Effects of dexamethasone and hydrocortisone in the control of
*Leishmania major* infection in BALB/c mice

Rose Magoma Nyamao

A thesis submitted in partial fulfilment of the requirements for the
award of the degree of Master of Science in Zoology (Immunology) of
Jomo Kenyatta University of Agriculture and Technology

2015
DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature.................................................. Date..........................................................

Rose Magoma Nyamao

This thesis has been submitted for examination with our approval as University supervisors:

Signature.................................................. Date..........................................................

Prof. Rebecca Waihenya

JKUAT, KENYA

Signature.................................................. Date..........................................................

Dr. Zipporah Osiemo Lagat

JKUAT, KENYA
DEDICATION

This thesis is dedicated to my beloved parents, Mr. Naftal Nyamao Nyantika and Mrs. Hellen Gesare Nyamao, for their immeasurable support both spiritual and financial and for their unfailing faith in me. I am greatly indebted to your love, may God bless and preserve you two.

"Don't be afraid to give up the good to go for the great." ~ John D. Rockefeller
ACKNOWLEDGEMENT

I am very grateful to the Almighty God for the gift of health, power of will and patience that he granted me throughout this period of study. All this wouldn’t have been without your help and may your name be praised, Amen.

Extraordinary thanks to Dr. Robert Karanja, Prof. Rebecca Waihenya, and Dr. Zipporah Osiemo-Lagat who patiently and persistently provided their expertise, resources, and time to make this work worth the while. May you live as long as you want, and never want as long as you live.

I would also like to extend my deep felt appreciation and thanks to Dr. Shadrack Muya, the post graduate advisor, Zoology department for his friendly attitude, helpful suggestions and valuable information.

I give enormous thanks to Mr. Johnstone Ingonga, Mr. Lucas Ogutu, and Ms. Sarah Nyasende, for their tremendous help and encouragement while carrying out laboratory work.

The academic journey was long, tiresome and mostly discouraging at many points. It would not have been possible without the love, support and constant encouragement and reassurance from Tom Kibet. I owe you the worth of this work.

This chapter will be incomplete without mentioning my classmates and friends Simon Mogaka, Charlotta Ndichu, Robert Kagali, Ednah Ongaga, Ruth Onchwari, Agnes Omire, Catherine Wachira, Edith, Christopher Khayeka, Bernard Jumba, Samuel Kiige, Daniel Kwalimwa, and Eliud Wafula for their moral support and encouragement during the search for knowledge.

Last but not least, I would also like to highly appreciate my siblings Isaac Nyantika, Lucky Moraa, Jeff Nyamo, Sirmeboy, nephew Bobo Jessie and niece Kate Mwango for their love, encouragement and patience during all the previous years that went into the completion of this work that makes them co-workers of this study.

iv
TABLE OF CONTENT

DECLARATION ..................................................................................................................... ii

DEDICATION ....................................................................................................................... iii

ACKNOWLEDGEMENT ......................................................................................................... iv

TABLE OF CONTENT .......................................................................................................... v

LIST OF TABLES .................................................................................................................... ix

LIST OF FIGURES ................................................................................................................ x

LIST OF APPENDICES ......................................................................................................... xii

ABBREVIATIONS AND ACRONYMS .................................................................................. xiii

ABSTRACT ............................................................................................................................... xiv

CHAPTER ONE ....................................................................................................................... 1

1.0 INTRODUCTION AND LITERATURE REVIEW ......................................................... 1

1.1 Background of the study ................................................................................................. 1

1.2 Literature review ........................................................................................................... 5

1.3 Statement of the problem .............................................................................................. 19

1.4 Justification ................................................................................................................... 19

1.5 Hypotheses ................................................................................................................... 20

1.6 Objectives of the study ................................................................................................. 20
CHAPTER TWO

2.0 MATERIALS AND METHODS .................................................. 21

2.1 Study site ............................................................................. 21

2.2 Experimental design............................................................. 21

2.3 Sample size calculation ......................................................... 22

2.4 Experimental animals............................................................ 22

2.5 Parasite culture .................................................................... 23

2.6 Drug preparation .................................................................. 23

2.7 Macrophage harvesting .......................................................... 23

2.8 Treatment of macrophages with dexamethasone and hydrocortisone (macrophage assay) ................................................................. 24

2.9 Enzyme linked immunosorbent assay ..................................... 24

2.10 Macrophage infection ............................................................ 25

2.11 In vivo studies ..................................................................... 26

2.12 Quantifying parasite burden .................................................. 26

2.13 Statistical analysis ............................................................... 27

CHAPTER THREE ........................................................................ 28

3.0 RESULTS .............................................................................. 28
3.1 Effects of glucocorticoids on production of MIP 1A -1α and MCP 1 chemokines before and after infection with L. major in macrophages of BALB/c in vitro ...........28

3.2 Effects of glucocorticoids on Leishmania parasite multiplication and infection rates in macrophages ........................................................................................................33

3.3 Determination of LDU ..............................................................................................................36

3.4 Effects of glucocorticoids on levels of MIP 1A -1α, MCP 1 before and after infection with L. major and effects on IFN γ after infection in vivo .........................................................37

3.5 Effects of glucocorticoids on lesion development in BALB/c mice ..........................42

CHAPTER FOUR ................................................................................................................................44

4.0 DISCUSSION ................................................................................................................................44

4.2 Efficacy of glucocorticoids on production of MIP 1A -1α and MCP 1 chemokines before and after infection with L. major in macrophages of BALB/c in vitro ...........44

4.3 Effects of glucocorticoids on Leishmania parasite multiplication and infection rates in macrophages ..............................................................................................................46

4.4 Determination of LDU ..............................................................................................................49

4.5 Effects of glucocorticoids on levels of MIP 1A -1α, MCP 1 before and after infection with L. major and effects on IFN γ after infection in vivo .........................................................50

4.6 Effects of glucocorticoids on lesion development in BALB/c mice ..........................53

CHAPTER FIVE ................................................................................................................................55

5.0 CONCLUSIONS AND RECOMMENDATIONS .........................................................................55
5.1 Conclusions .................................................................................................................. 55

5.2 Recommendations ....................................................................................................... 56

5.3 Study limitations or challenges .................................................................................. 57

REFERENCES ..................................................................................................................... 58

APPENDICES...................................................................................................................... Error! Bookmark not defined.
LIST OF TABLES

Table 1: Body weight, weight of spleen, spleeno-somatic index, number of parasites and LDU in BALB/c mice following treatment with hydrocortisone, dexamethasone, LPS and PBS

................................................................................................................. 36
LIST OF FIGURES

Figure 1: Map of Kenya showing leishmaniasis endemic regions in Kenya (survey of kenya) ........................................................................................................................................................................5

Figure 2: Concentration of MIP 1α in the macrophage of BALB/c mice after treatment with hydrocortisone before and after infection with L. major promastigotes in vitro ........................................................................................................................................................................29

Figure 3: Concentration of MIP 1α in the macrophage of BALB/c mice after treatment with dexamethasone before and after infection with L. major promastigotes in vitro ........................................................................................................................................................................30

Figure 4: Concentration of MCP 1 in the macrophage of BALB/c mice after treatment with hydrocortisone before and after infection with L. major promastigotes in vitro ........................................................................................................................................................................31

Figure 5: Concentration of MCP 1 in the macrophage of BALB/c mice after treatment with dexamethasone before and after infection with L. major promastigotes in vitro ........................................................................................................................................................................32

Figure 6: Parasitic infection rates in the macrophages of BALB/c mice subjected to various drug therapies ........................................................................................................................................................................33

Figure 7: Parasite multiplication rates in the macrophages of BALB/c mice subjected to various drug therapies ........................................................................................................................................................................34

Figure 8: Concentration of MIP 1α in the serum of BALB/c mice after treatment with hydrocortisone before and after infection with L. major promastigotes in vivo ........................................................................................................................................................................38

Figure 9: Concentration of MIP 1α in the serum of BALB/c mice after treatment with dexamethasone before and after infection with L. major promastigotes in vivo ........................................................................................................................................................................39

Figure 10: Concentration of MCP 1 in the serum of BALB/c mice after treatment with hydrocortisone before and after infection with L. major promastigotes in vivo ........................................................................................................................................................................40
Figure 11: Concentration of MCP 1 in the serum of BALB/c mice after treatment with dexamethasone before and after infection with L. major promastigotes in vivo ................................................................. 41

Figure 12: Serum IFN-γ levels of BALB/c mice infected with L. major and treated with dexamethasone and hydrocortisone ................................................................. 42

Figure 13: Lesion development in BALB/c mice subjected to different drug treatments ......................................................................................................................... 43
LIST OF APPENDICES

Appendix 1: Visceral leishmaniasis epidemiology in Kenya .................................................... 73
Appendix 2: Cutaneous leishmaniasis epidemiology in Kenya.................................................. 74
Appendix 3: Ethical clearance.................................................................................................. 75
### ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACUC</td>
<td>Animal care and use committee</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>CL</td>
<td>Cutaneous leishmaniasis</td>
</tr>
<tr>
<td>CBRD</td>
<td>Center for Biotechnology Research and Development</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>Iκκ</td>
<td>Inhibitor of kappa beta protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of kappa beta protein kinase</td>
</tr>
<tr>
<td>JAK 2</td>
<td>Janus activated kinase 2</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>LDU</td>
<td>Leishman Donovan Units</td>
</tr>
<tr>
<td>LHFD</td>
<td>Left hind footpad</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MIP 1α</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MCP 1</td>
<td>Monocyte Chemo-attractant Protein</td>
</tr>
<tr>
<td>NF-κκβ</td>
<td>Nuclear Factor kappa beta</td>
</tr>
<tr>
<td>NGHW</td>
<td>National Guidelines for Health Workers</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>STAT 1α</td>
<td>Signal transducer and activator of transcription 1α</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial Institute</td>
</tr>
</tbody>
</table>
ABSTRACT

