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Short communication

Peripheral T-cell apoptosis is not differentially affected by antiretroviral regimens in HIV-infected patients

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Background: HIV-induced CD4⁺ and CD8⁺ T-cell apoptosis decreases upon start of combination antiretroviral therapy (cART). Although *in vitro* evidence suggests an anti-apoptotic effect of protease inhibitors (PIs) as opposed to non-nucleoside reverse transcriptase inhibitors (NNRTIs), *in vivo* studies are inconclusive about effects of differential cART regimens on T-cell apoptosis.

Methods: Peripheral T-cell apoptosis was evaluated in a cross-sectional study including 20 patients on PI- and 19 on NNRTI-based combination antiretroviral therapy (cART), all with backbone therapy of tenofovir and emtricitabine and undetectable viral loads 6 months before inclusion. Spontaneous T-cell apoptosis was measured in freshly isolated peripheral blood

mononuclear cells (<4 h after venipuncture) using annexin V, propidium iodide and staining for caspase activity and levels of the anti-apoptotic protein Bcl-2. **Results:** The groups were comparable in general- and HIV-specific characteristics. In addition, T-cell activation was similar in both groups. We observed no difference in T-cell apoptosis as measured by annexin V, propidium iodide or caspase staining between PI- and NNRTI-treated patients. Interestingly, the level of anti-apoptotic protein Bcl-2 was higher in PI-treated than in NNRTI-treated patients. **Conclusions:** In this cross-sectional study on HIV-infected patients, direct *ex vivo* spontaneous T-cell apoptosis rates are not differentially affected by NNRTI- or PI-based cART.

Introduction

Currently, standard combination antiretroviral regimes for treatment of HIV can be divided in protease inhibitor (PI)-based regimens and non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimens, both with a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs) [1]. Both regimens are known to be similarly effective on suppression of HIV RNA but differ in profile of adverse effects; therefore, first choice between both treatment options is still under debate [2,3].

Treatment of HIV results in gradual reversal of HIV-induced immune distortions including a reduction of excessive HIV-related T-cell apoptosis [4,5]. While PIs may inhibit T-cell apoptosis *in vitro*, even in the absence of HIV, NNRTIs exhibit pro-apoptotic effects *in vitro* [6–8]. However, only a few clinical studies have evaluated the impact of different combination antiretroviral therapy (cART) regimes on peripheral T-cell apoptosis [9–12]. Two of these studies showed comparable levels of peripheral T-cell apoptosis among PI- and non-PI based cART [10]. By

contrast, a recently published small but carefully performed longitudinal study revealed a favourable effect of PI-based cART ($n=8$) on peripheral T-cell apoptosis in comparison to NNRTI-based cART ($n=8$) [11]. However, the use of frozen samples, lack of specifying backbone NRTI therapy as well as baseline differences in T-cell apoptosis between the patient groups hampered their conclusions [11,13].

In the present study, we characterized T-cell apoptosis directly *ex vivo* (<4 h from venipuncture) in patients with PI- or NNRTI-based cART with identical NRTI backbone therapy. Only patients with undetectable HIV viral loads and without concomitant infections or any other medical conditions likely to affect T-cell viability were included.

Methods

Patients

A total of 40 HIV-infected patients, of whom 20 on boosted PI- and 20 on NNRTI-based cART, all with

identical backbone therapy consisting of tenofovir and emtricitabine, were recruited from the outpatient clinic of the University Medical Center Utrecht (Utrecht, the Netherlands) based on undetectable viral loads (HIV RNA <50 copies/ml) for at least 6 months before inclusion, last CD4⁺ T-cell counts above 350 cells/mm³ and without concomitant infections or any other medical condition likely to interfere with T-cell activation or apoptosis. HIV viral loads were measured using AmpliPrep/COBAS Taqman PCR (lower limit of detection 50 copies/ml; Roche, Woerden, the Netherlands). Informed consent was obtained from all patients in compliance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th revision, 2008). The local Medical Ethical Committee approved the study protocol.

