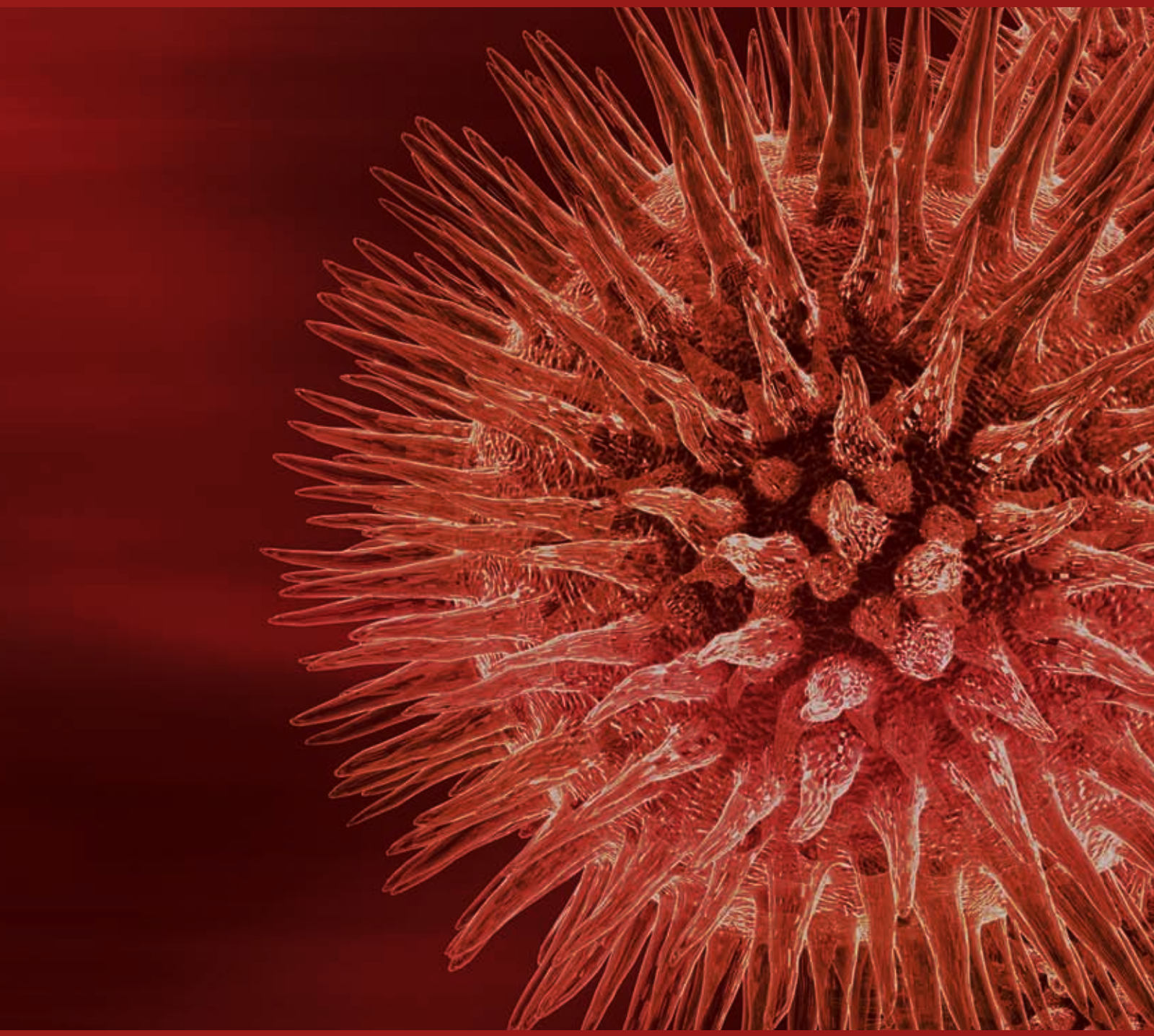
Journal of Biomedicine and Biotechnology

Immunology and Molecular Biology of Protozoan Infections

Guest Editor: Ali Ouaissi



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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 67210, 1 page
doi:10.1155/2007/67210

Editorial
Immunology and Molecular Biology of Protozoan Infections

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Received 6 December 2007; Accepted 6 December 2007

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The protozoan parasites, which cause important diseases affecting million of people worldwide especially in the tropical and subtropical areas, are responsible for high mortality and morbidity. Most of these parasites are transmitted by insect vectors and invade a range of different tissues in their mammalian hosts. Prophylactic and therapeutic strategies are far from satisfactory. Indeed, although significant progress has been made in our understanding of the immune response to parasites, no definitive step has yet been successfully done in terms of operational vaccines against parasitic diseases. Moreover, some drugs are available, but there are concerns over their effectiveness, toxicity, and emergence of resistant strains.

An acquired immune response is the expected outcome of invasion by any living foreign organism, yet in many hosts the parasites are able to survive for long periods and most of the host-parasite systems explored demonstrated their own peculiarities to reach this equilibrium.

In this issue of the Journal of Biomedicine and Biotechnology, functional lessons learned from a number of parasitic models: (Malaria, leishmaniasis, Chagas'disease, and toxoplasmosis) have been gathered. Analysis of plasmodium falciparum parasitized red blood cells cytoadherence to host cell receptors, a key step in the progression of parasitic infection, revealed that in the case of pregnancy-associated malaria (PAM), *P. falciparum* isolates from infected pregnant women and children have distinct adhesive and antigenic properties from that of parasites causing cerebral malaria. This strengthened the possibility that antigens expressed by parasites causing PAM could be potential targets for vaccine development. Furthermore, recent advances in DNA vaccines against protozoan parasites especially Leishmania and Trypanosoma cruzi have been reviewed in this special issue. The parasite and their released products elicit a complex series of cellular interactions leading to the activation/inhibition of the host immune system. This relies on ligand (s)-receptor (s) association among which CD40/CD40L costimulatory signaling, induction of immune cells such as dendritic cells, macrophages and T and B lymphocytes producing themselves various cytokines: IL-10, IL-12, IL-23, IL-27, IL-17, among others which create a microenvironment that may promote the development of defined Th cells. The balance between cytokine producing Th cells will determine the outcome of parasitic infection. This up-to-date information has been discussed in terms of physiological processes and therapeutic implications. The contributors convey both the state of the art and the direction of future studies in this particular area of research.

ACKNOWLEDGMENTS

As an Editor, I express my gratitude to the contributing authors as well as to the Editor-in-Chief, Dr. Abdelali Haoudi, for the opportunity provided by the Journal of Biomedicine and Biotechnology to present to the scientific community various facets of protozoan parasites-host interplay.

Ali Ouaissi

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 43785, 13 pages
doi:10.1155/2007/43785

Review Article

Molecular Aspects of *Plasmodium falciparum* Infection during Pregnancy

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Received 29 December 2006; Accepted 21 March 2007

Recommended by Ali Ouaissi

Cytoadherence of *Plasmodium-falciparum*-parasitized red blood cells (PRBCs) to host receptors is the key phenomenon in the pathological process of the malaria disease. Some of these interactions can originate poor outcomes responsible for 1 to 3 million annual deaths mostly occurring among children in sub-Saharan Africa. Pregnancy-associated malaria (PAM) represents an important exception of the disease occurring at adulthood in malaria endemic settings. Consequences of this are shared between the mother (maternal anemia) and the baby (low birth weight and infant mortality). Demonstrating that parasites causing PAM express specific variant surface antigens (VSA_{PAM}), including the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) variant VAR2CSA, that are targets for protective immunity has strengthened the possibility for the development of PAM-specific vaccine. In this paper, we review the molecular basis of malaria pathogenesis attributable to the erythrocyte stages of the parasites, and findings supporting potential anti-PAM vaccine components evidenced in PAM.

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1. THE IMPORTANCE OF CYTOADHERENCE IN THE PATHOPHYSIOLOGY OF *PLASMODIUM FALCIPARUM* MALARIA

P. falciparum infection encompasses a full range of clinical presentations, from asymptomatic infection to severe disease, including cerebral malaria, severe anemia, acute respiratory failure, hypoglycemia, renal failure, and pulmonary edema. Severe malaria, in particular cerebral malaria and severe anemia, constitutes one of the main causes of hospitalization in nonimmune individual from malaria endemic areas [1]. Patients with cerebral malaria present with a loss of consciousness and a coma, related to vascular obstruction by aggregated parasitized red blood cells (PRBCs), rosettes, and other fibrillous components. Parasite factors, such as GPI anchors elements (glycosylphosphatidylinositol), induce TNF-α and INF-γ, production that in turn will induce overexpression and relocalization of endothelial receptors, such as ICAM-1 and PECAM-1. In severe anemia, human and parasite factors play major roles. Anemia for instance is the consequence of PRBCs destruction, insufficient erythrocyte production, and increased clearance of both infected and noninfected RBCs by the spleen and the macrophages [2–5].

To survive, most microorganisms proceed to evolutionary adjustments in their virulence factors. In *Plasmodium spp*, these changes allow the parasite to sustain a chronicity inside its host by means of constant antigenic variations, allowing its transmission to the mosquito. Two virulence factors have been described in *P. falciparum*. Firstly, the growing rate, as parasites isolated from patients presenting with severe malaria express an in vitro multiplication rate higher than that of parasites isolated from nonsevere malaria patients [2].

This suggests that parasites causing severe disease multiply in their host faster than parasites associated with nonsevere disease. Factors controlling parasite multiplication rate still are not identified. The other *P. falciparum* virulence factor is the cytoadherence phenomenon (for review, see [6–8]). The nature of the *Pf*EMP1 protein expressed on the surface of PRBCs plays a key role in this process. Parasites unable to adhere to vascular endothelium are eliminated from the blood stream by the spleen filter. Indeed, erythrocytes do lose their deformability when parasitized, facilitating their clearance by the spleen. (for review, see [9]). RBCs surface expression of variant antigens constitutes an evasion strategy from the immune system, used by all *Plasmodium* species studied, and this may

probably represent a common feature within the *Plasmodium* genus. The expression of antigens unknown from the MHC would represent an excellent way to escape the immune system, but would also constitute a threat for species survival. The alternate expression of RBCs surface antigens thus is one of the intrahost mechanisms used by parasites for controlling their own population, while avoiding their host's death related to an excessive parasite multiplication [10, 11].

In *P. falciparum*, at least two variable surface antigens (VSAs), *Pf*EMP1 and RIFINs, are expressed on the surface of PRBCs [12]. Although members of the STEVORs family have been identified in Maurer's dots, a network of parasite microtubules inside the cytoplasm of PRBCs, these proteins may not be surface-exposed [13, 14]. All these three proteins are encoded by multigene families, and most of the genes composing each family are in a sub-telomeric location, an area subjected to a high level of recombination. Variations affecting VSAs suggest that they are necessary for the parasite survival. Despite the changes needed for immune evasion, the limited number of host receptors imposes the parasite to maintain a minimum stability between structure and function of its surface proteins by maintaining selected amino acids residues.

2. PARASITIZED RED BLOOD CELLS ADHERENCE

Adherence of PRBCs to endothelial receptors is a characteristic of *P. falciparum* infections [15]. While PRBCs containing young stages (ring) of the parasite do circulate in the blood flow without concern, those RBCs infected by mature stages (trophozoites and schizonts) of the parasite are sequestered in the microvasculature of deep organs [16], thus avoiding passage through the spleen. Parasite-encoded adhesines involved in the RBCs cytoadherence have been associated to protrusions (knobs) at the surface of erythrocytes (Figure 1). Even though adherence under physiological conditions may require knobs, it is now admitted that these knobs are not essential, as knobless parasite lines have been observed in vitro to bind endothelial cells [17, 18]. However Knobs are the location where most parasite ligands are expressed [19]. Following the demonstration of the major role the *Pf*EMP1 protein plays in the mechanisms of PBRCs binding to endothelial cells in 1984 [20], distinct adhesive properties of these parasite proteins to various receptors were also reported. The variations in the *Pf*EMP1 binding properties originate different types of interactions, such as deep organs tropism of PRBCs, agglutination with uninfected RBCs (rosetting) [21] or with other PRBCs (auto-agglutination) [22]. These various facets of PRBCs cytoadherence are in close relation with malaria pathophysiology. The withdrawal of mature forms from the blood flow, and their accumulation in deep organ microvessels may represent a pathologic event more or less well tolerated, according to the target organ and the level of PRBCs accumulation. Sequestration may be the key factor involved in vital organs failure, in particular during cerebral malaria.

3. PREGNANCY-ASSOCIATED MALARIA

In areas endemic for malaria, the pregnant woman is at high risk for malaria. Every year, twenty-five millions of pregnant women are exposed to malaria in sub-Saharan Africa, and pregnancy-associated malaria (PAM) is of serious public health concern [23]. In areas where malaria transmission is intense, its main consequences are a low birth weight (LBW) for the baby and a severe anemia for the mother. During pregnancy, massive sequestration of *P. falciparum* parasites in the placenta is likely to reduce maternofetal exchanges, explaining the frequency of LBW babies born from infected mothers. However, two recent studies have shown that pregnant women infected with *Plasmodium vivax* were also likely to give birth to LBW babies, suggesting that local or systemic production of selected inflammatory cytokines may also play a role in the pathological process [24, 25].

Recent data show that *P. falciparum* parasites infecting pregnant women express an antigenic profile different from that of parasites involved in cerebral malaria, and more generally, from parasites encountered in nonpregnant hosts [26]. This characteristic of PAM parasites is related to placenta-expressed receptors that participate in the selection of parasite phenotypes with a given specificity for these receptors. Chondroitin-sulfate A (CSA) is the major receptor for placenta sequestration [27, 28], and the number of parasite ligands involved in placenta sequestration is consequently highly restricted as compared to those implicated in cerebral malaria where several endothelial receptors may be involved. Although PAM parasites do preferentially bind to CSA, variable abilities were described among different placental isolates [29, 30]. Distinct subpopulations composed of strong and weak binders have been observed in $FCR3_{CSA}$ (a sub-line of FCR3 selected for its adhesion to CSA) using a model of adhesion under flow conditions [31]. Demonstration of different binding abilities among placental isolates showed particular interest as high binders were associated with high risk of LBW [30], and transcribed higher level of *var2csa* compared to low binders [32], emphasizing the role of *var2csa* in PAM.

4. CHONDROITIN-4-SULFATE (CSA) AND PLACENTAL RECEPTORS FOR SEQUESTRATION

Although glycosaminoglycans (GAG) have previously been shown to be involved in sporozoite adhesion to hepatocytes by binding to heparin-like motifs of the heparan sulfate (HS) [33], CSA is the first such receptor involved in RBCs sequestration. GAGs polysaccharides chains are usually composed of repeats of disaccharides units formed by one hexuronic acid and one hexosamine. At least one of the disaccharide elements has a carboxyl or a sulfate negatively charged. Among major GAGs are hyaluronic acid (HA), chondroitin sulfate, keratin sulfate, heparan sulfate, and heparin. Heparin is mostly a component of intracellular granules of mast cells lining the arteries of the lungs, liver, and skin while heparan sulfate is a component of the cell surface found in the basement membrane. HS contains heparin-like motifs that are enriched with *N*-sulfated glucosamine and 2-sulfated acids

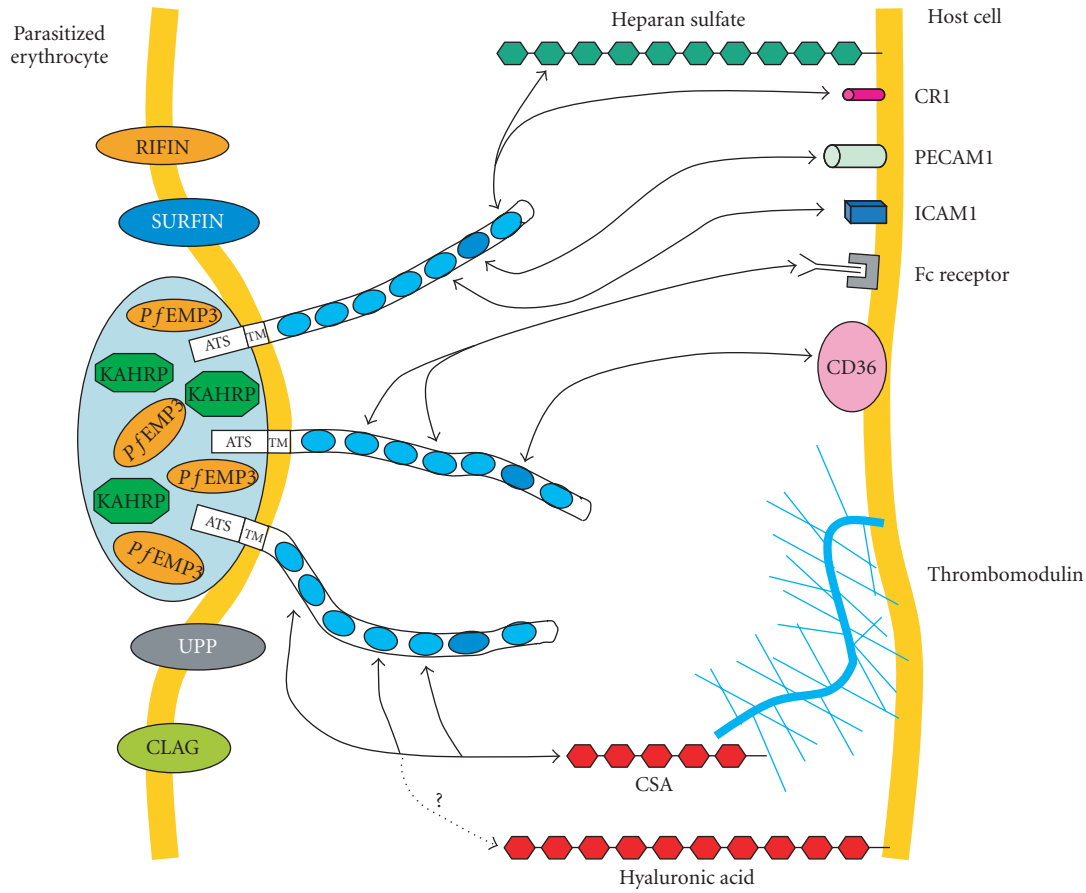

Figure 1: Schematic diagram of knobs showing potential intermolecular interactions between parasites proteins exported on the surface of PRBC and receptors on the host cell surface. *Pf*EMP, *Plasmodium falciparum* erythrocyte membrane protein; KHARP, knob-associated histidine-rich protein; RIFIN, repetitive-interspersed family proteins; CLAG, cytoadherence-linked asexual protein; CR1, complement receptor 1; ICAM1, intercellular adhesion molecule 1; PECAM1, platelet endothelial cell adhesion molecule 1; CSA, chondroitin sulphate A; UPP, uncharacterized parasite proteins. The question mark "?" means that the binding to hyaluronic acid is controversial.

[34]. Classical structure of CSA is tandem repeats of glucuronic acid [1–3] and *N*-acetylgalactosamine-4-sulfate [1–4]. Figure 2 illustrates structures of the different kinds of disaccharides composing GAGs of physiological significance. It is more and more obvious that GAGs structure is much more heterogeneous than previously thought [36, 37].

Chondroitin sulfates (CS) are mosaics formed by C4S (CSA) or C6S (CSC) types of disaccharides. The belonging to a CS type depends on the most abundant disaccharide. In chondroitin sulfate B (CSB), glucuronic acid is changed to iduronic acid, and in CSC, the *N*-galactosamine sulfate group is in position 6, while CSD and CSE are usually hyaluronate mix. CSB and CSC are not implicated in PBRC adherence [38].

Other GAGs of physiological significance include Type III TGF-β receptor, also called betaglycan, that contains both heparan and chondroitin sulphate chains [39]. CD44 family is composed of molecules that can exist in the proteoglycan and nonproteoglycan forms. CD44 is a cell surface receptor for hyaluronan [40] and is synthesized by lymphocytes, epithelial cells, fibroblasts, glial cells, Kupfer cells, and mesangial cells of the kidney. Like syndecans it has a short intracellular *C*-terminal and highly-conserved domain and a large extracellular domain [41]. Extracellular domain contains three disulfide-bonded loops, and it has a high homology with the hyaluronan binding region of aggrecan, link protein, neurocan, and versican [42].

The nervous tissue well-characterized proteoglycans include phosphacan, NG2 proteoglycan, agrin, receptor-type protein tyrosine phosphatase, and the aggregating proteoglycans neurocan and brevican. NG2 proteoglycan is a cell membrane-associated chondroitin sulphate proteoglycan present in nervous tissue cells that have not yet specialized into oligodendrocytes [43], but it has been found also in developing mesenchyme and human melanoma cells. The primary structure of NG2 proteoglycan consists of 2325 amino acids that code a 252 kd core protein [43].

In the vascular bed, thrombomodulin (TM) is a transmembrane glycoprotein containing high CSA levels [44]. CSA is involved in TM function, mainly by linking and inactivating the circulating form of thrombin, a coagulation factor [45]. TM is highly present in vascular endothelia

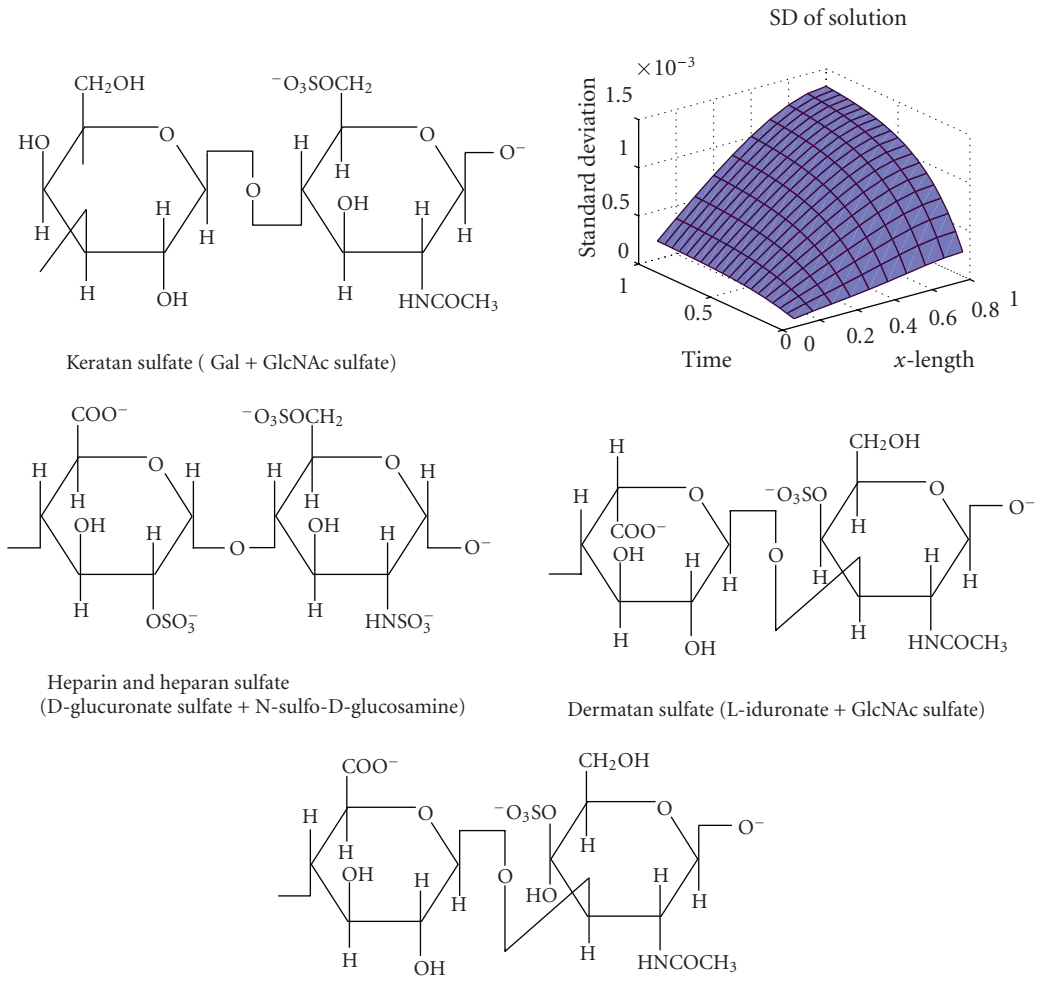

Figure 2: Structure of different kinds of GAG disaccharides of physiological significance.

(~100.000 molecules per cell), but also at the syncytiotrophoblast surface [46, 47], to which PRBCs bind in placenta [26]. The specificity of CSA binding of PRBCs is demonstrated by its full inhibition by either a minimum motif of 7 disaccharide units of the 4S type [48] or by chondroitinase ABC treatment. CSA is also present in pulmonary and cerebral vascular endothelium, suggesting a possible role in other severe forms of malaria [49].

Optimal binding of PRBCs in the placenta is observed in the presence of ~30% 4S disaccharides and ~70% nonsulfated disaccharides with a minimal motif of 6 disaccharide units [50]. This has been confirmed with a C4S/C6S bovine copolymer, although the minimal motif included 4 C4S units instead of 2 in the case of that from human origin [51]. Chondroitin sulfate proteoglycans (CSPG) isolated from human placenta are low sulfated, with around 8% of chains with a sulfate in position 4 (C4S) and the most part being nonsulfated [37]. This apparent discrepancy is explained by the fact that, in the CSPG structure, sulfated groups are concentrated in domains formed with 6 to 14 disaccharides [52]. These sulfate-rich domains include 20 to 28% of C4S, as opposed to the other regions.

