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Anti-HIV drugs, lopinavir/ritonavir and atazanavir, modulate innate immune response triggered by *Leishmania* in macrophages: The role of NF-κB and PPAR-γ

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ABSTRACT

This study evaluated the influence of HIV protease inhibitors lopinavir/ritonavir (LPV/RTV) and atazanavir (ATV) on macrophage functions during their first interaction with *Leishmania*. Macrophages from BALB/c mice treated for 10 days with LPV/RTV and ATV, infected or not *in vitro* with *L.* (*L.*) *amazonensis*, were used to investigate the effects of these drugs on infection index, leishmanicidal capacity, cytokine production and PPAR-γ and RelB expression. LPV/RTV and ATV treatments significantly increased the infection index and the percentage of *Leishmania*-infected macrophages compared to untreated infected macrophages. There was no correlated increase in the production of NO and H_2O_2 leishmanicidal molecules. Promastigotes derived from *Leishmania*-infected macrophages from LPV/RTV and ATV-treated BALB/c mice had an *in vitro* growth 45.1% and 56.4% higher in groups treated with LPV/RTV and ATV than with PBS in culture. ATV treatment reduced IL-12p70 and IL-10 secretion in *Leishmania*-infected macrophages, but had no effect on IL-23 and TNF production. LPV reduced IL-10 and had no effect on IL-12p70, TNF and IL-23 secretion. ATV treatment decreased PPAR-γ expression in *Leishmania*-infected macrophages compared to untreated infected macrophages. In addition, LPV/RTV, but not ATV, reduced RelB cytoplasm-to-nucleus translocation in *Leishmania*-infected macrophages. Results showed that LPV/RTV and ATV HIV protease inhibitors were able to modulate innate defense mechanisms against *Leishmania* via different intracellular pathways. Although HIV protease inhibitors are highly efficient to control the Human Immunodeficiency Virus, these drugs might also influence the course of leishmaniasis in HIV-*Leishmania*-co-infected individuals.

1. Introduction

The cruel synergism between HIV and *Leishmania* increases the risk of leishmaniasis development in HIV-infected individuals by several hundred-fold [1], through either decreased resistance to a new primary infection or reactivation of a previous subclinical infection [2]. At the same time, *Leishmania* infection promotes the clinical progression of HIV disease and the development of AIDS-defining conditions [3]. This occurs because of a synergistic detrimental effect on cellular immune response by targeting similar immune cells. $CD4^+$ T cell depletion and/or functional impairments observed in HIV infection contribute to the proliferation and spread of *Leishmania* parasites, reduce the likelihood of a therapeutic response and greatly increase the probability of relapse [4]. In addition, the chronic immune activation determined by *Leishmania* infection generates the intracellular signaling necessary to HIV replication and, consequently, increases viral load and a more rapid progression to AIDS, which reduce life expectancy in HIV-infected individuals [4].

The introduction of highly active antiretroviral therapy has changed the clinical course of HIV infection and its associated illnesses [2]. However, prevention of leishmaniasis relapses remains a challenge in the care of HIV and *Leishmania* co-infected individuals [2]. Between 38 and 70% of co-infected individuals who received highly active antiretroviral therapy (HAART) relapsed during the 24 month-period following anti-*Leishmania* treatment. Relapses occurred independently of an increase in the number of $CD4^+$ T cells and even with an undetectable viral load. However, it should be observed that HAART treatment improved the evolution of co-infection because the average time of relapses was 7 months longer in patients who had received HAART than in those who had not received it [5]. Nevertheless, recovery of the immune system related to HAART was also associated with

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exacerbation and dissemination of cutaneous lesions in some individuals with HIV/*Leishmania* co-infection [6].

In experimental mouse infections, leishmaniasis is healed when macrophages are activated by products of Th1 cells, such as IFN-γ and TNF-α, which stimulate NO production, and IL-1, IL-12, IL-23 and IL-27 [7]. On the other hand, uncontrolled parasite proliferation is observed when macrophages are activated by products of Th2 cells, such as IL-4 and IL-10, which stimulate polyamine production, which is an important nutrient used for the intracellular growth of *Leishmania* [8].

NF-κB transcription factor regulates the expression of numerous genes that participate in inflammatory, immune and acute phase responses [9]. Active DNA binding forms of NF-κB are dimeric complexes composed of various combinations of proteins that constitute the NF-κB/Rel family. Inactive forms of NF-κB are associated with inhibitor subunit IκB in cell cytoplasm. NF-κB dimers can be activated by a variety of stimuli, among which are cytokines, such as TNF-α; it then translocates towards the nucleus to regulate gene expression [9]. The RelB member of this family transcriptionally binds to the active kappaB motifs in the TNF-alpha promoter in normal cells, and *in vitro* studies with macrophages isolated from RelB-deficient animals revealed impaired production of TNF-alpha in response to LPS and IFN-gamma [10].

An early intracellular signaling event that follows phagocytosis of *Leishmania* by macrophages is NF-κB transcription factor activation, which regulates pro-inflammatory cytokine production. In turn, the NF-κB pathway is regulated by PPAR-γ. PPAR-γ, a member of the peroxisome proliferator-activated receptor family, which is a transcriptional regulator of inflammation that has anti-inflammatory effects in various cell types by inhibiting the expression of pro-inflammatory genes such as cytokines, metalloproteases and acute-phase proteins [11]. Furthermore, PPAR-γ activity is regulated by TNF-α [12], a key molecule involved in defense against *Leishmania*.