Blood samples and flow cytometry

Within 4 h of venous puncture, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque following standard procedures. Cells were incubated with antibodies against CD3 (AF700; Biolegend, San Diego, CA, USA), CD4 (PB; Biolegend), CD8 (V500; BD Biosciences, San Diego, CA, USA), CD38 (PE; Caltag Laboratories, San Francisco, CA, USA) and CD95 (APC; BD Biosciences) for 20 min at 4°C. After washing, cells were incubated with fluorescent inhibitor of caspases (FLICA) for caspase 8, caspase 9 or caspases 3 and 7 (FITC; ImmunoChemistry Technologies; Bloomington, MN, USA) following product guidelines, at 37°C (1×10⁶ cells per sample). Another sample was stained for annexin V (FITC; Biolegend) and propidium iodide (Biolegend) at room temperature for 15 min and directly analysed by flow cytometry. For staining of Bcl-2, samples were incubated with Cytofix/Cytoperm solution (BD Biosciences), washed with Perm/Wash buffer (BD Biosciences) and subsequently stained with antibodies directed against Bcl-2 (PE, 4G7, BD Biosciences) for 30 min at room temperature. All samples were washed, fixed with Cellfix (BD Biosciences) and directly analysed on a LSRII flow cytometry (BD Biosciences). Per sample, 250,000 cells were analysed. Maximally 6 patients were sampled at once; laboratory procedures were started within 4 h and finished within 8 h after venipuncture.

Statistics

Data were normally distributed and therefore expressed as mean with standard deviations. Means were compared using Student's *t*-test. χ^2 test was used to test relation of categorical variables. Dependence of variables was tested with Pearson's correlation coefficient. Statistical analysis was performed with IBM SPSS Statistics version 20 (IBM SPSS Inc., Chicago, IL, USA) and

GraphPad Prism 5 for windows version 5.03 (Graphpad Software, Inc., San Diego, CA, USA).

Results

A total of 40 patients participated in the study. Of those, one was excluded because of detectable viral load in combination with decreased CD4⁺ T-cell count at the day of inclusion (CD4⁺ T-cell count 342 cells/mm³ and HIV viral load 161 IU/ml). Patients on PI- (*n*=20) and NNRTI-based cART (*n*=19) were comparable in gender, age and HIV-specific parameters (Table 1). At the time of inclusion, viral loads were undetectable (<50 copies/ml) in all patients. The majority of patients in the PI group received ritonavir-boosted atazanavir (*n*=18), whereas efavirenz (*n*=18) was the preferred drug in the NNRTI group. The NRTI backbone consisted of tenofovir and emtricitabine in all patients, without addition of other antiretrovirals.

No differences in CD4⁺ T-cell activation were observed between PI- and NNRTI-treated patients (CD38⁺: 43% versus 43% respectively, *P*=0.98; Figure 1A). In both groups, T-cell activation correlated negatively with the duration of cART (CD4: *r*=−0.51; *P*=0.0010; CD8: *r*=−0.51, *P*=0.0008; Figure 1B).

PI- and NNRTI-treated patients depicted similar levels of directly *ex vivo* CD4⁺ T-cell apoptosis as measured by annexin V and propidium iodide (PI: 1.9%, NNRTI: 1.8%; *P*=0.80). In addition, no differences were observed in activation of the extrinsic apoptosis pathway, indicated by expression of death receptor CD95 (PI: 45%, NNRTI: 45%; *P*=0.60) and activated initiator caspase 8 (PI: 13%, NNRTI 15%; *P*=0.35). However, with regard to the intrinsic apoptosis pathway, expression of anti-apoptotic protein B cell lymphoma 2 (Bcl-2) was elevated in CD4⁺ T-cell of PI- compared with NNRTI-treated patients (5.4% versus 3.0%, *P*<0.001), but this was not accompanied by decreased activation of downstream intrinsic apoptosis pathway since activation

Table 1. Patient characteristics

Characteristic	PI, <i>n</i> =20	NNRTI, <i>n</i> =19	Statistical significance <i>P</i> -value
Gender (% male)	18 (90%)	16 (84%)	0.59
Age, years	48 (9)	48 (8)	0.94
Years since diagnosis HIV	10 (7)	9 (5)	0.64
Years since start antiretroviral therapy	6 (4)	7 (5)	0.34
CD4 ⁺ T-cell count, cells/ml	615 (165)	711 (231)	0.14
Prescription of statins	6 (30%)	4 (21%)	0.52

Characteristics of patients treated with protease inhibitor (PI)- or non-nucleoside reverse transcriptase inhibitor (NNRTI)-based combination antiretroviral treatment are given in means and standard deviations or number and percentages.

