

Abstract #F12c

Poster 398-T

**Antagonism of the CCR5 Receptor by SCH-C Leads
to Elevated β -Chemokine Levels and Receptor Expression
in Chronically Treated PBMC Cultures**

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Abstract

Background: SCH-C, a small molecule antagonist of the CCR5 receptor, has demonstrated potent *in vitro* antiviral activity against R5 viral isolates and is currently being evaluated in clinical trials. In this study we investigate the potential effects of CCR5 blockade on chemokine production and receptor expression *in vitro*.

Methods: Human PBMCs were cultured in the presence or absence of SCH-C or a CCR5 monoclonal antibody, 2D7, and supernatants tested for β -chemokine levels by ELISA and cells for CCR5 and CXCR4 expression by FACS. Quantitative RT-PCR analysis was used to quantitate β -chemokine and CCR5 mRNA levels in the cultures. Susceptibility studies were done by preincubating PBMCs with compound prior to infection with serially diluted R5, X4 or R5/X4 viral isolates and p24 production measured on day 4-6.

Results: Our results showed that cultures treated with SCH-C or the CCR5 antibody contained significantly higher levels of MIP-1 α , MIP-1 β and RANTES compared with untreated cultures. Elevated chemokine levels were sustained over the course of the cultures (15-20 days). Analysis of CCR5 receptor expression showed a significant increase in CCR5 staining on cells treated with SCH-C while no effect was seen on CXCR4 expression. Quantitative RT-PCR analysis of β -chemokine and CCR5 mRNA levels revealed no significant difference between the control and treated groups for any of the genes tested. This result suggests that the higher chemokine levels in the cultures resulted from inhibition of ligand uptake by the CCR5 receptor and not from upregulation of chemokine gene expression. Similarly, the increase in CCR5 surface expression observed may be due to inhibition of receptor recycling in the absence of ligand induced signaling. Since CCR5 receptor density can influence viral infectivity, we also tested the susceptibility of SCH-C treated cells to infection with R5, X4 and R5/X4 viruses. As expected, the CCR5 antagonists did not alter the susceptibility of cells to X4 or R5/X4 virus infection or influence the level of viral replication in treated cells. Similarly, R5 virus infection was not significantly changed by the pretreatment of cells with SCH-C.

Conclusions: CCR5 antagonists can effectively increase β -chemokine levels in long term PBMC cultures, likely due to inhibition of ligand uptake by the receptor. In addition CCR5 receptor expression is increased by SCH-C treatment, presumably resulting from inhibition of chemokine-induced receptor cycling. Additional data from clinical trials will be required to assess the potential of SCH-C to increase β -chemokines *in vivo* and whether this effect will provide added antiviral benefit.

INTRODUCTION

SCH-C is a novel small molecule antagonist of the CCR5 receptor. *In vitro* and *in vivo* this compound has demonstrated potent antiviral activity against HIV-1 infection (PNAS 2001 98:12718). SCH-C differs from other antiretroviral agents in that it targets a cellular receptor utilized by HIV-1 and not a viral protein like reverse transcriptase and protease inhibitors. Therefore, it is important to consider the potential biological effects mediated by its antagonist activity on CCR5 expressing cells. In this study we evaluated the effect of SCH-C on β -Chemokine and CCR5 mRNA and protein expression in PBMC cultures. In addition we tested whether long-term treatment with SCH-C could affect the susceptibility of cells to infection by R5, R5/X4 and X4 viruses. Our results indicate that SCH-C increases both CCR5 and chemokine expression in PBMC cultures and that this effect is mediated by blocking receptor down-regulation, not by upregulating gene expression.

Materials and Methods

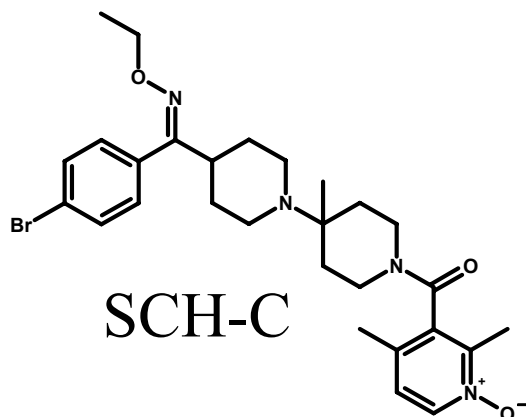
Chemokine Measurement: MIP-1 α , MIP-1 β , and RANTES concentrations were quantitated in culture supernatants by capture ELISA (R & D Systems).

FACS Analysis: PBMCs or BAF550 cells were incubated with a cocktail of 2 CCR5 antibodies, 5 μ g/ml each of 2D7 (Pharmingen) and MAB 182 (R&D Systems) or isotype controls for 30 min at 4°C. Cells were then washed and incubated with a biotinylated rabbit anti-mouse monoclonal followed by detection with a streptavidin-RPE conjugate. Fluorescence was detected using a Becton-Dickinson FACS Caliber flow cytometer.

RNA Quantitation: Total cellular RNA was isolated from PBMC cultures treated with or without SCH-C using the RNA Easy Kit from Qiagen. mRNA was measured using a quantitative RT-PCR Taqman assay kit (PE Biosystems). GAPDH was used as an internal control for input RNA.

