**UNIVERSIDAD DE SEVILLA**

# THE ROLE OF PLASMACYTOID DENDRITIC CELLS IN THE SPONTANEOUS CONTROL OF HIV INFECTION

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# *INTRODUCTION*

## INTRODUCTION

In the absence of antiviral therapy, the large majority of HIV-infected individuals have uncontrolled vireamia and undergo progressive immune impairment that leads to AIDS, opportunistic infections, and ultimately death. However, a very small percentage (≤ 1 %) of HIV-infected persons known as “HIV controllers” are able to spontaneously control viral replication during a long period of time in the absence of therapy [1]. These individuals can be divided into two subgroups according to the control of HIV-1 vireamia, “HIV elite controllers” (EC) are those subjects capable to maintain undetectable viral loads (<50 HIV RNA copies/ml), and the “HIV relative controllers” (RC) those who maintain viral loads between 50-1000 HIV RNA copies/ml. On the contrary of the long-term non-progressors (LTNP) subjects [2], the definition of “HIV controllers” is virological whereas that of LTNP is immunological, based on maintained CD4 lymphocyte count >500/mm$^3$ for several years without therapy. Besides the virological control, it has been shown that “HIV controllers” are also able to maintain higher CD4 T cells levels during a long period of time compared with other groups of HIV infected subjects [3]. However, some of these individuals experience CD4 loss and progression to AIDS [4]. The mechanisms responsible for this spontaneous control have been studied with the hope of developing a therapeutic vaccine against HIV. The principal mechanisms associated to the spontaneous control of HIV-1 until now are host genetic factors and adaptive immunity [5-9]. Nevertheless, these mechanisms do not explain entirely this phenomenon.

In relation to host genetic factors, large-scale human genome analyses, as Genome-Wide Association Studies (GWAS), have been performed in an attempt to identify gene polymorphisms associated to the spontaneous viral control. In particular some alleles of the Major Histocompatibility Complex (MHC) class I, as the HLA-B5701, are associated to the spontaneous HIV viral control [5, 8]. In a recent study, Kosmrlj et al suggested a mechanistic explanation to this association [6], by a more efficient antigen presentation by these HLA to immature T cell in the thymus, leading to the generation of more reactive clones who would exercise a higher pressure on the virus. This pressure would be achieved in a major context by CD8 T cells that inhibit virus replication in autologous HIV infected CD4 T cells [9], and an efficient elimination of these cells, contributing to the virus control [7] and the stability of the CD4 T cell levels in most of HIV controllers subjects [10].

The host genetic factors and the adaptive immunity do not seem sufficient to explain this spontaneous control of HIV infection. However the innate immunity responses in HIV controllers need to be characterized. The Plasmacytoid Dendritic cells (pDCs) are innate immune cells that are specialized to produce interferon-alpha (IFN-α); producing up to 1000 fold more interferon type I than other type of blood cells [11]. The pDCs produce IFN-α in response to pathogens containing ssRNA by the stimulation of the Toll-Like Recepto-7 (TLR-7) and unmethylated CpG DNA motifs through TLR-9 [12]. The IFN-α produced by pDCs not only participates in the inhibition of viral replication but also have an adjuvant effect on different immune cells types, like monocytes [13], natural killer cells [14], and T cells [15], providing a link between innate and adaptive immunity. The role of pDCs in HIV infection is not well understood.

The first studies reported that the number of circulating pDCs was decreased in HIV infection [16]. This blood-depletion correlates with high plasma viral load and low CD4 T cell count, probably due to the viral cytopathicity as found in vitro [17] or a lack of survival linked to a lower IFN production or to defective CD4 T cell helper functions [18, 19]. Another suggested cause of this depletion might be the migration of HIV-activated pDCs from blood to lymphoid organs [20] where massive CD4 T cell depletion occurs [21, 22]. A recovery in pDCs counts, at least a partial one, seems to be obtained through highly active antiretroviral therapy (HAART) during chronic infection as well as during primary infection [23], sometimes this increase of pDCs counts during HAART correlates with a lower virus set point after structured therapy interruption [24]. Furthermore, HIV-activated pDCs also express the apoptotic ligand TRAIL (tumor necrosis factor-related apoptosis inducing ligand) providing them potential killer activity (interferon-producing killer pDCs) [25]. These TRAIL-expressing killer pDCs (iKpDCs) were demonstrated to be in close proximity to apoptotic CD4 T cells in tonsils from HIV-infected viremic patients [22]..

TRAIL selectively induce apoptosis of HIV-exposed CD4 T cells in vitro [26]. TRAIL induces apoptosis of death-receptor 5 (DR5)-expressing cells [27]. The percentage of CD4 T cells coexpressing TRAIL and DR5 is elevated in the blood of viremic patients [28, 29] and is reduced by successful HAART [30]. Previous study on TRAIL regulation reveals that type I interferon produced by pDCs after HIV endocytosis regulates TRAIL on CD4 T cells [31].

In HIV controller subjects, the functionality of pDCs; IFN-α production and TRAIL expression by pDCs have not yet been investigated, thus our first objective was to assess pDCs levels and to analyze their functionality in HIV controllers. This objective was addressed in these two articles "**Plasmacytoid dendritic cells reduce HIV production in elite controllers**" (J Virology 2012) and "**Plasmacytoid dendritic cells (pDCs) from HIV controllers produce interferon-α and differentiate into functional killer pDCs under HIV activation**" (JID 2012).

On the other hand, some of the genetic and adaptive immunity factors involved in the spontaneous control of HIV mentioned above, are also implicated in the spontaneous clearance of hepatitis C virus (HCV) [32, 33]. The overrepresentation of the HLA-B57 in addition to the HIV spontaneous control, have also been associated to the spontaneous clearance of HCV in monoinfected subjects [32], as well as a HCV-specific polyfunctional CD8 T cell response [32-34]. Given the shared transmission routes of both HIV and hepatitis C virus (HCV), the rates of co-infection are elevated in HIV infected cohorts and HCV natural history is accelerated in HIV-coinfected patients [35]. Regarding the HIV/HCV coinfection in HIV controllers, an increase in the spontaneous clearance of HCV in a cohort of African-American infected with HCV genotype 1 has been reported [36], suggesting that the ability of some HIV controllers to successfully control 2 agents of chronic viral illness (HIV-1 and HCV), implies that a common genetic factors linked to either the adaptive or innate immune system are responsible for the viral control in these individuals.

The HIV/HCV co-infection in Caucassian HIV controllers has not yet been investigated. Thus, our second objective was to assess to what extent Caucasian HIV

controllers are able to control HCV replication and the potential associated factors. This objective was addressed in this article "**Hepatitis C virus replication in Caucasian HIV controllers**" (J Viral Hep 2011).

Interestingly, a single nucleotide polymorphism (SNP) rs12979860 near the IL28B gene, which codes for interferon λ3 ( also known as IL28B) have been recently associated with clearance of HCV [37] and sustained virological response after HCV-specific treatment [38]. Several studies have demonstrated the antiviral activity of INF-λ3 against different virus including HIV [39], although it is still unknown how this SNP affects the antiviral activity of INF-λ3. However, a higher prevalence of protective allele IL28B-CC in African-American elite controllers compared to HIV non-controllers subjects was not observed and no association was found between the IL28B rs12979860 and HIV spontaneous control in African-American individuals [40, 41]. Nonetheless, we have to take into account the small number of HIV controller patients included in these studies and the ethnicity of the cohort; composed only by African-American individuals who shown a distinct prevalence of IL28B-CC than Caucasian subjects [37]. The protective allele IL28B-CC has not been overrepresented in LTNP subjects neither [42], probably because the selection of the patients was based on immunological criteria (CD4 levels) and not virological ( undetectable viral load).

The association of the protective allele IL28B-CC and the spontaneous control of HIV infection in Caucasian individuals constituted the third objective of this Doctoral Thesis, and was addressed in this study "**IL28B single-nucleotide polymorphism rs12979860 is associated with spontaneous HIV control in white subjects**" (JID 2013).

Recently, a new transiently induced region that harbours a dinucleotide variant ss469415590 (TT or ΔG), which is in high linkage disequilibrium with IL28B rs12979860, has been discovered [43, 44]. ss469415590 [ΔG] is a frameshift variant between interferon lambda 2 (IFNL2) and IFNL3 genes that creates a novel gene, designated IFNL4, encoding the IFN-λ4, which structure is moderately similar to IFN-λ3 [43]. Although the relevance and the function of this protein are unknown, IFNL4 ss469415590-ΔG seems to be the functional variant as is associated with the production of IL28B [44]. When compared to rs12979860, ss469415590 is more strongly associated with the spontaneous clearance of HCV infection and the response to HCV treatment [43-46]. Whether ss469415590 is associated with clinical and immunovirological parameters in HIV-infected patients, is unknown.

In accordance with all these data it is provocative to think that ss469415590 polymorphism is associated with disease progression-related parameters in other virus infections, e.g. HIV. The fourth aim of the present Doctoral Thesis was to analyze the association of the IFNL4 variants with clinical and immunovirological parameters in HIV-infection. This objective was addressed in this study "**IFNL4 ss469415590 polymorphism is associated with unfavourable clinical and immunological status in HIV-infected individuals**" (Submitted Manuscript).

# ***OBJECTIVES***

## OBJECTIVES

**Objective 1:** To analyze the frequency and functionality of plasmacytoid Dendritic cells in relation to the spontaneous control of HIV infection. This objective was addressed in these two articles "**Plasmacytoid dendritic cells reduce HIV production in elite controllers**" (J Virology 2012) and "**Plasmacytoid dendritic cells (pDCs) from HIV controllers produce interferon-α and differentiate into functional killer pDCs under HIV activation**" (JID 2012).

**Objective 2**: To analyze the ability to control other chronic infections, such as hepatitis C virus infection in HIV controllers. This objective was addressed in this article "**Hepatitis C virus replication in Caucasian HIV controllers**" (J Viral Hep 2011).

**Objective 3**: To analyze the association of the IL28B SNP with the spontaneous control of HIV infection. This objective was addressed in this study "**IL28B single-nucleotide polymorphism rs12979860 is associated with spontaneous HIV control in white subjects**" (JID 2013).

**Objective 4**: To analyze the association of the IFNL4 variants with clinical and immunovirological parameters in HIV-infection. This objective was addressed in this study "**IFNL4 ss469415590 polymorphism is associated with unfavourable clinical and immunological status in HIV-infected individuals**" (Submitted Manuscript).

## ***METHODS,***

## ***RESULTS***

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# Plasmacytoid Dendritic Cells Reduce HIV Production in Elite Controllers

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**HIV elite controllers (EC) are a rare group of HIV-infected patients who are able to maintain undetectable viral loads during a long period of time in the absence of antiretroviral treatment. Adaptive immunity and host genetic factors, although implicated, do not entirely explain this phenomenon. On the other hand, plasmacytoid dendritic cells (pDCs) are the principal type I interferon (IFN) producers in response to viral infection, and it is unknown whether pDCs are involved in the control of HIV infection in EC. In our study, we analyzed peripheral pDC levels and IFN-$\alpha$ production by peripheral blood mononuclear cells (PBMCs) in EC compared to other groups of HIV-infected patients, the ability of pDCs to reduce HIV production *in vitro*, and the mechanisms potentially involved. We showed preserved pDC counts and IFN-$\alpha$ production in EC. We also observed a higher capacity of pDCs from EC to reduce HIV production and to induce T cell apoptosis, whereas pDCs from viremic patients barely responded without previous Toll-like receptor 9 (TLR-9) stimulus. The preserved functionality of pDCs from EC to reduce viral production may be one of the mechanisms involved in the control of HIV viremia in these subjects. These results demonstrate the importance of innate immunity in HIV pathogenesis, and an understanding of pDC mechanisms would be helpful for the design of new therapies.**

HIV elite controllers (EC) are a rare group of HIV-infected patients who are able to maintain undetectable viral loads during a long period of time in the absence of antiretroviral treatment (ART) (2, 9). Mechanisms responsible for this spontaneous control have been studied with the hope of developing a therapeutic vaccine against HIV.

Preserved T cell functionality has been described for EC (16, 22, 24); however, the innate immunity response needs to be characterized. Plasmacytoid dendritic cells (pDCs) are innate immune cells that respond to viral infections, producing up to 1,000-fold more alpha interferon (IFN-$\alpha$) than other cell types (31) through the stimulation of Toll-like receptor 7 (TLR-7) and TLR-9. The IFN-$\alpha$ produced by pDCs not only participates in the inhibition of viral replication but also has an adjuvant effect on different immune cells types, like monocytes (20), natural killer cells (14), and T cells (10), providing a link between innate and adaptive immunity. In the viral infection scenario, HIV stimulation of pDCs induces high levels of IFN-$\alpha$ production and the rapid expression of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), transforming them into IFN-producing killer pDCs (iKpDCs) (5). TRAIL was shown previously to be involved in the selective induction of apoptosis of uninfected CD4$^+$ T cells (12). Furthermore, TRAIL-expressing iKpDCs were shown previously to be in close proximity to apoptotic CD4$^+$ T cells in tonsils of HIV-infected viremic patients (30).

In a clinical setting, the number of pDCs has been shown to be preserved in long-term nonprogressor subjects and to be decreased in patients progressing to AIDS (29). Highly active antiretroviral therapy (HAART) induced the recovery of pDC numbers and IFN-$\alpha$ production earlier and at a higher level than CD4$^+$ T cell recovery (27, 28). This increase in the number of pDCs during HAART correlates with a lower virus set point after structure therapy interruption (21).

All this knowledge prompted us to hypothesize whether pDCs could be involved in the spontaneous control of HIV viremia experienced in EC. The aim of this study was to quantify pDC levels and to analyze pDC functionality in relation to the spontaneous control of HIV viremia observed for HIV elite controllers.

## MATERIALS AND METHODS

**Study subjects.** The study was performed at the Infectious Diseases Services of the Virgen del Rocío University Hospital (Seville, Spain) and the CNRS-Unite Mixte de Recherche UMR-8147 at the Necker Hospital (Paris, France). HIV-infected patients came from the Infectious Diseases Services HIV-infected patient cohort (26) and from the French ANRS CO18 HIV controller cohort. Buffy coat from HIV-1- and hepatitis C virus (HCV)-seronegative blood bank donors was obtained from the Centro Regional de Transfusión Sanguínea de Sevilla-Huelva y Banco de Tejidos (Seville, Spain) and from the Etablissement Français du Sang (Paris, France). In Spain, samples from patients were kindly provided by the HIV BioBank, integrated within the Spanish AIDS Research Network (RIS).

In this study, we included 14 EC, who were defined as HIV-infected subjects with undetectable viral loads (<40 HIV RNA copies/ml) in at least three determinations during the last year in the absence of any anti-

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retroviral therapy. The EC were compared with 13 relative controllers (RC), defined as HIV-infected subjects without any antiretroviral therapy and with viral loads of between 40 and 1,000 HIV RNA copies/ml in at least the last three determinations during the last year. There were also 19 treated subjects (HAART), defined as HIV-infected subjects on HAART and with undetectable viral loads at least during the last year; 25 viremic subjects (VIR), defined as HIV-infected subjects with high viral loads ($>$10,000 HIV RNA copies/ml) and naive for any antiretroviral therapy; and 22 HIV- and HCV-seronegative donors (HD). All participants gave written informed consent prior to blood sampling. Experimental procedures with human blood have been approved by the Virgen del Rocío, Bicêtre, and Necker Hospital Ethical Committees for human research and were done according to European Union guidelines and the Declaration of Helsinki.

**Laboratory measurements.** Absolute CD4 T cell numbers in fresh blood were determined with an Epics XL-MCL flow cytometer (Beckman Coulter). Plasma HIV-1 RNA levels were measured by quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1 test; Roche Molecular Systems) according to the manufacturer's instructions. The detection limit was 40 HIV RNA copies/ml. HCV RNA was detected with a commercially available PCR procedure (Cobas Amplicor; Roche Diagnosis) with a detection limit of 15 IU/ml.

**Isolation and culture of blood leukocytes.** *In vitro* experiments were performed with peripheral blood mononuclear cells (PBMCs) freshly isolated by density gradient centrifugation. Primary $CD4^+$ T cells were negatively isolated (purity of $>$90%) from whole blood (RosetteSep human $CD4^+$ T cell enrichment cocktail). Fresh pDCs (purity of $>$90%) were isolated from 450 ml of whole blood after density gradient centrifugation by use of an EasySep Human Plasmacytoid DC enrichment kit (StemCell) according to the manufacturer's instructions. All cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin-glutamine (Invitrogen).

**IFN-$\alpha$ production by PBMCs.** Freshly isolated PBMCs ($1.5 \times 10^6$ cells) were cultured in a 48-well plate overnight and stimulated with 1 $\mu$M CpG ODN 2216 (Invivogen), a TLR-9 ligand. The amount of IFN-$\alpha$ in the supernatants was assessed by an IFN-$\alpha$ multisubtype enzyme-linked immunosorbent assay (ELISA) kit (PBL Interferon Source) according to the manufacturer's instructions.

**Primary $CD4^+$ T cell infections.** Purified $CD4^+$ T cells were stimulated during 3 days with phytohemagglutinin (PHA) (5 $\mu$g/ml). $CD4^+$ T cells ($10^6$ cells/ml) were infected with HIV-1 BaL, a CCR5-tropic strain, at a multiplicity of infection (MOI) of 0.01 in 6-well plates by spinoculation at 2.5 krpm for 2 h at room temperature (19). After challenge, the cells were washed and cultured during 6 days in 5 ml of culture medium containing interleukin-2 (IL-2) (100 U/ml). Viral replication was measured by quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1 test; Roche Molecular Systems) according to the manufacturer's instructions. Levels of virus production in the supernatant after 6 days of infection ranged from $10^3$ to $10^6$ HIV RNA copies/ml, depending on the donor.

**pDC-mediated suppression and apoptosis assays.** Purified pDCs (effectors cells) were incubated overnight with or without 1 $\mu$M CpG ODN 2216 (Invivogen). The endosomal acidification inhibitor chloroquine diphosphate salt (CQ) at 1 $\mu$M (Sigma-Aldrich) and 10 $\mu$g/ml of anti-IFN-$\alpha$ antibody (R&D Systems) were used. In a 96-well plate, $50 \times 10^3$ pDCs per well were cocultivated with the chronically HIV-infected H9 T cell line (23, 25) at a 2:1 ratio of effector cells/target cells. After 5 days of coculture, the supernatants were collected to assess p24 (Innogenetic) and IFN-$\alpha$ levels by an ELISA (PBL Interferon Source). To analyze the ability of pDCs to suppress viral production, we calculated the index of suppression in the supernatants [index of suppression = log HIV p24 (T cells) $-$ log HIV p24 (T cells + pDCs)]. Apoptosis determined by annexin V/Topro-III staining and intracellular p24-positive ($p24^+$) cells were measured by flow cytometry with H9 T cells of the coculture. To analyze the antiviral effect of IFN-$\alpha$, in a different experiment, we cultured HIV-infected H9 T cells alone and in the presence of recombinant IFN-$\alpha$ (R&D Systems); after 1 and 5 days of culture, $p24^+$ H9 T cell percentages were assessed by flow cytometry. In a different experiment, HIV-infected primary autologous $CD4^+$ T cells were used as target cells and cultured in a 96-well plate in the presence of $50 \times 10^3$ unstimulated and CpG-stimulated pDCs per well at a ratio 1:2 (effector cells/target cells). After 24 h of coculture, the cells were washed with annexin buffer, and HIV-infected primary autologous $CD4^+$ T cell apoptosis rates were analyzed by annexin V/Topro-III staining.

**Flow cytometry.** Freshly isolated PBMCs were incubated for 20 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-BDCA2 (Miltenyi Biotec) and phycoerythrin (PE)-conjugated anti-CD123 (BD Bioscience) antibodies. pDCs were defined as $BDCA2^+$ $CD123^+$. This analysis was performed with a Cytomics FC500 flow cytometer, and data were analyzed by use of CXP software (Beckman Coulter). To measure apoptosis rates, cocultured cells were washed with annexin buffer (BD Bioscience) and incubated for 15 min at 4°C with FITC-conjugated anti-annexin V (BD Bioscience), PE-conjugated anti-CD123 (BD Bioscience), and allophycocyanin (APC)-conjugated anti-Topro-III (Invitrogen) antibodies. For intracellular p24 detection, after extracellular staining with PE-conjugated anti-CD123 antibodies, cells were incubated in permeabilization buffer containing 1% saponin with monoclonal anti-p24 (FITC-KC57; Beckman Coulter) or control isotype antibodies. Annexin V/Topro-III or intracellular p24 was measured in H9 T cells and in HIV-infected autologous $CD4^+$ T cells defined as being CD123 negative. Fluorescence-activated cell sorter (FACS) analysis was performed on a FACS Canto 7 color flow cytometer using FACS Diva software (BD Bioscience). FlowJo software (Treestar, Ashland, OR) was used to analyze flow cytometry data.

**Three-dimensional (3D) microscopy.** Purified pDCs from HD, VIR, and EC subjects were plated onto poly-L-lysine-coated slides (Sigma) and then fixed in 4% paraformaldehyde, quenched with 0.1 M glycine. Cells were incubated in permeabilizing buffer containing 1% saponin with mouse anti-TLR-7 (Cliniscience) and Alexa 547-labeled anti-CD4 (BD Bioscience) antibodies. The nucleus was stained by using 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Mounted slides were scanned with a Nikon Eclipse 90i upright microscope (Nikon Instruments Europe, Badhoevedorp, Netherlands) using a 100× Plan Apo VC Piezo objective (numerical aperture [NA], 1.4) and Chroma Bloc filters (ET-DAPI, ET-green fluorescent protein [GFP], and ET-Cy3) and were subsequently deconvoluted with a Meinel algorithm and 8 iterations and analyzed by using Metamorph (MDS Analytical Technologies). The TLR-7/DAPI/CD4/overlay/confocal plane was made by using ImageJ software (NIH, Bethesda, MD).

**Statistical analysis.** Statistical analyses were performed by using Statistical Package for the Social Sciences software (SPSS 17.0; SPSS Inc., Chicago, IL). The Spearman test was used to analyze the correlation between continuous variables. Differences between groups were analyzed by Mann-Whitney U tests. The Wilcoxon test was used to analyze related samples. All differences with a $P$ value of $<$0.05 were considered statistically significant.

