Maraviroc Reduces the Regulatory T-Cell Frequency in Antiretroviral-Naive HIV-Infected Subjects

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Background. Maraviroc is the first antiretroviral (ART) drug to target a human protein, the CCR5 coreceptor; however, the mechanisms of maraviroc-associated immunomodulation in human immunodeficiency virus (HIV)–infected subjects remain to be elucidated. Regulatory T cells (Tregs) play a key role in HIV-associated immunopathology and are susceptible to maraviroc-mediated CCR5 blockade. Our aim was to evaluate the effect of maraviroc on Tregs.

Methods. We compared the effect of maraviroc-containing or -sparing combination ART (cART) on Tregs in ART-naive, HIV-infected subjects. Tregs were characterized as CD4+CD25hiFoxP3+ on day 0, 8, and 30. Additional analysis on week 48 was performed in a subgroup of patients. The potential reduction in the frequency of Tregs among maraviroc-treated peripheral blood mononuclear cells (PBMCs) was also tested in vitro. The suppressive function of Tregs was also analyzed in maraviroc-treated Tregs.

Results. We found that maraviroc significantly reduced the Treg frequency in both the short term and 1 year after treatment initiation. In vitro experiments showed a dose-dependent reduction in the Treg frequency after treatment of PBMCs with maraviroc, although their in vitro suppressive function was not altered.

Conclusions. These findings partially explain maraviroc-associated immunomodulatory effects and open new therapeutic expectations for the development of Treg-depleting immunotherapies.

Keywords. regulatory T cells (Tregs); maraviroc; HIV; ART naives.

Maraviroc is the first antiretroviral drug to target a human protein, the CCR5 chemokine receptor. This protein also serves as a coreceptor for the entry of R5-tropic human immunodeficiency virus (HIV) strains into host cells. CCR5 plays a key role in lymphocyte activation, homing, and proliferation [1]; priming adaptive immune responses; and promoting the migration of CCR5-expressing cells to sites of infection and inflammation [2, 3].

This particular mechanism of action confers exceptional properties to maraviroc. Thus, independently of its antiviral effect [4], maraviroc may also have immunomodulatory effects [5]. Accordingly, maraviroc has been related to increased CD4 T-cell counts [6, 7] and reduced CD4 and CD8 T-cell activation [8], soluble CD14 levels [9], HIV-associated chronic inflammation [3] and apoptosis [10]. Moreover, it was recently found that maraviroc increases antigen-specific T-cell responses against several vaccine-related antigens by altering the cytokine profile [11]. Despite this evidence, the cellular and molecular basis of maraviroc-associated immunomodulation in HIV-infected subjects has not yet been revealed.
Regulatory T cells (Tregs), which suppress the intensity and duration of immune responses [12], play a key role in HIV-associated immunopathology and are involved in the suppression of T-cell activation, proliferation, inflammation, and cytokine production [13]. An increased proportion of Tregs has been described in HIV-infected subjects, although it is still not clear whether Tregs are detrimental or beneficial [13]. We have shown an inverse relationship between the frequency of Tregs and the qualitative and quantitative response to the hepatitis B virus (HBV) vaccine in HIV-infected subjects [14], suggesting that therapeutic strategies directed at reducing Treg levels in HIV-infected subjects before HBV vaccination are advisable.

Combination antiretroviral therapy (cART) has been reported to have an impact on Tregs, but there are contradictory results about its capacity to normalize Tregs levels [15–20], most likely because of different characterization approaches for this subset [21]. Moreover, no current information regarding the effect of new antiretroviral drugs, such as maraviroc, exists. It should be noted that, like other immune cells, Tregs express CCR5 [8], which makes them susceptible both to HIV infection and to maraviroc-mediated CCR5 blockade. Interestingly, the level of expression of this receptor is variable in different Treg subsets (eg, higher in effector Tregs than in naive Tregs) [22]. Together, these data prompted us to hypothesize that Tregs could be modulated by maraviroc, which could partially explain the maraviroc-associated immunomodulatory effects on HIV-infected subjects. Our aim in the present study was to analyze the effect of maraviroc on the Treg subset in ART-naive HIV-infected subjects starting ART.

