VITAMIN D EFFECT ON THE HIV-1 TRANSFER FROM DENDRITIC CELLS TO CD4+ T CELLS.

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DEDICATORIA

A la persona que me ha enseñado el amor más desinteresado que he conocido, que me ha querido incluso conociendo mi lado menos noble, que es protección y hogar y a la que le debo todo lo que soy; mi madre.

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GENERAL ABSTRACT

Background: HIV infection is one of the main health problems worldwide. Far from finding a preventive cure, the search of immunomodulatory compounds that may reduce the risk of acquiring the infection, or delaying AIDS progression, is one of the priorities in the field. The VitD could have a protective role in the context of the HIV infection; it has been observed that this hormone reduces the infection of $CD4^+$ T cells *in vitro,* and promotes the expression of antiviral factors. On the other hand, since this vitamin may decrease the activation and maturation profile of DCs, it is plausible to think that it could also reduce the ability of these cells to transfer viruses to $CD4^+$ T cells. However, the effect of the VitD in this critical step and the possible mechanism behind remains unknown.

Aim: To evaluate the effect of the VitD on the following parameters: i) the phenotype of DCs, particularly the activation/maturation markers; ii) the DCs capacity to transfer the HIV-1 to $CD4^+$ T cells, and iii) the expression of molecules related to HIV-1 transfer by DCs.

Methods: In this study 16 healthy donors were included. We differentiated immature and mature monocyte-derived dendritic cells (iDCs and mDCs respectively) from PBMCs or monocytes, in the presence/absence of VitD to carry on the following evaluations. The phenotype, using flow cytometry; their capacity to transfer HIV-1 in a co-culture with autologous $CD4^+$ T cells, using flow cytometry and ELISA, and the expression of genes related with viral transfer, potentially modulated by this hormone, using q-PCR. Finally, we evaluated the protein expression of DC-SIGN and SIGLEC-1, receptors associated with HIV-1 transfer, by flow cytometry.

Results: We observed that the iDCs and mDCs, differentiated in the presence of VitD, reduce the expression of activation and maturation markers. Likewise, a reduction in viral transference was observed in co-cultures performed with iDCs and mDCs, differentiated with this vitamin, by flow cytometry but not by ELISA. This reduction was not observed in trials performed with DCs treated only for two days with VitD precursor forms. On the other hand, we observed that both iDCs and mDCs, differentiated in presence of VitD, exhibit a similar gene expression profile. Lastly, we found a significant reduction in the expression of DC-SIGN and SIGLEC-1, by flow cytometry, in cells differentiated with this hormone.

Conclusion: Our findings suggest that, beyond the ability of the VitD in reducing the activation and maturation profile in DCs, it can also reduce HIV-1 transfer to $CD4^+$ T cells, probably by reducing the expression of DC-SIGN and SIGLEC-1. For our knowledge, the present work represents the first study using VitD as a modulator of HIV-1 transfer, mediated by DCs, and exploring the mechanism behind its effect.

SYSTEMATIC SUMMARY

This degree work consists of a general introduction (Chapter I), followed by three chapters that included two original articles (Chapters II and III) and one review paper (Chapter IV), and finally a general discussion (Chapter V), including a proposed model.

The first chapter comprises four main topics: First, a background, addressing the epidemiology of HIV-1 infection and the relevance of the research work. Second, general aspects of HIV-1 infection, including replication cycle facts, transmission mechanisms, and the immunopathogenesis. Third, dendritic cells and their role during HIV-1 infection including the characteristics of the subsets and the mechanisms involved in viral transference; and finally, the role of VitD during HIV-1 infection, in particular its immunomodulatory proprieties and the potential steps that could be targeted by this hormone for reducing mucosal viral transmission are addressed.

The second chapter consists of an original article that explores the *in vitro* effect of VitD precursor forms on DCs phenotype and on their ability to mediate viral transfer to $CD4^+$ T cells. In this article, published in Hormone Molecular Biology Journal, I had the opportunity to participate in the experimental development of some assays.

The third chapter corresponds to the main original article, summarizing most of the work carried out during my graduate studies. This paper, addresses the *in vitro* effect of calcitriol (the VitD active form) in the ability of DCs, differentiated in the presence of this hormone, to mediate HIV-1 transfer to $CD4^+$ T cells, exploring the mechanism behind its effect. It will be submitted to Scientific Reports journal.

The fourth chapter corresponds to a review paper, published in Frontiers in Immunology. It summarizes the potential beneficial effects of VitD supplementation in HIV-1 infected individuals, showing an approximation of the clinical application of this vitamin, as treatment, in the context of HIV-1 infection. This review represents a fundamental part of the master's process; it was the first approach to scientific writing.

Finally, the fifth chapter is a general discussion around the main findings of this research, proposing a model regarding the role of VitD on reducing HIV-1 transfer from DCs to $CD4^+$ T cells.

1. CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

The Human immunodeficiency virus 1 (HIV-1) infection remains a global public health issue that has been the cause of approximately 32 million (23.6 – 43.8) deaths since the begging of the pandemic. It is estimated that about 38 million people were infected worldwide in 2018, and 1.7 million new infections occurred, while in the same year, 770,000 people died from causes related to this infection [1]. In Colombia, there were around 160,000 people living with the virus in 2018, and approximately 6,900 acquired the infection [1,2].

Around the world, including Colombia, HIV-1 infection has a significant social impact and constitutes a considerable economic burden on the health system, highlighting the need to generate high-quality scientific knowledge that contributes to the well-being of the population through promotion, maintenance, and improvement of their health condition [2].

The major impact on the quality of life of HIV-1 infected people was the introduction of the combined antiretroviral therapy (cART). Nonetheless, the coverage of cART is not complete, and in 2018, only 23.3 million HIV-1-infected individuals were under cART globally [1]. Notably, cART has decreased deaths associated with the acquired immune deficiency syndrome (AIDS), reducing them by 33% in 2018 compared to 2010 [3]; in fact, cART is associated with an increase in the life expectancy of HIV-1-infected individuals [4]. Nonetheless, despite cART-induced viral suppression, these individuals have an increased frequency of non-AIDS conditions, such as cardiovascular, dyslipidemia, loss of bone mineral density, all of them associated with persistent inflammation [5,6]. This underlines the importance of searching for low-cost immunomodulatory therapies that can complement cART, further reducing viral burden, but mainly targeting the persistent inflammation.

A few decades ago, it was found that vitamin D (VitD) has pleiotropic effects on the immune system and on the antiviral response [7–9]. Consistently, *in vitro* studies in our group have shown the protective role of the VitD, by decreasing HIV-1 infection, promoting the production of antimicrobial peptides, and inducing an anti-inflammatory environment [7,10,11]. In addition, high levels of VitD and VitD receptor (VDR) have been found in HIV-1–exposed seronegative individuals (HESN) compared to seropositive and healthy individuals [12]. Certainly, it has been described that approximately 3% of the human genes have VitD response elements, and in particular, the presence of these elements has been demonstrated in genes that code for proteins involved in viral dissemination. Therefore, it is plausible that these proteins could be modulated by VitD, establishing new therapeutic targets.

1.2 General aspects of the HIV-1 infection

HIV is the etiologic agent of AIDS; it belongs to the *Retroviridae* family and the genus Lentivirus [13]. Two antigenically different types of HIV have been described, called HIV-1 and HIV-2, the latter characterized by producing a less aggressive disease, a seemingly slower progression towards AIDS and being restricted mainly to West Africa; therefore, this work is focused on HIV-1, the cause of the global pandemic [14,15].

During the HIV-1 replication cycle, the initial stage corresponds to the interaction between the glycoproteins gp120/gp41 of the viral envelope with its primary CD4 receptor and the coreceptors CXCR4 or CCR5 (according to viral tropism X4 or R5), located at the surface of the target cell. Then, there is a dissociation between gp120/gp41, allowing the insertion of a fusion peptide into the cell membrane, facilitating viral entry and the release of the virion components into the cytoplasm of the cell. Subsequently, the viral RNA is retro-transcribed by the viral reverse transcriptase (RT) that is packed within the virion, generating viral DNA that is imported into the cell nucleus where it is integrated into the host genome. At this point, the virus can remain in a period of "latency" with absence or low expression rate of some of its proteins, or it may start replicating, producing new viral progeny [16,17]. The duration of the HIV-1 replication cycle can take between 24 and 52 hours, depending on the cell type, the activation state and if the measurement is *in vitro* or *in vivo* [18,19].

HIV-1 infection can be acquired parenterally, transplacentally, or through mucosal tissue (oral, genital, intestinal, and conjunctive). The risk of acquiring the infection after exposure to the virus depends on factors such as the viral load of the source, viral fitness, the transmission pathway, and the presence of co-infections and inflammatory processes. Thus, there is a risk of 90% of transmission after a blood transfusion, between 10 and 35% in vertical transmission during pregnancy, childbirth or breastfeeding, and between 0.01 and 1% after a single sexual intercourse [20,21]. Of note, although sexual transmission represents a lower risk of transmission after a single exposure, the co-factors above mentioned increase the susceptibility up to 11-fold [22–24], partially explaining why sexual route remains the most frequent route of HIV-1 transmission, representing a challenge in controlling the incidence of this infection.

At genital mucosa, HIV-1 must pass the epithelial barrier, and for this purpose the virus takes advantage of the micro-abrasions occurring during the sexual encounter; also, the presence of intraepithelial $CD4^+$ T cells may favor the establishment of infection [25]. In addition, dendritic cells (DCs) also have an important role in viral

dissemination, as they can capture the virus, located at the genital surface, using their extensions that are emitted towards these cavities [26]. Once in the submucosa, the first round of replication may occur, generating new virions, for further dissemination. Mucosal-associated lymphoid tissue, particularly the gut (GALT), are sites of heightened viral replication, as they contain more than 60% of the body $CD4^+$ T cells, the vast majority of the effector memory profile [27]. The massive viral replication in GALT is also associated with the depletion of Th17 cells , which are essential to maintain homeostasis of the gastrointestinal tract [28]. These alterations lead to anatomical destruction and functional barrier alteration, resulting in translocation of microorganisms and their products to the systemic circulation. Pathogen-associated molecular patterns (PAMPs) are thus recognized by immune cells bearing pathogen recognition receptors (PRRs), generating a persistent and abnormal activation state that has additional deleterious effects, inducing immune exhausting, increase apoptosis and associated comorbidities [27]. The effector mechanisms of the immune system can partially control viral replication, allowing a temporary recovery of the blood $CD4^+$ T cell count, but not in GALT, whereas there is an establishment and increase of viral reservoirs. Subsequently, due to the inability of the immune system to completely eliminate the virus, chronic infection is established, eventually leading to the AIDS stage that is characterized by the appearance of opportunistic diseases and development of malignant processes [20,29,30].

1.3 Dendritic Cells and their role in HIV-1 infection

Dendritic cells exert multiple functions such as the detection of microorganisms, cytokine secretion, antigen processing, and T-cell priming. Thus, they play a crucial role in the development of immunological memory and tolerance [31]. In the periphery, DCs can be found in an immature state that is characterized by an increased ability to capture and process antigens. Once they capture the antigen, the maturation process begins while they migrate to the lymphoid organs to present the antigen. At this point, they express high levels of the costimulatory molecules CD40, CD80, and CD86, as well as the maturation marker CD83, promoting $CD4^+$ T cell activation [32].

Dendritic cells can be classified according to their morphology, origin, function, and anatomical location. However, the most extended DCs classification is based on their origin: i) from a common myeloid progenitor; plasmacytoid DCs (pDCs), and myeloid DCs (miDCs), also called conventional DCs ii) Langerhans cells (LC) with the earliest origin from primitive yolk sac hematopoiesis. iii) Monocyte-derived DCs (MDDCs) from monocyte lineage [33].

In humans, pDCs can be recognized by the expression of CD123 (IL-3R), CD303 (BDCA-2) and CD304 (BDCA-4 or neutrophilin-1) and are characterized by the expression of the Toll-like receptor (TLR) 7 and 9, and by the production of high quantities of type I and type III interferons (IFN) in response to viral infections [34]. There are two subsets of miDCs that are identified by the expression of CD1c (BDCA-1) and by the expression of CD141 (BDCA-3) (these cells also called DC2 and DC1 respectively) [33]. The miDCs CD1c+ secrete higher levels of IL-12 compared to miDCs 141+ and are good stimulators of naïve CD4+ T cells; they are also inducers of Th1, Th2, and Th17 responses [35,36]. The miDCs 141+ are characterized by the high capacity to cross-present antigens via class I MHC to activate CD8+ T cells and to promote T helper type 1 (Th1) and natural killer responses. Langerhans cells are located in basal epidermis and other stratified epithelia and express the C-type lectin langerin, and the invariant class I MHC molecule CD1a. Finally, MDDCs exist in human steady-state tissues, and during inflammation they expand considerably [33,37].

Since sexual intercourse is the main route of HIV-1 transmission, and due to the strategic location that DCs have in mucosal tissues, they are one of the first cells that get in contact with the virus after mucosal exposure [38]. Unfortunately, DCs play an antagonistic role in the context of the spread of HIV-1, since they can act as Trojan horses, capturing and transporting infectious viral particles, facilitating infection of $CD4^+$ T cells that are the primary target cells [39,40]. Therefore, DCs are critical in determining HIV-1 dissemination control or the establishment of the infection.

Although the rate of HIV-1 replication in DCs is 10 to 100 times lower than in $CD4^+$ T cells [41], infection is facilitated when the viral transfer occurs from a DCs to a $CD4^+$ T cell, compared to $CD4^+$ T cell infection with free viral particles [42]. The factors that explain the lower susceptibility of DCs to HIV-1 infection include: i) Reduced expression of the viral receptor and co-receptors [43], ii) Rapid degradation of internalized viral particles [40,44], iii) High expression of restriction factors that block viral replication, such as SAMHD1, APOBEC3F/G, and TRIM5α [45–47].

Respect to HIV-1 transfer from DCs to $CD4^+$ T cells, two models have been proposed:

1. Trans-infection, which occurs without the generation of new viral progeny in DCs [26]. In this case, DCs capture viral particles through receptors (described below) at the cell surface and subsequently transfer them to $CD4^+$ T cells, either by a virological synapse facilitated by the interaction between ICAM-1 (Intercellular Adhesion Molecule 1) in DCs and LFA-1 (Lymphocyte function-associated antigen 1) in $CD4^+$ T cells [39], or through secretion of exosomes [48]. In the first mechanism, the viruses that remained attached to the DCs membrane are

transferred once they are in proximity to the target cell. In the second mechanism, the viral particles are internalized in non-lysosomal compartments, preventing virus degradation, and are subsequently released into endocytic vesicles that fuse with the membrane of the target cell [49].

2. Cis-infection that occurs 24 hours after virus entry, in which there is a productive infection in DCs. In this case, the virus is transferred to $CD4^+$ T cells as a result of *de novo* replication. However, given the low frequency of productively infected DCs *in vivo,* the importance of this mechanism is controversial [44,50].

Two DCs receptors have been described as responsible for HIV-1 trans-infection; one of them is DC-SIGN, a C-type lectin, also denoted as CD209, which functions as an adhesion molecule required for the stabilization of the immunological synapse between DCs and $CD4^+$ T cells [51]. DC-SIGN also interacts with the HIV-1 gp120 surface glycoprotein, mediating viral transfer directly after the contact between DCs and the $CD4^+$ T cells at the virological synapse, or it can be involved in the internalization of the virion in protective endosomes [52,53]. The other receptor corresponds to sialic acid-binding immunoglobuline-like lectin 1 (SIGLEC-1) that binds to sialyllactose motifs in the HIV-1 membrane gangliosides [54].

It is important to highlight that the process of trans-infection during the virological synapse is more efficient than through the exosome secretion pathway [55]. Also, the dendrites formation is related to enhanced viral transference, suggesting that the mechanisms that allow the retention of the virus at the DC surface favor viral transmission [56]. Indeed, in a study of M. Ménager *et al*, it was observed that in the absence of the tetraspanin TSPAN7 (protein with multiple functions in adhesion, fusion, mobility and cell signaling, that facilitate the aggregation of other proteins at the cell membrane), dendrite formation and trans-infection are reduced [57]. In the same study, the process of silencing Dynamine- 2 (DNM2) (GTPase that controls endocytosis and the stabilization of actin networks) and TSPAN7 in DCs, induced the redistribution of viral particles in micropinosomes, reducing trans-infection. Altogether, this evidence indicates that the increase of endocytosis affects HIV-1 transference, while a stable formation of actin and dendrites in DCs allow viral permanence at the cell surface favoring its transference to $CD4^+$ T cells.

The maturation state, subtype, and localization of DCs are also critical factors to determine viral transference efficiency. Most evidence indicates that mature dendritic cells (mDCs) are better at transferring viral particles to CD4 + T cells by trans-infection [55,58], using mainly SIGLEC-1[59], which is preferentially expressed in these cells over DC-SIGN, mainly expressed by immature dendritic cells (iDCs) [54]. The mDCs has a lower degradation of internalized viral particles compared to iDCs [60]. Concerning to subtypes, LCs are less efficient in viral transference because HIV-

1 captured by langerin is internalized into Birbeck granules and partially degraded [61]. Likewise, in pDCs, the type I IFN production is induced by HIV-1 infection, resulting in the inhibition of viral replication [62].

On the other hand, MDDCs degraded most of the incoming viruses within the first 24 hours, suggesting that any virus transmitted from MDDCs to $CD4^+$ T cells must be a newly synthesized progeny or correspond to viral particles kept attached to the MDDCs membrane [63]. This last DCs subset has been widely used as a model to study many diseases, including HIV-1, in particular the process of viral transference from DCs to $CD4^+$ T cells *in vitro* [40]. Given the low frequency of DCs in blood, MDDCs are obtained in sufficient quantities after stimulating blood-derived monocytes with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In fact, MDDCs has similarities with miDCs in phenotype and function, since both express SIGLEC-1 and DC-SIGN [64,65], and possess potent $CD4^+$ T cell stimulatory capacity; in addition, MDDCs are able to directly cross-present cell-associated antigens to naïve T cells, inducing CD8+ T cell priming and activation [66,67]. Concerning anatomical location, the use of MDDCs in the context of the HIV-1 infection is pertinent, since it has been described that, at the female reproductive tract, a key subpopulation of DCs, derived from monocytes, capture and respond quickly to HIV-1, validating their importance during HIV-1 infection [68].

From the above, one can conclude that DCs have a crucial role in HIV-1 dissemination, underlying the importance of accurately defining this process for exploring new control or preventive strategies. Despite intensive research in this area, there is still much to learn, regarding the process of HIV-1 transfer from DCs to $CD4^+$ T cells, mainly considering that viral transfer mechanisms occur simultaneous and related to cellular complex processes.

1.4 Vitamin D and its role in HIV-1 infection

Vitamin D is a hormone that can be obtained from synthesis dependent on ultraviolet B (UVB) light from the sun; also from different food and nutritional supplements [69]. The synthesis under the influence of UVB light represents 90% of the source of this vitamin and is obtained by transforming at the skin the 7-dihydrocholesterol to an inactive form called pre-vitamin D3 and then to vitamin D3 or cholecalciferol. Subsequently, the cholecalciferol is hydroxylated in the liver, to obtain calcidiol (25 (OH) D), through the action of the 25-hydroxylase enzyme that is mainly encoded by the CYP2R1 gene, or alternatively by isoforms encoded by the CYP27A1, CYP3A4, and CYP2J3 genes. Then, the metabolism of this vitamin continues in the kidney, where the enzyme 1α-hydroxylase, encoded by the CYP27B1 gene, transforms

calcidiol into 1,25-dihydroxycholecalciferol (1,25 (OH) 2D), which is the physiologically active form of VitD, also known as calcitriol. On the other hand, the enzyme 1,25-dihydroxyvitamin D3 24-hydroxylase, encoded by the CYP24A1 gene, is responsible for initiating the degradation of calcitriol through hydroxylation of the side chain to form calcitroic acid, thus allowing its regulation [70,71]. Calcitriol is the ligand for the VitD receptor (VDR), found in the cytoplasm. Once attached to the VDR, this complex translocates to the nucleus, where it binds to the X retinoid receptor (RXR), acting as a transcription factor that binds to specific sites in the DNA, known as VitD response elements (VDRE) [71,72].

