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Extravirologic modulation of immune response by an NRTI-sparing antiretroviral regimen including darunavir and maraviroc

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SUMMARY

Dual therapies, including protease inhibitor + maraviroc (MVC), may represent an alternative to traditional regimens for management of HIV infection. The aim of this in vitro study was to assess the effects of darunavir (DRV) alone or in combination with MVC on cell apoptosis and chemotaxis. A significant decrease of cell apoptosis was found after DRV treatment. The addition of MVC to DRV also had an in vitro down-regulating effect on cell migration. The combination of an NRTI-sparing regimen including DRV+ MVC may have a potential role in immune system modulation by the direct down regulation of apoptosis and chemotaxis.

KEY WORDS: Darunavir, Maraviroc, HIV, Apoptosis, Cell migration.

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HIV infection is characterized not only by the development of profound immunodeficiency but also by a marked and persistent cellular immune activation and inflammation (Klatt *et al.,* 2013a). The generalized immune activation can induce failure of CD4+ T-cell homeostasis and impair the regenerative capacity of the immune system. Chronic inflammation might also be involved in the pathogenesis of some organ and metabolic disorders associated with HIV infection, including liver and kidney diseases, atherosclerosis and neurocognitive impairment.

The central role of immune activation and inflammation in the pathogenesis of HIV has stimulated the search for new approaches for managing HIV infection (Klatt *et al.,* 2013b). A variety of studies have indicated that part of the efficacy of protease inhibitor (PI)-based therapy in controlling HIV infection and progression might be due to the extravirologic properties of PI leading to immune system modulation, blocking inflammation, T-cell activation and cell apoptosis, and inhibiting matrix metalloproteinase activity (Badley, 2005; Latronico *et al.*, 2007; Mastroianni *et al*., 2007). Recently, it has been shown that CCR5 antagonists could also have immunomodulant properties beyond their capacity to inhibit virus entry (Corbeau *et al.,* 2009). Pharmacologic inhibition of CCR5 may protect CD4+ T-cells from immune activation resulting from HIV infection and disease progression. By preventing signaling through the CCR5 receptor, CCR5 antagonists may down-regulate immune activation, T-cell apoptosis and cytokine expression. Given the central role of CCR5 in governing both trafficking and recruitment of leukocytes into inflamed tissue, CCR5 antagonists could modulate cell chemotactic activity (Rossi *et al.,* 2010).

The development in clinical practice of dual therapies based on the use of PI/r + raltegravir (RAL) or MVC may represent an alternative to traditional regimens for the long-term man-

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agement of HIV-infected individuals. The use of NRTI-sparing regimens including PIs and CCR5 antagonists not only could reduce the risk of NRTI toxicity, but could have a potential beneficial effect on immunologic response.

The aim of this vitro study was to assess the effects of PI darunavir (DRV) alone or in combination with MVC on apoptosis and chemotaxis of human peripheral blood mononuclear cells (PBMC) and human neutrophils (PMN).

After isolation from healthy donors, the cells (10^6/ml) (PBMC and PMN) were suspended in RPMI-1640 medium supplemented with 10% FCS and cultured for 24h at 37°C and 5% CO_2 in the presence of the following drugs: DRV (0.1 µg, 1 µg, 10 µg), DRV (0.1 µg, 1 µg, 10 µg) + MVC (10 µM). We used indinavir (IDV) as a positive control, because it has been reported to have a potent anti-apoptotic effect on cells (Mastroianni *et al*., 2000). All drug concentrations were chosen on the basis of the therapeutically achievable levels unless otherwise specified. After incubation the cells were evaluated for apoptosis and chemotaxis. Drug-treated

