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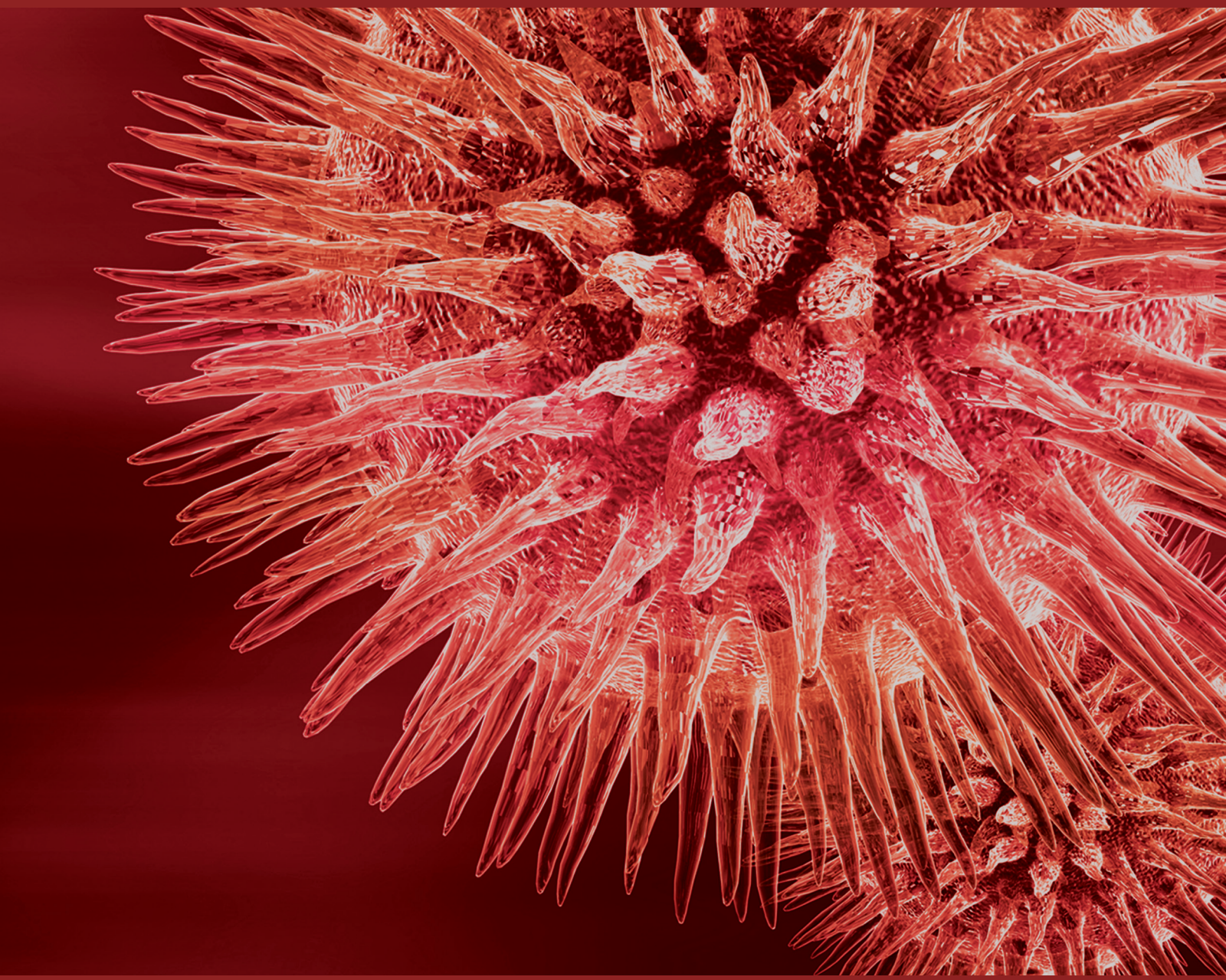
Guest Editors: Orlando Paciello, Chiara Palmieri, Iwona Otrocka-Domagala, Laura Rinaldi, Jorge Morales-Montor, and Peter Geldhof



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Editorial

Immunopathology of Parasitic Infections and Therapeutic Approaches in Humans and Animals

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Many studies of the immune system in human and animals have been focused on how it protects the body from parasitic and infectious agents [1], while, recently, many researches were oriented on how defects in the immune system can lead to immunopathological disorders, including autoimmune diseases, immunodeficiencies, and hypersensitivity reactions [1].

Many parasitic diseases are characterized by the long-term persistence of parasites in the host, due to the not fully effective host immunity or inadequate therapy. These conditions have created a particular and somewhat complicated host-parasite relationship supported by mechanisms of immune evasion by parasites, such as parasites covered by self-substances of the host, antigenic variations, and others [2].

Moreover, the first contact between the host and the parasite stimulates the innate immunity, the first immunological, nonspecific mechanism for fighting against infections. This immune response is rapid and is mediated by numerous cells including phagocytes, T-cells, mast cells, basophils, and eosinophils, as well as the complement system [1].

However the infection is established when innate immunity failed in eliminating parasitic and infectious agents and in that case adaptive immunity develops. The primary functions of the adaptive immune response are the recognition of specific "non-self"-antigens, the generation of immunologic pathways for specific pathogens, and the development of an immunologic memory [3]. The cells of the adaptive immune system include T-cells, which are activated through the interaction with antigen presenting cells (APCs), and B-cells [3].

Immune mechanisms are involved in the pathology of many parasitic infections. The main factors responsible for the tissue injury during parasitic infections are chronicity of the infections, release of parasites or host cells in tissues and within the blood, alteration and destruction of the host tissue, presence of antigenic components shared by the host and the parasite, and relative inefficiency of the host in eliminating the antigens or cross-reacting antibodies. The three main pathogenic mechanisms proposed to explain the role of infectious factors as triggers of autoimmune diseases during parasitic infections are (1) polyclonal B- or T-cell activation, (2) increased molecular mimicry, or (3) immunogenicity of self-antigens secondary to the infection-mediated inflammation [4–7].

Even though both immune mechanisms contribute to the development of immunopathological lesions in parasitic

diseases, in the past more attention has been given to the humoral response, especially immune complexes and complement.

Immune complexes (ICs) are formed when antibodies bind to relevant antigens: in parasitic diseases, this can occur at the site of parasite penetration, in the circulation, and within tissues or organs. Normally, during the course of the infection, the excess of ICs formed is removed by specific cells, while in pathological conditions the release of massive amounts of antigens from dying parasites which can substantially influence the ratio between antigen and antibody in favour of an antigen excess leads to high amount of ICs that can be detrimental. Furthermore, failure of the reticuloendothelial system to remove ICs from the blood can be another reason of the excess of circulating ICs [7].

Circulating ICs may localize in the walls of blood vessels or in the organs as described in the kidney during leishmaniasis, malaria, trypanosomiasis, schistosomiasis, and other parasitic infections. The local formation of ICs and related diseases has been also observed in several organs such as heart and skeletal muscle lesions in canine leishmaniasis and others [8].

Increased levels of IgE in the host infected with different parasites are a well-known process and they have been used as helpful diagnostic criteria. Their significance in pathology was usually attributed to a type I hypersensitivity reaction, while more recent data have shown that the biological importance of IgE includes other mechanisms [1].

Cell-mediated mechanisms of immunopathological lesions in parasitic diseases are mainly represented by delayed hypersensitivity reactions. A typical example is the granulomatous reaction around eggs or parasites entrapped in the liver, lung, gut, or intestine [9].

It is evident that immune-reactive cells including T-cells, B-cells, and macrophages play important roles in the pathogenesis of these immune-mediated diseases [10]. Recently, it has been shown that several cytokines are responsible for the development and progression of these diseases.

One example is interleukin- (IL-) 6 that is considered one of the major proinflammatory cytokines. It acts on a variety of cells, including immune-competent cells and hematopoietic cells, causing their proliferation and differentiation [11]. The overproduction of IL-6 may be associated with many clinical symptoms observed in inflammatory immune-mediated diseases such as during leishmaniasis [12].

Based on these findings, IL-6 was thought to be a valuable and attractive therapeutic target for drug discovery and some research groups have developed anti-IL-6 receptor antibodies for the treatment of patients with inflammatory autoimmune diseases.

Based on these considerations, much attention has to be given to the response of the immune system during parasitic infections considering immune-mediated associated diseases in the host and considering new targets for combined therapeutic approaches in such conditions.

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Review Article

Role of Macrophages in the Repair Process during the Tissue Migrating and Resident Helminth Infections

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The Th1/Th2/Th17 balance is a fundamental feature in the regulation of the inflammatory microenvironment during helminth infections, and an imbalance in this paradigm greatly contributes to inflammatory disorders. In some cases of helminthiasis, an initial Th1 response could occur during the early phases of infection (acute), followed by a Th2 response that prevails in chronic infections. During the late phase of infection, alternatively activated macrophages (AAMs) are important to counteract the inflammation caused by the Th1/Th17 response and larval migration, limiting damage and repairing the tissue affected. Macrophages are the archetype of phagocytic cells, with the primary role of pathogen destruction and antigen presentation. Nevertheless, other subtypes of macrophages have been described with important roles in tissue repair and immune regulation. These types of macrophages challenge the classical view of macrophages activated by an inflammatory response. The role of these subtypes of macrophages during helminthiasis is a controversial topic in immunoparasitology. Here, we analyze some of the studies regarding the role of AAMs in tissue repair during the tissue migration of helminths.

1. Introduction

Helminth infections are a worldwide public health and economic problem due to their high morbidity rather than mortality. These infections are associated with socioeconomic (poor hygiene), demographic (living in endemic zones), health (obesity, diabetes, and viral infections such as human immunodeficiency virus (HIV)), and biological (raw meat consumption, sex, age, and immune response) factors, among others [1].

The clinical manifestations are diverse and include self-limited diarrhea, respiratory symptoms such as cough, wasting syndrome, and anemia. In severe infections, some people develop asthma-like symptoms [2] and neurologic disorders when the pathogen has the ability to migrate into the brain, such as in neurocysticercosis by *Taenia solium* [3, 4] and *Toxocara canis* infection [5], or motor disorders such as those occurring in *Trichinella spiralis* [6] and *T. canis* infections [7]. The diversity of symptoms caused by helminths is related to the organs they migrate to during their life cycle, such as the lung (*Ancylostoma duodenale*, *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Brugia malayi*, *Dirofilaria immitis*, *T. canis*, *Schistosoma mansoni*, *Echinococcus granulosus*, and *Nippostrongylus brasiliensis*) [2], gall bladder and liver (*Schistosoma* sp., *Toxocara* sp., and *A. lumbricoides*), muscle (*T. canis*, *T. spiralis*) [6, 7], and brain (*T. canis*, *Taenia* sp.) [3, 4].

In contrast to the prevailing consensus, many helminth infections are not permanent residents of the bowels; instead, they have the ability to migrate through different organs or persist at a location for weeks, months, or years. During their migration, helminths secrete proteolytic enzymes, such as serine proteases and cysteine proteases, with fibrinolytic

effects that cause the disruption of cell junctions and the extracellular matrix, enabling entry to different organs [8–10] and causing tissue damage along the path of the migration.

2. Tissue Damage and Repair or Healing

When the larvae of helminths migrate through the host's body and destroy cells and tissues in their path, these cells and damaged tissue must be rapidly repaired. To achieve this, the host must turn on a series of coordinated biochemical and cellular events focused on the replacement of the destroyed tissue with living and functional tissue. Such a process is called tissue repair or tissue healing [11] and includes at least 4 events: bleeding, inflammation, proliferation, and remodeling (Figure 1) [12]. Bleeding and inflammation are early events that occur over hours or a few days, whereas proliferation and remodeling take weeks or even months. Vasculature changes such as vasodilatation and vasopermeability are rapidly induced to recruit cells to the site of damage, where prostaglandins and serotonin play an essential role. Once sufficient cell populations arrive at the site of the damaged tissue, the inflammatory stage ensues, with mast cells, neutrophils, and platelets as well as inflammatory $Ly6C^{hi}$ monocytes and resident macrophages playing an important role in containing bleeding and initializing the first steps of bleeding control. Some of these cells (neutrophils and macrophages) have strong phagocytic activity and help to remove debris in the wound, an essential step in the repair process. This stage is mainly driven by macrophages and fibroblasts, which become activated and initiate the production or expression of different molecules such as platelet-derived growth factor (PDGF). PDGF induces fibroblast proliferation and promotes fibrogenesis, collagen precursors, and integrins that help in the communication among the extracellular matrix, inflammatory cells, fibroblasts, and parenchymal cells [12, 13]. Here, macrophages play a critical role as providers of many of the molecules necessary for tissue repair, including arginase-1, transforming growth factor- (TGF-) β, FIZZ-1 (found in inflammatory zone), and fibrin, which are involved in fibrosis by inducing myofibroblast differentiation, key elements in collagen (mainly type III collagen) and fibrin deposition [14]. Another cell population recently implicated with the repair processes in the lungs and intestines are the type 2 innate lymphoid cells (ILC2s), which are a source of cytokines and other components that participate in the early steps of the healing process [15]. This inflammatory stage must be mainly acute (1–4 days) to avoid excessive collagen accumulation and fibrosis, elements that may alter tissue architecture. Thus, these early events prepare the tissue for the next step in healing-proliferation. The proliferation of fibroblasts is a key event in this stage because they will migrate to the damaged area from similar neighboring tissue. Here, macrophages again play a primary role as providers of macrophage-derived growth factors (including fibroblast growth factor) that activate and induce proliferation in fibroblasts to provide new cells to repopulate the affected tissue [16]. The final and longest step in the repair and healing of the tissue consists of remodeling, which involves the reorganization of collagen fibers, which are "weak or fine" fibers occurring during the inflammation and proliferation stages. However, in the remodeling step, these fibers become strong because collagen type III is replaced with collagen type I, with more cross-links, as it enters into a reorientation process [14]. Thus, collagen synthesis continues during this final step to "mimic" the damaged tissue as much as possible indicating that tissue repair is a dynamic and long-lasting process.

3. Immune Activation by Helminths

In addition to proteolytic enzymes, helminths generate immunomodulatory antigens that induce predominantly Th2-biased responses. This type of immunity is characterized by the production of different immunoglobulin (Ig) subclasses, such as IgE, IgG1, and IgG4, as well as interleukins, IL-4, IL-13, IL-5, and IL-10, which benefit the expansion of diverse cellular subpopulations such as eosinophils, mast cells, helper T cells, and alternatively activated macrophages (AAMs). The interactions among these different cell types and antibodies promote allergy and hypersensitivity, which are related to the increase in vascular permeability, angiogenesis, cellular recruitment, smooth muscle contraction, mucus secretion by goblet cells, and collagen deposits, which together are important mechanisms of defense against invasive helminth infections [17].

Another cell population associated with these parasites is the $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs), which are essential to maintain immune homeostasis and prevent autoimmunity; however, they are also involved in the control of T_H1 and T_H2 immune responses in some infectious diseases. In fact, several reports have confirmed that an increase in Treg numbers is associated with different helminth infections, where they play a dual role. Such an increase in the regulatory cells appears to be permissive for parasites, whereas these high numbers of helminth-induced $Foxp3^{+}$ Tregs dampen potentially pathogenic inflammatory responses or exacerbate Th2 responses in the tissue where the helminth has been allocated. Such conclusions were obtained by removing, stimulating, or transferring $Foxp3^{+}$ Tregs using different experimental designs [18].

More recently, other cell populations were related to helminth infections, ILC2s. This cell population comprises a limited number of cells but appears to play an important role in both protective and repair processes in mucosal tissues. They are classified into two different populations: natural ILC2s (nILC2s) and inflammatory ILC2s (iILC2s). ILC2s have been found in the steady state in many organs, such as the lungs, liver, spleen, intestinal lamina propria, skin, bone marrow, and adipose tissue. These cells are stimulated by thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 and after helminth infection produce effector cytokines, such as IL-4 and IL-13, and provide an important source of Th2-type cytokines. Thus, this process triggers the cell recruitment of eosinophils and macrophages, increasing the production of IL-13, IL-5, and IL-9, mucus production by goblet cells, muscle contraction, mastocytosis, tissue repair, and metabolic homeostasis [19]. The repair process by ILC2s is mostly associated with the early activation of AAMs, which can trigger the repair process via arginase-1 (Arg-1).

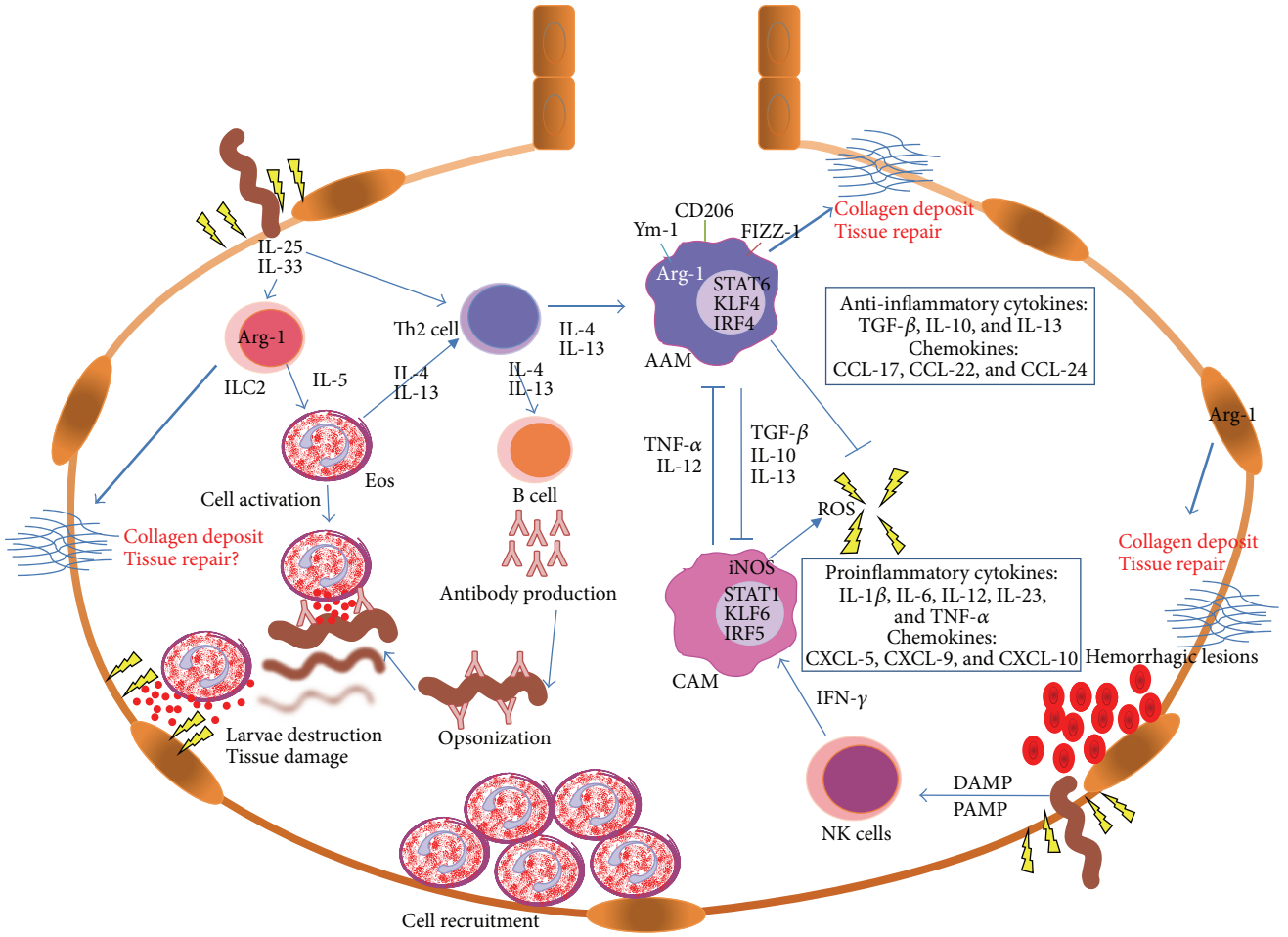

Figure 1: Immune response in tissue helminth infection. Lung tissue is usually affected during helminth migration; therefore, many repair mechanisms have been described for pulmonary tissue. The immune response is triggered when helminths disrupt the epithelial barrier. Helminths are a source of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs), which activate various cells such as NK cells, epithelial cells, and innate lymphoid cells (ILCs). The production of IL-25 and IL-33 also activates ILCs, which are a main source of IL-5 that is important in eosinophil (Eos) activation. Eos bind antibodies linked to the parasite surface and release their intracytoplasmic enzymes during the acute phase of the infection, allowing parasite elimination; however, the surrounding tissue is also damaged. IL-25 and IL-33 also activate T helper lymphocytes type 2 (Th2), which in turn secretes IL-4 and IL-13, which promote B cell activation, antibody production, and the induction of alternative activated macrophages (AAMs). AAMs have two important mechanisms to decrease tissue damage. First, they inhibit the cytotoxic effect produced by classically activated macrophages (CAMs). Second, they produce enzymes, such as arginase-1 (Arg-1), that promote collagen production and deposition on damaged tissue, therefore restoring the function lost during CAMs and parasite-induced injury. AAMs also produce various cytokines (IL-10 and TGF-β) and chemokines (CCL-17, CCL-22, and CCL-24) and express markers such as YM-1, FIZZ-1, and MMR. On the other hand, CAMs are activated through IFN-γ production by natural killer (NK) cells and produce proinflammatory cytokines (IL-1β, IL-6, IL-12, IL-23, and TNF-α) and chemokines (CXCL-5, CXCL-9, and CXCL-10) and express iNOS that produces reactive oxygen species (ROS) and causes tissue damage. It is important to notice that although AAMs are fundamental in tissue repair, other cells such as ILCs and epithelial cells, which constitutively express Arg-1, may aid in tissue repair and produce collagen deposits in the damaged tissue.

