UNIVERSIDAD DE ALCALÁ DE HENARES

FACULTAD DE MEDICINA

Departamento de Medicina y Especialidades Médicas

TESIS DOCTORAL

PERSISTENT IMMUNE ACTIVATION IN HIV-1 INFECTED INDIVIDUALS: TRIGERING MECHANISMS

Hiperactivación inmune en pacientes infectados por VIH-1: Origen multifactorial

Autora: María Abad Fernández

Director: Alejandro Vallejo Tiller

2013

A Carmen y Julio,

por su cariño e incondicional apoyo

AGRADECIMIENTOS

A mis compañeros del piramidín y piramidón, con quienes compartimos largas jornadas, incluyendo clínicos, investigadores y personal del hospital. Destaco la labor de mi director de Tesis, Alex, por la formación y apoyo que recibí, del Jefe de servicio, Santi, de quien aprecio sus valiosos consejos, y de las amistades que entablé, especialmente con mis "anatómicas". A profesores e investigadores que me transmitieron su entusiasmo por la Ciencia. Gracias.

A mi familia en general, con la que mantengo una estrecha relación y de la que me siento muy orgullosa. Destaco a mi abuela, por su jovialidad y sentido del humor que tanto nos divierte. Gracias.

A mi cuadrilla de Huesconsin, por las bonitas y buenas amistades. A pesar de la distancia y paso del tiempo, continuamos siendo las de siempre, con entrañables encuentros casuales o tradicionales en nuestro infalible 9 de agosto. Gracias.

A mi pequeña "familia madrileña", que hicieron de palabras típicas en Aragón, un diccionario, de Alcalá de Henares y Madrid, mis ciudades, y de su preciada amistad, un periodo en mi vida único e irrepetible. A mis compañeras de piso, por aprender juntas a sacar lo positivo de nosotras en convivencia. Gracias.

El valor y calidad de las estancias reside en las personas que te rodean, con quienes descubrir otras lenguas y culturas integrándome en sus círculos de amigos y familiares, conocer el entorno acumulando anécdotas, expandir mis conocimientos mediante colaboración y compañerismo y entablar amistades a lo largo y ancho de este mundo. A todos los que hicieron distinguible mi paso por Verona y Salt Lake, consiguiendo incluso que me olvidara de la gélida nieve de las Rocosas y del extravío de mi maleta a esas temperaturas. Gracias.

A mi portugués, por ser como es, y hacer que todo parezca fácil y sencillo.

Un buen concierto finaliza con una buena pieza musical, dejo así en esta posición a dos personas a las que admiro profundamente, a mis padres, Carmen y Julio, que además de ser financiadores de excelencia, recibí de ellos las mayores lecciones en esta escuela de vida. La tenacidad de herencia maternal y el optimismo de herencia paternal, fue la mejor combinación para mi incorporación en la investigación. Muchas gracias.

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ABBREVIATIONS

AEMPS: Spanish Agency for Medications and Health Products
AIDS: Acquired Immunodefiency Syndrome
ALT: Alanine transaminase
APC: Allophycocyanin
APC700: Allophycocyanin 700
APC-Cy7: Allophycocyanin-Cyanin 7
ART: Antiretroviral therapy
AST: Aspastate transaminase
bp: base pair
cART: Combination antiretroviral therapy
CCL3: CC-Chemokine ligand 3
CCR5: C-C chemokine receptor type 5
CD: Cluster of Differentiation
CDC: Centers for Disease Control and Prevention
cDNA: copy DNA
CR: Concordant patients
CTL: Cytotoxic T cells
CTLA-4: Cytotoxic T-Lymphocyte Antigen 4
DS: Discordant patients
EDTA: Ethylenediaminetetraacetic acid
eF450: eFluor™ 450
ELISA: Enzyme-linked immunosorbent assays
env: Envelope
FITC: Fluorescein Isothiacyanate
Flc: Fluorescein
FRET: Fluorescence Resonance Energy Transfer
FSC: Forward scatter
gag: Group antigens
GALT: Gut-associated lymphoid
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GI: Gastrointestinal Tract
GM-CSF: Granulocyte-macrophage colony stimulating factor
GOT: Glutamic oxoloacetic transaminase
gp120: Glycoprotein
GPT: Glutamic pyruvic transaminase
HAART: Highly active antiretroviral therapy
HCV: Hepatitis C Virus
HERN: HTLV European Research Network
HIV-1 : Hunam Immunodeficiency Virus type 1
HLA: Human leukocyte antigen
HSx: Unprotected heterosexual contact
HTLV: Human T-lymphotropic Virus
IDU: Injecting drug user
IFN: Interferon
Ig: Immunoglobulin
IL: Interleucin
ILC: Idiopathic CD4 lymphopenia
INR: Immunological non-responders
IQR: Interquartile range
KO: Krome™ Orange
LB: Luria Broth
LBP: Lipopolysaccharide binding protein
LFA-1: Lymphocyte function-associated molecule 1
LPS: Lipopolysaccharide
LTNP: Long-term non-progressors
LTR: Long terminal repeat
MIP-1: Macrophage Inflammatory Protein
mRNA: messenger RNA
MSM: Men who have sex with men
MVC: Maraviroc
NK: Natural killer
NNRTI: Non-nucleoside reverse transcriptase
nPCR: Nested PCR
NRTI: Nucleoside reverse transcriptase inhibitor
OD: Optical density
PBMCs: Peripheral Blood Mononuclear cells
PBS: Phosphate Buffered Saline
PCR: Polimerase chain reaction
PD-1: Programmed cell death 1
PE: Phycoerythrin
PE-Cy7: Phycoerythrin-Cyanin7

PerCP: Peridinin Chlorophyll Protein Complex
PI: Protease inhibitor
pVL: Proviral load
RAL: Raltegravir
RANTES: Regulated upon activation, normal T cell expressed and Secreted
rDNA: Ribosomal DNA
RM: Rhesus macaques
RT-PCR: Real-time polimerase chain reaction
sCD14: Soluble CD14
SIV: Simian Immunodeficiency Virus
SM: Sooty mangabeys
SSC: Side scatter
STLV: Simian T-lymphotropic virus
TCM: T central memory
TEM: T effector memory
Th: T helper cells
TLR: Toll-like receptor
TNF: Tumor necrosis factor
VL: Visceral Leishmaniasis
WB: Western Blot

ABSTRACT

The use of combination antiretroviral therapy (cART) in individuals infected by human immunodeficiency virus type 1 (HIV-1) resulted in substantial reductions in morbidity and mortality in many countries worldwide.

Successful treatment increases levels of $CD4^+$ T cells and suppresses virus replication. However, persistent immune activation and inflammation remained significantly elevated in these individuals. Several studies associated chronic immune activation with an increased risk of non-AIDS associated morbidity and mortality during treated HIV infection, such as cardiovascular disease, metabolic disorders, neurocognitive abnormalities, liver and renal diseases, osteoporosis and neoplasias.

The exact mechanisms responsible for persistent immune activation in HIV-1 infected individuals are not entirely understood. Some studies proposed ongoing viral replication in anatomical sites such as the gastrointestinal tract, where antiretroviral therapy may not reach inhibitory levels. Others, however, suggested that microbial translocation through damaged gut mucosa was the main driver mechanism of inflammation and immune activation via secretion of pro-inflammatory cytokines. While others highlighted the influence of frequent HIV-1 coinfections, such as with hepatitis C virus (HCV), as key potential determinants of persistent immune activation.

The aim of the study was to further investigate which factors could contribute to chronic immune activation and disease progression in HIV-1 infected individuals. Microbial translocation, inflammation, immune activation, coinfection with *Leishmania* and the influence of HTLV-2 coinfection frequently found in HIV-1/HCV coinfected and HIV-1-monoinfected patients, were analyzed.

HIV-1 infected patients with regular medical appointments in the Hospital Ramón y Cajal, in Madrid, Spain, were analyzed in this study. All participants were grouped based on immunological and virological characteristics into three well-differentiated scenarios. Within the first scenario, the effect of treatment intensification was evaluated on HIV-1 infected individuals with good immune recovery. The second scenario included patients with leishmaniasis compared to two control groups that encompassed both immunological discordant responders and concordant patients to cART. Finally,

the third scenario included the influence of HTLV-2 infection on HIV-1/ HCV coinfected and HIV-1 monoinfected individuals.

Results showed reduced immune activation in HIV-1 infected individuals ongoing treatment intensification. However, no parallel decrease was found on microbial translocation, which in fact increased during the maraviroc intensification follow-up period. There was good correlation between the techniques employed to measure microbial translocation, including levels of LPS, LBP and sCD14, though no correlation was found between these measurements and immune activation in long term suppressed patients.

HIV-1 infected individuals coinfected with *Leishmania* showed higher levels of activated $CD8^+$ T cells, IL-6, sCD14 and activated $CD8^+$ T cell senescence when compared to immunological non-responders to antiretroviral treatment.

HTLV-2 infection in HIV-1 infected individuals showed lower plasma HIV RNA levels before starting antiretroviral therapy, and higher levels of $CD8^+$ T cell count when compared to individuals non infected by HTLV-2. Moreover, HIV-1/HCV coinfected individuals also infected by HTLV-2 showed decreased levels of activated $CD8^+$ T cells, and also lower levels of both alanine transaminase and liver fibrosis.

In conclusion, this study demonstrated that microbial translocation is associated with inflammation and immune activation in HIV-1 infected patients. However, this association was not so evident in long-term suppressed individuals with relatively good immune recovery on treatment intensification. Besides, coinfection with Leishmania aggravated the HIV-1-associated immunosuppression with immune characteristics worse than those found in immunological non-responders to antiretroviral treatment. And finally, we corroborated the protective role of HTLV-2 infection on HIV-1 infection and disease progression, and evidenced further beneficial role of HTLV-2 infection in HIV-1/HCV coinfected individuals, reducing immune activation and decreasing risk of HCV-associated liver diseases.

RESUMEN

La implantación de tratamiento antirretroviral de alta eficacia (TARGA) en pacientes infectados por el virus de la inmunodeficiencia humana tipo 1 (VIH-1) ha reducido considerablemente la morbilidad y mortalidad de estos pacientes a nivel mundial. La terapia antirretroviral controla la replicación viral y contribuye a la recuperación inmunológica del individuo.

No obstante, a pesar de disminuir los niveles de inflamación y activación inmunológica, estos niveles permanecen significativamente elevados. Varios estudios han relacionado la expresión de marcadores de inflamación con un peor pronóstico clínico y eventos no-SIDA como enfermedades cardiovasculares, neurocognitivas, metabólicas, osteoporosis, enfermedades hepáticas y renales, y cáncer.

Los mecanismos responsables de la activación inmune en individuos infectados por VIH-1 continúa siendo tema de controversia. Algunos estudios señalan continuos ciclos de replicación viral como posible factor desencadenante de una activación crónica generalizada. Esta replicación viral, a niveles indetectables por métodos convencionales, podría estar sucediendo en lugares anatómicos como el tejido linfoide asociado al intestino, donde el tratamiento antirretroviral podría no alcanzar los niveles inhibitorios óptimos. Otros estudios, en cambio, proponen la translocación bacteriana como un proceso desencadenante de inflamación local y activación inmune mediante la secreción de citoquinas proinflamatorias. Estos fenómenos se agravarían en presencia de coinfecciones, frecuentemente producidas en individuos infectados por VIH-1, entre las que destaca el virus de la hepatitis C (VHC).

La finalidad de este estudio es contribuir a clarificar los efectos que pueden tener varios factores biológicos en la activación inmune persistente asociada a la infección por VIH-1, en particular, la translocación bacteriana y subsecuente expresión de citoquinas proinflamatorias, y la influencia de algunas coinfecciones frecuentes en este tipo de pacientes, ya sea parasitaria, como la infección por *Leishmania*., o víricas como la infección por VHC y el virus linfotrópico de células T humanas (HTLV).

En este estudio se incluyeron individuos infectados por VIH-1 que acuden periódicamente a revisiones médicas en el Hospital Ramón y Cajal, en Madrid. Los pacientes se analizaron de manera separada atendiendo a características inmunológicas y virológicas en un total de tres grupos claramente diferenciados. El

primero de ellos incluía individuos con una buena recuperación inmunológica que comenzaron intensificación con tratamiento como estrategia para reducir la replicación viral residual. El segundo grupo lo componen individuos con leishmania visceral cuyos parámetros analizados los comparamos con dos grupos control formados por individuos con respuesta concordante o discordante a tratamiento. Finalmente, el último grupo de estudio se compone de una gran cohorte de individuos infectados por VIH-1 coinfectados por HTLV-2 y VHC.

Los resultados muestran una reducción en la activación inmune de individuos infectados que se someten a intensificación de tratamiento con maraviroc o raltegravir. Sin embargo, sólo en aquellos pacientes que recibieron maraviroc se observó un incremento en los niveles de translocación bacteriana. Las técnicas utilizadas para medir el grado de translocación bacteriana correlacionan entre sí, sin embargo no se observa correlación entre la translocación bacteriana y la activación inmune.

Los individuos coinfectados por *Leishmania* muestran niveles de activación inmune, inflamación, translocación bacteriana y senescencia celular superiores a los individuos inmunológicamente discordantes a tratamiento.

Por otro lado, la coinfección por HIV-1/HTLV-2 muestra menores niveles de carga viral por VIH-1 previo inicio de tratamiento, además de mayores niveles de células T $CD8^+$ en comparación con individuos negativos para HTLV-2. En individuos coinfectados por HIV-1/HCV, la infección por HTLV-2 muestra menores niveles de activación celular, alanina transaminasa y fibrosis hepáticas que aquellos individuos no infectados por HTLV-2.

En conclusión, este estudio demuestra que los niveles de translocación bacteriana se asocian con la inflamación y activación inmune en individuos infectados por VIH-1. Sin embargo, esta asociación no se evidencia en individuos virológicamente suprimidos en tratamiento bajo un largo periodo de tiempo. Por otro lado, la coinfección por *Leishmania* acelera el nivel de inmunosupresión relacionado con la infección por VIH-1 con características inmunológicas peores que individuos inmunológicamente discordantes a tratamiento. Por último, la infección por HTLV-2 ejerce una función protectora en la infección por VIH-1 ralentizando la progresión a SIDA, y se evidencia por primera vez que esta función protectora es extensible a la infección por VHC, disminuyendo la hepatotoxicidad asociada.

Introduction

ANTIRETROVIRAL THERAPY: SUCCESSES AND CHALLENGES

Thirty decades after the recognition of acquired immunodeficiency syndrome (AIDS) in United States, HIV-1 has spread throughout the world, with growing heterogeneity. In 2010, the estimated number of people with HIV-1 world-wide was over 34 million people[1], more than 95% living in low-and middle-income countries. The number of people infected by HIV-1 is expected to increase, mostly because of inadequate access to prevention and treatment[2].

1.1 From lethal disease to chronic pathology: 1996

The AIDS mortality in 1993 was the leading cause of death among persons ages 25 to 44 years[3]. However, after 1996, the number of deaths among people with AIDS drops every year since then. According to the Centers for Disease Control and Prevention (CDC), AIDS mortality fell from more than 51,000 in 1995 to about 16,000 in 2002 (Figure 1.1). These dramatic declines in AIDS mortality rate were due largely to the availability of combination antiretroviral therapy[4-5], although with differences among demographic areas[6].

In this way, the development of antiretroviral therapy (ART) has been one of the greatest accomplishments of basic and translational research of the late 20th century. Combination ART (cART) with at least three drugs has resulted in substantial reductions in morbidity and mortality in many countries worldwide[7].

Successful cART increases levels of $CD4^+$ T cells and reduces HIV-1 viral load indefinitely to undetectable levels (below 40 HIV-1 RNA copies/ml)[8]. Besides, cART has proven efficacious in HIV prevention, reducing the risk of HIV-1 viral transmission from mother-to-child and serving as post-exposure prophylaxis for individuals exposed to HIV-1[9-10].

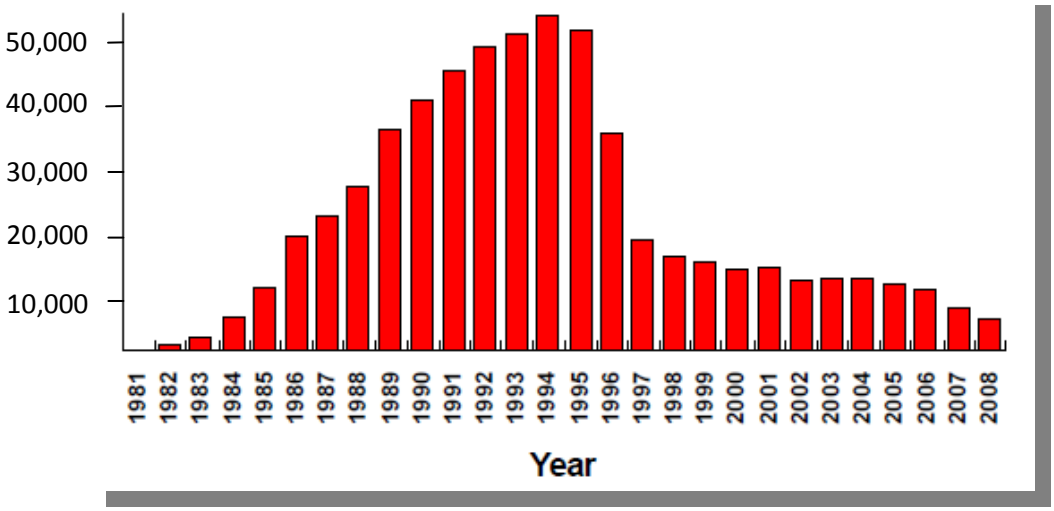

Figure 1.1 The estimated AIDS mortality from 1981 until 2008 in United States (www:cdc.gov)

1.2 Limitations of antiretroviral therapy

Although lifelong suppression of HIV-1 replication with cART should be possible in adherent patients, as soon as treatment is stopped, HIV-1 viremia rapidly rebounds[11-12].

Additionally, despite the inherent potency of cART to suppress virus replication, full life expectancy for patients living with HIV-1 has not been restored, remaining significantly less than population controls. Although ongoing refinements to improve the increased risk of morbidity and mortality, HIV-1-associated complications persist, including increased risk of cardiovascular disease, metabolic disorders, neurocognitive abnormalities, liver and renal disease, osteoporosis, and cancer [13-21].

The exact mechanisms responsible for this increased risk are not entirely understood, however, toxic effects of long-term antiretroviral treatment[22] or persistence of immune activation and inflammation emerged as key potential determinants of non-AIDS associated morbidity and mortality during treated HIV-1 infection[23].

PERSISTENT INMUNE ACTIVATION

Chronic immune activation is a characteristic feature of progressive HIV-1 disease[24-25]. Generalized immune activation contribute to the inefficiency of the HIV-1 immune response and to the impairment of the regenerative potential of the entire immune system, including deleterious effects on generation of effective immune responses and on T-cell homeostasis and changes in the composition of lymphoid tissues that impede immune function[26]. The increase in immune activation and inflammation is also accompanied by HIV-1 specific exhaustion of $CD4^+$ and $CD8^+$ T cells, defined by decreased proliferative and effector functions, alterations in homing receptor expression patterns consistent with migration to sites of inflammation and increased expression of inhibitory receptors such as PD-1, or CTLA-4 on T cells[27-28].

The most widely accepted evidence that immune activation plays a major role in HIV-1 immunopathogenesis is the nonpathogenic outcome of SIV infection in its natural host. SIV-infected sooty mangabeys (SM) have strikingly low levels of immune activation in the chronic stage of infection and almost never develop immunodeficiency despite high levels of SIV viremia[29]. Conversely, SIV infection of rhesus macaques (RM) and other non-natural hosts results in high levels of systemic immune activation despite having lower or similar levels of SIV viremia, $CD4^+$ T cell depletion, and rapid progression to clinical manifestations similar to AIDS[30-31]. It remains unclear, however, how the natural hosts of SIV infection are able to maintain low levels of immune activation despite high levels of viral replication.

1.3 Immunological non-response to cART

Abnormally high levels of T cell activation persist despite years of ART-mediated viral suppression compared to uninfected individuals[32]. Control of HIV-1 replication reduces $CD4^+$ T cell loss from direct cytolysis[33-34], and partially restores T cell homeostasis[35], increasing the number of $CD4^+$ T cell count. Although many patients continue to have $CD4^+$ T cell recovery for several years after receiving ART[36], from 6% to 30% patients known as immunological non-responders (INR) or immunologic discordant patients (ID) failed to increase their absolute counts of $CD4^+$ T cells[37-39],

having increased risk of non-AIDS-related complications[40-41] and detrimental clinical consequences[42-43].

Elevated immune activation has been established as a marker of disease progression in untreated patients[44-45]. Even though its role during viral suppression may be less significant[46], compelling evidences for decreased thymic production[47] and increased $CD4^+$ T cell hyperactivation that undergo rapid proliferation and subsequent cellular apoptosis seems to have a significant effect on $CD4^+$ T cell recovery during ART[48-49] (Figure 1.2).

These findings were consistent with other studies in which elevated $CD4^+$ T cell activation was observed in patients with idiopathic CD4 lymphopenia (ILC) who have low $CD4^+$ T cells counts in the absence of HIV-1[45], suggesting that $CD4^+$ T cell activation may be at least partly related to lymphopenia-driven T cell proliferation[50-51]. Although highlighting the possible pathogenic role of enhanced $CD4^+$ T cell activation in well suppressed HIV-1-infected individuals, these findings also raise the questions on the driver mechanisms of persistently elevated $CD4^+$ T cell activation[52].

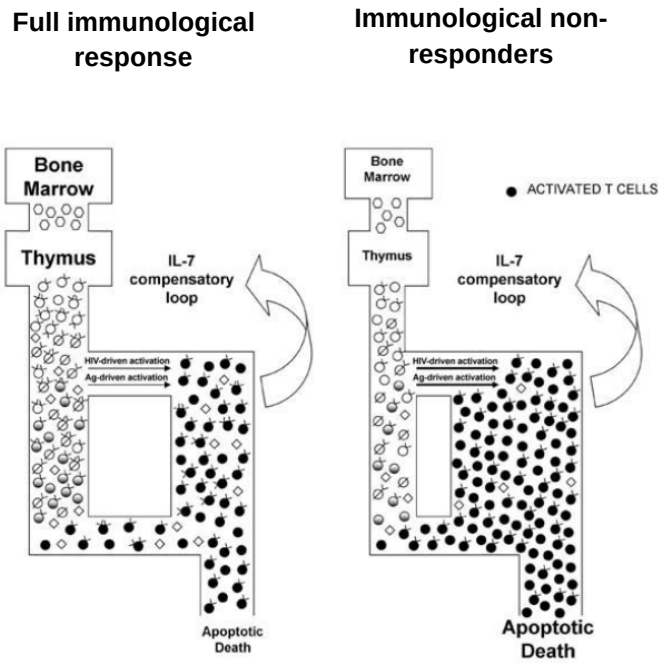

Figure 1.2 Activated $CD4^+$ T cells undergo proliferation and apoptosis having a significant effect on $CD4^+$ T cell recovery during HAART. (Modified from Gazzola et al, 2008 [52]).

1.4 Immunosenescence

Ongoing HIV-1-related immune dysfunction and inflammation during ART underlies premature aging in HIV-1-infected individuals[53-55]. Several physiological alterations and comorbidities related to old ages, such as osteoporosis, atherosclerosis, and neurocognitive decline, occurs in HIV-1 disease[56-59].

During aging, a reduction in T cell renewal together with progressive enrichment of terminally differentiated T cells occurs. Remarkably similar, HIV-1 reduces the capacity of the thymus to produce new cells[60-62]. Continuous stimulation of the immune system in HIV-1 disease with loss of $CD4^+$ T cells by activation-induced cell death coupled with poor T-cell restoration due to lower thymic function results in an impaired T-cell homeostasis. The naïve T-cell pool cannot be replenished efficiently, and therefore old senescent $CD8^+$ T cell clones or depleted $CD4^+$ T cells cannot either be replaced, leading to an imbalance in T-cell phenotypes, similar to that observed in the elderly[63-65].

HIV-1 infection induces premature aging of naïve $CD4^+$ T cells and both memory $CD4^+$ and $CD8^+$ T cells. Levels of cellular activation ($CD38^+$ $HLA\text{-}DR^+$)[66-68] are the major driving factor of proliferation and T-cell differentiation, resulting in the generation of antigen-experienced cells with limited proliferative potential that eventually lose expression of CD28 and increase the expression of CD57, which is a key predictor of immune incompetence in the elderly and HIV-1-infected individuals[69-71].

The association of HIV-1-related immune dysfunction and non-AIDS defining co-morbidities and premature aging is represented in the figure 1.3. The accelerated aging model in HIV-1 infection would start with ongoing HIV-1-related immune activation despite cART as the central event in the senescent pathway. Activated T cells undergo clonal expansion due to circulating antigen, resulting in differentiation and accumulation of nonfunctional end stage senescent cells that cannot be replenished due to the loss of thymic function altering T cell homeostasis increasing the risk of developing non-AIDS morbidities associated to HIV-1-associated immune activation and inflammation[72].

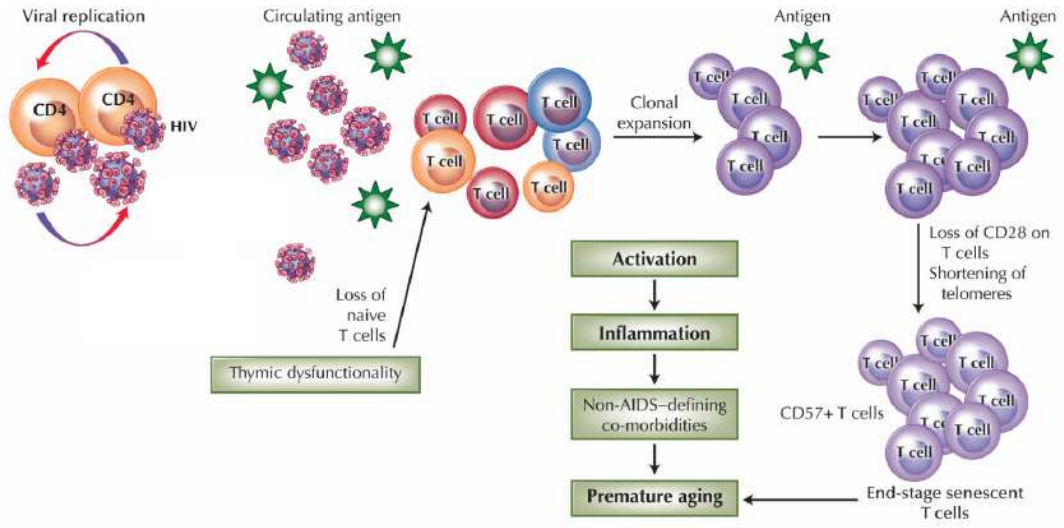

Figure 1.3 Accelerated aging model in HIV-1 infection (Modified from Desai et al, 2010[65]).

PERSISTENT IMMUNE ACTIVATION: CAUSES

The underlying causes of persistent immune activation during cART-mediated viral suppression are likely multifactorial but remain incompletely defined. Several potential causes have been proposed, highlighting HIV-1 ongoing replication, microbial translocation and co-infections, as continuous trigger mechanisms of immune activation[73].

RESIDUAL VIREMIA: ONGOING VIRAL REPLICATION

First, the effectiveness of current therapeutic regimens has been monitored by using HIV-1 RNA assays which have a sensitivity of 40 to 50 copies/ml. Suppression of plasma viremia below detectable levels can be sustained in HIV-1 infected individuals by antiretroviral regimens currently in use[74-75]. Nevertheless, replication-competent cell-

free virions can be found for many years in the plasma of most patients on cART, and viremia rapidly rebounds when antiretroviral suppression is interrupted[76-79].

Still infected individuals remain clinically stable on cART, persistent virus expression may contribute to long-term complications[53]. Using recently developed real-time HIV-1 RNA assay, with sensitivity below one HIV-1 RNA copy per ml, allows a more detailed analysis of the viral decay kinetics in patients on suppressive cART[80,81]. Several studies revealed persistent low-level viremia of around 3-5 copies/ml in plasma of most patients on cART and persists in patients even after years of therapy[82].

The source and dynamics of this residual viremia are currently unknown, representing a challenge to future attempts to induce a drug-free remission of HIV-1 disease and eradication of the infection[83]. Viremia could arise from ongoing cycles of viral replication in a sanctuary site where there is suboptimal drug penetration, from long-lived productively infected cells, or from activation of virus expression from latently infected cell reservoirs[84-87].

1.5 Generation of 2-LTR circles: recent infection events

Efforts to characterize the extent of ongoing viral replication in suppressed individuals have been hampered by a lack of convenient surrogates of ongoing viral replication[88]. Viral episomes containing one or two long terminal repeats (1-LTR or 2-LTR-containing episomes) are formed after completion of viral cDNA synthesis and translocation to the host cell nucleus, where recombination and direct ligation lead to the formation of episomes containing one and two LTRs, respectively. These episomal cDNA forms are labile and, as such, their presence is indicative of recent infection events[89-91].

1.6 Anatomical sites: HIV-1 reservoirs

Persistent ongoing viral replication may remain in anatomical sites immunologically sheltered from the blood and lymphoid systems with low antiretroviral

drug penetration[92-93]. These anatomical sites, including the lymphoid tissue, central nervous system, respiratory, gastrointestinal and genitourinary tracts, may act as reservoirs of HIV-1 replication[94-96].

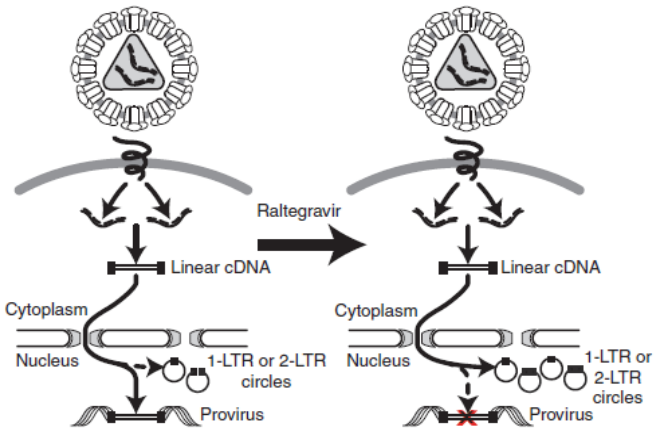

Figure 1.4 Generation of 2-LTR and 1-LTR circles after intensifying suppressive regimen with raltegravir (Buzon et al, 2010[110]).

1.7 Antiretroviral treatment intensification: maraviroc and raltegravir

Several studies have looked at the intensification treatment with an additional antiretroviral drug, not previously used by the patients, as a possible strategy to reduce low-level viremia and subsequent immune activation whether ongoing new cycles of viral replication were contributing to residual viremia[97]. Additionally, the emergence of new antiretroviral drugs with different mechanisms of action, such as CCR5 antagonists and integrase inhibitors, provides new opportunities to assess the viral reservoirs and residual viremia that persist in suppressed individuals[99-100].

Particularly, these new antiretroviral drugs referred are; maraviroc (MVC), a potent new antiretroviral agent approved for the treatment of HIV-1 infection that blocks interaction between the virus and the CCR5 co-receptor, targeting HIV-1 entry process, a crucial step in the HIV-1 life cycle[101-102], and raltegravir (RAL), the first-in-class integrase inhibitor approved for the treatment of HIV-1 infection. It blocks integration of linear viral cDNA that is subsequently circularized by host DNA repair enzymes to form

episomes containing two copies of 2-LTR circles or undergoes recombination to form a 1-LTR circle[103-104], as represented above (Figure 1.4).

Disappointingly, the vast majority of treatment intensification trials to date have failed to show any decline in low-level viremia or cell-associated HIV-1 DNA[105-107], highlighting two studies that showed no change in the gastrointestinal tract[108] or in the cerebrospinal fluid[109]. Nevertheless, the addition of raltegravir lead to an increase in 2-LTR circles within two weeks in one-third of the suppressed HIV-1-infected individuals, consistent with evidence of residual viral replication, and a significant reduction on T cell activation after 48 weeks intensifying with RAL, although there was still no change in persistent low-level HIV-1 RNA or cell-associated DNA, following intensification[110].

MICROBIAL TRANSLOCATION

Secondly, microbial translocation has been pointed as a possible mechanism underlying immune activation and non-AIDS-associated morbidity in HIV-1 disease during suppressive regimen[111-112].

1.8 Gut-associated lymphoid: anatomical viral reservoir

The gastrointestinal (GI) tract continues to serve as an important viral reservoir of HIV-1-infected cells even during long-term cART and contributes to viral persistence[113-114]. The GI tract harbors most of the total lymphoid tissue in the body and an abundance of activated effector memory $CD4^+$ T cells that are $CCR5^+$ and their predominantly activated state make them highly sensitive to HIV-1 infection[115-117]. Of these $CD4^+$ $CCR5^+$ T cells, Th17 cells, which also expressed the gut homing marker CCR6, are even more susceptible to HIV-1 infection and subsequent cellular depletion[118-119].

1.9 Intestinal epithelium: HIV-1 associated $CD4^+$ T cell depletion

HIV-1 infection cause massive depletion of $CD4^+$ T cells from the gut-associated lymphoid (GALT) tissue in the first few weeks of the infection, attributable to the cytopathic effects of the viral infection or the activation-induced uninfected $CD4^+$ T cell death[120-121]. Insufficient production and tissue delivery of $CD4^+$ memory T cells may contribute to sustained $CD4^+$ T cell depletion during disease progression[122]. The loss of $CD4^+$ T cells was coincident with increased prevalence of $CD8^+$ T cells in GALT and the subsequent marked changes in the T-cell subset distribution in GALT, that were not adequately reflected in peripheral blood[123]. This dysregulation in T-cell homeostasis leads to increased levels of pro-inflammatory cytokine[124-125], increased apoptosis of epithelial cells, and altered tight junction protein composition[126] resulting in a functional degradation of the intestinal barrier[127]. The mucosa damage leads to a process known as microbial translocation, whereby microbial products from the intestinal lumen cross the disrupted mucosal barrier into the systemic circulation in the absence of overt bacteraemia[128-129].

Additionally, the cytokines IL-17 and IL-22 produced by Th17 cells enhance epithelial regeneration and defend against microbial translocation by recruiting neutrophils to the GALT to clear microbial products and by stimulating epithelial cell proliferation and antibacterial defensin expression[130-132]. Therefore, loss of Th17 cells may cause impaired mucosal healing increasing intestinal permeability and the subsequent microbial translocation[133-134]. Hence, both HIV-1-infected individuals and SIV-infected rhesus macaques have preferential significant depletion of gut Th17 $CD4^+$ T cells with a sequential increase in Th1 $CD4^+$ T cells, contributing to epithelial injury and enhance local inflammation and immune activation[135]. However, SIV-infected sooty mangabeys and African green monkeys, the natural hosts for SIV infection that do not have increased microbial translocation and do not develop AIDS, maintain, strikingly, Th17 cells in the gut despite mucosal $CD4^+$ T cell losses[136 -137].

Taking together all data, intestinal epithelium in HIV-1 infected individuals is characterized by massive $CD4^+$ T cell depletion, particularly Th17 cells[138-140], abnormal enterocyte differentiation[141], enterocyte apoptosis and destruction of the tight junctions[142] caused by increased production of interferon-γ (IFN-γ) and tumor necrosis factor (TNF), contributing to increased microbial translocation[143] (Figure 1.5).

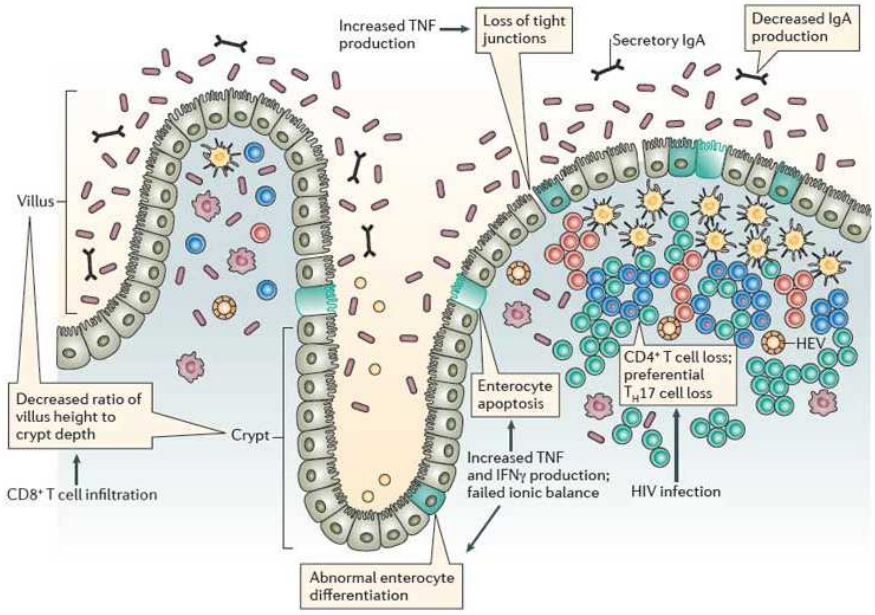

Figure 1.5. Intestinal epithelium in HIV-1-infected individuals (Sandler et al, 2012).

1.10 Microbial translocation

Increased levels of circulating microbial products in HIV-1-infected individuals, such as lipopolysaccharide (LPS), peptidoglycan, bacterial CpG DNA, flagellae, and viral genomes, molecules that can directly stimulate the innate immune system through Toll-like receptors (TLRs), are strongly associated with increased immune activation[144-146] (Figure 1.6).

Several parameters have been indistinctly used to determine bacterial translocation[147-149], though LPS and soluble CD14, have been the most commonly used[150]. Lipopolysaccharides (LPS) are the major component of gram-negative bacteria cell walls and a potent immunostimulatory product[151]. Circulating LPS promotes hepatic synthesis of LBP, a plasma protein that increases the binding of LPS to CD14/TLR4 receptor complex on monocyte/macrophages[152-153], triggering monocyte

activation and resulting in the release of soluble CD14 (sCD14) and the production of inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β), Tumor Necrosis Factor (TNF) and type I Interferons (IFN-I)[154-155], contributing to HIV-1-associated immune activation (Figure 1.6).

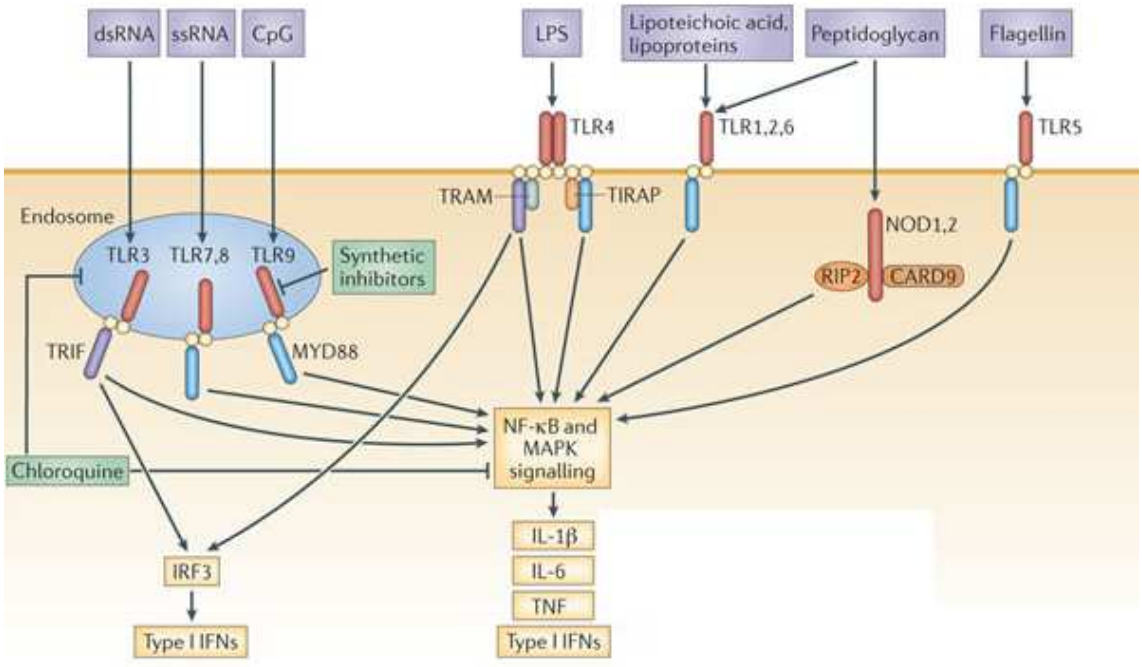

Figure 1.6. Intestinal epithelium in HIV-1-infected individuals (Sandler et al, 2012).

1.11 Possible driver mechanism of HIV-1 associated immune activation

The association between microbial translocation and immune activation were reported in several studies in the first years of chronic HIV-1 infection or in the most advance stages of HIV-1/AIDS[156-160].

Due to the ongoing viral replication in GALT and elevated chemokine production, there is a recruitment of $CD4^+$ T cells from the periphery into the gut via $\alpha_4\beta_7$, an integrin that mediates migration of lymphocytes to the gut[161]. Engagement of $\alpha_4\beta_7$ on

$CD4^+$ T cells by gp120 viral envelope protein leads to activation of lymphocyte function-associated molecule 1 (LFA-1) or $\alpha_L\beta_2$, facilitating formation of virologic synapses and increasing the efficiency of HIV-1 infection providing new targets for the virus[162].

1.12 Recovery of gut mucosa integrity

On the other hand, studies utilizing the SIV model showed that the major mechanism of early and near complete $CD4^+$ T cell restoration in GALT, involved trafficking of $CD4^+$ T cells from the periphery to gut mucosa during primary SIV infection[163-165]. Moreover, longitudinal follow-up studies revealed that the kinetics of restoration of the gut mucosal immune system in patients with HIV-1 infection during cART was substantially delayed and incomplete compared with that observed in the peripheral blood compartment and was independent of the time of initiation of cART[166]. This delay in mucosal immune restoration was attributed to incomplete suppression of viral replication in GALT during therapy and increased levels of local inflammation and immune activation[167].

Noteworthy, viral replication was detected in GALT of HIV-1-infected patients receiving cART despite undetectable viral loads in the peripheral blood[168]. The lack of complete viral suppression with the subsequent loss of $CD4^+$ T cells in the mucosa may contribute to discordant $CD4^+$ T cell restoration and viral suppression between GALT and peripheral blood[169].

HIV-1 COINFECTIONS

Thirdly, and finally, the vast majority of HIV-1-infected individuals are coinfected with other chronic viral infections or parasitic diseases, many of which get worse as a consequence of immunodeficiency during untreated HIV-1 disease, and may continue to contribute to systemic immune activation during treated HIV-1 infection[73].

1.13 Visceral leishmaniasis: *Leishmania infantum*

Visceral leishmaniasis (VL) caused by *Leishmania infantum* is a frequent disease among HIV-1 infected patients in countries of the Mediterranean basin[170-171] (*Leishmania* lifecycle in Figure 1.7). Despite cART, which indeed led to a reduction of its incidence, this disease still constitutes the third most frequent parasitic opportunistic infection in Europe[172]. Even in treated patients VL has case-fatality rates of 10-20%[173], especially in HIV-1-co-infected patients[174].

Leishmania and HIV-1 share some of the same target cells, namely macrophages and dendritic cells[175-176], and similar immune compromising mechanisms, most notably pro-inflammatory responses[177-178] and $CD4^+$ T cell lymphocyte depletion making immunosuppression as the hallmark of both *Leishmania*-HIV-1 pathogens[179].

The parasite infection in HIV-1-infected individuals is thought to induce chronic immune activation, viral replication and HIV-1 disease progression[180-181], diminishing their life expectancy[175]; whereas immunological disturbances caused by HIV-1, despite cART, are particularly favorable for the uncontrolled multiplication of the parasite[182], with increased parasite burden, drug resistance and frequent relapses[183-184].

Studies addressing microbial translocation in infectious diseases are scarce. Considering the involvement of microbial translocation in activation mechanisms in HIV-1-infected individuals[115], and the similar pathogenic features shared between VL and HIV/AIDS[185], it was also expected that gut parasitation by *Leishmania* amastigotes lead to mucosal barrier breach predisposing to microbial translocation in these patients. This phenomenon was, in fact, detected in VL[186-187], and may potentiated in co-infected patients contributing to the activation status by enhancing the plasma cytokine storm, aggravating the disease´s clinical outcome in *Leishmania*/HIV-1 co-infected individuals[188].

The contribution of microbial translocation to the pathogenesis of different infectious diseases with persistent immune activation is likely to vary. Thus, microbial translocation *per se* has been proposed as a common pathway causing disease progression that is shared by different pathogens, such as HIV-1, *Leishmania*, and HCV[189].

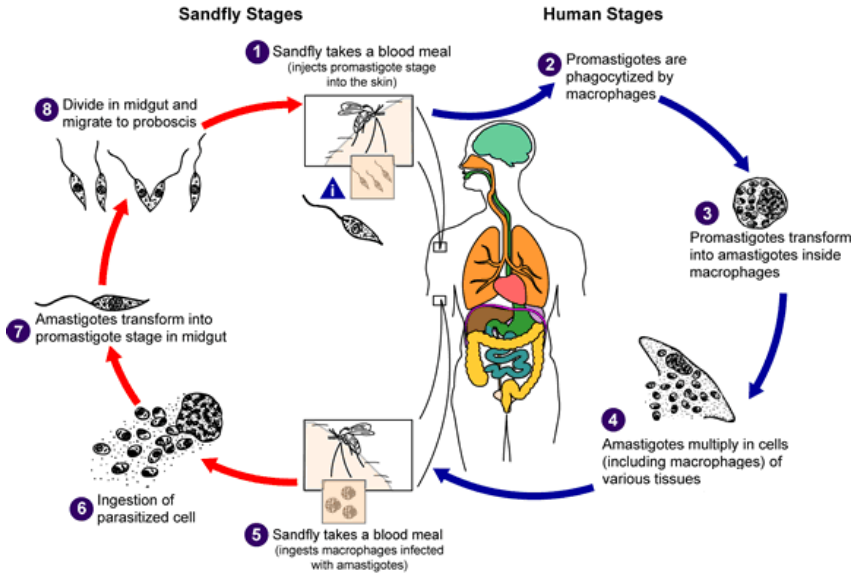

Figure 1.7 *Leishmania* life cycle (Centers for Disease Control and Prevention). Considering human stages, the female phlebotomies sandflies inject the promastigote form of *Leishmania* phagocytized by macrophages that reach the puncture wound. Promastigotes transform into amastigotes form within these cells, where they multiply and infect other mononuclear phagocytic cells and affect different tissues causing clinical manifestations of visceral leishmaniasis.

1.14 Hepatitis C virus (HCV)

The overall burden of HIV-1/HCV coinfection is estimated at 4 or 5 million people worldwide[190], finding the highest prevalence of HCV infection in HIV-1 infected individuals with a history of intravenous drug use, with a rate reported to be 82% to 93%[191-193]. In Europe, liver disease has emerged as a leading cause of death among HIV-1-infected individuals, mostly due to chronic viral hepatitis[194], increasing mortality from liver in coinfected individuals with the consumption of alcohol[195-196]. The increased mortality of coinfected individuals over that of HCV-monoinfected ones reflects accelerated progression of chronic liver disease with HIV-1-related immunosuppression[197-198].

The determinants of progression to cirrhosis and liver failure are poorly defined. One contributor to chronic inflammation and fibrosis in chronic HCV monoinfection is microbial translocation[199-202]. Microbial translocation may promote liver fibrosis either by direct interaction with Kupffer cells and hepatic stellate cells upregulating pro-inflammatory and pro-fibrogenic cytokines such as tumor necrosis factor TNF-α, IL-1, IL-6, and IL-12 or indirectly via induction of systemic immune activation and activation-induced apoptotic cell death[203-204].

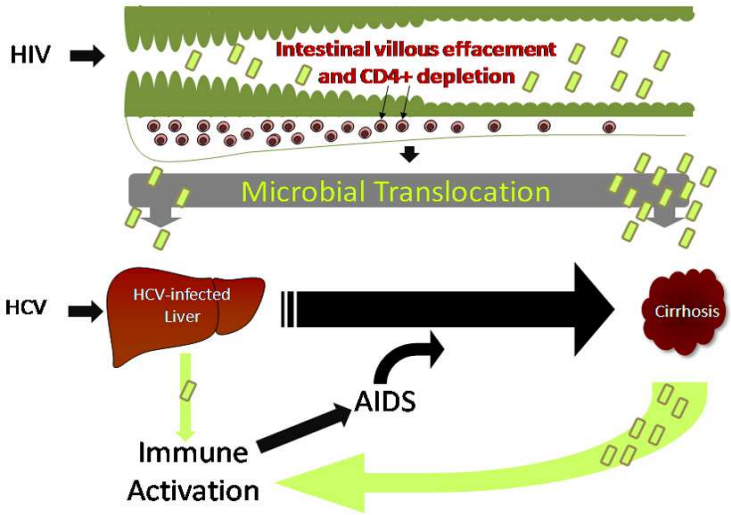

Figure 1.8 Microbial translocation due to HIV-1-related $CD4^+$ lymphocyte depletion contributes to both immune activation and progression of liver disease in HIV/ HCV coinfected individuals (Balagopal 2008[209]).

In addition, HIV-1/HCV seems to augment the local and systemic effects of microbial translocation caused by $CD4^+$ T lymphocyte depletion and immune activation, accelerating liver disease consistent with the chronic hepatic inflammation observed in chronic HCV infection[205-209] (See model below, Figure 1.8).

1.15 Human T-lymphotropic virus (HTLV)

During the 1980s and 1990s, Spain had one of the highest prevalence of drug injection in Europe[210]. Sharing needles practice led to the spread of different viruses among injection drug users (IDUs) and their sex partners, such as HIV-1, HCV and HTLV[211]. While the prevalence of HTLV-1 infection in Spain is low, there is a high prevalence of HTLV-2 infection specially in injecting drug users, reaching prevalence rates around 18% in prisoners, and around 5% outside the prison[212-214]. The molecular characterization of HTLV-2 isolates from North America and Europe demonstrated that HTLV-2a subtype was the predominant circulating variant in North America[215] and northern Europe[216], while HTLV-2b subtype was almost exclusive in southern Europe, particularly Spain and Italy[217-218], where co-infection with HCV and HIV-1 is frequent.

Up today, there is still no clear evidence that HTLV-2 causes any human disease, although it has been occasionally linked to neurological and lymphoproliferative disorders[219-221]. Several studies analyzing the effects of HTLV-2 on HIV-1 pathogenesis in dually infected HIV-1/ HTLV-2 individuals, revealed delayed progression of HIV-1 to AIDS[222-223] linked, in some cases, to a long-term nonprogressor phenotype[224-225].

This protective role is thought to be due to maintenance of $CD4^+$ T cells, lowering levels of HIV-1 replication and immune activation in dually infected HTLV-2/HIV-1 individuals compared to patients infected with HIV-1 only[226-228]. Additionally, several IDU long-term non-progressors (LTNPs) with stable CD4 counts, in the absence of ART, have been reported to be infected with HTLV-2[229], and the median value of HTLV-2 proviral loads (pVL) tended to be higher in the LTNP coinfected group compared to those who required antiretroviral treatment[230].

The possible mechanism underlying the milder HIV-1 disease course has been attributed to the modulation of cytokine and chemokine networks by HTLV-2 that would modify innate host immune responses. In this way, HTLV-2 induces the expression of three CC-chemokines, natural ligands for CCR5 from PBMCs and monocytes; Macrophage inflammatory protein 1-alpha (MIP-1α)/CC-Chemokine ligand-3 (CCL3); MIP-1β/ CCL4; and RANTES (Regulated upon activation normal T-cell expressed and secreted (RANTES)/CCL5 inhibiting HIV-1 entry by binding to CCR5[231-233], and the concomitant downregulation of the CCR5 receptor on lymphocytes[234-235], interfering with HIV-1 infection process (Figure 1.9).

Notably, different viruses have specific cytokine and chemokine requirements for maintenance in vivo. What appears to be optimal for one strain may be detrimental for another[236-238]. Therefore, the reported upregulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-γ secretion[239], which can also inhibit CCR5 expression contributing to slow HIV-1 disease progression[240]. In fact, it has been demonstrated in a clinical trial conducted on AIDS patients that GM-CSF decreases viral replication and increases the number of circulating $CD4^+$ T cells, the primary target of HIV-1[241]. In addition, IFN-γ is the pivotal cytokine triggering a phagocyte-dependent Th1 response leading to CTL response against invading pathogens[242].

In this regard, a poor Th1 response and a dominant Th2 response have been implicated in the pathogenesis and progression of HIV-1 infection[243-244]. Thus, HTLV-2 infection may exert protective effect on HIV-1 disease progression inducing Th1 response against invading pathogens via up-regulating IFN-γ expression[239,242].

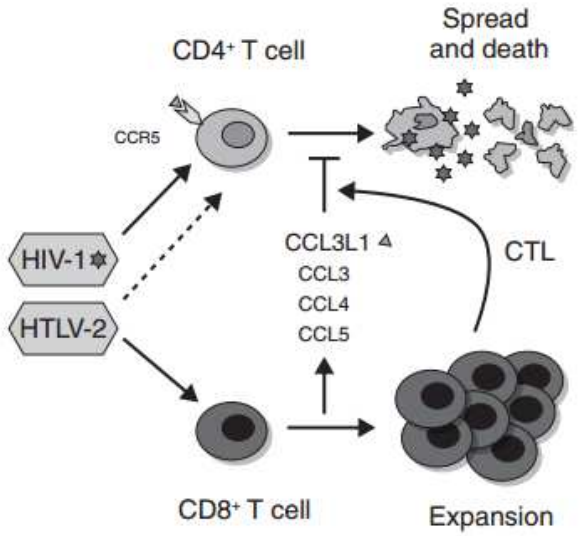

Figure 1.9 Scheme illustrating how HTLV-2 may interfere on HIV-1 pathogenesis. The HTLV-2 infected $CD8^+$ T cells and their expansion induces the secretion of CC-chemokines, which can reinforce the cytotoxic T lymphocyte response against $CD4^+$ HIV-1 infected cells (Casoli 2007[227]).

On the other hand, clinical features of coinfected individuals have been overlooked, despite its high frequency among injecting drug users[245]. Only higher frequencies of severe fibrosis has been found among HCV/HTLV-2 individuals compared to HCV-monoinfected group, although no significant[246]. Consistent with the fact that hepatic measures were reported to be worse in HTLV-1/ HCV coinfected individuals with more severe immunosuppression[247], cellular immune response should be analyzed in HCV/HTLV coinfected individuals to elucidate the effects on HTLV-2 on HCV infection and progression of liver disease[248].

From public health perspective, prevention of HTLV-1/2 is crucial as there is no current treatment for this infection. Several studies analyzed the effects of antiretroviral agents widely used for treating HIV-1 infection on HTLV virus expression, showing no effects on HTLV proviral burden or HTLV mRNA expression[249-251].

Raltegravir was an integrase inhibitor approved for HIV-1 infection treatment. Given the similarities between HIV-1 and HTLV integrases, raltegravir was thought to be effective against HTLV-1. Furthermore, two studies reported a decrease HTLV-1 pVL *in vitro* and also *ex vivo*[252-253]. Despite these promising *in vitro* results, no decrease on HTLV-1 pVL was reported in the unique *in vivo* study[254], attributed to the lack of new rounds of viral replication that is mainly maintained through cell division[255].

AIMS OF THE STUDY

Despite viral suppression and partially immune recovery, persistent increased immune activation continues in HIV-1-infected individuals. Microbial translocation and the subsequent pro-inflammatory cytokines expression has been proposed as the major driver mechanisms of HIV-1-associated immune activation and inflammation, also influenced by frequent HIV-1-coinfections.

The aim of the study was to analyze microbial translocation, cytokines profile, and immune activation in different scenarios, involving, HIV-1-infected individuals on treatment intensification, HIV-1-infected individuals with different immune response to antiretroviral therapy, and HIV-1-infected individuals with other coinfections, such as *Leishmania*, HCV and HTLV.

Objective 1. To evaluate in a longitudinal study the effects of treatment intensification with maraviroc or raltegravir and further intensifying drug discontinuation on immune activation, microbial translocation and dynamics of T cell subsets including expression of gut homing β7 receptor on T cells.

Objective 2. To determine correlations between different techniques employed to measure microbial translocation, including levels of LPS, sCD14, LBP and 16S rDNA, and between all these techniques and immune activation in HIV-1 infected patients on treatment intensification.

Objective 3. To investigate in a cross-sectional study how visceral leishmaniasis impacts immune activation compared to two control groups consisted of HIV-1 infected both immunological concordant and non-responders to antiretroviral therapy, and which factors could impair immune response in these individuals, including inflammation, microbial translocation and immunosenescence.

Objective 4. To investigate in a cross-sectional study how HTLV-2 infection influences both HIV-1 disease progression and liver disease associated to HCV infection. Immune activation, microbial translocation, cytokine profile and HTLV-2 proviral load are determined for this purpose.

Objective 5. Evaluation of HTLV-2 proviral load in coinfected HIV-1/HTLV-2 individuals ongoing treatment intensification with raltegravir.

Material and Methods

FLOW CYTOMETRY STAINING

Many immunologically important cells can be defined based on proteins present on their membrane. Human clusters of differentiation (CD) are used to classify many epitopes on the cell surface of leukocytes. The study of cellular characteristics using flow cytometry involves the use of fluorescent molecules such as fluorophore-labeled antibodies that binds to a specific molecule on the cell surface or inside the cell. In further analysis through flow cytometer, when laser light of the right wavelength strikes the fluorophore, a fluorescent signal is emitted and detected by the flow cytometer, demonstrating the presence of both superficial and intracellular proteins. The combination of several immunological markers is used to define cellular immune functions or properties.

The commercial antibodies used to study cellular characteristics using flow cytometry are shown in Table 2.6. These antibodies had the following fluorochromes FITC (Fluorescein Isothiacyanate), PE (Phycoerythrin), PerCP (Peridinin Chlorophyll Protein Complex), PE-Cy7 (Phycoerythrin-Cyanin7), APC (Allophycocyanin), APC700 (Allophycocyanin 700), APC-Cy7 (Allophycocyanin-Cyanin 7), eF450 (eFluor™ 450) and KO (Krome™ Orange) directly conjugated to the antibodies used as described above. Fluorocrhome peack excitation and emission wavelengths are also shown in Table 2.1. All antibodies against different CDs were purchased from Becton Dickinson (Becton Dickinson, NJ, USA).

CELL STAINING AND FLOW CYTOMETRY

Briefly, 100 µl of blood were lysed with 200 µl of FACS lysing solution (Becton Dickinson, NJ, USA) for 30 min at room temperature, incubated with the corresponding superficial antibodies during 20 min at 4ºC, washed twice with phosphate-buffered saline (PBS) containing 1% azida, fixed with BD Cytofix™ (Becton Dickinson, NJ, USA) for 10 min at 4ºC and finally washed and resuspended in PBS containing 1% azida. An unstained control was performed for all samples.

Table 2.1 Fluorochrome-conjugated antibodies used for flow cytometry staining.

Excitation Laser	Antibody	Fluorocrhome	Excitation Max (nm)	Emission Max (nm)
638 nm Red	HLA-DR	APC-Cy7	650	774
	CD38	APC700	696	719
	β7	APC	645	660
488 nm Blue	CCR7	PE-Cy7	496, 565	774
	CD8	PerCP	482	675
	CD45RA	PE	496, 565	575
	CD57	FITC	493	525
405 nm Violet	CD3	eF450	410	455
	CD4	KO	398	528

Cells stained with fluorochrome-conjugated antibodies were visualized using flow cytometry using a Gallios flow cytometer (Beckman-Coulter, California, US). Flow cytometry allows the analysis of multiple parameters of individual cells within hererogeneous populations. The flow cytometer analysis is performed by passing thousands of cells through a laser beam and capturing the light that emerges from each cell as it passes through one at a time. As a cell passes through the laser, it will refract or scatter light at all angles.

Forward scatter (FSC), or low-angle light scatter, is the amount of light that is scattered in the forward direction as the laser light strikes the cell. The magnitude of forward scatter is roughly proportional to the size of the cell.

Light scattering at larger angles, to the side (SSC), is caused by granularity and structural complexity inside the cell. The generation of two-dimensional dot or scatter plots, using forward and side scatter, allow us to distinguish between cell populations.

The scatter plot from a peripheral blood cell run is shown below (Figure 2.1). The populations that emerge include lymphocytes which are small cells possessing low internal complexity; monocytes which are medium-sized cells with slightly more internal

complexity, and neutrophils and other granulocytes which are large cells that have a lot of internal complexity.

A threshold was set to avoid information coming from a very large number of minute particles, like platelets and debris. Thereby, the majority of events that the cytometer collects are the cells of interest, despite small particles are still passing through the instrument.

Gallios flow cytometer can analyze 12 parameters, FSC, SSC and 10 different channels simultaneously, allowing to gather statistical data on large number of cells and to use that information to correlate multiple parameters within a cell population.

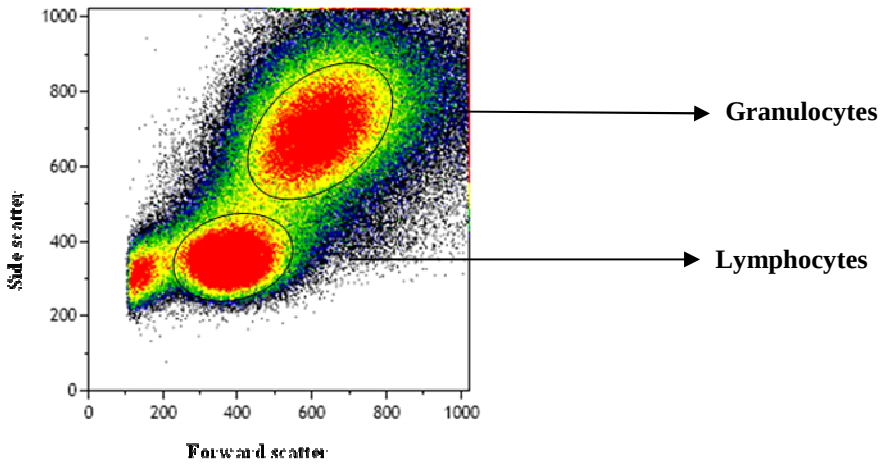

Figure 2.1 Populations emerged from a peripheral blood run defined by Forward and Side scatter.

At least 10^5 $CD3^+$ T cells were collected for each sample and analyzed up to nine different parameters with Kaluza software v.1.1 (Beckman coulter) by initially gating lymphocytes according to morphological parameters. Gating was always the same between the different time points. At least 20000 events were gated for the T cell subsets.

MICROBIAL TRANSLOCATION

CD14 is a glycoprotein that mediates the interaction of lipopolysaccharide (LPS, endotoxin) with cells, thereby signaling the presence of gram-negative bacteria. The binding of LPS to CD14 requires an acute phase protein, LPS-binding protein (LBP). These three factors appear to participate in a complex feedback mechanism of immune regulation involving both up-regulation and down-regulation of the inflammatory process triggered by LPS. The 16S ribosomal RNA, however is a component of the 30S small subunit of prokaryotic ribosomes, and thus, presented in all bacteria, gram-negative and gram-positive.

LIPOPOLYSACCHARIDE (LPS)

Lipopolysaccharides (LPS) are found in the outer membrane of Gram-negative bacteria. Levels of LPS are determined in duplicated 50 µl of plasma samples using a quantitative test QCL-1000 Limulus Amebocyte Lysate (Lonza®, Basel, Switzerland). This method utilizes lyophilized lysated prepared from the circulating amebocytes of the horseshoe crab *Limulus polyphemus* which was an extremely sensitive indicator of the presence of endotoxin. This technique requires to be used with appropriate precautions to avoid microbiological or endotoxin contamination.

SOLUBLE CD14 (SCD14)

CD14 is expressed mainly by macrophages, neutrophils and dendritic cells. CD14 acts as a co-receptor with Toll-like receptor-4 (TLR4) for the detection of bacterial lipopolysaccharide (LPS), to which it binds in the presence of lipopolysaccharide-binding protein (LBP). The soluble form of the receptor (sCD14) is secreted mainly by monocytes.

Levels of soluble CD14 were measured in duplicated 200-fold diluted plasma samples using the Quantikine® Human sCD14 Immunoassay (R&D Systems,

Minneapolis, Minnesota, US). This assay employs the quantitative sandwich enzyme immunoassay technique (ELISA), in which a monoclonal antibody specific for sCD14 has been pre-coated onto a microplate.

LIPOPOLYSACCHARIDE-BINDING PROTEIN (LBP)

Lipopolysaccharide-binding protein (LBP) is a soluble protein that binds to bacterial lipopolysaccharide (LPS) to elicit immune responses by presenting the LPS to the receptor CD14 and the pattern recognition receptor TLR4.

Levels of LBP were determined in 800-fold diluted plasma samples, run in duplicate, using the Enzyme Immunoassay for Quantification of free human LBP kit (Enzo Life Siences, Farmingdale, NY).

However, LBP levels were only determined at baseline, at week 48 after treatment intensification, and at weeks 12 and 24 after intensifying drug discontinuation.

BACTERIAL 16S RIBOSOMAL DNA (16S rDNA)

Because LPS is a component of cell wall of gram-negative bacteria, present on a proportion of enteric bacterial microbiota, the well-conserved 16S ribosomal DNA (16S rDNA) subunit common to most bacteria was also quantified during the follow-up 48 weeks of treatment intensification.

The bacterial 16S rDNA was quantified from the DNA extracted from 200 µl of plasma using QIAamp DNA kit (Qiage, Hilden, Germany). DNA concentrations were determined using ND-1000 spectrophotometer, according to manufacturer´s protocol. DNA was kept at -20ºC until further use.

Standard plasmids were generated through the amplification of partial 171bp 16S rDNA gene. Reaction mixtures were performed in a total volume of 25 µl containing PCR Buffer II (AccuPrime Taq High Fidelity, Bioline, London, UK), 0,5 U Taq

polymerase (AccuPrime Taq High Fidelity, Bioline, London, UK), 200 ng of genomic DNA from the plasma of diagnosed HIV-1-infected individual, and 20 pmol of the following primer pairs were used, (16S-1/ 16S-2) for partial bacterial 16S rDNA gene, in the Table below (Table 2.2). Thirty-five cycles of denaturation at 95ºC for 15s, annealing at 56ºC for 10s, and extension at 68ºC for 20s, followed by a final extension at 68ºC for 2min were performed.

Table 2.2 List of oligonucleotides used for generating the 16S rDNA standard plasmids.

Primer	Sequence	Length (bp)
16S-1	5´-TAGCGATTCCGACTTCATGGA-3´	171bp
16S-2	5´-ACACACGTGCTACAATGGC-3´	

Standard plasmids were generated by cloning PCR amplicon products in a pUC19 plasmid DNA following the instructions provided by TOPO® Cloning reactions (TOPO TA Cloning® kits, Invitrogen, Life Technologies, US) according to manufacturer´s protocol. Subsequently, competent One Shot® *Escherichia coli* were transformed and diluted bacteria were spread plated with Ampicillin (100 µg/ml) and incubated at 37ºC overnight (See Molecular Cloning 2.6.2).

The presence of the cloned amplicon was verified in some colonies by colony PCR using the same amplification conditions for 171 bp 16S rDNA gene explained above. The selected colonies were grown in 2 ml of LB liquid medium overnight, and plasmid DNA was extracted from transformed bacteria using the QIAprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany).

A standard curve for copy number quantification were generated in each run using six 5-fold serial dilutions of each plasmid specific amplicon template.

The bacterial 16S rDNA levels in plasma of HIV-1-infected patients were determined by real time PCR assay, with newly designed primers and probe are described in Table 2.3. A TaqMan® Probe (Sigma-Aldrich, Missouri, US) was used with 6-FAM™ and TAMRA as a fluorophore and a quencher, respectively.

Since any contaminating DNA is likely to be co-amplified and could interfere with quantification, we considered to perform a DNase treatment of commercial amplification FastSart Buffer kit prior to proceed with the real-time quantification. Thus, LightCycler FastStart DNA Master PLUS HybProbe 5X (Roche Diagnostics) was treated with DNase I (Invitrogen, Life Technologies, US) at 37ºC for 10 min followed by 10 min at 95ºC to inhibit the enzyme activity, according to manufacturer´s indications.

Table 2.3 Primers and probes used for partial 16S rDNA gene quantification by RT- PCR.

Primer	Sequence	Length (bp)
16S-3	5´-TTGCAGACTCCAATCCGGACT-3´	122bp
16S-4	5´-GCATAGAAAGAGAAGCGACCT-3´	
16S Taqman Probe	5´-CAGCGTCAAAGGTGAGGAGTGGGTGTCG-3´	

Since any contaminating DNA is likely to be co-amplified and could interfere with quantification, we considered to perform a DNase treatment of commercial amplification FastSart Buffer kit prior to proceed with the real-time quantification. Thus, LightCycler FastStart DNA Master PLUS HybProbe 5X (Roche) was treated with DNase I (Invitrogen, Life Technologies, US) at 37ºC for 10 min followed by 10 min at 95ºC to inhibit the enzyme activity, according to manufacturer´s indications.

The amplification reaction was carried out in triplicate using the capillary-based LightCycler® 2.0 (Roche). Standards curves were preparared for each amplification and analyzed at each PCR-run, including negative and positive controls. Reactions were carried out in triplicate in a total volume of 20 µl containing LightCycler FastStart DNA Master PLUS HybProbe 5X (Roche), 200 ng of DNA, 50 pmol of each primer, 16S-3/ 16S-4 along with 2 pmol of the following 16S Taqman probe. The cycling parameters included a hot start at 95ºC 10 min, and continued with 40 cycles of denaturation at 95ºC for 10s, annealing at 55ºC for 10s and extension at 72ºC for 15s.

The Ct values were converted into template quantity using the standard curve method, with PCR efficiency >90% and R^2 correlation superior to 0.9, avoiding replicates that have more than 0.5 Ct of difference. Levels of 16S rDNA were calculated and the final results were expressed as 16S rDNA DNA copies per microliter of plasma sample.

