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Evaluation of the Efficacy of Fusion or Combination Leishmania Donovani Peroxidoxin 1 and Superoxide Dismutase B1 Vaccine Candidates against Leishmaniasis in BALB/c Mice: Role of Granulocyte Macrophage Colony Stimulating Factor.

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by

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Abstract

Leishmaniasis is a vector-borne infectious disease that affects millions of people worldwide. Human leishmaniasis appears in three major clinical forms, cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL). Visceral leishmaniasis is the deadliest form with a mortality rate of about 100% in untreated clinically overt cases. The fact that people who are cured from CL develop durable protective immunity to re-infection has led to the assumption that developing effective vaccine to the disease should be feasible. However, there is no universally effective vaccine yet. Previous studies in our lab have demonstrated that amastigote-specific *Leishmania donovani* peroxidoxin 1 (LdPxn1) and iron superoxide dismutase B1 (LdFeSODB1) induce specific immune response and partially protect BALB/c mice when administered together with adjuvants. In this study, it was hypothesized that fusing these antigens or using them in a form of cocktail vaccine would further increase the immunogenicity and protective efficacy of the antigens. In addition, two forms of immunization strategies were compared; heterologous DNA/protein prime-boost and homologous protein/protein immunizations. Murine granulocyte macrophage colony-stimulating factor (mGMCSF) adjuvant was used in tandem fusion with the DNA vaccines. Generally, the fusion/cocktail vaccine significantly increased immunogenicity of the vaccines in both immunization protocols. However, the high immunogenicity result was not directly reflected in the protection. In DNA/protein approach, the fusion vaccine was found to be more protective than LdFeSODB1 but not LdPxn1. In protein/protein immunization, the cocktail vaccine showed lower protection than each of the individual antigens. As demonstrated by multiparameter flow cytometry, the increased immunogenicity and protection in DNA/protein immunization was correlated to induction of significantly higher number of antigen-specific $CD4^+$ helper T cells that individually

express IFN-γ, TNF-α, and IL-2 cytokines. In addition, the presence of mGMCSF adjuvant in DNA antigens generally increased immunogenicity and protective efficacy of individual or fusion vaccines as compared with the corresponding antigens without mGMCSF. Taken together, these results suggest that heterologous DNA/protein immunization with the fusion vaccine in the presence of mGMCSF adjuvant is more efficacious than protein/protein immunization with the cocktail vaccine.

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Dedication

To my mother, Melshiew Zerihun and my father, Genetu Bayih

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
α	Alpha
AIDS	Acquired Immuno Deficiency Syndrome
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
β	Beta
BALB/c	Bagg (Halsey J.) Albino c inbred mice
BCG	Bacille Calmette Guerin
C57BL/6	C57 Black 6
CHO	Chinese Hamster Ovary
CIAP	Calf Intestine Alkaline Phosphatase
CL	Cutaneous Leishmaniasis
Con A	Concanavalin A
CP	Cysteine Protease
CpG ODN	Cytosine-phosphate-Guanine Oligodeoxynucleotide
CTL	Cytotoxic T-Lymphocyte
DAT	Direct Agglutination Test
DC	Dendritic Cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FD-DAT	Freeze-Dried Direct Agglutination Test
Fe	Iron
γ	Gamma
GFP	Green Fluorescent Protein
GMCSF	Granulocyte Macrophage Colony-Stimulating Factor
gp63	Glycoprotein 63
GST	Glutathione-S-Transferase
His	Histidine
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IFAT	Indirect Fluorescent Antibody Test
IFN	Interferon
IgG	Immunoglubulin G
Ii	Ivariant chain
IL	Interleukin
IM	Intramuscular
IPTG	Isopropyl-β-D-1-Thiogalactopyranoside

kDa	Kilodalton
KMP	Kinetoplastid Membrane Protein
LACK	*Leishmania* Homolog of Receptors for Activated C Kinase
LB	Luria Bertani
LdFeSODB	*Leishmania donovani* Iron Superoxide Dismutase B
LdPxn	*Leishmania donovani* Peroxidoxin
LeIF	*Leishmania* Elongation Initiation Factor
LmSTI1	*Leishmania major* stress Inducible Protein 1
LN	Lymph Node
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
µ	Micro
MAC	Membrane Attack Complex
MCL	Mucocutaneous Leishmaniasis
MHC	Major Histocompatibility Complex
MnSOD	Manganese Superoxide Dismutase
MPL-SE	Monophosphoryl Lipid A-Squalene
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-reduced
NET	Neutrophil Extracellular Trap
NFk-β	Nuclear Factor kappa-B

Ni-NTA	Nickel-Nitrilo Triacetic Acid
NK	Natural Killer
NNN	Novy-MacNeal-Nicolle
NO	Nitric Oxide
OVA	Ovalbumin
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline – Tween
PCR	Polymerase Chain Reaction
Pen-Strep	Penicillin-Streptomycin
PKDL	Post-Kala-azar Dermal Leishmaniasis
PLGA	Polyactide-co-Glycolide
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PRR	Pattern Recognition receptor
PVDF	Polyvinyl Difluoride
RFLP	Restriction Fragment Length Polymorphism
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate
RPMI1640	Roswell Park Memorial Institute 1640
SC	Subcutaneous

SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulfate
SLA	Soluble *Leishmania* Antigen
SMT	Sterol-c-Methyl Transferase
TAP	Transporter Associated with Antigen Processing
TCA	Trichloroacetic acid
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-Beta
Th	T-helper
TIR	Toll/Interleukine 1 Receptor
TLR	Toll-Like Receptor
TMB	3,3’,5,5’-tetramethylbenzidine
TNF	Tumor Necrosis Factor
TSA	Thiol Specific Antioxidant
VL	Visceral Leishmaniasis
WHO	World Health Organization

Chapter One: **INTRODUCTION**

1.1 Leishmaniasis

Leishmaniasis is a vector-borne parasitic disease caused by an intracellular protozoan parasite belonging to the order *kinetoplastida* and genus *Leishmania*. More than 20 species have been reported as etiologic agent of the disease (El Hassan, 2001). The parasite is transmitted by the bite of female sandflies, which are of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. About 30 species of sandflies are proven vectors of leishmaniasis; the usual reservoir hosts include humans and domestic and/or wild animals (El Hassan, 2001, Desjeux, 2004).

Although leishmaniasis is predominantly a zoonotic disease where human beings are infected only accidentally, there are also anthroponotic forms, such as visceral leishmaniasis in India, where the disease is transmitted directly from an infected person to a healthy one through sandflies. In the zoonotic cases, rodents and dogs are major reservoir hosts and female sandflies get infected by feeding from reservoir hosts (Desjeux, 1996, Herwaldt, 1999) .

1.2 Types of Leishmaniasis

In humans, leishmaniasis presents itself in a spectrum of clinical manifestations. The three major clinical forms are visceral, cutaneous, and mucocutaneous leishmaniasis. Parasite factors are the major determinants for developing these forms. The parasite factors include tissue tropism, temperature sensitivity, as well as the ability to evade the immune response of and persist in the host (Murray et al., 2005). A genetic study on *Leishmania* amastigote-specific the A2 protein coding gene showed clear difference between visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) causing strains. It was found that A2 protein coding regions are present in VL-causing strains such as *L donovani*, *L chagasi*, and *L infantum* but absent in cutaneous

leishmaniasis-causing species such as *L major*, *L tropica*, *L aethiopica*, and *L braziliensis*. The only exception was CL-causing *L mexicana* that possesses the A2 gene and its protein product (Ghedin et al., 1997). Using anti-sense RNA technology, it was shown that the product of the A2 gene is important virulence factor for *L donovani*. A2-deficient amastigotes of *L donovani* injected in to BALB/c mice showed significantly lower survival as seen by lower parasite count in the liver (Zhang and Matlashewski, 1997). Interestingly, it was found that *L major* parasites that were transfected with plasmid containing the A2 gene showed significantly higher visceralization than wild-type parasites as seen by significant increase in the size of the spleen as well as three-fold increase in the parasite load in the spleen (Zhang and Matlashewski, 2001). On the other hand, transfection of *L major* with the A2 gene containing cosmid abrogated the ability of the parasite to cause cutaneous leishmaniasis. In BALB/c mice, subcutaneous infection with A2 transfected and control *L major* showed clear difference. Both groups showed similar footpad swelling during the early stage of infection. After eight weeks post-infection, mice infected with A2 expressing parasite showed significantly smaller lesion than those that were infected with the control *L major*. Similarly, intradermal infection of C57BL/6 mice with low dose metacyclic *L major* promastigote transfected with A2 gene containing cosmid failed to form any lesion on the infected ear and had much less parasite load than the control, wild type *L major* infected mice. Low dose infection of C57BL/c mice mimics the pathogenesis in humans. Moreover, A2 gene cosmid transfected parasites showed higher degree of migration out of the dermis than controls further strengthening the finding about the role of A2 gene encoded proteins in visceralization of leishmaniasis (Zhang et al., 2003). Although the different forms of leishmaniasis have different disease pathogenesis and manifestations, they also share similarities in that macrophages are

primary target cells and cure from the disease requires activation of these cells (Murray et al., 2005).

1.2.1 Visceral Leishmaniasis

Visceral leishmaniasis (VL), also known as Kala-azar, is the most severe form with a mortality rate of almost 100% in clinically overt untreated cases (Desjeux, 2004). It is caused by parasites of the *L donovani* complex: *L donovani* and *L infantum* in the Old World and *L chagasi* in the New World. *L donovani* is the cause of visceral leishmaniasis in Indian subcontinent and East Africa (Lukes et al., 2007). In southern Europe such as Spain, Italy, and France, visceral leishmaniasis is caused by *L infantum* (Dujardin et al., 2008). The clinical incubation period generally ranges from few weeks to several months. Visceral leishmaniasis is characterized by causing prolonged irregular fever, weight loss, splenomegaly, hepatomegaly, anemia, leucopenia, and thrombocytopenia. Unless treated, patients with these symptoms of active disease die within weeks to months as a result of severe anemia, secondary bacterial infection, and/or massive bleeding (Chappuis et al., 2007, Desjeux, 2004).

Some visceral leishmaniasis patients in Indian subcontinent and Africa develop a syndrome called post-kala-azar dermal leishmaniasis (PKDL). PKDL is a cutaneous manifestation of visceral leishmaniasis whose pathogenesis has not been fully understood. In India, it develops in 5 to 15% of individuals previously treated for visceral leishmaniasis. It appears 2 to 7 years after successful treatment of visceral leishmaniasis. In Sudan, more than 50% of patients develop PKDL within 0 to 6 months of visceral leishmaniasis diagnosis. Clinically, it is characterized by papular and nodular eruptions primarily on the face which later disseminates to the whole body. PKDL patients serve as a major reservoir of infection due to the presence of large number of the parasite in the nodular lesions (Murray et al., 2005, Croft, 2008, Ansari et al., 2006).

1.2.2 Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) produces skin lesions mainly on the exposed part of the body, such as the face, arms and legs, leaving permanent scar on the patient. The major etiologic agents of cutaneous leishmaniasis are *L tropica*, *L major*, and *L aethiopica* in the Old World and *L mexicana* complex and *L braziliensis* complex in the New World. Cutaneous leishmaniasis manifests itself in two different clinical forms; localized cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL) (Reithinger et al., 2007, Murray et al., 2005).

Localized cutaneous leishmaniasis is characterized by the development of a self-healing lesion on the exposed part of the body. It starts as a papule and develops into a nodule. Depending on the parasite strain, the nodules can develop into ulcerative lesions. Ulcerative lesions are more common in the New World cutaneous leishmaniasis. The clinical incubation period ranges from a few weeks to a few years. Most localized cutaneous leishmaniasis lesions heal spontaneously resulting in a life-long immunity to re-infection (Murray et al., 2005, Reithinger et al., 2007).

Diffuse cutaneous leishmaniasis (DCL) is a chronic progressive cutaneous form characterized by development of non-ulcerative, parasite filled, nodular lesions that occur in different parts of the body resembling lepromatous leprosy. The lesions never heal spontaneously and often relapse after treatment. DCL is caused by *L aethiopica* and *L mexicana* complex in the Old and New World, respectively. It has been shown that DCL is caused due to *Leishmania*-specific anergy (Murray et al., 2005, Reithinger et al., 2007, Akuffo et al., 1988).

1.2.3 Mucocutaneous Leishmaniasis

Mucocutaneous leishmaniasis (MCL) is caused mainly by parasites under *L braziliensis* complex. It mostly develops several years after the primary cutaneous lesion has healed. The parasite disseminated from the primary focus to mucous membranes through lymphatic or

haematogenous routes. It starts as erythema in the nostrils and progressively leads to perforation and destruction of the nasal septum and mutilation of the face. It can also disseminate to naso-oropharyngeal tissues causing further complications and extreme disfigurement. In contrast with DCL, MCL is caused by uncontrolled hyperactivity of cell-mediated immune response to the parasite. Moreover, lesions are characterized by having very low parasite load (Murray et al., 2005, Herwaldt, 1999, Reithinger et al., 2007).

1.3 Epidemiology of Leishmaniasis

According to the recent report by World Health Organization (Alvar et al., 2012), leishmaniasis is endemic in 98 countries in five continents. About three-fourth of these countries are developing or least developed. Thus, leishmaniasis is considered as the disease of the poor and disadvantaged people. Worldwide, a total of 350 million people live at risk of infection with an estimated prevalence of 12 million (Desjeux, 2004). A recent study has shown that the incidence of visceral and cutaneous leishmaniasis is estimated to be 0.2 to 0.4 million and 0.7 to 1.2 million per year, respectively. This report also pointed out that visceral leishmaniasis is responsible to an estimated 20,000 to 40,000 deaths per year (Alvar et al., 2012). The actual epidemiological figure of leishmaniasis is believed to be generally higher. This is due to low case detection and reporting in developing countries. These countries usually prefer passive case detection rather than active one due to lack of resources to conduct active epidemiological surveillance. In addition, leishmaniasis is sometimes misdiagnosed due to the similarity of early clinical symptoms with those of other diseases such as malaria (Desjeux, 2004, Alvar et al., 2006, WHO, 2007). For example, a recent epidemiological study in Bihar province in India has shown that visceral leishmaniasis reported by the national surveillance was 4.2-fold less than active case detection by house-to-house count (Singh et al., 2010).

The distribution of leishmaniasis is expanding due to changes in socio-economic activities such as rapid urbanization, massive migration, and development of extensive irrigation schemes in affected countries. Individual risk factors such as malnutrition and HIV-*Leishmania* co-infection are also attributed to the expansion in the geographical distribution of leishmaniasis in these countries. In South and Central American countries such as Brazil, the rapid and usually unplanned expansion of cities leads to creation of suburban settlements which exposes people to infection with zoonotic leishmaniasis which otherwise is limited to the rural areas (Desjeux, 2004). In East African countries such as Ethiopia, seasonal migration of non-immune labourers from the highlands to leishmaniasis endemic lowlands increases the transmission of the disease to previously unaffected areas (Bashaye et al., 2009). In addition, the ever-expanding of areas with *Leishmania* and HIV co-infection is contributing to the expansion of leishmaniasis. In East African countries such as Ethiopia and Kenya, HIV is becoming more common in rural areas subsequently increasing the overlapping geographical distribution of *Leishmania* and HIV infections (Desjeux, 1996, WHO, 2000).

Leishmaniasis is one of the most neglected diseases and is strongly associated with poverty. It has been shown that lack of access to treatment, poor housing conditions, migration of people for work or due to war and famine as well as malnutrition significantly increase morbidity and mortality due to leishmaniasis (Alvar et al., 2006). It has been shown that an estimated 100,000 people from a population of less than 500,000 died of visceral leishmaniasis in South Sudan in 1990s (Seaman et al., 1996). A 10 year prospective study conducted in Brazil indicated that malnutrition in children is an important risk factor for developing severe visceral leishmaniasis. Children with severe malnutrition were found to be nine times more likely to develop severe VL than normal children. The study found statistically significant increase in susceptibility to

developing VL in severely malnourished children than normal or mildly malnourished children. Interestingly, the study also showed that the level of malnutrition determines the severity of the disease. Severely malnourished children developed VL of significantly higher severity while normal and mildly malnourished children developed subclinical infection (Cerf et al., 1987).

On the other hand, compared to other tropical diseases such as malaria, the cost of leishmaniasis treatment is too high. It usually wipes out savings and assets of affected families further aggravating poverty and putting them in vicious cycle of poverty and disease (Alvar et al., 2006). Emergence and re-emergence of the disease, infections in travellers and soldiers, the spread in Europe and its strong association with HIV infection have led to renewed interest in studying leishmaniasis. In addition, *Leishmania* is used as a model organism to study intracellular pathogens and also skin/parasite interactions (Kautz-Neu et al., 2012, Herwaldt, 1999).

1.4 Life Cycle of *Leishmania*

Leishmania is an intracellular parasite. It has a digenic life cycle consisting of a uniflagellated promastigote stage, which resides within the alimentary tract of the sandfly vector and the amastigote stage, which multiplies inside phagolysosomal vacuoles of mammalian macrophages (Mosser and Brittingham, 1997).

As shown in **Figure-1.1** below, the life cycle begins when the vector introduces infective (metacyclic) promastigotes into the skin of the host during blood meal. The promastigotes are, then, quickly taken up mainly by localized tissue macrophages, Langerhans cells, and also by neutrophils and monocytes that are brought to the site due to inflammation caused by the bite wound. Within the early phagosome of macrophages, promastigotes lose their flagellum and start to develop into nonmotile amastigotes. The parasite containing phagosome fuses with lysosome to form a highly acidic phagolysosome. The amastigotes survive and replicate within the acidic

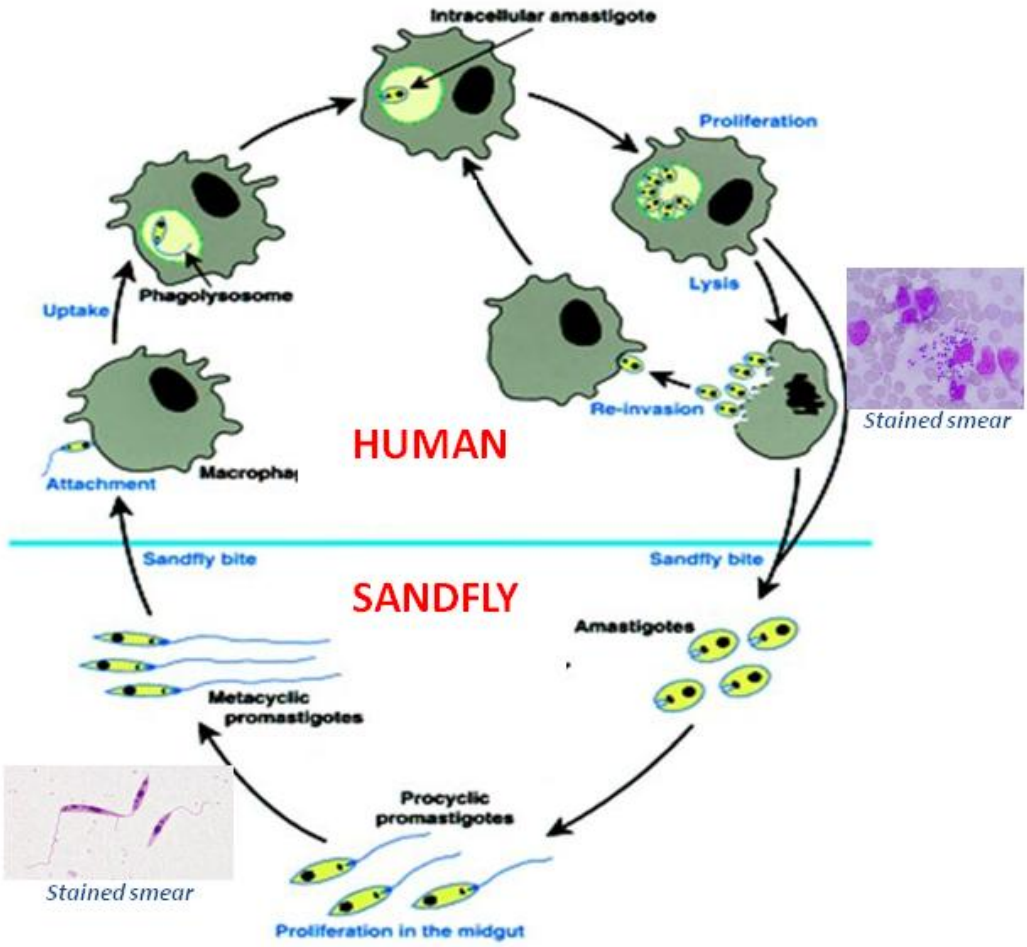
and hydrolytic environment of the phagolysosome. Multiplication of the parasite eventually leads to lysis of the infected cell freeing amastigotes to infect nearby cells. *Leishmania* parasites use different mechanisms and surface molecules to enter into host cells and cause infection. Metacyclic promastigotes fix components of the complement system such as C3b and C3bi on their surface, a process called opsonisation. The complement components, in turn, interact with their respective receptors on macrophages, CR1 and CR3, effectively mediating phagocytosis of the promastigotes by macrophages. The mannan residues on the LPG (lipophosphoglycan) of metacyclic promastigotes are also implicated for phagocytosis via their receptor on macrophages, the mannose-fucose receptor (Mosser and Brittingham, 1997, Olivier et al., 2005, Mougneau et al., 2011, Handman, 2001).

Upon taking a blood meal, the sandfly ingests either free amastigotes or amastigote-infected macrophages or both. Amastigotes in the blood meal develop into a multiplicative stage called procyclic promastigote and attach to epithelial cells in the midgut of the sandfly. The attachment is mediated by lipophosphoglycan molecules at the surface of promastigotes. From day-five after taking the blood meal, procyclic promastigotes progressively develop into non-replicating and highly motile metacyclic forms in the anterior midgut and foregut of the sandfly ready to infect a new host. Modification in the structure of LPG of promastigotes brings about loss of attachment so that the parasite can move to the foregut. Preferential selection of a certain strain of *Leishmania* by a sandfly species over another strain is attributed partly to the difference in the LPG structure of the parasite strains. Surface structural specificities also play role in withstanding digestive enzymes in the sandfly gut (Sacks and Kamhawi, 2001, Mosser and Brittingham, 1997, Olivier et al., 2005, Saporito et al., 2013). Kamhawi and colleagues (2004) have identified a receptor in the sandfly, *Plebotomus papatasi* that specifically binds to LPG of *L*

major. The receptor, PpGalec, is a tandemly repeating galectin expressed in the midgut of the sandfly and binds specifically to *L major* LPG.

Figure 1.1: Life cycle of *Leishmania*.

(Adapted from: (Handman, 2001)).


Intracellular amastigote
Proliferation
Lysis
Stained smear
Re-invasion
Phagolysosome
Uptake
Attachment
HUMAN
Sandfly bite
Sandfly bite
SANDFLY
Amastigotes
Metacyclic promastigotes
Procyclic promastigotes
Stained smear
Proliferation in the midgut

1.5 *Leishmania*-HIV Co-infection

Leishmania-HIV co-infection has become an important public health problem in places where the two infections overlap. As of 2007, 35 countries around the World have reported cases of co-infection. The overlapping geographical distribution of *Leishmania*-HIV co-infection is continuously increasing as a result of urbanization of visceral leishmaniasis as in Brazil and ruralisation of HIV as seen in East African countries such as Ethiopia and Kenya. Globally, *Leishmania*-HIV co-infection has been reported in 2-12% of all visceral leishmaniasis cases. This figure is much higher in some places such as Humera in Ethiopia where 15-30% of VL patients are also infected with HIV (WHO, 2000, WHO, 2007).

Leishmania and HIV, being intracellular pathogens, share a lot of similarities both in terms of the type of cells they invade and the immune response they modulate. As a result, the two pathogens together exert a synergistic deleterious impact on the cellular immune response. For example, it has been found that infection with VL-causing *Leishmania* strains activates HIV in latently infected cells. In addition, active VL significantly enhances the clinical progression of HIV infection to AIDS-defining conditions. On the other hand, HIV infection has been found to increase the risk of developing VL by a factor of between 100 and 1000 in endemic areas. It also reduces efficacy of drug treatment and greatly increases the rate of relapse (WHO, 2007). Studies have shown that greater than 90% of HIV associated visceral leishmaniasis in Mediterranean regions is due to re-activation of latent infections (Morales et al., 2002). As a result of high parasite load, *Leishmania*-HIV co-infected individuals remain to be important reservoirs of infection in places where *Leishmania* transmission is anthroponotic (WHO, 2007).

Co-infection with HIV also changes the pathophysiology and clinical manifestations of cutaneous leishmaniasis (CL). Cutaneous leishmaniasis patients co-infected with HIV show

unusual signs such as appearance of more than one clinical form in one patient and also visceralization of CL-causing strains (WHO, 2007).

1.6 Diagnosis of Leishmaniasis

Clinical diagnosis of leishmaniasis is usually a challenging exercise due to tremendous diversity of clinical signs and symptoms of the disease. For example, patients with cutaneous *Leishmania* infection could show a single nodular or necrotic lesion or multiple disseminated lesions all over the body resembling that of lepromatous leprosy. On the other hand, the signs and symptoms of visceral leishmaniasis are shared by other diseases such as malaria, tuberculosis, and typhoid fever which are also prevalent in areas where leishmaniasis is endemic (Sundar and Rai, 2002, Reithinger and Dujardin, 2007, Murray et al., 2005).

Rapid and effective diagnosis of leishmaniasis is necessary not only for proper management of patients but also for better control of the disease. Laboratory diagnostic techniques are said to be effective if they are easy to perform, specific, sensitive, and also affordable. Laboratory diagnosis of leishmaniasis involves several techniques involving direct microscopic examination of the parasite from samples taken from affected tissue, *in vitro* culture, serological methods detecting anti-leishmanial antibody or *Leishmania* antigens as well as molecular biological techniques such as polymerase chain reaction (PCR) (Reithinger and Dujardin, 2007, Srivastava et al., 2011).

The parasitological method involving microscopic examination of a stained smear is the gold-standard diagnosis for visceral and cutaneous leishmaniasis. It involves Giemsa- or Leishman-staining of aspirates of spleen, lymph node or bone marrow for VL and biopsy or skin scraping for CL followed by microscopic examination of the stained smears. Microscopic examination has a high degree of specificity but requires well-trained personnel (Reithinger and Dujardin,

2007). Spleen aspirate microscopy has a sensitivity of greater than 95%. However, smears of bone marrow and lymph node aspirates have sensitivities of 55-97% and 60%, respectively (Murray et al., 2005). Laboratory diagnosis of cutaneous leishmaniasis mainly involves identifying amastigotes in stained preparations of biopies, skin scrapings, or impression smears. Depending on the availability, *in vitro* culture of parasites is also used to diagnose cutaneous leishmaniasis. The use of combination of microscopy and culture has been found to increase the sensitivity of detection to more than 85% (Murray et al., 2005).

Serological techniques are useful methods to diagnose visceral leishmaniasis. In addition to being sensitive and specific, serological methods are relatively easy to perform even in places with minimal facility and also do not require highly qualified personnel. Because of low level antibody response and variable sensitivity, serological methods are not appropriate for the diagnosis of cutaneous leishmaniasis (Reithinger and Dujardin, 2007, Srivastava et al., 2011). Serological tests that involve antibody detection include Direct Agglutination Test (DAT), immunochromatic rk39 dipstick, Indirect Fluorescent Antibody Test (IFAT), and Enzyme Linked Immunosorbent Assay (ELISA). DAT test using freeze-dried *Leishmania* antigen (FD-DAT) and rk39 dipstick tests have become outstanding methods for the diagnosis of visceral leishmaniasis. They are not only sensitive but also simple and less expensive. However, they have inherent problem in that they are positive in 20 to 30% of healthy individuals who live in endemic areas. Furthermore, these techniques do not differentiate people having active infection from cured ones (Srivastava et al., 2011). Antigen testing from urine samples of VL patients has also been found to have more that 95% sensitivity and specificity (De Colmenares et al., 1995). Unlike the antibody tests, antigen tests are not positive in samples from cured individuals. These

tests are particularly important in HIV-visceral leishmaniasis co-infection cases because of the low antibody response in these patients (Srivastava et al., 2011).

Several molecular methods have been developed or are in the process of development to diagnose leishmaniasis from clinical samples and also to identify the type of infecting strain. Most molecular techniques involve amplification of DNA by polymerase chain reaction (PCR). The techniques include PCR-RFLP (restriction fragment length polymorphism), real-time PCR, and PCR-ELISA (Reithinger and Dujardin, 2007, Laurent et al., 2009, Adams et al., 2010). Molecular diagnostics techniques have several advantages over the classical techniques such as microscopy. They are generally rapid, more sensitive and are especially useful in samples with very low parasite load. Moreover, they can be employed in identifying the type of infecting *Leishmania* stain and quantifying the parasite load. However, these features come with some limitations in that molecular techniques are prone to false positive results due to contamination of lab-wares with amplicons from positive samples such as in simple PCR. Moreover, these techniques require high-tech and expensive equipment and highly skilled personnel which significantly increase the cost of the tests in developing countries (Reithinger and Dujardin, 2007, Srivastava et al., 2011).

1.7 Treatment of Leishmaniasis

Treatment of leishmaniasis relies on chemotherapeutic agents that are mostly not effective, costly, and/or associated with serious side-effects. The drugs include pentavalent antimonials (sodium stibogluconate and meglumine antimonate), pentamidine, amphotericin B, miltefosine, and paromomycin (Murray et al., 2005, Sundar and Chakravarty, 2013). As one of the most neglected diseases affecting the poor and disadvantaged, development of new anti-*Leishmania* drugs is not a priority for pharmaceutical industries. A retrospective study showed that out of

1393 drugs marketed between 1975 and 1999 only 16 were for tropical diseases including leishmaniasis, malaria, Chagas' disease, lymphatic filariasis, and schistosomiasis as well as for tuberculosis (Trouiller et al., 2002).

Pentavalent antimonials have long been used as first-line drugs to treat visceral leishmaniasis world-wide. The drugs are given parenterally for up to 20 to 30 days (Moore and Lockwood, 2010, Sundar and Rai, 2005). Treatment with pentavalent antimonial drugs is no longer in use in Bihar state in India where 45% of the world's kala-azar cases exist as a result of high degree of drug resistance. Antimonial resistance in Bihar state has been found to be greater than 60% (Croft et al., 2006).

Pentamidine which was once used as a second-line drug in patients who do not respond to pentavalent antimonials is no longer in use in most endemic regions. In addition to losing its efficacy, it showed unacceptably high toxicity. It causes cardiac toxicity, diabetes mellitus, hypotension, and gastrointestinal abnormality (Moore and Lockwood, 2010, Sundar and Rai, 2005).

Although amphotericin B is highly effective drug to treat visceral leishmaniasis, its use is limited because of the very high degree of toxicity it causes on patients. Treatment with amphotericin B has been found to cause irreversible toxicity such as renal failure. Liposomal amphotericin B, however, is very effective and shows less toxicity. It has higher tissue penetration and selectively targets macrophages. It is now the drug of choice to treat visceral leishmaniasis in Europe. However, extremely high cost of the drug impedes its use in poor countries in South Asia and East Africa (Murray et al., 2005, Sundar and Rai, 2005).

Miltefosine, is the first oral anti-leishmanial drug. Although miltefosine can be used as an outpatient drug reducing the treatment cost due to hospitalization, its efficacy is variable in

different endemic areas. For example, in Ethiopia, it was found to be less efficacious than sodium stibogluconate (Ritmeijer et al., 2006). As a result of reproductive toxicity (teratogenicity) seen in animal studies, miltefosine is contraindicated in pregnant women and is not recommended to women of child bearing age (Moore and Lockwood, 2010). Efficacy of oral miltefosine for the treatment of visceral leishmaniasis is declining in previously effective areas. It has been found that the cure rate at six months after treatment in India has declined from 94% to 90% in less than a decade. Moreover the relapse rate doubled from 3% to 6.8% (Sundar and Chakravarty, 2013).

Paromomycin is an aminoglycoside antibiotic that has generally shown good efficacy for the treatment of both visceral and cutaneous leishmaniasis. Although the efficacy is generally lower and variable between different areas in East Africa, it has shown more than 95% efficacy in highly endemic areas in India. The drug is approved in India for the treatment of visceral leishmaniasis (Sundar and Chakravarty, 2013).

Most cutaneous leishmaniasis lesions heal spontaneously in 2 to 15 month, some leaving permanent scar and cosmetic problem. Anti-leishmanial drugs are given to these patients in some leishmaniasis endemic regions to accelerate cure, reduce scarring, and reducing the likelihood of relapse. Drugs are also used to treat mucosal leishmaniasis. Pentavalent antimonials are the most commonly used drugs to treat cutaneous leishmaniasis (Murray et al., 2005). Antimonials alone or in combination with immunomodulators have been tried for the treatment of cutaneous leishmaniasis. Clinical trials in humans have been conducted to evaluate the efficacy of treatment of cutaneous leishmaniasis using a combination of pentavalent antimonials and an immunomodulator, imiquimod, in Peru. The results showed that combination of the drugs with topical 5% imiquimod cream modestly increased the effectiveness of the treatment and also

accelerate healing (Miranda-Verastegui et al., 2005, Miranda-Verastegui et al., 2009). Pentamidine is also used in fewer doses to effectively treat cutaneous leishmaniasis in different endemic regions. Studies on the use of miltefosine for the treatment of cutaneous leishmaniasis have shown a wide range of efficacy from 53% to 91%. In topical formulation, paromomycin has shown efficacy of between 74% and 86% for the treatment of cutaneous leishmaniasis in different endemic regions (Sundar and Chakravarty, 2013).

1.8 Host-Parasite Interactions in Leishmaniasis

As described in the life cycle, the infective metacyclic promastigote of *Leishmania* is introduced into the mammalian host via the bite of sandfly vector. Once in the mammalian tissue, the promastigote is exposed to a hostile host defense system. In order to combat leishmanial infection, the host mobilizes its non-specific and specific immune responses. The non-specific type ranges from the effect of complement to the different antiparasitic actions of immune cells. The specific immune response to *Leishmania* involves the use of antigen specific T lymphocytes. On the other hand, in order to survive and eventually cause infection, the parasite is endowed with an array of evasion mechanisms. The equilibrium of the two oppositely functioning processes determines the outcome of infection; either the parasite is destroyed and infection aborted, asymptomatic infection established or a full-blown infection ensue (Bogdan et al., 1990, Bogdan et al., 1996).

1.8.1 Immune Response to Leishmania Infection

Host immune responses to *Leishmania* infection can be broadly divided into two, innate and adaptive immunity. Innate immunity appears early in the process of infection and contributes in the control of infection in a non-specific manner. It is characterized by its ability to recognize the presence of “danger signals” and by its rapid response. The innate immune system includes

complement, phagocytic cells, and natural killer (NK) cells. The adaptive immune response, on the other hand, is specific. That is, it involves response to specific antigens. The specificity of adaptive immune response is the result of rearrangement of T cell receptors and immunoglobulin genes of the host which, in turn, brings about the creation of receptors each of which is specific to a specific epitope. Moreover, unlike the innate immune response, it comes later in the process and also induces immunological memory (Banchereau et al., 2000, Korbel et al., 2004, Cancro MP, 2005).

1.8.1.1 Innate Immune Response

The innate immune response is a part of the host defense system that appears in the acute phase of infection. In addition to mounting an antileishmanial effect, innate immunity also contributes in the initiation and modulation of the adaptive immune responses which comes at the later phase of infection. Innate immunity encompasses response by both soluble factors and cells. It includes the complement system as well as the cellular response by neutrophils, macrophages and natural killer cells. Dendritic cells serve as a bridge between innate and adaptive immune responses (Pearson and Steigbigel, 1980, Okwor et al., 2012, Mougneau et al., 2011).

1.8.1.1.1 Complement System

Upon entry into the mammalian host, extracellular promastigotes are exposed to the first-line of the host defense including the complement system. All *Leishmania* species can activate the complement system via the alternative pathway. This process may directly affect the survival of promastigotes or influence their subsequent interaction with macrophages (Mosser and Brittingham, 1997).

Pearson and Steigbigel (1980) investigated the action of serum from a non-immune person on promastigotes *in vitro*. While *Leishmania donovani* promastigotes exposed to fresh serum died,

those exposed to heat inactivated serum survived. They also showed that removal of the terminal complement component, C5b-C9, abolished the effect of serum on the promastigotes. The anti-leishmanial effect of host serum depends on the developmental stage of the parasite. Procyclic promastigotes fix the terminal component of complement and are readily lysed by non-immune serum whereas, metacyclic forms are partially resistant to serum-mediated killing (Bogdan et al., 1990).

1.8.1.1.2 Cells of the Innate Immune System

Cells of the innate immune system such as neutrophils, macrophages, and natural killer cells take part in host defense against *Leishmania* infection. Entry of the *Leishmania* parasite through a sandfly bite or needle inoculation triggers influx of cells of the innate immune system to the site of infection. The first group of cells to arrive to the infection site are polymorphonuclear leukocytes (neutrophils) followed by monocytes, dendritic cells and natural killer cells. These cells play roles not only in non-specific immune response but also in shaping the type of specific cell-mediated immune response that will dominate at the later stage of infection (Peters et al., 2008, Laskay et al., 2003, Kautz-Neu et al., 2012).

A. Neutrophils and Macrophages

In *Leishmania* infection, neutrophils play a role either in host defense against the parasite or in the dissemination of the parasite. These possibilities seem to depend on the strain of *Leishmania* parasite. Neutrophils exert antileishmanial effects in infection with strains of *L braziliensis* and *L donovani*. On the contrary, other strains such as *L major* use neutrophils as a “Trojan horse” to invade other cells and establish an infection (Novais et al., 2009, McFarlane et al., 2008, Peters et al., 2008, Laskay et al., 2003).

Neutrophils are the first wave of cells recruited to the site of infection. Within a few hours after parasite entry, massive numbers of neutrophils migrate into the skin. Acute local inflammatory response triggered during the sandfly bite induces the production of chemokines that recruit neutrophils to the site of infection. Neutrophils themselves also produce a chemokine, IL-8, and induce recruitment of more neutrophils amplifying their number at the infection site (Laskay et al., 2003). Moreover, it has been shown in mice that phagocytosis of promastigotes by macrophages induces the cells to produce a chemoattractant factor which recruits neutrophils to the site of infection (Racoosin and Beverley, 1997).

Peters and colleagues (2008) used *in vivo* imaging and flow cytometric methods to study the recruitment of different types of cells during the sandfly bite and also needle inoculation of *L major* promastigotes. In addition to clearly showing the dynamics of cell infiltration at the site of infection, they also demonstrated the role of neutrophils in establishing *L major* infection. Parasites expressing red fluorescent protein (RFP) were used to infect C57BL/6 mice that express Green Fluorescent Protein (eGFP). Both flow cytometric and imaging methods showed a high degree of neutrophil infiltration at the site of a bite by both infected and uninfected sandflies. Similar results were seen by needle inoculation of parasites to mice. After 2hr, a massive infiltration of GFP expressing neutrophils was seen at the site of the bite by both infected and uninfected sandflies. Eighteen hour post-infection (needle), the red fluorescent protein (RFP) signal was highly associated with neutrophils. Only a small number of macrophage/monocyte and Cd11c+ DCs were associated with the RFP signal. However, about 1 week later, the RFP signal was primarily associated with macrophages/monocytes and was significantly diminished in neutrophils. The RFP signal also increased on Cd11c+ DCs. After sorting neutrophils by a cell sorter, a limiting dilution assay was done using neutrophils. The

result showed that a large proportion of the infecting parasites were viable. This corroborates the notion that neutrophils act as "Trojan horse" for *L major* parasite to survive, enter into macrophages and dendritic cells and establish infection (Laskay et al., 2003, Kautz-Neu et al., 2012).

Neutrophils exert antileishmanial activity through different mechanisms. They phagocytose promastigotes and kill them through generation of reactive oxygen intermediates and lytic enzymes, release neutrophil extracellular traps (NETs), and indirectly kill by cooperating with macrophages. NETs are mesh like fibrous traps made from DNA and granule proteins of dead neutrophils. On the other hand, invasion of neutrophils by *Leishmania* inhibits apoptosis thus prolonging the life of the cell. Hence, infected neutrophils could act as reservoir of the parasite to infect new cells (Okwor et al., 2012, Mougneau et al., 2011).

The exact role of neutrophils in *Leishmania* parasite dissemination or host protection has been a contentious issue and appears to be species-specific (Okwor et al., 2012, McFarlane et al., 2008). It has been shown in genetically resistant mice that phagocytosis of *L major* by neutrophils helps the parasite to establish infection (Peters et al., 2008). However, in *L braziliensis*, neutrophils are involved in elimination of the parasite and inducing protective immunity in both genetically susceptible and resistant mice (Novais et al., 2009).

It has been shown both *in vivo* and *in vitro* experiments that neutrophils, in cooperation with macrophages, play a crucial role in host defense against *L braziliensis* infection through TNF-α and superoxide mediated mechanisms in both susceptible and resistant strains of mice. The protection was dependent on physical contact between neutrophils and macrophages as separation of these groups of cells with a semipermeable membrane did not reduce the proportion of infected macrophages (Novais et al., 2009). While live neutrophil co-inoculation

enhanced parasite death, *in vivo* depletion of these cells promoted parasite growth. Moreover, *in vitro* co-culture of live neutrophils with *L braziliensis* infected macrophages reduced the parasite load. The same treatment significantly increased the parasite load in *L major* infected macrophages. On the other hand co-culture of fixed neutrophils with *L braziliensis* infected macrophages did not reduce the parasite load. The effect of neutrophils was also similar on *L amazonensis* and *L chagasi* infected macrophages (Novais et al., 2009).

A neutrophil depletion experiment on BALB/c mice infected with *L donovani* using a specific monoclonal antibody indicated the protective role of neutrophils in visceral leishmaniasis. *L donovani* infected mice whose neutrophils were depleted in the first two weeks of infection showed significantly higher parasite load both in the spleen and bone marrow. Moreover, there was impaired expression of inducible nitric oxide synthase and granuloma formation in the liver. *In vitro* stimulation of spleen cells showed elevated levels of IL-4 and IL-10 and also reduced level of IFN-γ (McFarlane et al., 2008).

Macrophages play important role in the non-specific immunity against *Leishmania*. These cells devise several mechanisms that modify the physical and chemical environment in which the parasite lives. Phagocytosis of opsonised *Leishmania* promastigotes activates macrophages to produce massive amounts of reactive oxygen intermediates (ROIs) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$) with concomitant consumption of oxygen. This process is termed as oxidative/respiratory burst. The production of ROIs by activated macrophages is catalyzed by the NADPH oxidase enzyme system. The NADPH oxidase system is a complex consisting of multiple proteins. In unstimulated cells, this system exists in a dissociated form where the subunits reside in the cytoplasm and cell membrane. Upon stimulation, the subunits in the cytoplasm are assembled and translocated into the membrane that

forms a phagolysosome where together with the subunits in the membrane forms a functional oxidase (Robinson and Badwey, 1994, Robinson, 2008, Woods et al., 1994).

Infected macrophages also produce reactive nitrogen intermediates (RNIs) through enzymatically catalyzed reactions. It begins with the production of nitric oxide (NO) by oxidation deimination of L-arginine by nitric oxide synthase. The redox reaction of nitric oxide with superoxide radical produces other RNIs such as nitrogen dioxide (NO_2), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$). ROIs and RNIs exert their antiparasitic effect by damaging the parasite molecules such as enzymes of the central metabolic pathways, DNA and membranes (Woods et al., 1994, Robinson and Badwey, 1994).

B. Natural Killer Cells

Natural killer (NK) cells are bone marrow-derived lymphoid cells that directly kill tumor cells as well as cells infected with viruses and intracellular pathogens without prior exposure to them (Korbel et al., 2004). In addition, NK cells play important role in host defense against intracellular pathogens by releasing cytokines such as IFN-γ and TNF-α. As discussed in the next section, these cytokines activate the microbicidal activity of macrophages and neutrophils, stimulate the differentiation of Th-1 $CD4^+$ cells, and enhance antigen presentation by up-regulating expression of MHC molecules on antigen presenting cells. The latter two phenomena are important precursors for effective adaptive immune response against intracellular pathogens (Korbel et al., 2004, Prajeeth et al., 2011).

Like neutrophils, natural killer cells form part of the early inflammatory cell infiltrate at the site of *Leishmania* infection. Their activation is regulated by cytokines produced primarily by dendritic cells (DCs) and macrophages. IL-12, IL-15, IL-18, TNF-α, and IFN-α/β are implicated in activating NK cells, of which, IL-12 is the most potent one. IL-12 is produced by myeloid

dendritic cells (DCs) in a Toll-like receptor (TLR)-9-mediated mechanism. On the other hand, cytokines such as IL-4, IL-10, and TGF-β are known to suppress NK cell function (Prajeeth et al., 2011, Bogdan, 2012, Korbel et al., 2004). It has been shown that *Leishmania major* DNA activates DCs via TLR-9-dependent manner (Abou Fakher et al., 2009).

Although natural killer cells are not necessary for final control of both cutaneous and visceral leishmaniasis, they play important role in early resistance against leishmaniasis. The production of early IFN-γ by NK cells helps to shift the specific cellular response to a protective phenotype and establish a lasting protection (Bogdan, 2012). The early work of Laskay and colleagues (1993) demonstrated direct evidence that NK cells play crucial role in early resistance to *L major* infection in mice. Depletion of NK cells in genetically resistant C57BL/6 mice resulted in significantly higher footpad swelling and parasite load at two weeks post-infection than control mice. This difference was not maintained during the later course of infection as both normal and NK cell depleted mice healed after 16 weeks post-infection. Moreover, lymph node cells from NK cell depleted mice produced less IFN-γ than those of control mice *in vitro*.

Studies on human samples using *L aethiopica* whole cell antigen showed that NK cells play role in protection against cutaneous leishmaniasis. Stimulation of peripheral blood mononuclear cells of healthy individuals from a non-*Leishmania* affected country showed increased proliferation of NK cells indicating the role of these cells in innate immune response to *L aethiopica* (Akuffo et al., 1993). It was also shown in another human study that NK cells are involved in exerting protection against *L aethiopica* infection in endemic areas. Cell proliferation and phenotype analysis showed that people cured from cutaneous leishmaniasis caused by *L aethiopica* and healthy individuals living in endemic area have higher NK cell response than those actively infected ones (Maasho et al., 1998).

Recent *In vivo* and *in vitro* studies have shown that the anti-leishmanial activity of natural killer cells is not through direct cytotoxicity/lysis of infected cells. Rather, NK cells exert cell contact-independent killing of *L infantum and L major* in infected macrophages. By releasing cytokines such as IFN-γ and TNF-α, NK cells activate infected macrophages to kill the parasite through inducible nitric oxide synthase-mediated mechanism (Prajeeth et al., 2011).

C. Dendritic Cells - The Bridge to Specific Adaptive Immune Response

Dendritic cells (DCs) are a heterogeneous group of cells that have both lymphoid and myeloid origins. They are capable of interacting with cells of both innate and adaptive immune responses such as T cells, B cells, and NK cells through direct cell-cell contact or indirectly through cytokine production. Dendritic cells are sentinel cells that sample antigens in the peripheral tissues and transport, process, and present them to T cells inside lymphoid organs so as to initiate the adaptive immune response. Microbial invasion of tissue provides "danger signals" that activate DCs which, in turn, induce antigen specific T cells, consequently establishing specific immunity against the infectious agent. The danger signals include microbial products such as lipopolysaccharide and unmethylated CpG DNA. This feature of dendritic cells that involves recognition of pathogens based on pathogen-associated molecular patterns (PAMPs) and presenting pathogen molecules to variable and specific receptors on T cells makes them central cells linking the innate and adaptive immune responses (Shortman and Liu, 2002, Banchereau et al., 2000).

Dendritic cells are professional antigen presenting cells (APCs). Unlike other APCs such as macrophages, who present antigens only through MCH-II, DCs present via both MHC-I and MHC-II. Therefore, DCs are capable of inducing both $CD4^+$ and $CD8^+$ immune responses. In addition, while macrophages induce only previously primed $CD4^+$ cells, DCs can induce both

naive and primed $CD4^+$ cells. Dendritic cells are also important in the regulation of the type of T-cell immune response. In *Leishmania* infection, DCs are able to shift the immune response into a protective phenotype through the production of IL-12. Macrophages do not mediate this differentiation because they do not produce IL-12 at the early phase of *Leishmania* infection (Banchereau et al., 2000, Kautz-Neu et al., 2012). The interaction between dendritic cells and naive $CD4^+$ T cells is depicted in **Figure-1.2** below.

Dendritic cell progenitors in the bone marrow differentiate into precursors of DCs. Generally, DC precursor cells which circulate in the blood and lymphatic system develop into immature DCs in different peripheral tissues. Moreover, monocytes that are recruited to the site of pathogen entry or to the draining lymph node can also differentiate into dendritic cells. These cells are called monocyte-derived dendritic cells (mDC).

Immature DCs are endowed with the ability to capture antigens through a variety of mechanisms such as receptor-mediated endocytosis of microorganisms and phagocytosis of apoptotic or necrotic cell fragments containing antigens. After antigen uptake, DCs migrate to the lymphoid organs where they process the antigens and present them to T cells. Mature DCs in lymphoid organs are characterized by high level expression of MHC-II molecules as well as costimulatory molecules such as CD80 and CD86. Pathogen associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide and unmethylated CpG DNA as well as certain proinflammatory cytokines such as TNF, IL-1, and IL-6 are implicated in activation and maturation of dendritic cells (Banchereau et al., 2000, Leon et al., 2007).

Unlike neutrophils and monocytes, dendritic cells are involved in the late stages of interaction between *Leishmania* and the innate immune cells. Moreover, they take up only the amastigote stage of the parasite via a FcγR-mediated mechanism. In contrast, macrophages use complement

receptor-3 (CR3) to take up both promastigote and amastigote stages of *Leishmania* (Kautz-Neu et al., 2012).

Because dendritic cells are very diverse and heterogeneous, knowledge of the specific subset of DCs that induces protective immunity is of paramount importance in designing effective vaccines against leishmaniasis. Early studies suggested that Langerhans cells (LCs) play a critical role in inducing a Th-1 response against *L major* infection (Reviewed in: Mougneau and colleagues (2011). However, the most recent data clearly demonstrated that Langerhans cells inhibit induction of a Th-1 immune response and are responsible for exaggeration of the disease (Kautz-Neu et al., 2011). Depletion of all Langerin-positive cells from C57BL/6 resistant mice resulted in significantly reduced lesion size and parasite load after *L major* infection. On the other hand, the depletion of these cells significantly increased the IFN-γ response in the draining LN (Kautz-Neu et al., 2011).

Leon and colleagues (2007) did excellent work to demonstrate the role of monocyte-derived DCs in inducing a Th-1 protective immune response in *L major* infection in mice. During inflammatory reaction due to *L major* infection in resistant C57BL/6 mice, monocytes are recruited to the site of infection where they differentiate into dermal monocyte-derived DCs. After acquiring the parasite, these cells migrate to the draining lymph node (LN) and stimulate naive $CD4^+$ T cells to differentiate into a protective Th-1 type through the production of IL-12. It was also found that monocytes can migrate to the draining lymph node and differentiate to become “LN monocyte-derived dendritic cells” (LN mo-DCs). The result was that dermal mo-DCs showed a high degree of IL-12 production than LN mo-DCs. Based on analysis of the number of each subpopulation of dendritic cells of lymphoid or myeloid origins at different times after *L major* infection in mice, they showed that dermal mo-DCs and LN mo-DCs are more

important players in initiating the specific immune response against the parasite. Upon *Leishmania* infection, the number of all subpopulations of DCs increased during the first two weeks after infection. However, at week-3 and -4, the numbers of dermal mo-DCs and LN mo-DCs kept increasing while the others remained the same or reduced (Leon et al., 2007).

As compared with LN mo-DCs, dermal mo-DCs express higher level of MHC-II. Moreover, dermal mo-DCs express CD86 while the LN mo-DCs do not. The ability to induce a T cell response was analyzed by isolating dermal mo-DCs and LN mo-DCs at week-4 post infection and co-culturing them with preactivated $CD4^+$ and $CD8^+$ cells in vitro. It was found that dermal mo-DCs induced the production of significantly higher amount of IFN-γ by both $CD4^+$ and $CD8^+$ T cells than LN mo-DCs. Moreover flow cytometric analysis showed that about 10% and 2-3% of dermal mo-DCs and LN mo-DCs were positive for IL-12, respectively. The IL-12 expression was undetectable in other subpopulation of DCs found in the lymph node (Leon et al., 2007).

Past and recent studies have shown that dendritic cells are very heterogeneous group of innate immune cells that play central role in antigen processing and presentation. Thus, DCs serve as an important link between innate and adaptive immune responses to infection. As discussed in the next section, the phenotype of DCs and the cytokine environment determine the induction of either a non-protective Th-2 or a protective Th-1 specific immune response against *Leishmania* infection,

1.8.1.2 Antigen Processing and Presentation

Antigen processing and presentation is a crucial step in mounting effective specific immune response to infection. Antigen presenting cells (APCs) process antigens and present them to T cells in the context of class-I or class-II major histocompatibility complex (MHC). Antigen presenting cells include dendritic cells (DCs), macrophages, and B cells. These cells express

MHC-II and present antigens to helper T (Th) cells. They also express co-stimulatory molecules. Among them, dendritic cells are the most efficient APCs (Kindt, 2007a).

Two signals are required to induce effective acquired immune response to infection: signal-1 is the interaction of antigen loaded MHC molecule on antigen presenting cell to a specific T cell receptor whereas, signal-2 involves expression and engagement of co-stimulatory molecules on APCs with the respective receptors on T cells. The co-stimulatory molecules include CD86/CD80 and CD40 on APCs which interact with CD28 and CD40L on T cells, respectively. Unlike other APCs, DCs constitutively express both MHC-II and co-stimulatory molecules. They are therefore effective in inducing both naive and primed T cells. Macrophages express MHC-II and co-stimulatory molecules only after phagocytosis of particulate antigens. (Kindt, 2007a).

Antigen processing and presentation involves three interlinked processes: breakdown of proteins into small peptides, loading the peptide antigens onto MHC molecules and delivering the MHC-Peptide complex to the surface of plasma membrane of APCs. The effectiveness of these processes influences the quality of immune response to antigens (Mellman et al., 1998, van den Hoorn et al., 2011).

There are three antigen processing and presentation pathways: a) Endocytic pathway, b) Cytosolic pathway, and c) Cross-presentation.

1.8.1.2.1 Endocytic pathway

Endocytic pathway involves processing of exogenous antigens and presenting them in the context of class-II MHC molecule (MHC-II) to $CD4^+$ T cells. This pathway begins with uptake of antigens by APCs through pinocytosis, receptor-mediated endocytosis, or phagocytosis. The protein antigens are then broken down into short peptides mainly by cysteine proteases which are

active at acidic pH in the endosome. Peptides produced by this cleavage are loaded onto MHC-II molecule and transported to the plasma membrane to be presented to $CD4^+$ T cells (Banchereau et al., 2000, van den Hoorn et al., 2011).

MHC-II molecule is expressed only on antigen presenting cells. It is a heterodimeric membrane glycoprotein made up of noncovalently linked α and β subunits. Together, the two subunits form a peptide binding groove on membrane-distal domains. The sequence variation on this groove is attributed to the specificity in MHC-peptide interaction of each MHC-II molecule. Each MHC-II molecule assembled in the endoplasmic reticulum associates with a non-MHC product called invariant (Ii) chain. Invariant chain prevents the premature binding of MHC groove to peptides and also facilitates transport of MHC-II through Golgi apparatus (van den Hoorn et al., 2011).

Loading of peptide antigens onto MHC-II molecule takes place in a cytoplasmic region called MHC-II containing compartment (MIIC). After being assembled in the ER, MHC-II-Ii complex is transported to MIIC. In the MIIC, the Ii is degraded by proteases leaving a small fragment on the groove called CLIP. At the same time, the protein antigens in the endosome are also cleaved by proteases and this cleavage continues in the acidic MIIC producing peptide fragments that can bind to the groove of a specific MHC-II molecule. The dissociation of CLIP and its exchange for a peptide is facilitated by a chaperone molecule called DM (HLA-DM in humans or H2-M in mice) at acidic pH. The activity of DM is also modulated by another MHC product called DO (HLA-DO and H2-O in humans and mice, respectively). Both DM and DO are found abundantly in the lysosome. Once loaded with peptide, MHC-II is transported to plasma membrane ready to interact with T cell receptor (TCR) on $CD4^+$ T cells (Mellman et al., 1998, van den Hoorn et al., 2011).

1.8.1.2.2 Cytosolic pathway

Unlike the endocytic pathway, the cytosolic pathway involves processing of endogenous antigens and presenting them in the context of class-I MHC molecule (MHC-I) to $CD8^+$ T cells. Although MHC-I molecule is expressed in all nucleated cells, only antigen presenting cells such as DCs express costimulatory molecules. Thus, primary immune response involving $CD8^+$ T cells is restricted to DCs. Endogenous antigens (e.g. proteins from infecting viruses) are digested in the cytosol by proteasome system and transported to ER where they are loaded onto MHC-I molecule and transported to the PM to be presented to $CD8^+$ T (Pamer and Cresswell, 1998, Cresswell et al., 2005).

MHC-I molecule is a glycoprotein made up of α subunit heavy chain and β2 microglobulin. The alpha chain of MHC-I possesses three external domains (α1, α2, and α3), a transmembrane domain, and a cytoplasmic tail. The β subunit is a single external domain that is bound to α3 noncovalently. β2 microglobulin lacks transmembrane and cytoplasmic domains. MHC-I groove is made up of α1 and α2 domains and is capable of binding to peptides of 8 to 10 amino acids. The N- and C-termini of these peptides possess a few amino acids whose side chains interact with the complementary residues in the groove, forming a stable and high affinity interaction. These amino acids on both ends of the peptide are termed as anchor residues. Unlike the peptide binding groove of MHC-II, the MHC-I groove is closed at both ends. MHC-II binding groove is open at both ends allowing it to hold longer peptides of 13 to 18 amino acids. Moreover, MHC-Peptide interactions are done by few specific amino acids at both N and C termini of the groove of MHC-I. On the other hand, in MHC-II, the central 13 amino acids are used for interaction with the peptide (Pamer and Cresswell, 1998, Kindt, 2007a).

The cytosolic pathway of antigen presentation involves degradation of endogenous proteins by an enzyme system called proteasome, translocation of the peptides to ER via TAP (transporter associated with antigen processing), loading of MHC-I molecules and eventual transport and cell surface expression of MHC-I-peptide complex. Proteasome is a barrel-shaped proteolytic system in the cytosol made up of 14 subunits (Pamer and Cresswell, 1998, Kindt, 2007a).

The process of protein degradation begins with ATP-dependent covalent linkage of ubiquitin to a side chain of lysine residue on the target protein by a process called unbiquitination. This process unfolds the target protein and renders it to proteasome digestion. The resultant peptides are then translocated to rough ER through TAP in ATP-dependent manner. TAP is a heterodimer that contains two subunits, TAP1 and TAP2. Some of the peptides are trimmed by aminopeptidases in the ER to 8 to 10 amino acid long. Assembly of individual subunits of MHC-I (the α-subunits with β2 microglobulin) as well as MHC-I and peptide occurs in the rough ER. This process is chaperoned by three molecules, calnexin, calreticulin, and tapasin. The peptide loaded MHC-I molecule exits rough ER and is transported via the Golgi apparatus to the surface of the plasma membrane to interact with $CD8^+$ T cells (Pamer and Cresswell, 1998, Cresswell et al., 2005, Kindt, 2007a).

1.8.1.2.3 Cross-presentation

Certain viruses do not infect dendritic cells, cells that express costimulatory molecules in addition to MHC-I. $CD8^+$ T cell-mediated anti-viral primary immune response to these viruses cannot be, therefore, initiated by the cytosolic antigen processing pathway. Dendritic cells acquire these viral antigens from other cells mainly by phagocytosis and also by pinocytosis and receptor mediated endocytosis. Therefore, dendritic cells undergo presentation of exogenous

antigens in the context of MHC-I molecule. This process is called cross-presentation (Cresswell et al., 2005).

