**In vitro effects of the CCR5 inhibitor maraviroc on human T cell function**

H. Arberas¹², A. C. Guardo¹², M. E. Bargalló¹², M. J. Maleno¹², M. Calvo²³, J. L. Blanco²³, F. García²³, J. M. Gatell²³ and M. Plana¹²*

¹Retrovirology and Viral Immunopathology Laboratory, IDIBAPS, University of Barcelona, Villarroel 170, 08036 Barcelona, Spain; ²HIVACAT, Barcelona, Spain; ³Infectious Diseases Unit, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain

*Corresponding author. Retrovirology and Viral Immunopathology Laboratory, IDIBAPS, Hospital Clinic, University of Barcelona, C/Villarroel 170, 08036 Barcelona, Spain. Tel: +34-93-2275400, ext. 2884; fax: +34-93-4514438; E-mail: mplana@ub.edu

Received 27 July 2012; returned 8 September 2012; revised 3 October 2012; accepted 4 October 2012

**Background:** Several potential immunological benefits have been observed during treatment with the CC chemokine receptor 5 (CCR5) antagonist maraviroc, in addition to its antiviral effect. Our objective was to analyse the *in vitro* effects of CCR5 blockade on T lymphocyte function and homeostasis.

**Methods:** Peripheral blood mononuclear cells (PBMCs) from both HIV-negative (*n*=28) and treated HIV-positive (*n*=27) individuals were exposed *in vitro* to different concentrations of maraviroc (0.1–100 μM). Effects on T cell activation were analysed by measuring the expression of the CD69, CD38, HLA-DR and CD25 receptors as well as CCR5 density using flow cytometry. Spontaneous and chemokine-induced chemotaxis were measured by transwell migration assays, and polyclonal-induced proliferation was assessed by a lymphoproliferation assay and carboxyfluorescein succinimidyl ester staining.

**Results:** Maraviroc increases CCR5 surface expression on activated T cells, even at low doses (0.1 μM). Slight differences were detected in the frequency and mean fluorescence intensity of activation markers at high concentrations of maraviroc. Expression of CD25, CD38 and HLA-DR tended to decrease in both CD4⁺ and CD8⁺ T lymphocytes, whereas expression of CD69 tended to increase. Maraviroc clearly inhibits T cell migration induced by chemokines in a dose-dependent manner. Moreover, at 100 μM, maraviroc tends to inhibit T cell proliferation.

**Conclusions:** These data showed that *in vitro* exposure to maraviroc decreases some activation expression markers on T lymphocytes and also migration towards chemoattractants. These results support the additional immunological effects of CCR5 blockade and suggest that maraviroc might have potential capacity to inhibit HIV-associated chronic inflammation and activation, both by directly affecting T cell activation and by reducing entrapment of lymphocytes in lymph nodes.

**Keywords:** CCR5 blockade, T cell activation, immunological effects, chemotaxis

**Introduction**

Previous studies showing that CC chemokines could block virus replication served to aid the discovery of major coreceptors involved in HIV-1 entry. CC chemokine receptor 5 (CCR5) is the main coreceptor for HIV-1¹ and is relevant in the first steps of infection. An understanding of HIV-1 entry steps provided new opportunities for the development of therapeutic targets against HIV-1 infection and since then, new classes of drugs have emerged, such as CCR5 antagonists. In contrast to other antiretroviral drugs, these CCR5 antagonists target a human protein, inhibiting the replication of R5-tropic HIV variants by blocking viral entry into the host cell. Various clinical trials have demonstrated the efficacy of maraviroc in inducing HIV viral load suppression (maraviroc is the only CCR5 antagonist currently approved by the US FDA).¹²

It also has been suggested that maraviroc could induce immunological changes independent of its virological effect, and some authors have reported that maraviroc decreases immune activation³ and/or improves the CD4⁺ T cell count. On the other hand, some discordant results have also been described.⁴⁻⁶