ANTIRETROVIRAL TREATMENT INTENSIFICATION

In the frame of two pilot open-label phase II clinical trials, we analyzed the effects and dynamics of treatment intensification and intensified treatment discontinuation on immune activation, T cell subsets, expression of gut homing β7 receptor on T cells and microbial translocation in 16 HIV-1-infected patients of whom seven intensified with maraviroc, a CCR5 antagonist, and the other nine patients with raltegravir, an integrase inhibitor. We also analyzed the correlations between different measurements of microbial translocation including lipopolysaccharides (LPS), soluble CD14 (sCD14), lipopolysaccharide-binding proteins (LBP) and quantification of 16S ribosomal DNA (16S rDNA), and also the association between microbial translocation and either immune activation, T cell subsets or expression of gut homing β7 receptor on T cells.

2.1 Study design and ethic statement

Two independent pilot prospective open-label clinical trials were performed to evaluate the effect of maraviroc (MVC), a CCR5 antagonist (developed and provided by Pfizer, Inc., US) or raltegravir (RAL), an integrase inhibitor (developed and provided by Merck, New Jersey, US) on the HIV-1 latent reservoir in 16 HIV-1 infected patients. Both clinical trials (NCT00795444 and NCT00807443) were conducted at the Hospital Universitario Ramón y Cajal, in Madrid, Spain, between 2008 and 2011 with an intensification period of 48 weeks and a follow-up of 24 weeks after drug discontinuation. Biochemical, immunological and virological parameters were assessed at baseline and at weeks 12, 24, 36 and 48 of treatment intensification, and at weeks 12 and 24 weeks after intensifying drug discontinuation.

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our local Independent Ethics Committee (Hospital Ramón y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

2.2 Patients and specimen collection

Sixteen patients (seven from MVC trial and 9 from RAL trial) completed the intensification phase of the study; however one individual from RAL trial declined to participate in the follow-up 24 weeks after intensifying drug discontinuation, so finally fifteen patients completed the follow-up period of 24 weeks.

Plasma viral load was measured by quantitative RT-PCR with a detection limit of 40 copies/ml (Roche Taqman HIV-1 test; Roche Molecular Systems), and T-lymphocyte counts were determined by flow cytometry.

The main inclusion criteria were as follows: HIV-1 infected adults receiving antiretroviral treatment for at least 2 years; undetectable plasma viral load (below 40 copies HIV-1 RNA/ml) for at least two years; $CD4^+$ T-cell count above 350 cells/mm^3; and no previous experience with MVC or RAL. Patients were excluded if they had experienced virological failure, received any immunosuppressive or immunomodulatory therapy, were or planned to become pregnant, or planned to interrupt treatment for any reason. A total of 50 ml of whole blood with ethylenediaminetetraacetic acid (EDTA) was drawn for plasma and isolation of peripheral blood mononuclear cells (PBMCs). Plasma samples were stored at -80ºC and PBMCs in liquid nitrogen until further use.

2.3 Immune activation

Fresh EDTA anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell activation with the following antibody combination: CD3-eFluor™ 450 (eF450), CD4-Krome™ Orange (KO), CD8-peridinin chlorophyll protein complex (PerCP), CD38-

Allophycocyanin-700 (APC-700), and HLA-DR-allophycocyanin-Cy7 (APC-Cy7). Also staining with β7-allophycocyanin (APC) to analyze the expression of gut homing β7 on both T cells or activated T cells, respectively. All the antibodies (Becton Dickinson, NJ, USA) are shown in Table 2.1.

2.4 T cell subsets

Lymphocyte subpopulations were defined as naïve ($CD45RA^+CCR7^+$), T central memory ($CD45RA^+CCR7^+$, TCM), T effector memory ($CD45RA^+CCR7^+$, TEM), and T effector memory RA^+ ($CD45RA^+CCR7^+$, TemRA). To analyze lymphocyte subsets this antibody combination was used: CD3-eFluor™ 450 (eF450), CD4-Krome™ Orange (KO), CD8-peridinin chlorophyll protein complex (PerCP), CD45RA-phycoerythrin (PE) and CCR7-allophycocyanin (APC). Additionally, staining with β7-allophycocyanin (APC) was used to analyze the expression of gut homing β7 on both T cells or activated T cells, respectively. All the antibodies (Becton Dickinson, NJ, USA) are shown in Table 2.1.

2.5 Microbial translocation

Microbial trnalocation was measured in plasma by two commercial kit assays according to the manufacturer´s protocol. Plasma bacterial LPS was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza, Basel, Switzerland), plasma sCD14 was quantified using the Quantikine Human sCD14 Immunoassay (R&D Systems, Minneapolis, MN) and plasma LBP was measured by LBP soluble ELISA kit (Enzo Life Sciences, Farmingdale, NY). Real-time quantification was used to perform the bacterial 16SrDNA.

2.6 Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and discrete variables as percentages. The *t* test for independent samples was used to

compare normally distributed continuous variables and the Mann-Whitney U test to compare non-normally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. The Spearman correlation coefficient was used to analyze the correlations between continuous variables. Statistical analysis was performed using IBM SPSS software 21.0 (IBM, Chicago, Illinois, USA).Graphs were generated using GraphPad Prism 5.01 (La Jolla, California, USA).

COINFECTION WITH LEISHMANIA COMPARED TO IMMUNOLOGICAL DISCORDANT RESPONSE TO ANTIRETROVIRAL THERAPY

In this cross-sectional comparative study, we analyzed levels of microbial translocation, immune activation, inflammation through IL-6 levels, and immune senescence in HIV-1 infected patients with concordant immunological response to antiretroviral therapy, compared to infected individuals with discordant immunological response to treatment. We also studied correlations between microbial translocation measured by LPS and soluble CD14 levels, and either markers of inflammation or immune activation.

2.7 Study design, ethic statement and sample collection

A cross-sectional, descriptive and comparative study was performed in HIV-1 infected patients at the Hospital Universitario Ramón y Cajal, in Madrid, Spain. A total of 29 HIV-1-infected adults with undetectable viral load (lower than 40 HIV-1 copies/ml) and receiving antiretroviral therapy for at least one year were analyzed. 15 of these HIV-1 infected adults showed concordant immunological response to the therapy, with $CD4^+$ T cell count above 400 cells/mm^3, while the other 14 HIV-1-infected patients showed discordant immunological response with $CD4^+$ T cell count below 200

cells/mm^3. Whole blood was collected in Vacutainer-EDTA tubes (Becton-Dickinson, Madrid, Spain) for plasma and PBMCs isolation. Plasma samples were stored at -80ºC while the PBMCs were stored in liquid nitrogen until further use.

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the Institution Review Board and Ethics Committee (Hospital Ramón y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

2.8 Immune activation and Immune senescence

Fresh EDTA anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell activation with the following antibody combination: CD3-eFluor™ 450 (eF450), CD4-Krome™ Orange (KO), CD8-peridinin chlorophyll protein complex (PerCP), CD38-Allophycocyanin-700 (APC-700) and HLA-DR-allophycocyanin-Cy7 (APC-Cy7). Immunosenescence was defined as the expression of CD57-fluorescein isothiacyanate (FITC), and the absence of CD28-allophycocyanin-H7. All the antibodies (Becton Dickinson, NJ, USA) are shown in Table 2.1.

2.9 Inflammation

Inflammation was measured by plasma interleukin 6 (IL-6) quantification using hsIL-6 (Human IL-6 Quantikine High-Sensitive ELISA kit, R&D Systems). IL-6, along with TNF-α and IL-1, drives the acute inflammatory response and is important in the transition from acute inflammation to either acquired immunity or chronic inflammatory disease.

2.10 Microbial translocation

Microbial translocation was measured in plasma by the quantification of both lipopolysaccharide (LPS) using QCL-1000 Limulus Amebocyte Lysate (Lonza®, Basel,

Switzerland) and soluble CD14 using the Quantikine® Human sCD14 Immunoassay (R&D Systems, Minneapolis, Minnesota, US), according to manufacturer´s protocol. LPS-bound CD14 signals production of inflammatory cytokines and other inflammatory proteins.

2.11 Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables and the Mann-Whitney U test to compare non-normally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. The Spearman correlation coefficient was used to analyze the correlations between continuous variables. Statistical analysis was performed using IBM SPSS software 21.0 (IBM, Chicago, Illinois, USA).Graphs were generated using GraphPad Prism 5.01 (La Jolla, California, USA). Multivariate lineal regression analysis to identify variables associated with immune activation as dependent variable.

COINFECTION WITH HTLV-2 IN BOTH HIV-1/HCV COINFECTED AND HIV-1 MONOINFECTED INDIVIDUALS

In this cross-sectional comparative study, we evaluated the influence of HTLV-2 infection in both HIV-1/HCV coinfected and HIV-1 monoinfected individuals. With this purpose, immune activation, microbial translocation, cytokine profile, HTLV-2 proviral load, among other variables were studied.

2.12 Screening for HTLV infection among intravenous drug users infected by HIV-1

Following the diagnostic algorithm proposed by the HTLV European Research Network (HERN) criteria, the initial screening of HTLV-1/2 infection is based mainly on testing for antibodies by enzyme-linked immunosorbent assays (ELISA). Repeatedly reactive ELISA samples were confirmed by western blot. However, in some indeterminate cases, due to the showed bands pattern it is not possible to confirm HTLV-1 or HTLV-2 infection by WB and it is necessary to perform a supplementary assay such specific polymerase chain reaction (PCR) in order to further confirm HTLV-1 or HTLV-2 infection (HERN, 1996). Figure 2.2 summarizes the diagnostic algorithm proposed by the HERN for HTLV and other related infections.

A total of 1673 HIV-1 infected patients with regular medical appointments in the Hospital Ramón y Cajal, Madrid, Spain, at the end of the 2010, were analyzed. From these patients, 798 individuals had a history of intravenous drug use with undetectable viral load (<50 copies/ml) on antiretroviral therapy at least for one year. All these patients were included for the screening of HTLV-1/2 infection, following the aforementioned diagnostic algorithm proposed by the HTLV European Research Network (HERN) criteria.

- **ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)**

Qualitative detection of various specific IgA, IgG and IgM antibodies against HTLV-1 and HTLV-2 found in the collected human plasmas, using a commercial third generation direct sandwich immunoassay, BioELISA HTLV I+II 5.0 (Biokit, S.A., Barcelona, Spain), that utilizes a combination of recombinant proteins and a tri-fusion recombinant protein labeled with horseradish peroxidase.

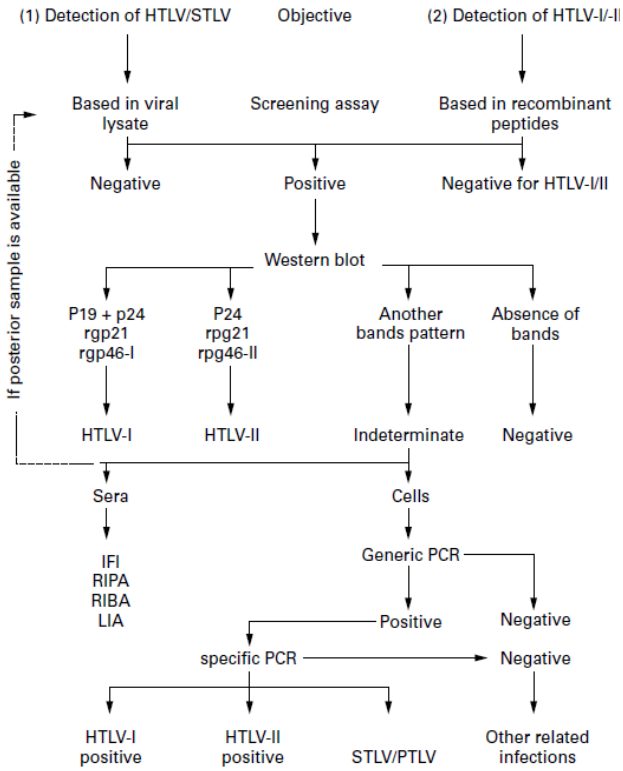

Figure 2.2. HERN algorithm for the diagnosis of HTLV-1/2 infections in seroprevalence studies.

- **WESTERN BLOT (WB)**

Repeatedly reactive ELISA samples were confirmed by western blot (WB) technique in plasma, using the commercial HTLV blot 2.4 Western Blot assay (MP Diagnostic Technology, Singapore). This test is a qualitative enzyme immunoassay for the *in vitro* detection of antibodies to core (*gag*) and envelope (*env*) proteins of HTLV-1 and HTLV-2.

A WB is considered HTLV-1 positive if band for *gag* proteins p19 with or without p24 and *env* recombinant proteins rgp21 (GD21) and rgp46-I (MTA-1), and it is HTLV-2 positive if gag proteins p19 with or without p24 and env recombinant proteins rgp46-I (K55). It is considered to be indeterminate when HTLV specific bands are detected but

does not meet criteria for HTLV-1 or HTLV-2 infection due to its reactivity to any other proteins (HERN, 1996) (Figure 2.3). In those cases of indeterminate WB, additional specific PCR for HTLV-1 or HTLV-2 has to be performed to obtain conclusive diagnosis of HTLV-1 or HTLV-2 infection.

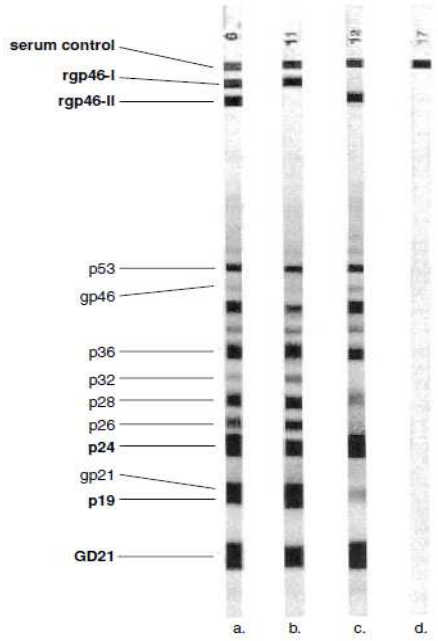

Figure 2.3. Interpretation of results based on bands pattern by WB technique. The specific bands as visualized with a- A HTLV-I/ II dual infection serum, b- Strong reactive control I (reactive for HTLV-1 only), c- Strong reactive control II (reactive for HTLV-2 only), d- Non-reactive control.

2.13 Patients`characteristics

Sixty-one HTLV-2-infected individuals with undetectable HIV-1 viral load on suppressive regimen at least for one year, were selected for the study. Forty of them were also infected by HCV.

Ninety HIV-1 infected individuals seronegative for HTLV-2 infection with undetectable HIV-1 viral load on suppressive regimen at least for one year, were selected as control group for this study. Forty-two of them were also infected by HCV.

Anticoagulated-EDTA whole blood drawn for plasma and isolation of peripheral blood mononuclear cells (PBMCs). Plasma samples were stored at -80ºC and PBMCs in liquid nitrogen until further use.

The clinical trials from which we obtained DNA samples were approved by the Ethics Committee of both Hospitals and all recruited patients signed an informed consent form.

2.14 HTLV-2 proviral load

Sixty-one HTLV-2-infected individuals with undetectable HIV-1 viral load on suppressive regimen at least for one year, were selected for the study. Forty of them were also infected by HCV.

- **STANDARD CURVES**

Partial 267 bp of HTLV-2 *tax* and 211 bp of cellular GAPDH genes were amplified by PCR. For both amplifications, reaction mixtures were performed in a total volume of 25 µl containing PCR Buffer II (AccuPrime Taq High Fidelity, Bioline, London, UK), 0,5 U Taq polymerase (AccuPrime Taq High Fidelity, Bioline, London, UK), 200 ng of genomic DNA from a diagnosed HIV-1/ HTLV-2b coinfected individual, and 20 pmol of the following primer pairs were used, (GAPDH-1/ GAPDH-2) and (Tx5/ Tx6) for partial cellular GAPDH and viral *tax* genes, respectively, as shown in the Table below (Table 2.4). For GAPDH gene amplification, thirty-five cycles of denaturation at 95ºC for 15s, annealing at 60ºC for 8s, and extension at 68ºC for 20s, followed by a final extension at 68ºC for 2 min were performed, while for *tax* gene amplification, thirty-five cycles of denaturation at 95ºC for 15s, annealing at 56ºC for 8s, and extension at 68ºC for 20s, followed by a final extension at 68ºC for 2 min were used.

Table 2.4 List of oligonucleotides used for generating the standard plasmids.

Primer	Sequence	Length (bp)
GAPDH-1	5´-GTGGACCTGACCTGCCGT-3´	211bp
GAPDH-2	5´-CCAGAAATGAGCTTGAC-3´	
Tx-5	5´- ACCCAGAGAACCGCCAAG-3´	267bp
Tx-6	5´-GTATAAGTGGCCAGGTCA-3´	

PCR amplicon products were cloned products in a pUC19 plasmid DNA following the instructions provided by TOPO® Cloning reactions (TOPO TA Cloning® kits, Invitrogen, Life Technologies, US) according to manufacturer´s protocol. Ligation between amplicon and plasmid vector was performed using the T4-DNA ligase enzyme (Sigma-Aldrich, Missouri, US).

Competent One Shot® *Escherichia coli* were transformed chemically through cells heat-shock with both cloning reactions containing one copy of HTLV-2 *tax* fragment or one copy of human genomic GAPDH gene fragment. Each transformation was spread in Luria Broth (LB) medium (pancreatic extract to casein, yeast extract, NaCl) plates to which Ampicillin (100µg/ml) was added (Invitrogen, Life Techonologies, US) and incubated at 37ºC overnight.

The presence of the cloned amplicon was verified in some colonies by colony PCR using the same amplification conditions for 211 bp GAPDH or 267bp *tax* partial genes explained above. The PCR amplicons were run on an agarose gel to check whether fragments of interest were amplified and, hence, inserted into the plasmid. The selected colonies were grown in 2ml of LB liquid medium overnight.

Plasmid DNA was extracted from transformed bacteria using the QIAprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany), allowing purification of high-purity plasmid DNA. Bacterial cultures are lysed and the lysates are cleared by centrifugation and the applied to the unique silica membrane that adsorbs plasmid DNA. Impurities are washed away and pure DNA is eluted in a small volume of UltraPure DNase/RNase-Free Distilled Water (Life Technologies, New York, US). DNA concentrations were determined using the NanoDrop® ND-1000 spectrophotometer.

A standard curve for copy number quantification were generated in each run using six 5-fold serial dilutions of each plasmid specific amplicon template.

- **PERIPHERAL PBMCS ISOLATION**

The Ficoll® density gradient separation (Lymphocytes Isolation Soution, Rafer, S.L., Zaragoza, Spain) was used for separation of mononuclear cells, such as monocytes and lymphocytes, with the lymphocyte population comprised of T cells, B cells and NK cells, from 4ml of heparinized whole blood.

Differential migration during centrifugation results in the separation of cell types into different layers. The bottom layer contains aggregated red blood cells. Immediately above this, a Ficoll® layer containing granulocytes and PBMC is found. Due to a slightly lower density, PBMCs sediment at the interface between the Ficoll® and the uppermost plasma/platelet layer (Figure 2.4). PBMCs were cryopreserved until needed.

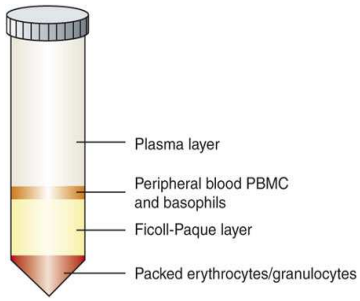

Figure 2.4 Layers after Ficoll®density gradient centrifugation.

- **DNA EXTRACTION**

DNA was extracted from cryopreserved PBMCs using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), allowing purification of high-quality genomic DNA.

In this system, DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors, such as divalent cations and proteins, are completely removed in thwo efficient wash steps, leaving pure DNA to be eluted in UltraPure DNase/RNase-Free Distilled Water (Life Technologies, New York, US).

DNA concentrations were determined using the NanoDrop® ND-1000 spectrophotometer by measuring the OD_{260} according to manufacturer´s protocol. The OD_{260}/ OD_{280} ratio was also calculated for an indication of nucleic acid purity. DNA was kept at -20ºC for further use.

- **REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)**

HTLV-2 proviral load from genomic DNA isolated from cryopreserved PBMCs from infected individuals was determinate by real time PCR assay, with newly designed primers and probes.

A TaqMan® Probe (Sigma-Aldrich, Missouri, US) was used for HTLV-2 *tax* gene quantification. The TaqMan method, first reported in 1991 by Holland et al., relies on the 5´-3´ exonuclease activity of the *Taq* DNA polymerase to cleave a dual labeled probe when it is hybridized to a complementary target during each annealing step of the PCR run. The double-strand-specific 5´-3´ nuclease activity of the *Taq* enzyme displaces the 5´end of the probe releasing the fluorophore (6-FAM™) from the quencher, (TAMRA) and leading to increase in fluorescence from the reporter.

Otherwise, hybridization probes (Sigma-Aldrich, Missouri, US) developed by Roche (Caplin et al., 1999) were used for partial GAPDH gene quantification. Two probes were designed to bind adjacent to one another on the amplicon during annealing step. One has a donor dye on its 3´end, Fluorescein (Flc), while the other has an acceptor dye on its 5´end, LightCycler® Red 705. The reporter is excited and passes its energy to the acceptor dye through Fluorescence Resonance Energy Transfer (FRET) and the intensity of the light emitted is measured by the second probe.

All sets of primers and probes for real time PCR quantification of both cellular 161 bp GAPDH and 171 bp *tax* genes are described in Table 2.5.

The amplification reaction was carried out in triplicate using the capillary-based LightCycler® 2.0 (Roche). Standard curves were preparated for each amplification and analyzed at each PCR-run, including negative and positive controls. Reactions were carried out in triplicate in a total volume of 20 µl containing LightCycler FastStart DNA Master PLUS HybProbe 5X (Roche), 200 ng of genomic DNA, 50 pmol of each primer, GAPDH-3/ GAPDH-4 or Tx-5/ Tx-6 for partial GAPDH or *tax* genes, respectively, along with 2pmol of the following fluorescent hybridization GAPDH or Taqman *tax* probes.

Table 2.5 Primers and probes used for partial GAPDH and *tax* genes amplification by RT-PCR.

Primer	Sequence	Length (bp)
GAPDH-3	5´-CCTGCCAAATATGATGAC-3´	161bp
GAPDH-4	5´-GTCGTTGAGGGCAATGC-3´	
GAPDH Hybridization Probe	5´-TGGAACCAACCCTCGGGGATCAGCTCCAGC [Flc]-3´ 5´-[LC640] TCCCTCGCCTTCCCTGAACCTG [Phos]-3´	
Tx-5	5´-CACTCCTGTCTCCCCCAAG -3´	171bp
Tx-6	5´-TACGGTTTTTCCCCAGGTG-3´	
Tax Taqman Probe	5´-[6-FAM] CACCCGCCTTCTTCCAATCAATGCGAAAG [TAMRA]-3´	

The cycling parameters for the both amplifications begun with hot start at 95ºC 10 min, and continued with 40 cycles of denaturation at 95ºC for 5s, annealing at 62ºC for 10s and extension at 72ºC for 20s for GAPDH gene, and 40 cycles of denaturation at 95ºC for 10s, annealing at 62ºC for 15s and extension at 72ºC for 20s for *tax* gene.

The Ct value is the PCR cycle in which there is a significant increase in reporter signal, above the threshold where signal can be considered not to be background. The Ct values were converted into template quantity using the standard curve method. Several parameters were evaluated each run to validate the assay performed, such as the PCR efficiency given by the slope of the standard curve, showing PCR efficiency >90%, R^2 correlation coefficients for all the standard curves greater than 0.9, avoiding replicates that have more than 0.5 Ct of difference. HTLV-2 copy number per genome

or cell was calculated as the ratio between the quantity of template for *tax* and GAPDH, multiplied by two (diploid genome in each cell). The final results were expressed as HTLV-2 DNA copies per million PBMCs.

2.15 Immune activation

Fresh EDTA anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell activation with the following antibody combination: CD3-eFluor™ 450 (eF450), CD4-Krome™ Orange (KO), CD8-peridinin chlorophyll protein complex (PerCP), CD38-Allophycocyanin-700 (APC-700) and HLA-DR-allophycocyanin-Cy7 (APC-Cy7). All the antibodies (Becton Dickinson, NJ, USA) are shown in Table 2.1.

2.16 Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables and the Mann-Whitney U test to compare non-normally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. The Spearman correlation coefficient was used to analyze the correlations between continuous variables. Statistical analysis was performed using IBM SPSS software 21.0 (IBM, Chicago, Illinois, USA).Graphs were generated using GraphPad Prism 5.01 (La Jolla, California, USA). Multivariate lineal regression analysis to identify variables associated with immune activation as dependent variable.

SUBSTUDY 1

Seventeen HTLV-2 infected individuals coinfected with HCV and HIV-1, and two other control groups notinfected by HTLV-2, including eight individuals coinfected by HIV-1/HCV and 10 HIV-1-monoinfected individuals, were analyzed. All three groups were receiving suppressive cART (HIV-1 viral load lower than 50 RNA copies/ml) with $CD4^+$ T cell count superior to 200 cells/mm^3.

2.18 Microbial translocation

Microbial translocation was measured in plasma by two commercial kit assays according to the manufacturer´s protocol. Plasma bacterial LPS was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza, Basel, Switzerland) and plasma sCD14 was quantified using the Quantikine Human sCD14 Immunoassay (R&D Systems, Minneapolis, MN).

2.19 Cytokine profile

Levels of the following cytokines: Inteleukin-6 (IL-6), tumor necrosis factor (TNF), interferon-γ (IFN-γ), and interleukin -17A (IL-17A) were measured by Flow cytometry using the BD™ CBA Human Th1/ Th2/ Th17 Cytkokine Kit (BD Biosciences, California, US).

2.20 Epidemiological characterization of the HTLV-2 isolates

Twelve HTLV-2-infected Spanish IDUs also infected by HIV-1 were phylogenetically analyzed through three viral genes, LTR, *env* and *tax* and compared with other reported isolates.

- **Nested polymerase chain reaction (nPCR)**

Partial LTR, *env*, and *tax* genes were amplified by nested PCR, a sensitive and selective method based on the amplification of the partial genes of interest followed by a second round of internal polymerase amplification for incrementing of the quantity of PCR product. For the first round of PCR, all reaction mixtures were performed in a total volume of 25 µl containing PCR Buffer II (AccuPrime Taq High Fidelity, Bioline, London, UK), 0,5 U Taq polymerase (AccuPrime Taq High Fidelity, Bioline, London, UK), 200 ng of genomic DNA and 20 pmol of the following primer pairs were used, (HT2-1/ HT2-2) for LTR, (Ev-2/ Ev-3) for the *env* gene, and (Tx3/ Tx4) for the *tax* gene, as shown in the Table below (Table 2.6). Thirty-five cycles of denaturation at 94ºC for 15s, annealing at 58ºC for 15s, and extension at 68ºC for 20s followed by a final extension at 68ºC for 2 min were used.

Table 2.6 Oligonucleotides used for partial HTLV-2 LTR, *env* and *tax* genes nPR.

Primer	**Sequence for the First PCR run**	**Target (bp)**	**Length (bp)**
HT2-1	5´-TAAAGGCTCTGACGTCTCC-3´	nt (89-684)	596 bp
HT2-2	5´-GCAGCAAGGGCTAGGGCT-3´		
Ev-2	5´-GTTCCAATAGCAGTGAGCCTTGT-3´	nt (5113-6129)	1017 bp
Ev-3	5´-AAAGCTGCATGCCCAAGAC-3´		
Tx-3	5´-CTGGTCTCCTAACGGCAATCTC-3´	nt (7142-8294)	1153 bp
Tx-4	5´-CAAGTAAAGGCTCTGACGTCT-3´		

Primer	**Sequence for the Second PCR run**	**Target (bp)**	**Length (bp)**
HT2-1	5´-TAAAGGCTCTGACGTCTCC-3´	nt (153-541)	390 bp
HT2-2	5´-GCAGCAAGGGCTAGGGCT-3´		
Ev-2	5´-GTTCCAATAGCAGTGAGCCTTGT-3´	nt (5180-5956)	777 bp
Ev-3	5´-AAAGCTGCATGCCCAAGAC-3´		
Tx-3	5´-CTGGTCTCCTAACGGCAATCTC-3´	nt (7213-8205)	993 bp
Tx-6	5´-CAGACCGTCTCACACAAACAATC-3´		

After the first PCR reaction, 2 µl of the amplified products were used for a second PCR run in the same amplification conditions, with the following primer pairs were used (HT2-1/ HT2-4) for the amplification of 390 bp of LTR, (Ev-2/ Ev-3) for 777bp of the *env* region, and (Tx3/ Tx4) for 993 pb of *tax* region were used, as also shown in the table 2.6. Thirty-five cycles of denaturation at 94ºC for 15s, annealing at 58ºC for 15s, and extension at 68ºC for 20s followed by a final extension at 68ºC for 2 min were used. The sequence positions are listed according to the Mo isolate (GenBank accession number M10060).

- **Sequencing and phylogenetic analysis**

PCR products are purified by ethanol precipitation method, to ensure good quality DNA templates for sequencing. After DNA precipitation, where competing ezymes or buffer components were eliminated, DNA pellet is air-dried and the DNA is resuspended in UltraPure DNase/RNase-Free Distilled Water (Life Technologies, New York, US).

Purified PCR products were sequenced in a final volume of 15µl by using the ABI Big Dye Terminator v3.1 cycle sequencing reaction kit (Applied Biosystems, Foster City, CA) and processed with the ABI 310 Genetic Analizer (Applied Biosystem).

Sequences were edited using SeqMan II Software version 5.0.1 (DNASTAR, Madison, WI) and aligned with MEGA software v4.0.2 together with reference sequences of the HTLV-2a subtype (Mo) and HTLV-2b subtype (G12 and NRA) whose GenBank accession numbers are shown in Table 2.7. Sequences from the samples studied were submitted to GenBank under the numbers shown in Table 2.7.

Phylogenetic trees were generated using the unweight pair group method with arithmetic mean (UPGMA) by Clustal W v5.8 and MEGA v4.0.2. The neighbor-joining method was also used to corroborate them. The topology of the trees was supported by 1000 bootstrap replicates. Genetic distance was estimated by Kimura´s two-parameter method. Some reported sequences from GenBank listed below (Table 2.8) were used for comparison purpose, and the simian T cell lymphotropic virus type 2 (STLV-2) was used as outgroup.

Table 2.7 GeneBank accession numbers of previously published HTLV-2 gene fragments used for comparison purpose.

GeneBank Accession Numbers of reported sequences	
Reference sequences	Mo (M10060), G12 (L11456), G2(AF07965), NRA (L20734), Gab (Y13051), Efe-2 (Y14365), STLV-2 (U90557).
For the LTR analysis	NAV.DS (U10357), SMH2 (Y09148), PH230PCAM (Z46838), LA8A (U10356), KAY73 (L42509), BRAZ.A21 (U10253), ATL18 (U10252), Dub805 (AF175467), AA (L7738), DP (L7737), JA (L7739), BF (L77236), SPAN129 (U10265), SPAN130 (U10266), RVP (L77244), 130 (L77242), 324 (L77243), PortH1 (AY622977), PortNn (AY622978), PortVs (AY622979), Gu (X89270), I-GI (Y09153), I-OV (Y09155), I-OG (Y09154), WYU1 (U12792), BBD_3126 (FJ911656), BBD_2278 (FJ911644), BBD_2766 (FJ911652), BBD_2286 (FJ911645).
For the *env* analysis	WY100 (S69268), Gu (X89270), SP-WV (AF139382), RP329 (AF326583), K96 (AF326584), AF4122314.
For the *tax* analysis	SMH2 (Y09148), Gal (AF292002), SP-WV (AF139382), K96 (AF326584), RP329 (AF326583), KAY1 (U32874), KAY2 (U32875), SP1 (U32873), SP2 (U32872), PR-46 (DQ022075), FUC (U32882), PAR (U32880).

Table 2.8 GeneBank accession numbers of HTLV-2 samples.

GeneBank Accession Numbers of samples sequenced	
LTR fragment	MA_CPE (GU455203), MA_DGE (GU455204), MA_FRM (GU455205), MA_MSC (GU455206), MA_JSR (GU455207), MA_CGO (GU455208), MA_GRO (GU455209), MA_SPG (GU455210), MA_ROL (GU455211), MA_VMM (GU455212), MA_CPE (GU455213), MA_CAS (GU4586121).
Partial *env* gene	MA_BMJ (GU586109), MA_DGE (GU586110), MA_FRM (GU586111), MA_MSC (GU586112), MA_JSR (GU586113), MA_CGO (GU586114), MA_GRO (GU586115), MA_SPG (GU586116), MA_ROL (GU586117), MA_VMM (GU586118), MA_CPE (GU586119), MA_CAS (GU586120).
Partial *tax* gene	MA_BMJ (GU591294), MA_CGO (GU591295), MA_CPE (GU591296), MA_DGE (GU591297), MA_FRM (GU591298), MA_GRO (GU591299), MA_JSR (GU591300), MA_MSC (GU591301), MA_ROL (GU591302), MA_SPG (GU591303), MA_VMM (GU591304), MA_CAS (GU591305),

SUBSTUDY 2

As secondary study, in the frame of two clinical trials, the evolution of HTLV-2 proviral load was studied among HIV-1/HTLV-2 co-infected patients who intensified their cART with an integrase inhibitor (raltegravir) compared to those who did not. In this retrospective longitudinal study, DNA was extracted from cryopreserved PBMCs and total HTLV-2 proviral DNA was quantified by in-house real-time PCR. For viral and cellular gene quantification, two standard curves were generated in each run respectively, using recombinant plasmids containing one copy of HTLV-2 *tax* gene fragment or one copy of human genomic GAPDH gene fragment.

3.21 Patients`s characteristics

HTLV-2 proviral load was measured in this retrospective longitudinal study at baseline, week 24 and week 48 in HIV-1/ HTLV-2- coinfected patients with intensifying treatment with raltegravir compared and those without raltegravir intensifying therapy.

Four HIV-1/HTLV-2-coinfected patients who intensified their antiretroviral treatment with 400 mg of an integrase inhibitor, raltegravir, twice daily, were recruited from Hospital Germans Trias I Pujol (Badalona, Barcelona, Spain) and Hospital Ramón y Cajal (Madrid, Spain). One of these patients was analyzed in two further time points, a second baseline and at week 4. Besides, eleven HIV-1/HTLV-2-coinfected patients who did not intensified their cART were also analyzed for 48 weeks as control group.

The main inclusion criteria were as follows: HIV-1 infected adults receiving antiretroviral treatment for at least two years; undetectable plasma viral load (below 40 copies HIV-1 RNA/ml) for at least two years; $CD4^+$ T-cell count above 350 cells/mm^3; and no previous RAL in their cART.

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our local Independent Ethics Committee (Hospital Ramón y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

Results

ANTIRETROVIRAL TREATMENT INTENSIFICATION

3.1 Patients characteristics

Baseline characteristics of the 16 HIV-1-infected individuals that undergo treatment intensification are summarized in Table 3.1. Median age was 46 years (IQR 41–50) and no significant differences were found between $CD4^+$ and $CD8^+$ T-cell counts at baseline [676 cells/mm^3 (IQR 522–828) and 678 cells/mm^3 (IQR 567–1066), respectively, p=0.7] and at the end of 24-week follow-up [642 cells/mm^3(IQR 552–722) and 647 cells/mm^3 (IQR 439–886), respectively, p=0.1]. During follow-up, no changes were made in previous ART regimens. Seven of them intensified their treatment with maraviroc (MVC), while the other nine intensified with raltegravir (RAL). All individuals remained virally suppressed during the period of the study.

Within the MVC group, the median age was 46 years and six patients were male. The median baseline $CD4^+$ and $CD8^+$ T-cell counts were 711 and 784 cells/mm^3, respectively. All patients were receiving nucleoside reverse transcriptase inhibitor (NRTI)–containing regimens combined with non-nucleoside reverse transcriptase inhibitors (NNRTIs) in two cases (28%), with protease inhibitors (PIs) in five cases (43%), and with a third nucleoside in two cases (22%). The median duration of ART before study entry was 75 months with undetectable HIV-1 viral load, below 50 copies/ml maintained until the end of the study

While within RAL group, patients were predominantly male with a long history of ART (median 12 years). Median CD4 cell count was 655 cells/mm^3 and median CD8 cell count was 636 cells/mm^3. Only two patients were coinfected with HCV (22%). All patients were receiving nucleoside reverse transcriptase inhibitors combined with nonnucleoside reverse transcriptase inhibitors in five cases (55%) and with protease inhibitors in four cases (44%) (Table 3.1).

Table 3.1: Baseline characteristics of the 16 HIV-1-infected individuals including in the treatment intensification according to the intensifying drug administered.

Variable	**MVC (n=7)**	**RAL (n=9)**	**Total (n=16)**
Age (years)	46 [31-48]	48 [44-53]	46 [41-50]
Male (%)	85.7%	100%	93%
Risk factors (%)	IDU (42.8%)	IDU (25%)	IDU (33.3%)
	MSM (42.8%)	MSM (50%)	MSM (46.6%)
	HSx (14.2%)	HSx (25%)	HSx (20%)
Viral Load (copies/mL)	< 50 copies/mL	< 50 copies/mL	< 50 copies/mL
CD4 count (cells/mm^3)	711 [547-793]	655 [417-877]	676 [522-828]
CD8 count (cells/mm^3)	784 [673-1109]	636 [367-827]	678 [567-1066]
Duration of ART (months)	75 [38-144]	144 [70-167]	110 [52-144]
MVC intensification (%)	100%	-	46.6%
RAL intensification (%)	-	100%	53.3%
$CD4^+$ $CD38^+HLADR^+$ (%)	3.1 %	2.4%	-
$CD8^+$ $CD38^+HLADR^+$ (%)	5.3%	3.8%	-

MVC: Maraviroc; RAL: Raltegravir; IDU: Intravenous drug use; HSx, unprotected heterosexual contacts; MSM: men who have sex with men; ART: antiretroviral therapy. Continuous variables are expressed as median and interquartile range (IQR).

3.2 Effect of treatment intensification on T-cell count

Globally, no differences were observed in $CD4^+$ or $CD8^+$ T-cell counts either during follow-up 48 weeks of treatment intensification or after the 24 weeks of discontinuation with respect to baseline (Figure 3.1).

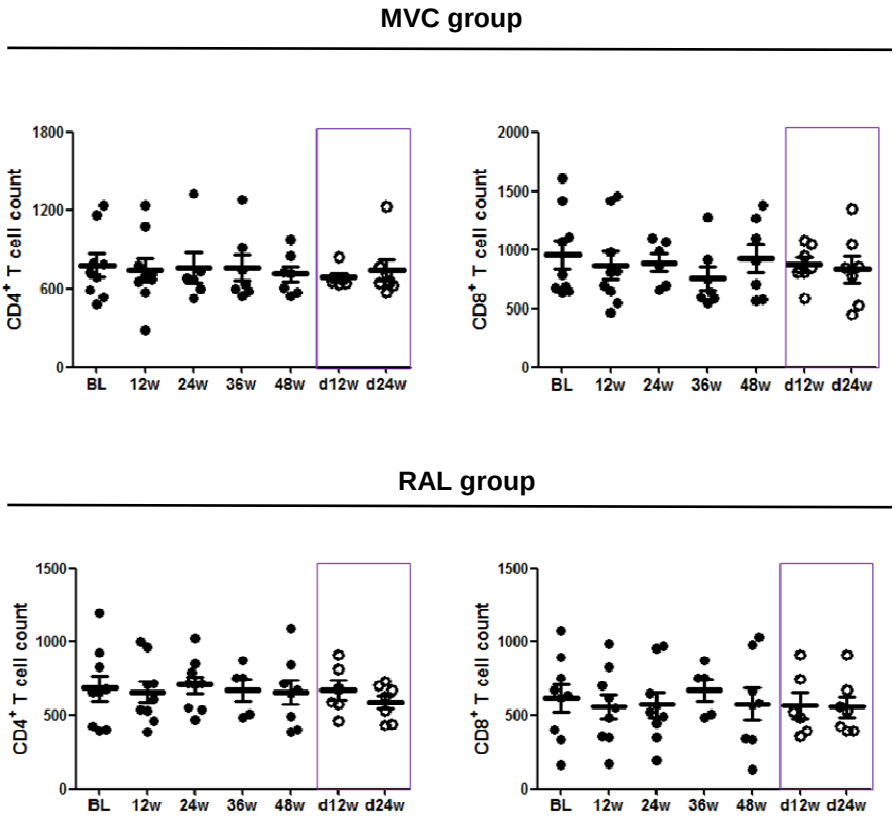

Figure 3.1: Effects of maraviroc or raltegravir intensification on $CD4^+$ and $CD8^+$ T cell count.

3.3 Effect of treatment intensification on T-cell immune activation

The level of $CD4^+$ T cell activation decreased significantly after 12, 23 and 36 weeks of maraviroc (MVC) intensification (p=0.028, p=0.027, and p=0.028, respectively), only to increase after 48 weeks, although the difference with baseline was not significant (p=0.6). Compared to HIV-1-negative subjects, $CD4^+$ T cell activation at baseline was significantly higher (p=0.001), with no significant differences at weeks 12 and 24 (p=0.950 and p=0.181, respectively). Despite no significant decrease in $CD4^+$ T-cell activation was observed at week 48 after intensification in the MVC group, these levels were significantly lower at week 24 after discontinuation than at baseline (p=0.028). The effect of intensification with MVC in reducing $CD8^+$ T-cell activation was maintained at week 12 and 24 after discontinuation compared with baseline (p=0.028 and p=0.028, respectively) (Figure 3.2a).

The level of activation of $CD8^+$ T cells also decreased significantly after 12 weeks of MVC intensification compared to baseline and continued diminished until the end of the study (p=0.043, p=0.025, p=0.028, and p=0.046, at weeks 12, 24, 36, and 48, respectively). The level of $CD8^+$ T cell activation at baseline was higher than in the HIV-1-negative subjects (p=0.001). And only at week 48 was this difference not significant compared to the HIV-1-negative group (p=0.181). Additionally, the effect of intensification with MVC in reducing $CD8^+$ T-cell activation was maintained at week 12 and 24 after discontinuation compared with baseline (p=0.028 and p=0.028, respectively) (Figure 3.2b).

However, within RAL group, $CD4^+$ T cell activation remained stable during intensification period or after RAL discontinuation, with no significant differences compared to baseline (Figure 3.2c). Nevertheless, $CD8^+$ T-cell activation decreased after intensification, the difference being statistically significant at week 36 (p=0.028), with a clearly decreasing trend at week 48 (p=0.093) compared with baseline. The slight waning effect at week 48 was due to the increase of cell activation in only three patients. Besides, only the levels of $CD8^+$ T-cell activation were significantly lower at week 24 after discontinuation than at baseline (p=0.042) (Figure 3.2d).

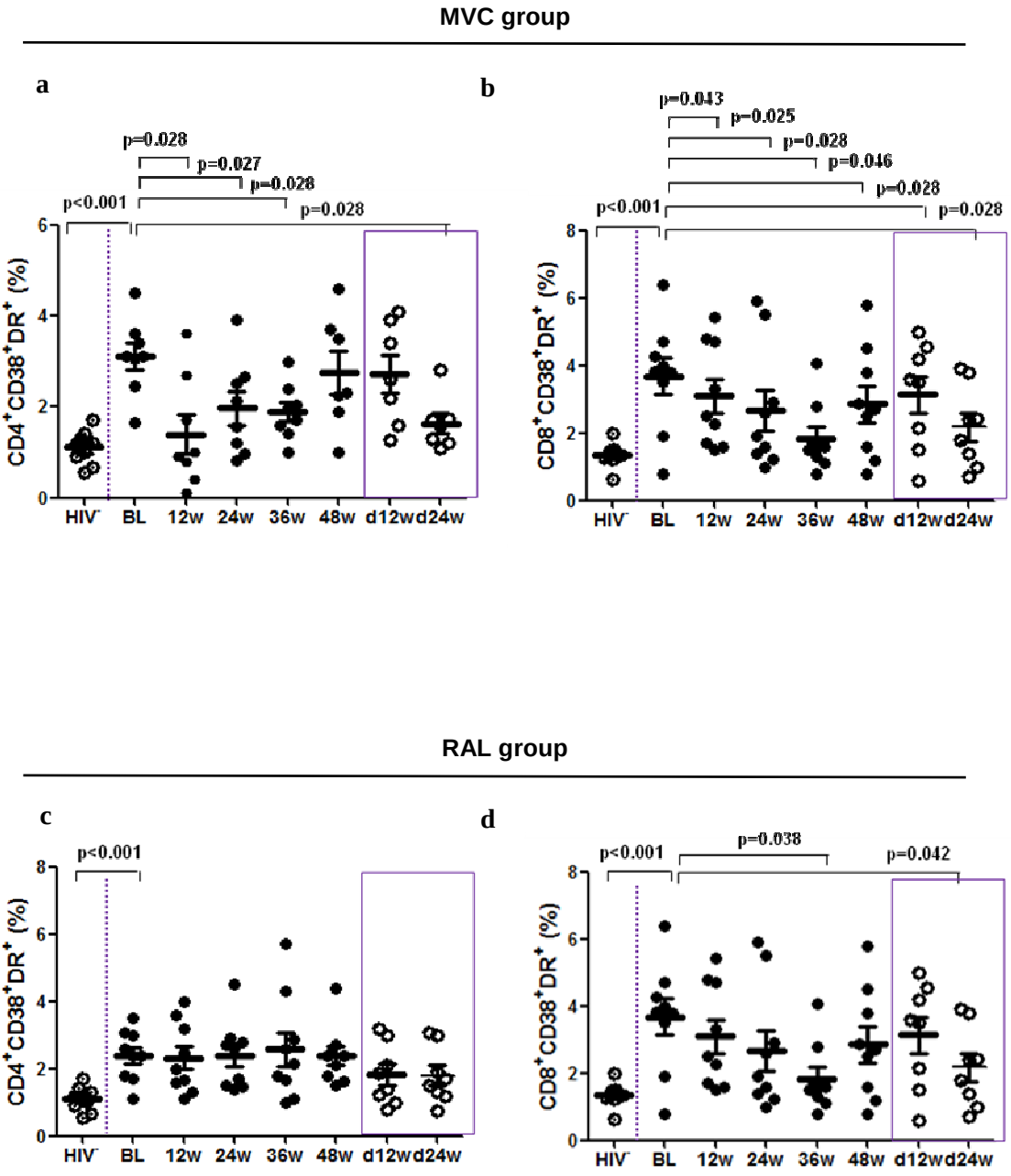

Figure 3.2: Effects of treatment intensification with maraviroc (a, b) or raltegravir (c, d) and intensifying drug intensification discontinuation on $CD4^+$ T cell (a, c) and $CD8^+$ T cell (b, d) immune activation.

3.4 Effect of treatment intensification on T cell subsets

Regarding to T cell subsets, a significant increase in $CD8^+$ effector memory and TemRA T cell count was found during the first 12 and 24 weeks after MVC intensification, respectively. Thereafter, the differences compared to baseline were not significant (Figures 3.3c and 3.3d). These increments are balanced by a significant decrease in $CD8^+$ central memory T cells at week 12 of MVC intensification (p=0.002), again with no differences thereafter compared to baseline (Figure 3.3b). Levels of naive $CD8^+$ T cells were similar during follow-up, at all time points (Figure 3.3a). Further, no significant differences were observed in any $CD4^+$ T cell subsets during follow-up (not shown).

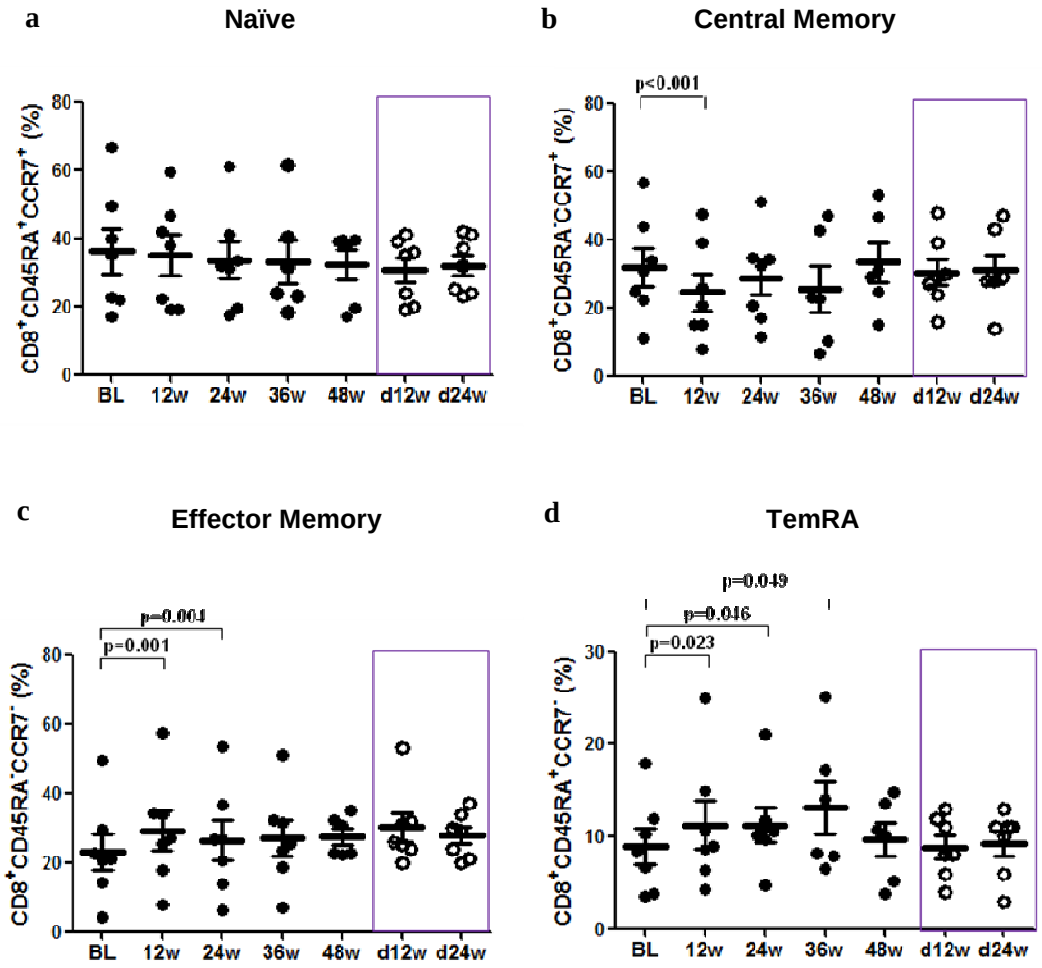

Figure 3.3: Effects of treatment intensification with maraviroc and intensifying drug discontinuation on naïve, and either central, effector or transitory memory $CD8^+$ T cells.

However, within RAL group, naïve $CD4^+$ and $CD8^+$ T cells remained stable during all time-points studied during treatment intensification and RAL discontinuation. Likewise, no other significant differences were found in the counts of either central, effector or transitory $CD4^+$ or $CD8^+$ T cells (not shown).

3.5 Effect of treatment intensification on microbial translocation

The effect of treatment intensification on microbial translocation was different whether individuals intensified with maraviroc or raltegravir.

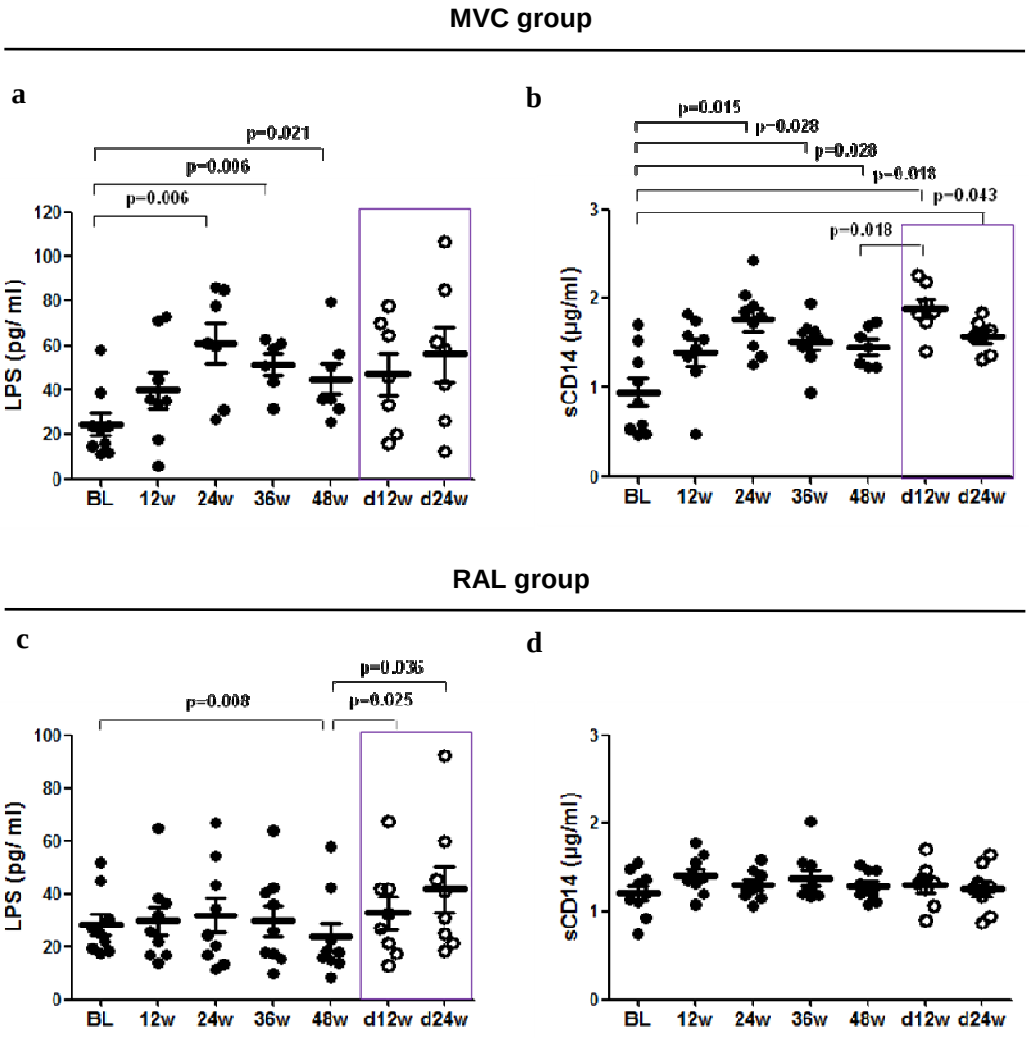

Figure 3.4: Effects of treatment intensification and drug discontinuation on either LPS or sCD14 levels.

Within MVC group, a significant increase in LPS level was observed after 24, 36 and 48 weeks of intensification with MVC compared to baseline (p=0.006, p=0.006, and p=0.021, respectively). After 12 and 24 weeks of discontinuation, LPS levels progressively decreased to levels similar to those found at baseline (Figure 3.4a). Besides, sCD14 levels were also significantly higher after 24, 36 and 48 weeks of MVC intensification compared to baseline (p=0.015, p=0.028 and p=0.028, respectively). However, these levels reverted but remained significantly higher after 12 and 24 weeks after drug discontinuation (Figure 3.4b).

On the other hand, within RAL group, LPS and sCD14 levels remained entirely stable during the follow-up period studied, except for the late decline in LPS with levels significantly lower at week 48 of intensification than baseline (p=0.008), decrease compensated after drug discontinuation with significant increase at week 12 and 24 compared to week 48 (p=0.025 and p=0.036, respectively), but no significance when compared to baseline (Figure 3.5c and 3.5d).

3.6 Effect of treatment intensification on the expression of gut homing β7 receptor on activated T cells

In individuals who received maraviroc intensification, the proportion of activated $CD8^+$ T cells expressing β7 receptor also increased in at week 12 compared to baseline (p=0.046), and a trend to increase was observed during the rest of the follow up 48 weeks of intensification. After MVC discontinuation, the levels of $CD8^+\beta7^+$ T cells remained stable at week 12 and 24, but significantly higher when compared to levels at baseline (p=0.042 and p=0.043, respectively) (Figures 3.5a). No change in activated $CD4^+\beta7^+$ T cells were observed during the analyzed follow-up (Figures 3.5b).

Contrarily, in individuals intensified raltegravir, the proportion of activated $CD4^+\beta7^+$ were stable during intensified period with a no significant decrease late at week 48. Those levels significantly increased at week 12 and 24 compared to week 48 (p=0.012 and p=0.017, respectively), with levels similar to those found at baseline (Figures 3.5c). No change in activated $CD8^+\beta7^+$ T cells was observed during the analyzed follow-up (Figures 3.5d).

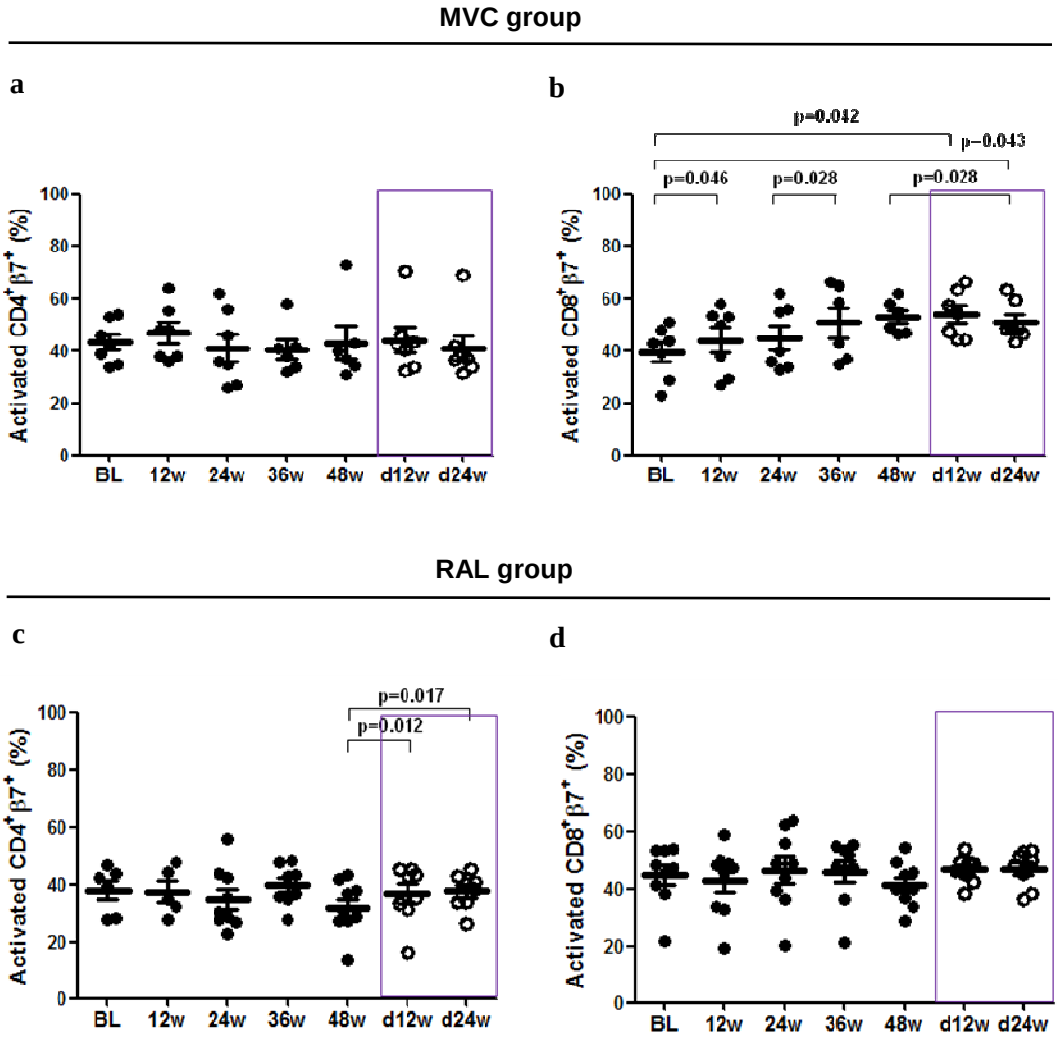

Figure 3.5: Effects of maraviroc or raltegravir intensification on the proportions of the expression of gut homing β7 receptor on activated $CD4^+$ or $CD8^+$ T cells.

3.7 Good correlation between microbial translocation measurements except for 16S rDNA quantification

Microbial measurements were performed in seven time points included in the study as follows: baseline, weeks 12, 24, 36 and 48 after the inclusion of the intensifying drugs, and weeks 12 and 24 after discontinuation of intensifying drugs from

the 16 patients recruited for the study. A total of 112 determinations performed from these patients at different time points were analyzed altogether.

Plasma LPS determinations positively correlated with levels of sCD14 and LBP found (p=0.001 and p=0.042, respectively) (Figures 3.6a,b). Similarly, high levels of sCD14 were associated with high levels of LBP detected (p=0.009) (Figure 3.6c). No correlation was found, however, between bacterial 16S rDNA either with LPS, sCD14, or LBP levels (p=0.346, p=0.405, and p=0.644, respectively) (Figures 3.6d, e,f).

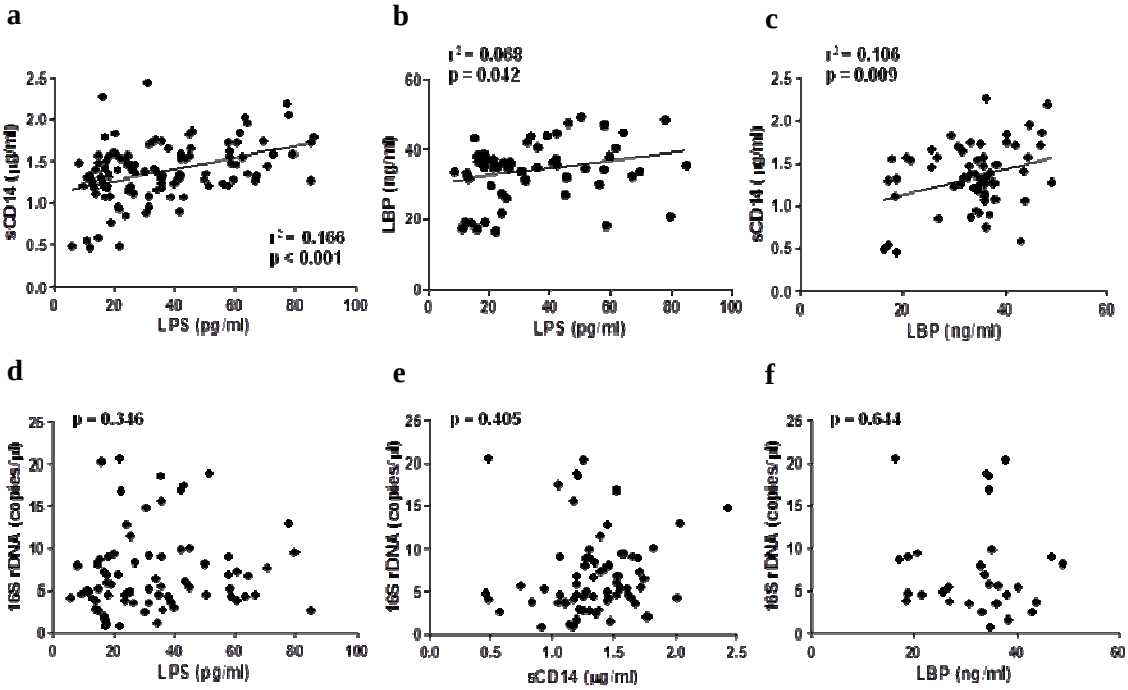

Figure 3.6: Correlation between different measurements of microbial translocation. Only significant data (p<0.05) are shown with regression lines and correlation coefficients, using Spearman correlation test.

3.8 16S rDNA levels correlated with activated $CD4^+$ T cells, while no correlation with $CD8^+$ T-cell activation was evident

No significant correlation was found between plasma levels of LPS, sCD14, or LBP and activated $CD4^+$ T cells (p=0.418, p=0.619, and p=0.728, respectively) (Figure

3.7a) or activated $CD8^+$ T cells (p=0.352, p=0.275, and p=0.124, respectively) (Figure 3.7b). However, high levels of bacterial 16S rDNA quantified correlated significantly with high levels of activated $CD4^+$ T cells (p=0.005) (Figure 3.7a) but not with activated $CD8^+$ T cells (p=0.171) (Figure 3.7b).

3.9 Association of microbial translocation and immune activation with T-cell subsets

No association between microbial translocation measurements and $CD4^+$ or $CD8^+$ T-cell count or CD4/CD8 ratio was observed (not shown). Similarly, T-cell immune activation showed no significant correlation either with $CD4^+$ or $CD8^+$ T-cell count or CD4/CD8 ratio (not shown).

No association was found between microbial translocation or immune activation with either naïve or memory CD4- or CD8- TCM, TEM or TemRA cells (not shown).

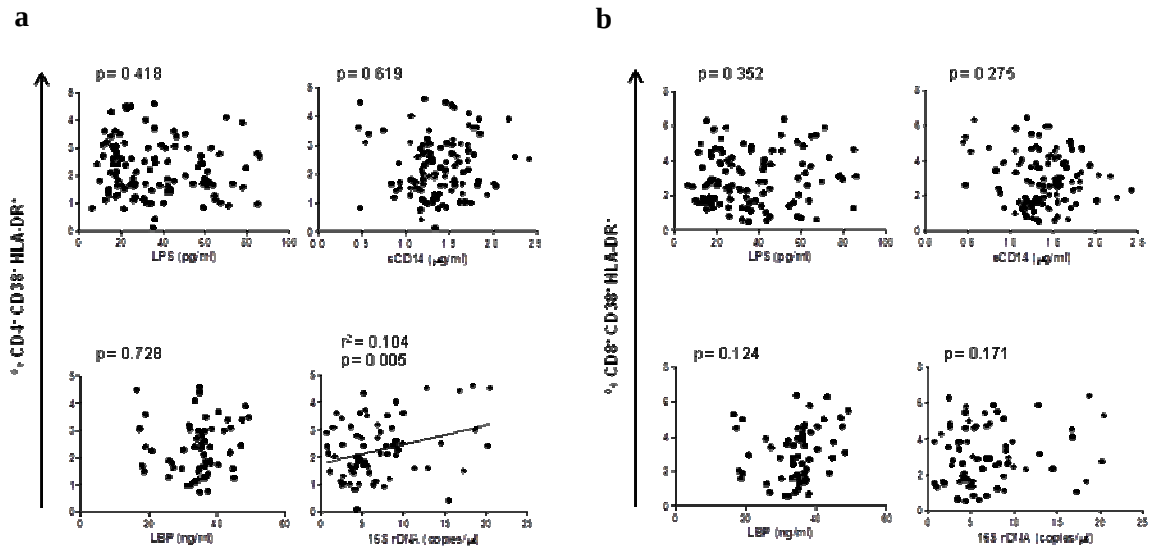

Figure 3.7: Correlation between different measurements of microbial translocation and immune activation. Significant data are shown with regression lines and correlation coefficients.

3.10 Association between microbial translocation and the expression of gut homing β7 receptor on activated $CD4^+$ and $CD8^+$ T cells

Despite no significant correlation was found between microbial translocation and expression of gut homing β7 receptor on activated T cells (not shown), similar trend was observed during the follow up period studied. Furthermore, within maraviroc group, LPS levels correlated with activated $CD4^+$ or $CD8^+$ T cells 48 weeks after MVC intensification. However, this correlation was lost after discontinuation of the drug (Figure 3.8).

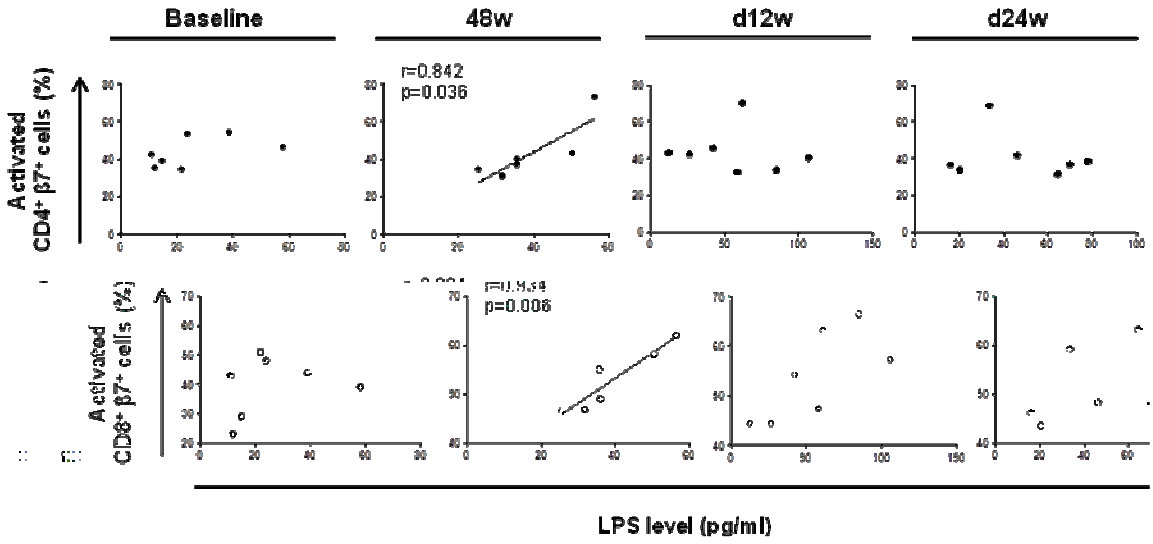

Figure 3.8: Correlation between LPS levels and activated $CD4^+$ or $CD8^+$ T cells bearing gut homing β7 receptor at baseline, after 48 weeks of MVC intensification and 12 and 24 weeks after drug discontinuation. Significant data are shown with regression lines and correlation coefficients.

COINFECTION WITH LEISHMANIA COMPARED TO IMMUNOLOGICAL DISCORDANT RESPONSE TO ANTIRETROVIRAL THERAPY

3.11 Patients´ characteristics

Eight HIV-1-infected individuals with visceral leishmaniasis, 62.5% males, had a median age of 45 years, similar to the age of the two control groups with no co-infection with *Leishmania* composed of 13 infected individuals with concordant response to antiretroviral therapy and 14 immunological non-responders or immune-discordant patients. All three groups were receiving suppressive cART (HIV-1 viral load lower than 50 RNA copies/ml) with similar duration of both cART and viral suppression.

