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Disease Susceptibility and Cytokine Production in F1 Generation of Mice Cross Breed of Swiss Albino with BALB/c Mice (SAB) following *Leishmania donovani* Infection

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ABSTRACT

The difference between Swiss albino (resistant) and BALB/c (susceptible) strains in response to L. donovani infection has been linked to inheritable genes, and the GATAq and Lsh group of genes plays a key role in disease transmission. This study sought to investigate effects of gene neutralization through cross breeding and back crossing. SAB mice were generated by cross breeding of BALB/c mice and Swiss Albino mice. This study aimed at investigating the susceptibility, immune response and pathology of SAB mice after L. donovani infection. Serum was collected from all mice groups 4 weeks post infection and levels of IFN-γ and IL-4 cytokine were determined using ELISA. Body and spleen weights were measured, and tissue section stained for histopathology examination. In this study the L. donovani infected swiss mice had significantly low amounts of IL-4 as compared to the SAB mice (P=0.0093). Whereas the IL-4 levels in BALB/c mice were not significantly different to that of SAB mice (P= 0.1507). Conversely infected swiss albino mice had significantly high amounts of IFN-γ compared to SAB (P=0.0216) and BALB/c (P=0.0326) mice. No significant difference in IFN-γ levels were between the groups. No significant difference in body and splenic weights between groups (P>0.05). Pathologically there was observed proliferation of kupffer cells, degenerated hepatocytes and fibrosis in BALB/c and SAB mice. In addition, there was chronic degeneration of structure of spleen of SAB mice characterized by disruption, an indication of severe infection. Results obtained in this study show that SAB mice can be used in studies involving L. donovani parasites. This mouse model can be used in the absence of BALB/c mice owing to their susceptibility to L. donovani. Further studies are needed to be carried out to determine other factors e.g genetic makeup that makes it susceptible to L. donovani parasites.

Keywords: *SAB Mice, Leishmaniasis, Hepatocytes, Fibrosis, Histopathology Examination*

INTRODUCTION

Leishmaniases are major tropical parasitic diseases caused by Kinetoplastida intracellular protozoan parasites of the genus *Leishmania* (Pigott et al., 2014). The parasites are transmitted following bites by infected female sand flies belonging to *Lutzomyia* and *Phlebotomus* genera in the new world and old world respectively. Infection of sandflies arises when uninfected sand fly feeds on infected people or infected animals such as rodents, dogs or foxes. Similarly, cases of *Leishmania* parasites transmission in humans is during blood transfusion and may also take place from infected pregnant mother to their fetus (Salam, 2004).

A report by World Health Organization (WHO, 2010) indicates that an average of 350 million people are at risk of being infected with leishmaniasis and according to Pigott *et al*., (2014), there are 2 million new cases of leishmaniasis annually and 15 million individuals are infected globally. The burden has been compounded by inadequacy of low-cost treatment and therefore, the need for more exploration especially vaccine-oriented research (Basu *et al*., 2005). Leishmaniasis consists of three main clinical forms: Cutaneous leishmaniasis (CL) affects the skin where the host is bitten by the infected sandfly; mucocutaneos leishmaniasis (MCL) is a disease that destroys the mucus membranes of the nose, mouth and pharynx while visceral leishmaniasis

(VL) also called kala-azar is the deadliest disease and is almost 100% fetal if untreated (Mutiso *et al*., 2013). Different *Leishmania* species can infect the skin macrophages leading to prognoses and variable clinical presentations (Lukes *et al*., 2007). The entry of *Leishmania* parasite into a macrophage cell triggers mechanism capable of killing up to 90% of invading parasites.

In the macrophage, *Leishmania* induces pro-inflammatory chemokines, causing chemotactic movement of phagocytic cells to the site of invasion. Infection with *Leishmania donovani* leads to a fatal disease in susceptible individuals or animals. The clinical signs associated with the disease are: fever, hepatosplenomegaly, anaemia, wasting, bone marrow destruction and high parasite infection is associated with high levels of antileishmanial antibodies (Mutiso *et al*., 2013).