One goal of current antiretroviral therapy, besides reducing the viral load to undetectable values, is to achieve a good...
and favour CD4+ increase cell proliferation of antigen-stimulated human T cells. CCR5 is expressed in several host defence cells, such as T cells, natural killer cells, monocytes, macrophages and dendritic cells, and belongs to the superfamily of the seven-transmembrane G-protein-coupled receptors. The expression of CCR5 plays an important role in priming the adaptive immune responses and is also involved in promoting the migration of CCR5-expressing cells to sites of infection and inflammation. It is known that CCR5 is also a coactivation molecule at the surface of CD4+ T cells and there is evidence that CCR5 is accumulated in the immunological synapse and should enhance T cell activation. CCR5 antagonist is administered. The three major CCR5 ligands [MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5)] increase cell proliferation of antigen-stimulated human T cells and favour CD4+ T cell differentiation into Th1 cells. Furthermore, it has been described that targeting CCR5 could be useful for the treatment of other autoimmune and infectious diseases in which CCR5 is accumulated in the immunological synapse and should enhance T cell activation, and which could explain the decrease of T cell activation when a CCR5 antagonist is administered. The three major CCR5 ligands [MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5)] increase cell proliferation of antigen-stimulated human T cells and favour CD4+ T cell differentiation into Th1 cells. Furthermore, it has been described that targeting CCR5 could be useful for the treatment of other autoimmune and infectious diseases in which CCR5 is accumulated in the immunological synapse and should enhance T cell activation, and which could explain the decrease of T cell activation when a CCR5 antagonist is administered.

Taking advantage of the importance that CCR5 has in several immune cells, the aim of this study was to analyse the in vitro effect of the CCR5 antagonist maraviroc on several parameters of T cell homeostasis and function. We examined the effect of CCR5 in vitro blockage on the activation, migration and proliferation of both CD4+ and CD8+ T lymphocytes. We also assessed the effect on the viability of the cells and the expression and density of the CCR5 receptor under exposure to different concentrations of maraviroc. In addition, and to avoid the influence of the known antiviral action of maraviroc, we have analysed these parameters on samples from HIV-1-infected subjects and those from healthy uninfected individuals.

**Materials and methods**

**Patients and samples**

In order to investigate the in vitro effects of maraviroc, we obtained peripheral blood mononuclear cell (PBMC) samples from Hospital Clinic of Barcelona. We included chronically HIV-1-infected patients (n=27) with current CD4+ T lymphocyte counts >400 cells/μm³ and pre-therapy viral loads >5000 copies/μL, all of them receiving antiretroviral therapy. We also included HIV-1-uninfected volunteers (n=28) to determine the effect of the use of the CCR5 antagonist without its antiviral effect. All patients and controls provided written informed consent and studies were approved by the institution’s ethics review board. PBMCs from both HIV-infected and healthy donors were isolated by Ficoll–Paque™ PLUS centrifugation gradient (GE-Healthcare Bio-Sciences AB, Uppsala, Sweden) and cultured in RPMI-1640 medium supplemented with 0.1% gentamicin and 10% fetal calf serum (R10 medium). The PBMCs were incubated with different maraviroc concentrations, ranging from 0.1 to 100 μM since blood levels range from 1 to 10 μM.

**Antibodies and reagents**

The antibodies used for the assays were fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (MAb), anti-CCR5 MAb and anti-CD14 MAb; phycoerythrin (PE)-conjugated anti-CD8 MAb, anti-CD4 MAb, anti-CXCR4 MAb, anti-CD69 MAb, anti-CD25 MAb, anti-CD38 MAb and anti-HLA-DR MAb; Peridinin-Chlorophyll Protein Complex (PerCP)-conjugated anti-CD3; allophycocyanin (APC)-conjugated anti-CD4 MAb and anti-CD8 MAb; and unconjugated CCR5 MAb. These and their respective isotype controls were purchased from Becton Dickinson (Mountain View, CA, USA). Maraviroc was kindly provided by Pfizer-ViV. Phyttohaemagglutinin (PHA) was from Murex Biotech Ltd (England, UK). OKT3 (10 ng/mL) was from Ortho Biotech Inc. (Raritan, NJ, USA). Anti-CD28 antibody (Ab) (100 μg/mL) was obtained by ammonium sulphate precipitation of culture supernatant of hybridoma 152-2E10 (IgG1), produced at our laboratory. [3H]Thymidine was supplied by Moravek Biochemicals (Brea, CA, USA).