Infection Assays: For chemokine combination studies, PHA stimulated PBMCs were pretreated with serially diluted SCH-C combined with different concentrations of RANTES or MIP-1 α prior infection with HIV-1 ADA for 4 hrs at 37°C. Replication was measured by p24 antigen on day 4 after infection. IC50 values were calculated using PRISM software and isobologram analysis performed by G. Hajian at SPRI. For susceptibility studies, unstimulated PBMCs were treated for 7 days with compound, washed and infected 24 hrs later with serially diluted R5 (JV1083), R5/X4 (P-17) and X4 (HC-4) viruses. Viral replication was determined as above.

Properties of the CCR5 Antagonist SCH-C



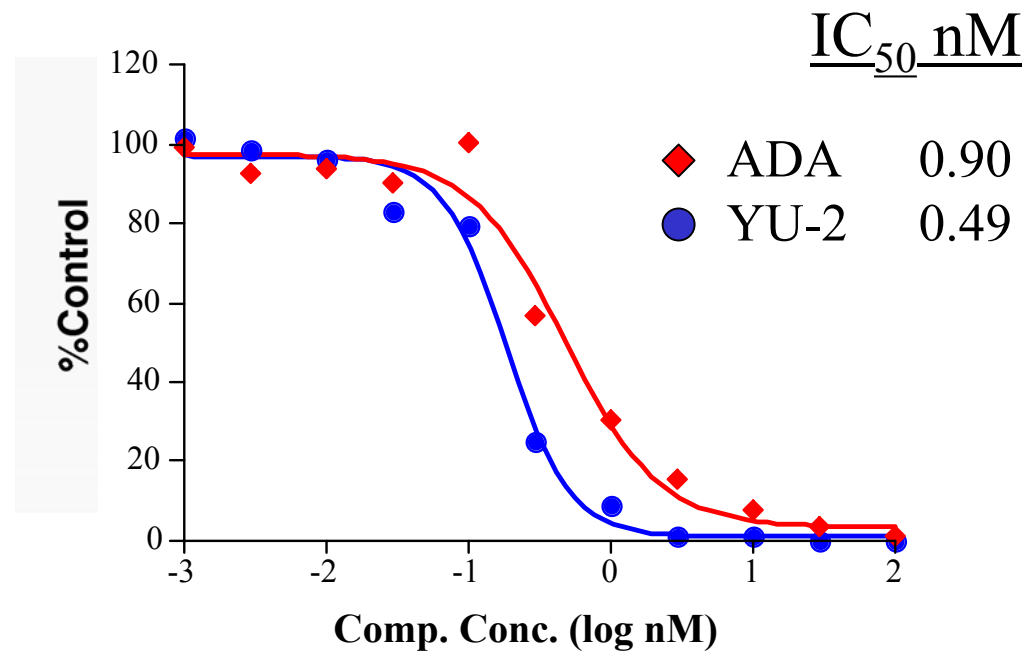
MW - 557.5