Leishmania is an obligate intracellular parasite that has to survive a hostile environment created by an infected host cell. There are many strains of Leishmania with different clinical presentations. Cutaneous leishmaniasis is the most common form of leishmaniasis and is manifested by skin lesions. The parasite has evolved many mechanisms to evade the immune system and propagate itself. One of the mechanisms is the induction of pro-inflammatory cytokines MIP 1α and MCP 1 which recruit more cells to area of infection leading to propagation of infection and pathology. This study was hereby aimed at determining the effects of dexamethasone and hydrocortisone in the control of L. major infection and their effect on the levels of MIP 1α and MCP 1 chemokines and the subsequent effect on the infection. The effects of glucocorticoids in the control of Leishmania major infection was investigated in both in vitro and in vivo experiments. Macrophages were treated with glucocorticoids (dexamethasone and hydrocortisone succinate). The macrophages were then infected with Leishmania major promastigotes and the levels of macrophage inflammatory protein 1α (MIP 1α), monocyte chemo-attractant protein-1 (MCP 1) chemokines were measured both before and after infection. Parasite multiplication in vitro was investigated by counting the number of amastigotes infecting treated and untreated macrophages (controls). For the in vivo experiment, Mice were treated with dexamethasone and hydrocortisone for 21 days then infected with Leishmania major parasites. Another group of mice was infected with Leishmania major promastigotes, monitored for lesion development for five weeks and then treated with glucocorticoids. Serum samples were collected from both arms of the experiment for cytokine analysis. Lesion development was also monitored for five weeks. Lesion sizes after infection of BALB/c mice were similar in all the experimental groups till the onset of treatment (P < 0.05). Two weeks after start of treatment, significant differences (P < 0.05) were discerned in the lesion sizes of the BALB/c mice in all the treatment groups. Both dexamethasone and hydrocortisone caused significant (P < 0.05) elimination of the parasites from the lesions and significantly reduced parasite burden in the spleen compared to the non-treated controls at the end of the experiment.
Hydrocortisone and Dexamethasone significantly (P < 0.05) reduced the production of MIP 1A 1α and MCP 1 1 chemokines both in experiments before infection and those after. They on the other hand significantly increased the production of IFNγ. Hydrocortisone gave the best results as compared to dexamethasone both in parasite elimination and in reduction of chemokine production with an exponential increase in IFN γ production. The results of this study demonstrate that Glucocorticoids control L. major infection in mice evidenced by reduction of parasite burden and elimination from lesions and also reduction of chemokine production limiting spread of infection. In this regard, i recommend the use of dexamethasone and hydrocortisone in the management of cutaneous leishmaniasis.
CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background of the study

Leishmaniasis, a vector-borne disease caused by obligate intracellular kinetoplastid protozoa of the genus *Leishmania*, is endemic in large areas of the tropics, subtropics and the Mediterranean basin (Chappuis *et al*., 2007; Banuls *et al*., 2007; Pearson and Sousa, 2009; Grimaldi & Tesh, 2012). In the Old World, leishmaniasis is caused by at least three main species of *Leishmania*: *L. aethiopica*, *L. major* and *L. tropica*. Cutaneous leishmaniasis of the Old World caused by *L. aethiopica* has been reported only in the highlands of Ethiopia and Kenya. In both areas, the disease is zoonotic; hyraxes act as reservoirs and the sandflies *Phlebotomus longipes* and *P. pedifer* serve as vectors (Sang *et al*., 1993).

Approximately 350 million people are considered at risk of infection by the disease which is endemic in 88 countries of the world. On average, 2 million new cases of leishmaniasis are reported every year (WHO, 2007). The WHO (2007) report indicates that more than 12 million people are currently infected with leishmaniasis worldwide. As per the report, the disease affects the poorest populations in the 88 countries most of which are developing countries.

Different species of *Leishmania* infect the macrophages in the dermis, with variable clinical presentations and prognoses (Dedet & Pratlong, 2003). Due to species differences in tissue tropism, virulence and their interaction with the host’s immune system, infection by *Leishmania* can result in a variety of clinical manifestations ranging from single self-healing ulcers in cutaneous forms to life threatening visceral infections. Manifestation of disease takes three main forms; visceral leishmaniasis (VL) or kala azar, mucocutaneous leishmaniasis (MCL) and cutaneous leishmaniasis (CL) (Tonui, 2006; Desjeux, 2004; Lukes, 2007).
Visceral leishmaniasis (VL) is characterized by a range of symptoms that include fever, weight loss, weakness, hepatomegaly, lymphadenopathy and splenomegaly with the incubation period ranging from three to eight months (Chappuis, et al., 2007). VL is also associated with high parasite numbers and the absence of an effective T helper cell type 1 (Th1) immune response (Gupta et al. 2011) The causative agents for this form of infection are; *L donovani*, *L infantum* and *L chagasi* (OIE manual, 2008)

Mucocutaneous leishmaniasis, also known as espundia is more prevalent in the New World with *Leishmania braziliensis* being the most common etiologic agent although *L. panamensis*, has also been reported to cause this form of disease. (Lerner & Choi, 2001). Fifty per cent of patients develop mucocutaneous lesions within 2 years of the initial cutaneous lesions, and 90% within 10 years (Lerner & Choi, 2001). Approximately one-third of patients have no prior history of skin lesions. Involvement of the mucous membrane is probably due to hematogenous or lymphatic dissemination, or occasionally from direct extension of nearby skin lesions (Lerner & Choi, 2001). The disease often begins in the nasal septum which becomes inflamed and infiltrated and subsequently perforates. The leading causes of death in MCL cases are malnutrition and pneumonia (Marsden, 1986; Lerner & Choi, 2001).

Cutaneous leishmaniasis is the most common form of leishmaniasis with an estimated annual incidence of 1.5 million cases worldwide according to WHO (2007) Its clinical manifestation is localized cutaneous leishmaniasis, which often heal without treatment, diffuse CL, which is very difficult to treat and mucosal leishmaniasis, which is the most severe form, producing disfiguring lesions (WHO, 2007). The causative agents for CL are; *L. major*, *L. braziliensis*, *L. Mexicana*, *L. peruviana*, *L. infantum*, *L. tropica* and *L. aethiopica* according to the OIE manual (2008).

During its life cycle, a *Leishmania* parasite presents basically three forms: promastigotes, paramastigotes and amastigotes. Promastigotes and paramastigotes are
flagellated, motile forms that are found in the alimentary tract of phlebotomine sandflies. Promastigotes are elongated extracellular organisms, with a body size of 15–20 × 1.5–3.5 μm with single flagellum 15–28 μm long, arising close to the kinetoplast at the anterior. Their nucleus is centrally located. Amastigotes are non-motile forms, which are rounded or oval, 1.5–3× 2.5–6.5 μm in size, found in vacuoles within the cytoplasm of the macrophages. They don’t have free flagellum, their nuclei are relatively large and their kinetoplasts consist of a rod-like body and a dot-like basal body (OIE manual, 2008). Metacyclic promastigotes are the form that infect and then multiply as amastigotes within macrophages (Molyneux & Killick-Kendrick, 2009; Killick-Kendrick, 2010).

Due to the absence of data on the effects of glucocorticoids in the control of leishmaniasis, the aim of this study was to find out the effects of glucocorticoids on Leishmania major infection focussing on production of MCP 1 and MIP 1α chemokines, parasite infectivity and multiplication index, lesion development and determination of leishman donovan units (LDU).

1.1.1 Leishmaniasis in Kenya
In Kenya, leishmaniasis was first reported in the early 20th century (Malaria Consortium, 2010; Tonui, 2006). Leishmaniasis remains of public health concern as it affects 22 districts in Kenya and severe, untreated forms of the disease can lead to mortality (National guidelines for health workers, 2012). The endemic areas for visceral leishmaniasis include Turkana, Baringo, Kitui, Machakos, Meru, West Pokot and Elgeyo Marakwet districts. Recent outbreaks of VL have however been reported in previously non-endemic districts like Wajir and Mandera in North Eastern Kenya between May 2000 and August 2001 (Tonui, 2006) with the vector for VL (Appendix I) being Phlebotomus martini although P. orientalis has also been reported to be a vector for VL. VL and CL have both been reported to occur in Baringo district. (Chan et al., 2006, Tonui, 2006).
The etiological agents for CL have been reported in different parts of the country: *L. major* has been reported in Baringo; *L. tropica* in Laikipia, Samburu, Isiolo, Nakuru and Nyandarua districts and *L. aethiopica* has being reported in the Mt Elgon area (Chan et al., 2006; Tonui, 2006). In Kenya, *P. duboscqi* and *P. guggisbergi* have been shown to be the vectors of *L. major* and *L. tropica* respectively while *P. pediffer*, *P. longipes* and *P. elgonensis* are vectors of *L. aethiopica* (Appendix I and II, plate 1); (Malaria Consortium, 2010; Tonui, 2006). The disease has also been reported to occur along the Kenya – Uganda and Sudan border, though little is known on its distribution because the area is expansive.

In Kenya, there are two main forms of disease which are; visceral and cutaneous leishmaniasis. Currently, the main control strategies being applied in kenya are; early case detection, treatment of existing cases and vector control which is mainly aimed at prevention and reduction of transmission (National guidelines for health workers, 2012; Malaria Consortium, 2010).
Figure 1: Map of Kenya showing leishmaniasis endemic regions in Kenya (survey of kenya)
1.2 Literature review

1.2.1 Leishmaniasis

Members of the *Leishmania* genus belong to a biologically diverse group of flagellate parasites of the trypanosomatidae family. Most of the species are pathogenic to both man and lower vertebrates; they have been differentiated by means of genetic, biochemical and immunological studies and grouped into different complexes of nine genera, two subgenera, nine species complex and sixteen specific species (Banuls *et al*., 2007; WHO, 2012).

One of the earliest events after promastigotes have entered the mammalian host is their contact with plasma proteins. It has been shown that fresh normal human serum (f-NHS) can cause the lysis of *Leishmania* spp. *in vitro* (Pearson & Steigbigel, 2008; Franke *et al*., 2009) through the alternative pathway of complement (Mosser & Edelson 2007; Brittingham *et al*., 2009). Therefore, they must have escaped the lytic effect of serum before they can invade macrophages (Jokiranta *et al*., 2005).

*Leishmania* parasites reside and multiply in their mammalian hosts within macrophages. Macrophages come to play first in the response against pathogenic infections. They are also very important because they link innate immunity to adaptive immunity. Infection of a macrophage leads to induction of numerous cellular genes, several of which encode cytokines that stimulate an inflammatory response and resistance to pathogens. To escape the host immune defense and to survive, *Leishmania* parasites have developed different strategies and inhibit several macrophage functions, including phagocytosis, nitric oxide generation, interleukin-12 (IL-12) production, and major histocompatibility complex class II expression (Belkaid *et al*., 1998 and De Souza-Leao *et al*., 1995).

The pentavalent antimonials; sodium stibogluconate and meglumine antimoniate, have been the first-line treatment for *Leishmania* in many areas for more than 70 years.
Cheaper generic forms of these drugs are available that have been shown to be equivalent to the branded products (Croft et al., 2006). Antimonials however, are toxic drugs with frequent, sometimes life-threatening, adverse side effects, including cardiac arrhythmia and acute pancreatitis. Patients under the age of 2 or aged 45 or over with signs of advanced disease and/or severe malnutrition are at higher risk of death during antimonial therapy owing to drug toxicity, slowness of drug action, VL complications or a combination of these factors (Seaman et al., 2006). Conventional amphotericin B has replaced antimonials as the first-line treatment for VL in some areas where treatment failure rates for antimonials reached >60% (Sundar, 2000). Since there is no vaccine in sight against *Leishmania*, the search for newer, safer and more efficacious drugs is still on (Croft et al., 2005; Handman, 2001).