**CCR5 density measurement**

CCR5 density on CD4+ and CD8+ T cells was quantified indirectly with QIFIKIT (Dako, Denmark), which allows calculation of the antigen quantity, expressed as the specific antibody-binding capacity (SABC). Briefly, in a 24-well flat-bottomed plate, 1.25×10⁶ cells per well were incubated with different concentrations of maraviroc. At 5, 24 and 48 h, the CCR5 receptor was marked as described in the manufacturer’s protocol with the QIFIKIT and after that cells were also incubated with the anti-CD3 PerCP, anti-CD4 PE and anti-CD8 APC. Data were expressed as SABC units.

**Determination of surface marker expression**

Briefly, 10×10⁶ of freshly isolated PBMCs were incubated with 0.5% PHA (45 μg/mL) and different dosages of maraviroc to measure the surface expression of CD69, CD25, CD38 and HLA-DR molecules. At 5, 24 and 48 h, four aliquots of cells from each condition were taken and incubated with anti-CD3 PerCP, anti-CD4 FITC, anti-CD8 APC and anti-CD69 PE, anti-CD25 PE, anti-CD38 PE or anti-HLA-DR PE. Percentage of expression and mean fluorescence intensity (MFI) of different surface markers were reported. In addition, to measure the expression of CCR5 and CXCR4, 1.25×10⁶ of freshly isolated PBMCs were incubated in a 24-well flat-bottomed plate with or without 10 ng/mL OKT3 and 100 μg/mL anti-CD28 and different concentrations of maraviroc, in a final volume of 500 μL. At 5, 24 and 48 h, two aliquots were taken and incubated with anti-CD3 PerCP, anti-CCR5 FITC, anti-CXCR4 PE and anti-CD4 APC or anti-CD8 APC. Samples were fixed and acquired. The expression of CCR5 and CXCR4 was determined and expressed as the mean of the percentage of total cell counts.

**Chemokinesis assay**

The chemokinesis of PBMCs in response to recombinant human CCL3, CCL4 and CCL5 (R&D Systems, Inc., Minneapolis, MN, USA) was analysed using 24-well, 3 μm pore size, polycarbonate membrane Transwell plates (Corning via Citek). R10 culture medium, supplemented or not with a cocktail of recombinant human CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES, was placed in the lower chamber of the plate and 0.5×10⁶ cells were added to the upper chamber with different dosages of maraviroc and left to migrate for 24 h at 37°C in a 5% CO2 humidified atmosphere. The samples of the lower chamber were pelleted down, resuspended in 100 μL of PBS–BSA and incubated with anti-CD3 PerCP MAb, anti-CD4 PE MAb and anti-CD8 APC MAb. Samples were placed in BD Trucount™ tubes (BD Biosciences, San Jose, CA, USA). Absolute counts of CD4+ and CD8+ T cells using Trucount tubes were calculated using the formula [(no. of events in quadrant containing cell population)/no. of events in absolute-count bead
Effects of maraviroc on T cell homeostasis

Viability test
Viability was measured using a far-red fluorescent reactive dye (LIVE/DEAD Fixable Dead Cell Stain Kits; Invitrogen, Eugene, OR, USA). In a 24-well flat-bottomed plate (Corning, NY, USA), 3.75 x 10^5 cells were incubated in culture medium with different concentrations of maraviroc. At 5, 24 and 48 h, 125 μL of the cultures was stained with the far-red fluorescent dye according to the manufacturer’s protocol. These cells were also stained with anti-CD3 PerCP MAb, anti-CD4 FITC MAb and anti-CD8 PE MAb surface markers.

