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► **To cite this version:**

Christian Körner, Benjamin Krämer, Daniela Schulte, Martin Coenen, Stefan Mauss, et al.. Effects of HCV co-infection on apoptosis of CD4+ T cells in HIV-positive patients. *Clinical Science*, 2009, 116 (12), pp.861-870. 10.1042/CS20080532 . hal-00483309

HAL Id: hal-00483309

<https://hal.science/hal-00483309>

Submitted on 14 May 2010

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Effects of HCV co-infection on apoptosis of CD4⁺ T cells in HIV-positive patients

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Keywords: HIV-1, apoptosis, CD4⁺ T cells, Hepatitis C virus, HAART, protease inhibitors

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Abstract

Background: Apoptosis importantly contributes to loss of CD4⁺ T cells in HIV infection, and modification of their apoptosis may explain why HIV/HCV-co-infected patients are more likely to die from liver-related causes, while effects of HCV on HIV infection remain unclear.

Methods: We studied in a cross-sectional and serial analysis spontaneous *ex vivo* CD4⁺ T cell apoptosis in HIV/HCV-co-infected and HIV-mono-infected patients before and after HAART. Apoptosis of peripheral blood CD4⁺ T cells was measured by both, a Poly [1] polymerase [1] and TdT-mediated dUTP-FITC nick end labelling (TUNEL) assay to detect cells with irreversible apoptosis.

Results: While hepatitis C alone did not increase CD4⁺ T cell apoptosis, HCV co-infection disproportionately increased elevated rates of apoptosis in CD4⁺ T cells from untreated HIV-positive patients. Increased CD4⁺ T cell apoptosis was closely correlated to HIV but not HCV viral loads. Under HAART increased rates of CD4⁺ T cell apoptosis rapidly decreased both in HIV-mono-infected and HIV/HCV-co-infected patients, without any significant difference in apoptosis rates between the two patients groups after 4 weeks of therapy. Nevertheless residual CD4⁺ T cell apoptosis did not reach the normal levels seen in healthy controls and remained higher in HIV patients receiving protease inhibitors than in patients with other antiretroviral regimens.

Conclusions: Our results suggest that HCV co-infection sensitizes CD4⁺ T cells towards apoptosis in untreated HIV-positive patients. However, this effect is rapidly lost under effective antiretroviral therapy.

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Introduction

The hallmark of HIV infection is gradual loss of CD4⁺ T cells, and apoptosis is now considered to contribute importantly to the declining numbers of CD4⁺ T cells in HIV-infected patients. Apoptosis is a highly regulated process which is characterised by specific physiological and morphological cell changes resulting in the formation of membrane-enclosed apoptotic bodies which are taken up by surrounding cells without an inflammatory reaction. Induction of apoptosis is mediated via different pathways. The extrinsic pathway is triggered by binding of ligands from the tumour-necrosis-factor (TNF) family to death receptors (Fas, DR4/5) which initiates activation of proteolytic enzymes, the cascade of caspases. Alternatively, apoptosis is induced in the intrinsic pathway via signals resulting from altered mitochondrial integrity. Both pathways converge at the level of caspase 3 activation, which induces irreversible steps of apoptosis such as cleavage of Poly-(ADP-ribose) polymerase (PARP).

Several mechanisms of apoptosis induction have been identified in HIV-infected individuals. Examples are killing by bystander cells due to the secretion of death ligands TRAIL and FasL, which are enhanced in HIV-infected patients. So called activation induced cell death (AICD) is another mechanism of apoptosis induction in HIV infection contributing to the loss of activated CD4⁺ T cells. Finally, HIV proteins themselves such as HIV Tat or Env proteins can directly interfere with host lymphocytes resulting in CD4⁺ T cell apoptosis [2-4].

Apoptosis is probably also a characteristic feature of hepatitis C virus (HCV) infection. Apoptosis of hepatocytes and peripheral blood lymphocytes has been reported in HCV-infected patients [5]. However, the underlying mechanisms have remained unclear so far. Moreover, both direct pro- and anti-apoptotic effects have been reported for single HCV proteins [6]. Thus, the role of apoptosis is discussed controversially in the pathogenesis of chronic hepatitis C.