1.2.2 Immunology of leishmaniasis

A protective host defense against *Leishmania* starts with the confinement of the parasite to the site of infection. This happens as a result of the expression of IFN-γ and the subsequent induction of antimicrobial molecules in infected macrophages. Also, within 24 hours of infection IFN-γ is up-regulated and upregulates the host response. The primary source of early IFN-γ is most likely NK cells which are usually the first to arrive at the site of infection.

An antigen-specific adaptive immune response is generated during the initial stages of immune response. The skin’s dendritic cells (DCs) take up the parasite and migrate to the draining lymph node differentiating into mature antigen presenting cells (APCs). The DCs present the parasite antigen to naive T cells and produce IL 12, which is most critical for a Th1 type response to be mounted. The activated Th1 cells are then attracted to the infection site and respond by inducing production of large amounts of IFN-γ, enhancing anti-*Leishmania* activities at the site of infection.
It is also well established that during *Leishmania* infections, like in many pathogenic invasions the host releases nitric oxide (NO), which acts as a potent cytotoxic effector molecule and inhibits the growth and function of many infectious agents, including both protozoan and helminthic parasites. Macrophage activation is associated with induction of nitric oxide (NO) synthase, which in turn leads to NO-mediated killing (Awasthi *et al.*, 2004). Tumour necrosis factor (TNF)-α, an anti-leishmanial cytokine is a co-factor with NO working in synergy (Awasthi *et al.*, 2004).

Complete healing of leishmaniasis depends more on the early presence of macrophages, their activation by type 1 interferon and, later, by low amounts of IFN-γ most likely produced by NK cells. The Th1 cells cause further killing of the parasites by releasing more IFN-γ. Important to note however, is that these immunological processes differ in effectiveness from one person to another their effectiveness between human beings, and these differences have clinical consequences which are evident in the manifestations of leishmaniasis from self-healing cutaneous lesions to severe progressive visceral disease with potentially fatal outcome.

*Leishmania* resistant mouse strains such as C57 BL/6 have immunity pegged on their ability to mount a type 1 helper T cell response with IFN-γ, the main cytokine activating macrophages to kill intracellular parasites. However, in susceptible mice, mainly BALB/c, susceptibility is attributed to a type 2 helper T cell response associated with the production of interleukin 4 (IL-4) and interleukin 5 (IL-5) (Noben-Trouth *et al.*, 2003; Mahmoodi *et al.*, 2005). This is evidenced by a report by Tonui (2006) indicating that immune response to leishmaniasis is not just a result of Th1/Th2 cytokine responses but also the genetic makeup of the host.

Cutaneous leishmaniasis has two main forms of disease. Localized Cutaneous leishmaniasis (LCL) is the most common form of cutaneous leishmaniasis and it usually heals spontaneously. In some individuals, however, the parasites spread over the body
and cause multiple non-healing lesions referred to as diffuse cutaneous leishmaniasis (DCL). The two forms of cutaneous leishmaniasis differ in severity, course of disease, chemokine profiles and the cells recruited at the site of infection. In lesions of LCL, Th1 cytokines such as IFN-γ are predominant whereas in DCL, Th2-type cytokines, such as IL-4 are more pronounced. Interestingly, the self-healing lesions of LCL exhibit a strong expression of Th1 associated chemokines, such as CCL2/MCP 1, CXCL9/MIG and CXCL10/IP-10, and small amounts of CCL3/MIP 1α. Chronic DCL is associated with an opposite expression pattern. CCL3/MIP 1α is dominant and CCL2/ MCP 1, CXCL9/MIG and CXCL10/IP-10 are expressed at low levels (Antoniazi et al., 2004).

The differences in the chemokine environment are reflected in the composition of the cells infiltrating the dermis. In LCL lesions, macrophages and numerous CD4 + T cells are clustered in the upper part of the dermis, whereas in DCL the infiltrate is evenly distributed over the whole dermal compartment. The immunological consequence of this change in the inflammatory infiltrate is the presence of a delayed-type hypersensitivity response against *Leishmania* antigen in LCL but not in DCL indicating normal and deficient Th1-type responses, respectively.

The role of B cells in *Leishmania* infection is still not very clear. Resistance is mostly identified with TH1 response and B cells do not affect T cell polarization. However, experiments with B cell knockout, wild type and susceptible mice exhibited a role of B cells in the IFN-γ production which influences the pathology of a secondary infection (DeKrey et al., 2003).

Furthermore, antibodies have been shown to be necessary for the development of an optimal protective immunity against *Leishmania major*, because DCs, when covered with parasite-reactive IgG take up the parasite more efficiently. The interaction of IgG with FcγRI and FCγRII on DCs provides an optimal recognition and ingestion of the parasite, a prerequisite to an immune response (Woelbing et al., 2006).
There is a crosslink in immune response between humoral and cell-mediated. It is interesting to note that Th2 lymphocytes are associated with humoral immunity and produce cytokines such as IL-4, IL-5, and IL-13, which act in promoting IgE production and activation of eosinophils and mast cells. Th2-cell-mediated immune responses predominate during infestations by gastrointestinal nematodes, but they have also a role in triggering responses against innocuous antigens in atopic allergy. (DeKrey et al., 2003)

1.2.3 MCP 1 and MIP 1α chemokines

Chemokines are small chemotactic proteins that are divided into four families based on the number and positioning of the N-terminal cysteines. They regulate both innate and adaptive immune responses by coordinating leukocyte trafficking with immune cell differentiation and effector functions (Conrad et al., 2007). As a result, Chemokines and their receptors play a critical role in the development of immunity against a wide variety of pathogens (Murdoch & Finn 2000). They recruit immune cells by chemotaxis, which also act in leukocyte activation, inflammatory diseases, and antimicrobial mechanisms.

They are involved in non-specific infiltration during inflammation and the cellular arrangement within the immune organs. They influence the presence or absence of an immune response, and also determine whether it is allergic, cell-mediated, cytotoxic, or humoral. To achieve the desired outcomes, many of them have to work in synergy, their actions leading to cascades of responses.

Both MIP 1α and MCP 1 are members of C-C group of chemokines. All the members of the group have attractive potencies for mononuclear cells with differing potencies for monocytes and various populations of lymphocytes. Some members can also attract basophils and eosinophils.
In an experiment of *in vitro* incubation of human peripheral blood leucocytes with parasites revealed that infected monocytes express CCL2/MCP 1 which could, in an *in vivo* situation, attract other monocytes and macrophages or other CCR2-positive cells to the site of infection.

The expression of MCP 1 is induced after stimulation with inflammatory stimuli including viruses, LPS, and cytokines such as TNF-α, IL-1, IFN-γ, and PDGF. MCP 1 is a potent chemo-attractant for monocytes/macrophages and lymphocytes. It has also been shown to be involved in the regulation of Th1/Th2 lymphocyte differentiation, enhancing Th2 development by increasing IL-4 production and inhibiting IL-12 production. The activities of mouse MCP 1 have been shown to be mediated by the chemokine receptor CCR2, a G protein-coupled, seven transmembrane domain receptor. Silencing of CCR5, a receptor for MIP 1α, leads to low parasite entry in the macrophages along with enhanced production of NO and proinflammatory cytokines in *L. donovani* infected macrophages (Bhattacharyya *et al*., 2008).

*Leishmania* parasites induce expression of both MIP 1α and MCP 1 (Teixeira *et al*., 2006). *Leishmania* virulence has been linked to the modulation of chemokine expression by macrophages (Liu *et al*., 2002). MCP 1 is known to be a potent chemo-attractant for macrophages activated during infection. MCP 1 has been found to be closely correlated with endothelial growth regulators including VEGF (vascular endothelial growth factors), and TP (thymidine phosphorylase), and IL-8. These endothelial growth regulators play a role in neovascularization and tumor progression and this has been demonstrated in many tumors (Ueno *et al*., 2000).

1.2.5 *Leishmania* parasite multiplication in macrophages

The first step in leishmaniasis is the attraction of cells acting as hosts for the parasites (i.e. macrophages). This attraction is mediated by sandfly saliva which is inoculated together with the pathogens into the dermal compartment. Once attracted, these
macrophages rapidly phagocytose the sandfly-transmitted pathogens. This is crucial for the survival of *Leishmania* parasites as it gives them the opportunity to replicate within the host cells.

1.2.6 Lesion development in BALB/c mice

Cutaneous Leishmaniasis in humans is caused by *Leishmania major*, a parasite of the skin. *L. major* infects mononuclear phagocytes of vertebrate hosts. In some mouse strains this infection causes no or only transient pathological changes, whereas some strains develop systemic visceral disease with splenomegaly, hepatomegaly, anemia, hypergammaglobulinemia and skin lesions.

Cutaneous lesions develop at sites of insect bite in *L. major* infection. Most of the time, the lesions heal spontaneously within weeks or even months. The infection may involve visceralization, in susceptible animal models BALB/c strain of mice but is highly uncommon in man though draining lymph nodes may be involved. Following self-healing, there results immunity. *L. major*, inoculated in the skin of BALB/c mice, produces large ulcers which do not resolve due to the susceptibility of this particular strain. The parasite metastasizes, has uncontrolled growth and eventually leads to death. (Maul et al 2002).

*Leishmania major* infects mononuclear phagocytes of vertebrate hosts. In most strains of experimental mice, *L. major* infection causes no or only transient pathological changes, whereas some strains develop systemic visceral disease with splenomegaly, hepatomegaly, anemia, hypergammaglobulinemia and skin lesions. The susceptible phenotype has been reported to be associated with high levels of IL-4 and IgE production, low levels of IFN γ production, and low macrophage anti-parasite activation by stimulated CD4+ T cells (Philips, 1993).

Mucocutaneous leishmaniasis lacks a good experimental animal model and therefore not much work has been done in relation to it. In BALB/c mice, *L. Braziliensis* causes very
mild and self-limited lesions (deMoura et al., 2005). Experimental infection with *L. amazonensis* causes persistent chronic lesions in C57BL/10 and C57BL/6 mice. The lesions last over 20 weeks but both animal models just like typical mucocutaneous leishmaniasis do not control the parasite in the tissue.

Unlike many strains of inbred mice that develop small, self-healing lesions following infection with *Leishmania major*, few strains like BALB/c develop large, non-healing lesions and eventually succumb to the infection (Li et al, 2002).

Mice that heal spontaneously develop Th1-type responses which are characterized by high production of the macrophage activating cytokines, IFN-γ, and TNF-α, as inducers of nitric oxide synthase 2 (NOS II) and NO synthesis from L-arginine, the main pathway responsible for the killing of *Leishmania*. However, susceptible mice like BALB/c develop Th2-type responses with interleukin-4 (IL-4) produced by CD4 cells as the dominant cytokine (Hollzmuller et al, 2002).