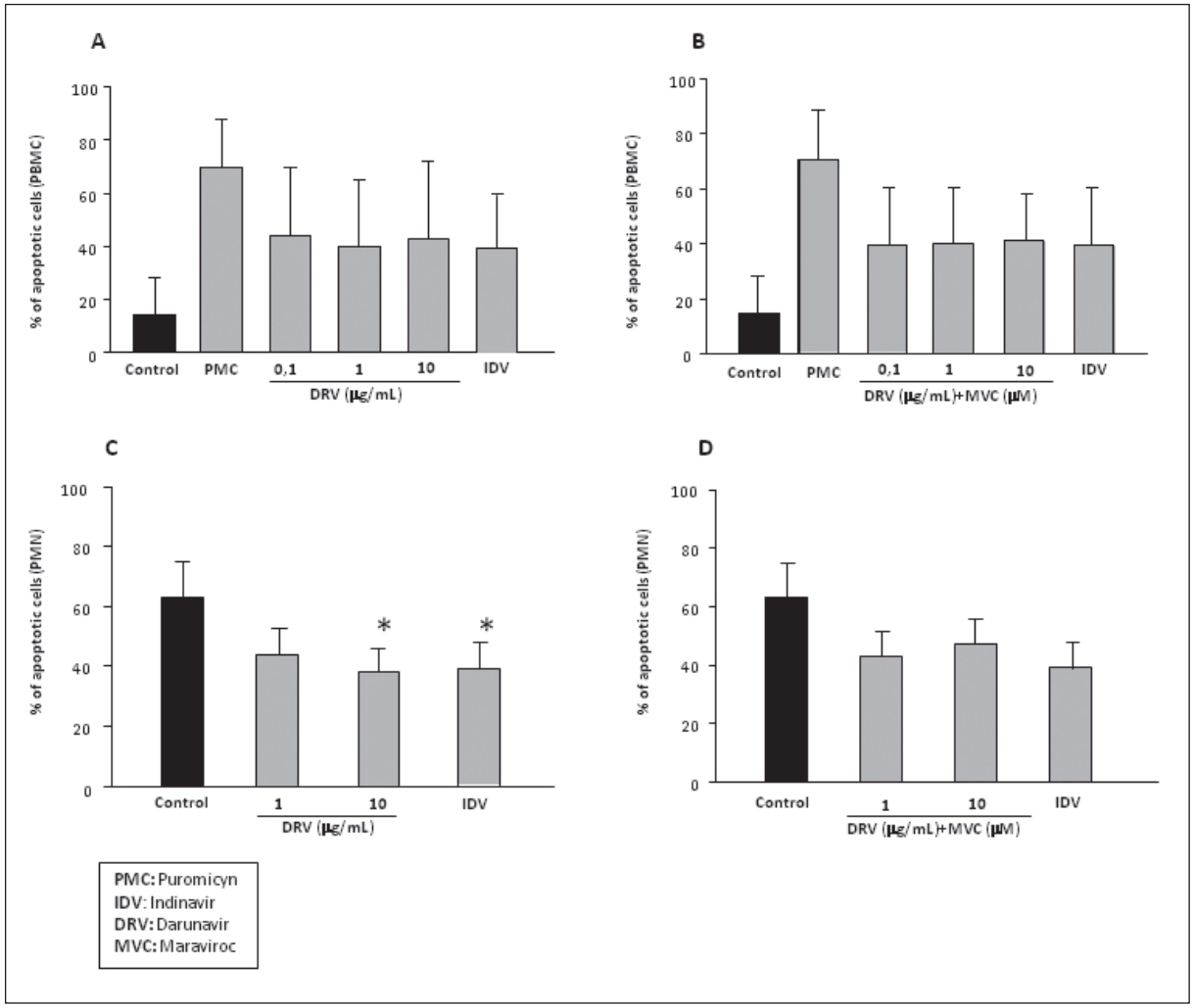

FIGURE 1 **- *Effect of darunavir and maraviroc on apoptosis of PBMC and PMN.*** *Panels A-B show the anti-apoptotic effect on PBMC. The apoptosis was evaluated in PBMC after 24h of incubation with DRV (panel A) and with DRV + MVC (panel B). In panels C-D, the apoptosis was evaluated in PMN (apoptosis spontaneous) after 24h of incubation with DRV (panel C) and with DRV + MVC (panel D). The control bars in each panel represent the cells incubated with medium alone. Data were expressed as mean ± SD of 3 independent experiments. Asterisks represent statistically significant (p<0.05, t-test) values compared with control.*

cells at all concentrations used showed a viability ≥95% as assessed by Trypan-Blue exclusion dye.