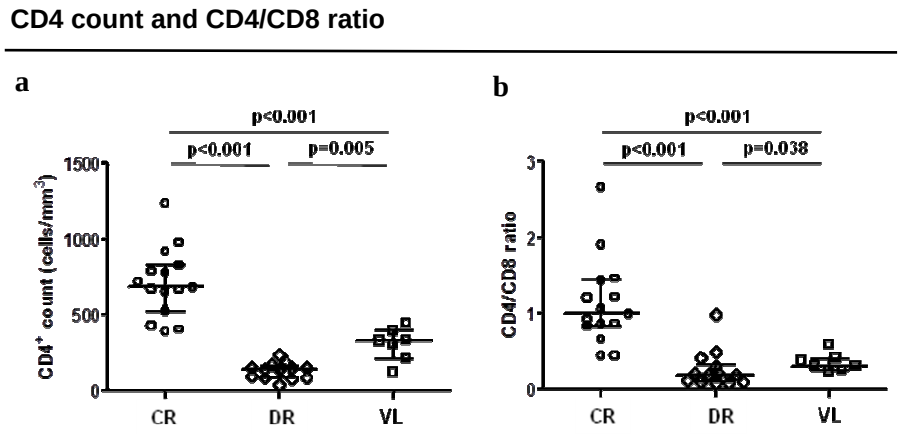

Figure 3.9: $CD4^+$ T cell count and CD4/CD8 ratio in patients with concordant response (CR), discordant response (DR) or with visceral leishmaniasis (VL).

All patients with VL but one were injecting drug users (IDUs) which is a different scenario compared to the risk factor of the other two control groups where patients with concordant response were predominantly men who have sex with men (MSM) (60%), and similar frequencies of IDUs and MSM were found within patients with discordant response (42.8% in both cases). Interestingly, while VL patients had significantly lower

levels of CD4 count, CD4/CD8 ratio and CD4 nadir compared to CR patients (p<0.001, p<0.001, and p=0.001, respectively), they had higher levels of CD4 count and CD4/CD8 ratio compared to DR patients (p=0.005 and p=0.038, respectively), while no significant differences were found in nadir CD4 count (Figure 3.9). Most of the VL patients were infected with VHC (75%) and had a time of diagnosis of VL similar to the duration of cART (75%). Clinical and laboratory characteristics are shown in Table 3.2.

3.12 Immune activation

$CD4^+$ T-cell activation in VL patients was similar to DR patients (p=0.402), but significantly higher compared to CR patients (p<0.001). On the other hand, $CD8^+$ T-cell activation was significantly higher in VL patients compared to both DR and CR patients (p=0.010 and p<0.001, respectively) (Figure 3.10a).

Table 3.2: Baseline characteristics of the HIV-1 infected patients included in the study. HSx, unprotected heterosexual contacts; MSM: men who have sex with men. Significant values when p<0.05, comparison VL vs CR and VL vs DR shown.

	VL patients N=8	CR patients N=15	p	DR patients N=14	p
Age (years)	45 [43-51]	45 [32-53]	0.728	47 [41-59]	0.664
Gender (male %)	62.5	86.6		92.8	
Risk factors (%)					
IDU	87.5	13.3		42.8	
MSM	-	60		42.8	
HSx	12.5	26.7		14.3	
CD4 count (cells/mm^3)	331 [145-667]	680 [527-825]	**<0.001**	143 [88-159]	**0.006**
CD8 count (cells/mm^3)	679 [546-1029]	675 [544-906]	0.776	691 [569-850]	0.815
CD4/CD8 ratio	0.42 [0.21-0.96]	1.0 [0.84-1.44]	**<0.001**	0.19 [0.09-0.32]	**0.035**
Nadir CD4 (cells/mm^3)	34 [16-68]	202 [62-317]	**0.001**	50 [26-99]	0.441
cART (months)	172 [74-203]	89 [43-144]	0.065	156 [97-197]	0.799
HIV-1 suppression (months)	59 [26-125]	63 [43-110]	0.824	69 [28-102]	0.799
HCV infection (%)	75	13.3		57	
VL diagnosis (months)	53 [36-165]	-		-	
Relapses of VL	3 [2-8.5]	-		-	

3.13 Inflammation and microbial translocation

Plasma IL-6 levels were significantly elevated in VL patients compared to both DR (p=0.035) and CR patients (p<0.001) (Figure 3.10b). While LPS levels in VL patients had significant higher levels compared to CR patients (p=0.001) and similar levels compared to DR patients (p=0.297) (Figure 3.10c), plasma sCD14 levels were significantly higher compared to both DR and CR patients (p<0.001 in both) (Figure 3.10c). Additionally, no association between time of VL diagnosis and number of VL relapses with inflammation and microbial translocation was found.

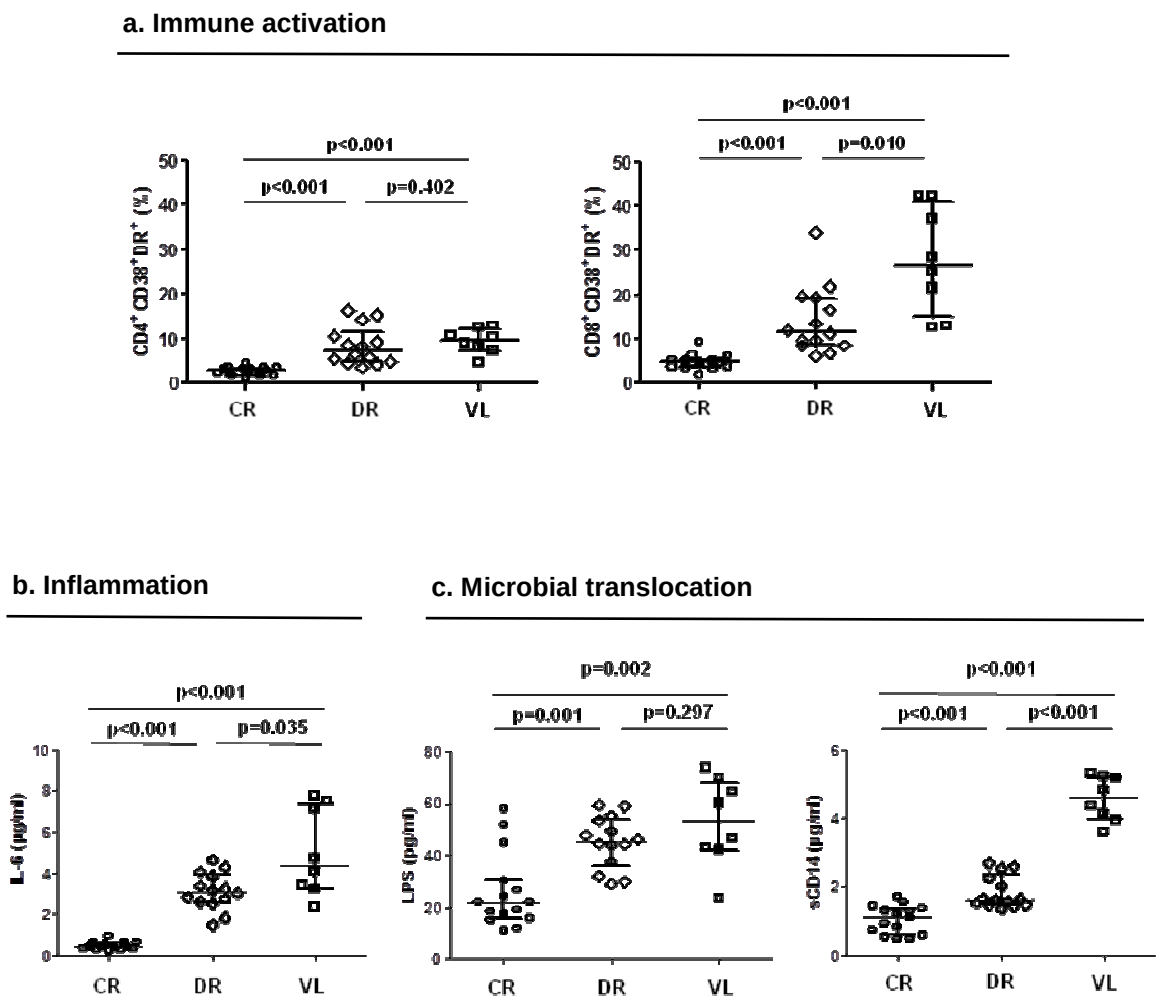

Figure 3.10: $CD4^+$ and $CD8^+$ T cell immune activation (a), levels of IL-6 immune inflammation (b) and microbial translocation measured by LPS and sCD14 levels (c).

3.14 Immune senescence

VL patients had similar level of $CD4^+$ T-cell senescence compared to DR patients, but significantly higher compared to CR patients (p=0.037), despite similar age of the patients in each of the three groups. Similarly, levels of activated $CD4^+$ T-cell senescence in VL patients were not different from those in DR patients, but again significantly higher compared to CR patients (Figure 3.11a).

Interestingly, $CD8^+$ T-cell senescence was higher in VL patients compared to DR patients, although not significant (p=0.059). However, significantly higher levels were found in activated $CD8^+$ T-cells compared to DR patients (p=0.005). Both senescence levels were significantly higher compared to CR patients (p<0.001 in both cases) (Figure 11b). No association between time of VL diagnosis and number of VL relapses with cellular senescence markers was found.

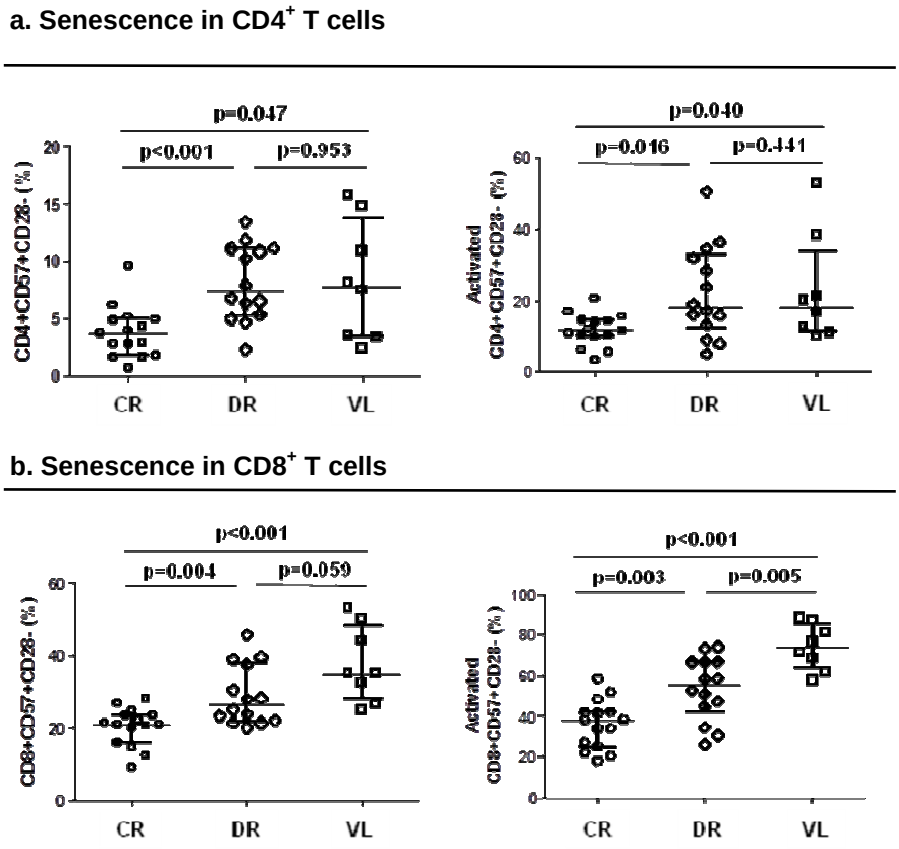

Figure 3.11: Immune senescence in both $CD4^+$ (a) and $CD8^+$ (b) T cells.

3.15 Factors independently associated with immune activation

Several factors were independently associated with immune activation (both $CD4^+$ and $CD8^+$ T-cell activation as dependent variables) to analyze whether HCV infection could influence the results of immune activation. As shown in table 3.3, CD4/CD8 ratio and sCD14 levels were independently associated with $CD4^+$ T-cell activation (p=0.045 and p=0.025, respectively), while leishmaniasis and sCD14 levels were independently associated with $CD8^+$ T-cell activation (p=0.046 and p=0.003, respectively). Therefore, leishmaniasis rather than HCV greatly contributes to the hyper immune activation. No association between both time of VL diagnosis and number of VL relapses with immune activation, CD4 count or CD4/CD8 ratio was found.

Table 3.3: Multivariate analysis: factors independently associated with immune activation (dependent variables $CD4^+$ and $CD8^+$ T cell activation).

	$CD4^+$ T cell activation		**$CD8^+$ T cell activation**	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p
CD4 count	-0.003 (-0.009-0.002)	0.234	-0.001 (-0.015-0.012)	0.838
CD4/CD8 ratio	-2.470 (-4.881- -0.058)	**0.045**	-5.216 (-10.817-0.384)	0.067
Nadir CD4 count	0.009 (-0.002-0.020)	0.105	0.018 (-0.008-0.044)	0.161
Leishmaniasis	-8.810 (-18.029-0.409)	0.060	-21.850 (-43.259- -0.440)	**0.046**
VHC infection	1.093 (-1.673-3.859)	0.424	-2.334 (-8.757-4.090)	0.462
IL-6	0.039 (-1.120-1.198)	0.945	0.843 (-1.849-3.535)	0.525
LPS	-0.064 (-0.191-0.063)	0.308	-0.173 (-0.468-0.122	0.238
sCD14	4.081 (0.546-7.617)	**0.025**	13.199 (4.988-21.410)	**0.003**

3.16 Correlation between immune activation, inflammation and microbial translocation

Among VL patients, there was a significant positive correlation between both $CD4^+$ and $CD8^+$ T-cell activation with sCD14 levels (p=0.048, r=0.711 and p=0.017, r=0.801, respectively). Moreover, IL-6 level correlated with LPS and sCD14 levels (p=0.001, r=0.935 and p=0.010, r=0.833 respectively), but not with T-cell activation (Figure 3.12a).

On the other hand, among DR patients, a significant correlation between $CD4^+$ T-cell activation and sCD14 levels (p=0.048, r=0.553), and between IL-6 and LPS levels (p=0.023, r=0.601) were found (Figure 3.12b).

Among CR patients no correlation was found between immune activation and inflammation or microbial translocation. Nevertheless, LPS correlated with sCD14 in the three groups of patients, i.e., VL (p=0.011, r=0.826), DR (p=0.001, r=0.779), and CR patients (p=0.046, r=0.659) (not shown).

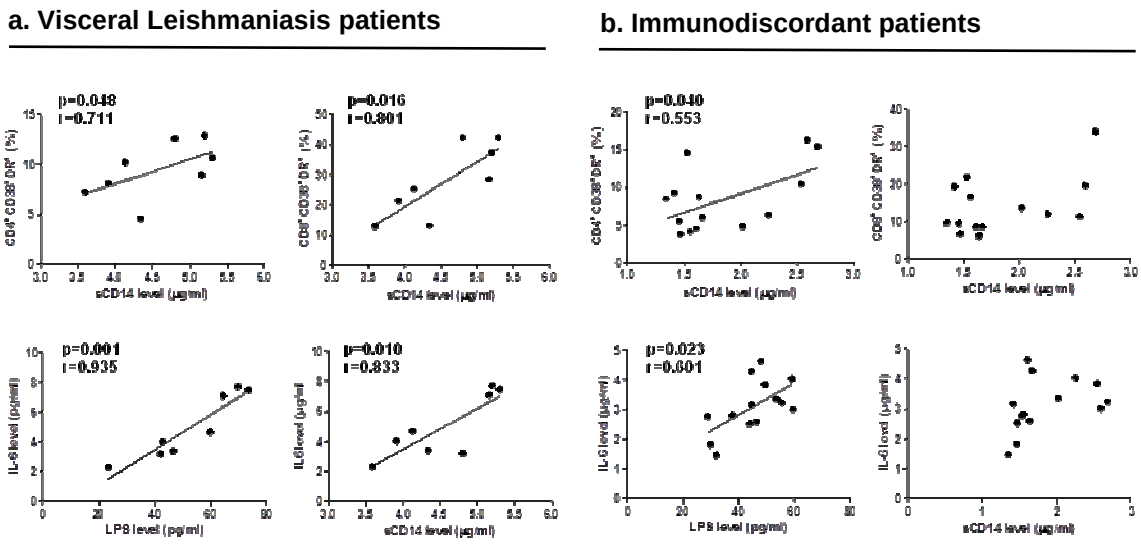

Figure 3.12: Correlation between immune activation or inflammation and microbial translocation, in VL patients (a) and immunodiscordant patients (b). Significant data are shown with regression lines and correlation coefficients.

COINFECTION WITH HTLV-2 IN BOTH HIV-1/HCV COINFECTED AND HIV-1 MONOINFECTED INDIVIDUALS

3.17 Screening for patients infected by HTLV-2

A total of 1673 HIV-1 infected patients with regular medical appointments are included in the HIV cohort in the Hospital Ramón y Cajal, Madrid, at the end of the 2010. Of them, 798 individuals had a history of intravenous drug use (Figure 3.14) and were included for the screening of HTLV-1/2 infection, following the diagnostic algorithm proposed by the HTLV European Research Network (HERN) criteria.

A total of 68 virologically suppressed HIV-1 infected patients (below 50 copies/ml) on cART, were diagnosed by HTLV-2 coinfection (Figure 3.13), 72% were men while 28% were women. They had a median age of 45 [IQR 42-49] years old with $CD4^+$ T cell count of 490 [IQR 297-599] and $CD8^+$ T cell count of 923 [IQR 625-1305]. From these patients, 67% were also infected by HCV, while the other 33% were HCV^- (Figure 3.13).

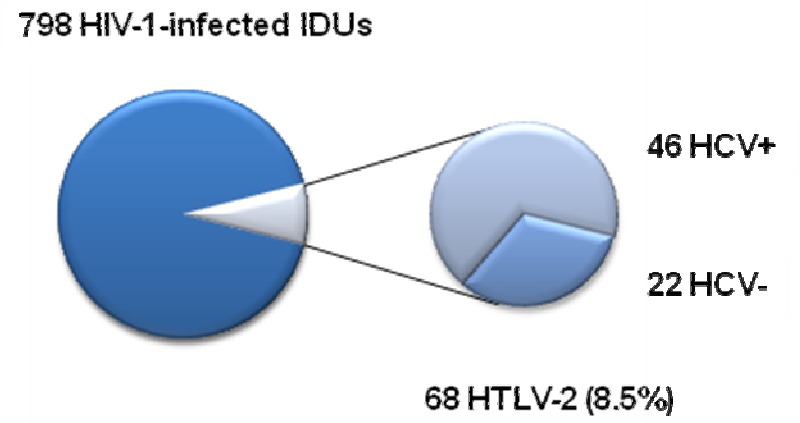

Figure 3.13: HTLV-2 infection among HIV-1 infected IDUs
In the Hospital Ramón y Cajal HIV cohort.

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3.18 HTLV-2 infected patients

Sixty-one HTLV-2-infected individuals with undetectable HIV-1 viral load on suppressive regimen at least for one year, were selected for the study. Forty of them were also infected by HCV, while the other 21 were not. However, no significant differences were found in age or T cell count among HTLV-2-infected individuals whether they were also infected with HCV or not. General clinical characteristics of HTLV-2 infected patients studied are shown in table 3.4

3.19 HTLV-2 proviral load

HTLV-2 proviral load (pVL) was determined in all these sixty-one HTLV-2-infected individuals. Univariate analysis was performed to evaluate which factors were independently and significantly associated to the HTLV-2 pVL. In this case, $CD8^+$ T cell count, both $CD4^+$ and $CD8^+$ T cells frequency and the CD4/CD8 ratio. Further, these four significant variables were analyzed simultaneously in a multivariate analysis showed that only $CD8^+$ T cell count was independently and significantly associated to HTLV-2 pVL (Table 3.5).

Additionally, we observed significantly higher HTLV-2 pVL in patients coinfected with HCV compared to those with no HCV infection (Figure 3.14). However, regarding to the gender of the participants, no difference in HTLV-2 pVL was found between male and women.

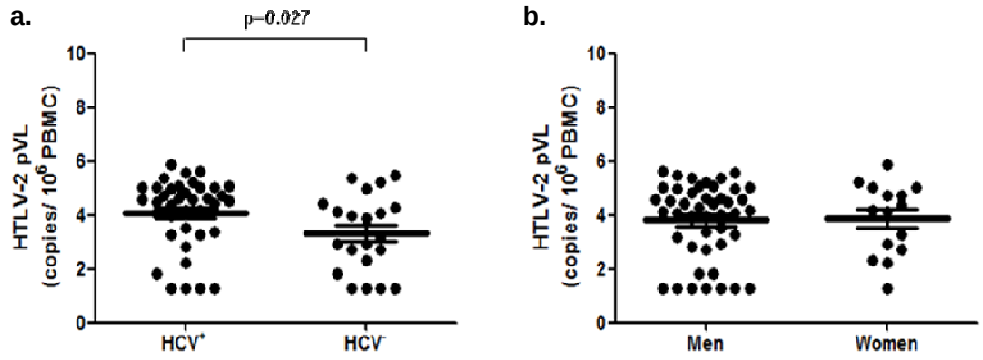

Figure 3.14: HTLV-2 pVL in HIV-1 infected patients regarding to HCV infection (a) and HTLV-2pVL variation whether infected individuals are women or men (b).

Table 3.4: Epidemiological and clinical characteristics of the HTLV-2-HIV-1-co-infected patients.

	HTLV-2/HIV-1 coinfected individuals				HIV-1 infected individuals				All	HCV+	HCV-
	All N=61	**HCV$^+$ N=40**	**HCV$^-$ N=21**	**p**	**All N=90**	**HCV$^+$ N=48**	**HCV$^-$ N=42**	**p**	**p**	**p**	**p**
Age (years)	46 [42-49]	45 [43-50]	46 [40-49]	0.246	47 [44-50]	47 [43-49]	47 [44-51]	0.967	0.081	0.203	0.144
Gender (male)	24.6 (75.4%)	27.1 (72.5%)	19 (81%)	0.459	26.7 (72.2%)	25 (75%)	28.6 (69%)		0.745	0.794	0.373
CD4 count	484 [298-610]	486 [297-599]	484 [276-674]	0.931	498 [299-702]	459 [235-654]	629 [361-727]	0.109	0.329	0.878	0.296
CD8 count	920 [645-1328]	988 [658-1477]	812 [592-1008]	0.130	821 [560-1041]	843 [621-1060]	709 [549-1041]	0.186	**0.041**	0.056	0.606
CD4 frequency	23.2 [14.7-31.6]	21.7 [14.2-30.5]	24.6 [18-34.8]	0.486	24.8 [17.5-32.9]	23.5 [16.9-30.2]	29.8 [19.0-38.3]	**0.024**	0.418	0.950	0.352
CD8 frequency	48.6 [37.7-58.3]	52.3 [39.5-59.6]	41.6 [31-57.5]	0.125	42.6 [33.9-51.2]	44.3 [36-54.5]	38.1 [30.1-48.7]	**0.013**	0.935	0.064	0.521
CD4/CD8 ratio	0.51 [0.28-0.81]	0.43 [0.26-0.78]	0.60 [0.42-0.84]	0.322	0.58 [0.37-0.98]	0.53 [0.36-0.70]	0.76 [0.40-1.17]	**0.011**	0.149	0.797	0.161
Nadir CD4	124 [41-214]	132 [66-189]	84 [37-236]	0.870	144 [65-222]	117 [66-169]	188 [50-244]	0.122	0.733	0.449	0.337
Time on cART	156 [116-195]	167 [115-195]	153 [117-167]	0.694	179 [118-211]	183 [163-213]	152 [97-196]	**0.014**	0.096	**0.015**	0.955
Time with HIV-1 suppression	51 [28-79]	52 [26-76]	48 [36-106]	0.315	85 [46-109]	76 [40-109]	94 [64-108]	0.522	**0.003**	**0.017**	0.158
HCV viral load	-	6.0 [5.5-6.7]	-	-	-	6.3 [5.4-6.6]	-		-	0.829	-
Pre-ART HIV1 VL	4.7 [3.9-5.2]	4.6 [4.0-5.2]	4.7 [3.6-5.1]	0.992	5.2 [4.7-5.6]	5.3 [4.8-5.6]	5.1 [4.6-5.6]	0.275	**<0.001**	**<0.001**	**0.005**

Table 3.5: Univariate and Multivariate analysis: factors independently associated with immune activation (dependent variable HTLV-2 proviral load).

	Univariate (p, r^2)			Multivariate (p, coefficient)	
HTLV-2 pVL dependent variable	All N=60	HCV^+ N=40	HVC^- N=21	All N=61	HCV^+ N=40
Age (years)	0.750	0.838	0.383		
Gender	0.941	0.858	0.863		
Time on cART (months)	0.506	0.539	0.604		
Time with undetectable HIV-1 viral load (months)	0.592	0.494	0.428		
Pre cART log HIV-1 viral load (RNA copies/ml)	0.837	0.215	0.096		
Nadir CD4 (cell/mm^3)	0.886	0.483	0.458		
CD4 count (cell/mm^3)	0.930	0.399	0.287		
CD8 count (cell/mm^3)	**0.001, 0.426**	**<0.001, 0.534**	0.871	**<0.001, 2.851**	**<0.001, 3.057**
CD4 frequency (%)	**0.020, -0.298**	**0.001, -0.499**	0.615	0.469	0.681
CD8 frequency (%)	**<0.001, 0.448**	**0.002, 0.469**	0.192	0.091	0.619
CD4/CD8 ratio	**0.002, -0.386**	**0.002, -0.482**	0.980	0.192	0.662
CD4 T cell activation (%)	0.548	0.398	0.393		
CD8 T cell activation (%)	0.852	0.716	0.366		
Log HCV viral load (IU/ml)	0.993				

3.20 HTLV-2 non-infected control groups

Ninety HIV-1 infected individuals seronegative for HTLV-2 infection with undetectable HIV-1 viral load on suppressive regimen at least for one year, were selected as control group for this study. Forty-two of them were also infected by HCV, while the other 21 negative for HCV infection. General clinical characteristics of HIV-1 infected patients studied are shown in table 3.4.

3.21 Characteristics associated to HIV-1 infection modified by HTLV-2 coinfection

Significant differences mainly in HIV-1 viral load previous treatment with antiretroviral therapy and in $CD8^+$ T cell count were found when compared the 61 HTLV-2/HIV-1 coinfected individuals with the 90 HIV-1-infected individuals (Table 3.4). Both differences were maintained among individuals coinfected with HIV-1/ HCV when compared based on HTLV-2 coinfection. However, when there was no HCV infection, only HIV-1 viral load previous treatment remained significantly different between HIV-1/HTLV-2 coinfected and those HIV-1-monoinfected individuals (Table 3.4).

Therefore, $CD8^+$ T cell count were significantly higher in HIV-1 infected individuals also infected by HTLV-2 compared to those HTLV-2 seronegative (Figure 3.15a). Despite HIV-1 viral load before starting antiretroviral therapy was significantly lower in HIV-1 who were also infected by HTLV-2 compared to those who were not, they had similar nadir $CD4^+$ T cells (Figure 3.15 b,c).

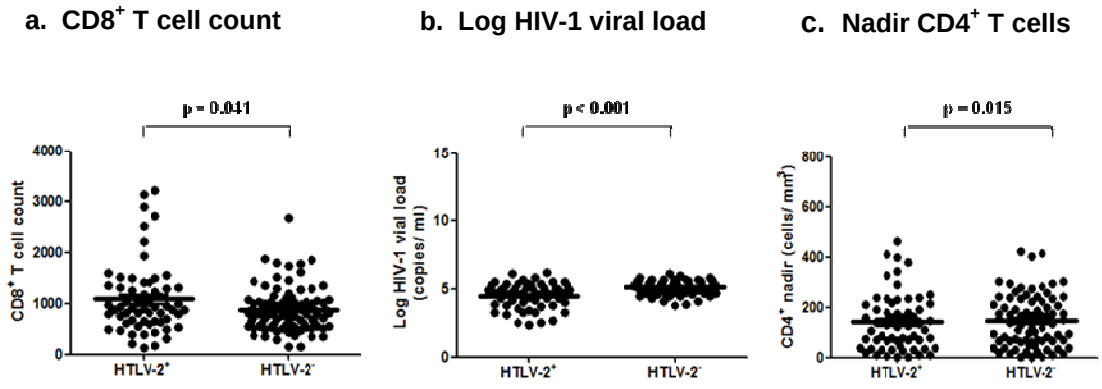

Figure 3.15: Differences in $CD8^+$ T cell count (a), log HIV-1 viral load (b) and nadir CD4+ T cells (c) in HIV-1 infected patients regarding to HTLV-2 coinfection.

3.22 Immune activation

Activated $CD8^+$ T cells were significantly elevated in HCV/HIV-1 coinfected individuals when compared to HIV-1 monoinfected ones. Strinkingly, those elevated

levels of activated $CD8^+$ T cells were significantly reduced with HTLV-2 infection. We further analyzed levels of activated $CD8^+$ T cells stratifying patients according to their $CD4^+$ T cell count. The decrease observed in percentage of activated $CD8^+$ T cells when HIV-1/HCV coinfected patients were also infected by HTLV-2 was mainly generated in individuals whose $CD4^+$ T cell count was above 500 cells/mm^3 (Figure 3.16a). This decrease was also observed in HIV-1/HCV coinfected individuals with $CD4^+$ T cells between 200 and 400 cells/mm^3, though no significant (Fig. 3.16b) and was completely lost in HIV-1/HCV coinfected individuals below 200 $CD4^+$ T cells/mm^3 (Fig. 3.16c). No significant differences were found in levels of activated $CD4^+$ T cells among HTLV-2 infection in any group studied based on $CD4^+$ T cell counts (not shown).

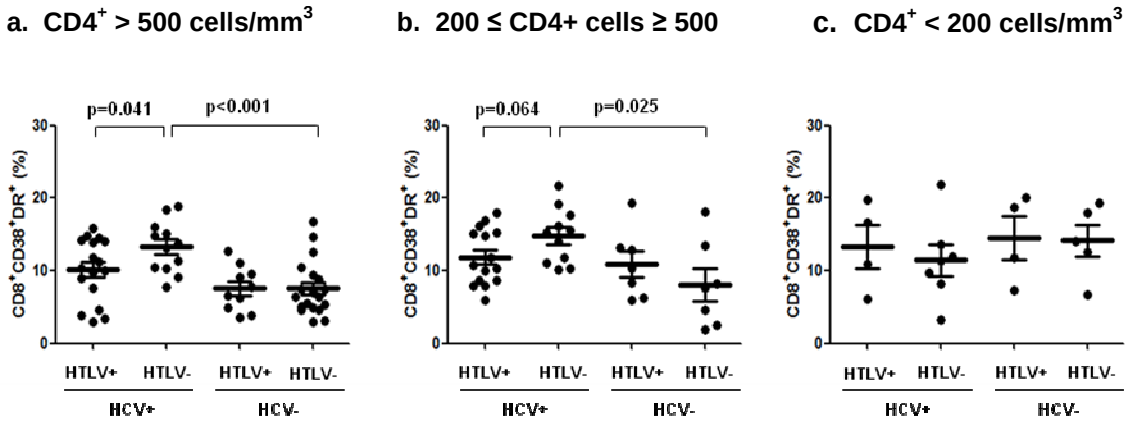

Figure 3.16: Differences in activated $CD8^+$ T cells among HIV-1-infected individuals stratified based on $CD4^+$ T cell count, superior to 500 cells/ mm^3 (a), between 200 and 500 cells/mm^3 (b) and below 200 cells/mm^3 (c).

3.23 Influence of HTLV-2 over HCV infection

Similar levels of log HCV viral load were found among HIV-1/HCV coinfected individuals whether they were coinfected with HTLV-2 or not (Fig. 3.17a).

However, when transaminase enzymes were analyze, similar levels of aspartate transaminase (AST) or glutamic oxoloacetic transaminase (GOT) were detected among HCV-infected patients, regardless of whether they were infected by HTLV (Fig. 3.17b). Interestingly, levels of alanine transaminase (ALT) or glutamic-pyruvic transaminase

(GPT) were significantly lower in HCV also infected by HTLV-2 when compared to those HCV-infected individuals negative for HTLV-2 (Fig. 3.17c).

Additionally, the analysis of Fibroscan values from HCV-infected patients showed a significantly lower degree of liver fibrosis in HCV-infected patients also infected by HTLV-2 than HCV-infected individuals without HTLV-2 infection (Fig. 3.17d).

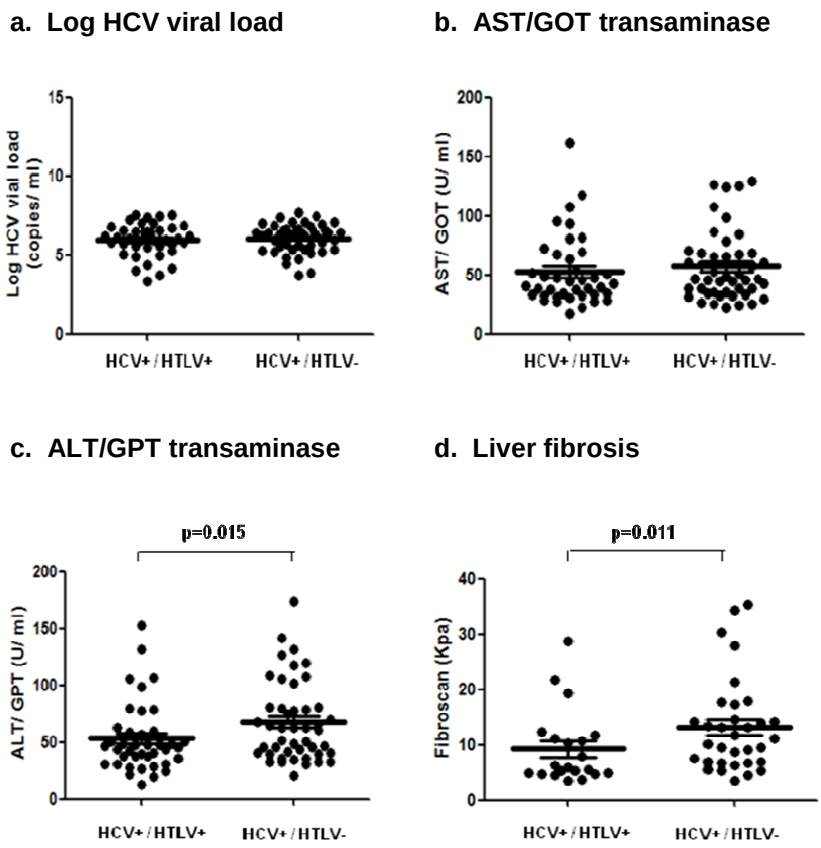

Figure 3.17: Log HCV viral load (a), levels of transaminase enzymes (b,c) and levels of fibroscan (d) analyzed in HIV-1/HCV-coinfected individuals also infected by HTLV-2.

SUB-STUDY 1

3.24 Patients´ characteristics

Seventeen HTLV-2 infected individuals coinfected with HCV and HIV-1 with a median age of 47 years, similar to the age of the two control groups non-infected by HTLV-2, composed of eight individuals coinfected by HIV-1/HCV and 11 HIV-1-monoinfected individuals, with a median age of 47 and 48, respectively. All three groups were receiving suppressive cART (HIV-1 viral load lower than 50 RNA copies/ml) with similar $CD4^+$ T cell count superior to 200 cells/mm^3 in all participants and similar $CD8^+$ T cell count.

We also analyzed twelve of the HTLV-2 infected patients studied epidemiologically through LTR, *env* and *tax* viral genes. All of them belonged to HTLV-2b subtype, closely related to HTLV-2b strains from Portuguese and Italian IDUs, and differentiated from HTLV-2a strains, the main subtype circulating in North Europe (Figure 3.18).

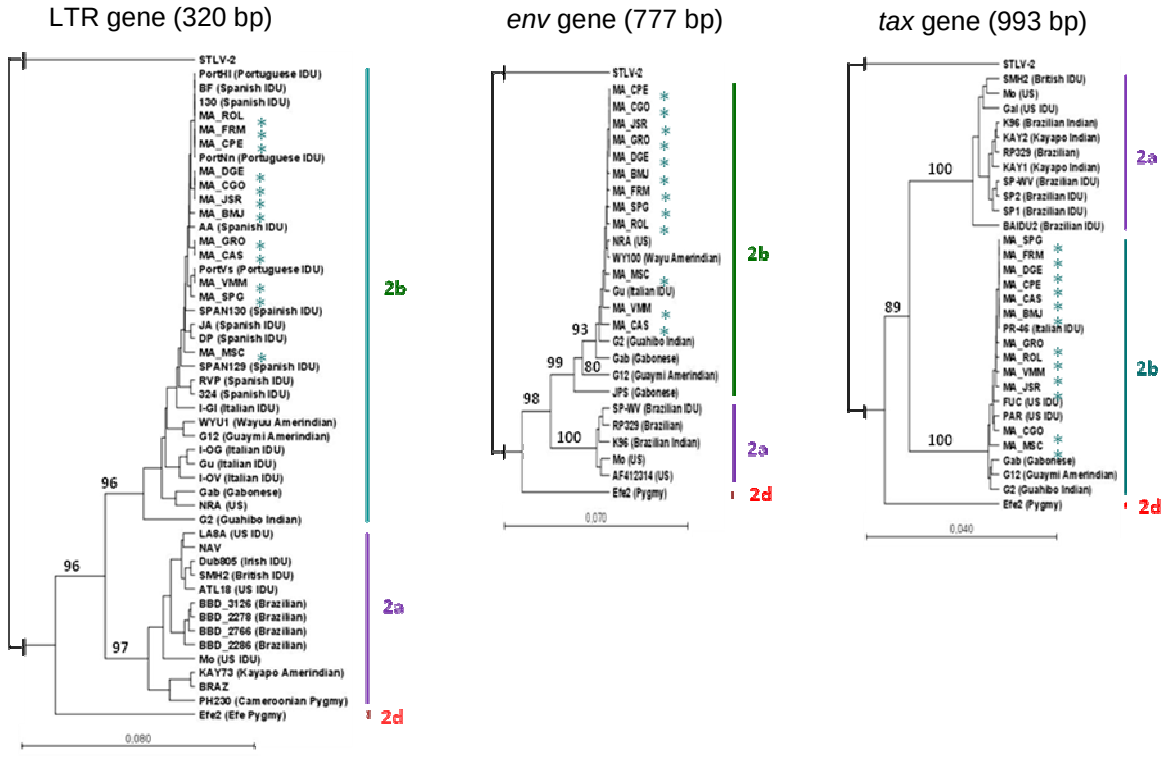

Figure 3.18: Epidemiological study of HTLV-2 subtypes among HIV-1 infected patients.

3.25 Microbial translocation

Plasma LPS levels in HIV-1 patients coinfected by HCV and HTLV-2 were significantly lower than either HIV-1/HCV coinfected (p=0.014) or HIV-1 monoinfected individuals (p=0.04). However, no difference was found in LPS between the HTLV-2-seronegative groups (Figure 3.19).

Similarly, levels of sCD14 were significantly lower than HIV-1-infected patients, but no significance was found when compared to HIV-1/HCV coinfected individuals (p=0.013). No difference was either found among HTLV-2-seronegative individuals.

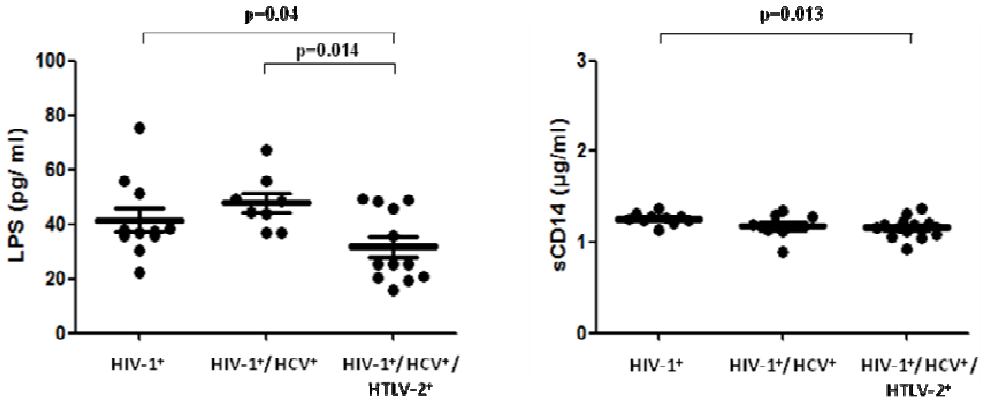

Figure 3.19: Microbial translocation in HTLV-2-infected individuals compared to both HIV-1/HCV-coinfected or HIV-1 monoinfected individuals.

3.26 Cytokine profile

Plasma IFN-γ levels were significantly higher in HTLV-2 infected individuals compared to both groups not infected by HTLV-2, either HIV-1/HCV infected (p=0.009) or HIV-1-monoinfected individuals (p<0.001) (Figure 3.20a). Similar levels of both IL-6 and TNF pro-inflammatory cytokines were found in plasma from the three groups of patients analyzed (Figure 3.20b and 3.20c).

Levels of IL-17 detected in plasma of HTLV-2-infected individuals were significantly higher than both HIV-1/HCV-coinfected (p=0.011) or HIV-1-monoinfected

individuals (p<0.001). Again, no significant differences were found among individuals not infected by HTLV-2.

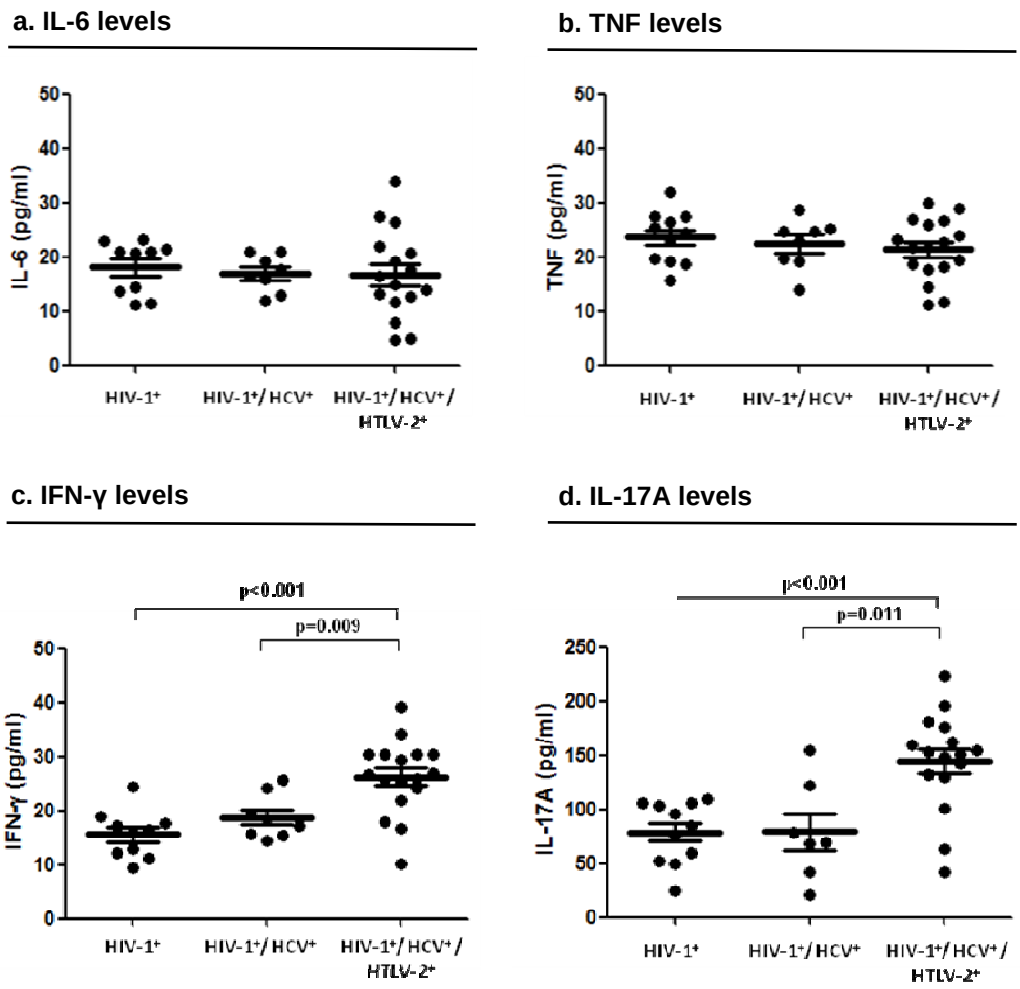

Figure 3.20: Levels of cytokines in plasma of HTLV-2-infected individuals compared to both HIV-1/HCV-coinfected and HIV-1 monoinfected individuals.

When analyzed levels of cytokines from HIV-1 patients analyzed all data together, only significant correlation was found between levels of IL-17A and IFN-γ (not shown). Neither significant correlation was found between levels of cytokines and levels of microbial translocation measured by LPS or sCD14 (not shown).

SUB-STUDY 2

3.27 Patients´ characteristics

Four patients who intensified their suppressive regimen with an integrase inhibitor (raltegravir) were analyzed. They had a median age of 51 [48-55], CD4 count of 215 [97-251] cells/mm^3, and CD8 count of 819 [658-929] cells/mm^3. Besides, a total of 11 HTLV-2-HIV-1-coinfected patients who did not intensify their cART with raltegravir were also analyzed as control group. The 11 patients from the control group had median age of 50 [46-56] years, CD4 count of 524 [307-1060] cells/mm^3, CD8 count of 980 [690-1308] cells/mm^3, and nine of them were male (82%). All the patients included in this study had undetectable HIV-1 plasma viremia (<50 HIV-1 RNA copies/ml) on cART for more than a year (Table 3.18).

3.28 T cell count

Neither CD8 count nor frequency, cells that are the main target for HTLV-2, showed any statistical change during the follow up among patients who received raltegravir as shown in figure 3.18. Among the control group, no significant variation in either CD8 count or frequency was found during the follow up (not shown).

Regarding CD4$^+$ T cells, other potential target for HTLV-2 infection, no significant variation of either counts or frequency was found. Only two patients increased either their CD4 counts or frequency (Ral1 and Ral3, table 1) at week 24 compared to baseline (Figure 3.22.). Among the control group, no significant variation in either CD4 count or frequency was found during the follow up (not shown).

3.29 HTLV-2 proviral load

During raltegravir intensification, HTLV-2 pVL significantly increased at week 24 compared to baseline in all four patients (p=0.003), as shown in figure 3.23a**.** At week

48 compared to week 24 a significant decrease of HTLV-2 pVL was found (p=0.020), reaching baseline levels. Among the control group, with no raltegravir in their cART, HTLV-2 pVL did not show any significant variation during the follow up, as shown in figure 3.23b.

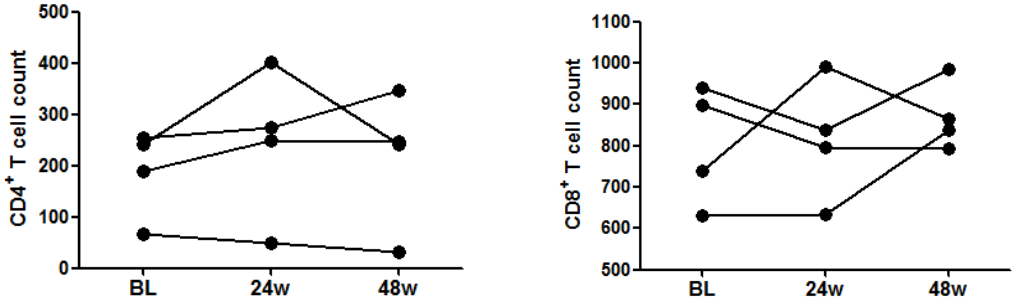

Figure 3.22: CD4 and CD8 T cell characteristics during the follow up and type of treatment of the patients who received raltegravir-based cART. Cell count in cells/mm3; cell frequency in percentage; IQR, interquartile range; Significant when p<0.05 (paired samples t test).

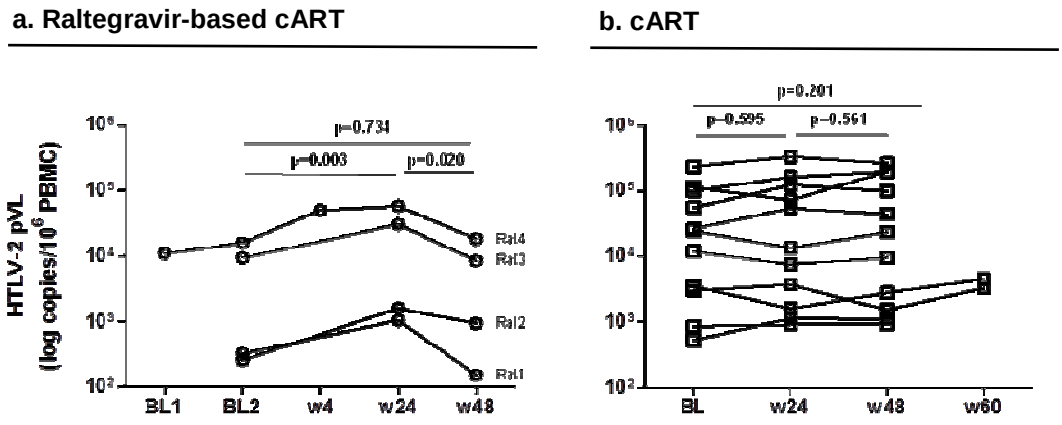

Figure 3.23: HTLV-2 pVL during the follow-up in patients with raltegravir-based cART (a) and patients with cART (b). Significant when p<0.05.

Discussion

ANTIRETROVIRAL TREATMENT INTENSIFICATION

Intensification of successful antiretroviral therapy has been evaluated as a strategy to help to eradicate HIV-1. The role of intensification in reducing residual replication and cell infection by the replicative virus has been supported by several studies[256-258]. One of them showed that intensification with raltegravir was associated with a significant decrease in proviral DNA in gut-associated lymphoid tissue (GALT)[108], and a decrease in unspliced HIV-1 RNA in $CD4^+$ T cells obtained from the terminal ileum[259], whereas no significant effect was observed on residual viremia. The hypothesis supported in these studies would be that the effect of intensification takes place mainly outside plasma sites, such as the GALT, where ART may not reach inhibitory levels, with the result that HIV-1 is able to replicate and infect new cells[260].

Activated $CD8^+$ T cells decreased during treatment intensification either with maraviroc or raltegravir. Therefore, the effect of treatment intensification seems to be not related to specific drug class. Moreover, this effect does not either seem to be due to a dilutional effect as a result of cell trafficking from plasma to the gut, because T-cell counts were stable during intensification.

The decrease observed in immune activation does not correlate with a decrease in bacterial translocation, which in fact increased in individuals who intensified their therapy with MVC, thus supporting increased viral replication and activation in the intestine resulting from the release of pro-inflammatory cytokines[261].

Within MVC group, the transient increase in TEM and TemRA $CD8^+$ T cell counts parallels the increase in bacterial translocation. These increments are balanced by a transient decrease in central memory $CD8^+$ T cell counts, while naive $CD8^+$ T cells remained stable[262].

Additionally, levels of microbial translocation increased during maraviroc intensification in parallel to levels of activated T cells bearing β7, favouring the idea of cells recruitment from peripheral blood to the gut tract as the major mechanism to recover the mucosa integrity. Hence, microbial translocation and systemic inflammation would be reduced[163-164]. Interestingly, this cell recruitment is reverted when microbial translocation is not longer increasing after 24 weeks of maraviroc discontinuation.

In accordance to these findings, within maraviroc group there is a strong correlation between both activated $CD4^+\beta7^+$ and activated $CD8^+\beta7^+$ T cells with LPS levels at the end of the intensification treatment, when the alteration of microbial translocation is the hieghest.

Within raltegravir group, bacterial translocation, levels of gut homing receptor β7 and T-cell subpopulations, that is naïve $CD4^+$ or $CD8^+$ T cells, TCM, TEM and TEMRA were stable during the follow-up period. No correlation between microbial translocation and either homing gut receptor expression on T cells or T-cell subsets were found.

On the other hand, analyzing patients on treatment intensification all together at the different time points, significant correlation between plasma LPS, LBP and sCD14 levels were found, in agreement with other studies[263]. Nevertheless, no correlation between 16S rDNA and the other measurements of microbial translocation was found in our study, although this correlation has been reported by other authors who evaluated both treatment-naive and -experienced patients[264]. The fact that our study included only patients on long-term cART who were virologically suppressed and with good immunological recovery may explain the discrepancy between our results and those of the other studies, including mixed populations.

Microbial translocation has been proposed as a possible driver mechanism of immune activation in HIV-1 infection[265-266], supported by the correlation between LPS levels and circulating $CD8^+$ T cells expressing $CD38^+$ and HLA-DR^+ observed in chronically infected HIV-1 patients, elite controllers, and naive patients[267]. Another group found a correlation between 16S rDNA and $CD8^+$ T-cell activation ($p=0.047$), but only when aviremic- and viremic-treated patients were analyzed together[145]. On the other hand, other studies failed to show this correlation during treatment interruption[268] or in patients undergoing raltegravir treatment intensification[269]. We did not observe any correlation between the levels of LPS, LBP, or sCD14 with immune activation.

Considering these data, we are unable to ensure that microbial translocation directly triggers T-cell immune activation, at least among these patients with relatively good immune recovery and under treatment intensification, although this process is clearly an important component of innate immune inflammation associated to HIV-1 infection.

COINFECTION WITH LEISHMANIA COMPARED TO IMMUNOLOGICAL DISCORDANT RESPONSE TO ART

Immunossupressed HIV-1-infected patients showed inefficient T-cell response compromising parasite control in *Leishmania* co-infection, resulting in frequent relapses especially in VL, despite antiretroviral therapy[270-272]. Conversely, *Leishmania* infection can promote HIV-1 replication[180] and increases the degree of immune system activation[273].

VL patients showed lower levels of CD4 counts compared to CR patients despite similar time on suppressive regimen, with levels similar to those observed in DR patients. This may be partially explained by the depletion of bone marrow precursor cells associated to *Leishmania* in individuals concomitantly infected with HIV-1 leading to deficiencies in the input of new lymphocytes into the periphery, though these values still remained superior to DR patients in some cases[274].

Another contribution for this immunosuppression is the elevated levels of T-cell activation in VL patients compared to those without leishmaniasis that lead to elevated T-cell depletion. This elevated immune activation was independently associated with CD4/CD8 ratio, leishmaniasis and sCD14 levels, but not with the presence of HCV infection. Therefore, the contribution of leishmaniasis to cell activation in VL patients was stronger than that observed in HCV infection.

Moreover, the elevated immune activation was associated to an increase of T-cell senescence, despite similar age of the groups studied, which might accelerate the impairment of the immune effector function, and could explain the lack of immunological recovery also observed.

On the other hand, the increased levels of sCD14 in VL patients may indicate higher levels of microbial translocation. However, *Leishmania* infect and may activate monocyte/macrophage linage increasing sCD14 secretion. For this reason, sCD14 levels in this subgroup of patients may is not an adequate indicator of microbial translocation, at least as a single measurement.

In any case, LPS levels in VL patients were higher than levels found in DR patients, though no significant. The overactivation of monocyte/macrophage lineage produced by *Leishmania* may impair the phagocitosis of microbial products leading to

the increase in LPS levels. Elevated levels of microbial translocation among VL patients, similar or even higher to those found in DR patients, suggest further deterioration of the mucosal tissue strongly associated to *Leishmania* infection that might increase proinflammatory response and immune activation. According to this hypothesis, we observed correlation between microbial translocation and inflammation in VL patients, only partially observed among DR patients. And furthermore, sCD14 correlated with immune activation in VL patients and, again, only partially among DR patients.

Our results show that VL is an independent cause of increased levels of immune activation, inflammation and microbial translocation compared to HIV-1 infected patients without leishmaniasis despite HIV-1 viral load suppression.

The main limitation in this study is the small number of VL patients although most of the published studies are performed with a limited number of patients.

COINFECTION WITH HTLV-2, HEPATITIS C VIRUS (HCV) AND HIV-1

HIV-1 and HTLV-2 coinfection is found with relatively high frequency among injecing drug users in North America and Western Europe[212-214]. We found prevalence rate for HTLV-2 infection of 8.5% in HIV-1-infected patients with a history of injecting drug use in our centre. Twelve of these patients epidemiologically analyzed revealed that HTLV-2b remained the prevalent circulating subtype in Spain, closely related to Italian and Portuguese IDUs.

Several studies emphasized the beneficial influence of HTLV-2 infection on HIV-1 disease progression[231-232,275].Even more, coinfection with HIV-1/HTLV-2 was found in many long-term non-progressors, where HTLV-2 infection was able to modify innate host immune responses by hampering the fusion and entry of the most commonly transmitted HIV-1 strains[234-235].

Supporting the protective role of HTLV-2 infection on the pathogenesis of HIV-1 disease, we found significantly lower plasma HIV-1 RNA levels in HTLV-2 coinfected individuals than individuals negative for HTLV-2 infection, despite having similar nadir $CD4^+$ T cell count and $CD4^+$ T cell count.

Additionally, we found higher levels of $CD8^+$ T cell count in HTLV-2 coinfected patients. Moreover, $CD8^+$ T cell count, the main target of HTLV-2 infection, was strongly and independently associated to HTLV-2 proviral load.

Further analysis of HTLV-2-coinfected patients regarding to HCV infection revealed that, within HCV-coinfected patients, the HTLV-2 proviral load was significantly higher than those negative for HCV infection. This finding was indicating that each HCV or HTLV-2 virus influenced the pathogenesis and natural history of the other. Despite elevated rates of HCV/HTLV coinfection, epidemiological and clinical features of coinfection have been overlooked.

Therefore, we evaluated the role of HTLV-2 infection in HIV-1 infected patients classified by HCV infection. Interestingly, we found that among HIV-1/HCV coinfected patients, HTLV-2 infection significantly reduced levels of activated $CD8^+$ T cells in patients with good immune recovery ($CD4^+$ counts greater than 500 cells/mm^3), levels of alanine transaminase and liver fibrosis, clearly indicating a further protective role of HTLV-2 infection in HCV-infection and liver disease, the current leading cause of death among HIV-1-infected individuals[194].

Further sub-studies evaluating the influence of HTLV-2 infection on microbial translocation and secretion of proinflammatory cytokines among HIV-1/HCV coinfected patients showed that HTLV-2 infection significantly decrease levels of LPS in plasma of HIV-1/HCV coinfected individuals. And, by contrast, levels of IFN-γ and IL-17A were significantly increased in presence of HTLV-2.

Taking all these findings together we proposed a model that supports the protective effect of HTLV-2 infection in the previously described HIV-1 disease progression, and moreover, HCV infection.

HTLV-2 infection in HIV-1/HCV coinfected individuals seemed to increase the secretion of IL-17A from Th17 cells. This interleukin-17A was reported to enhance epithelial regeneration in the GALT and defend against microbial translocation by recruiting neutrophils to clear microbial products[130-132]. According to its potential role on mucosa recovery, we found significantly lower levels of LPS in the HTLV-2 infected individuals.

Microbial translocation was suggested as one possible contributor to chronic inflammation and fibrosis in chronic HCV monoinfection[199-202]. Strikingly, in parallel to

decreased levels of LPS, we found significantly lower levels of both ALT, specifically released when hepatic injury[276] and hepatic fibrosis in presence of HTLV-2 infection.

Liver fibrosis is influenced by the Th2 response being associated with more active fibrogenesis[277-278]. Th2 response was also associated to HIV-1 disease progression[243]. The pattern of HTLV-2 infection may indeed contribute to induce a protective Th1 response upregulating IFN-γ secretion as observed in these HIV-1/HCV coinfected individuals and previously described[237], which can also inhibit CCR5 expression and is involved against invading pathogenes[240].

We are unable to ensure correlation between bacterial products and immune activation due to the small size of patients analyzed for microbial translocation. However, we observed significantly reduced levels of activated $CD8^+$ T cells in coinfected patients with good immune recovery ($CD4^+$ counts superior to 500 cells/mm^3), and elevated levels of $CD8^+$ T cells.

A vicious circle was reported in which inflammatory and fibrogenic cells were able to stimulate each other during hepatic fibrogenesis in coinfected patients[279]. HTLV-2 infection may disrupt this circle delaying HCV-disease progression and liver disease.

To end up, we performed another sub-study in which we evaluate the effect of treatment intensification with an integrase inhibitor, raltegravir, on HTLV-2 pVL in well-suppressed HIV-1-infected patients.

Up today, there is no effective treatment for the HTLV infection. However, given the similarities between HIV-1 and HTLV-1 integrase, raltegravir, was thought to be effective against HTLV-1. Supporting this idea, a decrease in HTLV-1 pVL *in vitro* and also *ex vivo* was detected in presence of raltegravir[252]. Furthermore, *in vitro* studies showed that raltegravir prevented the integration of HTLV-1 in both cell-free and cell-to-cell infection[253].

Despite these promising *in vitro* results, no decrease on HTLV-1 pVL was reported in the unique in vivo study on five infected patients[254]. The lack of any beneficial effect on HTLV-1 viral replication was attributed to the virus infection transmission mainly maintained through cell division instead of through new rounds of viral replication[255].

We observed a transient increase on HTLV-2 pVL in HTLV-2/HIV-1 co-infected patients when cART was intensified with raltegravir and no changes on HTLV-2 pVL in the HIV-1/ HTLV-2 coinfected patients on suppressive regimen without raltegravir.

This transient increase could be due to the activation and clonal expansion of the CD8 lymphocytes infected with HTLV-2[280-281], explaining the subsequent return to baseline proviral load levels by the CTL response that would rapidly eliminate infected cells once they start to express viral proteins. Nevertheless, this possible transient clonal expansion was not reflected on CD8 count or frequency that remained stable along the follow up studied.

On the other hand, it is also possible that a transient increase of episomal HTLV-2 2-LTR DNA circles might occur as seen in HIV-1 patients intensified with raltegravir [110]. This possibility implies an increase on HTLV-2 replication and a subsequent accumulation of episomal HTLV-2 2-LTR DNA circles due to raltegravir effects.

Two important limitations of this study were the limited amount of DNA sample from each patient to test these two given possibilities, and the small number of patients included for the analysis forcing to take these results with caution. Unfortunately, there is limited experience using specific antiretroviral drugs in these HTLV-2-infected patients and no treatment recommendations can be given.

CONCLUSIONS

1. Treatment intensification decreases immune activation in well-suppressed HIV-1 infected patients. However, parallel decrease was not found in microbial translocation which, in fact, increases within individuals who intensified with maraviroc. Effects on immune activation or microbial translocation during the intensification follow-up period were reverted after intensifying drug discontinuation.

2. Dynamics of microbial translocation are in parallel with expression of gut homing β7 receptor on T cells favoring the idea of T cells recruitment from the periphery to the GALT to recover gut mucosa.

3. The quantification of LPS, LBP and sCD14 are good measurements of microbial translocation and can be used interchangeably. However, poor association between microbial translocation and immune activation was found in long-term suppressed individuals.

4. Leishmaniasis is a factor that strongly contributes to increase the severity of immunodeficiency caused by HIV-1, with worse immunological characteristics than those observed in patients with discordant response to cART, showing increased levels of $CD8^+$ T cell activation, IL-6, sCD14 and activated $CD8^+$ T-cell senescence.

1. Protective role of HTLV-2 infection on HIV-1 infected individuals was corroborated in this study, with reduced plasma HIV RNA levels before starting antiretroviral therapy despite similar nadir CD4+ T cell count, and higher levels of $CD8^+$ T cell count in HIV-1/ HTLV-2 coinfected individuals compared to HIV-1 monoinfected patients.

2. Further protective role of HTLV-2 role on HCV infection and liver disease was observed in HIV-1/HCV-coinfected individuals. HTLV-2 infection significantly reduced levels of activated $CD8^+$ T cells in patients with good immune recovery ($CD4^+$ counts above 500 cells/mm^3), levels of alanine transaminase and liver fibrosis.

3. Sub-study from these patients further contribute to the protective role of HTLV-2 in HIV-1/HCV coinfected individuals, showing increased levels of IFN-γ (Th1 response) and IL-17 (Th17) in parallel with reduced levels of LPS.

4. A transient increase on HTLV-2 proviral load was found during raltegravir intensification.

Further investigations will be needed to prevent or attenuate microbial translocation and generalized immune activation in order to reduce the risk of developing non-AIDS defining comorbidities associated to HIV-1 related immune dysfunction and inflammation during antiretroviral treatment. Besides, further studies on the immunological interactions between HTLV-2 and HIV-1 or HCV would be useful to understand the mechanisms underlying the potencial protective effect of the HTLV-2 infection on other viral infections, such as HIV-1 and HCV.

DISCUSIÓN

INTENSIFICACIÓN DEL TRATAMIENTO ANTIRETROVIRAL

La intensificación de la terapia antirretroviral se ha evaluado como una estrategia para la erradicación de VIH-1. Numerosos estudios han defendido la intensificación de tratamiento como estrategia efectiva para reducir los niveles de replicación viral residual y la consecuente infección de nuevas células diana[256-258].

Uno de los estudios muestra una asociación entre la intensificación de tratamiento con raltegravir y una disminución de los niveles de ADN proviral en el tejido linfoide asociado a mucosas[108]. Otro estudio sostiene unos menores niveles de ARN no procesado en células $CD4^+$ procedentes del íleo[259]. Sin embargo, ninguno de ellos mostró cambios en la viremia residual. La hipótesis que sostienen para entender los resultados obtenidos, es que la intensificación de tratamiento tiene su efecto en determinados lugares anatómicos como la mucosa intestinal, donde el tratamiento no alcanza niveles inhibitorios esperados, con el consecuente establecimiento de zonas donde el virus continuaría replicando e infectando nuevas células[260].

Los niveles de células $CD8^+$ activadas se ven significativamente mermados durante la intensificación de tratamiento, independientemente del fármaco utilizado. Esta reducción podría ser causada por el reclutamiento de células $CD8^+$ activadas hacia el intestino, pero parece una opción poco probable teniendo en cuenta que la cantidad de linfocitos T permanecen estables durante el periodo de seguimiento.

Por otro lado, los niveles de activación inmune no se correlacionan con los niveles de translocación bacteriana. Por el contrario, aquellos individuos que intensifican su terapia con maraviroc, muestran un aumento de los niveles de lipopolisacáridos bacterianos a pesar de recibir tratamiento antirretroviral[261].

Sin embargo, cabe destacar que los niveles de translocación bacteriana varían paralelamente con la expresión de receptores β7 en la membrana de linfocitos T activados, ofreciendo evidencias de un posible reclutamiento de células T activadas como un mecanismo de recuperación de la integridad de la mucosa, para disminuir en consecuencia el proceso de translocación bacteriana e inflamación en el intestino[163-164].

Se puede observar una fuerte correlación entre las células T activadas expresando el receptor β7 y los niveles de lipopolisacáridos al final del periodo de intensificación (48 semanas), momento en el que el proceso de translocación bacteriana es máximo.

El mayor inconveniente en este estudio es el número reducido de pacientes que se someten a tratamiento de intensificación.

Si se analizan simultáneamente todas las determinaciones obtenidas de los pacientes incluidos en el estudio a lo largo del seguimiento, se observa una correlación positiva entre las diferentes técnicas para medir la translocación bacteriana, incluyendo lipopolisacáridos, proteínas de unión a lipopolisacáridos y CD14 soluble[263], con la excepción de los niveles de ADN de la subunidad 16 ribosomal[264].

La translocación bacteriana se ha propuesto como un mecanismo desencadenante de la elevada activación inmune de los individuos infectados por VIH-1 a pesar del tratamiento. Sin embargo, no se ha detectado correlación alguna entre los niveles de translocación bacteriana y la activación inmune. Esta ausencia de correlación se le ha atribuido a la propia naturaleza de los pacientes analizados, todos ellos con muy buena recuperación inmunológica y en tratamiento por largo periodo de tiempo.

INDIVIDUOS COINFECTADOS CON LEISHMANIA EN COMPARACIÓN CON INDIVIDUOS CON RESPUESTA DISCORDANTE A TRATAMIENTO

Los individuos infectados por VIH-1 con leishmaniasis visceral se caracterizan por una elevada activación inmune respecto a los individuos monoinfectados por VIH-1, más parecido su estado inmunológico a aquellos individuos infectados por VIH-1 con respuesta inmunológica discordante al tratamiento antirretroviral.

De este modo, los individuos coinfectados por VIH-1/*Leishmania* tienen menores niveles de células $CD4^+$, comparables a los niveles observados en individuos con respuesta inmunológica discordante al tratamiento antirretroviral.

Los niveles plasmáticos de CD14 solubles detectados en individuos infectados con leishmania visceral eran significativamente mayores que los niveles detectados en individuos monoinfectados. De todos modos, el parásito infecta monocitos, a los que activa pudiendo alterar los niveles de sCD14 liberados. Es posible que en este tipo de pacientes coinfectados, fuera más riguroso la utilización de una técnica alternativa para medir los niveles de translocación bacteriana.

Se han detectado mayores niveles de inflamación y activación de células T $CD8^+$ en individuos coinfectados con *Leishmania* en comparación con individuos inmunodiscordantes. La activación inmune elevada se asociaba con una mayor senescencia celular, a pesar de no haber diferencia de edades entre los grupos analizados.

COINFECCIÓN POR HTLV-2 Y HCV EN INDIVIDUOS INFECTADOS VIH-1

Las coinfecciones por HTLV-2 en individuos HIV-1 positivos, son relativamente frecuentes en usuarios de droga vía intravenosa. En el Hospital Ramón y Cajal, en Madrid, la prevalencia de infección por HTLV-2 que encontramos en este colectivo ascendió a un 8.5%.

Numerosos estudios han remarcado el posible papel protector que la infección por HTLV-2 ejerce en la infección por VIH-1 y progresión a SIDA[234-235]. Nuestros resultados corroborarían la ralentización de la progresión de la enfermedad en individuos coinfectados que tienen una menor carga proviral previo inicio del tratamiento antiretroviral, a pesar de contar con niveles similares de células $CD4^+$ T nadir. Además, los individuos coinfectados poseen mayores niveles de linfocitos T $CD8^+$ en comparación con individuos monoinfectados por VIH-1.