Due to similar routes of transmission 15-50% of HIV-infected patients are HCV co-infected, resulting in an estimated ten million patients worldwide who suffer from combined HIV and HCV infection. Of note, certain populations with special risk factors, such as HIV-positive haemophiles and HIV-positive intravenous drug users, have 70-90% prevalence of HIV/HCV co-infection among their individuals [7]. HIV/HCV co-infection has been identified to lead to increased liver-related mortality and is considered to accelerate the progressive course of HIV-infection towards severe immunodeficiency and AIDS resulting in an increased overall mortality [8-13].

However, subsequent studies failed to confirm differences in survival between HIV-mono- and HIV/HCV-coinfected patients. For instance, analysis of the EuroSIDA cohort did not reveal any significant differences in decreased HIV loads and increased CD4-counts between HCV-positive and HCV-negative patients after the initiation of effective antiretroviral therapy [14, 15]. Highly active antiretroviral therapy (HAART) enables effective control over HIV replication and leads to recovery of CD4⁺ T cell counts. Thus, HAART can substantially increase the life expectancy of HIV-infected patients. Moreover, effective HAART results in reduced progression of HCV-associated liver disease and improved liver-related mortality in HIV/HCV-co-infected patients [16, 17]. Finally, immune reconstitution under HAART is also associated with marked reduction of CD4⁺ T cell apoptosis [14, 15].

However, concerning HIV/HCV-co-infected patients it is still unclear, to which extent HCV co-infection can modify rates of apoptosis in CD4⁺ T cells and if HAART can reduce apoptosis of CD4⁺ T cells in these patients to the same degree as in HIV-mono-infected patients. Thus, applying two different assays for apoptosis detection in cross-sectional as well as longitudinal studies of HIV/HCV-co-infected and HIV-mono-infected patients before and after the initiation of HAART, we directly compared CD4⁺ T cell apoptosis between HIV- and HIV/HCV-infected patients.

Methods

Patients and controls

A total of 94 patients and 12 healthy controls were enrolled in this study comprising 47 HIV-mono-infected, 37 HIV/HCV-co-infected and 10 HCV-mono-infected patients. Of these, 25 HIV-mono-infected and 14 HIV/HCV-co-infected patients had never been treated with antiretroviral drugs or had been without any antiretroviral treatment for at least one year prior to the study. 22 patients with HIV mono-infection and 23 patients with HIV/HCV co-infection had been receiving effective HAART for at least one year with HIV loads being consistently below the level of detection (less than 50 copies/ml). All HIV patients were infected with HIV-1. All patients with chronic HCV infection had not received any anti-HCV therapy prior to inclusion.

In addition to a cross-sectional analysis over all individuals, 8 HIV-mono-infected and 5 HIV/HCV-co-infected patients could be studied sequentially during initiation of their first HAART (weeks 0, 4 and 12). Demographic and clinical details of all patients are summarized in table 1 and 2.

Informed consent was obtained from all patients; the study had been approved by the local ethics committee of the University of Bonn.

Isolation and short-term culture of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from peripheral blood samples with Ficoll Hypaque density gradients (Biochrom, Berlin, Germany). Isolated PBMCs were incubated for 20 h in 96 well plates at a concentration of 1×10^6 PBMC/ml in RPMI 1640 medium (PAA, Pasching, Austria) supplemented with 5% (v/v) autologous serum and penicillin/streptomycin.

Detection of apoptotic CD4⁺ T cells

Rates of CD4⁺ T cells apoptosis were measured by detecting cells via cleaved poly (ADP-ribose) polymerase (PARP) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-FITC nick end labelling (TUNEL), respectively. CD4⁺ T cells in early stages of apoptosis were detected with specific antibody against cleaved PARP [1]. A commercially available TUNEL assay was used to detect DNA-fragmentation characteristic for cells in the late stages of apoptosis [18-21].

In brief, 1×10^6 PBMCs were stained with PerCP-conjugated anti-CD3 (BD Biosciences, Heidelberg, Germany) and allophycocyanin-conjugated (APC) anti-CD4 (BD Biosciences, Heidelberg, Germany) for 15 min at room temperature, washed with PBS and fixed then with paraformaldehyde (CellFIX, BD Biosciences, Heidelberg, Germany) for 20 min at 4°C. Fixed cells were washed with PBSF [phosphate buffered saline supplemented with 0.01% (v/v) NaN₃, 2.5% (v/v) FCS (Biochrom, Berlin, Germany)] and then permeabilized with digitonin (100 µg/ml in PBSF) for 30 min at 4°C. After additional washing steps, apoptotic cells were detected by incubation with a FITC-conjugated antibody which was specific for cleaved PARP (Abcam, Cambridge, UK) for 30 min at room temperature.