Other studies demonstrate that HA is also involved in placenta sequestration of PRBCs, as most of *P. falciparum* placental isolates exhibit affinity for this GAG [53, 54]. However, not all agree with this finding [55]. With a similar structure as CSA, *N*-acetylgalactosamin in HA is not sulfated. Because HA is also present at the surface of syncytiotrophoblasts [56] and endothelial cells from microvessels [57], further studies are required to determine if HA represents a receptor in itself.

Despite the commonly admitted role of CSA in placental sequestration of *P. falciparum* infected RBCs, the overall process might be more complex involving multiple receptors such as (IgG, IgM, HA, CSA) rather than exclusive interaction with CSA [58]. A *P. falciparum* line selected according to the IgG-binding phenotype, was also shown to bind strongly to placental syncytiotrophoblasts, with a similar profile as wild isolates [59]. This binding is not inhibited by glycosaminoglycans or by chondroitinase ABC and

hyaluronidase treatment, but is inhibited by IgG-binding proteins, suggesting that *Pf*EMP1-containing domains that are able to bind CSA may also harbor IgG and IgM binding sites, offering another linking possibility between the PRBCs surface and Fc receptors expressed in the placenta.

5. *VAR* GENES FAMILY AND ANTIGENIC VARIATION

In *P. falciparum*, variant antigens (*Pf*EMP1) expressed at PRBCs surface are encoded by a family of genes called *var* composed of around 60 copies per haploid genome [60]. Members of this family are distributed among all chromosomes, most being localized in the subtelomeric regions and few in the central region of the chromosomes. Subtelomeric *var* genes are more vulnerable to the recombination phenomenon that affects their structure. Gene duplication phenomena are also frequent. These phenomena, known since the '30s [61], allow biological evolution and diversity. Selected genes are mutated after duplication or recombined with other members of the family, while transcription of the others is repressed following mutation. In the genome of the 3D7 *P. falciparum* strain, the high number of truncated *var* genes (pseudogenes) indicates the high frequency of gene deletion events occurring in the genome.

As for primary sequences, the number of domains may vary between *var* genes, as their size (from 3.9 to 13 kb). *Pf*EMP1 proteins encoded by these genes show differences that originate major antigenic variations at the erythrocyte surface. Each *Pf*EMP1 is constituted by an arrangement of distinct domains. The extracellular part is encoded by *var* gene exon 1, and possesses a variable *N*-terminal segment (NTS), several "Duffy-binding like" (DBL) domains (named following the Duffy-binding protein, the first such domain described, that allows *P. vivax* adherence to the Duffy antigen), and cysteine interdomain rich regions (CIDR) [62]. Each DBL domain is approximately 300 Aa long. Depending on their Aa sequence, DBLs as well as CIDRs have been classified into 5 types (α to ε). In selected *Pf*EMP1s, there is a small fragment after DBLβ originating a DBLβC2 structure. At the end of exon 1, there is a sequence of variable length (SVL) and a hydrophobic region with the characteristics of a transmembrane domain (TM) [63]. The entire *Pf*EMP1 molecule is anchored to the erythrocyte membrane by this TM domain, followed by an acidic *C*-terminal intracellular conserved segment (ATS), encoded by exon 2 (Figure 3).

Several studies have associated *Pf*EMP1s family to malaria pathogenesis, and the study of the function of the various *Pf*EMP1 molecules represents a research topic of high interest for the development of prevention strategies. The understanding of the mechanisms controlling *var* gene expression is of utmost importance for the control of their biological role. *Var* gene expression involves a set of regulation mechanisms implicating activation, switching, and silencing of localization sites. Studies of pre-erythrocytic maturation stages showed that *var* gene expression operates in a mutually exclusive fashion. Although several transcripts are detectable in a given parasite, a single one is massively transcribed as a full-length (untruncated) mRNA and expressed at the PRBCs surface while the others are kept inactivated (silencing) or give rise to truncated mRNA [64, 65]. Each *var* gene is a single transcriptional unit that can be activated in situ. The expression of the various members varies according to the development stages [66], but only the expression during the erythrocytic stages seems to play a major role in the parasite development in relation to the immune system escape. Although the expression profile may change in vitro without immune pressure, the expression of the same *var* gene during long periods of time has often been observed. The phenomenon appears to be highly different in vivo. Recent studies show a total change in the expression profile following passage of the parasite in the mosquito, suggesting a much higher in vivo switching rate [67, 68]. Selected genes with a physical colocalization show a tendency to be activated and expressed during the same development stage [66, 69].

Considering differences in gene structure, chromosomal organization, and sequences of untranslated regions, subgroupings of the *var* gene family have been proposed [60, 70, 71]. The analysis of gene upstream sequences allowed to define 3 major types of sequences (promoter-like): upsA, upsB, and upsC [60]. Two sequences belonging to the *var1* and *var2* subfamilies formed independent groups corresponding to upsD and upsE, respectively [71]. These sequences are associated to the localization and the orientation of each *var* gene. Subtelomeric genes orientated towards the telomere express promoter sequences of the upsA type, those orientated around the centromere express promoter sequences of the upsB type, and those located in the central part of the chromosome, upsC-type sequences. Differential transcription of *var* genes from different localizations inside chromosomes is likely to be a consequence of the differential expression of promoter-repressing elements. A silencing mechanism associated to the intron has also been suggested [72, 73]. Small size mRNA from some *var* gene introns, cooperating with 5′-UTR sequences, is able to inhibit expression of these genes. The expression of a conserved *var* gene lacking intron (var_{COMMON}) in 3D7 and 60 to 70% of wild isolates strengthens this hypothesis [74]. The high level of similarity in *var* genes intronic promoter motifs suggests this phenomenon is able to regulate *var* genes expression. Numerous queries are still remaining unanswered regarding the overall process regulating *var* genes expression and associated mechanisms. Changes in the chromatin structure have been associated with the switching phenomenon [75, 76]. A recent report by Chookajorn et al. [77] shows that an epigenetic memory that includes histone modifications reminiscent of those associated with gene transcription memory found in the homeotic genes of Drosophila melanogaster is involved in the control of *var* gene transcription. Specific epigenetic mark consisting in methylation of histone H3 and lysine K9 on chromatin seems to play a major role in transcriptional memory that can provide advantages to the parasites in pathogenesis and immune evasion.

In in vitro cultured *P. falciparum* strains, switch rate is higher in some lines (as ITG) than in others (as FCR3) [22]. In in vivo conditions, several factors may play a role in a given *var* gene type selection and expression. In children and

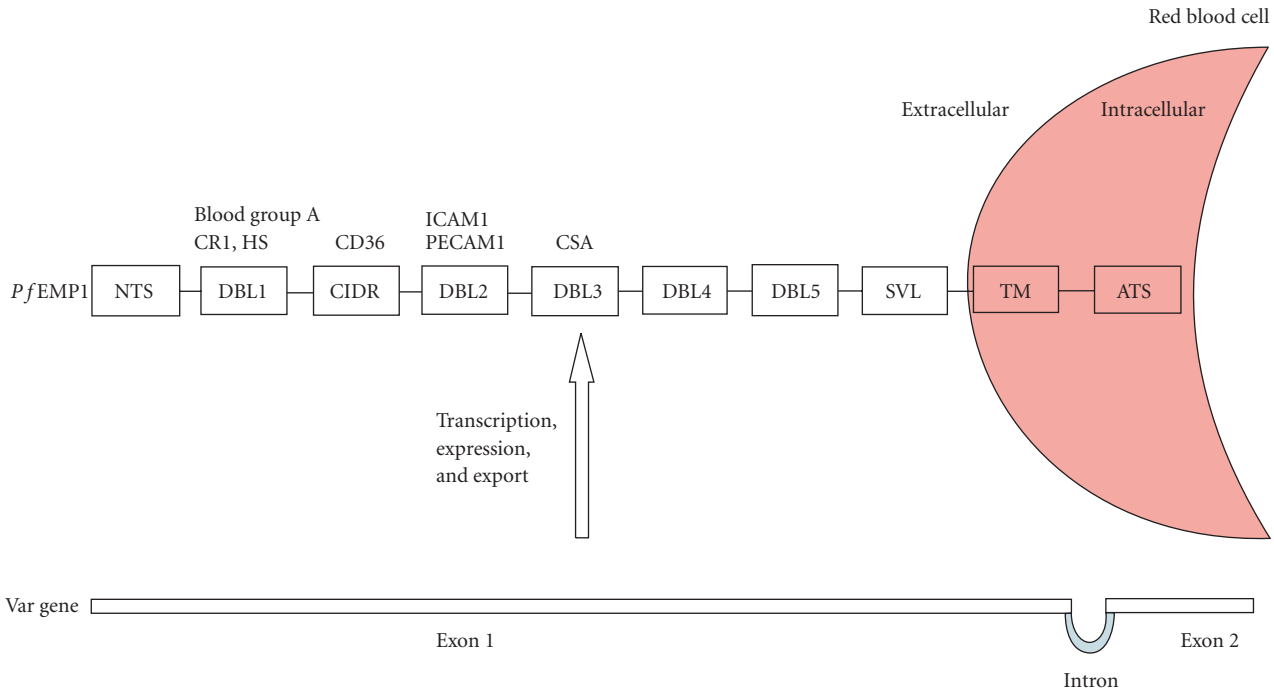

Figure 3: Schematic diagram illustrating *var* gene and *Pf*EMP1 organization. Domains with known binding properties are specified. NTS, *N*-terminal sequence; DBL, Duffy-binding like; CIDR, cystein-rich interdomain; SVL, sequence of variable length; TM, transmembrane; ATs, acidic terminal sequence.

malaria naive individuals, parasites tend to express selected VSA (mainly *Pf*EMP1) types at the surface of PRBCs (for review see [78]). In the pregnant woman, the placenta allows to select for parasite subpopulations expressing one (or several) *Pf*EMP1s able to bind receptors that are present on the syncytiotrophoblast surface. Mechanisms for selecting the *var* gene specifically expressed by parasites binding the placenta is currently unknown. Motifs of nuclear receptor sequences from hormones have been observed in the promoter regions of some *var* genes, but their putative function remains unknown [79], and the mechanism underlying *var* genes selection is unclear. Given its importance, there is an obvious need of investigation.

6. PARASITE LIGANDS INVOLVED IN PLACENTAL SEQUESTRATION

Studies of in vitro selected parasite lines evidenced three *Pf*EMP1 molecules that could be involved in placenta sequestration, through interaction with C4S receptors or with nonimmune Ig [59, 80, 81]. Although the CSA binding site has been localized inside the DBLγ domain of *Pf*EMP1 molecules encoded by the *FCR3varCSA* [81] and *varCS2* [80] genes, the role of these DBLγ domains in placenta binding is now questioned. Conversely to *CS2var* genes, *FCR3varCSA* genes are much conserved in various isolates and were named *var1csa* or var_{COMMON}. Transcription of these genes is not restricted to placental isolates [74, 82, 83], and analyses by Northern blot and real-time quantitative RT-PCR failed to demonstrate any overexpression of the *var1csa* or *varCS2* transcripts in parasite lines selected for CSA adhesion [84–86]. More recent works on laboratory-adapted parasite lines [85, 86] suggested that another *var* gene is involved in CSA adherence and placenta sequestration. This gene, termed *var2csa*, is localized on chromosome 12. Another gene with similar, but truncated, sequence is located on chromosome 13. The *Pf*EMP1 protein encoded by *var2csa* is constituted of 6 DBL domains, among which 3 remain unclassified. The sequence differs phylogenetically from that of other members of the family, and the DBLα and DBLγ are lacking [60]. *Var2csa* is structurally conserved between isolates and its overexpression by placental isolates is now confirmed [32]. However, the inability to find VAR2CSA *Pf*EMP1 in PRBCs membranes by proteomic approach was unexpected [87]. Moreover, a recent study based on a strategy of cross-linking PRBCs with a radioiodinated photoactivable C4S dodecasaccharide (representing the minimum requirement for efficient PRBCs binding) rather identified an $\sim$ 22 kd protein but no protein has been identified within the *Pf*EMP1 molecular weight range as a ligand for C4S [88]. This observation suggests that a low molecular weight PRBCs surface protein is involved in C4S binding. Even though these surprising findings do not exclude the role of VAR2CSA in the binding of PRBCs to C4S as possible technical insufficiencies in the experimental procedures would explain the inability to detect VAR2CSA, it likely appears that parasite binding to C4S not only might involve multiple binding sites within the VAR2CSA [89] but also might necessitate a multiprotein complex possibly comprising VAR2CSA *Pf*EMP1 and other

proteins for which identification remains an important goal to achieve.

7. IMMUNITY TO PAM

Initially thought that PAM was due to pregnancy-related immunomodulation and humoral alteration, studies have now established that PAM is caused by *P. falciparum* which express unique variant surface antigens (VSA_{PAM}) that allow the parasite sequestration in the placenta [90] by binding to CSPG receptors on syncytiotrophoblast [30, 91]. A number of studies have indicated that parasite-encoded VSA in the surface of PRBCs are important targets for acquired protective immunity that develops following exposure to *P. falciparum* parasites [92–95]. In the case of pregnancy malaria, women who have suffered from PAM develop VSA_{PAM}-specific anti-CSA adhesive antibodies which are associated with protection from malaria in subsequent pregnancies [96, 97]. The difference in susceptibility to PAM between primigravid women and multigravid women is attributed to the lack by primigravidae of antibodies against this particular VSA_{PAM}. These protective antibodies are thought to recognize reasonably conserved parasite antigens, because sera and parasites from pregnant women from different malaria areas cross-react [98]. This has raised hope for development of a vaccine to prevent PAM that should incorporate the PRBCs surface proteins expressed by placental parasites. Recent studies have shown that PAM parasites specifically transcribe high level of *var2csa*, one of the most conserved subfamily of *var* genes encoding a member of the *Pf*EMP1 family [32, 99]. Antibodies to this particular VSA_{PAM} was recently shown to specifically label the surface of in vitro adapted CSA-selected parasite [100]. Naturally acquired human monoclonal IgG1 antibodies were recently shown to react exclusively with intact CSA-adhering PRBCs expressing VSA_{PAM} [101]. Plasma samples from individuals from malaria endemic areas recognize VAR2CSA recombinant proteins in a sex- and parity-dependent manner [100, 102] and a kinetic study demonstrated that VAR2CSA-specific antibodies were acquired during pregnancy as an antiparasite response [102]. High plasma levels of anti-VAR2CSA antibodies early in pregnancy are associated with lower risk of LBW [100] and long lasting placental infections [102]. More recently it was shown that mouse antibodies raised against VAR2CSA DBL domains can inhibit adhesion of placental isolates to CSA as up to 60% [103]. These observations demonstrate that the antibody-mediated mechanism of protection against PAM can involve both adhesion-blocking antibodies as well as cytophilic process such as phagocytosis and complement activation. This is consistent with the finding by Megnekou et al. [104] that PAM IgG in Cameroonian women is predominantly composed of IgG1 and IgG3 subclasses.

It was previously suggested that *var* gene expression is hierarchically structured in field isolates, as the expression of certain *var* genes was found to be associated with severe malaria in young children [105]. An explanation would be that the progeny of parasites expressing *var* gene products that mediate the most effective sequestration outgrows the progeny of parasites expressing a molecule mediating less effective binding [105–107]. A similar process was observed in the expression of VAR2CSA molecules as some sequence motifs on DBL3X were more likely to occur distinctly in parasites isolated from primi- and multigravidae [108]. This sequence variation may have great consequence on the development of protective antibodies as PAM severe consequences are observed more among primigravidae.

The contribution of cell-mediated immunity in protection against PAM remains unclear. The maternofetal interface is a complex network where numerous cytokines are secreted. Immunomodulation during pregnancy was first considered to result from a Th1/Th2 bias to facilitate the fetal allograft development, and resulting in a decrease of Th1-type cytokines (TFN-α and IFN-γ) [109] and an increase of Th2-type cytokines (IL-4, IL-10, TGF-β) [110, 111]. Later, it was thought to result from monocyte activation and relative lymphocyte inhibition [112]. Placental cytokines modulate the antigen-presenting cell function by inhibiting or increasing the expression of various molecules on the monocyte surface. The development of *P. falciparum* in the placenta causes an immune imbalance with an increase of inflammatory cytokines, IFN-γ and TNF-α [113–115], explaining that immunomodulation is more important in the placental blood than in the peripheral blood [116]. This inflammatory response is responsible for functional damages in placental villi, and disturbances of the fetomaternal exchanges, leading to low birth weight [113, 117]. IFN-γ secretion by mononuclear cells of the intervillous blood is associated with protection against PAM [118], demonstrating the implication of the cell response. High level of anti-inflammatory cytokines is observed in multigravidae compared to primigravidae, suggesting that involvement of cell-mediated immunity in the mechanism of protection would necessitate a fine balance in timing and production of pro- and anti-inflammatory cytokines [119, 120].

8. TREATMENT AND PREVENTION: PERSPECTIVES FOR HUMAN APPLICATION

Initially the World Health Organization (WHO) recommended that pregnant women living in malaria-endemic areas receive chemoprophylaxis with a safe and effective antimalarial drug as part of routine antenatal care. Although this policy was widely adopted across sub-Saharan Africa, program implementation was often poor or nonexistent, especially in East Africa. Due to a number of difficulties encountered including the difficult deliverability of this strategy (poor adherence with weekly drug dosing) and rising rates of resistance to most chemoprophylaxis regimens, including chloroquine [121], WHO had to change its recommendations. In 2002, after studies conducted in Malawi and Kenya demonstrated that two treatment doses of sulfadoxine-pyrimethamine (SP) administered as intermittent preventive treatment (IPTp) during routine antenatal care decreased maternal anemia and diminished the frequency of low birthweight [122–124], WHO developed a strategic framework for the control of malaria during

pregnancy in Africa [23]. The document recommends that pregnant women receive at least two doses of IPTp during the second and third trimesters at routine antenatal care visits. The prevention strategies of malaria during pregnancy include IPTp, insecticide-treated nets, and effective case management of clinical malaria. The new policy leading to the adoption of IPTp is unique to pregnancy and is still under evaluation. In contrast, insecticide-treated nets and case management are strategies that are in use for all age and gender strata.

A recent study using depolymerized heparin demonstrated that these modified glycosaminoglycans (dGAGs) are able to disrupt binding properties of *P. falciparum* that form rosettes and employ heparan sulfate as a host receptor. Intravenous injection of these dGAGs could block up to 80% of PRBCs from binding in the microvasculature and release already sequestered parasites into the circulation in an in vivo model of severe malaria [125].

GAGs are structures contributing to host cell recognition and invasion by various infectious agents, including viruses (herpes simplex, viral hepatitis, HIV) and parasites (*Babesia*, *Leishmania*, *Plasmodium*) (for review see [126]). Three types of GAGs interact with *P. falciparum* endo-erythrocytic cycle: heparane sulfates [127] involved in rosette formation, CSA in PRBCs adherence, and heparine in RBCs invasion inhibition. As regards PRBCs adherence, it was shown that soluble CSA is involved both in vivo and in vitro. Intravenous injections of soluble CSA to monkeys infected with a CSA-binding *Plasmodium* strain are followed by the release of mature stages of the parasite in the peripheral blood [128]. Moreover, PBRCs in vitro binding is inhibited by almost 90% by purified CSA. These observations originated works related to the inhibitory capacity of CS. Hitherto, various polysaccharides have been tested for their ability to inhibit human erythrocyte invasion by *P. falciparum* merozoites, and PBRCs binding to various receptors [129–132]. Numerous sulfated polysaccharides, such as heparines, sulfate dextrans, fucoidans, and hyaluronates all exhibit inhibitory properties, but at different levels. Two carraghenate derivatives and cellulose sulfate (CS10) inhibit PRBCs binding to CSA [133]. Chondroitin-4-sulfate, a molecule already marketed (Chondrosulf, Structum), was unable to inhibit PRBCs binding to *Saimiri* endothelium [128], but this was probably related to the oral administration of the drug. Intravenous administration of Structum was effective in *Saimiri*, but the drug of bovine origin is not anymore available. Industrial synthesis of glycosaminoglycans cannot be currently achieved. It is necessary to conduct additional structural and toxicologic studies of CS, for this to be considered as a potential candidate for treating PAM.

ACKNOWLEDGMENTS

This work was supported by grants from the French Agence Nationale de la Recherche (MIME 2006), and the Institut de Médecine et d'Epidémiologie Appliquée. N. T. Ndam was the recipient of a postdoctoral fellowship from the Fondation pour la Recherche Médicale.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 90520, 11 pages
doi:10.1155/2007/90520

Review Article

DNA Vaccines against Protozoan Parasites: Advances and Challenges

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Received 8 December 2006; Accepted 21 March 2007

Recommended by Ali Ouaissi

Over the past 15 years, DNA vaccines have gone from a scientific curiosity to one of the most dynamic research field and may offer new alternatives for the control of parasitic diseases such as leishmaniasis and Chagas disease. We review here some of the advances and challenges for the development of DNA vaccines against these diseases. Many studies have validated the concept of using DNA vaccines for both protection and therapy against these protozoan parasites in a variety of mouse models. The challenge now is to translate what has been achieved in these models into veterinary or human vaccines of comparable efficacy. Also, genome-mining and new antigen discovery strategies may provide new tools for a more rational search of novel vaccine candidates.

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1. INTRODUCTION

In spite of the success of vaccines in public health, there are still numerous pathogens, and in particular protozoan parasites such as *Plasmodium falciparum*, *Trypanosoma* sp., or *Leishmania* sp. against which there are still no effective vaccine. However, the discovery that the direct injection of plasmid DNA encoding foreign proteins could lead to endogenous protein biosynthesis and a specific immune response against it opened new perspectives in vaccine development. Over 15 years later, DNA vaccines have gone from a scientific curiosity to one of the most dynamic fields of research and may offer new alternatives for the control of infectious diseases [1]. Indeed, the first two DNA vaccines have been licenced, in recent years, to protect horses from west nile virus and salmons from infectious hematopoietic necrosis virus, confirming the usefulness of this biotechnology. We review here some of the advances and challenges for the development of DNA vaccines against two well-studied protozoan parasites, *Leishmania* sp. and *Trypanosoma cruzi*. Both belong to the trypanosomatidae family and are ranked among the three major protozoan parasites affecting humans. Leishmaniasis is a complex disease caused by at least 18 species of parasites from the *Leishmania* genus and transmitted to humans by hematophagous sandflies. With an estimated 12 million cases, it has a major public health impact in several regions, and in particular in India, Sudan, and Brazil [2]. Clinical manifestations range from self-healing cutaneous lesion to fatal visceral form, and this variety can be attributed in part to the respective parasite species, and each presents specific relationships with the host and diverse mechanisms of pathogenesis [3, 4], which represents an additional difficulty for the development of treatments and vaccines. On the other hand, *T. cruzi* is the agent of Chagas disease, which is present from southern Argentina to the southern USA. An estimated 16–18 million persons are infected in the Americas and close to 100 million people are at risk of infection. After a short benign acute phase (a few weeks) and a very long (several years) asymtomatic phase, about 30–40% of infected patients develop chronic chagasic cardiomyopathy and eventualy die of heart failure. Current chemotherapy relies on nitrofurans (Nifurtimox), or nitroimidazoles (Benznidazole). However, the usefulness of these drugs is limited by their reduced efficacy (mostly during the early stages of the infection), serious side effects, and the emergence of drug-resistant strains of parasites, and new treatments are slow to develop [5].

2. WHY DNA VACCINES?

DNA vaccines induce a complete immune response against the encoded antigen. The exact mechanisms involved in this proccess are still poorly understood, and particularly the type of $CD4^+$ and $CD8^+$ effector and memory cells activated, and

some of these aspects have been reviewed in detail elsewhere [1]. Apart from their immunogenicity and efficacy that will be discussed below, there are several features of DNA vaccines that make them very advantageous against tropical diseases. First, they are extremely safe as they do not contain any pathogenic organism that may revert in virulence. The major concern of genomic integration of the plasmid DNA has also extensively been studied in safety studies and found to be rather unlikely [6]. Additional safety issues such as anti-DNA antibodies or autoimmunity have also been addressed in a growing number of preclinical and clinical studies [7], which confirmed the high safety of these vaccines. With respect to manufacturing, storage, and distribution, they also present major benefits in that the production process is the same for any DNA vaccine, which is not the case for other types of biologicals and vaccines, for which a specific protocol has to be developed for each. This makes production easy and costs will likely go down as this type of vaccines become mainstream and future technological improvements are implemented. Also, plasmid DNA is a very stable molecule, specially compared to recombinant or live attenuated vaccines, which would greatly facilitate storage and distribution of DNA vaccine in tropical settings with limited health infrastructure as the huge costs associated with the cold chain may be offset. Administration is also easy as simple IM or ID injections can be sufficient, and multiple plasmids can be combined for the elaboration of multivalent vaccines [1]. Overall, DNA vaccines may thus represent an ideally affordable alternative for disease control, which explains in part the growing interest in their development for the control of tropical parasitic diseases such as malaria, leishmaniasis, or Chagas disease.

3. DNA VACCINES AGAINST *LEISHMANIA*

3.1. Correlates for protection

As mentioned above, leishmaniasis is caused by at least 18 species of parasites with diverse relationships with the host and mechanisms of pathogenesis [3, 4]. Early studies of cross-protection between *Leishmania* species clearly showed that it is a complex problem, with infection by one species protecting or not from subsequent infection by another species, depending on the species and the order of infections. Most vaccine studies have thus been focusing on homologous protection, although a single vaccine able to protect against all pathogenic species would be ideal.