In recent decades, the perspective of the classical function attributed to VitD has changed dramatically since it was found that VDR and hydroxylases, responsible for giving rise to the active metabolite of VitD, could be expressed in many cell subpopulations, particularly those of the immune system [7–9,11]. Since then, studies of the VitD, as a potent immunomodulatory agent, attracted significant attention. The mechanism in which this vitamin might regulate the immune system is through transcription factors such as the nuclear factor of activated T-cells (NF-AT) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or by induction of inflammatory cytokines. Since VDR and CYP hydroxylases, involved in the metabolism of VitD, are expressed in monocytes, DCs, B and T-cells, this hormone can exert multiple effects on both the innate and adaptive immune system. In the innate response, it promotes the antimicrobial effects of macrophages and monocytes, by inducing the transcription of antimicrobial peptides such as defensins and cathelicidin (CAMP) [73]. Additionally, recent studies indicate that the activity of natural killer (NK) cells and the phagocytic activity of macrophages is increased after stimulation with VitD [74,75].

Vitamin D can also influence the DCs profile, favoring an immature state with tolerogenic properties characterized by decreased expression of IL-12 and costimulatory molecules, and increased IL-10 secretion [76–78]. These effects, in turn, promote a regulatory profile in $CD4^+$ T cell either by the direct action of IL-10 or by the lack of stimulation by DCs. Additionally, VitD has marked effects by suppressing the chemokines CCL17 and CCL22 in human miDC, and also by decreasing the expression of CCR7, a marker of homing and maturation in human miDC. On the other hand, no effect of this hormone has been observed in pDCs or in their ability to produce type I IFN [79]. In another study on human miDCs treated with calcitriol, a decrease in the nuclear translocation of the p65 subunit of NF-κB was observed, which may partly explain its anti-inflammatory role [80].

The VitD insufficiency has also been associated with disease progression in HIV-1 infected individuals. Several studies have shown that they are more susceptible to hypovitaminosis D, which is defined by serum levels of calcidiol (25 (OH) D) below

30 ng/L [69,81,82]. This condition is related to severe comorbidities; for instance, up to 60% of HIV-infected individuals develop osteopenia, and 15% of them may present osteoporosis [83]. Also, there is a relation between VitD insufficiency and cardiovascular diseases in this population [5]; additionally, HIV-infected patients with VitD deficiency exhibit more severe *Mycobacterium tuberculosis* disease [84]. The relationship between VitD insufficiency and $CD4^+$ T cell count, viremia, and advance disease remains unclear, although several studies have shown a positive association between the increase of calcidiol levels in plasma and the recovery on $CD4^+$ T cells count [85,86].

The origin of VitD insufficiency in individuals with HIV-1 is not entirely understood, but some evidence suggests that antiretroviral drugs, such as protease inhibitors, may decrease calcitriol levels due to suppression of the 1α-hydroxylase activity [81,87]. Another possible mechanism is the increase in the use of this vitamin for maturation and proliferation of T-cells during HIV-1 infection. In addition, proinflammatory cytokines, which are increased during this viral infection, might block Parathyroid hormone secretion, blocking renal hydroxylation of calcidiol [88–90].

Since several studies indicate a poor prognosis of HIV-infected individuals who have hypovitaminosis D, supplementation trials with this vitamin could have a potential benefit on immune reconstitution and prevention of comorbidities in HIV-infected individuals. In fact, some *ex vivo* studies also suggest potential beneficial effects of VitD supplementation in the setting of HIV-1 infection as we review in chapter 4. It has been found that this vitamin induces the expression of antiviral molecules, decreasing the expression of the viral coreceptor CCR5, and reducing the frequency of infected LT CD4 + [91]. In another study, the physiological concentration of calcidiol induced the production of the antimicrobial peptide cathelicidin (CAMP) in human macrophages coinfected with HIV-1 and *M. tuberculosis*, inhibiting the replication of both pathogens. Ligands of TLR8 inhibit HIV-1 through the mechanism of autophagy, which is dependent on VitD and CAMP [92]. Additionally, a positive correlation was found between the VDR expression and the expression of IL-10, elafin and cathelicidin in PBMCs from HESN [12].

The previous evidence suggests that VitD could be used in both HIV-1 pre-exposure therapy and as an adjuvant of cART, once the HIV-1 infection is established. However, much less is known on the effect of VitD during HIV-1 prophylaxis and what specific targets, related to infection control, could be modulated. Therefore, this area represents a promising research topic.

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1.6 PROBLEM DESCRIPTION

Although cART is efficient for the control of HIV-1 disease progression, the annual incidence of this infection remains high, with an increase in several comorbidities such as cardiovascular, renal, and hepatic diseases, most likely associated with the chronic inflammatory process. This underlines the importance of searching for adjuvant therapies to improve immune disturbances during this infection, as well as finding compounds for reducing the risk of HIV-1 transmission or viral replication.

Dendritic cells have a key role in HIV-1 infection since they transfer viral particles to CD4+ T-cells. Thus, compounds that can modulate this critical step is a relevant research field. In this sense, VitD could be a good candidate to study, since it promotes an immature profile of DC, which are less efficient for HIV-1 trans-infection. In addition, molecules required for trans-infection, such as TSPAN7, DNM2, DC-SIGN, and SIGLEC-1, among others, exhibit VDRE, suggesting that they could be modulated by this hormone.

Based on this evidence, we aimed to evaluate the effect of VitD on HIV-1 transfer from DCs to CD4+ T cells using an in vitro model of acute infection of primary cells. Also, the effect of Vit D on the expression of several genes that participate in this process, which exhibit VDRE, will be explored.

According to the results, this study might give the rationale for clinical studies evaluating the impact of VitD supplementation, as prophylactic or complementary therapy, in high risk or HIV-1 infected individuals.

HYPOTHESIS

VitD decreases the HIV-1 transfer from dendritic cells to $CD4^+$ T cells.

GENERAL OBJECTIVE

To evaluate the VitD effect on the HIV-1 transfer from DCs to $CD4^+$ T cells.

SPECIFIC OBJECTIVES

i. To evaluate the VitD effect on the activation/maturation of DCs.

ii. To evaluate the VitD effect on the DCs capacity to transfer the HIV-1 to $CD4^+$ T cells.

iii. To quantify the expression of molecules related to the HIV-1 transfer in DCs differentiated or not with VitD, by flow cytometry and q-PCR.

2. **CHAPTER 2: MAIN ORIGINAL ARTICLE IN PREPARATION:** This article includes the main experiments of the present degree work

Calcitriol decreases HIV-1 transfer from monocyte-derived dendritic cells to CD4$^+$ T lymphocytes, most likely, by decreasing DC-SIGN and SIGLEC-1

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ABSTRACT

Dendritic cells (DCs) promote HIV-1 transmission by acting as Trojan horses, capturing and transporting infectious viral particles, facilitating the infection of CD4$^+$ T cells. Vitamin D (VitD) decreases HIV-1 infection of CD4$^+$ T cells *in vitro*, most likely by decreasing activation and thus the susceptibility to infection; however, it is unknown if VitD could also decrease viral transfer from DCs to CD4$^+$ T cells. In this study, we co-cultured HIV-1-pulsed immature (iDCs) and mature (mDCs) monocytes-derived dendritic cells, differentiated or not in the presence of calcitriol (VitD active form), with VitD-untreated PHA-activated autologous CD4$^+$ T cells from 16 healthy donors. We observed that co-cultures performed with VitD-treated iDCs and mDCs significantly decreased the frequency of infected CD4$^+$ T cells, evaluated by flow cytometry. However, p24 levels, evaluated by ELISA, were not significantly reduced in culture supernatants. Regarding genes involved in HIV-1 transfer, in VitD-treated iDCs we observed a decreased expression of these genes compared to the control; moreover, VitD-treated iDCs and mDCs exhibit a similar gene expression profile, maybe related to a transcriptional balance achieved after a long treatment with VitD. Finally, we found that DCs differentiated with VitD reduced the surface expression of DC-SIGN and SIGLEC-1 receptors, widely associated with HIV-1 transfer, thus, it could be the most most likely mechanism by which VitD affects viral transfer.

Keywords: HIV-1, Dendritic cells, Vitamin D, HIV-1 transfer, DC-SIGN and SIGLEC-1.

INTRODUCTION

HIV-1 infection remains a global public health issue that caused of approximate 32 million deaths since the begging of the pandemic [1]. The failure to find a sterilizing cure has made the combined antiretroviral therapy (cART), the only current efficient alternative to treat infected individuals. Furthermore, far from finding a globañ preventive cure, the search of immunomodulatory compounds that may reduce the risk of acquiring the infection, or delaying AIDS progression, is one of the priorities in the field.

VitD arises as a good candidate since, beyond its role in mineral metabolism, it has anti-inflammatory [2,3] and antimicrobial functions [4,5]. Remarkably, higher VitD levels in plasma and expression of its receptor (VDR) in blood cells and mucosa were found

in HIV-exposed seronegative individuals (HESN) compared to seropositive and HIV-unexposed healthy subjects, suggesting that this vitamin is associated with natural resistance to HIV-1 infection [6]. Indeed, it has been observed that *in vitro* and *ex vivo* treatment with VitD reduces the frequency of HIV-infected $CD4^+$ T cells [7,8]. Although requiring confirmation, the reduction of immune activation and induction of antiviral genes expression seems to be part of the mechanisms behind the anti-HIV effect of VitD on $CD4^+$ T cells [8].

Since VitD may decrease the activation and maturation profile of DCs [9–11], it could reduce the ability of these cells to transfer viruses to $CD4^+$ T cells, contributing to reduce virus spreading, usually occurring during the initial stages of infection [12].

The viral transfer may occur by two mechanisms: *cis*- and *trans*-infection. *Cis*-infection may follow a productive infection in DCs, in which new viral progeny infects the $CD4^+$ T cells through the classical receptor molecules [13]. *Trans*-infection occurs when DCs transfer viral particles, without being infected, by keeping them attached to its cell membrane or by trapping them in non-lysosomal compartments until the viral synapse with $CD4^+$ T cells takes place [13]. In the *trans*-infection process, receptors such as the dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) and the Sialic acid-binding immunoglobulin-type lectin 1 (SIGLEC-1) are involved, which are mainly expressed in immature DCs (iDCs) and mature (mDCs) DCs respectively. Cellular processes and several proteins related to membrane trafficking, actin formation and stabilization, and vesicle and microtubule formation are critical to mediate *trans*-infection [14]. For example, DCs deficient in DNM2 and TSPN7, redistributed viral particles in pinocytic vesicles, exhibiting reduced trans-infection ability [14].

Based on this previous evidence, we evaluated the effect of VitD on HIV-1 transfer from DCs to $CD4^+$ T cells, using an *in vitro* model of acute infection. Monocytes-derived dendritic cells (MDDC) were stimulated with or without VitD. The relative expression of genes associated with vesicle trafficking and dendrites formation, potentially modulated by VitD, such as CD63, VAMP3, DNM2, MYO5, and TSPAN7 were evaluated. Finally, DC-SIGN and SIGLEC-1, key molecules mediating viral attachment were also explored.

METHODS:

Study population

Peripheral blood mononuclear cells (PBMCs) were isolated through a blood density gradient using the Histopaque reagent (Sigma-Aldrich) from 16 healthy donors from the blood bank of the “Escuela de Microbiologia, UdeA”, Medellín Colombia. The exclusion criteria were individuals with immunosuppressive or anticoagulant drug

treatments, who received dietary supplementation with VitD, or reported any chronic disease or pregnant women.

Preparation of monocyte-derived dendritic cells (MDDC)

Monocytes were obtained from PBMCs by a CD14 negative selection, using the Miltenyi, Human Pan Monocyte Isolation Kit, according to the manufacturer's instructions. The cells obtained were cultured for 6 days using RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotic (Penicillin-Streptomycin, ATB) (Sigma-Aldrich), GM-CSF at 75ng/mL and IL-4 at 50 ng/mL (Prepotech). Half of the cells were stimulated with 5×10^{-9} M of calcitriol (the active form of VitD) (Sigma-Aldrich) and the remaining cells with 0.01% vol/vol EtOH (Protokimica) as vehicle control. Half of the culture medium was changed every two days, maintaining the same concentration of its components. After 7 days of culture, and once the MDDCs were differentiated (hereafter referred as DCs), half of the cells from each treatment (VitD or EtOH) were treated with or without 5 µg/mL ultrapure lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 hours to obtain mature (mDCs) and immature (iDCs) DCs respectively. For practical purposes, all cells that were treated with LPS were named as mDCs regardless of the profile obtained after differentiating them or not with VitD.

Autologous $CD4^+$ T cells isolation and activation

Autologous $CD4^+$ T cells were obtained from frozen PBMCs by a CD4 negative selection, $CD4^+$ T cell Isolation Kit (Miltenyi Biotec), according to the supplier's instructions. The cells obtained were cultured for 48 hours in RPMI medium at 10% FBS, 1% ATB, supplemented with 8µg/mL phytohemagglutinin (PHA) (Sigma-Aldrich), and 50 IU/uL of IL-2 (Sigma-Aldrich) to activate them, increasing their susceptibility to HIV-1 infection.

HIV-1 transfer assay (co-culture):

VitD- or EtOH-treated mDCs and iDCs were pulsed for 2 hours with X4-tropic HIV-1 virions (13 ng of p24), obtained from supernatants of the H9HTLV-IIIB ell line (ATCC-CRL-8543). The mixture of viruses and cells were washed three times with PBS to remove non-absorbed virions, and DCs were co-cultured with activated autologous $CD4^+$ T cells in a 1:4 proportion (50.000 DCs and 150.000 $CD4^+$ T cells) in RPMI 1640 medium supplemented with 10% FBS and 1% ATB. After 72 hours, p24 levels were measured by ELISA (XpressBio, Frederick, MD) and by Flow Cytometry.

$CD4^+$ T cells infection with free viral particles

PHA/IL-2 activated $CD4^+$ T cells were pulsed for 2 hours with X4-tropic HIV-1 virions (13 ng of p24), then washed twice with PBS to remove non-absorbed virions. Cells were cultured for 7 days in RPMI 1640 medium supplemented with 10% FBS and

1% ATB. p24 levels were measured using the ELISA Kit XB-1000 (XpressBio, Frederick, MD), 3, and 7 days post-infection.

Flow cytometry:

For each assay, cell viability was tested with efluor 506 (eBioscience) Staining. Expression of activation and maturation markers in DCs was determined by staining with anti-HLA-DR FITC, anti-CD11c e fluor 450, anti-CD80 PE-cyanine5, anti-CD86 PE-cyanine7, anti-CD40 PE, and anti-CD83 APC (all from eBioscience). Also, the expression of DC-SIGN and SIGLEC-1 in DCs was determined using anti-CD209 Percp-cyanine5.5 and anti-CD169 super bright (eBioscience). The frequency of infected $CD4^+$ T cells was evaluated by detecting intracellular p24 by flow cytometry using the "Foxp3 staining kit" (eBioscience) and anti-p24 PE (Beckman-coulter, Brea, CA). Lectures were performed on an LSR Fortessa (BD) flow cytometer, and analysis was performed using the FlowJo v.7.6 software.

RNA extraction

RNA from cells was extracted with the Direct-zol RNA Kit (Zymo Research); following, DNAse I treatment (Thermo Fisher Scientific), the RNAs were retrotranscribed using a recombinant Moloney Murine Leukemia Virus retrotranscriptase (Thermo Fisher Scientific). Reverse transcriptase negative controls were performed to rule out contamination with genomic DNA in PCR amplifications.

Real-time PCR

A total of 7 genes implicated in HIV-1 transference; *CD63*, *VAMP3*, *DNM2*, *TSPAN7*, *MYO5*, *SIGLEC-1*, *DC-SIGN*, that according to the database of transcription factor binding profiles JASPAR, have possible VDREs, were selected for gene expression assays [14–16]. On the other hand, the gene expression of the viral restriction factors *APOBEC3G*, *TRIM5* and *SAMHD1* were evaluated in HIV-1 susceptibility tests of $CD4^+$ T cells. Real-time PCRs were performed using the Maxima SYBR Green q-PCR Master Mix 2x (Thermo Fisher Scientific), running melting curves to ensure specific amplification. The results are presented as the median of the relative expression units to the β-Actin reference gene calculated by the ΔCT method.

Statistical Analysis:

Data were analyzed on the GraphPad Prism V.7.05 software. Normality was tested by the Shapiro-Wilk test, and pairwise differences between treatments were tested by the paired t-test or Wilcoxon test, depending on the normality fulfillment. Correlations were evaluated using the Pearson coefficient rank (r). Since the number of cells of some of the 16 individuals was not enough to perform all the experiments,

sample sizes are specified in the figure legends. Results are presented as mean, and p-value < 0.05 was considered statistically significant.

RESULTS

VitD decreases maturation and activation in both iDCs and mDCs

According to previous evidences, VitD promote an immature profile in DCs with tolerogenic properties. We validated the effect of this vitamin, during the DCs differentiations process, by evaluating the expression of maturation and activation markers by flow cytometry, and using EtOH 0.01% as vehicle control. Firstly, the MDDCs cultured for 8 days with or without LPS at day 7 faithfully reproduce the two DCs phenotypes (mDCs and iDCs respectively) (Fig 1) required for subsequent experiments. As expected, VitD treatment reduced the expression of CD83, CD40, CD80, and CD86, measured by median fluorescence intensity (MFI), in both iDCs (67%, p= 0.0081; 67%, p= 0.0355; 70%, p= 0.0023; and 57%, p= 0.022 respectively) and mDCs (75%, $p < 0.0001$; 64%, p= 0.0625; 46%, $p = 0.0012$; and 40%, $p = 0.0132$ respectively) compared to EtOH treatment (Fig 2A-D).

Likewise, VitD treatment, during the differentiation process, also reduced the percentage of cells expressing the markers CD83, CD40, and CD80, but not those expressing CD86 in both, iDCs (90%, $p = 0.0025$; 83%, $p = 0.0252$; 36%, $p = 0.0248$; and 48%, $p = 0.519$ respectively) and mDCs (96%, $p = 0.0002$; 84%, $p = 0.0476$; 17%, p= 0.394; and 42%, p= 0.3125 respectively) (Fig 2E-F). Additionally, no significant differences were observed in the expression of these markers comparing iDCs and mDCs treated with VitD ($p > 0.062$ for each marker, Fig 2 C vs D and E vs F), suggesting that treatment with this vitamin generates a stable immature-like phenotype even after an inflammatory stimuli.

VitD reduces HIV-1 transfer from DCs to CD4$^+$ T cells, evaluated by flow cytometry.

