Clinical and Developmental Immunology

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Editorial Board

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Editorial

The Immunology of Zoonotic Infections

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Zoonotic infections are in general defined as infections transmitted from animal to man (and less frequently vice versa), either directly (through contact or contact with animal products) or indirectly (through an intermediate vector as an arthropod or an insect) [1]. Although the burden of zoonotic infections worldwide is major, both in terms of immediate and long-term morbidity and mortality [2, 3] and in terms of emergence/reemergence and socioeconomical, ecological, and political correlations [4], scientific and public health interest and funding for these diseases remain relatively minor.

Zoonoses include diseases induced by diverse pathogens (bacteria, viruses, fungi, and parasites), but a common pattern for the majority of them is their complexity: this term refers not only to their ecology, range of clinical characteristics, and diagnostic and therapeutic challenges, but foremost to their immunology. In fact, all other ecological, clinical, diagnostic and therapeutic complexities emerge from this multifaceted zoonotic pathophysiology, as certain papers of this special issue outline.

The paper by A. Agnone et al. in the present special issue underlines the complexity, as well as the derived therapeutic and diagnostic significance, of antigen-specific T-cell immune response in varying zoonotic infections. The authors underline the importance and difficulty of understanding these complex pathogenetic mechanisms, as well as their significance for the development of preventive vaccines. The paper by P. Skendros and I. Mitroulis focuses on another specific and increasingly recognized as important part of zoonotic pathophysiology, that of autophagic response in certain intracellular zoonoses, outlining how this autophagic machinery can be exploited by zoonotic pathogens, typically culminating in chronic infections.

Our ability to understand pathogenetic mechanisms in the subcellular level has greatly evolved in recent years, and the paper by P. Mansueto et al. demonstrates how this progress has allowed us to extensively understand the intracellular interactions observed in rickettsial infections, a group of zoonoses that includes diverse pathogens with certain common characteristics. The paper by C. F. Oliveira et al. is one of the papers in this special issue attempting to translate theoretical knowledge in experimental data: the authors demonstrate in a mice model how a particular cytokine receptor deficiency induces a specific default in immunity against *Leishmania major* that results in specific clinical manifestations.

Other three papers all deal with the immunology of cystic echinococcosis (CE), a worldwide prevalent parasitic zoonosis with major public health burden: the paper by W. Zhang et al. summarizes all novel developments in the understanding of host immune responses in CE and proposes potential translation of this understanding in the diagnostic field. The paper by A. Siracusano et al. adds a different angle at our understanding of these mechanisms

and discusses how the evolution of proteomics would further enhance our pathogenesis understanding. The paper by L. Piccoli et al. focuses on an aspect of immunologic diagnosis and activity evaluation of CE, outlining the difficulties such approaches may impose.

There are three papers that focus on parameters of the immunology of brucellosis, possibly the dominant bacterial zoonotic infection worldwide and known to induce a potentially noneradicable disease. The paper by F. S. Oliveira et al. is the first to demonstrate the critical role and the specific nature of nucleotide-binding oligomerization domain (NOD) receptors in the immune response against *Brucella abortus*. The paper by E. D. Avila-Calderón et al. focuses on the potential utility of certain outer membrane vesicles of *B. melitensis* in vaccine development, using an experimental mice model, while the paper by G. C. Macedo et al., in a similar experimental model, evaluates the exact role of interferon gamma in host protection against *B. abortus*.

Other two papers focus on viral zoonoses: the paper by J. Juno et al. is an up-to-date review of our knowledge of both intrinsic and pathogen-related immunomodulating factors affecting susceptibility to and clinical severity of pandemic H1N1 and avian influenza (H5N1) infection, while the paper by J. A. Silva Gomes et al. discusses the peculiarities of immune response induced by vaccinia virus (an orthopoxvirus similar to smallpox) both in humans naturally infected and in an experimental model.

The paper by M. Mosepele et al. focuses on the peculiarities of *Bartonella* infections outlining our limited knowledge of specific pathogenetic events taking place in immunocompromised patients, a subgroup of patients that is growing and is growingly susceptible to both *B. quintana* and *B. henselae*.

Altogether, these papers underline the multifactorial nature and the multiple potential applications of zoonotic pathophysiology knowledge. They further underline the need for enhanced scientific interest (also translated in enhanced funding) in zoonotic pathophysiology: this interest would not only allow the expansion of our theoretical knowledge but would eventually allow for improved understanding of host susceptibility, and thus prevention, improved diagnosis (since zoonoses do not fall into the typical microbiological diagnostic terms of culture positivity or eradication), and possibly novel therapeutic or preventive approaches through development of sophisticated vaccines.

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Review Article

Antigen-Specific T Cells and Cytokines Detection as Useful Tool for Understanding Immunity against Zoonotic Infections

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Zoonoses include a broad range of diseases, that are becoming of great interest, due to the climate changing, that cause the adaptation of vectors to new niches and environments. Host immune responses play a crucial role in determining the outcome of infections, as documented by expansion of antigen-specific T cells during several zoonotic infections. Thus, understanding of the contribution of antigen-specific T-cell subsets in the host immune response is a powerful tool to evaluate the different immunological mechanisms involved in zoonotic infections and for the development of effective vaccines. In this paper we discuss the role of T cells in some eukaryotic and prokaryotic infectious models.

1. Introduction

Zoonotic diseases are a significant burden on global economies and public health [1] and are due to the unaware role of wild and domestic animals, which act as reservoir or hosts of the etiological agents. More than 60% of emerging infectious diseases are constituted by zoonoses and the majority of these are increasing significantly over time [2]. In 2009 the World Organization for Animal Health (OIE) has commissioned Civic Consulting to conduct a study on the Cost of National Prevention Systems for Animal Diseases and Zoonoses, estimating that in developing and transition countries substantial differences in the public expenditure for the National Prevention System for Animal Diseases and Zoonoses exist, reaching from 10 million international dollars to 167 million international dollars [3]. The impair they cause should be attributed not only to human and animal suffering but also to the hampering agricultural production, the decreasing of food availability, and the creation of barriers to international trade [1], as well as the veterinary management, the maintenance of surveillance plans, and the capillary control in the food industry chain of production.

Many zoonotic agents are transmitted by vectors, others by contaminated water or food, and others by direct transmission. A broad range of pathogens can be responsible for zoonoses, ranging from virus to prokaryotic to eukaryotic (unicellular or multicellular), and the great difference in the antigenic input for the immune system of the hosts implies that many different branches of immunity could be involved in protection or pathogenesis.

T cells play a pivotal role in immune functions since they are able to act not only differentiating in different subsets (including $\gamma\delta$ T-lymphocytes and Cytotoxic T-Lymphocytes) but also inducing the production of antibodies that inhibit the pathogen spreading, both directly and with the help of other branch of the immune system.

Homeostatic cytokines are those factors able to regulate multiplication and differentiation of many cell types; T cells are dependent on contact with IL-2, IL-7, and IL-15, for their survival and intermittent homeostatic proliferation [4]. T-helper cell differentiation is instructed by distinct

environmental cytokines, that upregulate the expression of lineage-specific transcription factors and inhibit the alternate differentiation pathways [5]. The contact between the naïve T cell and the antigen induces the expression of IL-2 and IL-2 receptor leading to the entry of the T cell into several rounds of proliferation and to the differentiation in Th1, Th2, Th17, and induced regulatory T (iTreg) cells. The process consist of an intriguing cytokines puzzle, where IL-4 plays a major positive feedback role in Th2 differentiation, and IFN-γ, together with IL12, determines Th1 induction [6]. IL6 and IL1 are necessary for Th17 production, while the role of TGFβ needs still to be deeper investigated [7, 8]. Finally, activated naïve CD4 T cells stimulated by TGF-β in the absence of proinflammatory cytokines develop into iTreg cells [9].

The complex network of cytokines function is resolved in a balance from different T-cell activation pathways (Th1/Th2, Th1/Treg, Th2/Treg, Th1/NK, and/or $\gamma\delta$ T cells). Although T-cell-mediated immune response during zoonotic infections is poorly studied, the facilities in the setting-up experimental conditions make it good system for a deeper investigation on the specific activation of T-lymphocytes.

It is well known that protozoan, helminthic parasites, and intracellular bacteria are able to survive within the host, in spite of the activation of both innate and adaptive immune response [10]. Zoonotic infections caused by eukaryotic organisms are intriguing systems where the antigen-specific T-cell expansion can be studied [11].

Helminthes have the ability to drive the differentiation of naïve CD4 T cells to the Th-2 subset of effector cells which are able to eliminate the pathogens by the actions of antibodies induced by Th2 cytokines. During a protozoarian infection, protozoa are usually phagocytosed into macrophages, previously activated by Th1 lymphocytes, and are able to survive evading host immune response. As it happens in the case of intracellular bacteria, infected cells loose the ability to kill the pathogen, and Cytotoxic T-Lymphocyte- (CTL-) mediated immune response is needed for the elimination of microorganisms into macrophages [12] (Figure 1). The naïve T cells encounter the antigen in the peripheral lymph node, develop toward effector cells, and migrate to the site of infection for the killing of infected cells. This process is finely tuned by cytokines cross-talk and microbial ability to evade host immune response.

B cells and humoral response play the main role in the clearance of extracellular bacteria. Nevertheless, a certain enrolment of T-cells has been demonstrated [13]. In this paper, we draw attention on different mechanisms of T-cell-mediated immunity, in order to compare the mechanisms of immune modulation induced by various zoonotic agents.

2. T Cells and Cytokines Induced by Eukaryotic Zoonotic Agents

The nematode parasites *Toxocara (T.) canis* and *T. cati* choose dogs and cats as definitive hosts, respectively. Sometimes, when embryonated eggs are accidentally ingested by humans, larvae hatche in the small intestine, penetrate the intestinal wall, and cause the larva migrans syndrome [14]. Toxocariasis symptoms are classified according to the organs affected in visceral larva migrans (VLMs) and ocular larva migrans (OLMs). In the latter toxocariasis pathological effects on the host are restricted to the eye and optical nerve [15], while in the case of VLM, symptoms can persist for more than one year and include abdominal pain, coughing, headache, and normal or mildly elevated eosinophilia [16]. A recent survey [17] emphasizes that the seroprevalence value among humans is considerably high, thus demonstrating the relevance of this pathology. *T. canis* is able to control host immune response, through the modulation of cytokines produced by immune cells. The immunomodulatory effect has been demonstrated in mice, where the stimulation of normal macrophages with *T. canis* antigen *in vitro* induced IL-1α, IL-6, IL-10, and TGF-β, but not IL-12 and TNF-α [18]. Prototypical immune responses are characterized by increased lymphoproliferation of $CD4^+$ and $CD8^+$ T cells, increased production of IL-4 and IL-5, eosinophilia, and augmented production of IgE, as previously described in humans and mice [13–15]. As regards the immune response in dogs, it has been demonstrated that *T. canis* is able to induce antigen-specific IFN-γ production in pregnant dogs and in their puppies [19]. Blood mononuclear cells (BMCs) were isolated from pregnant dogs and their puppies and were cultured in the presence of ESAg (Excretory/Secretory Antigen of *T. canis*). Cytokine levels were tested in cultures' supernatants by ELISA, and it was noted that IL-10 concentration increases during pregnancy in infected animals while IFN-γ production decreases. On the contrary IL-10 concentration decreases with the age of infected puppies while IFN-γ amount increases. It appears clear that immune cells of infected dogs undergo *T. canis*-induced modifications. These modified pattern of cytokines detected in *T. canis* could be due to a synergistic effects of physiological changes of immunity during pregnancy and in the first month of life, and/or direct effects mediated by parasite interaction with host immunity. The finding that IL-10 and IFN-γ levels were significantly modified in infected pregnant dogs and their puppies provides new perspectives for immunotherapeutic interventions based on switch of Th2 to Th1 cytokine pattern in females before pregnancy.

Another system to understand the role of T cells in eukaryotic zoonotic infections is echinococcosis. Alveolar echinococcosis is caused by the metacestode stage of *Echinococcus multilocularis*. The definitive hosts are the foxes, which release Echinococcus eggs in the foecal matter, spreading them in the environment. Little rodents acquire the infection by ingesting eggs and carry the infection in their liver. Humans are aberrant intermediate hosts [20]. In humans, metacestode stage of the worm affects the liver, where an abdominal mass develops; other symptoms may arise like abdominal pain, jaundice, and liver failure [21]. The severity of the disease is dependent on the genetic background of the host and on the balance between the Th1-related immune response, associated with protection, and the induction of the immune tolerance by the parasite itself [22]. In experimentally infected C57BL/6J mice the

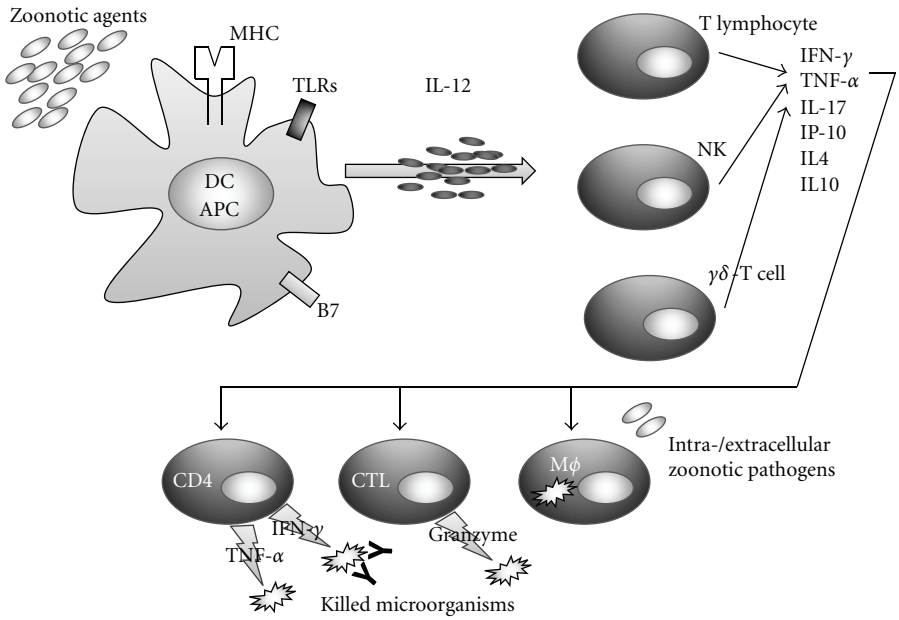

Figure 1: Schematic network of cells and molecules in response to zoonotic agents. An "oversimplified" scenario constituted by various cells and molecules involved both in binding of epitopes derived from pathogens and in the effector mechanisms hereby represented. APCs bind zoonotic derived epitopes and present them to various types of lymphocytes, in the context of MHC molecules and/or Toll-Like Receptors (TLRs). These subsets, producing different cytokines, could activate effector "protective" mechanisms involving macrophage killing, cytotoxic activity by CTL and/or CD4, and release of various cytokines, thus leading to the damaging of zoonotic pathogens. The killing by CTL, that could be not only CD8 but also NK cells, could be also due to an ADCC phenomenon with the contribution of antizoonotic epitopes,-specific antibodies.

promotion of the disease seems to be associated with the expansion of different T-cell subsets: spleen cells harvested at different time points after infection were stimulated *in vitro* with a crude parasite extract. A strong $CD4^+$ proliferative T-cell response was observed at the early stage of infection, and IFN-γ, IL-2, and IL-5 were produced within the first weeks after infection whereas the detection of IL-10 was slightly delayed [23]. Cystic echinococcosis is caused by *E. granulosus*. The main domestic cycle is maintained between dogs and sheep, with man as accidental intermediate host. The disease is acquired by ingesting eggs, originating from the faeces of definitive hosts (dogs, wolves, and other carnivores) [24], and it typically affect, the liver. It is often asymptomatic, but in case of rupture of the cyst, secondary infection and anaphylactic reaction can occur. The most frequent complications are pain, obstructive jaundice, cholangitis and sometimes shock [25]. It has been demonstrated that a restimulation of PBMC from affected patients with the crude antigen induces an upregulation of IL-5 and IL-10 [26] as well as a downregulation of IL-1 and TNF-α mRNAs [27].

The opportunistic parasite *Toxoplasma gondii* belongs to the phylum apicomplexa. Feline acts as definitive hosts in its life cycle, while mammalians, including humans, are intermediate hosts. Human toxoplasmosis is usually asymptomatic or paucisymptomatic, but the parasite is able to cross the intestinal barrier and disseminate through the body, reaching muscle, central nervous tissues, eyes, and placenta [28]. Congenital toxoplasmosis may hesitate in retinochoroiditis and/or mental abnormalities [29].

The infection by *T. gondii* induces a strong cellular response essential for the host resistance [30]. In particular, it has been noted since 1990 that upon an *in vitro* stimulation with *T. gondii* antigen, a strong $CD8^+$ T-cells response, sustained also by $CD4^+$cells expansion, is mounted [31]. The role of $CD4^+$ in the activation of $CD8^+$ has been demonstrated in mice [32], where the generation of optimal numbers of antigen specific $CD8^+$ effector T cells was found to require $CD4^+$ T-cells help. The parasite is also able to induce a strong natural killer (NK) cells activation and macrophages production of IL-12, both ending in a massive IFN-γ production. The IFN-γ production is sustained by $\gamma\delta$-T lymphocytes [33] that help $CD4^+$ and $CD8^+$T cells to restrict parasite growth until the emerging of the complete adaptive response. It has been recently demonstrated that the $CD8^+$ T-cells response is sustained both by "homeostatic cytokines" IL-15 and IL-7 and that the absence of IL-15 or IL-7 alone does not affect $CD8^+$ T cell activation during acute toxoplasmosis [34], thus suggesting that these cytokines could act in synergy. Immune response of congenitally infected newborns to *T. gondii* undergoes to a process that leads to anergy [35, 36], probably due to a developing immune system of the infant. In this case, both $\alpha\beta$- and $\gamma\delta$-T cells become unresponsive when stimulated with *T. gondii*-specific antigen. Nevertheless, $V\delta2^+$ $\gamma\delta$ T cells are able to

lose tolerance before $\alpha\beta$-T-cells, and to confer protection against the chronic phase of infection in congenitally infected children [37]. Indeed, $\gamma\delta$ T cells are considered to undergo peripheral tolerance, thus persisting in blood longer than $\alpha\beta$ T lymphocytes which are deleted in the thymus during *T. gondii* infection [37].

A useful model to better understand immune response to eukaryotic zoonotic agents is constituted by Leishmaniasis and its related immunity. Leishmaniasis is a vector-borne disease caused by obligate intramacrophage protozoan parasite of the genus Leishmania and its incidence is increasing in nonendemic areas due to changing patterns of international travel and to population migration [38]. Visceral leishmaniasis (VL) or kala-azar is one of several diseases caused by more than 20 species of the protozoan parasite Leishmania. The infection tends to affect mainly children, but immunosuppression and HIV increase the possibility to contract the illness. The common symptoms are fever, malaise, shivering or chills, weight loss, anorexia, and discomfort in the left hypochondrium [39]. In experimental *L. major* infections genetically resistant mice develop a T-cell response dominated by a $CD4^+$ (Th1) phenotype characterized by IFN-γ secretion while in susceptible mice the dominant response is a $CD4^+$ (Th2) phenotype characterized by interleukin IL-4, IL-5, and IL-13 secretion [40]. These observations of *L. major* in mice led to the emergence of the Th1/Th2 paradigm as opposing cytokine responses in the control of infections [41, 42]. The balance of Th1 to Th2 responses determines the outcome to infection. In the natural disease both Th1 and Th2 cellular subtypes are activated. Resistance to infection depends on production of cytokines such as IFN-γ, TNF, IL-2, and IL-12. These cytokines stimulate cell-mediated immunity which eliminates the infection activating leishmanicidal activity of macrophages [41, 42]. The infection in dogs shows different clinical presentations, from subclinical/asymptomatic to a fully developed disease, depending on the host's immune responses. The Th1/Th2 dichotomy is not clear in the different forms of canine leishmaniases, because it depends on physiological status of the infected subject. The production of IL-4, IL-5, IL-6, and IL-10, which in turn promote B-cell proliferation and antibody production, is the cause of susceptibility of dogs, which become not able to control the infection [43–45]. Our experience is focused to evaluate cytokine expression level with a quantitative real-time PCR assay to measure expression levels of cytokines relative to either Th1 or Th2 patterns in the blood of naturally infected asymptomatic dogs. High expression levels of IL-2 and IFN-γ were detected at the first observation, which decreased over time. Opposite cytokine-based effects were detected in infected dogs. In those that had a clinically evident outcome, IL-2 and IFN-γ were initially not expressed, but their levels suddenly increased with the appearance of clinical signs [43]. Furthermore from our study it was confirmed that IL-12 represents a marker of active disease, while IL-18 cannot be involved in the progression from asymptomatic to active disease. These data suggest that response to Leishmania in the dog does not fit into a specific cytokine profile.

3. Antigen-Specific T Cells and Derived Cytokines Detection in Prokaryotic Infections

Among prokaryotic microorganisms able to cause zoonotic disease, Leptospira, Brucella, and Mycobacteria offer suitable models to analyze the role of immune response against these pathogen since the related immunity could involve different antigen-specific T cell subsets. *Leptospira interrogans* is one of the main causative agents of leptospirosis. The pathogen is able to persist in the kidneys of infected (wild and domestic) animals and is spread in the environment through their urine. It is transmitted to humans through skin abrasions and causes haemorrhage, diarrhoea, renal impairment, and aseptic meningitis [46]. Phagocytosis is the main process that allows the clearance of the pathogen, and it has been recently demonstrated that the bacteria undergo a complex transcriptional regulation in order to evade host immune response [47]. In particular they downregulate the major OMPs (Outer Membrane Proteins) through the action of a hypothetical transcriptional factor. It is well accepted that humoral immunity has an important role for the elimination of extracellular bacteria, but sometimes antibodies alone could not be sufficient, especially in the case of *L. borgpetersenii* serovar Hardjo [48]. In this and other cases, IFN-γ plays an important role for the activation of macrophages and the production of IgG2 class of immunoglobulins [49, 50]. The involvement of a cellular immune response has been recently demonstrated: a strong Th1 response was recorded by the observation of the IFN-γ production following the *in vitro* stimulation of vaccinated bovine PBMC with the specific antigen [51]. The results from vaccinated animals indicated that approximately two-thirds of IFN-γ^+ cells were within the $CD4^+$ T-cell population while the remaining one-third were $\gamma\delta$ T cells [51]. Furthermore, Guo et al. have recently reported the existence of specific cytotoxic $CD8^+$ T cells in patients with leptospirosis and have detected a potential epitope of the leptospiral protein LigA, able to elicit specific cytotoxic T-lymphocyte (CTL) responses [13]. Naiman and Guo suggest that Th1 response to Leptospira requires the cooperation between two or more T cell subsets like $\gamma\delta$, $CD8^+$, $CD4^+$, and so forth. In Leptospira-infected hamsters a new soluble factor was shown to be important for the protection: IP-10 [52]. This evidence points to T cell-derived chemokines in zoonosis. These proteins are able to induce cell migration from lymphoid organs to affected tissues and they are also considered markers of T cell maturation [53]. Indeed, future approaches for a deeper analysis of T cell response in zoonoses could be comprehensive of the characterization of the released chemokines and their receptors.

A very hot field in veterinary immunology is represented by T cell responses against intracellular bacteria. Tuberculosis and Brucellosis remain major worldwide health emergencies among zoonotic bacterial infections, and a better understanding of the host immunological reactions to these pathogens is fundamental for improving both therapies and vaccines strategy, as well as to prevent dissemination of the

infectious agents in the herds. Tuberculosis causes in host mild fever and a wide range of symptoms depending on the localization of the Mycobacterium (pneumonia, kidney failure, meningitis especially in children, etc.) [54].

Animal tuberculosis is mainly observed in cattle (less frequently also in horses, swine, dogs, cats, sheep, and goats), caused by *Mycobacterium (M.) bovis*, and in birds, due to *M. avium*. Human tuberculosis is mainly caused by *M. tuberculosis*, but around 10% of total infections are due to *M. bovis*, typically as professional disease, while *M. avium* can cause disease in immunodeficient patients [55]. Dogs and parrots are highly susceptible to *M. tuberculosis* by the contact with infected humans. T-lymphocytes play a central role in the control of *M. tuberculosis* replication, as this infection evokes a strong cell-mediated immune response. Protective immunity against *M. tuberculosis* is due to adaptive cellular immune responses, and protective immunity correlates to the induction of T cell cytokines following antigen specific stimulation. $CD4^+$ and $CD8^+$ T cells are key components of anti-mycobacterial immunity [56, 57]. Both IFN-γ production and cytotoxic activity against infected target cells contribute to bacteria killing with lysis of infected cells [58, 59].

T cells response after *in vitro* stimulation of human PBMCs with *M. tuberculosis*-specific antigens (e.g., Purified Protein Derivative, or PPD) can be assessed by measuring intra- and extracellular IFN-γ [60]. The severity of *M. tuberculosis* infection may be detected by measuring $CD4^+$ and $CD8^+$ T cells, as their numbers markedly decrease in patients with severe tuberculosis, which can be a sign of suppressed cellular immunity in these patients [60]. Particularly, patients with active TB have a lower number of both $CD4^+$ T cells and their naïve, effector, and late differentiated memory subsets [61], with a drop in all the three phenotypic populations. Similarly, $CD8^+$ T cells counts were also significantly different between infected and negative patients. At least partially, these disturbances seem to be restored to baseline after successful therapies [61].

In our experience with cattle [62] it has been showed that cocktails of epitopes from ESAT-6 (the 6 kDa early secretory antigenic target of *Mycobacterium tuberculosis*) are recognized with high frequency by $CD8^+$ T lymphocytes of naturally infected cattle, thus confirming a role of ESAT-6 specific $CD8^+$ T cells in the response to *M. bovis*. Nevertheless, the number of IFN-γ-positive CD8-negative cells was larger than that of IFN-γ^+ $CD8^+$ T cells, indicating that IFN-γ^+ $CD8^+$ T cells are not the dominant subset responding to stimulation with ESAT-6-derived peptides. Nevertheless, ESAT-6-specific T-cell expansion could be useful to detect the early phase of the disease thus limiting the dissemination of *M. bovis*.

Other cytokines such as TNF-α, IL-2 [63], MCP-2 [64], and IP10 [65] were shown to be involved in the anti-mycobacterial immune responses in humans; Th1- and other cytokines interacting with macrophages are commonly considered as mediators of anti-mycobacterial biological agents. When reagents for the detection of these cytokines in vertebrates will be available, it could be intriguing to understand the role of these cytokines in mycobacterial immune response also in veterinary infections.

Brucellosis is a multisystemic disease with a broad range of symptoms, usually beginning with acute febrile illness, headache, malaise, and myalgia. Gastrointestinal signs as vomiting, anorexia, and nausea may also occur [66]. Humans are susceptible to *Brucella (B.) suis*, *B. Abortus,* and *B. canis*, and, more frequently, to *B. melitensis*. The disease can be transmitted by both direct and indirect contact with infected animals or secretions, or by eating contaminated food (especially unpasteurized milk and fresh cheeses). Interhuman transmission is extremely rare [67].

Brucella invades and proliferates within monocytes. In addition to the central role of monocytes/macrophages, other cells of the innate immune response are recruited and influence the interaction between bacteria and host. For instance, human Vγ9Vδ2 T cells play an important role in the early response to infection [67], and their number dramatically increases in the peripheral blood of patients with acute brucellosis [68], reaching 30% of the total T lymphocytes. Vγ9Vδ2 T cells are specifically stimulated by Brucella to secrete TNF-α, important for the autocrine activation of macrophage functions, IFN-γ, and other cytokines [69]. *In vitro*, Vγ9Vδ2 T cells exhibit a strong cytotoxicity against Brucella-infected cells. Vγ9Vδ2 T cells decrease the development of intracellular Brucella releasing lytic granules and/or acting through Fas-mediated signals to lyse infected macrophages. It was also shown that the recruitment of NKG2D by its ligands is sufficient to induce cytokine production and the release of lytic granules thus increasing the TCR-triggered responses of Vγ9Vδ2 T cells. The interaction between NKG2D and its main ligand expressed on Brucella-infected macrophages, UL16-binding protein 1 (ULBP1), is involved in the inhibition of bacterium development [69]. As demonstrated in the case of Vγ9Vδ2 T cells, it was shown that also NKT cells are able to exert an anti-Brucella *in vitro* activity, either secreting cytokines or killing infected macrophages [70]. NKT and Vγ9Vδ2 are considered as quite unrestricted T cells as they do not recognize MHC and peptides, but they expand following stimulation with nonpolymorphic MHC-like molecules CD1 and/or with nonpeptidic and glycolipid ligands. A cross-talk between Vγ9Vδ2 and NKT, due to cytokines released in the milieu, could be responsible for the activation of NKT in synergy with a possible upregulating role of CD1 molecules expression exerted by Brucella antigens. The previously described subsets activated during Brucella infection could exert a protective role during Brucella infection through their potent cytotoxic activity.

4. Concluding Remarks

Each microorganism hereby evaluated elicits a particular type of immune response. A "classical" Th1-mediated protective immune response was detected during zoonotic infections like leishmaniasis or tuberculosis. Toxoplasma-, Brucella- and Leptospira-induced immune response involves a wide range of T cells including $\gamma\delta$ and NKT cells. The *in vitro* and *ex vivo* detection of T cells upon stimulation with

the specific antigen allows going insight in the host/pathogen interaction. The equilibrium established after such dialogue is critical for the further ongoing of the infection. A complex network of T cells, cytokines, and chemokines could be studied to better understand the interactions between zoonotic agents and receptors of innate and adaptive immunity. This tool could be useful to develop vaccines and immunotherapies in the next future.

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Clinical Study

Serum Cytokine Profile by ELISA in Patients with Echinococcal Cysts of the Liver: A Stage-Specific Approach to Assess Their Biological Activity

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To investigate the usefulness of serum cytokine dosage in the clinical management of cystic echinococcosis (CE), we analyzed serum levels of Th1 and Th2 cytokines in patients with hepatic CE in different cyst stages, CE1-2 (active), CE3a-3b (transitional), and CE4-5 (inactive). *Ex vivo* assessment of Th1 (IFN-γ) and Th2 (IL-4, IL-13, and IL-10) cytokines in sera was carried out using ELISA. IL-10 was undetectable in all serum samples of patients and controls, while a few sera contained measurable amounts of IFN-γ, IL-4, and IL-13. No statistically significant difference was found between the percentages of positive samples for each cytokine and the different groups analyzed (patients/controls, stage, number, location, and size of the cyst, serology, and sex of patients), with the exception of the association of IL-4 and IL-13 with the cyst stage. Overall, this investigation showed many limits of serum cytokine dosage as a marker of biological activity of echinococcal cysts. Because of low sensitivity and lack of specificity of this test, we believe that other ways to evaluate *ex vivo* biological activity of the cysts should be explored.

1. Introduction

Cystic echinococcosis (CE) is a chronic infection caused by the tapeworm *Echinococcus granulosus*. In humans, the larval stage of the parasite can develop and form cysts in almost any organ, especially the liver and the lungs [1]. Diagnosis and clinical decision making of CE are currently based on imaging techniques, mainly ultrasound (US) and, to a lesser extent, on serological techniques.

To date, serology is not standardized, and specific anti bodies may persist for a long time, even after complete surgical removal of the cyst [2, 3]. Furthermore, biological activity of transitional cysts does not always match the US appearance of the echinococcal cyst [4]. As a consequence, cyst progression towards either inactivation or chronicization can be assessed only by changes in US appearance of the cyst and, to a lesser extent, by variation in antibody titers, which is demonstrated only over long-term followup, thus making serology less useful than US [5, 6].

To evaluate the biological activity of the cyst as a tool for clinical decision making, in the last decade several studies have tried to look for markers of the immune response against *Echinococcus granulosus*. Some *in vitro* studies, investigating cytokine production from peripheral blood mononuclear cells of CE patients, demonstrated the presence of both Th1 and Th2 response against the parasite. During chronicization of the infection, the more permissive Th2 response predominates in patients with active (or not cured) cysts over the parasite-damaging Th1 response which, on the contrary, is more active in patients with inactive (or cured) cysts [7–10].

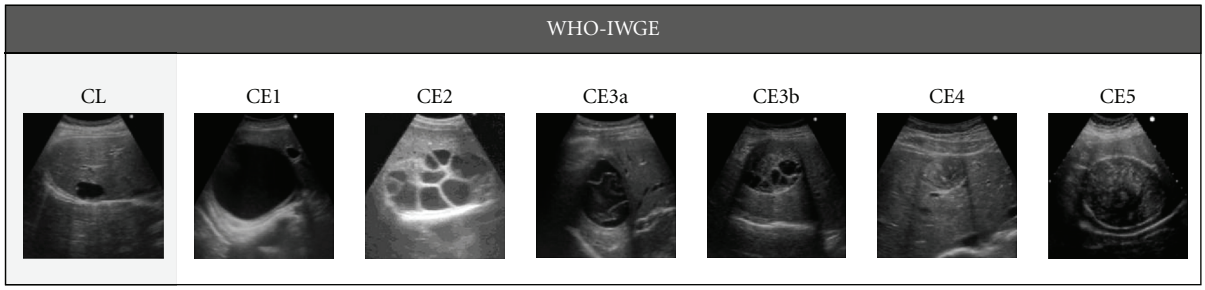

Figure 1: WHO-IWGE ultrasound classification of echinococcal cysts.

Other *ex vivo* studies, which detected cytokines in sera of CE patients, confirmed the association between cytokine production and outcome of the disease. Rigano et al. reported a higher serum level of Interleukin-4 (IL-4) and IL-10 in patients who did not respond to therapy compared to those who responded; Bayraktar et al. showed higher concentrations of IL-2, IL-4, and IL-10 in CE patients before treatment compared to those who were treated and to healthy controls; Mezioug and Touil-Boukoffa observed the coexistence of elevated levels of Interferon-γ (IFN-γ), IL-12, IL-16, IL-18, IL-4, IL-5, IL-10, and IL-13 in most sera of CE patients compared to healthy controls [11–13].

Although these studies showed an association between serum cytokine concentrations and active CE, in a recent study we could not confirm such association for all cytokines, as only a subgroup of CE patients with transitional cysts showed increased IL-4 levels compared to other subgroups and negative controls [14]. Additionally, these studies did not stratify patients according to the different cystic stages at US, which have been shown to correlate well with the biological activity of the cysts, with the exception of transitional stages [4, 15].

In this study, we analyzed serum levels of IFN-γ, IL-4, IL-13, and IL-10 in patients with hepatic CE in different US stages, to evaluate *ex vivo* the association of cytokine production and the stage of the infection. A second aim of the study was to assess whether serum cytokine dosage, which could be easily implemented in a clinical setting, can reliably assess the biological activity of CE cysts.

2. Materials and Methods

2.1. Subjects and Serum Samples. Serum samples were obtained from 53 CE patients seen at the Department of Infectious Diseases of the IRCCS San Matteo Hospital Foundation in Pavia, Italy, and from 20 healthy controls. The study protocol was approved by the ethical committee of our institution, and all subjects gave their informed written consent. Diagnosis of CE was made by ultrasound and serological assays, and patients were selected according to these inclusion criteria: (i) presence of at least one CE cyst localized to the liver, (ii) no previous surgery for CE, and (iii) no albendazole (ABZ) treatment or ABZ discontinuation at least 24 months before the time of serum collection. The control group was constituted by people for whom CE could be excluded by both abdominal US and serological assays. Serum samples were collected during a period of two years (from March 2009 to March 2011) and stored at −80°C until assayed.

2.2. Ultrasound. All patients and controls were examined by a clinician with long-standing experience in US and clinical management of CE (EB) using a commercially available US scanner with 3.5–5 MHz convex probes (Aloka ProSound ALPHA 10, Tokyo, Japan). For each patient, number, stage, size and location of the cysts were reported. Cysts were classified according to the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) standardized US classification for CE [16] (Figure 1) as CE1 and CE2 (active), CE3 (transitional), and CE4 and CE5 (inactive). Transitional CE3 cysts were further divided into 2 subgroups, CE3a and CE3b, based on their difference in response to nonsurgical treatments and biological activity [17]. Patients having multiple cysts were classified according to the more active stage, in accordance with the results of Hosch et al. [4]. Cyst size was reported as small, medium or large, if the greatest cyst diameter was lower than 5 cm, between 5 and 10 cm, or greater than 10 cm, respectively. Cyst location in the liver was classified as being in the right, left, or fourth segment.

2.3. Serology. All patients and controls were tested for anti-*Echinococcus* antibodies by IgG enzyme-linked immunosorbent assay (ELISA, Cypress Diagnostic, Langdorp, Belgium) and indirect hemagglutination (IHA, Cellognost Echinococcosis; Dade Behring, Newark, USA) by the Laboratory of Parasitology of our hospital. All controls and patients visited were tested for IgG western blot (*Echinococcus* western blot IgG, LDBIO, Lyon, France) during their first visit at our clinic. ELISA was considered positive if optical density was greater than 1.1, while IHA tested positive for dilution greater than 1/64. Serology was defined as either positive or negative, if both ELISA and IHA tested either positive or negative respectively, and, if necessary, were confirmed by WB; serology was defined as doubtful if one test was not congruent with the other one(s).

2.4. Cytokine Assays. Serum concentrations of IFN-γ, IL-4, IL-13, and IL-10 were determined by ELISA commercial kits (human IFN-γ, IL-4, and IL-10 high-sensitivity ELISA kits

Table 1: Distribution of positive samples for IFN-γ, IL-4, and IL-13 in the different groups analyzed (patients/controls, stage, number, location, and size of the cyst, serology, and sex of patients). *Difference statistically significant (P value < 0.05); the remaining differences are not significant.

Group	Total	IFN-γ positive		IL-4 positive		IL-13 positive	
	no.	no.	%	no.	%	no.	%
Total subjects	73	13	17.8	12	16.4	10	13.7
Controls	20	5	25	5	25	2	10
Patients	53	8	15.1	7	13.2	8	15.1
Cyst stage							
CE1-2	5	0	0	1	20*	1	20*
CE3a	8	3	37.5	4	50	3	37.5
CE3b	20	4	20	1	5	0	0
CE4-5	20	1	5	1	5	4	20
Cyst number							
1	46	7	15.2	6	13	6	13
2	7	1	14.3	1	14.3	2	28.6
Cyst liver location							
Right segment	41	6	14.6	7	17.1	6	14.6
Left segment	5	1	20	0	0	0	0
IV segment	7	1	14.3	0	0	2	28.6
Cyst size							
Small	17	1	5.9	2	11.8	1	5.9
Medium	27	5	18.5	4	14.8	6	22.2
Large	9	2	22.2	1	11.1	1	11.1
Serology							
Positive	29	6	20.7	6	20.7	4	13.8
Negative	19	2	10.5	0	0	2	10.5
Doubtful	5	0	0	1	20	2	40
Sex							
Male	25	4	16	5	20	4	16
Female	28	4	14.3	2	7.1	4	14.3

and human IL-13 ELISA kit, Gen-Probe Diaclone, France) according to the manufacturer's instructions. All tests were performed in duplicate. The ranges of the sensitivity standard curve of the ELISA kits were 0.78–25 pg/mL for IFN-γ, 0.31–10 pg/mL for IL-4, 3.12–100 pg/mL for IL-13, and 1.56–50 pg/mL for IL-10.

2.5. Statistical Analysis. Differences in percentages of patients and controls with detectable levels of each cytokine were assessed by Fisher's exact test. The same test was applied to assess any associations between cytokines and stage, number, location, and size of the cysts, serology, and sex of patients. A P value of less than 0.05 was considered statistically significant, and all tests were two sided. Data analysis was performed with the STATA statistical package (Ver. 10.0, 2009, Stata Corporation, College Station, TX, USA).

3. Results

The results are summarized in Table 1.

3.1. Subjects. This study included 73 subjects, 53 of whom were patients with liver CE cysts in different US stages, while 20 were healthy controls; in the control group CE could be excluded by both abdominal US and serological assays that tested negative. Of the 53 patients, 25 (47.2%) were males and 28 (52.8%) were females. Forty-six (86.8%) harbored one cyst each, while 7 (13.2%) harbored two cysts each. Five had active CE1-CE2 cysts (9.5%), 8 had transitional CE3a cysts (15.1%), 20 had transitional CE3b cysts (37.7%), and 20 had inactive CE4-CE5 cysts (37.7%). Forty-one patients (77.4%) had their cysts located in the right segments of the liver, 5 (9.4%) in the left segments, and 7 (13.2%) in the fourth segment. Seventeen patients (32.1%) had small-sized cysts, 27 (50.9%) had medium-sized cysts, and 9 (17.0%) had large-sized cysts. Serology was positive in 29 patients (54.7%), negative in 19 patients (35.9%), and doubtful in 5 (9.4%) patients.

3.2. Cytokine Dosages and Associations. IL-10 was undetectable in all 73 serum samples of patients and controls, while a few sera contained measurable amounts of IFN-γ,

IL-4, and IL-13; percentages of positive samples were 17.8%, 16.4%, and 13.7%, respectively. No statistically significant difference was found between the percentages of positive samples for each cytokine and the different groups analyzed (patients/controls, stage, number, location and size of the cyst, serology, and sex of patients), with the exception of the association of IL-4 and IL-13 with the cyst stage ($P = 0.010$ and $P = 0.033$, resp.). This was likely due to higher percentages of positive samples for IL-4 (50%) and IL-13 (37.5%) in the CE3a-stage group compared to the other cystic stages. The low number of patients in each group prevented us from evaluating any intergroup statistical differences.

4. Discussion

To date, ultrasound and serology are very useful to diagnose and monitor the evolution of cystic echinococcosis, but a marker of activity of the cyst is still lacking. Therefore, clinical decision making may be challenging, in particular for those cases that tend to relapse after an initial successful treatment [1, 17].

It is well known that CE patients generate both Th1 and Th2 immune responses, which skew towards the Th2 arm in the chronic phase [7, 18–22]. In this study, we evaluated the presence of Th1 (IFN-γ) and Th2 cytokines (IL-4, IL-13, and IL-10) in the sera of patients with different cystic stages according to the WHO-IWGE classification of echinococcal cysts [17]. Unlike previous publications [12, 13, 20], our results show that there is no difference in the presence of IFN-γ, IL-4, and IL-13 in sera of patients compared to negative controls. This is possibly because cytokines are not specific for a particular disease, as they are produced in every Th1- or Th2-mediated inflammatory process. Furthermore, Diaz et al. [23] recently reviewed the structure of the laminated layer of the echinococcal cyst and pointed out its possible role in downregulating both Th1 and Th2 response, thus allowing parasite survival. This aspect could explain the low percentage of positive samples containing detectable amounts of IFN-γ, IL-4, and IL-13. Interestingly, IL-10 could not be detected in any samples analyzed. This could be due to a sensitivity limit of the ELISA kit employed. Furthermore, in the literature reviewed by Diaz et al. [23], IL-10 is expressed by leukocytes in infected hosts, especially in the immediate vicinity of the parasite. This aspect could explain the difficulty in dosing IL-10 directly in serum samples.

The comparison of the percentages of positive samples for each cytokine and the different groups analyzed (patients/controls, stage, number, location, and size of the cyst, serology, and sex of patients) did not show any statistically significant differences, with the exception of the association of IL-4 and IL-13 with the cyst stage. This result indicates that the percentage of positive samples for both cytokines is not equal between the different cyst stages, perhaps because of the higher percentage of positive samples for IL-4 and IL-13 in the CE3a-stage group compared to the other cystic stages. Furthermore, these results do not confirm a previous finding by our research group, which showed that the percentage of IL-4-positive samples was higher in CE3b patients compared to other groups [14]. A limitation of these studies is the small sample size which is not sufficient to evaluate any intergroup statistical differences.

Cytokines are immune regulators, which are produced by many cells and have short half-lives. Therefore, their measurement in serum is difficult, and results of tests can be influenced by several variables, such as sample collection protocols (sample handling, processing, and storage) and patient behaviors prior to collection (dietary habits, food ingestion, physical activity, and stress) [24].

Overall, this investigation showed many limits of serum cytokine dosage as a marker of biological activity of echinococcal cysts. Because of low sensitivity and lack of specificity of this test, we believe that other ways to evaluate *ex vivo* biological activity of the cysts should be explored.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Characterization of Outer Membrane Vesicles from *Brucella melitensis* and Protection Induced in Mice

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The outer membrane vesicles (OMVs) from smooth *B. melitensis* 16 M and a derived rough mutant, VTRM1 strain, were purified and characterized with respect to protein content and induction of immune responses in mice. Proteomic analysis showed 29 proteins present in OMVs from *B. melitensis* 16 M; some of them are well-known *Brucella* immunogens such as SOD, GroES, Omp31, Omp25, Omp19, bp26, and Omp16. OMVs from a rough VTRM1 induced significantly higher expression of IL-12, TNFα, and IFNγ genes in bone marrow dendritic cells than OMVs from smooth strain 16 M. Relative to saline control group, mice immunized intramuscularly with rough and smooth OMVs were protected from challenge with virulent strain *B. melitensis* 16 M just as well as the group immunized with live strain *B. melitensis* Rev1 ($P < 0.005$). Additionally, the levels of serum IgG2a increased in mice vaccinated with OMVs from rough strain VTRM1 consistent with the induction of cell-mediated immunity.

1. Introduction

The release of outer membrane vesicles (OMVs) from bacteria is a phenomenon described about 40 years ago. OMVs are released spontaneously during the normal growth of Gram-negative bacteria [1–3]. OMVs have been described in both pathogenic and nonpathogenic Gram-negative bacteria such as *Escherichia coli* [4, 5], *Shigella* spp. [6, 7], *Neisseria* spp. [8], *Porphyromonas* spp. [9], *Pseudomonas aeruginosa* [7], *Helicobacter pylori* [10], *Vibrio* spp. [11], *Salmonella* spp. [12], *Brucella* spp. [13, 14], *Actinobacillus* spp. [15, 16], *Xenorhabdus nematophilus* [17], and *Pseudoalteromonas antarctica* [18].

OMVs possess a bilayer membrane and contain components such as lipoproteins, outer membrane proteins (OMP), lipopolysaccharide (LPS), and some periplasmic components [1–3]. OMVs have been implicated in many processes including the release of virulence factors such as proteases and toxins, signaling between bacterial and eukaryotic cells, DNA transfer, antibacterial activity, immunomodulation of the host, and facilitation of bacterial survival during envelope stress [2, 3, 19, 20].

Other studies have revealed that OMVs trigger the innate inflammatory response. For example, OMVs from *Salmonella typhimurium* are able to activate dendritic cells to secrete IL-12 and TNFα [12], and OMVs from *Pseudomonas aeruginosa* and *Helicobacter pylori* are able to elicit IL-8 production by epithelial cells [21, 22].

The use of OMVs from different Gram-negative bacteria as acellular vaccines has been explored in recent years [23–26]. OMV vaccines have been effective in the specific case of serogroup B of *Neisseria meningitis* [24]. More recently,

OMVs from *Vibrio cholerae* and *Bordetella pertusis* were demonstrated to elicit protection in mouse model [26, 27]. The interest in OMVs as vaccine carriers is increasing, and recent reports have showed that engineered OMVs were able to harbor overexpress antigens [28].

Brucellosis is a worldwide spread zoonotic disease transmitted from domestic animals to humans. It is frequently acquired by ingestion, inhalation, or direct contact of conjunctiva or skin-lesions with infected animal products. The human disease represents an important cause of morbidity worldwide whereas animal brucellosis is associated with serious economical losses caused mainly by abortion and infertility in ruminants [29].

The first effective *Brucella* vaccine was based on live *Brucella abortus* strain 19 (S19), a smooth strain attenuated by an unknown process induced by its subculturing. This strain induces reasonable protection against *B. abortus* in cattle, but at the expense of persistent serological responses that confound differential serodiagnosis of vaccinated and field- infected cattle. A similar problem occurs with the *B. melitensis* Rev.1 strain that is still the most effective vaccine against caprine and ovine brucellosis. This problem has been overcome in cattle by the development of the rifampicin-resistant mutant *B. abortus* RB51 strain. This strain has been proven safe and effective in the field against bovine brucellosis and exhibits negligible interference with diagnostic serology [30].

Currently, smooth live attenuated vaccines *B. abortus* S19 and *B. melitensis* Rev1 as well rough live attenuated vaccine *B. abortus* RB51 are used in the control of animal brucellosis. These smooth vaccines for animals may cause disease and considered unsuitable for use in humans; the rough strain RB51 is rifampin resistant and is considered unsuitable for humans as rifampin is one of the antibiotics of choice for therapy [31]. In the last few decades much research has been done in the attempt to develop safer *Brucella* vaccines [32]. It is important to mention that there is no licensed vaccine for prevention of human brucellosis. A human vaccine could be useful to protect farmers, veterinarians, animal care workers, and general populations living in endemic brucellosis areas [30].

Since OMVs from other bacteria have been used for development of acellular vaccines, we were interested in assessing the protective immune response induced by *Brucella* OMVs. The first studies related to OMVs isolated from *Brucella* spp. were limited to analysis of their protein profile using one-dimensional SDS-PAGE [14, 33]. More recently, Omp25 and Omp31 were identified in *B. suis* OMVs using monoclonal antibodies [13]. In 2007, Lamontagne et al. performed a proteomic analysis of a fraction they called outer membrane fragments from virulent *B. abortus* 2308 and attenuated BvrR/BvrS mutants [34]. To date the composition of OMVs from *B. melitensis* has not been yet explored.

In the attempt to increase the current understanding of the composition of *B. melitensis* OMVs, the proteome of OMVs isolated from smooth *B. melitensis* 16 M is described. Because of the distinct immunological role of the *Brucella* O-side chain in the host, mice were immunized with OMVs purified from smooth *B. melitensis* 16 M and the rough mutant *B. melitensis* VTRM1 (lacking the side O chain of LPS). The difference in dendritic cell cytokine expression and the serum IgG subtypes levels as well as the level of protection afforded to mice is also described.

2. Materials and Methods

2.1. Ethics Statement. The mice experiments were approved and conducted by Institutional Animal Care and Use Committee (approved protocol and 07-047CVM) at Virginia Tech.

2.2. Bacterial Strains and Growth Conditions. B. melitensis 16 M (ATCC 23456) and *B. melitensis* VTRM1 rough mutant derived from *B. melitensis* 16 M [35] were used. Both strains were cultured on tryptic soy agar (TSA) plates supplemented with 0.7% yeast extract and incubated 36 h at 37°C. A bacterial suspension was obtained from both strains, adjusted each to 0.5 g of cells per mL of tryptic soy broth, of which 0.5 mL was spread onto each of 100 TSA plates (10 cm diameter) and incubated at 37°C for 48 h.

2.3. OMVs Purification. The OMVs purification was performed according to the protocol described by Gamazo et al., 1989. Briefly, the bacteria were harvested with a rubber policeman and suspended in 250 mL sterile phosphate-buffered saline (PBS 0.1 M, pH 7.3). The bacterial suspension was centrifuged at 10,000 ×g for 30 min. The supernatant was passed through a 0.22 μm filter (Millipore Corp.), and a sterility test was performed by culturing an aliquot onto a TSA plate followed by incubation for 72 h at 37°C; the filtrate was stored at 4°C during the viability check. The filtered supernatant was centrifuged at 100,000 ×g for 2 h at 4°C. The pellet was washed twice with 25 mL of sterile PBS, and the OMVs were resuspended in 1 mL of sterile PBS. The total protein concentration was determined using PIERCE-BCA (PIERCE) reagents as per manufacturer's recommendations. The OMVs samples were divided into 0.5 mL aliquots and stored at −20°C until used [33].

2.4. Bone Marrow-Derived Dendritic Cells (BMDC). Dendritic cells were derived from 8 wk old, female BALB/c mice by *in vitro* culture of bone-marrow cells with 20 ng/mL rGM-CSF for 7 days as previously described [36, 37]. On day 7, cells showed differentiated morphology (BMDC) and expressed DC markers (CD11c+) in 75% of the population as assessed by flow cytometry (data not shown).

2.5. In Vitro Stimulation of BMDC, RNA Extraction, and Reverse-Transcription Polymerase Chain Reaction. Aliquots of 2.5×10^6 BMDC per well were plated in a 6-well flat-bottomed plate by triplicate and incubated overnight. Then 40 μg of purified OMVs from smooth *B. melitensis* 16 M or OMVs from rough *B. melitensis* VTRM1 were added to each well by triplicate. Total RNA (RNAeasy Qiagen) was extracted from BMDC (stimulated and unstimulated) at 1, 3, 6, and 12 h after induction. The DNA was removed with DNase I (DNA-free Kit Ambion). Then cDNA was prepared

from 1 μg of total RNA (Promega, A2500 kit). To verify the complete elimination of DNA, PCR for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene amplification was performed (data not shown).

2.6. Real Time-PCR. Templates cDNA were analyzed for IL-2, IL-6, IL-12p40 (IL-12), IL-10, IL-17, IL-23, INF-γ, TNF-α, and TGF-β (SABioscienes) expression using the PCR Array and the RT2 SYBR Green/Fluorescein qPCR Master Mix (SABiosciences) on the iCycler PCR System (Bio-Rad) as per recommendations of the manufacturer. Fold changes in gene expression were calculated using the $\Delta\Delta$Ct method in the PCR Array Data Analysis template. The amplification of house-keeping *gapdh* gene was used to normalize the fold changes in the cytokine expression.

2.7. Mice Immunizations. Female BALB/c mice of 6 weeks of age (5 per group) were vaccinated by two intramuscular inoculations, at day 0 and day 30, with 5 μg of purified OMVs from *B. melitensis* 16 M and *B. melitensis* VTRM1. Before the first dose, mice were prebled by puncturing retro-orbital plexus under anesthesia. Two weeks after boosting, the mice were bled by the same route. The serum was separated from the clotted blood and stored at -20°C until use for detection of IgG subtypes. As a positive control, a group of mice was vaccinated with 1.5×10^4 CFU of vaccine strain, *B. melitensis* Rev1. As a negative control, one group of mice was injected with saline. Mice were challenged at 6 weeks after the first vaccination dose with 5×10^4 CFUs of virulent strain *B. melitensis* 16 M by intraperitoneal route. At 2 weeks after challenge, all the mice were euthanized by CO_2 asphyxiation followed by cervical dislocation, spleens were collected aseptically, and colony-forming units (CFU) were determined.

2.8. Indirect ELISA. Levels of serum immunoglobulin IgG1 and IgG2a isotypes with specificity to OMVs from smooth *B. melitensis* 16 M and rough strain VTRM1 were determined by indirect ELISA. Sera from mice immunized with OMVs purified from *B. melitensis* 16 M were tested with OMVs purified from *B. melitensis* 16 M, and sera from mice immunized with OMVs purified from rough *B. melitensis* VTRM1 were tested with OMVs purified from rough strain. The OMVs were diluted in carbonate buffer, pH 9.6. The wells of polystyrene plates (Nunc-Immunoplate with maxisorp surface) were coated with 100 μL/well of the diluted antigens (2.5 μg/mL of protein from OMVs). Following overnight incubation at 4°C, plates were washed four times in wash buffer (Tris-buffered saline at pH 7.4, .05% Tween 20) and blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline. After 1 h incubation at 37°C, mice sera with appropriate dilution in blocking buffer were added to the wells (50 μL/well). Each serum sample was tested in triplicate wells; the plates were incubated for 4 h at room temperature and washed four times. Horse radish peroxidase-labeled anti-mouse isotype-specific conjugates (Southern Biotechnology Associates Inc, Birmingham, Alabama) were added (50 μL/well) at an appropriate dilution. After 1 h incubation at room temperature, the plates were washed four times. A 100 μL of substrate solution (TMB Microwell peroxidase substrate; Kirkegaard and Perry Laboratories, Gaithersburg, Md) was applied to each well. After 20 min incubation at room temperature, the enzyme reaction was stopped by adding 100 μL of stop solution (0.185 M sulfuric acid), and the absorbance at 492 nm was recorded using microplate reader (Molecular Devices, Sunnyvale, Calif).

2.9. Electron Microscopy. 20 μL of purified OMVs (25 μg of protein) or intact bacteria were placed onto copper grids coated with formvar and dried using filter paper. 40 μL of 1% phosphotungstic acid was added, and the grids were allowed to stand overnight at room temperature; they were observed under the transmission electron microscope (JEOL model JEM 10-10).

2.10. Denaturing Gel Electrophoresis. SDS-PAGE was performed in 15% acrylamide slab gels by the method of Laemmli [38]; the gels were stained with Coomassie blue. The apparent molecular masses of the OMV proteins were determined by comparing their electrophoretic mobility with that of the wide-range molecular mass markers [Sigma-Marker (Sigma)] using the computer program SigmaGel V. 1.0.

2.11. Enzymatic Digestion. After the separation of OMV proteins by denaturing electrophoresis, the acrylamide gel was cut into six sections. The excised samples were reduced with 50 mM dithiothreitol, alkylated with iodoacetamide and then "in gel" digested with trypsin. The peptides were desalted using a ZipTip (Millipore Corp) and then concentrated in a Speed-Vac SPD 1010 ThermoElectron (Instituto Nacional de Biotecnologia-UNAM, Cuernavaca, México).

2.12. LC-MS/MS. The samples were reconstituted to approximately 0.1–0.5 μg/μL in 50% acetonitrile containing 1% acetic acid and placed directly into a Finnigan LCQ ion trap mass spectrometer (Instituto Nacional de Biotecnologia-UNAM, Cuernavaca, Mexico), using a Surveyor MS syringe pump delivery system. The eluate at 10 μL/min was split to allow only 5% of the sample to enter the nanospray source (0.5 μL/min). LC-MS/MS analyses were carried out using a PicoFrit needle/column RP C18 from New Objective (Woburn, Mass, USA), with a fast gradient system from 5% to 60% of solution B (100% acetonitrile containing 1% acetic acid) for a period of 45 min.

The electrospray ionization source voltage was set at 1.8 kV and the capillary temperature at 130°C. Collision-Induced Dissociation (CID) was performed using 25 V of collision energy, 35–45% (arbitrary units) of normalized collision energy and the scan had the wide band activated.

All spectra were obtained in the positive-ion mode. Data acquisition and the deconvolution of data were carried out using Xcalibur software on a Windows XP PC system. The MS/MS spectra from enzymatically generated peptides were analyzed by Sequest software from Finnigan (Palo Alto,

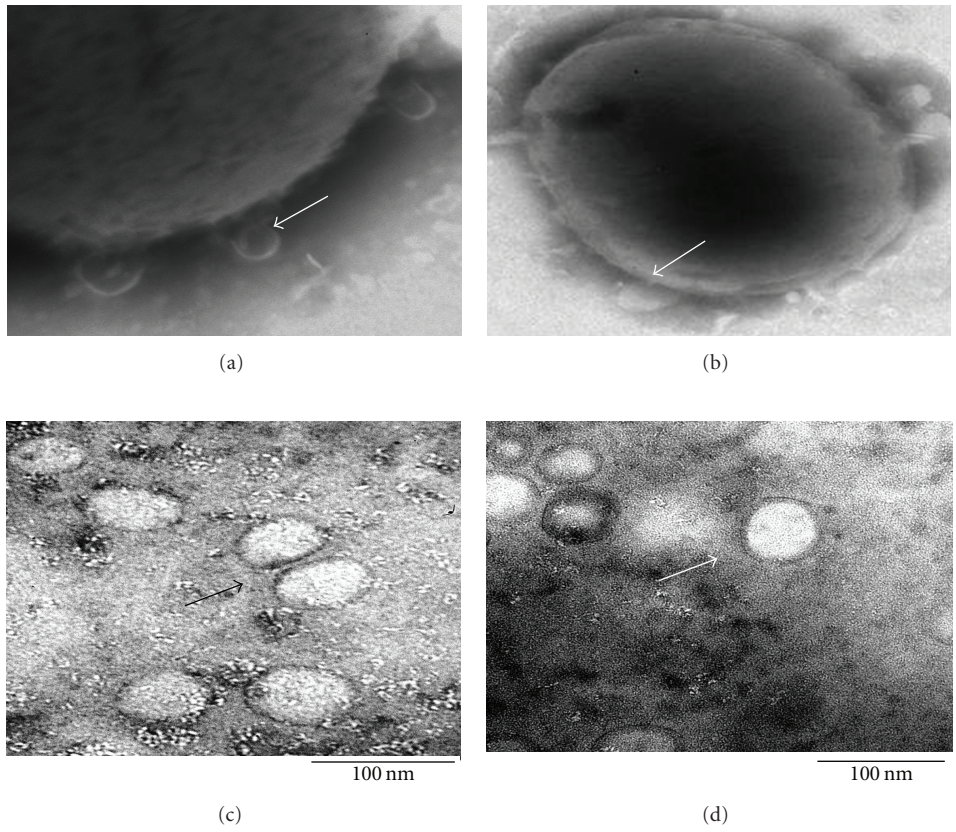

Figure 1: Electron microscopy of OMVs from smooth *B. melitensis* M16 and rough *B. melitensis* VTRM1. Negative stain of OMVs released from surface *B. melitensis* VTRM1 (a) and *B. melitensis* 16 M (b); negative stain of OMV purified by differential centrifugation from both strains; *B. melitensis* VTRM1 (c) and *B. melitensis* 16 M (d). The arrows point to the apparent shedding of the OMVs from the cell surface in (a) and (b); while the arrows in (c) and (d) point to spherical OMVs purified from both strains. The bars correspond to 100 nm.

Calif) and MASCOT search engine from Matrix Science Ltd (Boston, Mass).

2.13. In Silico Analysis. Once the proteins in the OMVs were identified, an *in silico* analysis was performed. Initially the amino acid sequences of the identified proteins were analyzed by BLAST comparing them to similar sequences from species of *Brucella* and other bacteria (http://www.ncbi.nlm.nih.gov/BLAST/). The isoelectric point and molecular weight were determined using Antheprot 2000 V. 5.2.

The prediction of "motif" sequences was performed by searching My Hits Motif Scan database (http://hits.isb-sib.ch/cgi-bin/PFSCAN) that uses different databases such as PROSITE, HAMAP, and Pfam. The prediction of the subcellular location of the proteins was carried out on the PSORT server available at http://www.psort.org [39] and Softberry database. The MatGAT V. 2.02 [40] program was used to determinate the similitude grade of the homologous sequences found by the BLAST search. The ProLinks database (http://dip.doe-mbi.ucla.edu/pronav/) [41] and Gene Ontology (http://www.geneontology.org) [42] were used to determinate the hypothetical function of proteins into OMVs.

