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Source / Izvornik: **Collegium Antropologicum, 2006, 30, 33 - 38**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:092867

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Download date / Datum preuzimanja: **2024-11-01**

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Coll. Antropol. **30** (2006) Suppl. 2: 33–38
Original scientific paper

Persistant Immunodominant Anti-Gag SLYNTVATL Responses in HIV-Patients with up to 7 Years of HAART

Snježana Židovec Lepej[1], Ela Kosor[2], Alenka Gagro[2], Adriana Vince[3], Anica Remenar[1] and Mario Poljak[4]

[1] Laboratory for Molecular Diagnostics and Cellular Immunology, University Hospital for Infectious Diseases »Dr. Fran Mihaljević«, Zagreb, Croatia

[2] Cellular Immunology Unit, Department for Research and Development, Institute of Immunology, Zagreb, Croatia

[3] Department for Viral Hepatitis and Laboratory for Molecular Diagnostics and Cellular Immunology, University Hospital for Infectious Diseases »Dr. Fran Mihaljević«, Zagreb, Croatia

[4] Institute of Microbiology and Immunology, Medical Faculty Ljubljana, Ljubljana, Slovenia

A B S T R A C T

We analyzed Gag-specific CD8$^+$ T-cells in HIV-patients on long-term HAART and in untreated chronically-infected patients by using iTAg MHC class I tetramers (HLA-A*0201) specific for SLYNTVATL. Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells were detectable in 18 of 26 treated patients (median 5.2 years of HAART) and in 10 of 14 untreated patients. Median percentage of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells in treated patients was 0.10 (range 0.00–0.70%). Median number of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells per 50,000 CD8$^+$ T-cells was 56.0 cells (range 2.0–344.0 cells) and was not significantly different compared with untreated patients (p=0.978). Numbers of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells were inversely correlated with the duration of undetectable plasma viremia (p=0.02, Rho= –0.430). Chronically-infected HIV-patients on HAART (for up to 7.7 years) maintained a stable subpopulation of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells. This finding is relevant for the analysis of treatment-induced immune reconstitution and, possibly, for future therapeutic strategies in HIV-disease.

***Key words**: HIV-1, MHC class I tetramer, HAART; CD8, immune reconstitution*

Introduction

Introduction of tetramer technology capable of direct ex vivo enumeration of antigen-specific cells enabled a detailed quantitative characterization of HIV-specific T-cell responses in different stages of infection and/or disease. Although HIV-specific CD8$^+$ T-cells can be detected in the majority of chronically infected individuals, they are unable to eradicate the virus and/or maintain a successful control of virus replication[1]. Limited efficacy of HIV-specific CD8$^+$ T-cell immunity can be, at least in part, attributed to loss of CD4$^+$ T-cell help, impaired antigen presentation and signal transduction, aberrations in surface phenotype and intracellular cytokine synthesis, viral evasion etc[2–5].

Nevertheless, antigen-specific CD8$^+$ T-cells appear to be critical for the partial containment of HIV-1/SIV replication at the chronic stage of infection[4,6]. Interestingly, several longitudinal and cross-sectional studies showed that HIV-specific T-cells impose a selective pressure on HIV-1 that results in viral evolution during chronic infection[7–10]. Moreover, it is possible that long-term efficient anti-HIV treatment will require augmentation of virus-specific T-cell responses as well.

Literature data regarding the impact of HAART on HIV-specific CD8$^+$ T-cell immunity at different stages of infection and/or disease are variable. Several studies showed HAART-induced decline in HIV-specific cellular immune responses in asymptomatic as well as advanced patients but other reports contradicted these findings[11–17]. For example, Appay et al. (2002) showed the presence of CD4$^+$ and CD8$^+$ HIV-specific responses in patients with 3–4 years of virologically and clinically successful HAART[16]. Similarly, Rinaldo et al. detected a sta-

Received for publication November 2, 2006

ble, residual population of potentially immunocompetent HIV-1-specific T-cells in advanced patients on long-term HAART[15]. Moreover, examples of de novo appearance of HIV-specific $CD8^+$ T-cells in patients with 6 months of successful HAART have been described[17].