Once it was confirmed that VitD reduced the expression of maturation and activation markers in DCs, we evaluated if this vitamin could modulate HIV-1 transfer from DCs to CD4$^+$ T cells, since iDCs have been associated with a lower ability of viral transfer [17,18]. For this purpose, we used an *in vitro* model, which consisted of HIV-1 pulsed VitD- or EtOH-treated iDCs and mDCs that were co-cultured with previously PHA-activated CD4$^+$ T cells. As described in a previous report from our team [19], no difference was found in viral transfer between iDCs and mDCs (1.69% p24+ CD4$^+$ T cells in iDCs vs 2.39% CD4$^+$ p24+ T cells in mDCs, p= 0.1053). However, VitD treatment in iDCs and mDCs significantly reduced the frequency of CD4$^+$p24$^+$ T cells

by 61% (*p*= 0.0049. Fig 3A) and by 38% (*p*= 0.0389. Fig 3B) respectively, compared to EtOH-treated counterparts, 3 days post-co-culture.

Likewise, the MFI of p24 in $CD4^+$ T cells co-cultivated with VitD-treated mDCs also decreased significantly by 20% (*p*= 0.0077. Fig 3C) but not in iDCs (4.5%, *p*=0.6979. Fig 3D). However, p24 levels in supernatants, detected by ELISA, were similar in co-cultures with VitD- and EtOH-treated DCs (Fig 3E and 3F).

The replicative capacity of HIV-1 in $CD4^+$ T cells did not correlate with the expression of restriction factors

The similar levels of p24 in culture supernatants, despite the reduced frequency of infected $CD4^+$ T cells after VitD treatment, could be explained by differences in the intrinsic susceptibility of $CD4^+$ T cells TO HIV-1 infection. To explore this hypothesis, we performed infection assays in $CD4^+$ T cells with free viral particles to evaluate gene expression of the viral restriction factors *SAMHD1*, *TRIM5*, and *APOBEC3G*. However, no correlations were found between the concentration of p24 (ng/mL) on day 3 (Fig 4A) or day 7 (Fig 4B) post-infection, with the expression of any of the restriction factors mentioned above, suggesting that expression of HIV restriction genes does not explain the similar p24 levels in supernatants.

iDCs and mDCs treated with VitD express similar levels of genes related to trans-infection

Because HIV transfer from DCs involved cell processes such as dendrites formation and vesicle trafficking, we analyzed the expression of some genes involved in these pathways by q-PCR, using RNA of VitD- or EtOH-treated iDCs and mDCs. We found a differential expression of these genes between EtOH-treated iDCs and mDCs (Fig 5. Solid white vs white dashed bars). In VitD-treated iDCs, a significant reduction by 48% in *DNM2* (*p*= 0.0202), 68% in *MYO5 (p*=0.0109), 54% *VAMP3 (p*= 0.0455), 53% *DC-SIGN* (*p*= 0.0318), and 92% *SIGLEC-1* (*p*= 0.001) was observed compared to EtOH-treated iDCs (Fig 5).

The VitD-treated mDCs increased the expression of some genes compared to the EtOH treated- mDCs, being significant for *CD63* and *TSPAN7* (183% *p*= 0.0399, 160% *p*= 0.0313 respectively. Fig 5 dashed bars). However, VitD treated- iDCs and mDCs maintain the gene expression at similar levels (Fig 5 Blue no dashed bars vs blue dashed bars).

DCs differentiated with VitD reduce the protein expression of DC-SIGN and SIGLEC-1

To go further in exploring the expression of DC-SIGN and SIGLEC-1 in VitD treated DCs, protein expression of both receptors was evaluated by flow cytometry in iDCs and mDCs, before being co-cultured with $CD4^+$ T cells. There was a significant reduction in the MFI and percentage of VitD-treated iDCs expressing DC-SIGN and SIGLEC-1 (Fig 6A, 6B and 6C) (DC-SIGN; MFI p= 0.0016, % p= 0.0182, SIGLEC-1; MFI p= 0.0026, % p= 0.0286) compared to EtOH treated iDCs. Similarly, in VitD-treated mDCs, there was a significant decrease in both MFI and in percentage (Fig 6D, 6E and 6F) (DC-SIGN; MFI p= 0.0009%, p= 0,015, SIGLEC-1; MFI p= 0.0007, % p= 0.0102). These results suggest that the expression of DC-SIGN and SIGLEC-1 in DCs can be modulated by VitD; most likely, resulting in a reduction in the viral transfer.

DISCUSSION

The search for immunomodulatory compounds that may reduce the risk of acquiring the infection is one of the priorities of the HIV research field. Based on previous evidence, showing the anti-HIV effects induced by the VitD on lymphocytes [7,8,20] as well as its tolerogenic properties on DCs [9], we evaluated the effect of this hormone on HIV-1 transfer from DCs to $CD4^+$ T cells, which is a critical step to establish the infection, using an *in vitro* model of acute infection.

Initially, we confirmed in our model that VitD significantly decreases the percentage and MFI of maturation and activation markers in DCs, in agreement with previous reports [10,11,21,22]. VitD-treated DCs maintained an immature-like profile even in the presence of an LPS stimulus.

Notably, by contrast with literature reports, in this study and in one, recently reported by us [19] there were no differences in viral transfer between iDCs and mDCs. One possible explanation for the discordant results might rely on differences in the tropism of the strain used and the sensitivity of the methods employed for detecting HV-1 infection. In fact, previous studies reporting higher HIV transfer to $CD4^+$ T cells by mDCs compared to iDCs used R5 strains and recombinant viruses with reporter genes [12,23]. Likewise, the duration of the co-culture assay also be also an important variable since in our model the co-culture time was 3 days, and it has been observed that iDCs are more capable of transferring HIV-1 by *cis*-infection, a mechanism that is favored after 24 hours; in contrast, trans-infection that occur in the first 24 hours is mostly used by mDCs [24].

More importantly, in the present study, we observed that when DCs were differentiated with calcitriol, the percentage of infected $CD4^+$ $p24^+$ T cells, evaluated by flow cytometry, decreased, regardless of LPS stimulus on DCs. However, the supernatants evaluated by ELISA showed no differences in p24 concentrations between co-cultures with EtOH- or VitD-treated DCs. These results might suggest that VitD can reduce viral transfer without altering the subsequent viral replication on pre-activated $CD4^+$ T cells. However, it is also possible that other variables had not allow observing differences in p24 levels between treatments; for example, a high productive infection in DCs, perhaps contributing to the p24 levels detected in the supernatants. In addition, the period of the co-cultures might have also influenced the results. To solve these hypotheses, it is necessary to carry out additional experiments with different co-culture times and with DCs treated with antiretrovirals to prevent their infection.

Viral replication on $CD4^+$ T cells seems not to be dependent on the viral restriction factors *SAMHD1*, *TRIM5*, or *APOBEC3G* since no correlation between their mRNA expression and p24 levels in supernatants of infected lymphocytes were found.

To elucidate the mechanism by which VitD-treated DCs decreased the frequency of infected $CD4^+$ T cells, the expression of some genes, previously reported to influence viral transfer, was evaluated [14,16,25]. Although all the evaluated genes, except *MYO5A*, are related to higher DCs mediated HIV-1 transfer [14], we observe that EtOH-treated iDCs present a higher relative gene expression compared to mDCs. This result could be related to the enhanced ability of iDCs to capture antigens, a process that also involve some of the evaluated genes. In addition, is important to note that even with an increase in the relative gene expression in VitD-treated mDCs of *CD63* and *TSPAN7*, genes that favor viral transfer, and a decrease in VitD-treated iDCs of *MYO5* that limit viral transfer, the overall result was a decrease in HIV-1 transfer. These results suggest that other mechanisms, not explored here, are involved in this process.

Additionally, we observed that VitD-treated iDCs and mDCs had similar relative expression levels for all genes, probably as a result of a transcriptional balance, achieved after the hormone treatment, related to a maturation and activation status more than by being a primary VitD modulation.

Finally, our results suggest that VitD can decrease the protein expression of SIGLEC-1 and DC-SIGN, receptors widely related to *trans*-infection [16,26], suggesting that this is at least one of the mechanisms by which VitD reduces viral transfer to $CD4^+$ T cells. To know if the VitD can impact additional routes is necessary to study more genes associated to related pathways.

To the best of our knowledge, this is the first study that relates VitD as a potential attenuator of the HIV-1 transfer process. This exploratory study serves as a basis to continue the investigation of the VitD as a potential therapeutic target for reducing the risk of acquiring HIV-1 infection. Particularly, considering that, beyond its anti-inflammatory and tolerogenic potential, related to HIV-1 resistance profiles in HIV-1-exposed seronegative individuals (HESNs) [27,28], this hormone might impact key steps associated directly with viral spread. Additionally, VitD could also have beneficial effects in HIV-1 infected individuals, since this hormone decreases the inflammation that is one of the main causes of the deleterious effects of the infection, pointing its likely use as an adjunctive therapy to cART. However, clinical studies exploring its therapeutic potential are required.

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Author contributions

Conceived and designed the experiments: NA, WAJ, and MTR. Performed the experiments and analyzed the data: NA. Contributed reagents/materials/analysis tools: NA, WAJ, and MTR. Wrote the manuscript: NA, WAJ, and MTR.

Competing interests

The author(s) declare no competing interests.

FIGURES AND LEGENDS

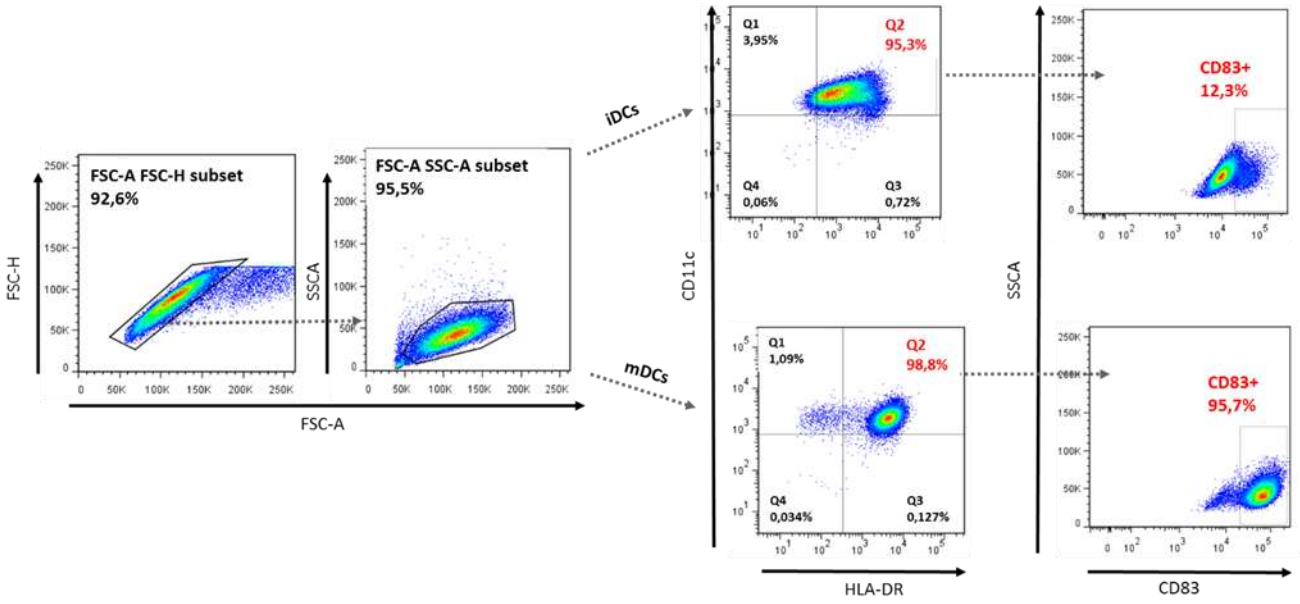

FIGURE 1. Representative gating strategy for the DCs population after 8 days of differentiation and maturation. Initially, the SSCA and FSC parameters were used to characterize the cellular physical properties, followed by a selection of the $CD11c^{+}$ $HLA\text{-}DR^{+}$ gate, corresponding to DCs. Mature (mDCs) phenotype of DCs was confirmed by the expression of the maturation marker CD83 after treatment with LPS at 5 µg/mL for 24 h. Non LPS-treated DCs were considered immature (iDCS). Indeed, the percentage (p = 0.0012) and median fluorescence intensity (MFI) (p = 0.0055) of CD83-expressing DCs increased significantly after the LPS treatment (mDCs) compared with those that were not LPS-treated (iDCs) (n=8), validating the presence of two DCs phenotypes (iDCs and mDCs) for the subsequent experiments.

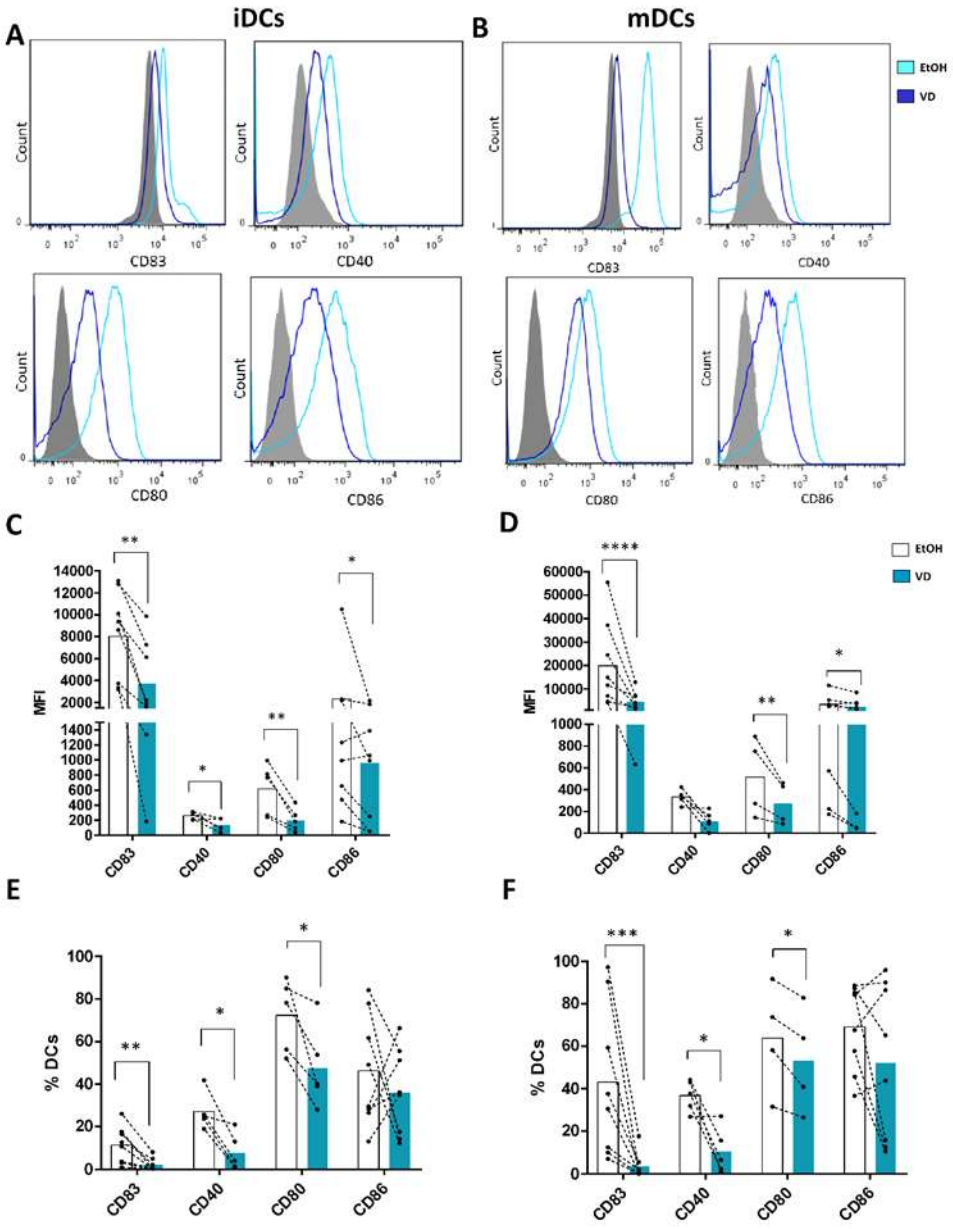

FIGURE 2. VitD reduces the maturation and activation profile in both iDCs and mDCs. iDCs (left panel) and mDCs (right panel) were differentiated in presence of 5×10^{-9}M of VitD or 0.01% vol/vol EtOH (control vehicle). (A and B) Representative overlay histograms comparing the expression of CD83, CD40, CD80 and CD86 markers on unstained cells (grey fill), VitD treated cells (dark blue lines), and EtOH treated cells (cyan lines) in both iDCs (A) and mDCs (B). In the bar graph, white bars correspond to cells treated with EtOH and blue bars to cells treated with VitD. MFI (C and D) and percentage (E and F) in iDCs and mDCs, correspond to the expression of different markers in 4 to 8 individuals. Statistical analysis was

performed using a Ratio paired test. Bars represent the mean,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

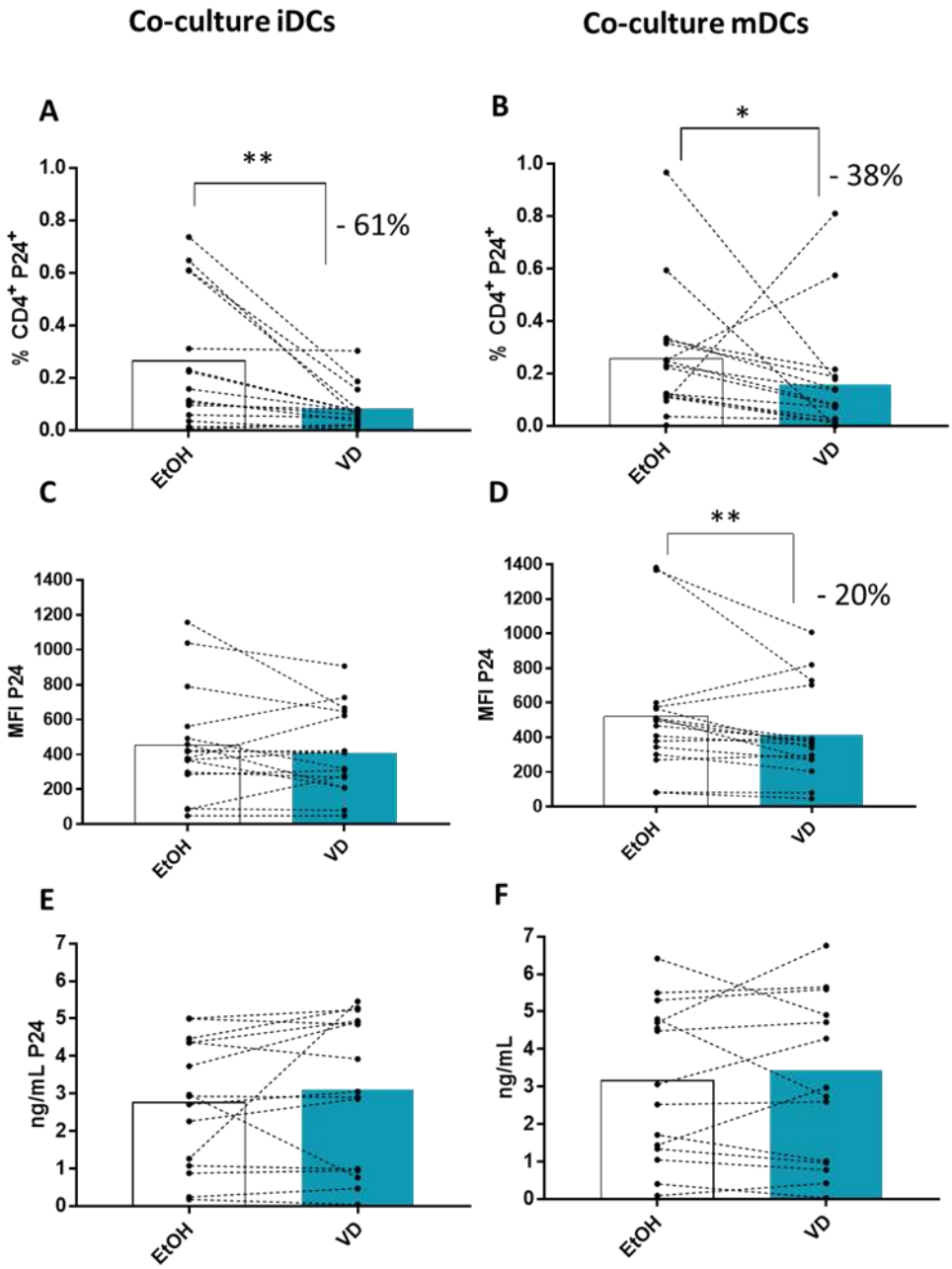

FIGURE 3. HIV-1 transfer from both VitD- and EtOH-treated iDCs and mDCs to CD4⁺ T cells. iDCs (left panel) and mDCs (right panel) were pulsed with X4 tropic virions (H9-HTLV-IIIB) and co-cultured with CD4⁺ T cells. After 72 h, the viral protein p24 levels were evaluated by flow cytometry (n = 16) and by ELISA (n = 15). The percentage of CD4⁺ p24⁺ T cells (A and B), p24 MFI (C and D) and p24 concentration

in ng/mL (E and F) in co-cultures with iDCs and mDCs were represented in bar charts, where white bars correspond to co-cultures made with DCs treated with EtOH and blue bars to those treated with VitD. The statistical analysis was performed using Ratio paired tests except for the percentage of $CD4^+$ $p24^+$ T cells and p24 concentration in co-cultures with mDCs where a Wilcoxon test was used due to non-normal distribution of differences. Bars represent the mean, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, and percent (%) decrease are depicted.