Although several possible mechanisms of cross-presentation have been extensively studied, no consensus has been reached yet. It is generally believed that the process of cross-presentation can occur in TAP-dependent and TAP-independent mechanisms (Banchereau et al., 2000). Two possible pathways have been suggested for the TAP-dependent mechanism of antigen presentation, ER-phagosome fusion and phagosome-to-cytosol pathways. In the ER-phagosome fusion model, the ER membrane provides a membrane transport system to export antigens from the phagosome to the cytosol and also to translocate proteasome-degraded peptides back into the ER where loading to MHC-I takes place. In the phagosome-to-cytosol model, antigens are leaked from phagosome into cytosol where they are degraded by the proteasome system and peptides are loaded onto MHC-I in the ER. On the other hand, the TAP-independent vacuolar pathway involves degradation of antigens in the phagosome and loading to MHC-I molecule there. It is not clear how MHC-I molecules are transported to the phagosome (Ramachandra et al., 2009).

1.8.1.3 Specific Adaptive Immune Responses

The adaptive immune response is antigen-specific and elicited as a result of processing and presentation of specific segments of the antigen to T cell receptors in the context of MHC-I and MHC-II molecules (Kindt, 2007a) .

In the adaptive immune response, B- and T-cells recognize specific antigenic determinants (epitopes) on antigens. The B-cell receptor (surface immunoglobulin molecule)- and T-cell receptor (TCR)- encoding genes undergo a series of recombinations to produce a repertoire of cells each expressing a receptor with unique specificity to an epitope (Cancro MP, 2005).

The adaptive immune response is broadly classified into humoral and cell-mediated types. The humoral immune response is effected by antibody molecules produced by B-cells. This response uses three mechanisms; virus neutralization, opsonisation, and complement activation (Bjorkman P, 2005).

Cell-mediated immunity is another arm of the specific immune response against pathogens. It is mediated by a repertoire of T lymphocytes each bearing a peptide specific T cell receptor (TCR). Based on their surface molecules, co-receptors, T cells can be identified as either $CD4^+$ or $CD8^+$. $CD4^+$ T cells, also called helper T cells, play a central role in the specific cell-mediated immune response. They execute their function by producing different types of cytokines. On the other hand, $CD8^+$ T cells (also called cytotoxic T cells) carry out antigen-specific lysis of cells infected with intracellular microorganisms such as viruses or of tumour cells. $CD8^+$ T cells also produce cytokines to activate other cells (Mosmann T, 2005). $CD4^+$ T cells play a central role in adaptive immunity by regulating the action of many other cells. For example, they activate naive B cells to proliferate and secrete antigen-specific immunoglobulin molecules. They also activate macrophages to kill intracellular pathogens. These effector functions of $CD4^+$ T-helper cells are performed by different subsets (phenotypes) (Mosmann T, 2005, Zhu and Paul, 2008).

By their pioneering work, Mosmann and colleagues (1986, 1989) classified helper T cells into two categories, T helper-1 (Th-1) and Th-2. *In-vitro* analysis of different clones of Th-cells demonstrated that $CD4^+$ cells consists of two distinct phenotypes each producing characteristic cytokines. Upon antigen or mitogen stimulation, each class of helper T-cells produced cytokines with distinct bioactivities. It was found that Th-1 cells produce IL-2, IL-3, and interferon-γ (IFN-γ) but not IL-4; whereas, Th-2 cells produce IL-3 and IL-4 but not IL-2 and IFN-γ. Moreover, it

was found that cell supernatants of Th-2 cells but not those of Th-1 cells induce B-cells to produce IgE and IgG1 isotypes. .

In their critical review about helper T-cells, Mosmann and Coffman (1989) not only meticulously described the two classes of helper T-cells but also raised several research questions that needed to be addressed in order to fully understand the different types of helper T-cells. For example, they raised the question regarding what regulates the activation of Th-1 or Th-2 cells and suggested that antigen presenting cells could influence the activation process.

By producing IFN-γ, Th-1 cells regulate cell-mediated immunity. They activate macrophages to kill intracellular pathogens such as *Leishmania* and also help $CD8^+$ T-cells to execute their cytotoxic function. On the other hand, by downregulating the production of Th-2 cytokines, IFN-γ inhibits the proliferation of Th-2 T cells. In addition, IFN-γ orchestrates isotope switching in B-cells to produce IgG2a (in mice). Whereas, Th-2 cells produce IL-4, IL-5, IL-10, as well as IL-13 which downregulate the antileishmanial function of macrophages and promote parasite persistence and multiplication inside macrophages. IL-4 induces B-cells to produce specific isotypes of antibodies such as IgG1 (in mice) (Paul and Zhu, 2010, Okwor et al., 2012, Stevens et al., 1988).

Early studies have clearly shown that IFN-γ production prior to *L major* infection determines susceptibility and resistance of mice to the parasite (Belosevic et al., 1989, Scott, 1991). Belosevic and colleagues (1989) demonstrated that a single injection of anti-IFN-γ renders naturally resistant mice susceptible to *L major* infection which resulted in development of cutaneous lesion and dissemination of the parasite to visceral organs such as spleen and liver. A different study found that administration of anti-IL-4 monoclonal antibody into *L major* infected genetically susceptible BALB/c mice stopped diseases progression and reduced parasite number

(Heinzel et al., 1989). In addition to IL-4, IL-10 also mediates disease progression in *L major* infection in mice. As reviewed in Okwor and colleagues (2012), IL-10 plays crucial role in susceptibility to *L major* infection in mice. It favors disease progression by inhibiting the development of Th-1 cells and by blocking activation of macrophage by IFN-γ.

In infections with intracellular parasites such as *Leishmania*, the balance between Th-1 and Th-2 responses determine the development of disease after infection. That is, Th-1 dominated response results in resistance to disease while Th-2 dominated response brings about clinically overt disease condition (Sacks and Noben-Trauth, 2002) . Recently, two additional groups (phenotypes) of $CD4^+$ T-cells were discovered with distinct functions: Th-17 and inducible regulatory T cells (Langrish et al., 2005, Zhu and Paul, 2008).

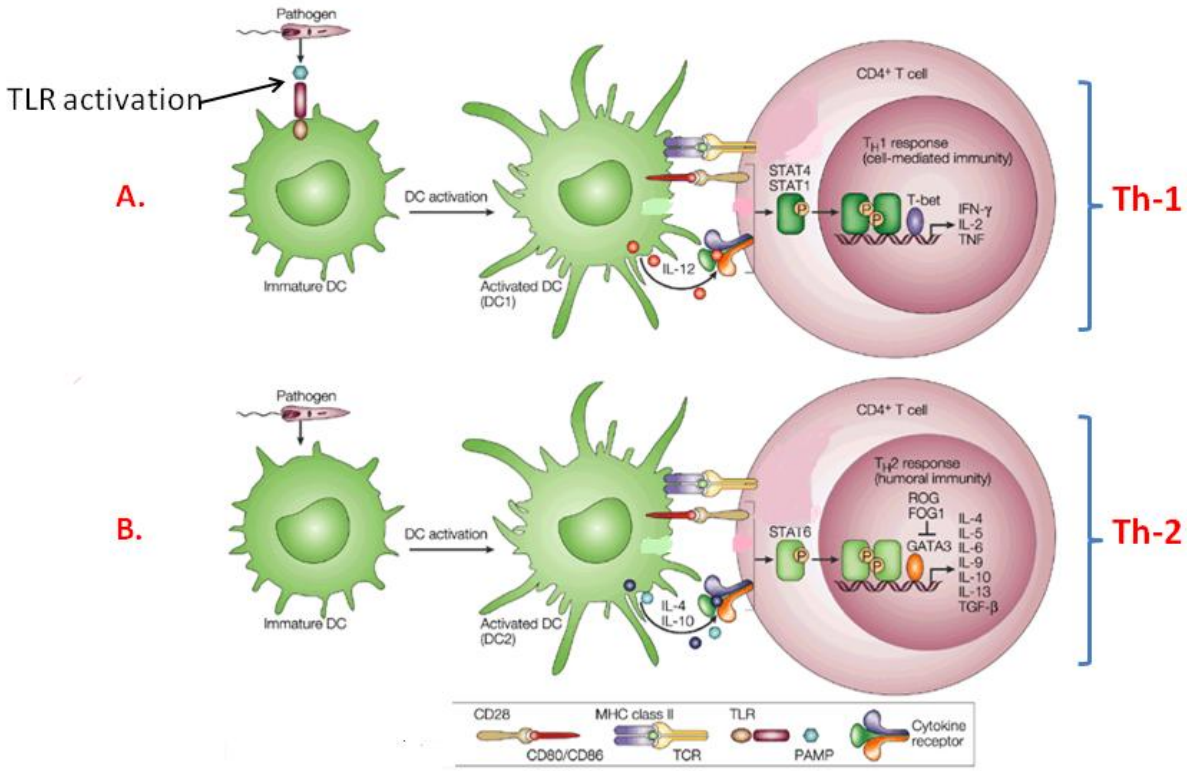
Figure-1.2 shows the widely accepted model of differentiation of naive $CD4^+$ T cells into a protective Th-1 or a non-protective Th-2 phenotype in *Leishmania major* infection. Factors that contribute to the differentiation of naive $CD4^+$ T cells in to Th-1 or Th-2 phenotypes fall in the following types: a) the presence of distinct pre-programmed dendritic cells (Maldonado-Lopez et al., 1999); b) affinity of specific T-cell receptors (TCR) for peptide-MHC complex on antigen presenting cells (APC) (Launois et al., 1997); c) specific interaction of microbial components with their respective receptors (for example Toll-like receptors) (Boonstra et al., 2003); d) the number of infecting parasite (Boonstra et al., 2003); and e) the route of antigen delivery (Constant et al., 2000). In all of these cases, cytokines play a central role. IL-12 production by dendritic cells directs the differentiation of naive $CD4^+$ T cells into Th-1. Whereas, an early IL-4 production by a few founder $CD4^+$ T cells activates the differentiation into Th-2 phenotypes. These cells were found to be specific to peptides from the *Leishmania* homolog of receptors for activated C kinase (LACK) protein. *Leishmania major* resistant C57BL/6 mice produce more IL-

12 than susceptible BALB/c strains. Moreover, T cells from BALB/c mice express only low level IL-12Rβ chain (Okwor et al., 2012, Gumy et al., 2004, Noben-Trauth et al., 2002).

Both the Th-1 and Th-2 immune response pathways share common features. Firstly, both are initiated by cytokines. Secondly, they follow JAK-STAT pathway of signal transduction in order to activate transcription factors that induce expression of the respective cytokine genes. On the other hand, the two systems use different mediators and transcription factors. As described in **Figure-1.2** below, Th-1 immune response uses JAK-2, STAT-1, STAT-4, and T-bet whereas, Th-2 system uses JAK-1, STAT-6, and GATA-3 (Sacks and Noben-Trauth, 2002, Paul and Zhu, 2010).

Figure 1.2: Differentiation $CD4^+$ T cells.

A) Th-1 $CD4^+$ and **B)** Th-2 $CD4^+$ (Adapted from: (Sacks and Noben-Trauth, 2002)**)**.


Pathogen
TLR activation
A.
DC activation
Immature DC
Activated DC
(DC1)
IL-12
CD4+ T cell
TH1 response
(cell-mediated immunity)
STAT4
STAT1
T-bet
IFN-γ
IL-2
TNF
Th-1
Pathogen
B.
DC activation
Immature DC
Activated DC
(DC2)
IL-4
IL-10
CD4+ T cell
TH2 response
(humoral immunity)
ROG
FOG1
GATA3
STAT6
IL-4
IL-5
IL-6
IL-9
IL-10
IL-13
TGF-β
Th-2
CD28
CD80/CD86
MHC class II
TCR
TLR
PAMP
Cytokine
receptor

Macrophages are the primary host cells of *Leishmania*. Different cytokines activate macrophages differently. IFN-γ and TNF-α-induced activation of macrophages elicits inducible nitric oxide synthase (iNOS) pathway to kill intracellular *Leishmania*. This process is called classical macrophage activation. On the other hand, IL-4, IL-10, IL-13 and TGF-β mediate disease progression by inducing the arginase pathway in macrophages and/or indirectly by blocking macrophage activation by IFN-γ. The arginase pathway is also called alternative macrophage activation (Stempin et al., 2010, Liew et al., 1990, Okwor et al., 2012).

In classical activation, iNOS changes L-arginine into nitric oxide and L-citrulline. Nitric oxide is potent nitrogen intermediate that kills *Leishmania* inside the phagolysosome of macrophages. Alternative pathway activates arginase-1 to convert L-arginine into L-ornithine. L-ornithine is a precursor for polyamines and collagen. The latter are used for production of extracellular matrix and are involved in wound healing. Macrophages activated by the alternative pathway as a result of Th-2 response promote multiplication of *Leishmania* inside the cells and brings about exacerbation of the disease (Stempin et al., 2010).

Knowledge of how the Th-1 and Th-2 immune responses are initiated and maintained in *Leishmania* infection is of paramount importance in the design of effective vaccine against leishmaniasis (Afonso et al., 1994).

As an intracellular parasite, *Leishmania* induces cell-mediated immunity. Early experiment on adoptive cell transfer into susceptible SCID (Severe Combined Immunodeficiency) mice demonstrated that resistance to *L major* infection is mediated by T cells (Varkila et al., 1993). $CD4^+$ T cells play a major role in mediating both resistance and susceptibility following *Leishmania* infection, Th-1 cells being responsible for resistance (Reed and Scott, 1993, Sacks

and Noben-Trauth, 2002). The contribution of $CD8^+$ T-cells to induce a primary immune response with consequent resistance to *Leishmania* infection is not clearly understood. Early studies involving $CD8^+$ T cell depleted mice reported that these cells are not important for eliciting protection against *L major* infection. $CD8^{+/-}$ or $CD8^{-/-}$ C57BL/6 mice infected with 2 x 10^7 stationary phase promastigotes of *L major* by the subcutaneous route controlled the infection for over one year with a strong $CD4^+$ T cell response (Huber et al., 1998). However, more recent studies on low-dose intradermal infection with *L major*, which mimics the natural infection, showed that IFN-γ producing $CD8^+$ T cells play an important role in inducing primary immunity to *L major* infection (Belkaid et al., 2002, Uzonna et al., 2004).

1.8.2 The Evading Strategies of Leishmania

In order to survive inside a mammalian host and cause infection, *Leishmania* parasites use different mechanisms to evade the host defense system. Immediately after entering into the host tissue, promastigotes encounter the lytic action of complement. The parasite has developed a system not only to escape the lytic complement but also to use it as a means of entry to the host macrophage cells. As described above, components of the complement system opsonise the parasite and render them susceptible to receptor-mediated endocytosis by macrophages. Parasite surface structures, lipophosphoglycan (LPG) and gp63 are effector molecules implicated in resisting the lytic effect of the complement system. LPG of metacyclic promastigotes prevents the attachment of C5b-C9 (also called membrane attack complex) onto the surface of the parasite effectively avoiding lysis. *Leishmania donovani* gp63, on the other hand, converts C3b into an inactive form (C3bi). C3bi is opsonic but prevents the formation of C5 convertase consequently avoiding the formation of membrane attack complex (MAC). Parasite-induced activation of complement also produces the chemotactic factors, C3a and C5a. These chemotactic factors

attract macrophages to the site of entry of the parasite and also up-regulate the expression of complement receptors on macrophages which, consequently, mediate infection of new batch of cells (Olivier et al., 2005, Mosser and Brittingham, 1997, Moradin and Descoteaux, 2012).

Once inside macrophages, *Leishmania* parasites are exposed to the harsh environment that has deleterious effect on them. In order to survive inside the cell and establish infection, the parasite has developed an array of evasion mechanisms which include: 1) inhibiting phagolysosome biogenesis by inhibiting phagosome-endosome fusion, 2) impairing NADPH oxidase and V-ATPase assembly; V-ATPase is a vacuolar type proton translocating ATPase (proton pump**)** by which macrophages keep phagolysosomes acidic, 3) scavenging reactive oxygen and nitrogen intermediates (ROIs and RNIs) of the host cell, 4) inhibiting phagolysosomal hydrolytic enzymes, and 5) inhibiting antigen presentation with subsequent inhibition of specific cellular immune response (Olivier et al., 2005, Moradin and Descoteaux, 2012).

Abundant lipophosphoglycan molecules on the surface of metacyclic promastigotes inhibit the phagosome-endosome fusion giving time for promastigotes to develop into amastigotes. Without fusion of late endosomes with phagosomes, macrophages cannot form phagolysosomes with an active NADPH oxidase system. Amastigotes, on the other hand, are capable of resisting the toxic environment of phagolysosomes (Olivier et al., 2005).

Inhibition of NADPH oxidase assembly is effected by inhibiting phosphorylation of the subunits of the enzyme system and by disrupting the lipid microdomain of the phagosome membrane. The later, executed by LPG, inhibits assembly of the NADPH oxidase and v-ATPase in the phagosome membrane effectively impairing the production of toxic ROIs and acidification of the phagolysosome (Moradin and Descoteaux, 2012).

In cases where the NADPH oxidase and v-ATPase systems are properly assembled into the membrane of a phagolysosome inside a macrophage, the parasite is exposed to toxic reactive oxygen and nitrogen intermediates. In order to survive in this hostile environment, *Leishmania* parasites have developed a variety of mechanisms including scavenging of oxidative metabolites and inhibiting their synthesis (Bogdan et al., 1996). Among other things, *Leishmania* produces antioxidant molecules such as superoxide dismutases (SODs) and peroxidoxins (Pxns) to defend itself from toxic ROIs and RNIs produced by infected host cells (Plewes et al., 2003, Barr and Gedamu, 2001, Barr and Gedamu, 2003)

As described above, antigen processing and presentation is a critical step in inducing a specific immune response against *Leishmania*. Once the parasite antigens are processed and presented effectively, cell mediated immunity is induced to exert a parasite-specific response. *Leishmania* parasites have developed several mechanisms to inhibit antigen presentation. Different species of *Leishmania* inhibit antigen presentation by different mechanisms including, inhibition of MHC-II expression, inhibition of antigen loading to MHC-II, sequestering the MHC-II molecule or the MHC-II-antigen complex within phagolysosomes, degradation of MHC-II by peptidases of amastigotes as well as inhibiting receptor-ligand interaction of costimulatory molecules (Olivier et al., 2005).

Leishmania parasites also modulate the immune response as an evasion mechanism. It has been shown that *L donovani* and *L braziliensis* promastigotes induce bone marrow derived murine macrophages to produce TGF-β, inhibitor of leishmanicidal activity of macrophages (Barral et al., 1993). It has been demonstrated that *Leishmania* cathepsin B cysteine proteases convert latent TGF-β into a biologically active form (Gantt et al., 2003, Somanna et al., 2002). Active TGF-β impairs the antileishmanial activity of macrophages by inhibiting the expression of

inducible nitric oxide synthase (iNOS) (Stenger et al., 1994). *Leishmania* produces cathepsin L-like and cathepsin B-like cysteine proteases that play role in host invasion and tissue degradation as well as in modulation of the host immune system (Somanna et al., 2002, Mundodi et al., 2005).

1.8.2.1 Superoxide Dismutases

Superoxide dismutases (SOD) are enzymes that serve as first-line antioxidants. By catalyzing the conversion of highly toxic superoxide anion to hydrogen peroxide, superoxide dismutases of *Leishmania* play an important role in the survival of the parasite inside macrophages. Based on the type of metal ion co-factor, superoxide dismutases are generally grouped into three major classes: iron-SOD, manganese-SOD, and copper/zinc-SOD. Of these, *Leishmania* possess iron-SODs (FeSODs). Human SODs, on the other hand, are mainly the Cu/Zn type and are different from *Leishmania* FeSODs (Paramchuk et al., 1997).

Studies in our lab have shown that *L chagasi* has two FeSOD genes; *LcFeSODA* and *LcFeSODB* which are located on different chromosomes. *LcFeSODA* is a single copy gene. Whereas, *LcFeSODB* is a two-copy gene: *LcFeSODB1* and *LcFeSODB2*. Expression studies have shown that *LcFeSODA* and *LcFeSODB1* are expressed at low level in the early logarithmic-phase promastigote and increase significantly towards the stationary-phase promastigote and amastigote stages. *LcFeSODB2*, on the other hand, is expressed at high level in the early promastigote stage and decreases towards the stationary-phase promastigote and amastigote stages. *L donovani* has also *FeSODA*, *FeSODB1/B2* which are identical to the *L chagasi* SODs (Paramchuk et al., 1997). It has been shown in our lab that FeSODB1 is important for the survival of *Leishmania* in macrophages (Plewes et al., 2003).

1.8.2.2 Peroxidoxins

Peroxidoxins (Pxns) also called peroxiredoxin or thiol-specific antioxidants are antioxidants found conserved in prokaryotes and eukaryotes. By detoxifying extremely reactive oxygen intermediates such as hydroxyl radicals and also hydrogen peroxide as well as reactive nitrogen intermediates, *Leishmania* peroxidoxins play a crucial role in evading host-defense system (Barr and Gedamu, 2003).

Leishmania has four types of peroxidoxins: Pxn1, Pxn2, Pxn3, and Pxn4. It has been shown in our lab that *L chagasi* Pxn1 (LcPxn1) is predominantly expressed in the amastigote stage, whereas LcPxn2 and LcPxn3 are expressed more in the promastigote stage. *L. chagasi* and *L donovani* have identical peroxidoxin genes and have the same expression patterns (Barr and Gedamu, 2001, Barr and Gedamu, 2003).

1.9 *Leishmania* Vaccine Development

Although huge information is available about the immunology of leishmaniasis and pre-clinical trials of vaccine candidates, no universally effective and safe vaccine has been developed against any form of leishmaniasis for general human use. The major factors attributed to this gap include 1) lack of defined and universally accepted correlate of protection of leishmaniasis in mice, 2) difference between human and mouse immune systems, and 3) use of different immunization protocols in different studies (Okwor et al., 2012).

Development and testing of vaccine candidates started as early as 1930's in Brazil. According to Khamesipour and co-workers (2006)**,** anti-*Leishmania* vaccines can be broadly classified into three categories: live *Leishmania* vaccines, first-generation vaccines, and second-generation vaccines.

Live *Leishmania* vaccines encompass natural parasites and those that are genetically modified and made avirulent. The deliberate injection of live virulent parasites as a vaccine to protect the individual from more serious forms of leishmaniasis is called leishmanization. Leishmanization relies on the fact that people recovered from cutaneous leishmaniasis develop strong and durable immunity to re-infection. Leishmanization was used in Israel and Iran as part of the vaccination campaign from 1950's to 1970's. However, this strategy failed due to two important reasons. First, it resulted in suppression of the immune response to diphtheria, pertussis and tetanus (DPT) vaccine. Second, some vaccinated individuals developed a chronic and non-healing lesion that did not respond to routine treatment (Khamesipour et al., 2006, Okwor et al., 2012).

As reviewed in Okwor *et al* (2012), several live attenuated parasites have been tested as vaccines with variable outcomes. Some of live attenuated *Leishmania* vaccines tested so far did not show high degree of protection in mice and some others failed to protect non-human primates. Attenuation of *Leishmania* could be done by continuous long-term *in vitro* culture, treatment of the parasites with chemical mutagenesis or radiation or by targeted gene deletion. The latter technique is more advantageous because of the reduced risk of reversal to a wild-type strain.

The first-generation vaccines include the use of killed parasites or fractioned preparations of the parasites as vaccines. The use of killed promastigotes as a vaccine was used in Latin American countries such as Brazil since 1930's. Killed *Leishmania* vaccine is easy to make and is less expensive. A clinical trial of a killed *Leishmania* vaccine found it to be safe and induced strong Th-1 immune response in humans. However, it lacked protection from clinical disease (Velez et al., 2005, Okwor et al., 2012). As a fractioned preparation, fucose mannose ligand (FML) was tested against VL in dogs (Khamesipour et al., 2006).

The second-generation vaccines include subunit protein and DNA vaccines. Unlike the whole parasite vaccines, subunit vaccines are composed of defined components. The inherent inability to cause disease makes subunit vaccines the most preferred forms over live pathogenic or attenuated vaccines. In addition, they are also relatively easy to produce and standardize. However, subunit vaccines are generally weak immunogens which necessitates repeated immunization and also the use of adjuvants. Several *Leishmania* proteins have been extensively studied as potential vaccines in the form of protein and DNA vaccines. These vaccines have shown variable level of immunogenicity and protection in animal models and humans. These subunit candidate vaccines include fusion *Leish111f*, *Leishmania* glycoprotein 63 (gp63), *Leishmania* homolog for receptors of activated C kinase (LACK), cysteine proteases, and A2 proteins (Reviewed in: (Khamesipour et al., 2006, Okwor et al., 2012).

Leish111f, a polyprotein fusion vaccine candidate made from *Leishmania* elongation initiation factor (LeIF), *Leishmania major* stress-inducible protein 1 (LmSTI1), and thiol-specific antioxidant (TSA; peroxidoxin2) is the only second generation *Leishmania* protein vaccine candidate that has reached human trial. *Leish111f*, has been tested as a vaccine candidate against both cutaneous and visceral leishmaniasis in animals. In BALB/c mice, immunization with *Leish111f* in the presence of MPL-SE (Monophosphoryl lipid A – sequalene) adjuvant resulted in significant and long-term protection of the immunized mice. It protected mice for at least 14 weeks (Coler et al., 2002). As shown in Coler *et al* (2007), experimental infection of immunized mice and hamsters demonstrated that *Leish111f*-MPL-SE induced significant protection against *L. infantum* infection, with reduction in the parasite loads of 99.6%, a level of protection greater than that reported for other vaccine candidates in animal models of visceral leishmaniasis.

Clinical trials have been done to test the safety and immunogenicity of *LEISH-F1*, a modified form of *Leish111f*, as a prophylactic and therapeutic vaccine in humans. It was tested in combination of meglumine antimonate or sodium stibogluconate for CL and MCL treatment, respectively (Nascimento et al., 2010, Llanos-Cuentas et al., 2010). The vaccine was also tested as a prophylactic vaccine for visceral leishmaniasis in humans (Chakravarty et al., 2011). In the entire three clinical trials, *LEISH-F1* antigen was used in combination with monophosphoryl lipid A-Sequalene (MPL®-SE) adjuvant. In all of the trials, *LEISH-F1* vaccine in combination with MPL-SE adjuvant has been found to be safe and immunogenic in humans. In addition, in CL trial, 80% and 38% of the vaccine recipients and placebo group were clinically cured at day-84, respectively (Nascimento et al., 2010). However, although the vaccine recipients were immunogenic and safe in MCL patients, no appreciable difference was seen in the cure rate between vaccine recipients and placebo groups (Llanos-Cuentas et al., 2010).

A phase-III clinical trial of *MML* (modified *Leish111f*) with MPL®-SE adjuvant against visceral leishmaniasis caused by *L infantum* in dogs was found to be non-protective. The study found no difference between the vaccine recipients and the control groups that received sterile saline with regard to protection against visceral leishmaniasis (Gradoni et al., 2005).

Leishmania glycoprotein-63 (gp63) is another subunit vaccine candidate that has long been tested in different forms against both cutaneous and visceral leishmaniasis forms. It is one of the major surface molecules and is expressed in both promastigote and amastigote stages. Moreover, gp63 is highly conserved across different *Leishmania* species. Different studies using gp63 showed variable degree of immunogenicity and protection in mice (Handman et al., 1990, Walker et al., 1998, Jaafari et al., 2007). Recently, the immunogenicity and protective efficacy of *Leishmania* gp63 was tested against both visceral and cutaneous leishmaniasis in mouse models

in DNA/DNA, DNA/Protein, and Protein/Protein immunization strategies in the presence of CpG ODN adjuvant. The result demonstrated that DNA/Protein immunization with gp63 results in a high degree of short-term and long-term protection of mice against visceral leishmaniasis. The parasite challenge was done 10 days and 12 weeks after the last immunization for short-term and long-term protection studies, respectively. Moreover, the same vaccine protected BALB/c mice from cutaneous leishmaniasis caused by footpad injection of *L major* (Mazumder et al., 2011).

Another fusion protein, KSAC, has been tested against both visceral and cutaneous leishmaniasis in mice. This polyprotein is made by fusing four *Leishmania* proteins; kinetoplastid membrane protein 11 (KMP 11), sterol 24-c-methyltransferase (SMT), A2, and Cysteine protease B (CPB). KSAC polyprotein vaccine was shown to be immunogenic in mice inducing significantly high number of multifunctional $CD4^+$ T cells capable of producing IFN-γ, TNF-α, and IL-2 simultaneously. Moreover, the vaccine showed high degree of protection of mice from both visceral and cutaneous leishmaniasis (Goto et al., 2011).

1.9.1 Superoxide Dismutase and Peroxidoxin as Vaccine Candidates

1.9.1.1 Superoxide Dismutase as Vaccine Candidate

Superoxide dismutase (SOD) has been tested as a vaccine candidate against a number of infectious diseases including leishmaniasis, tuberculosis, brucellosis, schistosomiasis and also against *Helicobacter pylori* infection (Daifalla et al., 2012, Park et al., 2008, Singha et al., 2008, Every et al., 2011).

Recently, we have shown that *L donovani* recombinant iron superoxide dismutase B1 (rLdFeSODB1) in the presence of CpG ODN adjuvant is immunogenic in BALB/c mice and partially protects mice against *L major* challenge infection. Immunization of mice with

rLdFeSODB1 antigen plus CpG ODN induced a significantly higher IgG2a/IgG1 ratio than the controls. Moreover, spleen cells from immunized mice produced appreciably higher IFN-γ upon *in vitro* stimulation with the recombinant protein (Daifalla et al., 2011, Daifalla et al., 2012).

DNA/DNA prime-boost immunization with *Schistosoma mansoni* SOD induced a significant increase in IFN-γ, and antigen specific IgG2a in immunized mice and a 39% protection from adult parasite, which fulfils the WHO standard of 40% protection from adult *Schistosoma* infection (Cook et al., 2004). In addition, it has been shown that the same DNA vaccine resulted in 53% (Shalaby et al., 2003) and 54% (LoVerde et al., 2004) protection from challenge with *S. mansoni* cercaria.

It has been also shown that SOD vaccines significantly protected mice against *Brucella abortus* infection (Onate et al., 2003, Singha et al., 2008). Liposome entrapped *Brucella abortus* SOD DNA vaccine induced a Th1 type immune response and protected mice from *B abortus* infection (Singha et al., 2008).

Park and colleagues (2008) conducted a protection study using a DNA vaccine cocktail containing *Mycobacterium avium* SOD in mice. The vaccine induced a strong Th1 response. The proliferative responses of spleen cells from vaccinated mice were 4 to 6-fold higher than the control group. Upon challenge with *Mycobacterium avium* subsp *paratuberculosis*, immunized mice showed significant protection.

1.9.1.2 Peroxidoxins as a Vaccine Candidate

Peroxidoxins have been used as vaccine candidates against leishmaniasis (Webb et al., 1998) and amoebiasis (Soong et al., 1995). Peroxidoxin-2, a homolog of human thiol-specific antioxidant (TSA), has been tested as a vaccine candidate against cutaneous and visceral leishmaniasis in mice and non-human primate models. Screening of a *L major* amastigote cDNA library with sera

from BALB/c mice that had been immunized with *L major* culture filtrate produced immunoreactive clone specific to *L. major* TSA (Pxn2). Then, the gene encoding TSA were isolated and cloned in a bacterial expression plasmid. The expressed recombinant protein was then purified and tested for its immunogenicity and protective efficacy in BALB/c mice. Immunization of mice with the recombinant TSA (rTSA) in the presence of IL-12 adjuvant resulted in the development of strong cellular immune responses and conferred partial protection against challenge infection with *L major*. It was also shown that rTSA elicited *in vitro* proliferative responses of PBMC of leishmaniasis patients and induced significant TSA protein-specific antibody titers in sera of both CL and VL patients (Webb et al., 1998).

Campos-Neto and co-workers (2001) investigated the vaccine potential of TSA when given in combination with *L. major* Stress Inducible Protein 1 (LmSTI1) and IL-12 adjuvant. In BALB/c mice, the cocktail antigen induced a strong Th1 response and significantly higher degree of protection whereas, TSA only induced partial protection. In rhesus monkeys, the same protein/protein immunization strategy induced a long-term protection as demonstrated by complete protection to re-challenge of the monkeys with *L. major*.

As a DNA vaccine, TSA induced a very high degree of protection that is higher than LmSTI1. The elevated protection is attributable to the increased $CD8^+$ response in addition to a $CD4^+$ type (Campos-Neto et al., 2002). As described above, *Leish111f*, a tri-protein fusion vaccine contains *L major* peroxidoxin-2 (TSA) as one of its components. *Leish111f* has been shown to be an effective vaccine against both CL and VL in animals (Coler et al., 2002, Coler et al., 2007).

1.9.2 DNA Vaccines

DNA vaccine is a plasmid vector that possesses antigen(s) cloned under a mammalian promoter. Unlike recombinant protein vaccines where the antigens are injected directly, DNA vaccine

antigens are expressed in transfected cells *in vivo*. The striking feature of DNA vaccines is their ability to elicit all forms of the adaptive immune response; antibody, $CD4^+$ T cell as well as $CD8^+$ T cell responses. The latter gives them a unique position in the development of vaccines for cancer and also for diseases caused by intracellular pathogens such as viruses and intracellular bacteria and parasites (Liu, 2011).

One of the early studies of *Leishmania* DNA vaccines clearly showed the importance of DNA vaccines in inducing a better quality immune response than that of recombinant protein vaccines. Immunization of susceptible BALB/c mice with LACK DNA conferred significantly greater degree of protection against *L major* infection than immunization with recombinant LACK protein. The level of protection was similar to the one induced by immunization with rLACK protein together with rIL-12 adjuvant. It was suggested that the increased protection by the DNA vaccine could be due to the immunostimulant CpG sequences on the DNA construct. The same study demonstrated that depletion of $CD8^+$ cells at the time of infection abrogated the protective capacity of the DNA vaccine indicating that $CD8^+$ cells play a crucial role in DNA vaccine induced protection (Gurunathan et al., 1997). Another study on testing *Leishmania* TSA as a DNA vaccine showed that high degree of protection of the vaccine was due to an increased $CD8^+$ response in addition to a $CD4^+$ type (Campos-Neto et al., 2002).

In addition to inducing all arms of adaptive immunity, DNA vaccines have other important features that make them more advantageous than protein vaccines. These include, 1) DNA vaccine antigens are expressed in small amounts over a longer period of time, a feature that enables them to induce a durable immune response, 2) DNA vaccines are amenable to modifications including fusion of cytokine and chemokine genes with the vaccine antigen, 3) They can be prepared without the need to handle infectious pathogens, 4) It is easier and faster to

prepare DNA vaccines, and 5) DNA vaccines are fairly stable at room temperature which makes global delivery easier (Liu, 2011, Tang et al., 1992, Gurunathan et al., 1997).

Although DNA vaccines have many advantages, they are not free from some potential drawbacks. These include the possibility of integration of the plasmid DNA into the chromosomal DNA of the recipient, and the possibility of causing an autoimmune response to DNA or tolerance. However, none of these has been seen in human clinical trials (Reviewed in: Liu *et al*, (2005). On the other hand, DNA vaccines have shown lower immune responses in humans than in small animals (Laddy and Weiner, 2006, Liu, 2011).

During intramuscular immunization of a plasmid DNA vaccine, only a very small portion of the vaccine transfects professional antigen presenting cells such as dendritic cells; the majority of the vaccine transfects myocytes. As myocytes lack the costimulatory signals, any presentation with myocytes brings about anergy. On the other hand, vaccine antigens expressed in myocytes can be taken up by professional APCs and presented in the context of MHC-I (cross presentation) and MHC-II. Thus, myocytes serve as antigen "factories". This phenomenon brings about more durable immune response to the vaccine antigens as compared to protein antigens (Liu, 2011).

Although no DNA vaccine has been approved for human use yet, three DNA vaccines have been licensed for veterinary use, two of them are for viral diseases of horse and fish and the third one is a therapeutic vaccine for melanoma in dogs (Liu, 2011).

Most vaccines are given in more than one dose in order to induce an effective immune response against the vaccine antigen. This strategy is called prime-boost immunization. Prime-boost immunization can be a homologous or heterologous type. A homologous prime-boost immunization involves administering all the doses in one form such as plasmid DNA containing

the antigen gene. On the other hand, heterologous immunization involves administering the first immunization in one form such as DNA and the subsequent immunizations with another form such as recombinant protein. Heterologous prime-boost immunization generally induces more effective and durable immune response than a homologous type involving repeated immunization with only one form of the antigen (Lu, 2009, Mazumder et al., 2011).

1.9.3 Vaccine Adjuvants

The term adjuvant comes from a Latin word "*adjuvare*" which means "to help". It includes a diverse group of compounds or macromolecular complexes that enhance or modulate the immune response to vaccine antigens. Adjuvants do not impart long-lasting effect on their own. They activate the innate immune system and increase the immunogenicity of vaccine antigens. Adjuvants that strengthen the vaccine antigen to induce high level immune response with minimum number of injections (immunizations) and those that improve the efficacy of vaccines in newborns, elderly and immune-compromised persons are generally regarded as effective adjuvants. Moreover, effective adjuvants induce minimum or no side effect on the vaccinee and have longer shelf-life (Lima et al., 2004, Reed et al., 2009).

Generally, live or live attenuated vaccines do not require adjuvants. Because these vaccines contain whole or large portion of the organism, immunization with live or live attenuated vaccine resembles infection with a pathogenic organism in eliciting strong immune response without help from external adjuvant. Different microbial components of a live attenuated vaccine activate innate immune system and trigger effective response against the cognate microorganism that the vaccine is intended for. However, live attenuated vaccines have the inherent problem of reversal in that they may become pathogenic to the vaccinated individual (Ishii and Akira, 2007, Jiang and Koganty, 2003, Reed et al., 2009).

On the other hand, subunit vaccines such as recombinant protein vaccines are generally safe and free from the problem of reversal. Nevertheless, because subunit vaccines are devoid of the complex components of microorganisms that stimulate the innate immune cells, they elicit a weaker immune response on their own. Subunit vaccines, therefore, need proper adjuvants to trigger a protective and long-lasting immune response in the vaccinees. The use of adjuvanted vaccines has several advantages over antigens alone. Besides stimulating the immune response, the use of adjuvant decreases the dose and number of immunizations of the antigen. Adjuvants also speed up the immune response to the vaccine antigens. Moreover, adjuvants can be used to modulate the immune response to the required quality. For example, they can be used to skew the immune response to humoral or cell-mediated type (O'Hagan and Rappuoli, 2004, Reed et al., 2009).

A variety of compounds such as proteins, carbohydrates, lipids and nucleic acids are being used as adjuvants in a wide range of candidate vaccines (Reed et al., 2009, Ishii and Akira, 2007). There has been accumulation of a great deal of evidence about how vaccine adjuvants use the innate immune system to exert their functions in enhancing the immune response to vaccine antigens. The innate immune system recognizes pathogens via pattern recognition receptors (PRR) that are expressed on immune and also non-immune cells. PRRs recognize specific molecular patterns called pathogen-associated molecular patterns (PAMPs) on pathogens. Toll-like receptor (TLR) system is the most widely known PRR that is involved in immune stimulation by adjuvants. But, recently it has been found out that adjuvants do also use non-TLR innate immune receptors. These include; cytosolic nucleotide oligomerization domain (NOD)-like receptors (NLR), and retinoic acid-inducible gene based-I-like (RIG-1) receptors (Ishii and Akira, 2007, Jiang and Koganty, 2003, Reed et al., 2009).

Based on their mechanism of action and physiochemical properties, vaccine adjuvants can be grouped into two broad categories: immunostimulants and vehicles. Immunostimulants such as TLR ligands are those that directly interact with the immune system to enhance the response to vaccine antigens. Whereas, vehicles ensure proper and stable presentation of the vaccine antigens to the immune system. For example, the use of biodegradable nanoparticles such as poly lactide – *co* - glycolide (PLGA) for vaccine delivery ensures slow release of antigens and induces long-lasting immune response. This phenomenon is called depot effect (Jilek et al., 2005, Reed et al., 2009, Ribeiro and Schijns, 2010).

Toll-like receptors (TLRs) are constitutively expressed membrane-spanning glycoproteins that are capable of recognizing pathogen-associated molecular patterns (PAMP) such as bacterial lipopolysaccharide (LPS). TLRs are structurally characterized by having two parts; the extracellular leucine-rich repeat (LRR) and the transmembrane and cytoplasmic TIR domain. The LRR domain contains repeating sequences xLxxLxLxx (where x is any amino acid and L is leucine). The TIR (Toll/interleukin-1 receptor) is so called due to the similarity between this domain and a region in of IL-1 receptor. The LRR domain is responsible for ligand binding while the TIR domain mediates signal transduction (Kindt, 2007b).

To date, ten and thirteen TLRs have been identified in humans and mice, respectively. Some of the TLRs form dimers while the others function as monomers. In humans, TLR1 together with TLR2 recognizes triacyl lipoproteins such as those of *Mycoplasma* whereas, TLR2 together with TLR6 recognize diacyl bacterial lipoproteins. TLRs 3, 4, 5, 7/8, and 9 recognize dsRNA, LPS, flagellin, ssRNA and unmethylated CpG respectively. In addition to ssRNA, TLR7 and/or 8 also recognize the synthetic molecule imidazoquinolines such as imiquimod and resiquimod. TLRs 3,

7, 8, and 9 are found on the phagosomal membrane while the rest are expressed on the cell surface (Kawai and Akira, 2006, Zhang and Matlashewski, 2008).

Binding of a specific TLR with its ligand triggers intracellular signal transduction that brings about activation of transcription factors and, in turn, expression of the genes that code for inflammatory cytokines, type-1 interferons (IFN-α, and IFN-β), and chemokines. Inflammatory cytokines produced through this signalling include TNF-α, IL-1β, IL-6, and IL-12. Moreover, the recognition of PAMPs by TLRs induces the expression of co-stimulatory molecules on antigen presenting cells such as dendritic cells (DCs). As a result, DCs mature and stimulate helper T-cells so as to elicit antigen-specific adaptive immune responses. Due to their role in activation and maturation of DCs, TLRs are involved in linking innate and adaptive immune responses (Kawai and Akira, 2006).

1.9.3.1 CpG ODN

Synthetic unmethylated CpG oligodeoxy nucleotide (CpG-ODN) has been used as adjuvant in viral and parasitic vaccines. It is a TLR-9 agonist and induces strong cellular immune response to the vaccine antigens. It has been shown in different vaccine trials that CpG ODN induces high level production of proinflammatory cytokines such as IFN-α, IFN-Y, and IL-12 (Reed et al., 2009, Ivory et al., 2006).

It has been shown in amoebiasis vaccine that CpG is a potent inducer of Th-1 response and protection against *Entamoeba histolytica* challenge in experimental gerbils. It was demonstrated that intramuscular immunization of gerbils with the vaccine antigen and CpG ODN protected all gerbils from amoebic dysentery and liver abscess while only 50% of those that received the antigen with a non-CpG ODN were protected from an intra-hepatical challenge with the parasite. Splenocytes from gerbils immunized with the vaccine antigen plus CpG ODN showed high

degree of proliferative response and IFN-γ and IL-12 production than the control animals (Ivory et al., 2006).

CpG ODN has been used in both systemic and mucosal vaccines. In HIV mucosal vaccine, CpG was found to induce both protective humoral and cell-mediated responses as seen by high level production of serum and vaginal IgG and IgA against the vaccine antigen. Moreover, *in-vitro* cultured splenocytes from immunized mice produced significantly higher IFN-γ than those immunized with the vaccine antigen without CpG adjuvant. The intranasally immunized mice were protected from intra-vaginal viral challenge (Dumais et al., 2002).

Several leishmaniasis vaccine studies have demonstrated that CpG ODN adjuvant enhances immunogenicity and protective efficacy of vaccine antigens. It skews the immune response to a Th-1 phenotype (Mazumder et al., 2011, Daifalla et al., 2011, Daifalla et al., 2012). In a DNA vaccine using *Leishmania* gp63, Mazumder and colleagues (2011) showed that CpG ODN increases the immunogenicity of the vaccine antigen, skews the immune response to Th-1 type and enhances the protective potential of the antigen.

In our lab, we investigated the role of CpG ODN adjuvant in enhancing the immunogenicity and protective efficacy of rLdFeSODB1 and LdPxn4 candidate vaccines in BALB/c mice. We found out that mice immunized with antigens together with CpG ODN adjuvant induced a significantly higher degree of a Th-1 biased immune response as seen by high level production of IFN-γ and also a high IgG2a/IgG1 ratio than those immunized with the antigen alone. Moreover, mice immunized with rLdSODB1 in the presence of CpG ODN were better protected against experimental *L major* infection (Daifalla et al., 2011, Daifalla et al., 2012).

1.9.3.2 GMCSF as a Vaccine Adjuvant and Secretory Module

Granulocyte-macrophage colony-stimulating factor (GMCSF) is a hematopoietic growth factor that stimulates multipotent progenitor cells to differentiate into macrophages/monocytes and also to granulocytes such as neutrophils and eosinophils. In addition to its role in hematopoiesis, GMCSF also functions as immunomodulator. GMCSF plays important role in activation, maturation, and function of dendritic cells. In addition, it recruits cells such as neutrophils and monocytes to the site it is produced in a paracrine fashion. As a result, GMCSF has been used as important adjuvant in infectious disease and cancer vaccine candidates (Disis et al., 1996, Shi et al., 2006).

GMCSF is produced by stimulated T-cells, macrophages, fibroblasts and endothelial cells in response to stimuli of microbial origin such as bacterial lipopolysaccharide and also cytokines such as TNF-α and IL-6. In an unstimulated condition, GMCSF is found in low to unidentified concentration in blood and tissue (Shi et al., 2006)

GMCSF is a glycoprotein that has pro- and mature-regions. The mature region of murine and humans GMCSF is 124 and 127 amino acids long, respectively. In a bacterial expression system it produces 14kDa recombinant protein. In mammalian cells, GMCSF is a highly glycosylated protein (Shi et al., 2006).

GMCSF has been used in the form of recombinant protein or plasmid DNA as an adjuvant in candidate vaccines for malaria, HIV, mycobacteria, *Leishmania*, as well as cancer (Weiss et al., 1998, Gonzalez-Juarrero et al., 2005, Follador et al., 2002, Higano et al., 2010, Lai et al., 2011).

In a malaria DNA vaccine study, a plasmid encoding mGMCSF was used together with another plasmid encoding *Plasmodium yoelii* sporozoite protein. Plasmid containing mutant mGMCSF and another plasmid DNA without the antigen and mGMCSF were used as controls. Parasite

challenge of mice with infectious sporozoite resulted in 54% and 28% protection in mice that received the combination of antigen and mGMCSF and antigen alone, respectively. Mice immunized with the combination showed increased IL-2 and IFN-γ production as well as higher $CD4^+$ T cell proliferation. Moreover, mice that received the combination showed significant increase in the production of antigen-specific antibody of IgG1, IgG2a and IgG2b phenotypes (Weiss et al., 1998).

A recent study with an animal model of HIV has showed a significant contribution of GMCSF as a vaccine adjuvant. A heterologous prime boost vaccination was followed using the antigen-GMCSF fusion and a modified vaccinia Ankara simian immunodeficiency virus (SIV) in macaques monkeys. Monkeys that were immunized with the fusion showed 71% protection as compared with 25% protection seen in those that received the antigen without GMCSF (Lai et al., 2011).

Gonzalez-Juarrero and co-workers (2005) showed the importance of GMCSF in *Mycobacterium tuberculosis* infection. Wild-type (WT) and GMCSF knock-out (KO) C57BL/6 mice as well as mice expressing GMCSF in the lung tissue on GMCSF KO background (GM+) were used. These mice were challenged with an infectious dose of *M tuberculosis* and the pathogenesis of the disease followed-up. While WT mice controlled the infection, the GMCSF KO ones were unable to contain it and succumbed to the infection by day-35. On the other hand, GM+ mice limited the bacterial infection during the early periods but began to die after 60 to 70 days. A significant increase in accumulation of IFN-γ producing $CD4^+$ and $CD8^+$ T cells was seen in the lungs of WT and GM+ cells but not in KO ones. Moreover, there was a significant increase in alveolar macrophages, DCs and monocytes in WT and GM+ cells.

Recombinant human GMCSF (rhGMCSF) was used in a clinical trial for American cutaneous leishmaniasis caused by *Leishmania amazonensis*. The adjuvant was given to healthy unexposed individuals together with parasite crude antigen. Individuals who received the antigen with rhGMCSF adjuvant generally produced more IFN-γ and IL-5 than the placebo groups (Follador et al., 2002).

Haddad and colleagues (2000) studied the detailed mechanism of action of GMCSF adjuvant in the form of plasmid DNA. Among other things, they investigated the type of cells recruited as well as whether or not the spatial and temporal separation of plasmids containing the vaccine antigen and GMCSF brings different results. They also investigated if injection of GMCSF plasmid can have systemic effects. Plasmids with genes of *Plasmodium yoelii* circumsporozoite protein, murine GMCSF, or mutant murine GMCSF were used on BALB/c mice. Injection of GMCSF plasmid alone or with the vaccine antigen resulted in large cellular infiltrate at the site of intramuscular injection and the vicinity. Histopathological and immunohistochemistry analysis of the infiltrate showed macrophage, neutrophil and dendritic cell (DC) but not T and B cell phenotypes. Macrophages were the dominant cells in the infiltrate throughout the 14 day follow-up. Dendritic cells, on the other hand, appeared on days 3 to 5 as immature state and disappeared faster than macrophages. Intramuscular (IM) co-injection of pGMCSF and pAntigen elicited strong cell- and antibody-mediated responses. On the other hand, intravenous (IV) co-injection did not elicit a response by week-8. Separation of the injection site of pGMCSF and pAntigen into two limbs or different sites in the same lymphatic drainage resulted in complete elimination of the immune response.

A granulocyte-macrophage colony-stimulating factor (GMCSF)-containing therapeutic vaccine was approved by US food and drug administration (FDA) for the treatment of asymptomatic or

minimally symptomatic prostate cancer in men. Sipuleucel-T (Provenge®, Dendreon) contains autologous DCs loaded with prostatic acid phosphatase (PAP) fused with GMCSF. PAP is specific to prostate tissue and is expressed in about 95% of prostate cancers. Because DCs express the receptor for GMCSF, the GMCSF in the fusion facilitates internalization of and processing of the antigen (Higano et al., 2010).

In addition to its role as a vaccine adjuvant, GMCSF can also be used as a secretory module for vaccines used in intramuscular injection. GMCSF possesses a 17 amino acid leader sequence that mediates secretion of the protein (Kaushansky et al., 1992).

1.10 Hypothesis

Knowing that superoxide dismutases and peroxidoxins are immunogenic and protective against different infectious diseases including leishmaniasis, we hypothesized that fusing these antigens or using them as a cocktail vaccine in a DNA/protein or a protein/protein immunization strategy further potentiates their immunogenicity and protective potential against leishmaniasis. Moreover, we hypothesized that fusing GMCSF to the vaccine antigens also increases the efficacy of the antigens.

1.11 Objectives

1.11.1 General Objective

The general objective of our study was to evaluate the immunogenicity and protective efficacy of a fusion (or cocktail) of *Leishmania donovani* superoxide dismutase B1 and peroxidoxin1 in the form of DNA/protein and protein/protein immunization approach in BALB/c mice.

1.11.2 Specific Objectives

The specific objectives of this study were:

1. To clone a fusion of the *Leishmania donovani* superoxide dismutase B1and peroxidoxin-1 genes in a plasmid vector and express it in a mammalian system *in vitro*.
2. To assess the immunogenicity and protective efficacy of a *Leishmania donovani* fusion of superoxide dismutase B1-peroxidoxin-1 in DNA prime/cocktail protein boost vaccination in BALB/c mice.
3. To assess the immunogenicity and protective efficacy of *Leishmania donovani* superoxide dismutase B1 and peroxidoxin-1 as cocktail vaccine in protein/protein vaccination strategy in BALB/c mice.

Chapter Two: **MATERIALS AND METHODS**

2.1 ANIMALS

Four to six week old female BALB/c mice were purchased from Charles River Laboratories (USA). The mice were maintained under pathogen-free animal facility of the Department of Biological Sciences, University of Calgary throughout the study period. Mice were acclimatized for two weeks before immunization with the vaccine candidates. The protocol for mice experiment was reviewed and approved by Life and Environmental Sciences Animal Care Committee (LESARC), University of Calgary, Alberta, Canada.

2.2 PARASITES

Leishmania major strain V1 (MHOM/IL/80/Friedlin) was obtained as a kind gift from Dr. Steven G. Reed, Infectious Disease Research Institute (IDRI) (WA, USA). In order to maintain the virulence, the parasites were injected into the footpads of BALB/c mice. Samples from infected footpad were inoculated into M199 medium containing 20% fetal bovine serum (FBS). Promastigotes were grown *in vitro* and stored at -80°C freezer until next use.

2.3 CULTURE MEDIA

2.3.1 Bacterial Culture Medium

Luria Bertani (LB) medium was used to grow different strains of *Escherichia coli* in solid or liquid culture.

One liter LB broth:

Tryptone (EMD) (USA).....10.0 gram

Yeast extract (EMD) (USA).....5.0 gram

Sodium chloride (EMD)(USA).....10.0 gram

The above ingredients were dissolved in double distilled water (ddH_2O), the pH adjusted to 7.25, heated on a hot plate until complete dissolution and autoclaved at 121^oC for 20 minutes. In order to make LB agar, 1.5% w/v granulated agar-agar (EMD, USA) was added to the LB broth medium before autoclaving.

2.3.2 Leishmania Culture Medium

M199 medium with 20% Fetal Bovine Serum was prepared for *L major* culture as follows:

M199 Medium, powder (GIBCO) (dissolved in ddH_2O)….....100.0ml

$NaHCO_3$(GIBCO)….....2.2g

[1]Fetal bovine serum (FBS) heat inactivated (GIBCO).....200.0ml

[2]Adenosine (Sigma) (100X)….....4.0ml

L-glutamine (200mM) (GIBCO)….....10.0ml

[3]Folic acid (1000X) (Sigma) ….....1.0ml

HEPES (without sodium salt) (GIBCO) …….....……..…5.96g

Penicillin – Streptomycin (100X) (GIBCO)…….....……2.0ml

Double distilled H_2O …….....683.0ml

The pH was adjusted to 6.8 with 5N HCl. Then the medium was filtered with 0.2µM filter (VWR) and stored in a refrigerator.

[1] FBS: Frozen FBS was thawed at 4^oC overnight and then heat-inactivated at 56 ^oC for 30min in a water bath.

[2]Adenosine: 0.134g Adenosine was dissolved in 20ml ddH_2O. It was then stored in 4ml aliquot in -20 freezer until use.

[3]Folic acid: 0.100g folic acid was dissolved in 10ml 1N KOH. One milliliter aliquot was stored in -20 freezer.

2.3.3 Chinese Hamster Ovary (CHO) Cell Culture Medium

CD CHO medium (1X) (GIBCO) was used to grow Chinese hamster ovary (CHO) cells. One liter of the medium was supplemented with 10ml HT supplement (100X) (GIBCO) and 40ml L-glutamine (200mM) (GIBCO).

2.3.4 Spleen Cell Culture Medium (complete RPMI, cRPMI)

RMPI 1640 HEPES modified (Sigma) 880ml

10% FBS (Heat Inactivated) (GIBCO) ..... 100ml (stock)

50µM B-mercaptoethanol (Sigma) .....3.5µl (Stock, 14.3M)

2mM L-glutamine (GIBCO)..... 10.0ml (200mM)

100U/ml Penicillin and 100µg/ml Streptomycin (100X) (GIBCO)..... 10.0ml (Stock).

The medium was filter sterilized with 0.2µM filter (VWR) and stored in a refrigerator.

2.3.5 Novy-MacNeal-Nicolle (NNN) Medium

Two hundred fifty millilitre NNN medium with 10% defibrinated rabbit blood was prepared and dispensed in 50µl per well into 96-well flat-bottom culture plate (Sarstedt, USA). Difco™ nutrient agar (BD) (6.0g), D-glucose (GIBCO) (0.375g), and sodium chloride (EMD) (1.5g) were dissolved, boiled in 225 ddH_2O, and autoclaved at 121°C for 20min. The medium was then cooled down and kept in a 45°C water bath. Then, 25ml of defibrinated rabbit blood (Quad Five, USA) was added to the medium aseptically. Finally, 50µl of the medium was applied into each well of 96-well flat-bottom culture plates that was kept tilted at 45° (Sarstedt, USA). After the agar solidified, the plates were sealed and kept refrigerated until use.

2.4 PLASMIDS, PRIMERS AND OTHER DNA MOLECULES

2.4.1 Plasmid Vectors

A modified pcDNA (Invitrogen) plasmid vector was previously developed in collaboration with Dr. Patrick Farrell, Schulich School of Engineering (Pharmaceutical Production Research Facility), University of Calgary, Canada. As described in **Figure-2.1** and **Figure-2.2** below, the plasmid DNA contains murine GMCSF (mGMCSF) and a spacer region cloned in a pcDNA 3.1 (+) frame (Invitrogen). The spacer region possesses six histidine residues, enteropeptidase cleavage site flanked by proline hinge at both ends. A GFP expression plasmid vector, pEGFPN3, was purchased from a commercial company (Clonetech, USA).

2.4.2 Primers

In this study, we cloned the fusion LdPxn1-LdFeSODB1 (LdVAC3) and LdFeSOB1 genes in modified pcDNA vector with or without mGMCSF fusion. LdPxn1 was previously cloned in pcDNA with/without mGMCSF in our lab (Dr. Tegegn, PhD thesis). The following primers were used to clone LdFeSODB1 gene and the fusion LdFeSODB1-LdPxn1gene (abbreviated as VAC3) in a modified pcDNA and pcDNA-mGMCSF vectors.: 1) *VAC3NotI-F-5'- GGA TCC GCG GCC GCC ATG CCG TTC GCT GTT CAG CCG CTG-3', 2) VAC3NotI-R-5'- CTC GAG GCG GCC GCC TTA CTT ATT GTG ATC GAC CTT CAG GCC AG, and 3) VAC2NotI-R-5'- CTC GAG GCG GCC GCC TTA AAG CTG GCT AGA GGC GAA ATC*. LdFeSODB1 gene was amplified using *VAC3NotI-F-5'* and *VAC2NotI-R-5'* as forward and reverse primers, respectively. The fusion gene (VAC3) was amplified using *VAC3NotI-F-5'* and *VAC3NotI-R-5'*, as forward and reverse primers, respectively.

2.4.3 CpG Oligodeoxynucleotide (CpG ODN)

A 20-mer CpG ODN 1826 with the sequence, 5'-tccatgacgttcctgacgtt-3' was purchased from InvivoGen (USA). Lyophilized CpG ODN was resuspended using 1X endotoxin free phosphate buffer saline (PBS) (Teknova, USA) and stored in -20°C freezer.

2.5 CHEMICALS AND REAGENTS

2.5.1 Buffers and Solutions

2.5.1.1 CHO Cell Lysis Buffer (TXSWB)

Cell lysis buffer was prepared according to the protocol on Tanudji *et al* (2002).

For 50ml:

1% Triton X-100…..... 0.5ml

100mM Tris-HCl, pH 8.0…….....……5.0ml (1M)

100mMNaCl….....**.....** 1.0ml (5M)

10mM EDTA…….....….....……... 0.5ml (1M)

2mM PMSF…..... 1.0ml (100mM)

Double distilled H_2O…..... 42.0ml

2.5.1.2 Protein Transfer Buffer

Towbin transfer buffer was used to transfer protein samples to polyvinylidene difluoride (PVDF) membrane (GE Healthcare) for Western blotting. The buffer contained 25mM Tris (pH 8.3), 192mM glycine, and 20% methanol.

2.5.1.3 Antibody ELISA Coating Buffer

Carbonate-bicarbonate buffer (pH 9.6) was used to coat ELISA plates with recombinant proteins for antibody ELISA. Two separate solutions were first prepared using sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$). Solution-1 was 100mM Na_2CO_3 and solution-2

was 100mM $NaHCO_3$. After the solutions were made, sodium carbonate was poured into sodium bicarbonate until pH reached 9.6. Then, the solution was filtered through 0.22µm filter and stored at 4^oC.