Existen pocos estudios, sin embargo, que estudien la influencia del virus HTLV-2 en la infección por VHC y el desarrollo de enfermedades hepáticas, a pesar del gran número de individuos VIH-1/HCV coinfectados en este sector.

Se propuso pues profundizar en las características inmunológicas y virológicas de estos pacientes para evaluar los efectos de HTLV-2. De este análisis se observó que la infección por HTLV-2 conduce a una reducción de los niveles de activación inmune de las células T $CD8^+$ y menores niveles de la enzima alanina transaminasa, indicadora de daño hepático, y de los niveles de fibrosis hepática.

Un sub-estudio realizado en estos pacientes VIH-1/HCV coinfectados, muestra además una menor translocación bacteriana en individuos HTLV-2^+, y un aumento de la secreción de la interleucina-17, precisamente encargada de la reparación de la mucosa intestinal.

Teniendo en cuenta los resultados obtenidos, la infección por HTLV-2 no sólo tendría un papel beneficioso en la infección por VIH-1, sino además ralentizaría además la aparición de enfermedades hepáticas asociadas a infección por VHC.

CONCLUSIONES

1. La intensificación de tratamiento disminuye activación inmune en individuos infectados por VIH-1 virológicamente suprimidos. Sin embargo, nose observó una disminución paralela con la translocación bacteriana, de hecho, estos niveles se incrementan en los individuos que intensificaron con maraviroc. Los efectos de la intensificación sobre la activación inmune y la trnanslocación bacteriana se revierten durante el periodo de desintensificación.

2. El proceso de translocación bacteriana varía paralelamente con la expresión del receptor β7 en la superficie de células T, favoreciendo el reclutamiento de linfocitos hacia la mucosa intestinal para restablecer su integridad.

3. Las técnicas para evaluar el proceso de translocación bacteriana, incluyendo niveles de LPS, LBP y sCD14 se correlacionan entre sí, permitiendo su utilización como marcadores intercambiables de translocación bacteriana. Sin embargo, no detectamos correlación entre translocación bacteriana y activación inmune, al menos en individuos suprimidos durante un largo periodo de tiempo.

4. La leishmaniasis visceral agrava severamente la inmunodeficiencia generada por VIH-1. Las características inmunológicas de los pacientes coinfectados por *Leishmania* son peores que las observadas en individuos monoinfectados con respuesta discordante a tratamiento. Entre estas características destacan una elevada activación de células T $CD8^+$, unos mayores niveles de inflamación, y sCD14 que puede explicar la deficiente respuesta inmunológica en estos individuos.

5. La función protectora de la infección por HTLV-2 en individuos VIH-1-positivos se ha corroborado en este estudio, con una reducida carga viral VIH-1 previa al comienzo de tratamiento a pesar de tener el mismo nadir CD4 con respecto a pacientes no infectados, y con un aumento de los niveles de células T $CD8^+$.

6. La función protectora de HTLV-2, se extiende a la infección por HCV y enfermedades hepáticas mediante la disminución de los niveles de activación de células T $CD8^+$ en individuos con niveles de $CD4^+$ superiores a 500 cells/mm^3, así como los niveles de enzima alanina transaminasa y de fibrosis hepática.

7. Un sub-estudio de estos pacientes coinfectados por HIV-1/HCV corrobora la función protectora de HTLV-2, mediante un aumento de la expresión de las citoquinas IFN-γ (Th1) y IL-17A (Th17) en paralelo con una disminución de los niveles de LPS.

8. Se ha observado un incremento transitorio de la carga proviral HTLV-2 durante la instensificación de tratamiento con raltegravir.

Posteriores investigaciones serían necesarias para desarrollar estrategias que puedan prevenir o atenuar la translocación bacteriana y activación inmune generalizada en individuos infectados por VIH-1, para lograr, si no su erradicación, una calidad de vida más próxima a individuos no infectados por VIH-1. Además, el entendimiento de los mecanismos que subyacen la ralentización de la progresión a SIDA y de enfermedades hepáticas en individuos coinfectados por HTLV-2 sería fundamental para reducir el riesgo de morbilidad y mortalidad en individuos infectados por VIH-1.

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APPENDIX 1: List of Publications

- **Abad-Fernández M**, Vallejo A, Hernández-Novoa B, Díaz L, Gutiérrez C, Madrid N, Muñoz MA, and Moreno S. Correlation between different methods to measure microbial translocation and its association with immune activation in long-term suppressed HIV-1-infected individuals. JAIDS 2013; 64: 149-153.

- **Abad M**, Dronda F, Dominguez E, Moreno S, Vallejo A. HTLV-2b among HIV-1-coinfected injecting drug users in Spain. AIDS Res & Hum Retrovir 2011; 27(5): 579-83.

- González-Serna A*, **Abad-Fernández M***, Soriano-Sarabia N, Leal M, Vallejo A. Alterations of CD8 T cell receptor Vβ chain repertoire are related with immunologic markers in HIV-1-infected patients during treatment interruption and are restored by antiretroviral therapy. J Clin Virol, in press.*, contributed equally.

- Gutiérrez C, Hernández-Novoa B, Vallejo A, Serrano S, **Abad-Fernández M**, Madrid N, Díaz L, Moreno A, Dronda F, Zamora J, Muñoz-Fernández MA and Moreno S. Dynamics of the HIV-1 latent reservoir after discontinuation of the intensification of antiretroviral therapy: Results of two clinical trials. AIDS 2013; 27(13): 2081-2088.

- Serrano-Villar S, Gutiérrez C, Vallejo A, Hernández-Novoa B, Díaz L, **Abad Fernández M**, Madrid N, Dronda F, Zamora J, Muñoz-Fernández MÁ, Moreno S. The CD4/CD8 ratio in HIV-infected subjects is independently associated with T-cell activation despite long-term viral suppression. J Infect 2013; 66(1): 57-66.

- Vallejo A, Gutiérrez C, Hernández-Novoa B, Díaz L, Madrid N, **Abad-Fernández M**, Dronda F, Pérez-Elías MJ, Zamora J, Muñoz E, Muñoz-Fernández MA, Moreno S. The effect of intensification with raltegravir on the HIV-1 reservoir of latently infected memory CD4 T cells in suppressed patients. AIDS 2012; 26 (15): 1885-94.

- Gutiérrez C, Díaz L, Vallejo A, Hernández-Novoa B, **Abad M**, Madrid N, Dahl V, Rubio R, Moreno A, Dronda F, Casado JL, Navas E, Pérez-Elías MJ, Zamora J, Palmer S, Muñoz E, Muñoz-Fernández MA, Moreno S. Intensification of antiretroviral therapy with a CCR5 antagonist in patients with chronic HIV-1 infection: Effect on T cells latently infected. Plos One 2011; 6(12): e27864.

- De Felipe B, Pérez-Romero P, **Abad-Fernández M**, Fernandez-Cuenca F, Martinez-Fernandez FJ, Trastoy M, Marta Rdel C, López-Cortés LF, Leal M, Viciana P, Vallejo A. Prevalence and resistance mutations of non-B HIV-1 subtypes among immigrants in Southern Spain along the decade 2000-2010. Virol J. 2011; 8: 416.

Correlation Between Different Methods to Measure Microbial Translocation and Its Association With Immune Activation in Long-Term Suppressed HIV-1–Infected Individuals

María Abad-Fernández, Alejandro Vallejo, PhD,* Beatriz Hernández-Novoa,* Laura Díaz,† Carolina Gutiérrez,* Nadia Madrid,* María Ángeles Muñoz,† and Santiago Moreno**

Introduction: Microbial translocation (MT) has been proposed as one of the triggering mechanisms of persistent immune activation associated to HIV-1 infection. Our objectives were to determine the correlation between different measurements of MT in suppressed HIV-1–infected individuals and to evaluate its correlation with immune activation.

Methods: Eighteen suppressed HIV-1–infected patients with $CD4^+$ T-cell count above 350 cells per cubic millimeter and undetectable plasma viral load, included in antiretroviral treatment intensification clinical trials, were evaluated. Samples obtained at baseline and at established time points during the trials were analyzed. Lipopolysaccharide (LPS), lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14), and bacterial 16S *ribosomal* DNA (16S rDNA), and markers of immune activation were determined.

Results: We analyzed 126 plasma samples from the 18 patients. LPS significantly correlated with sCD14 ($P < 0.001$, r = 0.407) and LBP ($P = 0.042$, r = 0.260). Also, a significant correlation was found between sCD14 and LBP ($P = 0.009$, r = 0.325) but not between bacterial 16S rDNA and LPS, sCD14, or LBP ($P = 0.346$, $P = 0.405$, and $P = 0.644$). On the other hand, no significant correlation was found between LPS, sCD14, or LBP and $CD4^+$ ($P = 0.418$, $P = 0.619$, and $P = 0.728$) or $CD8^+$ T-cell activation ($P = 0.352$, $P = 0.275$, and $P = 0.124$). Bacterial 16S rDNA correlated with activated $CD4^+$ T cells ($P = 0.005$, r = 0.104) but not with activated $CD8^+$ T cells ($P = 0.171$).

Conclusions: There is a good correlation in the quantification of LPS, sCD14, and LBP levels, but not with bacterial 16S rDNA, as measurements of MT. We are unable to ensure that MT directly triggers T-cell immune activation at least among these patients with relatively good immune recovery and under treatment intensification.

Key Words: microbial translocation, LPS, sCD14, LBP, 16S rDNA, immune activation

(*J Acquir Immune Defic Syndr* 2013;64:149–153)

INTRODUCTION

The mucosal damage associated to HIV-1 infection leads to translocation of microbial products from the intestinal lumen into the bloodstream.[1–5] Levels of microbial translocation (MT) in plasma of HIV-1–infected patients have been frequently determined by the quantification of the plasma levels of lipopolysaccharide (LPS), soluble CD14 (sCD14), lipopolysaccharide binding protein (LBP), or bacterial 16S *ribosomal* DNA (16S rDNA).

Increased MT has been proposed as one of the main trigger mechanisms for persistent immune activation in HIV-1–infected patients,[6–9] a strong predictor for disease progression.[10–14] Several studies have reported the association between MT and immune activation in the first years of chronic HIV-1 infection or in the most advanced stages of HIV/AIDS.[15–17] However, the dynamics of MT and immune activation in patients with high $CD4^+$ T-cell counts and undetectable plasma viral load are still poorly defined.

The aim of our work was to determine correlations between different techniques employed to measure MT in plasma of HIV-1–infected individuals under suppressive antiretroviral therapy (ART). For this purpose, we analyzed levels of bacterial products as LPS and 16S rDNA and sCD14 marker of monocyte activation and plasma LBP. As a secondary objective, we evaluated the correlation between MT and $CD4^+$ and $CD8^+$ T-cell activation.

MATERIALS AND METHODS

This study was performed within 2 pilot open-label phase II intensification clinical trials conducted at the Hospital Ramón y Cajal in Madrid, Spain, from 2008 to 2012.[18,19] The 18 patients included in this study had a median age of 46 (41–50) years and most were male (88%), with

Received for publication February 20, 2013; accepted May 1, 2013.

From the *Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria, Hospital Universitario Ramón y Cajal, Madrid, Spain; and †Immunobiology Laboratory, Hospital General Universitario Gregorio Marañón, Madrid, Spain.

Supported in part by the Spanish AIDS Network "Red Temática Cooperativa de Investigación en SIDA (RD06/0006), the Spanish National of Health, "Instituto de Salud Carlos III", grants (FIS-PI080958 and CP08/00046), and by the Foundation of Investigation and prevention of AIDS (FIPSE-36-0844/09).

The authors have no conflicts of interest to disclose.

Correspondence to: Alejandro Vallejo, PhD, Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria, Hospital Universitario Ramón y Cajal, Carretera de Colmenar Km 9, 28034 Madrid, Spain (e-mail: alejandro.vallejo@salud.madrid.org).

a median time on ART of more than 8 years (Table 1). The median CD4 count was 675 cells per cubic millimeter, and all of them had $CD4^+$ T-cell count above 350 cells per cubic millimeter and prolonged viral suppressive ART (<40 HIV-1 RNA copies/mL). Nine patients incorporated a CCR5 antagonist (maraviroc, provided by Pfizer, Inc., New York) to their current suppressive regimen, whereas the other 9 patients intensified their ART with an integrase inhibitor (raltegravir, provided by Merck Sharp and Dhome, Whitehouse Station, NJ).

The 7 time points included in this study were as follows: baseline, before the inclusion of intensifying drugs; weeks 12, 24, 36, and 48 after the inclusion of the intensifying drugs; and weeks 12 and 24 after discontinuation of intensifying drugs. All these 126 samples from the 18 patients at the different time points were analyzed altogether.

MT was measured in plasma by 3 commercial kit assays according to the manufacturer's protocol. Plasma bacterial LPS was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza, Basel, Switzerland), plasma sCD14 was quantified using the Quantikine Human sCD14 Immunoassay (R&D Systems, Minneapolis, MN) and plasma LBP was measured by LBP soluble ELISA kit (Enzo Life Sciences, Farmingdale, NY). All the samples were run in duplicate.

To perform the bacterial 16S rDNA quantification, DNA was extracted from 200 μL of plasma using QIAamp DNA kit (Qiagen, Hilden, Germany). A standard curve was generated using 10 to10^6 copies of a recombinant plasmid containing 16S gene fragment (171 bp) for quantification purpose. The amplification reaction was carried out in duplicate using LightCycler 2.0 (Roche Diagnostics, Manheim, Germany) in a total volume of 20 μL containing LightCycler FastStart DNA Master PLUS HybProbe 5X (Roche Diagnostics), 200 ng of DNA, 50 pmol of each primer, 16S-F (5′-AGGTCGCTTCTCTTTGTATGC) and 16S-R (5′-ATGCGCCATTGTAGCACGTGTGT), along with 2 pmol of the following fluorescent probe: 5′-[6FAM]-AAGTCCCGCAACGAGCGCAACCCT-[BQH1]. The cycling parameters included a hot start at 95°C for 10 minutes and continued with 40 cycles of denaturation at 95ºC for 5 seconds, annealing at 55ºC for 10 seconds and extension at 72ºC for 15 seconds.

Fresh ethylenediamine tetraacetic acid anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T-cell activation with the following antibody combination: CD3-allophycocyanin-Cy7, CD4-peridinin chlorophyll protein complex, CD8-phycoerythrin-Cy7, CD38-phycoerythrin, and HLA-DR-allophycocyanin. Antibodies were from BD (Becton Dickinson, Franklin Lanes, NJ), and an unstained control was performed for all samples. Briefly, 100 μL of blood were lysed with 200 μL of FACS Lysing Solution (Becton Dickinson) for 30 minutes at room temperature, incubated with the antibodies during 20 minutes at 4°C, washed, and resuspended in phosphate-buffered saline containing 1% of azida. Cells were analyzed using a Gallios flow cytometer (Beckman-Coulter, Brea, CA). At least 40,000 $CD3^+$ cells were collected for each sample and analyzed with Kaluza software (Beckman-Coulter) initially gating lymphocytes according to morphological parameters. The gating was always the same between different time points.

LPS and sCD14 levels and immunological parameters were assessed every 12 weeks during the 48 weeks of treatment intensification and also at weeks 12 and 24 after maraviroc and raltegravir removal. However, 16S rDNA levels were determined during the period of treatment intensification (baseline, 12, 24, 36, and 48 weeks during intensification), and LBP levels were assessed at baseline, at week 48 of treatment intensification, and at weeks 12 and 24 after discontinuation of intensifying treatment.

TABLE 1. Baseline Characteristics of the Patients

Number of determinations	126
Patients	18
Age (yrs)	46 (41–50)
Gender (male)	88%
$CD4^+$ count (cells/mm^3)	675 (570–775)
$CD8^+$ count (cells/mm^3)	693 (521–952)
Risk factors (%)	
MSM	50
IDU	28
HTx	22
Time on ART (mo)	99 (52–146)
Antiretroviral regimen (%)	
NRTIs	11
NRTIs + NNRTI	39
NRTIs + PI	50
$CD4^+$ T-cell activation ($CD38^+$ $HLA\text{-}DR^+$)	2.05 (1.44–2.95)
$CD8^+$ T-cell activation ($CD38^+$ $HLA\text{-}DR^+$)	2.8 (1.6–4.08)
sCD14 ($\times 10^6$ pg/mL)	1.4 (1.2–1.6)
LPS (pg/mL)	31.7 (18.4–50.9)
LBP (ng/mL)	34.9 (31.2–37.9)
16S rDNA (copies/μL)	5.4 (3.8–8.9)

IDU, intravenous drug users; HTx, heterosexual men; MSM, men who have sex with men; NNRTI, nonnucloeoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

Statistical Analysis

Continuous variables were expressed as the median and interquartile range and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables and the Mann–Whitney test to compare nonnormally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the χ^2 test. The Spearman correlation test was used. Statistical analysis was performed using SPSS software 21.0 (SPSS, Inc, Chicago, IL).

RESULTS

Good Correlation Between MT Measurements Except for 16S rDNA

We analyzed the associations among MT measurements performed on 126 determinations from 18 patients, altogether. Plasma LPS determinations positively correlated with levels of sCD14 and LBP ($P < 0.001$ and $P = 0.042$, respectively) (Figs. 1A, B). Similarly, high levels of sCD14 were associated with high levels of LBP ($P = 0.009$) (Fig. 1C). No

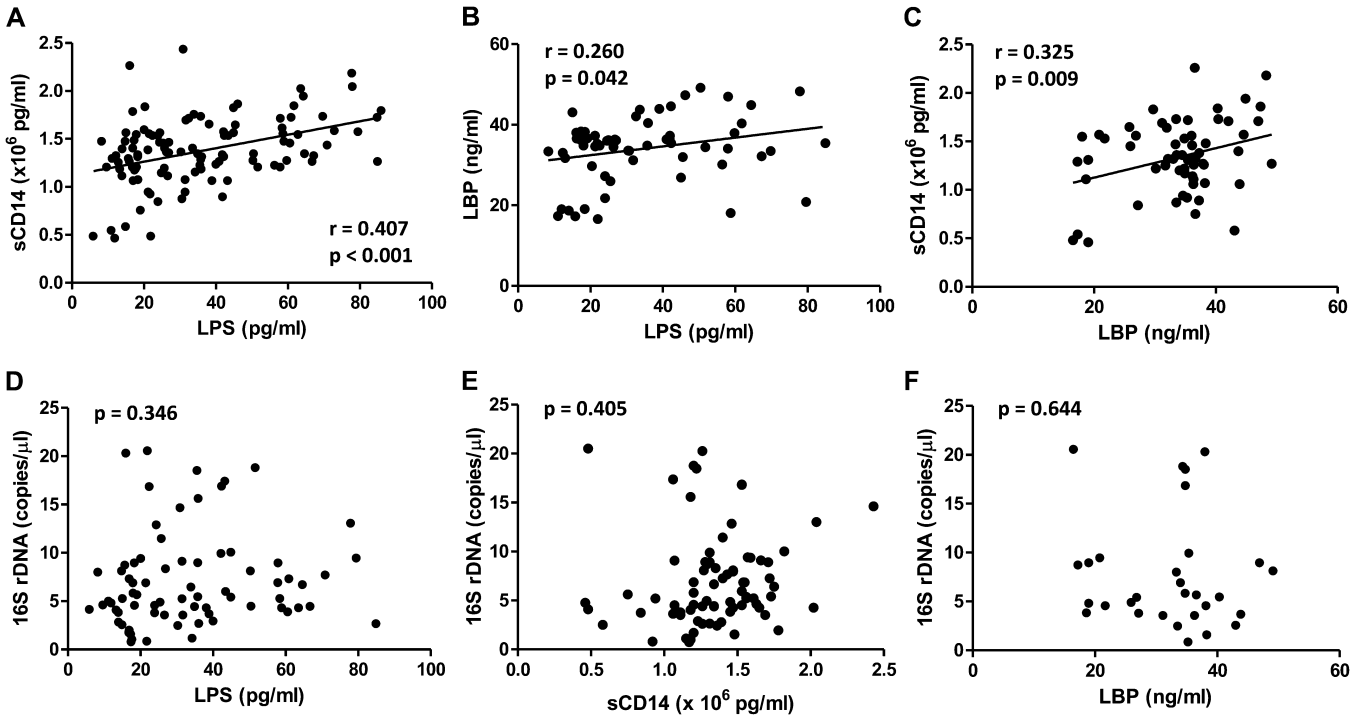

FIGURE 1. Correlation between different measurements of MT. Significant positive correlation between A, plasma LPS levels and sCD14; B, LBP and sCD14 levels; and C, LPS and LBP levels. No correlation between D, 16S rDNA and LPS levels; E, 16S rDNA and sCD14; and F, 16S rDNA and LBP. Only significant data ($P < 0.05$) are showed with regression lines and correlation coefficients. The Spearman correlation test was used.

correlation was found, however, between bacterial 16S rDNA either with LPS, sCD14, or LBP levels (Figs. 1D–F; $P = 0.346$, $P = 0.405$, and $P = 0.644$, respectively).

16S rDNA Level Correlated With Activated CD4+ T Cells, While No Correlation With CD8+ T-Cell Activation Was Evident

We also analyzed the associations between MT and $CD4^+$ and $CD8^+$ T-cell activation, $CD4^+$ and $CD8^+$ T-cell count, and the $CD4^+/CD8^+$ ratio. No significant correlation was found between plasma levels of LPS, sCD14, or LBP and activated $CD4^+$ T cells ($P = 0.418$, $P = 0.619$, and $P = 0.728$, respectively) (Fig. 2A) or activated $CD8^+$ T cells ($P = 0.352$, $P = 0.275$, and $P = 0.124$, respectively) (Fig. 2B). However, high levels of bacterial 16S rDNA correlated significantly with high levels of activated $CD4^+$ T cells ($P = 0.005$) (Fig. 2A) but not with activated $CD8^+$ T cells ($P = 0.171$) (Fig. 2B).

No association between MT measurements and $CD4^+$ or $CD8^+$ T-cell count or $CD4^+/CD8^+$ ratio was observed (data not shown). Similarly, T-cell immune activation showed no significant correlation either with $CD4^+$ or $CD8^+$ T-cell count or $CD4^+/CD8^+$ ratio.

DISCUSSION

In HIV-1–infected patients, elevated LPS in circulation binds the CD14 toll-like receptor 4 (TLR 4) through plasma LBP and triggers the activation of monocytes and macrophages that increase secretion of sCD14 and proinflammatory cytokines.[20] Several studies have reported the correlation between LPS and sCD14 levels in HIV-1 immune depressed patients[6,20,21] and in patients with undetectable HIV-1 viral load.[22] Besides, a correlation between LPS and both LBP and sCD14 levels was found and was associated to HIV-associated dementia in AIDS patients.[23]

We have observed a significant correlation between plasma LPS, LBP, and sCD14 levels in patients with optimal $CD4^+$ T-cell response (>350 cells/mm^3) during prolonged viral suppressive ART (<40 HIV-1 RNA copies/mL), in agreement with previous studies.[24] Nevertheless, no correlation between 16S rDNA and the other measurements of MT was found in our study, although this correlation has been reported by other authors who evaluated both treatment-naive and -experienced patients.[25,26] The fact that our study included only patients on long-term ART who were virologically suppressed and with good immunological recovery may explain the discrepancy between our results and those of the other studies, including mixed populations.

According to our data, and in this particular group of patients, these 3 markers (sCD14, LPS, and LBP) are suitable to measure MT. Nevertheless, each marker has limitations: sCD14 can be induced by different factors other than LPS in unsuppressed HIV-1 infection[27]; LPS is only present in Gram-negative bacteria and there are inherent technical difficulties in its measuring, that is, plasmas have to be stored in apyrogenic tubes and handled with care to prevent contamination. On the other hand, although 16S rDNA is present in both Gram-negative and

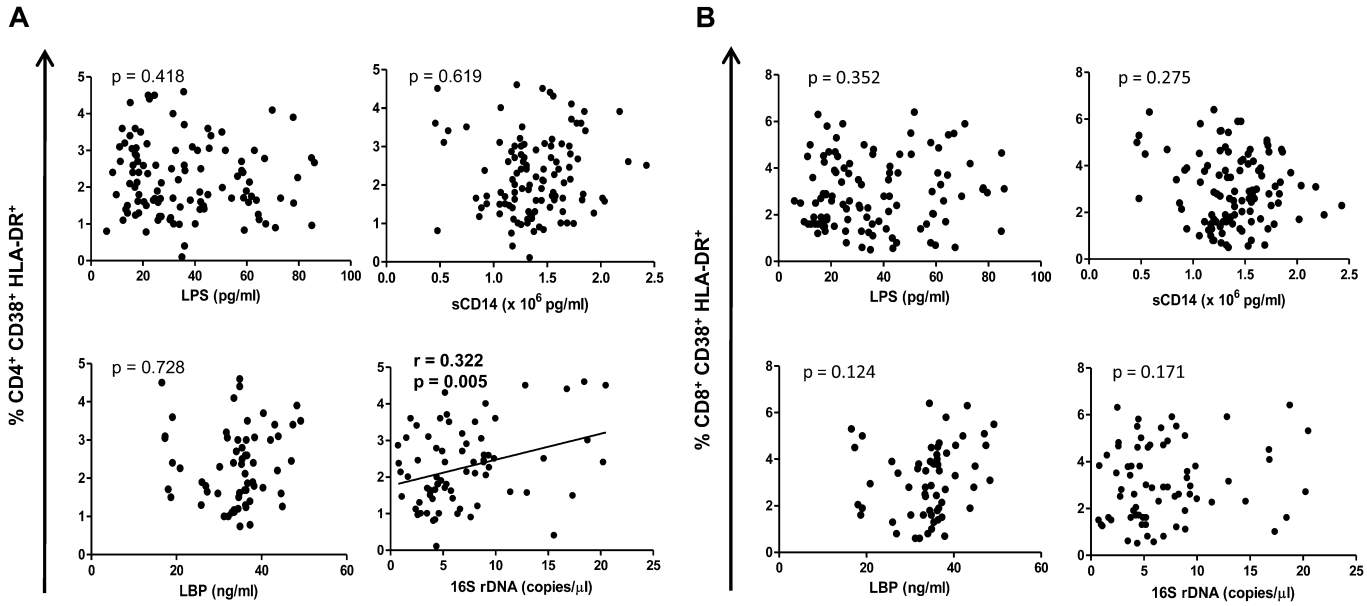

FIGURE 2. Correlation between measurements of MT and immune activation. Activated CD4+ T cells significantly correlated with plasma 16S rDNA (A), whereas no correlation between activated CD8+ T cells and either LPS, sCD14, LBP, or 16S rDNA was found (B). Only significant data ($P < 0.05$) are showed with regression lines and correlation coefficients.

Gram-positive bacteria, it has been technically hampered due to DNA contamination.

MT has been proposed as a possible driver mechanism of immune activation in HIV-1 infection,[28–30] supported by the correlation between LPS levels and circulating $CD8^+$ T cells expressing $CD38^+$ and $HLA\text{-}DR^+$ observed in chronically infected HIV-1 patients, elite controllers, and naive patients.[31] Another group has found a correlation between 16S rDNA and $CD8^+$ T-cell activation ($P = 0.047$), but only when aviremic- and viremic-treated patients were analyzed together.[25] On the other hand, other studies failed to show this correlation during treatment interruption[32] or in patients undergoing raltegravir treatment intensification.[33] After the scenario of treatment intensification, no correlation between the levels of LPS, LBP, or sCD14 with immune activation was found. Surprisingly, a significant correlation between levels of 16S rDNA and $CD4^+$ T-cell activation was found. We do not have a good explanation for this finding, but the type of patients and the fact that they had been in a treatment intensification scenario could account for this correlation.

Considering these data, we are unable to ensure that MT directly triggers T-cell immune activation, at least among these patients with relatively good immune recovery and under treatment intensification, although this process is clearly an important component of innate immune inflammation associated to HIV-1 infection.

We cannot discard that treatment intensification might affect MT and subsequent correlation with immune activation in different ways depending on the intensifying drug. To discard this possibility, we analyzed individuals who intensified with maraviroc separately from individuals who intensified with raltegravir, obtaining similar results. The discrepancies found in our study compared with others may be attributable to the good immunological characteristics of our cohort undergoing treatment intensification after at least 2 years of antiretroviral treatment maintaining HIV-1 viremia undetectable and high levels of $CD4^+$ T cells (>350 cells/mm^3).

In conclusion, the quantification of LPS, LBP, and sCD14 are good measurements of MT and can be used interchangeably, taking into account the technical difficulties in performing each measurement. On the other hand and studying this particular type of patients, long-term suppressed individuals, a poor association between MT and immune activation, was found because only a single correlation between activated $CD4^+$ T cell and 16S rDNA was found. Further studies focusing on the restoration of the gut mucosa in HIV-1 infection would help to clarify the role of MT in HIV-1–associated immune activation and the subsequent immune recovery.

ACKNOWLEDGMENTS

The authors thank C. Page, R. Lorente, E. Domínguez, and M. Coronel for the excellent technical assistance. We are deeply grateful to the patients and their families who participated in this study.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 27, Number 5, 2011

DOI: 10.1089/aid.2010.0263

HTLV-2b Among HIV Type 1-Coinfected Injecting Drug Users in Spain

María Abad,[1] Fernando Dronda,[2] Ester Dominguez,[1] Santiago Moreno,[2] and Alejandro Vallejo[1]

Abstract

Human T cell lymphotropic virus type 2 (HTLV-2) infection is endemic in the American Indian population and Pygmy tribes in Africa. Nevertheless, HTLV-2 infection has been predominantly detected in U.S. and European injecting drug users (IDU). Noteworthy is that the HTLV-2a subtype is the main circulating variant in North America and Eastern Europe whereas the HTLV-2b subtype is mainly found in Western Europe, particularly in Italy and Spain where coinfection with HIV-1 is frequent. Twelve Spanish subjects infected with HTLV-2 were recruited for the study. All of them were IDUs coinfected with HIV-1. Molecular epidemiology was done by sequencing the LTR, *env,* and *tax* regions and by generating phylogenetic trees. The present study showed that all the sequences belonged to the HTLV-2b subtype and were closely related to other Spanish and Portuguese reported sequences, clearly differentiated from those belonging to the HTLV-2a subtype from Eastern Europe. Therefore, infection with HTLV-2b remains prevalent in Spain based on previous studies.

The human T cell lymphotropic virus type 2 (HTLV-2) was initially identified and isolated from a patient with atypical hairy cell leukemia in 1981.[1] However, at present there is still no clear evidence that HTLV-2 causes any human disease, although it has been occasionally linked to neurological and lymphoproliferative disorders.[2] HTLV-2 infection is endemic in some American Indian populations and Pygmy tribes in Africa.[3] Nevertheless, HTLV-2 has been widely distributed in injecting drug users (IDUs) in the United States and Western Europe.[4]

Molecular epidemiological studies have distinguished three main HTLV-2 subtypes geographically dispersed. Subtype HTLV-2a is the main circulating variant in North America and Eastern Europe, while subtype HTLV-2b can be found in native Central and South American Indians as well as in Western Europe, particularly in Italy and Spain[5,6] where coinfection with HIV-1 is very frequent. Some Brazilian isolates clustered within subtype HTLV-2a but diverged by coding a 25 amino acid longer Tax protein. This diversity conceived as a molecular variant of HTLV-2a[7] was considered by some to indicate another subtype (HTLV-2c).[8] Finally, subtype HTLV-2d has been detected among African tribes.[9] Data from the Spanish HTLV national registry showed that a total of 717 HTLV-2-infected individuals had been diagnosed in Spain up to December 2009.[10] Despite of the continuing immigration from HTLV-2 endemic areas, most of the newly infected cases reported in Spain were native former drug users coinfected with HIV-1.[10]

In the present study, 12 HTLV-2-infected Spanish individuals (58% male) with a history of intravenous drug use and coinfected with HIV-1 were phylogenetically analyzed through three viral genes and compared with other reported isolates. The individuals analyzed were diagnosed between 2003 and 2009. All the selected samples were obtained from epidemiologically unlinked individuals from the Hospital Ramon y Cajal, located in Madrid, Spain.

Plasma samples from these individuals showed a complete positive HTLV western blot (WB) pattern (Bioblot HTLV, Genelabs, Singapore) with the exception of three samples that showed an indeterminate WB pattern, according to HTLV European Research Network criteria.[11] These indeterminate samples were later confirmed by HTLV-2-specific PCR. DNA was extracted from cryopreserved peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Partial LTR, *env,* and *tax* genes were amplified by nested polymerase chain reaction (PCR).

All reaction mixtures were performed in a total volume of 25 μl containing PCR Buffer II (AccuPrime Taq High Fidelity, Bioline, London, UK), 0.5 U Taq polymerase (AccuPrime Taq High Fidelity, Bioline), 200 ng of genomic DNA, and 20 pmol of each primer, HT2-1 (TAAAGGCTCTGACGTCTCC-3′) and

[1]Laboratory of Molecular Immunovirology and [2]HIV Clinic, Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitario (IRYCIS), Hospital Ramón y Cajal, Madrid, Spain.

HT2-2 (GCAGCAAGGGCTAGGGCT-3′) for LTR, Ev-2 (GTT CCAATAGCAGTGAGCCTTGT-3′) and Ev-3 (AAAGCTG CATGCCCAAGAC-3′) for the *env* gene, and Tx-3 (CTGG TCTCCTAACGGCAATCTC-3′) and Tx-4 (CAAGTAAA GGCTCTGACGTCT-3′) for the *tax* gene. Thirty-five cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 68°C for 20 s were used. After the first PCR reaction, 2 μl of the amplified products was used for a second PCR run in the same amplification conditions. Primer HT2-1 (TAAAGGCTCTGACGTCTCC-3′) and HT2-4 (AAC GAAACCTCAACGCCGCC-3′), Ev-3 (AAAGCTGCATGCC CAAGAC-3′) and Ev-4 (ATTGCTACCAACCTCGCCTAC -3′), and Tx-3 (CTGGTCTCCTAACGGCAATCTC-3′) and Tx-6 (CAGACCGTCTCACACAAACAATC-3′) were used for the amplification of 390 bp (nt 153–541) of the LTR fragment, 777 bp (nt 5180–5956) of the *env* region, and 993 bp (nt 7213–8205) for the *tax* region, respectively. The sequence positions are listed according to the Mo isolate (GenBank accession number M10060).

Purified PCR products were sequenced in a final volume of 15 μl by using the ABI Big Dye Terminator v3.1 cycle sequencing reaction kit (Applied Biosystems, Foster City, CA) and processed with the ABI 310 Genetic Analyzer (Applied Biosystem). Sequences were edited using SeqMan II Software version 5.0.1 (DNASTAR, Madison, WI) and aligned with MEGA software v4.0.2 together with reference sequences of the HTLV-2a subtype (Mo) and HTLV-2b subtype (G12 and NRA). Phylogenetic trees were generated using the unweight pair group method with arithmetic mean (UPGMA) by Clustal W v5.8 and MEGA v4.0.2. The neighbor-joining method was also used to corroborate them. The topology of the trees was supported by 1000 bootstrap replicates. Genetic distance was estimated by Kimura's two-parameter method. Some reported sequences from GenBank listed below were used in this study for comparison purpose, and the simian T cell lymphotropic virus type 2 (STLV-2) was used as the outgroup.

Within the LTR fragment (nt 153–541), the NF-κB binding site was highly conserved when compared to NRA except for the insertion of an A at position 183 in the MA_CGO, MA_DGE, and MA_JSR sequences. Another A was inserted at position 258 in the MA_MSC and MA_SPG sequences. All sequences showed a transition from G to A at position 305 except MA_GRO and MA_CAS, and two contiguous transversions from TC to GG at positions 323 and 324, respectively (data not shown). Similarity values when compared to prototypes NRA (HTLV-2b) and Mo (HTLV-2a) are shown in Table 1.

The splicing site within the beginning of the *env* fragment was conserved. This site is essential to generate the double-spliced messengers encoding the regulatory *tax* protein.[9] The nucleotide sequences were well conserved when compared to NRA, with high similarity values (Table 1). A total of 11 point mutations were found at positions 5231, 5358, 5827, 5832, 5945, and 5949 in MA_BMJ, MA_VMM, MA_SPG, MA_CAS, MA_ROL, and MA_FRM sequences, and at positions 5359 and 5413 in all sequences. The remaining three nucleotide point mutations at 5185, 5209, and 5437 were found in the same subject MA_VMM. Six of these mutations were related to an amino acid change in the coded protein at positions 10, 18, 59, 218, 256, and 257 in the MA_VMM, MA_BMJ, MA_MSC, MA_CAS, MA_ROL, and MA_FRM sequences, respectively (Fig. 1A).

Almost the complete *tax* region (nt 7213–8205) was analyzed. The percentage of nucleotide similarity with regard to both Mo and NRA prototypes is shown in Table 1. A total of 10 point mutations were found when compared to the HTLV-2b NRA prototype at positions 7400 in MA_GRO, 7475 in MA_ROL, 7824 and 8045 in MA_CGO, 7915 in MA_VMM, 8007 and 8030 in MA_MSC, 8010 in MA_JSR, and finally 8144 and 8182 in all sequences except in MA_MSC for the last position indicated, respectively. Generally, the amino acid sequences were well conserved, especially the nuclear location signal (NLS), nuclear export signal (NES), and the dimerization domain that remained unchanged for subtype b.[12] Nevertheless, six of the nucleotide mutations generated an amino acid substitution at positions 63, 88, 235, 273, and 278, in MA_GRO, MA_ROL, MA_VMM, MA_MSC, and MA_CGO sequences, respectively. All the sequences shared the amino acid change at position 323 except MA_MSC, which remained intact with regard to NRA prototype (Fig. 1B). The amino acid identity compared to Mo and NRA sequences is also shown in Table 1.

The phylogenetic tree for LTR showed that Spanish samples clustered with other reported sequences from Spanish (BF, AA, JA, DP, 130, 324, RVP, SPAN129, and SPAN130) and Portuguese (PortNn, PortHl, and PortVs) IDUs. Together these formed a subgroup closely related to sequences from Italian IDUs (I-GI, I-OV, I-OG, and I-OV Gu), and more distantly with sequences from American Indian (WYU1 and G12) and African (Gab) sequences. All these sequences clustered in the HTLV-2b subtype sustained by a bootstrap value of 85%. Subtypes 2a and 2d formed other two genetically distant clusters (Fig. 2).

Similar results were found for *env* gene analysis where Spanish sequences were highly related to reported sequences from Italian IDUs (I-OV). Together with sequences from Amerindian (WY100, G12, and G2) and African (Gab) individuals, these formed the HTLV-2b subtype supported by a bootstrap value of 99%. The MA_MSC and MA_VMM sequences were the most divergent sequences among the Spanish samples (Fig. 2).

Table 1. Comparison of HTLV-2 Nucleotide and Amino Acid Sequences of the Spanish Patients with HTLV-2 Mo (2a) and NRA (2b) Prototypes

	Nucleotide identity (%)			*Amino acid identity (%)*	
	LTR	*env*	*tax*	Env	Tax
Mo (2a)	99.941–99.931	99.955–99.951	99.962–99.960	99.969–99.965	99.976–99.973
NRA (2b)	99.990–99.982	99.999–99.995	99.998–99.996	99.996–99.992	99.997–99.994

FIG. 1. Alignment of Env **(A)** and Tax **(B)** amino acid sequences compared to prototypes HTLV-2a (Mo) and HTLV-2b (G12 and NRA).

Finally, the analysis of tax region showed three well-differentiated groups for HTLV-2a, HTLV-2b, and HTLV-2d subtypes, supported by high bootstrap values. The 2b subgroup included reported sequences from Italian (PR-46) and American (FUC and PAR) IDUs closely related to the Spanish sequences and the reported sequences from the Gabonese (Gab) and the American Indian prototypes (G12 and G2). Among Spanish samples, MA_CGO and MA_MSC were the most divergent (Fig. 2).

With respect to HTLV-2 infection, most of the cases reported in Spain were native IDUs and coinfected with HIV-1.[13] It has been speculated that HTLV-2 originated from the Amerindians and was introduced to European IDUs in at least two separate periods: one for the 2a subtype in Eastern Europe and another one for the 2b subtype in Western Europe.[14] During the 1980s and 1990s, HTLV-2 spread exponentially among Spanish intravenous drug users because of needle-sharing practices. The prevalence of HTLV-2 infection has been one of the highest in Europe, estimated at 18% among IDUs in prisons and at 1–5% outside the prison setting.[15] In the past decades, many injecting drug users have changed the route of drug usage, thus the number of new injecting drug users has decreased. Therefore, it is estimated that the number of new cases of HTLV-2 infection might be very low.[13]

The present study of 12 infected IDUs from Spain coinfected with HIV-1 showed that HTLV-2b remains the prevalent subtype in Spain. They also continue to be closely related to Italian and Portuguese IDUs, clearly differentiated from those belonging to the HTLV-2a cases from Eastern Europe.

Sequence Data

The following GenBank accession numbers for previously published HTLV-2 gene fragments were used in this study: Mo (M10060), G12 (L11456), G2 (AF07965), NRA (L20734), Gab (Y13051), Efe-2 (Y14365), STLV-2 (U90557). For the LTR analysis: NAV.DS (U10257), SMH2 (Y09148), PH230PCAM (Z46838), LA8A (U10256), KAY73 (L42509), BRAZ.A21 (U10253), ATL18 (U10252), Dub805 (AF175467), AA (L7738), DP (L7737), JA (L7739), BF (L77236), SPAN129 (U10265), SPAN130 (U10266), RVP (L77244), 130 (L77242), 324 (L77243), PortHl (AY622977), PortNn (AY622978), PortVs (AY622979), Gu (X89270), I-GI (Y09153), I-OV (Y09155), I-OG (Y09154), WYU1 (U12792), BBD_3126 (FJ911656), BBD_2278 (FJ911644), BBD_2766 (FJ911652), BBD_2286 (FJ911645). For the *env* analysis: WY100 (S69268), Gu (X89270), SP-WV (AF139382), RP329 (AF326583), K96 (AF326584), AF412314. For the *tax* analysis: SMH2 (Y09148), Gal (AF292002), SP-WV (AF139382), RP329 (AF326583), K96 (AF326584), KAY1 (U32874), KAY2 (U32875),

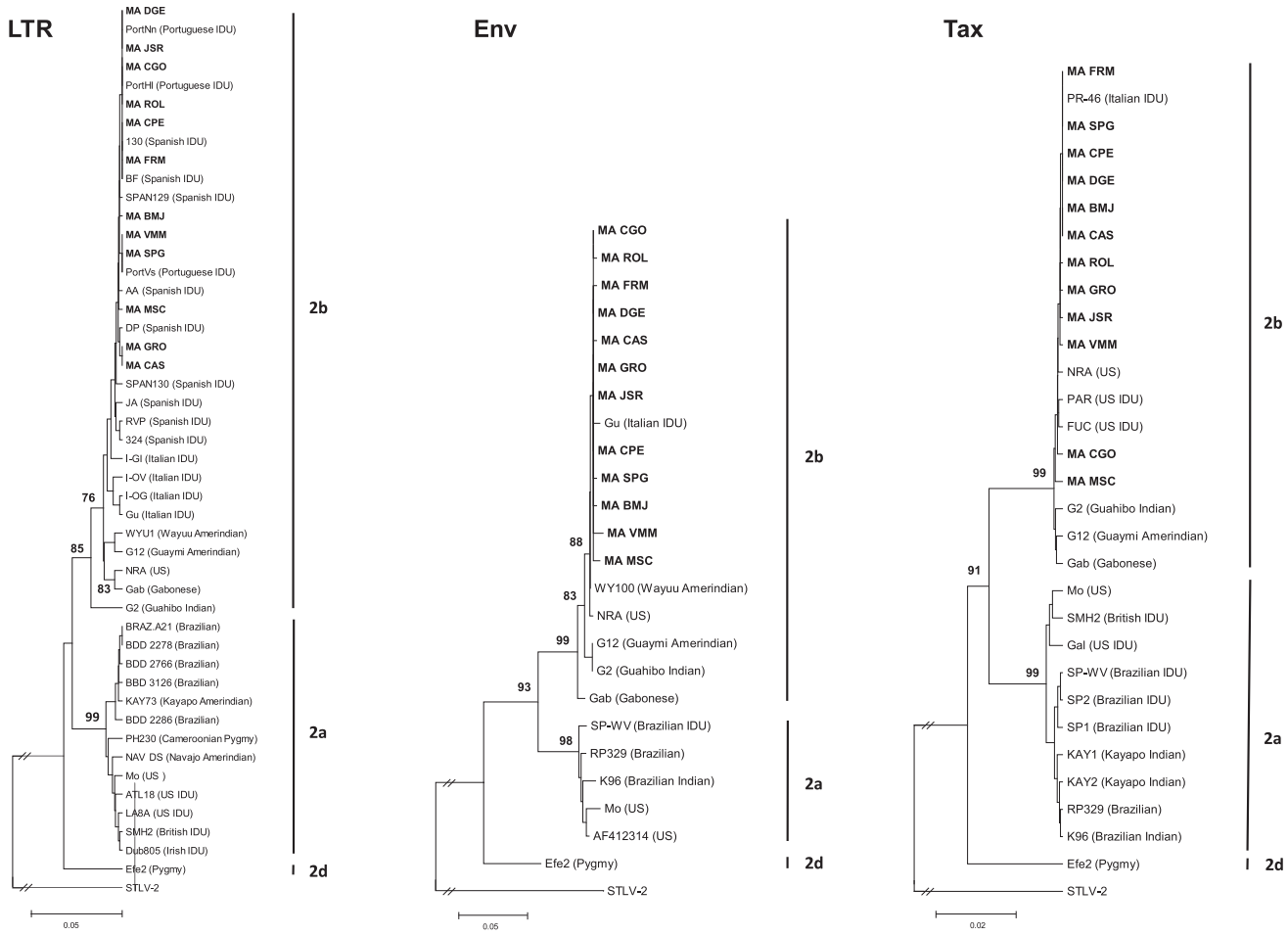

FIG. 2. Dendrograms showing the phylogenetic relationship between 320 bp of the LTR region, 777 bp of the *env* fragment, and 993 bp of the *tax* region of the HTLV-2 strains. Twelve Spanish sequences are shown in bold and compared to other reported sequences of which the simian T cell lymphotropic virus type 2 (STLV-2) was used as an outgroup for the analysis. Geographic and ethnic origins are shown in parentheses and 1000 bootstrap replicates were performed to support internal branches. The genetic distance bar is also shown.

SP1 (U32873), SP2 (U32872), PR-46 (DQ022075), FUC (U32882), PAR (U32880). Sequences from the samples studied in this work have been submitted to GenBank under the following numbers: LTR: MA_CPE (GU433203), MA_DGE (GU433204), MA_FRM (GU433205), MA_MSC (GU433206), MA_JSR (GU433207), MA_CGO (GU433208), MA_GRO (GU433209), MA_SPG (GU433210), MA_ROL (GU433211), MA_VMM (GU433212), MA_BMJ (GU433213), MA_CAS (GU586121). *env*: MA_BMJ (GU586109), MA_DGE (GU586110), MA_FRM (GU586111), MA_MSC (GU586112), MA_JSR (GU586113), MA_CGO (GU586114), MA_GRO (GU586115), MA_SPG (GU586116), MA_ROL (GU586117), MA_VMM (GU586118), MA_CPE (GU586119), MA_CAS (GU586120). *tax*: MA_BMJ (GU591294), MA_CGO (GU591295), MA_CPE (GU591296), MA_DGE (GU591297), MA_FRM (GU591298), MA_GRO (GU591299), MA_JSR (GU591300), MA_MSC (GU591301), MA_ROL (GU591302), MA_SPG (GU591303), MA_VMM (GU591304), MA_CAS (GU591305).

Acknowledgments

We want to thank Maria Pumares and Paloma Martí for their technical support. We thank all the participants of this study for their cooperation. This study was supported in part by Fondo de Investigación Sanitaria (FIS) 07/0070 and Redes Temáticas de Investigación Cooperativa en Salud (RETICS) Red de Investigación en SIDA (RIS) RD/06/0006/0034.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Alejandro Vallejo
Laboratory of Molecular Immunovirology
Servicio de Enfermedades Infecciosas
Planta 4 Centro, Control A
Instituto Ramón y Cajal de Investigación Sanitario (IRYCIS)
Hospital Ramón y Cajal
Ctra. Colmenar Km 9
28034 Madrid
Spain

E-mail: alejandro.vallejo@salud.madrid.org

Elsevier Editorial System(tm) for Journal of Clinical Virology
Manuscript Draft

Manuscript Number: JCV-2894

Title: CD8 TCR β chain repertoire expansions and deletions are related with immunologic markers in HIV-1-infected patients during treatment interruption

Article Type: Full Length Article (2500 words)

Keywords: CD8 TCR β repertoire; antiretroviral treatment interruption; T cell exhaustion; immune activation; effector memory cells

Corresponding Author: Mr. Alejandro Vallejo, Ph.D.
Corresponding Author's Institution: Hospital Ramón y Cajal
First Author: Alejandro González-Serna

Order of Authors: Alejandro González-Serna; María Abad-Fernández; Natalia Soriano-Sarabia; Manuel Leal; Alejandro Vallejo, Ph.D.

CD8 TCR β chain repertoire expansions and deletions are related with immunologic markers in HIV-1-infected patients during treatment interruption

Alejandro González-Serna [a*], María Abad-Fernández [b*], Natalia Soriano-Sarabia [c], Manuel Leal [a], Alejandro Vallejo [b]

a. Laboratory of Immunovirology, Department of Infectious Diseases, Hospital Virgen del Rocio, IBIS, Seville 41013, Spain.
b. Laboratory of Immunology and Molecular Virology, Department of Infectious Diseases, Hospital Ramón y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid 28034, Spain.
c. Department of Medicine, University of North Carolina. Chapel Hill, North Carolina, United States of America.

***** These authors contributed equally to this work.

Running title: CD8 TCR repertory in HIV infection

Corresponding author information: Alejandro Vallejo, Ph.D. Laboratory of Immunology and Molecular Virology. Department of Infectious Diseases. Instituto Ramón y Cajal de Investigaciones Sanitarias (IRYCIS), Hospital Ramón y Cajal. Ctra. Colmenar Km 9. Madrid 28034 - Spain.
Fax: +34 91 3368792
Tel: +34 91 3368711
Email: alejandro.vallejo@salud.madrid.org

Key words: CD8 TCR β repertoire, antiretroviral treatment interruption, T cell exhaustion, immune activation, effector memory cells.

Abstract word count: 199

Text word count: 2495

Abstract

Background: HIV-1-infected individuals progressively loss $CD4^+$ T cells leading to immunosuppression. $CD8^+$ T-cells play an important role in the immune response against virus infections through their TCR.
Objective: To evaluate the CD8-TCR repertoire and immunologic markers in HIV-1-infected patients.
Study design: Ten chronic HIV-1-infected individuals on prolonged effective ART were analyzed before treatment interruption (TI), after at least one year of TI and after at least one year from ART resume. Twenty-four TCR-Vβ gene families were analyzed by a modified CDR3 spectratyping method in isolated $CD8^+$ T-cells. Immune activation, exhaustion and subpopulation markers were analyzed by flow cytometry.
Results: Expansion of Vβ10, Vβ14 and Vβ15 families during TI that was associated with low cell activation and stable exhaustion markers. Moreover, an increment of effector memory cells was found. Besides, depletion of Vβ20, Vβ28, and Vβ29 families during TI was associated with an increase in cell activation and exhaustion markers. These alterations seemed to be more pronounced in patients who had longer time from diagnosis. ART seemed to restore altered $CD8^+$ T-cell repertoire and most of the immunologic markers.
Conclusions: Increased and depleted $CD8^+$ T-cell repertoire was related to increased or decreased levels of cell activation and exhaustion marker, respectively.

1. Background

HIV-1-infected individuals progressively loss $CD4^+$ T-cells leading to immunossuppression and raising the risk of opportunistic infections.[1] Factors that are critical for the function of the immune system include the frequency of naïve T-cells, premature T-cell senescence, cell exhaustion[2-4,] as well as the effector function of these cells through the repertoire of T-cell receptors (TCR).

TCR repertoire alterations have been observed in HIV-1-infected subjects, reflecting vigorous but not fully effective virus-specific cytotoxic T lymphocyte responses.[5-11] HIV-1 infection induces alterations in TCR repertoire during acute infection, as clonal expansion and exhaustion of HIV-1-specific T-cell clones[12-14], and also in chronic or progressive HIV-1 infection.[15] Although there is some controversy, some studies have observed that altered TCR repertoire is not fully reconstituted by the effect of an effective antiretroviral therapy.[12,16-21]

2. Objectives

In the present study, we examined the evolution of the $CD8^+$ T-cell receptor repertoire by CDR3-spectratyping technique in HIV-1-infected patients who received antiretroviral treatment after a prolonged period of treatment interruption. We also analysed the relationship between this evolution and the expression of cell activation, T-cell exhaustion, and memory cells in $CD8^+$ T-cells with specific TCR Vβ.

3. Study design

3.1 Patients

Up to February 2006, 55 Caucasian Mediterranean patients with plasma HIV-1 viral load under 50 copies/mL at least during the last 12 months, and CD4 count above 500 cells/mm^3 began a period of treatment interruption (TI). This cohort of patients has been described elsewhere.[22] Antiretroviral treatment was resumed either when CD4 count reached 250 cells/mm^3, clinical progression, or by self-decision. Ten patients had available samples to perform this retrospective study and were included in this study. Informed consent for the collection and analysis of blood samples was obtained from all of the patients.

Clinical characteristics of the patients are shown in table 1. CD4 count was determined in fresh samples by flow cytometry. Plasma HIV-1 RNA was measured by quantitative PCR assay (HIV MonitorTM Test Kit; Roche Molecular System, Hoffman-La Roche, Basel, Switzerland), according to the manufacturer´s instructions, every three months during the follow up. This assay has a lower detection limit of 50 HIV-1 RNA copies⁄mL. Twenty-four TCR Vβ gene families and T-cell subsets were analyzed in each patient in the three time points; after at least 12 months on effective antiretroviral treatment (median 90 months, baseline), at least 12 months on treatment interruption (median 22 months, TI), and at least twelve months after treatment resume, (median 30 months, TR). Viral load was undetectable at baseline and at TR time point, whereas viral rebound was observed at TI time point, with median 4.18 log RNA copies/mL (Table 1).

3.2 Isolation of $CD8^+$ T-cell subset

Peripheral blood mononuclear cells were isolated from heparinized whole blood by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway) and cryopreserved in liquid nitrogen. CD8 T-cell subset was isolated by immunomagnetic

separation technique (Dynabeads, Dynal, positive isolation kits, Paisley, UK) using monoclonal anti-CD3 and anti-CD8 antibodies for positive cell isolation, according to the manufacturer's instructions. Cell fraction contained at least 1-5 x 10^6 cells with purity above 98%.

3.3 Measurement of CDR3 length variation by multiplex RT-PCR

Total RNA was extracted from $CD8^+$ T-cell subset by using QIAamp Viral RNA Kit (Qiagen Diagnostics, Barcelona, Spain) following the manufacturer´s instructions, and RNA concentration was determined by spectrophotometry (NanoDrop ND-1000).

A modified multiplex RT-PCR was used to amplify the CDR3 region of 24 TCR Vβ families in order to minimize the number of reactions (filed patent number P201132088). Several forward primers and one reverse primer labeled with two different fluorochromes (C1 and C2) were designed to amplify three different molecular size ranges for 24 Vβ families (Table 2). To avoid artificially distorted CDR3 length distribution caused by insufficient mRNA template, an optimization was performed with cord blood cells purified from a healthy donor. Gaussian distribution of the amplified CDR3 length repertoires was guaranteed with 100 ng RNA (Figure 1A). To normalize and ensure a good quality of the mRNA template, a region of the GAPDH gene was amplified in each reaction. Also, a missing length was repeated twice to confirm the results.

High-temperature multiplex RT-PCR was performed in 20 µl reaction mixture containing 100 ng mRNA template, 1 µM dNTP, Buffer 5x, 20 U protector RNase inhibitor, 10 U transcriptor reverse transcriptase (Roche Applied Science), and 1 µM each primer (three Vβ forward primers and one C reverse primer). First cycle at 42ºC for 45 min, followed by inactivation step at 85ºC for 5 min, and 35 cycles consisting of 15 s at 94ºC, 15 s at 58ºC and 30 s at 72ºC, with a final extension step of 7 min at 72ºC.

To analyze the spectrum of each Vβ family we run samples using GeneScan-based spectratyping (Genetic Analysis System CEQ 8000, GenomeLab, Beckman Coulter). The calculated area under the curve for each CDR3 length in a given Vβ family was extrapolated to a proportion of expression (Figure 1B).

3.4 Flow cytometry analysis of T-cell subsets

Isolated PBMC from the subjects were immunostained with the respective antibody combinations for 30 min in phosphate-buffered containing 2% albumin and 0.1% sodium azide. Cells were analyzed by multiparametric flow cytometry (Gallios, Beckman Coulter). The following antibodies were purchased from BD Pharmingen (BD Biosciences): HLA-DR-allophycocyanin (APC)-Cy7, CD38-allophycocyanin (APC)-700, PD-1-peridinin chlorophyll protein complex (PerCP)-Cy5.5, CD45RA-phycoerythrin (PE)-Cy7, CCR7-allophycocyanin (APC), CD3-eFluor 450, and CD8-Krome Orange (KO).

The proportion of cells was determined initially gating lymphocytes according to morphological parameters and then gating on $CD3^+$ T-cells. $CD8^+$ T-cell subsets (at least 20,000 events were gated) were defined as follows: naïve cells, CD3+CD8+CD45RA+CCR7+; effector memory (EM) cells CD3+CD8+CD45RA-CCR7-; and central memory (CM) cells CD3+CD8+CD45RA-CCR7+. Activation levels were also analyzed by the co-expression of CD38 and HLA-DR, whereas PD-1 was used as an activation-induced inhibitory T-cell marker of exhausted T-cells. All these

parameters were analyzed in $CD8^+$ T-cells bearing three different TCR families using the following antibodies (co-staining): Vβ14-fluorescein isothiocyanate (FITC), Vβ20-FITC, and Vβ2-phycoerythrin (PE) (IOTest, Beckman Coulter).

3.5 Statistical analysis

Continuous variables were expressed as median and interquartile range, and discrete variables as percentages. The *t* test (paired samples) was used to compare normally distributed continuous variables and ANOVA test to compare non-normally distributed continuous variables in multiple groups. Statistical Package for the Social Sciences software package (SPSS 16.0, Chicago, Illinois, USA) was used.

4. Results

4.1 TCR CDR3 profile of Vβ families during the follow up

The overall median proportion of the expression of each TCR Vβ family from all the patients at baseline (BL), treatment interruption (TI) and treatment resume (TR) is shown in figure 1C. The expression of Vβ10, Vβ14, and Vβ15 families, significantly increased at TI compared to baseline (p=0.017, p=0.047, and p=0.007, respectively), and were restored at TR, with no statistical differences compared to baseline. The same profile was observed for Vβ9 and Vβ11 families, although with no statistical significance (Figure 1C). On the other hand, the expression of Vβ20, Vβ28, and Vβ29 families significantly decreased at TI compared to baseline (p=0.007, p=0.007, p=0.005, respectively), while a restoration in their expression was observed at TR, with no statistical difference compared to baseline (Figure 1C). A similar trend in Vβ families Vβ5 and Vβ24 was observed during the follow up, although with no statistical significance. The rest of the Vβ families showed no significant differences at TI compared to baseline.

4.2 TCR Vβ expression according to the time from diagnosis

Patients with longer time from diagnosis at baseline (greater than the median 105 months) showed significant higher levels of expression of Vβ10, Vβ14, and Vβ15 families (Figure 2A, p=0.035, p=0.034, and p=0.028, respectively) at TI compared to baseline, while patients with shorter time of infection at baseline (below the median 105 months) showed no significant differences. Interestingly, a significant increase of Vβ9 and Vβ11 families, and a significant decrease of Vβ5 and Vβ24 families were found at TI compared to baseline only in those patients with longer time of infection (data not shown).

On the other hand, a significant decrease of Vβ20, Vβ28, and Vβ29 families (Figure 2B, p=0.028, p=0.028, and 0.028, respectively) was found at TI compared to baseline, while patients with shorter time from diagnosis showed no significant differences. Again, a significant decrease of Vβ5 and Vβ24 families was found at TI compared to baseline only in those patients with longer time from diagnosis (not shown).

No influence of the nadir CD4 cell count or viral rebound at TI was observed in the expression of the Vβ families with the exception of the Vβ10 family. Within this family, only those patients with higher viral rebound (greater than 4.18 log copies/mL) had a significant increase (p=0.043) in its proportion at TI compared to baseline.

4.3 Immunophenotyping of CD8$^+$ T-cell subpopulations in altered Vβ families

The proportion of CD8$^+$ T-cell subpopulations, T-cell exhaustion, and memory cells within the expanded TCRVβ14 and depleted TCRVβ20 families were analyzed. As well, TCRVβ2 family was used as control. A comparison between the proportion of families Vβ14, Vβ20 and Vβ2 measured by spectratyping or by immunophenotyping showed no significant differences at any time point. This result validates the proportions obtained with this modified spectratyping method. The proportion at TI of CD8-Vβ14 cells significantly increased (p=0.023), while decreased in CD8-Vβ20 cells (p=0.04), compared to baseline. Instead, no significant difference in the proportion was observed in CD8-Vβ2 cells. These proportions were not different at TR compared to baseline with the exception of CD8-Vβ20 cells where it remains significantly lower (not shown).

No significant increase of cell activation was found in CD8-Vβ14 cells at TI among patients with shorter or longer time from diagnosis (Figure 3A, p=0.068 and p=0.144, respectively) compared to baseline. On the other hand, an increase was observed only in patients with longer time from diagnosis in CD8-Vβ20 cells at TI (p=0.003). Interestingly, activation increased in CD8-Vβ2 cells in all the patients. However, activation in CD8-Vβ20 cells was higher compared to CD8-Vβ14 cells in patients with longer time from diagnosis (p=0.003), and showed no difference with CD8-Vβ2 cells (p=0.353). Activation in CD8-Vβ14 cells at TI was significantly lower than in CD8-Vβ2 cells (p=0.018). At TR the level were restored in all patients but CD8-Vβ20 and CD8-Vβ2 cells in patients with longer time from diagnosis. Similarly, no increase in PD-1 level in activated CD8-Vβ14 cells at TI was found among patients with shorter or longer time from diagnosis (Figure 3B, p=0.461 and p=0.144, respectively). Besides, increased PD-1 level was observed in activated-CD8-Vβ20 cells in all the patients at TI (p=0.038 and p<0.001, respectively). Only patients with longer time from diagnosis showed increased PD-1 levels in activated-CD8-Vβ2 cells (p<0.001). Interestingly, PD-1 level in activated-CD8-Vβ20 cells in patients with longer time from diagnosis was higher at TI compared to that found in either activated-CD8-Vβ20 or CD8-Vβ2 cells (p=0.017 and p=0.008, respectively). Finally, the proportion of effector memory cells increased at TI only in patients with longer time from diagnosis in CD8-Vβ14 and CD8-Vβ20 cells (Figure 3C, p=0.046 and p<0.001, respectively), while increased in all patients in CD8-Vβ2 cells (p<0.001). Nevertheless, effector memory CD8-Vβ14 cells were lesser than either CD8-Vβ20 or CD8-Vβ2 cells (p=0.032 and 0.028, respectively). These levels were restored at TR in all patients.

5. Discussion

This study showed a common pattern of altered Vβ repertoire after treatment interruption in chronic HIV-1 infection among adults. This altered pattern consist on the one hand, in an increased transcript accumulation of families Vβ10, Vβ14, Vβ15, probably due to expansion of cytotoxic response due to the increment of either HIV antigens or other antigens, i.e. those produced by microbial translocation; and on the other hand, in a decreased transcription of families Vβ20, Vβ28, Vβ29, probably due to cell depletion after a direct cytolytic effect of the HIV-1 infection or the accumulation of other antigens. The fact that only the increase of Vβ10 family was related with the HIV-1 viral rebound suggest that other antigens produced during the treatment interruption period might play an important role in the alterations of the Vβ repertoire.

Previous works have reported the implication of most of these families in the response to HIV-1 exposure.[23-27] Nevertheless, few studies have analyzed the evolution of CD8$^+$

T-cell repertoire in the treatment interruption scenario.[28,29] No other previous studies have found a pattern of Vβ alterations similar to that found in this study maybe because neither of these studies analyzed the patients at three different time points, did not leave enough time between the studied time points or did not included enough patients to be able to observe a specific pattern.[30-34] A limitation of our study is the low number of patients eligible for the study. Also, functional studies and the contribution of HIV-1 specific responses are needed to further understand the fluctuations of Vβ families.

The expansion of the Vβ10, Vβ14, and Vβ15 families during treatment interruption were in parallel with a low increment in cell activation and no increment in T-cell exhaustion markers on these activated cells. These low parameters might not only prevent apoptosis and subsequent cell destruction of these cells but also allow them to proliferate. As well, an increment of effector memory cells coming from central memory cells was found that could be a consequence of antigen-specific stimulation.

On the other hand, the depletion of the Vβ20, Vβ28, and Vβ29 families during treatment interruption was in parallel with a remarkable increase in both cell activation and cell exhaustion markers that lead to an accelerated level of apoptosis. There is increasing evidence showing that up-regulation of PD-1 mediates HIV-specific $CD8^+$ T-cell functional exhaustion, including defective perforin and IFN-γ expression and impaired proliferative capability, resulting in immune deficiency of $CD8^+$ T-cells to control virus replication. Thus, the accumulation of PD-1 as well as the hyper-activation of T-cells prevent renewal of functionally competent $CD8^+$ T-cell repertoire.[7,35] Since only a limited correlation between this decreased families and HIV-1 viral load was found in this study, other antigens may have a role inducing PD-1 expression. A good candidate for this could be antigens derived from the increased microbial translocation driven by the viral load rebound after treatment interruption.[36-39] it is possible that microbial translocation can produce a generic stimulation of specific TRBV families that could account for these TRBV family-associated changes found in this study. Interestingly, we found that $CD8^+$ effector memory T-cells increased after treatment interruption. Some studies have suggested that PD-1 up-regulation is high in HIV-specific T effector memory cells and that may be associated with skewed maturation of virus-specific $CD8^+$ T-cells.[40-41] Despite an increase in cell activation was also found within the control Vβ2 family, the lower level of cell exhaustion determined that they were not depleted. A strong balance between cell activation and cell exhaustion is crucial to promote cell death or cell proliferation.

Interestingly, these TCR-Vβ fluctuations were significant only in those patients with longer time from diagnosis probably because they were prolonged exposed either to HIV-1 and, perhaps more importantly, to increased bacterial translocation.[36-39] Antiretroviral treatment reconstituted $CD8^+$ T-cell repertoire and most of immunologic markers that could be involved. Moreover, these fluctuations were not related with the nadir CD4.

Funding

This study was supported in part by Fondo de Investigación Sanitaria (FIS) 07/0070, Redes Temáticas de Investigación Cooperativa en Salud (RETICS) Red de Investigación en SIDA (RIS) RD06/0006/0021 and RD/06/0006/0034. We thank all the patients of this study for their cooperation.

Competing interests

The authors declare no financial or commercial conflict of interest.

Ethical approval

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation. It was approved by our local Independent Ethics Committee (Hospital Universitario Virgen del Rocío, Sevilla, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

Acknowledgements

AG-S, PhD student who received the DEA with AV as his supervisor, made the amplifications and analysis of the cell repertoire, and wrote a first draft of the manuscript. MA-F, PhD student, performed and analyzed all the immunologic parameters and helped in the revision of the manuscript. NS-S, postdoc fellow, helped in the interpretation of all the data, and in the revision of the manuscript. ML helped in the management and recruitment of the patients. AV designed and coordinated the study, helped in the analysis of the data, and finalized the manuscript in its final form. We want to thank all the patients who participated in this study.

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Figure legends

Figure 1: A) Proportion of expression (area under the curve) for each from cord blood sample. B) Gaussian distribution of the amplified CDR3 length repertoires in patient 8 in the different time points. C) Proportion of expression of each TCRVβ family in the different time points from all the patients. Black arrows highlight families with significant increased proportion of expression at TI, while grey arrows highlight families with significant decreased proportion of expression at TI.

Figure 2: Proportion of expression of disturbed TCRVβ families according to the time from diagnosis. A) Increased at TI, TCRVβ10, TCRVβ14, and TCRVβ15, and B) decreased at TI, TCRVβ20, TCRVβ28, and TCRVβ29. Filled boxes, patients with <105 months from diagnosis; empty boxes, patients with >105 months from diagnosis. Box represents interquartile range, black line represents median values, and also maximum and minimum values are shown. BL, baseline; TI, treatment interruption; TR, treatment resume. Significant when $p<0.05$.

Figure 3: A) Cell activation (CD38+HLA-DR+), B) level of cell exhaustion (PD-1+) of activated cells, and C) effector memory cells (CCR7-CD45RA-) of Vβ14, Vβ20, and Vβ2 cells. Filled boxes, patients with <105 months from diagnosis; empty boxes, patients with >105 months from diagnosis. Baseline (BL), treatment interruption (TI) and treatment resume (TR). A comparison betweendifferent cell populations is also shown. Significant when $p<0.05$.