### 1.2.7 Relevance of MCP 1 and MIP 1α in disease progression

In the macrophage, both *in vitro* and *in vivo*, *Leishmania* parasites induce pro-inflammatory chemokines, which promote the recruitment of phagocytic cells to the site of infection. These cells are then infected by the parasite as it propagates and spreads within the host. Since *Leishmania* parasites are virulent, the macrophages are armed with parasiticidal capabilities to inactivate and kill invading pathogens. In order to suppress host defense mechanisms and thrive in them, *Leishmania* parasites subvert the host immune system and hijack its resources for their survival and growth.

*Leishmania* parasites are capable of targeting the host immune surveillance by suppressing Janus-activated kinase 2 (JAK2)/STAT1α, while activating the nuclear factor kappa beta (NF-κβ) p65 RelA subunit. Activation of NF-κβ leads to the high
production of immunoglobulin G (IgG) which in normal circumstances opsonizes the parasite for destruction. However, *Leishmania* amastigotes bind to host derived IgG FcγR and use it to enhance parasite adhesion to macrophages (Peters et al., 1995). The ligation of the phagocytic receptor on macrophage to FcγR of the IgG induces synthesis and secretion of IL-10 (Sutterwala et al., 1998), which has the potential to inhibit the production of a Th1 immune response and prevent macrophage activation leading to persistent parasitemia in lesions.

Silencing of CCR5, a receptor for MIP 1α, leads to low parasite entry in the macrophages along with enhanced production of NO and proinflammatory cytokines in *L. donovani* infected macrophages (Bhattacharyya et al., 2008).

Chemokine expression reaches a peak within the first 24 h after infection and is sorely dependent on the virulence of the *Leishmania* strain used. Avirulent strains of *L. major* induce more CCL2/MCP 1 in murine macrophages than virulent parasites. Thus, it appears that *in vivo* the level of *Leishmania*-mediated release of CCL2/MCP 1 could directly, either favor the development of a ‘self-healing’ course of the disease or result in a ‘chronic’ form of leishmaniasis.

### 1.2.8 The NF-κβ pathway and leishmaniasis

NF-κβ is a critical transcriptional factor which regulates the expression of many gene encoding proteins involved in inflammation, macrophage functions, and plays an important role in the induction of specific chemokines. (Barnes, 1997). The NF-κβ -Rel family is composed of five different members, NF-κβ 1 (p50), NF-κβ 2 (p52), Rel A (p65), Rel B, and c-Rel (Ghosh et al., 1998). These proteins are present in resting cells as inactive complexes sequestered in the cytoplasm by tight binding to the inhibitory protein Iκβ (Baldwin et al., 1996).

NF-κβ is normally bound to an inhibitor protein known as Iκβ that sequesters NF-κβ in the cytoplasm. Phosphorylation of Iκβ by two Iκβ kinases (IKK-1 and IKK-2)
selectively leads to ubiquitination and subsequent degradation of this inhibitor protein by
the proteasome. Once NF-κβ is released, it migrates to the nucleus, where it binds to
specific promoter sites (kappa β binding sites) initiating genes transcriptions (Baldwin
et al., 1996; Mercurio & Manning, 1999).

Once activated, NF-κB binds to recognition elements in the promoter regions of
inflammatory and immune genes, such as proinflammatory cytokines, chemokines,
inflammatory enzymes and adhesion molecules. Glucocorticoids inhibit activated NF-κB
and this is likely to be important in the anti-inflammatory action of steroids. Novel
inhibitors of NF-κB are now under development for treatment of inflammatory diseases
(Barnes et al., 1997).

Besides Leishmania gp63, cleavage of NF-κβ can also be done by; LPS (lipopolysaccharide), cytokines like TNF-α and IL-1β, oxidative stress, and DNA-damaging agents present in most cells (Finco & Baldwin, 1995).

Infection with Leishmania parasites results in the specific cleavage of NF-κβ into
p35RelA and p65RelA subunits, in a process that is dependent on Leishmania gp63
(Gregory et al., 2005). The resultant p35RelA subunit then migrates to the nucleus,
where it binds DNA as a dimer with p50, inducing the expression of MIP 1α and IL-10
on the other hand blocking the induction of nitric oxide synthase and IL-2. This causes
subversion of the host defense response, promoting the survival and development of the
parasite inside infected macrophages. This effect has been observed to be specific for the
pathogenic species of Leishmania but not in a non-pathogenic one like L. tarentole
(Gregory et al., 2005 and Gregory et al., 2008).

Leishmania invasion induces the activation of the nuclear factor kappa β (NF-κβ)
transcription factor, which initiates innate immune responses. Infected macrophages are
refractory to activation by LPS and pro-inflammatory cytokines such as IFN-γ (Olivier
et al., 2005). IFN-γ pro-inflammatory mediators are induced in Leishmania-infected macrophages. These include specific chemokines, which are believed to play a crucial role in the recruitment of inflammatory cells, including macrophages that the parasite can subsequently invade to propagate the infection (Antoniazi et al 2004; Buates & Matlashewski, 2001; Dasgupta et al., 2003; Racoosin et al., 1997; Matte et al., 2002; Gregory et al., 2008).

Microorganisms take advantage of the NF-κβ system. Activation of the NF-κβ pathway is a strategy that protects the host cells from apoptosis, allowing pathogens to survive, replicate, and disseminate (Guizani-Tabbane et al., 2004).

According to Hadi et al., (2013), the progression of atherosclerosis is associated with activation of the NF-κβ pathway, thus understanding the pathway’s activation offers a unique opportunity as a target for interrupting parasite development.

1.2.9 The effect of glucocorticoids on the NF-κβ pathway

Glucocorticoids (GC) have profound effects on the development and homeostasis of the immune system. Physiological doses of glucocorticoids modulate the selection of thymocytes (Vacchio et al., 1994) and regulate immune responses by influencing the pattern of cytokine secretion (Brinkman et al., 1995).

Dexamethasone and Hydrocortisone inhibit the activation of NF-κβ by preventing the degradation of Iκβ, hence retention of NF-κβ in the cytosol (Kopp & Ghosh, 1994). Leishmania infection activates the NF-κβ pathway, resulting in increase of MIP 1α and MCP 1 chemokines and thus induction of macrophage chemotaxis therefore aggravating the infection though the inflammatory pathway is shut off.

Glucocorticoids are known to regulate gene expression either at the transcriptional or post-transcriptional level. Post-transcriptional regulation may involve alteration in
mRNA stability, translational efficiency or the secretion of proteins (Boumpas et al., 1993).

The glucocorticoid receptor complex has been shown to bind NF-κβ and therefore prevent it from accessing DNA sequences (Ikβ binding sites in the promoter region) important for gene expression (Ray & Prefontaine 1994; Mukaida et al., 1994 and Paliogianni et al., 1993). This has been suggested to occur in two ways. First, in the presence of glucocorticoids, the amount of NF-κβ that translocates into the nucleus is significantly diminished, suggesting an increased sequestration of the protein in the cytoplasm (Scheinman et al., 1995). Second, whereas glucocorticoids inhibits the transcription of the IL-2 gene by both nuclear factors AP-1 and NF-κβ, only the effect of NF-κβ requires new protein synthesis (Auphan et al., 1995).

Both observations suggest that increased transcription and protein levels of Ikβ X may be the most likely candidate mechanism. Indeed it has been demonstrated that glucocorticoids increase Ikβ X protein synthesis, which in turns traps NF-κβ in inactive form in the cytoplasm (Scheinman et al., 1995 and Auphan et al., 1995). This effect has been observed both in cultured cells Jurkat cells (a T-cell leukemia cell line) and HeLa cells (a cervical carcinoma cell line) and in mice. Similar to glucocorticoids, sodium salicylate and aspirin inhibit the activation of NF-κβ by preventing the degradation of Ikβ, leading to retention of NF-κβ in the cytosol (Kopp et al., 1994) leading to reduced production of genes such as IL-2 as observed in anergic T cells (Sundstedt et al., 1996).

It is envisioned that glucocorticoids could be used to limit the spread of *Leishmania* infection in macrophages by blocking the NF-κβ pathway. *Leishmania* parasites survive and proliferate in the host cell by deactivating the macrophage. Successful infection of *Leishmania* is achieved by alteration of signaling events in the host cell, leading to enhanced production of the autoinhibitory molecules like TGF-beta and decreased induction of cytokines such as IL12 for protective immunity. Nitric oxide production is also inhibited. In addition, defective expression of major histocompatibility complex
(MHC) genes silences subsequent T cell activation mediated by macrophages, resulting in abnormal immune responses (KEGG pathway).
1.3 Statement of the problem

Leishmaniasis is a neglected tropical disease which has been around since 1935. Chemotherapy of leishmaniasis relies mainly on pentavalent antimonials, amphotericin B (AMB), and pentamidine 3. These drugs are toxic and difficult to administer because of their long-term treatment and parenteral administration. Besides, they have frequent side effects and have been reported to face increasing resistance. They are also very expensive and considering the financial state of a big number of the population at risk, are therefore not tenable.

Many studies are ongoing in the search of a vaccine and alternative therapeutic strategies that are less expensive, easier to administer with a shorter period of administration and lower resistance rates.

Glucocorticoids have been successfully used in the management of many inflammatory conditions like asthma, arthritis and cytomegalovirus. The use of glucocorticoids in leishmaniasis has not been explored.

1.4 Justification

Glucocorticoids have been successfully used in the management of inflammatory conditions and are reported to have low toxicity levels with minimal side effects. They are also easily accessible and are not as expensive as pentavalent antimonials.

Glucocorticoids are known to block the NF-κβ pathway that induces chemokine gene expression. This results in the inhibition of chemokine production hence reduction of pathology.

This study sought to evaluate the effect of two glucocorticoids (dexamethasone and hydrocortisone) on production of MIP 1α and MCP 1 chemokines that are known to propagate infection by infiltration of macrophages to site of infection leading to increased pathology. The study also sought to determine the effects of the glucocorticoids on parasite infectivity, multiplication and lesion development in
BALB/c mice. The results of this study yielded a potential use of dexamethasone and hydrocortisone in the management of cutaneous leishmaniasis

1.5 Hypotheses
  i. Glucocorticoids do not reduce the production of MIP 1α and MCP 1 chemokines in macrophages of BALB/c in vitro.
  ii. Glucocorticoids do not affect Leishmania parasite multiplication in macrophages
  iii. Glucocorticoids do not affect the levels of MIP 1α and MCP 1 chemokines in vivo
  iv. Glucocorticoids do not affect lesion development in BALB/c mice.