DNA fragmentation was measured with the APO-Direct-Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 3×10^6 PBMC were stained with allophycocyanin-conjugated anti-CD4 antibody for 15 min at room temperature and then washed with PBS. Stained cells were fixed for 1 h with 1% (wt/v) paraformaldehyde in PBS on ice, rinsed two times with cold PBS and finally resuspended in 70% ethanol. Then, the cell suspension was stored for at least 24 h at -20 °C. After washing two times with washing buffer fragmented 3'-OH DNA ends were labelled by incorporating FITC-labelled dUTP by incubating the cells with a terminal deoxynucleotidyl transferase reaction mixture at 37 °C for 1 h. The reaction was stopped by washing two times with rinsing buffer and the cell suspension was incubated with RNase/PI buffer for 30 min at room temperature prior to flowcytometric analysis.

Flowcytometric analysis was performed on FACSCalibur flowcytometer (BD Biosciences, Heidelberg, Germany) using CellQuest pro software (BD Biosciences, Heidelberg, Germany) and Flowjo (Tristar, Ashland, USA).

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Determination of HIV and HCV load

HCV genotypes were determined using the INNOLiPA, HCV II kit (Innogenetics, Heiden, Germany). HCV-RNA and HIV-RNA were measured by transcription-mediated amplification and branched DNA assays (TMA Versant, HCV 3.0 and HIV 3.0 Versant; Bayer Diagnostics, Leverkusen, Germany).

Statistical analysis

Data are presented as median (interquartile range). Statistical comparisons between groups were done with the Mann-Whitney test using Prism 3.0 software (GraphPad, USA). Correlations between apoptosis, CD4⁺ T cell counts and viral loads were analyzed with Spearman rank correlation using Prism 3.0 software (GraphPad, USA).

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Results

Both, the PARP and the TUNEL assay consistently yielded identical patterns of CD4⁺ T cell apoptosis in the different patient groups (figure 1), confirming that our assays were actually measuring spontaneous *ex vivo* apoptosis. Of note, we found marked differences in the levels of CD4⁺ T cell apoptosis between the different patient groups: While the percentage of apoptotic CD4⁺ T cells was identical in patients with chronic hepatitis C and healthy controls (PARP: $P=0.77$; TUNEL: $P=0.32$), HIV-positive patients displayed significantly increased rates of apoptotic CD4⁺ T cells (PARP: $P=0.0002$; TUNEL: $P<0.0001$) (figure 2). Importantly, the percentage of apoptotic CD4⁺ T cells was approximately twofold higher in HIV patients with HCV co-infection (PARP: $P=0.0157$; TUNEL: $P=0.0089$) than in patients with HIV mono-infection. Rates of CD4⁺ T cell apoptosis were positively correlated to HIV viral loads in untreated patients with HCV coinfection as well as in untreated patients with HIV mono-infection (PARP: HIV $r_s=0.66$, $P=0.0003$; HIV/HCV $r_s=0.82$, $P=0.0002$; TUNEL: HIV $r_s=0.66$, $P=0.0003$; HIV/HCV $r_s=0.79$, $P=0.0003$). In contrast, neither in patients with HCV infection nor in patients with HIV/HCV co-infection CD4⁺ T cell apoptosis was correlated to HCV loads (data not shown). In addition, we could not identify any correlations between CD4⁺ T cell apoptosis and HCV genotypes, sex or age. However, the difference in CD4⁺ T cell apoptosis between HIV-mono-infected and HIV/HCV-co-infected patients was disproportionately greater in patients with high HIV loads indicating synergistic interactions between the two viruses.