Figure 1. Activation and apoptosis markers in CD4⁺ and CD8⁺ T-cells

(A) Percentages and standard error of the mean of CD38⁺ and CD95⁺ activated CD4⁺ (left) and CD8⁺ (right) T-cells in patients with protease inhibitor (PI)-based (grey bars) or non-nucleoside reverse transcriptase inhibitor (NNRTI)-based (black bars) combination antiretroviral treatment (cART). (B) Correlation of time since start antiretroviral therapy (ART; x-axis) with percentages of CD38⁺ activated CD4⁺ T-cells (y-axis) in patients on PI (grey dots) or NNRTI-based cART (black dots) and regression line. (C&D) Mean percentages and standard error of the mean of (C) CD4⁺ and (D) CD8⁺ T-cells positive for activated extrinsic activator caspases 8, intrinsic activator caspases 9, effector caspases 3 and 7, Bcl-2, annexin V and propidium iodide (Plo) in HIV-infected patients on PI- (grey bars) or NNRTI-based (black bars) ART. ^a $P < 0.001$. ^b $P = 0.02$.

of initiator caspase 9 (PI: 16%, NNRTI: 17%, $P = 0.64$) or effector caspases 3 and 7 (PI: 10%, NNRTI: 11%, $P = 0.98$; Figure 1C) were similar between both groups. CD8⁺ T-cells showed a similar pattern of significantly elevated Bcl-2 (4.6% versus 3.0%, $P = 0.02$) without other differences in expression of apoptosis- or activation markers (Figure 1D). Lastly, no correlations were found between levels of apoptosis or caspase-activity and duration of cART, age or CD4⁺ T-cell count (data not shown).

Discussion

This study shows no differences in directly *ex vivo* apoptosis levels between PI- and NNRTI-treated

patients, as measured by co-staining of annexin V and propidium iodide. Furthermore, the extrinsic apoptosis pathway (Fas and caspase 8) was not affected. With regard to the intrinsic apoptosis pathway, we found higher levels of the anti-apoptotic protein Bcl-2 in the PI- versus NNRTI-treated patients, but this was not associated with downstream activation of caspases 3 and 7.

Although excessive HIV-related T-cell apoptosis is reduced soon upon start of cART [5,14,15], *in vitro* studies indicate an anti-apoptotic effect of PI and pro-apoptotic effects of NNRTI in chronically treated patients, even in the absence of HIV [8]. However, this has not conclusively been observed in clinical studies

due to possible effects of cryopreservation and heterogeneity in cART backbones [6,7,9].

Our study is distinctive since we performed our apoptosis analysis on freshly isolated PBMCs (<4 h after venipuncture). Furthermore, by matching for gender, age and CD4⁺ T-cell counts, we minimized the potential for biased patient selection. Lastly, only patients without concomitant infections, malignancies, substance abuse or other medical conditions likely to interfere with T-cell apoptosis, were included [16].

Although one study showed that PIs (with the exception of atazanavir) were able to inhibit HIV glycoprotein 120-mediated T-cell death *in vitro* [17], other studies reveal that, even in the absence of HIV, susceptibility to apoptosis is affected by PIs [8]. Our aim was to evaluate pro- or anti-apoptotic effects of the long-term, aviraemic phase of treatment, since we expect that this is most relevant for possible long-term side effects of cART.

T-cell activation was similar in both groups, as indicated by the expression of the activation marker CD38. Furthermore, CD38 expression correlated negatively with time since start of cART, independent of cART regimen, suggesting that T-cell activation gradually decreases over years. This finding is in-line with findings from a larger cohort, indicating that our study population is representative for treated HIV patients [18].

Bcl-2 is an important anti-apoptotic protein that may influence mitochondrial membrane permeabilization, a key event of apoptosis, by interaction with other pro- and anti-apoptotic proteins. This mechanism may explain anti-apoptotic effects of PIs *in vitro* [9]. However, increased Bcl-2 alone does not necessarily result in reduced apoptosis [19,20]. We hypothesize that increased levels of Bcl-2 results in decreased apoptosis rates after stressing conditions like *ex vivo* cryopreservation, rather than affecting apoptosis in physiological conditions, which may explain the discrepancy between our findings and those of Jung *et al.*, [11] who observed increased apoptosis in cryopreserved T-cells of NNRTI- in comparison to PI-treated patients. Our data support another study analysing CD4⁺ and CD8⁺ T-cell apoptosis of whole blood and a longitudinal study comparing different cART regimens in which a combination of PI plus NNRTI showed similar levels of T-cell apoptosis as a regimen of NNRTI plus three NRTIs [10,12].

Based on our findings, we conclude that PI- and NNRTI-based cART regimens do not differentially affect levels of T-cell apoptosis in HIV-infected patients on stable cART regimens with undetectable HIV RNA and high CD4⁺ T-cell counts.

Disclosure statement

The authors declare no competing interests.

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