Review Article

Peroxisome Proliferator-Activated Receptor-γ-Mediated Polarization of Macrophages in Leishmania Infection

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Infection is the outcome of a contest between a pathogen and its host. In the disease leishmaniasis, the causative protozoan parasites are harbored inside the macrophages. Leishmania species adapt strategies to make the infection chronic, keeping a balance between their own and the host's defense so as to establish an environment that is favorable for survival and propagation. Activation of peroxisome proliferator-activated receptor (PPAR) is one of the tactics used. This ligand-activated nuclear factor curbs inflammation to protect the host from excessive injuries by setting a limit to its destructive force. In this paper, we report the interaction of host PPARs and the pathogen for visceral leishmaniasis, Leishmania donovani, in vivo and in vitro. PPAR expression is induced by parasitic infection. Leishmanial activation of PPARγ promotes survival, whereas blockade of PPARγ facilitates removal of the parasite. Thus, Leishmania parasites harness PPARγ to increase infectivity.

1. Leishmaniasis

Leishmaniasis is caused by parasitic protozoa of the genus Leishmania. The disease is found worldwide, with an estimated prevalence of 12 million cases, 50,000 annual deaths, and 350 millions of the world’s population at risk [1]. Leishmania has two stages in its life cycle: flagellated promastigotes that live within the alimentary canal of the insect vector and amastigotes that multiply within the phagolysosomes of mammalian macrophages. Infected female sandflies introduce saliva and promastigotes into the mammalian host during blood meals. The promastigotes are taken by leukocytes and differentiate into intracellular amastigotes within the macrophages. Then, infected macrophages carry the parasites to different organs. Over twenty species are known to infect humans. The cutaneous species reside and multiply within the skin tissue, whereas the visceral species predominantly accumulate in the liver, spleen, and bone marrow. These diverse species cause different clinical manifestations, varying from self-healing or metastasizing skin lesions to enlargement of visceral organs including the liver and spleen. The disease symptoms are classified as cutaneous, mucocutaneous, or visceral leishmaniasis.

2. Resistance versus Susceptibility

Immunity against all species of Leishmania uniformly relies on a type 1 immune response that produces interferon γ (IFNγ). Produced by T helper 1 cells, IFNγ activates macrophages to generate nitric oxide (NO), a free radical that can kill Leishmania. Type 2 immune response, on the other hand, is ineffective [2]. Production of interleukin-4 (IL-4) in Leishmania major infection, regulatory T cells in L. mexicana infection, and IL-10 in infection of various species are all associated with susceptibility [2–6]. It is well established that IL-4 exacerbates leishmaniasis when added exogenously, and IL-10 mutant mice become resistant to infection. However, to date, the reason why this cytokine promotes the pathogenesis of Leishmania infection remains partially understood [7–9].
3. M1 versus M2 in Disease Pathogenesis

Macrophages, the host of *Leishmania* parasites, are markedly heterogeneous. When stimulated by IFNγ, these macrophages differentiate into the classically activated (M1) phenotype, with inducible nitric oxide synthase (iNOS) which produces NO from arginine. Intracellular *Leishmania* parasites are eliminated by this subpopulation. On the contrary, IL-4 differentiates macrophages towards the alternatively activated (M2) phenotype, which promotes humoral immunity and tissue repair. This subpopulation produces IL-10, and transforming growth factor-β (TGF-β) [10].

In terms of signaling, IL-4 induces the expression of PPARγ and PPAR gamma coactivator-1 (PGC-1) β protein through the STAT-6 pathway [12]. This nuclear regulator polarizes the monocytes into alternatively activated (M2) macrophages with anti-inflammatory properties. By its transcriptional activity, it mediates the expression of arginase-1 (Arg1) and CD36 [13, 14]. Arginine metabolism away from production of NO compromises the ability of infected macrophages to clear the intracellular pathogens [2, 11]. CD-36 is a scavenger receptor that mediates phagocytosis and facilitates the removal of apoptotic cells. By its transrepressive action, PPARγ blocks the expression iNOS as well as nuclear factor kappa B (NFκB)-mediated transcription of pro-inflammatory mediators [15].

