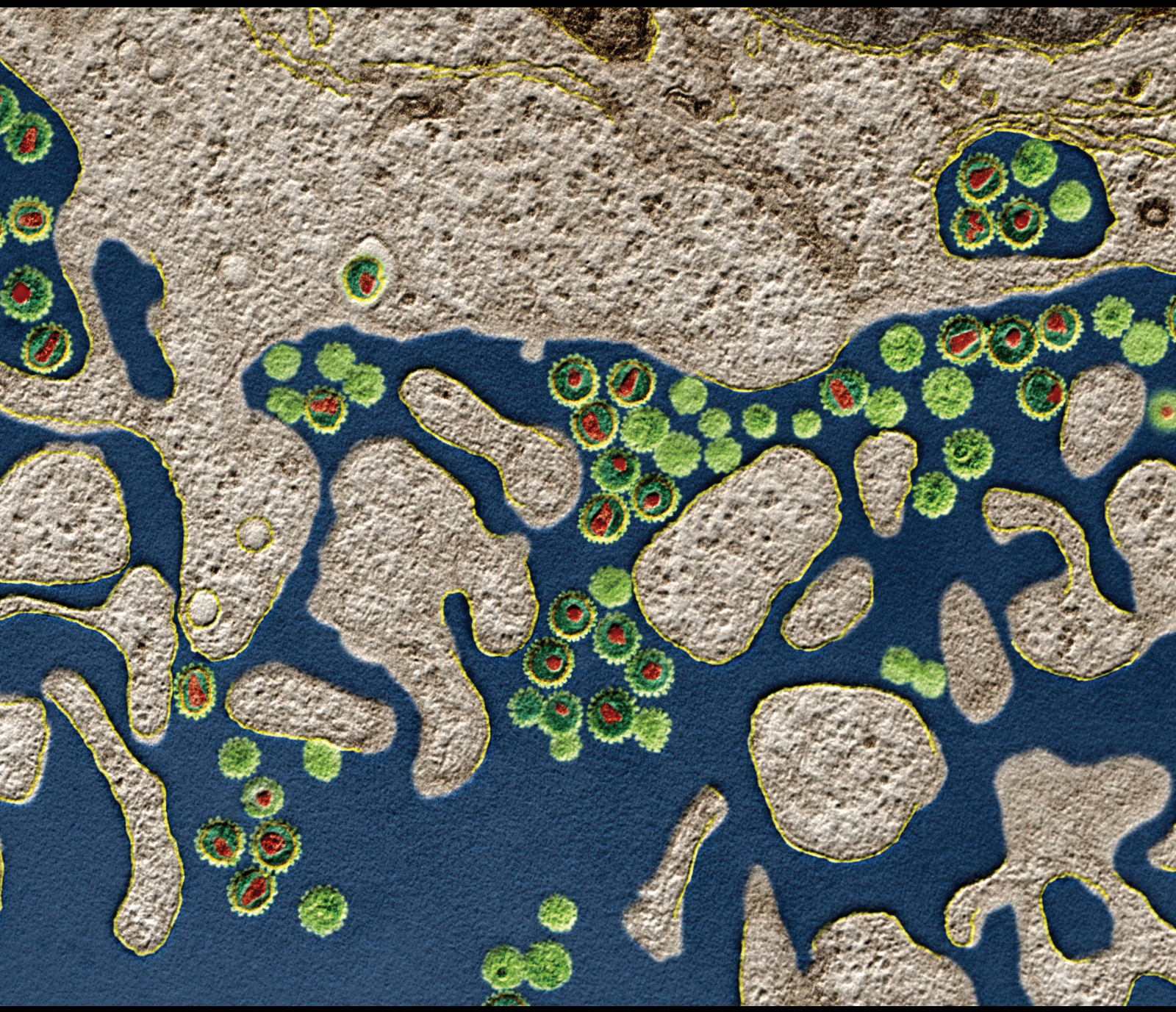
Journal of Immunology Research

Cellular Immunity-Pathogen Interactions in Infectious Diseases

Guest Editors: Muhammad Abubakar, Hasan T. Atmaca, Muhammad A. Zahoor, and Oguz Kul



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Editorial Board

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Editorial

Cellular Immunity-Pathogen Interactions in Infectious Diseases

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The interactions among host immunity, pathogens, and environment are ever-going process of disease and health. In nature, there is an ongoing equilibrium between infectious agents and their susceptible host organisms. While challenge of immune-competent individuals to low pathogenic strains usually results in elimination of the infectious agent, the other host-pathogen matches generally produce clinical courses of the disease. On the other hand, many pathogens produce certain enzymes, toxins, or proteins, which lead to the increased cellular adhesion and invasion abilities and also increased pathogenicity. However, in some instances, host innate immunity shows nonspecific resistance to such infectious diseases and it is called host resistance.

For example, *Bacillus anthracis* produces high mortality in its hosts, mammalian animals, including humans, but anthrax does not cause a fatal disease in the poultry. Otherwise, lineage 4 strain of the peste des petits ruminants which is prevalent in the Asian and Middle Eastern countries is responsible for the huge outbreaks among goat herds but not for sheep. These host resistance examples can be extended by host breed, age, and gender levels and the current knowledge has evidenced the presence of the disease resistance genes in the host genome.

The completion of full genome mapping in human and several animals allows the scientists to analyze host resistance genes against such a specific disease by the comparison of sick and healthy individuals' bioinformatics data. This means that the next discovery will be the definition of host resistance genes and their relations with cellular immunity in infectious diseases.

Keeping in view all these issues, we offered this special issue to the scientific community and we had an overwhelming response from them in form of 20 submissions including both research and review articles. Eight out of twenty submissions were accepted after a rigorous process of peer review.

This special issue incorporates four review papers and four original research papers. The review papers introduce concepts and themes underpinning the research papers including the immune invasion strategies by various pathogens including bacteria, virus, and parasite and also the interplay of host immune cells.

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Review Article

Helicobacter pylori and T Helper Cells: Mechanisms of Immune Escape and Tolerance

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Helicobacter pylori colonizes the gastric mucosa of at least half of the human population, causing a worldwide infection that appears in early childhood and if not treated, it can persist for life. The presence of symptoms and their severity depend on bacterial components, host susceptibility, and environmental factors, which allow *H. pylori* to switch between commensalism and pathogenicity. *H. pylori*-driven interactions with the host immune system underlie the persistence of the infection in humans, since the bacterium is able to interfere with the activity of innate and adaptive immune cells, reducing the inflammatory response in its favour. Gastritis due to *H. pylori* results from a complex interaction between several T cell subsets. In particular, *H. pylori* is known to induce a T helper (Th)1/Th17 cell response-driven gastritis, whose impaired modulation caused by the bacterium is thought to sustain the ongoing inflammatory condition and the unsuccessful clearing of the infection. In this review we discuss the current findings underlying the mechanisms implemented by *H. pylori* to alter the T helper lymphocyte proliferation, thus facilitating the development of chronic infections and allowing the survival of the bacterium in the human host.

1. Introduction

Helicobacter pylori is a human pathogen responsible for an infection involving nearly half of the world's population, frequently associated with chronic inflammation of the gastric mucosa that can lead to peptic ulceration and gastric cancer in particularly susceptible individuals [1, 2]. Infection is commonly acquired during childhood and, if not treated, the host can carry the bacterium even for life, mounting an innate and adaptive immune response which is unable to clear the pathogen [3]. Indeed the hallmark of *H. pylori* is its ability to escape host defence mechanism with several not yet entirely clarified strategies involving both the innate and adaptive immune systems of the host [4]. Many studies demonstrated that specific T helper (Th) cell subsets and their signature cytokines contribute to the control of the infection and sustain the development of the chronic inflammation. Most data support the critical role of these interactions in the pathogenesis of *H. pylori*-related diseases such as adenocarcinoma [5]. In this review, we discuss how *H. pylori* manipulates the responses of the T helper cells, avoiding its clearance by the host immune system.

2. The Interplay between *H. pylori* and the Effective T Helper Lymphocytes

2.1. T Helper-Mediated Cell Immunity in Chronic H. pylori-Induced Inflammation. Naïve T CD4+ helper (T CD4+) cells can be induced to differentiate towards T helper 1 (Th1), Th2, Th17, and regulatory (Treg) phenotypes according to the local cytokine milieu. T helper cells were historically divided into the two functional subsets, Th1 and Th2, characterized by distinct patterns of cytokine secretion. Th1 cells produce interleukin- (IL-) 2 and interferon- (IFN-) γ and promote cell-mediated immune responses, whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 and induce B cell activation and differentiation. In general, most intracellular bacteria induce Th1 responses, whereas extracellular pathogens stimulate Th2-type responses. Recently, the Th1/Th2 cell paradigm was enriched with another subset of T helper cells, called Th17, since they were identified as the source of IL-17. These cells are characterized as producers of IL-17A, IL-17F, IL-21, and IL-22 and are involved in host defensive mechanisms to various infections, especially extracellular bacterial infections, but also in the pathogenesis of autoimmune diseases [6].

Regulatory T cells (Treg) are naturally occurring T cells which are capable of suppressing effector T cell proliferation and cytokine production. Thereby they play a critical role in maintaining peripheral tolerance, moderate the immune response to pathogens by regulating the balance between immunity and inflammation, and prevent severe multiorgan autoimmune diseases [7]. The condition of chronic antral gastritis following *H. pylori* infection is characterized by a cellular inflammatory infiltrate which displays feature of both innate and adaptive immune response. Of the latter, the T CD4+ cells are considered the main actors in the establishment of chronic inflammation [8]. The adaptive immune response mounted by the host against *H. pylori* has been shown to include both Th1 and Th17 components, which are implicated in infection control through multiple pathways, as well as the Th2-derived cytokines, that have been detected in *H. pylori* infection although their role is not well understood [9].

2.1.1. Th1 Cells. Although the acquired immune response to *H. pylori* is composed of both Th1- and Th2-type cells, cytokine profiles indicate predominance of a Th1 response. Th1 are involved in immune response to many pathogens mostly by providing a source of IFN-γ. Indeed, as naïve T cells differentiate into Th1 cells, they will produce IFN-γ whose increased levels establish a Th1 dominant microenvironment and at the same time inhibit IL-2 production, which is necessary for Th2 response [10]. The Th1 proliferation in gastric mucosa infected by *H. pylori* involves signals provided by antigen-presenting cells and cytokines produced in response to the components of the pathogen, such as LPS, resulting in enhanced secretion of IFN-γ itself, IL-12, and IL-18 [11]. T-bet (T-box expressed in T cells) is a transcription factor that is required for differentiation of T CD4+ cells and their secretion of IFN-γ and hence holds a central role in the development of gastritis due to *H. pylori*. Mouse models have shown that T CD4+ cells from mice lacking T-bet fail to express IFN-γ and thus are limited to non-Th1-type responses [12].

2.1.2. Th2 Cells. Several reports indicated a role for Th2 phenotype in protection from infection. When a Th2 cell line from mice immunized/challenged with *Helicobacter felis* was transferred adoptively in naïve recipients before live bacterial challenge, they showed a dramatic reduction in bacterial load. On the other hand, increased numbers of bacteria were noted in IL-4-deficient mice [13]. Therapeutic mucosal immunization of mice with a recombinant *H. pylori* urease B subunit had been proven to induce progressively a Th2 cell response resulting in the elimination of the pathogen. Nevertheless, the mice did develop robust histologic gastritis upon challenge, consistent with a Th1-driven proinflammatory response, thus lessening the supposed role of Th2 pathway in preventing the *H. pylori* related diseases [14]. Consistent with these findings, Garhart et al. demonstrated that vaccine-induced protection is obtained in IL-4 deficient mice, suggesting that a Th2 response is not necessary for protection, although it could still play a role in the situation that some clearance mechanisms become redundant [15].

2.1.3. Th17 Cells. Many reports support the involvement of Th17 cells in the inflammation sustained by *H. pylori* in humans. Gray et al. showed that transfer of CD4+ T cells from mice that are deficient in IFN-γ or T-bet does induce gastritis, even if in a lower grade compared with C57BL/6 mice [16]. Thus, while IFN-γ clearly contributes to gastritis, Th1 cells do not appear to be essential, supporting the proinflammatory role of the IL-17. Also in human infection an enhanced production of IL-17 was found to perform a regulatory activity on the strong neutrophil chemoattractive cytokine IL-8, which plays a major role in the *Hp*-associated acute inflammatory response, as demonstrated by the significant inhibition of IL-8 production after neutralization of IL-17 [17].

2.1.4. Treg Cells. Since T effector cells are limited in their proliferation and function by Treg cells, elevated levels of Treg cells were verified in infected human gastric mucosa and also *H. pylori*-specific Tregs have been found in the circulation of infected individuals [18]. This evidence suggests that *H. pylori* colonization results in expansion of the Treg population and their recruitment to the site of infection limits the inflammatory response, thus representing a mechanism of pathogen persistence. However, reports investigating the role of Treg cells in *H. pylori* infection are controversial and far from a conclusion. Several studies suggest that the local Treg response protects the gastric mucosa from exaggerated inflammation and tissue damage, and the risk of *H. pylori*-related diseases is inversely related to Treg accumulation, even if the reduction of the inflammatory response achieved by Treg leads to increased bacterial density. The inability to mount a protective inflammatory response is responsible for the establishment of a chronic infection and in some patients for the development of atrophic gastritis and gastric cancer progression [19]. Other reports suggested a role for Tregs in modulating tumour growth. Indeed, Treg cells might be important in the cross-talk between neoplastic MALT B-cells and T-cells specifically activated by *H. pylori* antigens since the number of Treg cells had a positive influence in the response to antibiotic therapy [20]. These findings raised the possibility of a suppressive influence of these Tregs on the T-cell population responsible for tumour growth maintenance through the presentation of *H. pylori* antigens. Treg cells have been shown to downregulate the Th1 response toward *Bordetella pertussis* and *Leishmania major*, leading to prevention or retardation of pathogen eradication [21, 22]. In the context of *H. pylori* infection, Treg depletion led to an enhanced T CD4+ cell activation, which is responsible for the reduction in bacterial load. Consistently, *H. pylori*-infected mice lacking in Treg cells develop a severe gastritis, but not athymic mice, accounting for the suppressive role of Treg cells on Th1 response [23]. On the other hand, Treg depletion in BALB/c mice resulted in an increased production of Th2 cytokines by lymphocytes in response to *H. pylori* antigens, suggesting a skewing towards a Th2 response [24].

2.2. The Impaired Th1/Th2 Response as a Way of Escape for H. pylori. Studies based on animal models confirmed

the predominant Th1 phenotype, since infected or immunized/challenged mice demonstrated local and systemic production of IFN-γ and undetectable levels of IL-4 or IL-5. Cellular proliferation correlated with the severity of the gastritis score and *in vivo* neutralization of IFN-γ resulted in a significant reduction of gastric inflammation [25]. In humans, Th responses to *H. pylori* have been known for a long time to be strongly biased toward Th1. The pattern of cytokines produced by the immunologically active cells in the gastric antrum of infected individuals was analyzed and revealed the prevalence of IFN-γ, TNF-alpha, and IL-12, providing evidence for Hp-specific Th1 effector cells [26]. Despite these data, a growing number of reports, such as the one of Eaton et al. carried out in mice, suggest that this Th1-biased response is dysfunctional and may play an important role in pathogenesis of the *H. pylori*-related diseases [27]. The reasons for the impaired Th1 immune response could lie in the continuous process of virulence factor elaboration implemented by the bacterium over the thousands of years of coexistence with the human host.

2.2.1. The Vacuolating Cytotoxin Inhibits T CD4+ Cells Activation. The vacuolating cytotoxin (VacA), initially identified due to its ability to induce vacuolization of epithelial cells, has also been revealed as an inhibitor of T cells signaling and proliferation by inducing a G1/S cell cycle arrest through the interference with the T cell receptor/IL-2 signaling pathway at the level of the Ca2+-calmodulin-dependent phosphatase calcineurin. In this way, VacA avoids the nuclear translocation of nuclear factor of activated T cells (NFAT), the main regulator of the T cell pathway, resulting in the downregulation of IL-2 gene transcription [28]. Further experiments indicated that VacA suppresses IL-2-induced cell-cycle progression and proliferation of primary human T cells without affecting IL-2-dependent survival, but through its N-terminal hydrophobic region necessary for the formation of anion-selective membrane channels causing the arrest of the clonal expansion of T cells already activated by *H. pylori* antigens [29].

2.2.2. H. pylori Gamma-Glutamyl Transpeptidase and Arginase Impair T Cells Proliferation. A low-molecular-weight protein of *H. pylori* has been reported to inhibit proliferation of T cell lymphocytes by blocking cell cycle progression at the G1 phase through G1 cyclin-dependent kinase activity modulation [30]. Using functional experiments, Schmees et al. identified this suppression factor of T cells as the gamma-glutamyl transpeptidase (GGT) secreted by *H. pylori*. Since this enzyme mediates the extracellular cleavage of glutathione, with ROS production and consequently induction of cell cycle arrest in lymphocytes, the authors demonstrated that recombinantly expressed GGT showed antiproliferative activity while mutagenesis of GGT in different *H. pylori* strains completely abrogated this inhibitory effect [31]. An additional effective strategy of immune evasion implemented by the bacterium is attributable to *H. pylori* arginase, which is important for urea production by hydrolyzing L-arginine to urea and ornithine. Knowing that L-arginine is required for T cell activation and function, Zabaleta et al. incubated *H. pylori* wild type and arginase mutant bacteria with T cells and revealed that arginase caused a significant decrease in T cell proliferation by depleting L-arginine availability, but not in coculture with the arginase mutant strain. In addition, arginase inhibitors reversed these events. The results did not appear to be mediated by apoptosis because less than 10% of cells became annexin V positive in all experiments, but rather were correlated with a reduced expression of the chief signal transduction protein CD3ζ-chain of the T cell receptor (TCR), which is required for the initiation of T cell activation [32].

2.2.3. The Cytotoxin-Associated Gene Pathogenicity Island Induces T Cell Death. Another virulence factor of *H. pylori* involved in T cell function impairment is the cytotoxin-associated gene pathogenicity island (*cag* PAI), associated with a more aggressive phenotype of disease. Although the stimulation with viable strains of *H. pylori* with or without the *cag* PAI induced apoptosis in epithelial cells, T cell death was only observed using the *cag* PAI-bearing strains of the bacteria, through the induction of Fas ligand (FasL). This mechanism was found to be able to limit host immunity through the induction of T cell death in a Fas-dependent manner whereas inhibiting protein synthesis blocked FasL expression and apoptosis of T cells [33].

2.2.4. Modulation of Th1/Th2 Pathway by Cyclooxygenase and Indoleamine 2,3 Dioxygenase. Cyclooxygenase (COX) is an enzyme that catalyzes the conversion of arachidonic acid into prostaglandins. It exists in two isoforms, the constitutive isoform COX-1 and the inducible isoform COX-2, the latter being involved in the inflammatory response. Indeed, COX-2 activation suppressed Th1 polarization in response to *H. pylori* preparations in human peripheral blood mononuclear cells and has been shown to be upregulated in *H. pylori*-colonized gastric mucosa [34]. With this in mind, we provided evidence that an enhanced expression of COX-2 occurs during *H. pylori* colonization of the human stomach and may induce downregulation of Th1 signaling pathway, thus representing a mechanism by which *H. pylori* may actually interfere with normal T-cell activation in human gastric mucosa [35]. In a further study, we expanded this hypothesis showing that *H. pylori*-induced enhanced expression of indoleamine 2,3 dioxygenase (IDO) may modify the Th1/Th2 balance [36]. IDO is a heme-containing enzyme that catalyzes the first and rate-limiting step in tryptophan degradation via the kynurenine pathway. The consequent tryptophan starvation in the microenvironment limits T cell replication and induces T-cell apoptosis, hence impairing the Th1 response. Using functional experiments in *ex vivo* obtained gastric biopsies we demonstrated that the expression of IDO was higher in *H. pylori*-infected samples compared with uninfected samples and that its inhibition leads to increased levels of IFN-γ and T-bet while IL-4 production was reduced.

2.2.5. Stromal Factors Impair Th1 Response. Together with bacterial factors, local host factors can contribute to the permissive mucosal environment in *H. pylori* gastritis. Epithelial cells, resident immune cells, and lamina propria stromal cells

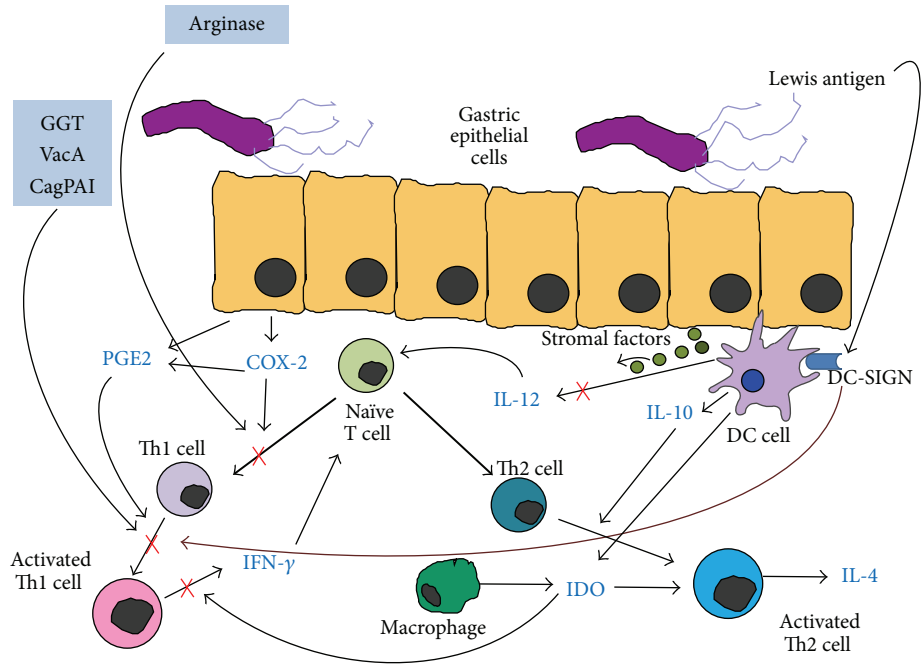

Figure 1: The biased Th1/Th2 cells response. The vacuolating cytotoxin (VacA) and the gamma-glutamyl transpeptidase (GGT) secreted by *H. pylori* inhibit Th1 cell proliferation by inducing a G1/S cell cycle arrest [28–31], while the cytotoxin-associated gene pathogenicity island (*cag* PAI) promotes Th1 death by the induction of Fas ligand [33]. The committed T naïve cells are unable to differentiate into Th1 line due to the enzyme arginase possessed by *H. pylori* [32]. *H. pylori*-induced cyclooxygenase- (COX-) 2 activation, alone and in conjunction with its product prostaglandin (PGE), suppresses Th1 polarization [34, 35, 39]. The *H. pylori*-induced expression of indoleamine 2,3 dioxygenase (IDO) limits the IFN-γ production by Th1 cells and favours the activation of Th2 cells [36]. Stromal factors suppress the IL-12 production by mucosal dentritic cells (DCs) [37], whose dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) receptor interacts with Lewis antigen expressed by *H. pylori* thus blocking Th1 cell recruitment [38].

secrete a series of cytokines, chemokines, and other soluble factors in the gastrointestinal mucosa, which are stored in the extracellular matrix, as a reservoir of immunoactive "stromal factors." Mucosal dendritic cells (DCs) are of particular importance in initiating the Th1 response to the bacterium, by releasing of IL-12. DCs were found to be suppressed in their activation against *H. pylori* by the presence of stromal factors in gastric and intestinal mucosa capable of downregulating DC responsiveness to *H. pylori* and thus resulting in a dampened gastric Th1 response [37]. *H. pylori* was found to induce tolerogenic DCs, unsuitable to elicit an effective and strong T cell recruitment also via the novel receptor for *H. pylori* on DCs called dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN). Indeed, Lewis antigen expression by *H. pylori* LPS was shown to bind to DC-SIGN C-type lectin present on gastric DCs and this interaction blocked Th1 cell recruitment, whereas Lewis-negative *H. pylori* strains enhanced Th1 cell development [38].

Prostaglandin (PGE) 2 is another important stroma-derived mediator, which, in addition to the inhibitory effect on *H. pylori*-induced DCs activation, has the capacity to downmodulate directly T lymphocytes. Indeed, in a mouse model, Th1 cells fail to migrate, proliferate, and secrete cytokines when exposed to PGE2 *in vitro* and *in vivo*, as the result of the silencing of interleukin-2 gene transcription [39].

The above-mentioned mechanisms, as a possible means of *H. pylori* evasion from the effective T helper lymphocytes, are summarized in Figure 1.

2.3. Does the Balance between Th17 and Treg Play a Key Role in the Tolerance? Recently it was assessed that *H. pylori* induces Th17 cell differentiation and impairment of this process could be involved in the shifting of T-cell responses which favours the persistence of the infection. IL-17 is the signature cytokine produced by Th17 cells and has a mediator role in the host inflammatory defence against bacterial and fungal pathogens, particularly at mucosal surfaces [40]. Although increased IL-17 expression is observed during chronic gastric inflammation, the levels produced are not sufficient to clear the infection. Accordingly, in *ex vivo* experiments on human gastric biopsy specimens, it was shown that IDO, which is highly expressed in *H. pylori*-infected gastric mucosa, downregulates IL-17 [36].

Commitment of Treg cells during *H. pylori* infection was demonstrated to be affected by B7 family ligands and their receptors, which are expressed on human epithelial cells and play important roles in the growth, development, and differentiation of T cells. At the same time, gastric epithelial cells (GECs) express enhanced levels of B7-H1 and this could

contribute to the suppression of CD4+ effector T cell activity and upregulation of Treg cells [41].

On the other hand, B7 family ligands are involved in Th17 response. Indeed, a subsequent study investigated the impact of *H. pylori* and its major virulence factor CagA on the modulation of B7-H2 in gastric mucosa. Using *in vitro* and *in vivo* studies, the authors showed that the downregulation of B7-H2 on GECs was operated by *H. pylori* through the presence of CagA cytotoxin, and that this fact correlated with the decrease in Th17 responses and the enhanced level of *H. pylori* colonization in mice [42].

Kao et al. suggest that a suboptimal Th17 response could lead to the failure of eradication and this would happen because *H. pylori* alters the DC-polarized Th17/Treg balance toward a Treg-biased response, thus suppressing the effective *H. pylori*-specific Th17 immunity. Using animal models, the authors showed that bone marrow-derived DCs pulsed with *H. pylori* skewed the response toward Treg differentiation by a VacA/CagA-independent, transforming growth factor-(TGF-) β/IL-10-dependent mechanism, rather than induction of a strong Th17 activation. Accordingly, the production of cytokines such as IL-17, IL-6, and IL-23 was found to be reduced. Moreover, functional experiments of Treg depletion showed the enhancement of the *H. pylori*-specific Th17 response. This correlated with decreased bacterial density and validated the major role of Th17 immunity in bacterial clearance [43]. Another report investigated the tolerogenic properties of *H. pylori* on DCs through the involvement of IL-18, a cytokine which acts directly on T cells and promotes their conversion to Tregs. The authors showed that the secretion of IL-18 is induced in DCs upon infection with *H. pylori* and suggest a key role for DC-derived IL-18 in skewing T cell differentiation away from Th17 and toward Treg responses [44]. The tolerogenic activity of DCs depends on inflammasome activation, which allows the release of interleukin-18 from preformed granules. Of note is the role of *H. pylori* in promoting the proteolytic processing of IL-1β and IL-18 induced by the inflammasome and caspase-1. This confirms the active presence of the bacterium in restricting pathogenic Th17 responses and favouring T-regulatory functions, supporting a pivotal role in the commitment of Treg cells [45]. New insights into the mechanisms underlying the development of *H. pylori*-associated complications derive from other experiments on Treg cells. Evidence that pathogens could take advantage of the suppressive function of Tregs on T effector cells has been shown previously in the context of *Leishmania major* and *Helicobacter hepaticus* infections, both conditions in which an increased number of Treg cells prevent the clearance of infection and limit the inflammatory response [22, 46]. However, the presence of chronic inflammation despite the existence of elevated numbers of Tregs suggests that these Tregs have impaired ability to suppress local inflammation.

Based on reports of elevated Treg numbers in *H. pylori* infected sites, a recent study investigated the direct and indirect effect of *H. pylori* on Treg proliferation and function *in vitro* as well as in gastric tissue biopsies from subjects infected with *H. pylori*. The hypothesis was that the bacterium is able to instruct DCs to stimulate proliferation of Tregs and that this happens together with a modulation acted by local factors which impairs the efficiency of Treg cells themselves. As expected, *H. pylori*-stimulated DCs drive Treg proliferation, but their suppressive function was expressed to a less extent and this was due to the production of IL-1β enhanced by the bacterium [47]. These data could conclude that Treg expansion in response to *H. pylori*-driven DCs is short-lived and the efficiency of Treg-mediated suppression might be expected to decline after the initial peak. Addressing the central role of IL-1β in mediating the effects of *H. pylori* on Tregs is of particular interest, because virulent strains of *H. pylori* expressing cagPAI are associated with elevated levels of IL-1β. In this context, further studies on polymorphisms in IL-1β could better define the mechanisms of immune evasion and interactions between *H. pylori* and the host.