Figure 1

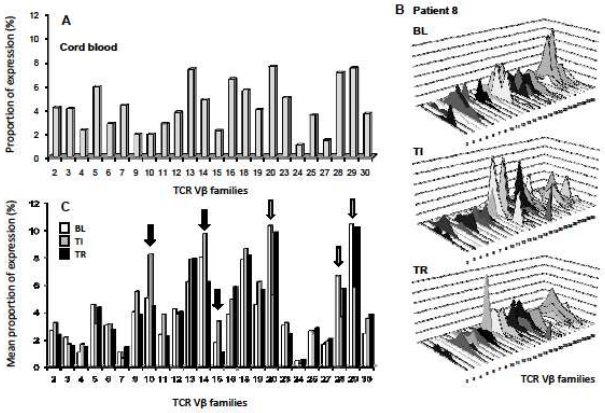


Figure 2

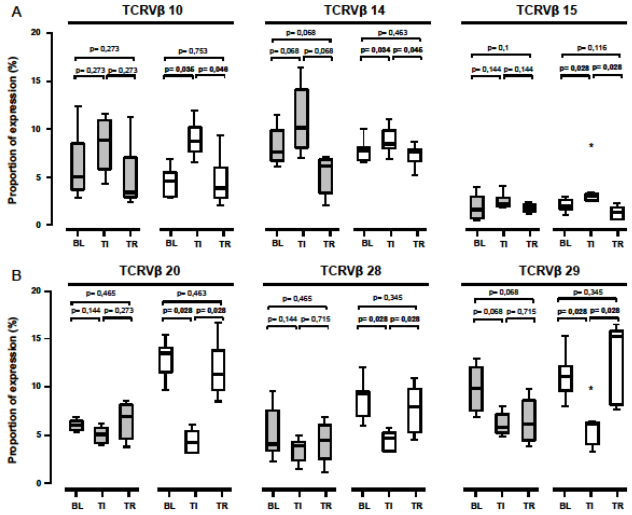
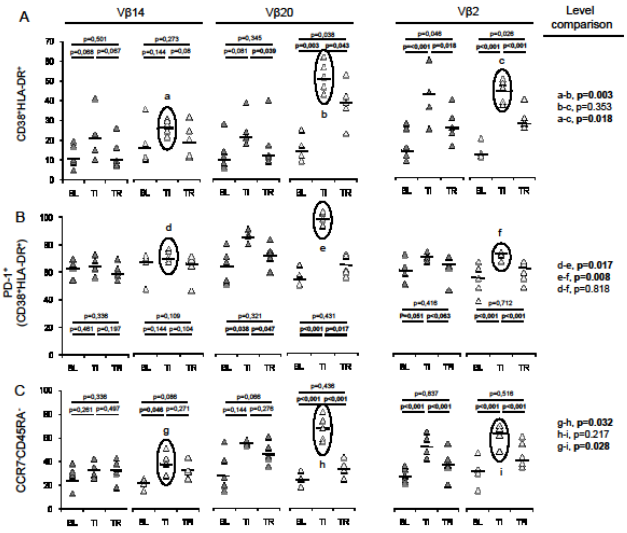


Figure 3



Dynamics of the HIV-1 latent reservoir after discontinuation of the intensification of antiretroviral treatment: results of two clinical trials

Carolina Gutiérrez[a], Beatriz Hernández-Novoa[a], Alejandro Vallejo[a], Sergio Serrano-Villar[a], María Abad-Fernández[a], Nadia Madrid[a], Laura Díaz[b], Ana Moreno[a], Fernando Dronda[a], Javier Zamora[c], María Ángeles Muñoz-Fernández[b] and Santiago Moreno[a]

Objective: Antiretroviral therapy (ART) intensification has been shown to reduce the reservoir of latently infected $CD4^+$ T cells. However, it is currently unknown whether this effect is maintained after discontinuation of the intensifying drug.

Design: The effect of ART intensification during 48 weeks with maraviroc or raltegravir in chronically HIV-1-infected patients was assessed in two previous clinical trials. In this study, we analysed this effect at week 24 after discontinuation of the intensifying drugs, at baseline and 48 weeks of intensification.

Methods: We measured the latently infected memory $CD4^+$ T cells carrying replication-competent virus, 2-long terminal repeat (2-LTR) circles and $CD4^+$/$CD8^+$ T cells activation.

Results: Fifteen patients were evaluated. After 48 weeks of intensification, HIV-1 reservoir size significantly decreased from 1.1 to 0.0 infectious units per million (IUPM) ($P = 0.004$). After 24 weeks of drug discontinuation, the median size of the reservoir was still significantly lower than at baseline ($P = 0.008$). 2-LTRs were undetectable in all individuals at baseline and after 48 weeks of intensification, continuing undetectable in all patients except two at week 24 after discontinuation ($P = 0.1$). $CD4^+$ and $CD8^+$ T-cell activation significantly decreased at 48 weeks after intensification, without further increase after discontinuation.

Conclusion: The effects of ART intensification with maraviroc or raltegravir persist at least 24 weeks after discontinuation of the drug. In a global strategy, ART intensification should be considered as part of a combination approach to achieve a functional cure or HIV eradication.

AIDS 2013, **27**:2081–2088

Keywords: 2-long terminal repeat circles, HIV-1 dynamics, HIV-1 reservoir, immune activation, treatment intensification

Introduction

Intensification of successful antiretroviral therapy (ART) with one or more drugs has been proposed as a potentially useful strategy to help achieve the eradication or a functional cure of HIV infection. The rationale for such intervention is based on the assumption that HIV replication persists in patients receiving effective ART [1–6].

[a]Infectious Diseases Department, Hospital Universitario Ramón y Cajal, and IRYCIS, [b]Immunobiology Laboratory, Hospital General Universitario Gregorio Marañón, and [c]Biostatistics Department, Hospital Universitario Ramón y Cajal, and IRYCIS, Madrid, Spain.

Correspondence to Dr Santiago Moreno, Department of Infectious Diseases, Hospital Universitario Ramón y Cajal, Carretera de Colmenar Km 9,1, 28034 Madrid, Spain.

Tel: +34 91 336 8710; fax: +34 91 336 8792; e-mail: smoreno.hrc@salud.madrid.org
Received: 13 February 2013; revised: 22 March 2013; accepted: 29 March 2013.

DOI:10.1097/QAD.0b013e328361d0e1

Indeed, a number of reports point in this direction, including the evidence provided by treatment intensification clinical trials [7], suggesting the gut-associated lymphoid tissue (GALT) as the site wherein HIV replication is more likely to persist [8]. As different intensification trials have failed to demonstrate a reduction in residual HIV found in plasma using ultrasensitive methods [5,8–12], other authors have argued against persistent replication of HIV and the utility of treatment intensification [13–15].

We and others have shown that intensification of antiretroviral treatment with different drug classes (reverse transcriptase inhibitors, CCR5 antagonists, integrase inhibitors) can lead to a reduction in the size of the latent cellular reservoir, as measured by the coculture assay, with no effect on residual viremia [16–18]. The most plausible mechanism for these findings would be the inhibition of ongoing viral replication in sites with suboptimal efficacy of the regimens, possibly due to subinhibitory concentrations of some of the drugs as suggested by other studies [3,4]. It is unknown, however, whether the effect achieved by treatment intensification persists after discontinuation of the intensifying drug. This could be important in order to better understand the mechanisms underlying the persistence of the HIV-1 cellular latent reservoir as well as for practical purposes.

To provide additional insight into the effects and dynamics of the cellular viral reservoir after treatment intensification, we amended two ongoing intensification clinical trials. The intensifying drugs used during these studies were discontinued to evaluate, after 24 weeks, whether the cellular reservoir was replenished or, alternatively, the reduction observed after intensification persisted. Together with the size of the HIV-1 cellular reservoir, we evaluated other markers of viral replication including the changes in the HIV-1 episomal DNA with 2-long terminal repeat (2-LTR) and markers of immune activation.

Materials and methods

Study design

We previously conducted two pilot open-label phase II clinical trials that evaluated the effect of two intensifying drugs [maraviroc (MVC) or raltegravir (RAL)] on the cellular HIV-1 reservoir in patients receiving ART [17,18]. The present study analyses the global effect after discontinuation of the intensifying drug (MVC or RAL). Both protocols were amended to include a measurement after 24 weeks of drug discontinuation (Fig. 1).

Both clinical trials (NCT00795444 and NCT00807443) were conducted at the Hospital Universitario Ramón y Cajal in Madrid, Spain, between 2008 and 2011 with an intensification period of 48 weeks, and a follow-up of 24 weeks after drug discontinuation. MVC was developed and provided by Pfizer, Inc, (New York, New York, USA) and RAL by Merck Sharp and Dome, (Whitehouse Station, New Jersey, USA).

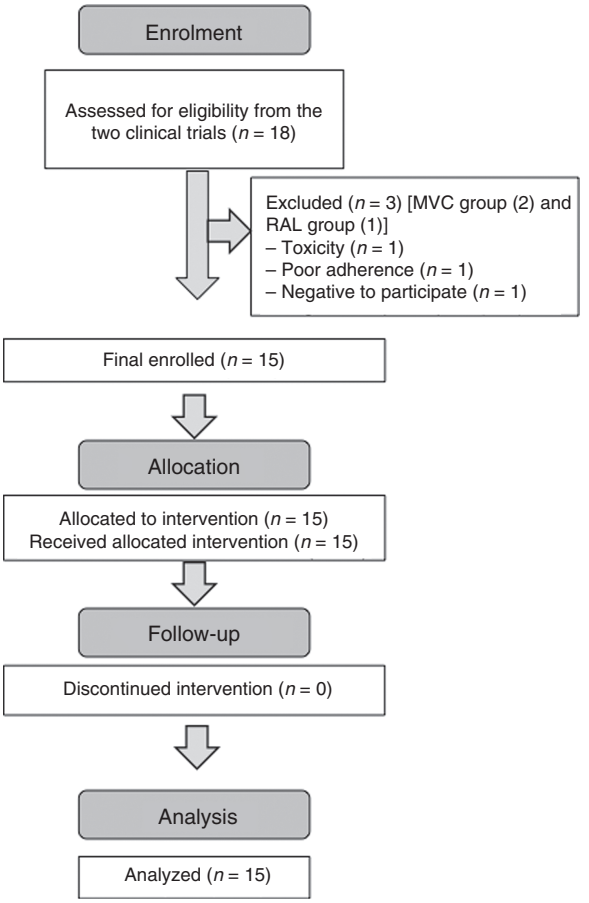


Fig. 1. Consort flow diagram.

Ethics statement

The two clinical trials were conformed according to the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines and were approved by the AEMPS (Spanish Agency for Medications and Health Products) and by the local Ethics Committee. All patients gave their written informed consent to participate in both trials once amended.

Patients and specimen collection

Sixteen patients (seven from MVC trial and nine from RAL trial) completed the intensification phase of the study; however, one individual from RAL trial declined to participate in the follow-up after drug discontinuation, so finally 15 patients completed the follow-up period of 24 weeks. The inclusion criteria were identical for the two clinical trials and included undetectable plasma viral load (pVL) by standard commercial assays

(<40 copies HIV-1 RNA/ml) for at least 2 years; ART with three or more drugs for at least 2 years; $CD4^+$ T-cell count > 350 cells/μl; R5 viral tropism using a phenotypic assay (Trofile; Monogram Biosciences, San Francisco, California, USA) in a pretreatment sample in the case of the MVC trial, and no previous treatment with any of the intensifying drugs (MVC or RAL). Patients were recruited from two hospitals (Hospital Ramón y Cajal and Hospital Doce de Octubre), in Madrid, Spain.

Blood samples were collected at baseline, after 48 weeks of intensification and after 12 and 24 weeks of drug discontinuation, apart from sampling for other determinations (data not shown). A total of 300 ml of heparinized whole blood was drawn to quantify the latent HIV-1 reservoir. Fifty millilitres of whole blood with EDTA were also drawn to isolate plasma and peripheral blood mononuclear cells (PBMCs).

HIV-1 cellular reservoir quantification: detection of cells carrying replication-competent HIV-1 virus

The detection of cells carrying replication-competent virus was determined using a previously described enhanced culture assay of highly enriched resting $CD4^+$ T cells that constitute the principal reservoir for the virus [19,20] with some modifications [17,18].

Briefly, PBMCs were isolated by Ficoll density gradient centrifugation (Lymphocytes Isolation Solution; Rafer, S.L, Zaragoza, Spain) from 300 ml of heparinized whole blood. Resting $CD4^+$ T cells were isolated from these total PBMCs by a negative selection of $CD3^+/CD4^+/HLA\text{-}DR^-/CD25^-$ cells using magnetic beads according to the manufacturer's recommendation (Miltenye Biotec, S.L. Bergisch Gladbach, Germany). The isolated resting cells (as minimum 63×10^6) yielded a purity greater than 99%.

Cells were plated in a duplicate five-fold serial dilution cultures from 25×10^6 to 320 cells. Ten-fold of allogenic irradiated PBMC from healthy donors was added to each culture with phytohaematoglutinin (PHA, 1 μg/ml) and recombinant interleukin 2 (IL-2, 100 U/ml) for efficient cell activation.

The maximum number of resting cells that were put into one well of a six-well plate was 10^6 in a final volume of 8 ml. Two individual wells of a six-well plate were labelled as 10^6 dilution plate. The 5×10^6 dilution was split into five wells with 1×10^6 cells/well and treated as a group, whereas the 25×10^6 dilution was split into 25 wells with 1×10^6 cells/well and again treated as a group. Cell concentrations below 1×10^6 were cultured in 24-well tissue culture plates with the appropriate number of resting cells, that is 2×10^5, 4×10^4, 8×10^3, 1.6×10^3 and 320 in a final volume of 2 ml.

Plates were placed at 37°C in a humidified 5% CO_2 incubator. On day 2, supernatants containing PHA were removed from the wells and replaced with fresh culture media (without PHA). Then, $CD8^+$ T-cell depleted PBMCs from healthy donors, prepared 2–3 days before in the presence of PHA (1 μg/ml), were added once a week to each culture. Depletion of $CD8^+$ T cells was performed using a positive selection method (CD8 positive selection; Miltenyie Biotec). These cells were obtained from different healthy donors each week and used fresh to avoid possible cell viability problems due to cryopreservation. Also, these cells were tested for fully permisivity for viral infection using a mixture of NL4-3 and IIIB HIV-1 strains. A total of 6×10^6 cells were added to each well of a six-well plate and 1×10^6 cells to each well of a 24-well plate.

On days 15 and 21, culture supernatants were tested for the presence of HIV-1 antigen using an HIV-1 p24 antigen assay kit (Innogenetics Diagnostica Iberia, S.L. Tarragona, Barcelona, Spain). Infected cell frequencies were determined using the maximum likelihood method and expressed as infectious units per million (IUPM) of resting $CD4^+$ T cells, with a limit of detection of 0.023 IUPM [21].

HIV-1 episomal DNA 2-long terminal repeat circles determination

The presence of 2-LTR circles was detected by a qualitative PCR, as described previously [17,18]. To maximize the recovery of 2-LTR circles and to overcome the lack of sensitivity of this technique [1,22,23], enriched 2-LTR circles were extracted selectively from approximately 5 million PBMCs using QIAprep Spin Miniprep (Qiagen, Valencia, California, USA) following the manufacturer's protocol for low copy number plasmids [1].

Immune activation and lymphocyte subsets

Fresh EDTA anticoagulated whole blood was used to analyse $CD4^+$ and $CD8^+$ T-cell activation using the following antibody combination: CD3-allophycocyanin (APC)-Cy7, CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin (PE)-Cy7, CD38-phycoerythrin (PE) and HLA-DR-APC, as previously described [18]. T-cell activation was characterized by $HLADR^+CD38^+$ expression. Antibodies were from Becton Dickinson (Becton Dickinson, Franklin Lakes, New Jersey, USA), and an unstained control was performed for all samples. At least 10^5 $CD3^+$ T cells were isolated for each sample and analysed with Kaluza software (Beckman-Coulter, Brea, California, USA) initially gating lymphocytes according to morphological parameters. Gating was similar between different time points.

Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and discrete variables as percentages. The *t*-test for independent samples was used to compare normally distributed continuous variables and

the Mann–Whitney test to compare nonnormally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. The Spearman correlation coefficient was used to analyse the correlations between continuous variables. Statistical analysis was performed using IBM SPSS software 16.0 (IBM, Armonk, New York, USA).

Results

Patient characteristics

Baseline characteristics of patients are summarized in Table 1. Median age was 46 years (IQR 41–50) and no significant differences were found between $CD4^+$ and $CD8^+$ T-cell counts at baseline [676 cells/μl (IQR 522–828) and 678 cells/μl (IQR 567–1066), respectively, $P = 0.7$] and at the end of 24-week follow-up [642 cells/μl (IQR 552–722) and 647 cells/μl (IQR 439–886), respectively, $P = 0.1$].

All were receiving nucleoside reverse transcriptase inhibitor (NRTI)-containing regimens combined with nonnucleoside reverse transcriptase inhibitors (NNRTIs) in seven cases (46.6%), with protease inhibitors in six cases (40%), and with one-third NRTI in two cases (13.3%). During follow-up, no changes were made in previous ART regimens. All individuals remained virally suppressed during the period of the study.

Effect of discontinuation of the intensifying drug on the HIV-1-cellular latent reservoir

The median size of the HIV-1-cellular latent reservoir at baseline including the 15 patients from the two clinical trials was 1.1 IUPM (IQR 0.023–3.2). At the end of the 48-week intensification period, the number of latently HIV-1 infected memory $CD4^+$ T cells decreased significantly, with a median of 0.0 IUPM (IQR 0.0–0.27) ($P = 0.004$). This effect in reducing the size of the HIV-1 latent reservoir was maintained at the end of the 24-week period after drug discontinuation, with a median of 0.0 IUPM (IQR 0.0–0.0), with no statistical differences with respect to the 48-week intensification period study point ($P = 0.9$), but significantly lower than at baseline ($P = 0.008$) (Fig. 2).

Effect of discontinuation of the intensifying drug on episomal 2-long terminal repeat DNA circles

All individuals showed undetectable episomal 2-LTR DNA circles at baseline and after the 48-week intensification period. As described, a transient increase was observed during intensification in patients in the MVC group at week 12 and 24 ($P = 0.037$ and $P = 0.012$, respectively, compared with baseline). After discontinuation, 2-LTR circles remained undetectable in all patients except two, without reaching a statistical significance compared with baseline and to the end of the 48-week intensification period ($P = 0.1$)

Effect of discontinuation of the intensifying drug on T-cell counts and T-cell activation

Globally, no differences were observed in the total $CD4^+$ and $CD8^+$ T-cell counts either after the 48 weeks of intensification or after the 24 weeks of discontinuation with respect to baseline.

We have previously reported a trend to decreased immune activation after intensification with both MVC and RAL. This effect was maintained at week 24 after discontinuation, with a significant decrease in $CD4^+$and $CD8^+$ T-cell

Table 1. Baseline characteristics of the 15 patients included in the desintensification study, according to the intensifying drug administered.

Variable	MVC ($n = 7$)	RAL ($n = 8$)	Total ($n = 15$)
Age (years)	46 [31–48]	48 [44–53]	46 [41–50]
Male (%)	85.7%	100%	93%
Risk factors (%)	IDU (42.8%)	IDU (25%)	IDU (33.3%)
	MSM (42.8%)	MSM (50%)	MSM (46.6%)
	HSx (14.2%)	HSx (25%)	HSx (20%)
Previous AIDS, *n* (%)	14.3%	37.5%	26.7%
HIV RNA (log copies/ml)			
Maximal	5.3 [4.1–5.5]	5.5 [4.8–5.6]	5.5 [4.8–5.6]
Before ART	5.3 [4.2–5.5]	4.6 [4.4–5.6]	4.8 [4.4–5.5]
At study entry	<1.7 (100%)	<1.7 (100%)	<1.7 (100%)
Duration of ART (months)	75 [38–144]	144 [70–167]	110 [52–144]
Duration of virological suppression (months)	70 [38–132]	55 [44.7–108.2]	62 [41–109]
CD4 cell count (cells/μl)			
Nadir	230 [171–416]	40 [17–297]	202 [62–317]
At study entry	711 [547–793]	655 [417–877]	676 [522–828]]
IUPM	0.41 [0.0–3.2]	1.4 [0.1–6.8]	1.1 [0.023–3.2]
Immune activation			
$CD38^+HLADR^+$ $CD4^+$ T cells (%)	3.3 [2.9–3.8]	2.4 [1.7–3.0]	3 [2.1–3.5]
$CD38^+HLADR^+$ $CD8^+$ T cells (%)	5.3 [5.05–8.0]	3.9 [3.5–4.5]	4.7 [3.7–5.7]

Continuous variables are expressed as median and interquartile range [IQR]. ART, antiretroviral therapy; HSx, unprotected heterosexual contacts; IUPM, infectious units per million cells; MVC, maraviroc; RAL, raltegravir.

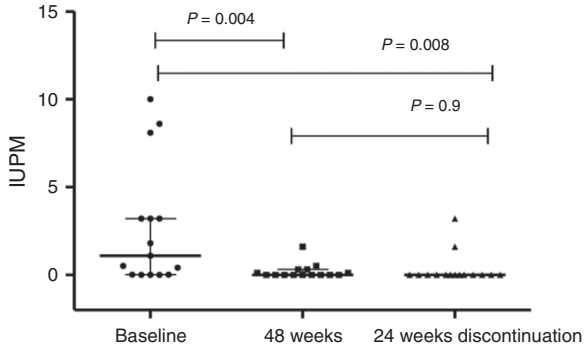


Fig. 2. Effect of discontinuation of the intensifying drug on the HIV-1 cellular latent reservoir. Median size of the HIV-1 latent reservoir at baseline, after 48 weeks of intensification and at the end of the 24-week period after drug discontinuation. The HIV-1 latent reservoir remained significantly lower after drug discontinuation than at baseline.

activation compared with baseline ($P = 0.003$ and $P = 0.042$, respectively), and a significant decrease in $CD4^+$ T-cell activation compared with week 48 after intensification ($P = 0.020$) (Fig. 3a).

Then, we analysed the differences in immune activation according to the intensifying drug. Although no significant decrease in $CD4^+$ T-cell activation was observed at week 48 after intensification in the MVC group, these levels were significantly lower at week 24 after discontinuation than at baseline ($P = 0.028$). The effect of intensification with MVC in reducing $CD8^+$ T-cell activation was maintained at week 12 and 24 after discontinuation compared with baseline ($P = 0.028$ and 0.028, respectively) (Fig. 3b). In the RAL group, no significant differences were observed in immune activation between baseline and week 48 after intensification, but only the levels of $CD8^+$ T-cell activation

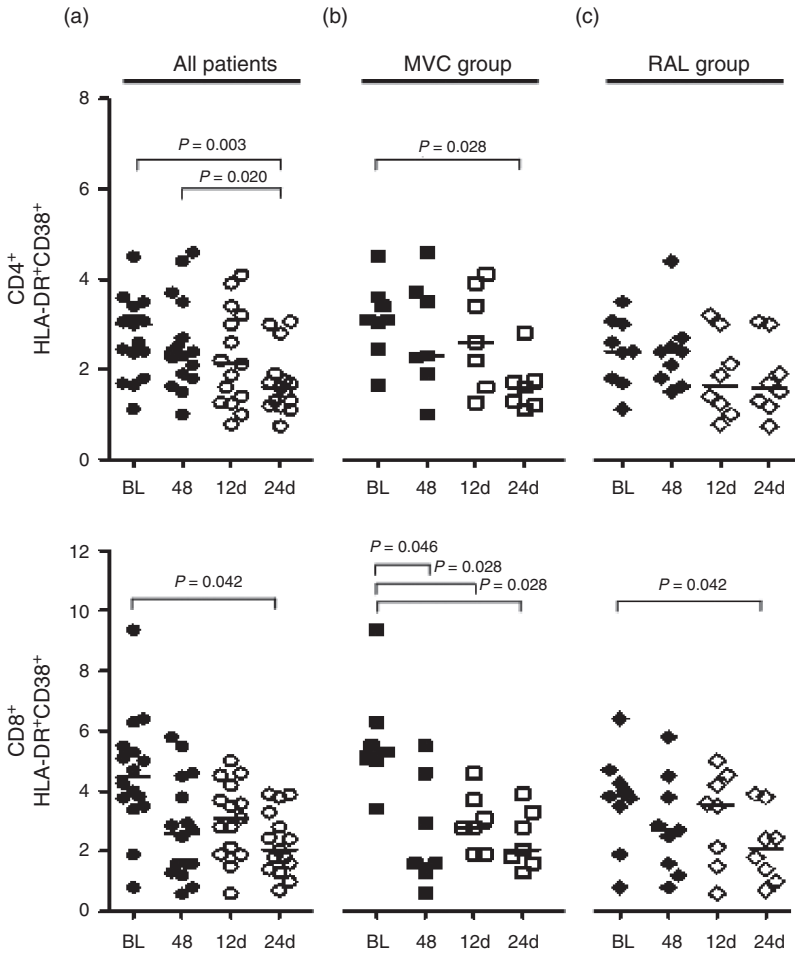


Fig. 3. Effect of discontinuation of the intensifying drug on T cell counts and T-cell activation. (a) $CD4^+$ and $CD8^+$ T-cell activation at baseline (BL), after 48 weeks of drug intensification (48) and after12 weeks (12d) and 24 weeks (24d) of drug discontinuation. (b) Levels of activated $CD4^+$ and $CD8^+$ T cells in the MVC group at baseline (BL), after 48 weeks of drug intensification (48) and after12 weeks (12d) and 24 weeks (24d) of drug discontinuation. (c) Levels of activated $CD4^+$ and $CD8^+$ T cells in the RAL group at baseline (BL), after 48 weeks of drug intensification (48) and after12 weeks (12d) and 24 weeks (24d) of drug discontinuation.

were significantly lower at week 24 after discontinuation than at baseline ($P = 0.042$) (Fig. 3c).

Discussion

To date, the pool of latently infected resting $CD4^+$ T cells has been the most profoundly analysed HIV-1 reservoir and is widely recognized as one of the major barriers to achieving eradication or a functional cure of HIV-1 [24–27]. In recent years, the absence of consensus on the causes of the stability of the latent viral reservoir originated a storm of controversy in relation to the possibility that residual HIV replication in subsets of $CD4^+$ T cells in the lymphoid tissue may contribute to replenishment of HIV-1 reservoir [8,13,15,28,29]. More recently, different groups have drawn attention to the GALT as a major HIV-1 reservoir in individuals receiving ART, as $CD4^+$ T-cell recovery is poorer in GALT and viral replication remains higher with respect to peripheral blood. This persistent viral replication in GALT probably contributes to maintenance of the reservoir despite peripheral viral suppression [7,8,30–32]. Therefore, intensification of conventional ART could further reduce the low-level ongoing viral replication and, subsequently, the reservoir replenishment.

Herein, we provide the first evidence that ART intensification can generate a significant and stable reduction of latently infected resting $CD4^+$ T cells that is maintained after discontinuation of the intensifying drug. Of note, in this pooled analysis of two ART intensification clinical trials of successful ART, participants showed a small size of the HIV-1 reservoir (median IUPM = 0.8 IUPM) at baseline, and low levels of $CD4^+$ and $CD8^+$ T-cell activation. Thus, this population had an optimal immunovirological status before the intervention, probably driven by the long-standing virological suppression [median 62 months (IQR 41–109)]. Even so, ART intensification was effective in reducing the size of the HIV-1 reservoir after 48 weeks, and strikingly, this effect was maintained after 24 weeks of discontinuation of the intensifying drug, with IUPM below the threshold of detection, and with undetectable 2-LTRs circles in the majority of patients. It could be argued that an increase in adherence could drive most of the impact of ART intensification on the size of the reservoir [33]. In our opinion, this seems unlikely based in two facts. First, in addition to long-standing virological suppression with no clinical rebound at any time, pharmacy records of adherence were excellent for patients included in the two studies of intensification. Only one patient had bad adherence during the MVC intensification clinical trials due to serious personal problems and he developed virological failure. Secondly, there was a clear relationship between the intensification of ART and the decrease of the reservoir.

Along with this efficacy in reducing the pool of latently infected resting $CD4^+$ T cells, a significant reduction in immune activation was also observed at the end of the follow-up, suggesting at least an indirect relation between $CD4^+$ and $CD8^+$ T -cell activation and the persistence of HIV-1 in the cellular reservoir.

So far, only three studies, including our clinical trials with MVC and RAL, have evaluated the effect of intensification strategies on the HIV-1-cellular latent reservoir in patients with virological suppression under ART by using the coculture method [16–18], which is currently considered the gold-standard technique to identify latently infected resting $CD4^+$ T cells. Importantly, all three studies revealed a decay of the reservoir. The fact that all three experimental strategies used drugs with different mechanisms of action (abacavir with/without efavirenz, MVC and RAL) imply that the reduction of the reservoir might be a consequence of a shrinkage in the viral replication in the anatomical reservoirs – such as the GALT – [7] rather than be driven by a specific drug effect of abacavir, MVC or RAL on the cellular reservoir. Thus, the rationale for ART intensification strategies would be an increase in drug concentrations up to inhibitory levels in sites with suboptimal efficacy of conventional ART regimens, as suggested previously [1–6,34].

In the light of these data, an important question remained to be answered. The proof that intensification strategies are effective in reducing the HIV-1 reservoir by inhibition of residual viral replication would be a decrease in IUPM after intensification and a subsequent rebound of IUPM and T-cell activation after discontinuation of the intensifying drug, due to an increase of the low-level viral replication and a replenishment of the HIV-1 reservoir [1–6,9–11,23]. This study was designed to provide new insight into this question. Although we found a decrease in IUPM and T-cell activation after intensification with two different drugs, the reservoir persisted undetectable and percentages of T-activated cells remained significantly lower after discontinuation of MVC/RAL with respect to baseline, contrarily to what we had hypothesized and expected. These surprising results deserve some considerations. The straightforward explanation is that replenishment of the HIV-1 reservoir might take more than 24 weeks after interruption of an intensification strategy. Against this reasoning, most immunovirological events such as plasma HIV-1 load rebound or CD4 cell count decline occur within the first weeks after ART interruptions [6,35]. Alternatively, intensification with MVC/RAL could have suppressed residual HIV-1 replication in tissues to an extent that may allow the immune response to exert some sort of control and prevent replenishment of the HIV-1 reservoir. The maintenance of low T-cell activation after MVC/RAL interruption supports this hypothesis. As IUPM and T-cell activation declined in parallel after intensification, an increase in T-cell activation after treatment discontinuation would have

suggested an increase in tissue HIV-1 load. However, both IUPM and T-cell activation remained at low levels after intensification interruption.

The findings regarding 2-LTRs circles also merit some considerations. These circles are episomal forms of nonintegrated HIV DNA with a relatively short half-life. Therefore, detection of 2-LTRs circles is generally considered a marker of recent infection [1,22,23,36] and do not necessarily reflect the size of the HIV-1 cellular latent reservoir [37]. As 2-LTRs were undetectable at baseline, after intensification and at the end of follow-up, the findings on 2-LTRs circles cannot lead us to any specific conclusion, other than they may indirectly reflect the absence of residual viremia in peripheral blood [12].

Our study is subject to a series of limitations. First, the small sample size demands a cautious interpretation; although our results were consistent across all comparisons and reached statistical significance, they should be reproduced in larger clinical trials. Second, the coculture assay is a technique of high sensitivity; however, an improvement of the threshold of detection might have enabled us to detect differences in the reservoir size between the determinations after intensification and at the end of drug discontinuation. Third, although we estimated that 24 weeks would be enough to detect a rebound of IUPM, this period might be insufficient to assess this outcome and future studies in this direction should be designed with longer periods of follow-up. The lack of a control group also limits the strength of the conclusions. However, the intensive laboratory work and high costs associated together with the fact that a spontaneous decrease of the reservoir was very unlikely made us decide not to include the control group. As stated in a previous report, the probability that the results be associated with hazard is really low ($P < 0.017$). Even so, the comparison with a control group would have led to more definitive conclusions.

In summary, our data provide new evidence that the effects of ART intensification with MVC or RAL in reducing the HIV-1 cellular latent reservoir persist at least 24 weeks after discontinuation of the drug. We believe that despite the great controversy on this field [1–6,9–12,23,38,39], this study shows that ART intensification strategies can alter the dynamics of the HIV-1 reservoir [23] and reduce its size to undetectable levels in selected patients. Thus, ART intensification strategies should be considered in future strategies – including the use of antilatency agents [40,41] – to achieve a functional cure or HIV eradication.

Acknowledgements

We would like to thank Carmen Page, Raquel Lorente, Ester Domínguez and María Coronel for the excellent technical assistance; the Radiophysics Departments at the Hospitals Ramón y Cajal and Gregorio Marañón for their help in the irradiation of cells, and the Regional Blood Transfusion Center in Madrid for providing the buffy coats from healthy donors. Specially, we thank all of our patients and their families for their participation in the study.

C.G., B.H., A.V., S.S., L.D., M.A.M.-F. and S.M. conceived and design the study and the experiments. A.M., F.D., S.S. and S.M. participated on the inclusion and follow up of the patients, and analysed clinical data. C.G., B.H., A.V., L.D., M.A. and N.M. performed all the experiments. C.G., B.H., A.V., S.S., L.D., M.A.M.-F., M.A., N.M., J.Z. and S.M. analysed all data. C.G., B.H., A.V., S.S. and S.M. contributed to the writing of the article. C.G., B.H., A.V., S.S., L.D., M.A., N.M., J.Z., A.M., F.D., M.A.M.-F. and S.M. approved the final version of the manuscript.

This work was supported in part by the Spanish AIDS Network 'Red Temática Cooperativa de Investigación en SIDA (RD06/0006)', the Spanish National of Health, 'Instituto de Salud Carlos III', grants (FIS-PI080958 and CP08/00046), and by the Foundation of Investigation and prevention of AIDS (FIPSE-36-0844/09).

Conflicts of interest

We declare that we have no conflicts of interest.

This work has been previously presented at the 19th Conference on Retroviruses and Opportunistic Infections (CROI); 5–8 March 2012; Seattle (Washington, USA).

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Journal of Infection (2013) **66**, 57—66

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The CD4/CD8 ratio in HIV-infected subjects is independently associated with T-cell activation despite long-term viral suppression

Sergio Serrano-Villar [a,d,*], Carolina Gutiérrez [a,d], Alejandro Vallejo [a,d], Beatriz Hernández-Novoa [a,d], Laura Díaz [b], María Abad Fernández [a,d], Nadia Madrid [a,d], Fernando Dronda [a], Javier Zamora [c,d], María Ángeles Muñoz-Fernández [b], Santiago Moreno [a,d,]**

[a] *Infectious Diseases Department, Hospital Universitario Ramón y Cajal, Madrid, Spain*
[b] *Inmunobiology Laboratory, Hospital General Universitario Gregorio Marañón, Madrid, Spain*
[c] *Biostatistics Department, Hospital Universitario Ramón y Cajal, Madrid, Spain*
[d] *IRYCIS, Madrid, Spain*

Accepted 28 September 2012
Available online 6 October 2012

KEYWORDS
HIV;
CD4/CD8 ratio;
Immune activation;
Immunosenescence;
Bacterial translocation;
HIV-1 latent reservoir;
CD4 count

Summary *Objectives:* HIV-infected subjects on antiretroviral therapy often fail to normalize the CD4/CD8 ratio despite CD4 count normalization. We aimed to analyze the biological significance of this finding.
Methods: Cross-sectional analysis in 20 HIV-infected subjects on stable triple-ART, plasma HIV RNA <40 copies/mL for at least 2 years and CD4 count >350 cells/mm^3. Laboratory measurements included T-cell activation (HLADR$^+$, CD38$^+$) and senescence (CD57$^+$), lipopolysaccharide (LPS), sCD14 and the HIV latent reservoir (number of latently infected memory CD4 cells carrying replication-competent virus).
Results: CD4/CD8 ratio was positively correlated with CD4 nadir ($r = 0.468$, $p = 0.038$) and accumulated ART exposure ($r = 0.554$, $p = 0.0011$), and negatively with viral load before ART initiation ($r = -0.547$, $p = 0.013$), CD4$^+$HLADR$^+$CD38$^+$ T-cells ($r = -0.428$, $p = 0.086$) and CD8$^+$CD57$^+$ T-cells ($r = -0.431$, $p = 0.084$). No associations with LPS, sCD14 or HIV latent reservoir were found. After the multivariate analyses, the CD4/CD8 ratio remained independently associated with CD4$^+$HLADR$^+$CD38$^+$ T-cells and CD8$^+$HLADR$^+$ T-cells.
Conclusions: In our study in subjects on suppressive ART the CD4/CD8 ratio was independently associated with T-cell activation. Our results must be confirmed in larger studies, as this

* Corresponding author. Infectious Diseases Department, Carretera de Colmenar Viejo, Km 9.100, 28034 Madrid, Spain. Tel.: +34 626 833 334; fax: +34 913 368 672.
** Corresponding author. Infectious Diseases Department, Carretera de Colmenar Viejo, Km 9.100 28034 Madrid, Spain. Tel.: +34 914 307 483; fax: +34 913 368 672.
E-mail addresses: serranovillar@gmail.com (S. Serrano-Villar), smoreno.hrc@salud.madrid.org (S. Moreno).

http://dx.doi.org/10.1016/j.jinf.2012.09.013

parameter might be a useful clinical tool to identify subjects with ongoing immune activation despite long-term viral suppression.

Introduction

Despite the dramatic success of combined antiretroviral therapy (ART) in the natural history of HIV infection, ART has failed in its attempt to completely restore normal health. Although recent evidence suggests that HIV-infected subjects with a good immunological recovery display mortality patterns similar to those in the general population,[1] most studies in patients with long-term viral suppression show increased morbidity and mortality,[2,3] essentially driven by non-infectious comorbidities,[4,5] a group of conditions generally associated to ageing, including cardiovascular disease, renal disease, liver disease, neurocognitive disorders, osteoporosis and non-AIDS malignancies.[5–10] Indeed, specific non-infectious comorbidities and pluripathology are more common among HIV-infected subjects than in the general population.[5,11,12] Thus, the great clinical challenge is no longer to reduce the incidence of opportunistic infections, but the early detection and management of subjects at risk of developing non-infectious comorbidities.

Most of these non-infectious conditions have been related to ongoing immune activation and low-level systemic inflammatory status that occurs in chronic HIV infection despite suppressive ART.[13,14] In fact, persistent immune activation in treated HIV infection is probably the major driving force of the accelerated immunological and systemic ageing in HIV infection.[13,15] Albeit our understanding of persistent immune activation is incomplete, different mechanisms such as viral persistence in cellular and anatomic reservoirs, increased bacterial translocation due to a chronically injured mucosa-associated lymphoid tissue (MALT) or asymptomatic cytomegalovirus (CMV) replication are likely to play a major role.[16–18]

The majority of ART-naive patients show an inverted CD4/CD8 T-cell ratio. In the general population, a low CD4/CD8 ratio is considered a surrogate marker of immunosenescence, and has been associated with persistent cytomegalovirus replication and with all-cause mortality.[19–22] In untreated HIV infection, the CD4/CD8 ratio has been investigated as a predictor of poor prognosis,[23–26] and has shown to independently predict immune restoration,[24] AIDS-related[25] and non-AIDS-related morbidities.[26] However, after several years of treatment, some patients still show an inverted ratio despite the normalization of CD4 counts, and the clinical and biological significance of this finding remains unknown. In aviremic HIV-infected subjects, the CD4/CD8 ratio has been associated with the frequency of $CD4^+$ T-cells carrying HIV-1 proviral DNA, used as an indirect measure of the size of the latent reservoir.[27,28] However, proviral DNA quantifies both replication of competent and incompetent virus, and the gold-standard technique for the evaluation of the HIV latent $CD4^+$ T-cell reservoir carrying replication-competent virus is currently considered the capacity of the latent $CD4^+$ T-cells to produce infectious virus.[29]

In this study, we analyzed the biological significance of the CD4/CD8 T-cell ratio, by evaluating the correlations with immune activation, immunosenescence, bacterial translocation and the size of the HIV latent reservoir measuring the infectious units per million resting $CD4^+$ T-cells.

Methods

Study design and patients

We combined the data of two pilot open-label phase II clinical trials to evaluate the effect of maraviroc (ERRADVIH-01 (MRV): NCT00795444)[30] and raltegravir (ERRADVIH-02 (RAL): NCT00807443)[31] on the HIV latent reservoir. The studies were conducted at the Hospital Universitario Ramón y Cajal in Madrid, Spain, between 2008 and 2010. Biochemical, immunological, and virological parameters were assessed at baseline and at weeks 12, 24, 36, and 48. For the present analysis, only baseline measurements — before ART intensification — were analyzed. Plasma viral load was measured by quantitative RT-PCR with a detection limit of 40 copies/mL (Roche Taqman HIV-1 test, Roche Molecular Systems), and T-lymphocyte counts were determined by flow cytometry.

The main inclusion criteria were as follows: HIV-1-infected adults receiving ART based on no less than three drugs for at least two years; undetectable plasma viral load (below 40 HIV-1 RNA copies/mL) for at least two years; $CD4^+$ T-cell counts above 350 cells/mm^3; and no previous experience with raltegravir or maraviroc. Patients were excluded if they had experienced virological failure or had received any immunosuppressive or immunomodulatory therapy. A total of 300 mL of heparinised whole blood was drawn from each patient for quantification of the HIV latent reservoir; 50 mL of whole blood with EDTA were drawn for plasma collection and isolation of peripheral blood mononuclear cells (PBMCs).

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials and was approved by our local Independent Ethics Committee (Hospital Ramón y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

T-cell activation and T-cell senescence

Fresh EDTA anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T-cells with the following antibody combination: CD3-allophycocyanin-Cy7 (APC-Cy7), CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin-Cy7 (PE-Cy7), CD38-phycoerythrin (PE), HLA-DR-allophycocyanin (APC) and CD57-fluorescein isothiocyanate (FITC). Antibodies were from Becton Dickinson (Becton

Dickinson, New Jersey, USA), and unstained controls were performed for all samples. Briefly, 100 μL of blood was lysed with FACS Lysing Solution (Becton Dickinson) for 30 min at room temperature, incubated with the antibodies for 20 min at 4 °C, washed, and resuspended in phosphate-buffered saline containing 1% azide. Cells were analyzed using a Gallios flow cytometer (Beckman Coulter, California, USA). At least 10^5 $CD3^+$ T-cells were collected for each sample and analyzed with Kaluza software (Beckman Coulter), by initially gating lymphocytes according to morphological parameters, to quantitate $CD4^+$ and $CD8^+$ T-cells expressing markers of activation ($CD38^+$, $HLADR^+$ or both) and senescence ($CD57^+$). Data on $CD38^+$, $HLADR^+$ and $CD57^+$ lymphocytes were expressed as the percentage of T-cells expressing these markers.

Bacterial translocation

Two commercial assays were used to evaluate bacterial translocation from plasma samples. Plasma bacterial lipopolysaccharide (LPS), gram-negative bacteria endotoxin, was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza®, Basel, Switzerland) according to the manufacturer's protocol. The plasma level of soluble CD14 (sCD14) was quantified using the Quantikine® Human sCD14 Immunoassay (R&D Systems, Minneapolis, Minnesota, USA), according to the manufacturer's instructions. All samples were run in duplicate.

Estimation of the HIV latent reservoir

The HIV-1 latent reservoir was determined using a modified co-culture assay of highly enriched $CD4^+$ T-cells, as previously described.[30,31] Briefly, PBMCs were isolated by Ficoll density gradient centrifugation (Lymphocytes Isolation Solution, Rafer, S.L., Zaragoza, Spain) from 300 mL of heparinised whole blood. Resting $CD4^+$ T-cells ($CD3^+/CD4^+/HLADR^-/CD25^-$) were then isolated and purified using magnetic beads according to the manufacturer's recommendations (Miltenyi Biotec GmbH., Bergisch Gladbach, Germany). Isolated resting $CD4^+$ T-cells (purity greater than 99%) were plated in serial limiting dilution cultures with allogenic irradiated PBMCs from healthy donors in the presence of phytohemagglutinin (PHA). To maintain cell cultures, $CD8^+$ T-cell depleted PBMCs from healthy donors were added once a week. On days 15 and 21, culture supernatants were tested for HIV-1 replication using an HIV-1 p24 antigen assay (Innogenetics Diagnostica Iberia, S.L. Barcelona, Spain). Infected cell frequencies were determined using the maximum likelihood method and expressed as infectious units per million (IUPM) of resting $CD4^+$ T-cells, with a limit of detection of 0.012 IUPM.

Statistical analysis

Continuous variables were expressed as median and interquartile range, and categorical variables as counts and percentages. Given the sample sizes and the non-normal distribution of some of the variables, non-parametric statistical tests were used. CD4/CD8 ratio was categorized by tertiles (0.8, 1.2) and patients were classified according to their CD4/CD8 ratio in two groups: low CD4/CD8 ratio (<0.8, first tertile) and normal CD4/CD8 ratio (≥ 0.8, second and third tertiles). Mann–Whitney tests were used for independent two-group comparisons and Spearman correlation coefficient was used to analyze the correlation between continuous variables. A series of multivariate linear regression models were built to explore simultaneous associations of any of the different phenotypes of activated and senescent T-cells, as dependent variables – $CD38^+$, $HLADR^+$, $CD38^+HLADR^+$ and $CD57^+$, in $CD4^+$ and $CD8^+$ T-cells – with the CD4/CD8 ratio, adjusting by a limited subset of independent variables. Assumptions for this analysis were checked by exploring regression residuals. All statistical analyses were performed using SPSS software 15.0 (SPSS Inc., Chicago, Illinois, USA).

Results

General characteristics

Median age was 45 years, median CD4 count 677 cells/mm^3 and median CD4 nadir 238 cells/mm^3. All patients displayed a CD4/CD8 ratio increase from baseline measurements before ART initiation (median, 0.5), with a median CD4/CD8 ratio at the inclusion in the study of 1.0. Median accumulated ART exposure was 73 months, with long-standing undetectable viral load (median, 60 months). Percentages of $CD4^+$ and $CD8^+$ T-cells with coexpression of HLADR and CD38 were low (3% and 4.5%, respectively), as well as the size of the HIV latent reservoir (median IUPM = 1.2). All patients were receiving nucleoside reverse transcriptase inhibitor (NRTI)-containing regimens, combined with non-nucleoside reverse transcriptase inhibitors (NNRTI) in eight cases (40%), with protease inhibitors (PIs) in ten cases (50%) and with a third NRTI in two cases (10%). Six subjects (30%) were coinfected by HCV. Table 1 summarizes the general characteristics of the 20 patients in the study.

CD4/CD8 ratio relations with therapy and immunovirological variables

CD4/CD8 ratio showed a positive correlation with CD4 nadir ($r = 0.468$, $p = 0.038$), accumulated ART exposure ($r = 0.554$, $p = 0.0011$) and a negative correlation with the peak of viral load ($r = -0.547$, $p = 0.013$). Despite the association with the accumulated ART exposure, no association was observed between the CD4/CD8 ratio and the time since HIV diagnosis ($r = 0.347$, $p = 0.133$). Importantly, the CD4/CD8 ratio correlated with $CD8^+$ T-cell count, but not with $CD4^+$ T-cell count ($r = -0.710$, $p < 0.001$; $r = 0.314$, $p = 0.177$, respectively) (Fig. 1). Thus, we explored the associations of $CD8^+$ T-cell count with CD4 nadir, accumulated ART exposure and the peak of viral load. $CD8^+$ T-cell count only showed a statistically significant correlation with accumulated ART exposure ($r = -0.612$, $p = 0.004$).

CD4/CD8 ratio, immune activation and immunosenescence

A negative correlation with border-line statistical significance was found between the CD4/CD8 ratio and the

Table 1 Characteristics of the study population ($N = 20$).

Variable	
Age (years)	45 (43–48)
Male, %/female, %	19 (95)/1 (5)
CD4 count (cells/mm^3)	676 (548–892)
CD8 count (cells/mm^3)	715 (626–1023)
CD4 nadir (cells/mm^3)	238 (79–358)
CD4 count at initiation of ART (cells/mm^3)	246 (160–378)
CD4/CD8 ratio	1.0 (0.7–1.6)
CD4/CD8 ratio before ART initiation	0.5 (0.2–0.8)
CD4/CD8 ratio increase from baseline	0.6 (0.2–0.9)
CD4$^+$HLADR$^+$CD38$^+$CD4$^+$ T-cells	3 (2.4–3.3)
CD8$^+$HLADR$^+$CD38$^+$ T-cells	4.5 (3.6–5.5)
Time on ART (months)	73 (49–143)
Time with viral suppression (months)	60 (40–107)
Time since HIV diagnosis (years)	9 (5–14)
HIV–HCV coinfection	6 (30%)
Risk factors	MSM 10 (50%)
	HSx 7 (35%)
	IDU 3 (15%)

All values are expressed as median and interquartile range, unless otherwise specified.
MSM, men who have sex with men; HSx, heterosexual; IDU, intravenous drug users.

percentages of CD4$^+$HLADR$^+$CD38$^+$ T-cells ($r = -0.428$, $p = 0.086$) and CD8$^+$CD57$^+$ T-cells ($r = -0.431$, $p = 0.084$). No significant correlations were found with other T-cell phenotypes of activation, or between the different phenotypes of activated T-cells and the CD4/CD8 ratio at ART initiation or the CD4/CD8 ratio increase from baseline (data not shown). Then, we analyzed the associations with the CD4/CD8 ratio categorized by tertiles (Fig. 2) with the percentages of the different T-cell phenotypes expressing markers of activation and senescence. Overall, subjects in the lowest tertile (CD4/CD8 ratio < 0.8) showed higher percentages of activated (Fig. 2A, B, D–F) and senescent CD4$^+$ and CD8$^+$ T cells (Fig. 2G, H, respectively). However, these differences only reached statistical significance for the percentage of CD4$^+$HLADR$^+$CD38$^+$ T-cells ($p = 0.049$) (Fig. 2A), with border-line statistical significance for the percentage of CD8$^+$CD38$^+$ T-cells ($p = 0.079$) (Fig. 2D). Given the strong negative correlation between the CD4/CD8 ratio and CD8$^+$ T-cell count, we also explored the associations between CD8$^+$ T-cell count and CD4$^+$ activated T-cells. CD8$^+$ T-cells did not correlate with the percentage of CD4$^+$HLADR$^+$CD38$^+$ T-cells ($r = 0.396$, $p = 0.115$), CD4$^+$HLADR$^+$ T-cells ($r = 0.137$, $p = 0.599$) or CD4$^+$CD38$^+$ T-cells ($r = 0.076$, $p = 0.772$).

CD4/CD8 ratio and bacterial translocation

No associations were found between the CD4/CD8 ratio and markers of bacterial translocation (LPS: $r = 0.192$, $p = 0.445$; sCD14: $r = -0.044$, $p = 0.861$). Similarly, we did not detect any association between these two variables and the two categories of CD4/CD8 ratio according to the CD4/CD8 ratio cut-off of 0.8 (Fig. 3). LPS and sCD14 correlated neither with the CD4/CD8 ratio before ART initiation ($r = -0.071$, $p = 0.811$ and $r = 0.042$, $p = 0.887$, respectively) or with the CD4/CD8 ratio increase from baseline ($r = 0.090$, $p = 0.759$ and $r = -0.235$, $p = 0.418$, respectively).

CD4/CD8 ratio and HIV latent reservoir

Seven (35%) patients had an undetectable HIV-1 reservoir (IUPM < 0.012). No association between the size of the HIV latent reservoir and the CD4/CD8 ratio was observed in the correlation analysis ($r = -0.322$, $p = 0.166$) (Fig. 4). The median values of IUPM were higher in subjects with CD4/CD8 ratio <0.8 compared to individuals with CD4/CD8 ratio ≥ 0.8; however, these differences did not reach statistical significance. IUPM correlated neither with the CD4/CD8 ratio before ART initiation ($r = -0.269$, $p = 0.314$) or with the CD4/CD8 ratio increase from baseline ($r = 0.335$, $p = 0.204$).

Multivariate analyses

A series of multivariate linear models were built to explore the associations between the different phenotypes of activated and senescent T-cells (dependent variables) and the CD4/CD8 ratio, adjusting for those variables most likely to exert confounding effects (Table 2). Thus, given the findings in the univariate analyses, the models were adjusted by CD4 nadir and accumulated ART exposure. After the multivariate analyses, both CD4 (HLADR$^+$CD38$^+$) and CD8 (HLADR$^+$) T-activated cells were independently associated with the CD4/CD8 ratio.

Discussion

Our results suggest that the CD4/CD8 ratio may provide relevant information in treated and virologically suppressed HIV-infected subjects, as it correlated positively with variables that summarize the immunological background – CD4 nadir and accumulated ART exposure and negatively with activation markers in CD4 and CD8 T-cells. No association with markers of bacterial translocation or the size of the HIV latent reservoir was observed.

Although failure to normalize the CD4/CD8 ratio despite peripheral CD4 count restoration is a common observation in clinical practice, few studies have addressed the biological or the clinical significance of this phenomenon. The outstanding importance of the viral load and the CD4 count as surrogate markers of ART efficacy in the global management of HIV-infected subjects could have led to overlook the significance of the CD4/CD8 ratio. Studies before the introduction of the highly active ART in 1996, recognized the CD4/CD8 ratio as a predictor of AIDS, providing similar prognostic information to that of CD4 count.[32] More recently, a low CD4/CD8 ratio at initiation of ART was found to be a predictor of Hodgkin lymphoma in the Swiss Cohort.[26] Remarkably, in a case–control study in aviremic subjects, the CD4/CD8 ratio was independently associated with subclinical atherosclerosis,[33] which in turn has been related to immune activation.[34] To our knowledge, no studies have addressed specifically the biological or clinical

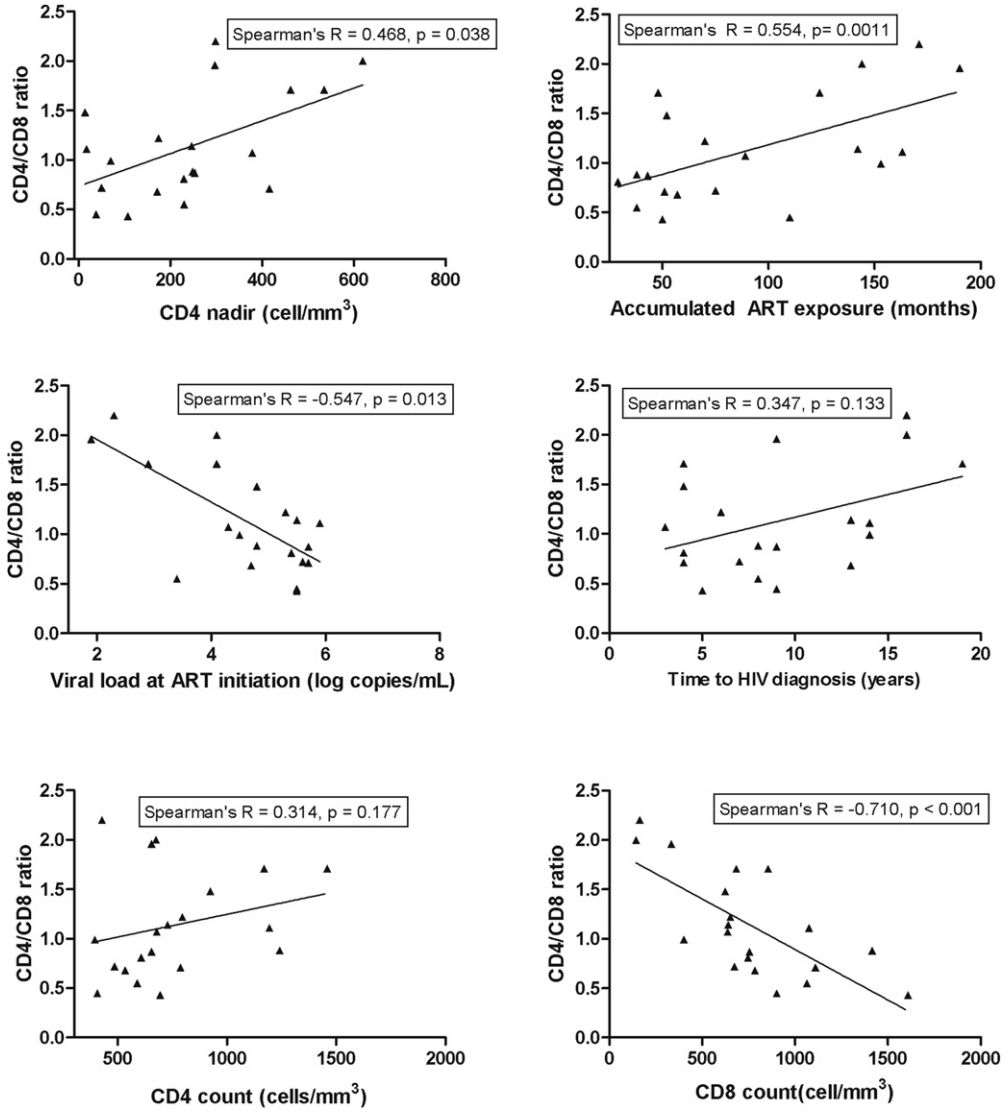

Figure 1 CD4/CD8 correlations with therapy and immunovirological variables.

significance of the CD4/CD8 ratio in HIV-infected subjects on suppressive ART. Most studies on this field have been conducted in the general population, where the CD4/CD8 ratio has been proposed as a surrogate marker of the immune risk profile, which entails a collection of immune-related defects that defines immune immunosenescence.[20,22] The immune risk profile was associated with all-cause mortality in two European cohorts[21,35,36] and is characterized by an increase in the number of $CD8^{+}CD28^{-}$ T-cells that result in a low CD4/CD8 ratio and it is associated to CMV-specific T-cells[19] or CMV seropositivity.[35] Remarkably, some of these immunological alterations accumulated with age in the elderly are similar to those that take place in HIV-infected subjects.[36] In fact, HIV infection is associated with expansion of CMV-specific T-cells, and CMV-specific T-cells are associated with atherosclerosis.[37] In our study, the CD4/CD8 showed a strong correlation with CD8 count, but not with CD4 count. The study design itself, which excluded patients with CD4 count <350 cells/mm^3 may explain this finding. While hallmarks of uncontrolled HIV infection are low CD4 count, high CD8 count and consequently a low CD4/CD8 ratio, subjects on ART usually display an increase of CD4 count and a decrease of CD8 count, to different extents. Thus, in individuals with successful CD4 count recovery we must assume that the CD4/CD8 ratio essentially depends on the CD8 count. In our view, a higher CD8 count among virally suppressed patients with CD4 count restoration might imply oligoclonal expansion of $CD8^{+}$ T-cells, reflecting an underlying immunosenescence.[19] Of note, we did not detect significant associations with the CD4/CD8 ratio before ART initiation or the CD4/CD8 ratio increase from baseline. This finding suggests that rather the total increase CD4/CD8 ratio due to ART, the homeostasis of absolute T-cell counts at a threshold between 0.8 — as suggested by our data, or 1 — as suggested by previous studies in the general population,[21,22] might correlate with immune activation and immunosenescence in HIV-infected patients.

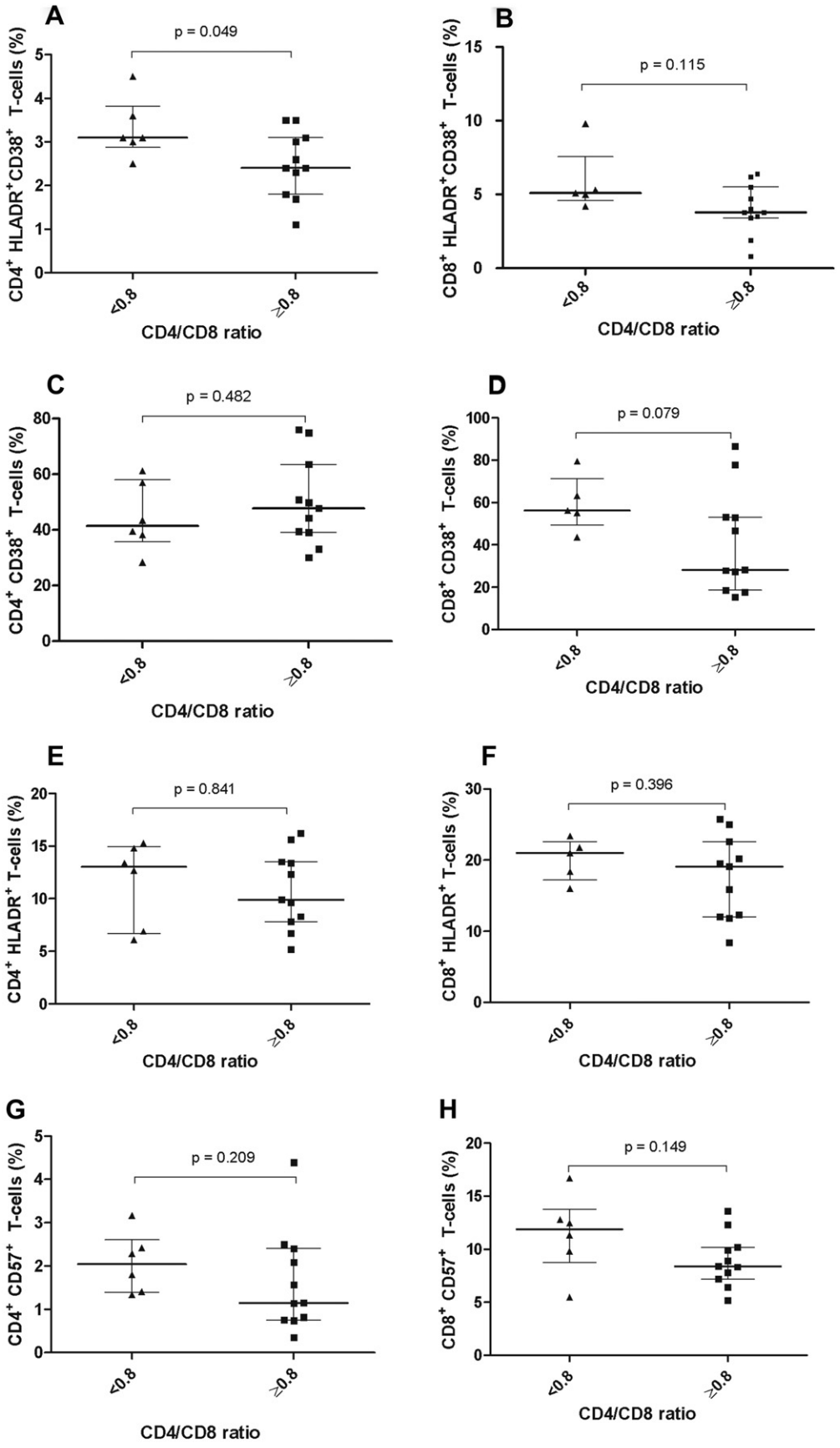

Figure 2 CD4/CD8 ratio, immune activation and immune senescence.

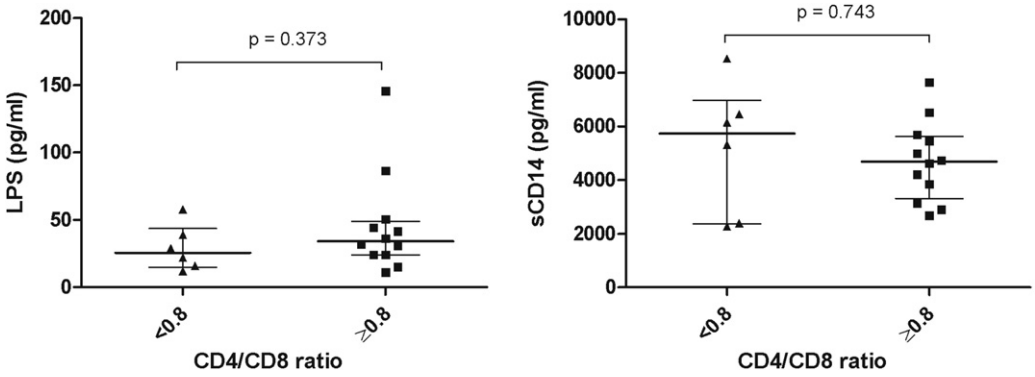

Figure 3 CD4/CD8 ratio and bacterial translocation.

The activated phenotype correlating with the CD4/CD8 ratio was different for $CD4^+$ and $CD8^+$ T-cells. While $CD4^+$ T-activated cells associated with the CD4/CD8 ratio showed coexpression of HLADR and CD38, only $CD38^+CD8^+$ T-cells correlated with the CD4/CD8 ratio. Although both markers are reliable surrogates of immune activation and are stronger predictors of disease progression than a viral load,[38] the coexpression of HLADR and CD38 is usually observed as the paradigm of late immune activation. Interestingly, recent evidence supports a different significance of the isolated expression of these activation markers. For example, patients with an inefficient response to ART display higher frequencies of $HLADR^+$ $CD4^+$ and $CD8^+$ T-cells[39] and the sole expression of CD38 has recently been shown to identify a subset of a hypoproliferative $CD4^+$ T-cells that does not fit into existing naïve and memory cell paradigms.[40] Besides, the association between the CD4/CD8 ratio and immune activation in HIV infection has a straightforward biological explanation and is in keep with what is known on the CD4/CD8 ratio. Considering that this parameter has been previously associated with immunological and systemic ageing, together with the fact that immunosenescence is considered to be driven by chronic immune activation,[41] we suggest that CD4/CD8 ratio might be a surrogate marker of immunosenescence also in HIV infection. Consequently, a point of paramount importance to be explored is if the persistence of a low CD4/CD8 ratio despite suppressive ART could also identify HIV-infected individuals with accelerated systemic ageing.

It is worth noting that, to our knowledge, only two studies have reported data on the association between the CD4/CD8 and the magnitude of the HIV latent reservoir, suggesting that failure to normalize the CD4/CD8 ratio in subjects on effective ART is explained, at least in part, by persistent, low-level, ongoing viral replication.[27,28] Remarkably, although there was a trend towards a higher size of the HIV latent reservoir at lower CD4/CD8 ratios, the difference did not reach statistical significance. The fact that a considerable proportion of patients (35%) showed an undetectable HIV latent reservoir, together with the small sample size makes these data difficult to interpret, preventing us from drawing conclusions on these particular data.

The lack of association between CD4/CD8 ratio and markers of bacterial translocation merits also some considerations. Since the CD4/CD8 ratio predicted T-cell activation, we would have expected to find an association with bacterial translocation. Importantly, both sCD14 and LPS levels were clearly lower to those in a HIV treatment-naïve control group already published elsewhere,[30] suggesting that the long-term virally suppressed subjects of this study might have experienced a certain degree of reconstitution of the MALT, resulting in decreased bacterial translocation. Again, given the small sample size and the low levels of bacterial translocation we cannot rule out an association between CD4/CD8 ratio and bacterial translocation; this association should be explored in larger studies in HIV-infected subjects with higher bacterial translocation.

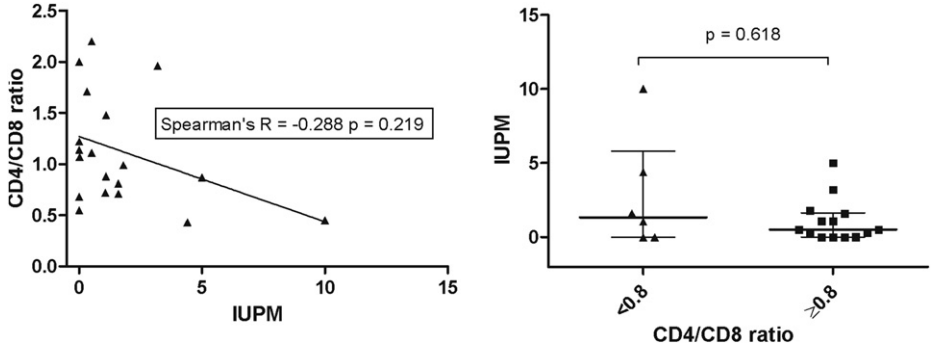

Figure 4 CD4/CD8 ratio and HIV latent $CD4^+$ T cell reservoir.

Table 2 Multivariate analyses: independent associations of activated and senescent T cells (dependent variable) with CD4/CD8 ratio (independent variable), adjusting by accumulated ART exposure and nadir CD4.

Dependent variable	Coefficient	Std. error	*p*
$CD4^+HLADR^+CD38^+$ T-cells	−1.197	0.387	**0.020**
$CD4^+HLADR^+$ T-cells	−7.082	9.659	0.476
$CD4^+CD38^+$ T-cells	−3.143	2.523	0.235
$CD4^+CD57^+$ T-cells	−0.799	0.632	0.228
$CD8^+HLADR^+CD38^+$ T-cells	−1.257	1.313	0.357
$CD8^+HLADR^+$ T-cells	−7.490	3.247	**0.040**
$CD8^+CD38^+$ T-cells	−19.169	14.793	0.219
$CD8^+CD57^+$ T-cells	−1.945	2.233	0.400

Our study is subject to a series of limitations. First, the sample size demands a cautious interpretation of the data. Only three independent variables could be included in the multivariate model, and we were forced to exclude other potential confounders as the time since HIV diagnosis. Second, we only measured the expression of CD57 in T-cells as a marker of immunosenescence. Increased expression of CD57 in T-cells reflects a state of replicative immunosenescence, an inability to proliferate and antigen-induced death of $CD8^+$ T-cells.[43] However, we did not quantify $CD28^-$ T-cells, which is an important limitation in the analysis of immunosenescence in our study. The lack of CD28 expression in $CD57^+$ T-cells is considered to identify the highly differentiated end-stage senescent cells, displaying shrinkage in the T-cell repertoire and a shortening of telomere lengths.[41] Third, we did not explore the relationship between CMV-specific T-cells and CD4/CD8 ratio. It would have been of great interest to analyze the relations between CD4/CD8 ratio, immune immunosenescence and CMV-specific T-cells in light of the evidence of an association in the general population.[19,20,22] Thus, our results warrant confirmation in larger studies in HIV-infected subjects on suppressive ART specifically designed to investigate the usefulness of the CD4/CD8 ratio as a marker of immune activation and immune immunosenescence.

In conclusion, our results suggest that the CD4/CD8 ratio in HIV-infected subjects on effective ART could be a surrogate marker of immune activation and possibly of immunosenescence. Since chronic immune activation seems to drive non-infectious associated mortality and morbidity in treated HIV infection,[34,42] subjects with higher immune activation despite ART might be the target of strategies aiming to prevent the development of these non-infectious conditions. Although these findings need to be confirmed in larger studies, the CD4/CD8 ratio merits further attention as this parameter, easily calculated in daily clinical practice, could help to identify subjects with ongoing immune activation despite long-term viral suppression.

Funding

This work was supported in part by the Spanish AIDS Network Red Temática Cooperativa de Investigación en SIDA (gs1) (RD06/0006), the Spanish National of Health, "Instituto de Salud Carlos III (gs2)", grants (FIS-PI080958 and CP08/00046), and by the Foundation of Investigation and prevention of AIDS (gs3) (FIPSE-36-0844/09). S.S-V is funded by grants from the Spanish Ministry of Science and Innovation (Ayudas para Contratos de Formación en Investigación Río Hortega). The Regional Blood Transfusion Center in Madrid provided the buffy coats from healthy donors. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflicts of interest

None.

