis. The PBMC of patients with chronic lesions produce IL-4 when stimulated with *Leishmania* antigens, in contrast to healthy controls or cured individuals (Coutinho et al., 1998; Ajdary et al., 2000). Likewise, the level of soluble IL-4 receptor is greatly increased in patients with visceral leishmaniasis (Sang et al., 1999). Unlike the trend in visceral or murine leishmaniasis, there does not appear to be any correlation between IL-10 production and cutaneous disease (Kemp et al., 1999; Ajdary et al., 2000). Once individuals have been exposed to the parasite and are immune to the disease, PBMC produce significantly higher levels of IFN-γ and TNF-α when stimulated with parasite antigen than the PBMC of

individuals who had never been exposed to the disease (Kemp et al., 1999). This cytokine profile is characteristic of a Type 1 response and suggests that the production of Type 1 cytokines in response to the parasite allows for protection.

In VL, there are some differences in Type 2 cytokine secretion patterns. However, in contrast to patients with CL, IL-10 production appears to be of importance in the severity of *L. donovani* and *L. chagasi* infections, possibly blocking the IFN-γ mediated activation of macrophages to kill engulfed parasites (Ghalib et al., 1993; Bacellar et al., 2000). This cytokine also appears to be responsible for a complication called post-kala-azar dermal leishmaniasis (PKDL), which causes papules or nodules in the skin (Gasim et al., 1998). High levels of IL-10 were found in the plasma of individuals who developed PKDL. T cells from PKDL patients are highly reactive to *Leishmania* antigens (Gasim et al., 2000). Antigen from *L. infantum*, another parasite which causes visceral leishmaniasis, elicits IL-10 production from PBMC of patients with visceral infections (Suffia et al., 2000).

The importance of CD4+ T cells in regulating the immune response to *Leishmania* is widely accepted (Kemp, 1997). CD4+ T cells from healed individuals have been shown to produce protective Type1 cytokines (Kemp et al., 1999; Ajdary et al., 2000). During infection, the concentration of peripheral blood CD4+ T cells can be depressed (Cenini et al., 1993). Similarly, indicating the importance of these cells in controlling infection, depleting CD4+ T cells responding to parasite antigen results in the loss of IFN-γ secretion and proliferative responses to *Leishmania* antigen (Cooper et al., 1994)

Not only are CD4+ T cells important in *Leishmania* infection, but CD8 T+ cells and NK cells also play a role in establishing and maintaining the cytokine milieu that is so important in determining disease outcome. Unexposed individuals and healed patients

showed increases in CD8+ T cells and NK cells in response to *L. aethiopica* antigen (Akuffo et al., 1993; Maasho et al., 1998). There were few NK cells in patients with ongoing infections, but these individuals had high levels of CD4+ cells. In American leishmaniasis, patients with active lesions had high levels of CD4+ T cells and following infection, there were both populations of CD4+ and CD8+ T cells (Coutinho et al., 1996; Coutinho et al., 1998). Overall, these studies suggest a role for CD8+ T cells in resolving infections in cutaneous leishmaniasis.

NK cells may contribute to early cytokine production and the subsequent polarization to Type1 or Type2 immunity. Human NK cells can proliferate (Maasho et al., 1998; 2001) and secrete IFN-γ and IL-10 (Maasho et al., 2001) in response to *Leishmania* antigens. Akuffo et al. (1999) has suggested that NK cells can influence a Type 1 or Type 2 response depending on the level of IL-12 present. NK cells require a threshold level of IL-12 in order to secrete IFN-γ, which could then in turn influence the development of Th1 cells (Akuffo et al., 1999). Below this IL-12 level, IL-10 is produced in levels capable of inhibiting IFN-γ production, and could polarize Th cells to a Type 2 response. In contrast to these findings, Kemp et al., (1997) determined NK cells had little role in leishmaniasis. T cells, not NK cells, were shown to be the main source of IFN-γ. The opposing findings of these studies indicate that the importance of NK cells in human leishmaniasis must be more thoroughly investigated and defined.

On the other hand, in mucocutaneous leishmaniasis, NK cells and CD8+ T cells most likely exacerbate infection. Both of these cell types were shown to cause cytotoxicity in a peripheral blood autologous system from mucocutaneous patients but did not cause the same

effect in a system from cutaneous leishmaniasis patients (Brodskyn et al., 1997), suggesting that these cells destroy cells in the lesion in mucocutaneous infections.

Not only has human leishmaniasis been studied using lesion material, serum and peripheral blood from patients, but also by infecting the cells of unexposed donors and measuring various immune responses. These studies allow for the investigation of the immune response to *Leishmania* under more controlled conditions, as the time and dose of the initial infection can be determined and modulated. The primary in vitro system can be used to dissect the interactions between different populations of host cells and the parasite. For instance, Brodskyn et al. (2001) used an in vitro system to study the effects of *L. major* on co-stimulatory molecules and found that during infection with the parasite, the expression of CD86 and CD40 was enhanced on macrophages. They also found that blocking these co-stimulatory molecules interfered with the production of IL-5 and IFN-γ by T cells and IL-12 in *L. major*-infected macrophages. The phenotypic changes in cells and modulation of cytokine production can also be explored in experimental in vitro infections (Hviid et al., 1990; Kurtzhals et al., 1995). Perhaps one of the most exciting aspects of a human in vitro system, is that it allows researchers the opportunity to screen potential vaccine and drug candidates to determine if there is an effect on the human response. As an example, an avirulent *L. major* parasite (*dhfr-ts)* stimulated a Th1 response in human cells, but did not divide within macrophages (Brodskyn et al., 2000). Likewise, specific parasite antigens can be tested to determine which can elicit an immunogenic response and may also be worthwhile for pursuing as a treatment option (Kemp et al., 1992; Probst et al., 2001).

Not only can in vitro systems be used for drug screening, but they are also helpful in examining and dissecting the immune response of human cells to infections. When naïve T

cells are sensitized with promastigote antigens and IL-12, they develop into cytotoxic T cells capable of IL-12 and IFN-γ secretion as well as lysis of *Leishmania* infected macrophages (Russo et al., 1998; Russo et al., 1999). These in vitro systems allow for the analysis of the interaction of human cells with the parasite and parasite antigens under controlled situations not possible using human donors from *Leishmania*-endemic areas.

Innate immunity and its effect on the adaptive immune response in Leishmania infections

In order for *Leishmania* to cause an infection in the host, the parasite must first survive the front line of host defense: innate immunity. Only when the innate immune response is overcome, is an adaptive immune response necessary to eradicate a pathogen. The first barrier of the innate immune system is the skin. When the epithelium is breached, most micro-organisms are recognized and controlled by the innate immune system. This system is based on non-clonally distributed receptors that recognize certain molecular patterns found in microbes, but not in self-tissues and are referred to as pathogen-associated molecular patterns (PAMPs)(reviewed in Janeway, 2001). The receptors that recognize these PAMPs are called pattern recognition receptors. These receptors do not recognize any self-structure (Janeway, 2001). The innate immune system is essential for the induction of adaptive immunity (Janeway, 2001). A particularly striking example of this is through Toll-like receptors (TLR), which have been evolutionarily conserved between insects and mammals (reviewed in Akira et al., 2001). Toll receptors were first described in the fruit fly, *Drosophila* and were later identified in mammals as TLR (reviewed in Akira et al., 2001). TLR are expressed on APC and can recognize a number of pathogen products including LPS (Hoshino et al., 1999), lipoproteins, glycolipids (Aliprantis et al., 1999; Brightbill et al., 1999; Hirshfeld et al, 1999;

Takeuchi et al., 2000), flagellin (Hayashi et al., 2001) and *Trypanosoma cruzi* GPI anchors (Campos et al., 2001). The maturation of DC can be induced not only by inflammatory cytokines and microbes, but by their products recognized by TLR (reviewed in Reis e Sousa et al., 1999). Mature DC can present antigen and stimulate T cells to respond to the pathogenic antigen. Thus, the TLR-mediated DC maturation is a crucial link between innate and adaptive immunity (Akira et al., 2001). TLR can also participate actively in the control of pathogens. For instance, TLR can also initiate killing of *M. tuberculosis* (Thoma-Uszynski et al., 2001). Although the interactions of TLR and *Leishmania* have not yet been demonstrated, it is likely that GPI-anchors on the parasite could activate TLR of APC.

Another potential mechanism thorough which pathogens can be recognized is via CD1. Similar to Toll receptors, the CD1 proteins are evolutionarily conserved (Porcelli and Modlin, 1999). The CD1 family of antigen presenting proteins, which is not linked to the MHC, despite having structural similarities to the MHC class I proteins, has evolved to present lipid and glycolipid proteins (reviewed in Jayawardenda-Wolf and Bendelac, 2001). CD1 molecules are found on APC, including DC and macrophages (Sieling et al., 1999; Porcelli and Modlin, 1999). Currently, CD1 presentation has only been reported in microbial infections, but since many pathogenic protozoan parasites express lipid molecules on their cell surface, such as the lipophosphoglycan molecules on *L. major*, it is conceivable that some of these organisms many also interact with CD1 molecules. CD1 molecules can present antigen to $\gamma\delta$ and $\alpha\beta$ T cells, as well as NK T cells (reviewed in Porcelli and Modlin ,1999).

There are two main ways which CD1 can influence an infection. First, pathogen reactive CD1-restricted T cells show a high degree of cytolytic activity against pathogen pulsed CD1+ phagocytes (Stenger et al., 1997). Hence, pathogens antigens presented through

CD1 could be used to tag cells for eradication. The interaction of CD1 and T cells can also influence cytokine secretion. CD1-restricted T cells secrete cytokines that can polarize Th cells towards a Type 1 or Type 2 response (Sieling et al., 1995; Yoshimoto et al., 1995; Exley et al., 1997). It has been proposed that IL-4 derived from CD1 autoreactive T cells influence the development of Th2 responses (reviewed in Maher and Kronenberg, 1997). This protein can also be modulated in response to pathogens. For instance, CD1 is highly expressed on DC of hosts effectively controlling leprosy (Sieling et al., 1999), but is down-regulated on APC infected with *Mycobacterium tuberculosis* (Stenger et al., 1998). In summary, CD1 can play a role not only in innate immunity, but can influence adaptive immunity as well.

During the innate response, certain cells such as macrophages and neutrophils can phagocytize *L. major* (reviewed in Titus et al., 1994; Tacchini-Cottier et al., 2000). Once the parasite is engulfed, it can be eradicated through the respiratory burst—the release of radical oxygen or nitrogen products into the PV. Neutrophils will die after a round of phagocytosis. In contrast, macrophages can become activated and secrete cytokines or express co-stimulatory molecules, enabling these cells to initiate an adaptive immune response (Aderem and Underhill, 1999).

NK cells can also play a role in innate immunity, as well as bridge innate and adaptive immunity. NK cells can kill cells by releasing cytotoxic granules onto the surface of a target cell that can induce cell death by apoptosis (reviewed in Biron et al., 1999). When NK cells are exposed to IL-12 or IL-18, which can be secreted by macrophages or DC, they become activated and produce IFN-γ (reviewed in Trinchieri, 1995; Fehniger et al., 1999). The cytokine production, primarily IFN-γ and IL-10, by NK cells can influence the type of Th cell produced during an adaptive immune response (Scharton and Scott, 1993; Scharton-Kersten

and Sher, 1997; Scott and Trinchieri, 1995). NK cells do not need to be previously primed to an antigen in order to quickly respond. These cells can be important in controlling *Leishmania* early in infection (Laskay et al., 1993) and an impaired NK response can result in increased susceptibility to the parasite (Vester et al., 1999). For instance, NK cells can lyse *Leishmania*-infected macrophages (Resnick et al., 1988). NK cells are also a source of IFN-γ (Scharton and Scot, 1993). Through the interactions of NK cells with infected cells, cytokines can be secreted by NK cells that can intertwine innate and adaptive immunity and assist in the activation of effector T cells.

The Major Histocompatibility Complex (MHC) and the T Cell Receptor (TCR)

The interactions between the MHC and the TCR serve as the initial mechanism leading to a cascade of immune responses. Before T cells can be activated in leishmaniasis, parasite antigens must first be processed through the MHC and then presented to the T cells and its receptor. Newly synthesized MHC II molecules are assembled in the endoplasmic reticulum (ER) together with an invariant chain (Ii) (Cresswell, 1996) to form a complex competent for transport to the Golgi. Upon exit from the trans-Golgi network (TGN), the Ii-αβ complex is directed to the endocytic pathway (Pieters et al., 1993; Odorizzi et al., 1994). When this complex enters the endosomes, most of the Ii chain is cleaved. However, an internal Ii fragment remains in the groove of the molecule (Cresswell, 1996) . This fragment is termed CLIP. CLIP will dissociate between a pH of 5-6, which is the pH of the endosome (Mellman et al., 1998) and antigenic peptide can be loaded onto the MHC. Each peptide binding site interacts strongly with a specific array of amino acids and thus, each allele of a MHC molecule

binds best to a certain defined subset of peptide sequences (Heinzel et al., 1998). The endosome compartments that the MHC are loaded into are called MHC II-like compartments (MIIC), and are similar to late endosomes or lysosomes (Harding et al., 1988; Kleijmeer er al., 1997). These compartments are eventually transported to the surface of the cell and their contents then available for interaction with T cells.

T lymphocytes recognize antigen as peptides bound to MHC molecules on the surface of APC. T cell epitopes are displayed on APC in low copy numbers, thus requiring T cell recognition to be extremely sensitive and highly specific. The T cell must also be able to determine if the antigen being presented is due to an infectious agent or is self-antigen (Lanzavrcchia et al., 1999). T helper cells recognize antigen via cell-surface TCRs that interact with complexes of antigenic peptides bound to MHC glycoproteins (Mannie et al., 1999). The TCR and its signaling module CD3, CD4 and the adhesion receptor/ ligand pair LFA-1/ICAM-1 all accumulate at the contact interface between CD4+ T cells and the APC surface (van der Merwe et al., 2000). These cell surface molecules segregate into distinct clusters on the surface of the T cell and the APC and are termed supramolecular activation clusters (SMACs), forming an organized interface termed an immunlogical synapse. CD4 aids in forming the immunological synapse. This synapse stabilizes the interactions between the TCR and the MHC and allows for sustained engagement and signaling (Grakoui et al., 1999). In order for an adaptive immune response to occur, T cells much receive at least two signals. The first signal is provided by the interaction of the TCR and the MHC. The second signal is non-antigen driven and consists of stimuli provided by costimulatory receptor-ligand interactions (Mattner et al., 1997). The interactions between the TCR and the MHC

initiate signal transduction events that will mediate immunogenic responses. Initial interactions of the TCR with MHC ligands result in the induction of CD40L on T cells, which interacts with CD40 on APC. Ligation of CD40 activates adhesion receptors and upregulates B7 molecules (CD80/CD86) on APC. The interactions of CD40 and CD40L greatly increase the length of time that DC can present antigen to T cells (Miga et al., 2001). T cells that encounter MHC ligands with sufficient co-stimulatory signals will undergo blastogenesis. T cells that have sufficient co-stimulatory stimulation (e.g. ligation of CD28 and B7s) will secrete IL-2, which is an autocrine T cell growth factor and causes clonal expansion of the responding cell population (reviewed in Lenschow et al., 1996). Late in the proliferative phase of the T cell response, activated T cells can differentiate into effector cells that can synthesize all the effector molecules required for their function and are now able to respond to its specific antigen without costimulation (London et al., 2000).

Antigen presenting cells (APC) in leishmaniasis

There are two types of antigen presenting cells that are of prime importance in leishmaniasis. These cells are dendritic cells (DC) and macrophages (MΦ). Each has a quite distinct role in infection—DC first come into contact with the parasite and stimulate naïve T cells, whereas MΦ harbor the parasite. DC have a low parasite load (Moll, 1993), whilst macrophages are avid scavengers of *Leishmania* and are permissive to infection unless they are stimulated by cytokines which will activate them to clear the infection (Stenger et al., 1994; Konecny et al., 1999). Both DC and MΦ interact with T cells and are essential for clearing the parasite via cellular immune responses. There are

two stages of this response in presenting antigen to T cells in leishmaniasis. In the first stage, naïve T cells are activated by peptide-loaded dendritic cells in the lymphoid organs, and in the second step, the primed T cells interact with infected target cells, such as MΦ at the infection site (Mellman et al., 1998).

DC are the only APC capable of inducing primary immune responses (reviewed in Banchereau et al., 2000). DC have an interesting maturation history. The progenitors of DC arise in bone marrow, leave and home to tissues throughout the body, and then transform into "immature DC" in the tissue where they are residing (Banchereau et al., 2000). These immature cells possess a high level of phagocytic activity. The DC in the skin are called Langerhans cells (LC). LC have been shown to phagocytize *L. major* (Blank et al. 1993) and then to migrate to the lymph node to present antigen to T cells (Larsen et al., 1990, Moll et al., 1993; Austyn, 1996; Moll, 2000). Mature LC possess a very high surface density of MHC molecules and are able to present a large peptide repertoire at the threshold concentration of 50-200 peptide-MHC complexes (Valitutti et al., 1995; Cella et al., 1997; Pierre et al., 1997). These cells are capable of stimulating T cells for several days (Cella et al., 1997).

After tissue damage, for instance a sand fly bite and dispersal of *L. major*, the immature DC capture antigen by macropinocytosis, receptor-mediated C-type lectin or phagocytosis (Ries e Sousa et al., 1993; Jiang et al., 1995; Sallusto et al., 1995; Engering et al., 1997) and migrate to the lymphoid organs via the afferent lymph (Moll, 1993). As the DC travel to the secondary lymphoid organs, they begin to mature. The maturation of DC is defined by several events: 1) the loss of endocytic/phagocytic receptors, 2) the up-regulation of co-stimulatory molecules such as CD40, CD80 and CD86, 3) changes in

morphology, 4) the up-regulation of DC-lysosome-associated membrane proteins (DC-LAMP) and 5) changes in class II MHC compartments (Banchereau et al., 2000). As the DC matures, the peptide-MHC class II complexes are clustered on the surface of the cell with co-stimulatory molecules, such as CD80 and CD86 (Turley et al., 2000). This arrangement will facilitate interactions with the TCR of the naïve T cell. The maturation process takes 24-48 hours (Pierre et al., 1997). These cells then present antigen to CD4+ T cells, initiating the immune response. MHC products and MHC-peptide complexes are 10 to 100-fold higher on DC than on MΦ (Inaba et al., 1997). The MHC class II peptide complexes are also much more stable in DC than they are in MΦ (Flohe et al., 1997).

In order for T cells to become activated, two signals are required. The first signal entails recognition of MHC-peptide complexes on DC by antigen-specific T cell receptors (TCR). The interaction between DC and T cells is mediated by adhesion molecules such as CD2, CD54, and intergrins β1 and β2 (Hart, 1997; Bell et al., 1999). The second signal helps to sustain T cell interaction with the DC. To maintain activation, there must be interaction between co-stimulatory molecules on the DC and their ligands on T cells. T cells can activate DC through the CD40 ligand (CD40L) interacting with CD40 on DC. This interaction triggers increased expression of CD80 and CD86 on DC, as well as secretion of IL-1, TNF and IL-12 (Sallusto and Lanzavecchia, 1994; Caux et al., 1994; Schoenberger et al., 1998).

Similar to polarized T cell subsets, the subset of DC that is activated may influence the formation of Type-1 or Type-2 immunity. In humans, naïve T cells interacting with monocyte-derived CD11c+ DC tend to develop a Th1 profile, while DC that are CD11c- cause T cells to produce Th2 cytokines (Rissoan et al., 1999; Robinson

et al., 1999). When DC internalize *L. major*, they stimulate naïve T cells to produce IFN-γ, while macrophages do not (Konecny et al., 1999). DC are most likely the major source of IL-12 during the initial response to the parasite, causing T cells to differentiate down the Th1 path (Konecny et a., 1999; von Stebut et al., 1998). In order to produce IL-12, not only does CD40 on the DC need to be ligated, but microbial stimuli are necessary (Bogdan et al., 1996; Marovich et al., 2000). Once naïve T cells are activated by DC, the effector cells then immigrate from the lymph nodes or spleen into the lesion where the cells will then interact with infected macrophages (Overath and Aebisher, 1999).

The MΦ is more important in immune defense after the initial activation of T cells by DC in *Leishmania* infections. Phagocytosis of pathogenic organisms induces macrophages to produce cytokines that coordinate various aspects of host defense such as chemotactic factors like chemokines, activating factors such as TNF-α, IL-12 and IL-1, acute phase stimulants like IL-6, modulatory factors such as IL-10 and hematopoietc stimulating factors such as M-CSF and GM-CSF (Reiner and Locksley, 1995). The MΦ is essential for the eradication of a *Leishmania* infection (Flohe et al., 1998). In order to circumvent the defenses of the MΦ, the parasite has evolved the ability to impair many functions of antigen presentation by MΦ by several strategies, including the internalization and degradation of MHC class II by amastigotes, the suppression of MHC synthesis, the inhibition of antigen processing and loading of the MHC class II, the deficient expression of co-stimulatory molecules and the inhibition of IL-12 production (reviewed in Moll, 2000). *L. major*-infected MΦs augment the activation of Th2 clones, most likely by interfering with costimulatory function (Chakkalath and Titus, 1994).

Saha et al (1995) later demonstrated that upon infection, MΦ were unable to deliver co-stimulatory signals to T helper cells.

The study of macrophages *in vitro* has elucidated three stages of activation of these cells (reviewed in Overath and Aebischer, 1999). In the first stage, termed the "resting MΦ", the infected cells are deficient in MHC class II molecules and thus, do not present parasite antigens; in stage 2, upon exposure to IFN-γ (such as from activated Th1 cells), the MΦ will synthesize MHC class II molecules and expose them on the surface, however, the viability of the parasite will be unaffected (Antoine et al., 1991; Fruth et al., 1993; Wolfram et al., 1995). This cell is now considered a "primed MΦ". Finally, in the presence of a second signal, such as TNF-α, the macrophages will become fully activated and will kill the parasite (Bogdan et al., 1990; Liew et al., 1990). As the parasite is killed, these antigens will then be able to be presented by the MΦ, albeit at a lower level than DC (Inaba et al., 1997), to primed T cells (Overath and Aebischer, 1999). Similar to DC, MHC class II antigen presentation requires ligation of CD80, CD86 and CD40 on the MΦ with CD28 and CD40L on the T cell (reviewed in Bogdan et al., 1996).

Costimulation in Leishmania infections

In order for the T cell to be activated appropriately, proper costimulation is necessary. The most important interactions among costimulatory molecules guiding the responses of APC and T cells are between CD40 and CD40L, B7 and CD28, and B7 and CTLA-4 on the APC and T cells respectively (Jenkins, 1994; Bluestone, 1995). Disruption of the CD40-CD40L interaction will critically affect cell-mediated immune response. Both CD40 and CD40L KO mice on resistant backgrounds are susceptible to

L. major (Campbell et al, 1996; Heinzel et al., 1998). Furthermore, macrophages of these mice were not able to produce IL-12 due to the inability of the T cells to activate the APC. DC are also dependant on CD40/CD40L interactions. Human DC which have internalized *L. major* produce IL-12 in a CD40/CD40L – dependent manner (Marovich et al., 2000). Ligation of CD40 on dendritic cells triggers the production of IL-12 and highly up-regulates the expression of CD80 and CD86 (Cella et al., 1996). The resultant increased production of IL-12 after CD40 ligation caused IFN-γ production enhancement of T cells, as well as T cell proliferation, biasing cells towards a Th1 response (Cella et al., 1996). CD40L may have value as a vaccine adjuvant as well (Chen et al., 2001). For instance, because CD40L is a potent inducer of IL-12, this ligand has been used in vaccine trials in mice. Mice vaccinated with CD40L and *Leishmania* antigen had much smaller lesions and a lower parasite burden than controls (Chen et al., 2001).

CD28 performs many roles in up-regulating and maintaining a cell mediated immune response. CD28 ligation with CD80 and CD86 acts to maximize T cell activation, promote T cell differentiation and expansion and to regulate survival of T cells (Mattner et al., 1997). However, in *L. major* infections, CD28 itself does not appear to play a major role, despite the importance of the modulation of B7s during *Leishmania* infections (Brown et al., 1196; Elloso and Scott, 1999; Gomes et al., 2000b).

Another important costimulatory protein is CTLA-4. CTLA-4 is a membrane protein on the T cell (Thompson and Allison, 1997). This molecule is a homolog of CD28 and binds CD80 and CD86 as well (Lindsey et al., 1991). In contrast to CD28, if CTLA-4 binds to CD80/CD86, TCR dependent cell activation is reduced (Krummel and Allison, 1995; Fleisher et al., 1996). The blockade of CTLA-4 in susceptible BALB/c

mice results in decreased parasite burdens and a skewing towards Type-1 immunity (Murphy et al., 1998; Gomes et al., 2000b). The engagement of this costimulatory molecule also induces CD4+ T cells to secrete TGF-β, a Th2-like cytokine which suppresses healing (Gomes et al., 2000b).

The final major group of costimulatory molecules important in leishmaniasis is the B7s. CD80 and CD86 (B7s), expressed primarily on APC, are ligands for CD28 and CTLA-4. CD86 expression is upregulated rapidly while CD80 is induced slowly and expressed for a longer duration (48 hours versus 4-5 days) when the APC is activated (reviewed in Chambers, 2001). There is controversy surrounding the importance of CD80 and CD86 in directing a Th1 or Th2 response. Elloso and Scott (1999) report that both co-stimulatory molecules can result in the production of either IFNγ or IL-4. On the other hand, there are reports of the importance of costimulatory molecules in the development of Th1 or Th2 responses. For instance, blocking CD86, but not CD80 significantly inhibited the ability of BALB/c LC to induce IL-4 production in Th2 cells (Mbow et al., 2001); however, Th1 responses could be altered as well. The production of IFN-γ by T cells of resistant mice was inhibited when either CD80 or CD86 was blocked on LC (Mbow et al., 2001). In contrast, LeIF, a *Leishmania* antigen that induces a Th1 response, up regulated CD80 and induced IL-12 production in human macrophages and dendritic cells (Probst et al., 1997). The intricate interactions between APC and T cells direct, via co-stimulation and the resulting secretion of cytokines in response to stimulation, the immune response to *L. major*.

Immunomodulatory effects of sand fly salivary glands and its lysate

The impact the sand fly vector has on the exacerbation of leishmaniasis must not be discounted. Over the past decade, several studies have begun to more thoroughly investigate the ability of sand flies to intensify and modulate *Leishmania* infection and establishment in the host. Sand flies are active participants in the disease process. The saliva secreted during a feeding may not only contain parasites, but pharmacologically active compounds to enable more efficient feeding. *Leishmania* species have evolved to utilize several of these compounds for the purpose of enabling the establishment of infections in the host. As a result, salivary gland products may be of use as vaccine targets. Studies have shown that pre-exposure to sand fly saliva or the salivary protein maxadilan can reduce the risk of severe leishmaniasis (Belkaid et al., 1998; Kamhawi et al., 2000; Morris et al., submitted).

Several different compounds have been isolated and characterized from the saliva of various sand fly species. A particularly interesting finding is that there is quite a lot of heterogeneity among the proteins various sand fly species possess. Surprisingly, there is a great difference in saliva composition between the Old and New World flies, despite their similar feeding patterns. One of the most widely studied salivary compounds to date is termed maxadilan (Max), which is only found in New World sand flies. Max is an extremely potent vasodilator (Lerner et al., 1991) and has many immunomodulatory properties that facilitate *Leishmania* infection (reviewed in Kamhawi, 2000; Gillespie et al., 2000). Several other salivary gland factors have also been identified. Charlab et al. (2000) have identified adenosine deaminase from the New World sand fly *Lutzomyia longipalpis*. This product possesses anti-inflammatory properties and is secreted during a blood meal. Hyaluronidase

has also been identified and is thought to assist in the diffusion of Max and other sand fly proteins into the mammalian host (Charlab et al., 1999).

Immunologically important compounds have also been identified in Old World sand flies. For example, salivary gland homogenates of *Phlebotomus papatasi* contain large amounts of adenosine and 5' AMP (Ribeiro et al., 1999). These substances have both vasodilatory and anti-platelet activities. It has been suggested these compounds could decrease the host's initial immune response to both the vector and the parasite (Ribeiro et al., 1999). Katz et al. (2000) suggested that salivary adenosine from *P. papatasi* is responsible for down-regulating nitric oxide (NO) synthesis in murine macrophages. In contrast, adenosine was not found in *L. longipalpis* salivary glands, and thus there was no evidence of NO down-regulation. Adenosine is released from several different cell types in response to metabolic stress. Extracellular adenosine reduces polymorphonuclear cell recruitment and suppresses inflammatory responses, such as NO and TNF-α production (Collis and Hourani, 1993). Both Old and New world flies possess apyrase, which is an anti-platelet aggregation enzyme that hydrolyses ATP and ADP to AMP and inorganic phosphate (reviewed in Kanhawi et al., 2000).

Numerous studies have characterized the impact of salivary gland lysates from primarily *P. papatasi* and *L. longipalpis* on *Leishmania* infections both *in vitro* and *in vivo*. Titus and Ribeiro (1988) were the first to report that salivary gland lysates from *L. longipalpis* enhanced *L. major* infection in both resistant and susceptible mice. They determined that lesions were larger in size and contained more parasites than uninfected controls. They also found that only when salivary gland lysate was co-injected, could a low number of parasites be detected at the injection site. Shortly thereafter, it was determined that *P. papatasi* salivary

gland lysate could also exacerbate *Leishmania* infections (Theodos et al., 1991). It was also demonstrated that live sand flies could inject substances while probing for a blood meal that in turn caused larger lesion development compared to the controls when parasites were injected into the feeding sites.

Since macrophages are one of the primary cells infected by *Leishmania* and are often at the site of a sand fly bite, the interactions between sand fly saliva, the parasite and murine macrophages have been investigated in several studies. The ability of murine macrophages to present parasite antigen to parasite specific T cells was inhibited by *L. longipalpis* salivary gland lysate (Theodos and Titus, 1993). The salivary gland lysate of *P. papatasi* inhibits the ability of IFN-γ stimulated macrophages to destroy *L. major* due to a down regulation of NO production (Hall and Titus, 1995). Interestingly, although saliva appeared to have a direct effect on macrophages, no modulation of I-A expression (class II) was observed in infected macrophages exposed to salivary gland lysate (Hall and Titus, 1995). These studies all indicate one of the primary cells affected by salivary compounds is the macrophage.

It is important to study not just the *in vitro*, but the *in vivo* effects of saliva on the immune response of an organism. The saliva of *P. papatasi* was found to increase parasite burdens of *L. major* in lesions (Mbow et al., 1998). Likewise, the production of cytokines by cells in the draining lymph node of mice co-injected with lysate and parasites was modulated. The Th2 cytokine IL-4 was higher in lysate treated mice. Conversely, IFN-γ and IL-12, which are Th1 cytokines, were inhibited. There was no effect on IL-10 or TGF-β. Similarly, infections in mice co-injected with *L. braziliensis* and saliva from sand flies have increased IL-4 production and larger lesions compared to mice injected only with parasites (Lima and Titus, 1996; da Bezzerra and Teixeira, 2001). Other studies have also observed system-wide

effects of salivary gland products in mice and have even shown that saliva or its products may be viable vaccine candidates. Pre-exposure to salivary gland products via sensitization injections neutralized the ability of salivary gland lysate to enhance *Leishmania* infection in the epidermis (Belkaid et al., 1998). In addition, when uninfected sand flies bite mice prior to infection with *L. major*, protection was conferred upon the mice. These "vaccinated" mice exhibited a strong delayed-type hypersensitivity response and IFN-γ production at the site of parasite delivery (Kamhawi et al., 2000). In a study using humans, Belkaid et al (2000) suggest that the DTH response may be used by the sand fly in order to promote faster feeding. Manipulating this response may be of use in designing a vaccine.

Immunomodulatory Effects of Maxadilan

Since there is a great deal of complexity within salivary glands, there was an effort to determine if there was a single protein that is responsible for many of the immunomodulatory effects reported when whole-lysate was used. The answer to this quest in the New World sand fly, *Lutzomyia longipalpis*, has been the discovery and characterization of Maxadilan (Ribeiro et al., 1989; Lerner and Shoemaker, 1992). Maxadilan (Max) is a 63 amino acid peptide that has potent vasodilatory properties (Lerner et al., 1991; Jackson et al., 1996). It is believed to cause the erythema which results from the bite of the sand fly (Ribeiro et al, 1989). Max most likely allows for the pooling of blood at a bite site, enabling the sand fly to take a blood meal (Lanzaro et al., 1999). The peptide has functional similarity to the human calcitonin gene-related peptide (CGRP), showing long lasting vasodilation and the ability to relax contracted aortic rings (Ribeiro et al., 1989). Max acts by raising intracellular levels of cyclic AMP in the

smooth muscle of blood vessels and macrophages (Grevilink et al., 1995; Soares et al., 1998). Since Max was found to bind to neural tissue, it was suggested that Max binds with cells through neuropeptide receptors (Moro et al., 1995; Moro and Lerner, 1997). Moro and Lerner (1997) determined that Max binds to cells via the pituitary adenylate cyclase activating peptide (PACAP) receptor. PACAP is a neuropeptide with vascular activity. Similar to Max, PACAP stimulates cAMP production in rat anterior pituitary cells (Miyata et al., 1989). PACAP is able to modulate immune responses and cells such as inhibiting lymphocyte mobility, increasing IL-6 production, and abrogating IL-12 production and STAT1 activation in macrophages (Tatsuno et al., 1991; Delgado et al., 1995; Delgado et al., 1999; Delgado and Ganea, 2000). Interestingly, PACAP and Max share little sequence homology (Moro and Lerner, 1997).

Researchers have characterized the immunological effects of Max. This peptide is able to modulate the immune response of cells to LPS stimulus. Soares et al. (1998) found that both Max and PACAP-38, an agonist for the PACAP receptor inhibit TNF-α production and induce IL-6 production in LPS-stimulated murine macrophages, indicating that these effects were modulated by the PACAP receptor. Max is also able to inhibit T cell proliferation in vitro and DTH responses in vivo (Qureshi et al., 1996). *L. major* infections are exacerbated when mice are co-injected with parasites and Max, similar to the effects when mice are exposed to salivary gland lysates and the parasite (Morris et al., submitted). This peptide appears to be one of the main components in *L. longipalpis* saliva that causes immunomodulatory effects.

Perhaps because Max is so potentially important to certain sand fly species, there may be selective pressure (e.g., immunological) against the peptide in hosts fed on by

sand flies. Supporting this concept, there is a surprising level of heterogeneity, up to 23%, in the amino acid sequence which make up the peptide in field populations and laboratory reared siblings of sand flies (Lanzaro et al., 1999). Despite this difference, all of the variants were capable of inducing IL-6 and had similar vasodilatory properties. Differences in the levels of Max may play a role in infection. In Costa Rica, infections with *L. chagasi* generally result in cutaneous leishmaniasis, while in South America, infections by the same parasite cause visceral leishmaniasis (Zeledon et al., 1989; Grimaldi et al., 1989). There are differences in the bites between *L. longipalpis* flies in these two different regions. In Costa Rica, the flies do not induce long-lasting erythemas, while in South America, the bites are characterized as being large, long lasting, and red (Warburg et al., 1994). Interestingly, the amount of maxadilan present from sand flies of these two regions appears to correlate with the differences in the disease manifestations (Yin et al., 2000). *L. longipalpis* from Central America had lower levels of Max mRNA expression compared to Max expression from South American flies. These reports strongly suggest that Max is capable of influencing the disease manifestations of *Leishmania* infection.

Because Max is so adroit at exacerbating leishmaniasis, Morris et al (submitted) hypothesized that vaccinating against it could neutralize the disease-enhancing effects of whole saliva and protect vaccinated mice against *L. major* infections. Their study found that lesions were much smaller in vaccinated mice and the parasite burden was greatly reduced. Max is particularly enticing as a vaccine candidate as opposed to salivary gland lysate or whole saliva. The reasons for this are that Max is a defined peptide and can be

synthesized. Further studies need to examine if Max can protect against a wide range of *Leishmania* species.

Aims of this project

The goal of the research in this dissertation was to establish a primary in vitro system reactive to *Leishmania major* using *Leishmania*-naïve peripheral blood mononuclear cells (PBMC) to elucidate the effects of the parasite on the early immune response of human cells. The three specific aims were:

Aim 1: To establish a primary in vitro system using human PBMC from *Leishmania*-naïve donors. The system was characterized phenotypically and via cytokine production.

Aim 2: To modulate the in vitro system to determine what cytokines play a role in the formation of early immune responses.

Aim 3: To use the in vitro system to determine the effects of sand fly salivary gland proteins on human cells.

Although the murine model of leishmaniasis is an integral tool in our understanding of the disease, it is also important to investigate how humans respond to the parasite. The human in vitro model allows us to study the immune response to *Leishmania* under controlled conditions and to determine how sand fly-specific proteins modulate this response early in infection. These types of studies would be difficult in human beings themselves due to not only ethical issues, but to the inability to precisely control experimental conditions. Our in vitro system allows us to add another dimension of study to the field of leishmaniasis.

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CHAPTER ONE

EARLY CELLULAR IMMUNE RESPONSES TO *LEISHMANIA MAJOR*: THE CHARACTERIZATION OF A HUMAN IN VITRO PRIMING SYSTEM

INTRODUCTION

Leishmaniasis, which infects approximately 12 million people world wide, results from infection with the protozoan parasite *Leishmania sp.* (WHO, 2000). The incidence of leishmaniasis is increasing, with many endemic areas reporting a 500% increase over the past several years (WHO, 2000). The clinical manifestations of the disease range from skin lesions, which can be self healing or ulcerative, to a visceral infection which can range from subclinical to fatal. The parasite resides within the macrophages of the host and is able to circumvent detection (Titus et al., 1994). Cell mediated immunity is essential for resistance to the parasite and recovery from the infection (Barrral-Netto et al., 1997). Cytokines produced by NK cells, T cells and antigen presenting cells play an integral role in this process.

There are three main subsets of T helper (Th) cells (Palmer and van Seventer, 1997). Mature, "unprimed" CD4+ T cells produce mainly IL-2 upon antigen-specific stimulation. Upon further antigen exposure, these Th cells then will differentiate into either 1) Th1 cells, which secrete IFN-γ and are involved in cell-mediated immunity; 2) Th2 cells, which secrete IL-4 and IL-5 and regulate humoral immune responses; or 3)

Th0 cells which secrete both Th1 and Th2 cytokines. The stimulation and proliferation of a particular Th-cell subset can determine the outcome of an infection.

Experimental murine cutaneous leishmaniasis induced by *L. major* is one of the best studied models in which the selective activation of Th-subsets occurs (Solbach and Laskay, 2000). In resistant mice such as the C3H strain, IFN-γ, which is produced by Th1 cells, is the primary mediator of resistance to *L. major*, as it can activate macrophages (MΦ) to destroy the parasite (Nacy et al., 1985; Sadick et al., 1986; Titus et al., 1984). In susceptible mice, such as BALB/c, IL-4, produced by Th2 cells, can block the ability of IFN-γ to activate MΦ to eradicate *L. major* (Liew et al., 1989). Cytokines can begin to influence the outcome of an infection shortly after exposure to the parasite (reviewed in Solbach and Laskay, 2000).

In the murine model, the CD4+ T cell is the primary cell driving the response to *L. major* (Scott et al., 1989). Similarly, in humans, CD4+ T cells are extremely important in controlling the infection (Kemp, 1997). In addition, CD8+ T cells appear to also influence the host response, particularly in mucocutaneous infections (Brodskyn et al., 1997; Russo et al., 1998) and chronic cutaneous infections (Da-Cruz et al., 1994).

Much of the information we have learned about leishmaniasis has involved the study of live animal models. These models are extremely valuable, however they do have some limitations. These constraints can be overcome by the use of in vitro models. For instance, it is difficult to isolate specific factors that contribute to early priming and commitment to either a Th1 or Th2-type response in vivo. To address this issue, Shankar and Titus (1993), developed a primary in vitro (PIV) assay system that yielded a large population of *L. major*-specific, CD4+, major histocompatibility complex class II-

restricted T cells from the lymphoid tissues of naïve BALB/c or C57 mice. The cell surface phenotype of these PIV T cells was identical to that of T cells which respond in mice infected with *L. major*. This system was further dissected by Soares et al. (1997) to determine the influence of cytokine production on the commitment of Th cells. These data were also comparable to results reported in in vivo studies. The influence of individual groups of antigen presenting cells, such as dendritic cells and macrophages, on priming and cytokine secretion of *Leishmania*-specific T cells was also examined using the PIV system (Shankar and Titus, 1993).

In human leishmaniasis research, most studies have involved the use of infected or recovered patients. These studies pose several limitations. For instance, in patients infected with *Leishmania*, it is difficult to determine when an individual was initially infected. Accordingly, it is not possible to study the interactions that initially occur between a host and the parasite. There are also confounding factors when investigating patients, particularly from poverty stricken regions. These issues can include secondary infections, lack of access to timely medical care, and malnutrition, all of which can influence the outcome of the disease. To start to address these limitations, some investigators have begun to develop in vitro systems. Brodskyn et al. (2000) developed a system using lymphocytes from *Leishmania*-naïve donors and exposing the cells to the parasite. Macrophages were used as APC for *Leishmania*-stimulated T cells. This system, however, is not well characterized. Another limitation of this in vitro system is that is has been reported that T cell subsets differ in their capacity to be stimulated based on the APC present (Shankar and Titus, 1997). Other laboratories have studied *Leishmania*-naïve donor cell responses to the parasite or parasite antigens. In these cases,

parasite-primed cells were not restimulated (Kemp et al., 1992; Akuffo and Britton, 1992; Akuffo et al., 1993; Kurtzhals et al., 1995; Nylen et al., 2001). These reports found that there are non-*Leishmania*-specific responses to the parasite or parasite antigen due to environmental and pathogen antigens cross reacting. The cells responding to the parasite were CD4+ T cells (Kemp et al., 1992; Kutzhals 1995) or NK cells (Akuffo et al., 1993). The responding cells produced IFN-γ (Akuffo and Britton, 1992; Akuffo et al., 1993; Kurtzhals et al., 1995; Nylen et al., 2001). These experiments did not involve priming cells to the parasite and then restimulation to dissect human immune responses. Russo et al. (1998;1999) derived *L. amazonensis*-specific T cells lines from *Leishmania*-naïve donors. The primary focus of these studies was on cytotoxic T cell responses.