The events described above could explain the state of tolerance implemented by the bacterium and are summarized in Figure 2.

2.4. The Immunological Context of Early and Late H. pylori Infection. Since most of infected persons acquire the bacterium during early childhood, the study of *H. pylori* infection in children offers the opportunity to investigate early mucosal responses to the bacterium in the human host. The majority of children are infected at a very young age and the risk of infection declines rapidly after 5 years of age. Age, a low socioeconomic status, limited living space, sharing of beds, a low parent education level, pollution of daily used water, and *H. pylori* infection in family members (especially the mother) are the known risk factors for infection [48]. Little is currently known about the immune response to the bacterium during early childhood, especially on factors promoting the spontaneous clearance of the infection that seems to be particularly high in this setting [49]. As in adults, gastric mucosal inflammation always characterizes *H. pylori* colonization in children although the degree of gastric inflammation is significantly less compared with that of adult subjects, in spite of the same *H. pylori* genotype and similar levels of colonization and CagA and VacA status [50]. Only a few studies evaluated the local cytokine profile in children. Results are somewhat conflicting, but they most consistently showed that *H. pylori* infection in children induces the production of proinflammatory cytokines according to a Th1 profile, similar to studies in adults, together with a higher IL-17 expression which correlated with bacterial density [51]. On the other hand, differences in mucosal immunopathology of infected children have been suggested. Lopes et al. found that local cytokine expression appeared to be smaller in *H. pylori*-infected children than in adults and did not correlate with antrum inflammation scores. Moreover, levels of IFN-γ were not so enhanced and moderate levels of IL-4, the main Th2 cytokine, were also found, in contrast to data from adult populations [52]. The fact that there was no clear Th1 dominance may indicate that children are more prone to mounting a gastric Th0 or Th2 response than adults or it may be due to a reduced capacity of T CD4+ cells from children to produce IFN-γ compared to adult T cells [53]. A study involving 245 children from Latin America showed a

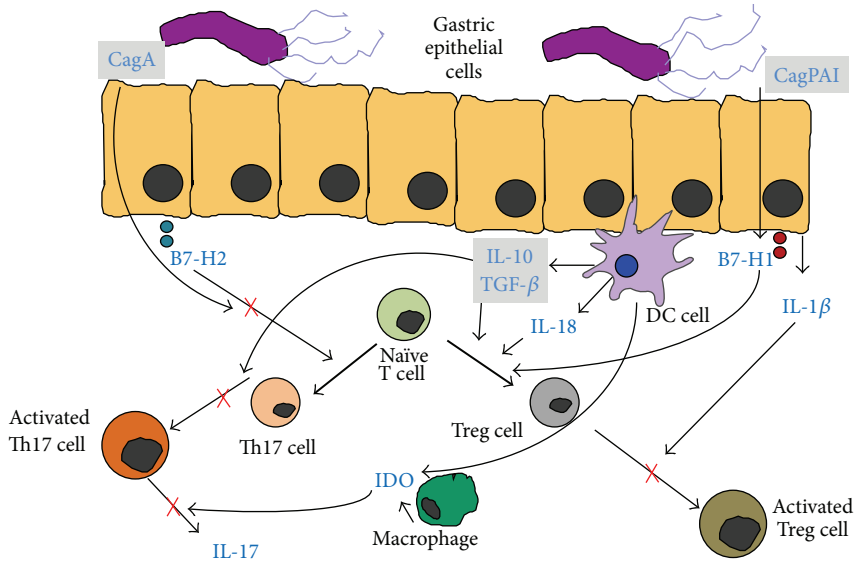

Figure 2: The impaired Th17/Treg balance. The induction of Treg cell differentiation is mediated by the overexpression of B7-H1 due to *H. pylori cag* PAI [41]. On the other hand, the CagA-induced lowered B7-H2 signaling inhibits Th17 cells development from naïve T CD4+ cells [42]. The higher expression of IDO downregulates IL-17 production [36]. DCs promote Tregs differentiation through higher production of IL-18 [44] as well as by a transforming growth factor- (TGF-) β/IL-10-dependent mechanism, which in turn blocks the Th17 cell proliferation [43]. The efficiency of Treg cells themselves is impaired by the production of IL-1β enhanced by the bacterium [47].

lower gastric concentration of IL-2 and IFN-γ in the infected children than in the infected adults, supporting the fact that Th1 immune response to *H. pylori* infection varies according to the age of the patients [54]. A reduced neutrophil accumulation in infected children gastric mucosa was found together with significantly lower levels of gastric Th17 cells and IL-17-specific mRNA and protein compared to infected adults [50]. Since IL-17 participates in the recruitment and activation of polymorphonuclear cells that are considered relevant to the clearance of the *H. pylori*, these findings may explain the lower degree of mononuclear and polymorphonuclear cell gastric infiltration observed in infected children than in adults, which is not attributable to differences in the gastric bacterium density. Nevertheless, further data has come from a parallel study recruiting infants, children, and adults. Indeed, PBMCs from infants showed the highest levels of production of IL-17 whereas cells from children produced slightly less and the lowest amounts were produced by adult cells from *H. pylori*-infected subjects. This could be due to the increased production of IL-1β by monocytes from infants, which is a strong activator of Th17 response [55]. A recent work compared the frequency of gastroduodenal ulcers in infected children and adults and investigated the effect of chronological age on severity of gastritis and also on NF-κB activation, a transcriptional factor for inflammatory genes induced by *H. pylori*. A positive correlation was found between age and densities of neutrophils and CD3, but not of CD8 or CD20 cells, while NF-κB-p65-positive cells were increased only in infected adults as well as NF-κB-binding activity. Moreover, peptic ulcer disease was less frequent in children than in adult infected subjects, maybe due to the lower mucosal immune response displayed in children [56]. Further intriguing data indicate that Tregs have an important role in regulating the early gastric mucosal inflammatory response to *H. pylori*. It seems that the gastric immune response is not only downregulated in children with *H. pylori* infection but also directed toward a sort of tolerance, which is relevant to the outcome of infection. Studies in animal models demonstrated that neonatally infected mice fail in the local and systemic responses to the bacterium but not adult-infected mice, providing evidence for a state of tolerance due to the induction of peripheral tolerogenic Treg, which efficiently control effector T-cell responses against *H. pylori* [57]. A Korean study demonstrated that the number of FOXP3-expressing Treg cells and the grade of TGF-β1 expression were significantly increased in *H. pylori*-positive children compared to the negative group and correlated positively with *H. pylori* density [58]. In children, the degree of generation of *H. pylori*-specific Treg cells seems to depend largely on the age at the time of infection, since *H. pylori* infected children have increased levels of FoxP3-expressing Treg cells and reduced gastric pathology compared with adults. Concurrent with the reduced gastric inflammation and the high number of Treg cells, the levels of Treg cytokines such as TGF-β and IL-10 were strongly increased in the gastric mucosa of *H. pylori*-infected children suggesting a pivotal role of Treg participation in the reduced Th1-mediated gastritis and ulceration in these subjects [59]. IL-23 participates in the expansion and maintenance of the Th17 lymphocytes. Children with *H. pylori* infection displayed lower gastric levels of IL-23 compared with adults, and this could prevent the amplification of the shifted Th17 cells, thus resulting in

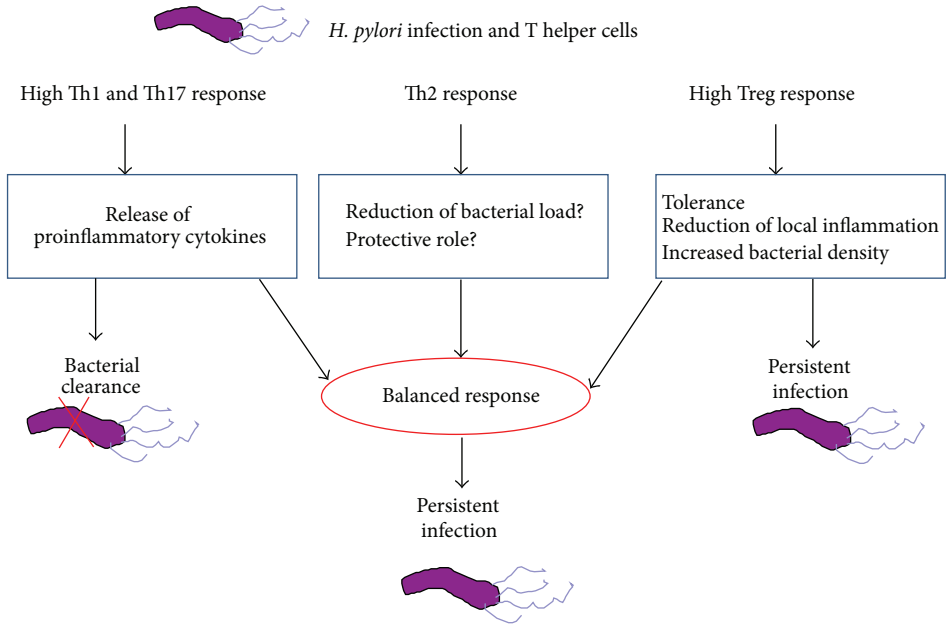

Figure 3: The interplay between *H. pylori* and the effective T helper lymphocytes. Although Th1 and Th17 pathways are both responsible for promoting inflammatory activities during *H. pylori* infection, neither Th1 nor Th17 cells are by themselves capable of a spontaneous clearance of the infection. It could be due to an impaired release of cytokines, suggesting that a more pronounced inflammation during the early phase of infection could switch the events towards eradication. Th2 response has been implicated in reducing bacterial load but its protective role is still controversial and deserves further investigation. Treg cells limit local inflammation and tissue damage but at the same time this fact favours a tolerogenic status which leads to a persistent infection. This complex interplay suggests that the conflict between persistent infection and clearance is decided in the early phase of infection.

Table 1: Children and adults *H. pylori*-related immunopathological features.

Feature	Children versus adults	References
Colonization level	=	[50]
Virulence factors (CagA/VacA)	=	[50]
Bacteria genotype	=	[50]
Polymorphonuclear and mononuclear cell infiltration	↓	[50]
Th2 response	↑	[52]
Th1 response	↓	[53]
T reg response	↑	[59, 60]
Th17 response	↓	[50]
Duodenal ulceration	↓	[56]
Gastritis score	↓	[56]

the predominance of Treg instead of Th17 cell differentiation and suggesting that the education of gastric Tregs to establish tolerance to the bacterium begins during early childhood infection [60]. Children and adults immunological features are summarized in Table 1.

3. Conclusions

H. pylori has coexisted with its human host for at least 30.000 years undergoing an evolutionary adaptation. In contrast to the majority of bacterial pathogens, which temporarily cause virulent disease and then are cleared by the pathogen-specific adaptive immune response, *H. pylori* successfully establishes a persistent infection in its host in spite of the presence of vigorous innate and adaptive immune response. The colonization can persist for decades or for life. *H. pylori* elaborated several evolutionary adaptations that allow the bacterium not only to escape detection by pattern recognition receptors on innate immune cells, but also to evade adaptive immunity. The host mediated immune response fails to clear *H. pylori* and also favours its colonization; hence bacterial virulence factors together with host factors determine the severity of disease. The role of T CD4+ cells is crucial in the immune response to the bacterium, with a Th1 polarized proliferation that was characterized first. Recently, studies showed that the impairment of this Th1 response may be implicated in maintaining the infection, along with the emerging role of other T CD4+ cell subsets, including Treg and Th17 cells. Although multiple studies investigated how all these events are possible, the full scenario is still unclear. Furthermore, little is currently known about the role of T cell subsets in controlling *H. pylori* infection in children, which with its intriguing mechanisms could provide a useful model into the early host response to the bacterium.

The exact role of these subsets of T helper cells in *H. pylori* infection is far from being fully understood. Indeed, the immunomodulatory properties of the pathogen reprogram the immune system towards immunological tolerance and *H. pylori* escape. Even if the Th1 and Th17 panel seems to be

involved in the proinflammatory activity of the bacterium, neither Th1 nor Th17 cells are by themselves capable of a spontaneous clearance of the infection. Realistically, a more pronounced inflammation during the early phase of infection could switch the events towards eradication. The increase in Treg cells and the parallel downregulation of Th1 response observed in children compared with infants suggest that the conflict between persistent infection and clearance is decided in the early phase of infection. In this context, Th2 response has been implicated in reducing bacterial load but its protective role is still controversial. On the other hand, the benefit in limiting local inflammation and tissue damage derives from the Treg pathway, but at the same time this leads to an increase in bacterial density and a persistent infection.

Studies on the relationship between *H. pylori* density and acute and chronic inflammation showed intriguing results but it is controversial whether the hallmark of protection is the degree of inflammation or the bacterial load. Indeed, *in vivo* depletion of Treg cells in infected mice was associated with an increased gastric inflammation and reduced bacterial colonization [61]. Enhanced inflammation in IL-10-knockout mice or in immunized mice leads to dramatic reduction of colonization and even clearance of *Helicobacter* species from the stomach [62]. However, a high grade of inflammation often progresses to gastric atrophy with disappearance of *H. pylori* from the stomach and development of an atrophic gastritis [63]. With regard to vaccination, the aim is to eliminate or reduce bacterial load when immunizing therapeutically and several reports showed that the immune responses are activated by the vaccination strategy, but there are few instances of achieving a reduced bacterial load [64]. Detailed studies on the protective T helper cell response have been published and have provided evidence that protection was dependent on Th1 responses. It is now generally accepted that the induction of increased inflammation by Th1 or Th17 cells is the starting point for a protective immunity [65]. Despite previous reports suggesting a protective role for Th2 cells [13, 14], more recent studies did not confirm this hypothesis [15, 66]. An intriguing argument is that protection could follow after an acute contact with *H. pylori*, sustained by an inflammation strong enough to clear the infection.

The events described above are summarized in Figure 3.

Conflict of Interests

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

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Research Article

The Potential Role of Th9 Cell Related Cytokine and Transcription Factors in Patients with Hepatic Alveolar Echinococcosis

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Human alveolar echinococcosis (AE) is a lethal parasitic infectious disease which may lead to liver failure if left untreated. It is caused by the larval stage of the fox tapeworm *Echinococcus multilocularis* and usually develops a substantial infiltrative occupation in solid organs. During the infection, T helper subsets are known to play crucial role in crosstalk between the parasite and human host. Th9 cells, a new member of $CD4^+$ T cell family which is characterized by its specific cytokine IL-9 and transcription factors PU.1 and IRF-4, have been known recently to have a critical role in allergic diseases, and cancers as well as the parasitic infection. To assess the potential role of Th9 cells during the infection, the mRNA levels of IL-9, PU.1, and IRF-4 both in peripheral blood mononuclear cells and in liver tissues were, respectively, detected by using real-time PCR. The plasma concentration levels of IL-9 were detected by using enzyme linked immunosorbent assay (ELISA). Th9 related cytokine IL-9 and transcription factors PU.1 and IRF-4 mRNA levels elevated both in PBMCs, and in hepatic lesion and paralesion tissues in AE patients. This may facilitate the infiltrative growth of the parasite and its persistence in human host.

1. Introduction

Human alveolar echinococcosis (AE) is a lethal parasitic infectious disease which may lead to liver failure if left untreated. It is caused by the larval stage of the fox tapeworm *Echinococcus multilocularis* and usually develops a substantial occupation in solid organs [1]. Liver is the most common targeted organ which accounts for nearly 95% of infections, with 5% percent to other organs including lung, brain, and bone. The parasite usually displays a cancer-like biological behavior and infiltrates to the liver tissue and major vasculature and is characterized by its granulomas formation. Previous studies have shown that $CD4^+$ T cell subsets play an active role in the development and immune response in AE and showed an upregulation of T helper (Th) 2 immune response which has been shown in the liver lesions locally and in circulating lymphocytes in animal models and patients. There is, however, evidence that cellular immunity and Th1 related cytokines also play a role by controlling totally the parasite growth in some individuals and limiting the size of the lesions in patients with the diseases. Clearly, Th1/Th2 imbalance plays an important role in controlling the immunopathogenesis of AE infection. Meanwhile, the immunological responses of host's Th17 and Treg cells against

AE have been extensively studied and displayed an important role of both T helper cells in immune tolerance and immune-pathological injury during the infection [2, 3].

Th9 cells, a new member of $CD4^+$ T cell family which is characterized by its specific cytokine IL-9 and transcription factors PU.1 and IRF-4, have been known recently to have a critical role in allergic diseases and cancers as well as the parasitic infection. Our previous study has shown that Th9 cells have been involved in the immune response on cystic echinococcosis [4]. However, whether this novel T cell subset is involved in regulating the immune response during the infection and development of AE is still unclear. Thus, we aimed to evaluate mRNA expression levels of IL-9, PU.1, and IRF-4 in PBMCs and circulating cytokine IL-9 in AE patients and healthy control, as well as the mRNA levels of IL-9, PU.1, and IRF-4 in lesion, paralesion, and normal hepatic tissues in hepatic AE patients in an effort to evaluate their potential role, if any, in the disease and their relation to disease activity.

2. Materials and Methods

2.1. Patients. This study was conducted in accordance with the Declaration of Helsinki (1964) and conformed to the approved institutional guidelines and was approved by the Ethical Committee of Xinjiang Medical University. Twenty-eight subjects were eligible for this study and informed consents were obtained from every single subject. They were divided into two groups: healthy control group (HC) and alveolar echinococcosis group (AE), with each group containing fourteen subjects. Healthy controls were qualified for medical testing and attended voluntarily, including 8 men and 6 women. All the patients with AE infection including 6 men and 8 women underwent surgery in the Department of Liver & Laparoscopic Surgery, First Affiliated Hospital of Xinjiang Medical University. The infection with *E. multilocularis* in all patients was confirmed by postoperative pathological examination.

All AE patients were classified according to the WHO/IWGE classification [5]. No patient was treated with anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs and corticosteroids. None had chronic inflammatory disease, cardiovascular disease, disseminated intravascular coagulation, advanced lung disease, renal failure, malignant disease, or other infectious diseases (such as septicemia and pneumonia).

2.2. Blood Samples. Blood samples were obtained from all the subjects in the recumbent position with a 21-gauge needle for clean venipuncture of an antecubital vein after admission in a fasting state on the following morning of the admission day. The samples were collected into collection tubes containing 0.2 mL sodium heparin. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient for analysis of real-time polymerase chain reaction (PCR). Plasma was obtained after centrifugation and stored at −80°C for the measurement of the cytokine and transcription factors.

2.3. Surgery and Liver Tissue Samples. All resections were performed via laparotomy. All surgical procedures were performed by three experienced hepatic surgeons. Experienced senior surgeons carried out all partial hepatic resections to guarantee at least one-centimeter safety margin. Frozen section examinations at the hepatic transsection line were performed in all cases.

All surgical specimens were reviewed by a senior pathologist. Clinical and pathologic staging were reassessed according to the World Health Organization on alveolar echinococcosis PNM classification of AE. Only one sample of lesion, paralesion (hepatic tissue within 1 cm from the AE lesion), and normal liver tissues was obtained for all the patients. The specimens had been snap-frozen in RNA later and liquid nitrogen for 15 seconds and were immediately stored at −80°C.

2.4. IL-9, PU.1, and IRF-4 mRNA Expression Determined by Real-Time PCR. Total RNA was extracted with TRIzol extraction (Invitrogen) according to the manufacturer's instruction. cDNA was synthesized using PrimerScript RT reagent kit (Takara, Biotechnology, Dalian, China). SYBR Green primers for human IL-9, PU.1, IRF-4, and GAPDH mRNA were purchased from Sangon, Shanghai. The samples were analyzed using the ABI Prism 7900 Sequence Detection System (BioRad, Life Science Research, Hercules, CA, USA). The following primer pairs were used: IL-9: F: 5′-CTCTAG CAGTCCACT TCACCAA-3′, R: 5′-ACAGCATGGGTC TGTCTTCT-3′; PU.1: F: 5′-GGAGCCCGGCTGGATGTT AC-3′, R: 5′-CACCAGGTCTTCTGATGGCTGA-3′; IRF-4: F: 5′-GACCCGCAGATGTCCATGAG-3′, R: 5′-TGTAGT TGTGAACCTGCTGGG-3′; GAPDH: F: 5′-GCACCGTCA AGGCTGAGAAC-3′, R: 5′-TGGTGAAGACGCCAGTGG A-3′. For each sample, mRNA expression was normalized to the level of GAPDH housekeeping genes (Table 1).

Both the housekeeping and the target genes for each sample were amplified in triplicate using the following procedure of initial denaturation at 95°C for 3 min, 39 cycles of 95°C for 10 s, 60°C for 30 s, and 65°C for 5 s, and after that they directly evolved to the ultimate temperature of 95°C. The $2^{-\Delta\Delta Ct}$ method was used to determine the cycle number Ct value corresponding to a specific fluorescence threshold and quantify the target genes.

2.5. ELISA Detection of Plasma Concentration Level of IL-9. The plasma levels of IL-9 were measured by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's protocols (all kits from Elabscience Biotechnology Co., Ltd.). The minimal detectable concentrations range was 15.625 pg/mL for IL-9, and the sensitivity was 9.375 pg/mL. Intra-assay and interassay coefficients of variation for all ELISA were <5% and <10%, respectively. All samples were measured in duplicate.

2.6. Statistical Analysis. Statistical analysis was performed using the SPSS 17.0, and values are expressed as median (P_{25} and P_{75}) in the text and tables. The data were analyzed by using nonparametric Friedman test. If significance was found, Wilcoxon signed ranks test and Mann-Whitney *U* test

Table 1: Primer sequence and amplicon of GAPDH, IL-9, PU.1, and IRF-4.

Gene	Sequences	Tm (°C)	Amplicon (bp)
GAPDH	F: 5′-GCACCGTCAAGGCTGAGAAC-3′	62.10	267
	R: 5′-TGGTGAAGACGCCAGTGGA-3′	61.70	
IL-9	F: 5′-CTCTAGCAGTCCACTTCACCAA-3′	59.70	112
	R: 5′-ACAGCATGGGTCTGTCTTCT-3′	58.64	
PU.1	F: 5′-GGAGCCCGGCTGGATGTTAC-3′	62.94	79
	R: 5′-CACCAGGTCTTCTGATGGCTGA-3′	62.00	
IRF-4	F: 5′-GACCCGCAGATGTCCATGAG-3′	60.53	82
	R: 5′-TGTAGTTGTGAACCTGCTGGG-3′	60.20	

Note. F: forward; R: reverse.

Table 2: Basic clinical characteristics of patients with hepatic alveolar echinococcosis.

Characteristics	HC ($n = 14$)	AE ($n = 14$)
Age (years)	36.5 (25.75–41.00)	38.0 (24.75–42.50)
Sex (male/female)	8 : 6	6 : 8
Location (right/left)	*N*	12 : 2
*Diameter of lesions (cm)	0	10.37 (7.9–14.21)
Previous operation	0 (14)	4 (14)

Values are expressed as median (P_{25} and P_{75}) or number; HC: healthy control; AE: alveolar echinococcosis; *for patient with multilesion, the average sizes were applied.

Table 3: The expression levels of IL-9, PU.1, and IRF-4 mRNA in PBMC in two groups.

	HC ($n = 14$)	AE ($n = 14$)
IL-9/GAPDH (copies/copies)	0.003 (0.002–0.008)	2.449 (0.693–5.706)***
PU.1/GAPDH (copies/copies)	0.771 (0.288–1.591)	313.105 (94.806–548.131)***
IRF-4/GAPDH (copies/copies)	0.0413 (0.0196–0.082)	21.583 (8.954–52.310)***

Values are expressed as median (P_{25} and P_{75}); HC: healthy control; AE: alveolar echinococcosis.

were performed to detect the difference among groups. Spearman correlation was used as a test of correlation between two continuous variables. Correlations were determined by Spearman correlation coefficients. A probable value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Basic Clinical Characteristics of Patients. Age, gender, diameters of lesions, and previous surgical history are listed in Table 2. There were no significant differences between the two groups. Four patients have undergone laparotomy for cholecystectomy in three patients and laparoscopic exploration in one patient at another institution. All patients in the AE group underwent hepatic resections. All AE patients were classified according to the PNM classification of WHO/IWGE as abovementioned. Among them, 12 patients were classified as P1N0M0 and the rest of them were classified as P2N0M0 (P1: peripheral hepatic lesion with no proximal hepatic vascular or biliary involvement; P2: central hepatic lesion with proximal involvement of vessels biliary ducts in one lobe; N0: no regional involvement; M0: no metastasis).

3.2. Expression of IL-9, PU.1, and IRF-4 mRNA in PBMCs in Two Groups. IL-9 is the main cytokine secreted by Th9 cells. PU.1 and IRF-4 are significant transcription factors for the differentiation of Th9 cells. We thus investigated the expressions of IL-9, PU.1, and IRF-4 mRNA in PBMCs from patients with AE and healthy controls. As shown in Figure 1 and Table 3, the expression levels of IL-9, PU.1, and IRF-4 mRNA were all significantly higher in AE group than those in HC group (***$P < 0.001$, ***$P < 0.001$, and ***$P < 0.001$, resp.).

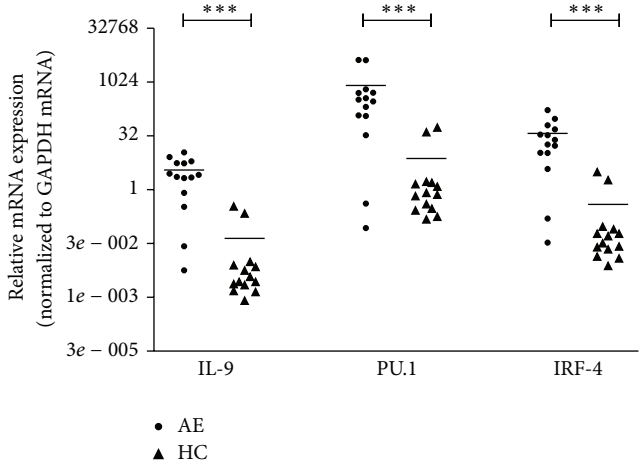

Figure 1: The expression levels of cytokine and transcription factors in PBMCs in AE and HC group. AE: alveolar echinococcosis; HC: healthy control. *P* value < 0.001 with marker ***; *P* value 0.001 to 0.01 with marker **; *P* value 0.01 to 0.05 with marker *; *P* value > 0.05 is not significant.

3.3. Expression of IL-9, PU.1, and IRF-4 mRNA in Different Liver Tissues of AE Patients. As shown in Figure 2, the relative

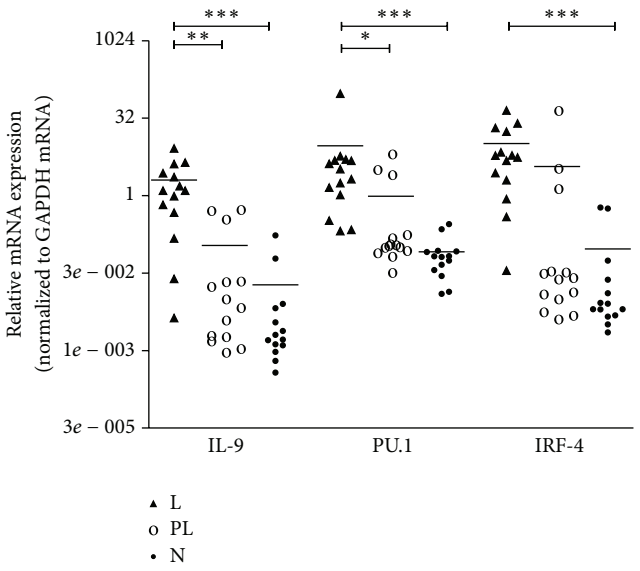

Figure 2: The expression levels of cytokine and transcription factors in different liver tissues. L: lesion group; PL: paralesion group; N: normal liver tissues. P value < 0.001 with marker ∗ ∗ ∗; P value 0.001 to 0.01 with marker ∗∗; P value 0.01 to 0.05 with marker ∗.