The majority of the abovementioned studies investigated the frequency of HIV-specific T-cell responses in patients with less than 4 years of HAART. The aim of our study was to investigate the frequency and absolute counts of Gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells in symptomatic HIV-patients who have been treated for long periods of time (median 5.2 years) and compare it with untreated chronically-infected patients. We also compared HIV-specific immunity in patients with and without detectable viremia during follow-up. In this study we focused on $CD8^+$ T-cell responses to Gag p17 of HIV-1.

Materials and Methods

Study design and patients

This prospective, cross-sectional study was done at the University Hospital for infectious diseases »Dr. Fran Mihaljević«, Zagreb, Croatia between the February and May 2005.

Sixtyeight HIV-infected patients were screened to obtain HLA-A2-positive group. Flow cytometric expression of HLA-A2 in the patients was determined by using anti-human HLA-A2 monoclonal antibody (Proimmune, Oxford, UK). A group of 40 patients (median age 43.1 years, range from 22.6–67.1 years) were selected for the study. The patients were divided into two groups: untreated HIV-infected individuals (n=14) and patients receiving long-term HAART (n=26). Twelve of 26 HAART-treated patients had detectable viremia during follow-up. A total of 4 treated HIV-patients experienced one virological failure. Four HLA-A*0201-positive HIV-negative individuals (n=4) and three HLA-A*0201-negative HIV-positive patients were also enrolled as controls for the specificity of tetramer assay.

Peripheral blood samples for immunological (flow cytometry) and plasma samples for virological (quantitative RT-PCR for HIV-1 RNA) analysis were collected as a part of routine diagnostics.

Informed consent was obtained from all patients and controls. The study was approved by the Ethics committee of the University Hospital for Infectious Diseases.

Viral load

The quantification of HIV-1 RNA in the plasma of HIV-1-infected persons was performed by using Amplicor HIV-1 monitor Test, version 1.5 (Roche Diagnostic Systems, Inc., Branchburg, New Jersey, USA) with lower limit of detection of 50 (ultrasensitive method) copies of HIV-1 RNA/ml.

Flow cytometry

Percentages of Gag-specific $CD8^+$ T-cells in the peripheral blood were determined by using phycoerythrin (PE)-labeled iTAg™ HLA class I tetramers (A*0201) specific for Gag p17 SLYNTVATL peptide, amino acid residues 77-85 (Beckman Coulter Immunomics Operations, USA). Additionally, we used conjugated murine anti-human monoclonal antibodies (mAb) specific for CD3 (FITC) and CD8 (RPE-Cy5) (Dako Cytomation A/S, Glostrup, Denmark).

Peripheral blood cells were first stained with HLA-tetramers for 15 min. in the dark and then for additional 10 min. with anti-CD3 and anti-CD8 mAbs, at room temperature. After incubation, cells were prepared for analysis by using whole blood non-wash ImmunoPrep Reagent System on Coulter TQ-prep (Beckman Coulter, Inc., Fullerton, CA, USA). For each sample, we collected 50,000 $CD3^+CD8^+$ events and analysed for tetramer expression on Cytomics FC500 flow cytometer via CXP 2.0 software (Beckman Coulter, Inc., Fullerton, CA, USA). Absolute counts of Gag-specific $CD8^+$ T-cells per microliter of peripheral blood as well as per 50,000 $CD8^+$ T-cells were determined by using Flow-Count Fluorospheres quantification reagent (Beckman Coulter, Inc., Fullerton, CA, USA).

Figures 1b and 1c show representative dot plots of $Gag_{77–85}$ tetramer staining in HLA-A*0201-positive HIV-positive patients. As negative controls we analysed HLA-A*0201-negative HIV-positive patients (n=4) and HLA-A*0201-positive HIV-negative healthy persons (n=3). Percentages of gag-specific $CD8^+$ T-cells in both control groups were lower than 00.001%.

Percentages of T-cell, $CD4^+$ and $CD8^+$ T-cells, B-cells and NK-cells in the peripheral blood of HIV-infected patients were determined by using a four-colour flow cytometry panel CYTO-STAT tetra CHROME (CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5) (Beckman Coulter, Inc., Fullerton, CA, USA) as recommended (CDC). Similarly to tetramer staining, the preparation of samples for $CD4^+$ T-cell quantification was performed automatically on Coulter TQ-prep and analysed on Cytomics FC500 cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Absolute counts of $CD4^+$ T-cells were also determined directly on the cytometer by using Flow-Count Fluorospheres (Beckman Coulter, Inc., Fullerton, CA, USA).