4. *Leishmania donovani* Infection Induces Host PPAR Gene Expression In Vivo and In Vitro

Our laboratory has been studying the pathogenesis of *Leishmania*, with particular interest in *L. donovani*. We investigated whether susceptibility to infection is associated with the activation of PPAR [16]. Mice of the susceptible BALB/c strain were infected with stationary phase promastigotes of *L. donovani*. After four weeks, their liver and spleen were excised, and PPARα and γ mRNA levels were analyzed, using the technique of quantitative real-time RT-PCR. Infection of *Leishmania* leads to increase in PPAR gene expression. We detected 3-fold increase in mRNA of PPARα in the liver (Figure 1(a)) and 3-fold increase for PPARγ in the spleen (Figure 1(b)), as compared to the uninfected control organs. At the cellular level, when resident macrophages from peritoneal exudates of BALB/c mice were infected, PPARγ gene expression was also increased. The increase of PPARγ mRNA was 2-fold for *Leishmania*-infected peritoneal exudate cells (PECs) (Figure 1(c)). Kinetics study was performed to examine whether PPAR expression correlates to parasite burden (Figure 2). The expression of PPARγ in the liver was found to be slightly ahead of the increase in parasites burden (Figure 2(a)). Both PPARα and parasite number peaked at 4 weeks, which is the time when granuloma will form and parasite growth will be quenched. As the infection in the liver decreases, the expression of PPARα subsides in coordination. In the spleen where the parasites will persist, the rise in PPAR expression correlates closely with the increase in parasite number (Figure 2(b)). Both parameters followed a logarithmic increase between weeks 2 to 4 until reaching a plateau at week 6.

5. Possible Mechanisms by Which *Leishmania* Induces PPAR Gene Expression

PPAR is a genetic sensor of fatty acids, and its ligands are produced during the course of *Leishmania* infection. Cyclooxygenase-2 (COX-2) is an enzyme that converts arachidonic acid into various bioactive lipids, including prostaglandin (PG) D2, PGE2, PGF2, thromboxane (TX) B2, 15d-PGJ2, and prostacyclin. Studies in the murine model of *L. donovani* infection have demonstrated that production of these bioactive lipids is enhanced upon infection [18, 19], and studies with *L. amazonensis* have revealed that COX-2 is needed for establishing infection [20]. Blockade of COX with indomethacin inhibits *L. amazonensis* infection of peritoneal macrophages in vitro and reduces the size of lesions in susceptible BALB/c mice.

Among the COX-2 products 15d-PGJ2 is a potent endogenous ligand for PPAR [21]. Moreover, in vitro addition of PGE2 increases the number of amastigotes within macrophages [22]. PGE2 can activate the generation of lipoxins, a relatively new class of eicosanoids that are also derived from arachidonic acid, but through lipxygenase or acylated COX instead [23, 24]. Lipoxin will shut off inflammatory response when bound to its receptor [25]. In *L. major* infection, addition of exogenous lipoxin A4 increases infectivity; this effect has been confirmed by receptor inhibition studies [26]. The eicosanoid downregulates inflammation by promoting clearance of apoptotic neutrophils [25]. *Leishmania* parasites (*L. major*, *L. donovani*, *L. mexicana*, etc.) are covered with phosphatidylserine (PS), a major surface characteristic of apoptotic cells, and engulfment of apoptotic cells leads to induction of PPAR [27, 28]. For these reasons, the parasitized macrophages would have activated PPAR and are likely to express an alternatively activated (M2) phenotype. Furthermore, PPAR turns on the expression of CD36, and this scavenger receptor would bind to thrombospondin and facilitate phagocytosis of the apoptotic neutrophils [29], the so-called Trojan horses for *Leishmania* parasites at the site of inoculation, reciprocally in a positive feedback manner [30–32]. Ligand activation of PPARγ augments phagocytic capacity of the alternatively activated macrophages.