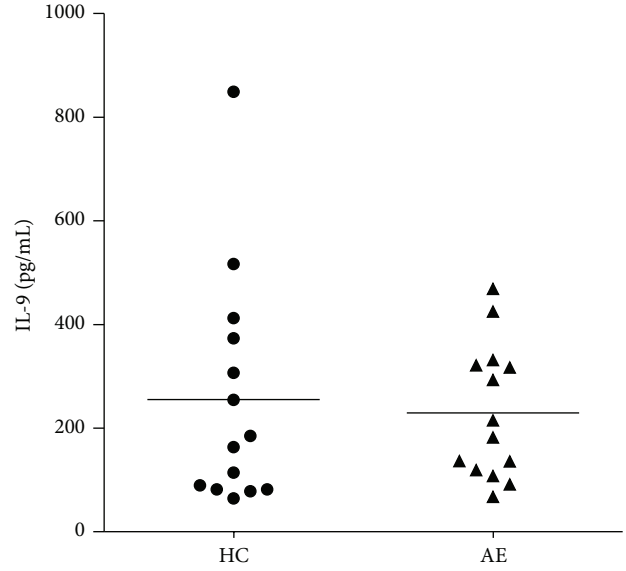

Figure 3: The plasma IL-9 concentration levels in AE and HC group. IL-9 concentrations in AE group were slightly lower than those in HC group, and there was no statistical significance found between the two groups ($P > 0.05$). HC: healthy control; AE: alveolar echinococcosis.

expression levels of IL-9 mRNA in lesion tissues (1.248; IQR, 0.389–3.071) were markedly higher than those in paralesion and normal tissues (0.008; IQR, 0.002–0.105; $^{**}P = 0.002$, 0.002; IQR, 0.001–0.007; $^{***}P < 0.001$), and there was no statistical significance between paralesion group and normal group ($P > 0.05$). PU.1 mRNA expression levels were elevated in lesion tissues (2.675; IQR, 0.851–4.865) as compared with those in paralesion and normal tissues (0.111; IQR, 0.085–0.782; $^{*}P = 0.013$, 0.065; IQR, 0.034–0.083; $^{***}P < 0.001$). Significantly elevated levels of IRF-4 mRNA were found in lesion group (0.025; IQR, 0.009–0.413) compared to those in normal group (0.007; IQR, 0.005–0.031; $^{***}P < 0.001$). However, there was no statistical significance between lesion group and paralesion group ($P > 0.05$).

3.4. Plasma Cytokine Concentrations in Two Groups. As shown in Figure 3, the plasma IL-9 concentration levels were slightly lower in AE group than those in HC group, and there was no statistical significance between the two groups ($P > 0.05$).

3.5. Correlation Analysis between IL-9 and PU.1 and IRF-4 mRNA Expression Levels in PBMCs and Liver Tissues. As showed in Figures 4(a) and 4(b), IL-9 mRNA expression in PBMCs had a positive correlation with PU.1 and IRF-4 mRNA expression ($r = 0.8228$, $^{***}P < 0.0001$; $r = 0.9332$, $^{***}P < 0.0001$, resp.). Spearman correlation coefficients indicated that IL-9 mRNA expression in the liver tissues had a positive correlation with both PU.1 and IRF-4 mRNA expression ($r = 0.7996$, $^{***}P < 0.0001$, $r = 0.4467$; $^{**}P = 0.003$, resp.) as shown in Figures 4(c) and 4(d).

4. Discussion

Along the years, the important role of both innate and acquired immunity in the field of *E. multilocularis* infection has been extensively studied. Among them, the role of $CD4^+$ T cells in the development of the parasitic lesion and immune evasion from human immune system's attack has been highlighted [6]. It is widely acknowledged that the parasitic infection can initiate a successful crosstalk between human immune system and parasite itself and actively modulate $CD4^+$ T cells development, thus guaranteeing its persistent survival. Both clinical and experimental studies displayed that *E. multilocularis* infection accompanies the imbalanced Th1/Th2 immune profile which is in favor of the parasitic survival [7–9]. Although different infection phase demonstrated various immune response patterns, the enhanced Th2 response and ameliorated Th1 response are considered to be linked with the survival of the parasite. Recent advances in the field of immunology have commenced new era for parasitic immunology. Further studies on novel T cell subset have unveiled the previously unknown phenomenon. Like other autoimmune diseases, cancers, fungal diseases, and parasitic infections, the reciprocal role of Th17 and Treg cells has drawn a great interest [10, 11]. Our previous animal and human studies have shown that the Treg cells overwhelmed immune response, although no significant alteration was found in terms of Th17 [2, 12]. As a new member of $CD4^+$ T cell family, Th9 cells have been known to play a significant role in some diseases, such as allergic diseases and cancers as well as the parasitic infections [13]. Furthermore, Th9 cells, through the expression of the effector cytokine IL-9, are more efficient than classical Th2 cells at orchestrating antihelminthic responses in helminthic infection in vivo [14].

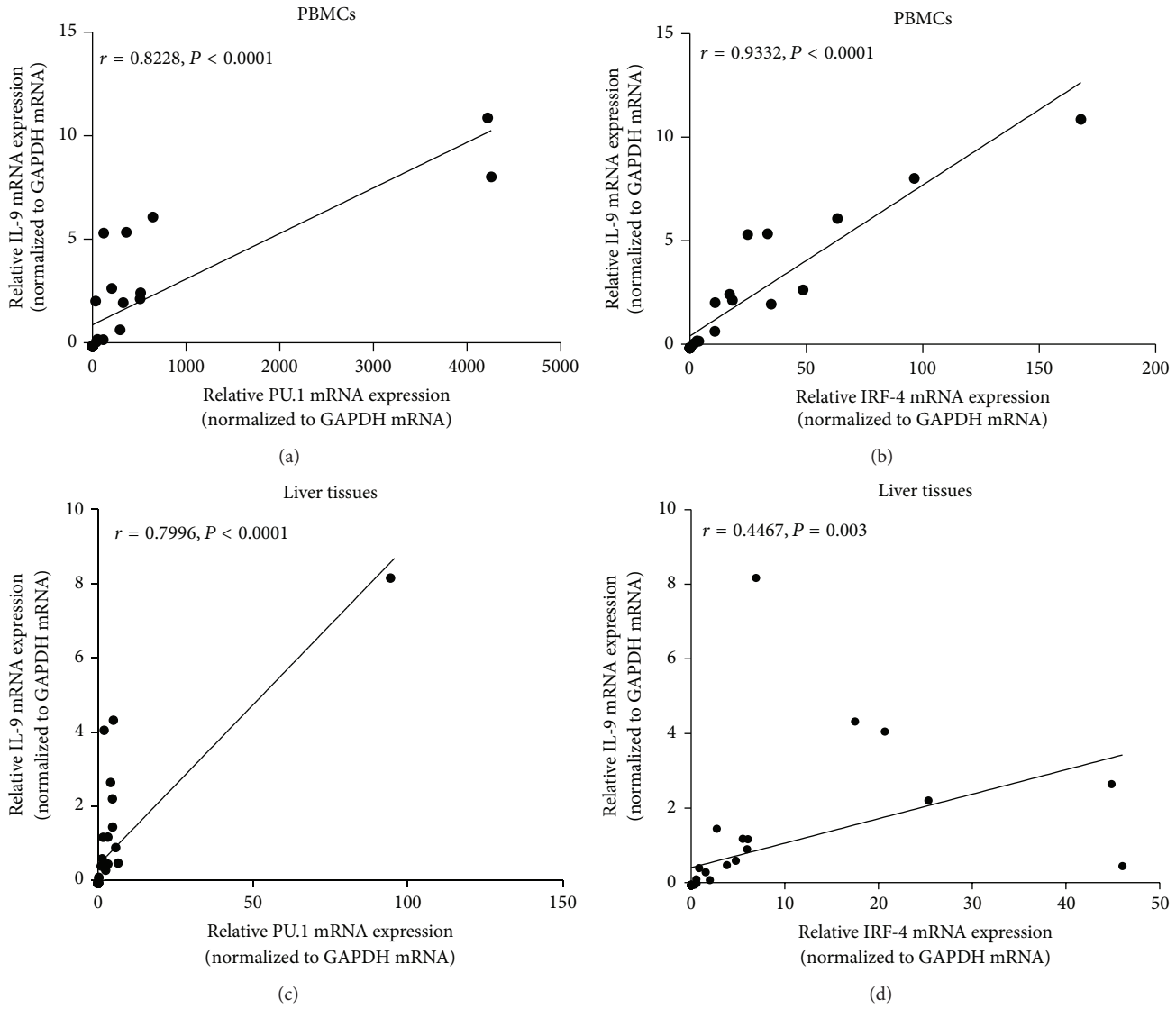

Figure 4: Spearman correlation of Th9 cells cytokine and transcription factors in PBMCs and liver tissues. ((a) and (b)) IL-9 mRNA expressions in PBMCs were correlated positively both with PU.1 and with IRF-4 mRNA expressions ($r = 0.8228$, $P < 0.0001$; $r = 0.9332$, $P < 0.001$, resp.). ((c) and (d)) The expression of IL-9 mRNA in liver tissues had a positive correlation with both PU.1 and IRF-4 mRNA expression ($r = 0.7996$, $P < 0.0001$; $r = 0.4467$, $P = 0.003$, resp.).

Our recent research has shown that Th9 and its functional cytokine IL-9 are upregulated in CE patients suggesting that they may be involved in regulating the immune response or even promote the process of *Echinococcus granulosus* infection [4]. However, whether Th9 cells also participate in the AE infection has not been studied yet, so we implemented this research to investigate its potential function in the AE infection.

Two decades before the description of Th9 cells, it was reported that IL-9 production by $CD4^+$ T cells depends on IL-2, is promoted by IL-4 and transforming growth factor TGF-β, and is further enhanced by IL-1, while IFN-γ represents a potent inhibitor of IL-9 expression [15]. As a great body of previous studies have revealed that IL-9 produced by Th9 cell promoted pathogenic processes in several autoimmune diseases, such as allergy and asthma [16]. Accumulating studies revealed a major role for IL-9 in protection against and expulsion of some helminthic parasites. As demonstrated in a recent study, IL-9 blockade prevents worm expulsion in mice infected with *Trichinella spiralis*, indicating that IL-9 was a pivotal cytokine to mediate effective expulsion of *T. spiralis* and was important for mediating an effective worm clearance [17]. In this study, IL-9 mRNA expression level was markedly increased in AE patient's PBMCs and, most intriguingly, the IL-9 mRNA expression levels were exponentially higher in AE lesions than in paralesion and normal liver tissues. However, the plasma IL-9 concentration levels in patients with AE were lower than in HC group

without statistical significance. This suggests that IL-9 may be involved in AE infection process and may have a potential role in parasite clearance as shown in previous studies [4].

PU.1 and another transcription factor IRF-4 are key transcription factors that induce differentiation of Th0 cells into Th9 cells. PU.1 plays a crucial role in the regulation and expression of CD80, CD86, and cells in the hematopoietic system and it can also block several autoimmune diseases and even induce donor-specific tolerance to allograft [18]. A recent research showed that modifications in the PU.1 promoter uniquely and dynamically control the threshold for Th9 cells development in naive and memory $CD4^+$ T cells [19]. Detailed analyses revealed that some cytokines play a pivotal role in Th9 development and function by inducing the expression of PU.1 [20]. Some studies have described PU.1 as a unique regulator of Th9 memory acquisition and Th9 immunity [21]. It has been reported to determine Th9 cells development and can enhance or be required for IL-9 production in Th9 cells [22]. T cell specific deletion of PU.1 results in decreased IL-9 production in vitro, as well as in vivo, coincident with diminished allergic inflammation [23]. IRF-4 was also found to be crucial in Th9 response, as IRF-4 deficient $CD4^+$ T cell failed to develop into IL-9-producing Th9 cells, and IRF-4 RNAi knockdown suppressed IL-9 production in WT $CD4^+$ T cells. Furthermore, chromatin immunoprecipitation analysis indicated that IRF-4 directly bound to the IL-9 promoter in Th9 cells [24].

Our previous study on cystic echinococcosis, which is caused by *E. granulosus*, has showed a distinct increase of both IL-9 and PU.I in infected patients' PBMCs [4]. In line with this, this study for the first time displayed the dramatic highly elevated levels of IL-9, PU.1, and IRF-4 mRNA in both PBMCs and hepatic tissues, albeit with no clear alteration in plasma levels. Hepatic AE patients in this study were all categorized according to the PNM classification of WHO/IWGE and 12 were categorized P1N0M0 and two were P2N0M0. All the cases were progressive AE patients. The regional discrepancies of IL-9, PU.1, and IRF-4 mRNA are quite clear in parasitic lesion, paralesion, and normal tissues. This may be because the parasite itself and its functional molecules modulate the systemic and regional T helper cell differentiation thus creating Th9 dominating immune response which is now known to be in favor of parasitic survival and, thus, may play a role in immune response and granuloma formation during the AE infection. Furthermore, as revealed in the correlation analysis in this study, IL-9 expression levels were positively correlated with the levels of PU.1 and IRF-4 expression, respectively, both in PBMCs and in local hepatic lesion tissues. These details further confirmed the significant roles of PU.1 and IFR-4 in the differentiation and function of Th9 cells.

In conclusion, the results reveal a rising trend in Th9 levels in both local and PBMCs in patients with AE. Moreover, a recent study implicated a potential role of Th9 cells promoting pathology in filarial disease and suggested it is dependent on the effect of IL-9 on promoting inflammatory responses in target cells [25]. These details suggest that Th9/IL-9 were involved in the AE infection and response during the *E. multilocularis*-host interplay and might play critical potentially beneficial roles in the parasite survival and pathogenesis in AE infection.

In our study, we disclosed for the first time that Th9 cell and its signature cytokine IL-9 and transcription factors PU.1 and IRF-4 were upregulated in peripheral and local hepatic tissues in AE patients. Our research strongly indicates that Th9/IL-9 may be associated with development of *E. multilocularis* infection and even may play a critical potential role to facilitate the infiltrative growth of the parasite and its persistence in human host. Further studies should be implemented for exploring the exact mechanism, and we believe it will deserve further attention in future studies.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

Conflict of Interests

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

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Research Article

The Interplay between Zinc, Vitamin D and, IL-17 in Patients with Chronic Hepatitis C Liver Disease

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Objectives. To assess zinc (Zn) and vitamin D (Vit. D) status in chronic Hepatitis C virus- (HCV) infected patients and their relationship to interleukin- (IL-) 17 and disease severity and then investigate whether Zn and Vit. D3 modulate IL-17 expression in chronic HCV patients. *Methods*. Seventy patients and fifty healthy subjects were investigated. Serum levels of Zn, Vit. D, and IL-17 were assessed in the patients group and subgroups. Patients lymphocytes were activated *in vitro* in the presence or absence of Zn or Vit. D3 and then intracellular IL-17 production was assessed using flow cytometry. *Results*. Zn and Vit. D were significantly decreased in HCV patients. Increasing disease severity leads to more reduction in Zn level opposed by increasing IL-17 level. Zn potently reduced IL-17 production in a dose-related fashion; however it did not exert any toxic effects. Although Vit. D apparently increases IL17 expression, it is unclear whether it is due to its toxic effect on cell count or lack of definite association between Vit. D and both IL-17 and disease severity. *Conclusions*. This study demonstrates that Zn modulates IL-17 expression and provides a rationale for evaluating this compound as a supplementary agent in the treatment of chronic HCV.

1. Introduction

Hepatitis C virus infects primarily the hepatocytes, leads to the development of fibrosis or cirrhosis of the liver, and is a significant risk factor for the development of hepatocellular carcinoma (HCC). Previous studies have demonstrated that T cell immunoregulatory cytokines contribute to liver damage [1].

Human interleukin-17 (IL-17) producing CD4 T cells, Th17, comprise a proinflammatory T cell subset. Previous studies have identified Th17 as a known arm of the $CD4^+$ T-cell effector response [2] and several key cytokines, including IL-1, IL-6, tumor necrosis factor alpha, and IL-23, create a cytokine milieu that regulates the differentiation and expansion of human Th17 cell [3]. IL-17A can mobilize, recruit, and activate neutrophils, leading to massive tissue inflammation, and promote the progression of autoimmune disease. Furthermore, serum IL-17 levels are increased and serve as a marker of the severity of acute hepatic injury [4, 5].

Zinc, one of the essential trace elements, is required by many enzymes and transcription factors for their activity or the maintenance of their structure. It has a variety of effects in the immune system. Zn deficiency causes an imbalance between Th1 and Th2 function in periphery. Production of IFN-gamma and IL-2, Th1 products, is decreased, whereas production of IL-4, IL-6, and IL-10 Th2 products is not affected [6].

The main zinc metabolism occurs in the liver hepatocytes. In patients with Zn deficiency, reduced Zn concentrations in the liver are one of the causes of impaired hepatocytes regeneration [7]. It has been demonstrated that Zn may play an important role as a negative regulator of HCV replication in genome length RNA-replicating cells. Thus zinc supplementations appear to offer a novel approach for further strategies in treatment of intractable chronic hepatitis C [8]. Zinc uses are claimed to suppress Th17-mediated autoimmune diseases at least in part by inhibiting the development of Th17 cells via attenuating STAT3 activation [9].

Vitamin D is emerging as a critical factor involved in the regulation of the immune system, inflammatory response, and fibrogenesis [10]. Several studies on Egyptian patients with hepatitis C virus showed a significant reduction of Vit. D and its active metabolite in HCVg4-infected patients compared to healthy controls [11, 12]. Moreover they found a significant negative correlation between viral load and Vit. D status. Interestingly, low vitamin D levels have been related to poor liver function and stage of cirrhosis [10, 13]. Vitamin D was shown to reduce the expression of collagen and profibrotic factors leading to decreased fibrosis [14]. The effect of Vit. D on the behavior of Th17 cells has been investigated in different diseases and Vit. D suppresses the expression of IL-17 and IL-23 [12, 15, 16], the regulatory effect on Th17 cells by Vit. D occurs through the reduction of retinoic acid-related orphan receptor (ROR)γt expression [15]. Therefore, this study was designed to assess Zinc and Vit. D status in chronic HCV-infected patients and its relationship to levels of IL-17 as immune inflammatory mediators and to clarify the effect of Zn and Vit. D in modulating the expression of IL-17 *in vitro*.

2. Materials and Methods

2.1. Subjects. Prior to initiation, this study received approval by the Ethical Committee of the Faculty of Medicine, Ain Shams University. The study recruited 70 patients with chronic HCV infection and they were diagnosed retrospectively by positivity of PCR and enzyme-linked immunosorbent assay (ELISA) HCV antibodies, who were selected from outpatients and inpatients of Internal Medicine and Tropical Medicine Departments at Ain Shams University Hospitals. In addition fifty healthy normal persons matched for age and sex as a control group were also included in the study. Inclusion criteria were based on a history of liver disease with HCV genotype 4 infection (as new patients or under follow-up). Patients with other causes of viral hepatitis: hepatitis B virus (HBV) or coinfection with HBV and human immunodeficiency virus (HIV), cytomegalovirus (CMV), and Epstein–Barr virus (EBV), or having criteria suggestive of fatty liver: Body Mass Index (BMI) >35, uncontrolled DM (HbA1c > 7), or history of taking hepatotoxic drugs for the previous 6 months, were excluded. BMI was calculated as weight in kilograms divided by the square of height in meters [17]. (Obesity was defined as BMI > 30 kg/m^2.)

All included patients underwent tests for liver function (ALT, AST, and bilirubin using Beckman Synchron CX7 Delta Clinical System, prothrombin time, and INR using stago analyzer), assessment of HCV levels, and abdominal ultrasonography for detection of cirrhosis. Based on ultrasonography results and liver function tests, the patients were classified into three subgroups: *Group 1* includes 16 recently diagnosed chronically HCV-infected patients showing no evidence of hepatic cirrhosis or liver cell failure. *Group 2* includes 37 compensated chronically HCV-infected patients showing an evidence of hepatic cirrhosis but no evidence of liver cell failure. *Group 3* includes 17 decompensated chronically HCV-infected patients showing an evidence of hepatic cirrhosis and liver cell failure.

2.2. Measurement of Serum Interleukin-17, Zinc Level, and 25-OH Vitamin D. After subclassification, venous blood samples (5 mL) were obtained (after overnight fasting) from all patients and controls. Samples were allowed to clot and sera were then separated by centrifugation (3500 rpm, 20 min, 25°C) and then stored at −20°C until used for serum analysis of the various parameters outlined below.

Commercially available ELISA kits (Labs Biotech, Inc, USA) were used for quantitative analysis of interleukin-17 while determination of zinc level was done by zinc colorimetric method (kit supplied from Química Clínica Aplicada S.A). Measurement of serum 25-OH vitamin D (as 25-OH vitamin D is the major circulating form of vitamin D and is used as an indicator of vitamin D status) using a commercially available (ELISA) kit supplied by Calbiotech's, Inc, USA, was done for 20 selected patients from Group 2. Vitamin D deficiency was defined as a 25(OH) D serum level < 12 ng/mL, vitamin D insufficiency as 25(OH) D level 12–32 ng/mL, and vitamin D sufficiency as > 32 ng/mL [18].

2.3. Assessment of Zn and Vit. D Effect on the Expression of IL-17 in Cultured PBMCs

2.3.1. Reagents. Phorbol 12-myristate 13-acetate (PMA) and ionomycin (IO) were both purchased from Serva Electrophoresis Germany, zinc sulphate was purchased from Elnasr, Pharmaceutical Chemical Industries, Egypt, vitamin D3 (Cholecalciferol) from Memphis, Pharmaceutical Chemical Industries, Egypt, and Intracellular Fixation & Permeabilization Buffer (plus Brefeldin A) kit from eBioscience, San Diego, CA, USA.

2.3.2. Monoclonal Antibodies. Antibodies used included CD3-PECy5, CD4-FITC, IL17-PE, and PE isotype control (eBioscience, San Diego, CA, USA).

2.3.3. Preparation of Peripheral Blood Mononuclear Cells (PBMCs). Twenty mL of peripheral blood were obtained by venipuncture from the 20 selected patients of Group 2 patients and collected into sterile EDTA tubes. The PBMCs were immediately separated by density gradient centrifugation over Ficoll–Hypaque (Lonza, BioWhittaker) and then washed twice with RPMI 1640. Cell count and viability were determined utilizing Guava ViaCount Flex Reagent for Flow Cytometry (Merck Millipore, France). Viability was exceeding 95% in all studied cases. PBMCs were suspended in RPMI 1640 medium, supplemented with 2 mM l-glutamine, 25 mM HEPES, 100 U/mL benzylpenicillin, 0.1 mg/mL streptomycin, and 10% ABserum (complete medium) (Lonza, BioWhittaker). All cultures were incubated.

2.3.4. Intracellular Cytokine Staining and Flow Cytometry. Peripheral blood mononuclear cells (PBMCs) from patients were cultured at a concentration of 5×10^5/well in 200 μL

of complete medium in 96-well U bottom cell culture plates (Corning Incorporated, Corning, NY) and stimulated with 10 ng/mL of PMA plus 1 μg/mL IO, in the presence or absence of vitamin D3 in two different concentrations (low 50 ng/mL and high 500 ng/mL) in some wells or Zn in the form of zinc sulphate in both low and high conc. (3 μmol/L and 30 μmol/L.) in other wells. The cells were incubated for 1 hour before the addition of Brefeldin A in a humidified incubator at 37°C and 5% CO_2. Then, the incubation was continued for an additional 72 hours in the same circumstances. After incubation, the cells were washed twice with FACS buffer and stained for surface markers by incubation with CD3-PECy5 and CD4-FITC antibodies for 20 min in the dark at 4°C. Cells were then washed twice with FACS buffer and resuspended in fix buffer for 30 min at 4°C in dark followed by 2-time wash with diluted PERM buffer. The permeabilized cells then stained for intracellular cytokine (IL17) using IL-17-PE antibodies then incubated in the dark at room temperature for 30 min. After intracellular cytokine staining, the cells were washed and resuspended in 200 μL phosphate-buffered saline. Flow cytometry was then performed and data was collected using a four-color Guava cytometer (Merck Millipore, France) and analysis was performed using FlowJo software (TreeStar, La Jolla, USA). The appropriate isotype-matched monoclonal antibodies were used to establish gating parameters.

2.4. Assessment of HCV Levels. Quantitative reverse transcription polymerase chain reaction (RT-PCR) for HCV was done using TaqMan technology by Stratagene Mx3000P Real-Time PCR System (Life Technologies, Applied Biosystems, USA); the RNA Isolation Kit (QIAamp minikit) and the reverse transcription and amplification Kit (Brilliant HCV QRT-PCR kit) were both purchased from Qiagen, Hilden, Germany. The RT-PCR had a limit of quantification (LOQ) of 25 IU/mL and a limit of detection (LOD) of 12 IU/mL.

2.5. Statistical Analyses. Data was analyzed using Prism 5 software (GraphPad, La Jolla, CA). Patient and control groups were compared using Student's t-test for parametric data while two-tailed Mann-Whitney and Kruskal-Wallis test were used for non parametric data. Correlations between parameters were determined using Spearman's correlation coefficient.

3. Results

3.1. Baseline Characteristics of Chronic HCV Patients. Seventy chronic HCV patients were enrolled. Demographical and clinical characteristics of patients and controls are reported in Table 1.

3.2. Elevated Levels of Serum IL-17 in Chronic HCV Patients Correlate with Severity of Liver Disease. Interleukin-17 is a potent mediator of delayed type reactions. It achieves this effect by elevating chemokine production in various tissues which, in turn, leads to recruitment of monocytes and neutrophils to the site of inflammation [19]. IL-17 was measured in the serum of chronic HCV patients ($n = 70$) and controls ($n = 50$). We observed significantly higher concentrations of IL-17 in patients group compared to control group ($P < 0.001$) (Figure 1(a)). To examine whether IL-17 was related to liver inflammation and fibrosis we stratified patients based on ultrasonography results and liver function tests. When comparing Strata, the recently diagnosed, compensated, and decompensated groups showed significantly higher levels of IL-17 as compared to controls; meanwhile the compensated and decompensated groups showed significantly higher levels of IL-17 when compared to recently diagnosed HCV group with P value < 0.001. Decompensated group also showed significantly higher levels of IL-17 as compared to compensated group (Figure 1(b)). In addition correlations between IL-17 and different laboratory parameters in patients group were done to show a significant negative correlation between IL-17 concentration and Albumin and a significant positive correlation with ALT, total and direct bilirubin, P.T, and INR ($P < 0.001$) (Table 2 and Figures 1(c) and 1(d)).

3.3. Zinc and Vitamin D Status in Chronic HCV Liver Disease and Their Relation with IL-17. The serum levels of zinc are often decreased in HCV patients, and serum levels also tend to negatively correlate with hepatic reserve [20]. This was obviously noticed in the patients group and subgroups which showed highly significant decrease in Zn level when compared to control group (Figure 2(a)). In addition data shown in Figure 2(b) indicate that compensated and decompensated groups had lower Zn levels compared to recently diagnosed group. Decompensated group also showed significantly lower Zn levels as compared to compensated group. Moreover there was a significant positive correlation between Zn and Albumin and significant negative correlation with ALT, total and direct bilirubin, P.T, and INR (Table 2). Because the liver plays a central role in Vit. D metabolism and its inadequacy is common in chronic liver diseases and correlates with disease severity [21], we selected 20 chronic HCV cases from the compensated group to assess Vit. D status which was significantly lower than the controls level (Figure 2(c)); however no correlations were found between vitamin D serum levels, biochemical and virological data of the patients (data not shown), as well as serum Zn level (Figure 2(d)). We determined a significant negative correlation between serum level of Zn and IL-17 (Figure 2(e)). There is also negative correlation between serum Vit. D level and IL-17; however, it is not significant (Figure 2(f)).

3.4. Role of Both Zn and Vit. D in Controlling IL-17 Expression. Both vitamin D and zinc play a role in innate and adaptive immune responses controlling inflammatory cytokine gene expression [15, 22]. To test the effects of Zn and vitamin D3 on IL-17 cytokine production, PBMCs from only 20 compensated cases were stimulated with PMA plus IO, in the presence or absence of Zn (3 and 30 μmol/L) or vitamin D3 (50 ng/mL and 500 ng/mL) then stained for intracellular IL-17, and analyzed by FACS (Figure 3). Data shown in Figure 4(a) indicate that the percentage of $CD3^+CD4^+IL17^+$T lymphocytes was significantly lower in the presence of Zn

Table 1: Demographical and clinical characteristic of patients and controls.

Clinical characteristics	All patients	Recently diagnosed	Compensated	Decompensated	Controls
Age, median (range)	48 (22–76)	30 (22–35)	48 (40–54)	63 (53–76)	31 (27–40)
Sex (M/F)	35/35	9/7	17/20	9/8	22/28
Albumin (g/dL), median (IQR)	3.6 (2.4–4)	4.2 (4–4.4)	3.6 (3.5–3.8)	2.3 (1.95–2.45)	>4.1
Total Bilirubin (mg/dL), median (IQR)	0.9 (0.7–1.7)	0.7 (0.5–0.8)	0.7 (0.6–0.9)	3.14 (1.7–5.1)	<1.2
Direct Bilirubin (mg/dL), median (IQR)	0.3 (0.2–0.9)	0.2 (0.1–0.3)	0.2 (0.1–0.3)	1.07 (0.8–2.35)	<0.2
P.T# (sec), median (IQR)	13 (11.8–16.6)	11.9 (11.63–12.48)	12.5 (11.8–13)	17.4 (16.3–19.9)	<12
INR, median (IQR)	1.015 (0.98–1.5)	0.99 (0.9–1.04)	1 (0.9–1.07)	1.5 (1.48–1.7)	0.93 (0.9–1.02)
ALT (IU/L), Mean ± SD	53.84 ± 12.36	56.5 ± 13	47.59 ± 11.87	58 ± 10.22	15.5 ± 7.65
AST (IU/L), Mean ± SD	42.74 ± 10.59	47.44 ± 8.78	38.35 ± 9.32	42.71 ± 11.93	15.5 ± 6.25
PCR (IU/mL), median (IQR)	31850 (22100–599000)	27600 (22100–646000)	36100 (22100–599000)	27600 (21200–630000)	—
IL-17 (pg/mL), Mean ± SD	776.3 ± 57.39	295.8 ± 48.04	766.1 ± 62.90	1207 ± 113.8	72.17 ± 4.622
ZN (μg/dL), Mean ± SD	59.01 ± 1.86	72.81 ± 2.679	58.13 ± 2.445	48.00 ± 2.727	91.28 ± 1.838
Vit. D (ng/mL), Mean ± SD[a]	—	—	18.65 ± 12.89	—	55.8 ± 16.27

[a]Data available for 20 patients.
P.T: prothrombin time; INR: international normalized ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IQR: the interquartile range; HCV: hepatitis C virus.