Effect of HAART on CD4⁺ T cell apoptosis

When we analysed untreated patients and patients on effective HAART in a cross-sectional comparison, both HIV-mono-infected and HIV/HCV-co-infected patients on HAART revealed significantly lower rates of CD4⁺ T cell apoptosis than untreated patients (HIV: PARP: $P=0.032$, TUNEL: $P=0.039$; HIV/HCV: PARP: $P=0.0005$, TUNEL: $P<0.0001$) (figure 3). Furthermore, CD4⁺ T cells apoptosis of HIV-mono-infected patients was not different from apoptosis in HIV/HCV-co-infected subjects under effective HAART. Nevertheless, rates of CD4⁺ T cell apoptosis in treated HIV patients remained still above the level of healthy controls (HIV: PARP: $P<0.0001$, TUNEL: $P=0.039$; HIV/HCV: PARP: $P<0.0001$, TUNEL: $P<0.0001$).

Stratification of all HAART-treated patients into PI- and non PI-based regimens revealed significant differences: Patients treated with protease inhibitors displayed higher rates of residual CD4⁺ T cell apoptosis than patients treated exclusively with NRTI or NNRTI/NRT combinations (PARP: $P=0.0033$; TUNEL: $P=0.0242$) (figure 3). This difference between PI- and non PI-based HAART was independent of HCV co-infection.

Changes of CD4⁺ T cell apoptosis during immune reconstitution

Longitudinal analysis of CD4⁺ T cell apoptosis during immune reconstitution revealed decreasing levels of CD4⁺ T cell apoptosis in both, HIV-mono- and HIV/HCV-co-infected patients. Mean levels of CD4⁺ T cell apoptosis patients had been higher in HIV/HCV-co-infected patients than in HIV-mono-infected patients before HAART (figure 4 C/D) but decreased more vigorously than in HIV-mono-infected patients during the first four weeks of treatment (HIV: PARP: -1.22%, TUNEL: -1.43%; HIV/HCV: PARP: -4.96%, TUNEL: -4.06%), while increases in CD4⁺ T cell counts (HIV: +124 cells/ml; HIV/HCV: +108 cells/ml) and inhibition of HIV replication (HIV: -2.47 log; HIV/HCV: -2.53 log) did not reveal significant differences between the two patient groups.

Measurements twelve weeks after initiation of HAART confirmed that levels of CD4⁺ T cell apoptosis were identical in HIV/HCV-co-infected patients and HIV-mono-infected patients (figure 4 C/D). In addition, increases in CD4⁺ T cell count (HIV: +135 cells/ml; HIV/HCV: +133 cells/ml) as well as drop in HIV loads (HIV: -3.49 log; HIV/HCV: -3.46 log) were equivalent in HIV- and HIV/HCV-co-infected patients 12 weeks after the start of HAART (figure 4 A/B).

Discussion

Apoptosis of CD4⁺ T cells has been repeatedly incriminated as a major process underlying the loss of CD4⁺ T cells in HIV-positive patients. However, the role of HCV co-infection for progression of HIV disease and particularly apoptosis of CD4⁺ T cells has remained controversial thus far.

In this study we investigated DNA fragmentation and cleavage of PARP in CD4⁺ T cells which together reflect irreversible changes associated with induction of apoptosis. Our data confirm high rates of apoptosis in CD4⁺ T cells of HIV-positive patients. Notably, apoptosis of CD4⁺ T cells in HIV/HCV-co-infected patients markedly exceeded the levels observed in patients with HIV mono-infection, whereas HCV infection alone resulted in rates of apoptosis, which were identical to healthy controls. Taken together, these findings indicate that although HCV infection alone did not induce apoptosis in CD4⁺ T cells, it sensitizes CD4⁺ T cells towards the apoptotic effects of HIV infection.

These results corroborate a previous observation reporting that HCV/HIV-co-infected patients had particularly high rates of annexin V binding in CD4⁺ T cells, a possible marker of early apoptosis [22]. However, unlike this previous study our data were based on markers of irreversible apoptosis and also differentiated rates of apoptosis between HIV-mono- and HIV/HCV-doubly-infected patients as well as treated and untreated patients, respectively. Our data seem also to correspond to findings in HepG2 cells, where collaborative induction of apoptosis has been reported for the interaction of HCV and HIV envelope proteins [23]. However, in HepG2 cells either viral protein alone did not induce apoptosis, whereas in our study apoptosis of CD4⁺ T cells was already increased in HIV mono-infection. Thus, further mechanisms must be involved, which sensitized CD4⁺ T cells towards apoptosis induction in patients with untreated HIV/HCV co-infection.