The available animal models of leishmaniasis include: mice, hamsters, non-human primates and domestic dogs and inbred mice strains have been extensively used probably because they are cheaper to breed, easy to handle and genetic manipulations can be carried easily in mice to produce a suitable disease model(Sacks and Melby, 2001). Both resistant and susceptible mice are available and these can be used to study protective and susceptible immune status respectively (Andrade-Narvaez, 2014). The BALB/c is highly susceptible to *Leishmania major* infection while the Swiss white and C57BL/6 are resistant. Swiss Albino mice on the other hand are well known for their resistance to both *L. donovani and L. major*(Santos *et al*., 2003). The susceptibility or resistance to mice *Leishmania* infection is dependent on the induced immune response. The susceptibility of BALB/c is associated with parasite specific T helper 2 cells (Th2) with production of IL-4, IL-10 and other type 2 cytokine as well as high levels of antleishmanial antibodies(Mutiso *et al*., 2012). Protective immunity in Swiss albino mice is associated with induction of specific T helper 1(Th1) cell with high levels of type 1 cytokines including interferon-gamma (IFN-γ) therefore Swiss Albino mice are able to control the disease. Although disease models of leishmaniasis exist, these may not provide adequate information on the events that take place in a host where there is a balance or near balance between Th1 and Th2 immune responses. This study therefore, sought to investigate disease susceptibility and antibody production in a SAB mice model, a cross breed between resistant Swiss albino mice and *Leishmania* susceptible BALB/c. The development of such a disease model enhances the understanding of interactions between protective and non- protective immune responses in clinical cases.

Materials and Methods

Study Area

This study was carried out in Leishmania laboratory at Centre for Biotechnology Research and Development (CBRD) and KEMRI where requisite facilities necessary for this study were provided.

Culture of *Leishmania* Parasites

A cryopreserved *Leishmania donovani*were cultured in Schneider Drosophila insect medium (Sigma, MI, Saint Louis in USA), augmented with 20 percent heat inactivated Foetal Bovine Serum (FBS) (Campinas, Cultilab, Brazil), 500 µg/ml streptomycin, 250 µg/ml 5-fluorocytosine arabinoside and 500 µg/ml penicillin (Gibco, Grand Island, NY, USA). Thereafter, the promastigotes were incubated at 25°C then developed to stationary stage to produce infective metacyclic structures. The stationary-phase promastigotes were harvested at the sixth day of culture and cleaned thrice with sterile phosphate buffered saline (PBS) and counted using a haemocytometer which is an Improved Double Neubauer chamber.

Animals and Experimental Design

Three strains of mice were used in this study and these included BALB/c, Swiss albino and SAB mice. The SAB mice, a cross breed between BALB/c and Swiss albino formed the test mice group with the parental mice forming controls. The mice all aged between eight and ten weeks old and of mixed sexes were obtained and maintained at KEMRI animal house. Fifteen mice, belonging to each of the three mice strain were infected intraperitoneally with 2×10^6 virulent *Leishmania donovani* parasites and monitored for a total of 8 weeks before termination of the experiment. Another three groups of 10 mice each from each of the three strains were used as uninfected controls.

Weights from all mice groups were measured once weekly during the experimental period. Eight weeks post infection all mice were anesthetized and sacrificed to obtain samples for measurement of various variables. Blood

samples were obtained from each mouse for quantification of IFN-γ and IL-4 cytokine analysis using ELISA. Spleen and liver samples were used to prepare sections for pathological examinations.

Sample Size Determination

The study's sample size was computed using the resource equation method (Mead R, 1988) which is dependent on the law of diminishing returns whereby, addition of one experimental unit to a small experiment yields good returns, while adding it to a larger experiment fails to do so;

E= N – B –T

Where;

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

N is the total degrees of freedom

B is the blocks degrees of freedom

T is the treatment degrees of freedom

For two treatment groups each with ten animals;

(3 x 6= 18 mice in total to be used per setup)

N=17, B=0, T=2 hence E= 17 - 0 - 2 =15

The experiment was done in triplicate hence a total of 15×6= 90 mice

Serum interferon-γ and IL-4 immunoassay

Serum interferon-γ immunoactivity was determined using a commercial single analyte ELISArray kit (SABiosciences, Canada) exactly according to the manufacturer's instructions (Appendix V). Outcomes were quantified by optical density at 450 nm, with background correction at 570 nm, using ELX 800, a universal microplate reader.

The detection levels of interferon-γ and IL-4 will be 39pg/ml and 32.8 pg/ml respectively.