Statistical analysis

The Wilcoxon Two-sample test was used for comparison of T-cell subpopulations in different patient groups. The correlation between variables was evaluated with the Spearman Rank test. Statistical analysis was performed with SAS software version 8.2. (SAS Institute, Cary, North Carolina, USA).

Results

Patients

Demographic, virological, immunological and clinical data on untreated HIV-infected patients and patients on long-term HAART are summarized in Table 1.

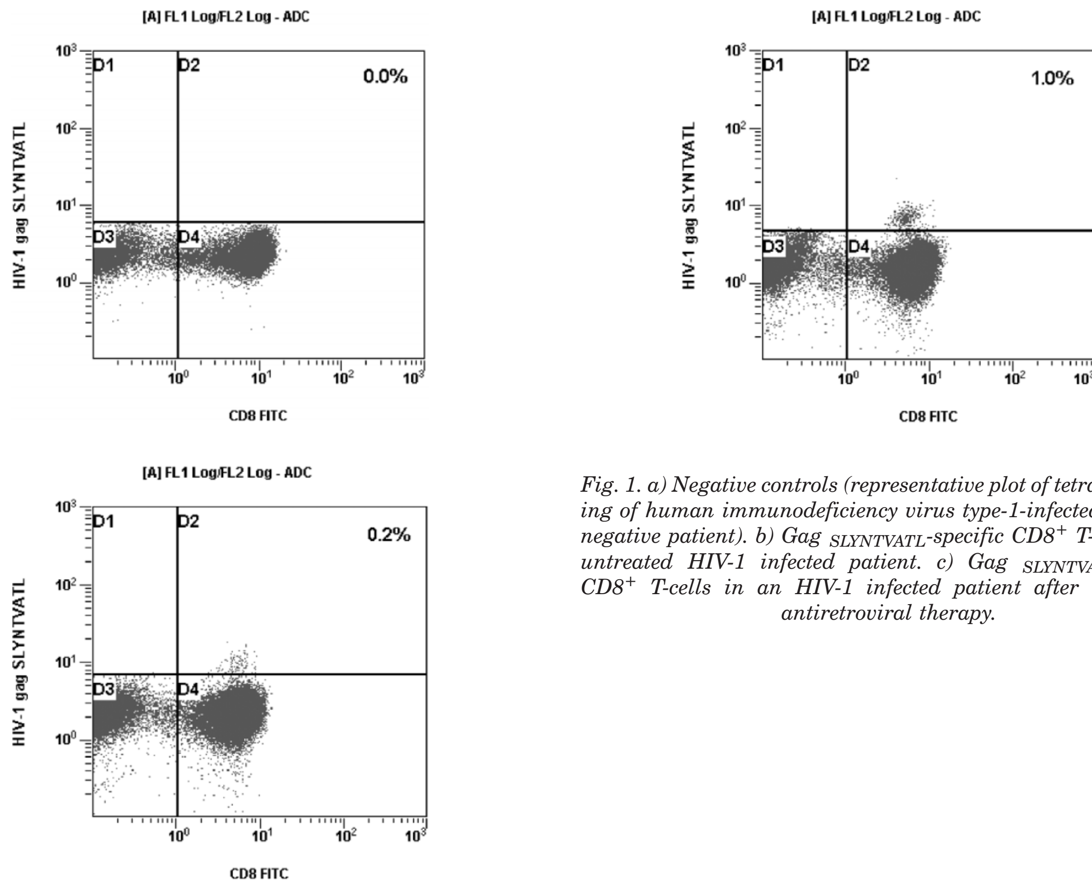

Fig. 1. a) Negative controls (representative plot of tetramer staining of human immunodeficiency virus type-1-infected HLA-A2-negative patient). b) Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells in an untreated HIV-1 infected patient. c) Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells in an HIV-1 infected patient after 7 years of antiretroviral therapy.

Duration of HAART in 26 selected patients ranged between 0.3 and 7.7 years (median 5.2 years). Median pre-treatment CD4$^+$ T-cell count in treated patients was 127 cells/μL (range 1–407 cells/μL). Median pre-treatment percentages of CD4$^+$ and CD8$^+$ T-cells were 8.95% and 54.1%, respectively. At the time of tetramer analysis, median CD4$^+$ T-cell count in treated HIV-patients was 396 cells/μL (range 117–965 cells/μL). Median percentages of CD4$^+$ and CD8$^+$ T-cells at the time of tetramer staining were 22.8% (range 10.6–44.4%) and 46.1% (22.2–70.4%), respectively. Median lowest recorded CD4$^+$ T-cell count in treated patients was 49 cells/μL. Median lowest recorded CD4$^+$ T-cell percentage was 7.0%.