Acknowledgments

We thank all of our patients and their families for their participation in the study.

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The effect of intensification with raltegravir on the HIV-1 reservoir of latently infected memory CD4 T cells in suppressed patients

Alejandro Vallejo[a,*], Carolina Gutierrez[a,*], Beatriz Hernandez-Novoa[a], Laura Diaz[b], Nadia Madrid[a], María Abad-Fernandez[a], Fernando Dronda[a], María J. Perez-Elias[a], Javier Zamora[c], Eduardo Muñoz[d], María A. Muñoz-Fernandez[b] and Santiago Moreno[a]

Objectives: The stability of the reservoir of latently infected memory $CD4^+$ T-cells may be associated with continuous replenishment from residual HIV-1, not completely eliminated by otherwise successful antiretroviral therapy (ART). Treatment intensification could help to control residual virus and to modify the latent reservoir. The objective of this work is to assess the effect of intensifying therapy with raltegravir on the HIV-1 cell reservoir.

Design: A pilot open-label phase-II clinical trial was performed to analyze ART intensification with raltegravir after 48 weeks in chronically HIV-1-infected patients on stable ART.

Methods: We measured the number of latently infected memory $CD4^+$ T cells, residual viremia, 2-long terminal repeat circles, $CD4^+/CD8^+$ T-cell activation, lymphocyte subpopulations, gut homing receptor, and bacterial translocation.

Results: A significant decay of HIV-1 latent reservoir was observed after intensification in the nine patients included ($P = 0.021$). No variation was found in either residual viremia or 2-long terminal repeat circles, whereas $CD8^+$ T-cell activation decreased at week 36 ($P = 0.028$). No differences were found in naive T-cell or effector memory cell counts, and the frequencies of gut homing receptor on activated or effector memory $CD8^+$ T cells. Bacterial translocation was stable, with the exception of a late decrease in lipopolysaccharide levels.

Conclusions: In this pilot noncomparative trial, treatment intensification with raltegravir significantly decreased the latent cellular HIV-1 reservoir and $CD8^+$ T-cell activation. Despite the limitations inherent to trial design, our results suggest that ART intensification should be considered as an adjuvant strategy to eradicate HIV-1 infection.

AIDS 2012, **26**:1885–1894

Keywords: bacterial translocation, gut-homing markers, HIV reservoir, immune activation, residual replication, treatment intensification

[a]Department of Infectious Diseases, Hospital Universitario Ramón y Cajal and IRYCIS, [b]Laboratory of Immunobiology, Hospital General Universitario Gregorio Marañón, [c]Department of Biostatistics, Hospital Universitario Ramón y Cajal, and IRYCIS, Madrid, and [d]Instituto Maimónides de Investigación Biomédica, Department of Cellular Biology, Physiology and Immunology, University of Córdoba, Córdoba Spain.

Correspondence to Santiago Moreno, MD, PhD, Department of Infectious Diseases, Hospital Ramón y Cajal, Carretera de Colmenar, Km 9,100, 28034 Madrid, Spain.

Tel: +34 91 336 8710; fax: +34 91 336 8792; e-mail: smoreno.hrc@salud.madrid.org

* A.V. and C.G. contributed equally to this work.

Received: 17 May 2012; revised: 16 July 2012; accepted: 18 July 2012.

DOI:10.1097/QAD.0b013e3283584521

Introduction

Successful antiretroviral therapy (ART) can reduce plasma viremia to below the limit of detection, leading to adequate immune recovery and clinical stability in most HIV-1-infected patients [1]. However, despite its well known benefits, lifelong ART is associated with important clinical problems. First, life expectancy may not be fully restored, and morbidity and mortality arising from non-AIDS events are increasingly frequent in patients with controlled viremia [2,3]. Second, successful lifelong ART is hampered by drug toxicity, resistance mutations, adherence, and cost, as well as limited access to treatment in the most affected developing countries [4]. Consequently, major efforts are being made in the search for new strategies to eradicate or cure HIV-1.

The main barrier to eradication of HIV-1 is virus persistence despite suppressive ART, which leads to pretherapy viremia levels when ART is interrupted [5,6]. The source (or sources) of the residual virus in patients receiving ART continues to be a matter of debate. Although nobody questions the importance of the pool of latently infected memory $CD4^+$ T cells [7,8], the possibility of ongoing viral replication resulting from incomplete inhibitory activity or penetration of ART in sanctuary sites remains controversial [9,10]. If the residual virus can replenish cell reservoirs, any attempt to eliminate these reservoirs should be preceded by complete inhibition of infection by the virus.

Intensification of treatment seems to be the most logical approach to eliminating residual HIV-1 replication. Previous studies using various drugs have shown conflicting results, possibly due to methodological differences in quantification of the latent reservoir, timing of administration of intensifying drugs (acute vs. chronic infection), baseline patient characteristics, duration of viral suppression, and sampling time. The emergence of new antiretroviral drugs with different mechanisms of action, such as integrase inhibitors and CCR5 antagonists, provide new opportunities in attempts to eradicate the latent reservoir [11–14].

We performed a pilot prospective open-label phase II clinical trial in chronically HIV-1-infected patients on suppressive ART in order to assess the effect of intensifying therapy with raltegravir on the HIV-1 cell reservoir. The main outcome measure was the number of memory $CD4^+$ T cells that were latently infected by HIV-1 with replicative capacity. The secondary outcome measures were residual viremia, episomal 2-long terminal repeat (LTR) circles, immune activation, and bacterial translocation.

Methods

Study design and patients

We performed a pilot open-label phase II clinical trial to evaluate the effect of raltegravir (developed and provided by Merck, Sharp and Dohme, Whitehouse Station, New Jersey, USA) on the HIV-1 latent reservoir. The study was conducted at the Hospital Universitario Ramón y Cajal in Madrid, Spain between 2008 and 2010. Biochemical, immunological, and virological parameters were assessed at baseline and at weeks 12, 24, 36, and 48. Plasma viral load was measured by quantitative RT-PCR with a detection limit of 40 copies/ml (Roche Taqman HIV-1 test; Roche Molecular Systems), and T-lymphocyte counts were determined by flow cytometry.

The main inclusion criteria were as follows: HIV-1-infected adults receiving ART based on no less than three drugs for at least 2 years; undetectable plasma viral load (pVL) (below 40 copies HIV-1 RNA/ml) for at least two years; $CD4^+$ T-cell count above 350 cells/μl; and no previous experience with raltegravir. Patients were excluded if they had experienced virological failure, received any immunosuppressive or immunomodulatory therapy, were or planned to become pregnant, or planned to interrupt treatment for any reason. A total of 300 ml of heparinized whole blood was drawn from each patient for quantification of the HIV-1 latent reservoir; 50 ml of whole blood with ethylenediaminetetraacetic acid (EDTA) was drawn for plasma and isolation of peripheral blood mononuclear cells (PBMCs).

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our local Independent Ethics Committee (Hospital Ramón y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

Quantification of latently infected resting CD4 T cells carrying replication competent virus

The quantification of latently HIV-1-infected cells was determined using a previously described enhanced culture assay of highly enriched resting $CD4^+$ T cells that represent the major long-term reservoir for the virus [15,16]. Briefly, PBMC were isolated by Ficoll density gradient centrifugation (Lymphocytes Isolation Solution; Rafer, S.L., Zaragoza, Spain) from 300 ml of heparinized whole blood. Resting $CD4^+$ T cells were then isolated by negative selection of $CD3^+/CD4^+/HLA\text{-}DR^-/CD25^-$ cells using magnetic beads according to the manufacturer's recommendations (Miltenyi Biotec, S.L. Bergisch Gladbach, Germany). Flow cytometry was next used to test the isolated resting cells that usually yielded a purity greater than 99%. A minimum of 63×10^6 resting $CD4^+$ T cells is needed to perform this assay. Cells were plated in duplicate five-fold serial dilution cultures from 25×10^6 cells to 320 cells. For efficient-cell activation, 10-fold allogenic irradiated PBMC from healthy donors, prepared the same day, were added to each culture in the

presence of phytohemagglutinin (PHA, 1 μg/ml) and recombinant interleukin 2 (IL-2, 100 U/ml).

The limiting dilution assay with five-fold serial dilution was set up as follows: The maximum number of resting cells that were put into one well of a 6-well plate was 10^6. Also, 10^7 irradiated PBMC were added to each well. Two individual wells of a 6-well plate were labeled as 10^6 dilution plate. Each of these wells had complete culture media (RPMI1640, 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin, 20 μg/ml streptomicin), PHA and IL-2 in a final volume of 8 ml. The 5×10^6 dilution were split into five wells with 1×10^6 cells/well and treated as a group, whereas the 25×10^6 dilution were split into 25 wells with 1×10^6 cells/well and again treated as a group. To prevent contamination of virus from one well into another, wells were separated by an empty well. Cell concentrations below 1×10^6 were cultured in 24-well tissue culture plates. Each well was set up with 2.5×10^6 irradiated PBMC and the appropriated number of resting cells, that is, 2×10^5, 4×10^4, 8×10^3, 1.6×10^3, and 320, in a final volume of 2 ml. Plates were placed at 37°C in a humidified 5% CO_2 incubator. On day 2 supernatants containing PHA were removed from the wells and replaced with fresh culture media (without PHA).

Then, $CD8^+$ T-cell-depleted PBMC from healthy donors, prepared 2–3 days before in the presence of PHA (1 μg/ml), were added once a week to each culture. Depletion of $CD8^+$ T cells was performed using a positive selection method (CD8 positive selection; Miltenyi Biotec). These cells were obtained from different healthy donors each week and used fresh to avoid possible cell viability problems due to criopreservation. Also, these cells were tested for fully permisivity for viral infection using a mixture of NL4–3 and IIIB HIV-1 strains. A total of 6×10^6 cells were added to each well of a 6-well plate and 1×10^6 cells to each well of a 24-well plate.

On days 15 and 21, culture supernatants were tested for the presence of HIV-1 antigen using an HIV-1 p24 antigen assay kit (Innogenetics Diagnostica Iberia, S.L. Tarragona, Barcelona, Spain). Infected cell frequencies were determined using the maximum likelihood method and expressed as infectious units per million (IUPM) of resting $CD4^+$ T cells, with a limit of detection of 0.023 IUPM [17].

Residual viremia

Residual viremia was measured using internally controlled ultrasensitive quantitative real-time RT-PCR (Single Copy Assay, SCA), as reported elsewhere [18]. Briefly, using improved nucleic acid isolation and purification techniques, as well as larger plasma volumes, the limit of detection was less than one HIV-1 RNA copy per ml, depending on the volume of plasma tested. When 5–7 ml of plasma is used the detection limit is 0.3 copies/ml. A median volume of 6.5 ml (interquartile range 4.6–7) was used for each patient; hence the limit of detection was 0.3 copies/ml in most patients.

Episomal 2-long terminal repeat circles

The presence of HIV-1 episomal 2-LTR circles was detected using in-house qualitative nested PCR. To maximize the recovery of 2-LTR circles and overcome the low sensitivity of this technique, enriched 2-LTR circles were extracted selectively from 5×10^6 PBMCs using QIAprep Spin Miniprep (Qiagen, Valencia, California, USA) following the manufacturer's protocol for low-copy-number plasmids, as previously described [19]. In the first round, 5 to 20 μl of episomal DNA were amplified in a 50 μl reaction with the following primers: forward, 5′-TAAGATGGGTGGCAAGTGGTCA; and reverse, 5′-TCTACTTGTCCATGCATGGCTT. The second round was performed using 1–2 μl of the first reaction product and primers spanning the unique junction formed by ligation of 5′ and 3′ LTR sequences, as follows: forward, 5′AATCTCTAGCAGTACTGG AAG; reverse, 5′GCGCTTCAGCAAGCCGAGTCCT. GCGCTTCAGCAAGCCGAGTCCT. PCR products were analyzed on 1% agarose gel stained with GelRed (Biotium, Hayward, California, USA).

T-cell activation, cell subsets, and gut homing receptor

Fresh EDTA anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cells with the following antibody combination: CD3-allophycocyanin-Cy7 (APC-Cy7), CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin-Cy7 (PE-Cy7), CD38-phycoerythrin, HLA-DR-allophycocyanin (APC), CD45RA-phycoerythrin (PE), CCR7-allophycocyanin (APC), and β7-APC. Antibodies were from Becton Dickinson (Becton Dickinson, Franklin Lanes, New Jersey, USA), and unstained control was performed for all samples. Briefly, 100 μl of blood was lysed with Lysing Solution (Becton Dickinson) for 30 min at room temperature, incubated with the antibodies for 20 min at 4°C, washed, and resuspended in phosphate-buffered saline containing 1% azide. Cells were analyzed using a Gallios flow cytometer (Beckman Coulter, Brea, California, USA). At least 10^5 $CD3^+$ T cells were collected for each sample and analyzed with Kaluza software (Beckman Coulter) by initially gating lymphocytes according to morphological parameters. Gating was always the same between the different time points. T-cell subsets (at least 20 000 events were gated) were defined as follows: naive cells, $CD3^+CD4^+(CD8^+)CD45RA^+CCR7^+$; effector memory cells $CD3^+CD4^+(CD8^+)CD45RA^-CCR7^-$; central memory cells $CD3^+CD4^+(CD8^+)CD45RA^-CCR7^+$; and transitional memory cells TEMRA $CD3^+CD4^+(CD8^+)CD45RA^+CCR7^-$. Cell activation levels were analyzed by the co-expression of CD38 and HLA-DR.

Bacterial translocation

Two commercial assays were used to evaluate bacterial translocation from plasma samples. Plasma bacterial lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza, Basel, Switzerland) according to the manufacturer's protocol. The plasma level of soluble CD14 was quantified using the Quantikine Humans CD14 Immunoassay (R&D Systems, Minneapolis, Minnesota, USA), according to the manufacturer's instructions. Samples were run in duplicate.

Statistical analysis

The frequency of latently infected cells was calculated using the parametric maximum likelihood method for limiting dilution experiments, as described elsewhere [16]. Continuous variables were expressed as median and interquartile range, and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables; the Wilcoxon test was used to compare non-normally distributed continuous variables. The association between categorical variables was evaluated using the chi-square test. The Spearman correlation coefficient was used to compare nonrelated variables. Statistical analysis was performed using SPSS software 16.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Patient characteristics

Nine HIV-1-infected patients under suppressive ARTwere included in this pilot study, and their baseline characteristics are summarized in Table 1. The patients were predominantly male with a long history of ART (median 12 years). Median CD4 cell count was 655 cells/μl and median CD8 cell count was 636 cells/μl. Only two patients were coinfected with HCV (RAL8 and RAL9, 22%). All patients were receiving nucleoside reverse transcriptase inhibitors combined with nonnucleoside reverse transcriptase inhibitors in five cases (55%) and with protease inhibitors in four cases (44%). Raltegravir was well tolerated by all the patients during the study.

Decreased frequency of latently HIV-1-infected cells

From each patient and time point at least 63×10^6 resting $CD4^+$ T cells were retrieved, with the exception of patients RAL5 and RAL6 at week 24 when the amount of resting cells were not enough to perform the assay. When the amount of resting cells allowed it, more than two wells of dilutions below 1×10^6 were put into culture, that is, a total of 20 wells were put into culture in each of the five next dilutions. All patients showed detectable IUPM at baseline including two with a value equal to the limit of detection of the assay (0.023 IUPM,

Table 1. Baseline characteristics of the patients.

Patient	Age (years)	Sex	Risk factors	Viral load (copies/ml)	CD4 cell count (cells/μl)	CD8 cell count (cells/μl)	Time on ART (months)	Antiretroviral regimen	Raltegravir dose (mg/BID)
RAL1	48	Male	MSM	<40	924	623	52	3TC+ABC+LPV/r	400
RAL2	36	Male	MSM	<40	678	636	89	FTC+TDF+EFV	400
RAL3	53	Male	HSx	<40	655	334	190	FTC+TDF+EFV	400
RAL4	45	Male	HSx	<40	406	901	110	FTC+TDF+EFV	400
RAL5	43	Male	MSM	<40	655	754	43	FTC+TDF+EFV	400
RAL6	68	Male	MSM	<40	830	675	144	FTC+TDF+ATV/r	400
RAL7	48	Female	HSx	<40	428	163	171	FTC+TDF+ATV/r	400
RAL8	46	Male	IDU	<40	1194	1074	163	FTC+TDF+EFV	400
RAL9	54	Male	IDU	<40	395	400	153	FTC+TDF+ATV/r	400
Median [IQR]	48 [44–53]	–	–	<40	655 [417–877]	636 [367–827]	144 [70–167]	–	–

ABC, abacavir; ART, antiretroviral therapy; ATV/r, atazanavir/boosted with ritonavir; ddI, didanosine; EFV, efavirenz; FTC, emtricitabine; HSx, Heterosexual; IDU, intravenus drug users; IQR, interquartile range; LPV/r, lopinavir/boosted with ritonavir; NVP, nevirapine; TDF, tenofovir.

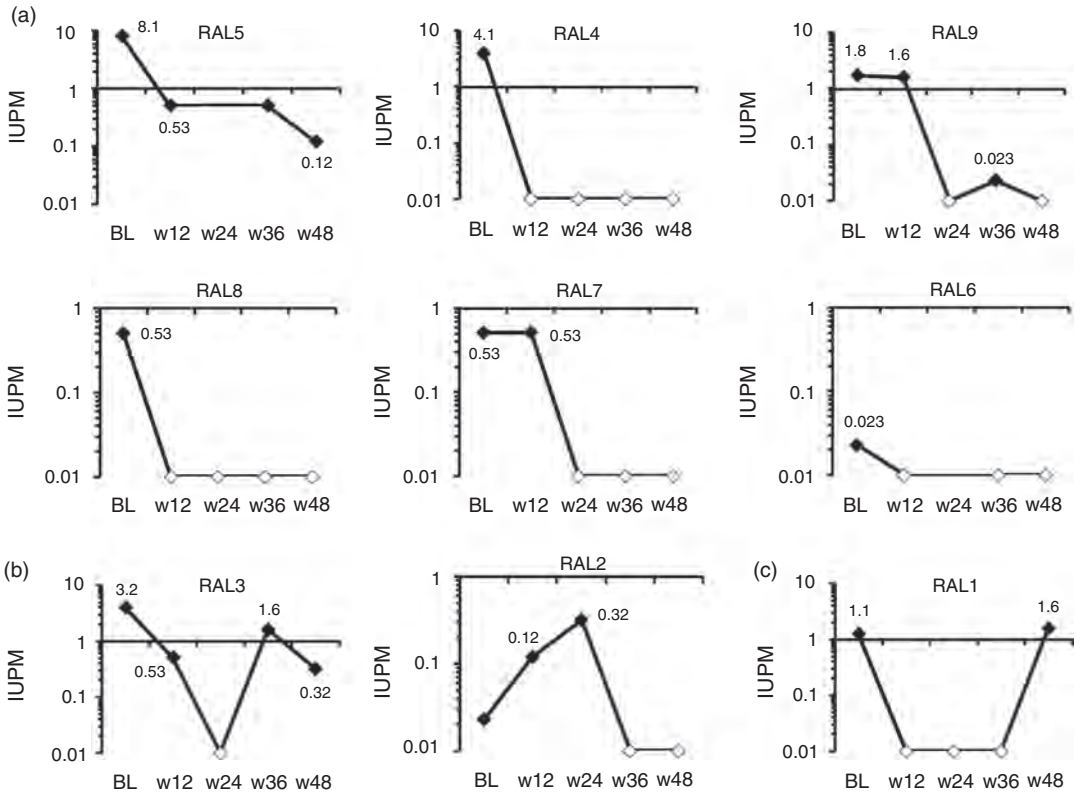

Fig. 1. Latently infected HIV-1 memory CD4$^+$ T cells after intensification with raltegravir (log infectious units per million cells, IUPM). All patients had detectable latent reservoir at baseline. (a) Patients with steady decline from higher to lower frequency of infection; (b) Patients with unsteady decline; and (c) Patient with higher frequency of infection at week 48 compared to baseline. Filled symbols: values above limit of detection. Open symbols: values under the limit of detection. Exact values of IUPM of each patient and time point are shown. The limit of detection was 0.023 IUPM.

patients RAL6 and RAL2). As shown in Fig. 1a, six patients showed a stable decline of IUPM during the follow-up, including three with only one positive time point (patients RAL4, RAL8, and RAL6). Two of the patients, RAL5 and RAL4, had a big decay of IUPM from basal to week 12. Patients with unstable decay of IUPM during the follow-up are shown in Fig. 1b (patients RAL3 and RAL2). Finally, only one patient showed a positive result after 48 weeks of treatment intensification (1.6 IUPM, RAL1, Fig. 1c).

We assume that this technique has some variability in culture recovery and to try to minimize it we always performed the assay with a minimum of 63×10^6 resting cells, added more than two wells in dilutions below 1×10^6 when possible, tested the permissibility of infection of the CD8$^+$ T-cell-depleted PBMC used to feed the cultures, and tested the viability of the cultures during the assay.

The decrease of IUPM during the follow-up was significant at week 12 [median 0.12 (0.0–0.51) IUPM, 24 median 0.0 (0.0–0.0), 36 median 0.0 (0.0–0.26), and 48 median 0.0 (0.0–0.22)] (Wilcoxon, $P = 0.025$, 0.028, 0.008, and 0.021, respectively). Assuming that the variation in the size of the cellular latent reservoir follows a binomial distribution, with parameter $P = 0.5$, the probability of such an observation, that is, spontaneous event, is as low as 0.017.

No effect of intensification on plasma residual viremia or 2-long terminal repeat circles

We measured plasma residual viremia by SCA at baseline and after 12 weeks of intensification with raltegravir. At baseline, residual viremia [median 0.4 copies RNA HIV/ml (0.3–1.4)] was detected in only three patients; at week 12, residual viremia [median 1.4 copies RNA HIV/ml (0.4–3.5)] was detected in five patients. Nevertheless, although there was a trend to an increase, it was not significant compared with baseline ($P = 0.08$) (Table 2).

Episomal 2-LTR circles were undetectable in all patients at baseline and remained undetectable after 2 weeks of intensification (table 2). Subsequently, 2-LTR circles could be transiently detected in four patients (44.5%); two at week 12, two at week 24, and one at week 36. Only one patient had detectable 2-LTR circles at two consecutive time points. 2-LTR circles were undetectable

Table 2. Effect of raltegravir intensification on residual viremia and episomal 2-long terminal repeat circles.

Patient	Single copy assay		Episomal 2-long terminal repeat circles					
	BL	w12	BL	w2	w12	w24	w36	w48
RAL1	<0.9	<0.4	Negative	Negative	Negative	Negative	Positive	Negative
RAL2	<0.3	<0.5	Negative	Negative	Negative	Negative	Negative	Negative
RAL3	2.0	1.4	Negative	Negative	Positive	Negative	Negative	Negative
RAL4	<0.3	4.0	Negative	Negative	Negative	Positive	Negative	Negative
RAL5	<0.3	<0.3	Negative	Negative	Positive	Positive	Negative	Negative
RAL6	<0.3	3.0	Negative	Negative	Negative	Negative	Negative	Negative
RAL7	0.4	2.4	Negative	Negative	Negative	Negative	Negative	Negative
RAL8	<0.6	<0.5	Negative	Negative	Negative	Negative	Negative	Negative
RAL9	6	11	Negative	Negative	Negative	Negative	Negative	Negative
p*		0.08		1	0.1	0.1	0.3	1

*Statistical significance compared to baseline (significant when $P < 0.05$).

in all patients at the end of the study. This transient detection of 2-LTR circles during follow-up was not significant compared with baseline.

Decrease in CD8$^+$ T-cell activation and no variation in T-cell subsets or gut homing receptor

CD4$^+$ T-cell activation remained stable during intensification with raltegravir. Nevertheless, CD8$^+$ T-cell activation decreased after intensification, the difference being statistically significant at week 36 ($P = 0.028$), with a clearly decreasing trend at week 48 ($P = 0.093$) compared with baseline. The slight waning effect at week 48 was due to the increase of cell activation in only three patients (Fig. 2a and b).

No differences were found in CD4 cell count (median 655, 610, 714, 755, and 703 cell/μl at baseline, 12, 24, 36, and 48 weeks, respectively) or CD8 cell count (636, 549, 522, 585, and 573 cells/μl at baseline, 12, 24, 36, and 48 weeks, respectively). The naive CD4 cell count (median 42.8, 41.8, 42.3, 38.2, and 43.2%, at baseline, 12, 24, 36, and 48 weeks, respectively) and the naive CD8 cell count (median 42.4, 36, 44.9, 42.3, and 45.9% at baseline, 12, 24, 36, and 48 weeks, respectively) were similar during the follow-up period ($P > 0.05$ at all time points in both cases). No significant differences were observed in the counts of either central, effector memory, or transitory CD4$^+$ and CD8$^+$ T cells (not shown). The proportions of activated CD8$^+$ T cells and effector memory CD8$^+$ T cells bearing gut homing β7 receptor did not vary significantly during the follow-up period (Fig. 2c and d, respectively). We also found no consistence differential effect between patients on nonnucleoside reverse transcriptase inhibitors vs. protease inhibitor.

Late decrease in bacterial translocation

At baseline, sCD14 and LPS levels were significantly lower than those of treatment-naive HIV-1-infected patients. Whereas sCD14 levels were stable during intensification, LPS levels decreased after 48 weeks of intensification ($P = 0.008$) (Fig. 3a and b).

Discussion

The results of this pilot clinical trial show that 48 weeks of intensification with raltegravir reduced the HIV-1 reservoir in latently infected memory CD4$^+$ T cells. However, this reduction was not associated with a reduction in residual viremia. The only associated finding was a decrease in CD8$^+$ T-cell activation, with no effect either on naive or memory T-cell counts or on gut homing receptor. A late decrease in a marker of bacterial translocation was observed as well.

The impact of intensifying ART on the HIV-1 latent reservoir in suppressed HIV-1-infected patients has been evaluated, and findings seem to support an effect of intensification on the latent reservoir. Only three studies, including the present one, have evaluated the impact of intensification by quantifying the number of IUPM in chronically HIV-1-infected patients using the coculture method [20,21]. All three studies revealed accelerated decay of the reservoir. As treatment was intensified with different drugs (abacavir with/without efavirenz, maraviroc, and raltegravir), the effect seems to be nonspecific and not related to a specific drug class. It must be noted that a different study showed no effect of intensification with raltegravir or efavirenz on the size of the latent reservoir [22]. However, some differences in the trial design could explain the different results. Although the enrolment criteria were similar to ours, the intensification included valproic acid either alone, or in combination with the intensifying drugs, and was administered during a shorter period of time.

The role of intensification in reducing residual replication and cell infection by the replicative virus is further supported by other findings. A comparative controlled clinical trial found a transient yet significant increase in 2-LTR circles in patients who had treatment intensification with raltegravir. This was interpreted as a clear sign of inhibition of residual replication [23]. Another study showed that intensification with raltegravir was associated with a significant decrease in proviral DNA in gut-associated lymphoid tissue (GALT) [24] and a decrease in

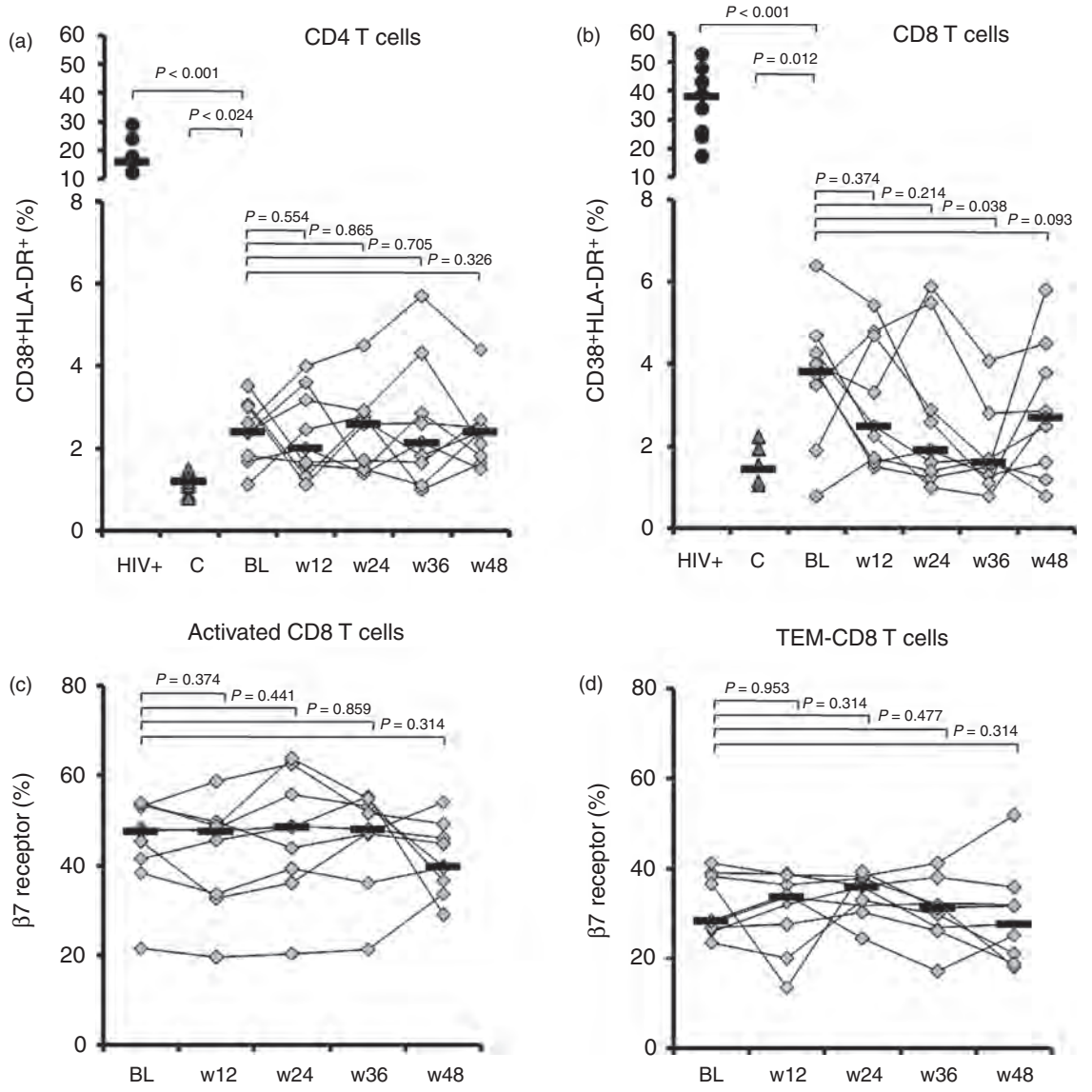

Fig. 2. (a) $CD4^+$ T-cell activation ($HLA\text{-}DR^+CD38^+$); (b) $CD8^+$ T-cell activation ($HLA\text{-}DR^+CD38^+$); and expression of gut homing receptor β7 on (c) activated $CD8^+$ T cells ($HLA\text{-}DR^+CD38^+$) and (d) effector memory $CD8^+$ T cells ($CD45RA^-CCR7^-$), after intensification with raltegravir. A group of naive HIV-infected patients (HIV+) and healthy individuals (c) were included as comparator groups.

unspliced HIV-1 RNA in $CD4^+$ T cells obtained from the terminal ileum [25], whereas no significant effect was observed on residual viremia. The preliminary results of an ongoing study show that ART may not reach inhibitory levels in tissues (lymph node and GALT), with the result that HIV-1 can replicate and infect cells, thus, increasing the possibility of replenishing the reservoirs [26].

In contrast, results from other studies have been interpreted as evidence against ongoing viral replication. No intensification study has been able to show any significant impact on residual viremia, as measured by SCA [9,27–29]. However, we feel that the lack of this effect is not surprising and does not contradict the potential action of raltegravir on residual replication. Intensification could act at extra-plasma sites with little impact on residual viremia measured in plasma. This indicates that residual viremia might arise from several different sources. In our study, residual viremia measured by SCA did not decrease during intensification. Therefore, the significant decay in the latent reservoir observed in the present study is not associated with decreased residual viremia, thus, implicating the long-standing cell reservoir as the probable source of such residual viremia. This observation is also supported by the results of studies that show a lack of genetic evolution in the persistent virus population [30]. The increase in 2-LTR circles have not been confirmed in other studies, although timing in measurements was different and could explain the

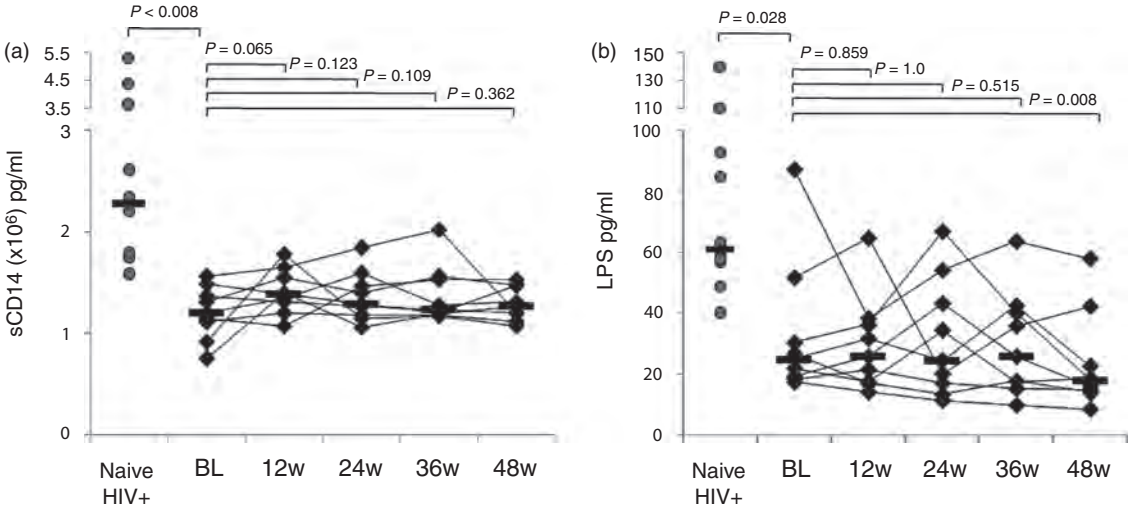

Fig. 3. Effect of intensification with raltegravir on bacterial translocation: (a) sCD14 levels, and (b) LPS levels during the follow-up period. A group of naive HIV-infected patients was included as comparator group.

discrepancy [22]. In our study, no significant changes were observed in 2-LTR circles after intensification with raltegravir at any time point, although the small sample size could at least in part explain the results.

Additionally, no clinical study has shown the impact of intensification on proviral DNA, which has been used to measure the HIV-1 reservoir. However, proviral DNA does not necessarily reflect the size of the latent reservoir, and its usefulness and validity for this purpose remain to be established. A final argument against the decay in the latent reservoir after intensification of ART is seen in the lack of an effect in patients with both acute and chronic infection who initiated therapy [12–14,28,31]. The discrepant results may be explained by the different situations of patients who initiate therapy and of those whose viral load has been suppressed for a long time. It is possible that initiation of ART could reduce the bulk of virions in tissues and plasma and that the effect on residual virus cannot be demonstrated months/years after the initial assessment.

$CD8^+$ T-cell activation decreased during intensification with raltegravir, as observed in other intensification studies [21]. This effect does not seem to be due to a dilutional effect as a result of cell trafficking from plasma to the gut, because T-cell counts were stable during intensification, and even T-cell subpopulations, that is, naive, central, and effector memory and transitory cells, were constant. We found no evidence of increased levels of gut homing receptor β7 in activated or memory T cells. Bacterial translocation was stable during intensification, with the exception of a late significant decrease in lipopolysaccharide levels. This finding is important, because we previously reported that intensification with maraviroc increased the levels of bacterial translocation that led to increased levels of gut homing receptor β7 in both activated $CD8^+$ T cells and effector $CD8^+$ T-memory cells and a subsequent decrease in $CD8^+$ T-cell activation, probably as a result of trafficking to the gut [21]. Hence, the mechanism by which $CD8^+$ T-cell activation decreased using either of these two drugs seems to be different.

Our study is limited by its small sample size and the lack of a control group. This trial was designed as a proof of concept study, and the absence of a control group was justified by the scarce possibilities of spontaneous decay of the reservoir, with no intervention. Apart from the frequency of infection within resting $CD4^+$ T cells is extremely stable (or decays very slowly), so any significant decrease that could be found might be attributed to the intervention. Another limitation of this study is the lack of two measurements at baseline. Nevertheless, in a previous intensification trial that we performed with a different drug [21], two baseline measurements 3 months apart were performed. As the values obtained in the two determinations were nearly identical, we decided to perform only single measurements due to resource constraints and work overload. In fact, the probability that this decrease was a random finding is very low. As with any study aimed at evaluating eradication strategies, our results are limited by the restrictions inherent to current methodological tools and the subsequent interpretation of results.

In summary, intensification with raltegravir can reduce the cellular viral reservoir without affecting residual viremia. Intensification with raltegravir or other drugs could increase the activity of ART and enhance inhibition of residual viral replication at sites such as the gut, where replication may persist due to limited penetration or suboptimal drug levels. If there is replenishment from low-level viremia to establish the equilibrium (or slow

decay) universally observed of the frequency of infection within resting $CD4^+$ T cells, there must be loss of latently infected cells at a roughly equivalent rate, Therefore, if there is depletion of resting cell infection by a period of raltegravir intensification as seen in this study, then the return to the prior level of resting cell infection after either an equal or greater period of unintensified ART could be one of two possibilities. The other possibility is that the level of resting cell infection is so low that the immune system can establish a new lower set point. Yet, it seems unlikely that intensification can eliminate HIV-1 infection, as existing cell reservoirs need to be purged. Other strategies, such as administering HIV-1 latency antagonists, need to be investigated as part of the work involved in eliminating HIV-1 infection.

Acknowledgements

We would like to thank Carmen Page, Raquel Lorente, Ester Domínguez, and María Coronel for their excellent technical assistance. We are grateful to the Radiophysics Departments at Hospitals Ramón y Cajal and Gregorio Marañón for their help with cell irradiation and to the Regional Blood Transfusion Centers in Madrid and Albacete for generously providing buffy coats from healthy donors. We are indebted to our patients and their families for their participation in the study.

Author contributions: A.V., C.G., L.D., B.H-N, M.A.M-F, E.M. and S.P. conceived and design the experiments. C.G. and L.D. performed the quantification of latently infected memory $CD4^+$ T-cells; A.V., L.D. and M.A-F. performed the cytometric analysis; M.A-F. performed the quantification of bacterial translocation; B.H-N and N.M. performed the detection of the 2-LTR circles. F.D., M.J.P-E and S.M. participated on the inclusion and follow up of the patients, and analyzed clinical data; C.G., A.V., L.D., B.H-N, M.A-F, N.M., J.Z. and S.M. analyzed all the data; C.G., L.D., A.V., B.H-N, N.M., M.A-F and S.M. contributed to the writing of the paper; C.G., L.D., A.V., B.H-N, M.A-F, N.M., E.M., M.A.M-F and S.M. approved the final version of the manuscript.

This work was supported by Spanish AIDS Network 'Red Temática Investigación SIDA' (RD06/0006); Foundation Investigation and Prevention of AIDS (FIPSE-36-0844/09).

This work has previously been presented at the 6th IAS Conference on HIV Pathogenesis, treatment and prevention 17-20 July 2011, Rome Italy.

Trial registration: ClinicalTrials.gov identifier: NCT00807443

Conflicts of interest

There are no conflicts of interest.

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PLoS one

Intensification of Antiretroviral Therapy with a CCR5 Antagonist in Patients with Chronic HIV-1 Infection: Effect on T Cells Latently Infected

Carolina Gutiérrez[1☯], Laura Díaz[2☯], Alejandro Vallejo[1], Beatriz Hernández-Novoa[1], María Abad[1], Nadia Madrid[1], Viktor Dahl[3], Rafael Rubio[4], Ana M. Moreno[1], Fernando Dronda[1], José Luis Casado[1], Enrique Navas[1], María Jesús Pérez-Elías[1], Javier Zamora[5], Sarah Palmer[3], Eduardo Muñoz[6], María Ángeles Muñoz-Fernández[2], Santiago Moreno[1]*

1 Infectious Diseases Department, Hospital Universitario Ramón y Cajal, and IRYCIS, Madrid, Spain, **2** Inmunobiology Laboratory, Hospital General Universitario Gregorio Marañón, Madrid, Spain, **3** Karolinska Institute, Stockholm, Sweden, **4** Infectious Diseases Unit, Hospital General Universitario Doce de Octubre, Madrid, Spain, **5** Biostatistics Department, Hospital Universitario Ramón y Cajal, and IRYCIS, Madrid, Spain, **6** Immunology Department, Universidad de Córdoba, Córdoba, Spain

Abstract

Objective: The primary objective was to assess the effect of MVC intensification on latently infected $CD4^+$ T cells in chronically HIV-1-infected patients receiving antiretroviral therapy.

Methods: We performed an open-label pilot phase II clinical trial involving chronically HIV-1-infected patients receiving stable antiretroviral therapy whose regimen was intensified with 48 weeks of maraviroc therapy. We analyzed the latent reservoir, the residual viremia and episomal 2LTR DNA to examine the relationship between these measures and the HIV-1 latent reservoir, immune activation, lymphocyte subsets (including effector and central memory T cells), and markers associated with bacterial translocation.

Results: Overall a non significant reduction in the size of the latent reservoir was found ($p = 0.068$). A mean reduction of 1.82 IUPM was observed in 4 patients with detectable latent reservoir at baseline after 48 weeks of intensification. No effect on plasma residual viremia was observed. Unexpectedly, all the patients had detectable 2LTR DNA circles at week 24, while none of them showed those circles at the end of the study. No changes were detected in $CD4^+$ or $CD8^+$ counts, although a significant decrease was found in the proportion of HLA-DR^+/$CD38^+$ $CD4^+$ and $CD8^+$ T-cells. LPS and sCD14 levels increased.

Conclusions: Intensification with MVC was associated with a trend to a decrease in the size of the latent HIV-1 reservoir in memory T cells. No impact on residual viremia was detected. Additional studies with larger samples are needed to confirm the results.

Trial Registration: ClinicalTrials.gov NCT00795444

Citation: Gutiérrez C, Díaz L, Vallejo A, Hernández-Novoa B, Abad M, et al. (2011) Intensification of Antiretroviral Therapy with a CCR5 Antagonist in Patients with Chronic HIV-1 Infection: Effect on T Cells Latently Infected. PLoS ONE 6(12): e27864. doi:10.1371/journal.pone.0027864

Editor: Rupert Kaul, University of Toronto, Canada

Received July 12, 2011; **Accepted** October 26, 2011; **Published** December 8, 2011

Funding: This work was supported in part by the Spanish AIDS Network "Red Temática Cooperativa de Investigación en SIDA (RD06/0006), the Spanish National of Health, "Instituto de Salud Carlos III", grants (FIS-PI080958 and CP08/00046), and by the Foundation of Investigation and prevention of AIDS (FIPSE-36-0844/09). Pfizer Inc provided the drug. The Regional Blood Transfusion Center in Madrid provided the buffy coats from healthy donors. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: Pfizer Inc. provided the drug. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: smoreno.hrc@salud.madrid.org

☯ These authors contributed equally to this work.

Introduction

Antiretroviral therapy (ART) can reduce plasma HIV-1 RNA levels to <50 copies/ml [1]. However, low residual viremia, which is only detectable using ultrasensitive assays, can persist despite ART [2–4]. The origin and clinical implications of persistent low-level viremia are uncertain. While some studies postulate that it may be the result of virus released from latently infected cells [5–7], others support that it could arise from ongoing viral replication, as a consequence of incomplete inhibitory activity or penetration of antiretroviral drugs [8–13].

Although intensification of current ART with potent drugs could potentially decrease residual viremia and prevent replenishment of viral reservoirs, prior intensification studies have not demonstrated any impact on residual viremia [12,14–17]. Only one study confirmed a transient increase in episomal 2LTR DNA circles after raltegravir intensification [18] as an indicator of recent HIV-1 replication [19–21], and another study showed a decrease

in the frequency of episodes of intermittent viremia [22]. To our knowlegede, only a few studies have previously evaluated the effect of intensification of stable therapy on the latent reservoir of resting $CD4^+$ T cells in chronically HIV-1-infected patients with controversial results. Ramratnam et al. found an accelerated decay of the HIV-1 latent reservoir after intensification therapy with abacavir with or without efavirenz [22]. Two more studies that measured the impact on the $CD4^+$ T cell reservoir in patients receiving a four-drug combination as initial antiretroviral therapy provided discordant results, namely, a reduction in the reservoir in patients treated during acute infection [23] and no changes in chronically infected patients [24].

Bacterial translocation and a low level of ongoing viral replication have been associated with an increase in immune activation in HIV-1-infected patients [25,26]. Evidence of increased bacterial translocation from damaged intestinal tissue was recently demonstrated in chronic HIV-1 infection [25,27]. In acute HIV-1 infection, intestinal $CD4^+$ T cells are rapidly depleted and T-cell activation is high. Since monitoring intestinal cells is difficult, expression of surrogate markers such as the mucosal homing receptor $\alpha4\beta7$ integrin on circulating T cells has been proposed to correlate with loss or restoration of intestinal $CD4^+$ T cells and could prove helpful in monitoring the success of therapeutic strategies [28–30]. Besides, recent studies have shown that HIV-1 utilizes $\alpha4\beta7$ integrin to bind to $CD4^+$ T cells [31].

Maraviroc (MVC) is a potent new antiretroviral agent approved for the treatment of HIV-1 infection that blocks interaction between the virus and the CCR5 co-receptor, a crucial step in the HIV-1 life cycle [32]. Previous clinical trials have demonstrated the safety, tolerability, and efficacy of MVC in both treatment-naive and treatment-experienced patients [33,34].

We performed a prospective open-label pilot phase II clinical trial to assess the effect of MVC intensification on latently infected $CD4^+$ T cells in chronically HIV-1-infected patients receiving antiretroviral therapy. We also analyzed residual viremia and episomal 2LTR DNA to examine the relationship between these measures and the HIV-1 latent reservoir, immune activation, lymphocyte subsets (including effector and central memory T cells), and markers associated with bacterial translocation. The hypothesis was that if these factors are causally linked, altering one of them with the intervention should result in alteration of the others.

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Study Design

We performed an open-label pilot phase II clinical trial to evaluate the effect of MVC on the cellular HIV-1 reservoir in patients receiving ART. The study was conducted at Hospital Universitario Ramón y Cajal in Madrid, Spain between 2008 and 2010. This independent clinical trial (NCT00795444) had a follow up of 48 weeks of intensification with MVC (developed and provided by Pfizer, Inc.).

Ethics Statement

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our Independent Ethics Committee (Hospital Ramón y Cajal, 28034 Madrid, Spain; ceic.hrc@salud.madrid.org). All patients provided their written informed consent for participation, which included sample collection and laboratory determinations.

Patients and specimen collection

Nine patients met all the following inclusion criteria: undetectable plasma viral load (pVL) by standard commercial assays (<50 copies HIV-1 RNA/ml) for at least two years; ART with three or more drugs for at least two years; $CD4^+$ T lymphocyte count > 350 cells/mm^3; R5 viral tropism testing a pretreatment sample using a phenotypic assay (Trofile®, Monogram Biosciences, C.A), and no previous treatment with MVC. Patients were recruited from two hospitals (Hospital Ramón y Cajal and Hospital Doce de Octubre), both in Madrid, Spain.

Patient's enrollment, allocation and follow up are described in figure 1.

Samples were collected at baseline and after 12, 24, 36, and 48 weeks of intensification with MVC. A total of 300 ml of heparinized whole blood was drawn to quantify the latent HIV-1 reservoir; 50 ml of whole blood with EDTA was drawn to obtain plasma and isolate peripheral blood mononuclear cells (PBMC). Samples were cryopreserved in the HIV Biobank of the Spanish AIDS Research Network (RIS) following current procedures [35].

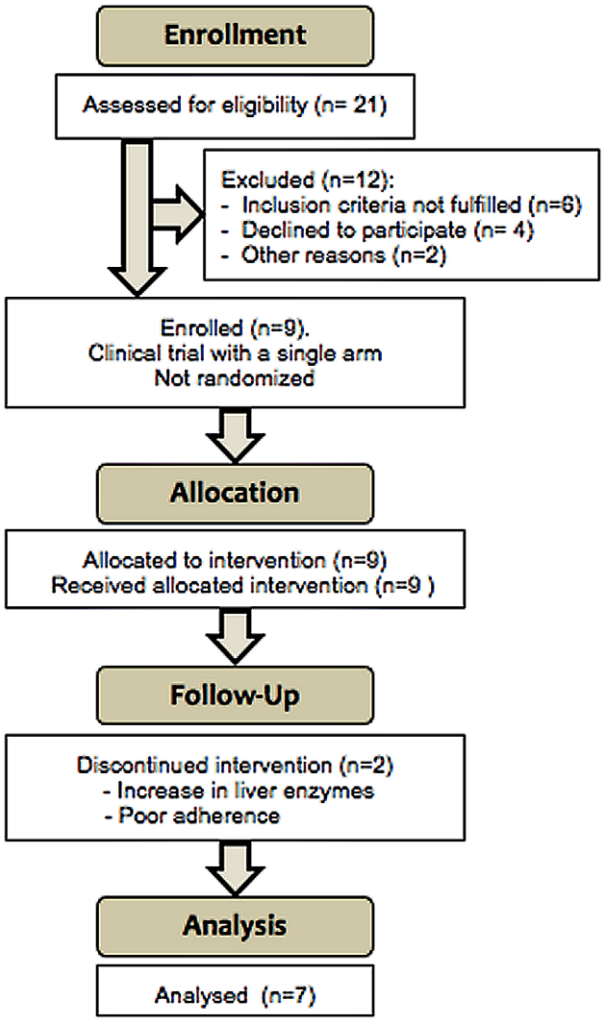

Figure 1. Consort Flow Diagram.
doi:10.1371/journal.pone.0027864.g001

In addition, two control groups (10 treatment-naive HIV-1-infected patients, and 10 HIV-1-negative subjects) were also analyzed for some measurements.

Latently HIV-1-infected memory T cells

Two baseline determinations (three months apart) were performed to investigate the accuracy of the method and the stability of the reservoir.

We carried out a previously described co-culture assay [36,37]. Briefly, PBMC were isolated from 300 ml of heparinized whole blood using Ficoll density gradient centrifugation (Lymphocytes Isolation Solution, Rafer, S.L Zaragoza, Spain). Resting $CD4^+$ T cells ($CD4^+$/HLA-DR^-/$CD25^-$) were isolated and purified using magnetic beads according to the manufacturer's recommendations (Miltenyi Biotec, S.L. Bergisch Gladbach, Germany). $CD4^+$ T-cell purity greater than 99% with less than 0.4% activation was confirmed by flow cytometry. The isolated resting $CD4^+$ T cells were plated in replicate limiting dilutions and co-cultured with allogenic irradiated PBMC from seronegative donors and phytohemagglutinin. The activated cell culture was fed once a week with PBMC from healthy donors after depletion of $CD8^+$ T cells. On days 15 and 21, culture supernatants were tested for HIV-1 replication using the HIV-1 p24 antigen assay (Innogenetics Diagnostica Iberia, S.L Tarragona, Barcelona, Spain). Infected cell frequencies were determined by the maximum likelihood method and expressed as infectious units per million (IUPM) resting $CD4^+$ T cells [38]. The millions of purified resting $CD4^+$ T cells obtained from the total PBMCs conditioned the number of replicates of each dilution. The assay was performed to each point as duplicate fivefold dilution series.

From baseline to week 12 of follow-up, the limit of detection of the assay was 0.12 IUPM resting $CD4^+$ T cells. Thereafter, the limit of detection decreased to 0.023 IUPM, since the total blood extraction volume was increased after approval of the amended protocol by the IEC.

Residual viremia

Residual viremia was measured with internally controlled an ultrasensitive quantitative real-time RT-PCR (single copy assay), as reported elsewhere [39]. The optimal HIV-1 RNA detection threshold was 0.3 copies/ml when 5 to 7 ml of starting plasma was available. We used a median of 6.5 ml (IQR: 4.6 to 7).

HIV-1 episomal 2LTR DNA circles

The presence of HIV-1 episomal 2LTR DNA circles was detected by qualitative PCR. To maximize the recovery of 2LTR DNA circles and overcome the lack of sensitivity of this technique [8,18,40] enriched 2LTR episomal DNA circles were extracted selectively from ~5 million PBMC using QIAprep Spin Miniprep (Qiagen, Valencia, California, USA) following the manufacturer's protocol for low-copy-number plasmids, as previously described [8].

A nested PCR flanking the episomal 2LTR DNA circle junction was designed. In the first round, 5–20 μL of episomal DNA were amplified in a 50-μL reaction with the following primers: forward, 5′-TAAGATGGGTGGCAAGTGGTCA; and reverse, 5′-TCTACTTGTCCATGCATGGCTT.

The second PCR round was performed using 1 to 2 μL of the first reaction product as the template and primers spanning the unique junction formed by ligation of 5′ and 3′ LTR sequences (forward, 5′-AATCTCTAGCAGTACTGGAAG; reverse, 5′-GCGCTTCAGCAAGCCGAGTCCT). PCR products were analyzed on a 1% agarose gel stained with GelRed (Biotium, Hayward, California, USA).

Immune activation and lymphocyte subsets

Fresh EDTA anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell activation with the following antibody combination: CD3-allophycocyanin (APC)-Cy7, CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin (PE)-Cy7, CD38-phycoerythrin (PE), and HLA-DR-allophycocyanin (APC). Lymphocyte subpopulations were defined as naïve (CD45RA+CCR7+), T central memory (CD45RA-CCR7+, TCM), T effector memory (CD45RA-CCR7-, TEM), and T effector memory RA+ (CD45RA+CCR7-, TemRA). To analyze lymphocyte subsets this antibody combination was used: CD3-allophycocyanin (APC)-Cy7, CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin (PE)-Cy7, CD45RA- phycoerythrin (PE), and CCR7-allophycocyanin (APC), β7-allophycocyanin (APC). Antibodies were from Becton Dickinson (Becton Dickinson, NJ, USA), and an unstained control was performed for all samples. Briefly, 100 μL of blood were lysed with 200 μl of FACS Lysing solution (Becton Dickinson) for 30 min at room temperature, incubated with the antibodies during 20 min at 4°C, washed and resuspended in PBS containing 1% azida. Cells were analyzed in a Gallios flow cytometer (Beckman-Coulter, CA, USA). At least 15000 $CD3^+$ cells were collected for each sample and analyzed with Kaluza software (Beckman-Coulter) initially gating lymphocytes according to morphological parameters. The gating was always the same between different time points.

Bacterial translocation

Two commercial assays were used to evaluate bacterial translocation from plasma samples. Plasma bacterial lipopolysaccharide (LPS) was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza®, Basel, Switzerland) according to the manufacturer's protocol. This test quantifies endotoxins from the cell wall of gram-negative bacteria. Plasma sCD14 was quantified using the Quantikine® Human sCD14 Immunoassay (R&D Systems, Minneapolis, Minnesota, USA), according to the manufacturer's instructions. Samples were run in duplicate in all cases.

Statistical analysis

This is an exploratory pilot study that is trying to prove a concept. A treatment group of 10 patients has been considered adequate, based on data from previous studies published by other authors on the same subject.

Continuous variables were expressed as the median and interquartile range and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables and the Mann-Whitney test to compare non-normally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. A Spearman correlation was used. Statistical analysis was performed using SPSS software 16.0 (Inc., Chicago, Illinois, USA).

Results

Patient characteristics

The baseline characteristics of the patients are summarized in table 1. The median age was 46 years and 8 patients were male. The median baseline $CD4^+$ and $CD8^+$ T-cell counts were 711 and 784 cells/mm^3, respectively. All patients were receiving nucleoside reverse transcriptase inhibitor (NRTI)–containing regimens combined with non-nucleoside reverse transcriptase inhibitors (NNRTIs) in two cases (22%), with protease inhibitors (PIs) in five cases (56%), and with a third nucleoside in two cases (22%). The median duration of ART before study entry was 75 months.

Table 1. Baseline characteristics of the patients.

Patient ID	Age (years)	Gender	Risk Factors	Viral load (copies/ml)	CD4 count (cells/mm^3)	CD8 count (cells/mm^3)	Duration of ART (months)	Current ART regimen	MVC intensification (mg/BID)
MVC 1	49	M	IDU	<50	1169	683	124	ddI+3TC+NVP	300
MVC 2	47	M	MSM	<50	486	673	75	FTC+TDF+ATV/r	150
MVC 3	46	M	IDU	<50	796	650	78	3TC+ABC+ATV	150
MVC 4	46	F	HSx	<50	534	784	57	AZT+3TC+ABC	300
MVC 5	45	M	IDU	<50	728	639	142	AZT+3TC+ABC	300
MVC 6	58	M	MSM	<50	787	1109	144	FTC+TDF+LOP/r	150
MVC 7	32	M	MSM	<50	694	1608	50	FTC+TDF+ATV/r	150
MVC 8	30	M	MSM	<50	589	1064	42	FTC+TDF+EFV	600
MVC 9	30	M	MSM	<50	1241	1414	38	TC+TDF+LOP/r	150
Median [IQR]	46 [31–48]	-	-	<50	711 [547–793]	784 [673–1109]	75 [38–144]	-	-

ddI: didanosine; NVP: nevirapine; FTC: emtricitabine; TDF: tenofovir; ATV/r: atazanavir/boosted with ritonavir; ABC: abacavir; AZT: zidovudine; LOP/r: lopinavir/boosted with ritonavir; EFV: efavirenz; F: Female; M: Male; HSx, Heterosexual; MSM: men who have sex with men.
doi:10.1371/journal.pone.0027864.t001

MVC was well tolerated. Two patients interrupted the study early. Data from these patients were included only until the study interruption. None of them was included in the final analysis at week 48.One patient, who was coinfected with hepatitis C virus, had a significant increase in liver enzymes after the visit at week 12; the other discontinued all antiretroviral drugs due to poor adherence after the visit at week 12. The remaining patients maintained an HIV viral load <50 copies/ml until the end of the study.

Effect of MVC intensification on latent HIV-1 reservoir

The number of latently HIV-1-infected memory CD4$^+$ T cells decreased after 48 weeks of MVC intensification, although the difference was not significant ($p = 0.068$) (figure 2). Three patients showed a reservoir below the limit of detection before receiving MVC (figure 2A), and this remained undetectable after 48 weeks despite a transient increase in two patients. The remaining patients, all of whom had a quantifiable reservoir at study entry, showed a decrease in the IUPM after 48 weeks of intensification (figure 2B) or after 12 weeks of intensification (figure 2C, two patients who discontinued the study after week 12). A mean reduction of 1.82 IUPM was found in the 4 patients with detectable latent reservoir at baseline after 48 weeks of intensification with MVC.

Effect of MVC intensification on residual viremia

The impact on the cellular reservoir was not associated with significant changes in residual viremia. At baseline, 8 of the 9 patients had undetectable residual viremia using the single copy assay. A significant increase in residual viremia was observed after 12 weeks of MVC intensification compared to baseline ($p = 0.027$). Afterwards, residual viremia decreased progressively until the end of the study, with no significant differences compared to baseline ($p = 0.08$, table 2).

Effect of MVC intensification on episomal 2LTR DNA circles

At baseline, episomal 2LTRs DNA circles were undetectable in all patients. However, at week 12 they were detected in 5 patients ($p = 0.037$, compared to baseline) and at week 24 in all 9 patients ($p = 0.012$, compared to baseline). At week 48, episomal 2LTR DNA circles were again undetectable in all patients (table 2).

Effect of MVC intensification on T-cell activation

The level of activation of CD4$^+$ T cells decreased significantly after 12, 24, and 36 weeks of MVC intensification ($p = 0.028$, $p = 0.027$, and $p = 0.028$, respectively), only to increase after 48 weeks, although the difference with baseline was not significant ($p = 0.6$) (figure 3). Compared to HIV-negative subjects, CD4$^+$ T-cell activation at baseline was significantly higher ($p<0.001$), with no significant differences at weeks 12 and 24 ($p = 0.950$ and $p = 0.181$, respectively).

The level of activation of CD8$^+$ T cells also decreased significantly after 12 weeks and until the end of the study ($p = 0.043$, $p = 0.025$, $p = 0.028$, and $p = 0.046$, at weeks 12, 24, 36, and 48, respectively) (figure 3). The level of cell activation at baseline was higher than in the HIV-negative subjects ($p = 0.001$). Only at week 48 was this difference not significant compared to the HIV-negative group ($p = 0.181$).

Effect of MVC intensification on T-cell subsets

No differences in the absolute number or percentage of CD4$^+$ or CD8$^+$ T cells were found during follow-up. A significant increase in CD8$^+$ effector memory and TemRA T cell count was found during the first 24 and 36 weeks after MVC intensification, respectively. Thereafter, the differences compared to baseline were not significant (figure 4). On the other hand, a significant decrease in CD8$^+$ central memory T cells was observed at week 12 ($p = 0.002$). The level of CD8$^+$ naive T cells was similar during follow-up ($p>0.05$ at all time points). No significant differences were observed in any CD4$^+$ T cell subsets during follow-up (data not shown).

The proportion of CD8$^+$ naïve and memory T cells expressing a β7 receptor significantly increased after 12 weeks of intensification ($p = 0.037$ and $p = 0.007$, respectively) (figure 5A and 5B respectively). The expression of β7 receptor also increased in activated CD8$^+$ T cells at week 12 compared to baseline ($p = 0.046$), and a trend was observed during the rest of the follow up (figure 5C).

Effect of MVC intensification on bacterial translocation

A significant increase in sCD14 level was observed after 24, 36 and 48 weeks of intensification with MVC compared to baseline ($p = 0.015$, $p = 0.028$ and $p = 0.028$, respectively) (figure 6). These

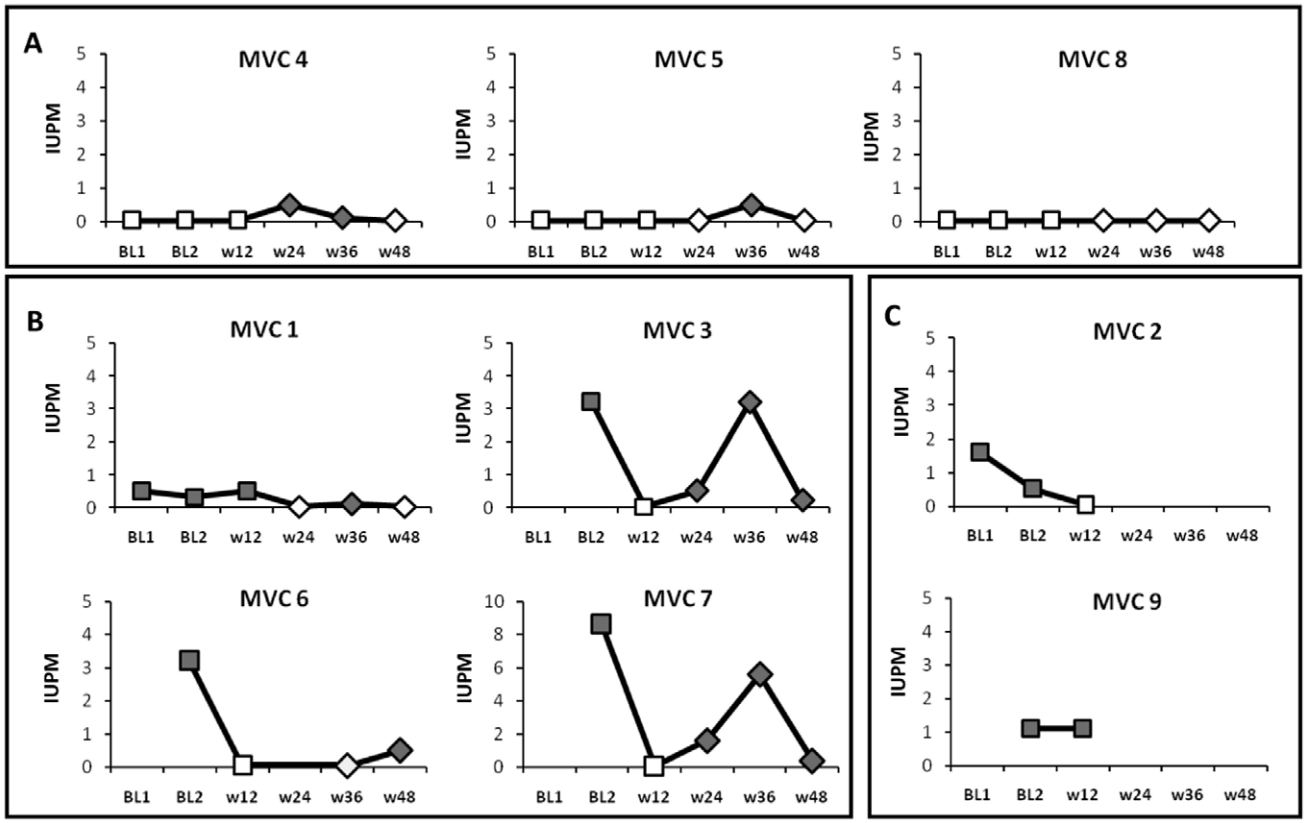

Figure 2. Effect of MVC intensification on HIV-1 latently infected memory CD4$^+$ T cells. (**a**) Patients who had a latent reservoir below the limit of detection at baseline, and remained undetectable after 48 weeks of intensification. (**b**) Patients with quantifiable latent reservoir at baseline, and decreased after 48 weeks of intensification, (**c**) patients with quantifiable latent reservoir at baseline but discontinuated the study after w12. Square symbols: detection limit of 0.12 IUPM. Diamond symbols: detection limit of 0.023 IUPM. Open symbols: under the limit of detection.
doi:10.1371/journal.pone.0027864.g002

levels were significantly lower than those of naive HIV-1-infected patients at all time points. LPS levels were also higher at weeks 24, 36, and 48 of intensification than at baseline ($p = 0.006$, $p = 0.006$, and $p = 0.021$, respectively) (figure 6). The level of LPS at baseline was significantly lower that that of treatment-naive HIV-1-infected patients; during follow-up, these levels were no different from those of the treatment-naive patients.

A significant direct correlation was found between sCD14 levels and LPS levels at baseline ($p = 0.035$, $r = 0.743$) while a similar trend was observed during the rest of the follow up. Furthermore,

Table 2. Effect of MVC intensification on residual viremia and episomal DNA circles.