The correlates for protection have been extensively studied in the case of *L. major*, and contributed considerably to the development of the Th1/Th2 paradigm [8]. Thus, there is a general agreement that a Th1-type immune response, characterized by a high IFNγ and low IL-4 and IL-10 production, leads to control of *L. major* infection, while a Th2-type immune response does not [8]. Antibodies may have an exacerbatory role [9], but may also contribute to T cell responses [10, 11]. Both IFNγ producing $CD4^+$and $CD8^+$T cells seem to contribute to protective immunity, and induction of NO production by macrophages is central to parasite elimiation [12, 13]. While it was assumed for a long time that this Th1/Th2 paradigm was applied to all *Leishmania* species, it has become clear in recent years that each species has a distinct relationship with the host, different mechanisms of pathogenesis, and possibly different correlates for protection [3, 4]. Nonetheless, IFNγ production seems to be a general requirement, although not necessarily sufficient, for protection against most if not all *Leishmania* species.

3.2. Single antigen DNA vaccines

The earliest DNA vaccine experiments against *Leishmania* used *L. major* GP63 antigen, which has been extensively used as a recombinant or peptide vaccine. Immunization with a plasmid encoding GP63 was able to induce a Th1-type cytokine profile and a significant reduction of lesion size after challenge of the immunized mice with *L. major* [14–17]. Subsequent studies investigated DNA vaccines encoding a large variety of *Leishmania* proteins (Table 1) and showed that many different DNA vaccines were able to induce a Th1 immune response, and confer variable degrees of protection as assessed by reduction in skin lesion size and/or parasite burdens in mouse models. However, given the large variety in experimental models and designs, it is difficult to compare the effectiveness of the different vaccines to induce a protective immune response. Nonetheless, it is clear from studies comparing different DNA vaccines that the nature of the antigen encoded by the vaccine is a key parameter for efficacy.

Also, a few studies provided interesting comparisons of the same antigens administered as recombinant protein or DNA vaccines and showed that the latter were overall more effective than their recombinant protein counterparts. Indeed, DNA vaccines were able to induce a stronger Th1 bias in the immune response, a longer-lasting immunity, and/or a better protection against disease progression [19, 31, 32, 35, 40, 44]. While most of these studies have used a rather artificial infectious challenge based on the injection via nonnatural routes of high parasite doses, an experimental system criticized by some authors, the superior efficacy of DNA vaccines was also observed using a low-dose intradermal challenge in the ear, which was proposed to more closely mimick natural infection [45]. In these studies, both DNA and protein vaccination were able to induce very similar level of short-term (2 weeks postvaccination) protection against infection with *L. major*, but only DNA vaccine was able to induce long-term (12 weeks postvaccination) protection [45].

These results thus confirmed the strong potential of DNA vaccines against *Leishmania*, but also indicated that in most cases only partial protection was achieved. Prime-boost immunization protocols have been tested with various antigens to increase vaccine potency (Table 1). They are based on priming the immune response with a DNA vaccine and boosting with the corresponding recombinant vaccine based on recombinant virus or protein (Table 1). In some studies, such immunization protocol resulted in increased immunogenicity of the vaccines and better protection levels [26, 27], but in others, DNA only remained the best formulation for optimum efficacy [32]. Nonetheless, a major drawback of

Table 1: DNA vaccines tested against murine leishmaniasis.

Antigen	Dose	Challenge	Immune response	Protection	Reference
GP63	$2 \times 100\,\mu$g IM	*L. major*	Th1	+++	[14–17]
LACK	$2 \times 100\,\mu$g IM	*L. major*	Th1, IFNγ	+++	[18–21]
LACK	$2 \times 30\,\mu$g IN	*L. amazonensis*		+++	[22]
LACK	$2 \times 100\,\mu$g IM	*L. mexicana*		–	[23]
LACK	$2 \times 100\,\mu$g IM or SC	*L. chagasi*	Th1, IFNγ	–	[24]
LACK	$2 \times 100\,\mu$g ID or SC	*L. chagasi*	Th1	–	[25]
LACK	Prime/boost	*L. major*	Th1	+++	[26]
LACK	Prime/boost	*L. major*	Th1	+++	[27]
LACK	Prime/boost	*L. major*	Th1, IFNγ	+++	[28]
LACK	Prime/boost	*L. infantum*	IFNγ	+++	[29]
TRYP	Prime/boost	*L. major*	IFNγ	+++	[30]
LiP0	$2 \times 100\,\mu$g im	*L. major*	Th1	++	[31]
SP1	$2 \times 100\,\mu$g SC or prime/boost	*L. major*	IFNγ	++	[32]
Histones mix: H2A, H2B, H3, H4	$3 \times 200\,\mu$g IM	*L. major*	Th1, IFNγ	+++	[22]
LmSTI1,TSA	$3 \times 100\,\mu$g IM	*L. major*	Th1	+++	[33]
LACK, LmSTI1, TSA	$1 \times 300\,\mu$g SC	*L. major*	Th1	+++	[13]
CPb/CPa	$2 \times 100\,\mu$g IM	*L. major*	Th1	+++	[34]
PSA-2	2×20–$50\,\mu$g IM	*L. major*	Th1	+++	[35]
LACK, PSA2, Gp63, LeIF, p20 Ribosomal like protein	$1 \times 50\,\mu$g IM	*L. major*		– to +++	[36]
Meta 1	$3 \times 100\,\mu$g IM	*L. major*	Th2	–	[37]
P4	$3 \times 100\,\mu$g various sites	*L. amazonensis*	Th1	+++	[38]
CPb, GP63, GP46	$2 \times 100\,\mu$g IM	*L. mexicana*		+ to +++	[23, 39]
NH36	$2 \times 100\,\mu$g IM	*L. domovani*		+++	[40]
		L. mexicana		++	
NH36, GP63	$2 \times 20\,\mu$g IM	*L. mexicana*	IFNγ	+++	[41]
CPa/CPb	Prime/Boost	*L. infantum*	Th1, IFNγ	+++	[42]
ORFF	$3 \times 100\,\mu$g IM	*L. donovani*		+++	[43]

IM: intramuscular; IN: intranasal; SC: subcutaneous; –: no protection; +: little protection; ++: fair protection; +++: very good protection.

such vaccine formulation remains its complexity, which may limit their practical use.

3.3. Multiple antigen DNA vaccines

An alternative way to broaden vaccine immunogenicity and increase its efficacy has been to use combination of plasmids encoding various antigens. For examples, cysteine proteinase (CP) a and b DNA vaccines are not protective when used individually, but immunization with a combination of both plasmids induces long-term protective immunity [34]. Alternatively, gene fusion has also been successively used to achieve expression of an antigenic fusion protein from a single plasmid construct [33]. Overall, expression of several antigens mostly resulted in increased efficacy, but this also depended on the antigen combination [13, 22, 23, 41, 45]. Most authors thus argue that a successful *Leishmania* vaccine is likely to be based on multiple antigens.

3.4. Antigen discovery

Immunization with large number of plasmids is also the basis for expression library immunization, a powerful but labor-intensive strategy for vaccine discovery [46], which has been used with *Leishmania*. Immunization of mice with *L. major* genomic expression library fractions was able to induce significant protection, but these authors did not pursue library fractionation further [47]. In another study, the identification of protective library subsets from an *L. donovani* amastigote cDNA library and their successive fractionation into smaller protective libraries lead to the identification of novel protective antigens [48]. Interestingly, most of the antigens identified would not have been predicted to be good

vaccine candidates. Indeed, they were not surface or secreted proteins, neither stage-specific, but were intracellular and some very conserved such as histones, or ribosomal proteins [48]. Vaccine discovery is also the next logical step following the recent completion of the *L. major* genome sequencing [49]. In one approach, the random screening of 100 genes upregulated in amastigotes tested as DNA vaccine allowed the identification of 14 novel protective and 7 exacerbating antigens [50, 51]. Again, function and cellular localization would have been poor predictors of the protective efficacy of these antigens, as most were not predicted to be localized on the surface, but shared similarity with ribosomal proteins, cytoskeleton, or metabolic enzymes [51]. It is thus becoming increasingly clear that there is little rationale to limit *Leishmania* vaccine discovery searches to surface or secreted antigens. Rather, new criteria need to be considered for the rational identification of vaccine candidates as strategies based on such random screening cannot be applied to large genomes such as that of *Leishmania*, with over 8000 annotated genes.

3.5. Therapeutic vaccines

An additional advantage of DNA vaccines is their potential as therapeutic vaccines, aimed at reinforcing or redirecting the immune response of an infected host to control disease progression [58]. The major advantage of this strategy in addition to its efficacy is that it relies on short treatment regimens, and it is thus an attractive alternative to chemotherapy, particularly in the case of *Leishmania* with so few chemotherapeutic options. Thus, administration of as little as two doses of a DNA vaccine encoding PSA-2 can control an ongoing infection with *L. major* in mice [59]. The therapeutic effect is due to a shift of the immune response towards a Th1 immune response [59]. Similarly, a DNA vaccine encoding *L. donovani* nucleoside hydrolase NH36 has therapeutic activity against murine visceral leishmaniasis caused by *L. chagasi* [60]. The simplicity of such treatment makes them very advantageous compared to chemotherapy. In addition, the fact that the same DNA vaccine can be effective for both the prophylaxis [40] and the therapy of *Leishmania* infection is thus very promising as this would provide a versatile tool for the control of this parasite.

3.6. Cross-protection against multiple Leishmania species

As mentioned above, an added challenge to *Leishmania* vaccine development is the large number of species, as well as the variability within species. Indeed, studies on the polymorphism of leading antigens such as GP63 quickly revealed that it was a very polymorphic [61, 62]. Such polymorphism has important implication for vaccine development as it may limit their efficacy against variant strains of parasites or novel escape mutants, and thus restrict vaccine protection to a single species [63, 64]. Antigen polymorphism between multiple strains and species is thus becoming a major issue in many vaccine development studies [65, 66]. In the case of *Leishmania*, few DNA vaccines have been tested against multiple species. LACK antigen, initially identified in *L. major*, and found to be very conserved between *Leishmania* species, can protect mice against *L. major* [20] and *L. amazonensis* [67], but not against *L. mexicana* [23], *L. donovani* [25], or *L. chagasi* [24]. On the other hand, *L. amazonensis* nuclease protein P4 can protect against both *L. amazonensis* and *L. major*, but cross-protection requires a different formulation (IL-12 or HSP70 as adjuvant, resp.) [38]. In other studies, antigens from one species were used to induce protection against another species [31], but the extent of cross-protection against various species was not investigated. More recently, a single formulation of *L. donovani* NH36 DNA vaccine was found to induce a very good protection against both *L. chagasi* and *L. mexicana*, suggesting that this DNA vaccine may be able to provide broad protection against various *Leishmania* species [40]. Importantly, no DNA vaccine has yet been tested against *L. braziliensis*, in spite of this species being responsible of most cases of cutaneous leishmaniasis in South America.

3.7. Non-Leishmania antigens as vaccines

While all the above DNA vaccines were based on *Leishmania* antigens, an alternative approach has used antigens derived from sand-fly saliva. Indeed, it has been shown that sand-fly saliva can exacerbate *Leishmania* infection [68, 69], and preexposure of mice to saliva components may be sufficient to induce protection against infection [70]. Thus, a number of salivary antigens have been tested as vaccines against *Leishmania*. Maxadilan is a potent vasodilator from sand-fly saliva and was found to be responsible of most of the exacerbatory effects of whole saliva on *Leishmania* infection [71]. Immunization with this antigen (as a recombinant vaccine) protected mice against *L. major* infection [71]. Other salivary components, such as *Phlebotomus papatasi* SP15, have been tested as DNA vaccines and found to protect mice against *L. major* and while the vaccine induced both humoral and DTH responses, protection seemed to be mostly accounted for by the latter, as B-cell deficient mice remain protected [72]. Thus, characterization of sand-fly salivary proteins may lead to the identification of new vaccine candidates [73, 74]. However, as for *Leishmania* antigens, salivary protein polymorphism remains an important issue and may limit the usefulness of such antigens as vaccine candidates [75, 76].

3.8. DNA vaccines against nonmurine leishmaniasis

Based on the success of many of these DNA vaccine studies in mice, a few vaccine candidates have been tested in additional animal models, possibly more relevants for the development of a veterinary or human vaccine (Table 2). PFR-2 and KMP11 antigens were tested as DNA vaccines in hamsters, a highly susceptible animal model. PFR-2 was tested as protein, DNA, or DNA-protein immunization, and protection levels against *L. mexicana* varied greatly depending on vaccine formulation, route of immunization, and sex of the animals [52]. Also, contrary to mouse studies, protein vaccination seemed more protective than DNA only vaccination.

Table 2: Preclinical studies *Leishmania* DNA vaccines in nonmurine models.

Antigen	Dose	Challenge	Host	Immune response	Protection	Reference
PFR-2	Variable	*L. mexicana* *L. panamensis*	Hamster		− to +++	[52]
KMP11	$2 \times 100\,\mu$g IM	*L. donovani*	Hamster	Th1-Th2	+++	[53]
PapLe22	$1 \times 100\,\mu$g IM	*L. infantum*	Hamster		+++	[54]
LACK	$2 \times 100\,\mu$g IM or Prime/boost	*L. infantum*	Dog	Th1/Th2 Th1	+ to +++	[55]
10 antigens mix	$2 \times 500\,\mu$g IM	*L. donovani*	Dog	Th1		[56]
CPa/b	Prime/boost	*L. infantum*	Dog	IFNγ	+++	[57]

−: no protection; +: little protection; ++: fair protection; +++: very good protection.

However, as in mouse studies, heterologous prime-boost vaccination with DNA and protein seemed better than DNA only [52]. Another DNA vaccine encoding PapLe22 was found to be immunogenic in hamsters and decreased parasitemia after infection with *L. infantum*, but further assessment of disease was not performed [54]. Immunization with KPM11 DNA induced a mixed Th1/Th2 response, but was able to protect hamsters against visceral leishmaniasis caused by *L. donovani* [53]. In dogs, while several protein vaccines have been tested and a purified protein vaccine has now been licenced for veterinary use [77], very few DNA vaccine studies have been performed. A heterologous prime-boost strategy using CPa and CPb DNA and protein was reported as immunogenic and protective [57], but the study was of limited power given the reduced number of animals. In another study, dogs were immunized with a mixture of DNA vaccines encoding 10 different antigens previously tested in mouse models, and this immunization induced a very good immune response, with a high production of IFNγ [56]. However, evaluation of protection was limited to an acute *in vitro* assay [56] and further studies will be required to assess the potential of this vaccine in dogs. In spite of their limitations, these studies clearly showed that several DNA vaccines can induce a potent immune response in nonmurine animal models, and it is likely that a good level of protection can be achieved in these as well, provided the correct antigens and vaccine formulation are used.

4. DNA VACCINES AGAINST *TRYPANOSOMA CRUZI*

4.1. Correlates for protection

Vaccine development against Chagas disease has been dramatically limited because of extensive debate on the mechanisms involved in this pathology [78, 79]. Indeed, some studies suggested that tissue damage was associated with the presence and replication of intracellular amastigotes, while others proposed that autoimmunity induced by parasite antigens mimicking host proteins was responsible for it. It was thus unclear if the immune response needed to be inhibited, to reduce autoimmunity, or stimulated, to eliminate the parasite. It is now accepted that the presence of parasites in cardiac tissue is necessary to initiate and maintain the inflammatory response, and that therapeutic treatments or vaccines aimed at eliminating *T. cruzi* would limit or prevent the progression towards chronic chagasic cardiomyopathy [80, 81]. There is a growing consensus that protection against *T. cruzi* relies on a Th1 immune response and the activation of cytotoxic $CD8^+$T cells [82–85].

4.2. Single antigen DNA vaccines

The first DNA vaccines to be tested against *T. cruzi* encoded an antigen from the well characterized trans-sialidase family of proteins. There are over 1400 members in this family, making it one of the largest protein families of the parasite, and they are very abundant surface proteins. Several studies have used different members of this family, such as TS or TSA-1 (Table 3) [84, 86–88]. Immunization with TS was found to induce significant antibody titers able to inhibit trans-sialidase enzyme activity, a strong DTH, and lymphoproliferative response [86]. This immune response was protective as determined by an increase in survival and a decrease in parasitemia. Immunization with TSA-1 DNA was found to induce a specific CTL response which also lead to a lower parasitemia and increased survival in both BALB/c and C57BL/6 mice [88].

As in *Leishmania* vaccine studies, a few authors addressed the question of comparing protein and DNA vaccines encoding the same antigen [90, 98]. In A/Sn mice, immunization with recombinant TS induced a higher antibody titer than TS DNA, but a comparable decrease in parasitemia. However, the DNA vaccine was unable to increase survival, which the author attributed to the strain of the mice used, since this DNA vaccine was protective in BALB/c mice [90]. On the other hand, immunization with recombinant CRP or CRP DNA induced a comparable Th1 immune response, but only the DNA vaccine was protective against infection [98].

A number of other studies showed that DNA vaccines encoding various antigens could induce significant protection against *T. cruzi* infection, as evidenced by decreased parasitemia and improved survival of vaccinated mice (Table 3). In addition, a few studies also presented evidence of a reduction in cardiac tissue damage and inflammation at the histopathologic level [87, 97]. Furthermore, T cell analysis confirmed that protection relied on $CD8^+$T cells [84, 91] and recent studies showed that these cells were very rapidly activated following infection of mice immunized with DNA vaccines [101]. DNA vaccines based on defined T cell epitopes from TS antigen have also been tested and it was found that

Table 3: DNA vaccines tested against *Trypanosoma cruzi*.

Antigen	Type of antigen	Dose	Mouse strain	Immune response	Protection	Reference
TS 154	TS family		BALB/c	Th1, CTL	+++	[84, 89, 90]
			A/Sn		−	
TSA-1	TS family	$2 \times 100\,\mu$g IM	C57BL/6 BALB/c	CTL	+++	[87, 91]
ASP-1	TS family	$2 \times 100\,\mu$g IM	C57BL/6	CTL	+++	[87]
ASP-2	TS family	$2 \times 100\,\mu$g IM	C57BL/6	CTL	+++	[87]
Tc13	TS family	$5 \times 50\,\mu$g IM	BALB/c		−	[92]
ASP-clone9	TS family	$4 \times 100\,\mu$g IM	BALB/c	IFNγ	+++	[93]
TSSA	TS family	2 to $4 \times 100\,\mu$g IM	BALB/c, C57BL/6 C3H/Hej	CTL	− to +++	[94, 95]
TS (7 members mix)	TS family	$2 \times 25\,\mu$g IM	C57BL/6		+++	[96]
ASP-clone9, TS	TS family	$4 \times 200\,\mu$g IM	BALB/c	IFNγ	+++	[97]
CRP		$2 \times 100\,\mu$g IM	BALB/c		+++	[98]
cruzipain			BALB/c	CTL		[99]
DHOD		2 to $4 \times 100\,\mu$g IM	BALB/c, C57BL/6 C3H/Hej		−	[94]
LYT1		$2 \times 25\,\mu$g	C57BL/6	IFNγ, CTL	+++	[96]
FCaBP/Tc24		$2 \times 25\,\mu$g	C57BL/6	IFNγ, CTL	−	[96]
Tcβ3		$2 \times 25\,\mu$g	C57BL/6	IFNγ, CTL	++	[96]
Mucin (6 members)		$2 \times 25\,\mu$g	C57BL/6		−	[96]
KMP11		4 doses IM	BALB/c	CTL	− to ++	[100]

IM: intramuscular; CTL: cytolytic activity; −: no protection; +: little protection; ++: fair protection; +++: very good protection.

both $CD4^+$and $CD8^+$T cell epitopes were necessary and sufficient to induce a protective immune response [102].

Taken together, these data clearly demonstrated that vaccination did not result in increased pathology, as initially feared, but allowed at least partial control of disease progression, thus confirming the central role of parasite persistence for Chagas disease pathogenesis and opening the way to further assessment of DNA vaccines against *T. cruzi*. However, it has to be noted that many of the antigens tested belonged to the trans-sialidase family of protein, so that there is still little diversity in terms of the antigens tested as vaccines against *T. cruzi* (Table 3).

4.3. Strategies for potentiating T. cruzi DNA vaccines

Because protection induced by single antigen DNA vaccine remained partial, a number of studies have evaluated strategies to increase vaccine efficacy. These include the use of cytokine/chemokine encoding plasmids to potentiate the immune response induced by the vaccine, and two of the most studied molecules have been IL-12 and GM-CSF, which both were generally able to potentiate protection (Table 2). Alternatively, mixtures of plasmids encoding distinct antigens were used for immunization, and as mentioned above for *Leishmania* vaccines. For example, immunization of mice with plasmids encoding TS and ASP-2 proteins resulted in a specific immune response against both antigens and an increased protection against infection [97]. On the other hand, an immunization with a mixture of DNA vaccines encoding up to 6 proteins from the mucin family resulted poorly protective, while a mixture of up to 7 proteins from the TS family was protective, but not as much as a single antigen vaccine encoding the TS-like antigen ASP-2 [96]. Similarly, a mixture of DNA vaccines encoding ASP-1, ASP-2, and TSA-1 had a similar protective activity as TSA-1 alone [87]. The lack of efficacy of these multivalent vaccines may be attributed to the presence of shared or immunodominant epitopes since they have significant sequence similarity that may not have resulted in a broader immune reponse.

Heterologous prime-boost approach has also been evaluated and immunization with some combinations of DNA and recombinant TS was found to enhance Th1 immune response, but protection was not significantly different from that obtained with DNA alone [103]. Taken together, these studies suggest that additional strategies need to be investigated to potentiate DNA vaccine efficacy against *T. cruzi*.

4.4. Therapeutic DNA vaccines

Therapeutic administration of DNA vaccines to control an ongoing infection with *T. cruzi* may represent an additional

alternative for Chagas disease control. The concept was demonstrated in mice acutely or chronically infected, and in both cases the administration of only two doses of DNA vaccine encoding TSA-1 or Tc24 antigens was sufficient to limit disease progression, as treated mice presented increased survival and reduced cardiac tissue damage, as assessed by histopathologic analysis [104]. A comparative study of different DNA vaccines identified Tc52 antigen as another therapeutic vaccine candidate, while DNA vaccines encoding antigens from the TS family previously found to be protective had no signifiacnt therapeutic effect [105]. It was found that therapeutic vaccination rapidly induced spleen cell proliferation, including IFNγ-producing $CD4^+$ and $CD8^+$T cells, while the effects on cardiac tissue inflammation and parasite burden take longer to be detectable [106]. Importantly, in all these studies, therapeutic vaccination of *T. cruzi* infected mice did not result in an increased inflammatory reaction in the heart, confirming that it is safe to stimulate the immune response of *T. cruzi* infected mice and that attacking the parasite can lead to a reduction of pathology. These studies thus open very attractive perspectives for the control of *T. cruzi* infection, and further studies on the efficacy of DNA vaccines encoding other antigens and on the immune mechanisms underlying their therapeutic effect should provide clues for the optimization of this strategy.

4.5. Antigen discovery

As for any vaccine, the nature of the antigen used remains a key factor for vaccine efficacy, and there is still little variety in terms of antigens evaluated as DNA vaccine candidates against *T. cruzi*. Thus, a number of studies have aimed at identifying novel antigens through various strategies. The most classical approach has been the screening of cDNA libraries using antibodies and screening an amastigote library allowed the identification of a novel antigen Tcβ3, and two previously characterized ones, LYT1 and FcaBP/Tc24 [96]. DNA vaccines encoding these antigens induced variable levels of protection, the best one being LYT [96]. Alternatively, expression-library immunization, described above for *Leishmania*, was also tested with *T. cruzi*, and found to be immunogenic, but there was no attempt at fractionating the library or identifying protective antigens [107]. A likely reason is that such strategy may be too labor-intensive for large genomes/libraries, and its usefulness may be limited to pathogens with small genomes. The availability of *T. cruzi* genome sequence also opens new possibilities for antigen discovery. In one of the first studies using such resource, a combination of bioinformatics analysis were used to identify GPI-anchored or secreted proteins, and most of the identified clones were immunogenic as DNA vaccines [108]. Further studies may confirm the usefulness of these new vaccines to protect against *T. cruzi* infection. Nonetheless, as discussed above for *Leishmania*, the rationale for limiting antigen searches to surface proteins may not be totally relevant, and additional strategies should also be used to include unbiaised genome-wide surveys for antigen discovery.

5. FUTURE DIRECTIONS

As detailed in this review, there have been considerable advances in DNA vaccines against *Leishmania* and *T. cruzi* in recent years. Taken together, these studies clearly validated the concept of using DNA vaccines for both protection and therapy against these protozoan parasites in a variety of mouse models. While sterile immunity seems to be an irrealistic goal for either *Leishmania* or *T. cruzi*, a reduction in disease severity and in the development of the pathology seems clearly within the reach of DNA vaccines. Nonetheless, the relevance of such mouse models for the development of veterinary or human vaccines against these parasites has been challenged by some authors. The few DNA vaccine studies in nonmurine models of leishmaniasis suggest that some extrapolation may be feasible, but certainly not completely. Additional advanced preclinical studies of DNA vaccine candidates in nonmurine animal models such as rats, hamsters, dogs, or monkeys are thus warranted in the next few years, to further explore the immunology and efficacy of DNA vaccines against these parasites. As already observed in such studies for other pathogens, this will lead to the challenge of achieving in these species an immunogenicity of comparable level and protective efficacy as that obtained in murine models. However, advances in adjuvants, DNA vaccine formulation, and delivery systems are likely to contribute to such results [1, 109].