2.14. Statistical Analysis. Statistical analysis, Two-Way-ANOVA, was carried out with SigmaStat statistical package V. 2.0 (SYSTAT).

3. Results

3.1. Isolation of OMVs and Electron Microscopy. OMVs were isolated from cell-free culture medium by differential centrifugation. In order to confirm purification of the OMVs from both strains, electron micrographs were performed using negative staining with phosphotungstic acid. In the micrographs (Figure 1), it is possible to see the bleb formation leading to the liberation of OMVs from the outer surface of the *Brucella*. In addition, the spherical morphology (average diameter 60–90 nm) of the purified OMVs including a double membrane can be observed as previously described [14, 33]. Moreover, no membrane debris bigger than 100 nm were observed by electron microscopy. In general, no differences, at least in the shape or in the number of OMVs released, were observed for the smooth or rough *Brucella* strains.

3.2. Cytokine Expression. In order to explore if OMVs could induce an immune response in antigen-presenting cells, we

used BMDC exposed to OMVs either from smooth or rough *B. melitensis*. At increasing times following exposure, the cytokines associated with the DC1-mediated Th1 (IFN-γ, IL-2, IL-6, IL-12, and TNF-α), DC2-mediated Th2 (IL-4 and IL-10), and DC17-mediated Th17 (IL-17, IL-23, and TGF-β) responses were measured by qRT-PCR. The results are shown in Table S1 (see in table S1 Supplementary Material available online at doi: 10.1155/2012/352493). The cytokine profiles elicited in the BMDC stimulated with smooth *B. melitensis* 16 M OMVs reached a maximum at 12 hours following stimulation with the highest production of IL-6, IL-4, IL-10, and IL-17 (Figure 2(a)). In contrast, the induction of cytokines by rough OMVs reached maximum expression at 1 h after stimulation and decreased over time, except for IL-10 (maximum expression at 3 hours) and for TNF-α (maximum expression at 3 hours) (Figure 2(b)). Using statistical analysis we compared the cytokine expression obtained from BMDC stimulated with OMVs from smooth and rough *Brucella* strains (Two-way-ANOVA analysis). Results showed significant differences between the cytokine profile induced by OMVs purified from rough and smooth *B. melitensis* ($P < 0.05$). OMVs from rough *B. melitensis* VTRM1 induced three cytokines that were significantly higher: IFN-γ, TNF-α, and IL-12 ($P < 0.05$, $P < 0.01$ and $P < 0.001$, resp.).

3.3. Protection against Challenge with Virulent B. melitensis 16 M. Mice were challenged with virulent strain *B. melitensis* 16 M to examine the protection induced by OMVs. In this experiment, protection was defined as a significant reduction in the number of bacteria in the spleens of immunized mice compared to the mice receiving saline. The *B. melitensis* Rev1 vaccine induced 2.64 log units of protection compared to saline control (Figure 3). In the case of mice vaccinated with OMVs, we observed that smooth OMVs induced 1.9 log units, and rough OMVs induced 3.08 log units of protection compared to saline control ($P < 0.005$).

3.4. Serology. Presence of antibodies specific to OMVs in serum of the mice vaccinated was determined by ELISA. The induction of IgG2a subclass during immune response should give an idea about Th1 or Th2 balance. As illustrated in Figures 4(a) and 4(b) OMVs purified from rough *B. melitensis* VTRM1 induced a higher IgG2a response than IgG1, suggesting a shift toward a Th1 response. In contrast, OMVs from smooth strain *B. melitensis* 16 M induced the same levels of IgG1 and IgG2a.

3.5. SDS-PAGE and Proteomic Analysis. The denatured electrophoretic protein profiles observed from OMVs obtained from rough *B. melitensis* VTRM1 and smooth *B. melitensis* 16 M show no discernable differences (Figure 5). The trypsin-generated peptide masses, as well as their fragment ions, were analyzed by LC-MS/MS. The resulting peptides sequences were used to query databases that led to the identification of 29 unique proteins (Table 1). A query result was only considered as significant if the overall score was higher than 25 and more than two tryptic peptides as well as their fragment ions matched to the protein and the calculated molecular weight corresponded to molecular weight in the original gel section [43].

3.6. In Silico Analysis. The results of the proteomic characterization of the *B. melitensis* vesicular proteins are summarized in Table 1. In addition, the complete results are available in supporting information Table S2 and Table S3. The identification score varied from 55 to 100%, with coverage from 3 to 50%. Besides the identification of each spot and calculation of its basic biochemical characteristics, the isoelectric point and molecular weight was obtained by means of analysis and search in databases. Subcellular location, protein motifs, immunogenic regions, signal peptide prediction, and closest homologues were also analyzed. Of the 29 proteins identified, approximately 52% belonged to the outer membrane, 17% to the periplasm, 20.6% to the cytoplasm, 2 proteins were from inner membrane, and 1 protein was predicted to be an extracellular protein. Using the Motif databases these proteins were classified into five groups: (i) structural and transport proteins (such as the outer membrane proteins), (ii) antigenic proteins, (iii) involved in metabolic processes (e.g., Frr, HU, GroES), (iv) involved in stress response (e.g., Dps, TrxC, and SOD), and v) invasion proteins (e.g., InvB and IalB). About 60% of the proteins were predicted to contain signal peptides and thus capable to be exported or targeted by the cellular machinery to the periplasm or outer membrane. Only twelve proteins did not contain signal peptides, five of these were predicted as cytoplasmic or mature periplasmic proteins in which the signal sequence was processed.

Additionally, the analysis of sequences using ProLinks [41] showed that with the exception of two proteins, the possible functions of 27 proteins were predicted. While the analysis of sequences using Gene Ontology terms showed that one half (14/29) of the proteins appeared to be involved in transport and/or integrity of the membrane.

All the proteins found in the *B. melitensis* OMVs were highly related to homologous proteins in other *Brucella* species (from 88 to 100% in similarity, data not shown). For most proteins, the closest non-*Brucella* homologues were found in *Rhizobia*, such as *Rhizobium* and *Bartonella* (50% to 90% in similarity). Interestingly, four proteins with a high degree of homology to those in *Escherichia coli* were found: FrpB (an iron-regulated outer membrane protein), a metal chelate outer membrane receptor, Dps (involved in DNA protection due to starvation or stationary phase), and SOD (a Cu/Zn superoxide dismutase).

4. Discussion

As has been described in other Gram-negative bacteria, OMVs are released from the *Brucella*'s outer membrane as we observed by electron microscopy (Figure 1). The OMVs are shed from both rough and smooth *Brucella* strains, grown in liquid or on solid media, and spontaneously released during the growth as observed previously [13, 14, 33].

OMVs can also strongly activate the host innate and acquired immune response pathways [23]. Based on this

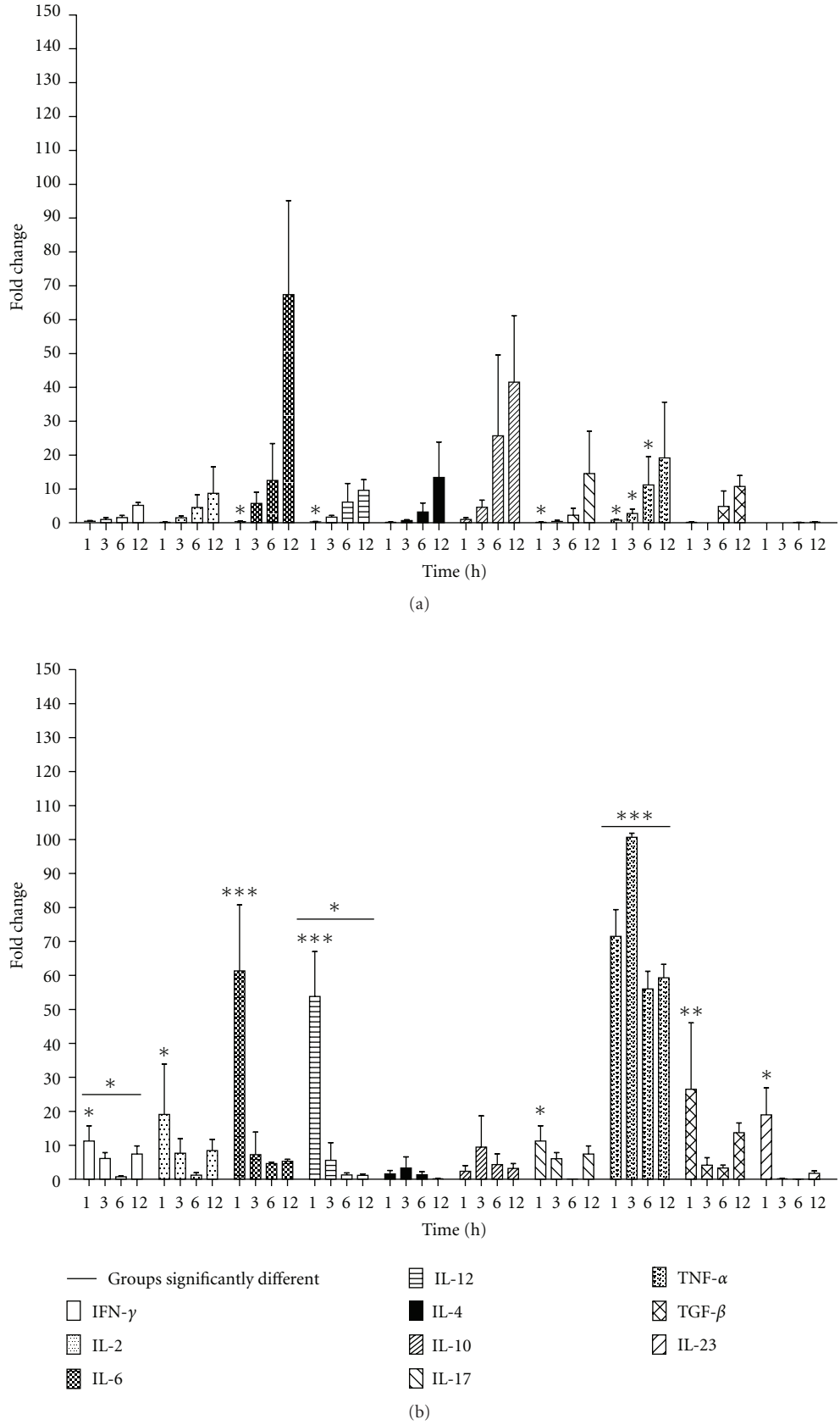

Figure 2: Cytokine expression of BMDC stimulated with OMVs. (a) BMDC stimulated with OMVs from smooth *B. melitensis* 16 M. (b) BMDC stimulated with OMVs from rough *B. melitensis* VTRM1. Two-way ANOVA analysis was performed to compare results. $^{*}(P < 0.05)$; $^{**}(P < 0.01)$; $^{***}(P < 0.001)$.

Table 1: *B. melitensis* 16 M OMVs proteins identified by 1D-SDS-PAGE coupled to LC-MS/MS.

Protein	*B. melitensis* denomination	Molecular weight (kDa)	Motif, subcellular localization	Closest ortholog, other than in Brucellae (% similarity)
Bacterial surface antigen	BMEI0830	85.90	Surface antigen, OM	Outer membrane protein *Bartonella henselae* str. Houston-1, (75.3%)
Iron-regulated outer membrane protein FRPB	BMEII0105	72.05	TonB-dependent receptor Plug Domain, OM	TonB-dependent receptor *Escherichia coli*, (41.3%)
Metal chelate outer membrane receptor	BMEI0657	64.80	TonB-dependent receptor Plug Domain, OM	Chain A, Outer Membrane Cobalamin Transporter (Btub) *Escherichia coli*, (46.2%)
Sugar-binding protein	BMEII0590	43.20	Bacterial sugar-binding extracellular protein, P	Probable sugar ABC transporter substrate binding protein *Rhizobium etli*, (75.7%)
Outer surface protein	BMEII0376	31.55	Surface antigen, OM	Probable heat-resistant agglutinin 1 protein *Rhizobium leguminosarum* bv. viciae, (54.9%)
D-Ribose-binding periplasmic protein precursor	BMEII0435	30.99	Periplasmic binding protein and sugar-binding domain of LacI family, P	Porin *Rhizobium leguminosarum* bv. trifolii, (59.2%)
Hypothetical protein BMEI0542	BMEI0542	30.04	Unknown, EC	Hypothetical protein *Rhizobium sp*., (48.3%)
25 kDa outer-membrane immunogenic protein precursor	BMEI1007	25.24	Porin type 2, OM	Hemin-binding C protein *Bartonella tricoborum*, (58.7%)
25 kDa outer-membrane immunogenic protein precursor	BMEI1249	23.18	Porin type 2, OM	Hemin-binding B protein *Bartonella henselae* str. Houston-1, (58.7%)
25 kDa outer-membrane immunogenic protein precursor	BMEI1829	24.58	Porin type 2, OM	Hemin-binding C protein *Bartonella tricoborum*, (45.4%)
25 kDa outer-membrane immunogenic protein precursor	BMEI1830	24.74	Porin type 2, OM	Outer membrane protein *Rhizobium etli*, (60.1%)
31 kDa outer-membrane immunogenic protein precursor	BMEII0844	23.27	OmpA-like domain profile, OM	Porin *Rhizobium leguminosarum* bv. trifolii, (52.5%)
BP26	BMEI0536	24.77	Protein of unknown function (DUF541), P	Unknown function protein DUF541 *Rhizobium leguminosarum* bv. trifolii, (64.9%)
Precursor YBIS protein	BMEI1369	23.51	Domain YkuD, C	Hypothetical protein *Rhizobium leguminosarum* bv. viciae, (67.2%)
OmpA family protein	BMEI0786	22.96	OmpA-like domain, OM	OmpA family protein *Rhizobium leguminosarum* bv. viciae, (81.4%)
Hypothetical lipoprotein	BMEI0785	21.91	Prokaryotic membrane lipoprotein lipid attachment profile, IM	Hypothetical protein *Rhizobium leguminosarum* bv. viciae, (74.7%)
Ribosome recycling factor	BMEI0826	20.66	Ribosome recycling factor (RRF-frr), C	Ribosome recycling factor *Bartonella henselae* str. Houston-1, (90.3%)
Hypothetical membrane-associated protein (BMEII0692)	BMEII0692	20.42	Invasion-associated locus B (IalB) protein, IM	Invasion-associated locus B family protein *Rhizobium leguminosarum* bv. trifolii, (62.4%)

Table 1: Continued.

Protein	*B. melitensis* denomination	Molecular weight (kDa)	Motif, subcellular localization	Closest ortholog, other than in Brucellae (% similarity)
22 kDa outer membrane protein precursor	BMEI0717	19.44	Unknown, OM	Outer membrane protein putative precursor *Rhizobium leguminosarum* bv. viciae, (48.6%)
DNA starvation/stationary phase protection protein Dps	BMEI1980	18.25	Dps protein family Ferritin-like domain, C	DNA starvation/stationary phase protection protein Dps *Escherichia coli*, (72.5%)
Peptidoglycan-associated lipoprotein	BMEI0340	18.23	OmpA family protein, OM	Outer membrane lipoprotein *Rhizobium etli*, (82.6%)
Invasion protein B	BMEI1584	18.03	Invasion-associated locus B (IalB) protein, P	Invasion-associated locus B protein *Bartonella quintana* str. Tolouse, (55.4%)
Outer membrane lipoprotein	BMEI0135	17.60	Bacterial outer membrane lipoprotein; Omp19, OM	Outer membrane lipoprotein *Bartonella henselae* str. Houston-1, (59.6%)
Chain A, Cu-Zn superoxide dismutase	BMEII0581	16.07	Cu-Zn superoxide dismutase, P	Cu-Zn superoxide dismutase *Escherichia coli*, (68.7%)
Thioredoxin C-1	BMEI2022	11.42	Thioredoxin active site, C	Putative thioredoxin *Rhizobium leguminosarum* bv. viciae, (88.8%)
Cochaperonin GroES	BMEII1047	10.39	10 kDa chaperonin protein Cnp10, C	Cochaperonin GroES *Bartonella tricoborum*, (91.8%)
DNA-binding protein HU	BMEI0877	9.07	Bacterial histone-like DNA binding protein signature, C	DNA-binding protein *Rhizobium sp*., (83.5%)
Hypothetical lipoprotein	Unknown	8.286	Prokaryotic membrane lipoprotein lipid attachment profile, OM	17 kDa surface antigen *Rhizobium leguminosarum* bv. trifolii, (61.2%)
Hypothetical protein BMEI0287	BMEI0287	8.596	Prokaryotic membrane lipoprotein lipid attachment profile, OM	Hypothetical protein *Rhizobium etli*, (56.8%)

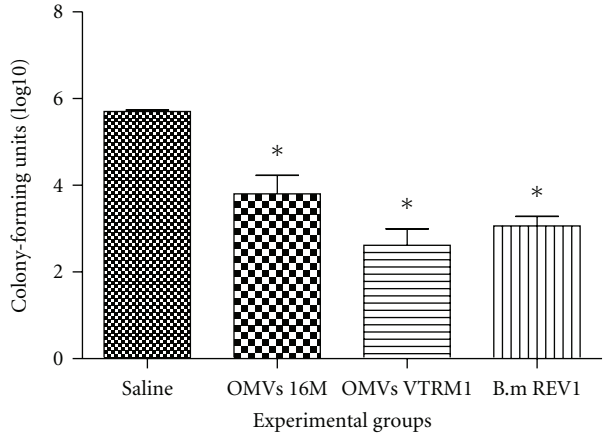

Figure 3: Analysis of IgG1 (a) and IgG2a (b) antibody responses of BALB/c mice to outer membrane vesicles from *Brucella*. Outer membrane vesicles (OMVs) were purified from *B. melitensis* 16 M and *B. melitensis* VTRM1, and mice were immunized. Sera from each mouse were collected and were assayed individually by ELISA. Antibody levels are expressed as optical density (OD) at 492 nm.

previous evidence, we stimulated BMDC with OMVs isolated from smooth and rough *Brucella* strains. At different time points, cytokines expression for DC1-mediated Th1 (IFN-γ, IL-2, IL-6, IL-12, and TNF-α), DC2-mediated Th2 (IL-4 and IL-10), and DC-mediated Th17 (IL-17, IL-23 and TGF-β) was analyzed by qRT-PCR. Interestingly, we determined that OMVs from rough *B. melitensis* VTRM1 induced significantly higher expression of IFN-γ, TNF-α, and IL-12.

TNF-α is necessary for full expression of the macrophage anti-*Brucella* activities. It also plays an important role in the triggering of specific immunity against several intracellular pathogens and positively controls early expression of IL-12 and IFN-γ in *Brucella*-infected mice [44]. Our results demonstrated an earlier expression of IL-12 and IFN-γ by OMVs from rough *B. melitensis* VTRM1 (Figure 2(b)). Clearly TNF-α participates in the establishment of acquired immunity of the Th1 response, with the generation of IFN-γ-producing $CD4^+$ cells and $CD8^+$ cytotoxic cells, two outcomes crucial for the complete killing of the intracellular

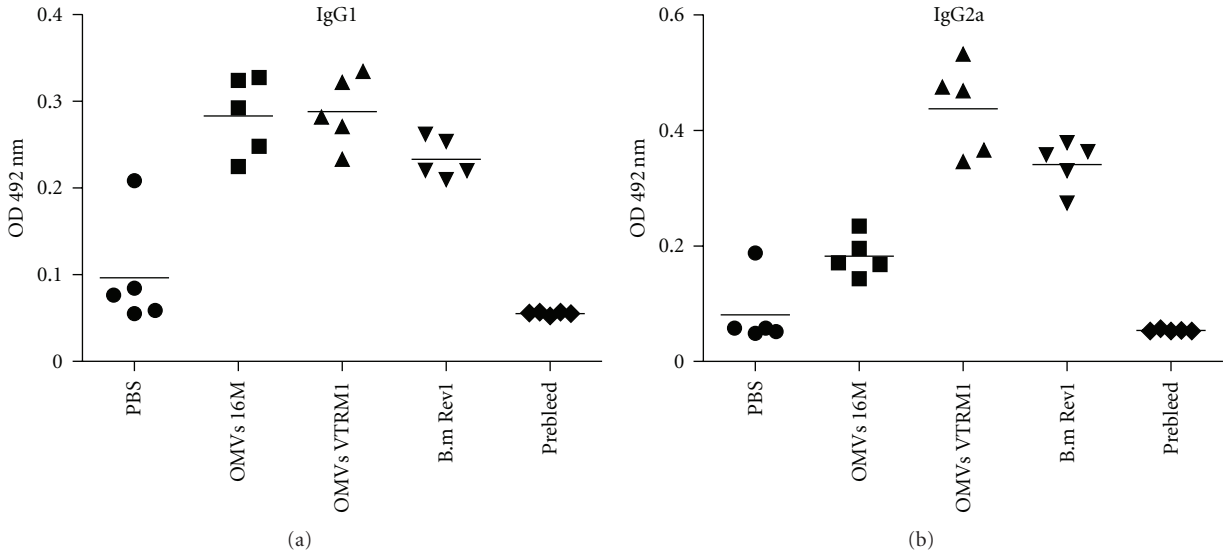

Figure 4: Level of protection against *B. melitensis* 16 M conferred by outer membrane vesicles purified from *B. melitensis* 16 M and *B. melitensis* VTRM1. In this experiment vaccine strain *B. melitensis* Rev1 was used as a positive control of vaccination, as negative control was used saline. $n = 5$ ($^{*}P \leq 0.005$ comparisons were OMVs versus saline, and *B. melitensis* Rev1 versus saline).

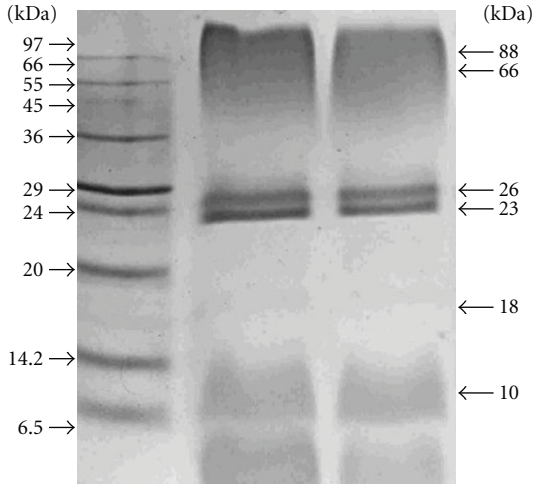

Figure 5: Electrophoretic profiles of OMVs purified from *B. melitensis*. Lane 1, molecular weight markers; lane 2, OMVs from smooth *B. melitensis* 16 M (80 μg); lane 3, OMVs from rough *B. melitensis* VTRM1 (80 μg). SDS-PAGE gels were stained with Coomassie blue.

Brucella. TNF-α produced a synergistic effect in presence of IFN-γ for the ultimate clearance of the infection [45].

The early expression of IL-6, IL-23, and TGF-β genes was observed in BMDC stimulated with OMVs from rough *B. melitensis* VTRM1. In contrast, in the case of smooth *B. melitensis* 16 M OMVs, the same cytokines genes were expressed after a longer time with a maximum expression at 12 h following induction. The early induction of the cytokine expression could offer a fast immune response against *Brucella*, for example, if IL-6 promotes a proinflammatory environment. However, the role of the IL-23 and TGF-β as part of the Th17 response in *Brucella* infection is not very clear to date. Some data suggest that a Th17 response is favored when IL-6 is present in high quantities, especially in aged mice [46].

The determination of whether Th17 response is merely present as epiphenomena or truly playing a role in the host defense is a focus of current research. We speculate that these differences could be the effect of the O-side chain in the LPS present in the OMVs from the smooth strain but not in the OMVs derived from rough *B. melitensis* VTRM1. In contrast to the infection with smooth *B. abortus* and *B. suis* strains and purified smooth LPS, infection of human DCs with rough mutants of *Brucella* leads to both phenotypic and functional maturation of infected cells [47].

Recently, Surendran et al. observed phenotypic maturation and production of IL-12 and TNF-α when murine BMDC were stimulated with live *B. abortus* RB51, a rough vaccine strain approved by the USDA for use in cattle and with *B. abortus* RB51SOD, which overexpressed a Cu-Zn superoxide dismutase (SOD) [37]. In contrast no maturation or secretion of either cytokine occurred when *B. abortus* strain 2308 was used to stimulate murine BMDC [47]. While Billard et al., [47] observed higher production of TNF-α and human DC maturation using rough mutants but not with smooth strains of *B. suis,* our cytokine expression results are in agreement with the works published by Billard et al., and Surendran et al. Those are in conflict with the reports by Zwerdling et al. and Macedo et al., where it was observed that smooth *B. abortus* exposure induced human and murine DC maturation, respectively [48]. Zwerdling et al. could only speculate the reasons for these discrepancies and some considerations were made such as: the different method for cell isolation, the concentration of the DC and the interactions between themselves in each experiment, and the type of strains used for those experiments [48].

The direct effect of purified rough or smooth LPS molecules on human DC maturation also has been explored [47]. However no difference could be determined between DC responses to rough or smooth LPS of *B. abortus*. These results are in line with the very low endotoxic properties of *Brucella* LPS and with the equivalent stimulation of macrophages by rough and smooth LPS [47]. This means that the ability of rough *Brucella* strains to induce human or murine DC maturation is not related to a direct effect of their LPS but maybe the absence of the O-side chain could allow the exposure of bacterial surface molecules that should normally be hidden. It is also possible that the absence of O-side chain in the rough OMVs permits a higher exposure to the outer membrane proteins. For example, it is well known that Omp16 and Omp19 interact with the TLR2 receptor and induce the production of IL-12, which is important for the control of infection [44].

Recently, it was demonstrated that Omp16 requires TLR4 interaction for the activation of DC and macrophages and elicits a Th1 and protective immune response [49]. On the other hand TLR4 is not involved in the Th1 and protective response induced by Omp19 [32].

After intramuscular administration of OMVs from *B. melitensis* 16 M and rough *B. melitensis* VTRM1, challenge with virulent *B. melitensis* 16 M was performed in BALB/c mice. Relative to saline control group, mice immunized intramuscularly with rough OMVs were protected from challenge with strain virulent *B. melitensis* 16 M just as well as the groups immunized with live strains *B. melitensis* Rev1 or OMVs from rough strain *B. melitensis* VTRM1 ($P < 0.005$) (Figure 3).

In addition to protection, in this study, we analyzed the humoral immune response induced in mice by OMVs isolated from smooth and rough *Brucella* strains. Since the subclass of IgG response is determined by the pattern of cytokines secreted by CD4 helper T cells, we measured the titers of both the IgG1 and IgG2a antibodies produced against OMVs. As has been observed (Figure 4(b)), OMVs from the rough strain were able to induce a higher IgG2a subclass. This IgG2a isotype is important because of the binding of their Fc portion to Fc receptors on the surface of phagocytes that activates a broad spectrum of antimicrobial responses (e.g., phagocytosis, cytokine synthesis, release of inflammatory mediators, and generation of reactive oxidant species) [50].

The information related to the immune response of OMVs from *Brucella* is limited to observe the antigenicity of these entities in rabbits as reported previously [33]; on the other hand using monoclonal antibodies other authors were able to identify two proteins in OMVs from *B. suis* [13]. In the same line, there are no reports regarding the immune protection of vaccines based on OMVs against *Brucella* infection in mouse model.

Many vaccine candidates for human brucellosis involve live attenuated *Brucella* strains, subunits vaccines, recombinant proteins, and DNA vaccines which have shown to be protective in a mouse model [31].

The biophysical properties of vesicles, as heterogeneous, proteinaceous, amphipathic structures, may allow greater movement through tissues. As a result, vesicles could travel deeper into tissues where resident phagocytes are located [2]. As mentioned previously, the practical application of OMVs as acellular vaccines has been exploited in other pathogens [24, 26, 27]. The advantage to make vaccines based on *Brucella* OMVs could be that they are able to carry many antigens exposed naturally in the outer membrane and periplasm of *Brucella*; on the other hand, OMVs are acellular entities that could give an alternative to make safer vaccines instead of using live *Brucella* vaccines, which have the additional potential complications of replication, virulence, and side effects due to induction of a strong immune response (i.e., fever).

Proteomics approaches have been used to identify the protein components of vesicles in attempts to provide clues to the mechanisms of vesicles production and cargo loading [51]. In this study conventional SDS-PAGE coupled to LC-MS/MS was used to identify the composition of *B. melitensis* 16 M OMVs. The denatured electrophoretic protein profiles observed from OMVs obtained from rough *B. melitensis* VTRM1 and smooth *B. melitensis* 16 M showed no discernable differences (Figure 5). The method selected for OMVs purification was that one reported for *Brucella* spp by Gamazo et al. in 1989, in which the authors observed a range of sizes in OMVs when *Brucella* was grown in solid media [33]. We also observed a slightly better yield of OMVs when solid medium was used instead of liquid medium to grow *Brucella*.

In comparing our results with Gamazo and Moriyon [33], in 1987, they observed an electrophoretic profile that showed two major bands (25 and 30 kDa) and several minor bands (18, 22, and 84 kDa) in the OMVs of both smooth *B. melitensis* 16 M and a rough strain *B. melitensis* B115. Our results showed bands similar to those observed by Gamazo and Moriyon, [14] in both smooth and rough OMVs. However, the same research group in 1989 working with OMVs purified from field strains of *B. melitensis* and *B. ovis* observed different electrophoretic profiles divided in four groups of proteins: A (25.5–32 kDa), B (21.5–22.5 kDa), C (18–19.5 kDa), and D (13–15.5 kDa); these profiles were different from that reported in 1987. The differences may be due to inherent differences between field strains compared with the reference strain.

After electrophoretic separation, the OMVs proteins from *B. melitensis* 16 M were excised and digested with trypsin. The trypsin-generated peptide masses, as well as their fragment ions, were analyzed by LC-MS/MS. The resulting peptides sequences were used to query databases led to the identification of 29 unique proteins (Table 1). Our results showed that the outer membrane proteins are the principal components of *Brucella* OMVs as has been reported previously for other Gram-negative bacteria [23, 41, 52]. However, Lamontagne et al. (2007) found that periplasmic and cytoplasmic proteins as the principal components of OMVs from *B. abortus* while outer membrane proteins were present as a smaller proportion.

In OMVs from *B. melitensis* 16 M we identified Omp25 and Omp31 that belong to the major outer membrane protein family [53]. Boigegrain et al. (2004) identified Omp25

and Omp31 in OMVs from *B. suis* using monoclonal antibodies. Also, Lamontagne et al. (2007) using mass spectrometry were able to identify the Omp31b and Omp25 (Omp3a) in OMVs from *B. abortus*. The spontaneous release of the OMVs and gentle isolation procedures should minimize cytoplasmic leakage and prevent the contamination that follows cell disruption [51]. Our analyses did not show the presence of inner membrane markers in OMVs composition including NADH-cytochrome C-oxidoreductase or succinate dehydrogenase.

In our study, outer membrane proteins with an OmpA motif were identified; these have been involved in immunostimulatory activities and induce leukocyte migration [54]. The presence of the family Ton B-dependent receptor proteins could be an alternative mechanism for *Brucella* survival in nutrient-limiting conditions such as found in macrophages [18, 55]. Additionally, Ton B-dependent receptors have been involved in siderophore internalization [18].

Omp16, and the Omp19 are lipoproteins that induce immunological protection very similarly to that elicited by the live vaccine *B. abortus* S19 with the induction of IFN-γ and $CD4^+$ as well as $CD8^+$ T-cells [29, 48, 56]. Also, the Omp16 shows significant similarity to the peptidoglycan-associated lipoprotein (PALs) of many Gram-negative bacteria [57].

Lipoproteins Omp10, Omp16 and Omp19 were previously identified in OMVs from *B. abortus* by Western blot [58].

OMVs from *B. melitensis* 16 M contain Cu-Zn SOD, Dps, and GroES that are part of the antioxidant defense system that protects bacteria from the toxic effects of reactive oxygen intermediates (ROIs) [59, 60]. The Dps protein has been reported in other pathogens (*Escherichia coli*, *Campylobacter jejuni,* and *Salmonella enterica)* to be responsible for resistance to oxidative stress and protecting the DNA against ROIs. The Dps protein has a ferritin-like domain (Table 1) and is thought to nullify the toxic combination of Fe (II) and peroxide [60, 61].

5. Conclusion

In summary, we identified 29 proteins in OMVs released by *B. melitensis* 16 M, some of them are well-known *Brucella* immunogens such as SOD, GroES, Omp31, Omp25, Omp19, bp26, and Omp16. Additionally, we determined that rough OMVs both stimulate a stronger innate response, as well as protective immunity against *B. melitensis* 16 M challenge. Based on these data, the potential of using rough OMVs of *Brucella* as an acellular vaccine should be considered.

Acknowledgments

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Review Article

Immunology and Immunodiagnosis of Cystic Echinococcosis: An Update

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Cystic echinococcosis (CE) is a cosmopolitan zoonosis caused by the larval cystic stage of the dog tapeworm *Echinococcus granulosus*. This complex multicellular pathogen produces various antigens which modulate the host immune response and promote parasite survival and development. The recent application of modern molecular and immunological approaches has revealed novel insights on the nature of the immune responses generated during the course of a hydatid infection, although many aspects of the *Echinococcus*-host interplay remain unexplored. This paper summarizes recent developments in our understanding of the immunology and diagnosis of echinococcosis, indicates areas where information is lacking, and suggests possible new strategies to improve serodiagnosis for practical application.

1. Introduction

Two neglected parasitic diseases, of both medical and public health importance, are cystic echinococcosis (CE) and alveolar echinococcosis (AE), caused by *Echinococcus granulosus* (Eg) and *E. multilocularis*, respectively. CE is a near-cosmopolitan zoonosis and responsible for most of the burden of echinococcosis globally [1], although AE is endemic in Europe [2, 3] and is problematic in China [4–6].

The immunology and serodiagnosis of echinococcosis have been reviewed previously [7–10]. In this review, we summarize the general consensus of the immunology and immunodiagnosis of CE, and reinforce previous findings with observations from some recent studies.

The *Echinococcus* organisms have a complex life cycle involving two hosts, a definitive carnivore host and an intermediate herbivore host. Intermediate hosts become infected by ingesting the parasite's eggs, which are released in the faeces of definitive hosts. The eggs hatch in the gastrointestinal tract and become activated larvae which penetrate the intestinal wall and enter the bloodstream, eventually locating in internal organs where they develop into hydatid cysts.

Hydatid cysts of *E. granulosus* develop in internal organs of humans and intermediate hosts (herbivores such as sheep, horses, cattle, pigs, goats, and camels) as unilocular fluid-filled bladders. These consist of two parasite-derived layers, an inner nucleated germinal layer and an outer acellular laminated layer surrounded by a host-produced fibrous capsule as the consequence of the host immune response [10]. Brood capsules and protoscoleces bud off from the germinal membrane. Carnivores such as dogs, wolves, and foxes act as definitive hosts. Sexual maturity of adult *E. granulosus* occurs in the host's small intestine within 4 to 5 weeks of ingesting offal containing viable protoscoleces. Gravid proglottids or released eggs are shed in the feces. An intermediate host is infected by taking an egg or eggs orally.

The intermediate host produces a significant immune response against *E. granulosus* infection [10]. However, the parasite has developed highly effective strategies for escaping the host defences and to avoid clearance. These mechanisms can be classified as antigenic mimicry, antigenic depletion, antigenic variation, immunologic indifference, immunologic diversion, and immunologic subversion [10]. Understanding how these immune responses are produced has been of

fundamental importance in developing immunodiagnostic kits and highly effective recombinant vaccines against *E. granulosus* infection.

There are three significant features of *E. granulosus* infection: (1) the parasite uses a large number of different mammalian species as intermediate hosts. Additional species can become quickly adapted as new intermediate hosts with the production of highly fertile cysts. Examples are Australian marsupials, which have become highly susceptible to CE after *E. granulosus* was introduced into Australia at the time of European settlement [11], and now plays a major role in the transmission of CE on this continent [12, 13]. (2) The resulting chronic cyst-forming disease in the intermediate host is characterized by long-term growth of the metacestode (hydatid) cysts in internal organs for as long as 53 years [14]. (3) The unilocular fluid-filled cysts can be located in most organs, with about 70% found in the liver, 20% occur in the lungs, with the remainder involving other organs such as the kidney, spleen, brain, heart, and bone. These distinct features combined with the multicellular nature of *E. granulosus* make CE a good general model for studying the immunology of chronic infections.

Cysts of *E. granulosus* can grow to more than 20 cm in diameter in humans, but the clinical manifestations are generally mild and remain asymptomatic for a considerable period. Consequently, serodiagnostic tools are important for screening populations at high risk of infection.

2. Host Immune Responses to Hydatid Infection

2.1. Antibody Responses. The earliest immunoglobulin (Ig) G response to CE hydatid cyst fluid and oncospheral antigens appears after 2 and 11 weeks, respectively, in mice and sheep challenged with eggs or oncospheres of *E. granulosus* [15, 16]. These antioncospheral antibodies play a major role in parasite killing and are central to the protective immune response against *E. granulosus* [17]. Although antibody levels against the oncosphere are low [15] in the early stages of infection, the parasite killing mechanisms may involve antibody-dependent cell-mediated cytotoxicity reactions [18, 19].

In the chronic phases of CE, there is frequent occurrence of elevated antibody levels, particularly IgG, IgM, and IgE [20–24], with IgG1 and IgG4 IgG subclasses being predominant [21, 25–29]. This antibody production is essential for the development of serodiagnostic tests.

About 30–40% of patients are antibody-negative for CE. In many of these patients, however, varying levels of circulating antigens (CAg) and circulating immune complexes (CIC) are measurable [30]. This phenomenon suggests that B cell activity and proliferation may be regulated and inhibited by *E. granulosus* antigens. It is not known whether these antigens directly target B cells or via T cell regulatory mechanisms.

2.2. Cellular Responses and Th2 Regulation. During the early stages of an echinococcal infection, there is a marked activation of cell-mediated immunity including cellular inflammatory responses and pathological changes [10, 31]. Cellular infiltration of eosinophils, neutrophils, macrophages, and fibrocytes occurs in humans [32, 33] and sheep [34] infections. However, this generally does not result in a severe inflammatory response, and aged cysts tend to become surrounded by a fibrous layer that separates the laminated cystic layer from host tissue.

There are very few reports on T cell cytokine profiles in an early primary (oral challenge with eggs) *E. granulosus* infection. Infection with *E. multilocularis* eggs induced low levels of interferon- (IFN-) gama, IL-2, and IL-4 at the beginning and high levels at the end of the infection [35, 36], and a similar immune profile in the early stage of CE infection is likely.

Given the recent advances in understanding the immunoregulatory capabilities of helminthic infections, it has been suggested that Th2 responses play a crucial role in chronic helminthiasis [37]. However, a remarkable feature of chronic CE infection is the coexistence of IFN-gamma, IL-4 and IL-10 at high levels in human echinococcosis [38]. It is unclear why hydatid infection can induce high levels of both Th1 and Th2 cytokines [39] since they usually downregulate each other [40]. Antigen and the amount of antigens released may play key roles. For instance, *E. granulosus* antigen B skewed Th1/Th2 cytokine ratios towards a preferentially immunopathology-associated Th2 polarization, predominantly in patients with progressive disease [41].

The role of IL-10 in chronic infection largely remains unclear, although one report showed that IL-10 may impair the Th1 protective response and allow the parasite to survive in hydatid patients [42]. The interaction of the *Echinococcus* organisms with their mammalian hosts may provide a highly suitable model to address some of the fundamental questions remaining such as the molecular basis underpinning the different effects of IL-10 on different cell types, the mechanisms of regulation of IL-10 production, the inhibitory role of IL-10 on monocyte/macrophage and CD4 T cell function, its involvement in stimulating the development of B cells and CD8 T cells, and its role in the differentiation and function of T regulatory cells.

2.2.1. Correlation of Cytokines with Antibody Production. Studies with mouse models to overexpress cytokines by inducing cytokine expression vectors showed that IL-12 and IFN-gamma induce a parasite-specific IgG2a response in mice infected with protoscoleces of *E. granulosus* whereas in IL-4-gene-transfected mice, IgG1 was elevated, indicating that IgG1 and IgG2 antibody isotypes are regulated by Th1 and Th2 cytokines, respectively [43].

When patients with relapsing disease or with viable, growing cysts, IgG1 and IgG4 are elevated and maintained at a high level [21, 44], whereas a low level of IFN-gamma produced by peripheral blood mononucleocytes (PBMC) in vitro compared with patients with a primary infection [45, 46]. For some relapsed cases, IFN-gamma levels were undetectable in the sera of patients [47] whereas the concentrations of specific IgG1 and IgG4 declined in cases characterized by cyst infiltration or calcification [44].This indicates that the IgG4 antibody response is also associated with cystic development, growth, and disease progression

whereas IgG1, IgG2, and IgG3 responses occur predominantly when cysts became infiltrated or are destroyed by the host [21].

2.2.2. T Cell Profile, Cyst Progression, and Efficacy of Treatment. The polarized Th2 cell response is a significant feature of the chronic stage of *Echinococcus* infection which is modulated by the developmental status of the hydatid cyst. In vitro T cell stimulation showed that cell lines from a patient with an inactive cyst had a Th1 profile while the T-cell lines derived from patients with active and transitional hydatid cysts had mixed Th1/Th2 and Th0 clones [48]. When CE patients were drug-treated with albendazole/mebendazole, a Th1 cytokine profile, rather than a Th2 profile, typically dominated, indicating that Th1 responses have a role in the process of cyst degeneration [46].

Mice injected with a vector expressing IL-4 displayed six times higher cyst load than the load in control mice [43], indicating IL-4 plays an important role in hydatid cyst development in the mammalian host.

Cytokine analysis of 177 CE patients showed that Th1 cytokines were related to disease resistance; in contrast Th2 cytokines were associated with disease susceptibility and chronicity [38]. Both in vitro and in vivo studies have shown that high levels of the Th1 cytokine IFN-gamma were found in patients who responded to chemotherapy, whereas high levels of Th2 cytokines (IL-4 and IL-10) occurred in patients who did not [46, 49–51], indicating IL-10/IL-4 impairs the Th1 resistant response allowing *E. granulosus* to survive [42, 52].

Self-cure of CE is common in sheep [53], and it most likely also happens in human populations in hyperendemic areas as patients with calcified cysts are reported [54, 55]. It would be of value to consider the T cell profiles of these self-cure patients as this may impact on future treatment approaches and vaccine development.

2.2.3. Dendritic Cells. More studies have focused on dentritic cells (DC) and their regulation on other immune responses in CE. *E. granulosus* antigens influence maturation and differentiation of DC stimulated with lipopolysaccharide (LPS) [56]. This includes downmodulation of CD1a expression and upregulation of CD86 expression, a lower percentage of CD83(+) cells present and, downregulation of interleukin-12p70 (IL-12p70) and TNF alpha [57]. In addition, hydatid cyst fluid (HCF) modulates the transition of human monocytes to DC, impairs secretion of IL-12, IL-6, or PGE2 in response to LPS stimulation, and modulates the phenotype of cells generated during culture, resulting in increased CD14 expression [56].

HCF antigen B (AgB) has been shown to induce IL-1 receptor-associated kinase phosphorylation and activate nuclear factor-kappa B, suggesting that Toll-like receptors could participate in *E. granulosus*-stimulated DC maturation [57].

E. multilocularis infection in mice induced DC expressing high levels TGF and very low levels of IL-10 and IL-12, and the expression of the surface markers CD80, CD86, and CD40 was downregulated [58, 59]. However, the higher level of IL-4 than IFN-gamma/IL-2 mRNA expression in AE-CD4+pe-Tcells indicated DC play a role in the generation of a regulatory immune response [59].

Different *E. multilocularis* antigens have been shown to stimulate different expression profiles of DC. Em14-3-3-antigen induced CD80, CD86, and MHC class II surface expression, but Em2(G11) failed to do so. Similarly, LPS and Em14-3-3 yielded elevated IL-12, TNF-I+/−, and IL-10 expression levels, while Em2(G11) did not. The proliferation of bone marrow DC isolated from AE-diseased mice was abrogated [60], indicating the *E. multilocularis* infection triggered unresponsiveness in T cells.

2.2.4. Summary of Immunological Responses in Echinococcosis and Directions for Further Study. Human helminth infections exhibit many immune downregulatory characteristics, with affected populations showing lower levels of immunopathological disease in cohort studies of allergy and autoimmunity. Model system studies have linked helminth infections with marked expansion of populations of immunoregulatory cells, such as alternatively activated macrophages, T regulatory cells (Tregs), and regulatory B cells [37].

In the established *Echinococcus* cystic stage, the typical response, in both humans and animals, is of the Th2 type and involves the cytokines IL-4, IL-5, IL-10, and IL-13, the antibody isotypes IgG1, IgG4, and IgE, and expanded populations of eosinophils, mast cells, and alternatively activated macrophages [10, 31]. The precise role of Th2 responses in parasitic infections is still not very clear. It is likely that *E. granulosus* controls the dialogue between cells of the immune system through the release of antigens which induce Th2 responses and suppression of others involving regulatory T and B cells. Th2 is significantly associated with chronic infection and may regulate the establishment of the parasite infection. More details are needed of the regulation of Th2 cytokines on antibody production, echinococcal cyst growth, and the efficacy of treatment. The role of the antibody responses in the host parasite interaction and chronic infection remains unknown in CE.

It has been shown that in vivo depletion of DC inhibits the induction of a Th2 immune response in chronic helminth infection and DC alone can drive Th2 cell differentiation [37]. It is not known which DC signals induce the Th2 differentiation programme in naïve T cells [61] but CE represents a good model to address this issue.

As well, a number of other critical questions remain that are important for studying the role of Treg cells in the chronic infection resulting from echinococcosis such as whether Treg cells present in greater frequencies in echinococcal infections as other infections [62, 63], whether *Echinococcus* can expand T reg cell populations, and whether the parasites secrete factors which can directly induce the conversion of naïve T cells into functional Treg cells. There are no studies in echinococcosis on regulatory B cells, which are populations of B cells that downregulate immune responses. These cells are most often associated with production of the immunosuppressive cytokine IL-10.

Moreover, many allergic and autoimmune inflammatory conditions can be ameliorated by a range of different helminth infections [64–66], so the question arises: can echinococcal infection reduce the allergic condition?

3. Serological Diagnosis

Typical asymptomatic features in the early stages of infection and for a long period after establishment makes early diagnosis of echinococcosis in humans difficult. Physical imaging to diagnose the CE infection, is usually used in the late stages of infection. Early diagnosis of CE by serology may, therefore, provide opportunities for early treatment and more effective chemotherapy. Another practical application of serology in human echinococcosis is the followup of the treatment.

Although hydatid disease is an asymptomatic infection, the host does produce detectable humoral and cellular responses against the infection. Measurement of these responses is a prerequisite for developing effective serodiagnostic tools.

3.1. Antibody Detection. Infection with larval cysts of *Echinococcus* in humans and intermediate animal hosts results in a specific antibody response, mainly of the IgG class accompanied by detectable IgM, IgA, and IgE antibodies in some patients [9, 31, 76, 77].

In terms of methodology, almost all serological tests developed for immunodiagnosis of human CE cases have incorporated the detection of antibodies. There are considerable differences between the various tests both in specificity and sensitivity. As the sensitivity of a test increases, so generally does the demand for improved antigens in order that sufficient specificity can be achieved to take advantage of the greater sensitivity. An optimum test should be specific with high sensitivity. Insensitive and nonspecific assays including the Cassoni intradermal test, the complement fixation test (CFT), the indirect haemagglutination (IHA) test, and the latex agglutination (LA) test have been replaced by the enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence antibody test (IFAT), immunoelectrophorosis (IEP), and immunoblotting (IB) in routine laboratory application [78].

A comparison of the diagnostic sensitivity and specificity of IEP, ELISA, and IB, in detecting IgG antibodies in patient sera to native and recombinant AgB and a hydatid fluid fraction (HFF), showed that HFF-IB gave the highest sensitivity (80%) followed by ELISA (72%) and IEP (31%). The diagnostic sensitivity significantly decreased as cysts matured (from type I-II to type VII, classified by ultrasound). Recombinant and native AgB-IB yielded similar levels of sensitivity (74%) but a large number of clinically or surgically confirmed CE patients (20%) were negative. In these patient sera, IB, to assess the usefulness of another recombinant *E. granulosus* molecule (elongation factor-1 beta/delta) in detecting IgE antibodies, yielded a positivity of 33%. Serological tests developed for determining anti-*Echinococcus* IgE in serum usually express results qualitatively or semiquantitatively in titres or units specific for the test kit [20, 79, 80].

The serodiagnostic performance of a range of different antigens and the various methods available for immunodiagnosis have been reviewed in depth [10, 31]. Some recent studies are referred to in Table 1 with the sensitivity and specificity of individual tests listed. Some antigens, such as native AgB and its recombinant proteins, yielded reasonable diagnostic performance using panels of sera from clinically confirmed cases of echinococcosis and other helminth infections. However, when the antigens were used for screening human populations in hyperendemic communities, they showed high seropositivity rates, although these rates had a low correlation with US monitoring of individual subjects [81].

Recently developed dipstick assays [82] are considered to be valuable methods for CE serodiagnosis. One dipstick assay has been developed that exhibited 100% sensitivity and 91.4% specificity when tested on sera from 26 CE patients and sera from 35 subjects with other parasitic infections using camel hydatid cyst fluid as antigen [83]. Since the dipstick assay is extremely easy to perform with a visually interpretable result within 15 min, in addition to being both sensitive and specific, the test could be an acceptable alternative for use in clinical laboratories lacking specialized equipment or the technological expertise needed for western blotting or ELISA. Similarly, a new 3-minute rapid dot immunogold filtration assay (DIGFA) for serodiagnosis of human CE and AE has been developed using four native antigen preparations crude and partially purified hydatid cyst fluid extracts from *E. granulosus* (EgCF and AgB), *E. granulosus* protoscolex extract (EgP), and *E. multilocularis* metacestode antigen (Em2) [70]. Like the dipstick assay, the test incorporates a simple eye-read colour change and achieved an overall sensitivity of 80.7% for human CE and 92.9% for human AE in a hospital diagnostic setting [70]. These rapid test scan be used for both clinical diagnostic support, as well as combining with ultrasound for mass screening in areas endemic for CE and AE.

Standardization of techniques and antigenic preparations and the characterization of new antigens are urgently required to improve the performance of hydatid immunodiagnosis. Antigens used in current tests are either cyst fluid or crude homogenates of the parasite collected from domestic animals. However, the supply of antigenic sources can often be limited, even for laboratory use. Since the preparation of purified echinococcal antigens relies on the availability of parasitic material and the quality control of this material is difficult to standardize for a large scale production, this can impact substantially on sensitivity and specificity of the available immunodiagnostic tools.

3.2. Antigen Detection. Antibody detection is likely to indicate exposure to an *Echinococcus* infection, but it may not necessarily indicate the presence of an established and viable infection, or the disease. Serum antibodies may persist for a prolonged period, reaching up to 10 years after hydatid cyst removal [84]. In addition, the degree of antibody response may be related to the location and condition of a mature

Table 1: Characteristics of assays using different antigens from *E. granulosus* developed after 2003 for immunodiagnosis of cystic echinococcosis.

Number of subjects tested			Antigen	Assay method	Sensitivity (%)	Specificity (%)	Ig isotype	Refs.
CE	Healthy controls	Other diseases						
44	—	43	8 kDa	WB	47.7	51.2	IgG	[67]
44	—	43	16 kDa	WB	45.5	67.4	IgG	[67]
44	—	43	24 kDa	WB	68.2	62.8	IgG	[67]
36	36	—	AgB	ELISA	91.7	97.2	IgG	[68]
102	95	68	rAgB1	ELISA	88.2	80.9	IgG	[69]
102	95	68	rAgB2	ELISA	91.2	93	IgG	[69]
875	5	739	AgB	Dot-WB	68.4	93.4	IgG	[70]
857	5	739	AgB	ELISA	57.4	93.4	IgG	[70]
324	70	500	AB	WB	86.4	92	IgG	[71]
155	110	58	?	ELISA	73.6	99.1	IgE	[72]
875	5	739	AgB	Dot-WB	68.4	93.4	IgG	[70]
857	5	739	AgB	ELISA	57.4	93.4	IgG	[70]
324	70	500	AB	WB	86.4	92	IgG	[71]
155	110	58	?	ELISA	73.6	99.1	IgE	[72]
155	110	58	?	ELISA	90.3	90.9	IgG	[72]
155	110	58	HCF	WB	90.1	94.5	IgG	[72]
324	70	500	EpC1	WB	88.7	95.6	IgG	[71]
95	37	—	HSP20		64		IgG1,4	[73]
97	37	58	Eg19	WB	10	100	IgG	[74]
102	95	68	E14t	ELISA	35.3	91.7	IgG	[69]
102	95	68	C317	ELISA	58.8	80.9	IgG	[69]
60	—	—	P5	WB	97	1	1	[75]

ELISA: enzyme-linked immunosorbent assay; WB: western blotting; dELISA: dot enzyme-linked immunosorbent assay.

hydatid cyst. For instance, hydatid cysts in human lung, spleen, or kidney tend to be associated with lower serum antibody levels [9]. Furthermore, in *Echinococcus*-endemic villages, up to 26% or more of the general population may have antibodies to HCF antigens, but with only about 2% of the villagers having hydatid cysts [81, 85, 86], indicating that the antibody levels may not necessarily reflect the true prevalence of CE.

Antigen detection may provide a suitable alternative. Serum antigen detection may also be less affected by hydatid cyst location and provides a tool for serological monitoring of antiparasitic therapy [87]. Circulating antigen (CAg) in CE patient sera, can be detected using ELISA directly or indirectly, and against titrated cyst fluid standards, CAg concentrations have been shown to vary from 100 to 700 ng/mL [88].

Antigen detection assays depend principally on the binding of specific polyclonal or monoclonal antibodies to parasite antigen present in serum or urine. A number of different assays have been developed to detect echinococcal antigens. The standard double antibody sandwich ELISA is a common method for measuring the presence and/or concentration of circulating parasite antigens. In the test, antibody raised to the targeted protein is coated onto a microtiter plate to capture antigen (Figure 1). The same antibody, which is enzyme labelled, is commonly used in the tertiary layer of the assay. This type of antigen capture therefore relies on the presence of multiple binding sites on the target antigens(s). Efforts to detect CAg in CE patients have been reviewed extensively by Craig et al. [85].

CAg in serum is normally in the form of a circulating immune complex (CIC) with some in free form. Therefore, the serum needs to be treated with acid buffer or polyethylene glycol (PEG) to release and concentrate the circulating antigens. Acidic treatment (0.2 M glycine/HCl) of CE patient serum is quite straightforward to dissociate CIC [85]. In a comparison of acid-treatment and PEG precipitation methods, all the sera of 30 confirmed positive cases of CE had detectable levels of antigen in the acid-treated sera [30]. However, 23 (77%) and 26 (87%) sera of 30 confirmed cases had free antigen as well as CIC of an 8 kDa antigen in the untreated and in the polyethylene glycol (PEG) precipitated sera, respectively. None of the sera from other patients with parasitic infections or viral hepatitis had any detectable levels of 8 kDa antigen in the untreated, acid-treated, or PEG-precipitated serum samples. These

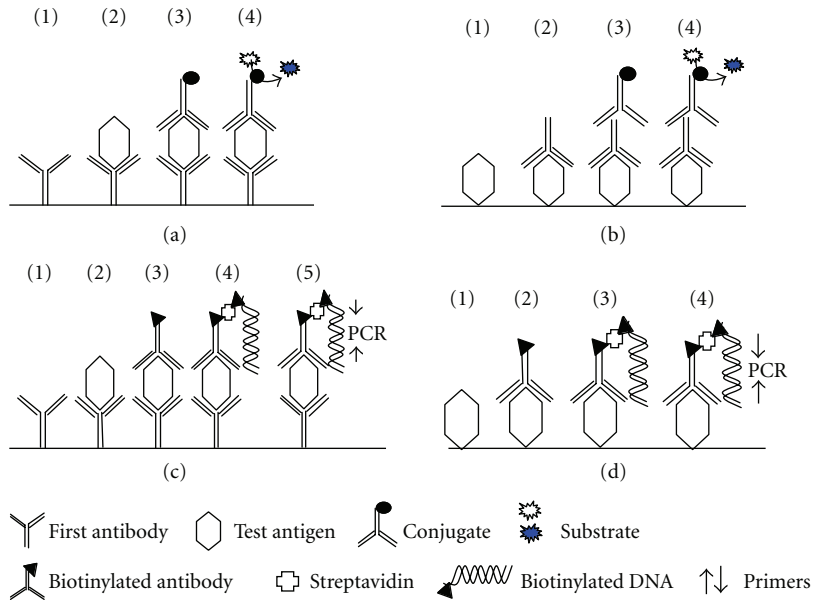

Figure 1: Schematic of ELISA and immuno-PCR for detecting circulating antigen in serum. (a) Sandwich ELISA. (1) Plate is coated with a capture antibody; (2) serum sample is added, and any antigen present in the serum binds to the capture antibody; (3) detecting antibody conjugate is added and binds to the antigen; (4) substrate is added, and is converted by the enzyme to a detectable form. (b) Direct ELISA. Plate is coated with diluted serum containing antigen; (2) detecting antibody is added, and binds to antigen; (3) enzyme-linked secondary antibody is added, and binds to detecting antibody; (4) substrate is added and is converted by the enzyme to a detectable form. (c) Capture immuno-PCR. (1) Plate is coated with capture antibody; (2) serum sample is added; (3) biotinylated detecting antibody is added and binds to antigen; (4) Streptavidin and biotinylated reporter DNA are added, and the biotinylated antibody and biotinylated reporter DNA are linked by streptavidin; (5) Primers and PCR components are added and PCR or real-time PCR undertaken to quantify antigen. (d) Non-capture immuno-PCR. Serum sample is coated on the plate and the remainder of the steps are as for the capture-immuno-PCR (C).

investigations, therefore, suggested that the demonstration of circulating antigen employing monospecific antibodies to affinity purified 8 kDa antigen in acid-treated sera is more efficient than the detection of free circulating antigen or CIC in untreated or in PEG-precipitated sera [89].

IgM CICs tend to be positively associated with active hydatid disease [85, 90]. Combining measurement of circulating antibody, CICs, and CAg resulted in an increase from 77% to 90% compared to measurement of serum antibody alone [91]. Antigens in soluble CICs from CE patients have been characterized by separating them on SDS-PAGE [85] or by ion-exchange fast protein liquid chromatography (FPLC) [92]. Both studies indicated a candidate antigen detectable in serum with an approximate relative molecular mass of 60–67 KDa, and which is also present in cyst fluid.

Comparison of CAg and IgG antibody using ELISA, together with western blotting, showed a relatively low sensitivity (43%) for detection of specific serum antigen in CE, compared to 75% for IgG antibodies [93]. However, the specificity of this CAg ELISA was 90% when tested against sera from AE patients and 100% against human cysticercosis sera. The limited cross-reactivity may be a way for practical diagnosis of CE in areas where AE and cysticercosis are coendemic. The advantage of CAg detection is its high sensitivity for detecting CE in 54–57% of patients who are serum antibody negative [91, 93]. CAg detection does appear, therefore, to be potentially useful as a secondary test for some suspected CE cases where antibody titers are low [85, 94].

A combination of CAg and antibody detection has been shown to increase the sensitivity from 85% (antibody only) to 89% (antibody+CAg) in ELISA of 115 surgically confirmed hydatid patients, 41 individuals exhibiting other parasitic and unrelated diseases, and 69 healthy subjects [95].

Although there has been no application to date for echinococcal diagnosis, a technique for antigen detection, called immunopolymerase chain reaction (immuno-PCR), was developed by Sano et al. [96]. It combines the molecular recognition of antibodies with the high DNA amplification capability of PCR. The procedure is similar to conventional ELISA but is far more sensitive. And, in principle, could be applied to the detection of single antigen molecules. Instead of an enzyme, a DNA molecule is linked to the detection antibody and serves as a template for PCR (Figure 1). The DNA molecule is amplified and the PCR product is measured by gel electrophoresis. An improvement of this method is to amplify the DNA fragment by real-time PCR, thereby eliminating post-PCR analysis. Furthermore, real-time PCR is extremely accurate and sensitive, which should make it possible to quantitate very low amounts of DNA-coupled detection antibody with high accuracy.

3.3. Serodiagnosis: The Future. Almost all available immunodiagnostic techniques, including methods for detecting specific antibodies and circulating parasite antigens in serum or other body fluids, have been applied for diagnosing echinococcosis. However, all the tools developed to date are

generally applicable for laboratory research purposes only. None of the available diagnostic tools, kits, or methods are generally accepted by clinical physicians. Nevertheless, such serological tools are potentially important for epidemiological studies, confirmation of infection status, and treatment and the monitoring of control programs, and efforts should continue so that new assays for improved, practical diagnosis of echinococcosis are developed.

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Clinical Study

Immune Modulation in Primary *Vaccinia virus* Zoonotic Human Infections

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In 2010, the WHO celebrated the 30th anniversary of the smallpox eradication. Ironically, infections caused by viruses related to smallpox are being increasingly reported worldwide, including *Monkeypox*, *Cowpox,* and *Vaccinia virus* (VACV). Little is known about the human immunological responses elicited during acute infections caused by orthopoxviruses. We have followed VACV zoonotic outbreaks taking place in Brazil and analyzed cellular immune responses in patients acutely infected by VACV. Results indicated that these patients show a biased immune modulation when compared to noninfected controls. Amounts of B cells are low and less activated in infected patients. Although present, T $CD4^+$ cells are also less activated when compared to noninfected individuals, and so are monocytes/macrophages. Similar results were obtained when Balb/C mice were experimentally infected with a VACV sample isolated during the zoonotic outbreaks. Taking together, the data suggest that zoonotic VACVs modulate specific immune cell compartments during an acute infection in humans.

1. Introduction

Thirty years after smallpox eradication the interest in *Orthopoxvirus* infections has been renewed by the potential use of Smallpox as a biological weapon [1] and the substantial increase in reports of zoonotic poxvirus infections throughout the world [2, 3], including the emergence of *Monkeypox virus* (MPV) in Africa and the USA [4], the emergence of *Vaccinia virus* (VACV) infections in Brazil [5–10], the maintenance of VACV in milking buffaloes in India [11, 12], and the increasing numbers of *Cowpox virus* (CPV) infections in Europe and Central Asia [13].