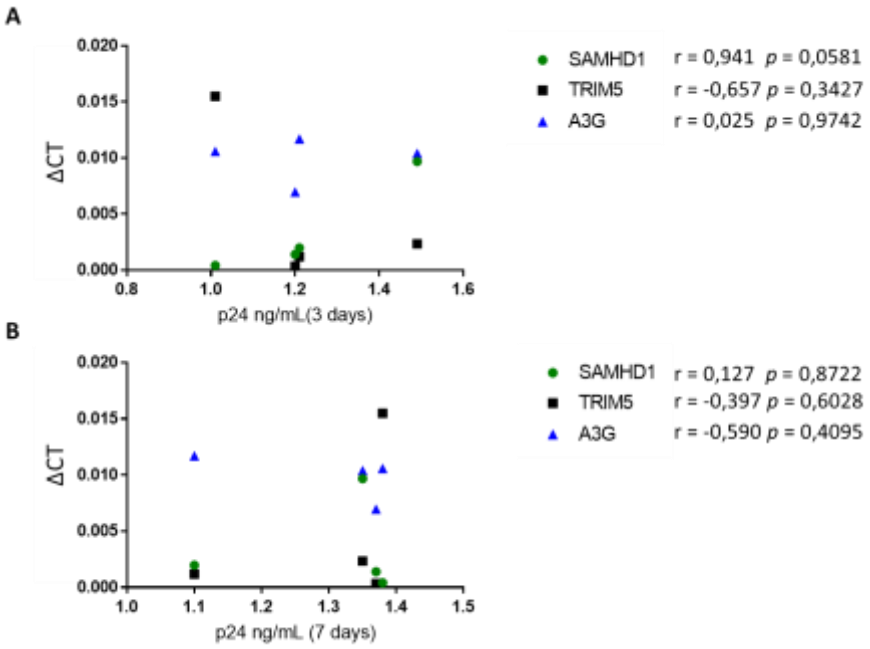

FIGURE 4. **Correlations between the concentration of p24 (ng/mL) and the relative expression of viral restriction factors in infected $CD4^+$ T cells.** The p24 levels were measured by ELISA test from the supernatant of the infected $CD4^+$ T cells cultures (n=4) at 3 (A) and 7 days (B) pos-infection. The gene expression levels for SAMHD1 (green circles), TRIM5 (black square) and APOBEC3G (A3G) (blue triangle) were measures by q-PCR using β-Actin as a reference gene. Correlations were evaluated using the Pearson coefficient rank (r).

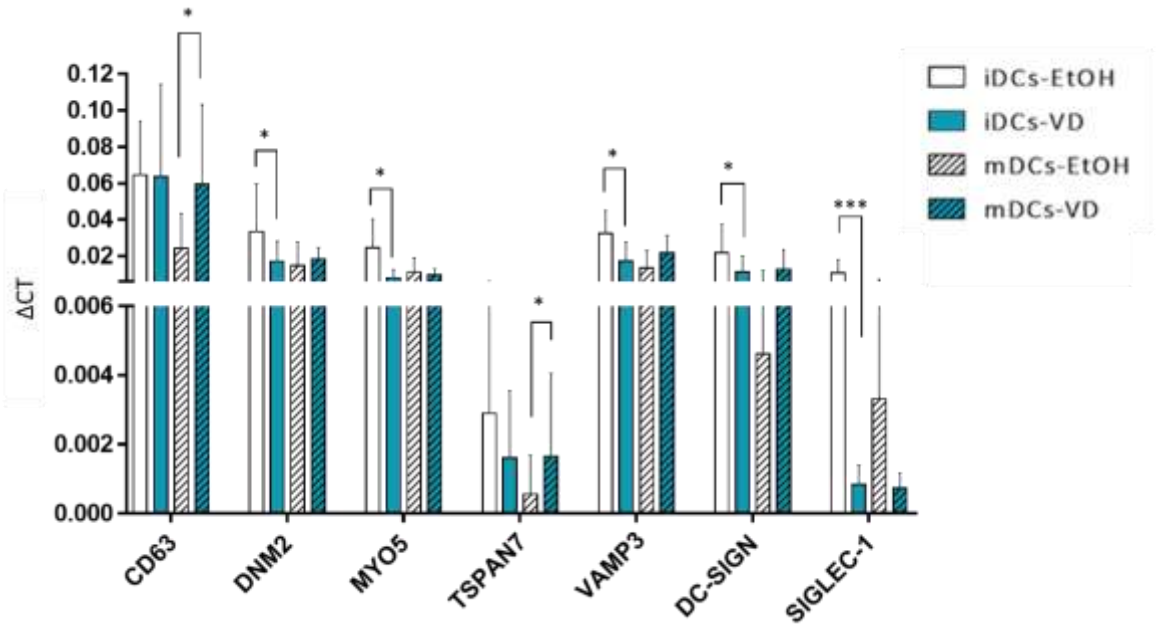

FIGURE 5. Relative expression of genes that participate in pathways related to trans-infection. The gene expression levels were measured by qPCR using β-Actin as reference gene, showing the results in bar graphics that represent the median of the relative expression in 7 individuals. Solid bars correspond to iDCs and dashed bars to mDCs, where white bars correspond to EtOH- treated cells and blue bars to VitD-treated cells in each one. The statistical analysis was performed by using a Ratio paired test; however, due to non-normal distribution of data a Wilcoxon test was used for the DNM2, TSPAN7, and VAMP3 analyzes mDCs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

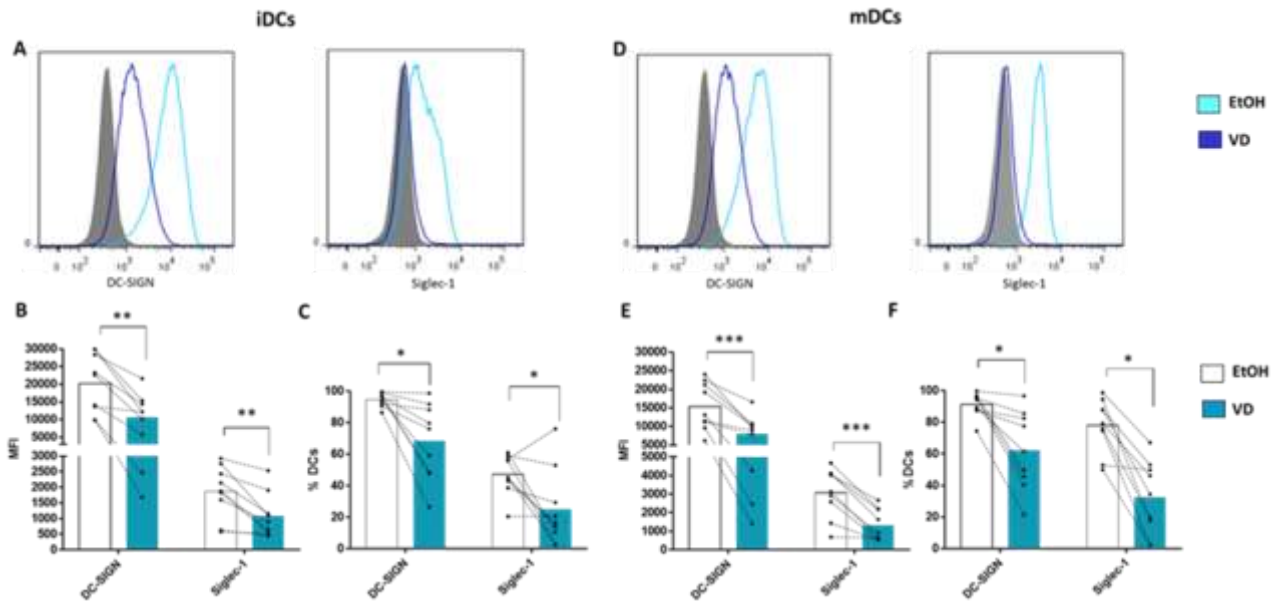

FIGURE 6. Expression of DC-SIGN and SIGLEC-1 in both VitD- and EtOH-treated iDCs and mDCs. (A and D) Representative overlay histograms comparing the expression of DC-SIGN and SIGLEC-1 on unstained cells (grey fill), VitD treated cells (blue lines), and EtOH treated cells (cyan lines) in both iDCs (A) and mDCs (D). In the bar graph, white bars correspond to cells treated with EtOH and blue bars to cells treated with VitD, where the MFI and percentage in iDCs (B and C) and in mDCs (E and F) of DC-SIGN and SIGLEC-1 are represented as the mean of 8 individuals. Statistical analysis was performed using a Ratio paired test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

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REVIEW
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3. CHAPTER 3: REVIEW PAPER

The Potential Protective Role of Vitamin D Supplementation on HIV-1 Infection

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HIV infection remains a global and public health issue with the incidence increasing in some countries. Despite the fact that combination antiretroviral therapy (cART) has decreased mortality and increased the life expectancy of HIV-infected individuals, non-AIDS conditions, mainly those associated with a persistent inflammatory state, have emerged as important causes of morbidity, and mortality despite effective antiviral therapy. One of the most common comorbidities in HIV-1 patients is Vitamin D (VitD) insufficiency, as VitD is a hormone that, in addition to its physiological role in mineral metabolism, has pleiotropic effects on immune regulation. Several reports have shown that VitD levels decrease during HIV disease progression and correlate with decreased survival rates, highlighting the importance of VitD supplementation during infection. An extensive review of 29 clinical studies of VitD supplementation in HIV-infected patients showed that regardless of cART, when VitD levels were increased to normal ranges, there was a decrease in inflammation, markers associated with bone turnover, and the risk of secondary hyperparathyroidism while the anti-bacterial response was increased. Additionally, in 3 of 7 studies, VitD supplementation led to an increase in CD4+ T cell count, although its effect on viral load was inconclusive since most patients were on cART. Similarly, previous evidence from our laboratory has shown that VitD can reduce the infection of CD4+ T cells *in vitro*. The effect of VitD supplementation on other HIV-associated conditions, such as cardiovascular diseases, dyslipidemia or hypertension, warrants further exploration. Currently, the available evidence suggests that there is a potential role for VitD supplementation in people living with HIV-1, however, comprehensive studies are required to define an adequate supplementation protocol for these individuals.

Keywords: HIV, vitamin D supplementation, comorbidities, immune modulation, metabolic homeostasis, antibacterial response, parathyroid hormone, bone turnover

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INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) infection is one of the most important public health problems worldwide, affecting approximately 38 million people and having caused over 32 million deaths. In 2018, 1.7 million people became infected, whereas 1 million died due to HIV-related causes (1). CD4+ T lymphocytes are the primary target cells of HIV, followed by dendritic cells, monocytes, and macrophages. The acute infection is characterized by the destruction of gut-associated lymphoid tissue (GALT) that harbors a high number of CD4+ effector memory

cells. Destruction leads to both anatomical and functional alterations of the gut mucosal barrier, facilitating the passage of commensal microorganisms into the circulation system, which in turn, promotes continuous immune activation. This process leads to immune exhaustion, or the inability to respond to infection leading to the destruction of the immune system and uncontrolled viral replication, resulting in increased tumor rates and opportunistic infections characteristic of acquired immunodeficiency syndrome (AIDS) (2, 3).

HIV-1 infection has also been associated with several metabolic disorders, including vitamin D (VitD) deficiency. Different studies have reported insufficient VitD levels [calcidiol serum levels <30 ng/mL (4–6)] in up to 100% of HIV-1 infected individuals and VitD deficiency [calcidiol serum levels <20 ng/mL (4–6)] in at least 30% of infected individuals (3). Even with combination Antiretroviral Therapy (cART), decreased VitD levels have been associated with comorbidities such as osteoporosis, cardiovascular diseases, type II diabetes mellitus, and infections (i.e., tuberculosis) (3, 7–10) all of which can be explained by looking at the immunomodulatory, anti-inflammatory, and antimicrobial properties of this hormone (11–13).

Alterations in VitD metabolism during HIV-1 infection is associated with an increase in proinflammatory cytokines which block the effect of the parathyroid hormone (PTH) and the hydroxylation of calcidiol in the kidney, preventing the synthesis of active VitD (14–17). Furthermore, certain non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) affect the function of hydroxylase enzymes from the Cytochromes P450 (CYP450) complex, inducing a marked decrease in calcitriol production, the active form of VitD (7).

Several trials have explored the beneficial effects of VitD supplementation in VitD deficient HIV-1 infected patients, focusing on the role of immune activation in HIV pathogenesis as well as the modulatory role of VitD. Therefore, this work aims to review the causes and comorbidities related to hypovitaminosis D during infection, with an emphasis on VitD supplementation in HIV-1 infected individuals. Consequently, we conducted a search using different databases such as PubMed, Scopus, Web of Science and Science Direct, with the search terms HIV-1 with vitamin D supplementation, cholecalciferol dose, vitamin D trial, cholecalciferol supplementation, and 25-Hydroxyvitamin supplementation. We excluded case reports, studies with <15 individuals, studies which supplemented with several micronutrients at once or did not report on VitD supplementation, as well as those that were conducted in a non-HIV population. In addition, to control for variability, a supplementation trial was also excluded due to low patient adherence (18).

COMORBIDITIES DURING HIV-1 INFECTION

While the current use of cART has dramatically decreased AIDS-related morbidity and mortality, its long-term use does not lead to viral eradication (19, 20) and is associated with side-effects (21) and viral drug-resistance (22), making long-term management of HIV-1 infection challenging to achieve. Moreover, persons living with HIV-1 often develop complications related to infection and treatment, with increased risk of complications associated with patient lifestyle, aging, and persistent inflammation (characteristic of HIV-1 infection). Complications include diabetes mellitus, chronic kidney disease, cardiovascular disease, and dyslipidemia (23), loss of bone mineral density (24), as well as a higher susceptibility to bacterial infections (such as Tuberculosis, a leading cause of death among people with HIV) (25, 26). However, to date, despite global efforts, interventions to effectively reduce HIV-related inflammation and comorbidities beyond effective and safer cART remain elusive.

The immunological component in HIV-1 pathophysiology suggests that endogenous immunomodulators, such as VitD, may have a beneficial impact on the infection. VitD is a hormone that, in addition to its physiological role on mineral metabolism, has pleiotropic effects on immune regulation. Indeed, one of the most frequent comorbidities during HIV-1 infection is VitD deficiency, highlighting a niche for a potential intervention which could significantly improve patients, health.

VITAMIN D

Metabolism and Function

Around 90% of VitD is obtained from UVB sunlight, with the remaining amount obtained from diet or nutritional supplementation (6). As was widely explained by a recent review by Jiménez-Sousa et al. (27), the natural process of VitD synthesis occurs in the skin by transforming 7-Dihydrocholesterol into vitamin D3 or cholecalciferol. Subsequently, cholecalciferol is hydroxylated to 25-hydroxycholecalciferol or calcidiol (25OHD) in the liver by the enzyme 25-hydroxylase, which is encoded by the CYP2R1 and CYP27A1 genes. Within the kidney, 1α-hydroxylase, encoded by the CYP27B1 gene, then transforms calcidiol into 1,25-dihydroxycholecalciferol (1,25 (OH) 2D), the physiologically active form of vitamin D (i.e., calcitriol). On the other hand, the enzyme 1,25-dihydroxyvitamin D3 24-hydroxylase, encoded by the CYP24A1 gene, is responsible for initiating calcitriol degradation and regulation.

Calcitriol is the ligand for the VitD receptor (VDR), which is located in the cytosol. Once calcitriol binds the VDR, the complex is translocated into the nucleus where it forms a secondary complex with the retinoid X receptor (RXR). Together, this complex acts as a transcription factor binding specifics sites within the DNA, known as VitD response elements (VDRE), which are located in a significant number of genes, emphasizing their essential role in gene expression regulation (16, 28–30).

VitD function is associated with mineral metabolism as well as bone maintenance. In these processes, VitD directly suppresses PTH release and regulates osteoblast and bone resorption (31). It also improves the absorption of calcium and phosphorus, promoting bone matrix mineralization. Clinical trials have demonstrated an essential role for VitD in preventing osteoporosis, bone breakage, and rickets (32).

Studies have also shown that VDR is expressed on pancreatic β cells as well as on adipocytes indicating a role for calcitriol in insulin secretion and insulin resistance (33). In *in vitro* and *in vivo* cancer therapy experiments, calcitriol has been reported to delay metastasis development by blocking the cell cycle, stimulating DNA repair, and inducing apoptosis (34, 35). VitD also plays a role in cardiovascular diseases, as VDR and CYP27B1 are expressed on myocytes and heart fibroblasts and the inhibition of VDR in mice has been correlated to cardiac hypertrophy (36).

Effects of Vitamin D on the Immune System

VitD influences both the innate and adaptive immune responses through the expression of its receptor on various immune cells such as monocytes, dendritic cells, and lymphocytes (37–40). VitD modulates the immune system by regulating transcription factors such as NF-AT and NF-kB, and by directly binding VDRE. During the innate response, VitD improves the antimicrobial effects of macrophages and monocytes by promoting transcription of antimicrobial peptides such as defensins (DEF) and cathelicidin (CAMP) (11). Recent research shows enhanced phagocytic and cytolytic activity in VitD-treated macrophages and NK cells, respectively (12, 41).