CCR5 Ki - 2.9 nM

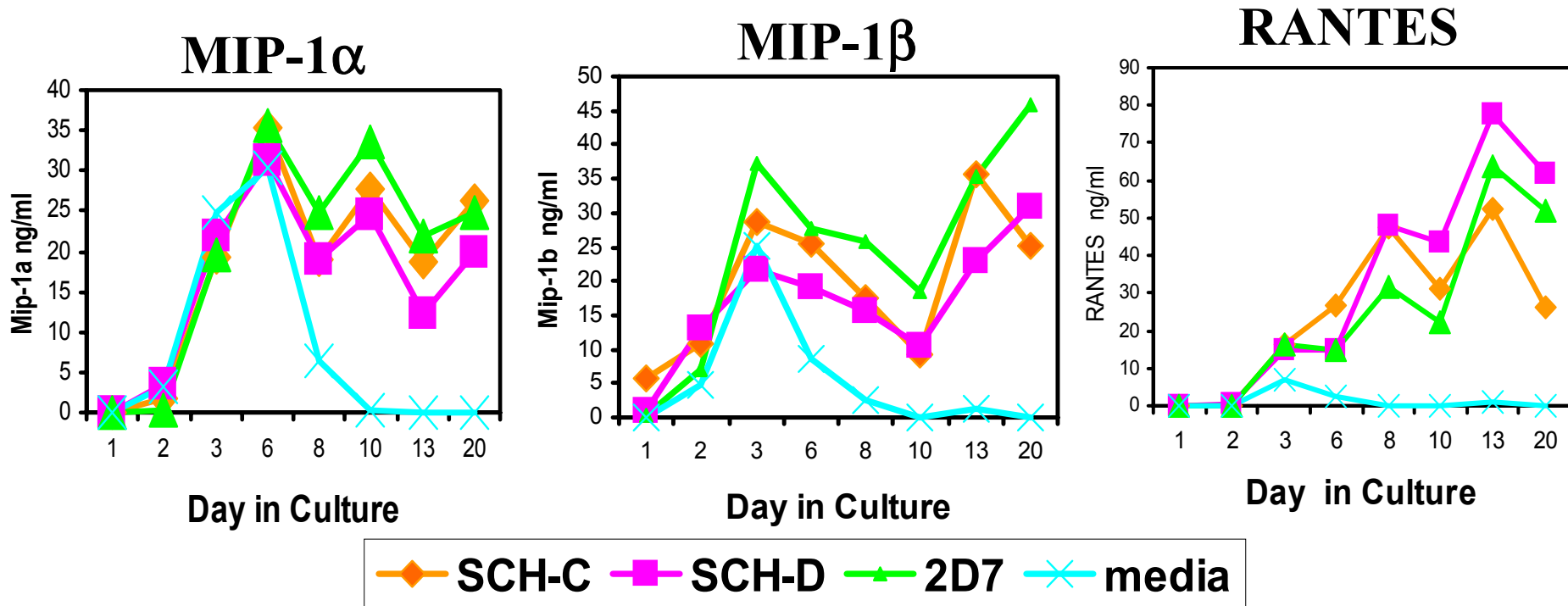
Bioavailability - >50%

AUC(0-24hr)(h.μM) - 6.9

Antiviral Activity

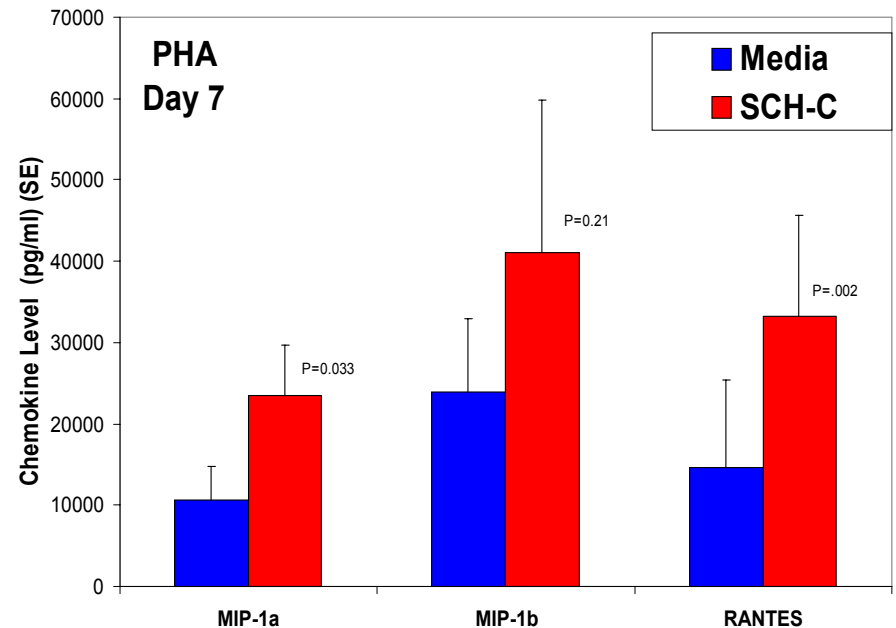
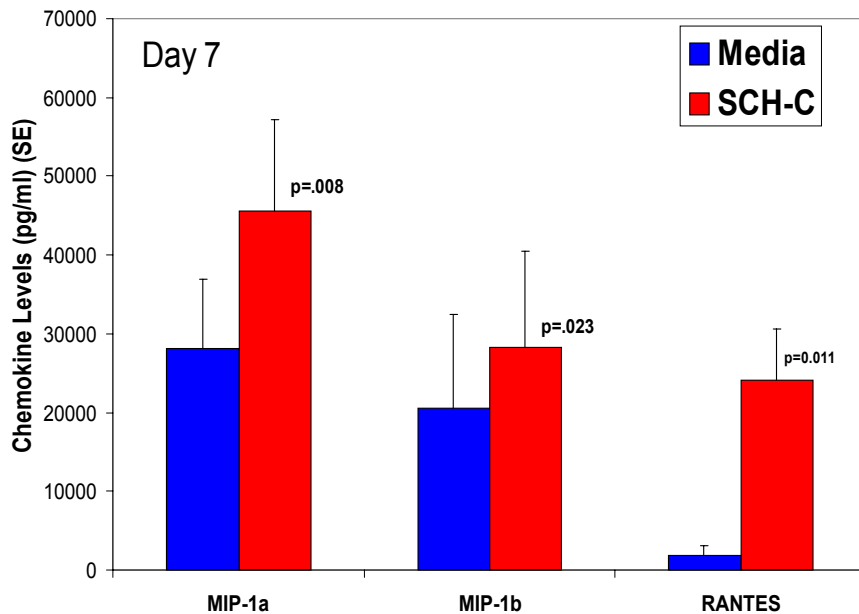


Chemokine Production by PBMCs is Increased by CCR5 Antagonists



PBMCs were cultured in the presence or absence of SCH-C (500 nM), SCH-D (500 nM) or the monoclonal antibody 2D7 and chemokine levels were measured over time by ELISA. Cultures treated with CCR5 antagonists were similar to the untreated controls during the first few days of culture, however, after 3-6 days chemokine concentrations decreased in the untreated cultures while treated cultures maintained relatively high levels of all three chemokines.

