

Review articles

The role of toll-like receptor agonists in the immunotherapy of leishmaniosis. An update and proposal for a new form of anti-leishmanial therapy.

Subhajit Dasgupta[1], Sanaz Aghazadeh-Dibavar[1], Mausumi Bandyopadyay[2]

[1]Department of Microbiology and Immunology, Saint James School of Medicine (Anguilla Campus), Albert Lake Drive, The Quarter, A-I-2640, P.O. Box 318, Anguilla
[2]Storm Eye Institute, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425, USA

Corresponding author: Subhajit Dasgupta; e-mail: subhajitdasgupta@outlook.com

ABSTRACT. The use of toll-like receptor agonists in immunotherapy is a new approach in the prevention of immunosuppression during fatal *Leishmania* parasite infection. The objective of such immunotherapy is to activate specific cell-mediated immune responses, macrophage activation and antigen-responsive inflammation, to kill intracellular amastigotes. Toll-like receptor agonist-based treatment in immunocompetent hosts can be effective either by selective use of the agonists alone or in combination with the anti-leishmanial drug stibanate. Recent investigations suggest that toll-like receptor signal pathways constitute a possible new mode of anti-leishmanial treatment. This article describes the prospect of toll-like receptor – mediated signal pathways in the immunotherapy of cutaneous and visceral leishmaniosis, as well as post kala-azar dermal leishmaniosis (PKADL), a skin-sequel of visceral infection. Suitable synthetic agonists need to be developed for toll-like receptors to overcome immunosuppression.

Key words: TLR, *Leishmania*, immunotherapy, resiquimod

1. Toll-like receptors in microbial infection

Toll-like receptors (TLRs) are the innate immune responders of mammals. The unique feature of TLRs is that, these versatile groups of receptor proteins respond to selective microbial Pathogen-Associated Molecular Patterns (PAMPs) [1–3]. The sequence and molecular structure of the ligands or agonists determine TLR activation and the subsequent signal pathways in the cells of different tissues. TLR expression has been found in myeloid and lymphoid progenitor derived cells, as well as natural killer (NK) cells. Their presence indicates the importance of TLR-signals in maintaining a bridge between innate and adaptive immune systems, although the mechanisms of action are not yet known. In addition, TLR-signal pathways play a significant role in brain development [4–7].

The TLRs in mammals are analogous to fruit fly *Drosophila* Toll protein, which confers antifungal properties to the fly [8,9]. The first discovered TLR was TLR1, attached to the interleukin 1 receptor (IL1R) [10]. The TLRs were divided into two groups: (A) intracellular TLRs, like TLR 3, 6, 9, 7, 8 and (B) extracellular TLRs, such as TLR 1, 2, 4, 5 [10,11]. The uniqueness of these TLR responses critically depend on ligand specificity, receptor-ligand interaction, and the type of cell signal events that eventually induce downstream activation of transcriptional regulators to promote inflammatory responses (Fig. 1). Though the TLRs are expressed in almost all mammalian cells, their mode of action varies depending on the cell and tissue.

The TLR-mediated immune responses involve upstream activation of multiple signal cascades, which lead to the induction of downstream nuclear factor κB (NF-κB) and AP-1 activation [3,12,13].

The structure of TLRs includes Leucin-rich repeat sequences. These sequences recognize molecular pattern specific ligands and trans-