However, a recent report has indicated that ILC2s can produce this enzyme both under basal conditions and in response to helminth infection; thus, ILC2s have the potential to participate in tissue repair [20].

During helminth infections, cytokines are essential to activate various cell types, including eosinophils, which are involved in the mediation of most helminth infections [21]. Eosinophils are mainly activated by IL-5 and are an important source of IL-4 and IL-13, which enable the activation of Th cells, AAMs, and mast cells. In addition to cytokine production and cell activation, eosinophils can neutralize and eliminate tissue parasites through the secretion of granules that contain proteins, such as eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil derived neurotoxin (EDN), and eosinophil cationic protein (ECP) (Figure 1) [22].

These parasite-killing mechanisms have been described in multiple studies. For example, Masure et al. measured the survival of *Ascaris suum* second-stage larvae in the presence of eosinophils, and they observed an important reduction in larvae survival associated with the degranulation

of eosinophils. The authors concluded that eosinophils are important immune cells in the defense against *A. suum* invasive larvae [23]. Other experimental models confirmed the protective role of eosinophils against multiple helminths, including *Strongyloides stercoralis* [24], *N. brasiliensis* [25], and *Heligmosomoides polygyrus* [26]. Nevertheless, during *T. canis* and *T. spiralis* infection, eosinophils do not seem to have such a protective role. For instance, in an *in vitro* model, Rockey et al. observed that eosinophils could attach to *T. canis* larvae and secrete granules; however, the larvae could separate from their sheaths and move away from eosinophils [4]. In another study, Takamoto et al. did not find a difference in the larvae burden of IL-5 deficient mice (characterized by having 3-fold lower circulating numbers of eosinophils), although eosinophils in WT mice were increased tenfold in the bone marrow and twenty-seven-fold in peripheral blood, and concluded that eosinophils do not play an important role in the clearance of *T. canis* larvae [27]. Another study using *T. spiralis* reported similar findings [28], suggesting that eosinophils do not enhance protective immunity also against this nematode.

Although it is clear that eosinophils play an important role in the protection against most helminths through the secretion of effector proteins, paradoxically, the proteins they release are sometimes harmful to the surrounding host tissues [29–32]. Evidence of this side effect was demonstrated in a model of *T. canis* infection, in which BALB/c mice were transfected with a plasmid encoding the IL-12 gene (pcDNA-IL-12) that inhibits the recruitment of eosinophils. The authors showed that transfected mice displayed reduced airway inflammation associated with a reduced eosinophilic infiltrate in the lungs and an increase in the Th1-type immune response characterized by elevated amounts of IL-12 and interferon-γ (IFN-γ) in this tissue [33]. In line with this idea, recently, a double-edged sword effect of eosinophils was demonstrated in experimental neurocysticercosis caused by *Mesocestoides corti*. Here, the authors used eosinophil-deficient mice to show that eosinophils are important cells for reducing the parasite load in the brain; however, these cells also intensify tissue damage and consequently worsen the disease outcome with more severe pathology [34].

Thus, after the damage to the host caused by helminth migration and immune cell activation, it is imperative that the tissue repair type 2 immune response plays a direct role in wound healing through the production of mediators that directly enhance the tissue repair process and through the control of inflammation, in which AAMs appear to have a central role [35]. However, a type 2 immune response takes several days; thus, a rapid mechanism for tissue repair is imperative early during the infection by helminths, when AAMs and their products appear to be crucial. In this regard, innate immune cells such as ILC2s may participate as an early source for IL-4 and IL-13 to rapidly induce AAMs.

4. Repair and Damage Mechanisms of Macrophages

Macrophage polarization to AAMs is related to the Th2 immune response and associated AAM cytokines, such as IL-4 and IL-13. Furthermore, diverse transcription factors, such as PU.1, signal transducer and activator of transcription 6 (STAT6), Kruppel-like factor (KLF) 4, and interferon regulatory factor (IRF) 4, are related to this type of macrophage [36]. Particularly, AAMs can be distinguished by their expression of diverse molecular markers, including the enzyme arginase-1 (Arg-1), members of the chitinase family (YM-1, YM-2, and AMCase), resistin-type molecules (FIZZ-1/Retnla/Relm-α, FIZZ-2/Retnlb/Relm-β, FIZZ-3/Retn/resistin, and FIZZ-4/Retnlg/Relm-γ), and TGF-β and mannose receptor (MMR/CD206) [37].

Moreover, classically activated macrophages (CAMs) are induced by Th1 immune responses, wherein IFN-γ plays a crucial role, and the transcription factors STAT1, KLF6, and IRF5 are implicated in their activation [36]. In contrast to AAMs, CAMs have enhanced antimicrobial actions mediated by the secretion of molecules such as nitric oxide (NO) and reactive oxygen species (ROS) that are essential for the destruction of intracellular pathogens (bacteria, viruses, and protozoan parasites). Additionally, CAMs are characterized by the production and secretion of proinflammatory cytokines, such as tumor necrosis factor- (TNF-) α, IL-12, and IL-1β [38]. Another difference between AAMs and CAMs is the expression by AAMs of Arg-1, which functions to metabolize L-arginine into L-ornithine. By contrast, CAMs use L-arginine to synthesize L-citrulline through inducible nitric oxide synthase (iNOS), producing NO and ROS, both mediators of their cytotoxic activity against intracellular pathogens and tissue damage [38].

5. Alternatively Activated Macrophage Functions

As previously mentioned, if macrophages are stimulated by IFN-γ, L-arginine is metabolized by iNOS, and the main cytokine induced is TNF-α or IL-12, macrophages will be classically activated. However, if there is predominance of Th2 cytokines, such as IL-4 and IL-13, there will be more AAMs that express Arg-1. Hence, AAMs expressing Arg-1 produce L-ornithine that can be metabolized into L-proline through ornithine aminotransferase (OAT), and L-proline is essential for collagen synthesis and tissue repair and regeneration [39]. AAMs also produce other elements involved in tissue repair, such as TGF-β and PDGF, which induce fibroblast proliferation and promote fibrogenesis and collagen production [40].

Another protein yielded by AAMs is YM-1, a member of the family of mammalian proteins that share homology to chitinases, which can bind chitin without chitinase activity. This protein has been associated with cellular recruitment and extracellular matrix deposition during tissue repair. Furthermore, FIZZ-1 is secreted in high amounts during inflammation, and it has been observed that diverse cells express this protein, including pneumocytes, alveolar epithelial cells, and macrophages. FIZZ-1 is also involved in fibrosis by inducing myofibroblast differentiation, key element in collagen and fibrin deposits [37, 41, 42]. Another immune factor related to AAM activation is antibody production. It has been described that some subclasses of antibodies

can reprogram macrophage gene expression and induce the production of repair-related molecules [43].

6. Alternatively Activated Macrophages in the Repair Process in Diverse Pathologies

The presence of AAMs in the repair process has been extensively studied in various pathologies where they play an active role and could be beneficial or harmful depending on the pathology. In a collagenase-induced intracerebral hemorrhage (ICH) mouse model, an increasing number of CX3CR1 macrophages were revealed to have AAM markers. When these macrophages were depleted, an increase in the ICH lesion volume was observed, and neurological deficits were more severe compared to those of control mice, indicating a protective role of these macrophages in ICH. From such data, the authors concluded that brain-infiltrating macrophages after ICH are polarized to the AAM phenotype, thereby contributing to recovery from such injury [44].

In asthmatic airway inflammation, the presence of AAM activated via PU.1 has a harmful effect on promoting the pathological progress of asthmatic airway inflammation. Such an effect was measured in conditional PU.1-deficient ($PU/ER(T)^{+/-}$) mice in response to the challenge of DRA (dust mite, ragweed, and *Aspergillus*) allergens, displaying attenuated allergic airway inflammation, decreased alveolar eosinophil infiltration, and reduced IgE production, changes associated with decreased mucus glands and goblet cell hyperplasia. To prove that AAMs were involved in the asthmatic airway pathology, macrophages from wild-type (WT) mice were differentiated with IL-4 and transferred to $PU/ER(T)^{+/-}$ mice showing an increase in asthmatic airway inflammation. When the mice were treated with tamoxifen to rescue PU.1 function, the pathology was worsened compared with that in mice transferred with macrophages. The data indicated that PU.1 plays a critical role in AAM polarization and, indeed, in the exacerbation of asthmatic inflammation pathology [45].

Other pathologies such as obesity and resistance to insulin are closely associated with inflammation. Obesity causes increased classical and decreased alternative macrophage activation, which in turn induces insulin resistance in target organs, as observed in a study where A_{2B} adenosine receptor (AR) activation results in important regulators of macrophage activation. A_{2B} AR deletion results in impaired glucose and lipid metabolism associated with increased inflammatory classical macrophage activation and inhibition of anti-inflammatory alternative macrophage activation. The expression of AAM transcription factors was also decreased in the adipose tissue of A_{2B} ARs-deficient mice, indicating that AAMs may play an important role in obesity and insulin resistance, and therapeutic strategies targeting A_{2B} ARs could be a preventive therapy for those pathologies [46].

In a recent report, the role for AAMs has also been highlighted in the reparative processes after myocardial infarction in adult mice, where higher numbers of AAMs were recruited to the infarcted area; such mechanism was IL-4-dependent [47].

In general, in pathologies different from those generated by tissue migrating or resident helminths, the presence of AAMs in the repair process is beneficial to counteract the effects of CAMs associated with a Th1 inflammatory immune response, with the exception of asthma where their presence seems to be more harmful than beneficial.

7. Role of Macrophages during Tissue Migrating and Resident Helminths

7.1. Nippostrongylus brasiliensis. This nematode, like many others, has a tissue migration phase in which it migrates throughout the lungs, causing alveolar hemorrhage and inflammation. The role of the immune response during acute lung injury caused by *N. brasiliensis* has been studied (Table 1). In the experimental model using BALB/c mice, it was observed that IL-17 contributes to the inflammation. On the other hand, an increase in IL-4 receptor activation causes the reduction of IL-17 and enhances the expression of insulin-like growth factor-1 (IGF-1) and IL-10, with consequent AAM activation and tissue repair. These data highlight the essential role of Th2 cytokines and AAMs in limiting lung damage [48].

Another study using FIZZ/Retnla gene KO mice, a Th2-inducible gene, showed that there was greater lung and liver damage in $Retnla^{-/-}$ mice infected with *N. brasiliensis* or *S. mansoni*, concomitant with exacerbation of fibrogenesis and increased IL-4 and IL-13 production, which in turn reduced parasite burden. These data suggest that the Retnla gene downregulates the Th2 immune response and suppresses resistance to nematode infection, granulomatous inflammation, and fibrosis [49].

The role of YM-1 during *N. brasiliensis* infection was also investigated. Sutherland et al., using neutralizing antibodies against YM-1 in an *in vivo* model, observed that YM-1 neutralization caused a decrease in neutrophils from bronchoalveolar lavage and lung tissue at 2 and 4 days after infection, followed by less inflammation but an increase in macrophages that was associated with lung healing [50]. Thus, even in the absence of YM-1, AAMs can still fulfill their repair function, and YM-1 appears to be implicated in neutrophilia and acute lung damage. However, other studies have confirmed that neutrophils are key elements in parasite neutralization and mediators of repair through AAM activation [51].

Other cells associated with tissue repair during *N. brasiliensis* infection include ILC2s, which are mainly induced by IL-25 and IL-33 [19] and to a lesser extent by IL-9, and appear to play an autocrine role amplifying ILC2s. The study was carried out on a model of $IL\text{-}9R^{-/-}$ mice, in which $IL\text{-}9R^{-/-}$ mice showed a significant decrease in ILC2s, IL-5, IL-13, and amphiregulin (a member protein of the epidermal growth factor family that promotes bronchoalveolar epithelium repair). Such a decrease was correlated with deficient tissue repair in the absence of IL-9. The tissue repair deficiency observed in this experimental setting was associated with a decrease in AAM markers, particularly Arg-1, Retnla, and YM-1, suggesting that ILC2s may induce AAMs

Table 1: Alternatively activated macrophages (AAMs) in the repair process during tissue migration and resident helminthes.

Helminth	Experimental model and mice strain	AAMs role	Ref.
Nippostrongylus brasiliensis	BALB/c	IGF-1 production is increased by IL-4, inducing AAM activation followed by tissue repair	[48]
	FIZZ/Retnla$^{-/-}$	The Retnla gene downregulates the Th2 immune response and suppresses resistance to nematode infection, granulomatous inflammation, and fibrosis	[49]
	BALB/c	In the absence of YM-1, AAMs can still fulfill their repair function	[50]
	BALB/c	Neutrophils are key elements in parasite neutralization and are mediators of repair through AAM activation	[51]
	IL-9$^{-/-}$	ILC2s may induce AAMs, which mediate adequate tissue repair	[52]
	STAT6$^{-/-}$ and Arg-1^{flox}	ILC2s constitutively express Arg-1, and they can repair lung tissue during acute inflammation in the absence of AAMs	[20]
Toxocara canis	STAT6$^{-/-}$	STAT6 absence may cause delayed wound healing by the reduction of the AAM population	[54]
Schistosoma mansoni	LysM^{cre}IL-4$^{-/flox}$ and IL-4$^{-/-}$	Polarization to the Th1 immune response, associated with CAM activation and NOS production, is related to hepatic damage and death	[57]
	IL-4R$\alpha^{flox/\Delta}$LysM^{Cre}	AAMs are necessary for pathogenic Th1/CAM suppression	[58]
	FLH$^{-/-}$	The absence of FHL induces Th1 polarization, which is associated with an increase in granuloma hepatic formation	[62]
	Arg-1$^{-/-}$	Arg-1 production by AAMs may play an important role in *S. mansoni* infection control and diminish intestinal damage	[63]
Heligmosomoides polygyrus bakeri	$J_H{}^{-/-}$ and FcR$\gamma^{-/-}$	Antibodies cause AAM activation, enhancing the expression of genes associated with the repair mechanisms	[43]
	C57BL/6 and Cx3Cr1$^{GFP/+}$	Infection increases the expression of Ym-1, RELM-α, and CD206, enhancing collagen deposition and fibrosis in heart tissue	[68]
Trichinella spiralis	Ob/ob and C57BL/6	AAMs mediate inflammation in adipose tissue, insulin resistance, and glucose control	[69]
	BALB/c	The rTsP53 protein appears to be beneficial during colitis, with an increase in AAMs	[70]
Taenia crassiceps	BALB/c	AAMs have immunomodulatory effects in experimental colitis and colon cancer models	[71, 72]
	C57BL/6	A decrease in inflammation and EAE symptoms is associated with AAM activation	[73]
Trichuris muris	Arg-1$^{flox/flox}$, Tie2-cre, and C57BL/6	Arg-1 is dispensable for tissue repair, but its absence is not related to damage	[74]

to mediate the tissue repair process [52]. Nevertheless, there is recent evidence indicating that ILC2s constitutively express Arg-1 [20]. Moreover, Monticelli et al. demonstrated that ILC2s are the major source of Arg-1 in the lungs even more than alveolar macrophages in basal conditions; however, their role in lung inflammation is controversial [15].

7.2. Toxocara canis. Toxocariasis is a worldwide zoonotic parasitic disease caused by the nematode *T. canis*. In humans, the infection is caused by accidental ingestion of embryonated eggs from contaminated soil: the eggs hatch, and the liberated larvae migrate to different organs, producing various disorders. Murine models have shown the presence of transitory hemorrhagic pulmonary lesions associated with strong Th2 responses and heavy parasite burdens. During *T. canis* infection, there is predominance of a Th2 immune response, characterized by the production of IL-4, IL-5, IL-13, and immunoglobulin subclasses IgG1 and IgE, as well as an increase in peripheral blood eosinophils and eosinophilic granuloma in the lung and liver [53].

Although *T. canis* can migrate through diverse organs and the immune response described during this infection is prone to induce AAM, there are very few studies assessing their role in tissue repair. Only one study has evaluated the possible role of AAM markers during this infection. In this study, STAT6$^{-/-}$ and WT mice were challenged orally with *T. canis* larvae, where longer persistence of hemorrhagic pulmonary lesions and inflammation in STAT6$^{-/-}$ mice was observed to be associated with a weak Th2 immune response as a consequence of the inhibition of the STAT6 signaling pathway. By contrast, WT mice displayed strong Th2 immune responses, associated with high levels of IgG1, IgE, and IL-4 and the presence of AAM markers in lung tissue. Additionally, these WT mice resolved the lung lesions faster than STAT6$^{-/-}$ mice. Interestingly, STAT6$^{-/-}$ mice displayed significantly lower parasite loads. These data suggest that the severity in lung damage and persistence of lesions are associated with the absence of AAMs, as suggested for other helminth infections (Table 1) [54]. Strong inflammatory lung reactions in human toxocariasis have been well described, which may trigger chronic hypersensitivity mediated by an eosinophilic environment and granulomatous inflammation. Eosinophilic granuloma enables pathogen neutralization; however, it may have deleterious effects on the host, and AAM may play an important role in decreasing inflammation and favoring tissue repair, although its role in toxocariasis is yet to be determined [33, 55, 56]. Similarly, studies on the role of ILC2s in tissue repair during toxocariasis are lacking.

7.3. Schistosoma mansoni. AAMs are an essential cell type during schistosomiasis (Table 1), which are involved in the reduction of tissue inflammation and associated injury triggered by *S. mansoni* eggs deposited in liver tissue. To prove the role of this cell type, Herbert et al. used an experimental model of LysM^{cre}IL-4$^{-/flox}$ and IL-4 deficient mice, both with impaired activation of AAMs and with the enhanced ability to induce CAM expressing iNOS2. The authors also observed that WT mice had smaller liver granulomas and higher expression of Arg-1 than LysM^{cre}IL-4$^{-/flox}$ and IL-4 deficient mice [57]. Similar results were published by Vannella et al. using IL-4R$\alpha^{flox/\Delta}$LysM^{Cre} mice, concluding that AAMs are necessary to suppress pathogenic Th1/CAM responses without a significant impact on fibrosis, although fibrosis was slightly higher in IL-4R$\alpha^{flox/\Delta}$LysM^{Cre} mice [58].