## RESULTS

**Quantification of peripheral pDCs and IFN-$\alpha$ production.** Characteristics of the study subjects are summarized in Table 1. pDC percentages were assessed for 14 EC, 13 RC, 19 HAART, 25 VIR, and 22 HD subjects (Fig. 1A). We observed preserved pDC levels in EC (0.38%) compared to HD (0.4%) subjects and pDC higher levels than those of noncontroller groups (Fig. 1A). These results were similar when absolute pDC number were analyzed (Fig. 1B); the only difference was that EC presented a trend of higher pDC numbers than HAART subjects (6.9 pDCs/$\mu$l and 4.5 pDCs/$\mu$l, respectively; $P = 0.065$). Interestingly, when both controller groups were analyzed, we observed a trend of higher pDC percentages in EC than in RC subjects ($P = 0.076$), and no differ-

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**TABLE 1** Characteristics of the study subjects[a]

| Parameter | Value for group | | | | |
|---|---|---|---|---|---|
| | HD ($n$ = 22) | EC ($n$ = 14) | RC ($n$ = 13) | HAART ($n$ = 19) | VIR ($n$ = 25) |
| No. (%) of female subjects | 9 (41) | 8 (57) | 5 (38) | 8 (42) | 10 (40) |
| Median age (yr) (interquartile range) | 40 (35–50) | 45 (40–48) | 42 (33–44) | 46 (40–49) | 38 (30–44) |
| Median time from diagnosis (yr) (interquartile range) | NA | 18 (8–21) | 9 (2–19) | 13 (7–16) | 2 (1–5) |
| No. (%) of naive patients | NA | 7 (50) | 7 (54) | 0 | 25 (100) |
| Median no. of $CD4^+$ cells/$\mu$l (interquartile range) | NA | 583 (408–938) | 522 (397–741) | 572 (355–706) | 409 (235–520) |
| No. (%) of $HCV^+$ $PCR^+$ subjects[b] | NA | 4 (29) | 6 (46) | 4 (21) | 4 (16) |
| Median no. of HIV RNA copies/ml (interquartile range) | NA | <40 | 201 (130–360) | <40 | 41,600 (15,534–65,000) |
| No. (%) of patients with risk | | | | | |
| IDU | | 7 (50) | 3 (23) | 9 (47) | 2 (8) |
| Sexual | | 5 (36) | 10 (77) | 9 (47) | 15 (60) |
| Others | | 2 (14) | 0 | 1 (5) | 8 (32) |

[a] NA, not applicable; IDU, injection drug use.

[b] Percentage of subjects with antibodies who were PCR positive for hepatitis C virus.

ences were observed when RC subjects were compared to HAART and VIR subjects ($P$ = 0.788 and $P$ = 0.175, respectively) (Fig. 1A). We also analyzed IFN-$\alpha$ production by PBMCs after TLR-9 stimulation (Fig. 1C). The level of IFN-$\alpha$ production was significantly higher in EC patients than in all other groups, even HD and RC subjects ($P$ = 0.038 and $P$ = 0.030, respectively). The level of production of IFN-$\alpha$ was not statistically different for other groups. As previously described (29), we also observed that peripheral pDC percentages correlated positively with IFN-$\alpha$ production ($r$ = 0.427; $P < 0.001$) (Fig. 1D) and negatively with viral load ($r$ = −0.307; $P$ = 0.005) (Fig. 1E).

**Preserved pDC-mediated HIV suppression in EC.** In order to analyze the capability of pDCs to reduce HIV production, we designed a coculture system using pDCs from 7 EC, 8 VIR, and 7 HD subjects as effector cells and HIV-infected H9 T cells (23, 25) as target cells. The EC included in these experiments were all naive for ART and had a time from diagnosis of over 5 years. First, we analyzed unstimulated pDCs (Fig. 2A) cocultured with H9 T cells. Because H9 T cells produce HIV-1 particles, pDCs should be activated during the 5 days of coculture. We observed that pDCs from EC subjects were able to induce levels of viral suppression similar to those of pDCs from HD subjects, while pDCs from VIR subjects were not able to reduce viral production in H9 T cells. Interestingly, when pDCs were previously stimulated with CpG (Fig. 2B), we did not observed any difference among the groups. pDCs from VIR subjects responded as well as pDCs from HD or EC subjects by inducing similar levels of viral suppression.

These data are in accordance with IFN-$\alpha$ production (Fig. 2C); pDCs from HD and EC subjects produced 100-fold more IFN-$\alpha$ than did pDCs from VIR subjects when pDCs were not previously stimulated. On the other hand, there were no differences in IFN-$\alpha$ production by pDCs among the different groups when pDCs were previously stimulated with TLR-9 (Fig. 2D).

We also observed that anti-IFN-$\alpha$ antibodies partially reverted the reduction of p24 production induced by CpG-stimulated pDCs (Fig. 2E). We also analyzed whether endocytosis was involved in this process. We observed a restoration of p24 levels when chloroquine (CQ) was used in the coculture. The levels of p24 in CQ-treated cultures were similar to those in H9 T cells alone, demonstrating a full recovery of p24 (Fig. 2F). These data show that the antiviral effect of pDCs is at least in part due to IFN-$\alpha$ and that endocytosis is necessary for pDC responses and IFN-$\alpha$ production.

In an attempt to reproduce the results found with the supernatants and to examine the mechanisms involved in viral suppression, we analyzed by flow cytometry $p24^+$ H9 T cell percentages in the coculture and the antiviral effect of IFN-$\alpha$ by adding recombinant IFN-$\alpha$ to H9 T cells (see Fig. S1 in the supplemental material). We used CD123 staining to distinguish between the two cell types (see Fig. S1A in the supplemental material). We observed a reduction in numbers of $p24^+$ H9 T cells when all cells were gated; this effect was abolished when pDCs were treated with CQ (see Fig. S1B in the supplemental material). In contrast, when we gated on live cells, we did not observe any change in $p24^+$ H9 T cell percentages, suggesting that the reduction of viral production was possibly due to the apoptosis of H9 T cells (see Fig. S1C in the supplemental material). In order to analyze the antiviral effect of IFN-$\alpha$, HIV-infected H9 T cells were cultured with and without recombinant IFN-$\alpha$. After 1 day of culture with recombinant IFN-$\alpha$, we observed a reduction of $p24^+$ H9 T cell percentages, as we observed when H9 T cells were cocultured with pDCs. However, this effect disappeared after 5 days, because no additional recombinant IFN-$\alpha$ was added to the culture, showing the transient effect of IFN-$\alpha$ (see Fig. S1D in the supplemental material). These results strongly suggested that IFN-$\alpha$ is responsible for the suppression of p24, probably due to the apoptosis of target cells.

**Preserved capability of pDCs from EC to induce HIV-infected T cell apoptosis.** To confirm that pDC-mediated apoptosis was involved in the reduction in p24 levels, we investigated the efficiency of pDCs to induce H9 T cell apoptosis. First, by analyzing cocultures of unstimulated pDCs and H9 T cells (Fig. 3A), we observed that pDCs from EC subjects induced similar annexin V-positive T cell percentages and a tendency to induce higher Topro-III-positive T cell percentages than pDCs from HD subjects. When EC and VIR pDCs were compared, a greater capacity of pDCs from EC to induce T cell apoptosis was observed (Fig. 3A). However, when CpG-stimulated pDCs were analyzed, no differences were observed among the groups; CpG-stimulated pDCs from EC, HD, and VIR subjects induced quite similar levels

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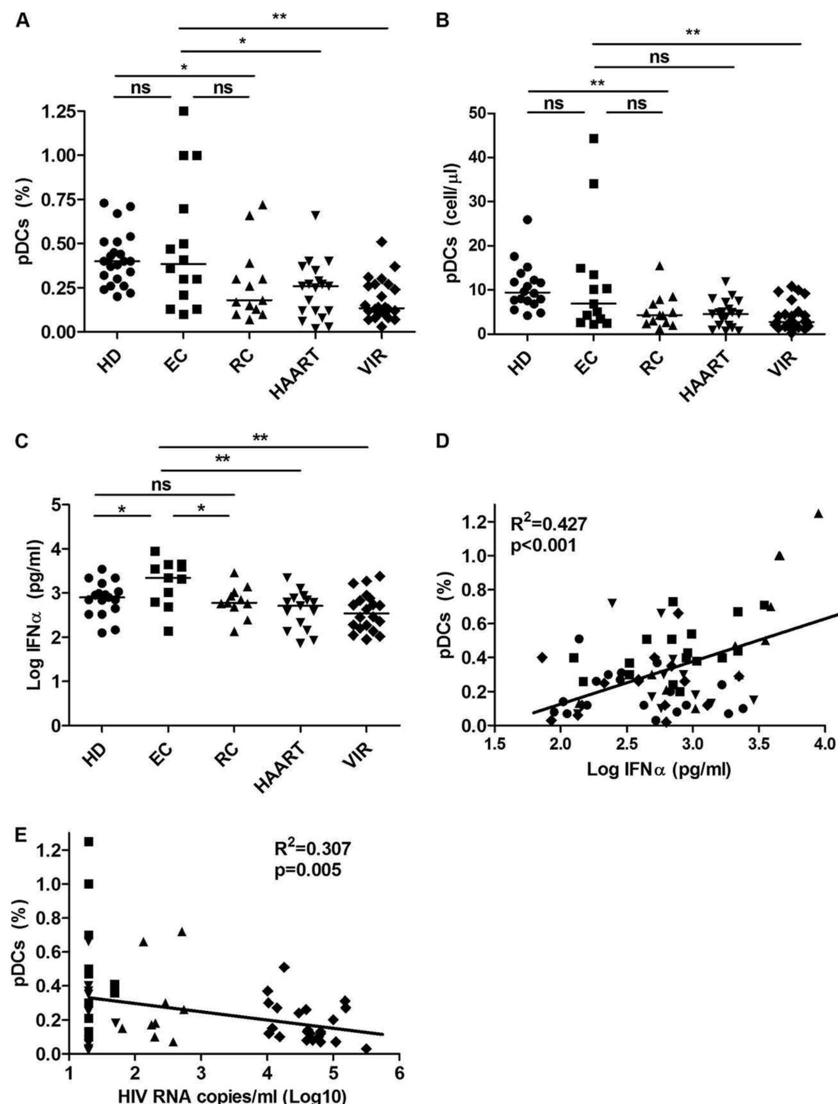


**FIG 1** Quantification of peripheral pDCs and IFN-$\alpha$ production. (A and B) pDC quantification. Percentages (A) and absolute numbers (B) of pDCs (BDCA2$^+$ CD123$^+$) from 22 healthy donors (HD), 14 elite controllers (EC), 13 relative controllers (RC), 19 HIV-treated patients (HAART), and 25 viremic subjects (VIR) were determined. (C) IFN-$\alpha$ production measured in the supernatants of PBMC cultures, in the presence of a TLR-9 ligand (CpG ODN 2216), from 18 HD, 11 EC, 11 RC, 15 HAART, and 22 VIR subjects. The level of IFN-$\alpha$ production by PBMCs cultured in medium alone was in all the cases below the detection limits (data not shown). (D) Correlation between numbers of peripheral pDCs and HIV RNA copies/ml ($\log_{10}$) in 11 EC, 11 RC, 15 HAART, and 22 VIR subjects. (E) Correlation between numbers of peripheral pDCs and IFN-$\alpha$ produced by CpG-stimulated PBMCs from 18 HD, 11 EC, 11 RC, 15 HAART, and 22 VIR subjects. $P$ values were determined by using a Mann-Whitney U test. ns, not significant (a $P$ value of $<0.05$ was considered statistically significant); *, $P < 0.05$; **, $P < 0.005$.

of H9 T cell apoptosis. It should be noted that there was a tendency for pDCs from EC to induce greater levels of annexin V-positive T cells than those from VIR subjects (Fig. 3B). We also analyzed the ability of pDCs to induce HIV-infected primary autologous CD4$^+$ T cell apoptosis in 8 healthy donors (Fig. 4). Both unstimulated and CpG-stimulated pDCs induced HIV-infected autologous CD4$^+$ T cell apoptosis.

**Reduced response of pDCs from VIR subjects is associated with CD4 expression.** According to the results of suppression and apoptosis assays, unstimulated pDCs from VIR subjects showed low degrees of responses to HIV. We also showed by the addition of CQ that the endocytosis pathway is needed for pDC activation. HIV binding to the CD4 expressed by pDCs is essential to activate the endocytosis pathway (7). Thus, we studied CD4 expression on pDCs from HD, EC, and VIR subjects using 3D microscopy (Fig. 5). CD4 was homogeneously expressed on the cell surface of pDCs from EC and HD subjects. In contrast, pDC from VIR subjects showed low levels of membrane CD4. We also observed intracellular CD4 expression in pDCs from VIR subjects, contrasting with pDCs from HD or EC subjects. When pDCs from HD subjects were exposed to $HIV_{MN}$ overnight, we observed the intracellular expression of CD4, as we observed for pDCs from VIR subjects. We also wanted to corroborate these results by flow cytometry; a decrease in CD4 cell surface expression levels in pDCs from VIR compared to HD subjects was observed (see Fig. S2 in the supplemental material). This finding suggests that continuous exposure



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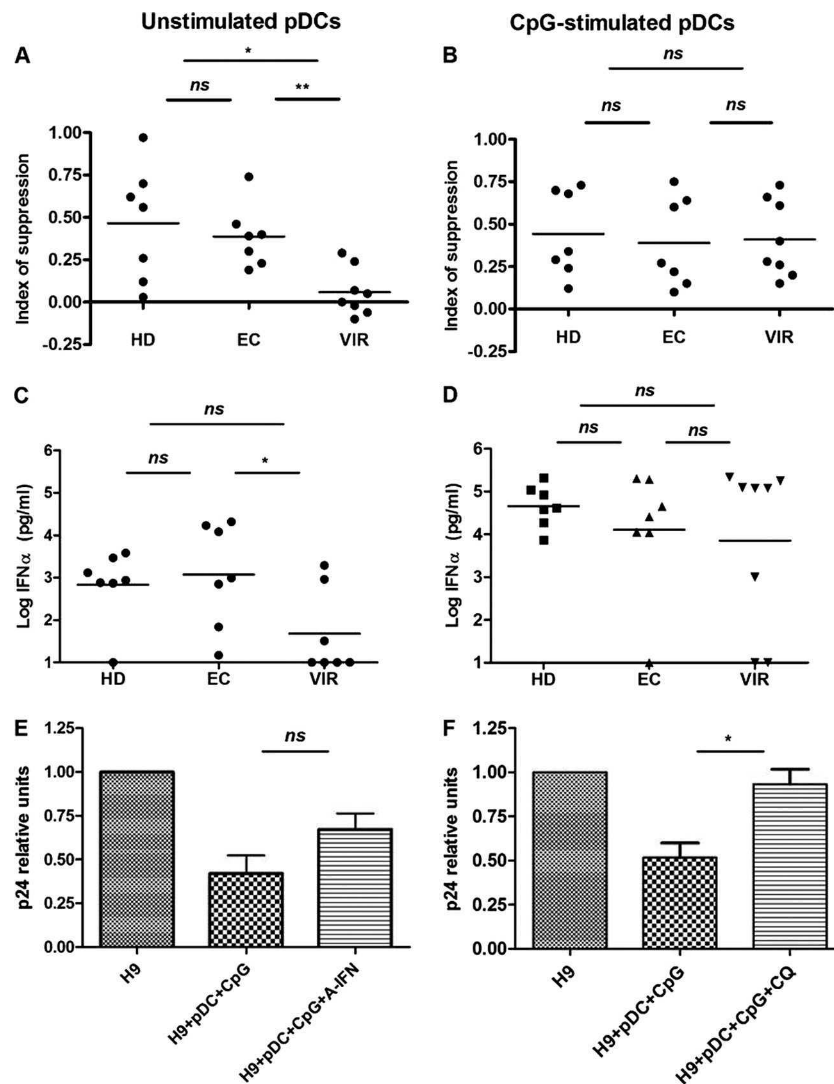


**FIG 2** pDC-mediated suppression of HIV production in H9 T cells. Purified pDCs from 7 HD, 7 EC, and 8 VIR subjects were plated overnight at $5 \times 10^4$ pDCs/well with or without CpG and cocultured in the presence of HIV-infected T cells. After 5 days of coculture, amounts of p24 and IFN-$\alpha$ in the supernatants were measured by an ELISA. (A and B) Effects of unstimulated (A) and CpG-stimulated (B) pDCs on HIV-infected T cell line virus production. The index of suppression by pDCs is expressed as the difference of log HIV p24 production between the culture containing T cells alone and the coculture of T cells and pDCs [index of suppression = log HIV p24 (T cells) − log HIV p24 (T cells + pDCs)]. (C and D) IFN-$\alpha$ production measured in the supernatants of the coculture. (E) Implication of IFN-$\alpha$ in pDC-mediated viral suppression. IFN-$\alpha$ produced by pDCs was partially neutralized by the addition of an anti-IFN-$\alpha$ antibody. Data are representative of 5 different experiments (pDCs from 3 HD and 2 EC subjects). (F) Blocking of endocytosis by the addition of CQ at 1 $\mu$M to wells of CpG-stimulated pDCs. Data are representative of 7 different experiments (pDCs from 2 HD, 1 EC, and 4 VIR subjects). *P* values were determined by using a Mann-Whitney U test. ns, not significant (a *P* value of $<0.05$ was considered statistically significant); *, $P < 0.05$; **, $P < 0.005$.

to HIV in VIR subjects is associated with the internalization of CD4 in pDCs.

## DISCUSSION

In the present study, we show the preserved frequency and functionality of pDCs in relation with the capacity to control HIV infection by EC. The decreased peripheral pDC count observed for VIR subjects was described previously (29) and is possibly due to the migration of these cells to lymph nodes (11, 13). We observed that after successful HAART, the loss of pDC numbers in the chronic phase of HIV infection seems not to be restored entirely, similar to data from a previous study (3). We also observed preserved IFN-$\alpha$ production in PBMCs from EC at even higher levels than in PBMCs from HD subjects. Indirectly, we can assume that this IFN-$\alpha$ production is due mainly to pDC stimulation. Indeed, only pDCs and B lymphocytes express TLR-9 (8), and only pDCs express 1,000 times more IFN-$\alpha$ than other cell types, including B lymphocytes (31). This finding was supported by the correlation observed between IFN-$\alpha$ production by PBMCs after TLR-9 stimulation and pDC counts ($r = 0.464$; $P < 0{,}001$). The higher level of IFN-$\alpha$ production by PBMCs through TLR-9 stimulation observed for EC may be due to the preserved pDC levels and not to a better functionality of pDCs in these patients. In fact, when isolated pDCs were stimulated with TLR-9, we observed similar levels of IFN-$\alpha$ production in HD, EC, and VIR subjects (Fig. 2B). However, when we analyzed IFN-$\alpha$ production by pu-



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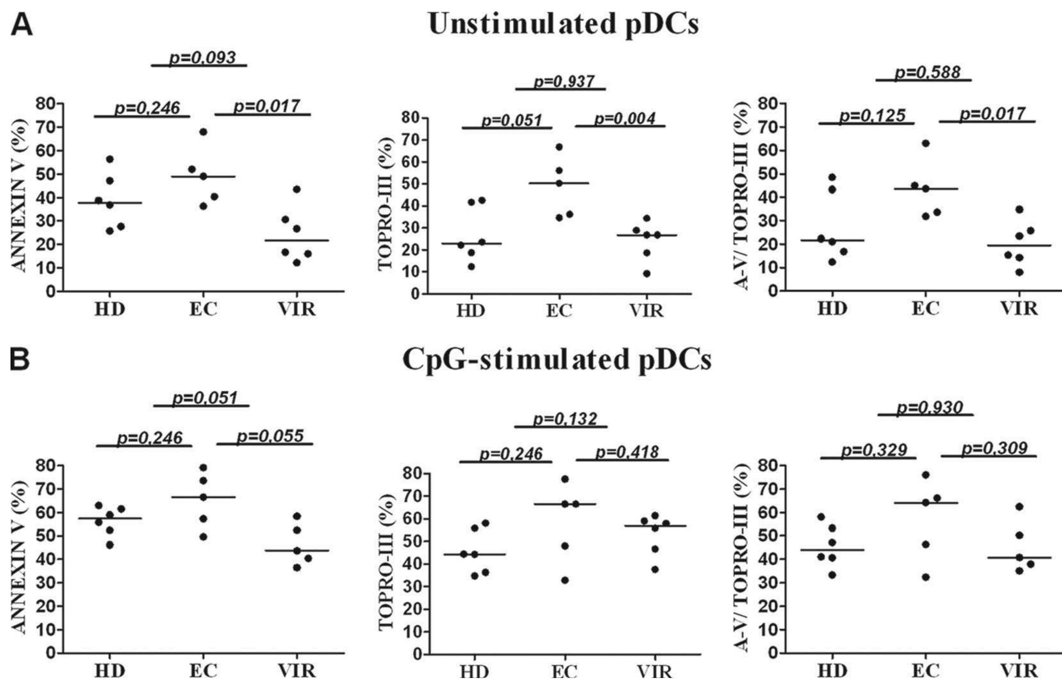


**FIG 3** pDC-mediated H9 T cell apoptosis. Unstimulated (A) and CpG-stimulated (B) pDC-induced apoptosis was assessed on H9 T cells from the cocultures of the suppression assays. The data represent data from 6 HD, 5 EC, and 6 VIR subjects. Left panels represent annexin V (A-V)-positive T cells, middle panels represent Topro-III-positive T cells, and right panels represent annexin V-positive/Topro-III-positive T cells. *P* values were determined by using a Mann-Whitney U test. A *P* value of $<0.05$ was considered statistically significant.

rified pDCs after HIV stimulation, we observed comparable amounts of IFN-$\alpha$ between HD and EC subjects and considerably reduced amounts for VIR subjects. These results demonstrate a preserved pDC response to HIV in EC.

On the other hand, when both HIV controller groups were compared, despite the lower difference in viral loads, we showed differences in pDC percentages and IFN-$\alpha$ production ($P = 0.076$ and $P = 0.030$, respectively) between them; these data demonstrated how the presence of detectable viral loads, even at lower levels, could affect peripheral pDC levels. Interestingly, when we compared RC and HD subjects, despite the lower pDC levels, PBMCs from RC subjects produced levels of IFN-$\alpha$ similar to those produced by PBMCs from HD subjects. Thus, we can conclude that the preserved pDC numbers are associated with the spontaneous control of HIV viremia.

We demonstrated a preserved capacity of pDCs from EC to suppress viral production in H9 T cells. This result is in agreement with data from a previous study (15), where a higher capacity to suppress HIV replication in autologous $CD4^+$ T cells was found for antiretroviral-naive low-viremic subjects ($<$12,500 HIV RNA

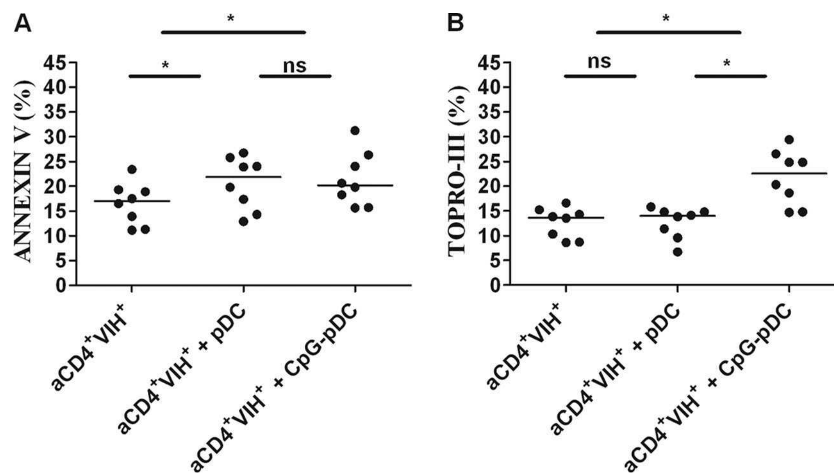


**FIG 4** pDC-mediated HIV-infected primary autologous $CD4^+$ T cell apoptosis. Isolated primary autologous $CD4^+$ T cells from 8 healthy donors were infected for 6 days with HIV BaL. HIV-infected primary autologous $CD4^+$ T cells (a$CD4^+$ $HIV^+$) were cultured in the presence of purified pDCs at a ratio of 1:2. After 24 h, annexin V-positive $CD4^+$ T cells (A) and Topro-III-positive $CD4^+$ T cells (B) were assayed by flow cytometry. Differences were analyzed by using a Wilcoxon test. A *P* value of $<0.05$ was considered statistically significant. ns, not statistically significant; $*$, $P < 0.05$.