6. Blockade of PPAR Reduces *Leishmania* Infection

Since PPAR is upregulated with *Leishmania* infection, we proceeded to determine whether the activation of PPAR is essential for infection. Studies assessing the effect of PPAR blockade on *Leishmania* infectivity have been conducted with *L. major*. Our laboratory studied *L. donovani* using PECs from the C57BL/6 mice, a strain that is susceptible to *L. donovani* infection though not *L. major*. A reason for selecting this strain is that it does not have a deficiency in T helper 1 cells and thus is capable of producing IFNγ, which is necessary for generation of the parasitoidal NO molecule. Nonelicited resident macrophages from the peritoneum were infected with *L. donovani* promastigotes, and IL-4 was added to activate PPARγ [33]. Then, PPARγ transcriptional
activity was blocked with SR202, an antagonist which efficacy and specificity have been shown in adipocytes [34–36]. The effect of PPARγ on parasite survival and proliferation in the host macrophages was assessed by enumerating the number of amastigotes per macrophage. In the absence of IL-4, the number of amastigotes per infected macrophage was $4.94 \pm 0.44$ (Figure 3(b)). IL-4 activated PPARγ (Figure 3(a)), and this resulted in an increase in infectivity (Figure 3(b)). There were $7.10 \pm 1.82$ amastigotes per macrophage. This enhancement by IL-4 was reversed by blocking PPARγ (Figure 3(b)). Addition of SR202, at 25 and 50 μM, in a dose-dependent manner, reduced the number of amastigotes per macrophage to $5.05 \pm 1.38$ and $2.02 \pm 0.81$, respectively. This reduced infection was further correlated to an increase in nitric oxide level in the cultures (Figure 3(c)). The effect of SR202 at 50 μM or lower is specific to the infectious process for, at these concentrations, the compound did not affect the survival of promastigotes or mammalian cells (Figures 3(d) and 3(e)). Figure 4 shows the cultures that had been stained with Diff-Quik for microscopic enumeration. The macrophages that had been treated with 50 μM of SR202 presented a healthy morphology with many empty parasitophorous vacuoles freed of parasites.

Complementary results were obtained with *Leishmania major* in bone marrow-derived macrophages from the resistant C57BL/6 mice in a study by Gallardo-Soler et al. (2008). Conversely, Gallardo-Soler et al. (2008) also demonstrated that the PPAR agonists GW1929 and GW7845 (for PPARα) and GW0742 (for PPARγ), at 1 μM concentration, increased

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**Figure 1:** *Leishmania* infection activates PPAR gene expression. In (a) and (b), BALB/c mice were infected i.v. with $10^7$ stationary phase promastigotes of *L. donovani* for 4 weeks, and then liver and spleen, respectively, were harvested. In (c), peritoneal exudate cells (PECs) were obtained from the peritoneum of normal BALB/c mice, infected with *L. donovani* at 1 : 10 ratio, and then harvested for RNA isolation after 2 days. PPAR activation was measured by real-time RT-PCR with normalization to 18S or actin RNA, and modulation was compared and expressed relative to the uninfected control using the delta-delta Ct method. The infected groups (black bars) showed higher levels of gene expression in liver (a), spleen (b), and PECs (c) in comparison to uninfected controls (open bars). Statistical analysis was performed by Mann-Whitney U test, and a *P* value of less than 0.05 was considered as significant.

The PPAR antagonists, GW9662 and GW5393, reduce infectivity [13]. Whereas we correlated PPAR activity in our *L. donovani* infection to the transrepressive action on iNOS-mediated NO production (Figure 3), this study correlated infectivity to the transcription of arginase. The enzyme is a bona fide marker for PPAR-mediated transcription and alternatively activated (M2) macrophages. Its mRNA level was decreased in coordination to the decrease in infection. In addition to pharmacological inhibitors, Odegaard et al. (2007) have examined *L. major* infectivity in mice with macrophages that do not express PPARγ (Mac-PPARγ KO, PPARγfl/fl LysMcre) [37]. The mutant mice have impaired M2 macrophage activation, a delayed disease progression, and a lower parasite load (less footpad swelling) compared to the wild type [37]. Henceforth, PPARγ plays an essential role in the pathogenic process of both *L. major* and *L. donovani*. In the absence of PPARγ activity, the balance shifts from the arginase producing M2 phenotype to that of nitric oxide producing, type 1, response.

7. **PPAR Activation Enhances *Leishmania* Infection**

Conversely, Gallardo-Soler et al. (2008) also demonstrated that the PPAR agonists GW1929 and GW7845 (for PPARα) and GW0742 (for PPARγ), at 1 μM concentration, increased
intracellular growth of *Leishmania major* in bone marrow-derived macrophages [13]. When both PPAR/RXR ligands were coadministered, the degree of infection was similar to those infected in the presence of IL-4. This increased number of intracellular amastigotes can be correlated to the levels of arginase activity.