By contrast, previous studies have shown that IL-4 and IL-13 (Th2-type response) may play dual roles in lung granuloma formation, which is necessary for *S. mansoni* egg containment, suggesting that Th2 immune responses may produce tissue damage rather than repair. For example, IL-13 induces tissue eosinophilia and high levels of IgE enhancing lung granuloma formation, whereas IL-13 blockage in mice was accompanied by changes in eosinophil accumulation and reduced granuloma size [59]. This is in line with another study that has established the fact that IL-13 exhibits chemotactic activity for human eosinophils; therefore, schistosome granulomas are rich in eosinophils [60]. Thus, damaged tissue is associated with eosinophil recruitment without participation of AAM, although both cell types are part of the Th2 immune response [59]. However, it has been recently observed that AAMs are also important in maintaining local Th2 responses in general and IL-13 production in particular during *S. mansoni*-induced granuloma formation, as demonstrated by partial AMM depletion that overall reduces lung fibrosis and pulmonary inflammation, as described by Borthwick et al. [61].

In an *in vitro* study, using bone marrow macrophages differentiated with IL-10 and IL-4 to AAMs, upregulation of FHL2 (a protein structural domain, also called LIM) was observed. However, when the bone marrow macrophages from FHL2$^{-/-}$ mice were similarly stimulated, the AAM genes were downregulated, and CAM markers seemed to be upregulated, proving the expression of FHL2 induced in mouse marrow-derived macrophages following stimulation with AAM-inducer cytokines. To prove the importance of FHL2 in AAM activation, FHL2$^{-/-}$ mice were challenged with *S. mansoni* showing higher numbers of granulomas and reduced expression of AAM markers, which correlate with an enhanced Th1 immune response. These data suggest a role for FHL2 in the pathogenesis of pulmonary granulomatous inflammation through AAM polarization and Th1/Th2 balance [62].

During intestinal schistosomiasis by *S. mansoni*, the use of *S*-(2-boronoethyl)-L-cysteine (BEC), an Arg-1, and Arg-2 antagonist, was related to impaired elimination of *S. mansoni* eggs that correlated with an increase in disease severity and mortality compared with that in nontreated mice. In the same study, now using Arg-1$^{-/-}$ mice, the authors observed hemorrhagic lesions in the intestinal mucosa that were not observed in WT mice. These data confirmed that Arg-1 production by AAMs is important for both *S. mansoni* infection control and reducing intestinal damage, and the absence of Arg-1 causes Th1 polarization associated with a proinflammatory cytokine profile [63].

Evidence that a decreased Th1 immune response and a reduced number of CAMs and therefore indirect stimulation

of AAM contribute to repair mechanisms has been shown in CD14 (a TLR-4 coreceptor) deficient mice. These mice have fewer and smaller hepatic granulomas and an increase in $CD4^+$IL-4, IL-5, IL-13^+, and $CD4^+Foxp3^+$IL-10^+ cells that correlate with collagen deposition and wound healing. This effect was associated with STAT6 signaling, suggesting that the absence of CD14 has an impact on the ILR4α/STAT6 pathway and macrophage polarization during infection [64, 65]. Moreover, the inhibition of cytokines or factors related to AAM polarization causes a decrease in the protective role of AMM. In conclusion, most data point out these cell types as vital for the successful repair of lesions during schistosomiasis [66, 67].

7.4. Heligmosomoides polygyrus bakeri. As mentioned above, antibodies also mediate AAM activation. This fact has been observed during *H. polygyrus bakeri* infection (Table 1). Esser-von Bieren et al. described that complement-dependent antibodies that bind to FcRγ cause macrophage adherence to *H. polygyrus* larvae *in vitro*, immobilizing the parasite, triggering macrophage reprogramming, and enhancing the expression of genes associated with repairing mechanisms. Such mechanisms were independent of the IL-4Rα signaling pathway, suggesting a different AAM activation mechanism [43].

Other studies have proven that cardiac resident macrophages, in the absence of infection, expressed classical (IL-1β, TNF-α, and CCR2) and alternative (Ym-1, Arg-1, RELM-α, and IL-10) markers. Nevertheless, during *H. polygyrus* infection, there is an increase in the expression of Ym-1, RELM-α, and CD206 and enhanced collagen deposition, causing fibrosis in heart tissue. Although *H. polygyrus* is a local migratory tissue parasite (which penetrates submucosal layer of the small intestine to the muscularis externa and later towards the lumen), polarization of AAM is induced by the immunologic activation of infection [68]. However, there is a lack of information regarding the role of AAMs in cardiac tissue repair. Although it has been suggested that fibrosis is a mechanism of tissue repair, it is still necessary to determine the positive or negative fibrotic effect on the heart during *H. polygyrus* infection. However, the idea that an intestinal helminth infection could have an effect on an organ as the heart is very interesting to explore.

7.5. Trichinella spiralis. During trichinellosis by *T. spiralis*, it has been reported that macrophages are important mediators of inflammation in adipose tissue, insulin resistance, and glucose control (Table 1). Therefore, the role of AAMs in obesity has been studied in the context of helminth infection. Using an experimental model of obese mice infected with *T. spiralis*, the induction of AAMs triggered by helminth infection led to decreased glucose intolerance and consequent lowering of the blood glucose levels which was associated with AAM markers such as Arg-1, CD206, and IL-10, as well as adipocyte death [69]. These results suggest that AAMs, which are induced by *T. spiralis* infection, have a beneficial role during obesity through the regulation of the inflammatory process in adipose tissue.

In other inflammatory diseases, such as colitis (Table 1), it has been observed that excretory/secretory (ES) proteins produced by these parasites have immunomodulatory effects. Among those ES proteins produced by *T. spiralis*, the recombinant 53 kDa protein rTsP53 was found to polarize the immune response to the Th2 phenotype. Using this protein during experimental colitis caused a Th2 immune response that correlates to reduced inflammation and the enhanced expression of the AAM markers Arg-1, FIZZ-1, TGF-β, and IL-10 [70]. These anti-inflammatory effects triggered by rTsP53 appear to be helpful in repairing tissue during colitis.

7.6. Taenia crassiceps. *T. crassiceps* is a cestode that has been extensively studied. These parasites induce a population of AAMs with suppressive activity and have been shown to have immunomodulatory effects on experimental colitis and colon cancer models (Table 1) [71, 72]. In this regard, AAMs play a central role in modulating both colonic inflammation and colitis-associated tumorigenesis. During experimental colitis, it has been shown that *T. crassiceps* infection induces the expression of Arg-1, YM-1, and FIZZ-1, which is related to increased collagen deposition in the intestine that does not cause fibrosis but diminishes intestinal inflammation and hemorrhage. Moreover, when AAMs isolated from *T. crassiceps*-infected mice were adoptively transferred to colitic mice, these cells could ameliorate ongoing colitis [71, 72].

A similar effect was observed in experimental autoimmune encephalomyelitis (EAE), in which the presence of *T. crassiceps* causes a decrease in inflammation and symptoms of encephalomyelitis with repair in the spinal cord. Such effects were associated with anti-inflammatory cytokine production and expression of AAM markers [73]; together, these data indicate that sometimes helminth infections generate improved side effects.

7.7. Trichuris muris. There is limited information regarding the role of AAMs during infection of the intestinal nematode *Trichuris muris* (Table 1), but it has been established that it induces a Th2 immune response and therefore induction of AAMs. The only study that has investigated the role of AAM during *T. muris* infection showed that Arg-1 is dispensable for tissue repair, and its absence was not related to more damage [74]. However, it was not determined whether other mechanisms associated with AAM or not were responsible for tissue healing.

8. Concluding Remarks

The type 2 immune response has evolved to direct the wound-healing machinery not only to repair and remodel tissue but also to mediate the containment, destruction, or expulsion of helminths. Both effects have been associated with the presence of AAMs; particularly, the issue related to tissue repair has also been related to other mechanisms independent of AAMs, such as ILC2s and epithelial cells from lung tissue that constitutively express Arg-1 for collagen production. Consequently, those cells could also be involved in tissue repair. Given such information, another way to

prove the importance of AAMs and other sources of collagen production in the repair process during helminth tissue migration could be depleting macrophages from lung tissue and measuring Arg-1 and collagen deposition associated with ILC2s or pulmonary epithelial cells. Even though there has been great progress in the understanding of AAMs functions and their role in tissue repair, a full depletion of AAMs from lung tissue has not been achieved, and there are still points of uncertainty and controversies that must be resolved in the future.

Competing Interests

The authors have no competing financial or commercial interests.

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Research Article

Immunopathological Features of Canine Myocarditis Associated with *Leishmania infantum* Infection

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Myocarditis associated with infectious diseases may occur in dogs, including those caused by the protozoa *Neospora caninum*, *Trypanosoma cruzi*, *Babesia canis,* and *Hepatozoon canis*. However, although cardiac disease due to *Leishmania* infection has also been documented, the immunopathological features of myocarditis have not been reported so far. The aim of this study was to examine the types of cellular infiltrates and expression of MHC classes I and II in myocardial samples obtained at necropsy from 15 dogs with an established intravitam diagnosis of visceral leishmaniasis. Pathological features of myocardium were characterized by hyaline degeneration of cardiomyocytes, necrosis, and infiltration of mononuclear inflammatory cells consisting of lymphocytes and macrophages, sometimes with perivascular pattern; fibrosis was also present in various degrees. Immunophenotyping of inflammatory cells was performed by immunohistochemistry on cryostat sections obtained from the heart of the infected dogs. The predominant leukocyte population was CD8+ with a fewer number of CD4+ cells. Many cardiomyocytes expressed MHC classes I and II on the sarcolemma. *Leishmania* amastigote forms were not detected within macrophages or any other cell of the examined samples. Our study provided evidence that myocarditis in canine visceral leishmaniasis might be related to immunological alterations associated with *Leishmania* infection.

1. Introduction

Canine leishmaniasis is a zoonotic disease caused by the protozoan parasite *Leishmania* spp. [1]. The disease has a worldwide distribution and is considered endemic in more than 70 countries, mainly distributed in Africa, Asia, Latin America, and Mediterranean regions [1, 2]. The domestic dog is confirmed to be the most important reservoir of human infection; in the Mediterranean region and New World, the one responsible for canine visceral leishmaniasis (CVL) in dogs is *Leishmania infantum* (syn: *L. chagasi* in the New World) [3, 4]. Even if congenital and sexual transmission have been demonstrated, the main route of transmission of the parasite among dogs, and from dogs to humans, is the bite of infected female phlebotomine sandflies [5, 6]. CVL is a multisystemic disease usually characterized by chronic progression with cutaneous and visceral clinical signs which become more and more evident as the infection progresses [7]. Enlargement of lymph nodes, dermal and ocular lesions, splenomegaly, pale mucous membrane, and weight loss are

the main clinical findings [8, 9], whereas the most common laboratory abnormalities are hypoalbuminemia, hyperglobulinemia, anemia, azotemia, and proteinuria [10, 11].

Canine myocarditis is a rarely diagnosed disease which can be caused by noninfectious (e.g., autoimmune reactions, toxins, trauma, and heat stroke) or infectious agents such as bacteria (i.e., *Staphylococcus*, *Streptococcus*, *Citrobacter*, *Bartonella*, and *Borrelia*), viruses (e.g., parvovirus), fungi (i.e., *Coccidioides*, *Cryptococcus*, and *Aspergillus*), and protozoa (*Leishmania*, *Toxoplasma*, *Hepatozoon*, and *Babesia*) [12–16]. Depending on the aetiology, myocarditis can have various histopathologic patterns. It is usually nonspecific and, although it is stated in the histopathologic examination, its direct cause can rarely be determined as discussed by Janus et al. [12]. The aim of this study is to evaluate the phenotype of inflammatory cells and define the immunopathological features of myocarditis associated with *L. infantum* infection in dogs.

2. Materials and Methods

2.1. Animals and Sampling. Fifteen crossbreed dogs, 8 males and 7 females, aged 7 to 11 years, living in an endemic area for *Leishmania* in southern Italy, were selected for the study. All dogs had an established intravitam diagnosis of leishmaniasis by serological and parasitological methods [17]. Moreover, laboratory abnormalities and clinical signs characteristic of visceral leishmaniasis were also found in all dogs.

The dogs were serologically negative for the main infectious agents responsible of myocarditis (*Ehrlichia canis, Toxoplasma gondii, Babesia canis, Rickettsia rickettsii, Leptospira, Borrelia burgdorferi,* and *Neospora caninum*) and did not show any other clinical signs of heart failure.

As a control, 3 crossbreed dogs, 2 males and 1 female, aged 7 to 11 years, living in the same endemic area of southern Italy, without clinical or laboratory evidence of leishmaniasis were used in the study; these dogs were serologically and parasitologically negative for *L. infantum* infection. Each animal used in the study died naturally or was humanely euthanized due to severe clinical conditions and poor prognosis and underwent full necropsy which confirmed the absence of concomitant diseases. Control group dogs died because of road accident trauma and underwent full necropsy which excluded the presence of any infectious or noninfectious disease.

At necropsy, specimens of myocardium, about 1 cm × 1 cm × 1 cm (L × W × H), were collected from the right atrium, ventricular free walls, and the interventricular septum as described by Rosa et al. [18]. Samples were frozen in isopentane precooled in liquid nitrogen and stored at −80°C.

2.2. Histopathology and Immunohistochemistry. Cryostat sections (5 μm thick) were stained with hematoxylin and eosin (H&E) for histopathological examination to assess a definitive diagnosis of myocarditis.

Immunohistochemical examination was carried out as previously described [19]. In brief, frozen myocardial specimens were sectioned (5 μm thick), dried at room temperature for 1 hour, fixed in acetone at 4°C for 5 minutes, and then blocked for endogenous peroxidase in 0.3% H_2O_2 in methanol solution for 20 minutes. Sections were incubated overnight at 4°C with primary antibodies against canine leukocyte antigens diluted in 0.01 M phosphate-buffered saline (PBS), pH 7.2–7.4, as follows:

(i) CD3 (mouse monoclonal antibody against canine CD3, T lymphocytes: from P. Moore, UC Davis) diluted 1 : 50.

(ii) CD4 (mouse monoclonal antibody against canine CD4, MHC class II-restricted cells, T, tissue macrophages: from P. Moore, UC Davis) diluted 1 : 50.

(iii) CD8α, CD8β (mouse monoclonal antibody against canine CD8α, CD8β, MHC class I- restricted cells; cytotoxic T lymphocytes: from P. Moore, UC Davis) diluted 1 : 50.

(iv) CD79α (mouse monoclonal mouse anti-human CD79α, B-linage cells clone HM57, DAKO A/S, Denmark) diluted 1 : 50.

(v) MHC I (mouse monoclonal antibody against MHC class I, clone H58A: from VMRD, Inc., USA) diluted 1 : 100.

(vi) MHC II (mouse monoclonal antibody against MHC class II, clone H34A, from VMRD, Inc., USA) diluted 1 : 50.

Slides were washed with PBS, then incubated with biotinylated secondary antibody, and labeled with streptavidin biotin for 30 minutes at room temperature, followed by incubation with streptavidin conjugated to horseradish peroxidase (LSAB Kit, DakoCytomation, Denmark). The reaction was revealed by diaminobenzidine treatment (DakoCytomation, Denmark) and finally, sections were counterstained with Mayer's haematoxylin. In the negative control sections, the primary antibody was either omitted or replaced with normal serum.

Approximately 20 fields at 20x magnification were evaluated for each section by two independent pathologists (AC, OP) with a concordance rate of 97%.

The inflammatory cell immunoreactions were scored as follows:

0 (not detected).

1 (percentage of immunoreactive inflammatory cells per section 1–25%).

2 (percentage of immunoreactive inflammatory cells per section 26–50%).

3 (percentage of immunoreactive inflammatory cells per section >50%).

2.3. Statistical Analysis. The relationship between the different types of infiltrating immune cells and MHC class I expression was evaluated using Spearman's Rho correlation (Past 3.10 software).

2.4. Ethics Statement. Necropsies were performed for diagnostic purposes after receiving the consent of the owner. Each owner consented to the use of tissues for research purposes, according to the internal rules of the Diagnostic Service of Pathology and Animal Health of the University of Naples Federico II. All the procedures were performed for diagnostic purpose; thus the study did not require any consent or ethical approval according to the European Directive 2010/63/EU.

3. Results

3.1. Noninfected Control Dogs. Myocardial samples from healthy control dogs, serologically and parasitologically negative for *L. infantum*, showed neither morphologic alterations nor inflammatory cells infiltrates. MHC classes I and II were expressed by endothelial cells of arterioles, venules, and capillaries.

3.2. Leishmania infantum Infected Dogs. At H&E stain all affected dogs (100% of studied cases) showed variable numbers of mononuclear cells, represented by lymphocytes and some macrophages, sometimes in a perivascular pattern (35.7%, 5/14). In some cases (14.3%, 2/14) a nonsuppurative granulomatous myocarditis, characterized by severe interstitial infiltration of mononuclear cells, was identified (Figure 1). Inflammatory cells infiltration was present in 71.4% (10/14) of the cases with cardiomyocytes hyaline degeneration and necrosis. Furthermore, we observed fibrosis in 9/14 (64.3%) cases; in 5 out of 9 cases (55.5%) fibrosis was mild and in other 4 cases (44.4%) fibrosis was moderate. In none of the studied cases *L. infantum* amastigotes were detected within macrophages.

Inflammatory cells phenotype was identified based on staining pattern of monoclonal antibodies against cell surface proteins. In all cases, independently of the severity and the pattern of inflammation, the predominant cell populations were CD3, CD8, and CD4 positive with predominance of CD8+ T cells (Figure 2(a)) compared to CD4+ cells (Figure 2(b)). Only in few cases were a small number of CD79α+ cells rarely detected within the inflammatory infiltrates. Vascular adventitia, endothelial cells, and cellular infiltrates within the myocardium stained intensely for MHC classes I and MHC class II antigens. In addition, many cardiomyocytes had MHC class I (Figure 2(c)) and class II positivity on the sarcolemma (Figure 2(d)).

A positive relationship was observed between the CD8 positive cells and MHC class I expression ($\rho = 0.854$; $P < 0.05$).

Results of the immunohistochemistry are summarized in Table 1.

4. Discussion

Canine myocarditis associated with *L. infantum* infection in dogs has been already described [20, 21]; however, the inflammatory pattern and its immunopathological features have never been fully investigated so far.

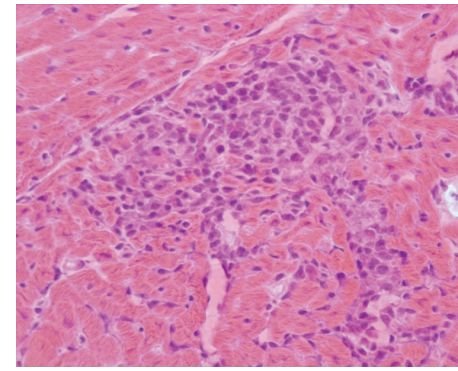

Figure 1: Myocardium, histopathological findings of a dog infected by *L. infantum*. Severe interstitial infiltration of mononuclear cells. H&E original magnification 40x.

Table 1: Immunohistochemical results: scoring of inflammatory cells immunoreactions and MHC classes I and II expression.