There is a need to develop an in vitro (IV) system from *Leishmania*-naïve donors which is well characterized so that the system can then be used as a tool to study the early immunological interactions between human cells and *L. major*. We developed an in vitro priming system in which peripheral blood mononuclear cells (PBMC) were isolated from *Leishmania-naïve* donors and exposed to the parasites for an initial priming period. This approach allowed the opportunity for macrophages, B cells and dendritic cells present in the periphery to present antigen to T cells. The responding T cells were then collected and exposed to irradiated autologous PBMC, which provided a source of antigen presenting cells, and pulsed with *L. major*. Both the primary and the secondary stimulation phases were characterized phenotypically and via cytokine production. We hypothesized that the cells generated would be primarily class II-restricted, *L. major*-specific CD4+ T cells.

MATERIALS AND METHODS

Parasites

L. major (isolate LV39, Rho/SV/59/P) parasites were grown on biphasic NNN medium (Titus et al., 1984) and passed through mice every two weeks to maintain virulence. Promastigotes were harvested in the stationary growth phase. *L. amazonensis* (LTB0016) and *L. mexicana* (WHO strain MNYC/BZ/62/M379, a gift from Dr. D. Russell) were maintained the same way as described for *L. major*. *L. donovani* (2S strain, a gift from Dr. J Farrell) that had been passed through a hamster and frozen was thawed and cultured in Schneider's insect medium (Sigma) (Howard et al., 1991). *L. major* lysates were prepared by rapidly freezing and thawing the parasites as described previously (Titus et al., 1985).

In vitro system

The *in vitro* system we developed was patterned after the murine *in vitro* system described by Shankar and Titus (1993). Human blood was obtained from healthy individuals at the Hartshorn Health Center at Colorado State University. Blood was drawn after informed consent was obtained from each donor. All of the procedures were approved by the Human Research Committee at Colorado State University. 100 ml of blood was collected during each blood draw. PBMC were isolated from heparinized venous blood by passage over a Ficoll-Hypaque gradient (Pharmacia; Upsala, Sweden) (Goldrosen et al., 1977). PBMC were washed three times and resuspended at a concentration of 2.5×10^6 cells/ml in complete medium consisting of RPMI-1640 medium

supplemented with 2 mM L-glutamine, penicillin (100 U/ml), gentamycin (100 µg/ml), and 10% heat inactivated human AB serum (Pel-Freeze; Brown Deer, WI). The cells were plated in 24-well tissue culture plates (Costar; Corning, NY) at 1 ml/well. *L. major* promastigotes were added to some of the cultures at a concentration of $2.5x10^5$ parasites/ml. To study early time point interactions between the PBMC and *L. major*, culture supernatants and/or cells were harvested for either cytokine analysis or phenotypic characterization via flow cytometry at 1, 3, 5 and 7 days.

For restimulation experiments, the culture plates were incubated for 8 days at 37°C with 5% CO_2. After the incubation period, non-adherent cells were removed (PIV cells). On day 8, 100 ml of blood was drawn again from the same donor and these cells were processed the same way as previously described. The freshly isolated PBMC were then irradiated (1500 rads, ^{137}Cs source), washed 3 times and resuspended at a concentration of $5x10^6$ cells/ml. These cells provided a source of antigen presenting cells (APC) for the PIV cells. To identify the functional phenotype of the cells, 8 day PIV cells (10^6/ml) were restimulated in 24-well plates with $5x10^6$ irradiated cells and *L. major* (10^6/ml). Supernatants were harvested 48 h later for cytokine analysis. All experiments were run a minimum of two times for each donor. In total, 15 donors were used in this study.

Proliferation assay

Eight day PIV cells were harvested and rested for 2 days to allow the cells to become quiescent. In order to rest the cells, the non-adherent cells were harvested, washed and replated at $2x10^6$/ml in 24 well plates. After rest, the cells were collected

from the wells and washed. Triplicate wells were prepared on 96 well microtiter plates (Corning Costar; Corning, NY) with $5x10^4$ PIV cells, 10^6 irradiated (1500 rads) autologous APC and 10^6 *Leishmania*/ml or 10^6 *L. major* equivalents/ml for the lysate preparation. Ovalbumin (OVA;100 µg/ml),(Sigma), *Mycobacterium bovis* Bacillus-Calmut-Guerin (BCG; a gift from Dr. A. Cooper) ($2x10^5$ bacteria/well) and gp63 (TEX 48, a gift from Dr. W.R. McMaster)(500 ng/ml) were also used in this assay. Proliferation was measured by the addition of 1µCi[^{3}H]-thymidine 48 h after plating. The degree of proliferation was assessed by scintillation counting. Cultures were harvested 24 h later on glass fiber filters (Wallac; Turku, Finland), using a Tomtech Harvester (Tomtec Inc., Hamden, CT). Incorporation of radioactivity into DNA was measured with a Wallac 1450 MicroBeta™ PLUS liquid scintillation counter (Wallac; Turku, Finland). All experiments were repeated two times.

Reagents

The following reagents were used in cell cultures: the monoclonal antibody to human HLA-DR,DP,DQ (clone TÜ39, mouse IgG_{2a} isotype; Pharmingen Beckton Dickenson; San Diego, CA) at 10 µg/ml, the monoclonal antibody to human HLA-A,B,C (clone G46-2.6, mouse IgG_1 isotype; Pharmingen) at 10µg/ml. Isotype matched antibodies specific for irrelevant antigens from the same supplier were used as controls. The following reagents were used for flow cytometry: fluorescein isothocyanate (FITC)-labeled anti-human CD3 (clone UCHT1, mouse IgG_1 isotype; Pharmingen), FITC-labeled anti-human CD25 (clone M-A251, mouse IgG_1 isotype; Pharmingen), Phycoerythrin (PE)-labeled anti-human CD4 (clone RPA-T4, mouse IgG_1 isotype; Pharmingen),

Cychrome (CY)-labeled anti-human CD8 (clone RPA-T8, mouse IgG_1 isotype; Pharmingen), CY-labeled anti-human CD19 (clone HIB19, mouse IgG_1 isotype; Pharmingen), FITC-labeled anti-human HLA-DR,DP,DQ (clone TÜ39, mouse IgG_{2a} isotype; Pharmingen), PE-labeled anti-human CD14 (clone M5E2, mouse IgG_{2a} isotype; Pharmingen), CY-labeled anti-human CD19 (clone HIB19, mouse IgG_1 isotype; Pharmingen), PE-labeled anti-human CD56 (clone B159, mouse IgG_1 isotype; Pharmingen). Appropriately labeled irrelevant isotype-matched antibodies from the same supplier were used as controls.

Cytokine assays

Concentrations of IFN-γ, IL-12 and IL-5 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (Genzyme; Cambridge, MA). The IL-12 kit was specific for both IL-12p40 and p70 subunits. The limits of detection for these assays were 10 pg/ml for IFN-γ and IL-12 and 2 pg/ml for IL-5. Supernatants were either measured at 24, 48 and 72 h after initial stimulation or at 48 h after restimulation.

Flow cytometry

The PBMC exposed to *L. major* were characterized phenotypically to determine if there were any changes in the cell populations when they were exposed to the parasite. Cells ($1x10^6$/sample) were prepared for analysis as previously described (Brodskyn et al., 2001). Briefly, cells collected from the 24-well tissue culture plates were resuspended in PAB (phosphate-buffered saline, 1% bovine serum albumin, 0.05%

sodium azide) and blocked with mouse IgG (20μg/ml) and 10% fetal bovine serum for 30 min on ice. Cells were then incubated with labeled antibodies or corresponding isotype controls for an additional 30 min. Cells were fixed with 1% paraformaldehyde in PAB and analyzed on a Coulter EPICS XL flow cytometer (Coulter Corp.; Miami, FL). Fluorescence data were obtained on 10,000 cells. As determined by forward light scatter, a gate was drawn around live cells (excluding debris and doublets) during data collection. Analyses of flow cytometry results were performed using FlowJo software (TreeStar; Palo Alto, CA).

CD4 or CD8+ T cell depletion assay

To determine if both CD4 and CD8+ T cells were secreting IFN-γ, CD4+ cells or CD8+ cells were depleted from the eight day *L. major*-stimulated PIV cultures. The cells were depleted using magnetic separation columns (Miltenyi Biotech; Auburn, CA), following the manufacturer's recommended protocol. PIV cells were labeled with either mouse anti-human CD4 monoclonal antibody or mouse anti-human CD8 monoclonal antibodies (Pharmingen) for 15 min and then washed two times with degassed wash buffer (PBS pH 7.2, supplemented with 0.05% bovine serum albumin and 2mM EDTA). Cells were resuspended and magnetically labeled with MACS goat anti-mouse IgG microbeads (Miltenyi) and incubated for 15 min on ice. Cells were washed and resuspended in 500 μl of wash buffer and then depleted on MACS MS+ separation columns using the supplied flow-resistor (Miltenyi) following the manufacturer's recommended protocol. Columns were washed three times with wash buffer and the total effluent was collected as a depleted fraction. These cells were then washed three times.

Purity of the cell fractions was then determined by flow cytometry. The cells from the CD4- or CD8- fractions (10^6/ml) were cultured with irradiated autologous PBMC ($5x10^6$/ml) with or without *L. major* (10^6)/ml. The cultures were incubated for 48 h and then the culture supernatants were collected. IFN-γ concentrations were analyzed by ELISA.

Macrophage killing assay

In order to determine if macrophages could kill live *L. major* in this system, macrophage killing assays were performed. This assay was patterned after Titus et al. (1984) and Melby et al. (1996). Tissue culture-treated glass coverslips were placed at the bottom of 24 well plates (Costar) and then PBMC were plated at $5x10^6$/ml in each well. Monocytes were allowed to adhere to the coverslips overnight and then non-adherent cells were washed away. The remaining cells were grown in culture for 8 days to allow the monocytes to develop into macrophages.

L. major was then added to the cultures at a ratio of 10 parasites to one macrophage. Any parasites that had not yet entered the cells were washed away after 8 hours. At this time, *L. major*-stimulated primary in vitro cells, which had been exposed to the parasite for 8 days (see above methods for protocol), from the same individual were harvested and placed in the macrophage cultures at a ratio of 10 PIV cells to one macrophage. Control wells did not have any PIV cells added to them, but were exposed to *L. major*.

In addition to macrophages, PIV cells and *L. major*, some culture also received addition stimuli (e.g. conditioned medium) or neutralizing antibodies. Conditioned

medium was prepared by collecting the supernatant from the PIV cells once the cells were centrifuged. Anti-human IFN-γ antibody (clone 25718.111, mouse IgG_{2a} isotype; R&D Systems, Minneapolis, MN) or the appropriate isotype control was added at 10μg/ml to designated cultures. The coverslips were then removed 18 h later and stained with Diff Quick (Scientific Products; McGraw, IL) and affixed to glass slides. The macrophages on the coverslips were then examined by light microscopy for the presence of intracellular parasites. The results are expressed as number of intracellular parasites per 100 MΦ counted. These studies were repeated two times for each donor.

Statistical Analysis

Statistical analyses were performed using Sigma Stat (SPSS, Chicago, IL) and InStat (Graph Pad Software, San Diego, CA) software. Due to the small sample sizes and the inherent variances in human responses, the data were not distributed normally, thus non-parametric analyses were necessary. For restimulation studies, data were compared with Wilcoxon matched pairs tests. For proliferation studies, cell depletion studies and macrophage killing assays, Kruskal-Wallis one way ANOVAs were performed. When $p<0.05$, Dunn's Multiple Comparisons Test was used to determine the significance of the difference between samples. For early time point studies and the time course analysis of macrophage killing, Friedman's nonparametric repeated measures test was used to analyze the data, followed by Dunn's Multiple Comparison Test when $p<0.05$. Overall, results were considered significantly different when the p value was <0.05.

RESULTS

Cytokine production at early time points

In order to begin to characterize the early immune response of PBMC from unexposed donors to stimulation with *L. major*, the ability of the donor cells to produce cytokines was measured. The amount of secretion of the Type-1 cytokines IFN-γ and IL-12 and the Type-2 cytokine IL-5 was determined by ELISA after PBMC had been exposed to *L. major* for 24, 48 or 72 hours. No IL-5 production was detected at any time point (data not shown), suggesting that a Type 2 response is delayed in onset, or that IL-5 is secreted in a level below the limit of detection for the ELISA. In contrast, both IL-12 and IFN-γ were detected. Although IL-12 was produced, there was no significant difference (due to the wide ranging variation in production of IL-12 among the donors) between controls (PBMC without *L. major*) and *L. major*-exposed cells, although a trend of increased IL-12 production when cells are stimulated with *L. major* is noted (Figure 1.1). PBMC produced IFN-γ at 24 hr after exposure to the parasite, however, at this time point, the difference is not statistically different from the controls (Figure 1.2). On the other hand, at 48 and 72 hr, there are significant differences ($p<0.05$) when the cells were exposed to *L. major* compared to the unstimulated controls. Although there is a trend of progressively increasing IFN-γ levels at each time point, these differences were not significant. These results suggest that Type 1 cytokines contribute to the early cellular response to the parasite.

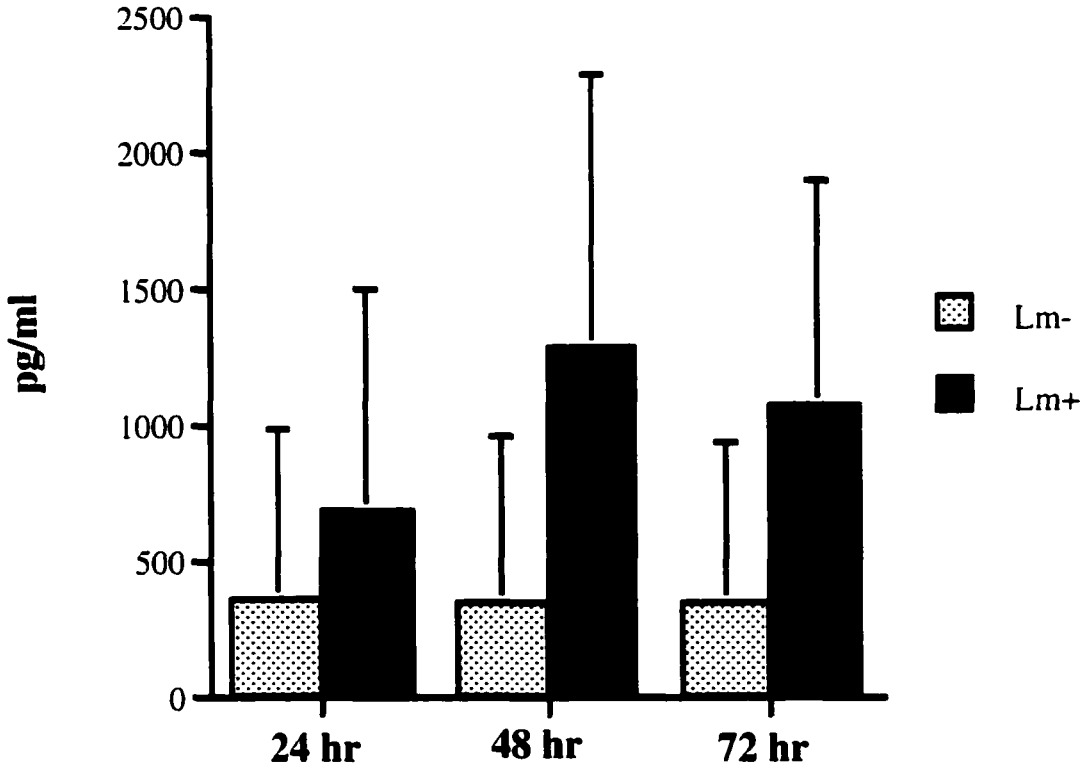


Figure 1.1 IL-12 production of PBMC at 24, 48 and 72 hr as measured by ELISA. Results are expressed as means±SD. N=6 donors.

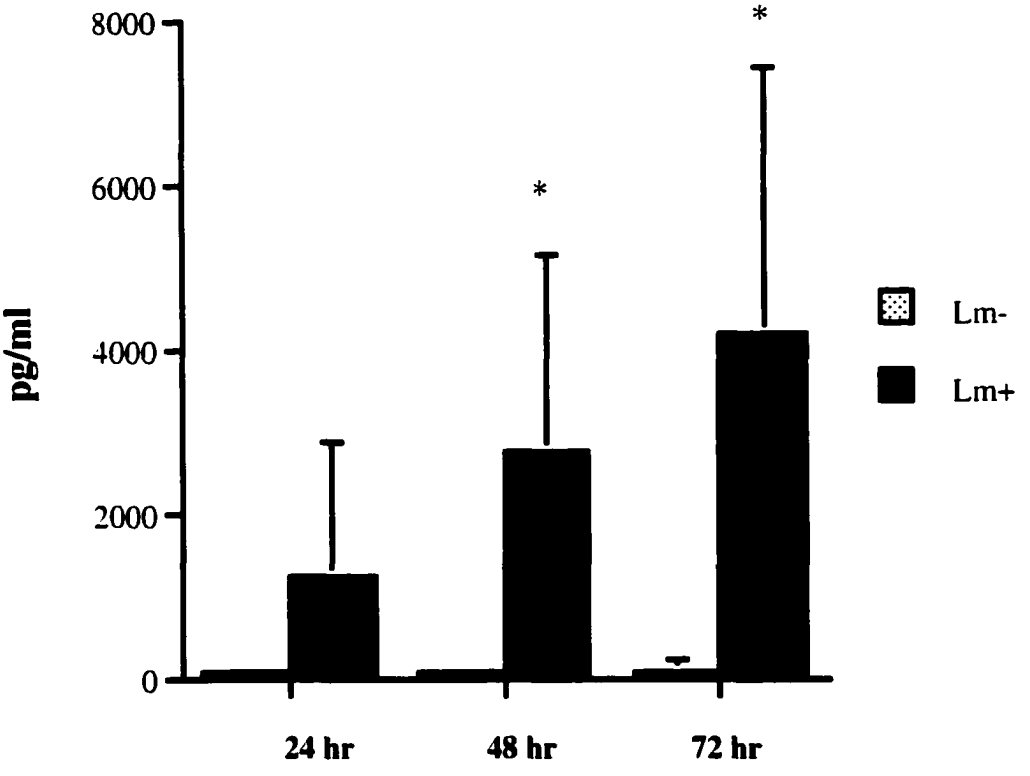


Figure 1.2 IFN-γ production of PBMC cultured for 24, 48, or 72 with or without *L. major* . Data shown as mean±SD. Significant differences ($p<0.05$) between controls and *L. major* -exposed cells are noted by *.

Phenotypic analysis of PIV cells at early time points

As difference was detected in IFN-γ production, suggesting the cells are responding to the parasite, we wanted to determine if we could detect phenotypic changes in the cell populations present in culture as the parasite and PBMC interacted. PMBC from *Leishmania*-naïve donors were exposed to the parasite for 1, 3, or 5 days, and controls were not exposed to *L. major*. The PIV cells were harvested at each time point and stained with conjugated antibodies against CD4, 8, 25, 56, 19 or HLA class II. The results are summarized in Table 1.1. To determine the phenotype of responding lymphocyte populations, a combination of CD3, CD8 and CD4 markers were used. A gate was drawn around the CD3-positive population and then those cells that stained double positive for CD3 and either CD4 or CD8 were evaluated. There was no difference in the percentage of cells that stained positive for CD4 or CD8 when comparing the cells that were exposed to the parasite or the control cells. Likewise, there is little difference in the percentage of cells positive for CD4 or CD8 over the time course studied.

To determine if the population of cells being activated could be detected, the cells were stained with antibodies against CD25, part of the IL-2 receptor complex. CD25 is found on activated T lymphocytes. Very few cells stained positive for CD25 (under 3%) and of those, less than 1.2% were double positive for CD4 and CD25 and less than 1% were double positive for CD8 and CD25. Again, when the number of cells staining positive for HLA Class II or CD19 (found on B cells) were determined, there were no differences over time or between treatment groups. Interestingly, although there were no differences between the percentages of *L. major* positive or negative cells expressing CD56, there was a decrease in percentage of cells expressing CD56, which is found on

Table 1.1 Time course evaluation of cell-surface markers expressed by PIV cells exposed to *L. major* or not (control)*.

	Day 1		Day 3		Day 5	
	Lm-	Lm+	Lm-	Lm+	Lm-	Lm+
CD3+CD4+	28	28.3	28.9	26.9	32.4	29.3
CD3+CD8+	9.5	10.6	9.3	8.2	8.9	6.6
CD4+CD25+	0.54	0.85	0.85	1.16	1.13	1.14
CD8+CD25+	0.5	0.17	0.32	0.32	0.17	0.53
HLA Class 2+	5.9	5.9	6.6	6.4	7	6.5
CD56+	13.9	14	10.3	6.4	6.8	6.8
CD19+	2	2.6	4	3.2	4.1	3.4

*The values in the table are the percent of the positive cells as detected by flow cytometry. Data are expressed as median values of 4 donors.

natural killer (NK) cells, over the time course studied.

Next, we compared the phenotype of *Leishmania*-naïve cells freshly isolated from donors to cells that had been exposed to the parasite for seven days (Table 1.2). Similar to the results found in the time course study, we detected few differences between the populations. In this case, we measured the percent of CD4+ and CD8+ cells that were in a CD3+ gate and found few differences. The percentages of CD56 and CD19 were not different between the sample groups. The only difference detected was when we compared the HLA class II expression of the two populations. The median of the *L. major* naïve cells was 7% (range 5-17.3%) and for the cells exposed to *L. major*, the median was 13.2% (range 6.3-34.1).

Cytokine production of L. major-primed cells upon restimulation with the parasite

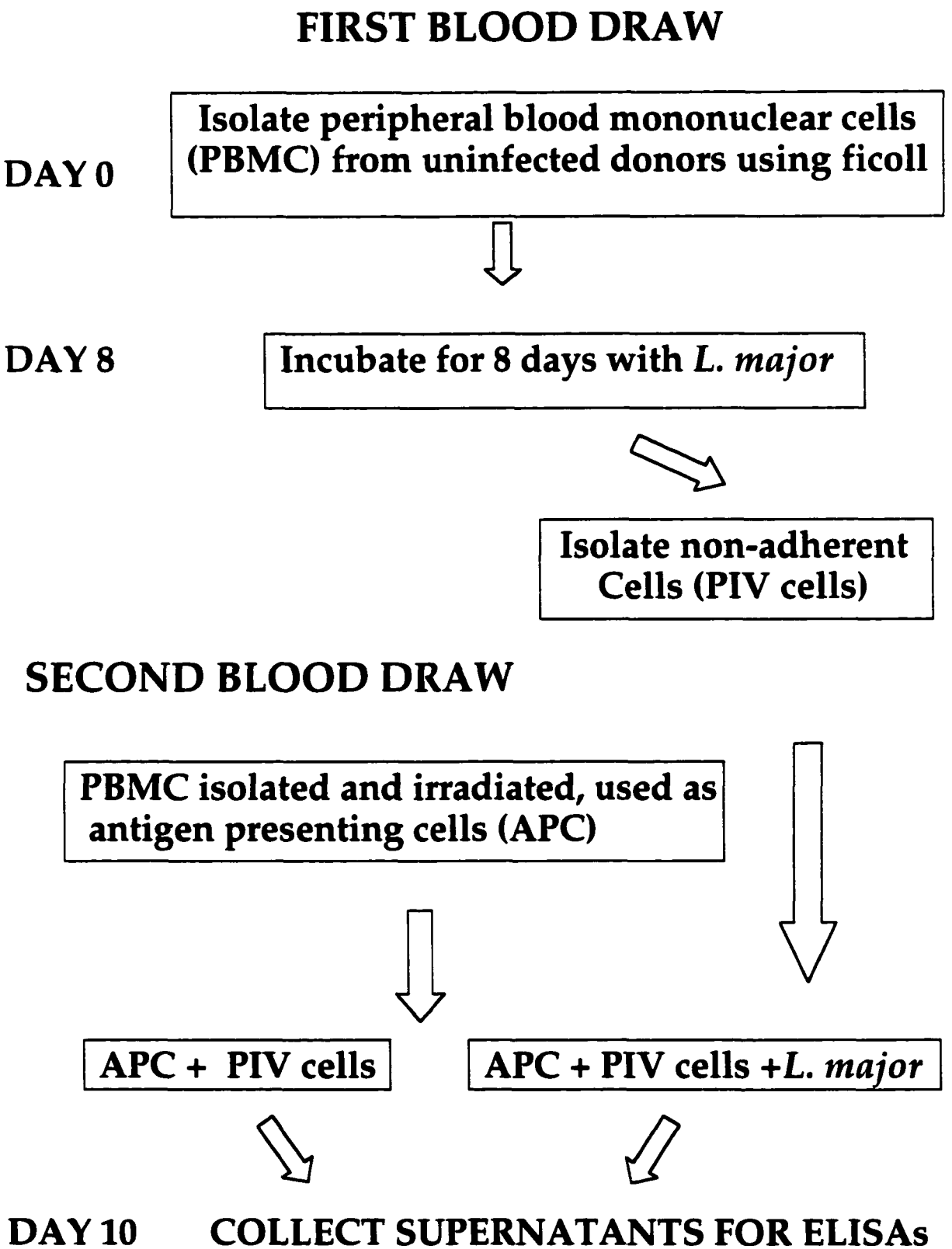
To determine whether antigen-specific cells had been induced during the priming period, we first exposed PBMC from *Leishmania*-naïve donors to the parasite for 8 days (Figure 1.3), then collected blood from the same donor and irradiated the PBMC to provide a source of antigen presenting cells (APC). The eight-day primary in vitro (PIV), parasite-exposed cells were then harvested and re-exposed to *L. major* and autologous APC and the supernatants sampled at 48 hr were measured by ELISA. We measured IFN-γ and IL-12 as representatives of Type 1 cytokines and IL-5, a Type 2 cytokine. Upon restimulation, all of the donors were able to produce IL-5 (Figure 1.4), albeit most donors at very low levels (<100 pg/ml). The differences between the controls (APC+PIV cells) and the restimulated cells (APC+PIV+*L. major*) are statistically significant

Table 1.2 Comparison o f the percent of *L. major*-naïve cells freshly drawn from donors and PBMC stimulated with *L. major* for 7 days stained for phenotypic markers, as evaluated by flow cytometry*.

Marker	*L. major* naive	*L. major* +
CD4 (Median)	49.8	52.9
Range	35.9-59.3	34.9-64.3
CD8 (Median)	21.4	28.0
Range	17.4-32.3	22.3-44.6
CD56 (Median)	17.4	17.2
Range	8.0-23.4	10.1-24.5
CD19 (Median)	11.0	12.8
Range	7.2-14.4	11.1-20.4
HLA Cl II (Median)	7.0	13.2
Range	5.0-17.3	6.3-34.1

*Data are expressed as median percent of positive cells on the first line and the range of percentages of positive cells on the second line. N=8.

Figure 1.3 Methods for in vitro stimulation



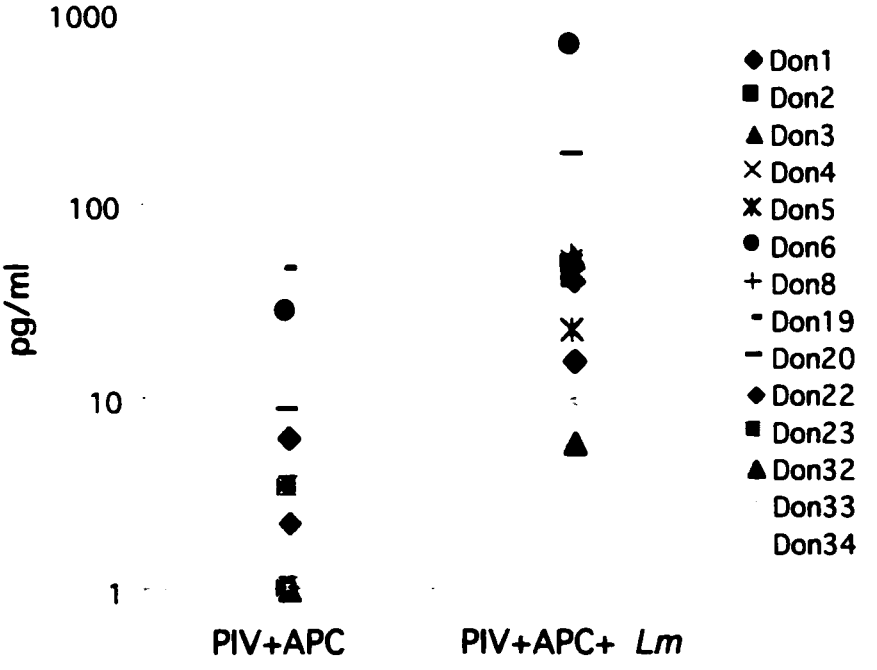


Figure 1.4 IL-5 production by *L. major*-primed cells as measured via ELISA. Results are expressed as means. For clarity, deviation bars are not shown. N=14. The PIV+APC+*Lm* treatment group is statistically different from the PIV+APC control ($p<0.001$)

(P<0.001). Two of the donors (Donor 6 and 20) were very high IL-5 producers.

We then determined the amount of IL-12 production as the primed cells were exposed a second time to the parasite (Figure1.5). As in the early time point study, the cells produced IL-12. However, in this experiment there was a significant difference in IL-12 production when the control samples and the experimental samples were compared (P<0.001). Two donors (Donor 5 and 33) produced twice as much IL-12 compared to the other donors stimulated with the parasite. Although not tested, the IL-12 is most likely being produced by macrophages and dendritic cells present in the cultures.

Most striking, however, when PIV cells were restimulated with the parasite, all donors secreted high amounts of IFN-γ (Figure 1.6). The production of IFN-γ was statistically significant when compared to the controls (P<0.001). These results indicate that IFN-γ is most likely the principal cytokine produced in the cellular response to the parasite, even in the two donors that produce high levels of IL-5. These high IL-5 producers (Donors 6 and 20) also produced fairly high levels of IFN-γ (>15,000 pg/ml). In summary, each of the donors tested produced IL-5, IL-12 and IFN-γ.

Proliferation of L. major-primed cells upon restimulation with the parasite

After determining the production of cytokines in our system, we used a functional assay approach to determine the effects of the parasite on T cell responses to *L. major*. First, we needed to establish the conditions required for an optimal response to the parasite. As shown in Figure 1.7, for optimal proliferation, *L. major*-primed cells require a source of antigen presenting cells, as well as the parasite (far right column). This group responded in a statistically greater fashion than any other group tested (ie: PIV cells

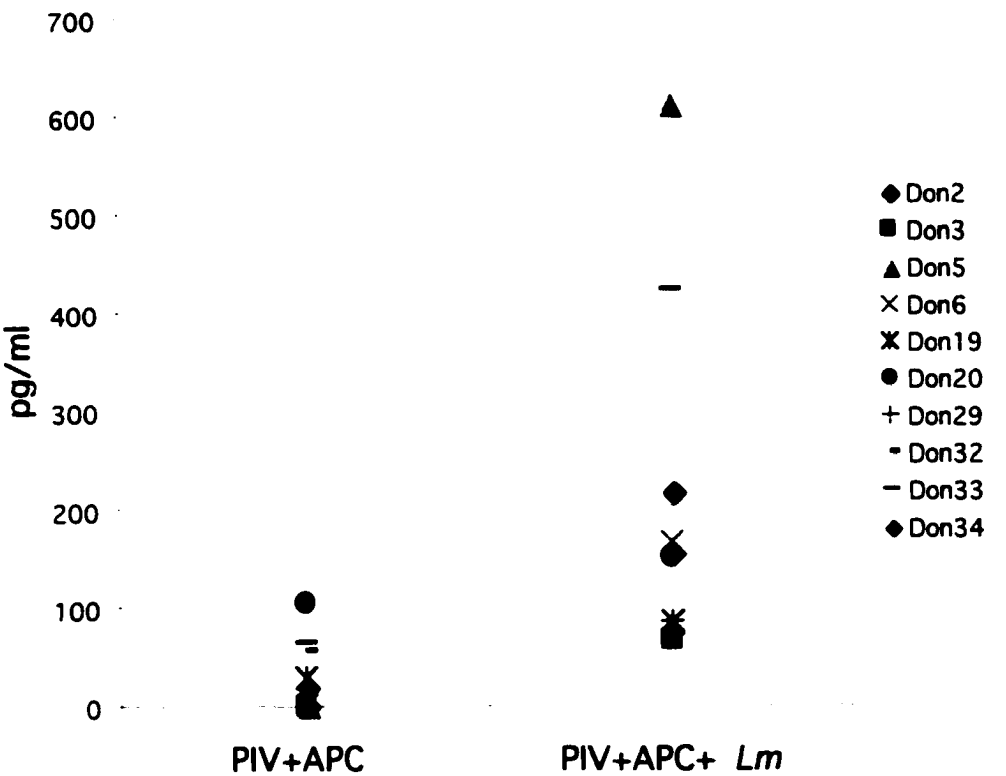


Figure 1.5 IL-12 production of *L. major*-primed PIV cells restimulated with the parasite. Data are expressed as means. For clarity, bars expressing deviation are not shown. N=10 PIV+APC+*Lm* treatment is significantly different from control ($p<0.001$).

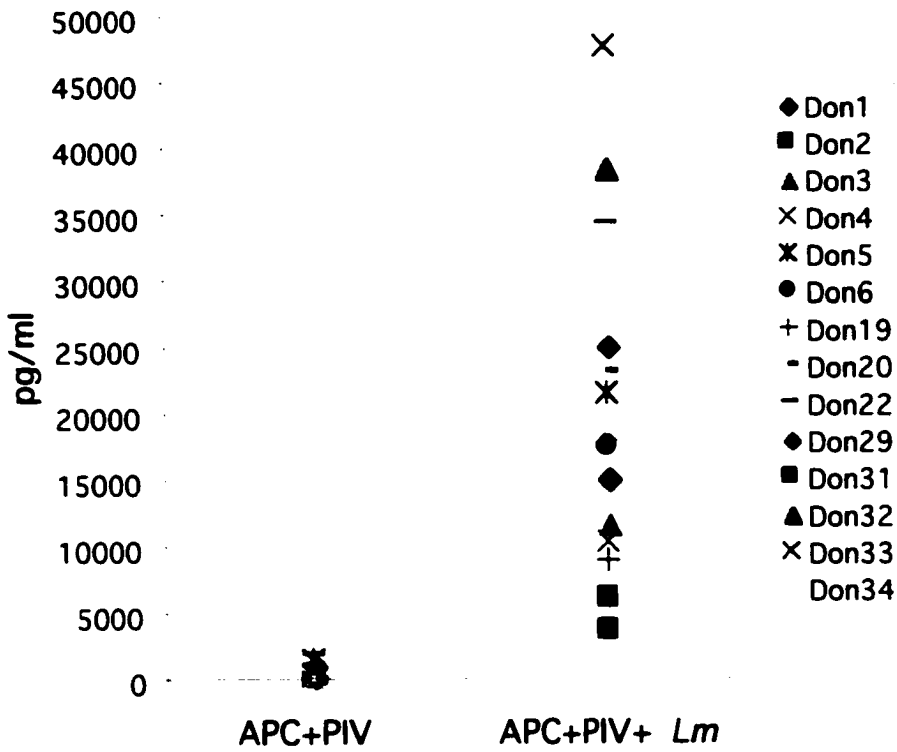


Figure 1.6 IFN-γ production of PIV cells restimulated with *L. major* as measured by ELISA. Results are expressed as means. For clarity, deviation bars are not shown. N=14. APC+PIV+*Lm* treatment group is statistically different compared to the control ($p<0.001$)

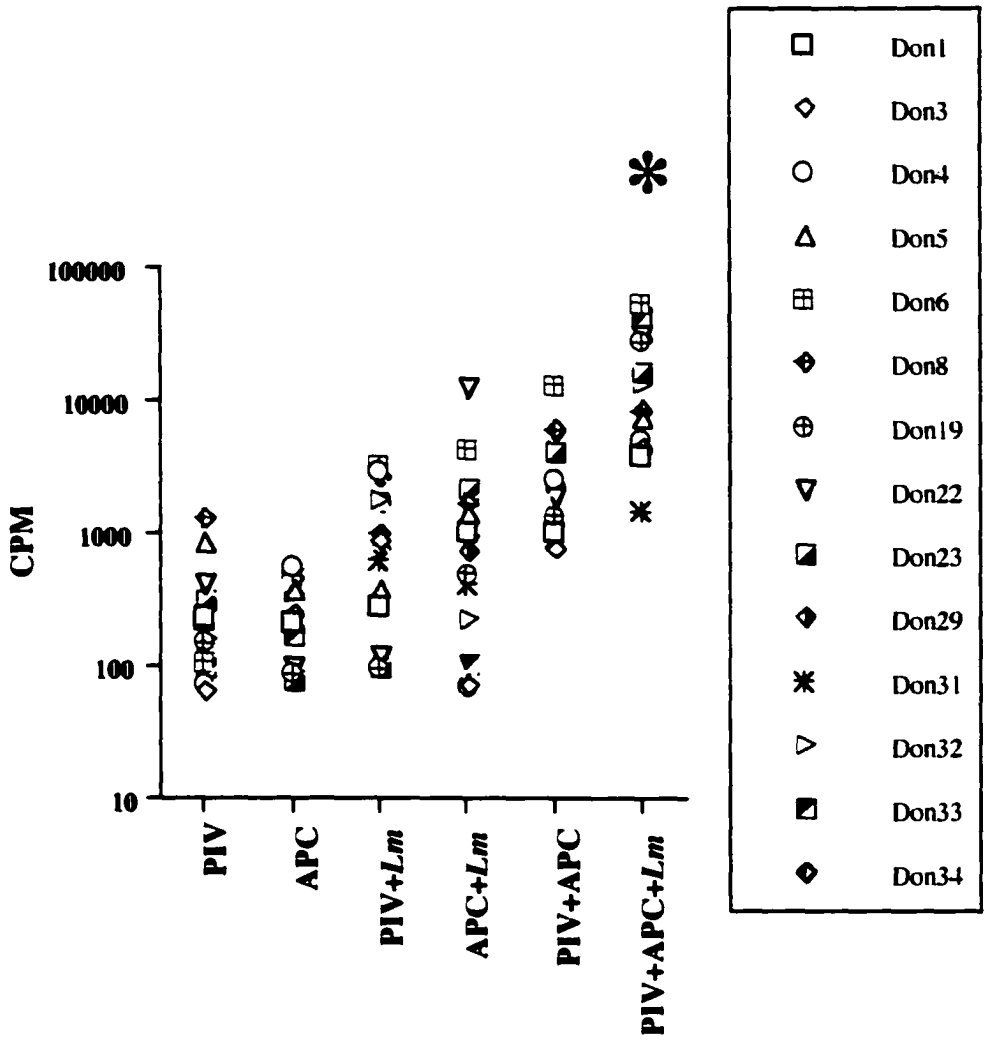


Figure 1.7 Measurement of ^{3}H-thymidine incorporation (counts per minute). Results represent the median of each donor tested. N=14. The '*' denotes the significant difference ($p<0.05$) between the PIV+APC+*L. major* group and all other groups.

alone, PIV cells + *L. major*, PIV cells + APC cells) ($p<0.05$).

Next, we wanted to determine if the parasite-primed cells would proliferate only in response to *L. major*, as has been reported in the literature (Shankar and Titus, 1993) or if the PIV cells would respond to other antigens as well. Figure 1.8 shows the proliferation of PIV cells when combined with autologous APC and then exposed to BCG, a bacteria whose lipoprotein coat can cause immunogenic responses, GP63, a recombinant protein that can be found on the outer surface of promastigote *Leishmania* and finally, ovalbumin (OVA), a non-specific protein with little cross-reactivity with leishmanial antigens. There is a trend of reduction of proliferation when cells are exposed to antigens other than *L. major*, however, due to the individual variation between donors, only the response to GP63 was statistically reduced compared to the PIV cells stimulated with *L. major* ($p<0.05$).

Finally, we wanted to determine if the proliferation response was specific to *L. major* or if the primed PIV cells would respond to other live *Leishmania* parasites or to *L. major* lysate (prepared by freezing and thawing the parasite). As shown in Figure 1.9, the PIV cells not only proliferated when restimulated with *L. major*, but also when stimulated with the Old World parasite *L. donovani* and the New world parasite *L. mexicana*, which causes cutaneous leishmaniasis as well. In contrast, there was a significant decrease in proliferation when the cells were exposed to *L. amazonensis*, which is a New World parasite, or *L. major* lysate.

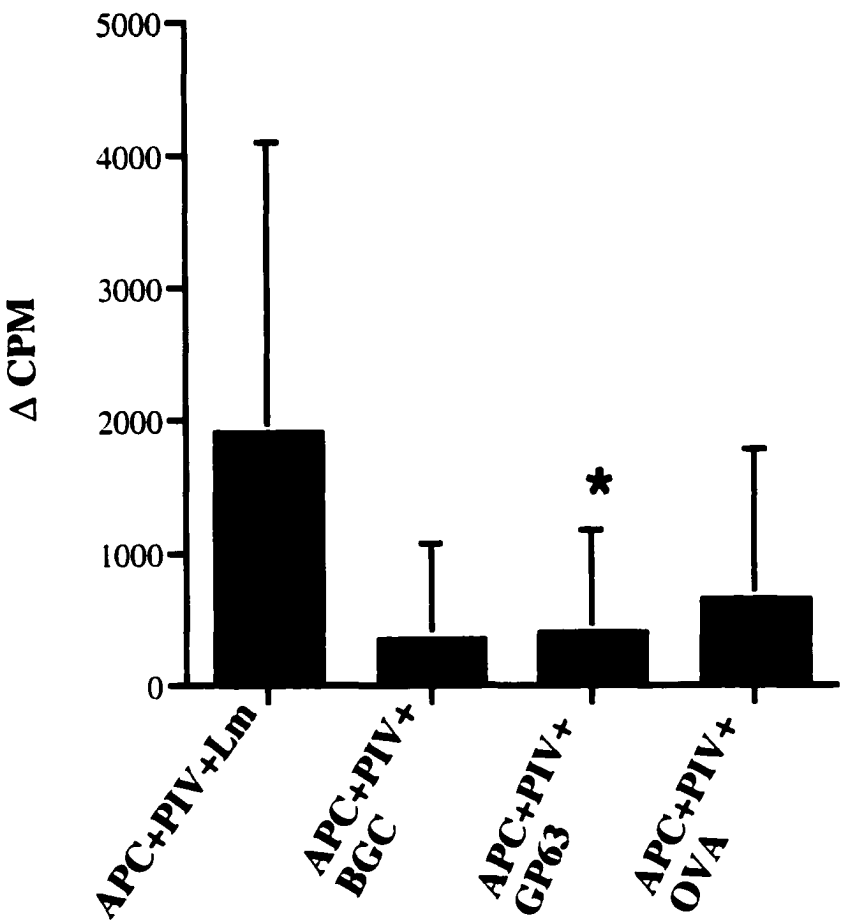


Figure 1.8 Proliferation, as measured by ^{3}H-Thymidine incorporation of *L.* major-primed PIV cells exposed to various antigenic stimuli. ΔCPM refers to the test condition CPM value once the background CPM (proliferation of APC+PIV) is subtracted. N=4. The * indicates a significant difference between the treatment and the APC+PIV+*Lm* control.