PPAR is also regarded as dietary-sensing nuclear receptors; many activators of PPARγ have been identified in foods [38]. Our laboratory is interested in the effect of curcumin (Figure 5), a dietary activator of PPARγ on visceral leishmaniasis [16, 39]. It is the active principle in the spice turmeric, which is used abundantly in India, where visceral leishmaniasis is endemic in the Bihar region. Curcumin is well known for its anti-inflammatory effect, and there is ample evidence that the activity can be attributed to the activation of PPARs [40–43]. Zheng and Chen (2007) have suggested that there is a curcumin-responsive element residing in the regulatory region of the PPARγ gene [44]. We examined the effect of curcumin on PPAR activation and *Leishmania* infection *in vivo* [16]. Susceptible BALB/c mice and resistant C3H mice were infected with *L. donovani*; immediately following inoculation, the mice were fed curcumin or phosphate-buffered saline (PBS) every other day. Then, at 4 weeks after infection, the livers and spleens were harvested and quantified for PPARγ, iNOS, cytokines, and parasite load. Parasite load was quantified by two complementary methods, limiting dilution analysis and real-time PCR detection, and compared by the parametric test ANOVA after data transformation. Figure 6 shows the results on PPARγ, iNOS, and *Leishmania* kinetoplast DNA.
Figure 3: Blocking PPARγ activation with an antagonist reduces *L. donovani* infectivity. In (a), peritoneal exudate cells were infected by *L. donovani* using a 1 to 10: PEC to promastigote ratio. The cultures were incubated with 4 ng/mL of IL-4 for 24 hours, then total RNA was harvested, and RT-PCRs were performed to quantify the copies of PPARγ and β-actin mRNA as described in Adapala and Chan [16]. In (b), PECs from C57/BL6 mice were attached to cover slips and infected with *L. donovani* promastigotes at 1 : 5 ratio. After 20 to 24 hours, 5 ng/mL of IL-4 and various concentration of SR202 were added. The infection was allowed to develop at 37°C in a 5% CO2 incubator for another 3 days. Then, the cover slips were fixed in methanol and stained with Diff-Quik. The degree of parasite burden was determined by enumeration under a microscope in a double-blind manner by at least two individuals. Uninfected macrophages, infected macrophages, and the number of amastigotes in these macrophages were counted. The result is reported as amastigotes/macrophage, and each data point was derived from counting at least about one hundred macrophages or one hundred infected macrophages, as appropriate. (c) shows the levels of nitric oxide in PECs that were similarly infected with *L. donovani* promastigotes, except that IFNγ was added instead of IL-4 to stimulated inducible nitric oxide synthase expression. On day 5, the amount of nitric oxide was determined with Griess reagent. Shown are the relative levels of nitrite, oxidized form of nitric oxide, in the culture supernatants. In (d), SR202 was added to freshly harvested, uninfected PECs and bone marrow cells at various concentrations. After 3 days, the number of live cells was determined by counting with trypan blue. In (e), SR202 was added to promastigotes, and after 5 days of proliferation, the number of parasites was determined by counting under a microscope. The results shown are representative of three independent experiments.
Figure 4: Morphology of the peritoneal macrophages after SR202 treatment. Micrograph of cover slips from the cultures described in Figure 3(b). The black arrows point to macrophages with phagolysosomes filled with amastigotes. The black arrows point to infected macrophages and the red arrow points to infected macrophages with phagolysosomes cleared of amastigotes.

Figure 5: Chemical structure of curcumin.

Quantification. Curcumin treatment led to a 5-fold increase in the gene expression for PPARγ and a 2-fold increase in the gene expression for PPARδ in the spleen. It also caused an 80% decrease in the expression of iNOS in the liver and 68% in the spleen (Figures 6(a) and 6(b)). Concomitant with these modulations, parasite burden was elevated compared to the untreated vehicle control, (results from limiting dilution were not shown).