In addition, during the adaptive response, VitD decreases dendritic cell maturation, reducing the expression of MHC class II and their co-stimulatory molecules (CD40, CD80, and CD86) decreasing their ability for antigen presentation and T cell activation. Therefore, VitD promotes a tolerogenic immune status with a lower inflammatory response, indirectly influencing the polarization of T cells (13). In fact, VitD decreases IL-12 and IFN-gamma production, while increasing IL-10, favoring the development of Th2 and Treg cells over Th1 and Th17 (42, 43). As a result, it has been proposed that VitD promotes tolerance and controls exacerbated immune responses.

Effects of Vitamin D Deficiency During HIV-1 Infection

Low VitD levels affect individuals of all ages in the general population and is a global issue. Indeed, it has been reported that over 75% of the US population has VitD deficiency (42, 44). Although the VitD deficit is widespread, people living with HIV-1 are more susceptible to hypovitaminosis D, with up to 100% prevalence reported in some HIV-1 infected cohorts across the world; a condition that has been correlated with comorbidities in seropositive individuals (9). In this population, osteopenia and osteoporosis have also been associated with hypovitaminosis D in up to 60 and 20% of infected individuals, respectively (45). Likewise, VitD may also contribute to the increased risk of cardiovascular disease (CVD) reported among HIV-1 infected patients (46). A similar finding has been reported in individuals with diabetes mellitus (10, 47). Lastly, in HIV+ individuals with tuberculosis, VitD deficiency has been associated with a worse clinical outcome (48).

Even though previous studies have associated the levels of VitD with CD4+ T cell recovery in individuals on cART (9, 49), the relationship between VitD deficiency and CD4+ T cell count remains unclear. Moreover, HIV-1 viral load and disease progression have been positively associated with low levels of VitD. Therefore, it is plausible that VitD supplementation may have a beneficial effect on immune recovery, which could decrease comorbidities among HIV-1 infected individuals (50).

VITAMIN D SUPPLEMENTATION IN HIV-1 INFECTED INDIVIDUALS

Characteristics of the 29 VitD supplementation trials included in this review are listed in **Table 1**. These studies were carried out in HIV-1 infected individuals, mainly of African-American or Afro-descendants, followed by Caucasians, and had a greater representation of men (60%). The number of individuals recruited for each trial ranged from 17 to 365, all of which were supplemented orally with cholecalciferol (Vitamin D3), except in the study by Falasca et al. in which individuals were also administered supplements via the intramuscular route (59). In approximately half (55%) of the studies, individuals were adherent to a cART regimen, while in the remaining studies, more than 65% of individuals were under a cART regimen and had an undetectable viral load. Prior to supplementation, the average VitD levels were <20 ng/mL, supporting that HIV infected individuals usually suffer severe hypovitaminosis D.

The variables that had the most heterogeneity among study populations were geographic origin and age, although most of the studies were carried out in America and Europe with little representation of the African and Asian continents (**Table 1**). All age groups were represented, but several trials were focused on infected children and youth due to the expectation that the infection would last longer leading to chronic and more profound immune dysfunction. The main objective in most trials was to determine whether VitD supplementation allowed individuals to attain normal VitD levels in serum. In most of the studies (93%), the effect on comorbidities and the association with CD4+ T cell count and viral load was also evaluated.

Safe and Efficient Doses of Supplementation

Despite the fact that most HIV-1 infected individuals suffer from hypovitaminosis D, no optimal, and safe supplementation dose has yet been established for this population. Generally, a healthy person should consume between 400 and 600 IU (International Units) of VitD daily to maintain sufficiency. However, currently, the Institute of Medicine recommends a standard dose of 600 IU to maintain the requirements of 97.5% of the population, with 4000 IU as the maximum daily dose (51). The North American Endocrine Society recommends three times the standard dose for cART-adhering individuals living with HIV (6). However, nine trials exceeded the maximum limits without adverse effects or associated toxicity **(Table 1)**. Supplementation represents a risk when an individual has calcidiol (25 (OH) D) levels higher than 100 ng/mL or when serum calcium levels exceed 2.70 mmol/L (51). Usually, in these instances, the skeletal system, cell membrane permeability, and nerve impulses are affected, leading to muscle weakness or spasms, constant fatigue, kidney conditions, as well as digestive symptoms such as nausea

TABLE 1 | Vitamin D supplementation studies in HIV-1 infected individuals.

References	Age [mean (range)]	(n)	The dose used in the study. (*Normalized to daily dose*). (IU)	Control group	%Subjects on cART/ virological status	Country	Ethnic group	Efficacy of VitD to restore levels	Main results	Topic of interest
Schall et al. (51)	20 (9–25)	58	7,000 daily for 52 weeks	Placebo and before vs. after supplementation	>76/–	USA	84% Black, 16% Hispanic	High	Supplementation was efficient in most participants	Supplementation
Havens et al. (52)	(18–25)	169	50,000 monthly (1,667 daily) for 12 weeks	Placebo and before vs. after supplementation	100	USA	Black 52%, White 22%, Mixed 26%	High	Supplementation was efficient regardless of the cART regimen	Supplementation
Longenecker et al. (53)	47 (39–55)	45	4,000 daily for 12 weeks	Placebo and before vs. after supplementation	100/78% undetectable	USA	78% Black, 15% White, 4% Hispanic, 3% other	Low	Individuals had severe VitD deficiency and did not reach sufficient calcidiol levels. FMD did not change, while PTH levels decreased	Cardiovascular
Muhammad et al. (54)	33 (25–47)	165	4,000 daily for 48 weeks	Placebo and before vs. after supplementation	100 recently	USA	27% Black, 20% Hispanic, 31% White	High	Supplementation did not change the lipid or glucose profile after starting therapy	Metabolic dysregulation
van den Bout-van den Beukel et al. (55)	>18	20	2,000 daily for 14 weeks, then 1,000 daily 48 weeks	Before vs. after supplementation	90	Netherlands	–	High	Insulin sensitivity and PTH levels decreased at week 24 but then returned to baseline levels	Metabolic dysregulation
Chun et al. (56)	<25	102	4,000 or 7,000 daily for 12 weeks	Placebo and before vs. after supplementation	75/50% undetectable	USA	–	High	CAMP expression increased but only 52 weeks after follow-up	Antibacterial response
Lachmann et al. (57)	35	17	200,000 once (6,667 daily) for 4 weeks	Before vs. after supplementation. cART-Naïve and uninfected individuals	65/–	England	18% Black, 63% White, 9% Asian, 9% Indian	High	The levels of CAMP and MIP-β, associated with an anti-HIV-1 effect, increased. Supplementation modestly reduced CD38+ T-cell frequency in HIV-infected patients on cART	Antibacterial response, Immune modulation
Noe et al. (58)	46	243	20,000 weekly (2,857 daily) for 52 weeks	Before vs. after supplementation	100/–	Germany	–	42 –78%	Between 42 and 78% of the individuals reached sufficient VitD levels after supplementation. There was no change in CD4 T cell counts	Immune modulation
Falasca et al. (59)	45 (34–56)	153	300,000 intramuscular every ten months (1,017 daily) or 25,000 oral monthly (892 daily), for 40 weeks	Supplemented vs. unsupplemented individuals	100/–	Italy	White	30–50%	Oral supplementation was more efficient than intramuscular administration; there was no change in CD4 T cell counts	Immune modulation
Fabre-Mersseman et al. (60)	49 (41–54)	53	100,000 every 14 days (7,142 daily) for 48 weeks	Before vs. after supplementation and deficient vs. sufficient individuals	100/–	France	–	High	The activation levels decreased, and the CD4/CD8 T cell ratio increased	Immune modulation

(Continued)

TABLE 1 | Continued

References	Age [mean (range)]	(n)	The dose used in the study. (*Normalized to daily dose).* (IU)	Control group	%Subjects on cART/ virological status	Country	Ethnic group	Efficacy of VitD to restore levels	Main results	Topic of interest
Eckard et al. (61)	20 (15–22)	51	18,000 (642), 60,000 (2,142) or 120,000 (4,285) monthly for 52 weeks	Before vs. after supplementation	100/–	USA	86% Black	71–92%	High doses diminished immune activation and exhaustion	Immune modulation
Stallings et al. (62)	(5–25)	58	7,000 daily per 48 weeks	Placebo and before vs. after supplementation	76/–	USA	85% Black	33–40%	RNA viral load decreased with increasing 25(OH)D, and CD4% and Th naive% were increased; NK% decreased short–term	Immune modulation
Dougherty et al. (63)	19 (8–24)	44	4,000 or 7,000 daily, for 12 weeks	Before vs. after supplementation	82/47% undetectable	USA	Predominantly Black	81%	There was a minimal increase in % CD4+ T cell, a decrease in viral load and the activation profile of CD8+ T cells in individuals receiving cART	Immune modulation
Kakalia et al. (64)	11 (7–15)	53	5,600 or 11,200 weekly (800 or 1600 daily), for 24 weeks	Before vs. after supplementation and Supplemented vs. no supplemented individuals	79/–	Canada	64% Black	67%	67% of the individuals reached sufficient VitD levels after supplementation, but there was no effect on CD4 T cell counts	Immune modulation
Giacomet et al. (65)	19 (14–23)	48	100,000 every 3 months (1,190 daily) for 48 weeks	Placebo and Before vs. after supplementation	85/81% undetectable	Italy	Predominantly white. Black were excluded	80%	There was no effect on CD4+ T cell count. However, the Th17/Tregs ratio decreased	Immune modulation
Coelho et al. (50)	45 (38–50)	97	100,000 weekly (14,285 daily) per 5 weeks; then 16,000 weekly (2,285 daily) for 19 weeks	Before vs. after supplementation and deficient vs. sufficient individuals	100/–	Brazil	53% White	83%	There was an association between CD4+ T cell recovery and VitD increase. Efavirenz use was associated with a higher increase in VitD levels	Immune modulation, Supplementation in cART
Steenhoff et al. (66)	19 (5–60)	60	4,000 or 7,000 daily for 12 weeks	Before vs. after supplementation	100/81% undetectable	Batswana	Black	80%	Only two individuals exhibited hypercalcemia after supplementation. Higher levels of VitD were achieved in individuals treated with efavirenz or nevirapine, compared with individuals treated with PI	Supplementation in cART
Lake et al. (67)	49 (41–55)	122	50,000 twice per week (14,285 daily) for 5 weeks; then 2,000 daily for seven weeks	Before vs. after supplementation	100/–	USA	60% White	81%	Tenofovir use did not affect levels reached after 24 weeks of treatment	Supplementation in cART

(Continued)

TABLE 1 | Continued

References	Age [mean (range)]	(n)	The dose used in the study. (*Normalized to daily dose).* (IU)	Control group	%Subjects on cART/ virological status	Country	Ethnic group	Efficacy of VitD to restore levels	Main results	Topic of interest
Lerma-Chippirraz et al. (68)	47 (41–52)	300	16,000 weekly or every 2 weeks (2,285 or 1,142 daily) for 104 weeks	Before vs. after supplementation	95/–	Spain	84,3% White, 9% Hispanic, Black 3%	82%	In 67% of individuals with secondary hyperparathyroidism, PTH levels decreased	PTH levels
Bañón et al. (69)	44 (22–75)	365	16,000 monthly (533 daily) for 36 weeks	Before vs. after supplementation and Supplemented vs. no supplemented individuals	98/–	Spain	90% White, 1% Black, 9% Hispanic	81%	The risk of secondary hyperparathyroidism decreased	PTH levels
Pepe et al. (70)	50	60	600,000 once (5,357 daily) for 16 weeks	Before vs. after supplementation	100	Italy	White	High	PTH levels decreased, and VitD levels increased regardless of the cART regimen	PTH levels
Havens et al. (71)	(18–25)	169	50,000 monthly (1,667 daily) for 12 weeks	Placebo and before vs. after supplementation	100/–	USA	Black 52%, White 22%, Mixed 26%	High	PTH and bone turnover markers (BAP and CTX) decreased only in individuals supplemented with VitD while on tenofovir	PTH levels and Bone composition
Quirico et al. (72)	46 (35–57)	79	3,200 daily for 96 weeks	Before vs. after supplementation	100/–	Italy	White	100%	Supplementation did not affect the bone mass but decreased PTH levels	PTH levels and Bone turnover
Puthanakit et al. (73)	(12–20)	24	400 daily for 24 weeks	Before vs. after supplementation	100/–	Thailand	Asian	Low	There was an increase in the BMDZ–score	Bone turnover
Overton et al. (74)	33 (25–47)	165	4,000 daily for 48 weeks	Placebo and before vs. after supplementation	100/ recently	USA	33% Black, 37% White, 25% Hispanic	High	Supplementation plus the start of cART attenuated the increase in bone turnover markers	Bone turnover
Piso et al. (75)	43 (34–52)	96	300,000 once (3,500 daily) for 12 weeks	Before vs. after supplementation	76	Switzerland	–	High	Bone replacement markers (BAP, PYR and DPD) decreased	Bone turnover
Etminani-Esfahani et al. (76)	40 (31–49)	98	300,000 once (3,500 daily) for 12 weeks	Before vs. after supplementation	100/–	Iran	–	100%	Osteocalcin increased in Efavirenz-treated individuals indicating improvement of bone formation	Bone turnover
Arpadi et al. (77)	10 (6–16)	56	100,000 every 2 months (1,785 daily) for 48 weeks	Placebo and before vs. after supplementation	–/36% undetectable	USA	64% Black, 36% Hispanic	High	Supplementation with calcium and cholecalciferol did not affect bone mass accumulation, despite a significant increase in serum calcidiol levels	Bone turnover
Rovner et al. (78)	21 (5–25)	54	7,000 daily for 48 weeks	Placebo and before vs. after supplementation	76/–	USA	86% Black	Low	No change in bone composition in infected children and youth	Bone turnover

IU, International Units; cART, Combination Antiretroviral Therapy; VitD, Vitamin D; PTH, Parathyroid Hormone; FMD, Flow Mediated Brachial Artery Dilation; CAMP, Cathelicidin; HBD, Human Beta Defensins; MIP-1β, Macrophage Inflammatory Protein beta; PI, Protease Inhibitor; BAP, Bone-Specific Alkaline Phosphatase; CTX, Carboxy-terminal Collagen Crosslinks; BMDZ-score, Body Mass Index Z-Scores; PYR, Pyridinolines; DPD, Deoxypyridinium.

and vomiting. Nonetheless, all of the supplementation studies reported herein were shown to be safe.

In the studies reported in this review, the supplementation schemes varied regarding the dose and frequency of administration. To make the data more homogeneous for ease of comparison, daily doses were calculated according to the equivalent in weeks or months used in each trial **(Table 1)**. The daily dose ranged from approximately 400 to 14,000 IU, with 4,000 and 7,000 IU as the most common doses. The duration of each trial varied from 4 to 104 weeks. Although most of the doses increased VitD levels, sufficiency was challenging to achieve due to the severe deficiency suffered by the HIV-1 infected population. The use of 7000 IU daily was the most effective dose (51, 56, 60, 63, 66, 78), and restored sufficiency [defined as calcidiol serum levels >30 ng/mL (4)] in 80% of treated individuals with higher levels seen following 12 months of treatment (61). Only 2 of the 215 individuals treated with this regimen had calcidiol levels >90 ng/mL and hypercalcemia (66). Once sufficiency is attained, a maintenance dose guaranteeing stable circulatory VitD levels should be established. Since the follow-up period was short during each of the trials, the long term effects of supplementation are still unclear; therefore, further studies will be required to evaluate the safety of long-term use.

VitD supplementation trials can be confounded by several aspects such as the season in which the study is carried out (78) or skin pigmentation since sunlight can affect vitamin levels. A study performed by Dougherty et al. showed that calcidiol basal levels were lower in individuals in winter than in other seasons (63). Additionally, in a healthy population, individuals with darker skin were reported to require higher doses of cholecalciferol (up to 2000 UI/day) to achieve VitD sufficiency (79). Ancestry may also play a key role in affecting the efficacy of supplementation since a study from Botswana reported that the VitD binding protein (DBP) was lower in plasma of individuals of African descent (1.8 umol/L) (66) compared to those which had an Afro-American background (3.3 umol/L) (80). Other factors, such as drug use as well as malabsorption syndromes and other unknown side effects associated with HIV-1 infection can also affect the results of VitD supplementation. Of note, no ethnic bias was identified during the review of the aforementioned studies as most of the results were obtained in trials which included individuals with varying ethnicities.

The Effect of Vitamin D Supplementation on CD4+ T Lymphocyte Count and Viral Load

CD4+ T cell counts and viral load are essential indicators for determining the clinical course of HIV-1 infection. However, since the mechanisms by which VitD influences HIV-1 disease progression, morbidity, and mortality are poorly understood, further investigations are required.

Currently, studies have shown that in HIV-1 infected individuals, VitD insufficiency is associated with low CD4+ T cell counts. In Coelho et al. 88% of individuals who had a CD4+ nadir count <50 cells/mm^3 had VitD insufficiency, while only 6% of participants with a similar nadir had VitD levels within normal range (50). In the same study, 1 ng/mL of calcidiol (25(OH) D) was shown to increase CD4 cell count by 3,3 cells/mm3, suggesting a beneficial role of VitD supplementation on immune recovery. Eckard and Dougherty reported similar results, showing a significant increase in CD4+ count after supplementation (11, 63). Likewise, Stallings et al. reported a reduction in viral load following supplementation (62). However, in other studies, VitD supplementation did not affect CD4+ T cell counts (58, 59, 64, 66).

It is important to note that in supplementation trials in which an increase in the CD4+ T lymphocyte count was observed, participants had remained on a cART regimen; therefore, it has been challenging to establish a causal relationship between VitD supplementation, immune recovery, and virological control. However, in a supplementation study in which 9 of the individuals were not on cART, an increase in CD4+ T cell count and differences in virological control were not seen (63). Although these findings still need to be corroborated, this evidence suggests that VitD may enhance immune recovery and viral control in combination with cART and may serve as an adjuvant to current therapy. Furthermore, none of the supplementation trials reviewed herein reported secondary side effects, supporting the safety of VitD treatment.

Supplementation Effects on Immune Activation

HIV-1 infected individuals have significantly higher levels of immune activation, even with cART, compared to their uninfected counterparts (81). Additionally, hypovitaminosis D has been associated with an increase in inflammatory markers, both in the general population, and in HIV-1 infected individuals (82, 83), therefore VitD insufficiency may facilitate the persistence of systemic immune activation. Taking into account that immune activation is the main mechanism associated with HIV progression and its associated comorbidities, it is necessary to continue the search for immunomodulators that can return the host to an immune quiescent state. Accordingly, it is interesting to speculate the role that VitD may play in this regard since it has been shown to promote the differentiation of naive T cells into Tregs or Th2 cells, inhibiting the development of Th1, and Th17 cells (13, 84). In fact, Fabre-Mersseman et al. reported that, after supplementing VitD insufficient patients with a dose of 7000 IU daily, immune activation levels, determined by measuring the expression of CD38 and Ki67 in CD8+ T lymphocytes, were reduced and there was an increase in the CD4+/CD8+ T cell ratio (60).

Similarly, in a trial by Eckard et al. looking at different doses of VitD supplementation, CD4+ and CD8+ T cell activation, frequency of inflammatory monocytes (CD14+ CD16+), and expression of PD1+ (an exhaustion marker) in CD4+ T cells decreased significantly in individuals treated with 4000 IU daily for 52 weeks (61). These results are in agreement with those reported by Dougherty et al. which showed a decrease in the percentage of activated cytotoxic T cells (CD8+ CD38+ HLA DR+) following a daily dose of 4000 or 7000 IU of VitD for 12 weeks, (63). These results support VitD supplementation as an adjuvant during routine clinical care of HIV-1 infected patients.