For PMN, spontaneous apoptosis was assessed after 24h of incubation with the different drugs. For PBMC we assessed the pro-apoptotic effect after 48h of drug incubation, while the anti-apoptotic effect was assessed after 24h of drug incubation, inducing apoptosis with the protein synthesis inhibitor puromycin (PMC, 10 µg/mL). Apoptosis was measured by morphological determination with acridine-orange/ethidium bromide. Briefly, after drug incubation, the cells ($2x10^6$ cells/ml) were stained with a solution of acridine-orange/ethidium bromide (both at 100 µg/ml) and observed at fluorescence microscopy. Cells with clear apoptotic morphology such us nuclear condensations, loss of nuclear lobularity or fragmentation into apoptotic bodies, were counted. The apoptosis rate was expressed as the percentage of those cells in the total cell counts. To confirm the data, apoptosis was measured by analysis of the activity of caspase 3 in cell lysates, using the caspase-3 colorimetric activity assay kits (Chemicon International) according to the manufacturer's instructions.

The *in vitro* chemotactic activity was measured in an 8 mm pore size Transwell system (Becton Dickinson Europe) for PBMC. The synthetic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) (10-5M) (Sigma, USA) was used as chemoattractant and CCL5/regulated upon activation, normal T-cell expressed and secreted (RANTES) (100 ng/ml) (R&D Systems). A bell-shaped curve described the typical migratory response of cells to increasing concentrations of chemoattractant. Cell suspensions in FCS-free RPMI-1640 were used at a concentration of $1x10^6$ cells/ml. After 90 min of incubation at 37°C in 5% CO_2, the migrated cells in the lower well were quantified by flow cytometry (FACS Calibur with Cell Quest software) using Trucount™ tubes (Becton Dickinson). To eliminate cellular debris, R1 gate was defined in a dot-plot of forward-scatter channel (FSC) *versus* a side-scatter channel (SSC). Random migration in the absence of chemoattractant was calculated and subtracted from migration in response to stimuli. Results were expressed as mean [stan-