Dog #	CD3+	CD4+	CD8(α, β)+	CD79+	MHC I	MHC II
1	2	0	2	0	2	2
2	3	2	3	0	3	2
3	1	0	1	0	1	1
4	2	2	3	1	3	2
5	3	1	2	0	2	1
6	1	1	1	0	1	1
7	2	0	2	0	2	2
8	3	1	3	1	3	3
9	2	2	2	0	1	2
10	1	1	1	0	1	1
11	2	1	1	0	2	2
12	1	1	1	0	1	1
13	3	1	3	0	3	3
14	2	2	2	0	1	2
15	3	2	3	1	3	2

Scoring system applied for inflammatory cells immunoreactions: 0 (not detected); 1 (percentage of immunoreactive inflammatory cells per section 1–25%); 2 (percentage of immunoreactive inflammatory cells per section 26–50%); 3 (percentage of immunoreactive inflammatory cells per section >50%).
Scoring system applied for MHC classes I and II expression: 0: absent; 1: mild; 2: moderate; 3: intense.

Pathological changes of myocardium observed in our cases including degeneration and necrosis of cardiomyocytes and interstitial infiltration of mononuclear inflammatory cells represented by macrophages and lymphocytes confirmed findings of previously published reports [15, 18]. In none of myocardial samples *Leishmania* amastigotes were detected, and this aspect seems to be in accordance with the report of Alves et al. [16].

The predominant inflammatory infiltrate cell types were CD8+ T lymphocytes and macrophages; macrophages were distinguished from lymphocytes, at light microscopy, by morphological features and they were MCH immunoreactives. However, CD4+ T cells were also found. It is now well accepted that the progression of *L. infantum* infection in dogs,

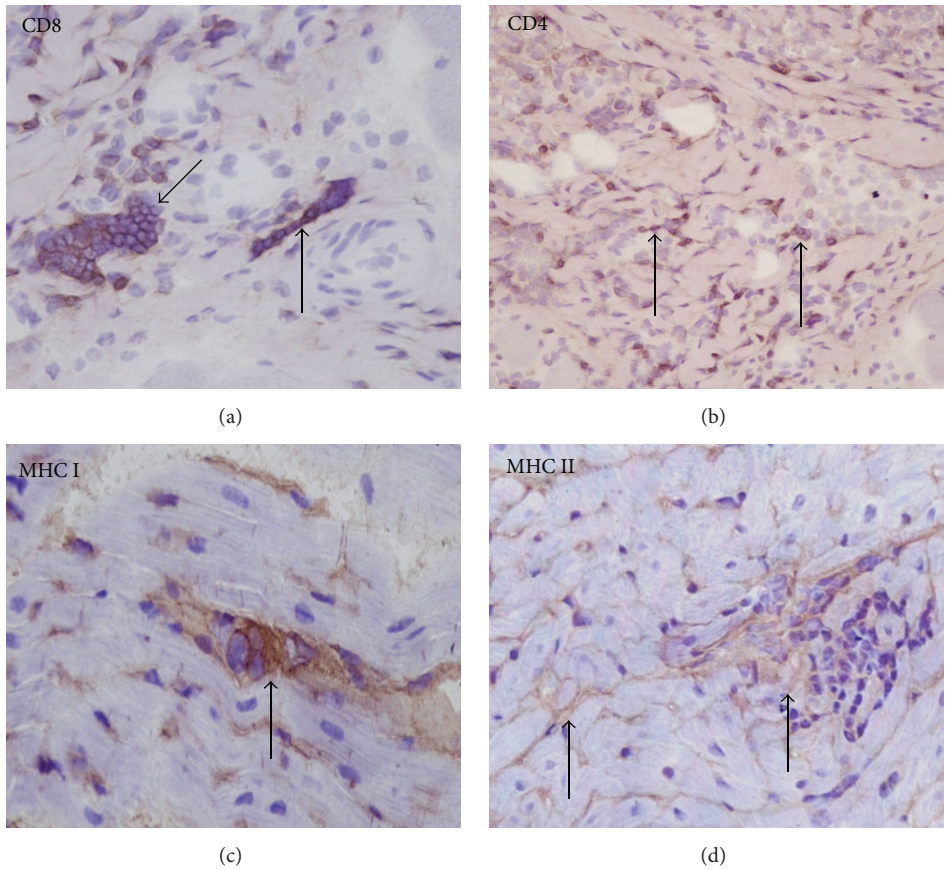

Figure 2: Myocardium, histopathological findings in a dog infected by *L. infantum*: (a) CD8 immunoperoxidase stain showing CD8+ T cells (arrows). (b) CD4 immunoperoxidase stain showing CD4+ T cells (arrows). (c) MHC class I immunoperoxidase stain showing abnormal positivity within cardiomyocytes (arrow). (d) MHC class II immunoperoxidase stain showing abnormal positivity within cardiomyocytes (arrows) (immunohistochemistry, HRP method, original magnification 40x).

notably the worsening or the regression of clinical signs, is the result of a multifactorial and complex interaction among the virulence of parasite, the environment (e.g., repeated bites by infected vectors), and the immune response of the host [22, 23]. The latter seems to play a key role, as *L. infantum* induces a mixed Th1 and Th2 response in CVL and the control of parasite replication, disease progression, or cure are strictly associated with the balance of these two patterns of immune system reaction [22]. The protective immunity against the parasite is mediated by CD4+ Th1 lymphocytes which release cytokines (γ-interferon, IL-2, and TNF-α) promoting macrophage anti-*Leishmania* activity through nitric oxide production that is responsible for parasite killing by apoptosis [24, 25]; moreover, macrophages infected by *Leishmania* amastigotes may also be lysed by CD8+ cytotoxic T lymphocytes even if this mechanism may be suppressed by the presence of high parasitic load [26, 27]. Contrarily, the Th2 humoral immune response, involving an increase of B cells and plasma-cells activity, is not protective and is associated with hyperglobulinemia and generation of autoantibodies, antihistone antibodies, and circulating immune complexes responsible for inflammation in almost every organ and tissue (e.g., glomerulonephritis, vasculitis, uveitis, polyarthritis, and myositis) [28–32]. The presence of both CD4+ and CD8+ inflammatory cells found in our study suggests that the dog immune system responds with a Th1/Th2 mixed response to *L. infantum* infection and this mechanism could be at the basis of myocardial injury and it was already demonstrated in canine inflammatory myopathy associated with *L. infantum* infection [19].

Detection of MHC classes I and II expression in cardiomyocytes was a common finding in the majority of samples from infected dogs [19]. Immunohistochemical detection of sarcolemmal MHC classes I and II is considered as a valid test for immune-mediated idiopathic inflammatory myositis in humans and dogs, in presence or absence of inflammatory cells infiltration [19]. Notably, MHC I and MHC II expression has been correlated to the active role of muscle fibers in antigen presentation and in initiating and maintaining pathological events in immune-mediated myositis [33–36]. CD8/MHC-I complex has already been described in other infectious and immune-mediated myositis of humans and dogs [19, 37, 38]. In dog the most common immune-mediated myopathies are masticatory muscles myositis, polymyositis, and dermatomyositis [39]. In 2009, Paciello et al. reported an immune-mediated inflammatory myopathy associated with

L. infantum infection [19]. Our data suggest that at least one pathologic mechanism resulting in myocardial inflammation in CVL can be an immune-mediated pathway as previously described in *L. infantum* infection associated myositis in dogs [28]. Furthermore, supporting this hypothesis, many parasites and viruses have been proposed as responsible factors of systemic diseases resulting in immune-mediated inflammatory myopathies in both humans and dogs [33, 34, 37].

5. Conclusion

Our data provided an initial antigenic characterization of infiltrating mononuclear cells and MHC classes I and II expression in myocarditis associated with *L. infantum* infection in dog. Our study provides evidence that during leishmaniasis myocarditis can occur with morphological and immunophenotypical pattern superimposable to canine myositis associated with *L. infantum* infection. Finally, our results, if confirmed on larger scale, could be used to improve therapeutic protocols for the management of dogs affected by leishmaniasis or to address the research towards new drugs useful to modulate immune-system response in order to reduce myocardial inflammation.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Histological Lesions and Cellular Response in the Skin of Alpine Chamois (*Rupicapra r. rupicapra*) Spontaneously Affected by Sarcoptic Mange

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Population dynamics of chamois (genus *Rupicapra*, subfamily Caprinae) can be influenced by infectious diseases epizootics, of which sarcoptic mange is probably the most severe in the Alpine chamois (*Rupicapra rupicapra rupicapra*). In this study, skin lesions and cellular inflammatory infiltrates were characterized in 44 Alpine chamois affected by sarcoptic mange. Dermal cellular responses were evaluated in comparison with chamois affected by trombiculosis and controls. In both sarcoptic mange and trombiculosis, a significantly increase of eosinophils, mast cells, T and B lymphocytes, and macrophages was detected. Moreover, in sarcoptic mange significant higher numbers of T lymphocytes and macrophages compared to trombiculosis were observed. Lesions in sarcoptic mange were classified in three grades, according to crusts thickness, correlated with mite counts. Grade 3 represented the most severe form with crust thickness more than 3.5 mm, high number of mites, and severe parakeratosis with diffuse bacteria. Evidence of immediate and delayed hypersensitivity was detected in all three forms associated with diffuse severe epidermal hyperplasia. In grade 3, a significant increase of B lymphocytes was evident compared to grades 1 and 2, while eosinophil counts were significantly higher than in grade 1, but lower than in grade 2 lesions. An involvement of nonprotective Th2 immune response could in part account for severe lesions of grade 3.

1. Introduction

Sarcoptic mange is a worldwide, highly contagious, burrowing mite infection in the skin of humans and animals, caused by *Sarcoptes scabiei* [1, 2]. Sarcoptic mange is responsible for epizootic disease in wildlife populations [2] and represents one of the most severe infections in Alpine chamois (*Rupicapra rupicapra rupicapra*, Linnaeus 1758). A study on chamois populations of Italian Alps [3] showed that the first impact of mange on naive host populations can be dramatic, with mortality rates of over 80%. After this mortality peak, populations tend to recover and the following peaks of the disease, generally 10–15 years after the first one, have a far less severe impact but could seriously affect fragmented populations with limited exchange with each other [2, 3]. Mortality in Alpine chamois has been used as a proxy of the sensitivity of different classes to the disease: no significant differences have been observed in mange-related mortality by gender, while an evaluation according to the age is still difficult due to the definitely lower probability of detection of smaller individuals (young chamois) in the field compared to the adult chamois in the framework of passive surveillance

Table 1: Scoring system used for classification of sarcoptic mange skin lesions determined by histologic assessment of skin sections from affected chamois.

Feature	Measure	Attributed scores			
		Absent (0)	Mild (1)	Moderate (2)	Severe (3)
Crusts	Thickness[a]	No crust	<2.5	2.5–3.5	>3.5
Alopecia	Percent of hair follicles containing hairs in histologic section	Normal hair	>55	45–55	<45
Mites	Average counts at 10x HPF[b]	0	1-2	3–6	>6
Eosinophils	Average counts at 40x HPF[c]	0-1	2–10	11–20	>20
Lymphocytes	Average counts at 40x HPF[c]	0	1–15	16–50	>50
Mast cells	Average counts at 40x HPF[c]	0	1–15	16–40	>40

[a]Thickness (mm) determined in eight randomly selected microscopic cross sections of skin. [b]Counts of mites in eight randomly selected fields. [c]Cell number in eight randomly selected fields. HPF: high-powered field.

[4]. On the other hand, mange cases show seasonality, being more frequent in late winter and spring [4, 5].

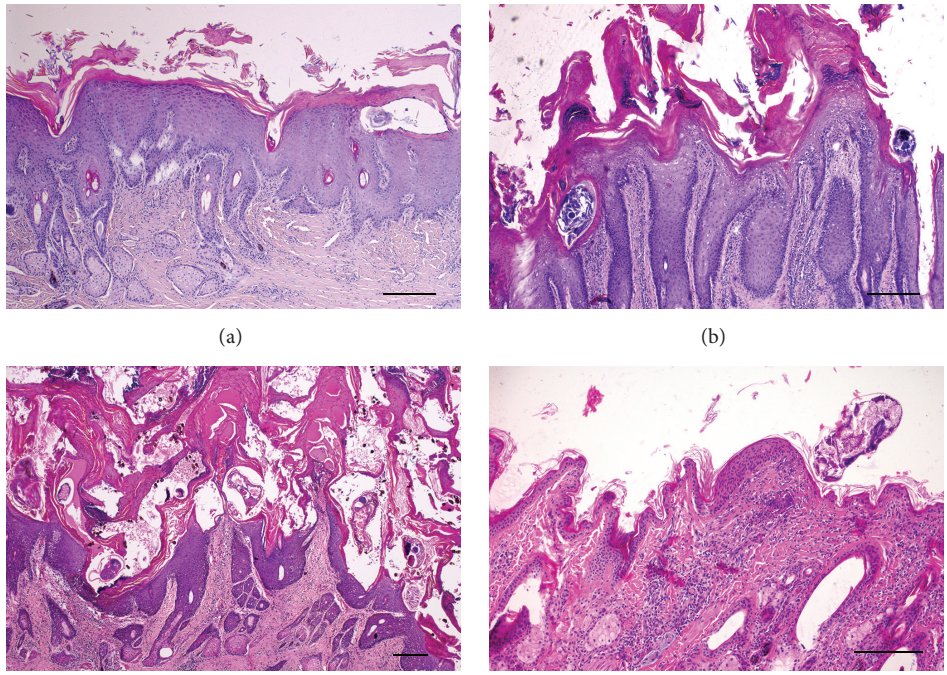
Sarcoptic mange skin lesions in wild and domestic animals are generally a combination of crusts and alopecia with dermatitis, orthokeratosis, and epidermal hyperplasia. However, severity and distribution of the lesions as well as the disease outcome vary among different host species and among individuals of the same species, probably due to different level of immune response and/or different clinical stages [1, 2]. Affected chamois showed clinical symptoms like restlessness, itch, and crusted alopecic skin, mainly involving head, neck, abdomen, and limbs [2, 3]. Histologically, orthokeratosis and parakeratosis, epidermal hyperplasia, and formation of crusts were observed. Vasodilation with perivascular and interstitial inflammatory infiltrates composed of lymphocytes, macrophages, eosinophils, plasma cells, neutrophils, and mast cells was detected suggesting a delayed hypersensitivity response [6, 7]. A study on *R. pyrenaica* suggested that sarcoptic mange infection elicits also a humoral immune response with increase of IgG levels in infected chamois associated with higher values of total proteins and gamma-globulin [8]. However, as in human scabies, it is uncertain whether increase of antibodies level can be specific or related to associated secondary bacterial infection [9]. In European wild ruminants, detailed histological features of sarcoptic mange have been described in Spanish ibex (*Capra pyrenaica hispanica*) [10], but *Sarcoptes* infection has been described also in ibex (*Capra ibex*) [3], Cantabrian chamois [7], Barbary sheep (*Ammotragus lervia*) [11], roe deer (*Capreolus capreolus*), and red deer (*Cervus elaphus*) [12]. An immunohistochemical study on formalin fixed skin specimens of normal and sarcoptic mange-infected chamois showed a progressive loss of cytokeratins in the epidermis and follicular epithelium in the orthokeratotic and parakeratotic form but failed to demonstrate reactivity of immune cells with a panel of anti-human antibodies [6].

In order to clarify the pathogenesis of dermal changes in Alpine chamois sarcoptic mange, the aims of this study were (1) to provide detailed histological features description of *Sarcoptes scabiei* var. *rupicaprae* infection in this species and (2) to evaluate dermal immune response in different forms of sarcoptic lesions. To evaluate and interpret inflammatory cells composition of mange cutaneous infiltrates, a group of chamois belonging to different districts and affected by trombiculosis was also evaluated.

2. Materials and Methods

2.1. Animals. Skin samples were collected from 75 Alpine chamois (*R. r. rupicapra*) ageing between one and thirteen years and culled during hunting seasons from 2013 to 2015. Three groups of chamois were selected: group 1 ($n = 44$) from Belluno province (46°53′N, 12°14′E) a mountainous area with altitudes ranging from less than 500 metres to over 3000 metres above the sea level (a.s.l.) affected by sarcoptic mange; group 2 ($n = 18$) from Lecchesi Alps and Pre-Alps hunting districts (45°59′N, 9°32′E), ranging from 300 to >2,000 m a.s.l. and from Val d'Ossola (46°07′N, 8°17′E) altitude ranging from 700 to 2,400 m a.s.l. affected by trombiculosis; group 3 consisting of 13 healthy chamois from all three previous districts. In each animal a 2 × 2 cm square skin sample was collected from affected skin or from lateral aspect of thigh in control animals. Samples were placed in a freshly prepared zinc salts fixative (ZSF) for 24–48 hours. All samples were processed routinely for paraffin embedding. Serial 5 μm sections from all specimen were stained for haematoxylin and eosin (HE) and Toluidine Blue (TB) and mounted on treated glass slides (SuperFrost Plus, Menzel-Glaser, Germany), respectively.

2.2. Histopathology. Histologic examination was performed on all 75 chamois. Characteristics of mange lesions were appreciated by describing crusting (thickness in mm), alopecia (percentage of hair follicle containing hairs in histologic skin sections), mites (counts at 10x HPF in three randomly selected fields), and dermal inflammatory cells infiltration using the scoring system proposed by Nimmervoll et al. [1] modified as proposed in Table 1. Data about the presence of bacteria in the crusts, orthokeratosis, parakeratosis, epidermal hyperplasia, spongiotic oedema, hypergranulosis, epidermal erosions, presence of pustule, sebaceous hyperplasia, and fibrosis were also recorded when observed.



(a) (b) (c) (d)

Figure 1: Histology of skin in sarcoptic (a: grade 1; b: grade 2; c: grade 3) and trombiculosis (d) affected chamois. Severe epidermal hyperplasia is evident in all grades of sarcoptic mange lesions with females within epidermal layers. Crusting with marked parakeratosis is progressively severe from grade 1 to grade 3. In grade 3 (c), crusts are associated with serum lakes, extravasated erythrocytes, and bacteria. Diffuse inflammatory infiltrates are evident in the dermis. Focal, mild epidermal hyperplasia is evident in trombiculosis (d) with a mite on the surface of the keratin layer and no crusts. Focal inflammatory infiltrates are evident in the dermis. Haematoxylin Eosin; bar = 100 μm.

2.3. Immunohistochemistry. Sections were dewaxed in xylene and rehydrated through graded alcohols prior to quenching endogenous peroxidase activity with H_2O_2 3% in methanol for 20 minutes. Immunohistochemical labelling was performed manually with the Sequenza slide rack and Coverplate system (Shandon, Runcorn, UK). Nonspecific antigen binding was blocked by incubation with 25% normal goat serum (code X0907, Dako UK Ltd., Ely, UK). Monoclonal anti-human CD3 (1 : 100, clone F7.2.38, Dako, UK Ltd., Ely, UK), CD79α (1 : 50, clone HM57, Dako, UK Ltd., Ely, UK), and CD68 (1 : 50, clone EBM 11, Dako, UK Ltd., Ely, UK) antibodies were applied to serial sections and incubated overnight at 4°C. Antibody binding was detected by the EnVision Plus System-HRP (DAB) (code K4007, Dako UK Ltd., Ely, UK) as indicated by manufacturer's instructions and slides were counterstained with haematoxylin. Substitution of the primary antibody with unrelated matched primary antibody was used to provide a negative control. Serial sections of chamois lymph node were used as positive control.

2.4. Cell Counting and Statistical Analysis. Four bright field images for each skin sample were acquired at ×20 magnification with a Leica Microsystem DFC490 digital camera mounted on Leica DMR microscope. Number of eosinophils, mast cells, CD3, CD79α, and CD68-positive cells were counted on eight 10,000 μm^2 random fields of each skin sample using a semiautomatic analysis system (LASV 4.3, Leica, Germany). Eosinophils were counted on HE sections and mast cells were counted on TB stained slides.