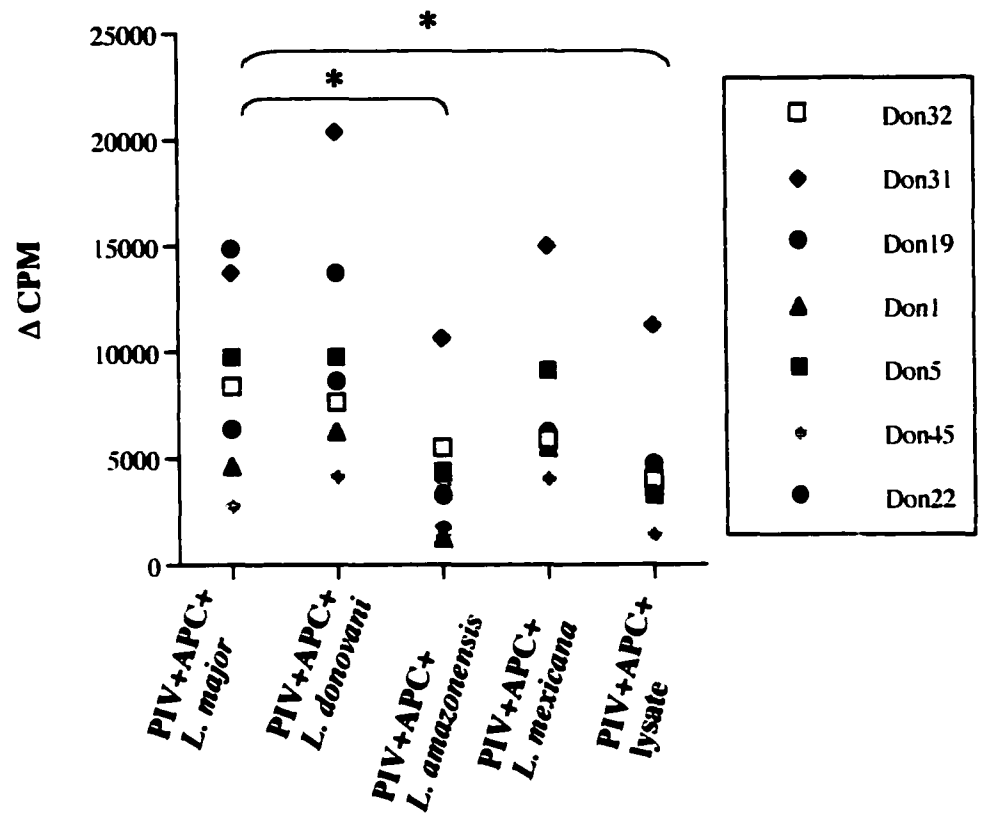


Figure 1.9 Measurement of ^{3}H-thymidine incorporation. Results represent the median of each donor tested. N=7. The lines and symbol '*' indicate significant differences ($p<0.05$) between the PIV+APC+*L. major* group and the other groups.

The phenotypic characterization of the PIV system

We wanted to determine what the population of the cells responding to *L. major* looked like phenotypically before we added the cells to restimulation experiments, as well as determine if the T cells were activated. We characterized the expression of a number of cell surface markers (CD4, CD8, CD56, CD19, HLA Class II and CD25) after the PIV cells had been exposed to the parasite for eight days. Control cells were cultured without the parasite. We chose to observe the cells at this time point and not later in culture, during the restimulation phase, due to concerns that we would not be able to differentiate between irradiated autologous PBMC and the PIV cells. Surprisingly, few differences were found in this study when we compared cells that had been in culture for eight days and either exposed to the parasite or not (Table 1.3), except for increased expression of HLA Class II (Figure 1.10) and CD25 (Figure 1.11) on the cell surface of the cells cultured with the parasite compared to the negative controls. The median percentage of control cells expressing HLA Class II was 12.9 (range 6.3-19.1%) and when the cells were exposed to the parasite, the percent of cells increased to 20% (range 6.6-29.5%). Similarly, the percent of CD25 expressing cells was 4.0% (range 1.4-7.2%) in the *L. major* -negative population and 10.3% (range 2.2-17.4%) in the parasite-positive population. We then wanted to further dissect the CD25+ population that appeared to be activated by *L. major*. A gate was drawn around either the *L. major*-stimulated or control CD4+ cells or the CD8+ cells and then the cells stained double-positive for CD4 or CD8 and CD25 were evaluated. We found that of the CD4+ control cells, 3.9% (median: range 2-7.4%) also expressed CD25+, while the median for the *L. major*-stimulated cells CD4+CD25+ cells was 11.1% (range 2.4-27.3%). When CD8+ cells were

Table 1.3 Phenotypic comparison of cell surface markers by flow cytometry from 7 day PIV cultures cells not exposed to *L. major* or cells exposed to the parasite.*

Marker	PIV *L. major* -	PIV *L. major* +
CD4 (Median)	51.4	49.5
Range	41.5-72.8	27.6-68.9
CD8 (Median)	15.2	13.6
Range	9.4-25.9	8.9-25.7
CD56 (Median)	10.1	12.1
Range	2.3-17.4	1.0-22.3
CD19 (Median)	5.1	5.3
Range	3.1-11.1	3.4-9.7
HLA Cl II (Median)	12.9	20.0
Range	6.3-19.1	6.6-29.5
CD25 (Median)	4.0	10.3
Range	1.4-7.2	2.2-17.4

*Data are expressed as the median percent of positive cells on the first line, and range of positive cells on the second line of the chart. N=8

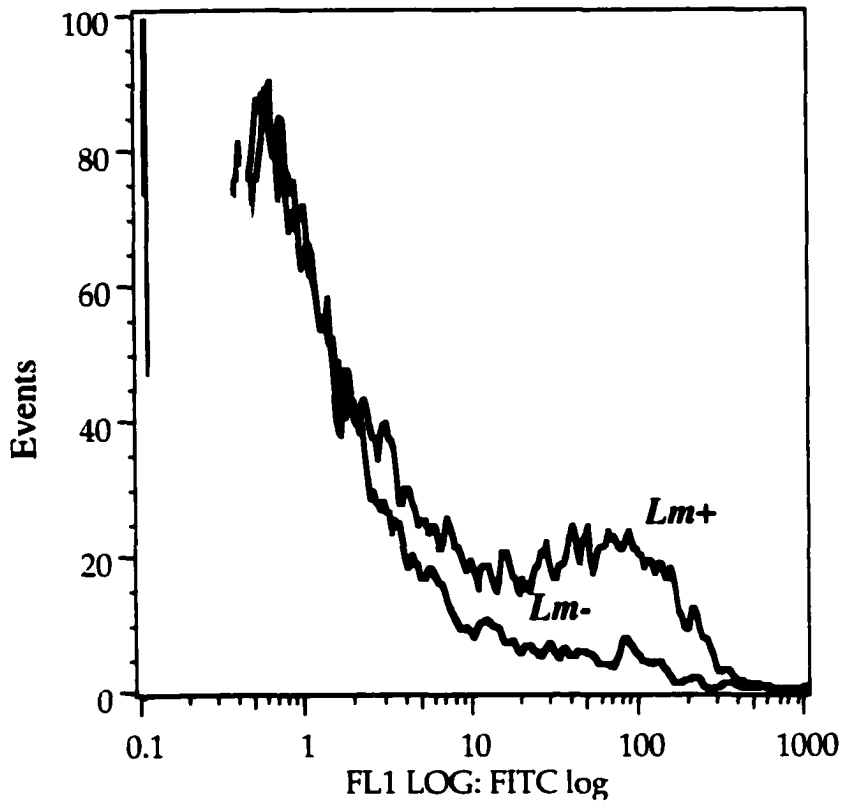


Figure 1.10 Expression of HLA CL II on PIV cells. Cells were either exposed to *L. major* (red line) or not (blue line) for 8 days and then stained for HLA CL 2. The shaded histogram represents nonspecific fluorescence of cells stained with an isotype control antibody. Data presented are representative of eight individual donors.

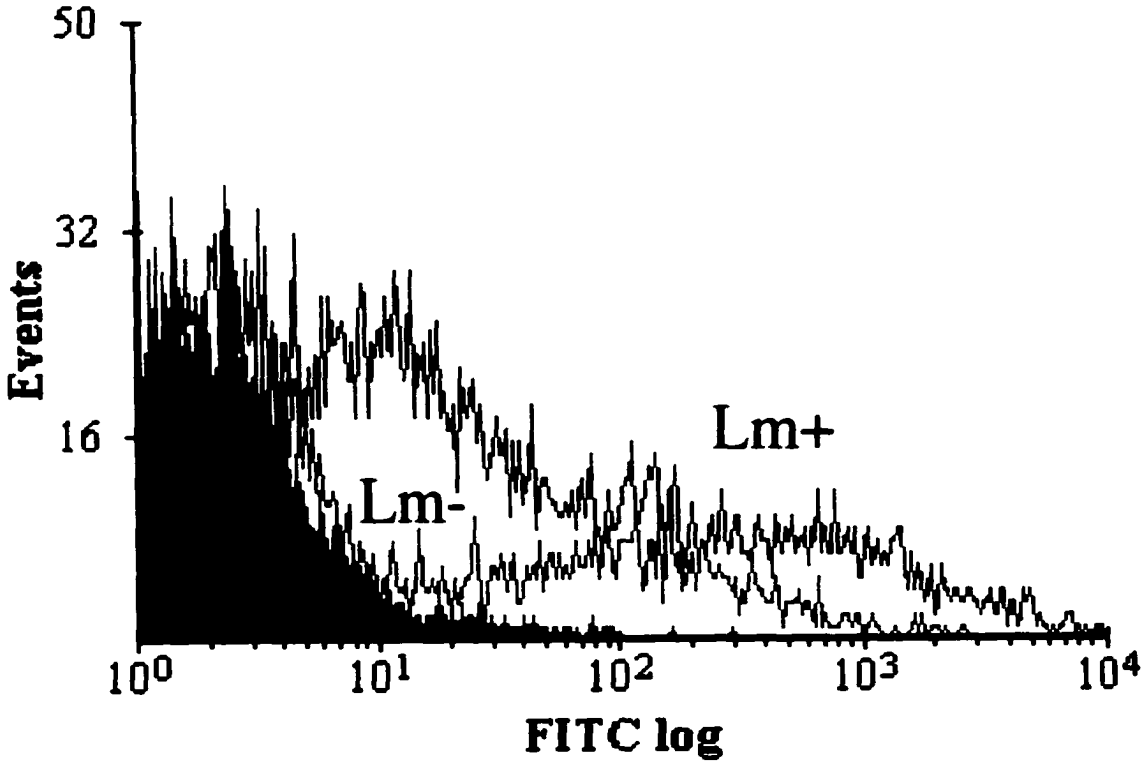


Figure 1.11 Expression of CD25 on lymphocytes. Cells were either exposed to *L. major* (blue line) or not (red line) for 8 days and then stained for CD25. The shaded histogram represents nonspecific fluorescence of cells stained with an isotype control antibody. Data presented are representative of eight individual donors.

measured, 1.3% (range 0.02-9.0%) of the control cells were CD25+. In contrast, 13.8% (range 2-37.5) of the *L. major*-stimulated cells were double positive for CD8+ and CD25. These results suggest that both CD4 and CD8+ T cells are being activated in our system.

The influence of CD4+ and CD8+ cells in the PIV response

Although it has been reported that CD4+ cells are the primary responding cell population and that the response to the parasite is MHC class II-restricted in the murine PIV system (Shankar and Titus, 1993), we wanted to determine the cells primarily influencing the response in the human PIV system, as well as to elucidate if antigen presentation is class I or class II restricted in our system. To this end, we first exposed *L. major*-naïve donor cells to the parasite for 8 days. Upon harvesting the PIV cells, we then depleted either CD4+ or CD8+ cells (purity ranged from 87-95%) and then cultured the remaining PIV cells with irradiated, autologous APC and in the presence or absence of *L. major*. IFN-γ production was then measured to determine if depleting CD4 or CD8+ cells affected the ability of the cell populations to produce IFN-γ. As Figure 1.12 indicates, cultures depleted of either CD4 or CD8+ cells are still capable of producing IFN-γ, suggesting that both of these cell populations contribute to the secretion of this cytokine.

To determine through which MHC pathway the PIV cells present *L. major* antigens, blocking antibodies against HLA Class II or HLA Class I were added to the cultures upon restimulation. Proliferation of the PIV cells was measured. The difference in proliferation between PIV cells not blocked with antibody and cells blocked with anti-HLA Class II antibody was not statistically different, although there was a tendency

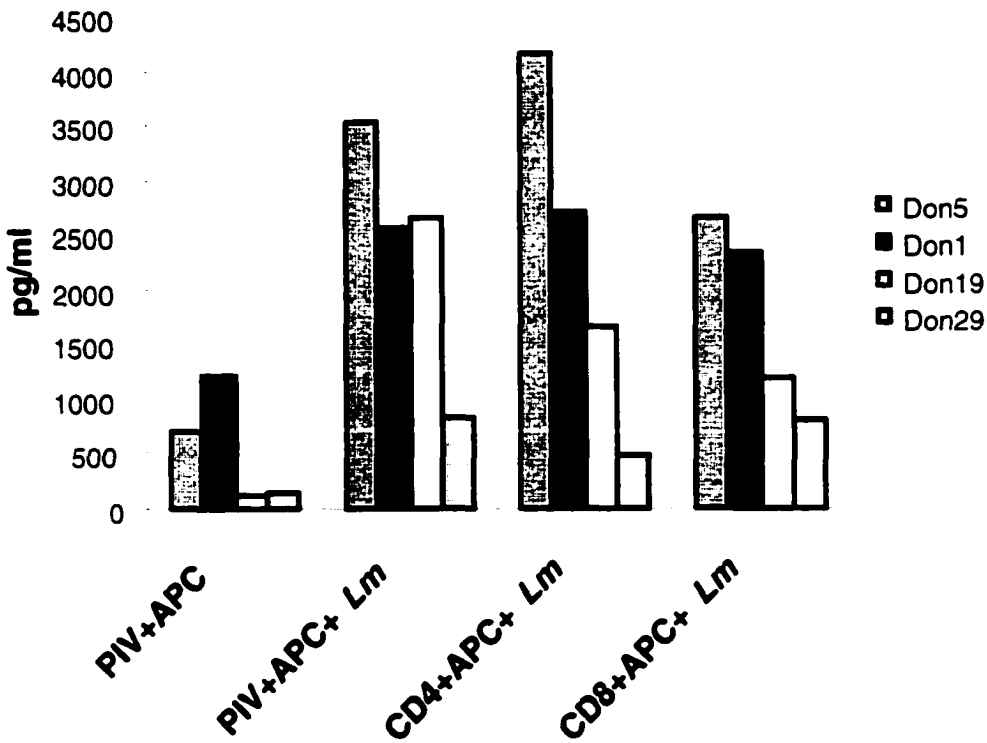


Figure 1.12 IFN-γ production by *L. major*-primed cells depleted of either CD4+ cells or CD8+ cells. There is no significant difference between the whole PIV population and the populations depleted of either CD4+ cells or CD8+ cells. Each of the cell populations produces significantly more IFN-γ ($P<0.05$) than the control (PIV+APC).

for reduced proliferation (Figure 1.13). However, cells exposed to anti-HLA Class I antibody proliferated significantly less than cells without the blocking antibody ($p<0.001$). There was no tendency for suppression of proliferation when an isotype control (IgG2a) for the antibodies was added to *L. major*-stimulated cultures.

We also wanted to determine the effects of blocking the HLA class I and class II pathways early after exposure to the parasite. Since the PIV cells do not proliferate significantly early after exposure to the parasite (<7 days) (data not shown), we decided to measure IFN-γ before the restimulation period as an indicator to how blocking antigen presentation may be affect the PBMC exposed to the parasite. In these experiments, PBMC were isolated from the donors and then cultured with the blocking antibody and parasite and then the supernatants measured after 3 (data not shown) and 7 days. Control wells did not contain *L. major*. Interestingly, when IFN-γ production of PIV cells exposed to the blocking HLA class II antibody was measured, the production of IFN-γ was significantly decreased ($p<0.01$) compared to cells exposed to the parasite (Figure 1.14a). When the class I pathway was blocked, the production of IFN-γ was also significantly decreased ($p<0.05$) as PIV cells were exposed to the *L. major* (1.14b). These findings suggest that not only are CD4+ cells important in responding to *L. major*, but CD8+ cells are also activated during parasite exposure. In order to determine if cytokine production is affected when the cells are stimulated in a non-HLA-dependent manner, PIV cells were stimulated with PHA (2 μg/ml; Sigma) and neutralizing HLA class II antibody was added to the cultures. There were no differences between the samples when IFN-γ was measured, suggesting that the neutralizing antibodies are

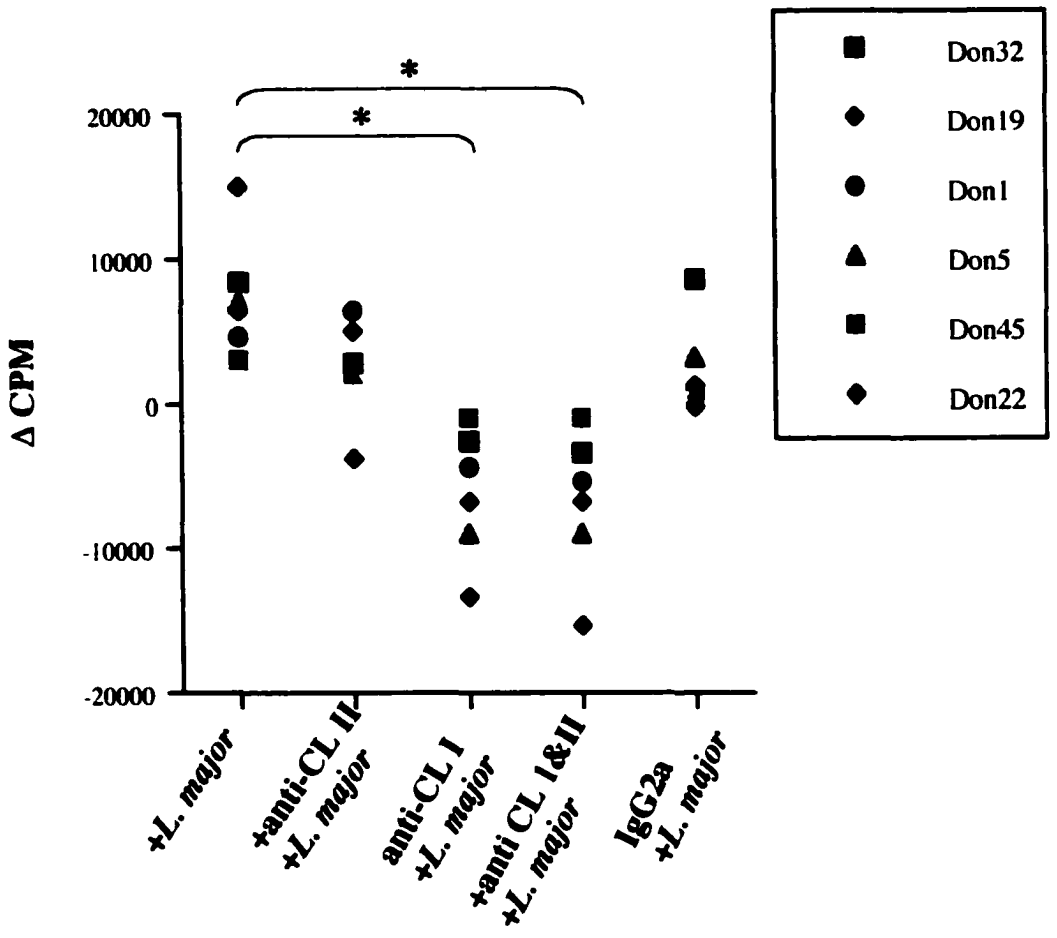


Figure 1.13 Proliferation of *L. major*-primed PIV cells. Cell populations With lines connecting them and marked with the symbol "*" indicate a significant suppression ($p<0.001$) of proliferation between cells exposed to only the parasite and cells exposed to blocking antibodies.

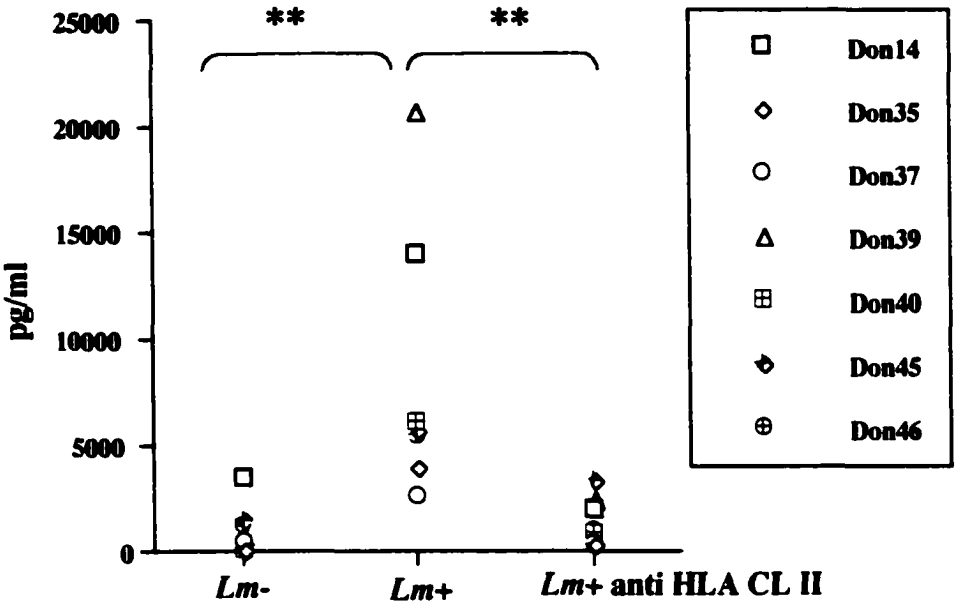


Figure 1.14a IFN-γ production is significantly decreased ($p<0.01$) in *L. major* exposed PIV cells where the HLA class II pathway has been blocked. The lines between groups indicate that the treatments resulted in significant differences in cytokine production. The symbol '**' indicated that $p<0.01$.

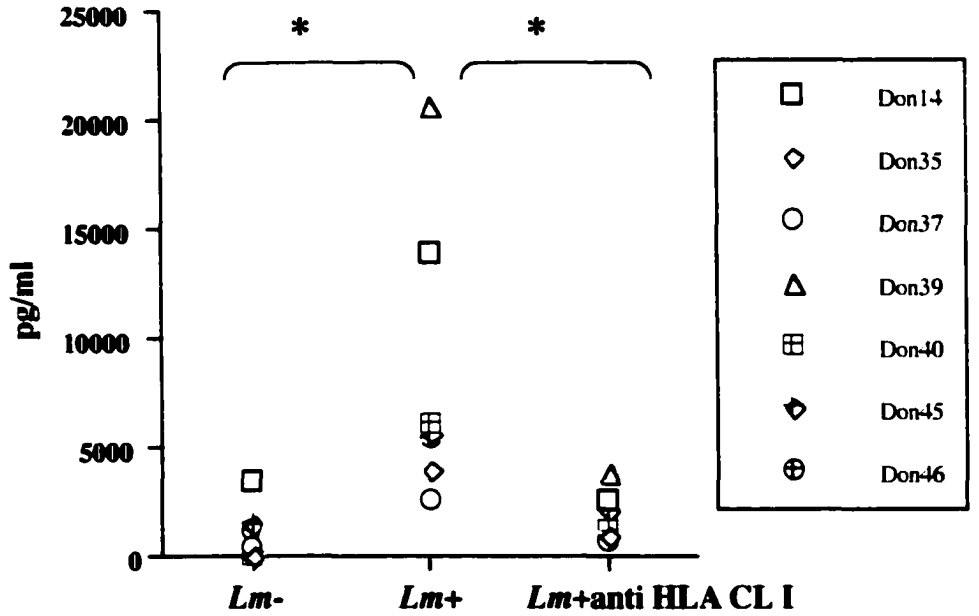


Figure 1.14b IFN-γ production is significantly reduced ($p<0.01$) in *L. major* exposed PIV cells where the HLA class I pathway has been blocked. The lines between groups indicate that the treatments resulted in significant differences in cytokine production. The symbol '*' indicated that $p<0.05$. The non-specific inhibition of the HLA-CL I antibody (15.9%) has been subtracted from the results.

interacting with HLA class II specifically and are not inhibiting IFN-γ production non-specifically (data not shown). On the other hand, when PHA stimulated cells were blocked with HLA class I, there was a 15.9% decrease of IFN-γ production, indicating the antibody is partially inhibiting IFN-γ secretion in a non-specific manner (data not shown). The data in Figure 1.14b have been adjusted to reflect this non-specific inhibition.

The ability of macrophages to kill parasites during infection

We wanted to establish if the parasites are being killed in our system. As it has been shown that macrophages play an integral role in harboring and killing the parasite, we wanted to analyze the mechanism by which human macrophages most efficiently kill *L. major*. We first tested the ability of the macrophages to become infected with the parasite (Figure 1.15a). Overall, the infection rates of the macrophages ranged from 10-50%. We then determined the ability of the macrophages to eradicate the parasite (Figure 1.15b). We found in our hands that the macrophages were extremely efficient at killing *L. major*. There was a significant reduction in parasite numbers between 48 and 72hr post exposure to the parasite ($p<0.01$) as well as a marked decrease in intracellular parasites at 72 hr compared to 24 hr post-exposure to the parasite ($p<0.001$). The macrophages were able to efficiently kill the parasite without additional activation, such as the addition of IFN-γ and LPS (data not shown). The addition of conditioned medium (collected from 8 day PIV cells), which contains all of the products PIV cells secrete while exposed to the parasite, did not influence the ability of the macrophages to destroy the parasite (data not shown).

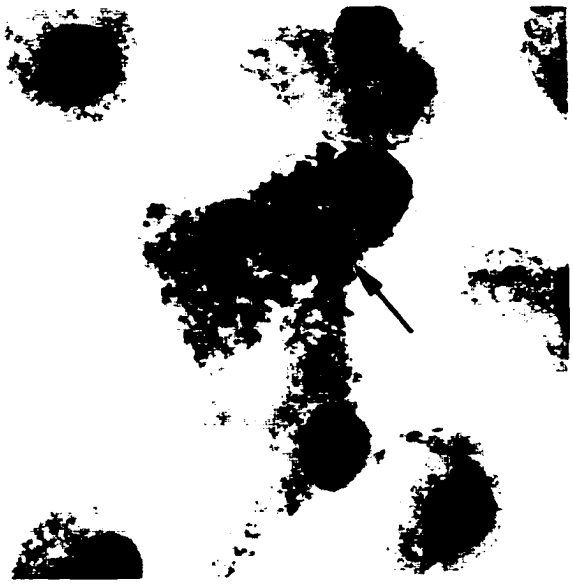


Figure 1.15a *L. major*-infected macrophage. Arrow indicates one of numerous parasites within the cell

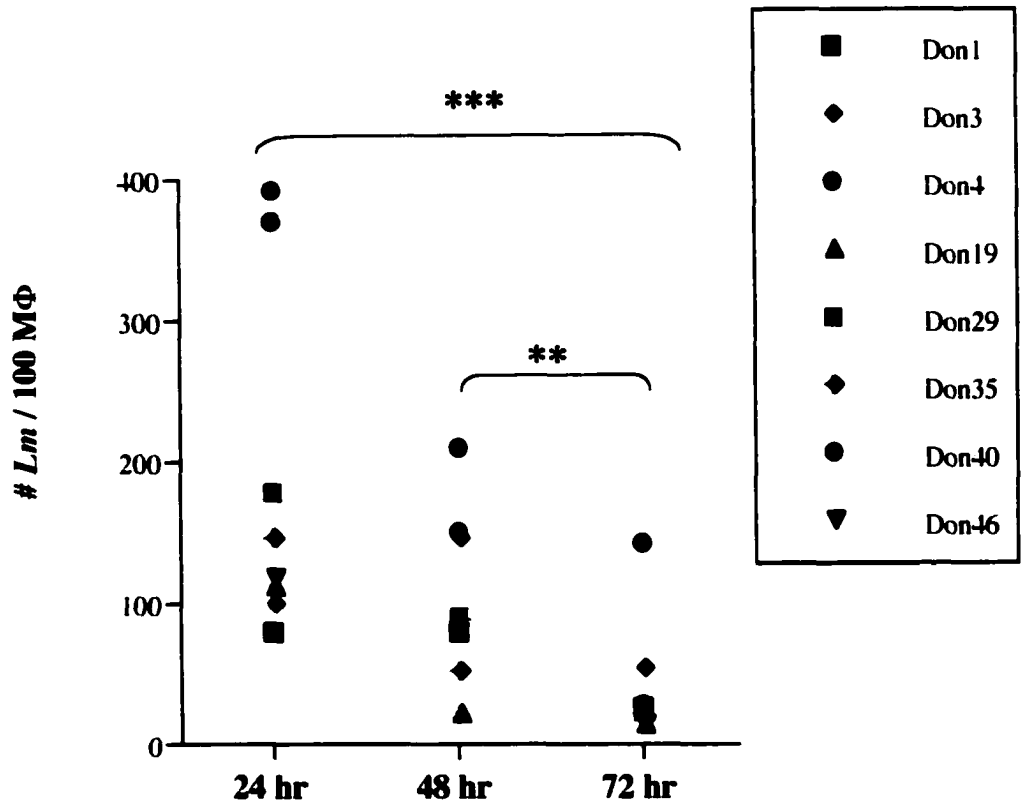


Figure 1.15b Macrophages eradicate *L. major* over time. The number of intact parasites contained within 100 macrophages was counted. The lines between groups indicate that the treatments resulted in significant differences in the number of parasites present in a macrophage. The symbol '**' indicates that $p<0.01$; the symbol "***' indicates that $p<0.001$.

In order to better model an in vivo system, we looked at the interactions of *L. major*-primed PIV cells and infected macrophages. We also wanted to determine if the interactions between the PIV cells and macrophages were dependent upon IFN-γ or cell to cell contact. Since macrophages appear to be able to effectively control the parasite at later time points, we decided to investigate the interactions of PIV cells and macrophages 8 hours after the macrophages had been stimulated by the parasite. Observation by light microscopy indicated that parasites were indeed within the macrophages by this time point. The macrophages were then exposed to PIV cells that had been exposed to *L. major* for 8 days and either conditioned medium collected from the PIV cultures in a ratio of 1:1 with fresh medium, or with anti-IFN-γ antibody, and the cultures were allowed to incubate for an additional 18 hr. When the macrophages, which were adhered to coverslips, were counted, it was determined that cultures to which PIV cells had been added had fewer parasites within the macrophages compared to macrophage cultures grown without T cells. Figures 1.16a and b demonstrate the potential importance of contact between PIV lymphocytes and macrophages in order to maximize parasite killing. The influence of T cells in efficient killing of *L. major* is shown in Figure 1.16b. There was no significant difference in the numbers of parasites when either medium or conditioned medium was added to the cultures. However, there was a striking decrease in parasite load when PIV cells were added to the cultures ($p<0.05$). The addition of a neutralizing IFN-γ monoclonal antibody did not abrogate the killing of the parasite, suggesting that cell-cell contact and activation, such as through co-stimulatory molecules is needed for the most efficient and quickest eradication of *L. major*.

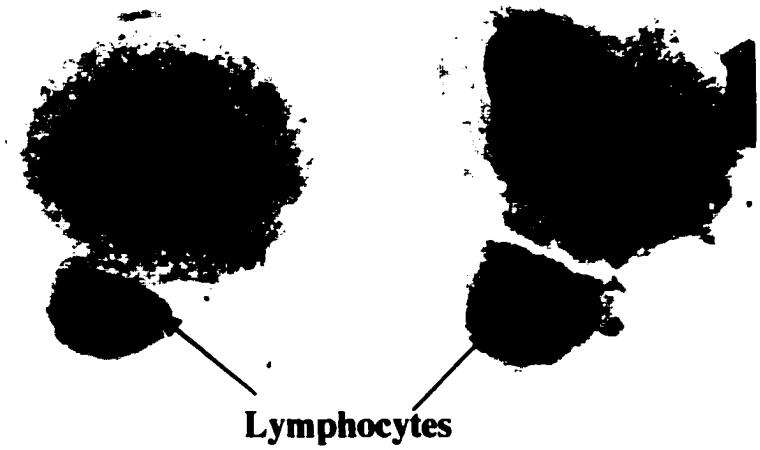


Figure 1.16a PIV lymphocytes interacting with macrophages exposed to *L. major*

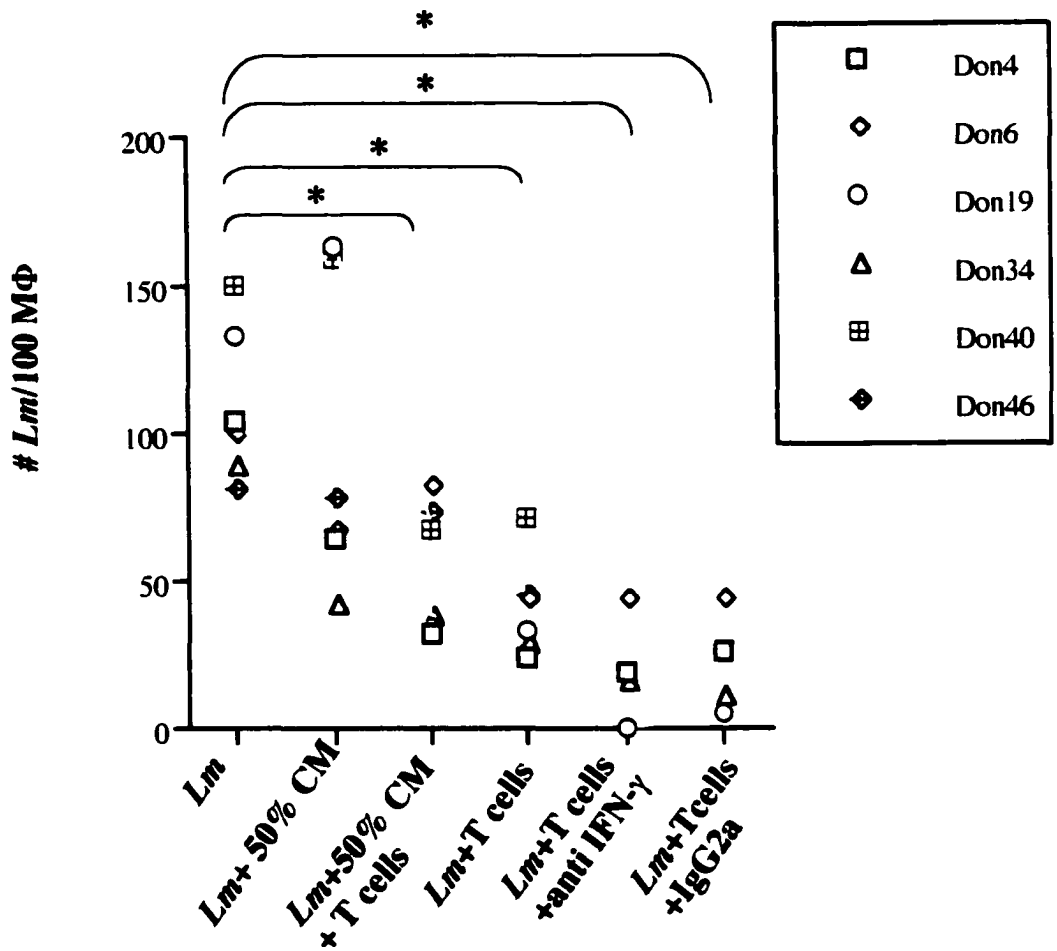


Figure 1.16b Macrophages can more efficiently eradicate *L. major* in the presence of PIV cells. The lines between groups indicate the treatments that cause significantly reduced numbers of parasites within macrophages compared to infected macrophages without further treatment. The symbol '*' indicates $p<0.05$.

DISCUSSION

In this study we developed and characterized a primary in vitro system using PBMC from *Leishmania*-naïve donors. Others have used cell lines and T cell clones developed from naïve donors (Russo et al., 1998; Russo et al., 1999; Raja Gabaglia et al., 2000; Probst et al., 2001) or different culturing methods, where macrophages were the only type antigen presenting cell present (Brodskyn et al., 2000) to study *Leishmania* interactions in vitro with human cells. We chose to pattern our system after a PIV model developed by Shankar and Titus (1993), which used splenocytes from *Leishmania*-naïve mice and primed the cells to *L. major*. When the murine PIV system was characterized, it was quite comparable to results reported in murine *Leishmania* infections (Shankar and Titus, 1993; Soares et al., 1997).

We hypothesized that our PIV system would result in *L. major*-specific, CD4+ and HLA- Class II restricted T cells that would produce IFN-γ. Our results indicate that human PBMC react to the parasite differently in several ways compared to results reported in the murine PIV system. These differences include cross reactivity in the PIV system with other *Leishmania* species and the apparent ability of not only CD4+ T cells, but also CD8+ T cells to recognize parasite antigens. In addition, both HLA class I and class II antigen presentation appear to play a role in the response to *Leishmania* antigens.

Not all humans who are exposed to *Leishmania* develop clinical infections. In fact, in endemic areas of the world, a significant number of individuals in the populations can harbor subclinical infections which may only become apparent upon immunosuppression, such as during HIV infection (Altages, 1991; Handman, 2001). Many people can heal cutaneous leishmaniasis without additional treatment (Herwaldt,

1999; Handman, 2001), however, the genetic make-up and immunocompetence of the infected individual are crucial in determining the outcome of infection.

As there is little information reported about the early immune response of humans when exposed to *L. major*, we wanted to develop a model where we would be able to study the parasite interactions with human cells. This approach leads to its own inherent problems, since there is fairly wide genetic diversity and daily individual variation in a donor's immunological status. Nonetheless, priming *Leishmania*-naïve PBMC with the parasite and studying the early interactions, as well as the immune response upon restimulation with the parasite has resulted in some useful findings.

First, we characterized the response of *Leishmania*-naïve PBMC to the parasite early after exposure. We evaluated the response both via cytokine production of Type 1 (IL-12 and IFN-γ) and Type 2 (IL-5) cytokines and the phenotypic expression of cell markers in order to monitor the effects the interactions were having on the cell populations present. It has been demonstrated in the mouse model that early interactions between the parasite, host cells and their resultant cytokine production leads to a Th1 or Th2 commitment and that once committed, this response is difficult to reverse (Chatelain et al., 1992; Mocci and Coffman, 1997). Although IL-4 is detectable in the murine model, it is difficult to detect this cytokine in human cell cultures without stimulating with phorbol ester and calcium ionophore (Kurtzhals et al., 1992; Palmer and van Seventer, 1997). Thus, we decided to evaluate IL-5, which is also a Th2 cytokine. This cytokine is found in humans in a "Th2 gene cluster", which consists of genes capable of regulating IL-4, IL-13, and IL-5, on chromosome 11 (Loots et al., 2000). These cytokines are co-activated in a Th2 response (Abbas et al., 1996). We found that we

could detect only Type 1 cytokines in the first 3 days after exposure to the parasite (Figures 1.1 and 2). We could not detect IL-5. As many individuals are capable of controlling infection without therapeutic interventions, our finding is not surprising.

In resistant mice, lymph node cells are able to bind IL-12 just two days after infections and drive a Type 1 response (Jones et al., 1998). Recently, Quinones et al. (2000) reported that macrophages and dendritic cells have membrane-associated stores of IL-12 which are released after only minutes of contract between the cells and *Leishmania donovani*. Our data suggests that in our PIV system IL-12 is also driving a Th1 response, as we could detect IL-12 shortly after exposure to the parasite. It is also possible, although it was not measured, that IL-18 could also be polarizing cells toward a Th1 response. IL-18 is an IFN-γ-inducing cytokine and acts in synergy with IL-12 to enhance IFN-γ production of T cells and NK cells (Okamura et al., 1995; Hyodo et al., 1999; Akira, 2000).

We were also able to detect IFN-γ early after exposure to *L. major*. IFN-γ is necessary to eradicate the parasite by activating macrophages to become microbicidal. This finding is similar to that reported by Soares et al. (1997) using the murine PIV system. They found that the splenocytes of resistant mice produced substantially more IFN-γ than cells from susceptible mice. As all of the donors produced this cytokine, we theorize that the donors in our study would be able to successfully control *Leishmania* infections. Interestingly, in contrast to results reported in the murine literature (reviewed in Lohoff et al., 1998), the Th1 cytokine pattern in human T cells is fixed; once the cells are committed to a Type 1 response, it is difficult to convert them to Th2 cells (Sornasse et al., 1996). Bradley et al. (1996) report that IFN-γ is capable of directly regulating

Type 1 responses in naïve cells, even in the absence of IL-12. IFN-γ has also been reported to be essential in maintaining a Type 1 response during *Leishmania* infections (Scott, 1991). These previously reported results suggest that in our system, the high production of IFN-γ, as well as the increased IL-12 production, is contributing to a positive feed-back loop for an enhanced Type 1 response. For instance, when IL-12 is secreted by antigen presenting cells, the cytokine can up-regulate IFN-γ production by NK cells and T cells and in turn the secreted IFN-γ will stimulate macrophages to eradicate intracellular pathogens (Melby et al., 1996; Akuffo et al.,1999).

Phenotypically, there were few changes in the cell populations present in the PIV system early after exposure to the parasite. We speculate that since cytokine production is just beginning to occur (at less than 7 days) and both populations of cells (*L. major* – and *L. major*+) are adapting to an in vitro environment, that the conditions are not favorable for changes in the make-up of the cell population. Indeed, when proliferation assays were performed on PIV cells less than 7 days after stimulation with *L. major*, there was little proliferation (data not shown). As the cells were stimulated with the parasite over time and cytokine production increased, then the populations of cells began to differentiate. For the most part, studies in human leishmaniasis which evaluate changes in cell population profiles generally use patients who have been infected for some period of time (Pimez et al., 1990; Coutinho et al., 1998; Ajdary et al., 2000) or observe populations after they have been restimulated with the parasite (Raja Gabaglia et al., 2000, Brodskyn et al., 2001), which could explain why we saw little phenotypic change compared to these studies.