MATERIALS AND METHODS

Patients, Samples, and Study Design

For the present study, we retrospectively selected consecutive ART-naive HIV-infected subjects that started cART between January 2010 and December 2012 at the Infectious Diseases Service of Virgen del Rocio University Hospital (Seville, Spain). We included patients who were initiating both types of cART: (1) a maraviroc-containing regimen including maraviroc (150 mg/24 hours) plus a ritonavir-boosted protease inhibitor (PI), and (2) a conventional cART regimen including a combination of 2 nucleoside analog reverse-transcriptase inhibitors and 1 nonnucleoside reverse-transcriptase inhibitor (in 10 of 12 subjects) or a PI (in 2 of 12 of subjects). Patients starting a maraviroc-containing regimen had first been tested for their sensitivity to this drug by the maraviroc clinical test, as previously described [23]. Briefly, the maraviroc clinical test consisted of an 8-day monotherapy exposure to maraviroc (300 mg every 12 hours) and was considered positive when a viral load reduction of at least 1 log₁₀ HIV RNA copies/mL was achieved at day 8 after exposure. On day 10, depending on the result of viral load test from day 8, a combination regimen including maraviroc was started in patients with a positive test result. Only asymptomatic ART-naive subjects without coinfection with hepatitis C virus (HCV) and/or HBV and with available frozen samples from baseline (day 0), day 8, and day 30 after antiretroviral exposure were included in the study (n = 21). Nine of them had started a maraviroc-containing cART regimen (the maraviroc group), and the remaining 12 had started a conventional maraviroc-sparing cART regimen (the cART group). Long-term follow-up data (from week 48) was also analyzed in patients without treatment changes during the follow-up period and with available cryopreserved samples (11 of 21 patients). All patients had an undetectable viral load at this time point.

For in vitro assays, 8 additional ART-naive patients were prospectively recruited, and blood specimens were collected and processed while fresh. Suppression assays were performed on these specimens if enough Tregs could be isolated (3 of 8 patients). All study participants gave informed consent, and the study was approved by the Committee of Ethics at the Virgen del Rocio University Hospital.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from samples and immunophenotyped using multicolor flow cytometry to characterize Tregs as CD4⁺CD25hiFoxP3⁺ T cells, as previously described [21]. Anti-FoxP3-PE (e-Bioscience, San Diego, CA), anti-IL2R1 (CD25)-FITC, and anti-CD4-ECD (Beckman-Coulter, Florida) were used. The intracellular FoxP3 staining was performed using the FoxP3 Staining Set (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Data were acquired on a Cytomics FC 500 flow cytometer (Beckman-Coulter, Florida) and were analyzed using CXP software (Beckman-Coulter, Florida). One million cells of each sample were stained, and a minimum of 200,000 events were acquired.

In Vitro Maraviroc Treatment of PBMCs

To analyze the effect of maraviroc on Tregs, PBMCs from ART-naive patients were isolated, seeded into 96-well U-bottomed plates (500,000 cells/well), and treated with different concentrations of maraviroc (UK-427857, kindly provided by Pfizer). Maraviroc was reconstituted in dimethyl sulfoxide (DMSO) and stored at 4°C until use. Maraviroc concentrations were chosen on the basis of pharmacokinetic published data [24, 25], and the final concentration of DMSO in all the wells was 0.1% for all concentrations. In preliminary time-course experiments (data not shown), we observed optimum effects after 3 days of incubation (1–5 days tested). The cells were incubated for 3 days with 1 µM, 10 µM, and 100 µM maraviroc in complete medium (Roswell Park Memorial Institute medium with 10% feta bovine serum, 1% L-glutamine, and penicillin [100 U/mL]/streptomycin [100 µg/mL]) at 37°C under 5% atmospheric CO₂. PBMCs
incubated with 0.1% DMSO in complete medium were used as controls. Viability of the cells was assessed by Trypan blue exclusion dye (≥85% at all maraviroc concentrations). After treatment of PBMCs, the Treg subset was quantified by flow cytometry for detection of CD4⁺CD25⁺FoxP3⁺ T cells, as described above.