1.6 Objectives of the study
1.6.1 General Objective
To determine the effect of glucocorticoid drugs on production of MIP 1α and MCP 1 chemokines and Leishmania parasitemia and infectivity

1.6.2 Specific Objectives
  i. To determine the effects of glucocorticoids on production of MIP 1α and MCP 1 chemokines in macrophages of BALB/c mice
  ii. To determine the effects of glucocorticoids on Leishmania parasite multiplication and infectivity in macrophages
  iii. To evaluate the effects of glucocorticoids on levels of MIP 1α and MCP 1 chemokines in vivo
  iv. To determine the effects of glucocorticoids on lesion development in BALB/c mice
CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study site

The study was carried out at the Kenya Medical Research Institute (KEMRI) Nairobi, at
the Centre for Biotechnology Research and Development (CBRD). *Leishmania*
laboratory and the animal house are the main points within CBRD where the study was
done.

2.2 Experimental design

This study was laboratory based with *in vitro* and *in vivo* studies. Experimental mice
were divided into two setups, chemotherapy and immunoprophylaxis, of 24 mice each.
There were four treatment groups (dexamethasone, hydrocortisone, lipopolysaccharide
and Phosphate Buffered Saline) in each setup, with six mice each. Each mouse of the
experimental group A was treated intraperitoneally with 0.69 mg/ml dexamethasone.
Group B experimental mice received 2 mg/ml of hydrocortisone each. Group C was the
positive control group which received 10 ng/ml of LPS while group D, the negative
control group was on PBS. In the first arm of the experiment (immunoprophylaxis/before infection), the mice were treated for 21 days and sacrificed on day 22. Blood was taken directly from their hearts through cardiac puncture. The
blood was thereafter analyzed for MIP 1α and MCP 1 chemokine expression. In the
second arm of the experiment (chemotherapy/after infection), the mice were inoculated
with $1 \times 10^6$ Promastigotes of *L. major* into the right footpad, and after five
weeks, with evidence of lesions, treatment was started. Group A was treated with 0.69 mg/ml
dexamethasone. Group B was treated with 2 mg/ml of hydrocortisone each. Group C
was the positive control group which received 10 ng/ml of LPS while group D, the
negative control group was on PBS.

For the *in vitro* studies, Macrophages were incubated with the drugs, dexamethasone and
hydrocortisone with the negative control group being incubated with complete RPMI
1640 media while positive control was incubated with LPS. Dexamethasone, hydrocortisone, LPS were serially diluted seven times to obtain eight sets of supernatants. After 48 hours, supernatants were collected for MCP 1 and MIP 1α chemokine analysis. RPMI media was replenished and then all the wells were seeded with $10^6$ parasites and incubated further for five days.

2.3 Sample size calculation
The sample size for this study was calculated using the resource equation method that depends on the law of diminishing returns in which, adding one experimental unit to a small experiment gives good returns, while adding it to a large experiment does not do so thus

$$E=N-B-T$$ (Mead, 1988).

Where

$E$ is the error degrees of freedom (df) which should be between 10 and 20

$N$ is the total degrees of freedom (df)

$B$ is the blocks degrees of freedom (df)

$T$ is the treatments degrees of freedom (df)

Therefore having four treatments, with six animals per group (24 mice total)

$N = 23$, $B=0$, $T = 3$ hence $E=N-T$; $E= 23-3 =20$

With two sampling points (before infection and after infection)

The setup thus had $24 \times 2 = 48$ mice.

2.4 Experimental animals
Eight week old, female BALB/c mice weighing 18-22g were used. The inbred BALB/c mice were obtained from the KEMRI animal facility. The mice were housed in 15cm × 21cm × 29cm transparent plastic cages with wood shavings and equipped with continuous-flow nipple watering devices. They were fed with pellets (Mice pellets UNGA® feeds) and water. The wood shavings in the cages were changed, and the water bottles re-filled every two days. The experimental room in the animal house was always
under lock and key and all cages clearly labeled with experimental details that included, name of investigator, assigned treatment groups and dates of procedures.

2.5 Parasite culture
Metacyclic promastigotes of *L. major* strain IDU/KE/83 =NLB-144 which was originally isolated in 1983 from a female *P. dubosci* collected near marigat Baringo County Kenya was used. The promastigotes were cultured in Schneider’s Drosophila medium supplemented with 20% fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Stationary-phase promastigotes was obtained from 5 to 7 day-old cultures. Parasites were maintained as described (Titus et al., 1984).

2.6 Drug preparation
Dexamethasone and hydrocortisone were dissolved in 15% RPMI 1640 and used at a final concentration of 10-100μM. LPS (5mg/ml obtained from Invitrogen™) was diluted with 15% RPMI1640 to a final concentration of 100 μM. (Lapara & Kelly, 2010)

2.7 Macrophage harvesting
Experimental animals were housed in designated animal holding facilities within KEMRI. Six mice were randomly selected for macrophage harvesting for the macrophage assay which was done as described by Delorenzi et al (2001) with minor modifications. Each individual cage was labeled using identification cards that had the following information; name of investigator, species of animal, gender of animal, type of experiment and length of study.

Six week old BALB/c mice were injected with 2% starch solution, using 18 gauge hypodermic needles in the peritoneal cavity for macrophage stimulation. After 48 hours the mice were placed in the anaesthetizing chamber with chloroform fumes, till unconscious. The body surface was disinfected with 70% ethanol and the skin was pulled back dorso-ventrally to completely expose the peritoneum. Ten milliliters of
sterile cold phosphate buffered saline (PBS) was injected using 18 needle gauge into the peritoneum and the abdomen softly massaged. Peritoneal macrophages were harvested by withdrawing the PBS into a plastic centrifuge tube placed on ice. The macrophages were then washed by centrifugation at 2000 rpm for 10 minutes at 4°C and the pellet obtained re-suspended in RPMI 1640 culture medium.

The macrophages were adsorbed in 24 well plates for 4 hours at 37°C in 5% CO₂ and 95% humidity. Non-adherent macrophages were washed with cold PBS and incubated overnight in RPMI 1640 culture medium. After macrophage harvesting, the mice were wrapped and disposed in a red bag labeled with biohazard symbol and taken for incineration; according to KEMRI animal care and use and guidelines.

2.8 **Treatment of macrophages with dexamethasone and hydrocortisone** *(macrophage assay)*

Before infection, macrophage numbers were determined on cultures run in parallel by counting nuclei as described by Antoine *et al.*, (1991). Macrophages were then incubated with the drugs for 12 hrs. Negative control group was incubated with complete RPMI media while positive control was treated with LPS. Dexamethasone, hydrocortisone, LPS were serially diluted seven times to obtain eight sets of supernatants (500µl) from each well.

2.9 **Enzyme linked immunosorbent assay**

Mouse MIP 1α and MCP 1 Kit control was reconstituted with 1.0 mL distilled water. 25 mL of wash Buffer to be added into distilled water to prepare 625 mL of wash Buffer. Color reagents A and B were mixed together in equal volumes within 15 minutes of use. 100µL of the resultant mixture was required per well. Mouse MIP 1α and MCP 1 standard was reconstituted with 5.0 mL of calibrator diluent RD5Z. This reconstitution produced a stock solution of 300 pg/mL. The standard was allowed to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions. 200 µL of calibrator diluent
RD5Z was pipetted into each tube. The stock solution was used to produce a 2-fold dilution series and each tube was mixed thoroughly before the next transfer. The undiluted mouse MIP 1α and MCP 1 Standard served as the high standard (300 pg/mL). Calibrator diluent RD5Z served as the zero standard (0 pg/mL). The ELISA was done following the instructor’s manual.

2.10 Macrophage infection
Promastigotes harvested in the exponential growth phase were re-suspended in fresh medium to achieve $10^6$ parasites/ml. Adherent macrophages (treated and non-treated) were infected with *L. major* promastigotes at a parasite/macrophage ratio of 10:1. They were then incubated at 37°C in 5% CO$_2$ and 95% humidity for 4 hours. Free promastigotes were removed by extensive washing with PBS, and the cultures incubated in RPMI 1640 medium for 24 hours.

After 5 days, the monolayers were washed with PBS at 37°C, fixed in methanol and stained with 10% Giemsa stain. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and results expressed as infection rate (IR) and multiplication index (MI) (Berman & Lee, 1984).

\[
IR = \frac{\text{No. of infected macrophages}}{100}\times 100
\]

\[
MI = \left(\frac{\text{No. of amastigotes in experimental culture}}{\text{No. of amastigotes in control culture}}\right) \times 100
\]

The infection rate was used in the calculation of the association index (AI). The AIs was determined by multiplying the percentage of infected macrophages by the number of parasites per infected cell. Association indices were the number of parasites that actually infected the macrophages.
2.11 *In vivo* studies

Female BALB/c mice, 6 to 8 weeks old were randomly assigned four groups of six mice each. They were treated with dexamethasone, hydrocortisone succinate, Lipopolysaccharide and plain media (RPMI 1640) from day 0 to day 10. On day 11, they were infected with Metacyclic promastigotes of *L. major* strain IDU/KE/83 =NLB-144 which was originally isolated in 1983 from a female *P. dubosci* collected near marigat Baringo County, Kenya. On day 20, blood was collected from the tails of the mice and the serum was analyzed for MCP 1 and MIP 1α cytokines using ELISA kits (R&D systems, USA) following the manufacturer’s instructions.

Inoculation of mice with *L. major* parasites was done on the left hind footpad (LHFD) and the right hind footpad served as the uninfected control. Lesion measurement was done using a direct reading of the vernier caliper using the method of Nolan & Farrell (1987)

Lesion size = size of infected footpad - size uninfected control footpad

2.12 Quantifying parasite burden

The mice were sacrificed by being exposed to chloroform fumes. The spleens were excised and weighed. Impression smears were then made from the spleens as described by Chulay & Bryceson (1983). The slides were fixed by methanol and then stained with 10 % Giemsa solution. They were examined under a compound microscope to enumerate the number of amastigotes per 1000 host nuclei. The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (LDU) and the total Leishman-Donovan Units (total LDU) respectively were both calculated according to the formula by Bradley & Kirkley (1977) where;

LDU = No. of parasites/1000 host nuclei
Total LDU = LDU x organ weight x 2 x 10^5

2.13 Statistical analysis
Data was recorded on MS excel sheets and on a hardcover laboratory workbook. This data was protected using a confidential password which was only accessed by an authorized person. Data was recorded as means and standard error of the mean of the amount of chemokines in the macrophage culture supernatants. ANOVA was used to compare the difference between means and all significantly different treatments were separated using the Student Duncan’s Multiple Range Test (DMRT) (SAS Institute, 2009). The level of confidence at which experiments were judged significant, was P ≤0.05.
CHAPTER THREE

3.0 RESULTS

3.1 Effects of glucocorticoids on production of MIP 1α and MCP 1 chemokines before and after infection with *L. major* in macrophages of BALB/c mice *in vitro*

There was a significant difference in the concentrations of MIP 1α produced before and after infection by *L. major* promastigotes among the various ranges of concentration tested (F = 32.222, df = 12, P = 0.0000). However, there was a higher reduction in the production of MIP 1α before infection as compared to reduction after infection. The efficacious ranges of hydrocortisone before infection ranged from 0.25 mg/l to 0.8 mg/l while the range after infection is from 0.4 mg/l to 0.9 mg/l. There was 80% reduction in the concentration of MIP 1α after infection as compared to 92.6% reduction in the chemokine before infection (Figure 2).
The concentrations of MIP 1α produced before and after infection by *L. major* among the various ranges of concentration tested (*F = 16.785, df = 12, P = 0.0001*) were significantly different (*P < 0.05*). Again, there was greater reduction in production of MIP 1α before infection as compared to the reduction after infection. The efficacious
range of dexamethasone before infection ranged from 0.16 mg/l to 0.85 mg/l while the efficacious range after infection was from 0.45 mg/l to 1.0 mg/l. percent reduction in the concentration of MIP 1α after infection was 80.5% as compared to 95.2% reduction before infection (figure 3).