Patient	SCA					Episomal 2LTR DNA				
ID	Baseline	w12	w24	w36	w48	Baseline	w12	w24	w36	w48
MVC 1	0.3	S.F.	S.F.	<0.3	<0.3	-	+	+	-	-
MVC 2	<0.3	<0.3	N.D.	N.D.	N.D.	-	+	N.D.	N.D.	N.D.
MVC 3	<0.3	SF	<0.5		0.5	-	-	+	-	-
MVC 4	<0.3	9.2	<0.4	<0.3	<0.3	-	-	+	-	-
MVC 5	<0.3	1.0	<0.5	<0.3	2.2	-	-	+	-	-
MVC 6	<0.3	3.0	3.0	1.1	3.3	-	+	+	-	-
MVC 7	<0.6	7.0	<0.5	<0.5	<0.5	-	-	+	+	-
MVC 8	<0.4	0.5	4.6	<0.5	3.7	-	+	+	-	-
MVC 9	<2.0	1.0	N.D.	N.D.	N.D.	-	+	N.D.	N.D.	N.D.
Median	0[0–0]	1[0.5–7]	0[0–3.4]	0[0–0.2]	0.5[0–3.3]					
p *		0.027	0.1	0.6	0.08		0.037	0.012		

SCA: Single copy assay; S.F.: Standard failure; N.D.: Not determined; *: Statistical significance compared to baseline (significance $p<0.05$).
doi:10.1371/journal.pone.0027864.t002

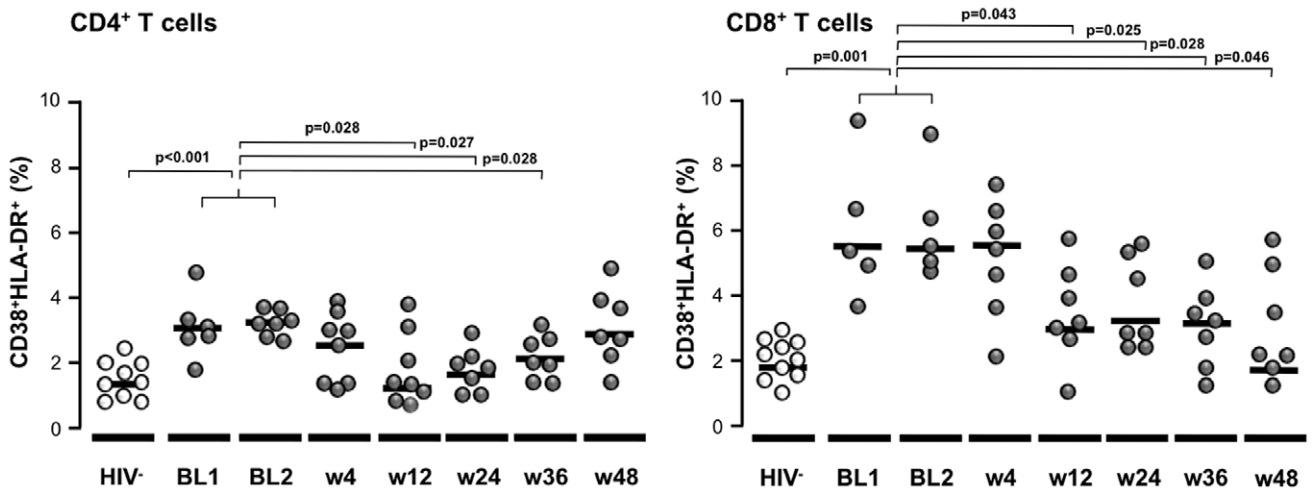

Figure 3. Immune activation during MVC intensification. $CD4^+$ and $CD8^+$ T cell activation of the patients during the follow up is shown. A group of HIV-1 negative individuals are also shown.
doi:10.1371/journal.pone.0027864.g003

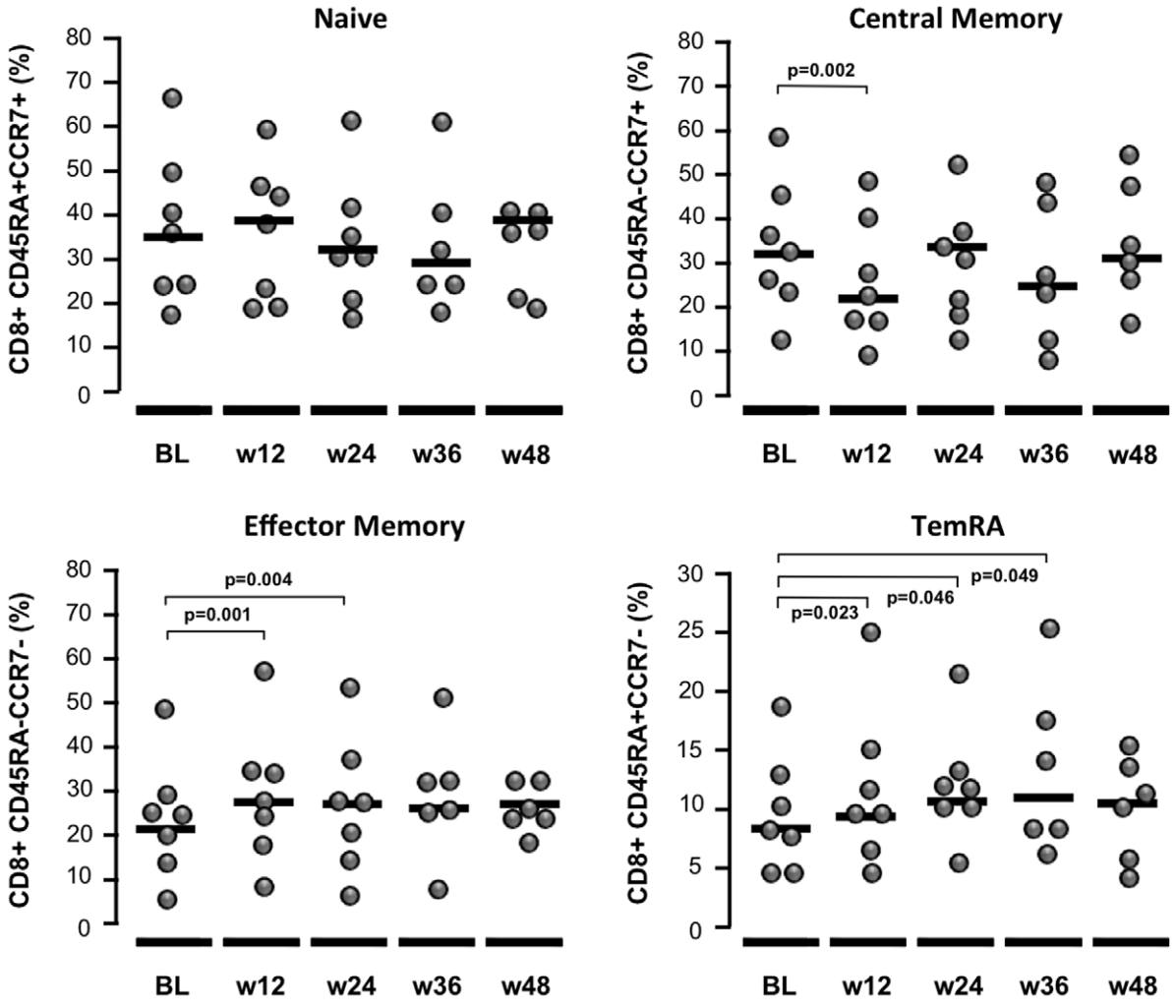

Figure 4. $CD8^+$ T cell subpopulations after MVC intensification. The proportion of naïve, central memory (TCM), effector memory (TEM), and effector memory RA^+ (TemRA) cells is shown.
doi:10.1371/journal.pone.0027864.g004

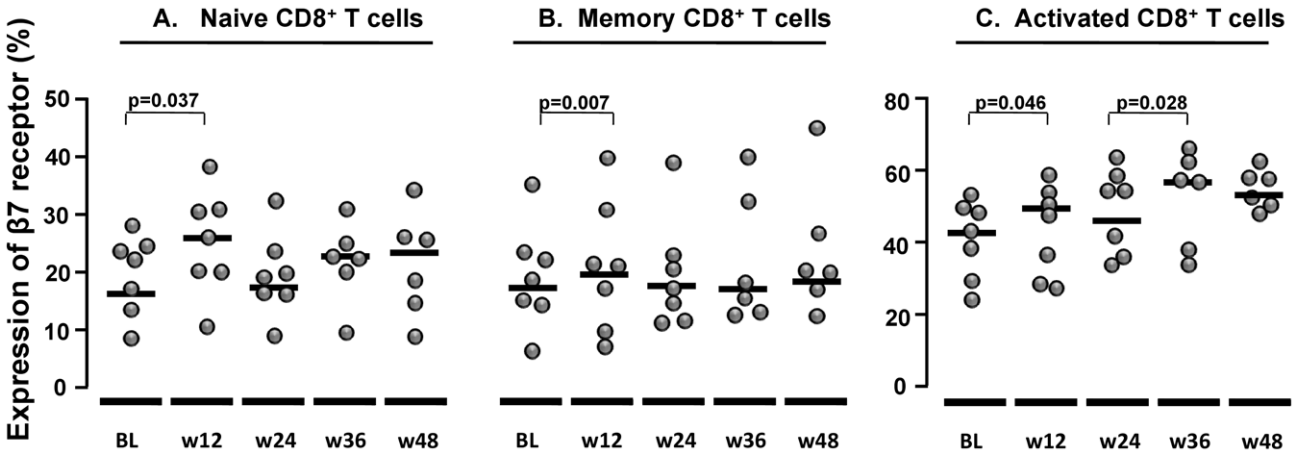

Figure 5. Proportion of CD8$^+$ T cells expressing β7 receptor. A) naïve T cells, B) memory T cells, and C) activated T cells.
doi:10.1371/journal.pone.0027864.g005

the LPS level correlated directly with both the proportion of CD4$^+$ T cells expressing a β7 receptor and the level of naive CD4$^+$ T cells expressing β7 receptor at week 24 ($p = 0.047$, $r = 0.817$). Similarly, the level of LPS at week 36 correlated directly with that of CD8$^+$ effector memory T cells ($p = 0.045$, $r = 0.818$).

Discussion

In this pilot study, intensification of treatment with MVC in patients chronically infected with HIV-1 receiving successful long-term ART was associated with a trend to an accelerated decay in the pool of latently infected CD4$^+$ T cells. No impact on residual viremia was observed.

Intensification of successful antiretroviral therapy has been explored in two different ways. Some studies have evaluated its role for clinical purposes, that is, to achieve better virological control or a greater increase in CD4$^+$ T-cell counts, or, more recently, to reduce immune activation. With the exception of one study that showed a decrease in HIV RNA levels after abacavir intensification, none of the studies has been able to show significant improvements with intensified therapy compared to the standard three-drug regimens [11,12,14–17,41]. As a consequence, most clinicians feel that no further research in this direction is necessary. Other studies have evaluated intensification as a strategy to help to eradicate HIV. The rationale for this approach is that suppression of putative replication could reduce residual viremia and replenishment of cellular reservoirs leading to a decrease in the total latent reservoir [8–18,41]. The results of all the studies are uniform in that no single group has shown a significant reduction in plasma HIV RNA as a result of intensification. This finding could be interpreted as confirmation of the lack of residual viral replication, thus supporting the hypothesis that residual viremia is the result of viral release from stable reservoirs and not the consequence of new rounds of viral replication [5–7]. An alternative hypothesis would be that the effect of intensification takes place mainly outside plasma sites and, therefore, the measurement of plasma viral load only could not detect this effect. Some of the studies that included measurements other than plasma HIV RNA seem to support this hypothesis [42–44].

We observed a reduction in the size of the latent reservoir after intensification of treatment with MVC, although this reduction was not significant, probably due to the small sample size. This finding is consistent with other studies that included patients with different baseline characteristics [22,23], although some authors do not support it [24]. The decline in the latent reservoir that we

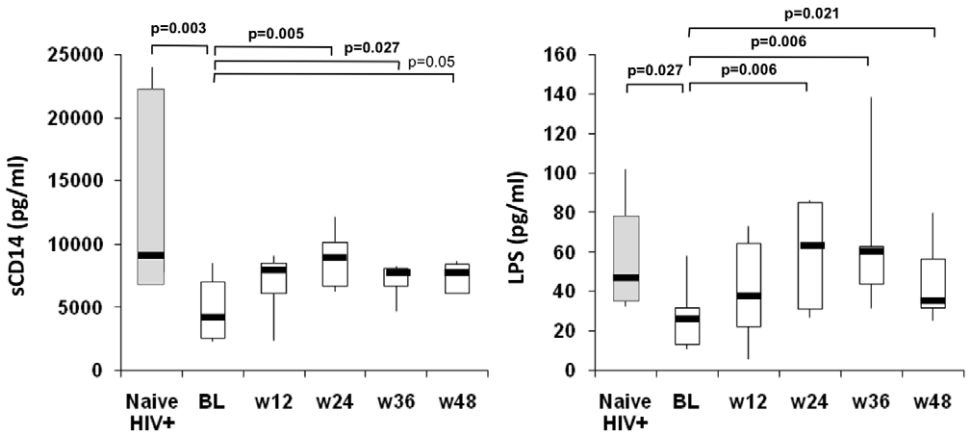

Figure 6. Bacterial translocation. Levels of sCD14 and LPS during the follow up are shown. The levels of a group of HIV-1 positive naïve for ART are also included.
doi:10.1371/journal.pone.0027864.g006

found does not appear to be secondary to a decrease in residual viral replication in plasma, since there were no detectable changes in the measurements of residual viremia. It has been suggested that a dilutional effect could account for the reduction observed, but the lack of an increase in the absolute count and the percentage of both $CD8^+$ and $CD4^+$ T cells strongly argues against this possibility.

Other findings in our study may help explain the effect on the latent reservoir. Unexpectedly, 2LTR DNA circles were transiently detectable during MVC intensification. Due to its mechanism of action, MVC is not expected to increase 2LTR DNA circles, even when inhibiting persistent viral replication. The increase in levels of this marker suggests that treatment with MVC in patients with undetectable viral load may induce viral replication. Preclinical studies showed that MVC had no agonistic activity on activated $CD4^+$ T cells and, therefore, could not induce viral replication [32]. Later reports, however, have shown that the possibility of acting as a partial agonist on CCR5 could be dependent on cell type [45]. Therefore, MVC could activate CCR5 in resting $CD4^+$ T cells and, through intracellular signalling, increase transcriptional activation of the latent virus. As an alternative hypothesis, CCR5 blockade by MVC could lead natural ligands or chemokines to bind to other receptors and induce latent HIV transcriptional activation through distinct signalling pathways. Both hypotheses would be consistent with the reduced number of latently infected memory T cells found in this study, the transient increase in the number of copies of HIV RNA seen in some patients, and the increased bacterial translocation plus migration of $\alpha4\beta7$ cells to the gut. This possibility is further supported by the increased immune activation in intestinal tissue biopsies that was observed in another MVC intensification study [46]. Finally, trafficking of cells to peripheral blood from some other compartment cannot be discarded to explain some of the results, including the increase in 2 LTR circles.

Neither of the above hypotheses can account for the diminished immune activation that we detected. MVC has been shown to decrease immune activation in clinical trials, and it could be assumed that a different mechanism drives the impact on immune activation [47]. The decrease in immune activation does not correlate with a decrease in bacterial translocation, which in fact increased during the study, thus supporting increased viral replication and activation in the intestine resulting from the release of proinflammatory cytokines [48].

The transient increase in TEM and TemRA $CD8^+$ T cell counts parallels the increase in bacterial translocation. These increments are balanced by a transient decrease in central memory $CD8^+$ T cell counts, while naive $CD8^+$ T cell levels remain stable [49]. In addition, the finding of increased $\beta7$ receptor levels on $CD8^+$ T cells and especially on activated CD8 T cells support the idea of migration of these cells to the intestine, where bacterial translocation takes place. This migration of activated cells to the gut could account for the reduced immune activation observed in the periphery. In this sense, immune events detected in peripheral blood may not reflect what takes place in the intestine, as is the case for the huge $CD4^+$ T-cell destruction that occurs in the intestine of the SIV rhesus macaque during acute infection, which is not reflected in lymph nodes or peripheral blood [50].

Our work has a series of limitations. First, it is a pilot study with a small sample size, thus limiting the strength of the conclusions. Second, no control group was included. The study was designed as a proof of concept with no guarantee of any spontaneous effect in the sample. Finally, the methodological tools used for the different measurements and their interpretations are still imperfect.

In summary, our study shows positive effects of MVC intensification on latently HIV-1-infected $CD4^+$ T cells. These effects are not reflected in reduced residual plasma viremia and may be associated with the activity of the drug in an anatomical reservoir, most likely the intestine. The mechanism by which MVC decrease the HIV-1 reservoir is under investigation. The decrease we observed, however, is by no means large enough to eradicate HIV. New therapeutic strategies, probably based on anti-latency drugs, are necessary to eradicate viral reservoirs. In this sense, complete inhibition of viral replication at all sites can be presumed to be necessary before these new treatment strategies can be applied. The exact role of treatment intensification in eradication remains undefined, and a large, comparative trial with adequate methodology is needed to provide a definitive answer to this relevant question.

Supporting Information

Protocol S1 Trial Protocol.
(DOC)

Checklist S1 CONSORT Checklist.
(DOC)

Acknowledgments

We would like to thank Carmen Page, Raquel Lorente and Ester Domínguez for excellent technical assistance; the Radiophysics Departments at the Hospitals Ramón y Cajal and Gregorio Marañón for their help in the irradiation of cells, and the Regional Blood Transfusion Center in Madrid for providing the buffy coats from healthy donors. Specially, we thank all of our patients and their families for their participation in the study.

Author Contributions

Conceived and designed the experiments: CG LD AV BH-N SP. Performed the experiments: CG LD AV BH-N MA NM VD. Analyzed the data: SM CG LD AV BH-N JZ. Contributed reagents/materials/analysis tools: CG LD AV BH-N SP. Wrote the paper: CG LD AV BH-N SM. Approved of the final version to be published: CG LD AV BH-N MA NM VD RR AM FD JC EN MP-E JZ SP EM MM-F SM. Conceived and design the study: SM MM-F EM. Inclusion and patients follow up: RR AM FD JC EN MP-E.

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VIROLOGY JOURNAL

RESEARCH Open Access

Prevalence and resistance mutations of non-B HIV-1 subtypes among immigrants in Southern Spain along the decade 2000-2010

Beatriz de Felipe[1], Pilar Pérez-Romero[1], María Abad-Fernández[2], Felipe Fernandez-Cuenca[3], Francisco J Martinez-Fernandez[1], Mónica Trastoy[1], Rosario del Carmen Mata[1], Luis F López-Cortés[1], Manuel Leal[4], Pompeyo Viciana[1] and Alejandro Vallejo[2,4*]

Abstract

Background: Most of the non-B HIV-1 subtypes are predominant in Sub-Saharan Africa and India although they have been found worldwide. In the last decade, immigration from these areas has increased considerably in Spain. The objective of this study was to evaluate the prevalence of non-B subtypes circulating in a cohort of HIV-1-infected immigrants in Seville, Southern Spain and to identify drug resistance-associated mutations.

Methods: Complete protease and first 220 codons of the reverse transcriptase coding regions were amplified and sequenced by population sequencing. HIV-1 subtypes were determined using Stanford University Drug Resistance Database, and phylogenetic analysis was performed comparing multiple reported sequences. Drug resistance mutations were defined according to the International AIDS Society-USA.

Results: From 2000 to 2010 a total of 1,089 newly diagnosed HIV-1-infected patients were enrolled in our cohort. Of these, 121 were immigrants, of which 98 had ethical approval and informed consent to include in our study. Twenty-nine immigrants (29/98, 29.6%) were infected with non-B subtypes, of which 15/29 (51.7%) were CRF02-AG, mostly from Sub-Saharan Africa, and 2/29 (6.9%) were CRF01-AE from Eastern Europe. A, C, F, J and G subtypes from Eastern Europe, Central-South America and Sub-Saharan Africa were also present. Some others harboured recombinant forms CRF02-AG/CRF01-AE, CRF2-AG/G and F/B, B/C, and K/G, in PR and RT-coding regions. Patients infected with non-B subtypes showed a high frequency of minor protease inhibitor resistance mutations, M36I, L63P, and K20R/I. Only one patient, CRF02_AG, showed major resistance mutation L90M. Major RT inhibitor resistance mutations K70R and A98G were present in one patient with subtype G, L100I in one patient with CRF01_AE, and K103N in another patient with CRF01_AE. Three patients had other mutations such as V118I, E138A and V90I.

Conclusions: The circulation of non-B subtypes has significantly increased in Southern Spain during the last decade, with 29.6% prevalence, in association with demographic changes among immigrants. This could be an issue in the treatment and management of these patients. Resistance mutations have been detected in these patients with a prevalence of 7% among treatment-naïve patients compared with the 21% detected among patients under HAART or during treatment interruption.

Keywords: non-B HIV-1 subtypes, immigrant, Spain, resistance mutation

* Correspondence: alejandro.vallejo@salud.madrid.org
[2]Laboratory of Immunovirology, Infectious Diseases Service, Instituto Ramón y Cajal de Investigaciones Cientificas (IRYCIS), Hospital Universitario Ramón y Cajal, Madrid, Spain
Full list of author information is available at the end of the article

Background

Human immunodeficiency virus type 1 (HIV-1) is the major pathogen responsible for the AIDS pandemic. Several genetic variants can be recognized within HIV-1 group M, including nine subtypes (A through K), at least 43 major circulating recombinants forms (CRFs), and multiple unique recombinant forms (URFs) (http://www.hiv.lanl.gov). The prevalence of HIV-1 subtypes varies greatly depending on the geographic region. Subtype B is predominant in North America and Western Europe, including Spain, although is responsible for only 10% of global infections [1]. Non-B HIV-1 subtypes and its recombinants, such as subtype C, A, CRF01_AE or CRF02_AG, are prevalent in Sub-Saharan Africa, Asia and Eastern Europe [2]. These subtypes cause up to 90% of the 36 million estimated infections, playing an important role in the HIV-1 pandemic [3,4]. Human migration produced in the last decade has contributed to the current spread of non-B subtypes in developed countries [5-7].

The extensive variability of HIV-1 has a potential impact on epidemiology, diagnosis, therapy and prevention of infection. In fact, faster progression to AIDS among individuals infected with non-B subtypes or recombinant variants enhances the importance of identifying these strains [8,9]. In addition, differences in the sensitivity to antiretrovirals in patients infected with non-B subtypes due to the high prevalence of polymorphisms in protease (PR) and/or reverse transcriptase (RT) associated with resistance to antiretroviral therapy has to be taken into account [10-16].

Finally, diagnostic tests, including viral load measurements, might be affected by the diversity of HIV-1 strains [17,18]. Therefore, HIV-1 subtype characterization is becoming an important aspect to adequate clinical management of HIV-1-infected individuals [3]. Sub-Saharan population in Spain has increased in recent years. Around 10-15% of HIV-1-infected immigrants are characterized with non-B subtype during the first medical evaluation [19-21].

Thus, the objective of this study was to analyse the prevalence of non-B subtypes in a cohort of HIV-1-infected immigrants in Southern Spain from 2000 to 2010, and to characterize drug resistance mutations associated to PR and RT.

Methods

Patients

From January 2000 to December 2010 a total of 1,089 new HIV-1-infected patients were included in the dynamic open cohort of the Infectious Diseases Service at the University Hospital Virgen del Rocio located in Seville, Southern Spain. Of these, 121 (11.1%) were HIV-1 infected immigrants. For the present retrospective study, immigrant patients of any nationality with available samples were selected. The study was approved by the Ethical Committee of the Hospital. A total of 98 patients were included and all signed an informed consent.

Laboratory determinations

$CD4^+$ T cell count was determined in fresh samples by flow cytometry. Plasma HIV-1 RNA was measured by quantitative PCR (HIV Monitor™ Test Kit, Roche Molecular System, Hoffman-La Roche, Basel, Switzerland), according to the manufacturer's instructions. This assay has a lower detection limit of 50 HIV-1 RNA copies/ml.

Subtyping and drug resistance profile

For genetic analysis, in patients with detectable viral load, HIV-1 RNA was extracted from plasma using a viral RNA purification kit (Qiagen, Diagnostics, Barcelona, Spain). cDNA was synthesized by avian myeloblastosis virus (AMV) reverse transcriptase. In patients with undetectable viral load and undergoing antiretroviral treatment at the time of the study, HIV-1 proviral DNA from cryopreserved peripheral blood mononuclear cells (PBMCs) isolated from heparinized blood by Ficoll density-gradient centrifugation, were used for DNA extraction (Qiagen DNA blood kit, Diagnostics, Barcelona, Spain), following the manufacturer's instructions.

PCR reactions were carried out in 50 μL final volumes containing 10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0,2 mM of each dNTP, 0.7 units Taq polymerase (Eurotaq, Euroclone, S.p.A, Syziano, Italy), 0.5 μM of each primer, and 1 μg purified DNA. Cycling parameters were 94°C for 5 min followed by 35 cycles of 94°C for 15 s, 55°C for 15 s and 72°C for 30 s, followed by a 10 min hold at 72°C. Identical conditions were used for the nested PCR. Outer primers PRF2 (5'-cagaagagagtctcaggtttggg-3') and PRF5 (5'-tggagtattgtatggattttcagg-3') for the PR-coding region, and 47RV (5'-gtattagtaggacctacacct-3') and Pol18 (5'-agactcacaatatgca-3') for the RT-coding region, were used. Inner primers Pol10 (5'-ccctcaagggcaggagc-3') and Pol14 (5'-gggccatccattcctgg-3') for the PR-coding region, and A35 (5'-ttggttgcactttaaattttcccattagtcctatt-3') and Lp2 (5'-atcaggatgagattcataacccatcca-3') for RT-coding region, were used. A fragment of 450 bp encompassing the entire PR-coding region and a fragment of 670 bp that includes the first 220 codons of the RT-coding region were generated by nested PCR.

Sequencing of PCR purified amplicons was performed using Applied Biosystems 310 Sequencer and BigDye deoxy terminator procedure as specified by the manufacturer.

Subtyping of the PR and RT sequences was determined using the Stanford University Drug Resistance

Database (http://hivdb.stanford.edu). Afterwards, the PR and RT sequences were aligned together with other HIV-1 group M reported sequences available in the GenBank by computer software CLUSTAL W. Phylogenetic trees were generated using the Neighbour-Joining method and bootstrap re-sampling of multiple alignments (1,000 data sets), included in the CLC DNA Workbench software, was employed to test the tree robustness. Drug resistance profiles in PR and RT-coding regions were defined according to the International AIDS Society-USA.

Statistical Analysis

Continuous variables are shown as median [interquartile range (IQR)], and categorical variables as number of cases (percentage). Chi square test were used to analyze differences between categorical variables. The statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS 16.0, Chicago, Illinois, USA).

Sequence Accession Number

GenBank accession numbers for consensus sequences: AM000054 (A); K03455 (B); AY563172 (C); DQ189088 (F); AY017457 (G); EF614151 (J); AJ249239 (K); FM252023 (CRF01_AE); FN557324 (CRF02_AG). Protease sequences: JF338626-JF338629 (PR_SE10-PR_SE13), JF338630-JF338633 (PR_SE15-PR_SE18), JF338634-JF338641 (PR_SE20-PR_SE27), JF338642-JF338653 (PR_SE29-PR_SE40), JF338653 (PR_SE40), JF338654-JF338658 (PR_SE42-PR_SE46), JF338659-JF338663 (PR_SE48-PR_SE52), JF338664-JF338686 (PR_SE54-PR_SE76), JF338687-JF338690 (PR_SE78-PR_SE81), JF338691-JF338716 (PR_SE90-PR_SE115). RT sequences: JF338717-JF338719 (RT_SE10-RT_SE12), JF338720-JF338731 (RT_SE14-RT_SE25), JF338732-JF338744 (RT_SE27-RT_SE39), JF338745-JF338761 (RT_SE41-RT_SE57), JF338762-JF338780 (RT_SE59-RT_SE77), JF338781 (RT_SE79), JF338782-JF338807 (RT_SE90-RT_SE115).

Results and discussion

Patients' characteristics

Baseline characteristics of the 98 immigrant individuals studied are summarized in Table 1. The study population was grouped into seven categories according to their home countries: Central-South America (Argentina, Peru, Brazil, Colombia, Cuba, Bolivia, Ecuador, El Salvador, Guatemala, Panama, Paraguay, Dominican Republic and Venezuela), Western Europe (France, Italy, Portugal, Belgium, Germany and Ireland), Sub-Saharan Africa (Nigeria, Cameroon, Angola, Uganda, Sierra Leone, Guinea, Cote d'Ivoire), Northern Africa (Morocco), Eastern Europe (Romania, Ukraine and Russia), North America (United States), and Asia (India). The largest immigrant group in Southern Spain was from Central-South America (47/98, 47.9%). In most cases, these individuals were infected with HIV-1 before moving to Spain.

Table 1 Characteristics of the 98 HIV-1-infected immigrant individuals studied

Male gender (%)	68 (69.4)
Age (years)	35 [30-38]
$CD4^+$ count (cell/mm^3)	321 [212-491]
HIV-1 viral load (log_{10}IU/mL)	4.28 [1.6-4.8]
Naïve ART patients (%)	62 (63,2)
Time in treatment (months)	25.1 [14.2-48.1]
Risk factor (%)	
Heterosexual	41 (41.8)
Bisexual	42 (42,8)
Injecting drug users	7 (7.1)
Others	8 (8.1)
Geographical Region (%)	
Central-South America	47 (47.9)
Western Europe	14 (14,3)
Sub-Saharan Africa	19 (19.4)
Northern Africa	5 (5.1)
Eastern Europe	10 (10.2)
North America	1 (1)
Asia	1 (1)
Not determined	1 (1)

The proportion of immigrants in our cohort of HIV-1-infected patients from 2000 to 2010 is shown in Figure 1. The proportion has been rising year by year from 2.1% in 2000 to around 12% in 2010. Notably, there has been a marked progressive increase up to 2008, which exceeded 20% (Figure 1). Regarding risk behaviour, injecting drug use was the most common route of transmission among native Spanish patients (around 40% vs. 7% among immigrants, $p < 0.001$ Chi Square test), while sexual transmission (hetero plus homosexual) was predominant (80% vs. 42% among native Spanish patients, $p < 0.001$ Chi Square test) among immigrants (Figure 1).

Subtype analysis

Both PR and RT sequences were obtained for 87 patients, while only PR or RT sequences were obtained for seven and four individuals, respectively. Twenty-nine of the overall sequences analyzed belonged to non-B subtypes (29.6%, Table 2). These included A, C, F, G, J, and K subtypes, and recombinant forms, such as CRF02_AG, as well as CRF01_AE. Besides, seven patients harbored different subtypes for each gene, including recombinants B/C, F/B, K/G, CRF02_AG/CRF01_AE, and CRF02_AG/G in PR and RT respectively. According to the geographical origin, the non-B

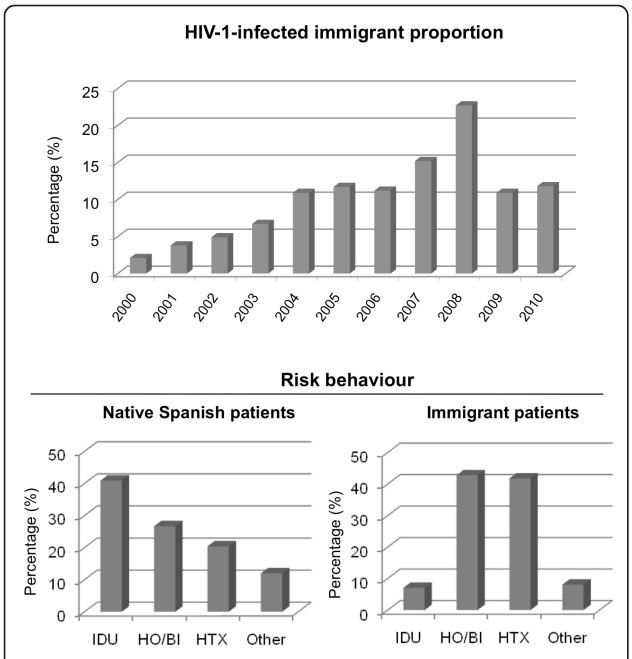

Figure 1 Proportion of HIV-1-infected immigrants from 2000 to 2010 in Southern Spain, and comparison of risk behavior between native Spanish and immigrant patients. IDU, injecting drug users; HO/BI, homosexual/bisexual; HTX, heterosexual.

subtype was detected in a total of 79% (15/19) of Sub-Saharan Africans, followed by 60% Eastern Europeans (6/10), 20% Northern Africans (1/5), 10.6% Central-South Americans (5/47) and 7.1% Western Europeans (1/14).

Phylogenetic analysis

Phylogenetic trees were generated based on PR and RT sequences and included reference sequences representing different HIV-1 subtypes. All sequences clustered within the correspondent subtype, which was concordant with the data provided by Stanford University (Figure 2). Only three of the RT sequences: SE16, SE113, and SE11 corresponding to subtype G were located slightly apart from this subtype although within the same branch. Noteworthy, no sub-clusters were formed based on the geographical origin of the patients. Sequences PR_SE15, PR_SE25, PR_SE51, PR_SE55, RT_SE14, RT_SE15, RT_SE27, RT_SE29, RT_SE45, RT_SE79, were excluded from the phylogenetic analysis due to the limited length that could have influenced the analysis.

Prevalence of resistance mutations

All patients infected with non-B HIV-1 showed at least one polymorphic mutation in PR (Table 2). Most of these mutations have been frequently found and reported amongst non-B HIV-1 subtypes [14,16]. The M36I mutation in protease was observed in all patients, whilst K20I/R, L63P, V77I and L10V/I mutations were present in 75%, 17.8%, 10.7% and 7.1% of patients, respectively. However, only patient SE59 had the major mutation L90M (1/28; 3.5%). This patient was infected with the recombinant form CRF02_AG and had been under HAART for at least two years.

On the contrary, only six patients showed RT mutations. Three of them (SE37, SE34 and SE60) showed major RT mutations (3/28, 10.7%, table 2). Patient SE37, naïve for antiretroviral treatment, had A98G and K70R major mutations associated with resistance to non-nucleoside RT inhibitors (NNRTI), and nucleoside RT inhibitors (NRTI), respectively. Patient SE34, in treatment interruption, had L100I mutation associated with NNRTI resistance, and finally patient SE60, receiving HAART, had the NNRTI resistance mutation K103N. Minor mutations or polymorphisms in RT were observed in patients SE71, SE91 and SE111 i.e.: V118I, E138A, and V90I mutations respectively.

Discussion

This study evaluates the prevalence of HIV-1 subtypes and PR and RT drug resistance mutations among HIV-1-infected immigrants living in Southern Spain. Immigration had been gradually increasing since 2000, with a peak of 22% in 2008. Since then, it has been slowly decreasing to 11.8% in 2010. The largest immigrant group was form Central-South America (47.9%) most of them infected with the HIV-1 B subtype. However, up to 25% of immigrants were from countries where HIV-1 non-B subtypes are predominant, as Sub-Saharan Africa with 79% of HIV-1 non-B subtypes (subtypes A, G, J or CRF02_AG), or Eastern Europe with 60% (subtypes A, F, CRF02_AG or CRF01_AE). This marked increase in individuals infected with non-B HIV-1 subtype in our society may have a direct impact on the spread of these subtypes among Spanish native individuals in addition to the changes in appropriate treatment regimes due to differences in genetic sequences amongst the different HIV-1 strains. These results are similar to those found in studies conducted in other regions in Spain, Madrid and Canary Islands [19,20,22].

Previous studies reported a high prevalence of minor resistance mutations in naïve patients infected with HIV-1 non-B subtype that could facilitate the emergence of major mutations [16,23,24]. However, the clinical relevance of minor mutations is unclear. Our results indicated that patients infected with HIV-1 non-B subtypes showed a high frequency of minor PR mutations (polymorphisms) as M36I, L63P, and K20R/I, in contrast to the low proportion of major resistance mutations. In fact, only one patient, subtype CRF02_AG, had L90M mutation probably as a result of receiving antiretroviral

Table 2 Characteristics of the immigrant patients infected with HIV-1 non-B subtypes (N = 29)

Patient code	Origin	Treatment	Subtype in PR gene	Protease inhibitor resistance mutations	Subtype in RT gene	RT inhibitor resistance mutations
SE58	EE	HAART	A	M36I, V77I	N.D.	N.D.
SE115	CSA	Naive	B	M36I	C	None
SE71	CSA	HAART	C	M36I	C	V118I
SE73	EE	Naive	F	L10V, K20R, M36I	F	None
SE43	CSA	HAART	F	M36I	B	None
SE99	EE	Naive	F	K20R, M36I	F	None
SE108	CSA	Naive	F	K20R, M36I	F	None
SE37	SSA	Naive	G	K20I, M36I	G	***K70R, A98G***
SE103	SSA	Naive	G	K20I, M36I	G	None
SE31	SSA	Naive	J	K20I, M36I, V77I	J	None
SE91	SSA	Naive	K	M36I, L63P	G	E138A
SE11	SSA	HAART	CRF02_AG	K20I, M36I, L63P	G	None
SE16	SSA	TI	CRF02_AG	K20I, M36I	G	None
SE113	N.D.	Naive	CRF02_AG	K20I, M36I	G	None
SE66	WE	HAART	CRF02_AG	K20I, M36I	CRF02_AG	None
SE59	NAF	HAART	CRF02_AG	K20I, M36I, ***L90M***	CRF02_AG	None
SE67	SSA	HAART	CRF02_AG	K20I, M36I, L63P	CRF02_AG	None
SE49	SSA	Naive	CRF02_AG	K20I, M36I, L63P	CRF02_AG	None
SE50	SSA	TI	CRF02_AG	K20I, M36I, L63P, V77I	CRF02_AG	None
SE33	SSA	TI	CRF02_AG	L10V, K20I, M36I	CRF02_AG	None
SE36	CSA	HAART	CRF02_AG	K20I, M36I	CRF02_AG	None
SE101	SSA	Naive	CRF02_AG	K20I, M36I	CRF02_AG	None
SE110	EE	Naive	CRF02_AG	K20I, M36I	CRF02_AG	None
SE111	SSA	HAART	CRF02_AG	K20I, M36I	CRF02_AG	90I
SE34	SSA	TI	CRF02_AG	K20I, M36I	CRF01_AE	***L100I***
SE96	SSA	Naive	CRF02_AG	K20I, M36I	CRF02_AG	None
SE62	EE	Naive	CRF01_AE	M36I	CRF01_AE	None
SE60	EE	HAART	CRF01_AE	M36I	CRF01_AE	***K103N***
SE19	SSA	HAART	N.D	N.D.	CRF02_AG	None

Protease and RT inhibitor resistance mutations.

CSA, Central-South America; SSA, Sub-Sahara Africa; EE, Eastern Europe; WE, Western Europe; NAF, Northern Africa. HAART, Highly Active Antiretroviral Therapy. TI, treatment interruption. Bold, major resistance. N.D., not determined.

therapy during two years (1/28, 3.5%). No difference between the number of patients with major or minor RT mutations (3/28, 10.7% in both cases) was found. Nevertheless, the proportion of patients with major RT mutations was higher compared to patients with major PR mutations.

It is of note that one treatment naïve patient, with G subtype, showed major RT mutations associated with resistance to NRTI (K70R) and NNRTI (A98G). This patient might have acquired these resistance mutations at the moment of infection. One limitation of our study was the impossibility to test plasma samples of antiretroviral-experienced patients before the initiation of antiretroviral therapy to determine primary resistance mutations.

An increasing effort has been done to characterize the difference in antiretroviral therapy responses within HIV-1 non-B subtypes. Although clinical evidence remains limited, response to antiretroviral therapy does not appear to differ significantly among subtypes [3]. In our study, 29 patients showed HIV-1 non-B subtype in PR and/or RT. Eleven of them were under antiretroviral therapy and had good virological and immunological responses. Nevertheless, larger cohorts are necessary to confirm our results. On the other hand, 14 immigrants infected by HIV-1 non-B subtype were naïve for antiretroviral therapy. Most of them with viral load around 4 log HIV-1 copies/ml, and two patients had undetectable viral load for a long period of time (one of them at least for one year). As previous studies have described the failure of laboratory techniques to detect HIV-1 non-B subtypes [25-27], however it would be interesting to establish whether the failure of the diagnosis may be due to a phenotype characteristic of viral load controller in this population.

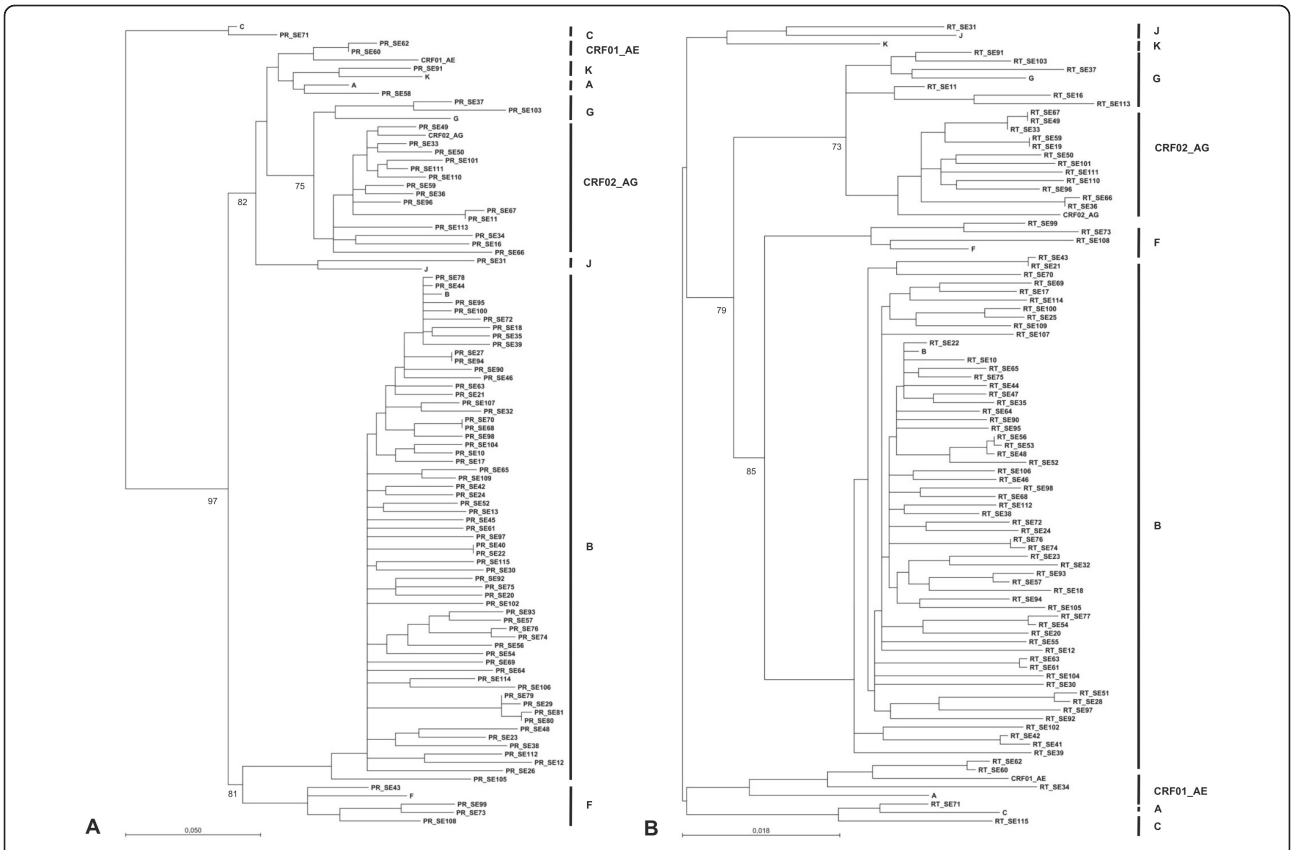

Figure 2 Phylogenetic analyses derived from protease (A) and reverse transcriptase (B) sequences from HIV-1-infected immigrants from Southern Spain. Trees were constructed using the neighbor-joining method. Consensus sequences of subtypes A, B, C, F, G, J, K, and circulating recombinant forms CRF02_AG and CRF02_AE are included. Bootstrap values at nodes are shown as percentage of 1000 resamplings (only values greater than 70% are shown). Central-South America (CSA); Sub-Saharan Africa (SSA); Western Europe (WE); Northern Africa (NAF); Eastern Europe (EE); North America (NAM); not determined (ND).

Conclusions

The circulation of HIV-1 non-B subtypes among immigrants, with a prevalence of 29.6%, has significantly increased in Southern Spain in the last decade, in association with demographic changes. Spread of CRF02-AG, G and F subtypes, and also some recombinant forms appear to predominate, which is consistent with previous findings [19,20,22,28]. This fact may affect the treatment and management of these patients, and the spread of these subtypes among native Spanish population. Furthermore, a low percentage of the immigrants with the non-B subtypes had major resistance mutations. Prevalence of these resistance mutations varies from 7% among treatment-naïve patients to 21% among patients under HAART or during treatment interruption.

Acknowledgements

We thank all the participants of this study for their cooperation. This study was supported in part by Fondo de Investigación Sanitaria (FIS) 07/0070, Redes Temáticas de Investigación Cooperativa en Salud (RETICS) Red de Investigación en SIDA (RIS) RD06/0006/0021 and RD/06/0006/0034, and Servicio Andaluz de Salud, Consejería de Salud 0407/2007.

Author details

[1]Infectious Diseases Service, IBIS, Hospital Univeritario Virgen del Rocio, Seville, Spain. [2]Laboratory of Immunovirology, Infectious Diseases Service, Instituto Ramón y Cajal de Investigaciones Cientificas (IRYCIS), Hospital Universitario Ramón y Cajal, Madrid, Spain. [3]Microbiology Service Hospital Virgen Macarena Seville, Spain. [4]Laboratory of Immunovirology, Infectious Diseases Service, IBIS, Virgen del Rocío University Hospital, Seville, Spain.

Authors' contributions

BF made most of the amplifications, sequencing, drug resistance profiles, sequence alignments and wrote a first draft of the manuscript. PPR, FJMF and FFC made some amplifications and sequencing. MAF helped in alignments of the sequences and revising the manuscript. MT and RCM helped in the management of the patients and collecting samples. LLC, ML, and PV recruited all the patients. AV designed and coordinated the study, helped in the alignment of the sequences and finalized the manuscript in its final form. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 14 June 2011 Accepted: 26 August 2011
Published: 26 August 2011

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doi:10.1186/1743-422X-8-416
Cite this article as: de Felipe *et al.*: Prevalence and resistance mutations of non-B HIV-1 subtypes among immigrants in Southern Spain along the decade 2000-2010. *Virology Journal* 2011 **8**:416.

APPENDIX 2: Articles submitted

- **Abad-Fernández M,** Gutiérrez C, Madrid N, Hernández-Novoa B, Díaz L, Muñoz-Fernández MA, Moreno S, Vallejo A. Gut homing β7 receptor and microbial translocation during antiretroviral treatment de-intensification of maraviroc and raltegravir in suppressed HIV-1-infected patients. Submitted to JAIDS.

- **Abad-Fernández M**, Cabrera C, García E, Vallejo A. Transient increment of HTLV-2 proviral load in HIV-1-co-infected patients during treatment intensification with raltegravir. Submitted to JAIDS.

- Casado JL, **Abad-Fernández M**, Moreno S, Pérez-Elías MJ, Moreno A, Bernardino I, Dronda F, Vallejo A. High levels of immune activation, inflammation, bacterial translocation and immunosenescence in Leishmania/HIV-1 co-infection. Submitted to Antivir Res.

- Vallejo A, **Abad-Fernández M**, Moreno S, Moreno A, Pérez-Elías MJ, Dronda F, Casado JL. High levels of CD4+ CTLA-4+ Treg cells and CCR5 density on T cells in HIV-1-infected patients with visceral leishmaniasis enable both persistence and relapses of the parasite. Submitted to Infect Immun.

Elsevier Editorial System(tm) for Journal of Infection

Manuscript Draft
Manuscript Number: YJINF-S-13-00774

Title: GUT HOMING B7 RECEPTOR AND MICROBIAL TRANSLOCATION IN SUPPRESSED HIV-1-INFECTED PATIENTS AFTER TREATMENT DE-INTENSIFICATION

Article Type: Full Length Article

Section/Category: Rest of the World Submissions

Keywords: HIV-1; maraviroc; raltegravir; gut homing receptor; microbial translocation

Corresponding Author: Dr. Alejandro Vallejo, Ph.D.

Corresponding Author's Institution: Hospital Ramon y Cajal

First Author: María Abad-Fernández

Order of Authors: María Abad-Fernández; Carolina Gutiérrez; Nadia Madrid; Beatriz Hernández-Novoa; Laura Díaz; María A Muñóz-Fernández; Santiago Moreno; Alejandro Vallejo, Ph.D.

Abstract:
Objectives: Mucosal damage associated to HIV-1 infection leads to microbial translocation despite successful antiretroviral treatment. Our objective was to analyze the gut-homing β7 receptor and microbial translocation on peripheral T cells after treatment de-intensification in the context of two clinical trials.
Methods: Fifteen long-term suppressed HIV-1-infected patients, seven intensified with maraviroc and eight with raltegravir, were included. Samples at baseline, week 48 of intensification, and weeks 12 and 24 after de-intensification were analyzed for soluble CD14, LPS, LPS-binding protein, gut-homing β7 receptor and T cell subsets.
Results: Our results showed a significant increase of both microbial translocation and gut-homing β7 receptor on activated CD8+ T cells was found during maraviroc intensification that decreased after deintensification. Moreover, significant correlations between activated β7+ T cells and LPS levels were found after intensification with maraviroc ($p=0.036$ and $p=0.010$, respectively), and were lost during de-intensification. In contrast, microbial translocation was stable during raltegravir intensification, with the exception of decreased LPS levels and activated CD4+β7+ T cells that reverted after deintensification.
Conclusions: De-intensification reverted the opposite effect of maraviroc and raltegravir after 24 weeks matching both gut-homing receptor and microbial translocation levels at baseline levels.

GUT HOMING B7 RECEPTOR AND MICROBIAL TRANSLOCATION IN SUPPRESSED HIV-1-INFECTED PATIENTS AFTER TREATMENT DE-INTENSIFICATION

María Abad-Fernández, B.Sc. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain.

Carolina Gutiérrez, Ph.D. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain.

Nadia Madrid, B.Sc. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain.

Beatriz Hernández-Novoa, Ph.D. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain.

Laura Díaz, Ph.D. Immunobiology Laboratory, Hospital General Universitario Gregorio Marañón, Doctor Esquerdo 46, 28007 Madrid, Spain.

María A. Muñoz-Fernández, Ph.D. Immunobiology Laboratory, Hospital General Universitario Gregorio Marañón, Doctor Esquerdo 46, 28007 Madrid, Spain.

Santiago Moreno, MD, Ph.D. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain.

Alejandro Vallejo, Ph.D. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain.

Running Head: Treatment de-intensification in HIV+ patients

Corresponding author: Alejandro Vallejo, Ph.D. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034 Madrid, Spain. Tel: +34 913368711; email: alejandro.vallejo@salud.madrid.org

Conflicts of Interest
The authors have no conflicts of interest

Source of Funding
This work was supported in part by the Spanish AIDS Network "Red Temática Cooperativa de Investigación en SIDA (RD06/0006)**,** the Spanish National of Health, "Instituto de Salud Carlos III", grants (FIS-PI080958 and CP08/00046), and by the Foundation of Investigation and prevention of AIDS (FIPSE-36-0844/09).

INTRODUCTION

A high percentage of $CCR5^+$-$CD4^+$ T cells, susceptible to HIV-1 infection, are localized in the gut mucosa,[1,2] which is believed to be an important viral reservoir in HIV-1 infected individuals.[3-7] Intestinal damage produced in acute HIV-1 infection leads to translocation of microbial products from the intestinal lumen into the systemic circulation, and persists despite successful antiretroviral treatment,[8-13] supported by the detection of increased levels of lipopolysaccharide (LPS) and soluble CD14 (sCD14) in plasma of HIV-1 infected individuals undergoing antiretroviral therapy.[14-17] Elevated circulating LPS levels in HIV-1-infected patients bind the CD14-toll-like receptor 4 (TLR 4) complex through plasma LPS binding protein (LBP) and trigger the activation of monocytes and macrophages that increase secretion of sCD14 and proinflammatory cytokines.[18-22]

Microbial translocation and persistent low-level viremia in HIV-1 infected patients despite successful treatment have been proposed as triggering mechanisms of increased immune activation detected in HIV-1 infection.[9,23-26] The ongoing and persistent low-level productive viral replication was expected to be reduced using intensification therapy with the new HIV-1 antiviral agents emerged, such as CCR5-receptor antagonists (maraviroc) and integrase inhibitors (raltegravir). Unfortunately, this strategy failed to show any effect in reducing peripheral low level HIV-1 viremia, although it is efficient in reducing HIV-1 replication in gut mucosa.[27-32]

The severe depletion of $CD4^+$ T cells in the gut mucosa during HIV-1 infection is restored much slower than in peripheral blood,[33-36] and studies using simian models have shown that T cell restoration in gut involved trafficking of $CD4^+$ T cells from periphery to gut mucosa.[37,38] Our previous studies of treatment intensification showed different behaviors of microbial translocation and the expression of gut homing β7 receptor on T cell subsets depending on the drug used, i.e. while raltegravir intensification reduced the levels of either microbial translocation and gut homing β7 receptor on peripheral T cells, the intensification with maravicoc led to an increase in these two parameters.[39,40] Hence, we wanted to further evaluate the impact of the treatment de-intensification on the alteration showed during treatment intensification on microbial translocation, measured by the quantification of plasma LPS, sCD14, and LBP levels, and the expression of the gut homing receptor β7 on T cells. The second objective was to analyze the association of microbial translocation with T cells bearing β7 receptor, and the dynamic of T cell subpopulations.

MATERIALS AND METHODS

Patients

This study was performed within two pilot open-label phase II clinical trials (ClinicalTrials.com NCT00795444 and NCT00807443) conducted at the Hospital Ramón y Cajal in Madrid, Spain, in order to evaluate the effect of treatment intensification with a CCR5-receptor antagonist (Maraviroc, Pfizer, Inc.) and a integrase inhibitor (Raltegravir, Merck Sharp and Dhome, Whitehouse Station, New Jersey, USA) on the HIV-1 latent reservoir. The initial intensification trial was amended to include a follow-up period of 24 weeks after de-intensification of maraviroc and raltegravir. Immunological and virological parameters were assessed at baseline, after 48 weeks of treatment intensification, and after 12 and 24 weeks of maraviroc and raltegravir de-intensification. Results of the intensification trials have been published elsewhere.[39,40]

Here we present the effect of de-intensification on measurements of microbial translocation, immune activation, and the dynamics of T cells subpopulations.

For this treatment de-intensification study, a total of 15 HIV-1-infected adult individuals recruited for the above clinical trials, initially with $CD4^+$ T cell count above 350 cells/mm^3 and undetectable plasma viral load (below 40 copies HIV-1 RNA/mL) for at least two years, remained in the trials and were analyzed. Seven patients discontinued maraviroc intensification, while other eight patients discontinued raltegravir intensification. None of them had had previous experience with any of these drugs.

The study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our local Independent Ethics Committee (Hospital Ramon y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

Flow cytometry

Fresh EDTA-anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell subpopulations with the following antibody combination: CD3-allophycocyanin (APC)-Cy7, CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin (PE)-Cy7, CD38-phycoerythrin (PE), and HLA-DR-allophycocyanin (APC), CD45RA-phycoerythrin (PE), CCR7-allophycocyanin (APC), and β7-APC. Antibodies were from BD (Becton Dickinson, Franklin Lakes, NJ, USA), and an unstained control was performed for all samples. Briefly, 100 µL of blood were lysed with FACS Lysing solution (BD) for 30 min at room temperature, incubated with the antibodies during 20 min at 4℃, washed and resuspended in PBS containin g 1% azida. Cells were analyzed in a Gallios flow cytometer (Beckman-Coulter, Inc, Brea CA, USA). At least 30000 $CD3^+$ T cells were collected for each sample and analyzed with Kaluza software (Beckman-Coulter, Inc) initially gating lymphocytes according to morphological parameters. T cell immune activation was analyzed by the co-expression of CD38 and HLA-DR, while T-cell subsets were defined as follows: naive cells, $CD3^+CD4^+(CD8^+)CD45RA^+CCR7^+$; effector memory T cells (TEM) $CD3^+CD4^+(CD8^+)CD45RA^-CCR7^-$; central memory T cells (TCM) $CD3^+CD4^+(CD8^+)CD45RA^-CCR7^+$; and transitional memory T cells (TEMRA) $CD3^+CD4^+(CD8^+)CD45RA^+CCR7^-$. The gating was sustained among different time points.

Measure of soluble factors

Microbial translocation was quantified in plasma by three commercial kit assays according to the manufacturer´s protocol. Plasma LPS was measured using QCL-1000 Limulus Amebocyte Lysate (Lonza®, Basel, Switzerland), plasma sCD14 was quantified using the Quantikine® Human sCD14 Immunoassay (R&D Systems, Minneapolis, MN, USA) and plasma LBP was measured by LBP soluble ELISA kit (Enzo® Life Sciences, Farmingdale, NY, USA). All the samples were run in duplicate.

Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and categorical variables as percentages. Mann-Whitney U test was used to compare medians between groups. Pearson´s χ^2 was used to compare differences in proportions between groups considering the continuity correction or the Fisher´s exact

test, as appropriate. The non-parametric Wilcoxon paired test was used to compare medians during the follow up. Correlations were analyzed with Spearman´s rank test. Statistical analysis was performed using SPSS software 21.0 (IBM, Chicago, IL, USA). Graphs were generated using GraphPad Prism 5.01 (La Jolla, CA, USA).

RESULTS

Effect of the treatment de-intensification on microbial translocation

Patients´ immunovirologic characteristics at baseline (before treatment intensification) as well as the characteristics before treatment de-intensification (after 48 weeks of treatment intensification) are shown in table 1. At 48 weeks of intensification, two patients of the maraviroc clinical trial and one patient of the raltegravir clinical trial were lost for the study. During the treatment de-intensification, one patient of the raltegravir clinical trial decided to drop out the study.

The effect of the treatment de-intensification on microbial translocation was different in patients who were intensified with maraviroc or with raltegravir. Overall, microbial translocation significantly increased during treatment intensification and then progressively decreased after drug discontinuation in patients who received maraviroc (Figure 1A). Hence, the levels of microbial translocation at the end of the clinical trial were not different from those levels found either at week 48 of intensification (LPS p=0.310, sCD14 p=0.892, and LBP p=0.866) or baseline (LPS p=0.091, and LBP p=0.846). Only sCD14 levels remained significantly increased compared to baseline (p=0.043).

In contrast, while the patients who were intensified with raltegravir had stable sCD14 and LBP levels during the treatment de-intensification, the levels of LPS significantly increased at week 12 and 24 of de-intensification when compared to week 48 of intensification (p=0.025 and p=0.036, respectively) (Figure 1B). Nevertheless, the significant increase in the levels of LPS during treatment de-intensification was a consequence of the significant decrease of these levels observed during intensification compared to baseline (p=0.008). Thus, at the end of the study LPS levels were not significantly different from baseline levels (before intensification).

Expression of gut homing β7 receptor on peripheral activated T cells during the treatment de-intensification

Again, the effect of intensification on the expression of gut homing β7 receptor on activated T cells was the opposite depending on the intensification treatment received. In patients who received maraviroc intensification, there was a significant decrease on activated $CD8^+\beta7^+$ T cells at week 24 of treatment de-intensification (p=0.028) compared to week 48 of intensification. However, activated $CD8^+\beta7^+$ T cells remained significantly higher at weeks 12 and 24 after treatment de-intensification compared to baseline (p=0.042 and p=0.043, respectively). In parallel, as observed with microbial translocation, the level of activated $CD8^+$ T cells expressing β7 receptor increased during intensification and decreased during maraviroc de-intensification. Nevertheless, no changes in activated $CD4^+\beta7^+$ T cells were observed during the analyzed follow-up (Figure 2A).

In contrast, in patients intensified with raltegravir, activated $CD8^+\beta7^+$ T cells were stable during the treatment de-intensification follow up, and no changes were observed compared to baseline (before intensification) (Figure 2B). However, activated $CD4^+\beta7^+$ T cells significantly increased at weeks 12 and 24 during raltegravir de-intensification compared to week 48 of intensification (p=0.012 and p=0.017, respectively). This increase was again a consequence of the decrease observed during the intensification period, although not significant (p=0.066).

Association between microbial translocation and gut homing β7 receptor

No correlation was observed between microbial translocation and gut homing β7 receptor at baseline, before intensification. However, in patients who received maraviroc, a correlation was found during the treatment intensification (week 48) between both activated $CD4^+\beta7^+$ and $CD8^+\beta7^+$ T cells, and microbial translocation that was significant when LPS levels were analyzed (Figure 3). Of note, this correlation was lost after drug de-intensification.

On the contrary, those who received raltegravir as treatment intensification showed no correlation between either activated $CD4^+\beta7^+$ and $CD8^+\beta7^+$ T cells with microbial translocation.

Dynamics of T cell subpopulations during the treatment de-intensification

$CD4^+$ or $CD8^+$ T cell counts were stable during the treatment de-intensification regardless the drug of intensification. Moreover, naïve $CD4^+$ and $CD8^+$ T cells also remained stable during the time-points studied regardless the drug used for treatment intensification, and no differences were found either in CD4- or CD8- TCM, TEM or TEMRA (not shown). T cell subpopulations did not correlate with microbial translocation (not shown).

DISCUSSION

We found opposite effects during de-intensification of maraviroc and raltegravir over the levels of microbial translocation and gut homing β7 receptor on T cells in long-term suppressed HIV-1-infected patients.

In patients who received maraviroc as intensification treatment, activated $\beta7^+$ T cells are probably recruited from peripheral blood to the gut tract as the major mechanism to recover the mucosa integrity, reducing microbial translocation and systemic inflammation.[35,37,38] This cell recruitment during the intensification treatment is reverted when microbial translocation is not longer increasing, i.e. during treatment de-intensification. In accordance with this, a strong correlation was found between both activated $CD4^+\beta7^+$ and activated $CD8^+\beta7^+$ T cells with LPS levels at the end of the intensification treatment. After the removal of maraviroc, these correlations were lost, as occurred at baseline (before intensification). Our study suggests that the expression of β7 receptor on peripheral T cells could be a good surrogate marker to monitor mucosal immune response. Our findings are consistent with other published maraviroc intensification study in which intestinal tissue biopsies were included. In this study, immune discordant HIV-1-infected patients were randomized to add maraviroc or placebo to their existing suppressive ART.[41] Histological examination of intestinal biopsies showed increased immune activation, which could be associated with the

increased bacterial translocation and gut homing β7 receptor on T cells, as that was found in our study.

The mechanisms by which maraviroc produces such effects remain unclear. CCR5 plays an important role in inflammatory function of several mucosal immune cell types (T cells, macrophage and dendritic cell subpopulations, innate lymphoid cells, etc). Blockade of this role is expected to interfere with mucosal immune cell functions required to control microbial intrusion, and indeed, these findings demonstrate microbial translocation, and β7 T cell activation, presumably in response to inflammatory activation in response to the intrusion. CCR5 blockade increase CCR5 on the cell membrane, increasing cell death and deteriorating the gut-associated lymphoid tissue, and also an increase of ligands such as MIP-1α, MIP-1β and RANTES that could signal through other receptors that eventually could increase gut immune activation. All these together could therefore increase the level of microbial translocation and gut homing β7 receptor.

On the contrary, raltegravir intensification showed a significant decrease in microbial translocation (measured by LPS levels) that was related with a significant decrease of $CD4^+\beta7^+$ cells. This effect was reverted during the treatment de-intensification. These findings are consistent with one study of intensification with raltegravir in HIV-1-infected adults with $CD4^+$ T cell count above 200 cells/mm^3, in which intestinal tissue biopsies were included.[5] No differences were shown in the gut immune activation, also consistent with our findings in peripheral blood. Raltegravir behaves as expected for a given antiretroviral drug, i.e. it did not appear to alter or tented to decrease microbial translocation and did not alter the level of gut homing β7 receptor on T cells, suggesting no alteration in the level of gut immune activation.
On the other hand, $CD4^+$ or $CD8^+$ T cell counts in peripheral blood did not change during the entire follow up period when they were intensified with maraviroc or raltegravir, showing no correlations with microbial translocation and supported by another study that failed to show this correlation during raltegravir intensification.[42] The fact that $CD4^+$ T cell count in peripheral blood was constant during the follow up period would confirm a delayed recovery of gut $CD4^+$ T cells compared to the $CD4^+$ T cell restoration in peripheral blood.[24]

Besides, no significant changes were observed in naïve $CD4^+$ or $CD8^+$ T cells, TCM, TEM and TEMRA during the intensification period with maraviroc or raltegravir or during de-intensification, with no correlation with microbial translocation or homing gut receptor expression. Despite the low number of patients included in this study, the levels of LPS, LBP and sCD14 detected in HIV-1-infected patients during maraviroc and raltegravir intensification, and after its discontinuation evidenced that the mucosal damage associated to HIV-1 infection persisted despite successful treatment allowing microbial translocation into the bloodstream that would cause gut immune activation and mucosal inflammation,[43-46] as evidenced by the association between microbial translocation and activated T cells expressing the gut homing receptor in periphery.

ACKNOWLEDGMENTS

We would like to thank Carmen Page, Raquel Lorente and Ester Domínguez for their excellent technical assistance. Specially, we thank all of our patients and their families for their participation in the studies.

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FIGURE LEGENDS

Figure 1. Effect of treatment de-intensification on microbial translocation. Levels of microbial translocation in both A) maraviroc and B) raltegravir groups during the follow up. Data related to the treatment de-intensification study are highlighted with a square. TI, treatment intensification; TDI, treatment de-intensification. *P* values were determined by the nonparametric Wilcoxon paired test and only significant values ($p<0.05$) are shown.

Figure 2. Effect of treatment de-intensification on the expression of gut homing β7 receptor on activated T cells. Activated $CD4^+$ and $CD8^+$ T cells expressing β7 receptor during the follow up in both maraviroc (A) and raltegravir (B) groups. Data related to the treatment de-intensification study are highlighted with a square. TI, treatment intensification; TDI, treatment de-intensification. *P* values were determined by the nonparametric Wilcoxon paired test and only significant values ($p<0.05$) are shown.

Figure 3. Association of activated $\beta7^+$ T cells with LPS levels. A positive correlation between LPS levels and either activated $CD4^+\beta7^+$ and $CD8^+\beta7^+$ T cells were found after 48 week of maraviroc intensification. These correlations were lost after the drug de-intensification. Data related to the treatment de-intensification study are highlighted with a square. TI, treatment intensification; TDI, treatment de-intensification. *P* values were determined by Spearman´s rank test and only significant values ($p<0.05$) are shown.

Figure 1

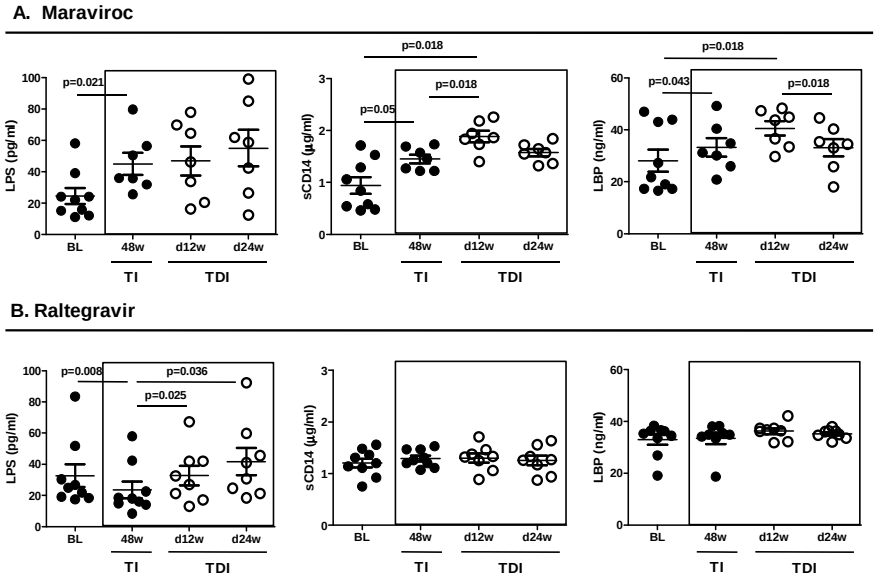


Figure 2

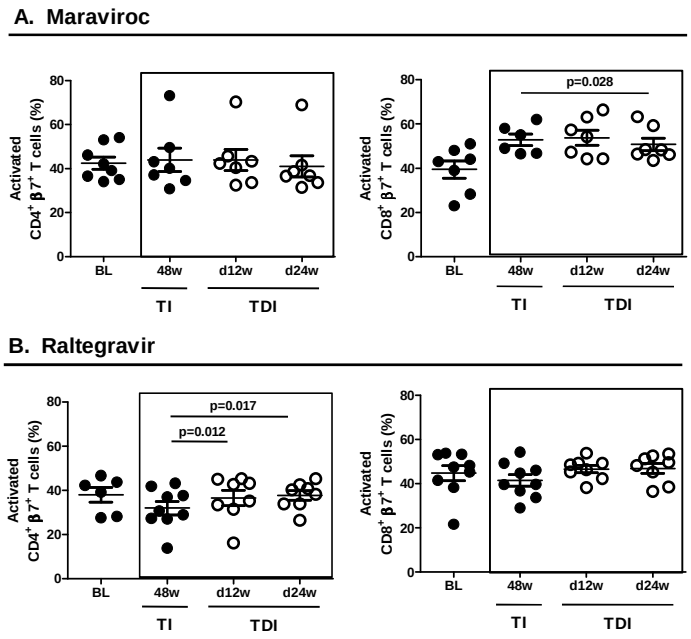
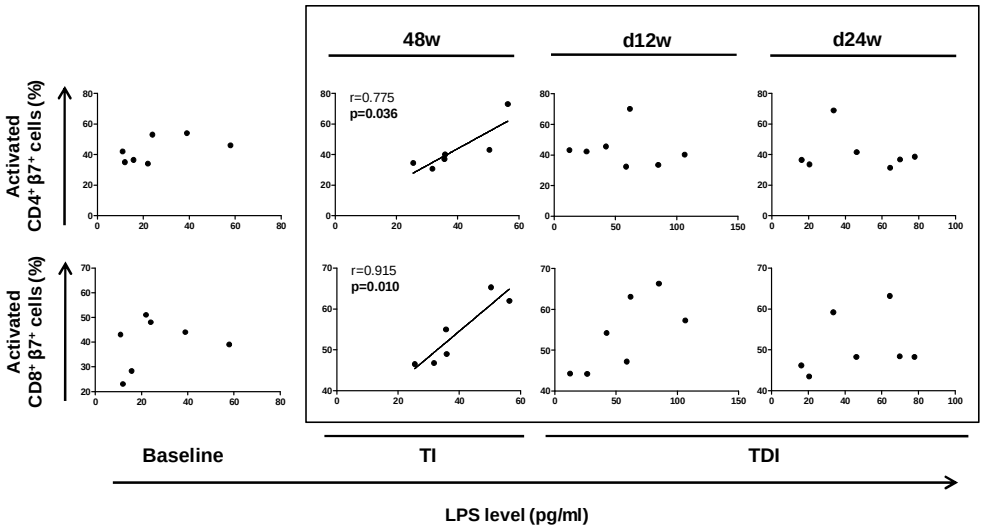


Figure 3


48w
d12w
d24w
Activated CD4+ β7+ cells (%)
Activated CD8+ β7+ cells (%)
r=0.775
p=0.036
r=0.915
p=0.010
Baseline
TI
TDI
LPS level (pg/ml)

Elsevier Editorial System(tm) for Antiviral Research

Manuscript Draft
Manuscript Number: AVR-S_13-00507

Title: Transient increment of HTLV-2 proviral load in HIV-1-co-infected patients during treatment intensification with raltegravir

Article Type: Research Paper

Section/Category: HIV and other retroviruses

Keywords: HTLV-2; proviral load; raltegravir

Corresponding Author: Mr. Alejandro Vallejo, Ph.D.
Corresponding Author's Institution: Hospital Ramón y Cajal

First Author: María Abad-Fernández

Order of Authors: María Abad-Fernández; Cecilia Cabrera; Elisabet García; Alejandro Vallejo, Ph.D.

Manuscript Region of Origin: SPAIN

Abstract: Numerous studies have analyzed the effects of raltegravir intensification on HIV-1 viral replication in infected individuals receiving suppressive combined antiretroviral treatment (cART). Nevertheless, there are only two studies on the effect of raltegravir in HTLV-1 infection, and none in HTLV-2. Hence, our objective was to study the effect of raltegravir on HTLV-2 infection in HIV-1-coinfected individuals. This study was a retrospective longitudinal study with a follow up of 48 weeks. HTLV-2 proviral load, CD4 and CD8 count and frequency of four HTLV-2-HIV-1-co-infected individuals who received raltegravir-based cART during 48 weeks and 11 HTLV-2-HIV-1-co-infected individuals under cART were analyzed. HTLV-2 proviral load significantly increased at week 24 compared to baseline among all the patients who received raltegravir ($p=0.003$), while no significant increases were found in the control group. No significant variation in either CD8 or CD4 counts were found during the follow up in both groups. Raltegravir intensification induced a transient increment on total HTLV-2 DNA proviral load in HTLV-2/HIV-1-coinfected individuals on suppressive cART. The period of follow up, nevertheless, did not permit to know whether the decrease observed at week 48 compared to week 24 would further continue over the time or would be stabilized at levels comparable to baseline. Further studies with larger number of individuals under raltegravir intensification would be required to understand the mechanisms used by raltegravir to transiently increase the HTLV-2 proviral load, as an important tool for the eradication of HTLV-2 infection and how it would affect HIV-1 infection in coinfected patients.