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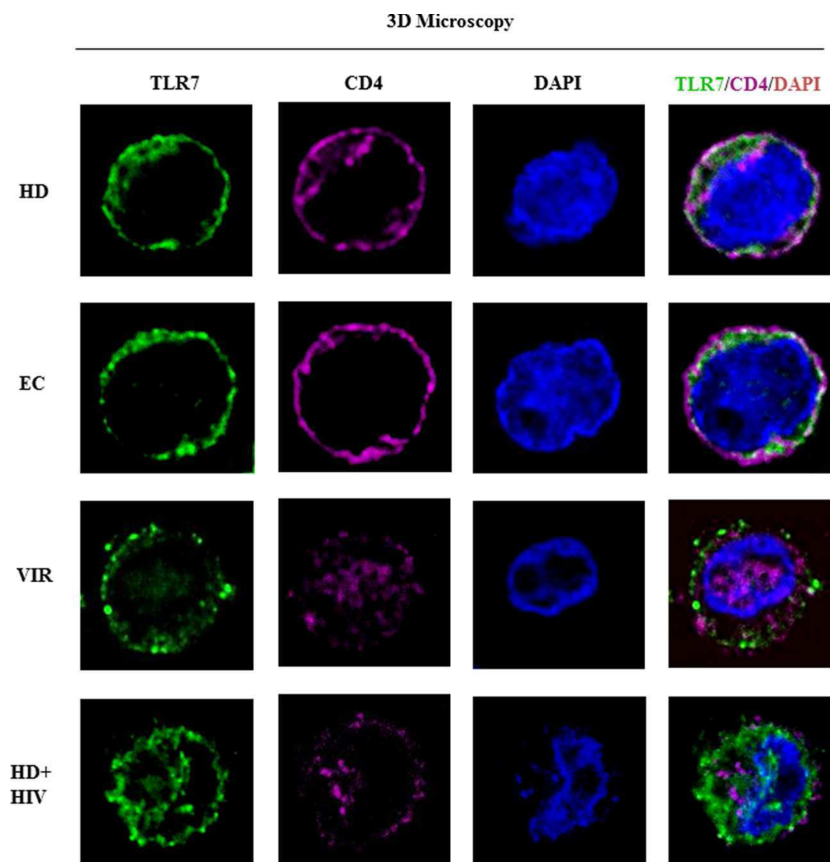


**FIG 5** Microscopy analysis of CD4 distribution in pDCs. Shown is three-dimensional microscopy of the CD4 distribution in pDCs. pDCs from HD, EC, and VIR subjects and HIV-activated pDCs from HD (HD+HIV) subjects were stained with anti-CD4 antibody (pink), anti-TLR-7 antibody (green), and DAPI (blue). pDC stainings (green, pink, and blue) were merged and analyzed by 3D microscopy.

copies/ml) than for treated subjects and high-viremic subjects (≥12,500 HIV RNA copies/ml). However, in our study, we used the same target cells (H9 T cell line) to compare the suppressive faculty of pDCs among the study groups. We demonstrated that pDCs from EC subjects had the same capacity to reduce HIV production as pDCs from HD subjects, whereas pDCs from VIR subjects could scarcely respond. In contrast, when pDCs from all groups were prestimulated with TLR-9, we observed the same behavior for the three groups. These results agree with previous data showing that CpG A- and HIV-stimulated pDCs are not refractory to IFN-$\alpha$ production after restimulation (18). In this work, we show that the magnitude of the response after continuous HIV stimulation is higher in EC than in VIR subjects. This result demonstrates that pDCs from VIR subjects are functional but are not able to be efficiently stimulated by HIV and need to be previously activated by an HIV-independent pathway to display antiviral activity against HIV-infected T cells. This activity was associated with the IFN-$\alpha$ produced by isolated pDCs. These results show that pDCs from EC have preserved functionality to suppress HIV.

The great majority of *in vitro* studies on pDCs suggested that IFN-$\alpha$ is the principal mechanism of viral suppression (4, 17). A previous report demonstrated that the HIV stimulation of pDCs induces a high level of production of IFN-$\alpha$ and a rapid expression of TRAIL, transforming them into IFN-producing killer pDCs (iKpDCs) (5). It was also shown previously that despite TRAIL expression, pDCs could not induce the lysis of autologous CD4$^+$ T cells (1). However, in the present work, when primary HIV-infected autologous CD4$^+$ T cells were used as target cells, we also observed apoptosis induced by both unstimulated and TLR-9-stimulated pDCs. This discrepancy can be explained because we investigated not lysis but apoptosis using early (annexin V) and late (Topro-III) apoptosis markers, and autologous CD4$^+$ T cells were productively infected and not only exposed to HIV; in addition, cocultures were maintained not for 6 h as in previous work but overnight in the case of autologous CD4$^+$ T cells or for 5 days in the case of H9 T cells. Thus, we demonstrated that besides IFN-$\alpha$, pDCs exert their antiviral effect by inducing T cell apoptosis. Indeed, when p24$^+$ T cell levels in the coculture were analyzed, we observed a reduction of p24$^+$ T cell percentages. This effect was also observed when recombinant IFN-$\alpha$ was added to H9 T cells. These observations suggest that the pDC-induced viral reduction is due mostly to H9 T cell-induced apoptosis, which is preserved in EC. This finding is in accordance with our results showing that purified pDCs from EC and HD subjects produced large amounts of IFN-$\alpha$ in response to HIV-1 particles produced by the T cell line. In contrast, the level of IFN-$\alpha$ production by pDCs from VIR subjects was very low and barely suppressed or induced T cell apoptosis, confirming the fact that VIR subjects had an impaired HIV-mediated activation of pDCs. Thus, in an at-



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tempt to understand why pDCs from VIR subjects were not able to respond to the HIV stimulus, we analyzed CD4 receptor expression on pDCs, which is necessary for HIV binding to pDCs and the subsequent activation of the endocytosis pathway by these cells (6, 7). The very low membrane CD4 expression level on pDCs from VIR subjects could explain the lack of responses when they were cultured in the presence of HIV. However, when pDCs from VIR subjects where stimulated by a CD4-independent activator (CpG), they responded similarly to those from HD or EC subjects. These data also explain the similar behaviors of pDCs among the different groups when a previous stimulation via a CD4-independent pathway, such as the TLR-9 ligand CpG, was performed.

In conclusion, this study shows the qualitative and functional involvement of pDCs in the spontaneous control of HIV viremia. These findings highlight the important role of innate immunity in HIV immunopathogenesis and could have important immunotherapeutic applications.

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MAJOR ARTICLE

# Plasmacytoid Dendritic Cells (pDCs) From HIV Controllers Produce Interferon-α and Differentiate Into Functional Killer pDCs Under HIV Activation

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***Background.*** Human immunodeficiency virus (HIV) controllers spontaneously control viremia and CD4 T-cell depletion in contrast to viremic patients. After HIV exposure, plasmacytoid dendritic cells (pDCs) produce high levels of interferon alpha (IFN-α) and express the apoptotic ligand TRAIL (tumor necrosis factor–related apoptosis inducing ligand). Simian models have shown that prolonged high levels of IFN-α production could be responsible for AIDS progression.

***Methods.*** We studied pDC activation in response to human immunodeficiency virus (HIV) using flow cytometry and 3D microscopy.

***Results.*** We show here that pDCs from controller patients produced higher levels of IFN-α in response to HIV than pDCs from viremic patients but similar levels to pDCs from healthy donors. Because binding of HIV to CD4 is essential for pDC activation, the low CD4 expression by pDCs from viremic patients may explain the weak IFN-α response to HIV. Three-dimensional microscopy revealed that pDCs from controllers and healthy donors expressed intracellular TRAIL that is relocalized to the membrane after HIV exposure. In contrast, pDCs from viremic patients expressed membrane TRAIL without any stimulation.

***Conclusions.*** We demonstrate that, in response to HIV, pDCs from controller patients produce IFN-α, express membrane TRAIL, and induce apoptosis of T-cell lines.

Human immunodeficiency virus type 1 (HIV-1) pathogenesis is characterized by high viral load and massive CD4 T-cell depletion [1, 2] in the vast majority of patients. However, a subset of HIV-1–positive individuals have been identified who do not progress and spontaneously maintain an undetectable viral load. This infrequent patient population is defined as HIV-1 controllers (hereafter called "controllers") [3–5] and represents <1% of HIV-1–infected patients [6, 7]. The majority of controllers are also defined by the absence of massive CD4 T-cell depletion, even after 10 years of infection [5, 8].

Plasmacytoid dendritic cells (pDCs) are innate immune cells [9, 10] and play a central role in host defense against viruses [11, 12] by producing high levels of interferon alpha (IFN-α) [13–16]. Ex vivo, a severe decrease of pDCs in blood from HIV-infected viremic patients is observed [17, 18]. This apparent blood depletion is probably due to migration of HIV-activated pDCs from blood to lymphoid organs [19, 20] where massive CD4 T-cell depletion occurs [21, 22]. Furthermore, HIV-activated pDCs also express the apoptotic

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ligand TRAIL (tumor necrosis factor–related apoptosis inducing ligand) providing them potential killer activity (interferon-producing killer pDCs) [15]. These TRAIL-expressing killer pDCs were demonstrated to be in close proximity to apoptotic CD4 T cells in tonsils from HIV-infected viremic patients [19]. In controller patients, IFN-α production and TRAIL expression by pDCs have not yet been investigated.

TRAIL selectively induces apoptosis of human HIV-exposed CD4 T cells in vitro [23]. TRAIL induces apoptosis of death receptor 5 (DR5)–expressing cells [24, 25]. The percentage of CD4 T cells coexpressing TRAIL and DR5 is elevated in the blood of viremic patients [26, 27] and is reduced by successful highly active antiretroviral therapy [28]. The study of TRAIL regulation reveals that type I interferon (IFN-α/β) produced by pDCs after HIV endocytosis regulates TRAIL on CD4 T cells [16]. IFN-α production by HIV-induced Toll-like receptor (TLR) 7 activation of pDCs correlates with disease progression and is sex dependent [29].

Only limited information is available about pDCs in controllers [30], and because it has not been yet reported, we investigated pDC response to HIV and TRAIL expression by controller patients.

## MATERIALS AND METHODS

### Patient Blood Samples

HIV controllers came from the French national cohort ANRS CO18 and from the Spanish cohort HUVR of HIV Controllers, previously described [31]. The controller cohorts were selected as HIV-1–infected patients who have never received any antiretroviral treatment with a follow-up >10 years and plasma HIV RNA measurements <400 copies/mL >90% of quantifications [32, 33] (Amplicor Monitor, Roche Diagnostics).

Viremic chronically infected untreated patients with CD4 count >400 cells/µL were selected at the Bicêtre Hospital in Paris (France) and at Virgen del Rocio University Hospital in Seville (Spain) for apoptosis assays (Table 1). Blood from 30 healthy donors was obtained from Etablissement Français du Sang (convention 07/CABANEL/106), Paris, France.

### Ethics Statement

The study was promoted by the Agence Nationale de Recherche pour le Sida (ANRS) under number ANRS Study CO18 and approved by the Comité de Protection des Personnes Ile de France VII under number 05-22. Experimental procedures with human blood have been approved by the Bicêtre and Necker Hospital ethical committees for human research and were done according to the European Union guidelines and the Declaration of Helsinki.

### Isolation and Culture of Blood Leukocytes

In vitro experiments were performed using peripheral blood mononuclear cells (PBMCs) freshly isolated by density centrifugation from peripheral blood lymphocyte separation medium (Cambrex). To preserve cell integrity and function, no frozen cells were used. CD4 T cells and pDCs were purified using a CD4 purification kit (Miltenyi Biotech) and a Human Plasmacytoid DC Negative Isolation Kit (StemCell Technologies), respectively. Cells were then cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Hyclone) and 1% Pen-Strep-Glut (Invitrogen).

### Viral Stimulation

Cells from healthy donors, controllers, or viremic patients were seeded at $5 \times 10^4$ cells/100 µL and cultured overnight with inactivated AT-2 HIV-$1_{MN}$ at 20 ng/mL $p24^{CA}$ equivalent, kindly provided by J. D. Lifson (SAIC-National Cancer Institute, Frederick, Maryland) or infectious influenza A virus (Flu) hemagglutinin A PR8, titer 1:8192 at dilution 1:1000. Supernatants were collected for cytokine detection. HIV-1/pDC interaction was inhibited using soluble CD4 (sCD4) (2 µg/mL) and recombinant gp120 (2 µg/mL) obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases.

### Flow Cytometry

Cells were incubated for 20 minutes at 4°C with fluorescein isothiocyanate–CD123 (MBL International), APC-BDCA-2/4 and Vioblue-CD4 (Miltenyi), and APC-Cy7-CD14 (BD Biosciences) or with appropriate isotype-matched control antibodies (5 µg/mL each) in phosphate-buffered saline containing 2% mouse serum (Sigma). The pDCs were tested for PE-TRAIL (eBioscience). Acquisitions were performed on a flow cytometer (FACSCanto 7 colors) using FACSDiva software (BD Biosciences). Flow cytometry data were analyzed by FlowJo software (Treestar).

**Table 1. Characteristics of HIV Controllers and Viremic Patients**

| Group | Age (y) | Year HIV Diagnosed | CD4 Count (Cells/mL) | Viral Follow-up (y) | RNA Viral Load (Copies/mL) |
|---|---|---|---|---|---|
| Controllers (n = 22) | 45 (42–69) | 1989 (1987–2000) | 718 (603–1140) | 11 (10–15) | <50 |
| Viremic patients (n = 18) | 42 (39–50) | 2007 (2005–2010) | 612 (539–796) | 2 (1–10) | 26 700 (5937–519 823) |

Data are median (interquartile range).

Abbreviation: HIV, human immunodeficiency virus.

### Cytokine Detection

Supernatants of PBMCs were tested for multispecies soluble IFN-α by enzyme-linked immunosorbent assay (Cell Sciences) according to the manufacturer's instructions.

### Apoptosis Assays

Purified pDCs were stimulated overnight or not with 1 µM CpG ODN 2216 (InvivoGen). Cells were cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Hyclone) and 1% Pen-Strep-Glut (Invitrogen). Freshly purified CD4 T cells or HIV-infected H9 T-cell lines were used as target cells and cocultured with pDCs. Unstimulated and CpG-stimulated pDCs were cocultured with the H9 T cells at cell effector/target ratio of 2:1, in a 96-well plate. After 24 hours of coculture, the supernatants were collected, and the cells were washed with Annexin buffer and incubated for 15 minutes with AnnexinV/Topro-III and CD123 antibodies at 4°C. AnnexinV/Topro-3 was measured on $CD123^{-}$ H9 T cells.

### Three-Dimensional Microscopy

Three-dimensional experiments were performed with pDCs cultured overnight with or without HIV except for pDCs purified from viremic patients (Supplementary Figure 1) that were directly stained with TRAIL (ex vivo). Purified pDCs from healthy donors, viremic patients, and controllers were plated on poly-L-lysine (Sigma)–coated slides and then fixed in 4% paraformaldehyde, quenched with 0.1 M glycine. Cells were incubated in permeabilizing buffer containing 1% saponin with mouse anti-TRAIL (clone RIK-2, eBioscience) and Alexa 547–labeled anti-CD4 (BD Biosciences). TRAIL staining was revealed using a secondary goat antimouse immunoglobulin G–Alexa 488 (Jackson ImmunoResearch). Nuclei were stained using DAPI (Molecular Probes). Mounted slides were scanned with a Nikon Eclipse 90i Upright microscope (Nikon Instruments Europe) and were subsequently deconvoluted (Meinel algorithm) and analyzed using Metamorph (MDS Analytical Technologies). ImageJ 3D viewer was used on an overlay stack on pDCs stained with TRAIL/CD4/DAPI.

### Statistical Analysis

Experiments were repeated 4 times. *P* values were determined using a 2-tailed Student *t* test. $P < .05$ was considered statistically significant. Univariate distributions of flow cytometry data were performed by probability binning, in 300 bins using FlowJo software.

## RESULTS

### Quantitative and Qualitative pDC Analysis

Plasmacytoid dendritic cell blood depletion is a serologic marker for AIDS progression [17, 34]. We determined the percentage of pDCs, among PBMCs, characterized by $CD4^{+}CD123^{+}CD11c^{-}BDCA\text{-}2^{+}BDCA4^{+}$ (Figure 1*A*) in blood from 11 untreated HIV-1–infected viremic patients and 13 controllers (Table 1) compared with 30 healthy donors. In contrast to the pDC depletion observed in viremic patients, controllers did not exhibit a statistically significant pDC percentage or absolute number decrease in blood compared to healthy donors. Also, the percentage and number of pDCs in controllers were significantly higher than in viremic patients (Figure 1*B*). We then tested whether purified pDCs from controllers or viremic patients were able to produce IFN-α when cultured with either HIV or Flu (influenza A virus).

HIV-stimulated pDCs from controllers and healthy donors produced similar levels of IFN-α (Figure 1*C*). However, HIV-1–induced pDC response of viremic patients was significantly lower than that of healthy donors ($P = .01$) and controllers (Figure 1*C*). The pDCs from controllers produced statistically higher levels of IFN-α in response to Flu than pDCs from healthy donors or viremic patients (Figure 1*D*). Thus, pDCs from controllers are able to produce IFN-α in response to both viruses, demonstrating that pDCs do not exhibit quantitative nor qualitative defects in the IFN-α pathway.

### TRAIL Expression and Regulation in pDCs From Controller Patients

For a complete functional analysis, pDCs from healthy donors, controllers, and viremic patients were purified from fresh blood, and TRAIL expression was studied. Cytometry analysis revealed that the mean fluorescence intensity (MFI) of membrane TRAIL expression by pDCs (Figure 2*A*) and the number of TRAIL-expressing pDCs (Supplementary Figure 1*A*) were higher in viremic patients compared with healthy donors and controllers. However, in vitro stimulation of pDC by HIV induced significant increase of membrane TRAIL (mTRAIL) MFI (Figure 2*A*) and number of TRAIL-expressing pDCs (Supplementary Figure 1*A*) in controllers ($P = .0001$) and healthy donors ($P = .001$). Surprisingly, HIV did not statistically increase mTRAIL MFI by pDCs but increased the number of TRAIL-expressing pDCs in viremic patients. This could be explained by the high levels of mTRAIL expressed by unstimulated pDCs from viremic patients, probably due to pDC activation in vivo.

Thus, we performed 3D microscopic experiments to study TRAIL expression and localization in unstimulated and HIV-activated pDCs from healthy donors (n = 3), controllers (n = 3), and viremic patients (n = 3). As shown in Figure 2, cytometry analysis revealed no expression of mTRAIL by unstimulated pDCs from healthy donors. Surprisingly, image plan analysis revealed that unstimulated pDCs from healthy donors expressed TRAIL in the intracellular compartment (Figure 2*B* and 2*C*). In this setting, TRAIL localization of pDCs from HIV Infected Controllers (HIC) was similar to that of healthy donors (Figure 2*B* and 2*C*). In contrast, pDCs from viremic patients expressed both intracellular and extracellular TRAIL (Figure 2*B*

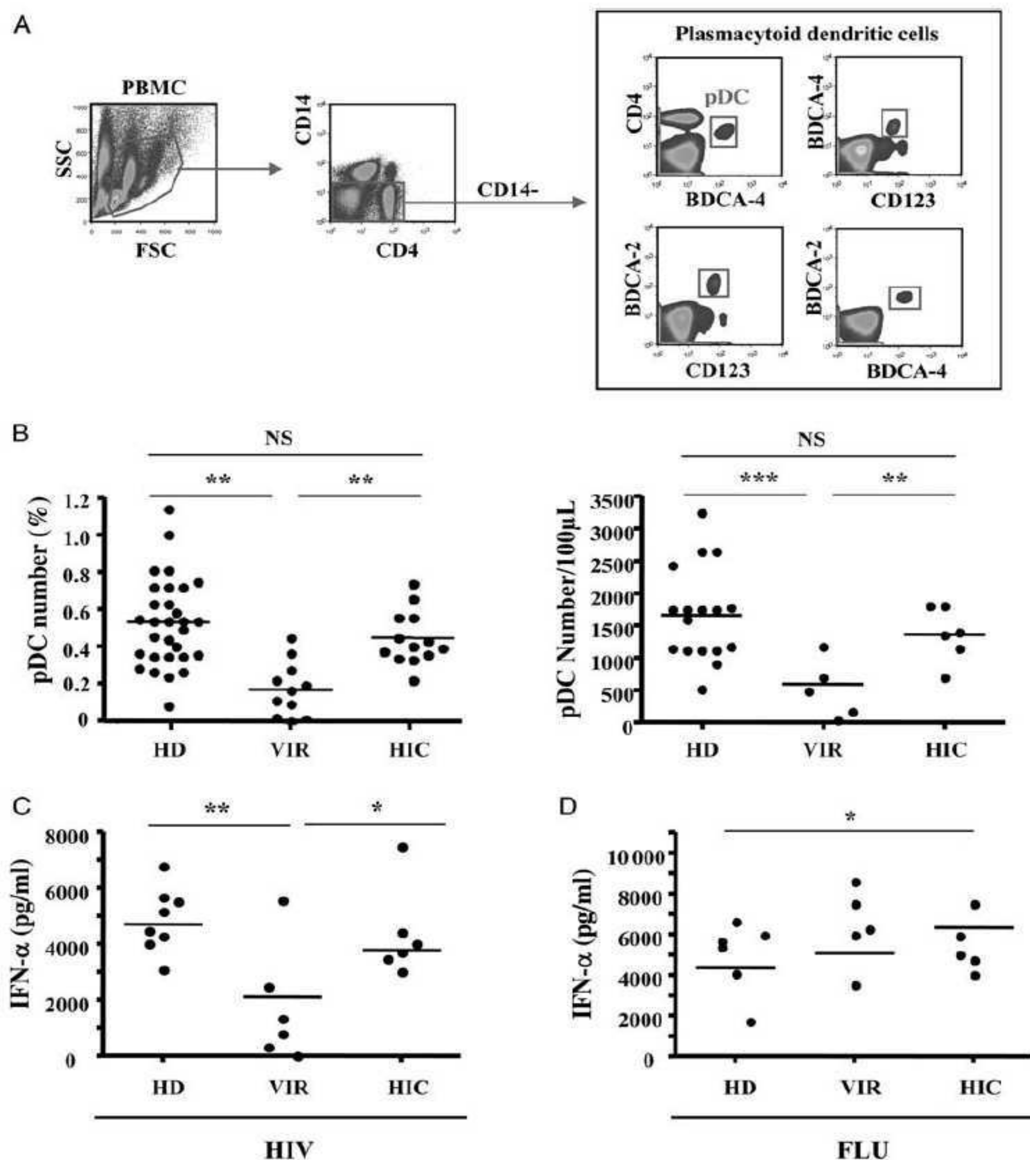


**Figure 1.** Quantitative and qualitative characterization of plasmacytoid dendritic cells (pDCs). *A*, Characterization of pDCs using CD14 exclusion and CD4, BDCA-2, BDCA-4, and CD123 stainings. *B*, pDC quantification. Percentage and number of pDCs (BDCA-2$^+$, BDCA-4$^+$, CD123$^+$, and CD4$^+$) contained in blood from healthy individuals, human immunodeficiency virus (HIV) controllers, and viremic patients. Statistical analysis of pDC number in peripheral blood mononuclear cells (PBMCs) from healthy donors (n = 30), viremic patients (n = 11), and controllers (n = 13). *C* and *D*, Interferon (IFN)–α secretion by virus-stimulated pDCs. IFN-α production was measured in supernatants of pDCs among PBMCs from healthy donors (n = 13), viremic patients (n = 9), and controllers (n = 11) and cultured with AT2 HIV-$1_{MN}$ (*C*) or influenza A virus (Flu) (*D*). *P* values were determined using Mann–Whitney (*A* and *B*) and 2-tailed Student *t* test (*C* and *D*). Abbreviation: NS, not significant. $P < .05$ was considered statistically significant. *$P < .05$; **$P < .01$.

and 2*C*), confirming fluorescence-activated cell sorting analysis (Figure 2*A* and 2*B*). Similarly, intracellular TRAIL expression of HIV-1–stimulated pDCs from HIC and healthy donors appeared to be decreased in favor of the membrane compared with unstimulated pDCs from healthy donors or controllers (Figure 2*B* and 2*C*).