SCH-C Treatment Increases β -Chemokine Levels in PBMC Cultures



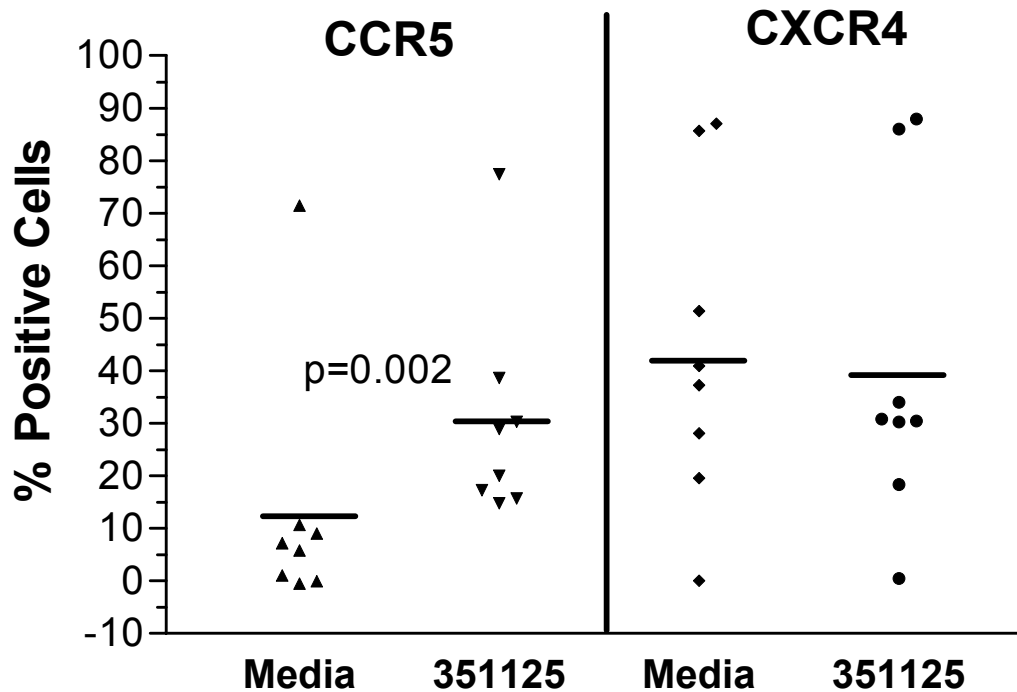
PBMCs from 6 donors were incubated in the presence or absence of SCH-C (500 nM) and chemokine levels determined by ELISA. Both unstimulated and PHA blasted cultures treated with SCH-C showed higher levels of chemokines on day 7 of culture compared with untreated cells.

Combination of SCH-C and β -Chemokines Show Additive Antiviral Activity

+MIP 1α (ng/ml)	IC 50 (nM)	+RANTES (ng/ml)	IC 50 (nM)
0	1.5	0	1.2
5	0.5	2	0.2
15	0.3	6	0.01

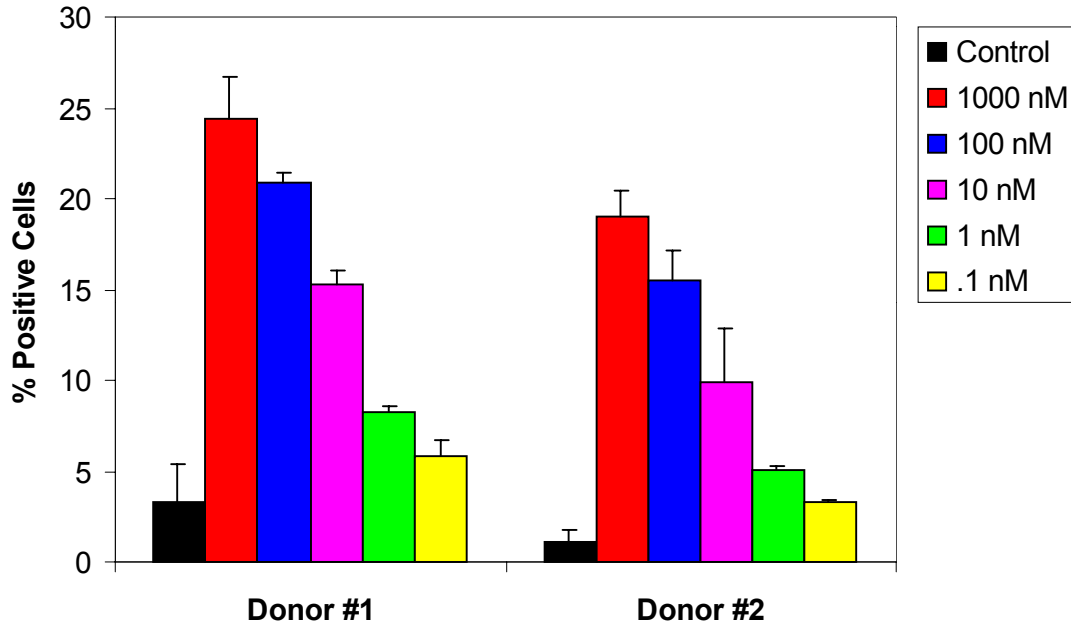
PBMC cultures were pretreated with increasing concentrations of SCH-C in the presence or absence of different concentrations of RANTES or MIP-1 α . Cells were infected with HIV-1 (ADA) for 4 days and p24 antigen measured by ELISA. IC₅₀ values were calculated using PRISM software and isobologram analysis was performed by G. Hajian (data not shown). Both RANTES and MIP-1 α showed additive antiviral activity when used in combination with SCH-C.

SCH 351125 Increases CCR5 but not CXCR4 Expression in Long-Term PBMC Cultures



CCR5 and CXCR4 expression was measured by FACS staining on PBMCs from 8 individual donors cultured between 6-20 days with or without SCH-C. The horizontal bars represent the mean % positive cells from all 8 donors. The results clearly show that SCH-C increases expression of CCR5 on PBMCs relative to untreated cultures. In contrast CXCR4 expression was not affected.