Another major issue is that of antigen discovery, and while a number of DNA vacines tested so far against *Leishmania* or *T. cruzi* have shown promise, we are still unsure if these are the best possible antigens, particularly since these parasites have relatively large genomes, and only a limited variety of antigens have been tested. The availability of the genome sequences of these parasites will without doubt be a key resource for genome-wide screenings for new protective antigens. A key lesson from the initial studies reviewed here [48, 51, 108], together with other similar antigen discovery studies, seems to be that cellular localization and protein function are poor predictors of the antigenicity and protective efficacy of a protein. Alternative criteria should thus be used so that potent vaccine candidates are not missed, and the important development of genome-mining and bioinformatic tools is providing new tools for a more rational search of vaccine candidates [110].

To conclude, those DNA vaccines represent a promising approach for the control of *Leishmania* sp. and *T. cruzi*, and such vaccines would have a major impact in developing endemic countries. Thus the question does not seem to be if DNA vaccines can control these parasites, since many studies have clearly showed that this is the case, but how to translate what has been achieved in mouse models into veterinary or human vaccines of comparable efficacy.

ACKNOWLEDGMENT

This work was funded by Grant SEP-2004-C01-47122 from the Consejo Nacional de Ciencia y Tecnología (CONACYT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 85154, 10 pages
doi:10.1155/2007/85154

Review Article

Immune Response Regulation by *Leishmania* Secreted and Nonsecreted Antigens

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Received 26 December 2006; Revised 6 March 2007; Accepted 29 April 2007

Recommended by Ali Ouaissi

Leishmania infection consists in two sequential events, the host cell colonization followed by the proliferation/dissemination of the parasite. In this review, we discuss the importance of two distinct sets of molecules, the secreted and/or surface and the nonsecreted antigens. The importance of the immune response against secreted and surface antigens is noted in the establishment of the infection and we dissect the contribution of the nonsecreted antigens in the immunopathology associated with leishmaniasis, showing the importance of these panantigens during the course of the infection. As a further example of proteins belonging to these two different groups, we include several laboratorial observations on *Leishmania* Sir2 and *Lic*TXNPx as excreted/secreted proteins and *Lm*S3arp and *Li*mTXNPx as nonsecreted/panantigens. The role of these two groups of antigens in the immune response observed during the infection is discussed.

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1. INTRODUCTION

Leishmaniasis are parasitic diseases, caused by protozoan parasites of the *Leishmania* genus, associated with significant morbidity and mortality in tropical and subtropical regions and in the Mediterranean basin. The disease has a wide range of clinical manifestations that depend not only on the infecting *Leishmania* species but also on the immune status of the host [1]. The most extensively studied leishmanial disease is the cutaneous form caused by *L. major* or *L. tropica* in the old world and *L. braziliensis* in the new world. It usually appears as a skin ulcer or dermal granuloma, which may take up to several months or years to heal [2]. With *L. braziliensis*, the infection may also spread to other cutaneous sites, like mucosal membranes giving origin to the mucocutaneous form of the disease. The most serious form of the disease is the visceral one that, if untreated, gives rise to a high mortality rate. It is characterized by fever, cachexia, hepatosplenomegaly, and hypergamaglobulinemia and is caused by members of the *L. donovani* complex (*L. donovani* in the old world, *L. infantum* in the Mediterranean basin and *L. infantum chagasi* in the New World) [3].

Leishmania is a digenetic protozoan that is transmitted to the mammalian host by sandflies of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world. In the alimentary tract of the insect vector, the parasite exists extracellularly as a flagellated motile form, the promastigote. During the insect blood meal, the infectious developmental form, metacyclic promastigotes, is injected into the dermis and phagocyted by resident macrophages within which the parasite differentiates into the nonmotile amastigote form and multiplies. Moreover, other cells such as fibroblasts and dendritic cells may also harbour parasites [4]. The cycle is completed when the sandfly takes another blood meal recovering free amastigotes or infected macrophages.

During an infection, the parasites have a remarkable adaptative capacity as they are able to survive inside phagocytic cells. These cells are responsible for the microbicidal and antigen-presenting functions however they serve as a safe habitat for the parasite. The existence of inbred mice, which are either susceptible (Balb/c) or resistant to infection (C57BL/6, CBA, C3H.HeJ) has helped to elucidate the protective or nonprotective role of cytokine and T-helper cell subsets and also the role of different leishmanial antigens in the immune evasion mechanism. Thus, it became generally accepted that resistance against leishmaniasis is associated with the production of IL-12 by antigen presenting cells (APC) macrophages and dendritic cells, leading to the

differentiation and proliferation of the Th1-subset of $CD4^+$ T-cells producers of IFN-γ. This will ultimately lead to the activation of parasite-infected macrophages that, through the induction of effector molecules as nitrogen and oxygen reactive species, will kill the intracellular parasites [5]. In contrast, failure to control the infection has been associated with the production of anti-inflammatory cytokines as IL-4, IL-10, IL-13 and TGF-β [6]. Given the ancient evolutionary divergence in *Leishmania* species, it is not surprising that the control of the different *Leishmania* driven diseases is related to different immunological properties. Hence, while in cutaneous leishmaniasis, IL-4 has been implicated in disease progression, in visceral leishmaniasis its importance has been ruled out [7]. In the latter, IL-10 has been shown to be the major immunosuppressive cytokine along with TGF-β. Overall, it suggests that it is the overshadowing of the Th2 response by a Th1 cell associated response that leads to the control of the infection [8]. Moreover, the real contribution of the humoral response is still under debate, however studies in different intracellular pathogens have shown that antibodies can also have a function in restricting the infection when the parasite is exposed to the extracellular milieu [9]. Consequently, in leishmaniasis, the induction of specific humoral responses to parasite antigens would, theoretically, be able to neutralize the parasite whether as free promastigotes, after the inoculum, or as amastigotes, when released from the infected macrophages, contributing to develop a protective response [10]. However, until now, no effective vaccine against human leishmaniasis is available for clinical use [3].

Leishmania parasites inside their hosts do not behave inertly. Rather, the virulence related to their pathology seems to be linked to an induced lack of immune response control. The parasite actively secretes proteases and other molecules that affect host immune system (cells and cytokines) facilitating the infection process. In addition, the parasite possesses intracellular nonsecreted antigens, members of conserved protein families, which are believed to contribute to the chronic immunopathology, observed in leishmaniasis. Here, we review these two groups of relevant parasite molecules, illustrated with laboratory observations of proteins belonging to the secreted and nonsecreted groups of antigens. Finally, we discuss their differential role in *Leishmania* infection and persistence as well in the development of a protective immune response.

1.1. The importance of the secreted versus nonsecreted antigens

Leishmania virulence has been explained using two different groups of parasite molecules, the secreted and surface and the intracellular molecules [11]. This model proposes that the secreted and surface molecules will be mostly important for the establishment of infection, protecting the parasite from the early action of the host immune system, acting as invasive/evasive determinants. According to this model, the intracellular molecules will be ultimately responsible for the disease phenotype [11].

1.2. Surface and secreted molecules

The secreted proteins have distinct functions during *Leishmania* infection. First, they play a role in the establishment of the infection [12] in conjunction with important elements existent in the saliva of the sandfly vector [13, 14]. In a second phase, they contribute to the maintenance of the infection by interfering with the macrophagic microbicidal functions, cytokine production, antigen presentation, and effector cells activation. This is achieved by repression of gene expression, post-translation protein modification or degradation, and by activation of suppressive pathways and molecules [15]. This macrophagic anergy enables the continuous multiplication of the amastigote form. The bulk of the knowledge on surface and secreted molecules of *Leishmania* is focused on lypophosphoglycan (LPG), on the promastigote surface protease named glycoprotein 63 (gp63), glycosylinositol phospholipids (GIPLs), cysteine peptidases and on a few others like β-mercaptoethanol activated proteases, acid phosphatases and chitinases. The importance of some of these molecules in the establishment of the infection is well documented [15, 16], but the real contribution of the secreted molecules remains elusive due to the difficulty of the intramacrophagic studies.

After entrance into a susceptible mammalian host, the *Leishmania* promastigotes are targeted by the host immune system. Serum components, like the complement system represent the first challenge following entrance into the bloodstream. Procyclic promastigotes are highly susceptible to complement action, unlike the metacyclic that can avoid complement mediated lysis [17]. This remarkable difference is mostly due to the surface molecule in *Leishmania*, the LPG. Composed mainly of repetitive units of a disaccharide and a phosphate, LPG is linked to the membrane by a glycosylphosphatidylinositol anchor [18]. The LPG is longer in metacyclic promastigotes preventing the attachment of C5b-C9 subunits of the complement complex avoiding its lytic action [17]. The relevance of LPG is not limited to complement resistance. Its importance is stated by several studies using either purified LPG or mutant strains. The LPG is implicated in several processes including the binding to the epithelial cells of the sandfly midgut [19], receptor mediated phagocytosis of macrophages through the CR3/CR1 ligand or the manose-fucose receptor (in conjunction with gp63) [20, 21], toll-like receptor 2 signalling [22], stimulation of NK cells [23], inhibition of phagosome-endosome fusion [24–26], and inhibition of phagosome-derived superoxide [27]. Several attempts to use LPG to confer protection were unproductive [28, 29]. Constitutively shed by several *Leishmania* species, the LPG is the paradigm molecule referred to as evasive and invasive. After the initial steps of infection, LPG is downregulated being almost absent from amastigotes [30].

Another molecule implicated in the invasive and evasive mechanisms is gp63. This protein is the most abundant in the parasite surface, although 10 fold less abundant than LPG [30]. In the promastigote form, gp63 is in the surface of the parasite under the LPG coat and is involved

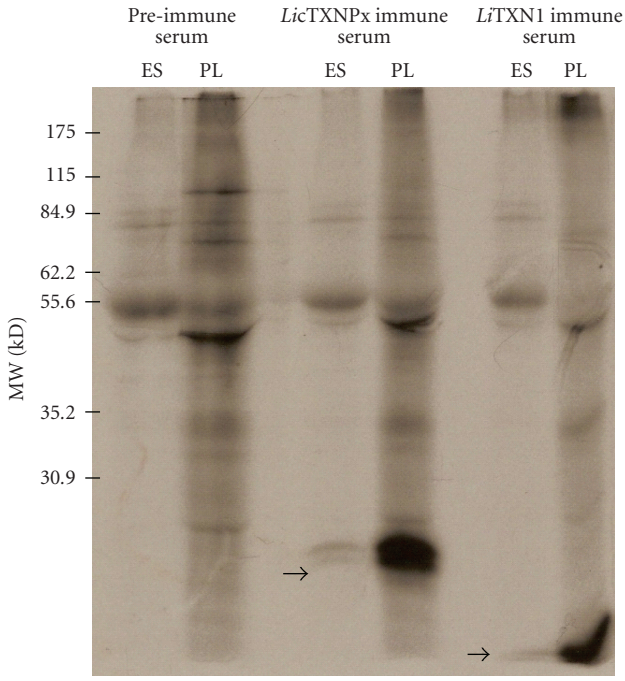

Figure 1: *The LicTXNPx and LiTXN1 are excreted/secreted proteins.* Autoradiography of [^{35}S] methionine labelled *L. infantum* promastigotes lysate (PL) and excreted/secreted antigens (ES), after 3 hours of incubation experiments, immunoprecipitated in the presence of immune anti-*Lic*TXNPx or anti-*Li*TXN1 sera or with a preimmune serum.

in *L. donovani* promastigote multiplication [31]. Like LPG, gp63 was shown to be implicated in complement resistance, in *L. major* and *L. amazonensis*, by mediating the interconvertion of C3b to C3bi [32]. This interconvertion favours the internalization via CR3 avoiding the oxidative burst. The binding of gp63 to fibronectin receptors favours the parasite uptake into the macrophage [33]. Furthermore, gp63 is an endopeptidase with the potential to degrade immunoglobulins, complement factors, and lysosomal proteins [34]. The optimal proteolitic activity of gp63 is at pH 4 that may indicate some active proteolitic function in the amastigote stage [34, 35]. Despite this, gp63 expression is downregulated in amastigotes [36]. In spite of being a virulence factor in most *Leishmania* species, immunization trials with gp63 were unable to protect mice from infectious challenge [37]. Moreover, gp63 mutation in *L. major* did not impair in vitro intramacrophagic survival [38]. So the importance of gp63 in the course of the infection remains elusive. The GIPLs are molecules 10 times more abundant than LPG on the parasite surface, although like gp63 they are physically under the LPG coat [39]. The GIPLs were described in *L. major* as having a protective role at the parasite surface by modulating the expression of nitric oxide synthetase in murine macrophages [40, 41]. Another interesting group of proteins are the cysteine proteases. In *L. mexicana,* this family of proteins seems to be associated with disease progression [42]. Cysteine protease activity can be found at the parasite surface or inside the macrophage endoplasmatic reticulum, probably associated with proteases released in the phagolysosome by *Leishmania*. The inhibition of major histocompatibility complex class II molecules in macrophages seems to involve, in *L. amazonensis*, the direct sequestering of these molecules following cysteine-peptidase-dependent degradation [43, 44]. Also, cysteine peptidase activity was demonstrated in *L. mexicana* to induce IL-12 repression and degradation of NF-kB [45]. It is still worthy to mention some other secreted proteins described as virulence factors, like the *L. mexicana* chitinase [46] and the *L. donovani* acid phosphatases [47–50]. An in depth study of the *Leishmania* secretome is missing. The most remarkable effort was done by Chenik and colleagues that were able to screen 33 different proteins using an *L. major* cDNA library and a rabbit immune sera raised against the secreted proteins [51]. Nine of them were already described as excreted/secreted proteins in *Leishmania* or other species, 11 corresponded to known proteins but not characterized as secreted and the other 13 were completely new and uncharacterized proteins [51]. This shows how little is known about the *Leishmania* secretome since only a few proteins are extensively characterized [52–56]. It is already known that total *L. major* secreted molecules, described as highly immunogenic [54, 57–59], can confer protection from infectious challenge [57, 59]. So it is obvious that somewhere among the *Leishmania* secreted proteins exist future candidates for vaccine design and drug targets. Nonetheless, one of the problems in vaccine design using surface or secreted/excreted proteins is the fact that these proteins are naturally exposed to the immune system. Chang et al suggest that these secreted/excreted proteins were evolutionarily selected becoming immunologically "silent" [60]. This fact implies that secreted proteins that have a specific function in the establishment of the infection will be "silent," allowing them to perform their vital functions unchecked by the host immune system [11, 12]. This will be more significant for the proteins involved in the first steps of infection, while the parasite is still exposed to the extracellular environment. As an example of this fact, we present three distinct proteins: a cytosolic tryparedoxin peroxidase of *L. infantum* (*Lic*TXNPx) [61], the *Leishmania* silent information regulator 2 (Sir2) [52], and a tryparedoxin of *L. infantum* (*Li*TXN1) [62]. All are *Leishmania* secreted proteins (Figure 1) [52], that show distinct immunological properties. A high antibody titre against the *Lic*TXNPx was detected in children [63]. This antibody titre is maintained during the *Leishmania* infection and decreases after its resolution [63]. Despite its high immunogenicity when tested in vitro or in vivo using the Balb/c model, this excreted/secreted protein did not show immunomodulatory properties (Figures 4, 5, and Table 1) and provided no protection against the infectious challenge (data not shown). On the other hand, the *Leishmania* Sir2 is a typical poorly immunogenic secreted antigen (Figure 2) characterized as a virulence factor [64]. Infectious challenge after *Leishmania* Sir2 immunization results in a decreased infectivity in the acute phase (Figure 3). This could be partially due to the production of lytic and neutralizing antibodies [65]. The immunization leads to a significant decrease of the spleen

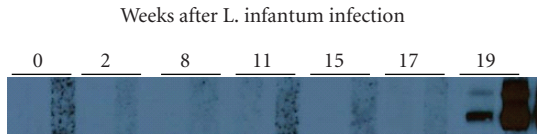

Figure 2: *Antibodies against Leishmania SIR2 protein in the sera of chronically L. infantum infected Balb/c mice.* Sera from 10^8 intraperitoneal (i.p.) *L. infantum* promastigotes infected Balb/c mice after 2, 8, 11, 15, 17, and 19 weeks, were used in a western blot against 1 μg of r*Li*SIR2 at two different dilutions, 1 : 200 and 1 : 50 (left and right lanes, respectively, for each different serum). A 0 weeks serum was obtained from noninfected mice.

and liver parasite load at two weeks post infection (Figure 3) [65]. However, it is incapable by itself of resolving the infection, as seen six weeks after infection, where there is no significant difference between the immunized infected group and the infected control group (Figure 3). Certain secreted proteins seem to function as immunomodulatory components, acting as host immune evasive proteins. As an example, another excreted/secreted *Leishmania* protein, *Li*TXN1 (Figure 1), is capable to increase IL-10 splenocyte secretion (Table 1), a major immunosuppressive cytokine (manuscript in preparation). *Li*TXN1 can be among the proteins responsible for a transient immunosuppressive state that can favour the parasite internalization and colonization of the host cells. These examples show that among the secreted proteins we can find proteins naturally immunogenic, albeit nonprotective, like *Lic*TXNPx while others less immunogenic show interesting properties in terms of protection probably due to the disruption of their in vivo functions, *Leishmania* Sir2, or by their immunomodulatory properties, *Li*TXN1. Unfortunately, the reduced immunogenicity of the most interesting secreted proteins probably will prevent their identification by serological based approaches [51].

The reduction of the secreted/excreted proteins to the given examples is an oversimplification. However, it is obvious that much more work is needed in this area, especially in the huge black hole of knowledge that concerns the interaction between host cell and *Leishmania* at a molecular level. Since most of the studies have been done using infection-phenotype approaches, little is known about the true agents involved in macrophagic disruption [16, 58, 68, 69]. We suggest that amastigote secreted proteins will be more immunogenic and can have interesting immunomodulatory properties since they have not been under the selective pressure as the promastigote secreted proteins. The selective pressure of the host immune system is a powerful driving force in evolution, as demonstrated in the case of *Schistosoma mansoni* that has the ability to completely evade the host immune system rendering itself "invisible" [70].

1.3. Panantigens—nonsecreted proteins

Human visceral leishmaniasis, unlike cutaneous leishmaniasis is characterized by high anti-*Leishmania* antibody titres [71, 72]. The role of these antibodies is still unclear as there

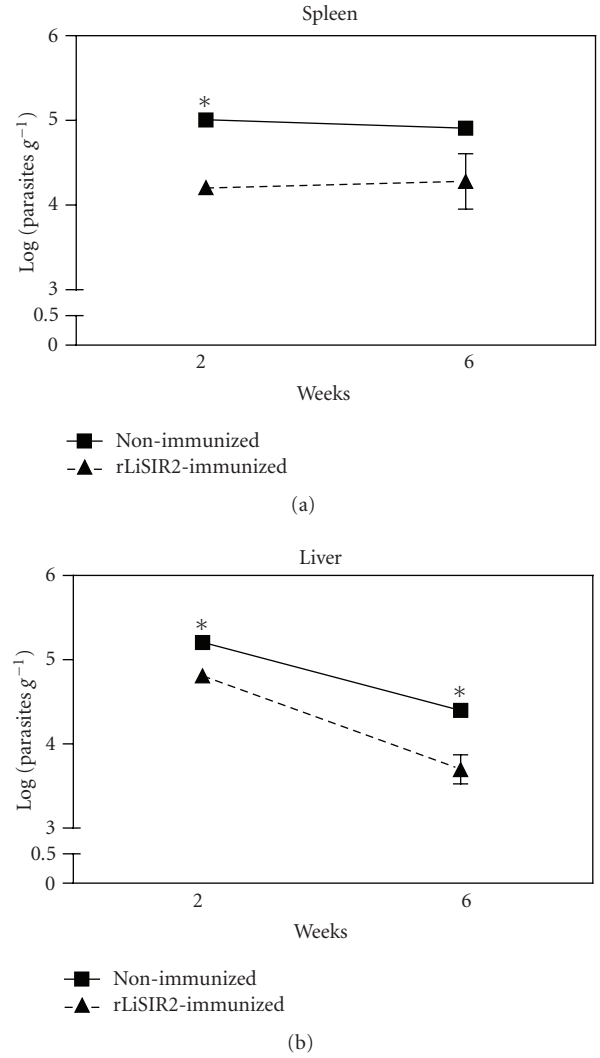

Figure 3: *The recombinant Leishmania SIR2 immunization reduces the parasite load in an acute phase of L. infantum Balb/c mice infection.* The immunized mice (▲) received 3 i.p. injections of recombinant *Leishmania* SIR2 (50 μg) once a week and infected 2 weeks after the last immunization with 10^8 *L. infantum* stationary phase promastigotes. The nonimmunized mice (■) were subjected to the same protocol but received PBS instead of recombinant *Leishmania* SIR2. The mice were sacrificed after 2 and 6 weeks of infection and the parasite load in the spleen and liver determined by the organ limiting dilution method [66]. The data represent means and standard deviations for three mice and are representative of two independent experiments. Statistical analysis was performed using Student *t*-test. Statistically, significant differences between immunized and nonimmunized mice are indicated. $^*P < .05$.

seems to be no relation with the progression or resolution of the infection[58, 73, 74]. This exuberant humoral response against promastigote and amastigote antigens (fractions or total protein extract or specific *Leishmania* proteins) has been exploited for serodiagnosis with different degrees of success [58, 63, 74, 75]. Interestingly, one of the most sensitive techniques using recombinant *Leishmania* proteins does not involve surface molecules like LPG or gp63 but

Table 1: Immunomodulatory properties of several *Leishmania* proteins

Protein	Properties	References
Leishmania Sir2	Secreted, B-cell activator, induces lytic, and neutralizing antibodies	[64, 65]
*Lic*TXNPx	Secreted, elicits strong humoral response and has no influence on cytokine production	[63]
*Lim*TXNPx	Nonsecreted, decreases IL-4 secretion both in vitro and in vivo	Figure 3
*Li*TXN1	Secreted, poorly immunogenic, induces IL-10 secretion both in vitro and in vivo	(Manuscript in preparation)
*Lm*S3arp	Nonsecreted, B-cell polyclonal activator, inhibits T-cell proliferation, and downregulate IL-2, 12 and IFN-γ in splenocytes	[67]

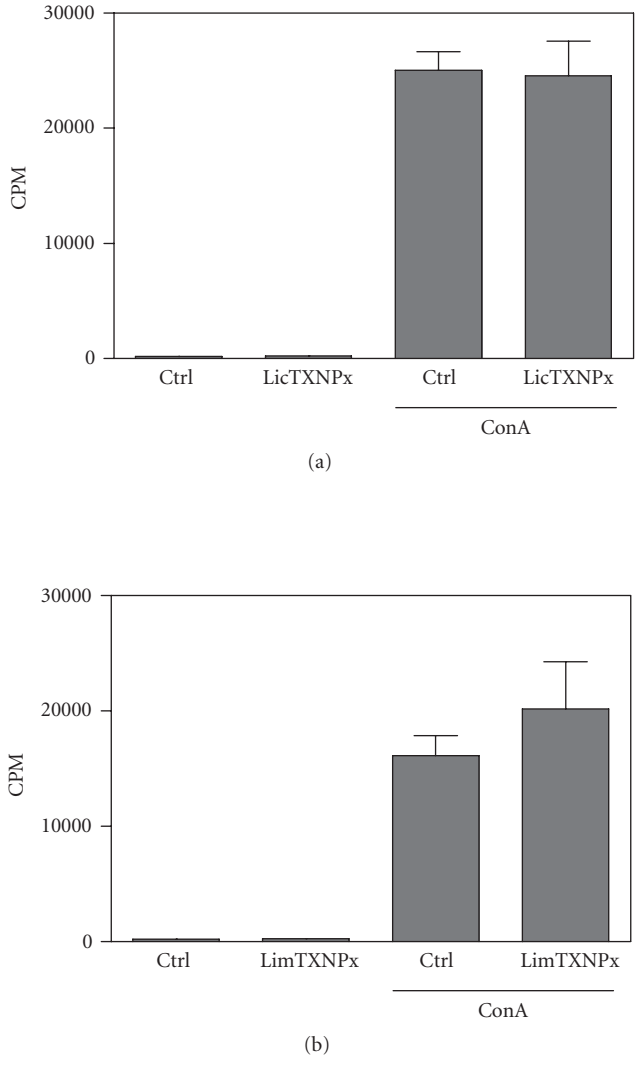

Figure 4: *No effect of rLicTXNPx and rLimTXNPx (a) on spleen cell proliferation.* Spleen cells from normal Balb/c mice were cultured for 48 hours (2.5×10^5 cells/well) in the presence or absence of concanavalin A (ConA) (5 μg/ml) with or without r*Lic*TXNPx and r*Lim*TXNPx (b) (10 μg/ml). The cells were pulsed with [methyl-^{3}H] thymidine in the last 8 hours of culture, and cpm (scintillations per minute) were determined. The data represent mean cpm and standard deviations from triplicate cultures of spleen cells from three mice analyzed individually. One of three independent experiments is depicted.