Proliferation assays
PBMCs were incubated with different dosages of maraviroc with polyclonal-inducing stimuli (0.5% PHA (45 μg/mL) or OKT3 (10 or 1 ng/mL) in the presence of anti-CD28 (100 μg/mL)). Proliferation was determined by both tritiated thymidine uptake proliferation assays and carboxyfluorescein succinimidyl ester (CFSE) staining and further flow cytometry analysis. [3H]Thymidine uptake assays were performed essentially as previously described elsewhere. PBMC proliferation was reported as the stimulation index, determined by dividing the mean counts per minute for the stimulated wells by the mean for the unstimulated control wells without maraviroc. Proliferation of T cells was also assessed by CFSE (Invitrogen, Eugene, OR, USA) staining. A 10 mM stock solution of CFSE was prepared by dissolving in DMSO and stored at −80°C. PBMCs were labelled with CFSE as is described in the manufacturer’s protocol in a final concentration of 2 μM. After washing three times, cells were resuspended in R10 medium for culture. These cells were incubated in 96-well round-bottomed plates in the absence or presence of 0.5% PHA (45 μg/mL) or OKT3 (10 or 1 ng/mL) with anti-CD28 (100 μg/mL). Cells were cultured in the presence of different concentrations of maraviroc, ranging from 0.1 to 100 μM. After 72 h, two aliquots of cells from each condition were stained with CD3-PerCP MAb and CD4-APC MAb or CD8-APC MAb. The proliferative capacity for each condition was reported as the percentage of divided cells corrected with the basal proliferation that occurred without stimulation and without maraviroc.

In all cases, samples were acquired using a FACSCalibur® flow cytometer (Becton Dickinson, San José, CA, USA) and data were analysed with FlowJo software version 7.2.5 (TreeStar, Ashland, OR, USA). CD4+ and CD8+ T lymphocytes were gated on the basis of forward- and side-scatter and their CD3+CD4+ or CD3+CD8+ phenotype, respectively.

Statistical analysis
Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). The normality of the distribution was tested using the Kolmogorov–Smirnov (KS) test. Differences between two groups (negative control versus different dosages of maraviroc) were assessed with a paired t-test for normally distributed data and with the Wilcoxon matched-pairs test for non-normally distributed data.

Results
Maraviroc modulates CCR5 but not CXCR4 expression on T cell surface
HIV-1 chemokine receptor CCR5 expression was measured on unstimulated and stimulated CD4+ and CD8+ T cells in the presence or not of different doses of maraviroc. Resting cells of HIV-uninfected individuals showed a slight tendency to down-regulate CCR5 expression over time when cultured without maraviroc (Figure 1a, white bar) and exposure to CCR5 antagonist had no effect on this expression. However, in HIV-infected individuals, CCR5 expression did not decrease in both resting CD4+ and CD8+ T cells. In fact, in the latter group, there was a CCR5 increment that was slightly higher than in cells treated with maraviroc (Figure 1c).

Treatment of cells with anti-CD28 promoted internalization of CCR5, decreasing the percentage of CCR5 expression (Figure 1b (HIV uninfected) and d (HIV infected)). Exposure to maraviroc positively modulated CCR5 expression on both the CD4+ and CD8+ T cell surface after being stimulated with anti-CD28 plus anti-CD3 in both HIV-uninfected and HIV-infected individuals (Figure 1b and d).

Additionally, the density of the receptor on CD4+ and CD8+ T lymphocytes was also measured in non-stimulated PBMCs. CCR5 density tended to rise in both T cell subsets of HIV-negative and HIV-positive PBMC samples when these were cultured in the presence of maraviroc (data not shown). We also assessed the expression of the receptor CXCR4 in the PBMCs and no differences were found either in HIV-infected or -uninfected CD4+ or CD8+ cells (data not shown).