2.5.1.4 GST Cleavage Buffer

10x GST cleavage buffer was prepared for cleavage of GST using thrombin. The buffer contains 500mM Tris-Cl (pH=8.0), 1M NaCl, 25mM $CaCl_2$, and 1% beta-mercaptoethanol in sterile distilled water. The buffer was used at final 1X dilution.

2.6 METHODS

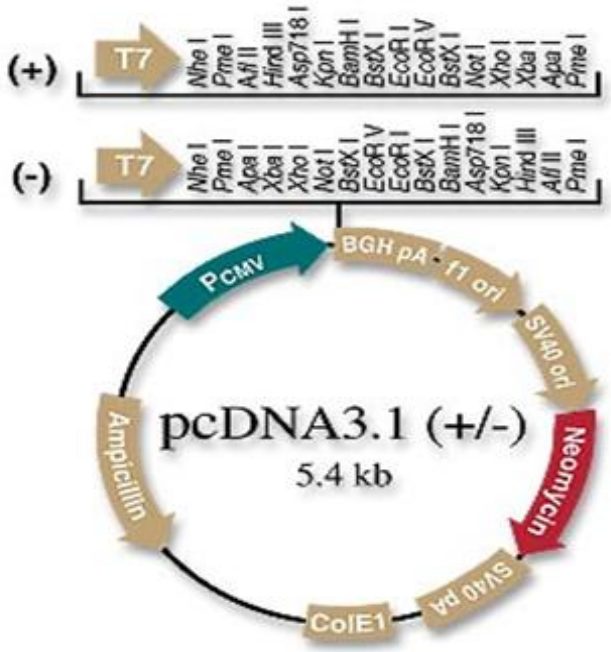
2.6.1 Cloning of DNA Vaccine Constructs

DNA vaccine genes were cloned in a modified pcDNA and pcDNA-mGMCSF plasmid vectors. pcDNA plasmid is a mammalian expression vector that is used to express genes under human cytomegalovirus (CMV) immediate-early promoter/enhancer (**Figure-2.1**). It also possesses T7 promoter sequence, multiple cloning sites, and bovine growth hormone (BGH) polyadenylation signal sequence. T7 promoter sequence was used to confirm the cloning of vaccine candidate genes.

As shown in **Figure-2.2**, the hydrophilic spacer region consists of six histidine residues and enteropeptidase recognition site flanked by proline residue at both ends. The six histidine residues and enteropeptidase recognition sequences were included for Ni-NTA purification and cleavage of the fusion proteins, respectively. By spatially separating the GMCSF from the fused vaccine candidate (s), the spacer region prevents any possible interference between them. In addition, the flanking proline residues allow the spacer region to form its own domain which, in turn, allows the Ni-NTA to bind to six histidine residues in situations where purification of the recombinant protein is needed.

Figure 2.1: Map of pcDNA 3.1 (+/-) Plasmid Vector

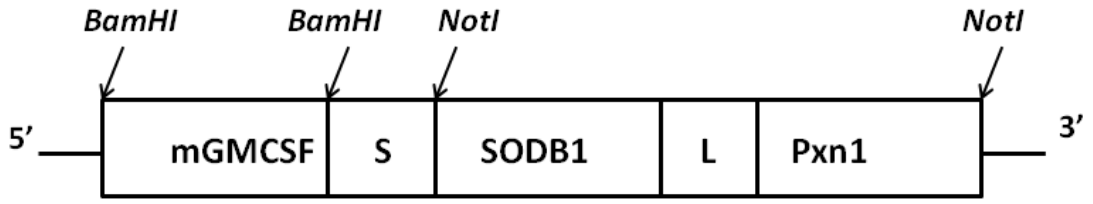
(*Invitrogen*)



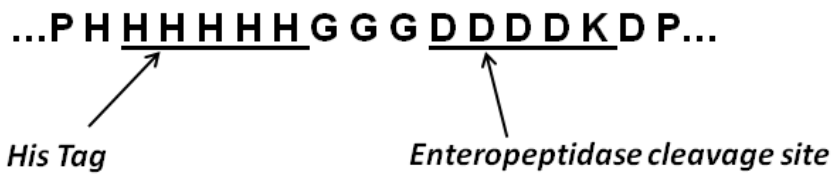
Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of the recombinant protein
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
Multiple cloning site	Allows insertion of the gene and facilitates cloning
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen
Neomycin resistance gene	Selection of stable transfectants in mammalian cells
SV40 early polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

Figure 2.2: Design of Fusion Vaccine Candidates for DNA Vaccines.
A) mGMCSF fused with vaccine candidate genes, **B)** Amino acid sequences of spacer region, and **C)** Amino acid sequences of linker. Note: The stop codons of mGMCSF and SODB1 were mutated.

A.



B.



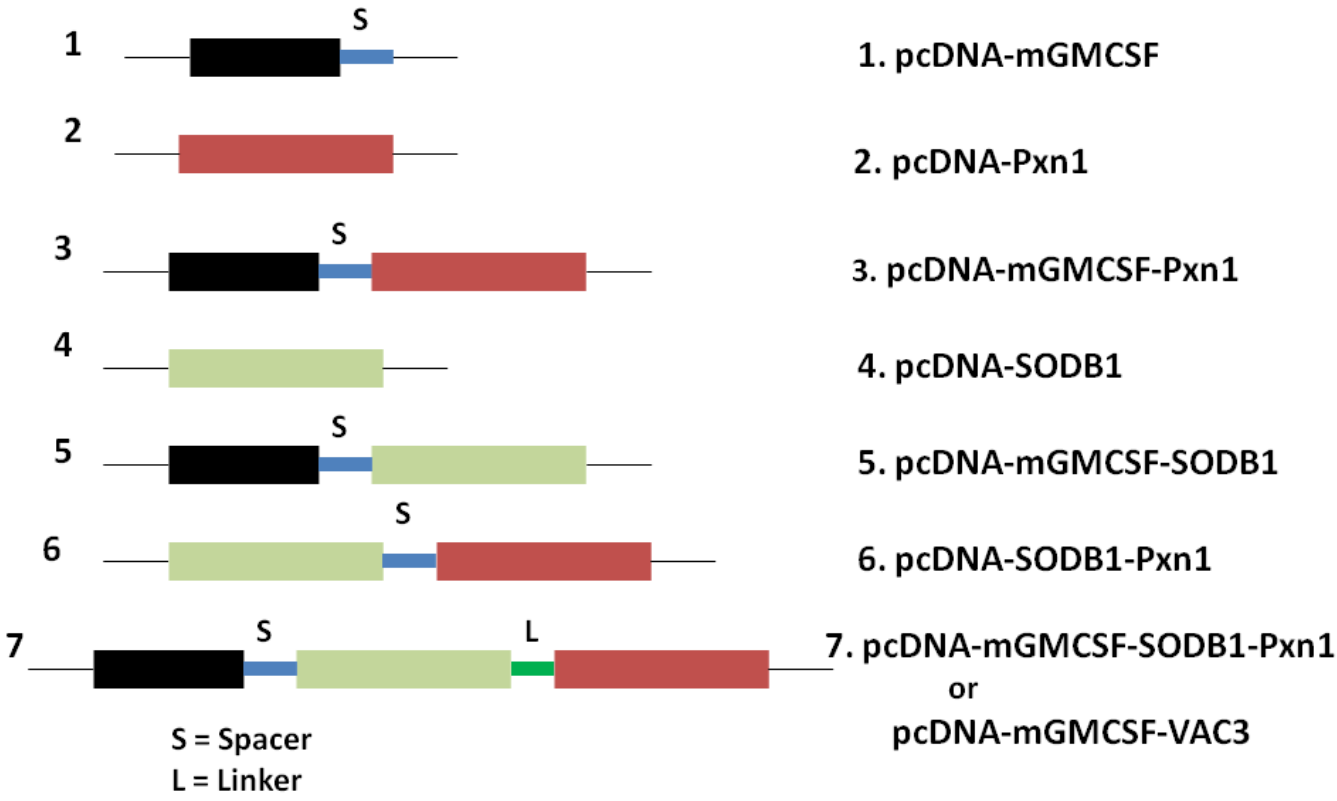
C.

...PGTSPGTSPGTSGPP...

2.6.1.1 Fusion Gene Synthesis and Cloning

Fusion LdFeSODB1-LdPxn1 (also named VAC3) was designed *in-silico* using Vector-NTI software (Invitrogen). A linker sequence was added in between SODB1 and Pxn1 sequences (**Figure-2.2**). Like in the spacer sequence, the linker sequence also possesses flanking proline residues. The fusion gene sequences were synthesised and cloned on pBSK plasmid vector (pBSK-VAC3) by a commercial company (Epoch Biolabs, USA). Individual and fusion vaccine candidate genes were then sub-cloned into the modified pcDNA plasmid vector. The list of vaccine candidates is illustrated in **Figure-2.3**.

Figure 2.3: Schematic Illustration of DNA Vaccine Candidate Constructs


1
S
1. pcDNA-mGMCSF
2
2. pcDNA-Pxn1
3
S
3. pcDNA-mGMCSF-Pxn1
4
4. pcDNA-SODB1
5
S
5. pcDNA-mGMCSF-SODB1
6
S
6. pcDNA-SODB1-Pxn1
7
S
L
7. pcDNA-mGMCSF-SODB1-Pxn1
or
pcDNA-mGMCSF-VAC3
S = Spacer
L = Linker

2.6.1.2 PCR Amplification

PCR amplification of VAC3 and SODB1 was done using pBSK-VAC3 template DNA. *VAC3NotI-F* and *VAC3NotI-R* primers were used to amplify VAC3 gene. On the other hand, SODB1 gene was amplified using *VAC3NotI-F* and *VAC2NotI-R primers. Each PCR mixture contained 100ng of template DNA, 20 picomoles of each forward and reverse primers, 250µM dNTPs (dATP, dTTP, dCTP, and dGTP), 1X high fidelity PCR buffer,* 5mM $MgSO_4$ (final), and 2 units Platinum® *Taq* DNA polymerase-high fidelity (Invitrogen) in a 100µl total reaction volume. PCR was done with one cycle of initial denaturation at 94°C for 5 minutes followed by thirty cycles of 94°C denaturation for 1min, 60°C annealing for 30seconds, and 68°C extension for 90 seconds. One final cycle was done at 68°C for 10minutes in Mastercycler PCR machine (Eppendorf, Germany). To check the PCR result, electrophoresis was done using 5µl PCR product on 1% agarose gel for 1hr. The DNA on the gel was visualized on Molecular Imager® Gel Doc™ XR (BIO-RAD, USA).

2.6.1.3 Restriction Digestion and Ligation

The PCR products were subjected to purification using QIAquick® PCR purification kit (QIAGEN, Canada). Then, restriction digestion was done on the PCR products and the plasmid vectors (pcDNA and pcDNA-mGMCSF) with high fidelity (HF) *NotI* enzyme (New England Biolabs, USA) at 37°C for 1hr in the presence of 1X *NotI* buffer (New England Biolabs, USA) and 1X bovine serum albumin (BSA) (New England Biolabs, USA). After digestion, the plasmid DNA was subjected with dephosphorylation using Calf Intestine Alkaline Phosphatase (CIAP) (Invitrogen, Canada). The digested DNA (plasmid and PCR product) was run on 1% low melting point agarose for 1hr and purified using QIAquick® gel extraction kit (QIAGEN, Canada) following the manufacturer's instruction. Finally, ligation reaction was done between the

digested PCR product and vector using T4 DNA ligase (Invitrogen, Canada) in the presence of 1X (final) ligase buffer (Invitrogen, Canada) at 16°C water bath overnight.

2.6.1.4 Competent Cell Preparation

Escherichia coli DH5α strain competent cells were prepared using $CaCl_2$ method (Mandel and Higa, 1970). Frozen *E coli* DH5α culture stored in -80°C freezer was used to inoculate 5ml LB medium. The inoculated medium was incubated at 37°C overnight in a shaking incubator (Orbital shaker, Forma Scientific, USA). Next morning, 500µl of the saturated culture was used to inoculate 30ml of fresh LB medium. The bacteria were allowed to grow at 37°C to approximately 10^8 cells/ml (equivalent to OD_{600} = 1). The cells were then recovered by spinning at 6,000rpm for 10min at 4°C using Sorvall® RC-5B centrifuge (Mandel, Canada). The media was decanted and the remaining media removed by inverting the tube on a paper towel. Then, the bacterial pellet was resuspended in 10ml of sterile, ice cold 50mM $CaCl_2$. The bacteria were then kept on ice for 30min before centrifuged at 6,000rpm for 10min at 4°C. Finally the bacteria were resuspended in 1ml sterile, ice cold 50mM $CaCl_2$ containing 15% glycerol, kept at 4°C for 1hr, aliquoted in 100µl in sterile microtubes, and stored in -80°C freezer.

2.6.1.5 Transformation

Transformation experiment was done using heat-shock method. One hundred micro-litre of competent *E coli* DH5α cells was mixed with 150ng of plasmid DNA and incubated for 10min on ice. The bacteria were then subjected to heat shock at 45°C for 2 min in a water bath. Then, the bacteria were kept at room temperature (RT) for 5min. One millilitre of fresh LB medium was added to each tube and incubated at 37°C for 45min in a shaking incubator (Orbital shaker, Forma Scientific, USA). The bacterial suspension was then spun for 30 seconds in a tabletop centrifuge (Eppendorf, Germany). The pellet was resuspended in 200µl LB medium and cells

spread on pre-warmed LB agar plate containing ampicillin. The plates were then incubated at 37°C for 12hr in ISOTEMP® incubator (Fisher, USA).

2.6.1.6 Cloning Confirmation

In order to confirm the correct cloning, plasmid DNA was isolated from transformed pure *E coli* colony using QIAGEN miniprep kit (QIAGEN, Canada). The DNA was then subjected to restriction digestion following exactly the same procedure as the cloning. In addition to restriction digestion, the cloning was checked by DNA sequencing of VAC3 and SODB1 genes on plasmid clones. The sequence data was compared with expected sequences using Vector NTI (Invitrogen, USA) and BioEdit Sequence Alignment Editor (Ibis Biosciences, USA) softwares.

2.6.1.7 Endotoxin-Free Plasmid DNA Preparation

Endotoxin-free vaccine candidate plasmid DNA was isolated from the transformed *E coli* using EndoFree® plasmid purification kit (QIAGEN, Canada) following the manufacturer's instruction. Glass tubes used for centrifugation were all baked at 180°C overnight in an oven to avoid possible endotoxin contamination. Endotoxin-free plasmid DNA samples were diluted to appropriate concentration using endotoxin-free PBS (Teknova, USA) before injection into mice. The integrity of plasmid DNA clones was checked once again by restriction digestion with *NotI* and *BamHI* enzymes for clones containing the vaccine antigens and mGMCSF, respectively.

2.6.2 Cloning of Peroxidoxin-1 and Superoxide Dismutase B1 in Bacterial Expression Plasmids

Leishmania donovani peroxidoxin 1 (LdPxn1) was previously cloned as a GST fusion and expressed in our lab by Dr. Stephen Barr. LdPxn1 gene was cloned into pGEX-2T plasmid (Amersham Biosciences) and transformed into *E coli* DH5α.

Leishmania donovani superoxide dismutase B1 (LdFeSODB1) was cloned and expressed in *E coli* as His-tagged protein by Dr. Ajay Bhatia (IDRI, USA). LdFeSODB1 gene was cloned into pET17b plasmid and expressed in *E coli* Tuner^{TM} (DE3) *pLysS* competent cells (Novagen, USA).

2.6.3 Purification of Recombinant Proteins

Glutathione S-transferase (GST) fused recombinant peroxidoxin 1 (rPxn1-GST) and His-tagged recombinant superoxide dismutase B1 (rSODB1) were purified from transformed *E coli* using Glutathione Sepharose^{TM} 4B beads (Amersham GE Healthcare, UK) and Ni-NTA agarose (QIAGEN, Germany), respectively.

2.6.3.1 Purification of Recombinant Peroxidoxin-1

LdPxn1-GST was purified from *E coli* following a modified procedure of Smith and Johnson (1988). LdPxn1 transformed *E coli* DH5α that had been stored in -80°C freezer were used to inoculate 5ml LB broth containing 100µg/ml ampicillin and incubated at 37°C in a shaking incubator (Orbital shaker, Forma Scientific, USA) overnight. Next day, 5ml bacterial culture was used to inoculate a 50ml fresh LB broth. The culture was incubated at 37°C overnight. Then, 450ml of fresh LB medium was inoculated with 25ml of the culture and incubated at 37°C. When the growth reached OD_{600} = 0.6, expression of the recombinant protein was induced using 0.2mM IPTG (final) and incubated for additional 5hr at 37°C in a shaking incubator. The culture was then spun at 6000rpm in Sorvall® RC-5B centrifuge (Mandel, Canada) and the supernatant discarded. The bacterial pellet was then stored in -20°C freezer until purification of the recombinant proteins. The expression of the recombinant protein was checked by running electrophoresis of bacterial lysate from aliquot of the cultures on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).

Purification of rPxn1-GST was done using Glutathione Sepharose™ 4B beads (Amersham GE Healthcare, UK). Briefly, the bacterial pellet was thawed on ice and resuspended in lysis buffer in MTPBS (150mM NaCl, 12.5mM Na_2HPO_4, 2.5mM KH_2PO_4, pH 7.3). The lysis buffer contained 1mg/ml lysozyme, 1% Triton-X 100, 0.05mM PMSF (phenylmethylsulfonyl fluoride) and 1mM DTT (dithiothreitol) in MTPBS. The mix was then centrifuged at 12,000g (Sorvall® RC-5B centrifuge, Mandel, Canada) for 10min and the suspension transferred to a new tube. Two millilitre of 50% v/v Glutathione Sepharose™ 4B beads (in MTPBS) (Amersham GE Healthcare, UK) was added to the protein suspension and incubated at room temperature for 30min with gentle shaking on Compact Rocker (Mandel, Canada). The beads were washed with 15ml 1X MTPBS three times followed by two washes with 50mM Tris-Cl pH 8.0. Finally, the rPxn1-GST protein was eluted by incubating the beads at room temperature for 10min with one bead volume of 50mM Tris-Cl (pH 8.0) containing 10mM reduced glutathione.

2.6.3.2 Purification of Recombinant Superoxide Dismutase B1

Histidine-tagged recombinant LdFeSODB1 protein was purified from *E coli* Tuner™ (DE3) *pLysS* cells that had been transformed with pET17b-SODB1 using Ni-NTA agarose (QIAGEN, Germany) column chromatography. The recombinant protein was purified from inclusion bodies. The bacterial pellet was thawed and subjected to lysis with mild sonication in 20mM Tris-Cl, pH 8.0 containing 1mM PMSF and EDTA-free Complete Mini protease inhibitor cocktail tablet (Roche, Germany). The suspension was treated with 1% CHAPS (Sigma) in Tris-Cl and incubated for 4hr in the cold room. Then, it was spun down at 10,000rpm for 20min (Sorvall® RC-5B centrifuge (Mandel, Canada) and supernatant discarded. The protein was purified from the inclusion bodies using Ni-NTA affinity chromatography (QIAGEN, Germany). The pellet was resuspended in binding buffer containing 8M urea (Sigma) in Tris-Cl followed by mixing it

with 5ml Ni-NTA agarose and incubation at room temperature for 1hr on a rotating Compact Rocker (Mandel, Canada). The mixture was then transferred into Purification Column (Invitrogen), washed once with buffer containing 8M urea and then treated with 12mM sodium deoxycholate (Sigma, USA) in 20mM Tris-Cl pH 6.3 and followed by serial washes with Tris-Cl pH 8.0 without urea. Finally, the rLdFeSODB1 was eluted with Tris-Cl pH 8.0 buffer containing 0.4M imidazole (Sigma, USA). Both rLdPxn1-GST and rLdFeSODB1 proteins were dialyzed using 1X PBS, pH 9.0.

2.6.4 Protein Concentration

Recombinant proteins in all elution fractions were pooled and were concentrated using Macrosep 10K Omega (Pall Life Sciences, USA) by spinning 5000rpm (Sorvall® RT6000 Refrigerated Centrifuge, Mandel, Canada).

2.6.5 Endotoxin Removal

Endotoxin was removed from the recombinant proteins using Detoxi-gel™ affinity Pak™ pre-packed columns (Pierce Biotechnology, USA) following manufacturer's instruction. Briefly, the columns containing endotoxin removing gel were regenerated by washing with five column volumes of 1% sodium deoxycholate followed by 3 to 5 column volumes of endotoxin-free water to remove the detergent. After all the water passed through the column, the solution containing recombinant proteins was applied into the gel in the column and incubated for 1hr at room temperature. Finally, the recombinant proteins were collected on the bottom end of the column.

2.6.6 GST Cleavage of Recombinant Pxn1-GST and Purification of Cleaved Protein

Recombinant LdPxn1-GST was subjected to thrombin cleavage. About one milligram of rLdPxn1-GST in 1ml buffer was mixed with 1X GST cleavage buffer and 20U thrombin (Amersham GE Healthcare, UK) and incubated at room temperature for 20hr. Then,

electrophoresis was done on aliquot of the cleaved protein and control samples of rLdPxn1-GST and rGST on 12% SDS-PAGE to check the degree of cleavage. After complete cleavage was achieved, cleaved rLdPxn1 was purified from the digestion mixture by passing through columns containing glutathione sepharose beads as above. Unlike purification of fusion rLdPxn1-GST, the cleaved rLdPxn1 was collected from the flow-through without adding reduced glutathione. The GST remained attached to the glutathione beads and was eluted with reduced glutathione in order to recycle the glutathione beads. The concentration of the protein was finally measured using Pierce® BCA protein assay kit (Thermo Scientific, USA).

2.6.7 Preparation of Soluble Leishmania Antigen (SLA)

Soluble *Leishmania* antigen (SLA) was prepared from promastigotes of *L major*. Promastigotes were seeded in 1 x 10^6 parasites into 10ml M199 medium (20% FBS) and incubated in 26°C incubator for six days. The stationary phase promastigotes were harvested at day-6 by spinning at 3000rpm using Sorvall® RT6000 refrigerated centrifuge (Mandel, Canada) at 4°C. The pellet was washed twice with endotoxin free PBS (Teknova, CA, USA) and finally resuspended at 7 x 10^8 parasites per millilitre. Then, the promastigotes were subjected to eight cycles of freeze-thaw with liquid nitrogen and 37°C water bath. The partially lysed parasites were then sonicated three times each for 30 seconds. Prior to sonication, the sonicator was treated with 1M NaOH followed by wash with endotoxin free water (Sigma, USA) and 70% ethanol to remove possible endotoxin contamination. The lysate was then spun at 10,000rpm for 30minutes in the cold room using table-top microcentrifuge (IEC Micromax, USA). Finally, the supernatant was taken in a new microtube and the concentration measured using Pierce® BCA protein assay kit (Thermo Scientific, USA). The SLA was stored in -80°C freezer until use for cell stimulation.

2.6.8 Expression of DNA Vaccine Candidates in Mammalian Expression System

Leishmania donovani Pxn1, SODB1, and the fusion (VAC3) genes each cloned in pcDNA and pcDNA-mGMCSF as well as controls (*pEGFPN3* (Clonetech, USA), pcDNA, and pcDNA-mGMSCF) were used for transfection and expression experiment on Chinese Hamster Ovary (CHO) cells. Expression of the DNA vaccine candidates involved growing and transfection of CHO cells, fluorescent microscopy, and Western blotting. CHO cells stored in liquid nitrogen were thawed, washed once and transferred into 15ml pre-warmed CD CHO medium supplemented with HT supplement and L-glutamine following the procedure on GIBCO manual in T75CN tissue culture flasks (Sarstedt, USA). After incubating for seven days at 37°C, the cells were subcultured in CD CHO medium and incubated for additional three days before transfection. Two sets of transfection were done; one for fluorescent microscopy and the other for Western blotting.

Cationic lipid-mediated transfection of CHO cells with DNA vaccine candidates was done using Lipofectamine® 2000 transfection reagent (Invitrogen) following the manufacturer's instruction. 6 x 10^5 cells were plated per well in a 24-well plate (Sarstedt, USA). One to three ratio of DNA and lipofectamine were used for transfection. That is, 0.8µg DNA to 2.0µl lipofectamine was used to transfect cells in 500µl/well CD CHO medium. The DNA and lipofectamine were dissolved separately in 50µl OPTI-MEM medium (GIBCO) each and incubated at room temperature for 5min. The diluted DNA and lipofectamine were mixed and incubated for additional 20min at room temperature to form DNA-lipofectamine complex. Finally, the DNA-lipofectamine complex was added to each well directly in a drop-by-drop fashion and incubated for 72hr in 37°C incubator with 5% carbondioxide.

Fluorescent microscopy was done to check the transfection of CHO cells. For this, cells were transfected with a mixture of each of the vaccine candidate constructs or control plasmid DNA and pEGFPN3 following the above procedure. After 72hr incubation, wet-mount was prepared from transfected CHO cells on a microscope slide. Then, expression of *pEGFPN3* was investigated using a fluorescent microscope (Leica, DMR) at 1000X magnification and images were taken with a cooled CCDl camera (Retiga 1350 EX, Qimaging). Another picture was taken on the same microscope field on bright light.

After 72hr incubation, the culture supernatant and cell lysate were collected and Western blotting was done using different antibodies. Culture supernatant from each well was transferred into separate microtube and centrifuged at 14000rpm for 15min using a table top centrifuge (IEC Micromax, USA) in the cold room to separate the floating cells from culture supernatant. The supernatant was then transferred to new microtube. Cells in each well were treated with 1ml of CHO cell lysis buffer and transferred to the microtube that had been used to pellet floating cells and mixed by pipetting up and down few times. Then, the cell lysate was centrifuged at 14000rpm as above and cell debris removed. Secreted and cellular proteins were isolated from the culture supernatant and cell lysate, respectively. Total proteins in the culture supernatant and cell lysate were precipitated using trichloroacetic acid (TCA). Briefly, 1 volume of TCA was mixed with 4 volumes of culture supernatant, incubated for 10min on ice and centrifuged at 14000rpm for 5min using a table-top microcentrifuge (IEC Micromax, USA). Then, the supernatant was discarded and the protein pellet washed twice with ice cold acetone. Traces of acetone were removed by heating the tubes in a 95°C heating block for about 5min. Finally, the proteins pellet was resuspended with 1X Laemmli sample buffer (Bio-Rad, USA) with 5% fresh beta-mercaptoethanol and boiled for 5min in a water bath.

Cell viability was assessed by staining by 0.4% trypan blue (GIBCO) of the transfected cells at 72hr post-transfection.

Western blotting was done on culture supernatant (SUP) and cell lysate (Lys) samples taken from transfected CHO cells. Three groups of CHO cells were used for this analysis. Group-1 contains cells transfected with plasmid clones of LdPxn1with or without mGMCSF and controls. Group-2 and Group-3 possess LdFeSODB1 and VAC3 clones with and without mGMCSF as well as controls, respectively. *pEGFPN3* (Clonetech, USA) was also used as a transfection control. Supernatant and lysate proteins from transfected CHO cells as well as recombinant protein controls were loaded into 12% SDS-polyacrylamide gel. The protein controls were rmGMCSF (AbCam, Canada), rLdPxn1 and rLdFeSODB1. Page Ruler™ Plus Prestained Protein Ladder (Fermentas, USA) was used as protein molecular size marker. Electrophoresis was done at 20V for 1hr. The gel was washed with distilled water for 3min on a shaking Compact Rocker (Mandel, Canada) and then equilibrated for 5min using protein transfer buffer. Meanwhile, Hybond-P PVDF (Amersham GE Healthcare, UK) membrane was pre-treated with absolute methanol for 1min, washed in distilled water for 5min and equilibrated in transfer buffer for 5min. The protein on the gel was then transferred to the membrane on 25V and 1A for 30min on Trans-Blot® Turbo™ transfer system (BIO-RAD, Canada). After transferring the proteins to the membrane, Western blotting was done following the instruction on ECL Western blotting detection system manual (Amersham GE Healthcare, UK). Briefly, the membrane was rinsed once with PBS and blocked with 5% skimmed milk in PBS containing 0.1% (v/v) Tween 20 (PBST) overnight in the cold room. Next morning, the membrane was rinsed twice with PBST wash buffer. The membrane was then incubated with primary antibody in 5% skimmed milk in PBST for 2hr at room temperature : 1) rabbit anti-mGMCSF polyclonal antibody (AbCam,

Canada) in 1:2000 dilution, 2) anti-GFP rabbit serum (Invitrogen, USA) in 1:1000 dilution, or 3) Pooled sera from mice immunized twice with pcDNA-Pxn1 and boosted once with rLdPxn1 in 1:500 dilution. The membrane was rinsed twice and washed three times with PBST each for 10min. The membrane was then incubated with secondary antibody for 1hr: 1) ECL^{TM}-anti-rabbit IgG-HRP (donkey) (GE Healthcare, UK) in 1:5000 dilution, or 2) ECL^{TM}-anti-mouse IgG-Peroxidase conjugate (GE Healthcare, UK) in 1:3000 dilution. Then, the membrane was rinsed and washed as above and incubated for 1min in ECL detection solution (Amersham GE Healthcare, UK). Finally, the signal was detected on Amersham Hyperfilm™ MP (GE Healthcare) and the photo developed and fixed in dark room.

2.6.9 Mice Immunization

2.6.9.1 DNA/Protein Immunization

After two weeks of acclimatization, one hundred female BALB/c mice were injected with DNA vaccine antigens or control. Fifty mice were used to study the immunogenicity and another fifty for protection of vaccine candidate. Five mice were used in each group. The names of different groups and the vaccination strategies are summarized on **Table-2.1**. As shown in **Appendix-B1**, mice were immunized three times in three weeks interval. Two DNA immunizations were given at week-0 and week-3 followed by one recombinant protein boost at week-6. Except the PBS group, all mice that received the vaccine candidates and controls also received 25µg CpG ODN (InvivoGen, USA) with each immunization. All immunizations were prepared in 50µl solution in endotoxin-free PBS (Teknova, USA). One hundred microgram plasmid DNA containing vaccine candidate with 25µg CpG ODN in 50µl total volume was injected intramuscularly (IM) into the thigh muscle of the right hind foot aseptically. Three weeks later, the same DNA vaccine candidates and adjuvant were used for the first booster immunization. Six weeks after the first

immunization, the second booster dose was given to the vaccine groups by subcutaneous injection (SC) of the respective recombinant proteins in combination with 25µg CpG ODN in the right hind footpad. Mice that were primed with the fusion LdFeSODB1-LdPxn1 DNA in the first and second immunizations were given a combination of 12.5µg of each of rLdFeSODB1 and rLdPxn1. On the other hand, mice immunized with individual DNA vaccine candidates received 12.5µg of the respective recombinant protein. However, pcDNA and pcDNA-mGMCSF immunized control mice received all the three immunizations in the form of DNA. Cleaning of the injection site was done by rubbing with a sterile piece of gauze soaked in 70% ethanol. All injections were done using a sterile syringe fitted with 25 gauge needle.

Table 2-1: DNA/Protein Immunization Strategy

Group	First Immunization Week-0	Second Immunization Week-3	Third Immunization Week-6
1	PBS	PBS	PBS
2	CpG ODN	CpG ODN	CpG ODN
3	pcDNA	pcDNA	pcDNA
4	pcDNA-mGMCSF	pcDNA-mGMCSF	pcDNA-mGMCSF
5	pcDNA-Pxn1	pcDNA-Pxn1	rLdPxn1
6	pcDNA-mGMCSF-Pxn1	pcDNA-mGMCSF-Pxn1	rLdPxn1
7	pcDNA-SODB1	pcDNA-SODB1	rLdFeSODB1
8	pcDNA-mGMCSF-SODB1	pcDNA-mGMCSF-SODB1	rLdFeSODB1
9	pcDNA-VAC3	pcDNA-VAC3	rLdPxn1 and rLdFeSODB1
10	pcDNA-mGMCSF-VAC3	pcDNA-mGMCSF-VAC3	rLdPxn1 and rLdFeSODB1

2.6.9.2 Protein/Protein Immunization

Female BALB/c mice were injected subcutaneously three times in three weeks interval with recombinant proteins in combination of CpG ODN adjuvant or controls. The strategy used for protein/protein immunization study is summarized on **Table-2.2**. Three and five mice per group were used to study the immunogenicity and protective efficacy of the recombinant proteins, respectively.

After two weeks of acclimatization, mice were randomly assigned to each group and injected with recombinant protein vaccine candidates or controls three times at week-0, week-3, and week-6. The injections were administered subcutaneously in the right hind footpad. Unlike DNA/protein immunization strategy where the fusion LdFeSODB1-LdPxn1 was used for the priming and the first boost, all three immunizations of the Protein/Protein study were given by combining rLdFeSODB1 and rLdPxn1 proteins (cocktail). The protein/protein immunization strategy is diagrammatically described in **Appendix-B2**.

Table 2-2: Protein/Protein Immunization Strategy

Group	First Immunization Week-0	Second Immunization Week-3	Third Immunization Week-6
1	rLdPxn1-GST	rLdPxn1-GST	rLdPxn1-GST
2	rLdFeSODB1	rLdFeSODB1	rLdFeSODB1
3	rLdPxn1-GST and rLdFeSODB1	rLdPxn1-GST and rLdFeSODB1	rLdPxn1-GST and rLdFeSODB1
4	rGST	rGST	rGST
5	CpG ODN	CpG ODN	CpG ODN

2.6.10 Challenge Infection with Leishmania major

Leishmania major strain V1 (MHOM/IL/80/Friedlin) was kept virulent by passing on BALB/c mice. For this study, stationary phase promastigotes were injected into BALB/c mice and re-isolated after four weeks post-infection. M199 medium containing 20% FBS was inoculated with tissue sample from infected footpad and incubated at 26°C for six days. Then, the parasites were subcultured into fresh medium and incubated for additional six days. Then, the stationary phase promastigotes were stored in -80°C freezer in 1ml aliquot. For infection of immunized mice for protection study, promastigotes of *L major* which had been stored in -80°C freezer were thawed and inoculated into fresh M199 medium with 20% FBS and incubated at 26°C for six days and subcultured once more before they were seeded into fresh medium to prepare them for infection. One week before infection, 1 x 10^{6} promastigotes per millilitre were seeded in 10ml M199 20% FBS and incubated for six days at 26°C. Then, the parasites were transferred into 15ml conical tube (Sarstedt, USA), centrifuged at 2000rpm using a refrigerated centrifuge (Sorvall® RT6000, Mandel) for 10min and supernatant discarded. The parasites in the pellet were washed once with endotoxin-free PBS and spun at the same speed for 10min. Promastigotes were then mixed with 0.4% trypan blue stain (Gibco) and counted using phase-contrast inverted microscope (Nikon, Japan). The parasites were then diluted at a concentration used for infection. In DNA/protein immunized groups, 3 x 10^{6} stationary phase live promastigotes in 40µl endotoxin-free PBS were injected subcutaneously into the footpad of the hind left foot of each mouse. The thickness and width of the left and right footpads were measured weekly until euthanasia using an electronic digital caliper (VWR, USA). Mice that showed a net footpad swelling of more than 3mm thick or those that developed necrotic lesions were euthanized even before the end date of week-17. In

protein/protein immunization study, 5 x 10^6 stationary phase live promastigotes in 40µl endotoxin-free PBS were injected to each mouse.

2.6.11 Mice Sample Collection

2.6.11.1 Blood Collection

Before euthanasia, blood samples were collected at different time points by retro-orbital sinus bleeding. At the time of euthanasia, blood samples were collected by heart bleeding. At all times, blood collection was done under anesthesia (**Appendix-B1 and B2**).

2.6.11.2 Serum Isolation

Serum was collected from blood samples and stored at -20°C until use. After collecting, the blood was kept at room temperature for 1hr. Then, it was spun at 5000rpm using a table-top microcentrifuge (IEC Micromax, USA). The serum was transferred into a new microtube and stored at -20°C freezer until use.

2.6.11.3 Spleen and Footpad Isolation

Mice were euthanized by inhalation of the anesthesia, isoflurane, followed by cervical dislocation. Then, the mice were immersed in 70% ethanol disinfectant for 1min followed by washing in sterile distilled water before subjected to dissection. By dissecting the abdomen, intact spleen was removed and kept in 1ml of cRPMI on ice until further processing. In addition, swollen footpad tissue was collected by de-skinning the foot and cutting the toes followed by cutting the footpad on the ankle. The footpad tissue was then stored in 1ml M199 medium containing 20% FBS and transported to the lab for processing.

2.6.12 Spleen Cell Culture and Stimulation

Mouse spleen isolated aseptically was kept in 1ml cRPMI in a microtube and transported to the lab within 30min. The medium was then discarded and fresh 1ml cRPMI medium was added.

Intact spleen was transferred into a 70µm Nylon BD Falcon™ cell strainer (BD, USA) that is placed on 60 x 15mm petridish (Sarstedt, USA). Then, cells were isolated by smashing the spleen using a 3ml syringe plunger on a cell strainer and flushing the cells with 4ml cRPMI. The cell suspension was transferred into a 15ml conical tube. The tube was filled up to 12ml with cRPMI and spun at 1500rpm for 10min using Sorvall® RT6000 refrigerated centrifuge at 4°C (Mandel, Canada) with break off. The supernatant was discarded, 1ml RBC lysis buffer (Sigma) added and incubated at room temperature for 3min with periodical agitation. Nine millilitre cRMPI was added and spun for another 10min. Then, the supernatant was discarded and the cells washed once more with 12ml cRPMI. Finally, the cells were resuspended in 10ml cRPMI medium and counted using trypan blue exclusion method on Double Neubauer Counting Chamber (VWR, USA).

Spleen cells were cultured in cRPMI medium and stimulated with concanavalin A (ConA) (Sigma), *L major* SLA, and the respective recombinant protein. 2 x 10^5 cells per well in 100ul cRPMI medium were seeded in triplicate in a 96-well tissue culture plate (Sarstedt, USA). The cells were stimulated with ConA (5µg/ml), *L major* SLA (40µg/ml) or recombinant protein (10µg/ml). Negative control cells received medium alone. Finally, the cells were incubated for 72hr at 37°C and 5% carbondioxide (CO_2). In order to prevent evaporation, each well on the edge of the plate was filled with 150µl cRPMI. After 72hr, cell culture supernatant was taken and transferred into another plate with appropriate labelling. The plate containing the supernatant was then sealed and stored in -80°C freezer until cytokine ELISA was done.

2.6.13 Footpad Tissue Homogenization and Parasite Culture

Infected footpad collected following the procedure described above was crushed using grinding chamber and pestle (VWR, USA) with 3ml M199 medium containing 20% FBS. The tissue was

allowed to settle and the parasite suspension taken for limiting dilution assay. The assay was done in a 96-well plate containing biphasic medium prepared using NNN agar slant with 10% rabbit blood overlaid with M199 medium in each well. One hundred eighty micro litres of undiluted tissue suspension was applied into duplicate wells and then transferred 30 times in 10X serial dilution. The wells on the edge of the plate were filled with sterile dH_2O to prevent evaporation of the medium in the samples. The plates were incubated at 26^oC incubator for 10days and microscopically examined for the presence of motile promastigote using inverted microscope. Finally, the parasite load was calculated based on the highest dilution that gave positive result.

2.6.14 Measurement of Antibody Responses

Antibody response to vaccine antigens was evaluated by measuring the magnitude of antigen-specific mouse total IgG, IgG1, and IgG2a antibody from sera isolated from mice immunized with the vaccine antigens and controls using indirect enzyme-linked immunosorbent assay (ELISA). Moreover, the titer of IgG1 and IgG2a was determined by performing eight twofold serial dilution starting at 1:100.

Ninety six-well flat-bottom Nunc MaxiSorp ELISA plates (eBiosciences, USA) were coated with recombinant protein in 50µl/well antibody ELISA coating buffer (carbonate buffer, pH 9.6) and incubated overnight (O/N) at 4^oC. The optimum amount of protein antigen used for coating was determined by performing optimization experiment. In this experiment, we used 250ng/well Pxn1, 500ng/well SODB1, or 250ng/well Pxn1 combined with 500ng/well SODB1 to measure the level of antibody response from serum samples collected from mice immunized with LdPxn1, LdFeSODB1, or the fusion/cocktail LdPxn1-LdFeSODB1, respectively. The cocktail antigen was used to measure antibody response in serum samples collected from control mice.

Next morning, the coating solution was discarded by flicking-out and blocking was done with 200µl/well 5% milk in 1X PBS and incubated at RT for 1h. Then, the blocking solution was discarded and the plates washed 3 times for 10 min each with 1X PBS-Tween 20 (0.1% Tween 20 in PBS, PBST). Meanwhile, serum samples were diluted at 1:100 in blocking buffer and 50µl/well was added to each well. For titration experiment, each serum sample was subjected to eight times two-fold serial dilution. The reaction was then incubated at RT for 1 h on a rotating Compact Rocker (Mandel, Canada). The plates were then washed 3 times as above and 100µl/well of goat biotinylated anti-mouse IgG, IgG1 or IgG2a (SouthernBiotech, USA) secondary antibodies were added into the respective plates at 1:1000 dilution in blocking buffer and incubated at RT for 1 h on a shaker. Again, the plates were washed and 1:1000 diluted Streptavidin-HRP was added at 100µl/well and incubated at room temperature for 1 h. The plates were then washed as above and incubated with 100µl/well TMB substrate (3,3',5,5'-tetramethylbenzidine solution) for 30min at RT in the DARK. Then, reaction was stopped by adding 50µl/well of 1N H_2SO_4. Finally, the absorbance was read at 450nm and also at 570nm as a reference wavelength using a microplate reader spectrophotometer (Molecular Devices, USA). Duplicate wells containing everything except serum samples were used as blank. Another duplicate wells with pooled sera from previously known positive samples were run ever time to check the batch-to-batch and day-to-day variations. Any reading where the positive samples showed a variation of greater than 10% was discarded.

2.6.15 Measurement of Cytokine Responses

The level of interferon-gamma (IFN-γ) and IL-10 was measured from supernatant of antigen/mitogen stimulated and unstimulated spleen cells using BD OptEIA™ Set Mouse IFN-γ and BD OptEIA™ Set Mouse IL-10 (BD Biosciences, USA) kits, respectively. Capture

antibodies (anti-mouse IFN-γ and anti-mouse IL-10), biotinlylated detection antibodies (biotinylated anti-mouse IFN-γ and anti-mouse IL-10), horseradish peroxidase (HRP) conjugated streptavidin and recombinant mouse IFN-γ and IL-10 standards were all included in the kit. Ten percent FBS in PBS was prepared as assay diluent. The stock protein standards (2000pg/ml) were 2-fold serially diluted until 31.3pg/ml in assay diluent. The last tube contained only the assay diluent (i.e. zero standard (0pg/ml)). Cytokine ELISA was performed on the culture supernatant samples according to the manufacturer's instruction.

Ninety six-well NUNC Maxisorp ELISA plates (Thermo scientific, USA) were coated with 100µl per well of capture antibody diluted 1:250 in coating buffer (0.2M sodium phosphate, pH 6.5), sealed and incubated at $4^{o}C$ overnight. Next morning, the wells were aspirated, washed five times with 300µl per well wash buffer (1X PBS with 0.05% Tween 20), blocked with 200µl per well assay diluents and incubated at room temperature (RT) for 1hr. Then, the plates were aspirated and washed five times. One hundred micro litre per well of each sample, standard, and control was added, plate sealed and incubated at RT for 2hr. After aspiration and washing five times, 100µl of working detector was added to each well and incubated at RT for 1hr. Working detector comprised of detection antibody and streptavidin-HRP conjugate. After the plates were aspirated and washed for 10 times, 100µl per well of substrate solution was added and incubated at RT in the dark for 30min. TMB (SouthernBiotech, USA) was used as substrate solution. Fifty micro litre of 2N H_2SO_4 was added to each well to stop the reaction. Finally, the absorbance was read on a microplate reader spectrophotometer (Molecular Devices, USA) at 450nm and also at absorbance at 570nm as reference wavelength. The concentration of the cytokines in the sample was calculated against the standard concentration using SoftMax Pro 5 software (Molecular Devices, USA).

2.6.16 Intracellular Cytokine Staining and Flow Cytometry

A seven color flow cytometry was performed on stimulated cells using a three laser BD FACSAria II machine. The following antibody-fluorochrome combinations was used: V450 rat anti-mouse CD3, V500 rat anti-mouse CD4, and APC-Cy7 rat anti-mouse CD8α for surface staining as well as PE-Cy7 rat anti-mouse IFN-γ, FITC rat anti-mouse TNF-α, PE rat anti-mouse IL-2 and APC rat anti-mouse IL-10 for intracellular staining.

All reagents for intracellular staining and flow cytometry were purchased from BD Biosciences (CA, USA) unless otherwise specified. The procedure involved interlinked steps; stimulation of spleen cells with antigen/mitogen, cell surface staining, fixation and permeabilization, intracellular cytokine staining as well as data acquisition and data analysis. 1 x 10^6 cells in 100µl cRPMI per well were seeded in a flat-bottom 96-well plate (Sarstedt, USA) and stimulated with phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml) (Sigma), 10µg/ml recombinant protein antigen, 50µg/ml *L. major* SLA or medium alone (unstimulated). Then, 2µg/ml of antibody to CD28 was added. Cells were incubated at 37°C and 5% CO_2 for 2hr. One microlitre of brefeldin A (GolgiPlug) was added into each well and incubated at 37°C for additional 12hr. The cells were transferred to a 96-well Vee-bottom plate (Sarstedt). In order to transfer all the cells, the wells were washed with 150µl cRPMI. The cells were spun down at 2000rpm for 7min at 4°C using Allegra™ 6R Centrifuge (Beckman Coulter, USA). After discarding the supernatant, the cells were blocked with 0.75µg anti-CD16/32 (Mouse BD Fc Block) in 50µl of staining buffer and incubated at 4°C for 20min. The plates were then centrifuged at 2000rpm for 5min.

After washing with 250ul staining buffer, the cells were stained with fluorochrome-conjugated antibody specific for surface antigens. Fifty microlitre of 1:400 diluted surface staining

fluorochrome conjugated antibodies was used. This dilution was determined by performing pilot experiments prior to the actual experiment. 1 x 10^6 cells were stained with appropriate amount of V450 rat anti-mouse CD3, V500 rat anti-mouse CD4, and APC-Cy7 rat anti-mouse CD8α in 50µl staining buffer and were incubated at 4^oC for 40min in the dark (wrapped in aluminum foil). Isotype control staining was done on antigen stimulated cells (in separate wells) with equal concentration of isotype-matched control of irrelevant specificity. For unstained control, antigen stimulated cells were treated with staining buffer devoid of any antibody. The cells were then washed 2 times with staining buffer (250µl/wash per well) and centrifuged at 2000rpm for 5min.

Perm/wash buffer rather than staining buffer was used to dilute the blocking and intracellular cytokine staining antibodies. Stock Perm/Wash buffer (10X) was diluted in 1:10 in distilled water. Fixation and permeabilization was done by thoroughly resuspending the cells in 100µl of Cytofix/Cytoperm solution and incubating at 4^oC for 20min. The cells were then washed 2 times with 250µl 1X BD Perm/Wash buffer.

Blocking was done by treating the cells with anti-CD16/32 as above except that Perm/Wash buffer was used instead of staining buffer. After blocking for 20min, the cells were washed once with Perm/Wash buffer. Then, cells were thoroughly resuspended in 50ul BD Perm/Wash buffer containing a predetermined concentration of a fluorochrome-conjugated anti-cytokine antibody or appropriate negative control. PE-Cy7 rat anti-mouse IFN-γ, FITC rat anti-mouse TNF-α, and PE rat anti-mouse IL-2 were used in 1:400dilution. Whereas, APC rat Anti-mouse IL-10 were used in 1:200 dilution. Isotype controls were treated with respective fluorochrome conjugated antibodies without specificity. The unstained control on the other hand was kept in buffer only solution. The cells were then incubated at 4^oC for 40min in the dark. After washing 2 times with 1XBD Perm/Wash buffer, the cells were resuspended in 250ul PBS, transferred into 12x75mm

tubes (BD Biosciences, USA) and immediately transported on ice to the flow cytometry facility (Microbial Communities Laboratory, University of Calgary) for analysis.

In order to perform fluorescence compensation, equivalent mixture of BD™ CompBeads Anti-Rat Ig, κ and BD CompBeads Negative Control (FBS) were used as positive and negative compensation beads, respectively, following the manufacturer's instruction. Seven tubes were used for seven fluorochrome-conjugated antibodies that were used to stain the cells. A single drop of each of the positive and negative compensation beads were mixed in 100µl staining buffer in each of the seven 12x75mm tubes. Then, 20µl of each prediluted antibody was added to the respective tubes and incubated at room temperature for 20min in the dark. The samples were kept on ice and transported to the flow cytometry facility. The flow cytometry results were analyzed using FlowJo software (Tree Star, Inc, USA).

2.7 Ethical Clearance

Ethical clearance was obtained from Life and Environmental Sciences Animal Care Committee (LESACC) and Conjoint Health Research Ethics Board, University of Calgary for studies involving mice and human samples, respectively. Ethical clearance for human work was also obtained from Science and Technology Agency, The Federal Democratic Republic of Ethiopia.

2.8 Statistical Analysis

The statistical differences between different groups of mice were analyzed using Kruskal-Wallis test. Whereas, the difference between means of any two groups were compared by Mann-Whitney U test. A p-value of < 0.05 was considered statistically significant. All statistical analysis was done using IBM SPSS Statistics 20 software.

Chapter Three: **CLONING AND EXPRESSION OF VACCINE CANDIDATES**

3.1 Experimental Rationale

To test the immunogenicity and protective efficacy of combination of LdPxn1 and LdFeSODB1, we have used a heterologous prime-boost immunization strategy using DNA priming using fusion DNA constructs followed by booster immunization using a mixture of recombinant LdPxn1 and LdFeSODB1. The first step in the preparation of DNA vaccine candidates is to clone the genes and confirm their expression in a mammalian system by performing an *in vitro* transfection experiments. As explained in Chapter-2, the fusion *Leishmania donovani* iron superoxide dismutase B1 (LdFeSODB1) and *Leishmania donovani* peroxidoxin-1 (LdPxn1) DNA was synthesised and then cloned into a modified pcDNA vector under the control of cytomegalovirus (CMV) promoter. The CMV promoter allows expression of the vaccine candidate (s) in mammalian cells including mouse muscle cells. Upon intramuscular immunization with DNA vaccine candidates, muscle cells are transfected *in vivo* and express the cognate proteins. In order to test the expression *in vitro*, Chinese Hamster Ovary (CHO) cells were transfected with the fusion DNA constructs, individual DNA constructs, and the vector alone and the expression of the cognate proteins was assessed by Western blotting. Transfection with pEGFPN3 was also performed to check the efficiency of Lipofectamine 2000-based transfection procedure.

In order to potentiate the immunogenicity of DNA vaccine antigens, we included the Granulocyte-Macrophage Colony-Stimulating Factor (GMCSF) gene fused to the vaccine candidate gene (s). We proposed that, in addition to being a cytokine adjuvant, GMCSF can also mediate the secretion of the fused vaccine antigen proteins. GMCSF protein has a 17 amino acid leader sequence which facilitates the secretion of the protein from expressing cell (Kaushansky

et al., 1992). Intramuscular (IM) injection of plasmid DNA into mouse muscle primarily transfects myocytes. As these cells lack the antigen presentation machinery, the antigen has to reach to professional antigen presenting cells for an effective immune response to be established (Liu, 2011). It has been shown in other studies that GMCSF is effectively secreted out from a mammalian cell. Farrell and colleagues (2000) used GMCSF and Juvenile Hormone Esterase (JHE) fusion to secrete cytoplasmic and nuclear proteins in mammalian and insect cell lines, respectively. They found that both GMCSF and JHE mediate the secretion of the proteins which otherwise remain intracellular. As compared to JHE, GMCSF was five times more efficient to secrete a cytoplasmic protein. Another study demonstrated that the secretory role of GMCSF, not its adjuvancy, is essential for antibody production against cytosolic proteins by fusing the cognate DNA with GMCSF (Farrell et al., 2013). *Leishmania chagasi* Cysteine Protease (CP) DNA was cloned in pcDNA-based vector separately or in fusion with either murine or human GMCSF. The constructs were then injected into mice intramuscularly. The result showed that only GMCSF fused CP induced the production of antigen-specific antibody in mice. Interestingly, both murine and human GMCSF fusions induced the antibody production showing that the secretory role, not the adjuvancy, was responsible for induction of antibody production (Farrell et al., 2013).

To test the role of GMCSF in the secretion of our vaccine candidate antigens, we transfected CHO cells with plasmid DNA containing vaccine candidate antigen genes with or without GMCSF fusion and the presence of the respective proteins was determined by performing Western blotting on both cell culture supernatants and cell lysates.

For immunization with *Leishmania* protein antigens, we needed to express the vaccine antigens in *Escherichia coli* and purify recombinant proteins *in vitro*. The His-tagged LdFeSODB1 was

cloned in pET17b and expressed in *E coli* Tuner™ (DE3) *pLysS* cells. The recombinant protein was then purified using Ni-NTA column chromatography. On the other hand, LdPxn1 was cloned as a GST fusion and purified with a glutathione sepharose gel. Then, GST was cleaved from the LdPxn1 protein using thrombin and the pure rLdPxn1 protein was separated from GST.

3.2 Experimental Results

3.2.1 Cloning and Expression of Vaccine Candidate Genes in a Mammalian Cell Line

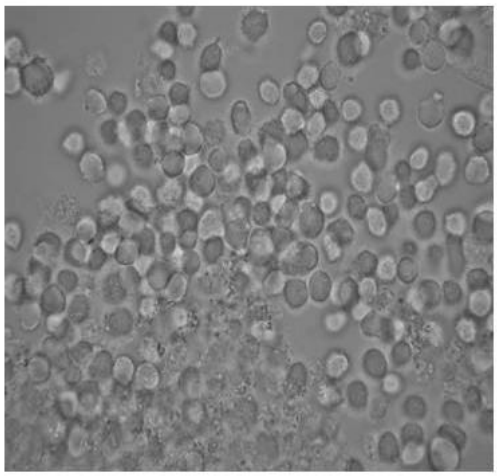
Restriction digestion analysis and nucleotide sequencing confirmed that all the vaccine constructs, fusion and individual genes, were successfully cloned in the modified pcDNA plasmid vector with and without murine-GMCSF (mGMCSF).

In order to check expression of the genes in mammalian system, cationic lipid-mediated transfection of CHO cells was performed using Lipofectamine 2000 reagent. Chinese hamster ovary cells were transfected with vaccine candidate genes and controls. The efficiency of the transfection procedure was checked by co-transfecting CHO cell with a mixture of each vaccine construct and pEGFPN3. After 72hr, a wet mount was prepared using the transfected cells and was examined under fluorescent microscope. **Figure-3.1A** shows co-transfected cells under bright field filter**.** Under green fluorescent filter, some of the cells appeared green indicating that the cells were transfected with pEGFPN3 plasmid DNA and the GFP protein expressed (**Figure-3.1B**). Those cells transfected with plasmid DNA constructs without pEGFPN3 did not appear green (data not shown). This confirmed that the Lipofectamine 2000 based protocol was effective to transfect CHO cells.

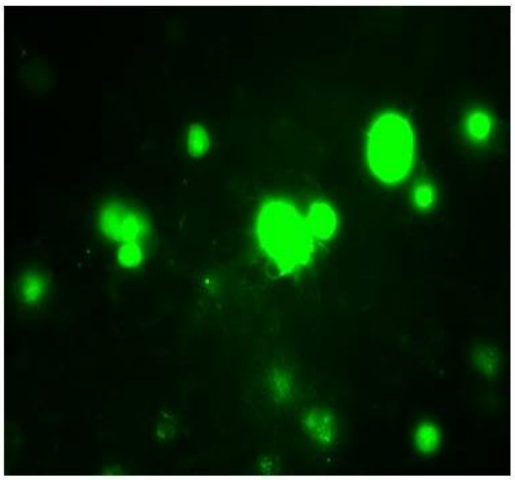
Figure 3.1: Fluorescent microscopy of CHO cells co-transfected with antigen construct and pEGFPN3.

A) Bright Field **B)** Green Fluorescent Field

A



B



Western blotting was done on the culture supernatant (SUP) and cell lysate (LYS) samples collected from CHO cells that were transfected with LdPxn1 in pcDNA and pcDNA-mGMCSF plasmid vectors. Two different primary antibodies were used. Pooled sera collected from mice that had been immunized with two doses of pcDNA-Pxn1 plasmid and boosted with one dose of recombinant LdPxn1 protein was used to check the expression and secretion of LdPxn1 in transfected CHO cells. In addition, rabbit anti-mGMCSF antibody was used to check the expression of pcDNA-mGMCSF-Pxn1.

Figure-3.2A shows the expression of pcDNA-Pxn1 in CHO cells. The use of mouse-anti-LdPxn1 serum as primary antibody produced signals on both culture supernatant (SUP) and cell lysate (LYS) samples taken from pcDNA-Pxn1 transfected CHO cells. As shown in lane-3 and -4, pcDNA-Pxn1 transfection produced protein of size about 22kDa. More intense band appeared on the lysate sample (lane-4) than that of culture supernatant (lane-3). The recombinant LdPxn1 protein gave a positive signal of slightly lower size (lane-8). No band was seen on samples taken from pcDNA-mGMCSF-Pxn1 transfected cells (**Figure-3.2A**, lane-5 and lane-6).

As shown in **Figure-3.2B**, pcDNA-mGMCSF-Pxn1 was expressed in CHO cells (lane-5 and-6). Sample taken from culture supernatant (SUP) gave a protein band of about 52kDa (lane-5), whereas, the lysate sample produced a band slightly lower in size (lane-6). pcDNA-mGMCSF control was also expressed and almost all of the protein was secreted into the culture supernatant (lane-1). As expected, no band was seen on samples from pcDNA-Pxn1 transfected cells using anti-mGMCSF antibody (lane-3 and -4) (**Figure-3.2B**).

Figure 3.2: Western blotting of samples from CHO cells transfected with LdPxn1 gene cloned in pcDNA and pcDNA-mGMCSF plasmid vector.

Cell culture supernatant (SUP) and cell lysate (LYS) proteins of transfected cells were run on a 12% denaturing polyacrylamide gel and Western blotting was done using: **A)** Pooled sera from mice immunized with pcDNA-Pxn1 and ECL-anti-mouse IgG-peroxidase primary and secondary antibodies, respectively, and **B)** Rabbit-anti-mGMCSF and ECL-anti-rabbit IgG-HRP (donkey) primary and secondary antibodies, respectively and Lanes: 1) pcDNA-mGMCSF-SUP, 2) pcDNA-mGMCSF-LYS, 3) pcDNA-Pxn1-SUP, 4) pcDNA-Pxn1-LYS, 5) pcDNA-mGMCSF-Pxn1-SUP, 6) pcDNA-mGMCSF-Pxn1-LYS, 7) pEGFPN3-SUP, 8) Recombinant LdPxn1 protein. The results in (A) and (B) confirm that LdPxn1 alone or in fusion with mGMCSF was expressed in CHO cells.