To better characterize TRAIL localization in pDC, 3D reconstruction analysis was performed (Figure 3). YZ and XZ stacks showed the relocalization of TRAIL at the membrane of HIV-1–stimulated pDCs from healthy donors and controllers (Figure 3*C* and 3*D*) in contrast to intracytoplasmic TRAIL repartition of unstimulated pDCs from controllers or healthy

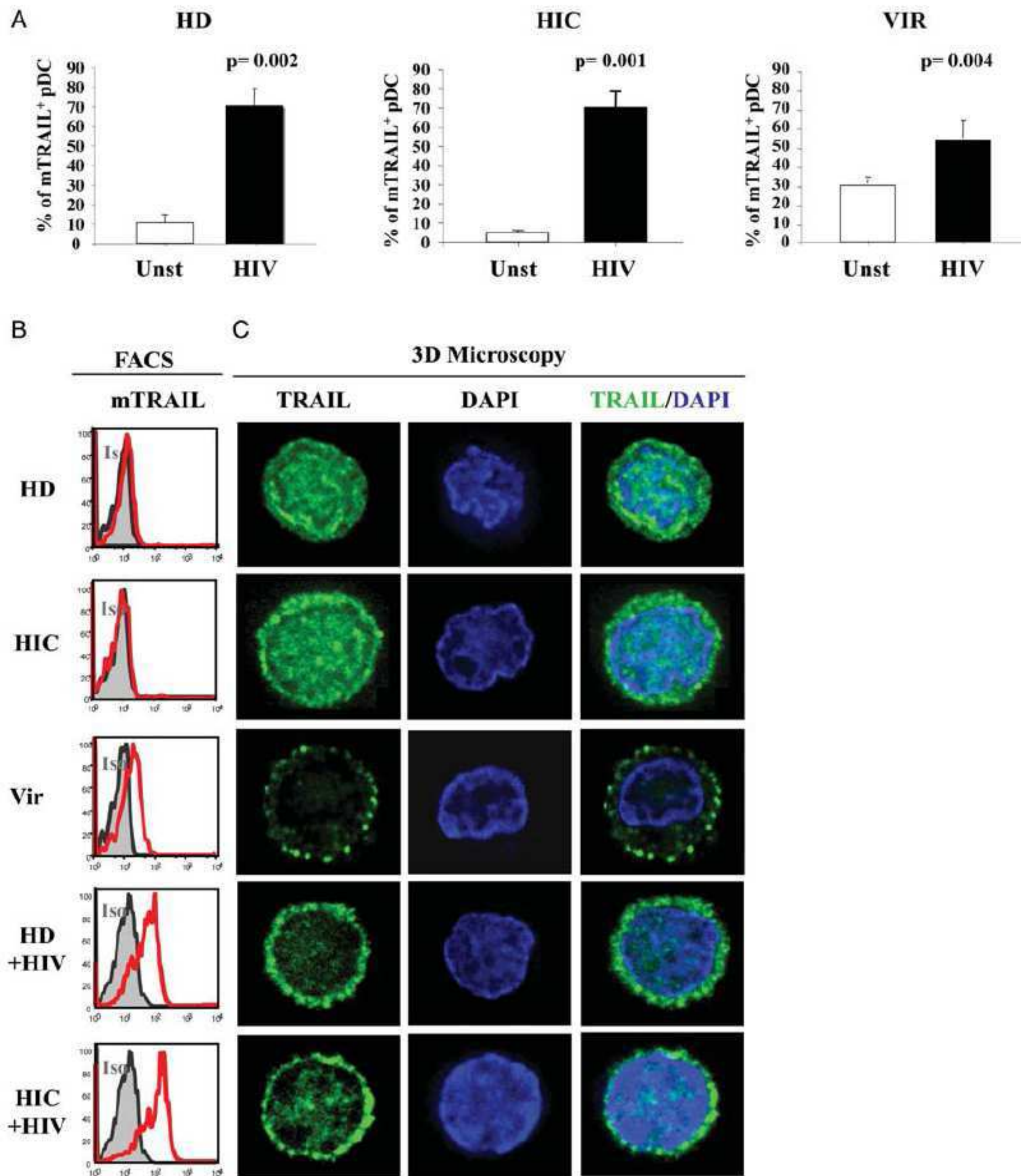


**Figure 2.** Cytometry and 3D analysis of TRAIL (tumor necrosis factor–related apoptosis inducing ligand) localization in plasmacytoid dendritic cells (pDCs). *A*, Freshly purified pDCs from healthy donors, viremic patients, and human immunodeficiency virus (HIV) controllers were cultured with (HIV) or without (Unst) HIV overnight. Cells were stained with anti-TRAIL antibody and analyzed by fluorescence-activated cell sorting (FACS). Panels represent membrane TRAIL mean fluorescence intensity (MFI) expressed by pDCs. *B*, pDCs from healthy donors, viremic patients, and controllers and pDCs from healthy donors and controllers cultured in vitro with HIV (healthy+HIV) were analyzed for TRAIL expression by flow cytometry and simultaneously by 3D microscopy. *C*, pDCs from healthy donors, viremic patients, and controllers and HIV-activated pDCs from healthy donors (healthy + HIV) and controllers (controller + HIV) were stained with anti-TRAIL (*green*) and DAPI (nucleus staining) and analyzed by 3D microscopy. pDC stainings (*green* and *blue*) were merged (*right panels*). pDCs from viremic and HIV-activated pDCs showed a different TRAIL localization compared with unstimulated pDCs from healthy donors or controllers (*C*).

donors (Figure 3*A* and 3*B*). A 3D interactive surface plot of ImageJ software allowed us to visualize with precision internal or external localization of TRAIL combined with phase contrast acquisition (membrane delimitation) (Figure 3*A–D*). The number of cells expressing membrane or intracellular TRAIL was quantified by counting a total of 50 pDCs per condition from healthy donors (n = 3), controllers (n = 3), healthy donors + HIV (n = 3), and controllers+ HIV (n = 3) (Figure 3*A–D*). It

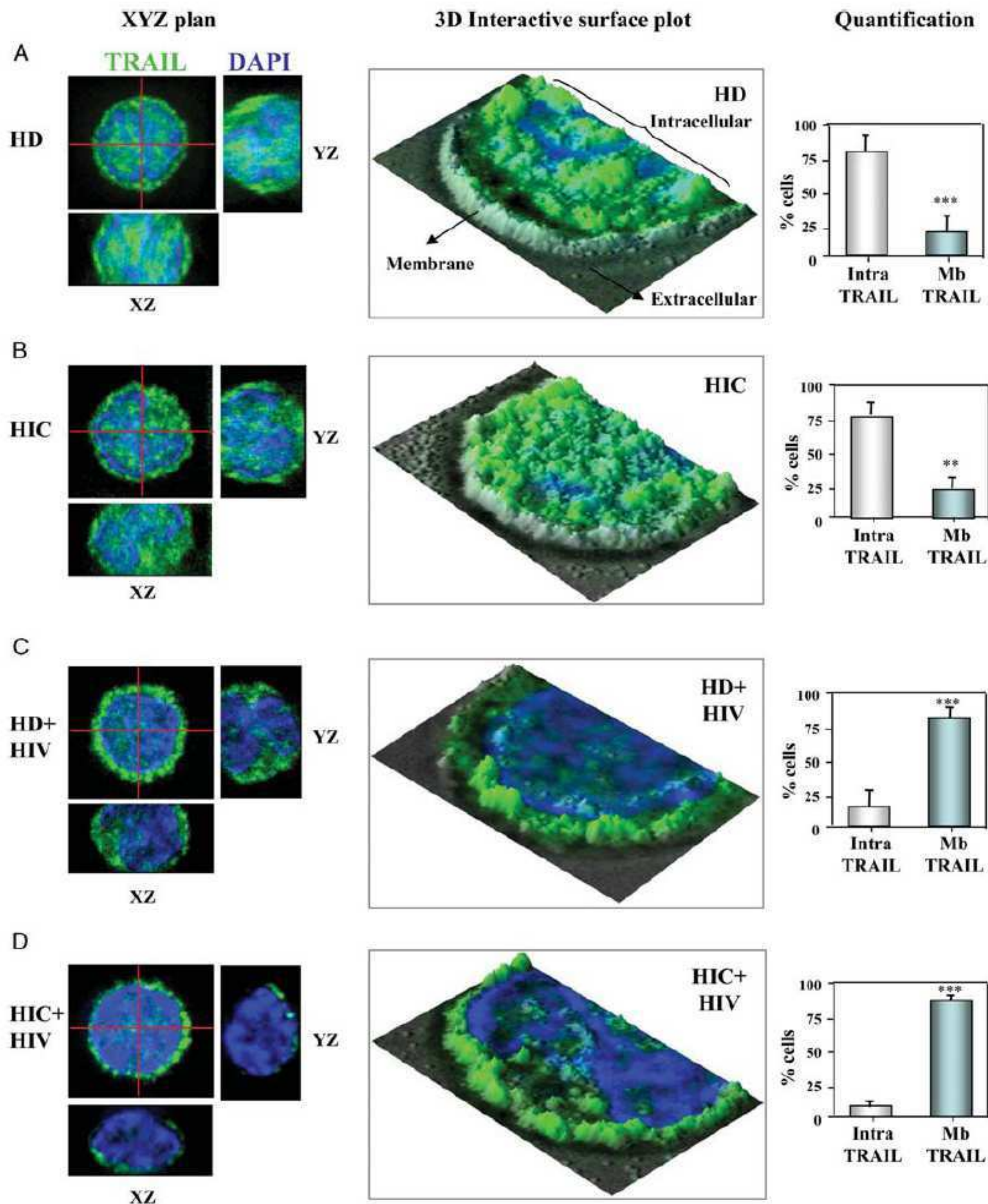


**Figure 3.** Microscopy analysis of TRAIL (tumor necrosis factor–related apoptosis inducing ligand) localization in plasmacytoid dendritic cells (pDCs). To better characterize TRAIL localization, images were analyzed using 3D interactive surface plot analysis. *A*, Deconvolution overlays of representative 2D red cross axis XY focal plan with XZ/YZ view of focal plane and projection overlay of cell stainings (analyzed by Metamorph software). pDCs from healthy donors (*A*) and controllers (*B*), human immunodeficiency virus (HIV)–stimulated pDCs from healthy donors (*C*), and HIV-stimulated pDCs from controllers (*D*) were stained with anti-TRAIL (*green*) and DAPI (nucleus staining). Deconvolution of representative 2D XY focal plane was treated to allow Z scaling of pixel intensity (interactive 3D surface plot analyzed by ImageJ software). Each staining, anti-TRAIL (*green*) and DAPI (nucleus staining) were merged together with phase contrast (*grey, defined cell surface*). This 3D surface plot representation was used to enhance the observation of intracellular TRAIL localization or its relocalization to the membrane (Mb). The vast majority of pDCs from healthy donors and controllers statistically expressed intracellular TRAIL (*green*) in contrast to pDCs from viremic patients and HIV-stimulated pDCs that expressed TRAIL at the cell surface. Fifty cells per condition—healthy (n = 3), controller (n = 3), healthy + HIV (n = 3), and controller + HIV (n = 3)—were quantified regarding their expression of membrane or intracellular TRAIL expression. Abbreviation: NS, not significant. $P < .05$ was considered statistically significant). *$P < .05$; **$P < .01$.

should be noted that cells expressing both intracellular and membrane TRAIL were counted as membrane TRAIL–positive cells. Our results clearly demonstrated that HIV-1–stimulated pDCs from controllers harbored membrane TRAIL localization (Figure 3*C* and 3*D*) in contrast to the restrictive intracellular TRAIL expression of the vast majority of pDCs from unstimulated healthy donors and controllers (Figure 3*A* and 3*B*). We also found that ex vivo pDCs from viremic patients expressed membrane TRAIL in contrast to pDCs from healthy donors or controllers (Supplementary Figure 1).

Thus, these results demonstrate that pDCs from controller patients are able to respond to HIV in vitro by expressing TRAIL but also activation markers such as HLA-DR, CD80, CD83, CD86, CD40, and CCR7 (data not shown).

### CD4 Expression on pDCs From Healthy Donors, Controllers, and Viremic Patients

We showed in Figure 1*C* that pDCs from viremic patients produced less IFN-α than pDCs from healthy donors or controllers in response to HIV, but they responded equally to Flu stimulation (Figure 1*D*). We and others previously reported that the binding of viral gp120 to cellular CD4 expressed by pDC was essential to activate the endocytosis pathway and the TLR response [15, 35, 36]. Thus, we quantified CD4 expression on freshly purified pDCs from all individuals in each group. CD4 expression was significantly lower on unstimulated pDCs from viremic patients than controllers or healthy donors. In contrast, CD4 expression was similar on pDC cell surface from controllers and healthy donors (Figure 4*A*).

Thus, purified pDCs were stained ex vivo with anti-CD4, anti-TRAIL, and DAPI (Figure 4*C*). Similar levels and repartition of CD4 were observed on pDCs from controller patients and healthy donors. CD4 was homogeneously expressed at the cell surface and TRAIL was localized into the cell. However, pDCs from viremic patients clearly showed membrane TRAIL and reduced CD4 expression. Confirming our results from Figure 3, we found that TRAIL was stored into intracellular compartments under the CD4 "cover." In contrast, pDCs from viremic patients showed very low CD4 expression and high membrane TRAIL expression, as expected.

We tested whether CD4 blocking by sCD4 or recombinant gp120 reduced IFN-α production by pDCs. Figure 4*D* shows that both sCD4 and gp120 significantly reduced IFN-α production by HIV-stimulated pDCs. IFN-α production by Flu-stimulated pDCs was not affected by sCD4 nor gp120. In contrast to HIV, Flu does not require cellular CD4 to activate pDCs.

### Efficiency of Apoptosis Induction by pDCs From Healthy Donors, Controllers, and Viremic Patients

Blood pDCs were purified from 8 viremic patients, 9 controllers (Table 1), and 8 healthy donors . To compare the efficiency of pDCs, we cultured them with a CD4 T-cell line (H9) chronically infected by HIV and expressing TRAIL death receptors (DR4 and DR5) but not membrane TRAIL (Figure 5*A*). However, coculture with pDCs induced mTRAIL expression by H9 cells (Figure 5*B*). This cell line allowed us to standardize the activity of killer pDCs, thereby avoiding variability of T-cell death due to differences in patient disease stage. CD4 T cells from viremic patients died faster in vitro than cells from healthy donors [27]. We show that recombinant TRAIL induced H9 cells apoptosis in dose- and time-dependent manners, and the addition of soluble DR5 (TRAIL inhibitor) dramatically inhibited TRAIL-induced H9 cell death (Figure 5*C*). These results clearly established the H9 cell line as target for pDC killer activity.

Our previous results show that the level of pDC activation by HIV was dependent on CD4 expression on the cell surface (Figure 4). Thus, we cocultured H9 cells with stimulated or unstimulated pDCs (Figure 5*D*) using a CD4-independent activator, the TLR9 stimulatory molecule CpG. We verified that CpG induced TRAIL expression on pDCs (Figure 5*E*) and that CpG-stimulated pDC–induced infected H9 cell apoptosis was significantly inhibited by TRAIL blocker soluble DR5. It should be noted that unstimulated pDCs were able to induce significant apoptosis of H9 cells (Figure 5*F*) due to the release of viruses from HIV-infected H9 cells.

Thus, we prestimulated (or not) pDCs from healthy donors, viremic patients, and controllers with CpG overnight and then cultured them with HIV-infected H9 cells. Unstimulated pDCs induced significant apoptosis of H9 cells, irrespective of their origin (Figure 5*G*). As expected, CpG-stimulated pDCs induced higher levels of apoptosis by H9 cells compared with unstimulated pDCs, in all groups of patients (Figure 5*H*). Interestingly, pDCs from controllers seemed to induce stronger H9 cell apoptosis compared with pDCs from healthy donors. In summary, we found that pDCs from controllers are functional and both HIV and CpG-activated pDCs can efficiently kill HIV-infected CD4 T cells.

## DISCUSSION

Plasmacytoid dendritic cells produce IFN-α in response to viral infection [12, 13, 16, 37]. There is now a debate in the literature concerning the activation of pDCs in AIDS-resistant simian models. One study showed that the resistance to AIDS progression in a sooty mangabey model was explained by a deficient IFN-α production by pDCs in response to simian immunodeficiency virus (SIV) [38]. In contrast, it was recently shown that pDCs could be activated by SIV [39] and produce high levels of type I IFN similarly in AIDS-resistant and AIDS-susceptible simian models [40–42]. The kinetic study of acute and chronic SIV infection demonstrated that the difference between pathogenesis-resistant African green monkey and susceptible Rhesus macaque resides in the kinetics of

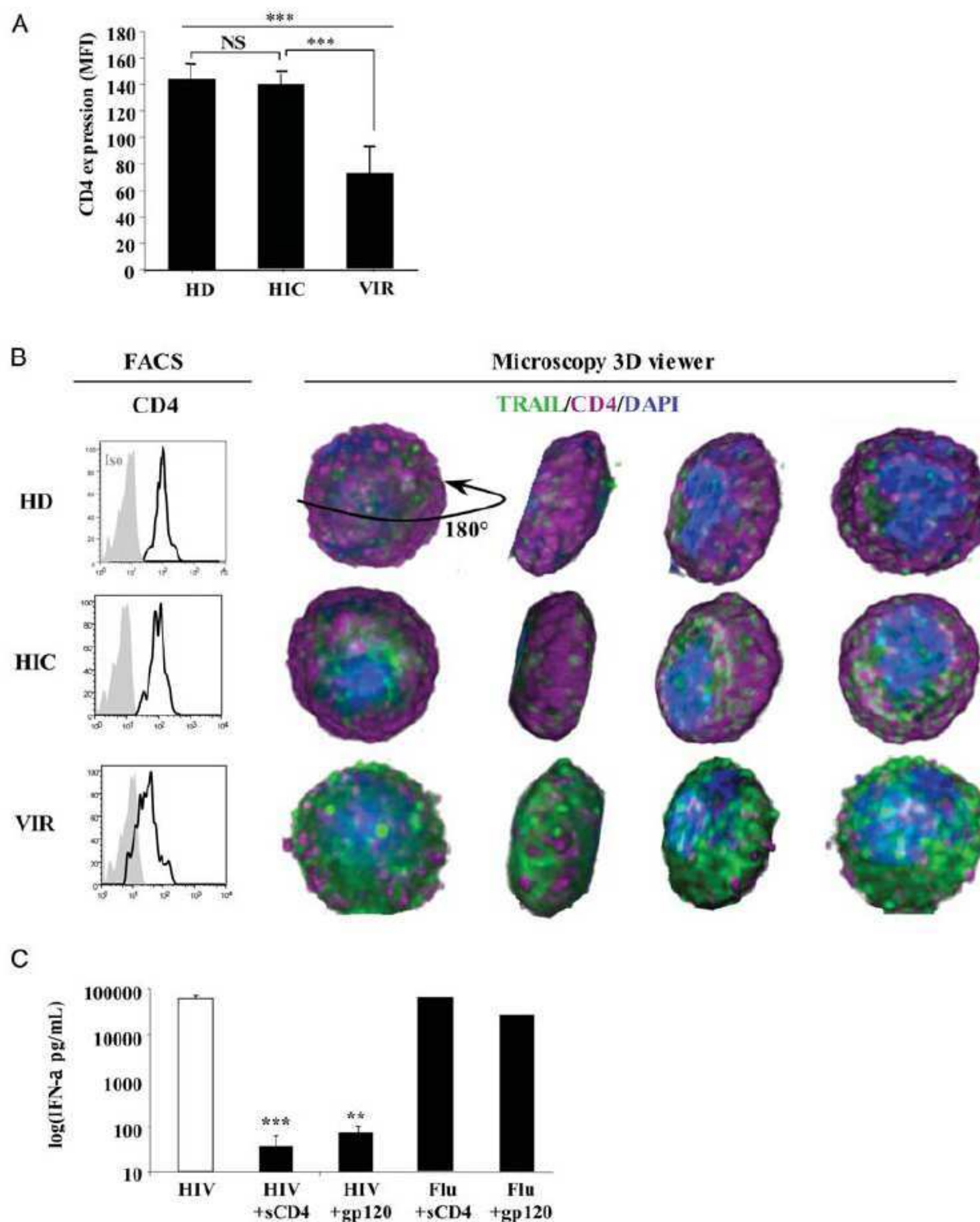


**Figure 4.** CD4 expression by plasmacytoid dendritic cells (pDCs). *A*, CD4 cell surface expression by pDCs from 12 healthy donors, 11 viremic patients, and 10 controllers was quantified by fluorescence-activated cell sorting (FACS) and expressed in mean fluorescence intensity (MFI). *B*, 3D microscopy of CD4 and TRAIL (tumor necrosis factor–related apoptosis inducing ligand) expression by pDCs. pDCs from healthy donors, controllers, and viremic patients were stained with anti-TRAIL (*green*), CD4 (*pink*), and DAPI (*blue*). pDC stainings (*green*, *pink*, and *blue*) were merged and analyzed using 3D viewer by ImageJ. CD4 expression by the same pDCs was also analyzed by FACS (*left panels*). *C*, Purified pDCs were cultured in presence of human immunodeficiency virus (HIV), with or without soluble CD4 (sCD4) and recombinant gp120 (gp120), and with influenza A virus (Flu) with or without sCD4 and gp120. Interferon (IFN)–α levels were measured by enzyme-linked immunosorbent assay after 12 h of culture.

interferon-stimulated gene expression. Resistance was associated with a peak at around 2 weeks after infection, followed by a decline to near-baseline levels by 4 weeks [41, 42]. In contrast, Rhesus macaque followed a generally similar kinetics until the decline, which leveled off well above the baseline to become chronic [40]. In humans, the role of IFN-α in HIV pathogenesis seems to be complex, and it has been recently demonstrated that activated pDCs and IFN-α contribute to chronic immune activation and CD4 T-cell depletion [19, 43].