At the time of smallpox eradication, human immune responses to the *Variola virus* infection were not well understood, nor the response against live VACV strains used for vaccination. In the last years, however, our knowledge on how humans respond immunologically to *Orthopoxvirus* infections was greatly improved [14–20]. Most efforts have been directed to understand the mechanisms of protection against subsequent infections conferred by previous vaccination. In this respect, it is now clear that antibodies have a major role in long-term protection against Orthopoxviruses, with relatively high titers that remain stable for decades, whereas $CD8^+$ and $CD4^+$ T cell responses decline slowly

over time [14, 21–23]. On the other hand, the immune mechanisms involved in replication control and clearing of an *Orthopoxvirus* primary infection are still not fully understood, but essential roles for the innate and adaptive immunity have been demonstrated [24]. In the innate immunity context, studies have shown that the complement system and NK cells are important to control these infections until the adaptive responses arise; the loss of these functions results frequently in host death [25–27]. For the adaptive responses, the importance and efficiency of $CD8^+$ T cells to control and clear poxviruses in the absence of antibody production depend greatly on the animal model and the virus species used [20]. Studies using VACV inoculation in mice have shown that $CD4^+$ T and B cells are able and sufficient to eliminate the virus. However, this is apparently not the case when animals are infected with species-specific orthopoxviruses such as *Ectromelia virus* [28–30]. In the latter case, $CD8^+$ T cells are vital to contain the virus early in infection, as mice lacking $CD8^+$ T cells succumb early due to high viral load. However, CTL function alone is insufficient to clear the virus. At later stages, antibodies become essential for virus elimination and survival. Evidences come from studies showing the inability of animals deficient in B or $CD4^+$ T cells to effectively control and clear the infection [29–31]. Moreover, passive transfer of B cells or immune serum restores virus elimination capabilities in infected animals [30]. The requirement for $CD4^+$ T cells is clear as robust-specific antibody responses fail to develop in animals lacking these cells [28]. Similarly, $CD4^+$ T cell function is essential for an optimal CTL response [20]. The most likely scenario is that both cell and humoral immunities work complementarily to contain *Orthopoxvirus* acute infections.

The active circulation of orthopoxviruses in Brazil has been reported since the early 1960s. From 1999 onwards, many outbreaks of an exanthematic disease affecting humans and cattle alike were associated with such viruses. As isolates became available, the agent of such outbreaks was demonstrated to be the VACV. Infections are usually zoonotic, as the virus spreads from sick lactating cows to their handlers, leading to the formation of vesicle and ulcers on the hands, arms, torso and face of sick individuals (reviewed in [32, 33]).

Here we analyzed aspects of the cellular immune responses in patients acutely and naturally infected by VACV during zoonotic outbreaks taking place in Brazil. Our results indicate that these infections trigger a virus-specific immune modulation biased mainly towards macrophage and T $CD4^+$ and B cell functions.

2. Materials and Methods

2.1. Study Population. The study population consisted of 53 individuals showing signs of poxvirus infection, ages between 18 and 70 years, both genders, all living in the outbreak areas. Patients were classified as acutely infected on the basis of the occurrence of typical clinical symptoms (mainly the presence of nonhealed pustules and vesicles), VACV DNA detection in serum samples or lesion fluids, and virus isolation from lesion swabs. Eighteen healthy individuals, with no signs of infection, 29 to 55 years old, also residing at the outbreak areas, were enlisted and included in the study as a noninfected control group. All patients were properly examined by a physician and those presenting apparent clinical signs of any other disease, infectious or not, were not included in the study.

2.2. Virus Isolation from Animals and Humans. Fluid from suppurated lesions was collected using a sterile swab and maintained in MEM culture media for transportation. Viruses were isolated by inoculation in chorio-allantoic membranes of embryonated chicken eggs (CAMs) and amplified in VERO cells. Viruses were purified and characterized by neutralization tests using anti-VACV antibodies and by nucleotide sequencing of *Orthopoxvirus*-specific genes after PCR amplification using VACV-specific primers [34].

2.3. Phylogenetic Analysis. The hemagglutinin (HA) gene nucleotide sequences from the isolated viruses and from other Orthopoxviruses (retrieved from GenBank) were aligned on the basis of codon positions using the CLUSTAL W software. Alignments were manually edited and used to perform phylogenetic analyses using the Neighbor-joining and Maximum-likelihood methods implemented in Mega3 and Paup*4.0b10. GenBank accession numbers are as follows: Vaccinia virus: Western Reserve (VACV-WR) (AY243312), VACV-Lister (AY678276), Modified virus Ankara (VACV-MVA) (AY603355), Copenhagen (VACV-COP) (M35027), VACV-Wyeth (Z99051), VACV-TTan (U25662), VACV-Malbran (AY146624), Br-Hu-1 (FJ173000), Br-Hu-2 (EF063677), Br-An-1 (FJ173001), Br-An-2 (FJ173002), Br-An-3 (FJ173003), Passatempo (VACV-PSTV) (DQ070848), Cantagalo (VACV-CTGV) (AF229247), Araçatuba (VACV-ARAV) (AY523994), Guarani P2 (VACV-GP2V) (DQ206437), Muriaé (VACV-MURV) (DQ247770), VACV-BeAn58058 (DQ206442), Belo Horizonte (VACV-VBH) (DQ206435), VACV-IOC (AF229248), Guarani P1 (VACV-GP1V) (DQ206436), VACV-SPAn232 (DQ222922), Lister Butantan (VACV-LTBUT) (EF175985); Buffalopox virus (BFPV-3906) (AF375077); Cowpox virus Brighton Red (CPXV-BR) (AF482758)'Rabbitpox virus rev (RBPV-rev) (AY484669); Ectromelia virus Moscow (ECTV) (AF012825); Camelpox virus: CMS (CMLV-CMS) (AY009089), M-96 (CMLV-M96) (AF438165); Variola virus: Garcia-1966 (VARV-GAR) (U18338) and Bangladesh-1975 (VARV-BSH) (L22579).

2.4. Cell Preparation and Proliferation Assay. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-diatriazoate density gradient centrifugation (LSM, Organon Teknica, Charleston, SC), cultured in triplicate (10^6 cells/well) in 96-well flat-bottom plates, and stimulated with either UV-inactivated VACV-WR for 6 days or PHA (at 2.5 μg/mL to test cell viability) for 3 days. Tritiated thymidine (1 μCi/well) was added to the cultures for 6 hours before completion of the incubation period. Incorporation of [^{3}H]thymidine was determined by liquid scintillation counting. Data were analyzed and presented as countings per minute (CPM) (calculated as the mean experimental cpm ± SD − mean control cpm ± SD).

2.5. Detection of Cytokine Levels by Cytometric Bead Array Immunoassay (CBA). Microbeads consisted of six distinct populations, unique on their Type 3 fluorescence intensity (FL-3), each coupled to mAb against one of the six Th1/Th2 and regulatory cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ). Captured cytokines were detected using six different mAbs coupled to PE (FL-2). A total of 1,800 events/gate were acquired. Standard curves were plotted using a four-parameter logistic curve fitting model. Cytokine concentrations were determined using standard curves. If a sample had a cytokine concentration below the detection limit for the assay, a value of 0 was attributed for statistical purposes.

2.6. Cell Phenotype Analysis. Cells were quantified after *in vitro* antigenic stimulation with UV-inactivated VACV using mouse anti-human monoclonal antibodies (MoAbs) conjugated with FITC or PE, specific for cell-surface markers. Cultured cells were washed in PBS containing 1% BSA plus 0.1% sodium azide (FACS buffer) and stained with monoclonal antibodies against CD3, CD4, and CD8 for T cell populations, CD19 for B cells, CD16 and CD56 for NK cells, and CD14 for monocytes. Same cells were labeled simultaneously with antibodies against costimulatory molecules (HLA-DR, CD25, CD69, CD28, CD80, and CD86). Cell preparations were fixed in FACS fix solution and stored at 4°C in the dark. A total of 30,000 events/tube were acquired using a FACScalibur flow cytometer (Becton Dickinson) set up to measure forward (FSC), side (SSC) light scatters, FITC (FL-1), and PE (FL-2) fluorescence. CELLQuest software was used for data acquisition and analysis.

2.7. Statistical Analysis. Analyses were performed using GraphPad Prism version 3.0 software. The following non-parametric tests were performed: (1) Mann-Whitney test to compare two groups (noninfected $\times$ infected individuals); and (2) Wilcoxon test to compare cultures stimulated and nonstimulated. The statistical analysis was performed by using the median values of each group.

2.8. Animal Experiments. Groups of 10 four-week-old male Balb/C mice were used. Animals were intranasally infected with 10^4 PFUs of a zoonotic VACV sample in PBS. Ten days after infection, animals were anesthetized and blood was collected. PBMCs were obtained as mentioned and cell surface markers (CD4, CD14, CD25, and CD69) were detected as described above.

2.9. Ethics Statement. The human study protocol complied with the Brazilian National Council of Health regulations and was approved by the Instituto René Rachou Review Board (IRR IRB) under protocol number 03/2006. All patients signed informed consents. Animal experiments were conducted in accordance with the Brazilian Federal Law number 11.794 (October 8th, 2008), which regulates the scientific use of animals, and IACUC guidelines. All protocols were approved by the Committee of Ethics for Animal Experimentation (CETEA) at UFMG under permit 9/2009, valid through April 2014. The CETEA-UFMG is affiliated to the National Council of Animal Experimentation Control (CONCEA).

3. Results

3.1. Characterization of the Population Involved in the Study. Since 1999, yearly outbreaks of an exanthematic disease affecting humans and cattle have been reported among poor pockets of population in the rural countryside of Southeast Brazil. In most cases, the isolated infectious agent causing the zoonotic outbreaks was the VACV. We have followed outbreaks taking place in farms at the Minas Gerais State, SE, Brazil, from 2005 to 2009. The studied group consisted of 53 affected human patients, who were clinically evaluated, and 18 noninfected and healthy individuals living at the same areas affected by the outbreaks (control group). Clinical symptoms of the infection included high fever, headache, muscle pain, nausea, lymphangitis, and the appearance of pleiomorphic lesions on hands, forelimbs and eventually in the face, torso, and genitals. In all patients, acute lesions were associated with a roseolar erythema and localized edema leading to the formation of vesicles [8]. As pointed out in previous studies [5–8], the disease is occupational, as persons dealing with infected dairy cattle were those presenting signs of infection. Importantly, out of 53 infected patients, at least 10 were vaccinated against smallpox in the past, as confirmed by visualization of a typical vaccination scar in their left arm.

3.2. Virus Samples Isolated during the Studied Outbreaks Are Genetically Consistent with Previously Described Circulating VACV Isolates. Two virus isolates were obtained from human patients during the studied outbreaks and characterized, together with three other samples isolated from cattle at the same areas (herein referred as VACV-Br-Hu-1, VACV-Br-Hu-2, VACV-Br-An-1, VACV-Br-An-2, and VACV-Br-An-3). The hemagglutinin (HA) genes from all viruses were sequenced, and they presented a signature of 18-nucleotide deletion also observed in previously isolated Brazilian VACVs [5–7, 32, 34]. Phylogenetic analyses based on the HA nucleotide sequences demonstrated that all isolated viruses cluster together with other Brazilian VACV samples isolated in the past, and none cluster with attenuated vaccine strains including VACV-Lister, the Lister-derived Butantã strain (LT-BUT), MVA, or Wyeth (Dryvax) (Figure 1). The data confirmed that the viruses involved with the studied outbreaks are consistent with those involved in past described VACV zoonotic outbreaks in Brazil.

3.3. PBMCs from Infected Individuals Proliferated and Produced IFNγ after Stimulation with VACV Antigens. In order to evaluate the immune responsiveness of the infected individuals to VACV antigens, we stimulated their peripheral blood mononuclear cells (PBMCs) *ex vivo*. Cells were expose to either PHA (Figure 2(a)) or UV-treated VACV (Figure 2(b)), and cell proliferation was determined by [^{3}H] thymidine incorporation. Upon mitogenic or antigenic stimulation, PBMCs from VACV-infected individuals presented a significantly ($P = 0.01$) higher cellular proliferative response

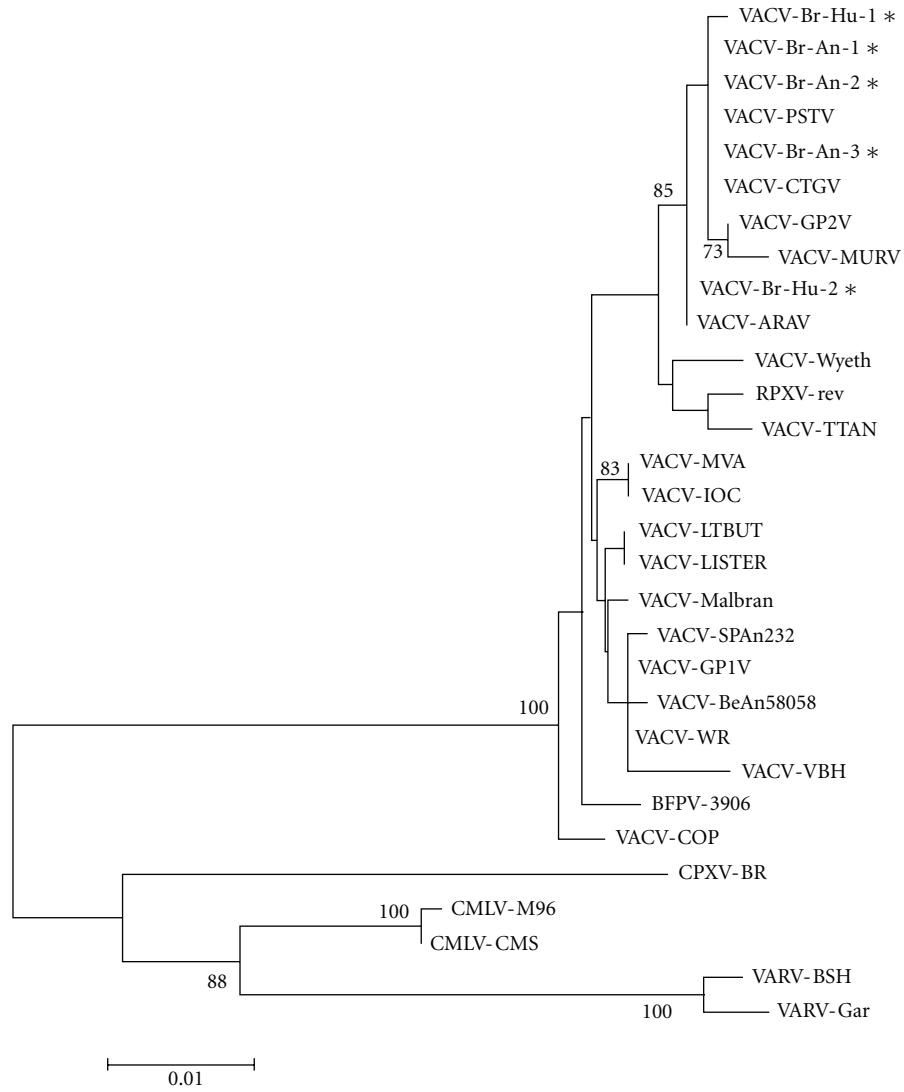

Figure 1: Phylogenetic relationships between the VACV samples obtained in this study and other relevant poxviruses. The phylogenetic tree was constructed by the Neighbor-joining method and used hemagglutinin gene nucleotide sequences from various orthopoxviruses, including Brazilian *Vaccinia virus* (VACV) isolates and other poxviruses. The Tamura3-parameter nucleotide substitution model was used and the reliability of the branching patterns was tested by 1000 bootstrap pseudo-replicates. Bootstrap values above 70% are shown. The scale bar represents 1% nucleotide sequence divergence. Samples are as follows: Zoonotic Brazilian Vaccinia virus isolated from humans (VACV-Br-Hu-1, VACV-Br-Hu-2) or cattle (VACV-Br-An-1, VACV-Br-An-2, VACV-Br-An-3)—labeled with stars (∗); other *Vaccinia virus* strains isolated in Brazil—Passatempo (VACV-PSTV), Cantagalo (VACV-CTGV), Araçatuba (VACV-ARAV), Guarani P2 (VACV-GP2V) (DQ206437), Muriaé (VACV-MURV), VACV-BeAn58058, Belo Horizonte (VACV-VBH), VACV-SPAn232, Guarani P1 (VACV-GP1V); reference *Vaccinia virus* strains—Western Reserve (VACV-WR), VACV-Lister, Modified virus Ankara (VACV-MVA), Copenhagen (VACV-COP), VACV-Wyeth, VACV-TTan, VACV-Malbran, Lister Butantan (VACV-LTBUT); VACV-IOC (AF229248), Buffalopox virus (BFPV-3906) (AF375077); other Orthopoxviruses—Cowpox virus Brighton Red (CPXV-BR), *Rabbitpox virus* rev (RBPV-rev), Ectromelia virus Moscow (ECTV), *Camelpox virus* CMS (CMLV-CMS), *Camelpox virus* M-96 (CMLV-M96), *Variola virus* Garcia-1966 (VARV-GAR), and *Variola virus* Bangladesh-1975 (VARV-BSH).

when compared to PBMCs from noninfected subjects. Cells from both groups showed lower proliferative responses when mock-treated with culture medium, as expected (Figures 2(a) and 2(b)). Mathew and coworkers [35] demonstrated that PBMCs from individuals who received VACV immunization presented transient decreased proliferative responses to PHA, anti-CD3, and VACV antigens when comparing the proliferative responses from single individuals before and after vaccination. However, this was not observed when we compared antigen-induced proliferation in PBMCs from naturally infected patients to noninfected individuals. Next, we evaluated the levels of cytokines secreted by PBMCs on the culture supernatants of cells obtained from all subjects. The amounts of secreted IFNγ produced after VACV antigenic stimulation were significantly higher in infected individuals ($P < 0.001$) (Figure 3). This result differs

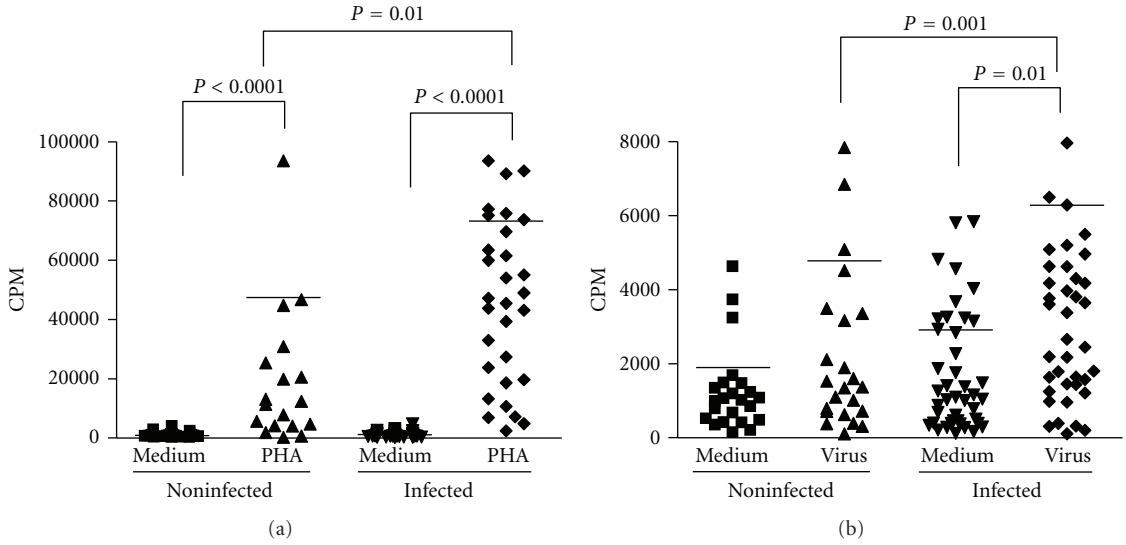

Figure 2: Proliferative responses in PBMCs of individuals infected or not by zoonotic *Vaccinia virus.* Peripheral blood mononuclear cells (PBMCs) from patients infected or not with zoonotic *Vaccinia virus* were cultured in the presence of PHA (a), UV-inactivated virus (b) or mock-treated (medium). After 6 days of stimulus, the cell proliferation was determined by [^{3}H]thymidine incorporation. Statistical significance (P values), based on the median values of each group, is presented on the graphs. CPM: counts per minute.

Table 1: Mean percentage of T (CD3$^+$, CD4$^+$, CD8$^+$) and B (CD19$^+$) lymphocytes, NK cells (CD16$^+$) and monocytes (CD14$^+$) on PBMCs from noninfected or zoonotic *Vaccinia virus* infected individuals after stimulation with virus antigens.

	Cell phenotype					
Groups	CD3$^+$	CD4$^+$	CD8$^+$	CD19$^+$	CD16$^+$	CD14$^+$
Noninfected	72.5 ± 3.9	52.1 ± 5.2	19.2 ± 2.1	16.4 ± 3.3	22.8 ± 6.5	2.9 ± 1.4
Infected	79.2 ± 4.9*	58.3 ± 3.2	20.3 ± 2.0	11.4 ± 2.5**	19.8 ± 7.4	4.4 ± 1.4

PBMCs: peripheral blood mononuclear cells.
*$P = 0.008$, when infected and noninfected groups were compared.
**$P = 0.03$, when infected and noninfected groups were compared.

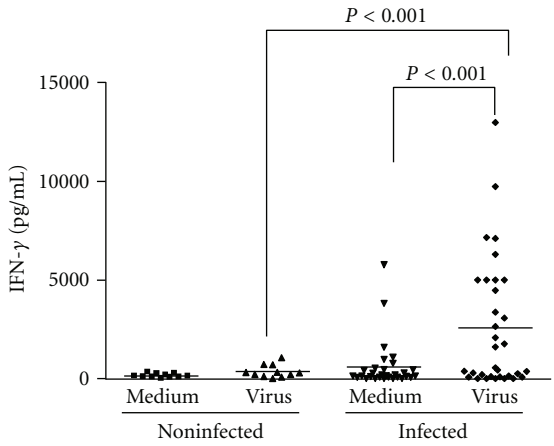

Figure 3: Interferon-gamma production in PBMCs of individuals infected or not by zoonotic *Vaccinia virus.* Peripheral blood mononuclear cells (PBMCs) from patients infected or not with zoonotic *Vaccinia virus* were cultured in the presence of UV-inactivated virus or mock-treated (medium). After 6 days of stimulus, the amount of IFN-γ produced in the cultures' supernatants was measured by cytometric bead array immunoassay. Statistical significance (P values), based on the median values of each group, is presented.

from a previous observation in which a single VACV-infected patient was studied and presented diminished amounts of IFNγ in comparison to noninfected controls [8]. Mock-treated cells did not produce significant amounts of IFNγ (Figure 3). Analysis of IL-2, IL-4, IL-5, IL-10, and TNF-α from both groups did not show detectable amounts of these cytokines in culture supernatants.

3.4. CD4$^+$ T Cells, but Not CD8$^+$ T Cells, Are Less Activated in Infected Patients When Compared to Noninfected Individuals. Analysis of the mean percentage of T and B lymphocytes, NK cells and monocytes were performed on PBMCs from both groups after *ex vivo* stimulation with UV-treated VACV. These analyses demonstrated an increase on the mean percentage of T cells (CD3$^+$) ($P = 0.008$) and a surprisingly lower mean percentage of B lymphocytes (CD19$^+$) ($P = 0.03$) in infected individuals (Table 1). Therefore, these results were associated with a significant increase in CD3$^+$: CD19$^+$ ratio in infected individuals when compared to the noninfected group ($P = 0.01$). No significant differences were observed in the mean percentage of T lymphocyte subsets (CD4$^+$ and CD8$^+$) and in the mean values of monocytes (CD14$^+$) and NK cells (CD16$^+$)

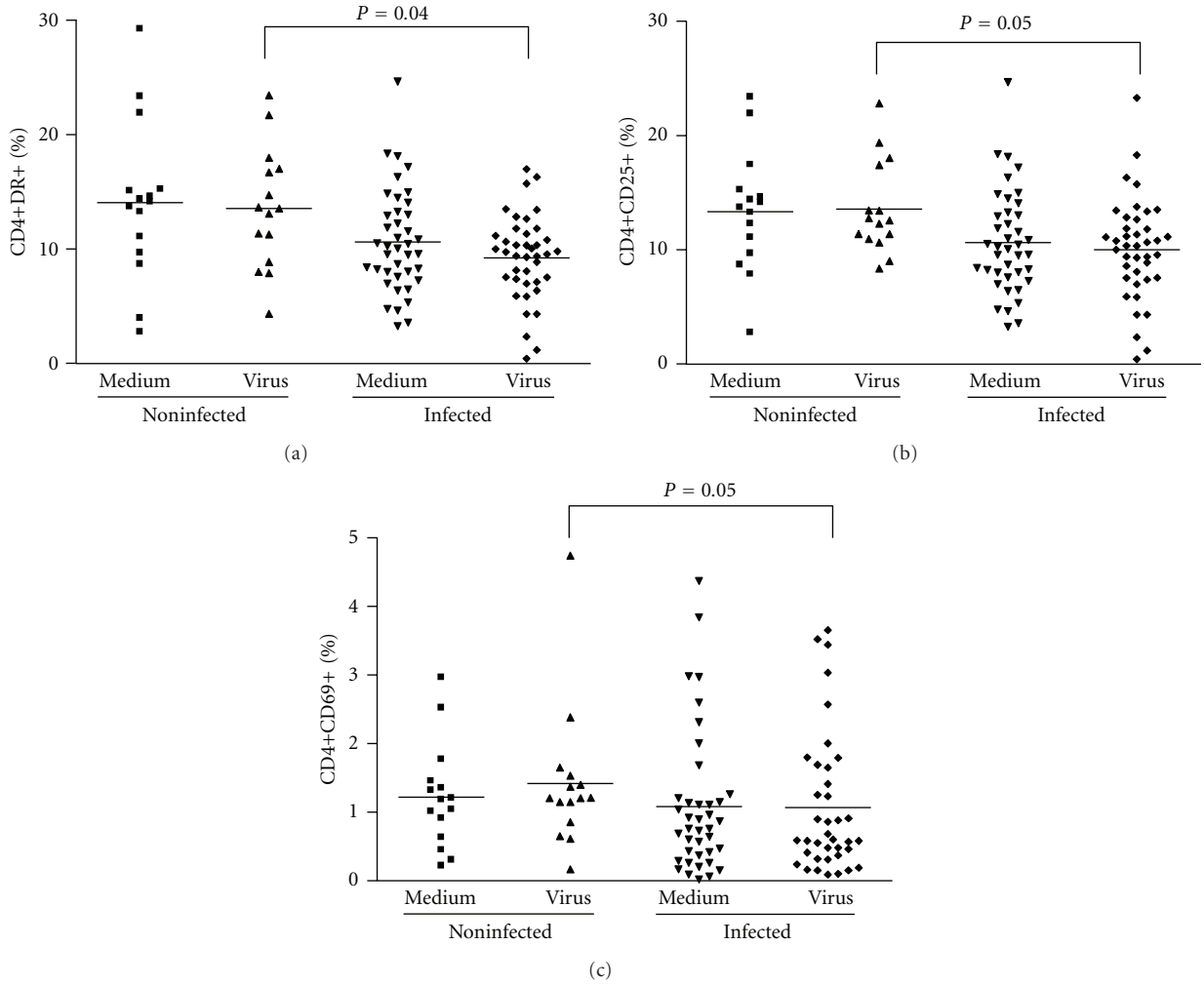

Figure 4: $CD4^+$ T-cell activation status in PBMCs from individuals infected or not by zoonotic *Vaccinia virus*. Peripheral blood mononuclear cells (PBMCs) from patients infected or not with zoonotic *Vaccinia virus* were cultured in the presence of UV-inactivated virus or mock-treated (medium). After 72 hours of stimulus, cells were fixed, counted, and the following parameters were evaluated by flow cytometry using specific mouse anti-human antibodies: (a) percentage of $CD4^+$ T lymphocytes expressing HLA-DR surface activation marker; (b) percentage of $CD4^+$ T lymphocytes expressing CD25 surface activation marker; (c) percentage of $CD4^+$ T lymphocytes expressing CD69 surface activation marker. Statistical significance (P values), based on the median values of each group, is presented on the graphs.

(Table 1). Apart from the unexpected decrease in total B cell amounts in infected patients, other results seemed to be consistent with typical late immune responses during an acute viral infection, especially considering T-cell responses. However, when T-cell subsets coexpressing CD25, CD69, CD28, CTLA-4, and HLA-DR, as activation markers, were analyzed on PBMCs from infected and noninfected individuals, a different picture emerged. Expression of HLA-DR, CD25, and CD69 on the surface of $CD4^+$ T lymphocytes was lower in infected patients ($P = 0.04$, $P = 0.05$, and $P = 0.05$, resp.) (Figure 4). Importantly, we observed no differences in the cell activation status when PBMCs were either stimulated with VACV antigens or mock-treated. Expression levels of CD28 and CTLA-4 on $CD4^+$ T lymphocytes from the two human groups were not significantly different (not shown). On the other hand, $CD8^+$ T lymphocytes from infected individuals presented a significant increase in CD28 expression ($P = 0.03$) (Figure 5(a)). No significant differences were observed when cells were stimulated with VACV antigens or mock-treated. Analysis of HLA-DR, CD25, and CD69 on $CD8^+$ T lymphocytes did not show any statistical differences between the groups (not shown).

3.5. $CD14^+$ Cells and B Cells Are Less Activated in Infected Patients When Compared to Noninfected Individuals. Relative Amounts of Regulatory CD8 T Cells Are Also Smaller in Infected Subjects. Some Orthopoxviruses, such as the CPV and VACV, are known to interfere with the APC's functions by disrupting MHC classes I- and II-mediated antigen presentation [36–39]. Therefore, although monocytes were present in PBMCs from infected and noninfected patients in comparable amounts, it seemed appropriate to check whether these cells were activated. Thus, the expression of the activation markers CD80 and CD86 on the surface

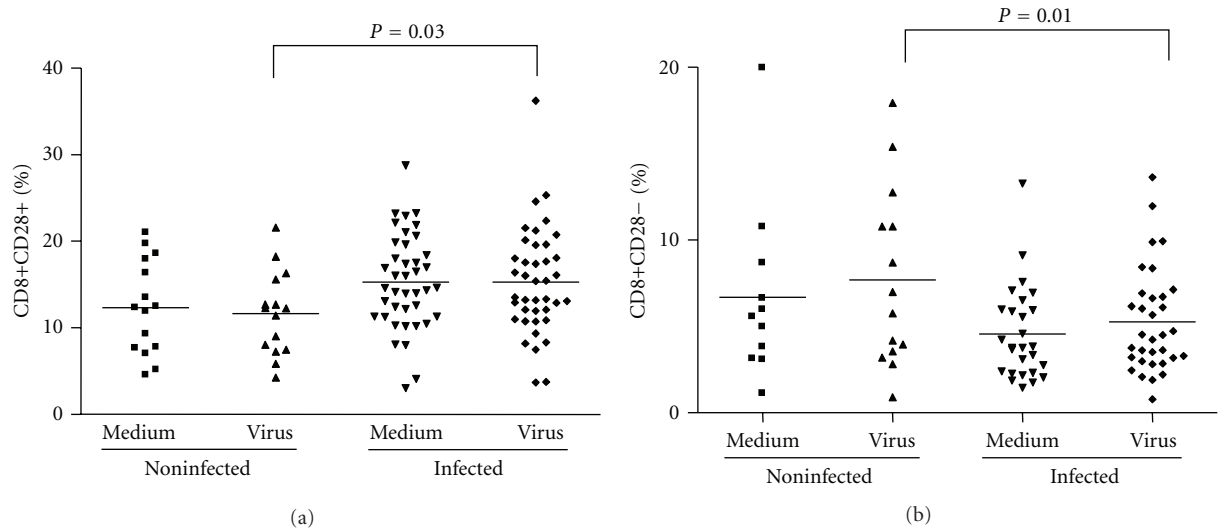

Figure 5: CD28 molecule expression and evaluation of regulatory T $CD8^+$ cells in PBMCs from individuals infected or not by zoonotic *Vaccinia virus*. Peripheral blood mononuclear cells (PBMCs) from patients infected or not with zoonotic *Vaccinia virus* were cultured in the presence of UV-inactivated virus or mock-treated (medium). After 72 hours of stimulus, cells were labeled with mouse anti-human CD8 antibodies and anti-human CD28 antibodies. The percentages of $CD8^+$ $CD28^+$ (a) and $CD8^+$ $CD28^-$ (b) T-cell subsets were determined by flow citometry. Statistical significance (*P* values), based on the median values of each group, is presented on the graphs.

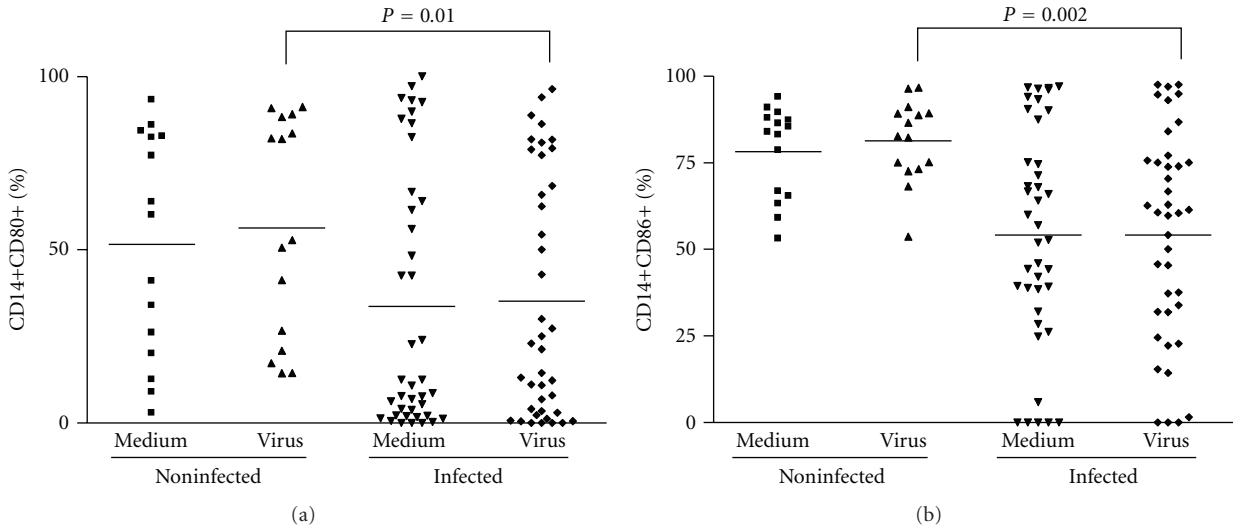

Figure 6: Monocyte ($CD14^+$) activation status in PBMCs from individuals infected or not by zoonotic *Vaccinia virus*. Peripheral blood mononuclear cells (PBMCs) from patients infected or not with zoonotic *Vaccinia virus* were cultured in the presence of UV-inactivated virus or mock-treated (medium). After 72 hours of stimulus, cells were fixed, counted, and the following parameters were evaluated by flow cytometry using specific mouse anti-human antibodies: (a) percentage of monocytes ($CD14^+$) expressing CD80 surface activation marker; (b) percentage of monocytes ($CD14^+$) expressing CD86 surface activation marker. Statistical significance (*P* values), based on the median values of each group, is presented on the graphs.

of macrophages/monocytes ($CD14^+$) after stimulation with VACV antigens were evaluated. The percentage of monocytes expressing such markers was significantly lower in infected patients ($P = 0.01$ and $P = 0.002$, resp.) (Figures 6(a) and 6(b)). Likewise, the expression of CD80 and CD86 on the surface of B lymphocytes ($CD19^+$) was measured. Not only the total amounts of B lymphocytes were lower in infected patients (Table 1), but these cells were also less activated when compared to uninfected individuals, as judged by the low percentage of $CD19^+$ $CD80^+$ and $CD19^+$ $CD86^+$ cells on PBMCs from the first group ($P = 0.01$) (Figures 7(a) and 7(b)). Finally, we also evaluated relative amounts of regulatory $CD8^+$ T Lymphocytes ($CD8^+/CD28^+$ to $CD8^+/CD28^-$ ratio) in PBMCs from all subjects. PBMCs from infected subjects presented significant decrease ($P = 0.01$) in the relative amounts of regulatory CD8 T Lymphocytes when

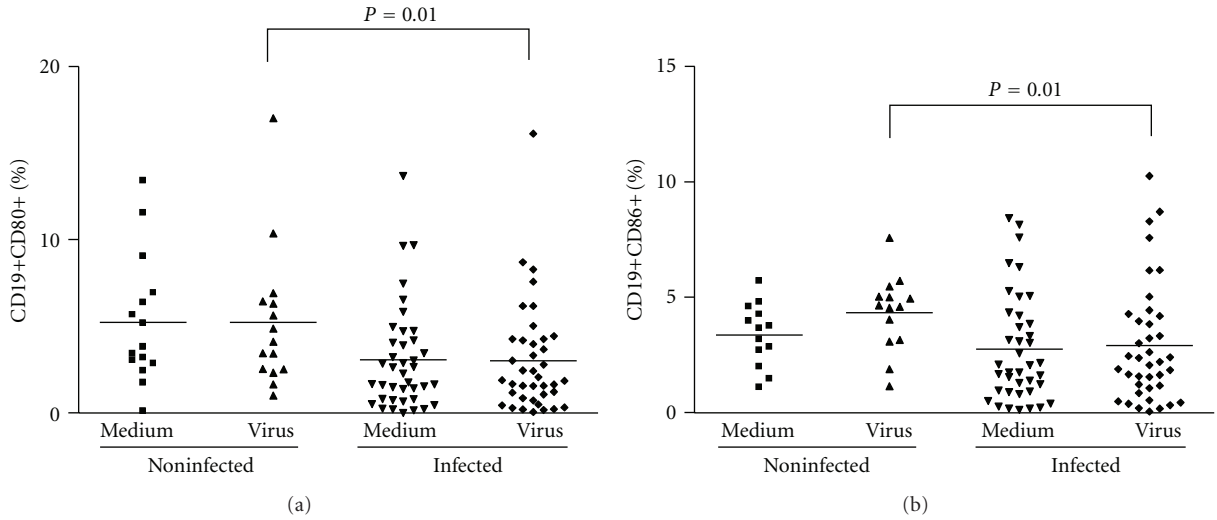

Figure 7: B cell ($CD19^+$) activation status in PBMCs from individuals infected or not by zoonotic *Vaccinia virus*. Peripheral blood mononuclear cells (PBMCs) from patients infected or not with zoonotic *Vaccinia virus* were cultured in the presence of UV-inactivated virus or mock-treated (medium). After 72 hours of stimulus, cells were fixed, counted, and the following parameters were evaluated by flow cytometry using specific mouse anti-human antibodies: (a) percentage of B lymphocytes ($CD19^+$) expressing CD86 surface activation marker; (b) percentage of B lymphocytes ($CD19^+$) expressing CD80 surface activation marker. Statistical significance (P values), based on the median values of each group, is presented on the graphs.

compared to noninfected individuals (Figure 5(b)). This is an interesting finding since increase in $CD8^+$ regulatory cells have been demonstrated to inhibit CD4 proliferation. Overall, no significant differences were seen between cells stimulated with VACV antigens or mock-treated.

3.6. Mice Infected with a VACV Zoonotic Isolate Also Show Modulation in Specific Compartments of Their Immune Responses. In order to further confirm the zoonotic VACV's ability to modulate specific immune responses, we infected mice with an isolated VACV sample and analyzed the cellular immune response elicited during the onset of the acute disease. Although not all parameters studied for humans were evaluated in the mice experiments, the obtained results were comparable to those observed in infected persons. Total counts of macrophages/monocytes ($CD14^+$ cells) were lower in the infected group ($P < 0.001$) (Figure 8(a)), and the expression of CD25 surface activation marker was diminished on $CD4^+$ T cells when compared to noninfected animals ($P = 0.01$) (Figure 8(b)). Relative amounts of specific $CD8^+$ T cells were higher in infected animals when compared to the noninfected group ($P = 0.01$) (Figure 8(c)).

4. Discussion

Orthopoxvirus-mediated modulation of immune responses in humans is poorly demonstrated due to the inherent difficulties to study populations infected by virulent virus strains. In this respect, Hammarlund and colleagues [19] have shown that MPV infecting human cells is able to evade $CD4^+$ and $CD8^+$ T cell responses through an MHC-independent mechanism, although, in this case, they did not evaluate immune responses in acutely infected patients.

On a previous work we had shown that one studied VACV-infected patient produced low amounts of IFNγ [8]. However, when a larger group of infected patients were analyzed in the current study, this pattern was not observed. Variations on the levels of IFNγ produced by different individuals is usually high, as exemplified by the individual dot distribution in Figure 3, and that may explain the fact that this cytokine level was low on the study performed before. Indeed, cytokine production may vary enormously in field patients as a result of different ages; different genetic backgrounds; other existing microbial infections; immunological and nutritional status; general health conditions. Such variations in IFNγ production has been seen in field studies of malaria, for instance, among other infectious diseases [40, 41].

We have also shown that specific compartments of the human immune response to zoonotic VACV acute infections are virus-modulated. That could be inferred from a marked virus-induced decrease in the activation status of $CD4^+$ T cells, B cells, and macrophage/monocytes. This apparent bias in the virus immune evasion strategy may suggest that these specific responses are those responsible for the impairment of VACV replication success in the human host. Indeed, viruses in general, and specially poxviruses (due to their large coding capacity), encode a multitude of proteins that interfere with diverse immunological functions of the host [42–44]. Moreover, viruses are usually very didactical in demonstrating which components of the host immune system are determinants to their replicative success by encoding evasion proteins that specifically affect such components. The fact that humans acutely infected by virulent VACV showed an apparent virus-induced modulation of macrophages/monocytes, $CD4^+$ T and B cells

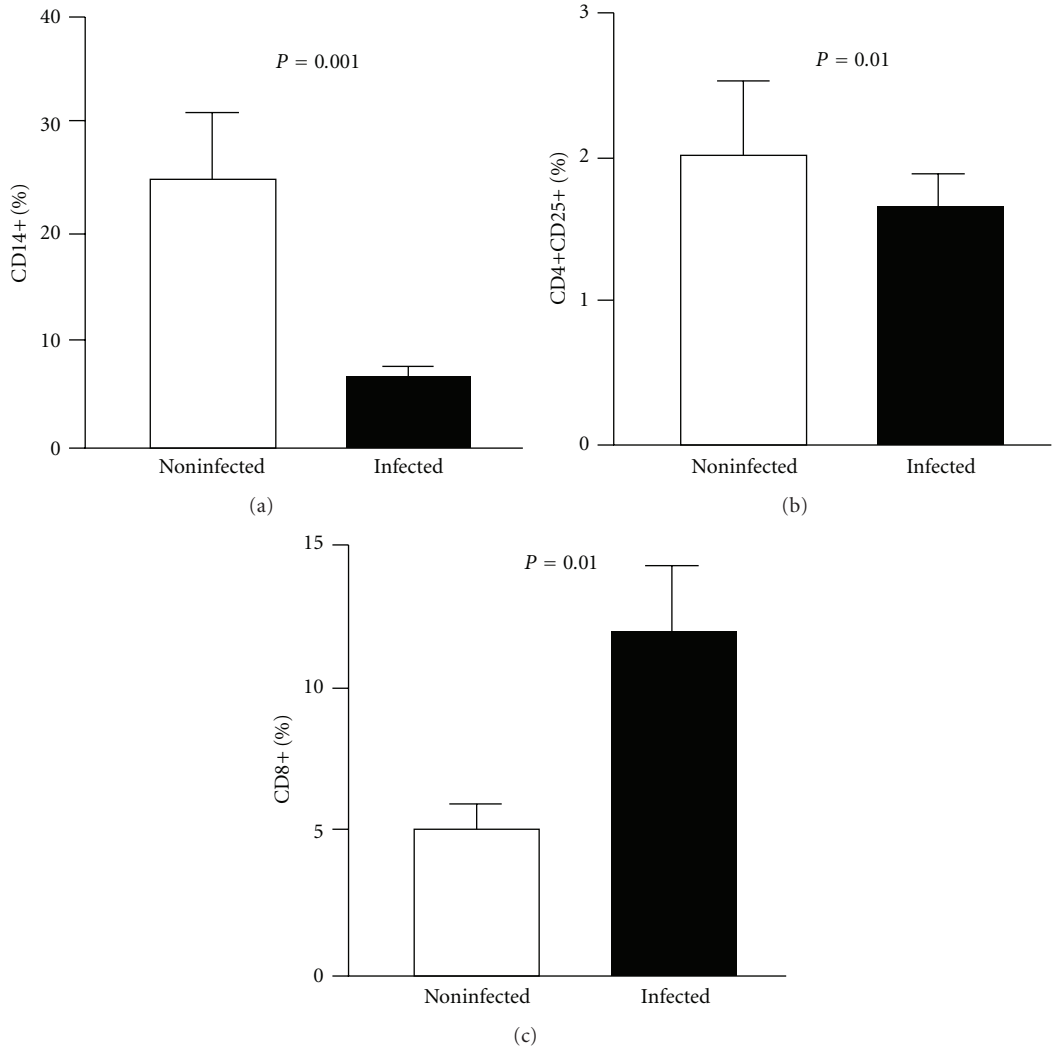

Figure 8: Aspects of the immune responses in mice infected or not with zoonotic *Vaccinia virus*. Peripheral blood mononuclear cells (PBMCs) from mice either infected with a zoonotic *Vaccinia virus* isolate or noninfected were cultured for 72 hours in the presence of UV-inactivated VACV. After incubation, cells were labeled with specific antibodies and analyzed by flow cytometry. (a) Percentage of total monocytes ($CD14^+$); (b) percentage of $CD4^+$ T lymphocytes expressing the CD25 surface activation marker; (c) percentage of $CD8^+$ T lymphocytes. Bars represent the mean results from 10 animals. Error bars and *P* values are indicated.

suggests that these may be essential and perhaps sufficient for virus clearing and disease resolution during an acute, primary infection. Indeed, VACV disruption of MHC class-II-restricted antigen presentation has been described [37–39]. On the other hand, we found that, $CD8^+$ T cell responses are apparently not modulated by the infections, suggesting that $CD8^+$ T cell responses may have a lesser impact on the resolution of VACV primary infections, as hypothesized by some authors [20, 28–31]. One limitation to the study is the size of the analyzed group, which comprises 53 orthopoxvirus-infected human patients and 18 noninfected individuals living in the same affected areas. Notification of human poxvirus infections is not mandatory in Brazil, and identification of patients is done through active random search. The fact has impaired our ability to enroll a larger number of patients. At this point, we cannot provide mechanistic explanations for the findings presented here. To that end, mice infections with mutant viruses lacking specific immune evasion genes could help to understand how orthopoxviruses induce such specific and polarized modulation of the host immune response.

The VACV outbreaks taking place in Brazil represent an important opportunity to understand aspects of naturally acquired *Orthopoxvirus* infections in a population scale. Although of undeniable importance, most studies on human immune responses to primary *Orthopoxvirus* infections have been carried out in voluntary recipients of the smallpox vaccine. Because *Orthopoxvirus*' infections in humans are relatively rare nowadays, vaccinated persons represent the most obvious alternative to study immune responses to these viruses in a population scale. However some aspects must be considered. First, vaccine strains are usually

chosen because they are attenuated and avirulent, and only cause adverse events on a small number of individuals under very specific conditions [45, 46]. Thus, the responses elicited against them may not be necessarily identical to the immune responses generated during infection with a more virulent *Orthopoxvirus* strain. Secondly, the attenuation process frequently leads to the loss of genes that are not essential for virus growth *in vitro*, such as those involved in host's immune evasion mechanisms. Therefore, VACV vaccine strains generally lack many such genes. This is the case of the Dryvax strain, which lacks genes coding for the interferon (IFN)-α/β viroceptor, an ankyrin-like protein (ortholog of VARV-BSHB18R) and tumor necrosis factor (TNF)-α receptor homolog [47]; and the Modified Vaccinia Ankara (MVA) strain, which lacks many immune-evasion genes and is not replicative in most mammal cells [48]. These viruses' relative deficiency in modulating host's immunity may result in infections whose patterns are definitely different from those caused by more virulent *Orthopoxvirus* strains. Finally, most studies are carried out in controlled environments and conditions which include predetermined human populations; inoculum size; sites of inoculation; careful followup of vaccinees. These conditions, although necessary, do not mimic natural infections, and that may have an impact on how the individual's immune system responds to the infection.

5. Conclusions

Despite the fact that immunomodulatory genes are present in the genome of VARV, the most notorious human poxviral pathogen [49], the impact of virus-encoded immune evasion strategies during human infections by poxviruses is largely unknown. We have studied 53 patients acutely infected by zoonotic VACVs and analyzed their innate and adaptative immune responses. Our results suggested that specific cell subsets including B cells, $CD4^+$ T cells, and macrophage/monocytes are specifically modulated by the infection, in comparison to uninfected patients. On the other hand, $CD8^+$ T cell responses seem to be unaltered, mirroring typical CTL response patterns during a viral infection. These results are compatible with a model in which $CD4^+$ T cell-dependent antibody responses are the main responsible for disease control and virus clearance during primary Orthopoxvirus' infections [28, 30]. This study represents the first attempt to analyze aspects of the human immune responses during the onset of acute, naturally acquired infections by orthopoxviruses.

Acknowledgments

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Research Article

Host Susceptibility to *Brucella abortus* Infection Is More Pronounced in IFN-γ knockout than IL-12/β2-Microglobulin Double-Deficient Mice

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Brucella abortus is a facultative intracellular bacterial pathogen that causes abortion in domestic animals and undulant fever in humans. IFN-γ, IL-12, and CD8+ T lymphocytes are important components of host immune responses against *B. abortus*. Herein, IFN-γ and IL-12/β2-microglobulin (β2-m) knockout mice were used to determine whether CD8+ T cells and IL-12-dependent IFN-γ deficiency would be more critical to control *B. abortus* infection compared to the lack of endogenous IFN-γ. At 1 week after infection, IFN-γ KO and IL-12/β2-m KO mice showed increased numbers of bacterial load in spleens; however, at 3 weeks postinfection (p.i.), only IFN-γ KO succumbed to *Brucella*. All IFN-γ KO had died at 16 days p.i. whereas death within the IL-12/β2-m KO group was delayed and occurred at 32 days until 47 days postinfection. Susceptibility of IL-12/β2-m KO animals to *Brucella* was associated to undetectable levels of IFN-γ in mouse splenocytes and inability of these cells to lyse *Brucella*-infected macrophages. However, the lack of endogenous IFN-γ was found to be more important to control brucellosis than CD8+ T cells and IL-12-dependent IFN-γ deficiencies.

1. Introduction

Brucella is a Gram-negative bacterium which is pathogenic to humans and animals [1]. The establishment on infection depends of entrance of this bacterium through the nasal, oral, and/or conjunctival mucosa. After entering into the host cells, *Brucella* has the ability to infect and multiply in phagocytic and nonphagocytic cells [2, 3]. However, macrophages are considered the main cells of *Brucella* residence in the host [4]. The immune response against *Brucella* infection involves many molecules and cells to trigger a Th1 immune response and activation of CD8+ T cells [5–7].

IFN-γ is a critical cytokine for host control of *Brucella* infection [8–10]. The importance of IFN-γ to control *Brucella* was first shown in vivo with monoclonal antibodies that depleted or neutralized IFN-γ in mice [10–12]. Subsequently, a more dramatic role was shown by using IFN-γ KO mice when both BALB/c and C57BL/6 mice died after infection with *B. abortus* strain S2308 [8]. CD4+ T cells are the major producers of IFN-γ in brucellosis, although other subsets such as CD8+ T cells also contribute [7, 13]. A number of studies have demonstrated a role for either CD4+ or CD8+ T cells in the control of brucellosis [7, 14]. In adoptive transfer studies, CD8+ and CD4+ T cells have been shown to be equally protective for resistance to infection with virulent

B. abortus [15]. Using β2-microglobulin (β2-m) gene KO mice, our group has demonstrated that CD8+ T cells have an additional role of lysing infected macrophages and thus either killing intracellular *Brucella* or exposing them to IFN-γ-activated macrophages [7].

IL-12 is a proinflammatory cytokine that has a profound effect on the induction of IFN-γ-producing type 1 pattern of immune response during *Brucella* infection [9, 16]. Since CD8+ T cells and IL-12 are important immunological components during brucellosis, we decided to investigate the course of *Brucella* infection in IL-12/β2-microglobulin double KO mice compared to IFN-γ KO animals. This study was designed to determine whether CD8+ T cells and IL-12-dependent IFN-γ deficiencies would be more critical to control *B. abortus* infection compared to the lack of endogenous IFN-γ. Our results revealed that IFN-γ and IL-12/β2-m KO mice died from *Brucella* infection. However, all IFN-γ KO were dead at day 16 postinfection (p.i.) whereas death within the IL-12/β2-m KO group was delayed and occurred at day 32 until day 47. These results suggest that lack of endogenous IFN-γ is more important than CD8+ T cells and IL-12-dependent IFN-γ deficiencies to control murine brucellosis.

2. Materials and Methods

2.1. Mice. IL-12/β2-microglobulin double-deficient mice (IL-12/β2-m$^{-/-}$) were provided by Dr. Leda Quercia Vieira (UFMG, Belo Horizonte, Brazil), and IFN-γ, deficient mice (IFN-$\gamma^{-/-}$) were provided by Dr. João Santana Silva (USP, Ribeirão Preto-SP, Brazil). The wild-type strain C57BL/6 mice were purchased from the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). Genetically deficient and control mice were maintained at UFMG and used at 6–8 weeks of age.

2.2. Bacteria. Brucella abortus S2308 strain was obtained from our laboratory collection [17]. The strain S2308 was grown in Brucella Broth liquid medium (BB) (DIFCO) at 37°C under constant agitation (200 rpm). After three days of growth, the bacterial culture was centrifuged and the pellet was resuspended in saline (NaCl 0.8% wt/vol), divided in aliquots, and frozen in 20% glycerol (vol/vol). Aliquots of these cultures were serially diluted and plated on BB medium containing 1.5% bacteriological agar (wt/vol). After incubation for 72 hours at 37°C, bacterial numbers were determined by counting colony forming units (CFU).

2.3. Infection and Brucella Counting in Spleens. Five mice of each strain (IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, or C57BL/6) were infected intraperitoneally with 1×10^6 CFU of *B. abortus* virulent strain S2308. These mice were sacrificed at 1- and 3-weeks after infection. The spleen harvested from each animal was macerated in 10 mL of saline (NaCl 0.8%, wt/vol), and it was used for counting of CFU and splenocyte culture. For CFU determination, spleen cells were serially diluted and were plated in duplicate on BB agar. After 3 days of incubation at 37°C in air with 5% CO_2, the number of colony forming units (CFU) was determined. Results were expressed as the mean log CFU of each group. The experiment was repeated three times.

2.4. Measurement of Cytokines and NO into Splenocyte Culture Supernatants. Spleens cells from IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, and C57BL/6 mice obtained after maceration were treated with ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) to lyse red blood cells. After that, the cells were washed with saline (NaCl 0.8%, wt/vol) and suspended in RPMI 1640 (Gibco, Carlsbad, Calif) supplemented with 2 mM L-Glutamine, 25 mM HEPES, 10% (vol/vol) heat-inactivated FBS (Gibco, Carlsbad, Calif), penicillin G sodium (100 U/mL), and streptomycin sulfate (100 μg/mL). To determine cytokine concentration by ELISA, 1×10^6 spleen cells were plated per well in a 96-well tissue culture-treated dish. Murine splenocytes from infected animals were stimulated with *B. abortus* S2308 (MOI 100 : 1), Concanavalin A (5 μg/mL Sigma, Sigma-Aldrich, St. Louis, Mo), or *E.coli* LPS (1 μg/mL, Sigma, St. Louis, Mo). Unstimulated cells were used as negative control. Spleen cells were incubated at 37°C in 5% CO_2, and aliquots of the supernatant were collected after 48 and 72 hrs of culture for TNF-α and IFN-γ measurements, respectively. Levels of TNF-α and IFN-γ were measured into cell supernatants by ELISA using the Duoset kit (R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions. To assess the amount of NO produced, splenocyte culture supernatants from IFN-$\gamma^{-/-}$, IL-12/β2-m$^{-/-}$, and C57BL/6 mice were assayed for accumulation of the stable end product of NO, NO_2^- which was determined by the Griess reaction. Briefly, culture supernatants (50 μL) from spleen cells stimulated as above mentioned for cytokine measurement were mixed with 50 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethyline diamine dihydrochloride, and 2.5% phosphoric acid) into plates. The OD at 550 nm was then measured. NO_2^- was quantified by comparison with $NaNO_2$ as a standard.

2.5. Survival Curve. Five mice of each strain (IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, or C57BL/6) were infected intraperitoneally with 1×10^6 CFU of *B. abortus* virulent strain S2308. Percentage of mouse survival was observed during 50 days postinfection. The experiment was repeated twice.

2.6. Generation and In Vitro Stimulation of Bone Marrow-Derived Macrophages (BMDMs). Macrophages were derived from bone marrow of IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, and C57BL/6 mice as previously described [18]. Briefly, bone marrow (BM) cells were removed from the femurs and tibias of the animals. Each bone was flushed with 5 mL of Hank's balanced salt solution (HBSS). The resulting cell suspension was centrifuged, and the cells were resuspended in DMEM (Gibco, Carlsbad, Calif) containing 10% (vol/vol) FBS (HyClone, Logan, Utah), 1% (wt/vol) HEPES, and 10% (vol/vol) L929 cell-conditioned medium (LCCM) as source of M-CSF, in 24 well plates (5×10^5 cells/well). After 4 days, 100 μL/well LCCM was added. At day 7, the medium was renewed. At day 10 of culture, when the cells had

completely differentiated into macrophages, the medium was harvested, and we added supplemented DMEM (500 μL/well) containing *B. abortus* S2308 (MOI 1000 : 1) or *E. coli* LPS (1 μg/mL, Sigma, St. Louis, Mo). Culture supernatants of BMDMs were collected after 24 hours of stimulation and assayed for the concentrations of IL-12 and TNF-α by ELISA (R&D Systems) according to the manufacturer's instructions.

2.7. Cytotoxic Assay. To determine the cytolytic activity of splenocytes from *Brucella*-infected mice, we used the CytoTox 96 Nonradioactive Cytotoxicity Assay (Promega, Madison, USA) that is based on the colorimetric detection of the released levels of the LDH enzyme. Macrophages differentiated (5×10^5 cells/well) from IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, and C57BL/6 mice were infected with *B. abortus* (MOI 100 : 1). After 24 hours of infection extracellular bacteria was removed. Macrophages infected were used as target cells for cytotoxic assay. Splenocytes (1×10^6 cells/well) obtained from IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$ and C57BL/6 mice at one week p.i. were used as effector cells and were cocultured with macrophages in 24 well plates in DMEM medium. Effector cells were added to target cells in duplicate at 2 : 1 ratio. Culture was maintained at 37°C in 5% CO_2 for 24 hours, and 50 μL of supernatants were harvested and placed in 96-well flat-bottom plate. Controls for spontaneous LDH release from effector and target cells, as well as target maximum release, were also added in the experiment. The cell supernatants were assayed for lactate dehydrogenase (LDH) activity following the manufacturer's protocol. The percentage of specific lysis was calculated according to the following formula: [(Experimental−Effector Spontaneous−Target Spontaneous)/(Target Maximum−Target Spontaneous)] × 100%.

2.8. Statistical Analysis. The results of this study were analyzed using the Student's *t*-test, using GraphPad Prism 4 (GraphPad Software, Inc). The level of significance in the analysis was $P < 0.05$.

3. Results

3.1. Increased B. abortus CFU in Spleens of IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ Mice. The level of systemic infection in murine brucellosis is detectable by enumerating the number of residual *Brucella* CFU in mouse spleens [19]. Thus, C57BL/6, IFN-$\gamma^{-/-}$, and IL-12/β2-m$^{-/-}$ mice were infected with *B. abortus* virulent strain, and splenic CFU were counted at 1 and 3 weeks postinfection (Figure 1). At one week postinfection, IL-12/β2-m$^{-/-}$ and IFN-$\gamma^{-/-}$ mice displayed increased numbers of *Brucella* CFU (7.28 ± 0.21 and 8.08 ± 0.07, resp.) compared to wild-type animals (6.36 ± 0.11). Additionally, the CFU difference observed between IL-12/β2-m$^{-/-}$ and IFN-$\gamma^{-/-}$ mice was statistically significant. At 3 weeks postinfection, the difference in *Brucella* CFU from IL-12/β2-m$^{-/-}$ animals compared to C57BL/6 increased from 0.92 to 3.76 logs. As for IFN-$\gamma^{-/-}$ mice, at 3 weeks after infection, all animals were dead. These results demonstrated enhanced susceptibility of IL-12/β2-m$^{-/-}$ and IFN-$\gamma^{-/-}$ mice to brucellosis, being more prominent in IFN-$\gamma^{-/-}$ animals.

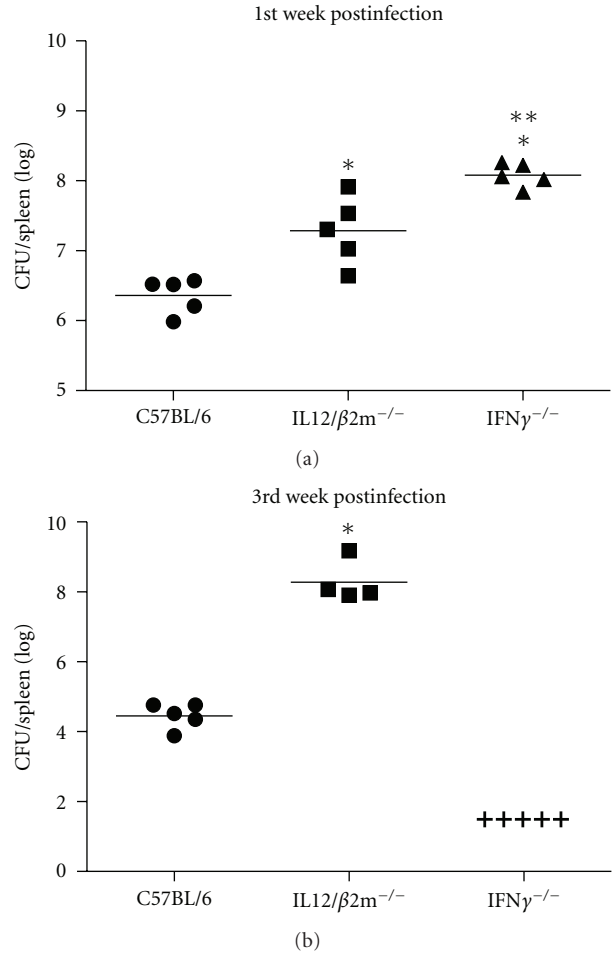

Figure 1: Growth of *B. abortus* in spleens of C57BL/6, IFN-$\gamma^{-/-}$, and IL-12/β2-m$^{-/-}$ mice at the first (a) and third (b) week postinfection. IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, and C57BL/6 mice were intraperitoneally inoculated with 10^6 CFU of *B. abortus* S2308. (+) This symbol shows that all IFN-$\gamma^{-/-}$ mice died before the CFU count at the third week postinfection. Data are expressed as mean ± SD of five animals per time point. These results are representative of three independent experiments. Significant difference in relation to C57BL/6 for $P < 0.05$ is denoted by an asterisk and in relation to IL-12/β2-m$^{-/-}$ by two asterisks.