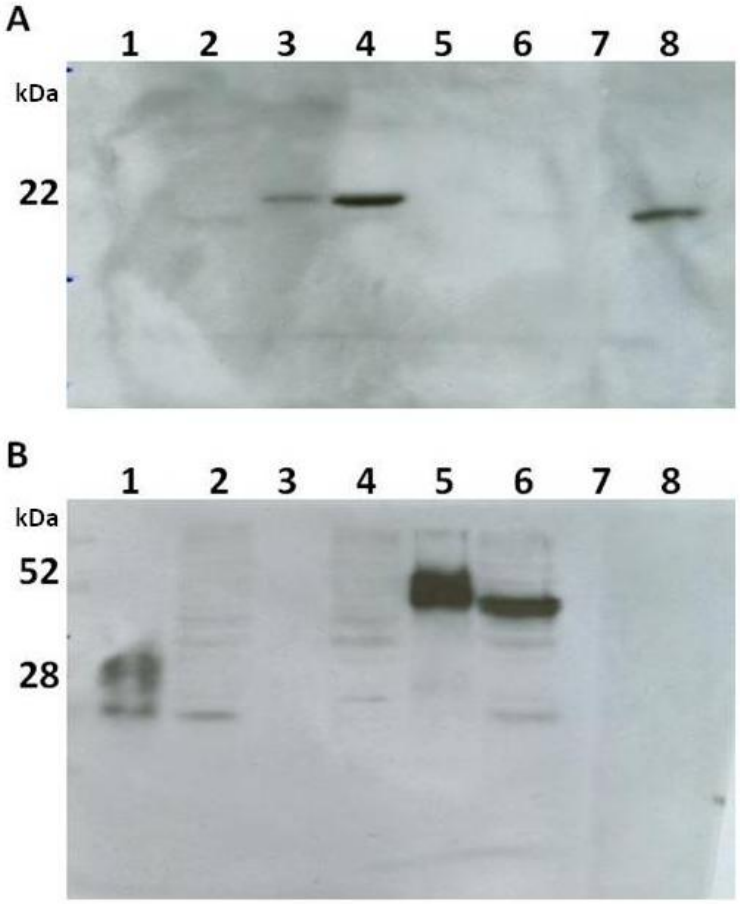

A
1 2 3 4 5 6 7 8
kDa
22
B
1 2 3 4 5 6 7 8
kDa
52
28

Figure-3.3 shows expression of LdFeSODB1 gene cloned in pcDNA-mGMCSF-SODB1. Murine GMCSF-LdFeSODB1 fusion was expressed in CHO cells and most of the protein was secreted out into the culture medium. Lane-5 represents the fusion mGMCSF-LdFeSODB1 protein of about 52kDa size. The lysate proteins shows only very faint band (lane-6). On the other hand, pcDNA-mGMCSF transfected cells produced two bands with size of about 25kDa and 30kDa in the culture supernatant. No band (signal) was seen on Lanes-3, -4, -7, and -8. Whereas, lane-9 gave a band of about 14kDa size.

Like individual constructs, the fusion LdFeSODB1-LdPxn1 (VAC3) cloned in pcDNA-mGMCSF was expressed in CHO cells (**Figure-3.4A**). Western blotting using rabbit anti-mGMCSF primary antibody demonstrated that culture supernatant samples from cells transfected with pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF contained the respective recombinant proteins. Supernatant from pcDNA-mGMCSF-VAC3 transfected cells gave a band of about 70kDa (lane-5). The control transfection with pcDNA-mGMCSF produced proteins of two bands with size of about 25kDa and 30kDa (lane-1). On the other hand, samples from pcDNA-VAC3 transfected cells did not give any specific band. Using anti-GFP antibody, Western blotting on cell supernatants and lysates of CHO cells co-transfected with pEGFPN3 gave bands of about 26kDa (**Figure-3.4B**).

Figure 3.3: Western blotting of samples from CHO cells transfected with LdFeSODB1 gene cloned in pcDNA and pcDNA-mGMCSF plasmid vectors.

Cell culture supernatant (SUP) and cell lysate (LYS) proteins of transfected cells were run on a 12% denaturing polyacrylamide gel and Western blotting was done using rabbit-anti-mGMCSF and ECL-anti-rabbit IgG-HRP (donkey) primary and secondary antibodies, respectively. Lanes: 1) pcDNA-mGMCSF-SUP, 2) pcDNA-mGMCSF-LYS, 3) pcDNA-SODB1-SUP, 4) pcDNA-SODB1-LYS, 5) pcDNA-mGMCSF-SODB1-SUP, 6) pcDNA-mGMCSF-SODB1-LYS, 7) pcDNA-SUP, 8) pcDNA-LYS, and 9) Recombinant mGMCSF protein. The result confirms that the fusion mGMCSF-LdFeSODB1 was expressed in CHO cells.

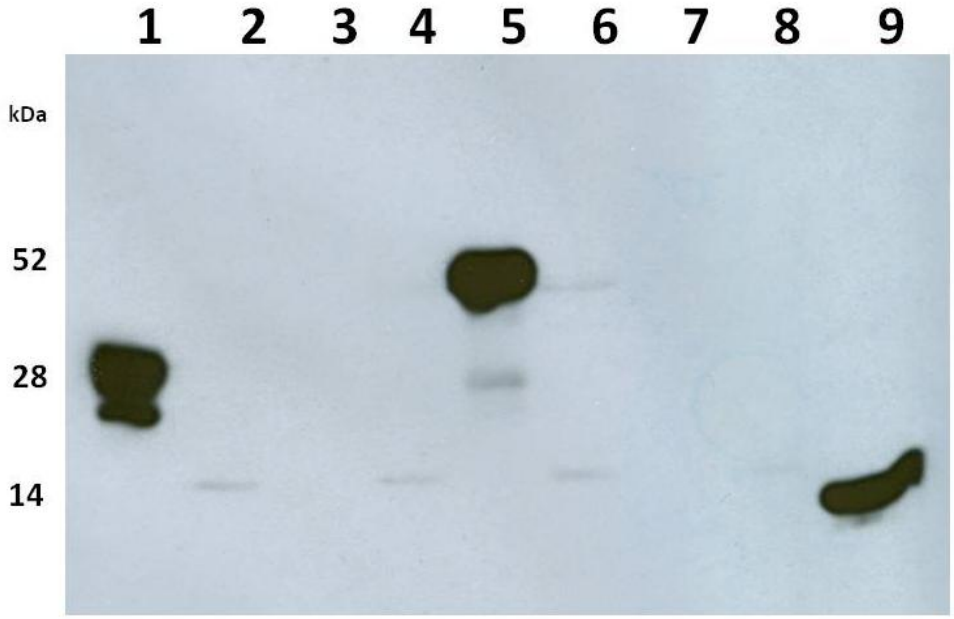
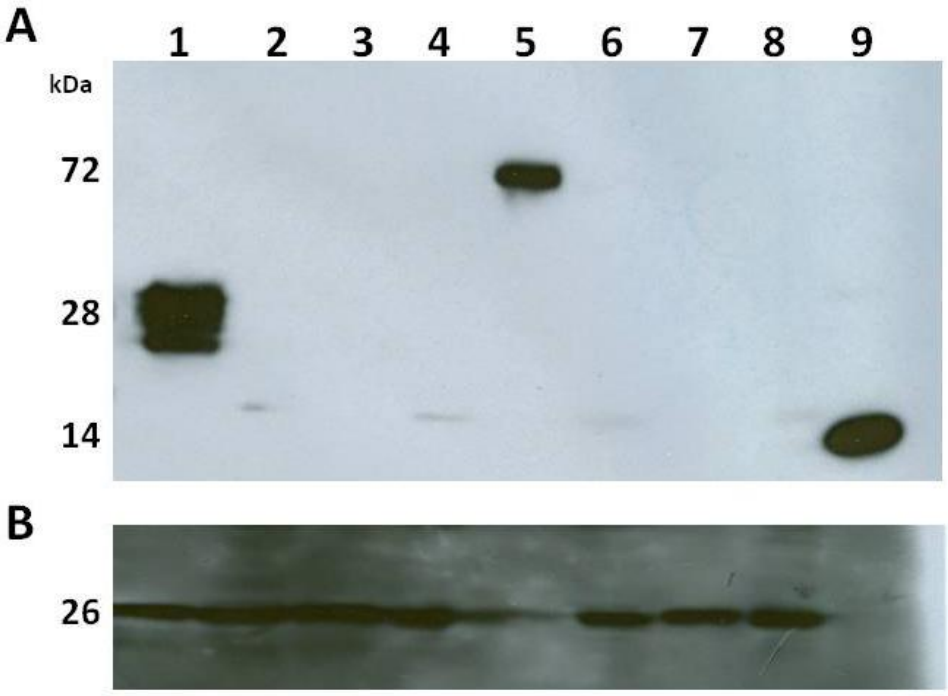

1
2
3
4
5
6
7
8
9
kDa
52
28
14

Figure 3.4: Western blotting of samples from CHO cells transfected with fusion LdFeSODB1-LdPxn1 (VAC3) gene cloned in pcDNA and pcDNA-mGMCSF plasmid vectors.

Cell culture supernatant (SUP) and cell lysate (LYS) proteins from cells co-transfected with VAC3 constructs and pEGFPN3 were run on a 12% denaturing polyacrylamide gel and Western blotting was done using: **A)** Rabbit-anti-mGMCSF and ECL-anti-rabbit IgG-HRP (donkey) primary and secondary antibodies, respectively, and **B)** Rabbit anti-GFP and ECL-anti-rabbit IgG-HRP (donkey) as primary and secondary antibodies, respectively. Lanes: 1) pcDNA-mGMCSF-SUP, 2) pcDNA-mGMCSF-LYS, 3) pcDNA-VAC3-SUP, 4) pcDNA-VAC3-LYS, 5) pcDNA-mGMCSF-VAC3-SUP, 6) pcDNA-mGMCSF-VAC3-LYS, 7) pcDNA-SUP, 8) pcDNA-LYS, and 9) Recombinant mGMCSF protein. This experiment confirms that the fusion antigen, VAC3, was expressed in CHO cells. Moreover, expression of rGFP in all co-transfected cells suggests that Lipofectamine transfection protocol effectively mediated transfection of CHO cells


A
1
2
3
4
5
6
7
8
9
kDa
72
28
14
B
26

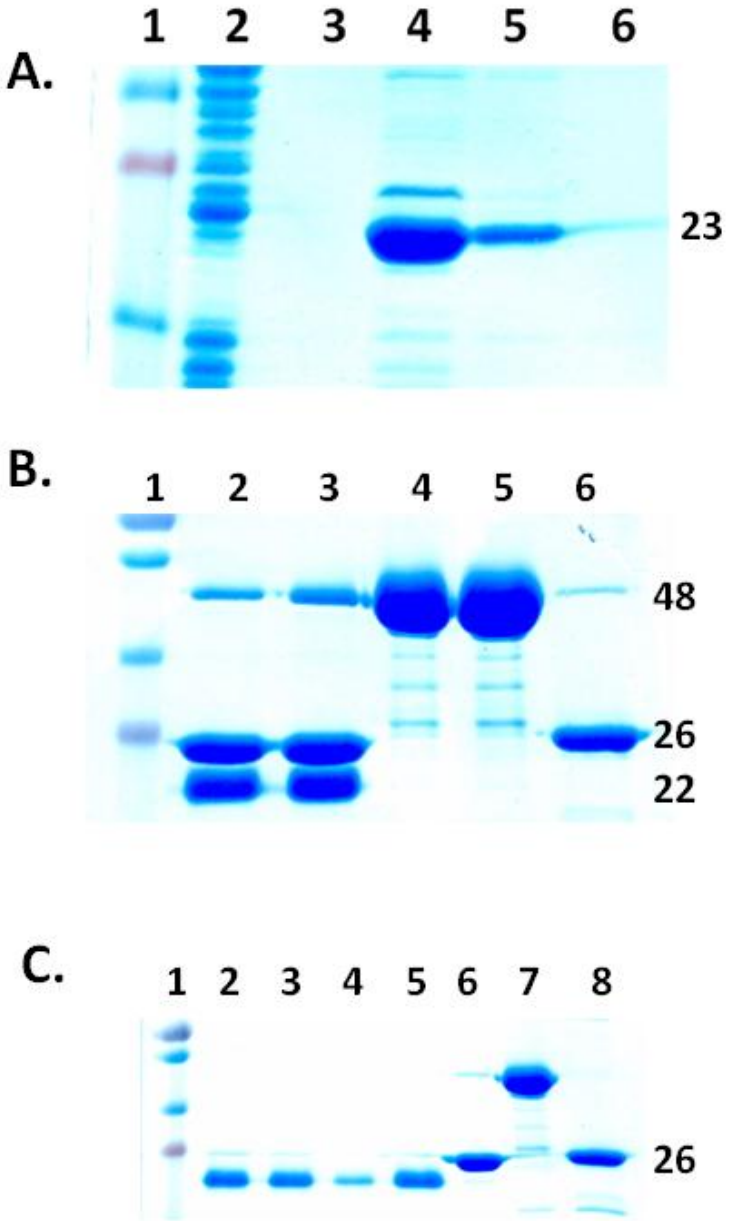
3.2.2 Purification of Recombinant Proteins

For immunization involving proteins, we needed to express recombinant LdFeSODB1 and LdPxn1 proteins in *Escherichia coli*. LdFeSODB1 and LdPxn1 proteins were expressed as His-tagged and GST-fusion forms, respectively. As shown in **Figure-3.5A**, recombinant LdFeSODB1 protein was purified by metal ion affinity chromatography using Ni-NTA agarose column. The recombinant protein was subjected to Ni-NTA agarose binding and followed by collection of the flow-through (lane-2). After washing with Tris-buffer, the pure recombinant protein was eluted using 0.4M imidazole. Most of the rLdSODB1 was collected from second and third elution fractions (lanes-4 and 5).

Recombinant LdPxn1 was expressed as a GST fusion protein and was purified using glutathione sepharose beads. Cleavage of GST from the fusion recombinant protein using thrombin resulted in two bands of about 22 and 26KDa (**Figure-3.5B**, lane-2 and lane-3). Lane-4 and -5 show un-cleaved rLdPxn1-GST protein. The rGST control produced a band of about 26KDa (lane-6). As shown in **Figure-3.5C**, the cleaved GST was separated from LdPxn1 by binding with fresh glutathione sepharose. By allowing the GST to bind to glutathione, the purified rPxn1 was collected from the flow-through (**Figure-3.5C**). Lane-2 through lane-5 are the flow-through fractions collected after binding the GST with glutathione. Elution with reduced glutathione resulted in a 26KDa protein (lane-6). Proteins on lane-7 and lane-8 represent intact rLdPxn1-GST and rGST controls, respectively.

Figure 3.5: Purification of recombinant *Leishmania donovani* recombinant proteins and cleavage of GST from fusion rPxn1-GST.

Coomassie blue stained SDS-PAGE gels showing: **A)** His-tagged recombinant LdFeSODB1 purification. Lanes: 1) PageRuler™ Plus Prestained Protein Ladder (Fermetas), 2) Flow-through, 3) Elution fraction-1, 4) Elution fraction-2, 5) Elution fraction-3, and 6) Elution fraction-4, **B)** GST cleavage of recombinant LdPxn1-GST. Lanes: 1) PageRuler™ Plus Prestained Protein Ladder (Fermetas), 2 and 3) Cleaved recombinant LdPxn1, 4 and 5) Uncleaved fusion rLdPxn1-GST, and 6) rGST control, **C)** Purification of cleaved rLdPxn1. Lanes: 1) PageRuler™ Plus Prestained Protein Ladder (Fermetas), 2 to 5) rLdPxn1, 6) Cleaved rGST, 7) rLdPxn1-GST control, and 8) rGST control.


A.
1 2 3 4 5 6
23
B.
1 2 3 4 5 6
48
26
22
C.
1 2 3 4 5 6 7 8
26

3.3 Experimental Discussion

As seen in the results above, expression of the vaccine candidate genes in mammalian system was confirmed by Western blotting on samples taken from transfected cells. The presence of green fluorescence in CHO cells that had been transfected with combination of the vaccine candidate and pEGFPN3 plasmid vectors shows that the Lipofectamine 2000 based transfection procedure worked (**Figure-3.1**). This was further confirmed by Western blotting using anti-GFP antibody. A 26kDa recombinant protein appeared in all supernatant and cell lysate samples of co-transfected cells (**Figure-3.4B**). Although GFP is a cytosolic protein which does not have secretory signal sequence, the protein appeared on both supernatant and cell lysate samples. Tanudija and colleagues (2002) clearly showed that improperly folded GFP protein is secreted by a non-classical pathway and does not need a signal sequence. They also found that improperly folded GFP protein does not fluoresce. The other possibility is that the cytosolic GFP could appear in the culture supernatant if the cells die and free their contents to the culture medium. In order to address this issue, the cell survival was measured by trypan-blue exclusion assay. In all experiments, the viability of transfected cells was more than 90%.

The expected size of the expression products of the vaccine candidates is indicated on the **Table 3.1** below. Western blotting experiment on cells that were transfected with plasmids containing mGMCSF showed recombinant proteins larger than expected. The expected size of expressed protein from pcDNA-mGMCSF, pcDNA-mGMCSF-Pxn1, pcDNA-mGMCSF-SODB1, and pcDNA-mGMCSF-VAC3 were 16.9, 36, 36.4, and 57kDa, respectively. However, as shown in **Figure-3.2B**, **Figure-3.3**, and **Figure-3.4A**, expression of these genes resulted in bands of about 25 to 30, 52, 52 and 72kDa in size, respectively. It appears that the difference between the

expected and actual sizes in each of these clones is similar clearly indicating that the size increase is due to mGMCSF but not related to the vaccine candidate antigens.

Expression of pcDNA-mGMCSF in CHO cells resulted in at least two bands with size ranging from 25 to 30kDa. The expected size of mGMCSF with the spacer sequence expressed in *E coli* was about 17kDa. In CHO expression experiments, we found at least two bands with bigger molecular size (approximately 25kDa and 30kDa). Similar results were seen in other studies involving expression of GMCSF in eukaryotic expression systems (James et al., 2000, Kaushansky et al., 1992, Tenbusch et al., 2008). James and colleagues (2000) showed that expression of human GMCSF in plant cells produced multiple bands in the supernatant. An intense double band of size 20 to 30KDa and faint bands of 30 to 40KDa were seen from cell culture supernatants. In another study, ovalbumin (OVA) gene was cloned in a pcDNA vector as a separate antigen or in fusion with GMCSF. Expression of pcDNA-OVA and pcDNA-GMCSF-OVA in 293T cells resulted in a 50kDa and 75kDa proteins, respectively. The expected size of GMCSF-OVA fusion was about 65kDa (Tenbusch et al., 2008). The increase in size of GMCSF expressed in eukaryotic cells from the one expressed in *E coli* is attributed to glycosylation. Granulocyte-macrophage colony-stimulating factor has two N-linked and several O-linked glycosylation sites. Depending on the level of glycosylation, GMCSF expressed in eukaryotic cells gives rise to multiple bands (Gora-Sochacka et al., 2010, Forno et al., 2004, Kaushansky et al., 1992). Expression of mGMCSF in plant cell culture produced a faint band of about 15kDa and two intense bands of size 17 and 22kDa. Upon treatment of the protein with endoglycosidase H (Endo H) and Peptide-*N*-Glycosidase F (PNGase F), all the bands were reduced into a 15kDa band indicating that the carbohydrate is cleaved and mGMCSF has N-linked glycosylation sites

(Gora-Sochacka et al., 2010). Studies on human GMCSF showed that glycosylation does not affect activity, level of secretion, and transit time of the protein (Kaushansky et al., 1992).

As shown in **Figure-3.2A**, expression of LdPxn1 in the absence of mGMCSF did not result in increase in the size. As expected, the expression of pcDNA-Pxn1 resulted in a protein of about 22kDa in size indirectly indicating that the discrepancy from the expected size in mGMCSF fusion constructs came from the size increase in mGMCSF. On the other hand, *E coli* expressed recombinant LdPxn1 (control, lane-8) produced a band slightly smaller than the one expressed in CHO cells. This is due to the presence of 17 amino acid spacer and another 11 amino acid equivalent of the multiple cloning site, together made up a total of 28 amino acid spacer in pcDNA-Pxn1.

Table 3-1: Expected Size of Recombinant Proteins Expressed in Chinese Hamster Ovary (CHO) Cells.

Vaccine Candidate Plasmid	Size of Vaccine Candidate (amino acid)	Size of Spacer (amino acid)	Size of Linke (amino acid)	Total Size (amino acid)	Total Expected Size (kDa)
pcDNA-mGMCSF	141	28	0	169	16.9
pcDNA-Pxn1	191	28	0	219	21.9
pcDNA-mGMCSF-Pxn1	332	28	0	360	36
pcDNA-SODB1	195	28	0	223	22.3
pcDNA-mGMCSF-SODB1	336	28	0	364	36.4
pcDNA-VAC3	386	28	15	429	42.9
pcDNA-mGMCSF-VAC3	527	28	15	570	57

It is not clear why the pooled sera from mice immunized with LdPxn1 did not produce any signal on samples from pcDNA-mGMCSF-Pxn1 transfected cells (**Figure 3.2**). This could be due to changes on rLdPxn1 that occurred as a result of fusion with mGMCSF. Fusing LdPxn1 with mGMCSF might have formed a recombinant protein that the specific epitopes of LdPxn1 are not exposed to the surface and that treatment with beta-mercaptoethanol could not fully denature the fusion protein and expose the reactive epitopes. Other studies showed a similar result. That is, antibodies raised against *E coli*-derived GMCSF did not recognize CHO cell-derived GMCSF. It was suggested that glycosylation of GMCSF expressed in mammalian cell masks the epitopes which are specific to antibodies to *E coli*-derived GMCSF (Wadhwa et al., 1996).

Granulocyte-macrophage colony-stimulating factor (GMCSF) protein possesses a 17 amino acid leader sequence that mediates secretion of the protein (Kaushansky et al., 1992). Fusion of different cytosolic protein coding genes with GMCSF gene and cloning in eukaryotic expression system produces a fusion protein that can be secreted via the classical pathway (Kaushansky et al., 1992). In our study, we have used mGMCSF fusion to secrete the vaccine antigens from transfected muscle cells in intramuscular immunization. In order to confirm whether the mGMCSF-fused antigens can be secreted from the transfected cells, we did Western blotting on cell culture supernatants (SUP) and cell lysates (LYS) of CHO cells transfected with mGMCSF-fused vaccine antigens. The result indicated that fusing the antigens with mGMCSF secrets the fusion protein. **Figure-3.2B** showed that mGMCSF-fusion LdPxn1 is predominantly secreted into the culture medium although some of the expressed proteins remained in the cell lysate. The protein in the lysate showed a slightly lower molecular size than the one in the supernatant. Drew and colleagues (2000) showed similar phenomenon with secreted form of *Taenia ovis* 45W antigen protein. The difference could be due to difference in the level of glycosylation between

the secreted and non-secreted forms of the protein. It was suggested that more glycosylation occurs as secreted proteins travel through the secretion pathway of the endoplasmic reticulum and Golgi apparatus (Drew et al., 2000). On the other hand, almost the whole fusion protein is exported out of the cells in pcDNA-mGMCSF-SODB1 and pcDNA-mGMCSF-VAC3 transfected cells (**Figure-3.3** and **Figure-3.4A**).

As shown in **Figure-3.2A**, in the absence of mGMCSF the majority of expressed LdPxn1 remains in the cytosol. Unexpectedly, some of the expressed LdPxn1 protein appeared on the supernatant. Two possible explanations can be given to why a portion of the expressed protein appeared in the supernatant. The first reason is that cells might be dead and released their contents to the culture. This was not the case in our experiment that, in all transfections, the viability of cells after transfection was greater or equal to 90%. The other possibility is that the cytosolic LdPxn1 could be secreted from the cell via a non-classical pathway. Similar result was obtained with expression of GFP protein in CHO cells (Tanudji et al., 2002). GFP is normally a cytosolic protein devoid of leader sequence and is expected to remain in the cytosol in mammalian expression cells. However, expression of the protein in CHO cells produced recombinant protein both on the culture supernatant and cell lysate. A more detail study using inhibitor of the classical pathway of protein secretion confirmed that the GFP protein was secreted to the cell supernatant via non-classical pathway (Tanudji et al., 2002). This finding also corroborates our result that GFP expressed in CHO cells appeared on both culture supernatant and cell lysate (**Figure-3.4B**).

In summary, the expression study have shown that our vaccine candidates were cloned in correct orientation in pcDNA based vector and are expressed in mammalian system. This result paved

the way to use the DNA antigens for vaccine studies in mice. Moreover, the experiments confirmed the role of GMCSF as a secretory module for the vaccine candidates.

Chapter Four: **ASSESSMENT OF THE IMMUNOGENICITY AND PROTECTIVE EFFICACY OF FUSION/COCKTAIL *LEISHMANIA DONOVANI* PEROXIDOXIN1 AND SUPEROXIDE DISMUTASE B1 IN DNA-PROTEIN PRIME-BOOST IMMUNIZATION IN BALB/c MICE**

4.1 Experimental Rationale

As described in Chapter-1, *Leishmania* superoxide dismutases and peroxidoxins play crucial role for the survival of *Leishmania* parasite inside host cells. These molecules detoxify toxic oxygen and nitrogen intermediates produced by host cells. *Leishmania* superoxide dismutase B1 and peroxidoxin 1 are expressed predominantly in amastigote stage of the parasite. On the other hand, superoxide dismutase B2 and peroxidoxin 2 (homolog of *L major* thiol-specific antioxidant, TSA) are primarily expressed in promastigote stage. It is suggested that parasite proteins that are expressed predominantly in the amastigote stage of the parasite are generally more useful antigens than those that are exclusively or predominantly expressed in the promastigote stage. In human host, promastigotes exist only in the early stage of infection. Amastigotes, on the other hand, persisted throughout the infection period and are the main target of the host immune system (Rafati et al., 1997, Handman, 2001). Moreover, as described in Chapter-1, dendritic cells (major antigen presenting cells) take up amastigotes but not promastigotes (Kautz-Neu et al., 2012).

A study on the immunogenicity of amastigote and promastigote fractions in human samples supported this idea. *In vitro* stimulation of peripheral blood mononuclear cells (PBMCs) from active cutaneous leishmaniasis patients or cured individuals with *Leishmania major* amastigote fraction induced higher response than stimulation with promastigote fraction as demonstrated by higher proliferation and IFN-γ production (Rafati et al., 1997).

Superoxide dismutases and peroxidoxins have been tested as vaccine candidates for various infectious diseases. Superoxide dismutases have been used as vaccine candidates against various diseases such as leishmaniasis (Daifalla et al., 2011, Daifalla et al., 2012), tuberculosis (Park et al., 2008), schistosomiasis (Cook et al., 2004), brucellosis (Onate et al., 2003, Singha et al., 2008, Saez et al., 2012), and against *Helicobacter pylori* infection (Every et al., 2011). As explained in Chapter-1, these studies have shown that superoxide dismutase is important antigen that induces a Th-1-biased immune response in experimental animal models. The challenge experiments have also shown that the antigen is at least partially protective against infection with the cognate microorganism.

Recently, we have shown that recombinant LdFeSODB1 protein is immunogenic in BALB/c mice. In the presence of adjuvants, mice immunized with rLdFeSODB1 in a protein/protein immunization strategy were immunogenic and partially protective against experimental *L major* infection (Daifalla et al., 2011, Daifalla et al., 2012).

Like superoxide dismutases, peroxidoxins have been also tested as vaccine candidates against infectious diseases such as leishmaniasis (Webb et al., 1998), amoebiasis (Soong et al., 1995) and *H pylori* infection (Stent et al., 2012). *L major* thio-specific antioxidant (TSA) is a homolog of *L donovani* peroxidoxin 2. In mice models of leishmaniasis, together with adjuvants such as rIL-12, TSA has been shown to be immunogenic and partially protective against both cutaneous and visceral leishmaniasis. The antigen induces a Th-1 biased immune response (Webb et al., 1998). Most importantly, TSA has been used as one of the three components of *Leish111F* (or LEISH-F1), the *Leishmania* polyprotein candidate vaccine that has reached clinical trial in humans. These trials have shown that the vaccine is safe and immunogenic in humans (Llanos-Cuentas et al., 2010, Nascimento et al., 2010). Peroxidoxins have also been shown to be

immunogenic and partially protective against other diseases such as amoebiasis (Soong et al., 1995) and *Helicobacter pylori* infection (Stent et al., 2012).

Studies have shown that both *Leishmania* superoxide dismutase and peroxidoxin are recognized by sera from leishmaniasis patients (Yeganeh et al., 2009, Santarem et al., 2005). Sero-reactivity of recombinant *L major* SODB1 was found to be 62.5% and 13.3% in VL and CL patient samples, respectively. On the other hand, only serum samples from 2.5% of non-leishmaniasis patients gave a positive sero-reactivity to rLmFeSODB1 indicating that the antigen is specific to *Leishmania* (Yeganeh et al., 2009). Santarem and colleagues (2005) investigated the reactivity of VL serum samples to *L infantum* peroxidoxins. Sera from more than 70% of active VL patients reacted to each of the three *Leishmania* peroxidoxins tested. In our hand, we have found that the antigens are recognized by sera collected from leishmaniasis patients in Ethiopia (**Appendix-A**).

Having these results, we wanted to test the immunogenicity and protective efficacy of fusion/cocktail LdFeSODB1-LdPxn1 in DNA/protein prime-boost immunization strategy in BALB/c mice. Studies have shown that the type of immune response generated by antigens prior to infection determines the outcome of *Leishmania* challenge infection (Scott, 1991, Gurunathan et al., 1997, Darrah et al., 2007). Therefore, we evaluated the immune response before and after infection as well as measured the protective efficacy of the antigens eight weeks after infection with *L major*.

Using more than one antigen in a form of fusion or cocktail vaccine is generally considered more effective than using individual antigen in that the former elicits higher immunogenic response capable of protecting immunized animals from developing disease. Moreover, using multiple antigens in one formulation is considered to be better suited to develop a vaccine that is effective in genetically diverse populations (Goto et al., 2011, Coler et al., 2007). Among others, the

fusion vaccine antigens *Leish111f* and *KSAC* have been found to be more effective than each of the antigens that constitute the fusion (Goto et al., 2011, Skeiky et al., 2002). A recent study by Goto and co-workers (2011) compared a fusion *Leishmania* vaccine (*KSAC*) or cocktail of the four antigens that make up the fusion with individual ones *vis-a-vis* immunogenicity and protection against VL and CL in mice. The fusion *KSAC* antigen consisted of four *Leishmania* proteins namely, kinetoplastid membrane protein 11 (*KMP11*), sterol 24-c-methyltransferase (*SMT*), A2, and cysteine protease B. The result showed that the fusion/cocktail vaccine is more immunogenic inducing a large number of multipotent $CD4^+$ T cells that can simultaneously produce IFN-γ, TNF-α, and IL-2 cytokines. Moreover, the fusion antigen induced higher protection against challenge infection than individual ones.

In our study, we used DNA/protein prime-boost immunization strategy. As described in Chapter-2, the DNA vaccine consists of fusion of LdFeSODB1 and LdPxn1 genes cloned in a mammalian expression plasmid vector, pcDNA. Each of the fusion and individual DNA antigens was cloned into a modified pcDNA vector that contains the murine granulocyte macrophage colony-stimulating factor (mGMCSF) gene. The same antigens were also cloned into pcDNA vector in the absence of mGMCSF.

We proposed that fusing GMCSF to the vaccine antigens has dual advantages. First, as discussed in Chapter-3 above, fusing the vaccine antigens with GMCSF helps to secrete the protein antigens from transfected muscle cells upon intramuscular immunization which makes vaccine antigens available for uptake by antigen presenting cells. Secondly, GMCSF itself acts as adjuvant. GMCSF is a chemoattractant factor and immunomodulator. As a chemoattractant factor it recruits neutrophils and monocytes to the site where it is produced in a paracrine manner. As immunomodulator cytokine, it activates dendritic cells and mediates their

maturation. Thus, GMCSF has been used as an important adjuvant in vaccines against various infectious diseases and cancer (Disis et al., 1996, Shi et al., 2006, Weiss et al., 1998, Haddad et al., 2000).

In our vaccine constructs, we preferred to fuse the gene encoding GMCSF with that of the vaccine antigen rather than cloning the two in separate plasmid vectors and co-administering into mice. The former strategy enables a simultaneous expression of GMCSF and the vaccine antigen (Liu, 2011).

As discussed in Chapter-1 above, CpG ODN adjuvant has been used together with various vaccine antigens against infectious diseases. Moreover, CpG ODN is well tolerated and is safe in humans (Klinman et al., 2004). Our previous work on individual rLdFeSODB1 and rLdPxn4 vaccine antigens in BALB/c mice also showed that the adjuvant plays important role in shifting the immune response to a protective Th-1 phenotype (Daifalla et al., 2011, Daifalla et al., 2012). Therefore, in this study, we used CpG ODN adjuvant in a DNA/protein immunization of the fusion LdFeSODB1-LdPxn1 in BALB/c mice.

In this part of our study, we used heterologous prime-boost immunization. Heterologous immunization regimen is more effective than homologous one in inducing stronger immune response and better protection. Comparison of the two vaccination strategies were made on testing vaccines against various infectious diseases such as HIV, tuberculosis, leishmaniasis. These studies have clearly shown that heterologous prime boost immunization is more effective than homologous one (Hu et al., 1991, Cai et al., 2006, Mazumder et al., 2011). In one of the pioneering studies on heterologous prime-boost immunization, Hu and colleagues (1991), demonstrated that priming with live recombinant vaccinia virus expressing HIV-1 envelop glycoprotein followed by recombinant HIV-1 gp160 protein is more effective in inducing

neutralizing antibody with the capability of cross reactivity on different isolates. This response was not obtained with homologous prime-boost immunization with recombinant gp160. Similar finding was obtained with a heterologous prime-boost immunization of a candidate tuberculosis vaccine in cattle. Cells from calves immunized with cocktail of DNA vaccine encoding Ag85B, MPT-64, and MPT-83 of *M tuberculosis* followed by BCG boost produced significantly higher IFN-γ than those from calves immunized with homologous strategy. Moreover, the former strategy elicited stronger proliferation of antigen-specific $CD4^+$ T cells. Calves immunized heterologously showed a 10 to 100-fold increase in protection against *M bovis* challenge infection (Cai et al., 2006). Mazumder and colleagues (2011) compared the efficacy of a *Leishmania* gp63 antigen in DNA/DNA, DNA/protein, and protein/protein immunization strategies. It was clearly demonstrated that DNA/protein heterologous immunization of *L donovani* gp63 induced immune response with increased protective Th-1 type than the homologous strategies as shown by increased production of IFN-γ, IL-12, nitric oxide as well as higher IgG2a/IgG1 ratio. It also showed significantly higher degree of protection even when the parasite challenge was done twelve weeks after the last immunization indicating that the immunization strategy confers durable immunity against the parasite. Moreover, the heterologous immunization showed protection of mice against both *L donovani* and *L major* infections.

In order to assess the degree of protective immunity induced by the vaccine antigens, we performed cytokine ELISA to measure the level of IFN-γ and IL-10 produced by antigen-stimulated spleen cells. We also performed multiparameter flow cytometry to further analyse the quality of the response induced by the vaccine antigens. Multiparameter flow cytometry is considered as a powerful technique to analyse vaccine induced immunity with respect to the

expression of cytokines that are attributed to protection in *Leishmania* infection in mice (Darrah et al., 2007). In this study, we used multiparameter flow cytometry to: 1) better characterize the phenotype of vaccine-induced T cells and to identify the expression of multiple cytokines i.e. to determine the expression of TNF-α and IL-2 in addition to IFN-γ and IL-10, 2) determine the proportion of T cells that produce more than one cytokines simultaneously, and 3) to determine the level of expression of cytokines that predict the protective efficacy of vaccine antigens.

Detailed analysis of vaccine antigen-induced T cell response in *Leishmania major* infection in mice has demonstrated that protection of immunized BALB/c mice from *L major* challenge infection is strongly correlated with the frequency of $CD4^+$ T cells that express three Th-1 cytokines simultaneously, IFN-γ, TNF-α, and IL-2 (Goto et al., 2011, Darrah et al., 2007). Therefore, in this study, we assessed the frequency of T cells that express these Th-1 cytokines and also IL-10 as well as the level of expression of these cytokines in antigen-stimulated spleen cells from mice immunized with the vaccine antigens and also controls.

Part of the data about the immunogenicity and protective efficacy of the antigens was presented (oral presentation) on the Fifth World Congress on Leishmaniasis in Porto de Galinhas, Pernambuco, Brazil, May, 2013 (Abstract # 174).

4.2 Experimental Results

4.2.1 Evaluation of the Immunogenicity of Vaccine Candidates in DNA/Protein Immunization

In this study, we hypothesized that fusing LdFeSODB1 and LdPxn1 would induce stronger immune response and bring about better protection than individual candidates. To evaluate the effectiveness of the fusion LdFeSODB1-LdPxn1 vaccine candidate in heterologous DNA/protein prime-boost immunization strategy, the fusion gene was cloned in a modified pcDNA plasmid vector in the presence or absence of fusion GMCSF gene. In order to compare the immune response induced by the fusion antigen with individual ones, LdFeSODB1 and LdPxn1 were also separately cloned in the pcDNA plasmid. Furthermore, each of the vaccine antigens was cloned into pcDNA-mGMCSF to evaluate the adjuvant role of GMCSF in mice.

Immunogenicity of the vaccine candidates and phenotype of helper T cell response they induced were then assessed by measuring antibody response from sera collected just before immunization and every three weeks thereafter. In addition, the cytokine response was evaluated by measuring IFN-γ and IL-10 production in antigen-stimulated spleen cells collected, at the time of euthanasia, from immunized mice and controls. Finally, the phenotype of T cells was determined by performing multiparameter flow cytometry on antigen stimulated $CD4^+$ and $CD8^+$ T cells stained with fluorochrome conjugated anti-mouse IFN-γ, anti-mouse-TNF-α, anti-mouse IL-2, and anti-mouse IL-10 antibodies.

Statistical comparisons were done between mice that were immunized with fusion pcDNA-mGMCSF-VAC3 antigen and those that were immunized with pcDNA-mGMCSF-SODB1 or pcDNA-mGMCSF-Pxn1; between the fusion pcDNA-VAC3 and pcDNA-SODB1 or pcDNA-Pxn1 immunized mice; as well as between mice immunized with each antigen (fusion or individual) and those injected with the control.

4.2.1.1 Pre-Challenge Antibody Response in DNA/Protein Immunization

In order to examine immunogenicity of the vaccine antigens, the level of antigen-specific total IgG, IgG1 and IgG2a was measured using ELISA from sera collected before and after immunization. As shown in **Figure-4.1A**, no antigen specific total IgG was seen in pre-immune sera (Week-0) from experimental groups except those immunized with LdFeSODB1. Three weeks after the first immunization (Week-3), mice vaccinated with the fusion, pcDNA-mGMCSF-VAC3, produced the highest antigen-specific total IgG response. The response was significantly higher than that induced by immunization with pcDNA-mGMCSF-Pxn1 ($p<0.05$) but not pcDNA-mGMCSF-SODB1. In the absence of mGMCSF, the fusion DNA did not induce detectable IgG response at Week-3.

Three weeks after administration of two doses of DNA antigens (i.e. Week-6 after first immunization), immunization with the fusion pcDNA-mGMCSF-VAC3 produced higher IgG response ($OD_{450nm} = 1.8 \pm 0.7$) than with either pcDNA-mGMCSF-Pxn1 (1.4 ± 0.7) or pcDNA-mGMCSF-SODB1 (0.4 ± 0.13). Likewise, in the absence of mGMCSF, the fusion construct induced higher IgG response than either of the individual vaccine antigens. However, the difference was not statistically significant. On the other hand, all the individual antigens induced significantly higher total IgG response to than the respective controls, pcDNA or pcDNA-mGMCSF ($p<0.05$) (**Figure-4.1A**).

At Week-9 after the first immunization (i.e. three weeks after receiving the respective recombinant protein antigen boost), mice immunized with the fusion antigen with or without mGMCSF produced significantly higher total IgG than those immunized with LdFeSODB1 in the presence or absence of mGMCSF, respectively ($p<0.05$). However, no difference was observed between mice immunized with the fusion antigen and those immunized with LdPxn1 as

the later also produced high level total IgG (**Figure-4.1A**). On the other hand, immunization with each of the individual or fusion vaccine antigens induced significantly higher antigen-specific total IgG response than the respective controls ($p<0.05$). For instance, mice immunized with pcDNA-SODB1 and pcDNA-mGMCSF-SODB1 in the first two immunizations followed by a boost with rLdFeSODB1 protein produced significantly higher anti-LdFeSODB1 total IgG response than those immunized with pcDNA and pcDNA-mGMCSF, respectively ($p<0.05$) (**Figure-4.1A**). Total IgG response suggested that the fusion antigen is more immunogenic than each of the individual antigens. The difference was more pronounced before administering booster immunization with the recombinant protein. Furthermore, the presence of mGMCSF increased the antibody response to the vaccine antigens.

Antigen-specific IgG1 production was also assessed on sera collected from mice before and after DNA/protein immunization with the fusion and individual antigens. As shown in **Figure-4.1B**, no detectable level of antigen-specific IgG1 response was seen on pre-immune sera (Week-0). At Week-3 post immunization, pcDNA-mGMCSF-VAC3 induced significantly higher IgG1 response than each of the individual antigens ($p<0.05$). It also induced significantly higher antigen-specific IgG1 response than the same antigen without mGMCSF ($p<0.05$).

At Week-6, pcDNA-mGMCSF-VAC3 elicited more IgG1 response (1.4 ± 0.68) than pcDNA-mGMCSF-SODB1 (0.02 ± 0.02) or pcDNA-mGMCSF-Pxn1 (0.8 ± 0.71). But the difference was not statistically significant ($p>0.05$) (**Figure-4.1B**).

At Week-9 post-immunization, mice immunized with the fusion pcDNA-mGMCSF-VAC3 antigen produced significantly higher IgG1 response than those immunized with pcDNA-mGMCSF-SODB1 or pcDNA-mGMCSF-Pxn1 ($p<0.05$). The antigen also induced significantly higher IgG1 immune response than pcDNA-VAC3 ($p<0.05$). Without mGMCSF, mice

immunized with the fusion produced significantly higher antigen-specific IgG1 than those immunized with pcDNA-SODB1 ($p<0.05$). Mice immunized with pcDNA-SODB1 and pcDNA-mGMCSF-SODB1 did not produce significantly higher IgG1 than those mice injected with controls ($p>0.05$) (**Figure-4.1B**).

Antigen-specific IgG2a level in mice immunized with the fusion and individual antigens is depicted in **Figure-4.1C**. The fusion pcDNA-mGMCSF-VAC3 resulted in significantly higher response at Week-3 post-immunization than individual antigens or controls ($p<0.05$). Like total IgG and IgG1 response, immunization with pcDNA-mGMCSF-VAC3 induced significantly higher IgG2a response than pcDNA-VAC3 ($p<0.05$).

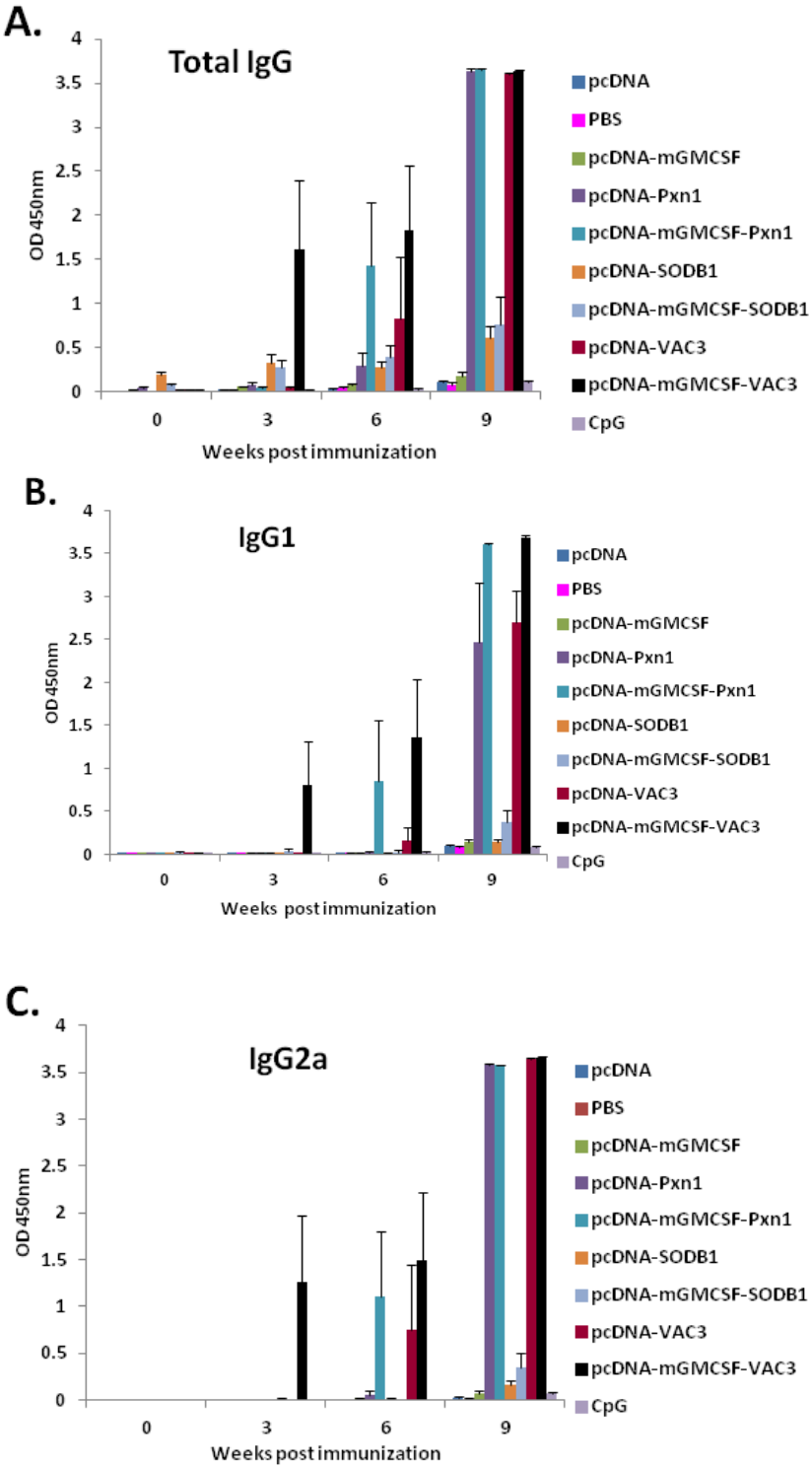
At Week-6 post immunization, the fusion antigen in the presence of mGMCSF induced more antigen-specific IgG2a response than each of the individual antigens. However, only the difference between the fusion and pcDNA-mGMCSF-SODB1 is statistically significant ($p<0.05$). Mice immunized with the fusion antigen without mGMCSF and those immunized with pcDNA-mGMCSF-Pxn1 also produced appreciably high amount of IgG2a (**Figure-4.1C**). At Week-6, in the absence of mGMCSF, immunization with pcDNA-VAC3, pcDNA-Pxn1, and pcDNA-SODB1 showed IgG2a to IgG1 ratio of 4.8, 2.2 and 0.8, respectively. The ratio is 1.1, 1.3, and 0 for pcDNA-mGMCSF-VAC3, pcDNA-mGMCSF-Pxn1, and pcDNA-mGMCSF-SODB1, respectively.

At week-9 post-immunization, the fusion pcDNA-mGMCSF-VAC3 induced significantly higher IgG2a production than with either pcDNA-mGMCSF-Pxn1 or pcDNA-mGMCSF-SODB1 ($p<0.05$). All the individual antigens also induced significantly higher IgG2a than the respective controls ($p<0.05$). However, no statistically significant difference was seen between either the fusion or individual antigens containing mGMCSF and their respective antigen without

mGMCSF (**Figure-4.1C**). At Week-9, the difference in IgG2a/IgG1 ratio in mice immunized with different antigens without mGMCSF is not as big as seen at Week-6. The ratio of IgG2a to IgG1 in mice that were immunized with pcDNA-VAC3, pcDNA-Pxn1, and pcDNA-SODB1 is 1.3, 1.45, and 1.15, respectively. In the presence of mGMCSF the ratio is 1.0, 1.0, and 0.93 for pcDNA-mGMCSF-VAC3, pcDNA-mGMCSF-Pxn1, and pcDNA-mGMCSF-SODB1, respectively. The IgG1 and IgG2a response suggests that both the fusion and individual antigens induce a mixed Th-1/Th-2 response. In the absence of mGMCSF, administration of two doses of fusion DNA appears to induce a Th-1 biased immune response in BALB/c mice. The result also shows that LdFeSODB1 is weak immunogen to induce antibody response both in the form of DNA and protein antigen.

Figure 4.1: Pre-challenge antibody response in DNA/protein prime-boost immunization in BALB/c mice.

Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All immunizations were given in three week intervals. Blood samples were collected before immunization, at the time of each immunization and upon euthanasia. Total IgG **(A)**, IgG1 **(B)**, and IgG2a **(C)** were measured using ELISA and the result is depicted as mean OD450nm of five mice per group and standard error of the mean (SEM). Statistical comparison between groups was performed using Mann-Whitney U test. The result shows that the fusion antigen is more immunogenic than either of the constituent antigens and induces a mixed Th-1/Th-2 response in DNA/protein immunization regimen in BALB/c mice.


A.
Total IgG
OD 450nm
Weeks post immunization
pcDNA
PBS
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
CpG
B.
IgG1
OD 450nm
Weeks post immunization
C.
IgG2a
OD 450nm
Weeks post immunization

As shown in **Figure 4.1B** above, at Week-9, the level of antigen-specific IgG1 response on serum samples from mice immunized with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 was high. Similar result was also seen with regard to the level of IgG2a response in mice immunized with the fusion and LdPxn1 antigens (**Figure 4.1C)**. In these experiments, the level of IgG1 and IgG2a was determined on serum samples at a specific dilution (i.e. 1:100). In order to examine if there is difference in the titer of these antibody responses, a two-fold serial dilution of the serum samples was performed. **Figure-4.2A and Figure-4.2B** shows the titer of antigen-specific IgG1 and IgG2a in two-fold serially diluted serum samples.

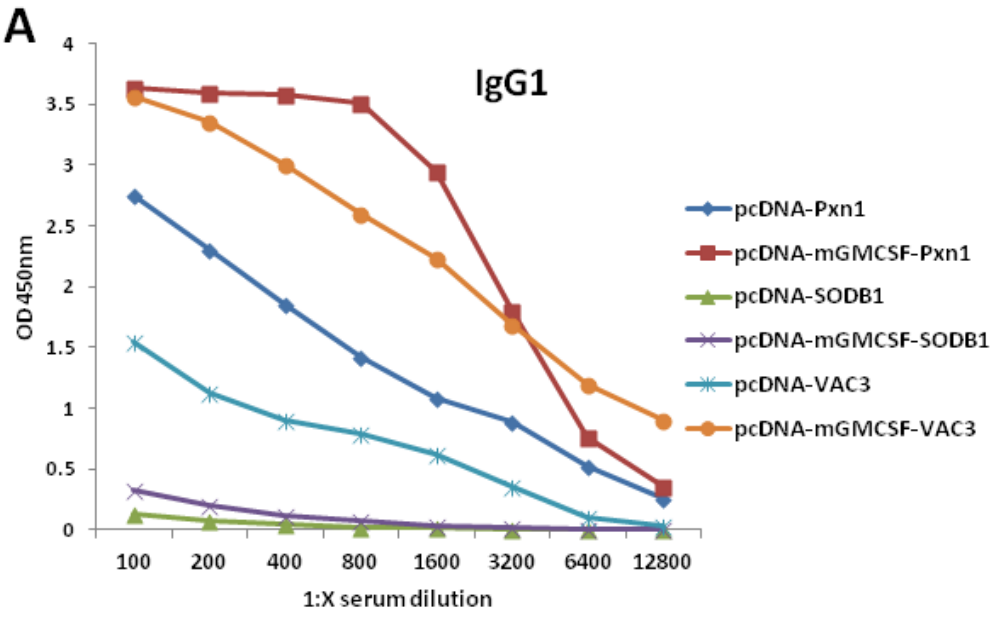
Figure-4.2A shows antigen-specific IgG1 titer in mice immunized with the individual and fusion antigens. Generally, antigens that contain mGMCSF fusion showed appreciably higher titer than their counterparts without mGMCSF. However, the difference was not statistically significant. Sera from mice that were immunized with pcDNA-mGMCSF-Pxn1 antigen showed higher IgG1 reading than that of pcDNA-mGMCSF-VAC3 antigen and remained higher at lower dilutions. However, at 1:800 dilution onwards, the IgG1 level in this group decreased sharply. On the other hand, sera from mice that were immunized with pcDNA-mGMCSF-VAC3 showed equal reading at 1:100 with sera from pcDNA-mGMCSF-Pxn1 immunized mice but decreased steadily until the last dilution. Statistical analysis between the two groups at 1:800 and 1:12800 dilutions did not show significant difference ($p>0.05$). On the other hand, in the presence of mGMCSF, sera from mice immunized with either the fusion or LdPxn1 showed significantly higher IgG1 reading than that of pcDNA-mGMCSF-SODB1 ($p<0.05$).

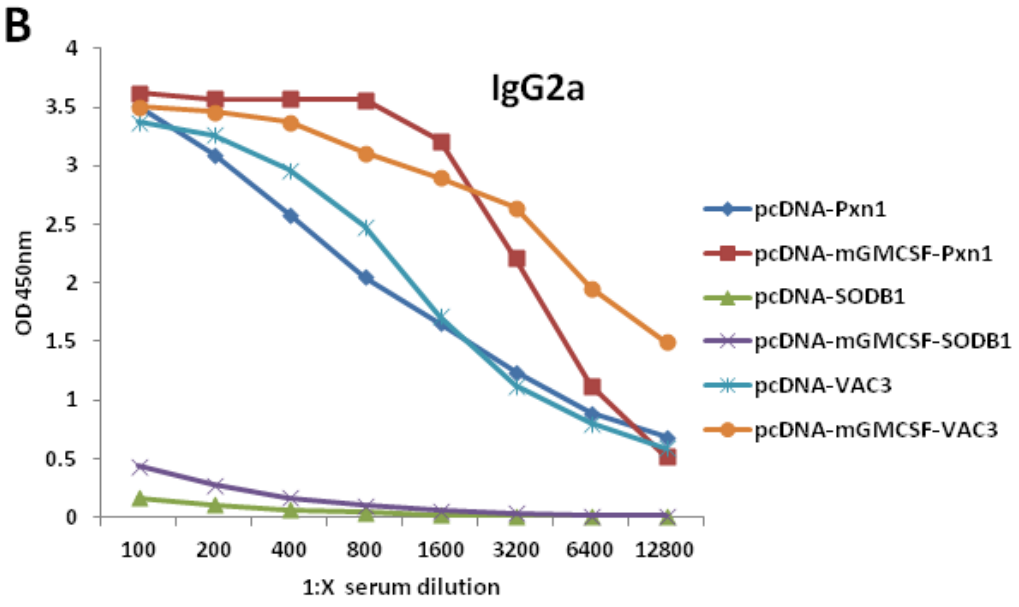
Titration of antigen-specific IgG2a clearly showed that although sera from mice immunized with LdPxn1 and LdVAC3 antigens with or without mGMCSF resulted in almost the same OD for IgG2a at 1:100 dilution, the antigens that contain mGMCSF showed a titer that remained high at

higher dilutions than antigens without mGMCSF. Sera from mice that were immunized with pcDNA-SODB1 and pcDNA-mGMCSF-SODB1 produced significantly lower IgG2a titer than the other antigens ($p<0.05$) (**Figure-4.2B**). There was no detectable antigen-specific IgG1 or IgG2a in serum samples collected from mice immunized with the controls (data not shown). Titration of sera further confirms that mGMCSF increases antigen-specific antibody response in both fusion and individual antigens.

Figure 4.2: Titration of pre-challenge sera from mice immunized with antigens in DNA/protein prime-boost immunization regimen.

Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Blood samples were collected at Week-9 after the first immunization. The level of IgG1 **(A)** and IgG2a **(B)** was measured using ELISA in a two-fold serially diluted sera. The result is depicted as mean OD450nm of five mice per group. Statistical comparison between groups was performed using Mann-Whitney U test. Immunization with pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 induced high level antigen-specific IgG1 and IgG2a titer. The data suggests that the presence of mGMCSF increased immunogenicity of the vaccine antigens.


A
IgG1
OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
12800
1:X serum dilution
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3


B
IgG2a
OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
12800
1:X serum dilution
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3

4.2.1.2 Pre-Challenge Cytokine Response in DNA/Protein immunization

Cell mediated immune response to vaccine antigens was evaluated by measuring the level of IFN-γ and IL-10 from supernatant of spleen cells cultured *in vitro* and stimulated with rLdPxn1, rLdFeSODB1, a combination of rLdFeSODB1 and LdPxn1 or soluble *Leishmania major* antigen (LmSLA). As positive control, cells were stimulated with mitogen (concanavalin A). Samples from unstimulated cells were included as negative control.

Figure-4.3A shows the IFN-γ response in spleen cells of immunized mice stimulated with the respective recombinant protein antigen. Cells from mice that received controls, pcDNA, pcDNA-mGMCSF, PBS, or with CpG ODN and stimulated *in vitro* with a combination of rLdFeSODB1 and rLdPxn1 did not produce detectable IFN-γ. On the other hand, cells from mice immunized with the vaccine antigens produced significantly higher IFN-γ than their respective controls ($p<0.05$). In other words, mice that were immunized with pcDNA-Pxn1, pcDNA-SODB1, or pcDNA-VAC3 showed significantly higher response than those that received the control, pcDNA ($p<0.05$). Likewise, mice that were immunized with pcDNA-mGMCSF-Pxn1, pcDNA-mGMCSF-SODB1, or pcDNA-mGMCSF-VAC3 resulted in significantly higher IFN-γ than those mice that were injected with pcDNA-mGMCSF control ($p<0.05$). However, there is no statistically significant difference in IFN-γ production between cells from mice immunized with pcDNA-mGMCSF-VAC3 (7.2ng/ml ± 1.2) and pcDNA-mGMCSF-Pxn1 (5.8ng/ml ± 0.64) or pcDNA-mGMCSF-SODB1 (6.7ng/ml ± 0.14). Cells from mice immunized with vaccine antigens containing mGMCSF produced significantly higher IFN-γ than those from mice immunized with the respective antigen without mGMCSF ($p<0.05$) (**Figure-4.3A**).

In order to analyze the contribution of each antigen in the fusion, spleen cells from mice that were immunized with the fusion antigen with or without mGMCSF were stimulated *in vitro* with

rLdPxn1, rLdFeSODB1 or a combination of rLdPxn1 and rLdFeSODB1. The result shows that stimulation with rLdPxn1 or rLdFeSODB1 induced comparable level of IFN-γ recall response in spleen cells isolated from mice that were immunized with the fusion antigen with mGMCSF. Spleen cells that were isolated from mice that were immunized with the fusion pcDNA-mGMCSF-VAC3 produced 5.0ng/ml ± 1.5, 5.3ng/ml ± 1.6 or 7.2ng/ml ± 1.2 when stimulated with rLdPxn1, rLdFeSODB1, or a combination of rLdPxn1 and rLdFeSODB1 antigens, respectively. However, cells from mice that were immunized with pcDNA-VAC3 in the absence of mGMCSF produced 0.32ng/ml ± 0.2, 0.6ng/ml ± 0.5 or 1.98ng/ml ± 1.3 upon stimulation with rLdPxn1, rLdFeSODB1, or a combination of rLdPxn1 and rLdFeSODB1 antigens, respectively. This indicates that the IFN-γ from mice immunized with the fusion came more from LdFeSODB1 portion than LdPxn1.

Upon stimulation with *L major* SLA, cells from mice immunized with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 showed high level IFN-γ recall response indicating that *L major* SLA possesses more proteins that cross-react with LdPxn1 vaccine antigen. Stimulation with the mitogen, ConA, induced high level IFN-γ production in spleen cells from both immunized and control groups.