Figure 3: Concentration of MIP 1α in the macrophage of BALB/c mice after treatment with dexamethasone before and after infection with L. major promastigotes in vitro
A significant difference between the concentrations of MCP 1 before and after infection by *L. major* among the various ranges of concentration tested was noted (F = 29.542, df = 12, P = 0.0000). The efficacious ranges of MCP 1 production by hydrocortisone before infection ranged from 0.08 mg/l to 0.72 mg/ml while the efficacious range after infection were from 0.17 mg/l to 0.67 mg/ml. There was 80% reduction in the concentration of MCP 1 after infection while that before infection was 92.6% reduction (Figure 4).

![Concentration of MCP 1 in the macrophage of BALB/c mice after treatment with hydrocortisone before and after infection with *L. major* promastigotes in vitro](image)

**Figure 4:** Concentration of MCP 1 in the macrophage of BALB/c mice after treatment with hydrocortisone before and after infection with *L. major* promastigotes in vitro
There was a significant difference (P < 0.05) in the concentrations of MCP 1 before and after infection with *L. major* promastigotes among the various ranges of dexamethasone concentrations tested (F = 56.115, df = 12, P= 0.0000). The efficacious ranges of dexamethasone before infection ranged from 0.08 mg/l to 0.67 mg/ml while that after infection was from 0.17 mg/l to 0.67 mg/ml. Reduction in the concentration of MCP 1 after infection was 80% that before infection was 92.4% (Figure 5).

**Figure 5:** Concentration of MCP 1 in the macrophage of BALB/c mice after treatment with dexamethasone before and after infection with *L. major* promastigotes in vitro
3.2 Effects of glucocorticoids on *Leishmania* parasite multiplication and infection rates in macrophages

Parasite infection rates displayed exponential reduction. The decay constant for dexamethasone (-0.4053) was slower than that of hydrocortisone (-0.7697) suggesting higher rate of reduction in parasitic infections in treatments using hydrocortisone as compared to dexamethasone. The control used in this study had an infection rate of 85.6%. An increase in concentration gives better reduction of parasite infection rates (figure 6).

![Graph showing infection rates](image)

**Figure 6:** Parasitic infection rates in the macrophages of BALB/c mice subjected to various drug therapies

- Hydro: \( y = 93.48e^{-0.40x} \), \( R^2 = 0.999 \)
- Dexa: \( y = 90.38e^{-0.77x} \), \( R^2 = 0.996 \)
- LPS: \( y = 90.38e^{-0.40x} \), \( R^2 = 0.999 \)
Parasite multiplication index displayed exponential reduction. The decay constant for hydrocortisone (-0.9982) was twice the decay constant dexamethasone (-0.4983) suggesting that hydrocortisone reduced the multiplication of parasites twice as much as dexamethasone. Higher concentrations offered higher reduction of parasite multiplication as opposed to lower concentrations (figure 7).

Figure 7: Parasite multiplication rates in the macrophages of BALB/c mice subjected to various drug therapies
3.3 Determination of LDU

There were no significant differences in the body weights of BALB/c mice among the various treatments (P > 0.05). Weight of the spleen, spleno-somatic index, number of parasites and LDU followed similar trends where hydrocortisone had the lowest value followed by values of dexamethasone while PBS has the highest values (Table 1).

Table 1: Body weight, weight of spleen, spleno-somatic index, number of parasites and LDU in BALB/c mice following treatment with hydrocortisone, dexamethasone, LPS and PBS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight</th>
<th>Weight of spleen</th>
<th>Spleno-somatic index</th>
<th>No of parasites</th>
<th>LDU (10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>23.75 ± 0.48</td>
<td>0.14 ± 0.012b</td>
<td>0.61 ± 0.06b</td>
<td>71.75 ± 2.98b</td>
<td>2.01 ± 0.41b</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>23.52 ± 0.65</td>
<td>0.13 ± 0.006a</td>
<td>0.55 ± 0.02a</td>
<td>28.33 ± 2.38a</td>
<td>0.74 ± 0.14a</td>
</tr>
<tr>
<td>LPS</td>
<td>23.50 ± 0.35</td>
<td>0.25 ± 0.05c</td>
<td>1.08 ± 0.08c</td>
<td>92.40 ± 12.25c</td>
<td>4.62 ± 1.21c</td>
</tr>
<tr>
<td>PBS</td>
<td>23.52 ± 0.35</td>
<td>0.30 ± 0.03d</td>
<td>1.31 ± 0.13d</td>
<td>196.50 ± 6.50d</td>
<td>11.79 ± 2.64d</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-value</td>
<td>0.987</td>
<td>45.895</td>
<td>65.125</td>
<td>76.125</td>
<td>74.122</td>
</tr>
<tr>
<td>P-value</td>
<td>0.762</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Means with the same letters as superscripts are not significantly different (P > 0.05) based on ANOVA followed by Duncans Multiple Range Test (DMRT).
3.4 Effects of glucocorticoids on levels of MIP 1α, MCP 1 before and after infection with *L. major* and effects on IFN γ after infection *in vivo*

As shown in the figure below, there was a significant difference in the concentrations of MIP 1α produced before and after infection by *L. major* promastigotes among the various ranges of concentration tested (F = 35.426, df = 12, P = 0.0000). However, there was a higher reduction in the production of MIP 1α compared to before infection as compared to reduction after infection. The efficacious ranges of hydrocortisone before infection ranged from 0.38 mg/l to 0.92 mg/l while the range after infection is from 0.54 mg/l to 0.97 mg/l. There was 82% reduction in the concentration of MIP 1α after infection as compared to 94.3% reduction in the chemokine before infection (Figure 8).
Figure 8: Concentration of MIP 1α in the serum of BALB/c mice after treatment with hydrocortisone before and after infection with L. major promastigotes in vivo

Based on repeated measure ANOVA, there was a significant difference between the concentrations of MIP 1α produced before and after L. major infection among the various ranges of dexamethasone concentrations tested (F = 14.439, df = 12, P = 0.0001). A greater reduction in production of MIP 1α was recorded before infection as compared to the reduction after infection. The efficacy of dexamethasone before infection ranged from 0.95 mg/l to 0.65 mg/l while its efficacious range after infection was from 0.6 mg/l to 0.9 mg/l. Percent reduction in the concentration of MIP 1α after infection was 85% while that before infection was 92.5% (figure 9).
The effect of hydrocortisone in the production of MCP 1 in macrophages of BALB/c mice both before and after infection with *L. major* promastigotes is provided in Figure 10. These results show a significant difference in the concentrations of MCP 1 before and after *L. major* infection among the various ranges of hydrocortisone concentrations tested (F = 35.497, df = 12, p = 0.0000). The efficacy of hydrocortisone before infection ranged from 0.06 mg/l to 0.69 mg/l while its efficacious range after infection was from 0.19 mg/l to 0.71 mg/l. Percent reduction in the concentration of MCP 1 after infection was 82% while that before infection was 96.4% (figure 10).
There is a significant difference in the concentrations of MCP 1 before and after infection with *L. major* promastigotes among the various ranges of dexamethasone concentrations tested \((F = 45.256 \, df = 12, \, p = 0.0001)\). The efficacy of dexamethasone before infection ranged from 0.89 mg/l to 1.2 mg/l while its efficacious range after infection was from 0.78 mg/l to 0.99 mg/l. Percent reduction in the concentration of MCP 1 after infection was 84% while that before infection was 90.6% (figure 11)
Figure 11: Concentration of MCP 1 in the serum of BALB/c mice after treatment with dexamethasone before and after infection with L. major promastigotes in vivo

There were significant changes in IFN-γ levels in serum of BALB/c mice infected with L. major among the two treatments, hydrocortisone and dexamethasone (F = 8.745, df = 7, P = 0.0001). There were increased IFN-γ levels from the start of the experiments in the L. major infected BALB/c mice albeit the hydrocortisone treated mice produced larger quantities of IFN-γ (Figure 12).
3.5 Effects of glucocorticoids on lesion development in BALB/c mice
Differences in lesion sizes between week 0 to week 5 were subjected to repeated measure ANOVA, which indicated that there were significant differences in lesion sizes among different treatment ($F = 21.1223$, $df = 35$, $P = 0.0000$). The untreated controls for infection of BALB/c mice with *L. major* (PBS) displayed the highest increase in lesion size after infection as was lesion in *L. major* treated with LPS. However, the low rates of increase in lesion was recorded in dexamethasone, which was significantly ($P < 0.05$) higher than lesion size in BALB/c mice treated with hydrocortisone (figure 13).
Figure 13: Lesion development in BALB/c mice subjected to different drug treatments
CHAPTER FOUR

4.0 DISCUSSION

4.1 Overview

Cutaneous Leishmaniasis in humans is caused by Leishmania major, a parasite of the skin. L. major infects mononuclear phagocytes of vertebrate hosts. In some mouse strains this infection causes no or only transient pathological changes, whereas some strains develop systemic visceral disease with splenomegally, hepatomegaly, anemia, and skin lesions. The aim of this study was to determine the effects of glucocorticoids in controlling L. major infection. This was done by calculating the LDU and determining the parasite multiplication indices and infectivity. This study contributes to the understanding of the role played by glucocorticoids in controlling Leishmania infection by inhibition of production of chemokines (MIP 1α and MCP 1) that propagate the infection by activating the NF-κβ pathway, and the control of lesion development in BALB/c mice.