Both *Leishmania* and HIV share target cells. *Leishmania* infects and multiplies inside macrophages, and HIV can invade and replicate in these cells, as well as in $TCD4^+$ cells [4]. Both infections change the predominant cellular immune response of Th1/Th0 to Th2 through complex mechanisms mediated by cytokines, conferring susceptibility to both infections [13]. Among cytokine changes, an inhibition in the production of IFN-γ has been recorded in HIV/*Leishmania* co-infected individuals, which may explain disseminated cutaneous infection and visceralization of species typically causing only cutaneous disease in the setting of HIV and *Leishmania* co-infection [14].

Antiretroviral therapy has evolved considerably over the last three decades, and introduction of HAART changed the clinical course of HIV. Current antiretrovirals have significantly prolonged the time for both AIDS development and death in those infected with HIV [15]. In 2011, of the 34 million people with HIV infection, eight million (23%) were receiving antiretroviral treatment and an additional seven million people have advanced HIV and need treatment [16]. This superlative figure concerning the prevalence of HIV/AIDS, mainly in places where *Leishmania* infection is endemic, pose tremendous challenges related to the treatment of co-infected individuals, mainly because antiviral drugs also act on immune mechanisms engaged in defense against *Leishmania*.

Protease inhibitors arrest maturation of HIV-1, blocking its infectivity and preventing subsequent infections of susceptible cells [16]. Furthermore, several HIV protease inhibitors directly inhibited *Leishmania* growth in a dose-dependent manner [17–19]. In addition to their antiviral and anti-*Leishmania* properties, antiretroviral drugs may also up or down-modulate monocyte/macrophage functions involved in defense against *Leishmania*. Ritonavir and indinavir decreased macrophage phagocytic capacity in relation to *Plasmodium falciparum*-parasitized erythrocytes due to a reduction in the expression of CD36 [20]. Lopinavir and nelfinavir increased the release of MIP-1α, MCP-1, IL-1β and TNF-α by PMA-stimulated THP-1 macrophages [21]. Indinavir, saquinavir and ritonavir decreased the expression of CD40, CD86 and CD80 in monocyte-derived dendritic cells [22]. After incubation with saquinavir, lopinavir, ritonavir, nelfinavir and amprenavir, neutrophils had their phagocytic capacity to opsonized zymosan reduced; chemotaxis to formilated peptides (fMLP) and superoxide anion production were also reduced [23]. Furthermore, ritonavir inhibited antigen presentation mediated by major compatibility complex class I and TCD8 + cell proliferation induced by the lymphocytic choriomeningitis virus [24].

The mechanisms involved in phagocyte antimicrobial capability and the molecules released during the initial interaction between *Leishmania* and macrophage may determine disease evolution to healing/cure or to a pathologic process. It is possible that protease inhibitors may positively or negatively influence this interaction. Therefore, the aim of this work was to evaluate the undetermined influence of lopinavir/ritonavir and atazanavir on functions of macrophages involved in innate defense during its first interaction with *Leishmania*. We assessed the macrophage infection index by *Leishmania*, NO and H_2O_2 production, IL-12, TNF-α, IL-23 and IL-10 secretion and PPAR-γ and RelB expression in peritoneal macrophages from BALB/c mice treated or not with ATV and LPV/RTV in their first interaction with *L.* (*L.*) *amazonensis*.

2. Material and methods

2.1. Study groups and ethical issues

Three groups of BALB/c female mice, 8–12 weeks old, were used to evaluate the influence of lopinavir/ritonavir and atazanavir treatment on the *in vitro* functions of *L.* (*L.*) *amazonensis*-infected macrophages. Group LPV/RTV consisted of 10 healthy mice treated with 200 mg/50 mg/kg lopinavir/ritonavir (Abbott, Illinois, USA), pipetting 60 μL per mouth once a day for 10 days [25]. Group ATV consisted of 10 healthy mice treated with 90 mg/kg atazanavir (Bristol-Myers Squibb, New York, USA), pipetting 60 μL per mouth once a day for 10 days [22]. Control group consisted of 10 healthy mice treated with 60 μL phosphate buffered saline (PBS) per mouth given once a day for 10 days. Doses of LPV/ATV and ATV correspond to the values when these drugs are used in humans.

On the 10th day of treatment of healthy mice with the drugs, macrophages were recovered by washing peritoneal cavity, and the *in vitro* phagocyte functions were assessed for each group described above with or without co-culture macrophages with *L.* (*L.*) *amazonensis*.

The isolate IFLA/BR/67/PH8 of *Leishmania* (*L.*) *amazonensis* was kept cryopreserved in liquid nitrogen until transferred to NNN-LIT medium and cultured at 26 °C for 48 h. In each experiment conducted, aliquots were added to Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 20% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and 40 μg/mL gentamicin (Novafarma, Anápolis, GO, Brazil), and cultured for six days until *Leishmania* promastigotes reached the stationary phase.

This study was carried out in accordance with the recommendations of the Brazilian National Council for Control of Animal Research (CONCEA). The Animal Research Ethical Committee at the University of Brasilia previously approved the experimental protocols (process number: 12673/2012).

2.2. Infection index

Mouse macrophages were obtained from all mice in each study group by washing their peritoneal cavity with 8 mL cold PBS, pH 7.2, and they were individually assessed. Macrophages were then washed with cold PBS (400 ×*g*, 10 min), quantified in a hemocytometer and suspended into cold RPMI 1640 (Sigma, St. Louis, MO, USA), pH 7.2, supplemented with 20 mM Hepes (Sigma), 2 mM glutamine (Sigma) and 40 μg/mL gentamicin. Cell viability was assessed with 0.05% nigrosin solution in 0.15 M PBS, pH 7.2, and it was always higher than 97%. To evaluate the infection index, samples of 2×10^5 macrophages in RPMI 1640 were placed on 13 mm-diameter glass coverlips in 24-well plastic plates (TPP, Switzerland, Europe), and incubated for 2 h in a wet