Immunophilin Ligands Modulate Phagocytosis and Viral 2 Replication in HIV-Infected Macrophages

Anish Sathe (✉ axs969@students.jefferson.edu)

University of California, San Diego

Ana Sanchez

University of California, San Diego

Benchawanna Soontornniyomkij

University of California, San Diego

Mary Swinton

University of California, San Diego

Shibangi Pal

University of California, San Diego

Cristian Achim

University of California, San Diego

Research Article

Keywords: rapamycin, HIV, cART, macrophages, amyloid

Posted Date: September 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-860160/v1>

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Immunophilin ligands modulate phagocytosis and viral replication in HIV-infected macrophages

Anish Sathe, Ana B. Sanchez, Benchawanna Soontornniyomkij, Mary Swinton, Shibangi Pal and Cristian L. Achim

University of California San Diego, Department of Psychiatry, 9500 Gilman Dr., La Jolla, CA 92093, USA

Email questions or concerns to Anish Sathe (axs969@students.jefferson.edu)

Abstract: (1) Background: HIV-associated neurocognitive disorders (HAND) can occur as a result of HIV-mediated neuroinflammation and affect people living with HIV (PWH) despite advances in combination antiretroviral therapy (cART). Brain macrophages have been implicated as a source of virus and neurotoxic factors. In this study, we examined the potential role of the immunophilin ligands rapamycin and FK506 in modulating neuroinflammation caused by infected macrophages. (2) Methods: Monocytes were isolated from blood samples from three different blood donors and were differentiated into macrophages (MDMs). These cells were subsequently infected with HIV and treated with combinations of an antiretroviral (ARV) cocktail (raltegravir, emtricitabine, and tenofovir), FK506, and rapamycin. Immunocytochemistry and RT-qPCR were used to analyze the phagocytosis of amyloid beta and the expression of macrophage phenotype-associated markers such as Iba1, TREM2, and IL-6. Viral replication was measured using p24 ELISA. (3) Results: Viral replication among infected MDMs as indicated by p24 levels was positively correlated with Iba1 levels and negatively correlated with IL-6 expression. However, infected MDMs showed lower Iba1 levels than non-infected cells. Rapamycin treatment appeared to lower p24 levels across all donors. Phagocytosis was associated with higher Iba1 levels and was impaired in rapamycin-treated MDMs. (4) Conclusions: Rapamycin seemed to protect against viral replication. However, decreased replication was correlated with a decrease in phagocytic activity. Iba1 may be involved in phagocytosis and HIV infection while IL-6 appeared to indicate protective effects against replication.

Keywords: rapamycin; HIV; cART; macrophages; amyloid

Funding

This work was supported by National Institutes of Health grant MH105319

Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare.

Availability of data and material

Code availability

Not applicable

Authors' contributions

All authors contributed to the study conception and design. Anish Sathe and Ana Sanchez have made equal contribution to this study as first authors. Material preparation, data collection and analysis were performed by Anish Sathe, Ana Sanchez, and Cristian L. Achim. The first draft of the manuscript was written by Anish Sathe and Ana Sanchez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Consent to participate

Consent for publication

1. Introduction

Immunophilins are a class of peptides that display cis-trans peptidyl-prolyl isomerase/rotamase activity. These proteins act as molecular chaperones by assisting with proper protein folding. Immunophilins such as FKBP52 and 51 respond to levels of steroids such as cortisol to affect glucocorticoid receptor (GR) activity. The GR normally translocates to the nucleus after binding with cortisol to increase the expression of genes related to neuronal metabolism and plasticity. The introduction of the immunophilin ligand (IPL) FK506 reduces this activity through immunophilin binding and interaction, therefore reducing the stress response in the brain [16, 17].

The binding of immunophilins to their specific IPLs can trigger different molecular pathways, such as the inhibition of rotamase activity. Through these pathways, immunophilins and IPLs can mediate the immune response. Different IPLs, such as FK506 and rapamycin, may act with similar mechanisms but ultimately different downstream pathways to produce their effects. Due to the immunosuppressive effects promoted by immunophilin-IPL binding, IPLs have been used in clinical applications such as organ transplants to prevent host rejection [18].