intracellular proteins like histones [75]. The screening of *Leishmania* expression libraries or total protein extract with serum from infected patients has unveiled several major immunogens [76–79]. Among these immunogens, nonsecreted proteins like heat shock proteins, ribosomal proteins and histones were described [76, 77, 80]. These highly-conserved proteins that elicit strong immune responses are generally designated as panantigens [81]. The elevated antibody titre against conserved proteins can be the direct result of B-lymphocytes polyclonal activation similar to what is found in Chagas disease [82, 83] or in autoimmune diseases [84]. Furthermore, in the Balb/c mouse model, an *L. major* protein homologue to the mammalian ribosomal protein S3a, *Lm*S3arp, (Table 1) is able to elicit an unspecific activation of B-lymphocytes with the production of autoreactive antibodies [67]. Despite this, in natural infections, the humoral and cellular responses are highly specific with no significant autoantibody production [80, 81, 85]. Moreover, the epitope mapping of several *Leishmania* panantigens tends to reveal *Leishmania* unique epitopes that elicit strong immune responses [79–81, 86, 87]. There is practically no response to the homologous regions in these proteins, which argues against the nonspecific polyclonal activation as the source of reactivity against *Leishmania* panantigens [11, 81]. So, it is expected that these proteins are presented to the immune system during the natural course of the infection. Unlike secreted and surface proteins that are exposed and can be processed by the host immune system, the intracellular proteins are not. One must expect that the contact between the immune system and these proteins happens only upon the parasite destruction. Subsequently, one obvious source of intracellular proteins is the parasites from the initial inoculum some of which are destroyed. Furthermore, it was recently demonstrated that the presence of apoptotic parasites in the initial inoculum is a requisite for disease development [88]. Albeit the small number of parasites in the initial inoculum is not sufficient to explain the physical expansion of cell populations and immune mediators during the course of infection, it is a fact that panantigens are exposed long before the onset of any visible symptoms [88]. This initial release of panantigens may function in conjugation with the secreted and surface proteins acting as a transient "smoke screen" that enables the onset of the initial infection by viable parasites. The immune response developed against the panantigens may contribute to hide the parasite molecules

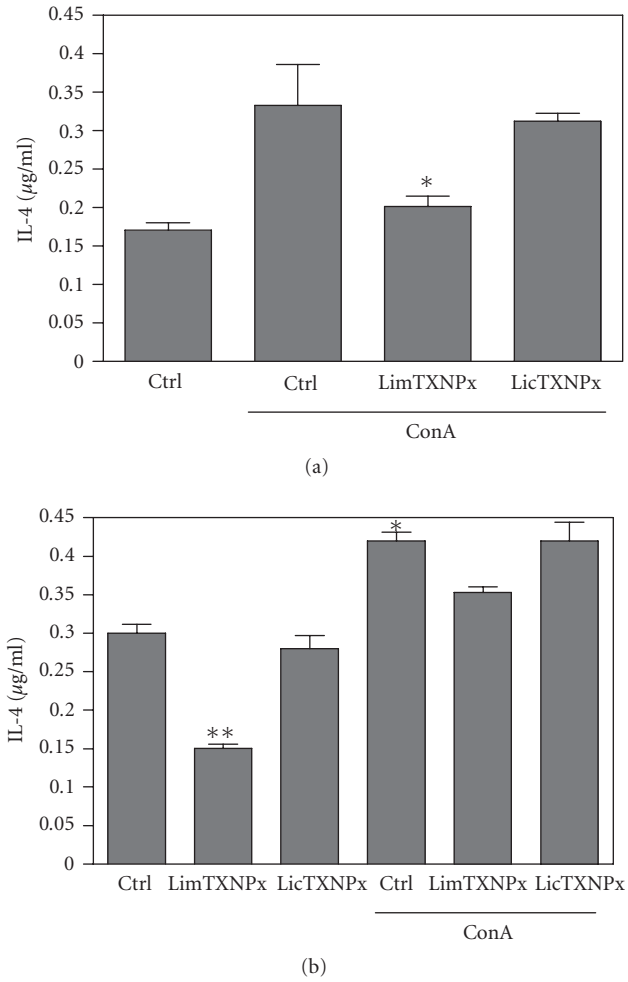

Figure 5: *Levels of IL-4 in the supernatants of spleen cells from rLicTXNPx or rLimTXNPx treated and untreated Balb/c mice.* The spleen cells from untreated (a) and treated (b) Balb/c mice (50 μg of r*Lic*TXNPx or r*Lim*TXNPx i.p. injected once a week for 3 weeks followed by 2 weeks before the spleen cells were recovered) were incubated with r*Lic*TXNPx or r*Lim*TXNPx (10 μg/ml) in the presence or absence of ConA (5 μg/ml) for 48 hours. The levels of IL-4 were determined by ELISA in comparison with a standard curve using the recombinant IL-4. The data represent means and standard deviations for triplicate cultures of spleen cells from three mice. The results are from a representative experiment of three carried out independently. Statistical analysis was performed using Student *t*-test. Statistically, significant differences is indicated. $^{*}P < .05$ and $^{**}P < .01$.

involved in the invasion of the phagocytic cells. Moreover, the humoral profile suggests a steady release of panantigens during the infection [58, 73, 74]. It is also [81] suggested that panantigens originate from the residing parasite population either by the destruction of intracellular amastigotes by active macrophages or by the destruction of amastigotes that burst from macrophages or even by the spontaneous cytolysis of amastigotes inside the infected cells [11]. In active leishmaniasis, there seems to be a general anergy in infected macrophages that leads to impaired functioning [16, 89–92]. So, in this case, it is not expected that panantigens may result from the macrophage mediated elimination of *Leishmania*, as it will lead to the resolution of the infection. Although free amastigotes can infect macrophages directly, they are almost undetectable even in heavily infected hosts. Thus, their contribution to the pool of panantigens should be diminished [11]. The low speed of intracellular amastigotes multiplication and their capacity to delay apoptosis in heavily infected phagocytes [60] enables a lasting coexistence in infected macrophages. The most viable theory for the phased release of panantigens would be the spontaneous cytolysis (described as apoptosis by some authors) of intracellular amastigotes [93]. The effect of the panantigen release is gradual and more significant as the infection develops and the parasite burden augments explaining the increasing intense immunopathology associated with *Leishmania* infection [11]. This increase in panantigen release can be extrapolated in correlation with panantigen antibody titres and parasite burden as seen for the *Leishmania* kinesin like protein, k39 [81, 94]. Another protein that shows similar characteristics to k39 is the *Lic*TXNPx which has also the ability to induce a high quantity of nonprotective antibodies both in natural or experimentally infected dogs (unpublished data) and in infected humans [63]. This induction can be done by direct activation on B-cell populations with clonal expansion as described for *Leishmania* Sir2 [65], which seems not to be the case since little or no antibodies for *Lic*TXNPx are seen in HIV patients with leishmaniasis (unpublished data), as was observed for k39 [95]. This suggests the existence of specific T-cell epitopes in *Lic*TXNPx. The nature of these epitopes will not be similar to those of k39, because the latter contain repetitive motifs that will contribute significantly to the clonal expansion of B-cells. For *Lic*TXNPx, the strong immune response observed should be due to the formation of highly stable multimeric structures characteristic of this protein [96]. The nonprotective antibody titres induced by *Lic*TXNPx seem to be transient and associated only with the immunopathology as they disappear after a period of time, unlike other *Leishmania* specific antibodies simultaneously in circulation [63]. These antibodies may contribute to the impairment of bone marrow and spleen [11].

The capacity of panantigens to modulate the immune system can be related to the fact that these intracellular proteins were not selected by the immune pressure, unlike the secreted and surface proteins. Hence, in the right conditions, they can provide the immunomodulatory properties needed for vaccine design. The most prominent intracellular proteins used in vaccine design are still LACK and *Lm*STI1 that are able to induce protective responses with a parasite-specific Th1 immune response (high IFN-γ but not IL-4 secretion) [87, 97]. Among the *Leishmania* proteins studied by our group, a mitocondrial tryparedoxin peroxidase (*Lim*TXNPx; Table 1), homologous to *Lic*TXNPx, is able to induce down regulation of IL-4, a Th2 cytokine, in splenocytes both in vitro and in vivo (Figure 5) though unable to induce significant protection (data not shown). It is noteworthy that similar proteins such as *Lic*TXNPx and *Lim*TXNPx are able to elicit distinct immune responses.

*Lic*TXNPx is secreted inducing only the production of nonprotective antibodies, while its related intracellular counterpart *Li*mTXNPx has immunomodulatory properties interfering with cytokine production (Figure 5). This can be a good example of the type of evolutionary pressure induced by the immune system, in which two related proteins have distinct immunomodulatory properties (Figures 4, 5). It suggests that the host immune system selects characteristics in the exposed proteins that are either innocuous or nondeleterious to the parasite. Since this does not occur in the intracellular proteins they can retain distinct immunoregulatory properties that could be useful in vaccine design.

2. CONCLUDING REMARKS

Taken altogether, these observations support the idea that secreted and surface proteins tend to be poor or nonprotective immune modulators, like *Lic*TXNPx. Nonetheless, their use in vaccine could induce short-lived protection probably due to the disruption of their biological activity or by production of lytic antibodies, as seen with *Leishmania* Sir2. Intracellular components like *Lm*S3arp and *Li*mTXNPx tend to have defined immunomodulatory properties. *Lm*S3arp is able to induce polyclonal activation of B lymphocytes while *LiLi*mTXNPxTXNPx confers a nonprotective dowregulation of IL-4 secretion by splenocytes.

Using the basic knowledge acquired in the study of the immune response against *Leishmania* in different murine models, one can look for proteins that induce the immunological phenotype needed for protection. Therefore, our data suggests that in vaccine development, the conjugation of secreted and surface proteins with intracellular components should provide a more efficient protection. Hence, the impairment of the parasite entrance in the host cells, either by lytic antibodies or by the disruption of protein function, will delay the onset of the immune suppression associated with *Leishmania*. The parasite elimination could be achieved through a protective cellular response, induced by the intracellular parasite components present in the vaccine.

ACKNOWLEDGMENTS

This work was supported by FCT and Programa Operacional Ciência e Inovação 2010 (POCI 2010) and FEDER in the projects: POCI/SAU-FCF/59837/2005 and POCI/CVT/59840/2004. R.S. and J.T. are supported by fellowships from FCT and FEDER number SFRH/BD/13120/2003 and SFRH/BD/18137/2004, respectively. The proteins, *Lic*TXNPx, *Li*mTXNPx, and *Li*TXN1, used in this works were purified from clones provided by A. Tomás.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 59430, 6 pages
doi:10.1155/2007/59430

Review Article

CD40-CD40L Interaction in Immunity Against Protozoan Infections

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Received 1 December 2006; Accepted 2 February 2007

Recommended by Ali Ouaissi

Activation of the immune system against protozoan infections relies particularly on two specific signals provided by cognate interaction of T cells with antigen presenting cells (APCs). The first signal is attributed to binding of the T-cell receptor (TCR) to peptide/MHC complexes on the surface of APCs, whereas the second signal is triggered through binding of several costimulatory molecules on the surface of APCs with their corresponding receptors on T cells. Among these costimulatory signallings, CD40/CD40L interactions have been particularly investigated in protozoan infection models with regard to their potential to amplify cell-mediated immunity against intracellular parasites. This article reviews current studies of the potential role of CD40/CD40L interaction in the modulation of immune responses against some protozoan parasites and highlights recent developments regarding manipulation of this interaction for promoting control of parasite infections.

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1. INTRODUCTION

A rational design of new approaches aiming to control protozoan infections depends not only on advances in our knowledge of virulent factors, molecular pathogenesis, and immune responses involved in the host defence, but also on our understanding of finely tuned co-stimulatory signallings that play a key role in immunity against infections. CD40-CD40L interaction has emerged in the last decade as an essential system that regulates host immune defence against infectious and noninfectious diseases. CD40, a surface glycoprotein receptor belonging to TNF receptor family is expressed by a number of cells including immunocompetent cells such as professional APCs, B lymphocytes, activated CD4+ and $CD8^+$ T lymphocytes as well as nonhematopoetic cells [1–5]. Its ligand, CD40L, is a co-stimultory molecule that can be expressed on various cells such as CD4+ T lymphocytes, B lymphocytes, natural killer (NK) cells, dendritic cells (DCs), monocytes and macrophages [6–9]. CD40-CD40L interactions play an important role in the regulation of thymus-dependant humoral immune responses through cognate interaction of B and T cells which promotes B cell proliferation, Ig class switching, and generation of B cell memory [10, 11]. On the other hand, CD40-CD40L interactions drive also cell-mediated immune responses. Engagement of CD40 present on APCs with CD40L on T cells is crucial for the priming and expansion of antigen-specific CD4+ T cells and for the induction of costimulatory molecules on APCs [12]. It is well known that triggering of CD40 on the surface of APCs leads to the production of cytokines, particularly IL-12 which plays a central role in the activation of T cells to produce IFN-γ, thereby directing cell-mediated immunity towards Th1 subset that is required for effective immunity against intracellular pathogens [13–15]. Naturally occurring mutation in human CD40L gene results in a defect in CD40 signalling and leads to hyper IgM syndrome. Theses patients not only are defective in humoral immunity but also exhibit impaired T cell-mediated immunity and therefore are susceptible to infections [16, 17]. In this review, we focus on insights provided by different studies arguing that cell-mediated immunity against intracellular parasites depends upon CD40-CD40L interactions.

2. CD40/CD40L INTERACTION PROMOTES CELL-MEDIATED IMMUNITY AGAINST PROTOZOAN INFECTIONS

2.1. *Leishmania*

Major insights gained into paradigms of Th-subset came from studies on immunity to *Leishmania* infection. Resistance to *Leishmania* which multiplies as an amastigote within macrophages depends on polarization of the immune response towards Th1 type in which IL-12 and IFN-γ play a pivotal role, while disease progression is linked to development of Th2 type response [18]. Since CD40-CD40L interaction is crucial for promoting Th1 immune response, various studies were focused on the impact of such interaction on the outcome of experimental infection. The first evidence for a direct role of these molecule pairs in promoting immune responses against protozoan infections came from studies showing that CD40- and CD40L-deficient mice are susceptible to *Leishmania* infections [19–21]. T cells from these mice fail to produce IFN-γ suggesting a defect in Th1 response. Conversely, administration of IL-12 in these deficient mice prevents disease progression. These deficient mice were unable to mount an effective immunity against parasite infection due to a defect in T cell mediated activation of macrophages. Beside their role in Th1 immune response, CD40-CD40L interactions were shown also to stimulate macrophages to produce number of cytokines and inflammatory mediators among which Nitric Oxide (NO) plays a key role in parasite killing [22]. Theses basic studies on *Leishmania* infections clearly pointed towards a major role of CD40-CD40L interactions in skewing immune response to Th1 type that is required for antiparasite host defence.

2.2. *Trypanosoma cruzi*

T. cruzi is an obligate intracellular parasite that invades several types of cells in vertebrate hosts. Development of a Th1-like immune response was sown to be associated with the control of infection in mice [23]. In particular, IL-12, IFN-γ, and TNFα play a crucial role in the development of cell-mediated immunity against the parasite [24]. Indeed, treatment of *T. cruzi*-infected mice with anti-IL-12 MAb increases parasitemia and mortality while an exogenous supply of IL-12 confers protection against infection [25, 26]. In view of the important role of IL-12, it seemed likely that CD40 ligation is important for induction of effector phases of immune response. The potent effect of the CD40-CD40L pathway in *T. cruzi* infection was first assessed by using CD40L-transfected 3T3 fibroblasts to monitor parasitological and immunological parameters in infected mice [27]. This study indicated that supernatants of murine spleen cells stimulated with CD40L-transfected cells prevent infection of macrophages in vitro and this phenomenon depends on de novo production of nitric oxide (NO). Anti-IL-12, anti-IFN-γ, and anti-TNFα MAb neutralize the effect of supernatants suggesting the importance of these cytokines in the prevention of macrophage infection. This in vitro data were further supported by in vivo experiments showing that coinoculation of CD40L-transfected 3T3 fibroblasts and *T. cruzi* into mice leads to reduced parasitemia and mortality and this effect is abolished by injection of anti-IL-12 MAb. Recently, we examined further the role of CD40 ligation in *T. cruzi* infection by using a new approach based on generation of CD40L-transfected parasite strain [28]. Mice inoculated with this recombinant strain exhibit a very low parasitemia and no mortality associated with preserved production of IFN-γ by spleen cells compared to wild-type strain. These findings highlight the potent role of CD40-CD40L interaction in the stimulation of an effective immunity against *T. cruzi*.

2.3. *Toxoplasma gondii*

Tachyzoites of *T. gondii* can disseminate in the host because of its ability to infect many nucleated cells. Since IFN-γ is the major cytokine required for the activation of a cell-mediated immunity against *T. gondii* [29], and giving the importance of IL-12 in the stimulation of early IFN-γ synthesis [30], one may suspect a critical role of CD40-CD40L interaction in the control of *Toxoplasma* infections. Studies performed in human with hyper IgM syndrome due to a natural CD40L mutation revealed that these patients exhibited a defect in IFN-γ secretion in response to *T. gondii* [31]. The lack of IFN-γ production was linked to impaired IL-12 secretion, indicating that CD40-CD40L signalling was required for an optimal T cell activation and production of IFN-γ. On the other hand, *Toxoplasma* infection was also investigated in CD40L-deficient mice [32]. This study showed that these mice produced less IL-12 than wild type when infected with *T. gondii*. Moreover, CD40L-deficient mice succumbed to toxoplasmic encephalitis indicating that these mice were not able to control parasite replication in the brain and suggesting an important role of the CD40-CD40L interaction in this process. Furthermore, CD40 signalling was shown to regulate IFN-γ-independent host protection against *Toxoplasma* infection through TNF-α-dependant induction of macrophage antimicrobial activity [33]. Susceptibility of both patients with hyper IgM syndrome and CD40L-deficient mice to *Toxoplasma* infection argues for the requirement of CD40/CD40L signalling for resistance to parasite infection.

3. MANIPULATION OF CD40 SIGNALLING AS A POTENTIAL TOOL TO IMPROVE CONTROL OF PROTOZOAN INFECTIONS

As reported above, CD40-CD40L interaction is crucial for the outcome of infection in a number of intracellular parasite models. Stimulation of CD40 on APCs has proved to be useful for amplification of Th1-type response in which IL-12 and IFN-γ play a cardinal role (Figure 1). The first approach used to modulate CD40 signalling was based on agonistic anti-CD40 Ab. Injection of these Ab in mice infected with *Leishmania* stimulates IL-12 and IFN-γ production and induces killing of the parasites within macrophages [34, 35]. Similarly, administration of anti-CD40L MAb in mice infected with *T. cruzi* results in a stimulation of IFN-γ-activated macrophages to produce NO and tocontrol parasite

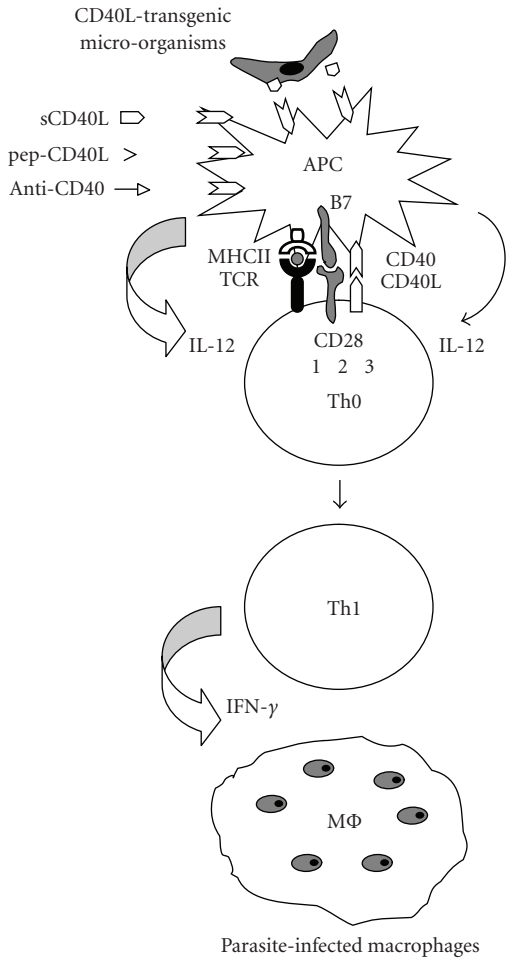

Figure 1: *Schematical representation of enhanced cell-mediated immunity against protozoan parasites through CD40-CD40L interaction*. Following the first signal (1) illustrated by the recognition of parasite peptides combined with major histocompatibility complex II (MHC II) on antigen presenting cells (APC), such as dendritic cells, by T cell receptor (TCR) on naïve T helper cells (Th0), and the second signal (2) which is attributed mainly to binding of CD28/B7 molecule pairs, interaction of CD40-CD40L (3) activates APC to produce IL-12 which promotes Th1 cell differentiation and secretion of IFN-γ. This leads to stimulation of macrophages to control parasite replication. This activation process can be amplified by agonistic anti-CD40 antiboy, by soluble CD40L (sCD40L) or by CD40L peptide mimetics (pep-CD40L). Transgenic parasites expressing CD40L can also activate CD40 signalling through membrane-bound or secreted CD40L.

infection [27]. Although CD40L exists in nature predominantly as a membrane-anchored molecule, the molecular characterization of CD40L molecule indicated that the extracellular carboxy-terminal region can be soluble and biologically active [6, 36]. Therefore, a variety of reports were focused on this agonistic molecule with the aim to assess its stimulatory role in the control of parasite infection as it is the case for other infectious diseases [14, 37]. Studies in patients with hyper IgM syndrome that exhibit deficient secretion of IL-12 showed that in vitro incubation of peripheral blood mononuclear cells (PBMC) with a soluble CD40L resulted in enhanced IL-12-dependant production of IFN-γ in response to *Toxoplasma gondii* [31]. Soluble CD40L was also shown to activate murine macrophages in vitro to control the replication of *T. gondii* [32].

As for many adjuvant proteins, the major obstacles related to stability and issues of in vivo delivery of CD40L had to be faced. In this regard, host cells transfected with CD40L gene were developed as a way of delivery in different parasitic models [27, 38]. Interestingly, Chen et al. described a strategy based on directing CD40L to macrophages by co-expressing the molecule on the surface of a cell line along with gp63 recombinant *Leishmania* antigen and showed that mice treated with these cotransfected cells produce higher amounts of IL-12 and control the disease progression [38]. Delivery of CD40L expressed by transfected 3T3 fibroblasts was shown to reduce parasitemia in *T. cruzi*-infected mice [27]. Recently, we developed a new concept based on the use of the pathogenic organism as a vehicle for CD40L delivery [28]. Following transfection of *T. cruzi* with CD40L gene, the encoded molecule was found properly processed and secreted across the parasite membrane. Notably, CD40L recombinant strain exhibited lower virulence and induced higher INF-γ production when injected into mice. Moreover, surviving mice resisted a challenge infection with wild-type strain, thereby confirming the vaccine adjuvant capacity of CD40L.

The molecular characterization of CD40L and the analysis of its binding domains were determinant steps towards the manipulation of CD40 signalling [39]. As for TNF receptors family, the signalling through CD40 depends upon the formation of a CD40L trimer complex that can each bind three CD40 molecules [40]. Incorporation of an isoleucine zipper motif that improves trimerization of the CD40L was shown to enhance its biological activity [41]. Advances in the molecular structures of CD40L binding domains allowed a conception of small CD40L mimetic molecules that could compete with the binding of CD40L homotrimers and induce IL-12 secretion by DCs [42]. Interestingly, recent findings indicate that when these mini-CD40L synthetic peptides were coinjected with *T. cruzi* into mice, a low parasitemia associated with enhanced $CD8^+$ T cells producing IFN-γ was observed [43]. These recent reports further support the importance of CD40L delivery as an adjuvant that can be used to drive type 1 immune response against intracellular parasite infection (Figure 1).

Protozoan parasites have a remarkable ability to adapt to different host microenvironments. To make the host "as safe as possible" for them, they have evolved many devises among which the immunosuppression is considered as a powerful mechanism to subvert the host's immune response [44]. Interfering with CD40 presentation or signalling can be one of the powerful arms used by the parasite. Indeed, CD40 molecules were found to be reduced in the surface of macrophages and DCs following their infection by *T. cruzi* [45, 46]. Recently, the CD40 was defined as a central molecule through which counteractive immune responses can be triggered [47]. Based on the cross-linking experiments

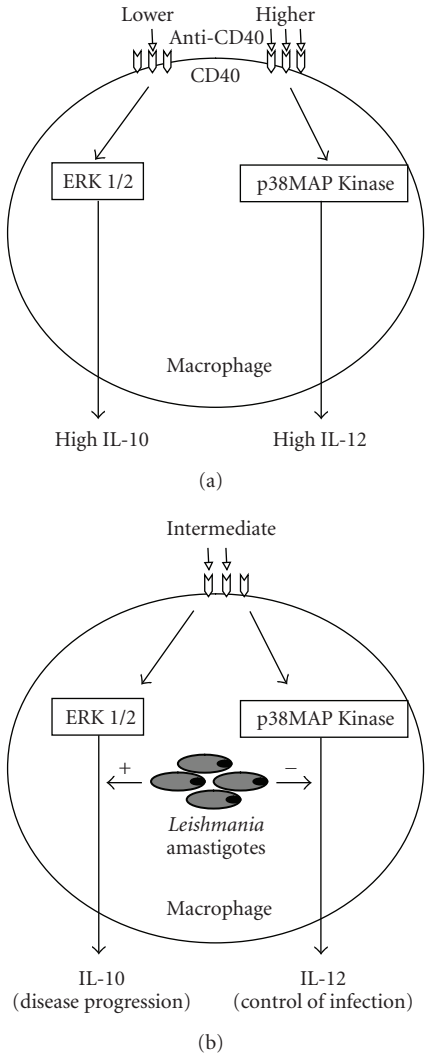

Figure 2: *Strength of CD40-CD40L interaction can influence the outcome of parasite infection*. (a) Cross-linking of CD40 with lower doses of agonistic anti-CD40 antibody (< 3 μg/mL) on noninfected macrophages increases phosphorylation of extracellular stress-related kinase 1/2 (ERK 1/2) and consequently stimulates high production of IL-10, whereas cross-linking with higher doses (> 3 μg/mL) increases phosphorylation of p38-mitogen activated protein kinase (p38MAPK) and therefore stimulates high production of IL-12. (b) Cross-linking of *Leishmania*-infected macrophages with intermediate dose of anti-CD40 antibody (3 μg/mL) leads to production of more IL-10 and less IL-12 than uninfected macrophages. This suggests the potential involvement of parasite factors in disease progression through stimulation of IL-10 production.

with anti-CD40 Ab on macrophages, this study indicated that the strength of CD40 signalling activates p38-mitogen activated protein kinase (p38MAPK) or extracellular stress-related kinase 1/2 (ERK-1/2) signalling molecules leading to a differential expression of IL-12 or IL-10. CD40 cross-linking at lower doses induces activation of ERK-1/2 and production of IL-10, a cytokine which promotes immunosuppression, whereas at higher doses it induces activation of p38MAPK and secretion of IL-12 (Figure 2(a)). How such strength of CD40 signalling is operating through interaction of the immunocompetent cells and whether it can influence the Th1 and Th2 immunoregulatory processes are still unclear. Interestingly, *Leishmania*-infected macrophages treated with an intermediate dose of anti-CD40 Ab produced more IL-10 and less IL-12 than uninfected macrophages (Figure 2(b)), suggesting that *Leishmania* infection promotes IL-10 production that would favour disease progression [47]. This study is in line with a previous report indicating that p38MAPK-dependant CD40 signalling is impaired in *Leishmania*-infected macrophages [35]. Overall, the involvement of CD40 signalling in the host-parasite interaction further exemplifies the refined nature of the host-pathogen crosstalk.