Table 2: Correlations between IL17 and Zn and different parameters in hepatitis C virus infected subjects.

	IL-17		Zn	
	R	*P* value	*R*	*P* value
Albumin (gm/dL)	−0.561	<0.001	0.616	<0.001
Total Bilirubin (mg/dL)	0.792	<0.001	−0.678	<0.001
Direct Bilirubin (mg/dL)	0.649	<0.001	−0.584	<0.001
P.T (sec)	0.656	<0.001	−0.632	<0.001
INR	0.484	<0.001	−0.557	<0.001
ALT (IU/L)	0.34	0.004	−0.304	0.032
AST (IU/L)	−0.059	0.684	−0.042	0.773
PCR (IU/mL)	−0.018	0.903	0.054	0.711

than in its absence and became more lower with increasing Zn concentration which further support our suggestion regarding the link between Zn and IL-17. On the opposite side addition of Vit. D (in high concentration 500 ng/mL) leads to a significant increase in the percentage of $CD3^+CD4^+IL17^+$ cells compared to its absence (Figure 4(b)).

3.5. Effect of Zn and Vitamin D3 on Lymphocyte Count. In order to assess the *in vitro* effect of Zn and Vit. D on cell count we compared the count of cells which stimulated in the presence of Zn or Vit. D with those which stimulated in their absence. We noticed that addition of Zn leads to increase in the cell count especially with increasing the dose; however this effect was not significant; contrary to this result, addition of Vit. D leads to significant lowering in the cell count compared to cells stimulated in its absence or in the presence of Zn, and this effect seemed to be also dependent on the dose of vitamin D; however the *P* value was nearly significant = 0.057 (Figure 5).

4. Discussions

Hepatitis C virus infection is a significant global public health problem. Persistent HCV infection eventually develops into liver cirrhosis or hepatocellular carcinoma [23]. Many reports in HCV infections indicate a close correlation between virus-induced liver inflammations, infiltration, and activation of Th17 cells and the amount of liver damage caused by the antiviral immune response. Moreover a shift from Th1 to Th17 seems to be potentially disadvantageous for the patient in terms of antiviral defense and liver disease progression [24, 25]. The present study showed that IL-17 was markedly increased in HCV-infected patients in comparison to controls and this elevation became more evident with the progression of the disease as shown upon comparing patients' strata. These results were supported by the finding that increasing circulating Th17, intrahepatic IL-17 positive cells, and HCV-specific Th17 cells were correlated with severity of liver inflammation in chronic HCV patients [26]. IL-17 and Th17 seem to have an important role in viral infections and stronger Th17 responses are associated with higher viral plasma load, increased levels of serum transaminases, and enhanced activation of blood monocytes as well as liver macrophages [25]. Finally, it has been reported that antiviral therapy with pegylated interferon and ribavirin in HCV-infected patients leads to a reduction of both Th1 and Th17 responses, ameliorating HCV-mediated liver inflammation

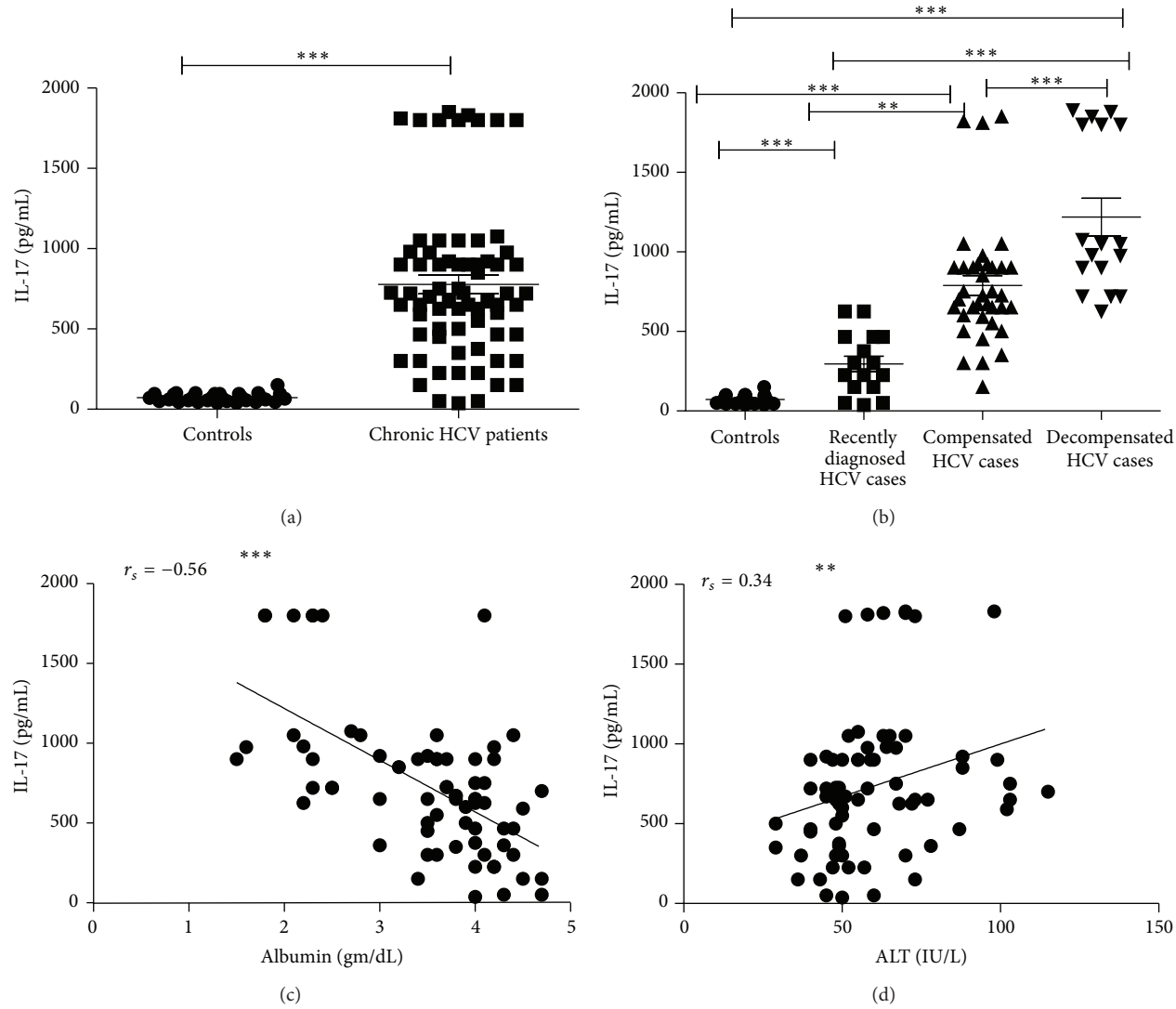

Figure 1: Serum IL-17 comparative results from chronic HCV patients and uninfected controls (a) and comparison between patients subgroups and controls (b). $*$ indicates $P < 0.05$; $**$ indicates $P < 0.01$; and $***$ indicates $P < 0.001$. IL-17 correlation with Albumin (c) and ALT (d). Spearman coefficients were determined ($r_s = -0.56$ for Albumin; and $r_s = 0.34$ for ALT).

[27]. These studies explain the significant negative correlation between serum IL-17 and Albumin and the significant positive correlations with serum bilirubin, prothrombin time, ALT, and AST which were evident in the current study; however, we could not find any significant correlation between viral loads and serum IL-17 level. This finding was strengthened by Chang et al. [26] who found that no association was found between serum IL-17 concentration and viral load as viral loads fluctuate in chronic HCV infection. Taken together that many IL-17 sources might account for serum IL-17 levels, since IL-17 is produced by various cells (e.g., neutrophils, $CD8^+$ T cells, $\gamma\delta$ T cells, natural killer T (NKT) cells, and Tregs) [28], this in turn could account for the lack of correlation between serum IL-17 levels and viral load.

Zinc is a micronutrient influencing growth and affecting the development and integrity of the immune system. Furthermore, it plays an important role in the function of the liver [29]. Liver disease has been associated with hypozincemia and zinc deficiency [30]. Confirming prior observations we detected significant decrease in Zn level in the patients group compared to controls which was prominently noticed in decompensated disease stage compared to earlier stages. We explained these results on the basis that, in liver disease, there is increase in gut permeability with endotoxemia, infections such as spontaneous bacterial peritonitis, and release of stress hormones which could be the causes of Zn deficiency [31]. Moreover this stress response is often associated with hypoalbuminemia [32]. In consistency with this finding, we observed a positive correlation between serum Zn and Albumin levels as it is a major binding protein for Zn; however serum Zn concentration can decrease with an inflammatory stimulus

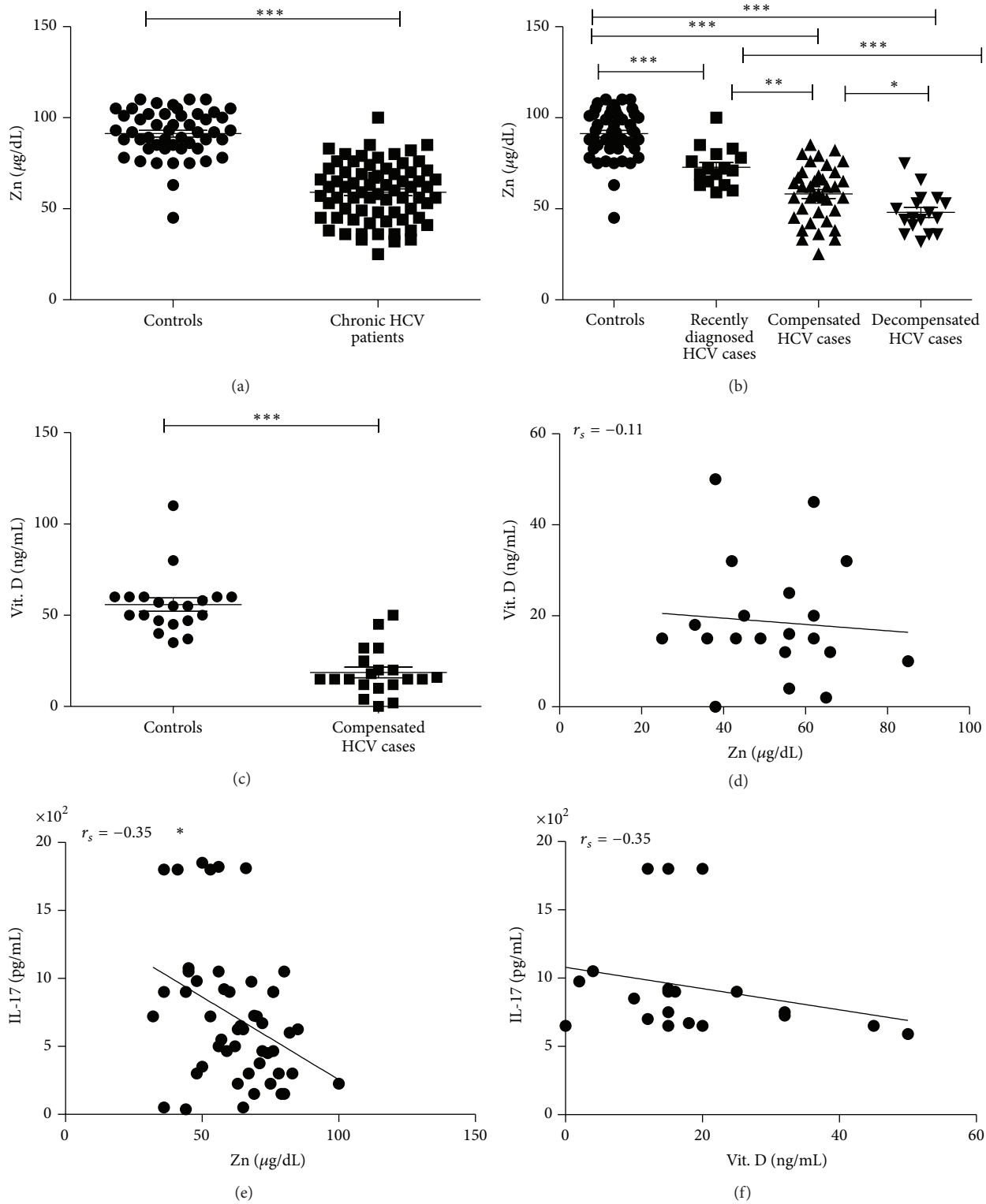

Figure 2: Lower Zn level in chronic HCV patients (a). Statistically significant differences in Zn level across the different groups are indicated (b). Lower Vit. D level in the selected compensated patients compared to controls (c). Correlation between Zn and Vit. D was nonsignificant Spearman coefficient ($r_s = -0.11$) (d). IL-17 was correlated with Zn (e) and Vit. D (f) and Spearman coefficient was calculated ($r_s = -0.35$). $*$ indicates $P < 0.05$; $**$ indicates $P < 0.01$; and $***$ indicates $P < 0.001$.

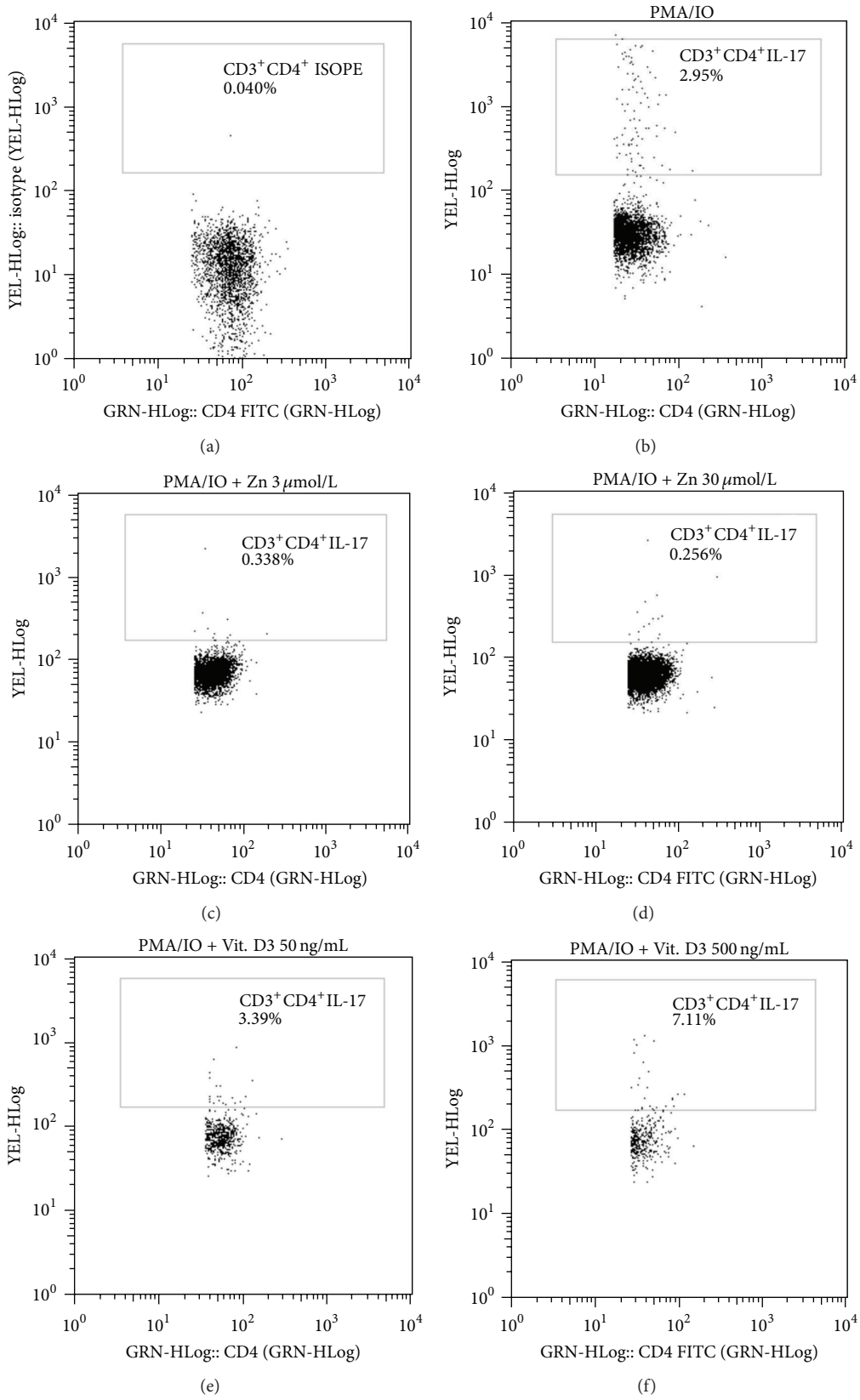

Figure 3: Representative flow cytometry plots for one patient are shown and gating of $CD3^+CD4^+$ ISOPE is indicated (a). Phorbol 12-myristate 13-acetate and ionomycin-stimulated peripheral blood mononuclear cells were stained and analyzed for IL-17 production (b). Inhibitory effect of Zn on IL-17 production with 3 μmol/L concentration (c) and 30 μmol/L concentration (d). Vitamin D3 effect on IL-17 production with 50 ng/mL concentration (e) and 500 ng/mL concentration (f).

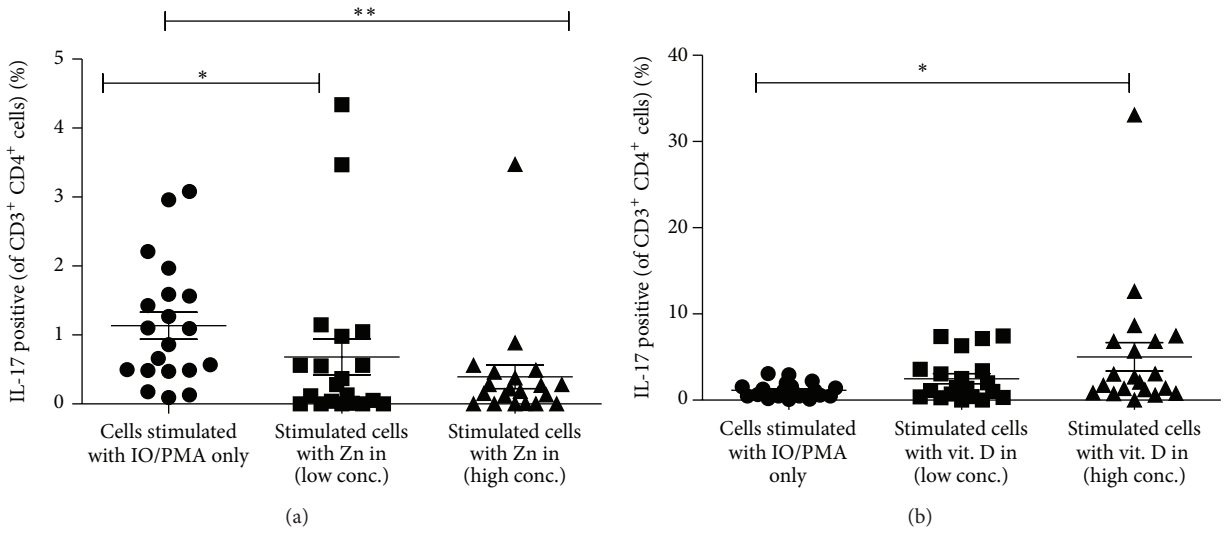

Figure 4: Peripheral blood mononuclear cells were stimulated with Phorbol 12-myristate 13-acetate and ionomycin in the presence or in the absence of Zn and Vit. D and stained and analyzed as described in the legend of Figure 3. Inhibition of IL-17 production by Zn in low (3 μmol/L) and high (30 μmol/L) concentrations (a). Significant increase in IL-17+ cells in the presence of high concentration (500 ng/mL) of vitamin D3 (b). $*$ indicates $P < 0.05$ and $**$ indicates $P < 0.01$.

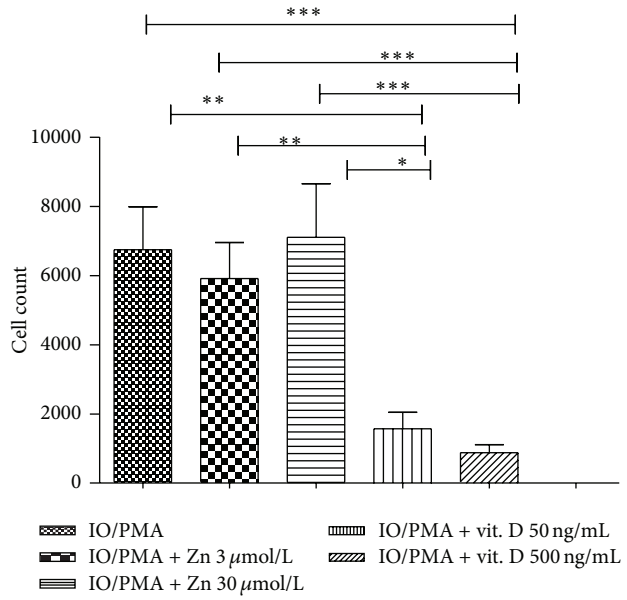

Figure 5: Different effect of Zn and Vit. D on cell count. Significant decrease in cell count in the presence of Vit. D in either concentration compared to its absence or compared to the presence of Zn.

even in the absence of hypoalbuminemia due to the effect of proinflammatory cytokines believed to play an important role in determining trace element concentration [33]. All in all from the previous observations we can understand the bases of the significant negative correlation between Zn and IL-17 which was obviously noticed in our result.

Regarding the role of Zn in HCV infection it may act as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells; thus its deficiency may increase the severity of the case [8]. In addition it was found that Zn levels depend on the severity of liver disease; thus, in patients with decompensated liver cirrhosis, Zn concentrations are reduced by up to 75%, this is explained by changes in the protein and amino acid metabolism and by disturbances in intestinal resorption and hepatic Zn extraction [33]. These data explain the highly significant negative correlation we found between zinc level and the other liver function tests; however we could not mount a significant correlation between viral loads and serum Zn level.

Vitamin D is a potent immunomodulator that favors innate immunity and cell differentiation [34, 35]. Increased production of 1,25-dihydroxy vitamin D3 results in the synthesis of cathelicidin, a peptide capable of destroying many viral infectious agents. Low serum levels of vitamin D prevent macrophages from initiating innate immune response [36]. Vitamin D deficiency is very common (92%) among patients with chronic liver disease, and at least one-third of them suffer from severe vitamin D deficiency [37]. Moreover levels of Vit. D and its active form are significantly lower in advanced liver disease (hepatic cirrhosis and/or carcinoma) patients, compared to those with bright hepatomegaly and perihepatic fibrosis [12]. In the light of these findings we assessed 25-OH Vit. D level in twenty compensated HCV patients which showed a significant reduction compared to healthy controls. This decrease in Vit. D was concomitant with an increase in IL-17 levels (however the negative correlation between them was not significant); this could be attributed to the finding that Vit. D suppresses proinflammatory cytokines and increases anti-inflammatory cytokines [38]. Vit. D regulates the growth and differentiation of multiple cell types and displays immunoregulatory and anti-inflammatory properties. Cells involved in innate and adaptive immune responses including macrophages, dendritic cells, T cells, and B cells express vitamin D receptor (VDR) and can both produce and

respond to 1,25$(OH)_2D_3$ [39]. Hepatocytes express only low levels of VDR mRNA [40], so that vitamin D effects on the liver are most probably not conferred by direct signalling in parenchymal liver cells. In contrast, nonparenchymal hepatic cells such as sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells (HSC) do express VDR mRNA and functionally active VDR protein [41]. Many reports indicate that 1,25$(OH)_2D_3$ suppresses Th17 driven cytokine responses, induces Treg cells, induces IL-4 production (Th2), and enhances natural killer T-cell function [11, 42] but the key immunomodulatory property of 1,25$(OH)_2D_3$ is its ability to inhibit expression of Th1 cytokines, whilst augmenting Th2 cytokines, with 1,25$(OH)_2D_3$ acting either directly via effects on T lymphocytes or indirectly via effects on antigen-presenting cells (APCs). Moreover, elevated VDR expression is also found on differentiated Th17 cells [43]. More recent studies showed marked anti-inflammatory and antifibrotic effects of VDR-signalling in HSC. During inflammatory liver injury after endotoxin injection, the activation of VDR signalling by vitamin D attenuated liver damage *in vivo*. Vit. D provides protection against autoimmune and inflammatory diseases, such as multiple sclerosis, type 1 diabetes, and inflammatory bowel disease partially due to its inhibitory effects on Th17 cells. The antiproliferative, prodifferentiative, antibacterial, immunomodulatory, and anti-inflammatory properties of synthetic VDR agonists could be exploited to treat a variety of inflammatory and autoimmune diseases [39]. Treatment with VDR agonists inhibits the T-cell production of IL-17. Furthermore, IL-17 production is sustained by IL-23, an IL-12 family member, the latter of which is strongly inhibited by VDR agonists [11]. It was clearly demonstrated that enhanced progression of liver fibrosis is significantly and independently associated with both genetic VDR variants and low 25-OH vitamin D plasma levels. This suggests vitamin D substitution as a preventive measure for patients with liver fibrosis [44].

In order to gain additional insight into the interplay between Zn, Vit. D, and IL-17 we have examined the ability of Zn and Vit. D3 to interfere with Th17 activation and expression of IL-17 *in vitro*. Noteworthy, IL-17 was suppressed significantly when Zn was added in both concentrations and this was claimed to its suppressive effect on IL-6/STAT3 (signal transducer and activator of transcription) signaling pathway which is a critical step for Th17 development. Zn binding changed the *a*-helical secondary structure of STAT3, disrupting the association of STAT3 with JAK2 kinase (Janus kinase 2) and with a phosphopeptide that included a STAT3-binding motif from the IL-6 signal transducer gp130 [22]. To test whether the effects of Zn on cytokine production were independent of toxic effects, cell count was examined and interestingly we observed that, with addition of Zn, the count was increased compared to its absence especially with increasing Zn concentration, although this observation was not significant; this effect could be mediated through Zn enhancement of DNA synthesis and RNA transcription, cell division, and cell activation as apoptosis (programmed cell death) is potentiated by Zn deficiency [45]; additionally Zn is an inhibitor of NADPH oxidases which catalyze the production of reactive oxygen species (ROS); on the other side, Zn activates the dismutation of $O2^{-}$ to H_2O_2 by super oxide dismutase which contains both copper and Zn [46]; it also negatively regulates gene expression of inflammatory cytokines such as TNF-α and IL-1β, which are known to generate (ROS) and this may be one additional mechanism by which Zn may be functioning as an antioxidant in humans [47].

There is promising evidence that zinc may decrease liver injury and provides antifibrotic effects in patients with chronic HCV. Himoto and Coworkers [20] used polaprezinc as an antifibrotic therapy in patients with chronic HCV and showed a decrease in noninvasive fibrosis markers.

One caveat of our studies was the effect of Vit. D on IL17 production. For reasons that remain unclear and despite the apparent negative association between serum Vit. D and IL17, addition of Vit. D showed an apparent increase in the percent of IL-17^+ cells which was significant only with high concentration of Vit. D. Contrary to our finding an experimental study on healthy human donor using $CD4^+$ T cells and mouse model for multiple sclerosis showed that 1,25$(OH)_2D_3$ inhibits human IL-17A and suppresses mouse IL-17A [48]; other studies in mice imply this regulatory effect on Th17 cells by Vit. D through the reduction of (ROR)γt expression [15].