3.2. IFN-$\gamma^{-/-}$ Are More Susceptible to B. abortus Infection Than IL-12/β2-m$^{-/-}$ Mice. IFN-γ and IL-12/β2-m KO on a C57BL/6 background were compared to their ability to survive *Brucella* infection. As shown in Figure 2, all IFN-γ KO succumbed at 16 days p.i. whereas death within the IL-12/β2-m KO group was delayed and occurred at 32 days until 47 days postinfection. In contrast, at 50 days p.i., 100% of C57BL/6 mice were still alive. These results suggest that IFN-γ and IL-12/β2-m are critical immune components to combat *Brucella* infection. However, the lack of endogenous IFN-γ is more important than CD8+ T cells and IL-12-dependent IFN-γ deficiencies to control murine brucellosis.

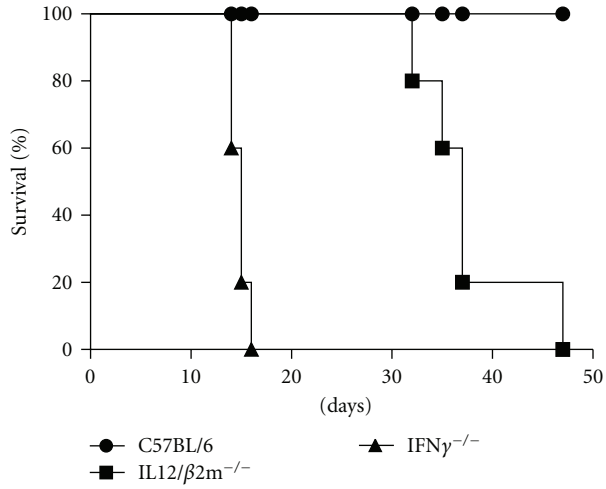

FIGURE 2: IFN-γ is critical for efficient control of *B. abortus* in vivo. Groups of 5 mice were injected intraperitoneally with 10^6 CFU of *B. abortus* S2308. Each mouse strain was monitored daily for survival during 50 days postinfection.

3.3. IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ Mice Showed Reduced Specific Type 1 Immune Response to B. abortus. Protective immunity against infection by *B. abortus* is directly related to the induction of a type 1 pattern of immune response. IL-12 and IFN-γ are key cytokines involved in this type of immunity [20]. Thus, we evaluated the production of IFN-γ, TNF-α, and NO in spleen cells from IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ mice. As expected, no detectable IFN-γ production was observed in IFN-$\gamma^{-/-}$ and also in IL-12/β2-m$^{-/-}$ mice when compared to wild-type animals at one week after infection (Figure 3(b)). Furthermore, a dramatic reduction on TNF-α and NO production was observed in IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ mice when compared to wild-type animals (Figures 3(a) and 3(c)). Additionally, the levels of NO produced by IFN-$\gamma^{-/-}$ cells were reduced when compared to IL-12/β2-m$^{-/-}$ mice. These results demonstrate that type 1 cytokine profile is compromised in IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ mice during *Brucella* infection.

3.4. IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ Mice Produce Normal Levels of TNF-α Levels in Macrophages. The recognition of *Brucella* by innate immune cells, such as macrophages and dendritic cells, results in activation and the concomitant production of proinflammatory cytokines [21]. In this study, we evaluated the proinflammatory cytokine production by macrophages from bone-marrow cells of IFN-$\gamma^{-/-}$ and IL-12/β2-m$^{-/-}$ mice when stimulated with live *B. abortus* or *E. coli* LPS. As shown in Figure 4, no IL-12 was detected in IL-12/β2-m$^{-/-}$ mice as expected but normal levels of this cytokine were measured in IFN-$\gamma^{-/-}$ cells. Regarding TNF-α, no statistically significant difference in production of this proinflammatory mediator was detected in knockout mice compared to C57BL/6.

3.5. Cytotoxic Activity of B. abortus-Induced Splenocytes. The ability of *B. abortus*-primed splenocytes from IFN-$\gamma^{-/-}$,

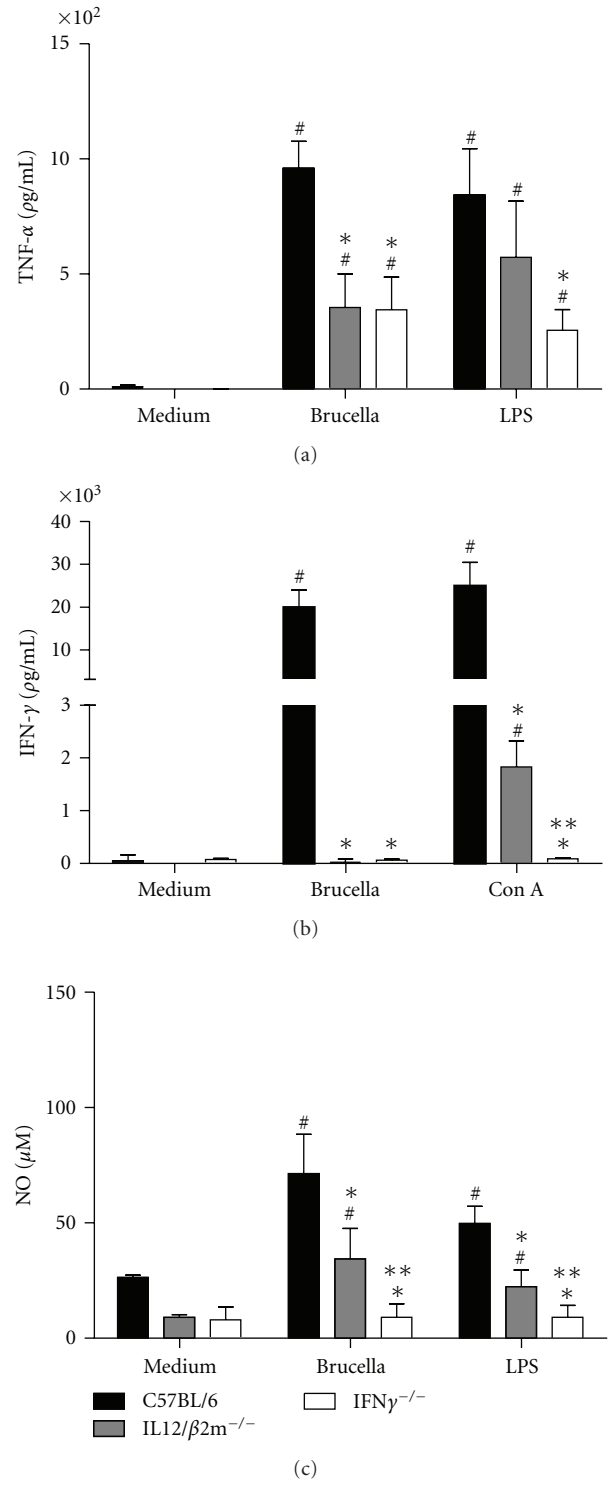

FIGURE 3: IFN-γ, TNF-α, and NO production induced by *B. abortus* in IFN-γ KO or IL-12/β2-m KO splenocytes. Spleens cells (1×10^6 cells) were stimulated with *B. abortus* S2308 (MOI 100 : 1), Con A (5 μg/mL), or *E. coli* LPS (1 μg/mL). Levels of TNF-α (a) and IFN-γ (b) were measured by ELISA after 48 and 72 hrs, respectively. Levels of NO_2^- (c) were measured by Griess reaction after 48 hrs of antigen stimulation. Statistically significant differences in relation to C57BL/6 mice are indicated by an asterisk ($P < 0.05$), in relation to IL-12/β2-m$^{-/-}$ by two asterisks, and those between unstimulated and stimulated spleen cells are indicated by the symbol # ($P < 0.05$).

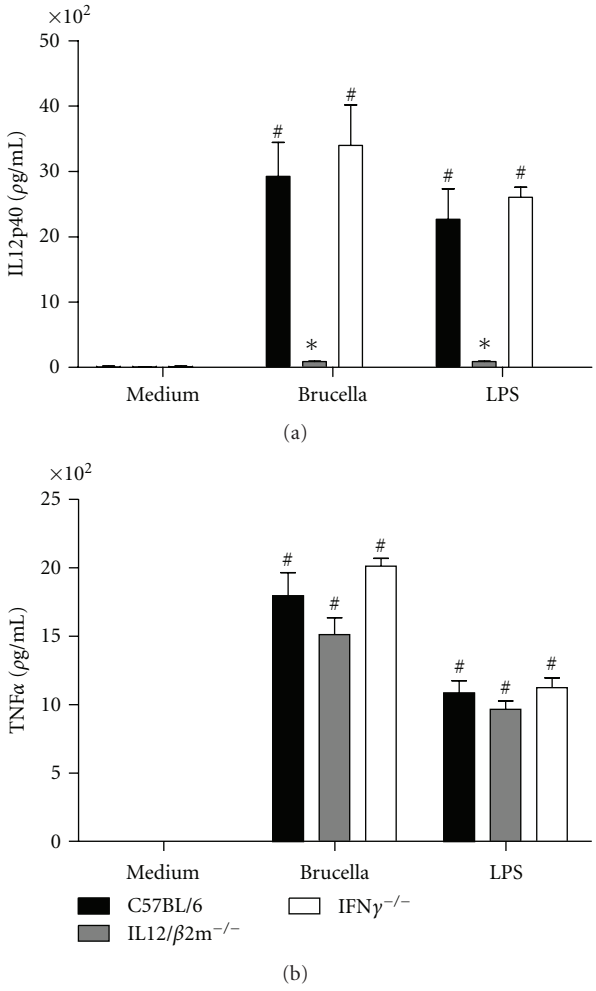

Figure 4: IL-12 and TNF-α production induced by *B. abortus* in IFN-γ KO or IL-12/β2-m KO macrophages. Bone marrow from C57BL/6, IFN-$\gamma^{-/-}$, and IL-12/β2-m$^{-/-}$ mouse cells were differentiated in macrophages and stimulated with *B. abortus* S2308 (MOI 100 : 1) or *E. coli* LPS (1 μg/mL). Supernatants were harvested for measuring IL-12 (a) and TNF-α (b) after 24 hrs by ELISA. Significant difference in relation to nonstimulated cells is denoted by the symbol # and an in relation to C57BL/6 mice is denoted by an asterisk ($P < 0.05$). Results are means ± standard deviations of experiments performed with three animals. Data shown are representative of two different experiments.

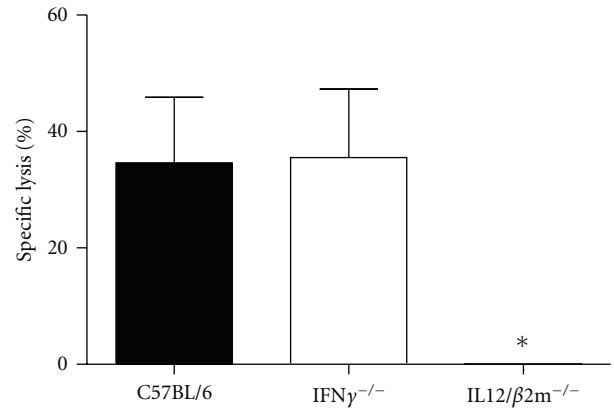

Figure 5: Lysis of *B. abortus*-infected macrophages by splenocytes from IFN-γ KO and C57BL/6 mice. Macrophages differentiated (5×10^5 cells/well) obtained from IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, and C57BL/6 mice were infected with *B. abortus* (MOI 100 : 1) and used as target cells. Splenocytes (1×10^6 cells/well) obtained from IL-12/β2-m$^{-/-}$, IFN-$\gamma^{-/-}$, and C57BL/6 mice at one week of infection were used as effector cells for cytotoxic assay and were cocultured with macrophages in 24 well plates in DMEM medium. Effector cells were added to target cells in duplicate at 2 : 1 ratio. Significant difference in relation to C57BL/6 and IFN-γ KO mice is denoted by an asterisk ($P < 0.05$). Results are means ± standard deviations of experiments performed. Data shown are representative of two different experiments.

IL-12/β2-m$^{-/-}$, and C57BL/6 mice to lyse infected bone-marrow-derived macrophages was assayed. Specific lysis of *Brucella*-infected macrophages was detected in IFN-$\gamma^{-/-}$ (35.5 ± 5.8) and wild-type (34.6 ± 5.6) mice but not in IL-12/β2-m$^{-/-}$ animals (Figure 5). This result suggests the lack of functional CD8+ CTL in IL-12/β2-m$^{-/-}$ mice what is one of the reasons for enhanced susceptibility to *B. abortus* infection in these animals.

4. Discussion

Typical host immunity to *Brucella* is based on a Th1-dependent immune response. Previously immunity to intracellular bacteria was considered to be exclusively dependent on CD4+ T cells [22]. However, later studies have emphasized the role of CD8+ T cells in protection against *Brucella* infection. [7, 8]. The purpose of this study was to compare the susceptibility of IFN-γ KO versus IL-12/β2-m KO animals, defining the importance of these immune components on host resistance to *B. abortus* infection.

Previous studies have demonstrated that IFN-γ was indeed crucial for the control of *Brucella* infection [8, 10]. Additionally, our group and others have established that CD8+ T cells are critical components of host resistance to *Brucella* [7, 15]. Herein, we determined that IFN-γ KO mice had increased numbers of *Brucella* CFU compared to IL-12/β2-m KO at one week postinfection. Furthermore, all IFN-γ KO died of infection at 16 days p.i. whereas death within the IL-12/β2-m KO group was delayed and occurred at 32 days until 47 days postinfection. In contrast, at 50 days p.i., 100% of C57BL/6 mice were still alive. Ko et al. [23] have previously demonstrated that IL-12 KO mice infected with *B. abortus* did not control infection and maintained high plateau of bacteria; however, the animals did not die at fours week postinfection. In contrast, in our study, IL-12/β2-m mice succumbed to infection as a result of combined IL-12 and β2-m deficiencies. Taken together, these results suggest that IFN-γ and IL-12/β2-m are important components to host control of *Brucella* infection. However, the lack of endogenous IFN-γ is more important than CD8+ T cells and IL-12-dependent IFN-γ deficiencies to induce immunity to brucellosis.

In order to determine which factors could be involved with enhanced susceptibility to *Brucella* infection in IFN-γ KO and IL-12/β2-m KO mice, we determined the concentration of IFN-γ, TNF-α, and NO in spleen cells of these animals. Splenocytes from both KO mice stimulated with live *Brucella* produced undetectable levels of IFN-γ and reduced amounts of TNF-α and NO. In the case of NO, this reduction was prominent in IFN-γ KO. Recently, Norman et al. have identified IFN-γ-based mechanisms that regulate NO production [24]. Furthermore, Yagi et al. [25] have demonstrated that deletion of Gata 3 allowed the appearance of IFN-γ-producing cells in the absence of IL-12. Thus, the Runx3-mediated pathway, actively suppressed by GATA3, induces IFN-γ production in a STAT4- and T-bet-independent manner. Another study using *Listeria monocytogenes* at low dose revealed that splenocytes of IL-12 KO mice produced only 10% of the amount of IFN-γ detected in wild-type mice in response to antigen [26]. They suggested that NK cells or other cells have the potential to produce residual but substantial amounts of IFN-γ independent of IL-12. Since *Listeria*-infected mice showed enhanced IL-18 expression, this cytokine may stimulate NK cells for IFN-γ production in the absence of IL-12. Additionally, Freudenberg et al. [27] demonstrated the existence of an IL-12-independent pathway of IFN-γ induction by Gram-negative bacteria in mice in which IFN-β and IL-18 act synergistically. *Brucella* has induced the production of IL-18 and IFN-β in mice [28, 29]. Therefore, this pathway could be used to produce IFN-γ during *Brucella* infection in absence of IL-12. Even though IFN-γ can be produced independently of IL-12, we did not detect this cytokine in IL-12/β2-m KO spleen cells activated with live *Brucella* at one week postinfection. It is possible that IFN-γ increases in IL-12/β2-m KO after one week postinfection. Another possibility is that IFN-γ production by NK and other cells that are present in low numbers in spleens is underestimated when we analyzed whole splenocytes. Further, it is possible that other cell types present in other organs than spleen are responsible for residual IFN-γ production in IL-12/β2-m KO.

Macrophages are key elements in innate immune responses and recognition of *Brucella* components [30]. Herein, we investigated the involvement of IFN-γ and IL-12/β2-m in *Brucella*-induced IL-12 and TNF-α production by macrophages. As expected, macrophages from IL-12/β2-m KO mice showed no production of IL-12 when they were stimulated with live *Brucella* compared to normal synthesis of this cytokine by IFN-γ KO and wild-type cells. As for TNF-α production, no statistically significant difference was observed between KO mouse macrophages compared to C57BL/6. Since macrophages are considered the main cells of *Brucella* residence in the host, we infected these cells and tested them as targets for primed splenocytes from KO and wild-type mice in a cytotoxic assay. Pathogenesis induced by *Brucella* is the product of a complex series of interactions between the bacteria and different components of the immune system. One interaction of interest is between CD8+ CTL and *Brucella*-infected macrophages. In this study, specific lysis of infected macrophages was detected in wild-type and IFN-γ KO but not in IL-12/β2-m KO mice. IL-12/β2-m KO mice fail to assemble and express MHC class I molecules on the cell surface, and, therefore, these animals are devoid of functional CD8+ $\alpha\beta$ T cells. Thus, the lack of functional CD8+ T cells might be the reason why we did not detect macrophage lysis by IL-12/β2-m KO splenocytes. Recently, Durward et al. [31] have identified two CD8+ T cell epitopes in *B. melitensis* that induced IFN-γ production and specific killing in vivo. Their work reinforced the important aspect of inducing *Brucella*-specific CD8+ T cells to achieve an efficient host response to this pathogen.

Collectively, we have demonstrated that IFN-γ and IL-12/β2-m are important components of host immune response to control *Brucella* infection. However, lack of endogenous IFN-γ is more crucial to immunity against this pathogen than lack of functional CD8+ T cells and IL-12.

Acknowledgments

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Research Article

Characterization of Chronic Cutaneous Lesions from TNF-Receptor-1-Deficient Mice Infected by *Leishmania major*

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Leishmania major-infected TNF receptor 1 deficient (TNFR1 KO) mice resolve parasitism but fail to resolve lesions, while wild-type mice completely heal. We investigated the cell composition, cytokine production, and apoptosis in lesions from *L. major*-infected TNFR1 KO and wild-type (WT) mice. Chronic lesions from *L. major*-infected TNFR1 KO mice presented larger number of CD8+ T and Ly6G+ cells. In addition, higher concentrations of mRNA for IFN-γ CCL2 and CCL5, as well as protein, but lower numbers of apoptotic cells, were found in lesions from TNFR1 KO mice than in WT, at late time points of infection. Our studies showed that persistent lesions in *L. major*-infected TNFR1 KO mice may be mediated by continuous migration of cells to the site of inflammation due to the presence of chemokines and also by lower levels of apoptosis. We suggest that this model has some striking similarities to the mucocutaneous clinical form of leishmaniasis.

1. Introduction

Parasites of the genus *Leishmania* cause a spectrum of cutaneous manifestations ranging from limited cutaneous lesions that heal spontaneously to the more severe mucocutaneous form. These different clinical manifestations depend on the species of *Leishmania* and the host immune response [1, 2]. In experimental models, it is established that BALB/c mice are susceptible to infection by *Leishmania major*. This mouse strain develops progressive lesions, uncontrolled growth of parasites, visceralization, and death. The C57BL/6 strain is resistant to infection by *L. major*, controls parasite replication, and heals lesions [3]. However, despite clinical and pathological cure of the disease, the parasite remains latent in the host. Resistance to infection by *L. major* is mediated by IFN-γ, TNF-α, and activation of macrophages to produce nitric oxide [4–6].

Mucocutanesous leishmaniasis is caused mainly by *L. braziliensis*. It is characterized by control of parasite growth in the tissue, but persistent chronic inflammation that commonly affects mucosal tissue causing severe disfiguration and social stigma to the patient [7]. High concentrations of inflammatory cytokines, namely, IFN-γ and TNF-α, are found in these patients [8]. The study of mucocutaneous leishmaniasis is hampered by lack of a good experimental model. Experimental infection with *L. braziliensis* causes a very mild and self-limited lesion in C57BL/6 and BALB/c strain mice [9, 10]. In addition, *L. amazonensis* causes a persistent chronic lesion in C57BL/10 and C57BL/6 mice that lasts over 20 weeks, but both animal models fail to control

the parasite in the tissue, a hallmark of mucocutaneous leishmaniasis. Moreover, IFN-γ and TNF production is impaired in these infection models [11–13]. The closest animal model for mucocutaneous disease would be the infection of C57BL/6 TNFR1-deficient (TNFR1 KO) mice with *L. major*. TNFR1 KO mice control tissue parasitism similarly to the wild-type resistant mouse, but develop nonhealing lesions. However, these lesions do not increase in size progressively. On the contrary, they remain chronic and small, but last for at least 20 weeks afterinfection [14, 15].

In experimental infection by *L. major,* TNF-α is important for activation of macrophages, in cooperation with IFN-γ, and elimination of intracellular parasites [4, 5, 16–18]. Another important fact is the involvement of TNF in the induction of apoptosis in lymphocytes from lesions from wild-type *L. major*-infected mice [19]. This information may suggest that TNF-α may play a key role in the healing of *L. major* lesions. However, this important phenomenon was not described in the chronic stage of infection to explain the persistent lesions in *L. major*-infected TNFR1 KO. Thus, the aim of this study was to characterize events in the chronic phase of *L. major* infection in TNFR1 KO mice.

2. Materials and Methods

2.1. Animals. C57BL/6 wild-type (WT) mice, 6 to 10 weeks old, were obtained from CEBIO (Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). TNFR1 KO mice were originally obtained from the University of Pennsylvania (Philadelphia, Pa, USA, a kind gift from Dr. Phillp Scott and Dr. Klaus Pfeffer) and maintained in Laboratory of the Gnotobiology and Immunology of the Instituto de Ciências Biológicas (UFMG, Brazil). All the procedures involving animals were in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation and were approved by the UFMG animal experimentation ethical committee at UFMG (CETEA), protocol number 55/2009.

2.2. Parasites and Infection. A clone of *Leishmania major* (WHO MHOM/IL/80/Friedlin) was used in this study. Parasites were maintained in Grace's insect medium (GIBCO BRL Life Technologies, Grand Island, NY, USA), pH 6.2, supplemented with 20% fetal bovine serum (Nutricell, Campinas, SP, Brazil), 2 mM L-glutamine (SIGMA Chemical Co., St. Louis, Mo, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO BRL Life Technologies). Mice were injected in the hind footpads with 1×10^6 *L. major* metacyclic promastigotes. Footpads were measured weekly with a caliper (Mitutoyou, Suzano, SP, Brazil). Lesion sizes are expressed as the difference between infected and uninfected footpads.

2.3. Parasite Load. Parasite load in infected footpads was determined by limiting dilution [14]. Results were expressed as the negative log of the last positive dilution.

2.4. Histological Analyzes. Infected footpads from WT and TNFR1 KO mice were removed at 6 and 15 weeks after infection and fixed in 10% of formalin. Tissues were processed and embedded in paraffin and 5 μm thick sections were stained with hematoxylin and eosin and analyzed by light microscopy. At least 10 microscopic fields measuring 250,000 micrometers, representative of lesions, were automatically analysed by KS 300 (Carl Zeiss, Germany) for determining the comparative cellularity of lesions at 15 wks.

2.5. Apoptosis Analysis. Infected footpads from WT and TNFR1 KO mice were removed at 6 and 11 weeks after infection and fixed in 4% formaldehyde. Apoptotic cells were assessed *in situ* by the TUNEL reaction, an *in situ* cell death detection kit (POD, Roche Applied Science, Penzberg, Germany). The results were obtained by counting the number of stained cells per 100 cells (600 cells counted per animal) in 6–10 random areas per histological section.

2.6. Flow Cytometry. Infected footpads from WT and TNFR1 KO mice were removed at 6 and 15 weeks after infection. Collected tissues were incubated for 90 minutes with 1.5 mg/mL of collagenase (Sigma-Aldrich, Mo, USA) in RPMI 1640 without supplements at 37°C, homogenized using a tissue grinder and centrifuged at 2000 g. Single-cell suspensions were stained with fluorochrome-conjugated antibodies (eBioscience, San Diego, Calif, USA) against CD4 (RM4-5), CD8 α (53-6.7), CD3 (17A2), F4/80 (BM8), and Ly6G (RB6-8C5) in PBS containing 1% FBS for 20 min on ice and then washed and fixed with 2% formaldehyde. Stained cells were analyzed using an FACScan flow cytometer equipped with cellQuest software (Becton Dickinson, Heidelberg, Germany). Statistical analyses of mean fluorescence intensity (MFI) were performed using the FlowJo v7.6.5 software (Tree Star Inc., Ashland, Ore, USA).

2.7. Chemokines and Cytokines Analysis. Chemokines and cytokines were analyzed by two methods: detection of mRNA by reverse transcription polymerase chain reaction (RT-PCR) and ELISA, at different times of infection (1 and 2 days, 2, 6, and 11 weeks). The footpad was excised and total protein and RNA were extracted with Trizol (GIBCO BRL Laboratories), as previously described. Cytokine and chemokine detections by RT-PCR were performed as previously described [20]. Briefly, *L. major*-infected and noninfected footpads were harvested and placed in 0.5 mL TRIZOL (GIBCO BRL) solution and homogenized, and RNA extraction was performed according to the manufacture's instruction. RNA was quantitated spectrophotometricaly and 1 μg of RNA was reverse transcribed using 25 U of M-MLV Reverse Transcriptase (Promega Corp., Madison, Wis, USA) in 12.5 μL reaction containing 2.5 mM dNTPs, 50 mM TRIS/HCl (pH8.3), 75 mM KCl, 2 mM $MgCl_2$, 10 mM DTT (GIBCO BRL Laboratories, Grand Island, NY, USA), 250 mM dNTP (Promega), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 10 U RNAsin (GIBCO BRL), and 7.5 pM of oligo dT_{15} (GIBCO BRL). The mixtures were incubated for 5 min at 95°C, 5 min at 4°C, and 5 min at 25°C; at this step 25 U of reverse transcriptase was added to each sample and the reaction mixture was incubated

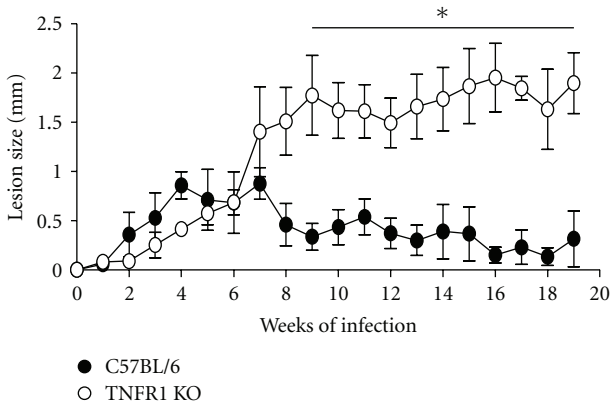
Table 1: Primers, temperature, and number of cycles used for RT-PCR.

Primer	Sequence	Length (bp)
HPRT	58°C 32 cycles	217
	FW GTT GGA TAC AGG CCA GAC TTT GTT	546–569
	RV GAT TCA ACT TGC GCT CAT CTT AGG C	763–739
IL-4	60°C 33 cycles	292
	FW TTT GAA CGA GGT CAC AGG AG	195–214
	RV TGC TCT TTA GGC TTT CCA GG	487–468
IFN-γ	60°C 32 cycles	202
	FW GGT GAC ATG AAA ATC CTG CAG	290–310
	RV GCG CTG GAC CTG TGG GTT GTT GAC C	493–469
TNF-α	62°C 30 cycles	300
	FW CGC TCT TCT GTC TAC TGA AC	293–312
	RV TGT CCC TTG AAG AGA ACC TG	593–574
CCL2	62°C 30 cycles	488
	FW CCG GAA TTC CAC TCA CCT GCT GCT ACT CAT TCA	179–205
	RV CCG GAA TTC GGA TTC ACA GAG AGG GAA AAA TGG	667–644
CCL5	54°C 26 cycles	309
	FW CGC GGA TCC CCA CGT CAA GG A GTA TTT CTA CAC C	184–209
	RV CGC GAA TCC CTG GTT TCT TGG GTT TGC TGT TG	493–473
CCL12	54°C 29 cycles	380
	FW GTT CCT GAC TCC TCT AGC TTT C	11–32
	RV ACG TAA GAG TTT TTG GAA CTC	391–371
CXCL9	54°C 30 cycles	398
	FW GAT CAA ACC TGC CTA GAT CC	302–321
	RV GGC TGT GTA GAA CAC AGA GT	700–681
CXCL10	62°C 27 cycles	383
	FW CGC GGA TCC TGA GCA GAG ATG TCT GAA TC	250–271
	RV CGC GGA TCC TCG CAC CTC CAC ATA GCT TAC AG	633–611
CXCL1	62°C 34 cycles	521
	FW CGC GGA T:CC TGG ACC CTG AAG CTC CCT TGG TTC	226–251
	RV CGC GGA TCC: CGT GCG TGT TGA CCA TAC ATT ATG	731–708

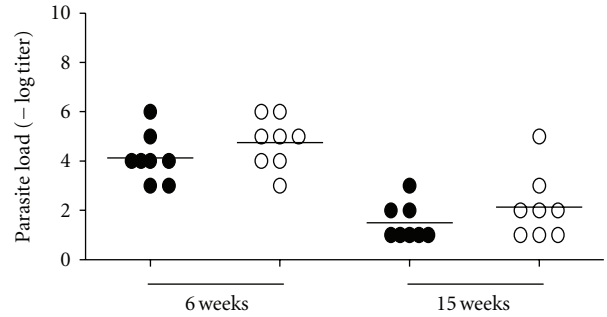
for 60 min at 37°C. The temperature was then elevated to 95°C for 5 min and cooled again at 4°C for 5 min. The PCR was performed in 10 μL reaction of samples diluted in the following buffer: 250 mM dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$, 5 mM of each primer and 0.05 U of TaqPolimerase (Phoneutria, Belo Horizonte, MG, Brazil). For each pair of primers, at least three different numbers of cycles were tested and the best nonsaturating condition was chosen. The PCR was standardized using the house-keeping gene HPRT. The primer sequences (sense and antisense sequence), PCR product size, number of cycles, and temperature of annealing used for each primer are listed in Table 1. The products were electrophoresed in 6% polyacrilamide gels and developed by silver staining. Primers and PCR conditions are listed in Table 1. For ELISA, footpads were homogenized in PBS (0.4 M NaCl and 10 mM de Na_2HPO_4, pH 7.2) containing antiproteases (0.1 mM phenylmehtylsulfonyl fluoride, 0.1 mM benzethoinium chloride, 10 mM EDTA, and 20 mM KI aprotin A/100 mL) and 0.05% tween-20. Homogenized tissues were kept on ice for 30 min and subsequently centrifuged (3000 × g for 10 min). The preparations were assayed using RD Systems kits (Duoset-RD Systems, Minneapolis, Minn, USA) according to instructions. The sensitivity for CCL-2 and CCL-5 was 16 and 32 pg/mL, respectively.

3. Results

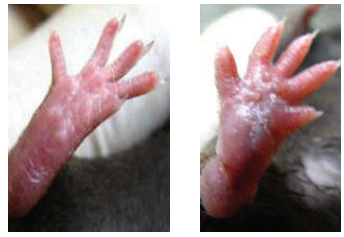
In this work, we characterized the lesions in *L. major*-infected TNFR1 KO mice and attempted to explain the reason why these lesions fail to heal. As shown previously [14] *L. major*-infected TNFR1 KO mice develop chronic nonhealing lesions, while WT mice heal lesions over time (Figure 1(a)). In addition, as previously described [14], parasite growth is controlled at the site of infection (Figure 1(b)). WT mice had completely healed lesions at 15 weeks of infection; however, TNFR1 KO mice had conspicuous lesions that led to some loss of function (Figure 1(c)). When histological examination of lesions was performed, we found that, albeit at 6 weeks of infection the inflammatory infiltrate was similar in both groups, at 15 weeks very few inflammatory cells were observed in WT mice, while lesions from TNFR1 KO



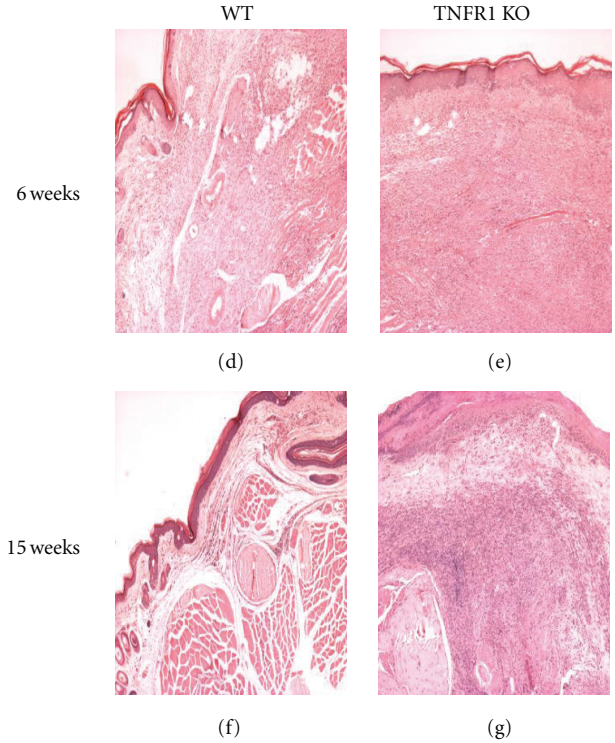
(a)



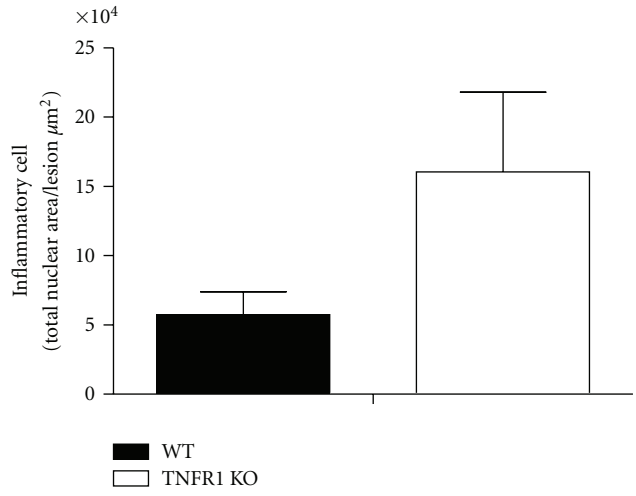
(b)



(c)



(d) (e) (f) (g)



(h)

Figure 1: Lesions and parasite burdens in TNFR1 KO mice with *L. major*. Mice were infected with 1×10^6 metacyclic stationary forms of *L. major* in footpads. (a) The footpads were measured weekly and the value for uninfected mice was subtracted from each infected footpad to estimate lesion size. (b) Parasite burden in WT and TNFR1 KO mice. Mice were sacrificed at 6 and 15 weeks afterinfection and parasite burden was determined by limiting dilution analysis ($n = 5$ mice per time point). (c) Lesions from WT and TNFR1 KO mice infected with *L. major* 15 weeks afterinfection. (d–g) Histological aspect of tissues from lesions from WT and TNFR1 KO infected for 6 weeks (d and e) or 15 weeks (f and g), stained with hematoxylin and eosin. (h) Morfometric quantification of inflammatory cells from WT and TNFR1 KO lesions 15 weeks afterinfection ($n = 4$ mice per group). *indicates $P < 0.05$. Data are from one experiment of three performed independently.

displayed a high number of inflammatory cells (Figures 1(d)–1(h)).

We further characterized the inflammatory infiltrate by flow cytometry. As seen in Figure 2(a), a higher percentages of CD4+ T lymphocytes was found in lesions from WT mice at 15 weeks of infection. However, since there were more cells in the inflammatory infiltrate in TNFR1 mice, when we calculated the absolute numbers of cells, similar numbers of CD4+ cells were observed in lesions from both groups of mice (Figure 2(b)). Higher percentages and absolute numbers of CD8+ T cells were found in lesions from TNFR1 KO mice (Figures 2(a) and 2(b)). Also, at 15 weeks of infection a higher percentage of macrophages (F4/80 positive cells) was found in WT mice (Figure 2(c)), while a higher percentage of Ly6G positive cells was found in TNFR1 KO mice (Figure 2(c)). In absolute numbers, we found similar numbers of CD4+ T cells and F4/80 cells in both groups of mice (Figure 2(d)) while a higher absolute number of Ly6G+ cells were found in lesions from TNFR1 KO mice (Figure 2(d)). In addition, expression of Ly6G on a per cell basis, indicated by the mean fluorescence intensity (MFI), was increased in these mice (Figure 2(e)).

We proceeded to investigate the expression of cytokines at the site of infection. As seen in Figure 3(a), similar concentrations of mRNA for TNF-α were found in both groups of mice. Similar concentrations of IFN-γ mRNA were found in lesions from both groups at 24 and 48 hours of infection, but higher concentrations were found in lesions from TNFR1 KO mice at 11 weeks of infection (Figure 3(b)). In addition, similar concentrations of IL-4 message were found in both groups at all time points tested. These data were confirmed by ELISA at 15 weeks of infection. We found higher TNF-α and IFN-γ protein concentrations in *L. major*-infected TNFR1KO mice (Figures 3(d) and 3(e)) than in WT mice. No differences were found for IL-4 protein expression between groups of mice (Figures 3(c) and 3(f)).

Since there were more cells at the site of infection in TNFR1 KO mice, we investigated the expression of chemokines by RT-PCR and ELISA. When we assayed CC cytokines, as seen in Figure 4, we found that at 11 weeks of infection there was higher expression of CCL2 and CCL5 mRNA in *L. major*-infected TNFR1 KO mice than in WT mice (Figures 4(a) and 4(b)). No differences were found in expression of CCL12 mRNA between the groups of mice (Figure 4(c)). We confirmed the results found for CCL2 and CCL5 by ELISA and found higher concentrations of protein (Figures 4(d) and 4(e)) at 20 weeks of infection in lesions from TNFR1 KO mice. In addition, no differences at later time points were found for CXCL1, CXCL9, and CXCL10 mRNA (data not shown).

In addition to more recruitment, a larger cellular population at the site of infection may be explained by a defective removal of inflammatory cells. Since TNFR1 mediates apoptosis [19, 21], we investigated apoptosis at the site of infection at 6 and 11 weeks by the TUNEL reaction. As seen in Figure 5 and quantified in Table 2, at six weeks of infection we saw TUNEL-positive cells in both TNFR1 KO and WT mice. However, at 11 weeks of infection we found higher numbers of TUNEL positive cells in WT mice.

4. Discussion

Cutaneous leishmaniasis is characterized by lesions that are self-limited and may heal spontaneously over time. However, approximately 3% of infected individuals develop the mucocutaneous form of the disease, with chronic ulcerative lesions affecting mucosal tissues and cartilage. One of the features in mucocutaneous leishmaniasis in humans is the prolonged maintenace of lesions [7, 22]. These tissues present an inflammatory infiltrate rich in mononuclear and polymorphonucelar cells [23–26]. High concentrations of IFN-γ, TNF-α, and IL-17 are found in affected tissues and are produced by peripheral blood monocuclear cells from these patients [8, 26, 27]. Another feature is a deficiency in the regulation of the inflammatory processes in mucocutaneous patients, when compared to patients who developed the cutaneous form of the disease [27]. Tissue damage is caused apparently by the inflammatory response, since parasites are detected in low concentrations or not at all in lesions [23–25, 28]. In this work, we studied the chronic cutaneous lesion developed by *L. major*-infected TNFR1 KO mice. This model presents some of the features found in mucocutaneous patients: control of parasite replication, high concentrations of IFN-γ and TNF-α, and a large uncontrolled inflammatory infiltrate. Hence, *L. major*-infected TNFR1 KO mice can control parasite replication, but do not overcome the intense inflammatory process that is observed even after 20 weeks of infection. Thus, lesions in *L. major*-infected TNFR1 KO mice present some similarities to mucocutaneos lesions in patients infected (Table 2). This model may be useful for studies aimed at interfering with the development of chronic nonhealing lesions caused by *Leishmania.*

Resolution of *L. major* growth in mice is mediated by nitric oxide produced by nitric oxide synthase 2 (NOS2), which is induced by IFN-γ and TNF-α [4–6]. Given the high concentrations of IFN-γ and TNF-α present in lesions from TNFR1 KO mice infected with *L. major,* it is not surprising that these mice control parasite growth. The reason for discrepancy between mRNA and protein expression of TNF-α is not known, but we could speculate that there is posttrancriptional regulation or that protein is accumulated at the site of infection, since there is no signaling through TNFR1. Albeit TNF-α was shown to signal through TNFR1 to induce NOS*2*, alternative pathways in TNFR1 KO mice have been proposed [15, 29, 30]. In fact, mice deficient in both receptors for TNF are capable of controlling parasite burdens [15], and it has been proposed that direct contact with T cells (via CD40L and LFA1) would substitute for TNF-α [29]. Thus, as previously described [14, 15], we show here that persistence of lesions in TNFR1 KO is not mediated by the persistence of parasites at the site of infection.

TNF-α is the starting factor that mediates cell infiltration to a site of infection or tissue damage [21]. Accordingly, it has been shown that in an HSV-1 encephalites mouse model, TNFR1 mice present a smaller cellular infiltrate in response to tissue damage [31]. In other models, it has been shown that TNFR1 KO mice do not control the inflammatory infiltrate and damage tissue [19, 30, 32–35]. In this work, we characterized the cellular infiltrate in lesions from TNFR1

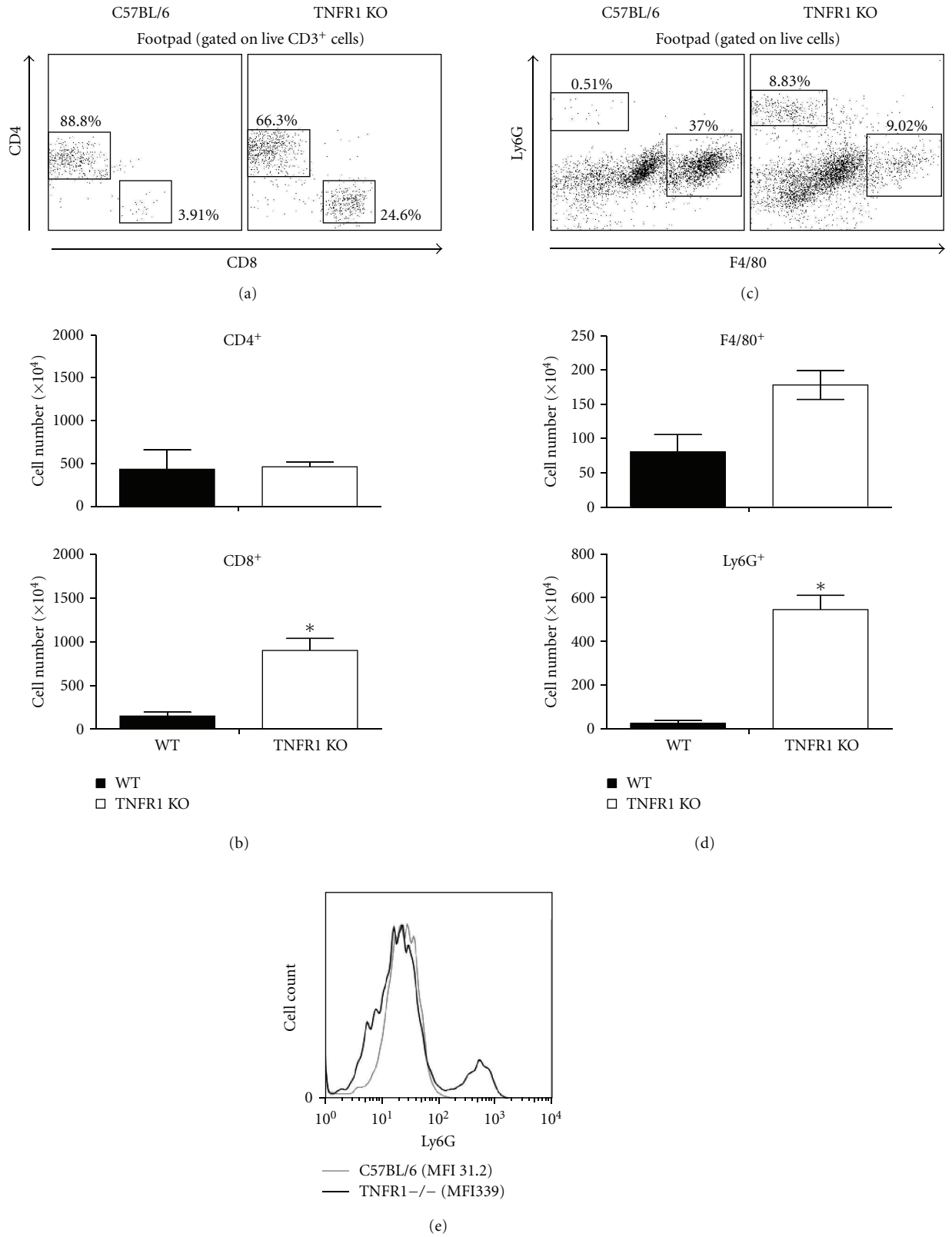


Figure 2: Cell populations in lesions from WT and TNFR1 KO mice infected with *L. major*. Mice were infected in the footpad with 1×10^6 *L. major* and 15 weeks post-infection the inflammatory cells were isolated from lesions and characterized by flow cytometry. (a) Dot plots and (b) absolute cell numbers of CD4+ and CD8+ T cells from pooled lesions from 4 mice per group. (c) Dot plots and (d) absolute cell numbers of F4/80+ and Ly6G+ cells from pooled lesions from 4 mice per group. (e) Ly6G fluorescence intensity. $^*P < 0.05$. Data are from one experiment.

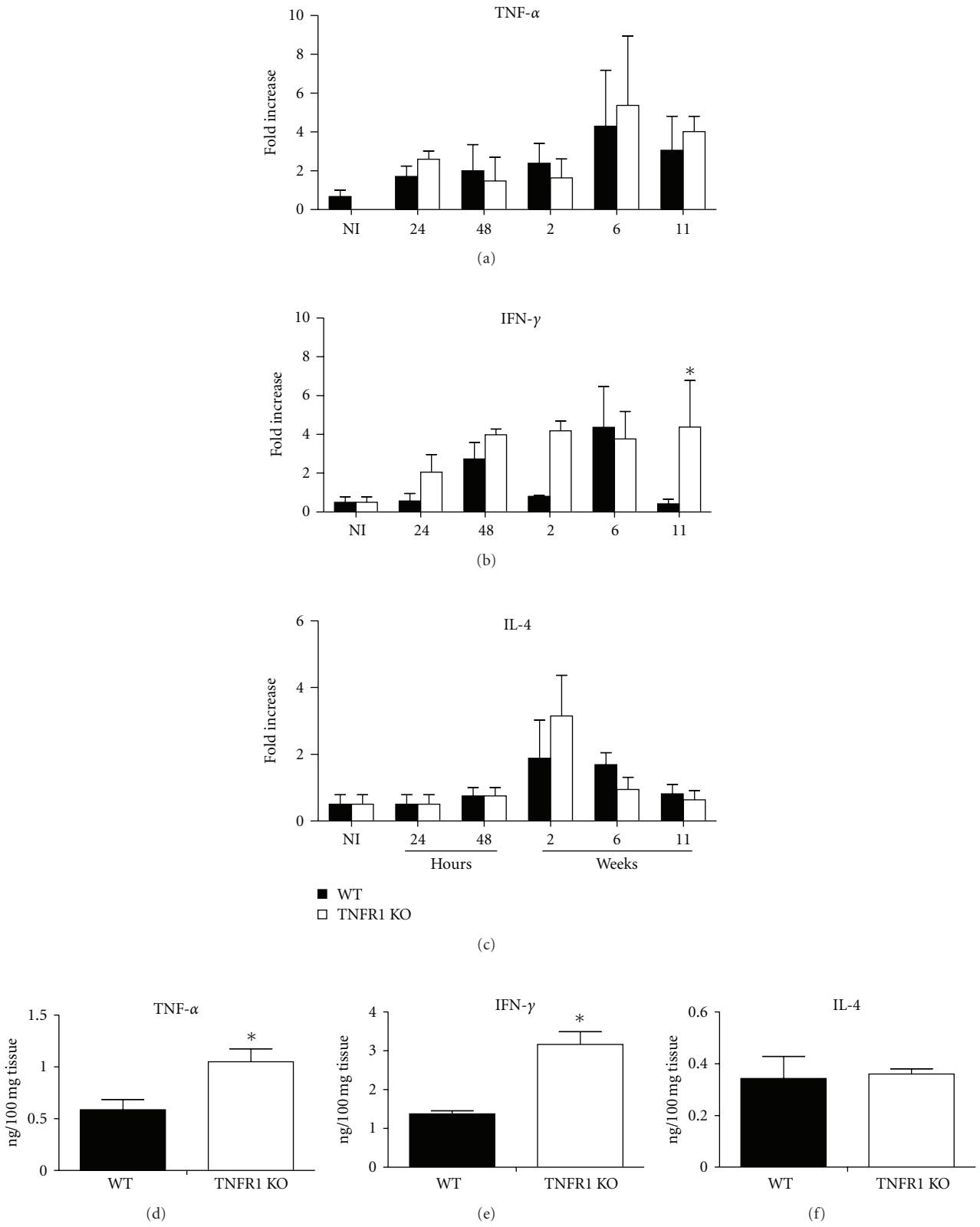


Figure 3: TNF-α, IFN-γ, and IL-4 in lesions from WT and TNFR1 KO mice infected with *L. major*. Mice were infected in both footpads with *L. major* (1×10^6). At 24 and 48 h and at 2, 6, and 11 weeks after infection, mice were killed, lesions were harvested, and RT-PCR reactions performed for cytokines. At 15 weeks protein was assayed by ELISA. (a–c) Fold increase in TNF-α, IFN-γ, and IL-4 mRNA, obtained by densitometry of bands in gels, as described in Section 2. (d–f) TNF-α, IFN-γ, and IL-4 protein was determined by ELISA 15 weeks after infection. Data shown are from one experiment representative of two indenpendently performed ones, with four mice per group. $^*P < 0.05$.

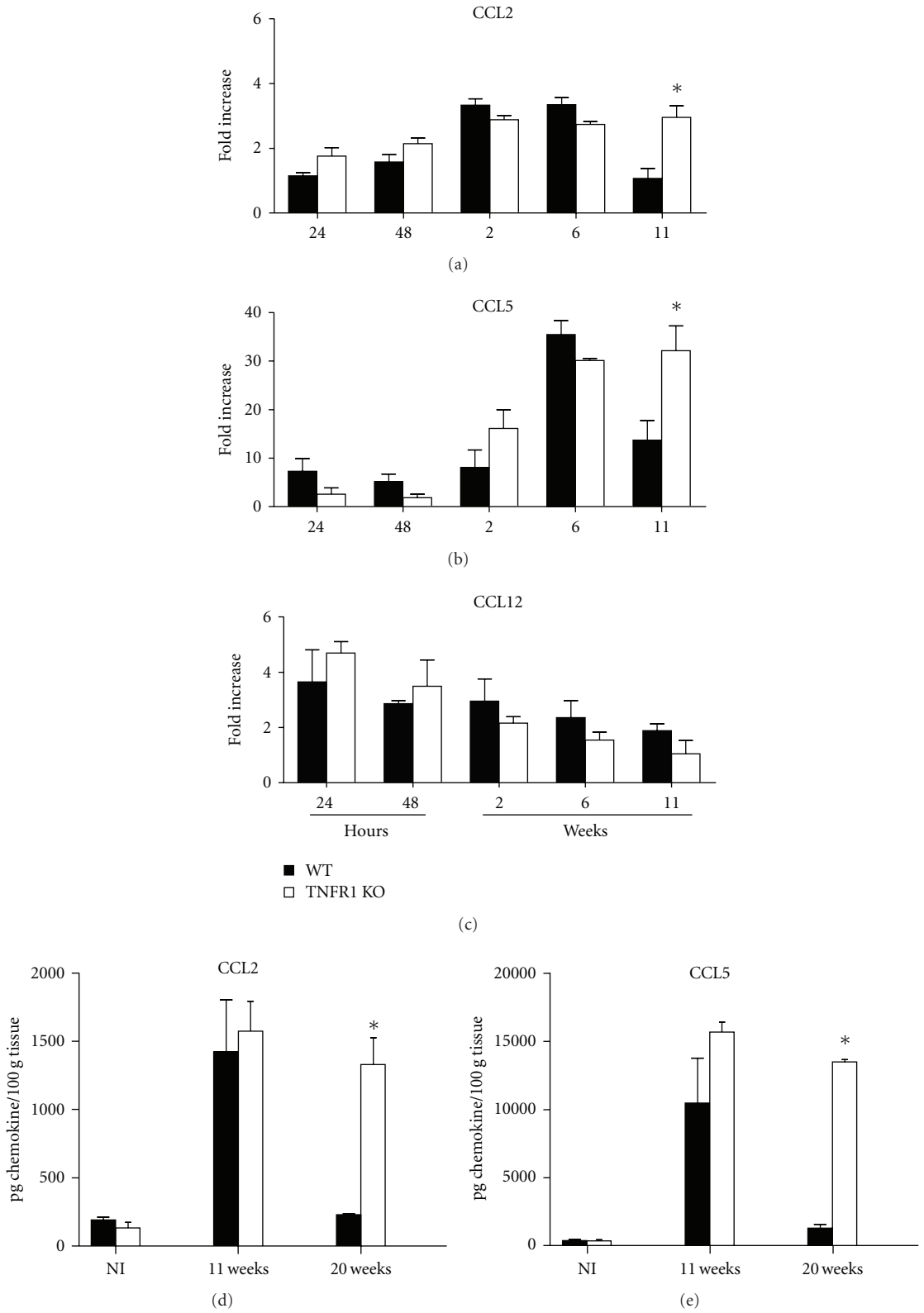


Figure 4: CCL2, CCL12, and CCL5 in lesions from WT and TNFR1 KO mice infected with *L. major*. Mice were infected in both footpads with *L. major* (1×10^6). At 24 and 48 h and at 2, 6, and 11 weeks after infection, mice were killed, lesions were harvested and RT-PCR reactions were performed for chemokines. At 15 weeks, protein was assayed by ELISA. (a–c) Fold increase for CCL2, CCL12, and CCL5 mRNA, obtained by densitometry of bands in gels, as described in Section 2. (d–e) CCL2 and CCL5 determined by ELISA in footpads of naïve mice (NI), and mice infected for 11 and 20 weeks. Data shown are from a single experiment representative of two separate experiments performed with four mice per group. $^*P < 0.05$.

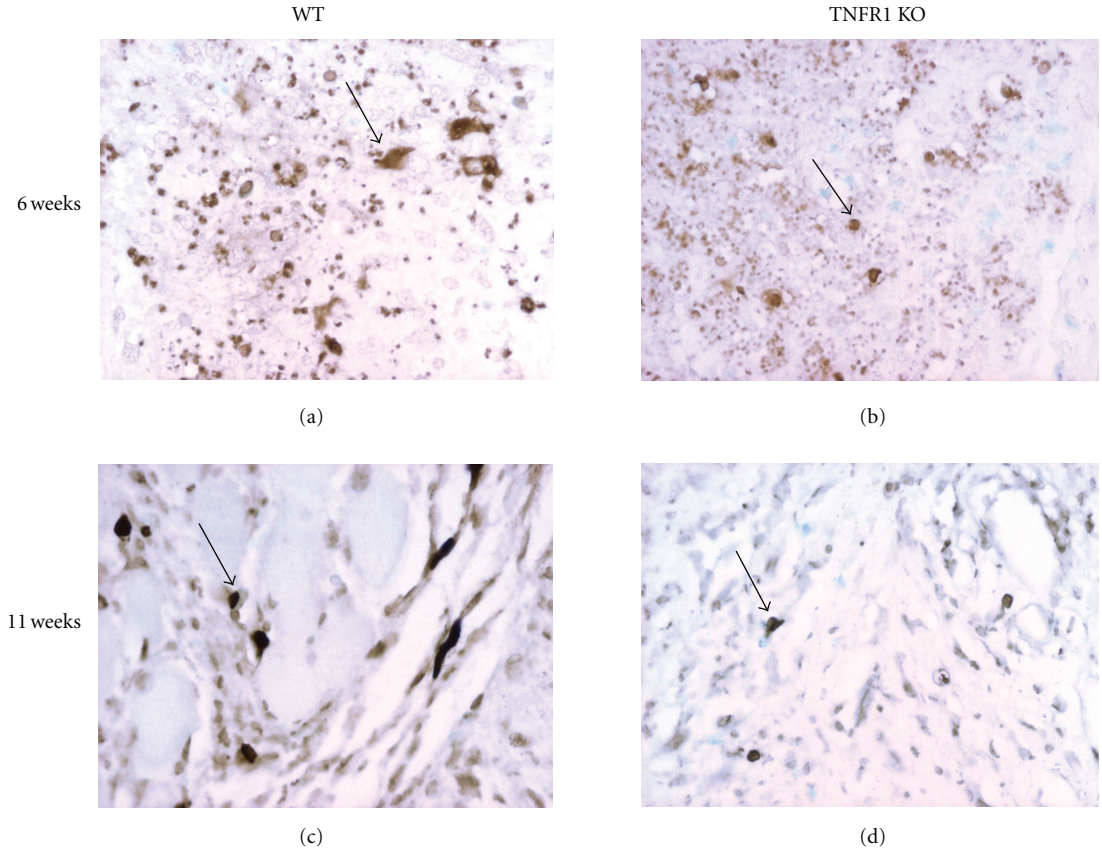


Figure 5: Apoptosis in lesions from WT and TNFR1 KO mice infected by *L. major*. Mice were infected with *L. major* in the footpad (1×10^6) and after 6 weeks (a and b) and 11 weeks (c and d) of infection. Tissues were stained by TUNEL, as described in Section 2. Arrows indicate TUNEL positive cells.

KO mice. We found that, at later times of infection, there were more neutrophils and CD8+ T cells in lesions from TNFR1 KO than in WT mice. CD8+ T cells have been implicated in the exacerbation of tissue injury in patients suffering from mucocutaneous form of leishmaniasis [36–38]. Likewise, the presence of neutrophils has been associated with a high concentration of IL-17 in areas of tissue damage in patients infected with *L. braziliensis* who developed mucocutaneous leishmaniasis [25, 26]. We also found lower percentages of F4/80-expressing macrophages in lesions from TNFR1 KO mice at 15 weeks afterinfection. This seems to be due to the presence of other cells in the same gate (e.g., neutrophils), since absolute numbers of macrophages were similar in both groups of mice. However, the smaller percentage of macrophages (which is not a smaller absolute number) did not compromise the control of parasites by TNFR1 KO mice.

Cell recruitment to sites of infection or tissue damage is mediated by chemokines [39]. In an attempt to explain the larger cellular infiltrate in TNFR1 KO mice, we determined the concentrations of mRNA for chemokines at several times of infection. At the earlier times of infection the concentrations of the chemokines assessed were similar in both groups. This observation is in accordance with our previous observations that initial cellular recruitment is not affected by the TNFR1, in a model of angiogenesis [40]. However, at 11 weeks of infection, when lesions were significantly different, we found higher expression of CCL5 and CCL2 mRNA and, at 20 weeks afterinfection, we found higher concentrations of CCL5 and CCL2 protein in lesions. This could be simply due to a larger number of cells at the site of infection; however, this is unlikely, since for CCL12 and other chemokines (not shown) we failed to observe the same effect. CCL5 and CCL2 are implicated on the migration of macrophages, monocytes, NK, and T cells to sites of injury [41–43]. Thus, it may be that the persistence of exacerbated inflammatory cells at the site of infection with *L. major* in TNFR1 KO is related with high concentrations of these two chemokines that are promoting the recruitment of more cells.

Apoptosis is required for the clearance of inflammatory cells from tissues, once the stimulus for recruitment of pathogens or damage is gone [44, 45]. TNFR1 mediates apoptosis, since it contains a cell death domain [46, 47]. In fact, it had previously been shown that there was defective apoptosis at the site of infection of *Rhodococcus equis*. Moreover, cells from *R. equis*-infected lungs of WT mice and from footpads of *L. major*-infected WT were sensitive only to TNF-mediated apoptosis. However, cells from TNFR1 KO mice infected with either *R. equis* or *L. major* were not sensitive

Table 2: Quantification of apoptosis at the site of infection by *L. major* in WT and TNFR1 KO mice.

	C57BL/6[a]		TNFR1 KO	
	Mean	Standard error	Mean	Standard error
6 weeks	**7.04**[b]	0.72	**6.75**	1.00
11 weeks	**8.15**[c]	1.01	**2.91**	0.52

[a]Mice were infected with *L. major* in the footpad (1×10^6) and after 6 and 11 weeks of infection animals were sacrificed and the lesions collected. Tissues were stained by TUNNEL, as described in Section 2. Apoptotic nuclei were counted and results expressed as apoptotic nuclei per 100 nuclei. Results represent the mean of 3–6 animals; six sections were counted per mouse.
[b]$P = 0.37$ Student's t-test.
[c]$P = 0.0005$ Student's t-test.