When we compared populations of freshly isolated cells or cells cultured for the whole priming period without the parasite to cultures where the parasite was present, we noted an increase in HLA-class II expression in the *Leishmania*-stimulated cells (Tables 1.2 and 3; Figs. 1.10 and 11). Cells which could be expressing class II include dendritic cells, macrophages, B cells and activated T cells. We also noted an increase in CD25+ staining cells. Since both CD4 and CD8 cells expressed CD25, we suggest that both types of lymphocytes are being activated in our system. The observation that HLA-class II expression increases in *L. major*- exposed cells also suggests that cells are activated in our system.

In humans, IFN-γ can induce activated T cells to express increased HLA Class II levels (Hurme and Sihvola,1989), which could be the reason we saw increased expression of HLA Class II after *Leishmania* priming. There are mixed reports on the ability of *Leishmania sp.* to down-regulate class II expression on antigen presenting cells. Several studies have shown that the parasites can interfere with ability of antigen presenting cells to properly process and present antigen (Reiner et al., 1987; Fruth et al., 1993; Kima et al., 1997). When the interactions between MHC Class II knock out mice and the parasite were investigated, the mice were unable to resolve the infections, again pointing to the importance of MHC class II in the immune response against *L. major* (Chakkalath et al., 1995). Other laboratories have reported the ability of *Leishmania*-infected APC to continue to express class II in the mouse model (Flohe et al., 1997; Lang et al., 2000) and in infected human patients (El Hassan et al., 1995). As our macrophage data suggest that human APC are capable of eradicating the parasite quickly in our system, it is possible that *L. major* is unable to down-regulate HLA class II since the parasite is being killed so

quickly. As the IFN-γ has been shown to cause increased expression of HLA class II (Steimle et al., 1994), it is likely that the IFN-γ in the *L. major*-primed cultures is also causing increased expression of HLA-class II in the human PIV system.

Next, we wanted to characterize our system after the *L. major*-naïve cells were restimulated with the parasite. In the murine PIV system, *L. major*-primed PIV cells derived from splenocytes of uninfected mice were reported as *L. major*-specific, only proliferating in response to live *L. major*, and had fairly little cross-reactivity to other *Leishmania sp* (Shankar and Titus, 1993). The PIV cells did not proliferate in response to the *Leishmania* surface protein gp63, nor to BCG. These cells were CD4+, MHC class II restricted and produced IL-4 and IFN-γ. Further investigation of this system (Soares et al., 1997) revealed that PIV cells from resistant CBA mice produced more IFN-γ than cells from *Leishmania*-susceptible BALB/c mice, whereas the PIV cells from BALB/c mice produced more IL-5 than resistant mice. Interestingly, in this report, no correlation between IL-12 production and development of a Th1 or Th2 response could be determined, in contrast to studies demonstrating the importance of IL-12 in in vivo systems (Jones et al., 1998, Park and Scott, 2001).

In our system, we first evaluated the production of IFN-γ, IL-12 and IL-5 of *Leishmania*-naïve human PIV cells that were then restimulated with the parasite (Figs. 1.4-1.6). In all cases, the donors tested were able to produce each of the cytokines. When we measured IL-5 levels, two donors (Donors 6 and 20) produced much higher levels of IL-5 compared to the other donors. These donors however, produced fairly high IFN-γ levels and moderate concentrations of IL-12. We are classifying these two donors as Th0 producers—donors whose PIV cells produce high concentrations of both Th1 and

Th2 cytokines. A similar phenomenon was reported by Russo et al. (1998) in their culture system which also derived *Leishmania* exposed cells from *Leishmania*-naive donors. Upon restimulation, all of the donors evaluated produced IL-12. Again, two donors (Donors 5 and 33) produced higher levels of IL-12 compared to the other donors. These donors also produced high levels of IFN-γ (median: Donor 5: 11870 pg/ml and Donor 33 20000 pg/ml). The PIV cells produced very large concentrations of IFN-γ upon restimulation (up to 49,000 pg/ml). Three donors (Donors 33, 32, and 22) secreted higher levels of this cytokine than the other donors. In the mouse model, the level of IFN-γ production correlates with the ability of mouse strains to resist infection (Lehmann et al., 2000). In human leishmaniasis, the presence of Type I cytokines correlates with control of the disease and healing (Kemp et al., 1994; Akuffo et al., 1997; Adjary et al., 2000), while the presence of Type 2 cytokines is generally associated with active or chronic infections (Melby et al., 1994; Gaafar et al., 1995). Our data suggests that 2/14 donors could be classified as Th0 donors and the remaining donors as Th1 donors. We speculate that the donors with the Th1 responses would most likely be able to control a *Leishmania* infection or have a subclinical infection and the Th0 donors may be more susceptible to developing a detectable disease.

We then wanted to evaluate the ability of the *Leishmania*-primed T cells to proliferate in response to the parasite and to determine how specific that response is. In order to obtain the highest proliferative response upon restimulation, it is necessary to not only have *L. major*-primed lymphocytes, but also the parasite and a source of antigen presenting cells, which in this case were irradiated autologous PBMC. We hypothesized that the proliferative response of the PIV cells would be *L. major* –specific. Our data is

less clear-cut than in the murine PIV system. In the first study, we exposed the *L. major*-primed PIV cells to gp63, BCG or OVA (Fig. 1.8). We found that the PIV cells proliferated significantly less when exposed to gp63, a glycoprotein found on the surface of promastigotes, compared to cells restimuated with the whole parasite. A possible reason for inability of this parasite antigen to stimulate *Leishmania*-primed cells could be due to the fact that once the promastigotes enter the phagolysosome, they covert to amastigotes and gp63 would not be present on the surface of the amastigotes and thus not processed by the class II pathway. A similar result was found in the murine PIV system (Shankar and Titus, 1993). Although not statistically significant, there was a trend of less proliferation when the cells were exposed to BCG or OVA. As our data suggested that the proliferative response of *L. major*-primed cells might be parasite dependant, we restimulated the cells with Old and New world parasites, as well as *L. major* lysate. When we exposed the PIV cells to *L. donovani, L. mexicana, L. amazonensis*, or *L. major* lysate, we found that the cells responded only significantly less to *L. amazonenesis* and *L. major* lysate compared to cells restimulated with *L. major* (Fig 1.9). In the murine PIV system, Shankar and Titus (1993) reported that their PIV cells also had a reduced proliferative response when restimulated with lysate. The proliferative profile of the PIV cells suggests that the responding cells are reacting primarily to live parasites and are *Leishmania*-dependant and not *L. major*-specific. The fact that the PIV cells did not proliferate significantly in response to *Leishmania* antigen could be due to differences in the way parasites and soluble antigen are processed. It is possible that the soluble antigens, such as gp63 and the parasite lysate are being pinocytosed and the whole parasites are being phagocytosed and these difference may be causing presentation via

the class I or class II pathway respectively, as was reported by Peppelenbosch et al. (2000). Our results comparing the decreased proliferation of PIV cells when exposed to *L. amazonenesis* compared to cells stimulated with *L. major* are particularly interesting considering a recent paper where it was reported that *L. major*-resistant mice of the CBA strain were susceptible to *L. amazonensis* (Lemos de Souza et al., 2000), indicating there may be significant differences between the way the parasites infect and interact with hosts.

It has been reported in folk medicine, as well as in limited studies using human subjects, that cross-protection can occur (reviewed in Melby, 1991; Jaffe, 1999). Individuals "vaccinated" with parasites from cutaneous lesions were protected in some cases from diffuse cutaneous or visceral leishmaniasis. Other studies have shown that various parasite antigens, such as parasite surface antigen (PSA)-2 are capable of cross-species protection in mouse models (reviewed in Handman, 2001). Using the murine model, there have been several reports of cross-protection when the mice are exposed to one species and challenged with a different species of *Leishmania* (Veras et al., 1999; Lima et al., 1999; Bebars et al., 2000).

There is precedence for *Leishmania*-naïve human PBMC to proliferate in response to *Leishmania* parasites or parasite antigen (Akuffo et al., 1992; Kemp et al., 1992). Kemp et al. (1992) suggested that antigens recognized may be epitopes shared by *Leishmania* parasites and other environmental microorganisms. This phenomenon has been reported in a study comparing *Trypanosoma cruzi* and *Leishmania* antigens (Carvalho et al., 1987). Similar to our findings, human T cell clones that were *L. braziliensis*-primed proliferated in response to several other *Leishmania sp.* (Raja

Gabaglia et al., 2000). Because humans are exposed to hundreds, if not more, antigens on a daily basis, it is not at all surprising to find that the PIV cells we developed will respond to other antigens. However, as the proliferative response to non-leishmanial antigens is lower than the response to *L. major* and since restimulation with some of the parasite antigens (gp63, lysate) was not effective, we feel that the PIV proliferative responses are dependant on living *Leishmania* parasites.

In the continued analysis of our PIV system, we wanted to determine if the PIV cells responding to the parasite were CD4+ or CD8+ and if antigen processing was restricted to the HLA class II pathway. Our hypothesis, similar to that of the murine PIV system, was that the responding cell population would be primarily CD4+ T cells and HLA class II restricted. Our results indicate otherwise. In order to better assess which cell population (CD4+ or CD8+ T cells) produced IFN-γ in this system, we depleted each population from the *L. major* stimulated population and restimulated the remaining cells with the parasite and irradiated autologous APC (Fig 1.12). When either CD4+ cells or CD8+ cells were depleted from the assay, the remaining population was still capable of producing IFN-γ, indicating that in our system, both CD4+ cells and CD8+ cells secreted IFN-γ. It is also possible that NK cells could be producing IFN-γ in our system.

We next evaluated whether blocking the HLA class I or class II pathway would have any effect on antigen presentation (Figs. 1.13 and 14). To evaluate the effects, we measured cell proliferation of *L. major*-stimulated PIV cells when the cells were exposed to neutralizing antibodies during restimulation. Surprisingly, our data indicate that blocking the HLA class I pathway decreased proliferation compared to *L. major* stimulated cells. There was also a tendency for decreased proliferation when HLA class II

was neutralized. To investigate these findings further, we measured IFN-γ production of *L. major*-stimulated cells that were exposed to neutralizing antibodies for HLA Class I or Class II. In this case, blocking either the class I or class II pathways significantly decreased IFN-γ production compared to cells stimulated with only the parasite. Both of these studies suggest that the parasite is being presented through both the class I and class II pathways.

In the PIV cultures, the suppression of IFN-γ production and proliferation of the PIV cells could be attributed to several factors. It is possible that the ability of NK cells to produce IFN-γ is being reduced or abrogated by the blocking of Class II. NK have been shown to be important during the early immune response in human infections (Akuffo et al.1993; 1999). The proliferation of CD8+ cells is dependant on the production of IL-2 by CD4+ cells (reviewed in Fitch et al., 1995), and if the proliferation and activation of CD4+ cells is being abrogated by the blocking of Class II, this could be a reason why both CD4 and CD8 cells are not proliferating or producing IFN-γ in response to *L. major*. IL-15, which is produced by macrophages, has a similar function as IL-2 and utilizes the IL-2R on lymphocytes (Waldmann and Tagaya ,1999). This cytokine could also possibly be abrogated by the blocking of HLA Class II and resulting in decreased activation and hence, IFN-γ production of CD8+ cells. Unlike the production of IFN-γ when HLA class II is blocked, the production of IFN-γ and the proliferation of the PIV cells is not completely inhibited when HLA class I is blocked. Taken together all three of these studies indicate that in the PIV system CD4+ and CD8+ T cells are producing IFN-γ. Hand in hand with this finding, our data suggest the parasite is being presented through both the class I and class II antigen processing pathways.

Cross-presentation allows a means whereby APC can present antigen to both naïve CD4+ and CD8+ T cells. This pathway favors presentation of high dose antigens or those derived from apoptotic cells (Albert et al.,1998: reviewed in Heath and Carbone, 1999). Although the predominant site for *Leishmania sp.* to be harbored within APC is within the parasitopherous vacuole leading to class II antigen processing, there is some evidence that promastigotes can localize within the cytosol. Therefore, the parasite would be available for class I antigen processing (Rittig and Bogdan, 2000). Several parasites activate CD8+ T cells, including *Toxoplasma gondii, Plasmodium berghei,* and *Trypansoma cruzi* (reviewed in Harty et al., 2000). *Mycobacterium tuberculosis*, which primarily activates CD4+ T cells is also capable of activating CD8+ T cell effectors (reviewed in Harty et al., 2000; Serbina et al., 1999).

There are conflicting reports in the *Leishmania* literature regarding the ability of CD8+ T cells to be activated, as well as the importance of HLA or MHC (in the mouse) class I antigen processing and presentation. In human PBMC in vitro cultures from *Leishmania*-unexposed donors, as well as the murine PIV system developed from naive mice, the responding cells were found to be CD4+ (Shankar and Titus, 1993; Kurtzhals et al., 1995; Raja Gabaglia et al., 2000) and class II restricted (Shankar and Titus, 1993). In studies analyzing blood from humans immune to *Leishmania*, CD4+ cells were found to be the primary effector cells secreting IFN-γ (Kemp et al., 1999). In the murine model, several studies have reported that mice immunized with *Leishmania* antigens (gp46-M2, recombinant hydrophilic acylated surface protein B1–dHASPB1, lysates) were shown to induce class I restricted CD8+ CTL that produced IFN-γ (Kima et al., 1997; Lehmann et al., 2000; Stager et al., 2000). Similarly, Muller et al (1993) demonstrated that CD8+ T

cells played a role in the memory response of *L. major* vaccinated mice, contributing to the IFN-γ production upon rechallenge. Interestingly, cytotoxic T cell activity and IFN-γ secretion of CD8+ T cells is enhanced in IL-4 deficient mice, suggesting that Type 2 cytokines may play a role in down-regulating the ability of CD8+ cells to react to pathogens (Villacres et al., 1999). Although the dogma in murine *Leishmania* research indicates that CD4+ T cells are the primary effector cells in the infection, evidence is accumulating that CD8+ T cells may participate in at least a limited role in *Leishmania* infections.

Similar to murine studies, some studies using in vitro systems to evaluate the interaction between either *Leishmania*-stimulated cells or PBMC collected from infected donors, report the stimulation of CD8+ T cells. Particularly, Russo et al. (1999) sensitized naïve human T cells with promastigote antigen and IL-12. The responding population of cells included a population of CD8+ T cells, suggesting in human *Leishmania* infections, CD8+ T cells were stimulated in response to the parasite. Not all of these cells, however, secreted IFN-γ, but they did have potent cytotoxic effects. A report in which patient cells were used determined that control of *L. infantum* infection was associated with CD8+, IFN-γ producing T cells, as well as IFN-γ producing CD4+ cells (Mary et al., 1999). The mucosal destruction evident in mucocutaneous infections may be due to the cytotoxic activity of CD8+ cells (Brodskyn et al., 1997). Da Cruz et al. (1994) also report a higher proportion of *Leishmania*-reactive CD8+ T cells compared to CD4+ T cells after eradication of the disease. In contrast, in another study, higher levels of CD8+ T cell apoptosis compared to CD4+ cells were found in donors with active lesions, reducing the level of CD8+ T cells after infection (Bertho et al., 2000). Overall,

the data reported thus far suggests that CD8+ cells most likely do play a role in *Leishmania* infections, perhaps more so upon restimulation with the parasite. Our data suggests that CD8+ T cells do indeed contribute to the immune response. The CD8+ cells can participate in enhancing a CD4+ Th1 response though the secretion of IFN-γ. CD8+ cells could also potentially influence the infection by eradicating macrophages via cytotoxic responses. The role of CD8+ T cells in leishmaniasis requires further investigation.

It is also possible that non-classical antigen recognition and presentation is occurring in our system, although we did not examine this area of study in our experiments. Two of the most probable mechanisms are antigen presentation through CD1 and recognition through Toll-like receptors (TLR). CD1 molecules form an antigen presentation system that specializes in the presentation of lipids and glycolipids for T cell recognition (reviewed in Porcelli and Modlin, 1999). *Plasmodium falciparum*-derived glycophosphatidylinositol (GPI) and *Trypanosoma brucei* variable surface antigen glycoprotein can be processed through the CD1 pathway (Schofield et al., 1999). *Leishmania sp.* also possess GPI, and it is probable that the CD1 pathway could be used to present antigen to T cells. TLR have been identified as ancient receptors that recognize "pathogen associated molecular patterns" (Kopp and Medzhitov, 1999; Hoffmann et al., 1999; Aderem and Ulevitch, 2000) including glycolipids and lipopeptides from a number of bacteria (Hoshino et a., 1999; Brightbill et al., 1999) and the GPI anchors from *T. cruzi*, a protozoan parasite (Campos et al., 2001). TLR activate the innate immune response, resulting in the production of IL-12, TNF-α and NO by

macrophages (Campos et al., 2001). As *L. major* also has GPI, it is possible that TLR are used in the PIV system to generate an initial innate immune response.

In our final study, we observed the interactions between macrophages and the parasite. In leishmaniasis, the parasite resides primarily within the mononuclear phagocytes of the host (reviewed in Kane and Mosser, 2000). The parasite is engulfed via receptor-mediated phaogocytosis. Once inside the macrophages, *Leishmania* can grow in non-activated macrophages, however, in activated macrophages, the parasite will be killed by either a respiratory burst from oxygen or nitrogen radicals (Murray, 1982; Wei et al., 1995). Although the presence of nitric oxide appears to be essential for control of parasites in the murine model (Murray and Nathan 1999), in human infections, the importance of NO is less clear (Ritter and Moll, 2000; Kane and Mosser, 2000). We found that macrophages isolated from *Leishmania*-naïve human PBMC were capable of significantly reducing their parasite burdens over a 72 h time period, without any additional stimuli. The parasites were probably eradicated by production of oxygen radicals. It is possible that the glycoproteins or lipophosphoglycans, which facilitate parasite binding to the macrophages (Chang et al., 1990; Chen et al., 2000), and are present on the surface of the parasite, are also capable of activating macrophages. Another possible candidate for macrophage activation are the glycoinositolphospholipids which are present on the surface of amastigotes (reviewed in Alexander et al., 1999). There is precedence for activation of macrophages by pathogens and pathogen-related factors. For instance, human macrophages can be activated by surface lipoproteins of bacteria such as *Borrelia burgdorferi* and *Treponema palladium* (Sellati et al., 1999).

We wanted to determine if the addition of *L. major* primed T cells would be able to modulate the interactions between the parasite and the macrophages. We found that if PIV cells are added to *L. major*–infected macrophages 8 h after the macrophages are exposed to the parasites, and then allowed to incubate for an additional 18 h, there is a significant reduction in the number of parasites present within the macrophages (Fig 1.16b). Attempts to modulate this effect by adding a neutralizing antibody for IFN-γ were not successful, suggesting that the response is most likely governed by interactions with the lymphocytes themselves. Most likely, the macrophages were activated by the interaction with *Leishmania*-specific T cells that recognized pathogen-derived peptides presented by the major histocompatibility complex at the surface of the infected macrophages. A similar finding was reported by Wyler et al. (1987). They found that there appeared to be two pathways for macrophage killing of *Leishmania*. The first pathway involves soluble mediators, such as cytokines and the second pathway depends on direct membrane interactions. Kima et al. (1996) report that promastigote infected macrophages present endogenous parasite antigens to CD4+ T cells for only a limited time, with maximal presentation occurring within 24 hr of infection. The efficient killing we saw when we added T cells to the macrophage cultures could also be due to efficient early processing of the parasite. As the macrophages quickly present antigen to the *Leishmania*-primed PIV cells, which include CD4+ and CD8+ T cells, the T cells can interact with the macrophage and stimulate the infected cells to eliminate the parasite via the production of cytokines, or in the case of CD8+ cell, through killing the macrophage itself.

It is possible that other factors we did not investigate play a role in the destruction of *L. major*. Due to the enhancement of killing when T cells are added to the cultures, it is likely that co-stimulatory molecules are necessary in the interactions between the two cell populations. As CD40L plays an important role in cell mediated immune response (Chen et al., 2001), it is possible that the PIV cells expressed CD40L and that a CD40-CD40L interaction assisted in eradicating the parasites more efficiently in the macrophage cultures. Nashleanas and Scott(2000) found that CD40-CD40L interactions can activate macrophages infected with *L. major*. The interactions between B7 molecules on the macrophage surface and CD28 on the PIV T cells may also enhance parasite killing by macrophages. Brodskyn et al. (2001) report that the expression of CD86 on *L. major* infected macrophages is critical in the initiation of anti-leishmanial T cell activation. Another potentially important factor is the monocyte chemotactic protein-1 (MCP-1), which has been reported to stimulate the elimination of parasites in human monocytes from patients with cutaneous leishmaniasis (Ritter and Moll, 2000). Costimulatory molecules most likely play an essential role in the interactions between the infected macrophages and the *L. major*-primed lymphocytes.

To summarize the results of this study, we developed a PIV system which we characterized based on cytokine secretion, phenotype and proliferation. We found that although both Type 1 and Type 2 cytokines are produced, we could only detect IL-5 upon restimulation and just at low levels for most donors. IL-12 is also produced at low levels, but is detectable after just 24 h of stimulation. IFN-γ, on the other hand, is detectable at fairly high levels 24-72 hr after parasite exposure and at extremely high levels upon restimulation. These results suggest that most of the donors in our study would be quite

capable of curing leishmaniasis efficiently. When antigen-specific proliferation of *Leishmania*-primed T cells were measured, we determined that although the PIV cells respond to other *Leishmania sp*, there does appear to be a tendency for the response to be dependent on live *Leishmania* parasites , as the cells responds significantly less to gp63, parasite lysate. We have determined that antigen presentation in the PIV system is not restricted to the class II pathway, but the class I pathway for antigen presentation is also important in responding to the parasite, implicating both CD4 and CD8+ T cells in responding to the parasite. Finally, we determined that macrophages from *Leishmania*-naïve donors are capable of reducing parasite burdens without other stimulation, but that the eradication of parasites is greatly enhanced by the addition of T lymphocytes. The macrophage-T cell interaction appears to be contact mediated, perhaps through the interaction of co-stimulatory molecules. Overall, this human PIV system reproduces the host-parasite interactions expected from the literature. Because of this, the PIV system will allow further study of human-parasite interactions, such as the interactions of *Leishmania*-primed T cells with antigen presenting cells and the further exploration of co-stimulatory molecules necessary for an immune response. This system will enable the dissection of individual parts of the immune response during *Leishmania* exposure not possible using infected patients. The effects of parasite and vector proteins can also be evaluated. For instance, the study of salivary gland proteins and their effects upon the human immune response can also be undertaken in this type of system.

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CHAPTER TWO

THE EFFECTS OF CYTOKINES ON THE PRIMARY IN VITRO SYSTEM

INTRODUCTION

In *Leishmania* infections, be it in mouse or man, cytokines play an essential role in determining the outcome of infection. The parasite is controlled by cell mediated immune responses and to that end, the maturation and differentiation of an appropriate subset of CD4+ T cells is key. T helper cell (Th) subsets are defined primarily by their cytokine secretion patterns (reviewed in Seder and Paul, 1994; Romagnani, 1994), although recent evidence also indicates that the subsets exhibit particular cell surface markers as well (Rogge et al., 1999; Lim et al., 1999; Nagata et al., 1999). Th1-type T cells secrete IFN-γ, which can activate macrophages to eradicate the parasite via nitric oxide or reactive oxygen intermediates (Murray et al., 1983; Corradin et al., 1991; Titus et al., 1994). IFN-γ also upregulates the expression of class I and class II major histocompatibility molecules, enhancing antigen presentation to T cells (Volk et al., 1986).

Type1 immunity can be defined as cell mediated immunity, which is generally extremely well suited to eradicating intracellular pathogens such as *Leishmania major*.

On the other hand, Th2-type T cells secrete IL-4, IL-10 and IL-5 and activate B cells to produce an antibody mediated response. IL-4 has been shown to control the polarization of undifferentiated T cells, termed Th0 cells, into Th2 cells (Swain et al., 1990; Le Gros et al., 1990). IL-5 stimulates bone marrow production of eosinophils (Parsons et al., 1993). This cytokine is also upregulated when IL-4 is produced (Verheyen et al., 2000).

IL-10 inhibits the synthesis of cytokines by Th1 cells and can decrease proliferation of these cells (reviewed in Mosmann and Moore, 1991). This cytokine also inhibits antigen presentation by macrophages and dendritic cells (Buelens et al., 1995; Creery et al., 1996), as well as prevents macrophages from killing bacteria (Fleming and Campbell, 1996). Type 2 immune responses are generally efficient at eliminating extracellular pathogens, such as helminth worms, through coating the parasites with antibodies that will subsequently activate appropriate cells, such as eosinophils to respond to the infection (Capron and Dessaint, 1992). Certain viruses (e.g. papilloma viruses) and bacteria (e.g. *Helicobactor pylori, Neisseria gonorrhoeae*) and toxins (e.g. Diptheria or Tetanus toxins) they may produce, can be neutralized or opsonized by antibodies resulting from activation of Type 2 immunity and subsequently removed by phagocytic cells (Konya and Dillner, 2001; reviewed in Janeway et al, 2001). A Type 2 immune response can also cause allergic reactions, such as to birch pollen (Moverare et al., 2000).

Cytokines, such as IL-12 and TGF-β, secreted by cells such as macrophages and dendritic cells, can also influence the immune response and profoundly affect whether Type1 or Type 2 immunity will occur. In particular, IL-12 plays a critical role in promoting Th0 cells to differentiate into IFN-γ secreting Th1 cells, as well as augmenting

the production of IFN-γ from differentiated cells (Hseih et al., 1993; Yang et al., 1999). In contrast, TGF-β production results in a shift to Type 2 immunity. TGF-β causes suppression of Th proliferation (Rodrigues et al., 1998) and suppression of IFN-γ production (Wilson et al., 1998).

The secretion of either subset of Type 1 or 2 cytokines can down-regulate the other. For instance, the production of IFN-γ will result in T cell unresponsiveness to IL-4 (Gajewski and Fitch, 1988; Romagnani, 1994; Oriss et al., 1997). IL-4 production can suppress phagocytosis and intracellular killing (Oswald et al., 1992; Cenci et al., 1993), as well as reduce IL-12 and IFN-γ production (Swain et al., 1990; Launois et al., 1997; Sasaki et al., 2000). Several factors, such as the local cytokine environment (reviewed in Spelberg and Edwards, 2001; Solbach and Laskay, 2000) and the dose of antigen (Bretscher et al., 1992; Doherty et al., 1996; Memon and Bretscher, 1998) can influence the secretion of cytokines and the polarization of Type 1 or Type 2 immunity.

The murine leishmaniasis model has allowed for the close examination of the Th1/Th2 paradigm. In *Leishmania* infections, the central tenet is that the production of cytokines causes the polarization of Th cells to either a Type1 response, which will allow for control of the disease, or a Type 2 response, which results in disease exacerbation. The BALB/c strain of mouse cannot control the infection, while many other strains, such as C57Bl/6 and C3H/HeN can heal spontaneously (reviewed in Reiner and Locksley, 1995; Launois et al., 1998). An early study demonstrated that T cells are essential in resistance to *L. major* infections (Mitchell et al., 1981). Soon thereafter, it became clear that CD4+ T cells are also the primary contributor to susceptibility to infection (Milon et al., 1986; Titus et al., 1987). Sadick et al., (1990) then demonstrated that susceptibility to

L. major depended on the production of IL-4 and the development of the Th2 subset. The murine model has allowed for the indentification of cytokines important in controlling and exacerbating *Leishmania* infections. IL-12 has been shown to promote Th1 differentiation in murine infections. When exogenous IL-12 is injected into mice during the first week of infection with *L. major*, susceptible BALB/c mice are able to control the parasite (Heinzel et al., 1993; Sypek et al., 1993). In contrast, mice generally resistant to infection were made susceptible by the addition of antibodies blocking IL-12 (Sypek et al., 1993; Heinzel et al., 1995). Belkaid et al. (1998) demonstrated that the parasite can actively suppress IL-12 production of *L. major*-infected macrophages. IFN-γ also plays an essential role in controlling murine *Leishmania* infections (reviewed in Reiner and Locksley, 1995; Launois et al., 1998). Mouse strains that have a resistant background, but lacking the IFN-γ or IFN-γ receptor gene are quite susceptible to *L. major* infections (Wang et al., 1994; Swihart et al., 1995).

The production of Type 2 cytokines results in the exacerbation of leishmaniasis. The study of murine leishmaniasis has demonstrated the dominant role IL-4 plays in Th2 cell differentiation (Heinzel et al., 1989; Morris et al., 1993). Susceptible BALB/c mice produce a burst of IL-4 shortly after infection, that rapidly returns to basal levels and then five days after parasite exposure, returns to high levels of IL-4 (Launois et al., 1995, 1997). The increase in IL-4 production in susceptible mice has been attributed to CD4+ T cells expressing the Vβ4-Vα8 T cell receptor (TCR) chain (Launois et al., 1997).

Other cytokines, such as IL-5, TGF-β and IL-10 also contribute to Type 2 immunity in murine leishmaniasis. These cytokines are produced in large concentrations by non-healer mice during *Leishmania* infection (Chatelain et al., 1999; Nashed et al.,

2000). TGF-β has been found to inhibit *L. major* killing by macrophages (Nelson et al., 1991), while the neutralization of this cytokine augments IFN-γ and diminishes IL-4 mRNA levels (Barral-Netto et al., 1992). In murine visceral leishmaniasis, TGF-β inhibits Th1- associated resolution (Wison et al, 1998). Both IL-10 and IL-5 contribute to Th2-like immune responses early in infection. The IL-10 in resistant mice can be down-regulated during infections lasting less than two weeks (Reiner et al., 1994). When IL-10 is altered, mice can be more susceptible to infection (Groux et al., 1999). IL-10 also appears to play an important role in regulating immune responses to *Leishmania*. IL-10 transgenic mice, which have elevated IL-10 production, created from a resistant strain of mice are susceptible to *L. major* infections (Groux et al., 1999). IL-10 can also affect lesion development, as susceptible mice lacking IL-10 have smaller lesions compared to wild-type controls (Kane and Mosser, 2001). Finally, this cytokine is also produced by macrophages upon infection and inhibits macrophage activation (Bhattacharyya et al., 2001; Kane and Mosser, 2001).

Using a murine in vitro model, Soares et al. (1997) dissected the cytokine response of resistant and susceptible mice to determine the factors which cause naïve T cells to commit to the Th1 or Th2 subset. Splenocytes from BALB/c mice produced 10-fold more IL-4 than cells from resistant CBA mice during the first 7 days of infection. Conversely, CBA mice produced 10-fold more IFN-γ than BALB/c mice during the same time period. Interestingly, they did not find any influence of IL-10 early after exposure to the parasite, suggesting that IL-10 may play a more important role in chronic infections.

Cytokines also play a very important role in human *Leishmania* infections. The well characterized Th subsets found in the mouse model for leishmaniasis are less clear in human studies, particularly when focusing on Type 2 immunity. For instance, elevated IL-4 levels have been found in patients infected with visceral leishmaniasis (VL) or cutaneous leishmaniasis (CL) (Swingenberger et al., 1990; Sundar et al., 1997; Adjary et al., 2000). However, in some studies focusing on New World VL infections, IL-4 was not shown to be involved in *L. chagasi* infections (Carvalho et al., 1994; Bacellar et al., 2000). There is some evidence that in contrast to murine infections, IL-10, not IL-4, may be responsible for uncontrolled infections in humans VL infections (Ghalib et al., 1993; Carvalho et al., 1994; Bacellar et al., 2000), although this may not be the case in Old World CL infections (Adjary et al., 2000). In contrast, Rocha et al (1999) reports IL-10 is responsible for down-regulating T cell responses in early-phase cutaneous leishmaniasis infections caused by the New World parasite *L. braziliensis*. Similarly, peripheral blood mononuclear cells (PBMC) from VL patients with active infections produce IL-10 in response to *Leishmania* antigen and after treatment, production of this cytokine ceases (Ghalib et al., 1993; Holaday et al., 1993; Ghalib et al., 1995). Other Type 2 cytokines can also be present in human leishmaniasis. IL-5 and TGF-β have been detected in chronic CL and mucocutaneous lesions (Pirmez et al., 1993; Melby et al., 1994; Barral et al., 1995). In cell culture studies, TGF-β is produced by human macrophages infected with *Leishmania* and allows for greater numbers of parasites within the phagocytic cell (Barral et al.,1995). IL-5 has been shown to be decreased by IL-12 in *L. major* infected PBMC (Brodskyn et al., 2000). The studies in humans

indicate that during infection, the production of Type 2 cytokines may delay healing or exacerbate disease.

The production of IFN-γ and IL-12 are essential in order to develop Type 1 immunity in humans. The presence of IL-12 is necessary to stimulate T cells to produce IFN-γ and thus, it is not surprising that in human *Leishmania* infections, as has been shown in mice, IL-12 and IFN-γ production go hand in hand. For instance, PBMC from patients treated for VL produced both IL-12 and IFN-γ in response to *L. donovani* lysate (Ghalib et al., 1995). It has also been demonstrated that the addition of IL-12 restored lymphocyte proliferation and IFN-γ production by PBMC from patients with active VL (Carvalho et al., 1994; Ghalib et al., 1995). The importance of IFN-γ in controlling infection has been demonstrated by studies showing that if IFN-γ is not adequately produced, there is an inability to properly control the infection (Akuffo et al., 1997; Weinstock et al., 1997). Similarly, in endemic regions, individuals who do not produce IFN-γ in response to *Leishmania* antigen have a significant chance of developing leishmaniasis (Carvalho et al., 1992). In contrast, peripheral blood from patients with active or healed localized cutaneous lesions produce IFN-γ in response to *Leishmania* antigens in vitro (Carvalho et al., 1994; Kemp et al., 1994; Kemp et al., 1999; Adjary et al., 2000). Based on such reports, it has been suggested, as in the mouse models, that resolution of *Leishmania* infections depends on a Type 1 immune response (Bottrel et al., 2001).

We have developed a human in vitro system that enables us to dissect the human immune response to *L. major*. In order to better use our system in the future to test possible immunomodulatory factors which may allow for better control of the parasite, it

is necessary to more thoroughly investigate the interactions of cytokines in our system. This was achieved by isolating PBMC from *Leishmania*-naïve donors and exposing the cells to *L. major*, then examining the effects of adding exogenous cytokines or neutralizing antibodies on Type 1 or Type 2 cytokine production. The exogenous cytokines and neutralizing antibodies used in this study included IL-12, anti-IL-12 monoclonal antibody (MAb), IL-4, anti-IL-4 MAb, IL-10, anti-IL-10 MAb, TGF-β and anti-TGF-β MAb. We measured the production of IFN-γ, IL-5 and IL-10 at 3 and 7 days after exposure to *L. major* and the cytokine in question or its blocking antibody. We chose to study the immune responses early after parasite exposure, since commitment to a Type 1 response or a Type 2 response occurs early in infection. We formulated several hypotheses for this study. First, we hypothesized that adding Type 1 cytokines would enhance the IFN-γ response and that blocking Type 1 cytokines would abrogate IFN-γ production and enhance IL-5 and IL-10 production. Also, we speculated that adding Type 2 cytokines would enhance IL-5 and IL-10 production and abrogate IFN-γ production. As expected, in our system it appears that IFN-γ is the dominant cytokine produced by PBMC in response to the parasite. This cytokine was modulated more greatly than the IL-10 and IL-5. In contrast to the IFN-γ data, few discernable patterns, could be detected when secretion of Type 2 cytokines were measured.

MATERIAL AND METHODS

Parasites

L. major (isolate LV39, Rho/SV/59/P) parasites were grown on biphasic NNN medium (Titus et a., 1994) and passed through mice every two weeks to maintain virulence. Promastigotes were harvested in the stationary growth phase.

In vitro system

The *in vitro* system we developed was patterned after the murine *in vitro* system described by Soares et al (1997). Human blood was obtained from healthy individuals at the Hartshorn Health Center at Colorado State University. 100 ml of blood was collected during each blood draw. PBMC were isolated from heparinized venous blood by passage over a Histopaque-1077 (Sigma Diagnostics; St. Louis, MO) gradient (90). PBMC were washed three times and resuspended at a concentration of $2x10^6$ cells/ml in complete medium consisting of RPMI-1640 medium supplemented with 2 mM $_L$-glutamine, penicillin (100 U/ml), gentamycin (100 µg/ml), and 10% heat inactivated human AB serum (Pel-Freeze; Brown Deer, WI). The cells were plated in 24-well tissue culture plates (Costar; Corning, NY) at 1 ml/well and incubated at 37°C with 5% CO_2. *L. major* promastigotes were added to some of the cultures at a concentration of $4x10^5$ parasites/ml. To study early time point interactions between the PBMC, cytokines and *L. major*, culture supernatants were harvested for cytokine analysis at 3 and 7 days.

Cytokine assays

Concentrations of IFN-γ, IL-10 and IL-5 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (Pharmingen Beckton Dickenson; San Diego, CA). The limits of detection for these assays were 10 pg/ml for IFN-γ, 6.3 pg/ml for IL-10 and 2 pg/ml for IL-5. Supernatants were measured at 3 and 7 days after stimulation. All experiments were repeated two times.

Reagents

The following cytokines or monoclonal antibodies against a particular cytokine were used in cell cultures: anti-human IL-4 antibody (clone 34019.111, mouse IgG_{2b} isotype; R&D Systems Inc.; Minneapolis, MN) at 10 μg/ml, recombinant human (rh)IL-4 (PeproTech Inc.; Rocky Hill, NJ) at 10 ng/ml, monoclonal anti-human IL-10 antibody (clone 23738.11, mouse IgG_{2b} isotype; R&D Systems) at 10 μg/ml, rhIL-10 (PeproTech) at 10ng/ml, monoclonal anti-human IL-12 antibody (clone 24910.1, mouse IgG1 isotype; R&D Systems) at 10 μg/ml, rhIL-12 (supplied by the Bioanalytical Science Department of Genetics Institute, Inc., Cambridge, MA) at 10 ng/ml, monoclonal anti-human TGF-β1 antibody (clone 9016.2, mouse IgG1 isotype; R&D Systems) at 10μg/ml and rhTGF-β1 (R&D Systems) at 100 pg/ml. Irrelevant isotype matched antibodies from the same supplier were used as controls.

Statistical analyses

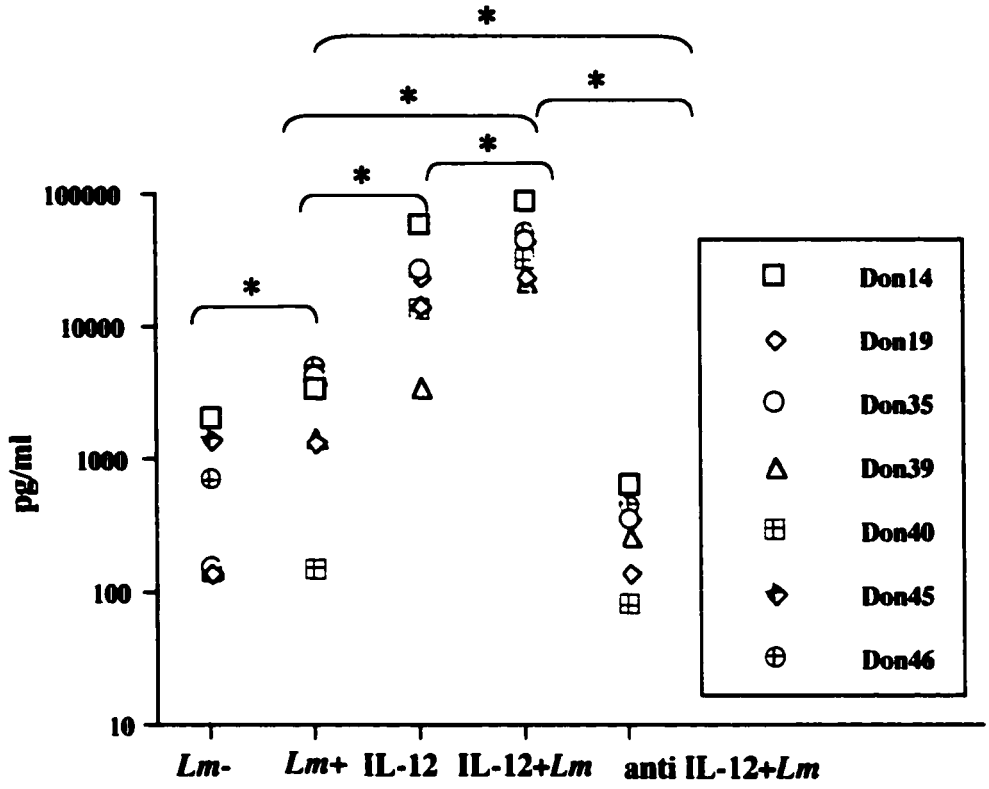
Statistical analyses were performed using Sigma Stat (SPSS, Chicago, IL) software. Due to the small sample sizes and the inherent variances in individual human responses, the data were not distributed normally, thus non-parametric analyses were necessary. Data were compared using Kruskal-Wallis one way ANOVA on ranks. When $p<0.05$, the Student-Newman-Keuls Multiple Comparisons Test was used to determine the significance of the differences between samples. Overall, results were considered significant when the p value was <0.05.