Statistical analysis was performed using the statistical package SPSS Advanced Statistics 21.0 (SPSS Inc., Chicago, IL, USA). ANOVA test was used to compare the composition of cell infiltrates detected in the skin of animals with sarcoptic mange, trombiculosis, and controls. Post hoc analysis was made by Bonferroni Test. Statistical significance was based on a 5% (0.05) significance level. The statistical correlation between mite counts and crust thickness was performed using the Pearson correlation test.

3. Results

3.1. Histopathologic Findings

3.1.1. Sarcoptic Mange. In all sarcoptic mange cases, variable crusting was observed (Figure 1). Crusts thickness, measured on histologic sections, ranged from mild to severe. Mild crusting (grade 1) was observed in 7/44 chamois (15.9%), while moderate crusting (grade 2) and severe crusting (grade 3) were detected in 17/44 (38.6%) and 20/44 animals (45.5%), respectively. Crusts, generally, containing a large number of mites and multifocal to diffuse colonies of bacteria, showed severe parakeratosis and moderate orthokeratosis, occasionally serum lakes, and extravasated erythrocytes. Generally, crust thickness increased with mite counts within the crusts

($r = 0.677$, $p = 0.000$) and cases with severe crusting showed higher presence of bacteria. Alopecia was commonly seen in chamois with severe crusting; however, correlation between crusting and alopecia was not significant.

In all mangy forms (all grades of crusting severity), moderate to very severe epidermal hyperplasia was observed with conspicuous, diffuse rete ridge formation. Diffuse spongiotic oedema of the epidermal layer was observed in 9 animals (9/44, 20.5%) (Figures 1(b) and 1(c)) and mild hypergranulosis was observed in 18 chamois (18/44, 41.0%). Focally extensive to multifocal epidermal erosions were observed in 13 (13/44, 29.5%) animals. Focal or multifocal pustules with neutrophils and/or eosinophils were present in 10 (10/44, 22.7%) animals and generally involved cases with focal to multifocal epidermal erosions. Moderate sebaceous gland hyperplasia was observed in 31 (31/44, 70.5%) cases but was not correlated with crusting severity. Varying degrees of inflammatory cells infiltration were present, mainly in the superficial and also in the deep dermis with a diffuse and less perivascular distribution. Eosinophils, lymphocytes, plasma cells, and macrophages were detected with few numbers of mast cells. Dermal fibrosis was frequently observed and was more pronounced in grade 3 lesions.

3.1.2. Trombiculosis. Trombiculid mites were localized on the surface of the epidermis, over the keratin layer (Figure 1(d)). Generally, a low number of mites (1-2 mites at 10x HPF) were detected with moderate histological lesions. Moderate parakeratosis with slight crusts (1.3 mm of thickness) and multifocal erosions was observed only in a chamois with high number of mites (3 mites/HPF), but generally no crusts or orthokeratosis was evident in chamois with trombiculid mites. No epidermal hyperplasia and alopecia were observed in all chamois with trombiculosis. In the superficial dermis, an eosinophilic stylostome, with a variable angle with skin surface, was associated with mites and surrounded by a focal granulomatous reaction, with macrophages, lymphocytes, and eosinophils. Inflammatory cells were also slightly diffuse in the surrounding superficial dermis.

3.1.3. Control Chamois. In nonaffected chamois, no epidermal hyperplasia, crusts, orthokeratosis, or alopecia was detected. In the superficial dermis, mild infiltration of lymphocytes, macrophages, and rare, single eosinophils were detected (not shown).

3.1.4. Inflammatory Cells. T and B lymphocytes and macrophages were correctly identified in chamois skin with immunohistochemistry (Figure 2). Counts of the different cell subsets present in the inflammatory infiltrates are presented in Table 2 and Figure 3. In the skin of parasitized chamois, with both sarcoptic mites and trombiculid, there was a significant increase of dermal inflammatory cells such as T and B lymphocytes ($p = 0.000$ and $p = 0.000$, resp.), macrophages ($p = 0.000$), eosinophils ($p = 0.000$), and mast cells ($p = 0.000$), when compared with control chamois. Moreover, there was a significantly higher number of T lymphocytes and macrophages in chamois with sarcoptic mange compared to chamois with trombiculosis ($p = 0.000$ and $p = 0.001$, resp.). Finally, in sarcoptic mange cases there was higher number of eosinophils (6.5 *versus* 5.5 eosinophils/0.01 mm^2) compared to animal with trombiculosis, although this difference was not statistically significant.

3.1.5. Sarcoptic Mange. Epidermal exocytosis were observed mainly for T lymphocytes and CD68-positive cells. The last cells were interpreted as exocytosis of macrophages or Langerhans cells. However, no inflammatory cells within epidermal layer were counted in this study. T lymphocytes and macrophages were the prevalent cells in inflammatory infiltrates of mangy chamois with higher number of CD68-positive cells in 0.01 mm^2. These cells were present in dermal infiltration with comparable levels in different crusting severity. Counts of the different cell subsets present in the inflammatory infiltrates are presented in Table 2 and Figure 4. Indeed, T lymphocytes lightly increased from grade 1 to grade 3 but differences were not significant. Conversely, B lymphocytes were significantly more numerous in animals with grade 3 lesions than in animals with grades 1 and 2 lesions ($p = 0.05$). Significantly a higher number of eosinophils were evident in grade 2 (7.8 eosinophils/0.01 mm^2) compared to grade 3 lesions (6.2 eosinophils/0.01 mm^2; $p = 0.05$) and grade 1 form (4.0 eosinophils/0.01 mm^2; $p = 0.001$). Moreover, a significant higher number of eosinophils were evident in grade 3 compared to grade 1 lesions ($p = 0.05$). Mast cells were present with comparable numbers in all three groups of sarcoptic lesions.

4. Discussion

In this study, histopathological features and inflammatory infiltrates in Alpine chamois skin affected by sarcoptic mange were characterized. ZS fixed specimens allowed a complete evaluation of inflammatory cells. With a comparison purpose, trombiculosis skin lesions were also characterized. As expected, an increase of dermal inflammatory cells such as eosinophils, T and B lymphocytes, and macrophages was observed in the skin of parasitized chamois with both sarcoptic mites and trombiculid mite larvae. Generally, a lower number of mites and less severe lesions were observed in trombiculosis than sarcoptic mange. Indeed, in trombiculosis, focal lesions with one or two parasites were associated with moderate inflammatory infiltrates with granulomatous features. However, being trombiculid mites on the surface of the epidermis (so they could be removed during fixation and embedding) and stylostomes not always detectable, the impact of trombiculosis could be histologically underestimated.

Lesions due to sarcoptic mange were consistent with other studies in chamois and other species [1, 2, 6, 7, 13–15]. Crusts, parakeratosis, severe epidermal hyperplasia, abundance of mites, bacterial colonies, mixed dermal infiltration, sebaceous gland hyperplasia, and fibrosis were the most frequent histopathological features in affected chamois. Inflammatory infiltrates had a diffuse and perivascular distribution and

Table 2: Subsets of inflammatory cells in skin samples from controls and trombiculid and sarcoptic mange-infected chamois.

Examined subjects	Classification of sarcoptic mange skin lesions	$CD3^+$ lymphocytes/10,000 μm^2 Mean ± SD	$CD79\alpha^+$ lymphocytes/10,000 μm^2 Mean ± SD	$CD68^+$ macrophages/10,000 μm^2 Mean ± SD	Eosinophils/10,000 μm^2 Mean ± SD	Mast cells/10,000 μm^2 Mean ± SD
Controls		5.4 ± 4.8	1.3 ± 1.9	5.0 ± 4.0	0.9 ± 1.7	0.1 ± 0.3
Trombiculosis		$10.6 \pm 7.1^{*}$	$2.9 \pm 3.0^{\dagger}$	$13.0 \pm 11.7^{+}$	$5.5 \pm 3.8^{\#}$	0.3 ± 0.4
Sarcoptic mange	Grade 1	14.1 ± 7.5	2.3 ± 2.3	16.5 ± 10.0	4.0 ± 2.4	0.9 ± 0.7
	Grade 2	15.2 ± 7.6	2.4 ± 2.4	15.2 ± 10.1	$7.8 \pm 6.8^{\dagger\dagger\dagger}$	1.0 ± 0.6
	Grade 3	16.7 ± 10.3	$3.3 \pm 3.0^{***}$	16.9 ± 9.2	$6.2 \pm 3.8^{\ddagger\ddagger\ddagger}$	1.0 ± 0.6
	Total	$15.7 \pm 8.9^{*\ddagger}$	$2.9 \pm 2.8^{\dagger}$	$16.2 \pm 9.7^{+\dagger\dagger}$	$6.5 \pm 5.2^{\#}$	$1.0 \pm 0.7^{**\ddagger\ddagger}$

Significant differences among $CD3^+$ T lymphocyte (∗), $CD79\alpha^+$ B lymphocyte (†), $CD68^+$ macrophages (+), eosinophils (#) and mast cells (∗∗) with controls. Significant differences between $CD3^+$ T lymphocyte (‡), $CD68^+$ macrophages (††) and mast cells (‡‡) in chamois with sarcoptic mange and subjects with trombiculosis. Significant differences among $CD79\alpha^+$ B lymphocyte (∗∗∗) in chamois with grade 3 and subjects with grade 1 and 2; significant differences among eosinophils (†††) in chamois with grade 2 and animals with grade 1 and 3; significant differences between eosinophils (‡‡‡) in chamois with grade 3 and grade 1.

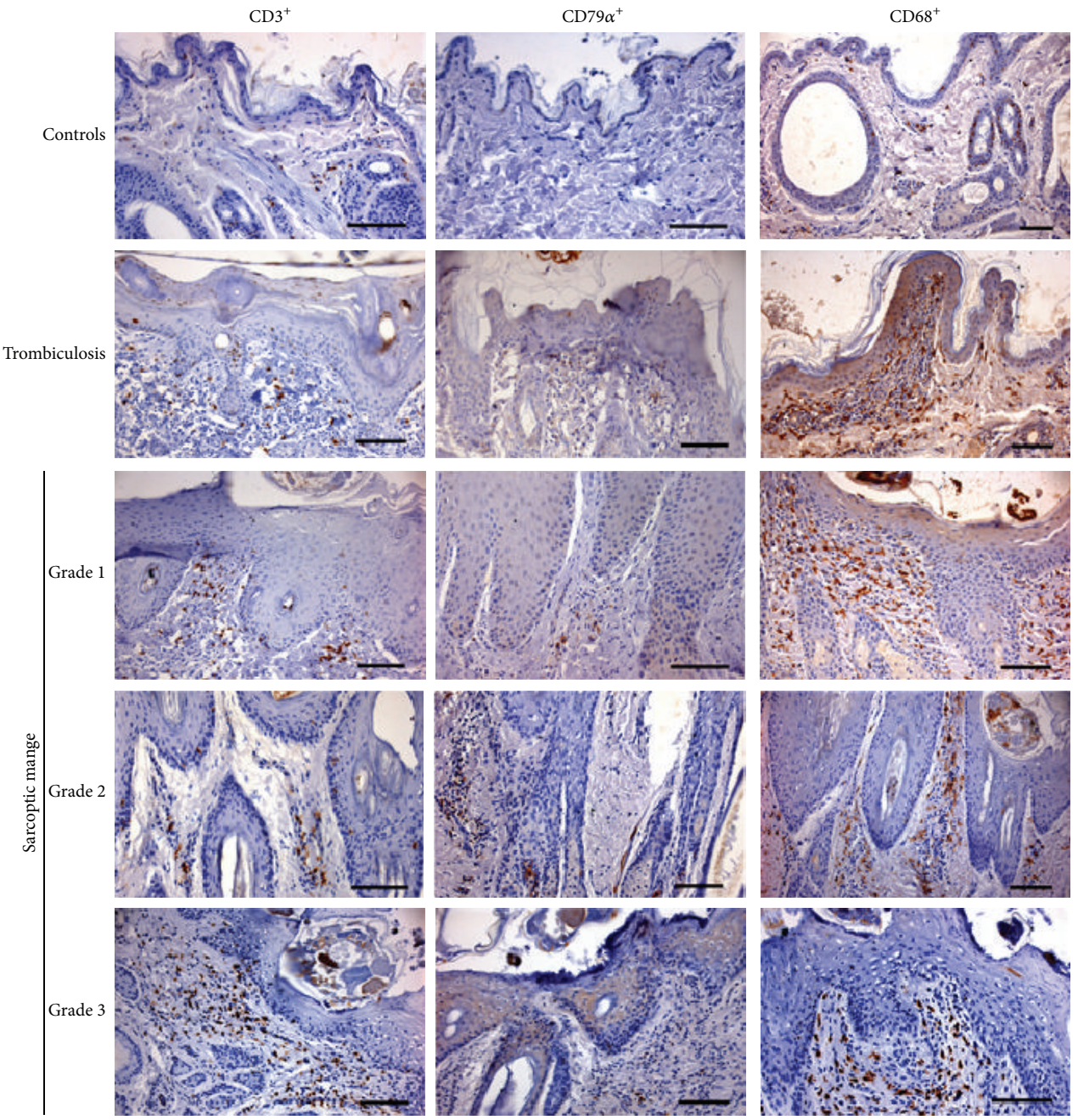


FIGURE 2: Immunohistochemical staining of skin of control, trombiculosis, and mange affected chamois. Scanty T lymphocytes ($CD3^+$ cells) and macrophages ($CD68^+$ cells) and very rare B lymphocytes ($CD79\alpha^+$ cells) are evident in control chamois. Focal inflammatory infiltration with a prevalence of macrophages is evident in trombiculosis-affected chamois. A prevalence of macrophages and T lymphocytes is evident in all three groups of sarcoptic mange affected chamois. DAB chromogen and haematoxylin counterstain. Bar = 100 μm.

were characterized by a prevalence of macrophages and lymphocytes, with eosinophils and rare mast cells. Epidermal hyperplasia was a common feature in all three grades of crusting severity. Indeed, epidermal disruption by burrowing female mites is known to initiate keratinocyte hyperproliferation also in early stages of sarcoptic infection [16]. Subsequently, in severe cases, activated CD8+ lymphocytes may induce dysregulated keratinocyte apoptosis contributing to the elicitation and progress of epidermal hyperproliferation [9]. In sarcoptic chamois, the number of T lymphocytes and macrophages in the dermis was significantly higher than in chamois with trombiculid mites and controls animals, consistent with a dermal inflammatory response. Indeed, the mite secretes compounds that dissolve the stratum corneum of the epidermis, burrowing through this dead cell layer until it reaches and consume live cells of the underlying layers of the epidermis. In all stages, mite and its products such as secretory products, exuvia, faeces, and eggs present antigens

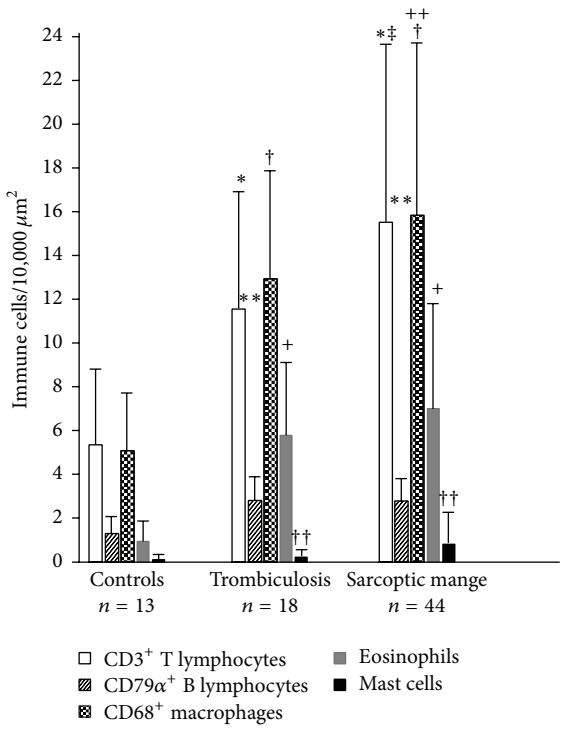


Figure 3: Histogram of number of immune cells in 10,000 μm^2 of skin in control, trombiculosis, and mange affected chamois. Significant differences among CD3$^+$ T lymphocyte (∗), CD79α^+ B lymphocyte (∗∗), CD68$^+$ macrophages (†), eosinophils (+), and mast cells (††) with controls. Significant differences between CD3$^+$ T lymphocyte (‡) and CD68$^+$ macrophages (++) in chamois with sarcoptic mange and subjects with trombiculosis.

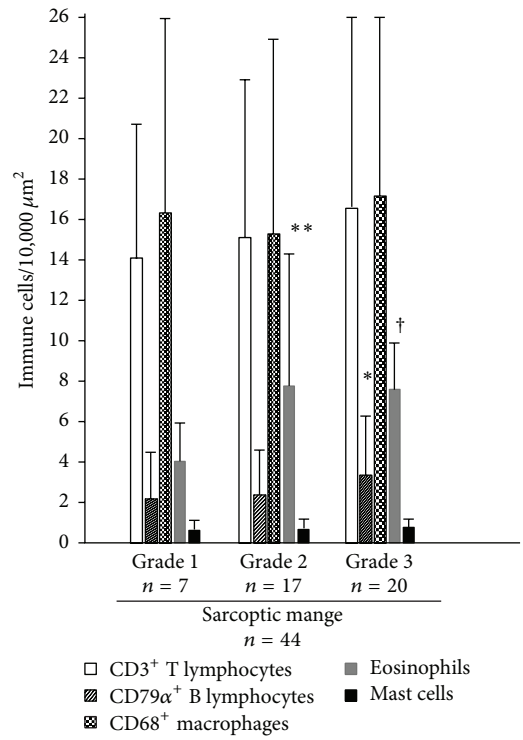


Figure 4: Histogram of number of immune cells in 10,000 μm^2 of skin in mange affected chamois, regarding different grading of sarcoptic mange lesions. Significant differences among CD79α^+ B lymphocyte (∗) in chamois with grade 3 and subjects with grades 1 and 2; significant differences among eosinophils (∗∗) in chamois with grade 2 and animals with grades 1 and 3; significant differences between eosinophils (†) in chamois with grade 3 and grade 1.

towards which hosts produce hypersensitivity reaction [14, 15]. Infiltration of Langerhans cells was observed in the epidermis, and it is assumed that epidermal Langerhans cells internalize sarcoptic antigen, migrate to draining regional lymph node, and stimulate T cells [17].

Inflammatory infiltrates varied between grades of severity. Grade 3 lesions were characterized by an increase of T and B lymphocytes, macrophages, and eosinophils compared to grade 1. However, only the increased numbers of B lymphocytes and eosinophils of grade 3 compared to grade 1 were significant. Moreover, in grade 3 lesions eosinophils were significantly lower than grade 2. In all stages, both type I (immediate) and type IV (delayed) hypersensitivity cells are evident, eosinophils and mast cells and lymphocytes and macrophages, respectively. In several cases we observed also signs of cytotoxicity (diffuse epidermal spongiosis). Involvement of immediate and delayed hypersensitivity immune response had been described also in other wild and domestic animals [2] as well as in humans [17]. In all mange stages, we detected a moderate number of B lymphocytes and plasma cells, higher than in controls and similar to trombiculosis-affected chamois. This feature could be indicative of hosts that have been exposed fairly recently to *Sarcoptes*. Indeed, in other free living animals such as foxes or wombats, rarely plasma cells or B lymphocytes were detected [1, 13], because some tolerance to infection with *S. scabiei* could occur with a reduction of the humoral response. However, during the immune response to arthropod antigens, desensitisation with exhaustion or suppression of antibody-producing could be the last event [13]. No significant differences were observed in the numbers of mast cells in the three grades forms of sarcoptic mange. Also, in experimentally infected Cantabrian chamois (*R. pyrenaica parva*) no mast cells hyperplasia was observed [7]. This finding differs from studies on foxes or wombats, where mast cells increased with severity of sarcoptic lesions suggesting that mast cells could not have a determining role in the pathogenesis of disease [1, 2].