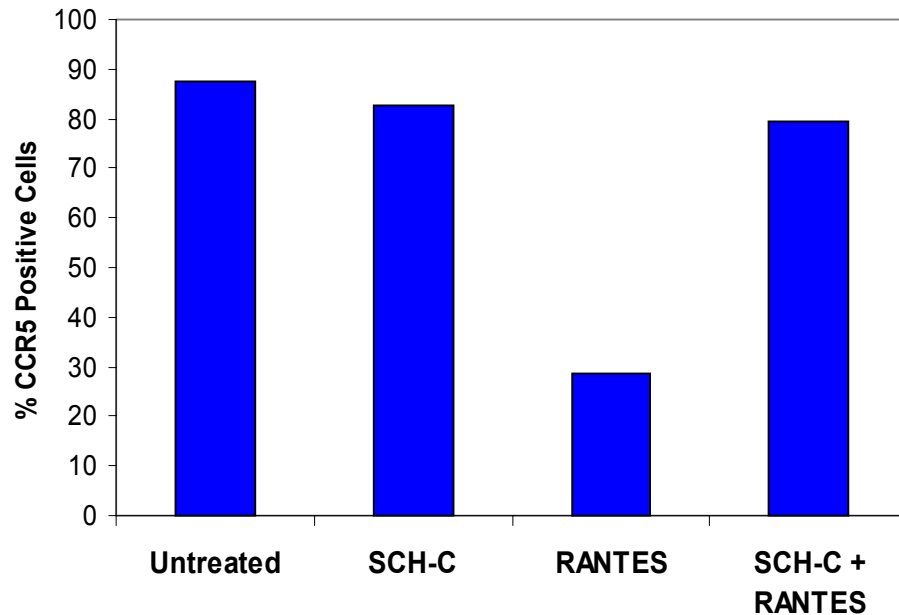
CCR5 Up-Regulation by SCH-C is Dose Dependent



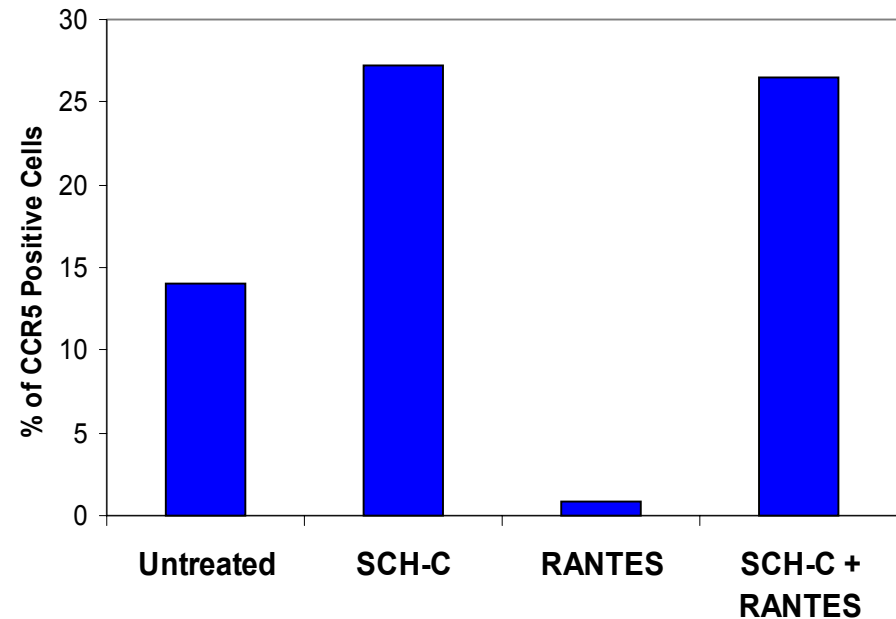
PBMCs from 2 donors were incubated for 8 days with increasing concentrations of SCH-C and CCR5 expression measured by FACS analysis. SCH-C caused a dose dependent increase in CCR5 expression on cells from both donors.