Although it was shown that HIV-driven immune activation is correlated with apoptosis of CD4⁺ T cells in HIV mono-infection, Nunez *et al.* did not find any association between T cell activation and CD4⁺ T cell apoptosis in HIV/HCV-coinfected patients [22]. This does not rule out increased production of pro-inflammatory cytokines or altered expression of death receptors and their corresponding ligands. Indeed, up-regulated expression of death receptors FAS as well as TRAIL receptors I and II has independently described in HCV and HIV mono-infected patients and seems to correspond to increased serum levels of death receptor ligands FasL, TRAIL and TNF alpha [24-30]. In particular, soluble TRAIL is increased in HIV infection closely related to the level of HIV replication. Moreover, HIV-Tat-stimulated monocytes have been demonstrated to kill CD4⁺ T cells via TRAIL-dependent apoptosis [31, 32]. In this context it is noteworthy that HCV core protein has been shown to sensitize Huh7 cells towards apoptosis mediated by TRAIL [33]. This finding has been attributed to enhanced apoptosis signalling via sequential induction of caspase 8, Bid cleavage, activation of the mitochondrial pathway and activation of effector caspase 3 as well as direct activation of caspase 9. Since cleavage of PARP and DNA fragmentation are apoptotic events downstream of caspase 3 activation, we could not differentiate which pathway of apoptosis activation was enhanced in CD4⁺ T cells from HIV/HCV-co-infected patients.

Although the exact mechanisms underlying apoptosis sensitisation remain unclear thus far, our results suggest a pivotal role for HIV replication. First, rates of apoptosis in HIV/HCV-co-infected patients were increased the more over HIV-mono-infected patients the higher the level of HIV replication. Next, this effect was fully reversed when HIV replication was suppressed by effective HAART, as shown by our cross-sectional comparison and the individual serial longitudinal studies in single patients. Of note, the decline of apoptosis rates was more pronounced in HIV/HCV-co-infected patients at week 4 than in HIV-mono-infected patients, although HIV replication had been inhibited to the same degree in both groups. Although rates of apoptosis further declined in the subsequent weeks on HAART, residual apoptosis in CD4⁺ T cells still remained above the level of healthy controls and HCV-mono-infected patients but did no longer reveal any significant differences between HIV- and HIV/HCV-co-infected patients. This may reflect effects resulting from ongoing HIV replication below the level of detection on one hand. On the other hand unexpected adverse effects due to the anti-retroviral agents must also be considered. In this context it was an intriguing finding, that residual CD4⁺ T cell apoptosis was significantly higher in patients on PI-based HAART regimens than on non PI-regimens. Although PIs block cellular apoptosis at the level of mitochondrial pore proteins, paradoxical pro-apoptotic effects have been reported *in vitro* at high concentrations both in

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hematological cell lines and solid tumors [34, 35]. Increased *in vitro* apoptosis from PIs was either associated with inhibited transcriptional activation of NF- κ B together with inhibited expression of its targets Bcl-X_L, survivin, c-Myc and cyclin D2 [34] or an increase in the cellular concentration of the antiproliferative and proapoptotic proteasome substrate cdK inhibitor p21 [35]. This reasoning is consistent with an earlier report that HIV PI have an impact on proteasome activity [36]. Furthermore, *in vitro* inhibition of cells with ritonavir, saquinavir or nelfinavir was associated with decreased expression of the anti-apoptotic protein Mcl-1 as well as blocked IL-6 mediated phosphorylation of ERK 1/2 and STAT3 [37].

Unfortunately, however, we do not have data to clarify if resolution of hepatitis C by anti-HCV treatment can reduce CD4⁺ T cell apoptosis to the same degree as antiretroviral therapy, since all our interferon-treated patients had been on antiretroviral therapy prior to anti-HCV treatment. Thus, the relationship between anti-HCV treatment and CD4⁺ T cell apoptosis still needs to be clarified in future projects.