4.2 Effects of glucocorticoids on production of MIP 1α and MCP 1 chemokines before and after infection with L. major in macrophages of BALB/c in vitro

This study determined how application of glucocorticoids modulates production of MIP 1α and MCP 1 in BALB/c mice infected with L. major. It was established that the use of two glucocorticoid drugs dexamethasone and hydrocortisone resulted in significant reduction of MCP 1 and MIP 1α when applied pre- and post-infection. Application post infection proved to be most effective in reducing the MCP 1 and MIP 1α levels. Comparatively, hydrocortisone was the most effective drug in reducing the chemokine levels during the pre- and post-infection of macrophages. As reported by Bhattacharyya (2008) silencing of CCR5, a receptor for MIP 1α, leads to low parasite entry in the macrophages along with enhanced production of NO and proinflammatory cytokines in L. donovani infected macrophages. This confirms that low production of MIP 1α reduces parasite infectivity.
Previous research shows that wild type *Leishmania* parasites induce MCP 1 production as early as one hour post-infection in mice (Matte & Olivier, 2002). This study also determined the effects of the glucocorticoids drugs on the concentration of MCP 1 in macrophage of BALB/c mice before and after infection with *L. major* promastigotes. It was established that the two drugs resulted in significant reduction of MCP 1 when applied pre- and post-infection with application post infection being most effective in reducing the MCP 1 levels. This study revealed that hydrocortisone was the most effective drug in reducing MCP 1 during the pre- and post-infection of macrophages with efficacious ranges of hydrocortisone being much lower than those of dexamethasone for effective reduction of the MCP 1 in the macrophage before infection and after infection with *L. major* promastigotes.

Scheinman *et al.*, (1995) has well documented that glucocorticoids cause a level of immunosuppression, which is therapeutic by causing reduction in the function and numbers of lymphocytes, including both B cells and T cells. The main mechanism for this immunosuppression is through inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a critical transcription factor involved in the synthesis cytokines and proteins, for example adhesion proteins that promote the immune response. Being steroids, glucocorticoids regulate transcription factors (Liu *et al.*, 2010) like, in this case NF-κB.

Induced expression of MCP 1 is strongly dependent on activation of the transcription factor NF-kB. Exposure of cells to TNFα results in the sequential activation of NF-kB–inducing kinases (NIKs) and IkB kinases A and B (IKKa/b) which promote phosphorylation that eventually leads to IkBa degradation and p50/p65 translocation to the nucleus. Recent studies demonstrate that activation of p38 even inhibits TNFα-induced NF-kB activation under certain conditions. Thus, the cross-talk between the p38 MAP kinase cascade and the NF-kB–activating pathway may occur at different levels,
depending on the extracellular stimuli, the individual gene, and the cell type. Interestingly, p38 significantly contributes to TNFα-inducible expression of MCP 1 at a transcriptional level in the vascular endothelium.

Leishmania parasites are known to activate the NF-κB pathway, causing subversion of the host defense response, while promoting the survival and development of the parasite inside infected macrophages (Gregory et al., 2008). Hadi et al., (2013) also reports that progression of atherosclerosis is attributed to activation of the NF-κB pathway. The parasites use activation of the NF-κβ pathway as a strategy to protect the host cells from apoptosis, therefore allowing pathogens to survive, replicate, and disseminate (Guizani-Tabbane et al., 2004). This makes the use of glucocorticoids in control of leishmaniasis very viable.

4.3 Effects of glucocorticoids on Leishmania parasite multiplication and infection rates in macrophages
For Alexander et al., (2005), the paradigm Th1/Th2 of resistance/susceptibility to intracellular parasites appears to be an oversimplification of one of the most complex immune chains of regulatory/counter-regulatory interactions, which differ mainly according to the Leishmania species and experimental model employed. Leishmania parasites have the ability to survive and to multiply within macrophages and their capacity to circumvent the leishmanicidal activities of macrophages have been linked to alterations in key signaling cascades (Guizani-Tabbane et al., 2004).

In leishmaniasis, the dose of parasites used to initiate infection is particularly important. Thus, high doses of parasites can convert normally resistant animals to a susceptible phenotype, while low doses of parasites can promote healing and Th1-cell development in normally susceptible mice. Besides determining the course of infection, parasite
numbers also play a role in regulating T-helper cells once they have developed. This study determined the effects of glucocorticoids on parasite loads in the macrophage. Based on the study, the parasite infection rates displayed exponential reduction with reduction rates being higher in the macrophages treated with hydrocortisone as compared to those treated with dexamethasone.

Presently, the mechanism by which a high parasite dose inhibits the development of a protective type 1 response is unknown. Parasites, or products of infected cells, such as IL-10, transforming growth factor (TGF), and prostaglandin E2, might directly act to down-regulate Th1 cells and alternatively, the influence of parasite dose may relate to the amount of antigen presented to the immune system. Many studies to address the role of an antigen in Th-cell differentiation have been designed, but the conclusions reached vary; for example, a study with T-cell-receptor-transgenic mice found that high doses of peptide favored a Th1 response but that low doses favored a Th2 response (Antoniazi et al., 2004). In contrast, in T-cell-receptor-transgenic mouse, high and very low concentrations of peptide favored a Th2 response while intermediate levels of antigen favored a Th1 response (Berman & Lee, 1984). The parasite-macrophage ratio used in this study was 10:1 which is the optimum for such experiments. (Pinelli et al., 1999)

An extremely high parasite load can block the development of a protective immune response. In the presence of a large number of parasites, a type 1 response would be suppressed, probably be due to a high antigen load associated with the infection or the ability of parasites to stimulate the production of inhibitory factors by the infected cells. In addition to regulating high antigen levels, a large parasite load might regulate type 1 cells by stimulating the production of inhibitory cytokines due to the increase in the number of infected macrophages or dendritic cells. L. major-infected cells produce several factors, such as IL-10, TGF-β, and prostaglandin E2, that have been shown to inhibit Th1 responses. However, it is clear that a high parasite load has no overriding influence on the ability of a type 1 cell population to resolve an established infection.
The potency of a type 1 cell population may also be enhanced by the presence of dendritic cells in the donor population that have been exposed to IL-12 and can present *Leishmania* antigens.

Dendritic cells exposed to leishmanial antigens and IL-12 are known to induce substantial protection when transferred to immunocompetent mice and this is probably by ensuring that any *Leishmania*-specific T cells develops into an IFN-γ producer. Type 2 cell populations regulate type 1 responses in the presence of high numbers of parasites but not in the presence of low numbers of parasites, the mechanism has not been known though. Noben-Trauth *et al.*, (2003) reported that cells from *L. major*-infected BALB/c mice contained both Th2 cells and precursors of Th1 cells. It is therefore possible that in a low-level-antigen environment, these Th1 precursors may have an advantage in developing hence resistance to infection.

The study also determined how glucocorticoids influenced the multiplication rates of the parasites and found that the drugs resulted in exponential reduction rates. As shown in the results, reduction in multiplication rates of the parasites was higher in macrophages treated with hydrocortisone compared to those treated with dexamethasone. Hydrocortisone reduced the multiplication rate of parasites twice as much as dexamethasone. Glucocorticoid potency, duration of effect, and overlapping mineralocorticoid potency varies from one to another. Hydrocortisone (cortisol) is reported to be the standard of comparison for glucocorticoid potency. Oral potency may be less than parenteral potency because significant amounts, up to 50% in some cases may not be absorbed from the intestine. This potency explains the ability of hydrocortisone to significantly reduce parasite multiplication as compared to dexamethasone.
4.4 Determination of LDU

Treatment with various glucocorticoids drugs resulted in some noticeable physiological changes. Although the treatment did not reduce the body weights of the mice, weight of the spleen, spleno-somatic index, number of parasites and LDU were found to be affected the application of the drugs. Hydrocortisone exhibited highest reduction of the spleen weight, number of parasites and LDU when compared to dexamethasone, and they were both significantly different from those of the control. The LDU values obtained in this study indicate the route of administration of the drugs did not have a significant effect on its efficacy. Compared to the negative control, hydrocortisone and dexamethasone reduced parasitaemia significantly with hydrocortisone controlling Parasitemia better than dexamethasone.

The time taken for the glucocorticoid drugs to take effect, demonstrated by a significant reduction in lesion size, was the same (14 days of treatment) compared to the controls. After 21 days of treatment, there was no significant difference in the lesion sizes of treated animals, inferring that the healing time is similar. Apparently, all the treatment drugs were well-tolerated by the animals as the body weights were not significantly different at termination.

The spleen is a major site of *Leishmania* multiplication in the natural infection in susceptible hosts. In BALB/c mice, the splenic parasite burden is initially quite low, but it increases steadily for at least 3 months, and unlike the hepatic burden, it does not decline spontaneously without treatment (croft et al., 2003) The splenic efficacy of the test compounds should be emphasized, since until recently splenectomy was performed as the last recourse for cases of antimony resistant leishmaniasis. The efficacy of the glucocorticoids in the spleen is compatible with the available data on its tissue distribution (Marschner et al., 1992)
4.5 Effects of glucocorticoids on levels of MIP 1α, MCP 1 before and after infection with *L. major* and effects on IFN γ after infection *in vivo*

This study determined how application of glucocorticoids modulates production of MIP 1α and MCP 1 in BALB/c mice infected with *L. major*. It was established that the use of two glucocorticoid drugs dexamethasone and hydrocortisone resulted in significant reduction of MCP 1 and MIP 1α when applied pre- and post-infection. Application post-infection proved to be most effective in reducing the MCP 1 and MIP 1α levels. Comparatively, hydrocortisone was the most effective drug in reducing the chemokine levels during the pre- and post-infection of macrophages. According to Bhattacharyya (2008) silencing of CCR5, a receptor for MIP 1α, leads to low parasite entry in the macrophages along with enhanced production of NO and proinflammatory cytokines in L. donovani infected macrophages. This confirms that low production of MIP 1α reduces parasite infectivity.

In this study, there was a significant (P < 0.05) reduction in the production of MCP 1 and MIP 1 chemokines both in the *in vivo* and the *in vitro* studies. However, there were noticeable differences in the concentrations at which the reduction was highest, the efficacy ranges, and the percent reduction. Before infection of the macrophages with promastigotes of *L. major*, application of the glucocorticoids resulted in significantly lower production of MIP 1α. It was observed that hydrocortisone resulted in the highest reduction in production of MIP 1α with its efficacious ranges from 0.25 mg/l to 0.8 mg/l compared to the efficacious range of dexamethasone. Also in terms of percentage reduction; dexamethasone resulted in 80% reduction in the concentration of MIP 1α compared to 92.6% reduction in the concentration in hydrocortisone.

Pro-inflammatory cytokines are mainly produced by mononuclear phagocytic cells but many other cells can also produce these mediators (Borish & Steinke, 2003). Bacterial components, such as lipopolysaccharide (LPS), are potent inducers of pro-inflammatory cytokines. Their production is regulated by several cytokines and other inflammatory mediators (Steinke & Borish, 2006). Proinflammatory cytokines also contribute to the
adaptive immune response by; stimulating the migration of dendritic cells to lymphoid organs, activating T-cells, promoting B-cell proliferation and maturation and synthesis of immunoglobulins (Borish & Steinke, 2003; Janeway et al., 2005).