High concentrations of maraviroc modified expression of T cell activation markers
The effects on T cell activation after in vitro exposure to different dosages of maraviroc (0, 0.1, 1, 10 and 100 μM) were analysed by measuring the frequency and MFI of CD69, CD38, HLA-DR and CD25 activation markers on CD4+ T lymphocytes after PHA stimulation of PBMC samples from both HIV-infected and -uninfected individuals. Data were compared using the Wilcoxon matched-pairs test for non-parametric data and no significant differences were found. Despite this, some activation markers modified their expression after 48 h of in vitro maraviroc exposure. A decrease in the CD25 receptor, in both frequency and MFI, was observed, mainly at high doses of maraviroc, on both CD4+ (Figure 2a and b, respectively) and CD8+ (data not shown) T lymphocytes from HIV-positive and -negative subjects. The frequency and MFI of the CD38 marker tended to decrease after 48 h of maraviroc exposure in HIV-negative CD4+ and CD8+ T lymphocytes (Figure 3a), but not in T cells from HIV-positive individuals (Figure 3b and Table 1). This different behaviour between infected and uninfected individuals could originate from the fact that CD38 levels have been traditionally associated with viral replication and their presence is substantially up-regulated in HIV-infected individuals. In turn, CD69, an early activation marker, increased its frequency and MFI on CD8+ T lymphocytes (Table 1), but on CD4+ T lymphocytes this increment was more notably observed in HIV-uninfected individuals (Table 1). In HIV-uninfected individuals there was a slight decrease of HLA-DR expression on CD4+ T lymphocytes, but no differences in MFI were found (Table 1). This decrement was more pronounced in CD8+ T lymphocytes than in CD4+ (Table 1). In fact, in HIV-infected patients, we observed preserved frequency and MFI of the HLA-DR marker on CD4+
Figure 1. CCR5 expression after in vitro exposure to different doses of maraviroc (MVC) in CD4+ T cells. Expression of CCR5 after 5, 24 and 48 h of culture without stimulation (a and c) or in the presence of anti-CD3 plus anti-CD28 (b and d) is represented in HIV-uninfected (n=10) (a and b) and HIV-infected (n=10) individuals (c and d). Dashed lines indicate CCR5 expression at baseline before culture was initiated. Values are expressed as the mean±SEM of CCR5 percentage. Asterisks show different levels of significance (*P<0.05 significant, **P<0.01 highly significant and ***P<0.001 very highly significant).

Figure 2. Effect of exposure to maraviroc (MVC) on expression of the CD25 receptor. CD25 activation marker frequency (a) and MFI (b) on CD4+ T lymphocytes from HIV-negative (n=5) and HIV-positive (n=5) individuals after 48 h of maraviroc in vitro exposure. A reduction in the frequency and MFI of the CD25 receptor on CD4+ cells from HIV-uninfected and HIV-infected individuals (a) was detected at high doses of maraviroc. This decrease was more appreciable in CD4+ T lymphocytes of HIV-infected individuals at 100 µM (P=0.0625 compared with the control group without maraviroc). The horizontal bar shows the median, the box spans the 25th to 75th percentiles, and the whiskers show the minimum and maximum values.
T lymphocytes during 48 h culture with different dosages of maraviroc (Table 1).

**Maraviroc inhibits T lymphocyte chemotaxis in a dose-dependent manner**

The chemotactic activity of CD4+ and CD8+ T lymphocytes in response to medium alone or a cocktail of recombinant human CCL3, CCL4 and CCL5 was analysed after culture in the absence or presence of different concentrations of maraviroc. We observed a significant reduction of T cell migration towards those chemoattractants from a concentration of 1 μM in both HIV-infected and -uninfected individuals (P<0.05) in a dose-dependent manner (Figure 4). On the other hand, spontaneous migration of T cells was, in general, not affected after exposure to different concentrations of maraviroc (0.1 and 1 μM), CD38 expression is maintained as on cells cultivated without maraviroc (continuous line), whereas treatment in vitro of cells with higher concentrations (10 and 100 μM) provokes a decrease in the intensity of the CD38 marker (dotted and thick dashed lines, respectively).