Another potential use for the immune-modulating function of IPLs is in HIV infection. As many as 30-60% of HIV-infected patients display HIV-associated neurocognitive disorders (HAND). The development of combination antiretroviral therapy (cART) has improved quality and expectancy of life for many people living with HIV (PWH). However, despite a reduction in the severity of HAND among PWH, the prevalence remains similar to the pre-cART era [4].

The Trojan Horse model of neurological HIV infection holds that virus integrates and replicates in macrophages, which can provide a route for the virus to cross the blood-brain barrier and reach the brain [1]. This increase in the presence of infected macrophages can lead to a heightened inflammatory response, eventually causing neuronal damage and degradation [1]. Furthermore, the virus can remain with the brain as a reservoir. All of these factors may contribute to the development of HAND in PWH despite cART. Macrophages which carry the virus and modulate the inflammatory response to neurological infection make up a potential target for therapy.

IPLs may be able to modulate the inflammatory response of macrophages by stimulating neuroprotective effects against damaging inflammation [5]. As the neurological effects of HIV depend on the activity and function of macrophages, we examined whether IPLs could alter the macrophage response to infection and offer protective effects in HIV infection. To understand the impact of IPLs on HIV infection in macrophages, we studied degree of viral replication using %increases in p24 levels, cytokine expression of IL-6, phenotype markers with Iba1 and TREM2, and phagocytic activity using amyloid-beta in an *in vitro* experimental system.

2. Materials and Methods

Monocyte isolation and monocyte-derived macrophage (MDM) cultures

Human buffy coats were obtained from healthy donors from the San Diego Blood Bank. Human peripheral mononuclear cells (PBMCs) were isolated from buffy coat diluted in PBS, underlaid with Histopaque-1077 (Sigma, 10771), and centrifuged for 30 min at 400xg. PBMCs, at the interface layer, were collected, washed with PBS, and counted. Monocytes from PBMCs were isolated by negative selection using

the Pan Monocyte Isolation kit magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Viable monocytes were counted, and resuspended in IMDM culture media (Iscove's Modified Dulbecco's Medium, Gibco-ThermoFisher Scientific, 12440-053) containing 10% Human serum AB (GeminiBio, 100512), 2 mM GlutaMAX (Gibco-ThermoFisher Scientific, 35050-061) and 1% Penicillin/Streptomycin (Corning, 30-001-Cl). Monocytes were seeded at 105 cells/well, in clear-bottom 96-well plates for imaging (Corning Falcon, BD Biosciences, San Jose, CA) and 106 cells/well in 12-well plates (Falcon). The monocytes were allowed to adhere and differentiate into MDMs in the absence of any stimulant for at least 5 days before use.

HIV-1 infection

HIV-1 (JRFL strain) was obtained from the NIH AIDS Research and Reference Reagent Program. For this study, MDMs were seeded and infected at 1000 pg/mL of p24 for 12 hours, followed by three washes. Supernatants were collected at 0, 3 and 4 days post infection and viral replication was measured using a HIV-1 p24 Antigen ELISA assay (ZeptoMetrix Corp).

Antiretroviral treatments

Two-nucleoside reverse transcriptase inhibitors (NRTIs), Tenofovir (TDF) and Emtricitabine (FTC), and the integrase inhibitor Raltegravir (RAL) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. MDMs, after being infected with HIV-1 for 3 days, were treated with a cocktail composed of TDF, FTC, and RAL at concentrations of 20ng/mL, 20ng/mL, and 20nM, respectively. The concentrations were chosen based on patient pharmacokinetics, choosing the average 24hr serum concentrations of these three antiretroviral drugs.

Immunophilin ligand treatments

Cells were treated, after 3 days of HIV infection, with FK506 (Sigma; F4679) and rapamycin (Sigma; R8781) at 1 μ M for 24 hours.