According to our results, grades 1 and 2 with slight and moderate crusting and lower number of mites could be initial stages and grade 3 could be the following, generalized, severe stage. However, analogously to ordinary scabies in human beings, grade 1 and grade 2 could be suggestive of skin immune response to *S. scabiei* with adequate presence of macrophages, T lymphocytes, and eosinophils that allows a parasite control with reduction of mite numbers and limitation of lesions. Differently, grade 3 form with significant increase of B lymphocytes and decrease of eosinophils not associated with a significant increase of macrophages and T lymphocytes could be related with an involvement of nonprotective Th2 response that could be unable to control or reduce the mite burden particularly in sequential infestations [9].

However, evaluation of genetic polymorphism, in particular of MHC (major histocompatibility complex) class II, could be worthy to elucidate if animals with severe crusting really have a different genotype that account for a nonprotective immune response.

In conclusion, our study allowed us to characterize in detail the histopathological changes associated with *Sarcoptes* infection in the chamois, to classify these changes accordingly to their severity, and to define, for the first time, the cellular subsets present in the inflammatory infiltrates of lesions of different grade. The increase of B lymphocytes and eosinophils in grade 3 lesions highlights how an involvement of a nonprotective Th2 response could in part be responsible for the development of these more severe forms.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Biosensor for Hepatocellular Injury Corresponds to Experimental Scoring of Hepatosplenic Schistosomiasis in Mice

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Severe hepatosplenic injury of mansonian schistosomiasis is caused by Th2 mediated granulomatous response against parasite eggs entrapped within the periportal tissue. Subsequent fibrotic scarring and deformation/sclerosing of intrahepatic portal veins lead to portal hypertension, ascites, and oesophageal varices. The murine model of *Schistosoma mansoni* (*S. mansoni*) infection is suitable to establish the severe hepatosplenic injury of disease within a reasonable time scale for the development of novel antifibrotic or anti-infective strategies against *S. mansoni* infection. The drawback of the murine model is that the material prepared for complex analysis of egg burden, granuloma size, hepatic inflammation, and fibrosis is limited due to small amounts of liver tissue and blood samples. The objective of our study was the implementation of a macroscopic scoring system for mice livers to determine infection-related organ alterations of *S. mansoni* infection. In addition, an *in vitro* biosensor system based on the detection of hepatocellular injury in HepG2/C3A cells following incubation with serum of moderately (50 *S. mansoni* cercariae) and heavily (100 *S. mansoni* cercariae) infected mice affirmed the value of our scoring system. Therefore, our score represents a valuable tool in experimental schistosomiasis to assess severity of hepatosplenic schistosomiasis and reduce animal numbers by saving precious tissue samples.

1. Introduction

In the host adult *Schistosoma mansoni (S. mansoni)* worms are found within the mesenteric blood vessels around the sigmoid colon and the rectum, where the oviposition takes places [1]. Schistosomal eggs induce a strong antibody response against soluble egg antigens that corresponds to a type 4 hypersensitivity reaction mediated by T-helper 2 (Th2) cells and alternatively activated macrophages. Via blood-stream the lateral-spined eggs get into the liver and become entrapped within the periportal tissue [2]. Th2 response directs periocular inflammation towards fibrous scarring with subsequent deformation and sclerosing of the intrahepatic portal veins whereby the arterial and ductular structures remain largely unaffected [3]. Fibrotic septa sprout from the periportal area into the parenchyma, while the acinar architecture is preserved. Due to high infection rates or repeated infections periportal (Symmers' pipestem) fibrosis [4] occurs as mild chronic granulomatous hepatitis or advanced hepatosplenic manifestations with massive fibrosis, splenomegaly, portal hypertension, ascites, and esophageal varices.

By virtue of intact liver function serum biochemistry is inconspicuous. During the acute stage of disease (Katayama syndrome) eosinophilia and a slight increase of alanine aminotransferase (ALT) can be seen. Later in the chronic stage of disease aminotransferase levels are often within the normal ranges and alkaline phosphatase might be slightly elevated.

Apart from discrepancies regarding susceptibility for infection between mice and man [5], immunological reactions to parasite eggs run similarly in terms of involved cells types and regulating cytokines, granuloma formation, disease progression, and clinical symptoms [6]. In man, disease progression towards advanced hepatosplenic schistosomiasis depends on the age, gender, infection rates, and duration of infection [7]. The mouse model is suitable to establish severe hepatosplenic manifestations within eight weeks by infection with low numbers of worms [3]. Therefore the murine model is used to study the complex immune reactions to egg and worm antigens within a reasonable time scale and to develop novel antifibrotic or anti-infective strategies against *Schistosoma* infections [8]. However, complex analysis of egg burden, granuloma size, hepatic inflammation, and fibrosis is limited due to small amounts of liver tissues and blood samples. Therefore usually large numbers of animals are needed, in order to address scientific issues adequately.

The objective of our study was the implementation of a macroscopic scoring system determining infection-related organ alterations as markers of disease severity in mice infected with *S. mansoni*. An *in vitro* biosensor system detecting hepatocellular injury in HepG2/C3A cells affirmed our scoring system.

2. Material and Methods

2.1. Schistosoma mansoni Mouse Model. Schistosoma mansoni (*S. mansoni*, Brazilian strain) was held in a life cycle with *Biomphalaria glabrata* fresh water snails (*B. glabrata*, Brazilian strain) as intermediate hosts and female NMRI mice as definite hosts. Mice were kept with a 12:12 hour light/dark cycle, standard mouse chow (SSNIFF, Soest, Netherlands), and water *ad libitum*. *B. glabrata* were kept in aquarium water at 25°C and a lettuce diet. Cercariae were obtained by mass shedding after light exposure and the number of cercariae/mL was determined using a conventional light microscope (magnification 100-fold). To generate pathologies of different severities, 6- to 8-week-old NMRI mice were infected with either 50 *S. mansoni* cercariae ($n = 9$) for a moderate infection or 100 *S. mansoni* cercariae ($n = 9$) for a heavy infection. Mice were exposed to *S. mansoni* cercariae by sitting in a water bath, except the healthy control-group ($n = 5$). Twelve weeks after infection mice were sacrificed via cervical dislocation under Ketamine/Xylazine anesthesia. Collection of sterile plasma for biosensor testing was performed by immediate retrobulbar exsanguination using lithium/heparin blood collection tubes (Sarstedt, Hannover, Germany). Within the experimental groups plasma samples were pooled and frozen at −80°C. The experiments were performed according to the German animal protection law and approved by the local animal care and use committee (file number 7221.3-2.5-003/10).

2.2. Serum Biochemistry and Liver Histology. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), and bilirubin were measured in serum with the UniCel® *DxC 800* Synchron® Clinical System (Beckman Coulter GmbH).

Table 1: Description of the macroscopic score.

Parameter	Modality	Score level
Spleen weight	<0,1 g (healthy)	−
	>0,11 g	+
	>0,4 g	++
Liver weight	<1,2 g (healthy)	−
	>1,21 g	+
	>2 g	++
Color	Red/glossy	−
	Dark red/fade	+
	Greyish/pale	++
Nodules	None	−
	Occasional	+
	Area-wide	++
External surface	Regular	−
	Furrows/bosselation	+
	Macronodular	++
Consistency	Soft/elastic	−
	Firm	+
	Rigid	++

One-half of the right liver lobe was fixed in 10% neutral buffered formalin solution (Sigma Aldrich, Germany) and embedded in paraffin. Five μm thin sections were stained with either hematoxylin/eosin (HE) or Sirius red (SR).

2.3. Macroscopic Evaluation of Liver Injury. Twelve weeks after infection, liver and spleen weights were determined and the extent of liver damage was assessed macroscopically by using a minus (−)/plus (+) scoring system: (−) unobtrusive for the evaluated parameter and (+) obtrusive for the evaluated parameter. The score ranges from 1− = healthy to 12+ = heavily affected by *S. mansoni* infection. The scoring system determines the extent of liver damage and considers infection-related organ alterations as color, stiffness, and prevalence of nodules compared to naive livers (Table 1).

2.4. Human Hepatocyte Based Biosensor. Two mL heparinized plasma was drawn from all three experimental groups for testing with the *in vitro* hepatotoxicity test [9, 10]. To determine the toxicity of animal plasma the human hepatocytes cell line HepG2/C3A obtained from the American Type Culture Collection (ATCC CRL-10741) was used. The cells were cultivated in Dulbecco's modified Eagle's Medium (Dulbecco's MEM, GIBCO Life Technologies, Eggenstein, Germany). HepG2/C3A cells were seeded in 24-well cell culture plates in a density of 250.000 cells/well; then the cells were cultured for three days with 0.5 mL heparinized plasma from each animal. Subsequently, cells were rinsed once with Dulbecco's MEM and incubated with 1 mL fresh Dulbecco's MEM for three days. Cells, respectively, supernatants, were tested for viability using the XTT test (dehydrogenase activity in the mitochondria) and measurement of cell count was indicated with the trypan blue-staining. The XTT test was carried out according to the protocol of Scudiero et al. [11].

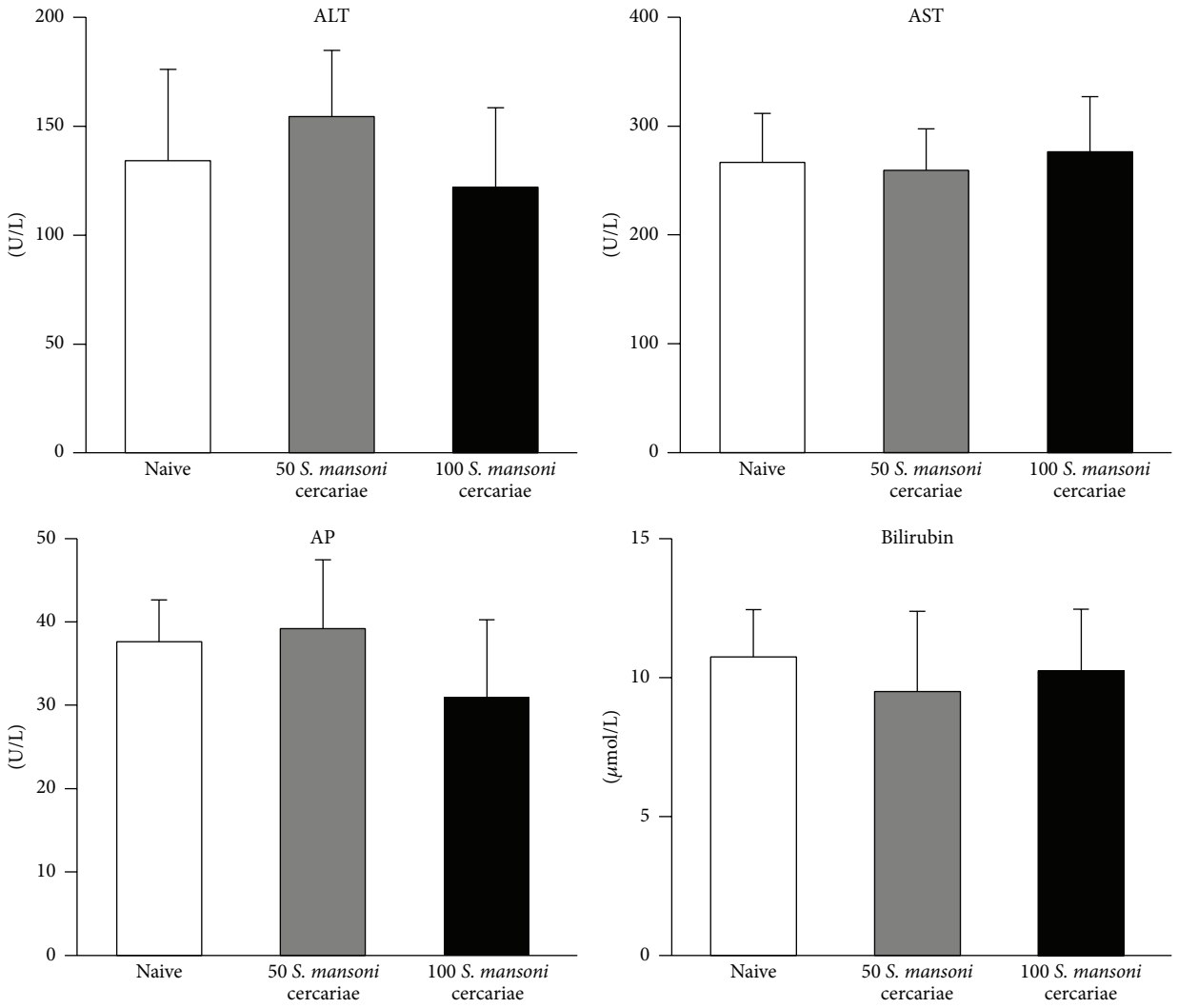

Figure 1: Serum biochemistry of mice infected with 50 or 100 *S. mansoni* cercariae. Serum biochemical markers of hepatocellular injury (ALT and AST) and cholestasis (AP and bilirubin) remain unchanged in mice infected with 50 or 100 *S. mansoni* cercariae compared to naive control mice.

Additionally, the release of lactate dehydrogenase (LDH) into the cell culture supernatants was measured. Each test batch with plasma from one group was tested in four experiments ($n = 4$), Dulbecco's MEM control was used and each measurement was performed twice.

2.5. Statistical Analysis. Significance between the groups was analyzed with the Kruskal-Wallis one way and the two-tailed Mann-Whitney U-test, using the Statistical Package for the Social Sciences (SPSS). The results are expressed as median and range. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Serum Biochemistry. Serum levels of ALT, AST, AP, and bilirubin were not increased by the different *S. mansoni* infection intensities compared to the naive control (Figure 1).

3.2. Scoring System. *S. mansoni* infection resulted in significant increase of liver and spleen weights compared to healthy control mice (Figure 2(a)). The different infection intensities were reflected by marked differences in the macroscopic appearance of infected mice livers in mice infected with 100 *S. mansoni* cercariae (Figure 2(b)). Livers of these mice appeared greyish and pale; bosselations of the capsule with star-like depression and wide spread nodules were clearly visible. A semiquantitative evaluation of the infection-related organ alteration was performed according to the scoring system described in Table 1. The highest score levels with a mean of 10 ± 0.1 were found in mice infected with 100 *S. mansoni* cercariae compared to mice infected with 50 *S. mansoni* cercariae and score levels of 7 ± 1.9 (Figure 2(c)).

3.3. Microscopic Appearance. Histological survey staining (HE, magnification 2.5-fold) of mice livers revealed clearly visible hepatic granulomas within enlarged portal spaces.

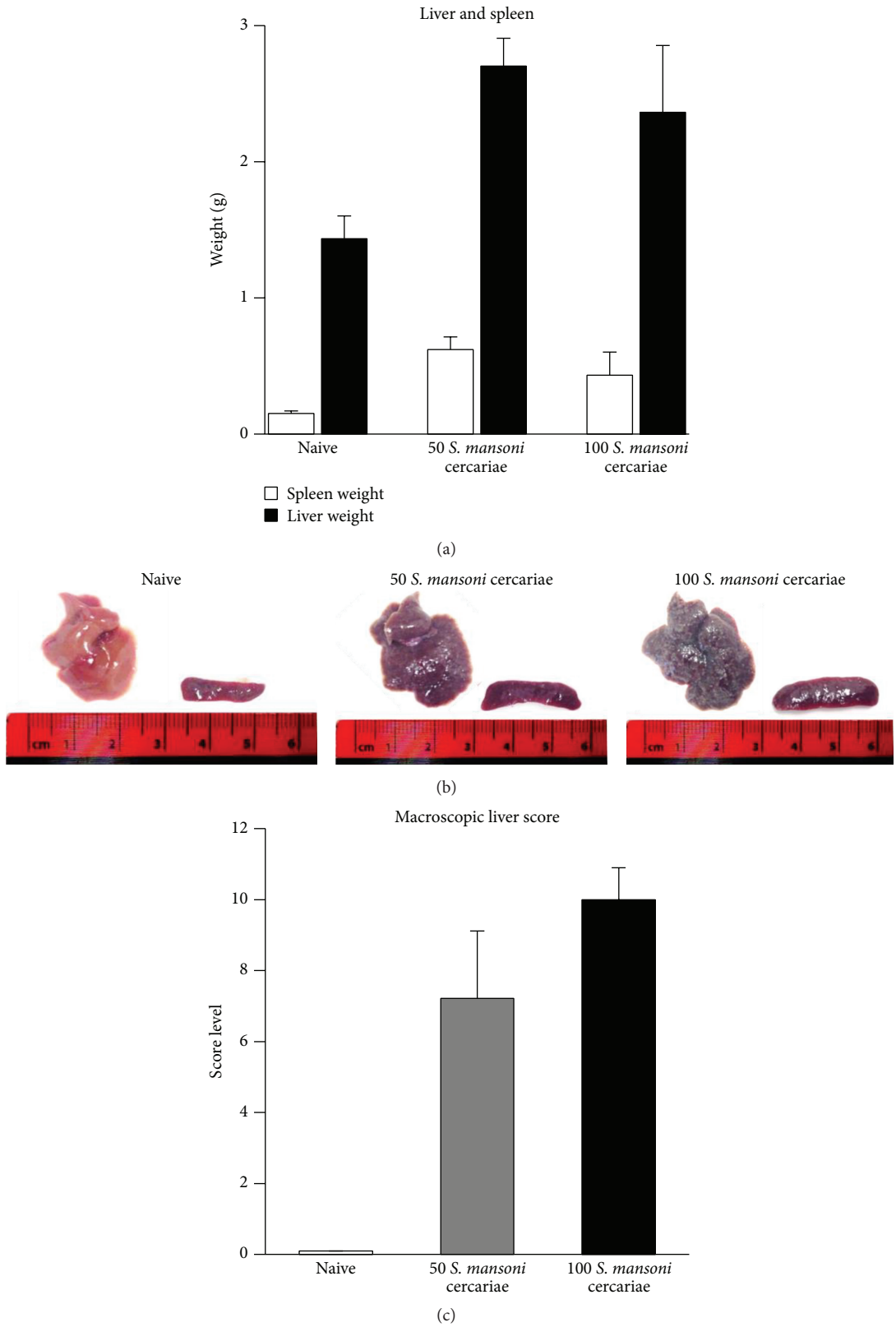

Figure 2: Pathological changes of the liver due to different *S. mansoni* infection intensities. (a) Weights of spleens and livers of infected mice groups are significantly increased compared to naive mice. Infection with 50 or 100 cercariae results in a comparable increase of spleen and liver weights. (b) The outer appearance of mice livers following infection with 50 or 100 cercariae is markedly different. Compared to the livers of naive and mice infected with 50 cercariae, the livers of mice infected with 100 cercariae appear firmer and paler with area-wide nodules, bosselations, and star-like depressions. (c) Scoring of parameters describing the outer appearance of all mice livers reveals highest score levels in mice infected with 100 cercariae compared to livers of naive mice and mice infected with 50 cercariae.

Table 2: Biosensor results (median and range).

	Naive ($n = 4$)	50 cercariae ($n = 4$)	100 cercariae ($n = 4$)	Medium-control ($n = 4$)
Number of cells (×1.000)	*833* (825–840)	*610* (575–645)	*560* (545–575)	*1111* (535–1.355)
Vitality (%)	*95.2* (93.3–97.1)	*82.8* (80.6–84.9)	*73.8* (64.4–83.2)	*94.9* (91.1–97.3)
XTT (extinction/well)	*1.48* (1.25–2.37)	*0.92* (0.84–1.00)*	*0.84* (0.51–0.90)*	*2.13* (1.72–3.27)*
Release of LDH (U/L)	*135* (134–136)	*145* (139–150)*	*157* (152–161)*	*113* (110–123)

* $P \leq 0.05$ versus naive; LDH: lactate dehydrogenase.