Transient increment of HTLV-2 proviral load in HIV-1-co-infected patients during treatment intensification with raltegravir

María Abad-Fernández, BSc. Laboratory of Immunovirology, Department of Infectious Diseases, Universitary Hospital Ramón y Cajal, 28034 Madrid, Spain.

Cecilia Cabrera, PhD. IrsiCaixa-HIVACAT, Institut de Recerca en Ciències de la Salut Germans Trias i Pujol (IGTP), Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, 08916 Barcelona, Spain.

Elisabet García, BSc. IrsiCaixa-HIVACAT, Institut de Recerca en Ciències de la Salut Germans Trias i Pujol (IGTP), Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, 08916 Barcelona, Spain.

Alejandro Vallejo, PhD. Laboratory of Immunovirology, Department of Infectious Diseases, Universitary Hospital Ramón y Cajal, 28034 Madrid, Spain.

Running title: Raltegravir increases HTLV-2 proviral load

Key words: HTLV-2 proviral load, HIV-1 coinfected, raltegravir

Corresponding author: Alejandro Vallejo, Ph.D. Laboratory of Immunovirology, Department of Infectious Diseases, Ramón y Cajal University Hospital, Ctra. Colmenar Km 9, 28034 Madrid, Spain. Phone: +34 91 336 8711. Fax: +34 91 336 8792. e-mail: alejandro.vallejo@salud.madrid.org

Abstract

Numerous studies have analyzed the effects of raltegravir intensification on HIV-1 viral replication in infected individuals receiving suppressive combined antiretroviral treatment (cART). Nevertheless, there are only two studies on the effect of raltegravir in HTLV-1 infection, and none in HTLV-2. Hence, our objective was to study the effect of raltegravir on HTLV-2 infection in HIV-1-co-infected individuals. This study was a retrospective longitudinal study with a follow up of 48 weeks. HTLV-2 proviral load, CD4 and CD8 count and frequency of four HTLV-2-HIV-1-co-infected individuals who received raltegravir-based cART during 48 weeks and 11 HTLV-2-HIV-1-co-infected individuals under cART were analyzed. HTLV-2 proviral load significantly increased at week 24 compared to baseline among all the patients who received raltegravir ($p=0.003$), while no significant increases were found in the control group. No significant variation in either CD8 or CD4 counts were found during the follow up in both groups. Raltegravir intensification induced a transient increment on total HTLV-2 DNA proviral load in HTLV-2/HIV-1-coinfected individuals on suppressive cART. The period of follow up, nevertheless, did not permit to know whether the decrease observed at week 48 compared to week 24 would further continue over the time or would be stabilized at levels comparable to baseline. Further studies with larger number of individuals under raltegravir intensification would be required to understand the mechanisms used by raltegravir to transiently increase the HTLV-2 proviral load, as an important tool for the eradication of HTLV-2 infection and how it would affect HIV-1 infection in co-infected patients.

1. Introduction

Since the isolation of human T cell lymphotropic virus type 2 (HTLV-2) from a patient with atypical hairy cell leukaemia in 1981 (Kalyanaraman et al., 1982), no clear evidence of human disease has been linked to HTLV-2 infection, except for a few cases of neurological and lymphoproliferative disorders (Araujo and Hall 2004; Biswas et al., 2009; Orland et al., 2003; Poiesz et al., 2000; Thomas et al., 2010). Even though HTLV-2 infection is endemic in American Indian population and Pygmy tribes in Africa (Switzer et al., 1995), HTLV-2 has been widely distributed in injecting drug users (IDU) in USA and Western Europe, including Spain where co-infection with HIV-1 is frequent (Abad et al., 2011; Beilke et al., 2007; Beilke, 2012; Lowis et al., 2002; Saito and Bangham, 2012; The HTLV European Research Network, 1996; Treviño et al., 2010; Treviño et al., 2012; Vallejo et al., 1996; Vallejo et al., 2003).

While HIV-1 infection causes the depletion of the infected $CD4^+$ T cells leading to immunosupression, HTLV-2 infection triggers the expansion of $CD8^+$ infected T cells, with a possible potential to cause lymphoproliferative disorders (Simonis et al., 2012). Both retroviruses require the integration of the viral genome into the cell DNA as part of its life cycle. During chronic HTLV-2 infection, the low viral replication would drive infected cells to proliferate and propagate the infection via the clonal expansion with concomitant propagation of integrated proviral DNA (Asquith et al., 2007). The reported stable proviral load (pVL) over time would support a balance between virus-induced clonal proliferation and the immunologic control of HTLV infection (Demontis et al., 2013; Furtado Mdos et al., 2012; Kwaan et al., 2006; Olavarria et al., 2012). On the other hand, HTLV-1 pVL seems to be determined by the infection route, which

suggests that the infectious dose at the time of infection might determine the chronic pVL, and/or time of infection, where carriers by vertical transmission have significantly higher pVL than carriers by sexual transmission (Murphy et al., 2004; Ueno et al., 2012). It has also been reported increased HTLV-2 pVL after the initiation of antiretroviral treatment in HIV-1-coinfected patients (Murphy et al., 2003).

Raltegravir was the first integrase inhibitor approved for HIV-infection treatment that blocks integration of linear viral cDNA into the host chromosomal DNA. This blocked cDNA subsequently circularize to form episomal DNA (2LTR DNA circles). Since then, numerous studies have analyzed the effects of raltegravir intensification on HIV-1 viral replication in infected individuals receiving suppressive combined antiretroviral treatment (cART) (Gandhi et al., 2010; Gandhi et al., 2012; Markowitz et al., 2006; Markowitz et al., 2007; Markowitz et al., 2009). For this purpose, some of them measured the amount of HIV-1 proviral DNA viral as reflect of viral reservoir (Chege et al., 2012; Nicastri et al., 2011; Delaugerre et al., 2010; Negredo et al., 2013; Buzón et al., 2010).

There are only two studies on the effect of raltegravir in HTLV-1 (Seegulam et al., 2011; Treviño et al., 2012). The first one is an in vitro study supporting that integrase inhibitors were effective in inhibiting in vitro cell-free and cell-to-cell transmission of HTLV-1 in lymphoid and non-lymphoid cells. The authors suggested that raltegravir and other integrase inhibitor could be administered as treatment for patients with HTLV-1-associated diseases (Seegulam et al., 2011). The other study was an *in vivo* study of five naive HTLV-1 carriers. The authors concluded that antiretroviral treatment with raltegravir does not result in a reduction of HTLV-1 pVL after 6 months of treatment (Treviño et al., 2012). No studies on the effect of raltegravir on HTLV-2 have been reported yet, neither in vitro nor in vivo.

In this retrospective longitudinal study, our objective was to study the effect of treatment intensification with raltegravir on HTLV-2 pVL among HTLV-2/HIV-1-co-infected individuals.

2. Materials and Methods

2.1 Patients

This was a retrospective longitudinal study aimed to analyze the effect of the intensification of the cART with an integrase inhibitor (raltegravir) on the level of HTLV-2 pVL measured at baseline, week 24 and week 48 in HIV-1-co-infected patients. Four HTLV-2-HIV-1-co-infected patients who received 400 mg of raltegravir twice daily were recruited from Hospitals Germans Trias I Pujol (Badalona, Barcelona, Spain) and Hospital Ramón y Cajal (Madrid, Spain) and analyzed. One of these patients was analyzed in two further time points, a second baseline and at week 4. Besides, a total of eleven HTLV-2-HIV-1-co-infected patients who did not intensify their cART with raltegravir were also analyzed as control group. All the patients included in this study had a history of intravenous drug use and have been under cART for more than one year with undetectable HIV-1 plasma viremia (<50 HIV-1 RNA copies/ml). The clinical trials from which we obtained the DNA samples were approved by the Ethics Committee of both Hospitals and all recruited patients signed an informed consent form.

2.2 Quantification of HTLV-2 proviral load

Total HTLV-2 DNA pVL was quantified by in-house real-time PCR. DNA was extracted from the 10^6 cryopreserved peripheral blood mononuclear cells (PBMCs) using QIAamp DNA kit (Qiagen GmbH, Hilden, Germany). A standard curve was generated in each run using 5-fold dilution of 10^6 copies of a recombinant plasmid (TOPO TA cloning, Invitrogen) containing one copy of HTLV-2 *tax* gene fragment (171 bp). Besides, another standard curve was generated using 5-fold dilution of 10^6 copies of a recombinant plasmid containing one copy of human genomic GAPDH gene fragment (162 bp).

The amplification reaction was carried out in triplicate using LightCycler 2.0 (Roche Diagnostics,Manheim, Germany). All reaction mixtures were performed in a total volume of 20 µL containing LightCycler FastStart DNA Master PLUS HybProbe 5X (Roche Diagnostics), 200 ng genomic DNA, 50 pmol each primer, Tax-F (5´TACGGTTTTTCCCCAGG) and Tax R (5´ACTCCTGTCTCCCCCAAG) for *tax* gene, GADH-F (5´ CTGACCTGCCGTCTAGA) and GADH-R (5´GTCGTTGAGGGCAATGC) for GADH gene, along with 2 pmol of the following fluorescent probes: 5´-FAM-CAC CCG CCT TCT TCC AAT CAA TGC GAA AG-TAMRA and 5´-CAG GTG GTC TCC TCT GAC TTC AAC-[Flc] / 5´-LC705-CGA CAC CCA CTC CTC CAC CTT TG-[Phos]-3´ for *tax* gene and GADH gene, respectively.

The cycling parameters for the both amplifications begun with hot start at 95℃ 10 min, and continued with 40 cycles of denaturation at 95ºC for 10s, annealing at 62ºC for 15s and extension at 72ºC for 20s for *tax* gene, and 40 cycles of denaturation at 95ºC for 5s, annealing at 62ºC for 10s and extension at 72ºC for 20s for GADH gene. The results were expressed as HTLV-2 DNA copies per million PBMCs.

2.3 Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and categorical variables as proportions when appropriated. The comparison of the HTLV-2 pVL between time periods was performed using a generalized estimating equations (GEE) model to take into account the within patient correlation observed in the repeated measures. This model includes terms for time as an indicator variable with baseline as reference level, group (treated or control), and time-by-group interaction term. All statistical tests used 0.05 as significance level. Analyses were performed using Stata software version 12.0 (StataCorp. College Station, TX, USA)

3. Results

The four patients who received raltegravir-based cART were men with median age of 51 [48-55], CD4 count of 215 [97-251] cells/mm^3, and CD8 count of 819 [658-929] cells/mm^3. The 11 patients from the control group had median age of 50 [46-56] years, CD4 count of 524 [307-1060] cells/mm^3, CD8 count of 980 [690-1308] cells/ mm^3, and nine of them were male (82%).

The statistical GEE model found a significant interaction between HTLV-2 pVL and time (repeated measures) among the patients who received raltegravir (Figure 1A), while found no statistical significance in the control group (Figure 1B). Hence, the model found a p-value of <0.001 in both the increase found at week 24 compared to

baseline and in the decrease found at week 48 compared to week 24 among the patients who received raltegravir.

When HTLV-2 pVL increments were analyzed, these were above 0.5 log copies/10^6 in the four patients who received raltegravir-based cART at week 24 (Δ24-BL), followed by a significant (GEE model) decrement of HTLV-2 pVL at week 48 (Δ48-24), as shown in figure 2A. The increment levels at Δ24-BL were significantly higher to those found at either Δ48-24 ($p<0.001$) or Δ48-BL ($p=0.020$), and the decrement levels found at Δ48-24 were significantly lower compared to those found at Δ48-BL ($p=0.003$). Instead, HTLV-2 pVL increments were between ±0.5 log copies/10^6 in the control group at week 24 (Δ24-BL, figure 2B). Seven patients of those (63%) showed HTLV-2 pVL increments while the rest showed decrements. This random behavior was observed along the follow up, as shown in figure 2B. Thus, the increment levels in the control group were stable along the follow up ($p>0.05$ in all cases). Of note, the increment level of HTLV-2 pVL at Δ24-BL in patients who received raltegravir was significantly higher compared to those found at any time point during the follow up in the control group ($p<0.005$). On the other hand, the increment level at Δ48-24 was significantly lower to those observed at any time in the control group ($p<0.005$ at each time point).

Neither CD8 count nor frequency, cells that are the main but not the only target for HTLV-2, showed any statistical change during the follow up among patients who received raltegravir as shown in table 1. Among them, only one patient increased CD8 count (Ral1) while CD8 frequency decreased in three out of four patients after 24 weeks. Among the control group, no significant variation in either CD8 count or frequency was found during the follow up (not shown).

Regarding CD4 T cells, other potential target for HTLV-2 infection, no significant variation of either counts or frequency was found. Only two patients increased either their CD4 counts or frequency (Ral1 and Ral3, table 1) at week 24 compared to baseline. Among the control group, no significant variation in either CD4 count or frequency was found during the follow up (not shown).

4. Discussion

During the last few years, the effect of raltegravir has been analyzed in several clinical studies in HIV-1-infected patients receiving cART. Most of them showed no significant changes in total or integrated HIV-1 DNA levels during the follow-up period. Moreover, no changes in $CD4^+$ or $CD8^+$ T cell counts were demonstrated. Only one study showed a marked HIV-1 proviral decay after 24 weeks with raltegravir-based cART (Markowitz et al., 2006), while another demonstrated transient increase in episomal HIV-1 DNA (2LTR) despite no changes in total proviral DNA in a large percentage of HIV-1-suppressed subjects (Buzón et al., 2010).

Given the similarities between HIV-1 and HTLV-1 integrases, raltegravir was thought to be effective against HTLV-1. Rabaaoui et al observed a decrease in HTLV-1 pVL *in vitro* and also *ex vivo*. However, the effects detected *ex vivo* were significantly lower than those observed *in vitro*. Further *in vitro* studies showed that raltegravir prevented the integration of HTLV-1 in both cell-free and cell-to-cell infection (Ratner, 2005).

Despite these promising *in vitro* results, no decrease on HTLV-1 pVL was reported in the unique in vivo study on five infected patients (Treviño et al., 2012). In this report, no episomal DNA (2LTR) was quantified. This lack of any beneficial effect on HTLV-1 viral

replication was attributed to the virus infection transmission mainly maintained through cell division instead of through new rounds of viral replication (Pique et al., 2012).

No *in vivo* studies on the effect of raltegravir over the HTLV-2 pVL in infected patients have been performed yet. Thus, this is the first study that shows that HTLV-2 pVL is transiently increased in HTLV-2/HIV-1 co-infected patients when cART was intensified with raltegravir. This increase observed at week 24 after raltegravir intensification was followed by a significant decrease at week 48, reaching similar levels compared to baseline.

Since all four patients who received raltegravir behaved similarly, we wanted to discard two possible issues, i.e., a generic effect of the intensification treatment (regardless the antiretroviral used), and a random fluctuation of the proviral load over the time. For the first issue, we only could analyze one HTLV-2-HIV-1-co-infected patient who received maraviroc (CCR5 antagonist) as intensification treatment along with the initial suppressive cART. Since we found no significant changes on the HTLV-2 pVL during one year of follow up (Δw24-BL -0.16, Δw48-w24 -0.05, and Δw48-BL 0.23 log copies/10^6 PBMC), a generic effect of intensification seemed not to account for the variation observed with raltegravir. For the second issue and despite that a stable level of HTLV-1 pVL over the time has been reported (Demontis et al., 2013; Furtado Mdos et al., 2012; Olavarria et al., 2012), we analyzed a group of HTLV-2-HIV-1-co-infected patients who received cART without raltegravir. No significant changes were found in the HTLV-2 pVL during the follow-up in this group, with variations (increments and decrements) below 0.5 log. Again, this does not support a random fluctuation of the magnitude observed in the patients intensified with raltegravir.

One possible explanation for the increase in the HTLV-2 pVL would be that raltegravir intensification interferes in the host immune response leading to the activation and clonal expansion of the CD8 lymphocytes infected with HTLV-2. The $CD8^+$ T cell division would trigger a burst of horizontal viral replication and the consequent increase on the DNA proviral load (Cimarelli et al., 2012; Kannian et al. 2012). Nevertheless, this transient clonal expansion had to be not very large given that significant increases in CD8 count or frequency were not observed along the follow up. The subsequent return to baseline proviral load levels at week 48 would be explained by the CTL response that would rapidly eliminate infected cells once they start to express viral proteins as it has been previously described for HTLV-1 infection (Bangham, 2008). Besides this possible expansion of infected-CD8 lymphocytes, it is also possible that a transient increase of episomal HTLV-2 2LTR DNA circles might occur as seen in HIV-1 patients intensified with raltegravir (Buzón et al., 2010). This implies an increase of HTLV-2 replication and a subsequent accumulation of HTLV-2 episomal 2LTR DNA circles by the effect of raltegravir. Unfortunately, the limited amount of DNA sample for each patient made it impossible to test either cellular clonality or HTLV-2 episomal 2LTR DNA circles quantification. On the other hand, no increase in CD4 counts was found in these patients suggesting that this potential cell target did not account for the increase of HTLV-2 pVL.

One important limitation of this study is the small number of patients included for the analysis forcing to take these results with caution. Unfortunately, there is limited experience using specific antiretroviral drugs in these HTLV-2-infected patients and no treatment recommendations can be given.

In summary, for the first time we showed that raltegravir intensification induced a transient increment on total HTLV-2 pVL in HTLV-2/HIV-1-coinfected individuals on suppressive cART. We do not know whether the decrement observed at week 48

compared to week 24 would further continue over the time or the level of would be stabilized at levels comparable to baseline. Further studies with larger number of individuals under raltegravir intensification would be required to understand the mechanisms used by raltegravir to transiently increase the HTLV-2 pVL, as an important tool for the eradication of HTLV-2 infection and how it would affect to the HIV-1 infection in co-infected patients.

Acknowledgements

We want to thank Dr. J Zamora for the statistical analysis and all the patients for their participation.

Financial support

This work was supported in part by Spanish AIDS Network “Red Temática Cooperativa de Investigación en SIDA (RD06/0006).

Transparency Declaration

The authors declared no financial or commercial conflict of interest.

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Figure legends

Figure 1: HTLV-2 pVL during the follow up in patients with raltegravir-based cART (A) and patients with cART (B). Generalized estimating equations (GEE) model was used for repeated measures. Only significant p values when $p<0.05$ are shown.

Figure 2: HTLV-2 pVL increments during the follow up in both individual patients and mean with interquartile range under raltegravir-based cART (A), and individual patients and mean interquartile range under cART (B). The shadowed square represents increments of ±0.5 log copies/10^6 PBMC. Generalized estimating equations (GEE) model was used for repeated measures. Only significant p values when $p<0.05$ are shown.

Table 1: CD4 and CD8 T cell characteristics during the follow up and type of treatment of the patients who received raltegravir-based cART.

		Ral1	Ral2	Ral3	Ral4	Median [IQR]	p
CD4 count (frequency)	BL	242 (18)	255 (21)	188 (11)	67 (3.3)	215 [97-252] (14.5 [5.2-20.2])	
	w24	401 (21)	275 (20)	248 (16)	48 (2.7)	261 [98-369] (18.0 [6.0-20.7])	0.246 (0.354)
	w48	241 (15)	346 (21)	246 (13)	32 (1.9)	243 [84-321] (14.0 [4.6-19.5])	0.393 (0.605)
CD8 count (frequency)	BL	740 (55)	631 (52)	940 (55)	898 (45)	819 [658-929] (53.5 [46.7-55.0])	
	w24	992 (52)	633 (46)	837 (54)	795 (50)	816 [673-953] (51.0 [47.0-53.5])	0.895 (0.618)
	w48	866 (54)	839 (51)	984 (52)	792 (47)	852 [803-954] (51.5 [48.0-53.5])	0.385 (0.498)
cART		ABV, 3TC, ATV	ATV, FTC, RTV, TDF	3TC, LPV, RTV, TDF	FTC, TNF		

Cell count in cells/mm3; cell frequency in percentage; IQR, interquartile range; Significant when $p<0.05$ (paired samples t test).

Figure 1

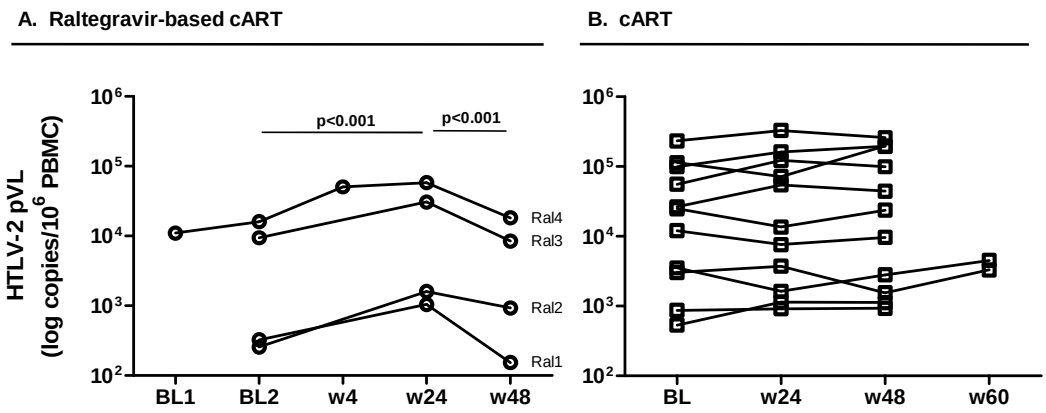
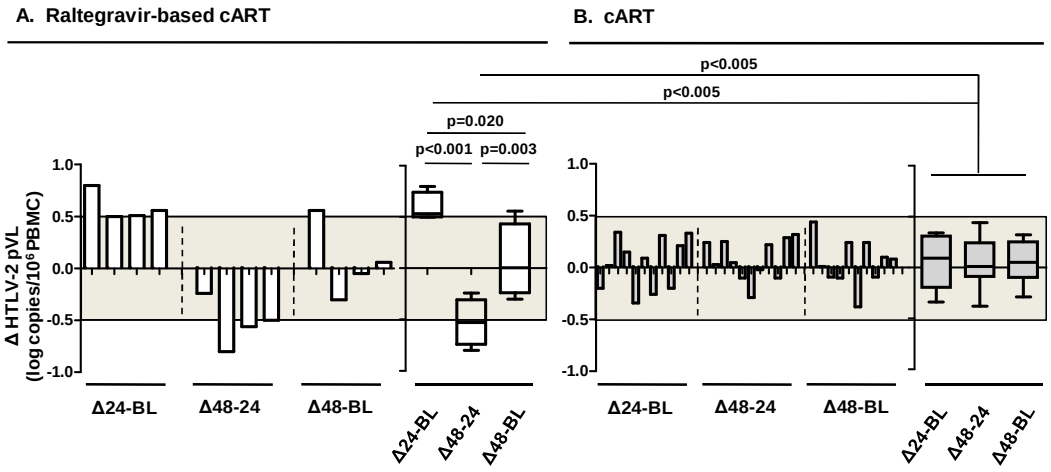


Figure 2



High levels of immune activation, inflammation, microbial translocation and immunosenescence in Leishmania/ HIV-1 co-infection

Order of Authors: José L Casado; María Abad-Fernández; Santiago Moreno; María J Pérez-Elías; Ana Moreno; Ignacio Bernardino; Fernando Dronda; Alejandro Vallejo, Ph.D.

1. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Ramón y Cajal University Hospital, Madrid, Spain.
2. Department of Infectious Diseases, La Paz University Hospital, Madrid, Spain

Running title: Immune impairment of VL/HIV co-infection.
Key words: Visceral Leishmaniasis, HIV-1, immune activation, microbial translocation, immunesenescence, inflammation.
Corresponding author: Alejandro Vallejo, Ph.D. Laboratory of Immunovirology, Department of Infectious Diseases, Ramón y Cajal University Hospital, Ctra. Colmenar Km 9, 28034 Madrid, Spain.
Phone: +34 91 336 8711.
Fax: +34 91 336 8792.
e-mail: alejandro.vallejo@salud.madrid.org

Abstract

Visceral leishmaniasis (VL) in HIV-1-infected patients has been associated with poor immunological recovery and frequent disease relapses. The aim of this study was to compare the immunological profile of VL in HIV-1-infected-patients with immunodiscordant HIV-1-infected patients. A cross-sectional study of eight patients with VL with suppressive cART for at least one year, and both 14 patients with discordant immunological response (DR) and 15 patients with concordant immunological response (CR) without leishmaniasis was performed. T-cell subpopulations, immune activation, microbial translocation, and immunosenescence were analyzed. CD8+ T-cell activation was higher compared to both DR and RC patients, and independently associated to VL and sCD14 levels. Plasma IL-6 levels and sCD14 were increased and was not related to time of VL diagnosis nor the number of relapses. In addition, VL patients had similar level of CD4+ and higher CD8+ T-cell senescence compared to DR patients, and greater than the observed in CR patients, despite similar age of the patients. In conclusion, VL was associated with immune activation, microbial translocation, and immunosenescence in HIV-1-infected patients, higher than those observed in patients with discordant immunological response to cART. These data could explain the poor immunological recovery of these patients and the persistence of relapses.

INTRODUCTION

Visceral Leishmaniasis (VL) is an infectious disease caused mainly by *Leishmania donovani* and *L. infantum* (*L. chagasi*). It is a common co-infection in HIV-1-infected individuals in the Mediterranean basin, including Spain with a prevalence of up to 9% mainly among intravenous drug users with a mortality rate of up to 50% (1,2). These protozoans are intracellular parasites that infect macrophage cell lineages from lymphoid organs. The immunological response triggered after infection is directly associated to the clinical outcome, but the pathogenic mechanisms are still controversial.

Leishmania and HIV-1 infections share immune-compromising mechanisms that may affect the parasite control by the immune system. It is demonstrated that VL accelerates the progression of HIV-1 disease, increasing chronic immune activation

and accelerating T cell depletion (3). In addition, it has been shown that HIV-1 infection inhibits the intracellular killing of *Leishmania*, interfering in parasite clearance (4-7).

On the other hand, the gut parasitation by *Leishmania* amastigotes in VL would accelerate the mucosal damage associated to HIV-1 infection increasing the levels of microbial translocation into the bloodstream causing a systemic proinflammatory response and increasing immune activation (8-15). It has also been observed higher Th2 cytokine production in PBMCs from HIV-1-*Leishmania* co-infection compared to HIV-1-infected patients, although clinical consequences remain unclear (16,17).

While combination antiretroviral treatment (cART) has been shown to decrease the incidence of leishmaniasis in HIV-infected patients, it does not seem to prevent relapses (18-20). Thus, the parasite persistence may still impact the pathogenesis of HIV disease.

Both immunological alterations could explain the high prevalence of impaired immunological response to cART in patients with VL, in a similar manner to that described with immunodiscordant patients.

Our objective was to investigate how VL could impact immune activation and which factors could impair the immune response to control the parasite infection in suppressed HIV-1-infected patients, including inflammation, microbial translocation, and immunesenescence, in comparison with both HIV-1-infected patients with immunodiscordant response and with a concordant response to cART.

MATERIALS AND METHODS

Patients

A cross-sectional, descriptive, comparative study was performed in eight HIV-1-infected individuals with active visceral leishmaniasis (VL) from the Department of Infectious Diseases at the Universitary Hospital Ramón y Cajal, Madrid, Spain. All of them had been diagnosed for VL by parasitological diagnosis by direct visualization of amastigotes in bone marrow and peripheral blood, positive *Leishmania*-specific PCR on peripheral blood (21), as well as by clinical symptoms. All of them were treated with anti-*Leismania* treatment (liposomal amphotericina B, miltefosine or meglumine antimoniate, according to local guidelines (22)) and most of them received secondary

prophylaxis based on monthly amphotericina B, or miltefosine). At inclusion, all of them had received cART for at least one year with sustained undetectable plasma HIV-1 RNA (<50 copies/ml).

As comparison groups of HIV-1-infected patients, we analyzed 13 patients with concordant response (CR, CD4 count above 400 cells/mm^3) and 14 patients with discordant response (DR, CD4 count below 250 cells/mm^3) with no coinfection with *Leishmania* and receiving suppressive cART (HIV-1 viral load lower than 50 RNA copies/ml) for at least one year.

A single blood extraction was performed in all the patients. Blood was collected in Vacutainer tubes (Becton Dickinson, Madrid, Spain) and processed for both plasma and cytometry analysis.

This study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our local Independent Ethics Committee (Hospital Ramon y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

Analysis of inflammation and microbial translocation

Inflammation was measured by plasma IL-6 quantification using hsIL-6 (human IL-6 Quantikine High Sensitivity ELISA Kit, R&D Systems). Microbial translocation was measured in plasma by the quantification of both lipopolysaccharide (LPS) using QCL-1000 Limulus Amebocyte Lysate (Lonza®, Basel, Switzerland), according to manufacturer´s instructions, and soluble CD14 (sCD14) using the Quantikine® Human sCD14 Immunoassay (R&D Systems, Minneapolis, Minnesota, USA), according to manufacturer´s protocol. Samples were tested in duplicate.

Flow cytometry analysis of blood samples

Fresh EDTA-anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell subpopulations with the following antibody combination: CD3-V500, CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin (PE)-Cy7. Cellular activation was defined as the co-expression of CD38-phycoerythrin (PE) and HLA-DR-allophycocyanin (APC). Immunesenescence was analyzed on T cells with the

expression of CD57-Fluorescein isothiocyanate (FITC), and the absence of CD28-APC-H7.

Antibodies were from Becton Dickinson (Becton Dickinson, NJ, USA), and an unstained control was performed for all samples. Briefly, 100 µL of blood were lysed with FACS Lysing solution (Becton Dickinson) for 30 min at room temperature, incubated with the antibodies during 20 min at 4°C, washed and resuspended in PBS containing 1% azida. Cells were analyzed in a Gallios flow cytometer (Beckman-Coulter, CA, USA). At least 30000 $CD3^+$ T cells were collected for each sample and analyzed with Kaluza software (Beckman-Coulter) initially gating lymphocytes according to morphological parameters.

Statistical analysis

Continuous variables were expressed as the median and interquartile range and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables and the Mann-Whitney test to compare non-normally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. Spearman´s rank correlation coefficient was used to analyze continuous variables. Statistical analysis was performed using SPSS software 16.0 (Inc., Chicago, Illinois, USA).

RESULTS

Patients with VL had a median age of 45 years, similar to the age of the two control groups, with 62.5% of males. All but one were injecting drug users (IDUs) which is a different scenario compared to the risk factor of the other two control groups where patients with concordant response were predominantly men who have sex with men (MSM) (60%), and similar frequencies of IDUs and MSM were found within patients with discordant response (42.8% in both cases). Interestingly, while VL patients had significantly lower levels of CD4 count, CD4/CD8 ratio and CD4 nadir compared to CR patients ($p<0.001$, $p<0.001$, and $p=0.001$, respectively), they had higher levels of CD4 count and CD4/CD8 ratio compared to DR patients ($p=0.005$ and $p=0.038$, respectively), while no significant differences were found in nadir CD4 count (**Figure 1A**). On the other hand, all three groups of patients had similar duration of both cART and viral suppression. Most of the VL patients were infected with VHC (75%). Interestingly, most of the VL patients had a time of diagnosis of VL similar to the duration of cART (75%). Clinical and laboratory characteristics are shown in **table 1**.

Immune activation

The level of CD4 T cell activation in VL patients was similar to that found in DR patients (p=0.402), but significantly higher compared to CR patients (p<0.001). On the other hand, CD8 T cell activation is significantly higher in VL patients compared to both DR and RC patients (p=0.010 and p<0.001, respectively) (**Figure 1B**).

We also analyzed which factors were independently associated with immune activation (both CD4 and CD8 T cell activation as dependent variables) to analyze whether VHC infection influence immune activation. As shown in **table 2**, CD4/CD8 ratio and sCD14 levels were independently associated with CD4 cell activation (p=0.045 and p=0.025, respectively), while leishmaniasis and sCD14 levels were independently associated with CD8 cell activation (p=0.046 and p=0.003, respectively).Therefore, leishmaniasis rather than VHC greatly contributes to the hyper immune activation.

Levels of inflammation and microbial translocation

Plasma IL-6 levels were significantly increased in VL patients compared to both DR (p=0.035) and CR patients (p<0.001) (**Figure 2A**).

While LPS levels in VL patients had significant higher levels compared to CR patients (p=0.001) and similar levels compared to DR patients (p=0.297) (**Figure 2B**), plasma sCD14 levels were significantly higher compared to both DR and CR patients (p<0.001 in both).

Correlation between immune activation, inflammation and microbial translocation among VL patients

Among VL patients, there was a significant positive correlation between both CD4 and CD8 T cell activation and sCD14 levels (p=0.048, r=0.711 and p=0.017, r=0.801, respectively) (**Figure 3A**). On the other hand, IL-6 level correlated with LPS and sCD14 levels (p=0.001, r=0.935 and p=0.010, r=0.833 respectively), but not with T cell activation.

On the other hand, among DR patients, a significant correlation between CD4 T cell activation and sCD14 levels (p=0.048, r=0.553), and between IL-6 and LPS levels (p=0.023, r=0.601)) were found (**Figure 3B**). Among CR patients no correlation was found between immune activation and inflammation or microbial translocation. Nevertheless, LPS correlated with sCD14 in the three groups of patients, i.e., VL (p=0.011, r=0.826), DR (p=0.001, r=0.779), and CR patients (p=0.046, r=0.659).

Immune senescence

VL patients had similar level of CD4 T cell senescence compared to DR patients, but significantly higher compared to CR patients (p=0.037), despite similar age of the patients in each of the three groups. Similarly, VL patients had levels of activated CD4 T cell senescence not different from those in DR patients, but again significantly higher compared to CR patients (**Figure 4A**).

Interestingly, CD8 T cell senescence was higher in VL patients compared to DR patients, although not significant (p=0.059), while significantly higher levels were found in activated CD8 T cells compared to DR patients (p=0.005). These levels of senescence were significantly higher compared to CR patients (p<0.001 in both cases) (**Figure 4B**).

DISCUSSION

Our study demonstrates that VL patients are a subgroup of HIV-1-infected patients with special immunological characteristics, worse that those observed in patients with discordant response to cART. Thus, the increased levels of $CD8^+$ T cell activation, IL-6, sCD14 and activated CD8 T cell senescence found in this group of patients could fairly explain the lack of immune response observed in some cases, and the persistence of relapses.

It is known that *Leishmania* can persist in patients after treatment and may further reactivate under immunossupression (4). Immunossupressed HIV-1-infected patients showed inefficient T cell response compromising parasite control in *Leishmania* co-infection, resulting in frequent relapses especially in VL. Even though antiretroviral therapy has been shown to decrease the incidence of leishmaniasis in these patients, it does not seem to prevent relapses (18,20,23). *Leishmania* infection can promote HIV-1 replication (17) and increases the degree of immune system activation (11).

Our results show that VL patients have increased levels of immune activation, inflammation and microbial translocation compared to HIV-1-infected patients without leishmaniasis despite HIV-1 viral load suppression. All these effects may impair the immune effector function providing a mechanism for the parasite to relapse.

We found that VL patients had lower levels of CD4 T cell counts compared to CR patients despite similar time under suppressive cART. This may be partially explained by the depletion of bone marrow precursor cells caused by *Leishmania* in individuals

concomitantly infected with HIV-1 leading to deficiencies in the input of new lymphocytes into the periphery, though these values still remain superior to DR patients (24,25).

Another contribution for this immunosuppression is the fact that VL patients have elevated levels of T cell activation compared to patients without leishmaniasis that lead to elevated T cell depletion. This elevated immune activation was independently associated with CD4/CD8 ratio, leishmaniasis and sCD14 levels, but not with the presence of VHC infection. Thus, the contribution of leishmaniasis to cell activation is stronger than that observed in VHC infection. Besides, this elevated immune activation was associated to an increase of T cell senescence, despite similar age of the groups studied, which might accelerate the impairment of the immune effector function.

In addition, we have found that microbial translocation correlated with inflammation in VL patients that was only partially found among DR patients. Moreover, immune activation correlated with sCD14 in VL patients and again only partially among DR patients. sCD14 levels seem to be an important factor that reflects an increased monocyte/macrophage activation, as it has been described (11), and probably it could not be an adequate marker of bacterial translocation in patients with VL. These findings are in accordance to other reported works that shown an association between cellular activation and LPS levels in co-infected patients (26). The overactivation of monocyte/macrophage lineage could impair the phagocitosis of bacterial products leading to the increase of LPS levels observed in VL patients which might increase proinflammatory response and immune activation.

In conclusion, leishmaniasis is a factor that strongly contributes to increase the severity of immunodeficiency caused by HIV-1, despite viral load suppression. Compared to DR patients, VL patients also have increased levels of T cell senescence that accelerates the deterioration of the immune system. Also, increased levels of inflammation and bacterial translocation, especially sCD14 levels, contribute to the persistent immune activation. Therefore, VL should be considered a possible independent cause of bacterial translocation, immune activation and impaired immune response in HIV-1 infection, despite viral load suppression.

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Table 1

	Visceral Leishmaniasis (VL) patients								N=8	DR patients N=14	p	CR patients N=15	p
	1	2	3	4	5	6	7	8					
Age (years)	55	45	44	43	41	51	45	51	45 [43-51]	47 [41-59]	0.664	45 [32-53]	0.728
Gender (male %)	M	M	M	F	F	M	M	F	62.5	92.8		86.6	
Risk factors (%)													
IDU	-	1	1	1	1	1	1	1	87.5	42.8		13.3	
MSM	-	-	-	-	-	-	-	-	-	42.8		60	
HSx	1	-	-	-	-	-	-	-	12.5	14.3		26.7	
CD4 count (cells/mm^3)	208	204	122	144	397	451	331	336	331 [145-667]	143 [88-159]	0.006	680 [527-825]	<0.001
CD8 count (cells/mm^3)	564	1449	207	455	1587	1107	1063	912	679 [546-1029]	691 [569-850]	0.815	675 [544-906]	0.776
CD4/CD8 ratio	0.37	0.21	0.59	0.23	0.25	0.41	0.31	0.37	0.42 [0.21-0.96]	0.19 [0.09-0.32]	0.035	1.0 [0.84-1.44]	<0.001
Nadir CD4 (cells/mm^3)	70	13	8	27	61	35	89	33	34 [16-68]	50 [26-99]	0.441	202 [62-317]	0.001
cART (months)	62	200	111	205	38	177	215	168	172 [74-203]	156 [97-197]	0.799	89 [43-144]	0.065
HIV-1 suppression (months)	59	125	26	203	16	28	76	126	59 [26-125]	69 [28-102]	0.799	63 [43-110]	0.824
HCV infection (%)	No	Yes	Yes	Yes	Yes	No	Yes	Yes	75	57		13.3	
VL diagnosis (months)	65	181	20	35	39	170	42	153	53 [36-165]	-		-	
Relapses of VL	2	27	2	3	3	9	1	7	3 [2-8.5]	-		-	

cART, combination antiretroviral therapy; CR, concordant response; DR, discordant response; significant when p<0.05, comparison VL vs CR and VL vs DR.

Table 2 Multivariate analysis: factors independently associated with immune activation (dependent variables CD4 and CD8 T cell activation).

	CD4 T cell activation		CD8 T cell activation	
	Coefficient (95% CI)	**p**	**Coefficient (95% CI)**	**p**
CD4 count	-0.003 (-0.009-0.002)	0.234	-0.001 (-0.015-0.012)	0.838
CD4/CD8 ratio	-2.470 (-4.881- -0.058)	**0.045**	-5.216 (-10.817-0.384)	0.067
Nadir CD4 count	0.009 (-0.002-0.020)	0.105	0.018 (-0.008-0.044)	0.161
Leishmaniasis	-8.810 (-18.029-0.409)	0.060	-21.850 (-43.259- -0.440)	**0.046**
VHC infection	1.093 (-1.673-3.859)	0.424	-2.334 (-8.757-4.090)	0.462
IL-6	0.039 (-1.120-1.198)	0.945	0.843 (-1.849-3.535)	0.525
LPS	-0.064 (-0.191-0.063)	0.308	-0.173 (-0.468-0.122	0.238
sCD14	4.081 (0.546-7.617)	**0.025**	13.199 (4.988-21.410)	**0.003**

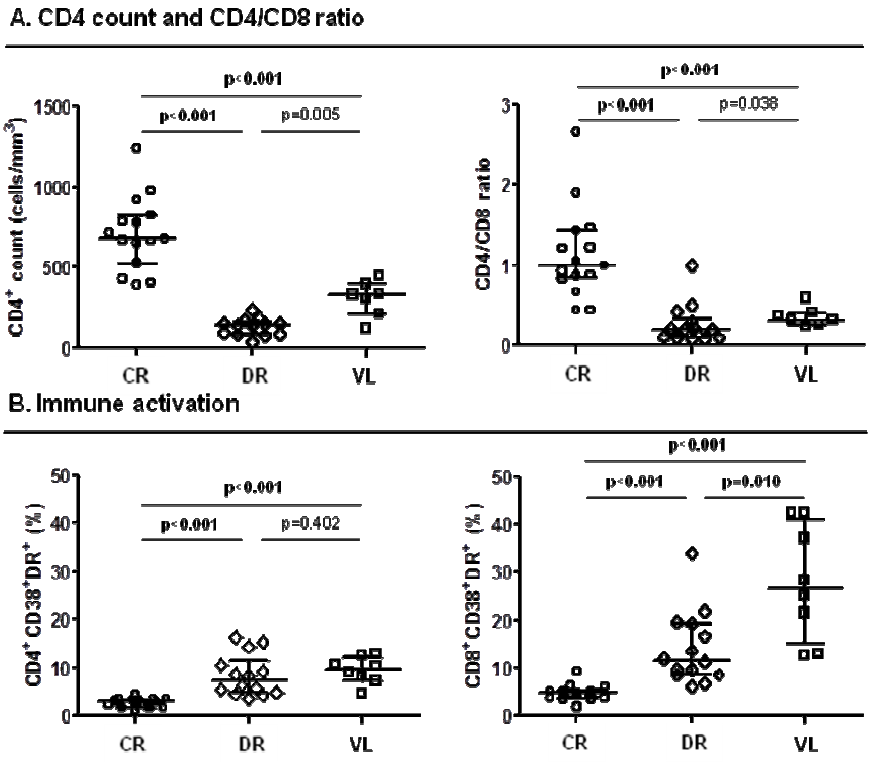

Figure 1: Levels in VL, DR and CR patients of CD4 count and CD4/CD8 ratio (A), and immune activation in CD4 and CD8 T cells (B). VL, visceral leishmaniasis patients; DR, discordant response patients; CR, concordant response patients. Significant when p<0.05.

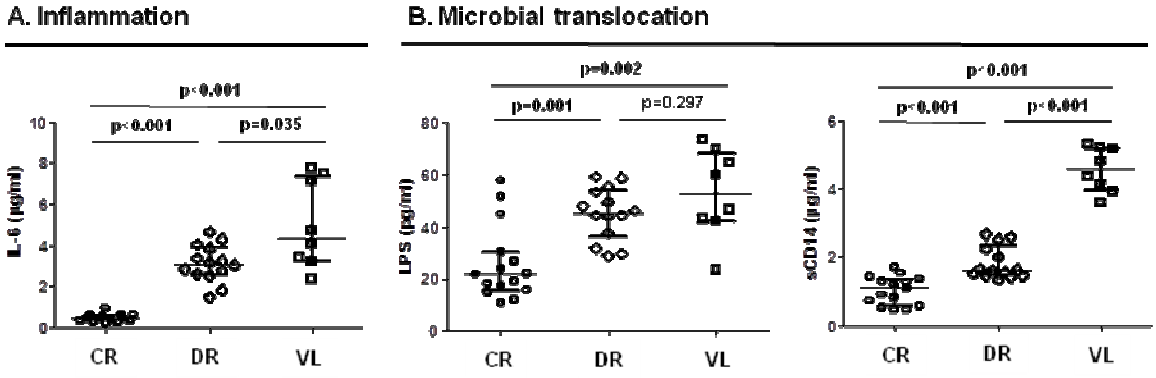

Figure 2. Inflamation (A) and microbial translocation (B) of VL patients and control groups. VL, visceral leishmaniasis patients; DR, discordant response patients; CR, concordant response patients. Significant when p<0.05.

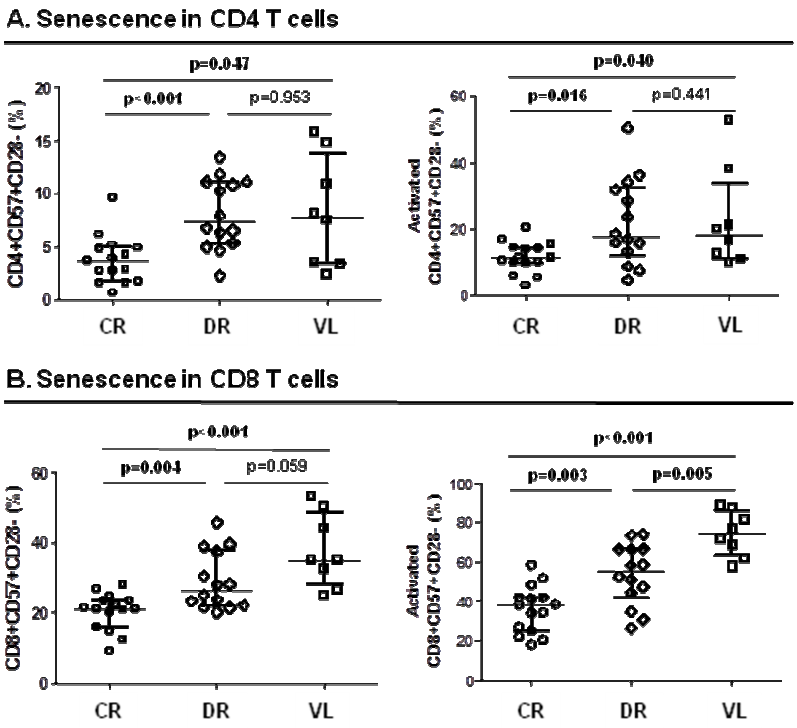

Figure 3: Levels of immunosenescence in CD4+ T cells (A) and CD8+ T cells (B). VL, visceral leishmaniasis patients; DR, discordant response patients; CR, concordant response patients. Significant when p<0.05.

High levels of CD4+ CTLA-4+ Treg cells and CCR5 density on T cells in HIV-1-infected patients with visceral leishmaniasis enable both persistence and relapses of the parasite

Alejandro Vallejo1, María Abad-Fernández1, Santiago Moreno1, Ana Moreno1, María J. Pérez-Elías1, Fernando Dronda1, José L. Casado1

1. Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Ramón y Cajal University Hospital, Madrid, Spain.

Running title: Immune impairment of VL/HIV-1 co-infection.

Corresponding authors:

José Luis Casado, M.D. Department of Infectious Diseases, Ramón y Cajal University Hospital, Ctra. Colmenar Km 9, 28034 Madrid, Spain. Phone/Fax: +34 913368672. e-mail: jcasado.hrc@salud.madrid

Alejandro Vallejo, Ph.D. Laboratory of Immunovirology, Department of Infectious Diseases, Ramón y Cajal University Hospital, Ctra. Colmenar Km 9, 28034 Madrid, Spain. Phone: +34 91 336 8711. Fax: +34 91 336 8792. e-mail: alejandro.vallejo@salud.madrid.org

ABSTRACT

Background: Visceral leishmaniasis (VL) in HIV-1-infected patients has been associated with poor immunological recovery and frequent disease relapses. The aim of this study was to analyze the role of changes in T cell populations, Treg cells and CCR5 density compared with immunodiscordant HIV-1-infected patients without leishmaniasis.

Methods: Cross-sectional study of eight VL patients receiving suppressive cART for at least one year, and both 14 HIV-1-infected patients with discordant immunological response (DR) and 13 HIV-1-infected patients with concordant immunological response (CR) without leishmaniasis. T-cell subpopulations, Treg cells, and CCR5 expression were analyzed.

Results: In VL patients, CD8 effector cells were higher compared to CR patients ($p=0.016$), and CD4 Treg cells were similar compared to DR patients, but higher compared to CR patients ($p<0.001$). Interestingly, CD4 Treg CTLA-4+ cells were higher compared to either DR or CR patients ($p=0.048$ and $p<0.001$). The CD4 Treg/TEM ratio was similar to DR patients but higher compared to CR patients ($p=0.038$). While CD4+CCR5+ T-cells was higher compared to the DR patients ($p=0.087$) and CR patients ($p<0.001$), CD8+CCR5+ T-cells was higher compared to CR patients ($p<0.001$). Interestingly, CCR5 density in CD4+ and CD8+ T-cells among VL patients were higher compared to both DR and CR patients ($p<0.005$ in all cases).

Conclusion: Leishmania/HIV-1-co-infected patients had worse immunological profile than the observed in immunodiscordant patients. A lower number of naïve T-cells, higher Treg inhibitory activity and a higher density of CCR5 receptors could explain the persistence and relapses of the pathogen and the associated immunodeficiency in these patients.

INTRODUCTION

Leishmania spp are intracelular protozoan parasites which cause two common diseases, i.e. cutaneous leishmaniasis and visceral leishmaniasis (VL). This last disease is common among HIV-1-infected individuals in the Mediterranean basin, including Spain with a prevalence of up to 9%, mainly among intravenous drug users, with a mortality rate of up to 50% (1,2). These protozoans are intracellular parasites that infect macrophage cell lineages from lymphoid organs and frequently produce persistent infection. Reactivation commonly occurs under conditions of immunossuppression associated with HIV coinfection.

The development of effector T cell response to leishmaniasis and how memory T cells are generated and maintained in humans is not fully understood.

Naturaly occurring regulatory T (nTreg) cells play a central role in maintaining self-tolerance. The regulation of adaptive immune responses is indispensable for the

effective clearance of antigen without harm to self tissues. Although the importance of Tregs in this process is well established, evaluation in human disease is challenging because of the increasing recognition of the complexity of their phenotype and function.

Persistent infections may represent a balance between pathogen and host. While the host attempts to maintain a strong effector immune function, pathogens strive to subvert host immunity to actively promote immunossuppression and pathogen persistence. nTreg cells may also suppress potencially beneficial immune responses, such as those directed against microbial pathogens as Leishmania and HIV.

In animal models, a definitive role of Treg cells in causing suppression of local effector immune response by altered ratio of Treg and effector T cells was reported (suffi). Moreover, studies on the Treg cells and their role in suppression of effector T cells response among human VL patients are limited.

Therefore, our objective was to analyze the balance of T cell subpopulation, especially effector memory cells, and to understand the status of Treg cells in terms of their frequency and function among VL patients. Since CCR5 expression on cells, including Treg cells, is essential for their trafficking from peripheral lymphoid tissues into infected sites driven by CCR5 ligands, we wanted to analyze whether the frequency and density of such receptor is altered in VL patients.

MATERIAL AND METHODS

Patients

A cross-sectional, descriptive, comparative study was performed in eight HIV-1-infected individuals with active visceral leishmaniasis (VL) from the Department of Infectious Diseases at the Universitary Hospital Ramón y Cajal, Madrid, Spain. All of them had been diagnosed for VL by parasitological diagnosis by direct visualization of amastigotes in bone marrow and peripheral blood, positive *Leishmania*-specific PCR on peripheral blood (21), as well as by clinical symptoms. All of them were treated with anti-*Leismania* treatment (liposomal amphotericina B, miltefosine or meglumine antimoniate, according to local guidelines (22)) and most of them received secondary prophylaxis based on monthly amphotericina B, or miltefosine). At inclusion, all of them had received cART for at least one year with sustained undetectable plasma HIV-1 RNA (<50 copies/ml).

As comparison groups of HIV-1-infected patients, we analyzed 13 patients with concordant response (CR patients, CD4 count above 400 cells/mm^3) and 14 patients with discordant response (DR patients, CD4 count below 250 cells/mm^3) with no coinfection with *Leishmania* and receiving suppressive cART (HIV-1 viral load lower than 50 RNA copies/ml) for at least one year.

A single blood extraction was performed in all the patients. Blood was collected in Vacutainer tubes (Becton Dickinson, Madrid, Spain) and processed for both plasma and cytometry analysis.

This study was carried out according to the recommendations of the Declaration of Helsinki and current Spanish legislation on clinical trials. It was approved by the AEMPS (Spanish Agency for Medications and Health Products) and our local Independent Ethics Committee (Hospital Ramon y Cajal, Madrid, Spain). All patients provided written informed consent for participation, sample collection, and laboratory determinations.

Flow cytometry analysis of blood samples

Fresh EDTA-anticoagulated whole blood was used to analyze $CD4^+$ and $CD8^+$ T cell subpopulations and quantify CCR5 density.

Antibodies were from Becton Dickinson (Becton Dickinson, NJ, USA), and an unstained control was performed for all samples. Briefly, 100 µL of blood were lysed with FACS Lysing solution (Becton Dickinson) for 30 min at room temperature, incubated with the antibodies during 20 min at 4°C, washed and resuspended in PBS containing 1% azida. Cells were analyzed in a Gallios flow cytometer (Beckman-Coulter, CA, USA). At least 30000 $CD3^+$ T cells were collected for each sample and analyzed with Kaluza software (Beckman-Coulter) initially gating lymphocytes according to morphological parameters.

T cells were stained to define subpopulations with the following antibody combination: CD3-allophycocyanin (APC)-Cy7, CD4-peridinin chlorophyll protein complex (PerCP), CD8-phycoerythrin (PE)-Cy7, CD45RA-phycoerythrin (PE), CCR7-allophycocyanin (APC). The different subpopulations were defined as follows: naive cells, $CD3^+CD4^+(CD8^+)CD45RA^+CCR7^+$; effector memory T cells (TEM) $CD3^+CD4^+(CD8^+)CD45RA^-CCR7^-$; central memory T cells (TCM) $CD3^+CD4^+(CD8^+)CD45RA^-CCR7^+$; and transitional memory T cells (TEMRA) $CD3^+CD4^+(CD8^+)CD45RA^+CCR7^-$.

Regulatory T cells (Treg) and their inhibitory function were analyzed using a combination of CD25-APC, FoxP3-PE (Human regulatory T cell staining kit, eBioscience), CD3-eFluor, CD4-PerCP and CD8-V500, CTLA-4-APC-Cy7 in permeabilized cells.

The frequency of T cells expressing CCR5 receptor was analyzed with CCR5-FITC, in combination with CD3-eFluor, CD4-PerCP and CD8-V500. Also, cell surface CCR5 density was quantified (Quifikit, Dako) using a series of 6 bead populations coated with different well-defined quantities of a mouse monoclonal antibody. The number of this monoclonal antibody molecules ranges from 0 to 500.000. Cells are labeled with primary mouse monoclonal antibody (purified CCR5, BD Pharmingen)) at saturating concentration. Then, cells are incubated, in parallel with the beads, with FITC-conjugated polyclonal anti-mouse immunoglobulins at suturing concentrations. A calibration curve is constructed using the fluorescence intensity of the individual bead populations against the number of molecules on the beads. The density on the specimen cells is then calculated by interpolation.

Statistical analysis

Continuous variables were expressed as the median and interquartile range and discrete variables as percentages. The *t* test for independent samples was used to compare normally distributed continuous variables and the Mann-Whitney test to compare non-normally distributed continuous variables. Categorical variables were described as proportions. The association between categorical variables was evaluated using the chi-square test. A Spearman correlation was used. Statistical analysis was performed using SPSS software 16.0 (Inc., Chicago, Illinois, USA).

RESULTS

Patients with VL had a median age of 45 years, similar to the age of the two control groups, with 62.5% of males. All but one were injecting drug users (IDUs) which is a different scenario compared to the risk factor of the other two control groups where patients with concordant response were predominantly men who have sex with men (MSM) (60%), and similar frequencies of IDUs and MSM were found within patients with discordant response (42.8% in both cases). Interestingly, while VL patients had significantly lower levels of CD4 count, CD4/CD8 ratio and CD4 nadir compared to CR patients ($p<0.001$, $p<0.001$, and $p=0.001$, respectively), they had higher levels of CD4 count and CD4/CD8 ratio compared to DR patients ($p=0.005$ and $p=0.038$, respectively), and no significant differences in nadir CD4 count. On the other hand, all three groups of patients had similar duration of both cART and viral suppression. Most of the VL patients were infected with VHC (75%). Interestingly, most of the VL patients had a time of diagnosis of VL similar to the duration of cART (75%). Clinical and laboratory characteristics are shown in **table 1**.

T cell subpopulations analysis

VL patients had significant lower levels of naïve $CD4^+$ T cells compared to both DR and CR patients ($p<0.001$ in both cases). This loss of naïve cells was recovered by a significant increase of TemRA ($p=0.042$) and an increase of TCM, although not significant ($p=0.059$). The level of TEM cells was not different compared to both DR and CR patients (**Table 2**).

In the same way, naïve $CD8^+$ T cells were lower in VL patients compared to DR and CR patients ($p=0.024$ and $p<0.001$, respectively). This was balanced by a significant increase of TCM cells ($p<0.001$ compared to DR and CR patients). Although TEM cells were not different from DR patients, they were higher compared to CR patients ($p=0.016$). No differences were found in the level of TemRA cells compared to any of the other two groups of patients (**Table 2**).

Regulatory T cells and immune inhibitory function

VL patients had no difference of either CD4 or CD8 Treg cells compared to DR patients. Nevertheless, they had significant higher levels of CD4 Treg cells compared to CR patients ($p<0.001$) (**Figure 1A**).

Interestingly, CD4 Treg CTLA-4^+ cells were significantly higher compared to either DR or CR patients ($p=0.048$ and $p<0.001$, respectively) (**Figure 1B**). No

significant differences were found in CD8 Treg CTLA-4$^+$ cells compared to either DR or CR patients.

The CD4 Treg/TEM ratio in VL was significantly higher compared to CR patients (p=0.038), but not different compared to DR patients. No differences were found in CD8 Treg/TEM ratio in VL compared to the other group of patients (**Figure 2**).

CCR5 frequency and receptor density

While the frequency of CD4$^+$ T cells bearing the CCR5 receptor in the VL patients was slightly higher compared to the DR patients, although not significant (p<0.087), it was significantly higher compared to CR patients (p<0.001). There was also a significant difference between the frequency found in DR patients compared to CR patients (p=0.005), lower in this last group (**Figure 3**). The frequency of CD8$^+$ T cells expressing CCR5 in VL patients was again similar compared to DR patients (p=0.137), but significantly higher compared to CR patients (p<0.001). The frequency was also significantly higher in DR patients compared to CR patients (p=0.046).

Interestingly, CCR5 density in CD4$^+$ T cells among VL patients was significantly higher compared to both DR patients (p=0.004) and CR patients (p<0.001). On the other hand, the difference of CCR5 density between DR and CR patients was not significant (p=0.060). CCR5 density in CD8$^+$ T cells was significantly higher in VL patients compared to both DR (p=0.003) and CR patients (p<0.001). In these cells, CCR5 density was significantly higher in DR patients compared to CR patients (p=0.028).

DISCUSSION

The frequent relapses observed in VL/HIV-1-infected patients could be evidence of a reduced ability to maintain the parasite under control due to an impaired immune function. Despite our results showed higher levels of TEM cells compared to CR patients but similar compared to DR patients, VL patients had higher inhibitory activity exerted by Treg cells and a higher CD4 Treg/TEM ratio compared to HIV-infected patients without leishmaniasis.

The lower level of naïve T cells found in VL patients may evidence a poor renewal of T cell repertoire that can fight against the parasite. On the other hand, this low level of naïve T cells could be a consequence of the infection of Leishmania into the bone marrow impairing the maturation or even favoring the premature death of stem cells.

Compared to DR patients, the naïve T cell deep depletion found in VL patients were balanced with two different mechanisms in CD4 or CD8 T cells, i.e., while TemRA increased in CD4 T cells, TCM increased in CD8 T cells. An expected increase in TEM cells, since they are coinfected with another pathogen, was not found in these individuals.

Other factor that could contribute to the inefficient cell immune responses to maintain Leishmania under control is that despite similar levels of Treg cells, VL

patients have higher levels of CD4 Treg inhibitory activity compared to other HIV-infected patients without leishmaniasis, according to other works (Rai AK 2012). This elevated inhibitory activity affects also to HIV infection deteriorating the T cell homeostasis since Treg suppression affects not only to CD4 T cells, but also CD8 T cells, B cells, and innate cells, impairing both Leismania and HIV infection. Persistent presence of Treg cells and their inhibitory activity may play critical role in persistence of residual parasite burden even after clinically effective treatment, which may result in the relapse of the disease.

T cell maturation from naïve to memory cells is characterized by an increase in CCR5 expression. Leishmania amastigotes might avoid activating macrophages by mimicking apoptotic cells and, therefore, inhibit macrophage activity by exposing phosphatidylserine (de Freitas). CCR5 plays a significant role in the entry and establishment of parasite infection in monocytes/macrophages.

CCR5 receptor is generally displayed on all types of activated T cells, and its role is particularly important in the recruitment of overall T cell population to in inflammatory sites. Furthermore, CCR5 expression results upregulated in both *in vitro* (Dasgupta) and *in vivo* in a number of co-infections characterized by the accelerated disease progression of HIV-1 infection (Nigro). The expression of CCR5 on CD4+ T lymphocytes of HIV-1-infected subjects has been proved to be strongly related to HIV viral load (Yang). Furthermore, in subjects expressing a high density of CCR5 co-receptor on CD3+ T cells, the progression of HIV-disease is accelerated in comparison with patients expressing a low quantity of CCR5 (Reynes 2). In our study, despite VL patients have similar levels of CCR5 on T cells compared to DR patients, but higher compared to CR patients, CCR5 density in T cells is higher compared to patients without Leishmania that might contribute to accelerate the progression of HIV disease and favors the infection of monocyte/macrophage by Leishmania.

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Table 1

	Visceral Leishmaniasis (VL) patients								N=8	DR patients N=14	p	CR patients N=15	p
	1	2	3	4	5	6	7	8					
Age (years)	55	45	44	43	41	51	45	51	45 [43-51]	47 [41-59]	0.664	45 [32-53]	0.728
Gender (male %)	M	M	M	F	F	M	M	F	62.5	92.8		86.6	
Risk factors (%)													
IDU	-	1	1	1	1	1	1	1	87.5	42.8		13.3	
MSM	-	-	-	-	-	-	-	-	-	42.8		60	
HSx	1	-	-	-	-	-	-	-	12.5	14.3		26.7	
CD4 count (cells/mm^3)	208	204	122	144	397	451	331	336	331 [145-667]	143 [88-159]	0.006	680 [527-825]	<0.001
CD8 count (cells/mm^3)	564	1449	207	455	1587	1107	1063	912	679 [546-1029]	691 [569-850]	0.815	675 [544-906]	0.776
CD4/CD8 ratio	0.37	0.21	0.59	0.23	0.25	0.41	0.31	0.37	0.42 [0.21-0.96]	0.19 [0.09-0.32]	0.035	1.0 [0.84-1.44]	<0.001
Nadir CD4 (cells/mm^3)	70	13	8	27	61	35	89	33	34 [16-68]	50 [26-99]	0.441	202 [62-317]	0.001
cART (months)	62	200	111	205	38	177	215	168	172 [74-203]	156 [97-197]	0.799	89 [43-144]	0.065
HIV-1 suppression (months)	59	125	26	203	16	28	76	126	59 [26-125]	69 [28-102]	0.799	63 [43-110]	0.824
HCV infection (%)	No	Yes	Yes	Yes	Yes	No	Yes	Yes	75	57		13.3	
VL diagnosis (months)	65	181	20	35	39	170	42	153	53 [36-165]	-		-	
Relapses of VL	2	27	2	3	3	9	1	7	3 [2-8.5]	-		-	

cART, combination antiretroviral therapy; CR, concordant response; DR, discordant response; significant when p<0.05, comparison VL vs CR and VL vs DR.

Table 2

	VL patients N=8	DR patients N=14	CR patients N=15	P value VL vs DR	P value VL vs CR	P value DR vs CR
CD4 T cell subpopulations						
Naive	8,42 [6,21-11,46]	15,25 [13,21-21,70]	33,40 [27,36-40,44]	**<0.001**	**<0.001**	**<0.001**
TCM	46,29 [45,88-48,30]	43,66 [24,20-46,41]	35,88 [26,35-41,86]	0,059	**0.002**	0.354
TEM	32,09 [30,64-34,90]	28,99 [25,13-50,17]	23,72 [22,09-26,71]	0,365	**0.001**	**0.008**
TemRA	11,51 [9,88-13,41]	9,86 [8,66-10,74]	5,20 [3,30-7,39]	**0.042**	**<0.001**	**<0.001**
CD8 T cell subpopulations						
Naive	10,03 [5,94-11,74]	14,56 [10,84-24,04]	26,72 [21,89-36,35]	**0.024**	**<0.001**	**0.001**
TCM	44,31 [43,68-44,89]	34,39 [29,24-37,26]	33,65 [28,51-35,23]	**<0.001**	**<0.001**	0.652
TEM	32,90 [30,26-35,04]	35,48 [26,27-38,73]	25,46 [22,50-30,21]	0.330	**0.016**	**0.007**
TemRA	14,11 [12,63-14,86]	14,24 [12,41-18,54]	11,93 [7,43-16,64]	0.664	0.548	0.146

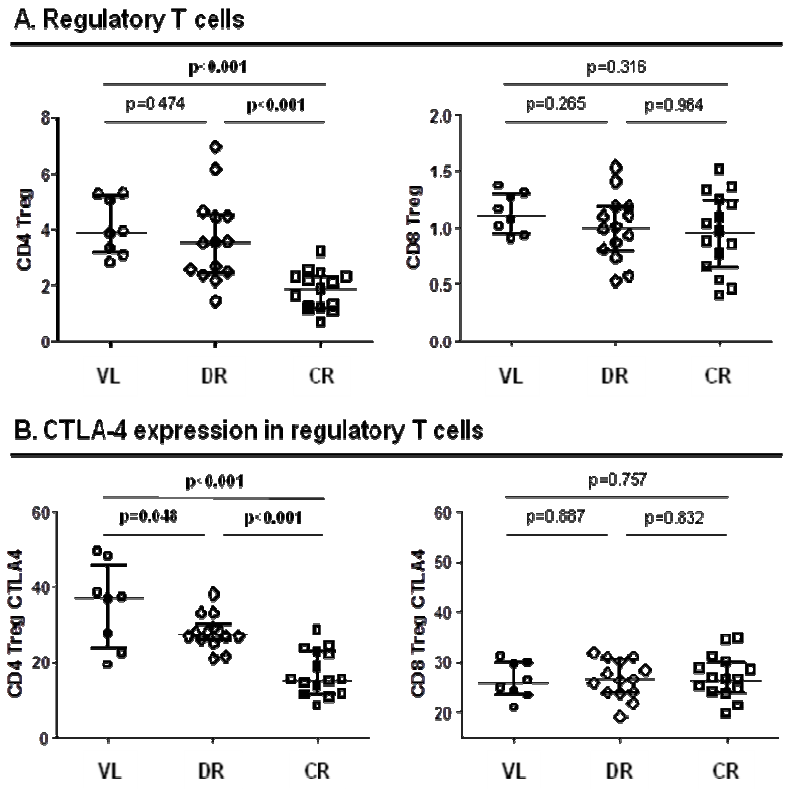

Figure 1: Regulatory T (Treg) cells (A) and Treg inhibitory activity (B) in patients with visceral leishmaniasis (VL), with immunodiscordant response (DR) and with concordant response.

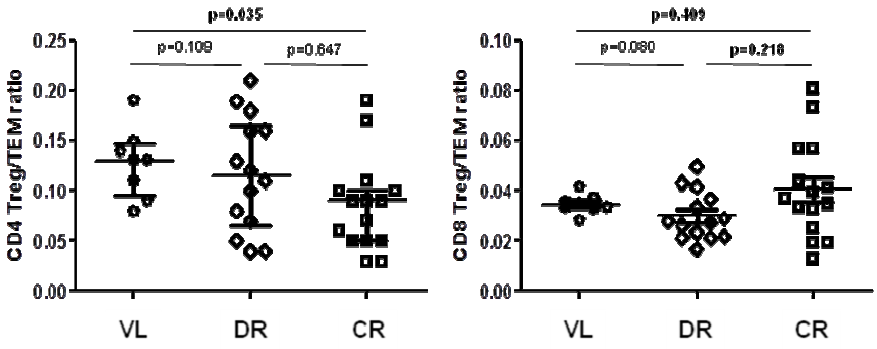

Figure 2: Treg/TEM ratio in CD4+ and CD8+ T cells in patients with visceral leishmaniasis (VL), with immunodiscordant response (DR) and with concordant response.

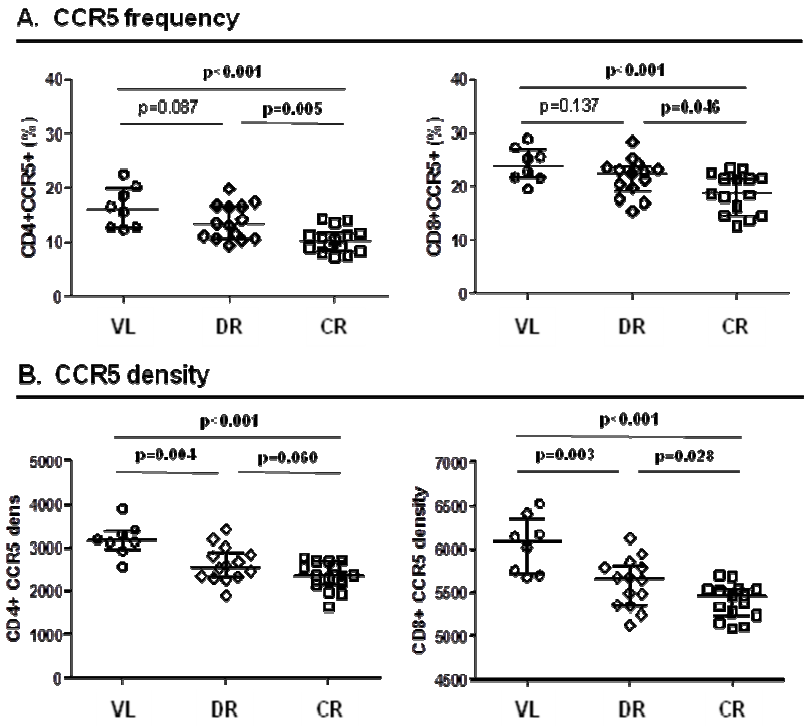

Figure 3: Frequency of CCR5+ T cells and receptor density in patients with visceral leishmaniasis (VL), with immunodiscordant response (DR) and with concordant response.