SCH-C Inhibits RANTES-Mediated CCR5 Down-Regulation

BAF 550 Cells

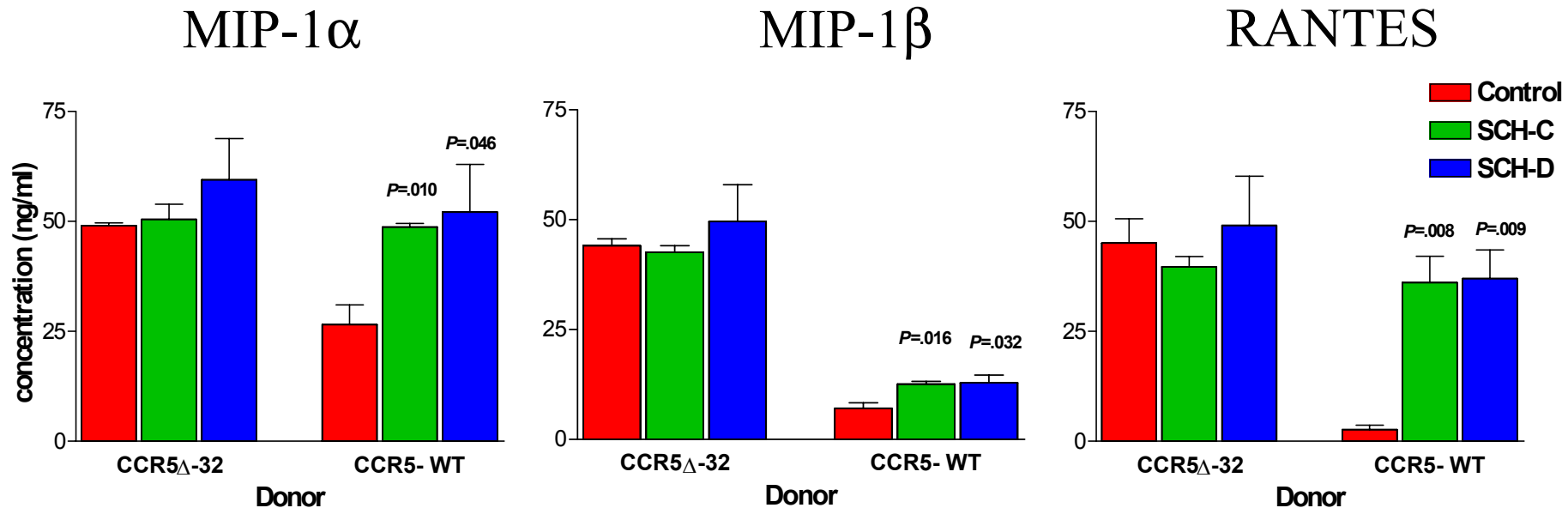


PBMCs



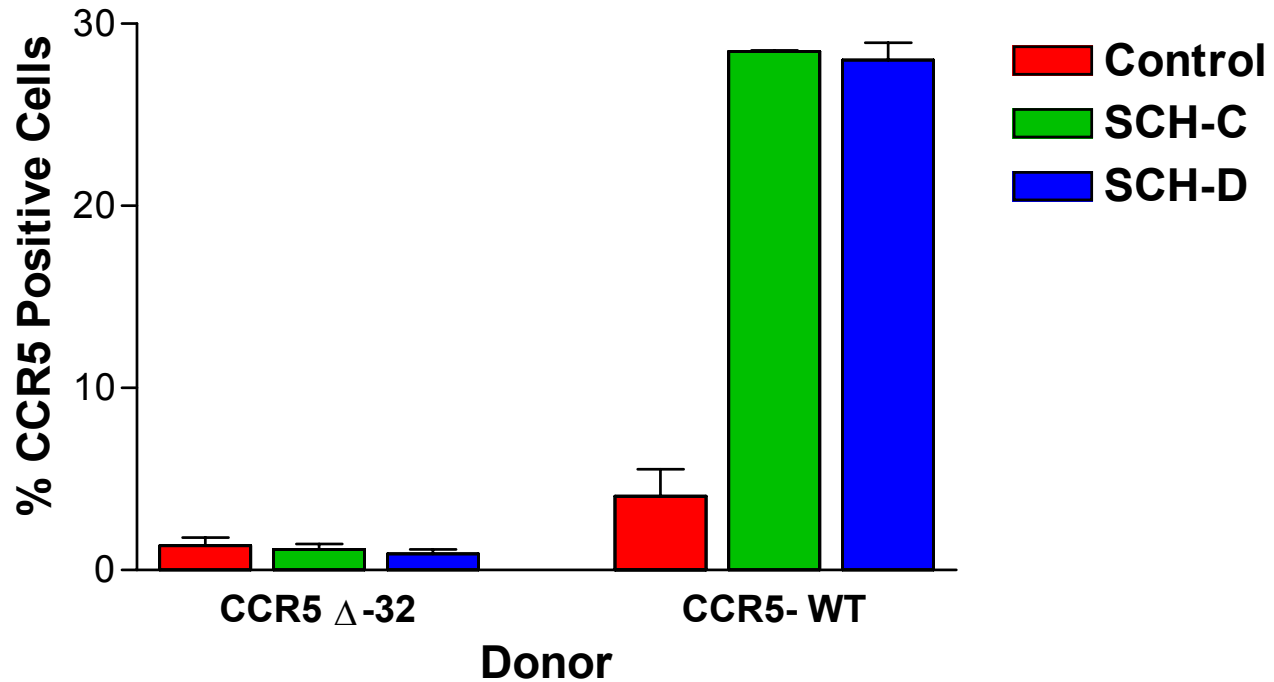
To determine if SCH-C could inhibit RANTES mediated CCR5 down regulation, BAF550 cells or PBMCs were cultured in the presence of SCH-C (100 nM), RANTES (1 μ g/ml) or both for 48 hrs. CCR5 expression was determined by FACS analysis and expressed as % of positive staining cells. The results clearly demonstrate that in both cell types, SCH-C can block RANTES-mediated receptor down regulation.

Chemokine Production in CCR5 Δ -32 and Wild Type PBMC Cultures Treated with CCR5 Antagonists