**High concentrations of maraviroc affect T cell proliferation but not their viability**

PBMCs were incubated with different dosages of maraviroc and stimulated with different polyclonal stimuli. Using the [3H]thymidine uptake assay, we observed a reduction of T cell proliferation induced by all the stimuli tested only at the highest dose (100 μM; data not shown) in both HIV-infected and -uninfected individuals in a dose-dependent manner.
Table 1. CD69, HLA-DR and CD38 marker frequency and MFI in CD4+ and CD8+ T lymphocytes of HIV-negative and HIV-positive individuals

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<td>0 µM</td>
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<td><strong>CD4+</strong></td>
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<tr>
<td>CD69 frequency (%)</td>
<td>73.01 (71.58–79.32)</td>
<td>73.47 (71.8–76.81)</td>
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<tr>
<td>CD69 (MFI)</td>
<td>159.7 (133–264.2)</td>
<td>156.5 (147.6–268.4)</td>
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<td>HLA-DR frequency (%)</td>
<td>53.24 (40.35–60.88)</td>
<td>53.81 (39.26–56.77)</td>
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<td>HLA-DR (MFI)</td>
<td>183.2 (178.2–237.2)</td>
<td>183.5 (169.1–219.7)</td>
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<td>CD38 frequency (%)</td>
<td>89.33 (80.69–91.72)</td>
<td>89.04 (79.46–90.96)</td>
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<td>CD38 (MFI)</td>
<td>789.8 (409.4–1150)</td>
<td>635.0 (441.1–1072)</td>
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<td><strong>CD8+</strong></td>
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<tr>
<td>CD69 frequency (%)</td>
<td>70.21 (67.34–77.97)</td>
<td>74.29 (66.2–76.12)</td>
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<tr>
<td>CD69 (MFI)</td>
<td>130.3 (90.17–142.1)</td>
<td>144.3 (96.56–148.9)</td>
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<td>HLA-DR frequency (%)</td>
<td>62.47 (54.13–70.93)</td>
<td>62.23 (52.47–67.64)</td>
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<td>HLA-DR (MFI)</td>
<td>180.3 (162–242.8)</td>
<td>165.9 (155.4–226.8)</td>
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<td>CD38 frequency (%)</td>
<td>92.55 (84.61–96.67)</td>
<td>94.26 (82.12–96.06)</td>
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<td>CD38 (MFI)</td>
<td>954.6 (409.1–1115)</td>
<td>792.8 (414.9–1077)</td>
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<tr>
<td><strong>CD4+</strong></td>
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<tr>
<td>CD69 frequency (%)</td>
<td>49.66 (47.25–63.59)</td>
<td>49.76 (45.78–58.26)</td>
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<tr>
<td>CD69 (MFI)</td>
<td>145.5 (83.85–208)</td>
<td>133.4 (82.86–224.4)</td>
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<tr>
<td>HLA-DR frequency (%)</td>
<td>57.03 (52.09–64.87)</td>
<td>54.86 (49.83–62.42)</td>
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<td>HLA-DR (MFI)</td>
<td>308 (233.5–450.1)</td>
<td>314.7 (234.1–440.2)</td>
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<td>CD38 frequency (%)</td>
<td>70.01 (59.56–75.54)</td>
<td>68.92 (58.88–75.63)</td>
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<td>CD38 (MFI)</td>
<td>390.8 (367.8–741.7)</td>
<td>401.6 (365.4–727.3)</td>
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<tr>
<td><strong>CD8+</strong></td>
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<tr>
<td>CD69 frequency (%)</td>
<td>55.15 (43.97–64.45)</td>
<td>47.21 (43.25–58.42)</td>
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<tr>
<td>CD69 (MFI)</td>
<td>104.8 (70.19–149.2)</td>
<td>93.04 (68.49–161)</td>
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<td>HLA-DR frequency (%)</td>
<td>68.02 (54.28–73.32)</td>
<td>67.57 (56.66–70.09)</td>
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<td>HLA-DR (MFI)</td>
<td>468.5 (273.5–875)</td>
<td>510.7 (263.1–893.9)</td>
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<tr>
<td>CD38 frequency (%)</td>
<td>79.81 (74.82–81.73)</td>
<td>78.6 (73.9–79.7)</td>
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<tr>
<td>CD38 (MFI)</td>
<td>562.2 (497.7–752.4)</td>
<td>535.3 (458.9–735.4)</td>
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Mean and range (25%–75%) are indicated.
individuals. Further experiments using CFSE staining in the same culture conditions indicated that this tendency to inhibition occurred in both CD4+ (Figure 5) and CD8+ (data not shown) T lymphocytes of both groups when cells were stimulated with anti-CD3 and anti-CD28, as well as with PHA, and also at the highest concentration of maraviroc (Figure 5a and b).
The effect of maraviroc on cell viability was assessed using a LIVE/DEAD Fixable Dead Cell Stain Kit, which provides the percentage of live and dead cells. The reactive dye permeates membranes of necrotic cells, resulting in intense fluorescent staining, and also reacts with the surface of viable cells, resulting in slight fluorescence. Results showed that maraviroc did not alter the cell viability unless high concentrations of the drug (100 µM) were used. In the latter case, we observed a raised mortality tendency (up to 15%) in CD4+ and CD8+ T cells (data not shown). These undesirable effects were only observed when using higher concentrations (100 µM) than normal maraviroc blood levels (1–10 µM).13