As shown in **Figure-4.3B**, cells from all groups of mice immunized with the antigens produced lower IL-10 than IFN-γ. Cells from pcDNA-mGMCSF-VAC3 immunized mice produced a relatively higher amount of IL-10 than those from mice immunized with individual antigen. Nevertheless, the difference is not statistically significant. On the other hand, except cells from mice immunized with pcDNA-Pxn1 and pcDNA-VAC3, cells from all other groups of antigen immunized mice produced significantly higher IL-10 response than their respective controls ($p<0.05$) (**Figure-4.3B**). Upon *in vitro* stimulation with the recombinant protein antigens, spleen

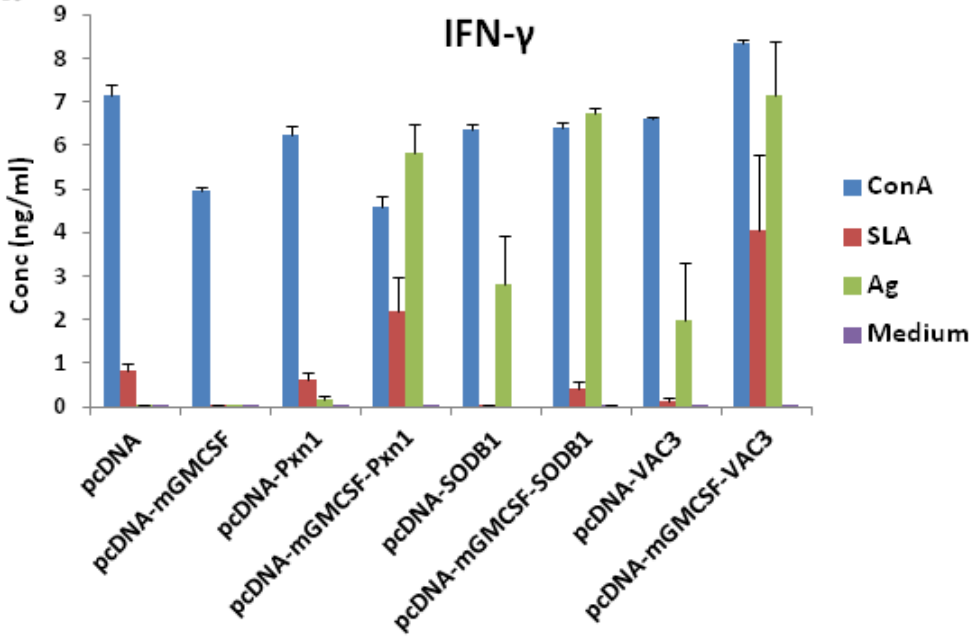
cells isolated from control mice that were injected with PBS or CpG ODN did not produce detectable levels of IFN-γ or IL-10.

Considering the IFN-γ/IL-10 ratio, mice immunized with the vaccine antigens generally induced a predominantly Th-1 response than mice that received each of the control injections (**Table-4.1**). That is, the ratio is significantly higher in vaccine antigen immunized mice than the controls. However, within antigen immunized groups, the ratio does not perfectly reflect the level of protection induced by these antigens as shown in **Figure-4.24**. First, the antigens with the highest protection did not show proportionally high IFN-γ/IL-10 ratio. Second, the non-protective pcDNA-mGMCSF-SODB1 showed the highest ratio. The possible explanation for this phenomenon is explained in the discussion section below.

Figure 4.3: Pre-challenge cytokine response of mice immunized with antigens in DNA/protein prime-boost immunization regimen.

IFN-γ **(A)** and IL-10 **(B)** production in stimulated spleen cells was measured using cytokine ELISA. Mice were immunized twice with DNA antigens or controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from mice (five mice per group) three weeks after the last immunization and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or concanavalin A (5µg/ml). Unstimulated cells were included as negative control. Cells were cultured at 37°C and 5% CO_2 and culture supernatant was collected at 72hr. The level of IFN-γ and IL-10 was measured using cytokine ELISA kit (BD Biosciences). The concentration of the cytokines (ng/ml) was calculated by correlating the optical density to concentration of the protein standard included in the kit. The mean concentration and standard error of the mean (SEM) of five mice per group was shown. Statistical comparison between groups was performed using Mann-Whitney U test. Cells from PBS and CpG ODN groups did not result in detectable amount of IFN-γ and IL-10 (data not shown). The result clearly showed that the presence of mGMCSF fusion increased cytokine response to both the fusion and individual vaccine antigens.

A.


IFN-γ
Conc (ng/ml)
0
1
2
3
4
5
6
7
8
9
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
ConA
SLA
Ag
Medium

B.

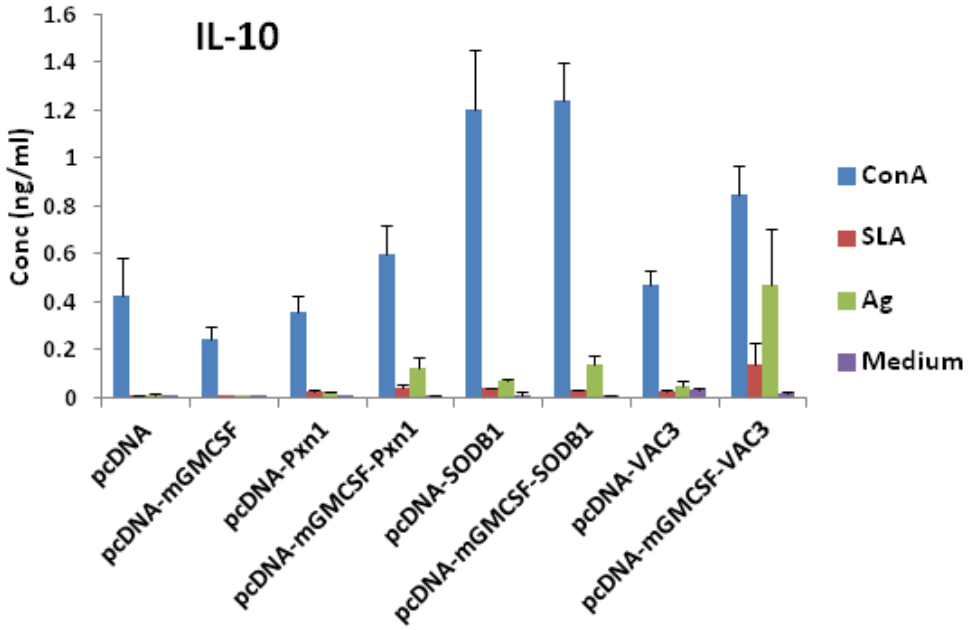

IL-10
Conc (ng/ml)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
ConA
SLA
Ag
Medium

Table 4-1: Pre-challenge IFN-γ/IL-10 ratio.

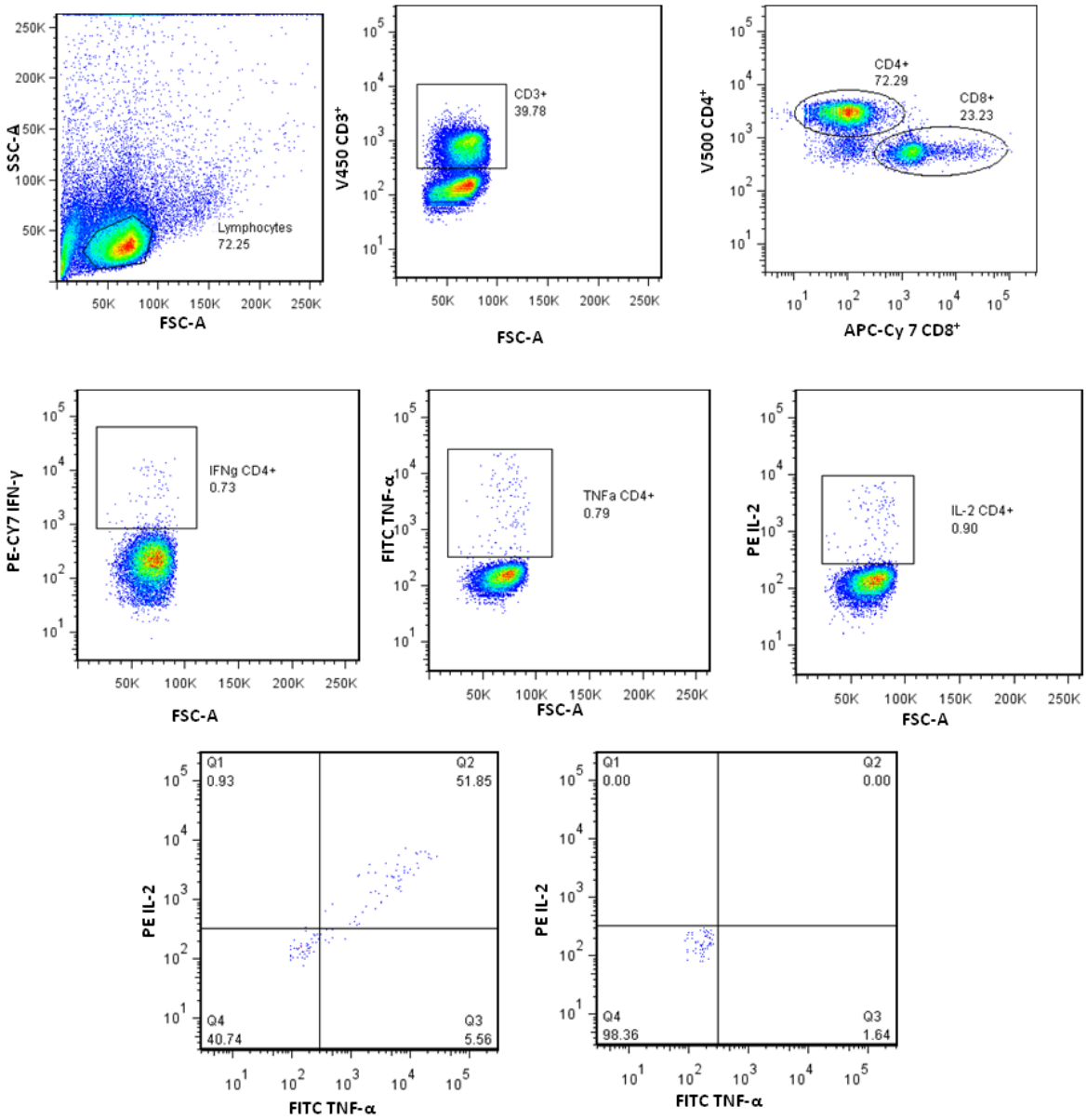
Group	IFN-γ (ng/ml)	IL-10 (ng/ml)	IFN-γ/IL-10 ratio
PBS	0.0222	0.0372	0.6
CpG	0.04575	0.019	2.4
pcDNA	0.026	0.012	2.16
pcDNA-mGMCSF	0.012	0.0044	2.73
pcDNA-Pxn1	0.1724	0.024	7.2
pcDNA-mGMCSF-Pxn1	3.58	0.121	29.6
pcDNA-SODB1	2.82	0.07	40.3
pcDNA-mGMCSF-SODB1	6.72	0.14	48
pcDNA-VAC3	1.976	0.05	39.5
pcDNA-mGMCSF-VAC3	7.166	0.4676	15.3

4.2.1.3 Phenotype of Antigen-Specific T cells

The type of cell-mediated immune response elicited by the vaccine antigens was further characterized using intracellular cytokine staining followed by multiparameter flow cytometry analysis. Antigen-stimulated spleen cells were analyzed based on $CD4^+$ and $CD8^+$ surface staining as well as intracellular staining for IFN-γ, TNF-α, IL-2, and IL-10 using fluorochrome conjugated antibodies. $CD4^+$ and $CD8^+$ T cells that produce specific cytokine were analyzed based on the percentage of cells expressing a specific cytokine and also median fluorescent intensity (MFI) of each cytokine expressed on these cells. In order to understand the quality of cytokine production in a population of $CD4^+$ or $CD8^+$ T cells, we calculated the product of the percentage of these cells and MFI. The product is termed as integrated MFI (iMFI) (Darrah et al., 2007). **Figure-4.4** shows the gating strategy we used to characterize the phenotype of antigen stimulated spleen cells and controls.

Figure 4.4: Gating strategy used in multiparameter flow cytometry.

Spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37°C and 5% $CO_{2.}$ Stimulated and unstimulated cells were stained first with a cocktail of V450 rat anti-mouse CD3, V500 rat anti-mouse CD4, and APC-Cy7 rat anti-mouse CD8α followed by another cocktail containing PE-Cy7 rat anti-mouse IFN-γ, FITC rat anti-mouse TNF-α, PE rat anti-mouse IL-2, and APC rat Anti-mouse IL-10. Multicolor flow cytometry was then performed using FACSAria II machine (BD, USA). Data was analysed using FlowJo software (Tree Star Inc, USA). Gating of different lymphocyte populations was performed based on surface marker and intracellular cytokine expression. $CD3^{+}$ cells were gated from the total lymphocyte population. In turn, $CD4^{+}$ and $CD8^{+}$ cells were gated from $CD3^{+}$ cell population. Then, the frequency of each cytokine expressing $CD4^{+}$ and $CD8^{+}$ cells was determined. Finally, the proportion of multifunctional Th-1 cells was determined by subdividing IFN-γ expressing cells into individual or a combination of TNF-α and IL-2 expressing cells.


SSC-A
FSC-A
Lymphocytes
72.25
V450 CD3+
CD3+
39.78
V500 CD4+
CD4+
72.29
CD8+
23.23
APC-Cy 7 CD8+
PE-CY7 IFN-γ
IFNg CD4+
0.73
FITC TNF-α
TNFa CD4+
0.79
PE IL-2
IL-2 CD4+
0.90
Q1
0.93
Q2
51.85
Q4
40.74
Q3
5.56
Q1
0.00
Q2
0.00
Q4
98.36
Q3
1.64
FITC TNF-α

The frequency of cytokine expressing $CD4^+$ T cells and the level of expression of the cytokines were analyzed from surface and intracellular stained spleen cells from mice that were immunized with the vaccine antigens and also controls. **Figure-4.5** represents the IFN-γ production by $CD4^+$ T cells stimulated with antigens and controls. With regard to the percentage of $CD4^+$ T cells expressing IFN-γ, immunization with pcDNA-mGMCSF-VAC3 resulted in a significantly higher value than pcDNA-mGMCSF-SODB1 or pcDNA-mGMCSF-Pxn1 ($p<0.05$). However, no difference was observed between cells from mice immunized with the fusion pcDNA-VAC3 and those from mice immunized with individual antigens devoid of mGMCSF. On the other hand, immunization with pcDNA-mGMCSF-VAC3 induced significantly higher percentage of IFN-γ producing $CD4^+$ T cells than pcDNA-VAC3 ($p<0.05$) (**Figure-4.5A**).

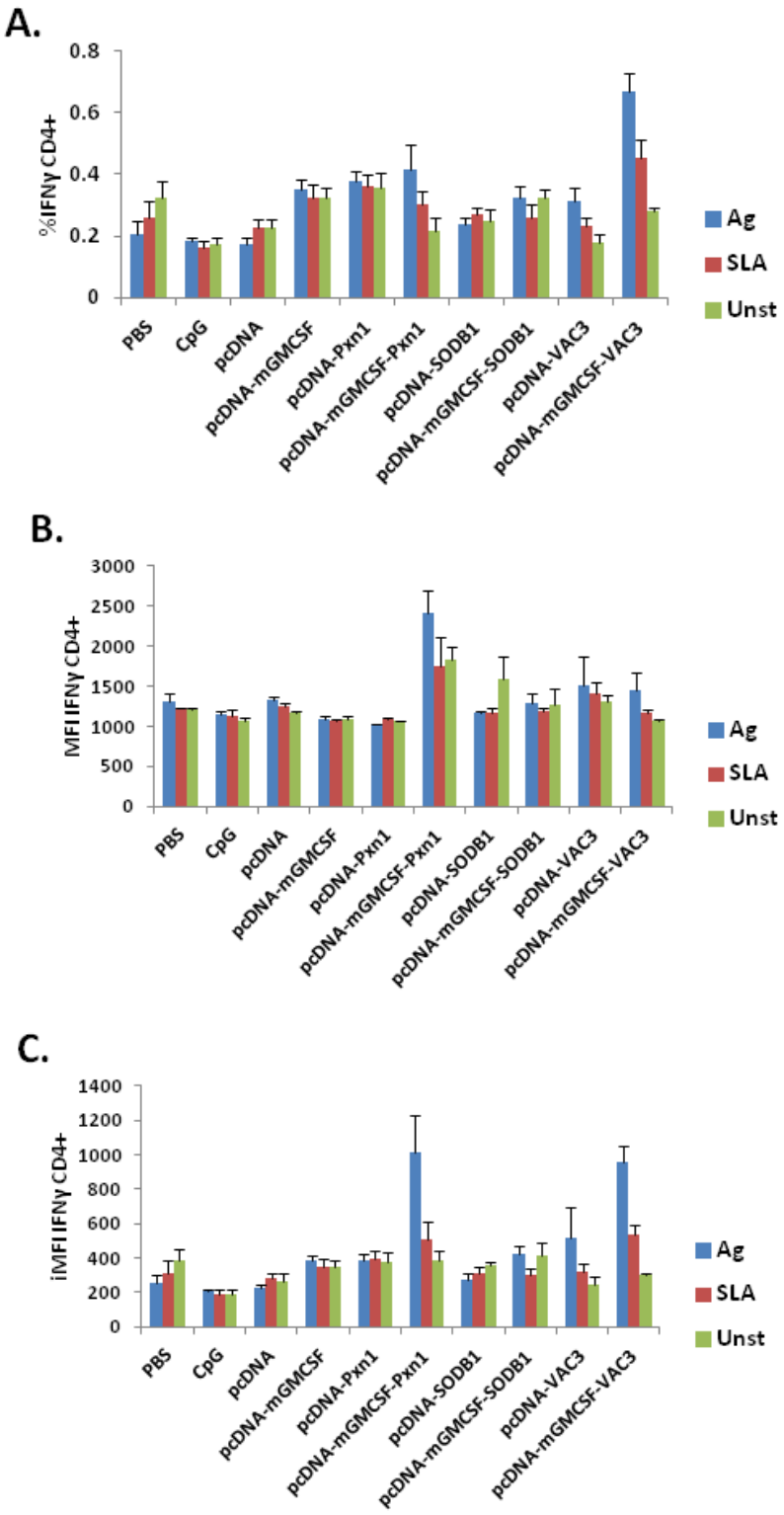
As shown in **Figure-4.5B**, cells from mice immunized with pcDNA-mGMCSF-Pxn1 showed the highest MFI for IFN-γ expressed on $CD4^+$ cells. The expression was significantly higher than cells from mice immunized with the control pcDNA-mGMCSF ($p<0.05$). Immunization with the fusion antigen did not elicit significantly higher degree of IFN-γ expression than even the control, pcDNA-mGMCSF.

Analysis of the integrated MFI for IFN-γ shows that cells from mice that were immunized with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 elicited significantly higher degree of cumulative IFN-γ (iMFI) than cells from any other groups ($p<0.05$). In addition, immunization with pcDNA-Pxn1 or pcDNA-VAC3 induced significantly higher iMFI than injection of the control, pcDNA ($p<0.05$) (**Figure-4.5C**). The data suggests that immunization with the fusion or LdPxn1 antigens induces high quality IFN-γ response by $CD4^+$ T cells than immunization with

LdFeSODB1. Moreover, it shows that mGMCSF significantly increases IFN-γ expression in antigen-specific $CD4^+$ T cells.

Figure 4.5: Antigen-specific IFN-γ expressing $CD4^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IFN-γ expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from mice (five mice per group) three weeks after the last immunization and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). The data show that immunization with pcDNA-mGMCSF-VAC3 induced large number of IFN-γ expressing $CD4^+$ T cells; whereas, immunization with pcDNA-mGMCSF-Pxn1 resulted in high degree of expression of IFN-γ on peroxidoxin 1-specific $CD4^+$ T cells.


A.
%IFNγ CD4+
0.8
0.6
0.4
0.2
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
B.
MFI IFNγ CD4+
3000
2500
2000
1500
1000
500
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
C.
iMFI IFNγ CD4+
1400
1200
1000
800
600
400
200
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

Like in IFN-γ response, spleen cells from mice immunized with pcDNA-mGMCSF-VAC3 had the largest percentage of TNF-α producing $CD4^+$ T cells (**Figure-4.6A**). $CD4^+$ T cells from these mice possessed significantly higher percentage of TNF-α expressing phenotype than from mice immunized with pcDNA-mGMCSF-Pxn1 ($p<0.05$). However, the difference with mice immunized with pcDNA-mGMCSF-SODB1 was not statistically significant ($p>0.05$). On the other hand, antigen stimulated $CD4^+$ T cells from mice immunized with pcDNA-mGMCSF-VAC3 contained significantly higher TNF-α^+ $CD4^+$ phenotype than those from mice that were immunized with pcDNA-VAC3 ($p<0.05$). The percentage of TNF-α^+ $CD4^+$ phenotype among antigen-stimulated $CD4^+$ T cells from mice that were immunized with each of the individual antigens with or without mGMCSF were significantly higher than the cells from mice that received the respective controls ($p<0.05$) (**Figure-4.6A**).

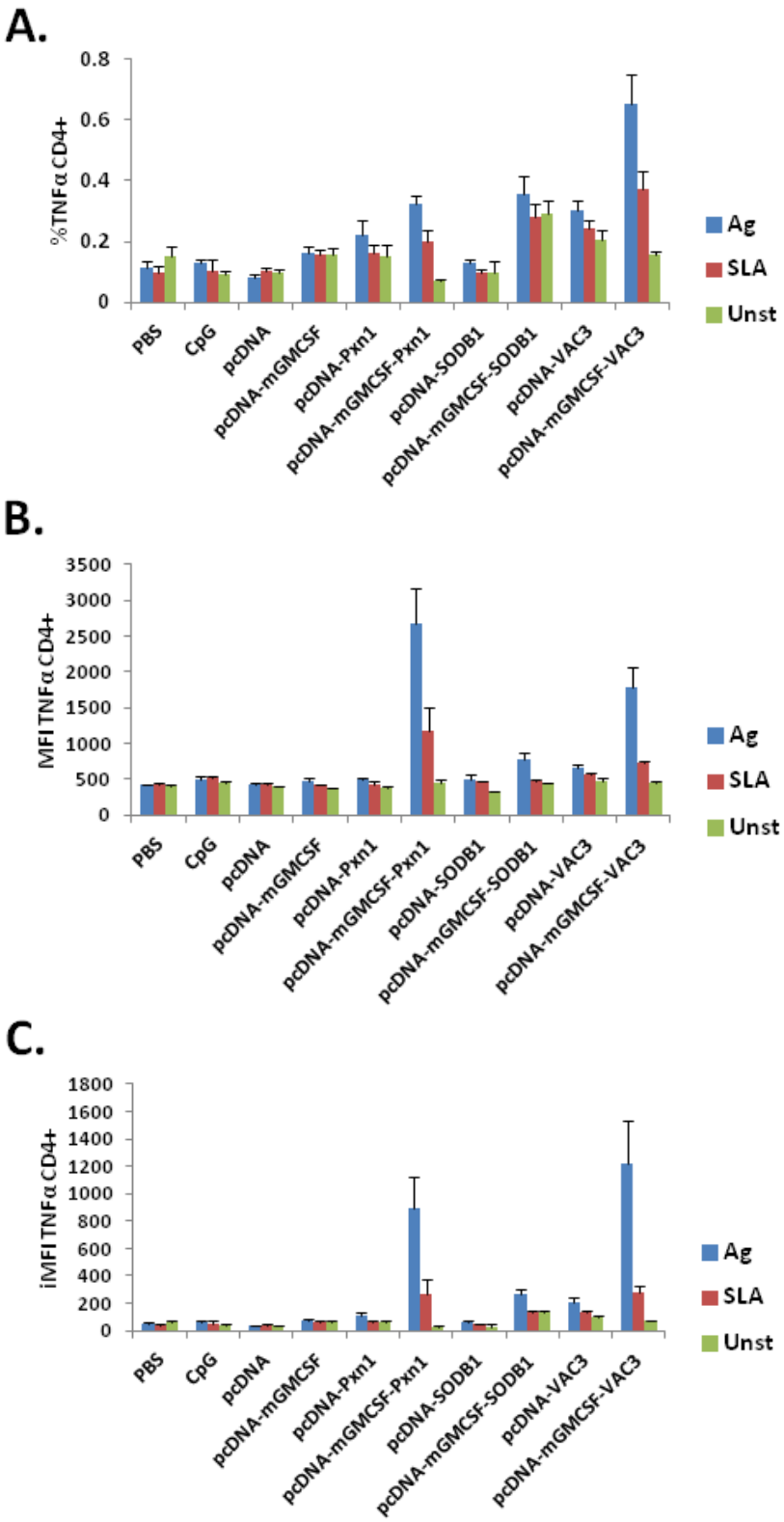
With regard to MFI for TNF-α, spleen cells from mice that were immunized with pcDNA-mGMCSF-VAC3 showed significantly higher response than those from mice immunized with pcDNA-mGMCSF-SODB1 ($p<0.05$) (**Figure-4.6B**). However, immunization with this antigen resulted in a response that was lower than that of pcDNA-mGMCSF-Pxn1. In other words, cells from mice immunized with pcDNA-mGMCSF-Pxn1 gave the highest TNF-α MFI on $CD4^+$ T cells. As compared to pcDNA-mGMCSF control group, cells from mice immunized with pcDNA-mGMCSF-SODB1 also showed significantly higher MFI for TNF-α ($p<0.05$). Moreover, pcDNA-mGMCSF-VAC3 immunization resulted in significantly higher MFI TNF-α than that of pcDNA-VAC3 ($p<0.05$) (**Figure-4.6B**).

Integrated MFI (iMFI) was also calculated for TNF-α on $CD4^+$ cells. As shown on **Figure-4.6C**, the highest iMFI for TNF-α was seen on cells from mice that were immunized with the fusion

antigen, pcDNA-mGMCSF-VAC3, followed by pcDNA-mGMCSF-Pxn1. Spleen cells from mice that were immunized with pcDNA-mGMCSF-VAC3 fusion antigen gave significantly higher iMFI for TNF-α than those from mice that were immunized with pcDNA-mGMCSF control or pcDNA-mGMCSF-SODB1 antigen ($p<0.05$). However, the difference between pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 antigens is not statistically significant ($p>0.05$). Moreover, the presence of mGMCSF contributed to the statistically significant increase iMFI for TNF-α in cells from mice that were immunized with the fusion antigen ($p<0.05$). On the other hand, each of the individual antigens with or without mGMCSF induced significantly higher iMFI for TNF-α than their respective control ($p<0.05$) (**Figure-4.6C**). The result shows that the vaccine antigens are capable of eliciting TNF-α expression in $CD4^+$ T cells. It also further strengthens the hypothesis that GMCSF enhances the immune response against the vaccine antigens.

Figure 4.6: Antigen-specific TNF-α expressing $CD4^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of TNF-α expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from mice (five mice per group) three weeks after the last immunization and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). The result shows that pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 are capable of eliciting high degree of expression of TNFα, on antigen-specific $CD4^+$ T cells. Moreover, it shows that mGMCSF increases Th-1 immune response to the vaccine antigens.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%TNFα CD4+
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0.6
0.4
0.2
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PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
B.
MFI TNFα CD4+
3500
3000
2500
2000
1500
1000
500
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
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SLA
Unst
C.
iMFI TNFα CD4+
1800
1600
1400
1200
1000
800
600
400
200
0
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CpG
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pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

In addition to IFN-γ and TNF-α, the expression of IL-2 on $CD4^+$ T cells was also assessed in stimulated spleen cells. The frequency of IL-2 expressing $CD4^+$ cells is shown in **Figure-4.7A**. Like other Th-1 cytokines, cells from mice that were immunized with the fusion antigen in the presence of mGMCSF showed the highest percentage of IL-2^+ $CD4^+$ T cells. The difference is statistically significant as compared with those of mice immunized with each of the individual antigens or controls ($p<0.05$). pcDNA-mGMCSF-VAC3 immunization also showed significantly higher percentage of IL-2^+ $CD4^+$ T cells than the same antigen without mGMCSF ($p<0.05$) (**Figure-4.7A**).

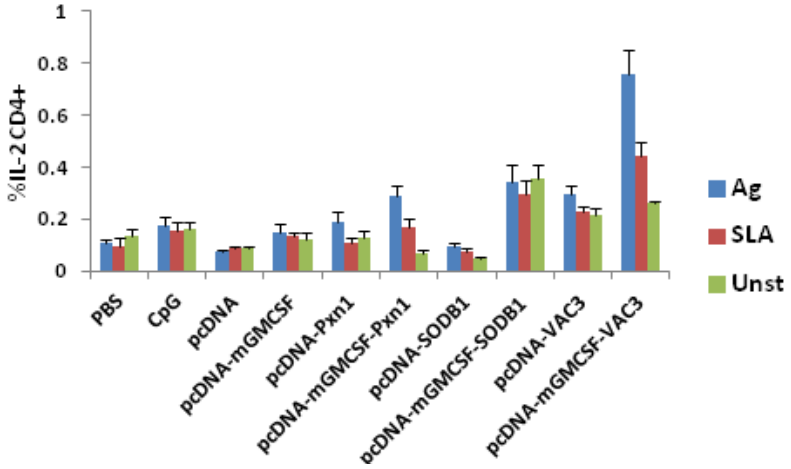
Like that of IFN-γ and TNF-α, MFI for IL-2 on $CD4^+$ T cells from mice that were immunized with pcDNA-mGMCSF-Pxn1 showed the highest value (**Figure-4.7B**). Immunization with the fusion antigen did not show significantly higher MFI than that of pcDNA-mGMCSF-SODB1 immunization. On the other hand, each of the individual or fusion antigens with or without mGMCSF showed significantly higher MFI for IL-2 expressed by $CD4^+$ T cells than the respective controls ($p<0.05$) (**Figure-4.7B**).

Figure-4.7C shows integrated MFI of IL-2 expressed on $CD4^+$ cells from immunized mice and controls. The highest iMFI for IL-2 was seen in cells from mice that were immunized with the fusion pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1. The fusion pcDNA-mGMCSF-VAC3 showed significantly higher iMFI for IL-2 than pcDNA-mGMCSF-SODB1 ($p<0.05$). Moreover, the fusion antigen with mGMCSF induced significantly higher iMFI response than the fusion without mGMCSF ($p<0.05$). Cells from mice immunized with each of the fusion and individual antigens with or without mGMCSF induced significantly higher iMFI response than the respective controls ($p<0.05$) (**Figure-4.7C)**.

Figure 4.7: Antigen-specific IL-2 expressing $CD4^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IL-2 expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from mice (five mice per group) three weeks after the last immunization and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). The data show that although both the fusion and individual antigens induced high IL-2 response than controls the highest quality IL-2 response was induced by immunization with pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1. Like in other Th-1 cytokines, the presence of GMCSF fusion increases IL-2 production by antigen-specific $CD4^+$ T cells.

A.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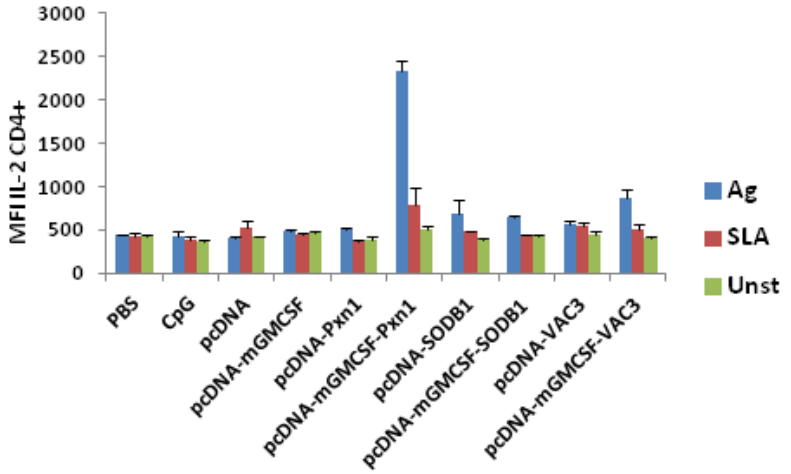
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

B.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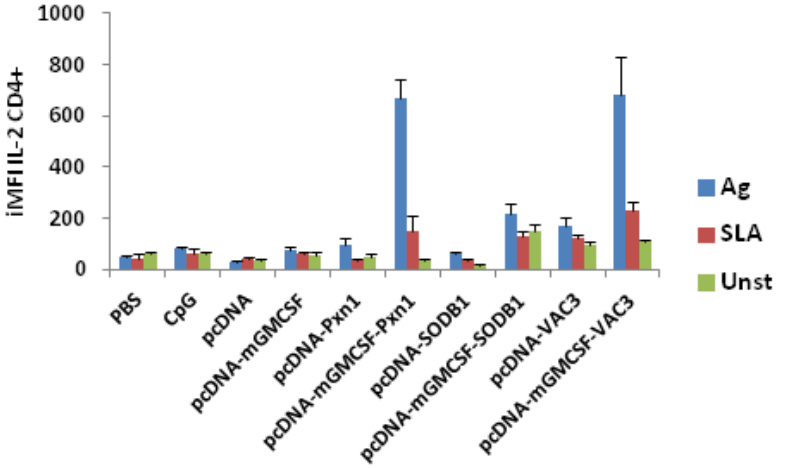
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

C.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800
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400
200
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pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

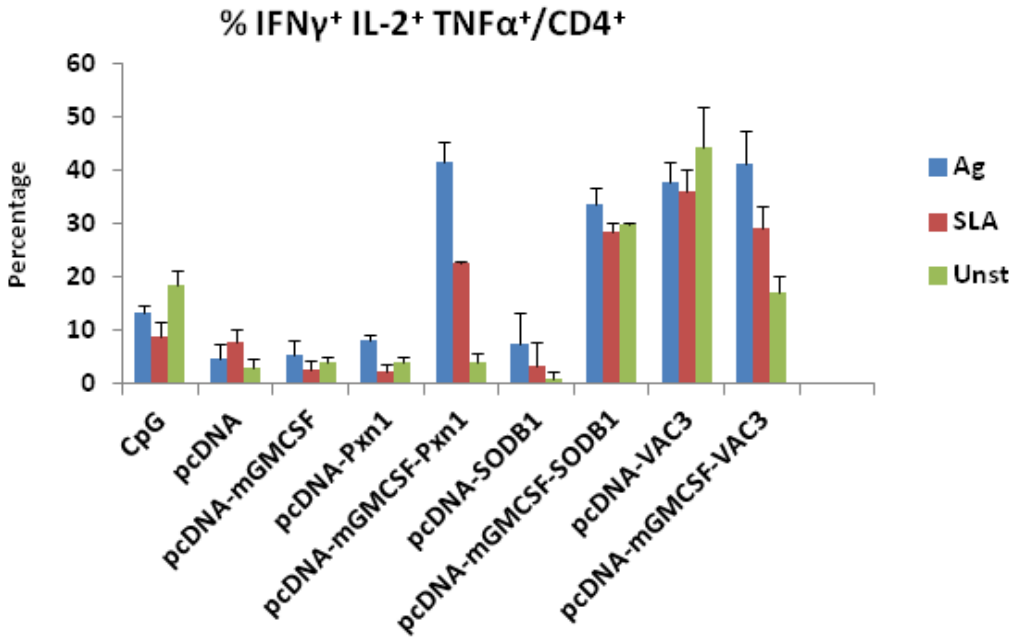
In order to assess the proportion of multifunctional T cells which simultaneously produce more than one cytokine, we further analyzed IFN-γ $^{+}$ CD4^{+} T cells for TNF-α and IL-2 expression. As shown on **Figure-4.4** above, this was done by subdividing IFN-γ $^{+}$ CD4^{+} T cells based on expression of TNF-α and/or IL-2. The gating showed four groups of cells; 1) IFN-γ $^{+}$ TNF-α^{+} IL-2^{-} CD4^{+}, 2) IFN-γ $^{+}$ TNF-α^{-} IL-2^{+} CD4^{+}, 3) IFN-γ $^{+}$ TNF-α^{+} IL-2^{+} CD4^{+}, and 4) IFN-γ $^{+}$ TNF-α^{-} IL-2^{-} CD4^{+}. Group-4 (IFN-γ $^{+}$ TNF-α^{-} IL-2^{-} CD4^{+}) cells are the same as IFN-γ^{+} CD4^{+} single-positive cells that are described above.

Statistical analysis on the percentage of the four groups of cells that exist in stimulated CD4^{+} cells isolated from mice immunized with the individual or fusion antigens showed no significant difference between groups with respect to IFN-γ $^{+}$ TNF-α^{+} IL-2^{-} CD4^{+} and IFN-γ $^{+}$ TNF-α^{-} IL-2^{+} CD4^{+} cells (data not shown).

On the other hand, appreciable difference was seen between some of the antigens with respect to multifunctional CD4^{+} T cells simultaneously expressing all the three Th-1 cytokines, IFN-γ, TNF-α, and IL-2 (**Figure-4.8**). However, no difference was seen in the percentage of these triple positive cells between mice that were immunized with pcDNA-mGMCSF-VAC3 and those of mGMCSF containing individual antigens. On the other hand, mGMCSF containing individual and fusion antigens induced significantly higher percentage of triple positive CD4^{+} cells than pcDNA-mGMCSF control ($p<0.05$). Moreover, pcDNA-VAC3 also induced significantly higher number of triple positive CD4^{+} cells than pcDNA control ($p<0.05$) (**Figure-4.8**).

Figure 4.8: Frequency of multifunctional $CD4^+$ T Cells expressing IFN-γ, TNF-α, and IL-2 at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency of $CD4^+$ T cells simultaneously expressing IFN-γ, TNF-α, and IL-2 in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from mice (five mice per group) three weeks after the last immunization and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean percentage of positive cells and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Immunization with the fusion antigen in the presence or absence of mGMCSF elicits large number of antigen-specific multifunctional $CD4^+$ T cells that simultaneously express the three Th-1 cytokines. Immunization with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-SODB1 also induced these cells in a frequency comparable to that of the fusion.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Percentage
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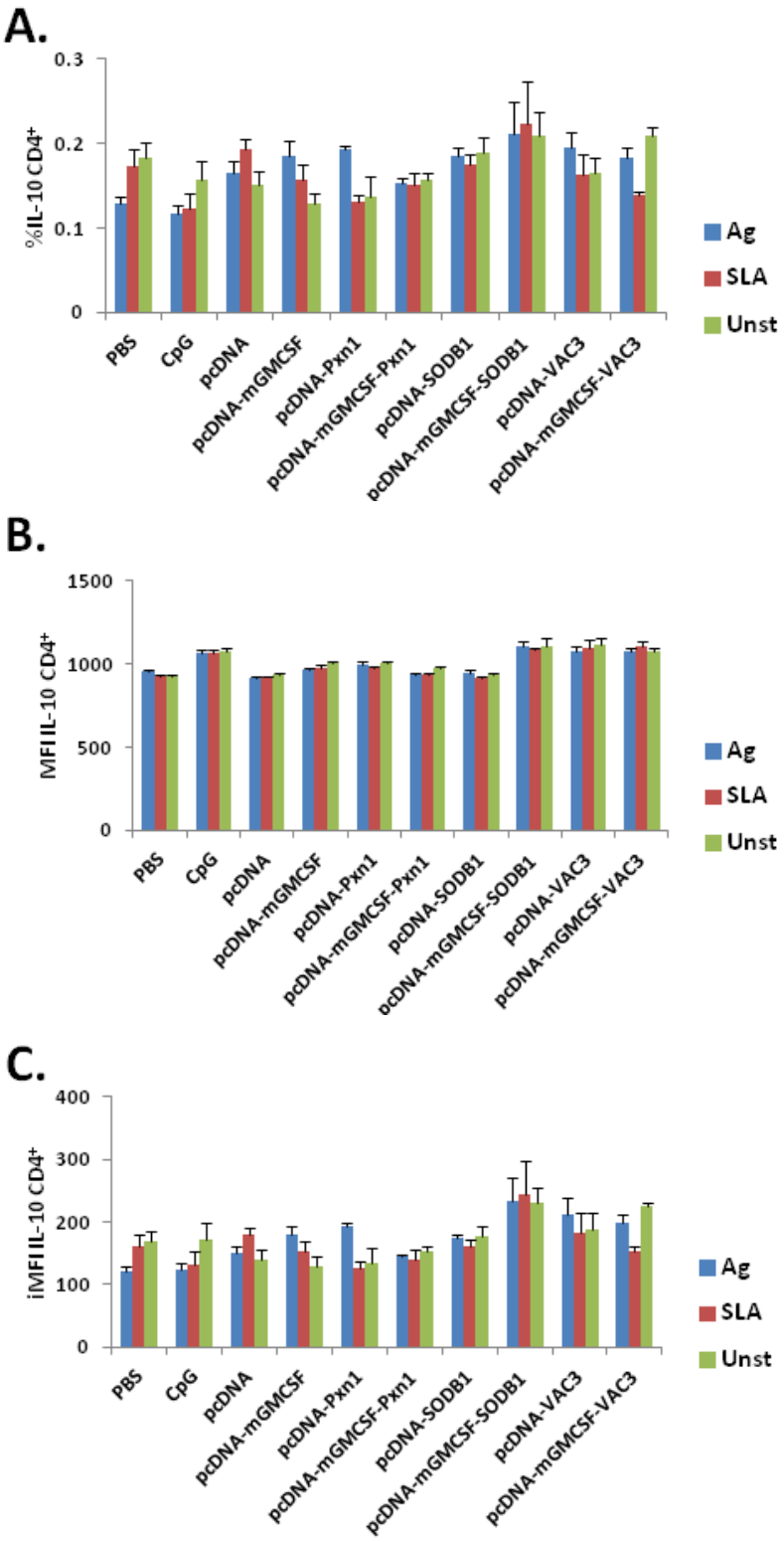
50
40
30
20
10
0
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

In addition to Th-1 cytokines, the level of a Th-2 cytokine was also examined on antigen stimulated spleen cells that were isolated from mice that received the vaccine antigens and controls. The percentage of IL-10 producing $CD4^+$ cells and the level of expression were determined from these cells using flow cytometry. Unlike Th-1 cytokine response, no difference was seen between mice that were immunized with vaccine antigens and controls with regard to the percentage of $CD4^+$ T cells that express IL-10 cytokine (**Figure 4-9A**). Similar pattern was seen with regard to MFI (**Figure 4-9B**) and integrated MFI (**Figure 4-9C**).

Figure 4.9: Antigen-specific IL-10 expressing $CD4^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IL-10 expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from mice (five mice per group) three weeks after the last immunization and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). No significant difference was seen between mice immunized with the vaccine antigens and those that received control injection with respect to IL-10 expression in antigen-specific $CD4^+$ T cells.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pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
B.
MFI IL-10 CD4+
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1000
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pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
C.
iMFI IL-10 CD4+
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300
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pcDNA-mGMCSF-Pxn1
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pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

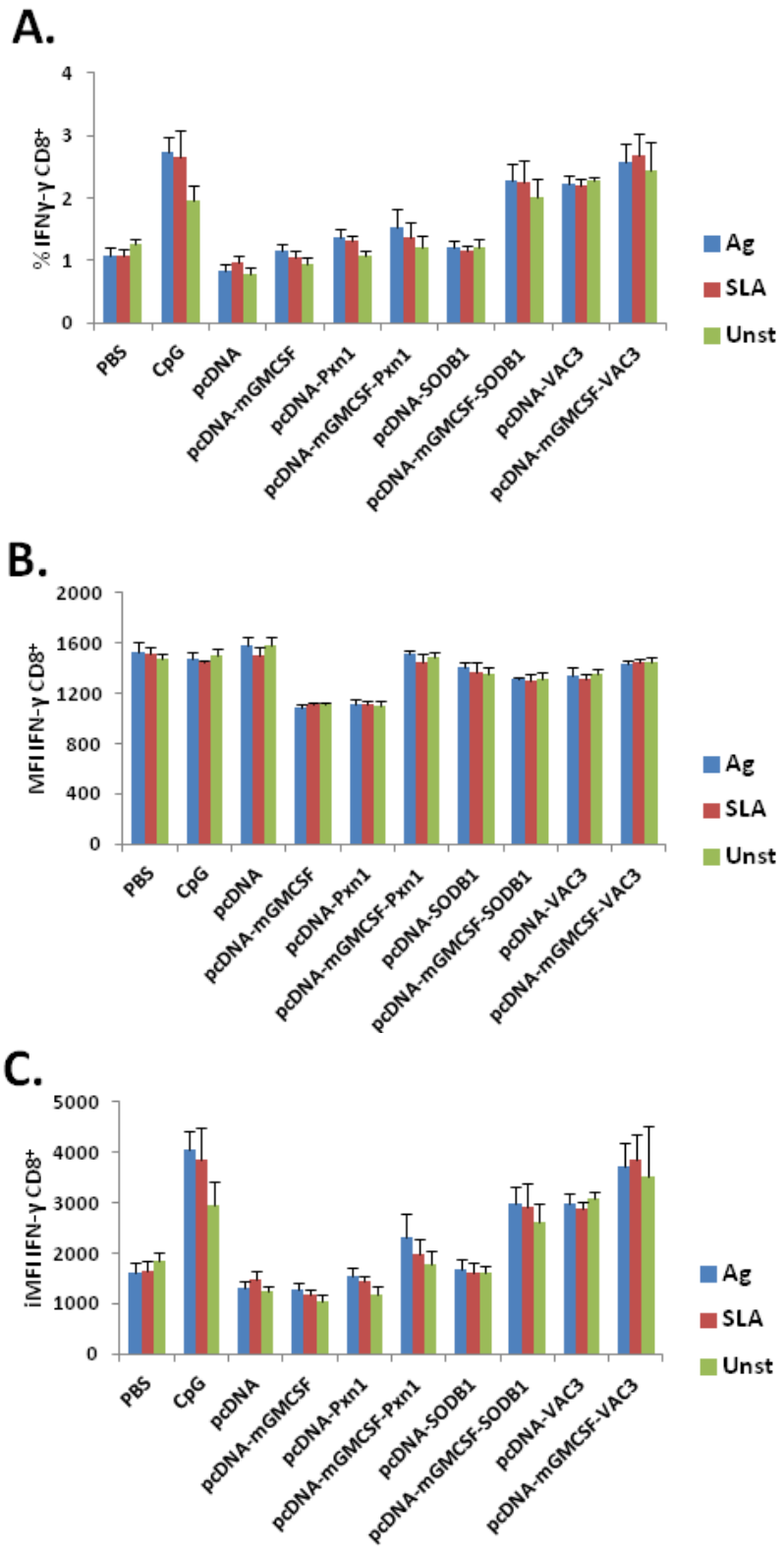
In addition to $CD4^+$ T cells, the percentage of $CD8^+$ T cells expressing different cytokines and the quality of expression was determined from spleen cells isolated from mice that were injected with the vaccine antigens and controls. Unlike the $CD4^+$ response, the $CD8^+$ response did not show clear difference between mice immunized with the vaccine antigens and those that received the control injections. **Figure-4.10A** shows the percentage of IFN-γ expressing $CD8^+$ cells. No statistically significant difference was seen between mice immunized with mGMCSF containing fusion and those that were immunized with individual antigens ($p>0.05$). Whereas, mice immunized with pcDNA-VAC3 induced significantly higher percentage of IFN-γ^+ $CD8^+$ cells than those immunized with individual antigens without mGMCSF ($p<0.05$). With the exception of mice that were immunized with pcDNA-SODB1 and pcDNA-mGMCSF-Pxn1, mice that were immunized with all the vaccine antigens (fusion of individual) induced significantly higher percentage of IFN-γ^+ $CD8^+$ cells as compared to the respective controls ($p<0.05$) (**Figure-4.10A**). With regard to MFI for IFN-γ on $CD8^+$ cells, no significant difference was seen between the vaccinated groups and controls. Only mice that received pcDNA-mGMCSF and pcDNA-Pxn1 showed relatively lower MFI for IFN-γ (**Figure-4.10B**).

Analysis of the integrated MFI for IFN-γ expressed on $CD8^+$ cells showed that cells from all the immunized groups except pcDNA-SODB1 and pcDNA-Pxn1 groups resulted in significantly higher values than the respective controls ($p<0.05$). Moreover, the fusion antigen in the absence of mGMCSF produced significantly higher iMFI for IFN-γ on $CD8^+$ cells than each individual antigen without mGMCSF ($p<0.05$). In the presence of mGMCSF, however, there was no statistically significant difference between the fusion antigen and each individual antigens ($p>0.05$). Mice that received CpG ODN control showed exceptionally high percentage and iMFI

of IFN-γ expressing $CD8^+$ T cells (**Figure-4.10C**). The data suggest that in the absence of mGMCSF fusion, immunization with the fusion antigen is better to induce IFN-γ expressing antigen-specific $CD8^+$ T cells that individual antigens.

Figure 4.10: Antigen-specific IFN-γ expressing $CD8^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IFN-γ expressing $CD8^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown).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pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
B.
MFI IFN-γ CD8+
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1600
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800
400
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
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pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
C.
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4000
3000
2000
1000
0
PBS
CpG
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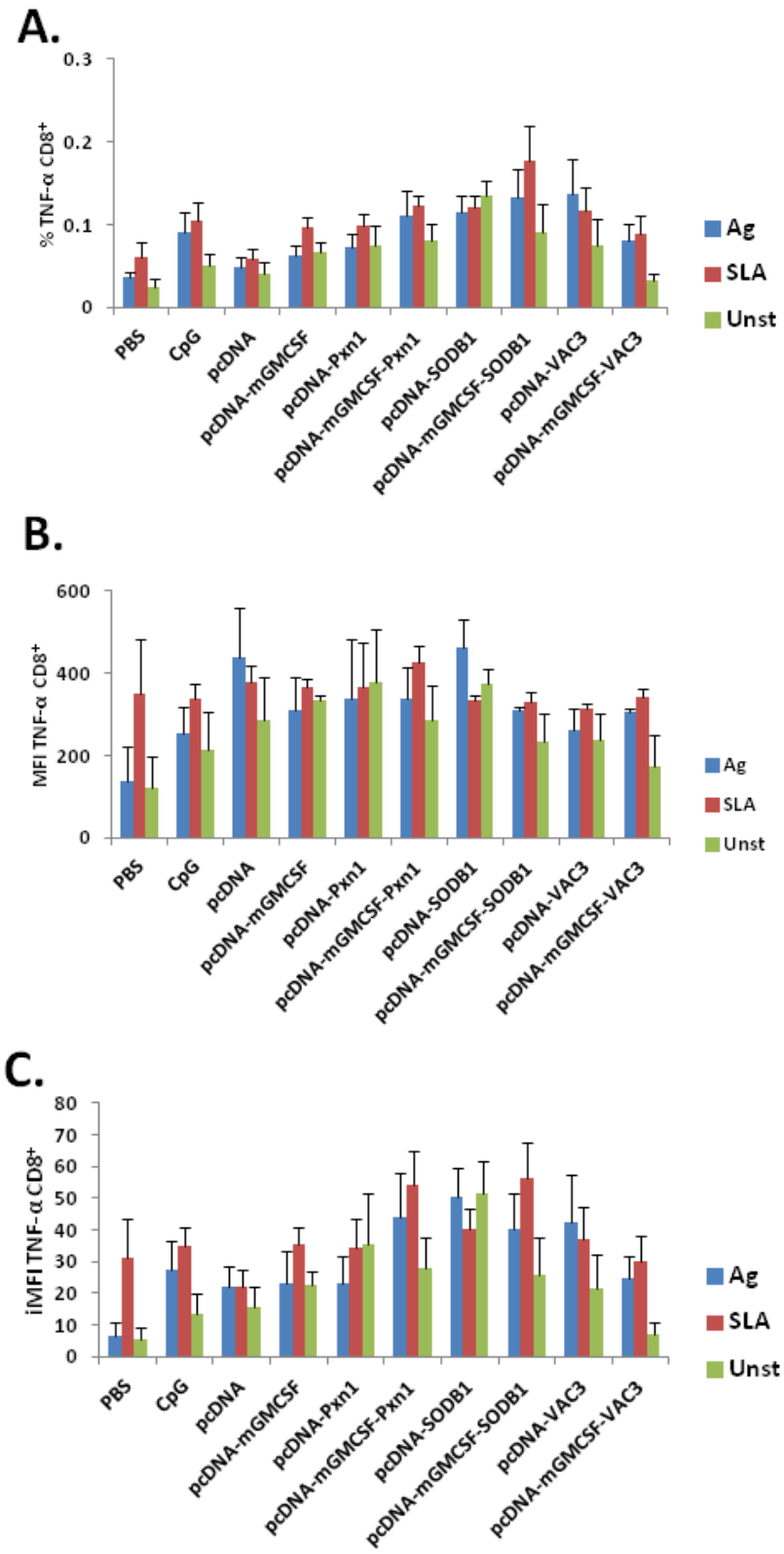
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

Analysis of the percentage of TNF-α expressing $CD8^+$ T cells from antigen-stimulated spleen cells that were isolated from mice immunized with the vaccine antigens and controls shows no statistical difference between any of the groups (**Figure-4. 11A**). Similar result was obtained with respect to MFI (**Figure-4. 11B**) and iMFI (**Figure-4. 11C**) for TNF-α.

With regard to IL-2 expression on $CD8^+$ cells, antigen-stimulated cells that were isolated from mice immunized with the fusion antigen did not show higher percentage of cells than the cell from mice immunized with each of individual antigens. Rather, antigen-stimulated cells that were isolated from mice immunized with pcDNA-mGMCSF-Pxn1 and pcDNA-SODB1 showed appreciably higher percentage of IL-2^+ $CD8^+$ cells. But the difference was not statistically significant (**Figure-4.12A**). MFI for IL-2 also showed that cells from mice immunized with pcDNA-mGMCSF-Pxn1 and pcDNA-SODB1 showed higher value than those cells from mice immunized with the fusion antigen (**Figure-4.12B**). The data from integrated MFI for IL-2 also showed similar pattern (**Figure-4.12C**).

Figure 4.11: Antigen-specific TNF-α expressing $CD8^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of TNF-α expressing $CD8^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Kruskal-Wallis analysis showed no statistically significant difference between any two groups.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pcDNA-mGMCSF-SODB1
pcDNA-VAC3
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SLA
Unst
B.
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pcDNA-mGMCSF-SODB1
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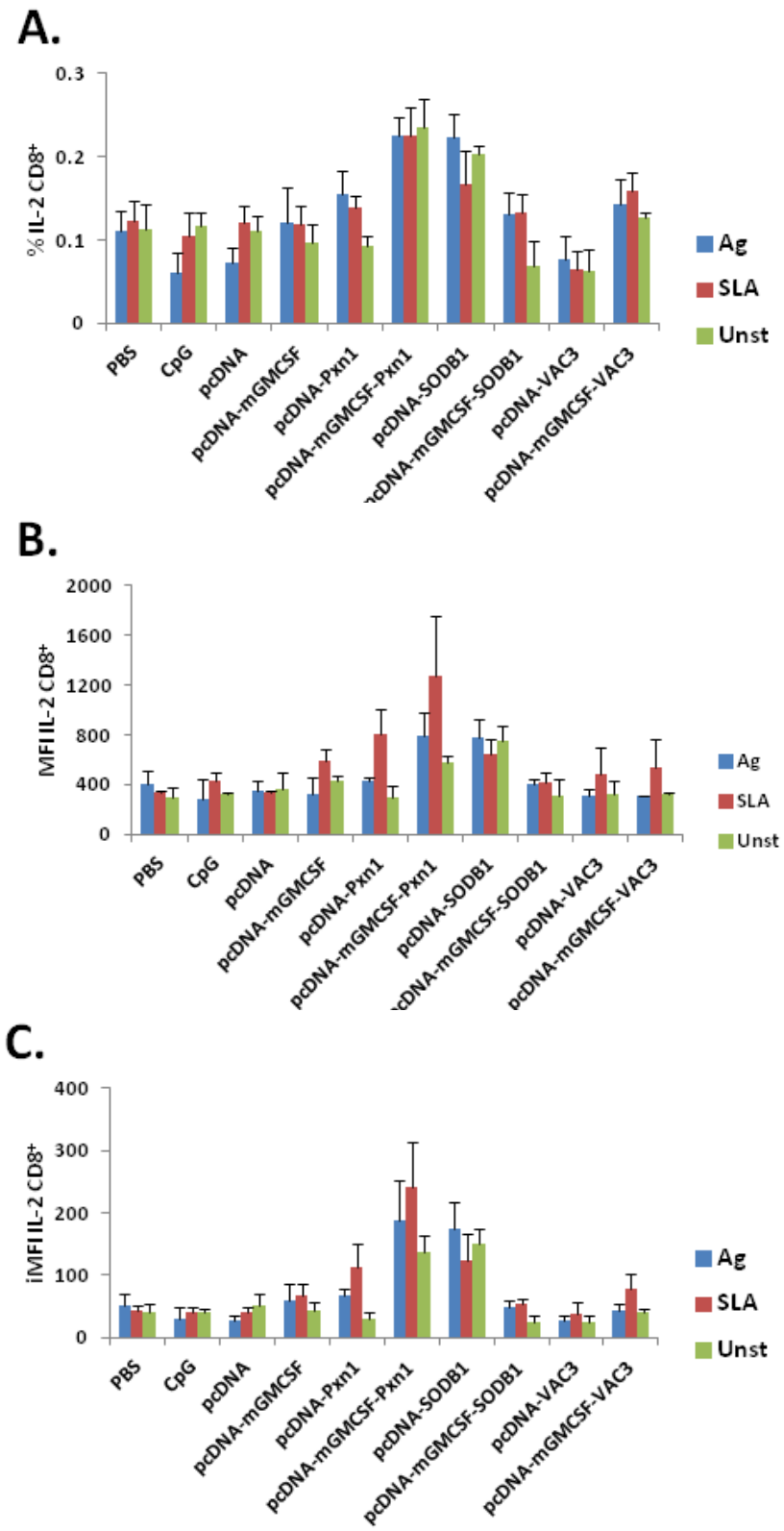
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

Figure 4.12: Antigen-specific IL-2 expressing $CD8^+$ T cells at the time of challenge.

Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IL-2 expressing $CD8^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. Spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Immunization with the fusion antigen did not induce higher number of IL-2 expressing $CD8^+$ T cells.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SLA
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pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

4.2.2 Evaluation of Protective Efficacy of Vaccine Candidates in DNA/Protein Immunization in BALB/c Mice.

4.2.2.1 Antibody Response in DNA/Protein Immunization before and after Challenge Infection

Antibody response to vaccine antigens was assessed on serum samples collected from mice that were injected with vaccine antigens and controls after *L major* challenge infection. The level of total IgG, IgG1 as well as IgG2a was measured on serum samples collected before immunization, three times after the first immunization but prior to challenge infection, three weeks after challenge (Week-12) as well as at the time of euthanasia (Week-17).

Antigen-specific total IgG, IgG1, and IgG2a responses observed prior to *L major* challenge infection were generally similar to what were seen in immunogenicity experiment. Mice immunized with pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 showed the highest antibody response.

At Week-12 (three weeks post parasite challenge), mice immunized with all the vaccine antigens showed high level antigen-specific total IgG (**Figure-4.13A**). Surprisingly, control mice that were injected with pcDNA, saline (PBS), or CpG ODN alone also showed high level total IgG response specific to a combination of rLdPxn1 and LdFeSODB1 protein antigens. However, statistical comparison of antigen-specific total IgG response between mice that were immunized with most of the antigens in the presence or absence of mGMCSF and those mice that received pcDNA and pcDNA-mGMCSF controls, respectively, shows significant difference. With the exception of pcDNA-SODB1 immunized mice, immunization with antigens in the absence of mGMCSF resulted in induction of significantly higher total IgG response than injection with pcDNA control ($p<0.05$). Moreover, all the vaccine antigens with mGMCSF fusion also induced significantly higher total IgG response than pcDNA-mGMCSF control ($p<0.05$). At the time of

euthanasia (labeled as Week-17), all experimental and control mice showed a very high degree of antigen-specific total IgG. There was no statistically significant difference between groups (**Figure-4.13A**).

Figure-4.13B shows the IgG1 response of mice immunized with antigens and those that received control preparations. Like total IgG response, both antigen immunized groups and control mice showed high level IgG1 response to vaccine antigens especially at Week-17. Although the control mice injected with pcDNA and pcDNA-mGMCSF control preparations showed high IgG1 response at Week-12 than Week-9, the level of response was significantly lower than that produced in mice immunized with the vaccine antigens except pcDNA-SODB1 ($p<0.05$). At Week-17, all experimental and control mice produced very high level of IgG1 response (**Figure-4. 13B**).