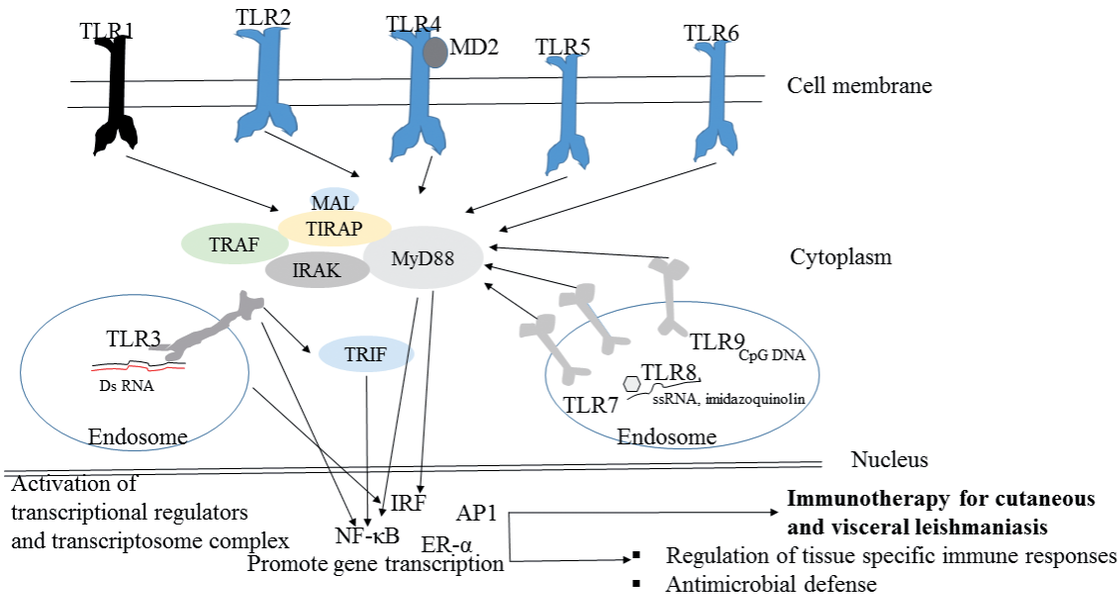

Fig. 1. **Signal events of extracellular TLR 2, 4, 5, 6 and intracellular TLR 3, 7, 8, 9.** The extracellular and intracellular toll-like receptors (TLRs) induce signals through activation of MyD88 adaptor protein and intermediate MAL/TIRAP, TRAF, IRAK signal adaptor protein molecules. The TLR3 signal pathway includes TRIF. The TLR-mediated signals differentially induce downstream activation of NF-κB, AP1, ER-α and IRF to promote gene expressions. The immunotherapy of cutaneous and visceral leishmaniosis depends on selective activation of TLR-signals for regulation of tissue specific immune responses and antimicrobial defense.

membrane TIR (Toll/Interleukin 1 receptor 1) domain. The TIR domain is linked with the adaptor molecules TIRAP, MyD88, IRAK4 and IRAK 1/2 [2,13,14]. Inhibition of MyD88 activation remarkably reduces TLR-mediated inflammation. Interestingly, intracellular TLR3-mediated cell signal events have been found to interact with TRIF, but not MyD88, in the upstream signaling pathway linking IRF transcription regulators to produce antiviral type 1 interferon [10,13]. The downstream of TLR3-mediated signal event is partially related with MyD88 dependent TLR4 signal pathway connecting activation signals for NF-κB as well as IRF3. The TLR3 agonist polyIpolyC (pIpC) induces mRNA expression of inducible nitric oxide synthase (iNOS) in macrophages [15,16]. The observations suggest that pIpC has the ability to cross-signal through TRIF and MyD88 which, in turn, activates the IRF-mediated signal pathway, or the down-stream cytoplasmic MAPK pathway, to activate NF-κB or AP1. The screening of agonists for TLR2 and TLR3, is thus an important part of determining a successful antimicrobial defense approach.

Recent advancements also highlight the importance of the TLR signal in inducing the formation of noncoding microRNA (miR-155) [17], which is TLR inducible and in turn modifies the function of MyD88. Apart from an inflammatory response, induction of miRNA provides an important modulatory mechanism in TLR function.

1.1. Impact of toll-like receptors in cutaneous leishmaniosis

Cutaneous leishmaniosis (CL) is manifested as nodular skin lesions in patients. The disease is endemic in Central and South America, Africa, the Middle East and Mediterranean regions [18–20]. It is caused by the blood-borne parasites *Leishmania tropica* and *Leishmania major* as well as a wide range of other species and subspecies. Although the mechanism of localized immune responses to CL-causing is not yet clear, a number of studies suggest that, the parasite and nature of cell-mediated immune response against the CL parasite antigen in skin nodular lesions has a distinct character, which vary between muco-cutaneous (MCL) or diffuse-cutaneous (DCL) leishmaniosis. The classic cutaneous leishmaniosis is self-healing and is associated with strong T cell-mediated immune responses following infection [21,22]. The persistence of *Leishmania* infection in CL depends

on ability of the *Leishmania* parasite to survive within macrophages and skin-tissue derived dendritic cells (Langerhan's cells). The self-healing infection persists for long periods in the skin as nodular lesions.