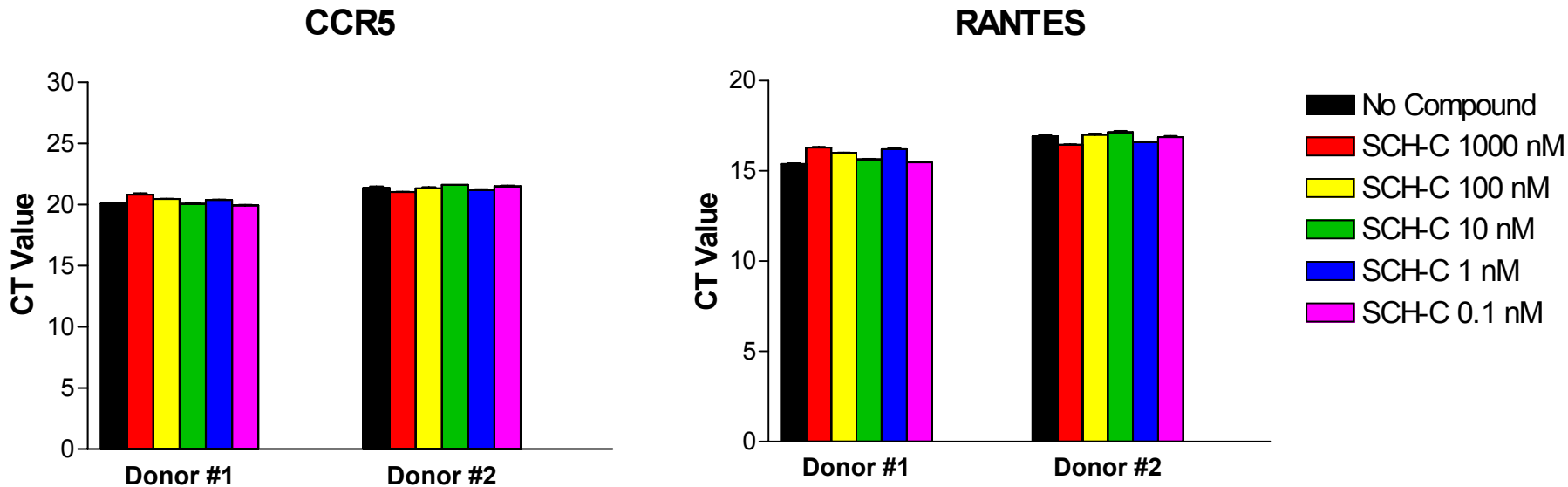
To demonstrate that SCH-C enhancement of chemokine levels is mediated by receptor blockade, we treated cells from a CCR5 Δ -32 donor with receptor antagonists and measured chemokine levels on day 7 after treatment. The CCR5 Δ -32 cultures had high levels of all three chemokines regardless of compound treatment. This is in contrast to CCR5-WT cultures which showed significant increases in chemokines in treated vs. control cultures. This result suggests that the absence or inhibition of CCR5 receptors, can lead to an accumulation of ligand due to inhibition of re-uptake by the receptor.

CCR5 Expression on $\Delta 32$ and WT PBMCs After Treatment with CCR5 Antagonists



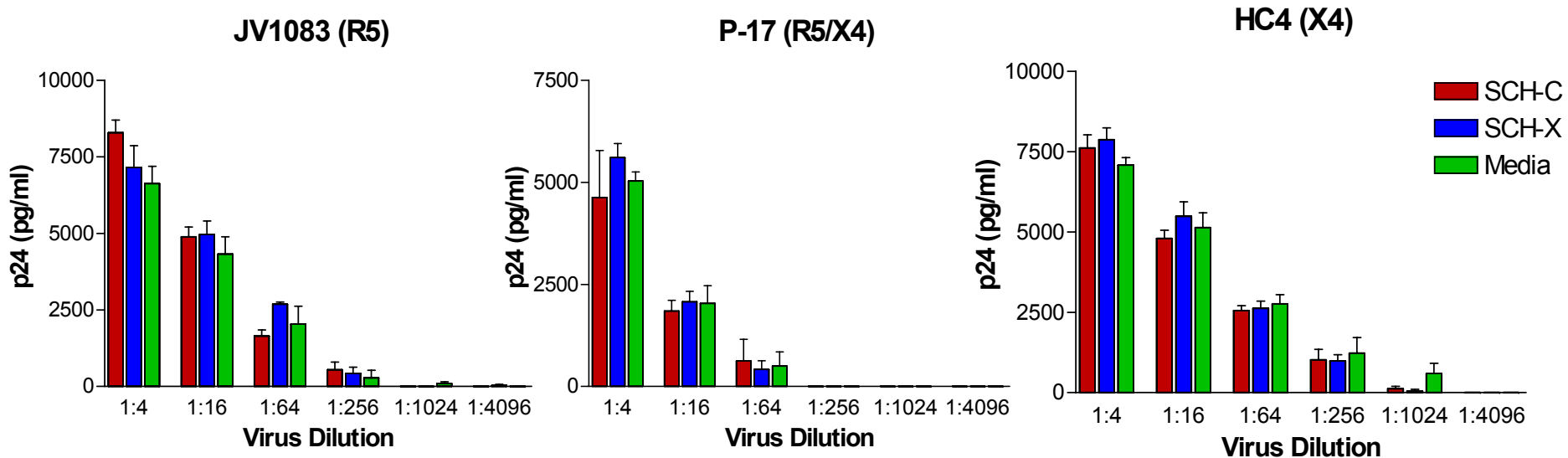
PBMCs from CCR5 wild type (wt) or $\Delta 32$ donors were cultured in the presence or absence of compound for 7 days and analyzed for CCR5 surface expression by flow cytometry. As expected the cells from the $\Delta 32$ donor were negative for CCR5 staining regardless of treatment. Cells from the wild type donor showed a significant increase in CCR5 expression following treatment with either SCH-C or SCH-D

SCH-C Does Not Up-Regulate CCR5 or RANTES mRNA in PBMCs



PBMC cultures from two donors were treated with decreasing concentrations of SCH-C for 8 days. Cells were harvested and total cellular RNA isolated. CCR5 and RANTES mRNA was quantitated using Taqman PCR (PE Biosystems). GAPDH primers were used as an control for input RNA (data not shown). No significant differences in CCR5 or RANTES mRNA levels were detected between the control and SCH-C treated cells. This result indicates that the increased protein expression measured in treated cultures is not a result of increased gene expression.