Immunocytochemistry and microscopy

After treatment and washing with phosphate-buffered saline (PBS), cell cultures were fixed for 20 min with 4% paraformaldehyde (PFA) at 4°C. Cells were permeabilized and blocked from non-specific antigens using 0.2% Triton X-100, 1% bovine serum albumin, and 5% normal donkey serum for 1 hour, then incubated overnight with primary antibody at 4°C. After washing in PBS, secondary antibodies were incubated at a 1:500 dilution in blocking buffer for 30 minutes, then washed 3 times and incubated in Hoechst H33342 (1:5000) for nuclear DNA staining for 5 minutes, and then washed 3 times with PBS. The following primary antibodies were used, diluted in blocking buffer: rabbit anti-Iba-1 (1:1,000) (Wako 019-19741, Richmond, VA, USA), mouse anti-TREM-2 (1:200) (Abcam, ab201621, Cambridge, UK), and mouse anti-Amyloid Beta (1:500) (BioLegend, San Diego, CA, USA). Secondary antibodies included donkey anti-rabbit Alexa Fluor 568, donkey anti-mouse Alexa Fluor 647, and goat anti-mouse Alexa 488 at a 1:500 dilution (Invitrogen, Life Technologies, Waltham, MA, USA). Controls were included in which primary antibodies were omitted. After washing, the cells were left in 1x PBS. Quantitative data acquisition and analysis was performed using the CellInsight CX5 High Content Screening (HCS) platform (ThermoFisher, Waltham, MA, USA).

Automated imaging and high-content analysis using the CX5 high content screening platform

Cells were imaged by CellInsight CX5 High Content Screening (HCS) equipment (Life Technologies). Images were analyzed using Cell Health Profile (CHP) and General Spot Measure Tool (GSMT) profiles. Results are presented as mean $\pm$ SEM of three independent experiments. Fluorescent signal from individual channels was quantified by designating cells of interest using the nuclear staining to find cells. Background signal was removed using reference wells which were not stained with primary antibodies.

Signal intensities reaching above a certain threshold determined by the reference wells were collected for each object.

Two methods of analysis were used: spot detection and intensity measurement. The spot detection method was used to measure amyloid beta phagocytosis. The method filtered through aggregation of signal below a certain area and counted the total number of spots associated with each cell, the total area covered by the spots in each cell, and the total signal intensity given off by the spots in each cell. These parameters were called Spot Count, Spot Total Area, and Spot Total Intensity, respectively. The intensity measurement method was used for the analysis of all other markers of interest. It picked up the total signal intensity given off by each cell and divided that by the total area of the signal. This value for each cell was then averaged over every single valid cell in the whole well. This parameter was called the Mean Average Intensity and represents an indication of signal density over the cell.

RNA isolation and quantitative RT-PCR (qRT-PCR-TaqMan)

RNA isolation was performed using the Qiagen RNeasy® Mini kit (Qiagen, #74136 Hilden, Germany) as per the manufacturer's instructions. RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). TaqMan gene expression assays were performed using StepOnePlus real-time PCR system (Applied Biosystems) using a primer specific to IL-6 (Taqman, cat. no. hs00174131) with ActB (TaqMan, cat. no. 99999903) used as a reference gene. The reactions were carried out at 48°C for 30min and 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. Samples were analyzed in duplicate. Fold changes were calculated using the comparative CT method.

Statistical analysis

The data are expressed as mean values +/- standard errors of the means (SEM) for three or more independent experiments. Statistical analysis was performed using GraphPad Prism 7. To detect statistically significant differences, we used a one-way analysis of variance (ANOVA) followed by the Fisher's PLSD post hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

3. Results

3.1. Iba-1 levels, but not TREM2, correlated with increased viral replication

We found that non-infected macrophages show higher Iba1 levels compared to infected macrophages of the same treatments, indicating a decrease in Iba1 expression associated with the infection (Fig. 1). On the other hand, HIV infection had no effect on levels of TREM2, a marker for macrophage phagocytic phenotype [6].

Fig. 1 Iba-1 and TREM-2 expression for HIV-infected MDMs exposed to FK506, Rapamycin, and ARV cocktail for 24 h Monocyte-derived macrophages (MDMs) from three independent donors were infected with HIV-1, followed 72 hours later by exposure to an ARV cocktail (TDF-20 ng/ml; FTC-20 ng/ml; RAL- 20 nM), FK506 (1 μ M), and rapamycin (1 μ M). After treatment for 24 hours, cells were fixed, permeabilized, and stained for Iba-1 and TREM-2. Images were taken and quantitative data acquisition was analyzed using CellInsight CX5 high content screening (HCS) platform (ThermoFisher) (A and B). Images were acquired with 20x-objective. Statistical significance was calculated from 3 biological replicates and 5 fields each (n=10) using one-way ANOVA followed by the Fisher's PLSD post hoc test (*p<0.05, **p<0.01, *** p<0.001, and ****p<0.0001). Error bars represent SEM.