Whatever the molecular and cellular mechanisms, our data clearly demonstrate that HIV/HCV-co-infected patients without effective HAART suffer increased apoptosis of CD4⁺ T cells and thus, are prone to progress more rapidly to severe immunodeficiency. Moreover, our data may help to understand why clinical data on HIV progression in patients with hepatitis C co-infection are controversial. For, in our comprehensive study a negative impact on disease progression was only noted in untreated patients, and rates of CD4⁺ T cell apoptosis became identical between HIV and HIV/HCV-co-infected patients shortly after initiation of HAART. Although differences in outcome between HIV and HIV/HCV-co-infected patients have been reported [8, 38, 39], our findings would support several recent longitudinal observations of HIV/HCV-co-infected patients which suggest there are no relevant differences in immune recovery between HIV and HIV/HCV-co-infected patients on long-term HAART [14, 40, 41]. In line with these results, early initiation of antiretroviral therapy in HIV/HCV-co-infected patients is not only a good way to reduce liver-related mortality but may also be an effective measure to prevent excessive loss of CD4⁺ T cells in HIV/HCV co-infection [42].

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Acknowledgements

All authors made substantial contribution to this research project. None of the authors has any conflict of interest related to this study.

We also are grateful for the contributions made by J.-C. Wasmuth, C. Schwarze-Zander and Jetske Emmelkamp.

Funding

This work was supported by a grant from the German Federal Ministry of Education and Research (BMBF) within the network for resistance in hepatitis C, grant number [01KI0792].

J. Nattermann was supported by a grant from the H.W. & J. Hector foundation, grant number [M42].

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Tables

Table 1. Patient characteristics

Characteristic	Healthy controls	HCV	HIV		HIV/HCV	
			no HAART	HAART	no HAART	HAART
Number	12	10	25	22	14	23
Female sex ^{a)}	5 (42.0%)	4 (40.0%)	5 (20.0%)	6 (27.3%)	2 (14.3%)	7 (30.4%)
Age (years) ^{b)}	36.7 (26-51)	40.5 (19-78)	42.5 (28-67)	44.8 (27-69)	42.8 (32-60)	42.5 (30-53)
Risk factors						
I.V. drugs ^{a)}	-	7 (70.0%)	0 (0.0%)	4 (18.2%)	7 (50.0%)	9 (39.1%)
Blood Transfusion ^{a)}	-	1 (10.0%)	0 (0.0%)	1 (4.5%)	0 (0.0%)	0 (0.0%)
Heterosexual ^{a)}	-	0 (0.0%)	5 (20.0%)	3 (13.6%)	0 (0.0%)	2 (8.7%)
Haemophilia ^{a)}	-	0 (0.0%)	1 (4.0%)	4 (18.2%)	2 (14.3%)	7 (30.4%)
MSM ^{a), c)}	-	0 (0.0%)	15 (60.0%)	6 (27.3%)	3 (21.4%)	2 (8.7%)
Endemic ^{a)}	-	0 (0.0%)	3 (12.0%)	3 (13.6%)	2 (14.3%)	0 (0.0%)
Unknown ^{a)}	-	2 (20.0%)	2 (8.0%)	1 (4.5%)	0 (0.0%)	3 (13.1%)
HIV status						
HIV load (x10 ³ copies/ml)	-	-	39.9 (0.05-375.9)	< 0.05	46.1 (0.45-548)	< 0.05
CD4 ⁺ cells /µl ^{d)}	n.a. ^{e)}	n.a. ^{e)}	371 (11-1235)	459 (134-1000)	350 (40-1000)	430 (38-1088)
CD8 ⁺ cells /µl ^{d)}	n.a. ^{e)}	n.a. ^{e)}	780 (324-1727)	815 (160-1491)	1034 (589-2229)	933 (410-1937)
HCV status						
HCV load (x10 ⁵ copies/ml)	-	5.8 (0.02-25)	-	-	7.7 (0.09-25.7)	15.5 (0.01-74)
Genotype 1	-	10 (100%)	-	-	8 (57.1%)	13 (56.5%)
Non genotype 1	-	0 (0.0%)	-	-	6 (42.9%)	10 (43.5%)

^{a)} number of cases (number/total) ^{b)} mean (range) ^{c)} MSM; men who have sex with men ^{d)} median (range) ^{e)} n.a. not analyzed

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Table 2. Characteristics of HAART-treated patients