The reasons of these conflicting results still need to be determined, although we would contend that the interpretation was somewhat misleading. Looking at the effect of Vit. D on cell count showed us a marked dose-related reduction in cell count compared to its absence or to the presence of Zn. Preclinical research indicates that vitamin D3 potently inhibited T cell proliferation in a dose-related fashion [49]. The active metabolite of vitamin D, 1 alpha,25$(OH)_2D_3$, also known as calcitriol, has antiproliferative effects, activates apoptotic pathways, and inhibits angiogenesis [50]. The common antiproliferative vitamin D receptor (VDR) functions are associated with arrest at G0/G1 of the cell cycle, coupled with upregulation of a number of cell cycle inhibitors including p21 and p27 [51]. On the other hand Th17 and other Th17 and other IL-17 secreting cells could play a part in hepatic viral persistence bymeans of antiapoptoticmolecules upregulation [26]. Consistently, IL-17 has been implicated in modulating the expression levels of prosurvival Bcl-2 family proteins, including Bcl-2 (B-cell lymphoma 2) [52] and Bfl-1/A1 (Bcl-2 family member) in some autoimmune diseases such as systemic lupus erythematosus [53]. Based on these observations it seems feasible that our preconceived notion regarding the impairment of apoptosis pathway in Th17 cells can contribute to the rise of IL-17^+ cells percent among the remained cells; however it remains to be determined whether the apparent increase is a true enrichment of IL-17 producing cells or simply due to survival of IL-17^+ cells from the toxic effect of Vit. D. However the clinical implication of low vitamin D levels and HCV severity is still not clear. At least one study has found that serum Vit. D levels were not associated with any parameter indicating disease severity. And they concluded that serum Zn but not serum vitamin D levels is strongly associated with disease severity and treatment response in chronic HCV [54]. This latter study may also account in part for the lack of significant correlations

between Vit. D serum levels, biochemical and virological data of the patients, and serum Zn and IL17 levels that have been investigated in the current study. However there are some limitations in this study. An important limitation is the lack of assessment of Vit. D status in both recently diagnosed and decompensated groups which would give us a better informative insight on the interplay between Vit. D and IL-17 in chronic HCV liver disease and whether it has a protective role in preventing liver fibrosis or not.

A final conclusion of our study concerns the potential use of Zn as an adjunct antifibrotic therapy and novel strategies for the treatment of chronic hepatitis C; it may be worthwhile exploring the benefit of zinc supplementation even with the advent of novel direct antiviral agents. Meanwhile the role of vitamin D is still a topic of debate and much work will be required to understand the perturbations found in IL-17 and its relation to vitamin D at a mechanistic and genetic levels.

Ethical Approval

Written informed consent was obtained from each of the participants after approving the study protocol by the Ethics Committee of Faculty of Medicine, Ain Shams University.

Conflict of Interests

There is no conflict of interests. All authors do not have a direct financial relation with the commercial identities mentioned in the paper.

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Review Article

Comparative Immune Response in Children and Adults with *H. pylori* Infection

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Helicobacter pylori (*H. pylori*) infection is generally acquired during early childhood; therefore, the immune response which usually takes place at this age may influence or even determine susceptibility to the infection contributing to the clinical outcomes in adulthood. Several cytokines including IL-6, IL-10, and TGF-β1 as well as Foxp3$^+$ cell numbers have been shown to be higher; however, some other cytokines consisting of IL-1β, IL-17A, and IL-23 are lower in infected children than in infected adults. Immune response to *H. pylori* infection in children is predominant Treg instead of Th17 cell response. These results indicate that immune system responses probably play a role in persistent *H. pylori* infection. Childhood *H. pylori* infection is also associated with significantly lower levels of inflammation and ulceration compared with adults. This review, therefore, aimed to provide critical findings of the available literature about comparative immune system in children and adults with *H. pylori* infection.

1. Introduction

Helicobacter pylori (*H. pylori*) infections usually occur during childhood, continue throughout the life, and cause severe diseases such as gastritis, gastric ulcer, gastric carcinoma, and duodenum ulcer in adulthood. *H. pylori* is a well-known gastric pathogen infecting more than half of the world's people [1]. The outcomes of *H. pylori* infection seem to be dependent on some factors like gene regulation factors, genetic predisposition of the patient, receptor gene polymorphisms, particular cytokine, constituents, and environmental influences [2, 3]. Fortunately, most of the infected children do not develop any complications; however, the immunological events which usually develop in the children gastric mucosa are probably decisive in the immune response determining the final outcome the infection. The colonization of the stomach by this pathogen bacterium causes an inflammatory response and recruits neutrophils, lymphocytes, dendritic cells, and macrophages, to the gastric mucosa [4, 5]. There are complex mechanisms by which *H. pylori* may start and maintain the local immune response; however, cytokines produced by both adaptive immune and innate systems may lead to the development of gastric mucosa-associated lymphoid tissue lymphoma, gastric adenocarcinoma, and other ulcerative diseases; studies in *H. pylori* infection have revealed that childhood *H. pylori* infection is usually associated with significantly lower levels of gastric inflammation and ulceration in comparison to adults. Therefore, this review study was aimed to provide the critical findings of the available literature about comparative immune system in children and adults with *H. pylori* infection.

2. Bacterial Virulence Factors

Helicobacter pylori may express the virulence factors associated with inflammation as well as inflammatory symptoms in infected patients. The main pathogenicity factors

of *Helicobacter pylori* include γ-glutamyl transpeptidase (GGT), cytotoxin-associated gene A (cagA) product, and virulence components vacuolating toxin (vacA), in addition to pathogen-associated molecular patterns (PAMPs) such as flagella and lipopolysaccharide (LPS) [6–9]. The cytotoxin-associated gene (cag) pathogenicity island (PAI) is one of these factors which has been extensively studied in regard to inflammation [10–12]. Colonization with the strains that possess cagA is more frequently associated with peptic ulceration gastric adenocarcinoma or other gastric mucosal complications than the cagA strains [13, 14]. It has been shown that cagA may play a role in production of IL-8 as well as activation of nuclear factor kappa-B (NF-κB) [15]. Furthermore, expression of cagA induces production of IL-8 and translocation of NF-κB nuclear in gastric epithelial cells [13, 16]. The vacA from *H. pylori* is capable of inducing intracellular vacuolation in gastric epithelial cells. Hence, it has been hypothesized that it may contribute in damage of gastric and duodenal mucosa which ultimately leads to ulcer formation, *in vivo* [14]. Moreover, the bacterial virulence factors vacA and cagA have important roles in pathogenesis of *H. pylori* infection. Others like blood group antigen-binding adhesion (BabA), outer inflammatory protein (oipA), sialic acid-binding adhesion (sabA), iceA (induced by contact with epithelium), and duodenal ulcer promoting gene (dupA) may promote colonization of the mucosa, too [17]. In regard to virulence factors cagA and vacA, these bacteria are very heterogeneous [18]. A lot of evidences have revealed that these genetic variations may have an important role in the outcome of infection [19, 20].

3. T Cell Subsets

T helper (Th) cells have been shown to differentiate into functional classes of two major $CD4^+$ including Th1 cells (able to produce some cytokines such as IL-2 and IFN-γ) and Th2 cells (producing cytokines like IL-4, IL-5, and IL-10) [21, 22]. Th1 cells mediated cell immunity, which has an important role against intracellular parasites. However, Th2 generates humoral immunity as well as prevention of intestinal helminthes [23]. Other than Th1/Th2 paradigm, a unique subset of IL-17 producing Th17 cells has been discovered [24–26]. IL-23 has a crucial role in differentiation of Th17 cells. However, IL-4 and IL-12 promote, respectively, Th1 and Th2 cell differentiation [27]. It has been revealed that IL-17 possesses 6 family members (IL-17A–F), IL-17A (simply called IL-17) being the prototypic IL-17 family member [28, 29]. Furthermore, IL-17A exerts proinflammatory effects by stimulation of the production of chemokines such as IL-1, IL-6, cytokines, monocyte chemoattractant protein-1, and upregulation of cell adhesion molecules like vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. The IL-17A plays a crucial role in induction of autoimmune diseases such as inflammatory bowel disease (IBD), experimental autoimmune encephalomyelitis (EAE), and rheumatoid arthritis (RA), as well as chronic inflammatory diseases [30–33]. Regulatory T (Treg) cells, by proliferation of antigen specific T cells and suppressing the activation, have important role in chronic inflammation. It should be noted that depletion or dysfunction of Treg cells is usually associated with inflammatory bowel disease, allergy, and autoimmune disease [34]. Treg cells comprise different subsets: Tr1 cells secreting interleukin IL-10, Th3 cells characterized by transforming growth factor (TGF-β1) secretion, and naturally occurring FOXP3-expressing $CD4^+CD25^{high}$ Treg cells [35, 36]. The $FOXP3^+CD4^+CD25^{high}$ Treg cells are further divided into two subsets: thymus derived naturally occurring $FOXP3^+CD4^+CD25^{high}$ Treg cells and peripherally induced $FOXP3^+CD4^+CD25^{high}$ Treg cells [37].

4. Differences in Immunity of Children and Adults Infected and Uninfected with *H. pylori*

The human gastric mucosal biopsies revealed that people who were persistently infected with *H. pylori*, in comparison to uninfected ones, show an increased and higher level of infiltrated various types of leukocytes [38]. In these specimens, lymphocytes (T and B cells), monocytes, mast cells, neutrophils, macrophages, eosinophil, and dendritic cells are usually present [2, 4]. $CD4^+$ T cells, B cells and dendritic cells may be organized in lymphoid follicles [39] indicating ongoing chronic immune responses and antigen presentation. In peripheral blood and gastric mucosa of infected humans, the *H. pylori*-specific $CD4^+$ T cells are detectable which is not detectable in uninfected individuals [40]. The cytokines such as TNF-α, IFN-γ, IL-1, IL-6, IL-7, IL-8, IL-10, IL-17, IL-18, and IL-23 have usually increased levels in the stomach of *H. pylori*-infected patients in comparison to the uninfected subjects [2, 41, 42]. IL-4 is not usually detectable in the gastric mucosa of *H. pylori*-infected patients [43]. Hence, to show, in children, the mucosal regulation of *H. pylori* infection, which can provide a window to the early host response to bacteria, the mucosal cytokine response to the infection, the associated cellular infiltrate, and the characterized bacteria might be helpful. Studies on *H. pylori*-infected children and adults have shown that children possess reduced gastric inflammation in comparison to the infected adults, in spite of similarity in *H. pylori* colonization level. Furthermore, inflammation in children has been shown to be less in comparison to that of adults, indicating a downregulation in immune response to infection in children [44, 45]. Moreover, the sequence analysis revealed that the bacteria isolated from the infected children and adults might have similar *cagA* and *vacA* gene profiles. The difference in bacterial strains and common virulence factors were not the cause of low level of inflammation in infected children in comparison to adults [44]. *H. pylori*-infected children in comparison to infected adults possess lower levels of protein and gastric IL-17-specific mRNA as well as fewer gastric Th17 cells, indicating more reduction in the mucosal Th17 response in infected children. Moreover, the gastric mucosa of the infected children has lower level of IFN-γ mRNA, confirming the findings indicated of reduced Th1 response in children with *H. pylori* infection [46, 47]. Recent study indicated that the gastric concentrations of cytokines

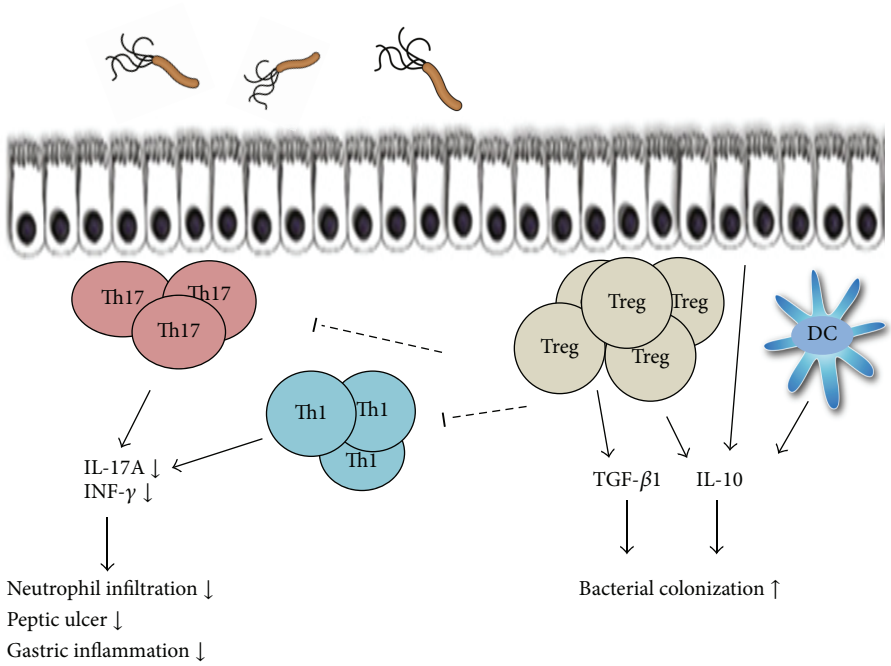

Figure 1: Diagram of how the Treg cell response may influence inflammation, bacterial colonization density, and occurrence of *H. pylori*-mediated disease.

representative of the innate and Th1 response were higher in the *H. pylori*-positive than in the *H. pylori*-negative children and adults. The gastric concentrations of IL-1α and TNF-α were significantly higher, while those of IL-2, IL-12p70, and IFN-γ were lower in the infected children than in the infected adults. In the infected children, the gastric concentration of IL-1α, IL-2, IL-12p70, and IFN-γ increased, whereas in adults the gastric concentrations of IFN-γ and IL-12p70 decreased with aging. Increased gastric concentration of Th1-associated cytokines correlated with increased degree of gastritis, that is, the background lesion for the development of the *H. pylori*-associated severe diseases [48]. Treg cells are described as the key regulator of the immune system in the maintenance of immunologic tolerance. Recently, the close relationship between *H. pylori* infection and immunosuppressive Treg cells has been reported in animal and human models [49]. Treg cells suppress *H. pylori*-induced Th1-mediated immune response to contribute to the bacteria's persistent colonization in the gastric mucosa and therefore may play a major role in inducing chronic gastritis. The TGF-β1 and IL-10 gastric levels and the gastric number of Treg Foxp3$^+$ cells in *H. pylori*-positive groups are higher in children than in adults (Table 1) [44, 46, 49–51]. The consensus is that Treg cells and Th17 commitments might be mutually controlled. TGF-β is required for the differentiation of both Treg cells and Th17 by inducing key transcription factors, Foxp3 and RORγt/RORc, respectively [52–54]. But, in absence of IL-6, an exclusive Treg differentiation might occur as Foxp3 is capable of associating with and inhibiting the RORγt. In contrast, in presence of IL-6, this inhibition might be abrogated allowing Th17 differentiation [55]. In a paradoxical pattern, the gastric concentration of IL-6 is usually less in infected adults than in infected children. In this regard, it might be hypothesized that these results are due to the higher gastric levels of IL-23 in adults, compared with children, which might prevent the amplification/stabilization of the shifted Th17 cells. The other possibility might be the higher level of TGF-β in the gastric milieu of infected children. It should be noted that, at low levels, TGF-β synergizes with IL-6 to promote IL-23 receptor expression in favor of Th17 cell commitment. However, the high level of TGF-β represses IL-23 receptor expression favoring Foxp3$^+$ Treg cell differentiation [49, 56]. A recently published study revealed that IL-6 overproductions by IL-6 transgenic mice do not affect the function and development of natural Treg [57]. In this regard, the predominant Treg differentiation in children infected with *H. pylori* might account for more susceptibility of children to the *H. pylori* infection as well as to the bacterium persistence. Study in mouse stomach showed that *H. pylori*-induced dendritic cells skew the Th17/Treg balance toward a Treg-biased response that suppresses Th17 immunity through a cagA and vacA independent, TGF-β and IL-10-dependent mechanism [58, 59]. In support of these findings, recent study showed that *H. pylori* was capable of stimulating human gastric dendritic cells to produce IL-10, potentially supplementing Treg suppression of inflammation in the gastric mucosa [60, 61] (Figure 1). From these findings we might conclude that *H. pylori*-induced gastritis in adult is the consequence of both Th1 and Th17 immune-mediated inflammatory pathway involvement and that both pathways might be downregulated in the gastric mucosa of infected children.

Table 1: Comparative immune response and clinical outcome in children and adults infected with *H. pylori*.

	Children	Adults
Th1	↓	↑
Th17	↓	↑
Treg	↑	↓
TGF-β1	↑	↓
IL-10	↑	↓
Gastric inflammation	↓	↑
Neutrophil infiltration	↓	↑
Peptic ulcer	↓	↑
Virulence factors	Similar	Similar

5. Conclusion

In conclusion, *H. pylori* infection in children is associated with high Treg response, as well as low Th1 and Th17 response [44, 46]. But, *H. pylori*-specific Th17/Th1 detection in chronically infected patients may reveal that the initial response is progressively lost [43, 62], indicating that, with progression of time, the mucosal immune system probably identifies *H. pylori*, as a pathogen. Hence, Th1, Th17, and Treg results may imply gastric mucosal response to *H. pylori*. More data from immune-mediated mechanism(s) of mucosal inflammation is required to provide strategies against this challenging pathogen, particularly for children who are living in countries with high rate of gastric cancer and/or *H. pylori* infection.

Conflict of Interests

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

Authors' Contribution

Alireza Razavi and Nader Bagheri contributed equally in preparation of this paper.

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Research Article

Potential Use of Interleukin-10 Blockade as a Therapeutic Strategy in Human Cutaneous Leishmaniasis

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Interleukin-10 overproduction has been associated with worse prognosis in human cutaneous leishmaniasis, while IFN-γ-dependent responses are associated with parasite killing and host protection. Innovative strategies are needed to overcome therapeutic failure observed in endemic areas. The use of monoclonal antibody-based immunotherapy targeting IL-10 cytokine was evaluated here. Partial IL-10 blockade in *Leishmania braziliensis* whole soluble antigen-stimulated cells from endemic area CL patients with active or healed lesions and asymptomatic controls was evaluated. Overall decrease in IL-10, IL-4, and TNF-α production was observed in all groups of subjects. Only patients with active lesions still produced some levels of TNF-α after anti-IL-10 stimulation in association with *Leishmania* antigens. Moreover, this strategy showed limited modulatory effects on IFN-γ-dependent chemokine CXCL10 production. Results suggest the potential immunotherapeutic use of partial IL-10 blockade in localized cutaneous leishmaniasis.

1. Background

Infections due to the protozoa from the genus *Leishmania* still constitute a major public health problem worldwide. Patients suffer from all clinical forms of the disease, without a specific vaccine or a safe and effective treatment. Interleukin-10 was implicated on T cell unresponsiveness observed in visceral leishmaniasis (VL) patients infected with *L. donovani* [1]. Cutaneous leishmaniasis (CL) is believed to present an unbalanced Th1/Th2 response during its acute phase with clinical resolution being an IFN-γ-dependent event, whereas lesion progression and therapeutic failure are related to IL-10 overproduction [2–6]. The reduction of IL-10 levels using neutralizing anti-IL-10 or anti-IL-10R monoclonal antibodies (mAb) might be useful as immunotherapeutic adjuvants immunotherapies to prevent or treat experimental infections by many pathogens by favoring the production of IFN-γ and TNF-α [7]. Anti-IL-10 mAbs when added to cell cultures restored the proliferative response of peripheral blood mononuclear cells (PBMC) from a VL patient [1] and increased the IFN-γ production by $CD4^+CD25^-$ T cells cocultured with intralesional Treg cells of *L. guyanensis* infected CL patients [2]. Furthermore, PBMC from unexposed subjects showed an increase on IFN-γ, TGF-β, and reactive nitrogen production when cultured in presence of *Leishmania* antigens and anti-IL-10 mAb [8]. All these data suggest that new CL vaccines and therapies should involve an IL-10-neutralizing strategy. Considering that IFN-γ-dependent responses are essential anti-*Leishmania* defense mechanisms and that their magnitude is modulated by IL-10, the evaluation of any product of the IFN-γ signaling cascade, such as CXCL10, rather than the cytokine alone,

would improve data interpretation of how successful this network is modulated in CL patients. Our results suggest that partial IL-10 neutralization using anti-hIL-10 mAb is able to reduce Th2 profile and increase protective IFN-γ-related response in peripheral PBMC from subjects living in areas where *Leishmania braziliensis* (Lb) is endemic.

2. Findings

2.1. Materials and Methods

2.1.1. Study Population. For this study, 18 male individuals were selected from a previously characterized CL endemic area located in Buerarema Village, Bahia State, Brazil [6]. The groups consisted of 6 patients with active lesions (aCL), 6 patients with chemotherapeutically healed lesions (hCL), and 6 asymptomatic uninfected endemic area subjects (asymptomatic). The mean age of these individuals was 33, 39, and 35 years, respectively. The evolution time of the lesions in the aCL group was between 1 and 2 months, while hCL group presented healed lesions with more than 1 year. All individuals, including asymptomatic ones, lived for at least 22 years in the area, without any migratory event within this period. The aCL and hCL patients were treated with meglumine antimoniate following Brazilian Ministry of Health procedures, as previously described [4].

2.1.2. Mononuclear Cells Isolation and Culture. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque centrifugation (Pharmacia, Uppsala, Sweden), at 400 ×g, 20 min at room temperature, washed three times in RPMI medium (Gibco, Grand Island, NY), and suspended in DMEM medium (Gibco), supplemented with 50 μM 2-mercaptoethanol, 2 mM L-glutamine (Gibco), 40 μg/mL gentamicin, and 5% fetal calf serum (Gibco). Cell cultures (2×10^6 cells/well) were stimulated with 5 μg/mL whole soluble Lb antigens in presence or not of 10 μg/mL of anti-human IL-10 monoclonal antibody (anti-hIL-10 mAb) (R&D Systems, MAB217, Clone 23738) for 24 h at 37°C, 5% CO_2.

2.1.3. Cytokine and Chemokine Detection by ELISA. Cytokines (IL-10, IL-4, and TNF-α) and CXCL10 levels were measured by ELISA using pair-matched antibodies (Pharmingen, San Diego, CA) as described [4]. Briefly, 96-well ELISA microplates (Nunc, Denmark) were sensitized overnight with 100 μL of 1 μg/mL specific monoclonal antibody (Mabtech, USA, and Pharmingen, USA). The plates were then washed with PBS 0.05% Tween-20 (Sigma) and incubated with 2% bovine serum albumin (BSA; Sigma) in PBS. Plates were incubated overnight with 100 μL of 1 : 2 dilutions of culture supernatants in 2% BSA-PBS or with recombinant human cytokine (Pharmingen). Then, 1 μg/mL of appropriate biotinylated anti-cytokine monoclonal antibody was added (Mabtech and Pharmingen) for 2 h at 37°C, followed by washing and incubation with alkaline phosphatase-conjugated streptavidin for 2 h at 37°C. Finally, enzymatic activity was developed by incubation with p-nitrophenyl phosphate (Sigma). Absorbance was read at 405 nm in a microplate reader (BioRad, USA).

2.1.4. Statistical Analysis. Comparison of cytokines and chemokine production among groups of patients was performed by Kruskal-Wallis followed by Dunn's post hoc test. Statistical significance adopted $P < 0.05$. The equation used for data analysis was

$$\Big\{\big[(\text{cytokine detected in AgLb} + \text{aIL10 supernatant}) - (\text{cytokine detected in AgLb supernatant})\big] \cdot (\text{cytokine detected in AgLb supernatant})^{-1}\Big\} \times 100. \quad (1)$$

3. Results

The potential use of anti-IL-10 blocking mAb was tested in PBMC from individuals living in an endemic area of cutaneous leishmaniasis. As shown in Figure 1, there was only a partial blockade of the anti-IL-10 mAb used indicated by the remaining IL-10 detection in all groups. Decreased IL-4 levels were observed in cultures from all studied subjects, while decreased TNF-α production was observed in healed and asymptomatic individuals only. Patients with active lesions, however, presented a concomitant increase in TNF-α and in CXCL10 after IL-10 blockade.

The percentage of inhibition of cytokine production in Lb-stimulated PBMC cocultured in the presence of anti-IL-10 mAb was also evaluated (Figure 1(e)). Interestingly, anti-IL-10 mAb induced an overall decrease of IL-10, IL-4, TNF-α, and to a lesser extent CXCL10 production by cells from all subjects. Patients with active lesions were less affected by the IL-10 blockade, showing increased capacity of cytokine production in this condition. Moreover, CXCL10 production by healed patients and IL-4 production by cells from asymptomatic individuals showed not to be modulated by IL-10 blockade.

4. Discussion

Strategies focusing on the control of the cytokine milieu are really important in combating many infectious and noninfectious diseases. One of the most immunomodulatory cytokines to be elected for such control is IL-10. It is produced by different cell types with effects that vary from the activation to the downregulation of many cell types depending on cellular conditions and activation status [7]. One important aspect of the IL-10 biology is that this cytokine is able to modulate immunological responses of both Th1 and Th2 patterns. Effective cellular immune *response* and clearance of *Leishmania* infection in humans have shown to be dependent on Th1 cytokines like TNF-α and IFN-γ, while parasite persistence and disease establishment are favored by IL-4 and IL-10 overproduction [1–6]. It is noteworthy that the control of the IL-10 production would bring new insights to the therapy of both cutaneous and visceral forms

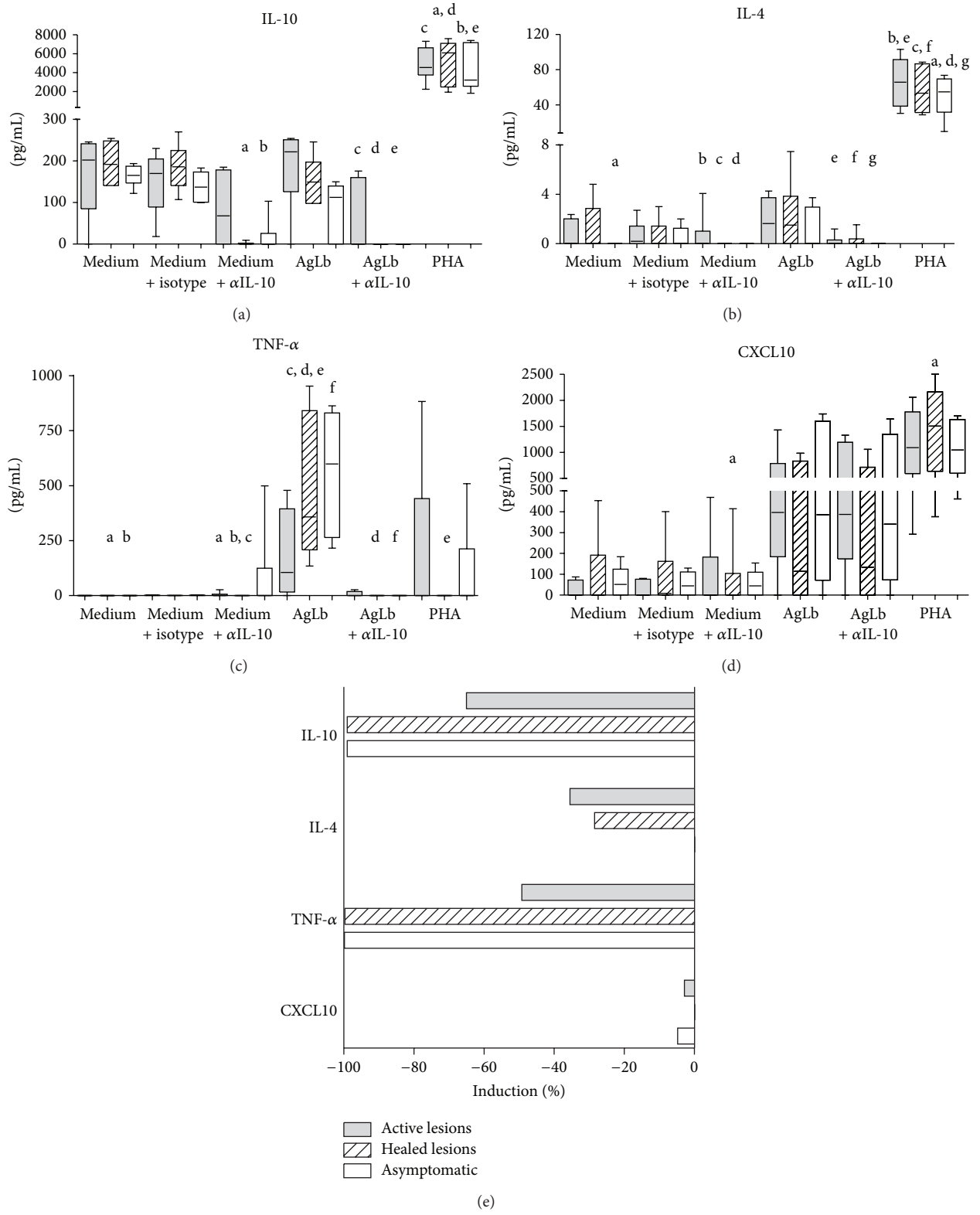

Figure 1: Modulatory effects of *in vitro* IL-10 blockade over T cell response in patients with cutaneous leishmaniasis. Cytokines IL-10, IL-4, TNF-α, and chemokine CXCL10 produced by PBMC were measured by ELISA. Cells were cultured for 24 h in the presence of 5 μg/mL *L. braziliensis* antigens alone or in combination with 5 μg/mL anti-human IL-10 mAb. Patients were grouped according to the presence of active lesions ($n = 6$), healed lesions ($n = 6$), or lack of any disease history ($n = 6$). (a)–(d) Levels of each cytokine production are plotted. The horizontal line represents the median, the bar 25th–75th percentiles, and the vertical line the 10th–90th percentiles. Equal letters mean Kruskal-Wallis test, $P < 0.05$, and post hoc Dunn test statistically significant. (e) Percentage of induction in the production of each cytokine and chemokine by IL-10 blockade was evaluated considering $\{[(\text{AgLb} + \alpha\text{IL-10}) - \text{AgLb}]/\text{AgLb}\} * 100$. Bars indicate median inhibition values for each group. $^{*}P < 0.05$.

of the disease especially in those cases where parasites present drug resistance or when patients display low tolerance to therapy. However, few data is available on the effect of IL-10 neutralization in human leishmaniasis. In VL form of the disease, the administration of an anti-IL-10 mAb to PBMC cultures restored the unresponsiveness of T cell proliferation against *Leishmania* antigens in one patient [1]. More recently, a similar neutralization strategy in cultures of splenic aspirate cells from VL patients promoted a decrease in the number of amastigotes concomitantly with an increased production of IFN-γ and TNF-α [9]. Moreover, PBMC from unexposed subjects produced higher levels of IFN-γ, TGF-β, and reactive nitrogen species when cultured in the presence of *Leishmania* antigens and anti-IL-10 mAb [8]. In cutaneous leishmaniasis, the only existing data is a recent report on which the addition of an anti-IL-10 mAb abrogated the *in vitro* modulatory effect of intralesional $CD4^+CD25^+Foxp3^+$ Treg cells and promoted an increase in IFN-γ production by effector T cells from *L. guyanensis* infected individuals [2]. Recent data suggested that human IFN-γ-producing $CD4^+$ T cells seem to be essential for inducing parasite killing by macrophages, *in vitro*. These cells lost their activity after anti-IFN-γ mAb addition to the culture [10]. On the other hand, $CD8^+$ T cells have been associated with tissue damage, local necrosis, and lesion progression in CL patients and infected mice [10, 11]. In both papers, the cytolytic activity of $CD8^+$ T cells observed in CL patients seems not to be directed against parasite killing but to tissue destruction. Inhibition of IFN-γ in the cell cultures did not modulate the cytolytic activity of $CD8^+$ T cells but increased the infection index of cocultured macrophages infected with *L. braziliensis*. These data suggest that $CD4^+$ T cells are the main sources of anti-*Leishmania* IFN-γ-dependent protective immune responses, which indicates that increasing the activity of Th1 cell population would function as an important strategy in CL therapy. The importance of IL-10 in modulating the cytolytic activity of $CD8^+$ T cells in human leishmaniasis is unclear.