4. CONCLUDING REMARKS

Increasing evidence points towards the crucial role of CD40-CD40L for the development of a cellular host protective immune response against intracellular parasites. Although the role of CD40-CD40L signalling in B cell maturation and isotype switching is well documented, little is known about the potent stimulation of CD40 signalling that can promote humoral immune response against extracellular parasites. A study on African *Trypanoma* infection in a model of SCID mice reconstituted with a bovine immune system indicated that administration of an agonistic antibody against CD40 enhanced mice survival to infection with *Trypanosoma congolense*, and was associated with increased production of specific IgG [48]. Further studies aiming to depict accurately the CD40 signalling-dependant protective Th2 immunity against protozoan parasites are yet to be investigated. The stimulation of CD40 signalling by CD40L and its derivatives can be considered as a useful adjunct in a vaccine strategy against protozoan infections. Current knowledge in host-parasite interaction includes a breakthrough in the modulation of CD40 signalling brought about by using soluble CD40L, CD40L-transgenic microorganisms or small peptide mimetics of the CD40L. However, recent findings outlined the possible regulation of CD40 signalling by the parasite and therefore stressed the need of a further understanding of the host-pathogen crosstalk that could lead to novel approaches for disease control.

ACKNOWLEDGMENT

The author would like to thank Drs B. Vray, M. Goldman, Y. Carlier, and A. Allaoui for discussions and support.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 94971, 10 pages
doi:10.1155/2007/94971

Review Article

Regulatory Cells and Immunosuppressive Cytokines: Parasite-Derived Factors Induce Immune Polarization

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Received 27 November 2006; Accepted 19 March 2007

Recommended by Abdelali Haoudi

Parasitic infections are prevalent in both tropical and subtropical areas. Most of the affected and/or exposed populations are living in developing countries where control measures are lacking or inadequately applied. Although significant progress has been made in our understanding of the immune response to parasites, no definitive step has yet been successfully done in terms of operational vaccines against parasitic diseases. Evidence accumulated during the past few years suggests that the pathology observed during parasitic infections is in part due to deregulation of normal components of the immune system, mainly cytokines, antibodies, and immune effector cell populations. A large number of studies that illustrate how parasites can modify the host immune system for their own benefit have been reported in both metazoan and protozoan parasites. The first line of defense against foreign organisms is barrier tissue such as skin, humoral factors, for instance the complement system and pentraxin, which upon activation of the complement cascade facilitate pathogen recognition by cells of innate immunity such as macrophages and DC. However, all the major groups of parasites studied have been shown to contain and/or to release factors, which interfere with both arms of the host immune system. Even some astonishing observations relate to the production by some parasites of orthologues of mammalian cytokines. Furthermore, chronic parasitic infections have led to the immunosuppressive environment that correlates with increased levels of myeloid and T suppressor cells that may limit the success of immunotherapeutic strategies based on vaccination. This minireview briefly analyzes some of the current data related to the regulatory cells and molecules derived from parasites that affect cellular function and contribute to the polarization of the immune response of the host. Special attention is given to some of the data from our laboratory illustrating the role of immunomodulatory factors released by protozoan parasites, in the induction and perpetuation of chronic disease.

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1. INTRODUCTION

There is increasing evidence that immune mechanisms are involved in the pathogenesis of many parasitic infections. The initial stages of the disease are generally characterized by the induction of a nonspecific lymphoproliferation, which is believed to disrupt antigen recognition and interfere with protective immune responses. Paradoxically, in most cases a state of immunosuppression can be evidenced. This hyporesponsiveness to antigen-specific and polyclonal stimuli in chronic parasitic infections could be related to immunosuppressive cytokines (i.e., IL-10 and TGF-β) secreted by antigen presenting cells and regulatory T cells (Treg cells). A growing list of parasite-derived molecules able to exert immunomodulatory activities on the cells of the innate immunity leading to such polarized cytokine secretion has been reported [1, 2]. Interestingly, these immunosuppressive regulatory responses resulting from repeated exposure to pathogens and/or their released products have been postulated to be responsible for protection against inflammatory diseases such as allergy or autoimmunity leading to the germless theory of allergic diseases and the hygiene hypothesis [3, 4]. This has led investigators to search for parasite molecules which could be used as a new therapy for immunological disorders [5].

In fact, complex feedback loops could explain the properties of a suppressor activity seen in parasitic infections. For instance, the parasites cannot only induce the production of host immunomodulatory lipids, the best characterized being the endogenous eicosanoids, but are also able to synthesize/secrete their own glycoproteins and lipids, which in turn activate cells of innate immunity towards the anti-inflammatory cytokine response. This physiological microenvironment may favor the development of Treg cells. A number of excellent reviews were devoted to the Treg cells

and their physiological role at the level of peripheral self-tolerance, avoidance of autoimmune diseases, tumor, and infectious disease immunology [6–13].

The purpose of this minireview is to analyze some of the current data related to the regulatory components or processes originating from the parasite (protozoa and some helminth pathogens) that affect host cellular functions leading to an immunosuppressive state that counteract proinflammatory cytokine production which may lead to excessive host tissue damage. The outcome of a parasitic infection will depend on the final balance of the protective and pathological properties of the cytokine network.

2. INDUCTION OF SUPPRESSOR CELLS

Immunosuppression involves an act that reduces the activation or efficacy of the immune system. Some components of the immune system itself have immunosuppressive effects on other parts of the immune system, and immunosuppression may occur as an adverse reaction to treatment or other conditions like infection processes. The ability of parasites to survive in the immune hosts depends on a variety of escape mechanisms. One of these is the inhibition or the suppression of the immune responses of their hosts. Several possible explanations have been put forward, such as antigenic competition, acquired tolerance, as well as the possible blocking role of soluble antigens or circulating immune complexes. The release by the parasites themselves of excretory-secretory products, which have potent immunosuppressive activity, represents another possible explanation. Moreover, a large number of reports have described the presence of suppressor cells (T lymphocytes and macrophages) in humans and animals infected with various parasites.

For instance, during onchocerciasis due to a pathogenic human filarial worm, *Onchocerca volvulus*, a state of cellular immune unresponsiveness develops in patients with the generalized form of the disease [14]. Moreover, it has been reported that parasite antigens are present in breast milk of *O. volvulus*-infected women which activated cells that suppressed the proliferative response of autologous lymphocytes to mitogens and antigens, suggesting therefore that this may induce tolerance and/or suppression in infants born of infected mothers [15].

A number of years ago, we have shown that total antigens from *O. volvulus* (OVA) markedly inhibited the proliferation of normal human lymphocytes stimulated with polyclonal activators such as phytohaemagglutinin (PHA). The inhibition was not due to a cytotoxic effect of OVA and was not abrogated by removal of the adherent cell population. Interestingly, we showed that the in vitro response of normal human lymphocytes was suppressed by coculture with allogeneic or syngeneic lymphocytes, which had previously been exposed to OVA. A significant reduction of the suppression was however observed when OVA pretreated cells were depleted of T cells by centrifugation of E rosettes. Moreover, the passage of OVA through an immunoadsorbant column containing a monoclonal antibody to OVA epitope abrogated its immunosuppressive effect. These observations allowed us to postulate that a parasite antigen(s) was responsible for the induction of T suppressor cells [16]. Since the molecular mechanisms of suppressor cells were difficult to characterize, interest in such cells was lost.

Recent progress in the identification of $CD4^+$ T cell populations, together with the use of genetically modified animal models have led to significant advances in the understanding of the immunosuppression phenomenon at the cellular and molecular levels. The concept of T regulatory cells (Treg) suppressing immune responses via cell-cell interactions and/or the production of suppressor cytokines are currently well documented [6–9]. At least two main Treg cell populations were defined: "naturally" occurring regulatory T cells ($Foxp3^+$ $CD4^+$ $CD25^+$) and the "adaptive" regulatory T cells (e.g., T_R1 or T_H3) [10–12]. Although some controversy has been reported in the literature, evidence which accumulated over the years has undoubtedly shed light on the importance of Treg cells in health and disease. Thus, in the case of onchocerciasis, a series of reports has shown that the hyporesponsiveness in individuals with the generalized form of the disease is not due to a shift towards a T_H2 response. Rather, it results from *O. volvulus* antigen-specific T cells having a cytokine profile with no IL-2 and high IL-10 and TGF-β production similar to the adaptive Treg cells also known as T_R1 and T_H3 which suppress ongoing inflammation [17, 18]. Cloning procedures allowed obtaining T cell clones bearing T_R1 suppressor cytokine profile producing significant amounts of IL-10 but no IL-2 or IL-4 and expressing high levels of cytotoxic T lymphocyte antigen (CTLA-4) after stimulation.

Although the examination of T cell lines or clones derived from the peripheral blood mononuclear cells of infected individuals has the great advantage that the cells producing the cytokines can be accurately defined, in this case the cytokines derived from "neighboring" cells in vivo are no longer represented in the system. Nevertheless, the generation of T cell clones has provided valuable information on human responses in general and to infections in particular.

However, when considering a wide range of autoimmune and inflammatory manifestations, the mechanisms by which regulatory Treg cells exert their activity remain unclear. For instance, studies in vivo have demonstrated that regulation is dependent on cytokines such as IL-10 and TGF-β as well as the expression of CTLA-4 molecule [19, 20]. However, in vitro studies have shown that neither soluble cytokines nor CTLA-4 is required for the suppressive effects of Treg [21–23]. Moreover, it has been shown that regulation of the ileal inflammatory process resulting from *Toxoplasma gondii* in murine model is dependent on TGF-β producing intraepithelial lymphocytes suggesting therefore that these cells represent an essential component in gut homeostasis after oral infection with this parasite [24]. Furthermore, in the susceptible BALB/c mouse experimental model of filarial infection with *Litomosoides sigmodontis*, a parasite closely related to *Brugia* and *Wuchereria* species causing human lymphatic filariasis, the Treg cells have been shown to be responsible for susceptibility to parasite. However, although treatment of infected mice with antibodies to CD25 and

glucocorticoid-induced TNF receptor family-related gene reduced Treg activity and led to increased antigen-specific immune responses, this results in the significant reduction of parasite numbers, in vivo neutralization of IL-10 receptor, but did not restore the ability of the immune system to kill parasites, supporting the notion that Treg cells act in an IL-10-independent manner [25].

Nevertheless, a recent study has shown that in a murine model of coinfection with a gastrointestinal nematode parasite *Heligmosomoides polygyrus* and the blood-stage malaria parasite *Plasmodium chabaudi* increased levels of IL-10. This occurred in concurrent infections, whereas high levels of TGF-β were seen during *P. chabaudi* single infections [26]. Interestingly, anthelminthic drug treatment of mice before *P. chabaudi* infection reduced TGF-β levels and restored antimalarial immunity. Thus, induction/expansion of the suppressor function is a complex process depending on multiple factors, among which concurrent infections are highly prevalent in many endemic tropical and subtropical regions of the world.

Hyporesponsiveness also occurs during human schistosomiasis. In fact, the infection downregulates both T_H1 and T_H2 cytokines [27]. In more recent studies, it has been shown that in a mouse schistosomiasis, Treg cells and IL-10 inhibited T_H1 development [28] and egg-induced pathology [29]. However, a number of studies have pointed to the role of IL-10 and TGF-β (two cytokines being released by adaptive Treg cells) as immunomodulatory cytokines in helminth infections. Parasite-specific activation of natural Treg cells has been reported in mice *Leishmania major* infection [30]. The cells were positive for Foxp3, produce IL-10 in response to *Leishmania*-infected dendritic cells, and exerted strong suppressive activity in vitro. In fact, previous observations have shown that in the case of *L. major* infection of genetically resistant C57BL/6 mice which spontaneously heal their dermal lesions with persistence of latent parasites, $CD4^+$ T cells are the main producers of IFN-γ and IL-10 in the dermis, although $CD8^+$ T cells were also able to produce either cytokine with appropriate stimuli [31]. Similar to T_R1 cells, the majority of $CD4^+$ T cells in the dermis and a proportion of $CD4^+$ T cells in the draining lymph nodes were able to produce both IL-10 and IFN-γ. Thus, in the chronic sites of infection, the release of IL-10 and IFN-γ by T cells led to the establishment of a latency with persistence of low number of viable parasites within lymphoid tissue and skin lesion after self-cure.

Although the T cell network seems to play a key role in the immunosuppression process, the existence of other T-cell-independent mechanisms has been clearly demonstrated. Indeed, recent investigations have shown that helminth and protozan infections can elicit a myeloid population characterized as $Gr\text{-}1^+/CD11b^+$ cells that substantially impaired antigen-specific T cell responses [32–34].

The myeloid suppressor cell-induced immunosuppression is mediated by nitric oxide production (NO), a messenger known to be involved in diverse signaling pathways including smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, and destruction of microbes and tumor cells [35]. The participation of NO in the suppression of T cell activation has been reported in a number of biological systems (reviewed in [36]). In fact, NO production during toxoplasmosis in C57BL/6 mice has two opposite effects being protective against *Toxoplasma gondii* and downregulating the immune response, suggesting its possible contribution in the establishment of chronic infections [37]. In the case of *Trypanosoma cruzi*, previous studies have shown that IFN-γ and nonoxidative molecules (TNF-α and NO) could play a role in the control of *T. cruzi* infection in mice [36]. Furthermore, a series of experiments supports the notion that IFN-γ and TNF-α mediated activation of macrophages which leads to increased production of NO, and in turn suppresses T cell activation [38]. The involvement of NO in apoptosis of thymocytes and macrophages has also been documented [39, 40] and NO markedly inhibits the induction of IL-2 promoter, which can account for most of the reduction in IL-2 production, and weakly increases the activation of IL-4 promoter [41]. This mechanism could be involved in the downregulation of IL-2 gene expression observed during *T. cruzi* infection [42]. Therefore, it is likely that NO production during the initial phase of acute infections might participate in the clearance of parasites by macrophages, whereas its overproduction during the late phase of acute infection would account for the immunosuppression observed.

Although significant advances have being made regarding the Treg subsets, a recent exiting review pointed to the importance of B cells possessing regulatory functions and suggested these be called Breg cells (reviewed in [43]). Indeed, in addition to the pathogenic role of B cells, which produce autoantibodies that contribute to the development of autoimmune diseases, the existence of regulatory B cells capable of inhibiting inflammatory responses through the production of regulatory cytokines IL-10 and TGF-β has been demonstrated in a number of experimental models of chronic inflammation [43].

In the case of parasitic infections, a number of studies have reported several alterations in B cell functions. For instance, in the mouse model of *Schistosoma mansoni* infection, splenic B cells have been shown to proliferate in response to an oligosaccharidic antigen (see also the next section) and were triggered to secrete high levels of IL-10 [44]. Furthermore, spleen B cells from mice infected with the L3 larval stage of filarial nematode *Brugia pahangi* contributed substantially to IL-10 production that in turn downregulated the expression of B7 molecules on the B cell surface [45]. This led to the decrease of their efficiency as antigen presenting cells to $CD4^+$ T cells and restricting their expansion, suggesting therefore that B-cell-derived IL-10 could participate in the regulation of proinflammatory CD4 responses as in other various models of autoimmune diseases [46].

2.1. Induction of apoptosis in the host immune cells

Another mechanism leading to the homeostasis disorder in the host is the fact that the invading parasites can release factors which kill the cells of the immune system by

activating the cellular death machinery, thus inducing apoptosis. Therefore, apoptosis seems to represent a fundamental feature with particular relevance in the maintenance of protozoan infections [47]. Indeed, studies in experimental mouse *T. cruzi* infections have shown that apoptosis of T cells play an important role in the immunossupression that occurs during the acute phase of Chagas' disease [48]. In a canine model of acute Chagas myocarditis, Zhang *et al.* [49] have reported that programmed cell death (PCD) occurs in cardiac myocytes, endothelial cells, macrophages, interstitial dendritic cells, and lymphocytes, suggesting that parasite-derived factors could be responsible of the apoptosis observed in the immune cells. In the same way, a correlation between the extent of PCD and the level of suppression of $CD4^+$ T-cell proliferative responses was observed [50]. Moreover, it has been suggested that the upregulation of Fas and Fas ligand play an important role in the induction of $CD4^+$ T cell death. This pathway is able to control and modulate the immune response against *T. cruzi* [51].

Parasite-derived substances are believed to be key factors in the immunossupression phenomena observed by exerting a proapoptotic activity against some immune cells. Thus, it has been reported that the *T. cruzi*-secreted trans-sialidase (TS) was able to induce apoptosis features in cells of the immune system in vivo [52]. Furthermore, evidences reported support that TS is a virulence factor responsible for thymic alterations via apoptosis of "nurse cell complex" [53]. Moreover, the glycoinositolphospholipid (GIPL) from *T. cruzi*, in the presence of IFN-γ, induced murine macrophage apoptosis leading to increased parasite release from these cells [54]. Surprisingly, internalization of apoptotic T lymphocytes by macrophages increased the replication of *T. cruzi* amastigotes inside macrophages [55]. Another additional mechanism that may amplify the immune suppression process is the fact that apoptotic cells release their TGF-β during their suicidal act. They flip intracellular phosphatidylserine onto the outer leaflet of their membranes rendering them target of phagocytic cells signaling the macrophages to release significant amounts of TGF-β which in conjunction with IL-10 and PGE-2 may act as a feedback amplification loop mediating immune suppression.

2.2. PD-1 as active suppressor of T regulatory cells

Programmed death-1 (PD-1) [56], a member of the CD28 family, is an immunoreceptor tyrosine-based inhibitory-motif- (ITIM-) containing receptor induced on T, B, and myeloid cells upon activation in vitro [57]. Interaction of PD-1 ligands (PD-L1 and PD-L2, members of the B7 family) with PD-1 may lead to the inhibition of proliferation and cell division of activated T cells expressing PD-1. Thus, the absence of PD-1 induced proliferation of effector T cells in the adenovirus-infected liver and resulted in rapid clearance of the virus. The blockage of the PD-1 pathway can augment antiviral immunity [58]. Recent investigations have reported that PD-1/PD-L systems may play a role during parasitic infections. Indeed, it has been shown that PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis [59]. In fact when compared to wilt-type mice (WT), the PD-$L1^{-/-}$ and PD-$L2^{-/-}$ exhibited distinct disease outcomes following infection with *L. mexicana*.

PD-$L^{-/-}$ mice developed resistance, whereas PD-$L2^{-/-}$ showed exacerbated disease. Although both PD-$L1^{-/-}$ and PD-$L2^{-/-}$ produced similar levels of IFN-γ as the WT mice, the development of IL-4 producing cells was reduced in PD-$L1^{-/-}$ mice suggesting that impairment of T_H2 response due to PD-L1 deficiency could be related to increased resistance to *L. mexicana* infection. Furthermore, in the case of CBA/J mouse *Schisitosoma mansoni* chronic infection, the parasites can induce increased expression of PD-L2 on splenic $CD11c^+/B220^-$ dendritic cells leading to moderate morbidity [60]. Taken together, these observations suggest that PD-1/PD-L systems may play a role in negative regulation of immune responses during parasitic infections.

2.3. Parasite released molecules as immunoregulatory factors

2.3.1. Lipids

A number of reviews pointed to the importance of endogenous as well as parasite-derived lipids as immunoregulatory factors [2, 62, 63]. It is well known that eicosanoids such as prostaglandins PGE2, PGD2, and LipoxinA4 can act not only on antigen-presenting cells through defined or putative surface receptors and strongly modify their pattern of cytokine synthesis (IL-1, TNF-α, IL-12, and IL-10), but also on T cells at the level of IL-2 synthesis, IL-2 receptor expression, and cellular proliferation [2]. Glycoinositolphospholipids (GIPLs) are some of the major glycoconjugates present on the cellular surface of *Leishmania* [63] and different strains of *T. cruzi* [64]. *T. cruzi* GIPL blocks T cell responses induced by different polyclonal activators, the suppressive domain being assigned to the ceramide portion of the molecule. Indeed, purified GIPLs from *T. cruzi* inhibit in vitro $CD4^+$ and $CD8^+$ T cell proliferation induced by bacterial superantigen and anti-TCR;CD3 antibodies. The inhibition leads to loss of IL-2 responsiveness, with inhibition of CD25 expression on both $CD4^+$ and $CD8^+$ subsets [65].

Dendritic cells (DCs) are crucial in the initiation of the immune response and are distinguishable from the other antigen presenting cells by their highly efficient antigen presentation. DCs are specialized to acquire and process antigen in peripheral nonlymphoid sites, and to transport the antigen to the secondary lymphoid organs where the stimulation of naïve lymphocytes occurs. During their migration, DCs enter a process of maturation that determines whether adaptive immune response occurs and the nature of that immune response. Studies with the glycoinositolphospholipid (GIPL) from *T. cruzi* have demonstrated that this molecule led to a downregulation of human DC surface antigens, such as CD80, CD86, HLA-DR, CD40, and CD57 that are important for T cell activation [66]. These observations allowed investigators to propose a novel efficient mechanism leading to the alteration of DC function and maturation that may be used by *T. cruzi* to escape the host immune response. However, although these investigations are interesting, it is important

to remember that the GPI anchors express biological activities similar to those of lipopolysaccharides (LPS). Given the fact that LPS induces the maturation of dendritic cells, one would expect that *T. cruzi* -derived LPS-like substances could activate rather than inhibit DC maturation [67]. Therefore, the observations showing DC inhibition await further explanation. In this regard, it is noteworthy that GPI anchors and GIPLs from *T. cruzi* are potent activators of the human and mouse macrophage toll-like receptor 2 (TLR2) [68].

2.3.2. Polysaccharides

A number of saccharides (from oligosaccharides to complex polysaccharides) derived from parasites have been identified and were shown to be implicated in host cell signaling systems. Complex polysaccharides for example which are not digested by the macrophage lysosomal enzymes can be retained intracellularly for a long period of time, interfering therefore with the presentation of peptide antigens to T cells [69]. Moreover, these molecules can act directly on the cell of the immune system. For instance, lacto-*N*-fucopentaose III (LNFP-III) and lacto-*N*-neotetraose (LNnT), sugars of egg antigens of *S. mansoni*, also found in human milk were shown to participate in the T_H2 polarization and immune suppression. Indeed, intraperitoneal injection of LNnT-Dex into mice expanded a cell population, phenotypically defined as $Gr1^+/CD11b^+/F4/80^+$ producing high levels of IL-10 and TGF-β ex vivo [32].

$Gr1^+$ cells suppressed naïve $CD4^+$ T cell proliferation in vitro in response to anti-CD3/CD28 antibody stimulation. Suppression involved cell contact and was dependent on IFN-γ and NO, with a discrete role played by IL-10 [32]. Furthermore, LNFP-III stimulated splenic B cells from parasite-infected mice to proliferate and produce IL-10 and PGE2, two molecules known to downregulate T_H1 cells [44]. The major source of IL-10 was the B-1 subset ($CD5^+$ $B220^+$) [70].

Oligosaccharide structures from other parasites have been shown to modulate B cell activity. Indeed, the *T. cruzi* GIPL was found to be a stimulatory factor for B cells, inducing the production of IgG3 in the absence of any costimuli, the active portion being present in the oligosaccharide fraction [71].

In the case of *Leishmania* parasites, the major cell surface molecule, phosphoglycan (PG), has been shown to selectively inhibit the synthesis of IL-12 (p. 40, p. 70) by activated murine macrophages. The inhibition was dependent on the galactose (beta1-4) mannose (alpha1)-PO4 repeating units and not the GPI lipid anchor of lipophosphoglycan [72].

2.3.3. Polypeptides

In addition to lipids and polysaccharides, parasite-derived proteins and even small RNA molecules could interfere with the cell of the immune system. Thus, the glutathione-S-transferases (GSTs), which are ubiquitous housekeeping enzymes found in nearly all animals and some parasites, appeared to have immunomodulatory functions [73]. Indeed, the dimeric form of GST present in the excretory-secretory products of *Fasciola hepatica* exerted a significant inhibition of rat T cell proliferation in vitro and a downregulation of NO production by normal peritoneal macrophages. Furthermore, in *O. volvulus*, a novel type of GST possessing the characteristic of secreted protein has been identified. In fact, the parasite has two GSTs (ovGST1 and ovGST2), the ovGST2 functions as an intracellular cytosolic housekeeping enzyme, whereas the ovGST1 is found in the media surrounding adult worms maintained in culture, suggesting therefore that the enzyme is released from the parasite. Recent investigations have shown that *O. volvulus* extracellular GST produces PGD2, a known anti-inflammatory molecule [74].