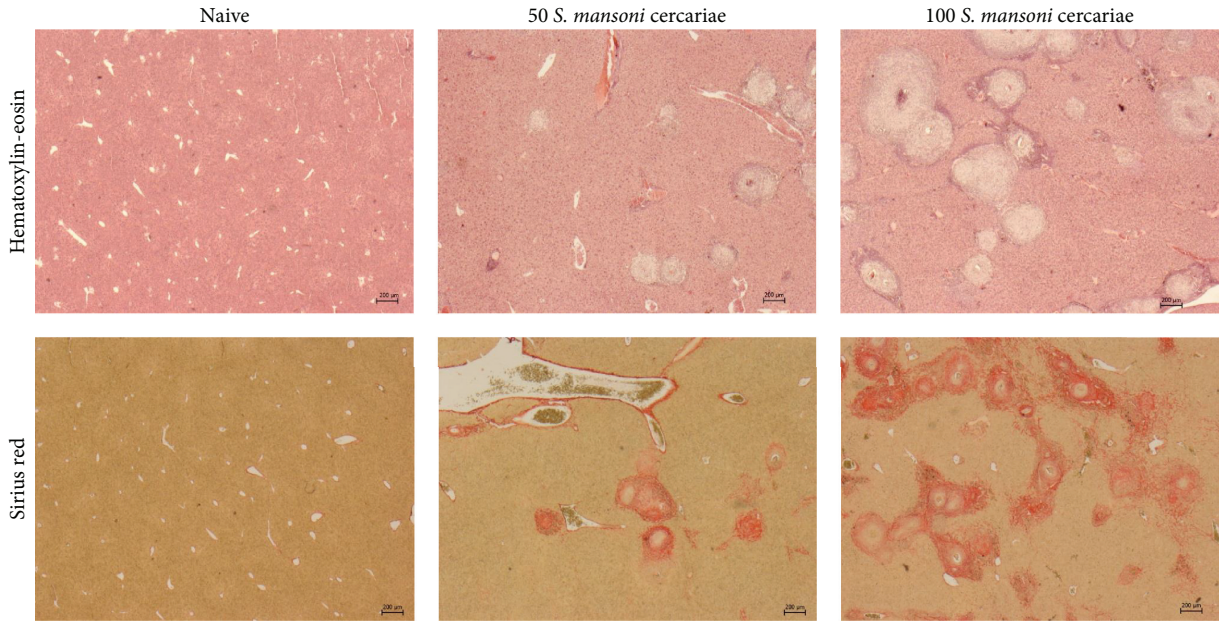

Figure 3: Liver histology of mice infected with 50 or 100 cercariae. Representative images of mice livers (HE, 2.5-fold magnification and SR, 2.5-fold magnification): uninfected mice (naive), mice infected with 50 cercariae (50 cercariae), and mice infected with 100 cercariae (100 cercariae). Livers of mice infected with 100 cercariae have considerably more periocular granulomas compared to the other mice. Higher infection rates result in tortuous vascularization of the portal area and pronounced portoportal bridging with markedly sclerosed hepatic veins.

Livers of mice infected with 100 *S. mansoni* cercariae displayed tortuous vascularization of the portal area with replaced blood vessels. Infiltration with inflammatory cells as macrophages/monocytes, granulocytes, and T-lymphocytes was not restricted to periocular regions but to blood vessels and was sporadically found within the parenchymal interface. The amount of hepatic granulomas was conspicuous higher following infection with 100 compared to 50 *S. mansoni* cercariae. Sirius red positive area was mostly distributed in livers of mice infected with 100 *S. mansoni* cercariae. Livers of these mice displayed pronounced portoportal bridging and markedly sclerosed hepatic veins. In animals infected with 50 *S. mansoni* cercariae connective tissue was restricted to periocular areas with dense connective tissue fibers spread into the parenchymal interface (Figure 3).

3.4. Biosensor of Hepatocellular Injury. The results of testing the toxicity of the animal plasma from the three experimental groups using human hepatocytes are displayed in Table 2. A markedly impairment of viability was only seen in the infected groups; however, the impairment of viability was more pronounced in the 100 cercariae group compared with the 50 cercariae group. The plasma of the animals from the control-group led to a slightly decrease of the number of cells and extinction in the XTT test and higher values of LDH in cell culture supernatants compared with the medium-control.

4. Discussion

Murine models of *S. mansoni* infection were used to imitate human disease patterns and remain indispensable for antischistosomal drug development. The presented experimental scoring system specifically pays attention to infection-related organ alterations resembling severity of disease. It comprises the evaluation of liver characteristics, which were commonly used for the assessment of hepatic fibrosis and related morbidity in humans such as liver stiffness, presence of nodules, and surface condition [12–14]. The different pathologies and therefore the usability of the score were proven by an *in vitro* biosensor system based on the detection of hepatocellular injury in HepG2/C3A cells.

Infection with fifty to hundred *S. mansoni* cercariae represents the usual amount of cercariae used for experimental schistosomiasis [3, 8, 15]. The presented score describes specific organ alterations following infection with either 50 or 100 *S. mansoni* cercariae, resembling moderate and heavy infections under experimental conditions. Both infection intensities are incomparable to human infection, since the heaviest infection found in man at autopsy rarely exceeded five worm-pairs per kilogram of body weight [16]. Experimental schistosomiasis is performed with considerably higher numbers of cercariae to provoke severe clinical patterns within a reasonable time scale.

Diagnostic methods in human as transient elastography (FibroScan) or magnetic resonance elastography are used to analyze liver stiffness, occurrence of nodules, or surface conditions to grade hepatic fibrosis and to assess morbidity. In mice these clinical changes have not attracted scientific interest so far. Common approaches to quantify hepatic fibrosis in mice are based on determination of hepatic hydroxyproline content, histological collagen staining, or measurement of profibrotic gene expression [8, 17]. Examining egg secretion in feces or detection of soluble egg antigen in serum seldom reflects real infection burden or organ alterations [18]. Moreover, determination of whole egg or worm numbers in mice livers requires processing of the whole liver but does not reflect the extent of liver damage.

To distinguish between moderate (50 cercariae) and heavy (100 cercariae) *S. mansoni* infection we performed a cell based analysis of hepatotoxicity in the HepG2/C3A hepatocyte cell line [9, 10]. The incubation with plasma of the three experimental groups (naive, 50 cercariae, and 100 cercariae) led to a significant impairment of cell viability in the infected groups only. According to the histological score, the impairment of test cells was more pronounced in the mice infected with 100 *S. mansoni* cercariae than in the mice infected with 50 *S. mansoni* cercariae. This might result from different levels of chemokines and cytokines in the plasma of the infected animals. In chronic schistosomiasis the degree of hepatic fibrosis correlates with serum levels of interleukin 13 and interleukin 4 [19, 20]. It is debatable to which extent proinflammatory cytokines are involved in the impairment of cell functions and viability in HepG2/C3A cells, since in chronic schistosomiasis Th1 response is suppressed by Th2 cytokines [21]. However, proinflammatory cytokines are known to affect cell viability by causing dysfunction of mitochondria [22], downregulating albumin synthesis [23], and diminishing function of P450 cytochromes like cytochrome P450-1A2, cytochrome P450-2E1, and cytochrome P450-7A1 [24, 25].

So far, this test system was tested in a prospective clinical study addressing hepatotoxicity of plasma from septic and nonseptic patients [9]. We found that the plasma of patients with septic shock impaired cellular functions and viability of HepG2/C3A cells. These values of biosensor-parameters were increased only in survivors compared to nonsurvivors in this study. Another clinical study in patients with severe sepsis or septic shock tested the influence of an extracorporeal granulocyte treatment on the biosensor cells. During the extracorporeal treatments a significant increase of vitality and function of the test cells was seen. These results suggest a positive impact of the extracorporeal granulocyte treatment on the liver cell vitality and function measured in this indirect cytotoxicity test. Against this background and regarding the fact that in schistosomiasis analysis of standard biochemical markers fails to assess severity of disease, it would be prudent to test the HepG2/C3A biosensor in human schistosomiasis.

In conclusion, the presented experimental scoring system displays a useful tool to assess infection-related alterations of the liver indicating severity of hepatosplenic schistosomiasis. Therefore, the score may help to determine effects of therapeutic interventions aiming at hepatic fibrosis or reduction of infection loads. In addition, improved exploitation of scientific information saves precious tissue samples and may reduce numbers of animals for experiments.

Abbreviations

ALT:	Alanine aminotransferase
AP:	Alkaline phosphatase
AST:	Aspartate aminotransferase
B. glabrata:	*Biomphalaria glabrata*
Th1:	T-helper cell response type 1
Th2:	T-helper cell response type 2
XTT:	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
LDH:	Lactate dehydrogenase.

Competing Interests

The authors declare no financial or commercial competing interests.

Authors' Contributions

Martina Sombetzki, Nicole Koslowski, Emil C. Reisinger, and Martin Sauer conceived and designed the experiments. Martina Sombetzki, Nicole Koslowski, and Martin Sauer performed the experiments. Martina Sombetzki, Nicole Koslowski, Micha Loebermann, and Martin Sauer analyzed the data. Martina Sombetzki, Nicole Koslowski, Emil C. Reisinger, Micha Loebermann, Martin Sauer, and Michael Trauner contributed reagents/materials/analysis tools. Martina Sombetzki, Nicole Koslowski, Emil C. Reisinger, and Martin Sauer wrote the paper.

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Research Article

Development of a Murine Infection Model with *Leishmania killicki*, Responsible for Cutaneous Leishmaniosis in Algeria: Application in Pharmacology

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In Algeria, *Leishmania infantum*, *Leishmania major*, and *Leishmania killicki* (*Leishmania tropica*) are responsible for cutaneous leishmaniosis. We established a murine model of *L. killicki* infection to investigate its infective capacity, some immunophysiopathological aspects, and its suitability for pharmacological purposes. Following the injection of *L. major* or *L. killicki* metacyclic promastigotes in the ear dermis of BALB/c mice, the course of infection was followed. The infection with *L. killicki* caused slower lesion formation than with *L. major*. The presence of *L. killicki* or *L. major* DNA and parasites was detected in the ear dermis and in lymph nodes, spleen, and liver. Lesions induced by *L. killicki* were nonulcerative in their aspect, whereas those caused by *L. major* were highly ulcerative and necrotic, which matches well with the lesion phenotype reported in humans for *L. killicki* and *L. major*, respectively. The treatment of *L. killicki* lesions by injection of Glucantime® significantly reduced the lesion thickness and parasite burden. Ear dermal injection of BALB/c mice constitutes a model to study lesions physiopathology caused by *L. killicki* and presents interest for *in vivo* screening of new compounds against this pathogen, emerging in Algeria.

1. Introduction

Leishmania are obligate intracellular parasites, which cause different forms of leishmanioses in humans, ranging from dermal ulcers to fatal visceral forms. Cutaneous leishmanioses (CL) are caused by several *Leishmania* species and display various clinical manifestations. In Algeria, *L. killicki* was discovered in 2005 in the southern province of Ghardaia and recently reported in the northern part of the country [1, 2]. Phylogenetic studies based on Multilocus Enzyme Electrophoresis showed that *L. killicki* strains were included in clearly distinct clades within the *L. tropica* complex. A recent Multilocus Sequence Analysis further evidenced the substructuration of *L. tropica* species and supported this proposal [3, 4]. In Algeria, up till now only strains belonging to the *L. killicki* subgroup within the *L. tropica* complex have been isolated. The pathogenicity and the infectivity of these strains have never been studied. Cutaneous lesions caused

by *L. tropica* tend to form dry ulcers that require a long time to heal, typically one year or more. Healing is often associated with disfiguring scars and papules that can also recur at the periphery of the original lesion and are called recurrent type lesions [5, 6]. Cutaneous lesions caused by *L. killicki* are called chronic cutaneous lesions because they persist for several years [7]. So clinical signs of cutaneous leishmaniosis due to *L. killicki* are restricted to a chronic cutaneous lesion, resistant to standard treatment in opposite to *L. tropica* cutaneous leishmaniosis [8–11]. In addition, the transmission of *L. killicki* seems to be strictly zoonotic, while it is anthroponotic or occasionally anthropozoonotic for *L. tropica*.

Animal models have been used in the drug discovery and development process to characterize disease physiopathology and to estimate clinical dosing regimens safety margins and toxicity and of course to validate targets and compounds. The ideal in an animal model is that it should replicate to a great extent a human disease phenotype and its underlying causality. Many experimental models have been developed on rodents to study CL. Mouse models were established for *L. major*, *L. tropica*, *L. amazonensis*, and *L. braziliensis*, each with specific features in order to characterize the immune response, but none of them reproduces the pathology observed in human disease [12–14]. For *L. tropica* and *L. major*, patterns of responses are species specific with different sex effects and largely different host susceptibility genes [6, 15, 16]. So, *L. killicki* causes in humans cutaneous lesions, which tend to form dry ulcers similar in their aspect to those caused by *L. tropica* [1, 6, 7]. Nevertheless, their healing requires a longer period of treatment. CL caused by *L. killicki* is called chronic CL because lesions persist for years, as opposed to CL caused by *L. major* for which lesions usually resolve with scare after few months only [7].

The aims of this work were therefore to develop an animal model that allows us to study basic physiopathological and immunological aspects of the infection caused by *L. killicki* and to evaluate its suitability for pharmacological purposes.

2. Materials and Methods

2.1. Parasites. *L. major* (MHOM/DZ/10/LIPA175/11) and *L. killicki* (MHOM/DZ/11/LIPA281/11) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 25 mM glucose, 100 μg/mL streptomycin, and 100 IU/mL penicillin at 25°C. Strain virulence was maintained by a regular passage in susceptible BALB/c mice.

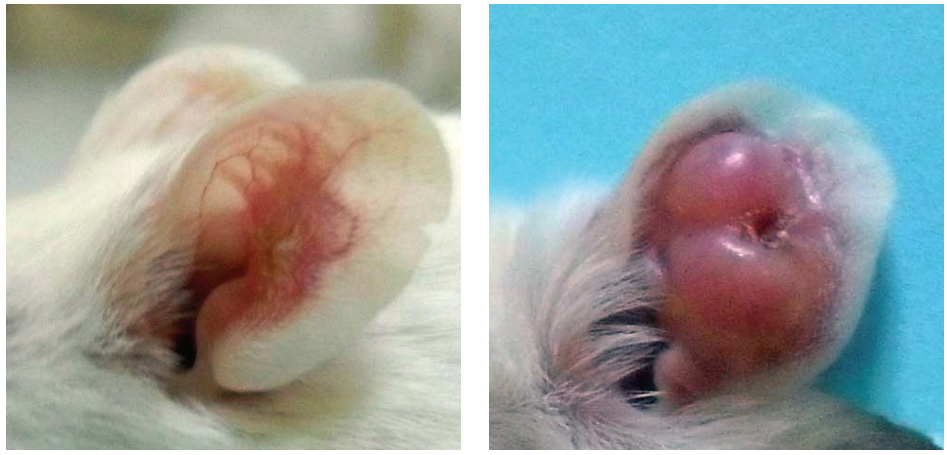
2.2. Ethics Statement. All experiments were carried out in compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and approved by the Ethical Committee of the Pasteur Institute in Algiers.

2.3. Mouse Infection. A total of 120 BALB/c mice were obtained from the animal breeding stock facility of Pasteur Institute in Algiers. Six-week-old females were kept in conventional conditions with barriers, controlled temperature, and light cycle. Food and water were provided *ad libitum*. Infective promastigotes were isolated at the metacyclic stage from stationary phase cultures (6 days old). Metacyclic promastigotes were isolated on a Ficoll gradient [17], washed once with phosphate buffered saline (PBS) (pH 7.3), and then resuspended in PBS (pH 7.3). 10^3 metacyclic promastigotes in 10 μL of PBS were injected in the left ear of each mouse [13].

2.4. L. killicki Animal Model. To establish an *in vivo* model of cutaneous lesions caused by *L. killicki*, we compared the appearance and the induration thickness of the lesion following intradermal inoculation with *L. killicki* or *L. major* into the ear dermis of BALB/c mice. To monitor lesion development, mice were divided into three groups: 5 mice for the control noninfected mice, 5 mice infected with *L. major*, and 5 mice infected with *L. killicki*. Experiments were conducted during 12 consecutive weeks after infection in mice infected with *L. major* and during 30 consecutive weeks after infection for mice infected with *L. killicki*. Lesion development was monitored by measuring the ear thickness using a digital micrometer caliper (Fisherbrand) at weeks 2, 4, 6, 10, and 12 after infection for *L. major* and at weeks 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 30 after infection for *L. killicki*. Results are expressed as the difference between thicknesses of the inoculated ear and the noninoculated contralateral ear (internal control).

2.5. Detection and Quantification of Parasites in Tissues. To look at the capacity of *L. killicki* and of *L. major* to disseminate into internal organ, the presence of parasite DNA and of live parasites was investigated at weeks 2, 4, 8, and 12 for *L. major* infected mice and at weeks 2, 4, 8, 12, 16, and 20 for the *L. killicki* group. Mice were euthanized, and the retromaxillary draining lymph nodes, spleen, and liver were collected to extract DNA. At the 12th week, an aliquot from each sample was seeded to LIT (Liver Infusion Tryptose) medium and incubated for 4 weeks in order to isolate live parasites. Samples of tissue were homogenized in PBS using potter grinders and 1.5 mL microtubes with single-use blue pellet pestles (Polylabo, France). Homogenates were then aliquoted and stored at −20°C until DNA extraction. Total DNA was purified using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's protocol. Parasite DNA was detected after amplification of the ribosomal internal transcribed spacer 1 (ITS1) using primers LITSR and L5.8S previously designed by Schönian et al. [18]. The PCR mix (25 μL) contained 2.5 μL of DNA, 10x buffer, 300 μM $MgCl_2$, 200 μM dNTP, 500 nM of each primer, and 2 U of Taq DNA polymerase. Amplification products were separated on a 1% agarose gel and visualized after staining with ethidium bromide.

To investigate parasite proliferation during lesion expansion, we measured the parasite load at the inoculation site. At weeks 4, 8, and 12 for *L. major* infected mice and at weeks 4, 8, 12, 16, 20, and 30 for *L. killicki* groups, mice were euthanized. Parasite proliferation within the lesion was monitored by counting the number of amastigotes in Giemsa-stained smears under 100x magnification. The number of



(a) (b)

Figure 1: Clinical evolution of the ear lesions in BALB/c mice. Lesions' appearance in mice infected with *L. major* (4th week after infection) (a) or *L. killicki* (20th week after infection) (b).

infected macrophages and the mean number of amastigotes per macrophage were determined in one hundred randomly selected fields. The results are expressed as the mean number of parasites per 100 macrophages.

2.6. Drug Treatment. In a first attempt to investigate the capacity of this new *in vivo* murine model of infection with *L. killicki* to be used for the screening of new antileishmanial compounds, we compared the outcome of Glucantime treatment which consisted in injecting drug into lesions at weeks 4 and 8 for *L. major* and *L. killicki*, respectively. In all experiments, the treatment was initiated when the infection was well established and when the lesions were obvious 4 and 8 weeks after the inoculation with *L. major* and *L. killicki*, respectively. Two days before drug administration, mice were randomly divided into 2 groups of thirty animals. N-Methylglucamine antimoniate (pentavalent antimony: SbV) was diluted in PBS and then administered to mice by injection directly into lesions at a dose of 28 mg per kg of body weight every 5 days for 15 days. Ear thickness was measured weekly during the treatment and after the end of the treatment. The antimony treatment efficiency was monitored by calculating ear thickness and parasite load indexes:

> Ear thickness index = mean thickness of ear from treated mice/mean thickness of ear from untreated mice.
>
> Parasite load index = mean parasite load in untreated mice/mean parasite load in treated mice.