Preparation and examination of liver and spleen tissues for pathology

Liver tissues were sectioned from three representative mice from each experimental and control groups. Tissues were fixed in 10% formalin and dehydrated using isopropyl alcohol in an increasing concentration (70%, 85%, 95%, and 100%) for 1 hour at each solution. Tissues were infiltrated with molted paraffin at 58°C and allowed to solidify at room temperature for embedding. Sectioning was done using a microtome to produce 10 µm thick sections. Sections were flattened by floating them in water held at 45°C. They were then mounted on a microscope slide with adhesive solution. Clearing of mounted sections to remove paraffin was achieved by passing the mounted sections through clearene that dissolved the paraffin. Staining of sections was done with hematoxylin and eosin (Fischer et al., 2008) before examination under the light microscope. Photographs were acquired using the spot camera specially made for taking such images for reporting.

Data Presentation and Analysis

Data was recorded as means, standard error of the mean of body and splenic weights and concentration of IFN-γ and IL-4 cytokines. Data was analyzed using excel and STATA softwares (version 14.2). Data was analysed using paired student t-test to statistically compare between SAB/Swiss mice, SAB//BALB/c mice and BALB/c/Swiss mice independently. P value of less than 0.05 was considered significant.

Ethical Approval

The study was approved by the Scientific Steering Committee (SSC) and Animal Care and Use Committee (ACUC) of Kenya medical research institute (KEMRI). The study was strictly confined to the stipulated guidelines.

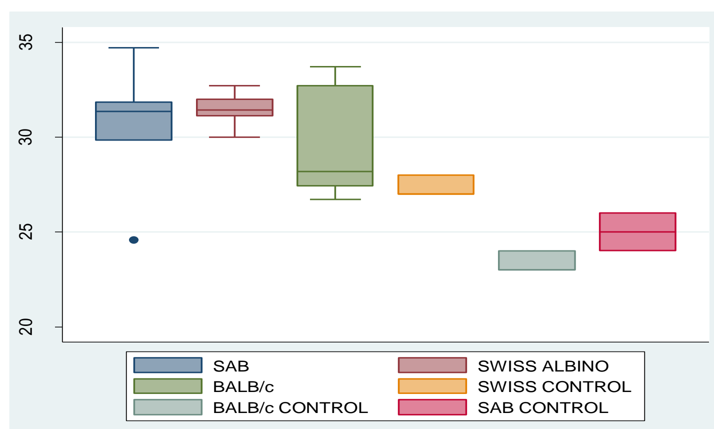

Figure1. *Body weights of L. donovani infected SAB, Swiss and BALB/c mice.*

RESULTS

Comparison of body weights of SAB, Swiss and BALB/c mice

There was no significant difference in body weights of infected SAB mice compared to infected BALB/c and Swiss mice (P=0.2254 and 0.31 respectively). Swiss albino mice had increased body weight compared to BALB/c mice but not significantly different (P=0.0925) (Table1). Infected SAB had significantly increased body weights compared to uninfected BALB/c and Swiss Albino mice (P=0.0112 and 0.0084 respectively) mice were significantly higher as compared to uninfected mice respectively (Figure1).

Table1. *The mean body weights of Swiss, BALB/c and SAB*

		Infected			Uninfected		
		Swiss	**BALB/c**	**SAB**	**Swiss**	**BALB/c**	**SAB**
Body weight	Mean±SEM	31.52±0.52	31.9±1.48	31.76±1.45	27.3±0.33	23.2±0.33	2.5±0.57
	STD	0.436	2.564	2.52	0.577	0.577	1
Spleen weight	Mean±SEM	0.157±0.019	0.173±0.007	0.46±0.33	0.11±0.25	0.11±0.25	0.081±0.002
	STD	0.032	0.115	0.563	0.0152	0.435	0.003

Comparison of splenic weights of cross breed, Swiss and BALB/c mice

Infected SAB mice had higher spleen weights compared toinfected BALB/c (0.16±0.1978, t (df=5) =0.8088, P=0.232) and swiss mice (0.145±0.17,t (df=5)=0.8522, P=0.2165) (Table 1, figure 2). Similarly, the splenic weights of BALB/c mice were significantly higher than that of swiss albino mice (0.032±0.0116, t (df=4) =2.7644, P=0.025).