Table 3: Comparison between human mucocutaneous and TNFR1 KO lesions.

Characteristic	Mucocutaneous leishmaniasis	*L. major*-infected TNFR1 KO
Chronic cutaneous lesions	Yes	Yes
Intense inflammatory cells infiltrate in chronic phase	Yes	Yes
Low parasite load in lesions	Yes	Yes
High production of proinflammatory cytokines (IFN-γ and TNF-α) in the lesions	Yes	Yes
Defect in the regulation of inflammatory response	Yes	Yes
Presence of high numbers of CD8+ T cells and neutrophils in lesions	Yes	Yes
High levels of IL-17	Yes	Not determined
Invasion of mucosal tissues	Yes	No

to TNF-α-mediated apoptosis [19]. Thus, we investigated apoptosis, *in situ*, in lesions from WT and TNFR1 KO mice. At 6 weeks of infection, both mouse strains showed similar concentrations of TUNEL positive cells, thus suggesting that at this time point apoptosis occurred independently of the TNFR1. However, at 11 weeks of infection, when WT mice were healing and TNFR1 KO mice had a large inflammatory infiltrate at the site of infection, lesions from WT mice had significantly more TUNEL positive cells than lesions from TNFR1 KO mice. This indicated to us that TNFR1 plays a crucial role in the clearance of the inflammatory infiltrate and in the healing of lesions.

5. Conclusion

In this paper, we implicated persistent chemokine production and defective apoptosis as the factors that prevent healing of lesions in TNFR1 KO mice. In addition, characterization of parasite burden, cellular infiltrate, and cytokine production allowed us to propose the TNFR1 KO infected with *L. major* as a model to study chronic nonhealing lesions found in patients that present the mucocutaneous clinical form of leishmaniasis (Table 3).

Acknowledgments

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Research Article

Nucleotide-Binding Oligomerization Domain-1 and -2 Play No Role in Controlling *Brucella abortus* Infection in Mice

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Nucleotide-binding oligomerization domain proteins (NODs) are modular cytoplasmic proteins implicated in the recognition of peptidoglycan-derived molecules. Further, several *in vivo* studies have demonstrated a role for Nod1 and Nod2 in host defense against bacterial pathogens. Here, we demonstrated that macrophages from NOD1-, NOD2-, and Rip2-deficient mice produced lower levels of TNF-α following infection with live *Brucella abortus* compared to wild-type mice. Similar reduction on cytokine synthesis was not observed for IL-12 and IL-6. However, NOD1, NOD2, and Rip2 knockout mice were no more susceptible to infection with virulent *B. abortus* than wild-type mice. Additionally, spleen cells from NOD1-, NOD2-, and Rip2-deficient mice showed unaltered production of IFN-γ compared to C57BL/6 mice. Taken together, this study demonstrates that NOD1, NOD2 and Rip2 are dispensable for the control of *B. abortus* during *in vivo* infection.

1. Introduction

The innate immune system plays a crucial role in host defense against invading pathogens and relies on pattern recognition receptors (PRRs), which detect conserved microbial- or danger-associated molecular patterns (MAMPs or DAMPs). Several PRRs have been identified, among them are the TLRs (Toll-like receptors), NLRs (nucleotide-binding and oligomerization domain-like receptors), and RLR (retinoic-acid-inducible gene-1-like receptors) [1].

Nod1 and Nod2 are NLR proteins that trigger nuclear factor-κB (NF-κB) signaling in response to bacterial peptidoglycan. Specifically, Nod1 recognizes muramyl peptides containing *meso*-DAP (diaminopimelic acid) found in the peptidoglycan of most Gram-negative bacteria and certain Gram-positive bacteria [2] whereas Nod2 recognizes muramyl dipeptide (MDP) produced in all bacteria [3]. Upon peptidoglycan detection, Nod1 and Nod2 recruit and associate with the adaptor protein Rip2, triggering proinflammatory pathways such as NF-κB and the mitogen-activated protein (MAP) kinases p38, JNK, and ERK [4]. Furthermore, activation of Nod1 and Nod2 by live bacteria triggers proinflammatory responses, leading to the induction of cytokine and chemokines [5, 6]. Using Nod-deficient mice, several *in vivo* studies have demonstrated a role for Nod1 and Nod2 in host defense against pathogens such as *Helicobacter pylori, Listeria monocytogenes, Staphylococcus aureus, and Legionella pneumophila* [7–10].

Brucella is a Gram-negative bacterium which is pathogenic of human and animals [11]. The immune response against *Brucella* infection involves many molecules and cells to trigger a Th1 immune response and activation of CD8+ *T* cells [12–14]. The innate immune response against *B. abortus* infection begins with the recognition of molecular structures related to this pathogen by receptors such as Toll-like receptors (TLRs) [15]. Some *in vitro* and *in vivo* studies have shown the involvement of TLR2, TLR4, and TLR9 in the recognition of *Brucella* and induction of

inflammatory response [16–20]. Moreover, our group and others have demonstrated that MyD88 is essential for host control of *Brucella* infection *in vivo* and the induction of proinflammatory cytokines [21]. So far, no study has demonstrated the role of NOD-like receptors in the control of *Brucella* infection. Herein, we have shown that NOD1, NOD2, or the adaptor molecule Rip2 plays no role in enhancing resistance to *B. abortus* infection *in vivo*. However, reduced production of TNF-α was detected in bone-marrow-derived macrophages (BMDM) from NOD1, NOD2, and Rip2 KO mice compared to C57BL/6.

2. Materials and Methods

2.1. Mice. NOD1, NOD2, and RIP2 genetically deficient mice ($NOD1^{-/-}$, $NOD2^{-/-}$, and $RIP2^{-/-}$) were kindly gifted by Dr. Richard Flavell (Yale University) and maintained in the animal facility of the University of São Paulo (FMRP/USP). The wild-type strain C57BL/6 mice were purchased from the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). Wild-type and deficient mice were maintained at UFMG and used at 6–8 weeks of age.

2.2. Bacteria. *Brucella abortus* virulent strain S2308 was obtained from our laboratory collection [22]. The strain S2308 was grown in *Brucella* Broth liquid medium (BB) (DIFCO) at 37°C under constant agitation. After three days of growth, the bacterial culture was centrifuged and the pellet was resuspended in phosphate buffered saline (PBS) 0.15 M pH 7.4 (2.8 Na_2PO_4 mM, 7.2 mM Na_2HPO_4, and 0.14 M NaCl). Aliquots of these cultures were serially diluted and plated on BB medium containing 1.5% bacteriological agar. After incubation for 72 hours at 37°C, bacterial numbers were determined by counting colony forming units (CFU).

2.3. B. abortus Infection. Five mice from each group C57BL/6, $NOD1^{-/-}$, $NOD2^{-/-}$, and $RIP2^{-/-}$ were infected intraperitoneally with 1×10^6 CFU of *B. abortus* strain S2308. These mice were sacrificed at 2 weeks after infection. The spleen harvested from each animal was macerated in 10 mL of saline (NaCl 0.8%), and it was used for counting of CFU and splenocyte culture. To count residual *Brucella* CFU, spleen cells were serially diluted and were plated in duplicate on BB agar. After 3 days of incubation at 37°C, the number of colony forming units (CFU) was determined. Results were expressed as the mean log CFU of each group.

2.4. Measurement of IFN-γ into Splenocyte Culture Supernatants. Spleens cells from C57BL/6, $NOD1^{-/-}$, $NOD2^{-/-}$, and $RIP2^{-/-}$ mice were treated with ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) to lyse red blood cells. After that, the cells were washed with saline (NaCl 0.8%) and suspended in RPMI 1640 (Gibco, Carlsbad, Calif, USA) supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated FBS (Gibco, Carlsbad, CA), penicillin G sodium (100 U/mL), and streptomycin sulfate (100 μg/mL). To determine cytokine concentration by ELISA, 1×10^6 spleen cells were plated per well in a 96-well tissue culture-treated dish. Splenocytes were stimulated with *B. abortus* S2308 (MOI 100 : 1) or concanavalin A (5 μg/mL Sigma, Sigma-Aldrich, St. Louis, Mo, USA). Unstimulated cells were used as negative control. Spleen cells were incubated at 37°C in 5% CO_2 for 72 h, after that supernatants were harvested for measuring IFN-γ levels. IFN-γ was measured into cell supernatants by ELISA using the Duoset kit (R&D Systems, Minneapolis, Minn, USA) according to the manufacturer's instructions.

2.5. Generation and In Vitro Stimulation of Bone-Marrow-Derived Macrophages- (BMDMs). Macrophages were derived from bone marrow of C57BL/6, $NOD1^{-/-}$, $NOD2^{-/-}$, and $RIP2^{-/-}$ mice as previously described [23]. Briefly, bone marrow (BM) cells were removed from the femurs and tibias of the animals and cultured in DMEM (Gibco, Carlsbad, Calif, USA) containing 10% FBS (HyClone, Logan, Utah, USA), 1% HEPES, and 10% L929 cell-conditioned medium (LCCM) as source of M-CSF, in 24-well plates (5×10^5 cells/well). After 4 days, 100 μL/well LCCM was added. At day 7, the medium was renewed. At day 10 of culture, when the cells had completely differentiated into macrophages, the medium was harvested and we added supplemented DMEM (500 μL/well) containing *B. abortus* S2308 (MOI 1000 : 1) or *E. coli* LPS (1 μg/mL, Sigma, St. Louis, Mo, USA). Culture supernatants of BMDMs were collected after 24 hours of stimulation and assayed for the concentrations of IL-12, IL-6, and TNF-α by ELISA (R&D Systems) according to the manufacturer's instructions.

2.6. Statistical Analysis. A previous analysis of normal distribution of the data was performed, and ANOVA was used followed by Tukey's test when we compared more than two variables. Furthermore, Student's *t*-test was applied when only two variables were compared using GraphPad Prism 4 (GraphPad Software, Inc.). The level of significance in the analysis was $P < 0.01$.

3. Results

3.1. NOD1, NOD2, and Rip2 KO Mice Control B. abortus Infection. To investigate the role of NOD1, NOD2, and Rip2 molecules during *B. abortus* infection, knockout and wild-type mice were infected with 1×10^6 CFU of *B. abortus* strain S2308 and the number of bacteria in mouse spleens was monitored by colony forming units (CFU) counting. As shown in Figure 1, there was no difference in bacterial load from NOD1, NOD2, and Rip2 KO mice compared to C57BL/6. These results indicate that NOD1, NOD2, and Rip2 are not important to *in vivo* host control of *Brucella*.

3.2. NOD1, NOD2, and Rip2 Do Not Account for IFN-γ Response to B. abortus. Protective immunity against infection by *B. abortus* is directly related to the induction of a type 1 pattern of immune response [24]. IFN-γ is a critical cytokine involved in this type of immunity. Thus, to evaluate the role of NOD1, NOD2, and Rip2 in inducing a type 1 immune response during *B. abortus* infection, splenocytes

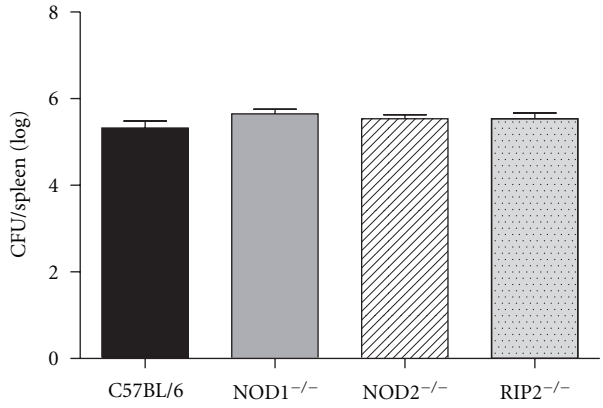

Figure 1: Control of *Brucella abortus* infection is NOD1 and NOD2 independent. C57BL/6, NOD1$^{-/-}$, NOD2$^{-/-}$, and RIP2$^{-/-}$ mice were intravenously infected with 10^6 CFU of *B. abortus* S2308, and the number of bacteria in the spleen was analyzed by counting CFU at 2 weeks after infection. Data are expressed as mean ± SD of five animals per time point.

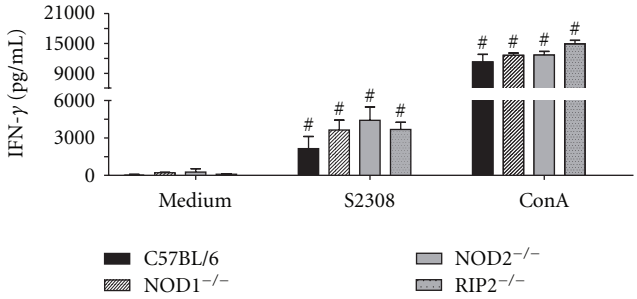

Figure 2: IFN-γ production by spleen cells induced by *B. abortus* in NOD1, NOD2, and Rip2 KO mice. C57BL/6, NOD1$^{-/-}$, NOD2$^{-/-}$, and RIP2$^{-/-}$ mice were infected with 10^6 CFU of *B. abortus* S2308, and 2 weeks after infection, spleen cells (1×10^6/well) were stimulated with *B. abortus* S2308 (MOI 100 : 1) or concanavalin A (5 μg/mL). Supernatants were harvested after 72 h for measuring IFN-γ levels by ELISA. Statistically significant difference in relation to nonstimulated cells is denoted with #. The significance of differences was compared by ANOVA followed by Tukey's test ($P < 0.01$).

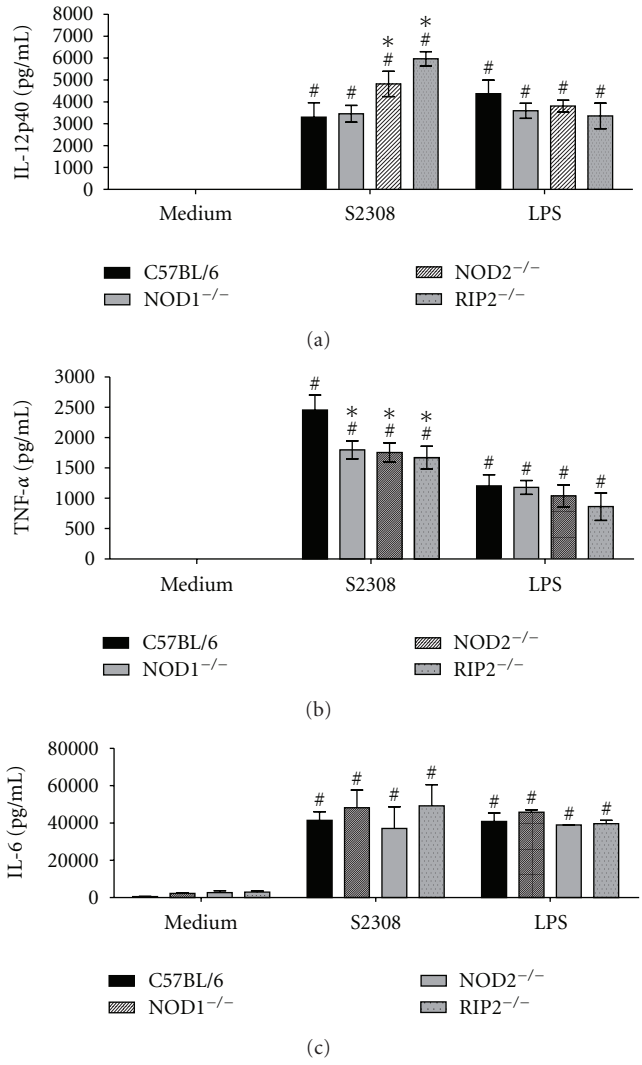

Figure 3: TNF-α production induced by *B. abortus* in macrophages, but not IL-12 and IL-6, requires NOD1, NOD2, and Rip2. Bone marrow from C57BL/6, NOD1$^{-/-}$, NOD2$^{-/-}$, and RIP2$^{-/-}$ mice cells was differentiated in macrophages and stimulated with *B. abortus* S2308 (MOI 100 : 1) or *E. coli* LPS (1 μg/mL). Supernatants was harvested for measuring IL-12 (a), TNF-α (b), and IL-6 (c) after 24 hrs by ELISA. Statistically significant difference in relation to non-stimulated cells is denoted with # and in relation to C57BL/6 mice is denoted with an asterisk (∗). The significance of differences was compared by ANOVA followed by Tukey's test ($P < 0.01$).

from *Brucella*-infected animals were stimulated with live *B. abortus*. After 72 hrs of cell culture, the supernatant was collected and the level of IFN-γ was analyzed. Herein, it was observed a similar level of IFN-γ production by NOD1, NOD2, or Rip2 KO mice when compared to wild-type animals (Figure 2). Taken together, these results suggest that the lack of NOD1, NOD2, and Rip2 causes no effect on induction of type 1 immune response by *B. abortus*.

3.3. Lack of NOD1, NOD2, and Rip2 Causes a Significant Reduction in TNF-α Production by Macrophages. The recognition of *Brucella* by innate immunity cells, such as macrophages and dendritic cells, results in activation and the concomitant production of proinflammatory cytokines [19]. In order to evaluate the role of NOD1, NOD2, or Rip2 in the proinflammatory cytokine production, bone-marrow-derived macrophages from NOD1, NOD2, or Rip2 KO and C57BL/6 mice were stimulated with *B. abortus*. As shown in Figure 3(b), NOD1, NOD2, or Rip2 deficiency reduced the production of TNF-α by macrophages from knockout mice compared to wild type cells. In contrast, IL-12 levels of NOD1 KO cells remained unaltered compared to wild type but were higher in supernatants of NOD2 and Rip2 KO macrophages (Figure 3(a)). Regarding IL-6, the levels of this cytokine produced by knockout macrophages were similar to C57BL/6 cells (Figure 3(c)). These results showed that

NOD1, NOD2, and Rip2 are important molecules involved in TNF-α synthesis induced by *Brucella* in macrophages but not in IL-6 and IL-12 production.

4. Discussion

Innate immune responses against intracellular pathogens are crucial to produce an efficient host response that triggers control of microbial replication and resistance to infection. NOD receptors are important molecules that play a key role in the induction of nitric oxide, a molecule that is known to be directly microbicidal [25]. Further, activation of NOD1 and NOD2, by live bacteria triggers proinflammatory responses, leading to the induction of cytokine and chemokine [5, 6]. In this study, we aimed to analyze the contribution of NOD1, NOD2, and Rip2 to the immune responses triggered by the intracellular bacterium *B. abortus*.

To examine the role of NOD1, NOD2, and Rip2 in control of *B. abortus in vivo*, we used knockout mice for these molecules. At 2-weeks after-infection, no significant differences in *B. abortus* CFU were observed between C57BL/6 and NOD1$^{-/-}$, NOD2$^{-/-}$, and Rip2$^{-/-}$ (Figure 1). We next examined the participation of NOD1, NOD2, and Rip2 in IFN-γ production during *B. abortus* infection. As observed in Figure 2, the level of IFN-γ produced by NOD1$^{-/-}$, NOD2$^{-/-}$, and Rip2$^{-/-}$ spleen cells was not different from wild-type mice. Consistent with these results, Rip2 was found to be dispensable for the induction of an effective Th1 response during *Toxoplasma gondii* infection [26], and mice double knockout to Nod1 and Nod2 respond similarly to wild type to restrict protozoan parasite infection by *Plasmodium berghei* [27]. Similarly, single deficiency in NOD1 or NOD2 had little or no effect on restriction of bacterial growth inside host cells during *L. pneumophila* or *M. tuberculosis* infection [10, 28]. In the case of *L. pneumophila*, NLR-dependent bacterial recognition triggers early responses that are further sustained by TLRs signaling pathways [29]. *Brucella* possesses both TLR and NLR agonists; however, it seems that they do not act synergistically to activate host cells. In situations where cells are rendered refractory to TLR agonists, it is possible that NOD1/2 signaling is increased [30]. Here, we speculate that during host responses to some pathogens that are strongly TLR dependent, NLRs become minor components of the pathogen recognition machinery. According to this hypothesis, we have previously determined the critical role of TLR/MyD88 axis to host control of *Brucella* infection [19].

Macrophages are key elements in the innate immune response and recognition of *Brucella* components resulting in the production of proinflammatory cytokines [19]. Herein, we investigated the involvement of NOD1, NOD2, and Rip2 in *Brucella*-induced IL-12, IL-6, and TNF-α by macrophages. Macrophages deficient in NOD1, NOD2, and Rip2 showed reduced production of TNF-α, but not IL-6, when they were stimulated with live *Brucella* as compared to C57BL/6 cells. Unexpectedly, we detected enhanced production of IL-12 for NOD2 and Rip2 KO macrophages. Berrington et al. [31] have also observed increased IL-6 and MCP-1 levels in NOD1 and NOD2 KO lung cells infected with *L. pneumophila*. They suggested that NOD1 and NOD2 regulate proinflammatory cytokine response by an unknown mechanism. One possibility is that, through heterotypic association of the caspase-1 recruitment domains, NOD1/NOD2 may inhibit inflammasome components or modulate cytokine production through interaction with TLR-pathway intermediates [32].

Taken together, the findings of this study provide evidence that NOD1, NOD2, and Rip2 may participate in innate immune signaling in response to *B. abortus*, but they are not essential for host defense against *B. abortus* infection *in vivo*. To the best of our knowledge, this is the first report that demonstrates the dispensable role of NOD1 and NOD2 to control *Brucella* infection.

Acknowledgments

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Review Article

Bartonella Infection in Immunocompromised Hosts: Immunology of Vascular Infection and Vasoproliferation

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Most infections by genus *Bartonella* in immunocompromised patients are caused by *B. henselae* and *B. quintana*. Unlike immunocompetent hosts who usually develop milder diseases such as cat scratch disease and trench fever, immunocompromised patients, including those living with HIV/AIDS and posttransplant patients, are more likely to develop different and severe life-threatening disease. This paper will discuss *Bartonella's* manifestations in immunosuppressed patients and will examine *Bartonella's* interaction with the immune system including its mechanisms of establishing infection and immune escape. Gaps in current understanding of the immunology of *Bartonella* infection in immunocompromised hosts will be highlighted.

1. Introduction

In this paper, we present a summary of the basic characteristics of *Bartonella* species which are important etiologic agents in human disease. The discussion focuses on a review of what is known of the immunology of *Bartonella*, particularly in immunocompromised hosts.

2. Classification

Bartonella are fastidious hemotropic facultative intracellular Gram-negative bacteria of the alpha-2 subgroup of Proteobacteria [1, 2]. Of the 24 species that comprise the genus *Bartonella, B. bacilliformis, B. henselae,* and *B. quintana* infections have been most commonly implicated in human disease and *B. henselae* and *B. quintana* account for the majority of morbidity and mortality among immunocompromised individuals [3–5].

3. *Bartonella* Epidemiology

Each of the major *Bartonella* species implicated in human disease has unique epidemiology. While the vast majority of *Bartonella* species have an identified zoonotic reservoir such as domesticated animals and farm animals, the animal reservoir for both *B. quintana* and *B. bacilliformis* have not yet been identified. While *B. bacilliformis* is transmitted by the sand fly and the major vector for *B quintana* is the human body louse, *B quintana* has also been found in rodent fleas, including rat fleas, and thus a potential for a rodent vector has been hypothesized [6, 7].

B. henselae occurs throughout the world. It is a zoonosis transmitted from the natural reservoir, cats (*Felis catus*), usually via cat scratch or bite and less commonly by a vector such as cat fleas (*Ctenocephalides felis*) or ticks [8]. Cross-sectional studies have reported as much as 81% seropositivity point prevalence among cats in different regions of the world. Bacteremia is more prevalent in cats that are less than 1 year old [2]. Humans and dogs are accidental hosts.

Although *B. quintana* occurs worldwide, with improved sanitation and living conditions, disease caused by *B. quintana* has dramatically decreased and now occurs primarily in small epidemics in conditions characterized by crowding and poor sanitation.

Humans are likely the natural reservoir for *B. quintana* which is spread by human body lice (*Pediculus humanis corporis*) [8]. Infected body lice excreta on human skin induce pruritis, and skin breakdown that follows scratching allows for *B. quintana's* pathway of invasion [9]. Wild rodents have also been identified as potential reservoirs [6, 7].

B. bacilliformis is endemic in mountainous areas of Peru, Columbia, and Ecuador [10]. It is transmitted by the *Lutzomyia* sandfly, but the animal reservoir has yet to be identified.

4. *Bartonella* and the Human Immune System

4.1. Establishing Infection: Evasion of the Immune System. Despite extensive knowledge about the disease syndromes caused by *Bartonella* species in humans, little is known about the humoral and cellular immune mechanisms involved in establishment and persistence of *Bartonella* infection in humans. Experimental studies using *B. tribocorum* inoculation onto an immune-competent rat model have shown that the brief bacteremia after inoculation is followed by a 3–5-day bacteremia-free window before resurgence of persistent intraerythrocytic bacteremia that lasts for 8–11 weeks [11]. This model highlights possible existence of a sanctuary site where the initial infection gets established before persistent intraerythrocytic infection manifests. So far, three such potential sanctuary sites include (a) extracellular matrix where high local bacterial replication may be achieved following initial infection [11], (b) bone-marrow stem cells such as erythroid progenitor cells where CD34+ cells in the bone marrow may be infected followed by release of infected erythrocytes into the blood stream [12] or endothelial progenitor cells which may also get infected in the bone marrow prior to their mobilization into circulation for endothelial repair [13], and, finally, (c) mature endothelial cells [14].

Bartonella Has Several Characteristics That Lead to Immune Evasion. Bartonella lipopolysaccaride (LPS) is composed of a unique combination of Lipid A and long-chain fatty acids and likely contributes to the bacteria's ability to evade the immune system [15]. Empiric data have shown that *Bartonella* initially evades the innate immune system because its surface molecules are not recognized by TLR-4 on dendritic cells or macrophages, [16], thereby allowing for establishment of what might lead to persistent infection. The LPS does not induce tumor necrosis factor alpha and has reduced stimulation of Toll-like receptor 2 (TLR2) and thus reduced endotoxicity [17, 18]. The LPS of *B. quintana* may even antagonize the Toll-like receptor 4 (TLR4) [19], which is a key component of innate immunity. Popa et al. and Matera et al. demonstrated that LPS from *B. quintana* resulted in the downregulation in human monocytes of nearly all cytokines normally produced by TLR4 in response to LPS [19, 20] and *B. quintana's* antagonism of TLR4 may be responsible for the absence of symptoms normally associated with bacteremia with Gram-negative organisms, such as septic shock.

Furthermore, *B. henselae* can avoid lysosomal fusion and acidification after the bacteria invades phagocytes such as endothelial cells and macrophages. For instance, *B. henselae* containing vacuoles (BCVs) had delayed development or complete lack of typical endocytic markers [21]. When the BCV contained inert or killed *B. henselae*, the endocytic markers were in normal ranges. Such Dendritic cells within cat scratch disease (CSD) granuloma in immune-competent humans that are chronically infected with Bartonella are also able to limit the infection to the same lymph node region via a localized B-cell-mediated humoral immune activation that is regulated by dendritic cells [22]. On the contrary, immunocompromised persons develop bacteremia, and distant seeding may occur, hence recovery of *Bartonella* in cardiac valves, other vasoproliferative lesions (bacillary angiomatosis or peliosis). Exactly how local bacterial control is lost in immunocompromised subjects is unclear.

4.2. Bacterial Persistence: Pro- and Anti-Inflammatory Response. Historically, clinical and in vitro studies implicated a Th1 immune response to *Bartonella* infection. The old *Bartonella* skin test involved an intradermal injection of suppurative material derived from lymph nodes from cat scratch disease patients and was interpreted as positive if their skin developed a large erythematous area within 48 hrs. This test is a classic delayed type hypersensitivity reaction, mediated through the Th1 immune response. In vitro studies have also supported the use of a Th1 response with mice splenocytes showing increased production of the Th1 cytokines IFNγ and IL-12 in response to *B. henselea* when compared with controls [23]. The role of innate immunity is also demonstrated through the repeated evidence of elevated IL-8 in response to *B. henselae* [24].

More recent studies have focused on the role of cytokines in acute and chronic *Bartonella* infection. Acute infection in immune-competent subjects with CSD have revealed upregulation of proinflammatory (IL-2, IL-6) and anti-inflammatory (IL-10) cytokines [25]. On the contrary, low CD4 counts (a marker of immune-compromise) have been associated with elevated IL-10 levels during acute infection with *Bartonella*, a cytokine milieu that may allow an acute infection to persist especially given the anti-inflammatory nature of IL-10 [16, 25, 26]. Chronic infection is associated with elevated levels of Interferon-alfa and IL-4 in both animal [16, 27] and human studies of the bacillary angiomatosis [16], but not in immune-competent subjects with CSD [25]. Thus, far, empiric data regarding *Bartonella* infection persistence and role in vasoproliferation is better understood from cytokine profiles, and less so from the traditional roles of humoral or cellular immunity.

Most of the research in infection involving immunocompromised hosts has focused on the establishment of bacterial stimulated angiogenesis. Understanding of the immune defense of *Bartonella* species in immunocompromised patients has been hampered by the lack of good animal model [28]. Since the response in the competent immune system involves Th1 and innate immunity through macrophages, a logical inference can be made that HIV-positive and other patients deficient in these immunological areas would have difficulty limiting the infection and thus would develop systemic manifestations. This deduction, however, has not yet been demonstrated as the mechanism by which HIV facilitates the spread of *Bartonella*. Chiaraviglio et al. described a murine model for chronic *Bartonella* infection using *B. taylorii* in SCID/Beige mice (B-, T-, NK-cell deficient) [29]. The group described that this model

approximated human bacillary peliosis and splenitis through *Bartonella* growing in extracellular aggregates in the spleen and liver. As the model is still relatively new, they did not present any further characterization of the immune response. However, it is clear that intraerythrocytic persistence is characteristic of infections in immune-competent hosts while endothelial/periendothelial persistence leading to vasoproliferation is characteristic of an infection in immune-compromised hosts [29].

5. Pathophysiology of *Bartonella* Disease/Lesions in Immunocompromised Hosts

5.1. Angiogenesis. Bacillary angiomatosis is the most common sequelae of *B. quintana* and *B. henselae* infections in patients with cell-mediated immunodeficiency such as HIV and posttransplant patients on immunosuppressive therapy [9, 23, 30]. *B. henselae* and *B. quintana* induce their characteristic vasoproliferative lesion, bacillary angiomatosis, or peliosis, in immunosuppressed patients, by direct and indirect effects on endothelial cells.

Whereas most pathogenic bacteria achieve tissue destruction and wider dissemination by inducing apoptosis on host cells, *B. henselae* and *B. quintana* infections persist within periendothelial extracellular matrix resulting in sustained, localized bacterial replication within collagen tissue [29]. This localized bacterial replication facilitates an antiapoptotic state in endothelial cells by secreting effector proteins (BepA and BepA2) which bind to the endothelial membrane receptor. The ensuing transmembrane signal transduction results in high cytoplasmic cAMP levels. The high cAMP levels upregulate cAMP responsive genes and induce an antiapoptotic state in the endothelial cells, resulting in their proliferation [31]. In addition, Schmid et al. in the same experiment showed that BepA can inhibit cytotoxic T-cell-mediated apoptosis of endothelial cells infected by *B. henselae* and *B. quintana*. A further analysis of the antiapoptotic mechanisms of some *B. henselae* strains isolated from HIV-infected patients revealed that an anti-apoptotic state may be effected through *B. henselae* strain-specific upregulation of antiapoptotic or downregulation of proapoptotic factors that work through the mitochondrial intrinsic apoptotic pathway [32]. As noted earlier, Interferon-alfa and IL-4 in chronic *Bartonella* infections may play a role, albeit unclear, in the development of bacillary angiomatosis given that elevated levels have been reported both animal [27] and human studies of the bacillary angiomatsois [25], but not in immune-competent subjects with CSD [25].

NF-κB is also upregulated by *Bartonella*, resulting in increased leukocyte rolling and adhesion and causes a proinflammatory environment [12]. Additional research is needed to explore this link between some *B. henselae* strains that infect HIV patients and vasoproliferation and inflammation. Such work may shed more light into pathogenicity of *B. henselae* and host susceptibility factors in HIV-positive patients.

6. Immunocompromise Induced by *Bartonella* Infections

B. bacilliformis itself induced an immunocompromised state. Although *B. bacilliformis* is not widespread in distribution and has not been described as a cause of bacillary angiomatosis or hepatic peliosis in immunocompromised patients, it is notable that the acute bacteremic phase of *B. bacilliformis* causes significant immunosuppression by overloading the reticuloendothelial system. These patients are predisposed to infections such as salmonella and tuberculosis [28].

7. Clinical Features of *Bartonella* Infections in Immunocompromised Hosts

Bartonella infections caused by *B. henselae* or *B. quintana* in the immunocompromised host may be characterized by fever of unknown origin, culture-negative endocarditis, osteomyelitis, or angioproliferative lesions that may affect virtually any organ system, but have a predilection for the skin, liver, and spleen.

7.1. Clinical Features of Bartonella Infections in HIV-Infected Patients. Bacillary angiomatosis lesions and fever are the most common manifestation of *B. henselae* or *B. quintana* infection in HIV/AIDS patients [3, 9]. The lesions appear as cutaneous nodular vascular lesions but may also be found in a variety of organs including the GI-tract where they may cause hematemesis [33–35], genitourinary system [36], and other organs including heart, spleen, bones, and central nervous system. The differential diagnosis for bacillary angiomatosis includes Kaposi's sarcoma, angiosarcoma, and pyogenic granuloma.

Similar vascular lesions are seen in both bacillary peliosis and splenitis. Patients present with hepato- and/or splenomegaly as blood filled cysts proliferate in these organs.

Confirming the diagnosis can be challenging as serology has a 50–95% sensitivity and demonstrates cross-reaction between *Bartonella*, *Coxiella,* and *Chlamydia* species. Results from tissue culture are not always positive and take a long time given the fastidious nature of the bacterium. PCR is favored as a faster, sensitive, and specific diagnostic test [37, 38].

7.2. Clinical Features of Bartonella Infections in Transplant Patients. Persistent fever following close contact with cats is the most common presenting symptom among posttransplant patients who are infected with *B. henselae* [39]. *B. henselae* infection was complicated by acute graft rejection in 2 renal transplant patients [40]. It is unclear if this rejection was a random occurrence or if it is associated with some unknown effect of *B. henselae* on decreasing the donor tolerance of the graft or enhancing graft antigenicity. In liver transplant patients, *B. henselae* can present with hepatic masses [41], hepatic granulomas [42, 43], or disseminated disease [44].

7.3. Clinical Features of Bartonella Infection in Homeless Patients with Chronic Alcoholism. Subacute, culture-negative

endocarditis is the most common clinical presentation of *B. quintana* (less so *B. henselae*) infection among homeless patients with chronic alcoholism [4, 45, 46]. Risk factors for *Bartonella* endocarditis include preexisting valvular lesions and exposure to human body lice.

B. quintana may be cultured from the blood, but given the prolonged time required for culture, other diagnostic tests are preferred. An indirect immunofluorescent IgG antibody test directed against *Bartonella* species with titer of 1 : 800 or higher has a 95% positive predictive value for diagnosing *Bartonella* endocarditis [47]. Diagnosis is usually confirmed by performing PCR testing on plasma or tissue samples following DNA extraction [6].

8. Conclusion

Bartonella species are important zoonotic agents of human disease. Immunocompromised hosts are more susceptible to infection from *B. henselae* or *B. quintana* which results in a more severe clinical picture as compared with the normal host. More recent work explores role of cytokines and chemokines in subverting the immune system, and subsequently a persistent infection characterized by vasoproliferation in immunocompromised humans. The mechanisms of establishing infection and immune evasion in immunocompromised hosts are still incompletely understood and are areas where further studies are needed.

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Review Article

Immunogenetic Factors Associated with Severe Respiratory Illness Caused by Zoonotic H1N1 and H5N1 Influenza Viruses

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Following the 2009 H1N1 pandemic and ongoing sporadic avian-to-human transmission of H5N1 viruses, an emphasis has been placed on better understanding the determinants and pathogenesis of severe influenza infections. Much of the current literature has focused on viral genetics and its impact on host immunity as well as novel risk factors for severe infection (particularly within the H1N1 pandemic). An understanding of the host genetic determinants of susceptibility and severe respiratory illness, however, is currently lacking. By better defining the role of genetic variability in influenza infection and identifying key polymorphisms that impair the host immune response or correlate with protection, we will be able to better identify at-risk populations and new targets for therapeutic interventions and vaccines. This paper will summarize known immunogenetic factors associated with susceptibility or severity of both pH1N1 and H5N1 infections and will also identify genetic pathways and polymorphisms of high relevance for future study.

1. Introduction

Transmission of zoonotic influenza A viruses to humans is commonly the cause of new pandemics, which typically result in high disease burden and increased symptomatic severity and mortality. In order to predict which populations may be at highest risk of infection and to develop more effective therapeutic interventions and vaccines, a thorough understanding of both viral and host contribution to pathogenesis is required. In both the recent 2009 H1N1 (pH1N1) pandemic and the on-going rare avian-to-human transmission of H5N1, numerous studies have taken an in-depth look at the impact of viral evolution and mutation on viral pathogenesis. Conversely, while both human and animal model studies of the host immune response to infection have identified correlates of severe disease, the contribution of host genetics to these correlates and to variability in susceptibility remains relatively unknown. Identification of host genetic polymorphisms contributing to altered susceptibility or disease severity has several benefits: identification of high-risk populations at greater need of prophylactic intervention, elucidation of host proteins important in virus-host interactions, and new targets for therapeutic interventions or vaccine development [1]. Studies of host genetics have provided important contributions to the study of other infectious diseases, including HIV, SARS, and HCV. This paper will describe what is currently known about the impact of host immunogenetics in both pH1N1 and H5N1 infections and will identify highly relevant polymorphisms and genetic pathways that could be investigated in future work.

2. 2009 Pandemic H1N1

H1N1 influenza viruses emerged as a result of a presumed or documented reassortment of segments from viruses of zoonotic origin with human-adapted influenza virus to cause pandemic spread in 1918 and again in 2009. The 2009 appearance of a swine-origin reassortant virus led to the first pandemic of the 21st century. During earlier pandemics, records indicate that certain individuals or populations appeared to be more susceptible to severe disease, but the

ability to conduct studies in order to understand the immune mechanisms that underlay the increased propensity for complications was limited. The 2009 H1N1 (pH1N1) pandemic was accompanied by improved surveillance, thereby facilitating better estimation of disease severity and methods to examine the immune mechanisms behind complicated disease [2–11]. This surveillance allowed for the identification of several novel risk factors among various populations, but with a limited understanding of the genetic variation that may contribute to those risk factors.

2.1. Novel Risk Factors Associated with Severity of Pandemic H1N1 Infection. The 1918 H1N1 as well as the recent 2009 pandemics were both notable for the comparatively high rates of morbidity among healthy, young adults not typically observed with seasonal influenza [11]. During the recent pandemic, several studies of confirmed pH1N1 cases in Canada and the US reported the median age of severe infections to be 23–27 years old [8, 10]. In Canada, 30%–48% of infections also presented in persons with comorbidities; diabetes, heart disease, and immunosuppression were associated with the highest risk of severe infection, while lung diseases and obesity were among the most common underlying conditions [10, 12–14]. The role of pregnancy as a risk factor, regardless of the stage, was also supported by a myriad of reports; among hospital admissions, pregnancy accounted for roughly 30% of female cases aged 20–39 years old [9, 12, 15].

Ethnicity was another major risk factor of pH1N1 susceptibility identified in several populations in North America and Australasia. The increased proportion of aboriginal individuals presenting with severe pH1N1 infection was not unique for this pandemic and was also seen in the 1918 H1N1 pandemic during which mortality in aboriginal communities in North America (3%–9%) was significantly higher than among nonaboriginal communities [16, 17]. In the 2009 pandemic, Pacific Islanders accounted for 2.5% of the Australian population but made up 9.7% of patients admitted to Australian ICUs with confirmed pH1N1. Maori individuals represent 13.6% of the New Zealand population, but accounted for 25% of ICU admissions in the ANZIC study [18]. Kumar et al. [12] also reported 25.6% of the individuals admitted to ICUs in Canada belonged to First Nations, Inuit, Metis, or aboriginal ethnicities; this is an overrepresentation compared to the 4.4% rate of self-reported aboriginal ethnicity according to the 2001 census (Statistics Canada). Similarly, pH1N1 mortality rates among American-Indian/Alaska Natives were four times higher than persons in all other ethnic populations combined in the United States [19].

None of these studies examined the causal factors that lead to the higher influenza mortality in the high-risk groups described. It is clear that multiple converging risks account for the high rates of complications, including socioeconomic factors such as inability to access care, delayed seeking of care, higher rates of poverty, and greater numbers of household members. A few of the risk factors listed in the previous sections, however, share a degree of immune system impairment. One can, therefore, speculate that the partial protection afforded by the immune system, primarily by cross-reactive CD8+ T-cells recognizing viral epitopes, is decreased in some of the previously described groups (pregnancy is a good example). Additionally, genetic variation in immune-related genes leading to either gain-of-function or loss-of-function phenotypes could contribute to the variation observed in pH1N1 susceptibility and disease severity.

2.2. Novel Immunogenetic Risk Factors Associated with Severity of Pandemic H1N1 Infection. When a novel strain of influenza emerges, the pre-existing antibody response directed largely at the surface glycoproteins is rendered ineffective. In these cases, the mechanisms underlying heterosubtypic cross-protection assume a dominant role and it is, therefore, not surprising that immune dysfunction caused by underlying genetic polymorphisms may lead to impaired responses and would, therefore, be associated with adverse outcomes. During the 2009 H1N1 pandemic, several immunogenetic determinants of severe disease were identified.

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2.2.1. CCR5Δ32 Allele. The CCR5 protein is a chemokine receptor expressed primarily on T cells, macrophages, and dendritic cells. CCR5 plays a pivotal role in mediating leukocyte chemotaxis in response to chemokines (including RANTES, MIP-1α, and MIP-1β) and is believed to be important in the homing of many immune cell subsets, including regulatory T cells and Th17 cells, to mucosal surfaces. Until recently, the purported role of CCR5 in supporting the antiviral immune response was limited to appreciation of the effect of receptor deficiency in protecting from HIV infection and disease progression among individuals homozygous for the Δ32 allele. The understanding of the roles played by CCR5 was expanded when the Δ32 allele was found to be associated with an increased risk of symptomatic and fatal West Nile Virus (WNV) infection [20–22], a severe adverse reaction to the live yellow fever virus vaccine, and with severe tick-borne encephalitis symptoms [23, 24]. Together, these data suggest that CCR5 may also play a critical role in the immune response to flavivirus infections. The spectrum of symptomatic severity observed during the 2009 H1N1 pandemic led our group to study CCR5 genotype among patients requiring intensive care admission and respiratory support for severe H1N1 symptoms. Among twenty samples of confirmed severe pH1N1 infection, the CCR5Δ32 allele was found in 5 out of 9 of the Caucasian individuals, giving a Caucasian allele frequency of 27.8% [25] (Table 1). This observed frequency is approximately 2.5

Table 1: Genetic polymorphisms of interest in H1N1 susceptibility and severity.

Gene	Polymorphism	Functional significance	Reference
CCR5	CCR5△32	Increased allele frequency among Canadian H1N1 ICU cases	[25]
FcγRIIa, IGHG2	IGHG2 *n/*-n FcγRIIa-R131H	Polymorphisms previously linked to IgG2 deficiency, but not corroborated in H1N1 patients	[28–30]
NLRP3	2107C/A (Q705 K) rs4612666 (intron 7) rs10754558 (3′ UTR)	Association with dysregulation of inflammatory response (2107), alteration of NLRP3 mRNA stability and enhancer activity	[33, 34]
HLA	Various alleles	Influenza-specific CTL responses exhibit varying frequency and magnitude across various HLA alleles	[35]

times higher than that reported for local North American Caucasian populations [26, 27]. Given the small sample size available in this cohort, further studies will be required to conclusively determine the impact of CCR5 deficiency on pH1N1 susceptibility and severity.

2.2.2. IgG2 Subclass Deficiency. Similarly to IgG1, IgG2a and IgG2b are able to bind to Fc receptors with high affinity and are thought to be important in protecting against influenza infection. A group of Australian investigators identified an index case of severe influenza in a pregnant woman with IgG2 subclass deficiency and subsequently measured total IgG and IgG subclasses in all patients with pH1N1 infection requiring ICU care (many of whom were pregnant) compared to less severe controls and asymptomatic pregnant women presenting to antenatal clinic. A low level of IgG2 was correlated with severe pH1N1 infection after multivariate analysis. Measurement of IgG2 after 90 days among 15 of the surviving IgG2-deficient patients showed that 11 remained IgG2 deficient despite albumin levels returning to baseline values [28]. Additionally, a case-control study from China enrolled 38 Asian patients with respiratory failure due to severe pandemic influenza and compared IgG2 levels with 36 mild cases. They did not find any cases of selective IgG2 deficiency, but did observe significantly lower levels of IgG2 among the severe cases (despite normal levels of the other IgG subclasses) [29]. The authors looked for the presence of *FcγRIIa* and *IGHG2* genotypes (Table 1) that were previously shown to be associated with IgG2 deficiency, but found similar rates among cases and controls. They did, however, corroborate the previously reported finding [30] of cytokine dysregulation among severe cases of infection and suggested that the mechanism responsible for the low IgG2 is the more robust Th1 response and a suppressed Th2 response. Given the lack of *FcγRIIa* and *IGHG2* genotype data available from the Gordon et al. study [28], the impact of these polymorphisms on IgG2 levels and severe pH1N1 infection remains to be determined.

2.3. Additional Candidate Polymorphisms Contributing to Severe Influenza. Genetic polymorphisms associated with pH1N1 susceptibility and disease severity identified to date are limited, and much of the data is derived from small cohorts. An improved understanding of the sequence of immune responses to influenza as well as the application of newer technologies that employ high-throughput expression array or sequencing technologies can be used to guide a more focused approach to identify specific pathways that may be differentially activated by individuals with severe disease. Based on available data, we can identify several immune pathways and their genetic variants that warrant further investigation.

2.3.1. NLRP Inflammasomes. Recently, emphasis has been given to the role of the inflammasome in viral infections and, specifically, influenza. NLRP3 (NOD-like receptor family, pyrin domain-containing 3) inflammasomes are multiprotein complexes containing NLRP3, ASC (or Pycard), and caspase 1. Activation of cytokine/chemokine release through NLRP3 requires a signal derived from Toll-like receptor (TLR) stimulation, with resultant production of pro-IL-1β, -IL-18, and -IL-33. The prointerleukins are in turn cleaved to their respective active forms by caspase 1, which requires the input of an additional signal. The second signal in the context of influenza has been elegantly demonstrated by Ichinohe et al. [31], who showed that golgi-localized H1N1 M2 is both necessary and sufficient to trigger inflammasome activation. Differential expression of the components of the two signaling cascades that are required for inflammasome activation may therefore explain differences in influenza disease severity. Indeed, mouse knockout studies have shown that intact inflammasomes are necessary for innate immune responses to influenza A, chemokine production, and late stage viral clearance (reviewed in [32]). Evidence suggests that NLRP3-mediated signaling is also important in cellular recruitment and tissue repair during infection. A similar requirement for proper ASC function was observed in adaptive influenza immune responses. Multiple *NLRP3* SNPs have been associated with dysregulated inflammation responses and NLRP3 mRNA stability in humans but have not been examined in the context of pH1N1 susceptibility or mortality [33, 34] (Table 1).

2.3.2. HLA Alleles. The CD8+ T-cell response is a strong predictor of vaccine-induced protection and is thought to be particularly valuable in the elderly. Because this response is focused on more conserved viral proteins, it has the additional benefit of providing some cross-reaction with new influenza strains [46, 47]. Undoubtedly, multiple factors underlie the differences in disease severity among ethnic

groups, as previously discussed. From an immunogenetic perspective, however, HLA alleles are among the most variable human genes, and it is therefore conceivable that variable proportions of HLA class I alleles among ethnic groups may lead to qualitatively and quantitatively distinct CD8+ T-cell responses, as well as differences in immunodominant epitopes (Table 1). Boon et al. [35] have demonstrated that the frequency of CTL responses specific for the HLA-B8-restricted epitope $NP_{380–388}$ was lower in HLA-B27-positive donors than in HLA-B27-negative donors. They also showed that the HLA-A1-restricted epitope $NP_{44–52}$ responses were higher in HLA-A1-, -A2-, -B8-, and -B35-positive donors than in other donors. These observations suggest that the epitope specificity and magnitude of the CTL response is related to the HLA class I genetic background [35].

2.3.3. Gene Expression Studies. The role of cell-mediated immunity in ameliorating infection caused by novel influenza strains has been the focus of intense study [48] and it is, therefore, the most compelling area to investigate in order to identify immunogenetic factors that predict severity of pandemic H1N1 influenza. A comprehensive investigation was undertaken by Bermejo-Martin et al. [49] in a study from Spain. They enrolled 19 critically ill patients with primary pH1N1 influenza pneumonia and used gene expression analysis in order to identify host immune responses associated with severe disease defined by illness requiring mechanical ventilation. They identified impaired expression of a number of MHC class II and MHC class I genes, T-cell receptor-associated genes, and also of a cluster of genes thought to be involved in dendritic cell maturation, indicating defective antigen presentation in the most severe group of patients. They found further evidence for the effect of altered antigen presentation on the development of an appropriate adaptive response against the virus in the impaired expression of a group of genes critical to the activation and function of both T and B cells. The group with severe illness also showed higher expression of genes involved in IL-6 and IL-10 pathways, and these results were in concordance with the high serum levels of IL-6 and IL-10 in the group dependant on mechanical ventilation. The authors concluded that severe disease is associated with an impaired transition from innate to adaptive immunity in response to the pH1N1 virus, similar to observations in the context of SARS and severe infections caused by H5N1. The impaired adaptive response was also associated with delayed viral clearance. This study did not, however, explore the role of genetic polymorphisms in this immune dysregulation.

3. H5N1 Avian Influenza

Pathogenic avian influenza A/H5N1 viruses are endemic among poultry populations across Asia and Africa and present an ongoing risk for avian-to-human transmission. As of June 22, 2011, the WHO reports a total of 562 confirmed H5N1 cases and 329 deaths across 15 countries worldwide [50]. Although human-to-human transmission of H5N1 has so far been rare, the potential for viral evolution into a more transmissible strain raises the possibility that H5N1viruses could cause a pandemic. Given the high mortality rate currently associated with human H5N1 infection, a thorough understanding of the immune response and the underlying mechanisms of viral pathogenesis is crucial to improve treatment and to identify highly susceptible populations. To date, much of the research into the immunobiology and pathogenesis of human H5N1 infection has focused on the H5 haemagglutinin protein and the impact of viral genetic polymorphisms on viral pathogenicity. Although studies have begun to characterize of the role of the host immune response in pathogenesis, the impact of genetic variability on susceptibility and disease severity remains an important gap in our current knowledge. By identifying host genetic polymorphisms that exacerbate immunopathology or provide protection, we will be able to improve treatment and future vaccines [1, 51].

3.1. The Case for Host Genetic Variation in H5N1 Susceptibility and Disease Severity. The precise impact of host genetic variability on H5N1 susceptibility remains somewhat controversial, given the limited case data available and the relatively low number of published studies. A case study in Indonesia [52] found evidence of clusters of H5N1 infection among blood relatives that may be indicative of shared genetic susceptibility, but the authors were unable to rule out a shared viral exposure or altered viral pathogenesis in any of the clusters. Similar observations in a number of additional studies have prompted several authors to suggest a potentially strong genetic basis for H5N1 susceptibility [53–57]. A compilation of confirmed H5N1 cases worldwide found that, on average, 22% of cases occurred in clusters, and only 6% of cases within the clusters were not genetically related to other cluster members [1]. While this data does not conclusively point to genetic variation as an important determinant of susceptibility, it is important to note that human-to-human transmission of H5N1 is very rare, and therefore does not likely explain the high degree of genetic relatedness among cases [1]. It would also be expected that clusters of nonrelated individuals working in the poultry industry would be more prominent than genetically related clusters, if people are at equal risk of infection [1]. Some studies, however, have suggested that the observed clustering of infections among families could occur due to chance alone at the low rates of infection that are observed in H5N1 and highlight the difficulty in drawing conclusions from the currently available data [58].

3.2. Candidate Genes Influencing Susceptibility. Despite the intense research into the effect of viral mutations on pathogenesis and viral fitness, very few, if any, studies have assessed the impact of specific host polymorphisms on human H5N1 infection. Although genetic association studies face a number of challenges with regard to H5N1 infection, including limited numbers of infected patients, difficulty in determining appropriate control populations, and limited human-to-human transmission with resultant high likelihood of exposure to unique virus, identifying genetic variants involved in increased susceptibility and/or disease outcome could provide important data regarding crucial

Table 2: Genetic polymorphisms of interest in H5N1 susceptibility and severity.

Gene	Polymorphism	Functional significance	References
MBL2	230G/A	Low serum MBL levels; increased susceptibility to SARS	[36, 37]
MxA	−88G/T (rs2071430) −123C/A (rs17000900)	Increased basal (−123 A) and IFN-stimulated (−88 T) MxA expression and activity *in vitro;* altered susceptibility to SARS	[38, 39]
OAS1	rs2660 (3′ UTR A/G) rs3741981 (Exon 3 A/G) rs1077467 (Intron 5)	Altered susceptibility to SARS (3′ UTR, Exon 3); West Nile susceptibility and reduced activity due to splicing (Intron 5)	[38, 40, 41]
CCR5	CCR5△32	Increased mortality among CCR5 knockout mice, increased allele frequency among severe H1N1 infections	[25, 42]
CCR2	190G/A (V64I)	Altered HIV progression; stabilization of CCR2a splice variant and binding to CCR5	[43, 44]
TLR3	908T/C	Missense mutation identified in a patient with influenza-associated encephalopathy	[45]

host/virus interactions and the qualities of a protective immune response. To date, a number of candidate genes have been identified from both human and mouse immunobiology studies. Mouse models of influenza infection have the advantage of being able to dissect gene expression kinetics and the characteristics of the immune response at various stages of infection. Comparison of infection across inbred mouse lines demonstrates significant differences in viral titre and core temperature, as well as distinct patterns of immune gene upregulation, suggesting an important contribution of host genetic background [59].

3.2.1. Host-Virus Interactions. Genetic variation affecting host proteins required for viral entry and pathogenesis may partially explain the sporadic and rare nature of avian-to-human H5N1 transmission. Although humans do express the SAα-2,3Gal molecules that are efficiently bound by avian H5N1, their expression is usually limited to the lower respiratory tract and has only occasionally been detected in the nasal mucosa and upper respiratory tract [60, 61]. Additionally, the binding of several influenza strains to human erythrocytes is highly variable, with up to a 40-fold difference between individuals tested in one study, suggesting a role for genetic polymorphism in regulating susceptibility [62]. Whether variation in the human *ST3 beta-galactosamide alpha-2,3-sialyltranferase 1* (*ST3GAL1*) gene that produces the SAα-2,3Gal linkage affects H5N1 susceptibility is not known, but remains a possibility [63]. Interestingly, a population genetics study designed to detect human SNPs under virus-driven selective pressure found a significant enrichment of glycan biosynthesis gene SNPs associated with viral selection, including rs3758105 (intronic A/G SNP) in the *ST3GAL1* gene [64] (Table 2). Data demonstrating the infection of upper respiratory tract cells with H5N1 *in vitro* also suggests the presence of additional cellular receptors for the virus [61].

Alternately, prevention of viral attachment in the respiratory tract is accomplished by host proteins that can sterically hinder viral HA binding, or aggregate and opsonize the virus. These proteins include serum mannose-binding lectin 2 (MBL2) and surfactant, pulmonary-associated protein A1 and D (SFTPA1 and SFTPD, resp.). A SNP in *MBL2* (230G/A), resulting in low serum MBL levels, is associated with SARS susceptibility [36, 37], while polymorphisms in *SFTPA1* and *SFTPD* are associated with other respiratory illnesses [65, 66] (Table 2). To date, none of these polymorphisms have been investigated with respect to H5N1 susceptibility.

3.2.2. Innate Immune Signalling. Induction of an innate immune response following infection can occur as a result of the activation of pattern recognition receptors (PRRs), commonly known as Toll-like receptors (TLRs). TLRs recognize many elements of foreign pathogens, including LPS, flagellin, and dsRNA, and initiate signaling cascades that result in the production of type I interferons. Accumulating data suggests that genetic variation in TLRs and their associated signaling components modulates the response to TLR ligands and, consequently, the inflammatory immune response [67]. TLR3 is constitutively expressed on lung alveolar and bronchial epithelial cells and has been shown to contribute to the secretion of multiple cytokines following influenza A infection [68]. Given the data suggesting that H5N1 pathogenicity is due in part to alterations in innate immune responses and hypercytokinemia, it is plausible that polymorphisms altering TLR function could contribute to susceptibility or protection from infection. This hypothesis is supported by a genetic study of a case of influenza-associated encephalopathy, a condition associated with apoptosis and hypercytokinemia [45]. In this case, a missense mutation (908T/C) in the TLR3 gene was identified and was shown to be a loss-of-function mutation, suggesting a protective role for TLR3 signaling in severe influenza infection [45] (Table 2). Although these results are consistent with studies suggesting a protective effect of TLR3 in West Nile infection [69], they are at odds with TLR3 null mouse studies, which have shown reduced proinflammatory cytokine production following cellular stimulation [70–72]. Consequently, the contribution of TLR genetic variants to H5N1 inflammatory responses remains to be resolved.

3.2.3. Interferon-Related Pathways. Induction of the type I interferon response during influenza infection appears to be important in both human and mouse models, as

evidenced by the increased expression of genes including *Irf1*, *Ifi202*, *Oas1*, and *Mx1* in mouse microarray studies [73–75] (reviewed in [76]). This is consistent with the observation that viral evasion and attenuation of the IFN pathway contributes to H5N1 pathogenesis in humans and suggests potential targets for genetic studies [75, 77]. A strong target for analysis includes the myxovirus resistance (*Mx*) gene, which encodes interferon-induced antiviral proteins that inhibit viral RNA transcription and consequently confer influenza resistance in mouse lines with functional *Mx1* alleles [78]. Polymorphisms in swine *Mx* genes have also been associated with influenza susceptibility [79] and multiple SNPs in the human *MxA* gene have been associated with variability in IFN responsiveness in Hepatitis C infection [80] and SARS susceptibility [38–40] (Table 2). Specifically, the −123C/A promoter SNP associated with SARS protection correlates with increased basal MxA expression, leading the authors to speculate that −123 genotype may be an important determinant of H5N1 susceptibility [39]. Although human MxA protein has been shown to inhibit influenza replication [81], no studies have looked for an association between *MxA* SNPs and H5N1 disease outcome or susceptibility. Because *MxA* is located on chromosome 21, studies have compared susceptibility to respiratory infections between wild-type and trisomy 21 patients (who exhibit increased MxA expression), but found greater susceptibility among the trisomy 21 group [82]. This group of patients is known to suffer from a multilevel T-cell dysfunction, however, making the role of MxA in respiratory immunity somewhat unclear.

Polymorphisms in *OAS1* (2′,5′-oligoadenylate synthetase 1; an interferon-induced antiviral protein) have also been associated with SARS susceptibility and progression [38, 40] and West Nile infection [41]. Consistent with the idea of increased H5N1 susceptibility and pathogenesis associated with poor IFN responses, the *OAS1* SNP rs1077467 is correlated with reduced OAS-1 protein activity and associated with increased susceptibility to West Nile infection and *in vitro* viral replication [41].

3.2.4. Cytokine Response. Comparison of severe H5N1 infections with uncomplicated seasonal influenza infections revealed a pattern of increased viral load and elevated cytokine production in the respiratory tract and serum [75, 83]. The robust cytokine/chemokine response often seen in H5N1 infected patients (hypercytokinemia) is believed to be at least partially responsible for the observed pathogenesis and high fatality of H5N1 infection. Elevated cytokines both *in vivo* and *in vitro* include IFNγ, sIL-2R, IL-6, IP-10, TNFα, and MCP-1 [75, 84–86]. Mouse models of H5N1 infection also demonstrate elevated levels of MCP-1, MIP-1α, IL-6, and IFNγ, even compared to 1918 H1N1 virus infection [87]. Knocking out IL-1R in mice exacerbates H5N1 pathology and suggests that IL-1β-mediated signalling may be important in protection [75]. In ferret models, IP-10 upregulation and signaling through CXCR3 was determined to be a major component of H5N1 disease severity and mortality [88]. Expression of many of these chemokines and cytokines in humans is modulated by SNPs in their promoter regions, including MCP-1 −2518 G/A [89], IP-10 −201G/A [90], and IL-6 −174G/C [91]. Genetic variants affecting expression and function of chemokine receptors may also modulate influenza pathogenesis, as CCR5 knock-out mice exhibit increased influenza mortality, whereas CCR2 knock-out strains show increased survival (Table 2); both of these effects appear to be related to the kinetics and strength of macrophage recruitment to the lung [42]. *In vitro* evidence further suggests upregulation of CCR5 on monocyte-derived macrophages that may enhance pathogenesis [92].

4. Conclusions

Although relatively few studies have systematically evaluated the influence of genetic polymorphisms on susceptibility and disease severity in zoonotic H1N1 and H5N1 infections, the data available suggest that host immunogenetic variation could play an important role in determining the outcome of the immune response. With improvements in surveillance and case confirmation as well as new sequencing and gene expression platforms, we now have the capability to study host genetic variants among severe respiratory illness cases. Although several challenges to conducting such a study include ethical permission to carry out genetic polymorphism studies, the need for large numbers of well-characterised clinical specimens with relevant clinical data, difficulty to obtain sufficient number of samples from severe and fatal cases at a single institution, and difficulty in identifying mild controls. The extreme cases of human H5N1 disease are very rare, sporadic, with scattered cases in different countries, adding economic and political sensitivities associated with this disease. Overcoming these barriers and conducting collaborative research can lead to insights that will shed light on the varying degree of susceptibility observed between populations during the recent H1N1 pandemic and will provide greater insight into the host-pathogen interactions that determine disease course during severe H5N1 infection.