RESULTS

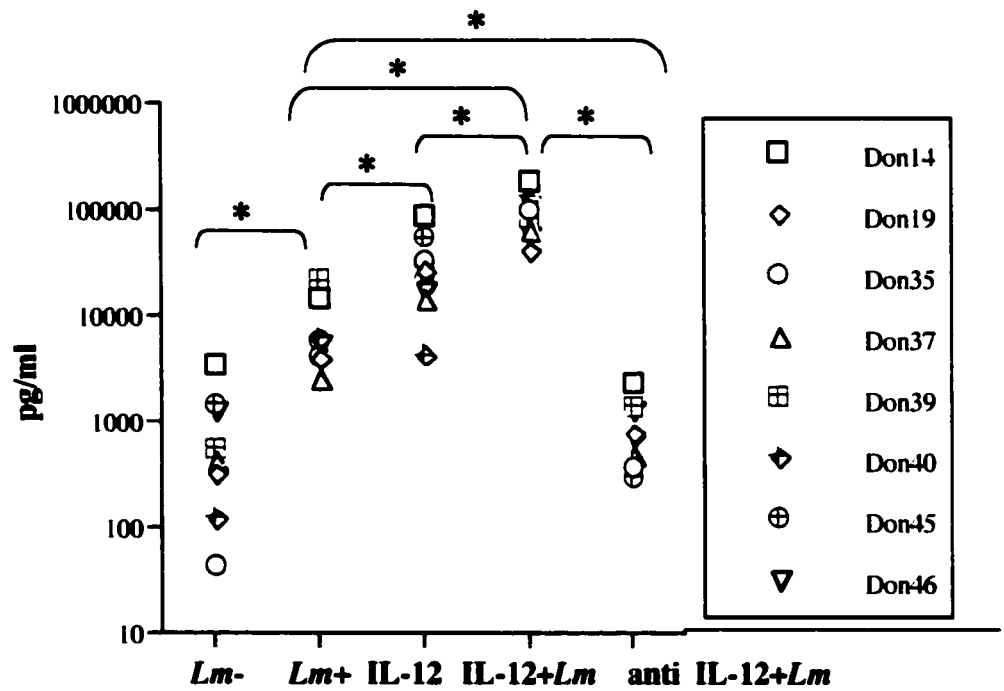
Effects of enhancing and blocking the Type 1 response on IFN-γ production

Since our initial characterization of the PIV system indicated that IFN-γ is most likely the cytokine driving the Type 1 response and it has been established that IL-12 production by macrophages is the primary inducer of IFN-γ production, we wanted to characterize the effects of IL-12 and conversely, blocking IL-12 on IFN-γ production. In order to determine the effects of cytokines early after exposure to *L. major*, we measured IFN-γ production by PIV cells at 3 and 7 days post-exposure to the parasite. Figures 2.1a (IFN-γ production at day 3) and 2.1b (IFN-γ production at day 7) show that at both time points, the addition of IL-12 increased IFN-γ production significantly compared to cells stimulated with *L. major* ($p<0.05$). The production was also significantly higher when the cells were stimulated with the parasite and IL-12 compared to stimulation with IL-12 only. Conversely, blocking IL-12 almost completely abrogated IFN-γ production ($p<0.05$). All of the treatment groups to which the parasites were added produced IFN-γ

A. Day 3



B. Day 7



Figures 2.1a and 2.1b The production of IFN-γ at 3 (2.1a) and 7 (2.1b) days after exposure to *L. major*. The bars and '*' denote differences between treatment groups ($p<0.05$).

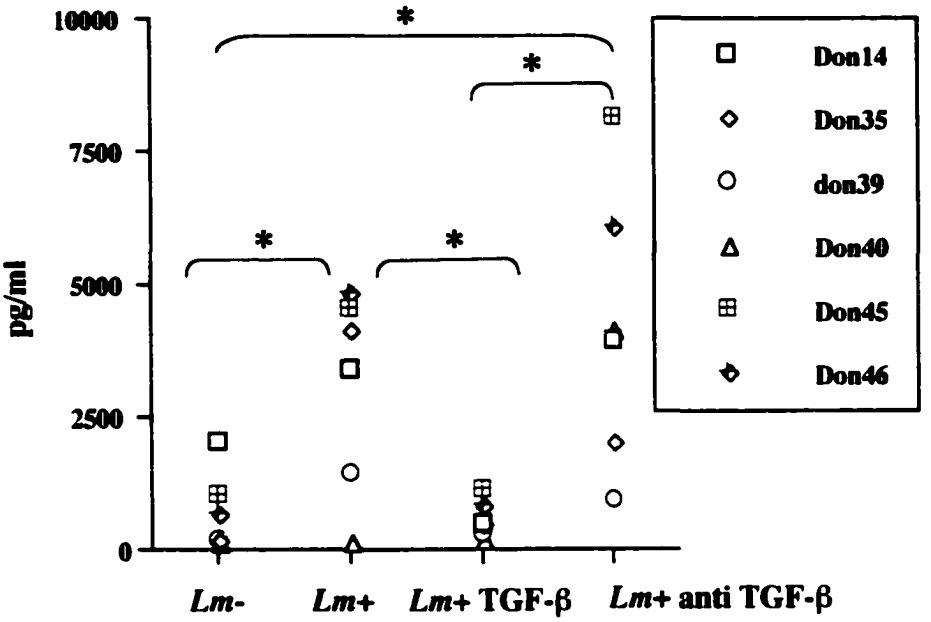
to a significantly greater level than the control samples (cells without *Leishmania* added) except for when the anti-IL-12 antibody was added to the cultures. The samples collected at day 7 had 1.5 to 2-fold more IFN-γ present in the culture supernatants than a day 3, indicating an accumulation of this cytokine.

Effects of enhancing and blocking the Type 2 response on IFN-γ production

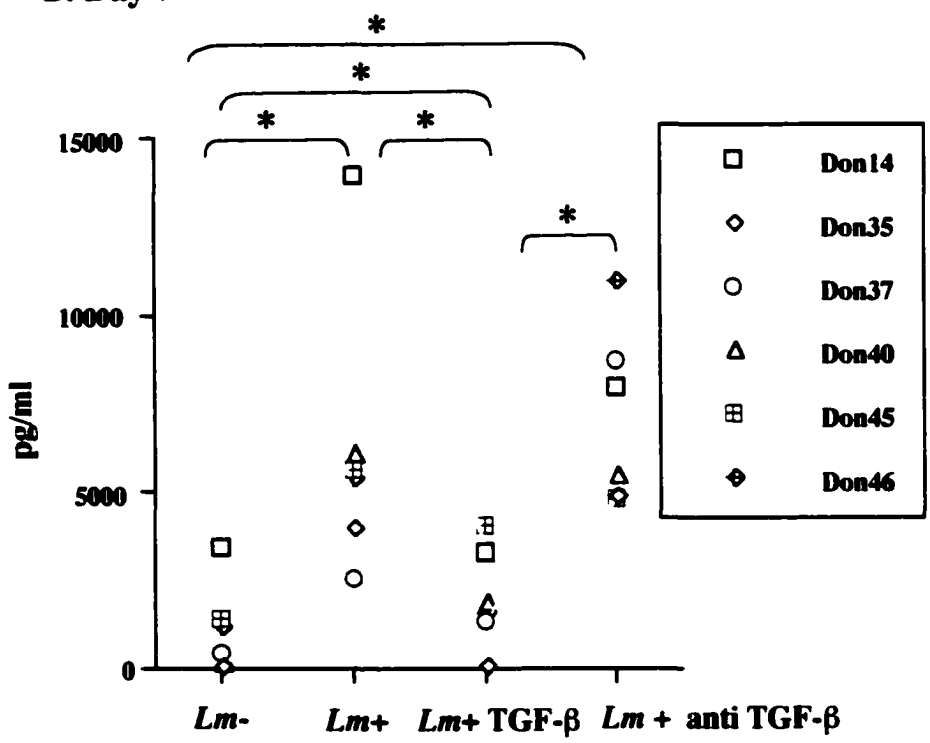
To determine the impact on IFN-γ production by cytokines capable of driving a Type 2 response, we examined the effects of three cytokines: TGF-β, IL-10 and IL-4. These cytokines have been shown to influence a Type 2 response in mice and patients. Since there have been mixed reports about these cytokines and their role in human leishmaniasis, we hoped to elucidate their effects using our PIV system. First, we examined the importance of TGF-β in modulating the production of IFN-γ. As shown in Figures 2.2a and 2.2b, both the 3 and 7 day samples trend in similar ways. There was no significant difference between the IFN-γ production of *L. major*- stimulated cells and when TGF-β was neutralized in parasite-exposed cultures. In contrast, when the PIV cells are exposed to recombinant TGF-β and the parasite, IFN-γ production is significantly decreased compared to cells stimulated with the parasite.

Next, we investigated the effect of IL-10 in our system. IL-10 has been shown to be important in chronic cutaneous and visceral leishmaniasis (Ghalib et al.,1993; Melby et al.,1996; Bacellar et al., 2000). In both the 3 and 7 day samples, the addition or the blocking of IL-10 had profound effects on IFN-γ production (Figure 2.3a and 2.3b). The addition of IL-10 significantly decreased IFN-γ secretion at both time points. In contrast, blocking IL-10 greatly enhanced the ability of the PIV cells to produce IFN-γ, even to

A. Day 3



B. Day 7



Figures 2.2a and 2.2b The production of IFN-γ at 3 (2.2a) and 7 (2.2b) days after exposure to *L. major*. The lines connecting the groups and the '*' denote differences between treatment groups ($p<0.05$).

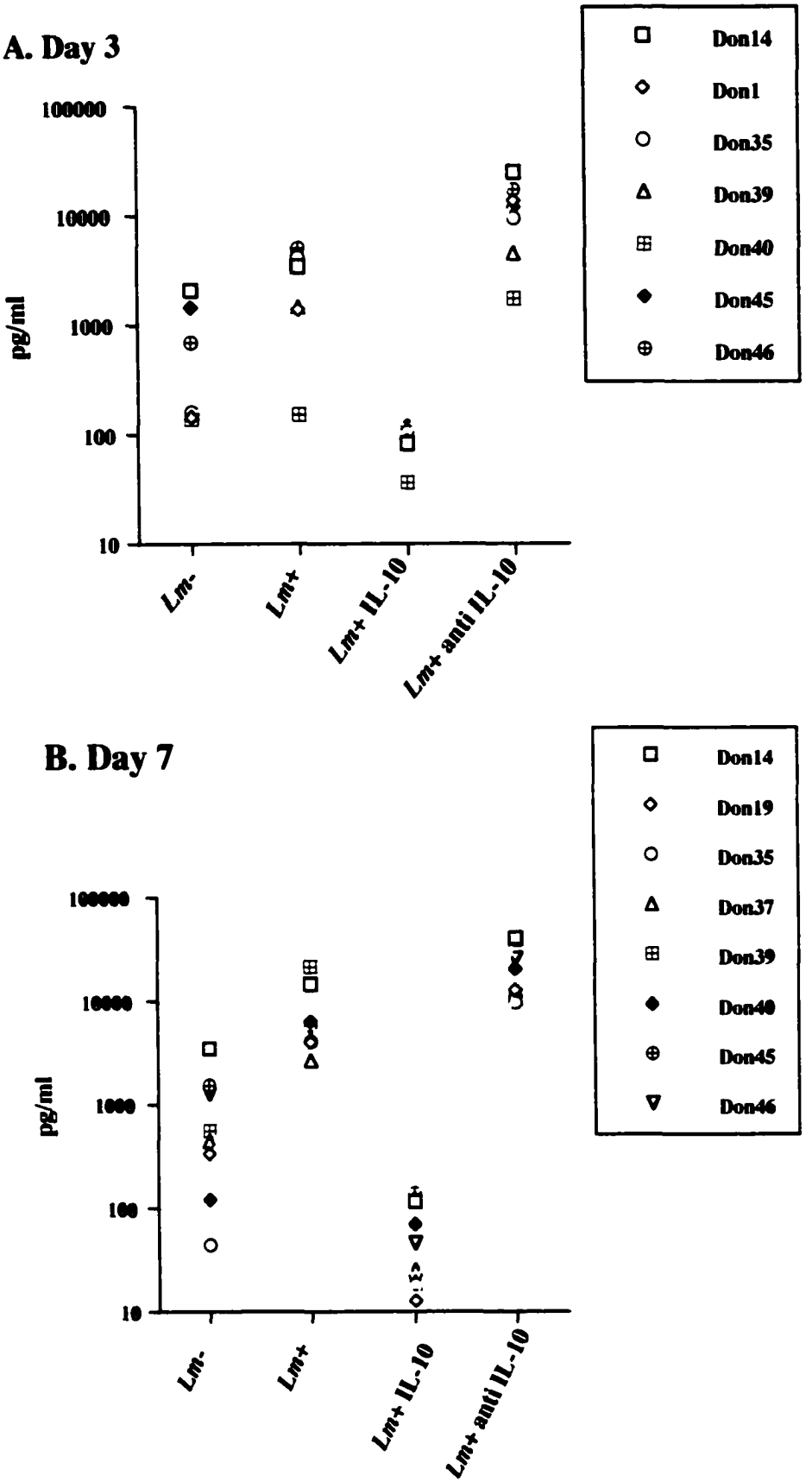


Figure 2.3a and 2.3b The production of IFN-γ at 3 (2.3a) and 7 (2.3b) days after exposure to *L. major*. Each treatment group is significantly different from every other treatment group ($p<0.05$).

levels significantly greater ($p<0.05$) than when the cells were exposed to the parasite alone. These results suggest that IL-10 is capable of modulating a *Leishmania*-related immune response soon after exposure to the parasite and may play a role in modulating the production of IFN-γ in this PIV system.

Finally, it has been shown that IL-4 plays an important role in exacerbating *L. major* infections in mice. However, the influence of IL-4 in infected human patients is less clear (Ghalib et al., 1993; Carvalho et al., 1994; Sundar et al., 1997). To test the relevance of IL-4 in our system, we exposed *L. major*-naïve cells to the parasite and to IL-4 or a neutralizing IL-4 monoclonal antibody. Unexpectedly, neither rhIL-4 nor anti-IL-4 antibody had any effect on IFN-γ production in our system (Figure 2.4a and 2.4b). We also tested rhIL-4 at higher concentrations (20, 50 and 100 ng/ml), as well as different lots of the cytokine and still did not see any effect on our system (data not shown).

Effect of Type 1 exogenous cytokines and neutralizing antibodies on IL-5 production

In order to determine how Th2 cytokines are modulated by Type 1 cytokines in our PIV system, we examined the effects of adding exogenous IL-12, or neutralizing monoclonal antibodies to IL-12 to PBMC that were exposed to *L. major*. Few donors produced IL-5 at detectable levels at day 3 (3 of 7 donors—data not shown), and 5 of 7 donors secreted IL-5 that was detectable by ELISA at day 7. Figure 2.5 illustrates the effects of IL-12 on IL-5 production after 7 days. Although the differences between the *L. major*-stimulated cells and those cells exposed to either rhIL-12 or neutralizing IL-12 antibody were not statistically significant ($p>0.05$), there were some

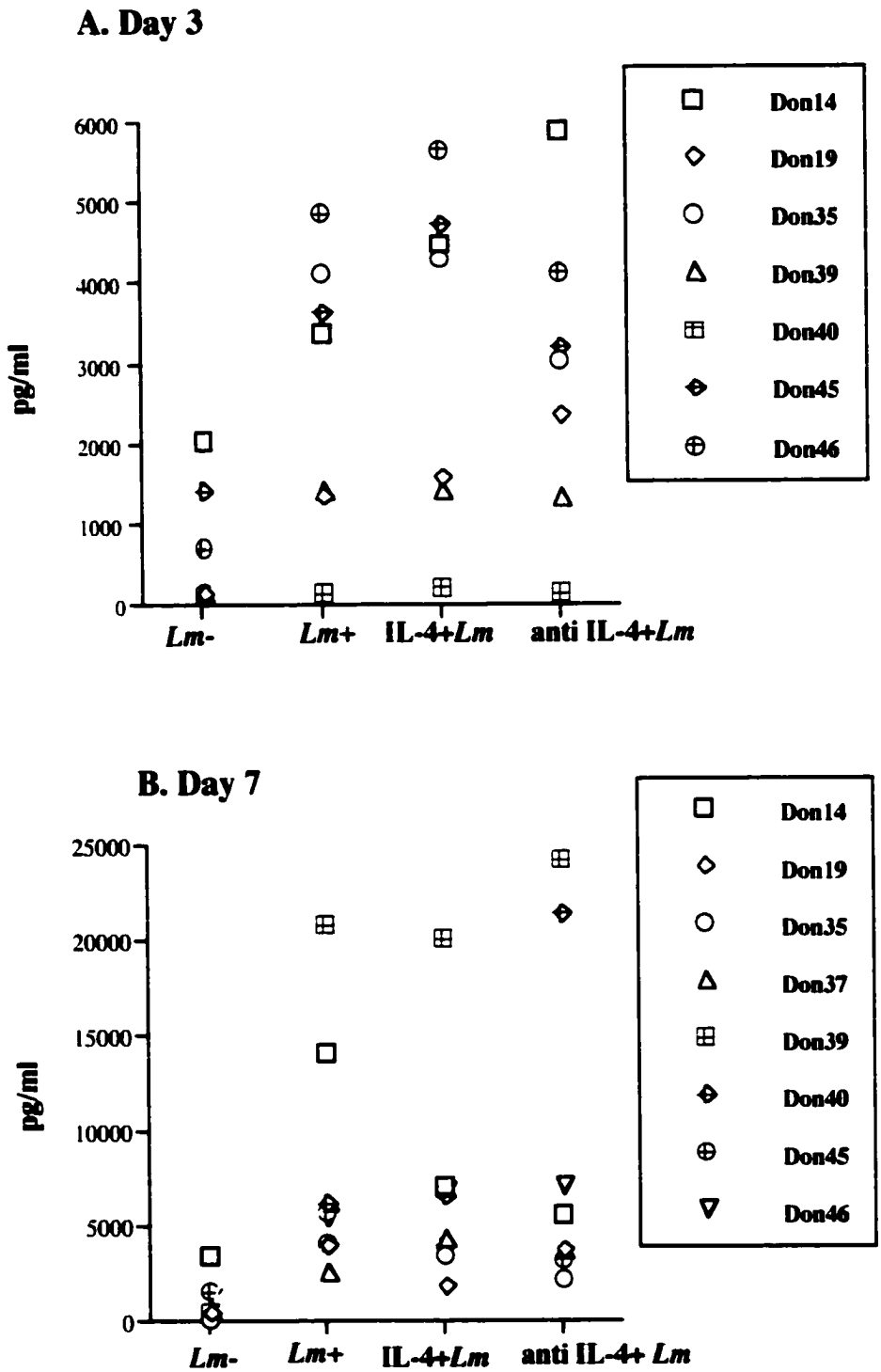


Figure 2.4a and 2.4b The production of IFN-γ at 3 (2.4a) and 7 (2.4b) days after exposure to *L. major*. There are no significant differences between any of the samples.

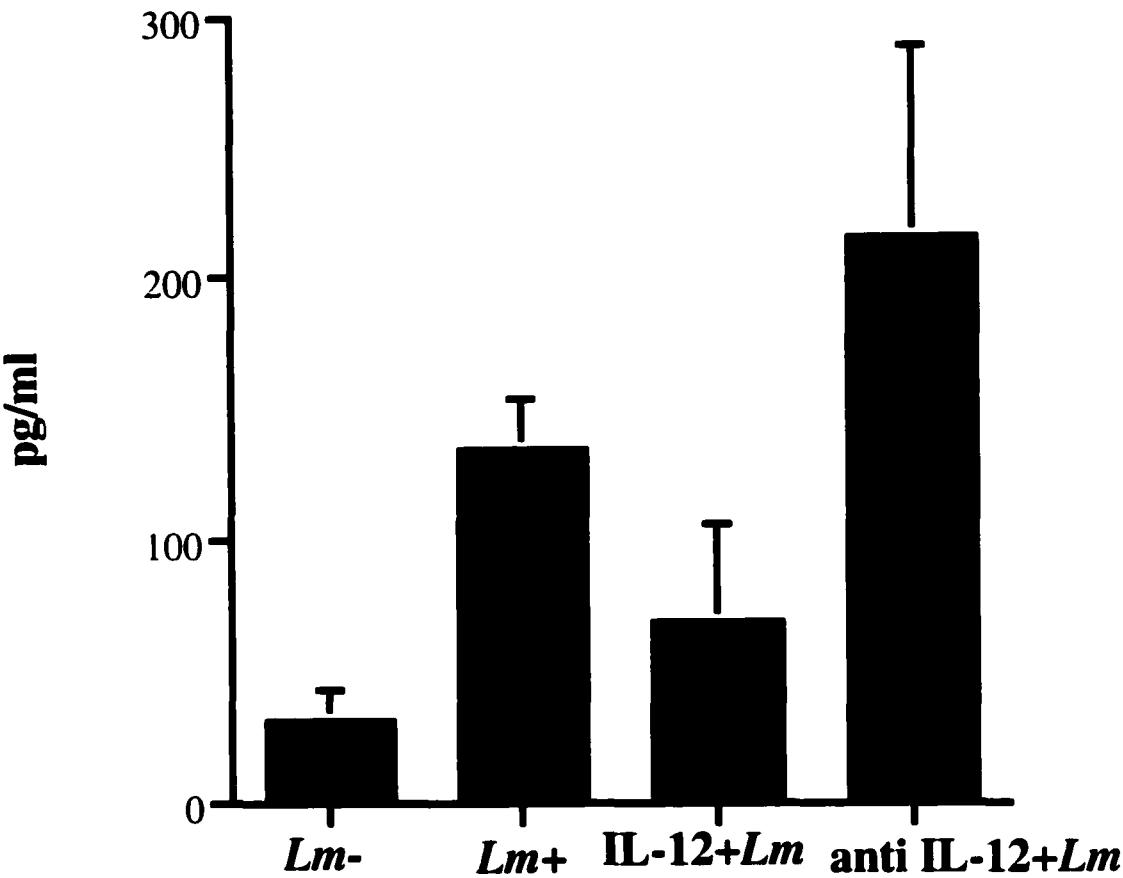


Figure 2.5 IL-5 production of PIV cells exposed to *L. major* for 7 days as measured by ELISA. Data shown as mean+SE. N=5.

marked trends in the data. The rhIL-12 appeared to decrease IL-5 production compared to parasite stimulated treatments. Conversely, neutralizing IL-12 had a tendency to enhance IL-5 production compared to samples with only *L. major* present.

Effect of Type 2 exogenous cytokines and neutralizing antibodies on IL-5 production

We also wanted to determine the effects of Type 2 cytokines on IL-5 production, so we examined the effects of IL-10, TGF-β and IL-4 in the PIV system. Although these cytokines have been shown to exacerbate *Leishmania* infections, there are few reports on the interplay between these cytokines. First, we wanted to determine the influence IL-10 or neutralizing IL-10 had on IL-5 production. The results of this experiment are shown in Figure 2.6. Surprisingly, both the addition of IL-10 and the neutralization of the cytokine significantly decreased ($p<0.05$) IL-5 production compared to the levels produced when the PIV cells were exposed only to *L. major.*

Next, we evaluated the role of TFG-β in modulating IL-5 production. To our knowledge, there have been few reports in the literature describing the interactions between these two cytokines, other than the fact that they can both be detected in some Type 2 responses (Melby et al., 1994; Foucras et a., 2000). The results of this experiment, shown in Figure 2.7 suggest that although the data are not significantly different, neutralizing TGF-β may reduce IL-5 production compared to samples where the cells were exposed to *L. major* only. The addition of TGF-β did not affect the production of IL-5.

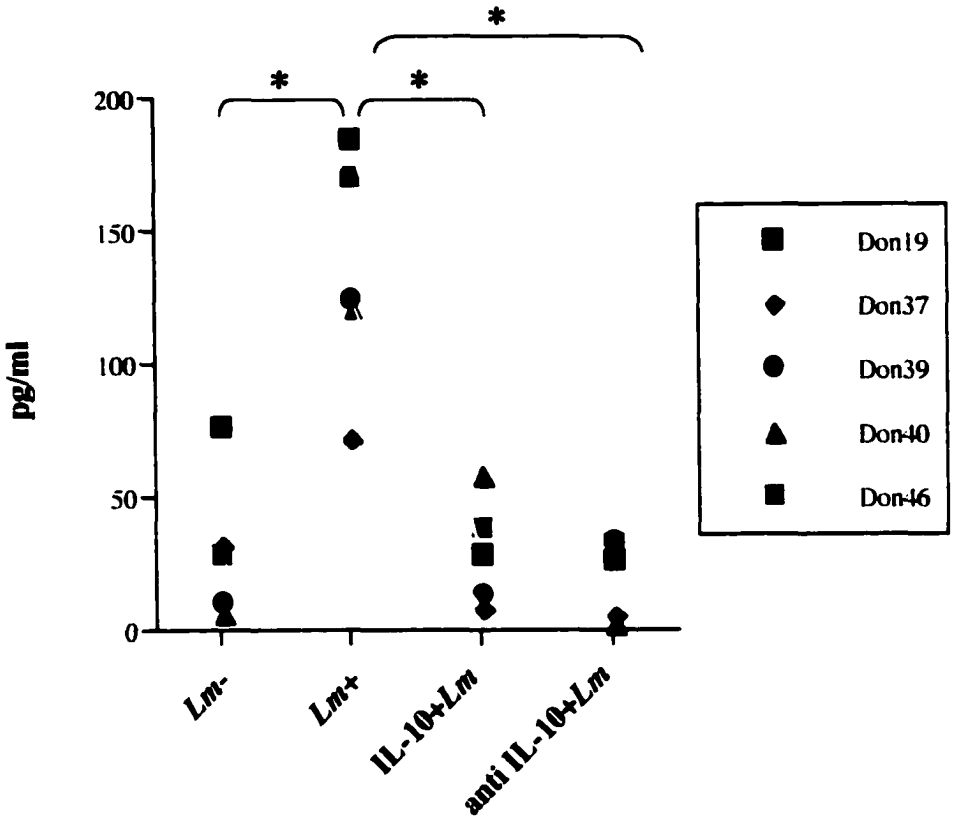


Figure 2.6 The production of IL-5 at 7 days after exposure to *L. major* as measured by ELISA. The lines connecting groups and the '*' denote statistical differences between the sample treatments ($p<0.05$).

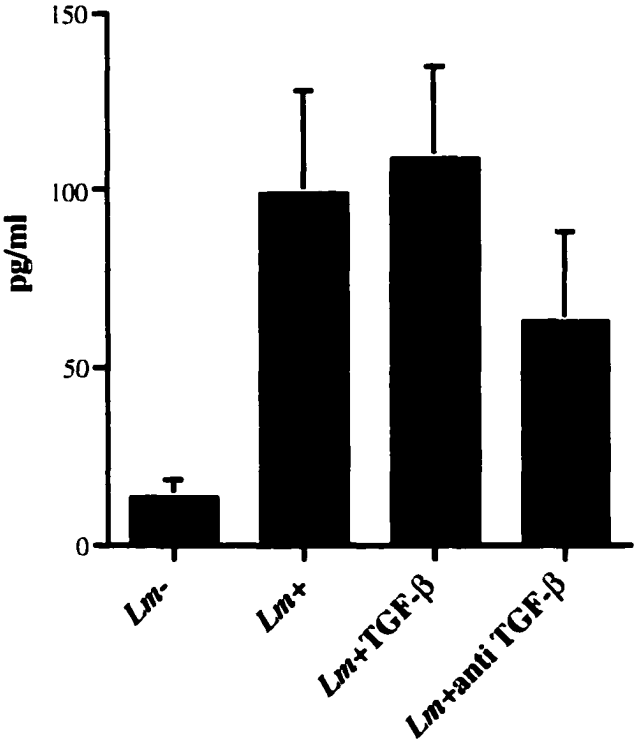


Figure 2.7 IL-5 production of PIV cells exposed to *L. major* for 7 days as measured by ELISA. Data shown as mean+SE. N=5.

Finally, we wanted to determine if exposing PIV cells to IL-4 would affect IL-5 secretion. Along the same line, we hypothesized that blocking IL-4 would also block IL-5 production, as production of these two cytokines are linked in leishmaniasis. Similar to the results we found when we measured IFN-γ production, IL-4 appeared to have little effect upon the PIV cells' ability to secrete IL-5 (Figure 2.8).

Effect of Type 1 cytokines and blocking antibodies on IL-10 production

Production of IL-10 appears to be of great importance in chronic human leishmaniasis, particularly in visceral infections. It is less well understood what role this cytokine plays in acute infections and after the initial exposure to the parasite. To attempt to elucidate the effects of IL-10 after a short-term exposure to the parasite (3 days and 7 days), we exposed PIV cells to *L. major* and either IL-12 or a neutralizing antibody for IL-12 and then measured IL-10 production. At 3 days, there were no differences between the samples (Figure 2.9a), however, by 7 days, there were some surprising differences (Figure 2.9b). Contrary to what we predicted, when PIV cells were exposed to IL-12, there was a significant increase in IL-10 production compared to cells exposed only to the parasite. When IL-12 was blocked, there was little effect on IL-10 production compared to parasite-only exposed cells, however, the secretion of IL-10 was significantly less than the cells exposed to IL-12.

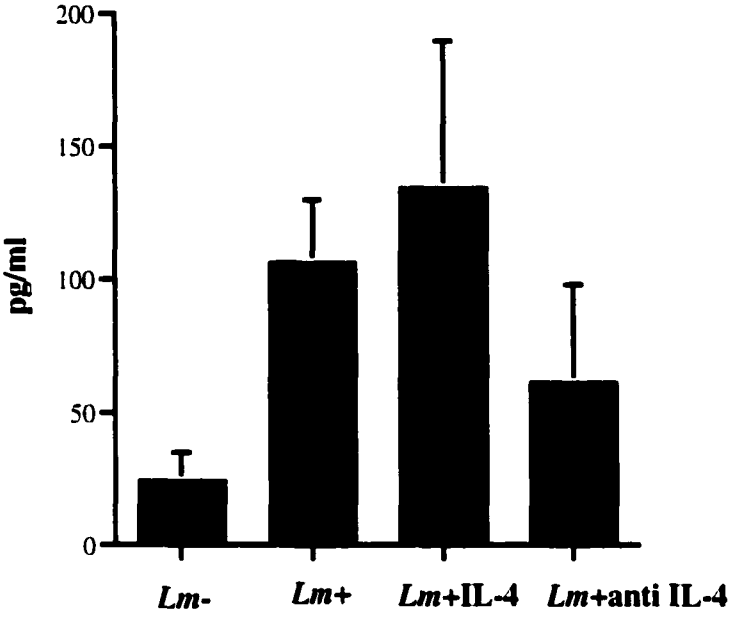


Figure 2.8 IL-5 production of PIV cells exposed to *L. major* for 7 days as measured by ELISA. Data shown as mean+SE. N=7.

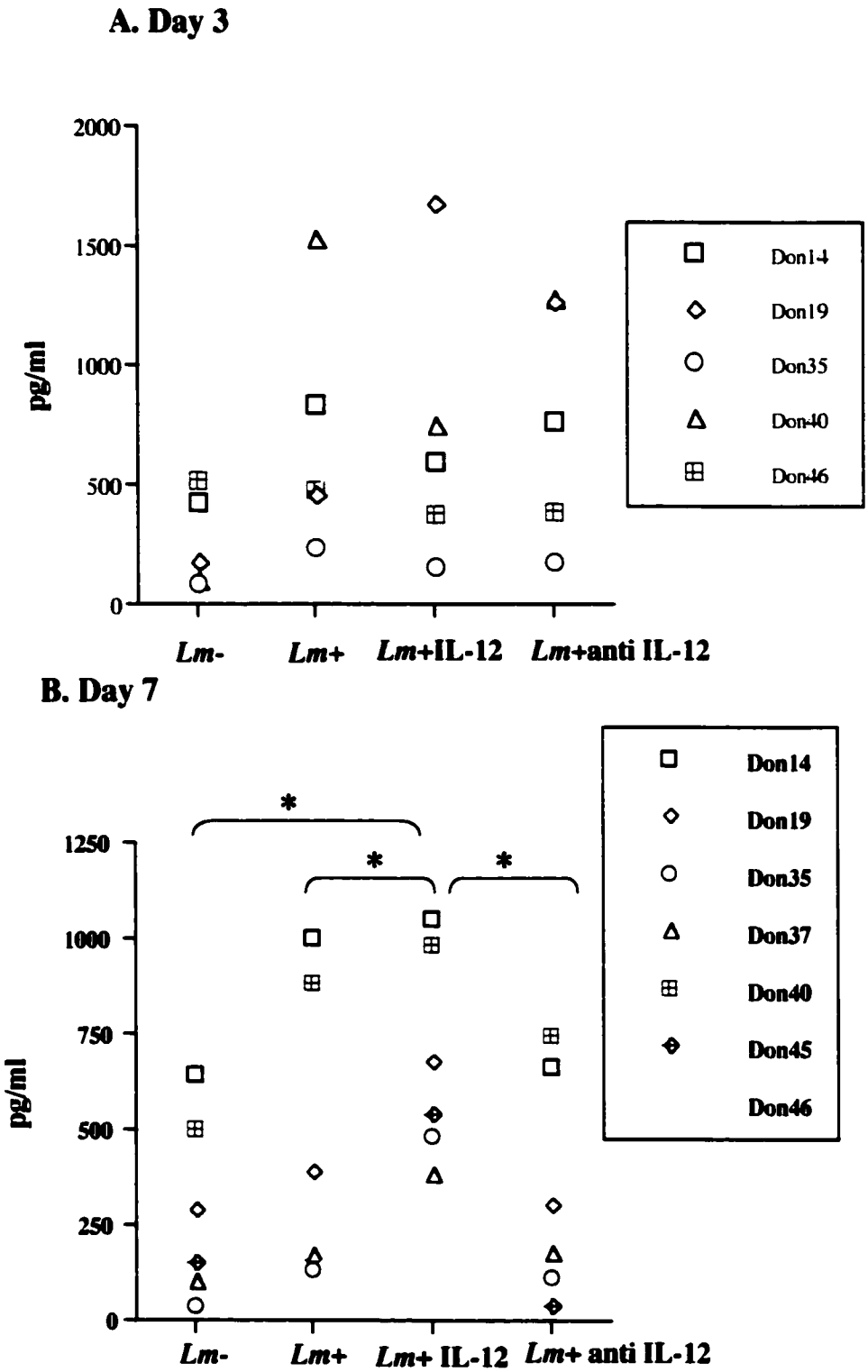


Figure 2.9a and 2.9b The production of IL-10 at day 3 (2.9a) and day 7 (2.9b) after exposure to *L. major*. Lines connecting the groups and the '*' symbol denote differences between treatment groups ($p<0.05$).

Effect of Type 2 cytokines and neutralizing antibodies on IL-10 production

As the interactions between Type 2 cytokines have not been well characterized, we wanted to determine the effects of adding exogenous Type 2 cytokines and their corresponding neutralizing antibodies on IL-10 production. This study was an attempt to determine in the PIV system if there were any notable relationships between IL-10 production and TGF-β or IL-4. As shown in Figures 2.10a and 2.10b, there was no effect on IL-10 secretion when TGF-β was added at concentrations that did significantly modulate IFN-γ production. At day 3, the production of IL-10 by PIV cells exposed either to TGF-β or anti-TGF-β antibody appeared to generally be lower than cells exposed to L. major only. However at day 7, these treated groups produced the same level of IL-10 as the parasite-only exposed cells.

Similar to the other cytokines tested in our PIV system, IL-10 did not appear to be greatly affected by the addition or the neutralization of IL-4 (Figures 2.11a and 2.11b). The data suggest a slight increase in IL-10 production at day 7 when IL-4 is neutralized. Overall, it is interesting to note that unlike the production of IL-5 and IFN-γ, IL-10 production appeared to decrease from the 3 to 7 day time point (the means are 780 pg/ml at 3 days and 419 pg/ml at 7 days).

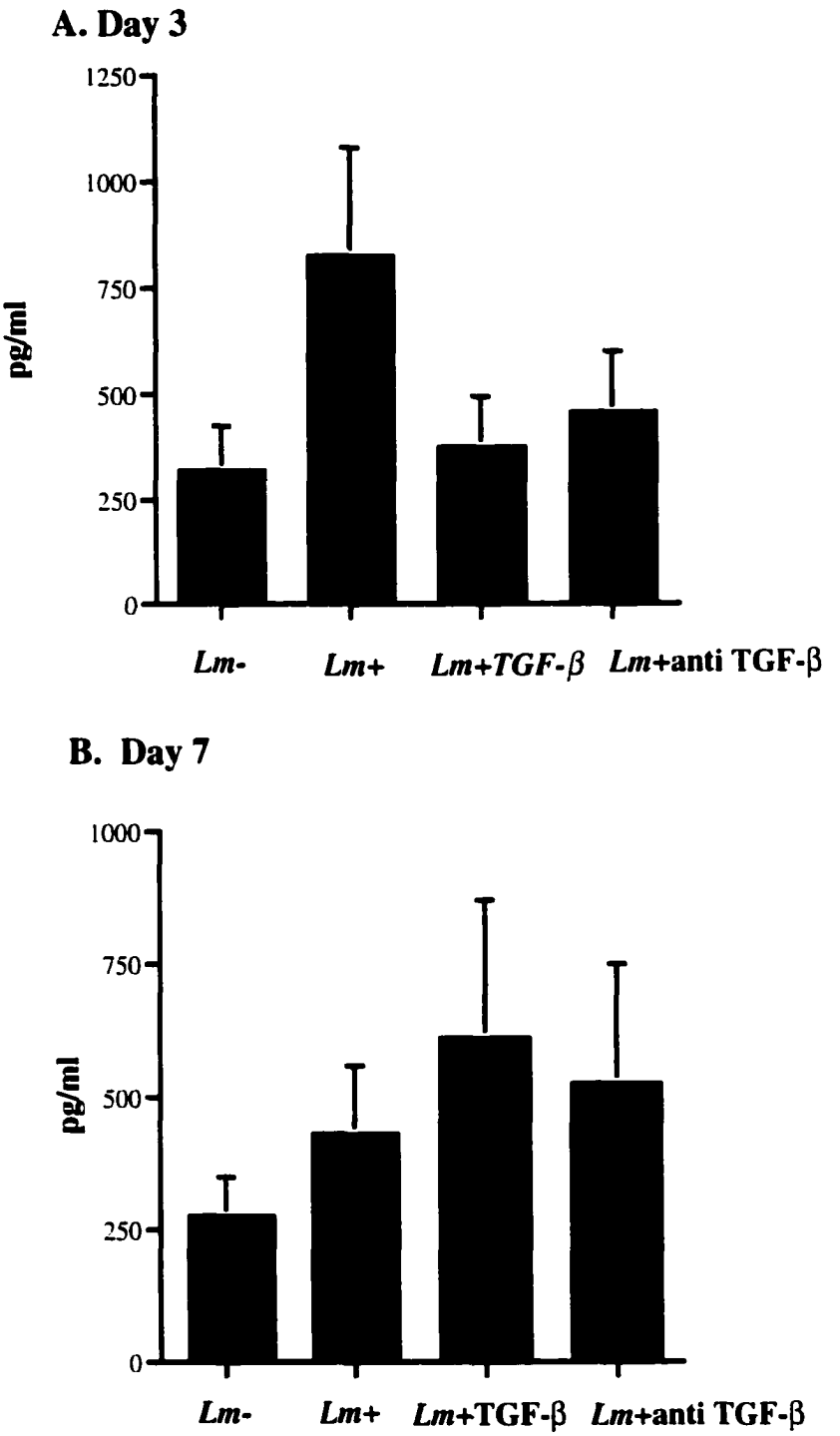


Figure 2.10a and 2.10b IL-10 production of PIV cells exposed to *L. major* for 3 (Fig. 2.10a) and 7 (Fig. 2.10b) days as measured by ELISA. Data shown as mean+SE. N=5 for Fig. 2.10a and N=7 for Fig 2.10b.

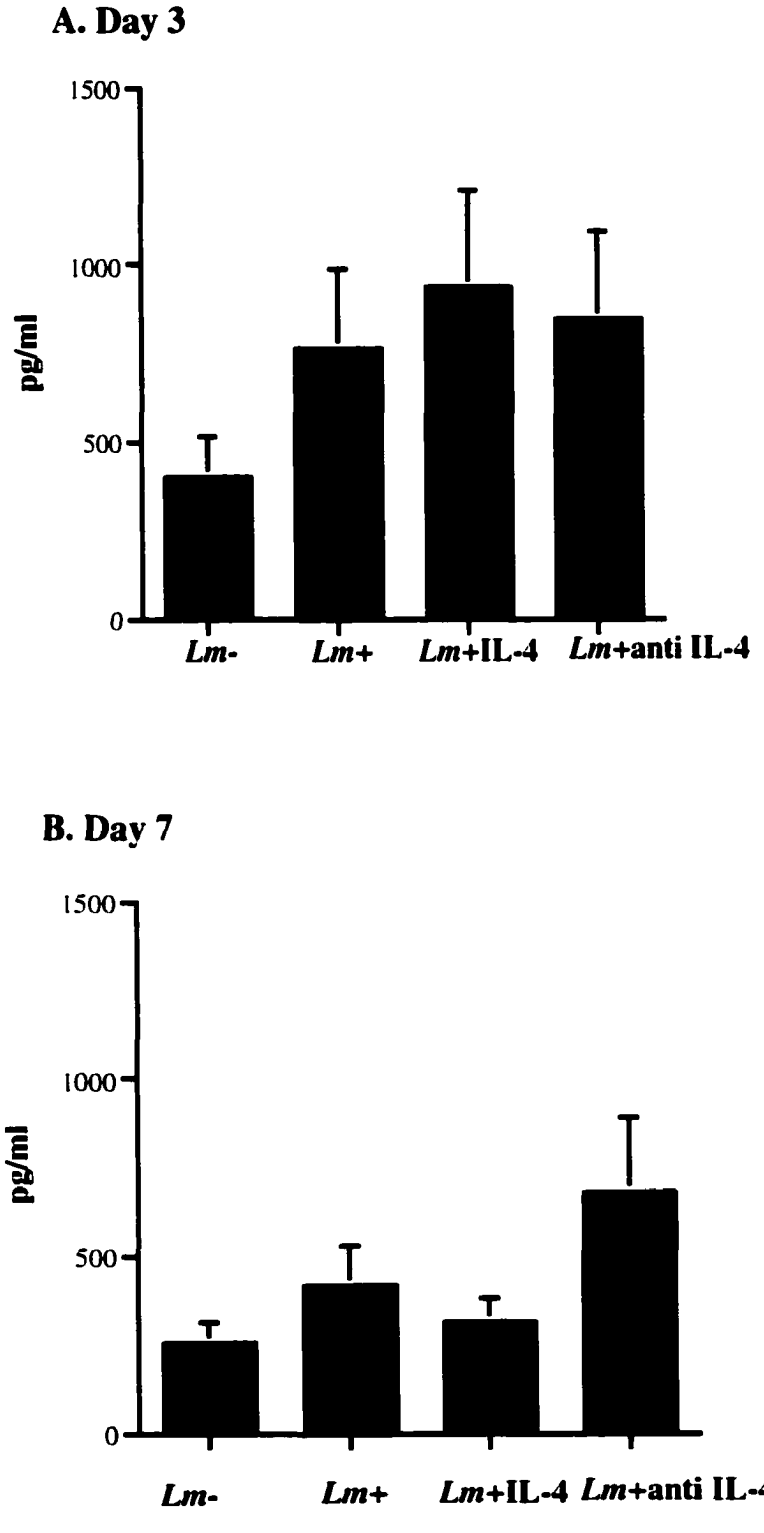


Figure 2.11a and 2.11b IL-10 production of PIV cells exposed to *L. major* for 3 (Fig. 2.11a) and 7 (Fig. 2.11b) days as measured by ELISA. Data shown as mean+SE. N=6 for Fig. 2.10a and N=8 for Fig 2.10b.