In accordance with these findings, we have also demonstrated the ability of a *T. cruzi* released protein, Tc52, containing a tandemly repeated structure characteristic of glutathione S-transferases (GSTs) to induce nonspecific suppression of T lymphocyte activation [75]. Furthermore, our studies have provided evidence demonstrating that purified Tc52 acted directly on macrophages to increase IL-10 gene expression [76]. In addition, experiments carried out with murine macrophages harboring a eukaryotic plasmid carrying Tc52 gene showed increased IL-10 mRNA levels [77]. Moreover, using synthetic peptides spanning the amino terminal or carboxy-terminal domain of Tc52 protein, we found that the sequence encapsing the carboxy-terminal residues 432–445 when coupled to a carrier protein, ovalbumin, exhibited increased inhibitory activity on T lymphocyte activation and significantly downregulated IFN-γ and IL-2 secretions [78].

The in vivo immunomodulatory effect of Tc52 has been investigated in mice. Given that we have already established by genetic manipulation *T. cruzi* clones lacking a Tc52 protein-encoding allele ($Tc52^{+/-}$) [79], we decided to examine the disease phenotype in $Tc52^{+/-}$-infected BALB/c mice, during the acute and chronic phases of the disease. The results obtained are in agreement with the observations made when using in vitro experimental models. Indeed, these studies showed a reversion of the suppressive phenotype in vivo during the infection with mutant parasites lacking one Tc52 gene allele. Moreover, a lack of increased secretion of IL-10 correlates with decreased in vivo Tc52 production [80]. Therefore, it is reasonable to suggest that this reduction by gene targeting which in turn downregulates the IL-10 synthesis could be among the immunregulatory mechanisms operating during *T. cruzi* infection. It is tempting to speculate that Tc52-inducing increased IL-10 secretion might participate in the downregulation of IL-2 production. This is in agreement with previous studies showing that murine IL-10 can downregulate the host immune response by decreasing the production of IL-2 and inhibiting mitogen-driven T cell proliferation [81, 82]. Furthermore, the effect of Tc52 on the allergic airway inflammation induced by OVA in the BALB/c strain of mouse was evaluated. While the OVA challenge induced increased cellular infiltrates in the bronchoalveolar lavage fluid, simultaneous injection of Tc52 with OVA significantly reduced inflammation (Lamkhioued, personal communication).

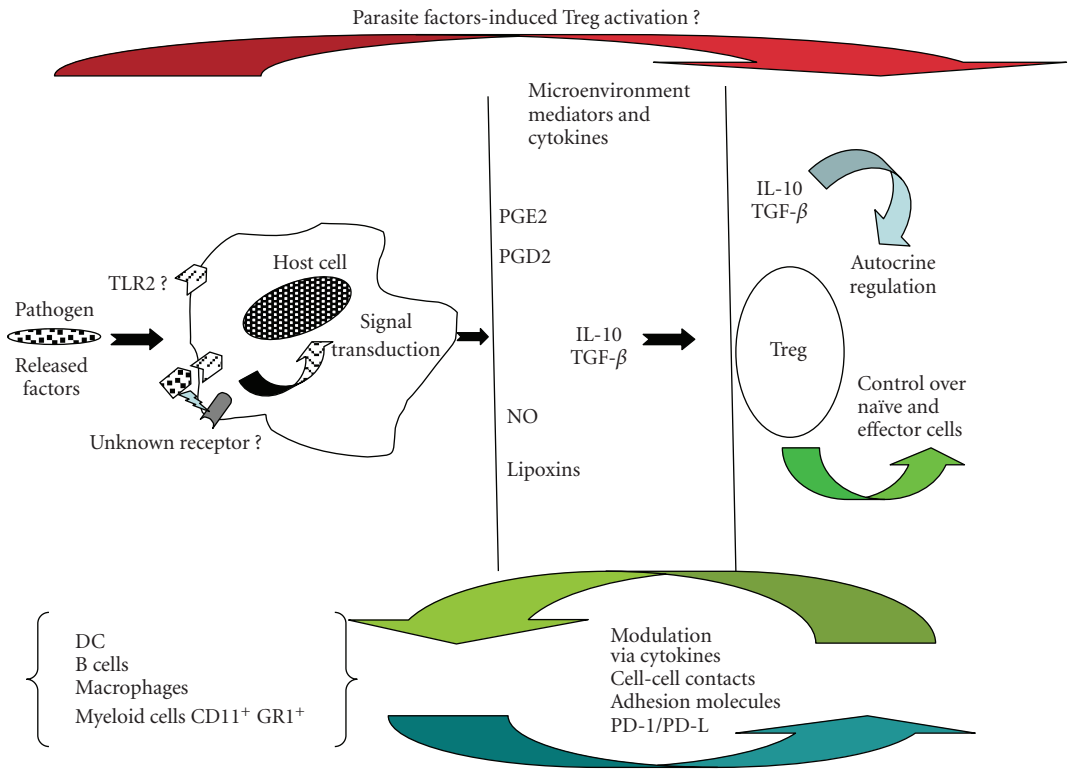

Figure 1: A model for parasite-derived factors-host cell interaction and signaling pathways.

In view of the fact that increased NO production by splenic macrophages has been involved in the suppression of lymphocytes proliferation in mice infected with *T.cruzi* [38], we have investigated whether Tc52 modified macrophage NO production. We first showed that Tc52 could be bound to the macrophage surface as evidenced by FACS analysis. Moreover, while Tc52 alone had no effect, addition of IFN-γ induced the production of high amounts of NO by macrophages correlating with increased levels of *iNOS* transcripts [76]. It is known that IFN-γ by itself is a relatively poor stimulator of NO production by macrophages and the addition of a second signal can significantly enhance NO production [83]. Since high levels of IFN-γ have been recorded during acute phase mouse *T.cruzi* infection, and that Tc52 could be detected in the blood of infected mice, it is reasonable to suggest that Tc52 may act as a "secondary signal" for NO secretion by macrophages in vivo, which in turn could modulate T cell function. This kind of mechanism also occurred in T. *brucei* infection. Indeed, it has been reported that NO-mediated suppression of T cells during *T. brucei* infection could result from a synergistic effect of soluble trypanosome products and IFN-γ on *iNOS* expression [84].

Modulation of *iNOS* gene and NO production by soluble proteins has also been reported in the case of *Entamoeba histolytica* [85].

Although Tc52 could induce the secretion of the suppressive cytokine IL-10 by macrophages, recent investigations have shown that the protein could trigger human and mouse DC maturation as evidenced by an upregulation of costimulatory surface antigens such as CD54, CD86, and HLA-DR molecules. Moreover, incubation of DC with Tc52 led to increased inflammatory chemokine synthesis (IL-8, monocyte chemoattractant protein-1, and macrophage-inflammatory protein-1α). Interestingly, binding experiments showed complex molecular Tc52-DC interactions that involved toll-like receptor 2 and Tc52 glutathione-binding site which mediated intracellular signaling, whereas another unidentified portion of the Tc52 molecule is involved in its binding to DC [86]. In fact, the Tc52 is made of two homologous domains comprising a glutathione binding site (G-site) and a hydrophobic C-terminal region (H-site). The molecule may act as a dimeric-like complex where the two "pseudo-subunits" areas are arranged in an antiparallel fashion separated by a strong β-turn motif (Ala225-Pro-Gly-Tyr228). The Tc52 G-site binds to TLR2, the other portion of the molecule, likely the H-site, interacts with a putative DC surface structure. Binding to the TLR2 activates the signaling cascade leading to NF-κB nuclear translocation and regulation of nuclear gene expression. It might be that the H-site interacts first with the still unknown DC surface structure, the membrane-bound receptor-Tc52 complex, then moves to reach the TLR2 and binds to it through the Tc52 G-site resulting in the activation of intracellular signaling cascades leading to NF- κB nuclear translocation and regulation of DC gene expression.

On first examination, the fact that Tc52 activity resulted in the induction of gene encoding both anti-inflammatory

(IL-10) and proinflammatory (IL-6, IL-8) mediators may appear paradoxical. Yet, in common with other physiological systems, it is apparent that a counter-regulatory mechanism is essential to provide the balance and regulation that are necessary to control the inflammation cascade. Within the site of an inflammation reaction, IL-10 produced locally would act to counter the stimulatory effects of IL-8 and IL-6, and thereby enable the balance to be established.

Other parasite-derived molecules which subvert immune regulation have been described: the *T. cruzi* antigen molecule SAPA (shed acute phase antigen) which exhibited a neuraminidase-transsialidase activity downregulated T lymphocyte proliferation as a consequence of T suppressor/cytotoxic cell activation and secretion of PGE2 [87]. A *T. cruzi* membrane glycoprotein inhibited the expression of IL-2 receptor chains and secretion of cytokines by subpopulations of activated human T lymphocytes [88] among others.

Moreover, parasite-derived polypeptides could act directly on B cells either as specific or nonspecific activators. Indeed, we have shown that in vivo treatment of mice with a flagellar Ca^{+}-binding protein, Tc24 from *T. cruzi,* induced a quick increase in the number of B cell secreted immunoglobulins of IgM isotype, suggestive of a mitogenic activity of Tc24 on B cells that is T cell independent [89]. Moreover, we have identified an *L. major* gene encoding a protein sharing significant homology to mammalian ribosomal protein S3a named LmS3a exhibiting dual activity being stimulatory and inhibitory towards T and B cells, respectively [90]. Analysis of cytokine production revealed a significant downregulation of IFN-γ, IL-2, and IL-12 secretion by LmS3a. These results are compatible with mitogenic induction of the immune system accompanied by a state of immunosuppression.

Another intriguing aspect in the parasite relationship is the fact that parasites could release factors that mimic host cytokines. For instance, (1) hydatid fluid fractions mimicked IL-1, IL-2, and IL-6 [91]; (2) an IFN-γ homolog that binds to the IFN-γ receptor and induced change in lymphoid cells has been identified in an intestinal nematode [92]; (3) two homologs of the human macrophage migration inhibitory factor (MIF) have been characterized in the human parasitic nematode *Brugia malayi* and termed Bm-MIF-1 and Bm-MIF-2, both having functional properties similar to the MIF human counterpart [93]; (4) *Toxoplasma gondii* releases cyclophilin-18 (C-18) that signals through the chemokine receptor CCR5 leading to the IL-12 synthesis by dendritic cells and a strong protective response [94]; (5) the tapeworm *Hymenolepis diminuta* has been shown to express an IL-12-like peptide, one of the suggested hypothesis being that the peptide could act as a competitive antagonist for the IL-12 receptor, thus contributing to the general immunosuppression [95].

Taken together, these examples raise the distinct possibility that the production of parasite factors that interact with cell surface receptors may be one mechanism whereby the parasite is able to interfere with the regulation of the induction/initiation phase of the host immune response that may protect the host from excessive inflammation and may potentiate the parasite's own survival.

2.4. Concluding remarks

The soluble parasite factors can elicit a complex series of cellular interactions leading to an immunosuppression state (Figure 1). The fact that immunoregulatory parasite-derived substances may have additional roles in driving early immunological events towards T_H2-type or anti-inflammatory responses has opened new areas of investigation looking for molecules that may represent novel potential therapeutic agents for the treatment of T_H1-mediated inflammatory and autoimmune diseases.

ACKNOWLEDGMENTS

Part of the studies described in this review was supported by grants from INSERM, IRD, and ACI Microbiology no. MIC 03 29. The author thanks M. John Bolger for editorial assistance.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 79401, 7 pages
doi:10.1155/2007/79401

Review Article

Regulation of Defense Responses against Protozoan Infection by Interleukin-27 and Related Cytokines

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Received 26 November 2006; Revised 2 February 2007; Accepted 26 February 2007

Recommended by Ali Ouaissi

Cytokine-mediated immunity is crucial in the defense against pathogens. Recently, IL-23 and IL-27 were identified, which along with IL-12 belong to the IL-12 cytokine family. IL-27 is pivotal for the induction of helper T cell (Th) 1 responses while IL-23 is important for the proliferation of memory type Th1 cells. Recent studies revealed that IL-27 also has an anti-inflammatory property. In some protozoan infection, various proinflammatory cytokines were over produced causing lethal inflammatory responses in IL-27 receptor-deficient mice. The anti-inflammatory effect of IL-27 depends, at least partly, on inhibition of the development of Th17 cells, a newly identified Th population that is induced by IL-23 and is characterized by the production of the inflammatogenic cytokine, IL-17. IL-27 thus has a double identity as an initiator and as an attenuator of immune responses and inflammation. With the discoveries of the new IL-12-related cytokines and Th17 cells, Th development is facing a new paradigm.

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1. INTRODUCTION

Immune responses against pathogens determine the course of infection as well as the pathogenesis of diseases caused by the pathogens. During the responses, immune cells, such as dendritic cells, macrophages, and T and B lymphocytes, are orchestrated by various cytokines produced by the immune cells themselves. For instance, during infections with some protozoa, such as *Leishmania major* or *Trypanosoma cruzi*, the importance of IFN-γ has been proven not only experimentally but also clinically [1, 2]. IFN-γ is produced mainly by helper T cell (Th) 1-differentiated $CD4^+$ T cells, a population induced by IL-12, although other types of cells, such as NK/NKT cells and $CD8^+$ T cells are also known to produce IFN-γ. While being critically important for the defense against pathogens, cytokines are sometimes notorious as causative agents for the development of immunopathology and inflammation, which may result in organ/tissue damage. TNF-α, a cytokine produced by Th1-type $CD4^+$ T cells and activated macrophages for defense against intracellular pathogens, may induce cell apoptosis or necrosis depending on situation. Therefore, it is essential that one should mount proper immune responses against pathogens, making sure the response is not too weak to eliminate pathogens or too strong to damage the host. Recently, two new cytokines, IL-23 and IL-27 have been identified as members of the IL-12 cytokine family. With IL-12, a cytokine known as the most potent Th1-inducing cytokine, the IL-12 cytokine family has been shown to be involved in various diseases such as infection, autoimmune diseases, and inflammations. This review will focus on the complicated roles of IL-27, as well as those of related cytokines, and their potential importance during some protozoan infections.

2. Th1 DIFFERENTIATION AND IL-12 CYTOKINE FAMILY

When stimulated through T-cell receptors, $CD4^+$ T cells proliferate and differentiate into either Th1 or Th2 cells, two functionally distinct subsets that produce characteristic cytokines, respectively [3]. Th1 cytokines, especially IFN-γ, are critical for the macrophage activation and nitric oxide production required for eliminating intracellular pathogens such as *L. major* [4]. In contrast, Th2 cytokines such as IL-4, IL-5, and IL-13 are important for inducing humoral immunity required to expel helminth from the digestive canal [5]. Th1 and Th2 cells develop from the same Th precursor (Thp or Th0) cells, but which subset differentiates in a given situation is driven by factors, particularly cytokines, in the surrounding microenvironment. IL-12 promotes IFN-γ

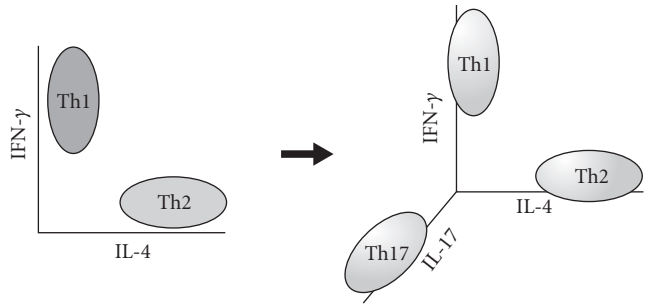

Figure 1: Th1, Th2, and Th17 differentiation. (Left) A traditional Th1 and Th2 differentiation model. Th1 population producing IFN-γ versus Th2 population producing IL-4 differentiate in a mutually exclusive manner. (Right) A new Th differentiation model. Additional population producing IL-17 (but producing neither IFN-γ nor IL-4) also differentiates from the same precursor cells.

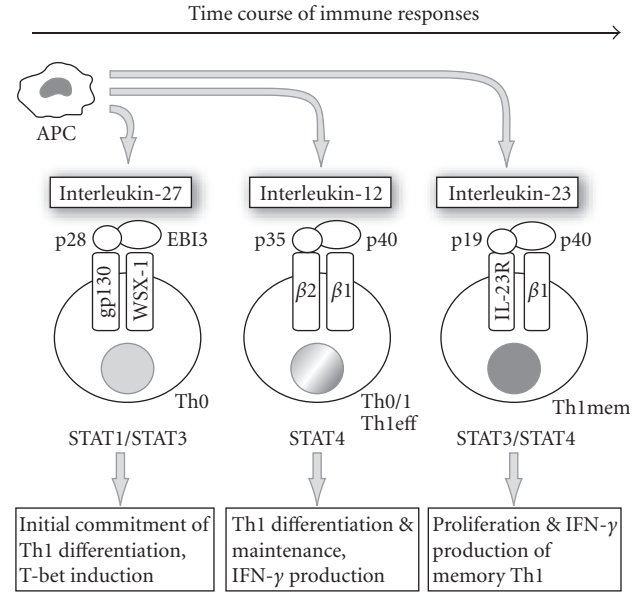

Figure 2: Differential roles and differential requirement of IL-12 family members during the time course of Th1 development (APC, antigen presenting cells; Th1eff, effector Th1 cells; Th1mem, memory Th1 cells).

production and Th1 development, whereas IL-4 binding to the IL-4 receptor promotes IL-4 production and Th2 development [6]. Developments of Th1 and Th2 are mutually exclusive and differentiation of one suppresses that of the other by various mechanisms. In addition to these two subsets, recent lines of evidence have shown that there is another Th subset, called Th17 or $Th_{IL\text{-}17}$ [7–9] (Figure 1). Th17 cells produce IL-17 for induction of various inflammatory responses and for defense against some pathogens [10, 11]. The differentiation of Th17 cells requires IL-6 plus TGF-β for commitment and IL-23 for development, while other cytokines such as IL-1 and TNF-α also support the development of Th17 [12–14].

IL-12 was originally identified as a potent inducer of IFN-γ production by T, NK, and other types of lymphocytes and was shown later to be a potent inducer of Th1 differentiation of $CD4^+$ T cells [15]. Until recently, IL-12 has been the only known heterodimeric cytokine, composed of two subunits, p35 and p40. The p35 subunit is homologous to IL-6 and G-CSF with a four-α-helix bundle structure, while the p40 subunit is homologous to the extracellular portion of IL-6Rα and related cytokine receptors. Thus, it was proposed that IL-12 evolved from a cytokine of the IL-6 family, which is covalently bound to the extracellular portion of its primordial α chain receptor [15]. Recently, IL-23 and IL-27 were identified as heterodimeric cytokines functionally and structurally related to IL-12 [16, 17] (see [18, 19] for review). Now along with two other cytokines, CLC/soluble CNTFR and CLC/CLF-1, IL-12, IL-23, and IL-27 compose a family of heterodimeric cytokines [18, 20]. IL-23, a heterodimeric cytokine composed of the IL-12p40 subunit and the IL-12p35-related molecule p19, preferentially acts on memory Th1 $CD4^+$ T cells for their proliferation [17], although IL-23 has recently been shown to promote Th17 differentiation. IL-27, another heterodimeric cytokine composed of the IL-12p40-related protein EBI-3 [21] plus the IL-12p35-related protein p28, acts on naïve $CD4^+$ T cells to induce the expression of the IL-12Rβ2 gene and make the cells responsive to IL-12. While IL-12 is the most potent inducer of Th1 differentiation and IFN-γ production acting on effector Th1 cells, chronologically differential roles and differential usage of IL-12, IL-23, and IL-27 have been proposed. First, IL-27 commits naïve $CD4^+$ T cells to differentiate into Th1 cells by inducing IL-12Rβ2, then IL-12 acts on committed effector Th1 cells for IFN-γ production, followed by IL-23 mediating the proliferation of memory Th1 cells [17] (Figure 2). While the Th1 immune response is pivotal for the host defense against pathogens including some bacteria and parasites such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *L. major* (reviewed by Romani et al. in [22]), as well as against tumors [23], excess of Th1 responses may cause some autoimmune diseases including rheumatoid arthritis and multiple sclerosis [24, 25]. It is of note that recent lines of evidence have shown that some of these "Th1-mediated" autoimmune diseases are actually mediated by IL-23-driven Th17 cell populations but not by IL-12-driven Th1 cell populations, as has been shown for experimental autoimmune encephalomyelitis, an experimental model for multiple sclerosis [7, 8] (see also [26, 27] for review). Nonetheless, proper initiation, reinforcement, and maintenance of the Th1 response seems to be ensured, and at the same time tightly regulated by related, but distinct, cytokines [28].

3. IL-27 AND ITS RECEPTOR, WSX-1, FOR Th1 DIFFERENTIATION

Sprecher et al. cloned an orphan cytokine receptor and named it WSX-1 (what is now called IL-27R α chain; also known as TCCR) after the WSXWS motif, a characteristic feature of cytokine receptors, in its extracytoplasmic portion

Table 1: Responses of IL-27/IL-27R-deficient mice to parasitic infection.

Mouse genotype	Pathogen	Helper T cell response	Outcome	Reference
WSX-1$^{-/-}$	*Leishmania major*	Initial Th1 impaired	Susceptibility to infection, normal Th1 at later phase	[33]
WSX-1$^{-/-}$	*Trypanosoma cruzi*	Th1, Th2, and Th17 enhanced	Increased hepatic immunopathology due to cytokine production	[45]
WSX-1$^{-/-}$	*Toxoplasma gondii* (acute)	IFN-γ high Proliferation high	Increased hepatic immunopathology	[46]
WSX-1$^{-/-(a)}$	*Leishmania donovani*	Th1 enhanced	Increased hepatic immunopathology	[47]
WSX-1$^{-/-}$	*Toxoplasma gondii* (chronic)	Th17 enhanced	Exacerbated encephalitis	[63]
EBI-3$^{-/-}$	*Leishmania major*	Initial Th1 impaired	Susceptibility to infection, enhanced Th1 at later phase	[34]
WSX-1$^{-/-}$	*Trichuris muris*	Th2 enhanced	Enhanced clearance	[35, 36]

[(a)]TCCR (another name for WSX-1)-deficient mice generated by Chen et al. [32].

[29]. WSX-1 binds to the gp130 (of IL-6R) to conform to a fully functional IL-27 receptor complex, downstream of which, STAT1 and STAT3 are activated by ligand binding [30, 31]. Although the function of WSX-1 was not clear in the original cloning paper, two groups including ours independently generated strains of mice with *WSX-1* (or *TCCR*) gene disruption and demonstrated that WSX-1 is critical for induction of Th1 responses and IFN-γ production. Chen et al. [32] demonstrated that *WSX-1*$^{-/-}$ mice showed impaired IFN-γ production and remarkable susceptibility to *L. monocytogenes*. In the report by Yoshida et al. [33], an impairment of Th1 development and IFN-γ production was also demonstrated in *WSX-1*$^{-/-}$ mice infected with *L. major*. Interestingly, impaired production of IFN-γ as only observed at early phases of *L. major* infection, and the IFN-γ production in *WSX-1*$^{-/-}$ mice was restored to the wild-type level at later phases of infection. In accordance with this "in vivo" observation, T cells from *WSX-1*$^{-/-}$ mice produced much less IFN-γ than wild-type cells during primary stimulation in the presence of IL-12 for Th1 differentiation, while fully differentiated *WSX-1*$^{-/-}$ Th1 cells produced a comparable level of IFN-γ upon secondary stimulation. These data, both "in vivo" and "in vitro," indicated that WSX-1 is required for the initial mounting of the Th1 response by naïve CD4^{+} T cells, but its role is later mitigated presumably by the IL-12 system in fully activated and differentiated Th1 cells. The importance of WSX-1 in Th1 differentiation was further substantiated by the discovery of IL-27, a ligand for WSX-1, as another Th1-inducing composite cytokine [16]. Early expression of p28, a component of IL-27, prior to that of IL-12 components, by activated dendritic cells also supported the idea that IL-27 was involved in the early phase of Th1 differentiation [16]. A report on the role of EBI-3, a subunit of IL-27, in which EBI-3-deficient mice showed susceptibility to *L. major* infection with impaired Th1 response also supported the importance of IL-27/WSX-1 in Th1 differentiation [34]. (See Table 1 for comparison.)

While resistance against *L. major* exclusively depends on proper Th1 responses, expulsion of *Trichuris muris*, a gastrointestinal nematode from digestive canals largely depends on Th2 responses. In our recent *T. muris* infection experiments [35, 36], *WSX-1*$^{-/-}$ mice successfully expelled the worm and failed to harbor chronic infection in contrast to wild-type mice. *WSX-1*$^{-/-}$ lymphocytes produced significantly less IFN-γ and much more IL-4 and IL-13 than wild-type cells in response to the worm antigen, reiterating that WSX-1 is important for the initial mounting of proper Th1 responses during infection. Another interpretation of the results, however, is also possible for the impairment of Th1 responses against *L. major* and *T. muris* infection. (See below in the following section.)