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Review Article

Host-Parasite Relationship in Cystic Echinococcosis: An Evolving Story

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The larval stage of *Echinococcus granulosus* causes cystic echinococcosis, a neglected infectious disease that constitutes a major public health problem in developing countries. Despite being under constant barrage by the immune system, *E. granulosus* modulates antiparasite immune responses and persists in the human hosts with detectable humoral and cellular responses against the parasite. *In vitro* and *in vivo* immunological approaches, together with molecular biology and immunoproteomic technologies, provided us exciting insights into the mechanisms involved in the initiation of *E. granulosus* infection and the consequent induction and regulation of the immune response. Although the last decade has clarified many aspects of host-parasite relationship in human cystic echinococcosis, establishing the full mechanisms that cause the disease requires more studies. Here, we review some of the recent developments and discuss new avenues in this evolving story of *E. granulosus* infection in man.

1. Introduction

Human immune system has evolved specialized mechanisms and cell populations to protect us from the full spectrum of pathogens that poses very different problems for the immune system. Helminthes have developed complex evasion strategies and, when the immune response falls short, it may be necessary for the host to enter a damage limitation state, accommodating infection in order to minimize pathology. Parasite immune evasion mechanisms themselves depend on a form of molecular dialogue between pathogen and host and, in turn, many parasites depend on host molecular signals for their development [1].

During cystic echinococcosis (CE) the host-parasite relationship is interactive and the outcome of infection depends on the balance achieved by the combination of the different variables involved with the host immunity and the *E. granulosus* avoidance strategies [2]. An understanding of the biological events occurring during infection is necessary to visualize the diverse immune stimuli to which the parasite subjects the host and to define diagnostic and therapeutic tools. We discuss in detail these topics in this review.

2. *E. granulosus* Epidemiology

CE, a chronic endemic helminthic disease caused by infection with metacestodes (larval stage) of the tapeworm *E. granulosus*, is one of the most widespread zoonotic diseases in humans in both developing and developed countries [3]. Recently, the World Health Organization included echinococcosis as part of a Neglected Zoonosis subgroup for its 2008–2015 strategic plans for the control of neglected tropical diseases [4, 5]. The distribution of *E. granulosus* is worldwide, with only a few areas such as Iceland, Ireland, and Greenland believed to be free of autochthonous human CE [6]. CE is prevalent in countries of the temperate zones, including South America, the entire Mediterranean region, Russia, central Asia, China, Australia, and parts of Africa [3, 7–9]. In the USA, most infections are diagnosed in immigrants from countries in which echinococcosis disease

is endemic. Sporadic autochthonous transmission is currently recognized in Alaska, California, Utah, Arizona, and New Mexico [10].

E. granulosus comprises a number of forms that exhibit considerable genetic variation [11]. Ten strains of *E. granulosus* (G1–10) have been described with molecular biology techniques using mitochondrial DNA sequences [12]. These include the common sheep strain (G1), a Tasmanian sheep strain (G2), two bovine strains (G3 and G5), a horse strain (G4), a camel strain (G6), a pig strain (G7), a cervid strain (G8), a Poland swine strain (G9) [12], and an Eurasian reindeer strain (G10). Recent molecular re-evaluation of *Echinococcus* species strongly suggests that *E. granulosus* is an oversimplified species. The genotypes G1 to G5 have been reclassified into *E. granulosus sensu stricto* (G1 to G3), *E. equinus* (G4), and *E. ortleppi* (G5). The genotypes G6 to G10 and the lion strain of *E. granulosus* (formerly *E. felidis*) have to be re-evaluated [13]. The sheep strain (G1) has a worldwide geographical distribution, specifically it is widely spread in North Africa and has a natural circulation in some European countries such as Italy [14]; this strain is commonly associated with human infections. G2 strain is geographical distributed in Tasmania and Argentina but there are recent observations of the emergence of the presence of this strain also in some endemic European countries as Bulgaria, Italy, France, Portugal, and Spain. G3 strain has a major distribution in Asia and in more endemic European countries. The "cervid" genotype (G8) cycle involves wolves, dogs, mooses and reindeers [12].

Despite the increasing epidemiological reports, available information on CE is still incomplete and is insufficient to assess properly its world epidemiology. CE importance tends to be underestimated due to underreporting and to the lack of compulsory notification. To note, the reporting of incidental cases is mandatory in most of the EU member countries except Denmark and Italy [15]. These facts strongly recommend the convenience of maintaining and/or intensifying the control measures currently in place in order to consolidate the progress achieved and to avoid the recrudescence of the disease.

3. *Echinococcus* Metacestodes

3.1. E. granulosus Biology. The complex cycle of the parasite can explain the intricate host-parasite relationship. *E. granulosus* is a small tapeworm (rarely exceeding 7 mm in length) that lives firmly attached to the mucosa of the small intestine in definitive hosts, usually dogs, where the adult-stage reaches sexual maturity within 4 to 5 weeks. This is followed by the shedding of gravid proglottids (each containing several hundred eggs) and/or of released eggs in the feces of definitive hosts. After being ingested by the intermediate host, eggs release embryos (oncospheres) that penetrate the gut wall, travel via blood or lymph, and are trapped in the liver, lungs, and other sites where cystic development begins. This process involves transformation of the oncospheral stage to reach the metacestode stage.

E. granulosus typically develops as a large unilocular, turgid cyst, which grows through an increase in diameter from less than 1 to 5 cm each year. This general structure can be thought to allow a permanent low ratio between total parasite cellular volume and host-exposed area, through linear growth that can exceed three orders of magnitude. Hydatid cyst is usually surrounded by a host-derived collagen capsule (adventitial layer), but can also been circled by host inflammatory cells. Metacestode (hydatid cyst) is bounded by the hydatid cyst wall, which comprises an inner cellular layer (germinal layer) and an outer protective acellular layer (laminated layer). The germinal layer (GL) gives rise towards the cyst cavity to cellular buds that upon vesiculation become brood capsules, and in turn bud towards their inside to generate protoscoleces. The GL exposes towards the outside the apical plasma membrane of its syncytial tegument, which carries truncated microtriches. The GL has additional, nonsyncytial cell types, including muscle, glycogen-storage, and undifferentiated cells. Towards the cyst cavity, there is neither a syncytial organization nor junctional complexes between cells, so that the intercellular fluid of the germinal layer is apparently continuous with the cyst/vesicle fluid [16, 17].

In spite of being widely considered the crucial element of host-parasite interfaces, the laminated layer (LL), a structure only found in the genus *Echinococcus*, is poorly understood. In fact, it is still often called "chitinous," "hyaline," or "cuticular" layer, or said to be composed of polysaccharides. However, over the past few years the LL was found to be comprised of mucins bearing defined galactose-rich carbohydrates, and accompanied by calcium inositol hexakisphosphate deposits. A recent review discusses the architecture and biosynthesis of this unusual structure [18]. The cyst cavity is filled with hydatid cyst fluid (HCF) that is the main factor responsible for the antigenic stimulation. The hydatid liquid is clean and clear, "as well as the clean water from its natural source," containing secretions from both the parasite and host and all the elements from the "inner wall" of the cyst, named hydatid sand [19]. It has an identical composition to that of the host's serum (Na, K, Cl, CO_2, a density between 1.008 and 1.015, alkaline pH) and some specific proteins that confer antigenic properties such as Ag5 and AgB.

3.2. E. granulosus Natural History. The natural history of *E. granulosus* cysts and its clinical implications comprises various developmental stages. The initial stage, primary infection, is always asymptomatic. During this stage, small (<5 cm) well-encapsulated cysts develop in organ sites, where they persist inducing no pathologic consequences. In humans, the hydatid cysts are localized in approximately two-thirds of cases in the liver and in about 20% in the lungs, and less frequently in the kidneys, spleen, heart, and bone. Some 20–40% of patients have multiple cysts or multiple organ involvement. After an undefined incubation period lasting months or years, if cysts exert pressure on adjacent tissue and induce other pathologic events, the infection may became symptomatic. Because hydatid cysts grow slowly, the host often tolerates it remarkably well. Patients with CE may come to clinical attention only when a large cyst mechanically alters body function, when allergic phenomena or other

miscellaneous symptoms such as eosinophilia develop, or when the cyst accidentally ruptures thus triggering acute hypersensitivity reactions. Cysts or a cystic mass may also be discovered by chance during body scanning or surgery, or for other clinical complications [19]. During the outcome of the infection, several events can occur into the cyst: the death of the parasite due to dysfunction of the GL (detachment or aging), the "cyst's wall" fissure due to detachment of membranes or micro traumatisms, the transformation of scoleces into vesicles (vesiculation). These new vesicles, called offspring or "daughter" vesicles, live into the hydatid fluid and have the same constitution as well the same mission of the mother vesicle and occasionally form within larger cysts. Therefore, in this way, protoscoleces may develop into either a new cyst or an adult parasite.

The extensive variation at the genetic level may influence *E. granulosus* life development rate, cycle patterns, host specificity, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents, and pathology with important implications for the design and development of vaccines, diagnostic reagents and drugs. To note, human infection with G8 strain presents a predominantly pulmonary localization, slower and more benign growth, and less frequent occurrence of clinical complications than reported for other strain genotypes [10]. Zhang and McManus have recently extensively reviewed a detailed account of genetic variation in *Echinococcus* and its implications [20].

4. *E. granulosus* Antigens

Since the 1960s, research on CE has been focused on the identification of immunologically important proteins, especially potential immunodiagnostic or vaccine candidates. Because of the expression of different antigens during the different developmental stages, the human host responds independently to antigenic stimuli of the invading oncosphere, the metacestode in transformation from the oncosphere, and finally, the mature metacestode (larvae) [2]. *E. granulosus* immunology has been divided into an "establishment" phase during which the parasite is most susceptible to host effectors, and an "established metacestode" phase during which the parasite elicits chronic disease. In the early stages of echinococcal development, cellular responses may play a crucial role in protection against infection [21].

Older studies reported that the oncospheres stimulate a strong immunity to a challenge infection [22]. Strong antibody responses against purified oncosphere proteins have been reported also in sera from experimentally infected sheep [23]. Most recent experiments in mice showed that a second oncospheral challenge 21 days after the primary infection with *E. granulosus* produced very high levels of protection but with a very low antibody response [24]. Therefore, because the oncosphere is known to be associated with the protective immune response, understanding the mechanisms whereby protective antibodies against the oncosphere act, is of fundamental importance in developing highly effective vaccine against *E. granulosus* [25]. The results of Heath and Lawrence [23] settled the basis for the development of the Eg95 vaccine in ruminants [26, 27].

The LL, an insoluble and unusual biological structure, is the crucial element of host-parasite interface in larval echinococcosis. Because of its massive carbohydrate-rich structure and resistance to proteolysis, it contains few T-cell epitopes and abundant T-independent anti-carbohydrate antibodies. Consequently, the innate immunity induces a noninflammatory response and the adaptative immunity induces a humoral response characterized by low-avidity antibodies specific for α-galactose [28]. The history of LL represents an example of our evolving knowledge in the immunological mechanisms that *E. granulosus* takes to survive in the host. It has been fascinating to arrive at explanations for observations that lay forgotten in papers published decades back [29, 30]. In particular, in 1974, we have observed that sera from patients with pulmonary cyst localization presented antibodies against a glycoprotein antigen (α-galactosyl residue) isolated from the hydatid membrane. This antigen showed a high P_1 blood group activity thus suggesting an intriguing role for the hydatid membrane in the host-parasite relationship [30]. Later, P1 blood antigen has been also identified in protoscolex [31]. A recent review describes in depth the modern studies on the biochemistry of LL that allowed a more informed analysis of its immunology [28]. The major immunodiagnostic protein antigens are present in HCF [32]. However, if T-independent anti-carbohydrate responses are included, the laminated layer may instead be the major source of antigens [28]. The GL of the cyst is a barrier against immune competent cells of the host. It is generally thought that damages in the GL, like fissures or rupture, induce an antigenic stimulation. When this antigenic stimulation occurs, there is a continuous elevation of the immunologic values for an indeterminate time. This elevation also happens after the cyst manipulation (surgery, puncture, etc.) [33].

Extensive studies have focused on hydatid fluid antigens that still represent the main antigenic source for hydatid disease diagnosis. At the present time, despite the large number of studies, the parasitic antigens present in HCF that have major immunodiagnostic value in detecting *E. granulosus* are antigen 5 (Ag5) and antigen B (AgB) [34, 35]. Native Ag5, a 400 kDa thermolabile glycoprotein produces two subunits at 55 and 65 kDa in sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and two subunits at 38/39 and 22–24 kDa under reducing conditions [36–38]. The biological role of Ag5 is almost completely unknown, although its elevated concentration in HCF suggests a relevant function in the development of the metacestode. The 38/39 kDa component with phosphorylcholine epitopes may be responsible for a large proportion of cross-reactions with sera from patients infected with nematodes, cestodes, and trematodes [36–39]. The 38 kDa subunit is closely related to serine proteases of the trypsin family, but has no detectable proteolytic activity [40]. Studies by sequencing of the N-terminal fraction of the 38 kDa subunit revealed a single amino acid sequence with alternative residues at some positions, demonstrating that Ag5 is present in different isoforms [41]. Regarding the 22 kDa subunit, the heparan sulphate proteoglycans and calcium-binding sites found in this component seem to

provide binding targets for the Ag5 molecule [40]. These would target the antigen and ensure its localization in the host tissue surrounding the metacestode, or otherwise, the mucosal epithelium of the *E. granulosus* definitive host. Ag5 has been widely used in the serodiagnosis of human CE, particularly by means of the identification of a precipitation line (arc 5) in immunoelectrophoresis assays [32]. González-Sapienza et al., identified and cloned a metacestode-specific component (named P29) immunologically related to, but distinct from, Ag5 [42]. This finding would imply that much of the information derived from studies carried out using antibodies to Ag5 could be equivocal because of the cross-reactivity between both Ag5 and P29 [43].

Native AgB, a 160 kDa thermostable lipoprotein, produces three main subunits at 8/12, 16, and 20 kDa in SDS-PAGE under reducing and nonreducing conditions as well as other mass subunits, probably polymers of the 8/12 kDa subunit [44]. The 8/12 kDa subunit induces a good humoral and cellular response [45]. Even though the 8/12 kDa subunit of AgB is cross-reactive in a high percentage of patients with alveolar echinococcosis sera and in a small percentage of patients with cysticercosis, native AgB is of high immunodiagnostic value [32, 39, 46]. The oligomeric organisation of the *E. granulosus* AgB (EgAgB) was further investigated by González et al. [47], who analysed the subunit composition of EgAgB in HCF by comparing the amino acid sequence of tryptic peptides isolated from the 8, 16, and 24 kDa subunit bands of native EgAgB with that of the 8 kDa subunit monomers and found that the 8 kDa band contained at least two components, which constituted the building blocks of the higher molecular weight subunit bands. Further progress towards characterising AgB came from experiments using DNA cloning [44, 48, 49]. Using this technique, Shepherd et al. [50] reported a cDNA clone encoding the carboxy-terminal of the 12 (8) kDa subunit of antigen B and Frosch et al. [51] described its complete sequence (AgB/8 or EgAgB8/1). Nucleotide variations are present at a conserved position between AgB/8 cDNA sequences from different isolates, indicating that this gene is polymorphic. Others later isolated a cDNA clone coding for a second 8 kDa subunit of AgB (EgAgB8/2) [52]. Specific antibodies against both antigens recognized all AgB bands in western blot, and peptide sequencing revealed that both antigens are components of the native AgB subunits [47]. Together these results show that AgB is made up of subunits encoded by at least two different genes. Molecular studies now show that *E. granulosus* AgB is encoded by a multigene family having at least five gene loci (B1–B5), each one consisting of several minor variants that phylogenetic tools grouped into two clusters: EgAgB1/B3/B5 and EgAgB2/B4 [51, 53–56]. A more recent phylogenetic analysis failed to discriminate between the isoforms EgAgB3 and EgAgB5 [57]. The putative protein isoforms encoded by the five EgAgB genes differ in amino acid sequence (44–81%). Switching from one isoform to another could be among the mechanisms parasites use to evade the host's immune response and to modulate periparasitic inflammatory reactions [55]. Recently, Muzulin et al. showed that *E. granulosus* strains differ in the type of genomic and transcribed EgAgB sequences, reinforcing previous evidence that the AgB gene family is highly polymorphic [49]. How this variation affects the way each strain adapts to its specific intermediate host, and whether it influences AgB's potential as a diagnostic tool remain matters for future studies**.** In contrast with previous data, showing that *E. granulosus* strains differ in the types of genomic and transcribed EgAgB sequences, Zhang et al. found that the EgAgB gene family comprises at least ten unique genes, each of them was identical in both larval and adult *E. granulosus* isolates collected from two different continents [58]. DNA alignment comparisons with EgAgB sequences deposited in GenBank databases showed that each gene in the gene family is highly conserved within *E. granulosus*, which contradicts previous studies claiming significant variation and polymorphism in EgAgB. Quantitative PCR analysis revealed that the genes were differentially expressed in different life-cycle stages of *E. granulosus* with EgAgB3 expressed predominantly in all stages. Finally, Chemale et al. [59] characterising the properties of native EgAgB to bind hydrophobic ligands and comparing the activity of two of the 8 kDa subunit monomers (rEgAgB8/1 and rEgAgB8/2), found that the hydrophobic ligand binding properties of EgAgB differ from the helix-rich hydrophobic ligand binding properties displayed by proteins from other cestodes. Because many of these proteins are immunogenic and some are involved in lipid detoxification, transport, and metabolism with their fatty acid binding properties, AgB could be involved in the process of parasite survival in host microenvironment.

Similar to *E. granulosus*, AgB also exists in the cyst fluid of *E. multilocularis* and AgB genes are expressed in a developmentally regulated manner in *E. multilocularis* vesicles, protoscoleces, and immature adult worms [44].

In the 1990s it had become apparent that the new techniques in molecular biology offered a new approach to overcome some problems and several recombinant antigens have been produced and used as molecular tools in the immunodiagnosis of CE [60] (Table 1). In a series of molecular studies, we screened an *E. granulosus* cDNA library with IgE from patients with CE who had acute cutaneous allergic manifestations and we identified three conserved constitutive proteins: EgEF-1 β/δ, EA21, and Eg2HSP70 [61–64].

Later, we screened an *E. granulosus* cDNA library with IgG4 from patients with active disease and with IgG1 from patients with inactive disease. By screening with IgG4 from patients with active disease, we obtained two proteins. The first is present on the protoscolex tegument and on the GL of cyst wall (EgTeg) and the second protein has 19.0 kDa (Eg19) [65, 66]. By screening the *E. granulosus* cDNA library with IgG1 from patients with inactive disease, we obtained EgTPx [67].

5. *E. granulosus* and Antibody Responses

There are extensive data on immune responses against the hydatid cyst both from studies on patients with *E. granulosus* infection and from experimentally infected animals [22]. The established parasite produces significant quantities of

Table 1: Main *Echinococcus granulosus* antigenic molecules identified and characterized, and/or recombinantly expressed.

Antigen	Name	References
Antigen 5	Ag5	Capron et al. [36]
Antigen B	AgB	Lightowlers et al. [35]
Echinococcus granulosus 29 kDa	P-29	Gonzáles et al. [42]
Echinococcus granulosus paramyosin	EG36	Mühlschlegel et al. [68]
rEgG5	rEgG5	Lightowlers et al. [26]; Li et al. [69]
Thioredoxin peroxidase	TPx	Salinas et al. [70]; Margutti et al. [67]
EgA31	EgA31	Fu et al. [71]
Elongation factor 1β/δ	EgEF-1 β/δ	Margutti et al. [61]
Cyclophilin	EA21	Ortona et al. [63]
EpC1	EpC1	Li et al. [72]
Tropomyosin	Trp	Esteves et al. [73]
Heat shock protein 70	HSP70	Ortona et al. [64]
Echinococcus granulosus Tegumental antigen	EgTeg	Ortona et al. [65]
Eg19	Eg19	Delunardo et al. [66]
Heat shock protein 20	HSP20	Vacirca et al. [74]

molecules that modulate the immune responses and these include both humoral and cellular immune response against the parasite.

Although the data are limited, there is, nevertheless, clear evidence from experiments with animals challenged with *E. granulosus* eggs or oncospheres that infected hosts produce significant immune responses, including antibodies and T cell responses generated by lymphocytes.

The earliest IgG response to oncospheral antigens appears after 11 weeks in mice and sheep challenged with eggs or oncospheres of *E. granulosus* [23]. These anti-oncospheral antibodies play a major role in parasite killing and are central to the protective immune response against *E. granulosus*.

Numerous studies demonstrated that *E. granulosus* HCF induces a strong humoral response in humans. Even if sera from patients with CE contain abundant circulating IgG, IgM, and IgE antibodies to *E. granulosus* antigens, none of these antibodies is associated with protection [75]. Because IgG antibodies, that retain floating levels for many years even after "cure," cannot be considered as immunological markers of the outcome of therapy, the analysis of IgG subclass, that vary during the outcome of the disease, has been considered for a long time useful in follow-up [18]. In contrast with these results, we demonstrated that the expression of the various IgG isotypes remained practically unchanged over a long-term follow-up, but antibody levels before therapy differed in the patients grouped according to the outcome of chemotherapy. IgG isotype expression differed also in its HCF and AgB binding profiles. Hence, although IgG isotypes cannot be considered as immunological markers of the outcome of chemotherapy, we concluded that they might be a useful guide to the clinical management of CE [76]. Recently Pan et al. demonstrated that because the expression of AgB2 declines with progression of the disease, this antigen is a suitable immunological marker for detection, diagnosis, and progression of the disease [77]. Given that the first studies of IgG subclass antibody responses in advanced human CE indicated a switch from predominant IgG1 response to IgG4 in CE patients with progress disease, the peculiar role of IgG4 during CE has been extensively studied and IgG4 actually are considered as immunological markers during CE [78]. IgG4 is a subclass associated with prolonged, chronic infection, that is neither cytophilic nor complement fixing, is nonfunctional, and binds weakly to receptors for the Fc portion of immunoglobulins, it may help the parasite to evade the host immune response [79]. Moreover, parasite-specific IgG4 antibodies can inhibit IgE-mediated degranulation of effector cells reducing allergic pathology in the host [80]. In agreement with these studies, we found that albendazole-treated patients, who exhibited a good therapeutic and clinical response to treatment, had significantly lower levels of serum IgG4 antibodies, than poor responders or nonresponders whereas IgG1 antibody levels showed a reverse trend [81, 82]. Later we confirmed the presence of higher IgG4 and IgE in patients with progressive disease and higher IgG1 and IgG3 in patients with stable disease [76].

6. *E. granulosus* and Cytokine Induction

A key question is how *E. granulosus* that encounter the immune system can influence the differentiation decision. Th1 and Th2 cells are not precommitted phenotypes but rather, represent endpoints of a multistep differentiative process, whereby a common precursor population acquires a distinct cytokine secretion profile [83]. During CE, the evidence concerning antibody levels of IgG4 and IgE isotypes and frequent eosinophilia, suggested that the immune response to established *E. granulosus* infection is Th2 dominated and that *Echinococcus* antigens modulate polarized T-cells. Immunological studies conducted in our laboratory,

showing high *in vitro* production of parasite antigen-driven IL-4, IL-5, IL-6, IL-10, and IFN-γ by peripheral blood mononuclear cells (PBMC) isolated from patients with CE, confirmed that the human immune response to *E. granulosus* infection is predominantly regulated by Th2 cell activation but also by the Th1 (or Th0) cell subset. [81, 82]. Data obtained in *E. granulosus* experimental infection supported the hypothesis that early IL-10, secreted by B cells in response to nonproteic antigens, may favor parasite-survival and the establishment of a polarized type-2 cytokine response [84]. Recent findings suggested that IL-4/IL-10 impairs the Th1 protective response and allows the parasite to survive in hydatid patients [85]. Experimental studies in mice supported the possible local immunosuppression mediated by IL-10 and TGF-β as possible mechanism that helps the parasite in escaping the host cell-mediated response [86].

To note, the probable immune-suppressing effects of TGF-β (and regulatory T cells) have been shown to be present in *E. multilocularis* experimental infection. Intraperitoneal murine *E. multilocularis* infection induces differentiation of TGF-β-expressing dendritic cells (DCs) that remain immature and modulates peritoneal $CD4^+$ and $CD8^+$ regulatory T-cell development [87].

Evidences highlighting crucial role of cytokines in the host-parasite relationship come from studies on parasite-driven cytokine production in a large number of albendazole-treated patients with CE. PBMC from patients who responded to chemotherapy produced high amounts of IFN-γ (Th1 derived) whereas PBMC from patients who did not respond produced IL-4 and IL-10 (Th2 derived). We later confirmed this finding in a molecular study by detecting IL-12 p40 mRNA in 86% of successfully treated patients at the end of chemotherapy. PBMC from patients in whom therapy failed, expressed weakly IL-4 mRNA before therapy, and strongly thereafter; PBMC from patients who responded to therapy expressed higher IFN-γ and TNF-α mRNA values than patients who did not [88]. Finally, T cell lines from a patient with an inactive cyst had a Th1 profile whereas T cell lines derived from patients with active and transitional cyst had mixed Th1/Th2 and Th0 clones [89]. Since PBMC from seronegative patients produced no parasite antigen driven-IL-5 and scarce IL-4 and IL-10, we suggested that during CE the seronegativity occurs because host or parasite factors or both preclude Th2 cell activation thus limiting or preventing production of IL-5, the cytokine that has a critical role in immunoglobulin expression [90].

Collectively our data indicated that in CE a strong Th2 response correlates with susceptibility to disease (active cyst) whereas a Th1 response correlates with protective immunity (inactive cyst) and that Th1 and Th2 responses coexist.

The role of DCs in the immunity of CE and in the host-parasite relationship has been recently evaluated. Inflammatory mediators or microbial agents promote the migration of DCs into the secondary lymphoid organs. As they migrate, DCs mature, lose their Ag-capture ability, and gain an increased capacity to prime T cells. DC-parasite interactions are pivotal in triggering and regulating parasite-induced immunity. DC function is itself modulated during parasitic infection for the mutual benefit of the host and of the parasite [91, 92]. *E. granulosus* hydatid fluid modulates DC differentiation and cytokine secretion [93]. We have demonstrated that *E. granulosus* hydatid fluid impairs monocyte precursor differentiation into immature DCs rendering them unable to mature when stimulated with lipopolysaccharides. The parasite modulates also sentinel DC maturation, priming them to polarize lymphocytes into Th2 cells [94]. Collectively, these cellular findings establish that *E. granulosus* can directly influence the components of host cellular response, T lymphocytes, and DCs.

7. *E. granulosus* and Immune-Modulating Molecules

Because *E. granulosus* inhabits immunocompetent hosts for prolonged periods it is not surprising that it should possess modulator molecules that remodel host responses to enhance its survival. AgB is the principal *E. granulosus* immune-modulant antigen [45]. Because it can modulate both innate and adaptive host immune responses, AgB plays a prominent role in the immunomodulatory mechanisms that *E. granulosus* uses to develop, progress, and cause chronic disease [2]. To survive in host tissues the parasite must be able to adapt metabolically to the host microenvironment, and plentiful AgB in HCF probably guarantees parasite survival. A large amount of data suggests that AgB directly immunomodulates the host immune response by inhibiting PBMC chemotaxis and indirectly by skewing the Th1 : Th2 cytokine ratio towards a preferentially Th2 polarization associated with chronic CE disease.

The 12 kDa subunit of AgB is a serine protease inhibitor with strong chemoattractant activity and with the ability to inhibit human neutrophil chemotaxis without altering either random migration or oxidative metabolism [50, 95]. In agreement with the negative immunomodulatory role suggested for AgB on human neutrophils, when accidentally released hydatid fluid activates neutrophils, AgB could act as an interference antigen allowing the released protoscoleces to develop into secondary cysts [96]. We investigated the role of AgB in acquired immunity by evaluating AgB-driven Th1 and Th2 cytokine production by PBMC from patients with CE [95–97]. Patients' PBMC stimulated with AgB produced IL-4, IL-13, and low IFN-γ concentrations, but did not produce IL-12. This Th2 polarization was more evident in patients with active disease, in whom the stimulus with AgB increased the imbalance observed in cultures from patients with inactive disease [89]. Finally, AgB modulates sentinel DCs maturation, priming those to polarize lymphocytes into an exclusive Th2 response that benefits the parasite (IL-4 expression). Our data offer a rationale for this polarization by showing that if AgB encounters immature DCs, it suppress IL-12p70 production by inducing the immunoregulatory cytokine IL-10. AgB reduces lipopolysaccharide-induced production of IL-12p70 but not of IL-6, providing further evidence that it actively modulates DC responsiveness in a manner favouring a Th2 outcome [94].

In a series of molecular studies, we screened an *E. granulosus* cDNA library and we identified constitutive proteins (EgEF-1 β/δ, EA21, Eg2HSP70, EgTeg, Eg19, and

EgTPx) that appear to have immunomodulant propriety. The EgEF-1 β/δ intervenes in immunomodulation because it continues to be released into the hydatid fluid after the protoscoleces degenerate; in fact, we found a higher percentage of antibodies specific against EgEF-1β/δ in patients with CE who had inactive cysts than in patients with active cysts [61, 62]. Also we found that a high percentage of sera from patients with CE without allergic manifestations had IgG4 antibodies specific to EA21 whereas patients with allergic manifestations showed IgE specific to EA21 we suggested that in CE, as in other parasitic diseases, IgG4 apparently acts to block pathogenic processes, minimizing severe pathology in the host [63]. Regarding Eg2HSP70, this antigen seems to elicit IL-4 production not through its intrinsic ability but by strengthening, the generalized Th2 polarization previously established [64].

EgTeg is an immunomodulatory molecule that, as AgB, contributes to chronic infection by inhibiting chemotaxis and inducing IL-4 and IgG4 [65]. Regarding Eg19 reactivity, the percentage of total IgG-, IgG1-, and IgG4-positive sera were significantly higher in sera from patients with active disease and cyst in multiple sites than from patients with inactive disease and cyst in the liver. Because anti-Eg19 antibody concentration decreased over the course of treatment in sera from patients with cured disease, our data, confirming the presence of antigens inducing both IgG1 and IgG4 during active CE, suggest that Eg19 might be a marker of disease status [66].

EgTPx seems to have an unclear role in immunomodulation, further researches are necessary to clarify precisely how EgTPx intervenes in immune evasion and whether anti-EgTPx antibodies can be used to counteract larval survival and development [67]. In a recent review about the *E. multilocularis* parasite-host interplay, Gottstein and Hemphill described the protein and glycoprotein composition of the laminated layer and the E/S fraction, including Em2- and Em492-antigens, two metacestode antigen fractions that exhibit immunosuppressive or -modulatory properties [98]. An important molecule is the 14-3-3 protein family, small proteins (30 kDa), described and characterized in several parasites and mostly studied in *E. granulosus* and *E. multilocularis.* In a recent review, Siles-Lucas et al. have deeply described new data about this protein and its important implications in the parasite biology and immunology in the frame of the host-parasite relationship [99].

8. New Perspectives from Proteomic

The advent of proteomics techniques, applied to the analysis of the protein content of biological fluids, has significantly improved the identification and characterization of proteins from *E. granulosus* metacestode to use as potential new diagnostic and prognostic indicators. The use of related analytical techniques also offers the opportunity to gain information on regulation, via post-translational modification, and on elucidation of the protein expression profile in different parasite stages and in different disease stages. Moreover, proteomics will certainly play an important role in the study of changes in the protein expression levels of protoscoleces in response to external factors, such as antihelmintic treatment, stress and in the comparative analysis of cysts from different hosts or between active and resting cysts [100, 101].

The lack of a complete sequenced genome and the presence of highly abundant host serum proteins prevented for long time the *E. granulosus* metacestode proteomic analysis. However, these negative factors have been at least in part compensated by the availability of a comprehensive *E. granulosus* EST database and by the use of an immunopurification approach to enrich samples with proteins from parasite origin, respectively. Therefore, the strategy of searching not only in the *E. multilocularis* EST database but also in the EST data available from other platyhelminthes allowed to extend the previously restricted overall repertoire of known proteins expressed and released by the *E. granulosus* metacestode [102].

Chemale et al. [100] reported for the first time the proteomic technique for identification of new proteins in *E. granulosus*. These authors, using a parasite-enriched fraction from a whole protoscoleces protein extract, identified and analyzed by MALDI-TOF-MS 100 prominent protein spots. They identified important proteins, such as actin, tropomyosin, paramyosin, thioredoxin reductase, antigen P-29, cyclophilin, and the heat shock proteins hsp70 and hsp20. Three different protein spots were identified as actins, and this data confirms previous results suggesting the existence of several actin genes in the *E. granulosus* genome [103].

More recently, Monteiro et al. [102] analyzed antigens of *E. granulosus* during infection of its intermediate bovine host. They used an immunoproteomic strategy joining immunoblot immune screening with proteome technologies involving 2-DE-PAGE and mass spectrometry for the identification of proteins. Parasite proteins were identified in different metacestode components (94 from protoscolex, 25 from GL, and 20 from HCF). The subsequent search for antigenic proteins by immunoblot resulted in the identification of many proteins recognized by cystic hydatid disease patient sera. As well as proteins previously identified as antigens (P-29, EgTPx, EgcMDH, HSP70, grp78, actin, calreticulin, tropomyosin, HSP20, and 14–3–3), the authors identified for the first time five antigens: enolase, GST, putative MVP protein, fructose-bisphosphate aldolase, and citrate synthase. Moreover, they found proteins that may contribute to immunoregulatory events such as paramyosin and tetraspanin, proteins contributing to the establishment of an *E. granulosus* chronic infection as AgB and EgTeg.

Aziz et al. [101] used a different proteomic approach: firstly, 1D SDS-PAGE gels were used to fractionate HCF and these were divided into thirty bands and subjected to LC-MS/MS after in-gel digestion. Secondly, a large quantity of HCF was analysed using peptide OGE and an LC-MS/MS protocol that incorporated an extended (2 h) LC step. Using these techniques, they were able to identify 130 protein constituents of HCF from three intermediate hosts of *E. granulosus*. Over forty parasite proteins were identified in HCF, the most abundant being AgB and Ag5, two known antigens. As in previous studies [91, 95], thioredoxin peroxidase and two isoforms of the low-density lipoprotein

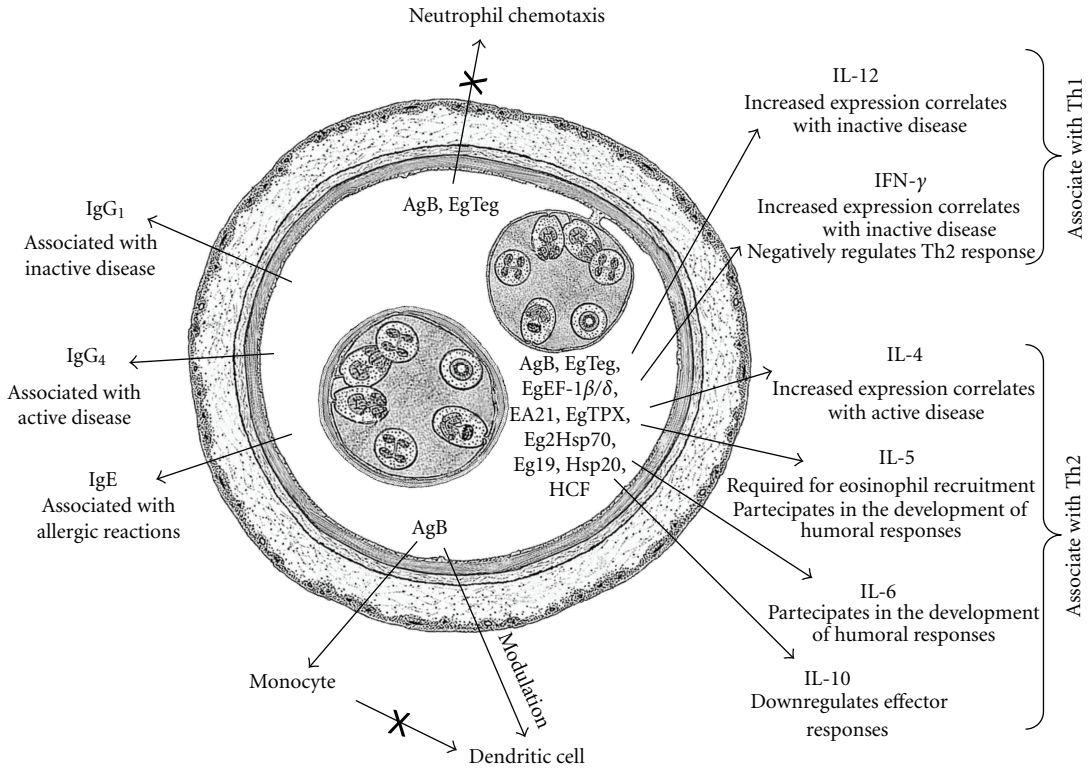

Figure 1: Major components of the immune response to hydatid cyst fluid in the host: *Echinococcus granulosus*-derived immune modulators and the main cytokines that regulate this response. Parasite-derived molecules as AgB, EgTeg, and EgEF-1β/δ could elicit a predominant Th2 activation whereas EgTPx and other HCF components can elicit a concomitant Th1/Th2 cell activation.

receptor were identified and these are likely to aid parasite survival by protecting against oxidative damage and in the uptake of sterols and fatty acids from the host, respectively. Other identifications included cyclophilin, ferritin, heat shock proteins, annexin A13, and cathepsin B.

We have exploited the classic immunoproteomic strategy to identify *E. granulosus* antigens distinctive of different stage of the disease [74]. Two-dimensional gel electrophoresis (2-DE) of HCF, followed by immunoblot analysis with sera from patients with distinct phases of disease enabled us to identify, by mass spectrometry, HSP20 as a potential marker of active CE. Immunoblot analysis revealed anti-HSP20 antibodies in a statistically significant higher percentage of sera from patients with active disease than in sera from patients with inactive disease. Anti-HSP20 antibody levels significantly decreased over the course of pharmacological treatment in sera from patients with cured disease, relative to sera from patients with progressive disease. This proteomic approach emphasizes the presence of a large number of antigenic proteins associate to parasite immune evasion during the development of the disease and highlights the difficulty in understanding the host-parasite relationship.

9. Conclusions

The hydatid cyst secretes and exposes numerous immunomodulatory molecules to the host's immune system. Throughout the past 30 years, experimental studies probing the immunobiology of *E. granulosus* have begun to uncover an evolving story in which parasite immunomodulating proteins actively interact with innate and adaptative human immune processes to reduce the impact of a host response (Figure 1). The natural history of cyst development indicates that each cyst is a story in itself and that significant efforts must be made to establish markers of cyst viability and of nature and intensity of immune response.

Clinical proteomic looks like one of the most conceptually and scientifically sound ways of generating and exploiting new biological insights and technologies for the benefit of patients. Alternative strategies, such as generation of multiplex quantitative immunoassays, may need to improve diagnosis, classification, prediction of treatment response, and prognosis of CE disease. In conclusion, the *E. granulosus* story is not over yet, but continues.

Conflict of Interests

The authors reveal no possible conflict of interests in the submitted paper.

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Review Article

Host Cell Autophagy in Immune Response to Zoonotic Infections

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Autophagy is a fundamental homeostatic process in which cytoplasmic targets are sequestered within double-membraned autophagosomes and subsequently delivered to lysosomes for degradation. Accumulating evidence supports the pivotal role of autophagy in host defense against intracellular pathogens implicating both innate and adaptive immunity. Many of these pathogens cause common zoonotic infections worldwide. The induction of the autophagic machinery by innate immune receptors signaling, such as TLRs, NOD1/2, and p62/SQSTM1 in antigen-presenting cells results in inhibition of survival and elimination of invading pathogens. Furthermore, Th1 cytokines induce the autophagic process, whereas autophagy also contributes to antigen processing and MHC class II presentation, linking innate to adaptive immunity. However, several pathogens have developed strategies to avoid autophagy or exploit autophagic machinery to their advantage. This paper focuses on the role of host cell autophagy in the regulation of immune response against intracellular pathogens, emphasizing on selected bacterial and protozoan zoonoses.

"Let your food be your medicine and your medicine your food"
—Hippocrates, 460-377 BC

1. Introduction

The term autophagy etymologically originates from the Greek "auto", meaning oneself, and "phagy", meaning to eat. Macroautophagy (hereafter simply referred to as autophagy) is a dynamic biological process in which various cytoplasmic targets are sequestered within double-membraned vesicles, called autophagosomes, and subsequently delivered to lysosomes for degradation. It constitutes an evolutionarily conserved, intracellular mechanism between all eukaryotes for the maintenance of cellular homeostasis. Although, at basal, constitutive level, autophagic activity is usually low, it is markedly upregulated in response to cell stress, nutrient starvation, and immunological stimuli [1, 2].

Recently, substantial evidence demonstrates the pivotal role of autophagy in host defense against infections implicating both innate and adaptive immunity. In particular, the induction of the autophagic machinery in macrophages is an important innate immune mechanism resulting in inhibition of survival and direct, through degradation (xenophagy) or indirect, *via* formation and release of antimicrobial peptides, elimination of various intracellular pathogens [3]. Ligation of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) results in the activation of autophagy [4, 5]. Conversely, autophagy contributes to the delivery of PAMPs to endosomal PRRs indicating a bidirectional relationship between autophagy and innate immune receptors [6]. In addition, several cytokines and reactive oxygen species (ROS) that are released during the immune response to infection have been found to trigger the autophagic process [5, 7, 8].

Notably, autophagy contributes to antigen processing and facilitates major histocompatibility complex (MHC) class II and probably I presentation, linking innate to adaptive immune mechanisms [1, 9]. Additionally, autophagy indirectly influences the adaptive immunity against microbes by regulating the development and survival of lymphocytes [1, 9]. Finally, autophagy is an effector of Th1 immune

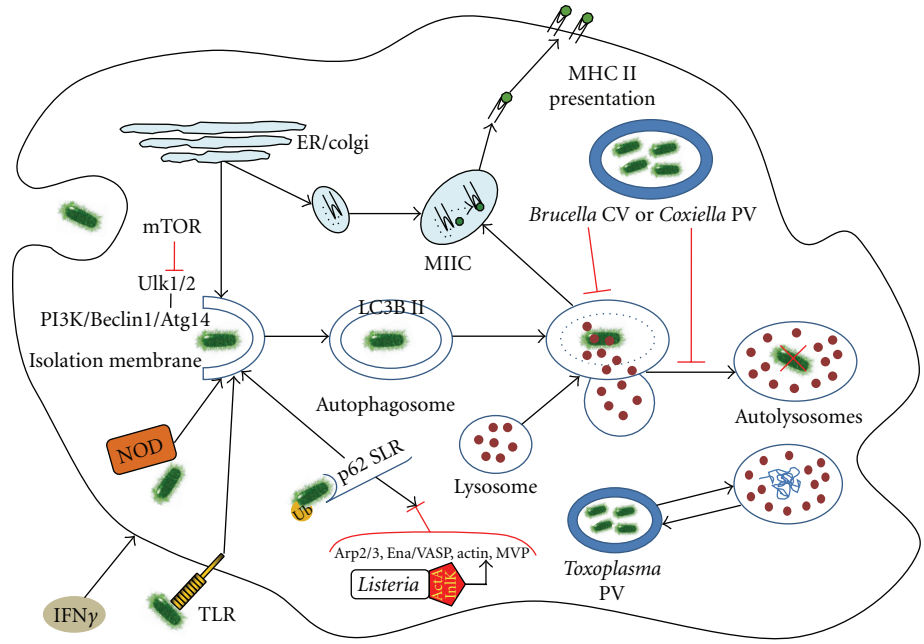

Figure 1: The interplay between autophagy and intracellular pathogens. Pathogen engulfment by antigen presenting cells (e.g., macrophage) and PRRs (TLR, NOD, p62) signaling induce the initiation of autophagic machinery (Ukl1/2 and PI3 K-Beclin1-Atg14 complex) and the formation of autophagosome (LC3B II), resulting in pathogen elimination by autolysosomal degradation (e.g., *Salmonella*). Th1 immune rensponse (IFNγ) further enhances the autophagic process. In parallel, autophagic pathway intersects the endosomal network and targets microbial antigens of phagocytosed pathogens to MHC II loading compartment (MIIC), promoting endogenous MHC II antigen presentation. Zoonotic intracellular pathogens juxtapose different mechanisms to manipulate autophagy aiming to survival and chronic parasitism, such as block (e.g., *Brucella*) or delay (e.g., *Coxiella*) of autophagolysosomal fusion, inhibition of the initiation of the autophagic machinery (e.g., cytoplasmic *Listeria*) and induction of autophagy in order to receive nutrition supplies (e.g., *Toxoplasma*). Red lines indicate negative effect. ER; endoplasmic reticulum, Ub; ubiquitin, CV; containing vacuoles, PV; parasitophorous vacuoles.

response, which is critical for the eradication of many intracellular microbes [7].

On the other hand, several intracellular pathogens have developed diverse evasion strategies against autophagy or exploit autophagic machinery, aiming to establish an intracellular niche for long-term survival and replication [10]. Many of these pathogens are responsible for common zoonotic infections, representing an important cause of morbidity and mortality worldwide. This review summarizes the role of host cell autophagy in the regulation of immune response against intracellular pathogens, emphasizing on bacterial and protozoan zoonotic infections.

2. Basics of the Autophagic Molecular Pathway

The autophagic pathway is unreeled in three principal stages: initiation, elongation, and maturation. Yeast genetic studies have identified more than 30 autophagy-related genes (*ATG*), which are responsible for the triggering and regulation of autophagic machinery, although the mammalian homologs are not completely identified yet [7].

At the initiation stage, the autophagosome begins to form as isolation membrane (phagophore), originating from rough endoplasmic reticulum or probable by other membrane sources such as Golgi apparatus, mitochondria, plasma, or nuclear membrane [2, 3] (Figure 1). Atg1 (Ulk1/2 for mammals) induces this step in cooperation with a key molecular complex that constitutes class III phosphatidylinositol 3 kinase (PI3 K) hVPS34 in association with Beclin 1 (homolog of Atg6) and Atg14. Mammalian target of rapamycin (mTOR) protein kinase is thought to be the master endogenous regulator of autophagy. mTOR is coupled to Ulk1/2 complex (Ulk1/2-Atg13-FIP200-Atg101) inhibiting the induction of autophagic machinery in a nutrient-dependent manner (Figure 1). Upon nutrient/energy starvation, mTOR dissociates from the Ulk1/2 complex, which translocates at early, preautophagosomal structures exerting its inductive effect. Rapamycin is a well-characterized inhibitor of mTOR and is commonly used for the *in vitro* induction of autophagy [11]. In addition, the binding of Bcl-2 antiapoptotic protein to Beclin 1 disrupts the association of Beclin 1 with hVPS34, leading to the inhibition of autophagy [12].

During the next step of elongation, isolation membrane enlarges and closes to form the double-membraned autophagosome that enwraps the cytoplasmic target (Figure 1). This process is regulated by two ubiquitin-like conjugation systems that are activated by Atg7, which is essential for both of them: (a) the Atg16/Atg5-Atg12 complex, which results from the Atg16 (Atg16L in mammals) in association with the Agt5-Atg12 conjugate and (b) the LC3B system which is

the human homolog of yeast Atg8. Specifically, Atg16/Atg5-Atg12 complex acts as E3-like enzyme of the ubiquitin system and induces the LC3B I lipidation with phosphatidylethanolamine, resulting in LC3B II generation. LC3B-II-lipidated protein is translocated at nascent autophagosomal membrane facilitating its growth, expansion, and closure [2, 11] (Figure 1). Finally, after autophagosomal maturation to autolysosome, LC3B undergoes lysosomal degradation. Thus, LC3 II is a tracker of autophagosomes and the conversion of LC3 I to LC3 II is a widely used marker to monitor autophagic activity [13].

However, it has been recently suggested an Atg5/Atg7-independent alternative pathway of the mammalian autophagy that seems to be regulated by Ulk1and Beclin 1 and generates autophagosomes in a Rab9-dependent manner by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes [14].

Maturation represents the final, degradative, step of autophagic molecular pathway when the autophagosomes loose the inner of the two membranes and fuse with late endosomal/lysosomal organelles, to form autolysosomes. Autolysosomes are single-membraned, acidic, vacuoles assigned to degrade sequestered material by lysosomal hydrolases [11] (Figure 1). Maturation depends on the molecular complex consisting of hVPS34—Beclin 1 in association with UVRAG (VPS38). UVRAG is a positive regulator of autophagic maturation activating Rab7 GTPase, a key element for the biogenesis and maintenance of the lysosomal compartment [15, 16]. Noteworthy, during maturation autolysosomes can also fuse with antigen processing and major histocompatibility complex (MHC) class II loading compartments, supporting MHC-II restricted endogenous antigen presentation (Figure 1) [17].

3. Autophagy and Immune Response to Intracellular Pathogens

The interplay between intracellular pathogens and host immune system is critical for the development of chronic parasitism or infection clearance. Five years ago, the term "immunophagy" was introduced to cumulatively describe the contribution of autophagic machinery to all aspects of immunity. In fact, autophagy possesses regulatory and effector role influencing immune response against intracellular pathogens in many different ways [18]. The established role of autophagy as an *in vivo* defense mechanism against intracellular bacteria and protozoa has been demonstrated by studies using Atg5 knockout mice infected by *Listeria* and *Toxoplasma*, two well-characterized zoonotic pathogens [19, 20].

3.1. Antigen-Presenting Cells, PRRs, and Autophagy. Macrophages of the reticuloendothelial system are activated by autophagy (autophagic macrophage activation, APMA) in order to properly modulate intracellular microenvironment and combat the invading pathogens [10]. Autophagic elimination of intracellular microbes by APMA implicates two main ways: xenophagy and release of neoantimicrobial peptides [3, 10].

The best studied and well characterized is xenophagy, where microbes undergo direct degradation by autolysosomes. In contrast to nonselective or bulk autophagy that induced by nutrient deprivation or rapamycin, xenophagy involves autophagic adaptors/receptors for selective degradation of foreigner invaders [21]. This process is triggered by innate immunity receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding, oligomerization-domain-(NOD-)like receptors (NLRs), following the detection of various PAMPs on cellular surface or into cytosol (Figure 1). Almost all members of the TLR family are thought to be directly or indirectly involved in the initiation and regulation of autophagic machinery against intracellular pathogens [3]. In most of these studies, the model of mycobacterial infection has been used. For example, TLR4 stimulation by lipopolysaccharide (LPS) induces autophagy in macrophages enhancing mycobacterium colocalization with the autophagosomes [22]. It seems that TLR4 signaling mediates the recruitment of Beclin-1 through dissociation of Bcl-2 inhibitor, promoting autophagy [23]. Moreover, TLR2/1 signaling regulates antibacterial autophagy pathway through functional vitamin D3 receptor activation and cathelicidin expression [24], while induction of autophagy in BCG-infected macrophages by TLR7 ligands results in pathogen elimination in a MyD88-dependent manner [25]. Of note, it is suggested that autophagy induction downstream of TLRs activation is balanced by the inhibitory effect of NF-κB [26], although this matter seems to be under controversy recently [27].

On the other hand, autophagy acts upstream to PRRs and mediates the delivery of microbial sensors to cytosolic receptors. This process is probably related to viral infections, given that ssRNA recognition of endosomal TLR7 and production of interferon-α (IFNα) by plasmacytoid dendritic cells are suggested to be autophagy dependent [6].

Recent evidence also links bacterial sensing by cytoplasmic NLRs with the induction of autophagy. It is proposed that NOD1/2 signaling recruits Atg16L1 to plasma membrane at the sites of bacterial entry [28]. Dendritic cells from individuals with Crohn's disease that express NOD2 or Atg16L1 risk variants perform defective autophagy [29]. Interestingly, Atg16L1 polymorphism has been recently associated with an excessive production of IL-1β and IL-6 in humans, further indicating the implication of autophagy in the pathophysiology of Crohn's disease [30]. Together these findings support a potential role of food-borne enterobacterial infections in Crohn's disease's pathogenesis.

Sequestosome-like receptors (SLRs) represent a new group of cytoplasmic PRRs, which serve as adaptors for selective autophagy. In particular, SLRs contain an LC3 interacting region (LIR), commissioned to recognize and capture ubiquitin-coated intracellular microbes or microbes-containing compartments for xenophagy (Figure 1). SLRs contribute to xenophagy against zoonotic bacteria such as *Salmonella* and *Listeria* [2].

Besides the active role of xenophagy in host defense as a "microbial killer", several lines of evidence also indicate its regulatory role as an "immune recognition enhancer" of host infected cells via the generation of antigenic microbial

peptides. It is well established that autophagic pathway intersects the endosomal network and targets microbial antigens of phagocytosed pathogens to MHC II loading compartment, promoting MHC II presentation to CD4+ T-lymphocytes (Figure 1) [17]. Dendritic cells that lack key autophagy proteins such as Atg5, Atg7, or Atg16L1 are characterized by disturbances of the MHC II presentation pathway [9]. Studies of *Mycobacterium* have demonstrated that rapamycin-induced autophagy enhances MHC II presentation by mouse dendritic cells and increases antigen specific CD4+ T-cells [31]. In addition, NOD2-mediated autophagy is required for the generation of MHC II antigen-specific CD4+ T cell responses in human dendritic cells [29]. Interestingly, recent studies, albeit limited, suggest the implication of autophagic machinery to MHC I presentation of phagocytosed pathogens [32, 33]. These results support the speculation that autophagy facilitates antigenic cross-presentation process, which is critical in promoting CD8+ T-cell responses to bacteria and virus [34].

In another way of autophagic clearance, antimicrobial peptides are generated, *via* a process that implicates p62/SQSTM1 SLR. Ribosomal proteins and ubiquitin are delivered to proteolysis in autolysosomes where they are proteolytically converted into potent neoantimicrobial peptides (cryptides), further reinforcing host immune arsenal [3]. These peptides exert their antibacterial activity following the fusion of autolysosomes with parasitophorous phagosomes. Although this microbicidal mechanism has been demonstrated for *M. tuberculosis*, it is also probably related to various other intracellular bacteria like *L. monocytogenes*, *S. typhimurium* [35].

Moreover, during the APMA, phagocytosis and autophagy pathway are interconnected in a process that involves TLRs engagement and signaling. Phagocytosis constitutes a fundamental antimicrobial mechanism whereby the engulfed microbe is targeted to specialized endocytic compartments, the phagosomes, and delivered to lysosomes for degradation. Translocation of Beclin 1 and LC3 to the phagosome is related to phagosome-lysosome fusion, leading to acidification and killing of the ingested organism [36].

3.2. Cytokines and Autophagy Regulation. Several cytokines modulate autophagic mechanisms to limit intracellular pathogens replication and disturb their lifestyle. In particular, Th1 immune response is thought to be critical in host protection against intracellular pathogens, while Th2 switch has been associated with the establishment of chronicity. Of note, the principal Th1 cytokine IFNγ induces the autophagic control of *M. tuberculosis*, whereas Th2 cytokines (IL-4, IL-13) yield an inhibitory effect [37]. Experimental studies on macrophages have demonstrated the implication of activating immunity related GPTases (IRGs) in the autophagic clearance of different intracellular pathogens [3]. Apart from the induction of the autophagic machinery, IRGs also promote the expression of host defense proteins, such as the phagocyte oxidase, and antimicrobial peptides [38]. In mouse, various IRGs (Irgm1, Igrm3, Irga6) are directly induced by IFNγ (IFN-γ-inducible GTPases), conferring immunity to different intracellular infections within macrophages and animals [20, 39, 40]. In human, IRGM is the only IRG that has been identified since today [3]. Although it is not directly IFNγ inducible, it is important for the autophagic elimination of mycobacteria upon stimulation of macrophages by IFNγ [41]. Moreover, human genetic studies have demonstrated the association between IRGM single nucleotide polymorphisms (SNPs) and predisposition to tuberculosis, further underscoring the role of autophagy in intracellular bacterial infection [42–44].

TNFα is another Th1 cytokine, which strongly enhances the bactericidal activity of macrophages. TNFα has been also reported to upregulate autophagy in a ROS-dependent manner, although this effect was demonstrated in tumor cells that lack NF-κB activity [26]. In addition, TNFα stimulates p62/SQSTM1-mediated autophagic activity and restricts the survival of *Shigella* and *Listeria* [45].

Furthermore, type I interferons have been implicated in antiviral autophagic response both *in vitro* and *in vivo* [46, 47]. Moreover, treatment of macrophages with interleukin IL-1 triggers the ubiquitination of Beclin 1 and the formation of autophagosomes [48].

4. The Crosstalk between Autophagy and Zoonotic Pathogens

Previous data strongly support the role of autophagy as an immune mechanism in the defense against intracellular pathogens. However, many pathogens successfully survive and replicate inside antigen-presenting cells (macrophages, dendritic cells) using different strategies to subvert innate immunity. Some intracellular pathogens parasitize in survival-permitting special phagosomes by remodeling the intracellular compartment to prevent phagosome maturation and phagolysosome fusion. Other intracellular microorganisms escape into the cytoplasm to avoid lysosomal degradation. A third evasion mechanism includes evasion from autophagic machinery or diversion from phagosomal to autophagic pathway and manipulation of host autophagy for microbial survival and replication [49].

Hereinafter, the role of host autophagy in zoonoses is described using selected examples of common bacterial and protozoan infections.

4.1. Salmonella: Autophagy Targets Enterobacterial Pathogens with Zoonotic Potential. Worldwide, foodborne diseases, and more especially diarrhoeal diseases, constitute an important cause of morbidity and mortality. *S. Typhimurium* is one of the most virulent foodborne pathogens causing gastroenteritis in humans [50].

S. Typhimurium invades nonphagocytic cells, such as epithelial cells and localizes within a membrane-bound compartment called Salmonella-containing vacuole (SCV) where the bacterium replicates, protected from the immune system. However, some of the SCVs are damaged and cytosolic-evaded bacteria can subsequently be targeted by ubiquitin system for autophagy [51]. Knockdown of mouse embryonic fibroblast for Atg5 was associated with increased intracellular bacterial growth, suggesting a role for autophagy in preventing bacterial escape into the cytoplasm and restricting its

survival [49]. However, the *in vivo* role of xenophagy against *Salmonella* infection has been demonstrated in experimental models using autophagy defective parasites. Mutations in autophagy genes rendered these parasites susceptible to *S. Typhimurium* lethal infection, allowing intracellular survival and replication [52]. Recent studies report that the autophagic adapters p62/SQSTM1 and NDP52 are involved in the autophagic clearance of ubiquitin-coated *Salmonella* from the cytosol [53, 54]. It has been also demonstrated that phosphorylation of the autophagy receptor optineurin *via* TBK1 kinase restricts *Salmonella* intracellular growth [55].

In macrophages, *S. Typhimurium* SipB bacterial protein activates the autophagic machinery, causing autophagic cell death through the disruption of mitochondria [56]. This process might represent a host defense mechanism that destroys the bacterial intracellular cycle or, in contrast, it could be a bacterial virulence strategy [57].

4.2. Listeria: Inhibition of Autophagic Machinery Favors Survival. L. monocytogenes is a Gram-positive facultative anaerobe bacterium that causes febrile enteritis and invasive listeriosis, a disease that occurs primarily in newborn infants, pregnant women, elderly, and immunocompromised patients. Listeriosis is associated with a high mortality rate especially when complicated with sepsis and central nervous system involvement. The main route of acquisition of *Listeria* is the ingestion of contaminated food products such as raw meat, dairy products, vegetables, and seafood [58].

L. monocytogenes, in contrast to *Salmonella*, replicates in the cytoplasm after escaping from the phagosome. For this purpose, *Listeria* secretes listeriolysin O (LLO) toxin which forms pores on the phagosome membrane. Listeria has been shown to induce autophagic response in fibroblasts, epithelial cells, and macrophages in the early phase of primary infection [59, 60]. Furthermore, bacterial expression of LLO was required for autophagy induction [60, 61]. A recent study using macrophages and gene-deficient animals supports the role of IFNγ-inducible IRGs to cell-autonomous immunity and autophagy response to listerial infection [38].

However, *Listeria* has developed sophisticated mechanisms to escape from the autophagic machinery elimination. Specifically, ActA virulence protein, which is responsible for the actin-based motility of *Listeria*, has been shown to protect the bacterium from autophagic degradation. ActA promotes the recruitment of host cytosolic actin polymerization components (Arp2/3, Ena/VASP and actin) to properly mask pathogen from the recognition of ubiquitin-p62/SQSTM1-LC3 autophagic system (Figure 1), whereas ActA mutants are efficiently targeted by selective autophagy [62]. In addition, NDP52 autophagic adaptor has also been reported to target *Listeria* ActA mutant, further indicating the crucial role of ActA in resistance to the autophagic machinery [45].

Noteworthy, a second "camouflage" strategy has been recently demonstrated in *L. monocytogenes* murine infection. *Listeria* internalin InlK recruits major vault protein (MVP), a mammalian cytoplasmic protein, which disguises intracytosolic bacteria from ubiquitination and autophagic recognition promoting survival [63] (Figure 1).

4.3. Brucella: Block of (Auto)Phagolysosomal Fusion for Chronic Parasitism. Brucellosis is the commonest bacterial zoonotic infection worldwide. *Brucella* infects humans by consumption of contaminated dairy products or by occupational contact with infected animals. In humans, the disease causes high clinical morbidity and protean clinical manifestations, as any organ may be affected. *Brucella* can survive and replicate for prolonged periods within host macrophages and dendritic cells, producing chronic, and even lifelong, infections. To achieve this, *Brucella* produces various virulence factors, such as smooth LPS and outer membrane proteins/lipoproteins (Omps) that modify phagocytosis, phagolysosome fusion, antigen presentation, cytokine secretion, and apoptosis [64].

Brucella containing vacuoles (BCV) are special tight phagosomes, which represent the intracellular replication compartments. The type IV secretion system (T4SS) is a membrane-associated transporter used to deliver substrate molecules to target cells. *Brucella* T4SS is crucial for the development of the BCV in host cells as it has been described to modify the bacterial intracellular trafficking [65].

The participation of autophagy in *Brucella* spp. intracellular trafficking remains a matter of controversy [66]. In the epithelial cell line HeLa, *B. abortus* can be found in autophagosomes-like BCVs, supporting the hypothesis that pathogenic *B. abortus* exploits the autophagic machinery to establish an intracellular replication niche within the endoplasmic reticulum [67]. However, autophagic BCVs were not detected in murine macrophages [68]. Moreover, studies in cultured human peripheral blood monocytes did not demonstrate any association between BCVs and rough endoplasmic reticulum or autophagosomes, even though BCVs avoid fusion with lysosomes [69] (Figure 1).