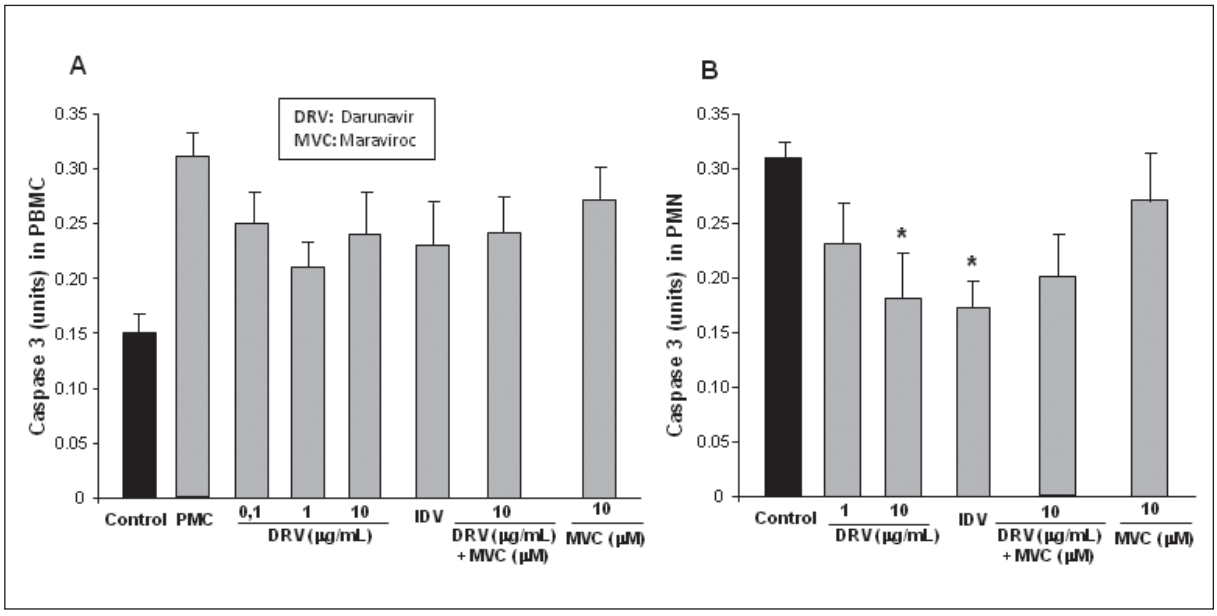

FIGURE 2 - ***Effect of darunavir and maraviroc on caspase activity in PBMC and PMN.*** *Panels A-B show the anti-apoptotic effect on PBMC (panel A) and on PMN (in panel B). In this experiment the cells were incubated for 24h with DRV alone and with DRV + MVC and the apoptosis was measured by analysis of the activity of caspase 3 in cell lysates. The control bars in each panel represent the cells incubated with medium alone. Data were expressed as mean ± SD of 3 independent experiments. Asterisks represent statistically significant ($p<0.05$, t-test) values compared with control.*

dard deviation (S.D.)] percentage of chemotaxis of three different experiments using different donors. The percentage was calculated using the following formula: %=N of migrated cells/N of 100% controls × 100. Control chemotaxis was set at 100% and drug treatments were represented as the percentage of control (cells incubated with medium alone).

Our results showed that no differences were found assessing the apoptosis of PBMC after 48h of drug incubation, thus showing that DRV alone or in combination with MVC did not have any pro-apoptotic effect on apoptosis of the PBMC ($p>0.05$ for each drug concentration). A modest but not significant decrease in apoptosis induced with PMC were observed after 24h of incubation with all concentrations of DRV (Fig. 1A). The addition of MVC did not modify the anti-apoptotic activity of DRV (Fig. 1B).

With regard to PMN we found that DRV at 10μg/ml concentration significantly inhibited apoptosis ($p=0.04$) with an efficiency similar to IDV ($p=0.05$) (Fig. 1C), whereas the addition of MVC did not affect the anti-apoptotic activity of DRV (Fig. 1D). All data were also confirmed by analysis of the activity of caspase 3 in cell lysates as shown in Fig. 2A and 2B.

The chemotactic responsiveness of PBMC was assessed towards FMLP as chemoattractant. Results showed that PBMC treated with DRV did not exhibit a significant inhibition of chemotactic activity (Fig. 3A). Conversely, the chemotaxis was significantly inhibited when cells were pre-treated with both drugs at a concentration of 10μg for DRV + 10μM for MVC ($p=0.04$) (Fig. 3B). Indeed, DRV+MVC affect cell migration by reducing the chemotactic activity of PBMC by 57%. A significant down-regulation of chemotaxis was also observed with cells pre-treated with MVC alone. The chemotactic activity was also assessed towards RANTES as chemoattractant, and we found that the chemotaxis of PBMC was reduced by 47%.

In summary, a significant decrease in spontaneous apoptosis of PMN was observed after in vitro treatment with DRV. These data confirm our previous study with IDV and ritonavir (RTV). Indeed the first generation of PI has been shown to reduce the apoptosis of T-cells and neutrophils (Mastroianni *et al.*, 2000; Phe-