Recent investigations suggest that TLR2 and TLR4 agonists play a protective role in cutaneous leishmaniosis. A number of studies on American Cutaneous Leishmaniosis (ACL) indicate that extracellular TLR2 agonist plays a protective role under active disease conditions [23–25]. Cezario et al. [26], as well as other studies [27,28], demonstrated an increase in mRNA expression of TLR 2 and 4, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) to be associated with parasite infection. These results indicate that, selective agonist-mediated TLR 2, 4 activation is a potential strategy for the development of anti-leishmanial vaccine. Recently, Raman et al. demonstrated a synergistic application of toll-like receptor 4 and 9 agonists with leishmanial antigen in effective anti-leishmanial immune responses [29].

In the search for possible therapeutic interventions with minimal side effects, the discovery of combination therapy of stibanate with TLR3 synthetic agonist polyIpolyC is seen as a vital step towards effective immunotherapy [30]. Likewise, the use of the bacterial membrane component muramyl dipeptide (MDP) as an immunoadjuvant is another important step in the development of more effective immunotherapy in animal models [31]. The activation of natural killer T cells (T cytotoxic cells) through a TLR-mediated signal is a classic stimulus to induce the killing of intracellular parasite loaded macrophages, thereby demonstrating the importance of TLR agonists in prevention of leishmaniosis [32]. In the context of searching TLR agonists the TLR 7/8 ligand, imidazoquinoline compound imiquimod or resiquimod, has been recognized as a potential anti-microbial agent. Imiquimod is approved by the FDA for topical treatment of genital warts [33,34] as well as in the treatment of leishmaniosis. Selective use of

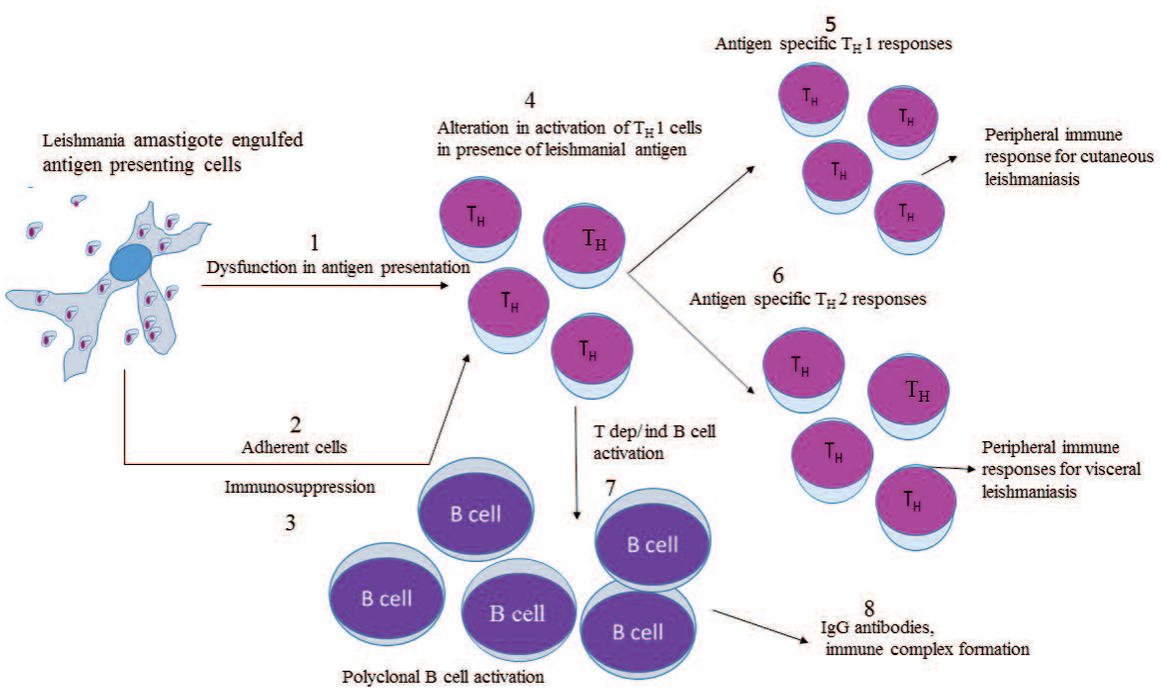

Fig. 2. Multifunctional immune responses following infection of leishmania parasites. Intracellular growth and multiplication of *Leishmania* parasites develop alteration in functional immune responses in immunocompetent hosts. There are different steps in antigen specific immune responses, alteration in any or more than one stage generate dysfunction in protective immune response to prevent infection. 1. Dysfunction in antigen presentation by antigen presenting cells; 2. Presence of adherent cells having suppressor cell property; 3. T-helper cell (T_H)-dependent immune responses include antigen specific and generalized immunosuppression); 4. Alteration in activation of T_H1 cells; 5. Antigen specific T_H1 cell responses; 6. Antigen specific T_H2 cell responses; and 7. T dependent as well as independent B cell activation. The polyclonal B cell activation and T cell-mediated peripheral immune responses are found during progression of leishmaniosis.