4.4. Coxiella: The Autophagolysosomal Fusion Delays to Benefit Survival. C. burnetii is an obligate intracellular pathogen that causes Q fever, a worldwide zoonose with acute and chronic stages, ranging from asymptomatic to fatal disease. Farm animals and pets are the main reservoirs of infection, although a variety of species may be infected. Infection of humans usually occurs *via* inhalation of contaminated aerosols by dried placental material, fluids, and excreta of infected animals [70].

Once *C. burnetii* is internalized by the host cell, it is localized in early phagosomes which fuse with other vesicles to form the large parasitophorous vacuoles (PV) where this pathogen multiplies [49]. Early engagement of the autophagic machinery in the PV was associated with a delay in lysosomal fusion that enables *C. burnetii* to replicate (Figure 1). This process closely depends on T4SS virulence factor, although its effectors have not been identified yet [71, 72]. In addition, recent findings indicate that *C. burnetii* infection modulates autophagy and apoptotic pathways through Beclin 1/Bcl-2 interplay to establish a persistent infection in host cell. It seems that both PV development and the antiapoptotic effect of *C. burnetii* on host cells are affected by Beclin 1 depletion and by the expression of a Beclin 1 mutant defective in Bcl-2 binding [73].

4.5. Toxoplasma and Leishmania: The Autophagic Machinery against Protozoa. Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan *T. gondii*. Toxoplasmosis is found in humans and in many species of animals worldwide. Cats are the primary source of infection to human hosts and fecal contamination of hands by *Toxoplasma* oocysts is a significant risk factor. Other routes of human infection include ingestion of undercooked meat and consumption of contaminated food or drink. The majority of primary infections produce no symptoms in immunocompetent persons; however, congenital toxoplasmosis may result in premature birth, hydrocephalus, chorioretinitis, deafness, or epilepsy [74].

Several lines of evidence indicate the role of autophagy in defense against *T. gondii*. In particular, CD40, a member of the TNF-receptor superfamily, signaling has been found to trigger autophagy, inducing macrophage anti-Toxoplasma gondii activity [75]. Moreover, Portillo et al. used *Toxoplasma* murine model to demonstrate the *in vivo* role of CD40-autophagic machinery for host resistance independently of IFN-γ. CD40 signaling upregulates Beclin 1 and triggers the elimination of *T. gondii* in microglia/macrophages by decreasing protein levels of p21, a molecule that degrades Beclin 1. These findings suggest CD40-p21-Beclin 1 as a pathway by which adaptive immunity stimulates autophagy [76]. Other animal studies also support the *in vivo* role of IFN-γ-inducible IRGs and ATg5 in autophagic host response against toxoplasma [20, 40].

Interestingly, a recent study presents evidence that Atg5-associated autophagic induction by *T. gondii* in HeLa cells and primary fibroblasts is independent of mTOR signaling, suggesting that *T. gondii* derives nutritive benefit from the upregulation of host cell autophagy to promote its intracellular growth [77]. Collectively these data probably indicate a dual role of host cell autophagy to *T. gondii* infection, acting either as a defense or as a protective mechanism (Figure 1).

Leishmania is an intracellular protozoan parasite that invades macrophages in the dermis after inoculation. Cutaneous leishmaniasis is the most common form of leishmaniasis, whereas visceral leishmaniasis is a severe form in which the parasites have migrated to the vital organs [78].

L. donovani promastigotes survive and evolve into amastigotes in phagolysosomes. Subsequently, amastigotes multiply and disseminate to the reticulo-endothelial system through vascular and lymphatic system, infiltrating the bone marrow macrophages. It is thought that the inhibition of autophagolysosome formation potentiates the survival of this parasite. Induction of autophagy by IFNγ or starvation increased *L. amazonensis* load and the percentages of infected macrophages from BALB/c but not from C57BL/6 mice, suggesting that autophagy may regulate the outcome of *L. amazonensis* infection in macrophages in a host strain specific manner [79]. Moreover, we have also reported the induction of the autophagic machinery during natural human bone marrow infection by *L. donovani* [78].

5. Conclusions

Autophagy is an important host cell defense mechanism against intracellular pathogens; many of them characterized by zoonotic potential and cause persistent/relapsing infections. The involvement of autophagy in both innate and adaptive immunity to infections is well established. On the other hand, several pathogens have evolutionary developed antiautophagic strategies or manipulate autophagic machinery for their own benefit to achieve survival and/or chronic parasitism. Further elucidation of the autophagic mechanisms implicated in the immune response or the cross talk between the immune system and pathogens is important for the discovery of biomarkers, concerning infection relapse and chronicity, as well as development of novel, autophagy-based, therapeutic approaches and vaccination strategies in livestock and humans [80].

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Review Article

New Insight into Immunity and Immunopathology of Rickettsial Diseases

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Human rickettsial diseases comprise a variety of clinical entities caused by microorganisms belonging to the genera *Rickettsia*, *Orientia*, *Ehrlichia*, and *Anaplasma*. These microorganisms are characterized by a strictly intracellular location which has, for long, impaired their detailed study. In this paper, the critical steps taken by these microorganisms to play their pathogenic roles are discussed in detail on the basis of recent advances in our understanding of molecular *Rickettsia*-host interactions, preferential target cells, virulence mechanisms, three-dimensional structures of bacteria effector proteins, upstream signalling pathways and signal transduction systems, and modulation of gene expression. The roles of innate and adaptive immune responses are discussed, and potential new targets for therapies to block host-pathogen interactions and pathogen virulence mechanisms are considered.

1. Introduction

The human rickettsial diseases are a variety of clinical entities caused by α-proteobacteria of the order *Rickettsiales* belonging to genera *Rickettsia* and *Orientia* (family: *Rickettsiaceae*) and *Ehrlichia* and *Anaplasma* (family: *Anaplasmataceae*) [1, 2].

Rickettsiae are small, obligately intracellular, gram-negative bacteria that reside free in the host cell cytoplasm, and are transmitted to human hosts by arthropod vectors. A modern classification based on whole-genome analysis, divides the species of the genus *Rickettsia* in four groups: spotted fever group (*R. rickettsii*, *R. conorii*, *R. parkeri*, and several others), typhus group (*R. prowazekii* and *R. typhi*), ancestral group (*R. bellii* and *R. canadensis*, not known to be pathogenic), and transitional group (*R. akari*, *R. australis*, and *R. felis*). Further investigation of a variety of hosts including nonhematophagous insects, amoebae, and leeches identified many novel *Rickettsia* clades opening a broader view of their evolution [3].

Rickettsia prowazekii is the only pathogen among various rickettsial species with acknowledged capacity to maintain persistent subclinical infection in convalescent patients, which can later manifest as recrudescent typhus or Brill-Zinsser disease.

Orientia tsutsugamushi is the etiologic agent of scrub typhus, a rickettsiosis that is widespread in Asia, the islands of the western Pacific and Indian Oceans, and foci in

northern Australia. The genus *Ehrlichia* is member of the family *Anaplasmataceae*, which also includes the genera *Anaplasma*, *Wolbachia,* and *Neorickettsia*. It consists of six formally named members: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, *E. Ovis,* and *E. ruminantium*. Human monocytic ehrlichiosis, caused by *E. chaffeensis*, and human granulocytic anaplasmosis, caused by *Anaplasma phagocytophilum*, are the most important ehrlichioses and are considered emerging diseases. Major findings in rickettsioses and ehrlichioses/anaplasmosis include fever in a patient with exposure to a potential vector that may be associated with rash, inoculation eschar, or localized lymphadenopathy. Laboratory studies commonly reveal neutropenia, thrombocytopenia, and moderate increases in hepatic transaminases. The severity of rickettsial diseases varies with the causative agent and the host. Older age, alcoholism, and deficit in glucose-6-phosphate dehydrogenase have been associated with more severe disease [1, 2].

For each genus, the main steps taken by the microorganism to play their pathogenic roles will be discussed with particular emphasis to the roles of innate and adaptive immune responses.

2. Rickettsia spp

2.1. Endothelial Cell Invasion, Injury, and Activation. Although pathogenic rickettsiae are able to infect and replicate *in vitro* in a great number of different cell types, during *in vivo* infection, both in humans and in established experimental models of infection, the pathogens invade and proliferate within vascular endothelial cells (ECs) lining small and medium-sized blood vessels, the major target cells of rickettsial infection, together, to a lesser extent, with perivascular cells, that is, monocytes and macrophages, and hepatocytes, destroying them, spreading infection to the endothelia of the vascular tree. The recently emerging concept of "ECs heterogeneity" considers the significant morphological and functional differences between ECs of small and large vessels and between cells derived from various microvascular endothelial beds, and the necessity for a more comprehensive analysis of the biological basis of rickettsial affinity for vascular ECs (see below). However, damage to the endothelium, and subsequent endothelial dysfunction and activation, is followed by acute phase responses and alteration in coagulation and in the cytokine network, together with transient immune dysregulation, characterized by reduction in circulating peripheral CD4+ T lymphocytes and perivascular infiltration by CD4 and CD8 T lymphocytes, B cells, and macrophages, all features collectively termed as "rickettsial vasculitis". The mechanisms of host defence are not yet completely understood, although cell-mediated immunity is thought to play a crucial role [4, 5].

2.1.1. ECs Invasion. Rickettsial entry into human ECs via induced phagocytosis requires rickettsial viability and effector function of the host cell cytoskeletal actin, implicating that adherence of a viable bacterium to the cell surface triggers intracellular uptake by a metabolically active host cell. Even though some signalling events involved in rickettsial entry into the ECs have been documented, that is, activation of phosphoinositide 3-kinase, Cdc42 (a small GTPase), src-family tyrosine kinases, and tyrosine phosphorylation of focal adhesion kinase (FAK) and cortactin, all of the effector host proteins mediating entry are not fully elucidated (Figure 1) [6].

Some studies, especially on spotted fever group rickettsiae *(R. rickettsii* and *R. conorii)*, have reported the Ku70 subunit of a mainly nuclear DNA-dependent protein kinase, located in cytoplasm and plasma membrane too, as a receptor involved in rickettsial internalization. Ku70 is recruited to rickettsial entry sites, and inhibition of its expression impairs rickettsial internalization. Rickettsial infection also stimulates the ubiquitination of this kinase. The ubiquitin ligase c-Cbl is recruited to rickettsial entry foci, and downregulation of c-Cbl blocks rickettsial invasion and Ku70 ubiquitination. Experimental approaches identified the rickettsial outer membrane protein B (OmpB) as a ligand for Ku70 and that Ku70-OmpB interactions are sufficient to mediate rickettsial invasion of nonphagocytic host cells. Furthermore spotted fever group rickettsiae use OmpA, Sca1, and Sca2 as adhesion proteins [7]. Antibodies to particular epitopes of OmpA and OmpB may protect against reinfection, but they appear not to play a key role in immunity against primary infection [8]. OmpA, OmpB, Sca1, and Sca2 belong to a family of outer membrane proteins, termed autotransporters, found in gram-negative bacteria. Bioinformatic analysis of sequenced rickettsial genomes has identified a family of at least 15 genes in addition to *ompA* (*sca0*) and *ompB* (*sca5*), termed *sca* (for surface cell antigens), whose predicted products resemble autotransporter proteins. The function of all of these Sca proteins remains unknown. However, it was hypothesized that they should contribute to the specific recognition of different sets of host receptors. It is possible that Sca1 and Sca2 may work in concert with other rickettsial proteins (i.e., OmpB and OmpA) to interact with target mammalian cells, especially ECs during the infection process. The interactions of Sca1 and Sca2 with their cognate receptors may be coordinated with other receptor-ligand pairs to trigger previously observed downstream signaling events, that is, phosphoinositide 3-kinase, Cdc42, and src-family tyrosine kinases that, ultimately, lead to localized actin recruitment and nucleation required for entry [9–11].

Other possible rickettsial adhesins, encoded by the genes RC1281 and RC1282 in *R. conorii*, and, respectively, designed as Adr1 and Adr2, have been proposed to be also involved in bacterial adhesion and entry into the host cells [12].

The rickettsiae induce internalization by phagocytosis. Then, to enter the cytosol of the host cell, where nutrients, adenosine triphosphate, amino acids, and nucleotides, are available for growth, and to avoid phagolysosomal fusion and death, rickettsiae must escape from the phagosome. Rickettsiae secrete phospholipase D and heamolysin C, encoded by the rickettsial genes *pld* and *thyC* which are capable of disrupting the phagosomal membrane and allowing a rapid

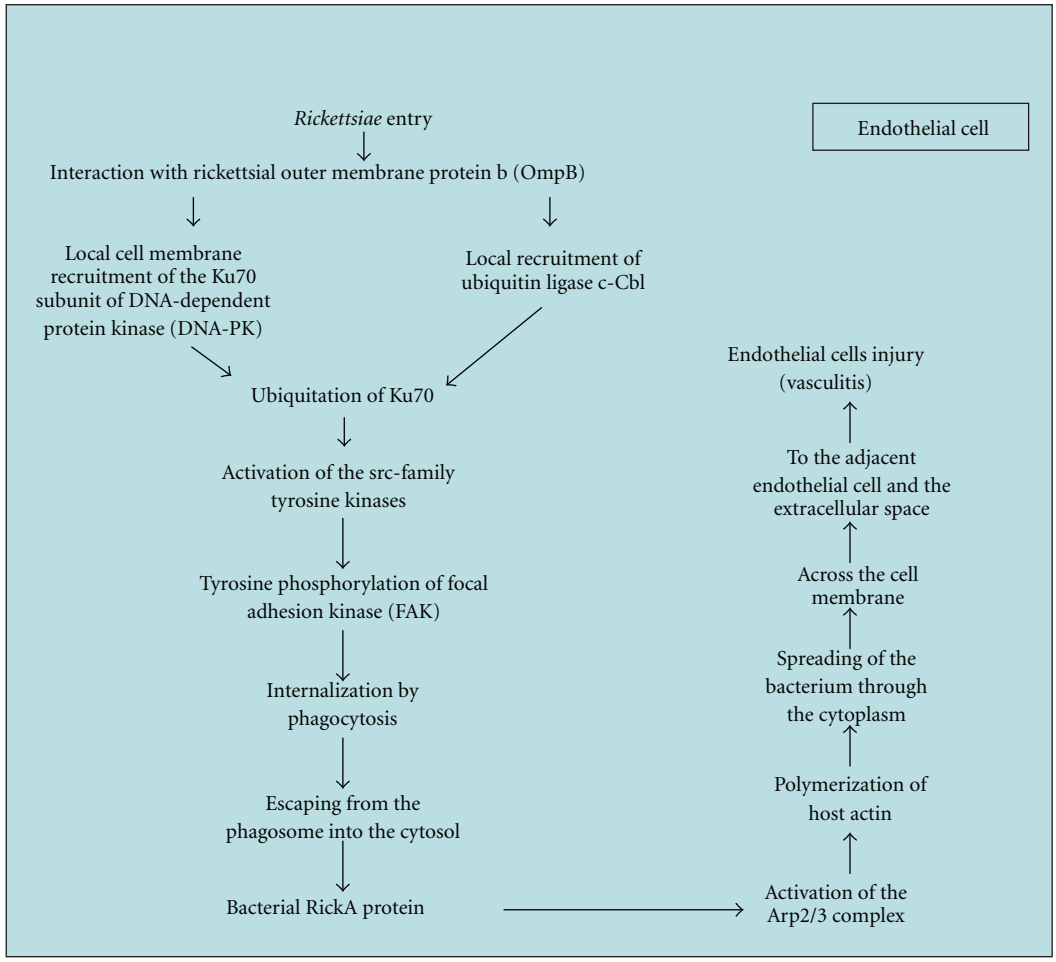

Figure 1: Rickettsia entry into endothelial cells.

escape. However, knockout of the *pld* gene alone does not prevent phagosomal escape [13].

In the cytosol, rickettsiae express a surface protein, Sca2, which recruits the Arp2/3 complex, activate it inducing directional host actin polymerization, which, finally, propels the bacterium through the cytoplasm and across the cell membrane into the adjacent endothelial cell or extracellular space. On its own, the Arp2/3 complex has no actin nucleation activity. It is activated by a class of cellular proteins called nucleation-promoting factors (NPFs), two examples of which are members of the Wiskott-Aldrich syndrome protein (WASP) family and of the cortactin family. RickA, a mimic of WASP family NPFs, has been proposed as the mediator of Arp2/3 recruitment and actin nucleation. However, the evidence for a role for Sca2 including gene knockouts and complementation is more extensive and compelling [14].

Then filaments of actin propel *Rickettsia* to the surface of the host cell, where host cell membrane is deformed outward and invaginates into the adjacent cell. Disruption of both cell membranes enables the *Rickettsia* to enter the adjoining cells without being exposed to the extracellular environment. However, some rickettsiae exit via the luminal surface of blood vessels into the bloodstream [15, 16].

Typhus group rickettsiae tend to accumulate within the cytoplasm until cell lysis as *R. prowazekii* does not stimulate actin-based mobility and *R. typhi* exhibits only erratic mobility. Spotted fever group organisms spread rapidly from cell to cell and usually accumulate intracellularly in significantly lower numbers than typhus group rickettsiae. It would be reasonable to hypothesize that the host cell is able to sustain growth, multiplication, and accumulation of progeny rickettsiae during infection with typhus group organisms, owing to significant differences in pathogenic mechanisms of cell injury in comparison to those triggered by spotted fever group rickettsiae, that is, differences in the intensity and/or kinetics of the nuclear-factor- (NF-) κB activation pattern in *R. Typhi*- versus *R. conorii*-infected host ECs (see below) or stimulation of oxidative stress [17].

In addition to the above-mentioned rickettsial mechanisms of EC invasion, several studies pointed out the role of T4SS as an additional virulence factor. T4SSs are complex multiprotein structures spanning the bacterial envelope and are composed of up to 12 individual protein components classified into three groups, each represented in this bacterial genus. The exact function of these transporters, which are ancestrally related to bacterial conjugation systems, diverged during evolution. Since T4SS components are retained in

rickettsial genomes, many of which lack plasmids; their primary suspected role is secretion of virulence factors rather than conjugation. Among rickettsial substrates susceptible to be exported by such a secretion system are sec7 proteins (effectors known to contribute to the establishment of a replicative organelle by inhibiting phagosome-lysosome fusion, even if these bacteria grow in the cytoplasm of infected cells), LepA (a protein promoting release of bacteria from protozoa by an exocytic pathway, a marker of probable ancestral location of rickettsiae within ameba), and, finally, VipD (a protein belonging to the patatin family of proteins, with phospholipase A_2 [PLA2] activity that perturbs membrane trafficking and modulates intracellular bacterial growth) [18].

Factors secreted by the T4SS most likely promote rickettsial survival by triggering synthesis of nutrients from the host cell or allowing adaptation of rickettsiae to the intracellular environment [19].

2.1.2. ECs Injury. The mechanisms responsible for cell injury and vascular denudation by rickettsiae remain poorly understood (Figure 2) [20]. A comprehensive analysis of damage to the membrane structure and integrity of subcellular organelles by electron microscopy indicated the possibility of involvement of reactive oxygen species (ROS) production, that is, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), induced by the combination of tumor-necrosis-factor- (TNF-) α and interferon- (IFN-) γ, in rickettsial pathogenesis, both as host defense and infection-induced injury mechanisms. *In vitro* and *in vivo* experiments demonstrated the induction of oxidative stress mechanisms, as evidenced by accumulation of ROS, altered levels of antioxidant enzymes, that is, superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase (G6PD), and catalase, alterations in mRNA expression of selected antioxidant enzymes, and depleted levels of intracellular reduced thiols, for example, reduced glutathione (γ-glutamylcysteinylglycine). These studies suggest an important role for oxidant-mediated cell injury in the pathogenesis of rickettsial infections. In addition, compared to subjects expressing physiologic levels of G6PD, the prognosis in G6PD- deficient patients is relatively poor, due to complications, such as hemolysis (involving red blood cells which lack the ability to synthesize G6PD to replace unstable prematurely denatured G6PD) and acute renal failure (as consequence of hemolysis), and enhanced severity of disease. These observations confirm the potential importance of antioxidant defence in the outcome of human rickettsial infections [21, 22]. In this context, it has also been demonstrated that rickettsial infection *in vitro* as well as *in vivo* induces the expression of hemeoxygenase-1 (HO-1), the inducible form of antioxidant and vasoprotective isozyme 1 of heme oxygenase, a rate-limiting enzyme in the pathway for heme catabolism, resulting in the release of ferrous iron and generation of carbon monoxide (CO) and biliverdin. The latter is subsequently converted to bilirubin. One of the most critical regulatory functions of HO-1 in the vasculature is to control the activity of the cyclooxygenase (COX) system, which is responsible for generation of a number of vasoactive substances, including prostaglandins (PGs), prostacyclin, and thromboxanes. Rickettsial infection of human ECs causes robust induction of COX-2 mRNA and protein expression but has no apparent effect on the constitutive COX-1 isoform. Induction of the endothelial COX-2 system and the resultant enhanced release of vasoactive PGs, especially PGE2 and PGI2, may contribute to the regulation of inflammatory responses and vascular permeability changes during rickettsial infection [23, 24].

Cell injury and vascular denudation of the endothelium may also result from direct damage to host cells. One among possible mechanisms of direct damage is a rickettsial phospholipase activity, that is, phospholipase D or phospholipase A_2, that may contribute to direct host damage. Spotted fever group rickettsiae may also damage cells at the time of exiting, when directional actin polymerization is followed by membrane rupture. Otherwise, typhus group rickettsiae, which lack directional actin polymerization (*R. typhi* polymerizes actin, but not directionally), grow inside infected cells until the cells burst, and, in this context, genes, encoding proteins with possible membranolytic activities, for example, pat1 and tlyC, could play a role [25]. Another widely accepted concept regarding the pathogenesis of EC injury and rickettsial diseases is increased vascular permeability, resulting in fluid imbalance and edema of vital organs, as a major feature of acute inflammation. *R. conorii* infection of human ECs induces changes in the localization and staining patterns of adherens junction proteins, and discontinuous adherens junctions lead to formation of interendothelial gaps. Interestingly, proinflammatory cytokines, that is, TNF-α and IFN-γ, during infection, exacerbate the effects of rickettsiae on endothelial permeability, further suggesting that the changes in the barrier properties of vascular endothelium are likely due to a combinatorial effect of intracellular rickettsiae and immune responses mediated by and/or directed against the infected host cell [26].

2.1.3. EC Activation: Intracellular Signaling Mechanisms. It is widely accepted that interactions between invading rickettsiae and host ECs constitute one of the most important aspects underlying the onset and progression of infection, replication within the intracytoplasmic niche, dissemination through the host, and the pathogenesis of resultant disease. *In vitro* studies suggest that ECs undergo a series of active cellular responses during infection with rickettsiae, resulting in significant alterations in the pattern of gene transcription and characteristic responses, a phenomenon referred to as "endothelial activation" [20].

Many pathophysiological situations affecting ECs of the vasculature, for example, rickettsial invasion, lead to the expression of genes dependent on the NF-κB family of transcription factors. Most of the early-response genes transcriptionally upregulated in response to rickettsial invasion, that is, IL-8 and Monocyte-Chemoattractant-Protein-(MCP-) 1 (see below), contain NF-κB binding sites in their promoter regions, indicating that infection-induced alterations in gene expression may be governed, at least in part, by activation of NF-κB. Human ECs infected with rickettsiae display nuclear translocation of NF-κB. This event is a consequence

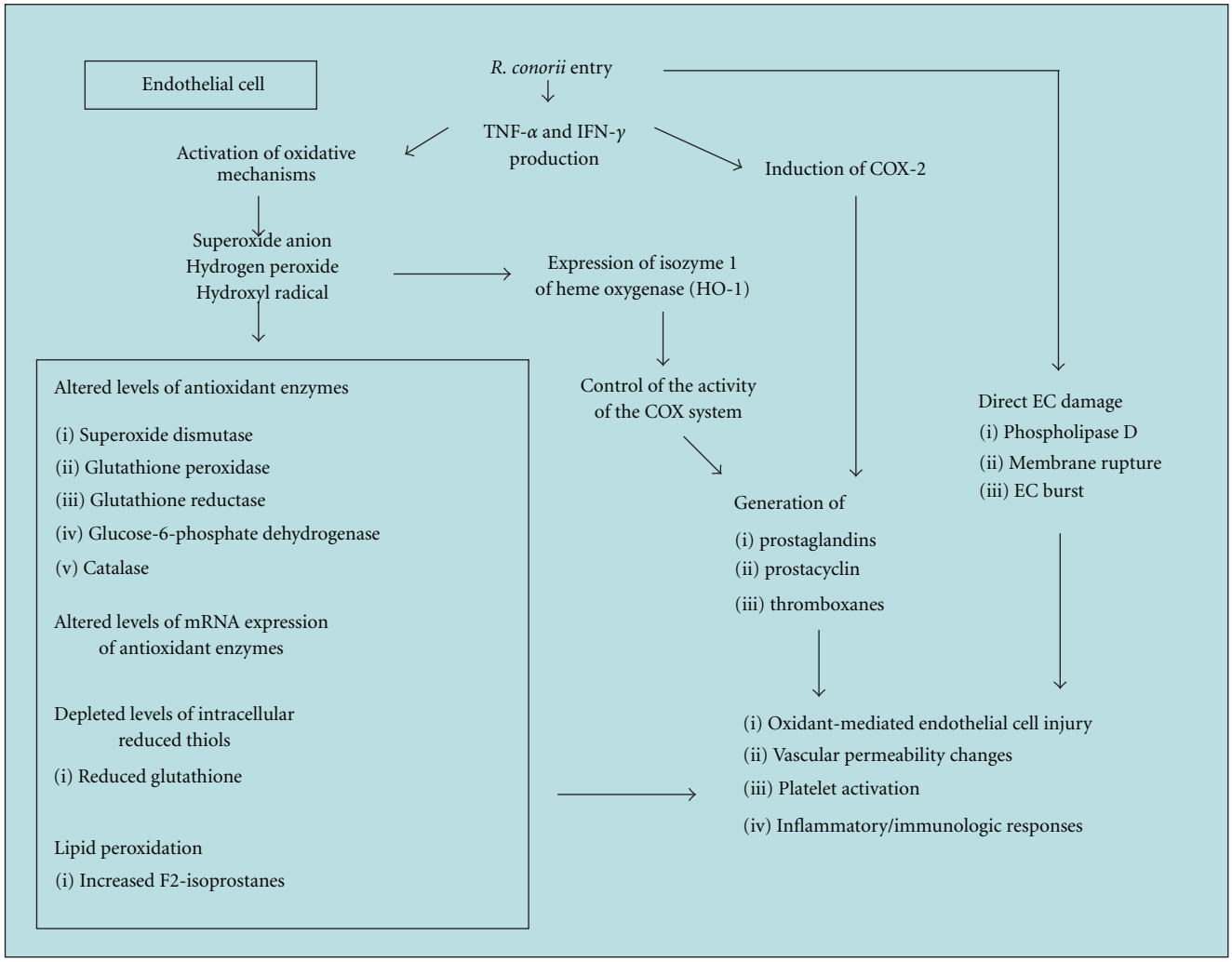

Figure 2: Rickettsia injury to endothelial cells.

of the degradation, by proteasomes, of masking regulatory proteins, termed inhibitors of NF-κB, or IκB, after their phosphorylation mediated by IκB kinase (IKK) complex, and the exposure of the nuclear location sequences. However, *R. rickettsii* is also capable of directly interacting with NF-κB in its inactive form in the EC cytoplasm by an unidentified bacterial protease activity [27, 28]. Several studies have also suggested a role for protein kinase C (PKC), in the activation of NF-κB by infectious agents, such as rickettsiae, and other stimuli [29].

Mitogen-activated protein kinases (MAPKs) also play a critical role in signal transduction events. Three major MAPK cascades, namely, extracellular signal-regulated kinases, c-Jun-N-terminal kinases, and p38 MAPK, have been identified and characterized, and they can be activated simultaneously or independently to constitute a central regulatory mechanism that coordinates signals originating from a variety of extracellular and intracellular mediators. Activation of enzymes in the MAPKs module by proinflammatory cytokines, reactive oxygen species (ROS), and shear stress transmits signals down the cascade, resulting in phosphorylation of many proteins with substantial regulatory functions throughout the cell, that is, NF-κB. *R. rickettsii* infection selectively induces activation of p38 MAPK in ECs. Activation of p38 MAPK depends on active cellular invasion by viable rickettsiae, appears to involve generation of ROS, and subsequently facilitates host-cell invasion by *R. rickettsii* [30].

2.1.4. ECs Production of Cytokines. Human umbilical vein ECs (HUVECs) infected by *R. conorii* actively secrete large amounts of IL-6 and IL-8 via an IL-1α-dependent pathway (Figure 3). Induced expression of genes encoding these proinflammatory cytokines occurs through signaling mechanisms that require activation of p38 MAPK and NF-κB (see above) [31]. IL-1α, produced by HUVECs, remains associated with the cells, anchored to the cell surface via fatty acid acylation or through a lectin-like mechanism, mainly in a precursor form. Therefore, IL-1α precursor, produced by HUVECs, can be secreted and immediately bound to type I IL-1 receptor. Thus, IL-1α can act as an autocrine and paracrine factor on HUVECs, without being detectable in the supernatants [32]. Alternatively, IL-1α can be active without being secreted, fixed on endothelial surface membranes, in a

cell-cell membrane interaction. IL-1α activates ECs, induces the production of other cytokines, such as IL-8 and IL-6, and increases the expression of adhesion molecules on both ECs and leukocytes [33].

IL-6 and IL-8 might play a role in the development of vasculitis induced by rickettsial infection. IL-6 may mediate acute-phase protein production associated with rickettsial infection. IL-6 might also be involved in the local differentiation and proliferation of T lymphocytes, through its stimulatory effects on IL-2 production and IL-2 receptor (IL-2R) expression on T cells and on B-lymphocytes stimulation [34]. IL-8 is a member of the chemokine family, a group of structurally related proteins with proinflammatory properties [35]. IL-8 is a powerful chemotactic agent for polymorphonuclear leukocytes, stimulates polymorphonuclear leukocyte transendothelial migration, and activates their functions, but it seems to have no effect on ECs [36].

2.1.5. EC Production of Cellular Adhesion Molecules and Chemokines. Rickettsial infection of ECs, through the release of early-response cytokines (IL-1α and IL-6) and other components of the acute phase reaction, enhances the expression on the surface of ECs and polymorphonuclear leukocytes of cellular adhesion molecules (CAM), that is, E-selectin, VCAM-1, and ICAM-1, and it has been demonstrated that the enhanced rolling and the adherence of mononuclear cells to infected ECs involves E-selectin-, VCAM-1-, and ICAM-1-dependent mechanisms (Figure 3) [37]. Selectin molecules (P, L, and E), interacting with cell surface carbohydrate counterreceptors (sialyl Lewis X family), mediate rolling of the leukocytes along the vascular endothelial surface, in such a way that slows the leukocytes in the circulatory flow [38]. This permits adhesion of leukocytes to endothelial -cell-expressed adhesion molecules at the site of inflammation [39].

During the acute phase of boutonneuse fever (BF) (i.e., within 2 weeks after the onset of symptoms), soluble forms of these adhesion molecules (sL-selectin, sE-selectin, s-VCAM-1, and sICAM-1) are shed into plasma by proteolytic cleavage of their membrane-bound counterparts from activated leukocytes and ECs and/or by differential splicing of their mRNA. The shed forms are functionally active and could be involved in the control of adhesive interactions between cells. In particular, the increase in their levels might reduce adhesion of leukocytes and exert anti-inflammatory effects, reducing local lymphocyte and monocyte infiltration. sL-selectin and s-VCAM-1 return to the normal range in the third week, whereas sE-selectin and sICAM-1 persist at significantly high levels even after the third week of disease. Since a direct correlation between sL-selectin and fever has been demonstrated, normalization of serum levels may result in good prognosis, whereas persistence of high levels of this CAM has negative prognostic value and could be useful to monitor disease evolution. The lack of correlation among these soluble CAMs, serum secretion cytokines (i.e., IL-1α and IL-6), and the absolute number of leukocytes can partly be explained by the observation that the effects of these cytokines on cell surface CAM expression do not parallel the soluble CAM production, and the absolute number of leukocytes depends on the variable sources of these soluble molecules, which, in turn, bind different cell types. Differing from other CAMs, levels of sE-selectin directly correlate with the number of circulating neutrophils. Therefore, sE-selectin might be an important factor in reducing the quantity of local neutrophils [37].

Recently, a greater inflammatory EC response to *R. conorii* than that to *R. africae* was observed. These are, respectively, the etiologic agents of severe (BF) and mild (African tick bite fever, ATBF) forms of spotted fever group rickettsioses, characterized by marked increase in IL-8, MCP-1, and adhesion molecules in EC, involving Toll-like receptor 4 (TLR4) activation (see below). Importantly, there is evidence of a similar pattern *in vivo*, with increased serum levels of IL-8, VCAM-1, and E-selectin in patients with BF as compared with those with ATBF. These findings suggest a greater *R. conorii* inflammatory potential in ECs than in infection with *R. africae* related to the more severe systemic inflammation and clinical disease in the former rickettsial infection [40].

Finally, in experimental models of *R. conorii* infection of endothelium, there is prominent expression of chemokines CCL2, CCL3, CCL4, CCL12, CX3CL1, CXCL1, CXCL9, CXCL10, and Regulated by Activation, Normal T-cell Expressed and Secreted (RANTES). In particular, increased expression of CXCL1 appears to coincide with infiltration of macrophages into *R. conorii*-infected tissues, whereas expression of CXCL9 and CXCL10, known to target activated T cells through CXCR3, is significantly increased in mice infected with *R. conorii*. Rickettsiae-triggered activation of p38 MAPK and NF-κB may trigger most of these responses [41].

2.1.6. ECs Intracellular Killing of Rickettsiae. Perivascular infiltrations of CD4+ and CD8+ T lymphocytes, natural killer cells, macrophages, marginated elements of blood, and infected ECs themselves secrete cytokines and chemokines that activate infected ECs, macrophages, and hepatocytes, by paracrine and autocrine stimulation, to kill intracellular rickettsiae [12]. Therefore, the mechanism of killing of *R. conorii* within human target cells, mainly endothelium, and, to a lesser extent, macrophages and hepatocytes, has not been fully determined. Human cells might be capable of controlling rickettsial infections intracellularly, the most relevant location in these infections, by a cytokine-, chemokine- (i.e., RANTES), and a nitric oxide-dependent mechanism, and, in particular, by one or a combination of three different possibilities involving, (1) nitric oxide synthesis, (2) hydrogen peroxide production, (3) tryptophan degradation [42, 43].

AKN-1 cells (human hepatocytes) stimulated by IFN-γ, TNF-α, and RANTES killed intracellular rickettsiae by inducible nitric oxide synthase (iNOS) expression and nitric oxide-dependent mechanism. HUVEC, when stimulated with the same concentrations of cytokines and RANTES, differed in their capacity to kill rickettsiae by a nitric oxide-dependent mechanism and in quantity of nitric oxide synthesized. Hydrogen-peroxide-dependent intracellular killing

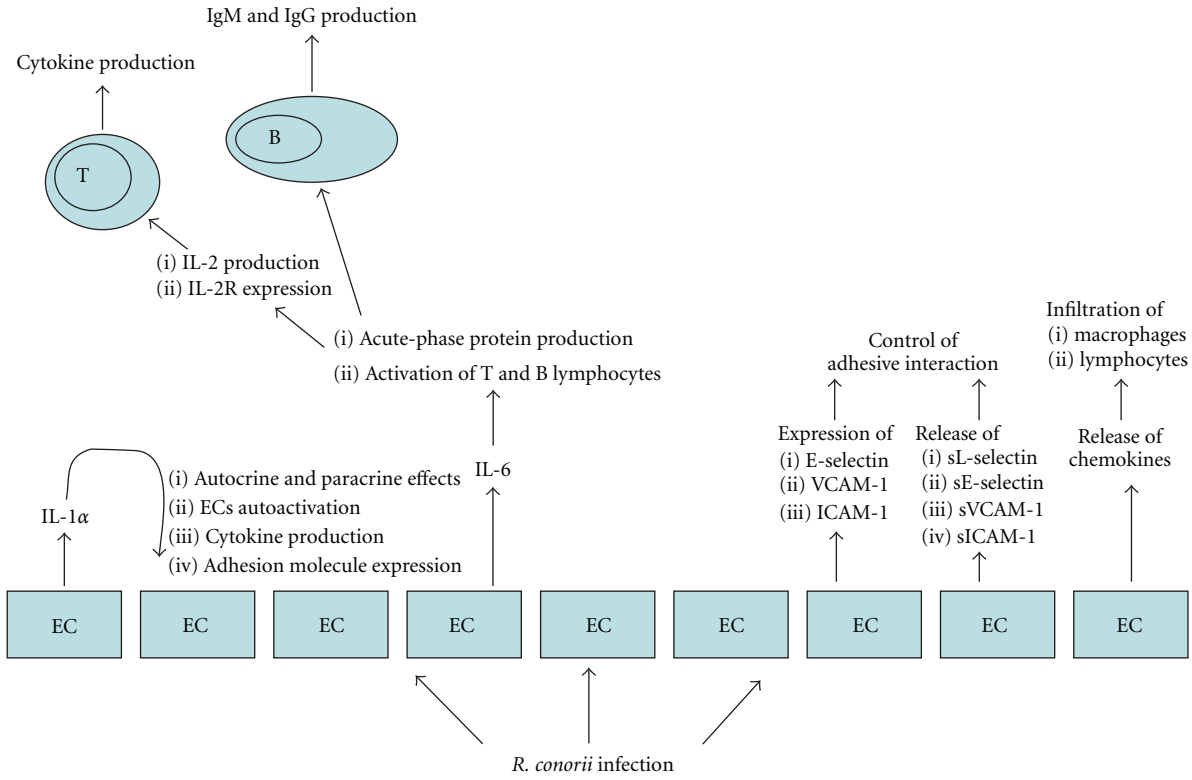

Figure 3: Cytokine production and induction of cellular adhesion molecules in rickettsial diseases.

of *R. conorii* was demonstrated in HUVECs, THP-1 cells (human macrophages), and human peripheral blood monocytes activated by cytokines. Rickettsial killing in a human macrophage cell line was also mediated by a limitation of the availability of tryptophan in association with expression of the tryptophan-degrading enzyme indoleamine 2, 3-dioxygenase (IDO), resulting in its intracellular depletion and starvation for the bacteria [44].

2.1.7. Regulation of Programmed EC Death . While apoptosis of infected cells is an important host defense mechanism for limiting spreading of infection, viruses and intracellular bacteria employ a variety of strategies to inhibit the host cell's apoptotic machinery to ensure and enhance the survival of the host, as a cell that dies upon infection is a cell that does not provide a niche for the intracellular pathogen's multiplication. The first evidence supporting this behaviour was demonstration that the antiapoptotic functions of NF-κB are essential for survival of ECs during *R. rickettsii* infection [45]. Infection of cultured human ECs with *R. rickettsii* with simultaneous inhibition of NF-κB induces activation of apical caspases-8 and -9 and also of executioner enzyme, caspase-3. The peak activity of caspase-3 coincides with the cleavage of poly-(ADP-ribose)-polymerase, followed by DNA fragmentation, mitochondrial damage, and, finally, apoptosis. Thus, activation of NF-κB is required for the maintenance of mitochondrial integrity of host cells and protects against infection-induced apoptotic death by preventing activation of the caspase-8- and -9-mediated pathways [46]. The B-cell lymphoma-2 (Bcl-2) family of proteins, which includes both pro- and antiapoptotic factors, plays a critical role in regulation of apoptotic cell death by controlling mitochondrial permeability [47]. Determination of the effects of NF-κB on the inhibition of proteins of the Bcl-2 family during *in vitro* EC infection by *R. rickettsii* further reveals significant changes in expression of various pro- and antiapoptotic proteins, the ultimate outcome of which is an "equilibrium shift" towards inhibition of apoptosis [48].

2.1.8. Heterogeneity of Macrovascular and Microvascular ECs Response to Rickettsial Infection. The recently emerging concept of "EC heterogeneity" dictates that, depending on their location and physiological functions, vascular endothelium of different organ systems displays significant differences in its biological properties and activation patterns in response to known stressors. In this contest, recent studies have provided evidence for relatively similar levels of infectivity and activation of both NF-κB and p38 MAPK, as common signaling mechanisms, and HO-1 mRNA expression in different types of macro- (e.g., pulmonary artery cells) and microvascular (e.g., human dermal, brain, and pulmonary endothelium) ECs infected with *R. rickettsii*. Although systematic comparative analysis of mechanisms underlying hosts cell's transcriptional activation illustrates activation of NF-κB and p38 MAPK in all microvascular cell types tested, that is, human dermal, brain, and pulmonary endothelium, the most striking changes in terms of intensity

of *R. rickettsii*-induced responses were observed in dermal ECs. Correspondingly, infected ECs were also found to secrete the largest amounts of IL-8 and MCP-1. Interestingly, refractoriness of brain-derived microvascular endothelium to secrete chemokines in response to infection was also revealed despite evidence for the activation of NF-κB and p38 upstream signalling mechanisms and reduced ability of microvascular ECs to induce COX-2 expression and, consequently, secretion of PGE2 in response to *R. rickettsii* infection. In contrast, *R. rickettsii* infection of large vessel ECs, that is, those isolated from human pulmonary artery, produces significant amounts of PGE2, which may be attributable to increased activity of COX-2. Absence of any change in COX-2 activity during microvascular *R. rickettsii* infection suggests that the observed alterations in vascular permeability of cerebral ECs (i.e., cerebral edema) apparently involve PG-independent mechanisms [49].

2.2. Platelet and Hemostatic/Fibrinolytic Systems' Activation. In the acute phase of BF, by measurements of a major metabolite of thromboxane (TX) in the urine (11-dehydro-TXB2), a marker of platelet activation and of plasma prothrombin fragment 1+2, the levels of which reflect activation of prothrombin to thrombin; the occurrence of TXA2-dependent platelet activation and thrombin generation has been demonstrated *in vivo*. These phenomena could, at least partly, reflect the clinical manifestations of BF, such as vasculitis and focal microthrombus formation [50].

In addition, recently, a putative biochemical link has been demonstrated between endothelial dysfunction and platelet activation by investigating *in vivo* the generation of F2-isoprostanes. This family of bioactive iso-eicosanoids is produced from arachidonic acid through a process of nonenzymatic free-radical-catalyzed lipid peroxidation, and their biological activities may transduce the effect of oxidant stress into specialized forms of cellular activation [51]. Oxidative stress, induced in ECs by *R. conorii*, with increased lipid peroxidation, as shown by increased generation of F2-isoprostanes, causes endothelial dysfunction, as demonstrated by enhanced ADMA (asymmetric dimethylarginine) [52] levels.

ADMA is an endogenous inhibitor of NO synthase, able to cause reduced NO bioavailability and, thus, endothelial dysfunction. The close relationship between production of F2-isoprostanes and ADMA levels seems to confirm the hypothesis that *R. conorii* infection, inducing oxidative stress, could, in turn, provoke endothelial dysfunction [53].

Furthermore, endothelial activation, induced by rickettsial infection, associated with systemic inflammation can induce platelet activation with consequent CD40L release, as demonstrated in African tick bite fever, by *R. africae*, and in BF [52, 54]. CD40L is a transmembrane glycoprotein, belonging to the tumor necrosis factor family, originally identified on CD4+ T cells, but also found in platelets. It is estimated that >95% of the circulating CD40L is derived from platelets. Binding of soluble CD40L (sCD40L) to various vascular cells (e.g., ECs or monocytes) contributes to the pathogenesis of inflammatory and thrombotic processes, as a further inflammatory stimulus to endothelial activation. Thus, once established, enhanced sCD40L shedding from platelets may sustain a vicious circle, persisting even after recovery, at a time when clinical manifestations of disease and the systemic inflammatory reaction apparently had waned [55, 56].

Rickettsial diseases are generally also associated with significant changes in the levels of hemostatic/fibrinolytic proteins, demonstrated by pronounced alterations in plasma concentrations of coagulation factors, natural anticoagulants, and components of the fibrinolytic system. An elevation in levels of plasma fibrin(ogen)-degradation products during *R. rickettsii* and *R. conorii* infection of humans indicates activation of the fibrinolytic system, as increased levels of circulating fibrinogen likely reflects enhanced synthesis of this acute-phase protein. The presence of circulating C1-inhibitor-kallikrein complex and lower concentrations of prekallikrein indicate activation of the kallikrein-kinin system during spotted fever rickettsioses. Rickettsia-infected ECs acquire a procoagulant phenotype. Rickettsial infection induces expression of tissue factor, secretion of plasminogen activator inhibitor, production of platelet-activating factor, expression of thrombomodulin, and release of von Willebrand factor. However, disseminated intravascular coagulation occurs rarely even in lethal cases and is not a common feature of rickettsioses [57].

2.3. Acute Phase Response. In BF, invasion and proliferation of rickettsiae in ECs are the events responsible for destruction of vessels and for acute-phase response activation. Some acute-phase responses appear to be involved in the promotion of inflammatory events (IL-1α, IL-8, IFN-γ, complement proteins, C-reactive protein (CRP), and fibrinogen), while others appear to moderate them (ceruloplasmin and α1-antitrypsin). After the resolution of infection, all mediators return to the normal range, acute-phase response subsides, and the homeostatic balance is restored (Figure 4). IL-1α is not detectable in the blood of acute BF patients, probably because stimulation of ECs causes the production of IL-1α predominantly in a cell-associated form, thus contributing to the localized procoagulant and inflammatory responses which occur during rickettsial disease. Undetectable serum levels of IL-8 may be due to its prevalent presence in endothelial protrusions, either in vesicles (the Weibel-Palade bodies) or on the plasma membrane, which could be responsible for neutrophil adhesion. As for IFN-γ, an increase has been detected in the first week, and it offers a good explanation of the complement data. In fact, IFN-γ is the main inducer of C4 gene expression, acting, at least partly, on control of its transcription. Concerning factor B, its rate of gene transcription is increased by IFN-γ, whereas IL-1 and IL-6 act synergistically at the pretranslational level. Since in BF IL-1 is not increased and IL-6 is very high during the first two weeks of infection, IL-6 could play a role with IFN-γ in active synthesis of factor B in the liver. The high levels of CRP detected in the acute phase of BF could by binding cells further induce activation of the classical complement pathway. This, by generating C5a, in turn, triggers the influx of neutrophils and enhances the phagocytosis of the cells that have bound CRP and complement, thereby enhancing

inflammation and tissue damage. On the other hand, CRP and complement could prove useful for elimination of infected, damaged or compromised cells, exerting anti-inflammatory activities. The increase in fibrinogen levels, in the first week of BF infection, constitutes the primary event inducing the local activation of haemostasis, through platelet activation, and it is a cursory indicator of inflammation in most human infections. The strong release of ceruloplasmin could minimize the inflammatory response, acting as a scavenger of oxygen free radicals produced by macrophages, neutrophils, and ECs. In fact, ceruloplasmin, in BF patients, returns to a baseline level nearly contemporaneously with the normalization of the white blood cell counts. α1-antitrypsin persists at high levels, in BF patients, until the third week, and this persistence could indicate that this proteinase inhibitor is required for a longer time, both to neutralize lysosomal hydrolases released by infiltrated macrophages and neutrophils and, more importantly, to induce release of anti-inflammatory cytokines. However, enhanced levels of ceruloplasmin, α1-antitrypsin, and, in general, plasma α fraction proteins have also been observed in several parasitic diseases, even though there was no relationship between their changes and disease evolution [58, 59].

2.4. Immunologic Transition from Innate to Acquired Immunity

2.4.1. Toll-Like Receptors, Dendritic Cells, and the Innate Immune System. One of the mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms is through pattern-recognition receptors (PRRs), which are germ-line-encoded receptors, including transmembrane Toll-like receptors (TLRs), and cytosolic nucleotide-binding oligomerisation-domain-(NOD-) like receptor (NLR) family proteins [48, 49]. Recognition of pathogen-associated molecular patterns by TLRs, either alone or as heterodimers with other TLR or non-TLR receptors, triggers signals which are responsible for activation of genes important for an effective host defense, especially those of inflammatory cytokines [60, 61]. There are at least 10 TLRs in humans. TLR signalling is mediated via interactions with adaptor proteins, including MyD88 and toll-receptor-associated activator of interferon (TRIF) [62]. Toll-like receptor 4 (TLR4) has an important role in inflammation and immunity, and its expression has been reported in most tissues of the body. TLR4 is well known as receptor for LPS, and its activation is critical for response to gram-negative bacteria. Recently, mutations in human TLR4 were found to be associated with hyporesponsiveness to LPS and increased risk of gram-negative infections both in humans and experimental animals [63]. The +896 A → G non-synonymous single-nucleotide polymorphism of the TLR4 gene, causing the Asp299 → Gly change in the extracellular domain of TLR4, attenuates receptor signalling, decreases endotoxin responsiveness, and determines poor outcomes from sepsis [64]. Interestingly, it has been demonstrated that the +869G single-nucleotide polymorphism of the TLR4 gene is overexpressed in BF patients with significant differences in the frequency of TLR genotypes and alleles between BF patients and age-matched controls. These data might be interpreted as one hypothetical basis for genetic susceptibility to BF [65].

The role played by dendritic cells (DCs), key initiators and orchestrators of the immune response, is important to understand in rickettsial infections. TLR4 signalling, due to ligation with rickettsial LPS, is important in activating antigen-presenting cells, for example, DCs, toward production of proinflammatory cytokines (i.e., IL-2, IL-6, IL-12, and IL-23), initiation of antirickettsial innate immunity (namely, expansion of the natural killer (NK) cell population and subsequent production of IFN-γ) and production of adaptive T_H1 type or T_H17 responses (which play a critical role in autoimmunity and are also implicated in protective immune responses to bacterial pathogens). Conversely, lack of TLR4 stimulation during antigen presentation leads to disproportionate expansion of CD4+ CD25+ Foxp3- T-regulatory lymphocytes (T_{reg}) and subsequent suppression of proinflammatory immune responses to rickettsiae. C3H/HeJ mice, which are naturally defective in TLR4 signaling, develop overwhelming, fatal rickettsial infections when given an inoculum that is nonfatal for TLR4-competent mice. C3H/HeJ mice have a reduced T_H1-type response and a significantly greater percentage of T_{reg} cells in their peripheral lymph nodes, which could potentially suppress proinflammatory responses by production of IL-10 or transforming growth factor-β (TGF-β), and limit the initiation of adaptive immunity, by decreasing the number of effector cells during antigen stimulation (see below). In contrast, mice with competent TLR4 responses are more resistant to rickettsial infection. Moreover, this resistance is associated with an increase in number of known antirickettsial effector cells, specifically T_H1- and T_H17-polarized CD4+ and CD8+ T lymphocytes [66, 67].

TLR4-ligated DCs induce recruitment of NK cells to draining lymph nodes. Furthermore, this recruitment and production of NK cell-derived IFN-γ in draining lymph nodes are important in augmenting the T_H1 immune response. It has been documented that TLR4 stimulation is also important in inducing NK cell activation *in vivo*. The number and percentage of NK cells in spleen are consistently lower in C3H/HeJ mice than in TLR4-competent ones after *R. conorii* infection. Additionally, splenocytes from C3H/HeJ mice have less NK cell cytotoxic activity *in vitro* than those from TLR4-competent mice. Moreover, TLR4-competent mice have significantly higher serum levels of IFN-γ during early infection, and subsequent investigation demonstrated that NK cells in TLR4-competent mice produce significantly more IFN-γ after *in vitro* stimulation. This IFN-γ production is important in inducing early NO production in infected ECs [68].

Taken together, all these data reveal an important role for DCs in recognizing rickettsiae through TLR4 and that a vigorous proinflammatory response induced by DCs is associated with protective immunity to rickettsiae. Subsequently, generation of antigen-specific immunity is crucial to complete protection [66–68].

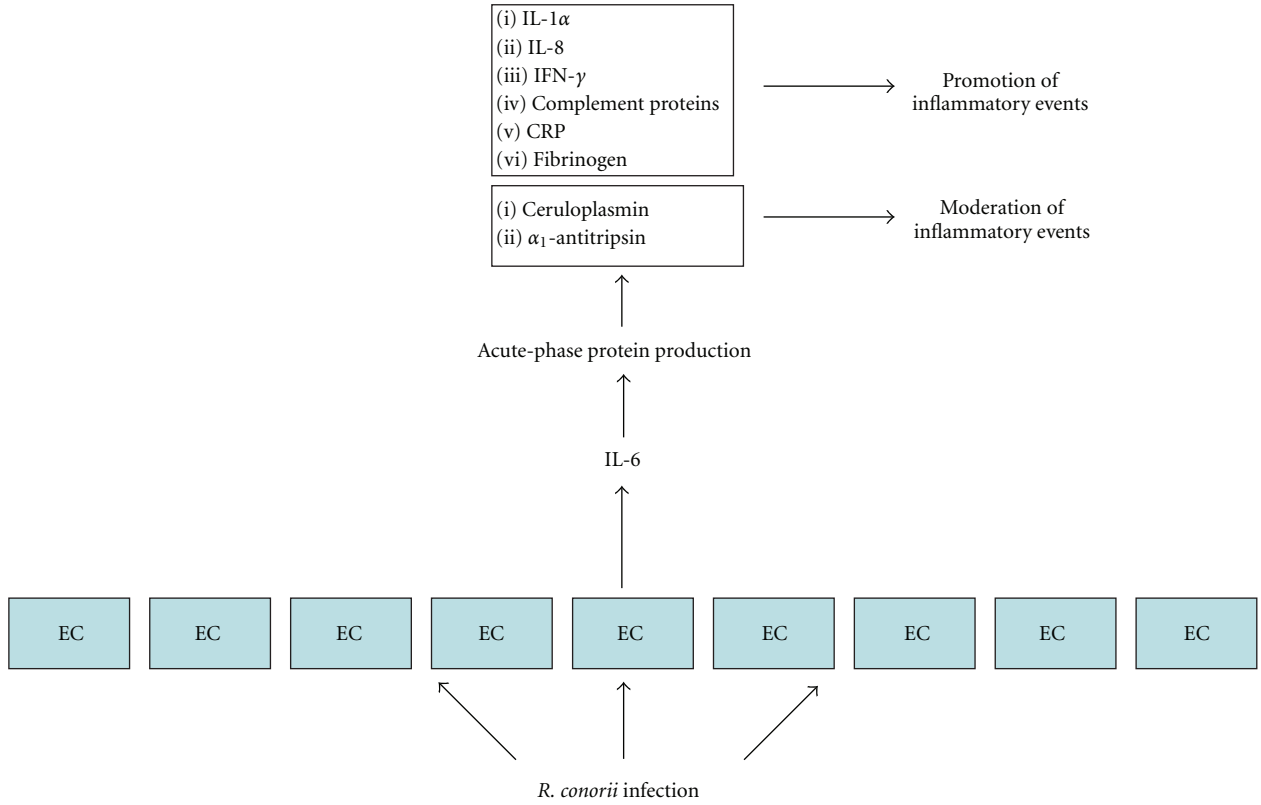

Figure 4: Acute phase response in rickettsial diseases.

2.4.2. CD4+ Lymphocytes and Related Cytokines. In acute BF patients, a reduction of circulating T cells, and, in particular, of CD4+ (helper/inducer T cells), CD4+/CD45RO+ (memory T cells), and CD4+/CD45+ (naive cells) subsets have been demonstrated. These modifications could be due to cell adhesion to vascular endothelium, followed by their entrance into the sites of inflammation. Apoptosis, spontaneous and/or activation-induced, could also have a role in this diminution [69, 70]. Other lymphocytic subsets (CD8+ (suppressor/cytotoxic T cells), CD16+ (NK cells), and CD20+ [B cells)) tend to decrease but not statistically significantly, whereas monocytes (CD14+/HLA-DR+) are significantly increased. All cell subsets return to normal levels after successful treatment except for monocytes, which persist at a high level after recovery. Inflammatory and immunologic responses, mediated by increased levels of T_H1-type (TNF-α and IFN-γ) and T_H2-type cytokines (IL-10 and IL-6), appear to be important for recovery from infection. In detail, serum levels of TNF-α, IFN-γ, IL-10, and IL-6 are significantly increased in patients with acute BF, compared with those from control healthy subjects. On the contrary, *in vitro* peripheral mononuclear cells from acute BF patients produce low levels of IFN-γ and IL-10 in response to mitogen or specific antigen, whereas TNF-α and IL-6 responses are in the normal range. It is possible that IFN-γ and IL-10 are produced at the sites of infection, as observed in leishmaniasis, as a result of local recruitment and accumulation of activated CD4+ T cells in the perivascular area adjacent to infected ECs. Furthermore this observation is compatible with observed reduction of circulating CD4+ cells. In contrast, normal production of TNF-α and IL-6 could be due to the presence of high levels of peripheral monocytes, important sources of TNF-α and IL-6 production. Dramatic increase in TNF-α in the first week after onset of symptoms, together with IFN-γ, is one of the early events observed in acutely ill BF patients, followed by increases of IL-10 and IL-6. IFN-γ levels drop sharply in the second week after onset of fever, whereas TNF-α, IL-10, and IL-6 gradually decline, reaching normal levels after the third week [69, 71].

Antigen-presenting cells activated by rickettsial antigens produce high levels of TNF-α and IFN-γ. TNF-α induces TNF-α and IFN-γ production from activated CD4+ T cells with T_H1 phenotype and NK cells. One important effect of TNF-α and IFN-γ on macrophages is induction of receptors for TNF-α, so the binding of TNF-α on macrophages might further activate these cells, by an autocrine TNF-α loop, thus increasing their antirickettsial activity via induction of NO synthesis (Figure 5) [72–74]. Persistence of high serum levels of TNF-α in recovered patients could be a sign of residual local lesions. Patients with severe forms of BF, associated with disseminated intravascular coagulation, had very high levels of TNF-α that persisted for a long time, and decreased only when the condition of the patients improved. A relationship has also been observed between serum TNF-α and a high level of production of one of two soluble TNF receptors, persisting high values of sTNF-RI in BF could be an indicator of disease activity [69, 75].

Because IL-10 (a T_H2-type cytokine) suppresses the ability of IFN-γ-activated macrophages to produce inflammatory mediators, its persistent high levels could be owing to its ability to downregulate the potentially tissue-damaging effects of responses mediated by increased T_H1-type cytokines [76, 77].

The profile of serum IL-6 (another T_H2-type cytokine) production in patients with acute BF and after recovery is similar to that of IL-10. In acute BF, high IL-6 levels could be due to synthesis at sites of infection by activated and damaged ECs, infiltrating monocytes, and T_H2 T cells. In acute BF, IL-6 could act as an inflammatory cytokine, rather than as a growth and differentiation factor for B cells and immunoglobulin production. The fact that B cells are reduced in acute BF in the face of high levels of IL-6 is consistent with this hypothesis [44, 69]. Later, when IL-10 begins to inhibit T_H1 cells that produce TNF-α and IFN-γ, favouring proliferation and secretion of T_H2 cytokines, such as IL-6, terminal differentiation of B cells to immunoglobulin-producing cells could be favoured [69, 78]. In agreement with this scenario, there is normalization of B cells in recovered patients. The specific antibody titer increases only 2 weeks after infection and peaks in 4 weeks. Afterwards, IgM decreases, and IgG remains high for several months [69, 79]. Since soluble IL-6R (sIL-6Rα) could enhance rather than inhibit the biological activity of IL-6 both *in vitro* and *in vivo*, the elevated levels of sIL-6Rα detected in the first weeks of infection in BF patients might result in increased IL-6 activity. In this case, IL-6-sIL-6Rα complexes could act as strong activators of the inflammatory response [69, 80].

Therefore, in BF patients, T_H1- and T_H2-type responses are not characteristically polarised, as both activating (i.e., TNF-α, IFN-γ) and suppressive (i.e., IL-10 and IL-6) cytokines are detected [81, 82]. It is possible that in BF, as in any other inflammatory environment, counterbalancing mechanisms are normally produced to curtail the process. IL-10 and IL-6 are capable of derailing T_H1-type responses and deactivating macrophages, thereby moderating tissue injury [83].

IL-12, a cytokine produced by B cells, phagocytic cells, and other antigen-presenting cell types, induces cytokine production, primarily IFN-γ, from T cells with T_H1 phenotype and NK cells, acts as a growth factor for activated T cells and NK cells, enhances the cytotoxic activity of NK cells, and favours cytotoxic T-lymphocyte generation, thus exerting stimulatory effects on T_H1-type responses and downregulating T_H2-type activity [84]. In BF patients, IL-12, predominantly produced by monocytes, appears to act very early, favouring T_H1 differentiation and T_H1-type cytokine production and contributing to phagocytic cell activation and innate resistance to intracellular pathogens. Therefore, IL-12 could, if produced in effective amounts, favour rickettsial clearance, activating the T_H1 responses, as observed in other intracellular infections [84, 85].

Finally, some authors have described the occurrence in patients with BF of haemophagocytic lymphohistiocytosis (or haemophagocytic syndrome), a potentially fatal hyperinflammatory syndrome, characterized by histiocyte proliferation and haemophagocytosis. The pathophysiology of haemophagocytic lymphohistiocytosis in BF patients is not fully understood. However, an uncontrolled immune response can lead to hypersecretion of cytokines, upregulation of adhesion molecules and MHC I and II molecules on monocytes-macrophages, and expansion of inflammatory monocytes, together with impaired or absent function of NK cells and cytotoxic T cells. This exaggerated inflammatory response could result in uncontrolled proliferation and phagocytic activity of histiocytes [86].

2.4.3. Role of CD8+ T Cells. CD8+ T lymphocytes, perhaps activated by antigen-presenting ECs, in association with antigens on class I of major histocompatibility complex (MHC-I), contribute to protective immunity to rickettsiae by both MHC-I-restricted cytotoxic T-lymphocyte (CTL) activity and production of IFN-γ. However, CTL activity might be more critical to recovery from rickettsial infection than the effects of IFN-γ production by CD8+ T cells (Figure 5). Therefore, CD4+ and CD8+ T lymphocytes are both a potentially rich source of IFN-γ, which could activate endothelial rickettsicidal activity and tip the balance in favour of survival. However, in mice, depletion of CD4+ cells has no observed effect on course or outcome of infection. In contrast, CD8+-depleted mice, infected with ordinarily sublethal dose of *R. conorii*, remain persistently infected and ill, and a high proportion of these animals die of uncontrolled rickettsial infection and its consequent pathologic effects. Rickettsia-infected ECs, and to a lesser extent, rickettsia-infected macrophages, are the targets of MHC-I-restricted CTL-mediated clearance of the infection. Perforin is one of the mechanisms of elimination of *R. conorii*-infected cells, but it is probably not the only one. As of yet, the roles of Fas-Fas ligand interaction and/or granulysin, in antirickettsial CTL activity, might also be important [87, 88].