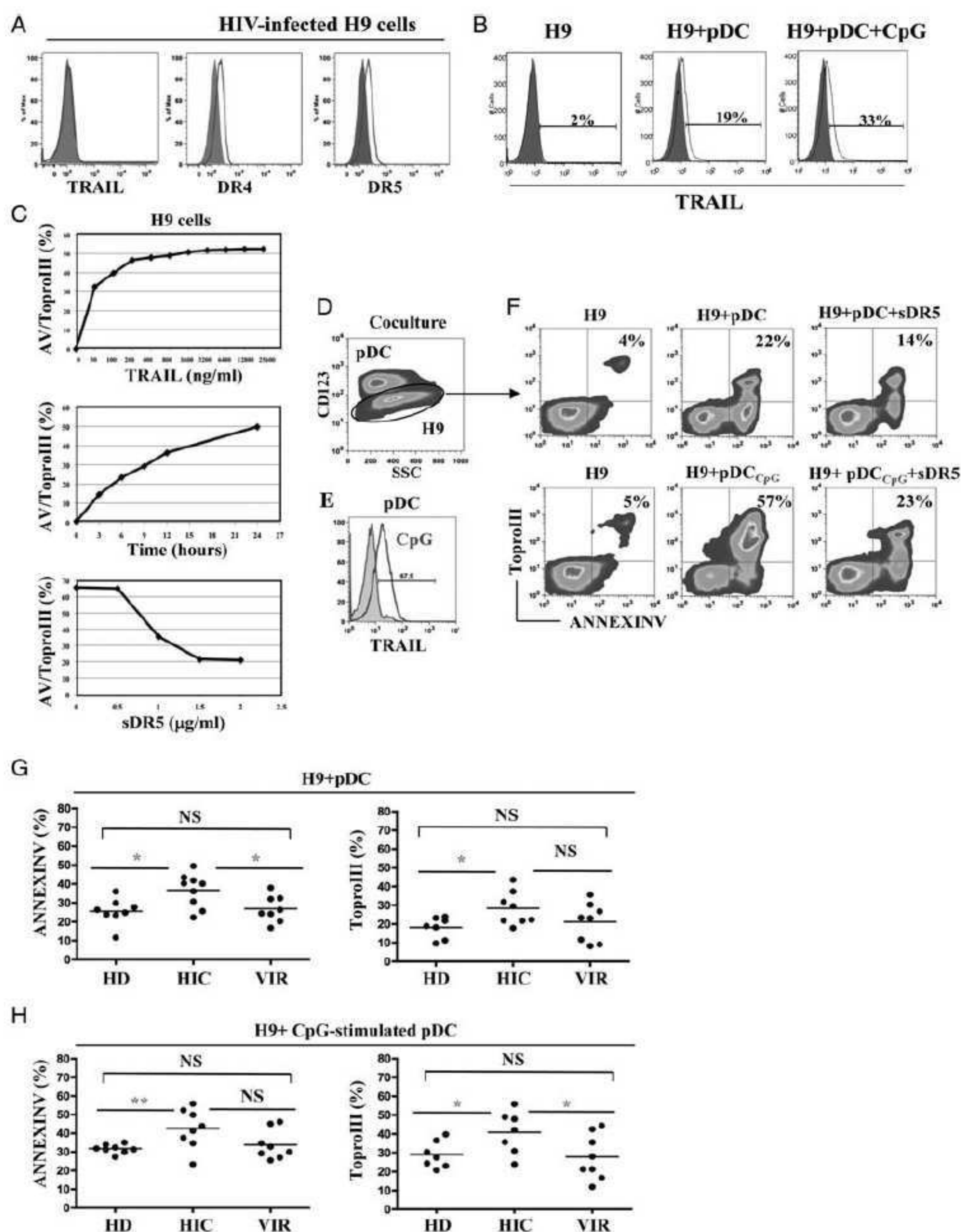


**Figure 5.** Apoptosis of H9 cells by plasmacytoid dendritic cells (pDCs). *A*, Human immunodeficiency virus (HIV)–infected H9 cell line was characterized by fluorescence-activated cell sorting (FACS) for TRAIL (tumor necrosis factor–related apoptosis inducing ligand) and TRAIL death receptor (DR4 and DR5) expressions. *B*, Membrane TRAIL expression on H9 cells cultured without pDCs (*left panel*), with pDCs (*middle panel*), or with CpG-activated (*left panel*) pDCs. *C*, HIV-infected H9 cells were cultured for 24 h in presence of increasing concentration of recombinant TRAIL (*upper panel*) and apoptosis was quantified by FACS using Annexin V/Topro III stainings. H9 cells were cultured for 24 h in presence of recombinant TRAIL (200 ng/mL), and apoptosis was measured using AnnexinV/Topro III every 3 h (*middle panel*). H9 cells were cultured in presence of TRAIL and soluble DR5 (sDR5) for 24 h and apoptosis was evaluated by AnnexinV/Topro III (*bottom panel*). *D* and *E*, H9 cells were cocultured 24 h with purified unstimulated or CpG-stimulated pDCs from healthy donors (*F*). Cells were discriminated using CD123-SSC parameters, and apoptosis was quantified by FACS. *F*, TRAIL expression by unstimulated or CpG-stimulated CD123$^{+}$ cells (pDCs) was quantified by FACS. *G* and *H*, pDCs were purified from 8 healthy donors, 8 viremic patients, and 9 controllers and stimulated or not overnight with CpG. Unstimulated pDCs (*G*) and overnight CpG-stimulated (*H*) pDCs from 8 healthy donors, 8 viremic patients, and 9 controllers were cocultured for 24 h in presence of H9 cells at ratio 2:1. Left panels represent Annexin V–positive H9 cells and right panels, Topro III–positive H9 cells. Abbreviation: NS, not significant. $P < .05$ was considered statistically significant. $^{*}P < .05$; $^{**}P < .01$.

Thus, we studied pDCs to clarify whether human pDC response to HIV is altered in HIV controllers. We found that controller patients maintained high blood number of pDCs, which were producing high levels of IFN-α in response to HIV exposure. In contrast, we found that pDCs from viremic patients statistically produced less IFN-α than pDCs from controllers or healthy donors in response to HIV, but surprisingly produced similar levels of IFN-α in response to Flu as controllers and healthy donors. Furthermore, we also found that CpG exposure induced similar levels of IFN-α by pDCs from viremic and controller patients or healthy donors (data not shown). Thus, our data support the recent findings of a hyperfunctionality of blood pDCs from viremic patients [30]. Furthermore, we found that pDCs from controllers did not express activation (CD40, HLA-DR), maturation (CD80, CD83, CD86), or migration markers (CCR7) in vivo, but expressed them after in vitro HIV exposure (data not shown).

Previous studies hypothesized that HIV-mediated pDC activation was dependent of viral gp120 binding to cellular CD4 [35, 44]. We demonstrate here that soluble CD4 or recombinant gp120 strongly abrogated IFN-α production by HIV-activated pDCs. Thus, we quantified CD4 expression on the pDC cell surface. Cytometry and microscopic study clearly demonstrate that pDCs from viremic patients statistically express lower levels of surface CD4 than pDCs from healthy donors. Furthermore, CD4 expression on pDCs from controllers was similar to that on pDCs from healthy donors. This could be explained by the very low levels of HIV-1 particles observed in these patients. Thus, very low CD4 expression could explain that pDCs from viremic patients produce less type I IFN in response to HIV than controllers or healthy donors, but similar levels when stimulated by a CD4-independent virus, such as Flu.

In addition to type I IFN production, we previously showed that HIV transformed pDCs into killer pDCs [15]. Thus, we tested whether HIV could induce activation of pDCs into TRAIL$^{+}$ killer pDCs in controllers. Our results showed that most pDCs from controllers did not express TRAIL on their membrane in vivo, contrasting with the 30% of TRAIL-expressing pDCs from viremic patients. Microscopic analysis revealed that pDCs from healthy donors possessed an intracellular pool of TRAIL, that is, relocalized to the cell membrane under HIV activation, transforming pDCs into killer pDCs. The pDCs from controllers exhibit an intracellular pattern and a same relocalization of TRAIL expression after viral exposure as pDCs from healthy donors. In contrast, ex vivo pDCs from viremic showed high levels of membrane TRAIL expression. In addition, 3D microscopy analysis of circulating pDCs from viremic revealed the existence of killer pDCs, confirming the in vivo generation of this cell subset during HIV infection [19].

We thus demonstrated that pDCs from controllers are functionally defined by IFN-α production and membrane TRAIL expression after viral stimulation, transforming them into killer pDCs. The fact that pDCs from controllers, in contrast with pDCs from viremic patients, do not express TRAIL ex vivo suggests that they are less stimulated in vivo, probably due to the nearly absence of HIV particles in the blood. Thus, pDCs are not activated and probably do not migrate to lymphoid organs, explaining the elevated number of pDCs in blood from controllers compared to viremic patients. These results are consistent with our previous study showing that tonsils from controllers do not exhibit IFN-α staining in contrast to tonsils from viremic patients [45].

Finally, we performed functional assays to test whether TRAIL-expressing killer pDCs could induce apoptosis of CD4 T cells. It has been shown that HIV induced TRAIL expression by pDCs; however, these TRAIL-expressing pDCs could not induce lysis of autologous HIV-infected CD4 T cells measured by $^{51}$chrome release after 6 hours of culture [46]. We did not investigate lysis in our study, but we investigated apoptosis using early (Annexin V) and late (Topro-III) markers to precisely determine apoptosis levels. Indeed, it is now clearly accepted that the vast majority of CD4 T cells undergo massive death by apoptosis in HIV-infected patients. We previously demonstrated that HIV-1–exposed CD4 T cells expressed high levels of DR5 after 24 hours of culture and consequently became sensible to TRAIL-mediated apoptosis [27]. We show here that, in coculture, TRAIL-expressing pDCs, and probably TRAIL-expressing H9 cells, induced apoptosis of the DR5-positive CD4 T-cell line H9. This result, combined with TRAIL expression and the high levels of IFN-α produced by pDCs from controller patients, suggest that pDCs from controllers are fully functional, acquire a killer profile. and are not defective in the ability to induce apoptosis via TRAIL death receptors.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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***Author contributions.*** L. B., K. M., and C. G. performed and analyzed research. J.-P. H. designed and analyzed research and wrote the paper.

J.-F. D., F. B., M. L., E. R.-M., and O. L. provided patient samples and analyzed the research.

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***Potential conflicts of interest*** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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# Hepatitis C virus replication in Caucasian HIV controllers

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SUMMARY. Whether HIV controllers, patients who spontaneously control HIV viraemia, are able to control hepatitis C virus (HCV) infection, in terms of spontaneous clearance or lower HCV replication, is not well understood. To assess to what extent Caucasian HIV controllers are able to control HCV replication and potential associated factors, plasma HIV-1 and HCV RNA levels, anti-HCV antibodies, HCV genotype and human leucocyte antigens (HLA) typing were determined in samples from 75 HIV controllers (33 viraemic controllers, <1000 HIV-1 RNA copies/mL, and 42 elite controllers, <40 HIV-1 RNA copies/mL) and compared with 261 HIV-infected noncontrollers. We did not find differences in the HCV spontaneous clearance rates between groups. However, we interestingly found a lower HCV viral load in HIV controllers, alongside a different distribution of HCV genotypes in relation to the comparison group. In addition, HLA-B57 was associated with a lower HCV viral load in the control group and HIV controllers, and conversely, HLA-B35 with higher HCV viral load in HIV controllers. The subrepresentation of HCV genotype 1 and the overrepresentation of HLA-B57 only partly explained the lower HCV viral load found in HIV controllers. In fact, HIV controller status was independently associated with lower HCV viral load, together with HCV genotype non-1, the presence of HLA-B57 and absence of HLA-B35. Caucasian HIV controllers are able to better control HCV replication, in terms of lower HCV viral load levels. These findings support the idea that some common host mechanisms are involved in the defence against these two persistent infections.

*Keywords*: elite controllers, hepatitis C virus, HIV, HIV controllers.

## INTRODUCTION

In HIV infection, there is a rare group of patients (1–5%) who are able to maintain very low levels of plasma viraemia over long periods of time in the absence of antiretroviral treatment. These patients are known as HIV controllers (normally <1000 HIV-1 RNA copies/mL) or elite controllers for those who maintain plasma viraemia below the detection limits [1]. The mechanisms involved in this outstanding phenotype are not well understood at the moment. The overrepresentation of different human leucocyte antigens (HLAs) such as HLA-B57 and the associated cytotoxic T-lymphocyte (CTL) response seems to be a relevant mechanism associated with the natural control of HIV replication [2]. However, important questions remain open concerning the extraordinary immunity of these patients. One important aspect is to know whether HIV controllers are able to control other persistent infections in the same way that they do HIV. Hepatitis C virus (HCV) is of particular interest, whose natural history is accelerated in HIV-co-infected patients [3]. Recently, an increase in the spontaneous clearance of HCV in a cohort of African-American HIV controllers infected with HCV genotype 1 has been reported [4]. The aim of the current work was to analyse whether Caucasian HIV controllers were able to control HCV infection in terms of spontaneous clearance and/or HCV viral load levels and whether this fact was associated with different HLAs and the distribution of HCV genotypes.

Abbreviations: CTL, cytotoxic T-lymphocyte; FIPSE, fundacion para la Investigacion y Prevencion del SIDA en España; HCV, hepatitis C virus; HLA, human leucocyte antigens; LTNP, long-term nonprogressors.

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## PATIENTS AND METHODS

### Patients

HIV controllers included in this study came from different cohorts of Spanish HIV-infected patients: the HIV-infected

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patients cohort (CoRIS) and HIV Biobank of the Spanish AIDS Research Network (RIS), as well as the HIV-infected patient cohorts of the Infectious Diseases Service at the Hospitals of Universitario Virgen del Rocio (Seville), Comarcal de Don Benito (Badajoz) and Navarra (Pamplona). Samples from patients were kindly provided by the HIV BioBank integrated within the Spanish AIDS Research Network (RIS). Blood samples were processed following standard procedures and frozen immediately after their reception. All patients participating in the study gave their informed consent, and protocols were approved by institutional ethical committees.

HIV controllers were defined as established by the International HIV Controller Consortium but with the following modifications [2]; these were subjects with positive antibodies for HIV-1, plasma viral load measurement <1000 HIV-1 RNA copies/mL in the absence of antiretroviral therapy (ART) for at least 12 months and with at least three plasma viral load determinations during this period of time. Seventy-five subjects that fulfilled these criteria were included in the present study. HIV-2-infected patients were excluded. HIV controllers were divided in two subsets: (i) 'HIV–elite controllers' were patients were able to maintain plasma viral load below the limit of detection, <40 HIV-1 RNA copies/mL (isolated viral rebounds 'blips' <1000 HIV-RNA copies/mL were allowed) ($n$ = 42) and (ii) 'HIV–viraemic controllers', patients who maintained plasma viral load between 40 and 1000 HIV RNA copies/mL ($n$ = 33). 'Loss of HIV control' was considered when two consecutive viral load measurements were >40 HIV-1 RNA copies/mL for HIV–elite controllers and >1000 HIV-RNA copies/mL for HIV–viraemic controllers.

Clinical and demographic characteristics of HIV controllers were compared with a group of 261 HIV-1-infected patients from our cohort (Hospital Universitario Virgen del Rocio) who consecutively visited our Unit until the study was censored, from now on refer to as the 'comparison group'. HIV controllers and HIV-2-infected patients were excluded from this comparison group. Patients were asymptomatic and in the chronic phase of HIV or HIV/HCV infection, at the time of the study.

## METHODS

### *Quantification of plasma viraemia*

Plasma HIV-1 RNA was measured in fresh samples by a quantitative RT-PCR (COBAS Ampliprep/COBAS Taqman HIV-1 test; Roche molecular systems, Basel, Switzerland) according to the manufacturer's instructions. The detection limit was 40 HIV-1 RNA copies/mL. Plasma samples were tested for anti-HCV antibodies using HCV-ELISA (Siemens Healthcare Diagnosis, Deerfield, IL, USA). HCV genotype was determined using a reverse-hybridization assay (InnoLiPA HCV II; Innogenetics, Barcelona, Spain). A quantitative RT-PCR amplification was performed for plasma HCV RNA amplification (COBAS Amplicor; Roche Diagnosis, Barcelona, Spain) with a detection limit of 15 IU/mL. Patients with positive antibodies for HCV and negative PCR for plasma HCV RNA levels in the absence of previous HCV-specific treatment were considered to have spontaneous clearance of HCV infection. In the case of several HCV-RNA determinations available during follow-up, the last determination was considered. In the case of HCV-specific treatment, the HCV-RNA determination just before the start of treatment with either pegylated interferon and ribavirin or alpha interferons alone was taken. Besides, for HIV controllers who lost control of HIV viraemia during follow-up, HCV-RNA determinations were considered only during the HIV control period.

### *HLA typing*

HLA-B* alleles were genotyped using a reverse stripdot-blot kit with sequence-specific oligonucleotide probes (Dynal RELI™ SSO HLA-A and HLA-B typing kits; Dynal Biotech, Bromborough, UK) following the manufacturer's instructions.

### *Statistical analysis*

Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 17.0, Chicago, IL, USA) using nonparametric statistical tests in all cases. Median and interquartile ranges were used to describe continuous variables and percentage for categorical ones. Spearman test was used to analyse correlation between continuous variables. Differences between groups were analysed with the Kruskal–Wallis, Mann–Whitney $U$-test, chi-square test or Fisher's exact test when appropriate. All the differences between groups with a $P < 0.05$ were considered statistically significant. To analyse the independent factors associated with HCV viral load, a multivariate linear regression analysis was assessed. Variables showing a $P$ value <0.05 were considered statistically significant.

## RESULTS

The characteristics of the patients are summarized in Table 1. A total of 75 HIV controllers, of whom 42 were elite controllers, were recruited. It is important to mention that there was a twofold higher proportion of women in the HIV controller groups compared to the comparison group. In this group, naïve patients (14.6%) had a median viral load of 4.8 [4.1–5.3] log HIV-1 RNA copies/mL. Sixty per cent of the patients on treatment had undetectable viral load, and the rest of the treated patients had a median viral load of 3.41 [2.8–4.3] log HIV-1 RNA copies/mL.

### *Low plasma HCV load in HIV controllers*

Owing to the high proportion of HCV infection in HIV controllers, we analysed whether HIV controllers were able to

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**Table 1.** Characteristics of the patients

| | Comparison group ($n$ = 261) | Elite controllers* ($n$ = 42) | HIV controllers† ($n$ = 75) | *P** | *P*† |
|---|---|---|---|---|---|
| Female sex (%) | 52/261 (20.0) | 17/42 (40.5) | 32/75 (42.7) | **0.005** | **<0.0001** |
| Caucasian race (%) | 258/261 (99.0) | 41/42 (97.6) | 73/75 (97.3) | 0.451 | 0.310 |
| Age (years) | 43.0 [38.3–47.1] | 43.0 [39.0–46.3] | 43.0 [38.0–46.0] | 0.926 | 0.211 |
| Time since diagnosis (years) | 12.1 [5.1–16.8] | 15.1 [6.0–20.8] | 13.9 [5.0–20.1] | **0.004** | **0.033** |
| CD4+ T-cells/mm$^3$‡ | 428.5 [247.8–594.3] | 649.3 [442.8–1028.0] | 664.0 [444.0–915.0] | **<0.0001** | **<0.0001** |
| Naïve for ART§ | 38/261 (14.6) | 31/42 (73.8) | 52/75 (69.3) | **<0.0001** | **<0.0001** |
| Clinical stage C (%) | 63/213 (29.6) | 2/40 (5.0) | 3/60 (5.0) | **0.007** | **<0.0001** |
| Risk group (%) | | | | | |
| IDU¶ | 120/261 (46.0) | 27/42 (64.3) | 41/75 (54.7) | **0.031** | 0.192 |
| Sexual | 131/261 (50.2) | 13/42 (31.0) | 32/75 (42.7) | **0.030** | 0.295 |
| Anti-HCV+ (%) | 134/261 (51.3) | 32/42 (76.2) | 50/75 (66.7) | **0.003** | **0.019** |
| HCV RNA+ (%)** | 95/134 (71.0) | 22/29 (75.9) | 35/47 (74.5) | 0.656 | 0.709 |
| HCV clearance with HCV treatment | 17/134 (12.7) | 1/29 (3.4) | 3/47 (6.4) | 0.485 | 0.584 |
| Spontaneous HCV clearance | 22/134 (16.4) | 6/29 (20.7) | 9/47 (19.1) | 0.581 | 0.670 |
| Log HCV RNA (IU/mL) | 6.4 [5.7–6.9] | 5.7 [4.8–6.5] | 5.9 [5.0–6.6] | **0.005** | **0.007** |
| HCV genotypes†† | | | | | |
| Genotype 1 | 56/96 (58.3) | 8/23 (34.8) | 15/36 (41.7) | 0.061 | 0.117 |
| Genotype 3 | 17/96 (17.7) | 10/23 (43.5) | 15/36 (41.7) | **0.013** | **0.006** |
| Genotype 4 | 22/96 (22.9) | 4/23 (17.4) | 5/36 (13.9) | 0.780 | 0.335 |
| HLA-B57+ (%) | 6/83 (7.2) | 6/31 (19.4) | 8/40 (23.3) | 0.085 | 0.065 |
| HLA-B35+ (%)‡‡ | 12/83 (14.5) | 5/31 (16.1) | 8/40 (20.0) | 1.000 | 0.448 |

*Elite controllers *vs* comparison group; †HIV controllers (elite plus viraemic) *vs* comparison group. Mann–Whitney *U*-test was used; ‡Last determination; §Antiretroviral treatment; ¶Injecting drug user; **Not available in three patients in the elite and HIV controller groups; ††HCV genotype determination available for those HCV RNA +, including patients with HCV clearance on HCV treatment, where HCV genotype was assayed before the start of HCV-specific treatment; ‡‡HLA typing was assayed in patients where DNA samples were available. *P* values depicted in bold for significant variables.

control HCV infection in terms of spontaneous clearance. Conversely, the proportion of HCV spontaneous clearance was not statistically different to the comparison group (Table 1). However, when HCV load was also quantified, a lower HCV load was found in HIV controllers in relation to the comparison group, with a decrease of 0.7 log HCV IU/mL in elite controllers (Fig. 1 and Table 1). This result was similar when viraemic and elite controllers were grouped together, although the difference decreased to 0.5 log HCV IU/mL (Fig. 1 and Table 1).

We also observed that some elite controllers lost the capability to control the virus during follow-up. We wondered whether this fact could be associated with plasma HCV RNA levels. A tendency to have higher HCV load in elite controllers who thereafter lost the capacity to control HIV was found (Fig. S1).

Additionally, it was found that HCV PCR+ HIV controllers had lower CD4+ T-cell levels than HCV PCR– HIV controllers ($P$ = 0.022, Mann–Whitney *U*-test). Although this tendency was repeated in the elite controllers, statistical significance was not reached ($P$ = 0.131, Mann–Whitney *U*-test). However, these differences were not observed in the comparison group (data not shown).

### *Unusual distribution of HCV genotypes in HIV controllers*

HIV controllers showed lower prevalence of HCV genotype 1, and reciprocally, the proportion of HCV genotype 3 increased when compared to the general HIV population. No differences were found in the distribution of genotype 4 (Table 1).