2.7. Statistical Analysis. Values are given as the mean ± SEM for groups of n samples. Analysis of variance (ANOVA) and Student's t-test were performed using GraphPad Prism Software (GraphPad Software Inc., San Diego, California, USA) and Microsoft Office Excel 2013 was used to determine the significance of differences.

3. Results

3.1. Lesion Appearance and Development. A striking difference in the onset, the type, and the severity of lesions was observed between both *Leishmania* species. Cutaneous redness, which is the first symptom of infection caused by tissue inflammation, was detectable 2 weeks after the infection of mice with *L. major* but 4 weeks after infection with *L. killicki*.

After 4 weeks, *L. major*-infected mice exhibited lesions with elevated borders and sharp craters (Figure 1(a)). Mice infected with *L. killicki* developed a detectable lesion later: the ear thickness increased progressively during the time course of the experiment (Figure 1(b)). Infection never caused lesion ulceration, which was observed in *L. major*-infected mice. Strikingly, lesion phenotypes induced by *L. killicki* were clearly distinct from those induced by *L. major*.

In mice infected by *L. major* metacyclic promastigotes, thickening of the ear was observed at the inoculation site as early as 3 weeks after infection. The ear thickness rapidly increased, reached a maximum of 2.4 mm at week 10, and then regressed (Figure 2). For *L. killicki*, the ear thickness expanded linearly and more slowly throughout the time course of the experiment to reach 1.4 mm and 4.3 mm at weeks 10 and 30, respectively.

3.2. Parasite Burden in the Ear Dermis and Occurrence in Other Organs or Tissues. The presence of parasites in the ear dermis of mice inoculated with *L. major* was observed as early as the 4th week (Figure 3). Moreover, the parasite burden steadily increased until the 8th week and the maximum was reached at the 10th week (Figures 1 and 3). As from this time, the ear thickness and the lesion size slightly decreased until the ear perforated.

The onset of parasite burden in *L. killicki*-infected mice was different. Parasite expansion began later than for *L. major*, 8 weeks after infection, reached its maximum at week 16, and was maintained until the end of the experiment

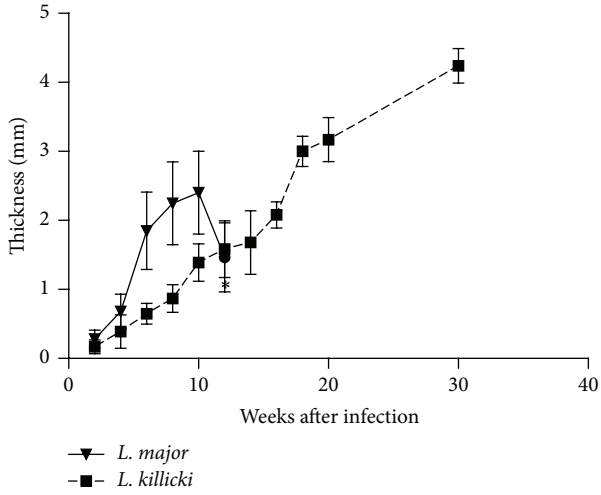


Figure 2: Evolution of indurations following infection of BALB/c mice with *Leishmania killicki* or *Leishmania major*. The ear thickness is expressed as the difference between the thicknesses of the inoculated ear and the noninoculated contralateral ear. The data represent the mean values of measures ± standard deviations ($n = 5$). Note: 12 weeks (∗) after infection with *L. major*, ears became necrotic and showed a loss of tissue which has prompted us to interrupt the experiment at this time.

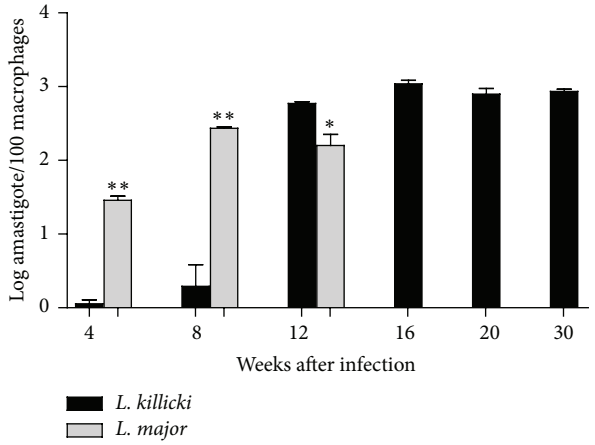


Figure 3: Parasite burden measured in ears of BALB/c mice infected with *Leishmania killicki* or *Leishmania major*. Following intradermal injection of 10^3 metacyclic promastigotes, the parasite load was estimated as described in Materials and Methods. Each bar is representative of the mean of five determinations ± standard deviations. Statistical analysis ($^*p < 0.05$ and $^{**}p < 0.01$) was performed using Student's t-test under GraphPad Prism ($n = 5$ mice/group).

at week 30. The maximal parasite burden was significantly higher than for *L. major*-infected mice ($p < 0.001$). In *L. killicki*-infected mice, no further parasite expansion was observed after the 12th week, while the induration thickness increased (Figures 1 and 3).

Beyond the capacity of *Leishmania* to replicate at the inoculation site, the dissemination of *L. killicki* and *L. major* in various tissues or organs was further investigated (Table 1). *L. major* and *L. killicki* were both detected in culture at 12 weeks. Indeed, DNA detection evidenced the presence of parasites at the inoculation site from the 2nd week after infection for both species. However, the major difference was the delay observed for the colonization of the organ following infection. *L. major* colonized draining lymph nodes and spleen more quickly, 2 weeks after infection compared to 4 weeks for *L. killicki* (Table 1). DNA was detected in liver 4 weeks after infection for both species. After this time point, the PCR remained positive until the 12th week for *L. major* and the 20th week for *L. killicki*.

3.3. Compared Clinical Evolution of L. killicki and L. major Lesions and Parasite Load under Antimonial Treatment. The ear thickness was roughly similar in *L. major*-infected mice when treated with drug or PBS (Figure 4(a)). The reduction in the lesions observed in *L. major*-infected mice 10 weeks after infection was due to tissue necrosis and loss. These results show that antimony has no or undetectable effect on *L. major* lesions after the end of the treatment, although at concentration we used in our experiment (Figure 4(c)). Interestingly, the expansion of lesion halted in treated *L. killicki*-infected mice submitted to chemotherapy (Figure 4(b)). Accordingly, the ear thickness index increased constantly during the time course of the treatment (Figure 4(c)).

Glucantime treatment slightly affects the parasite load with only 0.12-fold reduction of the mean number of *L. major* amastigotes/100 macrophages in lesions (see Figures 5(a) and 5(c)). However, it is more efficient in *L. killicki*-infected mice, which exhibited twofold reduction in *L. killicki* amastigotes (Figures 5(b) and 5(c)). Parasite loads were significantly different as early as one week after the beginning of the treatment, that is, at the third injection of Glucantime ($p < 0.001$). Overall, a better concordance between parasite load and ear thickness was observed in mice infected with *L. killicki* during antimony treatment than in mice infected with *L. major*.

4. Discussion

In this work we seek for the first time to establish a CL animal model for the emerging *Leishmania* parasite in Algeria: *L. killicki*. In an attempt to reproduce the natural biology of *Leishmania* transmission, Belkaid et al. [19] established a dermal model of infection in which low numbers of *L. major* promastigotes were injected into the ear. Based on this methodology, we established a dermal model of infection using *L. killicki,* which involved a cutaneous lesion in the ear dermis of mice, similar to those observed in patients with *L. killicki* infection, that is, localized lesions that do not heal spontaneously. The appearance of lesions induced by *L. killicki* is different from those produced by *L. major* or *L. tropica sensu stricto* [6, 16]. Experimental infection of BALB/c mice with *L. tropica* produced lesions that developed up to 3 months after infection and then regressed [6, 16]. In mice infected with *L. killicki*, we never observed regression of the ear thickness but a continuous extension of the lesion. Indeed, in humans, *L. killicki* induces chronic lesions that persist up

Table 1: Detection of *L. major* and *L. killicki* DNA and parasitesin various tissues of BALB/c mice. ND, not determined.

Leishmania	Tissue or organ	PCR after inoculation/LIT culture					
		2 weeks	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks
L. major	Ear (inoculation site)	+	+	+	+/ND		
	Draining lymph node	+	+	+	+/+		
	Spleen	+	+	+	+/+		
	Liver	−	+	+	+/+		
L. killicki	Ear (inoculation site)	+	+	+	+/ND	+	+
	Draining lymph node	−	+	+	+/+	+	+
	Spleen	−	+	+	+/+	+	+
	Liver	−	+	+	+/+	ND	ND

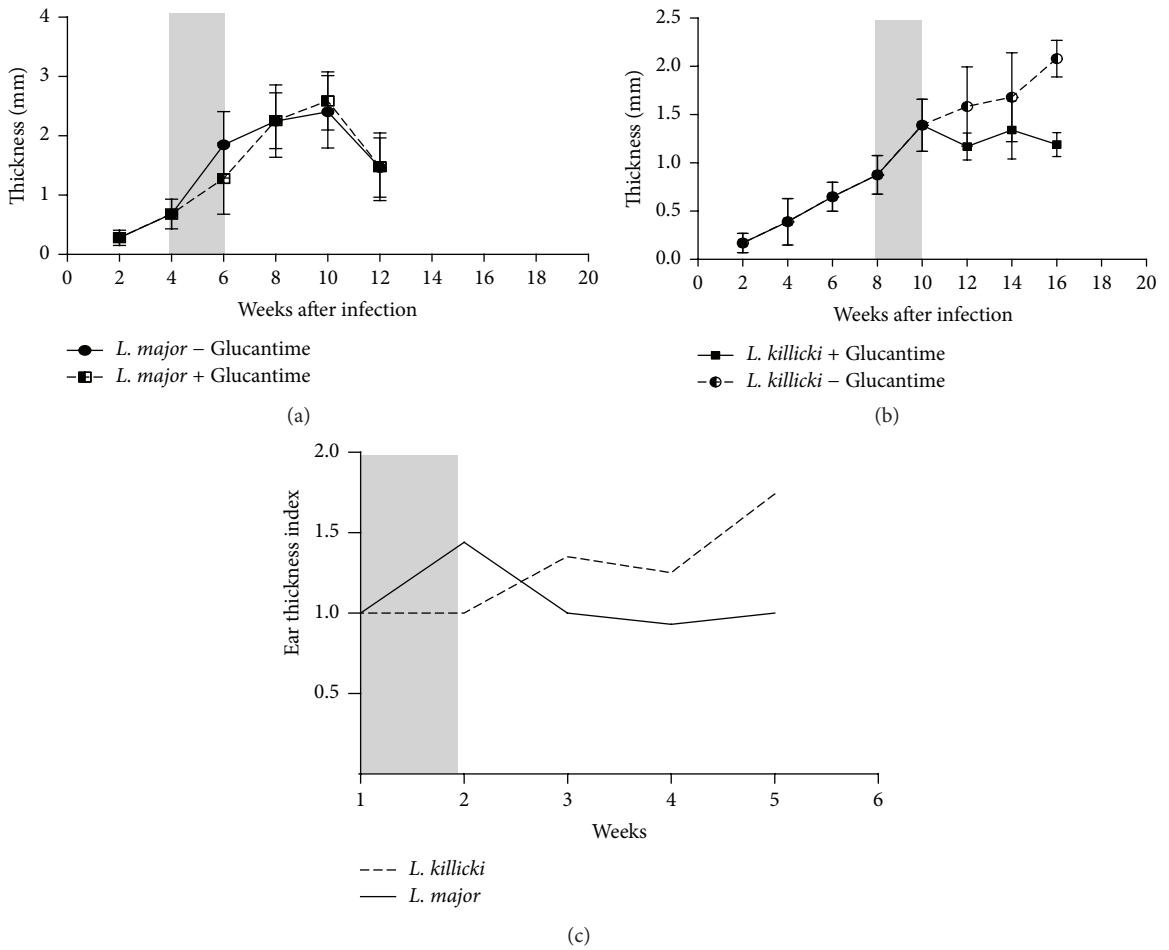


Figure 4: Effect of Glucantime treatment on ear induration in mice infected with *Leishmania major* (a) or *Leishmania killicki* (b) and on the ear thickness index (c). Grey bars indicate the Glucantime treatment period. The induration thickness is expressed as the difference of thicknesses between infected ears and contralateral noninoculated ear (control). Data are expressed as mean values ± standard deviations (error bars) ($n = 5$).

to one year [2]. In the mouse, we observed that *L. killicki* lesions do not spontaneously heal but persist and develop all along the experiment. Further studies will be required to understand the underlying immunological determinants allowing the long-term persistence of *L. killicki* in lesions. In the old world, only *L. infantum* and *L. donovani* are known to cause visceral forms of leishmaniosis (VL). Nevertheless, these two species can also be the causative agents of some forms of CL [20, 21]. In the same way, *L. tropica* as *L. major* cause CL in humans but different studies reported the isolation and characterization of *L. tropica* in patients with VL [8, 22–24]. In mice, *L. tropica* or *L. major* are also known to cause visceral infections; that is, they are detected in spleen and liver but the rapidity of the visceral dissemination,

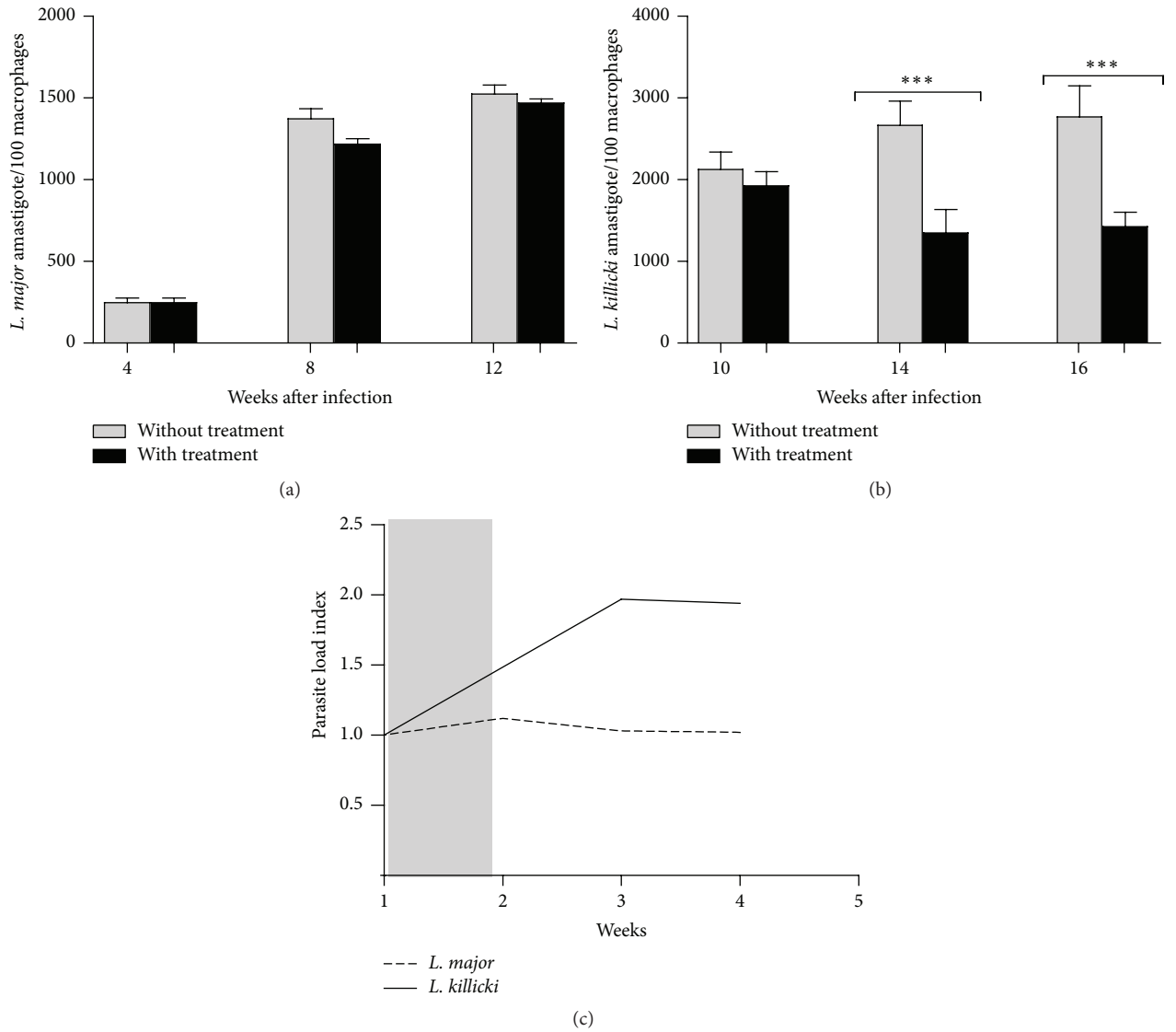


FIGURE 5: Parasite load in mice infected with *Leishmania major* (a) or *Leishmania killicki* (b) and evolution of the parasite load index following antimony treatment (c). The parasite load index was calculated as follows: mean parasite load in untreated mice/mean parasite load in treated mice. Each bar is representative of the mean parasite load determined in 5 mice ± standard deviations (error bars). Statistical analysis ($^{***}p < 0.01$) was performed using Student's t-test under GraphPad Prism ($n = 5$ mice/group). Grey bar indicates the Glucantime treatment period.

the symptoms observed, and the parasite load differ between *L. tropica* and *L. major* [25–27]. We observed that *L. killicki* had also the capacity to disseminate and to persist in internal organs of mice. Nevertheless, the ability of *L. killicki* to cause VL in humans remains unknown and has not been reported to date.

L. killicki is considered as an emerging pathogen resistant to pentavalent antimonial treatment [1, 2, 7, 10]. Therefore, it would be interesting to evaluate the suitability of this *L. killicki* infection model in experimental pharmacology. This infection model could test the leishmanicidal activity of known drugs and be predictive of their clinical efficacy. Currently, animal models of infection optimized to test antileishmanial compounds are available for *L. major* and *L. amazonensis* [28, 29]. Human infections caused by *L. tropica* complex are considered to be refractory to most classical treatments, including antimonial containing drugs, unlike *L. major* infections [22, 30, 31]. In our study, we have observed that antimony treatment does not affect the outcome of lesions induced by *L. major* in contrast to *L. killicki*. In fact, the outcome of the experiment cannot be further followed in mice infected with *L. major* because of lesions' necrosis. The treatment of mice infected with *L. killicki* resulted in the reduction of lesions size and of the thickness which were associated with a drastic diminution of the parasite load at the inoculation site.

5. Conclusions

Our observations support the notion that this *L. killicki* model of infection has several practical advantages over the *L. major* model. First, the drug regimen can be evaluated

over a longer time period (up to 30 weeks) as compared to *L. major*, where tissue loss and the appearance of ulcerative lesions limit the time course of the experimentation. Second, the intense and continuous parasite multiplication at the inoculation site makes it possible to more easily assess the leishmanicidal activity of new molecules in a simple way. The continuous emergence of antimony resistance in *Leishmania* spp. in various parts of the world necessitates the development of new alternative antileishmanial drugs [32–34]. To this end, this study supports the notion that this new *L. killicki* experimental model might be useful for screening and validating new compounds *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Naouel Eddaikra and Ihcene Kherachi Djenad contributed equally to this work.

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