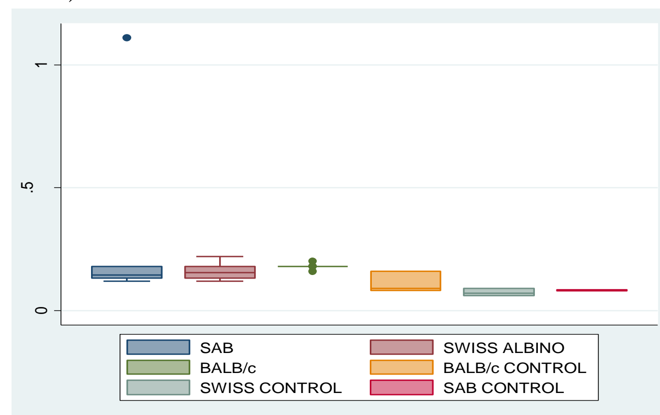

Figure2. *Graphical representation of splenic weigths of infected Swiss albino, BALB/c and SAB mice.*

Cytokine profiles by infected SAB mice depicts an impaired Th2 immune response in comparison with Swiss mice a strong Th1 immune response compared to BALB/c mice

Swiss mice infected with *L. donovani* had significantly high amounts of IL-4 cytokine levels compared to cross breed mice (SAB), ($3.7\times10^{-4}\pm5.13\times10^{-5}$, t (2) =7.2102, P=0.0093), conversely the *L.donovani* infected BALB/c mice had more IL-4 cytokine levels in the serum as compared to infected SAB mice ($1.6\times10^{-4}\pm1.159\times10^{-4}$, t (2) =1.3805, P=0.1507) but not significant as shown in table 2.

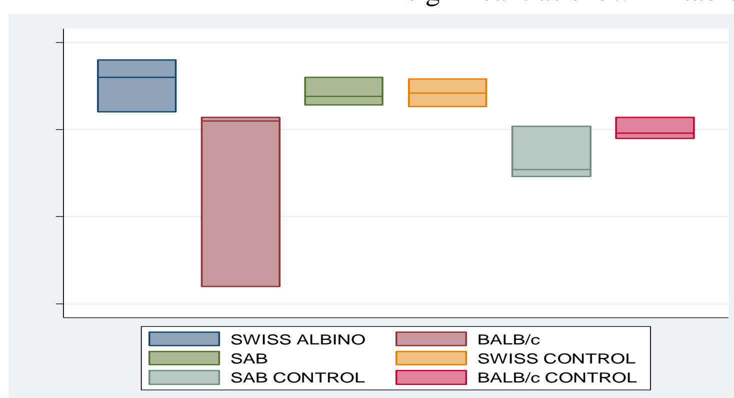

Figure3. *IL-4 levels in Swiss, SAB and BALB/c mice*

Table2. *Mean values of infected Swiss, cross breed (SAB), BALB/c mice*

	Swiss mice infected	BALB/c mice infected	Cross breed infected	Swiss mice uninfected	BALB/c mice uninfected	Cross breed mice uninfected
Means	0.0017667	0.00124	0.00171	0.00171	0.0015	0.00134
SEM	0.000882	0.0003201	0.0000473	0.0000462	0.0000907	0.0000361
Std Dev	0.0001528	0.0005543	0.0000819	0.00008	0.0000624	0.0001572

Infected Swiss mice had high amount of IFN-gamma in their serum than in the cross bred mice. The difference in levels of IFN-gamma was significantly different (0.0011133 ± 0.0002395, t (2) =4.6492, P=0.0216). There was a high amount of IFN-gamma levels in serum of infected cross bred mice as compared to BALB/c mice but the difference was not significant ($5.987\times10^{-4}\pm3.256\times10^{-4}$, t(2)=1.8385, P=0.10 37) The serum of *L. major* infected Swiss mice had significantly high amounts of FN-γ levels compared to BALB/c mice (0.001712± 0.0004602, t (2) =3.7205, P=0.0326).