Susceptibility of PBMCs to HIV Infection Following 7-Day Treatment with CCR5 Antagonists



The susceptibility of PBMCs to infection with R5, R5/X4 or X4 viruses was not altered, relative to control cultures, by treatment with SCH-C or a control compound SCH-X.

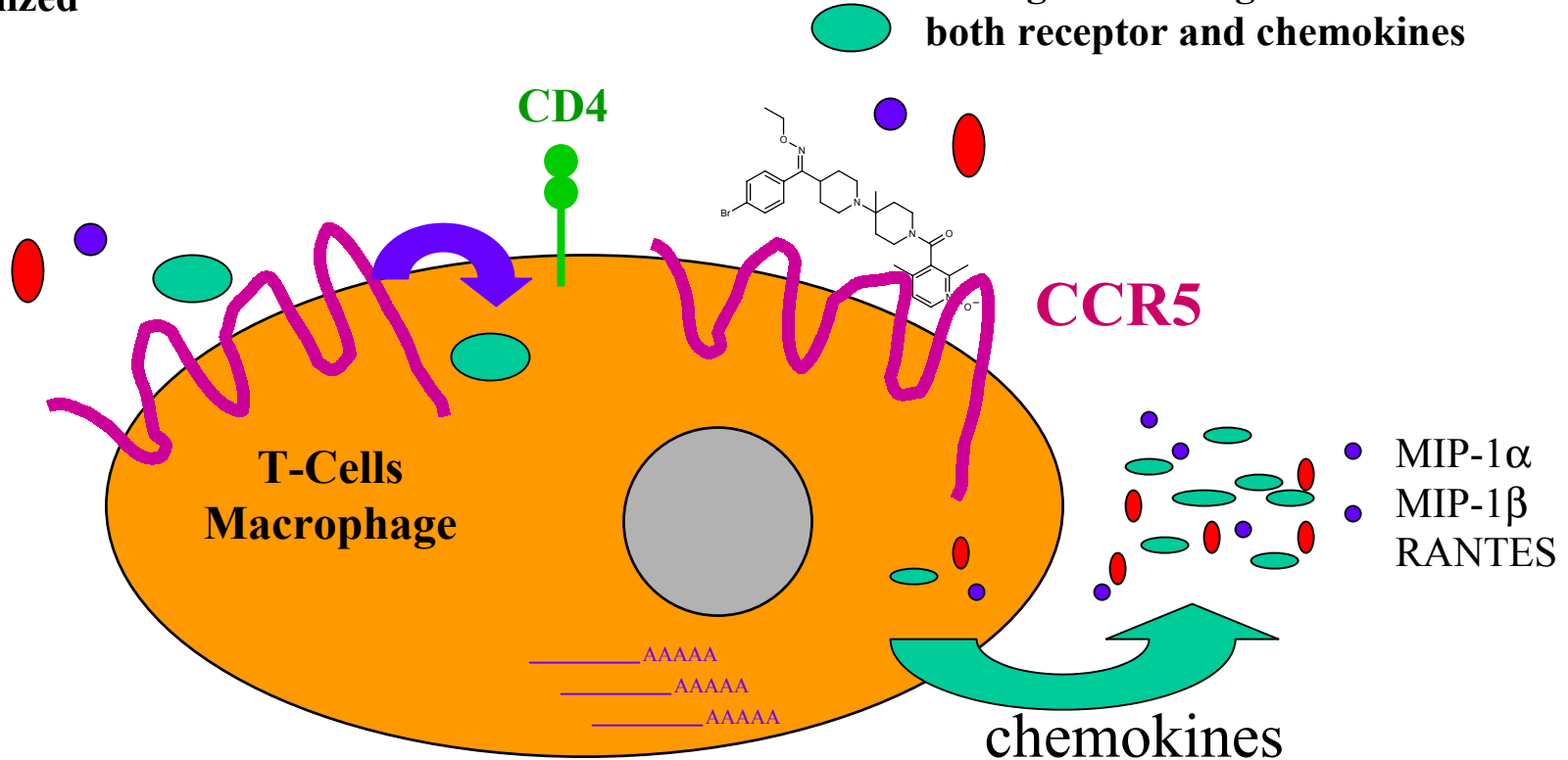
Model for SCH-C Increase of CCR5 and β -Chemokine Levels in PBMC Cultures

No SCH-C:

Chemokines bind to CCR5 and both receptor and ligand are internalized

+ SCH-C:

SCH-C blocks ligand binding and subsequent internalization of CCR5 and ligand leading to accumulation of both receptor and chemokines



Conclusions

We have shown that treatment of PBMCs with CCR5 antagonists (SCH-C, SCH-D) results in an increase in β -chemokine levels in culture supernatants. In addition CCR5 receptor expression is increased by SCH-C in these cultures. Quantitative RT-PCR measurement of cellular mRNA indicate that the increase in chemokine and receptor protein levels does not result from upregulation of gene expression by SCH-C, but is more likely due to inhibition of chemokine-induced receptor cycling. Despite the increase in CCR5 surface expression, SCH-C treated cells were not more susceptible to infection by R5, R5/X4 or X4 viral isolates. Combination studies demonstrated that SCH-C had additive antiviral activity in conjunction with RANTES or MIP-1 α . Taken together, these results suggest that antagonism of CCR5 by a small molecule antagonist can increase extracellular chemokine concentrations which, in the context of antiretroviral therapy, may provide additional antiviral benefit.