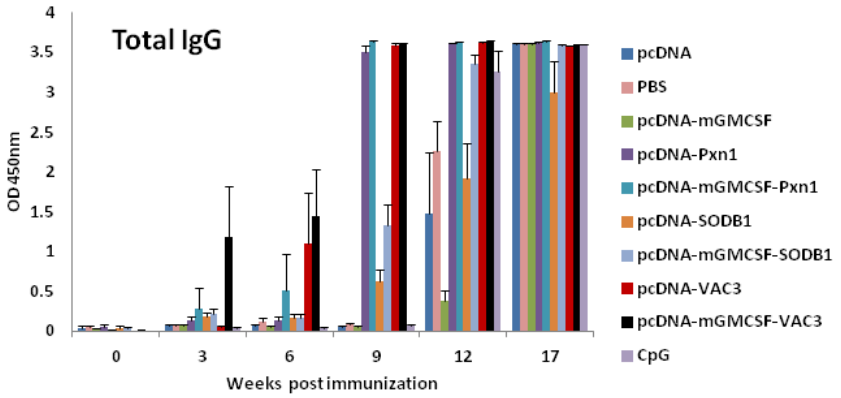
Like total IgG and IgG1 responses, the IgG2a response to vaccine antigens pre-challenge was similar to what was seen in immunogenicity experiment with pcDNA-mGMCSF-VAC3 inducing the highest response (**Figure-4.13C)**. Unlike total IgG and IgG1 response, however, there was very little or no IgG2a response at Week-12 in mice that received control preparations. Except mice that were immunized with pcDNA-SODB1, all the groups that were immunized with individual or fusion antigens produced significantly higher IgG2a response at Week-12 than the respective controls ($p<0.05$). Moreover, mice immunized with the fusion pcDNA-mGMCSF-VAC3 showed significantly higher IgG2a response than those that were immunized with pcDNA-mGMCSF-SODB1 ($p<0.05$). At Week-17, except mice that were immunized with pcDNA-SODB1, mice that were immunized with individual or fusion antigens in the presence or absence of mGMCSF showed significantly higher IgG2a response than pcDNA-mGMCSF or pcDNA, respectively ($p<0.05$). Among the control groups, mice that received CpG ODN control

induced the highest IgG2a response (**Figure-4.13C)**. The result suggests that the fusion antigen and LdPxn1 induce high degree of antigen-specific antibody response before and after infection with *L major*. High degree of total IgG and IgG1 response in the control groups indicates the presence of infection-induced immunity specific to the vaccine antigens.

Figure 4.13: Post-challenge antibody response in DNA/protein prime-boost immunization in BALB/c mice.

Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigote. Serum samples were collected before immunization, at the time of each immunization, at the time of infection, three weeks after infection with *L major* (Week-12) and upon euthanasia (Week-17). Total IgG **(A)**, IgG1 **(B)**, and IgG2a **(C)** were measured using ELISA and the result is depicted as mean OD450nm of five mice per group and standard error of the mean (SEM). Statistical comparison between two groups was performed using Mann-Whitney U test. The data suggests that immunization with the fusion or peroxidoxin 1 induced high level antibody response before challenge infection with *L major* and the response remained high after infection. Antibody response on the control mice after infection suggests the presence of infection-induced immune response specific to *Leishmania* peroxidoxin 1 and/or superoxide dismutase B1. The similarity of the pre-challenge result with the result obtained in the immunogenicity study confirms the reproducibility of the experiment.

A.


Total IgG
OD 450nm
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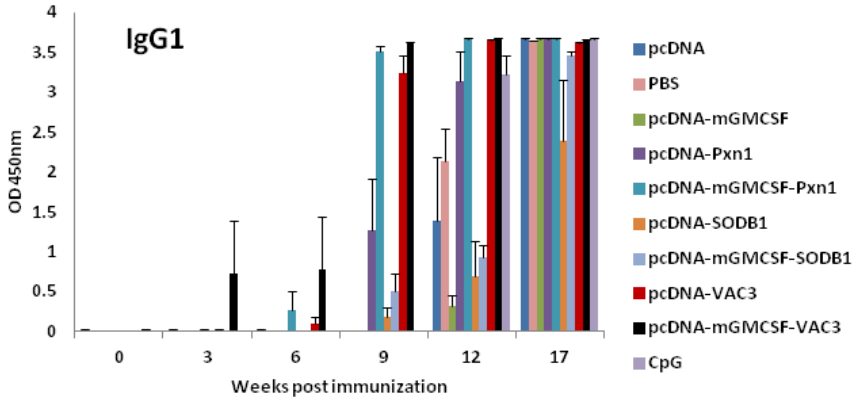
3.5
3
2.5
2
1.5
1
0.5
0
0
3
6
9
12
17
Weeks post immunization
pcDNA
PBS
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
CpG

B.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OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
0
3
6
9
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17
Weeks post immunization
pcDNA
PBS
pcDNA-mGMCSF
pcDNA-Pxn1
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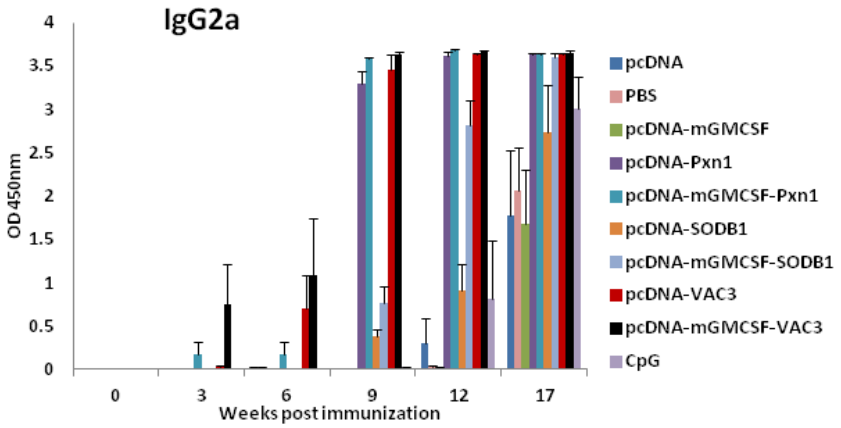
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
CpG

C.


IgG2a
OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
0
3
6
9
12
17
Weeks post immunization
pcDNA
PBS
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
CpG

The level of IgG1 and IgG2a response at Week-17 showed that control mice that were injected with pcDNA, pcDNA-mGMCSF, PBS, and CpG ODN resulted in high level response. The case is much pronounced in IgG1 response than IgG2a. In order to determine the titer of antigen-specific IgG1 and IgG2a isotypes, two-fold serial dilution was done on sera collected from immunized and control mice at Week-17 **(Figure-4.14A and Figure-4.14B)**.

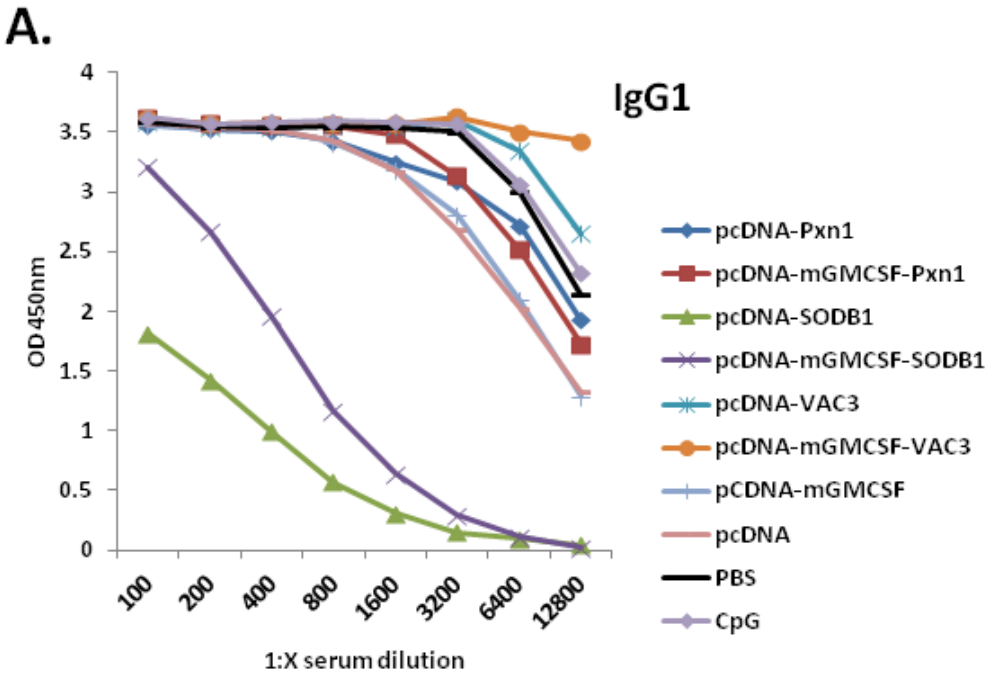
Except in mice that were immunized with pcDNA-SODB1 or pcDNA-mGMCSF-SODB1, sera from all immunized and control mice resulted in high titer for IgG1. The IgG1 level remained similarly high in all groups including controls until 1:1600 dilution. At 1:12800 dilution, sera from pcDNA-VAC3 and pcDNA-mGMCSF-VAC3 immunized mice showed the highest IgG1. At this dilution sera from pcDNA-mGMCSF-VAC3 immunized mice showed significantly higher IgG1 than sera from each group that was immunized with individual antigen or control ($p<0.05$). On the other hand, sera from mice that were immunized with pcDNA-Pxn1 and pcDNA-mGMCSF-Pxn1 showed higher IgG1 at 1:12800 dilution than some of the controls (pcDNA or pcDNA-mGMCSF) but lower than other control groups (PBS or CPG) (**Figure-4.14A**).

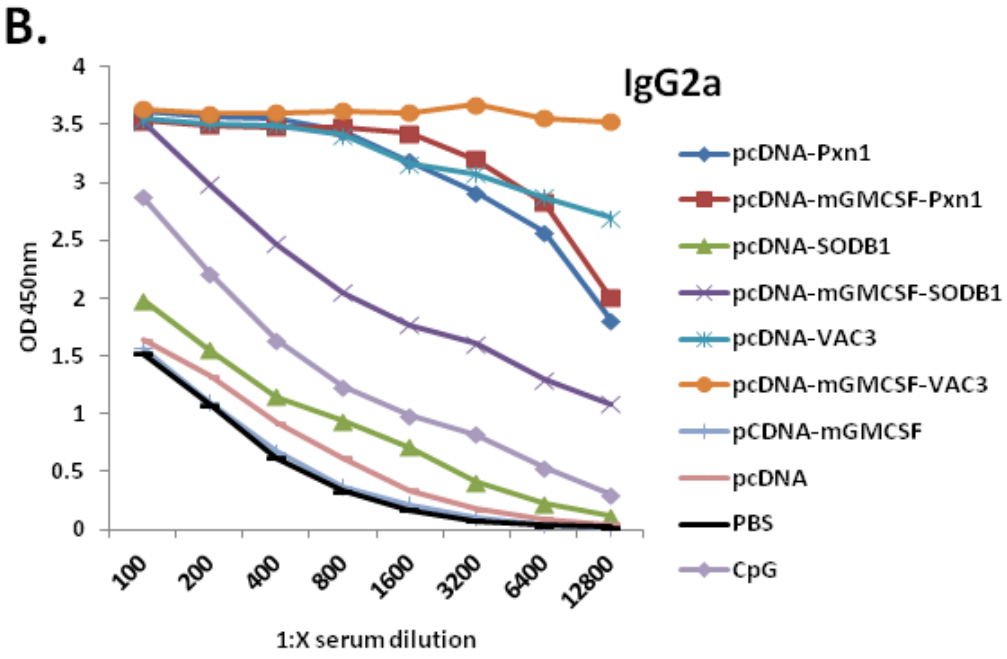
As shown in **Figure-4.13C** above, although it was not as high as most of antigen-immunized groups, control mice showed some degree of IgG2a response. However, titration experiment showed that sera from the control mice had generally lower IgG2a titer than those of antigen-immunized mice (**Figure-4.14B**). In other words, sera from mice that were immunized with the fusion antigen or pcDNA-Pxn1 with or without mGMCSF resulted in high IgG2a titer that remained high even at the highest dilution. On the other hand, the titer of IgG2a remained low in most of the control groups and decreased dramatically at higher dilutions. For instance, although CpG ODN group showed high IgG2a at 1:100 dilution, it decreased steeply in higher dilutions.

At 1:12800 dilution, pcDNA-mGMCSF-VAC3 immunization showed significantly higher IgG2a than immunization with each of the individual antigens or with pcDNA-VAC3 as well as injections with controls such as pcDNA-mGMCSF and CpG ODN ($p<0.05$). However, the difference between pcDNA-VAC3 and pcDNA-Pxn1 was not statistically significant ($p>0.05$). Like IgG1, the titer of IgG2a in sera from mice that were immunized with pcDNA-SODB1 and pcDNA-mGMCSF-SODB1 was generally low (**Figure-4.14B**). The high titer of IgG1 and IgG2a in sera from mice immunized with the fusion suggests that the antigen induces high level mixed Th-1 and Th-2 response. High IgG1 and low IgG2a titers in the control groups suggest that the infection-induced immune response in these groups is predominantly a non-protective phenotype.

Figure 4.14: Titration of post-challenge sera from mice immunized with antigens in DNA/protein prime-boost immunization regimen.

Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. Blood samples were collected at eight weeks after infection (Week-17). The level of IgG1 **(A)** and IgG2a **(B)** was measured using ELISA in a two-fold serially diluted sera. The result is depicted as mean OD450nm of five mice per group. Statistical comparison between groups was performed using Mann-Whitney U test. The data indicate that immunization with the fusion antigen induced high titer IgG1 and IgG2a response suggesting a mixed Th-1 and Th-2 phenotype.


A.
IgG1
OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
12800
1:X serum dilution
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
pCDNA-mGMCSF
pcDNA
PBS
CpG


B.
IgG2a
OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
12800
1:X serum dilution
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
pCDNA-mGMCSF
pcDNA
PBS
CpG

4.2.2.2 Post-Challenge Cytokine Response

In order to assess the cytokine response to vaccine antigens after parasite challenge, five mice per group were sacrificed either 6 or 8 weeks after challenge infection with *L major* promastigotes. Mice in most of the control groups were euthanized at Week-6 post-infection. Whereas, mice immunized with vaccine antigens were sacrificed at Week-8. As in immunogenicity study, spleen cells that were isolated from immunized mice were stimulated with recombinant antigens that correspond to the antigens used for immunization. On the other hand, cells that were isolated from the control mice were stimulated with individual as well as a combination of rLdFeSODB1 and rLdPxn1 proteins. In addition to stimulation with recombinant vaccine antigens, spleen cells from each group were also stimulated with *L major* SLA. Unstimulated cells were used as negative control. After 72hr, the supernatant was collected from each well and ELISA was done to measure IFN-γ and IL-10 recall response.

Figure-4.15A shows IFN-γ response of stimulated and unstimulated spleen cells that were isolated from mice immunized with vaccine antigens and controls. Statistical analysis of IFN-γ production by antigen-stimulated cells from experimental and control groups shows that immunization with most of the vaccine antigens induced significantly higher IFN-γ response than the respective control. In the presence of mGMCSF, both the fusion and individual vaccine candidates induced significantly higher IFN-γ response than pcDNA-mGMCSF control upon stimulation with recombinant proteins ($p<0.05$). Without mGMCSF, only cells from mice that were immunized with the fusion, pcDNA-VAC3, were able to produce significantly higher IFN-γ than cells from mice that were injected with the control, pcDNA ($p<0.05$). Immunization with the fusion pcDNA-mGMCSF-VAC3 also induced significantly higher IFN-γ than that of

individual antigens in the presence of mGMCSF ($p<0.05$). Moreover, this vaccine antigen also induced the production of significantly higher IFN-γ than pcDNA-VAC3 ($p<0.05$) (**Figure-4.15A)**.

Spleen cells from mice that were injected with the controls also produced high level IFN-γ upon stimulation with a combination of rLdFeSODB1 and rLdPxn1. Moreover, *L major* SLA stimulation of spleen cells that were isolated from mice that received the vaccine antigens or controls induced a very high degree of IFN-γ response (**Figure-4.15A)**.

Stimulation of spleen cells from control mice with individual or a combination of antigens resulted in different level of IFN-γ upon *in vitro* stimulation with either of the individual antigens or a combination of the two. In all cases, stimulation with rLdFeSODB1 gave higher IFN-γ than with rLdPxn1. For instance, spleen cells from control mice that were injected with pcDNA-mGMCSF produced 0.22 ± 0.06ng/ml, 1.14 ± 0.44ng/ml, and 2.44 ± 0.5ng/ml IFN-γ upon stimulation with rLdPxn1, rLdFeSODB1 or a combination of rLdPxn1and rLdFeSODB1, respectively. Likewise, in CpG ODN control groups, stimulation with rLdPxn1, rLdFeSODB1 or a combination of rLdPxn1and rLdFeSODB1 produced 0.63 ± 0.07ng/ml, 1.7 ± 0.05ng/ml, and 2.5 ± 0.09ng/ml IFN-γ, respectively. This indicates that more IFN-γ was produced in response to stimulation with rLdFeSODB1 than with rLdPxn1 (**Figure-4.15A)**.

Antigen-stimulated spleen cells from mice immunized with vaccine antigens and controls generally produced low level IL-10. However, as compared with immunization with individual antigens, the fusion antigen in the presence of mGMCSF induced higher IL-10 response. Spleen cells that were isolated from mice that were immunized with pcDNA-mGMCSF-VAC3 induced significantly higher IL-10 than those from pcDNA-mGMCSF injected mice ($p<0.05$). This

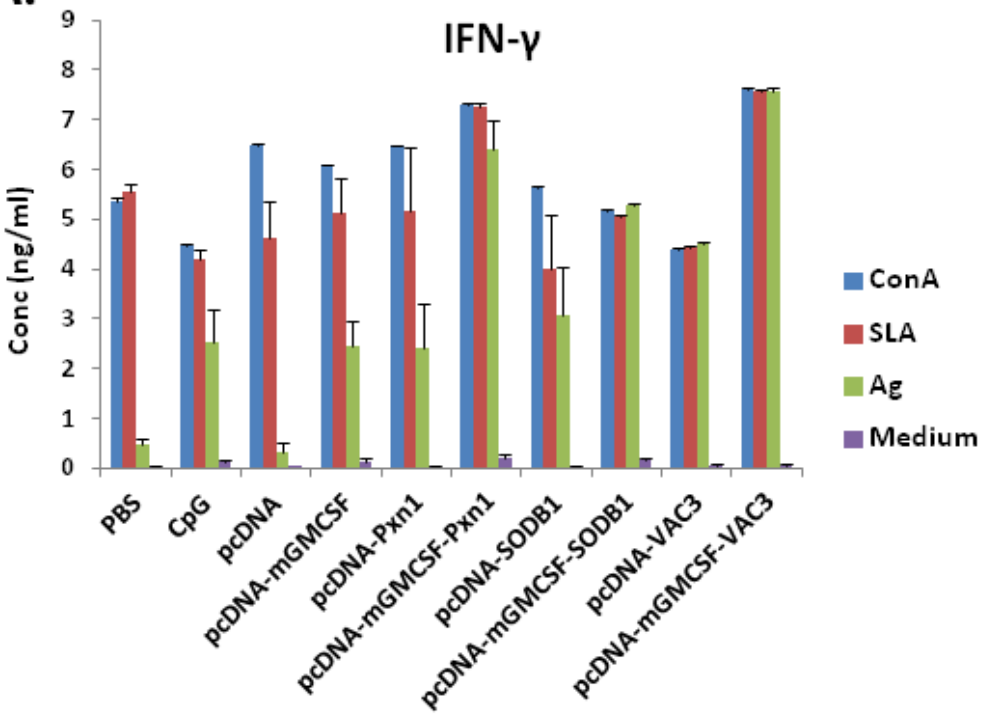
response was also significantly higher than that of pcDNA-mGMCSF-SODB1 immunized mice ($p<0.05$). Cells from both immunized and control mice produced relatively high IL-10 upon stimulation with *L major* SLA (**Figure-4.15B)**.

The ratio of IFN-γ to IL-10 was calculated on both antigen immunized and control mice and summarized in **Table-4.2** below. The data about IFN-γ and IL-10 of the control mice comes from stimulation with a combination of rLdPxn1 and rLdFeSODB1. Like in the pre-challenge experiment, the ratio of IFN-γ to IL-10 is generally higher in antigen immunized mice than those that were injected with controls. However, CpG ODN and pcDNA-mGMCSF controls also showed a high ratio as a result of higher production of IFN-γ.

Figure 4.15: Post-challenge cytokine response of mice immunized with antigens in DNA/protein prime-boost immunization regimen.

IFN-γ **(A)** and IL-10 **(B)** production in stimulated spleen cells was measured using cytokine ELISA. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or concanavalin A (5µg/ml). Unstimulated cells were included as negative control. Cells were cultured at 37°C and 5% CO_2 and culture supernatant was collected at 72hr. The level of IFN-γ and IL-10 was measured using cytokine ELISA kit (BD Biosciences). The concentration of the cytokines (ng/ml) was calculated by correlating the optical density to concentration of the protein standard included in the kit. The mean concentration and standard error of the mean (SEM) of five mice per group was shown. Statistical comparison between groups was performed using the Mann-Whitney U test. Data of antigen stimulated spleen cells indicates that immunization with the fusion or individual antigens induced high level IFN-γ and low IL-10 six or eight weeks after infection. Mice injected with the controls also showed some degree of IFN-γ and IL-10 responses upon *in vitro* stimulation of spleen cells with a combination of rLdPxn1 and rLdSODB1.

A.


IFN-γ
Conc (ng/ml)
9
8
7
6
5
4
3
2
1
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
ConA
SLA
Ag
Medium

B.

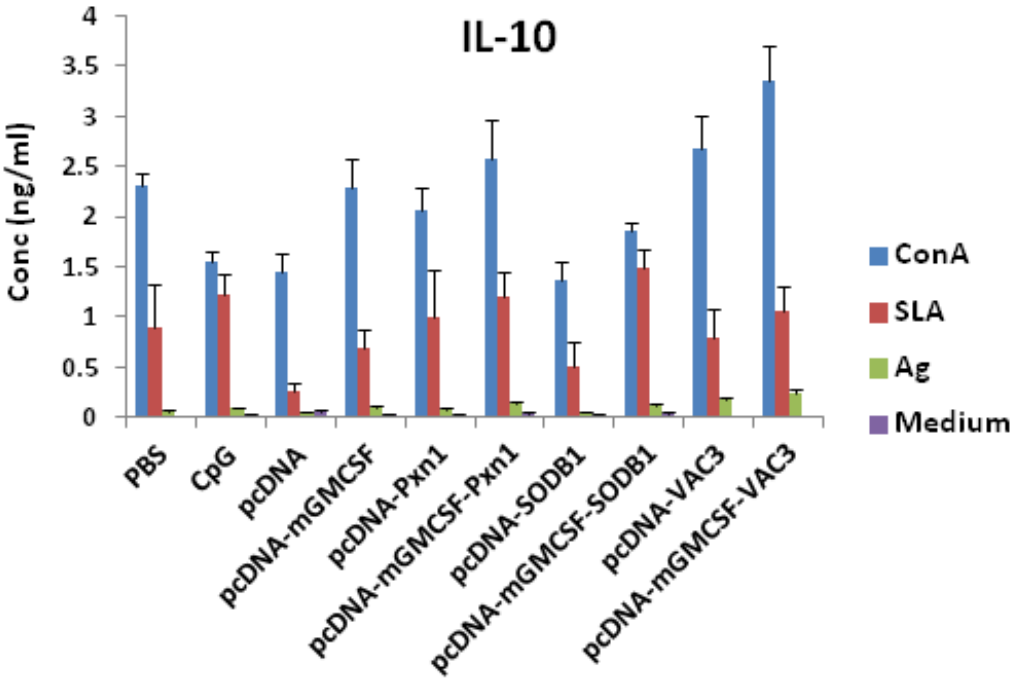

IL-10
Conc (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
ConA
SLA
Ag
Medium

Table 4-2: Post-challenge IFN-γ/IL-10 ratio.

Group	IFN-γ (ng/ml)	IL-10 (ng/ml)	IFN-γ/IL-10 ratio
PBS	0.48	0.055	8.7
CpG	2.54	0.09	28
pcDNA	0.319	0.047	6.78
pcDNA-mGMCSF	2.44	0.1	24.4
pcDNA-Pxn1	2.43	0.078	31
pcDNA-mGMCSF-Pxn1	6.39	0.13	49.2
pcDNA-SODB1	3.08	0.053	58
pcDNA-mGMCSF-SODB1	5.29	0.117	45.2
pcDNA-VAC3	4.5	0.168	26.8
pcDNA-mGMCSF-VAC3	7.59	0.233	32.6

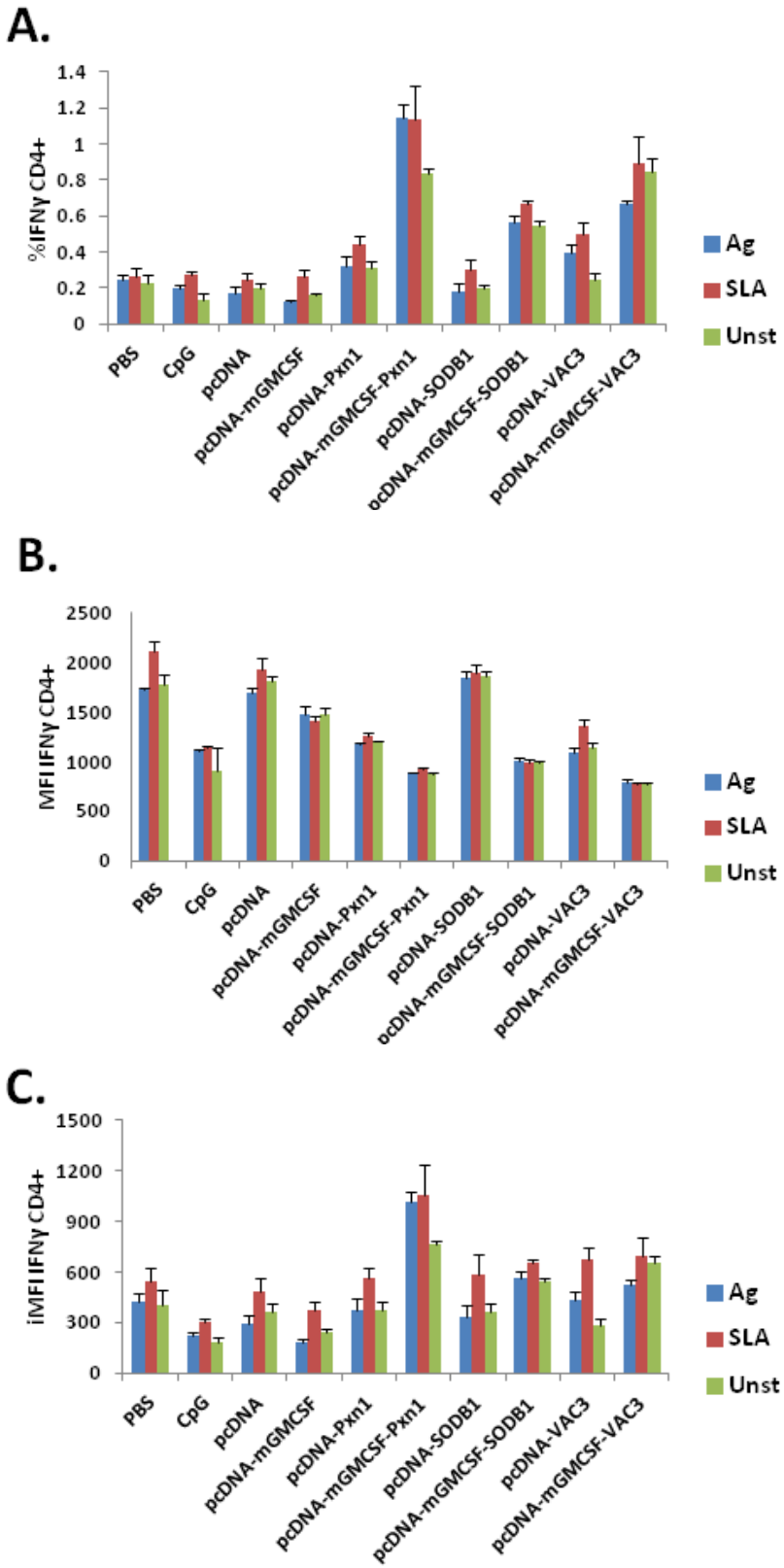
4.2.2.3 Phenotype of Antigen Specific T cells after *Leishmania major* Challenge Infection

In this experiment, we wanted to characterize the phenotype of T cells from spleen cells isolated from mice immunized with antigens and controls six or eight weeks after infectious challenge with *L major*. Like in the immunogenicity experiment, spleen cells were isolated, stimulated with antigen or SLA and stained with fluorescent conjugated antibodies to cell surface molecules and intracellular cytokines. The phenotype of stained cells was analyzed using multiparameter flow cytometry.

Figure-4.16A shows the percentage of IFN-γ^+ $CD4^+$ T cells in antigen or SLA stimulated spleen cells. Mice immunized with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 showed the highest percentage of IFN-γ^+ $CD4^+$ T cells in antigen-stimulated spleen cells. Each group of mice that were immunized with individual or fusion antigen in the presence of mGMCSF fusion showed significantly higher percentage of this phenotype of cells than the control mice that were immunized with pcDNA-mGMCSF ($p<0.05$). Spleen cells from these mice had also significantly higher percentage of IFN-γ^+ $CD4^+$ T cells than those isolated from mice that were immunized with the corresponding antigen without mGMCSF ($p<0.05$). Stimulation with *L major* SLA also showed equal or higher percentage of IFN-γ^+ $CD4^+$ T cells than stimulation with the recombinant protein antigens (**Figure-4.16A**). Unlike the percentage of IFN-γ^+ $CD4^+$ T cells, the MFI for IFN-γ is generally higher in control mice than those immunized with vaccine antigens (**Figure-4.16B**). The integrated MFI (iMFI) for IFN-γ expressed on $CD4^+$ T cells is generally a reflection of the percentage of the cells seen on **Figure-4.16A**. In other words, spleen cells from mice that were immunized with pcDNA-mGMCSF-Pxn1 showed the highest iMFI for IFN-γ. Immunization with both the individual and fusion vaccine candidates in the presence of

mGMCSF resulted in significantly higher iMFI for IFN-γ than pcDNA-mGMCSF control (**Figure-4.16C**). The data shows that the high quality IFN-γ response in mice immunized with pcDNA-mGMCSF-Pxn1 before infection persisted eight weeks after infection with *L major*.

Figure 4.16: Antigen-specific IFN-γ expressing $CD4^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IFN-γ expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Eight weeks after infection, mice immunized with the fusion or individual antigen with mGMCSF showed higher percentage of IFN-γ expressing $CD4^+$ T cells than those immunized with the corresponding antigen without mGMCSF.


A.
%IFNγ CD4+
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
B.
MFI IFNγ CD4+
2500
2000
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1000
500
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PBS
CpG
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pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
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pcDNA-VAC3
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SLA
Unst
C.
iMFI IFNγ CD4+
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1200
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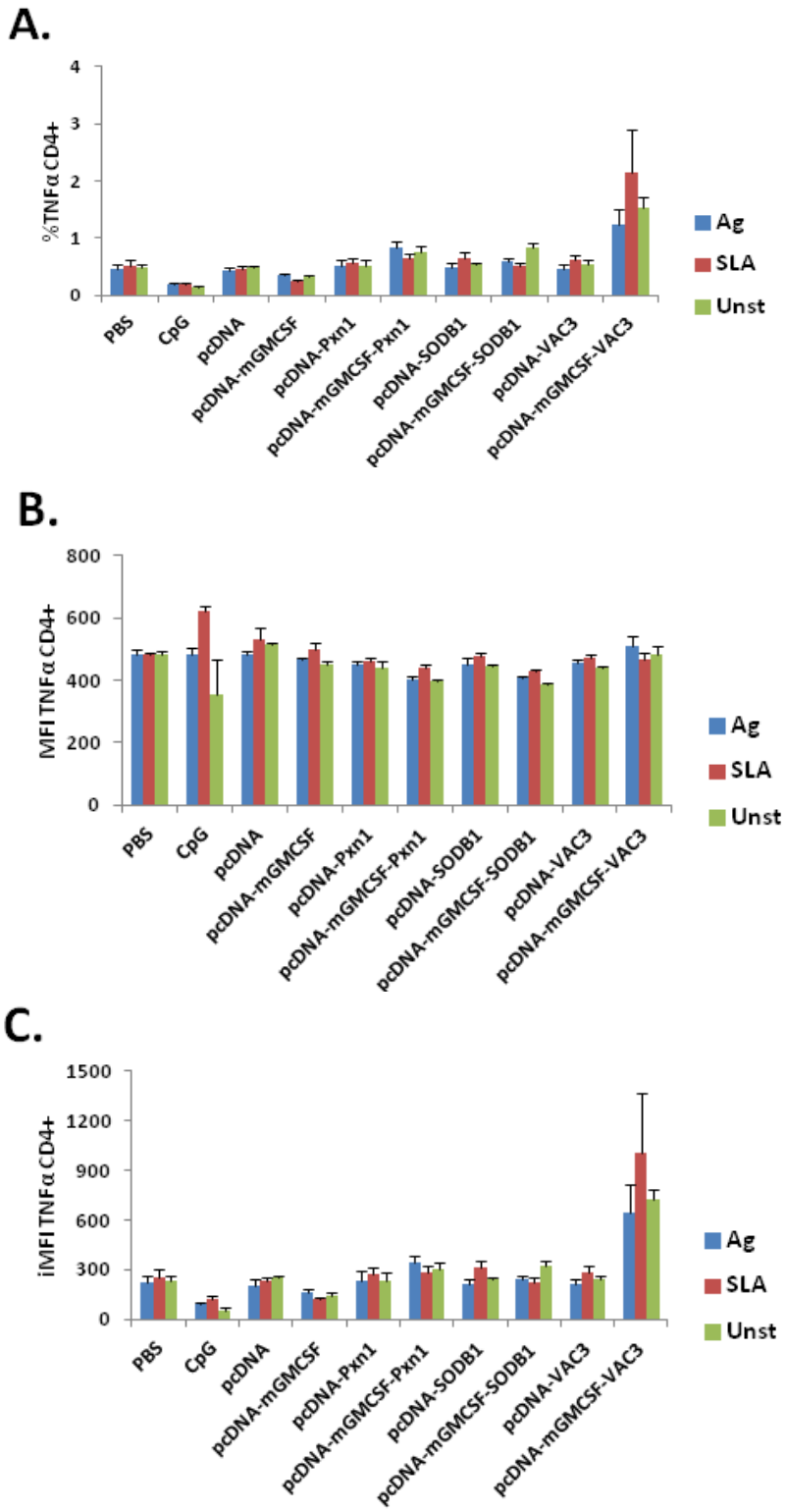
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

The percentage of TNF-α^+ $CD4^+$ T cells and the level of expression of the cytokine were also assessed on stimulated spleen cells. The highest percentage of this phenotype was seen in spleen cells that were isolated from mice immunized with the fusion, pcDNA-mGMCSF-VAC3 **(Figure-4.17A)**. All the vaccine antigens in the presence of mGMCSF adjuvant induced significantly higher percentage of TNF-α^+ $CD4^+$ T cells than the control injection with pcDNA-mGMCSF ($p<0.05$). Mice that were immunized with pcDNA-mGMCSF-VAC3 showed significantly higher percentage of these cells than those that were immunized with pcDNA-mGMCSF-SODB1 or pcDNA-VAC3 ($p<0.05$). Stimulation with *L major* SLA induced even higher response in mice immunized with the fusion antigen in the presence of mGMCSF **(Figure-4.17A)**. Unlike the percentage of TNF-α^+ $CD4^+$ T cells, there was no increase in MFI for TNF-α in spleen cells isolated from mice immunized with the vaccine antigens **(Figure-4.17B)**. With regard to integrated MFI for TNF-α, mice immunized with the pcDNA-mGMCSF-VAC3 and also with pcDNA-mGMCSF-Pxn1 showed higher value than those injected with the control, pcDNA-mGMCSF ($p<0.05$). Moreover, the fusion with mGMCSF induced significantly higher iMFI response that the corresponding antigen without mGMCSF ($p<0.05$) **(Figure-4.17C)**.

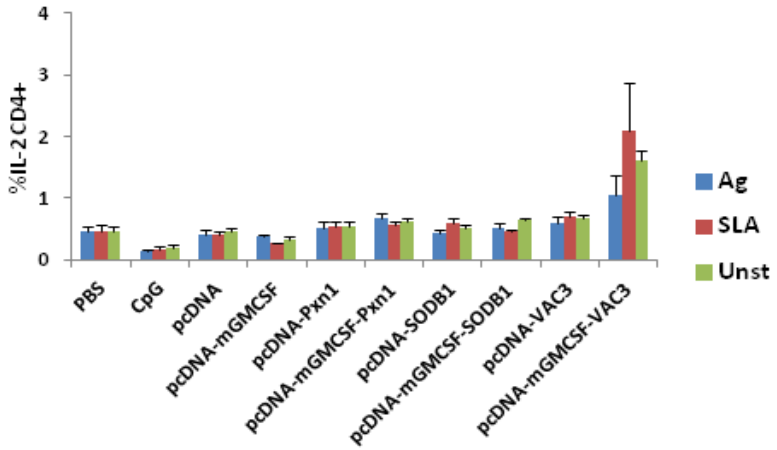
Figure 4.17: Antigen-specific TNF-α expressing $CD4^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of TNF-α expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). In the presence of mGMCSF, the fusion antigen maintained higher number of TNF-α expressing $CD4^+$ T cells eight weeks after challenge infection with *L major*.


A.
%TNFα CD4+
4
3
2
1
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
B.
MFI TNFα CD4+
800
600
400
200
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst
C.
iMFI TNFα CD4+
1500
1200
900
600
300
0
PBS
CpG
pcDNA
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

The pattern of IL-2 response by $CD4^+$ T cells is similar to what was seen in TNF-α response. As shown in **Figure-4.18A**, spleen cells from mice immunized with pcDNA-mGMCSF-VAC3 had the highest percentage of IL-2^+ $CD4^+$ T cells when stimulated with a combination of rLdPxn1 and rLdFeSODB1 or with *L major* SLA. Statistical analysis between experimental groups and controls showed that mice immunized with pcDNA-mGMCSF-VAC3 as well as pcDNA-mGMCSF-Pxn1 resulted in significantly higher percentage of IL-2^+ $CD4^+$ T cells than the control, pcDNA-mGMCSF ($p<0.05$) (**Figure-4.18A**). Like in TNF-α response, the MFI for IL-2 expressed by $CD4^+$ T cells shows no significant difference between antigen stimulated cells from experimental and control groups (**Figure-4.18B**). The integrated MFI for IL-2 is generally the reflection of the percentage of IL-2^+ $CD4^+$ T cells. In the presence of mGMCSF, each group of mice immunized with either of individual antigen or the fusion antigen showed significantly higher iMFI for IL-2 expressed in $CD4^+$ T cells than the control group injected with pcDNA-mGMCSF ($p<0.05$) (**Figure-4.18C**).

Figure 4.18: Antigen-specific IL-2 expressing $CD4^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IL-2 expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Immunization with pcDNA-mGMCSF-VAC3 induced the highest frequency of IL-2 expressing $CD4^+$ T cells.

A.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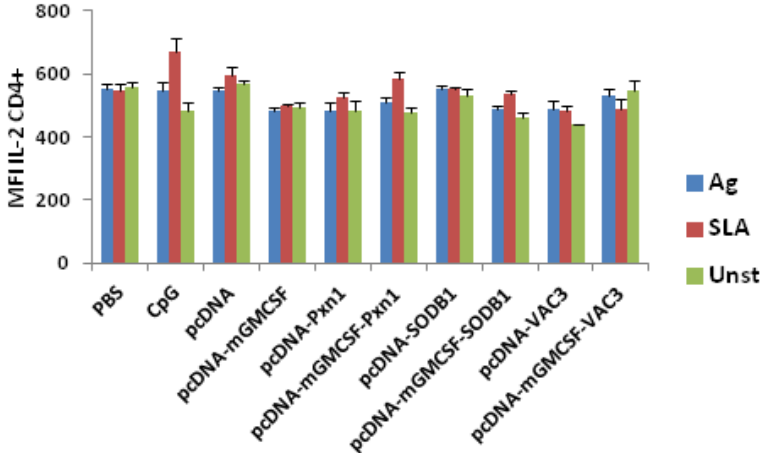
B.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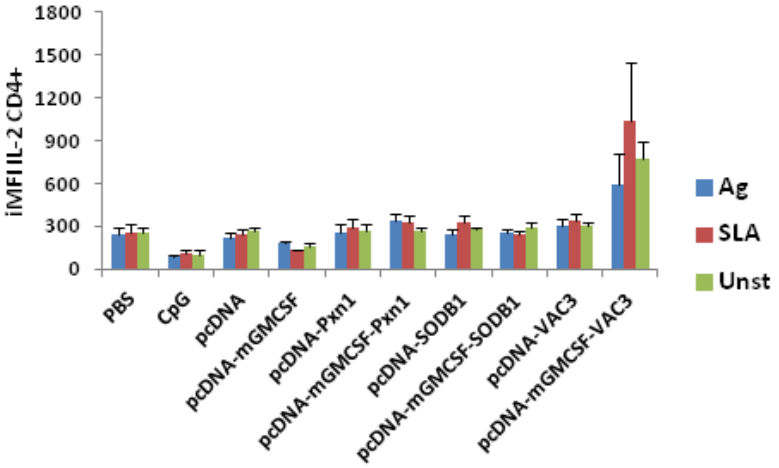
C.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pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
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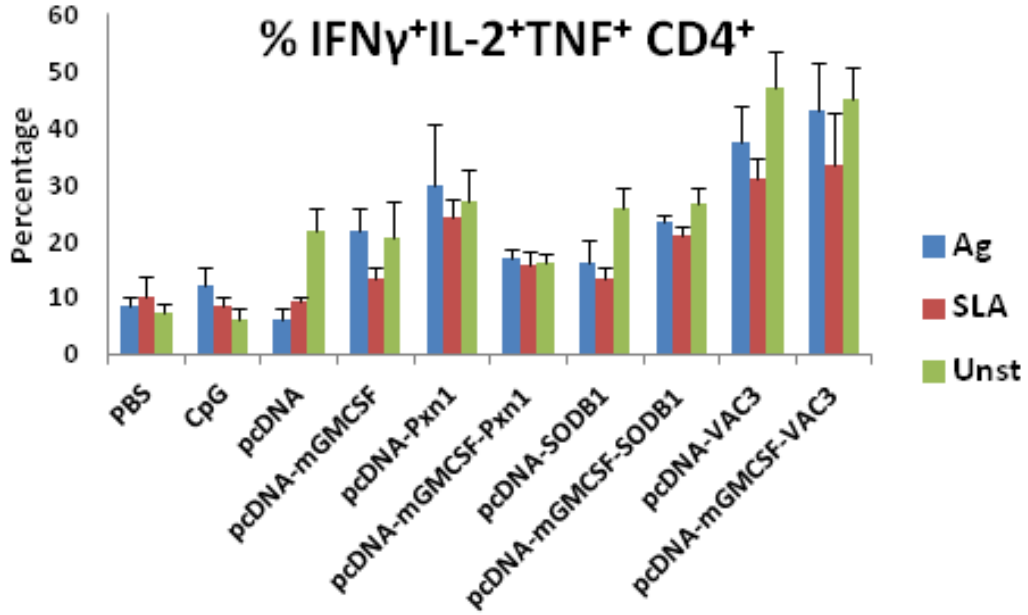
The proportion of multifunctional $CD4^+$ T cells was also analyzed on antigen stimulated spleen cells. $CD4^+$ T cells which were positive for IFN-γ expression was also screened for TNF-α and/or IL-2 expression. Then, the proportion of double positive and triple positive $CD4^+$ T cells was assessed. Statistical comparison shows no significant difference between experimental and control groups with regard to the percentage of IFN-γ^+ TNF-α^+ or IFN-γ^+ IL-2^+ T cells in antigen-stimulated spleen cells (data not shown). With regard to triple positive $CD4^+$ T cells, antigen-stimulated spleen cells that were obtained from mice immunized with pcDNA-Pxn1 and pcDNA-VAC3 showed significantly higher percentage of IFN-γ^+ TNF-α^+ IL-2^+ $CD4^+$ T cells than the control groups that were isolated from mice immunized with pcDNA vector alone ($p<0.05$). Unexpectedly, unstimulated spleen cells showed higher percentage of these cells than antigen or SLA stimulated ones (**Figure-4.19**).

Figure 4.19: Frequency of multifunctional $CD4^+$ T Cells expressing IFN-γ, TNF-α, and IL-2 eight weeks after infection.

Multiparameter flow cytometry was used to determine the frequency of $CD4^+$ T cells simultaneously expressing IFN-γ, TNF-α, and IL-2 in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). The fusion antigen with or without mGMCSF and pcDNA-Pxn1 showed the highest frequency of triple positive $CD4^+$ T cells. Unexpectedly, unstimulated cells showed higher number of these cells than antigen stimulated ones.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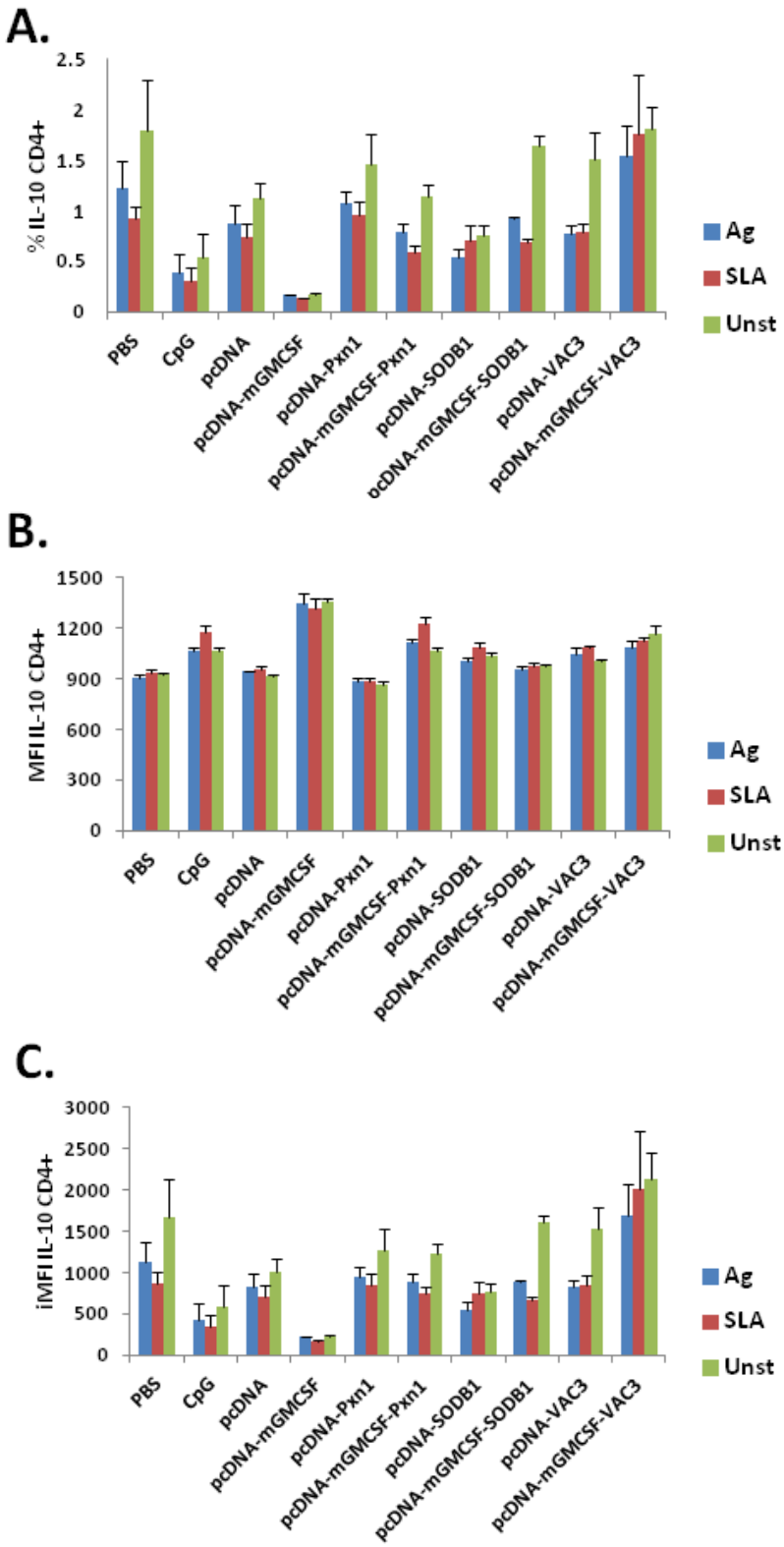
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
Ag
SLA
Unst

The proportion of $CD4^+$ cells expressing IL-10 and the degree of expression the cytokine was also assessed on spleen cells isolated from mice post-challenge with *L major*. **Figure-4.20A** shows the percentage of IL-10^+ $CD4^+$ cells among antigen-stimulated total $CD4^+$ T cells. Cells from mice immunized with pcDNA-mGMCSF-VAC3 had significantly higher proportion of IL-10 positive $CD4^+$ cells than those from mice immunized with either of the individual antigen in the presence of mGMCSF ($p<0.05$). The former also produced higher percentage of IL-10^+ $CD4^+$ T cells than those immunized with the fusion without mGMCSF ($p<0.05$). Cells from mice that were immunized with either the individual or fusion antigen in the absence of mGMCSF did not induce significantly higher percentage of IL-10^+ $CD4^+$ T cells than the respective control, pcDNA ($p>0.05$). Unstimulated cells resulted in higher percentage of these cells than those stimulated with antigen or SLA (**Figure-4.20A**).

Like other phenotypes of cells, the MFI for IL-10 in IL-10^+ $CD4^+$ T cells did not show an increase in experimental groups than controls after challenge infection. Cells from mice that were injected with pcDNA-mGMCSF control showed higher MFI (**Figure-4.20B**). As a result, the pattern of integrated MFI for IL-10 is similar to the one seen in the percentage analysis of these cells (**Figure-4.20C**). The high IL-10 response in mice immunized with the fusion antigen with mGMCSF corroborates the cytokine ELISA results obtained in spleen cells both before and after challenge.

Figure 4.20: Antigen-specific IL-10 expressing $CD4^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IL-10 expressing $CD4^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Immunization with the fusion, pcDNA-mGMCSF-VAC3, elicited the highest proportion of IL-10 expressing $CD4^+$ T cells than other antigens.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pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
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pcDNA-VAC3
pcDNA-mGMCSF-VAC3
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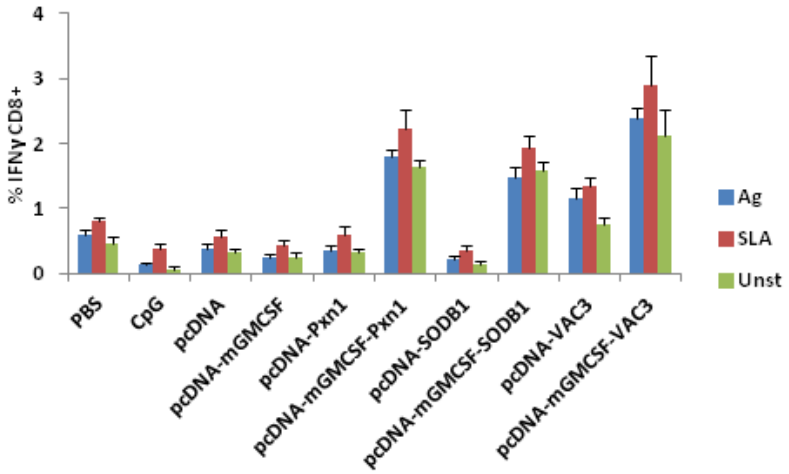
In addition to the $CD4^+$ response, the degree of contribution of $CD8^+$ T cells for cytokine production was also assessed after challenge infection with *L major* in immunized and control mice. Percentage of $CD8^+$ cells expressing different cytokines and the MFI for each cytokines was analysed from antigen/SLA stimulated and unstimulated spleen cells of immunized and control mice.

Figure-4.21A shows the percentage of IFN-γ expressing $CD8^+$ cells among the total $CD8^+$ cells in antigen stimulated/unstimulated spleen cells. Generally, cells from mice immunized with antigens in the presence of mGMCSF fusion showed higher percentage of IFN-γ^+ $CD8^+$ T cells than those immunized in the absence of mGMCSF. Among antigens that did not possess mGMCSF fusion, pcDNA-VAC3 showed the highest percentage of these cells. Statistical analysis shows that cells from mice immunized with each of the antigens showed significantly higher percentage of IFN-γ^+ $CD8^+$ T cells than the respective controls except those from mice immunized with pcDNA-Pxn1 and pcDNA-SODB1 ($p<0.05$). Moreover, the fusion antigen, pcDNA-mGMCSF-VAC3, induced significantly higher percentage of these cells than pcDNA-mGMCSF-SODB1 ($p<0.05$). Although this fusion antigen induced higher percentage of IFN-γ^+ $CD8^+$ T cells than pcDNA-mGMCSF-Pxn1, the difference is not statistically significant. Cells isolated from mice immunized with pcDNA-mGMCSF-VAC3 showed significantly higher percentage than those from mice immunized the same antigen in the absence of mGMCSF ($p<0.05$). On the other hand, the fusion antigen in the absence of mGMCSF induced higher percentage of IFN-γ^+ $CD8^+$ T cells than each of the individual antigens in the absence of mGMCSF ($p<0.05$) (**Figure-4.21A**).

MFI for IFN-γ^+ expressed on $CD8^+$ T cells showed generally similar data between samples taken from different groups of immunized and control mice. Only cells obtained from mice immunized with pcDNA-SODB1 showed unusually high MFI reading (**Figure-4.21B**). The value of iMFI for IFN-γ from cells that were isolated from mice immunized with individual and fusion antigens is consistent with the pattern seen in the percentage of IFN-γ^+ $CD8^+$ T cells. Comparison of iMFI of mice immunized with mGMCSF containing fusion antigen with each of individual antigens showed that the fusion antigen resulted in significantly higher iMFI than pcDNA-mGMCSF-Pxn1 but not pcDNA-mGMCSF-SODB1 (**Figure-4.21C**). Generally, mGMCSF containing antigens induced more IFN-γ expressing $CD8^+$ T cells than the corresponding antigens without mGMCSF.

Figure 4.21: Antigen-specific IFN-γ expressing $CD8^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IFN-γ expressing $CD8^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). The presence of mGMCSF appears to increase the frequency of IFN-γ expressing $CD8^+$ T cells.

A.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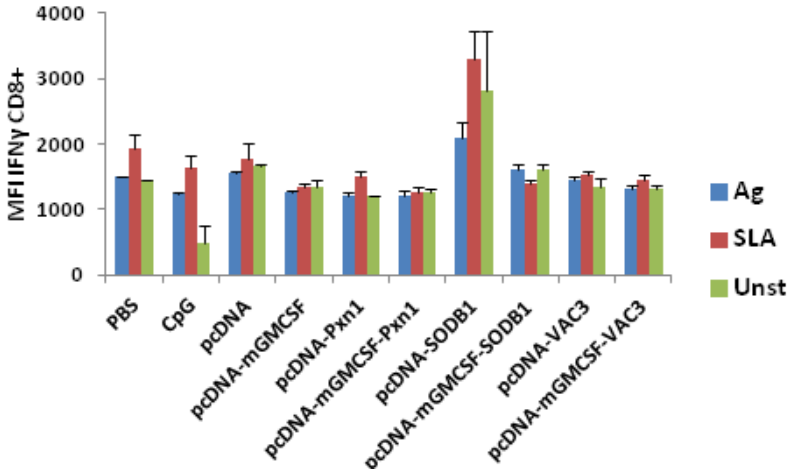
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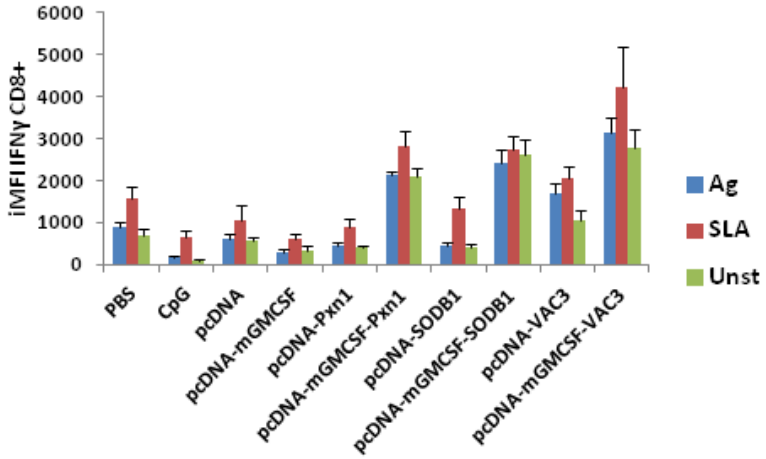
C.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pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
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Analysis of TNF-α expression by $CD8^+$ T cells showed that the percentage of TNF-α^+ $CD8^+$ T cells is highest in cells isolated from mice immunized with the fusion antigen in the presence of mGMCSF. As shown in **Figure-4.22A**, the percentage of TNF-α^+ $CD8^+$ T cells among antigen-specific $CD8^+$ cells is significantly higher in pcDNA-mGMCSF-VAC3 immunization than that of injection of the control pcDNA-mGMCSF or immunization with each of the individual antigens with mGMCSF fusion ($p<0.05$). In the absence of mGMCSF, each individual antigen induced significantly higher percentage of TNF-α^+ $CD8^+$ T cells than the control, pcDNA ($p<0.05$). However, immunization with pcDNA-VAC3 did not induce higher response than the control (**Figure-4.22A**). With regard to MFI, the fusion antigen with mGMCSF did not induce higher response (**Figure-4.22B**). The high frequency of TNF-α expressing $CD8^+$ T cells in pcDNA-mGMCSF-VAC3 immunized mice resulted in a proportional increase in the iMFI in spleen cells collected from these mice (**Figure-4.22C**). The data demonstrate that the fusion vaccine with mGMCSF induced better TNF-α response by $CD8^+$ T cells.

Figure 4.22: Antigen-specific TNF-α expressing $CD8^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of TNF-α expressing $CD8^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Mice immunized with pcDNA-mGMCSF-VAC3 resulted in higher number of TNF-α expressing $CD8^+$ T cells than those immunized with other antigens.