However, the data also show a correlation between the %increase in p24 levels with the Iba1 expression at day 4 of infection among all three donors. This trend suggests that heightened Iba1 expression indicates an increased replication of HIV in infected macrophages. Across all three donors, rapamycin-treated cells consistently displayed lower %increases in p24 compared to vehicle-treated cells along with lower Iba1 immunofluorescence signal (Fig. 2).

Fig. 2 Viral replication of HIV-1 JRFL in infected MDMs exposed to FK506, Rapamycin, and ARV cocktail for 24 h Supernatant samples of HIV-infected MDMs were taken at 0, 3, and 4 days of infection. MDMs were treated with none, one, or a combination of FK506, rapamycin, and ARV cocktail. p24 concentration for each sample was measured using p24 ELISA. The percentage increase in p24 concentration from day 3 to day 4 was calculated for each treatment. Statistical significance was calculated from 3 biological replicates using one-way ANOVA followed by the Fisher's PLSD post hoc test (*p<0.05, **p<0.01, *** p<0.001, and ****p<0.0001). Error bars represent SEM.

3.2. IL-6 expression is inversely correlated with HIV infection

We found that rapamycin-treated macrophages appear to express higher transcriptional levels of IL-6 than do respective vehicle or ARV-treated controls across all three donors (Fig. 3).

Fig. 3 IL-6 cytokine mRNA levels in infected and treated MDM cells mRNA expression of IL-6 was quantified by qRT-PCR assay. Statistical significance was calculated from 3 biological replicates using one-way ANOVA followed by the Fisher's PLSD post hoc test (*p<0.05, **p<0.01, *** p<0.001, and ****p<0.0001). Error bars represent SEM.

Rapamycin-treated macrophages also show a lower %increase from day 3 to day 4 of p24 levels post-infection compared to untreated cells. Furthermore, FK506-treated cells show a higher %increase in p24 levels from day 3 to day 4 of infection than the untreated controls, correlating with lower IL-6 transcriptional levels. This evidence suggests that IL-6 is involved in an anti-HIV mechanism and that IL-6 may act to modulate an anti-inflammatory response during HIV infection.

Furthermore, HIV-infected macrophages treated with rapamycin alone displayed higher IL-6 transcription levels and a lower %increase in p24 compared to infected cells treated with rapamycin and ARVs, and even more so compared to cells treated with just ARVs.

3.3. Rapamycin and FK506 differentially alter phagocytic activity in macrophages

Our data show that HIV infection lowers phagocytic activity compared to vehicle controls (Fig. 4). Our results further indicate a correlation between Iba1 levels and phagocytic activity. Rapamycin-treated macrophages expressed less signal from amyloid-beta aggregates compared to respective control treatments. This matches with the lower levels of Iba1 immunofluorescence expressed by rapamycin-treated macrophages compared to controls. Furthermore, in non-HIV-infected conditions, rapamycin-treated macrophages expressed significantly lower amyloid-beta signal than rapamycin and ARV-treated cells. On the other hand, FK506-treated cells from Donor 11 show similar phagocytic activity compared to controls, while FK506-treated cells from Donor 10 show heightened levels of phagocytosis compared to controls. These values also correspond to the Iba1 levels seen in FK506-treated macrophages which are generally similar or higher compared to controls.

Fig. 4 Amyloid-Beta phagocytosis in infected and treated MDM cells MDM cells were exposed to amyloid-beta peptide aggregates for 1 hour following 96 hours of infection including 24 hours of treatment with none, one, or a combination of ARV cocktail and IPLs. The cells were subsequently fixed, permeabilized, and stained for amyloid-beta. Images were taken and quantitative data acquisition was analyzed using CellInsight CX5 high content screening (HCS) platform (ThermoFisher). Images were acquired with 20x-objective. Statistical significance was calculated from 4 biological replicates and 5 fields each (n=20) using one-way ANOVA followed by the Fisher's PLSD post hoc test (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).