Corresponding to the feeding studies, we found that curcumin increased PPARγ and decreased iNOS gene expression in infected macrophages. At 10 μm, curcumin increased PPARγ mRNA levels in infected peritoneal macrophages from BALB/c by 1.5-fold Figure 7(a). The dose dependency of the curcumin actions was demonstrated by iNOS gene expression and nitric oxide production. The level of gene expression is shown in Figure 7(b). At 10 μm, curcumin reduced the level of steady-state RNA by 70%. The level of nitric oxide in the culture supernatants was also reduced. At 5, 7.5, and 10 μm of curcumin, the reduction was 18, 39.3, and 61.4%, respectively [16]. In parallel to the reduction, parasite infectivity increased. The number of infected macrophages increased dose dependently from 28 to 37% in the resistant C3H strain and from 35 to 48% in the susceptible BALC/c strain. The number of amastigotes per macrophage also increased dose dependently, as shown in the table in Figure 7(c).

8. Conclusion: Mechanisms of PPARs on Leishmaniasis

Taken together, these cumulative data from L. donovani and L. major infections indicate that PPAR plays a role in leishmaniasis, no matter in the liver where the PPARα forms predominate, in the spleen and residential macrophages from the peritoneum or the bone marrow where the PPARγ forms predominate [46]. Our perspective on how the nuclear factor is activated during infection and how its activation enhances the survival of Leishmania parasites is as follows. When the infected sandflies bite, an inflammatory reaction initiates
innate and adaptive immune response for protection against the parasites. Neutrophils and macrophages are recruited to the injection site, and promastigotes enter the phagocytes. Launching a type 1 immune reaction, with production of nitric oxide, would resist infection. However, the parasites and infected host cells can synthesize ligands that activate PPARy. Phagocytosis of apoptotic neutrophils and IL-4 from T helper 2 cells can do so as well. With the activation of PPARy, Leishmania parasites would benefit from infiltration of macrophages, inactivation of the destructive inflammatory response, and promotion of the resolution of inflammation. Activation of PPAR promotes differentiation.
Figure 7: Curcumin induces PPARγ mRNA expression and reduces iNOS mRNA expression in infected macrophages. In (a), PECs of BALB/c mice were infected with *L. donovani* promastigotes, and then 10 μM of curcumin (brown bar) or vehicle control (0.1% acetone) was added. After 2 days, the cells were harvested for RNA isolation to determine the level of PPARγ and β-actin expression by real-time RT-PCR. In (b), murine RAW264.7 cells were infected with *L. donovani* for 16–20 hours. Then, different concentrations of curcumin were added. Thirty minutes after curcumin treatment, IFNγ was added to activate the macrophages. At 5 hours after the addition of IFNγ, the cells were harvested, mRNA was extracted, and conventional RT-PCR was performed. The gel shows the end point PCR-amplified iNOS (496 bp) and β-actin cDNAs products. In (c), nonelicited PECs from resistant C3H and susceptible BALB/c strains were cultured with *L. donovani* promastigotes in wells that contained cover slips for a period of 16–20 hours; then curcumin and IFNγ and TNFα were added. On day 4-5, the coverslips were stained to enumerate the percent of infected macrophages and number of amastigotes per macrophage under a microscope, similar to steps described in Figure 3.

Currently, whether antagonists of PPAR would be therapeutic for leishmaniasis remains to be investigated. SR202, the antagonist that we used in our study, has been shown to prevent obesity in rats and therefore has *in vivo* efficacy. Ligands for PPARγ are drugs for type 2 diabetes, and ligands for PPARα are also currently in clinical use for obesity. How
Figure 8: Scheme of Leishmania interaction with mediators of the resolution process during inflammation. When infected sandflies bite, promastigotes enter the neutrophils and macrophages that are recruited to the inflamed site of injection. The parasite can, by itself, activate the infected host cells to produce PPAR activators. This includes PPARγ agonists such as the bioactive lipids 15d-PGJ2 and LXA4 from the arachidonic acid pathways. Engulfment of apoptotic neutrophils and IL-4 from T helper 2 cells can activate PPARγ as well. Activation of PPARγ polarizes the host macrophage towards the alternatively activated macrophage (M2) phenotype, which would produce arginase to divert substrate from iNOS and thus reduce the production of nitric oxide. As such, the parasite can survive and multiply within the host’s macrophages, and the infection becomes chronic.

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