TLR agonists exerts an anti-leishmanial effect with public health benefits. In cutaneous leishmaniosis, the topical use of TLR agonists is a noninvasive choice of immunotherapy.

1.2. Impact of toll-like receptors in visceral leishmaniosis

Unlike cutaneous leishmaniosis, visceral leishmaniosis (VL) is a fatal progressive disease caused by the Phlebotominae sandfly vector. Immunosuppression of the *Leishmania* specific antigen is a major event during progression of the disease [35–38].

Leishmania antigen-specific lymphoproliferative responses have been demonstrated by many investigators suggesting intrinsic impairment of T helper type 1 cell activation during progression of illness. *Leishmania donovani* infection eventually results in two different patterns of immune response: antigen specific immune suppression and antigen unspecific generalized immunosuppression. A longitudinal study using a hamster model indicated an overall decrease in lymphoproliferative responses with progressive *L. donovani* infection [38–41]. Adherent immune cells of macrophage origin have been shown to impair cell-mediated immune responses: their removal partially restored the lymphoproliferative responses [39].

Hence, the mechanistic possibilities for immunosuppression in susceptible hosts include (1) altered antigen presentation by APCs (antigen presenting cells) as well as (2) the inability of T helper cells to commit a T_H1 or T_H17 type response in the presence of the *Leishmania* antigen (Fig. 2). The findings from a hamster model allow the diversity of APCs to be determined, and for an effective population of antigen-specific T cells to be generated.

The importance of toll-like receptors is a challenging aspect in developing vaccinations for the management of visceral leishmaniosis (VL). Several investigators [42,43] have suggested that the induction of IL1, TNF-α, and IFN-γ reduces intracellular *Leishmania donovani* accumulation in monocytes *in vitro*. Kar et al. [44] suggested that cystatin plays a role in the prevention of visceral leishmaniosis via induction of NF-κB mediated pro-inflammatory responses downstream of the TLR/MyD88 signaling pathway.

1.3. Impact of TLRs on post-kala-azar dermal leishmaniosis (PKADL)

Post-kala-azar dermal leishmaniosis (PKADL) is a sequel of visceral leishmaniosis (VL) [45–47]. The disease is associated with nodular lesions on skin of VL patients develop due to improper drug treatment and the development of dysfunctional host immune responses against the *Leishmania donovani* parasite. The current medications based on stibanate (Pentostam) have limitations in curing *Leishmania* infection. Several reports [48-49] suggest the drug-resistant variety of *Leishmania donovani* generates skin nodular lesions in the immunocompetent hosts. PKADL is an example of a condition which demonstrates reversible immune responses to *Leishmania* antigen(s). However, the impact of TLRs on PKADL treatment is still unknown. Immunotherapy using selective TLR agonists would be a better choice for the treatment of the nodular lesions and the activation of localized immune responses in PKADL patients.

2. Prospect of TLR agonists as immuno-therapeutic agents for leishmaniosis

In spite of extensive research, the immunotherapeutic approach and vaccination strategy to prevent cutaneous and visceral leishmaniosis is still under investigation. The areas for improvement in the management of leishmaniosis concern vector biology, variation in parasite antigens, and the genetic susceptibility of hosts towards *Leishmania* infection [50,51]. However, the correlation between geographical location and host-parasite interaction pattern is still unclear. The *Leishmania* parasites grow and multiply intracellularly within the macrophage [52,53], and by virtue of this location, the parasites escape direct interaction with the drug. At the same time, dysfunctions in antigen presentation by amastigote-infected macrophages and professional antigen presenting cells impair the T helper type 1-mediated inflammatory response, which is required to destroy parasites. Since there is still no effective immunotherapy and sodium stibogluconate or stibanate, is still used as a drug of choice for leishmaniosis [54–56].