Interestingly, our data showing the partial neutralization of the IL-10 production in PBMC was divergent among habitants from the same area where CL is endemic. Infected individuals were still able to respond to the IL-10 neutralization by producing near to basal levels of cytokines as observed in the anti-*Leishmania* T cell response. Decreased CXCL10 modulation observed here indicates that IFN-γ-dependent responses could be restored in these individuals and would ensure disease recovery, parasite control, and a better prognosis. In contrast, there was a limited downmodulation on TNF-α production in aCL group in response to anti-IL-10 mAb. This result would be considered as a drawback of the potential therapeutic administration of anti-IL-10 mAbs to CL patients. Strong evidence suggests that excessive proinflammatory responses, especially those mediated by TNF-α, lead to tissue damage and mucosal commitment in American tegumentary leishmaniasis [12–15]. In this context, the adoption of therapeutic strategies aiming at the complete depletion of IL-10-dependent response should not be the best option for treating CL patients. For that reason, a partial blockade of IL-10 was adopted in this study. Overall decrease on cytokine production observed in uninfected individuals suggests that the adoption of this strategy is only effective on susceptible individuals whose immune response tends to be Th2-biased and modulated by IL-10 [4, 6]. Though limited to a small number of subjects, our work reinforces the idea that an unbalanced immunomodulatory response might be detrimental for successful CL treatment and lesion healing in *L. braziliensis* infection [4, 6, 13–16].

As discussed here, our data complements previous studies on IL-10 blockade strategy in human *Leishmania* infection. Considering the host-parasite interplay, independently on the clinical form of the disease, a partial blockade of the IL-10 would favour parasite clearance, lesion healing, and the establishment of an effective anti-*Leishmania* immune response. In human CL, our work is the first to adopt this strategy in *L. braziliensis* infection, which complements the previous data on IL-10 blockade in *L. guyanensis* infection [2].

Traditionally, it has been shown that VL patients treated with a combination of recombinant human INF-γ and pentavalent antimony had an increased successful rate and better disease recovery [17, 18]. In CL, the immunotherapeutic approaches already adopted were based mostly on the administration of killed or pasteurized *Leishmania* parasites alone or in addition to BCG showing elevated treatment efficacy and lesion healing [19]. One work adopted the administration of GM-CSF to a small number of patients showing a 100% cure of CL lesions [20]. Collectively, these data suggest that studies about new potential immunotherapies should be performed for both VL and CL treatments.

Here, we explore, for the first time, the potential role of IL-10 blockade in humans infected with *L. braziliensis* and bring some perspectives on the generation of new immunotherapies for cutaneous leishmaniasis.

Abbreviations

aCL: Active cutaneous leishmaniasis lesions
AgLb: *Leishmania braziliensis* protein extract
CL: Cutaneous leishmaniasis
hCL: Healed cutaneous leishmaniasis lesions
Lb: *Leishmania braziliensis*
mAb: Monoclonal antibody
Treg: T regulatory lymphocytes
VL: Visceral leishmaniasis.

Ethical Approval

The study protocol was approved by the Ethical Committee on Human Research of the Universidade Federal do Triângulo Mineiro.

Consent

Patients signed the informed consent before enrollment.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Lucio Roberto Castellano and Virmondes Rodrigues conceived the study and designed the study protocol; Dalmo Correia, Helia Dessein, and Alain Dessein carried out the clinical and epidemiological assessment; Lucio Roberto Castellano, Laurent Argiro, Marcos Vinícius da Silva, and Virmondes Rodrigues carried out the immunoassays and cytokine determination and analysis and interpretation of these data. Lucio Roberto Castellano, Marcos Vinícius da Silva, Dalmo Correia, and Virmondes Rodrigues drafted the paper; Dalmo Correia, Alain Dessein, and Virmondes Rodrigues critically revised the paper for intellectual content. All authors read and approved the final paper. Lucio Roberto Castellano and Virmondes Rodrigues are guarantors of the paper.

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Review Article

Complexity and Controversies over the Cytokine Profiles of T Helper Cell Subpopulations in Tuberculosis

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Tuberculosis (TB) is a contagious infectious disease caused by the TB-causing bacillus *Mycobacterium tuberculosis* and is considered a public health problem with enormous social impact. Disease progression is determined mainly by the balance between the microorganism and the host defense systems. Although the immune system controls the infection, this control does not necessarily lead to sterilization. Over recent decades, the patterns of CD4+ T cell responses have been studied with a goal of complete understanding of the immunological mechanisms involved in the maintenance of latent or active tuberculosis infection and of the clinical cure after treatment. Conflicting results have been suggested over the years, particularly in studies comparing experimental models and human disease. In recent years, in addition to Th1, Th2, and Th17 profiles, new standards of cellular immune responses, such as Th9, Th22, and IFN-γ-IL-10 double-producing Th cells, discussed here, have also been described. Additionally, many new roles and cellular sources have been described for IL-10, demonstrating a critical role for this cytokine as regulatory, rather than merely pathogenic cytokine, involved in the establishment of chronic latent infection, in the clinical cure after treatment and in keeping antibacillary effector mechanisms active to prevent immune-mediated damage.

1. Introduction

Tuberculosis (TB) is a contagious infectious disease caused by the TB-causing bacillus *Mycobacterium tuberculosis* and is considered a public health problem with enormous social impact. Approximately 8.6 million new TB cases and 1.6 million deaths are recorded annually; therefore, this illness is a major cause of death worldwide [1]. Transmission of *M. tuberculosis* occurs by inhalation of droplets containing these bacilli that are eliminated in the sputum of an individual with active disease. In most cases, approximately 90–95%, *M. tuberculosis* infection is clinically asymptomatic and not transmitted, a state referred to as latent tuberculosis. It is estimated that one-third of the world population is infected with *M. tuberculosis*, but only 5–10% will develop active disease at some point in their lives [1, 2].

Disease progression is determined mainly by the balance between the microorganism and host defense systems and major changes in the immune status of the individual potentiate TB activation or reactivation [3]. Although the immune system controls the infection, this control does not necessarily lead to sterilization. Once *M. tuberculosis* can be found in vacuoles of macrophages, the protective immune response against mycobacteria is dependent on

the interaction between these host cells and CD4+ T cells. Depletion of CD4 or MHC class II molecules in mice impairs control of bacterial growth, and animals succumb to the disease [4, 5]. Similarly, HIV patients with reduced CD4+ T cells are highly susceptible to tuberculosis [6].

Over recent decades, the patterns of CD4+ T cell responses have been studied, with the goal of complete understanding of the immunological mechanisms involved in the maintenance of latent or active tuberculosis infection and of the clinical cure after treatment. Conflicting results have been suggested over the years, particularly in studies comparing experimental models and human disease. In recent years, in addition to Th1, Th2, and Th17 profiles, new standards of cellular immune response, such as Th9 and Th22, have also been described (Table 1). Similarly, several studies have pointed IL-10 as a crucial regulator to determine the quality and intensity of the immune response, demonstrating a major role in the establishment of latent infection, to prevent immune-mediated damage and establishment of clinical cure after treatment keeping the antibacillary effector mechanisms.

2. T Helper Cells and Immune Response in Tuberculosis

T cell-mediated immune response begins after dissemination of *M. tuberculosis* to the lymph nodes [66, 67]. After activation and expansion of antigen-specific T cells, they then migrate to the infected lungs where they are found, together with other leukocytes, as part of granulomas. Several distinct types of T helper cells (such as Th1, Th2, Th17, and regulatory T cells) are present at the site of infection (Table 1); however, the Th1 subset is classically associated with impaired growth and dispersion of *Mycobacterium tuberculosis* [68]. Because each pattern of immune response culminates in different effector mechanisms, it is essential to understand the role of each one in response to *M. tuberculosis*.

2.1. Th1. IFN-γ, the main cytokine of the Th1 profile, enhances macrophage microbicidal mechanisms because it activates signaling pathways that include the iNOS pathway [39, 40] and induces the process of acidification and maturation of phagosomes and autophagy [41]. The main source of the IFN-γ that is responsible for the control of *M. tuberculosis* is CD4+ T cells [69]. Additional roles in the production of that cytokine are attributed to CD8+ T cells, natural killer cells, $\gamma\delta$ T cells, and CD-1 restricted T cells; however, none of them can compensate for the absence of CD4+ T cells [68]. The importance of IFN-γ in response to *M. tuberculosis* has been widely investigated in experimental models and in humans. Knockout mice for IL-12 [7], IFN-γ [8, 9], or T-bet [10] are highly susceptible to TB. It was also demonstrated that a reduction in IFN-γ may lead to an increased influx of neutrophils and extensive tissue damage resulting in tuberculosis in animal models [38]. Individuals with mutations in the IL-12/IFN-γ axis develop disseminated infection caused by BCG or nontuberculous species of mycobacteria [11]. Furthermore, results from our group and other studies have also demonstrated that peripheral blood mononuclear cells (PBMCs) from patients with active disease secrete lower levels of IFN-γ *in vitro*, both in unstimulated cultures or after stimulation with mitogens or mycobacterial antigens [13, 33, 58, 60, 70–74]. A recovery of the ability to produce IFN-γ after specific antituberculosis therapy was also demonstrated, but this production was found at low levels when compared with patients with latent tuberculosis [74, 75].

Despite the important role of IFN-γ in the fight against *M. tuberculosis*, some studies have shown that production of this cytokine is not sufficient to prevent active disease. Most people who develop active TB are able to activate IFN-producing T cells that are specific for *M. tuberculosis* at the site of infection [34–37]. It has been shown that patients whose T cells produce higher amounts of IFN-γ are more likely to progress to active disease than patients with weaker responses [76]. One possible explanation for the contradictory results regarding the production of IFN-γ at the site of infection and in peripheral blood is that the PBMCs of patients with active disease are more susceptible to apoptosis than healthy controls [77]. Moreover, in patients with active disease, T cells specific for mycobacterial antigens have been shown to be recruited and retained in lung tissues. In fact, several authors have shown that there is sometimes a positive relationship between circulating T-cell clones and those retained in the site of infection, especially in patients with active disease [78–80].

The notion that IFN-γ is necessary but not sufficient for bacterial control after infection is also supported by several studies in knockout mice for TNF-α, granulocytes, GM-CSF, IL-1, and IL-6, as they also die rapidly after *M. tuberculosis* infection. In other words, these results suggest that additional pathways are essential for immunity against *M. tuberculosis* [81]. Several experimental models have also linked the production of TNF-α with the maintenance of granuloma integrity, and changes in their levels have been correlated with disease susceptibility both in experimental models and in human patients [33, 42–44]. In fact, TNF-α acts synergistically with IFN-γ to stimulate the production of NO by macrophages and influences the expression of chemokines, such as CCL5, CCL9, CXCL10, and CCL2, which induce migration to and maintenance of immune cells in the infection site [82]. Blocking TNF-α, for example, in the treatment of rheumatoid arthritis, leads to a loss of granuloma structure and reactivation of the disease [45–48]. Conversely, *M. tuberculosis*-specific stimulation of IFN-γ (but not TNF-α) and IFN-γR signaling are significantly depressed in active TB, which correlates with TB disease severity and activity. Thus, the depression of both TNF-α and IFN-γ production and IFN-γR signaling may synergize to contribute to defective host control in active TB [74].

2.2. Th2. The role of Th2 cytokines, classical antagonists of the Th1 profile, has not been fully elucidated in experimental models or in patients with tuberculosis. Although these cytokines may be involved in mechanisms of evasion of *M. tuberculosis* from the immune system, their direct participation in disease reactivation is even more controversial [83].

Table 1: T helper cell (Th) subtypes and Th-related soluble mediators in human and experimental tuberculosis.

	Species	Putative role in tuberculosis	Reference
		T helper subtype	
Th1	Mouse and human	↓ the growth and dispersion of *M. tuberculosis*	[7–11]
Th2	Human	↑ in BALF associated with clinical progression	[12]
		↓ Th2 response in active disease	[13, 14]
	Mouse	↑ progression and reactivation of TB	[15]
T regulatory	Human	↑ Treg cells in more severe active disease	[16]
		↑ reactivation of latent TB	[17]
		↑ TB-MDR	[18]
	Mouse	↑ Treg cells in the early days of infection protects from severe disease	[19]
Th17	Human	↓ Th17 cells in active TB	[20, 21]
	Mouse	↑ neutrophil accumulation and tissue damage	[22, 23]
		↑ recruitment of IFN-γ producing cells	[24]
		Induce granuloma formation and remain as long-lived memory cells	[25–27]
Th22	Human	↑ in healthy *M. tuberculosis*-exposed individuals	[20]
		↑ in PBMC culture from patients with active TB	[28]
		↑ in pleural fluid from patients with active TB	[29–31]
Th9	Human	↑ Th9 cells in tuberculous pleural effusion	[32]
		T helper-related soluble mediator	
IFN-γ	Human	↑ *Mycobacterium*-specific production after clinical cure	[33]
		↑ in active TB patients at the site of infection	[34–37]
	Mouse	↓ influx of neutrophils and neutrophil-associated tissue damage	[38]
		↑ iNOS in infected macrophages	[39, 40]
	Human and mouse	↑ autophagy	[41]
NO	Mouse	↑ killing and growth inhibiting of virulent *M. tuberculosis*	[39]
TNF-α	Human	↑ *Mycobacterium*-specific production after clinical cure	[33, 42–44]
	Mouse and human	Maintenance of the granuloma integrity	[45–48]
IL-4	Human	↑ in the blood and BALF in TB patients with severe forms	[12, 49–53]
	Mouse	↑ progression and reactivation of TB	[15, 54]
		Without any influence in disease susceptibility	[55, 56]
	Mouse and human	↓ autophagic control of intracellular *Mycobacterium tuberculosis*	[57]
IL-10	Human	↑ *Mycobacterium*-specific production after clinical cure	[33]
		↑ in active disease	[14, 58, 59]
		No difference between active-TB and clinically cured individuals	[60]
	Mouse	↓ long-term lack of control of inflammatory responses and progression of the disease	[61]
IL-17	Mouse	Th1 induction following BCG vaccination	[62]
IL-9	Human	↑ in patients with pulmonary TB	[63]
		↓ IFN-γ expression by PBMCs in latent TB	[64]
IL-22	Human	↑ in pleural fluid from patients with active TB	[29–31]
	Mouse	IL-22 deficiency does not alter the outcome of infection	[32, 65]

In experimental models, some studies have shown an association of increased IL-4 with progression of tuberculosis and reactivation of the disease [15, 54], but other authors have shown that the absence of this cytokine does not influence susceptibility to the disease [55, 56].

In human tuberculosis controversial results are also observed with respect to induction of the Th2 subset of CD4+ T cells. Some authors have demonstrated increased levels of IL-4 in the blood [49–52] and in bronchoalveolar lavage fluid (BALF) in patients with TB, especially those with the more severe forms [53]. Ashenafi et al. have recently demonstrated an association between high levels of IL-4 and CCL4 in BALF, increased expression of SOCS3, and clinical progression of the disease. Furthermore, a positive

correlation was found between IL-4 and CCL4 levels and mycobacteria-specific IgG titers in BALF and plasma samples [12]. In other studies, it was observed that the decrease in Th1 response in patients with active disease is not accompanied by an increase in the Th2 pattern [13, 14]. When examining the kinetics of Th1 and Th2 cytokine production in patients with active disease, the secretion of both types of cytokines has been shown by using PBMCs from active TB patients [84]. Also, Marchant and colleagues demonstrated that, prior to therapeutic intervention, there is a predominance of "Th0" cells because concomitant expression of IFN-γ and IL-4 was found; however, with treatment progression, there was a decrease in clones producing IL-4. In contrast, patients with treatment-resistant tuberculosis showed an increase in "Th0" cells because there was persistent production of IL-4 regardless of the presence or absence of treatment [84].

The participation of Th2 cytokines in susceptibility to tuberculosis was reinforced by the description of the importance of autophagy in immunity against *M. tuberculosis* [41, 85]. It is known that IL-4 and IL-13 cytokines act as inhibitors of the autophagic process [57, 86], impairing antigen presentation, T cell clonal expansion, and consequently the organization of granulomas in tuberculosis [85–87]. However, it remains unclear whether the production of Th2 cytokines is a primary cause of reactivation of tuberculosis or just a consequence of progression of the active infection [83, 88].

2.3. T Regulatory. In many chronic infections, including TB, immune-mediated tissue injury can lead to greater damage to the host when compared with the presence of the pathogen itself. Thus, it is important that mechanisms are activated to counterregulate proinflammatory immune responses and prevent the harmful effects of excessive inflammation [81]. Regulatory T cells (Foxp3+ CD4+ and CD25+ (Treg)) suppress inflammation and limit immune responses by producing immunosuppressive cytokines, such as IL-10, IL-35, and TGF-β, and directly interacting with other cells by inhibiting surface molecules [89].

The role of Treg cells in tuberculosis, especially in human disease, still remains controversial. Some studies suggest that the involvement of Treg cells is observed more in individuals with more severe active disease [16], and depletion of these cells results in the production of high levels of IFN-γ [33, 90–93]. Previous results also suggest an important role for regulatory T cells in reactivation of latent tuberculosis and development of active tuberculosis by decreasing the production of IFN-γ, while production of IL-17 continues to induce the accumulation of polymorphonuclear leukocytes at lung sites [17]. Additionally, one study suggests that a concomitant increase in the proportion of CD4+ CD25+ and CD4+ CD25+ Foxp3+ cells are associated with active pulmonary tuberculosis [94], but the depletion of CD4+ CD25+ cells has no effect on bacterial load or lung damage due to infection [95]. A recent study by Chen and colleagues showed that, in experimental tuberculosis, concomitant expansion of regulatory T cells (Foxp3+ T cells) and effector T cells is already occurring in the early days of infection, and this increase is critical for control of the parasite because it protects from severe disease [19]. Despite these results, it is known that the persistent stimulation in multidrug-resistant tuberculosis increases Treg cells and could be a cofactor in clinical cure delay [18].

2.4. Th17. Activation of naïve T cells in the presence of TGF-β and IL-6 directs the differentiation of these cells into Th17 cells through activation of STAT-3. This in turn increases expression of the transcription factor RORγt and promotes the production of both proinflammatory cytokines IL-17 and IL-22 [96]. The induction of IL-21 mediated by IL-6 also strengthens the engagement of the Th17 strain in an autocrine fashion. It is known that TGF-β is produced in excess during tuberculosis and is expressed at sites of active *M. tuberculosis* infection [97], suggesting their involvement in the differentiation of Th17 cells in addition to its well-known immunomodulatory role. For tuberculosis, it has been shown that IL-23 is required for the development of Th17 cells because mice deficient in the p19 subunit of IL-23 are unable to maintain sustained expression of IL-17 during the course of infection [98]. In the same study, however, it was suggested that the Th17 response is dispensable for protection against infection.

Wozniak et al. suggest that cross-regulation of Th1 and Th17 populations is essential for conferring a significant protective effect against *M. tuberculosis* without excessive damage [25]. In agreement with these findings, another study showed that, during infection with *M. tuberculosis*, IFN-γ inhibits the production of IL-17 by CD4+ T cells, impairing the survival of neutrophils and the accumulation of these cells in infected lungs, which contributes to a reduction in inflammation [38]. Cruz et al. showed that IFN-γ-deficient mice infected with mycobacteria exhibit intensified accumulation of neutrophils and IL-17-producing T cells in the granulomatous lesions and that these cells did not control the growth of bacteria and yet compromised the integrity of the infected tissue [22]. These data suggest that IFN-γ appears to limit the population IL-17-producing cells. The involvement of IL-17 and IL-23 in mediating the immunopathology of TB has also been demonstrated by Cruz et al. [23].

In contrast, some studies have indicated that cells producing IL-17 may confer protection in patients with tuberculosis. Khader et al. demonstrated in an experimental model that IL-17 is required to accelerate the recruitment of cells producing IFN-γ in the lung, and this effect is a result of increasing concentrations of the chemokines CXCL9, CXCL10, and CXCL11 [24]. Recently, Gopal et al. suggested that an IL-17 mediated response is required for induction of a Th1 response following vaccination with BCG [62]. Other studies have also shown that Th17 cells, in addition to inducing the early events of granuloma formation [26] and remaining as long-lived memory cells [25, 26, 99], can also mediate mechanisms of protection independent of IFN-γ [25, 27]. In humans, some studies have noted that there is a deficient Th17 response in patients with active TB, especially when compared with latent TB, a finding that does not seem to be related to a large recruitment of these cells to the lung environment [20, 21].

2.5. Th22. IL-22 is a member of the IL-10 family of cytokines, which are mainly produced by Th17 cells. However, recently, a subpopulation of human T cells producing IL-22 has been described as a separate helper T cell line known as Th22. The differentiation of these cells occurs from naïve precursors and is dependent on IL-6 and TNF-α via activation of the transcription factor aryl-hydrocarbon receptor (AhR) [100]. It appears that IL-22 is important for inflammatory responses in the skin and mucosal surfaces because it has been reported in a number of human diseases, including inflammatory bowel disease, psoriasis, and rheumatoid arthritis [101]. Despite reports that IL-22 deficiency or neutralization does not alter the outcome of *M. tuberculosis* infection in mice [32, 65], studies in patients with tuberculosis have shown the presence of IL-22-producing CD4+ T cells: Scriba et al. demonstrated that a substantial proportion of mycobacteria-specific Th cells from healthy *M. tuberculosis*-exposed individuals produce IL-22 and are distinct from Th17 and Th1 cells, implicating IL-22 as an important cytokine axis in human antimycobacterial immunity [20]. Similarly, Qiu et al. demonstrated that there is an intense production of IL-22 and IFN-γ by distinct subsets of CD4+ T cells in cultures of PBMCs from patients with active TB, and these populations were reciprocally regulated after blocking such cytokines in culture with monoclonal antibodies [28].

Patients with tuberculous pleural effusion have an increased concentration of IL-22 and Th22 cells in pleural fluid samples that exceed the corresponding blood levels in the same patients, suggesting that this cytokine may be involved in pathogenesis of the disease [29–31]. Most Th22 cells in the pleural effusion exhibited a phenotype of effector memory cells, expressing high levels of CD45RO and low levels of CD45RA and CD62L. In addition, it was shown that IL-1β, IL-6, and TNF-α can promote the differentiation of Th22 cells from naïve CD4+ T cells and that combinations of these cytokines promote differentiation at more pronounced levels [63].

2.6. Th9. IL-9 has long been considered a Th2 cytokine due to its participation in processes of allergic inflammation. However, recent studies have revealed that this cytokine has other important functions and distinct subpopulations of CD4+ T cells, called Th9, are able to produce them. Th9 cells are characterized by production of IL-9 and IL-10 and develop from a naïve CD4+ precursor in the presence of TGF-β and IL-4 [102]. Some studies show that Th9 cells can trigger inflammation and contribute to the development of allergic diseases [64]; however, the roles of these cells in infectious diseases, including tuberculosis, are not well established.

Recently, Ye and collaborators demonstrated the presence of Th9 cells in patients with tuberculous pleural effusion. The differentiation of Th9 cells from CD4+ cells that were isolated from the pleural effusion or blood of these patients was dependent on TGF-β, and the production of IL-9 in the cultures was amplified by the addition of IL-4, IL-1β, and IL-6. It was also suggested in the same work that IL-9 may be, along with TGF-β, promoting the differentiation of Th17 cells because a positive correlation was observed between the number of Th9 and Th17 cells in the pleural effusion [63].

Although the participation of Th9 cells in tuberculosis has not been reported in other studies, production of the cytokine IL-9 has been widely demonstrated in some studies. One example is that patients with pulmonary TB had significantly higher levels of IL-6 and IL-9 compared to healthy controls [103, 104]. Hur et al. found that IL-9 production, together with IL-5, IL-13, and IL-17 in response to antigens ESAT-6/CFP-10, can potentially differentiate between latent *M. tuberculosis* infection and infections with environmental mycobacteria, such as *M. avium* and *M. kansasii* [105]. Moreover, in patients with latent TB, the addition of exogenous IL-9 reduced the expression of IFN-γ by PBMCs *in vitro*, and neutralization of IL-9 restored IFN-γ production, suggesting that IL-9 may contribute to the development of TB by promoting an impaired Th1 response [106].

3. IL-10 and Tuberculosis—A Delicate Balance between Bacillary Persistence and Reducing Damage

Several studies have indicated that the recurrence of tuberculosis is associated more with reemergence of a previous infection than with a new infection, reinforcing the concept that antituberculosis immunity that is generated after treatment culminates in clinical recovery but without resulting in a sterilizing cure. This aspect seems crucial because immune responses mediated by both effector (especially Th1 and Th17) and regulatory (T regulatory cells and IL-10) mechanisms are required to allow the patient to fight against the bacilli without suffering extensive lung damage or death. Indeed, because the activity of unrestrained TNF and IFN-γ can be detrimental to the host under conditions of infection or microbial colonization, including during *M. tuberculosis* infection, various mechanisms are in place to prevent immunopathology, including those mediated by Foxp3+ regulatory T cells [16, 90, 91, 93] and IL-10 [14, 58–60, 74]. Contrary to what was initially believed, the ability to produce IL-10 has been shown not only in Th2 and Treg [107, 108] cells but also in Th1, Th9, Th17, and CD8+ T cells, especially those that are long-lived [107–119], and the occurrence of these multifunctional populations in the context of IL-10 still needs to be correctly determined in human tuberculosis.

Previous results from our group [33] note an interesting time-dependent effect in the establishment of protective immunity after antimicrobial therapy against *M. tuberculosis*. We demonstrated that the establishment of a Th1 response, characterized by increased production of IFN-γ and TNF-α, occurs later, as evidenced in patients who had been cured for over 12 months, and is accompanied by increased IL-10 production [33]. This slow development of a Th1 response in human tuberculosis differs from that observed in cutaneous leishmaniasis, a protozoan infection in which the Th1 response is also associated with cure and the Th1 response is induced immediately after treatment [120]. Consequently, our results indicate that although the process of clinical cure progresses with potentiation of Th1 cytokine production (IFN-γ and TNF-α), the production of higher levels of IL-10 is important for regulating the production of these proinflammatory cytokines [110]. The balance between these

regulators and TNF/IFN-γ may determine if the immune system can eradicate *M. tuberculosis* with minimum associated damage.

There is a growing body of evidence suggesting that the relationship between IL-10 and Th1 cytokines is not as antagonistic as originally believed, and infectious diseases appear to act in a complementary form [121]. Studies show that, for some infectious diseases, an increase in IL-10 potentially acts to decrease the deleterious effects of inflammation derived from Th1 cytokines without affecting the clearance of infectious agents [121–124], such as *Listeria monocytogenes*, *Trypanosoma cruzi*, and influenza virus [125–127]. Similarly, the protective response to *Toxoplasma gondii* is associated with IFN-γ, although in the absence of IL-10, infected animals may die due to extensive early tissue damage [128, 129]. *In vitro* approaches suggest that the sources of IL-10 in these infections are Th1 cells and these IL-10-producing Th1 cells still maintain their ability to activate macrophages [121]. CD4+ CD25+ Treg cells are also related to this function, especially in *Leishmania major* infection [90, 117, 118]. Also, during *L. major* infection, although the absence of IL-10 enhanced pathogen clearance, mice displayed a loss of immunity to reinfection, suggesting that IL-10 does limit pathogen clearance but has a key role in the maintenance of effector memory populations via a mechanism that remains unknown [130]. Knockout mice for IL-10 that were infected with *M. tuberculosis* did not show increased IFN-γ production [131], although they did show long-term lack of control of inflammatory responses and progression of the disease [61]. Additionally, IL-10 can also enhance inflammatory mediators, especially in an environment that is rich in IFN-γ [119].