Discussion

According to several lines of evidence, the interaction of CCR5 with its ligands modulates T cell response7,8,10 and the blockade of this receptor by treatment with maraviroc gives potential immunological benefits in addition to its virological effect.14,15 In the present study, we have investigated the possible modulatory effects of this drug on T cell homeostasis in vitro, and the results we obtained support the hypothesis of an additional immunological advantage on human T cell function by a mechanism independent of its antiviral activity.

Besides evidence indicating that CCR5 is involved in modulating T cell responses, other studies have described a decrease in immune activation and inflammation markers.3 Here, we report that high concentrations of maraviroc tended to diminish CD38, CD25 and HLA-DR activation markers after 48 h of PHA treatment. This reduction was not statistically significant, probably due to the small sample size. Although PHA is a commonly used mitogen to stimulate T cells in vitro, further experiments should be done to determine whether we can extrapolate what occurred under in vitro PHA stimulation to in vivo T cell activation. Previous experiments using stimulated anti-CD3 and anti-CD28 human T lymphocytes showed no reduction in the expression of activation markers under maraviroc treatment. Although a significant reduction in CD25 expression was observed when CCR5 was blocked using an anti-CCR5-neutralizing Ab,17 we hypothesize that treatment with maraviroc partially impaired T cell activation. This could explain why CD69 expression levels (an early activation marker) increased after 48 h of culture whereas levels of the other markers (CD38, CD25 and HLA-DR) remained decreased. In fact, activated T lymphocytes, identified by expression of CD3818 alone or in combination with HLA-DR,19 are strongly implicated in the pathogenesis of HIV-1 infection. Furthermore, the percentages of activated lymphocytes in peripheral blood are increased during HIV-1 infection and correlated with the plasma HIV-1 RNA concentration.20 The lack of viral replication, either ongoing or subjacent, could also be the reason why maraviroc would reduce CD38 levels on T cells from healthy individuals but not in those from HIV-infected subjects (Figure 3). Additionally, our results established a possible link between activation marker reduction (especially CD25) and the modest response obtained against mitogens after exposure to maraviroc. This relationship could be explained by an imbalance in the interleukin 2 (IL-2)-dependent autocrine mechanism, which is required for proper T cell population expansion.21 However, to confirm this hypothesis, IL-2 levels should also be determined in the presence of maraviroc in vitro. All these data, while not decisive, provide an indication that CD4+ T cell restoration, besides of cellular redistribution, could also be reached by attenuating immune activation.

Stimulation of cells with anti-CD28 down-regulates CCR5 expression.22 We report here that during maraviroc treatment in vitro, this reduction caused by anti-CD28 on CD4+ and CD8+ T cells did not take place. This could be explained by the idea that maraviroc inhibits the internalization of the receptor caused by its natural ligands, as previously described for other CCR5 antagonists.23 Blocking interaction between CCR5 and its ligands should increase their presence in plasma and tissues, that also can bind other chemokine receptors24 producing an additional effect on the immune response. We also showed that HIV-negative T cells cultured with medium alone tended to decrease their CCR5 expression compared with cells cultured in the presence of maraviroc. On the other hand, T lymphocytes from HIV-infected individuals showed increased CCR5 expression whereas those from HIV-uninfected individuals did not. CXCR4 expression did not change after maraviroc exposure. It is known that culture conditions of PBMCs can up-regulate CXCR4 expression to >90%. Due to this effect, it could be difficult to find differences using such an approach.