Assessment of Suppressive Function of Tregs In Vitro
To evaluate the suppressive function of Tregs in vitro against total PBMCs, a Treg-suppression assay was performed following the manufacturer’s instructions (BD FastImmune Regulatory T Cell Function Kit). Briefly, PBMCs were isolated from cART-naive patients and incubated in X-VIVO-15 medium with 10% human AB serum in 96-well U-bottomed plates. To isolate Tregs, the Human Regulatory T cell Isolation Kit II (Miltenyi Biotech) was used. Purified autologous CD4⁺CD127⁻CD25⁺ T cells (>95% pure) were left untreated or were treated with 100 µM maraviroc for 24 hours before the assay. They were then added at a 1:1 ratio with resting autologous PBMCs. PBMCs alone were used as controls. The cells were then stimulated with anti-CD3 and anti-CD28 coated beads (Dynabeads Human T-Activator CD3/CD28, Invitrogen) at a ratio of 1:2 (beads to PBMCs) in the presence of CD154-APC reagent (supplied by the kit). After 7 hours of culture at 37°C under 5% atmospheric CO₂, the samples were stained with CD4-FITC/CD25-PE/CD3-PerCP-Cy5.5/CD69-PE-Cy7 (supplied by the kit), and the presence of early activation markers (CD154 and CD69) in PBMCs was assessed by flow cytometry.

Laboratory Measurements
Absolute numbers of CD4 T cells were determined with an Epics XL-MCL flow cytometer (Beckman-Coulter). Plasma HIV-1 RNA levels were measured by quantitative polymerase chain reaction (qPCR; Cobas Amplicrep/COBAS Taqman HIV-1 test, Roche Molecular Systems) according to the manufacturer’s instructions. The detection limit was <40 HIV RNA copies/mL. The plasma samples were tested for HBV-related markers (HBsAg, anti-HBs, and anti-HBc) and anti-HCV antibodies. A HBV enzyme-linked immunosorbent assay (ELISA) and an HCV ELISA, respectively (Siemens Healthcare Diagnosis). qPCR amplification was used for plasma HCV amplification (COBAS Amplicor, Roche Diagnosis), and the test had a detection limit of 15 IU/mL.

Statistical Analysis
Continuous variables were recorded as median values and interquartile ranges (IQRs), and categorical variables were recorded as numbers and percentages. For multiple longitudinal comparisons, the Friedman test and Bonferroni adjustment to a series of post hoc Wilcoxon matched pairs tests were applied. Comparisons between groups were made using the nonparametric Mann–Whitney U test for continuous variables and the χ² or Fisher exact test for categorical variables, as appropriate. Statistical analyses were performed using SPSS software (SPSS 18.0, Chicago, IL), and the graphics were generated with the Prism program, version 5.0 (GraphPad Software). Except for the Bonferroni correction (P < .017), P values of <.05 were considered statistically significant for all tests.