Recent investigations concerning leishmaniosis therapy indicate a need to develop noninvasive treatment procedure in skin lesions of cutaneous forms of *Leishmania* infection. Thus, a strategy to

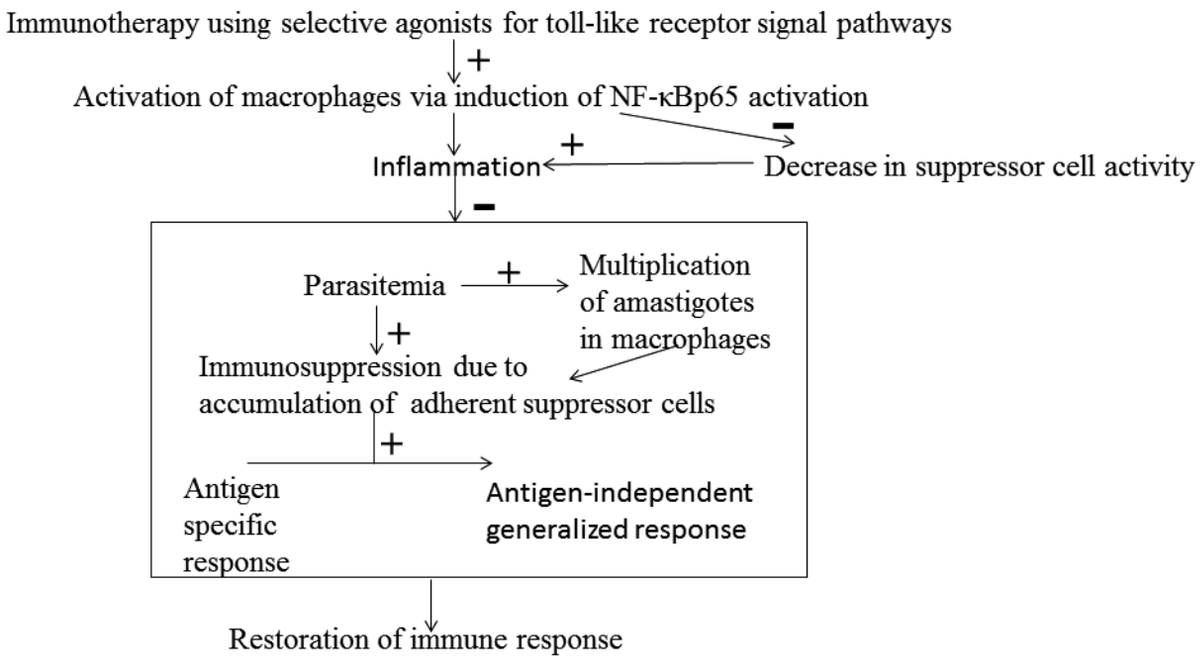

Fig. 3. Mode of action of toll-like receptor agonists in reversal of *Leishmania* parasite-induced immunosuppression. Mechanism of toll-like receptor agonists in reversal of immunosuppression via down regulation of adherent suppressor cells causing antigen specific and independent generalized immunosuppression during progression of disease. The increase in parasitemia gradually shifts antigen specific suppression of lymphoproliferative response to generalized antigen independent response in susceptible population. Removal of adherent cells restores functional immune response. The "+" sign indicates increase while the "–" sign indicates decrease in response.

produce effective topical medicine has acquired profound importance. The laboratory based investigations indicate that resiquimod alone or liposomal resiquimod (imidazoquinoline) may be a significant component of the treatment of visceral leishmaniosis [57,58]. The prospect of TLR agonist-based immunotherapy hence offers a promise in the treatment of leishmaniosis.

Conclusions

The successful treatment of leishmaniosis in various geographically-distinct areas depends on the susceptibility of infected populations towards parasite infection. While studying the mechanism of antigen specificity during *Leishmania* parasite infection, tests on a susceptible golden hamster model revealed that an increase in parasite load gradually decreases lymphocyte proliferation. However, such suppression of lymphocyte proliferation can be reversed, though partially, upon removal of adherent suppressor cell population from the spleen cells *in vitro* [39]. Hence, we propose that the down-regulation of adherent suppressor cell function using TLR-signal based immunotherapy is a worthwhile approach for overcoming immunosuppression and attaining a leishmaniacidal immune response.

The toll-like receptor agonists induce inflammatory responses through activation of NF-κBp65 and thereby decrease adherent cell populations which cause immunosuppression (Fig. 3). This proposed immunotherapy can prevent both antigen-specific and independent generalized immunosuppression in a susceptible population in endemic zones. Future research is necessary to screen potent TLR agonists for immunotherapy of *Leishmania* infection.

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