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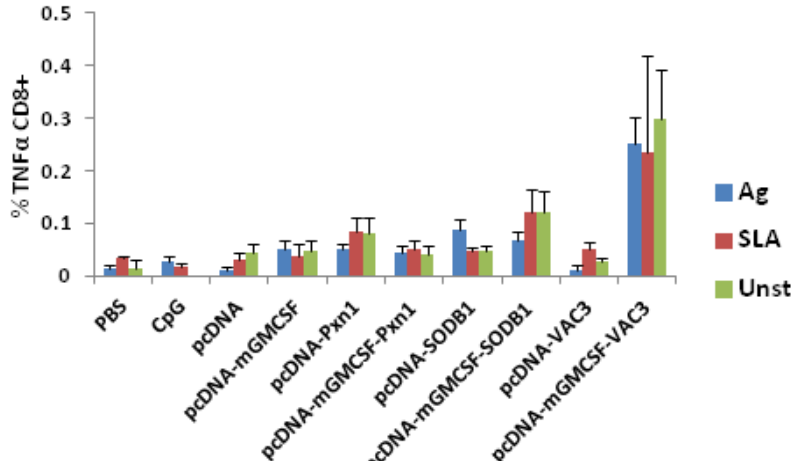
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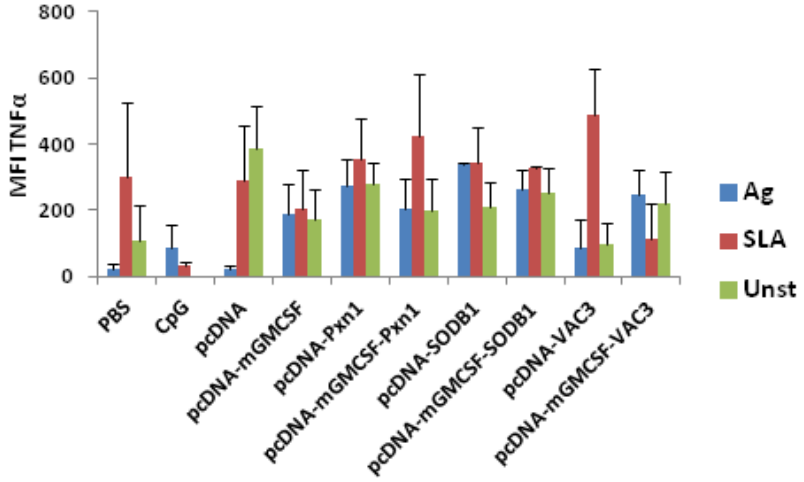
B.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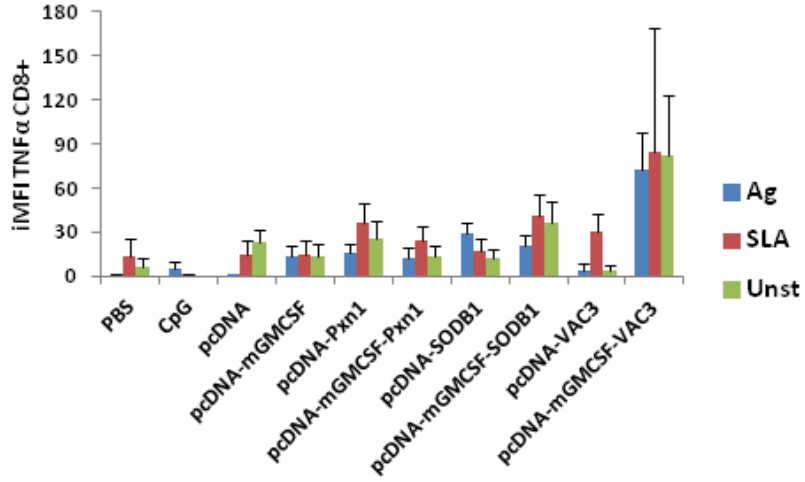
C.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pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3
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IL-2 expression on antigen stimulated $CD8^+$ T cells indicated that immunization with the fusion with mGMCSF, pcDNA-mGMCSF-VAC3, induced higher percentage of IL-2^+ $CD8^+$ T cells than the individual antigens or the control. However, this difference is not statistically significant ($p<0.05$). Stimulation with *L major* SLA induced a response higher than that of antigen stimulation (**Figure-4.23A**). With regard to MFI for IL-2 of antigen stimulated $CD8^+$ cells, the fusion antigen containing mGMCSF resulted in lower response that is even lower than the control. Again, SLA stimulation resulted in higher MFI for IL-2 than stimulation with the recombinant antigen (s) in samples from both experimental and control mice (**Figure-4.23B**). On the other hand, no statistically significant difference is seen between antigen immunized and control mice with regard to integrated MFI for IL-2 expressed on $CD8^+$ cells (**Figure-4.23C**).

Figure 4.23: Antigen-specific IL-2 expressing $CD8^+$ T cells eight weeks after infection. Multiparameter flow cytometry was used to determine the frequency **(A)**, median fluorescent intensity (MFI) **(B)**, and integrated MFI **(C)** of IL-2 expressing $CD8^+$ T cells in antigen-stimulated and control spleen cells. Mice were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigotes. After euthanasia (Week-17), spleen cells were isolated from immunized mice and controls (five mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or phorbol myristate acetate (PMA) (5ng/ml)/ionomycin (500ng/ml). Unstimulated cells were included as negative control. Cells were cultured at 37^oC and 5% CO_2. Multiparameter flow cytometry was performed on stimulated spleen cells that were stained with fluorochrome conjugated antibodies specific to surface markers and intracellular cytokines. The mean and standard error of the mean (SEM) of five mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. Spleen cells stimulated with PMA/ionomycin resulted in consistently high reading (data not shown). Antigen stimulated cells from mice immunized with pcDNA-mGMCSF-VAC3 induced higher frequency of IL-2 expressing $CD8^+$ T cells than those from mice immunized with individual antigens.

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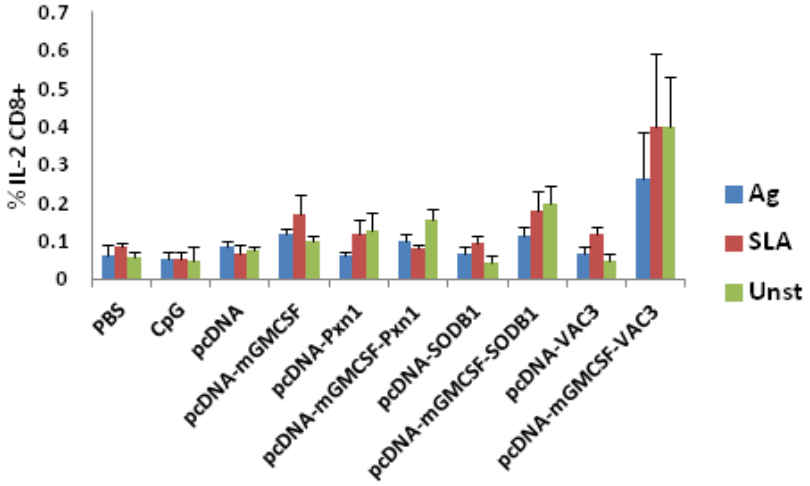
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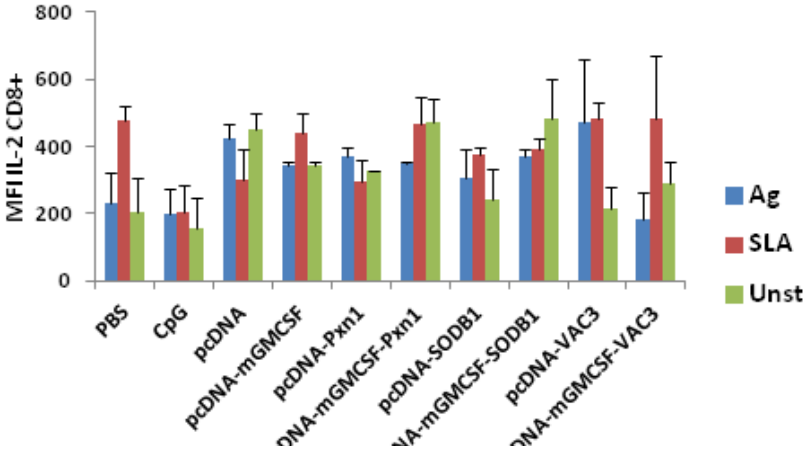
B.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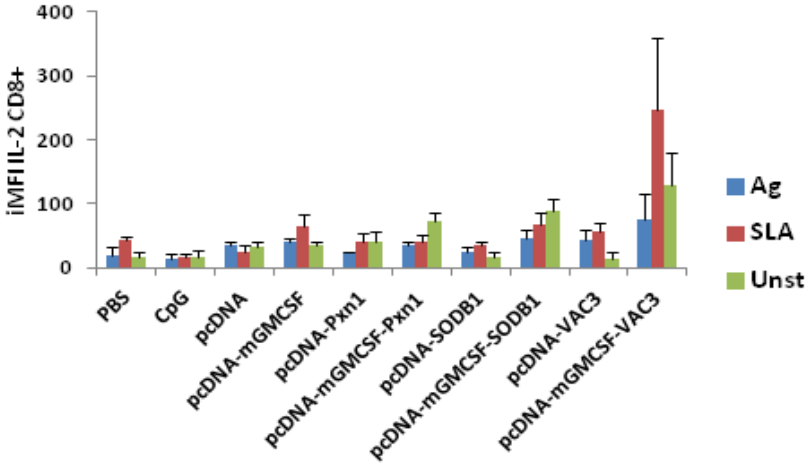
C.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pcDNA-mGMCSF-VAC3
Ag
SLA
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4.2.2.4 Footpad Swelling and Parasite Load in DNA/Protein Immunization

Protective efficacy of the vaccine antigens was evaluated by measuring footpad swelling every week for eight weeks after challenge infection with 3 x 10^6 stationary phase promastigotes of *Leishmania major* strain V1 (MHOM/IL/80/Friedlin). Moreover, the parasite load was determined by performing limiting dilution assay of infected footpad.

As shown in **Figure-4.24**, control mice that received pcDNA vector alone, pcDNA-mGMCSF, CpG alone, or saline (PBS) developed the highest footpad lesion that progressively increased over time. Except pcDNA-mGMCSF injected ones, all the control mice had to be euthanized at Week-6 post-challenge because most of them developed lesions with a net increase of about 3mm in thickness. This is in line with the ethical standard we put in our animal experiment protocol. On the contrary, mice that were immunized with vaccine antigens developed lower footpad lesion over the entire period of the experiment (**Figure-4.24**).

Until Week-5 post-immunization, mice that received both individual and fusion vaccine antigens in the presence or absence of mGMCSF fusion showed similar lesion size. At Week-6 post challenge, all the vaccine groups except pcDNA-mGMCSF-SODB1 immunized ones developed significantly smaller lesion than that of the control groups. At Week-8 post immunization, mice that were immunized pcDNA-mGMCSF-Pxn1 had the least footpad thickness followed by those immunized with pcDNA-mGMCSF-VAC3. Both groups developed lesions that were significantly smaller in size than those of control mice ($p<0.05$). In the absence of mGMCSF, only mice immunized with pcDNA-Pxn1 and pcDNA-SODB1 developed significantly smaller footpad lesion at Week-8 than pcDNA control at Week-6 ($p<0.05$). Although the lesions developed by mice that were immunized with pcDNA-VAC3 at Week-8 was smaller than lesions developed by mice that were injected with pcDNA control plasmid, the difference is not

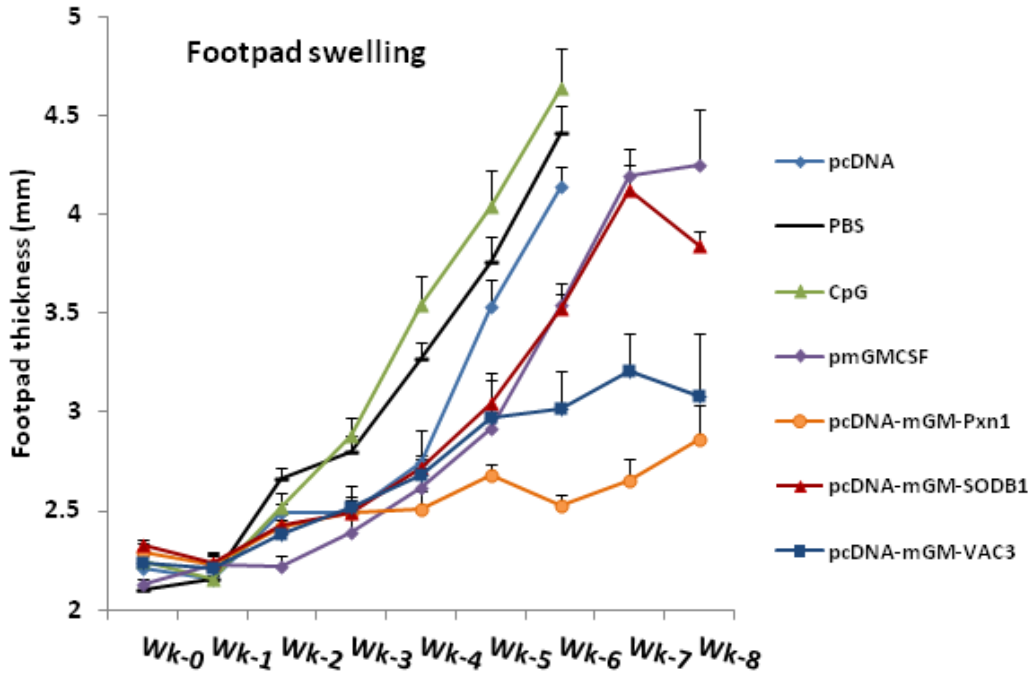
statistically significant ($p>0.05$) (**Figure-4.24**). The relatively higher protection seen mice immunized with pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 reflects the high Th-1 cytokine expression by $CD4^+$ T cells at the time of parasite challenge. That is, spleen cells from these mice showed high frequency of IFN-γ^+CD4^+, TNF-α^+CD4^+ and IL-2^+CD4^+ T cells.

Parasite load was determined from the whole footpad of infected leg using 10-fold serial dilution of the homogenate on a 96-well plate. The result showed that mice immunized with the fusion pcDNA-mGMCSF-VAC3 as well as those immunized with pcDNA-mGMCSF-Pxn1 antigens in DNA prime/recombinant protein boost regimen developed lesions with significantly lower parasite load than control mice that received pcDNA-mGMCSF alone ($p<0.05$). The parasite load in footpads of mice that received the other antigens did not show a significant reduction in the parasite number as compared to the respective control ($p>0.05$) (**Figure-4.25**). Lower parasite number in pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 immunized groups substantiates the footpad swelling result.

Figure 4.24: Footpad swelling of mice immunized with antigens and controls in DNA/protein immunization strategy.

BALB/c mice (five per group) were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with subcutaneous injection of 3 x 10^6 stationary phase *L major* promastigotes in the hind footpad. The footpad swelling was assessed by measuring the thickness of infected footpad weekly for eight weeks using electronic digital caliper. The data represents mean footpad size in millimetre of five mice and standard error of the mean. **(A)** Footpad swelling of mice immunized with antigens with mGMCSF fusion and controls; and **(B)** footpad swelling of mice immunized with antigens without mGMCSF. Immunization with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 resulted in the least footpad swelling.

A.


Footpad swelling
Footpad thickness (mm)
5
4.5
4
3.5
3
2.5
2
Wk-0 Wk-1 Wk-2 Wk-3 Wk-4 Wk-5 Wk-6 Wk-7 Wk-8
pcDNA
PBS
CpG
pmGMCSF
pcDNA-mGM-Pxn1
pcDNA-mGM-SODB1
pcDNA-mGM-VAC3

B.

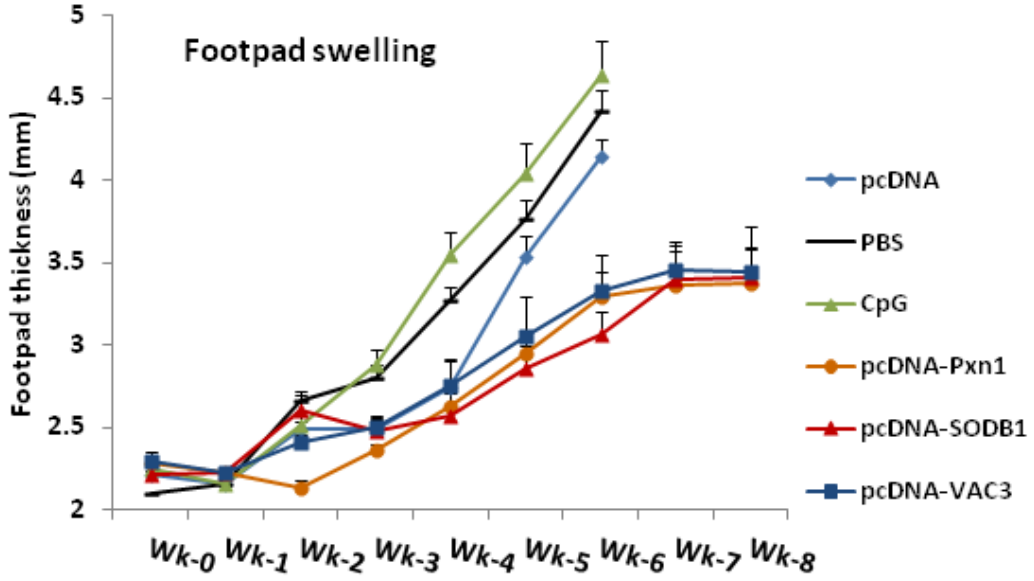
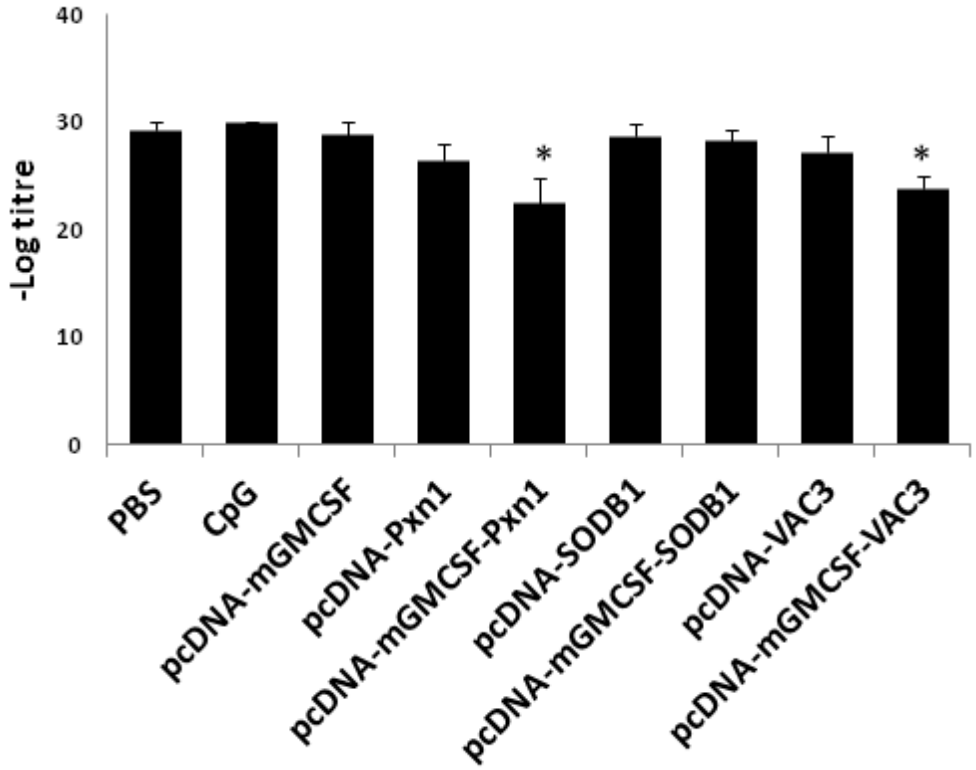

Footpad swelling
Footpad thickness (mm)
5
4.5
4
3.5
3
2.5
2
Wk-0 Wk-1 Wk-2 Wk-3 Wk-4 Wk-5 Wk-6 Wk-7 Wk-8
pcDNA
PBS
CpG
pcDNA-Pxn1
pcDNA-SODB1
pcDNA-VAC3

Figure 4.25: Footpad parasite load in mice immunized with antigens and controls in DNA/protein immunization strategy.

BALB/c mice (five per group) were immunized twice with DNA antigens and controls in the presence of CpG ODN adjuvant followed by a boost with the respective recombinant protein. All the immunizations were given in three week intervals. At Week-9, all mice were infected on the footpad with subcutaneous injection of 3 x 10^6 stationary phase *L major* promastigotes in the hind footpad. Eight (or six) weeks after infection, the footpad tissue was homogenized using grinding chamber and pestle. The homogenate was inoculated, in duplicate, into biphasic medium prepared using NNN agar slant with 10% rabbit blood overlaid with M199 medium in 96-well plate. The parasite load was determined by limiting dilution assay. The data represents mean and SEM of the reciprocal titer of five mice. Immunization with pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 showed significantly smaller footpad parasite load than other antigens.


40
30
20
10
0
-Log titre
*
*
PBS
CpG
pcDNA-mGMCSF
pcDNA-Pxn1
pcDNA-mGMCSF-Pxn1
pcDNA-SODB1
pcDNA-mGMCSF-SODB1
pcDNA-VAC3
pcDNA-mGMCSF-VAC3

4.3 Experimental Discussion

In this study, we assessed the immunogenicity and protective efficacy of fusion *Leishmania* peroxidoxin 1 and superoxide dismutase B1 in DNA priming and recombinant protein boost immunization strategy. In previous studies in our lab, we have shown that each of the individual antigens is immunogenic in mice and are capable of partially protecting susceptible BALB/c mice from experimental infection with *Leishmania major* (Daifalla et al., 2011, Daifalla et al., 2012). We, therefore, hypothesized that fusing the two antigens or using them in a form of cocktail vaccine would increase the immunogenicity and bring about better protection against *L major* infection.

For the DNA/protein heterologous prime-boost immunization, we cloned each of the individual and fusion antigens in mammalian expression vector. As discussed in Chapter-3 above, we confirmed the expression of these vaccine antigens in a mammalian cell line, Chinese Hamster Ovary (CHO) cells. However, repeated attempts to express the fusion DNA in bacterial expression system and purify the recombinant fusion protein was without success. Therefore, we resorted to use a combination of rLdPxn1 and rLdFeSODB1 proteins for booster immunization of mice that had been given fusion DNA injections in the first two doses. Several studies have used a similar approach to investigate the potential of using combination (cocktail) of antigens in the form of DNA or protein vaccines (Rafati et al., 2001, Campos-Neto et al., 2001, Campos-Neto et al., 2002, Goto et al., 2011). For example, using a combination of TSA and LmSTI1 DNA in the form of fusion or cocktail vaccine showed similar protection against *L major* infection (Campos-Neto et al., 2002). A recent study by Goto and colleagues (2011) also demonstrated that using four *Leishmania* antigens in the form of fusion or cocktail vaccine have similar immunogenicity and protective efficacy. However, using a fusion vaccine is more

advantageous over a cocktail vaccine in that standardizing a fusion vaccine is easier than standardizing individual vaccine antigens separately thus reduces the cost of manufacturing the vaccine. Moreover, using a fusion vaccine ensures equivalent uptake of the individual antigens by antigen presenting cells (Goto et al., 2011, Campos-Neto et al., 2002, Coler et al., 2007).

Murine granulocyte macrophage colony-stimulating factor (mGMCSF) was used as adjuvant and secretory module. The role of mGMCSF as a secretory module was discussed in Chapter-3 above. In order to investigate the adjuvant effect of this glycoprotein, individual or fusion vaccine antigens were cloned in tandem with mGMCSF. As a control, the vaccine antigens were also cloned in absence of mGMCSF. Then, immunogenicity and protective efficacy of the fusion antigen in the presence or absence of mGMCSF was compared with that of individual antigens in BALB/c mice. In addition, we used bacterial CpG ODN adjuvant to enhance the immune response to vaccine antigens.

In general, the magnitude and quality of immune response induced by *Leishmania* vaccine antigen(s) before infection is considered to determine the efficacy of the antigen(s) in protecting against infection with the parasite. In order to evaluate the potential of the vaccine candidates to induce a protective immune response before infection, we conducted an immunogenicity study by measuring antigen-specific antibody and cell-mediated responses prior to infecting mice with stationary phase *L major* promastigotes. Multiparameter flow cytometry was also performed to further analyse the quality of cell-mediated immune response that the vaccine antigens induced. Moreover, a separate study was conducted to evaluate the level of protection by measuring footpad swelling and parasite load. Antibody and cell-mediated immune responses were also evaluated at the end of the protection study in order to evaluate whether the antigens were targets of Th-1 immune response after infection with *L major*.

The data on footpad swelling measurement show that immunization with pcDNA-mGMCSF-VAC3 or pcDNA-mGMCSF-Pxn1 resulted in the highest degree of protection at eight week post-challenge (**Figure-4.24**). A similar pattern was also seen based on parasite load on an infected footpad (**Figure-4.25**). However, in the absence of mGMCSF, all the individual antigens as well as the fusion showed more or less similar level of protection. The level of protection by these antigens was lower than that of pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 but higher than that of controls such as pcDNA-mGMCSF or CpG ODN (**Figure-4.24**). Immunization with pcDNA-mGMCSF-SODB1 showed no protection in both footpad swelling and parasite load measurements.

Several studies have demonstrated that immunogenicity of an antigen and quality of response before infection generally predicts the protective potential of the antigen (Gurunathan et al., 1997, Coler et al., 2007, Darrah et al., 2007). In order to assess this response, antigen-specific immunoglobulin G (IgG) was measured from serum samples and IFN-γ and IL-10 from supernatant of antigen stimulated spleen cell cultures. In addition, antigen stimulated $CD4^+$ and $CD8^+$ cells were characterized with respect to expression of both Th1 and Th2 cytokines using multiparameter flow cytometry. Multiparameter flow cytometry enabled us not only to measure the extent of expression of cytokines that we did not measure by ELISA (TNF-α and IL-2) but also helped us to assess whether the vaccine antigens are capable of inducing multifunctional T cells capable of simultaneously expressing more than one cytokines.

As expected, immunization with the fusion antigen in the presence of mGMCSF induced higher antigen-specific total IgG response than the individual antigens that constitute the fusion. The difference was much more pronounced at early time points after immunization (**Figure-4.1A**). For instance, only a single injection of the fusion antigen in the presence of mGMCSF, pcDNA-

mGMCSF-VAC3, resulted in significantly higher IgG response than that of pcDNA-mGMCSF-Pxn1. However, the difference with pcDNA-mGMCSF-SODB1 immunization is not statistically significant. This is because the later induced some degree of response. This response, however, does not seem to be antigen-specific as there was a response from pre-immune sera too (Week-0). A big difference in IgG response was seen between each mouse within a group at Week-3 and Week-6. This could be due to difference in the level of transfection of cells and expression of the DNA vaccine antigens between each mouse.

After two doses of DNA immunization (Week-6), pcDNA-mGMCSF-VAC3 and also pcDNA-mGMCSF-Pxn1 antigens induced a high degree of antigen-specific total IgG. At this time point, the DNA vaccines without mGMCSF also induced some degree of an IgG response. Three weeks after recombinant protein boost (Week-9), all groups that were immunized with the fusion or individual peroxidoxin 1 antigens with or without mGMCSF induced high level total IgG while the response to pcDNA-mGMCSF-SODB1 remained low. Generally, this result indicates that the fusion antigen is more immunogenic than the individual antigens. The difference is more pronounced when given in the form of DNA vaccine. Secondly, the use of mGMCSF improves the immunogenicity of the vaccine antigens with respect to inducing antigen-specific immune response (Barouch et al., 2002, Lai et al., 2011, Weiss et al., 1998). The increase in antibody response in mGMCSF containing antigens could be a result of secretory and/or adjuvancy of mGMCSF. A study on the effect of secretion of proteins on antibody response showed that a secreted protein produced 18-times more antibodies than non-secreted form of the same protein (Drew et al., 2000).

Finally, the result clearly shows that superoxide dismutase B1 is weaker immunogen in eliciting antibody response in BALB/c mice in DNA/protein immunization strategy. The weak antibody

response in mice immunized with LdFeSODB1 could be due to the presence of fewer B-cell epitopes on the antigen. This study did not investigate the numbers of B-cell epitopes on the antigens. The presence of low level total IgG specific to superoxide dismutase B1 in pre-immune sera was unexpected.

Self-reactivity to mouse mGMCSF was tested on mice sera. Antibody ELISA performed on serum samples from immunized mice using recombinant mouse GMCSF capture antigen did not show any response indicating that antibody specific to mouse GMCSF was not produced (data not shown). The result rules out the possibility of induction of self reactive antibody (autoantibody) against mouse GMCSF.

In order to indirectly assess the degree of Th1 and Th2 immune responses that the vaccine antigens induced, we measured the proportion of antigen-specific IgG2a and IgG1 isotypes from sera of immunized and control mice. Early studies demonstrated that Th1 cytokine, IFN-γ, induces isotype switching to IgG2a while a Th-2 cytokine, IL-4, induces secretion of IgG1 isotype (Stevens et al., 1988). Therefore, by measuring the proportion of IgG2a and IgG1, we wanted to indirectly evaluate the degree of Th1 and Th2 responses induced by the vaccine antigens. Based on the proportion of IgG2a and IgG1, the study shows that the vaccine antigens induced a mixed Th1/Th2 response when given in DNA/protein strategy in BALB/c mice.

As shown in **Figure-4.1B and Figure-4.1C**, the fusion vaccine antigen induced slightly higher IgG2a than IgG1 prior to protein boost. After protein boost, mice that were immunized with the fusion or peroxidoxin 1 antigen produced high level IgG1 and IgG2a. In the absence of mGMCSF, these antigens produced relatively lower IgG1 than IgG2a. Consequently, mice that were immunized with antigens in the presence of mGMCSF fusion showed lower IgG2a/IgG1 ratio. Thus, based on the ratio, the presence of mGMCSF fusion appears to shift the immune

response to a slightly more Th2 type. This is not in line with the protection data in that mGMCSF containing fusion and peroxidoxin 1 antigens showed higher protection than their counterparts that do not have mGMCSF. Other studies showed that high level IgG1 is not necessarily an indicator of a non-protective response. A study of *Leishmania* DNA vaccine candidates demonstrated that the partially protective LmSTI1 antigen produced more IgG1 than IgG2a (Campos-Neto et al., 2002). Another study demonstrated that mouse IgG1 antibodies consists of two different subtypes; the production of one subtype is mediated by IL-4 (Th-2 cytokine) while the other subtype is dependent on IL-12 and IFN-γ (Th-1 cytokine) (Faquim-Mauro et al., 1999).

Titration experiment on Week-9 sera showed that in the presence of mGMCSF, the fusion antigen and peroxidoxin 1 alone showed higher titers of both IgG1 and IgG2a than their counterparts in the absence of mGMCSF. Other studies have also demonstrated similar results that protective antigens induce high titer of both IgG1 and IgG2a (Gurunathan et al., 1997, Campos-Neto et al., 2002, Skeiky et al., 2002). For instance, Gurunathan and co-workers (1997) showed that a *Leishmania* DNA antigen that protected mice from challenge infection resulted in high titer of antigen-specific IgG1 and IgG2a. In another study, a protective TSA DNA antigen induced similar titer of IgG1 and IgG2a (Campos-Neto et al., 2002).

As described in Chapter-1 above, susceptibility and resistance to *Leishmania* is mediated by different cytokines. Th1 cytokines such as IFN-γ, TNF-α, and IL-2 favor resistance to infection while IL-4, IL-10 and IL-13 mediate disease progression and parasite persistence. Interferon gamma and TNF-α work synergistically to activate macrophages to kill *Leishmania* parasite by producing nitric oxide. On the other hand, IL-10 inhibits IFN-γ-mediated activation macrophages (Okwor et al., 2012, Liew et al., 1990, Stempin et al., 2010).

Generally, vaccine antigens that induce more of Th1 cytokines prior to infection are considered to be more effective in protecting against infection with *Leishmania*. That is, production of high level Th-1 cytokines at the time of infection is supposed to be a predictor of protection against *L major* infection. However, the exact correlate of protection against *Leishmania* has not been fully defined. More recent studies have indicated that the degree of induction of multifunctional $CD4^+$ T cells that simultaneously produce the three Th1 cytokines is a better correlate of protection against experimental *Leishmania major* infection in mice (Goto et al., 2011, Darrah et al., 2007). Therefore, in our study, we assessed the efficiency of the vaccine antigens by measuring IFN-γ and IL-10 using ELISA and also by evaluating the expression of both Th-1 and Th-2 cytokines on antigen-stimulated spleen cells using multiparameter flow cytometry.

Cytokine production at the time of infection showed that both the individual and fusion antigens in the presence or absence of mGMCSF induced a mixed Th1/Th2 response. That is, the antigens induced the production of both IFN-γ and IL-10. This is in line with the result obtained in antibody ELISA.

With regard to the amount of IFN-γ produced by antigen-stimulated spleen cells, no significant difference is seen between mice that received the fusion antigen and those that were immunized with the individual ones except in mice that were immunized with pcDNA-Pxn1. In the presence of mGMCSF, the fusion antigen induced only slightly higher IFN-γ than the individual antigens. In the absence of mGMCSF, the fusion produced even lower amount of IFN-γ than pcDNA-SODB1. Goto and co-workers (2011) also showed similar finding in testing a fusion vaccine antigen. Out of four antigens that constitute the fusion antigen, KSAC, stimulation of spleen cells

with one of the constituent antigens induced the production of more IFN-γ than stimulation with the fusion antigen.

Cytokine response by spleen cells to the vaccine antigens indicated that mGMCSF containing antigens are generally more immunogenic than their counterparts without antigen. This was clearly seen by the level of IFN-γ production of spleen cells stimulated with the respective antigens. A similar pattern was also seen with respect to IL-10 production in that vaccine antigens that were fused with mGMCSF induced the production of more IL-10 that those without mGMCSF. These data corroborated the result seen in antibody ELISA. Moreover, mGMCSF containing antigens generally showed higher frequency of $CD4^+$ T cells expressing Th-1 cytokines than the corresponding antigen without mGMCSF. It is, therefore, safe to conclude that the presence of mGMCSF fusion increased immunogenicity of the vaccine antigens.

Based on our data, we do not know the exact mechanism of action of mGMCSF that enabled it to increase immunogenicity and protection of the antigens. As described in Chapter-1 and Chapter-3, the secretory and immunomodulatory roles of mGMCSF are important features that could enable the molecule to potentiate the vaccine antigens. In this study, we confirmed the secretory role of mGMCSF when fused with a vaccine antigen. Farrell and co-workers (2013) demonstrated that the increased production of antibody by GMCSF fusion is the result of secretory role rather than activity (adjuvancy). Immunization of mice with human or murine GMCSF induced antibody against the antigen fused with GMCSF. In our study, we did not address the immunomodulatory effect of the molecule. To do this, we needed to confirm the activity of the mGMCSF that was fused with vaccine antigens. We assumed that the spacer region between mGMCSF and the vaccine antigen physically separates the mGMCSF from the

vaccine antigen and prevents interference of the activity of mGMCSF by the vaccine antigen and vice versa.

Previous studies demonstrated that the presence of high level of IFN-γ at the time of infection predicts the outcome of infection by *L major (Gurunathan et al., 1997)***.** For example, the protective effect of LACK (*Leishmania* homologue of receptors for Activated C Kinase) antigen against *L major* was found to be correlated with elevated IFN-γ at the time of infection (Gurunathan et al., 1997). However, this was not always true in our study. Unexpectedly, in addition to the partially protective antigens, pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1, the non-protective antigen, pcDNA-mGMCSF-SODB1, also induced the production of high level IFN-γ. Several studies on vaccine antigens of intracellular microorganisms such as *Leishmania* and *Mycobacterium tuberculosis* have clearly indicated that high level IFN-γ production at the time of infection does not necessarily predict protection (Gicheru et al., 2001, Leal et al., 2001, Elias et al., 2005).

Gicheru and colleagues (2001) evaluated the safety and immunogenicity of killed *L major* parasite vaccine in the presence of IL-12 adjuvant in vervet monkeys. They compared the level of IFN-γ production between monkeys that received the vaccine and another group from a different study that were confirmed to resolve *L major* infection. Although the two groups showed comparable IFN-γ production in stimulated peripheral blood lymphocytes, monkeys that were immunized with the antigen were not protected against *L major* challenge infection. They showed only a slight reduction in lesion size. This is in agreement with our data that immunization with pcDNA-mGMCSF-SODB1 induced production of high level IFN-γ but fail to induce protection in mice. Similar data were obtained in testing vaccine antigens against

Mycobacterium tuberculosis, intracellular bacteria that show similar pathogenesis as *L major* infection. Although antigen stimulated spleen cells from immunized mice produced significantly higher IFN-γ than the controls, the immunized mice were not protected from intra-venous or aerosol challenge with pathogenic strain of *M tuberculosis* (Leal et al., 2001, Elias et al., 2005). Other studies demonstrated that the protective efficacy of vaccine antigens not only depend on the mere amount of IFN-γ but also the amount of IL-10 at the time of challenge infection (Stober et al., 2005). A study showed that high level IL-10 produced by regulatory T cells at the time infection predicts failure of a vaccine in protecting against *L major* infection. Thus, high IFN-γ to IL-10 ratio at the time of challenge is a better correlate of protection. Moreover, it was found that the ratio remained high after challenge infection with *L major* (Stober et al., 2005). Our result partially agrees with this finding. At the time of infection, mice immunized with partially protective vaccine antigens had IFN-γ to IL-10 ratio 5.6- to 49-fold more than mice injected with controls (**Table-4.1**). However, there are also discrepancies between our data and the above finding. First, although mice immunized with pcDNA-mGMCSF-VAC3 or pcDNA-mGMCSF-Pxn1 showed higher level of protection against *L major* infection than other antigens, the IFN-γ/IL-10 ratio is considerably lower than those antigens that elicited less protection. This is attributed more to the higher IL-10 production rather than low IFN-γ production. Secondly, pcDNA-mGMCSF-SODB1 did not produce any protection against *L major* infection yet it induced the highest ratio among all the vaccine antigens. Third, immunization with pcDNA-Pxn1 induced remarkably low IFN-γ response than other antigens which resulted in the lowest IFN-γ/IL-10 ratio. Co-incidentally, we found that *in vitro* stimulation of spleen cells of mice immunized with the fusion antigen with rLdPxn1 or rLdFeSODB1 showed similar pattern. That

is, stimulation with rLdPxn1 induced lower IFN-γ than stimulation with rLdFeSODB1. For example, spleen cells from mice immunized with the fusion, pcDNA-VAC3, produced 0.32 ± 0.23ng/ml and 0.6 ± 0.49ng/ml upon stimulation with rLdPxn1 and rLdFeSODB1, respectively.

The study shows that the high level expression of three Th-1 cytokines namely IFN-γ, TNF-α and IL-2 by antigen-stimulated spleen cells at the time of infection better correlates to protection against *L major* infection in mice. This was elucidated by the result of multiparameter flow cytometry in $CD4^+$ T cells. Helper T cell type-1 (Th-1) cytokine expression on $CD4^+$ T cells prior to *L major* infection clearly demonstrates that immunization with the fusion antigen, pcDNA-mGMCSF-VAC3, or pcDNA-mGMCSF-Pxn1 alone induced the highest expression of each of the Th-1 cytokines, IFN-γ, TNF-α and IL-2. Immunization with the fusion, pcDNA-mGMCSF-VAC3 induced higher frequency of cytokine producing $CD4^+$ T cells (**Figure-4.5A**). On the other hand, immunization with pcDNA-mGMCSF-Pxn1 alone resulted in increased expression of the respective cytokine on $CD4^+$ T cells that are positive for the cytokine. This was exhibited by high level MFI for each cytokine (**Figure-4.5B**). Taken the two data sets together, immunization with the fusion antigen or peroxidoxin 1 alone in the presence of mGMCSF resulted in high quality Th-1 cytokine response. This correlates with the highest protective efficacy of the two antigens in terms of both footpad swelling and parasite number.

In the absence of mGMCSF, the fusion antigen as well as individual peroxidoxin 1 or superoxide dismutase B1 induced significantly higher iMFI for TNF-α and IL-2 than the control, pcDNA ($p<0.05$). Moreover, the fusion and peroxidoxin 1 alone also induced significantly higher iMFI for IFN-γ than pcDNA ($p<0.05$). Thus, although in a lesser degree, the antigens that do not have mGMCSF also induced generally higher expression of Th-1 cytokines on $CD4^+$ T cells than

controls. This follows the pattern of footpad swelling data that immunization with pcDNA-mGMCSF-VAC3 or pcDNA-mGMCSF-Pxn1 induced the highest protection followed by all three antigens without mGMCSF (**Figure-4.24**).

Recent studies have shown that the frequency of multifunctional Th-1 T cells is a better predictor of protection against *L major* infection in mice. These cells simultaneously express IFN-γ, TNF-α, and IL-2 (Goto et al., 2011, Darrah et al., 2007). However, our data do not fully agree with these findings. Although the protective antigens, pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 induced the highest frequency of IFN-γ^+, TNF-α^+, IL-2^+ $CD4^+$ T cells (triple-positive), the non-protective antigen, pcDNA-mGMCSF-SODB1, also induced comparable percentage of these cells. Moreover, the fusion antigen without mGMCSF also elicited high frequency of the triple-positive cells.

Unlike $CD4^+$ response, the cytokine response of $CD8^+$ T cells before infection did not demonstrate clear reflection of the protection data. That is, the vaccine antigens that brought protection did not show as significantly higher response as seen in $CD4^+$ T cells. Taken together, based on flow cytometric data on pre-challenge samples, the increase protection by pcDNA-mGMCSF-VAC3 and pcDNA-mGMCSF-Pxn1 antigens appears to be mediated by $CD4^+$ T cells that express IFN-γ, TNF-α and IL-2 separately. Similar result was obtained with *Leish111f* polyprotein vaccine against visceral leishmaniasis in mice. That is, mice immunized with *Leish111f* in the presence of MPL-SE adjuvant protected mice against *L infantum* infection and induced high level expression of the three Th-1 cytokines on individual $CD4^+$ T cells (Coler et al., 2007).

This study did not investigate the cytotoxic role of $CD8^+$ cells. In addition to producing protective cytokines, $CD8^+$ T cells also mediate cytotoxic antileishmanial activity. In a protection study of individual and fusion vaccine antigens, Campos-Neto and colleagues (2002) found that the antigen with the highest protection showed significantly higher level of $CD8^+$ cytotoxic T lymphocyte (CTL) activity in addition to high $CD4^+$ mediated Th-1 cytokine response. Our study did not investigate the degree of induction of CTL by the vaccine antigens tested.

In the protection study, immunization with the fusion pcDNA-mGMCSF-VAC3 induced significantly higher protection against *L major* infection than immunization with pcDNA-mGMCSF-SODB1. However, it showed slightly lower protection as compared to immunization with pcDNA-mGMCSF-Pxn1. It appears that the presence of superoxide dismutase B1 antigen shifts the response into a relatively non-protective type. Similar phenomenon was seen in testing a cocktail DNA vaccine consisting of *Leishmania major* TSA (peroxidoxin 2) and LmSTI1 in BALB/c mice. When tested separately, TSA immunization resulted in better protection than LmSTI1. The cocktail TSA and LmSTI1 DNA immunization showed a level of protection equivalent to that of TSA but much higher than the protection seen in immunization with LmSTI1 DNA alone. (Campos-Neto et al., 2002).

In order to assess the immune response to the vaccine antigens after infection with *L major*, we evaluated antibody and cell mediated responses on serum samples and antigen-stimulated spleen cells after infection. Serum samples were collected four times before infection and twice after infection (Week-12 and Week-17). Spleen cells were isolated at the end of the protection experiment (Week-17). The level of antibody and cytokine responses as well as the phenotype of $CD4^+$ and $CD8^+$ T cells were assessed in the same way as in the immunogenicity study.

As described in section 4.2.2.1 above, antigen-specific antibody response at Weeks-0, -3, -6, and -9 seen in protection study is generally the same as that seen in the immunogenicity study. However, there are few differences. Firstly, the small antibody response against superoxide dismutase B1 antigen at Week-0 in immunogenicity study was not seen in the protection experiment. It appears that the response at Week-0 in immunogenicity experiment may not be actual response. Secondly, at Week-9, IgG1 level in mice immunized with pcDNA-Pxn1 is appreciably lower in the protection experiment. Repeating the experiment (ELISA) three times resulted in the same value.

Surprisingly, high level antibody response was seen at Week-12 and Week-17 not only in mice immunized with vaccine antigens but also in those that were injected with controls such as pcDNA, PBS, or CpG ODN. As a control for the fusion vaccine, antibody specific to a combination of rLdPxn1 and rLdFeSODB1 was measured on the serum samples from control mice. Therefore, the high response after infection with *L major* could be the result of parasite-induced antibodies that react with rLdPxn1 and rLdFeSODB1. *Leishmania* peroxidoxin is a multicopy gene and the product of expression of these genes could cross-react with rLdPxn1 used for ELISA. Therefore, the high antigen-specific antibody response appears to be infection-induced. Similar phenomenon was seen in other studies (Goto et al., 2011, Mazumder et al., 2011). Control mice produced high level of total IgG and IgG1 but low IgG2a. This shows that the antibody response seen in control mice is biased to a Th-2 type and agrees with the protection data. Titration experiment in Week-17 serum also corroborates this result. That is, the titer of IgG1 is high both in the vaccinated and control groups but the later produced significantly lower titer of IgG2a. Similar response was seen in other studies. Immunization of BALB/c mice with a DNA vaccine antigen, tryparedoxin peroxidase, and a control containing only the plasmid vector

showed that two weeks after infection with *L major*, mice in both experimental and control groups produced high IgG1 titer specific to the recombinant tryparedoxin peroxidase. However, the control mice produced significantly lower IgG2a titer than mice that received the vaccine antigen (Stober et al., 2007). In a study of a fusion vaccine, KSAC, in the presence of MPL-SE adjuvant, Goto and colleagues (2011) showed similar finding. Control mice injected with saline produced high IgG1 and low IgG2a against *L major* SLA while mice that were immunized with the vaccine antigen produced equivalent IgG1 and IgG2a eight weeks after infection with *L major*. In another study using *Leishmania* gp63 in DNA/protein immunization strategy, control mice that received saline produced antigen-specific IgG1 after infection with *L donovani* (Mazumder et al., 2011).

Like antibody response, after infection with *L major*, IFN-γ response in cocktail antigen or SLA stimulated cells was also high both in the vaccine as well as control groups. Among the control groups, mice that received pcDNA-mGMCSF or CpG ODN induced higher IFN-γ than those that were injected with pcDNA or PBS upon stimulation with a combination of rLdPxn1 and rLdFeSODB1. High level IFN-γ production by unimmunized mice is not correlated with the protection data as control mice did not show any sign of parasite control both on footpad swelling and parasite load measurements. Similar phenomenon was also seen in other studies in mice and also in human clinical trial (Llanos-Cuentas et al., 2010, Oliveira et al., 2005, Zhang and Matlashewski, 2008).

A study on evaluation of role of vaccine adjuvants in protection against *L major* infection demonstrated similar phenomenon. Subcutaneous immunization of heat killed *L major* together with R848 (resiquimod) adjuvant showed significantly higher degree of protection of mice from *L major* challenge infection than controls. However, the same antigen-adjuvant combination

injected intramuscularly failed to protect mice. Analysis of the cytokine production by spleen cells collected after *L major* challenge infection demonstrated that both protective and non-protective immunizations induced high degree of IFN-γ production. The non-protective immunization produced even higher IFN-γ than the protective one. However, clear difference was observed with regard to Th-2 cytokines, IL-4 and IL-10. While the protective immunization induced significantly low IL-4 and IL-10, the non-protective one induced appreciable amount of IL-4 and failed to suppress IL-10 production at all (Zhang and Matlashewski, 2008). The high level production of IFN-γ in mice immunized with both protective and non-protective immunizations agrees with our data. That is, as shown in **Figure-4.15A**, immunization with partially protective antigens such as pcDNA-mGMCSF-Pxn1 and pcDNA-mGMCSF-VAC3 as well as the non-protective antigen, pcDNA-mGMCSF-SODB1 and controls such as pcDNA-mGMCSF induced high IFN-γ recall response by antigen-stimulted spleen cells eight weeks after *L major* infection. However, clear discrepancy is seen with regard to IL-10 production. In our study, the non-protective antigen and the controls produced smaller IL-10 than the partially protective, pcDNA-mGMCSF-VAC3. Unfortunately, we did not measure the degree of IL-4 production

In a clinical trial of a therapeutic LEISH-F1 vaccine together with MPL-SE adjuvant for mucosal leishmaniasis, patients who received the placebo showed antibody response to LEISH-F1. Moreover, upon antigen stimulation, peripheral blood mononuclear cells from some of the individuals in the placebo group also produced elevated level of IFN-γ. One of the constituents of LEISH-F1 antigen is *Leishmania* TSA (a homologue of peroxidoxin 1). It was suggested that the high response in placebo groups is due to infection-induced immune response that cross-react

with the antigen (Llanos-Cuentas et al., 2010). As discussed above peroxidoxin and superoxide dismutase are multicopy genes and could be capable of inducing high level of IFN-γ upon *in vitro* recall experiment on cells isolated from *Leishmania* infected individuals. This is in line with the high IFN-γ seen in antigen-stimulated spleen cells from mice immunized with the vaccine antigen and controls after *L major* infection (**Figure-4.15A**). In pre-challenge experiment, stimulation with SLA produced higher IFN-γ by spleen cells from mice immunized with pcDNA-mGMCSF-Pxn1 and the fusion pcDNA-mGMCSF-VAC3 (**Figure-4.3A**). As explained in Chapter-1, LdPxn is a multicopy gene and the four copies are expressed by the parasite. Thus, these proteins in the SLA brought about high recall response in mice immunized with LdPxn1 or the fusion even before infection. The lower IFN-γ response in SLA stimulated cells from LdFeSODB1 immunized mice could be due to the fact that LdFeSODB gene has only two copies.

Similar result was also seen in a study on the immunoreactivity of antigens in visceral leishmaniasis patients in Iran. A recombinant protein from a multicopy gene showed higher immunoreactivity towards sera of leishmaniasis patients and cured individuals than the one from a single copy gene of same family (Rafati et al., 2003).

Although high level IFN-γ was produced by spleen cells from control mice, the mice were not protected from *L major* challenge infection. This is in line with previous study on Swiss/NIH mice (Oliveira et al., 2005). Upon infection with *L major*, conventional Swiss/NIH mice produce Th-1 response with high IFN-γ and are capable of controlling the parasite and the development of lesion. However, Swiss/NIH mice that were raised and maintained in a special germ-free condition did not control infection with *L major* and showed higher lesion size and parasite

number. However, lymph node cells from both conventional and germ-free mice showed comparable level of early IL-12 and IFN-γ (two days after infection). In addition, at the end of the experiment, spleen cells from germ-free mice produced elevated IFN-γ and low IL-4 that are comparable to the amount in conventional Swiss/NIH mice. Macrophages from the germ-free mice, however, could not kill *L major* parasite in the presence of IFN-γ *in vitro*. This suggests that high level production of IFN-γ after infection does not necessarily correlate to high degree of protection (Oliveira et al., 2005).

Close analysis of IFN-γ production by spleen cells from mice injected with CpG ODN and pcDNA-mGMCSF after *L major* infection clearly showed that much of the IFN-γ came from stimulation with rLdFeSODB1. In mice immunized with CpG ODN, stimulation with rLdPxn1 and rLdFeSODB1 resulted in IFN-γ to IL-10 ratio of 9.5 and 31, respectively. Similarly, the ratio in mice immunized with pcDNA-mGMCSF is 4.1 and 21 upon stimulation with rLdPxn1 and rLdFeSODB1, respectively. Thus, the high IFN-γ to IL-10 ratio seen in these control groups after infection is due to high IFN-γ production by rLdFeSODB1 stimulation (**Table-4.2**). On the other hand, flow cytometric data before and after infection showed that immunization with LdFeSODB1 resulted in stimulation of fewer number of IFN-γ expressing $CD4^+$ cells than immunization with the fusion or LdPxn1. The discordance between cytokine ELISA and flow cytometry results in LdFeSODB1 immunization suggests that the source of IFN-γ in mice immunized with LdFeSODB1 could be different from T cells and this IFN-γ might not be able to activate macrophages.

Generally, the result of multiparameter flow cytometry in protection study was a reflection of the immunogenicity study. One noticeable difference is that immunization with pcDNA-mGMCSF-

Pxn1 did not bring the level of expression of Th-1 cytokines in $CD4^+$ T cells (as depicted in MFI data, Figure-4.16) that was seen in immunogenicity experiment. That is, infection with *L major* somehow suppressed the level of expression of Th-1 cytokines in pcDNA-mGMCSF-Pxn1 immunized mice.

As expected, immunization with the fusion antigen with mGMCSF showed the highest frequency of each of the Th-1 cytokine producing $CD4^+$ T cells eight weeks after *L major* infection. This shows that immunization with pcDNA-mGMCSF-VAC3 antigen induces durable protective immune response in BALB/c mice. Infection with *L major* did not affect this feature of the fusion vaccine. This feature makes it a useful vaccine candidate against *L major* infection in mice. On the other hand, mice immunized with the fusion antigen in the presence of mGMCSF showed higher degree of expression of IL-10 on $CD4^+$ T cells. This corroborated the cytokine ELISA result that the fusion antigen induced the production of more IL-10 than the individual ones.

After *L major* infection, mice that were immunized with the fusion antigen with mGMCSF showed higher percentage of $CD8^+$ T cells that express individual Th-1 cells than mice that received the individual antigens with mGMCSF. This result is different from the result from pre-challenge experiment. Currently, we do not exactly know what increased the frequency of Th-1 cytokine expressing $CD8^+$ cells in pcDNA-mGMCSF-VAC3 immunized mice eight weeks after infection with *L major*.

In summary, the data show that using fusion/cocktail LdPxn1-LdFeSODB1 in DNA/protein prime-boost immunization increases immunogenicity of the vaccine antigens in BALB/c mice. It also shows that the use of GMCSF fusion generally increases the immunogenicity and protective efficacy of the antigens. Moreover, protective efficacy of antigens is better correlated to

frequency of $CD4^+$ T cells expressing individual Th-1 cytokines and the level of expression of these cytokines in $CD4^+$ T cells. The production of only IFN-γ by antigen-stimulated spleen cells before infection does not predict protection against *L major* infection.

Chapter Five: **ASSESSMENT OF IMMUNOGENICITY AND PROTECTIVE EFFICACY OF COCKTAIL *LEISHMANIA DONOVANI* PEROXIDOXIN 1 AND SUPEROXIDE DISMUTASE B1 IN PROTEIN-PROTEIN IMMUNIZATION IN BALB/c MICE**

5.1 Experimental Rationale

In addition to the DNA/protein approach, we also tested the vaccine antigens in a protein/protein immunization regimen. The benefit of this approach over DNA/protein immunization is that only one form of the antigen (recombinant protein) is used which makes preparation of the antigens easier. In addition, in using DNA vaccines one has to ensure the expression of the vaccine antigen in mammalian cells. As discussed in chapter-4 above, the level of expression of the DNA vaccine antigens might be different between individual mice which, in turn, may bring about variable immune response to the vaccine antigen. In this experiment, we used a combination of rLdPxn1 and rLdFeSODB1 proteins for both primary immunization and booster doses. We also used individual antigens as controls.

This experiment was done in collaboration with Dr. Nada Daifalla. As part of her PhD study in our lab, she tested rLdFeSODB1 and rLdPxn1 as individual vaccine antigens in BALB/c mice. In order to compare the efficacy of a cocktail antigen with individual ones, we used mice that were immunized with individual antigens as controls. Thus, part of the data from Dr. Daifalla's thesis on individual antigens is included in the analysis of the efficacy of the combination antigens and presented in this thesis. The result of the immunogenicity and protective efficacy of individual rLdFeSODB1 was published on two articles:

1. **Daifalla NS, <u>Abebe Genetu Bayih</u>**, and Gedamu L (2012) *Leishmania donovani* recombinant iron superoxide dismutase B1 protein in the presence of TLR-based adjuvants induces partial protection of BALB/c mice against *Leishmania major* infection. *Experimental Parasitology*, 131:317-24 (***The first and second authors contributed equally to the work***).

2. Daifalla NS, **Abebe Genetu Bayih**, and Gedamu L (2011) Immunogenicity of *Leishmania donovani* iron superoxide dismutase B1 and peroxidoxin 4 in BALB/c mice: the contribution of Toll-like receptor agonists as adjuvant. *Experimental Parasitology*, 129:292-98.

5.2 Experimental Results

5.2.1 Evaluation of the Immunogenicity of Vaccine Candidates in Protein/Protein Immunization

Like in DNA/protein approach, the immunogenicity of the vaccine candidates was also assessed in protein/protein immunization. In this experiment, all the three doses were given in the form of recombinant proteins. The level of total IgG, IgG1 and IgG2a was measured from serum samples collected before immunization (Week-0) and 3, 6, and 10 weeks thereafter. At Week-10, the mice were sacrificed and spleens isolated. Spleen cells were then stimulated with recombinant proteins, *L major* SLA or concanavalin A. Unstimulated cells were also included as negative controls. Seventy-two hour later, culture supernatants were collected from each well and the level of IFN-γ and IL-10 production measured using ELISA.

5.2.1.1 Pre-Challenge Antibody Response in Protein/Protein Immunization

At Week-3 post the first immunization, mice that were immunized with rLdPxn1 or the cocktail of rLdPxn1 and rLdFeSODB1 produced appreciably higher antigen-specific total IgG than those that were immunized with rLdFeSODB1 alone. The OD_{450}nm of total IgG was 2.02 ± 0.2, 0.42 ± 0.09 and 1.7 ± 0.29 in mice immunized with rLdPxn1, LdFeSODB1 or a combination of rLdPxn1 and LdFeSODB1, respectively (**Figure-5.1A**).

At Week-6, the biggest increase in antigen-specific total IgG was recorded in mice immunized with rLdFeSODB1. It increased from 0.42 ± 0.09 at Week-3 to 2.97 ± 0.41 at Week-6. As a result, no statistically significant difference was seen between different groups of mice that received either the individual or a combination of the antigens. At Week-10, all the vaccine antigens and the GST control induced high level total IgG. There was no appreciable difference between the different groups. The control mice that received CpG ODN alone did not produce

IgG specific to a combination of rLdPxn1 and LdFeSODB1 until Week-6. At Week-10, one out of a total of three mice produced elevated specific IgG which resulted an average total IgG of 0.37 ± 0.37 (**Figure-5.1A**). The total IgG response suggested that the cocktail vaccine and rLdPxn1 have similar level immunogenicity and the presence of rLdFeSODB1 in the cocktail did not contribute to increase the immune response in mice that received the cocktail vaccine. Moreover, a single injection with the cocktail vaccine or rLdPxn1 elicits antibody response as early as three weeks post-immunization. However, rLdFeSODB1 is a weaker immunogen that induction of a significant immune response could be possible only after two dose injections.

As shown in **Figure-5.1B**, a very small amount of antigen specific IgG1 was produced at Week-3 by mice that were immunized with either rLdPxn1 or a combination of rLdPxn1 and LdFeSODB1. Mice that were immunized with rLdFeSODB1 did not produce detectable specific IgG1. At Week-6, the highest level of IgG1 was produced by mice immunized with the combination antigen (OD_{450nm} 2.9 ± 0.32) followed by those that received rLdPxn1 antigen (2.2 ± 0.6). However, this difference is not statistically significant. On the other hand, mice that were immunized with the combination antigen produced significantly higher IgG1 than those that were immunized with rLdFeSODB1 alone ($p<0.05$). At Week-10, all the three groups of mice that received either the individual or combination of antigens showed high level IgG1. At this time, there is no statistically significant difference in IgG1 production between antigen immunized groups. Control mice that received CpG ODN alone did not produce detectable level of IgG1 even at Week-10 (**Figure-5.1B**).

Figure-5.1C shows antigen-specific IgG2a response in immunized and control mice. Like the total IgG, antigen-specific IgG2a response was higher in mice immunized with rLdPxn1 alone (OD_{450} 1.3 ± 0.19) or a combination of rLdPxn1 and rLdFeSODB1 (1.1 ± 0.13) than in mice that

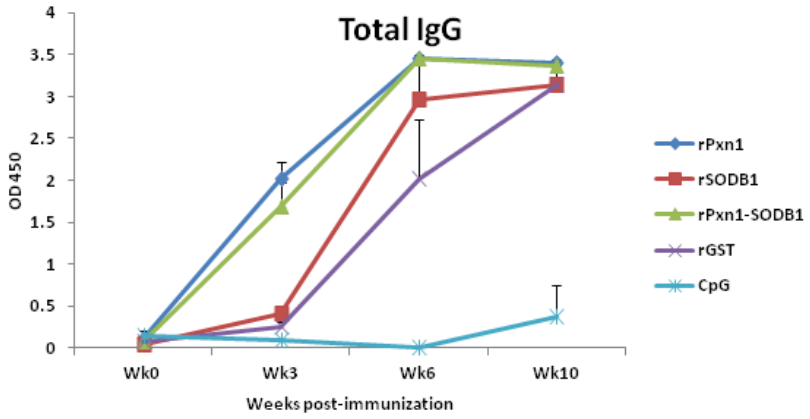
were immunized with rLdFeSODB1 alone (0.38 ± 0.09). At Week-6, immunization with rLdFeSODB1 alone also induced high IgG2a. However, the response was still lower than that seen in mice that were immunized with either rLdPxn1 or a combination rLdPxn1 and rLdFeSODB1. There is no statistically significant difference between the cocktail vaccine and either of the individual ones ($p>0.05$). At Week-10, all the groups that received either the individual or the combination antigens showed high level response. At all time points, the IgG2a response by the rGST control was significantly lower than that of rLdPxn1 or the cocktail vaccine ($p<0.05$) (**Figure-5.1C**).