We also provided data indicating that maraviroc diminished the in vitro chemotactic activity of T lymphocytes towards a mixture of chemokines in a dose-dependent fashion. These data support what has been described previously for other immune cells.25 The increase in the CD4+ T cell number associated with the use of the CCR5 antagonist could also be a consequence of blocking the trafficking of T cells between circulation, lymph nodes and peripheral tissues. On the other hand, this could explain why deficient CCR5 expression is associated with lower susceptibility to inflammatory disorders in which T lymphocyte recruitment could be involved.26 In this sense, both the toxicity of antiretroviral therapy and higher levels of T cell activation have been proposed as risk factors of atherosclerosis in HIV-infected individuals, so treatment with maraviroc could prevent higher levels of T cell activation, on the one hand, and migration and infiltration of activated cells into atherosclerotic lesions, on the other hand. Rossi et al.25 previously described that maraviroc blocked the migration of macrophages and dendritic cells, and proposed that blocking the accumulation of these populations in the atheroma could down-regulate T cell recruitment and activation, decreasing the release of inflammatory mediators involved in the development and progression of HIV-associated atherosclerosis. Although we have not yet evaluated the levels of inflammatory mediators in the culture supernatants from the in vitro studies presented herein, our data suggest that treatment with maraviroc could also diminish atheroma formation by decreasing the activation and recruitment of T lymphocytes directly. It has also been observed that in homozygous CCR5-Δ32 patients, renal transplant survival was increased,27 and it has been proposed that CCR5 should have a role in T cell recruitment in renal allografts during rejection episodes.28 We support this finding, since we observed that T cell recruitment is decreased by CCR5 blockade. Moreover, it has been described that targeting CCR5 could be useful for the treatment of other autoimmune and infectious diseases in which CCR5 has been reported to directly regulate T cell function. Among the pathologies where CCR5 seems to play a role are...
Effects of maraviroc on T cell homeostasis

multiple sclerosis, rheumatoid arthritis, type 1 diabetes and liver diseases, although the benefits after the use of CCR5 antagonists remain controversial.\textsuperscript{11,29}

The immunological effect of maraviroc is still elusive. Some studies showed a higher increase in the CD4+ T cell count that others did not achieve.\textsuperscript{4,6,30} This finding has been attributed to a mechanism independent of the antiviral effect of maraviroc. Our \textit{in vitro} results throw light on the existence of a relevant immunological role for maraviroc treatment that is absolutely independent of virus presence. The results obtained regarding T cell activation and chemotaxis in T cells from healthy individuals confirm this point of view. Nevertheless, our study has a number of drawbacks. First, the sample size of the experiments is small and should be increased to confirm our findings. Additionally, these effects in combination with the antiviral activity\textsuperscript{5} could be responsible for synergies that need to be better characterized. These results are submitted in order to provide an overview of the action of maraviroc on T lymphocytes. Nevertheless, further experiments should be done to clarify the effects on the immune system in different groups of HIV-infected patients, such as CD4 non-responders, and to know more accurately the mechanisms underlying the potential immunological effects of CCR5 blockade in the context of treatment for both HIV infection and other diseases.

Acknowledgements

We are grateful to Pfizer-ViiV for kindly providing us with maraviroc for our experiments. We especially thank Maite Garcia and Ana M. Garcia for their technical assistance. This study was presented in part at the 16th IAS Conference on HIV Pathogenesis, Treatment and Prevention (IAS 2011, Rome, Italy, 17–20 July 2011; Abstract TUPE060).

Funding

This study was partially supported by grants from Fondo de Investigación Sanitaria (FIS PI040503 and FIS PI070291 to M. P.) and Red Temática Cooperativa de Grupos de Investigación en Sida (RIS). Montserrat Plana is a researcher from the Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), and is supported by the ISCIII (Instituto de Salud Carlos III) and the Health Department of the Catalan Government (Generalitat de Catalunya).

Transparency declarations

None to declare.

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