### *HLA-B57 and HLA-B35 are associated with HCV load levels in an opposite way*

All the HLA-B alleles present in the study subjects were analysed and only HLA-B57+/− and HLA-B35+/− subjects correlated with HCV load levels. HLA-B57 was associated with lower HCV load levels in all the study subjects (Fig. 2a). This was true for the comparison group

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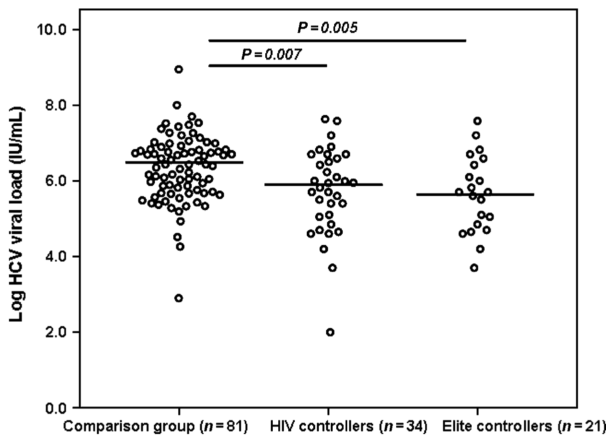


**Fig. 1** Influence of HIV controller status on HCV load. HCV viral was available in 81 patients in the comparison group, 34 patients in the HIV controller group and in 21 patients in the elite controllers group. HIV controllers, including the subgroup of elite controllers, showed lower levels of HCV load than the comparison group. Values represent median and interquartile ranges.

(a 0.6 log IU/mL decrease) (Fig. 2b). In HIV controllers, where this allele is overrepresented (Table 1), these results were reproduced and HCV load levels were even lower, with a 1.34 log IU/mL decrease (Fig. 2b). Besides, we found that HLA-B57+ subjects had a 3.6-fold increase in the proportion of spontaneous clearance of HCV in HIV controllers (43% in HLA-B57+ *vs* 12% in HLA-B57− subjects, $P = 0.101$, chi-square test).

On the other hand, HLA-B35 has been traditionally associated with disease progression in HIV infection [5]. However, the association of HLA-B35 with HCV is not clear. In the light of the results with HLA-B57, we also wondered whether HLA-B35 was reciprocally associated with higher levels of HCV. We found that HLA-B35+ HIV controllers had higher HCV load than patients negative for this allele (Fig. 3a). An additional interesting finding with HLA-B35 was based on the association of HLA-B35 with HIV disease progression [5]. This idea prompted us to analyse whether HLA-B35+ was associated with the loss of the capability to control HIV in controllers. The proportion of HLA-B35+ HIV controllers who lost the capacity to control HIV viraemia was higher than the proportion of HLA-B35+ HIV controllers who persistently controlled the virus (Fig. 3b). This loss of control was assumed when both elite controllers became viraemic controllers and viraemic controllers became non-controllers. When loss of HIV control was only considered for elite controllers (from elite controllers to viraemic controllers), this tendency was also maintained (43% of HLA-B35+ subjects lost control compared to 8.3% of HLA-B35+ elite controllers with persistent control, $P = 0.062$, Fisher's exact test).

### *Elite controller status, HCV genotypes, HLA-B57 and HLA-B35 are independently associated with HCV load levels*

Multiple linear regression analysis was performed to pinpoint which variables were independently associated with HCV

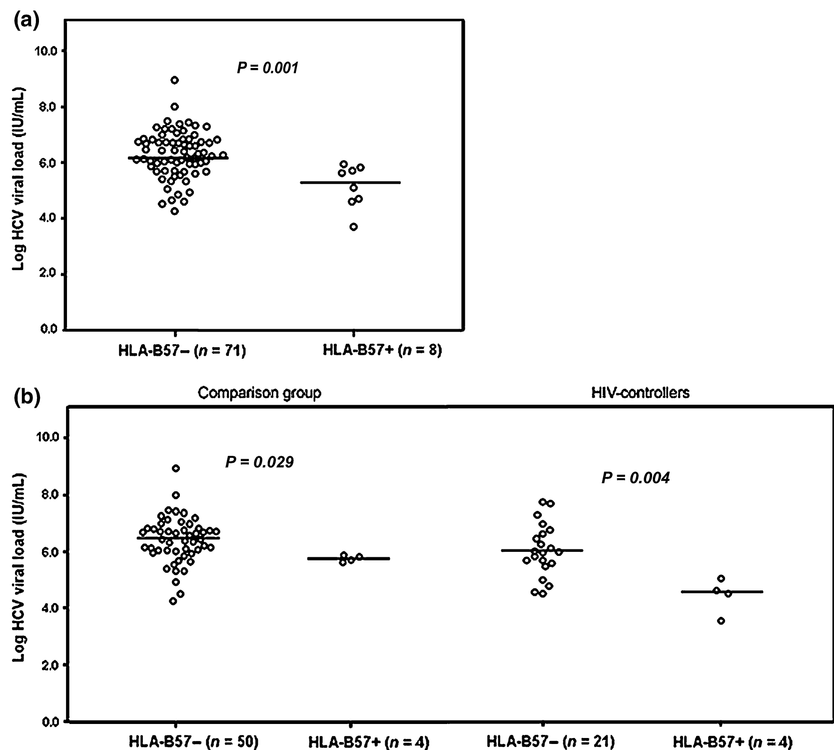


**Fig. 2** Influence of HLA-B57 on HCV viral load. HLA typing and HCV load were available in 54 patients in the comparison group and in 25 patients in the HIV controllers group. (a) HLA-B57+ patients showed lower levels of HCV load than HLA-B57− patients. (b) This was true for both patients in the comparison and HIV controller groups. Mann–Whitney *U*-test was used. Values represent median and interquartile ranges.

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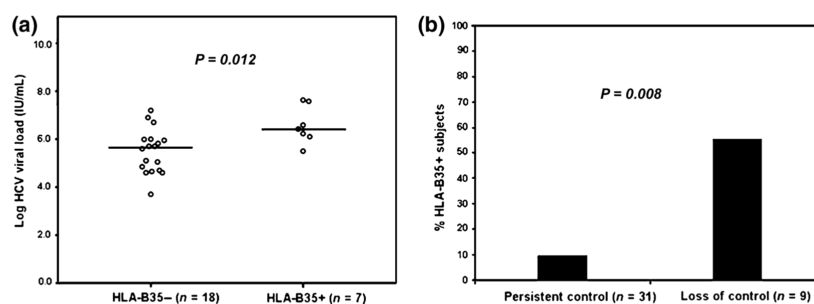


**Fig. 3** Influence of HLA-B35 on HCV load and loss of HIV control. HLA typing was available in 40 patients and both, HLA typing and HCV load were available in 25 patients in the HIV controllers groups. (a) HLA-B35+ HIV controllers showed higher levels of HCV load than HLA-B35- patients. Mann–Whitney *U*-test was used. Values represent median and inter-quartile ranges. (b) There was an overrepresentation of HLA-B35+ patients who lost HIV control. Fisher's exact test was used.

**Table 2** Results of the multivariate analyses

| | *P* | B (95% CI)* |
|---|---|---|
| Constant (Log HCV load)† | | 6.0 (5.6, 6.2) |
| Elite controller status | **<0.0001** | −0.577 (−0.973, −0.180) |
| HCV Genotype 1 | **0.005** | 0.690 (0.336, 1.044) |
| HLA-B57 | **0.024** | −0.625 (−1.164, −0.087) |
| HLA-B35 | **0.025** | 0.570 (0.073, 1.066) |
| CD4+ T-cells/mm$^3$ | 0.654 | <0.001 (−0.001, 0.001) |
| Time since diagnosis | 0.494 | 0.012 (−0.023, 0.047) |
| Female sex | 0.680 | 0.098 (−0.375, 0.570) |
| Naïve for ART | 0.401 | 0.298 (−0.408, 1.003) |

Multivariate linear regression analysis, *P* values depicted in bold for significant variables; *B, Regression coefficient, changes in the estimation of the mean of the Log HCV load (constant) when the rest of the variables do not change. CI, confidence interval; ART, antiretroviral treatment; †Dependent variable expressed as estimation of the mean.

load. Variables included in the model were elite controller status, HCV genotype 1, HLA-B57, HLA-B35, CD4+ T-cell counts, time since diagnosis, sex and naïve for antiretroviral treatment. This analysis showed that when we controlled for the above potential confounders, the variables elite controller status, the absence of genotype 1 and HLA-B35 and the presence of HLA-B57 were independently associated with lower HCV load ($n = 63$, $R^2 = 0.476$, Table 2). This analysis showed no colinearity among variables which means that each one exerts an additive effect in lowering HCV load.

## DISCUSSION

Results presented herein show how Caucasian HIV controllers exhibited better control of HCV replication, measured as plasma HCV loads, when compared with a comparison group. Furthermore, they presented a different distribution of HCV genotypes with an overrepresentation of genotype 3. Genetic determinants, associated with HIV progression, were also associated with HCV loads. These findings support the idea that some common host mechanisms are involved in the defence against these two persistent infections.

The absence of differences in the proportion of spontaneous HCV clearance between the comparison group and HIV controllers agrees with previous work that showed how different immune mechanisms are operating against HIV, cytomegalovirus and HCV, at least in relation to CTL responses [6]. However, we found that HIV controllers had lower levels of HCV loads. Hence, both persistent infections should share common control mechanisms although at a different level. On the other hand, the association between loss of HIV control and higher levels of HCV load can be related, although in a different scenario, with previous observations that showed increased disease progression in HIV–HCV-co-infected patients with higher viral load [7].

The clinical implications of this lower HCV load in the response to HCV-specific treatment and the natural history of HCV in HIV controllers are unknown at the moment, although recently no differences in liver histology between HIV controllers and non-HIV controllers with chronic HCV infection were reported [4]. In this same work, significantly lower CD4 cell counts in HIV controllers without chronic HCV infection compared to HIV controllers with chronic HCV infection were found. These data are in line with those presented in the current work.

In this study, another peculiarity of the HIV controller cohort was the different distribution of HCV genotypes compared to the general HIV-infected population. The reasons for this different distribution are unknown at the moment. One possible explanation could be related to the different distribution of specific HLAs in elite controllers. In this sense, a variable influence of HLA on various genotypes has been reported [8]. We can also hypothesize that the presence of genotype 1 would favour the loss of HIV control, and hence, this genotype could be underrepresented in the HIV controller group. This idea could be supported, although at a different level, by the higher progression rate in

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HIV-co-infected people with HCV genotype 1 found in some studies [9,10].

The lower HCV loads found in HLA-B57+ subjects point once again to some common immunogenetic host mechanisms involved in the defence against these two viruses. It has been widely documented that the HLA-B57 is overrepresented in HIV controllers [11]. The reasons why this host genetic factor has been associated with HIV control are beginning to be understood [12]. Recent studies point to the presence of a strong CTL response and a viral fitness cost to the virus that arises from HLA-B57 escape mutations [13]. This process could also take place in HCV infection and might be associated with a common immunological cellular mechanism such as efficient elimination of infected cells by HLA-B57-restricted CTLs recognizing distinct epitopes from HIV or HCV. Studies relating HLA-B57 with HCV are scarce, and an association has only been found between the presence of HLA-B57 and the spontaneous clearance of HCV [14–16]. This agrees with our results, although the contrast was not statistically significant because of the difficulty in finding a high number of patients who were HIV controllers, HLA-B57+ and exposed to HCV.

Following this reasoning, we found the opposite effect with HLA-B35, i.e. higher HCV loads in HLA-B35+ HIV controllers. In relation to this observation, HLA-Cw04 has been shown to be associated with HCV persistence and this allele was in strong linkage disequilibrium with HLA-B53 and HLA-B35. However, only HLAB-53 and the Cw04-B-53 haplotype were weakly associated with viral persistence [15]. This finding again shows how a genetic factor associated with progression in HIV infection shares features with HCV infection, at least in relation to HCV loads. Apart from this, the additional observation of the association between the presence of HLA-B35 with the loss of HIV control is in agreement, although in a different scenario, with the disease progression in HIV infection [5]. Apart from these HLAs, other genetic factors, such as IL28B polymorphism, which has been shown to be associated with HCV clearance, genotypes and response to treatment, should be explored to define their association with the lower HCV loads seen in Caucasian HIV controllers [17–20].

A limitation of this work is the low number of HLA-typed patients, and another inherent limitation of this work is its cross-sectional nature. In this sense, studies have shown loss of control and/or HCV re-infection when patients were followed longitudinally particularly in HIV-co-infected patients [21,22]. However, we have to assume that the re-infection frequency in these patients is anecdotal because they have abandoned injecting drug use and HCV re-infection by sexual transmission is rare.

Different variables as CD4+ T-cell counts [23], sex [24,25] and even the influence of antiretroviral therapy [26] have been associated with HCV load. These variables were statistically different between the comparison group and the HIV controller groups, the same happened with the time since diagnosis. To avoid potential confounders, all these variables were controlled for by their introduction in a multivariate model together with elite controller status, HCV genotype 1, HLA-B57 and HLA-B35. Only elite controller status, the absence of HCV genotype 1 and HLA-B35, and the presence of HLA-B57 were independently associated with the lower HCV load. The reason why elite controller status *per se* is independently associated with lower HCV load is not yet clear. One limitation of the present study is that we do not provide experimental data to answer this question. However, we can hypothesize, based on observations in long-term nonprogressors (LTNP), how common immunological control mechanisms against these two persistent viruses could explain the lower HCV load found in HIV controllers. In fact, a strong HCV Th1 cell response with higher HCV peptide recognition has been found in LTNP when compared to progressors [27,28].

In a recent study, Sajadi *et al.* [4] have shown an increased rate of HCV spontaneous clearance in 'natural viral suppressors' (<400 HIV-RNA copies/mL), when compared to HCV–HIV-co-infected and HCV-mono-infected control groups. In that study, the cohort was entirely composed of African-Americans. This fact could explain the discrepant results found in our cohort composed mainly of Caucasians. Another difference is that variations in HCV load between HIV controllers and reference groups were not found. This could be explained, apart from the race differences, by most of the subjects being HCV genotype 1 (approximately 90%) and, as we have shown, HCV genotype is associated with HCV load. In this regard, it is important to mention that HIV controllers from the Sajadi cohort had almost fourfold higher HCV load than elite controllers from our cohort owing to the prevalence of genotype 1. Although in this previous work genetic factors were not analysed, our findings underlined how different race groups of HIV controllers with different genetic and immunological backgrounds can influence HCV load levels.

In summary, the results presented herein show how Caucasian HIV controllers exhibit better control of HCV replication when compared with a comparison group. This is associated with a different distribution of HCV genotypes, absence of HLA-B35 and the overrepresentation of HLA-B57. Additional immunological mechanisms should be explored to explain how HIV controller status influences partial HCV control to design future therapeutic strategies against both infections.

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## CONFLICT OF INTERESTS

The authors do not have any association that might pose a conflict of interest.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** HCV load and loss of HIV control. HCV load was available in 21 patients in the elite-controller group. Some elite-controllers lost the capability to control HIV; these patients showed a tendency to have higher HCV load just before the loss of control compared to elite-controllers who did not lose the control of HIV. Mann–Whitney *U*-test was used. Values represent median and interquartile ranges.

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BRIEF REPORT

# *IL28B* Single-Nucleotide Polymorphism rs12979860 Is Associated With Spontaneous HIV Control in White Subjects

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**The single-nucleotide polymorphism (SNP) rs12979860 near the *IL28B* gene has been associated with the spontaneous clearance of hepatitis C virus. We sought to determine whether this SNP could be associated with the spontaneous control of human immunodeficiency virus (HIV) infection. We studied the prevalence of the *IL28B* CC genotype among 53 white HIV controllers, compared with the prevalence among 389 HIV-infected noncontrollers. We found that the *IL28B* CC genotype was independently associated with spontaneous HIV control (odds ratio [OR], 2.669; $P = .017$), as were female sex (OR, 7.077; $P \leq .001$) and the presence of HLA-B57 and/or B27 (OR, 3.080; $P = .017$). This result supports the idea that common host mechanisms are involved in the spontaneous control of these 2 chronic infections.**

***Keywords.*** HIV controllers; IL28B, and SNP.

The spontaneous control of human immunodeficiency virus (HIV) infection is observed in a rare group of individuals who have become known as HIV controllers. These individuals are able to maintain undetectable or very low viral loads in the absence of antiretroviral treatment over a long period [1]. Several genetics factors have been associated with this control, including the overrepresentation of different HLA antigens, such as HLA-B57 [1]. Curiously, the presence of HLA-B57 has also been related to the spontaneous clearance of hepatitis C virus (HCV) infection [2], suggesting shared mechanisms of viral control for both infections. We have previously reported the ability of white HIV controllers to maintain lower HCV loads than other noncontroller HIV-infected subjects, as well as an association of HLA-B57 with lower HCV levels in HIV controllers [3]. Recently, a single nucleotide polymorphism (SNP) near the *IL28B* gene, which codes for interferon λ3 (IFN-λ3), has been associated with the spontaneous clearance of HCV [4] and with sustained virological response (SVR) after HCV-specific treatment [5]. Although it is still unknown how this SNP affects the antiviral activity of IFN-λ, several studies have demonstrated the antiviral activity of IFN-λ against different viruses, including HIV [6]. However, no association was found between these protective alleles and spontaneous HIV control in African American individuals [7, 8]. We wondered whether this *IL28B* SNP would be overrepresented in white HIV controllers, and, thus, our aim was to analyze the association of the *IL28B* SNP with the spontaneous control of HIV infection.

## METHODS

All patients included in this study came from Spain and were white. The HIV controllers included in this study came from the HIV-infected patient cohort of the Infectious Diseases Service of Virgen Del Rocío University Hospital (Seville) and from a National Multicenter Cohort from the HIV Controllers Consortium of the AIDS Spanish Network (some of these subjects came from HIV-Infected Long-Term Nonprogressors Cohort and the prospective HIV-Infected Patients Cohort, both from the AIDS Spanish Network). The sample included 53 HIV controllers, defined as subjects who tested positive for antibodies against HIV had a plasma HIV-1 load of <1000 HIV-1 RNA copies/mL in the absence of antiretroviral therapy (ART) for at least 12 months, and had at least 3 plasma HIV-1 load determinations during this period. Thirty-four of these HIV controllers were naive to ART; the remaining 19 patients had received ART, with a median interval of 4.42 years (range, 2.76–7.83 years) between their most-recent use of ART and enrollment in the study. The HIV controllers

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were compared with 389 HIV-infected noncontrollers from our cohort who consecutively visited our unit (Virgen Del Rocío University Hospital, Seville) until the study was completed. These patients had had a confirmed plasma HIV-1 load of >1000 HIV-1 RNA copies/mL at ≥2 follow-up visit since entrance into the cohort. HIV type 2–infected subjects were excluded. We also studied in a prospective manner 61 HIV-infected noncontrollers who had data available on the primary infection. The time of HIV-1 infection was estimated as the midpoint between the last negative and the first positive HIV antibody test result, with maximum periods of 12 months between the HIV antibody tests and 15 days before onset of acute retroviral symptoms. The virus set point was defined as the median of at least 2 HIV-1 load determinations between months 3 and 6 after the estimated date of infection. All of these patients were asymptomatic at the time of the study. Samples were kindly provided by the HIV Biobank integrated within the Spanish AIDS Research Network. All of the patients participating in the study gave their informed consent, and the protocols were approved by the institutional ethics committees.

Routinely, the absolute numbers of $CD4^+$ and $CD8^+$ T cells were assayed from fresh whole blood samples, using the Epics XL-MCL flow cytometer (Beckman Coulter). Plasma HIV RNA load was measured in fresh samples by quantitative polymerase chain reaction (PCR; COBAS Ampliprep/COBAS Taqman HIV test; Roche molecular systems), according to the manufacturer's instructions. The detection limit was 40 HIV RNA copies/mL. Quantitative reverse transcription PCR was performed for plasma HCV RNA amplification (COBAS Amplicor; Roche Diagnosis, Barcelona, Spain), with a detection limit of 15 IU/mL. HCV genotype was determined using a reverse-hybridization assay (InnoLIPA HCV II; Innogenetics, Barcelona, Spain).

The HLA-B group alleles were genotyped using a reverse sequence-specific oligonucleotide bound to a fluorescently coded microsphere system (LABType SSO, RSSO1B, One Lambda, Canoga Park, CA), following the manufacturer's instructions. The genotyping of the *IL28B* SNP rs12979860 was performed as previously described [9], using a TaqMan 5′ allelic discrimination assay (Applied Biosystems, Foster City, CA).

The statistical analyses were performed using SPSS software, version 18.0 (SPSS, Chicago, IL). Median and interquartile ranges were used to describe continuous variables and percentages to describe categorical variables. To analyze the association of the *IL28B* SNP with spontaneous HIV control, in the multivariate analysis, HIV controller status was the dependent variable, and only innate host parameters (ie, sex, HLA-B57 and HLA-B27 alleles, and *IL28B* CC genotype) were included. All of the differences between the groups in the bivariate and multivariate logistic regression analysis with a $P$ value of $< .05$ were considered statistically significant. The studied population was in Hardy-Weinberg equilibrium in relation to the *IL28B* CC genotype ($P > .05$)

## RESULTS

The characteristics of the study subjects are summarized in Table 1. Some of these subjects were included in our previous studies [3, 10]. The frequency of women was significantly greater among HIV controllers, compared with noncontrollers. As expected, having one of the protective alleles (HLA-B57 and/or HLA-B27) was overrepresented in the HIV controllers (odds ratio [OR], 2.631; 95% confidence interval [CI], 1.151–6.015; $P = .022$). The HIV controllers showed lower rates of previous AIDS, compared with noncontrollers (8.8% vs 28.9%).

In relation to HCV coinfection, the spontaneous clearance rates were similar in both study groups, and the HIV controllers displayed a tendency to maintain lower levels of HCV as compared to noncontrollers. As we previously described [3], HCV genotype 3 was overrepresented among HIV controllers as compared to noncontrollers (46.4% vs 20.2%; OR, 3.792; 95% CI, 1.473–9.763; $P = .006$). The association of the *IL28B* CC genotype with spontaneous clearance of HCV, as previously reported [4], was also observed in our study when all patients were analyzed together (OR, 4.316; 95% CI, 1.723–10.575; $P = .002$). However, this association was not observed in the HIV controllers, most likely because of the HCV genotype heterogeneity observed in this group.