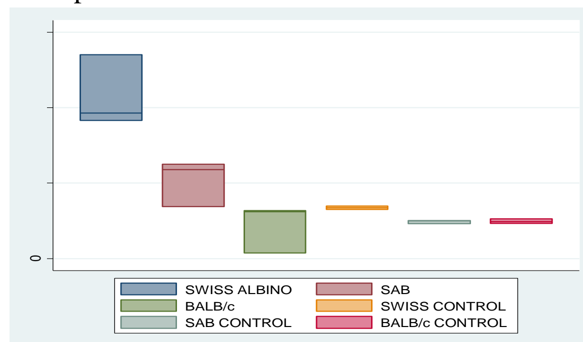

Figure4. *IF-γ levels in infected Swiss albino, BALB/c and SAB mice*

Uninfected Swiss mice had high amounts of IFN-gamma in their serum on the 4th week of infection as compared to cross breed mice. The difference in IFN-gamma levels were significant ($1.853\times10^{-4}\pm2.65\times10^{-5}$, t (2) =6.9845, P= 0.0099). The difference in the amounts of IFN-gamma in serum of uninfected BALB/c mice was not significant, however it was higher as compared to uninfected cross breed ($6.33\times10^{-6}\pm8.95\times10^{-7}$, t (2)=0.7076, P= 0.2763).

Table3. *IFN-gamma levels in Swiss, cross breed and BALB/c mice*

	Swiss mice infected	BALB/c mice infected	Cross breed infected	Swiss mice uninfected	BALB/c mice uninfected	Cross breed mice uninfected
Means	0.0021533	0.00104	0.0004413	0.0006737	0.0004947	0.0004883
SEM	0.0002749	0.0001762	0.0001862	0.0000144	0.0000175	0.0000138
Std Dev	0.0004761	0.0003057	0.0003225	0.000025	0.0000303	0.0000239

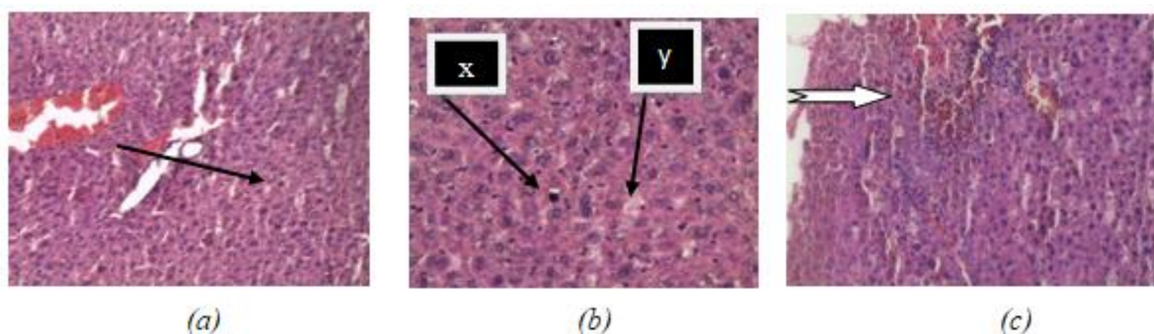

Figure5. *(A) Arrow shows perivascular infiltration of kupffer cells (B): Hepatocytes and Kupfer cells in SAB mice withx showing degenerative hepatocytes and y showing proliferation of kupffer cells.(C) shows parenchyma and perivascular in liver tissues,severe local infiltration in parenchyma and perivascular in liver tissues of BALB/c mice*

Histopathology of liver tissues of SAB mice.

There was observed Perivascular infiltration (magnification x200) on liver tissues of SAB mice suggesting evidence of infection with L. donovani (Figure 5A) with evidence of proliferation of kupfer cells and degenerative

hepatocytes in SAB mice (Figure 5B). There was severe local infiltration in parenchyma and perivascular in liver cells of BALB/c mice which is an evidence of severe infection with L. donovani. In Swiss albino mice there was no liver tissue infiltration an evidence of presence of parasite but no infection on the liver tissues. There was congestion of kupffer cells due to proliferation. (Figure 5C). Evidence of giant cells in parenchyma in SAB mice (figure 6) indicates there was chronic infection with *L. donovani*.