Median plasma viremia in untreated patients (n=14) at the time of tetramer staining was 4.78 $\log_{10}$ copies of HIV-1 RNA/ml of plasma. Median absolute count of CD4$^+$ T-cells in untreated patients was 256 cells/μL (range 31–525 cells/μL). Median percentages of CD4$^+$ and CD8$^+$ T-cells in untreated patients at the time of HIV-specific immunity analysis were 20.5% (range 1.6–31.0%) and 53.9% (range 43.1–77.1%), respectively. Median lowest recorded CD4$^+$ T-cell count in untreated patients was 200 cells/μL. Median lowest recorded CD4$^+$ T-cell percentage was 22.0%.

Cellular immune responses to HIV-1 Gag

Ex vivo tetramer staining of CD8$^+$ T-cells was used to monitor CD8$^+$ T cell responses specific for HLA-A$^+$0201-restricted HIV-1 gag antigenic peptide SLYNTVATL in patients on long term successful HAART as well as in untreated HIV-infected patients (Figures 1a, 1b and 1c). In addition to the percentage of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells, we determined absolute counts of tetramer-positive cells per μL of the peripheral blood (by using commercially available absolute counting reagent) and per 50,000 CD8$^+$ T-cells. Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells were detected in 18 of 26 treated patients and in 10 of 14 untreated patients.

Median percentage of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells in patients on long-term HAART was 0.10 (range 0.00–0.70%) (Table 2). Median number of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells per μL of the peripheral blood in treated patients was 1 cells/μl (range 0–6 cells/μL) and, when expressed per 50,000 CD8$^+$ T-cells, it was 56 cells (range 2–344 cells).

In untreated patients, median percentage of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells was 0.10% (range 0.00–1.10%) (Table 2). Median number of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells per μL of the peripheral blood in treated patients was 1 cells/μl (range 0–9 cells/μl) and, when expressed per 50,000 CD8$^+$ T-cells, it was 61 cells (range 10–573 cells).

Percentages, number per μL and per 50,000 CD8$^+$ T-cells of Gag $_{SLYNTVATL}$-specific CD8$^+$ T-cells in treated and untreated HIV-pateints were not significantly differ-

TABLE 1
STUDY POPULATION

Parameter	Patients receiving long-term HAART	Untreated HIV-infected patients
Number	26	14
Median age (years, range)	44.8 (33.9–62.2)	34.3 (22.7–67.1)
Sex ratio (males/females)	23/3	12/2
Median plasma viremia at the time of analysis (log_{10} copies/mL, range)	Undetectable	4.7 (3.5–6.0)
Median $CD4^+$ T-cell count at the time of analysis (cells/μL, range)	396.5 (117.0–965.0)	256.5 (31.0–525.0)
Median lowest $CD4^+$ T-cell count (cells/μL, range)	49.0 (1.0–352.0)	200.5 (31.0–525.0)
Median lowest percentage of $CD4^+$ T-cells (median, range)	7.0 (0.1–45.1)	22.0 (1.0–35.5)
Median duration of HAART-induced undetectable viremia (years, range)	5.2 (0.3–7.7)	n.a.
Pre-treatment median plasma viremia (log_{10} copies/mL, range)	5.19 (4.07–6.92)	n.a.
Pre-treatment median $CD4^+$ T-cell count (cells/μL, range)	127.0 (1.0–407.0)	n.a.

HAART – highly active antiretroviral therapy, n.a. – not applicable

TABLE 2
GAG $_{SLYNTVATL}$-SPECIFIC $CD8^+$ T-CELLS IN HIV-PATIENTS RECEIVING LONG-TERM HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) AND IN UNTREATED PATIENTS

Parameter of HIV-specific immunity (median, range)	Patients receiving long-term HAART (n=26)	Untreated HIV- -infected patients (n=14)	p*
Percentage of gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells	0.10 (0.00–0.70)	0.10 (0.00–1.10)	p=0.202
Number of gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells per μl of blood	1 (0–6)	1 (0–9)	p=0.579
Number of gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells per 50,000 $CD8^+$ T-cells	56 (2–344)	61 (10–573)	p=0.533

* between group comparisons, Wilcoxon Two – sample test, p<0.05 was considered

ent (p=0.202, p=0.579, p=0.533, respectively). Percentages, number per μL and per 50,000 $CD8^+$ T-cells of Gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells in treated patients with or without detectable viremia during follow-up were not significantly different (p=0.778, p=0.198, p=0.978, respectively).