With regard to the dynamics of IL-10 production in tuberculosis active disease and after clinical cure, the results are conflicting. Some studies indicate that there are higher levels of IL-10 in active tuberculosis [14, 58, 59], but Sahiratmadja and colleagues showed that this increase happens only after clinical cure [74]. Still, Zhang and colleagues showed no change in the levels of IL-10 among individuals with active disease and those who had been treated [60]. Recently, Siawaya and colleagues found that, during antituberculosis therapy, there were no major changes in the levels of IL-10; however, patients who had lower levels of this cytokine in the earlier stages had negative cultures for *M. tuberculosis*, indicating better bacterial clearance in these individuals [132]. Pereira and colleagues found that, in addition to an enhancement in the production of TNF-α, patients with systemic manifestations of tuberculosis have increased production of IL-10 [73]. These data suggest that IL-10 can have deleterious effects on the patient during active disease. Gerosa and colleagues have previously shown that there is concomitant production of IFN-γ and IL-10 by Th1 lymphocytes as well as by memory T cells in bronchoalveolar lavage fluid obtained from patients with active tuberculosis [133]. If the IL-10 somehow acts as a weapon used by the bacilli to interfere with proper macrophage activation during active infection, participation in controlling exacerbation of the immune response seems to be of vital importance to the infected individual [134].

In recent years, special attention has been given to so-called double-cytokine-producing effector T cells and their role in mediating the immune response in several infectious diseases, especially protagonists of the Th1 response, which is the case with toxoplasmosis, malaria, and leishmaniasis [33, 121, 135–137]. Initially, Th2 cells were the main populations of T cells that produced IL-10 in a sustained manner [138–140]. Later, several groups reported an associated production of IFN-γ and IL-10 [121, 141–144] and subsequently IL-17 and IL-10 [124]. These studies have not yet been able to determine if these populations are stable or are only transitory stages, where the production of IL-10 occurs only transiently and returns to its initial profile [145].

Experimental models suggest that the generation of IL-10-producing Th1 cells is due to chronic or repeated antigenic stimulation [146–149] because newly differentiated Th1 cells are apparently unable to secrete IL-10 due to the inaccessibility of the IL-10 gene promoter [139]. In fact, as mentioned earlier, several studies have indicated that the expression of IL-10 in cells that were initially committed to rigid profiles, such as Th1, Th2 or Th17, may reflect a mechanism of self-control after repeated antigenic stimulation where cell survival should be extended. Previous work in experimental models demonstrated that external factors, such as antigenic load and increased presence of IL-12, may direct Th1 cells to expression of IL-10, which returns to baseline upon removal of these stimuli [23, 150]. Moreover, this production is dependent on both the induction of STAT-1, which is classically associated with Th1 and IFN-γ production, and STAT-3, particularly via the IL-27-IL-27R axis [146, 147]. In Th17 cells, production is dependent on STAT-3 and IL-27-IL27R axis after induction by IL-6 and IL-21 [148–150]. Studies indicate that the IL-10/STAT-3 axis is essential for the development and function of CD8+ memory T cells [154–156] and expansion of these cells with IL-10 production capacity occurs during chronic infection with *M. tuberculosis* [157].

In addition to the aforementioned signaling pathways, the signaling receptor Notch has been shown to be essential for production of IL-10 by CD4+ T cells, as well as the IFN-γ+ and IL-10+ double-producing T cells [158, 159]. These receptors have also been associated with IFN-γ, especially via Notch1 and Notch2 during *Leishmania major* infection [160]. Indeed, stimulation of the Notch pathway via DLL-4, a prominent Notch receptor ligand, is described as guiding STAT-3, and consequently, IL-10 [156] and Notch signaling pathway has also been associated with increased survival of T cells [161], data that together could strengthen understanding of the occurrence of these double-producing cells in chronic pathological processes. Current results from our group indicate that a higher percentage of CD4+ cells express Notch1 in patients who are clinically cured of tuberculosis, a condition that is characterized by increased IFN-γ and IL-10.

Furthermore, FoxP3− CD4+ memory cells, especially those that express CCR6, are induced by IL-10 and secrete the same cytokines with the purpose of self-regulation [162]. We should also consider that the decrease in IFN-γ and TNF-α in the supernatants of these cell strains can only be the result of apoptosis of IL-10-producing Th1 cells after their

suppression. Several studies have also indicated that anti-apoptotic functions of IL-10 are important because blocking this cytokine makes non-Hodgkin's lymphoma cells more susceptible to apoptosis [163] and increases cell apoptosis in the retina [164]. In addition, IL-10 prevents T-cell mediated apoptosis by parainfluenza virus type 3 (PIV3) [165] and EBV [166] and inhibits apoptosis of cardiomyocytes, monocytes, and macrophages [167, 168], especially via the induction of STAT-3 [169].

Over the years, several studies have examined the ability of DNA vaccines based on mycobacteria HSPs, particularly HSPs from *M. tuberculosis* and *M. leprae*, to provide protection against pulmonary tuberculosis. Interestingly, although the results indicate their ability to induce protective immunity against *M. tuberculosis* by enhancing effector populations of CD4 and CD8+ T cells and increasing IFN-γ [170–175], they also induce regulatory T cells and IL-10 for potential inhibition of autoimmune manifestations [147–149, 176–178], demonstrating a practical situation of success in controlling infection with *M. tuberculosis* with effector/regulator concurrent mechanisms.

In the context of human infection with *M. tuberculosis*, we understand that the influence of IL-10 on inhibiting apoptosis of T cells is crucial for maintaining the repertoire of cells that are responsible for bacillary control, maintenance of clinical latency, long preservation of lymphocyte life and memory, and therefore the ability to produce those cytokines that are related to protection and preservation of granulomas.

4. Conclusions

Based on careful surveys of the literature and published works from our group, we have come to the following conclusions:

(i) The balance between resistance and susceptibility to infection with *M. tuberculosis* is a very complex mechanism and presents several controversies over the cytokine profiles of T helper cell subpopulations.

(ii) IL-10 appears to have an important role in shaping the repertoire of T helper lymphocytes, irrespective of other cytokines that define their subpopulations.

(iii) Chronic infection in the presence of IL-10 appears to exert a regulatory role and minimize tissue damage, but without compromising the immune mechanisms involved in the control of *M. tuberculosis* and other related microorganisms.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Human Gene Expression in Uncomplicated *Plasmodium falciparum* Malaria

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To examine human gene expression during uncomplicated *P. falciparum* malaria, we obtained three samples (acute illness, treatment, and recovery) from 10 subjects and utilized each subject's recovery sample as their baseline. At the time of acute illness (day 1), subjects had upregulation of innate immune response, cytokine, and inflammation-related genes (IL-1β, IL-6, TNF, and IFN-γ), which was more frequent with parasitemias >100,000 per μL and body temperatures ≥39°C. Apoptosis-related genes (Fas, BAX, and TP53) were upregulated acutely and for several days thereafter (days 1–3). In contrast, the expression of immune-modulatory (transcription factor 7, HLV-DOA, and CD6) and apoptosis inhibitory (c-myc, caspase 8, and Fas Ligand G) genes was downregulated initially and returned to normal with clinical recovery (days 7–10). These results indicate that the innate immune response, cytokine, and apoptosis pathways are upregulated acutely in uncomplicated malaria with concomitant downregulation of immune-modulatory and apoptosis inhibitory genes.

1. Introduction

Malaria is caused by intracellular protozoa of the genus *Plasmodium* [1]. Each year 300 million to 3 billion people are infected and more than 600,000 die, making malaria the most important vector-borne disease in the world [2]. Of the five parasites that infect humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*), *P. falciparum* causes the most severe disease and the most morbidity and mortality [3]. Its manifestations range from asymptomatic to fatal infection [4, 5] and there are major gaps in our understanding of its pathogenesis [6] and the changes in gene expression responsible for the host response that are the focus of this study [7, 8].

Previous studies have shown that humans with malaria release proinflammatory mediators, such as tumor necrosis factor (TNF), interferon gamma (IFN-γ), and nitric oxide (NO), which may damage organs such as brain, lung, and kidney [5], and suggest that the levels of these mediators are related to the severity of disease [9–13]. Potential parasite virulence factors include the glycolipids that anchor parasite proteins to the red cell surface [14, 15], *var* genes [16, 17], and hybrid sequences in parasite proteins on the surface of parasitized red blood cells (Block 2 region of merozoite surface protein 1 [OA Koita: personal communication]).

However, until now, it has been difficult to study host gene expression in malaria because methods available could

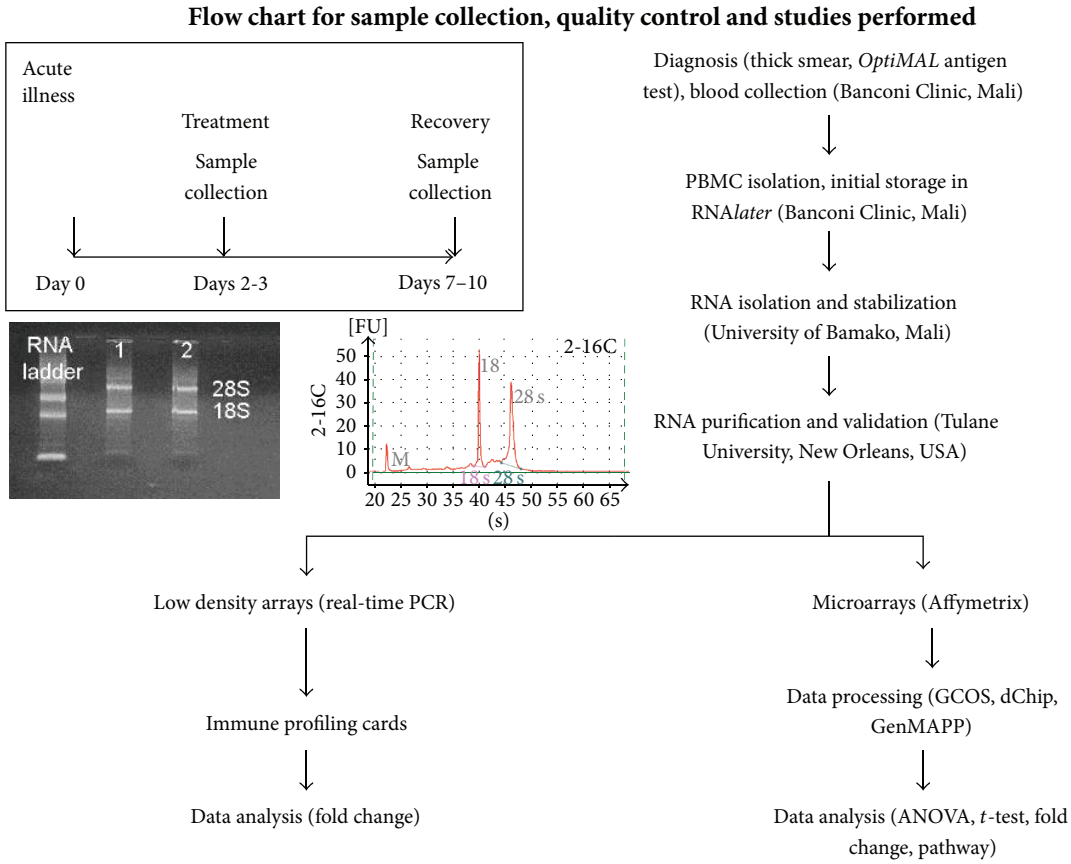


Figure 1: Study design and flow chart for sample collection. The time line (upper left) provides the times when the 3 samples were collected. The insets provide sample results of the procedures used to test RNA quality: agarose gel electrophoresis performed on-site in Mali (left) and capillary electrophoresis using the Agilent Biolanalyzer 2100 in New Orleans (right). Flow chart for sample collection, quality control, and studies performed.

examine only a few genes at a time (Northern blots, reverse transcriptase PCR). In contrast, cDNA [18] and oligonucleotide microarrays [19, 20] permit examination of the entire human transcriptome. To examine host gene expression in malaria, it was necessary to examine and resolve (1) effects of ambient temperatures > 45°C on RNA stability, (2) transport of RNA preparations from malarious areas to the U.S., (3) potential confounding by host genomic differences and host variation in gene expression, (4) the effects of antimalarial treatment on host gene expression, and (5) potential false-positive and false-negative microarray results.

2. Methods

2.1. Blood Collection and RNA Isolation. Venous blood samples (3 mL) were drawn from children 3–16 years of age with uncomplicated malaria and transferred to tubes containing Ficoll-Hypaque (CPT Tube 362760, Becton Dickinson). After centrifugation at 1,500 ×g for 20 min at room temperature, peripheral blood mononuclear cells (PBMCs) formed a 1-2 mm layer 5 mm above the gel separator which was aspirated manually (Samco Pipet 335, San Fernando, CA), mixed with 1.3 mL RNA*later* (Ambion, Austin, TX), and stored at 0°C (ice-water bath). Within 2 hours, RNA was isolated from this suspension using the RiboPure-Blood kit (Ambion).

2.2. RNA Quality. Was assessed by agarose gel (Figure 1) and OD 260/280 ratios in Mali. Samples with distinct 18S and 28S bands on agarose gel and OD 260/280 ratios ≥ 1.7 were stored in sodium acetate : ethanol (0.1 vol 3 M sodium acetate, [pH 5.2] : 2.5 vol 98% ethanol) at −20°C until transport to New Orleans where they were stored at −20°C for ≤ 4 weeks. RNA was purified by ethanol precipitation, followed by cleanup with the RNeasy Kit (Qiagen). RNA was then reexamined using the RNA 6000 Nano Marker Green kit (25–6,000 nt standards, Ambion) and the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, GERMANY) to identify 18S and 28S ribosomal peaks on the electropherogram and estimate the RNA Integrity Number (RIN) [21]. Samples with well-defined 18S and 28S peaks on the electropherogram, ≥1.0 μg RNA, and RINs ≥ 6.5 were converted to biotinylated cRNA and hybridized (Figure 1).

2.3. Conversion of mRNA to Double-Stranded Biotinylated cRNA. Double-stranded cDNA was synthesized using the Superscript system (Invitrogen), purified by phenol : chloroform

extraction, and concentrated by ethanol precipitation. *In vitro* transcription was used to produce biotin-labeled cRNA (GeneChip *In Vitro* Transcription Labeling Kit, Affymetrix).

2.4. Microarray Hybridization. Biotinylated cRNA was cleaned (RNeasy), fragmented, and hybridized on HG-U133 Plus 2.0 chips (Affymetrix). Each chip had > 54,000 probe sets for > 38,000 human genes [22]. After washing, chips were stained with streptavidin-phycoerythrin, amplified with biotinylated anti-streptavidin (Vector Laboratories), and scanned for fluorescence (GeneChip Scanner 3000) using the GeneChip Operating software, version 1.0 (GCOS).

2.5. Microarray Data Processing. Fluorescence intensities for perfect match (PM) and mismatch (MM) nucleotides were used to determine whether mRNA for specific genes was present (P), marginally present (M) or absent (A). Scanned images were transferred to dChip [23–25] and corrected for image discontinuities. To compare results across chips, one array was chosen as baseline (147C, median intensity 167) to which others were normalized by calculating expression values based on PMs and MMs. Negative results were assigned a value of one.

2.6. Filtering. Was performed to identify genes with changes in expression between (1) acute illness (A) and recovery (C), (2) acute illness (A) and treatment (B), or (3) treatment (B) and recovery (C) based on ≥ 90% confidence in a ≥ 1.5-fold change in fluorescence ($p < 0.01$) and a P-call ≥ 70% for samples with up- or downregulation. Samples were permuted 100 times to estimate the false discovery rate which was 0.0% (median), 90% CI: 16.4–25.2%. The three sets of genes were then combined by removing duplicate genes and genes with redundant probe sets.

2.7. Hierarchical Clustering in dChip. Expression values were standardized by adjusting samples linearly to a mean of zero with a standard deviation of one and by determining correlation coefficients (r values) for normalized expression (intergenic distances defined as $1-r$). Genes with the shortest distances between them were merged into supergenes, connected in dendrograms using lengths proportional to genetic distance (centroid-linkage), and repeated $n-1$ times to cluster all genes. A similar algorithm was used to cluster the samples [26, 27].

2.8. Gene Ontologies. Five patterns of expression on the heat maps were examined for Gene Ontology (GO) term enrichment [28] across the entire microarray by using the exact hypergeometric distribution (significant p values < 0.01).

2.9. Filtering Based on Parasitemia and Temperature (Table 1). Was based on acute illness samples and used analysis of variance (p values ≤ 0.01 and P-calls ≥ 20%); hierarchical clustering was used to identify patterns of expression and to test for GO term enrichment.

Table 1: Baseline information at the time of enrollment: subjects with uncomplicated *P. falciparum* malaria and uninfected controls.

Subjects			
Age (years)	Sex (M, F)	Temperature (°C)	Parasitemia (per μL)
6	F	37.9	1,400
13	M	38.0	35,575
14	F	38.0	6,100
9	F	38.6	10,625
7	M	39.4	61,125
16	M	39.6	14,675
14	M	39.6	53,350
3	M	39.7	113,000
9	F	40.0	166,500
11	M	40.0	29,525
Uninfected controls			
6	M	36.2	0 (Neg)
4	M	37.2	0 (Neg)
7	F	36.7	0 (Neg
7	F	36.8	0 (Neg)
2	M	36.0	0 (Neg)

2.10. Pathway Analysis and Filtering. Identified genes with differences in expression between acute illness and recovery based on (1) ≥ 90% confidence in ≥ 2-fold changes in expression (up or down) and (2) P-calls of 100% for the samples in which genes were up- or downregulated.

2.11. Samples from Uninfected Controls. Included 4 subjects who received chloroquine and one who received placebo (5% dextrose in saline). After normalization based on sample 2405A (median intensity 153), cRNA from these subjects was analyzed similarly using dChip.

2.12. Microarray Target Validation. Was performed with TaqMan low density arrays using cDNA prepared from total RNA (iScript kit, Bio-Rad), loaded in low density cards, and run on an ABI 7900 HT real-time thermocycler using 50–100 ng cDNA per well. Data were normalized using endogenous 18S cDNA as the control to calculate fold changes between samples with 95% CIs for genes with valid threshold cycle data for 3 or more of 4 replicates (based on acute illness or recovery samples). Genes with changes in expression that overlapped the baseline sample (based on the 95% CI) were excluded from the subsequent analyses.

2.13. Subject Identification, Informed Consent, and Study Design. Identification of infected subjects was performed at the Banconi Clinic in Bamako using Giemsa-stained thick blood smears, a positive parasite antigen test, and PCR for the polymorphic Block 2 region of *msp1* [29]. Informed consent was obtained before enrollment from the parents or guardians of children with uncomplicated *P. falciparum* malaria using a protocol approved by the Mali IRB in

Bamako and the Tulane IRB in New Orleans. To control for host genomic variation and variation in gene expression, three samples were obtained from each subject (Figure 1): (1) an acute sample at the time of diagnosis on day 1 before beginning treatment (*A*), (2) a treatment sample on day 3 or 4 after completion of the 3-day course of treatment (*B*), and (3) a recovery sample 7–10 days after treatment (*C*, no signs or symptoms of malaria, no asexual parasites on the thick smear). The recovery sample served as the baseline to which the other samples were compared to identify changes from baseline (normal) gene expression. Relevant clinical data, including parasitemia and temperature (Table 1), were obtained at the time of diagnosis and during follow-up visits.

To control for the effects of antimalarials on gene expression, uninfected healthy controls were identified in the village of Missira in the Kolokani District (160 km NW of Bamako) based on a negative Giemsa-stained thick smear, negative parasite antigen test, and the absence of symptoms or signs of malaria. After obtaining consent, these subjects were enrolled in the study and randomized to receive either chloroquine or placebo (5% dextrose in saline).

3. Results

3.1. Examination of Gene Expression Profiles. Identified three sets of genes with ≥1.5-fold changes between (1) acute illness and treatment ($n = 127$), (2) acute illness and recovery ($n = 67$), or (3) treatment and recovery ($n = 57$). These three sets of genes ($\Sigma = 251$) contained 219 unique genes, which were reduced to 182 by removing redundant probes. Conversely, when these criteria were used with samples from uninfected controls, only one gene was identified, indicating that antimalarial treatment produced no identifiable changes in gene expression. Gene expression patterns in the treatment and recovery groups (B and C) clustered closest to each other, with the acute illness group (A) being most distant. However, those patterns were not observed with the uninfected controls. Hierarchical clustering indicated that the uninfected controls were most similar to the recovery samples (C) and thus the recovery samples provided an accurate profile of gene expression in healthy subjects.

Hierarchical clustering of these 182 genes identified five expression profiles, four of which were linked to Gene Ontology (GO) categories such as host immune response or apoptosis (Figure 2). These four profiles included (1) genes upregulated during acute illness (A), (2) genes upregulated during both acute illness and treatment (AB), (3) genes upregulated during both treatment and recovery (BC) and (4) genes upregulated only at the time of recovery (C). The fifth profile (B), which contained genes upregulated only during treatment, was not included in the subsequent analyses because those genes were scattered across Gene Ontology categories.

Expression profile A (79 upregulated genes, 65 in 41 GO categories) included genes involved in the innate immune response, effector cell activation, and chemotaxis: leukocyte immunoglobulin-like receptor (LIR) family, neutrophil/monocyte/macrophage chemotactic chemokine receptor-like 2 (CCRL2), toll-like receptor 5 (TLR-5), aquaporin 9, complement component receptor C3AR1, acute-phase cytokine IL-6, and the IL-1β receptor antagonist.

Expression profile AB (44 upregulated genes, 41 in 25 GO categories) included genes involved in the immune response, apoptosis, and cell death. Immune response genes included complement components C1qA, C1qB, C1qG, and C2, chemokine ligand CXCL16, and Fc III γ-receptors CD16a and CD16b. Upregulated apoptosis- and cell death-related genes included BCL2-related X-protein (BAX), BH3 interacting domain death agonist (BID), baculoviral IAP repeat-containing 5 (survivin), and caspase 5.

Expression profile BC (7 upregulated genes, 7 in 13 GO categories) included genesinvolved in cellular development and the response to injury such as myosin regulatory protein MYL9, bone morphogenic protein 6, thrombospondin 1, and the oxytocin receptor.

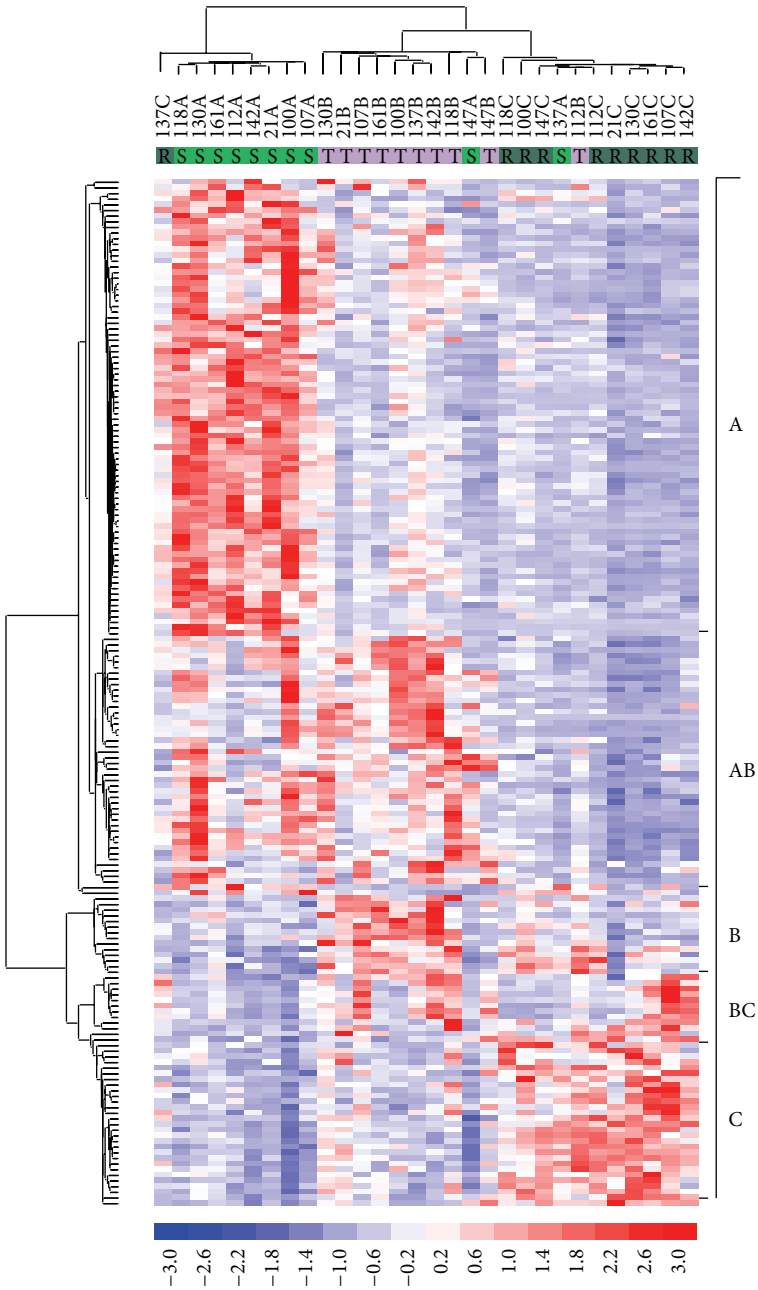
Expression profile C (31 upregulated genes, 27 in 7 GO categories) includedgenes upregulated at recovery (10–14 days after acute illness) such as human leukocyte antigens HLA-DOA and HLA-DOB, transcription factor 7, CD6, CD1C antigen, and the Fc IgE receptor, FCER1A.

3.2. Pathways (Gene Networks) Involved in Uncomplicated P. falciparum Malaria. Based on pathway analyses, a number of cytokine and inflammatory response genes were upregulated, including IL-6, IL-10, IL-1β, and TNF (Figure 3). Examination of the apoptosis pathway (Figure 4) demonstrated simultaneous upregulation of genes that facilitate apoptosis (BAX, NF-κ1B, TNF SF10, Gzm B, and TNF) and concomitant downregulation of genes that inhibit apoptosis (c-Myc, TNF R SF25, IRF4, KBBKG, caspase 8, and Fas Ligand G).

3.3. Pathway Analyses Based on Parasitemia and Temperature. Identified 55 genes based on parasitemia and 21 based on temperature. GenMAPP analyses revealed differences in the inflammatory and apoptosis pathways between subjects with low parasitemias and subjects with medium or high parasitemias. Within the inflammatory, immune response, and host response pathways, the differences were most striking for acute phase mediators such as IFN-γ, IL-1α, IL-1β, IL-6, IL-7, IL-10, IL-15, and TNF (Figures 3(a)-3(b)). Within the apoptosis pathway, the differences were most striking for TP53 (p53), Fas, Fas Ligand, MCL1, caspases 6, 8, and 10, TNF, and TNFSF10B and 25 (Figures 4(a)-4(b)). Similar results were obtained with increasing body temperature (data not shown).

3.4. Upregulation of the Inflammatory and Apoptosis Pathways with Higher Parasitemias. Was identified with ANOVA using Kruskal-Wallis. Similar upregulation was observed in subjects with elevated temperatures (< 39°C versus ≥39°C, data not shown).

3.5. Microarray Validation Using Low Density Arrays. Quantitative real-time PCR using TaqMan low density arrays demonstrated excellent correlations between the changes observed in the microarrays and low density arrays. The magnitudes of changes (fold increase or decrease) in gene expression in the inflammatory response and apoptosis pathways



GO group	*p* value
Expression profile A (79 total genes)	
Response to pest, pathogen, parasite	0.000000
Humoral immune response	0.000032
Cellular immune response	0.007245
Peptide receptor activity	0.005498
Expression profile AB (44 total genes)	
Response to pest, pathogen, parasite	0.001246
Complement activation	0.000001
Apoptosis	0.004142
Expression profile B (14 total genes)	
Hydrolase	0.005533
ATPase/transmembrane activity	0.005316
Expression profile BC (10 total genes)	
Response to wounding	0.009941
Hemostasis	0.000771
Development	0.000695
Expression profile C (31 total genes)	
Immune response	0.000190
Antigen presentation	0.003419
Immunoglobulin-like	0.001547

Figure 2: Heat map for changes in gene expression among subjects with uncomplicated malaria. Changes in gene expression with uncomplicated *P. falciparum* malaria revealed five major expression patterns: [A] genes upregulated at the time of acute illness, [AB] genes upregulated at the times of acute illness and treatment, [B] genes upregulated at the time of treatment, [BC] genes upregulated at the times of treatment and recovery, and [C] genes upregulated at the time of recovery. Based on Gene Ontology (GO) categories, profile A and AB genes were related to the immune response, AB genes to apoptosis, and C genes to immune-modulatory functions. In contrast, similar changes in gene expression were not observed in healthy control subjects without *P. falciparum* malaria (data not shown). Heat map for changes in gene expression among subjects with uncomplicated *Plasmodium falciparum* malaria.