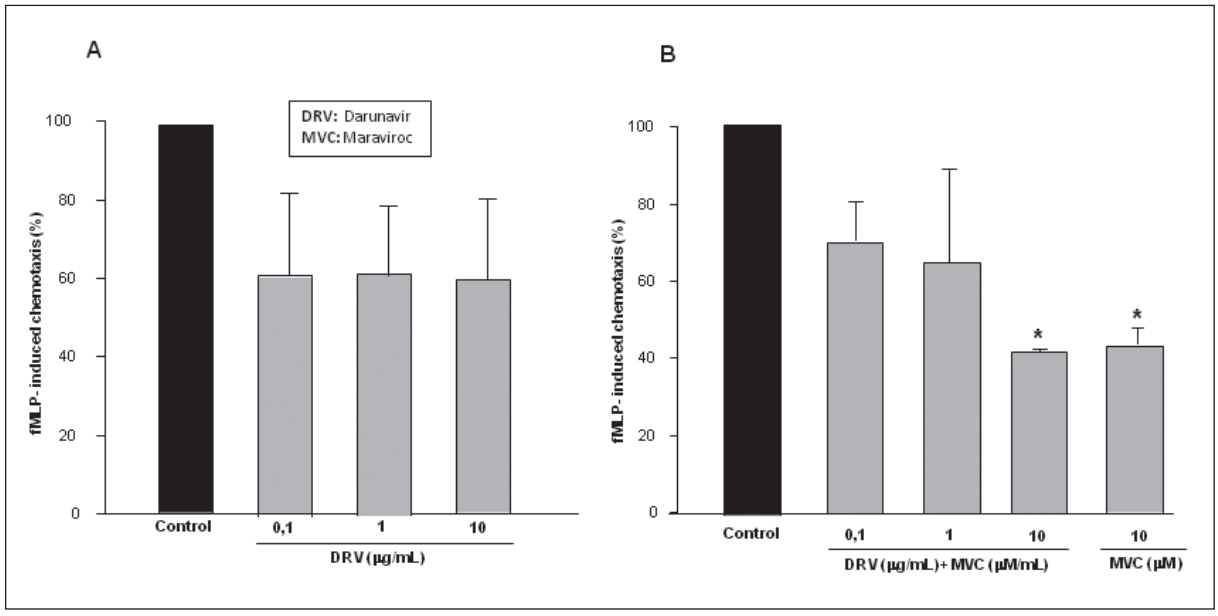

FIGURE 3 - ***Effect of darunavir and maraviroc on chemotactic activity of PBMC***. *The in vitro chemotactic activity in response to synthetic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) was evaluated in PBMC after incubation with DRV (panel A) and with DRV + MVC or MVC alone (panel B). Results were expressed as mean [standard deviation (S.D.)] percentage of chemotaxis of 3 different experiments. Control chemotaxis was set at 100% and drug treatments were represented as the percentage of control (cells incubated with medium alone). Asterisks represent statistically significant ($p<0.05$, t-test) values compared with control.*

nix *et al.*, 2002) in AIDS patients even in the absence of inhibition of viral spread, and increase in vitro cell viability by inhibiting apoptosis of infected and uninfected T-cells, suggesting a direct HIV-independent effect on apoptosis (Ghibelli *et al.*, 2003; Lichtner *et al.*, 2006).

Interestingly, our findings showed that the addition of MVC to DRV has an in vitro down-regulating effect on cell migration. It is likely that the effect induced by the treatment with the two drugs is completely attributable to maraviroc. These findings confirmed our previous data in which we demonstrated in vitro that the CCR5 antagonist MVC is able to inhibit the migration of macrophages and dendritic cells by mechanisms which could be independent from the pure anti-HIV effect (Rossi *et al.*, 2010; Rossi *et al.*, 2011). The drug might have a potential role in the down-regulation of HIV-associated chronic inflammation by blocking the recirculation and trafficking of innate immune cells. These in vitro experiments suggest that the combination of an NRTI-sparing regimen including DRV+ MVC may have a potential role in the modulation of apoptosis and chemotaxis. Enhancement of the combined effect of DRV+MVC on the immune system may deserve future ex vivo studies to assess the impact of this NRTI-sparing regimen on inflammation, immune activation, apoptosis, cytokine secretion and cell migration.

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