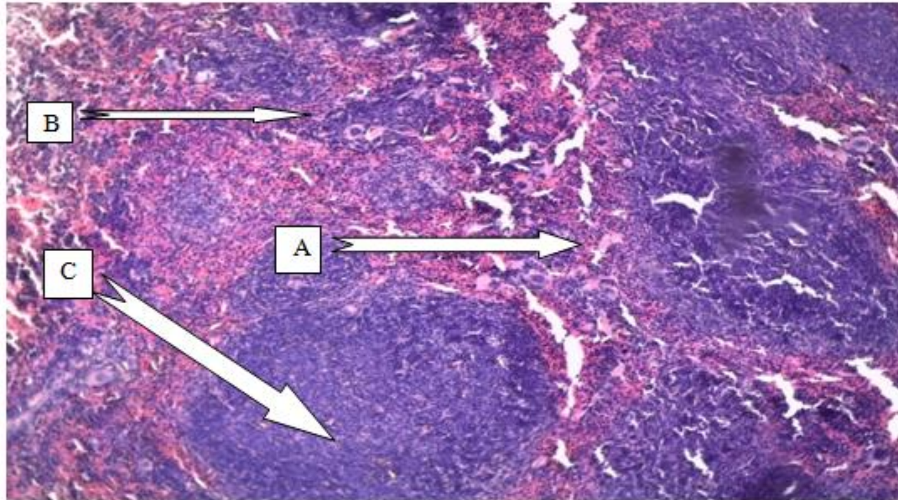

Figure6. *Giant cell, red pulp and white pulp of SAB mice. (A) giant cell, (B) unclear red pulp, (C) unclear white pulp (x100) in spleen tissues of SAB mice.*

Evidence of fibrosis was observed in spleen of BALB/c mice indicating infection (Figure 7A) with fibrosis seen in spleen of swiss albino mice (Figure 7B)

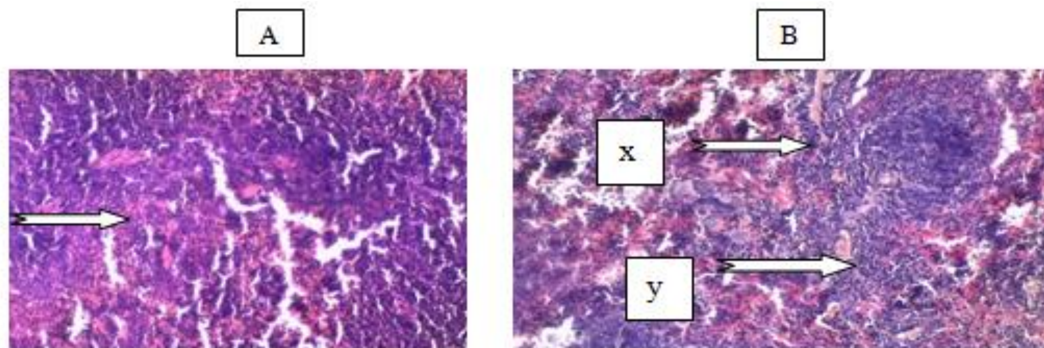

Figure7. *A) Fibrosis of spleen cells in BALB/c mice, B)Giant cells and fibrosis in Swiss Albino mice in spleen where x showgiant cell and y show Minimal fibrosis of Swiss albino spleen (mag. x200)*

Discussions

Studies on the susceptibility or resistance in mice have been found to be the determinant of the immune response generated in reaction to various forms of pathogenic invasion. An example of study on BALB/c susceptibility to *L. donovani are correlated* with emergence of parasite-specific (Th2) cytokine interleukin 4 (IL-4) T helper 2 cells, that has been shown to be responsible for differentiation of Th2 effector cells (Cotterell Sara E. J, 1999; Engwerda Christian R, 1998; Kane and Mosser, 2001).

Infected Swiss mice with *L. donovani* had significantly low amounts of IL-4 cytokine levels compared to cross breed mice (SAB). These results suggest that SAB mice were susceptible to *L. donovani* infection due to higher levels of IL-4 in their serum. IL-4 has been shown to markedly enhance Th2 priming through a Stat6-dependent pathway that involves the up-regulation of GATA-3 (Flavell, 1997; Wenjun Ouyang, 1998). TH2 cytokines suppress these inflammation mediators and promote the production of IL-10 by macrophages (Bogdan, 1998). However the IL-4 regimen is known to induce IL-12 production by DC from draining lymph nodes within hours and thus shifts the cytokine balance toward a TH1 response (Tilo Biedermann, 2001). This concept explains the higher amounts of IL-4 in both Swiss albino and SAB mice.

In comparison of the levels of serum IL-4 cytokines of infected Swiss mice with uninfected, our study found out they were high even though not significant. Our data suggests that infection triggers the stimulation of the IL-4 cytokines. There were high amounts of serum IL-4 cytokine levels in infected BALB/c mice as compared to uninfected one. This present study therefore explains why IL-4 application to infected mice increases susceptibility; however, when treatment to IL-4 is delivered during activation of DC and before T cell activation, it resistance to *L. donovani* is developed in these Swiss mice, resulting to parasite cure and clearance(Tilo Biedermann, 2001).