Gag-specific T-cell immunity and viremia

Numbers of Gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells were inversely correlated with the duration of HAART-induced undetectable plasma viremia (p=0.02, Rho= –0.430). Additionally, numbers of tetramer-positive cells positively correlated with a total number of $CD8^+$ T-cells (Rho= 0.520, p=0.007).

There was no correlation between pre-treatment plasma viremia and percentage of tetramer-positive cells (Rh0= –0.063, p=0.767), number of tetramer-positive cells per μL of blood (Rho=0.209, p=0.326) and number of tetramer-positive cells per 50,000 $CD8^+$ T-cells (Rho=–0.029, p=0.890) in treated patients.

In untreated HIV-infected persons, there was no correlation between plasma viremia at the time of analysis and percentage of tetramer-positive cells (Rh0=–0.059, p= 0.841), number of tetramer-positive cells per μL of blood (Rho=–0.036, p=0.902) and number of tetramer- positive cells per 50,000 $CD8^+$ T-cells (Rho=–0.235, p=0.418).

Discussion

Tetramer-based ex vivo analysis is a highly sensitive tool for the enumeration of antigen-specific cells without in vitro manipulation. This technique has proven to be a useful tool in monitoring vaccine and immunotherapy trials as well as in other types of basic and clinical research where a quantitative characterization of antigen-specific T-cell responses may be of importance. By using this technology, we demonstrated the presence of Gag-specific $CD8^+$ T-cell responses in the majority of HIV-patients who received HAART long periods of time (up to 7 years). The amplitude of Gag-specific $CD8^+$ T-cell responses (percentage and counts of tetramer-positive cells) in patients on long-term HAART and untreated HIV-infected patients was not significantly different. Similarly, HIV-specific responses in treated patients with and without detectable viremia during follow-up were not significantly different. Numbers of Gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells were inversely correlated with the duration of HAART-induced undetectable plasma viremia

Studies in animal and human models have shown that antigen-specific $CD8^+$ T-cells are critical for the controls of HIV-1/SIV replication in vivo[6]. $CD8^+$ T-cell responses specific for five HIV-1 proteins (Gag p17, Gag

p24, reverse transcriptase, Env, Nef) in acute and chronic infection/disease have been well characterized.

The majority of untreated chronically-infected patients who express the histocompatibility leukocyte antigen (HLA)-A*0201 recognize the Gag p17 SLYNTVATL (aa residues 77–85) epitope (SL9). Gag-specific T-cell responses can be detected in the majority of HIV-2-infected individuals as well[18].

The ability of HAART to suppress plasma viremia to clinically undetectable levels has been well documented. However, much less is known about the effect of long-term HAART on the evolution of HIV-specific immunity.

According to some studies, a decline in plasma viremia following HAART was accompanied by diminished HIV-specific T-cell responses[11–13]. However, other studies consistently showed the presence of HIV-specific T-cells during antiretroviral therapy[14–17]. Appay et al showed the maintenance of HIV-specific T-cell responses in patients who received HAART for up to 4 years[16]. Furthermore, Benito et al were able to demonstrate de novo appearance of HIV-specific $CD8^+$ T-cells in patients on successful HAART[17].

Our study has shown the presence of HIV-specific $CD8^+$ T-cell responses in the majority of patients who have been receiving HAART for long periods of time (median 5.5 years, maximum 8 years). Additionally, we did not observe any difference in the amplitude of HIV-specific T-cell responses in treated patients with and without detectable plasma viremia during follow-up.

The maintenance of HIV-specific T-cell immunity in patients treated for several years is particularly important in the context of viral evolution. Several studies have shown that HIV-specific T-cells exert a selective pressure on the virus that causes a dynamic viral evolution during chronic infection, which is an important issue in immune evasion as well as for resistance to antiretroviral drugs[7–10].