Characteristic	HAART all	HAART PI-based	HAART Non PI-based
Number	45	31	14
Female sex ^{a)}	13 (28.9%)	7 (22.6%)	6 (42.8%)
Age (years) ^{b)}	42.3 (27-69)	41.7 (27-69)	43.7 (30-55)
HCV-co-infected	23 (51.1%)	17 (54.8%)	6 (42.9%)
Protease inhibitors (PI)			
Atazanavir	9	9 (29.0%)	-
Lopinavir/Ritonavir	19	19 (61.3%)	-
Saquinavir	2	2 (6.5%)	-
Ritonavir + PI	5	5 (16.1%)	-
Frosamprenavir	1	1 (3.2%)	-
NNRTI			
Efavirenz	9	-	9 (64.3%)
NRTI			
Tenofovir	32 (71.1%)	24 (77.4%)	8 (57.1%)
Emtricitabin	24 (53.3%)	17 (54.8%)	7 (50.0%)
Lamivudin	24 (53.3%)	16 (51.6%)	8 (57.1%)
Abacavir	12 (26.7%)	7 (22.6%)	5 (35.7%)
Zidovudin	5 (11.1%)	0 (0.0%)	5 (35.7%)

^{a)} number of cases (number/total)

^{b)} mean (range)

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Titles and legends to figures

Figure 1. Flowcytometric analysis of CD4⁺ T cells. Representative dot plots from patients from each group with apoptosis determined by PARP (columns 2, 3) and TUNEL assay (columns 4, 5). The dot plot of the isotype control is representative for all controls from each patient (column 1). Lymphocytes were gated by side and forward scatter. CD4⁺ T cells were defined as CD3⁺/CD4⁺-positive lymphocytes. Apoptotic cells are PARP-positive as determined in the lower right quadrant. Late apoptotic cells show high levels of DNA fragmentation (TUNEL) as determined in the lower right quadrant.

Figure 2. (A/B) Percentage of CD4⁺ T cells with apoptosis in different patient groups. Figure 2A shows the results obtained with the PARP assay. Figure 2B illustrates rates of CD4⁺ T cell apoptosis determined by DNA fragmentation (TUNEL assay). Box plots (median, interquartile range) display levels of apoptosis in healthy controls (n=12), HCV-mono-infected patients (n=10) and HIV-mono-infected (n=25) as well as HIV/HCV-co-infected subjects (n=14). Statistical comparisons between groups were done with the Mann-Whitney test using Prism 3.0 software (GraphPad, USA). **(C/D) Associations between HIV load and CD4⁺ T cell apoptosis.** CD4⁺ T cell apoptosis of HIV-mono-infected and HIV/HCV-co-infected patients is plotted versus HIV loads. (C) CD4⁺ T cell apoptosis determined by PARP assay is correlated to HIV loads both in HIV-mono-infected and HIV/HCV-co-infected patients (HIV: $r_s=0.66$, $P=0.0003$; HIV/HCV: $r_s=0.82$, $P=0.0002$). (D) Levels of CD4⁺ T cell apoptosis determined by TUNEL assay is correlated to HIV loads (HIV: $r_s=0.66$, $P=0.0003$; HIV/HCV: $r_s=0.79$, $P=0.0003$). Correlations between apoptosis and viral loads were analyzed with Spearman rank correlation using Prism 3.0 software (GraphPad, USA).

Figure 3. Impact of effective HAART on CD4⁺ T cell apoptosis. Percentage of CD4⁺ T cells with apoptosis in HIV-mono-infected and HIV/HCV-co-infected patients with (n=22, n=23) and without effective HAART respectively (n=25, n=14). Columns A and C refer to the PARP assay, columns B and D to the TUNEL. In figure C and D patients were stratified with respect to the type of HAART, irrespective from HCV co-infection. Statistical comparisons between groups were done with the Mann-Whitney test using Prism 3.0 software (GraphPad, USA).

Figure 4. Changes of CD4⁺ T cell apoptosis during Immune reconstitution. (A) CD4⁺ T cell counts at baseline, 4 and 12 weeks after HAART initiation of HIV-mono (n=8) and HIV/HCV-co-infected (n=5) patients. (B) HIV loads at baseline, 4 and 12 weeks after HAART initiation of HIV-mono and HIV/HCV-co-infected patients. Percentage of CD4⁺ T cell apoptosis at baseline, 4 and 12 weeks after HAART initiation in HIV-mono and HIV/HCV-co-infected patients determined by PARP assay (C) and TUNEL assay (D).

Fig. 1