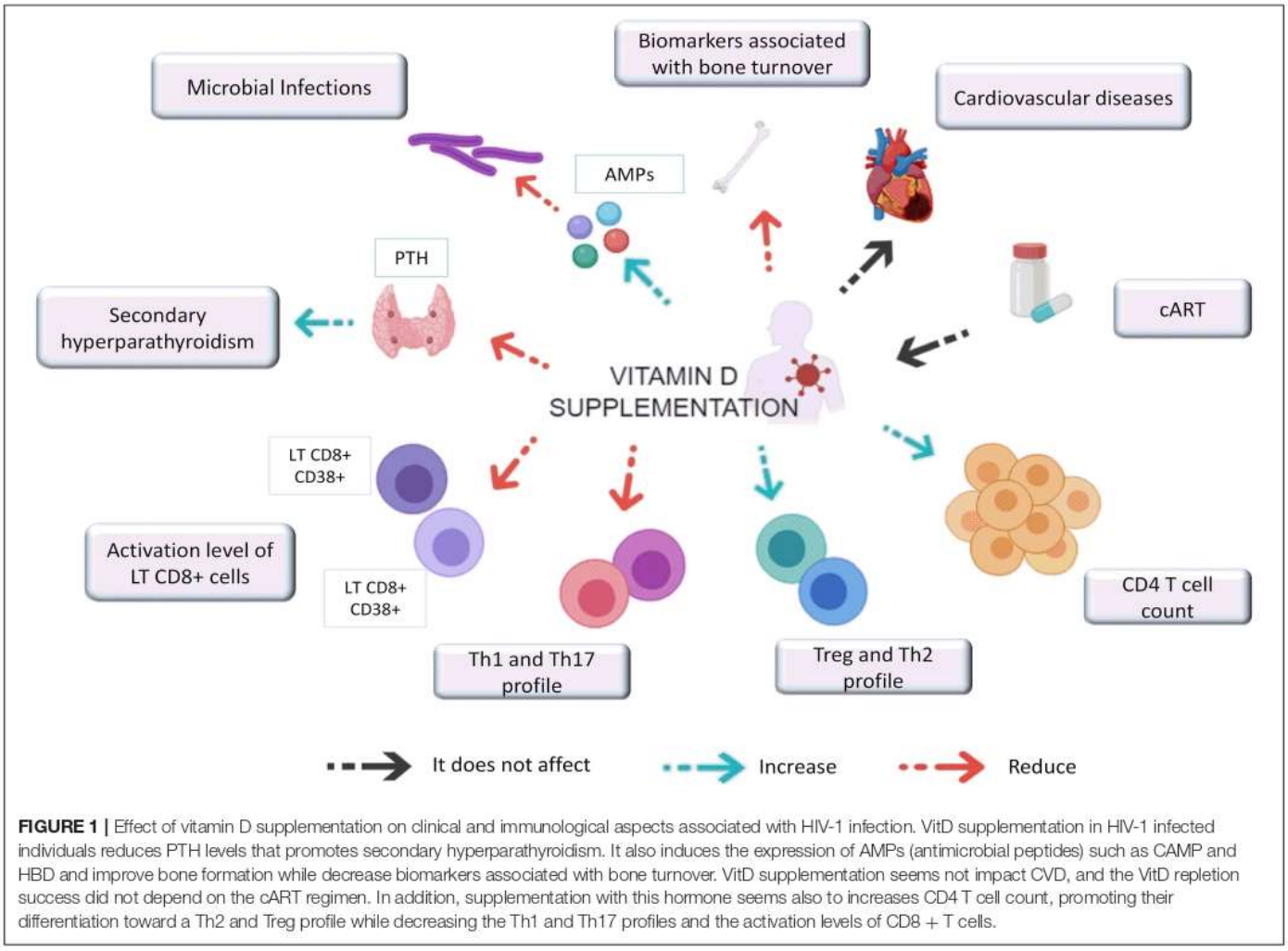

FIGURE 1 | Effect of vitamin D supplementation on clinical and immunological aspects associated with HIV-1 infection. VitD supplementation in HIV-1 infected individuals reduces PTH levels that promotes secondary hyperparathyroidism. It also induces the expression of AMPs (antimicrobial peptides) such as CAMP and HBD and improve bone formation while decrease biomarkers associated with bone turnover. VitD supplementation seems not impact CVD, and the VitD repletion success did not depend on the cART regimen. In addition, supplementation with this hormone seems also to increases CD4 T cell count, promoting their differentiation toward a Th2 and Treg profile while decreasing the Th1 and Th17 profiles and the activation levels of CD8 + T cells.

The Effect of Antiretroviral Therapy on the Response to VitD Supplementation

Although there is evidence suggesting that some antiretrovirals affect VitD metabolism, little is known regarding the effect of VitD supplementation on cART. Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI) have been associated with lower levels of VitD. For example, efavirenz has been suggested to increase VitD catabolism and disrupt 25(OH)D synthesis through the modulation of the cytochrome p450 system, which controls VitD hydroxylation (85–88). However, other trials do not support this hypothesis. Indeed, a study comparing several cART regimens showed that after receiving a daily dose of 4000 or 7000 UI of VitD for 12 weeks, VitD levels were 20 ng/mL higher among individuals on efavirenz compared to all other therapeutic regimens (63). In another study using a similar timeline and supplementation dose schedule, individuals treated with efavirenz reached VitD sufficiency. Of note, variations in baseline VitD levels were not associated with any antiretroviral drug (66). These results suggest that, although efavirenz has been associated with low VitD levels, it is possible to reach sufficient concentrations following supplementation. Moreover, once sufficient levels are reached, efavirenz could have additional benefits related to bone mass, as reported in a supplementation trial in South Africans children (89).

Conversely, zidovudine, a Nucleoside Reverse Transcriptase Inhibitor (NRTI), has been associated with lower levels of vitamin D, while tenofovir has not been associated with deficiency nor insufficiency and neither NRTI has shown significant effects during supplementation trials (9, 59, 90). The Protease inhibitors (PIs) have not yet been correlated with baseline VitD levels or with success or failure to achieve sufficient levels after supplementation (17, 91). No data is currently available for the effect of integrase inhibitors or CCR5 inhibitors on supplementation. In summary, according to previous evidence, the use of cART, even including efavirenz, does not limit the achievable objective of increasing levels of VitD during supplementation trials.

The Effect of Vitamin D Supplementation on HIV-1 Associated Comorbidities

Hypovitaminosis D has been associated with various comorbidities associated with HIV-1 disease progression resulting in higher mortality rates among infected individuals (92, 93). These individuals have an increased risk of osteomalacia

and osteoporosis, notable weight loss, low bone mineral density, and a reduction in muscle mass (78). In contrast, individuals with sufficient VitD levels have a low tendency for skeletal affections; however, the ideal level to minimize risk remains unknown (63).

Although a study in HIV-1 infected individuals with vitamin D deficiency showed a significant reduction in the risk of hypocalcemia after supplementation (58), 3 out of the 5 trials that evaluated bone composition found that, despite an increase in VitD levels following supplementation, bone mass did not change in children and adults (72, 78), even with the addition of calcium (89). In contrast, two studies showed that VitD supplementation decrease biomarkers associated with bone turnover (71, 75) while Etminani-Esfahani et al. reported an increase in Osteocalcin, biomarker associated with bone formation following a single high dose of VitD. Similar results were also noted in other studies (73, 94), where VitD supplementation was found to improve bone composition among HIV-1 infected individuals, albeit, this process might require more time than that seen in previously reported studies.

On the other hand, hypovitaminosis D is related to secondary hyperparathyroidism, a reversible state associated with excessive secretion (>65 pg/mL) of PTH (68), a known cause of decreased bone mineral density (95). Consequently, PTH can be an early indicator of vitamin D deficiency and is an essential criterion for determining if a person requires supplementation (65). Studies evaluating secondary hyperparathyroidism in HIV-1 infected persons are scarce, and as a result, there is little data on the impact of this condition on their clinical status. However, five supplementation trials evaluating PTH levels showed that while VitD levels increased, the levels of PTH decreased during the initial phases of the trials (53, 63, 65, 66, 72, 95).

Finally, although some studies have linked VitD deficiency to hypertension, cardiovascular disease, myocardial infarction, and metabolic syndromes in HIV infected individuals, few studies have evaluated the effect of VitD supplementation on these conditions. In a trial by Chris T Longenecker et al., VitD supplementation in HIV-1 infected patients with hypovitaminosis D did not affect endothelial function, measured by flow-mediated brachial artery dilation (FMD). Furthermore, changes in serum 25(OH)D or FMD were not correlated in the treatment group, although they had not reached sufficient levels of VitD. In Muhammad et al. the authors concluded that VitD supplementation is unlikely to be an effective strategy to attenuate metabolic dysregulation following cART initiation, since lipid and glucose profiles did not improve during treatment (54). These results suggest that VitD supplementation is not enough to avoid the development of these comorbidities, and cannot achieve vitamin sufficiency to improve health conditions (53).

Vitamin D and Bacterial Infections

VitD plays a key role in the effector activity of innate immune cells in response to microbial infections. During monocytes and macrophages activation, the VDR and the enzyme 1α-hydroxylase (CYP27B1), an activator of vitamin D, are expressed. During the intracrine conversion of the VitD precursor (25(OH)D) to its active form (1,25(OH)2D), it is possible to stimulate the expression of antimicrobial peptides such as cathelicidin (CAMP) and human beta defensins (HBD) (96). Some studies reported that VitD affects autophagy, supporting its anti-microbial properties, for example by promoting *Mycobacterium tuberculosis* clearance and antiviral responses (i.e., inhibiting HIV replication) (97). In a supplementation trial, treatment of HIV-1 infected individuals with VitD promoted CAMP expression, despite requiring longer treatment periods compared to uninfected individuals (56). Similarly, an increase in CAMP and macrophage inflammatory protein beta (MIP-1β) production was also reported in another trial (57). Further studies are needed to evaluate other antimicrobial molecules that can be modulated by vitamin D, such as β-defensin 2 or hepcidin.

CONCLUSION

VitD supplementation in HIV-1 infected individuals leads to an increase in VitD serum levels, regardless of cART, geographical location, and ethnicity of the individual being administered the supplementation. Increased VitD levels may have positive effects on several clinical and immunologic aspects which are summarized in **Figure 1.** Among them, the most striking results included the potential reduction in the likelihood of secondary hyperparathyroidism and microbial infections such as tuberculosis, as well as an increase in CD4+ T lymphocytes count and a decrease in biomarkers associated with bone turnover and chronic inflammation. However, the effect of VitD supplementation on viral load has not yet been established since the current guidelines for HIV patient management indicate initiation of therapy as soon as individuals are diagnosed, making it impossible to evaluate. Furthermore, the effect of VitD supplementation on the incidence of other comorbidities associated with hypovitaminosis D, such as metabolic syndromes has not yet been carried out.

Overall, evidence suggests that VitD supplementation may be a good adjuvant to cART. However, it is important to emphasize that the effects greatly depend on the dose quantity and duration of which the supplementation is given. In general, the dosages which showed the most success were 4000 and 7000 IU daily for at least 12 weeks. Studies with larger sample sizes are required to confirm the beneficial effects of VitD and to establish optimal supplementation and maintenance doses in the context of HIV-1 infection.

AUTHOR CONTRIBUTIONS

NA contributed with the literature search and reading and writing and correcting the manuscript. WA-J contributed with writing and suggestions and corrections. MR contributed to reviewing the manuscript and writing.

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4. CHAPTER 4: GENERAL DISCUSSION

Dendritic cells are among the first cells to get in contact with HIV after exposure at the mucosa; therefore, the role of these cells is crucial in controlling or allowing the spread of infection since they can transfer viral particles to $CD4^+$ T cells that are the primary target cells of the virus. Hence, an important study topic is the search for strategies that allow the modulation of this process, looking to decrease the infection incidence that remains far from the proposed objective. In the current study, we explored the role of the immunomodulatory hormone VitD in the HIV transfer process, using an *in vitro* model with MDDC.

Considering previous reports indicating that mDCs have a higher capacity to transfer HIV to $CD4^+$ T cells compared to iDCs, at least of R5 strains [1–3], we decided to use both profiles, iDCS and mDCs, to evaluate the potential effect of the VitD in the viral transfer process. The mature phenotype was reached successfully after LPS stimulation, which was reflected in an increase in the expression of the maturation marker CD83, similar to previous reports [4–6]. When DCs were treated for 24 h with Cholecalciferol (VitD precursor form), one day before receiving the LPS stimulus, a mild modulation of the iDC phenotype was observed, while no changes were perceived in the mDCs phenotype. This result might be explained by the reduced capacity of these cells to transform this metabolite into the active form [7]; in addition, the short pre-treatment time might have also influenced the outcome. Interestingly, after differentiating DCs in the presence of calcitriol, the maturation and activation markers decreased significantly in both iDCs and mDCs, in percentage and in MFI, evidencing the ability of the active form of VitD to induce an immature-like profile in these cells that is consistent with the literature [8–10]. The phenotype reached in DCs, differentiated in the presence of VitD, was stable; indeed, the expression of maturation and activation markers remained low, even after LPS stimulation, similar to an iDCs phenotype. Therefore, according to our findings and previous reports, this immature-like profile remains stable if the differentiated process occurs in the presence of VitD or at least if the hormone, in its active form, is present before the maturation stimuli [9].

Since iDCs are not skilled at activating $CD4^+$ T cells as mDCs, and the VitD precisely promotes an immature profile, we guaranteed that all lymphocytes were previously activated at the time of co-culture with previous stimulation of IL-2 and PHA to enhance T cell susceptibility and viral replication. Our findings showed, in contrast with the literature reports, no differences between iDCs and mDCs in their ability to transfer the HIV-1 to $CD4^+$ T cells, regardless of the VitD treatment [2,11]. This result could be explained by factors such as the tropism of the strain used, the method employed for detecting HIV-1 infection, and the length of the co-culture assay. In

fact, It has been observed that iDCs are more capable of transferring HIV-1 by cis-infection, and this mechanism is favored after 24 hours over trans-infection, which is the mechanism preferentially used by mDCs [1,12,13].

Interestingly, both iDCs and mDCs, differentiated in the presence of VitD, decreased the HIV-1 transfer, evaluated by flow cytometry, unlike the DCs treated for only one day with VitD precursor forms. At least *in vitro*, these results seem to be related to something beyond the maturation of DCs, since in our model there were no differences in viral transfer between iDCs and mDCs, as discussed above. However, the supernatants evaluated by ELISA did not show a reduction in p24 concentration in the co-cultures of T cells with DCs, differentiated in the presence of VitD. One possible explanation, related to the intrinsic ability of each individual to replicate the virus, was partially ruled out since there was not a correlation between the levels of p24 in culture supernatants with the expression of the viral restriction factors *SAMHD1*, *TRIM5*, and *APOBEC3G* in $CD4^+$ T cells. An additional possibility is that the DCs were productively infected, contributing to the levels of p24 in the co-culture supernatant. In fact, several reports suggest that, although DCs are lesser susceptible to replicate the virus, compared to $CD4^+$ T cells, they indeed support HIV-1 infection [14–16].

To elucidate the mechanism behind viral transfer from DCs to $CD4^+$ T cells, we evaluated the expression of some genes, previously reported to influence this process. These genes were *DNM2* related to actin nucleation processes, *TSPAN7* related to membrane protuberances, *MYO5A*, *VAMP3* and *CD63* related to vesicle trafficking, as well as *DC-SIGN* and *SIGLEC-1* with HIV-1 capture[17–19]. The selected genes exhibit VDREs, therefore are potentially modulated by VitD treatment. All genes, except *MYO5A*, enhance viral transfer [17]. Unexpectedly, in our model, iDCs exhibit higher relative expression of all genes in comparison to mDCs, despite the fact that we did not observe differences in viral transfer between both DCs subpopulations.

Important to point that the mechanisms used by iDCs and mDCs for viral transfer are different. The iDCs, for example, internalize HIV-1 in late endosomal compartments or multivesicular bodies, and their trans-infection to $CD4^+$ T cells is mediated by exosomes [2,12,13,20]; in contrast, in mDCs, viral transfer is mediated preferentially when viral particles remain bound to the membrane [21]. Therefore, it would be pertinent to search for additional molecules, modulated by VitD, with the potential effect on viral transference to clarify this process.

On the other hand, we observed that when iDCs were differentiated in the presence of VitD, the relative expression of *DNM2*, *MYO5*, *VAMP3*, *DC-SIGN*, and *SIGLEC-1*

decreased significantly compared to EtOH- treated DCs. These results suggest that VitD could decrease most of the genes related to enhance viral transfer; however, it is striking that *MYO5A* that is related to limit viral transfer is decreased in VitD-treated iDCs. Likewise, in VitD-treated mDCs, the relative expression of *CD63* and *TSPAN7* increased significantly, compared to EtOH-treated mDCs that as mentioned above, are related to an enhanced-viral transfer. Altogether, these results suggest that despite the fact that VitD did not modify the expression of all genes that limit viral transfer, the gene modulation induced by this hormone seems to be sufficient to decrease viral transmission, at least moderately, as observed in chapter III.

Finally, when iDCs and mDCs were differentiated with VitD, they achieved a similar expression profile in all evaluated genes. Although all these genes exhibit VDREs, the VitD effect in gene expression is opposite in most cases between iDCs and mDCs, suggesting that the expression of these genes is secondarily modulated, in other words, is influenced by other genes. Therefore, the observed outcomes might be due to the transcriptional balance achieved after a long treatment with VitD. Notably, SIGLEC-1 was the only gene that remains down-regulated in both iDCs and mDCs treated with VitD.

Due to the widely reported leading role of DC-SIGN and SIGLEC on HIV-1 binding during the trans-infection process, we decided to evaluate the expression of their protein product by flow cytometry. We observe that iDCs and mDCs, differentiated with VitD, significantly decreased the expression of both receptors compared to EtOH-treated cells, being the most likely mechanism by which VitD decreases viral transfer in our model. In fact, studies in which these receptors have been inhibited, HIV-1 capture and transference have been largely affected, although not totally blocked [18,22–24]. Actually, individuals with a mutation that produces SIGLEC-1 protein truncation can acquire HIV infection, exhibiting typical progression to AIDS [25].

Interestingly, the mRNA expression of *SIGLEC-1*, evaluated by q-PCR was higher in iDCs than mDCs, while the protein expression evaluated by flow cytometry was higher in mDCs than iDCs, which is similar to previous reports [18]. In this case, the mRNA expression is not predictive for protein expression, having a biological explanation in the sense that not all mRNAs are immediately or successfully translated, generating accumulation of these, just as not all proteins degrade in similar kinetics [26].

It is essential to note the limitations of this study. First, the methodology used to determine the percentage of $CD4^+$ T cells infected is not very sensitive; the use of an infectious virus with a reporter gene as GFP could have yielded more information.

Also, the use of qualitative techniques such as fluorescence microscopy could have given us more precise evidence on the role of the VitD effect in this process. In addition, as discussed above, the use of an ELISA test to evaluate viral transfer in co-culture is not the best strategy since DCs can also produce viruses as a result of a productive infection. To solve this issue would have been ideal to use virus-like particles (VLPs), without the ability to infect DCs. An additional strategy, that we are currently testing, is to treat the DCs with antiretroviral drugs to avoid a productive infection. Importantly, either with VLPs or using antiretroviral drugs, these results will only give information of *trans*-infection, since by avoiding *de novo* replication in DCs, the *cis*-infection mechanism is eliminated. Additionally, taking into account that early HIV-1 infection is characterized by the predominance of R5 tropic strains [27–29], additional experiments using R5 strains are highly recommended.