RESULTS
Baseline Characteristics of HIV-Infected Subjects
Baseline characteristics of patients included in the maraviroc and cART groups are summarized in Table 1. There were no differences between them with respect to sex, age, CD4 T-cell counts, nadir CD4 T-cell counts, viral load, or time from diagnosis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Maraviroc Group (n = 9)</th>
<th>cART Group (n = 12)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>10 (83.3)</td>
<td>8 (88.9)</td>
<td>.916</td>
</tr>
<tr>
<td>Age, y</td>
<td>40.5 (27.3–45.5)</td>
<td>32.0 (28.5–35.0)</td>
<td>.382</td>
</tr>
<tr>
<td>Time from diagnosis, wk</td>
<td>9.0 (7.0–16.0)</td>
<td>18.0 (4.50–52.5)</td>
<td>.931</td>
</tr>
<tr>
<td>CD4 T-cell count, cells/µL</td>
<td>409 (388–464)</td>
<td>360 (207–476)</td>
<td>.456</td>
</tr>
<tr>
<td>Nadir CD4 T-cell count, cells/µL</td>
<td>387 (363–468)</td>
<td>317 (139–441)</td>
<td>.169</td>
</tr>
<tr>
<td>Viral load, log RNA copies RNA/mL</td>
<td>4.8 (4.2–5.1)</td>
<td>4.8 (4.2–5.3)</td>
<td>.602</td>
</tr>
</tbody>
</table>

Data are no. (%) of participants or median value (interquartile range). Mann–Whitney U-test was used for comparison between the maraviroc and cART groups.
found when analyzing absolute Treg numbers (Supplementary Table 1). Conversely, decreases in viral load and increases in CD4 T-cell counts during treatment were similar between groups (Figure 1C–F).

Notably, graphs from both treatment groups showed very different profiles. Considering the period from day 0 to day 8, the maraviroc group showed a consistent and pronounced reduction, whereas the cART group showed a heterogeneous profile. We asked whether the absence of maraviroc could explain such a profile in the cART group. To address this issue, we analyzed the spontaneous variation of Treg frequency between baseline and day 8 in a group of 7 age- and sex-matched, cART-naive

Figure 1. Changes in regulatory T-cell (Treg) frequency, CD4 T-cell counts, and human immunodeficiency virus (HIV) load after the initiation of treatment. The start of treatment resulted in a rapid reduction in Treg cell frequency in the maraviroc group at days 8 and 30 (A), but no changes were observed in the combination antiretroviral therapy (cART) group at any time point (B). Both groups showed a similar sharp decrease in viral loads together with a similar increase in CD4 T-cell levels (C–F). Corresponding points for subjects who had increased Treg levels at day 8 have been highlighted with discontinuous lines in panels B and D. Despite some overlapping lines, especially in panel B, all patients have been represented in all panels. The Wilcoxon test was used for longitudinal comparisons.
HIV-infected patients not initiating treatment. We observed that the untreated cART-naive patients showed the same heterogeneous behavior for Tregs as the cART-treated group (Supplementary Figure 1A). These results support that a conventional cART regimen has no effect on the Treg subset, whereas maraviroc induced a regular trend to reduce the Treg frequency. Finally, to check whether HIV infection could explain the heterogeneous profile in the cART group, we analyzed the spontaneous variation of Treg frequencies between baseline and day 8 from 5 age- and sex-matched subjects, and we observed no modifications (Supplementary Figure 1B), although we cannot exclude that the absence of variability could be due to the much lower values in healthy subjects compared to HIV subjects.

**Long-term Reduction of Treg Frequencies in Maraviroc-Treated Patients**

We next assessed the long-term impact of either treatment, with or without maraviroc, on the Treg subset. A subgroup of 5 patients in the maraviroc group and 7 patients in the cART group were analyzed because they had available samples and no treatment changes during the 48 weeks of treatment. The baseline characteristics of both subgroups were similar to those of the overall study population (data not shown). All patients had undetectable viral load at this time point. Thus, a significant reduction in HIV viremia ($P < .001$ for both) and increase in CD4 T-cell counts was observed in the maraviroc and cART groups ($P = .043$ and $P = .013$, respectively). Both groups showed a reduction in Treg frequency (Figure 2A–B). However, Tregs returned to healthy levels after 48 weeks of treatment only in the maraviroc group (Figure 2C).