Interestingly, the *IL28B* CC genotype was associated with spontaneous HIV control (OR, 2.018; 95% CI, 1.118–3.641; $P = .020$). There was an overrepresentation of the *IL28B* CC genotype among HIV controllers, compared with noncontrollers (62.3% vs 45.0%). In a multivariate statistical analysis that used HIV controller status as the dependent variable and adjusted for sex, HLA-B57 and/or HLA-B27 positivity, and *IL28B* CC genotype, the *IL28B* SNP was still independently associated with the spontaneous control of HIV in white HIV controllers (Table 2). However, when HCV-related parameters (ie, log HCV RNA load and HCV genotype) were included in the multivariate statistical analysis, only the *IL28B* CC SNP (OR, 4.222; 95% CI, 1.360–13.109; $P = .013$) and female sex (OR, 10.977; 95% CI, 3.261–36.944; $P < .001$) remained independently associated with spontaneous HIV control, showing that this association was not mediated by HCV coinfection. We also sought to determine whether the *IL28B* CC genotype was related to a lower viral load set point in primary HIV infection. Therefore, we analyzed the prevalence of the *IL28B* CC genotype among 61 noncontrollers who had available data on primary HIV infection. We compared the median of HIV load set points within the first 12 months since primary infection between subjects with *IL28B* CC and subjects with *IL28B*

**Table 1. Characteristics of Controllers and Noncontrollers of Human Immunodeficiency Virus (HIV) Infection**

| Characteristic | HIV Controllers (n = 53) | HIV Noncontrollers (n = 389) | *P* | OR (95% CI) |
|---|---|---|---|---|
| Female sex | 23 (43.4) | 60 (15.4) | <.001 | 4.204 (2.287–7.729) |
| Elite controllers | 34 (64.2) | NA | NA | NA |
| White | 53 (100) | 389 (100) | NA | NA |
| Age, y | 43.0 (39.0–46.0) | 41.0 (35.0–46.2) | .305 | 1.016 (.985–1.049) |
| Time since HIV diagnosis, y | 15.4 (8.0–20.5) | 8.1 (2.0–15.4) | <.001 | 1.108 (1.059–1.160) |
| $CD4^+$ T-cell count, cells/µL | 642.0 (462.5–937.5) | 415 (239.0–582.0) | <.001 | 1.003 (1.002–1.004) |
| $CD8^+$ T-cell count, cells/µL | 726.0 (569.3–1100.0) | 747.5 (523.3–1032.8) | .464 | 1.000 (1.000–1.001) |
| Previous AIDS | 3/34 (8.8) | 109/385 (28.3) | .022 | 0.245 (.073–.818) |
| Injection drug use | 31 (58.5) | 143 (36.9) | .004 | 2.424 (1.352–4.347) |
| Anti-HCV detected | 37 (69.8) | 158/388 (40.7) | <.001 | 3.366 (1.810–6.260) |
| HCV RNA detected[a] | 24/36 (66.7) | 107/151 (70.9) | .622 | 0.822 (.378–1.788) |
| HCV spontaneous clearance[b] | 7/36 (19.4) | 22/151 (14.6) | .469 | 1.415 (.552–3.627) |
| HCV RNA load, log IU/mL | 5.88 (5.0–6.5) | 6.2 (5.6–6.8) | .058 | 0.636 (.398–1.016) |
| HCV genotype detected[c] | | | .019[d] | |
| 1 | 10/28 (35.7) | 70/119 (58.8) | | |
| 3 | 13/28 (46.4) | 24/119 (20.2) | .006 | 3.792 (1.473–9.763) |
| 4 | 5/28 (17.9) | 25/119 (21.0) | .572 | 1.400 (.436–4.496) |
| *IL28B* rs12979860 | | | | |
| CC | 33 (62.3) | 175 (45.0) | .020 | 2.018 (1.118–3.641) |
| CT | 18 (34.0) | 180 (46.3) | .093 | 0.597 (.327–1.091) |
| TT | 2 (3.8) | 34 (8.7) | .229 | 0.409 (.095–1.756) |
| HLA-B57 and/or HLA-B27 detected[e] | 13/ 41 (31.7) | 18/120 (15.0) | .022 | 2.631 (1.151–6.015) |
| HLA B27 detected | 6/ 41 (14.6) | 7/120 (5.8) | .084 | 2.767 (.872–8.779) |
| HLA B57 detected | 8/ 41 (19.5) | 11/121 (9.1) | .080 | 2.424 (.901–6.526) |

Qualitative data are no. (%) of individuals, and quantitative data are median (interquartile range).

Abbreviations: CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; NA, not applicable.

[a] Data were not available for 1 HIV controller and 7 noncontrollers.

[b] Refers to the subjects who cleared HCV infection without HCV-specific treatment. The remaining subjects (5 HIV controllers and 22 noncontrollers) showed a sustained virological response to HCV treatment.

[c] Data are for HCV RNA–positive patients, including those with HCV clearance on receipt of HCV treatment, for whom the HCV genotype was determined before the start of HCV-specific treatment.

[d] Associated with the specified HCV genotypes.

[e] HLA typing was performed for patients with an available DNA sample.

CT/TT, and we did not observe significant differences between the groups (Supplementary Figure 1).

## DISCUSSION

In this study, we demonstrate that the *IL28B* CC genotype is independently associated with spontaneous HIV control in white individuals. This finding adds to the suggestion of common shared mechanisms involved in the control of 2 persistent infections, HIV infection and HCV infection. In fact, we and others have shown how HIV controllers can control HCV infection in terms of lower HCV loads [3] and higher rates of spontaneous clearance [7], compared with non–HIV controllers.

The interest in the SNP in *IL28B* arose from its association with the control of HCV infection [4, 5]. However, this study is the first to show that this SNP is independently related to the control of HIV infection. Interestingly, previous work showed that the *IL28B* SNP was not associated with spontaneous HIV control in African American individuals [7, 8]. Racial differences may explain the discordant results found by the present work in white subjects. Examples of racial differences and associations with HIV disease outcome have been previously shown for SNPs in natural killer cell–related genes, where the association between these SNPs and HIV

**Table 2. Association Between *IL28B* rs12979860 CC and Spontaneous Control of Human Immunodeficiency Virus (HIV) Infection**

| Characteristic | *P* | OR (95% CI) |
|---|---|---|
| Female sex | <.001 | 7.077 (2.893–17.313) |
| *IL28B* rs12979860 CC | .017 | 2.669 (1.188–5.997) |
| HLA-B57 and/or HLA-B27 detection | .017 | 3.080 (1.225–7.744) |

Multivariate logistic regression analysis.

Abbreviations: CI, confidence interval; OR, odds ratio.

susceptibility were found in African Americans but not in other racial groups [11]. Rallon et al [12] analyzed the *IL28B* SNP prevalence among white long-term nonprogressors and HIV-exposed seronegative subjects and did not find any association of the *IL28B* SNP with HIV disease progression or HIV protection. This discrepancy could have arisen because long-term nonprogressors are defined by the maintenance of high levels of $CD4^+$ T cells, in contrast to HIV controllers, who are defined only by their virological control. In addition, a previous study analyzed the association of this SNP with HIV acquisition and AIDS progression, and no association was found [13]. However, the aim of the present study was different because it analyzed the relationship between the *IL28B* CC genotype and the ability to control HIV viremia.

The association of the *IL28B* SNP with HIV control was not discussed in a previous genome-wide association study [1], most likely because the *IL28B* SNP might be in strong linkage disequilibrium with other analyzed SNPs, which could explain the loss of the association with viral control in the statistical analysis. In relation to viral set point establishment, we did not find differences in HIV loads between the *IL28B* CC and *IL28B* CT/TT groups. Because this lack may be due to the small number of subjects studied, analysis of a large cohort may contribute to clarifying this issue.

The overrepresentation of HLA-B57 among HIV controllers has been widely documented [1], as has its association with HCV control [2, 3]. This suggests that some common host immunological mechanisms are involved in the response against these 2 viruses, such as the efficient elimination of infected cells by HLA-B57–restricted cytotoxic T lymphocytes that recognize distinct epitopes from HIV or HCV [2]. The association of the *IL28B* CC genotype with HIV control was observed even when we adjusted for sex and the presence of protective alleles, such as HLA-B57 or HLA-B27. In this regard, sex differences in the course of HIV infection have been already described in several studies, and we had previously observed a higher prevalence of female sex among HIV controllers [3, 10]. In agreement with this observation, lower HIV loads during an early stage of infection [14] have been shown in women. Further studies are necessary to clarify the extent to which female hormones may be implicated in spontaneous HIV control and the mechanisms involved.

In this report, we show for the first time an association between the *IL28B* rs12979860 SNP and spontaneous HIV control. The mechanistic explanation could be related to the antiviral activity of IFN-λ, which has been demonstrated against different viruses, including HIV [6]. However, the influence of the *IL28B* SNP on IFN-λ production and activity are still unknown. Further studies are needed to analyze the antiviral properties of IFN-λ in HIV controllers and their possible role in the spontaneous control of HIV infection, as previously shown for IFN-α [15].

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**IFNL4 ss469415590 polymorphism is associated with unfavourable clinical and immunological status in HIV-infected individuals.**

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**Summary:**

IFNL4-ss469415590 polymorphism has been associated to HCV clearance. We wondered whether ss469415590 was associated with clinical and immunovirolgical parameters in HIV infection. We found an association of IFNL4-ss469415590-ΔG allele with lower CD4+ T-cell levels and higher prevalence of AIDS-defining illness.

**Abstract**

**Introduction/Objective**: Recently, a new variant ss469415590 (TT or ΔG), in high linkage disequilibrium with IL28B rs12979860 genotype, which creates a novel gene, IFNL4, encoding the IFN-λ4, was shown to be more strongly associated with HCV clearance than IL28B genotype. We wondered whether IFNL4 ss469415590 is associated with clinical and immunovirological parameters in HIV-infected subjects.

**Methods:** A total of 388 asymptomatic HIV-infected patients, naive for antiretroviral treatment with available DNA samples were included. Clinical and immunovirological data including CD4 T-cell levels, AIDS-defining illness and HIV viral load were recorded.

**Results**: The percentage of AIDS-defining illness was elevated in carriers of ΔG variant (TT/TT: 4.9%; TT/ΔG: 9.6% and ΔG/ΔG: 19.5%). IFNL4-TT variant was the only variable associated with HCV spontaneous clearance [p=0.015, OR=5.176, 95%CI= 1.367-19.593]. IFNL4-ΔG/ΔG [p=0.001, OR=14.066, 95%CI=2.995-66.967], age [p=0.007, OR=1.063, 95%CI=1.017-1.112] and CD4 T-cell [p=0.003, OR=0.995, 95%CI=0.992-0.998], but not HIV viremia, were independently associated to present an AIDS-defining illness. Besides, IFNL4-TT variant [p=0.044, B=55.814, 95%CI=1.514, 110.114] and HIV viremia [p<0.001, B=-55.771, 95%CI=-86.310, -25.232] were independently associated to CD4 T cell counts in individuals with CD4 T cell≥150 cell/μl.

1 **Conclusion**: The association of IFNL4 genotypes with clinical and immunological
2 parameters related to HIV disease progression point out common host mechanisms
3 against different viral infections. The elucidation of IFN-λ3 and λ4 mechanisms of action
4 will enable the development of therapeutic interventions not only in HCV but also in HIV
5 infection.

## Introduction

Genome-wide association studies (GWAS) identified a single nucleotide polymorphism (SNP) rs12979860, located on chromosome 19 upstream the gene which encodes for interferon λ3 (IFN-λ3), also known as IL28B [1, 2]. The rs12979860 was associated to both spontaneous and treatment-induced clearance of hepatitis C virus (HCV) infection in HCV-monoinfected and HIV coinfected patients [3, 4]. Although not well understood, the antiviral properties of IL28B/IFN-λ3 have been demonstrated *in vitro* against different virus, including HIV [5-7]. We showed that the favourable genotype IL28B-CC was associated with the spontaneous control of viremia in HIV-infection [8].

Recently, a new transiently induced region that harbours a dinucleotide variant ss469415590 (TT or ΔG), which is in high linkage disequilibrium with IL28B rs12979860, has been discovered [9, 10]. ss469415590 [ΔG] is a frameshift variant between interferon lambda 2 (IFNL2) and IFNL3 genes that creates a novel gene, designated IFNL4, encoding the IFN-λ4, which structure is moderately similar to IFN-λ3 [9]. Although the relevance and the function of this protein are unknown, IFNL4 ss469415590-ΔG seems to be the functional variant as is associated with the production of IL28B [10]. When compared to rs12979860, ss469415590 is more strongly associated with the spontaneous clearance of HCV infection and the response to HCV treatment [9-12]. Whether ss469415590 is associated with clinical and immunovirological parameters in HIV-infected patients, is unknown.

In accordance with all these data it is provocative to think that ss469415590 polymorphism is associated with disease progression-related parameters in other virus infections, e.g. HIV. The aim of the present study was to analyze the association of the

1 IFNL4 variants with clinical and immunovirological parameters in HIV-infected

2 antiretroviral therapy-naïve patients.

## Patients and Methods

All patients included in this study were white and belonged to the HIV-infected patients cohort of the Infectious Diseases Service at Virgen Del Rocío University Hospital (Seville, Spain). A total of 388 asymptomatic, HIV-infected patients, naive for antiretroviral treatment with available CD4 T-cell data and peripheral blood mononuclear cells samples, were included. Clinical and immunovirological data including CD4 T-cell levels, AIDS-defining illness and viral load were recorded at the entry in the cohort. Some of these patients had been included in a previous study [8]. HIV-2-infected subjects were excluded. All the patients participating in the study gave their informed consent, and the institutional ethical committees approved the protocols.

Routinely, the absolute numbers of CD4 and CD8 T cells were assayed from fresh whole blood samples using the Epics XL-MCL flow-cytometer (Beckman Coulter). Plasma HIV RNA was measured in fresh samples by a quantitative polymerase chain reaction (COBAS Ampliprep/COBAS Taqman HIV test; Roche molecular systems), according to the manufacturer's instructions. The detection limit was 40 HIV RNA copies/mL. Quantitative reverse transcription PCR was performed for plasma HCV RNA amplification (COBAS Amplicor; Roche Diagnosis), with detection limit of 15 IU/mL

The HLA-B group alleles were genotyped using a reverse sequence-specific oligonucleotide bound to a fluorescently coded microsphere system (LABType® SSO, RSSO1B, One Lambda Inc), as previously reported [13]. IL28B rs12979860 genotyping were performed as previously described [8] using a TaqMan 5' allelic discrimination assay (Applied Biosystems,). The IFNL4 ss469415590 genotyping was assayed using TaqMan assays with Genotype Master Mix (Qiagen) on the ABI SDS7700 under standard conditions as previously described [9].

The statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 18.0). Statistical Chi square test and T student were used to analyze categorical variables and continuous variables respectively. All of the differences between the groups in the bivariate and multivariate linear and logistic regression analysis with a *p* value <0.05 were considered statistically significant.

1 **RESULTS**

2

3 **Characteristic of the subjects**

4 The characteristics of the subjects are summarized in Table 1. Among the 388
5 included subjects, thirty-six were IFNL4-ΔG/ΔG. Regarding CD4 T-cell levels and HIV
6 viremia, no differences were observed according to IFNL4 genotypes. The frequency of
7 AIDS-defining illness was increased in carriers of ΔG variant. In addition, from the
8 sixteen HCV spontaneous clearers, thirteen were IFNL4-TT/TT subjects. Linkage
9 disequilibrium between ss469415590 and rs12979860 was r=0.809. The distribution of
10 IFNL4 genotypes was under Hardy-Weinberg equilibrium.

11

12 **IFNL4-ΔG allele is associated with AIDS-defining illness.**

13 The percentage of AIDS-defining illness was elevated in carriers of ΔG variant
14 [TT/TT: 9/185; TT/ΔG: 16/167 and ΔG/ΔG: 7/36] (Figure 1A). Most of the AIDS-
15 defining illnesses observed were tuberculosis and Pneumocystis jiroveci pneumonia. In
16 addition to IFNL4-ΔG variant; age, CD4 T-cells and HIV viremia were associated to
17 AIDS-defining illnesses (Table 2). When these variables were included in the
18 multivariate analysis: age, CD4 T-cells and IFNL4-ΔG variant remained independently
19 associated with AIDS-defining illnesses (Table 2). The rs12979860 IL28B was also
20 independently associated with AIDS-defining illnesses [p=0.004, OR=0.161
21 95%CI=0.047-0.555].

22

23 **IFNL4-TT allele is associated with CD4 T-cell levels**

24 We also analyzed which factors were related to CD4 T-cell levels: age, HIV
25 viremia and AIDS-defining illness were associated; meanwhile IFNL4 genotypes

1 remained in a trend (data not shown). However, when we analyzed subjects with CD4 T
2 cell ≥ 150 cell/μl, we observed higher CD4 T-cell levels (Figure 1B). Regarding the
3 bivariate statistical analysis, HIV viremia, age, AIDS-defining illness and IFNL4-TT
4 allele were associated to CD4 T-cell levels (Table 3). Adjusting by these variables, only
5 HIV viremia and IFNL4-TT allele remained independently associated to CD4 T-cell
6 counts (Table 3). The rs12979860 IL28B was also independently associated with CD4 T-
7 cell counts in these individuals [p=0.019, B=64.773, 95%CI=10.571, 118.976].
8
9 **IFNL4 genotypes are not associated with HIV viremia**
10 HIV viral load was assessed in 287 patients, those who enter the cohort since
11 1997 when HIV viremia measurement was available. In the bivariate analysis, IFNL4
12 genotypes were not associated with HIV viremia (data not shown). After adjusting by
13 sex, CD4 T-cell levels, AIDS-defining illness and HLA-B57, only, CD4 T-cells
14 [p<0.001, B=-0.001, 95%CI= -0.002, -0.001] and HLA-B57 [p=0.012, B=-0.597,
15 95%CI= -1.059, -0.135] remained independently associated with HIV viremia.
16
17
18 **IFNL4-TT/TT is associated with HCV spontaneous clearance**
19 The IFNL4-TT/TT was associated with higher prevalence of HCV spontaneous
20 clearance [26.5% TT/TT vs 6.5% non-TT/TT subjects, p=0.009] (Table 1). Curiously,
21 none of the sixteen HCV clearers were IFNL4-ΔG/ΔG genotype. When we used the
22 logistic regression analysis, among all the included variables, the IFNL4-TT/TT was the
23 only variable independently associated to HCV spontaneous clearance [p=0.015,
24 OR=5.176, 95%CI= 1.367-19.593]. Moreover, the association was identical to IFNL4-
25 TT/TT when rs12979860 IL28B was analyzed.

## DISCUSSION

In this cross-sectional study, the results showed that IFNL4-ΔG variant was associated with unfavourable clinical and immunological status independently of HIV viremia. We also showed an association of IFNL4-TT/TT genotype with HCV spontaneous clearance. These results point out common host mechanisms against different viral infections which level of protection are related to the intrinsic characteristics and immunopathogenesis caused by each virus.

Remarkably, the most relevant and novel discovery in our study was the association of rs12979860 as well as ss469415590 with AIDS-defining illness prevalence. One open question remained whether this allele was associated with a specific opportunistic infection. Interestingly, the AIDS-defining illnesses observed were mostly tuberculosis and pneumonia. However, we were not able to observe a significant association between the IFNL4-ΔG and any of these two infections (data not shown). Nonetheless, the trend observed between tuberculosis and IFNL4-ΔG guarantee further analysis in other cohort of white subjects.

On the other hand, we also observed an association with a preserved immunological status in carriers of the favourable allele for both rs12979860 and ss469415590. The fact that this association was observed in subjects with CD4 T-cell ≥ 150 cell/µl, suggests that the protective effect of these alleles is transient and/or need the involvement of other genetic factors or immunological mechanisms. Regarding HIV viremia, the absence of differences between IFNL4 variants suggests that the effect of this polymorphism on HIV viremia depends on other factors e.g. HLA-B57 and/or may

1 be early in the infection. In fact, we previously showed a trend to higher HIV viral load in
2 carriers of the protective allele of IL28B in a different seroprevalent cohort during the
3 first 9 months of HIV infection [8]. These data were also described for acute HCV
4 infection [12]. The influence of these early differences on HIV viremia over CD4 T-cell
5 levels or AIDS-defining illness prevalence in HIV infection remains unknown.
6
7 In addition, we found that IFNL4-TT/TT was associated with HCV spontaneous
8 clearance, in agreement with a previous study where approximately 85% of the white
9 participants were HIV/HCV coinfected [11]. Moreover, the presence of the IFNL4-TT
10 allele seems almost a necessary determinant for spontaneous virus clearance in white
11 subjects, since 100% of the HCV spontaneous clearers, in our and the previous study
12 [11], carried this allele.
13
14 A possible explanation of our results may be a defect on IL28B/IFN-λ3
15 production and/or impaired activity in individuals carriers of unfavourable alleles of
16 rs12979860 and/or ss469415590 as previously suggested [10]. We suggest that besides
17 the antiviral activity [3], IL28B/IFN-λ3 may also be a regulator of the adaptive immune
18 response. In fact, Morrow et al, by using IL28B/IFN-λ3 as an adjuvant for HIV DNA
19 vaccination, demonstrated that IL28B/IFN-λ3 have potent effects on antigen-specific
20 responses; inducing an increase of T helper-1 cell response and simultaneously reducing
21 regulatory T cells [14]. On the other hand, Prokunina-Olsson et al, suggested that only
22 carriers of IFNL4-ΔG variant express IFN-λ4 protein which may compete with
23 IL28B/IFN-λ3 receptors; and apparently cause a preactivation of the interferon stimulated
24 genes which reduces the responsiveness to type I and III interferon [9]. These data may
25 explain antiviral and immunomodulatory defects in carriers of IFNL4-ΔG variant. In

these subjects IFN-λ4 production may impair an adequate immune response against opportunistic events, which explains the higher prevalence of AIDS-defining illness observed in our study.

In summary, the association of IFNL4 genotypes with clinical and immunological parameters related to HIV disease progression point out common host mechanisms against different viral infections, as we anticipated in our previous study [8]. The elucidation of IFN-λ3 and λ4 mechanisms of action will enable the development of therapeutic interventions not only in HCV but also in HIV infection.

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**CONFLICT OF INTERESTS**

The authors do not have any association that might pose a conflict of interest.

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1 **Table 1. Characteristics of the study subjects.**
2

| Characteristics | All subjects N (%) | IFNL4-ss469415590 TT/TT | ΔG/TT or ΔG /ΔG | *p* |
|---|---|---|---|---|
| Number of subjects | 388 | 185 (47.8) | 203 (52.2) | NA |
| Gender (male) (%) | 320 (82.5) | 159 (85.9) | 161 (79.3) | 0.086 |
| Age (years) | 32 [28 – 40] | 32 [ 27 – 40] | 32 [ 28 – 40] | 0.387 |
| CD4 T cell/ul | 397 [216 – 570] | 412 [198 – 593] | 372 [224 – 548] | 0.198 |
| Nadir CD4 T cell/ul | 308 [186 – 439] | 308 [177.5 – 442] | 310 [193 – 435] | 0.787 |
| Log HIV RNA copies/ml ¥ | 4.60 [4.05 – 5.12] | 4.56 [3.95 – 5.17] | 4.63 [4.05 – 5.06] | 0.634 |
| AIDS-defining illness | 32 (8.2) | 9 (4.9) | 23 (11.3) | 0.021 |
| Anti-VHC+ (%) | 95/359 (26.5) | 49/175 (28.0) | 46/184 (25.0) | 0.576 |
| PCR-VHC+ (%) | 67/95 (70.5) | 29/49 (59.2) | 38/46 (82.6) | 0.040 |
| HCV Spontaneous clearance § | 16/95 (16.8) | 13/49 (26.5) | 3/46 (6.5) | 0.009 |
| HLA-B57‡ | 18/255 (7.0) | 7/123 (5.7) | 11/132 (8.3) | 0.410 |
| IL28B.rs12979860 | | | | |
| CC | 186 (47.9) | 173 (93.5) | 13 (6.4) | NA |
| CT/TT | 202 (52.2) | 12 (6.5) | 190 (93.6) | |

3

4 Qualitative variables are shown as numbers and percentages of individuals; quantitative
5 variables are displayed as median and interquartile ranges (IQR). ¥ HIV viral load
6 measures were available in 287 subjects who visited our unit since 1997, HIV viral load
7 were not available before this date. § HCV spontaneous clearance refers to the subjects
8 who cleared HCV infection without HCV specific treatment, the data of the remaining 12
9 subjects were not available. ‡ HLA typing was assayed in patients where DNA samples
10 were available. NA, not applicable. HCV, hepatitis C virus. PCR, polymerase chain

reaction. HLA, human leukocyte antigens. Statistical Chi square test and T student were used to analyze differences between categorical variables and continuous variables, respectively. p value <0.05 were considered statistically significant.