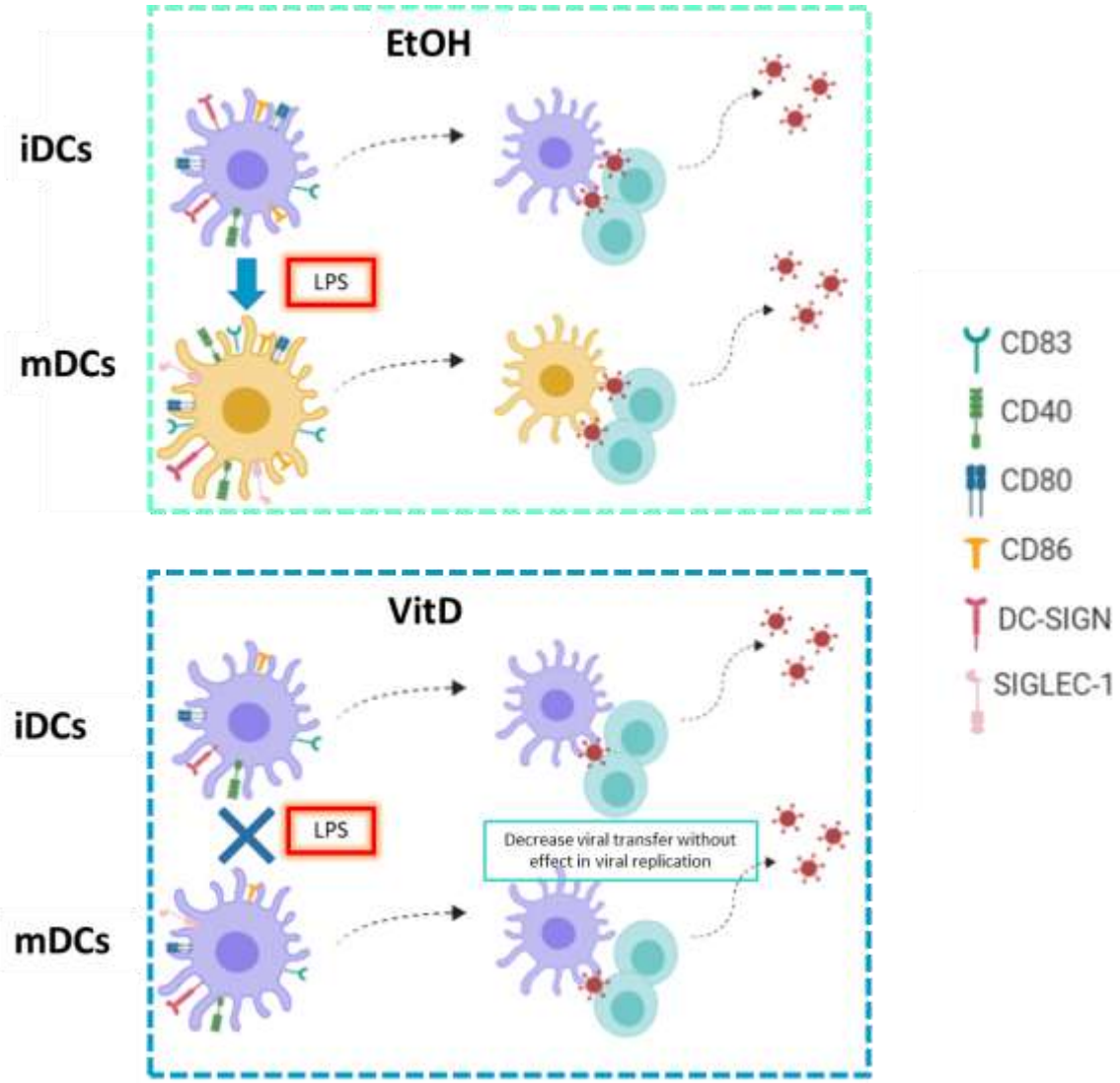
In summary, our results suggest a possible protective effect of VitD on HIV-1 transfer from DCs to $CD4^+$ T cells, most likely by decreasing *trans*-infection as a result of a reduction in the expression of SIGLEC-1 and DC-SIGN. However, as discussed, HIV-1 transfer occurs through several routes, independent of each other, which can be simultaneous. In addition to the modulation of DC-SIGN and SIGLEC-1, it is possible that VitD also, directly or indirectly, modulates some of the routes involved in acquiring viral particles.

To the best of our knowledge, this is the first study evaluating the VitD effect on viral transfer pathways. Therefore, now that we have evidenced that this process can be modulated by this vitamin, it is pertinent to expand the evaluation to other genes, trying to fully elucidate the mechanisms behind this process.

This study could have potential clinical implications on HIV transmission, underlying a rationale for a prevention strategy, since it reveals that this vitamin could impact the first steps of viral spreading at the mucosa, which is critical for the establishment of the infection. In addition to the modulatory effect of the VitD on viral transference, this hormone might have beneficial effects on mucosal tissues, providing other mechanisms for HIV resistance. This vitamin induces the expression of antimicrobial peptides, antiviral factors, decreases immune activation and promotes a tolerogenic environment, which has been associated with resistance phenotypes in HESN (32–35). Indeed, ongoing experiments of our group have shown that VitD treatment on genital epithelial cells led to a decreased infectious capacity of viral particles possibly by the increase of the antiviral peptide cathelicidin (manuscript in preparation). This evidence makes VitD a good candidate to be evaluated as an adjuvant to the pre-exposure prophylaxis (PrEP) therapy.

It has been reported that in HIV-infected individuals, LPS levels in plasma correlate with AIDS progression and systemic immune activation (36,37). Additionally, it is conceivable that increased LPS in HIV-infected individuals may induce DC maturation and potently stimulate HIV dissemination *in vivo*. Therefore, the use of VitD in individuals with HIV-established infection would also be useful for reducing the chronic immune activation, the main cause of viral pathogenesis. In fact, VitD levels have been positively correlated with decreased progression to AIDS (38). Likewise, as discussed extensively in chapter 4, VitD supplementation in HIV-infected population on cART, who frequently exhibit hypovitaminosis D, have several benefits such as a decrease in several comorbidities, as the secondary hyperparathyroidism, and microbial infections as tuberculosis; it also reduces bone resorption markers and immune activation, improving $CD4^+$ T cells counts in some cases (39). All this evidence supports the potential protective role that the VitD could have against HIV-infection *in vitro* and *in vivo*, underlying the importance of clinical trials to finally define the potential use of this hormone as a preventive therapeutic strategy toward HIV-1 infection.

5.1 Proposed model



Both, iDCs and mDCs, differentiated in the presence of the active form of VitD (calcitriol), significantly decrease the maturation (CD83) and activation markers (CD40, CD80, CD86) compared to DCs differentiate with EtOH (vehicle control). The iDCs and mDCs exhibit a similar capacity to transfer HIV-1 particles to $CD4^+$ T cells. However, co-cultures performed with both, iDCs and mDCs, differentiated with VitD, decreased the frequency of infected $CD4^+$ T cells, but not the viral replication as a means of the p24 levels in supernatants, evaluated by ELISA. One possible explanation for the lack of reduction in p24 levels, is that the amount of this protein in co-culture supernatans is the result not only of viral replication in T cells but also in DCs. The DCs differentiated with VitD reduced the expression of DC-SIGN and

SIGLEC-1, pointing this reduction as the most likely mechanism by which viral transference is decreased. The decrease in viral transfer was not observed when DCs were only transiently stimulated with a precursor form of VitD after the differentiation process have occurred, suggesting that these cells are incompetent to activate the precursor form and/or that the hormone should be present during the entire process of differentiation.

5. **SUPPLEMENTARY CHAPTER: ORIGINAL ARTICLE:** in this article, published in Hormone Molecular Biology Journal, I had the opportunity to participate in the experimental development of some assays.

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Original Article

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Cholecalciferol modulates the phenotype of differentiated monocyte-derived dendritic cells without altering HIV-1 transfer to CD4$^+$ T cells

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Abstract:
Background: Dendritic cells (DCs) play a crucial role during HIV-1 transmission due to their ability to transfer virions to susceptible CD4$^+$ T cells, particularly in the lymph nodes during antigen presentation which favors the establishment of systemic infection. As mature dendritic cells (mDCs) exhibit a greater ability to transfer virions, compared to immature DCs (iDCs), maintenance of an iDC phenotype could decrease viral transmission. The immunomodulatory vitamin D (VitD) has been shown to reduce activation and maturation of DCs; hence, we hypothesized that it would reduce viral transference by DCs.
Materials and methods: We evaluated the effect of in vitro treatment with a precursor of VitD, cholecalciferol, on the activation/maturation phenotype of differentiated monocyte-derived DCs and their ability to transfer HIV-1 to autologous CD4$^+$ T cells.
Results: Our findings show that although cholecalciferol decreases the activation of iDCs, it did not impact the maturation phenotype after LPS treatment nor iDCs' ability to transfer viral particles to target cells.
Conclusion: These findings suggest that despite cholecalciferol potentially modulates the phenotype of mucosal iDCs in vivo, such modulation might not impact the ability of these cells to transfer HIV-1 to target CD4$^+$ T cells.

Keywords: DC phenotype, HIV-1 infection, HIV-1 transference, vitamin D
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Introduction

During mucosal transmission of HIV-1 different types of cells play crucial roles influencing the outcome of viral exposure. Although CD4$^+$ T cells are the main target for HIV-1, dendritic cells (DCs) are considered the first immune cells to encounter the virus at the submucosa [1].

DCs may be infected by, or capture, the virus acting as Trojan horses for the infection by transporting viral particles to CD4$^+$ T cells located in the lymph nodes which favors the establishment of systemic infection [2], [3]. Such viral dissemination, mediated by DCs, has been described for male and female genital, as well as rectal, mucosa [4], [5], [6], [7], [8], [9]. Two mechanisms have been implicated during infection of CD4$^+$ T cells by DCs: in cis-infection, productively infected DCs transfer de novo produced viruses to CD4$^+$ T cells, most likely during immunological synapsis [10]; in trans-infection, DCs transfer captured viral particles without being infected. Viral particles may be endocytosed in non-lytic compartments [11], [12], or virions may bind to several receptors expressed on the surface of immature DCs (iDCs) and mature DCs (mDCs) [13], [14], [15]. This latter process is more efficient for viral transmission to CD4$^+$ T cells than internalized virions [16]. Based on available evidence, trans-infection is proposed to be the mechanism accounting for most in vivo infection [17].

In the trans-infection process, mDCs exhibit a higher ability to transfer viral particles than iDCs [18], most likely due to the stability of the immunological synapse established with CD4$^+$ T cells. This difference suggests that modulation of the activation/maturation state of DCs may influence viral transference by these cells.

Endogenous immunomodulator compounds such as vitamin D (VitD) have been investigated due to their potential for modulating the phenotype and function of different cell types, including DCs. DCs constitutively express the VitD receptor (VDR) [19], and treatment with VitD has been shown to reduce expression of the

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activation markers CD80 and CD86 and the maturation marker CD83 in these cells [20], [21], [22]. It has also been shown that VitD induces a tolerogenic phenotype in DCs with decreased production of IL-12 and increased production of IL-10, a phenotype that influences the activation level of T cells [23]. We previously showed that cholecalciferol reduced the susceptibility of $CD4^+$ T cells to HIV-1 infection in vitro [24], emphasizing its potential to be used as a preventive strategy to reduce viral transmission. In this regard, and considering all previous evidence, we proposed that through its effect on the DCs phenotype VitD could reduce DCs viral transfer to $CD4^+$ T cells and decrease HIV-1 transmission during mucosal exposure.

Methods

Study population

Ten healthy non-exposed HIV-1 negative individuals from Medellin, Colombia were included in this study. The study was performed according to the Helsinki declaration (1975, revised in 2000), and was approved by the Bioethics Board of the Instituto de Investigaciones Medicas, Universidad de Antioquia. All subjects gave written and signed informed consent to participate in the study.

Differentiation of monocyte-derived DCs and VitD treatment

Sixty milliliters of blood were obtained by venipuncture from all participants, and peripheral blood mononuclear cells (PBMCs) were isolated. Approximately 80% of the PBMCs were used to differentiate monocyte-derived DCs (hereafter referred to as DCs), while the remaining 20% were used to obtain purified $CD4^+$ T cells. The frequency of monocytes in PBMCs was evaluated by extracellular CD14 staining, and 5×10^5 $CD14^+$ cells were seeded per well in 24-well plates to obtain the monocytes by adherence after 2.5 h of culture; the purity was evaluated using flow cytometry by the exclusion of T cells ($CD3^+$), B cells ($CD19^+$), and NK cells ($CD56^+$) with a cut-off of an 85% pure culture. Monocytes were cultured with GM-CSF (75 μg/mL) and IL-4 (100 μg/mL) for 6 days at 37 °C and 5% CO_2 to obtain DCs. After differentiation into DCs, we induced two phenotypes: (i) iDCs were cultured for 48 h with the inactive form of VitD known as cholecalciferol (1×10^{-9} M, and 1×10^{-10} M), and (ii) mDCs were treated with cholecalciferol and after 24 h were treated with LPS (5 μg/mL) for an additional 24 h. Treatment with ethanol (EtOH) was used as vehicle control for VitD. Both types of DCs were used for viral transference assays with autologous $CD4^+$ T cells.

Phenotype of iDCs and mDCs

The differentiation of monocytes into DCs, and the effect of VitD treatment on the phenotype of iDCs and mDCs were evaluated by flow cytometry. Cells were stained with the following antibodies from eBioscience (Thermo Fisher, Waltham, Massachusetts, USA): anti-CD11c-efluor450, anti-HLA-DR-FITC, anti-CD80-PeCy5, anti-CD86-PeCy7, anti-CD40-PE and anti-CD83-APC. Isotype controls were used to define positive thresholds for each activation/maturation marker. DCs were defined as $CD11c^+HLA\text{-}DR^{low/high}$. Our gating strategy is shown in Figure 1.

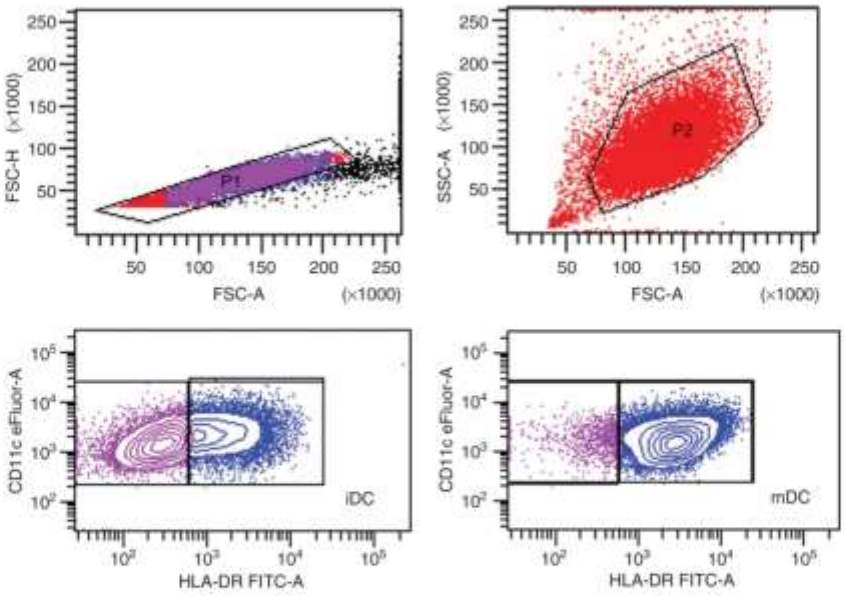

Figure 1: Gate strategy for selection of DC (CD11c$^+$ HLA-DR$^{low/high}$).
Upper left: aggregates exclusion. Upper right: DC region according to side scattered (SSC) and forward scattered (FSC). Lower left and right: DC selection according to CD11c and HLA-DR (low and high) in immature and mature cells, respectively.

Isolation of autologous CD4$^+$ T cells

Before the isolation of autologous CD4$^+$ T cells, the remaining PBMCs were frozen in liquid nitrogen for 5 days during the differentiation of monocytes to DCs. After, cells were thawed and rested overnight at 37 °C and 5% CO_2, and viability was evaluated by Trypan Blue staining. To purify CD4$^+$ T cells we used the negative selection CD4$^+$ T cell Isolation Kit (MACS – Miltenyi Biotec, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, 10 µL of CD4$^+$ T cell Biotin-Antibody Cocktail (Miltenyi Biotec, San Diego, CA, USA) was added for every 10^7 total cells, and the mix was then incubated for 5 min at 4 °C. Then, 20 µL of CD4$^+$ T cell MicroBead Cocktail (Miltenyi Biotec, San Diego, CA, USA) was added for every 10^7 total cells, and the cells were incubated for 10 min at 4 °C. We performed the magnetic isolation using the MACS LS columns (Miltenyi Biotec, San Diego, CA, USA), collecting the unlabeled cells that pass through and enriching the sample for CD4$^+$ T cells. Purity was evaluated by flow cytometry using the antibodies anti-CD3-PeCy5 and anti-CD4-APC from eBioscience, with final purities of approximately 95%.

The autologous CD4$^+$ T cells were polyclonally stimulated with PHA (10 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and IL-2 (50 IU/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 37 °C and 5% CO_2. Activated CD4$^+$ T cells were then used in co-cultures with DCs.

HIV-1 transfer from DCs to autologous CD4$^+$ T cells

The VitD-treated or EtOH-treated iDCs and mDCs (5×10^4 cells) were pulsed with 13 ng [by p24 enzyme-linked immunosorbent assay (ELISA)] of X4-tropic HIV-1 [obtained from H9-HTLV-IIIB cells (ATCC-CRL-8543)] for 1.5 h to allow viral binding to DC surface receptors. Cells were washed 3 times to remove unbound viral particles, and iDCs or mDCs carrying the virions were co-cultured with activated CD4$^+$ T cells at a ratio of 1 DC:3 CD4$^+$ T cells. A total of 50,000 DCs and 150,000 CD4$^+$ T cells were co-cultured for 72 h at 37 °C and 5% CO_2. Viral infection of CD4$^+$ T cells was evaluated by flow cytometry for intracellular p24; briefly, the cells were extracellularly stained with anti-CD3-PeCy5 (eBioscience-Thermo Fisher, Waltham, Massachusetts, USA) and anti-CD4-APC (eBioscience-Thermo Fisher, Waltham, Massachusetts, USA), and intracellularly with anti-p24-PE (Beckman Coulter, Atlanta, Georgia, USA). Cells were fixed and acquired on either a FACSCanto II (BD bioscience, New Jersey, USA) or a LSRFortessa cytometer (BD bioscience, New Jersey, USA). All data was analyzed using FACSDiva v.8.0.1 software (BD bioscience, New Jersey, USA). We established our lymphocyte population according to FSC and SSC, and determined the percentage of p24$^+$ CD4$^+$ T cells, and the mean fluorescence intensity (MFI) of p24, in total CD4$^+$ T cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v.7.05 software (San Diego, CA, USA). Parametric or non-parametric tests of paired samples were used for treatment comparisons, according to the normality of the data. As some individuals were excluded from analyses for technical issues, the sample sizes are specified in the figure legends. Results are presented as median or mean, and p-values < 0.05 were considered statistically significant.