In a murine model, protection against leishmaniasis can be measured by measuring infected footpad thickness in comparison with the uninfected control and enumeration of parasite load in infected tissue, either liver, spleen, or footpad. On infection with Leishmania, most isogenic mouse strains such as C57BL/6 and C3H develop an ultimately self-healing lesion around the inoculation site. Thereafter, these mice remain refractory to relapse of infection. BALB/c mice on the other hand are extremely susceptible to this infection and develop a non-healing lesion and subsequent visceral infection (Howard et al., 1980).

Chemokines are cytokines with important roles in cell migration and activation. Leishmaniasis induces the expression of various chemokine genes (Racoosin, & Beverley, 1997; Brenier-Pinchart et al., 2001; Ritter & Körner, 2002). Leishmania major infection induces expression of CCL2 (MCP 1), CCL3 (MIP 1α), CCL4 (MIP 1β), CCL5 (RANTES), CXCL2 (MIP 2α), and CXCL10 (γIP-10), along with the receptors CCR5, CCR2, and CCR1, in a time-dependent manner in mice (Matte & Oliver, 2002; Ji et al., 2003; ). Therefore, the capacity of glucocorticoids to significantly inhibit production of these chemokines indicates a therapeutic effect for leishmaniasis in this case.

There were increased levels of IFN-γ from the start of the experiments in the *L. major* infected BALB/c mice with hydrocortisone treated mice producing larger quantities of IFN-γ as compared to dexamethasone treated ones and the controls. In leishmaniasis,
IFN-γ is associated with resistance as reported by Noben-Trouth (2003) while in contrast, Mahmoodi (2005) reports that susceptibility to L. major as demonstrated by BALB/c mice is attributed to type 2 helper T cell response associated with production of IL-4 and IL-5. Infected macrophages produce IL-12 which induces natural-killer-cell (NK) activation, Th1 cell differentiation and IFN-γ production. INF-γ acts to stimulate production of nitric oxide by macrophages, which in turn is responsible for parasite destruction and infection resistance (Wilson et al., 2005).

IFN-γ pro-inflammatory mediators are induced in macrophages and they include specific chemokines, which are believed to play a crucial role in the recruitment of inflammatory cells (Gregory et al., 2008). The production of major Th1 cytokines is an indication of cell-mediated Th1-type immune response. IL-12 is the most significant cytokine in the development of Th1-type response, whereas IFN-γ mediates effector functions of Th1 lymphocytes. IFN-γ activates monocytes and macrophages to kill intracellular pathogens and promotes their cytokine production and antigen presentation. IFN-γ is also known to stimulate killing by neutrophils and natural killer (NK) cells and induces B-cells to produce IgG antibodies that mediate opsonization and phagocytosis. (Borish & Steinke, 2003; Janeway et al., 2005; Netea et al., 2005).

Rolão et al. (2007) reported that high INF-γ levels were accompanied by parasite burden reduction. However, Ansari et al. (2006) suggested that despite the presence of elevated INF-γ levels during infection, the host may fail to control the disease due to an incomplete response to INF-γ. The high levels of INF-γ may have been related to infection control, since some reports have shown that control of leishmaniasis initiates approximately four weeks after the infection (Melby et al., 2001; Engwerda et al., 2004).

Gomes-Pereira et al. (2004) concluded that IL-10 levels were not sufficient to inhibit IFN-γ production by hepatic leukocytes or subsequent parasite destruction, suggesting
that IL-10 may not directly inhibit IFN-γ function in experimental visceral leishmaniasis.

4.6 Effects of glucocorticoids on lesion development in BALB/c mice

In this study, the development of lesions in BALB/c mice infected with *L. major* and treated with two glucocorticoid drugs (hydrocortisone and dexamethasone) was monitored. This was done over a period of 5 weeks. The results indicated that there was a significant decrease in the lesion sizes between mice treated with glucocorticoids against the controls. These results suggest that glucocorticoids are able to reduce the development of lesions in BALB/c mice after infection with *L. major*.

In BALB/c mice, *L. major* produces visceral infection in addition to the local lesion at the point of inoculation. Thus, this was the most suitable model for this study. Also, the fact that a spontaneous healing cannot be achieved in these mice indicates that reduction of the lesions was due to the drug therapy administered only. Iniesta (2001) reiterates that experimental *L. major* infection is one of the best elucidated models for studying defense mechanisms in murine models of leishmaniasis.

Hydrocortisone was most effective in reducing lesion development in BALB/c mice compared to dexamethasone in this study. A variety of synthetic glucocorticoids, some far more potent than hydrocortisone, have been created for therapeutic use. They differ in both pharmacokinetics like absorption factor, half-life, volume of distribution, clearance and pharmacodynamics like retention of sodium and water. Because they permeate the intestines easily, they are administered primarily per by mouth, but some are applied topically.

In experimental infections by the genus *Leishmania*, host resistance or susceptibility to the parasite is usually determined by differentiation of T lymphocytes into the subpopulations Th1 or Th2 (Hoffman *et al.*, 2009). Recent studies especially concerning
leishmaniasis agents in humans, i.e. *L. major* define the Th1/Th2 paradigm as indicator of the resistance or susceptibility to the infection. The Th1/Th2 paradigm also indicates the role of IL-12 and IL-4, which is to elicit the development of Th1 and Th2 cells. In infections provoked by *L. donovani* and *L. chagasi*, which are agents of visceral leishmaniasis, such a pattern has not been observed (Alexander *et al.*, 2005; Pinto *et al.*, 2000). The resistance seen here, explained by healing of lesions albeit not completely, is attributed to the administration of the glucocorticoids and is evidenced by susceptibility of the control groups with emphasis to the negative control group which only received PBS.
CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Treatment with glucocorticoids reduced the production of MCP 1 and MIP 1α chemokines by inhibiting the NF-κβ pathway. Higher rates of decrease in lesion sizes were recorded in hydrocortisone, which was significantly better than lesion size in BALB/c mice treated with dexamethasone suggesting that hydrocortisone was the most effective in reducing lesion development in BALB/c mice.

Glucocorticoids reduced parasite multiplication and parasite infectivity and the parasite load (LDU) of *Leishmania major* parasites. This is probably due to a significantly high production of IFN-γ aided by application of the glucocorticoids. In this case too, Hydrocortisone gave better reduction of multiplication index, parasite infectivity and the parasite load when compared to dexamethasone.

Glucocorticoids significantly reduced lesion sizes in BALB/c mice infected with *Leishmania major* promastigotes.

Glucocorticoids have a potential use in the management of cutaneous leishmaniasis as evidenced by the results of this study.
5.2 Recommendations

i. I recommend that dexamethasone and hydrocortisone be used in the management of cutaneous leishmaniasis.

ii. More research on optimization of the anti-leishmanial effects of glucocorticoids is required.

iii. The association between the NF-κB pathway and disease progression in leishmaniasis, and the possible use of glucocorticoids to curb that should be further explored.
5.3 Study limitations or challenges

Procurement of the ELISA kit took very long halting the project for a while. Acquisition of experimental mice also took long as I had to apply and wait for them to be reared to the age I required.
REFERENCES


Bhattacharyya, S., Ghosh S., Dasgupta B., Mazumder D., Roy S., and Majumdar S (2002). Chemokine-Induced Leishmanicidal Activity in Murine Macrophages via the Generation of Nitric Oxide. The Journal of Infectious Diseases, 185, 1704–8


Dey, R., Sarkar A., Majumder N., Bhattacharyya S.M., Roychoudhury K., Bhattacharyya S., ... and Majumdar S., (2005). Regulation of Impaired Protein Kinase C Signaling by Chemokines in Murine Macrophages during Visceral Leishmaniasis. *Infection and Immunity*, 73(12), 8334–8344


Ghosh S., Bhattacharyya S., Sirkar M., Sa G.S., Das T., Majumdar D., ... and Majumdar S., (2002). *Leishmania donovani* Suppresses Activated Protein 1 and NF-kB Activation in Host Macrophages via Ceramide Generation: Involvement of Extracellular Signal-Regulated Kinase. *Infection and Immunity*, 70(12), 6828


Factor in Human Macrophages: Involvement in cytokine Synthesis.
*Infection and immunity, 72 (5), 2582-2589*


Murdoch, C. and A.Finn. (2000). Chemokine receptors and their role in

National guidelines for health workers (2012). Diagnosis and Management Of Visceral Leishmaniasis (Kala Azar)


## APPENDICES

### Appendix 1: Visceral leishmaniasis epidemiology in Kenya

<table>
<thead>
<tr>
<th>Province</th>
<th>Current foci (District)</th>
<th>Parasite</th>
<th>Confirmed vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rift valley</td>
<td>Turkana, Baringo, Marakwet and West pokot</td>
<td><em>L. donovani</em></td>
<td><em>P. martini (confirmed)</em></td>
</tr>
<tr>
<td>Eastern</td>
<td>Kitui, Meru and Machakos</td>
<td><em>L. donovani</em></td>
<td><em>P. martini (confirmed)</em></td>
</tr>
<tr>
<td>North Eastern</td>
<td>Mandera and Wajir</td>
<td><em>L. donovani</em></td>
<td><em>P. martini (confirmed), P. celiae (suspected), P. vansomeranae (suspected)</em></td>
</tr>
</tbody>
</table>
### Appendix 2: Cutaneous leishmaniasis epidemiology in Kenya

<table>
<thead>
<tr>
<th>Province</th>
<th>Current foci (District)</th>
<th>Parasite</th>
<th>Confirmed vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rift Valley</td>
<td>Baringo</td>
<td><em>L. major</em></td>
<td><em>P. duboscqui</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. guggisbergi</em></td>
</tr>
<tr>
<td></td>
<td>Laikipia, Samburu and Nakuru</td>
<td><em>L. tropica</em></td>
<td><em>P. duboscqui</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. guggisbergi</em></td>
</tr>
<tr>
<td>Eastern</td>
<td>Kitui</td>
<td><em>L. major</em></td>
<td><em>P. duboscqui</em></td>
</tr>
<tr>
<td></td>
<td>Isiolo</td>
<td><em>L. tropica</em></td>
<td><em>P. guggisbergi</em></td>
</tr>
</tbody>
</table>
Appendix 3: Ethical clearance

14TH August, 2012

Rose Magoma Nyamao

SC 351-1256/2011

RE: KEMRI ACUC approval for SSC 2373 ‘Efficacy of glucocorticoids in controlling Leishmania major infecting Balb/c protocol

Following the resubmission of the above mentioned proposal to the ACUC addressing the issues raised earlier, the committee recommends that you proceed with your work after obtaining all the relevant approvals.

The committee wishes you adhere to all the animal handling procedures on the mice you will use in your study as described in your proposal

The committee wishes you all the best in your work

Yours sincerely,

Dr. Konongoi Limbaso

Chairperson, KEMRI ACUC

Cc

Animal house