The molecular basis for the Th1-initiating property of IL-27/WSX-1 was then elucidated [37]. STAT1 was shown to bind to the cytoplasmic portion of WSX-1 in a tyrosine phosphorylation-dependent manner and following STAT1 activation by IL-27 stimulation, T-bet was activated to promote Th1 differentiation. The binding of STAT1 to WSX-1 is reasonable, since WSX-1 has pYEKHF motif at the conserved tyrosine residue, which shares amino acid similarities to the STAT1 docking site of the IFN-γR, pYDKPH [38, 39]. STAT1 activation per se by IL-27 stimulation has no direct effect on IFN-γ production but STAT1-mediated T-bet activation followed by IL-12Rβ2 expression was one of the critical events for Th1 differentiation. Other mechanisms, such as MHC class I augmentation, ICAM-1 expression, and suppression of the expression of GATA-3, a transcription factor pivotal for Th2 differentiation, were also involved for IL-27-mediated Th1 differentiation [40–42]. It is now clear that the molecular basis of IL-27/WSX-1-mediated Th1 initiation is the induction of T-bet, which is reminiscent of the finding that the IFN-γ stimulation of cells induces T-bet in a STAT-1-dependent fashion [43, 44]. Interestingly, even in the absence of WSX-1, T-bet was induced at later time points after T cell stimulation (our unpublished observation), presumably

due to the small amount of IFN-γ produced or a STAT1-independent mechanism yet to be determined [40]. This delayed induction of T-bet may explain the restoration of IFN-γ production and IL-12 responsiveness of *WSX-1*$^{-/-}$ T cells at later time points [45].

4. SUPPRESSION OF INFLAMMATORY RESPONSES BY IL-27

In sharp contrast to the finding that IL-27 is important for Th1 promotion, recent reports also demonstrated that IL-27 plays a regulatory role in immune responses. *WSX-1*$^{-/-}$ mice, when infected with *T. cruzi* or *Toxoplasma gondii*, showed remarkable sensitivity to the infection [45, 46]. This high sensitivity to infection was not due to impaired Th1 differentiation or IFN-γ production. Unexpectedly, over production of various proinflammatory cytokines including INF-γ, TNF-α, and IL-6 was observed in infected *WSX-1*$^{-/-}$ mice, followed by lethal liver necrosis. Rosas et al. also reported severe liver immunopathology in TCCR- (another name for WSX-1) deficient mice infected with *Leishmania donovani* [47]. Sources of the cytokines were not limited to T cells, but other types of cells such as NK/NKT cells and macrophages also produced various cytokines. In addition, *WSX-1*$^{-/-}$ CD4$^+$ T cells were more activated than wild-type cells in terms of cell cycle progression and expression of surface activation makers [46]. Similarly, *WSX-1*$^{-/-}$ NKT cells produced more of the proinflammatory cytokines, inducing lethal liver damages in Con A-induced liver damage experiment, which is an experimental model of viral or autoimmune hepatitis [48]. Although the development of NKT cells were normal in *WSX-1*$^{-/-}$ mice, these *WSX-1*$^{-/-}$ NKT cells produced more IFN-γ and IL-4 than wild-type NKT cells in response to Con A or α-galactosyl ceramide stimulation in vitro. When infected with *M. tuberculosis*, *WSX-1*$^{-/-}$ mice also suffered from liver damages and cachexia due to over production of inflammatory cytokines, such as TNF-α [49]. Interestingly, *WSX-1*$^{-/-}$ mice harbored significantly lower bacterial loads over wild-type mice due to higher amounts of TNF-α and IFN-γ. These results remind us of the fact that immunity against pathogens is a double-edged sword; it kills the pathogens in one way but may hurt the host in another way. Beside the observations described above, IL-27 actually suppressed cytokine production-activated CD4$^+$ T cells [31] in vitro. IL-27 also suppressed cytokine production such as TNF-α and IL-12 by macrophages [49, 50]. In these reports, IL-27 stimulation induced STAT3 activation and concurrently suppressed cytokine production by LPS-stimulated macrophages. Interestingly, IL-27 stimulation appeared less effective in macrophage suppression than IL-4 or IL-10. Stimulation of macrophages with IL-4, IL-10, or IL-27 resulted in differential gene induction, suggesting distinct suppression mechanisms by respective cytokines [50]. Wirtz et al. also reported the suppressive effect of IL-27 on monocytes and granulocytes reactive oxygen intermediate (ROI) production [51]. Administration of recombinant IL-27 to mice in the septic peritonitis model resulted in higher mortality than untreated mice due to reduced bacterial clearance by the down regulation of granulocyte/monocyte function. Collectively, these data revealed the novel role of IL-27/WSX-1 as an attenuator of proinflammatory cytokine production and cell activation of T cells as well as macrophages, to suppress excess of inflammation and/or to cease immune responses.

In this regard, a distinct (but not mutually exclusive) hypothesis on the impairment of Th1 responses in the *WSX-1*$^{-/-}$ mice should be described. Artis et al. reported an overproduction of IL-4 in *L. major*-infected *WSX-1*$^{-/-}$ mice and suggested that the overproduction of Th2 cytokines at early phases of infection reciprocally suppressed the following IFN-γ production and Th1 development [52]. Similarly, Artis et al. also reported that upregulation of Th2 responses is independent from Th1 impairment in *T. muris*-infected *WSX-1*$^{-/-}$ mice, claiming that primary role of IL-27 is the suppression of immune responses (including Th2 responses) but may not be the induction of Th1 differentiation (see [36] and see review by Hunter et al. [53]). Although reconciliation of the two seemingly conflicting ideas awaits further analyses of the role of IL-27 in various settings, a recent report by our group sheds light on this issue [31]. In this report, Yoshimura et al. demonstrated that IL-27 acts on naïve T cells for IFN-γ production while the same cytokine suppresses cytokine production by affecting fully activated cells. Therefore, the activation status of the cells may be the key determinant for the effects of IL-27.

The underlying mechanisms for this immune/inflammation suppression by IL-27 are not fully elucidated. It has been reported that IL-27 inhibited IL-2 production by CD4$^+$ T cells thereby regulating an excess of cell activation [54, 55]. In *WSX-1*$^{-/-}$ mice (or cells), however, not only IL-2 production but also the production of various types of cytokines were augmented [45]. Additionally, the source of cytokine production was not limited to CD4$^+$ T cells, but other types of cells were also affected by WSX-1 deficiency. IL-10 production was unexpectedly augmented similarly by WSX-1 deficiency [45, 46] and the so-called regulatory T cells (Treg) did not appear to be affected by the receptor deficiency [56]. A mechanism that collectively regulates cytokine production and/or cell activation should be taken into consideration.

5. Th17 AND IL-23 VERSUS IL-27

IL-23, another IL-12 cytokine family member, has recently been described to induce the differentiation of a new subset of Th cells, namely, Th17 [7, 8, 57]. Th17 cells differentiate from the same precursor cells as Th1 or Th2 cells, during which IL-6 and TGF-β plus IL-23 are required. Th17 cells exclusively produce IL-17, an inflammatogenic cytokine [58], but not IFN-γ or IL-4. IL-17 plays a pivotal role in the induction of some inflammatory diseases such as experimental autoimmune encephalomyelitis [7, 59], inflammatory bowel diseases [60], and rheumatoid arthritis [61]. It has also been reported that IL-17 is critically involved in some forms of infection, either by induction of inflammatory cytokines such as TNF-α or by recruiting neutrophils [10, 11], although not much is known about the role of IL-17 on the defense against parasite infection at this moment

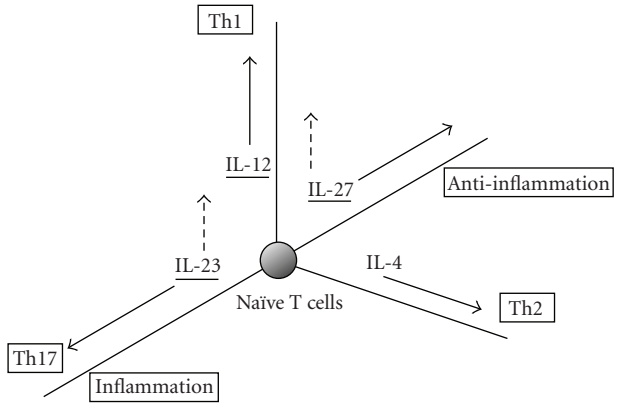

Figure 3: Multidimensional regulation of Th cell differentiation by IL-12 family members plus IL-4. Solid arrows show Th1, Th2, and Th17 differentiation axes induced by IL-12, IL-4, and IL-23, respectively, plus anti-inflammatory axis by IL-27. Dashed arrows show roles of IL-27 and IL-23 for Th1 induction. IL-12 cytokine family members are underlined.

[62]. A recent report by Stumhofer et al. revealed an intriguing function of IL-27 in view of its immune suppression [63]. In their report, Stumhofer et al. revealed that WSX-1-deficient mice chronically infected with *T. gondii* developed severe neuroinflammation with a prominent IL-17 response. We also reported that IL-27 receptor-deficient mice infected with *T. cruzi* produced more IL-17 in the sera than wild-type mice [31]. Although the precise role of IL-17 in pathogenesis of encephalomyelitis and infection-induced inflammation has yet to be elucidated, therefore, IL-17 produced in response to pathogens appears at least partially responsible for tissue damages induced by inflammation. In both reports and one by Batten et al. [56], IL-27 suppressed the development of Th17. It is interesting that IL-23, a member of IL-12 cytokine family, induces the development of Th17 for the induction of inflammatory responses while IL-27, another member of the family, inhibits the Th17 differentiation to suppress inflammation (Figure 3). Availability of IL-12 family cytokines plus other cytokines, such as IL-6 and TGF-β in the surrounding milieu, may determine the direction of immune responses toward either augmentation or regression. IL-27 thus may be a potential target for treating inflammatory diseases of infection and autoimmune origin mediated by IL-17. The molecular basis for the inhibition of Th17 differentiation is still unclear. While Stumhofer et al. and Batten et al. showed that the IL-27-meidated Th17 inhibition is dependent on STAT1 by taking advantage of STAT1-deficient mice, we demonstrated the requirement of STAT3 for this inhibition by using STAT3-deficient T cells. Although further elucidation of the molecular mechanisms for the IL-27-mediated Th17 inhibition is required, it would be reasonable to assume that appropriate balance of STAT1 and STAT3 activation downstream of the receptor determines the function of IL-27 signaling, as implicated by a transgenic mice study [64] or by examination of surface WSX-1 expression on naïve and activated T cells [65].

6. FUTURE PROSPECT

Since its discovery, IL-12 has been the only Th1-promoting cytokine for more than 10 years. It has been well recognized that the balance between IL-12-induced Th1 responses versus IL-4-mediated Th2 responses determines the immune responses against pathogens. Discoveries of two other IL-12-related cytokines along with the new Th cell population have been challenging and changing the paradigm. As discussed in this review, the IL-12 family members play differential roles in induction and maintenance of Th1 responses during the time course of infection/immune responses. IL-27 is critical in the commitment of Th precursor cells towards Th1 differentiation and IL-27 receptor deficiency resulted in impaired Th1 responses with high susceptibility to *L. major* infection. While having some overlapping functions, the members of the family also have distinct roles during immune responses. IL-23, one of the new comers to the family, augments inflammation by induction of Th17 cells, while IL-27, the other new comer, attenuates inflammation by suppressing cytokine production. These findings remind us of the intriguing fact that immune responses are elaborately regulated along the time course of infection to fight the dangerous pathogens and to simultaneously avoid hazardous damage to oneself. In this view, the multifaceted role of IL-27 is particularly notable. Although the induction of Th1 differentiation and suppression of cytokine production are seemingly conflicting, these distinct functions are quite reasonable when different target cells (naïve versus activated) and/or different time points (early versus late phase of activation) are taken into consideration. It would be premature to discuss the possibility of using IL-27 for treatment of disease because of the complexity of its role. However, further elucidation of the molecular mechanisms underlying the two distinct functions of IL-27 signaling as well as clarification of the situations where the two roles are differentially used will ensure that it will eventually be possible to use IL-27 either to reinforce defense against infectious agents or to treat infection-induced immunopathology and inflammatory diseases.

ACKNOWLEDGMENTS

The authors would like to thank members of the "Project W" for helpful discussion. The studies described in this review were supported in part by grants from the Ministry of Education, Science, Technology, Sports, and Culture of Japan (H. Yoshida, S. Hamano, and Y. Yoshiyuki), from the Japan Research Foundation for Clinical Pharmacology (H. Miyazaki), from the Sumitomo Foundation, a grant for Basic Science Research Projects (H. Yoshida), from the Naito Foundation (Y. Yoshida), and from the Takeda Science Foundation (H. Yoshida). S. Wang is a recipient of the Japan Society for the Promotion of Science (JSPS) postdoctoral fellowship for foreign researchers.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2007, Article ID 96410, 4 pages
doi:10.1155/2007/96410

Research Article

Enhancement of a T_H1 Immune Response in Amphotericin B-Treated Mucocutaneous Leishmaniasis

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Received 6 December 2006; Revised 2 February 2007; Accepted 7 March 2007

Recommended by Ali Ouaissi

In an attempt to investigate the effects of treatment of human leishmaniasis, the cytokines produced by peripheral blood mononuclear cells (PBMCs) of patients with cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) under treatment with amphotericin B were determined during the active disease from cocultures of cells and *Leishmania (Viannia) braziliensis* antigens. PBMC of these patients exhibited a nonsignificant marginal increased production of TNF-α upon antigen stimulation. However, under the same antigenic stimulus, patients with active MCL presented higher IFN-γ production compared to patients with CL. This increased IFN-γ production was accompanied by a drastically augmented IL-12 synthesis from cells of MCL patients. The highlighted T cell responses could be relevant for sound control measures of protozoan infections with emphasis on the combined usage of immunoenhancing agents and antiprotozoal drugs.

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1. INTRODUCTION

Leishmaniasis is a vector-borne disease caused by obligate intramacrophage protozoan parasites of the genus *Leishmania* [1, 2]. The infecting *Leishmania* species determines the clinical presentation of disease, of which there are three dominant clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis [1, 2]. In Bolivia, the etiological agent of both, CL and MCL is *Leishmania (Viannia) braziliensis*, formerly known as the *L. braziliensis* complex [3]. While CL is characterized by single or multiple ulcerated dermal lesions, MCL which develops as a complication of *L. (V.) braziliensis* CL in 5%–20% of patients [4] from parasite dissemination to the upper respiratory tract mucosa, involving the nasal, pharyngeal, and laryngeal mucosa, leads to extensive tissue destruction [5, 6]. CL either heals spontaneously or promptly responds to antimonial therapy but MCL usually evolves chronically and is difficult to treat [7]. Then, amphotericin B (amB) is an alternative for patients who fail to respond to pentavalent antimonial therapy.

It has been known that amB potentiates the antimicrobial and tumoricidal activities of macrophages [8], either directly [9] or via induction of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), as well as generation of a respiratory burst [10, 11]. Apart from these effects, little is known about the mechanisms associated with the efficacy of this compound in the treatment of MCL. Therefore, it was of interest to determine the participation of other soluble factors, apart from TNF-α, in amB-treated mucocutaneous leishmaniasis, keeping in mind that activation of the infected macrophages to kill intracellular parasites is carried out through a cell-mediated response that requires the classic features of antigen presentation and production of IL-12 by macrophages and activation of T_H1 lymphocytes with production of interferon-γ (IFN-γ) to activate the macrophages. The present study was aimed at elucidating the participation of critical soluble factors associated with amB treatment that could alleviate, in future, the collateral effects of this arduous treatment by combining immunochemotherapy with lower doses of drug.

In the present investigation, we present evidence for an exacerbated T_H1 immune response in MCL treated with amB, manifested by an elevated synthesis of IFN-γ which directly relates to a great increase in IL-12 production.

2. MATERIALS AND METHODS

Patients

Twenty four leishmaniasis patients were included in this study, 12 with CL and 12 with MCL, including male and

female, average age 30 years old. All of them acquired the disease in the Yungas Valley of La Paz Department, an endemic area for *L (V.) braziliensis* infection. Patients included in the study presented clinical features compatible with CL or MCL, were positive in both the Montenegro skin test and the serology for *L. (V.) braziliensis* antibodies (indirect immunofluorescence). At the moment of taking the blood samples, MCL patients were being treated with amphotericin B, at a dose of 1 mg/kg/day by infusion till a total dose of 1 to 3 grams, and had received mean doses of 7.5 (5–10 doses). CL patients were not receiving treatment when blood samples were taken. Informed consent was obtained from each participating donor before taking blood samples.

Antigens

The parasite lysate (ALb) utilized for cytokine production was obtained from an *L. (V.) braziliensis* strain (MHOM/BR/75/2903). The promastigotes were resuspended in phosphate-buffered saline (PBS) pH 7.2, at a concentration of 1×10^8 parasites per mL, and soluble antigens were prepared through seven cycles of freezing (−70°C) and thawing (37°C) the parasite suspension. This material was assayed for protein content, aliquoted, and stored at −70°C until used.

Culture of PBMC

PBMCs were purified by centrifugation (400 g, 20°C, 45 minute) over a mixture of Ficoll Hypaque at a density of 1.077 (Sigma, St. Louis, Mo, USA). After washings with serum free medium, the cells were resuspended at the desired concentration in RPMI medium containing 10% heat-inactivated human AB serum (Sigma), 100 IU of penicillin per mL, and 100 μg of streptomycin per mL (complete medium). Fresh PBMCs were cultured in duplicate in 24 well plates at a final concentration of 1.25×10^6 cells/mL in 2 mL complete medium for 3days (37°C, 5% CO_2), in the absence or presence of ALb, at a final protein concentration of 15 μg/mL.

Cytokine assays

Aliquots of cell-free supernatants from ALb in vitro-stimulated PBMC cultures were assayed for TNF-α, IFN-γ, and IL-12 by means of solid phase sandwich enzyme linked immunosorbent assays (ELISAs) (BioSource Europe, Belgium). All samples were tested in duplicate and cytokine concentrations were determined by comparison to standard curves. The sensitivity of each assay was as follows: TNF-α, 3 pg/mL; IFN-γ, 0.03 IU/mL; and IL-12, 1.5 pg/mL.

Statistical analysis

Statistical analysis was performed by the Wilcoxon nonparametric test using the Systat software, version 10.2 (Systat Software Inc., Richmond, Calif, USA). The level of significance was set at $P < .05$.

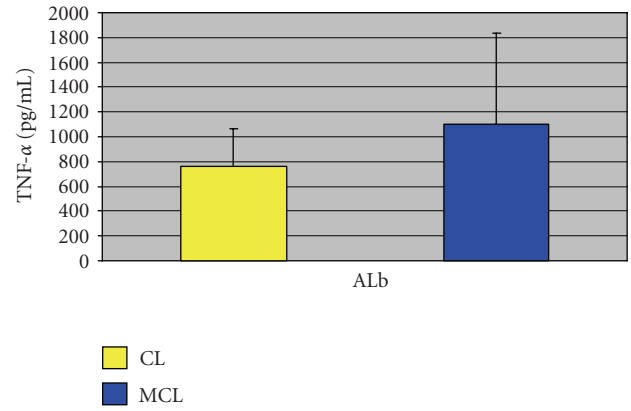

Figure 1: TNF-α production in cell-free supernatants of CL and MCL patients' PBMCs measured by ELISA upon ALb stimulation.

3. RESULTS AND DISCUSSION

The course of MCL has been associated with an unmodulated high production of the proinflammatory cytokines IFN-γ and TNF-α [12]. Considering the high activity of amB in the treatment of MCL, we decided to compare specific cytokine production between PBMC from CL and MCL patients, through an in vitro cell culture approach with ALb, that would recreate the status of patients' immune response.

Cytokine production by PBMCs from CL and MCL patients

The response of PBMC induced by ALb stimulation was evaluated in terms of TNF-α, IFN-γ, and IL-12 production, at 3 days of culture. While a 72-hour culture period has proven sufficient to stimulate production of TNF-α and IFN-γ in patients' PBMCs stimulated with leishmanial antigens [12], IL-12 production is specifically stimulated as early as 24 hours of PBMC culture from MCL patients [13].

Whatever the patient group, TNF-α, IFN-γ, and IL-12 were released at similar background levels. Therefore, in this study, the levels of cytokine released in unstimulated PBMC cultures did not reflect an activated state from contact in vivo with parasite antigens. Figure 1 reveals a nonsignificant slight increase in the production of TNF-α in MCL (1104 ± 732 pg/mL), comparing with CL (760 ± 307 pg/mL) patients ($P = .1$).

The reduced liberation of TNF-α in the supernatants of MCL patients is surprising considering that amB has been associated with its production [14] but it also reflects the beneficial effect of this drug in MCL as it has been reported that refractory mucosal leishmaniasis can be successfully treated through a combination of pentavalent antimony plus pentoxifylline, an inhibitor of TNF-α production [15]. Alternatively to the activation of macrophage microbicidal capacity through the induction of proinflammatory cytokines, amB can also exert its effect intracellularly. Intracellular accumulation of the drug in monocytes augmented the capacity of the cells to kill ingested *Candida albicans* [16]. However,

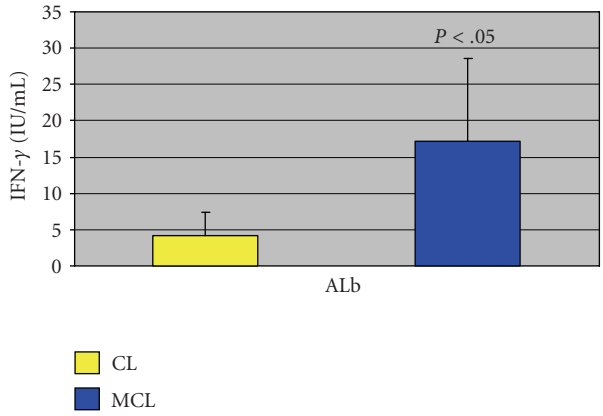

Figure 2: IFN-γ production in cell-free supernatants of CL and MCL patients' PBMCs measured by ELISA upon ALb stimulation.

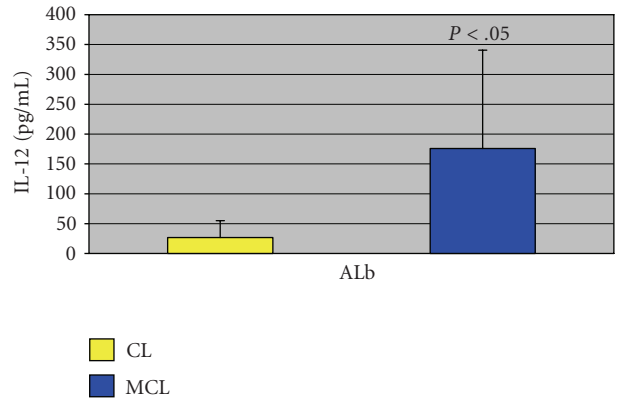

Figure 3: IL-12 production in cell-free supernatants of CL and MCL patients' PBMCs measured by ELISA upon ALb stimulation.

whether this mechanism of action is in fact operating in MCL treatment remains to be confirmed.

Contrary to the production of TNF-α, samples from MCL patients had the capacity to significantly augment synthesis of IFN-γ with regard patients with CL (17.1 ± 11.5 versus 4.2 ± 3.2 IU/m) [$P < .05$] (Figure 2). Furthermore, the observation of background levels of IL-4 despite stimulation with ALb (not shown) evokes a T_H1-type immune response.

Additionally, and in direct correlation to the increased production of IFN-γ, there was a much higher concentration of IL-12 when comparing MCL (175.7 ± 164.8 pg/mL) and CL (26.8 ± 28.2 pg/mL) patient cytokine responses ($P < .05$) (Figure 3). Future studies in vitro with amB will seek to verify the cellular source of IL-12 considering that, in human, peripheral blood monocyte/macrophages are the main producers of IL-12 [17]. A previous study [18] reporting suppression of IL-12 production by murine macrophages infected with *L. mexicana* amastigotes on interaction with T_H1 cells is of particular interest in the context of this study as it adds incentive to unveil through the present experimental system (ALb stimulation) the intracellular signals, likely set in motion by amB treatment, to increase production of IL-12, one of the two cytokines most clearly needed for protection in leishmaniasis.

In general, the present results are reminiscent of a previous investigation on the use of a recombinant leishmanial antigen from *Leishmania braziliensis* [13]. Apart from the production of IL-2, this antigen elicited also the production of IFN-γ dependent on IL-12, from PBMCs of patients with mucosal and cutaneous leishmaniasis. By analogy to our observations, it could be postulated that amB treatment would favor, preferentially, processing of antigens inducing T_H1-type immune responses, whereby stimulation of antigen-presenting cells by IFN-γ leads to IL-12 production, potentiating in this manner, a positive feedback loop. Even though amB killing of *Leishmania* parasites does not require a host immune response [19], we reason that similar targeting of the T_H1-cell mechanism might increase its efficacy and permit lower doses to be used with compatible activities.

An important aspect to consider in the present study is that cytokines are being compared in two patients groups differing by two parameters, MCL versus CL and treated versus untreated patients. Hence, there would be the possibility that the increase in IFN-γ and IL-12 relates to the different clinical forms rather than to amB treatment. However, it is interesting to note that samples from three MCL patients, not under treatment and excluded from this study, had the capacity to produce lower levels of IFN-γ compared with treated patients, and this production was not associated with an increased release of IL-12.

Therefore, manipulation of the host's immune response in favor of the T_H1-cell-associated mechanism may provide the opportunity to use amB-sparing regimens with lower doses of drug, fewer injections, and/or a shorter treatment duration, avoiding toxicity associated with the cumulative dose. Future studies will seek to improve understanding on the mechanisms of action of amB at the cellular level, particularly those associated with increased IL-12 production.

ACKNOWLEDGMENTS

Funding for this work was provided by Sustainable Sciences Institute, San Francisco, California, and by the French Embassy in La Paz, Bolivia.

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