Results

Cholecalciferol treatment modulated the activation/maturation phenotype of immature DCs

Dendritic cells from 10 healthy non-exposed individuals were treated with two concentrations of cholecalciferol, an inactive form of VitD, to evaluate the effect on the expression of activation and maturation markers. In iDCs, the treatment with the lower concentration of VitD (1×10^{-10} M) reduced the percentage of DCs expressing CD80 compared to EtOH (27.34% vs. 32.92%, p = 0.0353) (Figure 2A), whereas the expression of CD86, CD83, and CD40 was not affected. The treatment with the higher concentration of VitD (1×10^{-9} M) decreased the percentage of DCs expressing CD86 (12.57% vs. 15.72%, p = 0.0080) (Figure 2B), CD83 (9.96% vs. 11.99%, p = 0.0469) (Figure 2C), and CD40 (10.27% vs. 14.04%, p = 0.0383) (Figure 2D) compared to EtOH. The expression of CD80 was not affected.

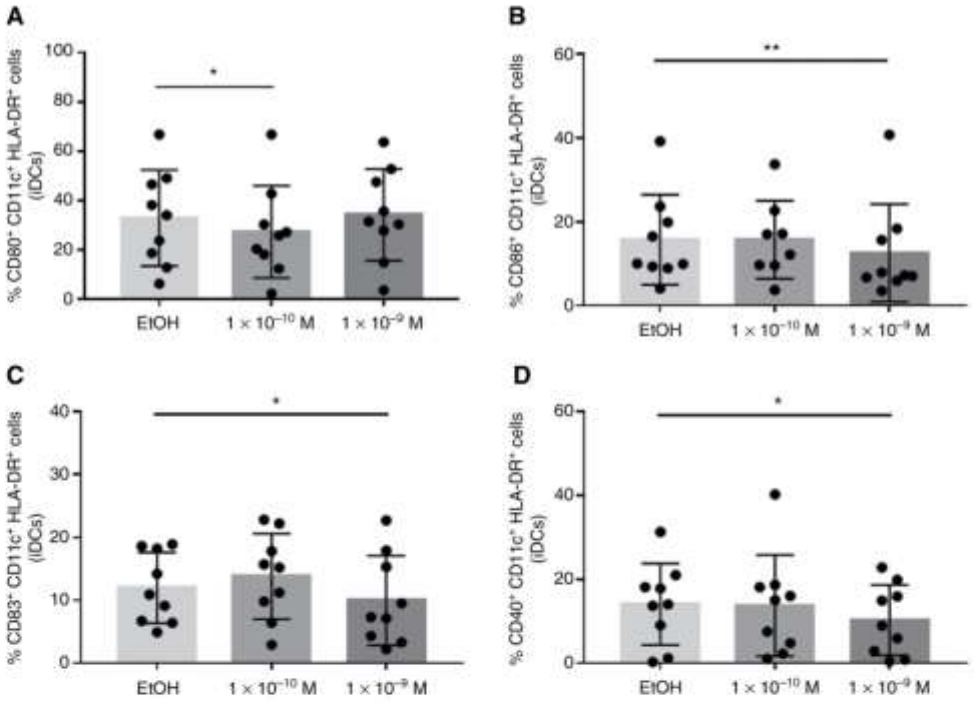

Figure 2: Frequency of iDC expressing activation/maturation markers.
Percentage of immature DC ($CD11c^{+}HLA\text{-}DR^{low/high}$) expressing CD80 (A), CD86 (B), CD83 (C) and CD40 (D) after cholecalciferol treatment at 1×10^{-10} M and 1×10^{-9} M (n = 9).
Comparison between treatments were made using the ratio paired t-test, (*) $p \leq 0.05$; (**) $p \leq 0.01$.

Similar results were observed for the MFI of these markers with decreased expression of CD80 with a cholecalciferol treatment at 1×10^{-10} M (662 vs. 884, p = 0.0491) (Supplementary Figure 1A), as well as reduced CD86 (2933 vs. 3617, p = 0.0263) (Supplementary Figure 1B) and CD40 (319 vs. 488, p = 0.0220) (Supplementary Figure 1C) with the 1×10^{-9} M treatment compared to EtOH. The MFI of CD83 was not affected by any concentration (Supplementary Figure 1D).

Cholecalciferol treatment did not affect the phenotype of mature DCs

To confirm the maturation status of DCs after LPS stimulus, we compared the frequency of cells expressing the maturation marker CD83 between iDCs and mDCs. As expected, we observed a higher frequency of $CD83^+$ cells in mDCs (11.99% vs. 66.31%, p = 0.0001) (Supplementary Figure 2).

In contrast to the results observed for cholecalciferol in iDCs, VitD did not modulate the percentage of mDCs expressing CD80 (39.17% vs. 40.87%, p = 0.3213, 1×10^{-10} M; and 41.33% vs. 40.87%, p = 0.2951, 1×10^{-9} M) (Figure 3A), CD86 (51.08% vs. 46.87%, p = 0.4564, 1×10^{-10} M; and 49.19% vs. 46.87%, p = 0.4660, 1×10^{-9} M) (Figure 3B), CD83 (60.67% vs. 66.31%, p = 0.1992, 1×10^{-10} M; and 62.23% vs. 66.31%, p = 0.1896, 1×10^{-9} M) (Figure 3C), or CD40 (56.17% vs. 56.29%, p = 0.3422, 1×10^{-10} M; and 54.16% vs. 56.29%, p = 0.2199, 1×10^{-9} M) compared to EtOH (Figure 3D). No changes in MFI were observed (data not shown).

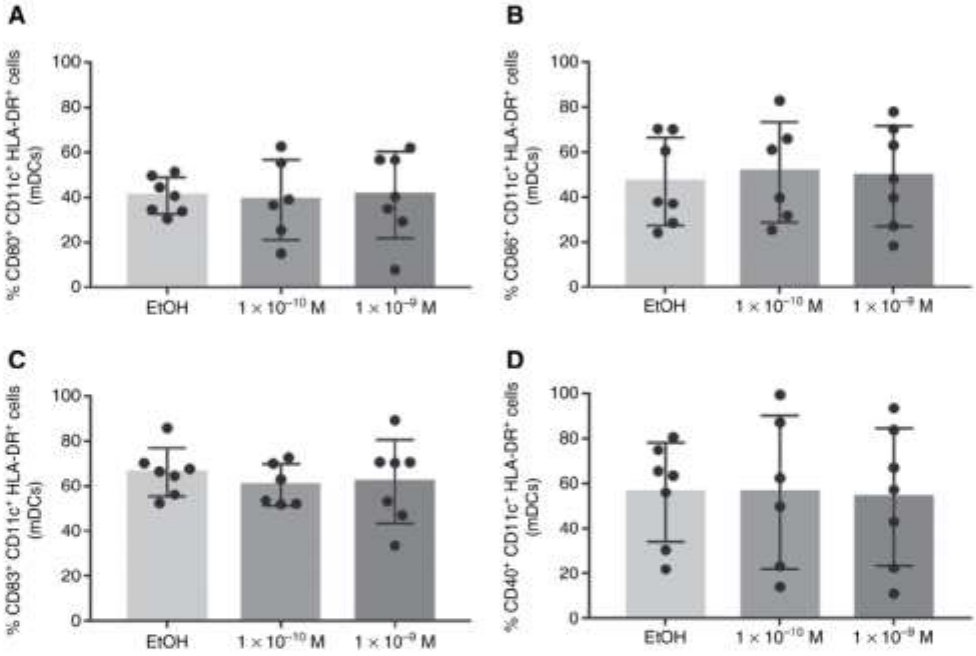

Figure 3: Frequency of mDC expressing activation/maturation markers.
Percentage of mature DC ($CD11c^+HLA\text{-}DR^{low/high}$) expressing CD80 (A), CD86 (B), CD83 (C) and CD40 (D) after cholecalciferol treatment at 1×10^{-10} M (n = 6) and 1×10^{-9} M (n = 7).
Comparison between treatments were made using the ratio paired t-test (variation in n are due to non-maturation of DC).

We also evaluated the spontaneously produced levels of the active form of VitD, calcitriol, in supernatants from iDCs and mDCs, observing a higher production by mDCs.

Immature and mature DCs exhibit a similar ability to transfer HIV-1 to $CD4^+$ T cells

As it has been described that mDCs exhibit a higher ability to transfer virions compared to iDCs, we compared the infection of $CD4^+$ T cells by virions transferred from either iDCs or mDCs in the absence of VitD and found no difference (1.69% vs. 2.39%, p = 0.1053) (Supplementary Figure 3A and B). We also evaluated the infection of iDCs and mDCs to establish the contribution of cis-infection to viral transference in our findings, and DC infection was lower than 0.1% in both populations.

VitD did not alter HIV-1 transfer from DCs to $CD4^+$ T cells

Considering our findings on the phenotypic modulation of iDCs by cholecalciferol, we evaluated its effects on HIV-1 transfer from DCs to $CD4^+$ T cells. VitD treatment of iDCs or mDCs did not affect their ability to transfer virions to $CD4^+$ T cells in terms of frequency of infected cells (iDCs, 1.95% vs. 1.68%, p = 0.3822, 1×10^{-10} M; and 1.73% vs. 1.68%, p = 0.5130, 1×10^{-9} M) (Figure 4A and B) (mDCs, 2.76% vs. 2.39%, p = 0.3456, 1×10^{-10} M; and 2.72% vs. 2.39%, p = 0.7476, 1×10^{-9} M) (Figure 5A and B), or number of viral particles per cell (iDCs,

142 vs. 142, p = 0.6943, 1 × 10^{-10} M; and 141 vs. 142, p = 0.2854, 1 × 10^{-9} M) (Figure 4C and D) (mDCs, 171 vs. 163, p = 0.8664, 1 × 10^{-10} M; 132 vs. 163, p = 0.4047, 1 × 10^{-9} M) (Figure 5C and D) compared to EtOH.

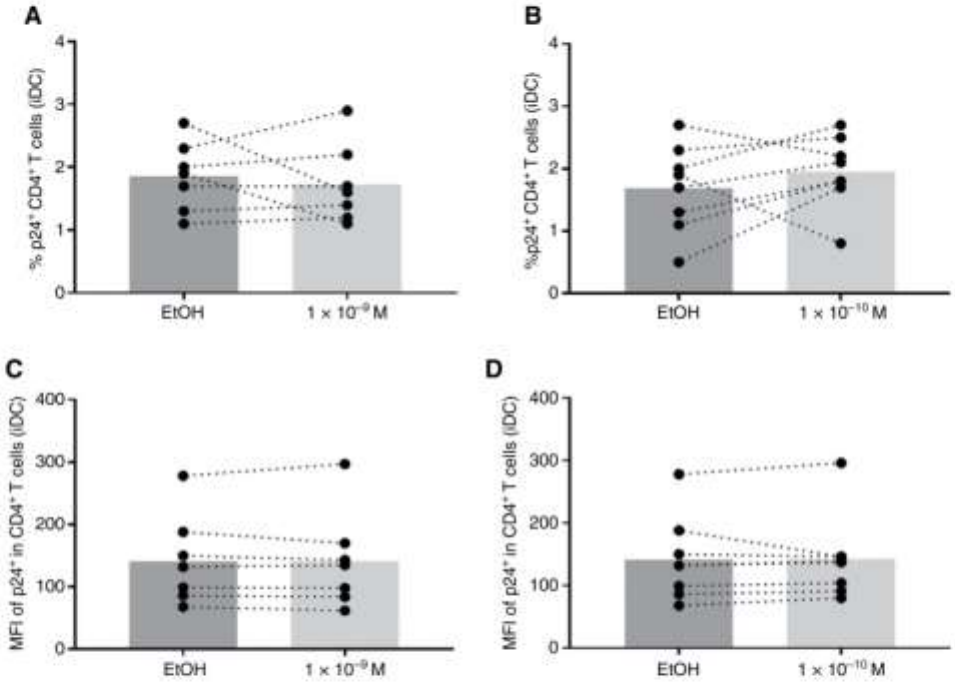

Figure 4: Transference of HIV-1 by iDC to CD4+ T cells.
Frequency of HIV-1 infected CD4+ T cells (p24+CD4+ T cells) and MFI of p24 in CD4+ T cells after co-culture with iDC-cholecalciferol treated at 1 × 10^{-9} M (A, C) and 1 × 10^{-10} M (B, D) (n = 8).
Comparison between treatments were made using the ratio paired t-test.

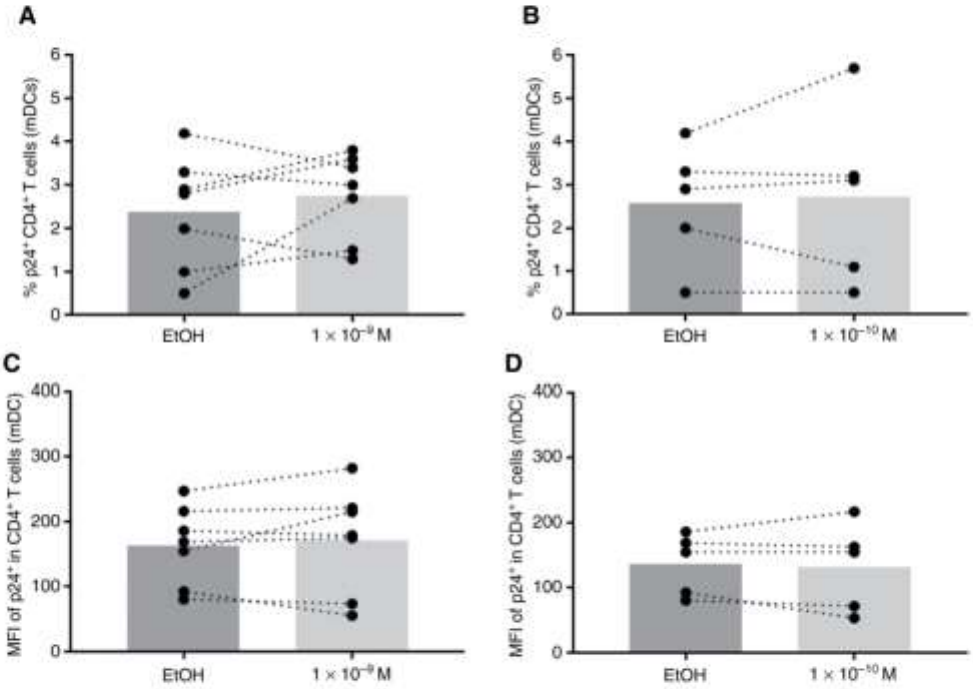

Figure 5: Transference of HIV-1 by mDC to CD4+ T cells.
Frequency of HIV-1 infected CD4+ T cells (p24+CD4+ T cells) and MFI)of p24 in CD4+ T cells after co-culture with mDC-cholecalciferol treated at 1 × 10^{-9} M (A, C) (n = 7) and 1 × 10^{-10} M (B, D) (n = 5).
Comparison between treatments were made using the ratio paired t-test.

Discussion

During genital exposure to HIV-1, once the virus reaches the submucosa, DCs are thought to be the first cells encountering the virus as they are located within the outermost genital epithelial layers [1]. Given their ability to migrate to the lymph nodes for antigen presentation, they can act as Trojan horses carrying viral particles and favor systemic dissemination [2], [3]. These cells may transfer viral particles to $CD4^+$ T cells through cis-infection after being infected [10], or trans-infection via internalized virions or virus bound to surface receptors, the latter considered the main transference mechanism [25].

It seems that activation level and maturation of DCs influence viral transfer, as it has been previously reported that iDCs exhibit a lower capacity to transfer virions compared to mDCs [16], [18], [26]. We did not observe such a difference; a result that could be explained by the use of an X4-tropic virus over the previously used R5-tropic HIV-1, as variations regarding the use of cis- and trans-infection have been described for both strains depending on the maturity phenotype of the DCs [16], [18]. These conflicting results may also be related to the method employed for detecting HIV-1 infection, as we used flow cytometry for p24 detection, whereas other authors have used a fluorescent tagged-virus [16], [18].

We evaluated whether cholecalciferol, an inactive precursor form of VitD, could modulate the activation/-maturation phenotype of differentiated monocyte-derived DCs and their impact on viral transfer to $CD4^+$ T cells to verify the potential use of this hormone as a preventive strategy against mucosal transmission. Indeed, fact, we have previously shown that cholecalciferol reduced the susceptibility of $CD4^+$ T cells to HIV-1 infection in vitro [24], and, to the best of our knowledge, this is the first study evaluating the effect of VitD during HIV-1 transfer mediated by DCs.

Interestingly, a mild effect of VitD on the phenotype of iDCs was observed. In these cells the lower concentration of cholecalciferol reduced the frequency and density of CD80 expression, whereas the higher concentration decreased CD86, CD40, and CD83, suggesting that specific gene modulation depends on the hormone concentration. For mDCs, the VitD precursor did not influence their phenotype in contrast to previous reports using calcitriol [21], [22]. This result is most likely due to the potent effect of LPS inducing maturation that could not be reversed by the short pre-treatment with this inactive form of VitD.

The mild modulation of the iDC phenotype, induced by the precursor form of VitD, could be related to the reported decreased ability of these cells to convert precursor forms into the active form calcitriol, compared to other cells like monocytes [22]; hence, they might require that calcitriol activation is carried out by other cells [22]. In fact, a ten-times higher concentration of calcidiol is required in order to reach similar effects on DCs to those observed for calcitriol [27]. Nonetheless, other authors have shown that DCs, at the latest stages of differentiation, produce high concentrations of calcitriol [27].

What is evident is that most of the studies evaluating the effect of VitD on the phenotype of DCs have been conducted with calcitriol treatment, as the active form of VitD might exert a more potent modulatory effect [27], [28].

The duration of cholecalciferol treatment could have also influenced our results, as we added the hormone at day 6 of monocyte culture to evaluate the effect on differentiated DCs, whereas other authors added the hormone at the beginning of the differentiation process [27]. It has been described that the modulatory activity of VitD is greater in monocyte precursors than in iDCs, with a higher reduction of CD86 expression and greater allostimulatory effect on DCs treated with VitD since the beginning of culture, compared to those treated 4–9 days after initiation of the differentiation process [27]. This observation is most likely due to the higher expression of VDR in monocytes than iDCs and CD40 ligation-matured DCs [27].

Interestingly, the stimulus of iDCs with LPS induced a higher expression of VDR and CYP27B1 [21], [28], suggesting that LPS could increase the sensitivity, at least of mDCs, to VitD. Indeed, we observed an increased release of calcitriol in supernatants from mDC compared to iDC.

Despite our findings of the phenotypic modulation of DCs by VitD, we did not observe an impact on HIV-1 transference to $CD4^+$ T cells. This could be related to the reasons already discussed, including the use of a precursor form that exhibits a limited effect compared to calcitriol, and treatment of DCs after differentiation instead of the monocytes at the beginning of the differentiation process. It is also possible there is simply no effect of VitD on HIV-1 viral transfer via DCs.

Our results suggest that in vivo supplementation with cholecalciferol may decrease the activation of iDCs, which are mainly located at mucosal sites. Although such modulation might not directly impact HIV-1 transfer to susceptible target cells, at least in vitro, it could modulate the local microenvironment, reducing the activation of $CD4^+$ T cells, and, thus, decreasing their susceptibility to infection [24]. This hypothesis requires further exploration. In addition, these experiments should be validated with a larger sample size and in iDCs and mDCs treated with calcitriol, as it exerts more potent effects than cholecalciferol. Day one treatment, at the beginning of the differentiation process, also may represent a more in vivo scenario.

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Informed consent: All subjects gave written and signed informed consent to participate in the study.

Ethical approval: The study was performed according to the Helsinki declaration (1975, revised in 2000), and was approved by the bioethics board of the Instituto de Investigaciones Medicas, Universidad de Antioquia.

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