**Maraviroc Reduces the Treg Frequency in a Dose-Dependent Manner In Vitro**

We analyzed the effect of culturing freshly isolated PBMCs from 8 cART-naive patients with maraviroc on the frequency of Tregs. As observed in Figure 3, a significant dose-dependent reduction in the frequency of Tregs (Figure 3A) and reduced FoxP3 expression

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**Figure 2.** Regulatory T-cell (Treg) frequency after 48 weeks of successful treatment in the maraviroc (A) and combination antiretroviral therapy (cART; B) groups. The frequency of Tregs was significantly higher at baseline in both study groups than in age-matched healthy subjects (HS), but no differences were found between the groups ($P = .89$). After 48 weeks of treatment, although all subjects showed undetectable viral load and increases in CD4 T-cell counts, Treg frequencies returned to healthy levels only in the maraviroc group, in which the levels were significantly lower than in the cART group ($P = .011$; C). Continuous lines indicate Mann–Whitney $U$ tests, and dashed lines indicate Wilcoxon tests.
by these cells (Figure 3B) was observed in all tested samples, while the CD4 T-cell frequency remained invariable (Figure 3C).

Maraviroc Treatment Does Not Affect the Suppressive Function of Tregs

We then investigated whether Tregs retain their suppressive activity after being treated with maraviroc. To do this, we measured expression of the early activation markers CD69 and CD154 on CD4 T cells after PBMC stimulation in the presence of Tregs with or without previous maraviroc treatment. As shown in Figure 4, maraviroc-pretreated Tregs showed the same suppressor ability as untreated Tregs.

DISCUSSION

In this study, we show that in ART-naive HIV-infected subjects, a maraviroc-containing cART regiment mediated both short- and long-term reductions of Treg frequencies and promoted a return to healthy values after 48 weeks of treatment. Furthermore, since such reduction was observed by the maraviroc clinical test even during the initial period of monotherapy, it is reasonable to think about a maraviroc-specific effect. Accordingly, in vitro experiments showed a dose-dependent reduction of Treg frequencies among maraviroc-treated PBMCs, while the suppressive function of maraviroc-treated Tregs was not disturbed by the treatment. Our results contribute to the understanding of the immunomodulatory properties of maraviroc, and they reveal a novel effect on Tregs.

Despite some discordant results [18–20], which are likely due to different methods of phenotyping Tregs [21], ART has been shown to reduce the Treg frequency after a long-term period, although no short-term variations in this subset have been reported in the literature [15–17]. Consistently, in our study, the
control cART group (under a maraviroc-sparing cART regimen) showed a long-term soft reduction in the Treg frequency, but there was no evidence of a reduction after either the first 8 days or the first 30 days of treatment. It is noteworthy that the control cART group regimen contained 3 active drugs instead of 1. Despite this, the maraviroc-mediated reduction in Treg frequency was evident as soon as day 8 after the initiation of treatment, when it was administered as monotherapy, allowing us to conclude that this effect was exclusive to the drug. Such a specific effect could be explained by the differential expression of CCR5 by Tregs compared to other cellular subsets. In fact, Tregs have been shown to express the CCR5/CXCR4 ratio of coreceptors differently than T effector cells [26], and activated Tregs more strongly upregulate CCR5 than activated T-helper type 1 and type 2 cells [27]. Furthermore, Tregs are constituted by functionally different subsets with different pattern of expression of CCR5, which could imply different susceptibility to maraviroc. Thus, effector Tregs express the highest levels of CCR5, whereas naive Tregs are mainly CXCR4-expressing cells [22].

Importantly, this maraviroc-mediated reduction of Tregs could partially explain the recent observation of maraviroc-mediated increases in cellular and humoral immune responses against vaccination [11]. Accordingly, our previous data suggested a negative role of Tregs in the response to HBV vaccination in HIV-infected subjects [14]. Future studies will definitely explore whether such defective responses could be improved by using maraviroc as a vaccine coadjuvant or by including maraviroc in the antiretroviral regimen of HIV-infected subjects undergoing HBV immunization.