DISCUSSION

In these experiments we characterized the influence of cytokines and *L. major* on the polarization of a Th1 or Th2 response in a human PIV system developed from PBMC of *Leishmania*-naïve donors. As we developed our culture system from uninfected donors, we were able to monitor the initial infection and response over a defined period of time. This feature of our PIV system is not possible when *Leishmania*-infected or exposed donor cells are used, as it is not known when the study subjects became infected. Most of the knowledge we have gained detailing the interactions of human cells and *Leishmania sp.* has been obtained by culturing PBMC from *Leishmania*-exposed or infected donors (Akuffo et al., 1997; Kemp et al., 1999; Ajdary et al., 2000; reviewed in Kemp, 1997), thus these responses are more akin to a secondary response.

The polarization of the initial response to the parasite appears to be directed by the local cytokine milieu. To our knowledge, few studies have been reported in the literature regarding the modulation of cytokines of unexposed human cells when these cells are first stimulated with the parasite. Nylen et al. (2001) compared the production of cytokines in cells stimulated with live parasites, parasite antigens, or dead *L. aethopica.* Other studies using naïve donor cells and exposing them to *Leishmania* have primarily focused on cytokine production and not modulation of cytokines during early responses (Young et al., 1995; Akuffo and Britton, 1992).

To address the question of how the addition of various cytokines or neutralizing antibodies to those cytokines will affect a Th1 or Th2 response in *Leishmania*-naive human cells newly exposed to the parasite, we developed two basic hypotheses. First, we hypothesized that adding Type 1 cytokines would enhance an IFN-γ response and that

neutralizing Type1 cytokines would decrease IFN-γ production and enhance IL-5 and IL-10 production. Secondly, we speculated that adding Type 2 cytokines would stimulate IL-5 and IL-10 production and neutralize IFN-γ production. Our results were not as clear cut as findings reported in the mouse model. However, one tenet that held true consistently throughout our study is the fact that in our system, it appears that IFN-γ is the dominant cytokine produced by PBMC responding to the parasite. When cytokines or neutralizing antibodies were added in our system, IFN-γ was consistently modulated. Surprisingly, we found little modulation of IL-10 and IL-5 when we added exogenous cytokines or neutralized them with monoclonal antibodies. In the IFN-γ studies, we did not find striking differences in the production of IFN-γ at days 3 and 7, other than the fact that the levels were higher at day 7. On the other hand, IL-5 was only detected at day 7. Most likely the cytokine was produced at levels below the limits of detection by ELISA at the earlier time points. In contrast, the concentration of IL-10 produced appeared to be slightly higher at day 3 compared to day 7. The transcription of each of these cytokines is regulated differently, which may be a factor in the differences in production. IFN-γ is regulated by T-bet, IL-5 is regulated by Gata-3 and IL-10 does not appear to be well defined, although Gata-3 may play a role (Reiner, 2001; Nawijn et al., 2001).

As our previous study (Chapter 1) indicates, IFN-γ appears to have significant influence in our PIV system. Similarly, in the murine model, IFN-γ production or the neutralization of the cytokine has been shown to influence the early development of Th1 and Th2 responses (Scott, 1991). For this reason, we first studied the effects of adding or neutralizing Type 1 and Type 2 cytokines on the ability of *L. major*-stimulated cells or cells without parasite (controls) to produce IFN-γ. Initially, we determined the effect of

adding IL-12 or neutralizing IL-12 on IFN-γ secretion (Fig. 2.1 a and b). At both 3 and 7 days, adding IL-12 to *L. major*-stimulated cultures significantly increased the production of IFN-γ compared to cells stimulated only with *L. major*. In contrast, when IL-12 was neutralized, secretion of IFN-γ was greatly reduced to the levels of the unstimulated controls. The effects of IL-12 and its influence on the polarization of the Type 1 response are well known (reviewed in Trinchieri,1995). The extremely high levels of IFN-γ produced are most likely a result of a positive feedback loop. When IL-12 is secreted by antigen presenting cells, the cytokine can up-regulate IFN-γ production by NK cells and T cells and in turn the secreted IFN-γ will stimulate macrophages to eradicate intracellular pathogens (Melby et al., 1996; Akuffo et al.,1999). Autocrine stimulation of IFN-γ can also occur (Bradley et al, 1996).

In murine leishmaniasis, the essential nature of IL-12 in biasing T cells to a Th1 response has been established (Sypek et al., 1993; Scharton-Kersten et al., 1995; Gately et al., 1998; Schopf et al., 2001). During the innate immune response or early infection in resistant mice, the IL-12 receptor is up-regulated within two days of parasite infection, whereas in susceptible mice, there is no increase in receptor expression (Jones et al. 1998). Neutralization of this cytokine blocks early IFN-γ production and NK cell responses in resistant mice (Scharton-Kersten et al., 1995). Even later in infection, IL-12 is necessary to maintain the Th1 response and resistance to *L. major* (Park et al., 2000; Stobie et al., 2000). When treating mice to eradicate *Leishmania* infections, this cytokine enhances the ability of leishmanicidal drugs to kill the parasite (Murray et al., 2000).

Despite the fact that several laboratories have reported IL-12 to be down-regulated the first few days after parasite infection (Reiner et al., 1994; Belkaid et al.,

1998; McDowell and Sacks, 1999), we found in our system that IL-12 appeared to have effects after three days. These differences may be due to the use of procyclic phase parasites or metacyclic phase parasites when stimulating the cells. If metacyclic parasites are used to infect cells, a delay in IL-12 production is noted, whereas there is little delay in IL-12 production when procyclic parasites are used (Sartori et al., 1997). We used stationary phase parasites, which had a mixture of both forms of parasites, in these experiments, thus inducting IL-12 production. When IL-12 is neutralized or absent in murine infections, parasite loads and delays in healing can be greatly exacerbated (Sypek, 1993; Engwerda et al., 1998).

The effects of IL-12 on human cells are similar to those reported in murine leishmaniasis. In in vitro systems, when naïve human T cells are sensitized with parasite promastigote antigen and IL-12, the T cells develop into Th1 effector cells (Russo et al., 1999). In studies using patient cells, it has been reported that the PBMC of individuals who have recovered from leishmaniasis produce both IL-12 and IFN-γ when re-exposed to the parasite (Ghalib et al., 1995). In contrast, PBMC from patients with on-going infections did not produce IFN-γ and IL-12 in response to the parasite. In order to properly clear an infection, IL-12 is essential. Evidence suggests that IL-12 can also be important in strategies to control leishmaniasis. Because IL-12 is such a potent inducer of IFN-γ, IL-12 is regarded highly as a potential adjuvant for vaccines and therapy (Herwaldt, 1999; Stobie et al., 2000; Gicheru et al., 2001). Our PIV system demonstrates and reinforces the evidence for the close regulation of IL-12 and IFN-γ because the modulation of IL-12 had such a striking effect on IFN-γ production.

Next, we wanted to determine if IL-12 or the neutralization of IL-12 would down regulate or stimulate, respectively the production of IL-5 and IL-10, which are two cytokines associated with a Th2 response. The results found when we investigated the effects of IL-12 on IL-5 production generally supported our hypothesis. When we measured IL-5 production, due to the wide variation in the production of this cytokine by our donors, our results were not statistically significant, however, there was a marked trend of suppression of IL-5 when IL-12 was added, compared to the *L. major*- stimulated cells (Fig 2.5). Conversely, there appeared to be an increase in IL-5 secretion when IL-12 was neutralized. Others have reported blocking IL-12 induces IL-5 responses in human PBMC and adding exogenous IL-12 has the opposite effect (Verheyen et al., 2000; Brodskyn et al., 2000).

In our study, we also examined the interactions of IL-10 and IL-12. Contrary to what we predicted, there was a significant increase in IL-10 production when IL-12 was added and a decrease in IL-10 production when the cytokine was neutralized (Fig 2.9b). IL-10 can be secreted by macrophages, B cells, dendritic cells and T cells (Moore et al., 2001). Interestingly, in the lesions of cutaneous leishmaniasis patients, similar levels of both IL-12 and IL-10 have been measured (Melby et al, 1996). It has been suggested that the IL-10 may promote disease persistence in cutaneous leishmaniasis by inhibition of macrophage activation rather than by suppressing the Th1 cytokine response (Melby et al., 1996; Vieth et al, 1994). The concentration of IL-12 in the system can also have an effect on IL-10 production. Akuffo et al. (1999) report that at higher exogenously added IL-12 levels, there is simultaneous secretion of IFN-γ and IL-10. In this case, the responding cells were found to be NK cells. Another condition under which both IL-10

and IFN-γ are produced is during the innate immune response to LACK antigen when *Leishmania*-naïve donors cells are used (Maasho et al., 2000). The responding cells in this case were CD8+ T cells and NK cells. It is possible that the responding cells in our PIV system when IL-12 is added are primarily CD8+ and NK cells. Since they were exposed to the parasite, and hence LACK antigen, there could have been an additive effect to increase IL-10 production.

The IL-10 produced in the PIV system may demonstrate the intricate balance of cytokines necessary to maintain control over the parasite. It has been reported that over-production of inflammatory cytokines, such as are produced in the Type 1 response may actually contribute to the pathology of leishmaniasis (Brodskyn et al., 1997; Melby et al., 1996). As IL-10 can downregulate the production of IL-12 and would assist in reducing an overabundance of IL-12, IL-10 may control the overproduction of inflammatory cytokines. Likewise, in the murine PIV model, Soares et al. (1997) suggest that IL-10 regulates IFN-γ production. In our system, the levels of IFN-γ secreted in the PIV system are extremely high when IL-12 is added. Hence, it is likely that the increased production of IL-10 measured when IL-12 is added is a result of IL-10 negatively regulating an extremely high Th1 response.

As Type 2 cytokines are reported to have the opposite effect as Type 1 cytokines in leishmaniasis and cause exacerbation of disease, we evaluated the effect of several Type 2 cytokines on IFN-γ production. Nashed et al. (2000) reported that IL-5 and IL-10 appeared to have an important role early in infection in establishing a Type 2 response in leishmaniasis in mice. To address the influence of Type 2 cytokines in the human system during early exposure, we observed the effects of TGF-β, IL-10 and IL-4 and their

respective neutralizing antibodies on the secretion of IFN-γ in *L. major*-stimulated cells. In cultures exposed to TGF-β or IL-10 or to a neutralizing antibody for TGF-β or IL-10, there were marked effects on IFN-γ secretion (Fig. 2.2 and 2.3). In both cases, when the Type 2 cytokine was added, the production of IFN-γ was greatly reduced in cells stimulated with the parasite. In contrast, when the cytokine of interest was neutralized, there was a significant increase in IFN-γ production compared to the controls. Surprisingly, despite the fact that IL-4 production is well known to cause the polarization of Th cells to Th2 cells in the mouse model, we did not observe an effect of this cytokine on our PIV system, either by adding exogenous IL-4 or neutralizing the cytokine (Fig. 2.4).

TGF-β is capable of modulating an immune response to pathogens by inhibiting T cell proliferation and macrophage activation (Ding et al., 1990; Omer et al., 2000). In animals infected with *Leishmania*, TGF-β production can be greatly enhanced (Rodrigues et al, 1998; Wilson et al., 1998) and resistant strains of mice exposed to exogenous TGF-β have exacerbated infections (Wilson et al., 1998). In contrast, the addition of anti-TGF-β promotes the healing of mice infected with *L. major*, even when only low concentrations of IFN-γ were present, suggesting a profound influence of TGF-β on cytokine production (Li et al., 1999). In human leishmaniasis, TGF-β is present in the lesion sites in patients with early stage infections of the disease (Barral et al., 1995). The addition of TGF-β can also increase parasite loads in infected macrophages, as well as suppress IFN-γ mediated killing. It is possible that in our system, TGF-β may suppress IFN-γ mediated parasite killing since TGF-β decreases IFN-γ substantially.

In light of the knowledge that TGF-β can interfere with Type 1 cytokine responses, an attempt was made by other researchers to determine the mechanism by which TGF-β down-regulates the Th1 response. First, it was discovered that TGF-β suppresses IFN-γ -induced class II MHC expression (Lee et al., 1997). It was then determined that TGF-β does not affect either IL-12 or IFN-γ induced activation of Janus kinases and STATs (Nandan et al., 1997; Sudershan et al., 1999). This suggests that TGF-β is down-regulating events of cell cycling and proliferation. Although we did not test the theory, it is possible in our system that TGF-β may be reducing proliferation of Th1 cells, either by interference with cell cycling or by reducing class II expression of APC and thus decreasing the probability of Th1 cells to become activated and responsive to the parasite.

We also measured the influence of Type 2 cytokines on IL-5 and IL-10 production. We hypothesized that the addition of a Type 2 cytokine would create a "Th2-like" environment and would result in the increased expression of Type 2 cytokines. Type 2 cytokines are often detected at elevated levels in susceptible mice and during chronic human infections. We also suggested that neutralizing the production of theses cytokines would result in the suppression of the Type 2 response. First, we measured the production of IL-5 when exogenous IL-10 was added or blocked (Fig. 2.6) and unexpectedly, either adding or neutralizing IL-10 significantly reduced the production of IL-5. These results suggest that IL-10 may have little effect on IL-5 production. Others have reported similar results. Melby et al. (1994) noted that in intralesional analysis of cutaneous ulcers, IL-5 production was absent, although the level of IL-10 was quite abundant (Melby et al.,1994). In visceral leishmaniasis, there has also been a report of

the absence of IL-5 in bone marrow cells (Karp et al., 1993). It is possible that another cytokine not tested is causing IL-5 suppression when IL-10 is added to the cultures. For instance, Schandene et al. (1996) showed that IFN-α inhibits the production of IL-5 by CD4+ T cells and concurrently there was up-regulation of IFN-α. Thus, it is possible that in the PIV system another cytokine, perhaps IFN-α, is down-regulating IL-5 when IL-10 is added to the cultures. In the case of the IL-10 blocking studies, we observed that there was a high concentration of IFN-γ measured in the supernatant and as a result, this IFN-γ could have decreased the secretion of IL-5.

We next measured the effects of adding or neutralizing TGF-β (Fig 2.7 and 2.10b) on the production of IL-5 and IL-10 and in all cases, TGF-β did not have a significant effect on cytokine secretion of the Type 2 cytokines. It has been reported in neonatal T cells, TGF-β suppresses IL-5 production and has an inconsistent effect on IL-10 production (Demeure et al., 1994). It is possible that TGF-β has no direct effect on IL-5 production. On the other hand, TGF-β has been shown to increase IL-10 mRNA in the draining lymph nodes of mice infected with *L. braziliensis* and decrease IFN-γ secretion (Barral et al., 1993). In our system, we used human PBMC and not mouse lymph node cells. This difference may explain the discrepancy in the results reported in mice and humans. In T cells responding to *Mycobacterium tuberculosis*, it has been reported that both IL-10 and TGF-β are capable of reducing IFN-γ, but that these effects are not synergistic (Rojas et al.,1999). It is likely that although we also show that IL-10 and TGF-β can effect IFN-γ production in *L. major*-exposed PBMC, that these Type 2 cytokines may have little regulatory effect on each other.

In leishmaniasis, IL-10 promotes disease exacerbation. IL-10 has been shown to be involved in IL-12 suppression and the subsequent inhibition of IFN-γ in human infections (Ghalib et al., 1995). In in vivo studies, IL-10 knock out BALB/c are able to control the infection and have smaller lesions while wild type mice are susceptible to disease (Kane and Mosser, 2001). IL-10 can also reduce the ability of macrophages to properly clear infections. Neutralizing IL-10 blocks the inhibition of nitric oxide production in infected macrophages (Bhattacharyya et al., 2001). In contrast, Soares et al (1997) found that resistant mice produced IL-10 after stimulation with *L. major*. However, unlike the Type 2 cytokine IL-4, IL-10 in mice does not appear to cause the differentiation of Th cells to Th2 cells, but instead, because IL-10 can effectively regulate IFN-γ, the cytokine does play a role in the continued polarization of a Th2 response (Chatelain et al., 1999). The importance of IL-10 in murine infections needs to be studied further.

The role of IL-10 in human visceral *Leishmania* infections appears to be more pronounced than in murine infections. The production of this cytokine has been correlated with the exacerbation of the disease in visceral leishmaniasis (VL) (Ghalib et al., 1993). IL-10 inhibits human macrophages from killing the parasite (Vouldoukis et al., 1997). In PBMC blocking studies performed using cells from patients infected with VL and then stimulated with *L. donovani* lysate, there was a role for IL-10 in down-regulating cytokine production (Ghalib et al., 1995). IL-10 abrogated the production of both IFN-γ and IL-12 in PBMC cultures from patients with VL, and conversely, neutralizing IL-10 in the PBMC cultures allowed a shift to a Type 1 response. In patients with post-kala-azar dermal leishmaniasis, an inflammatory complication of VL,

there are high serum levels of IL-10 (Gassim et al., 1998). IL-10 also assists in regulating innate immunity. Depending on the concentration of IL-12, NK cells will produce either IFN-γ or IL-10 (Akuffo et al., 1999). If IL-10 is produced, IFN-γ production and cellular proliferation are abolished. Interestingly, if high levels of IL-12 are secreted, IFN-γ can still be produced and can thus override the negative effects of IL-10 (Akuffo et al., 1999). The stimulation of production of IL-10 may be a mechanism the parasite uses early in infection to establish itself in susceptible hosts.

In contrast to the exacerbating effect IL-10 has in VL in humans, it has been reported that in cutaneous leishmaniasis, there does not appear to be as much of an effect of this cytokine (Akuffo et al., 1997; Kemp et al., 1999; Ajdary et al., 2001). Some of these studies used parasite antigen, however, not live parasite (Kemp et al., 1999; Ajdary et al., 2001) to restimulate cells from actively infected or healed donors. Live parasites may be able to modulate the immune system more than parasite antigen. Akuffo et al. (1997) used *L. aethopica* to stimulate cells from patients with localized leishmaniasis. These patients produced high levels of IFN-γ and low levels of IL-10 and controlled their infections, suggesting that IL-10 plays a limited role in cutaneous leishmaniasis.

Several laboratories have investigated the mechanistic interactions of IL-10 secretion in leishmaniasis. An interesting study by Kane and Moser (2001) indicates that *Leishmania* amastigotes utilize host IgG on their surface as an opsonin that allows them to ligate FcγR on inflammatory macrophages. This ligation induces the production of significant amounts of IL-10, which can then render the cells unresponsive to IFN-γ and could potentially result in decreased macrophage killing. Another IL-10-related mechanism of subversion used by the parasite during infection involves the impairment

of protein kinase C when infected macrophages are exposed to IL-10 (Bhatacharyya et al., 2001). This impairment suppresses the ability of the macrophage to control parasite replication within the host. It would, therefore, be of interest to use our PIV system to study the effects of IL-10 on parasite killing in infected human macrophages.

In our system, as IL-10 was able to greatly suppress IFN-γ production and the neutralization of the cytokine has the opposite effect on IFN-γ secretion, we suggest that IL-10 is indeed playing a role in the PIV system. Based on evidence in the literature (Akuffo et al, 1999) it is possible that IL-10 may affect IFN-γ secretion by NK cells in our PIV system. Although we did not test this theory, the suppression of the Th1 response by NK cells could result in a polarization to a Th2 response.

The lack of a clear pattern of IL-10 production as measured by ELISA in response to exogenous cytokines was unexpected, especially considering how well the IFN-γ data correlated in response to the cytokines we tested. As IL-10, similar to IFN-γ, has been reported to have a regulatory role in human infections, we expected to elucidate a discernable pattern of Type 2 secretion. However, similar to our findings, Brodskyn et al. (2001) also reported the lack of effect on IL-10 secretion when PMBC were stimulated with *L. major* and co-stimulatory molecules were blocked, even though IFN-γ, IL-5 and IL-12 were all affected. IL-10 production was reduced when PBMC were infected with metacyclic promastigotes (Sartori et al., 1997). Although we did not specifically isolate metacyclic populations of parasites in our cultures, based on visual inspection, metacyclics were certainly present. It is possible that the variation we see in the IL-10 data, as well as the lack of any definite trends in most cases, could be due to differences in the numbers of metacyclics present in the parasite cultures used to infect the PBMC.

Another possible explanation for the lack of conclusive IL-10 data using ELISA as a measurement of secreted protein may be that the biologically active IL-10 may be present on the surface of cells and that this IL-10, not secreted IL-10 is involved in stimulating macrophages to kill the parasite. This scenario has been reported in macrophages infected with *Listeria monocytogenes*, an intracellular bacterium (Fleming and Campbell, 1996). Another possibility for the lack of a trend in the IL-10 data is that IL-10 is secreted relatively late compared to other cytokines and appears to be less effective at altering the differentiation of Th cells from their precursors (reviewed in Mossman, 1994). Finally, IL-10 is most likely more important during chronic infections (Melby et al., 1994) and due to this, the effects of IL-10 may not be apparent at the early time points we were studying.

One of the surprising results with the PIV system was the inability of IL-4 to modulate cytokine production when we measured either IFN-γ or the Type 2 cytokines. We had expected the production of IFN-γ to abrogated when IL-4 was added and enhanced when IL-4 was neutralized (Fig. 2.4). We also had anticipated that the addition of IL-4 would polarize the PIV cells towards a Type 2 response and thus would increase levels of secretion of IL-5 and IL-10. IL-4 has been shown to enhance IL-5 and IL-10 production by T cells (Houssiau et al. 1995; Demeure et al., 1994). As with the IFN-γ data however, we again noted no response to IL-4 in this system (Fig 2.8 and 2.11b). Other laboratories report that cells committed to the Th1 pathway become refractory to IL-4 (Nakamura et al., 1997). It is thus possible that because we find such an overwhelming Th1 polarization, as suggested by high IFN-γ production in our system that the PIV cells are not able to respond to IL-4.

In the murine model, the regulatory effect of IL-4 production on the polarization of T cells to a Type 2 response has been well-documented (reviewed in Launois et al., 1998). In susceptible BALB/c mice and IL-12 deficient mice, there is an early peak of IL-4 mRNA in spleens 90 min and in lymph nodes 16 hr after infection with *L. major* (Mattner et al., 1996). This burst of IL-4 has been associated with a subpopulation of T cells expressing the Vβ4-Vα8 T cell receptor chains that recognize a single epitope of the *Leishmania* homologue of the mammalian receptor for activated kinase C, termed LACK (Mougneu et al., 1995; reviewed in Launois et al., 1999). However, in an attempt to determine how LACK affects human T cells, it was found that LACK did not induce IL-4 producing cells (Bottrel et al., 2001). This finding suggests human and mouse cells react to parasite epitopes in different ways and could possibly account for differences in cytokine responses.

When the importance of murine IL-4 was examined using IL-4 deficient mice, which were hypothesized to be resistant to infection, they were unexpectedly susceptible to infection (Noben-Trauth et al., 1996). IL-4 neutralization had little effect on parasite growth in mice (Mattner et al., 1996). A probable reason for these findings is that other Th1 inhibitory cytokines, such as IL-13, IL-10 and TGF-β can contribute to the reduced ability of BALB/c mice to produce IFN-γ (Scharton and Scott, 1993; Noben-Trauth et al., 1999). Other cytokines can also influence the production of IL-4. For instance, IL-6 was shown to initiate the differentiation of IL-4 producing T cells (Rincon et al., 1997).

There are a number of factors which can result in the evolution of a Type 2 response in mice. For instance, the timing of the production of IL-4 can be a factor. In the murine PIV system, cells from BALB/c mice produced 10-fold more IL-4 than cells

from resistant mice after *L. major* stimulation (Soares et al, 1997). The genetics of the mouse strain have been suggested to influence whether IL-4 or IL-12 is the dominant cytokine during the initiation of the immune response (Hondowicz and Scott, 1999). Also, evidence also exists that different parasite strains can also modulate the outcome of a host's response (Hondowicz and Scott, 1999).

The role of IL-4 in human infections is more controversial than in murine infections. IL-4 is generally more difficult to detect than IL-5, IL-10, or INF-γ. In some cases, stimulating cells with ionomycin and phorbol myristate acetate before ELISA measurement is used to detect IL-4 (Kurtzhals et al., 1992). When soluble IL-4 receptors (sIL-4R) were measured in the sera of infected patients, the levels were higher compared to those of uninfected controls (Sang et al., 1999). This receptor was shown to neutralize the biological activity and the immunologic detection of IL-4, thus contributing to low IL-4 measurements (Fernandez-Botran et al., 1996). In order for IL-4 to be detected in stimulated naïve T cells, the IL-4R has to be blocked (Bullens et al., 1999) as IL-4 consumption interferes with attempts to quantify production of this cytokine by human T cells (Bullens et al., 1998). The blocking of sIL-4R of naïve stimulated T cells decreased IL-5 production, but had little effect on IL-10 production. Since IL-5 is so closely associated with IL-4 genetically and the production of IL-4 and IL-5 is linked, this could be a possible reason for the effect seen on IL-5 production and not IL-10. IL-10 is regulated differently than IL-5 (reviewed in Moore et al., 2001). Some studies do report a correlation of IL-4 and the inability to heal in leishmaniasis (Kemp et al., 1993; Adjary et al., 2000). However, other studies have not detected this cytokine or been able to

correlate it with disease exacerbation (Ghalib et al., 1993; Kemp et al., 1994; Carvalho et al., 1994).

It is possible that IL-4 is not as much of a Type 2 regulatory cytokine in human leishmaniasis as it is in murine infections. In lesions of patients with cutaneous leishmaniasis, IL-13, not IL-4, was found to be the predominant cytokine (Bourreau et al., 2001). Similarly, when PBMC of infected donors and naïve donors were stimulated with parasite antigen and the production of IL-13 and IL-4 measured, only the levels of IL-13 appeared to be different between the to study populations (Bourreau et al., 2001). Neutralization of IL-4 had no effect on IFN-γ production, indicating that IL-4 may not be as important in directing the Th2 response in human leishmaniasis. Interestingly, the addition of IL-13, but not IL-4, was able to down-regulate the IL-12Rβ2 chain, a Th2 marker, on T cells. Palmer and van Seventer (1997) were unable to detect IL-4 from T cells isolated from PBMC even in the presence neutralizing anti-IL-4R. Thus there is increasing evidence that cytokines other than, or in addition to, IL-4 are necessary for the maintenance of the Type 2 response. Future studies should also focus on the effects of IL-13 and IL-6 in polarizing PIV cells towards a Th2 response.

The lack of an effect of IL-4 in our human system could potentially be due to the down regulation of IL-4 receptor on the surface of the PIV T cells. IFN-γ is capable of down regulating IL-4 receptor gene expression and can thus antagonize the IL-4 response in human cells (So et al. 2000). It is possible with the fairly high levels of IFN-γ being produced in the PIV system that the receptors to IL-4 are being downregulated, and thus the cells are not able to respond to exogenous IL-4. Another possible reason for the widespread variation in the effects of IL-4 between murine and human studies could be

due to where the cells are taken from for analysis. For instance, IL-4 production was found in spleen, but not kidney cells in mice infected with *Staphylococcus aureus* (Rincon et al., 1997). Sornasse et al., (1996) reported that human naïve neonatal T cells required multiple days of priming and additional stimulation in the presence of IL-4 in order to polarize into Th2 cells. It is possible in our system that the Th2 cells are not polarizing early after parasite exposure and thus the Type 2 response is different than what has been reported in the murine model.

In human leishmaniasis, Uyemura et al. (1993) reported the differences in the response of host cells to the parasite depending on if cells were located in the lesion or were PBMC. Similarly, in the murine model, depending on the site of inoculation and the tissue studied, there are differences in cytokine production (Melby et al., 1998). The differences in cytokine production noted in our PIV system, when comparing to the mouse PIV system could possibly be attributed to the fact that the murine PIV involves cells taken from the spleens of mice and the human PIV system uses PBMC. PBMC are differentiated mononuclear cells, while the spleen is a lymphoid organ where adaptive immunity can occur. Cells taken from different areas of the body can express different receptors, could be in different stages of maturity, be confined to different organ architecture and thus respond differently to parasite stimulation.

The lack of a Type 2 response could also be attributed the lack of neutrophils in the system. In BALB/c mice, neutrophils were shown to contribute to the early burst (24 h after infection) of IL-4 and that depleting neutrophils inhibited Th2 development (Tacchini-Cottier et a., 2000). As neutrophils are removed in our system, this could possibly explain the lack of a definitive Type 2 response early after parasite exposure.

Also, the chemokine monocyte chemoattractant protein-1 (MCP-1) has been implicated in the stimulation of IL-4 and the polarization of naïve Th cells to Th2 cells (Gu et al., 2000). MCP-1 knock out mice do not produce IL-5 and IL-10 and have reduced levels of IL-4 (Gu et al., 2000). MCP-1 can be produced by monocytes, fibroblasts and keratinocytes. It is possible that MCP-1 is not being activated in vitro as it can be in vivo and thus, is not stimulating the production of Th2 cells. Although our data suggests that Th1 cells are being activated, it is possible that the conditions are not optimal for the activation of Th2 cells.

Overall, this study indicates that the donors studied had rather potent Th1 responses to *L. major* and suggests that the donors would be able to clear in infection efficiently. IFN-γ appears to be the primary cytokine modulated during the PIV response to the parasite. Similar to the overwhelming IFN-γ response, IL-12, a cytokine which causes T cells to produce IFN-γ, appeared to have an effect on Type 2 responses, decreasing IL-5 production and stimulating IL-10 secretion. IL-5 and IL-10 were not affected substantially by the addition or neutralization of several Type 2 cytokines, suggesting that this group of cytokines is not involved in cross-regulating each other. Also, the PIV culture may not provide the optimal environment for Th2 development due to the lack of Type-2 driving cells or proteins. Another reason for the apparent lack of response to Type 2 cytokines and inability to discern any trends in cytokine secretion could be due to the fact that the IFN-γ being secreted under most test conditions suppresses the ability of Type 2 cytokines to function at their full potential. This PIV system will allow for the further investigation of the responses that occur during the initial priming of *Leishmania*-naïve cells to *L. major*.

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CHAPTER THREE

THE IMMUNOMODULATORY EFFECTS OF SAND FLY SALIVARY GLAND PROTEINS ON THE HUMAN IN VITRO IMMUNE RESPONSE

INTRODUCTION

Leishmania parasites are transmitted to mammalian hosts through the bite of an infected phlebotomine sand fly as the insect takes a blood meal. Only female sand flies are hematophagous (blood feeding) and require a blood meal every 4-5 days for egg development (reviewed in Kamhawi, 2000). As a sand fly attempts to feed, it lacerates dermal capillaries and subsequently feeds on the pools of blood that form (reviewed in Titus et al., 1994). When a sand fly probes the skin for a meal, a number of salivary factors that facilitate the taking of blood, such as anticoagulants, antiplatelet and vasodilatory molecules, as well as parasites, can be injected into the bite site (reviewed in Ribero, 1987; Gillespie et al., 2000; Kamhawi, 2000).

Once the parasites are injected into the bite wound, phagocytic cells such as macrophages and dendritic cells engulf the pathogen (reviewed in Rittig and Bogdan, 2000). In addition to dendritic cells, macrophages also engulf *Leishmania*. Macrophages are the primary cells of residence for *Leishmania* and are also the cells most capable of eradicating the parasite. *Leishmania*-exposed dendritic cells can interact with T cells to prime them to respond to *Leishmania* antigens and when the T cells are activated,

they secrete IFN-γ which will activate the infected macrophages to eradicate the parasite (Moll, 1993; Banchereau and Steinman, 1998).

In leishmaniasis, control of the infection is related to an appropriate cellular immune response. Cytokines play an essential role in the determination of either a Type 1 or Type 2 cytokine response. The Type 1 response, which will enable a host to heal, is characterized by the secretion of IFN-γ and IL-12, whereas in a Type 2 response, marked by production of IL-4, IL-5, IL-6 and IL-10, will result in exacerbation of the disease (reviewed in Launois et al., 1998; Etges and Muller, 1998). *L. major* is capable of decreasing the ability of host cells to respond to infection in a number of ways, including using host complement components, such as C3 to suppress the respiratory burst (Mosser and Edelson 1987), as well as reducing production of IL-12 and TNF-α (Reiner et al., 1994), which can then suppress activation of T cells. Despite *L. major's* ability to prevent the development of immunity in certain hosts, particularly BALB/c mice and immuno-compromised people, *Leishmania* is unable to cause serious infection in resistant strains of mice, such as C3H, and in many people. In endemic areas, a portion of the population has subclinical infections or is able to self-heal if the person does develop an infection (Herwaldt, 1999).

In order to propagate effectively, it is believed by many that the parasite has co-evolved with the sand fly vector (Kamhawi, 2000) to use the sand fly salivary gland proteins as a mechanism to better enhance infectivity. Sand fly saliva of both Old and New World sand flies is immunomodulatory. In concert with the parasite or other stimuli, the in vitro and in vivo immunological responses of the host can be altered dramatically. As *Leishmania* predominantly resides in the macrophage during infection,

many studies have focused on the interactions between the macrophage and salivary gland proteins (reviewed in Gillespie et al., 2000; Kamhawi, 2000). These proteins are capable of altering the early interactions between macrophages and parasites. For instance, macrophage chemotaxis occurs in the presence of salivary glands and a greater number of parasites are phagocytized when salivary gland protein is present (Zer et al., 2001). It was suggested that this may be a mechanism to enhance parasite access to the macrophage. The immunological relationship between the parasite and macrophage is also modulated by salivary gland proteins. For example, salivary gland lysate inhibits the ability of macrophages to present *L. major* antigens to parasite-specific T cells (Theodos and Titus, 1991). The ability of macrophages to be activated by IFN-γ (a crucial step to eradication of parasites) and to produce nitric oxide is suppressed by salivary gland proteins as well (Hall and Titus, 1995).

Salivary gland proteins are also capable of modulating responses in vivo in murine models. Titus and Ribeiro (1988) were the first to report that sand fly salivary gland lysates enhance *L. major* infectivity in both resistant and susceptible strains of mice. The presence of saliva enhanced the lesion size of mice injected with as few as 10^1 or 10^2 parasites. Cytokine production in infected mice can also be modulated by salivary proteins. IL-4 production is higher in saliva-exposed mice and IFN-γ and IL-12 are reduced (Lima and Titus, 1996; Mbow et al., 1998). Likewise, the actual bite of a sand fly causes similar kinds of reactions. The lesions that occur when a sand fly is allowed to probe for a blood meal resemble those caused when mice are injected with salivary gland lysates and parasites (Theodos et al., 1991). Vaccination with either salivary gland proteins or sand fly bites from uninfected sand flies offer protection upon subsequent

exposure to *L. major* (Belkaid et al., 1998; Kamhawi et al., 2000). These authors suggest that sand fly saliva may be a potential vaccine candidate.

The bite of sand flies causes a localized erythema that does not produce itching or edema. This erythema is caused by a vasodilator present in salivary gland extracts of the New World sand fly *Lutzomia longipalpis* (Ribeiro et al.,1986; Ribeiro et al., 1989). From the saliva of this sand fly, a 63 amino acid peptide was isolated, its gene cloned and based on that sequence, it was synthesized and shown to have potent vasodilatory properties (Lerner et al., 1991; Lerner and Shoemaker, 1992). This peptide is called Maxadilan (Max). Similar to whole salivary gland proteins, Max is also capable of influencing the immune response. Most strikingly, Max can exacerbate infection with *L. major* to comparable levels as whole saliva (Morris et al., submitted). In cell culture, Max inhibits proliferation of murine T cells responding to concanavalin A (Qureshi et al., 1996). The peptide also modulates cytokine production. In the presence of Max, murine macrophages, as well as in vivo studies using mice exposed to the peptide, exhibit suppressed TNF-α production and enhanced IL-6 production (Soares et al., 1998; Bozza et al., 1998). Max also diminishes delayed type hypersensitivity reactions in vivo (Qureshi et al., 1996). Similar to whole saliva, this salivary gland peptide is also a potential vaccine candidate. Morris et al. (submitted) have reported that vaccinating with Max protects against infection with *L. major*.

Interestingly, Max was found to bind to brain tissue and neural cells, and to be a potent vasorelaxant (Grevelink et al., 1995; Moro et al., 1995; Jackson et al., 1996; Eggenberger 1999). Due to these findings, the ability of Max to activate neuropeptide receptors was studied and surprisingly, Max was found to be an agonist of the type I

receptor for pituitary adenylate cyclase activating peptide (PACAP) (Moro and Lerner, 1997). These two peptides share little sequence homology, although both share similar immuno-modulatory properties. Receptors for PACAP are found on macrophages. PACAP stimulates IL-6 production by bone marrow stromal cells (Cai et al., 1997), inhibits the production of IL-12, IFN-γ and TNF-α (Delgado et al., 1999; Delgado and Ganea, 2000), and enhances IL-10 production (Delgado et al., 1999) in vitro and vivo. Similar to reports with Max and saliva, PACAP can also decrease T cell proliferation (Ganea and Delgado, 1996).

To our knowledge, little work has been published on the effects salivary gland proteins have on human cells. It is of interest to determine if the immunomodulatory effects that salivary gland lysate and Max have in murine systems in vivo and in vitro are also found in using human cells. If salivary gland proteins are to be considered as vaccine candidates, it is essential to know what effects, if any, these proteins have on a human immune response. Therefore, we hypothesized that Max or salivary gland proteins would suppress a Th1 response (i.e.: decrease IFN-γ, IL-12, TNF-α production) and would enhance a Th2 response (i.e.: increase IL-10, IL6) in human PBMC or macrophage cultures, as similar effects have been reported in the murine system. We also hypothesized that Max would interact with human macrophages using the PACAP receptor. To begin to address these hypotheses, we studied the interactions of *Leishmania*-naïve human PBMC, *L. major* or LPS and Max or salivary gland lysate. We found that IFN-γ secretion is reduced in salivary protein exposed donor cells. As much of the literature published used macrophage cultures to study the interactions between the host response to stimuli and salivary gland proteins, we continued our experiments using

similar cell cultures. We found that Max increases IL-6 secretion. Max also appears to interact with macrophages via the PACAP receptor.

MATERIALS AND METHODS

Parasites

L. major (isolate LV39, Rho/SV/59/P) parasites were grown on biphasic NNN medium (89) and passed through mice every two weeks to maintain virulence. Promastigotes were harvested in the stationary growth phase.

Sand fly salivary gland lysate, PACAP 6-38 and synthetic Maxadilan

Salivary glands of *Phlebotomus papatasi* (Israel isolate) were collected as previously described (Titus and Ribeiro, 1988). Briefly, salivary glands from non-blood fed 5-7 day old adult female sand flies were dissected out under saline, placed in 0.1% BSA in water (pH 7.0) and frozen to achieve complete disruption. The glands were brought to isotonicity by the addition of 10x PBS. The glands were stored at -80°C until use. Glands were used at a concentration of 0.5 gland per ml. Pitiutary adenlylate cyclase activating polypeptide (6-38) amide (PACAP-6-38) (Bachem Bioscience Inc: Kind of Prussia, PA), an antagonist for the PACAP-38 receptor was used at a concentration of10 μg/ml. Synthetic maxadilan was prepared by Macromolecular Resources (Colorado State University, Fort Collins, CO). The 63-mer amino acid sequence used was based on the predicted sequence of mature, secreted Max (Lanzaro et al., 1999).

(CDATCQFRKAIEDCRKKAHHSDVLQTSVQTTATFTSMDTSQLPGSGVFKECMK EKAKEFKAGK). Max was used at concentrations of 5 and10 ng/ml.

In vitro system-PBMC cultures

The in vitro system we developed was patterned after the murine in vitro system described by Soares et al (1997). Human blood was obtained from healthy individuals at the Hartshorn Health Center at Colorado State University. 100 ml of blood was collected during each blood draw. Blood was drawn after informed consent was obtained from each donor. All procedures were approved by the Human Research Committee at Colorado State University. PBMC were isolated from heparinized venous blood by passage over a Histopaque-1077 (Sigma Diagnostics; St. Louis, MO) gradient (Goldrosen et al., 1977). PBMC were washed three times and resuspended at a concentration of $2x10^6$ cells/ml in complete medium consisting of RPMI-1640 medium supplemented with 2 mM $_L$-glutamine, penicillin (100 U/ml), gentamycin (100 µg/ml), and 10% heat inactivated human AB serum (Pel-Freeze; Brown Deer, WI). The cells were plated in 24-well tissue culture plates (Costar; Corning, NY) at 1 ml/well and incubated at 37°C with 5% CO_2. Cells were pre-incubated for three hours with either Max or salivary glands before stimuli (*L. major* at $4x10^5$/ml or LPS at 200 ng/ml) were added. To study early time point interactions between the PBMC, Max or salivary glands and *L. major*, culture supernatants were harvested for cytokine analysis at 48 and 72 h after exposure.

In vitro system-monocyte/ macrophage cultures

For macrophage studies, after the PBMC from the above step were isolated by Histopaque, the cells were washed and resuspended at a concentration of $4x10^6$ cells/ml in RPMI complete medium in 24-well tissue culture plates. The macrophage/maxadilan system established by Soares et al. (1998) was used as a guide. The monocytic cells were allowed to adhere overnight and non-adherent cells were removed from the wells. The macrophages /monocytes were then pretreated with either Max at 5ng/ml or 10 ng/ml for 3 hours, followed by LPS stimulation (from *Escherichia coli* serotype 0111:B4, Sigma Chemical, St. Louis, MO; at 200 ng/ml) or parasite stimulation. *L. major* was added at a concentration of 5 parasites to 1 macrophage. In the case of the PACAP 6-38 experiments, 10µg/ml of PACAP 6-38 was added to the macrophage cultures and the cells were incubated for 2 hours. The cells were then pretreated with Max for 2 hours and then LPS added. Supernatants were collected at 6 and 18 h after stimulation.

Cytokine assays

Concentrations of IFN-γ, TNF-α, IL-10, IL-6 and IL-5 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (Pharmingen Beckton Dickenson; San Diego, CA). The limits of detection for these assays were 10 pg/ml for IFN-γ, 6.3 pg/ml for IL-10, 2 pg/ml for IL-5, and 10pg/ml for IL-6 and TNF. IL-12p40 and IL-12p70 concentrations were measured by ELISA using commercial kits from R&D Systems (Minneapolis, MN). The limit of detection for IL-12p40 and IL-12p70 was 6 pg/ml.