Additionally, the presence of a stable pool of HIV-specific T-cells in patients treated for long periods shown in this study is relevant for further therapeutic strategies that will combine antiviral and immunomodulatory approaches. It is reasonable to assume that long-term successful anti-HIV treatment will be focused on the augmentation or modulation of antigen-specific T-cell immunity. Similarities in the amplitude of Gag-specific $CD8^+$ T-cell responses in untreated and treated HIV-patients shown in this study support the relevance of this concept. Additionally, further phenotypical and functional (intracellular cytokine expression) analysis of HIV-specific T-cells detected in patients with long-term successful HAART are needed to fully evaluate their contribution to treatment-induced immune reconstitution[5].

It has been difficult to establish a quantitative correlation between the frequency of HIV-specific T-cells and viremia in humans. For example, Buseyne et al (2002) have shown a positive correlation between the frequencies of ex-vivo activated HIV-specific $CD8^+$ T-cells and viremia in children[19]. Contrary to this finding, Benito et al (2003) found a negative correlation between Gag- and pol-specific $CD8^+$ T-cell responses and viremia[17]. According to some studies, a negative correlation between the frequency of HIV-specific CD8+ T-cells and plasma viremia can be established only in patients with high CD4 T-cell counts (> 400/μL)[20]. Our study failed to show a significant correlation between percentage and number (per μL and per 50,000 $CD8^+$ T-cells) in both treated and untreated HIV-patients.

Oxenius et al (2002) showed an inverse correlation (in a non-linear manner) between the frequency of HIV-specific T-cells and pre-treatment viremia in patients receiving HAART (median time on HAART 21 months)[21]. According to our results, there is no correlation between the percentage and number of Gag-specific $CD8^+$ T-cells and pre-treatment viremia in patients on long-term HAART.

In conclusion, chronically-infected HIV-patients receiving HAART for long periods of time (up to 8 years) maintained a stable subpopulation of Gag $_{SLYNTVATL}$-specific $CD8^+$ T-cells. This finding is relevant for the analysis of treatment-induced immune reconstitution and, possibly, for future therapeutic strategies in HIV-disease.

Acknowledgments

This study was supported by grants from the Croatian Ministry of Science, Education and Sports to Dr. A. Gagro (TP-01/0021-05), Dr. J. Begovac (0108027) and Dr. T. Jeren (0143999).

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S. Ž. Lepej

Laboratory for molecular diagnostics and cellular immunology, University Hospital for Infectious Diseases »Dr. Fran Mihaljević«, Mirogojska 8, 10 000 Zagreb, Croatia
e-mail: snjezana.zidovec.lepej@bfm.hr

PERZISTENTNA IMUNOREAKCIJA NA GAG PROTEIN HIV-a U BOLESNIKA LIJEČENIH HAART-OM

S A Ž E T A K

U ovom smo istraživanju analizirali Gag-specifične CD8+ T-limfocite u HIV-bolesnika liječenih HAART-om kao i u neliječenih zaraženih osoba primjenom iTAg MHC tetramera klase 1 (HLA-A*0201) specifičnih za SLYNTVATL. Gag $_{SLYNTVATL}$-specifične $CD8^+$ T-limfocite dokazali smo u 18 od 26 liječenih bolesnika (medjian 5,2 godine HAART-a) i u 10 od 14 neliječenih pacijenata. Medijan postotka Gag $_{SLYNTVATL}$-specifičnih $CD8^+$ T-limfocita u liječenih bolesnika bio je 0,10 (raspon 0,00–0,70%). Medijan broja Gag $_{SLYNTVATL}$-specifičnih $CD8^+$ T-limfocita na 50.000 $CD8^+$ T-limfocita bio je 56,0 stanica (raspon 2,0–344,0 stanica) i nije bio značajno različit u odnosu na neliječene zaražene osobe (p=0,978). Broj Gag $_{SLYNTVATL}$-specifičnih $CD8^+$ T-limfocita negativno je korelirao s dužinom perioda nemjerljive viremije u plazmi (p=0,02, Rho= –0,430). U liječenih HIV-bolesnika (do 7,7 godina HAART-a) postoji stabilna populacija Gag $_{SLYNTVATL}$-specifičnih $CD8^+$ T-limfocita. Rezultati ovog rada značajni su za analizu liječenjem-potaknute imunološke rekonstitucije te za razvoj novih strategija liječenja u HIV-bolesti.