According to(Bogdan, 1998), TH1 cells mediate immune responses have been shown to control leprosy or leishmaniasis through TH1-derived IFN-γ that triggers the production of NO synthetase (iNOS), TNF, IL-1I and IL-12. The resistance of Swiss albino to *L. donovani* parasites has been attributed by inactive TH2 cells and activated Th1 cells. Swiss albino mice had high amounts of IFN-γ in the serum suggesting that is is able to clear *L. donovani*parasites whereas BALB/c mice had significantly low amounts of IFN-γ levels which explain their susceptibility to *L. donovan I* parasites. IFN-γ levels in SAB mice were intermediate between that of Swiss albino and BALB/c mice indicating that there was gene dilution when cross bred. Some studies have shown that depletion of IL-12, or disruption of IL-12 gene, STAT4 gene and IL-18 gene (critical to IL-12 signaling) inders TH1 cell development and makes the resistant mice more susceptible, which may explain susceptibility of SAB mice(TSAGOZIS PANAGIOTIS, 2003). This study supports the role of IFN- γ in the control of *Leishmania* infection is demonstrated by the fact that IFN-γ knockout (KO) mice do not cure the infection(Wang *et al*., 1994).

Higher susceptibility has been shown to be linked with high parasite loads in infected murine models (Yole, 2007). Parasite elimination always tends to change the situation. Spleen weight reduction in SAB mice inflated with *L. donovani* coherent to parent strains reveals the complexity of the genes that trigger infectious diseases. Patients with asymptomatic (subclinical) often have visceral leishmaniasis and epithelioid granuloma in the liver (Pampiglione, 1974). A drop of weight of body of infected mice was observed, reflecting the effect of immune suppression on the spleen. In our study, the body weights of SAB and BALB/c mice were lower as opposed to Swiss albino. Swiss albino mice have been known to control *L. Donovani* infection explaining why their weights were not affected, as persistent infection leads to decrease in body weight. The spleen weights of *L. donovani* infected BALB/c mice were higher compared to those of SAB and Swiss albino.

Morphological changes of the liver in visceral leishmaniasis involve hepatocytes, Kupffer cells, sinusoids, hepatic veins and portal tracts. The BALB/c mice uninfected had clear sinusoids, a clear indication of no infection as compared to infected BALB/c mice which had infiltrated parenchyma and perivascular cells with *L. donovani* parasites. The SAB mice had degenerated liver cells and infiltrated kupffer cells an indication of severe infection

The inflammatory cellular reaction, constitutes lymphocytes, parasitized macrophages and plasma cells affects the portal tracts and is present within the lobule either focally or diffusely in the sinusoid.

Kupfer cells act both as effector cells in destructing the hepatocytes through production of harmful soluble mediators and antigens that present cells during liver infections (Kolios, 2006). Furthermore, they tend to represent a significant source of regulatory T cells and chemoattractant molecules for cytotoxic CD8 suggesting the infiltration of these cells during *L. donovani* parasites. Kupffer cells are the main sources of TGFbeta1 production that result to change of stellate cells into myofibroblasts, explaining the observation their proliferation in SAB mice and eventual degenerated hepatocytes.

The spleen's histological structure is denoted by a lymphoid tissue referred to as the white smush which is submerged in trellis of capillaries, leukocyte-reticular cell cords referred to as red pulp. This white pulp constitutes of 3 distinct sections: (1) Periarteriolar lymphoid sheath (PALS), (2) discrete nodular lymphoid follicles (3) a Coverage of loosely packed marginal zone. Visceral leishmaniasis and other viral infections affect the spleen's histological structure thus disrupting the white pulp microenvironment (Benedict *et al*., 2006; Engwerda, 1998; Kumagai, 2004) The disruption of the architecture of both white and red pulps in both SAB and BALB/c mice indicates a severe infection of spleen with *L. donovani*. Uninfected

BALB/c mice showed clear white and red pulps suggesting that they were not interrupted therefore no infection of the spleen. Our study recommends that the SAB mice should be tested as a model for other *Leishmania* species which have no known laboratory models for instance *L. Aethiopica and L. tropica.* The molecular characterization of the SAB should be carried out to establish the role of genes in influencing immune responses and disease progression and the SAB mice should be developed to study the phenotype to reveal the behavior of genes.

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