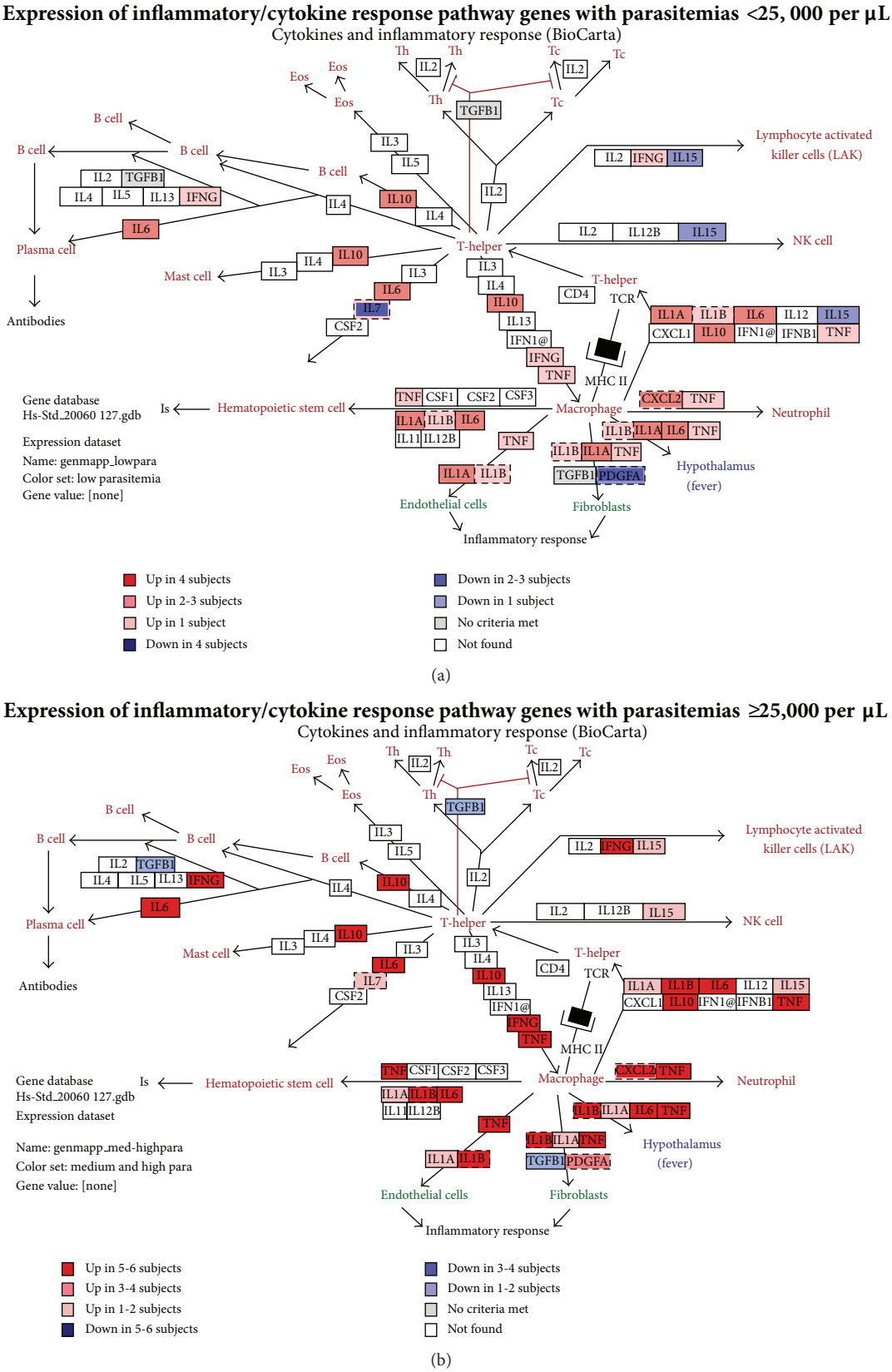
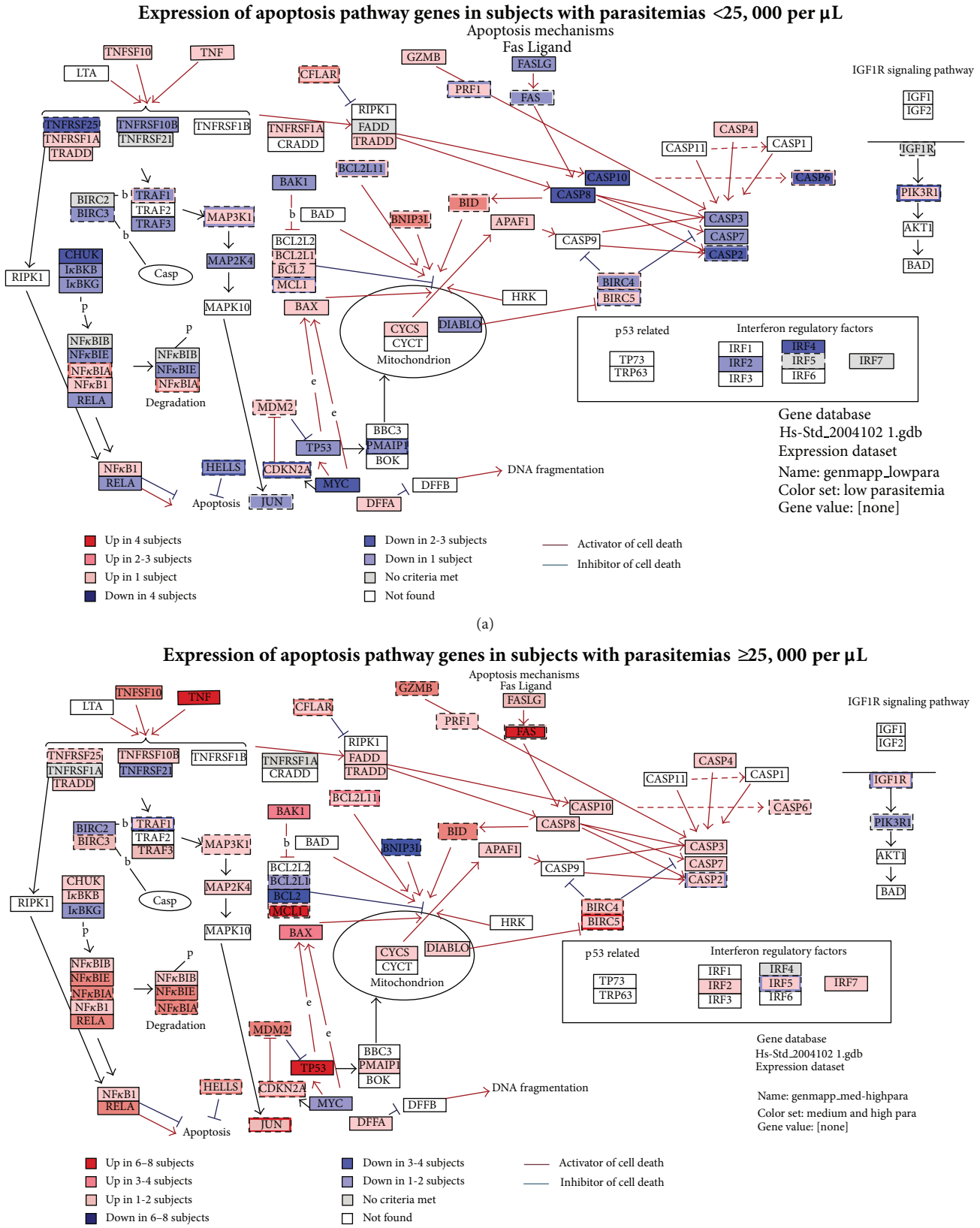


Figure 3: Inflammatory and cytokine response pathways. GenMAPP illustration of genes upregulated with low parasitemias (a) and medium to high parasitemias (b). Several genes were upregulated more frequently with medium or high parasitemias (b) than low parasitemias (a). Among these were IL-1β, IL-6, IL-10, TNF, and INF-γ. Additionally, a number of genes, including IL-7, IL-15, and PGDFA (platelet-derived growth factor alpha polypeptide), were downregulated with low parasitemias and upregulated with medium to high parasitemias. TGF-β was downregulated more with medium to high than low parasitemias.



(a)

(b)

FIGURE 4: Apoptosis response pathway. Expression of most apoptosis-related genes correlated with the parasitemia. Note that the majority of these genes, including Fas (first apoptosis signal), FasL (Fas Ligand), and TP53 (tumor protein 53), were upregulated in subjects with medium to high parasitemias. In contrast, the BCL2 interacting protein (BNIP) family gene and the BCL2 and BCL2L1 apoptosis inhibitory genes were upregulated in subjects with low parasitemias and downregulated in subjects with medium to high parasitemias.

varied similarly with parasitemia in both the microarrays (Figures 3-4) and the low density arrays (data not shown).

4. Discussion

4.1. Study Design, Obstacles, and Potential Confounders. The effects of ambient temperatures > 45°C in Mali on RNA quality were addressed by rapid sample processing, by storage of PBMC preparations at 0°C in RNA*later* until the time of RNA extraction, and by the evaluation of RNA quality on-site in Bamako and again in New Orleans. Confounding by individual variation in gene expression was addressed by using the recovery samples from each subject as their control. This study design also permitted the study of changes in expression over time in pathways that were initially up- or downregulated. Confounding by antimalarial treatment was addressed by demonstrating that antimalarial treatment alone had no effect on gene expression.

4.2. Comparison with Previous Reports. These results provide the first information of which we are aware for upregulation of the apoptosis pathway with concomitant downregulation of apoptosis inhibitory genes during acute malaria (Figure 4). In contrast, the upregulation of inflammatory, host response, and proinflammatory cytokine genes observed (TNF, IFN-γ) is consistent with previous reports (Figure 3) [5, 7, 9, 10, 15, 23, 24] and there was no upregulation of immune response genes in the uninfected controls (data not shown). Although some immune-related genes were downregulated, those downregulated genes had immune-modulatory functions (Figure 2, profile BC).

4.3. Pathway Analyses. Revealed upregulation of cytokine, immune response, inflammatory response, complement activation (Figure 3), apoptosis (Figure 4), and Fas pathways [30–32] in subjects with higher parasitemias (25,000–100,000 per μL and >100,000 per μL, Figures 3-4) and higher temperatures (≥39°C, data not shown).

4.4. The Role of the Innate Immune System in Uncomplicated P. falciparum Malaria [33–35]. Is supported by the upregulation of Toll-like receptors (TLR 5) [36, 37], NK cell receptors (LILRs, KIRs), and chemokines during the acute illness (day 1). Indeed, several studies have suggested that the glycosylphosphatidylinositol (GPI) lipopolysaccharide which anchors *P. falciparum* proteins to parasite and red cell surfaces may stimulate the production of proinflammatory cytokines such as TNF, and Zhu et al. [15] have shown that GPI induction of IL-6, IL-12, and TNF is dependent on TLR activation via the p38- and NF-κB pathways. In these studies, IL-1β, IL-6, and TNF were upregulated more frequently in subjects with higher parasitemias (Figures 3-4) and higher temperatures (data not shown). In addition, studies in Uganda suggest that individuals heterozygous for the TLR 2 Δ22 polymorphism may be protected from cerebral malaria [38].

4.5. Apoptosis in Malaria. The data reported here are likewise consistent with previous studies suggesting that apoptosis plays an important role in the pathogenesis of uncomplicated *falciparum* malaria [32, 39–41]. Because they indicate that genes involved in apoptosis, such as Fas, BAX, and TP53, are upregulated during uncomplicated *P. falciparum* malaria *in vivo* (Figure 4), they suggest that apoptosis may be initiated through several different pathways in uncomplicated malaria, including TNF binding to TNFRSF, Fas (which can be induced by TNF), TNF binding to FasL, and possibly HMOX in response to oxidative stress. In these studies, upregulation of apoptosis-related genes correlated with higher parasitemias (>100,000 per μL, Figure 4) and higher body temperatures (≥39°C, data not shown). These results are also consistent with the findings of Wassmer et al. [42] who suggested that TGF-β1 from activated platelets may stimulate endothelial cell apoptosis and death in cerebral malaria. Despite the evidence from these and other studies for apoptosis in *P. falciparum* infection, the actual role(s) of apoptosis in malaria remains unclear although a number of studies have reported that this pathway is upregulated in severe malaria [6, 43, 44].

5. Conclusions

The data reported here indicate that the immune response, proinflammatory, and apoptosis pathways are upregulated at the time of acute illness in uncomplicated *P. falciparum* malaria. The apoptosis pathway remains upregulated for several days (days 1–3) before returning to normal. In contrast, immune-modulatory and apoptosis inhibitory genes are downregulated initially (days 1–3) and return to normal by the time of clinical recovery 7–10 days later.

Abbreviations

Abs:	Absent
BAX:	BCL2 associated X-protein
CI:	Confidence interval
GCOS:	Gene chip operating system
GO:	Gene Ontology
GPI:	Glycosylphosphatidylinositol
HG-U133 Plus 2.0:	Gene chip for the study of human gene expression
ID:	Identification (as in Gene ID Number)
Marg:	Marginal
MM:	Mismatch
msp1:	Merozoite surface protein 1
PM:	Perfect match
Pres:	Present
RIN:	RNA integrity number
TNF:	Tumor necrosis factor
TP53:	Tumor protein 53 (*p53*).

Disclosure

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American Society of Tropical Medicine in Atlanta, GA, in November 2006 (Abstract 304).

Conflict of Interests

The authors of this paper state that they have no commercial or other associations which might pose a conflict of interests.

Authors' Contribution

James M. Colborn and Joni H. Ylöstalo contributed equally to this work.

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Review Article

Immune Evasion Strategies of *Trypanosoma cruzi*

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Microbes have evolved a diverse range of strategies to subvert the host immune system. The protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease, provides a good example of such adaptations. This parasite targets a broad spectrum of host tissues including both peripheral and central lymphoid tissues. Rapid colonization of the host gives rise to a systemic acute response which the parasite must overcome. The parasite in fact undermines both innate and adaptive immunity. It interferes with the antigen presenting function of dendritic cells via an action on host sialic acid-binding Ig-like lectin receptors. These receptors also induce suppression of $CD4^+$ T cells responses, and we presented evidence that the sialylation of parasite-derived mucins is required for the inhibitory effects on CD4 T cells. In this review we highlight the major mechanisms used by *Trypanosoma cruzi* to overcome host immunity and discuss the role of parasite colonization of the central thymic lymphoid tissue in chronic disease.

1. Introduction

Trypanosoma cruzi (*T. cruzi*) is a protozoan parasite that causes Chagas disease, which affects nearly 20 million people in the Americas [1, 2]. The disease progresses to a symptomatic chronic phase in which about 30% of patients develop cardiomyopathy or neuropathies and dilatations of the colon or esophagus at some time during their lifetimes [3]. Distinct hypotheses have been considered for the pathogenesis of Chagas disease, including autoimmune effects and parasite-driven tissue damage [4–6]. *T. cruzi* is a hemoflagellate parasite with a complex life cycle in which it enters vertebrates through the bite of a haematophagous triatomine (reduviid) insect. The life cycle has distinct stages involving epimastigotes and metacyclic trypomastigotes in the insect vector and blood-form trypomastigotes and intracellular amastigotes in vertebrate hosts [7]. In vertebrates, the parasite confronts a sophisticated immune system involving circulating cells and molecules as well as specialized tissues and organs [8–12].

To overcome host immunity, the trypanosome has an arsenal of evasion strategies linked to alternation between intracellular proliferative forms and nonproliferative, infective extracellular trypomastigotes. The different morphological life cycle forms are associated with adaptive changes in gene expression [13]. Genomic analysis has predicted the protein-coding sequences of *T. cruzi* and has annotated gene clusters/virulence factors implicated in evading host cell immunity. These factors are responsible for the wide range of host cells targeted by the parasite, mainly nonphagocytic cells [14]. Immune evasion by *T. cruzi* relies primarily on subverting the complement system and inhibitory effects on the mononuclear phagocyte system [15, 16].

Downregulation of phagocytic activity is also seen in other protozoan infections such as leishmaniasis and African trypanosomiasis, pointing to evolutionary convergence in the phylogeny of the protozoan parasites [17–19]. However, in contrast to other protozoan parasites that inhibit the maturation of phagolysosomes, *T. cruzi* evades macrophage microbicidal activity by escaping from the phagolysosome to the host cell cytoplasm where it replicates [20]. Moreover, it also interferes with the transcription of cytokines secreted by infected macrophages [21]. TLR activation by the parasite is weak

and a major parasite cysteine-protease prevents macrophage activation by blocking the NF-κB P65 pathway and shutting down the express ion of the proinflammatory cytokine, IL-12 [22]. In this scenario, the infection of macrophages favors the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β that impair the development of protective immune responses and favor the spread of infection [23, 24].

However, there are a variety of natural strains of *T. cruzi* and it appears that their immune modulatory effects are strain-dependent, a feature that may influence parasite-host interactions [25]. Phylogenetic reconstructions by comparative analysis of the RNA sequences of the various strains suggest that they diverged about 100 million years ago [26]. The different strains coexist dynamically in natural reservoirs. In fact, different combinations of *T. cruzi* strains have been found in the triatomine bugs from domestic and peridomestic areas [27]. Moreover, immune evasion may occur at the population level rather than at the level of a single strain. The $CD8^+$ T cell immunodominant epitopes encoded by the large trans-sialidase family of genes vary depending on the parasite strain [28]. As $CD8^+$ T cells are crucial for controlling the intracellular parasite, the T cell-mediated cytotoxic mechanisms that prevent parasite growth inside the host also vary.

2. Parasite-Associated Acute Phase Virulence Factors Can Overcome the Host Resistance Mechanisms and Establish Persistent Infections

The acute phase of Chagas disease is characterized by strong inhibition of the host immune response by the *T. cruzi* virulence factors, which are crucial for creating a persistent infection and establishing the chronic disease [5, 29, 30]. In both humans and experimental models, the acute phase is marked by a state of immunosuppression [5, 31–39] involving, among other things, the induction of anergy and clonal deletion in the T cell compartment, together with strong polyclonal B cell stimulation which ultimately restricts the development of antigen-specific lymphocytes [40, 41].

In fact *T. cruzi* provides a striking example of an immunosuppression strategy: thus, T cells from infected mice respond poorly to mitogens [33, 34, 37] and they also undergo enhanced apoptosis when the T cell receptor (TCR) is activated, hence increasing the unresponsiveness of host immunity [42–44]. *T. cruzi* membrane glycoproteins are critical for damping host protective immunity. The parasite surface is covered by mucin-like molecules with, attached to their terminal β-galactosyl residues, sialic acid residues which are transferred from host glycoconjugates by the parasite trans-sialidase [45–48]. These *T. cruzi* mucins are O-glycosylated Thr/Ser/Pro-rich proteins; they are the predominant glycoproteins on the parasite surface and are encoded by more than 800 genes comprising approximately 1% of the parasite genome [49–51].

The *T. cruzi* mucin-like molecules are key players in the host-parasite interplay, including invasion of the host and subversion of its immune system. Their sialylated forms are able to protect parasite antigenic determinants from host attack mediated by anti-galactosyl antibodies and complement factor B [52–55]. They also impair host dendritic cell function as demonstrated by inhibition of the production of IL-12 [56]. This inhibition may occur at the transcriptional level, since the *T. cruzi*-derived mucins are able to inhibit transcription of the IL-2 gene in T cells [33, 34], which also occurs when T cell activation and proliferation are blocked in response to mitogens and antigens [57]. The parasite sialoglycoproteins also inhibit early events in T cell activation, in particular tyrosine phosphorylation of the adapter protein SLP-76 and the tyrosine kinase ZAP-70 [37].

We have recently examined the inhibitory effects of the *T. cruzi* mucins *in vivo*. After exposure to these mucins during experimental infection in a murine model of Chagas disease, the mice displayed increased susceptibility to infection, with enhanced parasitemia and heart damage. These effects were associated with a reduction in IFN-γ-producing $CD4^+$ and $CD8^+$ T cell responses, together with decreased levels of both splenic IFN-γ and TNF-α [57]. With regard to the molecular mechanisms underlying these effects it has been shown that parasite-derived mucins bind to the mammalian acid-binding Tg-like lectin, Siglec-E (CD33) [58, 59], and our data suggest that binding of Siglec-E by *T. cruzi* mucins inhibits mitogenic responses in $CD4^+$ T cells. We showed that the impairment of TCR/CD3-mediated activation of $CD4^+$ T cells was correlated with arrest in the G1/S transition of the cell cycle and that interaction of the terminal sialyl residues of the *T. cruzi* mucins with $CD4^+$ T cells led to the induction of p27/Kip1, a cell cycle regulator that blocks the transition from G1 to S phase of the cell cycle [57].

The limited T cell responses contrast with the extensive polyclonal expansion of B cell lymphocytes in the acute phase of Chagas disease [41]. During infection an increased frequency of IgG2a- and IgG2b-secreting B cells can be observed in peripheral lymphoid organs. The majority of these cells are nonspecific and secrete antibodies with low affinity for *T. cruzi* antigens [60]; some cross-react with heart and neural tissue [61–63]. These autoantibodies are believed to play secondary roles in the pathogenesis of Chagas disease; they do not induce autoimmune effects because negative selection in the thymus during the process of central tolerance creates a peripheral lymphocyte repertoire with low affinity for cross-reacting autoantigens [64–66].

However, the polyclonal activation of the B cell compartment could restrict the size of the niche needed for optimal development of antigen-specific lymphocytes involved in protective responses to the infection by increasing competition for activation and survival signals in the lymphoid tissues [67, 68]. This phenomenon could have a role in the immunosuppression seen in both mice and humans in the acute phase of Chagas disease [5, 32–39]. Alteration of the homeostasis of the B cell compartment by the parasite has been attributed, at least in part, to parasite-derived glycoinositolphospholipids (GIPLs) [69], which are components of the dense glycolipid layer covering the parasite cell surface [70]. These GIPLs act as virulence factors that function as TLR4 agonists with proinflammatory effect [71]. There is also evidence that the proline racemase encoded by *T. cruzi*, which participates in arginine and proline metabolism, functions as a potent

mitogen for B cells and may therefore play a role in immune evasion by the parasite and its persistence in the vertebrate host [72, 73].

3. The Impact of Central Tolerance of Parasite-Specific T Cells Targeting the Thymus on Persistent Infection in Chronic Chagas Disease

Pathogens are able to interfere with vertebrate homeostasis at several levels. One important level involves the intersection between the three regulatory systems, neural, endocrine, and immune. These physiological networks can work together to recognize the danger of pathogen invasion. In most vertebrates threatened by a pathogen, acute short-term stress signals induce host responses that enhance innate defense mechanism [74]. A race between the pathogen-mediated evasion mechanisms and host immune responses then determines whether the invader will be rapidly eliminated or establish a persistent infection. In the latter case, the stress signals continue to suppress the host immune response, a scenario that favors the infection. It has been shown, for instance, that chronic stress causes a shift from T helper 1-mediated cellular immunity towards T helper 2-mediated humoral immunity, and this can influence the course of an infection and the susceptibility of the host to intracellular pathogens [75].

In infections caused by *T. cruzi*, TNF-α induces an inflammatory syndrome during the acute phase which activates the hypothalamus-pituitary-adrenal (HPA) axis leading to release of corticosterone. This stress hormone affects the disease outcome by its effect on the host immune system [76, 77]. Endogenous glucocorticoids also have an impact on the thymus, the central lymphoid organ controlling the continuous formation of T cells which are released to the periphery to form the host immunological repertoire [78]. The complex developmental processes in the thymus depend on direct contact between stromal cells and the thymocytes undergoing maturation, and disturbance of the thymic microenvironment can affect the T cell repertoire and thus the adaptive immune response [79].

Alterations of the thymic environment occur in infections involving many distinct pathogens (bacteria, viruses, parasites, and fungi) [80–88]. In most cases, disruption of thymic homeostasis can cause atrophy of the organ due to the apoptotic death of thymocytes [79]. This is the case in experimental models of *T. cruzi* infection, in which an imbalance between intrathymic and systemic stress-related endocrine circuits gives rise to high levels of intrathymic glucocorticoid hormones that mainly affect the viability of $CD4^+CD8^+$ thymocytes, but the populations of other subtypes such as double-negative (DN) T cells and SP cells are also reduced [76, 89]. This death mechanism is associated with the activation of thymocyte caspases 8 and 9, which promote apoptotic cell death [90].

Another contribution to thymic atrophy in Chagas disease is the premature export of immature thymocytes to the periphery. We have shown that the infection results in premature release of immature $CD4^-CD8^-$ double-negative thymocytes, as well as $CD4^+CD8^+$ double-positive thymocytes that have a proinflammatory activation profile [91–93]. We also found elevated levels of undifferentiated T lymphocytes in the peripheral blood of patients with severe cardiac forms of chronic Chagas disease and obtained evidence that the migration of very immature thymocytes from the infected thymus is due to sphingosine-1-phosphate receptor-1-dependent chemotaxis [94]; this points to an important role for sphingolipid signaling in the escape of undifferentiated thymocytes to the periphery in Chagas disease.

Although many pathogens induce thymic atrophy, until recently it was not clear whether negative selection eliminating T cells bearing TCRs against self-antigens was affected in the atrophic thymus. We answered this question by showing that the expression of peripheral antigens in the infected thymus is sufficient to promote negative selection in tolerance induction [93]. We provided evidence that immature thymocytes undergoing intrathymic maturation can be negatively selected during thymic atrophy. This corroborates the evidence that mature single-positive $CD4^+$ and $CD8^+$ T cells exiting the thymus do not harbor forbidden TCR genes [93, 95].

Although our data strengthen the notion that the infected thymus undergoing atrophy is still able to carry out negative selection, this matter should be thought of in the context of host-pathogen interactions. In Chagas infections, the parasites can colonize the thymus [96]. As a consequence, their antigens may be presented to recycling memory parasite-specific T cells moving from the periphery to the thymic microenvironment. The activation of these cells within the thymus could render them susceptible to the process of clonal deletion promoted by the thymic recognition of cognate antigens.

In addition, there is an alternative pathway in the thymus leading to the development of regulatory T cells recognizing specific antigens with high affinity TCRs [97]. Hence, the presence of *T. cruzi* antigens in the thymus could lead to the generation of parasite-specific regulatory T cells contributing to tolerance to parasites that target the thymus. If that process in fact occurs, it could induce central tolerance to the parasite antigens, thus undermining the establishment of protective immunity during the course of chronic disease. These issues are relevant to all host-pathogen interactions involving the thymus.

4. Concluding Remarks

The protozoa are the most ancient members of the animal kingdom and they have evolved to become one of the most dominant forms of life on earth. This evolutionary branch gave rise to the *Trypanosome cruzi*, a member of the kinetoplastid protozoa [98]. The survival of these parasitic unicellular organisms to the present day owes much to their efficient reproductive mechanism, with its short generation times and rapid developmental sequence producing large numbers of progeny [27]. These attributes lead to powerful infections, with an acute phase that strongly activates the host immune response. However, the acute phase involves complex molecular and cellular interactions between the

pathogen and its host that can be exploited to the parasite's benefit [5, 29, 30].

The systematic study of experimental *T. cruzi* infection models reveals that the parasite has ways to subverting immune defenses, and genomic studies have disclosed that it has evolved many genes devoted to this purpose [99]. However, important recent investigations focus on a new aspect of the parasite's evasion mechanism that may favor its chronic persistence in the host. These studies have shown that different parasite strains coexist in their natural reservoirs [27]. This creates a situation where the dynamics of antigen variation within a parasite population expressing distinct subdo minant T cell epitopes with low binding affinity to major histocompatibility complex (MHC) molecules could subvert host adaptive immune responses.

In addition, our studies have raised important questions about how the parasite undermines the host immune system at a more profound level and so increases its chance of persisting chronically. The finding that the parasite targets the thymus but does not alter the capacity of the organ to induce clonal deletion of antigen-specific T cells highlights the relevant issues to be approached [93]. It is reasonable to consider that the presence of pathogen antigens in the thymus may induce the recirculation of activated T cells from the periphery to the thymus in an attempt to prevent colonization of the organ. This scenario may induce clonal deletion of pathogen-specific T cells that recognize antigens via thymic dendritic cells involved in negative selection. Alternatively, it could lead to the generation of pathogen-specific regulatory T cells that induce tolerance to persistent infection. These possibilities are important for our understanding of the establishment of T cell protective immunity and the host's ability to control chronic persistent pathogen infections.

Conflict of Interests

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

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