2.4.4. Other Markers of Immunologic Activation. The strong T-cell activation, during BF, is also attested by observation of a very significant release of the α-chain of high-affinity IL-2R, in soluble form (s-IL2R), in serum and urine of acute BF patients [89]. IL-2R is the main cellular mediator of the actions of IL-2, as a growth and differentiation factor for T, B, and NK cells and as an activator for macrophages. sIL-2R, released both *in vitro* and *in vivo* following lymphocyte activation, binds IL-2, and, thus, it potentially downregulates IL-2-driven proliferation and IL-2-dependent cell-mediated responses [90]. sIL-2R is significantly increased in acute BF and returns to normal when patients recover from disease. Serum levels of sIL-2R correlate positively with those in urine, indicating that sIL-2R undergoes glomerular filtration [89].

Other markers produced by functionally active lymphocytes such as soluble CD4 (sCD4), soluble CD8 (sCD8), β2-microglobulin (β2-M), and soluble class I antigens (sHLA-I) are released, in the sera of patients with acute BF, and correlate with the evolution of infection [91]. The pathophysiological functions of these molecules are not

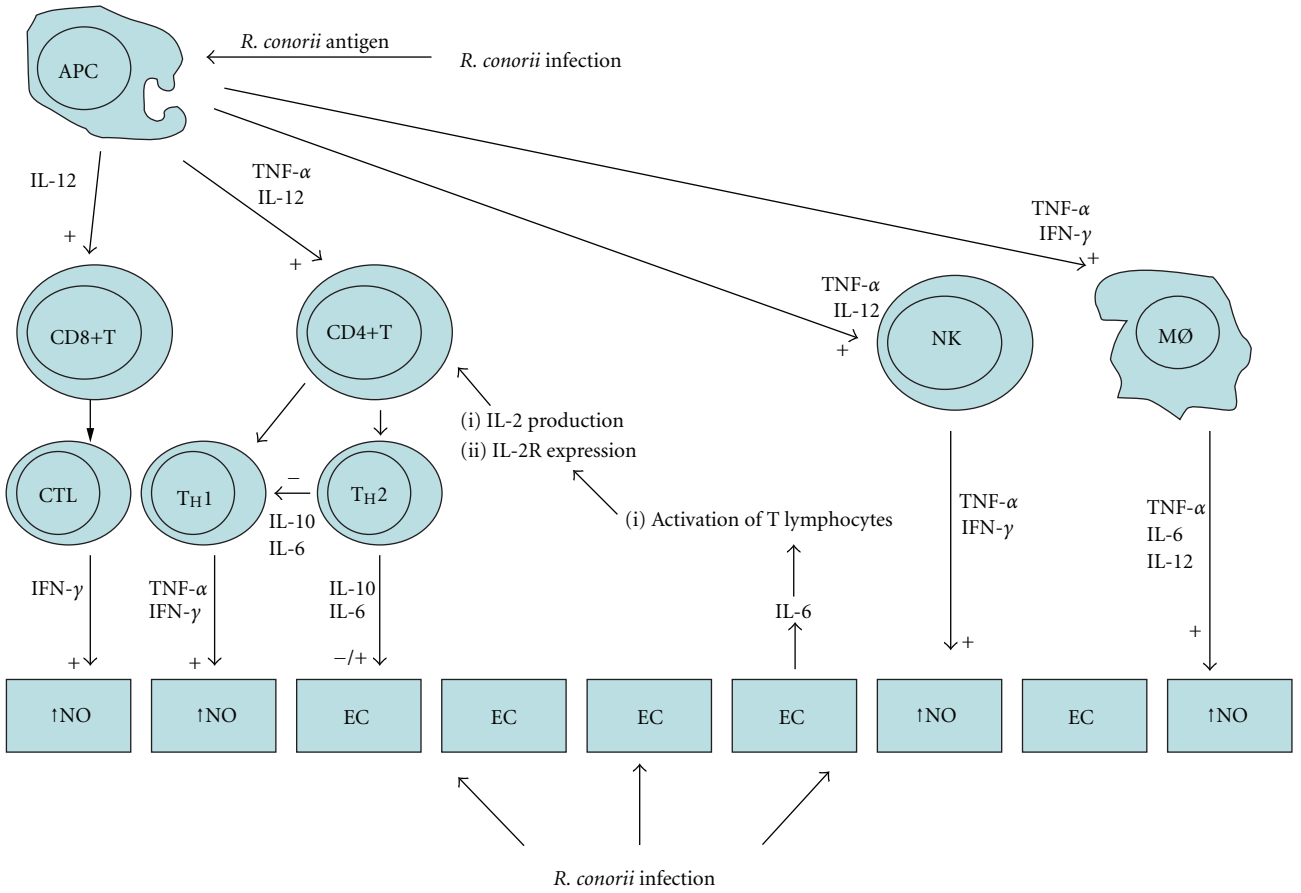

Figure 5: Immunologic alterations in rickettsial diseases.

known. It is likely that sHLA-I, shed as dimers with β2-M, contains the peptide of foreign rickettsial antigen within the groove, and in such a way it can potentially recognize reactive T cells. Thus, sHLA-I could play a relevant role in regulating the immune response, being released by activation and contributing itself to activation of other cells [92, 93].

2.4.5. Role of CD4+ CD25+ Foxp3- T Regulatory (T_{reg}) Lymphocytes. Acute severe human rickettsial diseases have been characterized as diseases that stimulate a dominant type 1 immunity and unresponsiveness or suppression of CD4+ T cells, with transient immune dysregulation. There is a suppressed CD4+ T_H1-cell response during lethal rickettsial infection in susceptible C3H/HeN mice (that mimic the pathogenesis of severe human rickettsial infection), including inhibition of IFN-γ production by spleen cells in response to rickettsial stimulation compared to production in sublethally infected mice, a serum level of IFN-γ significantly lower than that in sublethally infected mice, suppressed or unresponsive proliferation of CD4+ T cells in response to anti-CD3 or specific antigen stimulation, inhibition of IL-2 production by splenocytes, and, finally, a lower frequency of antigen-specific IFN-γ-producing CD4+ T cells than that in sublethally infected mice. Immunosuppression induced by a high dose of rickettsiae may account for the uncontrolled bacterial burden and overwhelming infection. The suppressed immune response observed in mice infected with a lethal dose of *R. conorii* is associated with substantial, significantly greater expansion of T_{reg} cells in the infection sites. Therefore, generation of T_{reg} cells might be a potential mechanism contributing to host susceptibility to acute severe rickettsiosis. In particular, it has been demonstrated that DCs from resistant C57BL/6 mice exhibit higher expression levels of MHC class II and higher IL-12 production upon rickettsial infection and are more potent in priming naïve CD4+ T cells to produce IFN-γ, whereas DCs from susceptible lethally infected C3H mice promote the induction and expansion of a novel phenotype of suppressive CD4+ CD25+ T-bet$^-$ Foxp3$^-$ CTLA-4^{high} T-reg cells, consisting of both IL-10-producing adaptive T_{reg} cells and IFN-γ-producing T-effector cells. This novel T_H1-cell-related adaptive T_{reg}-cell population contributes greatly to deep immunosuppression during acute severe rickettsiosis via as-yet-unidentified mechanisms that may involve IL-10 production, or CTLA-4 (cytotoxic T-lymphocyte antigen 4) function, or an indirect process via an influence on DCs. It is also possible that IL-10-producing antigen-presenting cells, such as DCs, are involved in immunosuppressive phenomena in addition to the suppression mediated by T_{reg} cells and that interactions between DCs and T_{reg} cells, rather than T_{reg} cells alone, may play a crucial role in the balance between

an efficient immune response and tolerance/susceptibility [94, 95].

2.4.6. Intralesion Production of Cytokines and Chemokines in BF. Very few human studies directly examined either the local mediators of inflammation or the immune response at the site of infection (e.g., skin) in rickettsial diseases. Skin-biopsy samples from patients with BF, collected 3–14 days after onset of fever, revealed a balanced mixed type 1, pro-inflammatory and anti-inflammatory responses, as reflected by elevated levels of mRNA expression of TNF-α, IFN-γ, and IL-10. Increased levels of IL-10 suggest the presence of an immunoregulatory mechanism that can help to avoid severe tissue damage due to excess intralesional production of proinflammatory type 1 cytokines. There is also a positive correlation between high levels of IFN-γ mRNA and TNF-α mRNA and the production of enzymes involved in limiting microbial growth (i.e., iNOS and IDO). Finally, although the median mRNA-expression levels of IFN-γ, TNF-α, IL-10, iNOS, and IDO were not statistically significantly different between patients with mild, moderate, and severe MSF, there was a trend toward expression of substantially higher levels of certain biomolecules in either mild or severe MSF. In detail, patients with severe BF showed higher levels of TNF-α mRNA and lower levels of IFN-γ mRNA compared with those of mild BF. All of the patients with severe BF had higher IDO and RANTES mRNA-expression levels and lower levels of iNOS mRNA compared with those of mild BF. The level of IL-10 mRNA expression was comparable in all 3 groups of patients. Therefore, IFN-γ is critical for protection against severe BF, and protection is dependent on the expression of iNOS mRNA, whereas TNF-α and RANTES may play a dual role, being involved in both protective antirickettsial immunity and the pathogenesis of severe BF [96].

3. Orientia tsutsugamushi

Orientia tsutsugamushi infects predominantly ECs, even though it may be found in several other cells, including DCs, macrophages, polymorphonuclear leukocytes (PMNs), and lymphocytes. The bacterium invades host cells by induced phagocytosis and then is taken into a phagosome. Loss of phagosomal membranes is frequently observed, suggesting a mechanism for *O. tsutsugamushi* escape from phagosome into cytoplasm. The virulence factors related to phagosomal membrane lysis, however, have not been identified. Then *O. tsutsugamushi* usually propagates in host cytoplasm via binary fission. Groups of budding *Orientia* (the budding process differs from that of enveloped viruses as the host cell membrane surrounds the bacterial cell wall rather than being incorporated into the organisms' structure) are clearly observed on the cell surface after 2-3 days of incubation. The released bacterium covered by host cell membrane may either directly infect neighboring cells by fusion of membranes or the surrounding host cell membrane may be lost, leaving naked *Orientia* to invade other cells [97].

3.1. Invasion, Injury, and Activation of Infected Target Cells

3.1.1. ECs, Dendritic Cells, and Macrophages Invasion. Bacterial invasion of host cells is primarily mediated by interactions between bacterial surface components and complementary host receptors, which stimulate host signal transduction pathways required for bacterial access. *O. tsutsugamushi* uses host fibronectin interactions with one of its outer membrane proteins, the 56-kDa type-specific antigen (TSA56), to carry out internalization. Then, fibronectin facilitates bacterial entry into the host cells via interactions with integrins, that is, $\alpha5\beta1$. After integrin engagement, signaling molecules at the inner surface of the plasma membrane, which act as integrators of responses to extracellular stimuli, are activated. In detail, focal adhesion kinase (FAK), Src kinase, and RhoA GTPase are activated by *O. tsutsugamushi* invasion, and the signaling adaptors talin and paxillin are recruited to the site of infection. Furthermore, extensive actin reorganization and membrane ruffling in the region surrounding the *O. tsutsugamushi* cells are induced within 10 min. after attachment. Therefore, *O. tsutsugamushi* exploits integrin-mediated signaling and rearrangements of the actin cytoskeleton for invasion of eukaryotic host cells. However, many other membrane proteins, as well as TSA56 (e.g., products of *sca* genes), may interact with other receptors to trigger the observed downstream signaling events, involving recruitment of host endocytic machinery components and their specific activation, which ultimately lead to the extensive actin reorganization required for pathogen entry [98, 99] (Figure 6).

3.1.2. ECs, Dendritic Cells, and Macrophages Activation. Inflammation is initiated by *O. tsutsugamushi*-infected cells in the dermis. Proinflammatory cytokines and chemokines secreted by activated DCs, that is, TNF-α, IL-13β, IL-6, macrophage inflammatory protein (MIP)-1 α/β, MIP-2, and MCP-1, are the main ones responsible for leukocyte recruitment into inflammatory tissues. Members of the CC chemokine subfamily, which include MIP-1 α/β (CCL3/CCL4), MCP-1 (CCL2), and RANTES (CCL5), preferentially attract monocytes and lymphocytes, while those of the CXC chemokine subfamily, such as IL-8 and MIP-2 (CCL8), are potent neutrophil attractants. While CC chemokines, such as MIP-α/β and RANTES, are efficient chemoattractants for T_H1 cells, T_H2 cells were not attracted by them. Stimulation of T cells in the presence of MIP-1α enhances production of IFN-γ by T_H1 cells, while stimulation of T cells in the presence of MCP-1 leads to an increase of IL-4 production (a T_H2 cytokine). Taken together, T-cell differentiation by a subset of chemokines produced by activated DCs might be a crucial factor in the induction of a resistant versus a susceptible immune response to *Orientia* infection (Figure 7). ECs are another key participant in the inflammation process. Activation of ECs is stimulated by proinflammatory cytokines, including TNF-α and IL-1, which are released from infected sites and result in the upregulation of cell adhesion molecules, such as P-selectin, E-selectin, ICAM-1, and VCAM-1, to promote cellular influx via transendothelial migration, as

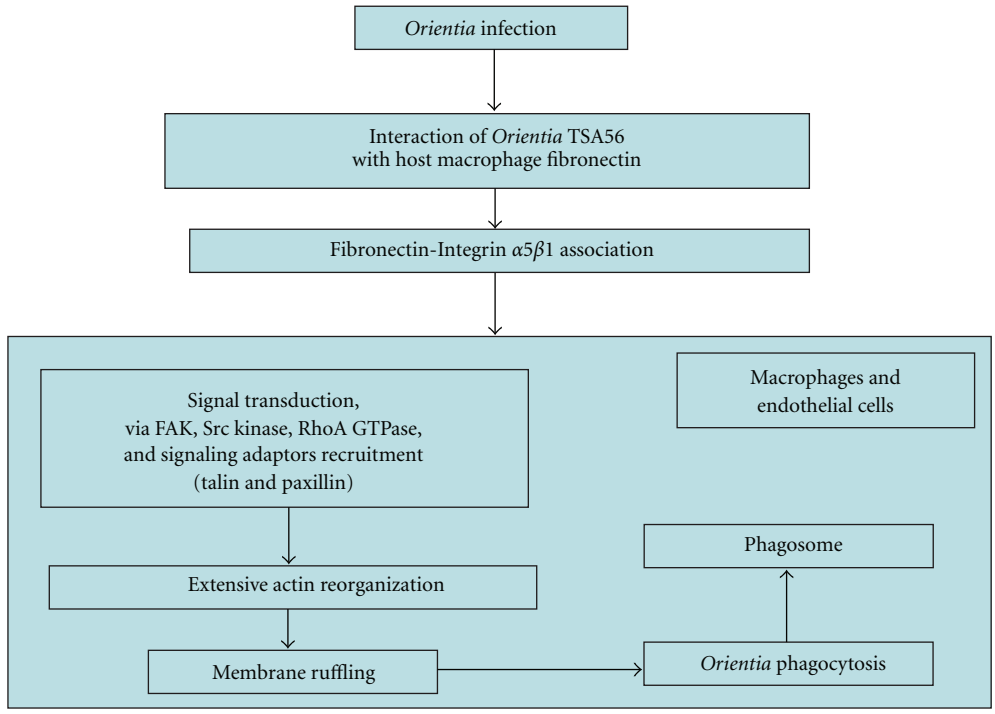

Figure 6: *Orientia* macrophages and endothelial cells invasion.

well as production of cytokines and chemokines, such as IL-1α, IL-6, IL-8 (CXCL8), IL-10, IL-15, TNF-α and TNF-β, CXCL1 to 3 (Gro), MCP-1 (CCL2), CCL5, and CCL17, to initiate and propagate local inflammatory responses (Figure 8). In addition, in patients with scrub typhus, the serum levels of soluble L- and E-selectin correlate with the symptoms of disease. MIP-1 α/β (CCL3/CCL4), MCP-1 (CCL2), and RANTES (CCL5), produced by activated macrophages, are induced via NF-κB activation, whereas activated ECs generate MCP-1 (CCL2) and CXCL8 (IL-8) independent of NF-κB activation [100, 101].

3.2. Immunologic Transition from Innate to Adaptive Immunity

3.2.1. Innate Immune System. As TLRs are important for innate immunity to rickettsiae, NLR family proteins are involved in innate immune mechanisms to *Orientia* infection. The NLR family is composed of 22 intracellular molecular-pattern-recognition molecules. One subfamily includes NOD1 and NOD2, which sense peptidoglycan polymers from cell wall components. Activation of NOD1 and NOD2 triggers recruitment of the adapter protein receptor-interacting serine-threonine kinase 2 (RIPK2 or RIP2), followed by activation of NF-κB and MAPKs. Another subfamily includes the NLRP proteins, which are essential for regulation of caspase-1 activation via the N-terminal pyrin domain by inflammasome formation, consisting of NALP3, caspase recruitment domain (ASC), and caspase-1. The NALP3 inflammasome then cleaves pro-IL-1β, pro-IL-18, and pro-IL-33 to mature IL-1β, IL-18, and IL-33, respectively. During *O. tsutsugamushi* infection, NOD1 senses an *Orientia* component in ECs and activates the downstream pathway of NF-κB, which leads to production of IL-32, an IL-18-induced cytokine, previously recognized as natural killer cell transcript 4 (NK4). Such increased IL-32 levels affect secretion of proinflammatory cytokines, that is, IL-1β, IL-6, and IL-8, as well as ICAM-1 expression in ECs. In addition, in ECs, IL-1β induces IL-32 production, followed by an increase in the levels of IL-1β, IL-6, IL-8, and ICAM-1. Therefore, IL-32-induced IL-1β expression in *O. tsutsugamushi*-infected ECs may reciprocally enhance IL-32 secretion. Consequently, IL-32 and IL-1β might form a positive feedback loop on ECs during the process of inflammation [102] (Figure 9).

3.2.2. CD4+ Lymphocytes and Related Cytokines. Type-1 cell-mediated immunity and IFN-γ production of T cells in response to *O. tsutsugamushi* antigen is essential for immune protection against infection, whereas the opposite T_H2 subset is considered to be detrimental. In animal models (intraperitoneal injection of *Orientia* in mouse, not perfectly corresponding to the natural modality of human infection, via intradermal penetration), T_H1- and T_H2-type responses are not clearly polarised, as both activating (i.e., IL-12, IFN-γ) and suppressive (i.e., IL-10) cytokines are simultaneously detected. It is possible in scrub typhus, like in other infectious disease (e.g., visceral leishmaniasis), that counterbalancing mechanisms are normally produced to modulate the process and ensure homeostasis within the host. Therefore, *O. tsutsugamushi* activates both proinflammatory and modulatory pathways, via IL-10, in the early stages of infection. An early step in generating T_H1 responses is IL-12 production by antigen-presenting cells (DCs, phagocytes, and mesothelium), which targets natural

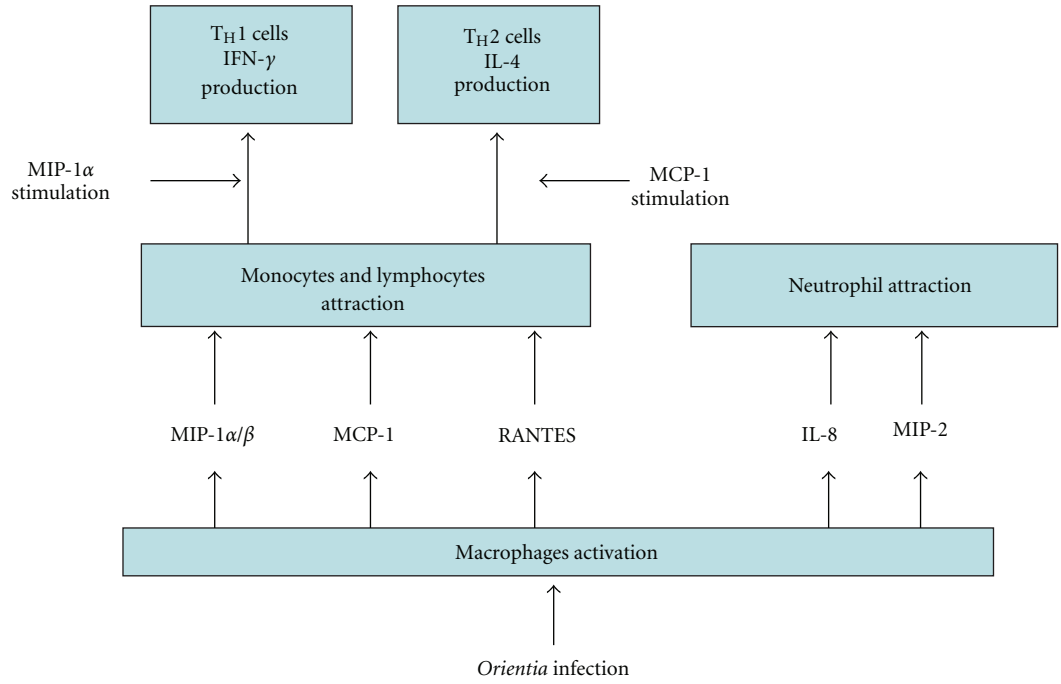

Figure 7: *Orientia* macrophages activation.

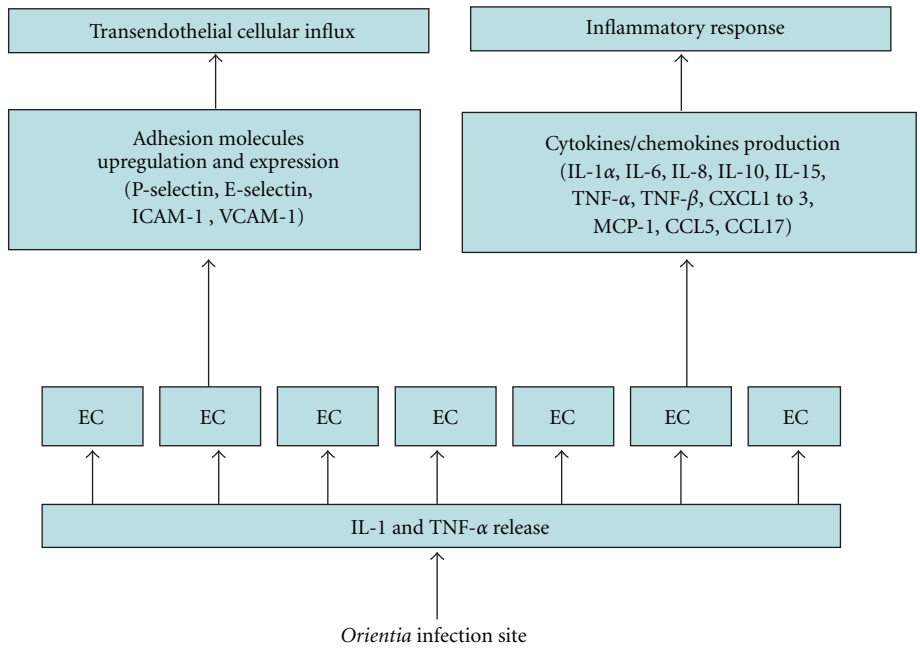

Figure 8: *Orientia* endothelial cells activation.

killer cells and T cells, inducing IFN-γ production, whereas early IL-10 production inhibits this response. IL-10 inhibits production of T_H1 type cytokines, including IFN-γ, IL-1, IL-2, IL-3, GM-CSF, and TNF-α and thereby inhibits the development of T_H1 immunity that is essential for clearing *O. tsutsugamushi* in infected ECs. In contrast, IFN-γ inhibits proliferation of T_H2 cells, thereby inhibiting production of T_H2-derived cytokines, including IL-4, IL-5, and IL-6 which are essential for B-cell differentiation and isotype switching [103].

Although the cytokine and chemokine profile demonstrates that *O. tsutsugamushi* infection results in immune reaction via soluble mediators from infected tissues or cells, this intracellular bacterium seems to exploit immune evasion mechanisms for survival. In addition, patients with tsutsugamushi disease sometimes suffer from severe clinical

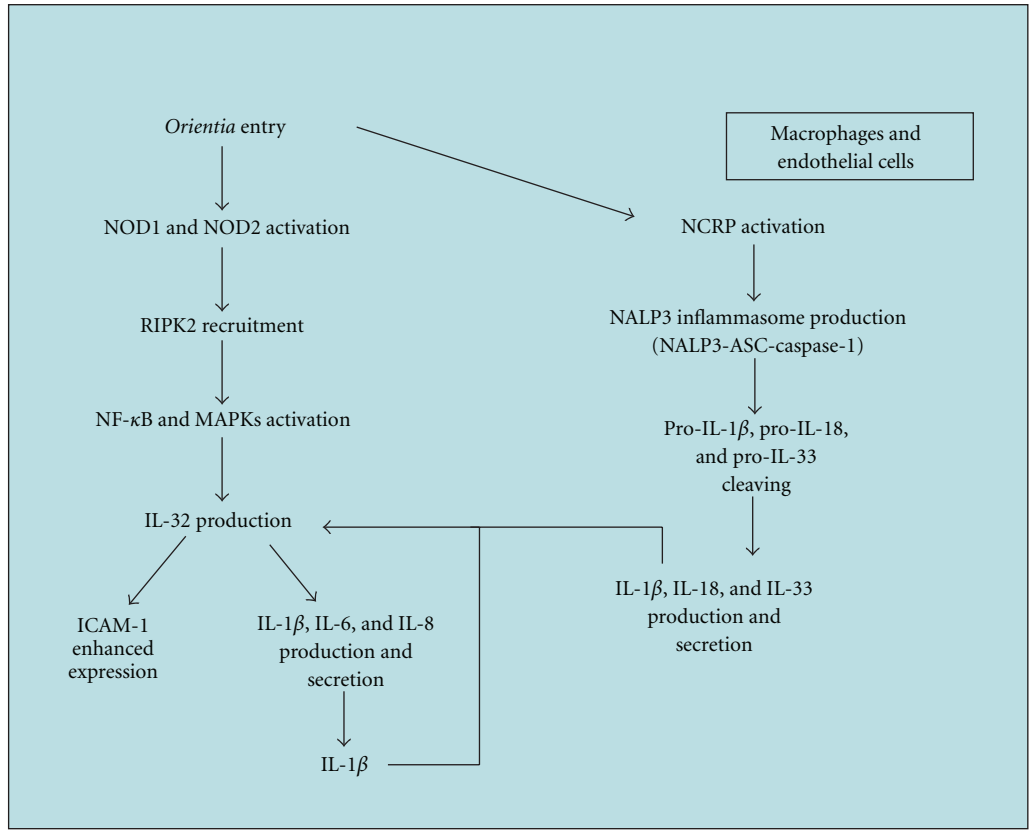

Figure 9: Innate immune system in oriential infection.

complications in the absence of appropriate antimicrobial chemotherapy. The exact mechanism leading to such severe complications is not well understood yet. In animal models (with the above-mentioned limitations), one of the possible mechanisms employed by *O. tsutsugamushi* involves the suppression of proinflammatory cytokine production, including TNF-α and IL-6, via induction of IL-10 secretion from infected macrophages and mesothelium. It has been reported that an imbalance of these two cytokines, with low levels of TNF-α and high levels of IL-10, may be important in worsening the condition of patients affected with bacterial infections. Alternatively, as an excessive inflammation-related cytokine response during the initial and essential host response to an infectious challenge develops, it may lead to harmful, or even fatal, consequences, as seen in septic shock or multiple organ failure, that is, SIRS. In a life-threatening infection, systemic release of several proinflammatory cytokines is not properly regulated, and higher serum concentrations of inflammatory cytokines have been strongly implicated in the development of SIRS. Some studies demonstrated that serum levels of TNF-α have some relationship with the severity of tsutsugamushi disease. The TNF-α levels in the acute phase could predict the severity of this infectious disease. As TNF-α is essential for host defense, TNF-α underproduction may help proliferation of the pathogen, whereas its overproduction may be harmful to the host [104] (Figure 10).

3.2.3. Humoral Immunity. It is unclear if anti-*Orientia* antibodies, appearing early enough in infection, really affect the course of infection, or if passive immunization only reflects what might be a benefit of vaccination. However, humoral immunity might play a role in protective immunity to *O. tsutsugamushi* by inhibiting the events required in attachment, entry, and/or trafficking and replication in the cytoplasm. It has been demonstrated that antibodies enhance opsonophagocytosis of *O. tsutsugamushi* by professional phagocytes, such as macrophages and PMN. In addition, antibodies inhibit invasion of *O. tsutsugamushi* into non-professional phagocytes, such as ECs, epithelial cells, and fibroblasts [105]. However, neither cytokines nor antibodies alone enable macrophage cultures to completely suppress oriential infection. This indicates that both cellular and humoral immunity play a role in clearing *O. tsutsugamushi* by facilitating uptake and ensuing intracytoplasmic destruction of the bacterium. Although identification of protective antigens is important to understand homotypic and heterotypic immunity to scrub typhus, the molecules that play important roles in generating protective immunity are partially known. Antigenic heterogeneity among *O. tsutsugamushi* strains is so pronounced that whole cell vaccines prepared against one strain generally fail to protect mice against infection from others. *Orientia* lacks both peptidoglycan and LPS, containing the major strain-variable 56-kDa protein, as well as the antigenically variable 110-,

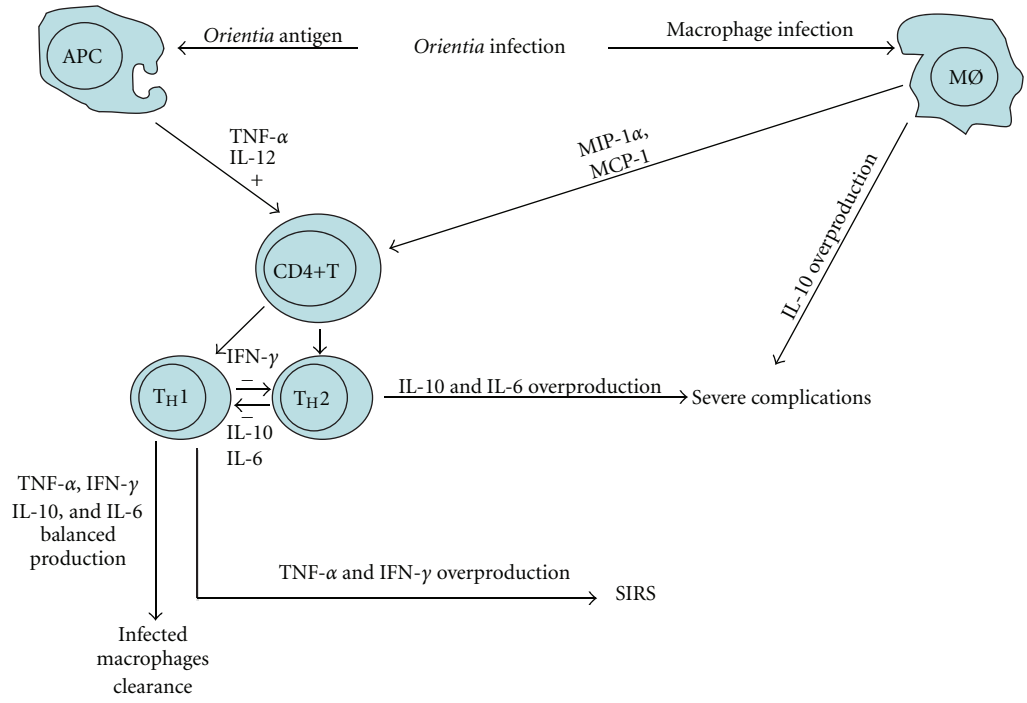

Figure 10: T lymphocytes and related cytokines in oriential infection.

47- and 25-kDa proteins. Among all antigens, the 56-kDa protein is often recognized by both human and animal host immune systems during infection. It is structurally and functionally nearly identical to the eukaryotes' protein known as Heat Shock Protein (Hsp) 60. There is substantial evidence indicating that heat shock proteins mainly serve as target molecules in the anti-infectious or autoaggressive immune responses. For these reason, the 58-kDa protein should be carefully considered as a recombinant vaccine candidate [106].

4. Ehrlichiosis

4.1. Target Cells. E. chaffeensis is confined to cytoplasmic membrane-bound vacuoles within monocytes/macrophages and DCs, replicating to form microcolonies, called *morulae*, that contain one to over 400 organisms. Morphologically, individual ehrlichiae are coccoid and coccobacillary and exhibit two ultrastructural cell types: a larger reticulate and a smaller dense-cored cell. *Ehrlichia* reticulate cells and dense-cored cells represent the bacterial replicating and infectious forms, respectively. The dense-cored cell form of *E. chaffeensis* binds to the host cell surface where it is rapidly internalised and completes the developmental cycle within 72 h [107].

4.2. Invasion, Injury, and Activation of Target Cells

4.2.1. Monocyte/Macrophage and Dendritic Cell Invasion. The presence of long-period tandem repeats distributed in intergenic regions of *Ehrlichia* is well recognised. They have also been found in a small group of ehrlichial proteins, named "tandem repeat proteins" (TRPs), that is, TRP120 and TRP47, associated with host-pathogen interactions, such as adhesion, internalisation, actin nucleation, and immune evasion [108].

Infection of the host cell involves dense-cored ehrlichiae that express TRP120 on the surface. It has important role in the binding and entry process, and a potential role has also been demonstrated for TRP47. Ehrlichiae binding to the host cell occurs through membrane receptors, such as E- and L-selectin and other glycosylphosphatidylinositol-anchored proteins located in caveolae (tiny indentations in the cell surface membrane), triggering receptor-mediated endocytosis that involves signalling transduction events, including transglutamination, tyrosine phosphorylation, phospholipase Cγ2 activation, inositol-(1,4,5)-trisphosphate (IP3)- production and increases in the intracellular calcium concentration. The vacuoles in which the organism enters contain caveolin-1, GM1 ganglioside, and phospholipase Cγ2. Later, after infection, vacuoles containing replicating ehrlichiae show the characteristics of early endosomes (i.e., the presence of Rab5A, early endosomal antigen 1 (EEA1), and vacuolar (H+) ATPase, together with vesicle-associated membrane protein 2, major histocompatibility class II and β2-microglobulin) and accumulate transferrin and its receptor. As a matter of fact, *Ehrlichia* survival depends on an available supply of intracellular iron, and the antiehrlichial activity of IFN-γ is mediated by limiting cytoplasmic iron availability (see below) [109–111] (Figure 11).

4.2.2. Modulation of Host Cell Gene Expression. Ehrlichia chaffeensis seems to actively modulate host cell gene transcription and function despite the fact that the bacterium

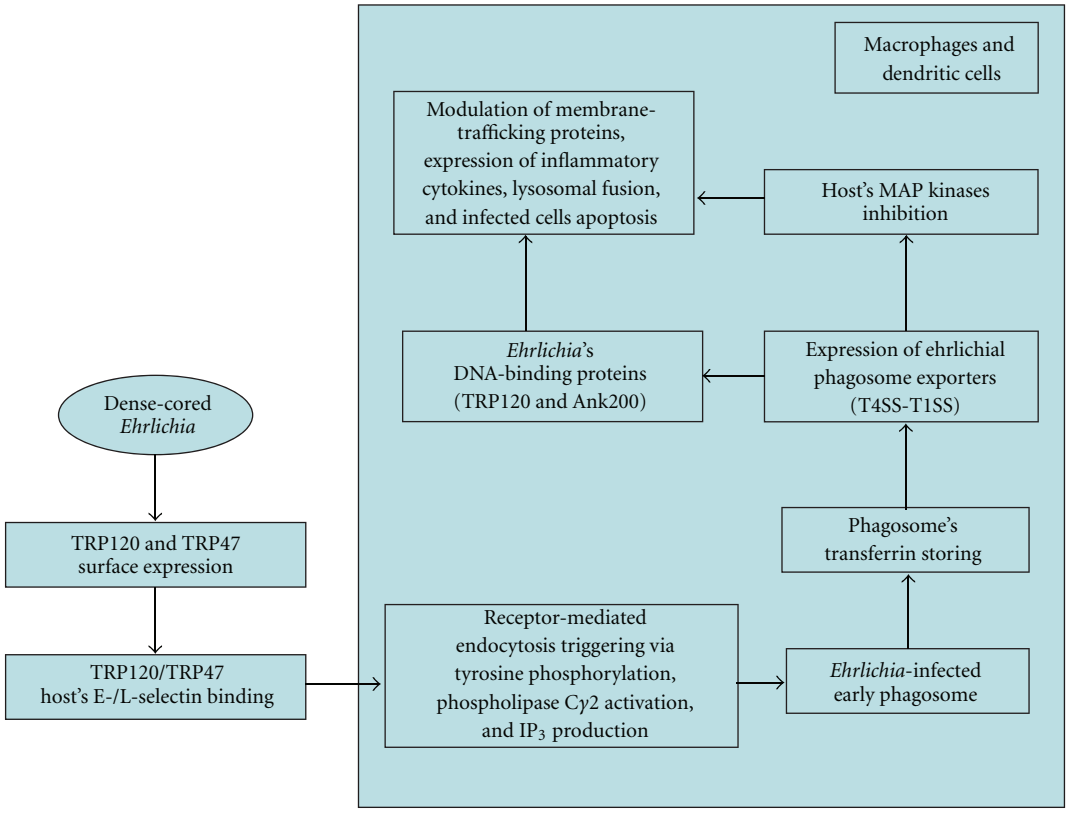

Figure 11: Ehrlichial monocytes/macrophages and dendritic cells invasion.

develops exclusively inside a vacuolar inclusion, separated from host cell cytosol by a host membrane. Therefore, host cell gene transcription modulation is related to secretion of specific ehrlichial effector proteins into the host cell cytosol, which requires active secretion systems. Such delivery mechanisms have been identified and include many of the known type IV secretion system (T4SS) components; Sec-dependent and Sec-independent protein export pathways for protein secretion across the membrane, as well as a putative type I secretion system (T1SS), have also been identified. A consequence of effector proteins-host-cell interaction is downregulation of transcription factors and transcription of target genes related to host defence and other cellular functions, at least in part by inhibition of host mitogen-activated protein (MAP) kinases. Discovery of *Ehrlichia* DNA-binding proteins (i.e., TRP120 and Ank200) provides another mechanism by which host cell gene transcription can be directly modulated. Specific cellular processes which appear to be modulated are membrane-trafficking proteins, expression of proinflammatory cytokines, and infected cell apoptosis [112, 113].

Ehrlichia chaffeensis lives in early endosome and inhibits its maturation to evade fusion with lysosomes and destruction by lysosomal enzymes. Moreover, *E. chaffeensis* inhibits transcription of genes involved in membrane trafficking and lysosomal fusion, such as Rab5, synaptosome-associated protein 23 (SNAP23), and syntaxin 16 (STX16) [114] (Figure 11).

Survival in mononuclear phagocytes requires the ability to evade the innate and adaptive immune responses (see also below). *Ehrlichia chaffeensis* inhibits transcription of cytokines involved in the early innate immune response and cell-mediated immune response to intracellular bacteria. As a matter of fact, this bacterium avoids stimulation of IL-12 production and represses IL-15 and IL-18 production; these cytokines play fundamental roles in stimulating T_H1 and NK cells to produce IFN-γ, which then promotes macrophages' killing of phagocytosed bacteria. IL-12 and IL-15 also activate destruction of cells infected with intracellular bacteria by NK cells and cytotoxic T lymphocytes. Thus, repression of IL-12, IL-15, and IL-18 may help *E. chaffeensis* to evade the host's innate and adaptive immunity [109].

Apoptosis is an innate cellular defence mechanism against microbes that is modulated by many bacterial pathogens, and there are new data that indicate that *Ehrlichia* also modulate host cell death. For most intracellular pathogens, apoptosis induction leads to pathogen killing and infection clearance, and this is thought to be beneficial to the host and to enhance the immune response to the infection. Delaying or preventing apoptosis could be a mechanism to enhance ehrlichial survival by preventing host cell death and subsequent immune recognition. In the case of ehrlichial infection, there seem to be several mechanisms involved in apoptosis modulation and inhibition. Modulation of genes associated with inhibition of apoptosis, cell cycle regulators, and mitochondrial function is observed early during *E. chaffeensis* infection and is probably necessary for delaying

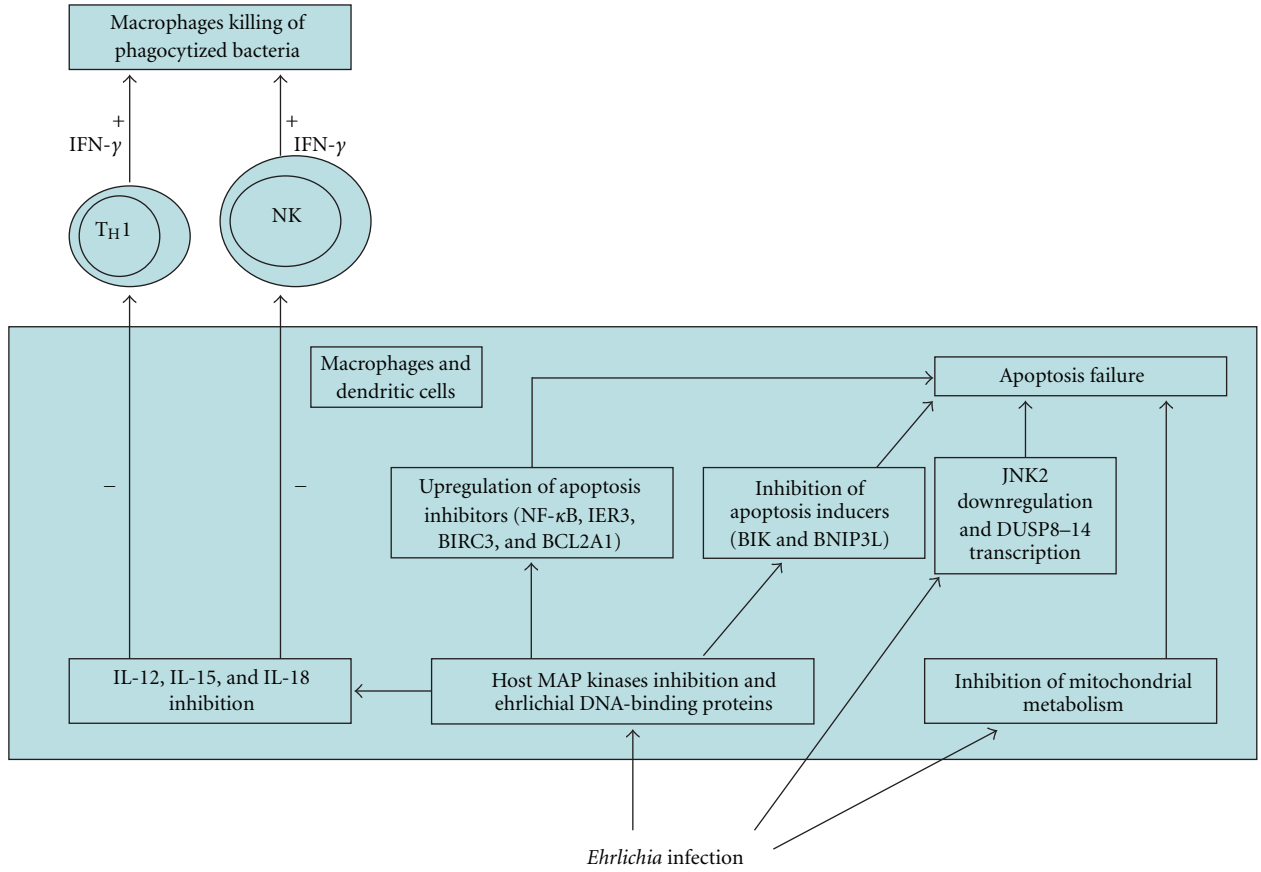

Figure 12: Modulation of host cell gene expression in ehrlichial infection.

host cell death. Particularly, *E. chaffeensis* infection both upregulates transcription of apoptosis inhibitors, such as NF-κB, IER3, BIRC3, and BCL2A1, and inhibits apoptosis inducers, such as BIK and BNIP3L, which initiate apoptosis by inactivating BCL2 proteins, thus impairing host cell apoptosis and maintaining a prolonged growth opportunity for ehrlichiae. At the same time, during the early infection stages, *E. chaffeensis* modulates genes related to the cell cycle, including cyclin G1 and CDC2-related protein kinases (CDKs). However, in the late stages of infection, *E. chaffeensis* upregulates cyclin E and CDC25, which activate cell replication. Thus, *E. chaffeensis* both arrests the host cell in the G1 stage during the early stages of infection to establish itself inside the cell and, in the late stages, upregulates cell proliferation to prevent the cell from dying because of progressive infection [115]. Finally, in *Ehrlichia*-infected cells, mitochondria are redistributed around *Ehrlichia morulae*, and mitochondrial metabolism is inhibited. A dormant mitochondrion may not trigger apoptosis in the infected cell. Thus, *Ehrlichia* recruits mitochondria to manipulate mitochondrial metabolism, which may result in host cell's apoptosis inhibition. Close apposition of mitochondria and *Ehrlichia* may be required for bacteria to effectively deliver proteins to mitochondria and inhibit the mitochondrial apoptosis pathway. In detail, *E. chaffeensis* downregulates JNK2 during the early stage of infection and upregulates DUSP8 and DUSP14, which dephosphorylate and inactivate JNK2. JNKs are basal in apoptosis regulation. As a matter of fact, absence of JNKs causes a defect in the mitochondrial death signalling pathway and cell apoptosis by inhibiting cytochrome *c* release [116] (Figure 12).

4.2.3. Exit Mechanisms. The mechanism by which *Ehrlichia* is released from host cells or transported between cells is not fully established. Recently, it was demonstrated that *Ehrlichia* is transported to neighboring cells through the host cell filopodium during the initial stages of infection but is released by local host cell membrane rupture adjacent to the morula during later stages of infection. Filopodia are thin cell surface protrusions containing bundles of parallel actin filaments. They are designed to explore the extracellular matrix and surfaces of other cells, identifying adhesion targets or navigating to its appropriate target. So cellular exit of pathogenic microorganisms could be supported by host cells' filopodia, and it has been observed that *Ehrlichia* uses this exit mechanism. An advantage of *Ehrlichia* transport through the filopodia is that the pathogen evades the host's immune system travelling cell to cell [117].

4.2.4. Ehrlichial Effectors and Molecular Host Interactions. Significant progress has been made in the identification of the host cell processes which *Ehrlichia* modulates. However, the effector proteins involved in manipulating these host cell

processes have been largely undetermined. Recent studies have focused attention on two small groups of *Ehrlichia*-encoded proteins: those containing tandem and ankyrin repeats. These proteins appear to be effectors involved in novel, complex, and multidimensional molecular strategies to reprogram host cell defence mechanisms. Particularly, two proteins, TRP47 and Ank200, have been emphasized in recent studies, demonstrating interactions with host cell's DNA and proteins associated with distinct host cell processes [118].

A recent study evaluating molecular interactions between TRP47 and host cells pointed out several interactions of this protein with several specific host cell targets. The strongest observed interaction between *Ehrlichia* and host proteins is between TRP47 and polycomb group ring finger 5 (PCGF5), which has important roles in regulation of HOX gene expression (involved in early embryonic development), X chromosome inactivation, maintenance of stem cell pluripotency, tumourigenesis, and stimulation of E3 ubiquitin ligase activity (involved in targeting proteins to be degraded within cells). Another interesting interaction is between TRP47 and immunoglobulin lambda-like polypeptide 1 (IGLL1), the surrogate light chain associated with the pre-B-cell receptor involved in signal transduction for cellular proliferation, differentiation from the pro-B-cell to pre-B-cell stage, allelic exclusion at Ig heavy-chain gene locus, and promotion of Ig light-chain gene rearrangements. Association of TRP47 with FYN tyrosine kinase, known to specifically phosphorylate caveolin-1 required for coxsackievirus internalisation and infection by caveolin-associated vesicles of epithelial cells, suggests that these proteins and their interaction might be involved in host cell attachment or entry. TRP47 also interacts with protein tyrosine phosphatase nonreceptor type 2 (PTPN2), also known as T-cell protein tyrosine phosphatase, which catalyses dephosphorylation of phosphotyrosine peptides and regulates phosphotyrosine levels in signal transduction pathways. Thus, it is involved in haematopoietic cell development and also in several human illnesses from autoimmune disease to cancer. PTPN2 has several substrates, including many JAK-STAT pathway proteins. Therefore, TRP47 might be involved not only in cellular developmental regulation but also in inhibition of IFN-γ-induced tyrosine phosphorylation of JAK and STAT proteins by interacting with PTPN2. Finally, TRP47 interacts with the multifunctional adenylate cyclase-associated protein 1 (CAP1), which has an active role in endocytosis, vesicle trafficking, actin turnover, and in apoptosis enhancement. Thus, in an effort to survive in the intracellular niche, *Ehrlichia* might manipulate cytoskeletal components of mononuclear phagocytes, such as actin, facilitating endocytosis and vesicle trafficking, and inhibiting apoptosis by modulating CAP1 activity [119] (Figure 13).

Ank200, one the four ehrlichial proteins with the ankyrin (Ank) repeat motif present in eukaryotic cells and in *Ehrlichia* species, has recently been detected in host cell nuclei of *E. chaffeensis*-infected cells, where it interacts with an adenine-rich motif in promoter and intronic *Alu* elements. *Alu* elements are short, interspersed mobile DNA elements distributed in a nonrandom scheme, which comprise approximately 5–10% of the human genome, and appear to be involved in transcriptional regulation. Global analysis of Ank200 binding sites demonstrates that this protein binds to several regions distributed on nearly every chromosome by direct DNA interaction or via other DNA-binding proteins. The host cell processes targeted by Ank200 include genes associated with transcriptional regulation, structural associations with the nucleus, and apoptosis. Several Ank200 target genes have been linked to pathogenesis and immune evasion, including JAK-STAT pathway proteins (see also above) and TNF-α. One of primary mechanisms by which *E. chaffeensis* survives in the host cell appears to be by blocking macrophage responsiveness to IFN-γ. Furthermore, JAK2 transcription appears to be silenced during *E. chaffeensis* infection, at least in part, by the the ehrlichial Ank200-host DNA interaction. TNF-α expression is not induced early in infection, but its expression is upregulated approximately 30-fold by the day 5 after infection. *E. chaffeensis* Ank200 might contribute to TNF-α induction by directly binding to the promoter and upregulating gene transcription. Overproduction and high serum concentration of TNF-α are closely associated with fatality in severe monocytotropic ehrlichiosis [115, 120].

4.3. Immunologic Transition from Innate to Adaptive Immunity

4.3.1. Ehrlichial Evasion of Innate Host Defences. *Ehrlichia* manipulates innate immune defence mechanisms, including apoptosis (see above), lysosomal fusion, production of reactive oxygen species (ROS), IFN-γ responsiveness by macrophages, and, in general, modulation of cell signalling pathways.

Phagolysosomes represent an important innate host defence mechanism against pathogens. *Ehrlichia* are able to inhibit fusion of phagosomes containing themselves with lysosomes and thus prevent their destruction by lysosomal enzymes. In this context, three two-component systems, named PleC-PleD, NtrY-NtrX, and CckA-CtrA, respectively, have an important role in preventing lysosomal fusion. These two-component systems, which control the response and adaptation to a variety of environmental conditions, consist of a sensor, associated with histidine kinase that detects environmental signals, and a cognate response regulator that has DNA-binding activity and regulates gene transcription. Cells treated with closantel, an inhibitor of the histidine kinase component, and incubated with *E. chaffeensis* have increased colocalisation between *E. chaffeensis* and lysosomes [121].

Ehrlichia chaffeensis is highly sensitive to O_2^- killing and lacks genes involved in detoxification of ROS. However, it actively blocks O_2^- generation and causes degradation of the NADPH oxidase subunit p22phox involved in ROS production. Degradation of p22phox is due to nonproteasomal proteolysis mediated by heat-labile factors or proteins from *E. Chaffeensis* [122].

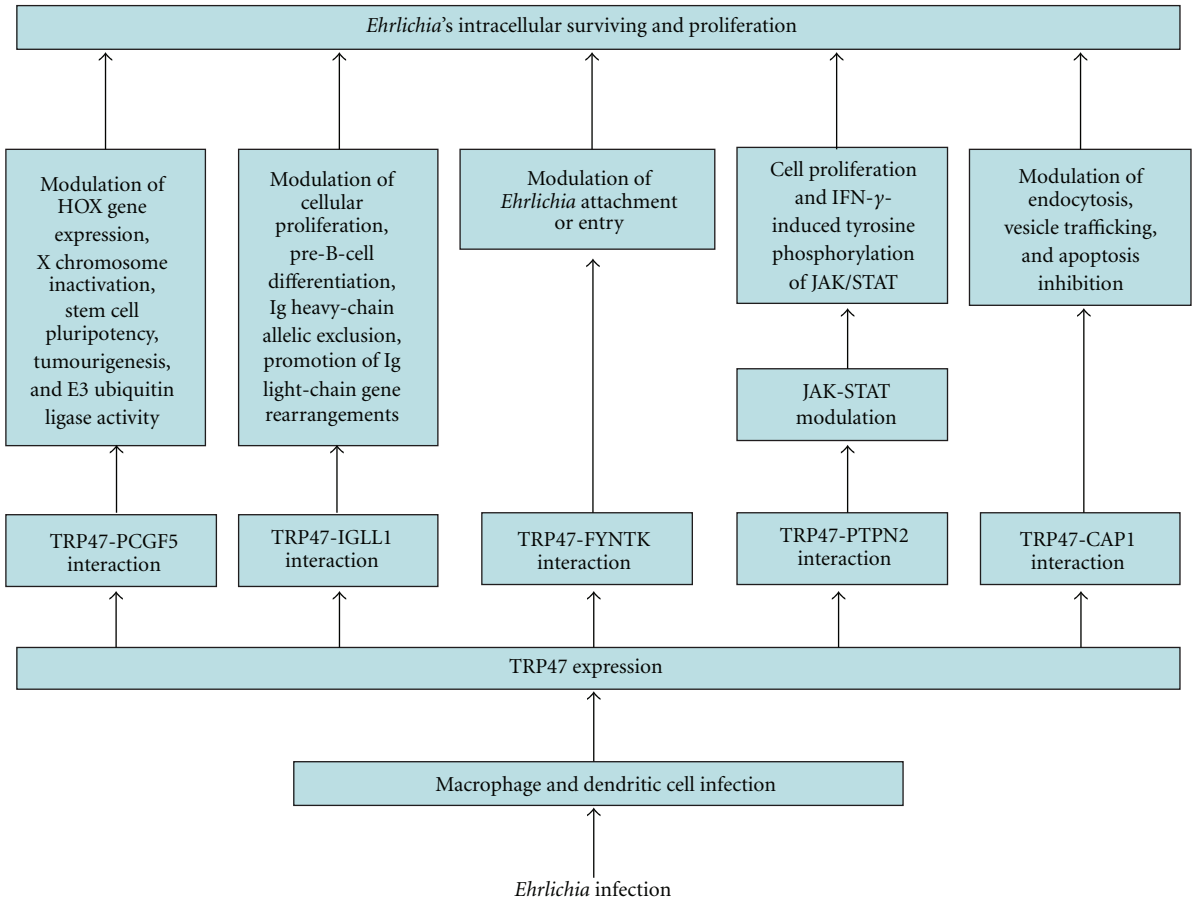

Figure 13: *Ehrlichia* effectors and molecular host interactions.

The macrophage-activating cytokine IFN-γ has an important role in innate and adaptive immune responses against intracellular pathogens. Activation of macrophages by IFN-γ induces several antimicrobial effector mechanisms, including regulation of iron homeostasis and ROS production. As aforesaid, the ability to acquire iron is important for survival of intracellular pathogens, such as *Ehrlichia*. The antiehrlichial activity of IFN-γ is mediated by limiting availability of cytoplasmic iron via reduction of surface transferrin receptor. However, *E. chaffeensis* upregulates transferrin receptor expression to counteract downregulation induced by the action of IFN-γ. The ability of *E. chaffeensis* to block the antimicrobial mechanisms of IFN-γ is mediated, at least in part, by inhibition of the JAK-STAT IFN-γ signal transduction pathway, that is, inhibition of tyrosine phosphorylation of JAK1 and STAT1, normally induced by IFN-γ and mediated by the ehrlichial protein TRP47, and/or transcriptional regulation of JAK-STAT expression by the DNA-binding protein and nuclear effector Ank200 (see below) [111, 115, 123].

Ehrlichia chaffeensis also appears to modulate the host innate immune response by influencing other cell signalling pathways. Antimicrobial activities of macrophages become progressively less responsive during *E. chaffeensis* infection in association with downregulation of pattern recognition receptors (PRR), including TLR2, TLR4, and CD14, which act as a PRR, and the activity of the PRR transcription factor PU.1, the activation of which has been linked to the p*38* MAPK-dependent pathway. Finally, *Ehrlichia* seems to target host tyrosine kinases and phosphatases, that is, FYN and PTPN2, but the downstream processes affected by these interactions are not known. In addition, *E. chaffeensis* infection downregulates protein kinases involving cell mobility and cytoskeletal changes, such as ITK, TXK, and PAKs (i.e., PAK1, PAK2, and PAK7) (Figure 14) [113].

4.3.2. Ehrlichial Immunopathological Mechanisms and Disease. During *E. chaffeensis* infection, there is a relatively low bacterial burden in blood and tissues in non-immunocompromised patients. However, the clinical manifestations, which may include fever, multiorgan failure, and adult respiratory distress syndrome, suggest that the pathogenesis of ehrlichiosis might involve immunopathological responses that are described as a toxic shock-like syndrome. Experimental lethal infection is accompanied by extremely high levels of serum TNF-α, high frequency of splenic TNF-α-producing CD8+ T cells, decreased *Ehrlichia-specific* CD4+ T-cell proliferation, low IL-12 levels in spleen, and decrease in the quantity of ehrlichial antigen-specific IFN-γ-producing CD4+ T_H1 cells. Recent studies have also

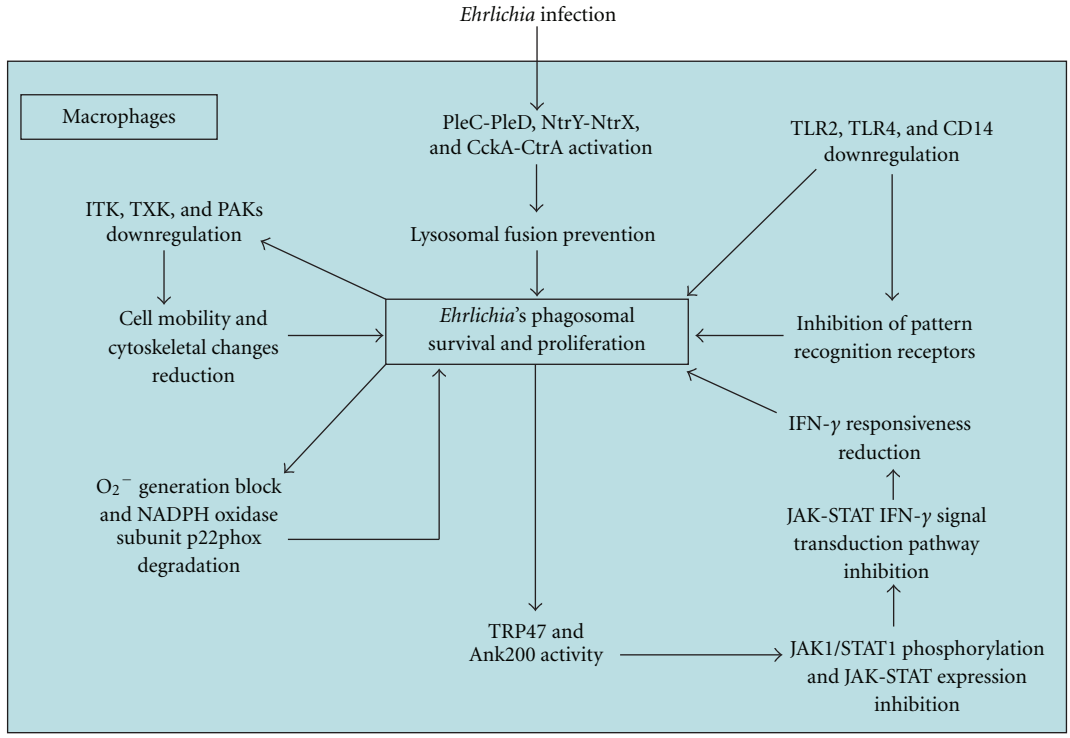

Figure 14: *Ehrlichia* evading innate host defences.

demonstrated that CD1d-restricted NK T cells, subsets of NK T cells that are key players in host defense against various microbial infections, might to be instrumental in induction of the immunopathological responses [124, 125].

5. Conclusion

Over the centuries, rickettsial, oriential, and ehrlichial diseases affected humankind all over the world, and they are still extremely widespread, remaining major health issues, with significant morbidity and mortality. They can be considered both reemerging and previously unrecognized diseases, likely owing to lack of in-depth epidemiologic surveys and application of appropriate tools for accurate diagnosis.

Advances in our understanding of *Rickettsia*-, *Orientia*-, and *Ehrlichia*-host interactions, target cells, virulence mechanisms, structures of bacterial effector proteins, upstream signalling pathways and signal transduction systems, modulation of gene expression, host immunologic responses, apoptotic elimination of infected cells, and, finally, immunopathological basis of clinical manifestations provided new targets for therapies to block host-pathogen interactions and pathogen virulence mechanisms.

Additional therapeutic approaches have been also identified, involving immunomodulatory therapies (i.e., antibodies-inhibiting interactions between effector proteins and host targets, inhibition of cytokine production, neutralization of cytokine effects, blocking specific cytokine receptors) and vaccine development, resulting in pathogen clearance by the immune system.

In rickettsial diseases, agents able both to quench harmful ROS production and to potentiate cellular protective mechanisms against oxygen radicals represent promising rationale for new and improved supplemental treatment. In this context, new therapeutic approaches in treating rickettsial infections and related vascular injuries are represented by the use of naturally occurring biologic antioxidants to preserve homeostasis at sites of vascular damage and by pharmacologic or genetic approaches targeting HO-1 or COX-2 in the vessel wall.

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