Statistical analyses

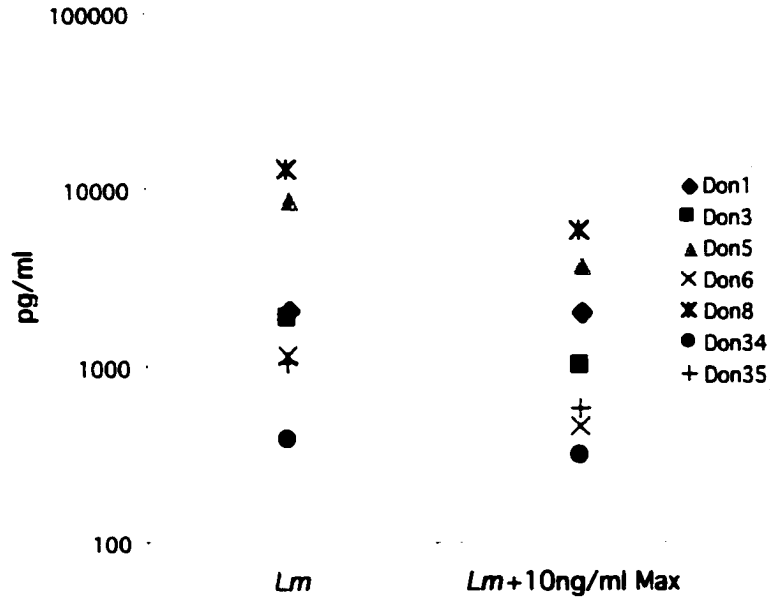
Statistical analyses were performed using Sigma Stat (SPSS, Chicago, IL) and InStat (Graph Pad Software, San Diego, CA) software. Due to the small sample sizes and inherent variances of human responses, the data were not distributed normally and thus, non-parametric analyses were necessary. For the experiments determining the effects of Max or salivary gland lysate on cytokine production, data were compared using the Wilcoxon signed ranked test. To ensure the pairing of data was effective, nonparametric Spearman correlations were performed. For comparison of multiple groups in the PACAP 6-38 studies, Kruskal-Wallis one-way ANOVAs were performed. When $p<0.05$, the Student-Newman-Keuls method was used to determine the significant differences between the samples. In all cases, results were considered significantly different when the p value was <0.05.

RESULTS

Effects of Maxadilan on Type 1 cytokine production of PBMC

We first wanted to determine the influence of the sand fly salivary gland protein, Maxadilan, on the production of cytokines. As IFN-γ has been shown in our other studies (Chapters 1 and 2) to be the cytokine primarily produced when cells are exposed to *L. major*, we examined the impact exposure to Max would have on cytokine production of *L. major*-stimulated cells. As shown in Figure 3.1, the addition of Max to the cultures causes a statistically significant reduction ($p<0.05$) in IFN-γ production at both 48 and 72 h after parasite exposure.

A. 48 h



B. 72h

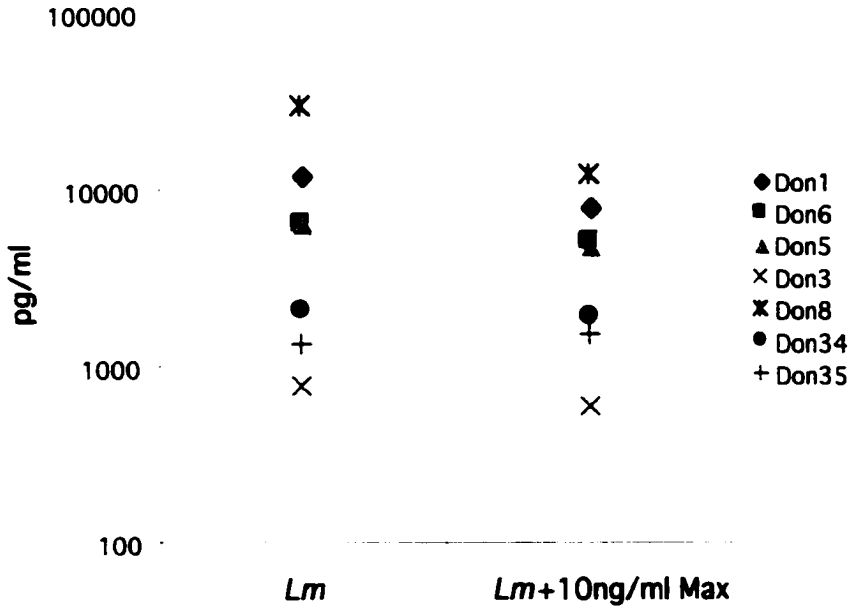


Figure 3.1a and 3.1b Max decreases IFN-γ production of *L. major*-stimulated PBMC at 48h (3.1a) and 72h (3.1b) after parasite exposure. There is a statistically significant difference of cells exposed to the parasite and Max compared to the cells exposed to *L. major* only. Background levels of IFN-γ were subtracted from the samples shown. Data are expressed as median values of duplicate experiments.

Since we had determined in previous studies that IL-12 is secreted early after exposure to the parasite (Chapter 1), and since IL-12 is produced by antigen presenting cells such as macrophages and dendritic cells that would likely first have exposure to the parasite and sand fly saliva in the case of natural transmission, we also wanted to evaluate the effect of Max on IL-12 production. As there have been suggestions that IL-12p70 and IL-12p40 are differentially detected in *Leishmania* infections (Gorak et al., 1998: Belkaid et al., 1998a), we decided to evaluate the secretion of both IL-12 molecules. IL-12 is a covalently linked 70 kDa heterodimer composed of two chains, p40 and p35. The p40 transcript is found only in cells producing biologically active IL-12 (reviewed in McDowell and Sacks, 1999). IL-12p40 is not bioactive, while IL-12p70 is. When we exposed PMBC to Max and *L. major*, we did not detect a significant difference at 48h in IL-12p40 production between the parasite-stimulated cells and cells exposed to Max and *L. major* (data not shown). However, at 72 h, there was a significant decrease in IL-12p40 production when the cells were exposed to 10ng/ml Max and *L. major*, compared to cells exposed to the parasite only (Figure 3.2). A distinct trend was not as apparent when 5 ng/ml Max was used. Although the data were not significantly different ($p=0.09$), Max also decreased IL-12p70 production when it was added at 5 ng/ml to cultures of PBMC stimulated with *L. major* for 48 h (Figure 3.3). No trends of IL-12p70 secretion were observed with other concentrations of Max or at the 72 h time point.

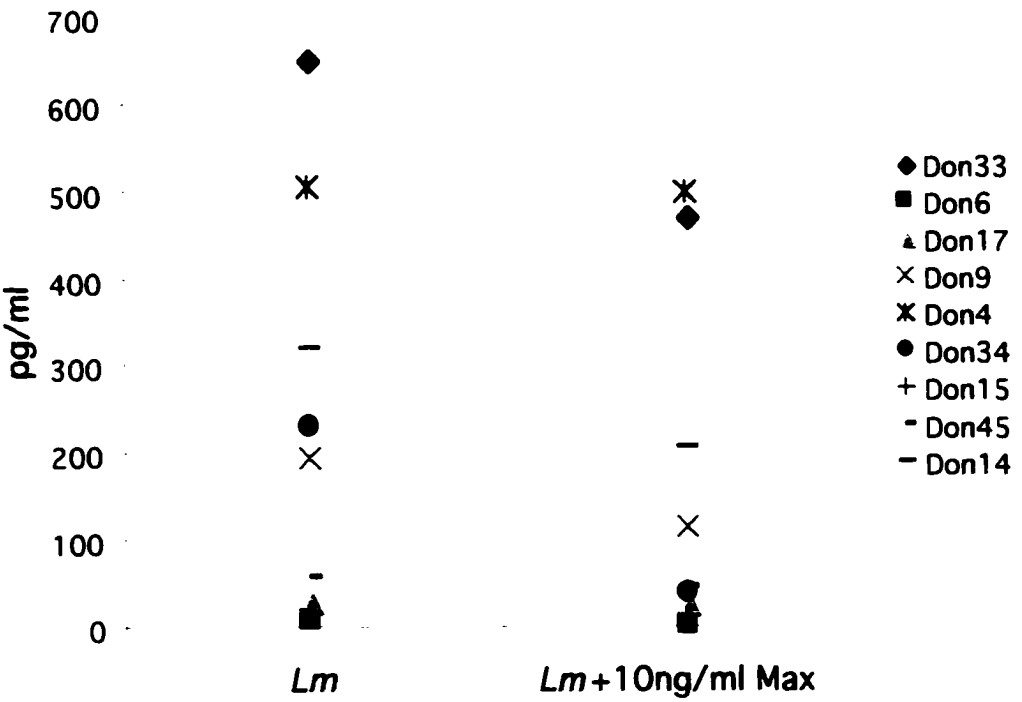


Figure 3.2 Maxadilan significantly reduces the secretion of IL-12p40 by PBMC from *Leishmania*-naïve donors exposed to the parasite and Max, compared to donor cells exposed only to *L. major* ($p<0.05$). These data are after 72 h of stimulation.

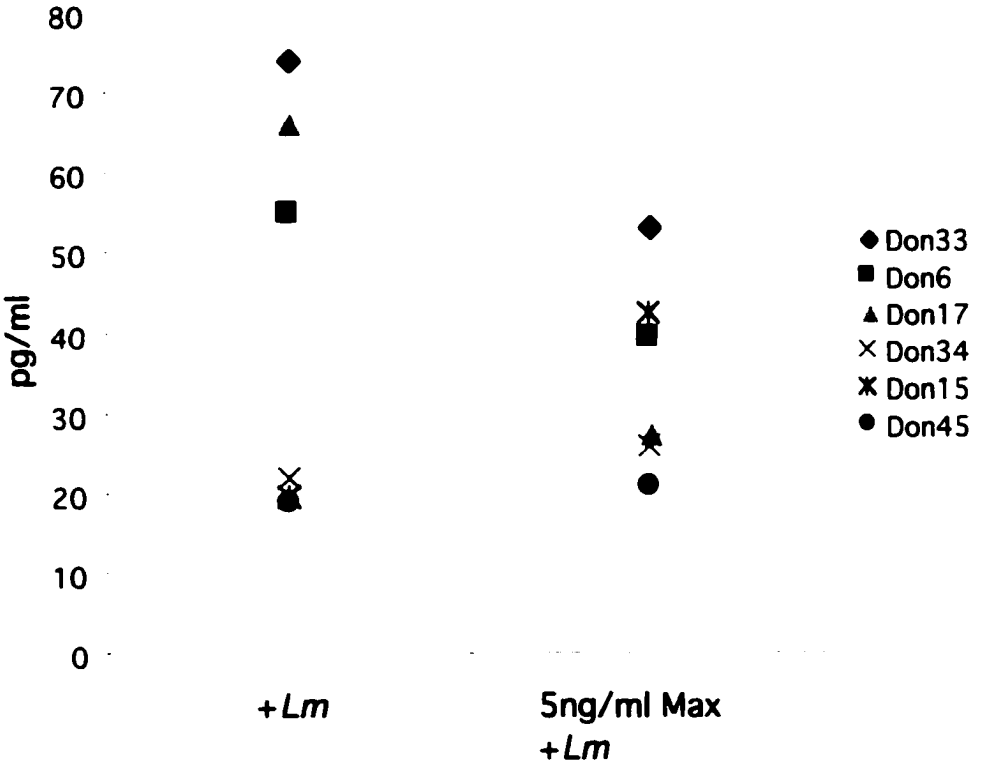


Figure 3.3 There is a trend of decreased IL-12p70 after 48 h of *L. major* stimulation when the cells are exposed to Max. Data are expressed as median values of duplicate experiments (p=0.09).

Effects of Maxadilan on Type 2 cytokine production of PBMC

We then wanted to determine if Max would have an effect on the secretion of Type 2 cytokines. We hypothesized that Max would increase the production of Type 2 cytokines. Thus, we measured IL-5 and IL-10 in the culture supernatants at 48 and 72 h after parasite exposure. At no time, could we detect IL-5 production in any of our experiments (data not shown). There was no significant difference nor any trend in the IL-10 levels of *L. major*-stimulated cells and parasite stimulated cells exposed to Max measured 48 h after the addition of parasites into the cultures (data not shown). However, at 72 h after exposure, there appeared to be a slight trend ($p=0.07$) of IL-10 reduction in PBMC of cells exposed to Max compared to the cells exposed only to the parasite (Figure 3.4).

Effect of Phlebotomus papatasi salivary gland lysate on the cytokine production of PBMC

Max is a salivary gland component found in New World sand flies. Max is not present in the salivary glands of Old World sand flies, however, Old World salivary glands also enhance infection with *L. major* and modulate immune responses (Mbow et al., 1998). Therefore, we wanted to test the ability of Old World whole salivary gland lysate to modulate the cytokine production of stimulated PBMC from *Leishmania*-naïve donors. First, we evaluated the capacity of salivary gland lysate (SGL) to modulate the IFN-γ production of *L. major*-stimulated PMBC. When we measured the production of

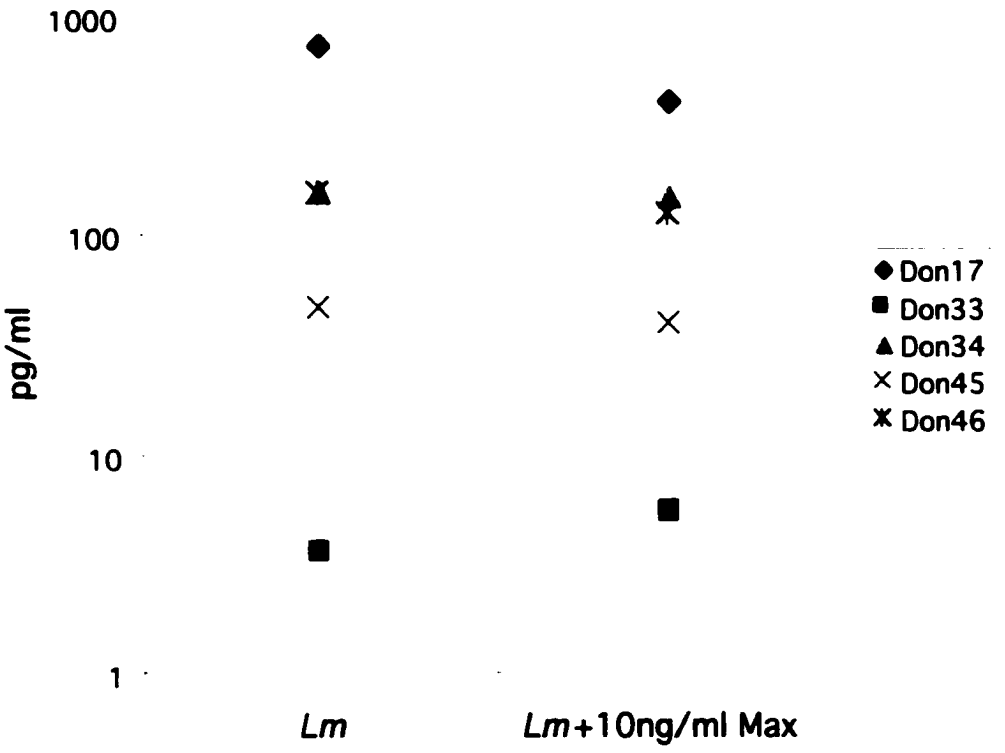


Figure 3.4 There is a trend of decreased IL-10 after 48 h of *L. major* stimulation when the cells are exposed to Max. Data are expressed as median values of duplicate experiments (p=0.07).

IFN-γ at 48 h (Figure 3.5), there was no significant difference detected. However, at 72 h, there was a trend (p=0.07) of slightly reduced IFN-γ production when *L. major*-stimulated cells are exposed to SGL (Figure 3.6). Next, we measured the effects of SGL on the IL-12p40 and IL-12p70 secretion of *L. major*-stimulated cells. There was no effect of SGL lysate on the production of IL-12p40 at either 48 or 72 h (data not shown). Similarly, SGL did not appear to have an influence on IL-12p70 production at 48 or 72 h (data not shown).

To examine the influence of SGL on Type 2 cytokines secretion, IL-10 and IL-5 concentrations were measured at 48 and 72 h. As with the Max study, no IL-5 could be detected (data not shown). When we evaluated the influence of SGL on IL-10 production, we did not detect any trends in the data (Figure 3.7).

Since *L. major* has been reported to not induce cytokine production of certain cytokines, such as IL-12, during initial infections (within the first week) in the mouse model (Reiner et al., 1994), we were concerned that the cells in our culture system were not being activated enough by infection with *L. major* to produce cytokines in great enough levels to be modulated. To address this concern, we decided to stimulate the cells with LPS. Although this is an artificial system from a disease standpoint, we were interested in determining the maximum effect SGL could have on cytokine production, since we were not able to detect many differences with *L. major* stimulation with cytokines other than IFN-γ. First, we evaluated the effect of SGL lysate on the secretion of IFN-γ from LPS-stimulated cells. As shown in Figure 3.8, there was a significant decrease in IFN-γ production between cells stimulated with LPS and cells exposed to

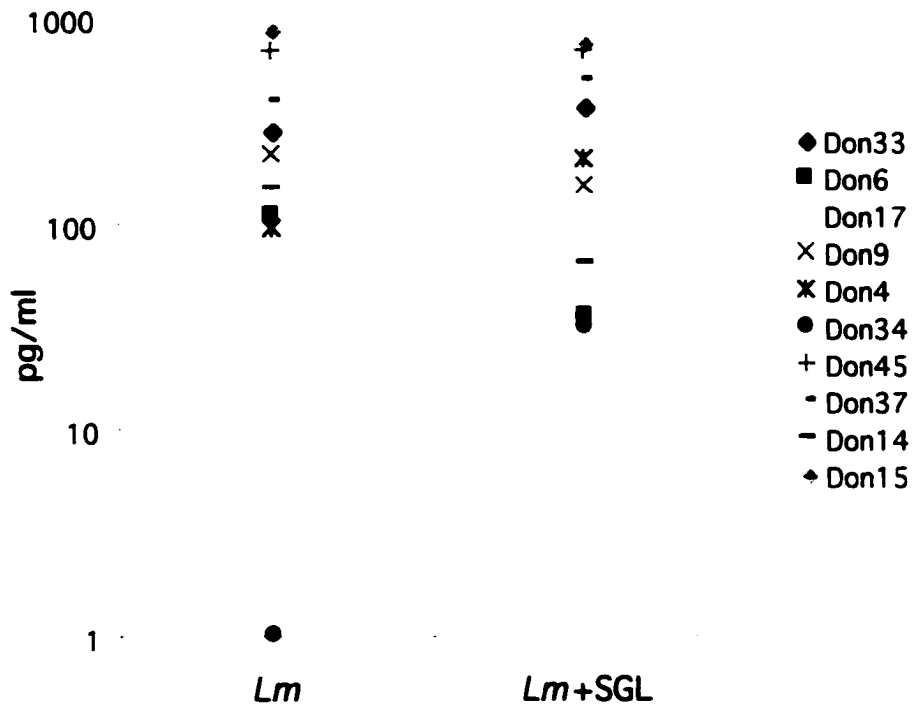


Figure 3.5 There is no significant difference in IFN-γ production after 48 h of *L. major* stimulation when the cells are exposed to salivary gland lysate.

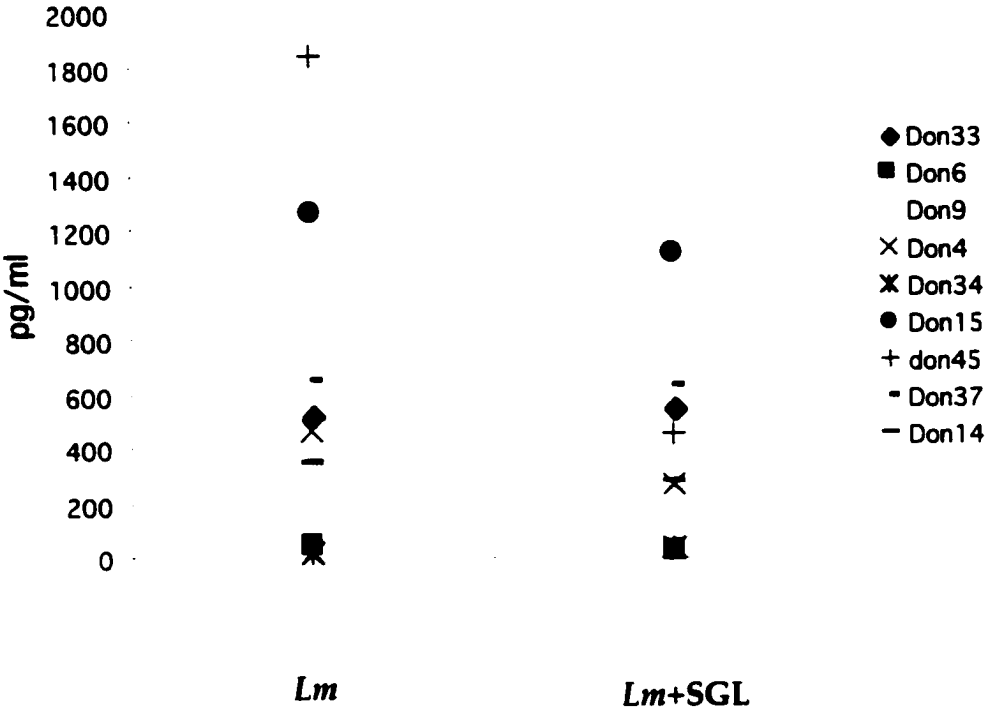


Figure 3.6 There is a trend of decreased IFN-γ secretion after 72 h of *L. major* stimulation when the cells are exposed to salivary gland lysate (p=0.07).

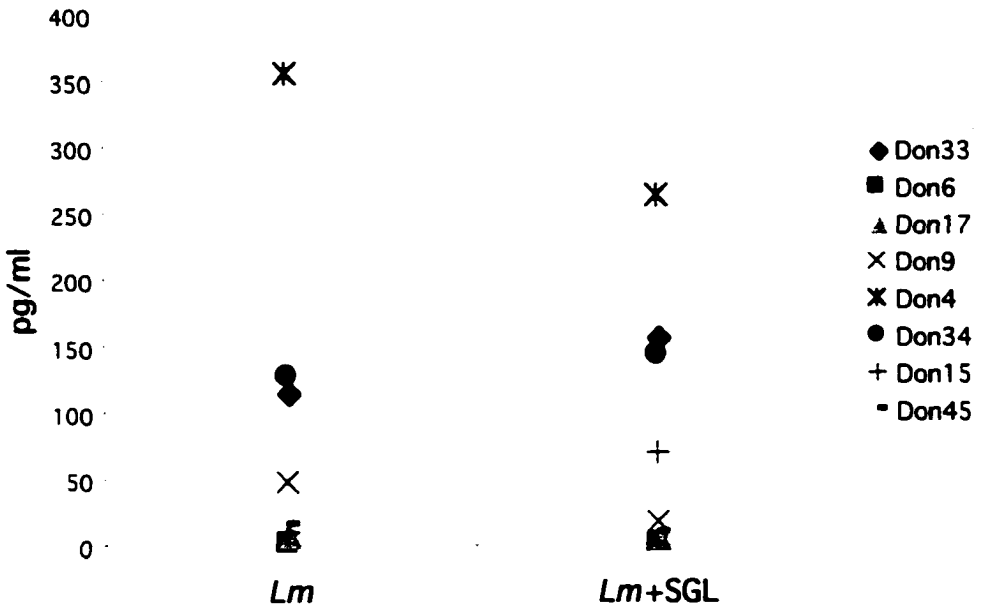


Figure 3.7 The addition of salivary gland lysate to *L. major*-stimulated PBMC had no effect on the production of IL-10. Data shown after 48h of stimulation.

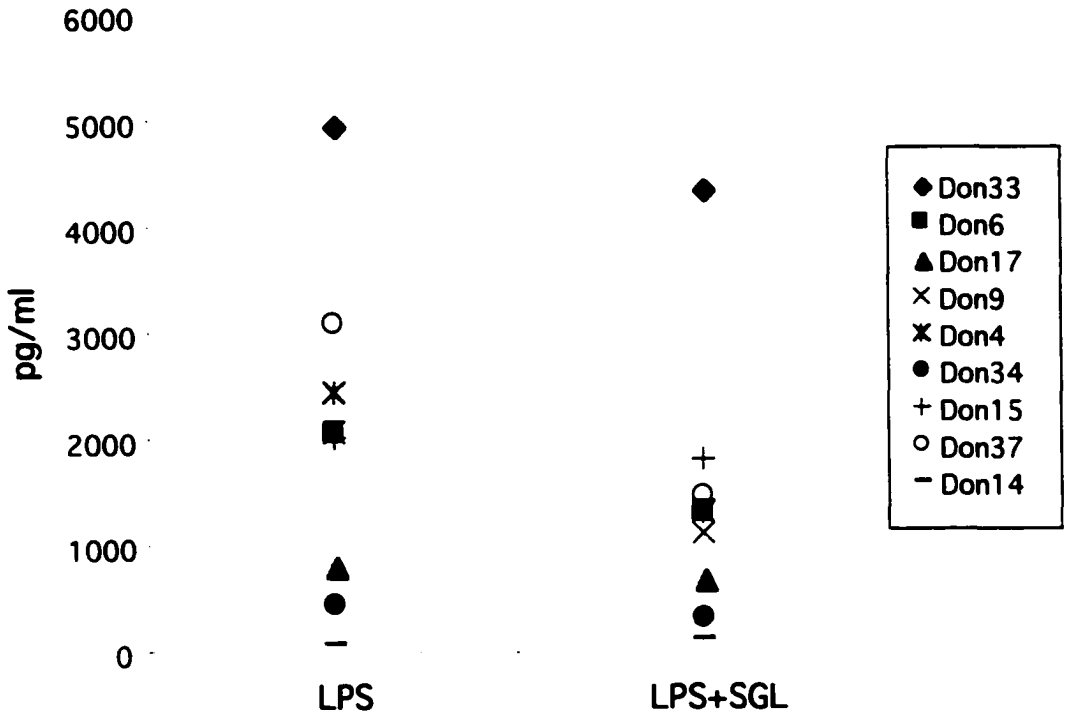


Figure 3.8 Salivary gland lysate significantly decreases IFN-γ production in LPS-stimulated PBMC ($p<0.05$) compared to the LPS stimulated controls. Data shown are after 48 h of stimulation.

LPS and SGL ($p<0.05$) after 48 h of stimulation. Unexpectedly, there was no effect of SGL on the LPS-stimulated cells 72 h after LPS stimulation (data not shown). We then determined the effects of SGL on IL-12p40 and IL-12p70 production at 48 and 72 h post-LPS stimulation. In contrast to the data observed when PBMC were stimulated with *L. major*, there was a significant decrease ($p<0.05$) of IL-12p40 when the LPS-stimulated cells were treated with SGL (Figure 3.9). There was little effect of SGL on IL-12 production (data not shown).

Finally, we measured the effect of SGL on the concentration of IL-10 and IL-5 in the supernatants of LPS-stimulated PBMC cultures. In contrast to the Type 1 cytokines, no discernable pattern of IL-10 secretion was detected at 48 h (data not shown). Instead, at 72 h, there was a decrease in IL-10 production (Figure 3.10) that was statistically significant ($p<0.05$).

Effect of Maxadilan on cytokine production of macrophages stimulated with L. major

Since we observed that Max decreases cytokine production in our whole PBMC cultures, we wanted to determine the effects of this salivary protein on macrophage function. Soares et al., (1998) characterized Max in vitro using LPS-stimulated macrophage cultures. To begin to compare the effects of Max in human cells with what has been reported in the literature in the murine system, we first evaluated cytokine production of *L. major*- stimulated macrophage cultures. To evaluate a Th1-type response, we measured the concentration of TNF-α, IL-12p40 and IL-12p70 in macrophage

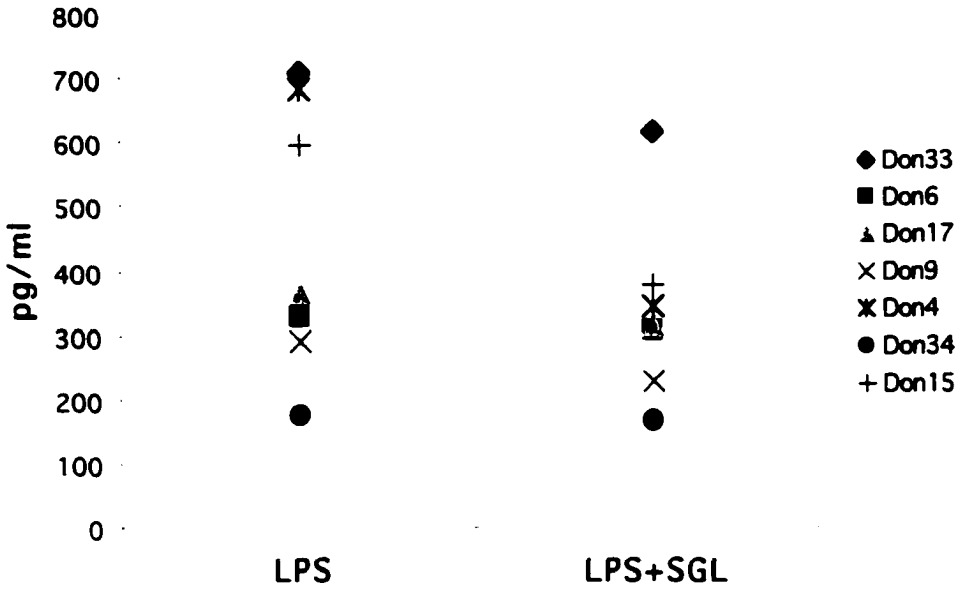


Figure 3.9 Salivary gland lysate significantly decreases ($p<0.05$) the production of IL-12p40 48 h after LPS exposure compared to cells stimulated with LPS. Background levels of IL-12 are subtracted from values shown.

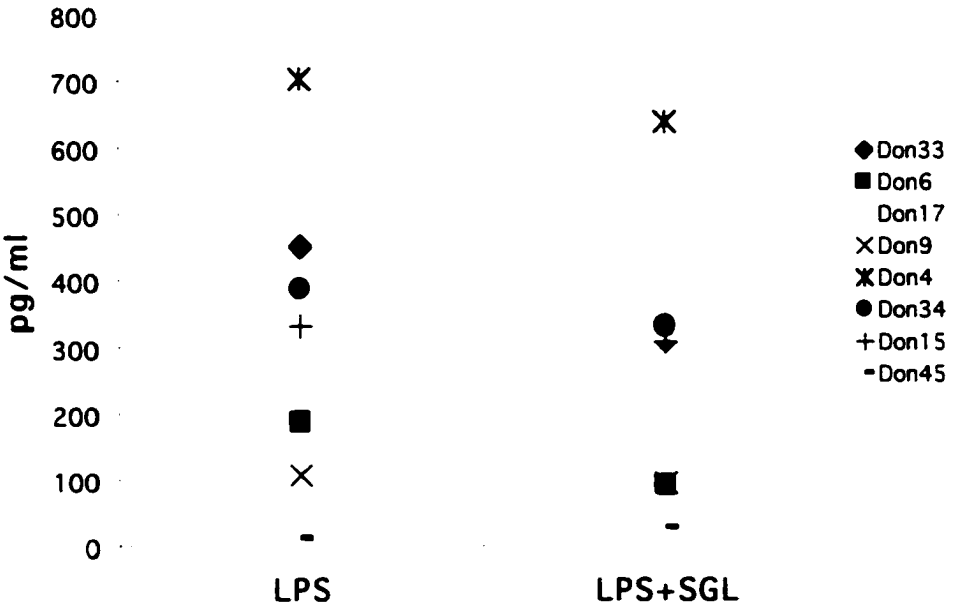


Figure 3.10 IL-10 production is significantly reduced ($p<0.05$) in PBMC stimulated with LPS and exposed to salivary gland lysate at 72 h, compared to cells exposed to LPS only.

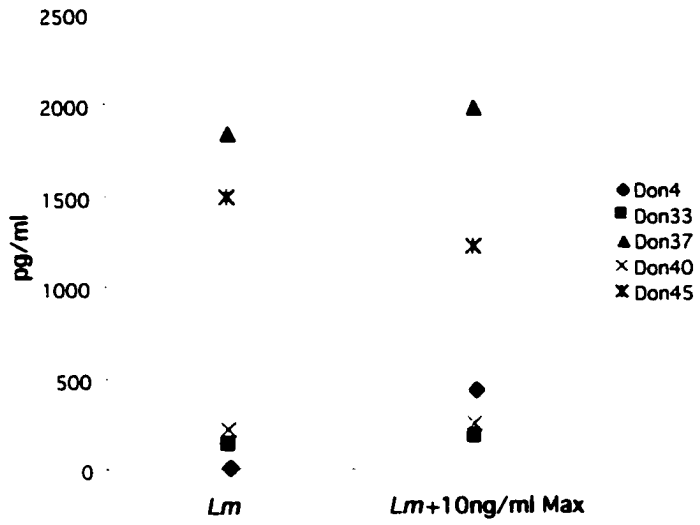
cultures stimulated with *L. major* at 6 and 18 h. Unexpectedly, we found that TNF-α is secreted at very low to undetectable level at both 6 h and 18 h and there was little discernable trend in the production of this cytokine (data not shown). Similarly, there was no significant difference in the production of IL-12p70 in samples stimulated with *L. major* and samples exposed to the parasite and Max (data not shown). Finally, there was little measurable production of IL-12p40 at either time point (data not shown).

In murine macrophage cultures, Max has been shown to have an impact on Type 2 cytokines, causing an induction of IL-6 (Soares et al., 1998). We wanted to determine if Max would have a similar effect on human macrophage cultures. We also measured the production of IL-10 in these cultures. After six hours of stimulation, there was no difference in the concentration of IL-6 detected in the parasite-stimulated cultures and the cultures in which the macrophages were exposed to both *L. major* and Max (data not shown). In contrast, at the 18 h time point, both 10ng/ml and 5 ng/ml of Max appeared to have some influence on IL-6 secretion. When the cells were exposed to the parasite and 10 ng/ml of Max, the data suggest ($p=0.08$) increased IL-6 production (Figure 3.11a). When the cells were exposed to 5 ng/ml of Max, there was a statistically significant increase in IL-6 secretion ($p<0.05$) (Figure 3.11b). There was little to no detectable IL-10 production in most of the macrophage cultures (data not shown).

Maxadilan modulation of cytokine production of LPS-stimulated macrophages

As the cytokine stimulating capacity of *L. major* was low, we wanted to stimulate the cells more fully and then determine if we could detect modulation of the

A. 10 ng/ml Max



B. 5 ng/ml Max

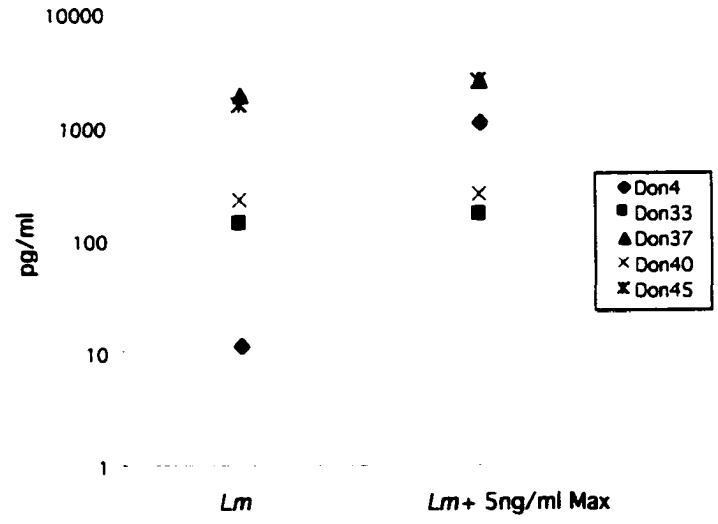


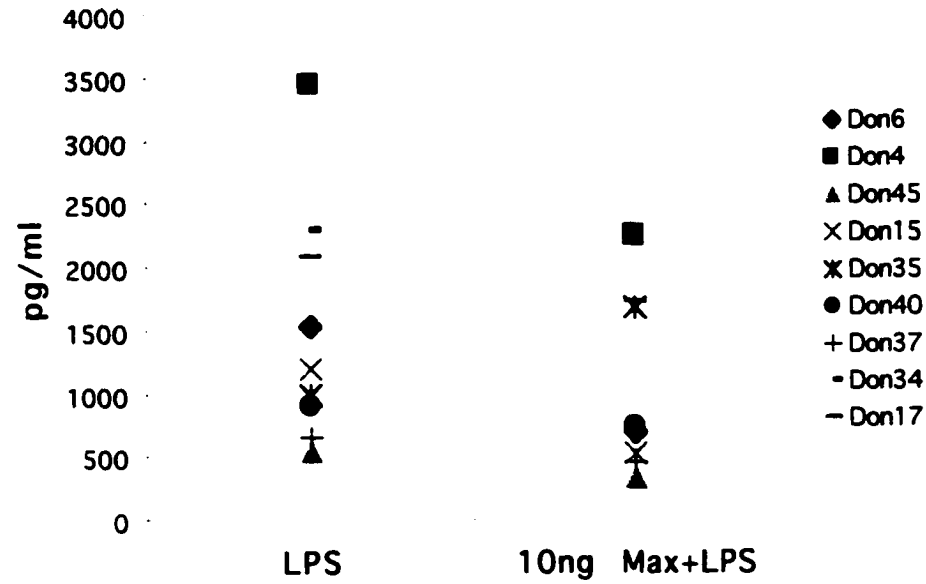
Figure 3.11a and 3.11b There is a trend of increased IL-6 production when 10ng/ml of Max is added to macrophage cultures ($p=0.08$) in Fig. 3.11a. In 3.11b, there is a statistically significant difference between the cells exposed to *L. major* only and those exposed to the parasite and 5 ng/ml Max ($p<0.05$). These data are at 18h post-exposure. Background levels of IL-6 were subtracted from the samples shown. Data are expressed as median values of duplicate experiments.

cytokines we were testing. We exposed the macrophages to Max and stimulated the cells with LPS and then measured the secretion of the cytokines of interest. First, we measured the Type 1 cytokines TNF-α and IL12p40 and p70. At 6 h post-stimulation, there was a trend of decreased TNF-α production (Figure 3.12a) ($p=0.07$). At 18 h there was a less marked pattern (Figure 3.12b) of reduction. When we measured the concentration of IL-12p40 and IL-12p70, there was little production of these cytokines (data not shown) and no remarkable tendencies in the data. We also measured the effect of Max on Type 2 cytokine production of LPS stimulated macrophages. Again, there was little detectable IL-10 (data not shown). IL-6, however was modulated by Max when the macrophages were stimulated with LPS. After 6 h, there was a trend ($p=0.09$) of increased IL-6 production (Figure 3.13a). At 18 h, there was a statistically significant increase in IL-6 production ($p<0.05$) in the macrophage cultures tested.

Effects of sand fly salivary gland lysate on the cytokine production of macrophages

We wanted to test to see if there were similar effects on macrophage cytokine secretion when stimulated macrophages were exposed to salivary gland lysate from *P. papatasi*. Therefore, we continued our experiments by exposing the macrophages to *L. major* and measured cytokine production. We did not detect any significant trends or differences when we evaluated the concentrations of TNF-α, IL-12p40 or IL-12p70 in the supernatants of the macrophage cultures (data not shown). Similarly, IL-10 was not detected (data not shown). In contrast, when IL-6 was measured, at 6 h there was a significant increase ($p<0.05$) in production from macrophages stimulated with SGL and

A. 6 hours



B. 18 hours

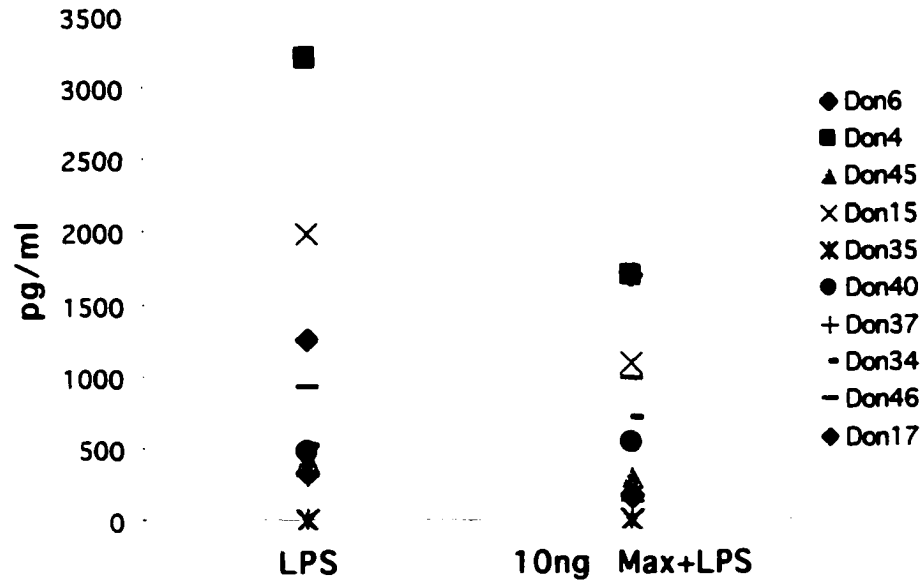
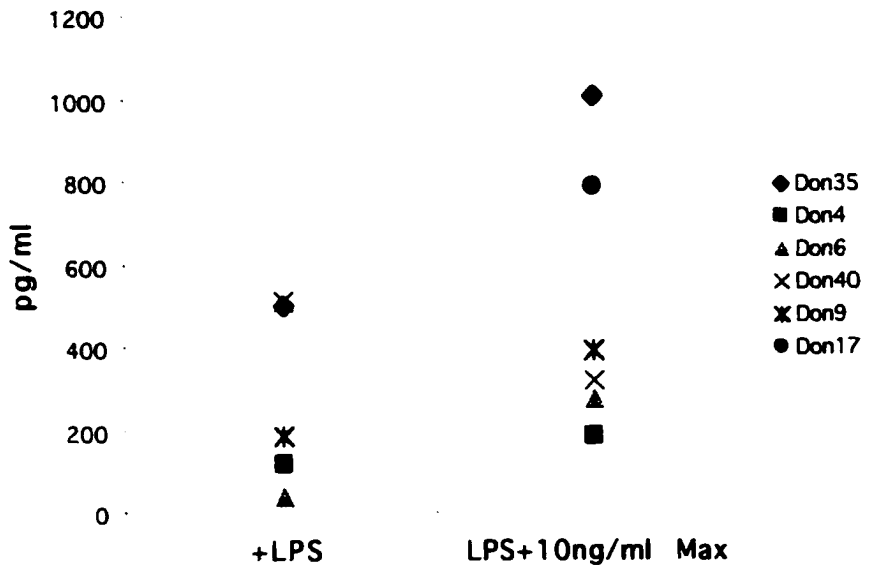


Figure 3.12a and 3.12b There is a trend of reduced TNF-α production when 10ng/ml of Max is added to macrophage cultures (p=0.07) in Fig. 3.11a at 6 h. In 3.11b, there is a less established trend of TNF-α reduction at 18 h when Max is added to LPS-stimulated macrophages Background levels of TFN-α were subtracted from the samples shown.