In order to analyze the Th-1/Th-2 balance, the IgG2a/IgG1 ratio was calculated for antigen stimulated groups. At Week-10, the IgG2a/IgG1 ratio in mice immunized with rLdPxn1, rLdFeSODB1, and the cocktail antigens is 1.17, 0.91, and 1.0, respectively. This suggests that immunization with the cocktail or individual recombinant proteins induced a mixed Th-1/Th-2 response and immunization with rLdPxn1 alone shifts the immune response to a slightly more Th-1 type. Immunization with rGST control shows the lowest IgG2a/IgG1 ratio, 0.75, indicating that it induced predominantly Th-2 response.

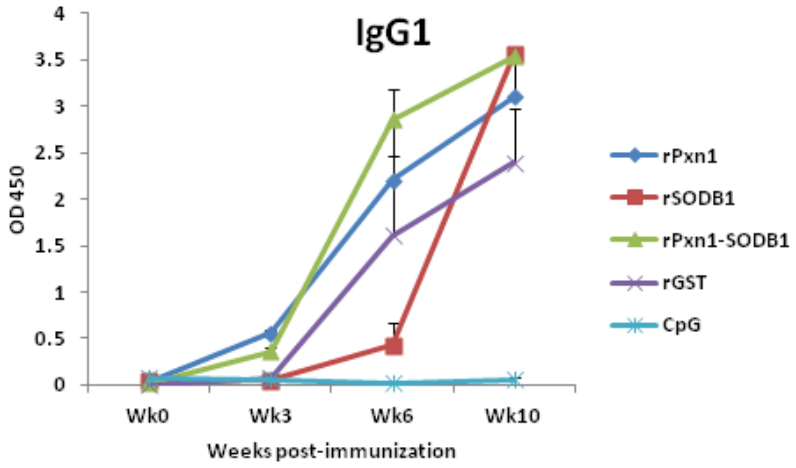
Figure 5.1: Pre-challenge antibody response of mice immunized with antigens in protein/protein immunization regimen.

Mice (three per group) were immunized with individual or cocktail recombinant protein antigens with CpG ODN adjuvant three times in three week intervals. Serum samples were collected before immunization (Week-0) and 3-, 6-, and 10-weeks after the first immunization. Total IgG **(A)**, IgG1 **(B)**, and IgG2a **(C)** were measured using ELISA. Data represents the mean OD_{450nm} of three mice and standard error of mean (SEM). The data show that rLdPxn1 and the cocktail antigens are more immunogenic in inducing antigen-specific antibody response than rLdFeSODB1.

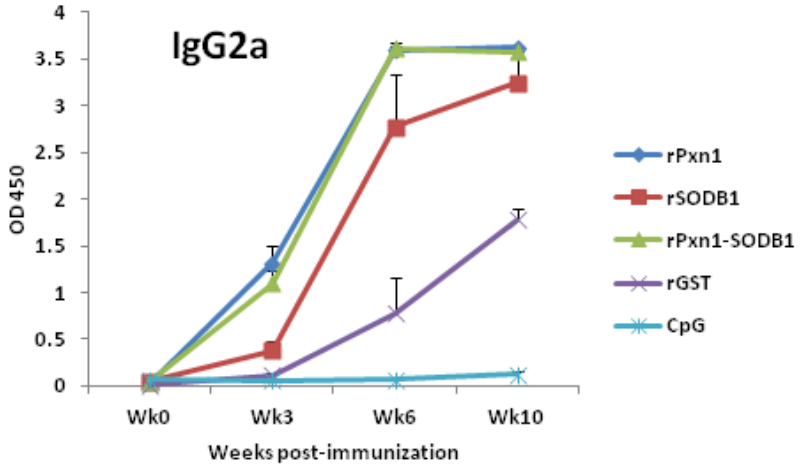
A.


Total IgG
OD 450
4
3.5
3
2.5
2
1.5
1
0.5
0
Wk0
Wk3
Wk6
Wk10
Weeks post-immunization
rPxn1
rSODB1
rPxn1-SODB1
rGST
CpG

B.


IgG1
OD 450
4
3.5
3
2.5
2
1.5
1
0.5
0
Wk0
Wk3
Wk6
Wk10
Weeks post-immunization
rPxn1
rSODB1
rPxn1-SODB1
rGST
CpG

C.


IgG2a
OD 450
4
3.5
3
2.5
2
1.5
1
0.5
0
Wk0
Wk3
Wk6
Wk10
Weeks post-immunization
rPxn1
rSODB1
rPxn1-SODB1
rGST
CpG

5.2.1.2 Pre-Challenge Cytokine Response in Protein/Protein Immunization

As mentioned above, spleens were isolated from immunized and control mice at Week-10 after the first immunization. Spleen cells were then stimulated with antigen or mitogen and the level of IFN-γ and IL-10 was determined from the culture supernatant. As shown in **Figure-5.2A**, mice immunized with a combination of rLdPxn1 and rLdFeSODB1 showed the highest level of IFN-γ response (12.16 ± 2.2ng/ml). Spleen cells isolated from mice immunized with of rLdPxn1 or rLdFeSODB1 produced 1.9 ± 1.3ng/ml and 0.7 ± 0.25ng/ml IFN-γ, respectively. The difference between the cocktail group and that of individual groups is statistically significant ($p<0.05$). Control mice that were immunized with CpG ODN alone did not produce detectable level of IFN-γ. On the other hand, mice that were injected with rGST protein in the presence of CpG ODN produced small amount of IFN-γ (0.56 ± 0.43ng/ml). Antigen-stimulated spleen cells isolated from mice that received CpG ODN alone did not produce detectable level of IFN-γ indicating that the IFN-γ response seen in the vaccine groups was the result of immunization. Based on the degree of IFN-γ production, the cocktail vaccine remains to be the most immunogenic as compared to either of the individual antigens.

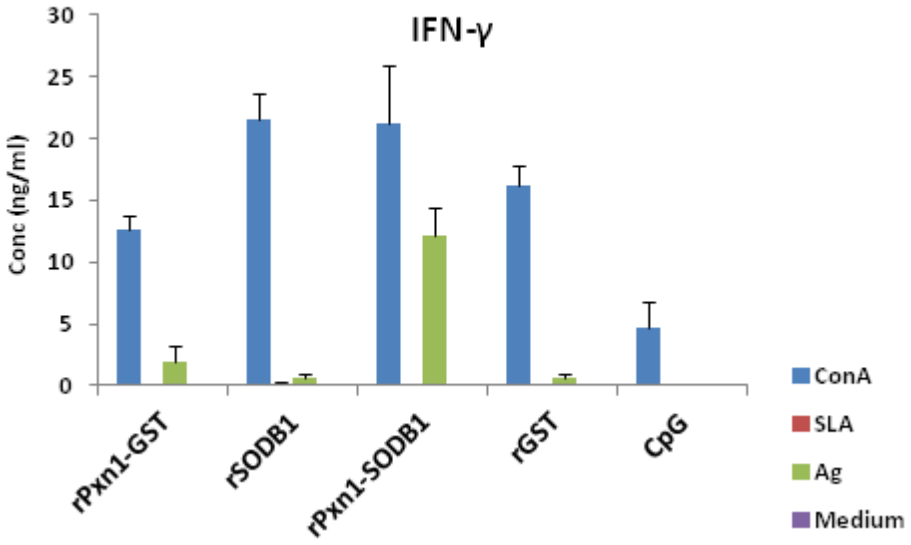
The level of IL-10 was also measured from culture supernatant of stimulated spleen cells. Like IFN-γ response, mice that were immunized with a combination of the antigens induced the highest IL-10 response (**Figure-5.2B**). Mice immunized with the combination of rLdPxn1 and rLdSODB1 antigens showed significantly higher IL-10 level than those immunized with either of the individual antigens ($p<0.05$). On the other hand, mice immunized with either rLdPxn1 or rLdSODB1 antigen produced significantly higher IL-10 than those that received CpG ODN control ($p<0.05$). The ratio of IFN-γ to IL-10 was calculated in mice immunized with the

individual or combination antigens. Mice immunized with rLdPxn1, rLdFeSODB1, or a combination of the two showed IFN-γ/IL-10 ratio of 5.1, 2.6, and 8.0, respectively. The ratio of IFN-γ to IL-10 suggests that immunization with the cocktail vaccine induced the highest level of Th-1 immune response which was higher than that induced by either rLdPxn1 or rLdFeSODB1 alone.

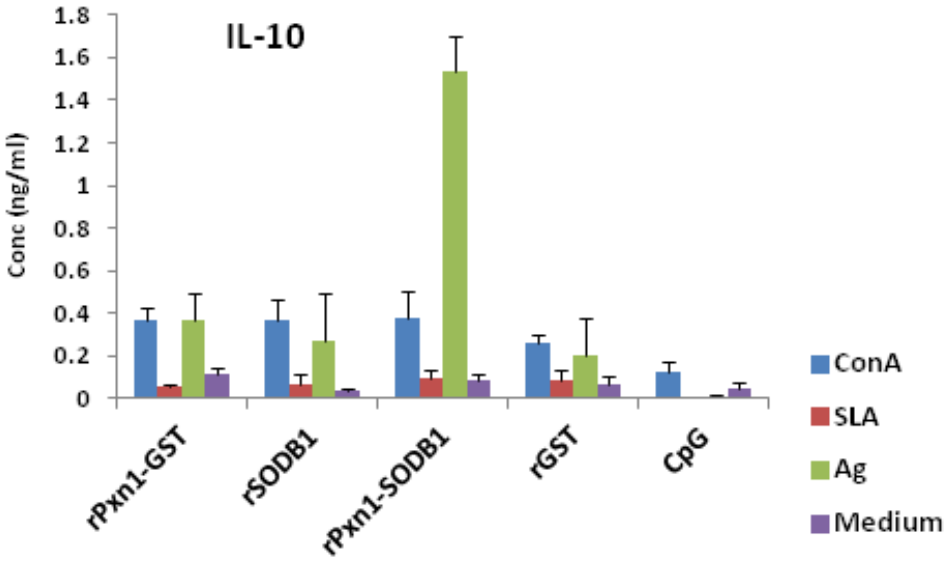
Figure 5.2: Pre-challenge cytokine response of mice immunized with antigens in protein/protein immunization regimen.

IFN-γ **(A)** and IL-10 **(B)** production in stimulated spleen cells was measured using cytokine ELISA. Mice (three per group) were immunized with individual or cocktail recombinant protein antigens with CpG ODN adjuvant three times in three week intervals. Four weeks after the last immunization, spleen cells were isolated from immunized mice and controls (three mice per group) and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or concanavalin A (5µg/ml). Unstimulated cells were included as negative control. Cells were cultured at 37°C and 5% CO_2 and culture supernatant was collected at 72hr. The level of IFN-γ and IL-10 was measured using cytokine ELISA kit (BD Biosciences). The concentration of the cytokines was determined using the standard proteins included in the kit and expressed in ng/ml. The mean concentration and standard error of the mean (SEM) of three mice per group is shown in the figure. Statistical comparison between groups was performed using Mann-Whitney U test. The data clearly show that with respect to cytokine response, the cocktail antigen is more immunogenic than either of the individual antigens.

A.


IFN-γ
Conc (ng/ml)
30
25
20
15
10
5
0
rPxn1-GST
rSODB1
rPxn1-SODB1
rGST
CpG
ConA
SLA
Ag
Medium

B.


IL-10
Conc (ng/ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
rPxn1-GST
rSODB1
rPxn1-SODB1
rGST
CpG
ConA
SLA
Ag
Medium

5.2.2 Evaluation of Protective Efficacy of Vaccine Antigens in Protein/Protein Immunization

A separate group of mice were used to evaluate the protective efficacy of a cocktail/combination of rLdPxn1 and rLdFeSODB1. The effectiveness of this immunization regimen was compared with immunization of each of the vaccine candidates separately. BALB/c mice (five per group) were immunized with the respective vaccine candidate three times in three weeks interval. Three weeks after the last immunization (Week-9), mice were given a challenge infection with stationary phase promastigotes of *L major* as described in Chapter-2. Then, footpad lesion was measured in each mouse every week for five weeks. Blood was drawn using retro-orbital sinus bleeding at Weeks-0, -3, -6 and -9. Moreover, heart blood was collected at the time of euthanasia (Week-14). Spleens were also collected and cells stimulated with antigen/mitogen. Stimulation and analysis of the culture supernatant was done in the same way as the immunogenicity study above.

5.2.2.1 Antibody Response in Protein/Protein Immunization before and after Challenge Infection

Antigen-specific total IgG response of immunized and control mice is depicted on **Figure-5.3A**. At Week-3, immunization with the rLdPxn1 or the combination of rLdPxn1 and rLdFeSODB1 induced specific IgG response. However, immunization with rLdFeSODB1 protein alone did not elicit any IgG response. Starting from Week-6 onwards, immunization with the combination antigen induced the highest response followed by rLdPxn1 antigen alone. Immunization with rLdFeSODB1 antigen alone resulted in the least IgG response. At Week-6, immunization with either of the individual antigen or the combination of antigens induced significantly higher IgG response than injection with the CpG ODN control ($p<0.05$). However, the difference with rGST control was not statistically significant. On the other hand, immunization with a combination of

antigens resulted in significantly higher IgG response than individual antigens at Week-6 and thereafter ($p<0.05$). At Week-14, a small response was seen in mice injected with CpG ODN. Similar pattern was seen with regard to IgG1 response (**Figure-5.3B**). That is, immunization with a combination of antigens resulted in the highest level of IgG1 response followed by immunization with rLdPxn1 alone. Although the IgG1 response was generally low at Week-3, immunization with rLdPxn1 alone or in combination with rLdFeSODB1 induced significantly higher IgG1 response than the control CpG ODN ($p<0.05$). Starting from Week-6 onwards, immunization with the combination antigen showed the highest IgG1 response. At Week-9, the level of IgG1 response induced by the combination antigen was significantly higher than that induced by either rLdPxn1 or rLdFeSODB1 alone ($p<0.05$). Similar result was also seen at Week-14. At this time, the difference in IgG1 induction between immunization with the combination antigen and that of rGST in the presence of CpG ODN is statistically significant ($p<0.05$). Immunization with rLdFeSODB1 alone also induced significantly higher IgG1 response than CpG ODN control at Week-6 and Week-9. However, at Week-14, CpG ODN control induced comparable level of IgG1 to that of immunization with rLdFeSODB1 antigen alone (**Figure-5.3B**).

Figure-5.3C shows antigen-specific IgG2a response in immunized and control mice. Immunization with individual antigens or a combination of the two showed significantly higher IgG2a response than that of CpG ODN control ($p<0.05$). Immunization with rLdPxn1 alone or in combination with rLdFeSODB1 resulted in the highest antigen-specific IgG2a response which was significantly higher than immunization with rLdSODB1antigen alone, rGST-CpG control or CpG ODN alone ($p<0.05$) at Weeks-6, -9, and -14. On the other hand, although the level of IgG2a induced by immunization rLdFeSODB1 antigen alone is generally low, it was

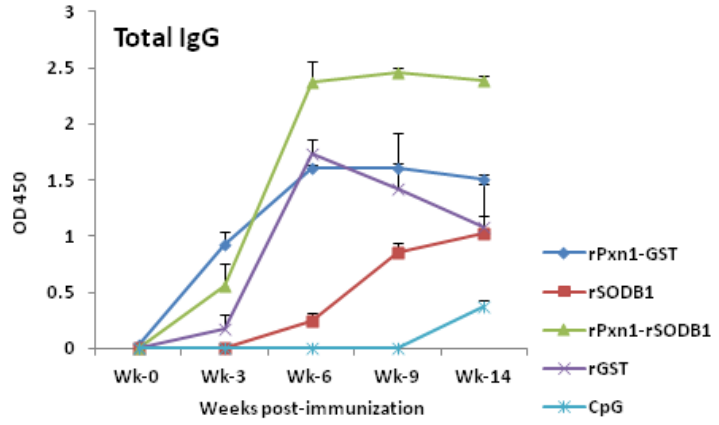
significantly higher than that induced by CpG ODN control ($p<0.05$). The results of antibody response suggest that combining rLdPxn1 and rLdFeSODB1 induces stronger immune response than using individual antigens alone.

IgG2a to IgG1 ratio was calculated from Week-14 sera from mice that were immunized with the individual and cocktail vaccine. Immunization with rLdPxn1, rLdFeSODB1 or a cocktail antigen resulted in IgG2a to IgG1 ratio of 1.1, 0.87, and 0.74, respectively. Moreover, the IgG2a/IgG1 ratio in mice that were injected with rGST control is 0.3. The data show that the cocktail vaccine induced immune response that is highly biased to the non-protective Th-2 type.

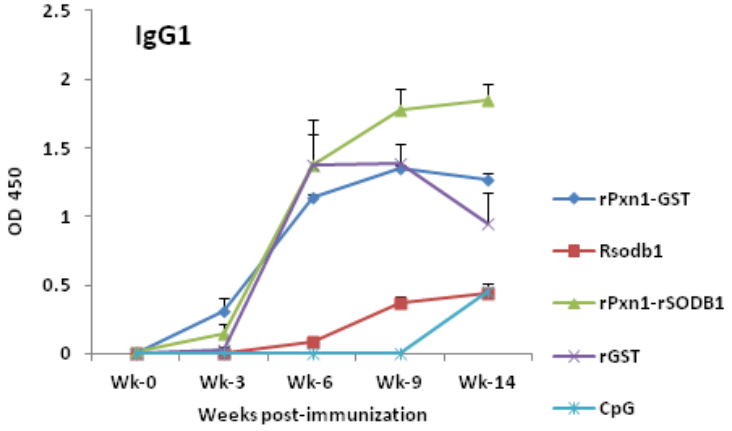
Figure 5.3: Post-challenge antibody response of mice immunized with antigens in protein/protein immunization regimen.

Mice (five per group) were immunized with individual or cocktail recombinant protein antigens with CpG ODN adjuvant three times in three week intervals. At Week-9, all mice were infected on the footpad with *L major* promastigote. Serum samples were collected before immunization, at the time of each immunization and infection, and upon euthanasia (Week-14). Total IgG **(A)**, IgG1 **(B)**, and IgG2a **(C)** were measured using ELISA. Data represents the mean OD_{450nm} of five mice and standard error of mean (SEM). Immunization with the cocktail antigen induced more antibody response than each of the individual ones.

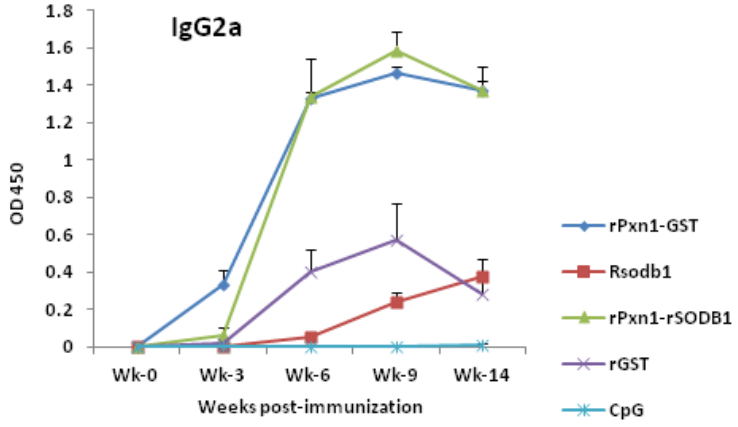
A.


Total IgG
OD 450
3
2.5
2
1.5
1
0.5
0
Wk-0
Wk-3
Wk-6
Wk-9
Wk-14
Weeks post-immunization
rPxn1-GST
rSODB1
rPxn1-rSODB1
rGST
CpG

B.


IgG1
OD 450
2.5
2
1.5
1
0.5
0
Wk-0
Wk-3
Wk-6
Wk-9
Wk-14
Weeks post-immunization
rPxn1-GST
Rsodb1
rPxn1-rSODB1
rGST
CpG

C.


IgG2a
OD 450
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Wk-0
Wk-3
Wk-6
Wk-9
Wk-14
Weeks post-immunization
rPxn1-GST
Rsodb1
rPxn1-rSODB1
rGST
CpG

5.2.2.2 Post-Challenge Cytokine Response

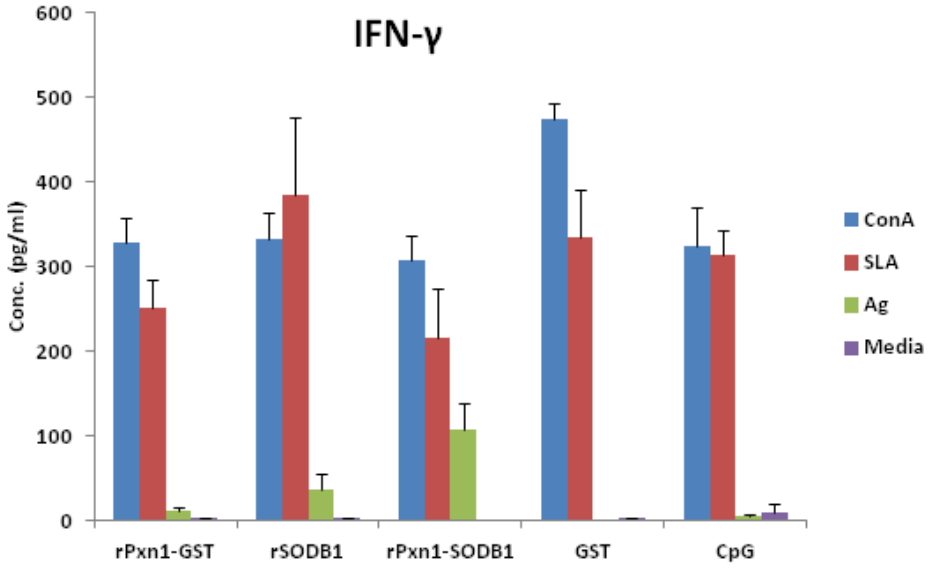
Five weeks after infection with stationary phase promastigotes of *Leishmania major*, both experimental and control mice were sacrificed and spleens isolated. Upon *in vitro* stimulation of spleen cells with antigen/mitogen, the level of IFN-γ and IL-10 was measured from culture supernatant samples.

Figure-5.4A shows the level of IFN-γ response in mice immunized with the recombinant protein antigens and also controls. Immunization with rLdPxn1, rLdFeSODB1, a combination of rLdPxn1 and rLdFeSODB1, GST-CpG or CpG ODN control resulted in 12 ± 3.5pg/ml, 36 ± 19pg/ml, 108 ± 30.5pg/ml, 0.9 ±0.4pg/ml, and 5.4 ± 2.4pg/ml IFN-γ, respectively. Immunization with a combination of rLdPxn1 and rLdFeSODB1antigens or with rLdSODB1 alone resulted in significantly higher IFN-γ than with the GST-CpG and CpG ODN controls, respectively ($p<0.05$). Immunization with rLdPxn1 antigen alone gave exceptionally low IFN-γ which is not even significantly higher than CpG ODN control ($p>0.05$). Spleen cell from all immunized and control groups responded with high IFN-γ upon stimulation with *L major* SLA (**Figure-5.4A**). On the other hand, no detectable level of IL-10 was detected in antigen-stimulated spleen cell cultures that were isolated from mice immunized with rLdPxn1, a combination of rLdPxn1 and rLdFeSODB1, or GST-CpG. Spleen cell from mice immunized with rLdFeSODB1 alone or the control CpG ODN produced 108.6 ± 5.0 and 17.7 ± 17.7pg/ml of IL-10, respectively. The IL-10 response seen in CpG ODN group was the result of only one out of a total of five mice (**Figure-5.4B**). Like the antibody ELISA data, the cytokine response show that the cocktail antigen is more immunogenic than individual antigens.

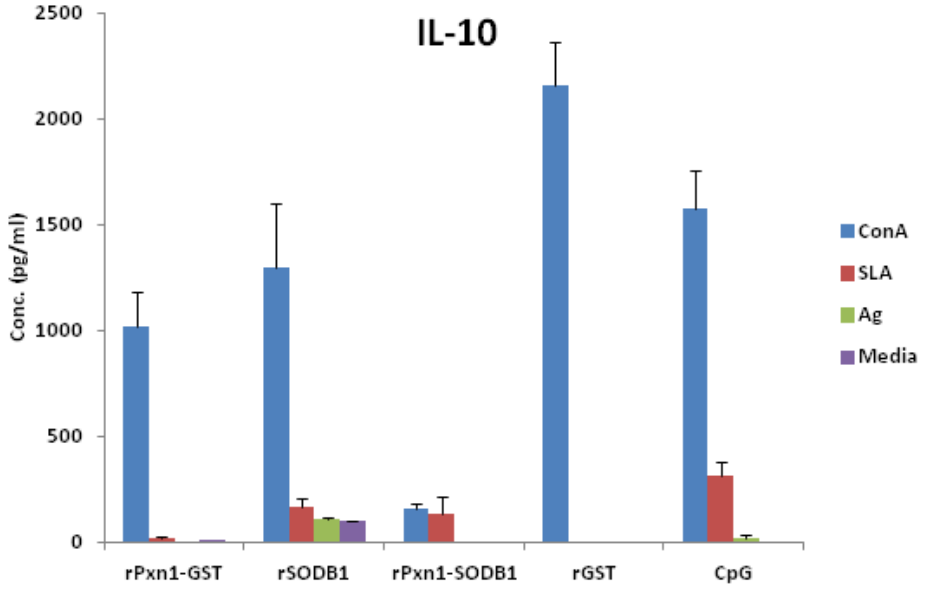
Figure 5.4: Post-challenge cytokine response of mice immunized with antigens in protein/protein immunization regimen.

IFN-γ **(A)** and IL-10 **(B)** production in stimulated spleen cells was measured using cytokine ELISA. Mice (five per group) were immunized with individual or cocktail recombinant protein antigens with CpG ODN adjuvant three times in three week intervals. Three weeks after the last immunization, all mice were infected on the footpad with *L major* promastigote. Five weeks after infection (Week-14), spleen cells were isolated from immunized mice and controls and stimulated *in vitro* with recombinant antigens (Ag) (10µg/ml), *Leishmania major* soluble *Leishmania* antigen (SLA) (50µg/ml) or concanavalin A (5µg/ml). Unstimulated cells were included as negative control. Cells were cultured at 37°C and 5% CO_2 and culture supernatant was collected at 72hr. The level of IFN-γ and IL-10 was measured using cytokine ELISA kit (e-Biosciences). The concentration of the cytokines was determined using the standard proteins included in kit and expressed in pg/ml. The mean concentration and standard error of the mean (SEM) of five mice per group is shown. Statistical comparison between groups was performed using Mann-Whitney U test. Immunization with the cocktail antigen induced the highest IFN-γ response.

A.


IFN-γ
Conc. (pg/ml)
600
500
400
300
200
100
0
rPxn1-GST
rSODB1
rPxn1-SODB1
GST
CpG
ConA
SLA
Ag
Media

B.


IL-10
Conc. (pg/ml)
2500
2000
1500
1000
500
0
rPxn1-GST
rSODB1
rPxn1-SODB1
rGST
CpG
ConA
SLA
Ag
Media

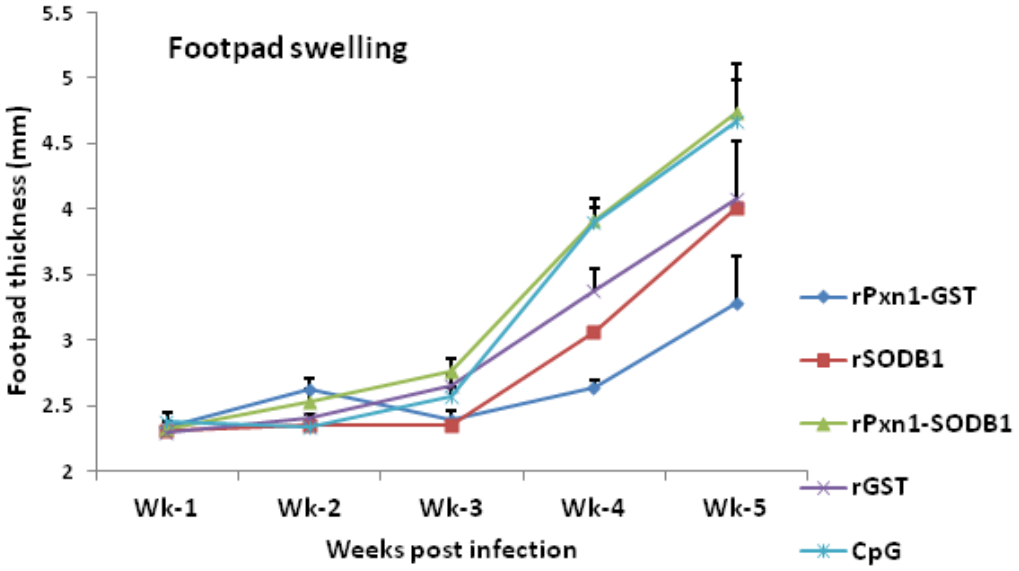
5.2.2.3 Footpad Swelling in Protein/Protein Immunization

Figure-5.5 shows the footpad thickness measurement of mice immunized with individual or cocktail antigens after infectious challenge with *L major*. Unexpectedly, mice that were immunized with the combination of rLdPxn1 and LdFeSODB1 developed the biggest footpad lesion throughout the study period than those immunized with individual antigens and also controls. For instance, at Week-4 post-infection, mice immunized with the combination antigen showed significantly bigger lesion than mice that were immunized with either rLdPxn1, rLdFeSODB1 or even with rGST control ($p<0.05$). On the other hand, mice that were immunized with rLdPxn1 showed the smallest footpad lesion which is significantly smaller at Week-4 than lesions of mice that were immunized with other antigens or controls ($p<0.05$). However, at the time of euthanasia (Week-5), there was no statistical difference between any of the groups.

Footpad parasite load measurement resulted in data that reflect the footpad lesion experiment. That is, mice immunized with the cocktail vaccine had the highest parasite load which was higher than mice immunized with either the rLdPxn1 alone or rLdFeSODB1 alone (data not shown).

Figure 5.5: Footpad swelling of mice immunized with antigens and controls in protein/protein immunization strategy.

Mice (five per group) were immunized with individual or cocktail recombinant protein antigens with CpG ODN adjuvant three times in three week intervals. Three weeks after the last immunization, all mice were infected on the footpad with subcutaneous injection of 5 x 10^6 stationary phase *L major* promastigotes in the hind footpad. The footpad swelling was assessed by measuring the thickness of infected footpad weekly for five weeks using electronic digital caliper. The data represents mean footpad size in millimetre of five mice and standard error of the mean. Immunization with the cocktail vaccine did not protect mice from *L major* infection.


Footpad swelling
Footpad thickness (mm)
5.5
5
4.5
4
3.5
3
2.5
2
Wk-1
Wk-2
Wk-3
Wk-4
Wk-5
Weeks post infection
rPxn1-GST
rSODB1
rPxn1-SODB1
rGST
CpG

5.3 Experimental Discussion

The result of the protein/protein immunization study clearly shows that using a combination of rLdPxn1 and rLdFeSODB1 as a cocktail vaccine increases the immune response than using individual ones separately. This was demonstrated by the higher degree of antibody and cell-mediated responses before infection. The high level immune response against the cocktail antigen remained high five weeks after infection with *Leishmania major*. However, this antigen failed to protect BALB/c mice from *L major* infection.

The cocktail vaccine or rLdPxn1 induced rapid and high level specific antibody response as seen in Week-3 total IgG response. However, rLdFeSODB1 showed significant antibody response only after two immunizations indicating that rLdFeSODB1 is the least immunogenic protein. This is in line with the results of DNA/protein immunization study (**Chapter-4**).

Four weeks after the last immunization, the cocktail antigen induced significantly higher IFN-γ and IL-10 than each of the individual antigens ($p<0.05$) demonstrating that combining rLdPxn1 and rLdFeSODB1 increases the specific cellular immune response. The level of IFN-γ production by the spleen cells isolated from mice immunized with the cocktail antigen was 6.4- and 17- times more than that from mice immunized with rLdPxn1alone or rLdFeSODB1 alone, respectively. Moreover, the result showed that the level of IFN-γ produced in mice immunized with the cocktail vaccine is much more than that the sum of IFN-γ produced by mice immunized with each of the individual antigens suggesting that the two antigens may act synergistically to induce strong immune response.

Prior to infection with *L major* infection, the cocktail vaccine also induced significantly higher IL-10 response than each of the individual antigens. This indicates that using a combination vaccine induces a mixed Th-1 and Th-2 response. Considering the high ratio of IFN-γ to IL-10

(8.0), it can be concluded that the cocktail vaccine induces a highly Th-1 biased immune response. This data, however, was not corroborated with the pre-infection antibody response. The IgG2a/IgG1 ratio in mice immunized with the cocktail vaccine was not significantly different from that in mice immunized with either of the individual antigens. Rather, the cocktail vaccine showed a slightly lower ratio than rLdPxn1. The discrepancy could be due to the fact that the level of IL-10 may not directly reflect the level of IgG1. As discussed in Chapter-1, the level of IgG1 is a direct reflection of the level of IL-4 rather than IL-10. In this study, we did not include the IL-4 data. No detectable amount of IL-4 was obtained in any of the immunized groups (data not shown). This phenomenon was also shown in other studies (Pascalis et al., 2003).

The high level of IFN-γ in mice immunized with the cocktail vaccine was also maintained until five-weeks after *L major* infection. However, the level of IL-10 after *L major* infection was undetectable in spleen cells from mice immunized with rLdPxn1, the cocktail antigen, or rGST control. Repeating the experiment produced the same result. We saw a clear discrepancy in IL-10 level between immunogenicity and protection experiments. We do not know if this difference was related to the cytokine ELISA kit used. Cytokine ELISA kits from BD-biosciences and e-Biosciences were used in immunogenicity and protection studies, respectively.

Although the cocktail vaccine showed a very high degree of immunogenicity with high IFN-γ, it failed to protect mice from infection with *L major*. As shown in **Figure-5.5** above, although not statistically significant, mice immunized with the cocktail vaccine showed the highest footpad lesion, as high as mice immunized with CpG ODN control. On the other hand, immunization with rLdPxn1 alone showed the least footpad swelling indicating that it is relatively more protective than the cocktail or rLdFeSODB1. This could be due to the same reason discussed in

Chapter-4. That is, induction of high level IFN-γ does not necessarily indicate protection against *L major* infection (Gicheru et al., 2001, Leal et al., 2001, Elias et al., 2005).

The protective efficacy of the cocktail vaccine in protein/protein immunization strategy is different from that of DNA/protein strategy. In the former, mice immunized with the cocktail vaccine developed footpad lesion appreciably higher than those immunized with either rLdPxn1 or rLdFeSODB1. In DNA/protein immunization, however, mice immunized with the fusion DNA in the presence of mGMCSF adjuvant followed by the cocktail recombinant protein boost developed smaller lesion than those immunized with rLdFeSODB1 but slightly bigger lesion than those immunized with rLdPxn1. In the absence of mGMCSF fusion, all the vaccine antigens including the fusion showed similar level of modest protection. At this point, we do not understand the exact reason for the discrepancy. Experimentally, there were three differences between the two studies. Firstly, in protein/protein immunization strategy, we used rLdPxn1 fused with GST where as in DNA/protein one, we used rLdPxn1 cleaved from rLdPxn1-GST fusion. However, the presence of GST does not appear to contribute for the high footpad lesion in mice immunized with the cocktail as rGST alone did show slightly lower footpad lesion than the cocktail. Moreover the rGST fusion was included not only in mice immunized with the cocktail but also in those immunized with rLdPxn1. Secondly, in protein/protein immunization, we used three doses of a cocktail of rLdPxn1 and rLdFeSODB1. Thirdly, 5×10^6 and 3×10^6 *L major* stationary phase promastigotes were used for infection in protein/protein and DNA/protein immunization regimens, respectively. Again, it is unclear if the high parasite load affected the mice immunized with the cocktail vaccine more than those immunized with individual antigens.

5.4 CONCLUSION AND FUTURE DIRECTION

The objective of this study was to evaluate the immunogenicity and protective efficacy of *Leishmania donovani* peroxidoxin 1 and superoxide dismutase B1 as a fusion or a cocktail vaccine in mice. To do this, DNA/protein and protein/protein immunization strategies were employed.

In DNA/protein immunization strategy, the fusion antigen generally induced higher immunogenicity than superoxide dismutase B1 antigen as shown by high antibody and cell-mediated immune responses. However, no significant difference was seen between the fusion and peroxidoxin antigens. Moreover, in the presence of mGMCSF, the fusion and peroxidoxin 1 antigens elicited a higher level of expression of Th-1 cytokines by $CD4^+$ T cells. The immunogenicity result was generally reflected in the protection of BALB/c mice from *L major* infection. That is, immunization with the fusion or peroxidoxin 1 antigen induced significantly lower footpad swelling and parasite load than the controls. On the other hand, immunization with superoxide dismutase B1 antigen did not protect mice against *L major* infection.

The use of mGMCSF in tandem with the vaccine antigen in DNA vaccines induced stronger immune response and better protection than the corresponding antigen without mGMCSF. Thus, GMCSF can be considered to be a good adjuvant in designing future vaccine antigens against leishmaniasis.

Like in DNA/protein immunization, protein/protein immunization using a combination of rLdPxn1 and rLdFeSODB1 showed a higher level of immune response than rLdFeSODB1 alone. However, the cocktail antigens did not protect mice from *L major* infection. On the other hand, immunization with rLdPxn1 alone resulted in a reduction in the footpad swelling of infected mice.

The results of DNA/protein and protein/protein immunization studies clearly showed that peroxidoxin 1 antigen is a more useful antigen and deserves a more detailed study in the future. It is, therefore, important to test the efficacy of LdPxn1 in long-term protection aginst *L major* infection. This can be tested by performing infection 10 to 12 weeks after the last immunization. Moreover, it will also be useful to test LdPxn1 as a component of multi-antigen fusion vaccine by fusing it with other antigens such as *Leishmania* cysteine proteases and A2 proteins.

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APPENDIX A: PILOT STUDY ON IMMUNOGENICITY OF VACCINE ANTIGENS IN LEISHMANIASIS PATIENT SAMPLES IN ETHIOPIA.

In order to be used as vaccine, an antigen (s) should be recognized by and elicit immune response in the host that it is intended to be used. Thus, *Leishmania* vaccines that are developed to be used in human should be capable of eliciting immune response in human host. Induction of response in experimental animals such as mice does not always guarantee effectiveness in humans (Handman, 2001). Moreover, different vaccine antigens induce variable level of recognition by human host. For example, Rafati and colleagues (2003) demonstrated that recombinant cysteine protease B (rCPB) is better recognized by sera from visceral leishmaniasis patients than rCPA. Therefore, in order to determine the effectiveness of a vaccine candidate, one has to first confirm whether the antigen is recognized by human immune system and assess the level of immunogenicity it elicits in humans. For this reason, we tried to assess the immunogenicity of rLdPxn1 and rLdFeSODB1 in Ethiopian cutaneous (CL) and visceral leishmaniasis (VL) patient samples.

To determine the immunogenicity of rLdPxn1 and rLdFeSODB1, venous blood was collected from CL and VL patients. Diagnosis of CL and VL was done following standard protocols. Diagnosis of cutaneous leishmaniasis was performed based on clinical and parasitological features. The parasitological technique involved *in vitro* culture of the parasite from samples taken from active lesion and/or microscopic identification of the parasite from stained smears. Skin scrape sample was inoculated to the liquid phase of NNN medium and cultured for at least one week. The presence of motile promastigote was considered positive. Likewise, diagnosis of visceral leishmaniasis was done by a combination of clinical and serological diagnostic techniques according to the guidelines of the Ministry of Health, Federal Democratic Republic of

Ethiopia. In addition to leishmaniasis patients, healthy controls from non-endemic area were also included in the study. Specimens from a total of eight CL and ten VL patients as well as nine healthy (uninfected) controls were used to assess the immunogenicity of the antigens in humans.

Ten to fifteen millilitres of venous blood was collected from each individual using heparinised vacutainer tubes. The blood samples were mixed thoroughly to prevent clotting and transported to the laboratory within 2-3 hours. Then, the blood was transferred into a 50ml conical tube (Sarstedt, USA). It was then centrifuged at 1200rpm for 5min using Allegra™ 6R Centrifuge (Beckman Coulter, USA). One millilitre plasma was transferred into a cryotube and stored in -20°C freezer until use for antibody ELISA.

Peripheral blood mononuclear cells (PBMCs) were isolated from the same blood samples. After the plasma sample was taken, the blood was diluted 1 to 1 in RPMI 1640 medium (Sigma, USA). Fifteen millilitre Ficoll-Paque Plus (GE Healthcare) was pipetted into a new 50ml conical tube. Then, the blood was layered onto the Ficoll by gently pipetting out the blood on the side of the tube at about 45°. Once layered onto Ficoll, the blood was centrifuged at 1800rpm for 20min with break off. Carefully, the PBMC layer at the interface between the Ficoll and plasma was taken and transferred into a new 50ml conical tube. Then, PBMCs were resuspended in 45ml RPMI 1640 medium and spun at 1800rpm for 10min with break off. The cells were then washed three times each with 45ml RPMI 1640 medium and the cell pellet was resuspended in 3ml RPMI 1640 with 20% FBS. After counting using Double Neubauer Counting Chamber (VWR), the cells were mixed with freezing medium (RPMI 1640 20% FBS 10% DMSO (Sigma) and kept in Mr Frosty freezing container (Thermo Scientific) and stored in -80°C freezer.

Total human IgG specific rLdPxn1 or rLdFeSODB1 was measured from plasma samples following the procedure in Chapter-2. The level of cytokine response was assessed by measuring

IFN-γ and IL-10 from supernatants of stimulated peripheral blood mononuclear cells (PBMCs) using cytokine ELISA procedure described in Chapter-2. PBMCs were stimulated with each of the recombinant proteins, *L major* SLA, or phytohaemagglutinin (PHA). A negative control well contained PBMCs in the absence of antigen/mitogen.

The results of antibody and cytokine ELISA are summarized in **Appendix-A1** and **Appendix-A2**, respectively. Plasma samples from cutaneous leishmaniasis patients reacted to both rLdPxn1 and rLdFeSODB1 as demonstrated by high OD_{450} for human total IgG specific to the antigens. The response to rLdPxn1 was significantly higher than that from plasma of healthy individuals ($p<0.05$). Plasma from visceral leishmaniasis patients also showed high OD_{450} for human total IgG specific to rLdPxn1. Unfortunately, plasma samples from visceral leishmaniasis patients were not tested against rLdFeSODB1 due to logistical constraint. These results demonstrated that rLdPxn1 and rLdFeSODB1 are recognized by human immune system and infection with *Leishmania aethiopica* and *Leishmania donovani* in humans elicits immune response against these antigens. *L aethiopica* and *L donovani* are the predominant species causing CL and VL in Ethiopia, respectively (Gadisa et al., 2007, Gelanew et al., 2010). Unexpectedly, rGST control was also equally recognized by CL and VL plasma.

Cytokine ELISA in PBMCs of CL patients showed that rLdPxn1 is the more immunogenic than rLdFeSODB1. *In vitro* stimulation of PBMCs with rLdPxn1 resulted in significantly higher IFN-γ than stimulation with rLdFeSODB1 antigen or rGST control ($p<0.05$). It also showed significantly higher IL-10 recall response than rLdFeSODB1 or the controls. Moreover, stimulation with *L major* SLA also showed high level IFN-γ response confirming active infection. Previous studies showed that different *Leishmania* antigens induce variable level of immune response in humans. Rafati and co-workers (2003) showed that the multicopy gene

product, *Leishmania* cysteine protease B (CPB), induced more immune response than the product of a single copy gene, CPA.

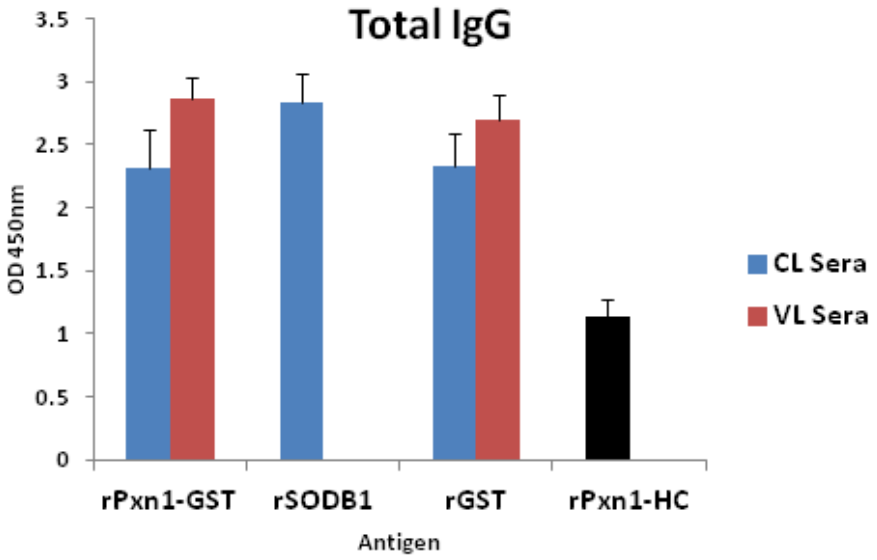
The presence of some degree of antibody response in sera from healthy individuals could be due to infection with other microorganisms that cross-react with rLdPxn1-GST. Moreover, although these individuals were recruited from a non-endemic area, the possibility of exposure to *Leishmania* infection was not completely ruled out.

In this preliminary study, we did not include samples from individuals cured from CL or VL. Theoretically, the presence of high level IFN-γ response in antigen stimulated PBMCs from cured individuals is an indication the presence of memory T cells specific to the vaccine antigen confirming the ability of the antigen to induce protective immune response (Rafati et al., 1997).

In conclusion, this preliminary study appears to show that rLdPxn1 and rLdFeSODB1 are recognized in humans infected with both CL and VL causing strains. However, in order to get a complete and better picture, test of the antigens in samples from larger number of cases taken from different stages of the disease is required.

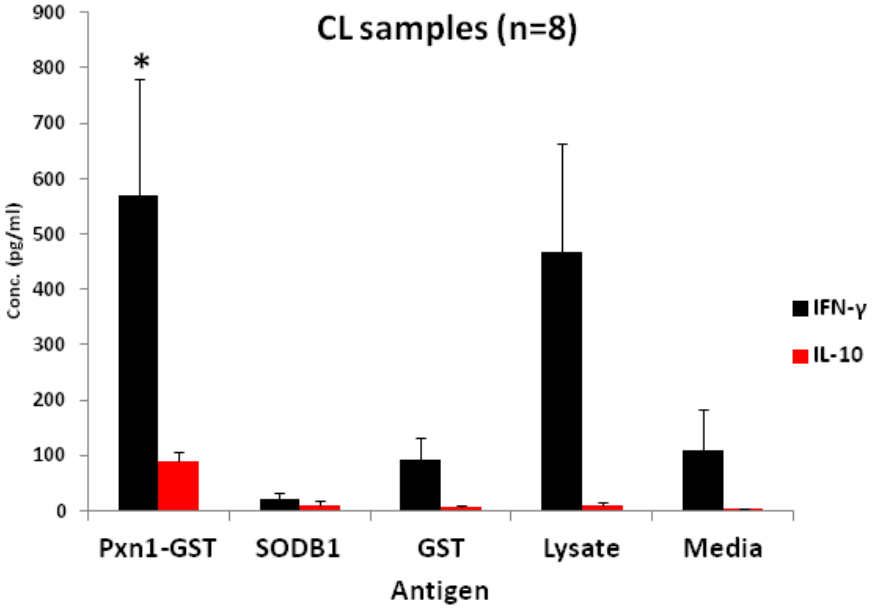
A.1.Immunoreactivity of cutaneous and visceral leishmaniasis patient plasma to rLdPxn1 and rLdFeSODB1.

The level of total IgG response was measured from plasma collected from cutaneous (n=8) and visceral leishmaniasis patients (n=10) as well as healthy individuals (n=9) using antibody ELISA. The result was expressed as mean OD_{450nm} and standard error of the mean. Statistical comparison between groups was performed using Mann-Whitney U test. Reactivity of rLdFeSODB1 was tested only in CL patient plasma. “HC” refers to “Healthy Controls”. The result showed that the level of antigen-specific IgG is significantly higher in both CL and VL plasma samples than in healthy controls ($p<0.05$).


Total IgG
3.5
3
2.5
2
1.5
1
0.5
0
OD 450nm
rPxn1-GST
rSODB1
rGST
rPxn1-HC
Antigen
CL Sera
VL Sera

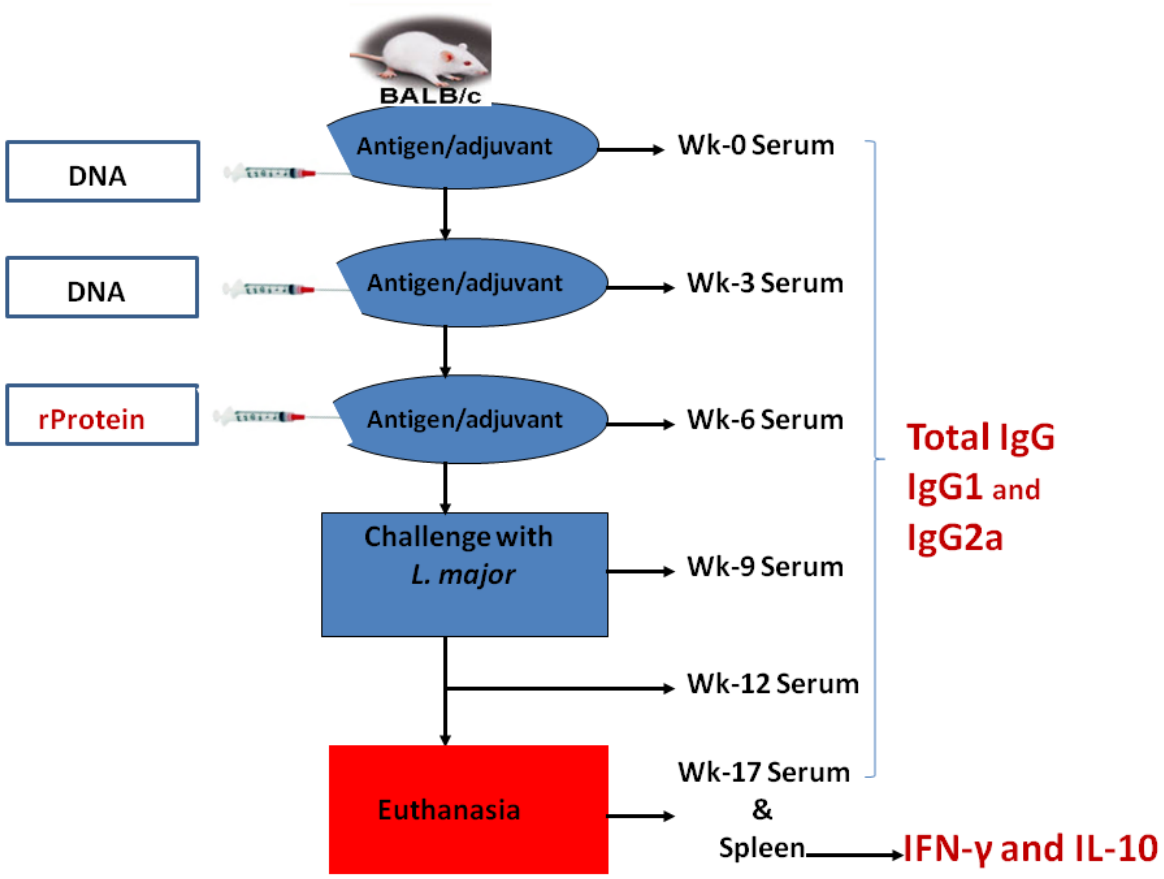
A.2.Cytokine response of PBMCs of cutaneous leishmaniasis patients.

Peripheral blood mononuclear cells were isolated from venous blood of cutaneous leishmaniasis patients (n=8) and stimulated with the rLdPxn1, rLdFeSODB1, rGST, and *Leishmania major* SLA. Positive and negative control cells were included by stimulation with phytohaemagglutinin (PHA) or adding medium only, respectively. The result represented as mean concentration of IFN-γ and IL-10 and standard error of the mean. Statistical comparison between groups was performed using Mann-Whitney U test. Stimulation with rLdPxn1 and SLA showed high degree of IFN-γ response. Recombinant Pxn1 induced statistically significant IFN-γ than rSODB1, rGST, or the negative control ($p<0.05$). Stimulation with PHA gave 1510.4 ± 67.3pg/ml IFN-γ and 380.3 ± 77.7pg/ml IL-10 (data not shown).

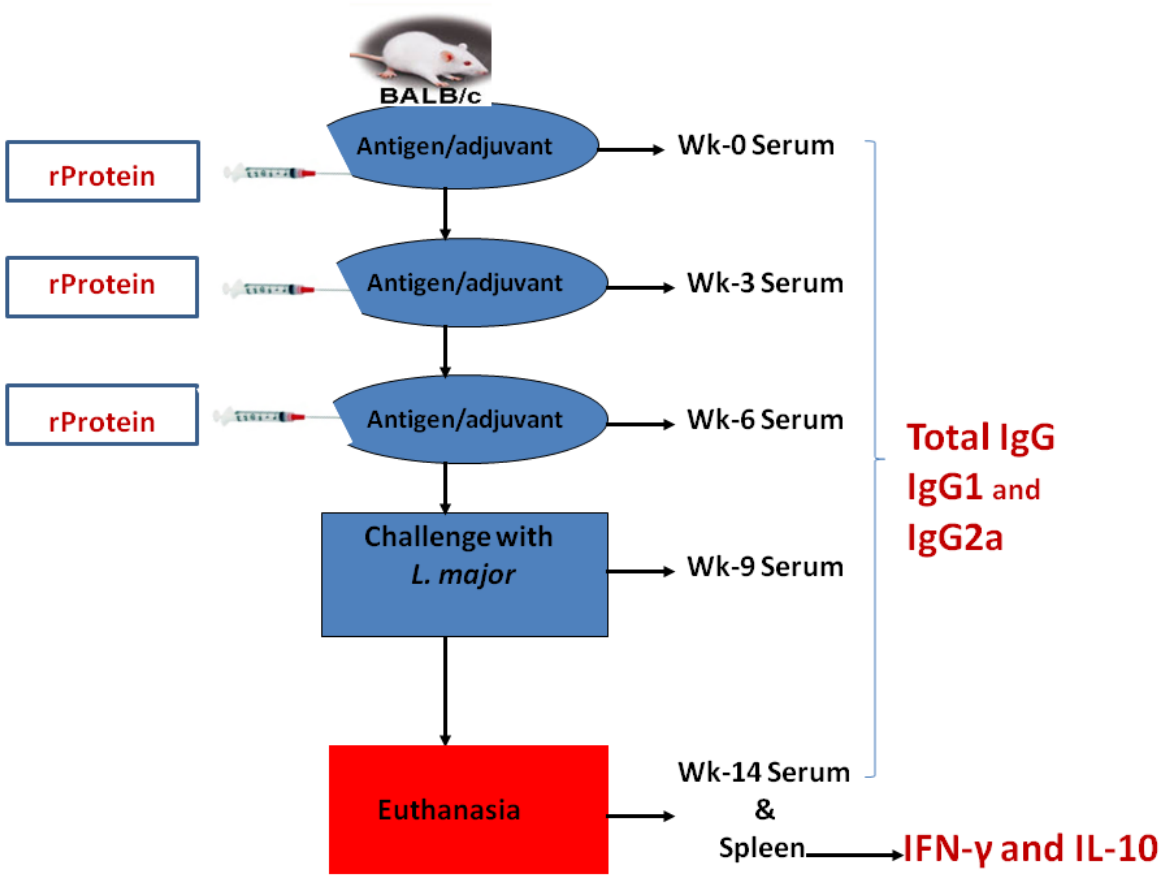

CL samples (n=8)
900
800
700
600
500
400
300
200
100
0
Conc. (pg/ml)
*
IFN-γ
IL-10
Pxn1-GST
SODB1
GST
Lysate
Media
Antigen

APPENDIX B: SCHEMATIC ILLUSTRATION OF IMMUNIZATION STRATEGIES

B.1.DNA/protein immunization protocol.



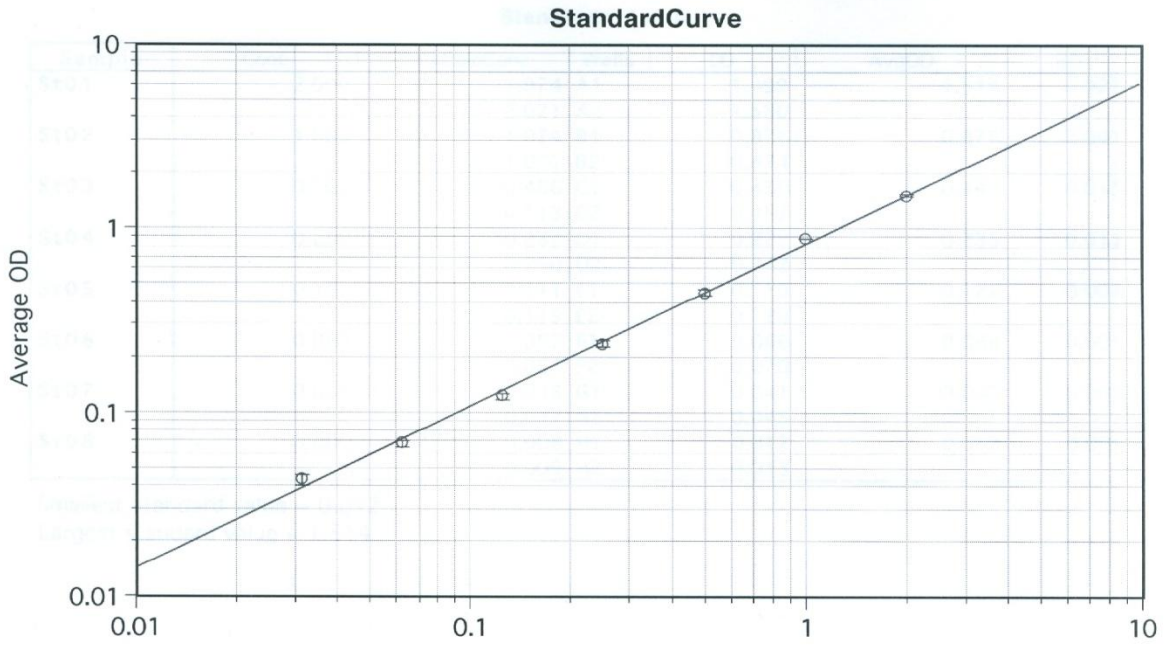
B.2.Protein/protein immunization protocol.



APPENDIX C: REPRESENTATIVE OF STANDARD CONTROL PROTEIN FOR CYTOKINE ELISA.

Standards (ng/ml)

Sample	Conc	BackCalcConc	Wells	OD	AvgOD	SD	CV
St01	2.000	1.974	A1	1.499	1.514	0.022	1.5
		2.021	A2	1.530			
St02	1.000	1.074	B1	0.876	0.875	0.001	0.2
		1.071	B2	0.874			
St03	0.500	0.486	C1	0.436	0.447	0.015	3.3
		0.513	C2	0.457			
St04	0.250	0.232	D1	0.228	0.235	0.011	4.5
		0.250	D2	0.242			
St05	0.125	0.111	E1	0.119	0.122	0.005	4.3
		0.119	E2	0.126			
St06	0.063	0.057	F1	0.066	0.068	0.003	4.1
		0.061	F2	0.070			
St07	0.031	0.033	G1	0.041	0.043	0.003	7.4
		0.037	G2	0.045			
St08	0.000	0.008	H1	0.012	0.012	0.000	4.1
		0.009	H2	0.013			



IFN-γ concentration (ng/ml)