1 **Table 2. IFNL4 ss469415590-ΔG allele is associated with a higher prevalence of**

2 **AIDS-defining illness**

| | AIDS-defining illness | | | | | |
|---|---|---|---|---|---|---|
| | Bivariate analysis | | | Multivariate analysis | | |
| Characteristics | *p* | OR | 95% CI | *p* | OR | 95% CI |
| Gender (male) | 0.438 | 1.534 | 0.520 - 4.527 | | | |
| Age (years) | <0.001 | 1.063 | 1.028 - 1.099 | 0.007 | 1.063 | 1.017 - 1.112 |
| CD4 T cell/ul | <0.001 | 0.994 | 0.991 - 0.996 | 0.003 | 0.995 | 0.992 - 0.998 |
| Log HIV RNA copies/ml | 0.002 | 2.804 | 1.464 - 5.374 | 0.249 | 1.546 | 0.737 - 3.242 |
| IFNL4-ss469415590 | 0.016* | | | 0.003* | | |
| - T/T | | | | | | |
| - ΔG/T | 0.091 | 2.072 | 0.890 – 4.824 | 0.010 | 5.482 | 1.503 – 19.991 |
| - ΔG/ ΔG | 0.004 | 4.720 | 1.631-13.665 | 0.001 | 14.066 | 2.955 - 66.967 |

3

4 Bivariate and Multivariate logistic regression analysis were performed using AIDS-

5 defining illness as dependent variables. *Associated with the specified ss469415590

6 genotypes. . p value <0.05 were considered statistically significant. Abbreviations: OR,

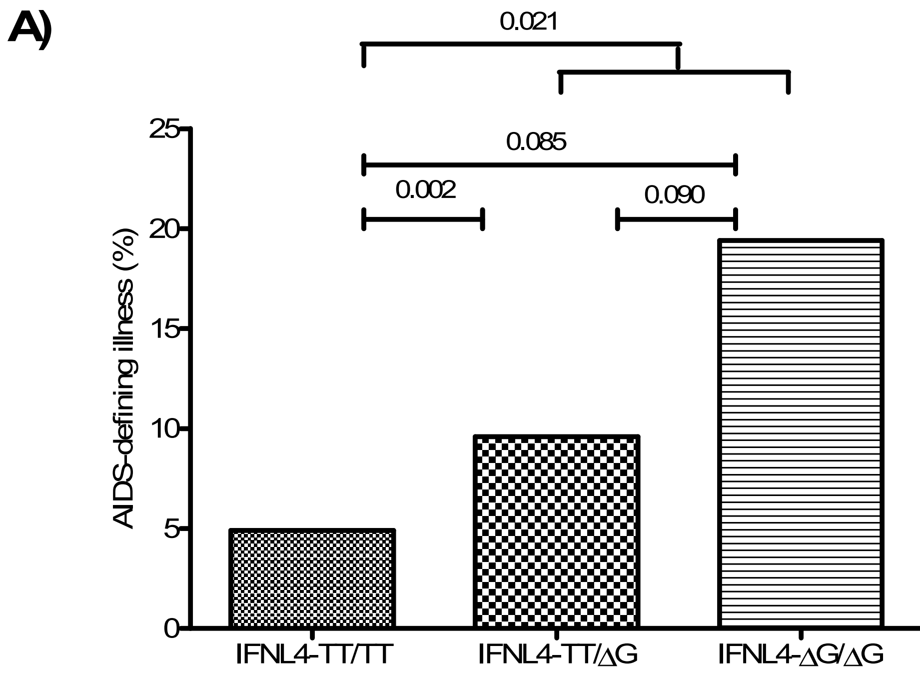
7 odds ratio; CI, confidence interval. NA, not applicable.

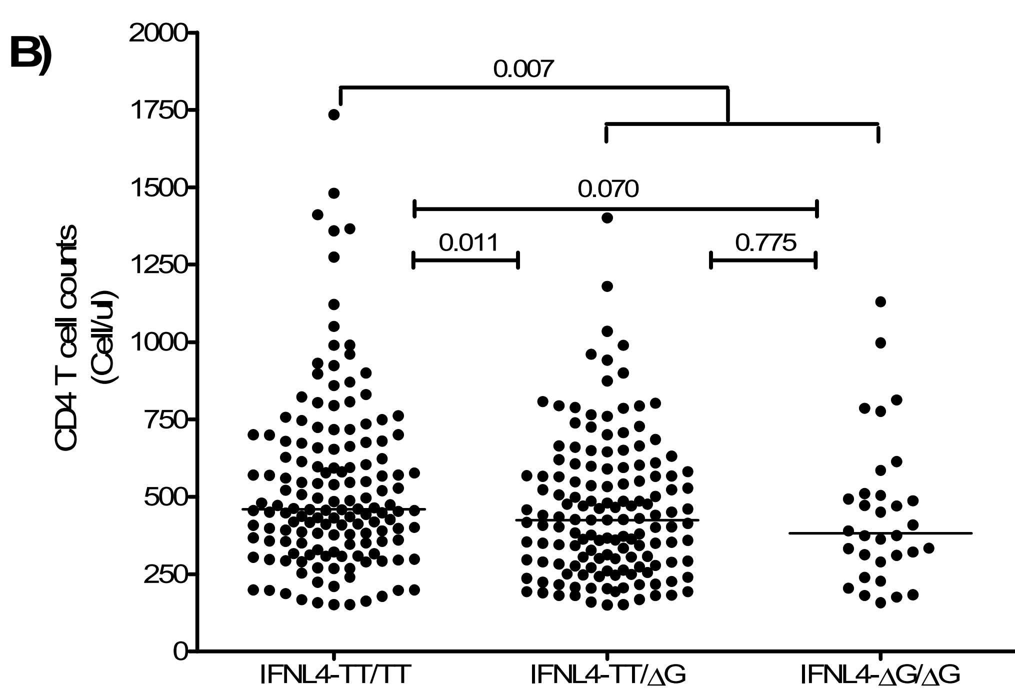
**Table 3. IFNL4 ss469415590-TT allele is associated with higher CD4 T cell level.**

| | CD4 T cell | | | | | |
|---|---|---|---|---|---|---|
| | Bivariate analysis | | | Multivariate analysis | | |
| Characteristics | *p* | B | 95% CI | *p* | B | 95% CI |
| Gender (male) | 0.905 | 4.407 | -67.902, 76.716 | | | |
| Age (years) | 0.002 | -4.897 | -7.914, -1.881 | 0.073 | -2.564 | -5.370, 0.241 |
| AIDS-defining illness | 0.020 | -161.412 | -297.556, -25.269 | 0.680 | -29.564 | -170.688, 111.559 |
| Log HIV RNA copies/ml | <0.001 | -57.818 | -88.349, -27.349 | <0.001 | -55.987 | -86.651, -25.324 |
| IFNL4-ss469415590 | | | | | | |
| - T/T | Ref* | | | | | |
| - ΔG/T | 0.011 | -75.685 | -135.795, -17.575 | 0.047 | -57.628 | -114.398, -0.858 |
| - ΔG/ ΔG | 0.072 | -88.929 | -185.882, 8.024 | 0.316 | -47.383 | -140.207, 45.441 |

Bivariate and Multivariate linear regression analysis were performed using CD4 T-cell as dependent variables. 325 subjects with CD4 ≥150 cell/μl were included: 148 were TT/TT; 145 TT/ΔG and 32 ΔG/ΔG. *Ref: Reference; Associated with the specified IFNL4-ss469415590 genotypes. p value <0.05 were considered statistically significant. Abbreviations: B, regression coefficient; CI, confidence interval. NA, not applicable.

1 **Figure 1: AIDS-defining illness prevalence and CD4 T cell levels according to**
2 **IFNL4 genotypes.**
3
4




5
6
7
8
9

1 **Figure legend**

2

3 Figure 1:

4 A) The percentage of AIDS-defining illness was elevated in carriers of ΔG variant:
5 TT/TT: 9/185; TT/ΔG: 16/167 and ΔG/ΔG: 7/36. B) higher CD4 T-cell levels were
6 observed in IFNL4-TT/TT compared to carriers of ΔG variant: TT/TT: 460 cell/μl , IQR:
7 352-671; TT/ΔG: 425 cell/μl , IQR: 285-585 and ΔG/ΔG: : 382 cell/μl , IQR: 294-509.
8 Statistical Chi square test and T student were used to analyze categorical variables and
9 continuous variables respectively. p value <0.05 were considered statistically significant.

# *DISCUSSION*

## DISCUSSION

The antiretroviral therapy (ART) for HIV infection needs lifelong adherence to expensive regimens with toxic effects. Despite the remarkable success of ART to control the viremia, the eradication of HIV remains challenging. Understanding mechanisms underlying the prolonged spontaneous control of viral replication by HIV Controllers may provide us with useful correlates of protection against HIV, which should help in the design of new vaccinal strategies.

The innate immune system is our first line of defense against invading microorganisms while the adaptive immune system acts as a second line of defense and also affords protection against reexposure to the same pathogen. One of the hallmarks of the innate immune response is the production of the antiviral cytokine type I IFN (IFN), which inhibits viral replication and spreading[47]. Plasmacytoid dendritic cells (pDCs) are the most potent IFN-$\alpha$-producing cells [48, 49] and serve as an essential link between innate and adaptive immunity [50].

Thus, we studied pDCs to clarify whether human pDCs response to HIV is altered in HIV controllers. We found that controller patients maintained high blood number of pDCs, which were producing high levels of IFN-$\alpha$ in response to HIV exposure. In contrast, we found that pDCs from viremic patients statistically produced less IFN-$\alpha$ than pDCs from controllers or healthy donors in response to HIV, but surprisingly produced similar levels of IFN-$\alpha$ in response to Flu as controllers and healthy donors. Furthermore, we also found that CpG exposure induced similar levels of IFN-$\alpha$ by pDCs from viremic and controller patients or healthy donors.

We demonstrated a preserved capacity of pDCs from EC to suppress viral production in H9 T cells. This result is in agreement with data from a previous study [51], where a higher capacity to suppress HIV replication in autologous CD4 T cells was found for antiretroviral-naive low-viremic subjects (<12,500 HIV RNA copies/ml) than for treated subjects and high-viremic subjects (≥12,500 HIV RNA copies/ml). However, in our study, we used the same target cells (H9 T cell line) to compare the suppressive faculty of pDCs among the study groups. We demonstrated that pDCs from EC subjects had the same capacity to reduce HIV production as pDCs from HD subjects, whereas pDCs from VIR subjects could scarcely respond. In contrast, when pDCs from all groups were prestimulated with TLR-9, we observed the same behaviour for the three groups. These results agree with previous data showing that CpG A- and HIV-stimulated pDCs are not refractory to IFN-α production after restimulation [52]. In this work, we show that the magnitude of the response after continuous HIV stimulation is higher in EC than in VIR subjects. This result demonstrates that pDCs from VIR subjects are functional but are not able to be efficiently stimulated by HIV and need to be previously activated by an HIV-independent pathway to display antiviral activity against HIV-infected T cells. This activity was associated with the IFN-α produced by isolated pDCs. These results show that pDCs from EC have preserved functionality to suppress HIV.

The great majority of in vitro studies on pDCs suggested that IFN-α is the principal mechanism of viral suppression [53, 54]. A previous report demonstrated that the HIV stimulation of pDCs induces a high level of production of IFN-α and a rapid expression of TRAIL, transforming them into IFN-producing killer pDCs (iKpDCs)

[25]. It was also shown previously that despite TRAIL expression, pDCs could not induce the lysis of autologousCD4+ T cells [55]. However, in the present work, when primary HIV-infected autologous CD4+ T cells were used as target cells, we also observed apoptosis induced by both unstimulated and TLR-9- stimulated pDCs. This discrepancy can be explained because we investigated not lysis but apoptosis using early (annexin V) and late (Topro-III) apoptosis markers, and autologous CD4+ T cells were productively infected and not only exposed to HIV; in addition, cocultures were maintained not for 6 h as in previous work but overnight in the case of autologous CD4+ T cells or for 5 days in the case of H9 T cells. Thus, we demonstrated that besides IFN-α, pDCs exert their antiviral effect by inducing T cell apoptosis. Indeed, when p24+ T cell levels in the coculture were analyzed, we observed a reduction of p24+ T cell percentages. This effect was also observed when recombinant IFN-α was added to H9 T cells. These observations suggest that the pDCs-induced viral reduction is due mostly to H9 T cell-induced apoptosis, which is preserved in EC. This finding is in accordance with our results showing that purified pDCs from EC and HD subjects produced large amounts of IFN-α in response to HIV-1 particles produced by the T cell line. In contrast, the level of IFN-α production by pDCs from VIR subjects was very low and barely suppressed or induced T cell apoptosis, confirming the fact that VIR subjects had an impaired HIV-mediated activation of pDCs. Previous studies hypothesized that HIV-mediated pDCs activation was dependent of viral gp120 binding to cellular CD4 [56, 57]. Thus, in an attempt to understand why pDCs from VIR subjects were not able to respond to the HIV stimulus, we quantified CD4 expression on the pDCs cell surface. Cytometry and microscopic study clearly demonstrate that pDCs from viremic statistically express lower levels of surface CD4 than pDCs from healthy donors. Furthermore, CD4 expression on pDCs from controllers was similar to pDCs

from healthy donors. The very low membrane CD4 expression level on pDCs from VIR subjects could explain the lack of responses when they were cultured in the presence of HIV. However, when pDCs from VIR subjects where stimulated by a CD4-independent activator (CpG), they responded similarly to those from HD or EC subjects. These data also explain the similar behaviors of pDCs among the different groups when a previous stimulation via a CD4-independent pathway, such as the TLR-9 ligand CpG, was performed.

In conclusion, our studies showed the qualitative and functional involvement of pDCs in the spontaneous control of HIV viremia. These findings highlight the important role of innate immunity in HIV immunopathogenesis and could have important immunotherapeutic applications.

However, important questions remain open concerning the extraordinary immunity of these patients. One important aspect is to know whether HIV controllers are able to control other persistent infections in the same way that they do HIV. Hepatitis C virus (HCV) is of particular interest, whose natural history is accelerated in HIV-co-infected patients [58]. Results presented in this Doctoral Thesis showed how Caucasian HIV controllers exhibited better control of HCV replication, measured as plasma HCV loads, when compared with HIV non-controllers group. Furthermore, they presented a different distribution of HCV genotypes with an overrepresentation of genotype 3. Genetic determinants, associated with HIV progression, were also associated with HCV loads. These findings support the idea that some common host mechanisms are involved in the defence against these two persistent infections.

The absence of differences in the proportion of spontaneous HCV clearance between the comparison group and HIV controllers agrees with previous work that showed how different immune mechanisms are operating against HIV, cytomegalovirus and HCV, at least in relation to CTL responses [59]. However, we found that HIV controllers had lower levels of HCV loads. Hence, both persistent infections should share common control mechanisms although at a different level. The clinical implications of this lower HCV load in the response to HCV-specific treatment and the natural history of HCV in HIV controllers are unknown at the moment, although recently no differences in liver histology between HIV controllers and non-HIV controllers with chronic HCV infection were reported [36]. In this same work, significantly lower CD4 cell counts in HIV controllers without chronic HCV infection compared to HIV controllers with chronic HCV infection were found. These data are in line with those presented in the current work.

Another peculiarity of the HIV controllers cohort was the different distribution of HCV genotypes compared to the general HIV-infected population. The reasons for this different distribution are unknown at the moment. One possible explanation could be related to the different distribution of specific HLAs in elite controllers. In this sense, a variable influence of HLA on various genotypes has been reported [60]. We can also hypothesize that the presence of genotype 1 would favour the loss of HIV control, and hence, this genotype could be underrepresented in the HIV controller group. This idea could be supported, although at a different level, by the higher progression rate in HIV co-infected people with HCV genotype 1 found in some studies [61, 62].

The lower HCV loads found in HLA-B57+ subjects point once again to some common immunogenetic host mechanisms involved in the defence against these two viruses. It has been widely documented that the HLA-B57 is overrepresented in HIV

controllers [63]. The reasons why this host genetic factor has been associated with HIV control are beginning to be understood [6]. Recent studies point to the presence of a strong CTL response and a viral fitness cost to the virus that arises from HLA-B57 escape mutations [64]. This process could also take place in HCV infection and might be associated with a common immunological cellular mechanism such as efficient elimination of infected cells by HLA-B57-restricted CTLs recognizing distinct epitopes from HIV or HCV. Studies relating HLA-B57 with HCV are scarce, and an association has only been found between the presence of HLA-B57 and the spontaneous clearance of HCV [65-67]. This agrees with our results, although the contrast was not statistically significant because of the difficulty in finding a high number of patients who were HIV controllers, HLA-B57+ and exposed to HCV.

In a previous study, Sajadi et al. [36] have shown an increased rate of HCV spontaneous clearance in “natural viral suppressors” (<400 HIV-RNA copies/mL), when compared to HCV–HIV-co-infected and HCV-mono-infected control groups. In that study, the cohort was entirely composed of African-Americans. This fact could explain the discrepant results found in our cohort composed mainly of Caucasians. Another difference is that variations in HCV load between HIV controllers and reference groups were not found. This could be explained, apart from the race differences, by most of the subjects being HCV genotype 1 (approximately 90%) and, as we have shown, HCV genotype is associated with HCV load. In this regard, it is important to mention that HIV controllers from the Sajadi cohort had almost fourfold higher HCV load than elite controllers from our cohort owing to the prevalence of genotype 1. Although in this previous work genetic factors were not analyzed, our findings underlined how different race groups of HIV controllers with different genetic and immunological backgrounds can influence HCV load levels.

Following the idea of common shared mechanisms of control, as the HLA B57, which is overrepresented in HIV controllers and also associated to HCV clearance. We were also interested in studying whether the IL28B SNP was associated to HIV control. We demonstrated that the IL28B CC genotype is independently associated with spontaneous HIV control in white individuals. This finding adds to the suggestion of common shared mechanisms involved in the control of 2 persistent infections, HIV infection and HCV infection. However, this study is the first to show that this SNP is independently related to the control of HIV infection. Interestingly, previous work showed that the IL28B SNP was not associated with spontaneous HIV control in African American individuals [40, 41]. Racial differences may explain the discordant results found by the present work in white subjects. Examples of racial differences and associations with HIV disease outcome have been previously shown for SNPs in natural killer cell–related genes, where the association between these SNPs and HIV susceptibility were found in African Americans but not in other racial groups [68]. Rallon et al [42] analyzed the IL28B SNP prevalence among white long-term nonprogressors and HIV-exposed seronegative subjects and did not find any association of the IL28B SNP with HIV disease progression or HIV protection. This discrepancy could have arisen because long-term nonprogressors are defined by the maintenance of high levels of CD4+ T cells, in contrast to HIV controllers, who are defined only by their virological control. In addition, a previous study analyzed the association of this SNP with HIV acquisition and AIDS progression, and no association was found [69].

The association of the IL28B SNP with HIV control was not discussed in a previous genome-wide association study [8], most likely because the IL28B SNP might

be in strong linkage disequilibrium with other analyzed SNPs, which could explain the loss of the association with viral control in the statistical analysis.

In a cross-sectional study, we studied the recently discovered IFNL4 ss469415590 polymorphism, which is in high linkage disequilibrium with IL28B rs12979860 [43, 44]. The results showed that IFNL4-ΔG variant was associated with unfavourable clinical and immunological status independently of HIV viremia. We also showed an association of IFNL4-TT/TT genotype with HCV spontaneous clearance. These results point out common host mechanisms against different viral infections which level of protection are related to the intrinsic characteristics and immunopathogenesis caused by each virus.

Remarkably, the most relevant and novel discovery in this study was the association of rs12979860 as well as ss469415590 with AIDS-defining illness prevalence. One open question remained whether this allele was associated with a specific opportunistic infection. Interestingly, the AIDS-defining illnesses observed were mostly tuberculosis and pneumonia. However, we were not able to observe a significant association between the IFNL4-ΔG and any of these two infections (data not shown). Nonetheless, the trend observed between tuberculosis and IFNL4-ΔG guarantee further analysis in other cohort of white subjects.

On the other hand, we also observed an association with a preserved immunological status in carriers of the favorable allele for both rs12979860 and ss469415590. The fact that this association was observed in subjects with CD4 T-cell ≥ 150 cell/μl, suggests that the protective effect of these alleles is transient and/or need the

involvement of other genetic factors or immunological mechanisms. Regarding HIV viremia, the absence of differences between IFNL4 variants suggests that the effect of this polymorphism on HIV viremia depends on other factors e.g. HLA-B57 and/or may be early in the infection. In fact, in the third objective of this Doctoral Thesis, we showed a trend to higher HIV viral load in carriers of the protective allele of IL28B in a different seroprevalent cohort during the first 9 months of HIV infection. These data were also described for acute HCV infection [45]. The influence of these early differences on HIV viremia over CD4 T-cell levels or AIDS-defining illness prevalence in HIV infection remains unknown.

A possible explanation of our results may be a defect on IL28B/IFN-λ3 production and/or impaired activity in individuals carriers of unfavourable alleles of rs12979860 and/or ss469415590 as previously suggested [44]. We suggest that besides the antiviral activity [39], IL28B/IFN-λ3 may also be a regulator of the adaptive immune response. In fact, Morrow et al, by using IL28B/IFN-λ3 as an adjuvant for HIV DNA vaccination, demonstrated that IL28B/IFN-λ3 have potent effects on antigen-specific responses; inducing an increase of T helper-1 cell response and simultaneously reducing regulatory T cells [70]. On the other hand, Prokunina-Olsson et al, suggested that only carriers of IFNL4-ΔG variant express IFN-λ4 protein which may compete with IL28B/IFN-λ3 receptors; and apparently cause a preactivation of the interferon stimulated genes which reduces the responsiveness to type I and III interferon [43]. These data may explain antiviral and immunomodulatory defects in carriers of IFNL4-ΔG variant. In these subjects IFN-λ4 production may impair an adequate immune response against opportunistic events, which explains the higher prevalence of AIDS-defining illness observed in our study.

In summary, the association of IFNL4 genotypes with clinical and immunological parameters related to HIV disease progression point out common host mechanisms against different viral infections, as we anticipated in our previous study. The elucidation of IFN-λ3 and λ4 mechanisms of action will enable the development of therapeutic interventions not only in HCV but also in HIV infection.

# ***CONCLUSIONS***

**CONCLUSIONS**

The conclusions obtained from the studies presented in this doctoral Thesis are presented below:

1. The preserved functionality of pDCs from EC to reduce viral production may be one of the mechanisms involved in the control of HIV viremia in these subjects. These results demonstrate the importance of innate immunity in HIV pathogenesis, and an understanding of pDC mechanisms would be helpful for the design of new therapies.

2. Caucasian HIV controllers are able to better control HCV replication, in terms of lower HCV viral load levels. These findings support the idea that some common host mechanisms are involved in the defense, against these two persistent infections.

3. The association of IL28B CC genotype with spontaneous HIV control, supports the idea that common host mechanisms are involved in the control of chronic infections, such as HIV and HCV.

4. The association of IFNL4 genotypes with clinical and immunological parameters related to HIV disease progression point out common host mechanisms against different viral infections. The elucidation of IFN-λ3 and λ4 mechanisms of action will enable the development of therapeutic interventions not only in HCV but also in HIV infection.

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