A. 6 hours



B. 18 hours

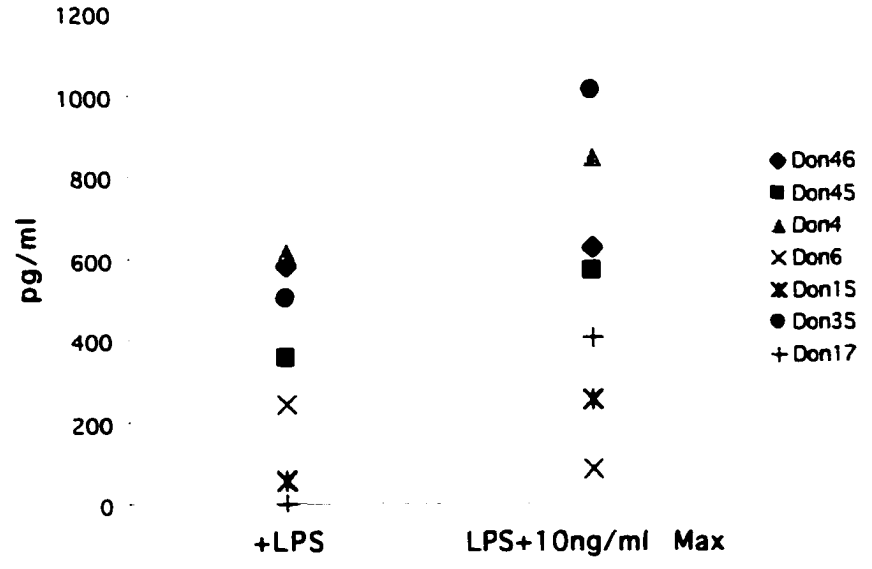


Figure 3.13a and 3.13b In Fig. 3.13a, after 6 h of exposure to Max, LPS-stimulated macrophages have a trend of increased IL-6 production ($p=0.09$). At 18h, the increase in IL-6 production is statistically significant ($p<0.05$) when comparing the difference between the cells exposed to LPS only and those exposed to LPS and 10 ng/ml Max ($p<0.05$).

L. major, compared to the parasite-only stimulated cells (Figure 3.14). This effect is abrogated after 18 h of parasite exposure.

In an attempt to maximize stimulation of the macrophages and then measure the effects of SGL on cytokine secretion, we stimulated the cells with LPS. However, similar to the results we found with *L. major* stimulation, only IL-6 cytokine production appeared to be modulated by SGL. There was a significant increase in IL-6 production when LPS-stimulated macrophages and LPS-stimulated macrophages exposed to SGL were compared ($p<0.03$) at both 6 and 18 h. These results are illustrated in Figures 3.15a and b.

Determining if Maxadilan is interacting with macrophages via the PACAP receptor

In mice, one of the primary receptors through which Max interacts with macrophages is the neuropeptide receptor PACAP. It is not known if this same receptor is used by Max to interact with human macrophages. We used the PACAP antagonist PACAP 6-38 to block the PACAP receptor and then exposed the macrophages to Max and stimulated the cells with LPS. As TNF-α and IL-6 were the cytokines that appeared to be affected in the macrophage system, we measured the secretion of these cytokines after 6 and 18 h. When the concentration of TNF-α was evaluated, it was determined that there was no difference in cytokine production between the cells stimulated with a). LPS alone, b). 10ng/ml of Max and LPS or c). LPS, 10ng/ml of Max and 10μg/ml PACAP 6-38. At 18 hr, although the medians of these three groups were different

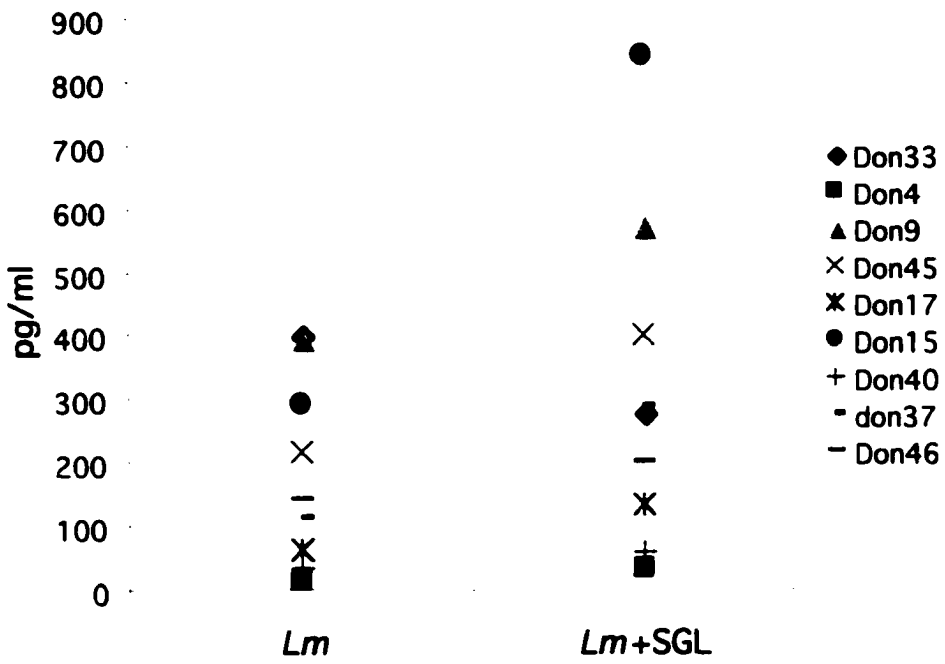


Figure 3.14 Salivary gland lysate significantly increases IL-6 production at 6 h in *L. major*- stimulated macrophages ($p<0.05$).

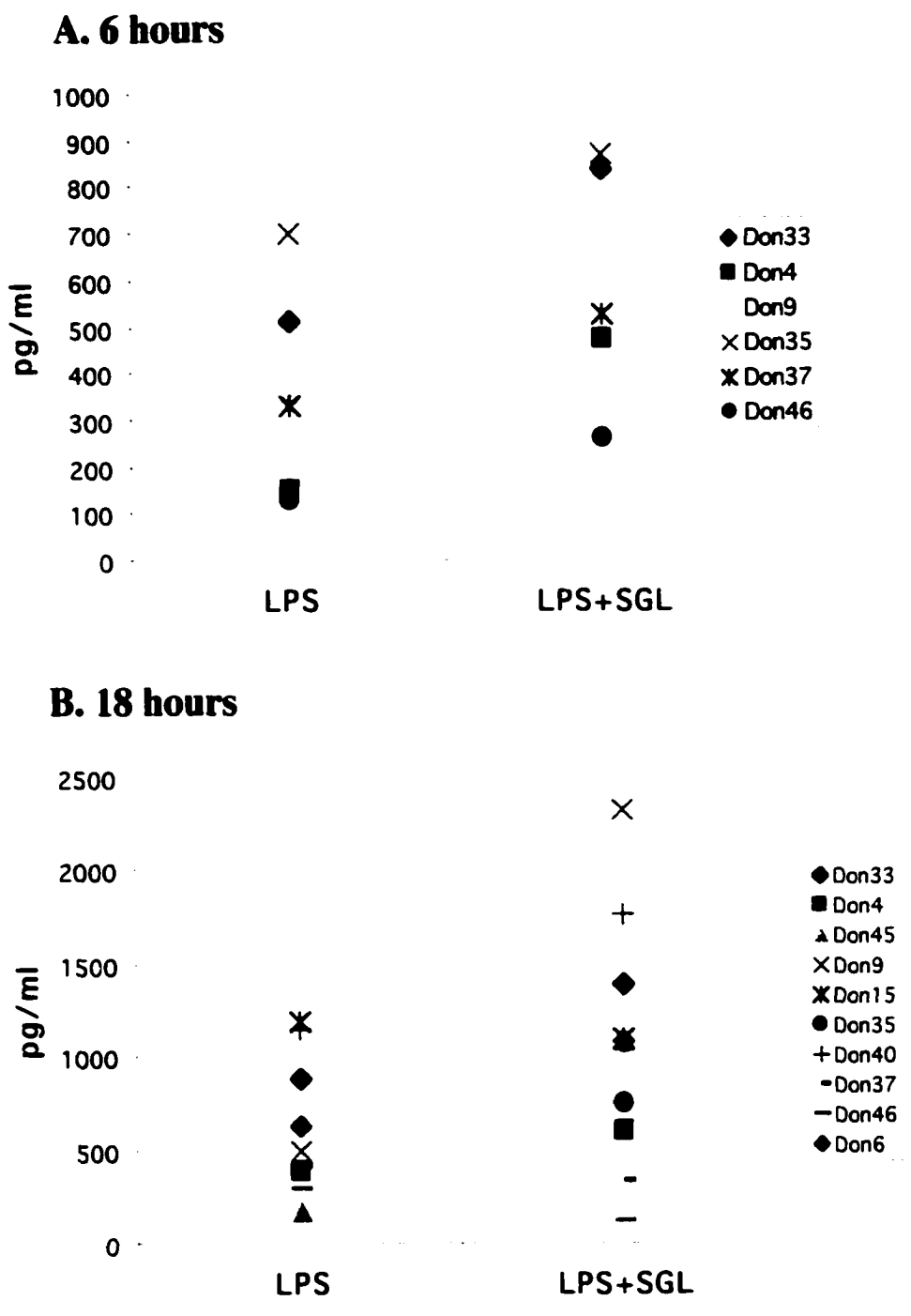


Figure 3.15 a and b. IL-6 production is increased in a statistically significant manner ($p<0.03$) after 6 h (fig. 3.15a) and 18 h (fig. 3.15b) after exposure to LPS and SGL compared to LPS stimulated macrophages.

(127pg/ml, 52.8 pg/ml and 173.2 pg/ml respectively), this difference was not significant. These data are shown in Figure 3.16. There was no trend where Max reduces TNF-α production and PACAP 6-38 abrogates the response apparent at 6 h (data not shown).

Since the greatest modulation in the macrophage system was apparent when IL-6 was measured, we expected to find that PACAP 6-38's effects would be most pronounced when we examined IL-6 production. Indeed, as illustrated in Figures 3.17 a and 3.17 b, at both at 6 and 18 h the Max related stimulation of IL-6 production was abrogated when the PACAP antagonist PACAP 6-38 is added to the macrophage cultures.

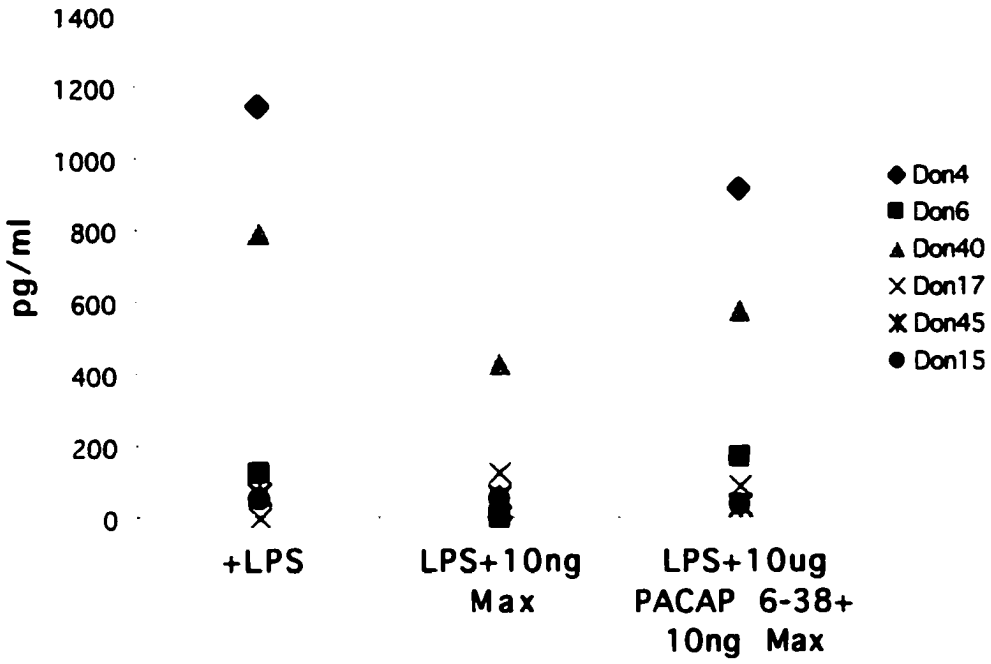


Figure 3.16 There is no statistical difference in TNF-α production between macrophages stimulated with LPS and then exposed to either Max or PACAP 6-38 and Max.

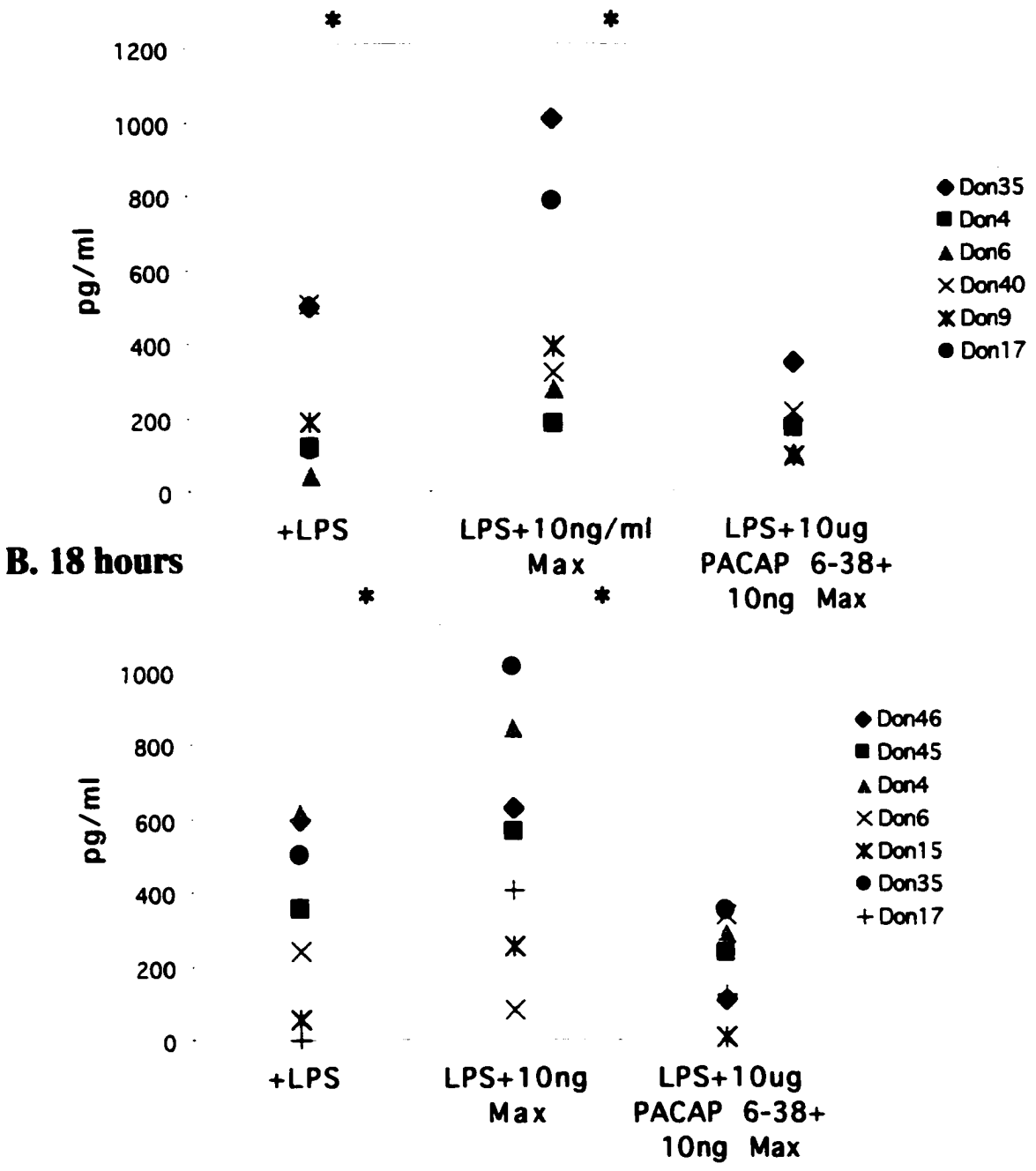


Figure 3.17a and 3.17b The addition of PACAP 6-38 decreases IL-6 production that is stimulated by the addition of Max to macrophage cultures. The lines drawn between samples denote groups that have significant differences. The '*' indicates a p value of <0.05.

DISCUSSION

In this study, we evaluated the influence of sand fly salivary gland proteins on the cytokine production of PBMC as well as macrophages, the primary cell that harbors the parasite during *Leishmania* infection. Without in vitro approaches, it is difficult, if not impossible to explore the influence of potentially immunomodulatory vector-related proteins on human cells. We obtained the cells from *Leishmania*-naive donors so that we could monitor the effect of both saliva and the parasite on the initial human response to infection. In our experiments we focused on Maxadilan, a peptide that was originally isolated from the New World sand fly *Lutzomyia longipalpis*, and salivary gland lysate, which contains numerous proteins, few of which have been characterized, from the Old World sand fly *Phlebotomus papatasi*, the vector of *L. major*. Although there have been several reports indicating the exacerbative effect of Max and sand fly saliva in establishing an infection in the murine model (Morris et al, 2001; Mbow et al.,1998; Theodos et al, 1991; Titus and Ribeiro, 1988), to our knowledge, this is the first report characterizing the effects of Max or sand fly salivary gland proteins on human cells.

In an effort to begin to dissect the immunomodulatory properties Max and salivary gland lysate (SGL) may have on cells responding to *L. major*, we hypothesized that these proteins would suppress the ability of stimulated PBMC to produce Type1 cytokines, such as IFN-γ and IL-12. Conversely, we speculated that salivary proteins would enhance a Type 2 response of PBMC and increase secretion of cytokines such as IL-10 and IL-5. As Max and saliva have been reported to act upon macrophages (Theodos and Titus, 1993; Hall and Titus, 1995; Soares et al., 1998), we also wanted to evaluate the influence of these proteins in modulating cytokine secretion of stimulated

macrophages. Similar to the PBMC study, we hypothesized that Max or SGL would decrease secretion of Type 1 cytokines, such as TNF-α and IL-12 and would increase the production of Type 2 cytokines such as IL-6 and IL-10. Finally, we wanted to attempt to determine if Max was able to modulate the function of macrophages through the PACAP receptor, as had been reported in the murine model (Soares et al., 1998). Overall, we determined that both Max and SGL are capable of modulating the immune response to either parasite or LPS-stimulation. Our data suggest that similar to findings reported in the murine model, Max also interacts with macrophages through the PACAP receptor. Table 3.1 summarizes the Max and SGL experiments.

As we have established and characterized a primary in vitro system to study the initial interactions between *L. major*-naïve human PBMC and the parasite, we wanted to first examine the interactions of the PBMC from *Leishmania*-naive donors with Max or SGL, and *L. major*. This experimental design allows for the opportunity to study the effects of vector components on the establishment of infection. It is important to not discount an influence of sand fly saliva in the initial exposure to the parasite, as it has been shown that the addition of saliva gland proteins allows for a much more effective infection (Titus and Ribiero, 1988; Belkaid et al., 1998b; Morris et al., 2001).

Exposure of PBMC to L. major

In the first set of our experiments, we exposed human PBMC to either Max or SGL and the parasite. As we predicted, Max decreased IFN-γ production at both 48 and 72 h in cells stimulated with *L. major* (Fig. 3.1 a and b). Similarly, the production of IL-12p40

Table 3.1 Summary of effects of salivary gland treatments on cytokine production . Results are compared to *L. major* or LPS-stimulated cells.

Treatment	IFN-γ	IL-12p40	IL-12p70	IL-10	TNF-α	IL-6
Max+PBMC+*Lm*	Decreased	Decreased	Trend to decrease	Decreased	---	---
SGL+PBMC+*Lm*	Trend to decrease	No effect	No effect	No effect	---	---
SGL+PBMC+LPS	Decreased	Decreased	No effect	Decreased	---	---
Max+MΦ+*Lm*	---	No effect	No effect	No effect	No effect	Increased
SGL+MΦ+*Lm*	---	No effect	No effect	No effect	No effect	Increased
Max+MΦ+LPS	---	No effect	No effect	No effect	Trend to decrease	Increased
SGL+MΦ+LPS	---	No effect	No effect	No effect	No effect	Increased

was reduced and there was a trend of reduction of IL-12p70 secretion in *L. major*–exposed PBMC after treatment with Max (Fig. 3.2 and 3.3). There were differences, however in the time points and concentrations of Max where an effect was observed. The decrease in IL-12p40 production occurred at 72 h and required 10ng/ml Max, while the influence of Max on IL-12p70 production was most pronounced at 48 h and 5 ng/ml of Max was the most effective. Several studies have described differences in IL-12p40 and IL-12p70 production during infections (Fernandez-Lago et al, 1999; Svensson et al., 2001). Likewise, the secretion of IL-12 is dependent on the subsets of APC present (i.e. dendritic cells or macrophages) (Gorak et al., 1998; Belkaid et al., 1998). It is possible in this system that APC are being activated at different levels, resulting in the differences seen in the secretion of these IL-12 subunits. The variations in IL-12 concentrations could also be related to Max itself. Max has been shown to have a bi-phasic dose-response in the lesion development of *Leishmania*-infected mice (Morris et al., submitted). Thus, we speculate that it is possible that in the human system, different concentrations of Max may be more effective than others in enhancing or suppressing cytokines. This effect could be cytokine-dependent.

Although we predicted that Max would have an exacerbative effect on the secretion of Type 2 cytokines, such as IL-5 and IL-10, we did not observe this result. We did not detect IL-5 by ELISA in our experiments. As we have shown that IL-5 is generally detectable during restimulation of cells to *L. major* (Chapter 1) and not as apparent during initial parasite exposure, the lack of IL-5 is not too surprising. Our data suggest that salivary gland proteins do not increase IL-5 secretion. Unexpectedly, the

levels of IL-10 actually decreased after exposure to Max. There have been few reports about the effects of Max on IL-10 production. Bozza et al. (1998) found that Max increased serum IL-10 levels in mice. However, using sand fly SGL, Mbow et al. (1998) found that there was no effect on IL-10 expression in lymph node cells of treated mice. It is possible that Max has a transitory suppressive effect on most inflammatory cytokines. This strategy could be used by the parasite to establish itself within the host with minimal detection.

Effect of SGL on PBMC cytokine production

In contrast to Max, there was a less pronounced effect of suppression of Type 1 cytokines when the cells were stimulated with *L. major* in the presence of SGL. However, some differences could be expected since Max is a New World sand fly protein and the SGL used was collected from Old World sand flies. In old world sand flies, instead of Max, adenosine (Ribeiro et al., 1999) as well as a recently isolated protein called SP15 (Valenzuela et al., 2001), are immunomodulatory. Our data suggest that there is a trend toward decreased IFN-γ secretion at 72h (Fig. 3.6). However no effect was observed when IL-12 was measured. Similarly, there was no effect on IL-10 production.

In an attempt to better determine the effects the salivary glands had on cytokine production, we stimulated the cells with LPS, which activates antigen presenting cells and allows for a greater baseline levels of cytokines in order to determine potential suppressive modulation of cytokines by saliva. When PBMC were stimulated with LPS and then exposed to salivary glands, IFN-γ, IL-12p40 and IL-10 were decreased. The

suppressive effect on the Type 1 cytokines appeared to be maximal at 48 h and the effect on IL-10 was apparent at 72 h post-exposure. There was no significant difference in IL-12p70 expression between cells stimulated with LPS and those exposed to LPS and SGL.

The effects of saliva and salivary gland proteins appears to be transitory in the human system. Similarly, after mice are bitten by sand flies or exposed to SGL, there is a transient inflammatory and cellular infiltrate response within 6h of exposure (Belkaid et al., 1998b; Kamhawi et al., 2000). We suggest, however, that even a short-term modulation of the cytokine milieu may be adequate to influence the immune response. The production of cytokines early after infection can play in important role in allowing the parasite to establish itself within the host, as well as influencing the cytokine milieu which can direct the polarization of a response to a Type 1 or Type 2 response. In murine leishmaniasis, it has been reported that an early burst of IL-4 directs BALB/c towards a Type-2 response, and results in unresponsiveness of T cells to IL-12 (Launois et al., 1995; Launois et al., 1997). In contrast, the early production of IL-12 and IFN-γ result in a Type 1 response (Scott, 1991; Sharton-Kerston et al., 1995). Costimulation of B7-1 and B7-2 can direct the early immune response to *L. major* and enable the development of cytokine responses (Elloso and Scott, 1999). It would be interesting to determine if these co-stimulatory molecules are affected by Max or SGL, and thus play a role in the modulation of the cytokine response. We speculate that Max could potentially play a role in the modulation of these early immune responses in natural disease conditions. For example, Max may inhibit Type 1 cytokines for a time period long enough to allow the parasite to establish an infection within macrophages.

Effect of Max or SGL on macrophages stimulated with L. major or LPS

Since we observed that Max and SGL decreased cytokine production in the PBMC cultures, we wanted to further dissect this response. As macrophages are the cells that primarily harbor the parasite (reviewed in Titus et al, 1994), and salivary gland proteins have been shown to modulate murine macrophage function (Hall and Titus, 1995; Soares et al., 1998; Zer et al., 2001), we wanted to determine if salivary gland proteins affected the cytokine production of stimulated human macrophages. In these experiments we measured IL-6 and TNF-α, two pro-inflammatory cytokines produced by macrophages, as well as IL-12p40, IL-12p70 and IL-10. Our hypothesis was that Max and SGL would suppress secretion of Type 1 cytokines such as TNF-α and IL-12 and conversely, would enhance Type 2 cytokine production of IL-10 and IL-6. When we stimulated the cells with *L. major*, we found little cytokine production of any of the Type 1 cytokines or IL-10. Similar results were found when we exposed SGL-treated macrophages to *L. major*.

In contrast, when we measured the secretion of IL-6 by macrophages exposed to the parasite and Max, we found that the cytokine concentration was increased compared to *L. major*-stimulated cells at 18 h (Fig 3.11b). When 10 ng/ml of Max was added to the macrophage cultures, there was a trend of increased production, however, when 5 ng/ml of Max was used, this resulted in a statistically significant increase in IL-6 production. When we examined the effect of SGL on IL-6 production, there was a significant increase in IL-6 production when macrophages were exposed to both *L. major* and SGL compared to parasite-only stimulated cells at 6 h (Fig. 3.14). This effect was not seen at 18 h.

It has been reported that in vitro, *Leishmania* are poor inducers of cytokine production in both human and murine macrophage cultures (Sartori et al., 1997; Reiner et al., 1994). Our results appear to substantiate these findings. In order to better study the effects of Max and SGL on cytokine production by macrophages, we stimulated the cells with LPS, a potent activator of macrophages. A similar approach has been used in our laboratory to study the interactions between Max and mouse macrophages (Soares et al., 1998). When we measured IL-12 or IL-10, we did not detect any significant trends in the data. On the other hand, when we evaluated TNF-α, there was a trend that suggested Max reduced TNF-α (8 out of 9 donors) after 6 hours of exposure to Max and LPS (Fig. 3.12a). However, after 18 h of exposure, only half of the donors were affected by Max, suggesting a transitory effect of Max on TNF-α. In contrast to the decrease in TNF-α production, when we examined the effect of Max on the production of IL-6 in LPS-stimulated cultures, we found that there was a trend after 6 h of exposure of increased IL-6 secretion, and by 18 h, this elevated level of IL-6 was greatly increased, as illustrated in Figures 3.13 a and b.

When we examined the effect of SGL on the production of cytokines, we observed very little effect of Old World sand fly salivary gland proteins on TNF-α, IL-12 or IL-10. However, similar to the results we found when we incubated the cells with Max and LPS, we determined a significant increase at both 6 and 18 h of IL-6 secretion when SGL was added to the LPS-stimulated macrophage cultures (Fig. 3.15 a and b). Although the effects of SGL on IL-6 has not been measured, when mice are exposed to SGL, IL-4 production is increased (Mbow et al., 1998). As IL-6 increases IL-4

production (Rincon et al., 1997), it is not unlikely that IL-6 production could have also been elevated in mice exposed to SGL.

Overall, the effects of Max and SGL, though sometimes quantitatively different, were similar qualitatively when stimulated PBMC are compared. There was a decrease in IFN-γ and IL-12p40 secretion, as well as IL-10 secretion. The most striking effects appeared to be when IFN-γ was measured. When the effects of salivary gland proteins on macrophage cultures were evaluated, there was little effect on IL-12 and IL-10 production. Max appeared to have a tendency to reduce TNF-α secretion in LPS-stimulated macrophages, whereas, there was no trend of suppression when SGL was added to the cultures. In contrast, under all conditions tested, IL-6 secretion was increased by Max or SGL.

When we compare the results found in the human studies to what has been reported in the murine literature, there are several similarities. Mbow et al. (1998) correlated the suppression of IFN-γ and IL-12 with exacerbated lesions in *Leishmania*-resistant mice exposed to SGL and *L. major*. They did not find any changes in IL-10 expression, however. Although it is not possible for us to do similar studies in humans, we did find similar patterns of Type 1 cytokine reduction. Likewise, the increases in IL-6 production when macrophage cultures are exposed to Max are comparable to the findings of Soares et al., (1998). In contrast, Soares et al. (1998) report a marked suppression of TNF-α production, whereas, we see only a tendency for decreased production when the macrophages are stimulated with LPS and Max.

It appears that the cytokines most affected by salivary gland proteins in the human in vitro system are IFN-γ, IL-12p40, and IL-6. As IFN-γ can activate human

macrophages to kill *Leishmania* (Murray et al., 1983), and IL-12 can activate CD4+ T helper cells, as well as NK cells to produce IFN-γ (Scharton-Kersten et al., 1995), it would be to the parasite's advantage to reduce IFN-γ and IL-12, potentially allowing for a better chance at establishing itself in the mammalian host. The role of TNF-α in human leishmaniasis is not as well established as it is in murine infections (Arena et al., 1997), and there is limited evidence that TNF-α may be more important in chronic infections (Melby et al., 1994). Similarly, evidence suggests that in human infections, IL-10 may play a more prominent role in chronic infections (Melby et al., 1996; Akuffo et al., 1997). This may be why we observed little of the predicted increase of IL-10 in our system.

The stimulation of IL-6 by saliva may also benefit the parasite, assisting the in the establishment of an infection. Rincon et al. (1997) demonstrated that IL-6 is able to initiate the polarization of naïve CD4+ T cells to effector Type 2 cells by inducing the production of IL-4 in murine cells. They also report that IL-6 antagonizes the IL-12-mediated differentiation of Type 1 cells. Likewise, IL-6 has also been shown to inhibit IFN-γ signaling (Diehl et al., 2000). Thus, it would be beneficial for the parasite to take advantage of the exacerbative effects of sand fly saliva to attempt to drive a Th2 response in infected hosts.

The differences in the responses of Max and SGL, particularly in the PBMC cultures stimulated with *L. major* could be attributed to different proteins acting upon the cells present. Maxadilan is a single, synthetic peptide from New World sand flies, whereas SGL is extracted from Old World sand flies and contains numerous proteins, such as adenosine and apyrase which can be vasodilatory and have other immunomodulatory properties (reviewed in Kamhawi, 2000). Another difference

between Max and saliva from *P. papatasi* is the fact that Max is an extremely potent vasodilator and a similar molecule has not been found in Old World sand flies (reviewed in Gillespie et al., (2000). Very recently, a protein called SP15 has been isolated from the saliva of *P. papatasi* and appears to protect mice vaccinated with it, however, few of its immunomodulatory properties are known (Valenzuela et a., 2001). It is possible that SGL is influencing the PBMC in a manner we did not measure. Most of the work reported in mouse models that characterize the cytokine response of salivary gland lysates has been done in vivo (Mbow et al, 1998) and not in vitro, which could also account for the differences noted in this study and murine studies.

In our last experiment, we wanted to determine if Max was interacting with macrophages through the neuropeptide receptor, PACAP. PACAP shares many of the same immunomodulatory traits as Max, such as the stimulation of IL-6 (Cai et al., 1997) and the inhibition of IL-12 and IFN-γ production (Delgado et al., 1999). Moro and Lerner (1997) reported that Max is an agonist for this receptor and subsequently, Soares et al. (1998) determined that Max interacts with the PACAP receptor on murine macrophages. Our hypothesis was that Max interacts with the PACAP receptor on human macrophages. To test this, we blocked the PACAP receptor with the antagonist PACAP 6-38 and then exposed the macrophages to LPS stimulation. We measured TNF-α production and did not see a statistically significant effect. As we measured little effect on TNF-α in our other experiments, this finding was not surprising. However, when we measured IL-6 production, we found that Max-induced stimulation of IL-6 was abrogated when PACAP 6-38 is added to the LPS-stimulated macrophage cultures. This finding suggests that Max interacts with human macrophages through the PACAP receptor.

The influence of salivary gland proteins is important in human infections. An interesting study by Yin et al. (2000) correlated the amount of Max present in the saliva of different colonies of sand flies in South and Central America with the distribution of human cutaneous and visceral leishmaniasis caused by *L. chagasi*. Humans can have immunological reactions to sand fly saliva. In endemic areas, humans can produce antibodies to salivary gland antigens if they have been exposed to the sand fly (Barral et al., 2000). Several researchers have suggested using Max or salivary gland proteins as a potential vaccine candidate for *Leishmania* infections (Belkaid et al., 1998b; Kamhawi et al., 2000; Morris et al., 2001). This approach to vaccinate is inviting, as it is difficult to target the mammalian stages of parasites, since the parasites are so effective at circumventing the host immune response. Jacobs-Lorena and Lemos (1995) suggest that since a vector has more limited contact with hosts and there is no direct contact between the internal organs of the vector and its hosts, viable vaccine candidates could be directed at the vector's internal organs. This vector-directed vaccine strategy has worked effectively in the control of cattle ticks (de Castro and Newson, 1993). In *Leishmania* infections, pre-exposure of a host to salivary gland proteins, saliva or Max decreases the severity of a subsequent infection with *Leishmania* (Belkaid et al., 1998b; Kamhawi et al., 2000; Morris et al., 2001). As the saliva of sand flies is immunomodulatory and can alter the host's response to the parasite, it is essential not to ignore vector factors in vaccine design, especially since the host would be exposed to saliva with each feeding by the sand fly.

In conclusion, we determined that Max and sand fly saliva do indeed have an immunomodulatory effect on human cells. These salivary gland proteins can decrease

secretion of protective, Type 1 cytokines, such as IFN-γ and IL-12 in PBMC cultures and enhance the secretion of IL-6, a Type 2 cytokine, in macrophage cultures. We also suggest that Max interacts with macrophages via the PACAP receptor. In order to further sand fly related vaccination research it is necessary to more completely determine the effects of salivary gland proteins on the primary host of medical importance, the human. The immunomodulatory effects of Max and sand fly saliva can be further investigated in a primary in vitro system.

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CONCLUSIONS

The murine model of leishmaniasis has become the primary paradigm to investigate cell mediated immunity. Depending on the stain of mouse infected with *Leishmania major*, the mice will either develop a cell mediated immune response (termed a Type 1 response), characterized by IFN-γ and IL-12 production, and recover from the infection or the mice will develop a humoral immune response (termed a Type 2 response), characterized by the production of IL-4 and IL-5 which will stimulate B cell production of antibodies such as IgE, and the mice will not be able to control the infection (reviewed in Solbach and Laskay, 2000). Despite the fact the disease is very well characterized in mice, leishmaniasis is not as well studied in humans. Most studies involve infected patients. Humans appear to have similar responses to the parasite as mice (Herwaldt, 1999). Little is known of the early interactions between the parasite and human cells, as most of the studies have focused on patients who have had the disease for weeks, if not months.

Shankar and Titus (1993) developed a murine in vitro system using splenocytes from uninfected mice stimulated *L major*. Using this system, it was possible to characterize the early immune interactions (under two weeks) of murine cells with *L. major* (Shankar and Titus, 1993; Shankar and Titus, 1997; Soares et al., 1997). Since it is generally difficult to determine when infected humans are first exposed to the parasite as well as the initial parasite doses, it is not possible to effectively study early

human interactions with *L. major*. There was a need therefore, to develop an in vitro system in which experimental parameters, such as dose and infection time could be controlled. There have been a few investigations published where *Leishmania*-naïve human cells were used to evaluate *Leishmania*-human interactions, but these studies have not been well characterized, or involved deriving cell lines over a several week time period (Brodskyn et al., 2000; Russo et al., 1998 and 1999).

The goal of the research in this dissertation project was to establish a human primary *in vitro* system to allow for the investigation of early cellular immune responses to *L. major*. In order to achieve this objective, we isolated peripheral blood mononuclear cells (PBMC) from *Leishmania*-naïve donors and exposed the cells to *L. major*. We also characterized the interactions of stimulated human PBMC, as well as macrophages with sand fly salivary gland lysate or salivary Maxadilan. We measured a number of parameters to elucidate the effects of the parasite on the early immune response of human cells, including cytokine secretion, phenotypic analysis and T cell proliferation. There were three specific aims in this project:

Aim 1: To establish a primary in vitro system using human PBMC from *Leishmania*-naïve donors. The system was characterized phenotypically and via cytokine production.

Aim 2: To modulate the in vitro system to determine what cytokines play a role in the formulation of early immune responses.

Aim 3: To use the in vitro system to determine the effects of sand fly salivary gland proteins on human cells.

In our first study, we developed an in vitro priming system and characterized the early immune response of *L. major*-naïve cells to the parasite. We hypothesized that the

parasite-primed cells generated would primarily be class II-restricted, *L. major*-specific and CD4+. We also speculated that the cells would produce primarily IFN-γ. We determined that IFN-γ was indeed the cytokine produced in the highest concentration and most consistently detected. IL-5 and IL-12 were also detected, albeit at much lower levels. This finding corresponds to studies using infected human patients, where it has been reported that the presence of IFN-γ correlates control of the disease and healing (Kemp et al., 1994; Akuffo et al., 1997; Adjary et al., 2000). In contrast to our hypothesis, the *Leishmania*-primed cells in our system did not proliferate in response to only *L. major*, but also to other *Leishmania* species, suggesting that human cells recognize cross-reactive leishmanial antigens. In humans, cross protection has been reported in limited vaccination studies (Melby, 1991), and our findings appear to substantiate these observations, as our PIV system was fairly cross-reactive with other live *Leishmania* species.

Both CD4+ and CD8+ T cells appear to respond to the parasite in our system, as their populations were much greater than any other cell type and they appeared to become activated, as shown by CD25 expression. Our studies also suggest that *L. major* is being presented by both HLA class I and class II. There is some evidence for cross presentation of *Leishmania* antigens in both the murine and human systems (Kima et al., 1997; Brodskyn et al., 1997; Russo et al., 1999) and this may have been responsible for presentation via class I. We also determined that human macrophages are extremely efficient at killing the parasite. Our data suggest that this killing may be enhanced by interactions between T cells and macrophages.

As cytokines are the primary determinants for the outcome of leishmaniasis (reviewed in Launois et al., 1998), we investigated the interactions between cytokines reported to be important in *Leishmania* infections and the resulting production of IFN-γ, IL-10 and IL-5. We chose to study the immune responses early after parasite exposure, since a commitment to a Type 1 or Type 2 response occurs within the first several days of infection (Solbach and Laskay, 2000). We hypothesized that adding Type 1 cytokines, such as IL-12, would enhance IFN-γ production and blocking Type 1 cytokines would abrogate the IFN-γ response and enhance IL-5 and IL-10 production. Second, we hypothesized that adding Type 2 cytokines to PBMC cultures would enhance IL-5 and IL-10 production and reduce IFN-γ secretion. Our results demonstrated that IFN-γ is the dominant cytokine affected by *L. major* in the in vitro system. Th2 responses were modulated by IL-12, a Type 1 cytokine. IL-5 production was decreased by the addition of IL-12 and IL-10 was increased. The addition of Type 2 cytokines, such as TGF-β had little effect on the production of IL-10 or IL-5, suggesting that these Type 2 cytokines may not cross-regulate each other. IL-4 had little effect on the system, either with Type 1 or Type 2 cytokines. Overall, these data suggest that IFN-γ has an important regulatory role in human *Leishmania* infections.

Finally, we used our PIV system to explore whether sand fly salivary gland proteins could modulate the human immune response. We examined the effects of salivary gland lysate (SGL) of *Phlebotomus papatasi*, which contains multiple proteins, as well as Maxadilan, a single immunomodulatory protein found within *Lutzomyia longipalpis* saliva. We hypothesized that Max or SGL would suppress a Type 1 response and would enhance a Type 2 response in human PBMC or macrophage cultures. We first

studied the interactions of stimulated-*Leishmania*-naïve human PBMC and Max or SGL. We found that salivary proteins reduce IFN-γ and IL-12. Similar effects were reported in the mouse model when mice were exposed to SGL (Mbow et al., 1998). When we measured cytokine production in the macrophage cultures, we found that salivary proteins enhanced IL-6, a cytokine which can promote a Type 2 response. Using murine macrophages, Soares et al., (1998) also reported that Max could exacerbate IL-6. The in vitro system allows for the effect of vector salivary proteins to be examined in human cells. The interactions between the parasite, its mammalian host, and the vector are understudied in *Leishmania* research. By studying mammalian /vector/parasite interactions, we may be able to obtain a more complete picture of the disease process.

Although the murine model of leishmaniasis is an essential tool to our understanding of the disease, it is also important to investigate how humans respond to the parasite. If we have a better understanding of the interactions between *L. major* and human cells during the early stages of parasite establishment, it may be possible to discover more efficient therapeutic drugs that target particular interactions between the parasite and human cells. The human in vitro model also allows us to determine how parasite or sand fly-specific proteins may modulate the immune response early in infection. These types of studies would be difficult in human beings themselves due to not only ethical issues, but to the inability to precisely control experimental conditions. Our in vitro system allows us to add another dimension of study to the field of leishmaniasis.

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