4. Discussion

Rapamycin and FK506 exhibit different effects on HIV replication in macrophages

For all three donors, we found that rapamycin-treated macrophages consistently showed lower %increases in p24 levels than vehicle, indicating that rapamycin effectively inhibited infection even when administered 72 hours after infection first began. This could have implications for chronic HIV infection, as rapamycin remained effective *in vitro* despite being administered well after the onset of the infection. Furthermore, rapamycin-treated cells expressed far higher levels of IL-6 transcription compared to controls. IL-6 is a cytokine that acts both in a pro- and anti-inflammatory manner depending on the particular transduction pathway through which it signals. Although the specific role of IL-6 in HIV infection is unclear, it may have anti-viral properties and act in an immunoprotective manner [13]. IPLs such as FK506 and rapamycin are known to increase IL-6 levels in plasma *in vivo* and stimulate IL-6 production via monocytes *in vitro*. These molecules have been shown to modulate an anti-inflammatory response. Decreasing IL-6 levels also reduces the effectiveness of certain anti-inflammatory, antiviral agents [14]. The higher levels of IL-6 expression and lower levels of HIV viral replication among rapamycin-treated macrophages indicate that rapamycin has antiviral properties.

FK506 displayed less consistent effects on modulating HIV replication. FK506-treated cells from donors 8 and 10 showed higher %increases in p24 levels compared to controls while cells from donor 11 acted in the opposite way, with treatments including FK506 having lower %increases in p24 levels. However, these results were not significant. As with rapamycin-containing treatment conditions, FK506-containing treatments saw Iba1 levels move in the same direction as p24 levels, although these differences often were not significant. Despite variation in its effects across the three donors and lack of statistically significant results, FK506 acted consistently within treatments for each donor, indicating that FK506 may be involved in modulating viral replication in macrophages in HIV infection depending on some individual host factors. The distinct effects of rapamycin and FK506 could be due to differences in the downstream pathways activated by the IPLs, such as the separate targets of the immunophilin-IPL complexes (mTOR and calcineurin, respectively).

Rapamycin lowers phagocytic activity in macrophages

Macrophages from both Donor 10 and Donor 11 had consistently lower counts and areas of amyloid-beta aggregates after being treated with rapamycin or rapamycin and ARVs compared to the respective vehicle non-infected and HIV-infected controls. These results demonstrate that rapamycin lowers the phagocytic ability of macrophages. Together with the decrease in viral replication and increase in IL-6 production in rapamycin-treated conditions, the data indicate that rapamycin may facilitate macrophage cytokine activation and shift macrophage function as a response to viral infection [9].

Iba1 may be involved in HIV infection

Iba1 is a calcium-binding structural protein expressed in macrophages and microglia involved in the activation of phagocytosis. Iba1 immunofluorescence levels across all three donors were positively correlated with an increase in p24 levels in HIV-infected macrophages. These results indicate that Iba1 may be involved in HIV infection of these cells. Previous reports suggest that Iba1 is involved in a pathway activating Rac1 GTPase [7], which is also activated during HIV infection [8, 12, 19]. Consequently, an increase in Iba-1 may represent shifts to allow virus entry into the cell. Rapamycin-treated cells from all three donors demonstrated lower levels of Iba-1 fluorescent signal and smaller %increases in p24 levels, indicating lower levels of viral replication.

Meanwhile, non-infected macrophages showed higher Iba1 levels than infected cells with the same treatments. The Iba1 levels were correlated with increased phagocytic activity in non-infected macrophages

compared to infected treatments. Prior studies have shown that the Iba1-Rac pathway may promote phagocytic activity [2, 10], suggesting the involvement of Iba1 in macrophage function.

These data also indicate that HIV infection could alter macrophage function by increasing antiviral activity but lowering phagocytic capability [3, 11, 15]. Rapamycin may also promote this shift, explaining the lower phagocytic activity, Iba1 levels, and %increases in p24 seen in rapamycin treatments in both HIV-infected and noninfected conditions.

Conclusion

We found that rapamycin treatment consistently lowers HIV replication in macrophages while FK506 may be involved in HIV infection depending on individual differences. These effects were seen despite the administration of treatments after the cells were already infected, indicating that rapamycin remains effective after the virus has already seeded into the cells. This provides promising results for rapamycin as an anti-HIV drug in chronic infection.

Rapamycin also lowers phagocytic activity in macrophages, indicating it pushes for a decrease or shift in macrophage function. Further studies may be done on the implications of this finding, such as how it may affect neurological function and development of disease in models of neurodegeneration.

Iba1 levels also differed between treatments, indicating that the protein may be involved in or a marker for HIV infection. Currently, Iba1 is used as a marker for macrophages/microglia, but further studies may reveal other functions related to phagocytosis or viral response.

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