Regarding the potential mechanisms involved in the maraviroc-mediated reduction in the Treg frequency, we can only speculate at this moment. Considering our in vitro experiments, we cannot exclude a direct cellular effect on Tregs. In fact, maraviroc seems to induce downregulation of FoxP3 expression on Tregs, and we cannot rule out an increase in the frequencies of apoptosis.

Figure 4. Regulatory T-cell (Treg) suppression of the expression of early activation markers CD154 and CD69 on CD4 T cells. The representative plots correspond to 1 experiment (A), although data were collected from 3 different experiments for CD154 (B) and CD69 (C). Peripheral blood mononuclear cells from 3 combination antiretroviral therapy–naïve human immunodeficiency virus–infected subjects were stimulated with anti-CD3/anti-CD28 in the absence or presence of autologous Tregs (both nontreated or maraviroc pretreated during 24 hours). Values are calculated following the manufacturer’s instructions (relative to the control:PBMCs stimulated). Maraviroc-pretreated Tregs maintained the ability to suppress CD4 T-cell activation. Abbreviations: ST, stimulated; Treg maraviroc, maraviroc-pretreated Tregs; Treg NT, nontreated Tregs; US, unstimulated.
among these cells, but it could also act preferentially on other conventional T cells that could mediate this Treg reduction. However, because of the high viability of cultured PBMCs and the absence of fluctuation in total CD4 T cells, a cytotoxic effect of maraviroc can be ruled out. On the other hand, our in vivo data could also be explained by the blockade of CCR5-dependent Treg migration, involving primary lymphoid tissue retention of Tregs, and hence, their peripheral reduction [28]. Notably, blockade of CCR5-dependent Treg chemotaxis has also been postulated as the main mechanism of its immunomodulation in other scenarios. Thus, maraviroc has already been used as an antitumor drug in mouse models [29, 30]. For example, in cancer, where Treg recruitment is associated with tumor progression [31–33], maraviroc blocks metastasis of basal breast cancer cells by blocking such recruitment [29, 34]. Furthermore, maraviroc blocked local Treg suppression of immune responses against fungal and Leishmania infections [35, 36], and it appears to be an effective new strategy against graft-versus-host disease [37], mediated by Treg recruitment during transplantation.

Conversely, it should be noted that the in vitro suppressive capacity of Tregs remained invariable after maraviroc pretreatment. However, as a consequence of decreased Treg frequency and numbers, global immune suppression would be likely reduced, and the effector response could be enhanced [11].

This work has several limitations and opens new questions. The small sample size could have prevented us from finding a higher increase in the CD4 T-cell count in the maraviroc group, which has been described by other groups [6, 7]. On the other hand, our results cannot be extrapolated to different scenarios of HIV infection other than ART-naïve subjects, in whom the effect of maraviroc should be investigated. Also, we do not know whether this effect is reversible, and a Treg expansion could be expected after interruption of maraviroc-containing cART [38]. Additionally, the following issues need to be addressed in future studies: (1) the specific effect of maraviroc on different Treg subsets (ie, naive and effectors), (b) the maraviroc-mediated mechanisms of the reduction in Treg frequency, and (c) the in vivo effect of maraviroc treatment on Treg function.

Finally, the observed dose-dependent in vitro effect of maraviroc on Tregs, as well as the in vivo effect during monotherapy (where the dose was 4 times higher than normal: 300 mg every 12 hours vs 150 mg every 24 hours for maraviroc-containing cART [39]), are important issues, considering that HIV-infected patients receiving maraviroc could be exposed to lower drug doses than those necessary for immunological benefit. We could speculate that a greater in vivo effect could be observed when used at higher doses.

Our results shed light on the immunomodulatory properties of maraviroc and have potential clinical implications for the development of Treg-depleting immunotherapies in different scenarios, such as chronic infections, cancer, and vaccination.

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 copyrighted. 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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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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